

Identification of a vicilin-like major allergen from *Prosopis juliflora* exhibiting cross-reactivity with legume food allergens

Bharti Arora^{a,b}, Swati Sharma^{a,b}, S.N. Gaur^c, Vikram K. Jain^d, Shakuntala Lavasa^e, Naveen Arora^{a,*}

^a Allergy and Immunology Laboratory, CSIR-Institute of Genomics and Integrative Biology, Mall Road, New Delhi, India

^b Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India

^c Department of Pulmonary Medicine, V.P. Chest Institute, University of Delhi, New Delhi, India

^d Department of Respiratory Medicine, Mahatma Gandhi Medical College, Jaipur, India

^e US Lavasa Medical Clinic, Chandigarh, India

ARTICLE INFO

Keywords:

Prosopis juliflora

Glycoprotein

Cross-reactivity

Legumes

ABSTRACT

Background: *Prosopis juliflora* is a clinically relevant allergic sensitizer worldwide and shares cross-reactivity with allergens from several tree pollen and food. The present study aims to purify and immunobiochemically characterize a major allergen from *Prosopis* pollen. The allergen was further investigated for its cross-reactivity with legume allergens.

Methods: *Prosopis* extract was fractionated by Q Sepharose and Superdex 75 gel filtration column to purify the allergen. Specific IgE against purified protein was estimated via ELISA and immunoblot. The protein was subjected to mass spectrometric analysis. Glycan characterization was performed by Schiff staining and lectin binding assay followed by deglycosylation studies. The functional activity of the purified protein was evaluated by the basophil activation test. Cross-reactivity was assessed by inhibition studies with legume extracts.

Results: A 35 kDa protein was purified and showed 75% IgE reactivity with the patients' sera by ELISA and immunoblot. Glycan characterization of protein demonstrated the presence of terminal glucose and mannose residues. A reduction of 40% and 27% in IgE binding was observed upon chemical and enzymatic deglycosylation of the protein, respectively. The glycoprotein allergen upregulates the expression of CD203c on basophils which was significantly reduced upon deglycosylation, signifying its biological ability to activate the effector cells. The identified protein shared significant homology with Lup an 1 from the lupine bean. Immunoblot inhibition studies of the purified allergen with legume extracts underlined high cross-reactive potential. Complete inhibition was observed with peanut and common bean, while up to 70% inhibition was demonstrated with soy, black gram, chickpea, and lima bean.

Conclusion: A 35 kDa vicilin-like major allergen was isolated from *P. juliflora*. The protein possesses glycan moieties crucial for IgE binding and basophil activation. Furthermore, the purified protein shows homology with Lup an 1 and exhibits cross-reactivity with common edible legume proteins.

1. Introduction

Prosopis juliflora is a xerophytic tree native to Central America with a widespread occurrence in tropical and subtropical regions (Singh and Kumar, 2003; Hussain et al., 2020). It belongs to Fabaceae (Leguminosae) family and exhibits an almost year-round flowering season. Its tremendous invasive capacity and high pollen burden are considered important factors in eliciting allergic reactions, including respiratory pollinosis, rhinitis, and rhino-conjunctivitis (Hussain et al., 2020). In

India, skin test positivity for *P. juliflora* among bronchial asthma and rhinitis patients was observed as 10% and 56% in semi-arid (Delhi) and arid regions (Bikaner), respectively. An extensive repertoire of 16 allergenic proteins was identified in *P. juliflora* of which an Ole e 1-like trypsin inhibitor protein (Pro j 1) and a profilin allergen (Pro j 2) were characterized (Dhyani et al., 2006; Dousti et al., 2016; Ali-Sadeghi et al., 2015). Earlier, a 66 kDa allergen from *Prosopis* exhibited humoral and cell-mediated cross-reactivity with allergens from predominating tree pollen species and lima bean (Dhyani et al., 2007; Dhyani et al., 2008).

* Corresponding author at: Allergy and Immunology Division, CSIR-Institute of Genomics and Integrative Biology, Mall Road Campus, New Delhi, 110007, India.
E-mail address: naveen@igib.res.in (N. Arora).

<https://doi.org/10.1016/j.molimm.2021.06.023>

Received 28 January 2021; Received in revised form 27 May 2021; Accepted 29 June 2021

Available online 6 July 2021

0161-5890/© 2021 Elsevier Ltd. All rights reserved.

Factors including phylogenetic relatedness among sensitizing species, homology in IgE binding epitopes, pan allergen families and presence of glycosylations have been implicated in cross-reactivity among pollen sensitizers and their subsequent homologs in plant-derived sources (Biedermann et al., 2019). Allergens from PR-10, profilin, and cupin family have been shown to induce cross-reactivity among homologous allergens from pollen and food sensitizers (Asam et al., 2015; Fernández-González et al., 2020; Smeekens et al., 2018). Major allergen from peanut, Ara h 1 cross-reacts with homologous legume allergens from several nuts (walnut, hazelnut, sesame) and soy (Cabanillas et al., 2018). The presence of carbohydrate moieties on allergens has also been demonstrated to influence sensitization patterns (Perusko et al., 2018; Ruan et al., 2013; Zhang et al., 2019), although their exact role in influencing cross-reactivity among pollen and food sensitizers is elusive.

Climate change has been recognized as an important factor responsible for novel sensitizations to previously unencountered allergen sources. With rising temperatures across the globe, pollen allergen sources native to tropical regions have gained clinical relevance world over. Recently, analysis of species distribution and climatic factors highlighted *Prosopis* (owing to its inherent efficiency in colonizing habitats) as a major invasive species with a projected massive territorial spread in Africa, North America, and Southern Europe by 2050 (Heshmati et al., 2019). Given the geographical diversity in prevalent pollen sensitizers and food sources, a molecular investigation of cross-reactive allergens is crucial. The present study is aimed at identification and characterization of a major *P. juliflora* allergen with an emphasis on its cross-reactivity with commonly consumed food sources, aiding accurate diagnosis, effective dietary restriction, and developing immunotherapy regimens.

2. Materials and methods

2.1. Study subjects

The present study included 20 patients of either sex (M/F), suffering from one or more of the symptoms like bronchial asthma, nasobronchial allergy, or allergic rhinitis. The diagnosis of asthma and rhinitis was ascertained following the guidelines by the American Thoracic Society (1991) and Allergic Rhinitis and its Impact on Asthma. Based on the patient history skin prick test (scratch test) with speculated allergens was carried out and histamine diphosphate and PBS in 50% glycerin were used as positive and negative controls, respectively. SPT reactions were observed and recorded after allergen administration and the diameter of the wheal was graded with respect to the reaction observed in positive control. Sera were provided by the laboratory and were stored at -70°C until further use.

2.2. Pollen extract preparation

Polleniferous inflorescence of *Prosopis juliflora* was collected from the field during the peak flowering season i.e., end week of March, and was freeze-dried. The pollen was separated from other plant parts by passing it through 300, 200, and 100-micron mesh. The processed pollens were observed under a microscope and purity percentage was calculated. Pollen material with > 95% purity was used for protein extract preparation. Fine pollen powder was defatted with 4–5 times changes of diethyl ether at 4°C . Protein extract was prepared using defatted pollen in 50 mM ammonium bicarbonate buffer pH 8.0, 1:20 (w/v) ratio containing 1 mM PMSF and 2 mM EDTA with continuous stirring for 6 h. at 4°C . The extract was centrifuged at 13,000 rpm for 30 min, the supernatant was filtered through a $0.45\ \mu\text{m}$ filter and then dialyzed against water. The protein concentration of the pollen extract was determined by Bicinchoninic acid assay (BCA). The protein extract was then lyophilized and stored at -70°C until use.

2.3. IgE specific immunoblotting

Specific IgE blot was performed using sera from SPT and specific ELISA positive *Prosopis* hypersensitive patients ($n = 20$). Purified protein was resolved on SDS-PAGE gel and electrophoretically transferred onto the nitrocellulose (NC) membrane. The membrane was blocked with 3% BSA in PBS for 2 h. at RT. Following three PBST washing it was incubated with sera (1:20 v/v in 0.5% BSA in PBS) overnight at 4°C . Following PBST washes, blotted strips were incubated for 1 h. at 37°C with horseradish peroxidase-conjugated anti-human IgE (1:5000 v/v in 0.5% BSA in PBS; Sigma-Aldrich) and the reaction was developed with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualized by chemiluminescence using ChemiDoc Imaging Systems (Bio-Rad). Sera from non-allergic subjects were used as control sera (normal human sera; NHS).

2.4. Purification of major IgE binding component from *P. juliflora*

To obtain protein up to homogeneity, *Prosopis* protein extract was fractionated by two chromatography steps using pre-packed chromatography columns on the BioLogic DuoFlow chromatography system (Bio-Rad). Briefly, 20 mg of protein extract resuspended in 20 mM Tris base, 20 mM NaCl, pH 8.0 buffer was loaded onto 5 mL Hi Trap Q Sepharose column (GE Life Sciences) initially equilibrated with Tris-NaCl buffer. Elution of the bound protein was done using a step-wise salt gradient using 0–0.5 M NaCl. The protein fraction resolved into four peaks corresponding to four fractions. The concentration of the fractions obtained was measured using the BCA method. The IgE binding ability of the fractions was assayed using *Prosopis* hypersensitive patients' sera. The fraction showing major IgE binding was further subjected to SuperdexTM 75 10/300 G L column (GE Life Sciences) (column volume 24 mL) previously equilibrated with. The partially purified protein was further resolved by isocratic elution with Tris-NaCl buffer (equilibration buffer); fraction volume 0.5 mL at a flow rate of 0.1 mL/min. Each of the peaks obtained was pooled separately and analyzed for its IgE binding activity by immunoblot. The fraction containing purified protein with maximum IgE binding activity were pooled, concentrated, and was further studied for immunobiochemical characterization. Protein concentration was determined by the Bicinchoninic acid (BCA) assay.

2.5. Gel electrophoresis

Reducing gel electrophoresis of pollen extract as well as protein fractions obtained following ion exchange and gel filtration chromatography was carried out on 12.5% polyacrylamide gel. The protein samples were mixed with reducing sample buffer and were heated at 90°C for 10 min. The protein bands on the gel were visualized by silver staining.

2.6. Carbohydrate detection

To detect the presence of carbohydrate moiety in the purified protein, the protein was subjected to periodic acid Schiff (PAS) staining. Briefly, the purified protein was resolved on 12.5% SDS-PAGE and transferred to the nitrocellulose (NC) membrane. Phytohemagglutinin (PHA; Sigma-Aldrich) was used as a positive control, while recombinant protein of *Periplaneta americana* was used as a negative control. The membrane was incubated in 1% periodic acid (w/v) (Sigma-Aldrich) in 3% acetic acid (v/v) (Sigma-Aldrich) for 15 min at RT in dark. Following this NC membrane was incubated in Schiff stain (Sigma-Aldrich) for 15 min in dark. The reaction was stopped by incubating the membrane in a 5% sodium metabisulfite (Sigma-Aldrich) solution. The membrane was rinsed with water to detect the pink color protein band confirming the presence of glycan moiety.

2.7. Identification of glycan moiety

The type of carbohydrate residues of the glycan structure was identified by the affinity detection method using *Concanavalin A* (Con A) lectin (Sigma-Aldrich) as per manufacturer instructions. Briefly, the purified protein was electrophoretically transferred to the NC membrane. After incubating membrane in TBST (TBS; 0.5% tween-20) for 1 h, it was incubated with biotin-conjugated *Concanavalin A* lectin (10 µg/mL) in Con A buffer (TBST, 1 mM MgCl₂, 1 mM MnCl₂) for 1 h, at RT. For specific binding control, the blotted membrane was incubated in Con A buffer containing 0.3 M α -methyl mannoside (Sigma-Aldrich) (Con A inhibitor). Ovalbumin (Sigma-Aldrich) from hen egg white was used as a positive control. Following this membrane was washed with TBST, and further incubated with streptavidin-HRP (Sigma-Aldrich) for 1 h, at RT. After washing the membrane with TBST, the reaction was developed with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualized by chemiluminescence using ChemiDoc Imaging Systems (Bio-Rad).

2.8. Specific IgE ELISA

To assess the specific IgE binding ability of *Prosopis* pollen extract and purified protein, a specific IgE ELISA was performed. Briefly, 0.1 µg of purified protein and 1 µg of *Prosopis* extract in 100 µL of carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃) pH 9.6 were coated in 96 well microtiter plate and kept overnight at 4 °C. Following incubation each well was blocked with 200 µL of 3% BSA in PBS for 3 h, at RT and further incubated with 100 µL of individual *Prosopis* hypersensitive patients' sera (1:10 v/v in 0.5% BSA in PBS) overnight at 4 °C. Following PBST washing, wells were incubated with horseradish peroxidase-conjugated anti-human IgE (Sigma-Aldrich) (1:1000 v/v in 0.5% BSA in PBS) and then developed using 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) as a substrate. The reaction was stopped using 2 N H₂SO₄ and absorbance was measured at 490 nm using Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, USA). Sera with OD values at 490 nm greater than three times that of OD value of control sera (Sera samples from non-allergic individuals were used as negative controls, also referred to as normal human sera or NHS) was considered positive.

2.9. Periodate oxidation

To determine the role of the glycan moiety in IgE binding, allergen was subjected to chemical de-glycosylation. NC membrane with blotted purified protein was incubated with 20 mmol/L sodium meta periodate in 50 mmol/L sodium acetate (pH 4.5) for 2 h. The reaction was carried out in dark and cold conditions to prevent protein oxidation by periodate. The reaction was stopped by adding a drop of ethylene glycol. The membrane was further incubated with 1 M sodium borohydride for 1 h, and the reaction was stopped by adding a drop of acetic acid. Following washing with PBST several times, the membrane was subjected to immunoblotting assay as described above. The extent of de-glycosylation was assessed by lectin binding assay using Con A lectin binding assay as described above. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualized by chemiluminescence using ChemiDoc Imaging Systems (Bio-Rad). The relative change in the intensity of deglycosylated protein bands with respect to the intensity of the native purified protein band in the immunoblot was quantified using Alpha Imager 3400 (Alpha Innotech Corporation, San Leandro, CA, USA).

2.10. Enzymatic deglycosylation

Purified protein was also subjected to enzymatic deglycosylation using PNGase A enzyme as per manufacturer instructions (Sigma-Aldrich). Briefly, 1 µg of protein was combined with glycol denaturing buffer and was denatured at 100 °C for 10 min. The denatured

glycoprotein was chilled on ice and was centrifuged for 10 s. To this glycol buffer 3 and 10% NP-40 (provided in kit) were added to make up the required reaction volume. The following reaction mixture was incubated with 1 unit of PNGase A enzyme for 1 h at 37 °C. The extent of deglycosylation was confirmed by lectin binding assay as described above. The deglycosylated protein was electrophoretically transferred onto the NC membrane and was immunoblotted to assess the IgE binding with patients' sera as described above. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualized by chemiluminescence using ChemiDoc Imaging Systems (Bio-Rad). The relative change in the intensity of deglycosylated protein bands with respect to the intensity of the native protein band in the immunoblot was quantified using Alpha Imager 3400 (Alpha Innotech Corporation, San Leandro, CA, USA).

2.11. Basophil activation test

A Basophil activation test was employed to study the functional relevance of purified allergen (GLY-PP) and its deglycosylated form (deGLY-PP) using a passive sensitization protocol (Santos et al., 2015). Briefly, PBMCs were isolated from healthy volunteers (n = 6) using Histopaque (Sigma-Aldrich). These were resuspended in lactic acid solution (13.4 mM Lactic acid, 140 mM NaCl, and 5 mM KCl) for 5 min at 4 °C to strip receptor-bound IgE. The reaction was stopped by the addition of RPMI media containing 0.25% Human serum albumin (HSA, Sigma-Aldrich) and washed with the same media. The cell pellet was resuspended in HSA supplemented RPMI media and incubated with pooled serum samples of *Prosopis* hypersensitive patients (n = 9) for 1 h, at 37 °C. After washing, the re-sensitized PBMCs were stimulated with a purified allergen (250 ng) and its deglycosylated form (by PNGase A method) for 30 min at 37 °C. Anti-human IgE (Sigma-Aldrich) at dilution of 1:100 was used as positive control while the unstimulated samples (US) served as a negative control. The cells were washed and stained with PE anti-human CCR3 and APC-anti human CD203c (BD Pharmingen). Basophils were selected as CCR3+ cells. Basophil activation was expressed as an increase in CD203c levels in CCR3+ basophils. Relative fold change in MFI in basophil activation is expressed as an increase in CD203c levels in CCR3+ basophils. The data is presented in graphical form depicting fold change in MFI as average $\pm$ SEM (p < 0.01).

2.12. Mass spectrometry

2.12.1. In gel trypsin digestion

To determine the identity of purified protein, it was subjected to mass spectrometric analysis. Briefly, 5 µg of purified protein was resolved on SDS-PAGE gel. The protein band stained with Coomassie brilliant blue was excised off from the gel, diced to small pieces, and was de-stained using ammonium bicarbonate: acetonitrile (1:1) until the gel pieces became translucent white. The gel pieces were dehydrated using acetonitrile and dried in a speed vac. They were then incubated for an hour with dithiothreitol (DTT) for rehydration. After removal of DTT, the gel was further incubated with iodoacetamide (IDA) for 45 min. The gel was further incubated with ammonium bicarbonate solution for 10 min followed by dehydration with acetonitrile for 10 min and was dried in speedvac. Gel pieces were incubated with trypsin solution (Sigma-Aldrich) 0.3 µg (20 ng/µL) overnight at 37 °C. The digested solution was transferred to fresh eppendorf tubes. The gel pieces were extracted thrice with extraction buffer (Acetonitrile and 0.1% Trifluoroacetic acid = 1:1 ratio) and the supernatant was collected each time into the eppendorf above and was speedvac.

2.12.2. MALDI analysis

The dried peptide mix was suspended in TA buffer (Acetonitrile and 0.1% Trifluoroacetic acid = 1:2 ratio). The peptides obtained were mixed with HCCA matrix in 1:1 ratio and the resulting 2 µL sample was spotted onto the MALDI plate. After air-drying the sample, it was

analyzed on the MALDI TOF/TOF MS BrukerDaltonics ULTRAFLEX III instrument and further analysis was done with Flex Analysis software. The masses of singly charged monoisotopic peptides obtained in the peptide mass fingerprint were submitted for Mascot search in the “CONCERNED” database for identification of the protein. Searches were performed against pollen proteins database using MASCOT Peptide Mass Fingerprint database search software (www.matrixscience.com). The possible modifications included were oxidation of methionine and alkylation of cysteines. Up to one missed tryptic cleavages was considered, and the mass tolerance for the monoisotopic peptide masses was set to ± 1.66 Da.

2.13. Immunoblot inhibition

To determine the cross-reactive potential of the purified protein, immunoblot inhibition was performed using six legume extracts. Briefly, 150 ng of the protein was resolved on SDS-PAGE and was transferred to the NC membrane. After blocking the membrane with 3% BSA in TBS for 2 h at room temperature, the membrane was probed with *Prosopis* hypersensitive patients' sera (1:50 v/v) inhibited with the following legume extracts overnight (500 μ g/mL): *Phaseolus vulgaris*, *Glycine max*, *Arachis hypogaea*, *Phaseolus lunatus*, *Cicer arietinum*, and *Vigna mungo*. Patient sera without inhibitor served as positive control and control sera (normal human sera) as a negative control. Following overnight incubation, the membrane was washed four times with TBST (TBS and 0.1% tween 20) and incubated with HRP- conjugated anti-IgE (1:8000 v/v; Sigma-Aldrich) for 30 min at RT and was developed as described earlier. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualized by chemiluminescence using ChemiDoc Imaging Systems (Bio-Rad). The relative change in the intensity of protein bands probed with respective inhibited sera with the extracts was quantified with respect to the intensity of protein band probed with un-inhibited sera using Alpha Imager 3400 (Alpha Innotech Corporation, San Leandro, CA, USA).

3. Results

3.1. Purification of *Prosopis* allergen

Prosopis extract was subjected to a strong anion exchanger. Protein elution was accomplished with a stepwise gradient of Tris-NaCl (0–0.5 M). The protein fractions obtained were analyzed for IgE binding using *Prosopis* sensitized patients' sera. Out of the four peaks obtained, protein fraction eluted at 0.3 M salt concentration yielded maximum IgE binding in immunoblot. This fraction was further resolved into three different peaks by the Sephadex column. The eluted peak with maximum IgE binding showed a protein band at 35 kDa molecular weight on reducing gel electrophoresis (Fig. 1).

3.2. Allergenicity assessment of purified protein

3.2.1. Specific IgE ELISA and immunoblot assay

Specific IgE reactivity of *Prosopis* extract and purified allergen with *Prosopis* hypersensitive patients' sera was assessed using specific IgE ELISA in subjects (n = 20) showing positive SPT to *Prosopis* extract. The serum IgE reactivity against *Prosopis* extract in terms of OD at 490 nm was from 0.059 to 0.1; cut-off set more than three times that of control sera, OD:0.018 [Fig. 2A(a)]. Fifteen out of twenty patients (75%) showed elevated IgE reactivity (OD 0.010 to 0.109; control sera: OD at 490 nm— 0.018) against the purified protein [Fig. 2A(b)]; Table 1). IgE binding of 35 kDa protein was further evaluated by immunoblot studies using individual *Prosopis* hypersensitive patient's sera (n = 20). Immunoblotting confirmed that 15 out of 20 ELISA positive patients exhibited IgE reactivity to the purified protein, demonstrating it to be a major allergen (Fig. 2B).

To eliminate the effect of non-specific sIgE reactivity, due to the

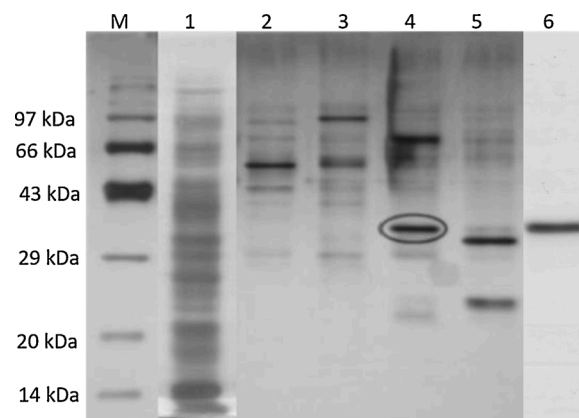


Fig. 1. SDS-PAGE profile of protein elutes fractionated by ion-exchange chromatography and gel filtration chromatography. Lane M: Protein marker, Lane 1: *Prosopis* extract, Lane 2-5: Protein fractions obtained after anion-exchange chromatography with a respective salt gradient of 10%, 20%, 30% and 40%, Lane 6: Protein fraction obtained after gel filtration Chromatography exhibiting maximum IgE reactivity.

presence of anti-CCD antibodies presented in patient's serum, we used an adsorption protocol incubating *Prosopis* sensitized patient sera with known marker and inhibitors of CCD epitopes Horseradish peroxidase (HRP) and Bromelain (Brl). Immunoblotting assay was then conducted with serum inhibited with HRP and Brl. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, USA) and visualized by chemiluminescence using ChemiDoc Imaging Systems (Bio-Rad). The relative change in the intensity of protein bands probed with HRP and Brl inhibited sera was quantified with respect to the intensity of protein band probed with un-inhibited sera using Alpha Imager 3400 (Alpha Innotech Corporation, San Leandro, CA, USA). The immunoblot images and reduction in the intensities of the purified protein bands probed with inhibited sera (in percentage) are presented in (Supplementary Fig. 1).

3.2.2. Detection and identification of glycan moiety

The cis-diol groups of glycan moiety of purified protein were oxidized by periodic acid and reacted with Schiff reagent to form a magenta color insoluble protein band, identifying it to be a glycoprotein (Fig. 3A).

The purified protein blotted on membrane-bound to *Concanavalin A* (Con A), thereby confirming the presence of terminal mannose and glucose residues in it. The absence of protein band in the membrane strip incubated with α -methyl mannoside along with biotin-Con A confirmed that the binding of Con A to the protein is glycan-specific (Fig. 3B).

3.3. Effect of deglycosylation on IgE binding of protein

The purified protein was deglycosylated by both enzymatic and chemical methods. Carbohydrate removal led to a reduction in IgE binding; 40% in periodate treated protein and 27% in PNGase A treated protein. A weaker band observed in treated protein than in untreated protein in lectin binding assay showed that both periodate oxidation and PNGase A treatment was effective in partial deglycosylation of the protein (Figs. 4 and 5). The greater reduction observed in periodate-treated protein may be due to distortion of peptide epitopes by the method employed.

3.4. Basophil activation test

To evaluate the clinical relevance of purified allergen in eliciting mediator release in effector cells, a basophil activation test was performed by isolating peripheral blood mononuclear cells (PBMCs) from

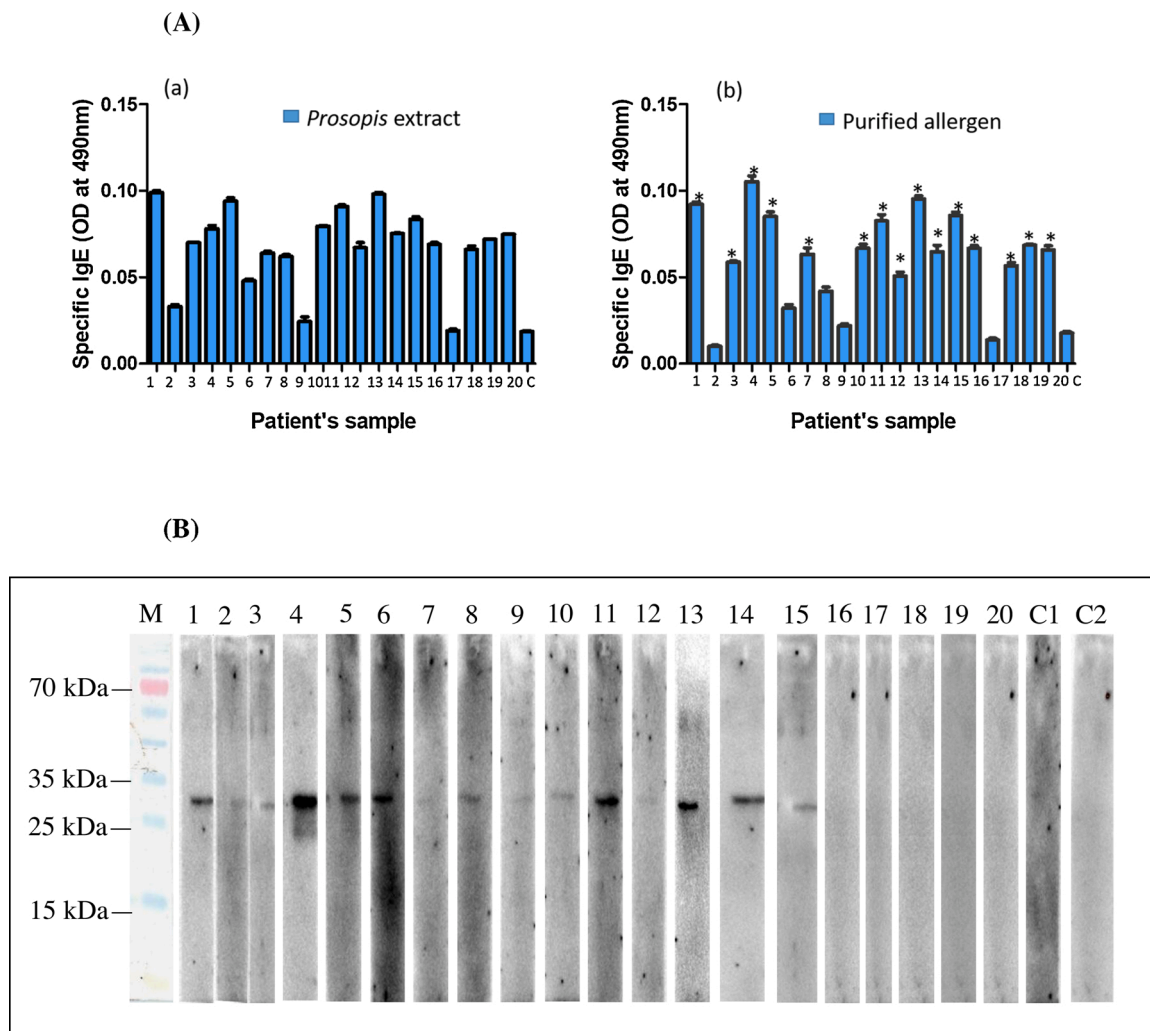


Fig. 2. A). Specific IgE ELISA of *Prosopis* extract and purified allergen with *Prosopis* hypersensitive patient's sera: IgE binding ability of *Prosopis* extract and purified protein was assessed by ELISA with serum samples of *Prosopis* hypersensitive patients. The data is presented in graphical form depicting IgE reactivity in terms of absorbance as average $\pm$ SEM. Sera showing specific IgE values (O.D. at 490 nm) $>= 3$ times that of control sera values (C: 0.018 O.D.) was considered as *Prosopis* sensitized. (a) Out of 20 *Prosopis* hypersensitive patients, all patients' sera showed IgE binding with *Prosopis* extract while (b) 15 patients demonstrate IgE binding with the purified allergen. Sera samples exhibiting immunoblot reactivity have been marked with *. B). Immunoblot of purified protein, Lane M: Pre-stain marker; Lane 1-20: purified allergen was resolved on SDS-PAGE and transferred on NC membrane. Nitrocellulose strips were blocked with 3% BSA and then probed with *Prosopis* hypersensitive individual patients' sera (1:20 diluted in 1%BSA (n = 20); C1, C2: Control Sera (NHS). Fifteen out of twenty *Prosopis* hypersensitive patients' sera showed IgE reactivity in immunoblot.

healthy volunteers followed by passive sensitization with *Prosopis* sensitized patient's sera containing specific IgE. Flow cytometric evaluation of basophil-specific marker CCR3 and activation marker CD203c revealed IgE-dependent up-regulation of basophil activation marker CD203c on CCR3+ basophils in PBMCs upon stimulation with the purified allergen. PBMCs were also sensitized with the deglycosylated purified allergen. The mean fluorescence intensity (MFI) was calculated and plotted relative to unstimulated samples. A significant MFI fold change of 2.6 ($p < 0.01$) was observed between purified allergen (GLY-PP; MFI fold change- 2.15) and its deglycosylated form (deGLY-PP; MFI fold change- 0.8) challenged PBMCs (Fig. 6).

3.5. Mass spectrometric analysis

The purified protein was identified by proteomic analysis of tryptic peptides obtained using MALDI-ToF/MS (BrukerDaltonik, Bremen, Germany) and the Mascot search engine (Matrix Science, Boston, Massachusetts, USA) using the database of Fabaceae pollen proteins. The mascot study results suggested protein scores greater than 29 to be

significant ($p < 0.05$). The top hit was obtained with Conglutin β 5 from *Lupinus angustifolius* (blue lupine bean) with a statistically significant score of 52. Out of 41 tryptic peptides of the purified protein, 13 of them showed homology to peptides of Conglutin β 5, with an e-value- 0.00028 (Fig. 7). SDAP analysis of short tryptic peptide sequence (25 amino acid stretch) revealed its homology with Lup an 1 (β -conglutinin allergen) with a sequence identity of 71%. Studies have reported 2.8–6.4% carbohydrate content in β -conglutinin (Eaton-Mordas, C. A. and Moore, K. A., 1978), Gueguen and Cerletti, 1994).

3.6. Cross-reactivity of purified protein

Immunoblot inhibition demonstrated allergenic cross-reactivity among purified allergen and the legume extracts. The purified protein showed reduced binding with the sera pre-inhibited with 500 μ g of extracts. The reduction in signal intensity was quantified in percentage using Alpha Imager 3400 (Alpha Innotech Corporation, San Leandro, CA, USA). Complete inhibition was obtained with the extracts of *Arachis hypogaea* (peanut), and *Phaseolus vulgaris* (common bean) followed by

Table 1

Clinical Characteristics of the Patients Selected for the Study.

Patient No.	Age/ Sex	Symptoms/ Clinical History	Reaction in SPT (mm)	Specific IgE reactivity (OD at 490 nm)		Allergen Sensitisation
				PE	PP	
1	45/M	AR	9	0.099	0.092	<i>A.alternata</i> , Prosopis
2	32/M	AR	7	0.033	0.010	Prosopis , <i>Rhizopus</i>
3	18/M	BA	11	0.070	0.058	Cockroach, Prosopis , <i>Rhizopus</i>
4	27/F	NBA	12	0.078	0.105	<i>A.fumigatus</i> , Prosopis , <i>Rhizopus</i>
5	45/M	BA, Angioedema	12	0.094	0.085	Cockroach, Prosopis , <i>Rhizopus</i>
6	35/F	BA	8	0.047	0.032	Cockroach, Dust mite, Prosopis
7	30/M	AR	10	0.063	0.063	<i>Parthenium</i> , Prosopis , <i>Rhizopus</i>
8	33/F	BA	9	0.062	0.041	Prosopis , <i>Parthenium</i>
9	46/M	AR	6	0.024	0.021	Cockroach, Prosopis , <i>Rhizopus</i>
10	26/M	AR	8	0.079	0.066	Prosopis
11	36/M	AR	5	0.090	0.082	Cockroach, <i>Parthenium</i> , Prosopis
12	37/F	NBA	9	0.067	0.050	Cockroach, <i>Parthenium</i> , Prosopis
13	29/F	AR, Urticaria	13	0.0981	0.095	<i>A.fumigatus</i> , <i>Parthenium</i> , Prosopis
14	35/M	AR, NBA	11	0.075	0.064	Cockroach, <i>Cynodon</i> , Dust mite, Prosopis
15	38/F	NBA	10	0.083	0.085	Cockroach, <i>Rhizopus</i> , Prosopis , Wheat
16	39/M	BA	9	0.068	0.066	<i>A.fumigatus</i> , Cockroach, Prosopis
17	54/M	AR	7	0.019	0.013	Cockroach, <i>Parthenium</i> , Prosopis
18	40/F	AR	14	0.066	0.056	Dust mite, Wheat, Prosopis
19	44/M	Urticaria	12	0.071	0.068	Prosopis , <i>Parthenium</i>
20	50/M	AR	13	0.074	0.065	Cockroach, <i>A. alternata</i> , Prosopis

Abbreviations: AR, Allergic Rhinitis; BA, Bronchial Asthma; NBA, Nasobronchial Asthma; PE, *Prosopis* Extract; PP, Purified Protein. Sera showing specific IgE values (O.D. at 490 nm) > or = 3 times that of control sera (NHS) O.D. values (O.D. at 490 nm: 0.018) was considered as sensitized to *Prosopis* extract and purified allergen.

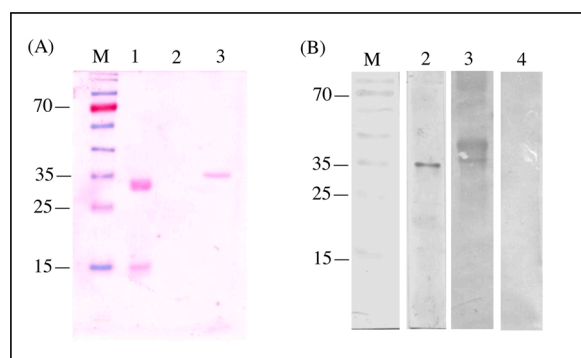


Fig. 3. (A) Periodic Acid Schiff staining, Lane M: Pre-stain marker, **Lane 1:** Phytohemagglutinin (positive control), **Lane 2:** recombinant protein from *Periplaneta americana* (negative control), **Lane 3:** Purified protein. **(B) Lectin Binding Assay; Lane 1:** Purified protein binding to Con A lectin, **Lane 2:** Ovalbumin (positive control), **Lane 3:** Purified protein incubated with Con A along with 0.3 M α -methyl mannoside (sugar inhibitor- specific binding control for lectin binding).

Vigna mungo (black gram) (78% inhibition), *Glycine max* (soybean) (72%), *Phaseolus lunatus* (lima bean) (64%) and *Cicer arietinum* (chickpea) (62%). Reduced inhibition of specific IgE reactivity observed suggested the presence of some unique allergens (Fig. 8).

4. Discussion

Pollen food allergy syndrome (PFAS) affects 45% of the patients exhibiting seasonal allergies (Geroldinger-Simic et al., 2011). Studies on major pollen sensitizers such as birch and cypress have demonstrated the importance of individual allergen components in eliciting immunological reactions to homologous proteins in food. Considering the

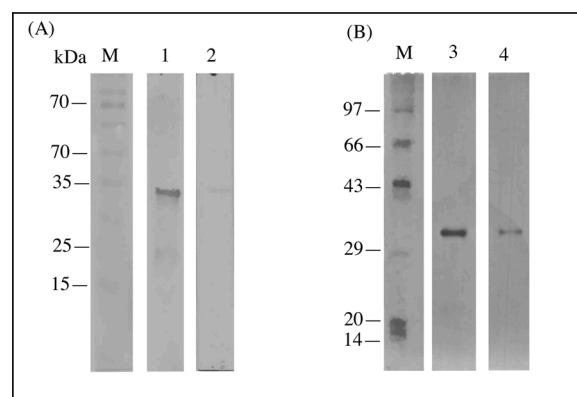


Fig. 4. Effect of periodate deglycosylation on IgE binding (A) Lectin binding assay of deglycosylated protein following periodate treatment; reduced binding with lectin ConA observed in periodate oxidized protein **Lane M:** Protein marker, **Lane 1:** Purified protein without periodate treatment, **Lane 2:** Purified protein with periodate treatment, **(B)** Immunoblot of deglycosylated protein following periodate treatment with pooled *Prosopis* hypersensitive sera; reduction in the IgE binding observed, **Lane 3:** Native protein without periodate treatment, **Lane 4:** Periodate oxidized purified protein.

diversity in geographical landscape and dietary habits, evaluation of clinically relevant cross-reactive allergens in pollen sensitizers prevalent in tropical regions becomes essential. Therefore, the present study was undertaken to identify an immunodominant allergen from *Prosopis juliflora* and investigate its cross-reactive potential with food allergens.

Prosopis pollen protein demonstrating the highest IgE binding with sera of SPT positive patients was selectively fractionated by employing a combination of anion exchange and gel filtration chromatography. Biochemical and immunological characterization of the obtained 35 kDa

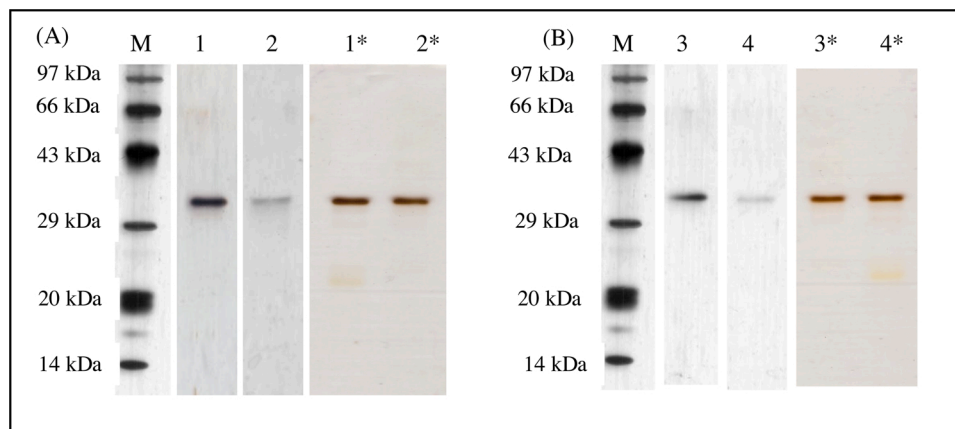


Fig. 5. Effect of enzymatic deglycosylation on IgE binding. (A) Lectin binding assay of deglycosylated protein following PNGase A treatment; reduced binding with lectin Con A observed in PNGase A treated protein **Lane M**: Protein marker, **Lane 1**: Purified protein without PNGase A treatment, **Lane 2**: Purified protein with PNGase A treatment, **Lane 1*** and **2***: loading control (silver-stained) (B) Immunoblot of deglycosylated protein following PNGase A treatment; reduction in the IgE binding observed after probing deglycosylated protein with pooled *Prosopis* hypersensitive sera, **Lane 3**: Native protein without PNGase A treatment **Lane 4**: PNGase A treated purified protein. **Lane 3*** and **4***: Loading control (silver-stained).

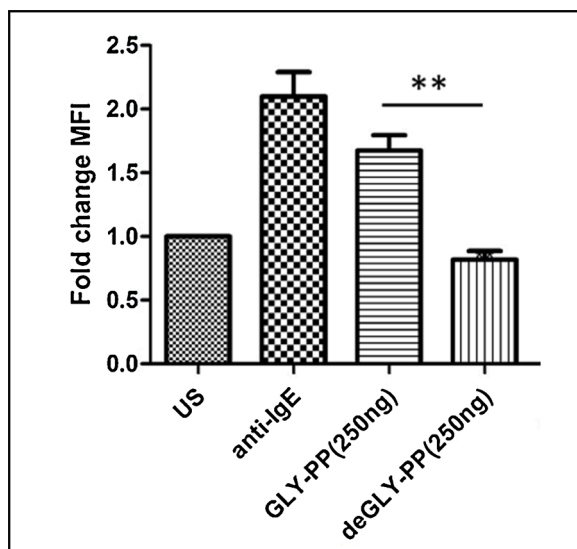


Fig. 6. Functional activity assay (Basophil activation Test).

The test was performed using passive sensitization protocol. PBMCs isolated from healthy volunteers ($n = 6$) were isolated and sensitized with pooled sera samples of *Prosopis* hypersensitive patients. PBMCs were then challenged with media (US), native purified protein (GLY-PP; 250 ng), and deglycosylated purified protein (deGLY-PP; 250 ng) for 30 min. Anti-IgE (1:100) sensitized cells were taken as a positive control. The cells were stained with PE anti-human CCR3 (BD Pharmingen) and APC-anti human CD203c (BD Pharmingen) and analyzed by Flow cytometry. Basophils were selected as CCR3+ cells. Relative fold change in MFI in basophil activation is expressed as an increase in CD203c levels in CCR3+ basophils. The data is presented in graphical form depicting fold change in MFI as average $\pm$ SEM ($p < 0.01$).

purified protein revealed it to be a major allergenic component with 75% IgE reactivity among *Prosopis* hypersensitive patients as exhibited by ELISA and immunoblot. Since, major allergens from various sources (including tree pollen allergens such as Pla a 2, Cry j 1, Jun a 1, Ole e1) tend to be glyco-proteinaceous in nature, we sought to examine the same using Schiff Assay. The purified protein demonstrated the presence of carbohydrate residues, upon further characterization by *Concanavalin A*, an unambiguous lectin, it demonstrated the presence of terminal mannose and glucose residues. Interestingly, the presence of mannosylated residues is a predominant feature of major allergenic proteins including Der p 1, Fel d 1, Ara h 1, Can f 1, cedar allergen Cry j 1, and pollen allergen Cha o 1 (Al-Ghoul et al., 2012).

The precise role played by carbohydrate residues in the allergenicity of proteins is unclear. Earlier studies on cod parvalbumin and celery allergen Api g 5, demonstrated the crucial role of periodate sensitive

glycan moieties in IgE binding and mediator release assays (Bublin et al., 2003; Bugajska-Schretter et al., 1998). Periodate treated deglycosylated purified 35 kDa protein exhibited a reduction in IgE binding (40%) thereby demonstrating the crucial role of glycan residues in IgE binding. Periodate treatment is known to remove carbohydrate residues of protein; however, it may lead to oxidation of certain amino acids and may render the tertiary structure unstable. To counter these possibilities in influencing the IgE binding ability of protein, the protein was also deglycosylated using PNGase A, a specific enzyme that cleaves N-glycosidic bonds of plant glycoproteins. A reduction of 27% was observed in enzymatically deglycosylated protein thereby demonstrating a substantial contribution of N-linked glycosylation in IgE binding. Basophil activation test was employed to assess the clinical significance of glycoprotein allergen. Compared to the deglycosylated form of the protein, the purified protein exacerbated effector cell response. A significant upregulation of CD203c expression on the surface of CCR3+ basophils in human PBMCs was observed signifying the biological ability of purified protein to bind and cross-link IgE bound on Fcε receptors on basophils.

To investigate the biological identity of purified protein, MALDI-TOF/MS was performed. It demonstrated the highest sequence identity with conglutin allergen, Lup an 1 of *Lupinus angustifolius*, a leguminous plant. β -Conglutins are generally glycosylated with 2.8–6.4% of carbohydrate content (Eaton-Mordas, C. A. and Moore, K. A., 1978), Gueguen and Cerletti, 1994). Lup an 1, a vicilin-like protein is known to be a clinically relevant cross-reactive allergen with peanut (Goggin et al., 2008; Mennini et al., 2016). Structural homology studies demonstrate high similarity of major allergen Ara h 1 with lupine conglutin. This is immunologically reflected in *in vivo* studies where a significant proportion (34%–52%) of peanut allergic patients demonstrated sensitization to lupine bean (Ballabio et al., 2013; Guarneri et al., 2005; Shaw et al., 2008). Further, *in silico* analysis of Lup an 1 reveals presence of common shared epitopes (B and T cell epitopes) that may be responsible for its cross-reactivity with major legume allergens including Ara h 1 and Pis s 1 (Lima-Cabello et al., 2016). These results suggest that allergenicity of purified *Prosopis* 35 kDa protein is substantially a function of inherent characteristics of protein with a minor yet significant contribution from attached N-linked glycan moieties.

MALDI-TOF/MS results prompted our investigation to determine the possibility of PFAS by *P. juliflora*. A considerable co-sensitization (82%) is reported between legume and pollen proteins (Mittag et al., 2006, 2004). Moreover, studies have established cross-reactivity among peanut, soy, and lupine bean allergens (Cabanillas et al., 2018). Allergic cross-reactions have been reported between cypress pollen and peach including oral allergy syndrome (Klingebiel et al., 2019). A common glycan motif GlcNAc[2]Man[3]Xyl[1]Fuc[1]GlcNAc[2] is found in cypress and cedar pollen allergens (Alisi et al., 2001; Maeda et al., 2005; Midoro-Horiuti et al., 1999; Osada et al., 2017). Though MS data

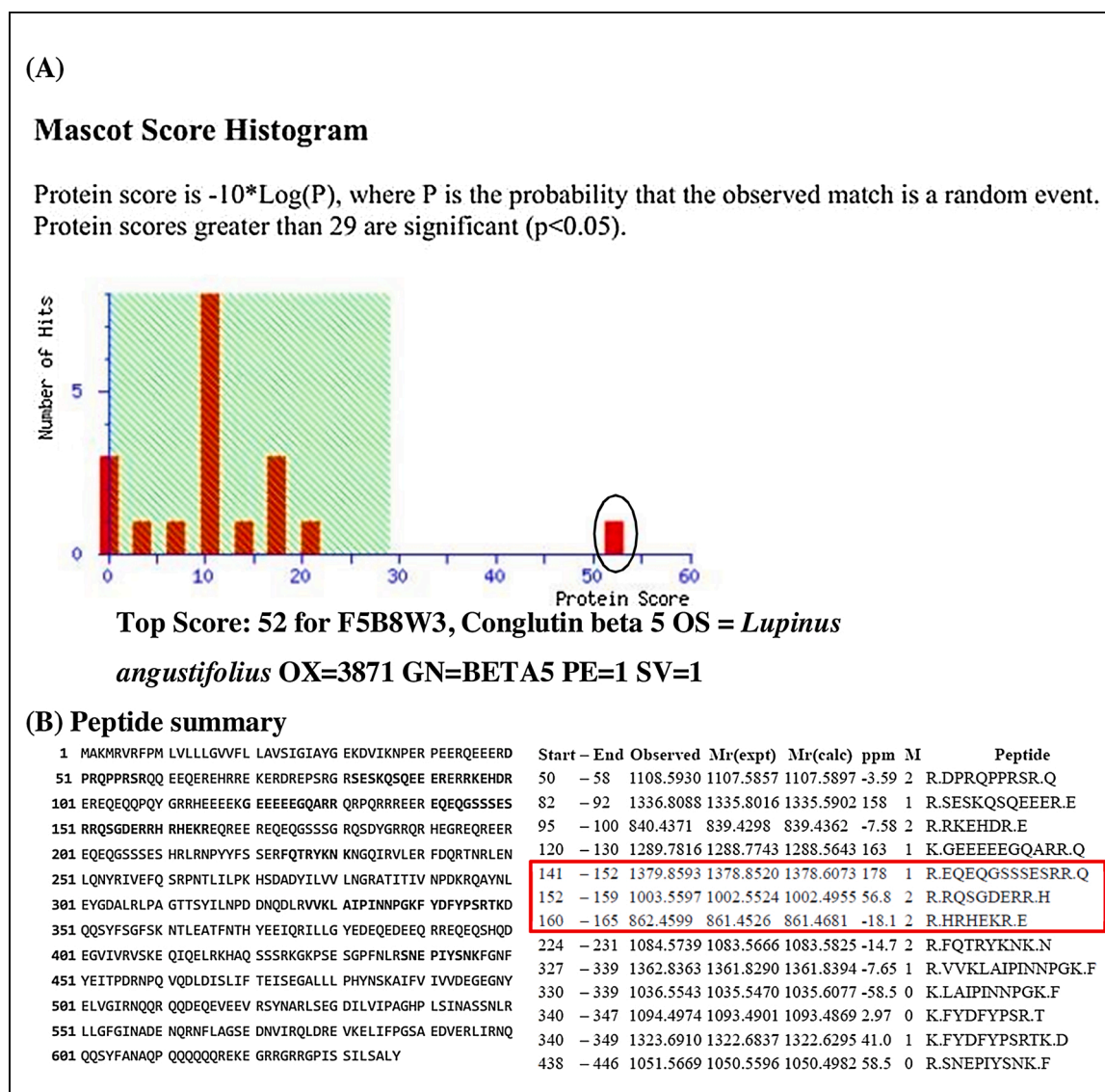


Fig. 7. Protein summary report for the mass spectrometry data. (A) Mascot score histogram- The significant protein score of the study obtained is 29. The top score obtained i.e. 52 following peptide mass fingerprint search corresponds to Conglutin beta 5 protein of *Lupinus angustifolius*. **(B) Peptide summary-** 13/41 peptides matched to the predicted sequence with e-value 0.00028, matched peptides shown in bold. SDAP analysis of short tryptic peptide sequence (25 amino acid stretches in red box) revealed its homology with Lup an 1 (β -conglutin allergen) with a sequence identity of 71%.

indicates a high similarity of purified protein with conglutin from lupine bean, the latter is barely consumed in India. Considering the abundance of *P. juliflora* in India, its cross-reactive potential was assessed with clinically relevant legumes widely consumed in the region. This study demonstrated complete inhibition with peanut and common bean extracts, wherein 60–78% inhibition was observed with legume extracts of black gram, chickpea, soybean, and lima bean. These results suggest the cross-reactive potential of purified protein with corresponding allergens of peanut and other legume allergens.

The present study highlights the presence of a major vicilin-like glycoprotein allergen in *Prosopis* and demonstrates its cross-reactivity with legume proteins including potent sensitizers such as peanut and soy. In addition, lupine bean is a superfood around the world due to its high nutritional content, nutraceutical properties, and low glycemic index (Lima-Cabello et al., 2018b, 2018a, 2017; “Lupin: From Stockfeed to Superfood? Australian Perspectives,” n.d.), hence a high sequence similarity of purified allergen with Lup an 1 is a matter of concern. *Prosopis* sensitized individuals may possess a great risk of developing systemic reactions upon consumption of foods containing cross-reactive

allergens. Our results further reinforce the need for molecular characterization of *P. juliflora* allergens ensuring improved diagnosis and development of therapeutic modalities.

Author statement

Bharti Arora: Conceptualization, methodology, investigation, validation, writing- original draft preparation, reviewing/editing, and visualization. **Swati Sharma:** Writing- original draft preparation and reviewing/editing. **SN Gaur:** resources and supervision. **VK Jain:** resources and supervision. **Shakuntala Lavasa:** resources and supervision. **Naveen Arora:** Conceptualization, writing- reviewing/editing and visualization, supervision and funding acquisition.

Funding

The study was funded by DBT, New INDIGO Project. Swati Sharma is a Senior Research Fellow of the Council for Scientific and Innovative Research, New Delhi. Bharti Arora is a DST-INSPIRE Senior Research

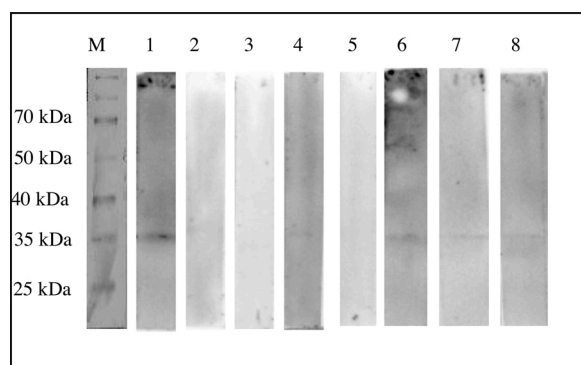


Fig. 8. Immunoblot inhibition using legume extracts, Lane M: Pre-stain marker, Lane 1: *Prosopis* hypersensitive patients' sera without inhibitor, Lane-2: Control sera (NHS), Lane3-8: purified protein probed with patient's sera pre-inhibited with 500 µg/mL of each *Phaseolus vulgaris*, *Glycine max*, *Arachis hypogaea*, *Phaseolus lunatus*, *Cicer arietinum* and *Vigna mungo* extract, respectively.

fellow of the Department of Science and Technology, New Delhi.

Ethics approval

The study was approved by Institutional Human Ethics Committee.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.molimm.2021.06.023>.

References

- Al-Ghoulh, A., Johal, R., Sharquie, I.K., Emara, M., Harrington, H., Shakib, F., Ghaemmaghami, A.M., 2012. The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent. *PLoS One* 7, e33929. <https://doi.org/10.1371/journal.pone.0033929>.
- Ali-Sadeghi, H., Khodadadi, A., Amini, A., Assarehzadegan, M.-A., Sepahi, N., Zarinhadideh, F., 2015. Pro j 2 is mesquite profilin: molecular characteristics and specific IgE binding activity. *Asian Pacific J. Allergy Immunol.* 33, 90–98. <https://doi.org/10.12932/AP0507.33.2.2015>.
- Alisi, C., Afferni, C., Iacovacci, P., Barletta, B., Tinghino, R., Buteroni, C., Puggioni, E.M., Wilson, I.B.H., Federico, R., Schinina, M.E., Ariano, R., Di Felice, G., Pini, C., 2001. Rapid isolation, characterization, and glycan analysis of Cup a 1, the major allergen of *Arizona cypress* (*Cupressus arizonica*) pollen. *Allergy* 56, 978–984. <https://doi.org/10.1034/j.1398-9995.2001.00125.x>.
- Asam, C., Hofer, H., Wolf, M., Aglas, L., Wallner, M., 2015. Tree pollen allergens-an update from a molecular perspective. *Allergy* 70, 1201–1211. <https://doi.org/10.1111/all.12696>.
- Ballabio, C., Peñas, E., Uberti, F., Fiocchi, A., Duranti, M., Magni, C., Restani, P., 2013. Characterization of the sensitization profile to lupin in peanut-allergic children and assessment of cross-reactivity risk. *Pediatr. Allergy Immunol.* 24, 270–275. <https://doi.org/10.1111/pai.12054>.
- Biedermann, T., Winther, L., Till, S.J., Panzner, P., Knulst, A., Valovirta, E., 2019. Birch pollen allergy in Europe. *Allergy* 74, 1237–1248. <https://doi.org/10.1111/all.13758>.
- Bublin, M., Radauer, C., Wilson, I.B.H., Kraft, D., Scheiner, O., Breiteneder, H., Hoffmann-Sommergruber, K., 2003. Cross-reactive N-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients. *FASEB J.* 17, 1697–1699. <https://doi.org/10.1096/fj.02-0872.fje>.
- Bugajska-Schretter, A., Elfman, L., Fuchs, T., Kapiotis, S., Rumpold, H., Valenta, R., Spitzauer, S., 1998. Parvalbumin, a Cross-reactive Fish Allergen, Contains IgE-Binding Epitopes Sensitive to Periodate Treatment and Ca²⁺ Depletion.
- Cabanillas, B., Jappe, U., Novak, N., 2018. Allergy to peanut, soybean, and other legumes: recent advances in allergen characterization, stability to processing and IgE cross-reactivity. *Mol. Nutr. Food Res.* 62, 1700446. <https://doi.org/10.1002/mnfr.201700446>.
- Dhyani, A., Arora, N., Jain, V.K., Sridhara, S., Singh, B.P., 2007. Immunoglobulin E (IgE)-mediated cross-reactivity between mesquite pollen proteins and lima bean, an edible legume. *Clin. Exp. Immunol.* 149, 517–524. <https://doi.org/10.1111/j.1365-2249.2007.03446.x>.
- Dhyani, A., Singh, B.P., Arora, N., Jain, V.K., Sridhara, S., 2008. A clinically relevant major cross-reactive allergen from mesquite tree pollen. *Eur. J. Clin. Invest.* 38, 774–781. <https://doi.org/10.1111/j.1365-2362.2008.02020.x>.
- DHYANI, A., ARORA, N., GAUR, S., JAIN, V., SRIDHARA, S., SINGH, B., 2006. Analysis of IgE binding proteins of mesquite (*Prosopis juliflora*) pollen and cross-reactivity with predominant tree pollens. *Immunobiology* 211, 733–740. <https://doi.org/10.1016/j.imbio.2006.03.003>.
- Dousti, F., Assarehzadegan, M.-A., Morakabati, P., Khosravi, G.R., Akbari, B., 2016. Molecular cloning and expression of Pro J 1: a new allergen of *Prosopis juliflora* pollen. *Iran. J. Allergy Asthma Immunol.* 15, 122–131.
- Fernández-González, M., Álvarez-López, S., González-Fernández, E., Jesús Aira, M., Rodríguez-Rajo, F.J., 2020. Cross-reactivity between the Betulaceae family and fallout in the real atmospheric aeroallergen load. *Sci. Total Environ.* 715. <https://doi.org/10.1016/j.scitotenv.2020.136861>.
- Geroldinger-Simic, M., Zelniker, T., Aberer, W., Ebner, C., Egger, C., Greiderer, A., Prem, N., Lidholm, J., Ballmer-Weber, B.K., Vieths, S., Bohle, B., 2011. Birch pollen-related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. *J. Allergy Clin. Immunol.* 127, 616–622. <https://doi.org/10.1016/j.jaci.2010.10.027>.
- Goggins, D.E., Mir, G., Smith, W.B., Stuckey, M., Smith, P.M.C., 2008. Proteomic analysis of lupin seed proteins to identify conglutin β as an allergen, Lup an 1. *J. Agric. Food Chem.* 56, 6370–6377. <https://doi.org/10.1021/jf800840u>.
- Guarneri, F., Guarneri, C., Benvenega, S., 2005. Identification of potentially cross-reactive peanut-lupine proteins by computer-assisted search for amino acid sequence homology. *Int. Arch. Allergy Immunol.* 138, 273–277. <https://doi.org/10.1159/000088864>.
- Gueguen, J., Cerletti, P., 1994. Proteins of some legume seeds: soybean, pea, fababean and lupin. *New and Developing Sources of Food Proteins*. Springer US, pp. 145–193. https://doi.org/10.1007/978-1-4615-2652-0_6.
- Heshmati, I., Khorasani, N., Shams-Esfandabad, B., Riazi, B., 2019. Forthcoming risk of *Prosopis juliflora* global invasion triggered by climate change: implications for environmental monitoring and risk assessment. *Environ. Monit. Assess.* 191. <https://doi.org/10.1007/s10661-018-7154-9>.
- Hussain, M.I., Shackleton, R.T., El-Keblawy, A., Del Mar, Trigo, Pérez, M., González, L., 2020. Invasive mesquite (*Prosopis juliflora*), an allergy and health challenge. *Plants (Basel, Switzerland)* 9 (Jan. (2)), 141. <https://doi.org/10.3390/plants9020141>.
- Klingebiel, C., Chantran, Y., Arif-Lusson, R., Ehrenberg, A.E., Östling, J., Poisson, A., Liabeuf, V., Agabriel, C., Birnbaum, J., Porri, F., Sarraf, A., Apoil, P.A., Vivinus, M., Garnier, L., Chiriac, A.M., Caimmi, D.P., Bourrain, J.L., Demoly, P., Guez, S., Boralevi, F., Lovato, B., Palussière, C., Leroy, S., Bourrier, T., Giovannini-Chami, L., Gouitaa, M., Aferiat-Derome, A., Charpin, D., Sofalvi, T., Cabon-Boudard, I., Massabie-Bouchat, Y.P., Hofmann, B., Bonardel, N., Dron-Gonzalez, M., Sterling, B., Carsin, A., Vivinus, S., Poitevin, B., Nicolau, L., Liautaud, G., Soler, C., Mezouar, S., Annesi-Maesano, I., Mège, J.L., Lidholm, J., Vitte, J., 2019. Pru p 7 sensitization is a predominant cause of severe, cypress pollen-associated peach allergy. *Clin. Exp. Allergy* 49, 526–536. <https://doi.org/10.1111/cea.13345>.
- Lima-Cabello, E., Robles-Bolivar, P., Alché, J.D., Jimenez-Lopez, J.C., 2016. Narrow leafed lupin beta-conglutin proteins epitopes identification and molecular features analysis involved in cross-allergenicity to peanut and other legumes. *Genomics Comput. Biol.* 2, 29. <https://doi.org/10.18547/gcb.2016.vol2.iss1.e29>.
- Lima-Cabello, E., Alche, V., Foley, R.C., Andrikopoulos, S., Morahan, G., Singh, K.B., Alche, J.D., Jimenez-Lopez, J.C., 2017. Narrow-leaved lupin (*Lupinus angustifolius* L.) β-conglutin proteins modulate the insulin signaling pathway as potential type 2 diabetes treatment and inflammatory-related disease amelioration. *Mol. Nutr. Food Res.* 61. <https://doi.org/10.1002/mnfr.201600819>.
- Lima-Cabello, E., Morales-Santana, S., Foley, R.C., Melsers, S., Alché, V., Siddique, K.H.M., Singh, K.B., Alché, J.D., Jimenez-Lopez, J.C., 2018a. Ex vivo and in vitro assessment of anti-inflammatory activity of seed β-conglutin proteins from *Lupinus angustifolius*. *J. Funct. Foods* 40, 510–519. <https://doi.org/10.1016/j.jff.2017.11.040>.
- Lima-Cabello, E., Morales-Santana, S., León, J., Alché, V., Clemente, A., Alché, J.D., Jimenez-Lopez, J.C., 2018b. Narrow-leaved lupin (*Lupinus angustifolius* L.) seed β-conglutins reverse the induced insulin resistance in pancreatic cells. *Food Funct.* 9, 5176–5188. <https://doi.org/10.1039/c8fo01164h>.
- Lupin: From Stockfeed to Superfood? Australian Perspectives [WWW Document], n.d.
- Maeda, M., Kamamoto, M., Hino, K., Yamamoto, S., Kimura, M., Okano, M., Kimura, Y., 2005. Glycoform analysis of Japanese cedar pollen allergen, Cry j 1. *Biosci. Biotechnol. Biochem.* 69, 1700–1705. <https://doi.org/10.1271/bbb.69.1700>.
- Mennini, M., Dahdah, L., Mazzina, O., Fiocchi, A., 2016. Lupin and other potentially cross-reactive allergens in peanut allergy. *Curr. Allergy Asthma Rep.* 16, 84. <https://doi.org/10.1007/s11882-016-0668-8>.
- Midoro-Horiuti, T., Goldblum, R.M., Kurosky, A., Goetz, D.W., Brooks, E.G., 1999. Isolation and characterization of the mountain cedar (*Juniperus ashei*) pollen major allergen, Jun a 1. *J. Allergy Clin. Immunol.* 104, 608–612. [https://doi.org/10.1016/S0091-6749\(99\)70331-3](https://doi.org/10.1016/S0091-6749(99)70331-3).
- Mittag, D., Akkeraas, J., Ballmer-Weber, B.K., Vogel, L., Wensing, M., Becker, W.M., Koppelman, S.J., Knulst, A.C., Helbling, A., Hefle, S.L., Van Ree, R., Vieths, S., 2004. Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *J. Allergy Clin. Immunol.* 114, 1410–1417. <https://doi.org/10.1016/j.jaci.2004.09.014>.
- Mittag, D., Batori, V., Neudecker, P., Wiche, R., Friis, E.P., Ballmer-Weber, B.K., Vieths, S., Roggen, E.L., 2006. A novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1 and homologous food allergens in individual

- patients. *Mol. Immunol.* 43, 268–278. <https://doi.org/10.1016/j.molimm.2005.02.008>.
- Osada, T., Maeda, M., Tanabe, C., Furuta, K., Vavricka, C.J., Sasaki, E., Okano, M., Kimura, Y., 2017. Glycoform of a newly identified pollen allergen, Cha o 3, from *Chamaecyparis obtusa* (Japanese cypress, Hinoki). *Carbohydr. Res.* 448, 18–23. <https://doi.org/10.1016/j.carres.2017.05.005>.
- Perusko, M., van Roest, M., Stanic-Vucinic, D., Simons, P.J., Pieters, R.H.H., Cirkovic Velickovic, T., Smit, J.J., 2018. Glycation of the major milk allergen β -lactoglobulin changes its allergenicity by alterations in cellular uptake and degradation. *Mol. Nutr. Food Res.* 62 <https://doi.org/10.1002/mnfr.201800341>.
- Ruan, W.-W., Cao, M.-J., Chen, F., Cai, Q.-F., Su, W.-J., Wang, Y.-Z., Liu, G.-M., 2013. Tropomyosin contains IgE-binding epitopes sensitive to periodate but not to enzymatic deglycosylation. *J. Food Sci.* 78, C1116–C1121. <https://doi.org/10.1111/1750-3841.12169>.
- Santos, A.F., Du Toit, G., Douiri, A., Radulovic, S., Stephens, A., Turcanu, V., Lack, G., 2015. Distinct parameters of the basophil activation test reflect the severity and threshold of allergic reactions to peanut. *J. Allergy Clin. Immunol.* 135, 179–186. <https://doi.org/10.1016/j.jaci.2014.09.001>.
- Shaw, J., Roberts, G., Grimshaw, K., White, S., Hourihane, J., 2008. Lupin allergy in peanut-allergic children and teenagers. *Allergy Eur. J. Allergy Clin. Immunol.* 63, 370–373. <https://doi.org/10.1111/j.1398-9995.2007.01568.x>.
- Singh, A.B., Kumar, P., 2003. Aeroallergens in clinical practice of allergy in India. *An overview. Ann. Agric. Environ. Med.* 10, 131–136.
- Smeekens, J.M., Bagley, K., Kulis, M., 2018. Tree nut allergies: allergen homology, cross-reactivity, and implications for therapy. *Clin. Exp. Allergy* 48, 762–772. <https://doi.org/10.1111/cea.13163>.
- Zhang, Z., Xiao, H., Zhang, X., Zhou, P., 2019. Insight into the effects of deglycosylation and glycation of shrimp tropomyosin on *in vivo* allergenicity and mast cell function. *Food Funct.* 10 (7) <https://doi.org/10.1039/C9FO00699K>.