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To cite this article: Sanaullah Khan, Syed Abid Ali, Tayyaba Yasmin, Mushtaq Ahmed & Hidayatullah Khan (2016): Purification and characterization of 2S albumin from *Nelumbo nucifera*, Bioscience, Biotechnology, and Biochemistry, DOI: [10.1080/09168451.2016.1158627](https://doi.org/10.1080/09168451.2016.1158627)

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Purification and characterization of 2S albumin from *Nelumbo nucifera*

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Received November 13, 2015; accepted January 12, 2016

<http://dx.doi.org/10.1080/09168451.2016.1158627>

The 2S albumins are a group of seed storage proteins that have recently attracted considerable attention in the field of allergen science due to their allergenic potential. A new 2S albumin from seeds of *Nelumbo nucifera* (Nn-2S alb) was purified to electrophoretic homogeneity by the combination of ammonium sulfate fractionation, gel filtration, and ion exchange chromatography. The protein has a molecular mass of about 12 kDa estimated by SDS-PAGE, in good agreement with 12.5 ± 0.01 kDa determined by ESI-MS. Circular dichroism data showed that protein contained about 66% α -helices as estimated by K2D3, indicating that the protein was predominantly helical. The sedimentation coefficient ($s_{20,w}^0$) of the predicted model was 1.72 ± 0.21 S. The predicted 3-dimensional structure of the Nn-2S alb revealed that the protein has a region of 12 amino acids which largely corresponds to the conserved immuno-dominant epitope of 2S allergens.

Key words: 2S albumins; purification; N-terminal amino acid sequence; CD spectroscopy; homology modeling

2S albumins are water-soluble storage proteins present in different seeds and nuts, and are defined on the basis of their sedimentation coefficient ($s_{20,w}^0$) value of 2.^{1,2} They belong to the superfamily of prolamins rich in sulfur containing amino acids with molecular mass of 12–15 kDa.^{2,3} They are typically heterodimer composed of 2 different polypeptide chains linked by 2 disulfide bonds (S–S), which are formed from post-translational proteolysis of a precursor protein.^{2,4–6} Napin is a well-characterized 2S albumin from oilseed rape, consisting of large and small subunits with molecular masses of 9 and 4 kDa, respectively, which are linked by interchain S–S bond.⁵ Some proteins such as 2S albumin from pea lack interchain S–S bonds and

exist as a single polypeptide. Although the 2S albumins differ in their subunit compositions and structures, their 3-dimensional structure is compact globular and enriched in α -helices.⁷

The physiological function of 2S albumins is not exactly known. However, it is believed that most of these proteins act as a nitrogen and sulfur donor based on their amino acid composition and their high contents in seeds and their mobilization during germination.^{8–11} Other activities such as antifungal activity, serine protease inhibition, and calmodulin antagonism have also been proposed for these proteins.^{11–16} In addition, this protein family has also attracted a significant research focus due to identification of several notable food allergens such as Sin a 1 and Bra j 1^{3,17} and due to their use for genetic engineering, as carriers for the synthesis of biologically active peptides.^{11,18}

Structural data are available for a number of 2S albumins and related proteins. These include napin, a 2S albumin from rape seeds,¹⁹ RicC3, from castor bean,¹¹ Ber e 1 from Brazil nuts²⁰ and a 2S albumin protein from sunflower seeds,²¹ bifunctional inhibitors (trypsin/ α -amylase inhibitor) from wheat and soya bean,^{22,23} and nsLTP from wheat,²⁴ maize,²⁵ barley,²⁶ and rice.²⁷ The characteristic features of 2S albumin proteins are the conserved fold 2S albumin and the 8 highly conserved cysteine residues (Fig. S1).

Given the fact that majority of these proteins (most plant allergens) can be classified into few protein families and superfamilies on the basis of sequence homology and conserved 3-dimensional structure,²⁸ it is becoming a great interest to purify and characterize these proteins to gain insight into their physiological role and many other biological activities. This study describes the purification and characterization of 2S albumin from seeds of *Nelumbo nucifera* (Nn-2S alb) which is an important edible plant, native to China, India, and Japan. Seeds and rhizomes of this plant have been widely used as traditional food in Asia.

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Abbreviations: Nn-2S alb, *Nelumbo nucifera* 2S albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MS, mass spectrometry; CD, circular dichroism; ESI-MS, electrospray ionization mass spectrometry.

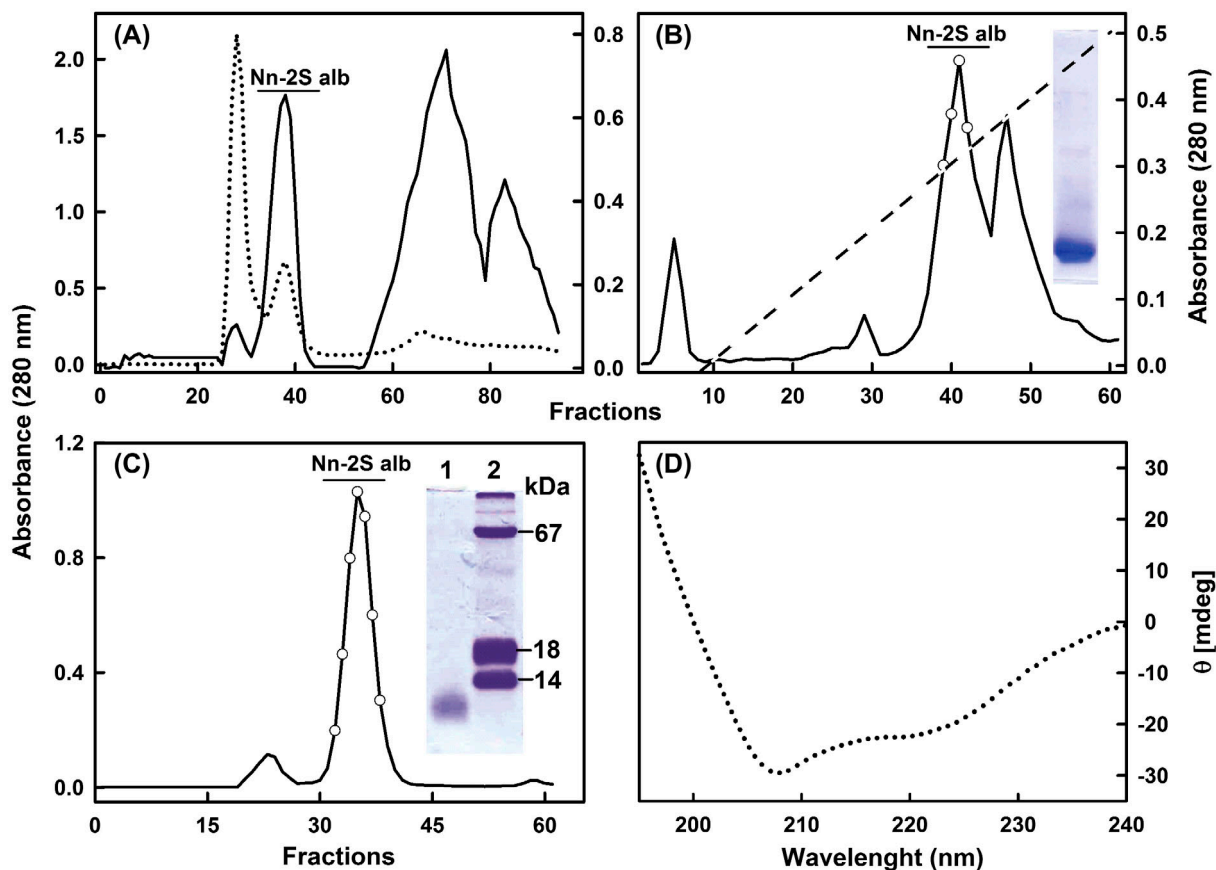


Fig. 1. Purification and CD analysis of Nn-2S alb.

Notes: (A) Elution profile of Nn-2S alb of ammonium sulfate precipitates on Sephadex G-50 column (with protein denaturation step: solid line, without denaturation: dotted line). (B) Elution profile of pooled fractions from peak 2 of Sephadex G-50 on DEAE-cellulose column. Elution was performed with a linear gradient of 0.0–0.25 M NaCl (dashed line). Inset, SDS-PAGE analysis of pooled fractions. (C) Elution profile of pooled fractions from peak 3 of DEAE-cellulose column on Sephadex G-50 column. Inset, SDS-PAGE (12%) of purified protein. Lane 1: purified Nn-2S alb, lane 2: protein standards. (D) Far-UV CD spectra of Nn-2S alb (0.5 mg/mL) in 10 mM Tris-HCl buffer pH 7.4.

Materials and methods

Seeds extraction. Proteins were extracted from 50 g of grinded seeds soaked in 500 mL of 10 mM Tris HCl buffer (pH 7.4) containing sodium azide (0.01%) for 24 h with constant stirring at 4 °C. The homogenate was filtered and centrifuged at 24,000 $\times g$ for 20 min at 4 °C.

Protein purification. The supernatant was evaporated to about 60 mL under reduced pressure at 30 °C, which resulted in insoluble precipitates of most high-molecular-weight proteins. The insoluble precipitates were removed by centrifugation at 24,000 $\times g$ for 20 min at 4 °C. For simplicity, we termed this step as protein denaturation step because around 85–90% of high-molecular-weight proteins were denatured. The proteins were then precipitated at 70% ammonium sulfate concentration with constant stirring at 4 °C. The saturated solution was kept at –20 °C for 30 min and the resulting precipitates were collected by centrifugation at 24,000 $\times g$ for 20 min. The precipitates were dissolved in 10 mM Tris HCl buffer pH 7.4. The dissolved 70% ammonium sulfate precipitates were applied to a Sephadex G-50 column (2.5 $\times$ 54 cm) that was pre-equilibrated with 10 mM Tris HCl buffer pH 7.4. Proteins were eluted with the running buffer at

flow rate of 12 mL/hr. The pooled fractions (opened circles) from the second peak of Sephadex G-50 were applied to a DEAE-cellulose column (2.5 $\times$ 24 cm) that was pre-equilibrated with 10 mM Tris HCl pH 7.4. The bound proteins were eluted with linear gradient of 0–0.25 M NaCl in the column buffer at flow rate of 24 mL/hr. Fractions from third peak were pooled and analyzed by SDS-PAGE. The SDS-PAGE showed that the Nn-2S alb contained trace amount of high-molecular-weight proteins, this partially purified Nn-2S alb that was re-loaded onto the same Sephadex G-50 column. This time, 50 mM NaCl was added in running buffer to reduce the interactions between gel matrix and the protein. For ESI-MS and protein sequence analyses, the protein was further purified by HPLC. The purified Nn-2S alb was then subjected to ESI-MS for molecular mass determination and for N-terminal sequencing using Edman degradation (supplemental materials).

Results and discussion

Protein purification and SDS-PAGE analysis

The protein was purified by combined use of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. The protein denaturation step under reduced pressure at 30 °C resulted in the

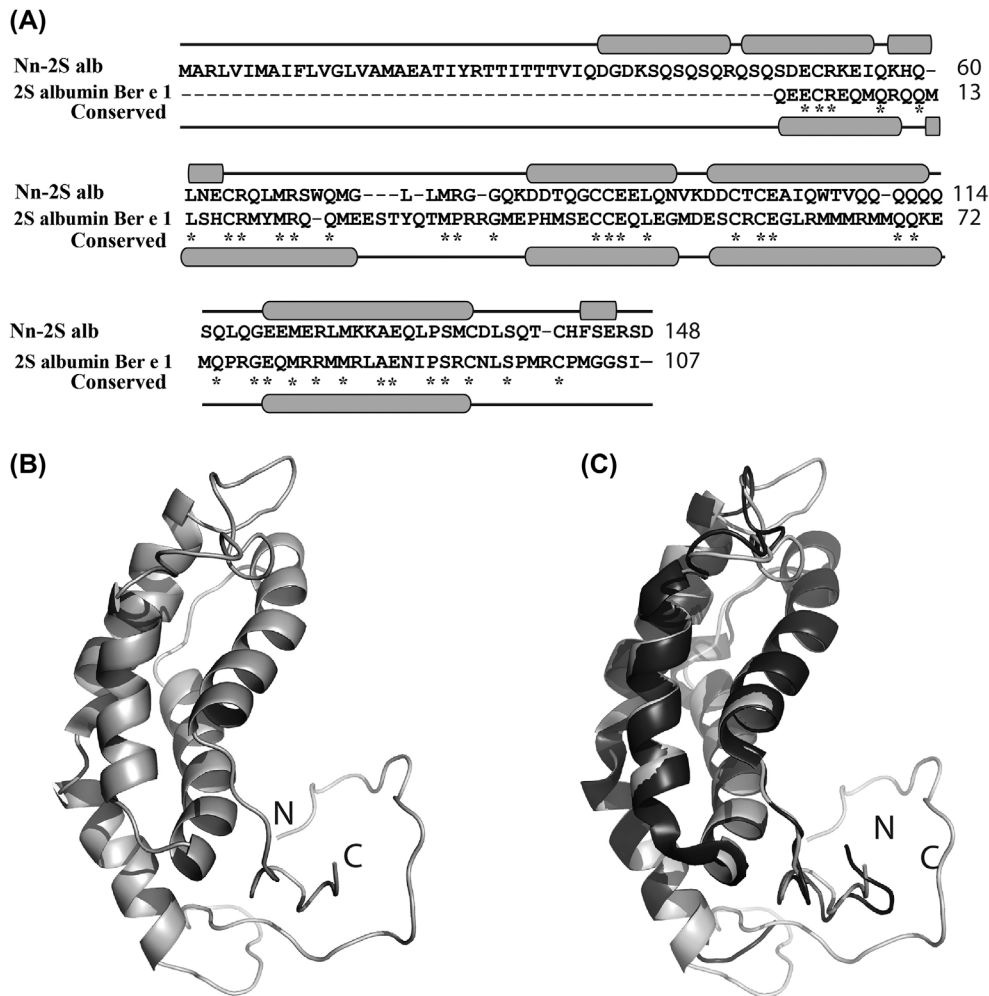


Fig. 2. Protein sequence alignment and predicted 3D structure of Nn-2S alb.

Notes: (A) Sequence alignment and structural conservation of Nn-2S alb and Brazil nut allergen Ber e 1. The secondary structural elements are shown above for Nn-2S alb and below for Ber e 1. (B) Predicted 3D structure of Nn-2S alb. (C) Superimposition of predicted model of Nn-2S alb (light gray) with Ber e 1 (dark gray).

removal (~90%) of high-molecular-weight proteins. The gel filtration profile of proteins with and without denaturation step is shown in Fig. 1(A). The intensity of first peak was greatly decreased compared to the first peak of the proteins without denaturation step, indicating that most of the high-molecular-weight proteins were removed. Fractions (open circles) from the peak 2 were pooled and applied to a DEAE cellulose column. Fractions corresponding to each individual peak were pooled and subjected to SDS-PAGE for the Nn-2S alb purification analysis (Fig. 1(B)). The Nn-2S alb was found in third peak. Fractions (open circles) from peak 3 of DEAE cellulose columns were analyzed by SDS-PAGE, which showed that these fractions contained some traces of high-molecular-weight proteins (Fig. 2(B), inset). These fractions were re-applied to the same Sephadex G-50 and were eluted with column buffer containing 50 mM NaCl to reduce the interactions between matrix and protein, which resulted in 1 major and 1 minor peak (Fig. 1(C)). The first peak contained traces of high-molecular-weight proteins, while the second peak contained pure Nn-2S alb. These steps yielded about 3.3 mg purified proteins from 120 mg crude proteins. The molecular mass of the purified protein was

estimated to be 12 kDa by SDS-PAGE under non-reducing condition (12%) (Fig. 1(C), inset). The purified protein in the presence of reducing agent (2-mercaptoethanol) runs 2 bands of ~8–9 kDa and ~3–4 kDa on SDS-PAGE, suggesting that polypeptides are linked together by disulfide bonds (Fig. S2). The molecular mass was lower than molecular mass derived from the sequence retrieved from BLAST, which may indicate that the sequence contains some amino acids that were cleaved off after its synthesis.

ESI-MS and amino acids sequence analyses

Ion spray mass spectrum of the purified protein gave 5 major peaks at m/z values of 1039.4, 1132.1, 1245.2, 1383.3, and 1555.8 (Fig. S3). The purified Nn-2S alb revealed a mono-isotopic molecular mass of 12.5 ± 0.01 kDa which is in excellent accord with molecular mass estimated by SDS-PAGE. The mono-isotopic molecular mass of Nn-2S alb was calculated according to a method described previously.²⁹ Partial N-terminal amino acid sequence of the protein was examined and 20 residues were determined. The amino acids sequence determined were GGQKDDTQGXREELQNVKDEXT, where X = not determined.

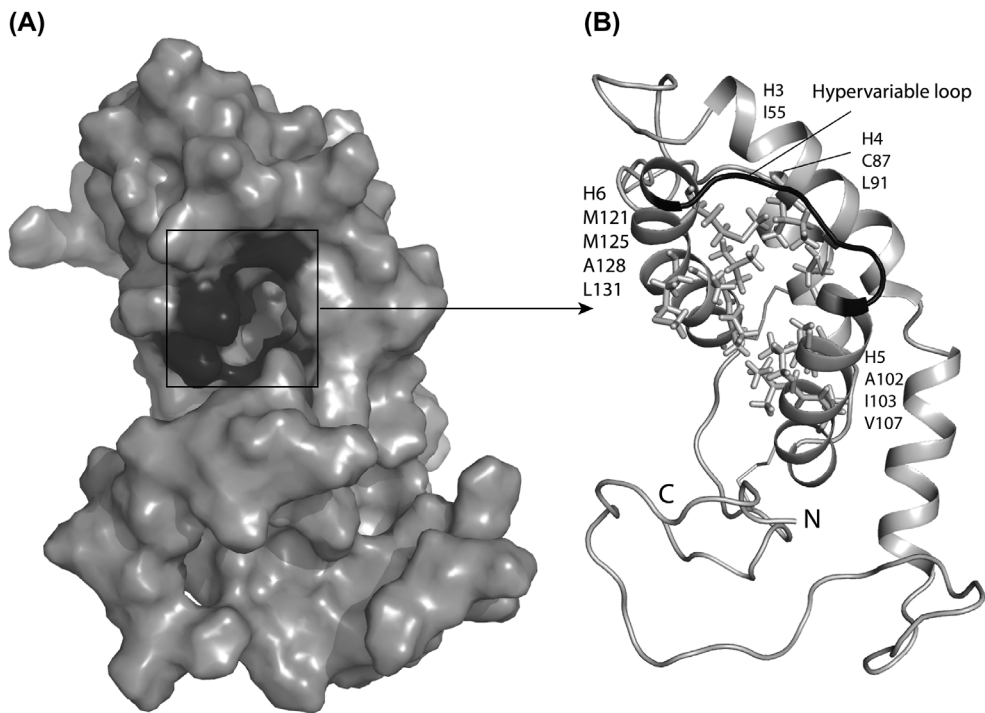


Fig. 3. Surface and ribbon representations of Nn-2S alb.

Notes: (A) Surface representation of the predicted model of Nn-2S alb, showing hydrophobic cavity in dark gray. (B) Ribbon representation of the Nn-2S alb model, showing helices (light gray), the hypervariable region (dark gray), and the amino acids residues (sticks) that constitute hydrophobic cavity.

Protein BLAST search of the sequence produced a top hit with 82% (excluding X) sequence similarity to the “predicted 2S sulfur-rich seed storage protein 2-like” from the same source (XP_010267886.1), indicating that protein is 2S albumin protein. The positions “X” corresponded nicely with cysteines of the predicted proteins which makes sense since cysteines are not identified in Edman degradation experiments due to their instability.

CD and sedimentation coefficient analyses

Secondary structural elements of purified Nn-2S alb were estimated by circular dichroism (CD) spectroscopy. Far-UV CD spectra for purified Nn-2S alb were recorded at concentrations of 0.3 mg/mL in 10 mM Tris HCL buffer pH 7.4. The spectra in Fig. 1(D) displayed 2 negative minima centered at 208 and 222 nm which is the characteristic of α -helical structure.³⁰ The protein contained about 66% α -helical contents as estimated by K2D3, which is consistent with the predicted Nn-2S alb structure, and the helical contents of template structure. The sedimentation coefficient $s_{20,w}^{\circ}$ calculated for Nn-2S alb model was 1.72 ± 0.21 S using HYDROPRO shell modeling program,³¹ which was in good agreement with s values reported for 2S albumins from other sources.³² The radius of gyration (R_G) value of the protein was 1.88 nm calculated by HYDROPRO program.

Homology-based model of Nn-2S alb

Blast search for amino acids sequence of the Nn-2S alb was run against a sequence database of PDB

templates. The search revealed 37% sequence identity with a recombinant 2S albumin from Brazil nut.²⁰ Brazil nut allergen Ber e 1 is a 12 kDa sulfur-rich protein composed of 2 polypeptides of 3 and 9 that are associated through disulfide bonds. The recombinant Ber e 1 was expressed as a single chain, similar to native protein (without removing the linker peptide between the subunits). Homology model for Nn-2S alb was constructed using the atomic coordinates of Ber e 1 as a template, assuming that the linker peptide between the 2 polypeptides of Nn-2S alb was not removed. The overall predicted 3D structure of Nn-2S alb was predominantly helical, composed of 7 α -helices: H1 (Asp24-Arg44), H2a (Gln47-Ile55), H2b (Lys57-Glu62), H3 (Asp82-Asn93), H4 (Asp96-Gln112), H5 (Glu119-Cys135) and H6 (Phe143-Glu147) (Fig. 2(A)), which is consistent with CD data. The template structure is also helical, composed of 5 α -helices, stabilized, and connected by 4 S-S bonds (Fig. 2(B)).²⁰ Superimposition of predicted Nn-2S alb model with structural allergen Ber e 1 showed that the helices of the Nn-2S alb were well matched but turns and loops of the predicted 3D structure showed slight structural deviations (Fig. 2(C)). The root mean square deviation was 0.56 Å, and all the residues of Nn-2S alb were in allowed region as validated by the program PROCHECK.³³

Six out of eight conserved cysteine residues form 3 S-S bridges between Cys51–Cys87, Cys88–Cys135, and Cys100–Cys141. These S-S bonds that connect H2 to H3, H3 to H5, and H4 to the loop between H4 and H6, stabilize further the protein 3D structure. Like most other members of 2S albumin family and related proteins, the main compact hydrophobic core that also

defines the global fold of 2S albumins is mainly surrounded by 4 helices (H2, H3, H4, and H5) (Fig. 3). The amino acid residues that constitute the hydrophobic core of the protein are shown in Fig. 3(B). The hydrophobic cavity of Nn-2S alb is analogous to the hydrophobic cavity of Ber e 1 and more interestingly to the lipid binding site in the non-specific lipid transfer protein (ns-LTP) reported from wheat,³⁴ revealing to accommodate large and small hydrophobic part of immuno-active lipid (Fig. 3(A)). The loop (Gln113-Gly118) in Nn-2S alb, that connects H5 to H6, corresponds to hypervariable region and is highly exposed (dark gray, Fig. 3(B)). This region is known to all 2S albumin proteins and is the immuno-dominant part of 2S albumins.^{7,20,35,36} The 2S albumins contain regions of 12–14 amino acids that are characterized as linear immuno-dominant epitopes. These include 2S albumin Jug r 1 allergen (QGLRGEEMEEMV), pecan nut Car i 1 allergen (EGIRGEEMEEMV), 2S albumin Ber e 1 allergen (EM(Q)PRGEQMRRMM), and castor bean Ricc 3 allergen (GQLHGEESEERVA).³⁷ The predicted Nn-2S alb model contains a region of 12 amino acids (SQLQGEEMERLM) on its molecular surface, which shared pronounced sequence and structural homology with the corresponding regions of most of these plant allergens. This region also contains hypervariable loop (Fig. 3(B)), thus predicting that this region of Nn-2S alb could be a linear immuno-dominant epitope and may account for cross reactivity between Nn-2S alb and other plant allergens.

Conclusion

This study described the purification and characterization of a new 2S albumin from seeds of *N. nucifera*. This study revealed that (1) the protein can be purified to electrophoretic homogeneity with high yield by simple chromatographic methods, (2) the predicted protein structure has a global 2S albumin fold that is identical to other members of the family, (3) the predicted protein structure has hydrophobic cavity that is analogous to 2S albumin proteins and to the lipid binding site in the non-specific lipid transfer protein, (4) the predicted protein structure has a region of 12 amino acids that shared sequence and structural homology to linear immuno-dominant epitopes of most characterized plant allergens, thus predicting cross reactivity between the Nn-2S alb and most other plant allergens. Further studies are needed to elucidate their antigenic and allergenic properties.

Author contributions

Sanaullah Khan designed and performed experiments, analyzed data and wrote the paper; Syed Abid Ali designed experiments and analyzed ESI-MS and amino acids sequence data; Tayyaba Yasmin, Mushtaq Ahmed and Hidayatullah Khan designed and performed experiments.

Supplemental materials

The supplemental material for this paper is available at <http://dx.doi.org/10.1080/09168451.2016.1158627>.

Acknowledgments

We are very thankful to Dr. Sajida Batool for reading of the manuscript and helpful suggestions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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