

Pepsin-Resistant 16-kD Buckwheat Protein Is Associated with Immediate Hypersensitivity Reaction in Patients with Buckwheat Allergy

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Key Words

Buckwheat · IgE-mediated allergy · ELISA · Immunoblotting · Food allergy

Abstract

Background: Buckwheat is becoming popular in many countries as a health food and the incidence of buckwheat allergy is increasing in Asia. The ingestion of small amounts sometimes provokes an anaphylactic reaction. However, it remains controversial which is the major allergen responsible for such reactions. **Methods:** The patients whose sera are positive for buckwheat-specific IgE antibody measured by the CAP system fluorescein-enzyme immunoassay (CAP-FEIA) were classified into two subgroups depending on the history of immediate hypersensitivity reactions (IHR). Major buckwheat allergens were identified with immunoblotting, ELISA and N-terminal amino acid sequencing. Various treatments such as pepsin digestion were added to characterize the proteins. **Results:** We found that the 24-kD protein that had previously been reported to be a major allergen reacted to IgE antibodies present in sera from almost all subjects (19/20) regardless of symp-

toms. On the other hand, 16- and 19-kD proteins were bound with IgE antibodies present in sera from 9 of the 10 patients with IHR including 8 patients with anaphylaxis but not in sera from buckwheat-specific IgE-positive subjects without IHR. After pepsin treatment, the 16-kD protein but not the 19- and 24-kD proteins remained undigested and preserved the capacity of IgE binding. This pepsin-resistant 16-kD protein had no homology with the 24-kD protein by the N-terminal amino acid sequencing. **Conclusions:** The 16-kD buckwheat protein was resistant to pepsin digestion and appeared to be responsible for IHR including anaphylaxis, while the pepsin-sensitive 24-kD protein was responsible for CAP-FEIA but not IHR.

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Introduction

Buckwheat (*Fagopyrum esculentum*) belongs to the Polygonaceae family and has no taxonomical relation to the Gramineae family that includes rice and wheat. The use of buckwheat flour is popular in soba noodles, dumplings and cookies especially in Asia. Ingestion of buck-

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wheat is known to cause immediate-type anaphylactic reactions through a specific IgE antibody [1–3]. Patients rarely grow out of this buckwheat-related reaction unlike IgE-mediated reactions caused by other kinds of food such as cow's milk [4, 5]. The incidence of buckwheat allergy in Japan is increasing and it was the fourth frequent food allergen according to the Japanese Ministry of Health and Welfare survey in 1999 [6].

Because of the risk of severe and dangerous reactions, skin tests and provocation tests should be limited. Thus, assays for serum buckwheat-specific IgE antibody are commonly used as a diagnostic tool. These *in vitro* assays such as CAP system fluorescein-enzyme immunoassay (CAP-FEIA) often show false-positive results especially for food allergens [7]. Among food allergens detected to be positive by CAP-FEIA, soybeans and wheat are often without symptoms, whereas digestive fluid-resistant allergens such as peanuts and egg often cause food hypersensitivity [7]. The majority of wheat and soybean allergens reacting with IgE antibody in *in vitro* testing may be easily digested *in vivo*, although a few allergens contained in wheat flour sometimes cause anaphylactic reactions. Thus, in general, food allergens are considered to be stable by various digestive treatments [8]. Indeed, the lipid transfer protein responsible for some fruit-induced anaphylaxis is highly resistant to pepsin digestion [9]. Regarding buckwheat, there are certain patients who have high buckwheat-specific IgE antibody titers but have no hypersensitivity reactions, while some exert immediate anaphylactic reactions [6].

In this study, we characterized buckwheat proteins using sera from subjects who have positive buckwheat-specific IgE antibody with or without immediate hypersensitivity reactions (IHR) to buckwheat. We found that the pepsin-resistant 16-kD protein appears to be responsible for IHR caused by buckwheat, while the pepsin-sensitive 24-kD protein is responsible for CAP-FEIA but not IHR.

Materials and Methods

Subjects

Twenty subjects, 14 males and 6 females ranging in age from 2 to 44 years old, median age 14.3 years, were selected for this study. Specific IgE levels to buckwheat were quantified by using CAP-FEIA (Pharmacia Diagnostic, Uppsala, Sweden). Five sera, from 3 atopic (who have highly positive IgE antibody against house dust mites and cedar pollen) and 2 nonatopic subjects who lack specific IgE against buckwheat, were used in this study as controls (age from 2 to 38 years, median 13.8 years).

Raw Buckwheat Extract

Five grams of finely ground buckwheat (Takizawa Food Industry, Koushoku, Japan) was extracted by overnight shaking at 4°C in 50 ml 0.086 M NaCl solution (Wako Chem., Osaka, Japan) containing 0.033 M NaHCO₃ (Nakarai Tesque, Kyoto, Japan). After centrifugation at 12,000 g for 30 min, the supernatant was dialyzed against PBS (Nissui Pharmaceutical, Tokyo, Japan) using dialysis tubes (Spectrum Lab., Dominguez, Calif., USA) with an MW cutoff value of 3,000 daltons and aliquoted for storage at –40°C until use.

Protein concentration was determined by the Bradford method [10] using Bio-Rad protein assay kit (Bio-Rad Lab., Hercules, Calif., USA) as a bovine serum albumin (Wako Chem.) standard.

Pepsin Digestion

Raw buckwheat extract was digested as described by Kato et al. [11] with slight modifications. The pH of raw buckwheat extract (protein concentration of 1 mg/ml) was adjusted to 3.0 with 0.1 N HCl (Wako Chem.) and preincubated at 37°C for 30 min. Digestion was initiated by the addition of pepsin (pepsin A from porcine stomach mucosa, Sigma Chemical, St. Louis, Mo., USA) dissolved in 0.01 N HCl (1:100, enzyme:protein ratio).

At 30, 60 and 120 min after pepsin addition, digested samples were aliquoted and adjusted to pH 8.0 with 1 N NaOH to stop digestion. The same procedure was performed with 0.01 N HCl instead of pepsin as a control.

SDS-PAGE and IgE Immunoblotting

SDS-PAGE was performed according to the procedure described by Laemmli [12]. Raw buckwheat and pepsin-digested buckwheat were dissolved in the same volume of sample buffer containing 2-mercaptoethanol (Nakarai Tesque) and were heated for 3 min. These samples were applied to the 4–20% gradient gel (Asahi Technos, Tokyo, Japan) by loading 20 µl/well at 125 V for 100 min. The gel was stained with Coomassie brilliant blue R-250 (Bio-Rad Lab). In some experiments, separated proteins in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Foster, Calif., USA) at 140 V for 75 min in 0.5 M CAPS (Dojindo, Kumamoto, Japan) with 7.5% methanol (Wako Chem.) at pH 10.5. The membrane was washed in PBS containing 0.1% Brij 35 (PBS/Brij, Sigma) and blocked with 1 mg/ml human serum albumin (Sigma) in PBS/Brij. After another wash with PBS/Brij, the membrane was cut into strips, which were incubated overnight with the serum of each subject diluted 1:10.

These strips were then washed and incubated with 1:5,000 dilution of alkaline-phosphatase-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA) and visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Promega, Madison, Wisc., USA).

Inhibition ELISA and Inhibition Immunoblotting

Inhibition ELISA for raw buckwheat extract was performed using sera from 3 subjects (No. 4, 5, 10) with IHR and 3 subjects (No. 11, 15, 18) without IHR. The microtiter plate (Costar, Cambridge, Mass., USA) was coated with raw buckwheat extract containing 10 µg/ml protein and incubated overnight at 4°C. The coated plate was washed with PBS/Brij containing 1 mg/ml human serum albumin and 0.1% Brij 35 detergent. Sera were usually diluted 1:10 (subjects No. 4, 5, 10, 18) with the above PBS/Brij and were treated with an equal volume of serially diluted pepsin-digested buckwheat (120 min digestion, protein concentration from 0 to 62.2 µg/ml)

Table 1. Clinical characteristics of 20 subjects with positive CAP-FEIA for buckwheat

No.	Age years	Gender	Buckwheat CAP-FEIA kU/l	Total IgE U/ml	IHR (symptoms)	Diagnosis
1	30	female	2.86	44	+ (APX)	FA
2	14	female	1.70	2,606	+ (UR)	FA, AD
3	5	male	25.7	318	+ (UR)	FA, BA
4	7	male	8.15	800	+ (APX)	FA, BA
5	14	male	44.6	640	+ (APX)	FA, AD, BA
6	19	male	32.9	600	+ (APX)	FA
7	44	male	6.75	411	+ (APX)	FA, BA
8	10	male	62.4	206	+ (APX)	FA
9	9	male	87.2	177	+ (APX)	BA
10	37	male	11.7	11,000	+ (APX)	FA, AD, BA
11	7	female	2.32	340	–	BA, AD
12	8	female	2.97	370	–	BA, AD
13	10	male	2.24	1,400	–	AD, AR
14	13	male	1.17	400	–	AD, BA
15	5	male	2.90	2,400	–	AD
16	2	male	2.14	12,746	–	BA, AD
17	14	male	18.7	2,300	–	BA, AD
18	7	male	6.97	2,800	–	AD
19	26	female	2.88	1,000	–	AR
20	3	female	14.3	24,068	–	AD

APX = Anaphylaxis including generalized skin, abdominal and respiratory symptoms; UR = generalized urticaria or angioedema only; AD = atopic dermatitis; BA = bronchial asthma; FA = food allergy; AR = allergic rhinitis.

overnight at 4°C. Sera obtained from subjects No. 11 and 15 were diluted 1:5, since the subjects had relatively low antibuckwheat IgE titers (table 1). Treated sera were added to the wells and the plate was incubated for 2 h at room temperature. After washing the plate, adsorbed IgE was detected with peroxidase-labeled goat anti-human IgE antibody (Kirkegaard & Perry Laboratories) diluted 1:3,000. The plate was further washed and developed with TMB solution (Kirkegaard & Perry Laboratories).

To terminate the reaction, 1 M phosphoric acid solution was added to the plate. Absorbance was measured at 450 nm by an MR 5000 Microplate Reader (Dynatech Lab., Chantilly, Va., USA). Percent inhibition was calculated as follows: % inhibition = $(OD_u - OD_i) \div OD_u \times 100$, where OD_u is absorbance with use of uninhibited serum and OD_i is absorbance with use of inhibited serum.

For inhibition immunoblotting, raw buckwheat extract (protein concentration of 1 mg/ml) was separated by SDS-PAGE on a 4–20% gradient gel under reducing conditions. An inhibition assay was performed using sera from 3 subjects (No. 4, 5, 10), which were selected due to strong binding to 8-, 16- and 24-kD proteins in IgE immunoblotting. Sera diluted 1:10 with the PBS/Brij containing 1 mg/ml human serum albumin were treated with an equal volume of serially diluted digested buckwheat (120 min digestion, inhibitor concentration from 0 to 62.2 µg/ml) for 2 h. Immunoblotting was then performed with treated sera as described above.

Anion Exchange Chromatography

Raw buckwheat extract dialyzed against 0.01 M Tris-HCl buffer (pH 7.8) was fractionated by anion-exchange chromatography on a column of Hitrap-Q (Pharmacia Biotech, Uppsala, Sweden). After the column was equilibrated with 0.01 M Tris-HCl buffer, samples were applied. The column was washed with 0.01 M Tris-HCl buffer and the absorbed proteins were eluted with a linear gradient of 0.5 M NaCl buffer in 0.01 M Tris-HCl buffer.

Gel Filtration by HPLC

Anion-exchange fractions of buckwheat proteins were concentrated by using Centriprep YM3 (3,000 MWCO, Millipore, Bedford, Mass., USA) and further purified by gel filtration. Concentrated fractions were dialyzed against 0.15 M Tris-HCl and injected on the column of Superdex 75 HR 10/30 (Pharmacia LKB Biotech, Uppsala, Sweden) and eluted with 0.15 M Tris-HCl buffer. Absorbance was detected at 280 nm. A calibration run was performed with standard proteins with known molecular weights (gel filtration calibration kits, Pharmacia Biotech).

N-Terminal of Amino Acid Sequence of Purified Buckwheat Proteins

The purified 16-kD buckwheat protein was separated by SDS-PAGE and blotted onto PVDF membranes. The membranes were cut out for protein sequence analysis. The N-terminal of amino acid sequence was analyzed with the Hewlett-Packard G1005A protein sequencing system.

Results

Characteristics of Subjects with IHR

We classified the subjects whose sera were positive for buckwheat-specific IgE antibody by CAP-FEIA into two groups based on clinical symptoms as to whether or not they had IHR to buckwheat. As summarized in table 1, 8 of the 10 subjects with IHR showed anaphylaxis including skin, gastrointestinal and respiratory symptoms, and the other 2 subjects had only generalized urticaria. Nine of the 10 buckwheat-specific IgE antibody-positive subjects without IHR had been diagnosed as moderate to severe atopic dermatitis, and none of them complained of symptoms related to buckwheat digestion, at least not within several hours after the digestion of buckwheat. We did not confirm this by double-blind placebo-controlled food challenge, since the 10 subjects with obvious IHR refused to do so. As negative controls, 5 subjects without a history of buckwheat allergy and antibuckwheat IgE were selected in this study. Three of the 5 subjects had positive anti-house dust mite and cedar pollen IgE antibodies (atopic controls).

Effect of Pepsin Digestion on IgE-Binding Buckwheat Proteins

Raw buckwheat extract and pepsin-digested buckwheat extract were analyzed with SDS-PAGE. Coomassie staining of the raw buckwheat proteins revealed multiple bands ranging in molecular weights from 6.5 to 70 kD. Buckwheat proteins larger than 16-kD proteins became invisible after 30 min of pepsin digestion indicating that high-molecular-weight proteins were pepsin-sensitive (fig. 1).

IgE immunoblotting revealed that IgE-binding patterns of sera from the IHR-positive group were distinct from those of IHR-negative groups (fig. 2). As shown in figure 2a, sera from the IHR-positive group reacted to the following major allergens, i.e. 24 kD (10 of 10 subjects, 100%), 19 kD (9 of 10 subjects, 90%), 16 kD (9 of 10 subjects, 90%) and 6.5 kD (5 of 10 subjects, 50%). On the other hand, the major allergens found using sera from the CAP-FEIA-positive IHR-negative group were 24 kD (9 of 10 subjects, 90%) and 83 kD (5 of 10 subjects, 50%). Only one serum sample (No. 20) reacted to the 32-, 36- and 45-kD protein. None of their sera (lanes 11–20, fig. 2) were bound to proteins of <24 kD in raw buckwheat extract.

We used IgE immunoblotting with pepsin-digested buckwheat extract to examine whether the buckwheat proteins are stable after the digestion. As shown in figure 2b, 16- and 6.5-kD proteins were bound with the sera

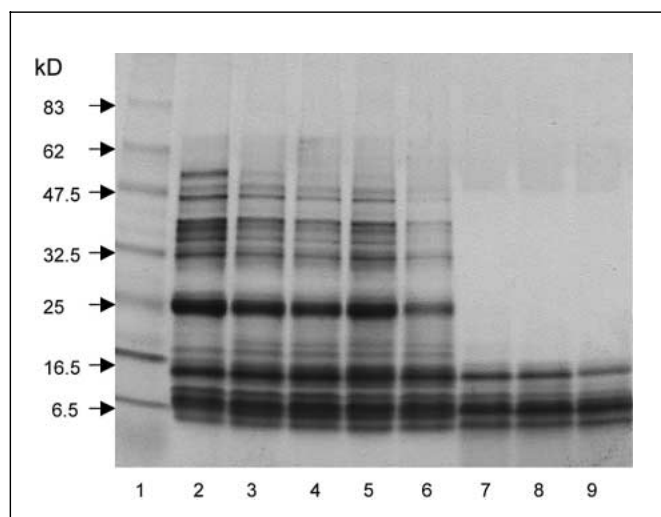


Fig. 1. SDS-PAGE analyses of raw buckwheat extract and pepsin-digested buckwheat. Lane 1 shows molecular weight marker and lane 2 shows protein fractions obtained from raw buckwheat extract. The buckwheat extract was incubated with 0.1 N HCl for 30 min (lane 3), or 0.01 N HCl for 30 min (lane 4), 60 min (lane 5) and 120 min (lane 6). Buckwheat was also digested with pepsin for 30 min (lane 7), 60 min (lane 8) and 120 min (lane 9).

diluted 1:10 from subjects with IHR. In contrast, none of the sera from subjects without IHR reacted to pepsin-digested buckwheat proteins. Since the sera from patients 5, 8 and 9 (table 1) had high CAP-FEIA titers against buckwheat, we performed the immunoblotting by diluting their sera at 1:100. We could similarly obtain the 16-, 19- and 24-kD bands (data not shown).

Buckwheat Proteins Remain Reactive to Sera from the Patients with IHR

As shown in figure 3, an inhibition ELISA test was performed to establish whether pepsin-digested buckwheat proteins can block raw buckwheat protein binding to serum IgE. The pretreatment with pepsin-digested buckwheat proteins at 60 µg/ml blocked the binding of raw buckwheat proteins to serum IgE from the IHR-positive group at 60–70%, whereas the same pretreatment blocked the protein binding in the case of the IHR-negative seropositive group only at <20%. In a preliminary experiment of inhibition immunoblotting, the pepsin-digested buckwheat extract at 6 µg/ml dilution failed to block the binding of the 3 sera from patients with IHR (No. 4, 5 and 10) to the 6.5- and 24-kD proteins in raw buckwheat extract, while it mostly blocked the binding to the 16-kD protein even at 0.6 µg/ml dilution (data not shown).

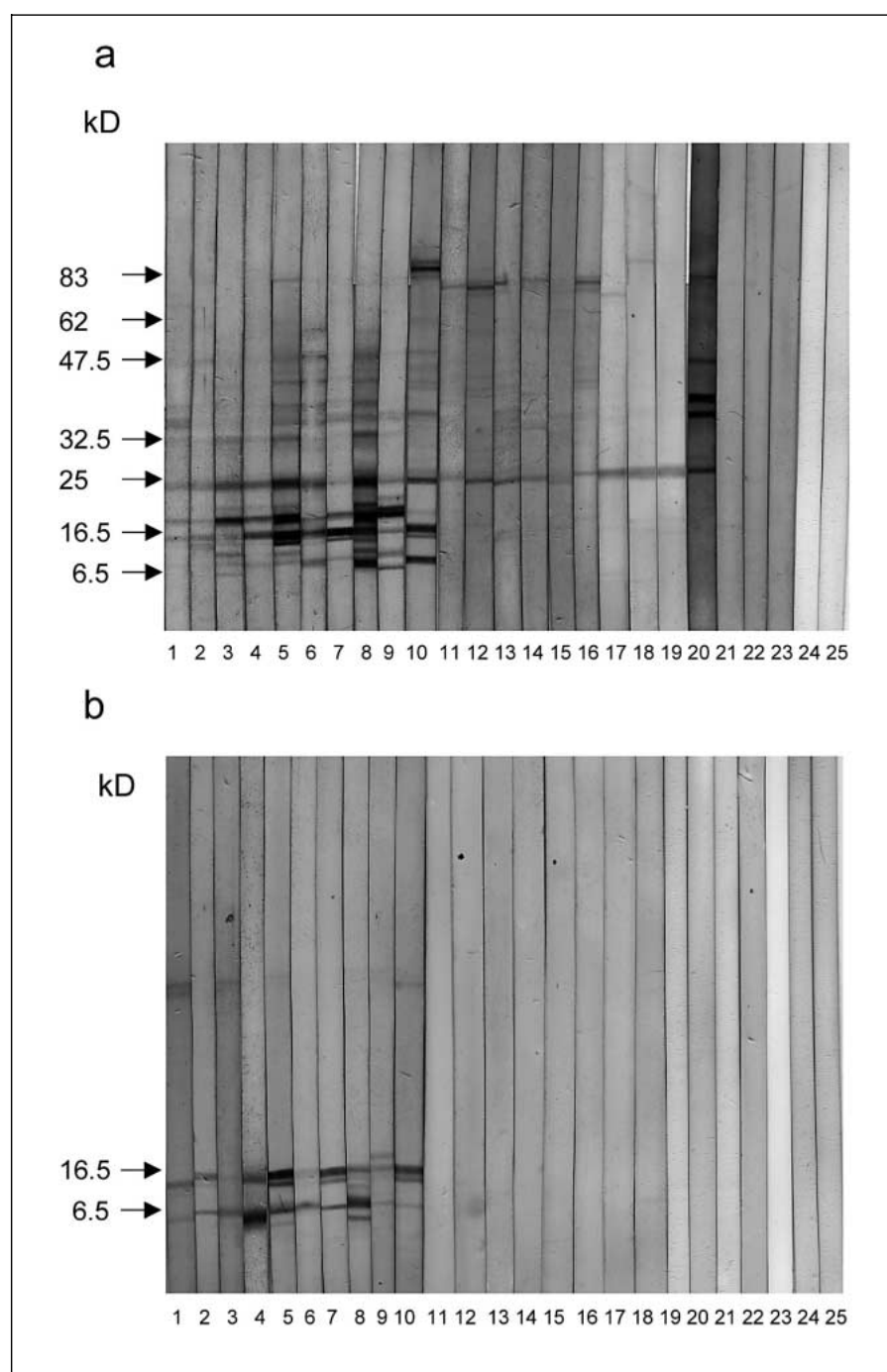


Fig. 2. IgE immunoblotting of buckwheat extract. Raw buckwheat extract (a) and pepsin-digested buckwheat extract (b) were fractionated and incubated with sera from 20 subjects with positive buckwheat-specific IgE (lanes 1–20). Lanes 1–10 were obtained using sera from IHR-positive patients. Lanes 11–20 were obtained using sera from IHR-negative subjects. Buckwheat-specific IgE-negative control subjects were shown in the lanes 21–25.

N-Terminal of Amino Acid Sequencing of the 16-kD Buckwheat Protein

As shown figure 4a, raw buckwheat extract was separated into six components with the anion-exchange column. SDS-PAGE revealed that fraction A contained the 16-kD protein and fraction B contained the 24-kD pro-

tein. Then, further purification was performed using gel filtration. Fraction A was found to contain two peaks of protein (fraction 40 and fraction 46, fig. 4b), and fraction 40 was found to contain the 16-kD protein.

The 16-kD protein and the 24-kD protein were then characterized by N-terminal sequencing. The sequence of

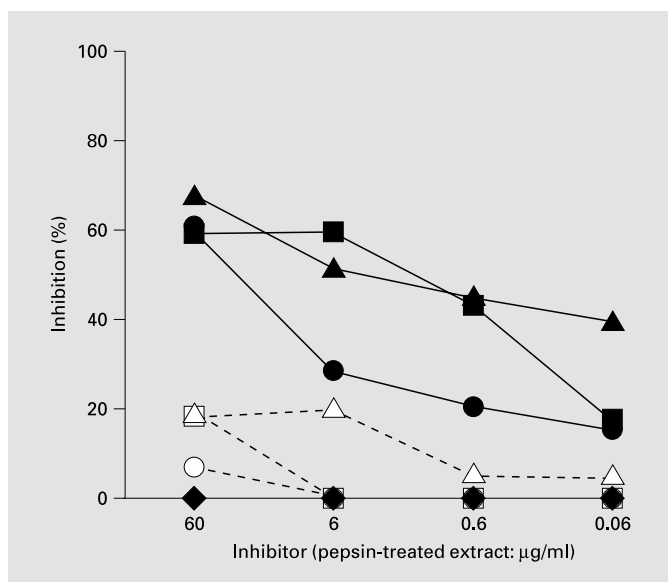


Fig. 3. Inhibition ELISA of raw buckwheat extract by pepsin-digested buckwheat extract. Three IHR-positive and buckwheat-specific IgE-positive serum samples (No. 4, 5, 10 in table 1; ■, ▲, ●), 3 IHR-negative but buckwheat-specific IgE-positive serum samples (No. 11, 15, 18 in table 1; □, △, ○) and a negative control serum sample (◆) were reacted with raw buckwheat extract after preincubation with the buckwheat extracts which had been digested by pepsin at the indicated concentrations.

the 16-kD protein was revealed to be 'RDEGFDDL-GETQMSSK'. The National Center for Biological Information databases (NCBI databases) indicated that it was 100% identical with the N-terminal sequence of the 18-kD buckwheat protein, which has recently been reported by others [13, 14]. The 16-kD protein was also found to have 80% homology with *Oriza sativa* dehydrin, a rice protein (Gene Bank Accession No. OSU 60097) and no homology with the 24-kD protein (GLEQAFXNLKF-KQNVN), which was identical with the major allergenic storage protein described by Nair and Adachi [15] (Gene Bank Accession No. AAD32713).

Discussion

We found in the present study that 24-kD buckwheat protein(s) were bound to IgE antibodies present in sera from almost all subjects with positive buckwheat-specific IgE. These results agreed with previous reports which found that the buckwheat protein of 24-kD is the major buckwheat allergen based on its IgE-binding capability

[16, 17]. We also found that 16- and 19-kD proteins reacted to IgE antibodies present in sera from 9 out of the 10 patients with IHR to buckwheat including 8 patients with anaphylaxis. The buckwheat protein of 16–18 kD is also reported to be one of the major allergens with buckwheat allergy [13, 14].

Park et al. [14] reported that the 19-kD protein was relatively specific for buckwheat allergy patients with IHR. Among the above three major allergens found in our samples derived from symptomatic patients, however, 19- and 24-kD proteins were easily digested by pepsin treatment. Raw buckwheat extract contained 24-kD proteins, which were often bound to IgE antibodies present in sera from antibuckwheat IgE-positive subjects without IHR. However, the pepsin-treated buckwheat extract was bound only to IgE antibodies present in sera from buckwheat allergy patients with IHR. These results suggest that the pepsin-treated buckwheat extract may be used as the buckwheat allergen for CAP-FEIA instead of the raw buckwheat extract. This may also be applied in the case of other food proteins causing IHR. Indeed, allergens causing anaphylactic reaction are known to be resistant to digestive fluid [7–9, 18, 19]. Van Ree et al. [20] also proposed that the treatment of food extracts with pepsin is a way to distinguish between mild and potentially severe food allergens. They also described in the same review [20] that IgE antibodies tend to react to carbohydrate moiety present in glycoprotein allergens, and thereby they are often cross-reactive to many plant-derived molecules. These IgE-cross-reactive carbohydrate-rich molecules are usually unstable and easily digested [7–9, 20, 21]. Therefore, it is of interest to establish in future studies whether the 19- and 24-kD proteins share carbohydrate-rich moieties with other plant-derived proteins.

In contrast with 19- and 24-kD proteins, the 16-kD protein was highly resistant to pepsin digestion. It was suggested on the basis of the inhibition ELISA that pepsin-resistant proteins in buckwheat were associated with the history of IHR to buckwheat. The inhibition immunoblotting suggested that the pepsin-resistant 16-kD protein is not degraded from 24-kD proteins. To confirm whether the 16-kD protein differs from the 24-kD proteins, we employed the N-terminal amino acid sequencing assay of the proteins. We found that the N-terminal amino acid sequence of the 16-kD buckwheat protein had 100% homology to the 16- to 18-kD buckwheat protein previously reported by others [13, 14]. The 16-kD protein had no homology with the 24-kD protein. Thus, the pepsin-resistant 16-kD protein seems to be a strong candidate for a major buckwheat allergen causing IHR.

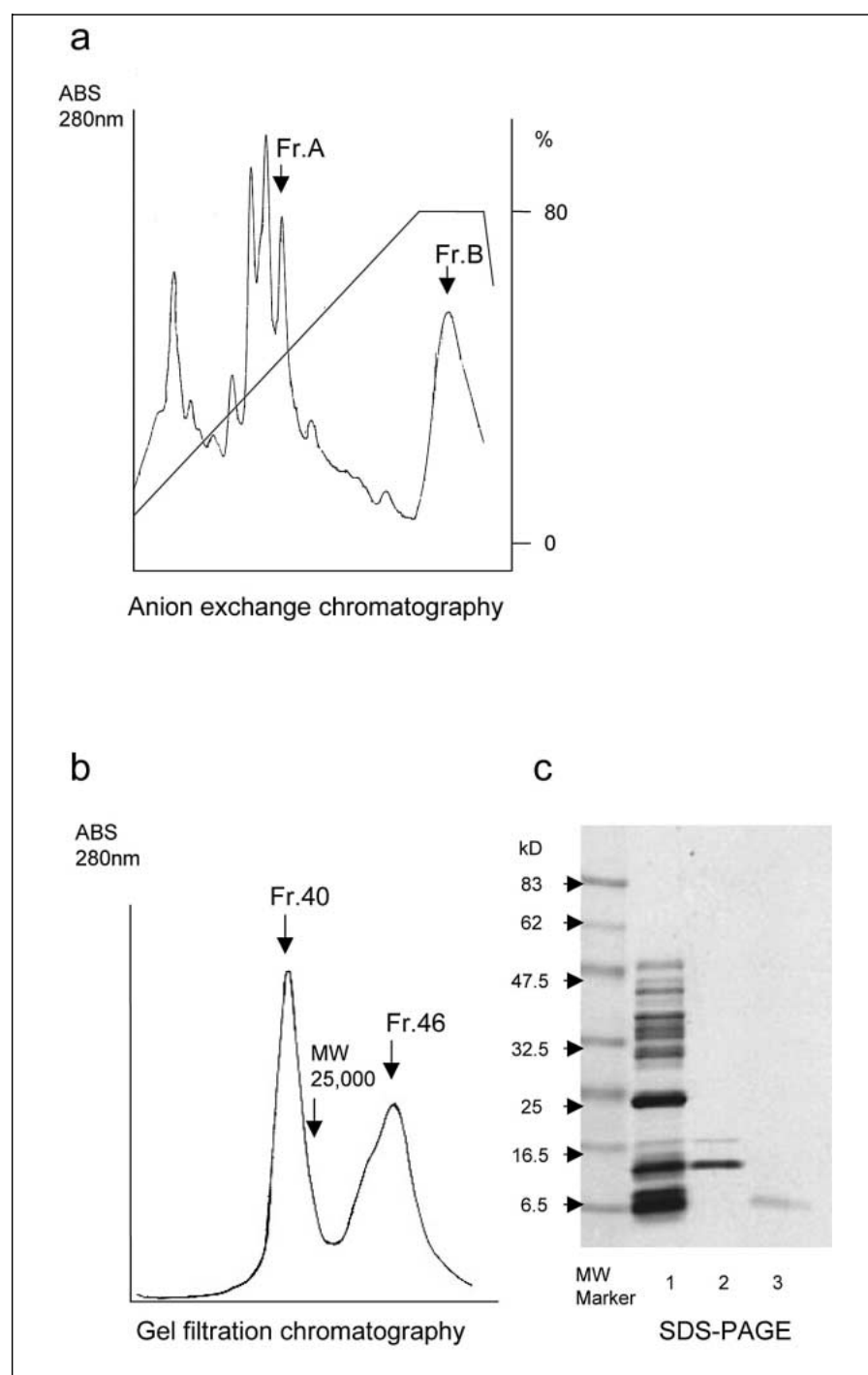


Fig. 4. Purification of the 16-kD protein. a Raw buckwheat extract was fractionated with anion exchange chromatography, and two fractions were obtained. Fr. B was found to contain a single peak of the 24-kD protein (data not shown). b Gel filtration chromatography revealed that Fr. A contained the two peaks, Fr. 40 and Fr. 46. c SDS-PAGE revealed that the 16-kD protein was found in Fr. 40 (lane 2) but not Fr. 46 (lane 3). The crude buckwheat extract is shown in lane 1.

The 16-kD protein also had 80% amino acid homology with the major allergen of rice [22, 23]. Cross-reactivity of IgE antibodies between buckwheat and rice was also reported [24]. However, none of the patients with IHR to buckwheat had any symptoms after rice digestion (data

not shown). The 20% sequence difference between these two proteins or the translational modification of the proteins such as glycosylation may be the key role molecules for inducing a severe anaphylactic reaction.

In conclusion, we demonstrated for the first time that the 16-kD buckwheat protein, which was highly resistant to pepsin digestion, appeared to be responsible for IHR after buckwheat ingestion. Buckwheat products are increasing in popularity in Russia, Italy, the USA and Canada as well as many Asian countries as part of the range of health food products based on their high protein content and due to the unusually short growing time of buckwheat. It is expected that the present study will help to diagnose severe forms of the allergy in Asian countries as well as in many other countries.

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