Research Square

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Sequence Analysis and Biochemical Characterization of Two Non-specific Lipid Transfer Proteins from Tartary Buckwheat Seeds

Xiaodong Cui (cuixiaodong@sxu.edu.cn)
Shanxi University
Wenhua Tian
Shanxi University
Wenhua Wang
Shanxi University
Jiao Li
Shanxi University
Zhuanhua Wang
Shanxi University
Chen Li
Shanxi University

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Abstract

Plant non-specific lipid transfer proteins (nsLTPs) exert various biological activities that are valuable for agronomy, industrial manufacturing, and medicine. In this study, two nsLTPs derived from Tartary buckwheat, namely FtLTP1a and FtLTP1b, were produced by gene cloning, expression and purification. Sequence analysis and biochemical characteristics of the proteins, including lipid binding ability, α-amylase inhibition activity, antifungal activity, and allergenic activity, were investigated. Both FtLTP1a and FtLTP1b exhibited similar lipid binding and antifungal properties, but only FtLTP1b showed weak inhibitory activity against α-amylase from the porcine pancreas. Finally, the binding ability of the two protein to IgE antibodies in serum samples from nine patients allergic to buckwheat was assayed using ELISA to investigate their allergenic activity. The data suggested that FtLTP1b not only specifically bound to IgE in serum but also cross-reacted with artemisia pollen allergen (W6). These findings can give constructive suggestions for understanding of cross-reactivity and specificity of nsLTPs.

1. Introduction

Non-specific lipid transfer proteins (nsLTPs) belong to the prolamin superfamily with conserved cysteine residues, abundant α-helices, and low molecular mass [1]. Usually, nsLTPs contain four or five α-helices stabilized by four disulfide bonds formed by a motif with the conversed sequence C–Xn–C–Xn–CC–Xn–CXC–Xn–C–Xn–C. In addition, a tunnel-like hydrophobic cavity exists in the nsLTPs, which is responsible for various lipids transportation [2]. According to the protein sequence length, nsLTPs are classed into two families, namely nsLTP1s with about 90 amino acid residues and nsLTP2s with about 70 amino acids [3]. The two families also have different subfamilies based on the particular pairing of cysteines in disulfide bridges. For instance, nsLTP1s are characterized by four disulfide bridges formed by Cys residues 1–6, 2–3, 4–7, and 5–8 [4], while disulfide bridges in nsLTP2s are paired by Cys residues 1–5, 2–3, 4–7, and 6–8 [5]. nsLTPs also belong to PR-14, a pathogenesis-related protein [6]. They involve in defending against viral plant pathogens, bacterial, or fungal, by activating the plant immune system through a receptor-dependent mechanism [7].

Many nsLTPs have been isolated and characterized from various plant species. They are reported to play essential roles in cutin formation, embryogenesis and response to stresses [8]. nsLTPs can reversibly bind to lipid molecules, including sterols, acyl-coenzyme A (CoA) and fatty acids [3]. This effect endows nsLTPs roles in the transmembrane transportation of lipids and permeabilization of phytopathogen membranes [9]. Many nsLTPs also have been considered to be allergens from different sources such as pollen, food or latex, causing various severe symptoms, such as anaphylaxis [10]. For example, Pru p3, a nsLTP from peach, is the first plant food allergen found in the nsLTPs family [4].

Buckwheat, which belongs to the family *Polygonaceae*, can be found in many parts of the world, but is grown mainly in the northern hemisphere [11]. With the increase in buckwheat food consumption, the cases of instant hypersensitivity caused by food containing buckwheat additives have also increased. Some natural allergenic proteins from common buckwheat and Tartary buckwheat have been isolated

and identified. Their molecular weights range from 10 to 100 kDa. Among them, proteins with molecular weights of 24 kDa, 19 kDa, 16 kDa and 10 kDa are considered the main allergens in common buckwheat, and these allergens can cause serious allergic reactions, such as asthma and anaphylactic shock [12]. The 24-kDa protein has been identified as a small subunit of 11S globulins and named Fag e1[13]. Moreover, the 16-kDa (Fag e2) and 19-kDa allergens have been identified as members of the 2S albumin family and 7S vicilin-like globulins, respectively. While, the 10-kDa allergen is the α-amylase inhibitor/trypsin inhibitor [14]. The 24-kDa allergen (Fag t1) isolated from Tartary buckwheat seeds is thermostable and homologous with the 24-kDa allergen from common buckwheat (Fag e1) [15]. A 56-kDa protein from buckwheat displayed allergic activity, and its nucleic acid sequence has been determined [16]. A 16-kDa allergenic protein (Fag t2) from Tartary buckwheat has been recombinantly expressed [17], and its core epitope has been identified [18]. A 9-kDa protein from buckwheat shows both allergic and trypsin inhibitory activity [12]. Research on a variety of allergens from buckwheat has been extensively carried out. However, nsLTPs from common buckwheat or Tartary buckwheat and their allergenic potentials have not been investigated.

In this article, the complete nucleic acid sequences of two nsLTPs from Tartary buckwheat seeds, namely FtLTP1a and FtLTP1b, were analyzed. The work aims to verify whether these nsLTPs are potential allergens. The two nsLTPs were recombinantly expressed in *Escherichia coli*. Their biochemical characteristics, including lipid binding ability, α-amylase inhibition and antimicrobial activities, were evaluated. The capacity of the two nsLTPs to bind IgE antibodies in serum of patients with allergy to the 24-kDa protein in buckwheat was also assessed.

2. Materials And Methods

2.1 Materials and reagents

The Tartary buckwheat strain Yunqiao No.1 was field-grown at the Science Academy of Agriculture in Yunnan Province, China. α-Amylase from porcine pancreas and Ni^{2+}-NTA agarose column were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The pET-32a vector was purchased from Promega Corporation (Madison, USA). T_4 DNA ligase and DNA restriction enzymes (*Xho* I and *Bam H*I) were obtained from Thermo Fisher Scientific (Waltham, USA). Sera from all recruited patients, aged 14 to 46 years, were tested for allergen-specific IgE using microarray immunoassay (Immuno CAP ISAC; Uppsala, Sweden). Information on the recruited patients, mugwort-specific IgE values, pollens causing respiratory symptoms, and other food allergen (based on CAP-system test), can be found in Table S1 and S2.

2.2 Sequence analysis

The open reading frame (ORF) of *FtLTP* genes was analyzed using the online software ORF finder (http://www.ncbi.nlm.nih.gov/gorf/). The identity of FtLTPs was determined by the BLAST tool available on the NCBI website. Amino acid sequence alignment of FtLTPs with other nsLTPs (or allergen) was

performed by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html)[19]. Phylogenetic relationships were determined according to the Maximum Likelihood (ML) method with 1000 replicated bootstraps using MEGA7.0 Software [20]. The midpoint rooted tree was generated using Interactive Tree of Life (iTOL, version 3.2.317, http://itol.emnl.de).

2.3 Expression and purification of FtLTPs

The sequence of FtLTPs (removed putative signal peptides) was optimized based on the preferred codon of *E. coli*., synthesized and then cloned into a pUC-57 vector. In the construction of plasmid, the *FtLTP* genes were first amplified using the following primers: 5'-GGATCCTTTGAATGTGGTGCAGTTAGCAAA-3' and 5'-CTCGAGTTAATGAACTTTGCTACAATCGGT-3' for *FtLTP1a*, and 5'-GGATCCGCACTGACCTGTGGTACCGTTACC-3' and 5'-CTCGAGTTAATTAAC TTTGCTACAAT CGGTGCT C-3' for *FtLTP1b*. The primers contained *Bam* HI and *Xho* I restriction sites. The purified PCR product was digested by *Bam* HI and *Xho* I, and then cloned into a pET32a vector to construct pET-32a-*FtLTP1a* and pET-32a-*FtLTP1b* recombinant plasmids. After transformation into *E. coli* BL21 (DE3) competent cells, clones were selected by ampicillin screening, and the accuracy of the two recombinants was verified by DNA sequencing.

Transformed *E. coli* was inoculated in 5 mL of LB medium containing 100 μg/mL ampicillin, and cultured at 37°C and 180 rpm to attain log-phase growth. Then, the culture was transferred to 1000 mL of LB medium and incubated. When the OD_{600} reached 0.6, protein expression was induced by IPTG (with a final concentration of 0.2 mM) at 16°C for 12 h. The cells were collected and subsequently sonicated. Finally, the cell lysate was analyzed by SDS-PAGE using a 12.5% separating gel.

The cell lysate supernatant was loaded onto a Ni^{2+}-NTA agarose column pre-equilibrated with buffer A (10 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole). After being thoroughly washed with buffer B (10 mM Tris-HCl, 0.5 M NaCl, 50 mM imidazole), target proteins were washed with buffer C (10 mM Tris-HCl, 0.5 M NaCl, 200 mM imidazole). Collected samples were subsequently dialyzed against water and then stored at -80°C.

2.4 Lipid binding assays

Fluorescence of recombinant FtLTP1a and FtLTP1b were measured at 37°C (310K) at the excitation wavelength of 280 nm and emission wavelengths of 300–420 nm using an LS55 fluorescence spectrometer (Perkin Elmer, USA). Two milliliters of 1 μM FtLTP1a or FtLTP1b were added into the cuvette and then titrated with lipid by stepwise adding lipid into the cuvette. Titration curves, which showed the variation of fluorescence intensity ($F-F_0$) at 342 nm with the molar ratio of lipid/protein, *R*i, were obtained.

2.5 α-Amylase inhibition assays

The enzymatic activity of α-amylase was assayed as per the reported method [21]. Briefly, the α-amylase activity was measured using starch as a substrate, and the product reducing sugar was quantified after reaction with 3, 5-dinitrosalicylic acid (DNS). The reaction mixtures containing 5 U of α-amylase and

diverse concentrations of FtLTP1a or FtLTP1b were preincubated for 15 min at 37°C. The reaction was initiated by adding a 400 μL substrate (1% starch; Sigma-Aldrich) and carried out for 15 min. After adding DNS, the reaction was terminated by boiling water treatment for 5 min. After cooling to room temperature (25°C), the absorbance at 540 nm of the mixture was measured. One unit of enzymic activity was defined as the amount of enzyme required for an increase in A_{540} nm by 0.1 in 30 min. The enzymatic activity of the control without FtLTP1a or FtLTP1b was considered to be 100%. The percent inhibition was calculated according to the following formula:

$[100 – (A_1 \times 100/A_2)]$, A_1 and A_2 are the absorbances of sample and control, respectively.

2.6 Antifungal assay

The antifungal activities of FtLTP1a and FtLTP1b were measured by spotting assay and a 2-fold microdilution assay [22, 23]. The hyphae of *Aspergillus niger, Aspergillus flavus*, or *Fusarium oxysporum* were added to sterilized ddH_2O to prepare mycelium suspension of 10^5-10^3 CFU/mL. A protein sample (100 μM) was added to the suspension and 10 μL of the resultant mixture was cultured on 1.2% potato dextrose agar (PDA) at 28°C. The colony growth was observed after 48 h. The mininum inhibitory concentrations (MIC) of FtLTP1a and FtLTP1b with respects to fungi were further determined by a 2-fold microdilution assay in RPMI 1640 medium. Briefly, mycelium suspensions with a turbidity of 0.5 McFarland standards were prepared. Cell suspension was diluted in RPMI 1640 medium, 50 μL was added to a 96-well plate, and protein samples with final concentrations of 0.25 to 256 μM were added. The plate was incubated at 28°C for 48 h and the fungal growth was observed. Medium alone and containing inoculum suspension were used as a negative control and positive control, respectively.

2.7 Enzyme-linked immunosorbent assay (ELISA)

An aliquot of purified FtLTPs (0.5 μg) was added to a 96-well plate containing 50 μL of TBS solution. After incubation for 1 h at 37°C, the plate was washed with TBST solution. Then, 1% BSA solution in TBS was added and incubated at 37°C for 2 h. After reaction with sera from allergic patients diluted in TBS at 37°C for another 2 h, the plate was washed with TBST. Anti-human IgE-horseradish peroxidase-conjugated antibody (Sino Biological Inc., China) was added to each well and carried out at 37°C for 1 h. The wells were rewashed with TBST, and bound antibodies were detected with TMB chromogen solution (Sigma). After termination by adding 4 N H_2SO_4, the absorbances at 450 nm were determined and analyzed.

2.8 Statistical analysis

In paper, all data were from at least three independent experiments and were presented as mean ± standard error of the mean (S.E.M). Analysis was evaluated using the SPSS version 20.0. One-way ANOVA test (Tukey's test) was used to determine the significant statistical differences. *p* value 0.05 or 0.01were considered as significantly different.

3. Results And Discussion

3.1 Sequence and phylogenetic analyses

Full-length cDNA and amino acid sequences of FtLTP1a and FtLTP1b are shown in Fig. S1. Both FtLTP1a and FtLTP1a contained 117 amino acid residues. FtLTP1a contained a 91-residue mature peptide and a 26-residue putative signal peptide (GenBank accession number: OK340654), whereas FtLTP1b contained a 92-residue mature peptide and a 25-residue signal peptide (GenBank accession number: OK340655). The molecular masses of mature FtLTP1a and FtLTP1b were estimated using DNAMAN software to be 9228.76 Da and 9038.55 Da, respectively. It has been reported that nsLTPs consist of two main subfamilies, namely the 9-kDa nsLTP1 family and the 7-kDa nsLTP2 family [24]. Moreover, both the two purified proteins share high sequence homology with nsLTP1 family. Furthermore, they contain two conserved motifs (T/SXXDR/K and PYXIS), which is also a characteristic of the nsLTP1 family [3]. Therefore, based on the above results, we may conclude that FtLTP1a and FtLTP1b belong to the nsLTP1 family.

The phylogenetic tree is a framework indicating genetic diversities and distances between different biological species and other entities. To explore the phylogenetic relationships between FtLTP1a/b from Tartary buckwheat and those from other species, multiple sequence alignments were analyzed, and a phylogenetic tree of 114 protein sequences was generated using a neighbor-joining method available in MEGA7 software (Fig. 1A). We found that nsLTPs from Tartary buckwheat were grouped with those from *Chenopodium quinoa* (XP 021723225.1, XP 021766670.1), *Spinacia oleracea* (XP 021851885.1), and *Beta vulgaris* subsp. *Vulgaris* (XP 010690793.1). Additionally, FtLTP1a and FtLTP1b were closely related to many allergens, such as Amb a6 (O04004.1, *Ambrosia artemisiifolia*), Zea m14 (P19656.1, *Zea mays*), Tri a14 (P24296.2, *Triticum aestivum*), Bra o3 (Q9ZSL7, *Brassica oleracea* var. *oleracea*), Pla a 3.02 (ALF00099.1, *Platanus x hispanic*), and Pla o3 (ABY21307.1, *Platanus orientalis*).

The amino acid sequences of FtLTP1a and FtLTP1b were also compared with three unique proteins, Api g2, Pru p3 and Tri a14, available in the UniProt database (Fig. 1B). The IgE-binding epitopes in Pru p3, Tri a14, and Api g2, the homologous nsLTP from peach, wheat, and celery, respectively, have been identified. FtLTP1a shared 54.9%, 47.3% and 41.1% sequence identity with Api g2, Pru p3, and Tri a14, respectively, and FtLTP1b shared 64.8%, 52.7% and 48.9%, respectively. The comparison of amino acid sequences revealed that FtLTP1b is more similar to Api g2, Pru p3, and Tri a14 than FtLTP1a. Previous studies have identified the IgE binding epitopes in Pru p3 using different methodologies [25–27]. Comparing the amino acid sequences of Pru p3 and FtLTP1a, it was found that there was high degree of diversity in the regions potentially involved in IgE recognition (Fig. 1B). Moreover, FtLTP1b had a high sequence identity with Pru p3, including a highly conserved region (GKCGVSIPYK) that can significantly contribute to IgE binding. This region in Api g2 and Tri a14 is also highly conserved. Therefore, the analysis above suggests that FtLTP1b is more likely to be a potential allergen than FtLTP1a.

3.2 Heterologous expression and purification of FtLTP1s

Recombinants of pET32a-FtLTP1a and pET32a-FtLTP1b were constructed, transformed into *E. coli* BL21(DE3) cells and induced expression. As shown in Fig. 2, the recombinant Trx-FtLTP1a and Trx-

FtLTP1a proteins were expressed in soluble form, and the target proteins were further purified by a Ni^{2+}-NTA column. Using this single-step procedure, the purity of both Trx-FtLTP1a and Trx-FtLTP1b proteins was about 95% and migrated as a single band on SDS-PAGE (Fig. 2). The estimated yield of the FtLTP1a and FtLTP1b was 2.14 mg and 2.46 mg per liter of culture, respectively(Table S3). The purity of FtLTP1a and FtLTP1b were 94.25% and 96.33% by gel scan.

3.3 Both FtLTP1a and FtLTP1b could bind lipid

Two highly conserved regions (T/SXXDR/K and PYXIS) play an essential role in lipid binding, and the fluorescence of tyrosine (Y, in PYXIS) is sensitive to lipid binding. Fluorescence spectroscopy is a tool for investigating the quenching mechanism and the binding properties. Intrinsic fluorescence of FtLTP1a was compared with that of FtLTP1b. As shown in Fig. 3A, the maximal fluorescence emission wavelength of both FtLTP1a and FtLTP1b was 342 nm. However, the intensity of FtLTP1b was dramatically lower than that of FtLTP1a. The difference in intensity may be due to the fact that FtLTP1a contains one tyrosine residue and two phenylalanine residues, while FtLTP1b only contains two tyrosine residues. When the purified FtLTP1a and FtLTP1b were incubated with oleic acid, the fluorescence intensity was associated with the increase in oleic acid concentration (Figs. 3B and 3C). According to the apparent saturation levels, the molar ratio of oleic acid/FtLTP1a or oleic acid/FtLTP1b (*R*i) was about 1.0 (Fig. 3D). The fluorescence of FtLTP1a and FtLTPlb with oleic acid was markedly increased by 160% and 144%, respectively, compared to that in the absence of oleic acid. In addition, the binding of FtLTPs with other two types of lipids, stearic acid and linolenic acid, was also studied, from which similar results were observed under the same test conditions (data not shown). The tyr-79 residue of the present nsLTPs and other corresponding residues of nsLTPs from other plant species are involved in increasing of fluorescence intensity when binding to lipids. Tyr-79, located in the C-terminal fragment, a part of the extreme hydrophobic cavity, should be perturbed by lipid-binding [28]. Maize nsLTP-fatty acid complex has shown that the carboxyl group in fatty acid is involved in forming hydrogen bonds with the hydroxyl group of Tyr81 [29]. Consequently, in a lipid-bound state, Tyr-79 in the protein may probably be less mobile, and thus could not be effectively quenched by a possible internal quencher.

3.4 FtLTP1b exhibits weak α-amylase inhibitory activity

According to the protein classification by NCBI, nsLTPs belong to the AAI_LTSS superfamily (protease inhibitor/seed storage/LTP family). However, only a few nsLTPs have been identified to exhibit α-amylase inhibitory activity till now. Thus, the α-amylase inhibitory activity of FtLTP1a and FtLTP1b was further tested. First, the secondary structures of FtLTP1a and FtLTP1b were verified by circular dichroism (CD) spectra. The CD spectra of two proteins showed typical peaks of α-helix, namely two minimum peaks at 208 and 222 nm (Fig.S2). This result is consistent with the CD spectrum of nsLTPs from red wine and grapes [30], peach, cherry, hazelnut [31], and mugwort pollen [32], which are all rich in α-helices. Thus, we conclude that recombinant FtLTP1a and FtLTP1a folded correctly and formed a natural conformation. As shown in Fig. 4, both FtLTP1a and FtLTP1b were unable to inhibit α-amylase from *Bacillus licheniformis*, and FtLTP1b could weakly inhibit α-amylase from porcine pancreas. It has been reported that lipid transfer proteins from *Coffea canephora* and *Vigna unguiculata* L. Walp showed inhibitory activity

against α-amylase from human saliva and *C. maculatus*, respectively [33, 34]. One study has reported that rVu-LTP purified from *Vigna unguiculata* L. Walp can inhibit the activity of HAS and intestinal α-amylase from *C. maculatus*, but cannot inhibit intestinal α-amylase from *T. maculates*[35].

Fragment Asp-Glu-Asp appears in the active site of almost all α-amylases [36]. Some studies have suggested that positively charged (argnine and lysine) amino acids in inhibitors can non-covalent interact with the negatively charged residues of Asp-Glu-Asp, resulting in an ultimate inhibition of the enzyme [37]. Two peptides from Vu-LTP, N_{29}-I_{47} (NGVKNILNGARTTADRRGI) and αG_{74}-N_{91} (GVNIPWKISSSTNαNTIN), have been identified and found to be responsible for HAS (human salivary α-amylase) inhibition [21]. The two regions of FtLTP1a did not contain positively charged amino acids, while there was lysine (K) residue in one region (G_{74}-N_{91}) of FtLTP1b. This may explain why FtLTP1b exhibited weak α-amylase inhibitory activity, whereas FtLTP1a did not.

3.5 Both FtLTP1a and FtLTP1b exhibit antimicrobial activity

Plant nsLTPs belong to pathogenesis-related protein, which are reported to display broad-spectrum antimicrobial activity. The antifungal activities of FtLTP1a and FtLTP1b against *Aspergillus niger, Aspergillus flavus* and *Fusarium oxysporum* were evaluated. As shown in Fig. 5, the two proteins exhibited inhibitory activity against *A. niger.* The MIC (mininum Inhibitory Concentration) values of the proteins were determined and tabulated in Table S4. The MIC values of FtLTP1a to *Aspergillus niger, Aspergillus flavus* and *Fusarium oxysporum* were 8 μM, 8 μM, and 64 μM, respectively, and those of FtLTP1b were 16 μM, 16 μM, and 64 μM, respectively.

The antimicrobial activities of nsLTPs from different plant species have been studied. Although the nsLTPs can inhibit both bacterial and fungal growths, the inhibition against bacterial growth is more potent than that against fungi growth [38]. Nevertheless, the mechanism of this occurrence remains unclear. The findings to date have indicated the ability to interact with lipids contributes to the antimicrobial activity of plant nsLTPs. Although the peptide Ace-AMP1 (from onion), also characterized as an nsLTP, cannot transfer phospholipid from liposome to mitochondria, it possesses antimicrobial activity [8]. It has been speculated this activity was due to their interaction with cell membranes, causing the loss of membrane integrity and permeabilization [39].

3.6 Immunological characteristics of FtLTP1a and FtLTP1b

Sera from 9 Chinese patients who tested positive for buckwheat allergen were used to screen IgE reactivity of FtLTP1a and FtLTP1b using ELISA. The clinical data of the nine patients who participated in this study are summarized in Table S1. All patients tested positive for IgE antibodies-specific 24-kDa buckwheat allergen and experienced allergic reactions after consuming buckwheat food. The P/N values > 2.1 were used to evaluate the allergenic activity of nsLTPs from buckwheat. As shown in Fig. 6A, all the 2-fold diluted sera from nine individuals (100%) were sensitive to FtLTP1b (P/N values > 2.1) but not FtLTP1a (P/N values < 2.1). In addition, only three 10-fold diluted sera were sensitive to FtLTP1b (Fig. 6B).

The fact that patients who are allergic to a particular allergen tend to be allergic to other agents indicates that the same IgE antibodies may recognize other homologous allergens which possess similar epitopes. As shown in Table S1, eight out of these nine patients were also allergic to the artemisia pollen (W6). FtLTP1a and FtLTP1b were tested on three sera that were sensitive to W6 and polyvalent grass pollen (WX7), to determine whether they share the same IgE epitopes with other allergens and further evaluate their potential cross-reactivity. As shown in Fig. 6C, FtLTP1b was able to bind IgE in these sera (P/N > 2.1). The majority (89%) of *Artemisia vulgaris* pollen-sensitized patients is also allergic to Art v3, a mugwort allergen [40]. Clinical studies have shown that Art v3 could cross-react with another homologous protein from peach (Pur p3). Patients with peach allergy and are primarily sensitive to Pru p3 can experience allergic response when exposed to mugwort pollen because of cross-reactivity with Art v3 [41]. In this study, certain cross-reactivity occurred between FtLTP1b and pollen allergen (W6 or WX7). However, clinical symptoms caused by this cross-reactivity may not be observable.

4. Conclusion

In this work, two recombinants of nsLTP1s (FtLTP1a and FtLTP1b) were prepared and purified by affinity chromatography. Moreover, their chemical properties and biological activities were characterized, including lipid binding ability, α-amylase inhibition, antifungal, and IgE-binding activities. The results showed that FtLTP1b from Tartary buckwheat is a novel allergenic member of the lipid-transfer protein 1 family, which can cross-react with homologous protein from pollen. The data may further help clinicians manage of patients allergic to buckwheat by avoiding being in unnecessary contact with pollen.

Declarations

Ethical Approval Not applicable.

Consent to participate Allauthors consent to participate.

Consent for publication All authors consent to publication.

Competing interests All authors declare that there are no conflicts of interests.

Authors' contributions Xiaodong Cui and Chen Li wrote the mauscript text. Wenhua Tian and Wenhua Wang prepared figures and formal analysis. Zhuanhua Wang and Jiao Li were responsible for supervision and methodology. All authors reviewed the manuscript.

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Availability of data and materials All data generated or analyzed during this study are included in this article and its supplementary materials files are available.

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Figures

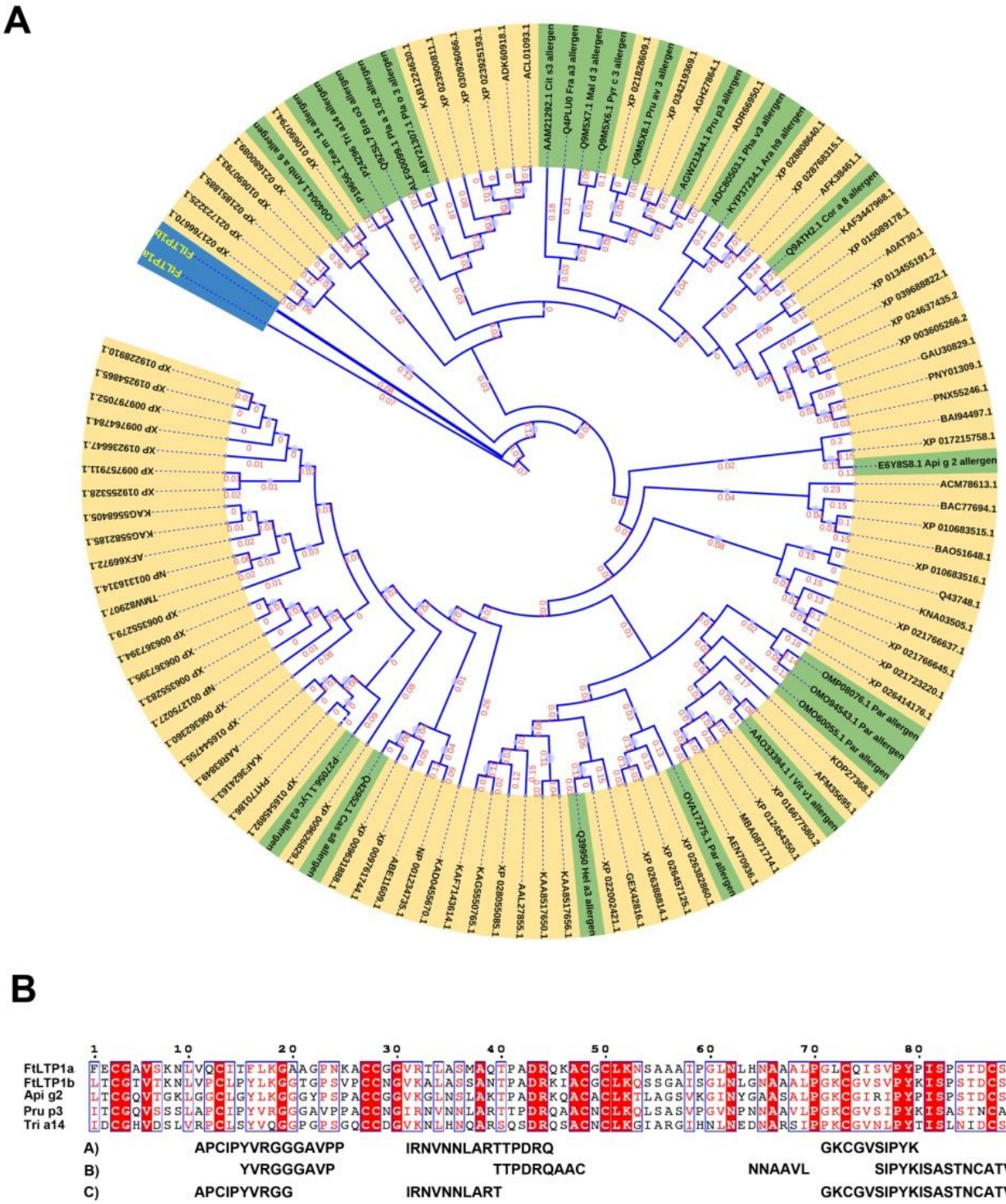

Figure 1

Phylogenetic and sequence analyses.(A) Phylogenetic analysis of FtLTP1a and FtLTP1b. The analysis was carried out by MEGA7 using 114 amino acid sequences and the Neighbor-Joining method. The figure is available in color at JXB online. (B) Multiple amino acid sequence alignment. Two nsLTPs in this work

were compared with sequences of Api g2, Pru p3, and Tri a14. The alphabets A-C represent the epitopes identified in Pru p3 by (A) Garcia-Casado *et al*. (2003), (B) Borges *et al*. (2003), (C) Tordesillas *et al*. (2003).

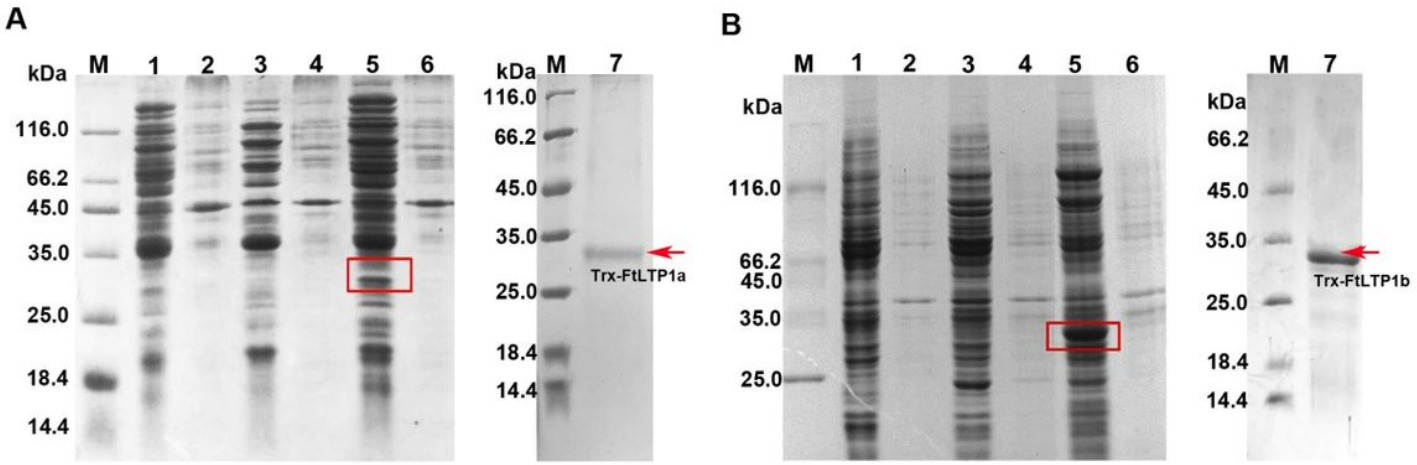

Figure 2

SDS-PAGE analysis of FtLTP1a (A) and FtLTP1b (B). Mr: molecular markers; lane 1: soluble protein (pET32a); lane 2: insoluble protein (pET32a); lane 3: soluble protein without induction by IPTG (pET32a-FtLTP1a/b); lane 4: insoluble protein induced by IPTG (pET32a-FtLTP1a/b); lane 5: soluble protein induced by IPTG (pET32a-FtLTP1a/b); lane 6: insoluble protein induced by IPTG (pET32a-FtLTP1a/b);; and lane 7: purified FtLTP1a or FtLTP1b containing Trx-His-tag.

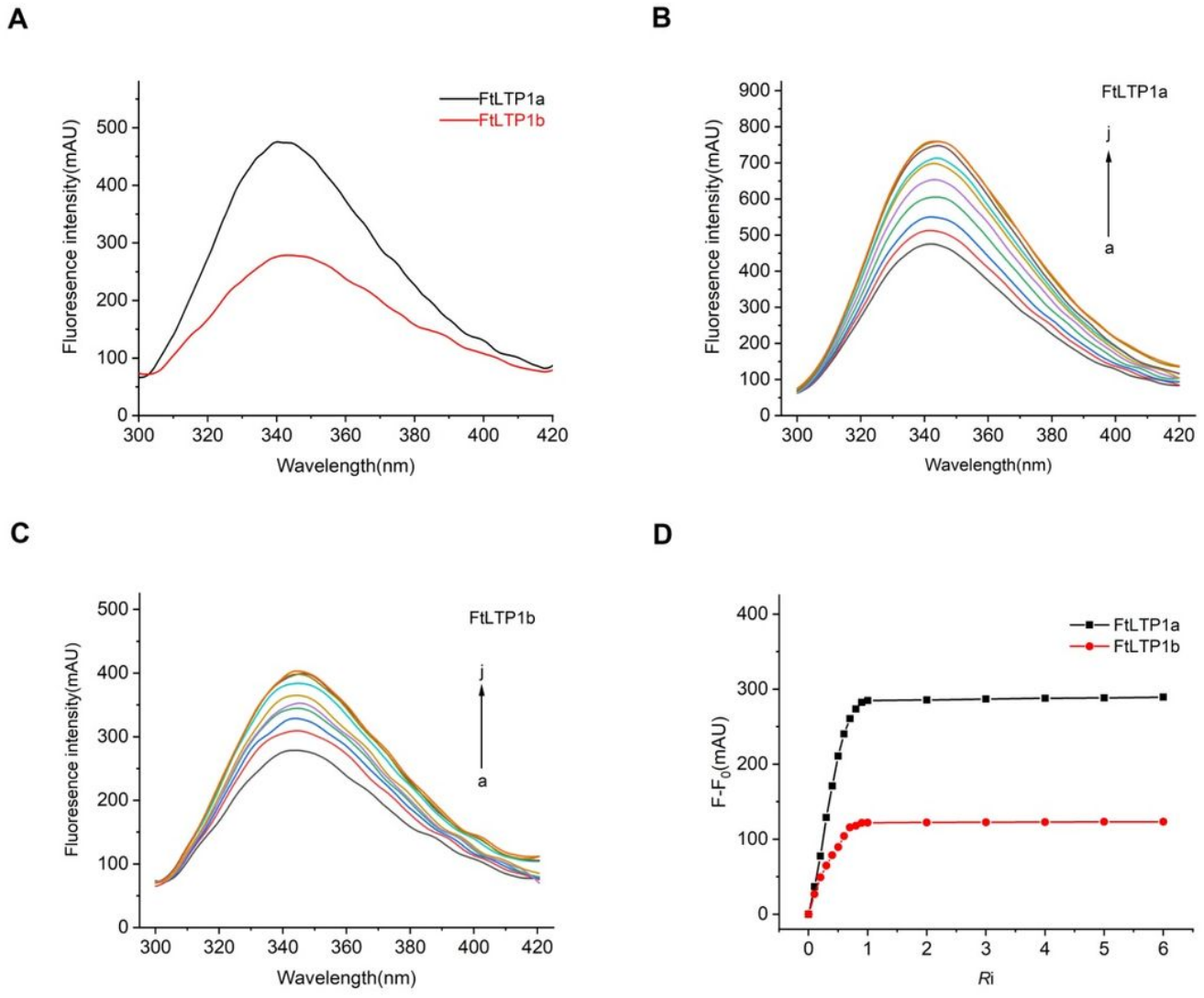

Figure 3

Lipid binding activity of FtLTP1a and FtLTP1b. (A) Intrinsic fluorescence spectra. (B) Intrinsic fluorescence spectra of FtLTP1a in the presence of oleic acid. a: without oleic acid; b-k: different concentrations of oleic acid. (C) Intrinsic fluorescence spectra of FtLTP1b in the presence of oleic acid. a: without oleic acid; b-k: different concentrations of oleic acid. (D) Variation of fluorescence intensity of FtLTP1a and FtLTP1b as a function of oleic acid concentration.

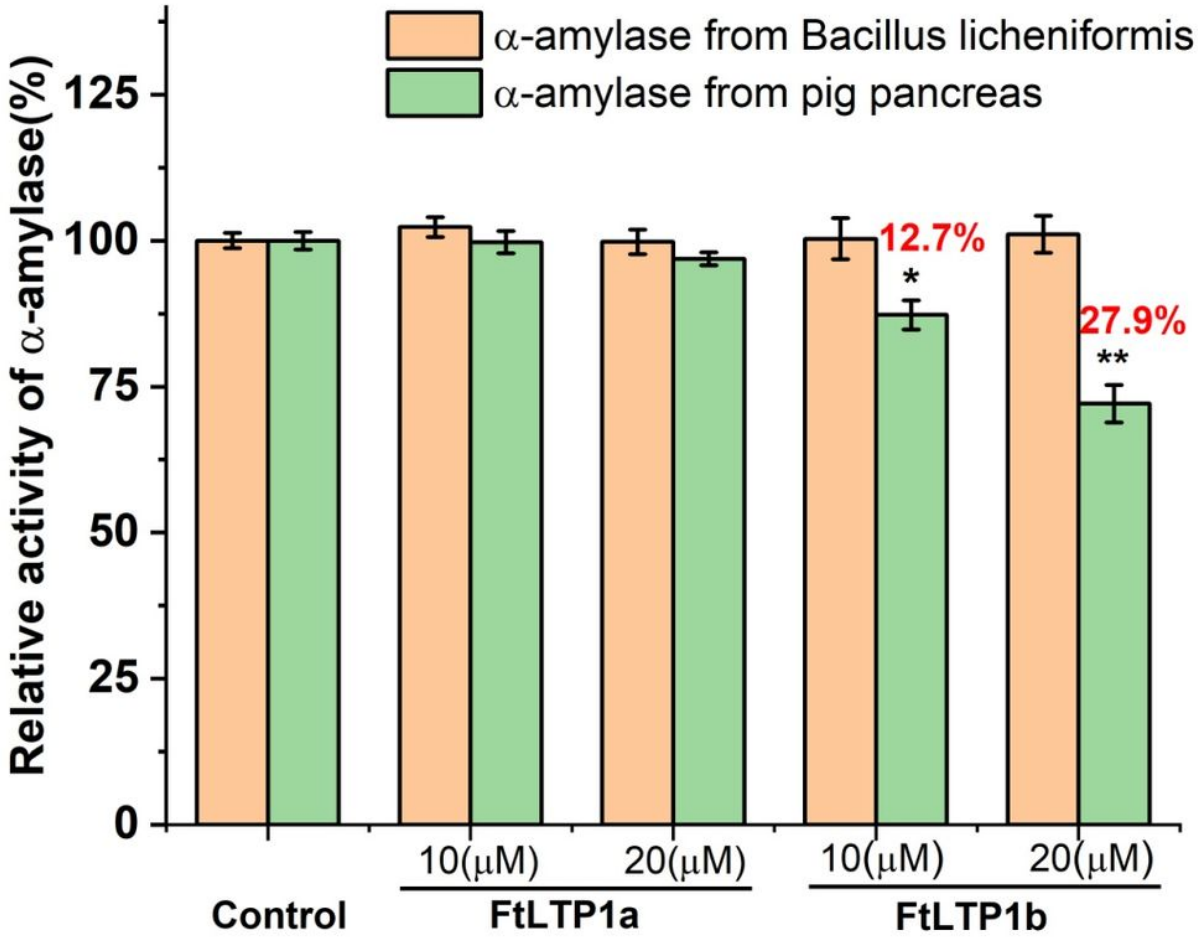

Figure 4

α-Amylase activity of FtLTP1a and FtLTP1b.

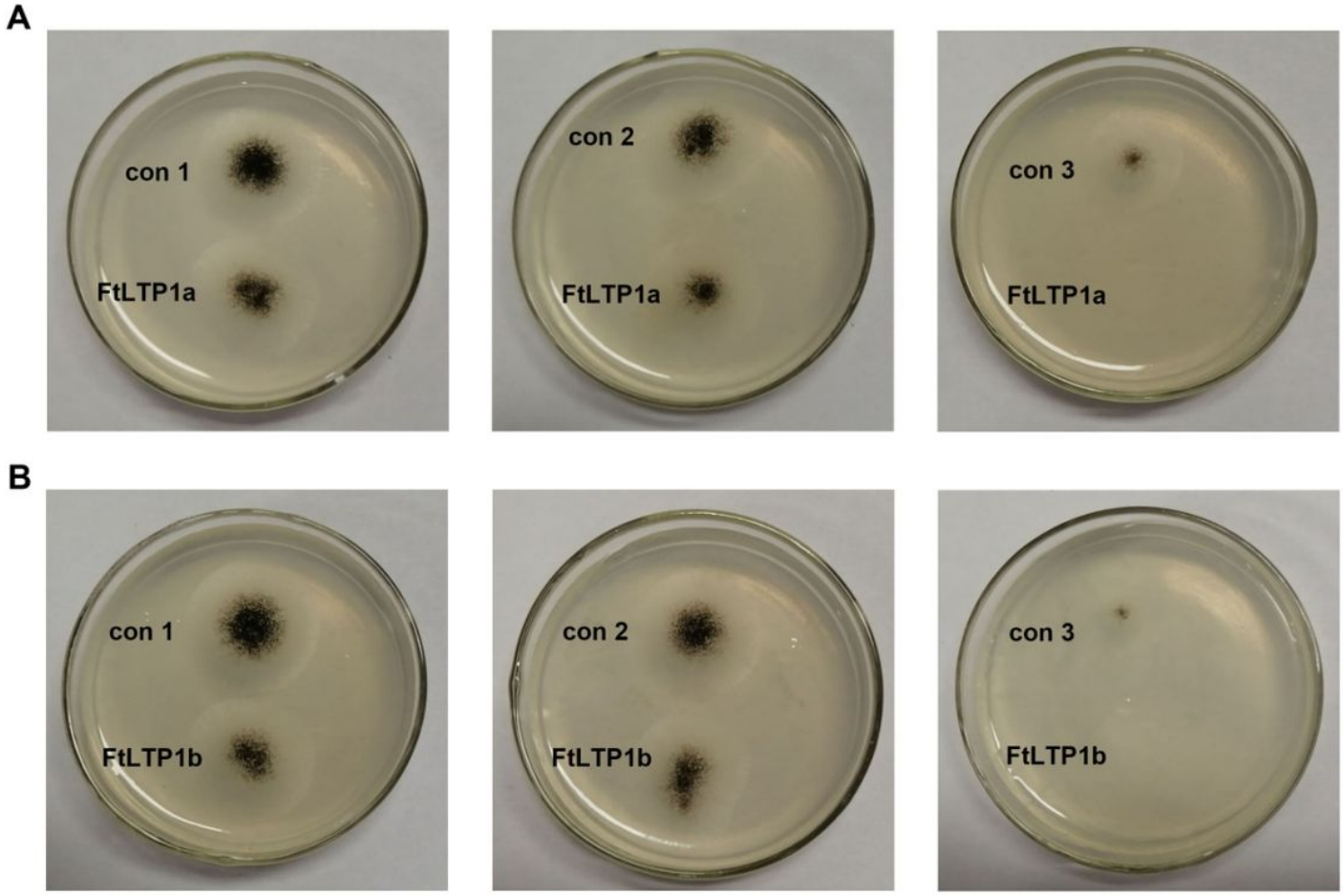

Figure 5

Inhibitory effects of FtLTP1a and FtLTP1b against *Aspergillus niger*. FtLTP1a or FtLTP1b was incubated with *Aspergillus niger* at different concentrations (1: 10^5 CFU/mL; 2: 10^4 CFU/mL; and 3: 10^3 CFU/mL), and those incubated with sterilized distilled water were used as controls. Growth inhibition was measured after 24 h of incubation at 28°C. The concentration of FtLTP1a and FtLTP1b was 100 μmol/L.

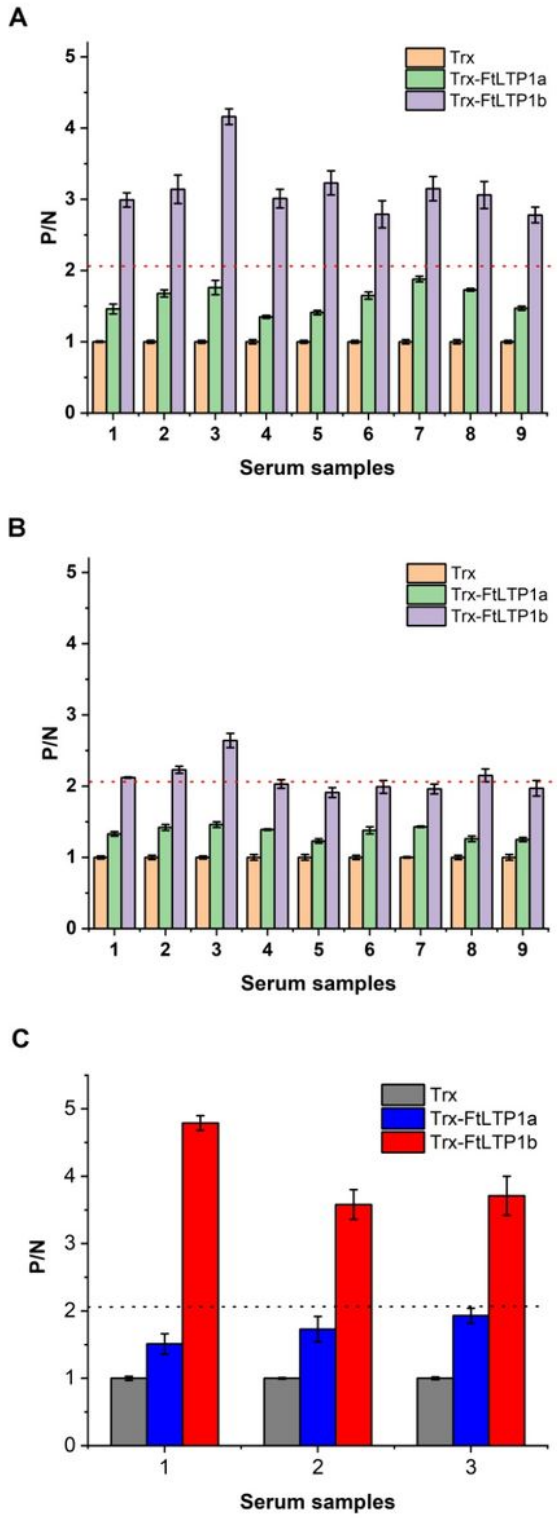

Figure 6

IgE-binding activity of purified FtLTP1a and FtLTP1b. (A) The binding of IgE in 2-fold diluted sera of patients allergic to buckwheat. (B) The binding of IgE in 10-fold diluted sera of patients allergic to buckwheat. (C) Cross-reactivity between FtLTP1a or FtLTP1b and pollen. The assay was performed using three replicate samples. P/N values > 2.1 were used as a benchmark for evaluating the presence of allergenic activity.

Supplementary Files

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