

Cloning, Expression, and Identification of Immunological Activity of an Allergenic Protein in Tartary Buckwheat

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A cDNA fragment encoding a 24 kDa allergenic protein in tartary buckwheat was obtained using reverse transcription PCR, 3'-rapid amplification of cDNA ends (RACE), and nest PCR. The cDNA clone contained 768 nucleotides, including 588 nucleotides in the open reading frame (ORF) and 180 nucleotides in the 3'-terminal sequence. The ORF encoded a functional protein of 195 amino acids. It shared 95% and 93% nucleotide homology with the allergenic storage protein and a legumin-like protein from common buckwheat respectively. The encoding region was expressed in host strain *Escherichia coli* BL21 (DE3) induced by IPTG at 28°C. The inclusion bodies of recombinant protein obtained were analyzed by western blot and purified by affinity chromatography. The purity of target protein reached above 95%. After they were refolded by step-wise dialysis, 68% of the inclusion bodies reached soluble state. An analysis of immunological activity showed that the recombinant protein had a specific IgE binding activity. This is the first report of the molecular cloning and expression of the major allergen from tartary buckwheat.

Key words: allergenic protein; gene sequence; expression; immunological activity; tartary buckwheat (*Fagopyrum tataricum*)

Buckwheat is a dicotyledonous crop and includes two cultivated species: common buckwheat (*Fagopyrum Esculentum*, CB) and tartary buckwheat (*Fagopyrum Tataricum*, TB). In some countries of Asia, Europea and Latin America, common buckwheat is more commonly grown, while tartary buckwheat is mainly grown in southwest and northwest areas of China.¹⁾ Buckwheat, containing up to 15% proteins and being rich in essential amino acids, such as lysine, methionine, and tryptophan,

is generally regarded as a plant with high nutritional value beneficial for health.^{2–4)} In addition, high contents of bioactive substances such as flavone-like compounds have showed significant effects in preventing diseases such as diabetes, hypertension, arthritis, etc.^{5,6)} But buckwheat can also cause allergy and even anaphylaxis in a few subjects.^{7–9)} The first report about buckwheat hypersensitivity was published in 1909.¹⁰⁾ One globulin protein with a molecular mass of 22 kDa or 24 kDa from common buckwheat was considered to be a major allergen,¹¹⁾ and its nucleotide sequence has been reported by Nair.¹²⁾ With high nutritional and medical values, tartary buckwheat has attracted more attention during recent years. Previous studies in our laboratory indicated that a 24 kDa protein from tartary buckwheat seeds was the major allergen.¹³⁾ In the present study, the gene encoding this 24 kDa protein was cloned (nucleotide sequence data reported are available in the GenBank databases under accession no. AY044918), and the protein was named tartary buckwheat allergen a (TBa). Then we successfully expressed the target protein in *Escherichia coli* BL21(DE3) and detected its immunological activity, which should lay a useful foundation for further study of the location of the epitope and reaction mechanism of this major allergen.

Materials and Methods

Materials. Tartary buckwheat seeds were harvested in Shouyang County of Shanxi Province, China. Avian myeloblastosis virus reverse transcriptase XL (AMV-RTase) was purchased from Promega (Madison, WI). *Taq* DNA polymerase for PCR was purchased from Takara (Dalian, China). Restriction endonucleases and other enzymes for molecular cloning were purchased from Sangon Company (Shanghai, China). A 3'-RACE

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Abbreviations: CB, common buckwheat; IgG-AP, IgG conjugated to alkaline phosphatase; IPTG, isopropyl-beta-D-thiogalactopyranoside; TB, tartary buckwheat; RACE, rapid amplification of cDNA ends; rTBa, recombinant tartary buckwheat allergen a; RT-PCR, reverse transcription PCR; TBa, tartary buckwheat allergen a

Kit was purchased from GibcoBRL (New York, USA). Expression vector pET-28a attached to a histidine tag at the N-terminal and host strain BL21(DE3) were kindly provided from Dr. Liang of Institute of Biotechnology, Shanxi University, China. Mouse anti-His antibody and Chelating HP Kit were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Goat anti-mouse IgG-AP and nitrocellulose membranes were purchased from Huamei (Shanghai, China). A microwell IgE EIA kit was purchased from Xinwan Company (Tianjin, China). Sera were from a patient allergic to buckwheat. All other reagents were of the highest purity available.

Total RNA extraction and cDNA synthesis. We immersed the tartary buckwheat seeds in distilled water for about 4 h, and then the seeds were germinated at room temperature in the following 2 d. The fresh radicles were collected and ground in liquid nitrogen, and the powder was then treated with RNA extraction buffer containing 100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 10 mM mercaptoethanol, and 1% sodium dodecylsulfate (SDS), pH 8.0. The total RNA was used to synthesize cDNA with AMV-RTase and an Oligo (dT).

Cloning of Tba cDNA. Based on the reported nucleotide sequence of allergenic protein of common buckwheat (CB)¹² and the N-terminal amino acid sequence of Tba obtained by MALDI-TOF-MS, two degenerated primers, primers I and II, were synthesized and used for PCR of the encoding region of Tba. PCR was carried out according to following amplification cycles: 94 °C, 1 min; 42 °C, 1 min; 72 °C, 1 min for 5 cycles; 94 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min for 30 cycles. The amplified PCR products were gel-purified and subcloned into pGEM-T Easy Vector for sequencing. The 3'-terminal nucleotide fragment was obtained by the 3'-RACE technique employing primer III and Abridged Universal Amplification Primer (AUAP). Analysis of the nucleotide and amino acid sequence homology was performed in GenBank by BLAST software. Comparison of amino acid sequence with that of other reported allergenic proteins was carried out in the Database of Food Allergens at AGMOBIOL (<http://ambl.lsc.pku.edu.cn>).

The primers used for cloning *Tba* cDNA were as follows:

Primer I: 5'-GGA TTG GAR CAA GCV TTC TGY AAC CT-3'

Primer II: 5'-AAC WAT RGA GAA ACG YTC CCT CTC CT-3' thereinto, R: (A + G) V: (A + G + C) Y: (C + T) W: (A + T)

Primer III: 5'-AACGCCATAACCGATCCGATT-3'

Primer AUAP: 5'-GGCCACGCGTCGACTAGTAC-3'

Construction of plasmid pET-28a-Tba and expression of recombinant Tba. According to the encoding sequence of Tba, two primers containing *Bam*HI and

*Hind*III sites were synthesized:

Sense primer: 5'-ATG GAT CCG GAT TGG AGC AAG CGT TC-3'

Antisense primer: 5'-ATA AGC TTT TAA ACT ATG GAG AAA CGT TCC-3'

The structure gene of Tba was PCR-amplified through 30 cycles of 94 °C 1 min, 60 °C 1 min, 72 °C 1 min. PCR products were subcloned into pGEM-T Easy Vector for sequencing. After it was found to be correct the vector was digested with *Bam*HI and *Hind*III. The fragment obtained was ligated into expression vector pET-28a to generate a recombinant plasmid, pET-28a-Tba. The plasmid generated was then transformed into host strain *E. coli* BL21 (DE3). The resulting engineered strain *E. coli* BL21(DE3)/pET-28a-Tba was grown in LB medium supplemented with kanamycin (50 µg/ml) at 37 °C. When the OD_{600nm} reached 0.6–0.8, IPTG was added to the medium at a final concentration of 0.1 mM and the culture was re-incubated at 28 °C for 3 h. The cells induced were harvested by centrifugation at 10,000 g for 30 min at 4 °C. Both the supernatant and the pellet were analyzed by SDS-PAGE using a 12.5% polyacrylamide gel.

Purification of the inclusion bodies of recombinant Tba. Purification of the inclusion body obtained from fragmentation and centrifugation described as above was first carried out by dissolving it with 8.0 M urea for 90 min at room temperature, and centrifuging it at 10,000 g for 30 min at 4 °C to collect the supernatant. Subsequently the supernatant was applied to a Ni²⁺-NTA agarose column pre-equilibrated with buffer I (20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 8.0 M urea, 1.0 mM 2-mercaptoethanol, pH 8.0). Finally, the bound proteins were eluted with buffer II (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 6.0 M urea, 1.0 mM 2-mercaptoethanol, pH 8.0).

Refolding of inclusion bodies of rTba. A step-wise dialysis method was adopted to refold the inclusion bodies.¹⁴ Dialysis buffers I, II, III and buffer IV (20 mM Tris-HCl, 500 mM NaCl, pH 9.0), containing 4 M, 2 M, 1 M, and 0 M urea respectively, were used to perform the refolding of the target protein *in vitro* at 4 °C for about 8 h. Then the solution was centrifuged at 10,000 g for 30 min at 4 °C to obtain the target protein and analyze it by SDS-PAGE. The protein concentration of purification steps was determined according to the method described by Bradford.¹⁵ Bovine serum albumin was used as the standard.

Western blot analysis of rTba. The purified protein was run on SDS-PAGE and transferred to nitrocellulose membrane using a TE-22 Blotter (Amersham Pharmacia Biotech, Uppsala, Sweden) at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) according to the manufacturer's guidelines. After blocking with defatted milk, the membrane

Table 1. Homologous Comparison of the Amino Acid Sequence of TBa with 11S Storage Globulin and Legumin-Like Protein

Protein source	Homology (%)
Allergenic protein; storage protein, legumin-like protein (<i>Fagopyrum esculentum</i>)	83–93
Citrin-sweet orange (<i>Citrus sinensis</i>)	57
Beta-globulin, legumin storage protein-upland cotton (<i>Gossypium hirsutum</i>)	49–58
Seed storage protein, legumin (<i>Ricinus communis</i>)	51–56
11S storage globulin, 11S storage protein-coffee (<i>Coffea arabica</i>)	58–59
Globulin, legumin (<i>Magnolia salicifolia</i>)	55–56
11S globulin (<i>Sesamum indicum</i>)	51
11S globulin-oat, 12S seed storage protein (<i>Avena sativa</i>)	48–52
11S globulin beta subunit-cucurbit (<i>Cucurbita pepo</i>)	51
Glutelin-rice (<i>Oryza sativa</i>)	51

was incubated with mouse anti-His antibody diluted (1:1,000) for 1 h and goat anti-mouse IgG-AP diluted (1:10,000) for 30 min at room temperature. In the end the membrane was stained for 1 h in AP color development solution.

Detection of immunological activity of refolded rTBa. The competitive ELISA method was used to detect the immunological activity of the rTBa as descriptions of the Microwell IgE EIA kit,¹⁶⁾ and modified slightly. The microwells were first coated with the crude natural allergen at 37 °C for 1 h. The allergen was previously obtained from tartary buckwheat seeds in our lab, then the purified rTBa mixed with the allergic patient's sera (1:1) were added into the well at 37 °C for a further 1 h. At the same time the control with purified natural TBa well was also incubated at 37 °C. After the reaction ended, the unemployed protein-sera mixture was washed off and the enzyme conjugate and substrate reagent were added successively. The color intensity of the mixture at 450_{nm} was detected with a micro titer plate reader (Bio-Rad, Co., California, USA). Inhibitory efficiency was calculated as the formula: $I = (W - X) / W$ (I, inhibitory efficiency; W, absorbance of the mixture of the natural allergen and sera at 450_{nm}; X, absorbance of the mixture of refolded protein and sera at 450_{nm}).

Results and Discussion

Cloning and sequence analysis of TBa cDNA

In the experiment, a 588 nucleotide fragment was cloned employing degenerate primers I and II. On sequence analysis, the fragment showed 95% homology with that of the gene encoding the 24 kDa allergenic protein from common buckwheat.¹²⁾ In addition, the start codon ATG was not found at the 5'-terminus of the fragment, it appears that the fragment encodes only a subunit or the C-terminal end of the legumin-like protein of tartary buckwheat. In many plants, the legumin-like protein is derived from a large protein precursor which is successively encoded and cleaved into two subunits in posttranslational processing.^{17,18)} To obtain a clone covering the 3'-terminal nucleotide sequence of TBa cDNA by the PCR technique, both primer III, designed

according to the 588 bp encoding sequence of TBa, and the 3'-RACE Abridged Universal Amplification Primer (AUAP) were employed. About 404 nucleotides with a 180 bp 3'-terminal nucleotide sequence were obtained. It was found by overlapping the two fragments of 588 bp and 404 bp that the full-length sequence of C-terminal of rTBa obtained was consisted of 768 nucleotides. The sequence was deposited in GenBank (accession no. AY044918).

Sequence analysis of the 768 bp fragment in GenBank with BLAST showed that the nucleotide sequence of TBa shared 95% and 93% homology respectively with the allergenic storage protein and legumin-like protein from common buckwheat. The deduced polypeptide contained 23 acidic amino acids (Asx and Glx) and 25 alkaline amino acids (Arg and Lys), and shared 93% and 83% homology with legumin-like proteins from common buckwheat and sword beans, 57% homology with citrin from sweet orange, and 59%–50% homology with a beta-subunit precursor of 11s globulin, legumin precursor, and seed storage protein from different plants such as castor-oil plant, coffee, magnolia, cucurbit, rice, and so on (Table 1). By comparing the deduced amino acid sequence of TBa with those of known allergenic proteins in the food allergen database (<http://ambl.lsc.pku.edu.cn>), it was found that the number of consecutive identical amino acids amounted to 5 or 6, EEAYK, EEEKEH, and KEEKE. Interestingly, the peptide of KEEKE at the site of 183–188 was almost common to allergens from different food materials, which suggests that this area might be the potential antigenic determinant in TBa.

Identification of recombinant pET-28a-TBa

Recombinant pET-28a-TBa was confirmed by restriction enzyme analysis. After plasmid pET-28a-TBa was digested by *Bam*HI and *Hind*III, the results showed that an anticipated 588 bp band was generated in the digested products. This indicates that the tartary buckwheat allergen a (TBa) gene was inserted into the expression vector pET-28a correctly. Furthermore, DNA sequence analysis of the recombinant plasmid also indicated that the reading frame was correct and that there was no mutation in the encoding region.

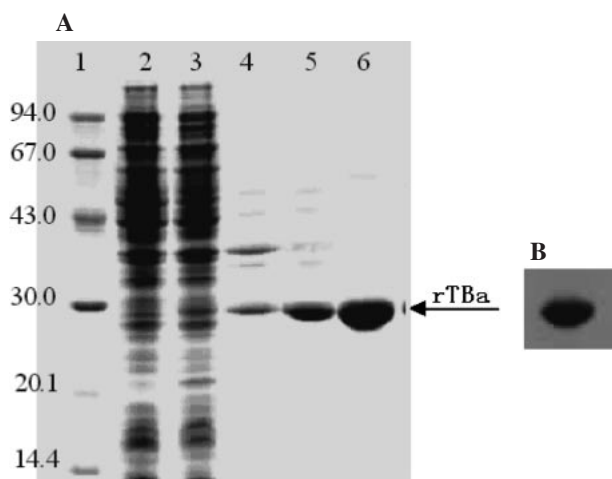


Fig. 1. SDS-PAGE Analysis and Western Blot of rTBa.

A, The total expression proteins were separated into supernatants and precipitates which were run in SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane 1, standard proteins marker (Pharmacia Bitotech, Uppsala, Sweden); lane 2, cell lysate of BL21(DE3)/pET-28a; lane 3, cell lysate of BL21(DE3)/pET-28a-TBa; lane 4, inclusion body of cell lysate of BL21(DE3)/pET-28a-TBa; lane 5, purified rTBa; lane 6, refolded rTBa. The arrow indicates the position of rTBa. B, Analysis of western blot of rTBa.

Expression and purification of rTBa

To find an optimal condition to increase the expression content of rTBa, different induction temperatures, of 16°C, 20°C, 28°C and 37°C, a gradient of IPTG concentration varying from 0.1 mM to 1.0 mM, and different induction times of 3 h, 4 h, and 6 h were tested. The results showed that the final concentration of 0.1 mM IPTG with an induction time of 3 h at 28°C was suitable to obtain enough rTBa. It was confirmed, at the same time, that rTBa was produced in the form of inclusion bodies under all available conditions. Comparing the expressed protein with the negative control from SDS-PAGE analysis, a new protein band with a molecular weight of about 24 kDa appeared in the sample precipitate (Fig. 1A, lane 3, 4), indicating that the protein was expressed mostly as inclusion bodies. Western blot analysis showed that the 24 kDa protein had a strong combination signal, implying that the induced *E. coli* BL21 (DE3) harboring pET-28a-TBa expressed the recombinant protein with 6 His-tags (Fig. 1B).

Refolding of inclusion bodies of rTBa

After two-step purification employing 8.0 M urea and affinity chromatography on an Ni²⁺-NTA agarose column, it was found that a large amount of non-target proteins were excluded from the expressed product. As Fig. 1A shows, purity of inclusion bodies increased greatly (lane 5), and succedent gel scanning analysis indicated that the content of inclusion bodies reached over 90%. During step-wise dialysis, to obtain a soluble target protein for immunological analysis, we chose a

Table 2. Detection of Immunological Activity of rTBa*

Sample	OD ₄₅₀	Inhibitory efficiency (%)
Crude allergen mixed with sera	0.373	—
Purified natural TBa mixed with sera	0.086	76.9
Recombinant TBa mixed with sera	0.184	50.7

*The concentrations of crude allergen, purified natural TBa, and recombinant TBa were all 0.4 mg/ml, and the patient's sera were diluted (1:5).

pH of 9.0 for dialysis solution. It was suitable to be kept away from the pI of the target protein (8.6) in case of protein aggregation. As Fig. 1A (lane 6) indicates some inclusion bodies became soluble after they were refolded by step-wise dialysis. In addition, determination of the protein concentration showed that the absorbance of the purified inclusion body solution at 595 nm was 0.338 and that the content of the rTBa was about 1.28 mg. The absorbance of the refolded protein solution at 595 nm was 0.326, and the content of the refolded rTBa was about 0.87 mg. So the soluble protein accounted for 68% of total inclusion bodies.

Detection of immunological activity of rTBa

To assay the immunological activity of soluble rTBa, the competitive ELISA method was used. As Table 2 shows, rTBa had a specific binding activity with IgE antibody in the sera of the patient allergic to buckwheat. The inhibitory efficiencies of purified natural TBa mixed with sera and recombinant TBa mixed with sera were about 80% and 51% respectively. The immunological activity of rTBa reached 66% of that of the natural TBa. The results described above indicate that the immunological activity of the recombinant allergen is similar to that of the natural allergen from tartary buckwheat seeds.

Tartary buckwheat is cultivated mainly in the southwest and northwest of China and some other Asian countries. Even though it is an underutilized crop, it remains important for food security in the temperate and hilly regions. Compared with common buckwheat, tartary buckwheat has special nutritional and medical values.^{19–21} It contains rich amino acids and abundant vitamin B1, B2, dietary fiber, proteins, minerals and vitamin P,^{22,23} which reduces the cholesterol concentration in the blood, but there are fewer reports concerning buckwheat allergy. In this investigation, cloning, expression, and identification of immunological activity of an allergenic protein (TBa) from tartary buckwheat is reported for the first time. Success in obtaining the valid cDNA sequence of TBa and the corresponding recombinant protein expressed in prokaryotic system make good preparation for subsequent research on locating the antigen epitope, analyzing the relationship between structure and function, and understanding the allergenic mechanism on the molecular level.

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