Characterization of buckwheat 19 kD allergen
and its application for diagnosing clinical reactivity

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Background: The 19 kD protein of buckwheat (BW) has been suggested to be a major allergen, but its characteristics and clinical significance are poorly defined.

Methods: cDNA of 19 kD BW allergen was cloned and expressed in E. coli. Allergenicity and cross-allergenicity were confirmed by inhibition immunoblotting or by ELISA inhibition. The recombinant protein (r19 kD) was assessed for clinical utility in the diagnosis of BW reactivity in 18 BW allergic and 19 BW asymptomatic sensitized subjects using ROC analysis.

Results: The 19 kD BW allergen, which is composed of 135 amino acids, has weak homology to the vicilin like allergens of cashew (Ana o 1), English walnut (Jug r 2) and 7 S globulin from Sesamum indicum. r19 kD can inhibit sIgE binding to native 19 kD BW allergen. The maximum % inhibition of sIgE binding to crude BW extract was 56%. About 83.3% of BW allergy patients had sIgE bound to r19 kD, compared to only 1 of the 19 BW asymptomatic sensitized subjects. The areas under the ROC curves for the skin prick tests [0.925 (95% CI: 0.839~1.012), p<0.001] as well as r19 kD sIgE ELISAs [0.860 (0.725~0.995), p <0.001] were higher than that of BW sIgE CAP test results [0.803 (0.661~0.945), p=0.002].

Conclusions: The 19 kD BW allergen may be the major allergen from BW. For the diagnosis of clinical reactivity to BW, the r19 kD sIgE ELISA test was more discriminative than the CAP sIgE measurement using whole BW extract.

Key Words: Buckwheat allergen, major allergen, 19 kD allergen, clinical reactivity

Introduction

Buckwheat (BW; Fagopyrum esculentum) belongs to the Polygonaceae group of weeds, and its fruit can be used as food. In recent years, BW has become more popular in many countries as a health food1, 2. Many cases of BW allergic subjects have been reported in Korea, Japan, the USA, and other western countries3-8. The 24 kD, 19 kD, 16 kD, 10 kD, and 9 kD proteins of BW are recognized as major allergens. The 24 kD BW protein was identified as the β subunit of 11 S globulin9-10, the 9 kD BW allergen as vicilin11-13, and the 10 kD BW protein as 2 S albumin14. Specific IgE antibodies (sIgE) to the 24 kD and 9 kD allergens can be found in both BW allergic and asymptomatic subjects, making these allergens poor candidate biomarkers for diagnosis of BW reactivity13, 15. We
Table 1. Clinical features patients allergic to buckwheat

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<th>Provocation</th>
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<th>SPT Wheal (mm)</th>
<th>r19 kD sIgE (AU/ml)</th>
<th>Other Food</th>
<th>Urti.</th>
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D=positive result to DBPCFC, Urti=urticaria, Resp.=respiratory, G-I=gastrointestinal, AU=arbitral unit

have reported that the 19 kD protein is one of the major allergens and have determined the N-terminal amino acid sequences of this protein. Interestingly, sIgE antibodies to the 19 kD allergens are found exclusively in patients who complain of allergic symptoms after ingestion of BW, but not in asymptomatic subjects with BW sensitization. These findings suggest that measurement of sIgE for the 19 kD allergen may be particularly useful for the diagnosis of BW clinical reactivity.

In our study, we first deduced the full amino acid sequence of the 19 kD allergen from its cDNA sequence, evaluated the allergenicity of a recombinant 19 kD (r19 kD) allergen, and then applied this information to diagnose BW reactivity in 18 BW allergic and 19 BW asymptomatic sensitized subjects.

Material and Methods

Subject

Eighteen BW allergy patients and 19 BW asymptomatic sensitized subjects were enrolled in our study, which had been approved by Yonsei University’s medical ethics committee (Number: 4-2006-0204). Four allergy patients were diagnosed with BW allergies by double-blind placebo-controlled food challenge (DBPCFC), and the other 14 patients complained of allergy symptoms such as urticaria, angioedema, dyspnea, and abdominal pain, immediately after eating BW noodles or jellies. The 14 patients did not undergo DBPCFC because their symptoms were too serious to be justified for the test. Clinical features of the BW allergy patients are summarized in Table 1. BW
asymptomatic sensitized subjects did not complain of any symptoms after ingestion of BW even though they had a positive skin prick test response (Bencard Co., England). The mean wheal diameter for all subjects was larger than 4 mm. Two of the asymptomatic sensitized subjects were remitted from BW allergy. Patient serum was kindly donated with permission from each subject. These samples were frozen at -70°C until further use.

**cDNA cloning**

100 mg of immature BW seeds were ground to a powder after being frozen in liquid nitrogen. Total RNA was isolated using RNeasy plant mini kit (Qiagen, Maryland, USA) according to the manufacturer’s instructions. To obtain the complete 3’ end of the sequence, we used rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR), and cDNA was generated from total RNA of BW following the manufacturer’s recommendation. Degenerated sense primer was synthesized based on the N-terminal amino acid sequence of the 19 kD BW protein, 5’-GGN GAY TAY CCN YTN GA-3’, and oligo (dT) conjugated adaptor (5’ CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTT 3’) was used for anti-sense primer. RACE-PCR was carried out in a 20 l reaction mixture containing 2 l of cDNA, 50 pmol of the degenerated sense primer, 2 pmol of the adaptor conjugated anti-sense primer, 2.5 mM of each dNTP, PCR buffer, and 1 unit of Intrn i-Pfu DNA polymerase (Intron biotechnology, Sungnam, Kyungkido, Korea). Reaction condition was 94°C for 5 min, followed by 35 cycles of 94°C for 20 sec, 44°C for 15 sec, and 72°C for 2 min. The amplified products were cloned with a Zero blunt TOPO PCR cloning kit (Invitrogen, Carisbad, CA, USA) and nucleotide sequences were determined with BigDye Terminator Cyclic Sequencing reaction system (ABI 3100 Genetic Analyzer; Applied Biosystems, Forster City, CA, USA). The clone representing the correct nucleotide sequence for the 19 kD BW protein was selected and used as a template for expression cloning.

**Expression of cDNA clones**

We performed PCR using the following primers: forward primer: 5’-CAA CCA TAT GGG GGA TTA TCC GTT TGA ACC-3’, and reverse primer: 5’-CGC AAG CTG TTC TCC GCA AAA AAG CGC GCA AA-3’. The forward and reverse primer contained the NdeI and Hind III restriction sequences, respectively, for subcloning into pET-21a. PCR was carried out in 50 l of PCR buffer containing 2.5 mM of each dNTP, 50 pmol of the forward primer, 10 pmol of the reverse primer, 1.25 units of i-Pfu DNA polymerase, and 1 l of the cDNA for 19 kD BW protein selected by 3’ RACE-PCR. Five annealing pre-cycles at 40°C were followed by 30 cycles of amplification (94°C for 20 sec, 44°C for 15 sec, 72°C for 2 min).

We used the pET-21a vector (Novagen, Madison, WI, USA) for protein expression. The amplified cDNA was digested with the NdeI and Hind III (TAKARA Korea biomedical Inc, Keumcheon, Kungkido, Korea), then ligated into pET-21a vector which was digested with the same restriction enzymes. The construct harboring cDNA insert with correct pET-orientation was selected by nucleotide sequencing. The pET-construct was transformed into BL21 cells, then the r19 kD BW protein was expressed by induction with 1 mM IPTG (Sigma, St Louis, MO, USA) at 30°C for 4 hours.

**Purification of r19kD BW allergen**

The induced and control flasks were placed on ice for 5 min before the cells were harvested by centrifugation at 5000xg for 5 min at 4°C. The pellets were thawed in ice for 15 min and then were resuspended in lysis buffer (100 mM NaH2PO4; 10 mM Tris-Cl; 8 M urea, pH 8.0) under denaturing conditions at 5 ml per gram wet weight. For complete lysis, cells were stirred for 30-60 min at room temperature and centrifuged at 10,000xg for 20-30 min in order to pellet cellular debris.
Based on the Ni-NTA affinity purification kit (Qiagen, Maryland, USA), 1 ml of the 50% Ni-NTA slurry was added to 4 ml 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 collected for SDS–PAGE analysis. After washing twice with wash buffer (five-fold resin volume; 100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 6.3), the recombinant protein was eluted by the subsequent use of two elution buffers: pH 5.9 and pH 4.5. The eluates were dialyzed with PBS before use. The protein concentrations of the recombinant allergen or the BW crude extract were assayed with a protein assay kit (Bio-Rad, Hercules, CA, USA).

SDS-PAGE and IgE/ inhibition IgE immunoblotting

Antigens were dissolved in reducing sample buffer (12.5 mM Tris–HCl; 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, CA, USA) at 50 V for 30 minutes followed by 180 V for 2 hours. Proteins were transferred onto nitrocellulose membranes (pore size 0.45 μm: Amersham, Buckinghamshire, UK) on a transfer unit (Hoeffer) in buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 350 mA for 2 hours. The membranes were blocked by 5% nonfat dried milk in TBS–T (50 mM Tris, 0.1% Tween, pH 7.5), incubated overnight at room temperature with 1:10 diluted atopic sera, and then washed with TBS–T. To inhibit immunoblotting, the atopic pooled sera were preincubated with appropriate concentration of r19 kD BW allergen at room temperature for 2 hours. This process bound up any reactive antibody. Membranes were incubated with 1:1000 diluted alkaline phosphatase conjugated goat anti–human IgE (Sigma) for 60 minutes. After washing, the colorimetric reaction was developed with the BCIP/NBT system (Promega, Madison, WI, USA).

ELISA specific IgE measurement and inhibition ELISAs

r19 kD sIgE was assayed by ELISA. A 96 well polystyrene plate was coated with 50 l of 10 g/ml of allergen in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. After washing with PBS–T (137 mM NaCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 27 mM KCl, 0.1% Tween–20, pH 7.4), each well was blocked with 1% bovine serum albumin in PBS–T. sIgEs were detected by incubating the plate with 50 l of BW atopic sera for 1 hour at room temperature. After washing, 1:1000 biotin labeled goat anti–human IgE (Vector, Burlingame, CA, USA) was added to each well and incubated with 1:1000 streptavidin–peroxidase (Sigma) for 30 minutes before another washing step. The colorimetric reaction was developed with an ABTS solution (25 mg of 2,2-azino-bis-3-ethybenzthiazoline-sulfonic acid in 50 mM citrate phosphate buffer, 50 l of 30% H₂O₂). The reaction was stopped by adding 100 l of 2 mM NaN₃ and afterwards we determined the optical density (O.D.) reading at the 405 nm UV wavelength by an automated microplate spectrophotometer (Dynatec, Alexandria, CA, USA). The r19 kD sIgE levels were expressed in arbitrary units (AU). The pooled sera (n=5) with high BW sIgEs were assumed to have 1000 arbitral units (AU)/mL. The threshold level for the presence of r19 kD sIgEs was 5.5 AU/mL, which was determined by the mean ±2 standard deviations of 15 non–atopic control subjects.

For ELISA inhibition tests, atopic sera were preincubated for 2 hours with each allergen concentration at room temperature before being measured in the ELISAs described above.

Statistical analysis

Statistical analysis was performed using SPSS 9.0 (SPSS Inc. Chicago, Illinois, USA). We analyzed the differences between groups with regards to mean wheal diameter as well as sIgE levels as determined by ELISA or CAP tests. Any group differences were analyzed with the Wilcoxon signed–rank test. The
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Results

Full amino acid sequence of 19 kD BW allergen and its homology to other food allergens

Based on the reported N-terminal sequence of the 19 kD BW allergen, we cloned the 19 kD BW allergen cDNA and deduced its full amino acid sequence (Genbank access No. EF532322). The 19 kD BW allergen consisted of 135 amino acids and had an estimated molecular weight of 16.05 kD (Fig. 1). Homology tests to other food allergens were done through the National Center for Biological Information program. The 19 kD BW allergen has a 35% homology to vicilin like allergens of English walnut (Jug r 2), cashew (Ana o 1), and 7 S globulin in Sesamum indicum.

Allergenicity of r19 kD BW allergen

The 19 kD BW allergen was expressed in E coli and its allergenicity evaluated with pooled sera from patients with allergies to BW (n=5, patient numbers 3, 10, 11, 15, and 17 in Table 1). The r19 kD allergen inhibited the binding of lgEs to the native 19 kD BW
Figure 3. ELISA inhibition of sIgE binding to BW crude extract (Fig. A) or the ELISA inhibition of sIgE binding to r19 kD BW protein (Fig. B) with pooled atopic sera. ELISA inhibitions of sIgE to BW crude extract were done with individual BW allergy sera (Fig. C patient 4; Fig. D patient 11; Fig. E patient 15; Fig. F patient 18 in Table 1). Closed and open circles represent r16 kD and BW crude extract as the inhibitor, respectively.

Figure 4. Mean wheal sizes from a skin prick test (A), levels of sIgE in BW crude extract as determined by CAP (B), and anti-r19 kD allergen sIgEs in BW allergic patients (n=18) and BW asymptomatic sensitized subjects (n=19). Closed squares represent the subjects who were remitted from BW allergies.

protein when IgE was used for immunoblotting (Fig. 2). The BW crude extract and r19 kD BW protein completely and dose-dependently inhibited sIgE binding to r19 kD in the ELISA inhibition assay. The r19 kD BW protein also inhibited sIgE binding to BW crude extract in a dose-dependent manner. For
Figure 5. ROC curves generated from skin prick tests (Fig. A), CAP IgEs in BW crude extracts (Fig. B) and anti- r19 kD IgEs ELISA (Fig. C).

example, 10 g/ml of r19 kD inhibited sIgE binding to BW crude extract by 56% (Fig. 3A). Individual variations for inhibition of crude BW extract sIgE with r19 kD were found (Fig. 3C-F).

Clinical application of SPT, CAP sIgE from whole extract, and sIgE from r19 kD allergen for BW reactivity diagnosis

BW allergy patients had stronger skin reactivity in prick tests (SPT) (p<0.001), higher CAP sIgEs in BW crude extract (P<0.001), and anti-r19 kD allergen sIgEs (p<0.001) compared to patients that were asymptomatic to the BW sensitizer (Fig. 4). The optimal cut-off values for the skin prick test, CAP sIgEs for the BW crude extract, and r19 kD sIgEs measured by ELISA in Figure 4 were determined by ROC curve analysis. These cut-off values were: wheal size of skin prick test 11.1 mm, BW CAP sIgE-3.4 kU/L, and r19 kD sIgEs by ELISA-5.5 AU/ml. For the diagnosis of BW allergic subjects, the presence of anti-r19 kD allergen sIgEs that were measured by ELISA were more discriminative than the CAP sIgEs measured by crude BW extract. 13 out of 18 (72.2%) BW allergy patients and 5 BW asymptomatic sensitized subjects were higher than the cut-off value of CAP sIgEs (Fig. 4B), while 83.3% of BW allergy patients and just one asymptomatic subject had sIgEs against r19kD allergen (Fig. 4C). The areas under the ROC curve for the skin prick test [0.925 (95% confidence interval: 0.839–1.012), p<0.001] and anti-r19 kD sIgE ELISAs to [0.860 (95% confidence interval: 0.725–0.965), p<0.001] were higher than that of a BW sIgE CAP test [0.803 (0.661–0.945), p=0.002] (Fig. 5). The levels of r19 kD sIgE were correlated with CAP sIgEs in BW SPT positive subjects (n=37, r=0.682, p<0.001) and BW allergic subjects (n=18, r=0.634, p=0.005), but not in BW asymptomatic sensitized subjects (n=19, r=0.420, p=0.863) (Fig. 5).

Discussion

Several BW proteins have been implicated as major allergens, however but no consensus has been reached regarding which proteins are the major allergens. Recently, the 19 kD protein has been identified as a major BW allergen candidate\(^{13, 15}\). Studies have shown that sIgE affinity to whole BW extract has different patterns in BW allergy patients compared to asymptomatic sensitized subjects. sIgEs to the 19 kD BW allergens were found specifically in symptomatic subjects, while the sIgEs to the 11 S globulin protein (Mw 22–24 kD) was found non-specifically in BW allergic patients and asymptomatic BW sensitized subjects. In our study, we are the first to identify the full amino acid sequence of the 19 kD BW allergen. We also confirmed allergenicity by immunoblotting and ELISA inhibition. As 83% of BW allergy patients...
Figure 6. Relationship between CAP sIgEs in whole BW extract and sIgEs against the r19 kD BW allergen. Closed circles represent BW allergy patients and open circles represent BW asymptomatic sensitized subjects.

have sIgEs that bind to the 19 kD BW protein and also had significant allergenicity as measured by ELISA inhibition, we consider the 19 kD allergen to be the major allergen produced by *Fagopyrum esculentum*.

Our results revealed weak amino acid sequence homology between the BW 19 kD allergen and the 7 S globulin from sesame, the vicilin like major allergens of cashew, and English walnut. These homology results suggest that the 19 kD BW protein may belong to the vicilin storage protein family. However, cross allergenicity is unlikely because the homologies are weak and we did not find sesame, cashew or walnut allergies in BW allergic patients. In this study we could not found homology between 19 kD allergen and other known BW allergens. However the inhibition immunoblotting with r19 kD showed that 13 kD BW allergen may share common IgE epitopes, but not the 9 kD allergen, which has been reported to be vicilin family protein. Shared IgE epitopes are not unique to 19 kD allergen. Marked homology and shared IgE epitopes between 16 kD BW and 10 kD BW allergens, which belong to 2 S storage protein, were also found.

For food allergies, SPT and sIgE measurements using whole extract are well known to have poor positive predictive values. The DBPCFC is the gold standard for diagnosing food allergies. Because DBPCFC is difficult and dangerous, the application of discriminating sIgE levels by using the CAP test or wheal size in SPT has been successfully evaluated for peanut, egg, and milk allergies. We found that all BW allergy patients had strong SPT responses, and the optimal differentiating mean wheal size between BW allergies and asymptomatic sensitizer was 11.3 mm. A large wheal size (>8 mm) in SPTs was also predictive of clinical reactivity in milk, egg, and peanut allergies with 95% accuracy. We also found that r19 kD sIgEs were more discriminative for diagnosis of BW clinical reactivity than the CAP sIgE measurement. The measurement of r19 kD sIgEs is especially useful for BW allergy patients with low CAP IgE levels in BW crude extract. The concentration of CAP IgEs for an 85% positive predictive level in our study group was 4.5 kU/L. Therefore, 6 out of 18 BW allergy patients were below the diagnostic decision level. Among these patients, 4 BW allergy patients had r19 kD sIgEs.

We do not know why the 19 kD allergen has more specific clinical reactivity to BW than the 24 kD 11 S globulin or 9 kD trypsin inhibitor BW allergens. Many food allergens have both conformational and sequential epitopes. Individuals who possess sIgEs to conformational epitopes may tolerate foods denatured by cooking or digestive enzymes, while allergies to sequential epitopes may react to the food in any form. Subjects with allergies to conformational epitopes may tend to develop clinical tolerance. Little information is available about the 19 kD allergen epitopes. Previously, we showed that the 19 kD allergen does not have carbohydrate epitopes by periodate oxidation of carbohydrates. Furthermore, the r19 kD BW protein in our study was expressed in *E.coli*, which produces non-glycosylated proteins. Thus, we can exclude the presence of anti-carbohydrate sIgEs to the 19 kD BW protein. sIgEs to carbohydrate epitopes are usually
cross-reactive in many plant-derived molecules. Frequently these epitopes do not have any clinical reactivity. The 19 kD allergen has been reported to be easily digested by pepsin. However, the allergenicity of the digested fragments of 19 kD BW has not been evaluated. Some investigators have suggested that in vivo or in vitro tests using pepsin-digested food extracts may be a useful tool to identify the food allergy patient against potentially dangerous stable allergens. The digestion of protein with pepsin might destruct epitopes of the allergen, but it also can convert the protein from non-sensitizing to sensitizing forms. Moreover, the fragments from the digested protein may maintain their allergenicity. Further studies are required to determine the IgE epitope structures of the 19 kD BW allergen.

In conclusion, we demonstrated that the 19 kD BW allergen is a major allergen of BW. The recombinant BW 19 kD might be particularly useful in the diagnosis of the clinical reactivity to BW.

REFERENCES
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