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ORIGINAL ARTICLE

A new pepper allergen Zan b 1.01 of 2S albumins: Identification, cloning, characterization, and cross-reactivity

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Abstract

Background: *Zanthoxylum bungeanum* (Sichuan pepper; 花椒 in Chinese) is used as a spice worldwide and is a potentially life-threatening allergenic food source, as first reported by our team in 2005. However, its allergen components are unknown.

Objective: We aim to identify and characterize its major allergen and determine its cross-reactivities with citrus seeds, pistachios, and cashew seeds.

Methods: Ionic exchange and molecular exclusion chromatography were used to isolate the protein components from Sichuan pepper seed. A protein fraction was characterized by SDS-PAGE, analytical ultracentrifugation, mass spectrometry, and circular dichroism spectroscopy. The coding region of it was amplified from the genome. ELISA and competitive ELISA assays were used to investigate the allergenicity and cross-reactivity of allergens.

Results: This protein allergen was around 14 kDa. It was a 2S albumin similar to an α-Amylase inhibitor (AAI) domain-containing protein of Citrus sinensis. Circular dichroism spectroscopy showed its thermal stability was high. A 303 bps DNA sequence of the AAI domain was cloned from the genome of the Sichuan pepper. Competitive ELISA assays showed positive cross-reactivities between this allergen and citrus seeds, pistachios, and cashew seeds.

Conclusions: A major allergen of around 14 kDa from Sichuan pepper seed was confirmed, which belongs to the 2S albumin of plant seed storage proteins. Based on the nomenclature of the IUIS Subcommittee for Allergen Nomenclature, this allergen is designated as Zan b 1.01. The cross-reactivities were demonstrated between Zan b 1.01 and citrus seeds, pistachios, and cashew seeds.

Key words: Zan b 1.01, *Zanthoxylum bungeanum Maxim*, 2S albumin, α-amylase inhibitor, pepper allergy, citrus allergy, cashew seed, IgE cross-reactivity

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Introduction

Food allergy is a worldwide public health problem. In addition to eight categories of globally recognized allergenic food groups,[1] regional or national unique allergen sources supplement the diversity of food allergens. Pericarpium Zanthoxyli (Sichuan pepper; 花椒 in Chinese) is the fruit of *Zanthoxylum bungeanum Maxim.*, which belongs to *Zanthoxylum Linn.* (Genus), *Rutaceae* (family). Although predominantly grown in China, Sichuan pepper is a spice that is widely used all over the world. The cultivated area suitable for growing Sichuan pepper accounts for 32% of China's total land area.[2]

Anaphylaxis as a response to Sichuan pepper was first reported in 2005.[3] Individuals who are allergic to Sichuan pepper often react to multiple foods such as cashew seeds, pistachios, oranges, and sesame seeds.[4] The allergenic protein's molecular features are critical for determining the pattern recognition receptors of innate immune cells to induce a Th2 polarization of the resulting immune response.[5] Many allergic protein components have been characterized.

Most known allergens are proteins present in foods.[6,7] Seeds and nuts belong to one large category of important allergic food sources.[8] Plant seeds contain a large quantity of seed storage proteins to provide nitrogen during germination. The protein content varies from 10% to 40% in dry seeds.[9] These seed storage proteins belong to limited protein families, including the prolamin superfamily (2S albumins, nonspecific lipid transfer proteins, α-amylase/trypsin inhibitors) or the cupin superfamily (7S and 11S seed storage proteins).[10] They are often major allergens in many plant seeds. The 2S albumins are important sources of nitrogen and sulfur during seed germination, with high contents of arginine, glutamine, cysteine, and methionine.[11] The 2S albumins, containing conserved cysteine residues, forming four disulfide bonds within the molecule, have high thermal and pH stabilities. For most 2S albumins, the precursor protein is cleaved into a small 3-4 kDa subunit and a large 8-10 kDa subunit during post-translational processing.[12] Many allergens of seeds are 2S albumins, including Pis v 1 from pistachios, Ana o 3 from cashew seeds,[13] Ber e 1 from Brazil nuts,[14] Ara h 2 from peanuts,[15] and Ses i 1, Ses i 2 from sesame seeds.[16] However, no Sichuan pepper allergens have been identified, and the cross-reactivity between Sichuan pepper and multiple food allergens needs to be evaluated. Here, we report the purification of a 14 kDa 2S albumin from Sichuan pepper and its identification as a new food allergen with its allergen name Zan b 1.01 approved by the WHO/IUIS Allergen Nomenclature Sub-Committee (http://allergen.org).

Materials and Methods

Sera samples

Sera from thirteen patients sensitized to Sichuan pepper (11 patients were allergic to Sichuan pepper and 2 were sensitive to Sichuan pepper without allergic symptoms; mean age: 50.2 years) and sera from nine non-allergic individuals (mean age: 50.0 years) were collected. This study was approved by the Institutional Review Board at Peking Union Medical College Hospital (NO.JS-3443).

Food allergy diagnosis was evaluated following relevant guidelines,[17,18] mainly based on the following factors: 1. Clinical manifestations of patients had IgE-mediated immediate food allergy characteristics and clear Sichuan pepper-induced allergic reactions occurred at least twice. 2. Patients had a convincing immediate history of anaphylaxis to Sichuan pepper (the allergic reaction was potentially life-threatening, i.e., laryngeal edema, severe wheezing, and/or hypotension.). 3. A positive immediate skin prick test to identify allergens in Sichuan pepper extract. 4. Positive Sichuan pepper specific IgE tests using the Immuno-CAP method. In addition, Open Oral Food challenge tests were done on two subjects with sIgE for Sichuan pepper but could tolerate the daily consumption of Sichuan pepper food products. They are sensitized to Sichuan peppers but not allergic.

Extraction and purification of protein

Sichuan pepper seeds were purchased from Hanyuan, Sichuan Province. Twenty grams of Sichuan pepper seeds were ground in 100 mL extraction buffer (20 mM NaAc, pH 4.6) and stirred after adding 50 mL n-hexane for 2 hrs. After centrifuging at 14,000 g for 20 min, the solution in the middle layer was collected and filtered using 0.22 μm filters.[19] Extracts of citrus, pistachio, and cashew seeds were prepared using the same method. The protein concentrations of the extracts were determined using a commercially available kit known as Modified Bradford Protein Assay Kit (Sangon Biotech, China). Then, the extract of Sichuan pepper was purified using a 6 mL RESOURCE™ S column (GE Healthcare) with a linear gradient of NaCl and an 80 mL elution volume by mixing the extraction buffer and an elution buffer (500 mM NaCl, 20 mM NaAc, pH 4.6). The major protein peak was concentrated to 1 mL, and then loaded onto a 24 mL Superdex 75 10/300 GL column (GE Healthcare) pre-equilibrated with a gel filtration buffer (200 mM NaCl, 20 mM Tris-HCl, pH 8.0). The gel filtration column was calibrated using a protein standard kit (BioRad, Hercules, CA). Protein concentration was determined by measuring its OD at 280 nm via a spectrophotometer (Analytik Jena).

SDS-PAGE analysis

Protein samples were mixed with a non-reducing SDS sample buffer (250 mM Tris pH 6.8, 2% SDS, 25% glycerol, 20 mM DTT, 0.01% Bromophenol Blue) or a reducing SDS sample buffer (non-reducing SDS sample buffer containing 0.5 M 2-Mercaptoethanol) at a ratio of 5:1 and boiled at 100°C for 15 minutes. Then, samples were loaded onto 4-15% gradient sodium dodecyl sulfate polyacrylamide gels, and the gels were run in a Tris-MES-SDS running buffer (50 mM Tris, 50 mM MES, 0.1% SDS, 1 mM EDTA). Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA) were used as references. After electrophoresis, the gels were stained in a Coomassie Blue Staining buffer (0.1% Coomassie Brilliant Blue R250, 40% ethanol, 10% acetic acid) for 15 minutes, and then destained in boiling water.[20]

SDS-PAGE for Western Blot: Protein samples were mixed with a reducing SDS sample buffer at a ratio of 5:1 and boiled at 100°C for 10 minutes. Then, samples were loaded onto 4-20% gradient sodium dodecyl sulfate polyacrylamide preformed gels (FuturePAGE™, ET12420Gel, Nanjing ACE Biotechnology Co., Ltd, Nanjing, China), and the gels were run in a MOPS-SDS Running Buffer (FuturePAGE™, F00004Gel, Nanjing ACE Biotechnology Co., Ltd, Nanjing, China) in a volt of 160V for 45 min.

Analytical ultracentrifugation

The analytical ultracentrifugation of the sample was carried out according to the method of Ni et al.[21,22] ProteomeLab XL-A (Beckman Coulter, Inc., Fullerton, CA) was used to perform the analytical ultracentrifugation of the sample. The protein solution was adjusted to 0.5 mg/mL using phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), and PBS was served as a control.

Mass spectrometry

After SDS-PAGE electrophoresis was performed, a 2 × 6 mm gel strip of 14 kDa protein was excised from the gel at the maximum concentration of 14 kDa protein. The gel was sliced into 1 mm^2 cubes and decolored with 50% ACN/25 mM NH_4HCO_3 (ABC), reduced with 10 mM DTT/25 mM ABC at 56°C and alkylated in dark with 50 mM iodoacetamide/25 mM ABC at room temperature for 1 h. Then the gel plugs were lyophilized, immersed in 10 ng/μL trypsin in 25 mM ABC and cleavage at 37°C for 16 h. Tryptic peptide mixtures were first extracted with 100 μL 5% TFA and then with the same volume of 2.5% TFA/50% ACN. The extracted solutions were mixed and lyophilized before MS analysis. Peptide samples were detected on a Q-Exactive™ Plus mass spectrometer (ThermoFisher Scientific, Pittsburgh, PA) connected with an EASY-nLC 1000 (Thermo Fisher Scientific, Massachusetts, USA). The PEAKS Studio X+ (ThermoFisher Scientific, Pittsburgh, PA) was used to analyze the raw file of mass spectrometry results to obtain the amino acid sequences of the peptides.[23] The data acquisition mode was data-dependent analysis (DDA). Capillary columns with 75 μm id × 150 mm were used. The mobile phase A was 99.9% water/0.1% FA, mobile phase B was 80% ACN/0.1% FA, and elution gradient was 60 min in total. The positive ion scan mode was used. For MS1, the mass scan range was 350-2000 Da. The automatic gain control (AGC) was set to 1e6 with resolution of 70,000 and the maximum ion injection time of 50 ms. For MS2, the AGC target value was set to 1e5 with resolution of 17,500 and the maximum ion injection time of 50 ms. The top 20 most intensive precursor ions were selected for MS/MS analysis. The normalized collision energy value was set as 27.

Circular dichroism spectroscopy

The secondary structures and thermal stability of the protein were determined using a Chirascan™-Circular Dichroism Spectrometer (Applied Photophysics, Ltd., Surrey, U.K.). The protein was adjusted to 0.2 mg/mL PBS. To reduce the sample, dithiothreitol (DTT) was added to a 2 mM final concentration. Circular dichroism was measured twice from 250 to 190 nm in 1 nm steps at 20°C, 95°C, and cooled down to 20°C again. For thermal titration, circular dichroism was measured from 250 to 190 nm between 20°C and 95°C in 5-degree steps. Each experiment was repeated twice. The 220 nm circular dichroism at different temperatures was fitted using Prism to calculate T_M and characterize conformational changes.[24]

Cloning, mammalian cells expression, and purification

Genomic DNA of Sichuan pepper was extracted from seeds using a Hipure tissue DNA kit (Magen, Guangdong, China). PCR primers (forward: CAGAGCTGCGAGCAG CAGATAC; reverse: CCTTGGAGAAATGTTGCAC) were designed based on the α-amylase inhibitor domain-containing protein of *Citrus sinensis* DNA sequence. After sequencing, the DNA sequence was inserted into a pTT5-based mammalian expression vector with a human IgG-Fc tag. Then, the plasmids were transfected to HEK 293-F cells via polyfectine, and the cell culture medium was collected for purification 1 day after transfection. The protein was purified using a 5 mL Protein A column (GE Healthcare, Piscataway, NJ) pre-equilibrated with a running buffer (150 mM NaCl, 20 mM Na_2HPO_4, pH 7.0) with a linear gradient of 30 mL 0.1 M acetic acid. The collected protein sample was mixed with 1 M Tris (pH 7.5) at a ratio of 5: 1 to adjust the pH to neutral.

Measurements of allergen-specific IgE via Western blot and ELISA in vitro

For Western blot, the reduced protein (20 μg) was electrophoretically transferred from SDS-gel to a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was used as a protein maker. The membrane was blocked with QuickBlock™ Blocking Buffer for Western Blot (Beyotime, Songjiang District, Shanghai) on a shaker at room temperature for 30 minutes. Then, the membrane was incubated with 1:20 diluted sera (QuickBlock™ Primary Antibody Dilution Buffer for Western Blot, Beyotime, Songjiang District, Shanghai) at 4°C overnight. After washing three times, the membrane was incubated with a 1:500-diluted HRP-conjugated anti-human IgE (epsilon) antibody (Sigma, St. Louis, MO). The results were collected by detecting the optical signal after an ECL substrate was added (Beijing Aoqiang Biotechnology CO., Ltd, Beijing, China) using a ChemiDoc Touch chemiluminescence imaging system (Bio-Rad, Hercules, CA).

The allergen-specific IgE in sera was quantitatively measured in vitro by enzyme-linked immunosorbent assay (ELISA).[20] Microtiter plates (ThermoFisher Scientific, Pittsburgh, PA) were coated with 0.2 μg protein per well hole in 50 mM $NaHCO_3$ (pH 9.6) at 4°C overnight. The plates were washed five times with PBST buffer and blocked with 5% skim milk in PBST buffer at 37°C for 1 h. Then, the plates were incubated with 1:20 diluted sera at 37°C for 2 h. 13 sera from allergic and sensitized individuals were used as the primary antibody. The negative control sera pool has come from non-allergic healthy individuals, and 1% skim milk (PBST buffer) was used as the blank control. After washing five times, the plates were incubated with a 1:2500-diluted Anti-Human IgE (epsilon) Antibody, Peroxidase-Labeled (SeraCare, Shanghai, China) 1 h. A TMB substrate solution (BioFX™ TMB, Eden Prairie, MN.) was added in the quantity of 100 μl per well, and the reaction was stopped by 50 μl per well 1 M H_2SO_4. Optical density at 450 nm was measured using a Synergy H1 microplate reader (Biotek). For cross-reactivity tests, sera were pre-mixed with 2 μg extract of citrus, pistachio, and cashew seeds at 4°C overnight before incubating with the 2S albumin protein from Sichuan pepper seed absorbed on the plate as the primary antibody. Then the other ELISA steps were performed as the same as those described before.

Multiple sequence alignment and homology tree

The AAI domain amino acid sequences of Sichuan pepper, 2S seed storage protein of citrus, allergen Pis v 1 from pistachio, and allergen Ana o 3 from cashew seed were aligned using the Multiple Sequence Alignment program of the DNAMAN software, and generated a homology tree.

Results

Sera samples

Sera from thirteen patients sensitized to Sichuan pepper (11 patients were allergic to Sichuan pepper and 2 were sensitized without allergic symptoms) were tested for specific IgE for Rf602 (Sichuan pepper), f202 (cashew seeds), f203 (pistachios), and f302 (mandarin) in the ImmunoCap format. All thirteen patients' sera had positive results for Rf602 with sIgE > 0.35 KUA/L (**Table 1**, **Table S1**). The results of f202, f203, and f302 tests showed varying levels of specific IgE. For healthy controls, total IgE and IgE levels specific to Rf602, f202, and f203 in a pool of sera from nine non-allergic individuals were assessed. No elevated levels were detected. (**Table S2**).

Table 1. Characteristics of subjects who are sensitive to Sichuan pepper.

General Information		Clinical features					SPT	
Sample	Gender/ Age (years)	Allergic to ScPS	ACi	ACa	AP	Other allergic diseases	ScPS	ScPP
A1	M/51	Anaphylaxis	Yes	Yes	Yes	AR, AS	++++	-
A2	F/48	Anaphylaxis	No	Yes	Yes	None	+++	-
A3	F/46	Anaphylaxis	No	Yes	Yes	AR	++++	-
A4	F/59	Anaphylaxis	Yes	Yes	Yes	AR, AS	+++	-
A5	F/43	Anaphylaxis	Yes	Yes	Yes	AR	++++	-
A6	M/46	Anaphylaxis	Yes	Yes	Yes	AR, AS, AA	ND	NO
A7	M/31	Anaphylaxis	No	Yes	Yes	AR, AS, AU	++++	+++
A8	F/56	Anaphylaxis	Yes	Yes	Yes	AR, AS	++++	+++
A9	F/57	Urticaria	No	Yes	Yes	None	++++	-
A10	F/62	Anaphylaxis	Yes	Yes	Yes	None	ND	NO
A11	F/60	Urticaria, oral allergic reactions	No	Yes	Yes	None	ND	NO
A12	M/44	None	No	No	No	AR, AS	ND	NO
A13	M/49	None	No	No	No	AR	ND	NO

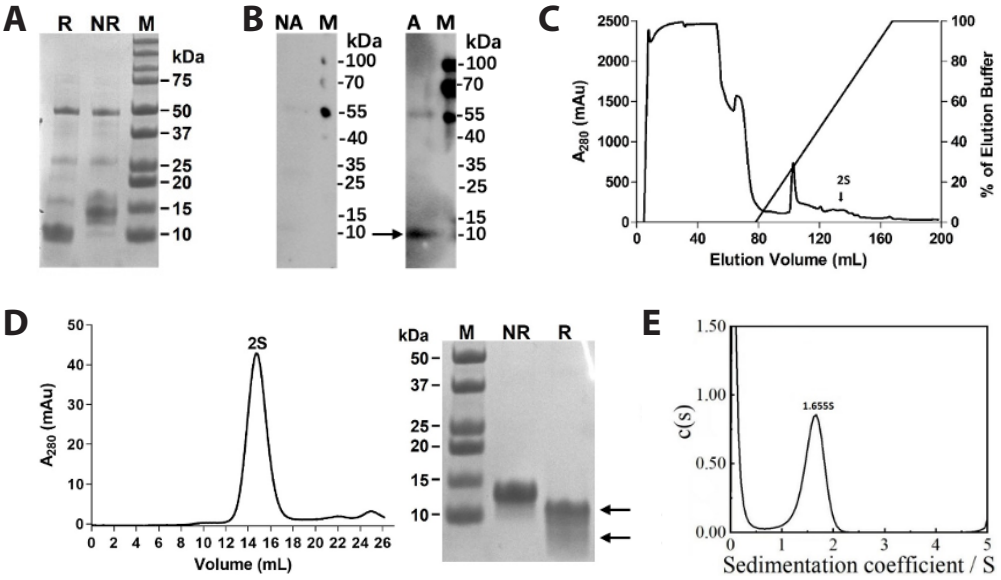
Abbreviations: ScPS, Sichuan pepper seed; ScPP, Sichuan pepper peel; SPT, skin prick test; ACi, allergic to citrus; ACa, allergic to cashew seed; AP, allergic to pistachio; ND, not done; AR, allergic rhinitis; AS, asthma; AA, amoxicillin allergy; AU, acute urticaria; MWD, mean wheal diameter; NC, negative control; PC, positive controls;

Note: The positive or negative SPT results are represented by the symbol "+" or "-" respectively. + (MWD > NC, ≥ 1 / 3 and < 2 / 3 PC); ++ (MWD ≥ 2 / 3 and < 1 PC); +++ (MWD = 1 PC); ++++ (MWD > 1 PC, or with pseudopods); - (MWD < 3 mm).

Purification and characterization of a 14 kDa component from Sichuan pepper

Sichuan pepper extract mainly had three prominent protein bands on a non-reducing (NR) gel: 50 kDa, 25 kDa, and 14 kDa. The protein mobilized at 14 kDa is the most abundant, and in a reduced form, this protein was mobilized at 10 kDa (**Figure 1A**). Western blot of the reduced extract showed that the sera pool from Sichuan pepper allergic individuals recognized the protein (**Figure 1B**).

To isolate this 14 kDa protein, we ground Sichuan pepper seeds in an acid-extraction buffer (20 mM NaAcO, pH 4.6), and a large quantity of protein was solubilized. The acid-extracted protein was further purified via ion exchange and analyzed using gel filtration chromatography (**Figure 1C, D**), where it was eluted at 14.74 mL as a single peak. Therefore, the protein was estimated to be 14 kDa and existed as monomers in the solution. The SDS-PAGE results of the 14 kDa fraction showed that the non-reducing (NR) sample had one band and reducing (R) sample had two bands (**Figure 1D**). Analytical ultracentrifugation showed this 14 kDa fraction with a sedimentation coefficient of 1.655 S (**Figure 1E**). These data suggest that the 14 kDa fraction belongs to the 2S albumin protein family which contains many allergens found in other seeds and nuts.



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Peptide	Modification#	Expected Mass	-10lgP	Actual Mass	Length	ppm	RT
QSCEQQIQQQQQLR	C	1801.92	42.51	1801.8376	14	5.9	25.93
CNISPR	C	745.82	27.71	745.3541	6	6.1	14.41
QQQQYLR, or QQQYLRQ	None	963.06	22.79	963.4774	7	7.3	23.94

Figure 1. Extract and purification of a 14 kDa protein component from Sichuan pepper seed. (A): SDS-PAGE results for the extract from Sichuan pepper seeds. R: reducing sample with 2-Mercaptoethanol, NR: non-reducing sample without 2-Mercaptoethanol, M: marker. (B): Western blot results for extract from Sichuan pepper seeds. NA: sera pool from non-allergic individuals, A: sera pool from Sichuan pepper allergic individuals. (C): Ion-exchange chromatography of acid-extracted protein. (D): Analytical exclusion chromatography and SDS-PAGE result. R: reducing, NR: non-reducing. (E): Analytical ultracentrifugation. (F): Supporting peptides of A0A067EPV8. -10lgP: This index defines the confidence of the corresponding spectrogram identification. Mass: monoisotope mass of the peptide segment, Length: amino acid number, ppm: deviation of peptide mass from the theoretical value, RT: retention time, #: C means S-carboxymethylation on reactive cysteine residue with an addition of 57.02 in mass; the expected mass was calculated using the Expasy server (https://web.expasy.org/compute_pi/) after considering a modification.

Identification of the 14 kDa protein component via Mass spectrometry

To further identify the 14 kDa protein, the SDS-PAGE band of the non-reducing sample was excised for mass spectrometry analysis. The raw file of mass spectrometry results was analyzed by PEAKS Studio using de novo sequencing to determine the amino acid sequences of peptide fragments resulting from trypsin digestion. Our results for the supporting peptides (**Figure 1F**) and best unique peptide–spectrum matches (PSMs) (**Figure S1**) suggest that the purified 14 kDa protein was similar to the α-amylase inhibitor domain-containing protein of *Citrus sinensis* (UniProtKB-A0A067EPV8), which belongs to the 2S albumin protein family.

Secondary structure and biochemical stability analysis

We used a circular dichroism spectroscopy analysis to investigate the secondary structure, thermal and chemical stability of the purified 14 kDa protein, The circular dichroism revealed a well-folded protein with a high content of helical structures as indicated by two minimal values (one at 222 nm and another at 208 nm), and one maximum value at 193 nm. Furthermore, when the temperature rose from 20°C to 95°C, and then returned to 20°C, the helical secondary structure also reappeared, suggesting it had similar thermal stability as the other 2S albumins (**Figure S2A**). For the sample with 2 mM dithiothreitol, since the two minima disappeared, the secondary structure was destroyed when the temperature rose to 95°C. Moreover, the helical secondary structure did not recover when the sample temperature returned to 20°C (**Figure S2B**). The circular dichroism signal at 220 nm is highly sensitive to secondary structural changes and is used to monitor the thermal transitions. Notably, this protein is less thermally stable in the presence of DTT: T_M for the protein with DTT (54.46 ± 0.38°C) is lower than the T_M without DTT (above 95°C) (**Figure S2C**).

Cloning and mammalian cell expression

As the genome sequence of the Sichuan pepper has not been published, we decided to clone it from the genomic DNA of the Sichuan pepper. We designed primers based on the possible coding sequences of the identified peptides analyzed by PEAKS Studio and the sequence of the α-amylase inhibitor domain-containing protein of *Citrus sinensis*. After many attempts, a partial sequence of the coding sequence was cloned. A 303 bp DNA fragment was amplified and sequenced. The results confirm that we obtained part of the DNA sequence of the α-amylase inhibitor (AAI) domain of this allergen (**Figure S3A, B**). A comparison of the cloned sequence and mass spectrometry results (**Table S3**) shows that the MS sequencing coverage of the protein is 61%. To express the complete protein in mammalian cells, we added 22-37 and 143-152 amino acids of the α-amylase inhibitor domain-containing protein of *Citrus sinensis* before and after the AAI domain of Sichuan pepper 2S albumin, respectively. After transfection and one day of expression, the protein with a human IgG-Fc tag was purified by a Protein A column (**Figure S3C, D**). The molecular mass of this chimeric protein (15.6 kDa) and human IgG-Fc fusion tag is 42.6 kDa.

IgE reactivity analysis

ELISA was performed to prove the specific IgE binding reactivity of the 14 kDa protein component. We used the purified natural 14 kDa protein component as antigens. The 14 kDa protein component allergen was named Zan b 1.01 by the IUIS Subcommittee for Allergen Nomenclature. Sera from thirteen patients sensitized to Sichuan pepper and a sera pool from non-allergic individuals were used as the primary antibodies. An HRP-conjugated anti-human IgE (ε-chain specific) antibody was used as the second antibody. ELISA results showed that among the 13 sera from allergic and sensitized individuals, 10 were significantly different from the sera from non-allergic individuals (**Figure 2A**).

Cross-reactivity between Zan b 1.01 and allergens in citrus, pistachio, and cashew seed extracts

The direct ELISA results showed that the sera from Sichuan pepper-allergic individuals recognized naturally purified 2S albumin from Sichuan pepper seed which was named Zan b 1.01, also reacted with the extracts of citrus seeds, pistachios, and cashew seeds (**Figure 2B**). In order to further investigate the cross-reactivity between Zan b 1.01 and crude extracts of citrus seeds, pistachios, and cashew seeds, competitive ELISA was performed. When sera were pre-mixed with the crude extracts of citrus seeds, pistachios, and cashew seeds, the binding between sera IgE and Zan b 1.01 was inhibited. (**Figure 2C**).

Both Sichuan pepper and citrus belong to the *Rutaceae* family. Sichuan pepper 2S albumin pre-mixed with extracts of citrus seed had the greatest degree of inhibition. We hypothesized a general cross-reactivity between similar allergens with similar sequences and structures. To prove our hypothesis, we compared the AAI domain amino acid sequences of the 2S albumin of Sichuan pepper, 2S seed storage protein of citrus, allergen Pis v 1 from pistachio, and allergen Ana o 3 from cashew seed via DNAMAN (**Figure 3A**). The homology tree of these four proteins built by DNAMAN shows the 2S albumin of Sichuan pepper and 2S seed storage protein of citrus had 80% similarity (**Figure 3B**). The phylogenetic tree of 2S albumin of Sichuan pepper, 2S seed storage protein of citrus, allergen Pis v 1, allergen Ana o 3, Ara h 7 from peanut, and allergen Cor a 14 from hazelnut was constructed using MEGA-X via the maximum likelihood method (**Figure 3C**). The phylogenetic tree showed 2S seed storage protein of citrus, Pis v 1, and Ana o 3 have a higher similarity with 2S albumin of Sichuan pepper than Ara h 7 or Cor a 14.

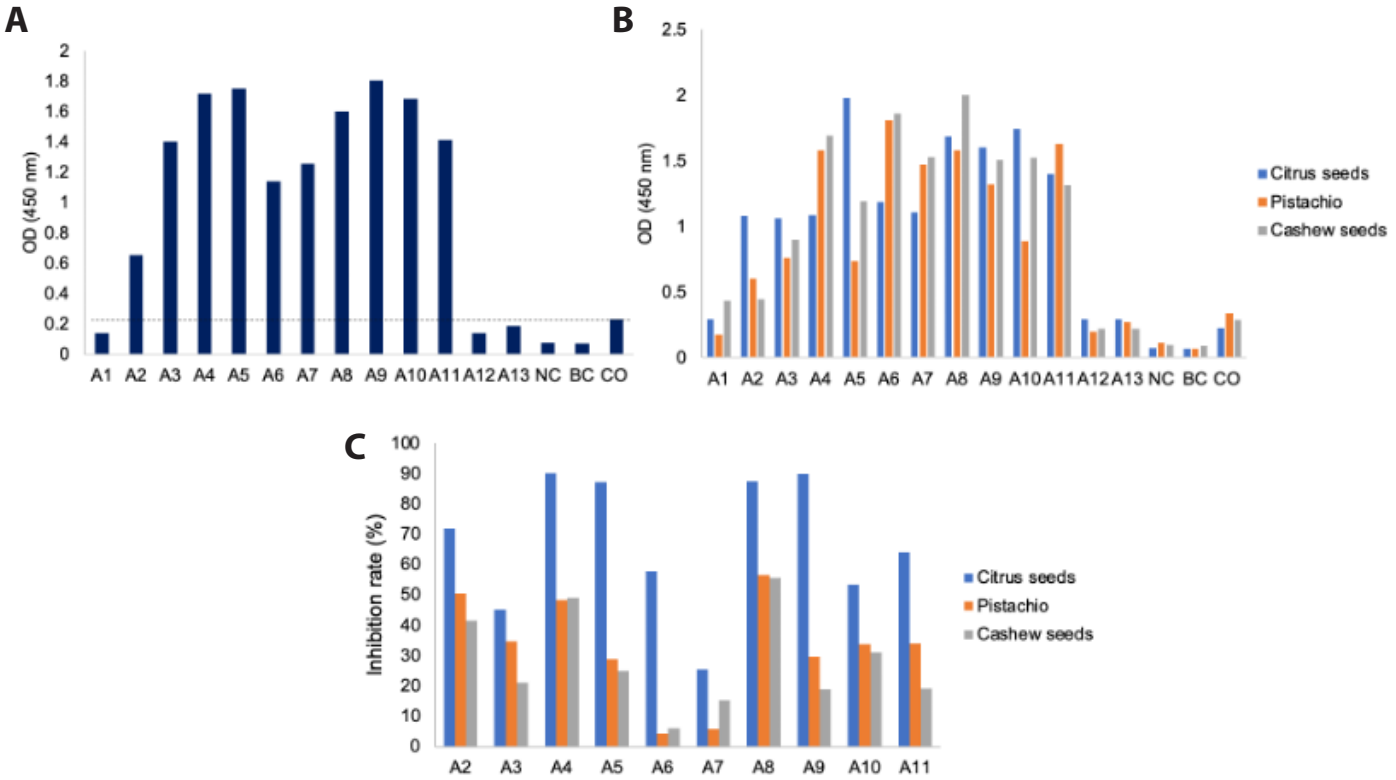

Figure 2. Cross-reactivity between 2S albumin from Sichuan pepper seeds and other nuts. (A) ELISA results of naturally purified 2S albumin from Sichuan pepper seeds. NC: negative control; BC: blank control; CO: cut-off value (which is three times of negative control). (B) ELISA results of citrus seeds, pistachio, and cashew seeds. (C) Inhibition rates of citrus seeds, pistachio, and cashew seeds, when Zan b 1.01 was used as the solid protein, and citrus seeds, pistachio, and cashew seeds were used as inhibitors.

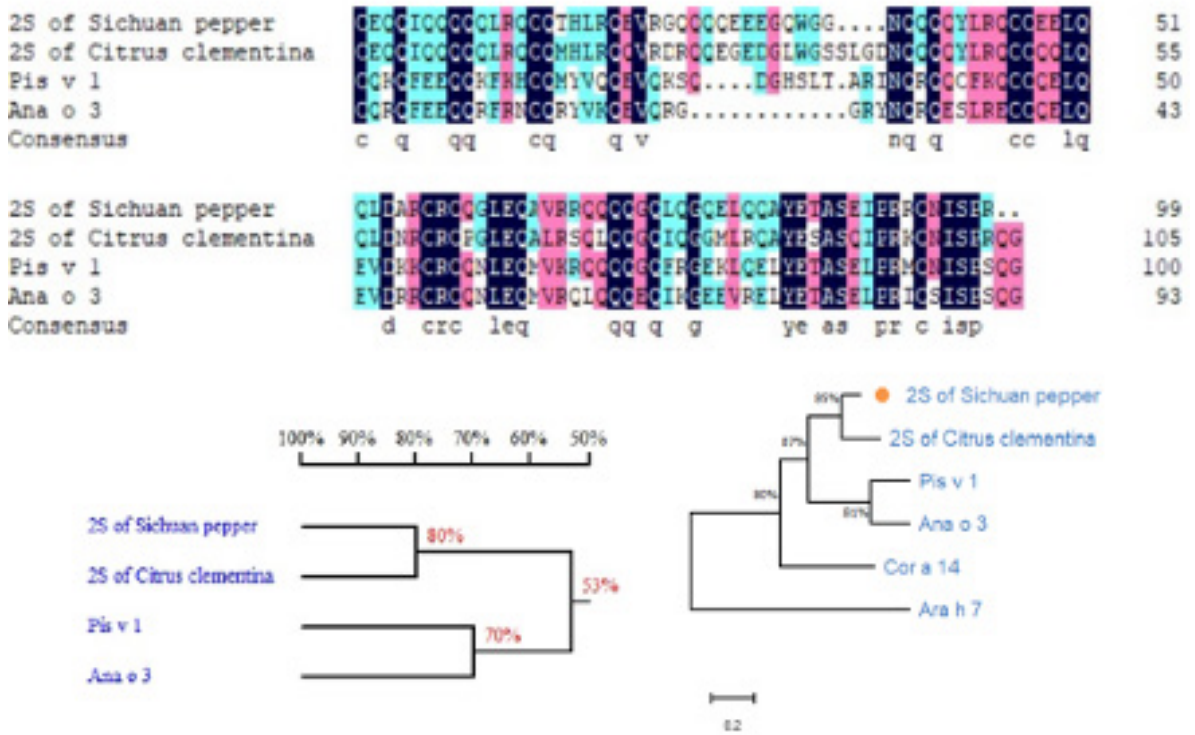

Figure 3. The 2S albumins of Sichuan pepper, citrus, pistachio, and cashew seeds have high sequence similarity. (A) AAI domain amino acid sequences comparison of 2S albumin of Sichuan pepper, 2S seed storage protein of citrus, Pis v 1 and Ana o 3 constructed via DNAMAN. (B) Homology tree of 2S albumin of Sichuan pepper, 2S seed storage protein of citrus, Pis v 1, and Ana o 3 constructed via DNAMAN. (C) Phylogenetic tree of 2S albumin of Sichuan pepper, 2S seed storage protein of citrus, Pis v 1, Ana o 3, Ara h 7 and Cor a 14.

Discussion

Sichuan pepper (*Zanthoxylum bungeanum Maxim.*) is a new food allergen source. In this study, we purified a protein of around 14 kDa from seeds of Sichuan pepper, and further identified it as a 2S albumin. Ten out of thirteen sera from Sichuan pepper-allergic and sensitive individuals recognize this 2S albumin, therefore, we identified the first member of allergens-Zan b 1.01 from Sichuan pepper. In addition to this, we proved this 2S albumin allergen had a regular α-helix structure and high thermal stability relying on disulfide bonds. The DNA sequence of the AAI domain was amplified from the genome. Since the genome sequence of the Sichuan pepper has not been published, the intron sequence before or after the coding region cannot be acquired; therefore, we cannot determine whether the cloned sequence is full-length. According to the comparison with other 2S proteins, we can only confirm that we obtained a majority DNA sequence without introns; therefore, we use "a 303 bp DNA sequence" not "the AAI domain" to describe it.

To prove the specific IgE binding reactivity of Zan b 1.01, ELISA was performed. Sera from Sichuan pepper-allergic individuals, but not those from non-allergic individuals, recognized Zan b 1.01 (**Figure 2A**), verifying that it is an allergen of Sichuan pepper.

Cross-reactivity between Zan b 1.01 and citrus seeds, pistachios, and cashew seeds was investigated via inhibition ELISA. In ELISA inhibition analysis (**Figure 2**), the binding of sIgE from all ten patients' sera to Zan b 1.01 was inhibited from 25.34% to 90% by citrus extracts. However, when the ten sera were premixed with the extract of cashew seeds or pistachio, the binding of sIgE from patients' sera to Zan b 1.01 was inhibited in nine out of the ten sera, and the inhibited rates varied from 15.2% to 55.47% by cashew seed. For pistachio, eight out of the ten sera were inhibited from 28.94% to 56.57%. Each serum from allergic individuals showed a different level of cross-reactivity between Zan b 1.01 and citrus seeds, pistachio, and cashew seeds allergens.

In 1997, we reported 30 adult cases of hypersensitivity to cashew seeds. Fourteen out of thirty of these adults had dysphagia and laryngeal edema reactions, and six out of thirty developed anaphylactic shock.[25] During the follow-up visits, an interesting phenomenon was observed—these patients usually had multiple food allergies. In addition to cashew seeds and pistachios, they often reacted to Sichuan pepper, orange, kumquat, sesame, etc. We initially discovered the main allergen in Sichuan pepper seeds.[4,26] In 2009, we reported 14 cases of anaphylaxis to Sichuan pepper;[4] 5 out of these 14 experienced anaphylactic shocks. Sichuan pepper had a high allergenic potency on some sensitized individuals. Accidental contact with the debris of Sichuan pepper may cause a life-threatening allergic reaction, and patients might accidentally consume Sichuan pepper powder in many foods. In patients with severe Sichuan pepper allergy, the initial symptoms are mainly oropharyngeal allergies, such as itching, tingling, edema of the mucous membrane of the throat, and a foreign body sensation in the throat. Patients who do not stop eating Sichuan peppers once they begin to experience mild discomfort may develop a more severe allergic reaction, such as anaphylaxis, as is the case in patients with anaphylaxis to cashew seeds.[27] Therefore, more attention should be paid to patients with Sichuan pepper allergy in terms of diagnosis, prevention, and patient education.

Tree nut (TN) allergy is common and often severe.[28-32] It is one of the most common causes of anaphylaxis in the US, UK, and Europe, accounting for up to 40% of cases in some series and, together with peanut allergy, up to 90% of food-related fatalities. The availability and consumption of various kinds of TN food products have increased. Unfortunately, there is a lower likelihood of resolution of TN allergy, roughly 10-20%.[33,34] In this study, we identified and characterized an allergen from Sichuan pepper seed and showed its high cross-reactivity with citrus, cashew seeds, and pistachio. Many allergens of seeds belong to 2S albumin, including cashew allergen Ana o 3, hazelnut allergen Cor a 14, walnut allergen Jug r 1, peanut allergens Ara h 2, Ara h 6, and Ara h 7,[15] sesame allergens Ses i 1 and Ses i 2, and pistachio allergen Pis v 1. 2S albumin allergens appear to be the most important candidates for explaining much of the observed co-sensitization among peanuts, tree nuts, and sesame seeds.[35,36] Anaphylaxis caused by Sichuan pepper has been clinically observed. Zan b 1.01 might be able to predict clinical severity. However, future research is needed to investigate the role of Zan b 1.01 in the accurate diagnosis of Sichuan pepper allergy.

Conclusions

In this study, we identified and characterized a 2S albumin allergen Zan b 1.01 from Sichuan pepper, and it showed high cross-reactivity with citrus seed, pistachio, and cashew seed. This new food allergen Zan b 1.01 might play an active role in individually precise diagnosis and better prevention of food allergies.

Author contributions

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Data Availability Statement

The data presented in this study are available on request from the corresponding authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Table S1. SIgE values for subjects sensitive to Sichuan pepper.

General Information	SIgE			
Sample ID	Rf602	f33	f202	f203
A1	2	1	3	3
A2	2	2	2	2
A3	5	3	6	6
A4	3	2	3	3
A5	2	2	3	3
A6	3	2	4	4
A7	4	3	5	5
A8	2	2	3	3
A9	2	2	3	3
A10	4	3	5	5
A11	3	1	3	3
A12	3	0	0	0
A13	3	2	0	2

Note: SIgE, Specific IgE values: 0 (0~0.35 kU_A/L);1 (≥ 0.35 KU_A/L, < 0.7 KU_A/L); 2 (≥ 0.70 KU_A/L, < 3.5 KU_A/L); 3 (≥ 3.5 KU_A/L, < 17.5 KU_A/L); 4 (≥ 17.5 KU_A/L, < 50 KU_A/L); 5 (≥ 50 KU_A/L, < 100 KU_A/L); 6 (≥ 100 KU_A/L). SIgE measured by ImmunoCAP.
Rf602: Sichuan pepper seed; f33: citrus; f202: cashew seed; f203: pistachio.

Table S2. Characteristics of healthy control subjects.

Sample	Gender/Age (years)	T-IgE (KU_A/L)	Phadiatop (KU_A/L)	Fx5	Allergies	S-IgE* (KU_A/L)
1	M/64	8.1	0.04	0.02	None	-
2	F/60	12.7	0.04	0.01	None	-
3	M/52	9.0	0.05	0.01	None	-
4	M/49	12.2	0.05	0.01	None	-
5	M/48	2.5	0.05	0.01	None	-
6	M/36	8.6	0.05	0.02	None	-
7	F/32	3.2	0.06	0.02	None	-
8	M/59	15.3	0.04	0.01	None	-
9	F/50	11	0.04	0.02	None	-
Sera pool	-	9	-	-	-	0

Note: Fx5: specific IgE of egg white, milk, Gadus, wheat, peanut, and soya bean. S-IgE*: specific IgE of Rf602, f33, f203, f202. KU_A/L: kilounit allergen/liter. Phadiatop: specific IgE of inhalant allergens screening tests. Allergies: Sichuan pepper allergy, citrus allergy, cashew seed allergy, anaphylaxis, food allergy, AR, AS, urticaria, eczema.

Table S3. Comparison results of cloned sequence and mass spectrometry.

Sequence	# PSMs	Theo. MH+ [Da]	XCorr
CNISPR	2	746.36138	1.81
CQGLEQAVR	3	1060.5204	1.93
CQGLEQAVRR	1	1216.62151	1.19
QCQTHLR	4	942.45741	1.51
QQQQGQLQGQELQQAYETASEIPR	3	2758.33907	1.44
QQQQGQLQGQELQQAYETASEIPRR	1	2914.44018	2.61
QSCEQQIQQQQQLR	8	1801.86097	2.71
RCNISPR	12	902.4625	2.71

Note: # PSMs: peptide–spectrum matches, Theo. MH+ [Da]: theoretical molecular weight.

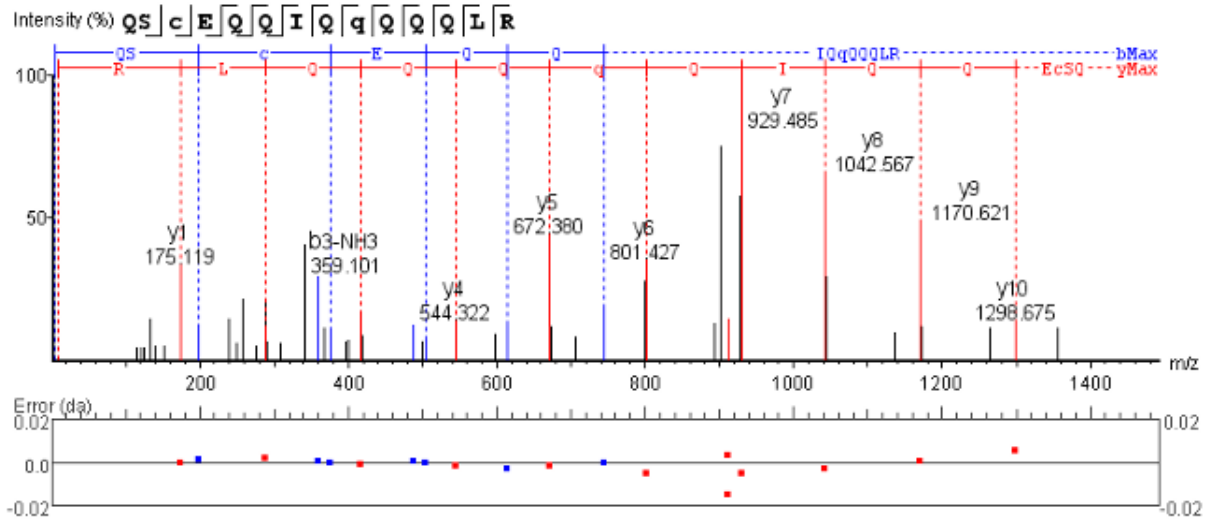

Figure S1. Best Unique PSM (Scan 6403, m/z = 901.9242, z = 2, RT = 25.93, ppm = 5.9) of A0A067EPV8.

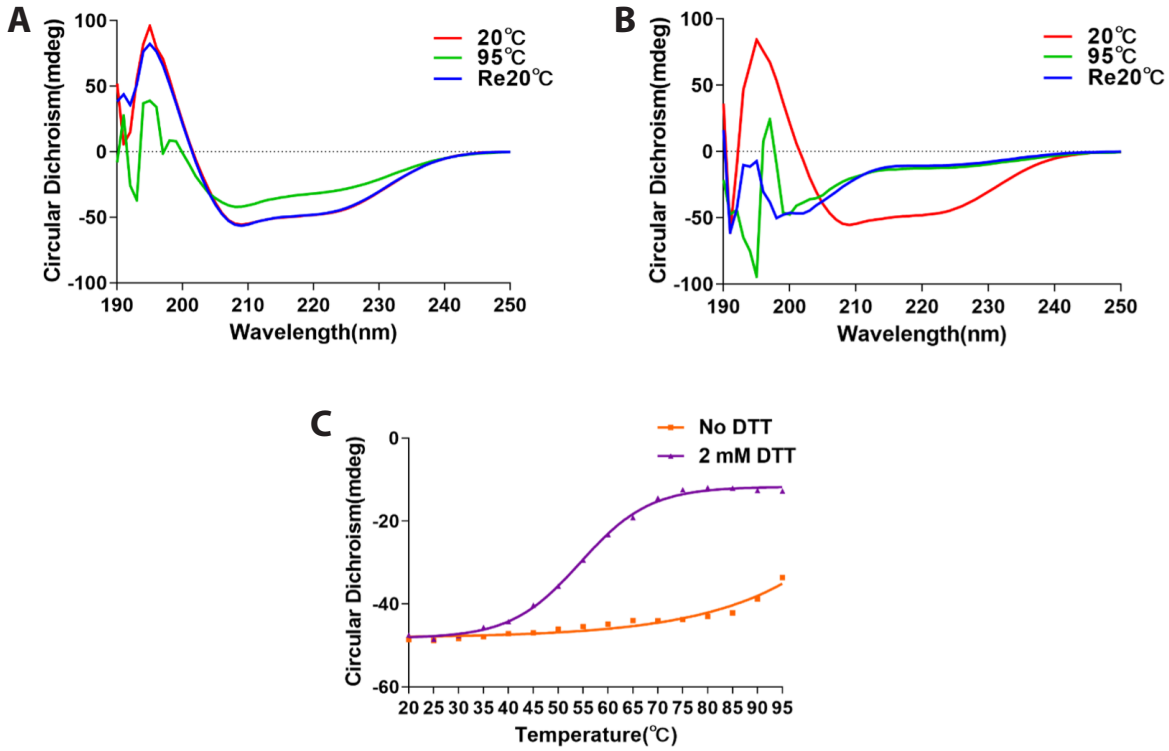

Figure S2. Secondary structure and biochemical stability analysis of 2S albumin. (A) Circular dichroism of 2S albumin without reducing agent. X-axes: wavelength from 190 nm to 250 nm, y-axes: circular dichroism (mdeg), red line: at 20°C, green line: at 95°C, blue line: sample heated from 20°C to 95°C and cooled to 20°C. (B) Circular dichroism of 2S albumin with 2 mM DTT. (C) Thermal denaturation of 2S albumin with 2 mM DTT and without DTT. Orange line: sample without 2 mM DTT, purple line: sample without DTT.

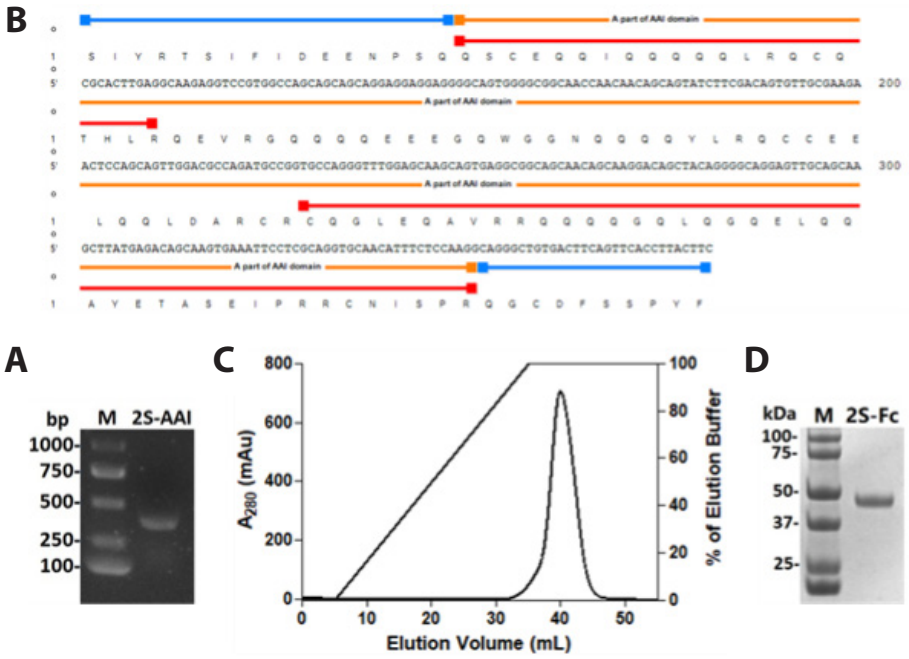

Figure S3. Cloning and mammalian cell expression of 2S albumin. (A, B) A 303 bp DNA sequence of α-amylase inhibitor (AAI) domain cloned from Sichuan pepper genomic DNA. M: marker, 2S-AAI: a part of the AAI domain of 2S albumin, orange line: amino acids of a part of AAI domain cloned from Sichuan pepper seed, red line: sequence detected by MS. (C) Protein A chromatography of 2S albumin expressed by mammalian cells. (D) SDS-PAGE result of 2S albumin-Fc (42.7 kDa). M: marker, 2S-Fc: 2S albumin with human IgG1 Fc.