

Characterization of 16-kDa major allergen with α -amylase inhibitor domain in tartary buckwheat seeds

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ABSTRACT

Tartary buckwheat (*Fagopyrum tataricum*, TB) is an important functional food containing proteins with balanced amino acid composition and more flavonoids than common buckwheat (*Fagopyrum esculentum*, CB). Buckwheat contains highly potent allergens that trigger an allergic reaction via an IgE mediated response. In this work, the full-length cDNA encoding *Fag t 2* from tartary buckwheat seeds was cloned by screening the cDNA library of seed-filling period. The recombinant *Fag t 2* (r*Fag t 2*) expressed in *Pichia pastoris* SMD1168H was purified by purified by immobilized metal affinity chromatography. It demonstrated that *Fag t 2* was a major allergen in tartary buckwheat with the activity of IgE binding and pepsin resistance, along with the thermal stability. The identification of natural *Fag t 2* (n*Fag t 2*) confirmed that the *Fag t 2* protein belongs to the 2S albumin family, only existing in embryo. Most interesting, we discovered that *Fag t 2* had a α -amylase inhibitor domain near the end of C-terminal. The possible activity of α -amylase inhibitor of *Fag t 2* will be detected in subsequent studies.

1. Introduction

Buckwheat belongs to the family of *polygonaceae*, with two buckwheat species cultivated around the world: common buckwheat (*Fagopyrum esculentum*, CB) and tartary buckwheat (*Fagopyrum tataricum*, TB). Tartary buckwheat contains proteins with balanced amino acid composition, and more edible fiber and flavonoids than common buckwheat (Guo and Yao, 2006; Wieslander, 1996; Yoshioka et al., 2004b). Furthermore, the bioactive flavonoids have beneficial effects on preventing diabetes, hypertension, rheumatoid arthritis and other diseases (Liu and Zhu, 2007; Ren et al., 2001;). Buckwheat-derived foods, however, contained a number of different allergens, for buckwheat-allergic patients, the ingestion causes immediate-type anaphylactic reactions (Noma et al., 2001; Park et al., 2016; Wieslander, 1996; Young Kim et al., 2013). A survey of food-allergic patients showed that the proportion of buckwheat allergy cases reached 0.2%, and buckwheat was one of the five major allergenic foods (eggs, milk, wheat, buckwheat, and peanuts) (Imai and Iikura, 2003) and has been deemed as the potential problem of food allergy in 21 st century Britain (Sammur et al., 2011).

Studies on the buckwheat allergens mainly focused on common buckwheat. Several common buckwheat allergic proteins have been reported, such as 24-, 19-, 16-, 10-, 9- and 8-kDa allergens, which have been identified as major buckwheat allergens (Fujino et al., 2001; Matsumoto et al., 2004; Park et al., 2000; Yoshioka et al., 2004a).

Among them, the 16-kDa allergic protein from common buckwheat has been considered to be a strong candidate allergen of buckwheat because of its strong anti-pepsin activity associated with immediate hypersensitivity reactions. In addition, the 16-kDa allergic protein was thought to be related to the allergic shock in buckwheat-allergic patients (Tanaka et al., 2002). Until now, there are few reports on the low molecular weight allergen proteins and their related biological functions in tartary buckwheat. Previously, a 24-kDa allergic protein was isolated from tartary buckwheat seeds (Wang et al., 2004), which shares 93% nucleotide homology with a legumin-like protein from tartary buckwheat (Wang et al., 2006). Recently, a 56-kDa allergen of tartary buckwheat was identified and it also shared a high homology with CB major allergenic storage protein and legumin-like 13S storage proteins (Zhang et al., 2008).

In this paper, we isolated n*Fag t 2* from tartary buckwheat and tried to clone the full-length cDNA of the 16-kDa allergen gene from tartary buckwheat seeds based on screening the cDNA library of seed-filling periods. Then, r*Fag t 2* were expressed in *Pichia pastoris* and purified. Patients IgE binding activity, pepsin resistance and thermal stability of r*Fag t 2* and n*Fag t 2* was characterized. All of these works will lay the foundation for the further mapping of allergenic epitopes and revealing the biological function of *Fag t 2*.

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2. Materials and methods

2.1. Construction of seed-filling period cDNA library

The total RNA of tartary buckwheat (Yu 6-21) seeds from seed-filling period was purified by using the Concert™ plant RNA Reagent (Invitrogen, USA) and treated with RNase-free DNase I (Takara Bio, Dalian, China) to avoid genome DNA contamination. A cDNA library was constructed using the SMART cDNA Library Construction Kit (Clontech, USA). The RT step was performed using PowerScript reverse transcriptase with the SMART IV oligonucleotide primer and the CDS III/3' PCR primer provided in the kit. The double-stranded cDNA (ds-cDNA) was synthesized by long distance PCR (LD-PCR) with the 5'PCR primer and the CDS III/3'PCR primer using the Advantage 2 PCR kit (Clontech, USA). After digestion of proteinase K at 45 °C for 20 min, the ds-cDNA was then digested by SfiI and fractionated by CHROMA SPIN-400 Column before directionally cloned into dephosphorylated λTriplEx2. The ligation mixtures were packed with MaxPlax™ Lambda Packaging Extracts (Epicenter, Madison, USA). The recombinant rate was 99.58%, the average insert cDNA size was 1.0 kb, and the size range from 0.5 to 3.0 kb, the titer of unamplification library was 1.3×10^6 pfu/ml.

2.2. Screening and molecular cloning of cDNA encoding a 16-kDa tartary buckwheat protein

The conversion of λTriplEx2 phagemid to a pTriplEx2 plasmid was carried out based on the manufacturers' instructions. One thousand well-isolated colonies were selected randomly for plasmid purification. Plasmid DNA was sequenced using an ABI 3730XL DNA Analyzer (Beijing AoKe Biotechnology). Screening primer X1 (TCCGAGATCTGGACGAGC) or primer X2 (TAATACGACTCACTA TAGGG) was used for sequencing the 5'- or 3'-end of the cDNA inserted, respectively. Removal of vector contaminated, the sequences were aligned to NCBI. The ORF of sequences were obtained with ORF Finder and then aligned to BlastP database. The information of query sequences was recorded and clustering analysis. One insert cDNA sequence analysis showed that the gene of 16-kDa protein was in high-abundance (0.19%). The isolated plasmid was named pTriTBW to be used for further analysis. The coding sequence was deposited in GenBank with accession No. HQ829975.

2.3. cDNA sequences and structure analysis of fag t 2

The cDNA sequences and deduced amino acids sequences were compared with sequences from GenBank database using Blast online software. Molecular weight and pI calculation were conducted using the ExPASy Proteomics online software. Signal peptide prediction was performed using SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). The prediction of secondary-structure, molecular modeling and functional structure were conducted using the PSIPRED protein structure prediction server (<http://bioinf.cs.ucl.ac.uk/psipred/>), Swiss-model (<http://swissmodel.expasy.org/SWISS-MODEL.html>) and Smart (<http://smart.embl-heidelberg.de>). The deduced amino acid sequences were compared with relative allergenic proteins. Phylogenetic analysis was constructed by CLUSTA LX 2.0 and Njplot 95 program. Antigenic epitope prediction was also performed using the MOBYLE online software (<http://bioweb.pasteur.fr/seqanal/interfaces/antigenic.html>).

2.4. Overexpression and purification of fag t 2 in Pichia pastoris

The cDNA with 384 bp (from 67 to 450 bp) encoding a functional protein of 127 amino acid residues (from 23 to 149) was amplified using the 5' primers (5'-GAAAAGAGAGGCTGAAGCTAGAGATGAAGGCTTCGATTTAG-3') and 3'primers(5'-TCAATGATGATGATGATGAT

GCACATAATACCAGTTTCCTCTAG-3') and the recombinant plasmid pET47b-Fag t 2 template. The PCR products contained the overlap fragments of pPICZ(α)A vector at the sides of Fag t 2 sequence. The PCR products were recombined with pPICZ(α)A vector using the SeamLess cloning kit (Takara Bio, Dalian, China). The recombinant products were transformed into *Escherichia coli* TOP10. Then recombinant plasmids were linearized and then transformed into *Pichia pastoris* SMD1168H by electroporation at 1.5 kV, 5 ms with Electroporator (Bio-Rad, USA). Positive clones were selected on YPD plates supplemented with 500 µg/ml Zeocin, and then transferred to the liquid YPD for growth. The clones incubated with liquid YPD were identified with sequencing primer 5'AOX1 and 3'AOX1. All the PCR reactions preformed using PfuTurbo DNA polymerase (Stratagene, USA). The PCR product was purified using QIA quick PCR Purification Kit (Qiagen, Germany).

The correct clone was inoculated in 20 ml BMGY medium and incubated in 28 °C, 200 rpm. And then the cell pellets were harvested by centrifugation at 4 °C for 10 min when the OD_{600nm} of the culture reached to 2.0–6.0. The cell pellets were resuspended with BMMY and inoculated to BMMY medium with inoculums size of 10%. The methanol were added at intervals of 24 h with the final concentration of 1% and induced for 72 h. The supernatant was harvested after 72 h by centrifugation at 4 °C, 8000g for 10 min.

The supernatant of Fag t 2 were dialyzed at 4 °C against dialysis buffer A (0.01 mol/L Tris-HCl, pH 8.0) for 12 h. And then dialyzed supernatant of Fag t 2 was purified by immobilized metal affinity chromatography. The Fag t 2 were eluted with the buffer B(0.01 mol/L Tris-HCl, pH 8.0, 150 mM NaCl) contained 150 mM imidazole at a flow rate of 0.5 ml/min. The Fag t 2 containing fractions were pooled and concentrated by ultrafiltration. The purified Fag t 2 were used for SDS-PAGE and stored at -20 °C for future use.

2.5. Specificity of rabbit polyclonal antiserum raised against rFag t 2 to nFag t 2

The production of rabbit (New Zealand white rabbits) polyclonal antiserum raised against rFag t 2 (recombinant Fag t 2) was performed by ProteinTech Group (WuHan, China). The serum from the immunized rabbit was used for Western blotting analysis.

The tartary buckwheat seeds protein extract was obtained by extracting in 1 × loading buffer (pH8.0 50 mmol/L Tris-HCl) at 4 °C overnight. The tartary buckwheat seeds protein extract and purified r Fag t 2 were mixed with an equal volume of 2 × SDS-PAGE sample buffer, boiled at 100 °C for 5 min, electrophoresed in 13.5% SDS-PAGE and transferred to a 0.15 µm nitrocellulose membrane (S&S, Dassel, Germany). The membranes were blocked with 5% defatted milk-PBS (pH 7.0) at room temperature for 10 h. After three times' washing (10 min/time), the membrane was incubated at room temperature for 3 h with diluted rabbit polyclonal antiserum (1:600). Then the washed membrane was incubated at room temperature with diluted (HRP)-conjugated Goat anti-Rabbit IgG (1:4000) for 3 h, followed by a reaction with a colorimetric substrate solution (0.1% DAB and 0.3% H₂O₂).

2.6. Location and identification of natural fag t 2 in seeds

0.5 g defatted seed flour was extracted by shaking with 2 ml distilled water at 20 °C for 4 h (albumin extract) and centrifuged at 10000g for 10 min. After water extraction, the flour was extracted with 2 ml of 50 mM NaCl at 20 °C for 4 h (globulin extract) and centrifuged at 10000g for 10 min. Each extraction was repeated two times in order to remove all the protein of each fraction. Seeds total protein was obtained by extracting in 1 × loading buffer at 4 °C overnight. The buffer of 10 mM HCl (pH 2.0) was also used for the protein extraction. All these extracts were subjected to 13.5% SDS-PAGE and western blotting analysis using rabbit polyclonal antiserum.

The defatted seed flour was extracted with HCl (pH 2.0, 10 mM HCl) and subjected to 13.5% PAGE followed by incubation with pepsin

GCC GGG GAT CAA CCA CAA CAT CAA ACC CTA AAA ACC CAG AAA <u>ATG</u> AAG CTC TTC	54
	M K L F 4
ATC ATC CTA GCA ACA GCC ACT CTC CTA ATC GCT GCA ACT CAA GCA <u>AAG</u> TAC CTG	108
I I L A T A T L L I A A T Q A <u>K</u> Y <u>L</u>	22
AGA GAT GAA GGC TTC GAT TTA GGC GAA ACC CAG ATG AGC TCA AAG TGC <u>ACG</u> CGA	162
R D E G F D L G E T Q M S S K C <u>T</u> R	40
CAA GTG AAG ATG <u>ATG</u> GAG CCA <u>GAA</u> CTC <u>GTG</u> AAA TGT AAC CGT TAC ATA GCC ATG	216
Q V K M <u>M</u> E P <u>E</u> L <u>V</u> K C N R Y I A M	58
GAT ATA <u>ATC</u> GAC GAC AAA TAC <u>GAA</u> GAA GCT TTG AGT AGG <u>ATT</u> CAA GGT GAA GGA	270
D I <u>M</u> D D K Y <u>E</u> E A L S R <u>I</u> Q G E G	76
TGC <u>GAG</u> AGT GAA GAG <u>AAG</u> TGT <u>CTG</u> AGA GGA <u>TGT</u> TGT GTG GCG ATG AAG GAG ATG	324
C <u>E</u> S E E <u>K</u> C <u>L</u> R G C C V A M K E M	94
GAA GAT GAA TGT GTT <u>TGC</u> GAG TGG ATG AAG ATG ATG GTT GAG AAT CAA <u>AAC</u> GGG	378
<u>E</u> D E C V C E W M K M M V E N Q K G	112
CGC ATT GGT GAA <u>ACG</u> TTG ATC <u>AGG</u> AAG GGG <u>ATT</u> AGG GAT TTG AAG GAA TTG CCT	432
R I G E <u>T</u> L <u>M</u> R <u>K</u> G <u>I</u> R D L K E L P	130
AAT AAG TGT GGG <u>ATT</u> AGT GAA <u>ATG</u> GAA TGT <u>CAC</u> TCT AGA GGA AAC <u>TGG</u> TAT TAT	486
<u>N</u> K C G <u>I</u> S E <u>M</u> E C <u>H</u> S R G N <u>W</u> Y <u>Y</u>	148
GTG <u>TAA</u> TTT GGT TGT TGA GTT GTT TGA TGA ATAAGT AGA TGA CTT GTT CTC TCT	540
V *	149
TGT GGC TAT ATA TGC TAT TGG AGA GGG AAG AAG TTT GTG TGT TGT AAG TTG TTG	594
TGT TAA GCA ACT GAA ATA TAT GAG CAC TTA TCA ACC GTA AAA AAA AAA AAA AAA	648
AAA AAA AAA AAA CAT GT - poly (A)	668

Fig. 1. Nucleotide sequence of *Fag t 2* cDNA and the deduced amino acid sequence.

The predicted signal peptide sequence is shaded, and the start and stop codons are underlined. Sequences different from the 16-kDa allergen sequence from common buckwheat (DQ304682) are indicated in white against black background.

solution (2 mg/ml) for 45 min. After pepsin digestion, the gel was used for western blotting analysis. A replica gel was stained by CBB R250, the band size corresponding to nFag t 2 (nature Fag t 2) was cut out and electric eluted using the Electro-Eluter (Model 422, Electro-Eluter, Bio-Rad). Eluted protein was confirmed by western blotting and Mass Spectrometric identification (MALDI-TOF-MS).

The eluted protein was also transferred to PVDF membrane using Bio-Rad semi-dry system for N-terminal amino acid sequencing with a PE Applied Biosystems Model 473A protein sequencer. In order to further identify the location of nFag t 2 in tartary buckwheat seeds, embryo and endosperm were separately isolated from tartary buckwheat seeds according to the method reported previously (Ličen and Kref, 2005), followed by SDS-PAGE and Western blotting analysis, and detected by eECL Western Blot Kit (Cwbio, CHN).

2.7. IgE binding assay based on immunoblotting and ELISA

SDS-PAGE for immunoblotting was carried out as described above. The transferred membranes were blocked by 5% defatted milk, and then incubated for 12 h at room temperature with serum from patients allergic to buckwheat or control serum (diluted 1:5 in PBS). After washing, these strips were incubated at room temperature with 1:1000 diluted HRP-conjugated goat anti-human IgE antibodies (KPL, Gaithersburg MD, USA) for 3 h, followed by reacting with 3, 3', 5, 5'-Tetramethylbenzidine (TMB) solution.

The IgE binding activity of rFag t 2 was determined by competitive ELISA as reported previously (Wang et al., 2006). ELISA plates (Corning, USA) were coated overnight at 4 °C with seed soluble protein (concentration of 1.5 µg/100 µl/well) extracted from tartary buckwheat seeds as described previously (Fujino et al., 2001) in coating solution (50 mM sodium carbonate buffer, pH 9.0). After washing, the plates were blocked with 1% BSA-PBS (pH 7.0) for 3 h at room temperature, then incubated with patient's serum (1:50) (provided by buckwheat allergic patients) which separately pre-mixed with 0.1% BSA-PBS, 150 µg/ml purified rFag t 2 and nFag t 2 extract (extracted with pH 2.0, 10 mM HCl, not fully purified allergen) diluted to 1:100 in 0.1% BSA-PBS (pH 7.0) at 37 °C for 1 h respectively. After washing, the plates were then sequentially incubated with HRP-conjugated goat anti-human IgE antibodies (1:1000) and reacted with a colorimetric

substrate solution (0.2% TMB and 0.03% H₂O₂). Reactivity was detected at 450 nm by using microplate readers (Spectra Max M2, Molecular Devices, USA). The calculation of inhibitory efficiency (I) was carried out according to the method described previously (Zhang et al., 2008). Formula: $I = (A_{\text{total protein}} - A_{\text{rTBW16}} \text{ or } A_{\text{nTBW16}}) / A_{\text{total protein}}$. "A" represents colorimetric intensity (OD₄₅₀).

2.8. Thermal stability and pepsin resistance test of fag t 2

Purified rFag t 2 protein and nFag t 2 extract were heated at 98 °C for 0, 30, 60, 90, 120 and 150 min, and the supernatant was analyzed by 13.5% SDS-PAGE. In addition, the gel of nFag t 2 extract proteins was subjected to western blotting analysis using rabbit polyclonal antiserum. The remaining samples heated at 98 °C for 120 min were used for ELISA analysis.

The purified rFag t 2 protein and nFag t 2 extract protein, which were heated at 98 °C for 120 min, were used for the pepsin digestion test according to the method reported previously (Koyano et al., 2006). Porcine pepsin (Sigma, USA) was prepared for 5 ml simulated gastric fluid (0.7 mg/ml pepsin; 2 mg/ml NaCl, pH 2.0). The simulated gastric fluid was incubated at 37 °C for 5 min before the addition of 100 µl pre-incubated recombinant Fag t 2 protein solutions (4.7 mg/ml), nFag t 2 extract protein (5.4 mg/ml) or BSA protein (4.5 mg/ml). At each scheduled time point (0.5, 2, 5, 8, 15, 30 and 60 min), 100 µl of the mixture was removed and added to a separate centrifuge tube containing 200 µl 2 × SDS loading buffer and 100 µl of 200 mM Na₂CO₃ followed by boiling at 98 °C for 5 min immediately and then SDS-PAGE was conducted. Two sample control tubes (without pepsin) and two pepsin control tubes (without sample) also preformed according to above procedure, and the corresponding volume in the control tubes were replaced by control solution (2 mg/ml NaCl, pH 2.0).

3. Results

3.1. Analysis of nucleotide sequence

The cDNA of *Fag t 2* (GenBank accession No. HQ829975) contained a 450 bp open-reading frame (ORF) encoding the protein of 149 amino acid residues (Fig. 1). The SignalP software (<http://www.cbs.dtu.dk/>)

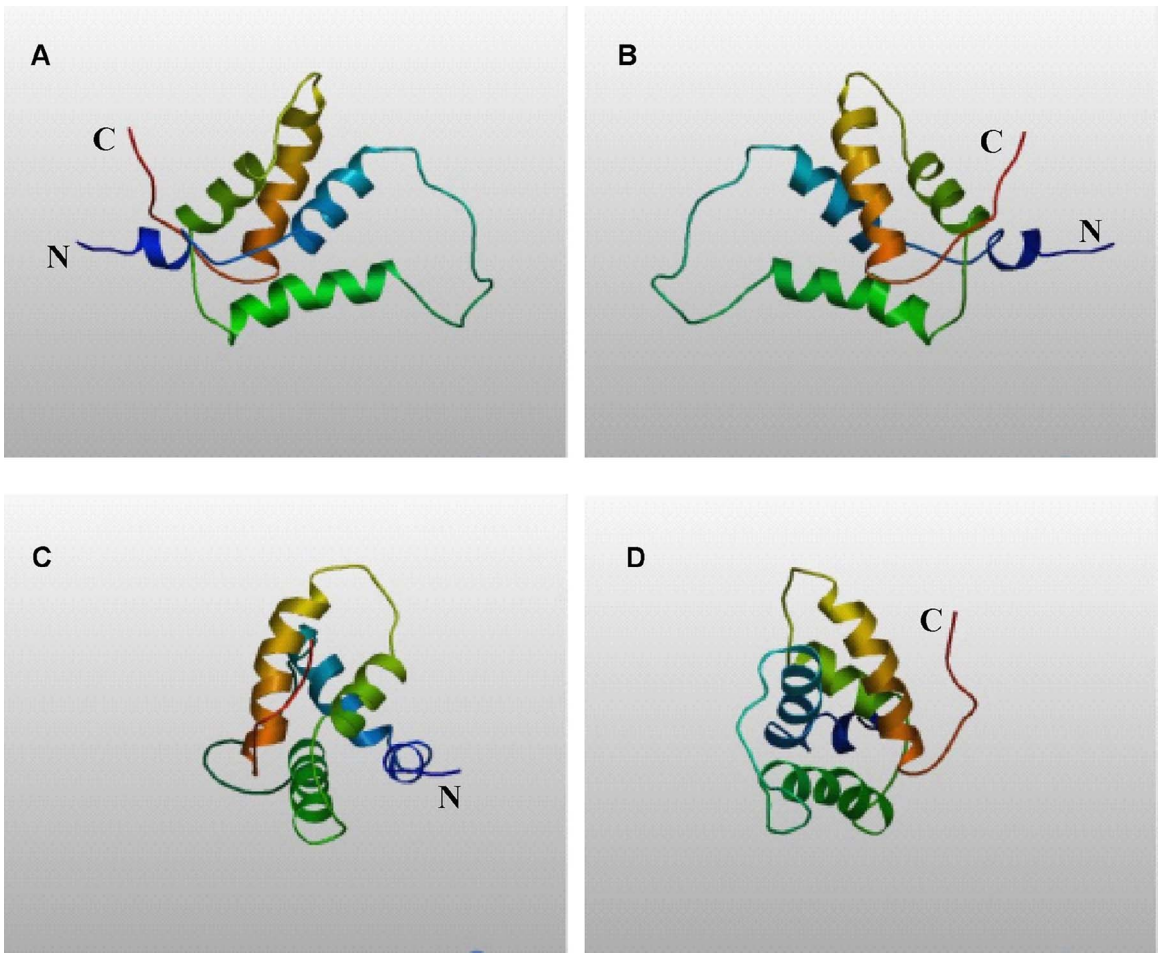


Fig. 2. Molecular modeling of Fag t 2 using SWISS-MODEL. A. The front of three-dimensional structure. B. The back of three-dimensional structure. C. The left of three-dimensional structure. D. The right of three-dimensional structure. N, N-termini; C, C-termini

Table 1
Antigenic epitopes prediction of Fag t 2.

Number	Sequences	Score	Length	Position	Max score position
1	EEKCLRGCCVAM	1.231	12	80–91	86
2	FIILATATLLIAATQAKY	1.127	18	4–21	5
3	PELVKCNRY	1.112	9	47–55	52
4	ICVCEW	1.101	7	96–102	101
5	LPNCCGI	1.065	7	129–135	132
6	EALSR	1.034	6	67–72	71

Note: The black shadow is antigenic epitopes predicted.

services/SignalP/) predicted that there were 19 residues (MKLFILATA TLLIAATQA) as the N-terminal signal peptide (shaded area in Fig. 1).

The cloned cDNA of *Fag t 2* showed 90% homologous to the *Fagopyrum esculentum* 16-kDa major allergen mRNA (GenBank accession No. FJ430161 and DQ304682), 89% homologous to the *Fagopyrum esculentum* BW 16-kDa allergen mRNA (partial cds) (GenBank accession number AY966013), and 70% homologous to the *Fagopyrum tatarZicum* BW10-kD allergen protein gene (GenBank accession No. EF488808).

3.2. Structure analysis and epitope prediction

The deduced amino acid sequence of Fag t 2 was used for computing pI/Mw by the ExPASy Proteomics Server (http://www.expasy.ch/cgi-bin/pi_tool), and the values of pI/Mw were 5.23 and 17218.13 Da, respectively. The secondary-structure prediction showed that the secondary-structures of Fag t 2 were mainly α-helices (68.64% α-helices and 37.54% random coils).

Molecular modeling result showed that the modeled residue range from amino acid residue 35–143, and the template, 1w2qA, was the allergen Ara h 6 from Peaunt (*Arachis hypogaea* L.). The Fig. 2 was the

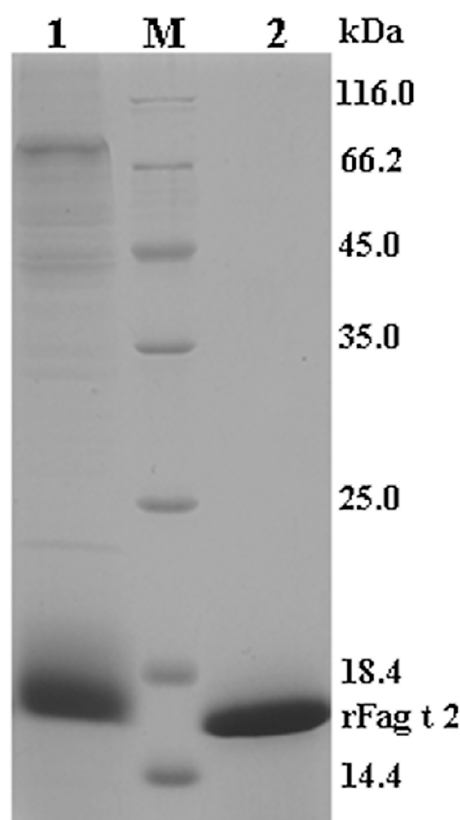


Fig. 3. SDS-PAGE analysis of recombinant Fag t 2 protein.

M: Molecular weight marker; lane 1: Expression of rFag t 2 in *Pichia pastoris*; lane 2: Purified recombinant Fag t 2 protein by metal affinity chromatography.

molecular model of Fag t 2 from different angles (A, B, C and D). Fag t 2 mainly contains four α -helices which flock together in parallel to form a compact and hollow barrel structure, and the N- and C-terminal of the molecule are in the bucket mouth of the barrel structure. Functional structure prediction showed Fag t 2 contained one α -amylase inhibitor (AAI) domain ranging from amino acid residue 38–140.

Epitope prediction showed that Fag t 2 contained 6 fragments as its epitopes, each epitope had one maximum score amino acid (the black against white back-ground one in Table 1), which were thought to be the central amino acids and considered to be the main parts which caused allergic reactions.

3.3. Expression and purification of recombinant fag t 2

Fag t 2 was expressed in *Pichia pastoris* SMD1168H (lane 1 Fig. 3) and purified with metal affinity chromatography. The purified rFag t 2 was detected mainly as a single band (lane 2 in Fig. 3) on 15% SDS-PAGE. The molecular mass of rFag t 2 was 16-kDa as detected on SDS-PAGE, and the result was corresponding to the molecular prediction of Fag t 2. There was no change in molecular weight of recombinant Fag t 2 after enzymolysis by PNGaseF (Data not shown), which indicate that recombinant Fag t 2 has no glycosylation expressed in *Pichia pastoris*. The result was consistent with the result of prediction of Fag t 2 without glycosylation site.

3.4. Western blotting analysis of n fag t 2 and r fag t 2

Seeds total protein extract and rFag t 2 were fractionated by SDS-PAGE and subjected to western blotting for identification the specificity of prepared rabbit polyclonal antiserum. The results showed that both rFag t 2 and total protein samples gave signal intensity band (Fig. 4). Nature Fag t 2 accounted for 4.2% and 22.0% of total protein and

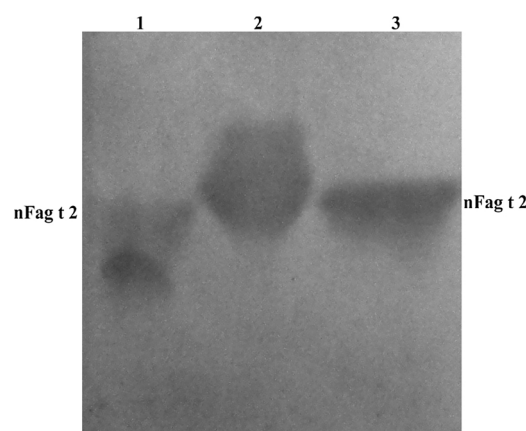


Fig. 4. Western Blotting analysis of nFag t 2 extract and rFag t 2. Lane 1 and 3: 40 μ l and 10 μ l nFag t 2 sample, respectively; lane 2: rFag t 2.

extracted albumin treated with HCl in buckwheat seeds, respectively (Data not shown). The contents of fraction extracted albumin treated with HCl from buckwheat seeds accounted for 14.6% in soluble total protein. And soluble protein accounted for around 50% in total protein of tartary buckwheat seeds (Guo, 2006). These results demonstrated that the 16-kDa allergen exists in high amounts in tartary buckwheat seeds. In addition, the extra band at the bottom of lane 1 (Fig. 4) may be corresponding to the tartary buckwheat 10-kDa allergen.

3.5. Location and identification of natural fag t 2 in seeds

The extracted albumin was treated with HCl (pH 2.0) and was subjected to 13.5% PAGE followed by pepsin digestion (2 mg/ml pepsin) and western blotting analysis. The comparison results of PAGE (Fig. 5A and B) and western blotting (Fig. 5C) showed that a band at the bottom of Fig. 5A and B were consistent with the hybridization signal at the bottom of Fig. 5C. Therefore, the band at the bottom of Fig. 5A and B might be the nFag t 2.

To further determine whether the band at the bottom of Fig. 5A and B was nFag t 2, the band was cut and electric eluted using the Electro-Eluter followed by western blotting analysis, N-terminal amino-acid sequencing and Mass Spectrometric analysis. Results of western blotting analysis indicated that the band obtained had a hybridization signal (Fig. 6), and that the molecular weight of the hybridization band was about 16-kDa (lane 1 in Fig. 6). The N-terminal amino-acid sequences of nFag t 2 were RDEGFDLGETQM, which was completely identical to that

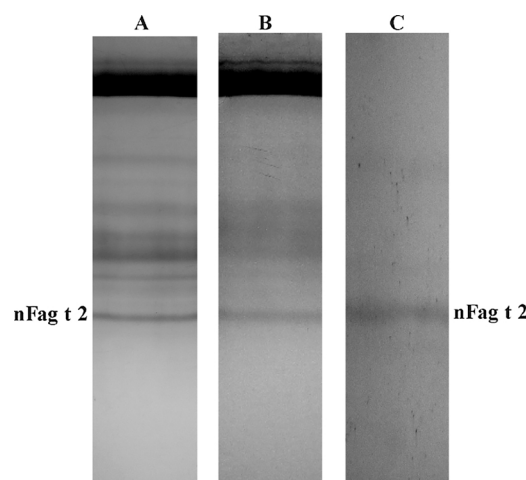


Fig. 5. PAGE and Western blotting of albumin from tartary buckwheat.

A. Albumin PAGE. B. Albumin PAGE gel treated with pepsin. C. Western blotting of pepsin digested albumin PAGE.

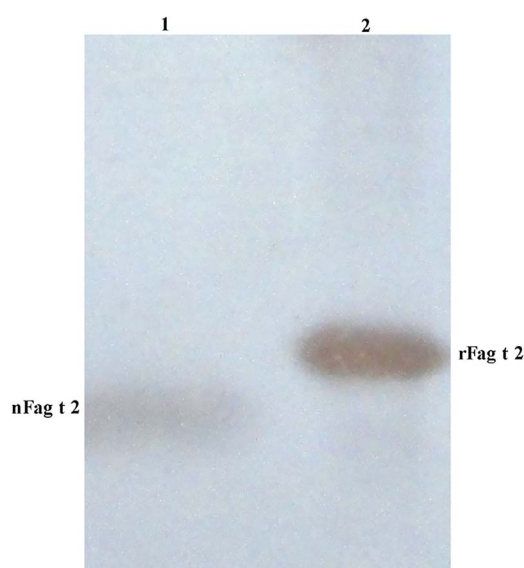


Fig. 6. Western blotting analysis of electric eluted nFag t 2. Lane 1: nFag t 2; lane 2: rFag t 2.

of rFag t 2 and common buckwheat 16-kDa allergen (Koyano et al., 2006; Tanaka et al., 2002). This indicates that the nFag t 2 is exactly the tartary buckwheat 16-kDa allergen. In addition, peptide mass fingerprint obtained from MALDI-TOF-MS also confirmed the same result above (data not shown).

Seed albumin, globulin and total proteins extracted by HCl (pH 2.0) were used for western blotting analysis. The results showed all samples had the bright hybridization signal except the globulin fraction (Fig. 7). In addition, there were two bright hybridization bands presented on the NC membrane. One was the nFag t 2, and the other might be the 10-kDa allergen (8-kDa allergen). The reason might be that the homology of those genes was high and also belong to the family of 2S globulin, which was similar to the 10-kDa and 16-kDa allergen in common buckwheat.

Western blotting analysis of embryo and endosperm protein was also carried out to determine the location of Fag t 2 in seeds. Result indicated that the nFag t 2 dose not exist in the endosperm (lane 1 and 4 in Fig. 8) but rather in embryo (lane 2 and 3 in Fig. 8). This result was consistent with the previous report (Ličen and Kreft, 2005).

3.6. IgE immunoblotting of fag t 2 protein

In order to clarify whether nFag t 2 and rFag t 2 react with serum from patients with positive IgE binding to buckwheat, IgE immunoblotting analysis was carried out by using sera from patients allergic to buckwheat, and 17 of 23 patient exhibits positive activity. And two representative positive sera (serum No.1 and No.2) from patients allergic to buckwheat and two control sera were chosen for illustrated the results. The results showed that both of the two positive sera had hybridization signals on NC membrane with nFag t 2 and rFag t 2 (lane 1 and 2 in Fig. 9A and B) but not for the two control sera. In

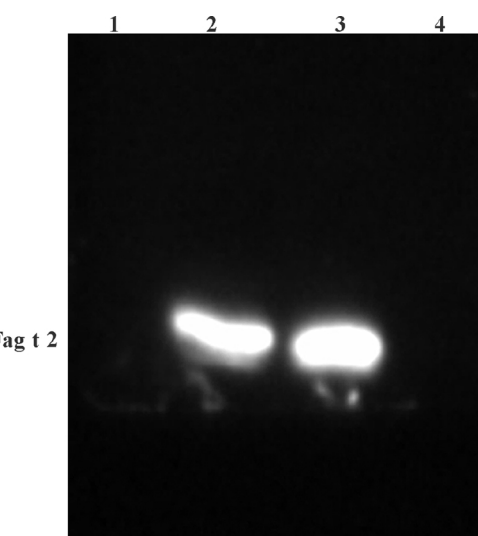
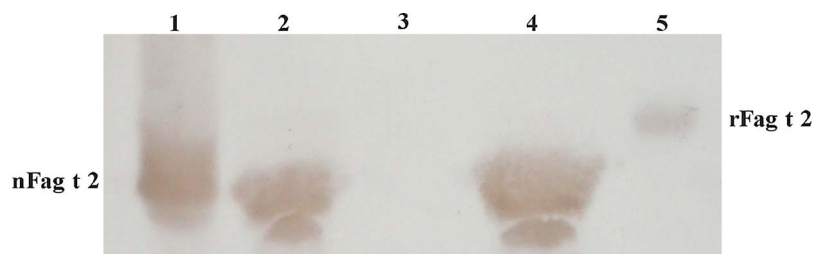


Fig. 8. Western blotting analysis of the embryo and endosperm protein from tartary buckwheat seeds.

Lane 1 and 4: Endosperm protein from tartary buckwheat seeds; lane 2 and 3: Embryo protein from tartary buckwheat seeds.

addition, the results also indicated that serum No.2 had stronger hybridization signals compared with serum No.1 (Table 2).

3.7. ELISA analysis of fag t 2

Competitive ELISA was performed to determine the immunological activity of purified rFag t 2 under nondenaturing conditions by using seeds total protein, rFag t 2 and seed albumin fraction, as well as two buckwheat allergic patients and two negative sera samples. Data in Table 3 indicates that seeds total protein had binding signals with No. 1 serum and No. 2 serum from patients with positive IgE binding activities to buckwheat but not with control sera (No. 3 serum and No. 4 serum). In addition, the inhibitory efficiency of rFag t 2 and albumin fraction were very similar (No. 1 serum: 42.3% and 45.1%, respectively; No. 2 serum: 42.5% and 44.2%, respectively), which indicated that purified rFag t 2, like nFag t 2, had the binding activity with IgE from allergic patients to tartary buckwheat, which indicated Fag t 2 was the allergenic component in albumin.

3.8. Thermal stability and pepsin digestion analysis

The purified rFag t 2 (Fig. 10A), as well as nFag t 2 (Fig. 10B and C) were being heated for 150 min at 98 °C and still kept in soluble state. To further determine whether heated Fag t 2 could react with the IgE from sera of patients allergic to buckwheat, ELISA analysis was conducted using purified rFag t 2 and nFag t 2 which were heated for 150 min at 98 °C. As shown in Table 4, heated rFag t 2 and nFag t 2 still kept IgE reactivity, which were 91.1%, 93.6% (No.1 and 2 serum) and 94.9%, 94.3% (No.1 and 2 serum), respectively.

We conducted a pepsin digestion test using natural and recombinant Fag t 2 to determine whether the Fag t 2 has pepsin resistance. In SDS-

Fig. 7. Western blotting analysis results of total protein, albumin and globulin. Lane 1: Tartary buckwheat seeds total protein; lane 2: Albumin; lane 3: Globulin; lane 4: Seeds total protein extracted by HCl; lane 5: rFag t 2.

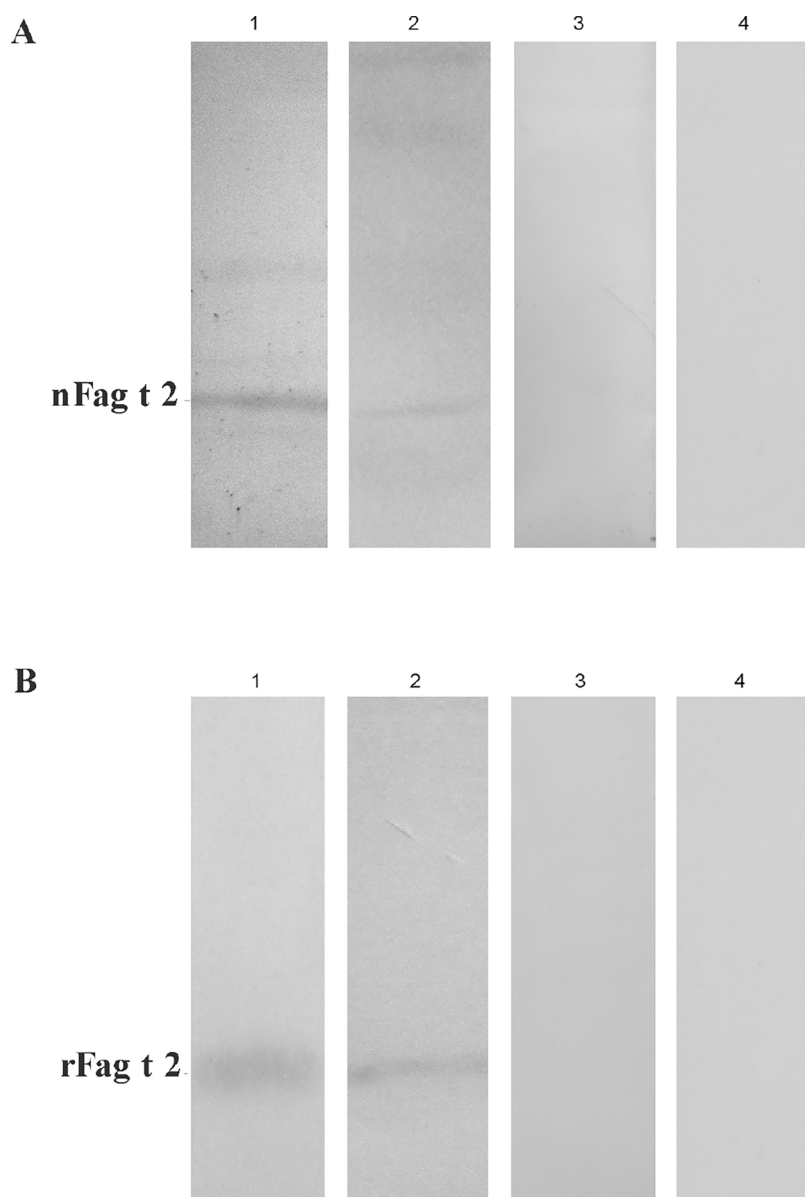


Fig. 9. IgE Immunoblotting of nFag t 2 and rFag t 2.

A. IgE Immunoblotting of nFag t 2 extract. B. IgE Immunoblotting of rFag t 2. Lane 1 and 2: IgE Immunoblotting of two positive sera (serum 1 and 2) from patients with positive IgE binding to buckwheat respectively; lane 3 and 4: IgE Immunoblotting of two control sera.

Table 2
Information of buckwheat allergy patients' serum.

Serum	Gender	Age	Allergy symptoms caused by tartary buckwheat
Number 1	male	25	appeared the symptoms of throat swollen, tongue numbness, mouth foaming, a burning sensation in the stomach, as well as tears and runny nose after eating the rice porridge containing buckwheat
Number 2	female	31	appeared the symptoms of tears, cough and asthma after contacting with buckwheat husk pillow; urticaria, vomiting and diarrhea after drinking the soymilk containing buckwheat

PAGE gel, both natural Fag t 2 band and recombinant Fag t 2 band gradually decreased after 0–8 min of pepsin digestion (Fig. 11A and B). However, other protein bands from tartary buckwheat seeds and the BSA band (Fig. 11C) disappeared after 0.5 min of pepsin digestion. It was indicated that nFag t 2 was resistant to pepsin digestion compared with other proteins in tartary buckwheat seeds.

4. Discussion

The consumption of products made from tartary buckwheat has increased in recent years due to its nutritive and health-promoting properties. While increasing incidence of allergy symptoms is observed in people who consumed buckwheat-containing foods (Sammur et al., 2011), the main reason for allergenic reactions is protein based allergen (Nakamura and Teshima, 2013). In many cases, food allergens are known to be a specific protein. Many allergenic proteins have been found in common buckwheat (CB) in the past few decades (Fujino et al., 2001; Sano et al., 2014; Shigemori et al., 2013; Yoshioka et al., 2004a).

The CB 16-kDa allergen had been identified as a major CB allergen with a strong pepsin resistance, and it was associated with IHR (immediate hypersensitivity reactions) including anaphylaxis (Ličen and Kreft, 2005). In this work, the full-length cDNA of TB 16-kDa allergen (Fag t 2) was isolated based on screening seed filling period of the cDNA library. Structure (including secondary-structure and tertiary structure) and antigenic epitope position prediction revealed that the allergenic protein mainly consisted of four α -helices (Fig. 2) which were the typical characteristics of members of 2S albumin family (Yoshimasu et al., 2000). The majority of predicted antigenic epitopes were located in the

Table 3
The ELISA results of recombinant Fag t 2.

Samples	Colorimetric intensity (OD ₄₅₀)/inhibitory efficiency (%)							
	number 1 serum		number 2 serum		number 3 serum		number 4 serum	
Total protein(control)	0.435	–	0.457	–	0	–	0	–
rFag t 2	0.251	42.3	0.263	42.5	0	–	0	–
nFag t 2	0.239	45.1	0.255	44.2	0	–	0	–

Note: The first columns data of each set of experiment are colorimetric intensity (OD₄₅₀), the second columns data of each set of experiment are inhibitory efficiency (%). Symbol “–”represents that the ratio has no value.

helices (Fig. 2 and Table 1), which suggested that these antigenic epitopes were linear epitopes. Western blotting indicated that Fag t 2 existed in the albumin fraction and located only in the embryo of TB seeds. Over-expression of Fag t 2 lacking its N-terminal signal peptide provided an active rFag t 2. The result of N-terminal amino-acid sequences and MALDI-TOF-MS of natural 16-kDa allergen of Tartary buckwheat was consistent with the recombinant Fag t 2. Proteins in the 2S albumin family with four disulfide bonds form a compact molecule and show very high thermal stability, resistance to extreme pH and the activity of protease hydrolysis (Dominguez et al., 1990; Sirvent et al., 2012). Characteristics analysis showed that nFag t 2 and rFag t 2 had both thermal stability and IgE binding activity. These data confirmed that Fag t 2 was a member of the 2S albumin family, which was consistent with previous result (Ličen and Kreft, 2005). Furthermore, the location of 16-kDa allergy in tartary buckwheat seeds provides a new thinking, which is that, the embryo of tartary buckwheat seeds were used for desensitization food of tartary buckwheat in food processing.

The stability including thermal and pepsin hydrolysis stability are the most basic features of plant allergens due to the compact and sturdy structure of allergens (Edreva, 2005). In neutral condition (Fig. 10 and Table 4) or acidic condition (data no shown), both rFag t 2 and nFag t 2 kept high soluble state and retained about 93% IgE reactivity after heating at 98 °C for 150 min. On the other hand, pepsin digestion results (Fig. 11) indicated that both rFag t 2 and nFag t 2 were resistant to pepsin, which was consistent with the results of CB 16-kDa allergen (Koyano et al., 2006; Tanaka et al., 2002). Therefore, we believe that thermal stability and pepsin resistance of Fag t 2 are important factors for causing IHR. These features indicated that Fag t 2 was major

Table 4
The ELISA results of heated rFag t 2 and nFag t 2.

Samples	Treatment	Colorimetric intensity (OD ₄₅₀)/ratio between heating and no-heating (%)			
		number 1 serum		number 2 serum	
rFag t 2	no-heating	0.460		0.456	
	heating	0.419	91.1	0.427	93.6
nFag t 2	no-heating	0.469		0.454	
	heating	0.445	94.9	0.428	94.3

Note: The first columns data of each set of experiment are colorimetric intensity (OD₄₅₀), the second columns data of each set of experiment are ratio between heating and no-heating (%). Ratio = A_{heating}/A_{no-heating}. The symbol “–”represents that ratio has no value.

allergen of tartary buckwheat, which reported was similar to CB 16-kDa allergen. Besides, we established the separation technology of natural 16-kDa allergen of buckwheat seed, which provides valuable reference information for further research in antigen epitope and molecular structure.

The application on allergy of buckwheat was more concentrate on diagnosis of allergic diseases caused by buckwheat (Choi et al., 2007; Heffler et al., 2011; Maruyama et al., 2016; Yanagida et al., 2018). There was little further study on allergy of buckwheat. Food allergy research is focused on eliminating or reducing allergic activity of food raw materials (Ree et al., 2015). One method to reduce activity of food allergies was allergic protein denaturation or antigen epitope changes in food processing. Whereas many food allergens are not sensitive to

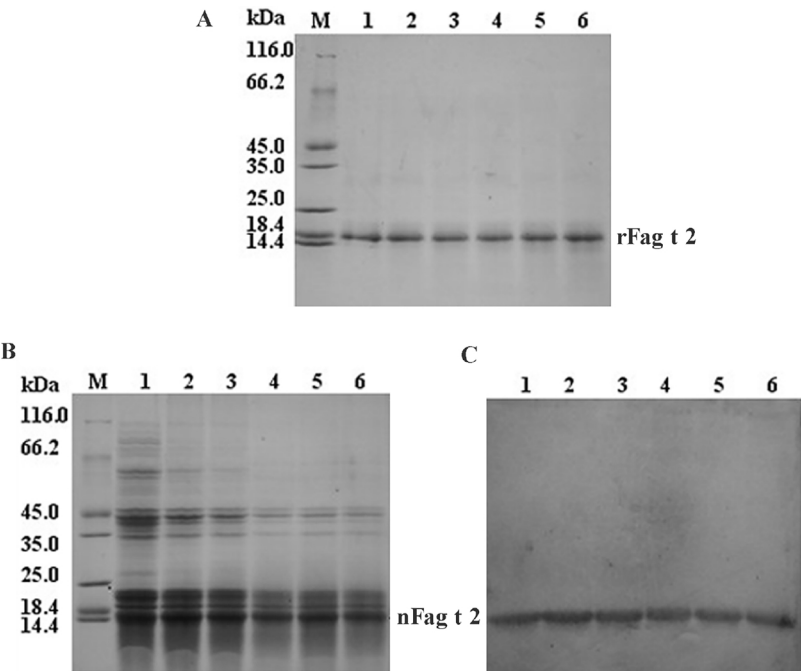


Fig. 10. SDS-PAGE and Western blotting analysis of heated rFag t 2 and nFag t 2 extract.

A. SDS-PAGE analysis of heated rFag t 2. B. SDS-PAGE analysis of heated nFag t 2 extract. C. Western blotting analysis of heated nFag t 2 extract. M: Marker; lane 1, 2, 3, 4, 5 and 6: Different time (0, 30, 60, 90, 120 and 150 min, respectively) of heating at 98 °C.

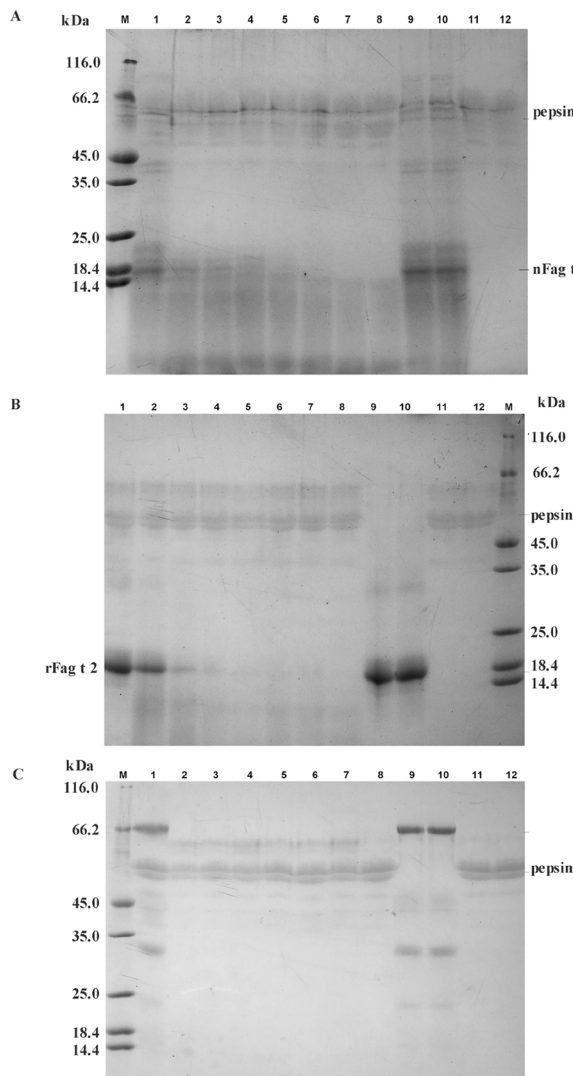


Fig. 11. Pepsin digestion test of natural and recombinant Fag t 2. SDS-PAGE analysis of nFag t 2 (A) extract, rFag t 2 (B) and BSA(C). M: Molecular weight markers; lanes 1–8: Pepsin digestion of nFag t 2, rFag t 2 or BSA controls at time: 0, 0.5, 2, 5, 8, 15, 30 and 60 min, respectively; lanes 9 and 10: nFag t 2, rFag t 2 or BSA controls at time: 0 and 60 min, respectively; lanes 11 and 12: Simulated gastric fluid controls at time 0 and 60 min, respectively.

degeneration of food processing, which is limited the development of this method (Ogo et al., 2014; Vissers et al., 2011). Another direct and economical method to reduce the activity of food allergy is screening and breeding the low sensitive varieties. Eliminating or inhibiting allergic protein expression by mutation or genetic engineering has being the main methods of breeding low sensitive varieties (Dodo et al., 2012; Ogo et al., 2014). It has been reported successful for obtaining low sensitive transgenic plants of peanut, soybean and rice by RNAi mediated gene silencing (Herman, 2003; Wakasa et al., 2011; Dodo et al., 2012; Ogo et al., 2014). There was not the relevant report on desensitization research of tartary buckwheat, which might be restricted by the establishment of genetic transformation system of buckwheat.

Functional structure prediction showed Fag t 2 contained α -amylase inhibitor (AAI) ranging from amino acid residue 38–140. Similar result was also reported on some nonspecific lipid-transfer proteins with predicted AAI domain (Breiteneder and Mills, 2005). α -amylase is the most important enzyme in the endosperm to catalyze the degradation of storage starch and to provide energy for the morphogenesis and growth of seedlings (Young et al., 1997). It is widely accepted that the expression of α -amylase is regulated by metabolic sugars and

phytohormones, such as gibberellin and ABA (Abscisic Acid) (Kashem et al., 2000). However, proteins involved in the regulation of starch degradation are poorly understood. So far, seven types of proteinaceous α -amylase inhibitors were found in nature as defined by similarities in sequence and three-dimensional structures, but most of them contain evolutionary related proteins without inhibitory activity (Svensson et al., 2004). In recent years, there were some reports related to α -amylase inhibitor with the activity of inhibiting fungi and *Tribolium castaneum* (Channale et al., 2016; Chen et al., 1999; Kasar et al., 2017; Oppert et al., 2003; Zottich et al., 2011). Therefore, we speculate that the 16-kDa allergen of tartary buckwheat with the AAI domain might play an important role in regulating the catalytic activity of amylase in starch hydrolysis and promoting the germination of tartary buckwheat seeds, or with function of inhibiting the catalytic activity of amylase and used for pest control. Those speculation functions of α -amylase inhibitor of Fag t 2 should be further detected.

In conclusion, we reported a low molecular weight member of 2S albumin family with α -amylase domain which restricted to the embryo. And also established the separation method of natural 16-kDa allergen of buckwheat seed, and identified the 16-kDa allergen of tartary buckwheat. Competitive ELISA and IgE immunoblotting indicated that Fag t 2 was represented IgE binding activity with the resistant to pepsin and heating, which implied that they were both the major allergy of tartary buckwheat. And the results are useful for understanding molecular mechanism of a major allergen in tartary buckwheat, as well as its application in the diagnosis of clinical reactivity and the development of a transgenic hypoallergenic crop. The purification of rFag t 2 can be obtained that allowed further detailed epitopes mapping and revelation of the biological function of Fag t 2.

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