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ORIGINAL RESEARCH PAPER

# Proteomic analysis of *Parietaria judaica* pollen and allergen profiling by an immunoproteomic approach

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**Abstract** *Parietaria judaica* pollen is a common cause of airway allergic disease in the Mediterranean area. Proteome analysis of mature *Parietaria judaica* pollen by two-dimensional gel electrophoresis (2-DE) and mass spectrometry has established the first reference proteome map of this weed. Proteins involved in a variety of cellular functions as well as the occurrence of allergens were detected. By using 2-DE and immunoblotting with sera from *Parietaria judaica* allergic patients we obtained a more detailed characterization of *Parietaria judaica* allergen profile so to improve our comprehension of the pathogenesis of pollen-induced allergic reaction.

**Keywords** Allergens · *Parietaria judaica* · Immunoproteome · Pollen

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## Introduction

*Parietaria judaica* belongs to the Urticaceae family and is commonly found in urban and rural areas. Its pollen is one of the main causes of allergy in the Mediterranean area (Masullo et al. 1996). *Parietaria judaica* pollen allergens have been identified and characterized by immunochemical and molecular-biological techniques (Amoresano et al. 2003; Stumvoll et al. 2003). *Par j 1* and *Par j 2* represent two major allergens belonging to a family of glycoproteins known as non-specific lipid transfer proteins (Salcedo et al. 2004). Recently, the presence of minor allergens like profilin (*Par j 3*) and 2-EF-hand (*Par j 4*) proteins have been also described (Asturias et al. 2004; Bonura et al. 2008). However, other proteins, specific isoforms or post-translational modifications (PTM) of known allergens, can contribute to hypersensitivity reactions in allergic individuals. The accurate diagnosis of hypersensitive patients as well as the in-deep knowledge of the mixtures for a effective immunotherapy depend on the molecular characterization of all allergenic components from a biological source.

Proteomic profiling of pollen extracts coupled with classical immunological approaches have been applied to examine the presence of novel allergenic components in maize, grass, and other species (González-Buitrago et al. 2007; Petersen et al. 2006). An understanding of *P. judaica* pollen proteome would provide insight in the molecular nature of allergens as well as of other proteins involved in the functional specialization of pollen. Using 2-DE followed by LC-MS/MS analyses, we generated the first reference map of the *P. judaica* mature pollen proteome. In total, we identified 70 protein spots including isoforms, and by using an immunoproteomic approach with serum from *P. judaica* allergic patients, we have obtained a more detailed characterization of *Parietaria judaica* allergen profile so to improve our comprehension of the molecular determinants of *P. judaica*-induced allergic reaction. In particular, we have evidenced that the IgE from patients recognise *Par j 1* and the *Par j 2* P2 allergens while they don't recognise the polypeptide *Par j 2* P8. These results could provide important indications to develop new and more efficient vaccines for allergic patients.

## Materials and methods

### Pollen collection and protein extraction

Pollen from *Parietaria judaica* was purchased from Allergon (Ängelholm, Sweden) and extracted as previously described (Alessandro et al. 2009). Briefly, the pollen grains were defatted with diethyl ether, extracted in PBS at 4°C, dialysed against water and finally lyophilised and stored at −80°C until use.

### 2-DE, image acquisition and analysis

Aliquots of the lyophilised proteins, 60 µg for analytical gels and 1 mg for preparative gels, were solubilized in a buffer containing 8 M urea, 4% w/v CHAPS, 40 mM Tris and 65 mM DTE and a trace amount of Bromophenol Blue.

The first dimension IEF was carried out using immobilized pH gradient (IPG) strips, pH 3–10 non-linear and pH 4–9 linear, 18 cm long. The first dimension, equilibration steps and the second dimension were performed as previously described (Fontana et al. 2007).

Gels were stained with ammoniacal silver nitrate for analytical gels (Hochstrasser et al. 1988) or Comassie staining for preparative gels. The gels were scanned by densitometer ImageScanner II (GE Healthcare Life Science, NJ, USA) and the ImageMaster 2D Platinum 6.0 software (GE Healthcare Life Science, NJ, USA) was used to perform the qualitative and quantitative analysis of the 2-DE maps. Human serum was used as internal standard for isoelectric point (pI) and Molecular Weight (MW) calibration of the 2-DE gel with pH 3–10 non linear gradient (Bjellqvist et al. 1993).

### *In-gel* protein digestion

Coomassie Blue or silver-stained protein spots were excised from the preparative gels and cut into 1-mm pieces. In-gel digestion was performed as described by Shevchenko et al. (1996) with minor modifications. De-staining was obtained by successive washes with 20 mM $NH_4HCO_3$ buffer and $H_2O$/acetonitrile (50/50 v/v) mixture for Coomassie Blue. For proteolytic digestion, the gel was treated by 5–15 µl trypsin (20 ng/µl in 50 mM $NH_4HCO_3$) for 5 h at 37°C. The resulting tryptic peptides were extracted from the gel by centrifugation and the supernatant fraction was recovered. A second extraction step was performed using 10–15 µl $H_2O$/acetonitrile/formic acid (60/36/4; by vol.) for 30 min and, finally, all extracts were pooled. For LC-ESI/MS/MS experiments, extracts were dried under vacuum and re-suspended in 0.1% TFA solution.

### NanoLC/nanospray/tandem mass spectrometry (LC-ESI/MS/MS)

Experiments were performed on a Q-STAR XL instrument (Applied Biosystems, Courtaboeuf, France) equipped with a nanospray source using a distal coated silica-tip emitter (FS 150-20-10-D-20, New Objective, USA) set at 2300 V. Information Dependent Acquisition (IDA) mode allowed +2 to +4 charged peptide ions within a m/z 400–2000 survey scan mass range to be analyzed for subsequent fragmentation. MS/MS spectra were acquired in the m/z 65–2000 range. The collision energy was automatically set by the software (Analyst 1.0) and was related to the charge of the precursor ion. The MS and MS/MS data were recalibrated using internal reference ions from a trypsin

autolysis peptide at m/z 842.510 $[M + H]^+$ and m/z 421.759 $[M+2H]^{2+}$. Deconvolution of the TOF MS spectra were done with Bayesian Peptide Reconstruct tool of Analyst. Multicharged ions in 700–1,800 m/z range with 0.2 Da mass tolerance were used to calculate glycopeptide masses from 2,000 to 4,000 Da.

Tryptic peptides were separated using an Ultimate-nanoLC (Dionex, Voisins Le Bretonneux, France) with a $C_{18}$ PepMap micro-precolumn (5 μm; 100 Å; 300 μm × 5 mm; Dionex) and a $C_{18}$ PepMap nano-column (3 μm; 100 Å; 75 μm × 150 mm; Dionex). After a 3 min wash with 0.05% TFA in $H_2O$/acetonitrile (98/2 v/v) of the injected sample solution on the micro-precolumn, the chromatographic separation was developed using a linear 60 min gradient from 0 to 50% B, where solvent A was 0.1% formic acid in $H_2O$/acetonitrile (95/5 v/v) and solvent B was 0.08% formic acid in $H_2O$/acetonitrile (20/80 v/v) at approx 200 nl/min.

Protein identifications were performed using the Paragon Algorithm thorough search mode in ProteinPilot™ software (Applied Biosystems, Courtaboeuf, France).

### Patient sera

For the identification of IgE-reactive proteins, sera from patients ($n = 10$) sensitized to *Parietaria judaica* whose radio-allergosorbent test (RAST) exhibited scores over 3, were used. Control sera were from healthy, non-allergic subjects and from subjects sensitized to other allergic sources (dust mite, Olea and Cupressus). All sera were stored at −80°C until use.

### Immunoblotting

For immunoblot analysis, 2-DE gel was transferred to nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Little Chalfont, UK) and the membrane was incubated in blocking solution (5% w/v non-fat dry milk, 20 mM Tris, 140 mM NaCl, 0.1% Tween 20) (TBST) at room temperature. After several washings in TBST buffer, the filters were incubated overnight at room temperature with sera from allergic patients diluted 1:10 followed by horseradish peroxidase (HRP)-conjugated rabbit anti human IgE (1:2,000 dilution). After washings in TBST buffer, immunocomplexes were detected by the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford, IL, USA).

## Results and discussion

### Proteome map of *Parietaria judaica* pollen

*Parietaria judaica* represents the main cause of allergy in the Mediterranean area where up to 80% of pollinosis patients are sensitized against the proteins composing the *P. judaica* pollen. With the aim of obtaining a comprehensive and detailed analysis of *Parietaria judaica* pollen content, we extracted total protein from 1.5 g mature pollen. Aliquots of the protein samples were subjected to 2-DE analysis and representative silver-stained 2D gel is shown in Fig. 1. Good reproducibility and resolution were achieved throughout the experimental procedures. On average 975 protein spots were clearly resolved using IPG strips pH 3–10 non-linear.

### Protein identification

To obtain a first reference proteomic map of the pollen, 100 protein spots were excised from Coomassie Blue-stained gels and among these, 70 protein spots (corresponding to 21 different proteins) were identified by LC-MS/MS technology (Supplementary Table 1). Identified proteins ranged in calculated molecular mass from 92 to 7.5 kDa and in calculated pI from 4.3 to 7.1. All proteins were designated with arbitrary spot numbers as shown in Fig. 1. While the identification of proteins by proteomics methodology has found extensive application with organisms which have a complete sequence of their genomes already available, cross species identification has been used to identify proteins from several plant species (Faulkner et al. 2005; Fernando 2005; Wang, et al. 2005). In fact, in our work, due to the poor protein and DNA sequence database coverage in *Parietaria judaica* (only 7 proteins are present in Uni-Prot database for this plant), MS analyses allowed the recognition of 25 protein spots from P.j and 45 from other species (Supplementary Table 1). Multiple spots corresponding to the same protein were also identified, as described in other proteomic studies, and reasonably corresponding to post-

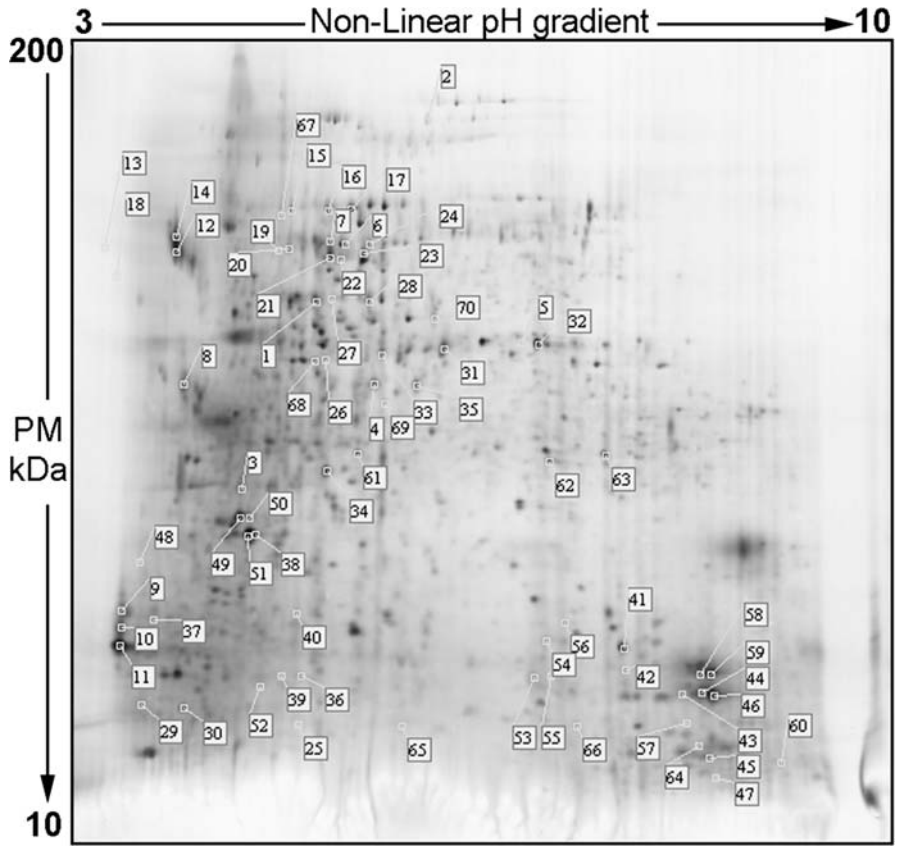


**Fig. 1** Representative proteomic map of *Parietaria judaica* pollen, proteins (60 μg) were loaded on IPG strips (18 cm, 3.5–10 non-linear pH). The second dimension was performed on a vertical linear-gradient slab gel (9–16%). Gel was silver stained

translational modifications, protein degradation, different isoforms derived from different genes of a multigene family or products of alternatively spliced mRNAs.

Figure 2 shows the distribution of P.j pollen proteins according to their functional classification. Briefly, the proteins were grouped in four distinct functional classes: proteins involved in carbon metabolism and energy generation, cytoskeletal and calcium-binding proteins, stress-related proteins, pollen allergens and proteins involved in the methionine metabolism. With respect to specific allergens, we identified 11 spots (37–47) corresponding to *Par j 2* P2 protein, 13 spots (49–61) corresponding to *Par j 2* P8 protein, while 1 spot (36) corresponded to *Par j 1* P1 protein. A search of *Par j 1* and *2* protein sequences, using specific softwares supported by ExPaSy Proteomics Server evidenced both O-glycosylation sites and phosphorylation sites (http://ogpet.utep.edu/OGPET, http://www.cbs.dtu.dk/services/NetPhos).

In particular, *Par j 1* P1 protein has one glycosylation sites and five phosphorylation sites, *Par j 2* P8

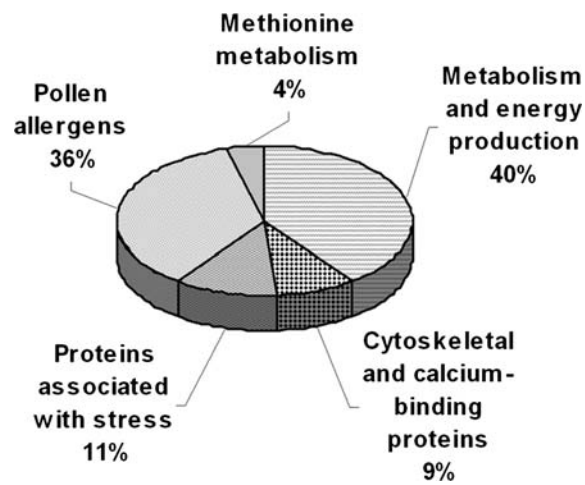


**Fig. 2** Pie-chart representing the percentage distribution of identified pollen proteins within functional classes

protein has five phosphorylation sites and *Par j 2* P2 protein has three phosphorylation sites. These post-translational modifications do not change significantly the molecular weight of a protein but they may

be responsible of the shifts in isoelectric points. At the same time, post-translational modifications might have an influence on the Ig binding so that the knowledge of the structure of allergens is important to improve diagnosis and therapy of allergy (Ferreira et al. 2006).

## Identification of IgE reactive spots by 2-DE and pollen allergens

Although molecular biology and immunological methodologies have allowed the identification of *Par j 1* and *Par j 2* as the major pollen components responsible of allergic reaction and profilin as a minor allergen (Asturias et al. 2007), the study of P.j pollen allergens have never been completed by using an immunoproteomic approach. Since *Par j 1* and *Par j 2* are basic proteins, to improve the separation of their several isoforms, the pollen protein samples were run on IPG strips 6–9 linear, which allows a better separation of polypeptides with a pI higher than 7. Figure 3a shows a representative Coomassie-stained 2 D gels and corresponding immunoblot obtained by using pooled sera of allergic patients (Fig. 3b). In order to better characterize the biochemical identities of immunoreactive spots (Fig. 3a, numbers 1–7) they were excised from Coomassie-stained 2-DE gel and subjected to LC-ESI MS/MS. Table 2 in Supplementary Material shows the results of protein spot identifications. Protein spots numbers 1 and 2 are isoforms of *Par j 1* allergen, protein spots numbers 3–5 are isoforms of *Par j 2* P8 while protein spots 6 and 7 are isoforms of *Par j 2* P2 allergen. Figure 3b, shows a representative western blot using the pooled sera from patients. In particular, the spot indicated with arrows corresponds to *Par j 1* and spot included in the square corresponds to *Par j 2* P2. Control experiments, carried out with pooled sera from 10 nonsensitized subject, indicated the specificity of antibody detection (Fig. 3c). Interestingly, no reaction was ever observed against *Par j 2* P8 suggesting that *Par j 2* P2, together with *Par j 1*, are the main allergenic components present in *Parietaria judaica* pollen.

The data presented in our study show for the first time the identification of *Parietaria judaica* pollen allergens through bidimensional western blot thus representing satisfactory tools for immunological characterization and classification of the proteins from a raw plant extract.

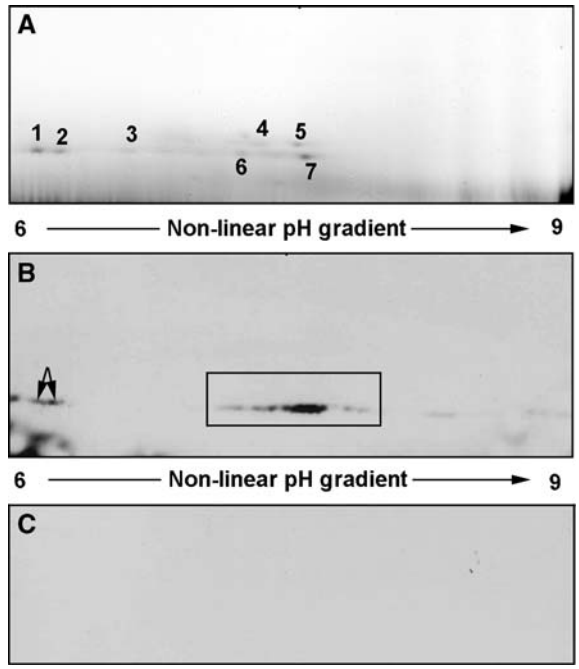


**Fig. 3** 2-DE analysis of *Parietaria judaica* pollen extract using a non linear pH 6–9 gradient in the first dimension followed by a 9–16% SDS-PAGE gradient in the second dimension. The separated proteins were stained by Coomassie (**a**) or transferred into a membrane for the detection with patients sera (**b**) or control subjects (**c**). In **b** *arrows* indicate *Par j 1* polypeptides detected by patients sera while spots corresponding to *Par j 2* P2 are bordered by a square

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