



Identification of a 53 kDa protein, as a new high molecular weight allergen from *Fraxinus excelsior* (Ash) pollen

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Received: 18 February 2020 / Accepted: 11 May 2020

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Summary

Background *Fraxinus excelsior* (Ash) is a common tree and is important cause of winter–spring pollinosis in many temperate regions in the world. In this study, a high molecular weight allergen from ash pollen was identified.

Methods In all, 20 individuals allergic to ash participated in the study. Characterization and immunoreactivity of ash pollen proteins was performed using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), two-dimensional (2D) gel electrophoresis and immunoblotting.

Results Immunoglobulin E (IgE)-binding proteins with apparent molecular mass ranging from 9 to 110 kDa were detected in ash pollen extract. Serum IgE of 7 (35%) patients reacted with a 53-kDa protein band. Analysis of 2D immunoblots showed several IgE-binding proteins. Moreover, mass spectrometry

analysis indicated that the 53-kDa allergen was homologous to calreticulin.

Discussion We defined a novel allergen from *F. excelsior* pollen with a molecular weight of about 53 kDa. This allergen could be considered as an important high molecular weight allergenic protein for further studies on cross-reactivity and development of diagnostic and therapeutic approaches.

Keywords Ash pollen · Allergen · 2D electrophoresis · IgE immunoblotting · Calreticulin

Abbreviations

2-DE	Two-dimensional electrophoresis
BSA	Bovine serum albumin
CBB	Comassie brilliant blue
CRT	Calreticulin
ER	Endoplasmic reticulum

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HMW	High molecular weight
IAARI	Immunology, Asthma and Allergy Research Institute
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IPI	International protein index
LiquidDAB+	High-sensitivity liquid diaminobenzidine
MALDI	Matrix assisted laserdesorption/ionization
MW	Molecular weight
PVDF	Polyvinylidene fluoride
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TOF	Time of flight

Introduction

Ash tree pollen is a principal representative of the Oleaceae family and according to Poncet et al., they are one of the main causes of respiratory allergies [1]. Their distribution extends in Europe from the Atlantic coast to Russia and to northern parts of Spain, Italy, Greece, and as far south as 37°N in Iran [2].

The genus *Fraxinus* is very diverse in terms of the morphology of its reproductive system and this diversity has led to the emergence of species with a high degree of adaptation to environmental conditions [3]. Previous studies noted great diversity within the same population of *Fraxinus* such as sexual phenotypes based on traits of pure male, female, and hermaphrodite flowers [2, 4].

In many Mediterranean countries and some parts of Europe, especially Grabs in Switzerland, knowledge of sexual phenotypes of *Fraxinus* is very important because sensitization to pollen has long been underestimated and the overlapping pollination period and cross-reactivity with other trees such as *Betula* (birch), *Populus* (poplar), and grasses [5, 6].

Ash pollen is typically abundantly found in the atmosphere in Tehran, the capital of Iran from the end of January to the start of April [7]. Thus far, previous studies have identified and reported several ash pollen allergens [1, 8–12].

Poncet et al. roughly categorized *Fraxinus* allergens into four major groups namely: (I) the major allergen *Fra e 1* (*Ole e 1*-like), which is a glycosylated protein that was sequenced and cloned [13], (II) *Fra e 2*, a member of the *Pan* allergen profilin family, (III) *Fra e 3*, an allergen belonging to the Ca²⁺-binding protein family and (IV) proteins which were indirectly identified as high molecular weight (HMW) proteins (>30 kDa) [1]. These high molecular weight proteins are the most important ash pollen allergens reported in a wide spectrum of patients around the world [9, 10, 14].

Objectives

Due to the relatively few studies on ash pollen HMW IgE-reactive proteins, in this study, we investigated one of the most IgE-reactive HMW allergenic proteins in mature *Fraxinus excelsior* pollen.

Materials and methods

Ash pollen sampling and protein extraction

Fresh pollen was collected during ash pollination season (mid-January to mid-March) from trees planted in an urban green space of Tehran, the capital city of Iran.

Pollen materials with more than 95% pollen and less than 5% floral parts of the same plant were taken for protein extraction. The samples were then stored at −20°C for further analysis. To extract proteins from ash pollen, 1 g of pollen was gently mixed with 20 ml of 0.01 M (pH 7.4) phosphate buffered saline (PBS) for 14 h at 4°C [15]. The mixture was then centrifuged at 13,000×g for 20 min at 4°C and the supernatant dialyzed against distilled water overnight. The extract was then freeze-dried and stored at −70°C until further use. Total protein content of pollen extract was determined by Bradford protein assay [16] using bovine serum albumin (BSA) as the standard.

Allergic patients' sera

Serum samples were obtained from 20 patients who were allergic to ash pollen, who had been referred to the Immunology, Asthma and Allergy Research Institute (IAARI) in Tehran. Blood samples (5 ml) were drawn from 20 patients and 5 non-allergic individuals who showed no specific IgE to ash pollen extract (as controls) after participants gave their written consent. Ethical approval was sought and granted by the human ethics committee of the IAARI. Sera samples from all patients and healthy individuals were stored at −20°C until use. Patients' data which included gender, age, and allergic reactions to a mixture of trees (cypress, olive, ash, acacia, poplar, planetree) were determined using RIDA panel and the recorded data are shown in Table 1.

The specific IgE for inhalant allergens were measured using a multiplex test kit on nitrocellulose membrane (RIDA qLine Allergy, Darmstadt, Germany). Based on the manufacturer's instruction, the allergen-specific IgE concentration was reported in IU/ml, and the test result was considered positive if the concentration of specific IgE was ≥0.35 IU/ml.

SDS-polyacrylamide gel electrophoresis

Aliquots of 60 µg of total protein were separated using 12% SDS gel under reducing conditions. Prior to separation, 2% 2-mercaptoethanol was added to the

Table 1 Clinical characteristics and specific IgE values of patients reactive to ash pollen extract

Sample No.	Gender ^a	Age (Years)	Symptoms ^b	Ash-specific IgE (IU/ml)
<i>Patients</i>				
P1	M	34	AR	1.04
P2	M	30	AR, AC	1.17
P3	M	36	AR, AC	0.53
P4	M	28	AR, AC	5.29
P5	M	43	AR, W	15.61
P6	M	38	AR, AC	1.54
P7	F	30	AR, AC	2.1
P8	M	37	AR	0.70
P9	M	27	W, AR, AC	1.76
P10	M	37	W, AR, AC	0.41
P11	M	23	AR, AC	2.39
P12	M	23	AR	2.62
P13	M	37	Asthma, AR	2.57
P14	F	37	W, AR, AC	8.44
P15	M	40	AR	8.2
P16	M	37	AR	7.5
P17	M	30	AR, AC	8.1
P18	F	21	W, AR, AC	1.88
P19	F	32	W, AR, AC	1.32
P20	F	19	AR	2.45
<i>Controls</i>				
C1	M	32	Negative	0.1
C2	F	23	Negative	0.1
C3	F	37	Negative	0.07
C4	F	32	Negative	0.2
C5	F	32	Negative	0.1

^aM Male; F Female, IgE Immunoglobulin E
^bAR Allergic rhinitis; AC Allergic conjunctivitis; W Wheezing

protein extracts and the samples were heated in an oven at 90 °C for 10 min before loading these onto the gels. The gel was initially run at 17 mA and after the tracking dye had moved down into the separation gel, the current was increased to 25 mA [17]. The sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed under denaturing condition using a vertical gel electrophoresis apparatus (GE Life Sciences, USA) along with a standard protein molecular weight (MW) marker (Fermentas, St. Leon-Rot, Germany). The protein bands were observed by CBB-R250 staining. The molecular masses of protein bands were estimated with Image J (<https://imagej.nih.gov/ij/>) analysis software and then compared with protein markers of known molecular weights (Fermentas, St. Leon-Rot, Germany).

Two-dimensional gel electrophoresis

We also investigated ash pollen protein extract using 2D gel electrophoresis as described in a previous study with some modifications [18]. Aliquots of

400 µg of pollen extract were reconstituted in 125 µl isoelectric focusing (IEF) buffer with 1% immobilized pH gradient (IPG) with cleaned wide strips of 7 cm, pH 3–11 NL (GE Healthcare, Uppsala, Sweden). Rehydration loading of the dry strips was done passively for 14 h in rehydration buffer containing 6 M urea, 2 M thiourea, 4% CHAPS (w/v), 2% DTT (w/v), 1.6% IPG buffer or carrier ampholytes and 0.4% bromophenol blue stock solution. IEF was performed using an Ettan IPGphor 3 system (GE Healthcare, Cytiva, Marlborough, MA01752, USA) at 20 °C and a maximum current of 50 µA/strip. After IEF, the strips were equilibrated for 20 min with equilibration buffer 1 (6 M urea, 0.003% Tris-HCl buffer pH 8.8, 29.1% glycerol, 2% SDS and 1% dithiothreitol) and then equilibrated for 20 min with equilibration buffer 2 (6 M urea, 0.003% Tris-HCl buffer pH 8.8, 29.1% glycerol, 2% SDS and 1.25% iodoacetamide). Electrophoretic separation in the second dimension was performed by laying strips on 12% SDS-PAGE gels (running at a constant voltage of 60 V for 3 h). Finally, 2-DE electrophoresis was run in duplicate and stained in CBB R250 [18].

IgE-specific 1D and 2D electrophoresis immunoblotting

Total protein profile in 12% SDS-PAGE was transferred from the gel on to membrane polyvinylidene fluoride (PVDF; GE Life Sciences, USA), IgE-specific western blotting was done according to a protocol previously described by Assarehzadegan et al. with some modifications [19]. The membrane was blocked for 1 h at room temperature with 3% BSA in tris-buffered saline (TBS) and then washed three times for 10 min in TBS-Tween 20 and incubated overnight with a primary antibody prior to adding 14 sensitive patients' sera diluted in 1:10 ratio in blocking solution at 4 °C. After the incubation, the membrane was washed three times and incubated for 1 h with 1:2500 goat anti-human IgE conjugated with peroxidase (Sigma, Munich, Germany) in PBS. The membrane was then washed again and the bound enzymatic activity of horseradish peroxidase was detected by high-sensitivity liquid diaminobenzidine (LiquidDAB⁺) chromogen (DAKO, Glostrup, Denmark). The spots in 2D gel, membrane stained with Ponceau S and immunoblotting and used to compare molecular weights and pI was determined in Quantity One Software (Bio-Rad). The ash pollen activity was visualized with Liquid DAB⁺. Imaging and statistical analysis of gels were scanned with an Image scanner (Amersham Pharmacia, Piscataway, NJ, USA), saved as TIFF images and analyzed with Image Master 2D Platinum 6.0 software (GE Healthcare) for spot detection, quantification, comparative, and statistical analysis.

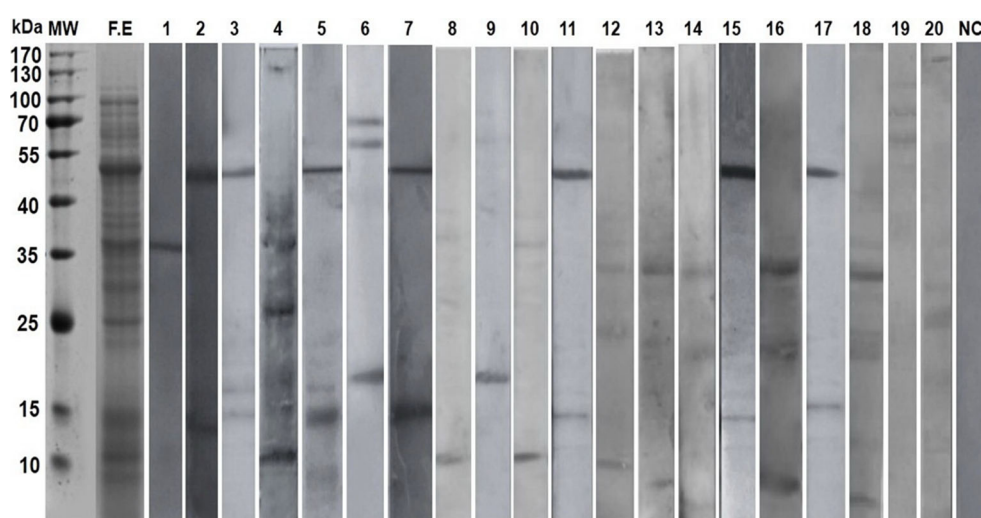


Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoreactivity of ash pollen extract Coomassie Brilliant Blue stained SDS-PAGE of the crude extract of *F. excelsior* pollen in reducing conditions on 12.5% acrylamide gel; lanes 1–20: Immunoblotting of ash

pollen extract. Each strip was first blotted with ash pollen extract. All strips were then incubated with allergic patients' sera and immunoglobulin E (IgE) reactive protein bands were detected. Lane MW: molecular weight marker (Fermentas, St. Leon-Rot, Germany); NC: negative control

Protein identification by mass spectrometric analysis (MALDI-TOF/TOF)

The differentially expressed IgE-reactive protein spot on one of the samples was selected and manually excised from preparative gels as candidate proteins for MALDI-TOF/TOF analysis. Analysis was carried out by the Proteomics Laboratory; University of York, U.K. The spots of interest were analyzed using a previously described protocol [20, 21]. Tandem mass spectral data were submitted to search in NCBI nonredundant protein database using MASCOT program from Matrix science (<http://www.matrixscience.com>) against the IPI (International Protein Index), through the Bruker Protein Scape interface (version 2.1). The parameters were set for MASCOT search were as follows: Database, NCBI nr; Taxonomy, Viridiplantae; Proteolytic enzyme, trypsin; Peptide tolerance, 100 ppm; Mass tolerance, 0.5 Da [18].

Results

SDS-PAGE and IgE-binding proteins of ash pollen extract

Analysis of the protein composition of ash pollen extract was performed by Coomassie Brilliant Blue staining (Fig. 1). The reducing SDS-PAGE of ash pollen extract revealed a number of protein bands with molecular weights (MWs) ranging from about 9 to 110 kDa. The prominent protein bands showed MWs of about 10, 13, 15, 36, 38, and 53 kDa (Fig. 1). Non-reducing SDS-PAGE results indicated that the pattern of migration of the ash pollen proteins in crude extract was not changed under non-reducing or reducing conditions (data not shown).

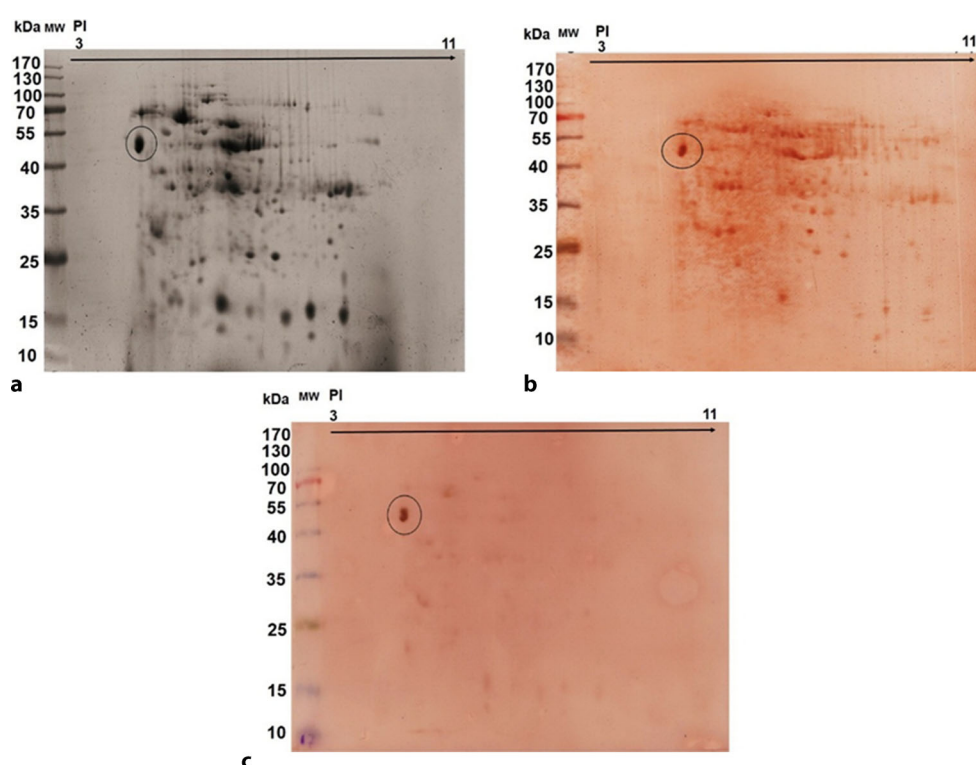
IgE binding reactivity of the protein from ash pollen extract was investigated using immunoblotting assays. Specific IgE reactivity fractions probed with sera from all 20 patients are shown in the Fig. 1. The results indicated several IgE reactive bands ranging approximately 10 to 65 kDa. The most frequent IgE reactive protein band among the patients with ash pollen allergy ($n=7$) showed a molecular weight of about 53 kDa (Fig. 1). No IgE-reactive band was identified when pooled sera of negative healthy control serum was assayed.

Two-dimensional gel electrophoresis analysis of ash pollen extract

Some spots observed after analyzing ash pollen extracts on a 2-DE were selected and excised upon CBB and analyzed by MALDI-TOF/TOF (Fig. 2). Immunoblotting assay of 2-DE was performed using the pooled sera of patients and one serum sample (patient no. 15 in Fig. 1) with high titer specific to IgE against ash pollen extracts.

A pattern of IgE reactivity was observed for ash pollen extracts in four areas: (1) mainly doublets MWs of around 16 to 21 kDa with a heterogeneous pI, (2) a series of 5 to 6 spots about MWs of 31 to 38 kDa, (3) a series of five spots MWs of 40 to 45 kDa with some differences, and (4) major spot at a MW of 53 kDa that are commonly observed for ash pollen extract. Moreover, 1D immunoblotting results revealed that the spot at a MW of approximately 53 kDa and also, from one patient was observed a single spot 53 kDa (Fig. 2a–c).

Fig. 2 Two-dimensional gel electrophoresis pollen extract from pollen of ash pollen extract. **a** Coomassie-stained two-dimensional gels, **b** Two-dimensional gel electrophoresis immunoglobulin E (IgE) immunoblot probed with the pooled 15 serum of ash pollen allergic patient, **c** A case report of a patient (patient No. 15 in Fig. 1) contains a 53-kDa spot. MW molecular weight marker (Fermentas, St. Leon-Rot, Germany)



Mass spectrometry analysis of the selected protein spot

The 2-DE IgE reactive spot at MW about 53 kDa was excised and subjected to MS/MS analysis using MALDI-TOF/TOF mass spectrometry. The results of mass spectrometry of 53 kDa spot for ash pollen extract are shown in Table 2.

Discussion

Pollen of ash tree is one of the important causes of pollinosis in various parts of temperate areas of Europe, the United States, Asia as well as in Iran [1, 9].

We carried out SDS-PAGE of ash pollen crude extract which revealed several bands with apparent MWs ranging from 9 to 110 kDa. There were some variations in molecular weights of ash pollen proteins of our study and those described by earlier studies [1, 22, 23]. This discrepancy could be due to differences in pollen extract serum samples, concentration of the gels and method of calculation of molecular weight [14].

SDS-PAGE results indicated that the pattern of migration of ash pollen proteins did not change in both reducing and non-reducing conditions (data not

shown). Therefore we postulated that the possible cysteine residues of ash pollen proteins may not be associated with interchain disulfide bonds.

While some of the low molecular weight (<30 kDa) of ash pollen proteins have been characterized [1, 11, 24], there has been limited data concerning the IgE-reactive proteins with relatively high molecular weight (HMW; >30 kDa) of ash pollen extract. In the present study, the 53-kDa protein showed specific IgE-reactivity to 35% (7/20) of patients' sera with ash pollen sensitization by immunoblotting assay.

We demonstrated that the 53-kDa protein is homologous to calreticulin (CRT). Calreticulin is a multifunctionality protein that has been identified as a key calcium binding molecular chaperone known to be involved in Ca^{2+} homeostasis and the proper folding of proteins in endoplasmic reticulum (ER) as well as in responses of plants to various stresses and binds its substrates at almost neutral pH in the ER and releases them in the Golgi because of a lower pH (about 6.5) [25, 26]. Calreticulin of the present study revealed significant immunoreactivity of the 53-kDa protein in patients with allergy, living in Tehran. A previous study by Poncet et al. in 2010 found a significant immunoreactivity in ash pollen extract proteins ranging 38 to 40 kDa. However, in some reports, this

Table 2 The 53 kDa immunoreactive protein identified from ash pollen extract using MALDI TOF/TOF MS

Protein name	Accession no.	Nominal mass (Mr)	Cal. pl	Taxonomy	Peptide sequence	Sequence coverage	Unique peptide	Protein score	Observed mass
Calreticulin isoform X1	Gil743830391	47.622	4.33	<i>Populus euphratica</i>	R.YVGIELWQVK.S	10%	2	224	1234.6

HMW protein, which has been identified as pectin methylesterase, was considered as an allergen in ash pollen and some other plants and foods [19, 26, 27]. The extended proteomic knowledge on major protein families triggering IgE-mediated allergies constitutes a good research basis to identify novel allergenic molecules from novel allergenic sources as well as predicting potential cross-reactivities among various allergenic species [13, 27].

Regarding high prevalence of ash pollen sensitization in many parts of temperate areas in the world and confirmed cross-reactivity among pollens of ash and allergenic pollens of some weeds (such as *Sal-sola kali*, *Kochia scoparia*) and trees (such as olive, a close taxonomically related tree) [10, 19, 22], it seems necessary to use purified, natural or recombinant ash pollen allergens for a more comprehensive allergen-based diagnosis and therapy. Moreover, in our study, pollen collection was performed from ash trees regardless of sex phenotypes; since there are different phenotypes of ash tree (pure male, pure female and hermaphrodite) [2], accordingly, it is suggested that in future studies on characterization of ash pollen allergens, the phenotypes of the tree could be considered.

Conclusion

This study revealed a 53-kDa immunoglobulin-E-reactive protein, as the first reported high molecular weight allergen from ash pollen extract that is homologous to calreticulin.

Acknowledgements This study was supported by Immunology, Asthma and Allergy Research Institute (IAARI), Children's Medical Center, Tehran University of Medical Sciences (grant # 93-03-40-27007).

Conflict of interest M. Sharif Shoushtari, A. Majd, M.-A. Assarehzadegan, S. Fanuel, M. Moin, T. Nejadshattari, R. S. Shoor-masti, M. Badalzadeh, S. Tajik, M.R. Fazlollahi, B. Tayebi, Z. Pourpak and G.A. Kardar declare that they have no competing interests.

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