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Application of an immunoproteomic approach to detect anti-profilin antibodies in sera of *Parietaria judaica* allergic patients

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Abstract

Pollen from grasses, weeds, and trees constitutes one of the main sources of inhalant allergens frequently associated with seasonal patterns of allergic diseases. Pollen allergens show some analogies in the amino acids sequence which determine immunological similarity and cross reactivity. *Parietaria judaica* (P.j) pollen represents one of the main sources of allergens in the Mediterranean area and its major allergens have already been identified (*Par j 1* and *Par j 2*). Recently, has been also described a minor allergen, profilin (*Par j 3*), an allergen present in pollen of trees, grasses and weeds. Allergenic plant profilins constitute a highly conserved family with sequence identities of 70% to 85% responsible for a wide range of cross-reactivity among pollens and plant foods. In this work we use an immunoproteomic approach to detect IgE antibodies against profilin in serum of P.j allergic patients.

Key words: pollen, allergen, profilin, 2D immunoblot

Introduction

Allergic airway disease attributable to inhalant allergens, such as pollen has been increasing steadily, with a recent population-based survey in Europe reporting a prevalence of 23%, with values as high as 40% in some countries [1]. *Parietaria judaica* (P.j) belongs to the Urticaceae family, is commonly found in urban and rural areas and its pollen is one of the main causes of allergy in the Mediterranean area, with a 60-80% prevalence in Italy and Greece and 25-40 % prevalence in Spain and southern France [2]. *P. judaica* pollen contains at least nine allergens, Par j 1 and Par j 2 represent two major allergens belonging to a family of glycoproteins known as non-specific lipid transfer proteins (LTPs) [3, 4]. However, other proteins, as profilin and calcium-binding protein, specific isoforms or posttranslational modifications (PTM) of known allergens can contribute to hypersensitivity reactions in allergic individuals [5]. Profilins are ubiquitous cytosolic actin-binding proteins implicated in the signal transduction cascade as well as in cytoskeletal rearrangements [6]. They have been identified as allergens in pollen of several species of trees, grasses, and weeds and in many fruits and vegetables. The widespread IgE cross-reactivity towards a broad range of profilins has led to the designation of profilins as pan-allergens [7].
Whereas the LTP-like allergens from *Parietaria judaica* are well described [8, 9], little information is available regarding other P.j allergens. Recently, using 2-DE followed by LC-MS/MS analyses, we generated the first reference map of the P.j mature pollen proteome, and by using an immunoproteomic approach with serum from P.j allergic patients we have evidenced that the IgE from patients recognize Par j 1 and the Par j 2 P2 allergens while they don't recognize the polypeptide Par j 2 P8 [10]. In the present work we used pooled sera of allergic subjects to test the presence of antibodies against a minor *Parietaria judaica* allergen, profilin (Par j 3) [11]. The obtained results have demonstrated that positive IgE reactions to profilin regards a pool of *Parietaria judaica* allergic patients, suggesting that the minor antigenic determinant may be useful to employ as a diagnostic agent or to develop new and more efficient vaccines.

Materials and Methods

Pollen collection and protein extraction

Pollen from *Parietaria judaica* was obtained from Allergon (Ängelholm, Sweden) and protein were extracted as previously described [12]. Briefly, the pollen grains were defatted with diethyl ether, and left in PBS at 4°C for 16h. Finally, after dialysis against water, the extracted proteins were lyophilized and stored at -80°C until use.

Patient sera

For the identification of IgE-reactive proteins, pooled sera from patients (n = 20) sensitized to *Parietaria judaica* whose radioallergosorbent test (RAST) exhibited scores over 3, were used. All sera were stored at -80 °C until use.

Two-dimensional gel electrophoresis (2DE)

Aliquots of the lyophilized proteins were solubilized in a buffer containing 8M Urea, 4% w/v CHAPS, 40mM TRIS and 65mM 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, 18 cm long. The first dimension, equilibration steps and the second dimension were performed as previously described [12].

After second dimension, the gels were stained with ammoniacal silver nitrate [14] or electroblotted to nitrocellulose membrane. 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 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 [15].

Western blot assay

For western blot (WB) assay, 60μg of proteins were separated by a 12% SDS–polyacrylamide gel electrophoresis or by 2D-WB and transferred to nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Little Chalfont, UK). After electroblotting, the membranes were incubated

in blocking solution (5% non-fat dry milk, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) at room temperature. Then the filters were incubated overnight at room temperature with specific primary antibodies against Par j 2 [12] diluted 1/10000, profilin (BIAL-Aristegui, Bilbao Spain) diluted 1/50000 or pooled sera from allergic patients diluted 1/10 in TBST. Then, the filters were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti human IgE (1:2000), or with horseradish peroxidase (HRP)-conjugated rabbit anti-profilin (1:10000). After washings in TBST buffer, immunocomplexes were detected by the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford, IL, USA). Both primary and secondary antibodies were diluited in blocking solution.

Results

To asses the presence of profilin-specific IgE antibodies in serum of *Parietaria judaica* allergic patients, *P. judaica* pollen protein extract was separated by SDS-PAGE and the gel was probed by sera from allergic patients by western blotting assay.

As shown in Figure 1, when pollen proteins were probed by pooled sera, a very intense immunoreactive band, between 10 to 14 kDa was revealed (line 1). Using specific antibodies we demonstrated that two different *Parietaria judaica* allergens were contained in immunoreactive band: Par j 2 and profilin (Figure 1, lane 2 and 3). Since major allergens of this pollen (Par j 1 and Par j 2) have a molecular mass similar to profilin, in the 10-14 kDa range, it was difficult to discriminate the presence of immunoreactive profilin using monodimensional western blot.

Thus, to improve the separation and discriminate the different allergens, *Parietaria judaica* pollen protein extract was resolved by 2-DE. In a previous work, we generated the first reference map of the P.j mature pollen proteome (Figure 2A) and, by using an immunoproteomic approach with serum from P.j allergic patients, we have demonstrated that the IgE from allergic patients recognize Par j 1 and the Par j 2 P2 isoforms [10]. To detect IgE specific antibody against profilin in *Parietaria judaica* allergic sera we performed 2-DE by using IPG strips with a pH range of 3-10 non-linear followed by western blot analyses with both sera from allergic patients and anti-profilin

antibody. Figure 2B shows a detail of silver-stained 2D gel and a corresponding immunoblot obtained by using pooled sera of allergic patients (Figure 2C). Pooled sera from *Parietaria judaica* allergic patients showed IgE reactivity to a spot (pI 4.53, MW 9) that corresponds with the basic profilin isoform [11]. The biochemical identity of this immunoreactive spot, was furtherly confirmed in the present work by using a specific antibody against plant profilin (Figure 2D).

Discussion

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.j pollen. An understanding of P.j pollen proteome would provide insight in the molecular nature of allergens as well as of other proteins involved in the functional specialization of pollen. *P. judaica* pollen contains at least nine allergens [16], and two of them, named Par j 1 and Par j 2, have been cloned and sequenced showing prevalences of 95% and 82% among *P. judaica* allergic patients [8, 9].

With the aim to obtain a comprehensive and detailed analysis of *Parietaria judaica* pollen content, in a previous work we generated the first reference map of mature pollen grains of *P. judaica* using 2-DE followed by LC-MS/MS analyses[10]. We identified 71 protein spots, including isoforms, and by using an immunoproteomic approach with serum from P.j 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.j-induced allergic reaction. In particular, we have evidenced that the IgE from patients recognize Par j 1 and the Par j 2 P2 allergens while they don’t recognize the polypeptide Par j 2 P8.

In 2004 Asturias et. al [11] described a minor *Parietaria judaica* allergen, named Par j 3, belonging to the conserved family of plant profilins. Profilins are ubiquitous cytosolic actin-binding proteins implicated in the signal transduction cascade as well as in cytoskeletal rearrangements [5]. They have been identified as allergens in pollen of several species of trees, grasses, and weeds and in many fruits and vegetables. The widespread IgE cross-reactivity towards a broad range of profilins has led to the designation of profilins as pan-allergens [6]. The prevalence of sensitization to

ubiquitous allergens, like profilins and calcium-binding proteins, is usually low. One reason for this low allergenic potential may be the fact that members of both protein families are found in human beings, leading to suppression of the immune response to these allergens in most individuals [17]. Since the pan-allergen are responsible for extensive cross-reactivity among pollen-sensitized patients, the identification and characterization of the allergenic molecules is an important goal to improve diagnosis and treatment of allergy.

In this study, proteomic strategy has been applied for the identification of allergenic proteins. Our data show that 2DE and subsequent western blot analysis represent satisfactory tools for immunological characterization of proteins from pollen extract. 2-DE technique improves the separation of proteins isoforms and discriminate between plant proteins which has similar molecular weight. By an immunoproteomic approach, we demonstrated that a pool of P.j allergic patients show a positive reaction to profilin. Profilin could be represented a potential diagnostic marker molecules by which to distinguish patients with primary sensitization to *Parietaria judaica* from those who exhibit IgE reactivity and clinical sensitivity (ie, skin sensitivity) to *Parietaria judaica* because of cross-reactivity with other weed species (eg, mugwort and ragweed).

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