

Purification and Characterization of a 24 kDa Protein from Tartary Buckwheat Seeds

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A 24 kDa protein was isolated from tartary buckwheat seeds by using chromatography of Superdex 75 gel filtration and Resource Q ion-exchange column. SDS-PAGE and Sephacryl S-200 gel filtration were used to provide information about the molecular mass of the protein purified from tartary buckwheat. The protein was composed of 215 amino acid residues and showed strong IgE binding activity in an ELISA test to the sera collected from two patients allergic to buckwheat. These results suggested that the purified 24 kDa protein from tartary buckwheat seeds was an important functional protein and was relatively specific for buckwheat-allergic patients. It should be a very useful tool in the diagnosis of buckwheat allergy in the future.

Key words: allergy; purification; immunoblotting; ELISA; *Fagopyrum tartaricum*

Buckwheat is widely grown in Asia and some other areas of the world. It is a good source of food with a higher protein content than rice, wheat, or sorghum.^{1–4)} It also benefits humans in preventing leg edema,⁵⁾ hypertension, and cardiovascular diseases.⁶⁾ In recent years, buckwheat has become much more popular in many countries as a kind of health food,^{7–10)} but the occurrence of buckwheat allergy is also increasing in Asia and Europe.^{11–13)} The major allergens of the common buckwheat (CB) have been characterized in detail by immunological methods,^{14,15)} while the allergens from tartary buckwheat (TB), one of the two cultivated varieties of buckwheat, have no original reports on their properties. Generally, allergenic disorders are caused by eating buckwheat food or using buckwheat pillows filled with buckwheat husks in Europe, Japan, and many other areas.^{16–18)} Some allergenic proteins with molecular masses of 8–9 kDa,

22–24 kDa, 34–38 kDa, and 69–70 kDa have been found in common buckwheat with IgE binding activity in allergic patients.^{19,20)}

Tartary buckwheat is traditionally cultivated in China, and grows mainly in high altitude mountain areas. It is a kind of dicotyledonous crop with high nutritional and medical values, especially efficient in preventing cardiovascular diseases and diabetes.^{1,17,18)} The earliest record of the medical function of tartary buckwheat in Chinese history traces to about two thousand years ago,¹⁾ and in recent years, both the active components of tartary buckwheat and some of its products have attracted more attention than ever before. Tartary buckwheat flour was used to produce various healthful foods including biscuits, noodles, and grain powder. The bran was also a good source to make buckwheat tea and polyphenol extracts. Many experiments in clinical medicine and pharmacology have proved that tartary buckwheat foods have a good effect in preventing and curing the “modern diseases of civilization,” including diabetes and hypertension.

Our previous studies have indicated that the protein complex from tartary buckwheat is a valuable product with high nutritional value.²¹⁾ The contents of protein, lipid, carbohydrate, ash, and crude fiber are 10.2%, 12.7%, 63.4%, 3.5% and 0.4% respectively. Flavonoid from the husks of tartary buckwheat can inhibit the growth of human acute myelogenous leukemia HL-60 by MTT assay,^{22,23)} suggesting that tartary buckwheat may potentially have a therapeutic function in human leukemia. In addition, the leaf extracts have an anti-senescence function in mice.²⁴⁾ Although much research has been carried out on tartary buckwheat, as mentioned above, fewer reports have been published on potentially allergenic proteins or on the differences in certain proteins between tartary buckwheat (TB) and common

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Abbreviations: CB, common buckwheat; ELISA, enzyme-linked immunosorbent assay; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TB24 kDa, tartary buckwheat 24 kDa protein; TBS, Tris-buffered saline; TBST, Tris-buffered saline-tween 20

buckwheat (CB). Hence, as a part of a study to make clear the allergenic mechanism of the allergens in TB, in this research we tried first to purify a major allergen in tartary buckwheat and second, to characterize its molecular mass, amino acid composition, biological function, and immunological properties.

Materials and Methods

Plant materials and reagents. Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) seeds were purchased from Academy of Agriculture Science in Shanxi province, China. Extract standard from common buckwheat was manufactured by ALK Laboratories Inc. Superdex G-75, Sephacryl S-200, and Resource Q, were obtained from Amersham Pharmacia Biotech of Sweden. A low molecular mass marker was purchased from the Sigma Company, St. Louis, Missouri, U.S.A. Mouse antihuman IgE was purchased from BETHYL Laboratories Inc. of U.S.A. The ELISA Detect kit was purchased from JingMei Company of China. All other reagents were of analytical level.

Human sera. Human sera were collected from 11 patients selected by investigating the clinical records on hypersensitivity kept at Shanxi Medical University, Shanxi province, China, and 2 patients sera were clinically reported as allergic to buckwheat.

Extraction of tartary buckwheat protein. The seeds were finely ground using a mortar and pestle and then defatted with acetone and thoroughly air-dried. 500 g of defatted tartary buckwheat flour was stirred with 10 times volume of extract solution containing 1.1% NaCl and 0.28% NaHCO₃ at room temperature for 24 h. The extracts were filtered through four layers of gauze and then centrifuged at 10,000 g for 30 min at 4 °C to remove precipitate. The supernatant was then ultrafiltered and concentrated with an Amicon Filter (molecular mass cut-off 30,000 Dalton). The protein content of the concentrated supernatant was determined by modified Bradford procedures,²⁵ and bovine serum albumin was used as the standard.

Purification of the major allergens by chromatography. The concentrated protein solution was applied to a Superdex 75 gel filtration column ($\phi 1 \times 30$ cm), equilibrated, and eluted with 50 mM phosphate buffer (pH 7.3). The fractions of the fourth peak were collected and concentrated to about 5 ml by PEG-20000, and then loaded onto a Resource Q column ($\phi 1 \times 10$ cm) pre-equilibrated with the same buffer. The column was eluted with a linear salt gradient of 0–1.0 M NaCl in the buffer. The fraction of major allergenic protein was combined and dialyzed against 10 mM phosphate buffer (pH 7.3) for 48 h. Finally, the protein solution was concentrated and freeze dried.

Estimation of molecular mass. The molecular mass of the major allergenic protein from TB was estimated with a Sephacryl S-200 gel filtration column ($\phi 1.6 \times 60$ cm). Bovine serum albumin (67,000), egg albumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and lysozyme (14,400) were used as standards. The molecular mass of the protein subunits was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli,²⁶ with a few modifications. The final gel concentration of the running gel was 12% acrylamide in the presence of both 0.1% SDS and 1% 2-mercaptoethanol, with a low molecular mass marker as the standard.

Amino acid composition analysis. 17.4 mg of the purified protein powder was hydrolyzed at 110 °C with 2 ml of 6.0 N HCl containing 1% reagent grade norleucine as an internal standard in thoroughly evacuated and sealed pyrex hydrolysis tubes. After 24 h, the tubes were opened and the contents evaporated on a rotatory evaporator at 45 °C. The dry residues were taken up in 50 ml sample application buffer and an aliquot of 25 μ l was analyzed with an LKB Alpha-Plus amino acid analyzer using the standard protein hydrolysate program with sodium citrate buffer. Data collection was done with a Shimadzu CR2AX integrator.

Immunoblotting. After SDS-PAGE, the proteins were electroblotted onto a nylon membrane in a Hoefer TE-22 Semi-Dry Transfer, Amersham Biotech, Sweden, using Tris-buffer saline (TBS, pH 7.5). Transfer was performed at 150 mA for 2 h. The membrane was washed three times with TBST (TBS+0.05% Tween-20, pH 7.5) and blocked with 5% defatted milk at 4 °C for 20 h. The membrane was sliced into pieces 5 mm wide, which then were further incubated with patients' sera diluted in TBST in the ratio of 1:30 at 4 °C for 18 h. Each strip was washed three times and incubated for 10 h at 4 °C with 1:1000 diluted mouse antihuman IgE, followed by a second wash with TBST and TBS, twice for each. The strips were then immersed in a buffer solution containing color developing reagents (0.14 mg/ml NBT and 0.5 mg/ml BCIP) and alkaline phosphatase substrate. After 30 min, the blots were rinsed in distilled water and air-dried.

ELISA. Purified protein dissolved in sodium carbonate buffer (pH 9.6) was used for antibody fixation. The wells on the ELISA plates were treated with solutions of different concentrations ranging from 300 μ g/ml, 100 μ g/ml, and 50 μ g/ml to 20 μ g/ml at 4 °C overnight. Incubation with the blocking solution was done at 4 °C for 12 h. The steps following the above procedures were carried out according to the directions for the ELISA Detect Kit.

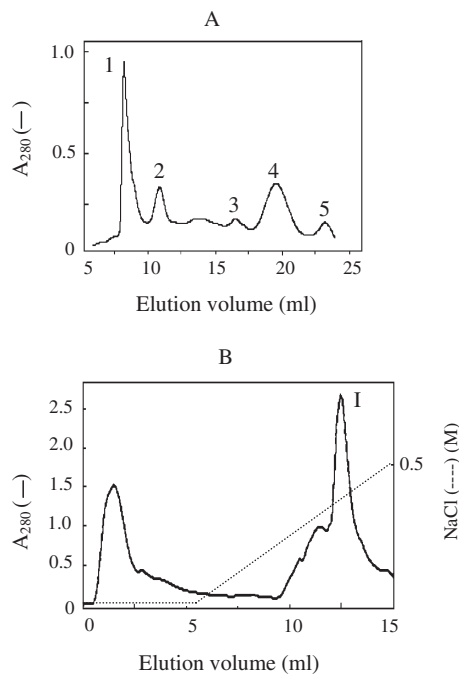


Fig. 1. Chromatograms for Allergenic Protein Purification.

(A) Gel filtration chromatogram. A crude extract preparation was passed through a Superdex 75 column ($\phi 1 \times 30$ cm) previously equilibrated with 50 mM phosphate buffer (pH 7.3). (B) Anion exchange chromatogram. The fractions of the fourth peak on the Superdex 75 column were collected and concentrated to about 5 ml by PEG-20000 and then loaded onto a Resource Q column ($\phi 1 \times 10$ cm) equilibrated with the same buffer. The column was eluted with a linear salt gradient of 0–1.0 M NaCl in the buffer.

Results and Discussion

Purification of 24 kDa protein from TB

The 24 kDa protein from tartary buckwheat seeds was purified by gel filtration and ion-exchange chromatography. In the gel filtration chromatogram by Superdex 75 column, five protein absorbance peaks appeared (Fig. 1A). The target protein (peak 4) was collected, concentrated, and then further separated on an anion exchange column (Resource Q). During the elution procedure with a linear salt gradient of 0–1.0 M NaCl, the target protein peak tuned out at a salt concentration of about 0.5 M NaCl, detected by the absorbance value at a wavelength of 280 nm (Fig. 1B). The recovery was 1.5% by comparing the protein content in each step, which showed a reasonable purification effect by the separation methods.

The results of the electrophoresis analysis and the gel filtration chromatography (Sephacryl S-200) showed that peak I on Resource Q column was the target protein and that its molecular mass was about 24 kDa. The homogeneity of the purified protein was confirmed by SDS-PAGE (Fig. 2).

Amino acid composition

Amino acid composition was analyzed according to the method described above. The data were calculated

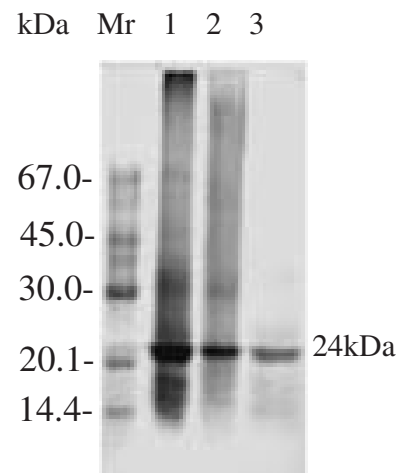


Fig. 2. SDS-PAGE Analysis of Purified Target Protein.

Line Mr, protein standard; line 1, crude extract; line 2, separated on gel filtration column; line 3, separated on anion exchange column.

Table 1. Amino Acid Composition of the TB 24 kDa Protein

Amino acid	Residues/molecule	Amino acid	Residues/molecule
Asx	21.6(22)	Thr	14.3(14)
Ser	17.1(17)	Glx	30.3(30)
Pro	8.3(8)	Gly	35.4(35)
Ala	18.8(19)	Val	13.6(14)
Ile	9.4(9)	Leu	13.4(13)
Tyr	3.2(3)	Phe	2.1(2)
His	3.5(4)	Lys	9.8(10)
Arg	15.2(15)	Cys	2.1(2)

on the basis of the protein molecular mass and the internal standard. An additional sample was hydrolyzed for 72 h in the same way to calibrate the sluggish release of valine and isoleucine. The values for threonine and serine were corrected for hydrolytic loss by linear and first-order extrapolation to hydrolysis respectively. The amino acid composition of the 24 kDa protein is shown in Table 1. It contained 215 amino acid residues and was particularly rich in glutamic acid plus glutamine and glycine. An approximate estimation of the value of the iso-electric point was about 5.6 with 30% of glutamic acid higher than 15% of arginine. The content of tryptophan was analyzed by the method of Edelhoch,²⁷⁾ but wasn't detected because it was extremely low.

Heat stability of the purified 24 kDa protein

To determine the heat stability of the purified protein kept in solution, a crude extract of 24 kDa protein from tartary buckwheat seeds was added to 50 mM phosphate buffer (pH 7.5) containing 1% EDTA. The mixture was incubated at 100 °C for 10, 20, and 60 min respectively. The protein composition before and after the incubation treatment was detected by polyacrylamide gel electrophoresis with a running gel of 12.5% polyacrylamide containing 0.1% sodium dodecyl sulfate (Fig. 3). Then

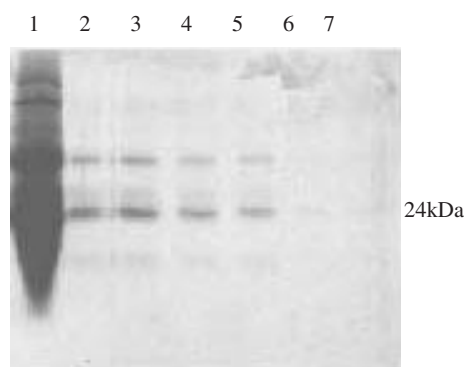


Fig. 3. Heat Stability of the 24 kDa Protein.

Line 1, crude protein; lines 2–3, sample incubated for 10 min at 100 °C; lines 4–5, sample incubated for 20 min at 100 °C; lines 6–7, sample incubated for 60 min at 100 °C.

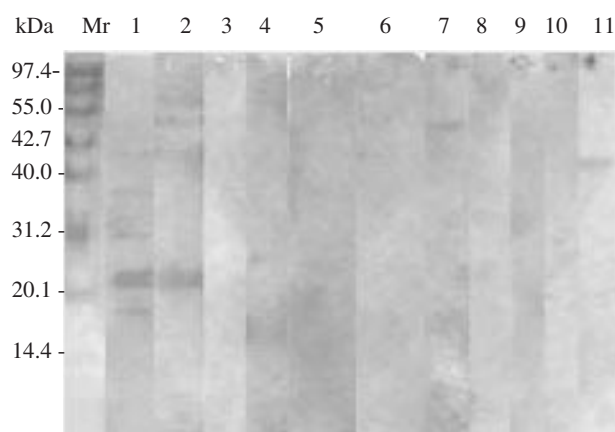


Fig. 4. IgE Immunoblotting Features of 11 Allergic Subjects.

Mr, protein standard; lines 1–2, sera of patients allergic to buckwheat; lines 3–11, sera of patients allergic to other plants.

the gel was stained with Coomassie Brilliant Blue (CBB) G-250. The final protein patterns were recorded using a Gel Documentation System. Figure 3 shows that all proteins except 24 kDa and 35 kDa disappeared on the SDS-PAGE profile (lines 2–5) after the protein solution was incubated for 10 and 20 min. Only after incubation for 60 min at 100 °C did the 24 kDa protein begin to disappear (lines 6–7), which indicated that the protein was thermostable under the heating conditions examined.

Immunoblotting analysis

Immunoblotting analysis showed the presence of the allergenic proteins with IgE binding activity (Fig. 4). The molecular mass of these proteins varied from 20 kDa to 90 kDa. The sera of the two patients allergic to TB showed the strongest IgE binding activity with the protein band of 24 kDa, indicating that TB24 kDa was the major allergen. In the sera of patients allergic to other plants, No. 7 and No. 11 showed a slight IgE binding activity with proteins, ranging from 40 kDa to

Table 2. Results of ELISA Analysis (OD₄₅₀ nm Absorbance Value)

	No. 1 serum	No. 2 serum	Negative Comparison
300 µg/ml	0.057	0.100	0.032
200 µg/ml	0.025	0.097	0.030
100 µg/ml	0.107	0.044	0.042
50 µg/ml	0.028	0.027	0.032
P/N	3.6 > 2.1	3.3 > 2.1	

55 kDa, but without specific IgE binding with 24 kDa protein from tartary buckwheat.

The results of ELISA analysis are listed in Table 2. Two sera named No. 1 and No. 2 from allergic patients showed positive reactions (P/N > 2.1), and an obvious protein band appeared at the site of 24 kDa. This indicated that the 24 kDa protein from tartary buckwheat seeds might be a major allergen with strong IgE binding activity to buckwheat-allergic patients. This appears to be very significant in the searching for the cause of allergy to buckwheat foods, and provided useful data for investigating the homology of allergens from indifferent plants.

Tartary buckwheat (*Fagopyrum tartaricum*) has been regarded as one of the most important food sources in the southwest and other regions of China.¹⁾ But there are fewer reports concerning buckwheat allergy. In this investigation, purification and identification of a protein with a molecular mass of 24 kDa from tartary buckwheat seeds is reported for the first time. An approximate estimation of the pI is about 5.6, with 30% of glutamine higher than 15% of arginine. The immunobiochemical results indicated that the extracts from tartary buckwheat seeds contained many kinds of allergenic proteins in the range 20–60 kDa, because they appeared as positive reactions on immunoblotting with sera of 11 patients allergic to different plants. But one protein component with a molecular mass of approximately 24 kDa was found to be the major allergen in tartary buckwheat because of the strongest binding activity in 2 sera of patients clinically reported as allergic to buckwheat.

After the crude protein was separated on the Resource Q column, fraction I with the 24 kDa protein was checked by ELISA against the collected sera of the patients. The results suggested that the 24 kDa protein had higher allergenic activity in comparison with other proteins of tartary buckwheat. For allergens from different origins in nature, it has been found that isoallergens with different molecular masses exist in the same organ or tissue, but they appear to have less similarity in allergic symptoms in patients.²⁸⁾ An estimate from many researchers considered that this might relate to different antigen determinant sequences in different allergens. This provides us reasonable suggestions for investigation of the reaction mechanism of allergy through studying the molecular characteristics of the major allergen in tartary buckwheat and the amino acid sequence homology analysis among different allergenic proteins in nature.

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