

Molecular and Immunochemical Characterization of Pop n 2: A New Allergen of *Populus nigra* Pollen

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Mohammad-Hossein Shams^{1,5}, Mohammad-Ali Assarehzadegan^{2,3*}, Nahid Eskandari ¹, Mohsen Masjedi ^{1*}, Farnaz Kheirandish ^{4,5}, Ramin Ghasemi ¹, Mazdak Ganjalikhani hakemi ¹, Ali-Mohammad Varzi⁶, Mojgan Safari⁷, Seyyed-Mohsen Sohrabi⁵, Haleh Abdoli Sereshki^{2,3}

Affiliations:

¹ Department of Immunology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

² Immunology Research Center, Institute of Immunology and Infectious Diseases, Iran University of Medical Science, Tehran, Iran.

³ Immunology Department, School of Medicine, Iran University of Medical Sciences, Tehran, Iran.

⁴ Department of Medical Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran.

⁵ Razi Herbal Medicines Research Center, Lorestan University of Medical sciences, Khorramabad, Iran.

⁶ Department of Medical Immunology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran.

⁷ Department of Pediatrics, School of Medicines, Hamedan University of Medical Science, Hamedan, Iran.

* Corresponding author (Mohammad-Ali Assarehzadegan):

1- Immunology Research Center, Institute of Immunology and Infectious Diseases, Iran University of Medical Science, Tehran, Iran.

2- Immunology Department, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

Postal address: Hemmat Highway, Iran University of Medical Sciences, Tehran, Iran.

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Office: +98 (21) 86703264

Postal code: 1449614535 Email addresses: assarehma@gmail.com & assareh.ma@iums.ac.ir

*** Co-Corresponding author (Mohsen Masjedi):**

Department of Immunology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

Postal code: 8174673461, Email addresses: mohsenmasjedi2013@gmail.com

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DR MOHAMMAD HOSSEIN SHAMS (Orcid ID : 0000-0002-1260-7251)

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Molecular and Immunochemical Characterization of Pop n 2: A New Allergen of *Populus nigra* Pollen

Abstract

Background: Pollen is one of the most common allergens that cause respiratory allergies worldwide. Pollen grains from poplars have been reported as important sources of pollinosis in many countries.

Objective: The aim of the present study was to determine the molecular and immunochemical characterization of Pop n 2, a novel allergen of *Populus nigra* (*P. nigra*) pollen extract.

Methods: In this study, the pollen extract of *P. nigra* was analyzed by SDS-PAGE, and the allergenic profile was determined by IgE immunoblotting and specific ELISA using the sera of twenty allergic patients. The coding sequence of Pop n 2 was cloned and expressed in the *Escherichia coli* BL21 (DE3) using plasmid the pET-21b (+). Finally, the expressed recombinant Pop n 2 was purified by affinity-chromatography.

Results: Pop n 2 belongs to the profilin family with a molecular weight of approximately 14 kDa. Pop n 2 is the most IgE-reactive protein (about 65%) in the *P. nigra* pollen extract. The cDNA sequencing results indicated an open reading frame 396 bp that encodes 131 amino acid residues. The results of ELISA and Immunoblotting assays showed that recombinant Pop n 2 could react with the IgE antibody in patients' sera, like its natural counterpart.

Conclusion: Our data revealed that Pop n 2 is a significant allergen in the *P. nigra* pollen extract. Moreover, we observed that the recombinant Pop n 2 produced by the pET-21b (+) vector in the *E. coli* system acts as its natural counterpart.

Keywords: Profilin, Pop n 2, Allergen, *Populus nigra*, Characterization

Abbreviation list: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SPT, skin prick test; ELISA, Enzyme linked immunosorbent assay; IgE, Immunoglobulin E; rPop n 2, recombinant Pop n2; 2-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TMB, tetramethylbenzidine; OD, optical density; PVDF, polyvinylidene difluoride

Introduction

Airborne allergens are common causes of respiratory allergic diseases such as asthma and allergic rhinitis (1). The most significant airborne allergens include pollen grains, dust mites,

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fungal spores, and animal dander (2). Pollen grains are one of the most common allergens that cause respiratory allergies worldwide (3).

Poplars are rapidly growing, weak-wooded trees that lack decorative flowers and fruits (4). The poplar genus has 35 species and is widely distributed in the Northern Hemisphere, including the throughout Europe, Central and West Asia, and North Africa (5, 6). Pollen grains from the poplars have been reported as important sources of airborne allergens that cause respiratory allergic diseases in countries such as United States, Iran, United Arab Emirates, Turkey, and Hungarian, and Eastern provinces of Saudi Arabia where the frequency of sensitization ranges from 20% to 30% (7-14). It has been indicated that poplar pollen is a major airborne allergen during the pollen season in Iran from the first week of February to the middle of October (15).

Despite a high rate of sensitization to poplar pollen in Iran and other countries, to our knowledge, only few studies have been conducted on the characterization of poplar pollen extract which have not been well documented. The identification of the allergenic ingredients of pollen grains is essential for diagnostic procedures, the design of allergen-specific immunotherapy, and the elucidation of sensitization mechanisms to various allergens (16, 17).

The aim of this study was to characterize and produce the first allergen from *Populus nigra* (black poplar) pollen, in *Escherichia coli* (*E. coli*), and subsequently evaluate the immunoreactivity of this recombinant allergen with *in vitro* assays using sera from allergic patients. The new and first allergen of *P. nigra* pollen, as a profilin protein, was nominated Pop n 2 by the WHO/IUIS Allergen Nomenclature Subcommittee (<http://www.allergen.org/>).

Methods

Preparation of the pollen Extract

P. nigra pollen grains were collected from February to the middle of October from provinces located in Central and Western Iran such as Tehran, Isfahan, and Lorestan by a trained pollen collector. Subsequently, to remove the impureness from the pollen grains, they were passed through sieves with pore diameters of 300, 200, and 100 μ m, respectively. Using a microscopic analysis, the final fine powder was examined for pollen purity and approved by a pollenologist. Pollen particles with purity greater than 95% and impurity less than 5% were selected for protein extraction. Pollen grains were defatted several times using diethyl ether buffer. Then, the extraction of pollen grains was performed from 0.4 mg of defatted pollen as described previously (18-20). Finally, the extract was lyophilized and kept at -70 °C. Moreover, the protein content of

the extract was determined using the Bradford method which is rapid and accurate for the estimation of protein concentration (21). The final total protein concentration of lyophilized *P. nigra* pollen extract was 1500 µg/ml.

Patients and Skin Prick Test

Using *P. nigra* pollen extract, grass mix extract (Kentucky blue/ June grass, Meadow Fescue, Orchard, Perennial Ryegrass, Redtop, Sweet Vernal, Timothy), tree mix (Acacia, Box Elder, Olive, Siberian Elm, Spring Birch, Western Juniper, Western Sycamore, Western White Oak, White Mulberry), and weed mix (Cocklebur, Giant Ragweed, Lamb's Quarter, Rough/Redroot Pigweed, Short Ragweed) (GREER, USA), skin prick test was performed on 200 patients who were referred to the Immunology and Allergy Department of Isfahan University of Medical Sciences and Hamedan University of Medical Sciences. Of these, 110 patients with symptoms of allergic rhinitis disease, and SPT positive for *P. nigra*, grass mix, tree mix and weed mix pollen extracts were selected. Subsequently, 20 patients with the symptoms of allergic rhinitis and positive SPT only with the *P. nigra* pollen extract were selected for this study (Table 1). Furthermore, ten healthy persons with negative SPT who lacked the symptoms of respiratory allergy diseases were considered as negative controls. To confirm the results of SPT, histamine 10 mg/ml was used as the positive control and glycerol saline as the negative control. Patients with a wheal diameter >3 mm were considered positive reaction compared to the negative control and were asked to donate a serum sample. The donated serum samples were kept at -20 °C until use. This research was approved by the Medical Ethics Committee (IR.mui.rec.1396.3.824), and all the patients completed the written consent form to participate in the study.

Total and Specific IgE Assays

With a commercial ELISA kit (abcam, Cambridge, MA, USA) and according to the manufacturer's instruction, the total IgE antibody levels of the patients were measured. Setting up and optimizing an indirect ELISA method, the level of specific IgE antibody against the proteins of *P. nigra* pollen extract was assessed. Indirect ELISA was performed according previous study (22). The OD of the plate wells at the wavelength of 450 nm was read by an ELISA reader. Sera from 10 control person was used as the negative control. Specific IgE antibodies were considered

positive in patients whose OD at wavelength 450 nm was three times higher than the mean of the negative control (The mean of negative controls= 0.046) (Cut-off: OD> 0.14) (23).

SDS-PAGE and IgE-Immunoblotting Assay

The proteins of *P. nigra* pollen crude extract was analyzed in reducing conditions using 2-ME 5% (v/v), and non-reducing conditions without 2-ME using discontinuous SDS-PAGE, as previously reported (22). At the end of electrophoretic separation, all the proteins sorted by size could be observed and analyzed by gel Coomassie Brilliant Blue staining.

To make separated proteins available for IgE antibodies in the sera of *P. nigra* pollen-sensitive patients, they were transferred to PVDF membrane by the electroblotting method and IgE-immunoblot experiment was performed as described previously (19) with some modification. The strips were probed with the biotinylated mouse monoclonal anti-human IgE (abcam, USA) with 1:4000 dilution and 1:20000 dilution streptavidin-HRP (abcam, USA). Finally, with HRP substrate luminol (Parstous Co, Iran), allergenic reactive proteins with serum IgE antibody were visualized by chemiluminescence using G-Box gel documentation system (Syngene, UK).

Cloning and Sequencing of the Gene Encoding Pop n 2

Initially, total RNA was extracted from 0.5 mg *P. nigra* pollen grains using a commercial kit. In this kit, the silica particles have been fixed inside the columns, enabling the quick and selective isolation of total RNA from different components of the plants (DENAzist Co, Iran). In the next step, single-stranded cDNA was synthesized using reverse transcriptase enzyme from the extracted RNA according to the instructions of a commercial kit (Parstous Co, Iran). Then, cDNA was amplified by PCR using *Tag* polymerase enzyme and degenerate primers (Forward: 5'-ATG TC (G/C) TGG CAG ACG TA (C/T) GT (A/C/T) GAT GA-3'; Reverse 5'- CAT GCC (C/T) TG TTC GAC CAG (A/G) TA (A/G) TC ACC-3') that were selected based on the profilin protein conserved sequence of the *P. nigra* related species in the NCBI database. Afterwards, the PCR product was extracted from the gel (Biosettia, Ca, USA) and cloned into the PTZ57RT T/A vector (Thermo Fisher Scientific, MA, USA). The resulting plasmids were transformed into TOP10 *E. coli* bacteria cells, and then the recombinant plasmid extracted from the gel by

plasmid extraction kit (Biosettia, USA) was sent to Swiss Microsynth Company to sequencing. Finally, the results of the DNA sequenced through ExPASy-translating tool (<https://web.expasy.org/translate/>) were converted to amino acid sequences. The complete amino acid sequence of the cloned Pop n 2 gene was used as the query to search for homologous sequences through the BLASTp in NCBI and UniProtKB databases. The alignment of the amino acid sequence of Pop n 2 with other homologous profilin sequences was also performed by CLC Genomics Workbench 3.0 software. Sequence identities and similarities between the Pop n 2 and homologous profilins from related and unrelated sources were calculated by SIAS (<http://imed.med.ucm.es/Tools/sias.html>) using the default parameters.

Construction of Expression Plasmid, pET-21b (+)-Pop n 2

To generate pET-21b (+)- Pop n 2 expression plasmid (Novagen, NJ, USA), the restriction sites of *Nde* I and *Xho* I restriction enzyme were introduced at the 5' and 3' ends of the Pop n 2 cDNA using the PCR method by two specific primers as follows: forward primer with restriction site for *Nde* I, 5'-**TCCCATATG**TCGTGGCAGGTGTACGTTG-3' and reverse primer with restriction site for *Xho* I, 5'-**TACTCGAGA**AAGGCCCTGGTCAATTAG-3' (restriction sites have been shown in bold form). Subsequently, both the PCR product and plasmid pET-21b (+) were doubly digested by restriction enzymes *Nde* I and *Xho* I, and then were ligated at 37 °C for 12 hours. The recombinant plasmid containing the Pop n 2 gene was transformed into the *E. coli* BL21 (DE3) and then the transformants were screened for ampicillin resistance. The presence of recombinant plasmid pET-21b (+)-Pop n 2 in resistant colonies was confirmed by enzymatic digestion and complete DNA sequencing.

Production and Purification of rPop n 2

A transformed colony from the expression host *E. coli* BL21 (DE3) containing the recombinant plasmid (pET-21b (+)-Pop n 2) was cultured in 2 ml lysogeny broth (LB) medium containing 100 µg/ml of ampicillin, and then incubated overnight at 37 °C until OD₆₀₀ reached 0.5, at which time, to induce expression of the rPop n 2, IPTG was added to a final concentration of 0.5 mM. Moreover, the incubation continued for 12 hours at 20 °C to increase the solubility of the rPop n 2. Afterwards, the cells were collected by centrifugation at 12,000g at 4 °C for 5 min, and resuspended using lysis buffer containing 50 mM/l HCL, 15 mM imidazole, 100 mM NaCl,

10% glycerol, and 0.5% Triton X-100, and then were disrupted by sonication. The rPop n 2 expressed was purified using Ni²⁺-NTA agarose columns (Invitrogen, CA, USA) according to the manufacturer's instructions. Finally, the quality and quantity of the purified rPop n 2 were evaluated by SDS-PAGE and Bradford method, respectively.

ELISA and IgE-Immunoblotting Inhibition Assays of rPop n 2

Levels of specific IgE antibodies against the rPop n 2 were measured by indirect ELISA as described above in specific ELISA assay. The only difference was that in ELISA inhibition experiment the ELISA microplate wells were coated with 130µl of coating buffer containing 1.5µg/well of purified rPop n 2.

For ELISA inhibition assay of the rPop n 2, pooled sera prepared from allergic patients with high IgE-specific titers against rPop n 2 (Patients no. 1, 2, 3, 6, 9, 12, 13, 14, and 20, as shown in Figure 1b and Table 1) was pre-incubated overnight at 4 °C with concentrations of 0.01, 0.1, 1, 10, 100 and 1000 µg of the purified rPop n 2 and *P. nigra* pollen extract both as inhibitor. Also, BSA 2% was used as a negative control. Finally, the percentage of inhibition was calculated by the following equation: [(OD of samples without inhibitor - OD of samples with inhibitor) / (OD of samples without inhibitor)] ×100.

The purified rPop n 2 was evaluated by SDS-PAGE under reducing conditions and was afterward transferred to the PVDF membrane by the Electroblotting method. Subsequently, the reaction of IgE antibody with PVDF membrane-blotted with purified rPop n 2 was analyzed as previously described above. To analyze the cross-inhibition between the natural pollen extract and rPop n 2, a mixture of 800 µl of pooled sera (diluted 5-fold) prepared from aforementioned patients was incubated with *P. nigra* extract (90 µg/mL, as an inhibitor), rPop n 2 (60 µg/mL, as an inhibitor), or BSA (as a negative control) overnight at 4°C with shaking. Afterwards, the reaction of pre-incubated sera with PVDF membrane-blotted with natural pollen extract was analyzed.

Physiochemical Analysis and Secondary Structure Prediction of rPop n 2

Using the default parameters ProtParam tool-ExPASy (<https://web.expasy.org/protparam/>) rPop n 2, physicochemical properties were calculated. The properties of the secondary structure of rPop n 2 was predicted using the Jpred server (<http://www.compbio.dundee.ac.uk/jpred/>).

Results

Patients, Controls and SPTs

This study included 20 patients, 14 men and 6 women (mean age of 33.25 ± 3.11 years, in the range of 9-59 years) (Table 1). All the patients suffered from seasonal allergy and rhinitis and none had asthma. SPT of these patients was positive with *P. nigra* pollen extract (Table 1).

Also, in this study, the sera of 10 subjects, 6 men and 4 women (mean age of 34.20 ± 4.57 years, range 14-53 years), without respiratory allergies and SPT negative for *P. nigra* pollen extract was used as a pooled sera and the negative control. It is noteworthy that the most common clinical manifestations in allergic patients participating in this project were nose (sneezing, runny, blocked) and eye symptoms (itching, dryness).

The mean diameter of positive SPT was 8.95 ± 0.5 compared to a negative control (Table 1). All the patients with positive SPT had approximately one or more of the clinical symptoms of allergic rhinitis (Table 1).

Serum Levels of Total and Specific IgE Antibodies

All the patients had high total IgE levels with a mean of 272 ± 29.35 (Table 1). Moreover, all the twenty patients had high specific IgE levels against *P. nigra* pollen proteins with a mean of 1.58 ± 0.13 (Table 1).

Protein and IgE-Binding Profiles of P. nigra Pollen Extract

The protein composition of *P. nigra* pollen extract was analyzed by SDS-PAGE and then Coomassie Brilliant Blue Staining. SDS-PAGE under reducing conditions showed that there were more than 10 protein bands with a molecular weight ranging from 8 to 100 kDa in the *P. nigra* pollen extract. The major bands had molecular weights of about 35, 40, 50, 60, 65, 72, and 100 kDa (Figure 1a). Other major bands had molecular weights of approximately 8, 10, 14, 17, and 27 kDa. Furthermore, SDS-PAGE of *P. nigra* pollen extract under non-reducing conditions showed that there were more than 8 protein bands with molecular weights around 12 to 90 kDa (Figure 1a).

The IgE reactivity of the separated protein bands of the *P. nigra* pollen extract were evaluated by immunoblotting using sera from allergic patients. Specific IgE binding protein bands probed with sera from all the twenty allergic patients have been indicated in Figure 1b. The results revealed that the IgE antibody-reactive protein bands had molecular weights between 14 to 100 kDa. Proteins with molecular weights about 14, 17, 27 and 72 kDa showed the greatest reactions with specific IgE antibodies in the immunoblotting assay. The IgE-reactive protein band with the molecular weight around 14 to 14.5 kDa from the *P. nigra* pollen extract was identified as profilin based on MALDI-TOF MS experiments (unpublished data).

Other IgE antibody-reactive protein bands also had molecular weights of approximately 30, 35, 40, 45, 58, and 100 kDa. Also, no protein bands reacted with the sera pool of negative controls in the immunoblotting assay (Figure 1b).

Sequence Analysis of the Full Length of Pop n 2

The result of the PTZ57R/T recombinant plasmid sequencing of Pop n 2 gene indicated an open reading frame of 396 bp encoding for 131 amino acid residues. The sequence of Pop n 2 was submitted to the NCBI GenBank with accession no. MN832852.1. Furthermore, the profilin of *P. nigra* (Pop n 2), as the first allergen, was submitted to the WHO/International Union of Immunological Societies (IUIS), Allergen Nomenclature Sub-committee (<http://www.allergen.org/>). Uniprot and BLASTp databases were used to search the homologous sequences of Pop n 2. The alignment of the amino acid sequence of Pop n 2 with homologous sequences was performed (Figure 2). It is noteworthy that some common allergenic profilins in our region and many other countries (7-14) were also considered for amino acid sequence alignment. SIAS results show that Pop n 2 has the most identity and similarity with allergenic profilins of *Hevea brasiliensis* (Hev b 8.0203) and *Arachis hypogaea* (Ara h 5) respectively, as it has been presented in Figure 2.

Production and Purification of rPop n 2

The expression of rPop n 2 was induced in *E. coli* BL21 (DE3) harboring the plasmid pET 21b (+)-Pop n 2 by the addition of 0.5 mM IPTG at 25 °C for 12 h. The results have been shown in Figure 3a. rPop n 2 was expressed as a fusion protein with a 6×His-tag at the carboxyl terminus for purification by affinity chromatography (Ni²⁺-NTA) resin. The rPop n 2 solubility

in the supernatant allows it to be further purified by affinity chromatography (Ni^{2+} -NTA) resin. The Bradford method revealed that approximately 7 mg of rPop n 2 was purified per liter of bacterial expression medium. SDS-PAGE was performed on the supernatant and lysate of the expressing cells, and a protein with a molecular weight of approximately 15 kDa was observed (Figure 3a).

Specific IgE Levels to rPop n 2 and ELISA Inhibition Assay

Specific IgE levels were measured in all serum samples of the patients against rPop n 2. Approximately, 65% of the patients (with a mean of $\text{OD}_{450} 0.86 \pm 0.14$) had significant levels of IgE antibody against the rPop n 2 (Table 1). Through ELISA inhibition, the binding capacity of the rPop n 2 to the IgE antibody was compared to its natural counterpart in the *P. nigra* pollen extract. It was found that the pre-incubation of pooled sera with 1000 $\mu\text{g}/\text{ml}$ of rPop n 2 and natural pollen extract significantly inhibited IgE antibody binding to rPop n 2 in the ELISA microplate wells (93% and 87%, respectively) (Figure 4).

Immunoblotting and Immunoblotting Inhibition of rPop n 2

In purified rPop n 2 immunoblotting (Figure 3b), a protein with a molecular weight of about 15 kDa was found to significantly responded to serum from eleven allergic patients (patients no. 1, 2, 3, 5, 6, 8, 9, 12, 13, 14, and 20, as shown in Figure 1b and Table 1). Moreover, immunoblotting inhibition results showed that pre-incubation of pooled sera (Patients no. 1, 2, 3, 6, 9, 12, 13, 14, and 20, as shown in Figure 1b and Table 1) with rPop n 2 significantly inhibited the IgE antibodies for binding to PVDF membrane-blotted with *P. nigra* pollen extract (Figure 5).

Physicochemical and Structural Analysis of rPop n 2

Analysis of the physicochemical properties of rPop n 2 using ExPASy tool showed that it had 131 amino acids, molecular weight was 14.01, hypothetical pI 4.49, instability index was 25.09, aliphatic index was 90.76, grand average of hydropathicity (GRAVY) was 0.028, and the total positive and negative charges were 8 and 16, respectively. Furthermore, the secondary structural properties of rPop n 2 were predicted using the Jpred server, and it was shown that it has 3 helices (28.24%), 7 β -sheets (31.30%), and 11 random coils (40.45%). In this analysis, random coils showed the highest number of residues followed by β -sheets and α -helices. The least

residues were detected in the α -helices, and the highest residues were detected in the random coils of the rPop n 2 (Figure 2).

Discussion

In many countries, including Iran, poplar pollen has been reported to be a significant source of airborne allergens, and its pivotal role in the intensification of respiratory allergies (7-12, 14, 24) has been approved. In a study, more than 28 proteins were predicted in *Populus deltoides* pollen as allergen candidates through the SDAP database (25). In another study, more than 27 allergenic proteins were predicted in *Populus tomentosa* pollen as allergens candidates through the SDAP database (26). *P. nigra* or *black poplar* (*Lombardy poplar*) is a species of poplar which is widely distributed throughout Europe, Central and West Asia, and North Africa (27). *P. nigra* is also abundant in Central, Northern and Western Iran (28).

The number of studies on the pollen allergenicity of poplar species is limited. In the present study, the immunochemical characterization of *P. nigra* pollen extract was performed in order to identify allergenic proteins that bind to IgE antibodies. In the next step, the gene sequence of the major reactive protein with IgE antibody was cloned, sequenced, and expressed.

All the selected patients showed a wheal greater than 3 mm in the SPT. It was also found in this study that the majority of patients with a positive SPT had a high level of specific IgE antibody against proteins present in *P. nigra* pollen extract. This implies that sensitive patients exhibit the same responses in the conditions of in vivo (SPT) and in vitro (specific ELISA). Also, the use of grass mix, weed mix, and tree mix pollen extracts in this study was to minimize the possibility of cross reactivity among allergenic profilins. Thereby, in this study, we selected patients who had an absolute sensitivity to *P. nigra* pollen extract.

SDS-PAGE electrophoresis showed that there were more than 10 protein bands with molecular weights of approximately 8 to 100 kDa in *P. nigra* pollen extract in reducing conditions. In non-reducing conditions, the patterns of bands were different, and approximately 8 bands with molecular weights ranging from 12 to 90 kDa were observed on SDS-PAGE gel. These distinct patterns of bands in reducing and non-reducing conditions imply the existence of cysteine amino acid disulfide bonds in the protein structure which is reduced in reducing conditions by 2ME buffer. One of the characteristics common to many allergens, particularly food allergens, is that they are resistant to denaturation due to the presence of disulfide bonds (29). Degradation of disulfide bonds has been shown to make available IgE-bound epitopes in

the allergen molecule, which may be beneficial for their allergenicity and identification in immunological procedures (29, 30). Generally, these observations suggest that certain proteins in *P. nigra* pollen extract are susceptible to degradation as a result of proteolysis or exposure to a reducing condition. Previous studies have also indicated that some pollen proteins, such as methionine synthase, are transformed into two fragments in reducing conditions (31). Furthermore, the number and size of degraded proteins may depend on the conditions of pollen extract preparation and storage.

Immunoblotting results have shown that several proteins with molecular weights between 14 to 100 kDa have the greatest reaction with the IgE antibody present in the sera of allergic patients. It has also been observed that proteins with molecular weights of approximately 14, 17, 27, and 72 kDa have the greatest reaction with specific IgE antibodies. As shown in Figure 1b, 65% of sensitive patients to *P. nigra* pollen showed severe responses to approximately 14.01 kDa proteins. According to previous studies conducted on the pollen of *Salsola kali*, *Amaranthus retroflexus*, *Acacia farnesiana*, *Fraxinus excelsior*, and *Prosopis juliflora* plants, this protein of about 14 kDa to 15 kDa is related to the profilin family (32-36). Profilins are a family of proteins (12 to 15 kDa) that are present in eukaryotic cells and have been identified in many plant sources such as weeds, fruits, and trees (37). Their highly conserved structure elicits cross-reactivity with IgE antibodies and is therefore known as pan-allergen, and they are responsible for most diagnostic errors in allergic patients (36, 38). Regarding the IgE binding frequency of the profilin (Pop n 2), this protein was considered as an important allergen in *P. nigra* pollen extract. The gene of this allergenic protein was amplified, cloned, sequenced, and the recombinant protein expressed was called rPop n 2. Moreover, the highest identity and similarity of Pop n 2 with other homologous profilin sequences demonstrate the conservative nature of the sequence of profilins among related and unrelated species (Figure 2).

In the present study, pET-21b (+) system was used to express the rPop n 2 in the *E. coli* BL21 (DE3), and after adding 0.5 mM IPTG, the temperature was lowered to 20° C for 12 h. Following this protocol, a significant increase in the soluble form of rPop n 2 was observed. This approves that clumping of protein decreased at a lower temperature. (32) Afterwards, the expression of the rPop n 2 was confirmed by SDS-PAGE and Bradford methods. Moreover, the rPop n 2 showed the reaction with serum IgE antibody of patients by ELISA and immunoblotting methods. Also, rPop n 2 significantly inhibited specific-IgE reactivity of pooled sera from the

selected patients in ELISA and immunoblotting inhibition assays. These results could indicate the correct folding of the rPop n 2 and appear to retain IgE-binding epitopes, like its natural counterpart in the *P. nigra* pollen extract. However, further studies are required for more precise investigation of this point.

The physicochemical analysis of rPop n 2 using the ExPASy showed that it has a molecular weight of 14.01. The pI 4.49 value indicated that rPop n 2 is an acidic protein. Furthermore, regarding the total number of positive and negative charges, it could be deduced that rPop n 2 is hydrophobic and fat-soluble. The rPop n 2 instability index is calculated to be 25.09, and since if the instability index value of a protein is greater than 40 it is an unstable protein, (39) rPop n 2 is a stable protein. The aliphatic index of rPop n 2 was 90.76, which indicates its relative thermostability. It was also shown that the GRAVY value of rPop n 2 was 0.028. Analysis of the secondary structure of rPop n 2 using the Jpred server showed that it contained 3 α -helixes, 7 β -sheets, and 11 random coils. The rPop n 2 protein, like all allergic profilins, consists of 3 α -helixes and seven β -sheets (40). α -helix and β -sheet regions are the most common domains located inside the proteins, while random coil regions are on the surface of the proteins. Hence, they can have epitopes that bind to antibodies (41).

The comparison of the amino acid sequence of Pop n 2 with common allergenic profilins indicated that there was a high level of identity among the profilin of *P. nigra* and other common allergenic plant profilins in our region (32, 33, 42, 43). This high degree of identity and similarity could play a pivotal role in IgE-cross reactivity in allergic patients.

The results of this study showed that proteins of the *P. nigra* tree pollen extract are allergenic, and a protein (Pop n 2) about 14 kDa is relatively high IgE reactive (65%, 13/20, figure 1). Moreover, Pop n 2 as the first allergen of *P. nigra* pollen grains, belongs to the family of profilin proteins. Considering the relatively high prevalence of allergy to pollen grains from the *P. nigra* in Iran and other countries (7-12, 14, 24), efforts are now underway to clone cDNA coding other allergic proteins such as 17, 27, and 72 kDa of the *P. nigra* pollen grains, that would lead to the exploration of new guidelines for diagnostic, therapeutic, and preventive purposes.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Table 1 Clinical features, total and specific IgE levels, prick skin test reaction of patients and controls

Patient No.	Age (years)/gender [†]	Symptoms [‡]	Total IgE (IU/ml)	<i>P. nigra</i> pollen extract		rPop n 2
				SPT (mm) [§]	Specific IgE	Specific IgE
1.	34/M	T,N,E	102	9	1.91	1.4
2.	19/M	T,N,E	465	10	1.73	1.0
3.	19/F	T,N,E	243	9	1.99	1.2
4.	41/M	N,E	117	5	0.65	0.06
5.	49/M	T,N,E	125	9	1.45	1.11
6.	32/F	T,N,E	435	11	2.25	1.57
7.	34/F	N,E	228	6	0.98	0.04
8.	11/M	T,N,E	465	12	2.24	1.7
9.	9/M	T,N,E	126	12	2.11	1.39
10.	41/M	T,N,E	381	9	1.01	0.11
11.	22/F	N,E	132	8	0.92	0.10
12.	48/M	T,N,E	286	12	2.24	1.35

13.	18/M	N,E	212	10	0.91	0.76
14.	41/M	T,N,E	417	10	1.8	1.34
15.	59/F	T,NE	340	11	2.38	1.5
16.	24/M	T,N,E	200	10	1.64	0.09
17.	38/F	N,E	253	5	0.83	0.12
18.	32/M	T,N,E	435	11	1.69	1.27
19.	51/M	N,E	113	6	0.76	0.04
20.	43/M	T,NE	368	12	2.14	1.15
Controls						
1.	14/F	-	51	0	0.02	0.02
2.	50/F	-	35	0	0.04	0.03
3.	32/M	-	63	0	0.05	0.04
4.	45/F	-	30	0	0.03	0.04
5.	53/M	-	74	0	0.06	0.03
6.	16/M	-	38	0	0.05	0.03
7.	20/M	-	57	0	0.02	0.02
8.	46/M	-	30	0	0.04	0.03
9.	27/F	-	45	0	0.06	0.04
10.	39/M	-	66	0	0.09	0.08

[†] M, male; F, female.

[‡] T, throat symptoms (itchy and sore throat); N, nose symptoms (sneezing, itchy, runny, and blocked); E, eyes symptoms (itching, and watery eyes).

[§] The mean wheal diameters are displayed in mm. Histamine diphosphate (10mμ) positive control; glycerin-negative control.

[†] Levels of specific IgE to *P. nigra* pollen extract by ELISA (optical density at 450 nm). Cut-off: OD > 0.14 (three times more than mean OD of negative controls).

Figure legends:

Fig. 1. SDS-PAGE and IgE immunoreactivity of *P. nigra* pollen extract. **(a)** Coomassie brilliant blue stained SDS-PAGE of the crude extract of *P. nigra* pollen extract in reducing and non-reducing conditions on 12.5% acrylamide gel. Lane MW, molecular weight marker (SMOBiO PM1700); R, reducing condition; NR, non-reducing condition. **(b)** Immunoblotting of *P. nigra* pollen extract (with reducing SDS-PAGE). Each strip was first blotted with *P. nigra* pollen extract. All strips were then incubated with allergic patients' sera and probed for IgE reactive protein bands. Lane MW, low molecular weight. NC, negative control.

Fig 2. Amino acid sequence alignment of Pop n 2 (MN832852.1) with other homologous profilin sequences from related and unrelated sources. *Hevea brasiliensis* (Hev b 8, AAF34343), *Arachis hypogaea* (Ara h 5, AAD55587), *Chenopodium album* (Che a 2, ACR77509), *Cucumis melo* (Cuc m 2, AAW69549), *Amaranthus retroflexus* (Ama r 2, ACP43298), *Betula pendula* (Bet v 2, ABG48509), *Salsola kali* (Sal k 4, ACS34771). The top line amino acid sequence alignment represents the secondary structure of Pop n 2 created by the Jpred server. The helixes are cylinder, β - sheets are arrows, and random coils are gray lines.

Fig. 3. SDS-PAGE and IgE-immunoreactivity of the rPop n 2. **(a)** Lane MW, molecular weight marker (SMOBiO PM1700). Lane 1, supernatant from cell culture containing pET 21b (+)-Pop n 2 along with IPTG. Lane 3, purified rPop n 2 (as an approximately 15 kDa recombinant protein along with 6 $\times$ His-tag). **(b)** IgE immunoblot of purified rPop n 2. Lanes 1–11 probed with sera from patients positive for rPop n 2 (These patients are the same patients with numbers 1, 2, 3, 5, 6, 8, 9, 12, 13, 14, and 20, respectively in Figure 1b); Lane NC, negative control.

Fig. 4. ELISA inhibition assay with rPop n 2 and *P. nigra* pollen extract. Inhibition of IgE binding capacity of rPop n 2 by ELISA using purified rPop n 2 and *P. nigra* crude extract. BSA 2% was used as a negative control. Also, the error bars show the standard deviation between the duplicated samples for rPop n 2, *P. nigra* pollen extract and BSA.

Fig. 5. Immunoblotting inhibition of rPop n 2. Lane MW, molecular weight marker (SMOBiO PM1700). *P. nigra* pollen extract after SDS–PAGE, blotted onto PVDF membrane (Lane 1, 2 and 3). Lane 1, Strip incubated with pre-incubated pooled sera with 90 µg of *P. nigra* pollen extract as an inhibitor (*P. nigra* pollen extract inhibit IgE-reactivity of pooled sera with blotted proteins of *P. nigra* pollen extract). Lane 2, Strip incubated with pre-incubated pooled sera with BSA 2% (as a negative control). Lane 3, Strip incubated with pre-incubated pooled sera with 60 µg rPop n 2 as an inhibitor (positive control). Lane 4, Strip blotted with rPop n2 that reacted with pooled sera without inhibitor (specific IgE against natural Pop n 2 in allergic patients' sera also reacted to blotted rPop n 2).

Appendices:

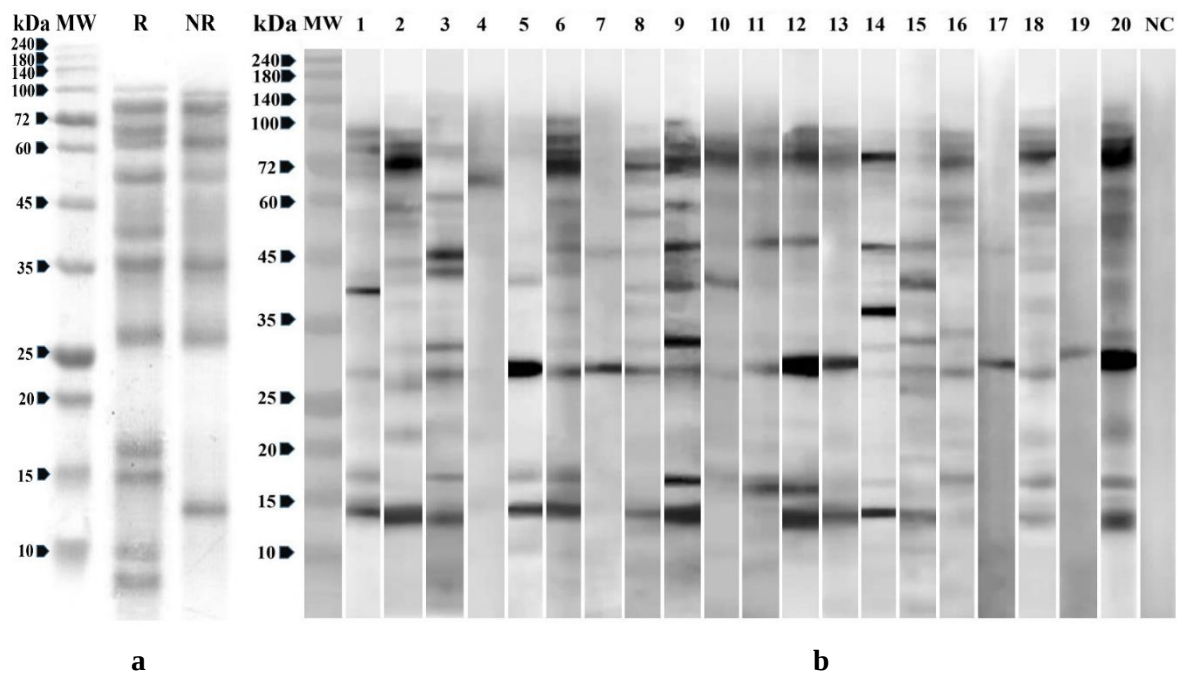


Figure.1

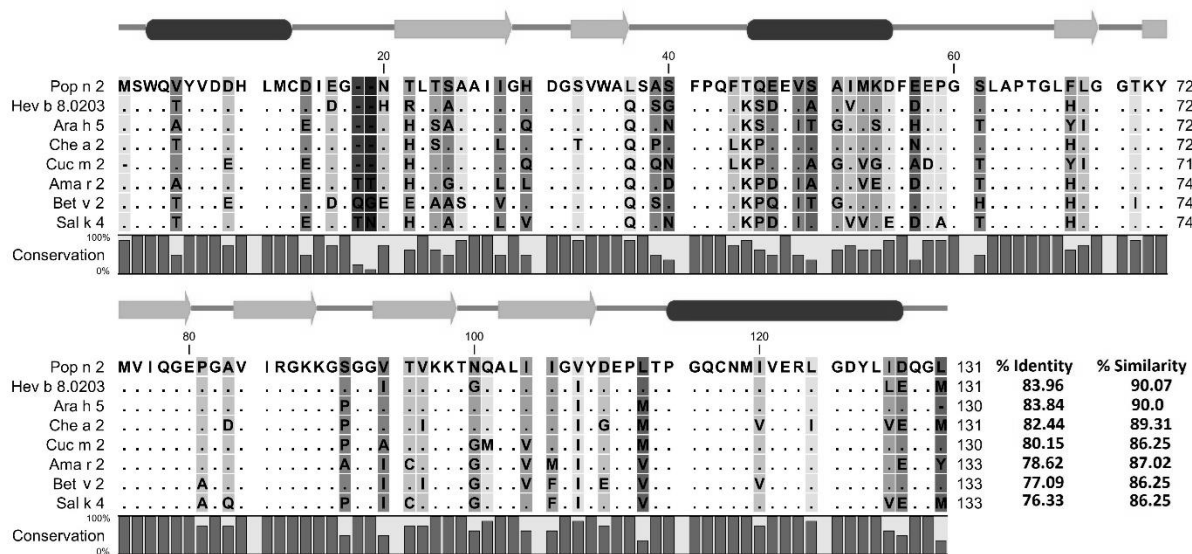
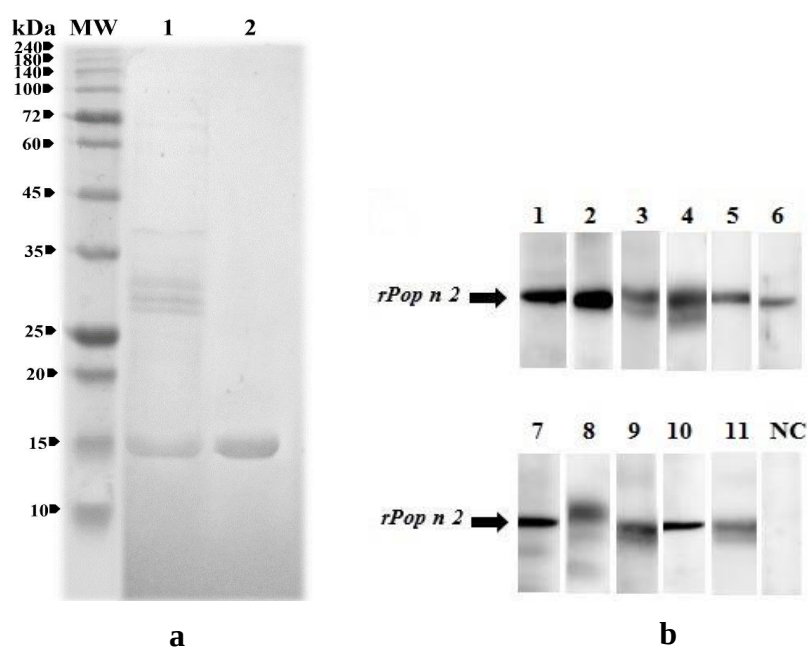


Figure.2

**Fig.3**

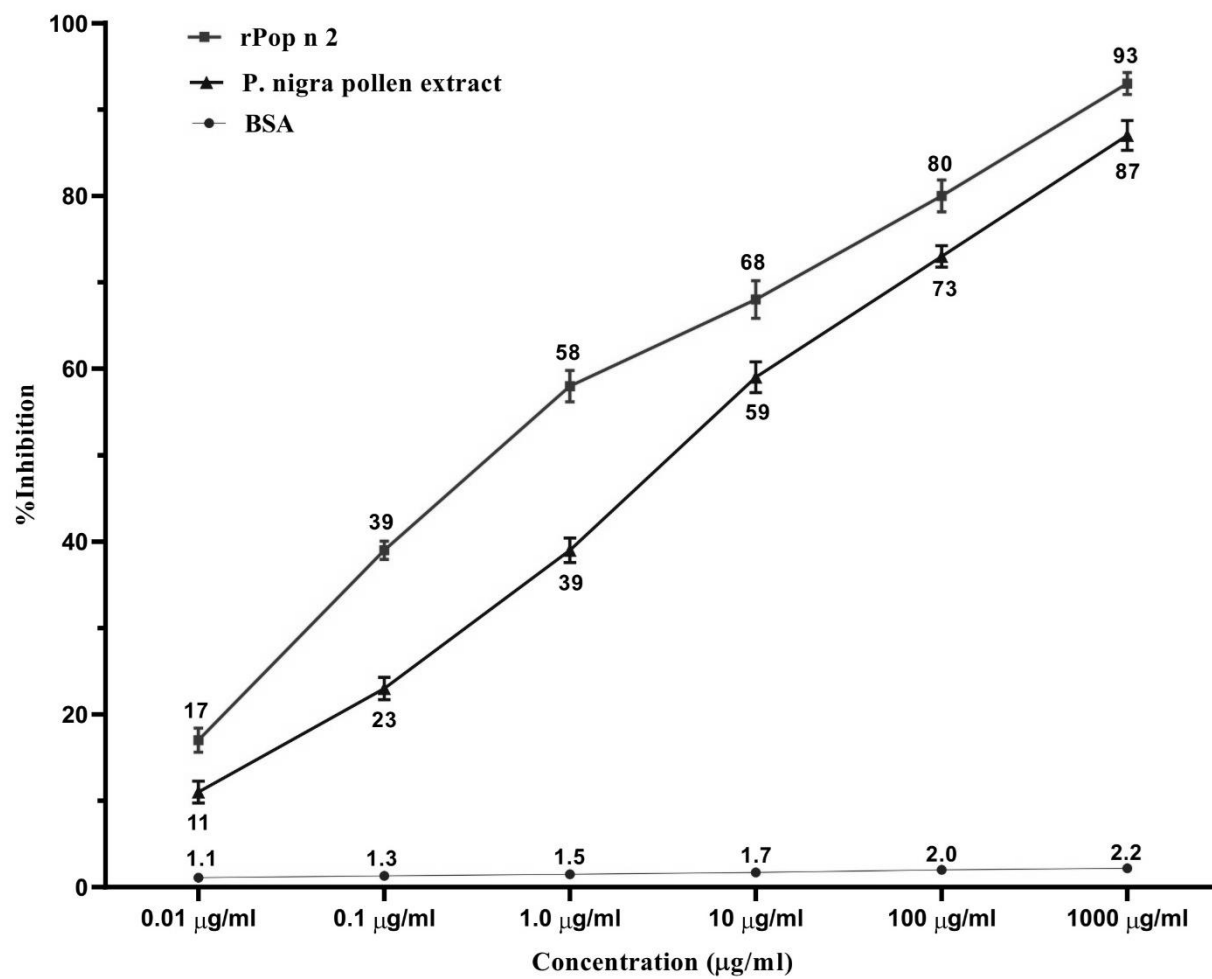
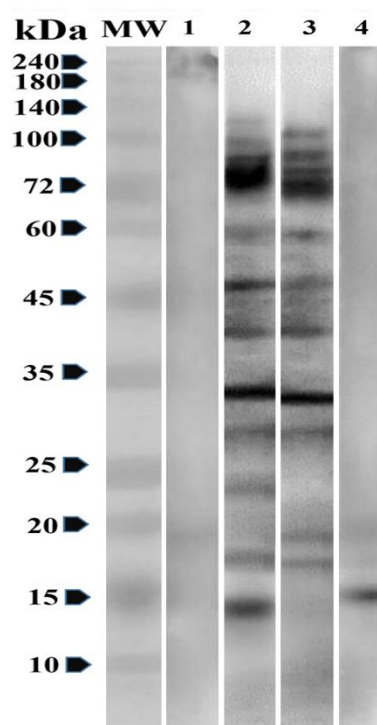


Fig.4

**Fig.5**