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# Molecular Cloning, Recombinant Expression, and Immunological Characterization of a Novel Allergen from Tartary Buckwheat

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Buckwheat is generally regarded as a nutritionally rich food source. However, earlier studies prove that it also causes allergies to subjects. Allergenic proteins with a strong IgE-binding activity have been identified in common buckwheat (CB) and a 24 kDa allergen (rTBa) in tartary buckwheat (TB). The objective of this research was to clone and express a novel allergen in tartary buckwheat and to evaluate its structure and immunological activity. The 1773 bp full-length cDNA was amplified and cloned from the total RNA of TB by polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) methods. Its nucleotide sequence had high similarity with legume-like 13S storage protein mRNA in CB. The deduced amino acid sequence included a putative signal peptide and 18 fragments as its epitope sites. The predicted full-length TB allergen sequence was found to have two domains, and the recombinant protein reacted with sera from patients with positive IgE binding to buckwheat and had a lower binding ability than the recombinant TBa and recombinant TBb (C- and N-terminal amino acid sequence of *TBt* codes for protein). This fact suggests that full-length TB allergen may hydrolyze to two domains *in vivo*, decreasing the IgE-binding ability.

**KEYWORDS: Allergenic protein; immunological characterization; protein expression; sequence analysis; tartary buckwheat**

## INTRODUCTION

Buckwheat belongs to the family Polygonaceae and includes two cultivated species: common buckwheat (CB, *Fagopyrum esculentum*) and tartary buckwheat (TB, *Fagopyrum tataricum*). The grain, which contains essential amino acids, such as lysine, threonine, and tryptophan, is a rich source of bioactive substances (*1*). Furthermore, buckwheat is likewise found to help cure hemorrhagic diseases and has beneficial effects on metabolism (*2, 3*). However, earlier studies on buckwheat hypersensitivity have noted that it is similar to allergies caused by soybean and peanuts (*4*), stressing that buckwheat could induce wheezing, urticaria, vomiting, and anaphylactic shock in certain people (*5, 6*).

In recent decades, scientists have identified allergens of CB (*7*) and confirmed that buckwheat allergy is an IgE-mediated immediate-type reaction. Some natural allergenic proteins from CB with different molecular weights of 6, 9, 19, 24, and 60−70 kDa have been identified (*8*−*10*), and among them, the 24 kDa protein was regarded as the main allergen (*10*). In addition, a seed storage protein in CB was identified, FA02, consisting of two separate components: a 41.3 kDa α subunit and a 21 kDa $\beta$ subunit (*11*). However, reports on the allergenic composition of TB are limited. Previously, a 24 kDa protein was isolated from TB seed (*12*), and the related gene was also cloned (GenBank accession number AY044918) and expressed (*13*). They were designated as TBa and rTBa, respectively. Analysis of their immunological activity showed that both proteins had a specific IgE-binding activity. Fujino and co-workers (*11*) reported that a bigger allergenic protein may exist in buckwheat, consisting of two domains sharing the same promoter in the nucleotide sequence. After expression and maturation of the intact protein in cells, it could be cleaved into two domains.

**Table 1.** Primers Designed for Cloning the Full-Length cDNA of Allergic Protein in Tartary Buckwheat

| fragments | primers | sequences (5′−3′) | position |
|---|---|---|---|
| TBa | P1 | GGA TTG GAR CAA | 960 |
| | | GCV TTC TGY AAC CT | |
| | P2 | AAC WAT RGA GAA | 1545 |
| | | ACG YTC CCT CTC CT | |
| | | [R (A + G) V (A + G + C) | |
| | | Y (C + T) W (A + T)] | |
| TBb | P3 | CAA CGC TCC AGG CAG AGT GAG | 417 |
| | P4 | TCC TTC GTC | 1224 |
| | | TCC AAC AAC CTG | |
| 3′ end | P5 | AAC GCC ATA | 1334 |
| | | ACC AGT CCG ATT | |
| 5′ end | P6 | CTC ACG GGA | 714 |
| | | TTG GCG GTC | |
| | P7 | TTC TGG TGC | 473 |
| | | TGG TCC CCG | |

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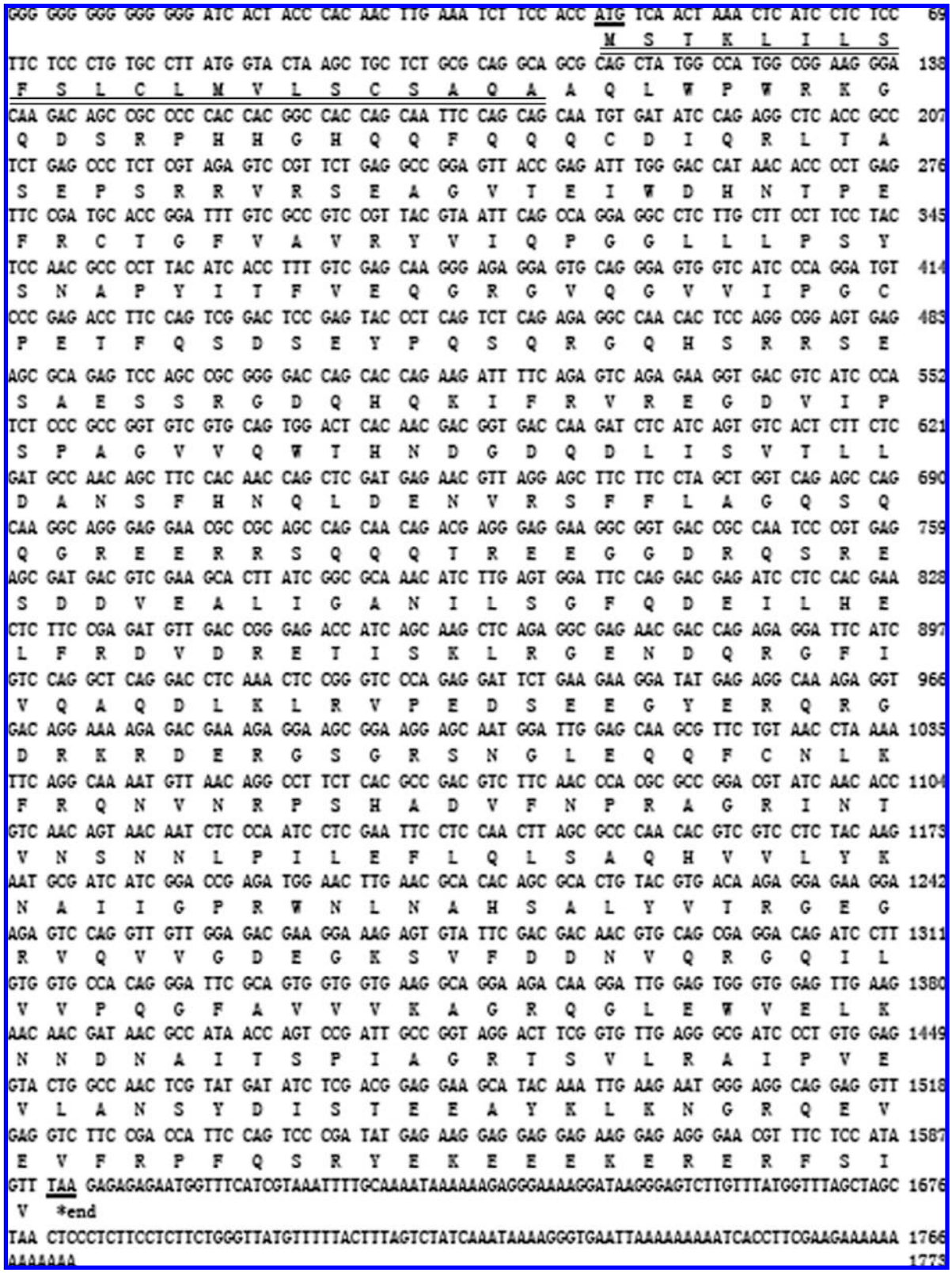


**Figure 1.** Nucleotide sequence of *TBt* and deduced amino acid sequence. The start and stop codons are underlined. The predicted signal peptide sequence is double-underlined.

In this study, we first cloned the full-length cDNA, designated *TBt*, encoded a 56 kDa allergen in TB based on the *TBa* gene, analyzed the homology of nucleotide, deduced amino acids, and carried out the prediction of disulfide bridges, signal peptide, secondary structure, and antigen sites. Then, we expressed and purified the recombinant protein. Specific IgE-binding activity with sera from patients who are hypersensitive to buckwheat was also performed.

## MATERIALS AND METHODS

**Materials.** Tartary buckwheat was field-grown at the Science Academy of Agriculture in Shanxi Province, China. Taq DNA

**Table 2.** Homology Comparison between *TBt* and Relative Gene from Common Buckwheat

| names | GenBank accession number | homology (%) |
|---|---|---|
| *F. esculentum* major allergenic storage protein (FAGAG1) mRNA, complete cds | AF152003 | 91 |
| *F. esculentum* mRNA for legumin-like protein, complete cds | D87980 | 91 |
| *F. esculentum* mRNA for legumin-like protein, complete cds | D87982 | 93 |
| *F. esculentum* allergenic protein gene, partial cds | AF216801 | 93 |
| *F. esculentum* legumin-type protein gene, partial cds | AY245536 | 90 |
| *F. esculentum* legumin-like 13S storage protein (LEG2) mRNA, partial cds | AY536050 | 89 |

polymerase for polymerase chain reaction (PCR) was obtained from TaKaRa (Dalian, China). Restriction endonucleases and other enzymes for molecular clone were purchased from Sangon (Shanghai, China). The 3′ rapid amplification of cDNA end (RACE) and 5′ RACE kits were purchased from GIBCOBRL (New York). All other reagents were ensured to be at their highest purity.

**Serum Specimens.** The sera of two patients were obtained from the Blood Center of Taiyuan, China, and with a history of respiratory, dermatologic, or gastrointestinal symptoms occurring within 1 h following ingestion of buckwheat. The CAP *in vitro* examination (CAP system; Pharmacia, Uppsala, Sweden) gave a value of 42.3 and 18.5 kilo-units/L, respectively, for buckwheat-specific IgE (normal value < 0.35 kilo-units/L). Two healthy sera from volunteers not allergenic to buckwheat were also used.

**Extraction of Total RNA.** Immature seeds at 20 days after flowering (DAF) of TB were collected. Total RNA was isolated as described in a previous report (*14*).

**Molecular Cloning of Full-Length cDNA Encoding TB Allergenic Protein.** On the basis of the nucleotide sequence of *TBa* and the known allergenic protein from CB, several primers were designed by ourselves (**Table 1**). Three templates of cDNA were synthesized as follows: cDNA I using the isolated total RNA and oligo dT primer, cDNA II for the 3′-end clone using adapter primer [5′-GGCCACGCGTCGAC-TAGTAC $(dT)_{17}$-3′] according to the direction of the 3′ RACE kit, and cDNA III for the 5′-end clone using primer P4 according to the direction of the 5′ RACE kit, respectively.

The C-terminal fragment (*TBa*) of allergenic protein in TB was synthesized by PCR through the P1 and P2 primers and by using cDNA I as the template. Similarly, the N-terminal fragment (*TBb*) of allergenic protein in TB was obtained with the P3 and P4 primers and by using cDNA I as the template. With a pair of primers P1 and abridged universal amplification primer (AUAP) (5′-GGC CAC GCG TCG ACT AGT AC-3′), the 3′ end of cDNA was first amplified using cDNA II as the template. Then, this first PCR reaction was used in a nested PCR with a set of primers P5 and AUAP. With a pair of primers abridged anchor primer (AAP) (5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′) and P6, the 5′ end of cDNA was first amplified using cDNA III as the template. Then, the obtained PCR products were used as a template for the nested PCR with primer AUAP and P7. PCR products were purified on a 1% agarose gel and cloned into the pGEM-T Easy vector (Promega, Madison, WI) for sequencing (ABI Prism, Foster City, CA). On the basis of the overlapping regions of four fragments, a full-length cDNA of TB allergen was then generated.

**Data Analysis.** A sequence comparison was performed in the GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). Signal peptide prediction was carried out using the SignalP 3.0 server. The PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred) was used for secondary-structure prediction. Clustalx 1.83 and Njplot 95 were used for phylogenetic analysis. Antigenic epitope prediction was also performed using the method of Kolaskar and Tongaonkar by the antigenic server (http://bioweb.pasteur.fr/seqanal/ interfaces/antigenic.html).

**Expression and Purification of the Full-Length TB Allergen.** The plasmid containing the *TBt* gene as the template and the forward primer (5′-AT GGA TCC GCG CAG CTA TGG CCA TGG-3′) and reverse primer (5′-AT AAG CTT TTA AAC TAT GGA GAA ACG TTC CC-3′) were used for PCR. The PCR product was digested and ligated between the *Bam*H I and *Hin*d III cloning sites of a modified vector pE-32m (the thioredoxin coding region was removed). After transformation into host strain *Escherichia coli* BL21 (DE3), the resulting engineered strain was grown in LB medium supplemented with ampicillin (100 $\mu$g/mL) at 37 °C. After the $OD_{600\ nm}$ reached 0.6−0.8, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 0.2 mM and the culture was reincubated at 28 °C for 3 h. After the total cell culture was lysed, the recombinant protein was harvested by centrifugation at 10000*g* for 30 min at 4 °C and purified using a HisTrap affinity purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). A recombinant TB allergen, designated rTBt, was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a 10% polyacrylamide gel.

**Western Blot Analysis of rTBt.** The purified rTBt was suspended in the SDS sample buffer with a standard reducing condition, boiled at 100 °C for 5 min, electrophoresed on a 10% SDS−PAGE with a 4% polyacrylamide stacking gel, and transferred to a nitrocellulose membrane using a TE-22 Blotter (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the guidelines of the manufacturer. After blocking with 5% (w/v) defatted milk, the membrane was incubated with mouse anti-His antibody (1:1000) for 30 min and then with goat anti-mouse IgG-AP dilution (1:10 000) for 30 min at room temperature. Finally, the membrane was stained for 1 h in AP color development solution.

**SDS−PAGE and IgE Immunoblotting.** SDS−PAGE was carried out as described above. The transferring membranes were cut into 4 mm widths, blocked by 5% defatted milk, and then incubated overnight at room temperature with the sera of patients and control sera (diluted 1:5 in TBS). After washing, the strips were incubated at room temperature for 60 min with horseradish-peroxidase (HRP)-conjugated mouse anti-human IgE (Southern Biotech, Birmingham, AL) and reacted with 2,2-azino-bis-3-ethybenzthiazoline-sulfonic acid (ABTS) solution.

**Enzyme-Linked Immunosorbent Assay (ELISA).** ELISA inhibition was performed to detect the immunological activity of the rTBt (*15*). The crude allergen extracted from tartary buckwheat seeds as described previously (*11*) was used as a control (inhibitor). After overnight coating with 40 $\mu$g/mL crude allergen at 4 °C, the 96-well polystyrene plate was washed with 10 mM phosphate-buffered saline at pH 7.4 containing 0.05% (v/v) Tween 20 (PBS−T) and blocked with 1% bovine serum albumin in PBS−T. Subsequently, it was incubated with serum samples (1:50) with PBS−T or 200 $\mu$g/mL purified rTBt, as well as rTBa and rTBb in PBS−T at 37 °C for 1 h. The plate was then incubated with 1:1000 HRP-conjugated mouse anti-human IgE. The plate was further washed and developed with ABTS solution. After the reaction, the color intensity of the mixture was detected at 405 nm with a microtiter plate reader (Bio-Rad, Hercules, CA). Inhibitory efficiency was calculated using the formula: $I = (W - X)/W$, where $I$ is the inhibition, $W$ is the absorbance with the use of un-inhibited sera, and $X$ is the absorbance with the use of inhibited sera.

## RESULTS

**Analysis of Nucleotide Sequence.** To obtain the full-length cDNA of TB allergenic protein, we first cloned four cDNA fragments by using 3′ RACE, 5′ RACE, and nested PCR. Then, on the basis of the overlapping region, four partial sequences amplified by PCR were combined to align the full-length cDNA (**Figure 1**). This full-length cDNA (GenBank accession number DQ849083) contained an open-reading frame (ORF) encoding

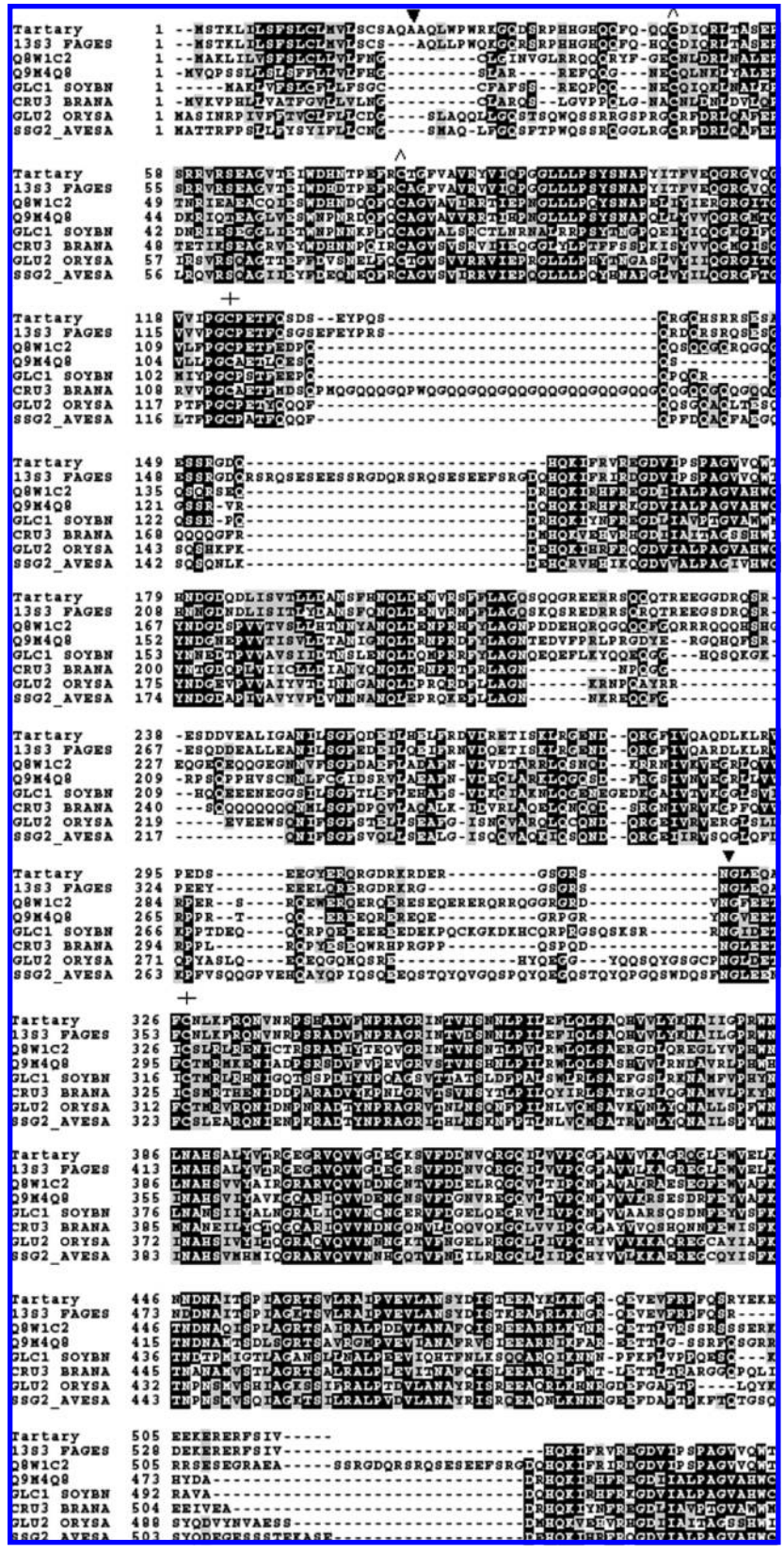

**Figure 2.** Alignment of amino acid sequences deduced from nucleotide sequences of TBt and other seed storage proteins. The identical and similar residues are shown on a black background and on shaded boxes, respectively. The arrow indicates the predicted cleavage site between the N and C domains. Cysteine residues marked by a plus (+) are involved in interchain bridge, and those marked by a caret (^) might be involved in the intrachain bridge. The text in the figure refers to the entry name of Uniprot Proteins databanks. Tartary (TBt), 13S3_FAGES (13S globulin seed storage protein 3 precursor from *F. esculentum*; NCBI TaxID, 3617), Q8W1C2 (11S globulin-like protein from *Corylus avellana*; NCBI TaxID, 13451), Q9M4Q8 (legumin-like protein from *Ricinus communis*; NCBI TaxID, 3988), GLC1_SOYBN (glycinin G1 precursor from *Glycine max*; NCBI TaxID, 3847), CRU3_BRANA (11S globulin from *Brassica napus*; NCBI TaxID, 3708), GLU2_ORYSA (glutelin type II precursor from *Oryza sativa*; NCBI TaxID, 4530), and SSG2_AVESA (12S seed storage globulin precursor from *Avena sativa*; NCBI TaxID, 4498).

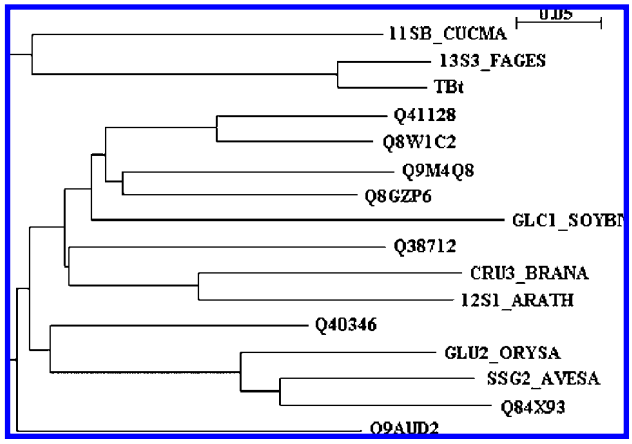


**Figure 3.** Phylogenetic tree is based on the amino acid sequences of the basic subunit of storage proteins and legumin-type proteins of various angio- and gymnosperms. The text in the figure refers to the entry name of Uniprot Proteins databanks.

**Table 3.** Antigenic Epitopes Prediction of *TBt*

| number | sequences | score | length | position | max score position |
|---|---|---|---|---|---|
| 1 | FRCTGFVAVRYVIQPGGLLL PSYSNAPYITFV | 1.200 | 32 | 78−109 | 87 |
| 2 | LPILEFLQLSAQHVVLYKNAI | 1.194 | 21 | 359−379 | 372 |
| 3 | VQGVVIPGCPET | 1.190 | 12 | 115−126 | 121 |
| 4 | RGQILVVPQGFAVVVKAG | 1.185 | 18 | 418−435 | 431 |
| 5 | DLISVTLLDAN | 1.172 | 11 | 185−195 | 189 |
| 6 | RTSVLRAIPVEVLANSY | 1.167 | 17 | 458−474 | 464 |
| 7 | AHSALYVT | 1.148 | 8 | 388−395 | 391 |
| 8 | EGDVIPSPAGVVQW | 1.121 | 14 | 164−177 | 172 |
| 9 | RVQVVGD | 1.112 | 7 | 400−406 | 402 |
| 10 | RSFFLAGQ | 1.109 | 8 | 206−213 | 208 |
| 11 | GFIVQAQDLKLRVP | 1.106 | 14 | 282−295 | 287 |
| 12 | EVEVFRPFQS | 1.105 | 10 | 490−499 | 494 |
| 13 | VEALIGANILSG | 1.091 | 12 | 242−253 | 245 |
| 14 | QAFCNLK | 1.088 | 7 | 324−330 | 328 |
| 15 | HADVFNP | 1.084 | 7 | 340−346 | 341 |
| 16 | HHGHQQFQQQCDIQRL | 1.083 | 16 | 37−52 | 49 |
| 17 | DEILHELFRD | 1.082 | 10 | 256−265 | 261 |
| 18 | QKIFRV | 1.078 | 6 | 157−162 | 159 |

a protein of 515 amino acid residues. By using the SignalP database (SignalP 3.0 server, http://www.cbs.dtu.dk/services/SignalP/), we found that the full amino acid sequence contained 22 residues (MSTKLILSFSLCLMVLSCSAQA) as the N-terminal signal peptide (double-underlined area in **Figure 1**), while the mature protein consisted of 493 residues with a calculated molecular weight of 56 026.8 Da. The predicted amino acid sequence of the TB allergen was 93% homologous to the *Fagopyrum esculentum* allergenic protein gene (GenBank accession number AF216801), 91% homologous to the *F. esculentum* major allergenic storage protein (FAGAG1) mRNA (GenBank accession number AF152003), and 89% homologous to the *F. esculentum* legumin-like 13S storage protein (LEG2) mRNA (GenBank accession number AY536050) (**Table 2**). This study has identified a full-length gene in TB.

**Primary and Secondary Structure Analyses.** A comparison of amino acid sequences between the TB allergen and other seed storage proteins showed that the deduced amino acid sequence of TB allergen contained less cysteines. Only one pair of disulfide bridges covalently linking the α and $\beta$ subunits in legumin-like proteins was reported (*16, 17*). In the case of the TB allergen, the cysteine residues were strictly conserved at amino acids 47, 80, 123, and 327 (**Figure 2**). These cysteines

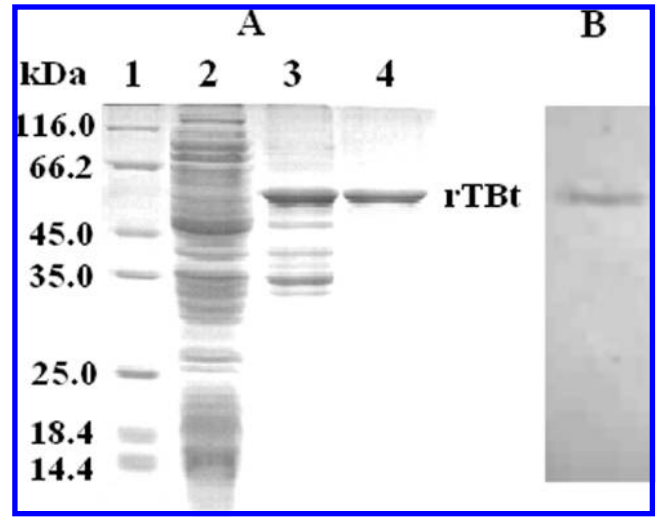


**Figure 4.** SDS−PAGE analysis and western blot of rTBt. (A) Total expression proteins are separated into supernatants and precipitates that were run in a 10% SDS−PAGE gel and stained with Coomassie Brilliant Blue. Lane 1, standard proteins marker (Pharmacia Bitotech, Uppsala, Sweden); lane 2, supernatant of BL21/pE-32m-*TBt*; lane 3, precipitate of BL21/pE-32m-*TBt*; lane 4, purified rTBt. (B) Western blot analysis of rTBt.

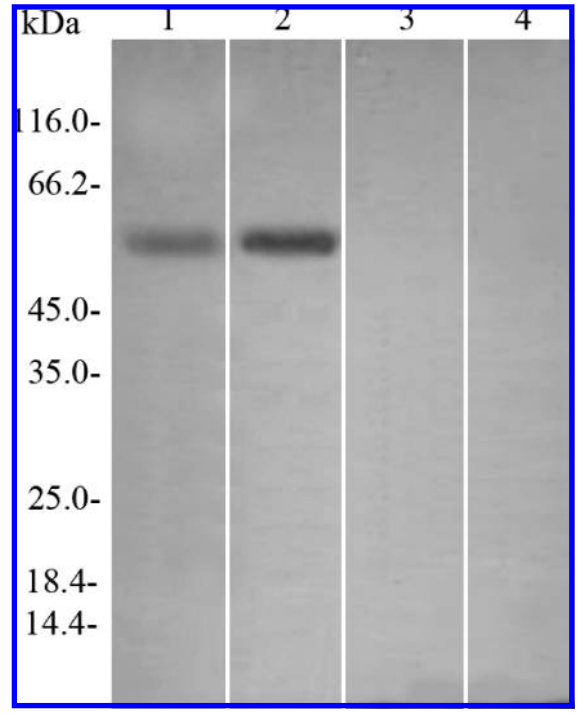


**Figure 5.** IgE immunobloting of rTBt using sera from allergenic and non-allergenic individuals. Purified rTBt was electrophoresed in 10% SDS−PAGE and immunoblotted using two buckwheat-allergenic sera (lanes 1 and 2) and two control sera (lanes 3 and 4). The standard proteins marker is indicated on the left.

formed an intrachain disulfide bridge ($Cys_{123}$−$Cys_{327}$) and an interchain disulfide bridge ($Cys_{47}$−$Cys_{80}$). In addition, the cleaved TB allergen generated two fragments, with a predicted molecular weight of 24 and 34 kDa. The secondary-structure prediction according to the deduced amino acid sequence of TB allergen indicated that the full-length protein has 13.79% α helices, 30.49% $\beta$ sheets, and 55.73% random coils.

**Phylogenetic Analysis and Prediction of Epitopes.** A phylogenetic tree was plotted on the basis of the amino acid

**Table 4.** Detection of Immunological Activity of rTBt, rTBa, and rTBb[a]

| | colorimetric intensity ($OD_{405}$)/inhibitory efficiency (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| samples | number 1 serum | | number 2 serum | | number 3 serum | | number 4 serum | |
| control | 0.505 | — | 0.411 | — | 0 | — | 0 | — |
| rTBt | 0.399 | 21 | 0.299 | 27.3 | 0 | — | 0 | — |
| rTBa | 0.256 | 49.3 | 0.233 | 43.3 | 0 | — | 0 | — |
| rTBb | 0.294 | 41.8 | 0.243 | 40.9 | 0 | — | 0 | — |

[a] The colorimetric intensity of all samples reacted with number 3 and 4 sera are nearly 0. The symbol "—" means the inhibitory efficiency has no value. Number 1 and 2 sera gave a value of 42.3 and 18.5 kilo-units/L, respectively, for buckwheat-specific IgE *in vitro* examination.

sequences of various storage proteins and legumin-type proteins. **Figure 3** showed that the TB allergenic protein was close to 13S globulin (seed storage protein) from CB and far from glycinin G1 precursor from the soybean and legumin precursor from anise magnolia.

On the basis of epitope prediction, the matured allergenic protein in TB included 18 fragments as its epitopes, and each epitope had a max score amino acid (the underlined one in **Table 3**), which is thought to be the central amino acid and considered to mostly result in allergy.

**Expression, Purification, and Western Blotting of rTBt.** The rTBt was expressed as 6× His-tagged protein according to the instructions of the manufacturer (Qiagen, Inc., Valencia, CA). In comparison of lanes 1 and 2, a new protein band with a molecular weight of about 56 kDa appeared in the precipitate (lane 3 in **Figure 4A**), indicating that the protein was expressed as inclusion bodies. After the recombinant protein was purified by affinity chromatography (as described in the Materials and Methods), SDS−PAGE analysis showed that the purity of rTBt was >95% (lane 4 in **Figure 4A**). Western blot analysis indicated that the recombinant protein has a strong hybridized signal, implying that induced bacterial cells harboring the recombinant plasmid, pE-32m-*TBt*, can express the target protein rTBt (**Figure 4B**).

**IgE Immunoblotting of rTBt.** Immunoblotting analysis was performed to clarify whether rTBt reacts with sera from patients with positive IgE binding to buckwheat. The purified rTBt was electrophoresed under a reduced condition and using two sera from patients allergenic to buckwheat and two control sera. As shown in **Figure 5**, recognition was observed around the 45.0−66.2 kDa region. It means that rTBt can react with IgE of the sera of buckwheat-allergenic individuals but not the control sera.

**ELISA.** To determine IgE reactivity of the rTBt under nondenaturing conditions, an ELISA inhibition was performed, using rTBt, rTBa, and rTBb, and above two buckwheat positive and two negative sera samples. The data in **Table 4** showed that two serum samples from patients allergenic to buckwheat were significantly reactive with the three recombinant allergens. The inhibitory efficiency of two positive samples mixed with rTBt was 20.9 and 27.2%, respectively, and the value was lower compared to the sera mixed with rTBa and rTBb. The other serum samples (numbers 3 and 4) from two normal laboratory volunteers were not significantly reactive with any of the three tartary buckwheat allergens (rTBt, rTBa, and rTBb). Previous reports have revealed that TBa and rTBa can be bound specifically by IgE from buckwheat-allergenic patients (*12, 13*). The present data indicated that rTBt, similar to rTBa, can be used to detect buckwheat-specific IgE, further implying that rTBa and rTBb had a higher IgE-binding activity than rTBt.

## DISCUSSION

Allergenic reactions to CB have been reported in many different fields (*10, 15, 18*), and the number of studies relating to TB have increased over recent years (*12, 19, 20*). In comparison to CB, TB was noted to have special nutritional and medical values (*21, 22*). In a previous study, we cloned and expressed a 24 kDa protein from TB and confirmed its immunological activity (*13*). In the present work, we isolated the full-length TB cDNA encoding a 56 kDa allergenic protein, and it was a longer version of the 24 kDa protein reported previously. It consisted of 1773 bp, which corresponds to 515 amino acid residues. Analysis of the nucleotide sequence and the deduced amino acid sequence revealed that this sequence shared a high homology with CB major allergenic storage protein mRNA and CB legumin-like 13S storage protein mRNA. This may be a 13S protein fragment in tartary buckwheat (*23*). To better understand the characteristics of the full-length allergenic protein in TB, structure and antigenic site prediction were performed. The secondary-structure prediction of the deduced amino acid sequence showed that the 56 kDa protein contained more $\beta$ sheets and random coils compared to $\alpha$ helices. We hypothesize that $\beta$ sheets and random coils in the structure play a more important role in the reaction to allergy (*24, 25*). The 18 central amino acids predicted by the antigenic server may be the key for the determination of the binding IgE ability (*26*). We are trying to analyze the immunological activity of rTBt with mutation at these specific residues.

Apart from this, the predicted structure of the TB allergen was also found to have two structural domains, which were cut at the putative cleavage site (**Figure 2**). It had the same cleavage pattern as other seed storage proteins (*16, 17*). The deduced N- and C-terminal domains were noted to be about 34 and 24 kDa, respectively. In dicotyledonous plants, the most common type of globulin is the 11−12S globulin (*11*), which is an oligomeric protein, composed of two polypeptides, a 30−40 kDa $\alpha$ subunit and a 20 kDa $\beta$ subunit, linked by a disulfide bond (*17*), which is similar to the results offered by our present study. This means that the 56 kDa protein in TB may hydrolyze into two domains (C and N domains) *in vivo*. Another of our research results also suggested that a protein with larger molecular weight (about 170−200 kDa) from tartary buckwheat seed was unstable and easily digested *in vitro* (data no shown) and that the degraded fragment could be continuously cut down to more low-molecular-weight peptides. It was earlier reported that allergenic proteins in separation may degrade rapidly, and protein degradation is considered a complex process involving a successive action of several proteolytic enzymes (*27, 28*).

Fujino's experiment has indicated that the 42−45 and 63 kDa polypeptides in CB were less reactive to the anti-serum compared to the 23−25 kDa ones (*11*). Our ELISA also showed that the inhibitory efficiency of either rTBa or rTBb was higher than that of rTBt in this study. The results demonstrated that the full-length allergenic protein (rTBt) was shown to be less reactive to the serum IgE of a patient compared to the rTBa and rTBb, which is in agreement with Fujino's reports that CB allergenic protein can hydrolyze into two domains. The possible reason for the increased IgE-binding ability of rTBa or rTBb is that these separated fragments from rTBt may possess higher immunological activity, with their epitope emerging from inside the molecule during cleavage.

In conclusion, we demonstrated that the 56 kDa protein in TB belongs to the 13S globulin family. It obviously reacted with serum from patients hypersensitive to buckwheat, but the

binding ability was lower than either rTBa or rTBb. Despite current limitations to explain the lower immunoreactivity of TBt compared to the possible degraded fragments, it may be considered as a new TB allergen. These results are very useful for further research or understanding the molecular mechanism of a major allergen in tartary buckwheat, as well as its application in the diagnosis of clinical reactivity.

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