

# Core epitope analysis of 16 kDa allergen from tartary buckwheat

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## ABSTRACT

Tartary buckwheat is widely accepted as its nutritional value. Some allergic reactions hinder its utilization. This research focused on evaluating the core epitope of 16 kDa allergen (Fag t 2) in tartary buckwheat. Six B- and seven T cell epitopes of Fag t 2 were predicted, and six B cell epitope-mutants were expressed in *Pichia pastoris*. Bioinformatics analysis and SDS-PAGE demonstrated that the molecular weight, isoelectric point and spatial structures of six mutant allergens were similar with Fag t 2, with the same signal peptide sequences and  $\alpha$ -amylase inhibitor domain. There was no significant change in mutants' spatial conformation confirmed by Circular Dichroism. The position K132N and peptides at 108–117 and 132–141 were the core B- and T cell epitopes of Fag t 2 confirmed by competitive inhibition ELISA and dot blot. This result was of great significance on the study of allergen epitopes in prevention and treatment of hypersensitivity.

## 1. Introduction

Buckwheat is a member of the *Polygonaceae* family including two cultivates, common buckwheat (CB, *Fagopyrum esculentum*) and tartary buckwheat (TB, *Fagopyrum tataricum*) (Hong, Ikeda, Kreft, & Yasumoto, 1996). Compare with common buckwheat, tartary buckwheat has special nutritional and medical properties (Kreft & Skrabanja, 2002). Due to its balanced amino acid composition and high content of flavonoids and dietary fiber, tartary buckwheat is not only widely eaten as global staple food, but also as a kind of functional food in some Asian countries (Nakamura et al., 2008).

With the increase of the buckwheat products consumption, the cases of immediate hypersensitivity triggered by buckwheat allergen have increased. The common symptoms of allergic reaction were urticaria, breathing, anaphylactic shock and other symptoms, even death (Li, Cui, Li, & Wang, 2011; Park, Jeong, & Lee, 2016). In 1990, Smith first reported the case of allergic symptoms caused by feeding buckwheat powder (Smith, 1990). The epidemiological survey results showed that the proportion of Korean primary and secondary school students who are allergic to buckwheat is 0.13%, and that of Japanese children is as high as 0.22% (Cho et al., 2015; Sung et al., 2014). The result of collaboration between Japanese hospitals and researchers had shown that buckwheat is one of the five main foods causing allergic reactions in Japan (Shigemori, Yonekura, Sato, Otani, & Shimamoto, 2013). Horesh and Sammut conformed that pathological mechanism of buckwheat allergy is the IgE-mediated type I allergic reaction, and the emergence of

buckwheat allergy is becoming more and more common (Horesh, 1972; Sammut, Dennison, Venter, & Kurukulaaratchy, 2011). Therefore, the study of buckwheat allergen is extremely urgent.

Research of buckwheat allergy focused on identification of allergens and antigen epitope on common buckwheat (Fujino, Funatsuki, Inada, Shimono, & Kikuta, 2001; Matsumoto et al., 2004). Some natural allergenic proteins with different molecular weights of 10 kDa to 100 kDa from common buckwheat have been isolated and identified (Choi et al., 2007; Park, Kang, & Kim, 2000; Yoshioka, Ohmoto, Urisu, Mine & Adachi 2004). Among them, the 16, 24 and 22 kDa allergens were considered the main allergen (Koyano, Takagi, Teshima, & Sawada, 2006; Wang et al., 2006). Subsequently, some allergens were characterized by SDS-PAGE, western blotting and enzyme linked immunosorbent assay (ELISA) (Cho et al., 2015; Yoshimasu, Zhang, Hayakawa, & Mine, 2000; Yoshioka et al., 2004).

However, there were few reports about the allergens in tartary buckwheat. Jeon first identified a 10 kDa allergen in tartary buckwheat (Jeon & Hong, 2008). The 24 kDa allergen with thermal stability isolated and purified from the seeds of tartary buckwheat was like to the common buckwheat (Wang et al., 2004). The nucleic acid and amino acid sequences of 56 kDa allergen were analyzed, and the signal peptides, disulfide bonds, secondary structure and antigenic sites were predicted (Zhang, Yuan, Cui, & Wang, 2008). In recent years, few studies have been conducted on the epitopes of buckwheat allergens (Cai, Bai, Li, Zhang, & Wang, 2006; Li et al., 2011). Our laboratory previously reported a 16 kDa allergen protein (Fag t 2) isolated from

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tartary buckwheat seeds and expressed in prokaryotic and eukaryotic cells. Fag t 2 belongs to the 2S albumin family, only existing in embryo, formed by a single polypeptide chain. Fag t 2 was secretory expression and soluble expression in eukaryotic with the activity of IgE binding, pepsin resistance and thermal stability. (Chen, Guo, Yan, & Li, 2011; Zheng, Zhang, Wang, Guo, & Chen, 2018).

The antigenic determinants of allergens with defined amino acid sequences are called epitopes. Antigen-specific IgE requires the antigen-specific T cells identifying the antigens presented by MHC. Exogenous allergens require binding of MHC-II molecules to the surface of antigen-presenting cells (APCs). In addition, B-cell epitopes cannot be ignored as B cells can activate T cells by capturing antigens through BCR and performing APC functions. In turn, activated T cells can also activate B cells to produce antibodies. Therefore, B- and T cell epitopes have to be carried out simultaneously, which is of great significance on the in-depth study of protein epitopes in the diagnosis and prognosis of diseases, the modification of protein molecules at fixed points to reduce the immunogenicity of protein drugs, the design of artificial non-toxic vaccines and immunological interventions, etc. (Shao & Fu, 2019).

In this study, Six B-cell epitopes and seven T cell epitopes of Fag t 2 were predicted by bioinformatics online software (MOBYLE and SYFPEITHI). And then six groups of amino acids were replaced by site-directed mutagenesis, seven peptides were synthesized. We obtained Fag t 2 and the six mutant allergens though eukaryotic expression from *Pichia pastoris*. Spatial structure simulation, functional domain prediction and CD detection of Fag t 2 and mutant allergens were to make sure that the introduction of mutations will not lead to significant changes in spatial conformation. Several amino acids positions played a critical role in the allergenic reactions of tartary buckwheat as the core B-cell epitopes of Fag t 2 determined by competitive inhibition ELISA. The core T cell epitopes of Fag t 2 were verified by dot blotting hybridization.

## 2. Materials and methods

### 2.1. Materials

cDNA library of Fag t 2 gene sequences in developing tartary buckwheat seeds (GenBank registration number: GO496294), and their corresponding amino acid sequence (registration number: HQ829975); The recombination plasmid pET47b-Fag t 2, *Escherichia coli* TOP10, *Pichia pastoris* SMD1168H were conserved by our laboratory. Kanamycin, Zeocin, Patient's serum (provided by buckwheat allergic patients), HRP-conjugated goat anti-human IgE and HRP-conjugated goat anti-rabbit IgG were purchased from Stratagene, TaKaRa, Wolsen, Gaithersburg MD. Rabbit (New Zealand white rabbits) polyclonal antiserum raised against Fag t 2 was performed by Protein Tech Group (Wu Han, China). All other reagents were of the highest purity available.

### 2.2. Bioinformatics analysis of Fag t 2 in tartary buckwheat

Previous studies have determined that Fag t 2 is the major allergen in tartary buckwheat (Zheng et al., 2018). The prokaryotic expression and purification system of Fag t 2 was not only successfully constructed, but also its immunogenicity was tested (Chen, Guo, Yan, & Li, 2011). With the extensive application of recombinant protein technology in the preparation of hypoallergenic allergens, the analysis of core antigenic epitopes has gradually been valued.

The amino acid sequence in B-cell epitopes of Fag t 2 were predicted with online software MOBYLE (<http://bioweb.pasteur.fr/seqanal/interfaces/antigenic.ht/ml>). The amino acids with higher scores were replaced according to the codon preferences, and then determined the feasibility of replaced amino acid. Furthermore, the online software ExPASy Proteomics ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)) was used for predicting the molecular weight and isoelectric point of Fag t 2 and its mutant allergens. Subsequently, the secondary structure, tertiary structure and functional domain of Fag t 2 mutants were predicted with

the PSIPRED server (<http://bioinf.cs.ucl.ac.uk/psipred>) and SWISS-MODEL (<http://swiss-model.expasy.org/SWISS-MODEL.html>) to ensure the change of amino acid in allergen epitope could not result in the change of spatial structure.

The amino acid sequences in T cell epitopes of Fag t 2 from tartary buckwheat were predicted with online software SYFPEITHI (<http://www.syfpeithi.de/scripts/MHCServer.dll/EpitopePrediction.htm>). In addition, the multiple sequence alignment and conserved domain prediction were conducted through online software ([http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST\\_PROGRAMS=blastp&PAGE\\_TYPE=BlastSearch&SHOW\\_DEFAULTS=on&LINK\\_LOC=blasthome](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)).

### 2.3. Vector construction and expression of Fag t 2 and mutant allergens

#### 2.3.1. Site-directed mutant vector construction of B-cell epitopes of Fag t 2

According to the theory of site-directed mutant we designed the mutant primer (Table S1) by PrimerX (<http://www.bioinformatics.org/primerx>) with TGC-to-GGC, TGT-to-GGT, AGG-to-AAC, TGTTGT-to-GCGGT, TGC-to-TAC and AAG-to-AAC mutations, respectively. After optimizing the PCR programs, the programs of six site-directed mutants with amino acids replaced were in different annealing temperature with the pET47b-Fag t 2 as template. The PCR programs were used for site-directed mutants as descriptions of the modified Quik Change Site-Directed Mutagenesis Kit. All PCR reactions were performed using PfuTurbo DNA polymerase (Stratagene, USA). PCR products were digested with DpnI (Takara Bio, Dalian, China) for 4–5 h and transformed into *E. coli* Top10. The mutations of were confirmed by sequencing after plasmid extraction with the Plasmid Purification Kit (Takara Bio, Dalian, China).

The cDNA of mutant allergens was amplified as the constructions of six mutant templates (Fag t 2<sup>C38G</sup>-pET47b, Fag t 2<sup>C52G</sup>-pET47b, Fag t 2<sup>R71N</sup>-pET47b, Fag t 2<sup>C87G-C88G</sup>-pET47b, Fag t 2<sup>C100Y</sup>-pET47b and Fag t 2<sup>K132N</sup>-pET47b), respectively. The PCR reaction procedure was consistent with the previous report of our laboratory (Zheng et al., 2018). The PCR products were recombined with pPICZ(α)A vector (Fig. S1) using the SeamLess Cloning Kit (Takara Bio, Dalian, China). The recombinant products were transformed into *Escherichia coli* TOP10, respectively. The correctly sequenced mutant plasmids were cloned into pPICZ(α)A vector to form recombinant plasmid described as Fag t 2<sup>C38G</sup>-pPICZ(α)A, Fag t 2<sup>C52G</sup>-pPICZ(α)A, Fag t 2<sup>R71N</sup>-pPICZ(α)A, Fag t 2<sup>C87G-C88G</sup>-pPICZ(α)A, Fag t 2<sup>C100Y</sup>-pPICZ(α)A and Fag t 2<sup>K132N</sup>-pPICZ(α)A.

#### 2.3.2. Expression and purification of Fag t 2 and mutant allergens in *Pichia pastoris*

The recombinant plasmids were linearized using 5' primers (5'-AGATGAGGGCTTTCTGAGTGTGGGG-3') and 3'primers (5'-GGAGT-GATGTTTCGGGTGTAATGCGG-3') and then transformed into competent cells of *Pichia pastoris* SMD1168H by electroporation at 1.5 kV, 5 ms with Electroporator (Bio-Rad, USA), respectively. The transformation of *Pichia pastoris* and overexpression of proteins with mutant recombinant plasmids were performed according to the method previous reported. *Pichia pastoris* with Fag t 2 and mutant allergens were expression induced by 1% methanol for 72 h in BMMY medium. The supernatant was harvested by centrifugation at 4 °C, 8000g for 10 min. The supernatants of Fag t 2 and six mutant allergens were dialyzed at 4 °C against dialysis buffer A (0.01 mol/L Tris-HCl, pH 8.0) for 12 h. And then the dialyzed supernatants were 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 mmol/L NaCl) contained 150 mmol/L imidazole. The Fag t 2 and six mutant allergens containing fractions were pooled and concentrated by ultrafiltration. (Zheng et al., 2018). And Fag t 2 and mutant allergens were used for quantitative analysis with Bradford method (Jeong et al., 2016).

## 2.4. Circular dichroism detection

The structural features of Fag t 2 and mutant allergens were analyzed by CD spectroscopy. Each mutant allergen was diluted to the concentration of 0.7–0.8 mg/mL with the 1 × PBS buffer as control. The scanning wavelength range was between 180 nm and 300 nm.

## 2.5. Identification of Fag t 2 epitopes

### 2.5.1. Competitive inhibition ELISA

The competitive inhibition ELISA was performed to detect the immunological activity of Fag t 2 and mutant allergens. 96-well ELISA plates (Corning, USA) were coated overnight at 4 °C with 100 µL crude protein extract from tartary buckwheat seeds, which diluted in coating solution (50 mmol/L sodium carbonate buffer, pH 8.0) to a concentration of 10 µg/mL. After washing 5 times with ELISA wash buffer, the 96-well plates were blocked with 1% BSA-PBS (pH 7.0) for 3 h at room temperature, then incubated with the 100 µL mixture of Fag t 2 or mutants allergens and patient's serum (1:5) (buckwheat allergic patients and control serum, voluntarily provided by buckwheat allergic patients and non-patients) at 37 °C for 1 h. Purified Fag t 2 and mutant allergens were diluted to 150 µg/mL with 0.1% BSA-PBS before preincubated with serum in equal volumes. The combination of Fag t 2 or mutant allergens and patient's serum were showed in Table 3. After washing, the plates were 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% H<sub>2</sub>O<sub>2</sub>). The optical density at 450 nm were detected with microplate readers (Spectra Max M2, Molecular Devices, USA). The calculation of inhibitory efficiency (I) was carried out according to the method described previously (Chen, Guo, Yan, & Li, 2011). Formula:  $I = (A_{\text{mutant}} - A_{\text{Fag t 2}}) / A_{\text{Fag t 2}}$ . "A" represents absorbance value (OD<sub>450</sub>).

### 2.5.2. Dot blotting

In order to confirm the specific IgE-binding capacity of the recombinant epitope-containing fragments and mutants, an immuno dot blotting assay was performed. The seven synthesized peptides were applied to a nitrocellulose membrane marked with 1 cm × 1 cm grid and blocked with PBS (pH 7.5) containing 5% skim milk. After washing 3 times the nitrocellulose membrane incubated with patient's serum (1:50) for 1 h at room temperature. After washing, the membrane was then sequentially incubated with HRP-conjugated goat anti-human IgE antibodies (1:1000) for 1 h at room temperature. DAB solution was used for chromogenic reaction.

## 3. Results and discussion

### 3.1. Bioinformatics analysis of Fag t 2 and mutant allergens

In this study, according to the MOBYLE software algorithm, the amino acids that play a critical role in the spatial structure of Fag t 2 were scored one by one. There were six B-cell epitopes predicted with high score in amino acids 38, 52, 71, 87–88, 100 and 132 using online software MOBYLE (Table 1) in Fag t 2, which considered to be the central amino acid caused allergy. The B-cell epitopes predictions with

**Table 1**  
B-cell epitopes prediction of Fag t 2.

| Number | Sequences    | Score | Length | Replaced amino acid | Position |
|--------|--------------|-------|--------|---------------------|----------|
| 1      | EEKCLRGCCVAM | 1.231 | 12     | GG                  | 87–88    |
| 2      | TQMSSKCTR    | 1.12  | 9      | G                   | 38       |
| 3      | PELVKCNRY    | 1.112 | 9      | G                   | 52       |
| 4      | DECVCEW      | 1.101 | 7      | Y                   | 100      |
| 5      | LPNKCIGI     | 1.065 | 7      | N                   | 132      |
| 6      | EALSRI       | 1.034 | 6      | N                   | 71       |

high score were replaced by the amino acids with decreasing the scores mostly (Table 1). And then the isoelectric points and molecular weights of six mutant allergens sequences were predicted using ExPASy Proteomics. There were few changes between Fag t 2 and six mutant allergens in isoelectric points and molecular weights (Table S2). It was indicated that the replacement of amino acids had few influences on the structures of Fag t 2 but for the allergenic properties.

The secondary structures and tertiary structure of Fag t 2 and mutant allergens were predicted using PSIPRED server and SWISS-MODEL. We analyzed the proportion of α-helix and β-folding, hydrophobicity, space conformation of mutant. It was found that the three-dimensional structures of six mutant allergens with replaced amino acids were similar with Fag t 2 (Fig. S2). Fig. S2a showed that Fag t 2 and six mutant allergens had the 19 amino acids as the same signal peptide sequence and α-amylase inhibitor domain. It demonstrated that the replacement of amino acids didn't destroy the secondary and tertiary structure of Fag t 2 of tartary buckwheat. Mutant allergens retained same helical structure and spatial conformation compared to Fag t 2 (Fig. S2 b–h), which indicated that the replacement of amino acids of B-cell epitopes predicted maintained its own space conformation. This could be determined, the amino acids of six groups mutant allergens did not change the original Fag t 2 protein structure.

BlastP software was used to search for the sequence with high amino acid sequence homology to Fag t 2. The amino acid sequence of the TB allergen was 83% homologous to the 16 kDa allergen protein of *fagopyrum esculentum* (GenBank Accession No.ABC18306), 51% homologous to the 10 kDa allergen protein of tartary buckwheat (GenBank Accession No.ABO93594), 48% homologous to 8 kDa allergen protein of *fagopyrum esculentum* (GenBank Accession No.BAB79444), 28% homologous to *arachis duranensis conglutin* (GenBank Accession No.ABW36073) and 26% homologous to *ricinus communis* 2S sulfur-rich seed storage protein (GenBank Accession No. XP002515708). In addition, multiple highly conserved single or grouped amino acid residues of Fag t 2 (Fig. S3) were obtained though aligning with the amino acid sequences of these five highly homologous proteins using BLASTP software, which were the potential T cell epitopes in Fag t 2. Seven peptides were identified as possible T cell epitopes of Fag t 2 predicted with SYFPEITHI software (Table 2).

In addition, the predictions of the secondary and spatial structures indicated the mutant allergens still retained the five α-helices of the original Fag t 2. The mutant allergens remained the α-amylase inhibitor domain. It was indicated that the mutated amino acids introduced insignificantly affected the spatial structure of Fag t 2.

### 3.2. SDS-PAGE of purified Fag t2 and its mutants

Comparing the function and structure of a protein mutant with the original protein is the most commonly method to determine the amino acids or domains in functional proteins that play an important role in structure and function. The vectors containing the genes of six mutant allergens were constructed successfully. Sequencing results confirmed that the six mutation sites were correct (Fig. S4). Then Fag t 2 and its mutant allergens were obtained by expressed in *Pichia pastoris* SMD1168H after purification. SDS-PAGE analysis showed that the molecular weights of mutants were in good agreement with the Fag t 2, and

**Table 2**  
T cell epitopes prediction of Fag t 2.

| Number | Predict peptides | Position | Length |
|--------|------------------|----------|--------|
| 1      | VKMMEPELVKCN     | 42–53    | 12     |
| 2      | ALSRIQEGGCESEE   | 68–81    | 14     |
| 3      | LRGCCVAMKEME     | 84–95    | 12     |
| 4      | ECVCEWMKMK       | 97–105   | 9      |
| 5      | ENQKGRIGET       | 108–117  | 10     |
| 6      | KGIRDLKELPN      | 121–131  | 11     |
| 7      | KCGISEMECH       | 132–141  | 10     |

**Table 3**  
ELISA result of Fag t 2 and six mutant allergens.

| Mixed samples              | A1    | A2    | A3    | A <sub>average</sub> | Inhibition rate (%) |
|----------------------------|-------|-------|-------|----------------------|---------------------|
| Fag t 2 + allergic serum   | 0.583 | 0.598 | 0.586 | 0.589                | 0                   |
| Fag t 2 + control serum    | 0.11  | 0.098 | 0.113 | 0.11                 | —                   |
| C38G + allergic serum      | 0.605 | 0.599 | 0.587 | 0.597                | 1.36                |
| C38G + control serum       | 0.099 | 0.114 | 0.122 | 0.112                | —                   |
| C52G + allergic serum      | 0.708 | 0.714 | 0.709 | 0.713                | 21.05               |
| C52G + control serum       | 0.112 | 0.125 | 0.126 | 0.121                | —                   |
| R71N + allergic serum      | 0.624 | 0.631 | 0.627 | 0.628                | 6.62                |
| R71N + control serum       | 0.111 | 0.115 | 0.112 | 0.114                | —                   |
| C87G-C88G + allergic serum | 0.775 | 0.783 | 0.788 | 0.782                | 32.77               |
| C87G-C88G + control serum  | 0.12  | 0.108 | 0.111 | 0.113                | —                   |
| C100Y + allergic serum     | 0.851 | 0.847 | 0.846 | 0.848                | 43.97               |
| C100Y + control serum      | 0.114 | 0.121 | 0.116 | 0.117                | —                   |
| K132N + allergic serum     | 0.917 | 0.911 | 0.913 | 0.915                | 55.35               |
| K132N + control serum      | 0.108 | 0.112 | 0.116 | 0.112                | —                   |

Inhibition rate formula:  $(A_{\text{mutant}} - A_{\text{Fag t 2}})/A_{\text{Fag t 2}}$ ; the symbol “—” represents that ratio has no value.

the purity of these recombinant proteins was high without any significant bands of other proteins (Fig. 1). This result was same as the related investigations (Cai et al., 2006; Li et al., 2011). In order to prepare for enzyme-linked immunosorbent assay, Fag t 2 and six mutant allergens were enriched using vivaspin ultrafiltration, respectively. And their concentrations were detected with method of Bradford with final concentration of 1 mg/mL (Table S3).

### 3.3. Circular dichroism

As proteins have UV absorbance at 185–240 nm and the asymmetry structure, the mutant allergens can be determined at 180–300 nm. The CD result indicated that spectrum of Fag t 2 and six mutant allergens almost overlapped, which fully showed that mutant allergens with the replaced amino acid did not change the original space structure (Fig. 2). Furthermore, there was a large negative absorption peak at 220–240 nm in Fag t 2 and mutant allergens. As we know, the absorption peaks of  $\alpha$ -helix and  $\beta$ -sheet are mainly at 208 nm (–) and 222 nm (–), and 215–217 nm (–), respectively, and the absorption peaks of unordered

loops are mainly at <200 nm (–). Although online prediction showed that  $\alpha$ -helix accounts for 68.64% in Fag t 2 and mutant allergens (Fig. S2), recombinant expression might affect the correct folding of Fag t 2 and mutant allergens, which resulted in secondary structure change. But the change of spatial conformation did not affect the analysis of epitopes, as shown in the CD result, the absorption peak of Fag t 2 and mutant allergens were similar. It was demonstrated that the introduced mutations did not cause changes in the spatial structure of Fag t 2. Nevertheless, based on the CD results, we suspected that there were structural differences between the overexpressed allergens and wide type allergens, and it was very likely that these epitopes are also different. Of course, there may be differences between the predicted allergen and the wide type or overexpressed allergen in structure.

### 3.4. ELISA assay

B-cell epitopes are mostly discontinuous epitopes and continuous epitopes, which must be located on the surface of the antigen and can be directly recognized by B cell receptors. Epitope residues may be the reason at these positions for IgE binding activity (Cheng, Tam, Chou, Tai, & Shen, 2008). Next, the core B-cell epitopes of Fag t 2 could be determined by testing whether the IgE binding of the mutant allergens was expected to decrease. The immunogenicity of the six mutant allergens and the binding activity of the synthetic polypeptide need to be verified by immunocompetence experiments. Compared with western blotting, the method of ELISA can more directly determine the binding efficiency of the antigen-antibody, and the operation is simple and efficient. According to the inhibition rate formula:  $(A_{\text{mutant}} - A_{\text{Fag t 2}})/A_{\text{Fag t 2}}$ , the reduction of mutant IgE binding was used to judge with the normal serum without allergic to tartary buckwheat as control. Compared to the original Fag t 2 of tartary buckwheat, the antigenicity of the six mutant allergens were reduced to some extent (Table 3). C38G and R71N were reduced insignificantly, which indicated that the two replacements had no significant effect on the anaphylaxis of Fag t 2, and should not belong to the B-cell epitope of Fag t 2. The inhibitory rates of C52G, C87G-C88G, C100Y and K132N were 21.05%, 32.77%, 43.07% and 55.35%, respectively, which indicated that those above sites played a certain role in the immunogenicity of Fag t 2. The inhibition rate of K132N was more than 50%, confirming that it played a critical role in the immunogenicity of Fag t 2, and should belong to the core B-cell

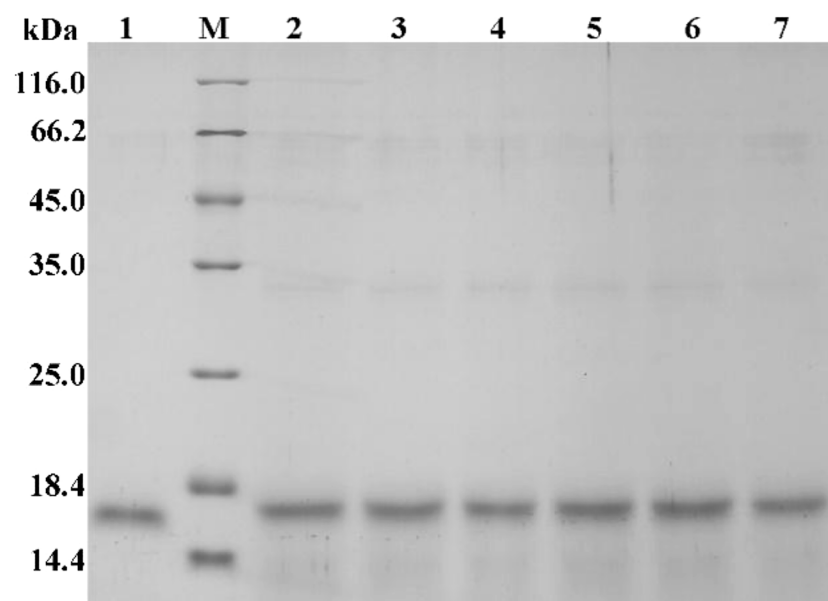


**Fig. 1.** Purification of Fag t 2 and six mutant allergens M: Molecular weight marker; lane 1: Fag t 2; lane 2: Fag t 2<sup>C38G</sup>(TGC-to-GGC); lane 3: Fag t 2<sup>C52G</sup>(TGT-to-GGT); lane 4: Fag t 2<sup>R71N</sup>(AGG-to-AAC); lane 5: Fag t 2<sup>C87G-C88G</sup>(TGTGT-to-GGCGGT); lane 6: Fag t 2<sup>C100Y</sup>(TGC-to-TAC); lane 7: Fag t 2<sup>K132N</sup>(AAG-to-AAC).

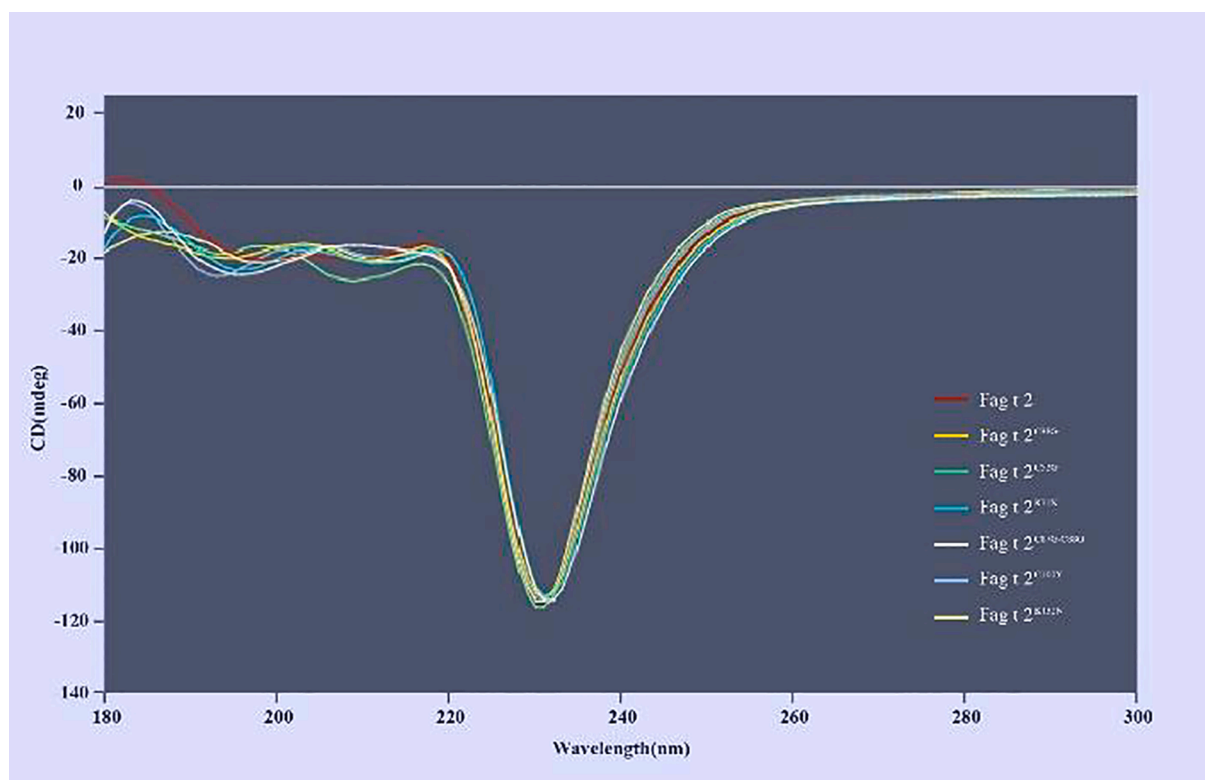


Fig. 2. Circular dichroism spectra of Fag t 2 and six mutant allergens.

epitopes of Fag t 2.

### 3.5. Dot blotting

T-cell epitopes are mostly continuous epitopes, which exist in any part of the antigen. The antigen needs to be processed by the antigen-presenting cells, and then the epitope can be exposed after treatment, and then transferred to the surface of the cell membrane after being combined with HLA molecules. Combined with T cell epitopes predictions and peptide synthesis techniques, dot hybridization can more rapidly and efficiently detect the antigenicity of amino acid fragments (Barre et al., 2009). It is particularly suitable for the study of linear epitopes of allergens. The complete allergenic protein peptide chain is divided into different short peptides, and the core linear epitope can be identified in a short time using point hybridization with sensitivity, efficiency and accuracy. Dot blotting was performed on six mutant allergens and Fag t 2 using the prepared patient's serum (voluntarily provided by buckwheat allergic patients). As shown in Fig. 3, Fag t 2 had a strong hybridization signal [Fig. 3(9)]. In addition, the fifth group of 108–117 ENQKGRIGET peptides and the seventh group of 132–141 KCGISEMECH peptides also had significant hybridization signals, but the hybridization signals were relatively weak compared to Fag t 2,

which indicating that the two groups of peptides segments were able to bind to patient's serum. While there were no obviously hybridization signals appeared in the other five groups. It could be concluded that the ENQKGRIGET peptide at position 108–117 and the KCGISEMECH peptide at position 132–141 were the core T cell epitopes of Fag t 2. At present, the studies on the allergen epitopes of tartary buckwheat mainly focus on the analysis of epitopes and the verification of allergic activity. There are few in-depth studies. For egg allergy, epitope mapping method were introduced to quantitative detection of the epitope and provided some reference for developing the egg allergens vaccine (Xiao et al., 2016). Modification of epitopes of tumor antigens and other disease-causing epitopes is a way to enhance their immunogenicity (Huang & Wei, 2016; Shi-Fang, Shao, & Chang, 2017). In our research, the research results provide a reliable theoretical basis for the further construction of low-sensitive buckwheat varieties and preparation of allergens vaccine.

In our results, the groups of 108–117 ENQKGRIGET peptides and 132–141 KCGISEMECH peptides had significant hybridization signals, there were no obviously hybridization signals appeared in the other five groups contained positions 52, 87–88 and 100 from predicted B-cell epitopes. This result was most likely because these sites located in the conformational epitopes rather than the linear epitopes.

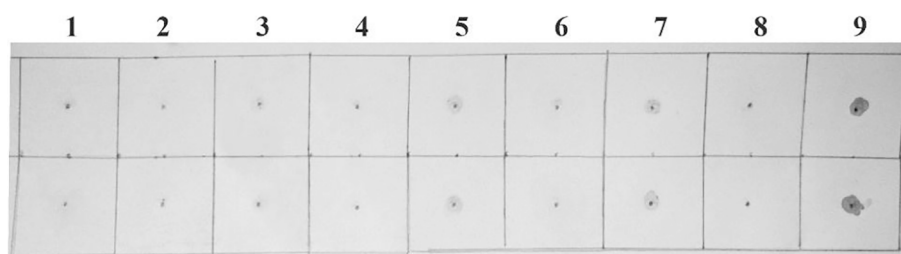


Fig. 3. Dot blotting of Fag t 2 and six mutant allergens 1:VKMMEPELVKCN(Pos.41–53), 2:ALSRIQEGEGCESEE(Pos.68–81), 3:LRGCCVAMKEME(Pos. 84–95), 4: ECVCEWMKM(Pos. 97–105), 5:ENQKGRIGET(Pos. 108–117), 6:KGIRDLEKLPN(Pos. 121–131), 7: KCGISEMECH (Pos. 132–141), 8: Blank control, 9: Fag t 2 control.

## 4. Conclusion

In this study, core epitopes of 16 kDa allergen (Fag t 2) in tartary buckwheat were evaluated based on the *Fag t 2* gene sequence and its corresponding amino acid sequence. The T- and B-cell core epitopes of Fag t 2 were predicted by bioinformatics. Six sets of mutation-substituted amino acids were designed based on predicted B-cell epitopes, namely C38G, C52G, R71N, C87G-C88G, C100Y, and K132N. Then Fag t 2 and its six mutant allergens were expressed in *Pichia pastoris*. Bioinformatic analysis and CD result showed that the secondary structure of the mutant allergens and the original allergen were almost unchanged, which visually confirmed that the mutation did not affect the spatial structure of the allergen. Competitive inhibition ELISA experiments showed that K132N was a core B- cell epitope. Dot blotting showed that the ENQKGRIGET peptide located at positions 108–117 and the KCGISEMECH peptide at positions 132–141 were core T cell epitopes of Fag t 2. This result contributes to the preparation of hypoallergenic vaccines and the acquisition of hypoallergenic plants.

## 5. Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors. Buckwheat allergy patients' serum were provided voluntarily, and the present study was approved by College of Life Science of Northwest A & F University.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128953>.

## References

- Barre, A., Culerrier, R., Granier, C., Selman, L., Peumans, W. J., Van Damme, E. J., ... Rouge, P. (2009). Mapping of IgE-binding epitopes on the major latex allergen Hev b 2 and the cross-reacting 1,3beta-glucanase fruit allergens as a molecular basis for the latex-fruit syndrome. *Molecular Immunology*, 46(8–9), 1595–1604.
- Cai, G., Bai, C., Li, Y., Zhang, Z., & Wang, Z. (2006). Fusion expression and analysis of the epitope regions in Tartary buckwheat allergen. *Food Science*, 27(11), 31–34.
- Chen, P., Guo, Y. F., Yan, Q., & Li, Y. H. (2011). Molecular cloning and characterization of Fag t 2: a 16-kDa major allergen from Tartary buckwheat seeds. *Allergy*, 66(10), 1393–1395.
- Cheng, T. T., Tam, M. F., Chou, H., Tai, H. Y., & Shen, H. D. (2008). Lys89, Lys90, and Phe91 are critical core amino acid residues of the Pen ch 18 major fungal allergen recognized by human IgE antibodies. *Biochemical & Biophysical Research Communications*, 375(4), 671–674.
- Cho, J., Lee, J.-O., Choi, J., Park, M.-R., Shon, D.-H., Kim, J., Ahn, K., & Han, Y. (2015). Significance of 40-, 45-, and 48-kDa proteins in the moderate-to-severe clinical symptoms of buckwheat allergy. *Allergy Asthma Immunology Research*, 7(1), 37–43.
- Choi, S. Y., Sohn, J. H., Lee, Y. W., Lee, E. K., Hong, C. S., & Park, J. W. (2007). Characterization of buckwheat 19-kD allergen and its application for diagnosing clinical reactivity. *International archives of allergy and immunology*, 144(4), 267–274.
- Fujino, K., Funatsuki, H., Inada, M., Shimono, Y., & Kikuta, Y. (2001). Expression, cloning, and immunological analysis of buckwheat (*Fagopyrum esculentum* Moench) seed storage proteins. *Journal of agricultural and food chemistry*, 49(4), 1825–1829.
- Hong, J.-H., Ikeda, K., Kreft, I., & Yasumoto, K. (1996). Near-infrared diffuse reflectance spectroscopic analysis of the amounts of moisture, protein, starch, amylose, and tannin in buckwheat flours. *Journal of Nutritional Science and Vitaminology*, 42(4), 359–366.
- Horesh, A. J. (1972). Buckwheat sensitivity in children. *Annals of Allergy Asthma & Immunology*, 30(12), 685–689.
- Huang, J., & Wei, M. (2016). Advances in research on modification strategies of CTL epitopes of tumor antigens. *Journal of Modern Oncology*, 24(13), 2170–2175.
- Jeon, Y. J., & Hong, K. W. (2008). A Duplex PCR assay for differentiating native common buckwheat and tartarian buckwheat, and its application for the rapid detection of buckwheat ingredients in food. *Food Science & Biotechnology*, 17(2), 357–361.
- Jeong, K. Y., Son, M., Lee, J. Y., Park, K. H., Lee, J.-H., & Park, J.-W. (2016). Allergenic characterization of 27-kDa glycoprotein, a novel heat stable allergen, from the pupa of silkworm, *Bombyx mori*. *Journal of Korean Medical Science*, 31(1), 18–24.
- Koyano, S., Takagi, K., Teshima, R., & Sawada, J. (2006). Molecular cloning of cDNA, recombinant protein expression and characterization of a buckwheat 16-kDa major allergen. *International archives of allergy and immunology*, 140(1), 73–81.
- Kreft, I., & Skrabanja, V. (2002). Nutritional properties of starch in buckwheat noodles. *Journal of Nutritional Science & Vitaminology*, 48(1), 47–50.
- Li, P., Cui, X., Li, Y., & Wang, Z. (2011). Epitope mapping and identification on a 3D model built for the tartary buckwheat allergic protein Tbb. *Acta Biochimica et Biophysica Sinica*, 43(6), 441–447.
- Matsumoto, R., Fujino, K., Nagata, Y., Hashiguchi, S., Ito, Y., Aihara, Y., ... Sugimura, K. (2004). Molecular characterization of a 10-kDa buckwheat molecule reactive to allergic patients' IgE. *Allergy*, 59(5), 533–538.
- Nakamura, S., Suzuki, Y., Ishikawa, E., Yakushi, T., Jing, H., Miyamoto, T., & Hashizume, K. (2008). Reduction of in vitro allergenicity of buckwheat Fag e 1 through the Maillard-type glycosylation with polysaccharides. *Food Chemistry*, 109(3), 538–545.
- Park, J. W., Kang, D. B., Kim, C. W., Koh, S. H., Yum, H. Y., Kim, K. E., ... Lee, K. Y. (2000). Identification and characterization of the major allergens of buckwheat. *Allergy*, 55(11), 1035–1041.
- Park, K., Jeong, K., & Lee, S. (2016). Clinical and laboratory findings of childhood buckwheat allergy in a single tertiary hospital. *Korean J Pediatr*, 59(10), 402–407.
- Sammur, D., Dennison, P., Venter, C., & Kurukulaarachy, R. J. (2011). Buckwheat allergy: a potential problem in 21st century Britain. *BMJ Case Reports*, 2011(nov 08 1).
- Shao, J. P., & Fu, J. (2019). Characteristics and prediction of B- and T-cell epitopes of the gp41 protein of HIV-1. *Journal of Pathogen Biology*, 3, 270–274.
- Shi-Fang, L. L., Shao, J. J., & Chang, H. Y. (2017). Research progress on the epitope-based vaccine against foot and mouth disease. *China Animal Husbandry & Veterinary Medicine*, 44(2), 396–401.
- Shigemori, S., Yonekura, S., Sato, T., Otani, H., & Shimosato, T. (2013). Expression of the immunoreactive buckwheat major allergenic storage protein in *Lactococcus lactis*. *Applied Microbiology & Biotechnology*, 97(8), 3603–3611.
- Smith, H. L. (1990). Buckwheat-poisoning with report of a case in man (1909). *Allergy proceedings: the official journal of regional and state allergy societies*, 11(4), 193–6; discussion 189–92.
- Sung, D.-E., Lee, J., Han, Y., Shon, D.-H., Ahn, K., Oh, S., & Do, J.-R. (2014). Effects of enzymatic hydrolysis of buckwheat protein on antigenicity and allergenicity. *Nutrition Research & Practice*, 8(3), 278–283.
- Wang, Z., Wang, L., Chang, W., Li, Y., Zhang, Z., Wieslander, G., & Norback, D. (2006). Cloning, expression, and identification of immunological activity of an allergenic protein in Tartary buckwheat. *Bioscience, Biotechnology, and Biochemistry*, 70(5), 1195–1199.
- Wang, Z., Zhang, Z., Zhao, Z., Wieslander, G., Norbäck, D., & Kreft, I. (2004). Purification and characterization of a 24 kDa protein from tartary buckwheat seeds. *Bioscience, Biotechnology, and Biochemistry*, 68(7), 1409–1413.
- Xiao, N., Liu, Y. Y., Liu, X. W., Tong, P., Zhou, H., Feng, Y. J., ... Gao, J. Y. (2016). Research of egg allergen epitope. *Science & Technology of Food Industry*, 37(3), 394–399.
- Yoshimasu, M. A., Zhang, J. W., Hayakawa, S., & Mine, Y. (2000). Electrophoretic and immunochemical characterization of allergenic proteins in buckwheat. *International archives of allergy and immunology*, 123(2), 130–136.
- Yoshioka, H., Ohmoto, T., Urisu, A., Mine, Y., & Adachi, T. (2004). Expression and epitope analysis of the major allergenic protein Fag e 1 from buckwheat. *Journal of plant physiology*, 161(7), 761–767.
- Zhang, X., Yuan, J. M., Cui, X. D., & Wang, Z. H. (2008). Molecular cloning, recombinant expression, and immunological characterization of a novel allergen from Tartary buckwheat. *Journal of Agricultural & Food Chemistry*, 56(22), 10947–10953.
- Zheng, B., Zhang, H., Wang, L., Guo, Y., & Chen, P. (2018). Characterization of 16-kDa major allergen with  $\alpha$ -amylase inhibitor domain in tartary buckwheat seeds. *Molecular Immunology*, 94, 121–130.

**Supplementary table 1** Mutation primers sequences

| Mutation site | Name       | Sequence                                 |
|---------------|------------|------------------------------------------|
| TGC-GGC       | C38G 1-1   | GATGAGCTCAAAGGGCACGCGACAAG               |
|               | C38G 1-2   | CTTGTCGCGTGGCCTTTGAGCTCATC               |
| TGT-GGT       | C52G 1-1   | CCAGAACTCGTGAAAGGTAACCGTTACATAGC         |
|               | C52G 1-2   | GCTATGTAACGGTTACCTTTCACGAGTTCTGG         |
| AGG-AAC       | R71N 1-1   | CGAAGAAGCTTTGAGTAACATTCAAGGTGAAGGATGC    |
|               | R71N 1-2   | GCATCCTTCACCTTGAATGTTACTCAAAGCTTCTTCG    |
| TGTTGT-GGCGGT | CC87GG 1-1 | GAAGTGTCTGAGAGGAGGCGGTGTGGCGATGAAGG      |
|               | CC87GG 1-2 | CCTTCATCGCCACACCGCCTCCTCTCAGACACTTC      |
| TGC-TAC       | C100Y 1-1  | GATGGAAGATGAATGTGTTTACGAGTGGATGAAGATGATG |
|               | C100Y 1-2  | CATCATCTTCATCCACTCGTAAACACATTCATCTTCCATC |
| AAG-AAC       | K132N 1-1  | GAAGGAATTGCCTAATAACTGTGGGATTAGTGAAATGG   |
|               | K132N 1-2  | CCATTTCACTAATCCACAGTTATTAGGCAATTCCTTC    |

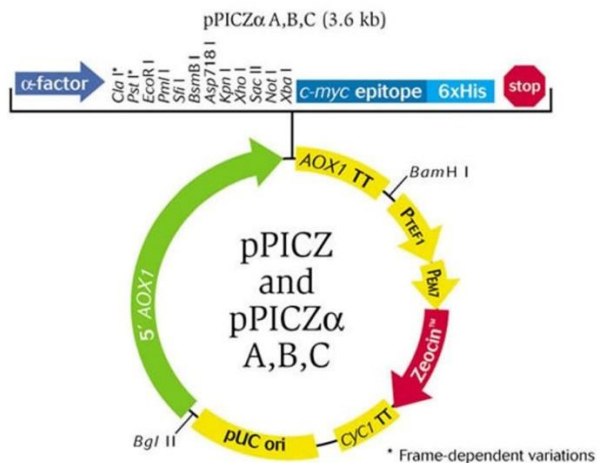
**Supplementary table 2** Isoelectric point and molecular weight of Fag t 2 and six mutant allergens

sequence

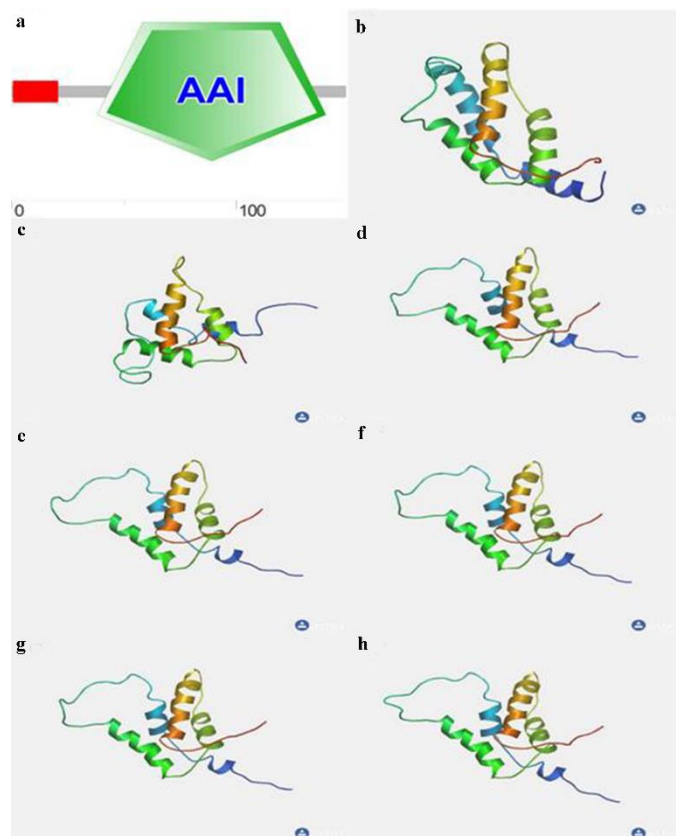
| Amino acid sequence | Isoelectric point | Molecular weight |
|---------------------|-------------------|------------------|
| Fag t 2             | 5.23              | 17280.21         |
| C38G                | 5.23              | 17234.12         |
| C52G                | 5.23              | 17234.12         |
| R71N                | 5.09              | 17238.12         |
| CC87GG              | 5.23              | 17188.03         |
| C100Y               | 5.23              | 17234.12         |
| K132N               | 5.09              | 17266.14         |

**Supplementary table 3** The concentration of six mutant allergens solutions

| Mutant protein  | C38G   | C52G   | R71N   | CC87GG | C100Y  | K132N  |
|-----------------|--------|--------|--------|--------|--------|--------|
| Absorbance      | 0.191  | 0.159  | 0.227  | 0.194  | 0.161  | 0.239  |
| Protein mass    | 25.624 | 21.172 | 30.253 | 25.829 | 21.434 | 31.922 |
| Solution volume | 25     | 20     | 30     | 25     | 20     | 30     |
| Protein content | 1.024  | 1.059  | 1.008  | 1.033  | 1.072  | 1.064  |



**Supplementary Figure 1** The map of expression vector pPICZ( $\alpha$ )A.



**Supplementary Figure 2** Spatial structure prediction of Fag t 2 and six mutant allergens

a.  $\alpha$ -amylase inhibitor domain predicted from Fag t 2 and six mutant allergens; b. Three-dimensional structure of Fag t 2; c. Three-dimensional structure of Fag t 2<sup>C38G</sup>; d. Three-dimensional structure of Fag t 2<sup>C52G</sup>; e. Three-dimensional structure of Fag t 2<sup>R71N</sup>; f. Three-dimensional structure of Fag t 2<sup>C87C</sup>-G88G; g. Three-dimensional structure of Fag t 2<sup>C100Y</sup>; h. Three-dimensional structure of Fag t 2<sup>K132N</sup>.

# CLUSTAL 2.0.12 multiple sequence alignment

```

TBW16 1 -----MKLFILATATLLIAATQAKYLRDEG-----FDLGETQMSKCTRQ
Bwp16 1 -----MKLFILATATLLIAATQATVPRDEG-----FDLGETQMSKCMRQ
TBW10 1 -----MKLFLILAAASLLIUASHA-----DSQMRSKCRKQ
Bwp8 1 -----MKLFLILAAASLLIUASHA-----DSQMRSKCRKQ
conglutin 1 -----MAKLTILUALAPFLAARASAR-----QQWELQGDARRCQ
Rc2S 1 MQKLYHTINNHLHLSNTHRSNSVFLSTARSQASSTMAKFALLASFIALLFLUDASIYRTTUIUD
      :  :::: *      :  :
      :  :::: *      :  :

TBW16 42 UKMMEPELUKCNRYIAMDIMDDKYEE-----ALSRIQEGGES-EEKFLRGCCUAMKEME
Bwp16 42 UKMMEPHLKKCNRYIAMDILDDKYAE-----ALS RUEGEGCKS-EESCHRGCCUAMKEMD
TBW10 31 MRMMEPQLQCEGYHTMDMMDA-----DSMRGQECRS-EESCHRGCCUAMKEMD
Bwp8 31 MRMMEPQLQCEGYHTMDMMDA-----DSMRGQECRS-EESCHRGCCUAMKEMD
conglutin 35 SQLERANLRPCEEHLHQKIQRDEDSYERDPYSPSPQDPYSPYDRRGAGS-SQHQR-CCNELNEFE
Rc2S 68 EEDANPSYQSCREQUUMRQVLSPCQEYIR-----QQVAGLGLSHGYNPRLDCCERIQSMQ
      .  .. * . :      .      :  * * *  ::::

TBW16 97 D--ECUCEWMKMHUENQK--GRI-GETLMRKGIRDLKELPNKCGIS-EMECHS-RGNWYVU--
Bwp16 97 D--ECUCEWMKMHUENQK--GRI-GERL IEGURDLKELPSKCGLS-ELECGS-RGNRYFU--
TBW10 80 D--ECMCEWMKMHUQQQR--GEM-GEEDMRMUMRKMQLPNKCGMG-HMRCHMGMTROYE--
Bwp8 80 D--ECMCEWMKMHUQQQR--GEM-GEEDMRMUMRKMQLPNKCGMG-HMRCHMGIGTRDYE--
conglutin 101 NNQRCEALQQIMENQS--DRLQGRQEQQFKRELRLNPQQCGLRAPQRCDLDVESGGDRDY
Rc2S 125 T--QCRCEGLRMAIDQKSKGILGQDS-RQAYNIAQDLPTYCGUS-PQKCRFGTRWGF----
      . * * *  ::  :::: *  . :  .  :::: * * :  . *

```

## Supplementary Figure 3 Amino acid sequences alignment of Fag t 2

TBW16 represents *Fagopyrum tataricum* 16 kDa allergen Fag t 2, Bwp16 represents *Fagopyrum*

*esculentum* 16 kDa allergen, TBW10 represents *Fagopyrum tataricum* 10 kDa allergen, Bwp8

represents *Fagopyrum esculentum* 8 kDa allergen, conglutin represents *Arachis duranensis* conglutin,

and Rc2S represents *Ricinus communis* 2S sulfur-rich seed storage protein.

"\*" indicates positions which have a single, fully conserved residue. ":" indicates that one of the

following 'strong' groups is fully conserved: STA/ NEQK/ NHQK/ NDEQ/ QHRK/ MILV/ MILF/HY/

FYW. "." indicates that one of the following 'weaker' groups is fully conserved: CSA/ ATV/ SAG/

STNK/ STPA/ SGND/ SNDEQK/ NDEQHK/ NEQHRK/ FVLIM/ HFY.

```

(1) C38G: TGC→GGC
Query 1 AGAGATGAAGGCTTCGATTAGGCGAAACCCAGATGAGCTCAAAGGCGACGACAAGTG 60
Sbjct 1 AGAGATGAAGGCTTCGATTAGGCGAAACCCAGATGAGCTCAAAGTGCACGCGACAAGTG 60

(2) C52G: TGT→GGT
Query 61 AAGATGATGGAGCCAGAACTCGTGAAGGTAACCGTTACATAGCCATGGATATAATCGAC 120
Sbjct 61 AAGATGATGGAGCCAGAACTCGTGAAGTGTAAACCGTTACATAGCCATGGATATAATCGAC 120

(3) R71N: AGG→AAC
Query 121 GACAAATACGAAGAAGCTTTGAGTAACATTCAAGGTGAAGGATGCGAGAGTGAAGAGAAG 180
Sbjct 121 GACAAATACGAAGAAGCTTTGAGTAGGATTCAAGGTGAAGGATGCGAGAGTGAAGAGAAG 180

(4) CC87G: TGTGT→GGCGGT
Query 181 TGTCTGAGAGGAGGCGGTGTGGCGATGAAGGAGATGGAAGATGAATGTGTTGCGAGTGG 240
Sbjct 181 TGTCTGAGAGGAGTGTGTGTGGCGATGAAGGAGATGGAAGATGAATGTGTTGCGAGTGG 240

(5) C100Y: TGC→TAC
Query 181 TGTCTGAGAGGATGTTGTGTGGCGATGAAGGAGATGGAAGATGAATGTGTTTACGAGTGG 240
Sbjct 181 TGTCTGAGAGGATGTTGTGTGGCGATGAAGGAGATGGAAGATGAATGTGTTTACGAGTGG 240

(6) K132N: AAG→AAC
Query 301 ATTAGGGATTGAAGGAATTGCCTAATAACGTGGGATTAGTGAATGGAATGTCACTCT 360
Sbjct 301 ATTAGGGATTGAAGGAATTGCCTAATAAGGTGGGATTAGTGAATGGAATGTCACTCT 360

```

**Supplementary Figure 4** The sequencing result of six mutant plasmids (Note: Red boxes indicate the mutated bases)