

Molecular Cloning of cDNA, Recombinant Protein Expression and Characterization of a Buckwheat 16-kDa Major Allergen

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Key Words

Allergen · Buckwheat · BWp16 · RACE · Recombinant protein · Signal peptide

Abstract

Background: Buckwheat is a common food in Japan, Korea and other countries. A candidate major buckwheat allergen, a 16-kDa protein (BWp16), was previously characterized as a pepsin-resistant protein associated with immediate-type allergies to buckwheat. However, whether recombinant BWp16 can react with a patient's IgE remains uncertain. **Methods:** The cDNA encoding BWp16 from Japanese buckwheat seeds was cloned based on the sequences obtained by the 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE PCR. Recombinant BWp16 protein expressed in *Escherichia coli* was purified using affinity chromatography. Western blotting, ELISA and cross inhibition tests of the purified recombinant BWp16 were performed using sera from patients with positive IgE binding to buckwheat and controls. Pepsin digestion experiments were also performed. **Results:** The full-length cDNA encodes 149 amino acid residues with a calculated molecular mass of 16.9 kDa. The deduced amino acid sequence included a putative signal peptide sequence. BWp16 showed significant homolo-

gies to the buckwheat 8-kDa allergen and *Ricinus communis* (castor bean) 2S albumin. Sera from patients with positive IgE binding to buckwheat reacted with the purified BWp16. Cross inhibition tests revealed immunological equivalence of the purified recombinant and natural BWp16. The recombinant and natural BWp16 were comparably resistant to pepsin digestion. **Conclusions:** BWp16 belongs to the 2S albumin family and is a buckwheat allergen. This purified recombinant BWp16 could be used in the diagnosis of buckwheat allergy.

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Introduction

Buckwheat (*Fagopyrum esculentum*) belongs to the Polygonaceae family, and its products are becoming popular in Russia, Italy, the USA and Canada as a health food with a high protein content and in Asian countries because of the unusually short maturation period. For buckwheat-allergic patients, however, its ingestion causes immediate-type anaphylactic reactions through the actions of specific IgE antibodies [1, 2]. Sicherer and Sampson [3] indicated that it is common and often informative to use skin prick tests with extracts, that particularly a negative result is useful in evaluating things the patient is not allergic to. They indicate that the rate of false-positive results is high. They also indicate that intradermal tests, where extracts are injected into the dermis, give higher

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1018–2438/06/1401–0073\$23.50/0

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false-positive rates and can be risky for the patient. However, many allergists around the world use skin prick tests in diagnosing food allergies. Commercial or laboratory extracts are commonly used for foods that are not fresh fruits (with good success). If the suspected food is a fresh fruit or a vegetable that is consumed raw, then fresh prick-prick tests are commonly used with greater effectiveness [4]. However, because of the risk of severe and dangerous reactions, skin tests and provocation tests should be avoided in case of diagnosing buckwheat allergy [5]. Thus, assays for serum buckwheat-specific IgE antibodies should be used as a diagnostic tool.

Available routine diagnostic methods for allergies to plant-derived foods, namely skin tests with fresh foods, oral challenges and the measurement of food extract IgE titers are often very sensitive, but none of these methods provide information on the allergenic molecule(s) causing sensitization. Generally, most clinicians do not have access to laboratories performing immunoblot analysis and/or N-terminal sequencing, and both natural and recombinant allergic molecules for routine diagnostic tools are lacking [6]. Buckwheat-derived foods may also contain a number of different allergens. The different allergenic proteins in these foods may have different physical/chemical characteristics that may strongly influence the clinical expression of buckwheat food allergy to buckwheat. Thus, at a molecular level the diagnosis of buckwheat allergy has a high clinical (both preventive and prognostic) priority.

Several buckwheat molecules have been reported to react with IgE from allergic patients. For instance, 24-, 19-, 16- and 9-kDa proteins have been identified as major buckwheat allergens [7–9]. Furthermore, Matsumoto et al. [10] have reported a 10-kDa protein and its allergenicity. The 10-kDa protein, which is identical to an 8-kDa buckwheat allergen (Genbank accession No. AB055892), is a member of the 2S albumin family [8]. Some of the 2S albumin family proteins have been identified as allergens in Brazil nuts (Ber e 1), English walnuts (Jug r 1), cashew nuts (2S albumins), sesame seeds (Ses i 2), yellow mustard (Sin a 1) and oriental mustard (Bra j 1) [6, 11, 12].

Recently, several researchers have characterized the allergenicity of a 16-kDa buckwheat protein (BWp16) [5, 7, 13]. BWp16 is thought to be a strong candidate buckwheat allergen in that it was identified as a pepsin-resistant protein associated with immediate hypersensitivity reactions in buckwheat-allergic patients [5]. However, whether purified recombinant BWp16 is immunoreactive to IgE from patients with positive IgE binding to buckwheat has not been confirmed. More recently, Park

et al. [7] cloned a partial cDNA encoding BWp16 (Genbank accession No. AY966013). However, cloning of the full-length cDNA, including translation initiation and termination codons, has not been conducted. In this paper, we cloned the full-length cDNA of BWp16, produced and purified the recombinant protein, and determined its reactivity with sera from patients with positive IgE binding to buckwheat.

Materials and Methods

Molecular Cloning of cDNA Encoding a 16-kDa Buckwheat Protein

Japanese buckwheat seeds were provided by Japan Agricultural Cooperatives in Fukui Prefecture. Total RNA was prepared from the seeds using a Sepazol RNA isolation kit (Nakarai Tesc, Kyoto, Japan). The isolated RNA samples were treated with RNase-free DNase I (Takara Bio, Otsu, Japan) to avoid genome DNA contamination. Two hundred nanograms of the total RNA was reverse-transcribed using BD Advantage 2 polymerase (BD Biosciences, Palo Alto, Calif., USA) according to the BD SMART RACE cDNA Amplification Kit protocol. Based on the reported partial cDNA sequence of BWp16 (Genbank accession No. AY966013), the 5'-RACE PCR reaction was performed using a universal primer mix (UPM, BD Biosciences) and BWp16-R3-5'-RACE primer (5'-TCATCTTCACTTGTCGCATGCACTTTG-3'). For the 3'-RACE PCR, the first round of PCR amplification was carried out using BWp16-F1-3'-RACE primer (5'-CGATTTGGGCGAAACCCAG-ATGAGC-3') and UPM. Next, the nested PCR was done using BWp16-F2-3'-RACE (5'-TGCTGTGTGGCGATGAAGGAGATGG-3') and nested universal primers (BD Biosciences) with 1/50 of the volume of the first-round amplification product. The PCR conditions were 94°C for 3 min followed by 35 cycles (5'-RACE), 25 cycles (the first 3'-RACE) or 20 cycles (the nested 3'-RACE) at 94°C for 30 s, 67°C for 30 s and 72°C for 3 min plus a final extension period at 72°C for 7 min. Each amplified product was subcloned into pT7Blue T-vector (Novagen, Madison, Wisc., USA), and the resultant plasmids were sequenced in both directions using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA) with the forward (5'-GTTTTCCCA-GTCACGACGT-3') and reverse (5'-ATGACCATGATTACGC-CAAG-3') primers. Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems).

To isolate the full-length BWp16 cDNA, we next cloned a gap region between the 5'-RACE and 3'-RACE clones. In view of the sequences of the 5'- and 3'-RACE clones, the gap region was amplified using BWp16-F1 (5'-GAAAGAGATGAAGGCTTCGATT-TAGGCGAAACC-3'; underline indicates artificial sequence added for cloning) and BWp16-R1 (5'-CCAAATTACACAAAATACCGATTTCCTCTC-3') primers. The amplified fragments were then sequenced as described above.

Finally, the full-length cDNA was amplified using BWp16-F2 (5'-AAAATGAAGCTCTTCATCATCCTAGCA-3') and BWp16-R1 primers, and the amplified PCR fragment was cloned into pT7Blue T-vector (Novagen). The full-length cDNA was verified by sequencing as described above.

Expression and Purification of Recombinant BWp16

The cDNA encoding amino acid residues 23–149 was amplified using forward (5'-GGGGGATCCAGAGATGAAGGCTTCGATTTAGGC-3'; underline denotes introduced *Bam*HI site) and reverse (5'-CCCGAATTCCTTACACAAAATACCGATTTCCTCT-3'; underline denotes introduced *Eco*RI site) primers and ligated between the *Bam*HI and *Eco*RI sites of pGEX-6P-1 (Amersham Biosciences, Little Chalfont, UK). The resultant plasmid was transformed into *Escherichia coli* BL21(DE3)pLysS, and the production of glutathione-S-transferase (GST)-BWp16 fusion protein was induced by culturing in L-broth medium containing 0.2 mM isopropyl β -D-1-thiogalactopyranoside (Takara Bio) for 4 h at 25°C. The total cell pellet of 200-ml culture was lysed in 20 ml of CelLytic B Cell Lysis Reagent (Sigma, St. Louis, Mo., USA) containing 1 $\times$ protease inhibitor cocktail (Sigma) and 500 units of benzonase (Novagen), and soluble supernatant was obtained by centrifugation at 15,000 *g* for 20 min. The overexpressed GST-BWp16 fusion protein was bound to glutathione Sepharose 4B (Amersham Biosciences) for 18 h at 4°C, extensively washed with wash buffer (20 mM Tris-HCl, pH 7.0), 1% Triton X-100, 1 mM EDTA, 1 mM DTT and 1 $\times$ protease inhibitor cocktail) and BWp16 was excised from the fusion protein by 80 units of PreScission Protease (Amersham Biosciences) in cleavage buffer (50 mM Tris-HCl, pH 7.0), 150 mM NaCl, 1 mM EDTA and 1 mM DTT for 4 h at 4°C, according to the manufacturer's instructions. After the cleavage, the buffer was replaced by PBS (pH 7.4) using Microcon YM-10 (Millipore, Bedford, Mass., USA) and the resultant protein solution was stored at 4°C. We quantified the protein concentration through a BCA protein assay (Pierce Biotechnology, Rockford, Ill., USA) according to the manufacturer's instructions. The purified recombinant BWp16 was subjected to 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by visualization with Coomassie brilliant blue (CBB) staining. Rainbow marker (Bio-Rad, Hercules, Calif., USA) and bovine serum albumin standard (Pierce Biotechnology) were used as standards.

Production of Mouse Polyclonal Antiserum Raised against Natural BWp16

Natural BWp16 was extracted from seeds according to a previously described method [5]. BALB/c mice were intraperitoneally immunized with 25 μ g of natural BWp16 plus 1 mg of alum 3 times. The serum from the immunized mice was used as a positive control in the Western blotting analysis.

Serum Specimens

Sera from 8 Japanese patients (sera Nos. 1–8), 9 commercially available sera (sera Nos. 9–17; Plasma Lab International, Everett, Wash., USA) and sera from 7 healthy volunteers were used. Informed consent was obtained from all the subjects and volunteers. This study was approved by the Institutional Review Board of the National Institute of Health Sciences. Buckwheat-specific IgE CAP RAST values of all the patient sera are shown in table 1.

SDS-PAGE and Immunoblotting

Buckwheat seed extract was obtained according to a previous report [5]. The buckwheat seed extract (10 μ g protein/lane) or purified recombinant BWp16 (0.5 μ g/lane) was suspended in the SDS sample buffer with standard reducing condition, boiled at 100°C for 5 min, electrophoresed on a 4–20% SDS-polyacrylamide gel (TEFCO, Tokyo, Japan) and transferred to a 0.22- μ m nitrocellu-

Table 1. Data on serum samples

Serum No.	Buckwheat-specific IgE by CAP RAST IU/ml	Other known food allergens
1	4.87	peanuts, wheat, rice, tomato, carrot, garlic, onion, bean, egg white, milk
2	1.13	peanuts, wheat, bean, egg white
3	12.6	egg white, milk
4	52.1	peanuts, wheat, bean, sesame, egg white
5	18	peanuts, wheat, rice, tomato, carrot, spinach, sesame, potatoes, egg white, milk
6	1.61	wheat, rice, sesame, egg white
7	17.5–49.9	egg white
8	3.50–17.4	peanuts, wheat, sesame, ragweed, Japan cedar, egg white, milk
9	12	
10	8.4	
11	28	
12	12.6	peanuts, wheat, garlic, bean, egg white, milk
13	6.38	peanuts, cobnut, Brazilian nuts, almond, tomato, carrot, potatoes, coconuts, wheat, strawberry, onion, apple, rye, barley, rice, corn, green peas
14	5.38	peanuts, cobnut, Brazilian nuts, almond, tomato, carrot, potatoes, coconuts, wheat, strawberry, onion, apple, rye, barley, rice, corn, green peas, orange
15	12.14	peanuts, cobnut, Brazilian nuts, almond, tomato, carrot, potatoes, coconuts, wheat, strawberry, onion, apple, rye, barley, rice, corn, green peas
16	11.7	peanuts, cobnut, Brazilian nuts, almond, tomato, carrot, potatoes, coconuts, wheat, strawberry, onion, apple, rye, barley, rice, corn, green peas, orange, egg white
17	5.57	garlic, onion, corn, sesame

All patients were male and are diagnosed as having multi-food allergy. Patients No. 1–8 were Japanese children aged 1–7 years and No. 9–17 were adults.

GCGGGACCCACAACAACACCTAAAAAACCCAGAAAATGAAGCTCTTCATCATCCTA	60
M K L F I I L	7
GCAACGGCCACTCTCCTGATCGCCGCAACTCAAGCAACGTACCCGAGATGAAGGTTTC	120
A T A T L L I A A T Q A T Y P R D E G F	27
GATTTAGGCGAAACCCAGATGAGCTCAAGTGCATGCGACAAGTGAAGATGAATGAGCCA	180
D L G E T Q M S S K C M R Q V K M N E P	47
CATCTGAAGAAATGTAACCGTTACATAGCCATGGATATACTCGACGACAAATACGCAGAA	240
H L K K C N R Y I A M D I L D D K Y A E	67
GCTTTGAGTAGGTTGAAGGTGAAGGATCAAGAGTGAAGAGTCGTGTATGAGAGGATGC	300
A L S R V E G E G C K S E E S C M R G C	87
TGTGTGGCGATGAAGGAGATGGATGAATGTGTTGTGAGTGGATGAAGATGATGTT	360
C V A M K E M D D E C V C E W M K M M V	107
GAGAATCAGAAAGGCGTATTGGTGAAGGTTGATTAAGGAGGGGTTAGGGATTGAAG	420
E N Q K G R I G E R L I K E G V R D L K	127
GAATTGCCTAGTAGTGTGGCTTAGTGAATTTGAATGTGGATCGAGAGGAAATCGGTAT	480
E L P S K C G L S E L E C G S R G N R Y	147
TTTGTGAATTTGGTGTGTGTGTGTGTGTGAATAAATAGTAGACTTGTGCC	540
F V *	149
TCTGTGGCTATGTATGCTATTGGAGAGGAAGAAGTTTGTCTGTTGAAGTTGTTGTGT	600
TAAGCACTGAAATATATGAGCACTTAACCTTTAATTTAAAAAG-poly(A)	645

Fig. 1. Nucleotide sequence of a cloned 16-kDa buckwheat protein cDNA and the deduced amino acid sequence. The predicted signal peptide sequence is shaded, and the putative polyadenylation signal is doubly underlined. Sequences different from the reference sequence (AY966013) are indicated in white against black background.

lose membrane (S&S, Dassel, Germany). The membranes were blocked, cut into ~4-mm strips, and then each membrane strip was incubated at room temperature for 1 h with each diluted serum from patients with positive IgE binding to buckwheat (1:4) or control serum (1:4) and then further incubated at 4°C for 18 h. Diluted mouse polyclonal anti-BWp16 serum (1:500) was incubated at room temperature for 2 h. After washing with PBS-T (10 mM phosphate-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20), the strips were incubated at room temperature for 1.5 h with horseradish-peroxidase (HRP)-conjugated goat anti-human IgE (Fc) (Nordic Immunology, Stockholm, Sweden; 1:500 dilution) or HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences) and washed again. The bound IgE or IgG antibodies were detected with Konica ImmunoStain HRP-1000 (Konica, Tokyo, Japan) according to the manufacturer's protocol.

Cross Inhibition Test of Recombinant and Natural BWp16 to Patient Serum IgE by Western Blotting

The diluted sera from patient Nos. 3 and 7 and control serum (1:20) were pre-incubated with PBS or 1 µM purified recombinant BWp16 in 0.2% casein in PBS at 4°C for 18 h. Buckwheat seed extract (10 µg protein/lane) was electrophoresed on a 4–20% SDS-polyacrylamide gel (TEFCO) and transferred to a 0.22-µm nitrocellulose membrane (S&S). The membrane was blocked with 0.5% casein in PBS (pH 7.4), cut at about 4 mm, and then each cut membrane strip was incubated at room temperature for 1 h with each of the pre-incubated diluted sera and then further incubated at 4°C for 18 h. After washing with PBS-T, the strips were incubated at

room temperature for 1.5 h with HRP-conjugated goat anti-human IgE (Fc) (Nordic Immunology, 1:500 dilution) and washed again. The bound IgE antibodies were detected as described above.

ELISA

BWp16-specific IgE antibodies were determined using ELISA as follows. Wells (96 wells; Costar 3590; Costar, Corning, N.Y., USA) were treated with purified recombinant BWp16 in binding buffer (50 mM sodium carbonate buffer, pH 9.6) at a concentration of 0.1 µg/50 µl/well. After washing, the wells were blocked with 0.1% casein-PBS (pH 7.0) for 2 h at room temperature and then incubated with serum samples diluted to 1:100 in 0.1% casein-PBS at 4°C overnight. The plate was then sequentially incubated with HRP-conjugated goat anti-human IgE antibodies (1:1,000) and reacted with a colorimetric substrate solution (TMB reagent; BD Biosciences). Reactivity was evaluated by comparing the colorimetric intensities (OD 450–570) with those for the sera from the 7 healthy controls.

Pepsin Digestion Test

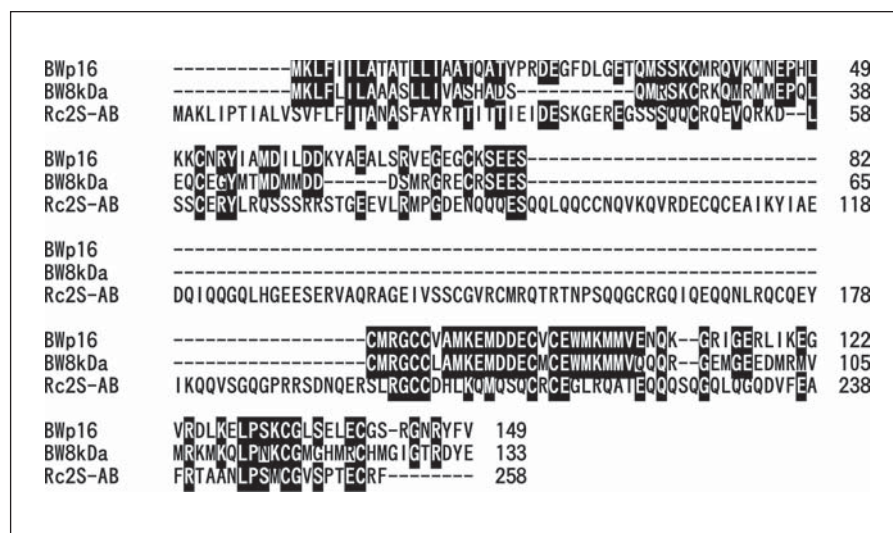
The pepsin digestion tests were performed as described previously [14]. Briefly, pepsin purchased from Sigma (3.8 mg; 13,148 units of activity) was dissolved in 5 ml of gastric control solution (2 mg/ml NaCl, pH 2.0). The resultant fluid (1,520 µl), simulated gastric fluid (SGF), was incubated at 37°C for 2 min before addition of 80 µl of buckwheat seed extract (6.16 mg/ml) or recombinant BWp16 protein solution (5 mg/ml) at time zero. At each scheduled time point (0.5, 2, 5, 10, 20, 30 and 60 min), 200 µl of the reaction mixture were removed and added to a separate sampling tube containing 70 µl of 5 × Laemmli buffer and 70 µl of 200 mM Na₂CO₃. In the zero-point samples, test protein solution (10 µl) was added to neutralize SGF (190 µl of SGF, 70 µl of 5 × Laemmli buffer and 70 µl of 200 mM Na₂CO₃). All neutralized samples were boiled at 100°C for 3 min and subjected to SDS-PAGE.

Results and Discussion

Sequence Analysis of the Full-Length cDNA

To obtain the full-length cDNA of BWp16, we first cloned and sequenced 5'-RACE and 3'-RACE clones. A sequence analysis revealed that an open reading frame (ORF) of the longest 5'-RACE clone contained the ATG codon (nucleotide positions 40–42 in fig. 1) at 66 bp upstream of the start nucleotide of the reference sequence. We used a database (SignalP 3.0 server; <http://www.cbs.dtu.dk/services/SignalP/>) to determine whether the translated N-terminal amino acid sequence of the longest cDNA contained the putative signal peptide sequence. The database program confirmed that the translated N-terminal amino acid sequence of the longest clone ORF did contain a putative signal peptide sequence (fig. 1). However, an in-frame stop codon in the 5'-untranslated region was not found in the ORF of the longest cDNA. The existence of the putative signal peptide sequence

Fig. 2. Alignment of deduced amino acid sequences of BWp16 (Genbank accession No. DQ304682), an 8-kDa buckwheat protein (BW8kDa, Genbank accession No. AB055892) and *R. communis* 2S albumin (Rc2S-AB, Genbank accession No. 1707274A). Gaps were introduced to optimize the alignment. Amino acid residues identical to those of BWp16 are indicated in white against black background.



strongly suggested that the first ATG codon in the longest clone sequence was the putative translation initiation codon.

From the 3'-sequence obtained by 3'-RACE, a TAA stop codon was shown to exist immediately after the 3'-end of the ORF of the reference sequence (nucleotide positions 487–489). In the 3'-untranslated region, a putative polyadenylation signal (AATATA, nucleotide positions 612–617) was located 123 bp downstream of the stop codon (fig. 1).

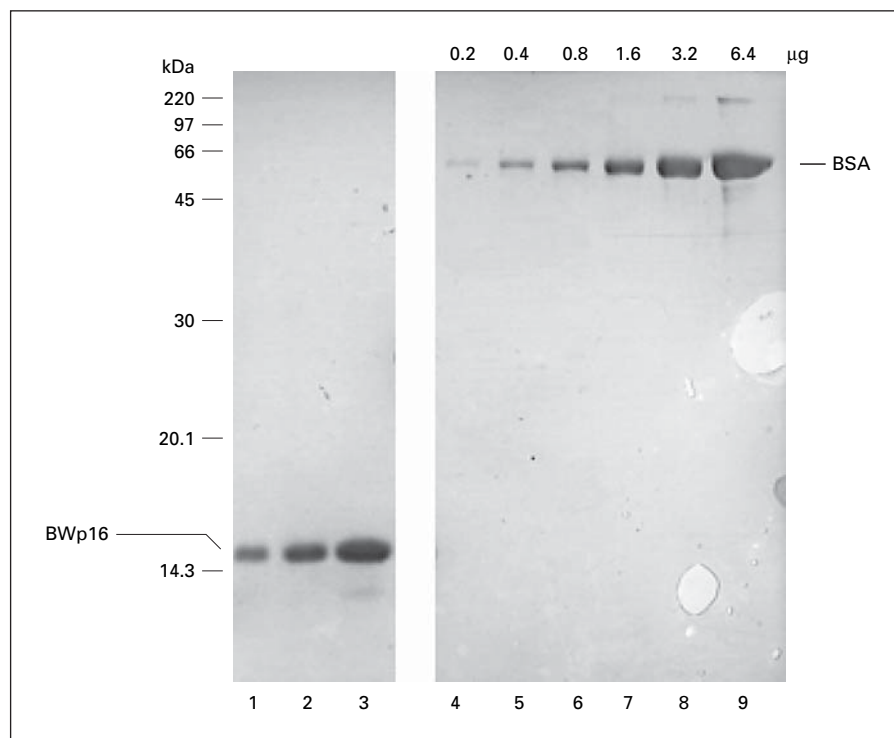
Next, we cloned the full-length cDNA encoding BWp16. Figure 1 shows the nucleotide sequence of the full-length cDNA and the deduced amino acid sequence. We have submitted the nucleotide sequence and predicted amino acid sequence to the Genbank with accession No. DQ304682. The full-length cDNA encodes an ORF of 149 amino acid residues with a calculated molecular mass of 16.9 kDa. Five nucleotide changes were found between the cloned cDNA and the reference sequences. Furthermore, one amino acid substitution (Met-133 in the reference sequence to Leu in the cloned sequence) was observed (fig. 1). A basic local alignment search tool (BLAST) search revealed that the buckwheat 8-kDa allergen (Genbank accession No. AB055892) had the highest degree of identity to BWp16 (54.1% amino acid identity, fig. 2), followed by *Ricinus communis* 2S albumin (Genbank accession No. 1707274A; 28.2% amino acid identity, fig. 2). Matsumoto et al. [10] reported that the 10-kDa buckwheat protein, which is identical to the 8-kDa buckwheat allergen, is a member of the 2S albumin multigene family. 2S albumin family proteins have been

identified as vacuolar-targeting storage proteins in many plants, and their N-terminal amino acid sequences include signal peptides that are absent in the mature proteins [15–18]. Moreover, 2S albumin has been reported to be a major allergen in nuts, sesame seeds and mustards [6, 11]. In the present study, the significant identity of BWp16 to 2S albumin, the existence of a signal peptide homologous to that of the 8-kDa allergen, the high methionine content (8.0%) and the conserved cysteine residues further demonstrated that BWp16 belongs to the 2S albumin family.

Expression and Purification of Recombinant BWp16

The N-terminal sequence analysis of the natural BWp16 was applied by the contractor (APRO Life Science Institute, Tokushima, Japan). Our preliminary data and previous reports showed that the N-terminal amino acid sequence of mature BWp16 protein was RDEGFDLGETQM (data not shown) [5, 7, 13]. Thus, we induced the expression of GST-mature BWp16 (amino acid residues 23–149 in fig. 1) fusion protein and excised the GST portion. The N-terminal sequence analysis of the purified recombinant BWp16 was applied by the contractor (APRO Life Science Institute). The result provides the sequence GPLGSRDEGF, in which the former five amino acids are derived from pGEX-6P-1 and the latter five amino acids are derived from mature BWp16. The fusion part cleaved by PreScission protease provides the residual defined five-amino-acid sequence prior to the N-terminal part of the cloned sequence. Thus, the N-terminal sequence analysis demonstrated that the purified recom-

Fig. 3. CBB staining of purified recombinant BWp16. Purified recombinant BWp16 was subjected to 18% SDS-PAGE and the resulting gel was stained with CBB. Lanes 1, 2 and 3 = 1, 2 and 4 μ l of the purified recombinant BWp16 solution, respectively. The bovine serum albumin standard is shown in lanes 4–9 (0.2–6.4 μ g). The molecular weight marker positions are indicated on the left.



binant BWp16 was almost equivalent to the natural one. Approximately 1 mg of a soluble form of purified BWp16 per 200 ml of culture was isolated. Figure 3 shows CBB-stained SDS-PAGE gels containing purified recombinant BWp16 and known standards (bovine serum albumin). This purified BWp16 was then used in Western blotting and ELISA analyses.

Western Blotting of Buckwheat Extract Using Sera from Patients with Positive IgE Binding to Buckwheat

To test whether sera from patients with positive IgE binding to buckwheat react with allergens in buckwheat extract, we performed a Western blotting analysis of buckwheat seed extract using sera from patients with positive IgE binding to buckwheat and control. The serum samples from all 17 patients reacted with a number of proteins in the extract (fig. 4). The patient IgE reacted with bands corresponding to 20 kDa (9/17 patients, 53%), 25 kDa (15/17 patients, 88%), 37 kDa (4/17 patients, 24%), 50 kDa (9/17 patients, 53%), 75–100 kDa (10/17 patients, 59%) and 150–250 kDa (6/17 patients, 35%; fig. 4). Such proteins have been reported to bind patient IgE [5, 19]. Thirteen (Nos. 1–7, 9, 10, 13–15 and 17) of the 17 sera (76%) from our patients had IgE binding to a band of about 15 kDa compared with control sera (fig. 4). Fur-

thermore, 4 (Nos. 3, 6, 7 and 13) of the 17 serum samples produced strong bands at around 15 kDa, compared to the other serum samples (fig. 4). These 4 serum samples were used in Western blots for purified recombinant BWp16.

Specific Binding of Sera from Patients with Positive IgE Binding to Buckwheat to Recombinant BWp16

To clarify whether the sera from patients with positive IgE binding to buckwheat could react with the purified form of BWp16, we performed a Western blotting analysis of purified recombinant BWp16 using the above 4 serum samples and 1 control serum sample. All 4 serum samples, but not the control serum, reacted with the recombinant BWp16 (fig. 5). Polyclonal mouse antiserum also reacted with the recombinant BWp16 (lane P in fig. 5).

Cross Inhibition Test of Natural and Recombinant BWp16

To confirm the immunological equivalence of the recombinant BWp16 to the natural one, we performed Western blotting of buckwheat extract using recombinant BWp16 as an inhibitor. The blotting data showed that only the 16-kDa band was diminished by pre-incubating

Fig. 4. Western blotting of buckwheat extract using sera from patients with positive IgE binding to buckwheat. Buckwheat extract (10 μ g/lane) was subjected to SDS-PAGE and immunoblotted using sera from patients with positive IgE binding to buckwheat (lanes 1–17) and a control serum (lane C). The molecular weight marker positions are indicated on the left.

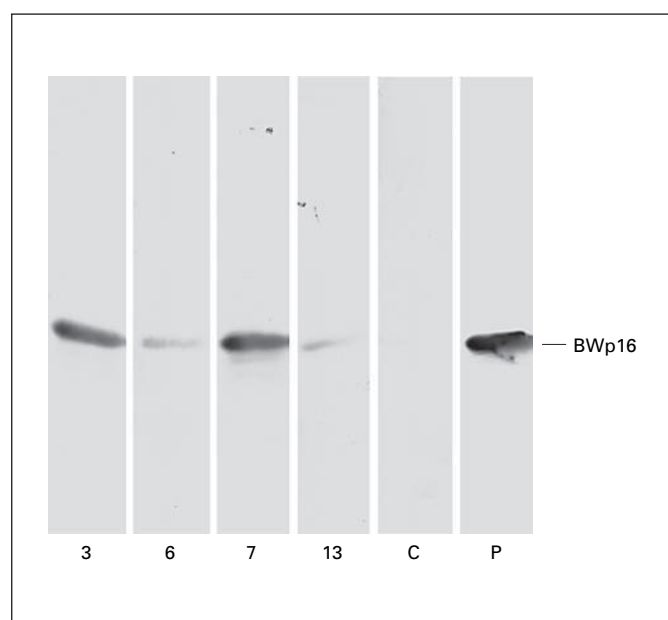
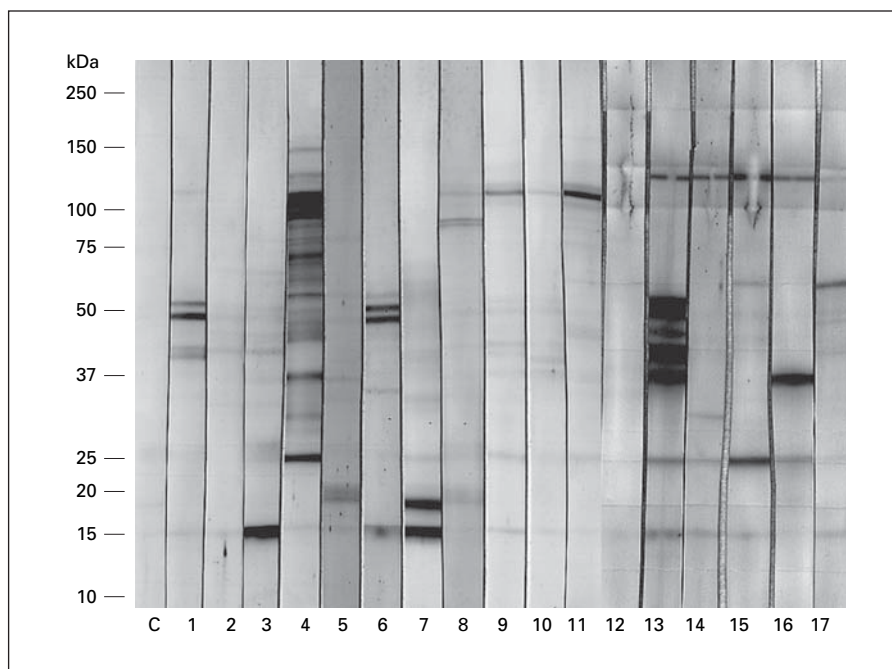


Fig. 5. Western blotting of purified recombinant BWp16 using sera from patients with positive IgE binding to buckwheat. Purified recombinant BWp16 (0.5 μ g/lane) was subjected to SDS-PAGE followed by immunoblotting using sera from patients with positive IgE binding to buckwheat. Lanes 1–4 = Serum Nos. 3, 6, 7 and 13, respectively; lane C = control serum. Mouse anti-BWp16 polyclonal antiserum was used as a positive control (lane P).

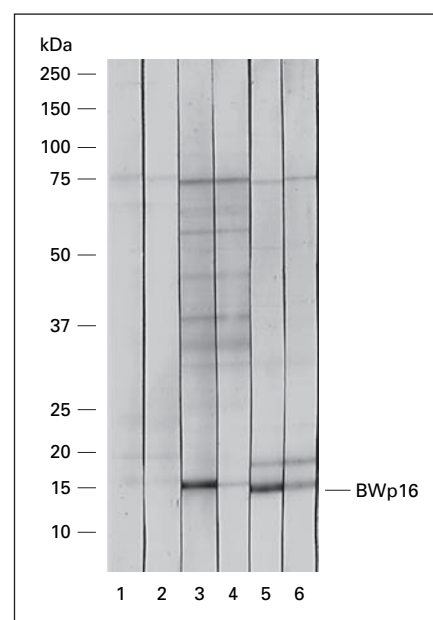


Fig. 6. Cross inhibition test of recombinant BWp16. The diluted sera from patients Nos. 3 and 7 or control serum was pre-incubated with PBS or 1 μ M purified recombinant BWp16. Buckwheat seed extract (10 μ g protein/lane) was subjected to SDS-PAGE followed by immunoblotting using the pre-incubated serum with recombinant BWp16. Lanes 1 and 2 = Control serum; lanes 3 and 4 = serum No. 3; lanes 5 and 6 = serum No. 7; lanes 1, 3 and 5 = serum pre-incubated with PBS; lanes 2, 4 and 6 = serum pre-incubated with 1 μ M purified recombinant BWp16. The molecular weight marker positions are indicated on the left.

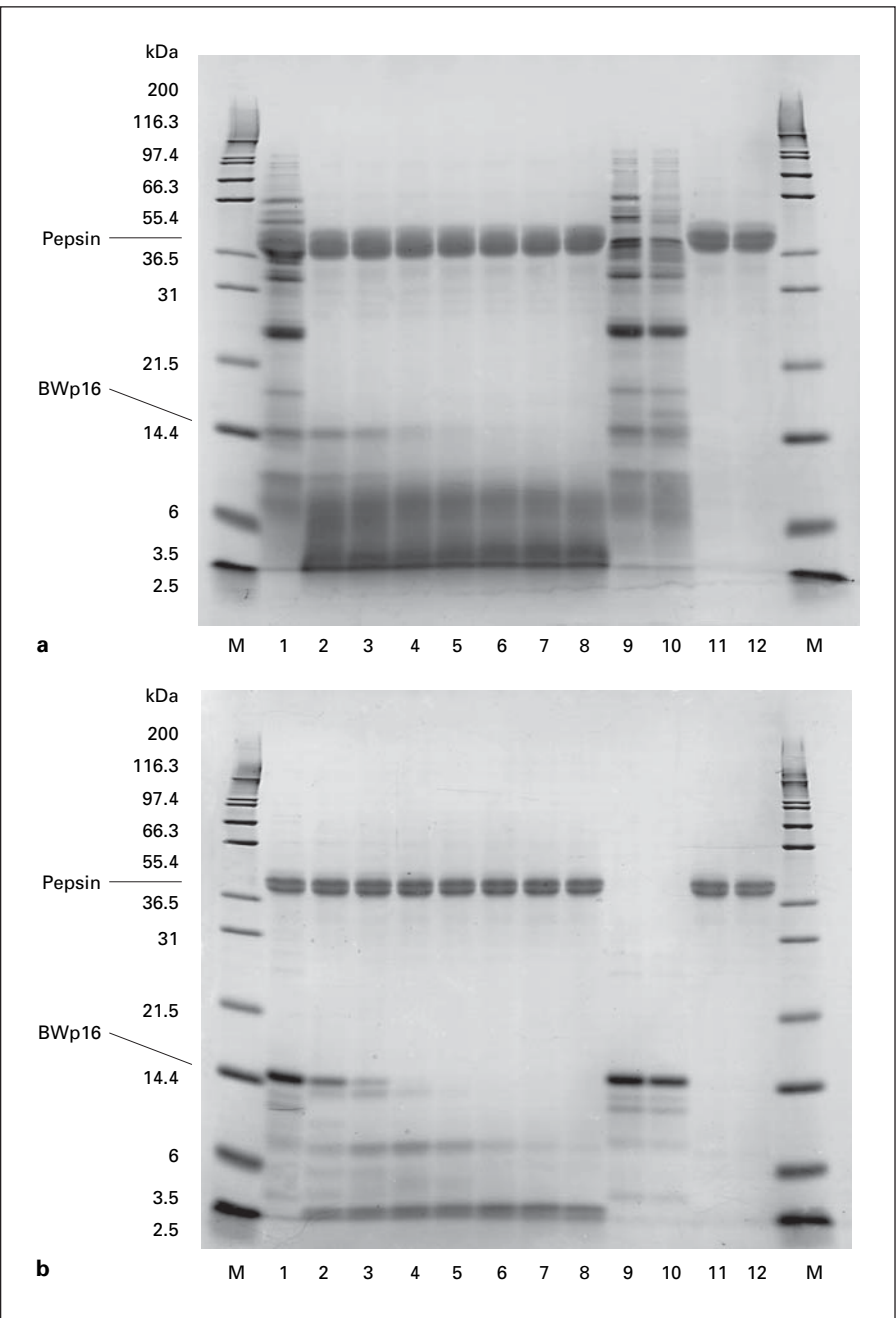
Table 2. ELISA of 4 patient serum samples

Serum No.	Colorimetric intensity (OD 450–570)
3	0.323 ¹
6	0.252 ¹
7	0.469 ¹
13	0.269 ¹

Average intensity of 7 control serum samples was 0.094, and the SD was 0.044. Average + 2 SD = 0.181.

¹ ELISA positive.

Fig. 7. Pepsin digestion of natural and recombinant BWp16. SDS-PAGE analysis of the SGF digestion of natural (**a**) and recombinant (**b**) BWp16. Lane M = Molecular weight markers; lanes 1–8 = SGF digestion pattern of BWp16 at t = 0, 0.5, 2, 5, 10, 20, 30 and 60 min, respectively; lanes 9 and 10 = BWp16 controls at t = 0 and 60 min, respectively; lanes 11 and 12 = SGF controls at t = 0 and 60 min, respectively.



patient serum with 1 μ M recombinant BWp16 (lanes 4 and 6), thus confirming the existence of the immunological equivalence of recombinant BWp16 to the natural one (fig. 6).

ELISA

To further clarify whether the sera from patients with positive IgE binding to buckwheat could react with the

recombinant BWp16 under non-denaturing conditions, we conducted an ELISA using the recombinant BWp16 and the 4 strongly positive serum samples or control serum samples. Seven control serum samples had an average colorimetric intensity of 0.094 ± 0.044 (table 2). All 4 serum samples were significantly reactive with the recombinant BWp16 (table 2). The other serum samples were not significantly reactive (their absorbance was

0.064–0.152) with recombinant BWp16 (data not shown).

Previous reports have revealed that natural BWp16 extracted from buckwheat seeds can be bound specifically to buckwheat-allergic patient IgE [5, 7, 13]. The present data indicate that recombinant BWp16, like natural BWp16, can be used to detect buckwheat-allergic patient IgE. Therefore, purified recombinant BWp16 could be used as a diagnostic standard for buckwheat allergies.

Pepsin Digestion

A previous report has shown that natural BWp16 was identified as a pepsin-resistant protein [5]. To examine whether the recombinant BWp16 has pepsin resistance, we performed pepsin digestion of natural and recombinant BWp16. A gradual decrease in the natural BWp16 band was noted after 0–10 min of pepsin digestion (fig. 7a). On the other hand, other protein bands decreased after 0.5 min of pepsin digestion, indicating BWp16 was resistant to pepsin digestion compared with other buck-

wheat proteins. Alternatively, the recombinant BWp16 band gradually decreased after 0–10 min of pepsin digestion similar to the natural one (fig. 7b). For recombinant BWp16, a 6- to 7-kDa band was clearly noticeable after 0.5–20 min of pepsin digestion, peaking at 5 min, and 2.5- to 3.5-kDa bands appeared after 0.5–60 min of pepsin digestion (fig. 7b). The data demonstrated that the natural and recombinant BWp16 were comparably resistant to pepsin digestion.

In conclusion, we molecularly cloned the full-length cDNA encoding BWp16. Purified recombinant BWp16 can react with sera from patients with positive IgE binding to buckwheat. The current data indicate that purified recombinant BWp16 could be used as a diagnostic standard for detecting buckwheat allergies.

Acknowledgments

This study was supported by a grant from the Ministry of Health, Labor and Welfare and by the Cooperative System for Supporting Priority Research of the Japan Science and Technology Agency.

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