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# Immunoproteomics of tree of heaven (*Ailanthus atltissima*) pollen allergens

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ABSTRACT

*Ailanthus altissima* pollen (AAP) is considered as an emerging cause of respiratory allergy in United States, Italy and Iran. However, the allergenic composition of AAP is still unknown and has yet to be characterized. The present study aimed to identify AAP allergens using a proteomics-based approach. For this purpose, optimized AAP protein extracts were analyzed using 1D- and 2D- gel electrophoresis and confronted to twenty sera from individuals with respiratory allergy during the AAP season. Candidate allergens were detected using the serum from an allergic patient with clinical history of AAP pollinosis. IgE-binding spots were identified using MALDI-TOF/TOF mass spectrometry and database searching. According to our results, AAP extracts were rich in proteins (up to 16.25 mg/ml) with a molecular-weight distribution ranging from 10 to 175 kDa. Two-D electrophoresis of AAP extracts revealed 125 protein spots from which 13 were IgE reactive. These IgE-binding proteins were identified as enolase, calreticulin, probable pectate lyase 6, conserved hypothetical protein and ras-related protein RHN1-like. By our knowledge, this study is the first report identifying AAP allergens. These findings will open up further avenues for the diagnosis and immunotherapy of the AAP allergy as well as for the cloning and molecular characterization of relevant allergens.

*Biological significance: Ailanthus altissima* colonizes new areas every year in Iran and is spreading aggressively worldwide. According to USDA, the tree of heaven is now present as an invasive plant in 30 states in US (www.invasivespeciesinfo.gov/plants/treeheaven.shtml) and come to dominate large areas in many regions. Up to now, several cases of allergy to *A. altissima* pollen have been reported in United States, Italy and Iran [1-4]. However, there is still no information on the sensitizing allergens and the molecular origin of these clinical symptoms, which constitutes a serious threat to patients suffering from respiratory allergies in these regions. To our knowledge, the current study describes, therefore, the first panel of proteins responsible for IgE-mediated *A. altissima* pollinosis by using a gel-based proteomic approach. This work represents the pioneer proteomic investigation on Simaroubaceae *spp.* and provides useful insights for further studies on the allergens of this widely distributed plant family.



*Abbreviations:* AAP, *A. altissima* pollen; I.U.I.S, International Union of Immunological Societies; 1D, one-dimensional gel; 2D, two-dimensional gel; 1-DE, one-dimensional gel electrophoresis; 2-DE, two-Dimensional Gel Electrophoresis; 2D-PAGE, two-dimensional- polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; CBB, coomassie brilliant blue; CHAPS, 3-(3-cholamidopropyl) diethyammonio-1 propanesulfonate; DTT, dithiothreitol; DTE, dithioerythritol; IEF, isoelectric focusing; MALDI, matrix assisted laser desorption/ionization; TOF, time of flight; PTM, post-translational modifications; TBS, tris-buffered saline; AP, alkaline phosphatase; IPG, immobilized pH gradient; kDa, kilodalton (molecular mass); MS/MS, tandem mass spectrometry; MS, mass spectrometry; *m/z*, mass-to-charge ratio; pI, isoelectric point; Mw, molecular weight; Pwt, pollen weight; VOEAC, volume of extract after centrifugation; EY, extraction yield; CPLL, combinatorial peptide ligand libraries; TP, total soluble protein content.

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## 1. Introduction

Native to China, the tree of heaven (*Ailanthus altissima*) represents a highly invasive tree which is now distributed in all continents from tropical and subtropical areas to temperate and arid regions worldwide. This species is spreading rapidly in many areas, particularly in United States, Europe and more recently in Iran [5,6]; crowding out native species and affecting urban infrastructures. Allergic reactions to *Ailanthus altissima* pollen (AAP) is another critical issue that poses a new threat to allergic population. From April to June, the *A. altissima* releases a large volume of elliptic and reticulate pollen grains (Fig. 1) that may cause severe allergic reactions in sensitized subjects [2,7]. Although reports suggest a sensitization prevalence of 36.5% among all tested allergic patients and 46.9% in atopic subjects living in Mashhad (northeastern Iran) [8], epidemiologic data from different regions worldwide are still missing because the AAP extract is not included in routine allergy diagnosis and its allergenic constituents have yet to be identified. Recently, two IgE-binding proteins of 42 and 52 kDa have been detected by means of IgE immunoblotting using the serum of an Iranian allergic patient [4]. In India, four IgE cross-reactive proteins in the range of 14 to 64 kDa have been detected in pollen extracts of the closely related species *Ailanthus excelsa* [9]. However, these potential allergenic proteins have not been characterized at molecular level and their identity remains unknown. Up to now, most of the researches on AAP allergy were confined to its clinical manifestations [1–3,8,10], with some of them pointing out the occurrence of extensive cross-reactivity with other allergenic family members [2,10]. A study conducted in Teheran, evaluated the cross-reactive profile for 19 patients with a clinical history of pollinosis to oriental plane (*Platanus orientalis*). On skin-prick tests, 16 out of 19 plane allergic patients were also positive to AAP extracts [11]. Thus, the identification of AAP allergens could be of great value to determine the molecular origins of these reactions as well as to predict the occurrence of clinically relevant cross-recognitions among homologous pollen allergens from various botanical sources.

To achieve this aim, electrophoresis appears to be a useful tool to separate proteins from an allergenic pollen extract and two-dimensional electrophoresis (2-DE) offers the best resolution [12,13]. Two-DE coupled with IgE immunoblotting and followed by mass spectrometry (MS) has thus been used as a powerful technique for the purification and identification of allergenic proteins from various sources. This approach, commonly known as "allergenomics", "allergomics" or "IgE immunoproteomics", enables both precise detection and identification of allergenic molecules from a complex allergenic mixture. However, the extraction of proteins from pollen grains has always been facing with hardships [14]. Pollen grains have proteins from 2.5% to 61% by dry mass and only a small portion of them are IgE-reactive and act as respiratory allergens [15,16]. In addition, the presence of a rigid cell wall, secondary plant metabolites such as phenolics, polyphenolics, pigments, lipids, polysaccharides and high proteinase concentrations may interfere with pollen protein extraction, purification and identification [14]. It has been shown that the optimization of the pollen extraction protocols and the use of protein enrichment techniques may considerably improve the quality of 2-DE separations from pollen extracts [12,13]. Besides, combinatorial peptide ligand libraries (CPLL) were fruitfully applied to allergy-causing substances such as latex [17], milk (whey proteins) [18] and maize [19] leading to the identification of new IgE-binding proteins which were previously poorly represented using conventional sample preparation protocols.

In the present study, immunoproteomic tools have been used to characterize and identify the AAP allergenic composition (Fig. 2). To our knowledge, this study represents the first proteomic study on AAP, as the most widespread and representative member of Simaroubaceae allergenic species. Up to now, no allergen from this botanical family has officially been identified and reported according to the allergen nomenclature of the International Union of Immunological Societies (I.U.I.S.; www.allergen.org) as well as in the Allergome database archiving allergenic molecules [20].

## 2. Materials and methods

### 2.1. Pollen sampling

Fresh pollens were collected during the 2014 AAP season (mid-April to mid-May) from male trees planted in an urban green space of Tehran, Iran. Collected pollen grains were then sieved through an appropriate mesh to achieve 99% purity. Pollen samples were then stored at −20 °C until use.

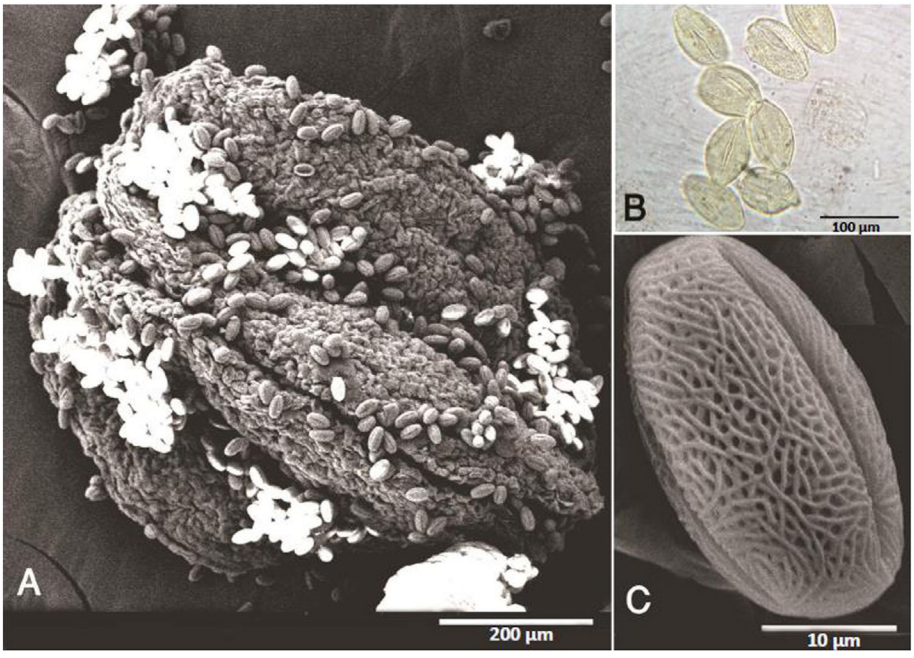



**Fig. 1.** Light and scanning electron micrographs (LM and SEM) of *A. altissima* anther and pollen grains. (A) Anther dehiscence and AAP release (SEM). (B) AAP under LM. (C) An elliptic and reticulate pollen grain of *A. altissima* under SEM. Bar = 200 µm, 100 µm, 10 µm, respectively.

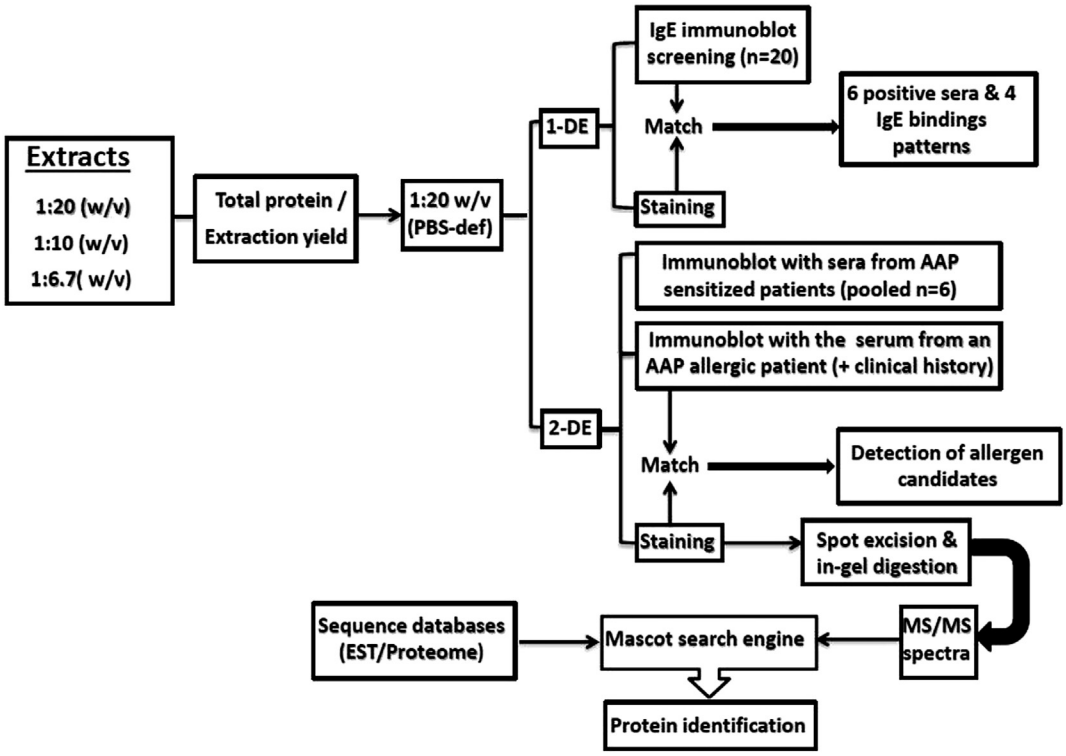



**Fig. 2.** Schematic illustration of successive analytical steps that were followed for the identification of AAP allergens.

### 2.2. Pollen protein extract and extraction yield

For the preparation of pollen extracts and the extraction yield optimization [21], 3 different pollen weights (Pwt) i.e. 50 mg (1/20 wt/vol), 100 mg (1/10 wt/vol) and 150 mg (1/6.7 wt/vol) of APP, either with or without prior defatting were incubated under stirring in 1 ml phosphate buffer saline (PBS) pH 7.4 at 4 °C overnight. Each extract was clarified by centrifugation at 12000 g for 30 min at 4 °C and desalted by dialyzing against distilled water overnight. The volume of recovered AAP extracts (expressed in ml) was measured before and after centrifugation (VOEAC). The total soluble protein content (TP) of AAP extracts was estimated using Bradford [22] protein assay with bovine serum albumin (BSA) as standard. Finally, the extraction yield (EY) has been calculated according to the following formula and expressed in mg of protein/g of pollen:

$$EY = \frac{VOEAC \times TP}{Pwt} \times 1000$$

All samples have been stored as aliquots at −20 °C until further use.

### 2.3. Patients sera

The allergy to AAP is currently difficult to diagnose because of the lack of standardized commercial extracts and specific diagnostics kits for this species worldwide. Therefore, for our preliminary sIgE immunoblot screening experiments on AAP PBS extracts, twenty sera were selected among atopic individuals suffering from respiratory allergy during the peak AAP season (mid-April to mid-May). The serum of a 31-year-old woman with a clinical history of AAP pollinosis and suffering from breathlessness, conjunctivitis, rhinitis, dry coughing, itching, and contact dermatitis after direct exposure to AAP, was used as positive control [4]. All 19 other sera represented residues of sIgE titer evaluations performed at the IAARI medical laboratory in Tehran (Iran). All blood samples were drawn at the time of the visit to the IAARI clinic and stored at −20 °C. In all sIgE screening experiments, the serum from a non-allergic healthy individual was chosen as negative control.

### 2.4. 1-DE (one-dimensional gel electrophoresis) separation

Extracted proteins were separated using 10% SDS-PAGE gels under reducing conditions [23]. Forty microgram proteins were loaded per lane. A reference protein standard (Fermentas, St. Leon-Rot, Germany) were applied to estimate the molecular weight of visualized protein bands. The gel was partly electro-transferred to a polyvinylidene difluoride (PVDF) membrane for western blotting assays and another part was stained with 0.1% coomassie brilliant blue R-250 (CBB-R250, Merck, Darmstadt, Germany).

### 2.5. Two-dimensional gel electrophoresis (2-DE)

Isoelectric focusing (IEF) was performed using immobilized gradient (IPG) strips, 7 cm pH 3–11 NL, (GE Healthcare, Uppsala, Sweden). Strips were rehydrated passively overnight (14 h) in 130 μg of AAP protein extracts mixed with 125 μl of 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 in an Ettan IPGphor 3 system (GE Healthcare) 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 strips were 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). 2D gels were run in duplicate and electrotransferred onto a PVDF membrane or stained with CBB-G-250. The molecular mass of protein bands was estimated using protein markers of known molecular weight (Fermentas).

### 2.6. Western blot

Following the electroblotting of 1-DE and 2-DE separations, PVDF membranes were dried and blocked with TBS-T (20 mM Tris, 150 mM NaCl with 0.3% Tween-20) for 1 h at room temperature. Membranes were then incubated overnight with the serum of the AAP allergic patient diluted TBS-0.3% Tween-20 (1/10 v/v) at 4 °C under shaking. After several washing, membranes were incubated during 1 h with 1/700 dilution alkaline phosphatase (AP)-conjugated goat anti-human IgE (Sigma-Aldrich, St Louis, Missouri). The AP activity was visualized with 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (Sigma-Aldrich).

### 2.7. Mass spectrometry and database searching

For mass spectrometric analyses, IgE-reactive protein spots were excised from CBB-stained 2D gel and in gel-digested with trypsin. In-gel tryptic digestion was performed after reduction with DTE and *S*-carbamidomethylation with iodoacetamide. Gel pieces were washed two times with 50% (v/v) aqueous acetonitrile containing 25 mM ammonium bicarbonate and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold with 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 mg/ml. Gel pieces were rehydrated by adding 10 ml of trypsin solution, and after 10 min, 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37 °C.

*A*1 ml aliquot of each peptide mixture was applied to a ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-a-cyano-cinnamic acid (Sigma) in 50% aqueous (v/v) acetonitrile containing 0.1%, trifluoroacetic acid (v/v).

Positive-ion MALDI mass spectra were obtained using a MALDI-TOF/TOF instrument (Bruker ultraflex III, Elmsford, New York) in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a range of 800–5000 *m/z*. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg$^1$-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu$^1$-Fibrinopeptide B, 1750.677; ACTH (1–17 clip), 2093.086; ACTH (18–39 clip), 2465.198; ACTH (7–38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP averaging algorithm (*C*4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

For each spot the ten strongest precursors, with an S/N greater than 30, were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 *m/z*, cycles 4); monoisotopic peak detection used a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.3) was used to perform spectral processing and peak list generation.

Tandem mass spectral data were submitted to database searching (NCBI nr database) using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.4), through the Bruker ProteinScape interface (version 2.1). Search criteria specified: Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M) and Deamidated (NQ); Peptide tolerance, 100 ppm; MS/MS tolerance, 0.5 Da; Instrument, MALDI-TOF-TOF. Results were filtered to accept only peptides with an expect score of 0.05 or lower. Homology searches and multiple amino acid sequence alignments were carried out by using the BLAST (http://expasy.org/tools/blast/) and CLUSTALW (http://services.uniprot.org/clustalw/) programs, respectively.

## 3. Results and discussion

The sensitisation to *A. altissima* pollen is now recognized as an emerging cause of respiratory allergy in many parts of the world. However, lack of proper information about allergenic components of AAP makes the diagnosis and treatment of this pollinosis difficult and has become a threat to patients suffering from pollen allergy in Iran [8].

The present study represents the first attempt to characterize allergenic proteins of *A. altissima* pollen. With the aim to precisely identify AAP allergens, we used an immunoproteomic approach based on 1-DE and 2-DE separation of pollen proteins followed by IgE immunoblotting.

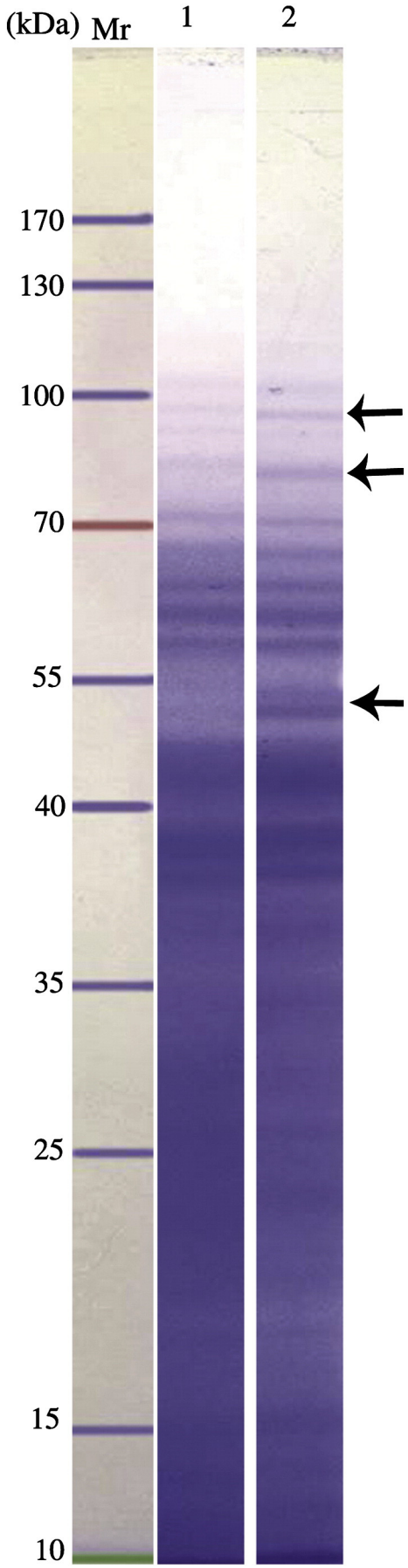
Overall, in plant proteomics and particularly for studying the proteome of pollen grains, the sample preparation represents the critical step. In order to establish the repertoire of AAP allergens, our approach consisted in setting up a protocol to optimally extract pollen proteins. For this purpose, different extraction conditions were used in order to improve the protein yield of AAP extracts (Table 1). Pollen extracts were labeled by weight-to-volume (*w/v*) based on the weight of the AAP to the volume of the extracting fluid (PBS). The total protein contents and extraction yields of 3 different weight-to-volume ratios were compared. As illustrated in Table 1, AAP extracts showed high soluble proteins content reaching 16.25 mg/ml in 1/6.7 *w/v* preparations. The highest and lowest extraction yields were obtained using 1/20 (*w/v*) and 1/10 (*w/v*) AAP extracts, respectively. Adding a preliminary acetone liquid extraction to dissolve lipids did not altered the total protein content of optimized 1/20 *w/v* extracts, despite a larger buffer absorption by pollen grains and a 10% drop in the protein yield (reducing the VOEAC from 0.9 to 0.8 ml). However, adding a defatting step prior to the AAP protein extraction has proved necessary to improve the quality of our electrophoretic separation by reducing smears and increasing the resolution of AAP protein profiles (Fig. 3).

Overall, our protein studies demonstrated that AAP is very rich in protein when compared to other allergenic pollen grains [24,25]. The 1-DE SDS-PAGE pattern of AAP extracts revealed about 19 protein bands ranging from 10 to 175 kDa. As shown in the Fig. 4A, 1D–E IgE immunoblotting led to the detection of 6 positive sera to AAP pollen. Four main IgE-binding patterns were distinguishable between positive patients. The first one (Fig. 4A, lane 2) represents a patient with a broad IgE reactivity to numerous proteins in the 25–100 kDa mass range. This kind of broad and diffuse IgE reactivity, was also found for some Oleaceae- and Cupressaceae-sensitized patients and reported to be associated with carbohydrate cross-reactive determinants or CCDs [25,26]. The CCDs appeared to have poor clinical relevance and are mainly linked to glycosylated high molecular weight proteins (HMW) contained in various pollen and foods [27]. In contrast, the second pattern (Fig. 4A, lanes 3 and 4) has been associated to a severe clinical history in a recent case report [4] and comprised 2 sera showing a strong and well-marked IgE reactivity to two proteins of 42 and 52 kDa. These results are partly in accordance with those of our previous animal study revealing a single IgE binding component of approximately 42 kDa [28] and they are also in line with 1D immunoblot experiments of Dhyani et al. [9] which led to the detection of IgE reactive proteins of 41 and 52 kDa in the pollen extracts of a closely related species belonging to Simaroubaceae, namely *Ailanthus excelsa*.

**Table 1**
Protein contents and extraction yields of *Ailanthus altissima* pollen extracts.

| Extracts (*w/v* mg/ml) | VOEAC (ml) | TP (mg/ml) | EY (mg protein/g pollen) |
|---|---|---|---|
| 1/20 | 0.9 | 4.3 | 77.4 |
| 1/10 | 0.75 | 8.5 | 63.75 |
| 1/6.7 | 0.65 | 16.25 | 70.4 |

w/v, wheight/volume (mg/ml); VOEAC, volume of extract after centrifugation; TP, total soluble protein content; EY, extraction yield.




The third (Fig. 4A, lane 5) and fourth (Fig. 4A, lanes 6 and 7) patterns were composed from strong single IgE-reactive bands of about 36 and 115-kDa, respectively.

The heterogeneity of the specific IgE repertoire of positive patients to AAP protein extracts was further investigated by 2-DE immunoblotting using the pool of sera from 6 AAP positive patients. As depicted in the Fig. 4B, the IgE repertoire of AAP sensitized patients was broadly directed against more than 59 protein spots, evenly distributed over a wide range of pI and Mr. Obviously, all these protein spots may not stand for allergenic components as the molecular basis of their IgE recognition including the presence of cross-reactive carbohydrate determinants (CCDs) as well as the correlation with clinical symptoms have yet to be assessed. It is now accepted that IgE from allergic individuals can recognize antigenic structures from allergens and non-allergens without any related symptoms [29]. Therefore, when defining a molecule as an allergen, both IgE-binding property and intrinsic capacity to trigger allergic symptoms should be taken into consideration.

In this respect and in order to identify allergen candidates, we used the serum from a patient with clinical history of severe allergy to AAP and representative of the second IgE binding pattern correlating with symptoms observed and listed at the IAARI allergy clinic after direct exposure to AAP and during the two following AAP season (during the springs 2014 and 2015). By using 2-DE approximately 125 protein spots distributed in a wide range of pI and molecular masses were visualized (Fig. 5A). Two-DE immunoblotting with the patient's serum detected 13 IgE reactive spots with molecular masses ranging from 35 to 62 KDa including one acidic (spots 1), nine basic (spots 2–6 and 7–10) and three very basic (spots 11, 12 and 13) protein spots (Fig. 5B). No IgE reactivity to AAP extracts has been found using the serum of a non-allergic/non atopic subject as negative control.

Two-DE IgE reactive spots (1, 2–5 and 7–10) were then excised and subjected to MS/MS analysis using MALDI-TOF/TOF mass spectrometry. Five allergenic protein of *A.altissima* pollen were identified including enolase, calreticulin (spot 1), probable pectate lyase 6 (spots 2–5 and 7–10), conserved hypothetical protein (spots 7–10) and ras-related protein RHN1-like (spots 7–10). Among these candidate allergens, the last two proteins displayed the lowest levels of identification confidence. The information related to identified proteins using MALDI-TOF /TOF mass spectrometry are listed in Table 2. MS/MS based MASCOT search using NCBInr database were statistically significant and identified all protein spots with a unique or two peptides and relevant MASCOT scores. The best matches were obtained against the NCBI database restricted to Viridiplantae (3796096 sequences). For the enolase and pectate lyase 6 probable, the highest score appeared to be highly significant. It is important to note that no EST data from members of the Simaroubaceae plant family was available to support our analyses. However, a search against the NCBI EST databases of the Rosids (10 million sequences) and Malvids (4.5 million sequences) plant families was performed without giving relevant matches.

In recent years, it was found that most allergens belong to a restricted number of protein families. 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 to predict potential cross-reactivities among various allergenic species. Enolases (spot 1), cytoplasmic house-keeping enzymes with a highly conserved structure, are involved in pathway of glucose metabolism (glycolysis and gluconeogenesis) in the cytosol of eukaryotic cells [30]. They are considered as significant inhalant allergens in various sources including fungi [31], yeast [32], pollen [33],

**Fig. 3.** Comparaison of 1-DE protein profiles of non-defatted and defatted AAP exracts. Lane 1: Coomassie-stained SDS-PAGE of 1/20 (*w*/*v*) non-defatted AAP PBS extract. Lane 2: Coomassie-stained SDS-PAGE of 1/20 (*w*/*v*) defatted AAP PBS extract. Additional or better resolved protein bands are shown by black arrows.

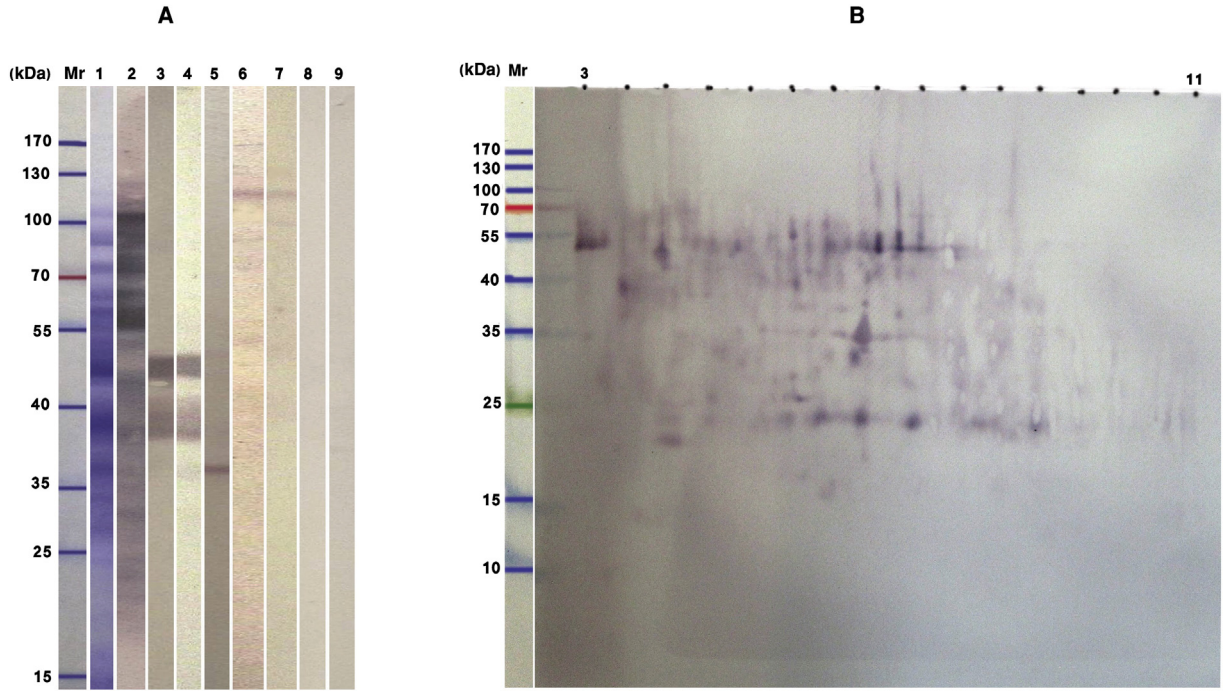



**Fig. 4.** IgE immunoblotting of *Ailanthus altissima* pollen extracts. (A) Lane 1: Coomassie-stained SDS-PAGE profile of the optimized AAP PBS extract. Lanes 2–7: IgE immunoblot patterns of 6 postive sera from patients exhibiting respiratory allergy during the AAP pollen season. Lane 8: IgE immunoblot with the serum of a non-atopic individual. Lane 9: Control without serum. (B) Two-DE immunoblotting using the pool of sera from 6 AAP positive patients. Mr.: Molecular weight marker.

cockroach [34], seafood [35] and latex [36] (Table 3). Enolases were initially described as pollen allergenic proteins from *Cynodon dactylon* [37] and were recently found in pollens from *Plantago lanceolata* [38], *Cocos nucifera* [39] and *Ligustrum lucidum* [40]. Interestingly, enolase protein sequence obtained from the spot 1 exhibited high homology with other enolase proteins reported in unrelated allergenic sources such as *Hevea brasiliensis* (Hev b 9), *Cynodon dactylon* (Cyn d 22) and *Zea mays* (Zea m 22) (Table 3). Another protein homologous to calreticulin has been identified from the same spot. Calreticulins are key calcium-binding molecular chaperones known to be involved in $Ca^{2+}$ homeostasis and the proper folding of proteins in ER as well as in responses of plants to various stresses. These proteins have previously been reported as allergens from *Penicillium chrysogenum* (Pen ch 31) and *Necator americanus* (Nec a Calreticulin). Calreticulin sequence from spot 1 showed 66.7 and 56.2% homology with Pen ch 31 and Nec a Calreticulin, respectively (Table 3).

Pectate lyase 6 probable protein belongs to a well-known allergenic family [41] and was identified from two groups of spots (spots 2–5 and 7–10). The existence of multiple allergenic isoforms for the same protein, were also reported in pollen proteome of *Fraxinus excelsior* [26] and *Cupressus sempervirens* [25]. Pectate lyases play an important role during plant development stages such as development of reproductive organs and fruit ripening. In pollens, they facilitate the pollen tube growth through the degradation of the cell wall pectin [41,42]. Up to now, these important allergenic proteins have been reported in the pollen of restricted plant families [41] (Table 3) i.e. Cupressaceae (including

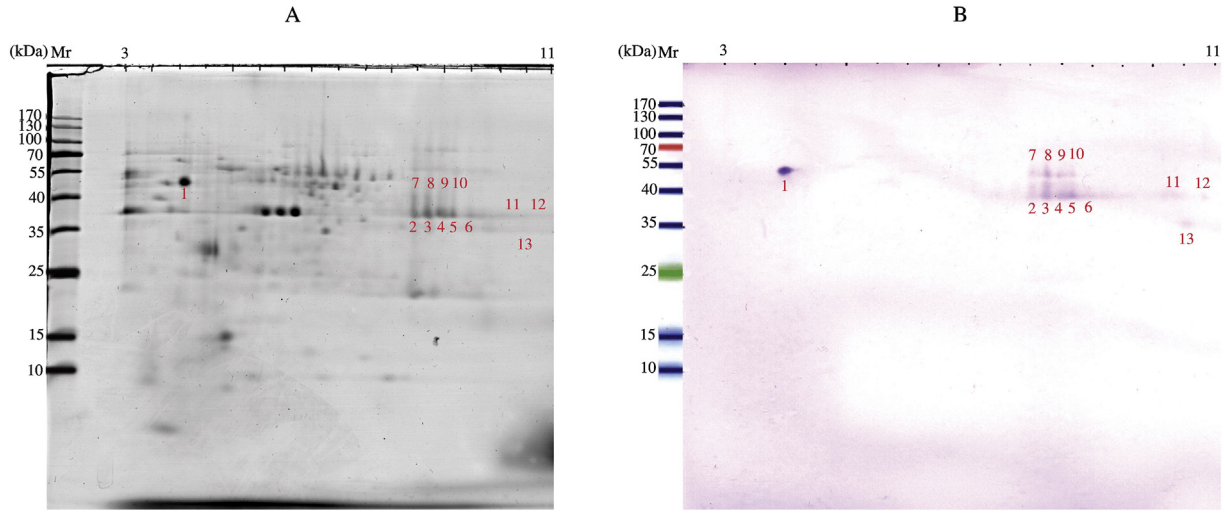



**Fig. 5.** 2-DE and immunoblot of the optimized pollen extract from *Ailanthus altissima* (A) Coomassie-stained 2-DE gel. (B) 2-DE IgE immunoblot probed with the serum of an AAP allergic patient.

**Table 2**
Immunoreactive proteins identified from *Ailanthus altissima* pollen extracts by MALDI TOF/TOF MS.

| Spot | Accession number (NCBI) | Protein | Taxonomy | Peptide sequence | Sequence coverage | Unique peptide | Score | Experimental mass/pI | Theoretical mass/pI |
|---|---|---|---|---|---|---|---|---|---|
| 1 | gi\|158144895 | Enolase | *Gossypium hirsutum* | R.IEEELGAE/AVYAGASFR.A | 3% | 2 | 136 | 52.964/4.00 | 47.982/5.49 |
| | gi\|1009712 | Calreticulin | *Arabidopsis thaliana* | R.FYAISAEFPEFSNK.D | 3% | 1 | 94 | 52.964/4.00 | 46.782/4.37 |
| 2–5 | gi\|685325501 | Probable pectate lyase 6 | *Brassica rapa* | K.LADCVLGFGR.K/K.NPTIISQGNR.F | 4% | 2 | 130 | 38.863/9.09 | 52.041/9.82 |
| 7–10 | gi\|685325501 | Probable pectate lyase 6 | *Brassica rapa* | K.LADCVLGFGR.K/K.NPTIISQGNR.F | 4% | 2 | 125 | 48.267/9.09 | 52.041/9.82 |
| | gi\|223542934 | Conserved hypothetical protein | *Ricinus communis* | K.QGMADALAEMTKR.S + Oxidation (M) | 2% | 1 | 56 | 48.267/9.09 | 64.915/6.64 |
| | gi\|743851961 | Ras-related protein RHN1-like | *Elaeis guineensis* | K.QGNSNMVTALAGNK.A + 3 Deamidated (NQ); Oxidation (M) | 6% | 1 | 55 | 48.267/9.09 | 22.195/5.78 |

*Chamaecyparis obtuse*,*Cryptomeria japonica*, *Cupressus arizonica*, *Cupressus sempervirens, Juniperus ashei)* and Asteraceae (including *Ambrosia artemisiifolia*, *Artemisia vulgaris*, *Senecio jacobae*). They have also been found in two fungi species i.e. *Penicillium citrinum* and *Aspergillus fumigatus*. Thus, Simaroubaceae *spp.* may constitute the third identified plant family expressing allergenic pectate lyases in their pollens. Results of alignment of pectate lyase amino acid sequence from spot 2–5 and 7–10 with the above-mentioned pollen grains and fungi species are provided in Table 3. As depicted in Table 3, the pectate lyase identified in AAP showed high homology whit pollen pectate lyase allergen from two members of Asteraceae (*A. artemisiifolia* and *A. vulgaris*) when compared to allergenic pectate lyase sequences from Cupressaceae family. Anyway, peptides resulting from our MS/MS analyses produced poor identities with allergenic pectate lyases of *P. citrinum* and *A. fumigatus* in protein BLAST. It has been shown that some pectate lyase proteins share high degree of identity and may represent a source of IgE cross-reactivity for allergic patients [41].

**Table 3**
Protein BLAST and sequence similarity between identified AAP IgE reactive proteins and known allergens from other species.

| Protein family | Allergens/ species | Sequence homologyy (%)* |
|---|---|---|
| **Enolase** | Hev b 9/*Hevea brasiliensis* | 94.4 |
| | Cyn d 22/*Cynodon dactylon* | 89.5 |
| | Zea m 22/*Zea mays* | 89.5 |
| | Alt a 6/*Alternaria alternate* | 77.8 |
| | Asp f 22/*Aspergillus fumigatus* | 77.8 |
| | Cla h 6/*Cladosporium herbarum* | 77.8 |
| | Bea b Enolase/*Beauveria bassiana* | 77.8 |
| | Asp c 22/*Aspergillus clavatus* | 77.8 |
| | Asp f 22/*Aspergillus fumigatus* | 77.8 |
| | Ict pu 2/*Ictalurus punctatus* | 77.8 |
| | Neo fi 22/*Neosartorya fischeri* | 77.8 |
| | Cla h 6/*Cladosporium herbarum* | 77.8 |
| | Pen c 22/*Penicillium citrinum* | 72.2 |
| | Thu a 2/*Thunnus albacares* | 72.2 |
| | Cand a Enolase/*Candida albicans* | 72.2 |
| | Bla g Enolase/*Blattella germanic* | 72.2 |
| | Tal st 22/*Talaromyces stipitatus* | 72.2 |
| | Thu a 2/*Thunnus albacares* | 72.2 |
| | Rho m 1/*Rhodotorula mucilaginosa* | 70.6 |
| | Sac c Enolase/*Saccharomyces cerevisiae* | 70.6 |
| | Rho m 1/*Rhodotorula mucilaginosa* | 70.6 |
| | Sal s 2/*Salmo salar* | 66.7 |
| | Ore m 2/*Oreochromis mossambicus* | 66.7 |
| | Cur l 2/*Curvularia lunata* | 58.8 |
| | Lig l enolase/*Ligustrum lucidum* | 28.6 |
| | Coc n enolase/*Cocos nucifera* | 25.0 |
| **Pectate lyase** | Art v 6/*Artemisia vulgaris* | 73.3 |
| | Amb a 1/*Ambrosia artemisiifolia* | 66.7 |
| | Sen j PL/*Senecio jacobaea* | 50.0 |
| | Cha o 1/*Chamaecyparis obtuse* | 47.4 |
| | Cry j 1/*Cryptomeria japonica* | 47.4 |
| | Cup a 1/*Cupressus arizonica* | 47.4 |
| | Cup s 1/*Cupressus sempervirens* | 47.4 |
| | Jun a 1/*Juniperus ashei* | 47.4 |
| | Jun v 1/*Juniperus virginiana* | 47.4 |
| | Jun o 1/*Juniperus oxycedrus* | 47.4 |
| | Pen c 32/*Penicillium citrinum* | 20.0 |
| | Asp f PL/*Aspergillus fumigatus* | 17.6 |
| **Calreticulin** | en ch 31/*Penicillium chrysogenum* | 66.7 |
| | Nec a Calreticulin/*Necator americanus* | 56.2 |

* Homology searches were performed by alignment (BLAST) of amino acid sequences obtained from AAP allergens (enolase, pectate lyase and carleticulin) with allergenic proteins which were already identified from other organisms. Allergens were ordered according to their respective level of homology.

Conserved hypothetical protein and ras-related protein RHN1-like were two other proteins identified in spots 7–10. So far, these two types of proteins were not reported as allergenic from other sources. However, they generated low MASCOT's protein scores in MS/MS analyses.

Taken together all the MS/MS results, homology driven proteomics has enabled us to identify novel IgE-binding proteins as candidate AAP allergens. To the best of our knowledge, this work represents the first proteomic investigation on Simaroubaceae *spp.* and provides useful insights for further studies on the allergens of this widely distributed plant family.

## 4. Conclusions

Despite the lack of information on Simaroubaceae genome and proteome sequences, our mass spectrometric analyses involving NCBI Viridiplantaea database allowed the fruitful identification of IgE binding proteins implicated in the emerging allergy to *A. altissima* pollen grains. Among identified allergen candidates, two (enolase and pectate lyase) belong to well-known allergenic protein families and have been detected as major sensitizers in other widespread pollen species. The clinical relevance of these allergens and their cross-allergenicity with enolases and pectate lyases from closely related species and other sources remains to be assessed. Considering the current lack of commercial diagnostic tools for this emerging allergy, the present report on the identification of allergenic proteins from AAP should pave the way for the standardization of AAP allergen extracts and for the diagnosis and immunotherapy of pollinosis to *A. altissima* and other Simaroubaceae *spp.* worldwide.

## Conflict of interest

The authors declare no conflict of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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