

Original article

Purification and characterization of Pla a 1, a major allergen from *Platanus acerifolia* pollen

Background: Plane trees, as *Platanus acerifolia*, are an important source of airborne allergens in cities of the United States and Western Europe. Little is known about the relevant allergens of this pollen. The aim of this study was to identify relevant allergens from *P. acerifolia* pollen and purify and characterize a major allergen of 18 kDa.

Methods: *P. acerifolia* pollen extract was fractionated using ion exchange, gel filtration, and reverse-phase chromatography. Analyzes were carried out by EAST, SDS-PAGE, isoelectric focusing, immunoblotting and amino-acid sequencing.

Results: An 18-kDa protein from the *P. acerifolia* pollen extract, which we named Pla a 1, was purified. This nonglycosylated protein had an isoelectric point value higher than 9.3 and was recognized by up to 92% of monosensitized *Platanus* allergic patients and 83% of polysensitized patients. Sequencing of its N-terminal yielded an amino acid sequence which showed no homology to the known proteins in the databases. Other relevant allergens detected in monosensitized patients were proteins of 43 and 52 kDa, with immunoglobulin (Ig)E-binding prevalences of 83 and 42%, respectively. Profilin was an important allergen in polysensitized patients.

Conclusions: The most relevant allergens from the *P. acerifolia* pollen have been determined. A major allergen, specific of this pollen, and named Pla a 1, has been purified and characterized.

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Plane trees, or sycamores, are widely grown as ornamental trees in the United States and Western Europe, being *Platanus acerifolia* (*P. hybrida*), called London plane tree, one of the most frequent trees in the majority of western European cities. High concentrations of its pollen are detected during the flowering season in several European countries (1–4), reaching up to 14% of the total pollen in Madrid, Spain, where about 73,000 plane trees were registered in 1994 (5). In spite of the high concentration of the pollen found, *P. acerifolia* has been described as a low allergenic pollen. However, recent studies performed in Spain showed a different prevalence of positive skin-prick tests (SPT) to *P. acerifolia* ranging from 5 to 56%, depending on the area of study (5, 6). Although the importance of *P. acerifolia* pollen in allergy has increased over the last years, few data on the description of IgE-reacting proteins from this pollen have been reported (6, 7).

In the present study allergens contributing to pollinosis symptoms on monosensitized and polysensitized *Platanus*-allergic patients were described. Furthermore, a major plane tree allergen has been purified for the first time, and its biochemical properties as well as its allergenic significance are presented.

Material and methods

Patients

Patients showed IgE-mediated allergy (8) to *Platanus* pollen according to a clinical history of seasonal or perennial rhinitis, asthma, or both, positive response to SPTs (Bial-Aristegui, Bilbao, Spain), and specific IgE levels to London plane tree pollen greater than enzyme-linked allergosorbent test (EAST) class 2 score. Twelve patients (five female and seven male patients, aged 25–50 years; mean age 42 years) were monosensitized to *Platanus* pollen. Eighteen patients (six female and 12 male patients, aged 14–66 years; mean age 28.5 years) allergic to *Platanus* pollen were polysensitized to other pollens as well, grass and olive pollen being the most important ones. Specific IgE levels were quantified by EAST (Hytec-specific IgE EIA; Hycor Biomedical Inc., Kassel, Germany), as described by the manufacturer, using *P. acerifolia* extract coupled to paper disks (9).

Pollen crude extract preparation

Crude extracts of pollen from *P. acerifolia*, *Lolium perenne*, and *Olea europaea* were prepared as previously described (10). Protein content of each extract was determined by the Bradford's method (11).

Purification of 18 kDa allergen and N-terminal amino acid analysis

Ion exchange chromatography: *P. acerifolia* pollen extract (88 mg protein) was fractionated on a HiTrap SP column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM

phosphate buffer pH 6.0 on a ÄKTA-prime System (Amersham Pharmacia Biotech) using an step gradient of 0.3, 0.5, and 1.0 M NaCl. Allergic activity of the fractions was analyzed by SDS-PAGE immunoblotting incubated with a patient serum (lane 2, Fig. 1A) that reacted exclusively to this protein.

Gel filtration chromatography: pooled reactive fractions were concentrated and applied to a Superdex 75 column on a SMART System (Amersham Pharmacia Biotech) equilibrated with 0.15 M NaCl in 20 mM Tris buffer pH 8.0. The fractions of all the resolved peaks were pooled separately and analyzed by SDS-PAGE immunoblotting.

Reverse-phase chromatography: pooled reactive fractions were applied to a μ RPC C2/C18 column (Amersham Pharmacia Biotech) on a SMART System using a gradient of 10–45% acetonitrile in 0.1% trifluoroacetic acid (TFA). Fractions reacting to the positive serum were pooled, dialyzed, concentrated, and kept at -20°C .

N-terminal amino acid of the Pla 1 was determined by Edman's degradation technique using a 494 Procise Protein Sequencing System (PE Biosystems, Weiterstadt, Germany).

Immunodetection and immunoblot inhibition assays

Proteins were analyzed by SDS-PAGE under reducing (12) and nonreducing conditions and visualized by Coomassie Blue R250 staining. Separated protein bands were electrophoretically transferred onto polyvinylidene difluoride (PVDF) (13) and blocked for 1 h at room temperature with 0.1% Tween-20 in Tris-buffered saline (TBS). Membranes were incubated overnight at 4°C with allergic patients sera (diluted 1 : 4 or 1 : 6 in 0.1% Tween-20 in TBS) followed by antihuman IgE-horseradish peroxidase conjugate incubation and detected by the chemiluminescence method as recommended by the manufacturer (ECL-Plus; Amersham Pharmacia Biotech).

Immunoblot inhibition assays were carried out using a pool of sera from patients. Samples of crude extracts were separated by SDS-PAGE and electrotransferred as above described. The serum pool (diluted 1 : 4 or 1 : 6 in blocking buffer) was preincubated overnight at 4°C with each inhibitor or bovine serum albumin (BSA), before exposure to the membranes.

Monospecific rabbit antisera against profilin, Ole e 1, and Lol p 1 were obtained as previously described (14, 15). Olive tree pollen profilin, Ole e 1, and Lol p 1 were purified using standard chromatographic methods (15–17).

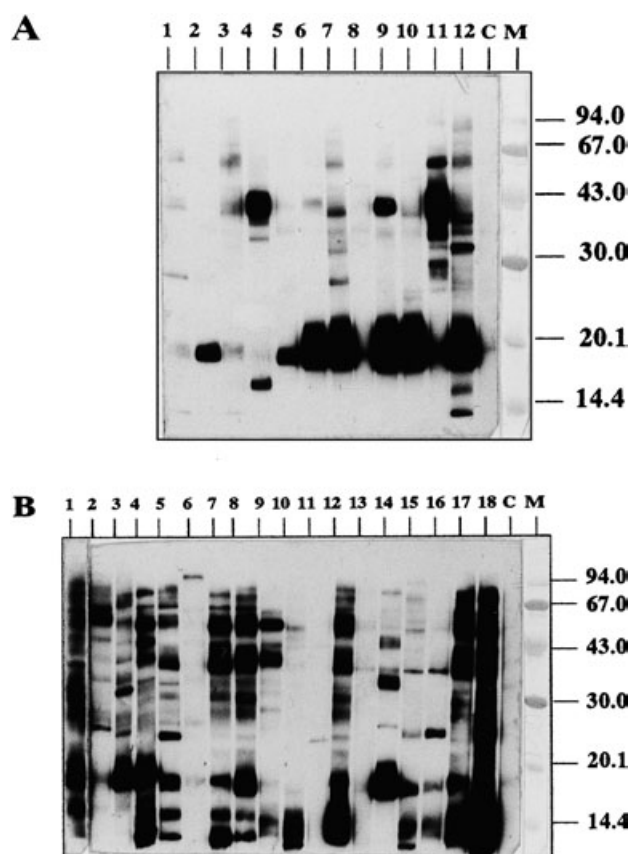


Figure 1. Immunoglobulin (Ig)E-immunoblot analysis of crude extracts from *Platanus acerifolia* (*P. acerifolia*). Samples (4 μg protein) were separated by SDS-PAGE, electrotransferred onto PVDF membranes, and incubated with individual sera from monosensitized (A) or polysensitized patients (B) to *Platanus* and serum pool from nonallergic patients (lane C).

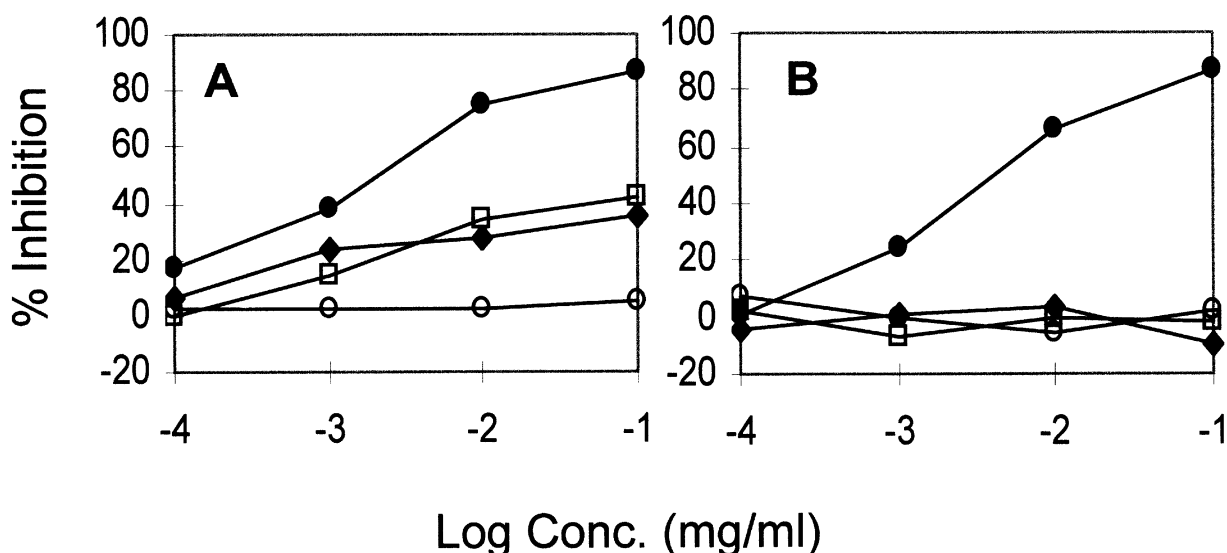


Figure 2. (A–B) IgE-EAST inhibition. Inhibition of IgE-binding of serum pool from polysensitized (A) and monosensitized (B) patients to *Platanus* after incubation with *P. acerifolia* (●), *L. perenne* (◆), *O. europaea* (□) and *P. judaica* (○) extracts.

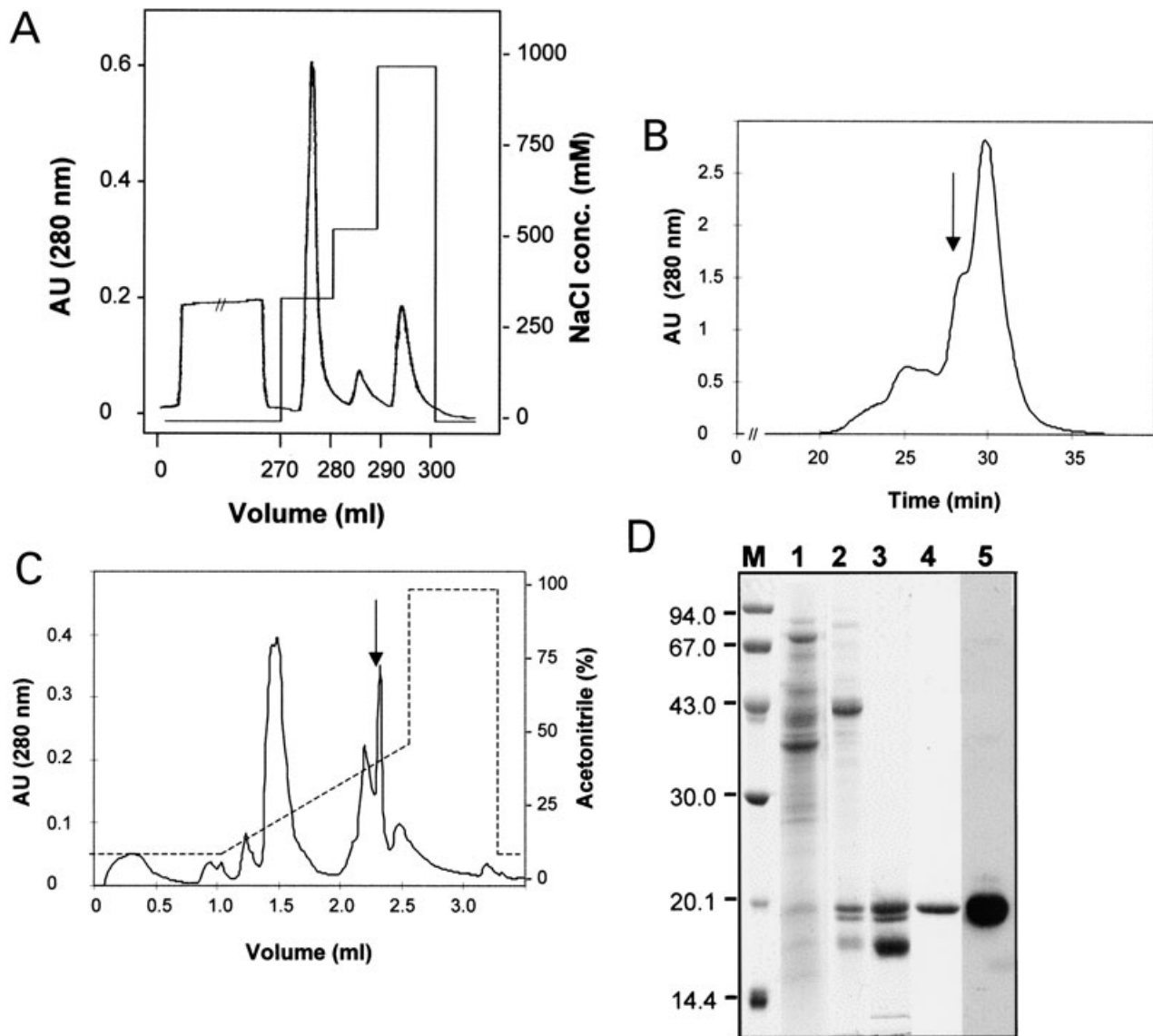


Figure 3. Purification of Pla a 1 from *P. acerifolia* pollen extract. (A) Elution profile of *P. acerifolia* pollen extract on HiTrap SP column. (B) Elution profile of IgE-reactive fractions on Superdex 75 column. (C) Elution profile of reactive fractions on μ RPC C2/C18 column. Arrows pointed to IgE-reactive fractions. (D) Coomassie R-250 stained of SDS-PAGE separation of pollen extract from *P. acerifolia* (lane 1) and active fractions after HiTrap SP column (lane 2), Superdex 75 column (lane 3), and μ RPC C2/C18 column (lane 4). IgE-immunoblotting of purified protein is shown in lane 5.

Agarose-isoelectric focusing

Isoelectric focusing (IEF) was performed on Isogel Agarose plates (FMC Bio Products, Rockland, MN, USA), pH 3–10, and separated proteins were capillary blotted onto PVDF (18), blocked for 1 h at room temperature with 0.1% Tween-20 in TBS, and incubated as described above. The resulting electrophoretic patterns were evaluated by image analysis in a GS-710 Image Analyzer (Bio-Rad Laboratories, Richmond, CA, USA).

Rotamase assay

Rotamase activity was assayed as described by Cadot et al. (19). The acceleration of the rate of chromogenic peptide (succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, 10 μ mol/l) cleavage by chymotrypsin (2 μ mol/l) in HEPES buffer (35 mmol/l, pH 8.0) was detected as the increase of absorbance at 390 nm.

Glycoprotein detection methods

Detection of glycoproteins of blotted purified 18 kDa protein was performed using the DIG Glycan Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the recommendations of the manufacturer. The specific binding of the 18 kDa protein to different agarose-coupled lectins was evaluated by the Hi-Trap Lectin Test Kit (Amersham Pharmacia Biotech) following the specifications of the supplier.

Results

IgE reactivity of sera from patients to *P. acerifolia* pollen

Crude extracts of *P. acerifolia* pollen was obtained by PBS extraction under standard conditions containing an average of 8.2% of protein. Sera from 30 patients

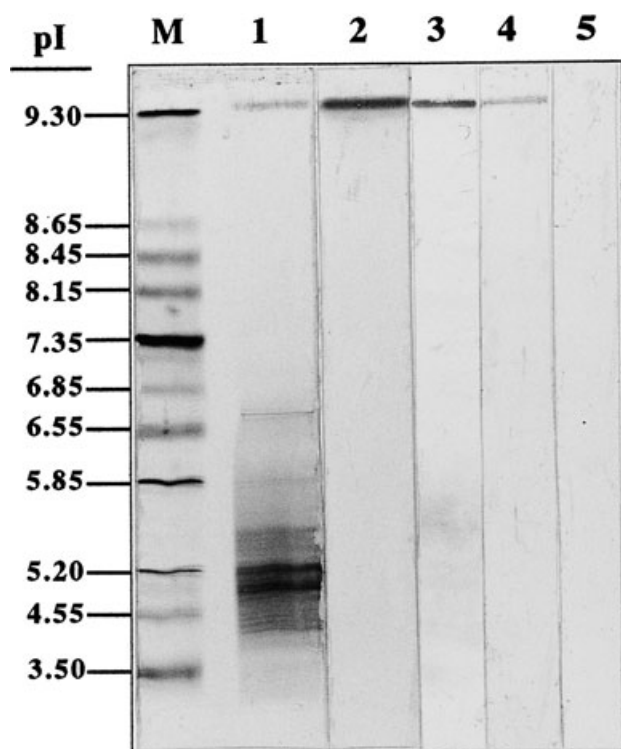


Figure 4. Coomassie-stained IEF (lanes 1, and 2) and IEF-immunoblotting (lanes 3, 4, and 5) of *P. acerifolia* extract (lanes 1, 3 and 5) and purified Pla a 1 (lanes 2 and 4). IEF-immunoblots were incubated with serum pool from patients monosensitized to *Platanus* (lanes 3 and 4) and with a serum pool from nonallergic individuals (lane 5). Lane M: pI markers.

allergic to London plane tree pollen were individually tested by specific IgE immunodetection after SDS-PAGE of the crude extract carried out under reducing conditions (Fig. 1). Monosensitized and polysensitized patients showed different IgE-binding patterns. The apparent molecular masses of the main allergens of *P. acerifolia* extract detected by sera from the monosensitized patients were 15, 18, 43, and 58 kDa with prevalences of 16, 92, 83, and 42%, respectively. Polysensitized patient sera revealed allergenic components with apparent molecular masses of 13, 15, 18, 43, 54–59 kDa, with prevalences of 50, 78, 83, 83, and 67%, respectively.

Cross-reactivity of *P. acerifolia* pollen

EAST-inhibition was performed to establish any cross-reactivity between *P. acerifolia* extract and pollen extracts from *L. perenne*, *O. europaea*, and *P. judaica*. IgE reactivity to *P. acerifolia* extract was partially inhibited by *L. perenne* and *O. europaea* extracts when serum pool from polysensitized patients was used (Fig. 2A). Incubation of *Platanus* extract with specific polyclonal antibodies against profilin demonstrated the presence of profilin in the *Platanus* extract at 15 kDa. No protein similar to Ole e 1 was detected in *Platanus* using specific Ole e 1-antiserum (data not shown). As

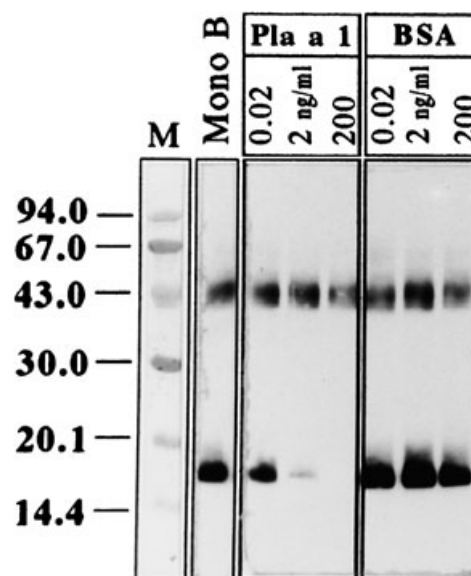


Figure 5. SDS-PAGE immunoblotting-inhibition of *P. acerifolia* pollen extract. Serum pool of monosensitized patients preincubated with buffer alone (Mono B) or different concentrations of purified Pla a 1 and bovine serum albumin (BSA).

expected, pool of sera from monosensitized patients showed no cross-reactivities between *Platanus* and *Lolium*, *Olea*, and *Parietaria* pollen extracts (Fig. 2B).

Purification of 18 kDa allergen

Crude pollen extract of *P. acerifolia* was applied to a cation exchanged column for a first enrichment in basic proteins, and eluted stepwise (Fig. 3A). Allergenic fractions appearing at 0.3 M NaCl were pooled, concentrated and applied to a Superdex 75 column. The 15–25 kDa fractions showing allergenic activity were further purified by reverse-phase chromatography. Seven peaks were resolved using this system, but only the peak eluted at 40% acetonitrile showed allergenic activity. The SDS-PAGE of the isolated protein showed a single electrophoretic band with an apparent molecular mass of 18 kDa under reduced conditions (Fig. 3D) and 16 kDa under nonreduced conditions (data not shown). The apparent molecular mass on gel filtration chromatography of purified 18 kDa protein was 18 ± 3 kDa, suggesting a monomeric nature of the protein. The final yield of the isolated *Platanus* allergen was 0.08% of the PBS-extracted protein.

According to the results obtained with sera from 30 allergic patients, and because more than 50% of these *Platanus*-allergic patients (87%) recognized this protein, it should be considered as a major allergen and was therefore denominated Pla a 1 in accordance with the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee.

Protein characterization

The presence of carbohydrate moieties in Pla a 1 was evaluated by its capacity of binding to agarose-coupled lectins from wheat germ, lentil, peanut, and jack bean (ConA), which showed different affinities against sugars. None of the lectins tested bound Pla a 1, excluding the presence of mannose, glucose, galactose, or N-acetyl glucosamine end groups coupled to the protein core. Further experiments on glycoprotein detection of blotted Pla a 1 using a commercial kit based on the mild periodate treatment, also gave negative results. These results suggested that Pla a 1 is less likely to be a glycoprotein. Partial purified preparations of Pla a 1, before phase-reverse chromatography, were tested for rotamase activity, but no rotamase activity was detected.

The high degree of purification obtained with Pla a 1 allowed the N-terminal amino acid sequencing of its 12 final residues. The resulted amino acid sequence was: ADIVQGTCCKVA. This sequence has been submitted to the SWISS-PROT database under accession number P82817. No sequence homology to other proteins could be found in the databases.

Isoelectrofocusing

Isoelectrofocusing experiments showed that the *P. acerifolia* extract is constituted mainly by acidic proteins with pI values ranging from 5.5 to 4.0 and a basic band of pI value higher than 9.3 (Fig. 4). Isoelectrofocusing-immunodetection with serum pool from monosensitized patients showed a strong IgE reactivity to the basic band and faint reactivity bands at pI values from 5.5 to 4.2. IEF of purified Pla a 1 showed a single band at pI value greater than 9.3, which reacted to pooled sera from monosensitized patients (Fig. 4).

Inhibition experiments

To demonstrate that the purified protein was the same as the one in the crude extract, immunoblot-inhibition experiments were performed by preincubating pooled sera from monosensitized patients allergic to plane tree pollen with purified Pla a 1. Pla a 1 concentrations higher than 2 ng/ml completely inhibited the IgE-binding to the 18 kDa protein present in the *P. acerifolia* pollen extract (Fig. 5). No inhibitory effect was detected in the control experiments with BSA as the control inhibitor.

Purified Ole e 1, olive profilin, and Lol p 1 (20 µg/ml) were used to inhibit pooled sera from polysensitized patients in order to study the presence of cross-reacting proteins. Olive profilin inhibited specific IgE-binding to 16–14 kDa band, while preincubation with Lol p 1 and Ole e 1 had no apparent effect on the IgE-binding to *Platanus* pollen extract proteins (data not shown).

Discussion

The occurrence of *Platanus* pollinosis is not generally considered although very large quantities of airborne *Platanus* pollen in many cities of the United States and Europe have been reported (1–5). Recent studies reported a high prevalence of positive SPT to *Platanus* in Spain, ranging from 5 to 56% (5, 6), but little is known about the relevant allergens of plane tree pollen. In this study, we characterized the *P. acerifolia* extract and purified a major allergen from this pollen.

SDS-PAGE immunoblotting patterns of *P. acerifolia* pollen incubated with sera from monosensitized and polysensitized *Platanus* patients were slightly different. Sera from monosensitized patients recognized four allergens of 18, 43, 58, and 15 kDa, but only the 18 and 43 kDa proteins were major allergens. IgE-reacting proteins of 17 and 45 kDa have previously been reported as allergens of *Platanus* pollen although no data on their biochemical or allergenic features have been described (20, 21). *Olea* and *Lolium* pollen extracts were able to partially inhibit IgE-binding to *Platanus* pollen extract in EAST using pooled sera from polysensitized patients, showing that these extracts share some common epitopes with the *Platanus* one. As expected, owing to the cosensitization with grasses, specific IgE antibodies against Lol p 1 are present in the sera of polysensitized patients (data not shown). Significant cross-reactivity to grasses have previously been observed by ELISA inhibition studies with serum from *Platanus*-allergic patients (21). No cross-reacting allergen to Ole e 1 was detected in *P. acerifolia* extract using anti-Ole e 1 rabbit serum and immunoblotting experiments, which suggested that the detected cross-reactivity should be ascribed to other olive allergens.

Profilins are prominent allergens in pollen of trees, grasses and weeds and are recognized by 20% of all pollen-allergic patients (22). As a result of this broad allergic incidence, profilins have been described as panallergens (21, 23). Immunoblotting-inhibition experiments with olive profilin showed that this panallergen is involved in the observed cross-reactivity of plane tree pollen. In these experiments olive profilin reduced the IgE-binding to 15 and 13 kDa bands, suggesting that the *Platanus* pollen extract could contain several profilin isoforms. The existence of microheterogeneity is already known in some plant profilins such as olive (16), sunflower (14), and soy bean (24). As the frequency of IgE reactivity to some well-characterized plant profilins is in general quite low, the observation that a plane tree profilin has a prevalence of 47% is of interest and needs to be further investigated.

The 18 kDa protein from the *P. acerifolia* pollen, with a 87% prevalence among plane tree pollen-allergic patients, was named Pla a 1 and purified by ion exchange, gel filtration and reverse phase chromatography. IEF-immunoblotting of crude extract and

purified Pla a 1 shown a similar pattern when incubated with serum pool from monosensitized patients, because Pla a 2, the other major allergen of *P. acerifolia*, has also got a pI value greater than 9.3 (J. Ibarrola, unpublished results), and the other allergens (very faint bands of acidic pI in Fig. 4) are almost lost by dilution in the pool of sera. The presence of isoforms of Pla a 1 can not be ruled out because as in Fig. 3(C), the elution profile after the reverse-phase chromatography indicates a significant peak overlap while the SDS-PAGE shows a single band of Pla a 1. Furthermore, Pla a 1 has a pI value >9.3 which is the limit of the range of the Isogel Agarose plates used for IEF; for this reason it is not possible to detect any putative bands corresponding to the Pla a 1 isoforms. In an earlier study, Anfosso et al. reported a 22-kDa glycoprotein, named allergen P, with a pI value of 4.2 present in the *Platanus* pollen extract (7, 25). Although this protein was reported as a major allergen and showed a molecular mass close to Pla a 1, the allergen purified here is a nonglycosylated and basic protein which suggested that Pla a 1 and allergen P may be different proteins. Recently, a new allergen from *Betula verrucosa* (Bet v 7), belonging to the cyclophilins family, has been reported (19). This allergen has a molecular mass of 18 kDa and a pI in the range of 9.0–9.3, similarly to Pla a 1, but as it is shown in the present study neither rotamase activity nor N-terminal amino acid sequence homology with cyclophilins were detected in Pla a 1. Furthermore, preliminary comparisons based

only on the N-terminal sequence of Pla a 1 to the SWISS-PROT database found no significant homology of Pla a 1 to any registered protein. All these results suggested that the Pla a 1 of *P. acerifolia* might be a new and unique allergen. Studies aimed at the elucidation of the complete sequence of Pla a 1 and its homology to other allergens are being carried out by isolation of mRNA and synthesis of the cDNA encoding allergen using specific primers derived from the N-terminal amino acid sequence here described.

In this report we have described the most relevant allergens from the *P. acerifolia* pollen and the purification and characterization for the first time of Pla a 1, a major allergen from the *P. acerifolia* pollen.

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