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

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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.

Ann Allergy Asthma Immunol. 2007;99:254–260.

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 allergens[8]; 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

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This study was supported by a grant from the Protein Chip Technology Program, Most (F104AB010007-06A0201-00711), Korea.
Authors have nothing to disclose.
Received for publication November 3, 2006.
Received in revised form April 23, 2007.
Accepted for publication April 30, 2007.

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′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTT-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, Sungnham, 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 *Nde*I and *Xho*I. 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 *Nde*I and *Xho*I, 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 5,000*g* 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 10,000*g* 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,