

# Research Article:

## Cloning and Expression of 2S Albumin As a Major Allergen of Persian Walnut



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## ABSTRACT

**Background:** Food hypersensitivity to walnut usually results in mild symptoms; however, several cases of anaphylactic reactions to this product have been observed. This study aimed to determine the immunochemical characteristics of the Persian walnut and provide the recombinant form of its main allergen.

**Materials and Methods:** The allergenic proteins of the Persian walnut were extracted by standard procedure. The IgE-binding profile was determined by common immunoassays, including ELISA and Western blotting. The characteristics of the main allergenic protein which showed a stronger and higher frequency of IgE-reactivity with the patient's sera was identified by MALDI-TOF-TOF method. The conventional PCR was used for the amplification of the coding sequence of the target protein which was then inserted into pET-21b(+) vector and expressed in *E. coli* BL21. The recombinant allergen was purified by metal affinity chromatography and the ELISA and immunoblotting assays were used for the evaluation of the IgE-binding capacity of the recombinant protein.

**Results:** All patients showed a considerable specific IgE-reactivity to total extract (OD 0.58±0.43 versus 0.047±0.026; P<0.05) in ELISA. Immunoblotting with crude extract indicated considerable IgE-reactivity of the patients' sera with a 15-kDa allergen which was characterized as 2S albumin by mass spectrometry methods. The refolded walnut recombinant 2S albumin showed considerable IgE-reactivity in ELISA and western blotting with patients' sera.

**Conclusion:** We demonstrated that the refolding of walnut recombinant 2S albumin could result in the reconstruction of an IgE-reactive allergen with a rather similar immunoreactivity to its natural counterpart. The refolded recombinant protein could be a suitable candidate for diagnostic and therapeutic approaches.

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## Introduction

Walnut is the edible seed of genus *Juglans* (*J*) trees, with more than 20 different species; however, only *J. regia* and *J. nigra* have economic interests [1]. *Juglans regia* is a famous species of walnuts, native to central Asia, Balkans region in South-east Europe, as well as Southwest China. Walnuts have achieved special worldwide attention because its intake is commonly related to healthy traditions and offset food diets [2]. However, it may induce hypersensitivity reactions in some individuals. Since 1993, walnut and other tree nuts were demarcated as responsible for nearly 90% of human food allergies [3-5]. Since then, most studies were focused on the molecular characterization of allergens from both *J. regia* and *J. nigra*. Although the exact prevalence of food allergy is not available, it seems that nearly 3%–4% of adults and 5%–6% of young children and up to 10% of the general population are affected by some types of food allergies [6-8].

Most walnut allergens, including legumins, 2S albumins, and vicilins are classified as seed storage proteins. Some other proteins such as profilins are regarded as minor allergens [9, 10]. Clinical features of IgE-mediated reactions to walnut, including oral pruritus, throat itching, and urticaria are usually mild; however, some more severe symptoms such as anaphylaxis have been observed, too [11].

Shared allergens of *J. nigra* and *J. regia* belong to prolamin, cupin, and profilin super-families. Well-known allergens of this genus, including Jug r 1, Jug r 3, and Jug n 1 belong to prolamin family whereas Jug r 2, Jug r 4, and Jug n 2 belong to cupin family of proteins [12, 13]. The proteins of 2S albumin superfamily, similar to prolamins are small globular, water-soluble proteins with 12-15 kDa molecular weight, and high content of asparagine, glutamine, cysteine, and arginine residues [14]. They are so named because of their sedimentation coefficient. Up to now, several 2S albumins, including Ara h 6, Ara h 7, and Gly m 2S albumin were classified as minor allergens and several other proteins, including Ana o 3, Bra j 1, Ara h 2 were classified as major allergens of this family of proteins [15].

Sensitization to 2S albumins is considered to occur through the gastrointestinal tract. High stability and structural resistance due to cysteine residues, make them sustainable in the harsh environment of the gastrointestinal tract [16]. Therefore, they may cross the gut mucosal barrier, presented to the immune system and trigger an allergic response [15]. Ber e 1 is the major 2S albumin

allergen from Brazil nuts which exhibits high stability to proteolysis so that 25% of the allergen remains intact following in vitro gastric digestion. Furthermore, comparable reactions to the recombinant form of 2S albumins and their native form are usually observed after standard skin prick test, showing appropriate folding of this type of recombinant proteins [17]. Therefore, based on the usefulness of 2S albumins in the diagnosis of allergic reactions, several 2S albumins from different species have been identified and produced by cloning techniques.

In one study, cloning of the 2S albumin of *J. regia* (Jug r 1) revealed that its DNA sequence consisted of 663 base pairs and encoded to 142 amino acids. Moreover, natural Jug r 1 and recombinant Jug r 1, despite their different lengths exhibited a very similar IgE-binding reactivity [18]. So far, a common IgE-binding epitope has been described in the large chain of 2S albumins, especially Jug r 1 [19]. As mentioned above, most previous studies introduce 2S albumin as the main allergen in walnut. Therefore, in this study, we focused on this protein and produced its recombinant form to apply it for in vitro diagnosis of Persian walnut allergy.

## Materials and Methods

### Protein extraction

Persian walnut, a morphologically similar cultivar to English walnut was obtained from Agricultural Research, Education, and Extension Organization local gardens in Tehran. The defatting of the nut powder was fulfilled by hexane (Sigma-Aldrich, Germany). Then, 1 g of the fine powder was solubilized in 5 mL of phosphate buffer saline (PBS) pH 7.4. The quality and quantity of the protein content of the extract were analyzed by Bradford and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods.

### Allergic patients' sera

Serum samples from 50 individuals who had clinical symptoms of walnut allergy such as itching of throat and nausea following walnut intake and demonstrated positive specific IgE with a commercial disk ELISA kit (Dr. Fooke, Germany) was collected from allergy clinic of Day General Hospital, Tehran, Iran. Furthermore, 10 healthy and non-allergic individuals with no clinical symptoms of respiratory or food allergy were included as negative controls.

### Assessment of specific IgE antibodies by ELISA

The indirect ELISA method was used to assess the specific IgE-reactivity of the extracted proteins with patients' sera. In brief, 3 µg of the extracted proteins (solubilized in 100 µL bicarbonate buffer pH 9.6) was coated in ELISA wells (Nunc, Denmark) at 4° C overnight. After washing with PBS-Tween 20 (0.05%), the wells were blocked with bovine serum albumin (BSA) at 4° C overnight. Then, 1:5 dilution of patients' sera (in PBS) was added to each well and the plates were incubated at 4° C overnight. After another wash, 1:1000 dilution of horseradish peroxidase (HRP)-conjugated anti-human IgE antibody (Sigma-Aldrich, USA) in 1% BSA was added to wells and incubated at 37° C for 2 h. After an extensive wash, TMB/H<sub>2</sub>O<sub>2</sub> substrate was added to each well and the plate was incubated in the dark at room temperature (RT) for 15 minutes. The reaction was stopped by 2M sulfuric acid and the absorbance was measured at 450 nm against a 630 nm reference filter.

### Determination of IgE-reactive bands by Immunoblotting

SDS-PAGE was performed under reducing condition in 15% separating gel [20]. Then, the proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane (Millipore, Bedford, USA) at 0.8 mA/cm<sup>2</sup> within 60 min using a semi-dry transfer apparatus (PeQlab, Belgium). Then, the membranes were stained with Ponceau S and cut into lanes. Following a destaining with distilled water, the lanes were incubated with 2% BSA at 37° C for 5 h on a rocker to block the protein-free spaces. Then, we incubated each lane with 1:2 dilution of the serum samples which have previously shown positive IgE-reactivity in disk ELISA, overnight on a rocker at 4° C. The lanes were then washed with PBS-T (3 times for a total of 15 min) and incubated with 1:1000 dilution of biotinylated anti-human IgE in 1% BSA at 37° C for 2 h. After another wash, the lanes were incubated with 1:5000 dilution of HRP-linked streptavidin (Sigma-Aldrich, USA) in 1% BSA at 37° C for 1 h. Following an extensive wash, the lanes were incubated with chemiluminescent substrate (Parsious, Iran) for 2 min at RT in dark and the IgE-reactive bands were visualized by Chemi Documentation System (Labtech, FUSION FX; England).

### Protein identification by mass spectrometry

The intended 2S albumin fraction was salted out by a 70% saturated ammonium sulfate solution. The precipitated proteins were solubilized in PBS containing 10 mM DTT and the content was subjected to SDS-PAGE

slabs. The resolved proteins were stained with colloidal Coomassie blue and destained by 20% methanol. The previously determined immunoreactive bands were spotted and manually excised from the gel and studied by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) at Yurk company (England) as previously described [21].

### Cloning and expression of walnut 2S albumin

Total RNA was extracted from the de-fatted walnut using CTAB and TRIzol® reagent (Invitrogen) according to the Chomczynski and Sacchi method with some modifications [22]. Then, cDNA was synthesized using a commercial kit (Fermentas, Vilnius, Lithuania) and applied as a template in a polymerase chain reaction with cloning primers. The cloning primers were designed based on the available Gen Bank sequence with the accession number of U66866 using Gene Runner software. Cloning specific primers, including F: 5'ATA GGA TCC AGC AGC TCT CCT TGT AGC CCT T3' and R: 5'ATA CTC GAG GAA CCA GCT TCT GCG AAT TT3' with restriction regions for Bamh 1 and Xho 1 regions were designed and applied in PCR cloning steps.

The target 2S albumin sequence was amplified by PCR under the following conditions: initial denaturation at 94° C for 5 min; 35 cycles of amplification (denaturation at 94° C, 30 s; primer annealing at 58° C, 30 s; extension at 72° C, 1 min) and a final extension at 72° C for 10 min. PCR product was visualized on 1% agarose gel in parallel with a 1 kb DNA ladder (Solis BioDyne, Tartu, Estonia). The amplicon was cut from the agarose gel and purified using a DNA extraction kit (YTA, Iran). Target bacterial vector (pET-21b(+), Invitrogen, USA) and the PCR product were both digested with XhoI and BamHI restriction enzymes, according to the manufacturer's protocols (Fermentas, Lithuania). After a cleanup, fragments were ligated with T4 DNA ligase (TAKARA, Japan) and the construct was transformed into *E. coli* BL21 competent cells.

Finally, ampicillin-resistant colonies were selected for plasmid extraction and DNA sequencing. The expression of recombinant protein was induced with 1mM isopropyl-1-β-D-thiogalactopyranoside (IPTG) overnight. Bacterial cells were then lysed and the recombinant 2S albumin containing the inclusion body was purified and solubilized. Partial refolding of the recombinant protein was performed through the stepwise addition of the proteins to the refolding buffer. In brief, 1 mL of solubilized inclusion bodies were added to in dropwise manner to 50 mL of buffer appropriate for refolding of cysteine-rich

proteins composed of 50 mM Tris-HCl pH 8.5, 0.5% Triton X-100, 0.4 M sucrose, 10% glycerol, 0.3 mM glutathione disulfide (GSSG), and 3 mM glutathione (GSH) at 4° C within an hour. Then, the mixture was stirred overnight in a cold room at 4° C. After a centrifugation step, the supernatant was applied to the Ni-IDA column for purification steps. Purification was fulfilled by nickel affinity chromatography with a HisTrap column (Ni-IDA Resin, ParsTous, Iran) according to the manufacturer's recommendation.

Native 2S albumin which shows a rather high expression rate in walnut seed was purified from walnut's total extract by electroelution. In brief, the total extract was subjected to SDS-PAGE and the corresponding 12-15 kDa band which is regarded as 2S albumin was horizontally removed from the slabs and placed in a dialysis tube and vertical electrophoresis apparatus supplemented with a cooling system. Finally, the eluted proteins were re-examined by SDS-PAGE.

#### Assessment of the IgE-reactivity of the recombinant and native 2S albumin

The indirect ELISA was carried out for the recombinant and native 2S albumin, as well as total extract pro-

teins. Approximately 25 µg of the recombinant or native proteins were subjected to SDS-PAGE 15 % acrylamide gel and electrotransfer of the resolved proteins and blotting was done as mentioned above.

#### Molecular modeling and plotting the 3D structure of the recombinant protein

The amino acid sequence of recombinant 2S albumin was considered as a query sequence, and then blasted in NCBI (<https://www.ncbi.nlm.nih.gov/>). The first eight seed storage proteins with high similarity to the query sequence were retrieved and along with Jug r1 were aligned by BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) for the conserved regions. Furthermore, the previously determined IgE epitopes of Jug r1 were considered on the aligned sequences to determine if they were placed in the mentioned conserved region. The three-dimensional structure of the recombinant protein was predicted by submitting its amino acid sequence in ITASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>).

#### Statistical analysis

The obtained data were analyzed in SPSS v. 20. The Mann-Whitney and t test were used for the analysis of non-parametric and parametric data, respectively. The data were presented as Mean±SD and Mean±SEM and P<0.05 were considered statistically significant.

## Results

#### Protein extraction

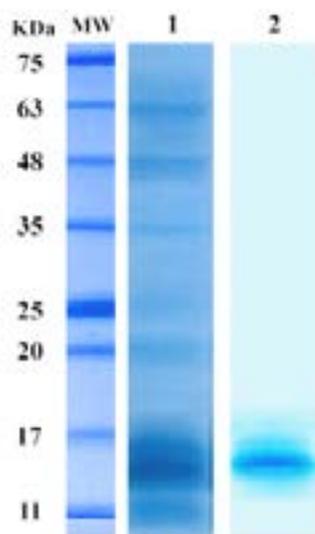
The electrophoretic patterns of total extract of walnut displayed protein bands ranging from 9 to 100 kDa with various intensities in addition to a faint smear of proteins. A rather pure native 2S albumin with 15 kDa molecular weight was separated by electroelution of the target band resolved from the total extract (Figure 1).

#### Assessment of specific IgE

Specific ELISA revealed that 18 out of 50 patients (36%) who declared walnut hypersensitivity had positive IgE-reactivity to walnut proteins. As Figure 2 demonstrates, most patients who showed specific IgE-reactivity were children.

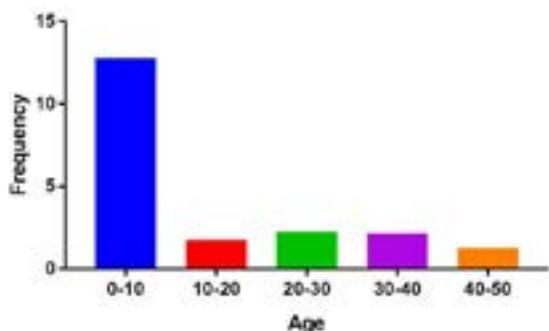
#### Immunoblotting

As demonstrated in Figure 3, sera from allergic patients mostly showed IgE-reactivity with proteins with a



**Figure 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Persian walnut total extract and native 2S albumin

The total extract of Persian walnut was prepared and its native 2S albumin was fractionated using ammonium sulfate precipitation method. Lane 1 and 2 show the Coomassie blue-stained SDS-PAGE of total extract and fractionated native 2S albumin on 15% gel, respectively. MW refers to the protein molecular weight marker (Sinaclone, Iran).



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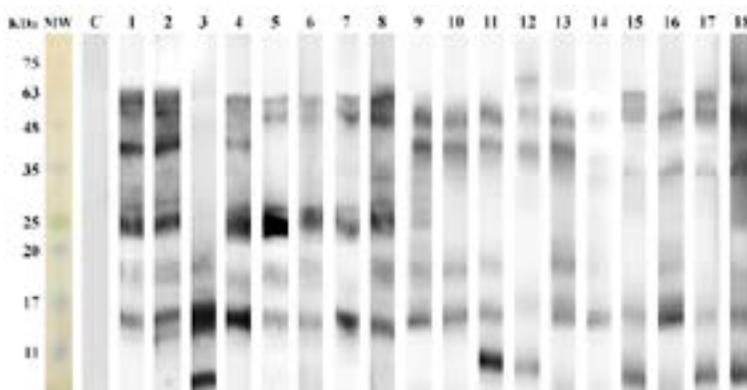
**Figure 2.** Frequency of the age of walnut allergenic patients in the studied group

Participants who complained from hypersensitivity to walnut was studied by ELISA. The Figure demonstrates the age-based categorization of the seropositive patients (18 out of 50 individuals), suggesting that specific IgE-reactivity is 5-6 fold greater in children less than 10 years old.

molecular weight of 9, 11, 18, 25, 44, and 54 kDa. Approximately all patients showed considerable IgE-reactivity with a 15 kDa protein band (Table 1), while the sera from non-allergic individuals did not show any significant reactivity with the electrophoretically resolved proteins.

### Mass spectrometry analysis

MALDI-TOF-TOF analysis confirmed that the 15 kDa protein belonged to 2S albumins and contained similar sequences to *J. regia* (English walnut 96%), *J. nigra* (black walnut 95%), pecan (American walnut 91%), and *Corylus* (Europe hazelnut 82%) (Table 2).



**Figure 3.** Immunoblotting of walnut extracted proteins with allergic patients' sera

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IgE-western blotting of walnut allergic patient's sera with total extract of Persian walnut confirmed the immunoreactivity of most of them with a 15 kDa protein which was a 2S albumin. C refers to the non-allergic controls pooled sera and MW to the molecular weight marker.

### Production and purification of r2S albumin

The target protein was cloned and expressed in the prokaryotic host. DNA sequencing of the cloned sequence shown 96% identity with previously-predicted sequences of walnut 2S albumin with EMBL accession number U66866. Lysis of the host bacteria showed that the recombinant protein was located in the inclusion body and only tiny amounts of the protein was found in the supernatant of the lysate. The purified inclusion bodies were solubilized and refolded and the expressed r2S albumin was successfully purified by immobilized metal affinity chromatography and applied in immunoassays (Figure 4).

### ELISA and immunoblotting using recombinant 2S albumin

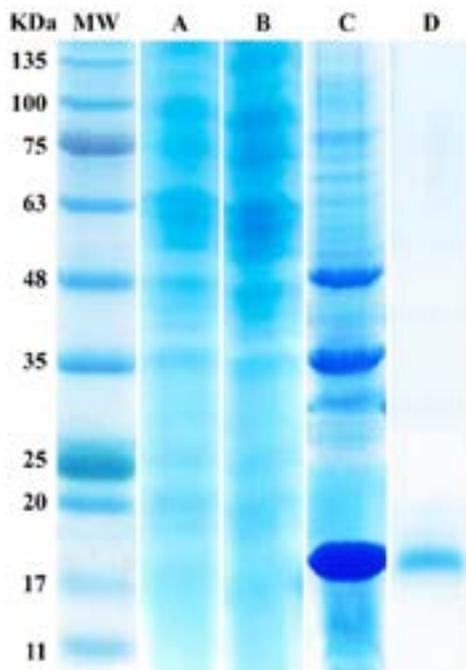
We found that 7 out of 18 total walnut extract reactive patient's sera (39%) show considerable IgE-reactivity with r2S albumin in ELISA. Western blotting showed that only 5 out of 7 ELISA positive patients (71%) had prominent IgE-immunoreactivity with the purified r2S albumin (Figure 5).

### Molecular modeling of 2S albumin

Molecular modeling revealed that the produced protein is composed of four alpha-helix structures and possess at least one accessible IgE-binding domain (Figure 6).

### Discussion

Large population studies show that allergy to walnut is the most common hypersensitivity reactions to tree nuts [23-25]. Vicilin-like protein and 2S albumin are two major allergens of walnut [26]. In this study, western blot-



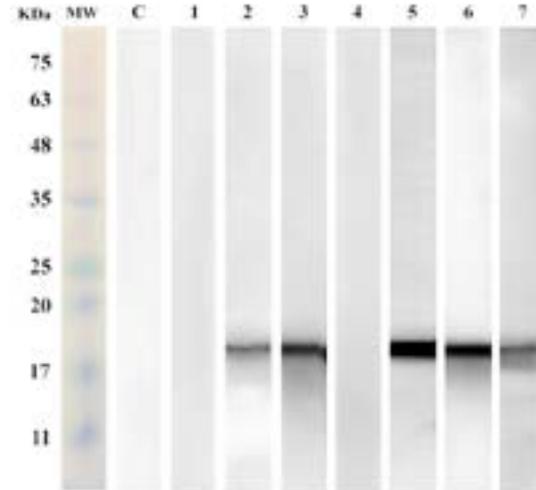
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**Figure 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified r2S albumin of Persian walnut

Walnut 2S albumin was cloned in pET-21b plasmid and expressed in a prokaryotic host (*E. coli* BL21). Following expression steps, the recombinant 2S albumin was purified by immobilized metal affinity chromatography.

MW refers to the protein molecular weight marker, lane A and B to the bacterial lysate of *E. coli* BL21 before transfection (control), lane C to the bacterial lysate expressing recombinant 2S albumin (18 kDa), and lane D to the purified recombinant 2S albumin.

ting with total extract demonstrated that several proteins of Persian walnut may contribute to these symptoms. Our results were rather similar to previous findings on other walnut species [25, 27, 28]. Western blotting of the total extract with the patient's sera revealed a specific IgE reactivity with 15 kDa protein which was later identified as 2S albumin. This protein could be found in the WHO-IUIS list of allergens as a typical IgE-reactive protein [12]. 2S albumins are allergenic proteins that were initially introduced in 1981 [29, 30]. The first walnut allergen, Jug r 1 (16.4 kDa), was reported in 1999 as one of 2S albumin family proteins. Other studies have reported the characteristics of other walnut allergens, including Jug r2, Jug r3, and Jug r4 [14, 25, 27, 31]. 2S albumins are the main storage proteins of nutrients that affect plant growth [32].



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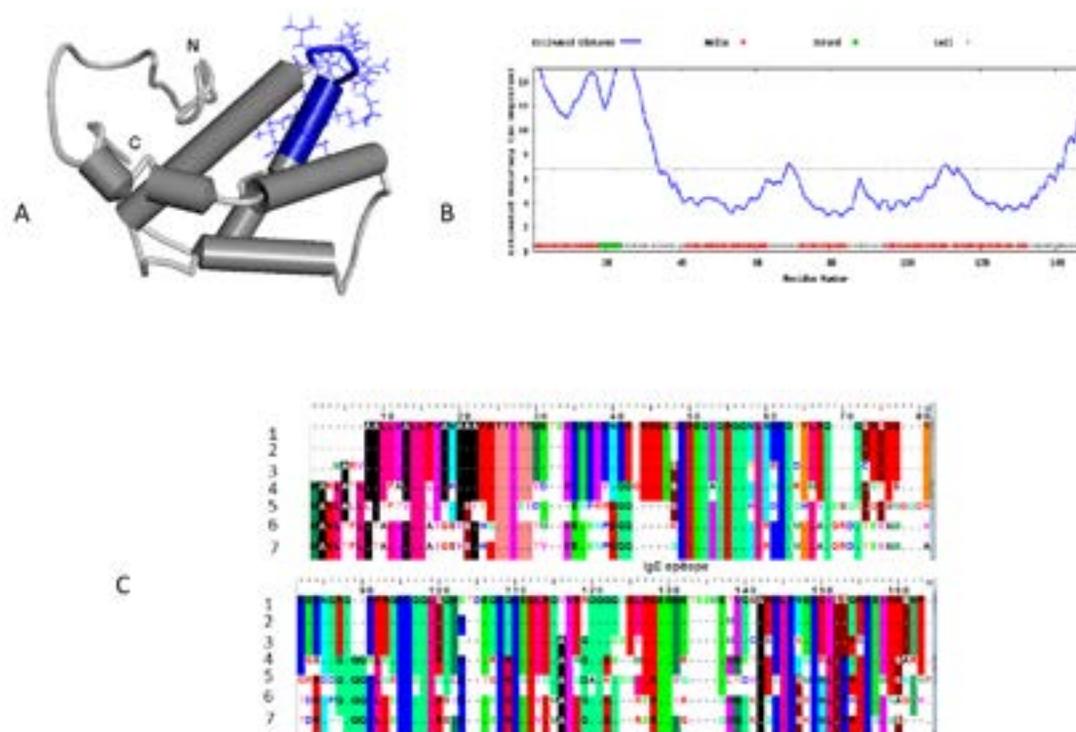
**Figure 5.** Immunoblotting of allergic patients' sera with purified walnut recombinant 2S albumin

Sera from individuals who showed IgE reactivity with a 15 kDa protein of walnut total extract in western blotting were also applied in western blotting assay with the produced recombinant form of walnut 2S albumin (18 kDa). Most patients showed significant reactivity (5 out of 7 studied samples), demonstrating the applicability of the produced protein in diagnosis.

MW refers to the protein molecular weight marker, and lanes 1-7 to the walnut allergic patients' sera.

In some studies even up to 91% of patients had specific IgE in their sera against some walnut allergens [26]. In this study, we described a 15 kD protein with high immunoreactivity with walnut allergic patient's sera.

The specific IgE antibody in walnut-sensitive patients' sera was a reliable factor for the evaluation of specific hypersensitivity to walnut and showed a significant titer of IgE-reactivity in ELISA and western blotting, compared with the controls. This finding was similar to the findings regarding the recombinant form of Jug 1 (15–16 kDa) from Brazil nuts, previously reported by Teuber and associates [27]. The recombinant Jug r 1 epitope mapping, confirmed that a linear 12 amino acid sequence of the large subunit of 2S albumin is responsible for strong IgE reactivity in walnut-sensitive patients [27]. This common epitope was also detected in pistachio, cashew, [33], and pecan, too [34]. Nuts sharing allergic proteins from other similar families, such as vicillins are also highly cross-reactive [35]. Allergy to certain well-defined mixtures of nuts may be due to the presence of similar or closely-related epitopes. Such epitopes are more common in phylogenetically related nuts. Therefore, the recombinant form of allergenic pro-



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**Figure 6.** Representation of structural properties and alignment of Persian walnut 2S albumin

A. Predicted ribbon structure of Jug r1 shows four alpha-helix and the possible IgE epitope is shown in blue color; B. The confidence score for estimating the quality of predicted models by I-TASSER based on the significance of threading template alignments and the convergence parameters of the simulations. The higher values point out to high confidence; C. Comparison of the amino acid sequence of Persian walnut 2S albumin with seed storage protein from other plants.

**Table 1.** Immunoreactivity of walnut proteins with allergic patients' sera

The Molecular Weight of the Reactive Band, kDa	Number of IgE-reactive Patients (Out of 18 Patients)	%
9	4	22
11	2	11
15	18	100
18	14	78
25	7	39
44	17	94
54	17	94

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**Table 2.** Mass spectrometry result for walnut 15 kDa protein

Protein Name/ Species	Peptide	Score	Expected Molecular Weight	Observed Molecular Weight	Amino Acid Start-End
2S albumin seed storage protein ( <i>Juglans nigra</i> )	RQQQQQLRGEEMEEMVQSARD	57	2361.0201	2362.0273	122-141
2S albumin seed storage protein ( <i>Juglans regia</i> )	RDLPKECGISSQRC	43	1388.6391	1389.6464	142-153

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tein can provide a suitable strategy for the detection and management of allergy.

Production of recombinant proteins which has more than one alpha-helix is rather difficult and the obtained protein is usually insoluble and shows lower functionality. Moreover, the refolding of the produced protein is usually not successful. Interestingly, in this study, we obtained a rather immunoreactive protein following step-wise refolding, demonstrating the appropriate refolding of the main epitopes. Although the refolded recombinant form showed a comparable immunoreactivity with native walnut 2S albumin, we did not compare their 3D structure through CD spectra analysis or UV spectrophotometry.

2S albumins have high structural stability that causes sensitization through direct absorption from the gastrointestinal tract, which classifies them as effective food allergens. Allergic reactions to 2S albumins from different plant sources, such as black walnut, Brazil nut, pecan, mustard, *Corylus*, and sesame show increasing frequency and should be further considered [15]. Therefore, the similarity between 2S albumin sequences of walnut and other plants could be distinguished using r2S albumin in order to improve prophylaxis and treatment of nut allergens.

We produced recombinant 2S albumin of walnut in the bacterial host and applied it for in vitro studies in the field of food allergy. We also showed that the refolded form of this protein could be a potent IgE-reactive molecule and due to its high amino acid homology with different edible allergenic plants could be a cross-reactive molecule. Comparison of the recombinant 2S albumin with its native form revealed a rather similar immunoreactivity, confirming that the recombinant form might be applied for evaluation of specific IgE reactions in the in vitro studies.

## Ethical Considerations

### Compliance with ethical guidelines

All experiments in this study conformed with guidelines from Research Ethics Committee of Iran University of Medical Sciences.

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### Authors' contributions

Conceptualization: Reza Falak, Mehran Gholamin; Methodology: Fereshteh Sadat Rasi Varae, Maryam Vakili Moghadam, Kobra Mokhtarian; Implementing the main experiments, sampling: Fereshteh Sadat Rasi Varae; Cloning steps: Fereshteh Sadat Rasi Varae, Kobra Mokhtarian; Purification of native protein steps: Kobra Mokhtarian; Bioinformatics analysis: Mohsen Mohammadi; Statistical analysis: Kobra Mokhtarian, Mohsen Mohammadi, Reza Jafari; Writing-original draft: Fereshteh Sadat Rasi Varae, Kobra Mokhtarian, Reza Jafari; Editing-final manuscript: Reza Jafari, Mehran Gholamin; Reading, approving the final manuscript: All authors.

### Conflicts of interest

The authors declared no conflict of interest.

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