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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  $\alpha$  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

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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
ТВа	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	

GGG	GGG	GGG	GGG	GGG	ATC	ACT	ACC	CAC	AAC	TIG	AAA	ICI	ICC	ACC	ATG	ICA	ACT	AAA	CIC	ATC	CIC	ICC	69
															M	S	T	К	L	I	L	S	
TIC	TCC	CIC	TGC	CII	ATG	GTA	CTA	AGC	TGC	TCT	GCG	CAG	GCA	GCG	CAG	CTA	TGG	CCA	TGG	CGG	AAG	GGA	138
F	S	L	С	L	M	v	L	S	с	S	A	0	A	A	Q	L	A	P	a	R	к	G	
CAA	GAC	AGC	CGC	CCC	CAC	CAC	GGC	CAC	CAG	CAA	TIC	CAG	CAG	CAA	IGI	GAT	ATC	CAG	AGG	CIC	ACC	GCC	207
Q	D	s	R	P	н	н	G	н	Q	Q	F	Q	Q	Q	с	D	I	Q	R	L	т	A	
TCT	GAG	CCC	TCT	CGT	AGA	GIC	CGT	TCT	GAG	GCC	GGA	GII	ACC	GAG	ATT	TGG	GAC	CAT	AAC	ACC	CCT	GAG	276
s	Ε	P	s	R	R	v	R	s	Е	A	G	v	т	Ε	I	a	D	н	N	т	P	Ε	
TIC	CGA	TGC	ACC	GGA	ш	GIC	GCC	GIC	CGT	TAC	GTA	ATT	CAG	OCA	GGA	GGC	CIC	TTG	CII	CCT	TCC	TAC	345
F	R	с	т	G	F	v	A	v	R	Y	v	I	Q	P	G	G	L	L	L	P	s	Y	
TCC	AAC	GCC	CCT	TAC	ATC	ACC	TTT	GIC	GAG	CAA	GGG	AGA	GGA	GTG	CAG	GGA	GIG	GIC	ATC	CCA	GGA	TGT	414
s	N	A	P	Y	I	т	F	v	Ε	Q	G	R	G	v	Q	G	v	v	I	P	G	С	
CCC	GAG	ACC	TTC	CAG	TCG	GAC	TCC	GAG	TAC	CCT	CAG	TCT	CAG	AGA	GGC	CAA	CAC	TCC	AGG	CGG	AGT	GAG	483
P	Ε	т	F	Q	s	D	s	Ε	Y	P	Q	s	Q	R	G	Q	H	s	R	R	s	Ε	
AGC	604	GLG	TCC	AGC	000	000	GLC	CIG	CAC	CAG	446	ΔΤΤ	TTC	AGA	GTC	AGA	GAA	COT	GAC	GTC	ATC	004	552
c		=		e c	P	6000	n	0	u	0	-		=	D	v	P	=	6	n	v	T	P	552
TCT	~~~~	2000	COT	CTC	CTC.	CLC.	TCC	ACT	CIC.		a.	COT.	cir.	~~~	CAT	CTC.	ATC	ACT	oto	ACT		ere .	621
	-		001			-	-	-		N.C.	D	001	D	Can	-		-			-			021
5					~~~~				A									5	~~~		-	-	
GAI	900	AAC	AGC	110	CAC	AAC	CAG		GAI	646	AAC	611	AGG	AGC	110	110	CIA	GCI	001	CAG	AGC	CAG	090
			5											5			-L	<u>.</u>		-	5	~	
CAA	GGC	AGG	GAG	GAA	CGC	CGC	AGC	CAG	CAA	CAG	ACG	AGG	GAG	GAA	GGC	GGI	GAC	CGC	CAA	ICC	OGI	GAG	759
Q	G	R	E	E	R	R	S	9	Q	Q	T	R	E	E	G	G	D	R	9	S	R	E	
AGC	GAT	GAC	GIC	GAA	GCA	CII	ATC	GGC	GCA	AAC	ATC	IIG	AGI	GGA	TIC	CAG	GAC	GAG	ATC	CIC	CAC	GAA	828
s	D	D	v	Е	*	L	I	G	*	N	I	L	s	G	F	Q	D	Ε	I	L	н	E	
CIC	TIC	CGA	GAT	GII	GAC	CGG	GAG	ACC	ATC	AGC	AAG	CIC	AGA	GGC	GAG	AAC	GAC	CAG	AGA	GGA	TIC	ATC	897
L	F	R	D	v	D	R	Е	т	I	s	K	L	R	G	Е	N	D	Q	R	G	F	I	
GTC	CAG	GCT	CAG	GAC	CIC	AAA	CIC	CGG	GIC	OCA	GAG	GAT	TCT	GAA	GAA	GGA	TAT	GAG	AGG	CAA	AGA	GGT	966
v	Q	A	Q	D	L	к	L	R	v	P	Ε	D	s	Ε	Ε	G	Y	Ε	R	Q	R	G	
GAC	AGG	AAA	AGA	GAC	GAA	AGA	GGA	AGC	GGA	AGG	AGC	AAT	GGA	TTG	GAG	CAA	GCG	TTC	TGT	AAC	CTA	AAA	1035
D	R	к	R	D	Ε	R	G	S	G	R	s	N	G	L	Ε	Q	Q	F	с	N	L	K	
TTC	AGG	CAA	AAT	GTT	AAC	AGG	CCT	TCT	CAC	GCC	GAC	GTC	TTC	AAC	OCA	CGC	GCC	GGA	CGT	ATC	AAC	ACC	1104
F	R	Q	N	v	N	R	P	s	H	A	D	v	F	N	P	R	A	G	R	I	N	т	
GTC	AAC	AGT	AAC	AAT	CTC	OCA	ATC	CTC	GAA	TTC	CTC	CAA	CTT	AGC	GCC	CAA	CAC	GTC	GTC	CTC	TAC	AAG	1173
v	N	S	N	N	L	P	T	L	E	F	L	0	L	S	4	0	н	v	v	L	Y	K	
AAT	GCG	ATC	ATC	GGA	OCG	AGA	TGG	AAC	TTG	AAC	GCA	cac	AGC	GCA	CTG	TAC	GTG	ACA	AGA	GGA	GAA	GGA	1242
N	4	T	T	G	P	R	W	N	1	N	4	н	s	4	1	v	v	т	R	G	F	G	
AGA	GTC	CAG	GTT	GTT	GGA	GLC	GLA	GGA	440	AGT	GTA	TTC	GLC	GLC	440	GTG	cic	064	GGA	CAG	ATC	CTT	1311
P	v	0	v	v	G	D	F	G	R	S	v	F	D	D	N	v	0	R	G	0	T	1	
CTC	GTO	000	CIG.	664	TTC		GTO	GTO	GTO	110		664	AGA	CIA	GCA	TTC	GIG	TOC	OTO	GIG	TTO	110	1380
		P	0	C	F	,		10		-	,	C	P	C	C		F		17	F		-	1000
ALC	110	CIT	ALC	~~~	174	100	ACT	~~~	ATT	~~~	ort.	100	ACT	TOO	oto	TTC	100		ATC	-	CTC	a	1440
N	No.	D			AIN .	ALCC -	A01	E	-	acc.	001	ROG	-	100			A00		AIC	P		-	1443
	N	0	N	Â.,	1		5	-	1			*		5	~		K	-	1	-		-	
GTA	CIC	CCC	AAC	ICG	TAT	GAT	ATC	ICG	ACG	GAG	GAA	GCA	TAC	AAA	TIG	AAG	AAT	GGG	AGG	CAG	GAG	GIT	1518
V	L	A	N	S	Y	D	1	S	1	E	E	A	Ŷ	x	L	K	N	G	R	Q	E	V	
GAC	GIC	TIC	CGA	OCA	TIC	CAG	ICC	CGA	TAT	GAG	AAG	GAG	GAG	GAG	AAG	GAG	AGG	GAA	CGI	TIC	ICC	ATA	1587
Ε	v	F	R	P	F	Q	s	R	Y	Ε	K	Е	Ε	Ε	K	Е	R	Е	R	F	s	I	
GII	TAA	GAG	ICTC!	ATG	GITIC	CATO	STAAL	IIII	IGCAL	LAAT.	LAAA	LAGAC	GGGA	AAGO	GATA	LCCC	GICI	TGT	TATO	GTTT	AGCI	TAGC	1676
v	*er	nd																					
TAA	CICO	CTCI	TCCT	ICTIC	CIGGO	TATE	IGITI	TTAC	TTT	GTCI	TATC	AATA	AAAA	GGT	GAATI	TAAAJ	LAAAJ	ATC	LCCTT	TOGAL	GAAA	AAA	1766
4444	4444																						1773

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 (%)
<i>F. esculentum</i> major allergenic storage protein (FAGAG1)	AF152003	91
mRNA, complete cds <i>F. esculentum</i> mRNA for legumin-like protein, complete cds	D87980	91
<i>F. esculentum</i> mRNA for legumin-like protein, complete cds	D87982	93
<i>F. esculentum</i> allergenic protein gene, partial cds	AF216801	93
<i>F. esculentum</i> legumin-type protein gene, partial cds	AY245536	90
<i>F. esculentum</i> 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)<sub>17</sub>-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 BamH I and Hind 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 µg/mL) at 37 °C. After the OD<sub>600 nm</sub> 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 10000g 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 antihuman 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 - 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

Tartary 1383 FAGES	1 NSTRLTISFELCINY SCSAQAAQLWPWRRGCOSRPHHOROCOCOCOCOTORTASE 1 NSTRLTISFELCINY SCS AQLIPWOROCORRPHHOROCOC- HOCDIORTASE
Q8W1C2 Q9M4Q8	1 MAKLILYSPSLOUTVIPNG
GLC1 SOYBN CRU3 BRANA	1 NARTY STATES
GLU2 ORYSA SSG2 AVESA	1 MASINEPITYPTTYCHPLYCCGSLAQQLLGCSTSQWQSSERGSPEGEEPEROAPE 1 MATTEPSELGYSYIFLCCGSMAQ-LFGCSPTPWQSSEGGLEGEEPEROAPE
	^
Tartary 1383 FAGES	58 SRRVRSEAGY DE INDENT PEPECTGFVAVRYVICPGGLLLPSYSHAPYI TEVEGGRGVCC 55 SRRVRSEAGY DE INDENT PEPECAGFVAVRYVICPGGLLLPSYSHAPYI TEVEGGRGVCC
Q8W1C2	49 THREFAEACOPSNDENDOOFCAOVAVEREDIEPHGLLLPCYSNAPDITTERGRGTTC 44 DIRFOTRAGAUSSNIPHEROOFCAOVAVEREDIEPHGLLLPSYSNAPCLYVVOGRGTTC
GLC1 SOYEN CRU3 BRANA	42 DIRRIES EGGLIETMERNINERS CAOVASRCTENRIAL REPSYING FOR TICOGIGIEG 48 TETTES FACENES WERENNED IRCACKENES I FOCOLUL PEPESED S SYNOCHOLOGIC
GLU2 ORYSA	57 TRSVRSCAGT BFFLYSMELFCCIOVSVVRRVIEPRGLLLPEVENCASLVVELOGRGITC 56 LEOVESCAGT BFFLYSMELFCCIOVSVVRRVIEPRGLLLPCVENAGASLVVELOGRGITC
	+
Tartary 1383 FAGES	118 WY INCOMPANY SIS EXPOS
Q8W1C2	
GLC1 SOYEN	
GLU2 ORYSA	117 PTFPCCPETYCQOF 116 TTPCCPETYCQOF 116 TTPCCPETYCQOF
BOOL_ATEDA	110 111 100 110 001
Tartary	149 ESEGDE
08W1C2	135 CSCRSEC
GLC1 SOYBN	121 CONTYR 122 CONTYR 122 CONTYNER 122 CONTYNER 122 CONTYNER 123 CONTYNER 124 CONTY
GLU2 ORYSA	168 GOOGOPR 143 SOSHXPK
SSG2_AVESA	142 SQBQNLK
Tartary 1383 FAGES	179 ENDOLODLISVINA ANSFERION ENVESTMACOSOOGREERRSOOTREEGGDROSR 208 ENNOUNDLISIII YLANSFORON ENVENTMACOSKOSREDRRSOROTREEGSDROSR
Q8W1C2 Q9X4Q8	167 YNDGE SPYVTYSLFHTNNYANOLE DYPRHTYLAGNPDDEHOROGOOF CORROQOUSHO 152 YNDGHEPVYTISYLETAN IGNOLERNPRDYLAGNTEDYPRLPRGDYE RGOHOF SR
GLC1 SOYBN CRU3 BRANA	153 YENEDT PWWAVSITETENSLENOLOOPERFYLAGNOLOEPLEYOOFGGHOSOKGE 200 YETGLOPLVIIGLLIAMYONOLEENPETFELAGN
GLU2 ORYSA	175 YNDGE YPWYAIYYTE INNGANOLEPRCEDILAGN
1383 FAGES	238 - ESDDVEALIGANNUSGIODIN HISTIRDVIRETISLIRGENI - ORGINVOADDLKLR 267 - ESODDEALLEANNUSGEEDINGIIRNVIQETISLIRGENI - ORGINVOARDLKLR
Q8W1C2 Q9H4Q8	227 EQGECEQQGEGNNYFSGADAAFT ADAAN -VEVETARRIGSNOE IRRNYKVEGRICVA 209 - RPSCPPHVSCNNEFGGIDSRVIAEAAN -VEEGLARRIGGGSE FRGSLVNVEGRIUVA
GLC1 SOYBN CRU3 BRANA	209 HQCEEENEGGSELSOFTLEFTEHASS-VERGENEGEDEGEDEGAAVTVRGGEST 240SCQQQQQQCHELSOFDPQVEACALX-IEVREAQEROROGESRCNAVRVRGPFQVA
GLU2 ORYSA SSG2_AVESA	219EVEEWSON HESOF STELL SEARCHIS CONBOLCONEORGEIVEWERGUSLI 217
Tartary	
1383 FAGES	
098408	265 RPR-T
CRU3 BRANA	294 REPLREPESEQUERPRGPP
SSG2_AVESA	263 EPPVSQQGPVERCAYOPIQSQ EQSTQYQVGQSPQYQEQSTQYQPGQSWDQSP
1383 FAGES	325 FONMAR SONVERPSEADULAPRAGENTUNSUNLPILESLOLSAGEVULTUNATCORNA 353 FONMAR SONVERPSEADULAPRAGENTULSUNLPILESLOLSAGEVULTUNATCORNA
28W1C2 29#4Q8	326 ICSIRINGNICTRSRADIYTEQVORINTVNSNILDVJR.LOLSARGDIOREGIYVPRWN 295 FCTMRHEDNIADPSRSDVFVPEVORVSTVNSENLPIIRWLOLSASEVVIRNDAVRPPRWE
CRU3 BRANA	316 ICTMRLEBNIGCTSSPLIYNFCACSVTTATSLDFPAFSWLRLSAEFOSFRINANFWPRYN 325 ICSMRTEBNIDDPARADVYNPNIGRVTSVNSYTLPILOYIRLSATROILGGNAMYPPLYN
SSG2_AVESA	312 POINEVERON IDNEMERATIVNER AGEVININGER DE LINLVOUS AVIVNINGENALLSE DE MA 323 POSLEARON IENPIRALTYNER AGEVININGEN DE LINLVOUS ATEVNINGEN ALLSE YMM
Tartary	386 INNESALWYIRGEGRVQVVGEEGRSVFFDENVQRGGIEVVFQGAVVVVKAGRGELENVELA
1353 FAGES 28W1C2	413 LNAHSALYVIRGEGRVQVVGLEGRSVPDDNVCRGQIDVVPQGAVVLXAGREGLENVELX 386 LNAHSVVYAIRGRARVQVVDDNGNTVPDDELRQQQVDTIDQNGAVAIRAESEGPENVAFX
298428 JLC1 SOYBN	355 INAHSVIYAVICCARIOVVDENCNSVPDGNVREGOVLTVPONSVVVRSESDRPSYVAPX 376 INANSXIYALNGRALIOVVNCNGERVPDGELOEGRVLIVPONSVVARSOSDNPSYVSSX
CRU3 BRANA GLU2 ORYSA	385 HNANEILYCTCCCARIOVVNDNCONVLDOOVOLCOLVVIPOGJAYWYOSHONNPEWISPX 372 INAHSIYYITCCBACVQVVNENGKTYPHGELRROOLDIVPOHYYVVAXACREGCAYIADA
SSG2_AVESA	383 INAHSVNHNIQGRARVQVVNNHGQTVFNDILRRGOLDITPOHYVULKABREGCQYISPX
Tartary	446 MIDHAITSPIAGETSVIENISVENISVENSVERSTEEAVKIMIGE-GEVEVERPOSKYEKE
1383 FAGES 28W1C2	473 NDDNAITSPIAGITSVLRAIPVEVLANSVDISTIEADRLENGR-CEVEVPRPOSR 446 TNDNACISPIAGRTSAIRALPDEVLANARCISREEARRLEVNR-CETTIVESSRSSSERI
298428 JLC1 SOYBN	415 TNDNANTSDISCRTSAVECHPVEVIANAPRYSIZEARRIIPAR - ESTTYG-SSRFCSCRR 436 TNETPHIGTLACANSLENALPEVICETPHILSSCARCIANN - PREPORT VPBORSC
CRU3 BRANA GLU2 ORYSA	445 TNANANYSTLAGETSALEALPLEVITNAPOTSLEEARRITENT-LETTITRARGOPOLI 432 TNPNSHYSHIAGESSIFRALPTOVLANAYRISREEACRITENEGDPGA5TDLOYK
SSG2_AVESA	443 INPRSEVECTACITEILERLEVEVELANAYRISROEACHLENNRGEEPEAFTPRETOTOSC
Tartary	
28W1C2	505 RRSESEGRAEA SSRGDQRSRQSESEEFSRGEQHQX IP IREGDV IP SPAGWYCH
JUC1 SOYBN	492 RAVA
JLU2 ORYSA	498 SYQDYNVAESS
10001	NU C NYCH POPERSTRY AS PARTY

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

					max
					score
number	sequences	score	length	position	position
1	FRCTGFVAVRYVIQPGGLLL	1.200	32	78-109	87
	PSYSNAPYITFV				
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	EVEVERPEQS	1.105	10	490-499	494
13	VEALIGANILSG	1.091	12	242-253	245
14	QAFC <u>N</u> LK	1.088	7	324-330	328
15	HADVFNP	1.084	7	340-346	341
16	HHGHQQFQQQCD <u>I</u> QRL	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  $\alpha$  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 nonallergenic 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<sub>123</sub>–Cys<sub>327</sub>) and an interchain disulfide bridge (Cys<sub>47</sub>–Cys<sub>80</sub>). 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%  $\alpha$  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<sup>a</sup>

		colorimetric intensity (OD405)/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	-				

<sup>a</sup> 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  $6 \times$  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 Nand 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 oligometric 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 lowmolecular-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

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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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