Purification and Properties of Allergenic Proteins in Buckwheat Seeds

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Three kinds of proteins (BA-1, BA-2 and BA-3) allergenic to the IgE antibody of allergic individuals were isolated from buckwheat seeds. These three proteins were essentially homogeneous as judged by both polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. The amino acid composition of BA-1 and BA-2 was very similar, and the molecular weight of each allergenic protein was between 8000–9000 by SDS-polyacrylamide gel electrophoresis. One of them was a trypsin inhibitor, and their immunoreactivity was quite stable to heating at 100°C for 60 min.

As with other common food protein sources such as cow's milk and eggs, cereal proteins cause an allergenic reaction in a small number of people.^{1,2)} Buckwheat is not a true cereal, but it is used extensively as a food both humans and domestic animals. The Japanese eat a food called soba which resembles spaghetti and is made of buckwheat flour. Little similarity has been noted between wheat and buckwheat endosperm proteins.³⁾

Although a buckwheat allergy is not very common, buckwheat has been considered as a highly explosive allergen.^{4–7)} Patients can be affected with severe symptoms by very small amounts of the allergen either by ingestion or inhalation. These properties of buckwheat allergen are very interesting, but few studies have been made about the physical, chemical and immunological properties of buckwheat allergen.

The aim of the present investigation was to isolate the allergenic protein in buckwheat seeds, using the antigenicity to the serum IgE antibody as an index, and to characterize some properties of the isolated allergenic protein.

Materials and Methods

Buckwheat protein Buckwheat seeds of Shinano No. 1 harvested in Nagano prefecture in 1988 were used in this experiment. Finely ground buckwheat seed meal was defatted with cold acetone and thoroughly dried. The proteins were extracted from the defatted powder (100 g) with 500 ml of distilled water for 24 hr at 4°C. After centrifuging the extract at 10,000 × *g* for 20 min, an equal volume of ethyl alcohol was slowly added to the supernatant at –20°C. After centrifuging at 10,000 × *g* for a further 20 min, the precipitate was dissolved in distilled water and dialyzed against 0.02 M Tris–HCl buffer (pH 8.6).

Mouse antiserum to buckwheat protein. Female C3H mice of approx. 5 weeks of age were immunized by the intraperitoneal injection of buckwheat protein (50 µg in 100 µl of phosphatebuffered saline per mouse) with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). They received a booster injection of 20 µg of the same protein with Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI) 3 weeks after the first immunization. The mice were bled 7 days after the booster injection, the serum being separated by centrifugation and stored at –80°C.

Allergenic activity measurements. The allergenic activity of buckwheat proteins was measured by an enzyme-linked immunosorbent assay (ELISA).⁸⁾ The sera with a high RAST score against buckwheat protein were selected from

the sera of patients with known clinical histories of allergies. Flat-bottomed microtiter plates were coated with an appropriate concentration ($1 \sim 10 \mu\text{g/ml}$) of the protein antigens. After incubating with human sera diluted appropriately ($1:100 \sim 1:1000$), the human IgE antibody which reacted with the plate-bound antigens was determined, using peroxidase coupled anti-human IgE antibody (goat, ϵ -chain specific, Cappel Laboratories Inc., Cochranville, PA) with *o*-phenylene diamine as the enzyme substrate as described previously.⁹⁾

For the competitive inhibitor assay, one buckwheat allergenic protein (BA-1) solution ($100 \mu\text{l}$) and $100 \mu\text{l}$ of the mouse anti-serum ($1:10000$ dilution) to buckwheat were added to the wells coated with another buckwheat allergenic protein (BA-2 or BA-3). The plates were incubated at 37°C for 3 hr, and the antibodies reacting with the plate-bound antigen were measured as already described.

Ion exchange chromatography. The buckwheat protein dialyzed against 0.02 M Tris-HCl buffer ($\text{pH } 8.6$) was fractionated at 25°C by ion-exchange chromatography on a column ($1.8 \times 3.5 \text{ cm}$) of DEAE cellulose (DE 52, Whatman Biochemicals Ltd.) that had been equilibrated with the Tris-HCl buffer. After applying the sample solution, the column was washed with the same buffer, and the proteins absorbed were eluted with a linear gradient of NaCl ($0 \sim 0.15 \text{ M}$ NaCl in 500 ml of the Tris-HCl buffer).

High-performance liquid chromatography (HPLC). Eluates of the buckwheat protein from DEAE cellulose column chromatography were further purified by two kinds of HPLC, namely, ion-exchange HPLC using a $7.5 \text{ mm} \times 7 \text{ cm}$ TSK gel DEAE-5PW column and reverse-phase HPLC using a $6.0 \text{ mm} \times 15 \text{ cm}$ Asahi pack ODP-50 column. Ionexchange HPLC was performed by injecting $40 \mu\text{g}$ of protein and eluting with a linear gradient ($0 \sim 50\%$) of 0.02 M Tris-HCl buffer ($\text{pH } 8.6$) containing 0.2 M NaCl. Reverse-phase HPLC was performed by injecting $40 \mu\text{g}$ of protein and eluting with a linear gradient ($20 \sim 55\%$) of CH_3CN containing 0.05% trifluoroacetic acid.

Trypsin inhibitory activity. Trypsin inhibitory activity was assayed by measuring the initial rate of increase in absorbance at 420 nm with *N*-benzoyl-L-arginine-p-nitroanilide as described by Waheed and Salahuddin.¹⁰⁾

Electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Laemmli,¹¹⁾ the gel sheet being stained with Coomassie R-250 Brilliant Blue. An amino acid analysis was performed with a Hitachi 650-15 HPLC, using a no. 694 column after hydrolyzing at 110°C for 24 hr in 6 N HCl in evacuated, sealed tubes.

Protein determination. Protein concentration was determined by the method of Lowry *et al.*¹²⁾ using ovalbumin

(SIGMA Chemical Co., Ltd.) as the standard.

Results and Discussion

Purification of the allergenic protein

Since the high viscosity of a buckwheat extract often hinders the purification and separation of an allergenic protein, it is desirable to reduce its viscosity at an early stage of the purification procedure. The high viscosity of the buckwheat extract is considered to be mainly caused by the polysaccharide component,¹³⁾ so we first tried to remove the polysaccharide from the aqueous extract by fractionation with ethyl alcohol. When an equal amount of ethyl alcohol was added to the solution, a large proportion of the buckwheat protein was precipitated, and most of the polysaccharide remained in the solution. The protein fraction precipitated with 50% ethyl alcohol showed strong allergenic activity against human IgE antibody of the patient serum, but the polysaccharide fraction which was not precipitated with 50% ethyl alcohol did not have any allergenic activity against human IgE antibody (Fig. 1).

The buckwheat protein fraction precipitated with 50% ethyl alcohol was dissolved in distilled water and fractionated by DEAE cellulose

Fig. 1. Immunoreactivity of the Water-extractable Fraction of Buckwheat Fractionated with Ethyl Alcohol.

1. Precipitated fraction with the addition of an equal volume of ethyl alcohol.

2. Supernatant fraction with the addition of an equal volume of ethyl alcohol.

●—●, ■—■, human patient serum; ○---○, healthy human serum.

Fig. 2. Ion-exchange Chromatogram of Buckwheat Proteins on a Column (1.8 × 35 cm) of DE 52, Pre-equilibrated with 0.02 M Tris-HCl Buffer (pH 8.6).

The proteins were eluted with a linear gradient (0 ~ 0.15 M) of sodium chloride.

Fig. 3. Ion-exchange High-performance Liquid Chromatogram of No. 1 Fraction (A) and No. 3 Fraction (B) Using a TSK-gel DEAE-5PW Column.

Twenty μ l of each fraction (0.2 mg/ml) were injected into the column and eluted with a linear gradient (0 ~ 50%) of 0.02 M Tris-HCl buffer (pH 8.6) containing 0.2 M NaCl. Peaks indicated by the arrow were used for further purification.

Fig. 4. Reverse-phase High-performance Liquid Chromatogram of No. 1 Fraction Using an Asahipack ODP-50.

A) Two hundred μ l of No. 1 fraction (0.2 mg/ml) were injected into the column and eluted with a linear gradient (20 ~ 55%) of CH_3CN containing 0.05% trifluoroacetic acid.

B) The peak indicated by the arrow in Fig. 4A was rechromatographed on the same column.

chromatography. As shown in Fig. 2, this protein fraction was separated into six components with this chromatography. Among the six components in Fig. 2, two fractions (No. 1 and No. 3) showed strong reactivity against human IgE antibody of the patient serum (data not shown). These two components were gathered and further purified by two kinds of HPLC. Fig. 3 shows ion-exchange HPLC patterns of No. 1 and No. 3 fractions. Although No. 1 fraction gave a sharp peak, No. 3 fraction gave several peaks. Reverse-phase HPLC was applied to both No. 1 fraction and the main component of No. 3 fraction, which was separated by ion-exchange HPLC (the arrowed peak in Fig. 3B). No. 1 fraction gave two well-separated peaks, of which the main peak was gathered and named BA-1 (Fig. 4). The main component of No. 3 fraction gave four well-separated peaks, of which two peaks were gathered and named BA-2 and BA-3, respectively (Fig. 5).

Properties of the allergenic protein

These three proteins were essentially homogeneous as judged by polyacrylamide gel electrophoresis (Fig. 6). The reactivity to human IgE antibody of the isolated proteins was

quantitatively analyzed by using sera from three individuals allergic to buckwheat (Fig. 7). The three proteins reacted well with IgE antibodies in all of the three sera, indicating that these proteins were a common allergen to the three individuals. The amino acid composition of BA-1 and BA-2 was very similar, although that of BA-3 was a little different (Table I). SDS-polyacrylamide gel electrophoresis of these proteins showed that they were homogeneous polypeptides of similar size. Since each protein band was rather broad, their exact molecular weight could not be

Fig. 5. Reverse-phase High-performance Liquid Chromatogram of the Arrowed Peak in Fig. 3 Using an Asahipack ODP-50.

A) Two hundred μ l of the arrowed peak in Fig. 3 (0.2 mg/ml) were injected into the column and eluted with a linear gradient (20~55%) of CH_3CN containing 0.05% trifluoroacetic acid.

B and C) Peaks indicated by the arrow in Fig. 5A were rechromatographed on the same column.

Fig. 6. Polyacrylamide Gel Electrophoregram of BA-1, BA-2 and BA-3.

Fig. 7. Immunoreactivity of BA-1, BA-2 and BA-3. A, BA-1; B, BA-2; C, BA-3.

■—■, ●—●, ▲—▲, human patient serum; ○—○, □—□, healthy human serum.

Table 1. AMINO ACID COMPOSITION OF THE BUCKWHEAT ALLERGENIC PROTEINS

Amino acid	Residues per molecule		
	BA-1	BA-2	BA-3
Asp	8	7	6
Thr	1	1	3
Ser	6	6	15
Glu	11	11	12
Pro	N.D.	N.D.	N.D.
Gly	7	12	14
Ala	5	5	7
Cys	N.D.	N.D.	N.D.
Val	9	11	7
Met	7	4	1
Ile	4	3	2
Leu	8	6	3
Tyr	<1	<1	<1
Phe	1	2	1
Lys	4	5	3
His	<1	<1	3
Arg	7	6	2
Total	78	79	79

N.D., not determined.

Fig. 8. SDS-Polyacrylamide Gel Electrophoregram of BA-1, BA-2 and BA-3.

assigned, although their approximate molecular weight was estimated to be between 8000~9000 (Fig. 8). It is unclear why the protein band was not sharp on the SDS-polyacrylamide gel electrophoregram. Since a

Fig. 9. Trypsin-inhibitory Activity of BA-1, BA-2 and BA-3.

●—●, BA-1; ○---○, BA-2; □—□, BA-3.

glycoprotein often shows a broad band on SDS-polyacrylamide gel electrophoresis, these proteins might have been glycoproteins.

Trypsin inhibitors in buckwheat have already been separated and their properties studied.^{14,15)} The molecular weight of these trypsin inhibitors was also shown to be between 8000~9000. There was a possibility that the allergenic proteins and trypsin inhibitors were the same proteins. Based on this assumption, we measured the trypsin-inhibitory activity of the allergenic proteins (Fig. 9). Although both BA-2 and BA-3 had no trypsin inhibitory activity, BA-1 was certainly a trypsin inhibitor. In spite of the similarity in amino acid composition between BA-1 and BA-2, BA-2 has no trypsin inhibitory activity. Proteolysis might have occurred during the preparation of BA-2 and its inhibitory activity lost. Further studies should be made about these proteins. However, it is interesting that one of the buckwheat allergenic proteins was trypsin inhibitor. Since these three allergenic proteins were immunologically cross-reactive, judging from the competitive inhibition analysis (Fig. 10), they are presumed to

Fig. 10. Competitive Inhibition Analysis for the Allergenic Reactivity of BA-2 and BA-3, Using BA-1 as a Competitor.

The amount of BA-1 was four times that of BA-2 or BA-3.

□, without the competitor; ■, with the competitor.

Fig. 11. Effect of Heating (100°C) on the Immunoactivity of BA-1, BA-2 and BA-3.

●—●, BA-1; ■—■, BA-2; ○—○, BA-3.

have a common structure for IgE binding. A similarity between the properties of BA-1,

BA-2 and BA-3 was also noted from measurements of their heat stability. As shown in Fig. 11, none of the allergenic proteins lost their allergenic activity by heating at 100°C for 60 min. An investigation is now underway in this laboratory about the chemical and physical properties of these allergenic proteins.

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