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Purification and Biochemical Characterization of a Novel Allergenic Protein from Tatar buckwheat Seeds

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Key words

- *Fagopyrum tataricum* Gaertn.
- Polygonaceae
- biochemical characterization
- immunological activity
- allergen

Abstract

▼ An allergenic protein from tatar buckwheat seeds, designated TBt, was purified by salting out, anion exchange chromatography and size exclusion chromatography (SEC). Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and SEC showed that the allergenic protein was composed of two subunits linked by disulfide bonds, which we named TBa and TBb. TBa, TBb and TBt have estimated molecular weights of 24, 34 and 56 kDa, and isoelectric points of 6.85, 5.85 and 7.00, respectively. The

immunological characteristics of the three proteins as analyzed by enzyme-linked immunosorbent assay revealed that TBa and TBb have comparable IgE binding activity, both higher than that of TBt. Determination of sulfhydryl (-SH) and disulfide (-S-S-) groups in TBt showed that most cysteine residues exist as disulfide linkages rather than free -SH groups. We calculated that TBt has an -S-S- content of 1.978 M/M protein, indicating that TBt contains one disulfide bond and could be the precursor of a 24 kDa allergenic protein from tatar buckwheat seeds.

Introduction

▼ Buckwheat (*Fagopyrum*), an annual melliferous crop, is grown in many countries of the world. In recent years, an increasing number of scientists have considered its nutritional components. The protein content of buckwheat flour is significantly higher than that of rice, wheat, millet, sorghum and maize [1], [2]. In addition to high-quality protein, buckwheat seeds contain many bioactive components such as flavonoids, phytosterols, fagopyrins and thiamine-binding proteins that have beneficial effects on the cardiovascular health [3]. However, buckwheat seed proteins can cause a hypersensitive reaction, which limits their use as a general food source and additive. Individuals in Europe, North America and some Asian countries have developed serious buckwheat allergy or allergenic symptoms [4], including wheezing, urticaria, vomiting and anaphylactic shock, even after ingestion of only a small amount by some individuals [5], [6]. Allergens from common buckwheat (*F. esculentum* Moench, CB) have been previously investigated and characterized [7], [8]. Electrophoretic immunoblotting demonstrated that proteins with molecular weights of 8–9, 10, 24, 36, 39–40 and

70–72 kDa displayed specific IgE binding activity to sera from patients allergic to buckwheat [9], [10], [11]. The 22–24 kDa protein in CB was considered to be the major allergenic protein [6]. The cDNA of another allergen in CB, named Fag e 1, was also isolated [12] and its epitopes analysed [13]. Limited studies on potentially allergenic proteins in tatar buckwheat (*F. tataricum* Gaertn, TB) are available [14]. Studies comparing specific proteins in TB with those in CB are also lacking. Tatar buckwheat has a long history of cultivation and usage in China, it is not only an ancient and specialty grain, but also a traditional Chinese herb. Current research on functional proteins in TB is very limited. The allergenic component and the exact allergenic mechanism involved are uncertain. In the present study, we isolated and purified one major allergen, here called TBt, from TB seeds. We further determined several structural characteristics of the 56 kDa TBt allergen.

Materials and Methods

▼ Materials

TB seeds were collected from ShouYang County, China, in October 2006, and were authenticated

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Bibliography

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by Prof. Rufa Lin (Agriculture Sciences Academy of Shanxi, China). A voucher specimen (No. 1015) was deposited in the National Germplasm Storehouse of China. Ampholine (pH range 3.5–10.0), isoelectric focusing (IEF) protein standards, molecular weight markers, the Resource Q anion exchange column and Sephacryl S-200 gel filtration columns were obtained from Amersham Biosciences. A microwell IgE ELISA kit was purchased from Xinwan. All other reagents were of the highest purity available.

Extraction of crude protein

Air-dried tatar buckwheat seeds were ground into powder in a miller, and 50 g of the powder was defatted in 200 mL of cold acetone at 4 °C. The defatted flour was immersed in 500 mL of 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl for 24 h. The precipitate was removed by centrifugation at 3000 rpm for 20 min and solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the supernatant to the point of 80% saturation. The precipitate was collected by centrifugation at 8000 rpm for 30 min at 4 °C, redissolved in 10 mM Tris-HCl (pH 8.0) buffer and dialyzed against water. The dialyzate was centrifuged and lyophilized to obtain crude protein (560 mg) for subsequent purification.

Purification of target protein

Crude protein (30 mg) as prepared above was dissolved in 10 mL of 200 mM phosphate buffer, pH 7.3, and centrifuged at 10,000 rpm for 20 min. The resulting supernatant (1 mL) was loaded onto a Resource™ Q anion exchange column (6.4 × 30 mm) pre-equilibrated with 15 mL 200 mM phosphate buffer, pH 7.3. Chromatography was carried out by initial elution with the same buffer (6 mL), followed by a linear gradient from 0 to 0.5 M NaCl in the same buffer (9 mL) at a flow rate of 0.5 mL/min. Two fractions (2.8 mL) from the anion exchange column were collected and 1 mL loaded onto a Sephacryl S-200 (1.6 × 60 cm) gel filtration column equilibrated with 200 mM phosphate buffer, pH 7.3 (150 mL) and then eluted with the same buffer (120 mL). The target fractions (5.2 mL), designated TBt, were collected and lyophilized after desalting.

Determination of TBt's molecular weight

The molecular weight of TBt was estimated by size exclusion chromatography (SEC) on a Superose 12 (1.0 × 30 cm) column using an ÄKTA purifier liquid chromatograph (Amersham Biosciences). The separation conditions were as follows: solvent system, 20 mM potassium phosphate buffer (pH 7.4) containing 0.2 M NaCl and 1 % Triton X-100 at a flow rate of 0.5 mL/min; standard proteins, α -lactalbumin (18 kDa), trypsin inhibitor (20 kDa), ovalbumin (45 kDa), and bovine serum albumin (68 kDa). K_{av} was calculated as follows: $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_o is the outer volume (7.71 mL) and V_t is the total volume (23.56 mL). SDS-PAGE was used to confirm the molecular weight.

Electrophoresis analysis

To estimate the possible state of TBt, SDS-PAGE was performed under reducing and non-reducing conditions using the methods of Laemmli [15]. Samples were dissolved in reducing or non-reducing sample buffer, heated at 100 °C for 3 min, and centrifuged at 10,000 rpm for 5 min. Electrophoresis was conducted under a constant current of 20 mA for 3 h. The gels were stained with Coomassie brilliant blue R-250. The molecular weight of individual protein bands was determined using a GeneGenius densitometric image analysis system with standard proteins.

Analysis of sulfhydryl and disulfide contents

The sulfhydryl (-SH) and disulfide (-S-S-) contents of TBt were determined using the method of Beveridge [16]. Protein samples (6.4 mg) were dissolved in 2 mL of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.04 M EDTA, pH 8.0) containing 8 M urea. The solution was gently stirred overnight until a homogenous dispersion was obtained. To determine the -SH content, 4 mL of Tris-Gly buffer (pH 8.0) were added to 1 mL of TBt protein solution. Then 50 μ L of Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid in Tris-Gly buffer, 4 mg/mL) were added and the absorbance was measured at 412 nm after 5 min. For analysis of the total -SH content [-SH and reduced -S-S-], 50 μ L of β -mercaptoethanol (β -ME) and 4 mL of Tris-Gly buffer (pH 8.0) were added to 1 mL of the TBt protein solution. The mixture was incubated for 1 h at 37 °C. After an additional hour of incubation with 10 mL of 12 % trichloroacetic acid (TCA), the mixture was centrifuged at 8,000 rpm for 10 min. The precipitate was resuspended twice in 5 mL of 12 % TCA and centrifuged to remove β -ME. The precipitate was dissolved in 5 mL of Tris-Gly buffer, pH 8.0. Then, 50 μ L of Ellman's reagent were added to 5 mL of the resulting TBt protein solution and the absorbance was measured at 412 nm after 5 min. Sample and reagent blanks were prepared for each determination. Results were calculated according to the formula $M\text{SH/g} = 73.53 \times A_{412} \times D/C$, where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/mL), and D is the dilution factor. The constant 73.53 is derived from $10^6/1.36 \times 10^4$, where 1.36×10^4 is the molar absorptivity and 10^6 is the conversion factor for changing from a molar basis to l M/mL basis and from mg solids to g solids. Half of the value after subtracting the -SH value from the total -SH value was defined as the -S-S- content.

Preparation of TBa and TBb by SEC

The purified TBt fraction was precipitated with cold acetone at a ratio of 1 : 3 for 30 min at -20 °C. After centrifugation at 8,000 rpm for 10 min at 4 °C, the precipitate was resuspended in 8 M urea, and β -ME and iodoacetamide were added to final concentrations of 2.88 and 0.1 M, respectively. The sample was injected onto a Sephacryl S-200 column (1.6 × 60 cm) and eluted with 200 mM Tris-HCl buffer (pH 7.3) as described above. These fractions were collected and analysed by SDS-PAGE. The isoelectric point (pI) of the proteins was analysed by isoelectric focusing under reducing conditions on a 7 % polyacrylamide gel according to the method of Hjerten [17].

Determination of immunological activity

Enzyme-linked immunosorbent assays (ELISA) were performed to determine the immunological activity of the three protein samples (TBt, TBa, TBb) [9]. Crude extract of TB flour was used as the control. Two positive sera (from patients with a history of buckwheat food intolerance) were obtained from the Blood Center of Taiyuan, China, and two negative sera from laboratory volunteers were used. A 96-well polystyrene plate was first coated overnight at 4 °C with 100 μ L of buckwheat crude extract (40 μ g/mL) in 50 mM carbonate buffer (pH 9.6). After washing three times with PBS-T (137 mM NaCl, 1.8 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 27 mM KCl, 0.1 % Tween 20, pH 7.4), each well was blocked with 1 % bovine serum albumin in PBS-T at 37 °C for 1 h. At the same time, purified proteins (TBa, TBb, TBt) were mixed with patient sera (1 : 1) at 37 °C for 1 h (serum samples were diluted 1 : 50 in PBS-T before use). After washing three times, the mixtures were added to the well at 37 °C for 1 h. The plate was

then incubated with 1:1000 horseradish-peroxidase (HRP)-mouse anti-human IgE. The plate was further washed and developed with ABTS solution (25 mg of 2,2-azino-bis-3-ethylthiazoline-sulfonic acid in 50 mM citrate phosphate buffer, 50 mL of 30% H₂O₂). After 20 min, 50 μ L of 1 mol/L H₂SO₄ were added to end the reaction and the color intensity of the mixture was measured at 450 nm using a microtiter plate reader (Bio-Rad). The percentage inhibition was calculated as follows: % inhibition = (ODu – ODi)/ODu $\times$ 100, where ODu is the absorbance of the control and ODi is that of the inhibited serum sample.

Results and Discussion

The crude proteins extracted by salting out were loaded onto an anion exchange column and washed, as described in the Materials and Methods section. As shown in **Fig. 1A**, the peak eluted at a volume of about 12.5 mL, corresponding to the target protein with the highest absorbance (peak 2). To further purify the protein for analysis of its properties and function, fraction 2 was collected from the anion exchange column and run on a Sephacryl S-200 column. The absorbance of the eluate was monitored at 280 nm and the target protein eluted at a volume of about 60 mL (**Fig. 1B**). SDS-PAGE revealed a highly purified target protein running as a single band (**Fig. 2**, lane 1) under non-reducing conditions (purity was > 95%), which we named TBt. The protein has an approximate molecular weight of 56 kDa. In the presence of β -ME, the protein electrophoresed as two bands (**Fig. 2**, lane 2). We named the lower and upper bands TBa and TBb, corresponding to estimated molecular weights of 24 and 34 kDa, respectively. The molecular weight of TBa is in good agreement with that reported previously [11], [14].

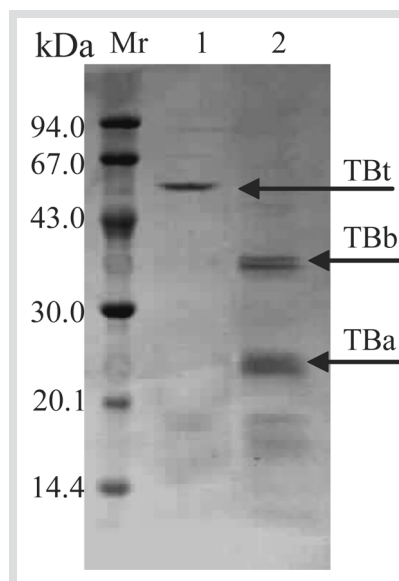


Fig. 2 SDS-PAGE analysis of the protein purified by gel filtration. A 12.5% solution of polyacrylamide gel (containing 1% sodium dodecyl sulfate) was used to separate the samples. Lane 1, molecular weight marker; lane 2, protein fraction purified by gel filtration under non-reducing conditions (sample buffer without β -ME); lane 3, protein purified by gel filtration under reducing conditions (sample buffer contains β -ME).

After treatment of purified TBt with β -ME and iodoacetamide at 37 °C for 1 h, the sample was injected onto a Sephacryl S-200 column. As shown in **Fig. 3**, two peaks eluted at volumes of 103 mL (F2) and 116 mL (F3), with a third small peak at approximately 60 mL (F1) that is similar to the peak in **Fig. 1B**. SDS-PAGE analysis of the fractions collected indicated that TBt had been separated into two fragments (TBa and TBb) at the expected positions (lanes 3 and 4 in the inset of **Fig. 3**). This result suggests that the two subunits might be linked by disulfide bonds.

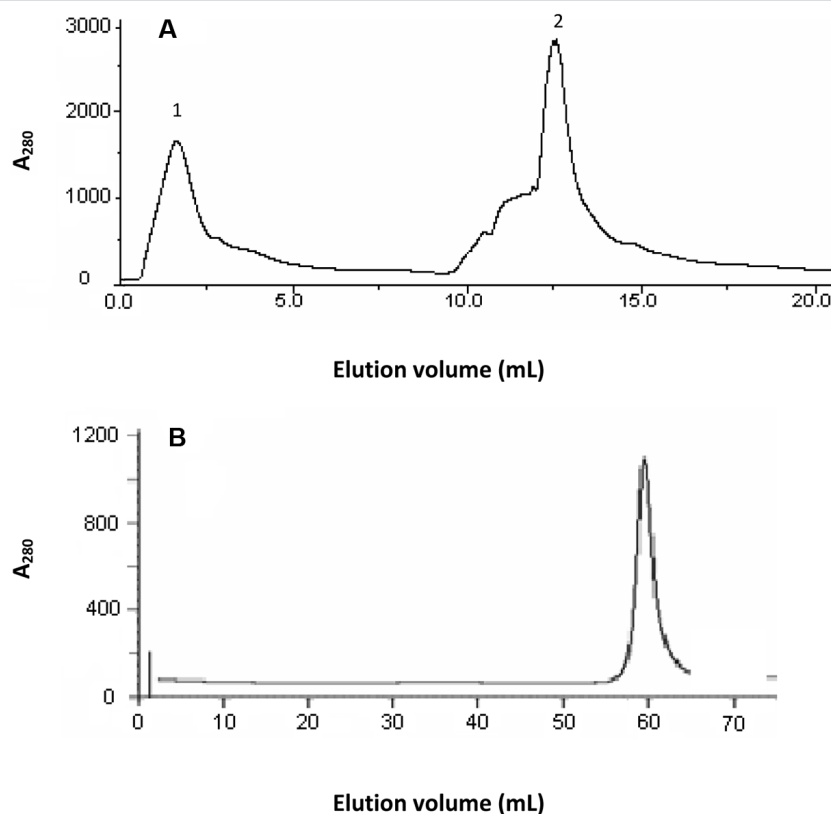


Fig. 1 Chromatographic characterization of TBt protein. (A) Anion exchange chromatography. Crude extracts were loaded onto a Resource Q anion exchange column (6.4 $\times$ 30 mm) pre-equilibrated with 200 mM phosphate buffer (pH 7.3) and eluted using a linear gradient from 0 to 0.5 mol/L NaCl in the same buffer. (B) Size exclusion chromatography. Fraction 2 recovered from the anion exchange column was loaded onto a Sephacryl S-200 (1.6 $\times$ 60 cm) gel filtration column equilibrated with 200 mM phosphate buffer (pH 7.3). The sample was eluted with the same buffer at a constant flow rate of 0.5 mL/min.

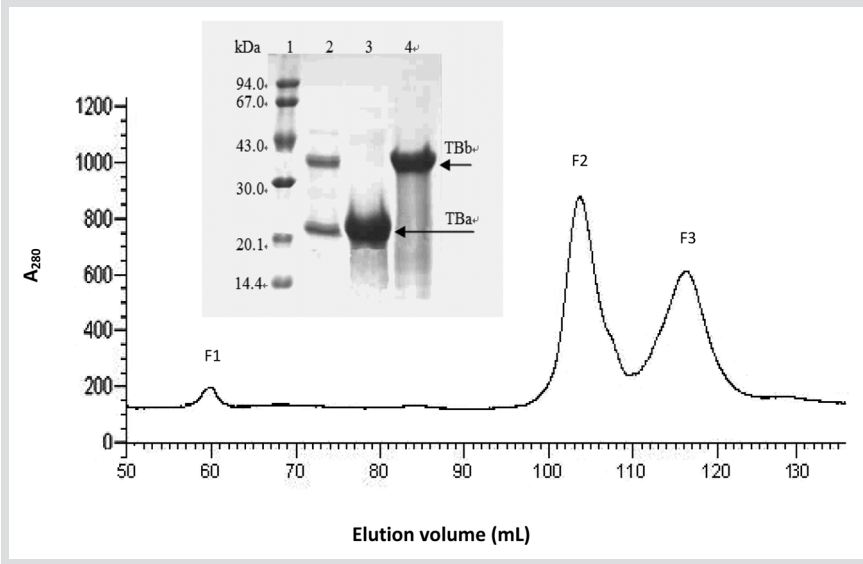


Fig. 3 Analysis of TBa and TBb. After treatment of purified TBt with β -ME and iodoacetamide, the sample was injected onto a Sephacryl S-200 column (1.6 $\times$ 60 cm) and eluted with 200 mM Tris-HCl buffer (pH 7.3). Two peaks eluted at volumes of 103 mL (F2) and 116 mL (F3), with a third small peak at approximately 60 mL (F1). These fractions were collected and analyzed by SDS-PAGE (inset). Lane 1, molecular weight marker; lane 2, fraction F1; lane 3, fraction F3; lane 4, fraction F2.

SEC and non-reducing SDS-PAGE were used to estimate the molecular weight of TBt as described in the Materials and Methods section. From the K_{av} and $\log M_r$ values shown in **Fig. 4**, we calculated a molecular weight of approximately 56 kDa for TBt. This result is consistent with the SDS-PAGE observations (**Fig. 2**), suggesting that TBt consists of two subunits with approximate molecular weights of 24 and 34 kDa in the native state. The pI of TBa, TBb and TBt was analysed by isoelectric focusing under reducing conditions on a 7% polyacrylamide gel. TBt had a pI of 7.0, which is close to the neutral region, while TBa and TBb had values in the acidic region, at approximately 6.85 and 5.85, respectively (data not shown). These values differ from those reported for storage proteins from CB seed [18], [19], but are similar to results for a 13S globulin in CB, where the larger polypeptide (TBb) was focused on the acidic region of the gel, and the smaller polypeptide (TBa) was focused at approximately pH 6.8 [20].

Table 1 shows that TBt has a total -SH content (-SH and -S-S-) of 37.91 μ M/g protein and a free -SH content of 1.95 μ M/g protein. Therefore, each TBt molecule has a total -SH content of

2.085 M/M protein and a free -SH content of 0.107 M/M protein. This suggests that most cysteine residues exist as disulfide linkages rather than as free -SH groups. From this fact we could also calculate that TBt has an -S-S- content of 1.978 M/M protein, indicating that TBt contains one disulfide bond. This result is compatible with the reducing and non-reducing SDS-PAGE patterns (**Fig. 2** and **Fig. 3**) indicating that the protein components were linked by disulfide bonds. This result suggests that most of the disulfide bonds in TBt exist in the form of inter-molecular disulfide linkages, similar to the globulin from CB [19].

ELISA was performed to establish whether the purified TBa, TBb and TBt could block a crude extract by binding to specific IgE. Sera from subjects with positive and negative IgE binding to buckwheat allergen were measured using an ELISA kit. As shown in **Table 2**, IgE results for sera numbers 1 and 2 were 42.3 and 18.5 kU/L, respectively. TBa and TBb appeared to have similar inhibitory ratios, blocking > 50% of the binding activity of crude extracts to IgE from TB flour. However, TBt exhibited only 20% inhibitory activity. We hypothesize that epitopes of TBa and TBb could be occluded when the subunits are linked by disulfide bridges to form a larger molecule (TBt). As revealed above, TBt contains two subunits and only one disulfide bond. Thus, the higher immunological activity of the individual subunits compared to the dimer might be attributable to exposure of some epitopes when the disulfide bond in TBt was reduced in the presence of β -ME to release the two subunits. In addition, by comparing the 56 kDa protein in TB with the 24 kDa protein in CB reported by Urisu [6], we feel it is a novel allergenic protein in TB, and here the ELISA experiments prove that the 56 kDa target protein could react with the sera from patients with positive IgE binding to buckwheat allergen.

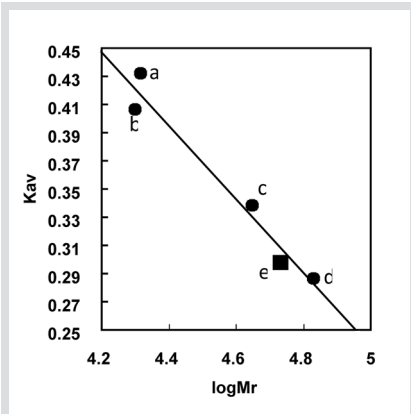


Fig. 4 Estimation of the molecular weight of TBt by SEC on a Superose 12 column (1.0 $\times$ 30 cm). The separation conditions were as follows: 20 mM potassium phosphate buffer (pH 7.4) containing 0.2 M NaCl and 1% Triton X-100 at a flow rate of 0.5 mL/min. The following protein marker standards were used: a, α -lactalbumin (18 kDa); b, trypsin inhibitor (20 kDa); c, ovalbumin (45 kDa); d, bovine serum albumin (68 kDa); e, sample (TBt, M_r 56 kDa). K_{av} was calculated as follows: $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_o is the outer volume (7.71 mL) and V_t is the total volume (23.56 mL).

Table 1 The content of -SH and -S-S- in TBt

	A 412nm	-SH content (μ M/g)	-SH content (M SH/M protein)
Free -SH (-SH)	0.017	1.95	0.107
Total -SH (-SH and -S-S-)	0.330	37.91	2.085

Serum No.	Buckwheat-specific IgE by CAP (kU/L)	Inhibition ratio (%) [*]			
		Control	TBt	TBa	TBb
1	42.3	–	22.0	59.3	56.2
2	18.5	–	19.6	54.3	52.1
3	0.21	–	–	–	–
4	0.16	–	–	–	–

^{*} % inhibition = (ODu - OD_i) ÷ ODu × 100, where ODu is absorbance of control and OD_i is absorbance with use of inhibited serum.

Table 2 Detection of immunological activity of allergens by inhibition ELISA

Although there have been many studies on different allergens from CB [6], [7], [8], less has been reported about the isolation, purification and characterization of allergenic protein from TB seeds. In the present study, a novel allergenic protein with an estimated molecular weight of 56 kDa from TB seeds was isolated for the first time. SDS-PAGE and SEC analyses revealed that the allergenic protein is composed of two subunits, with estimated molecular weights of 24 and 34 kDa, which are linked by disulfide bonds. It was suggested that the 56 kDa allergenic protein might be the precursor of a 24 kDa allergen in TB reported previously [11]. These findings are extremely significant in the investigation of the relationship between structure and function of the allergen in TB.

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