

Development of a Sandwich-Type ELISA for Measuring Pla a 1, the Major Allergen of *Platanus acerifolia* Pollen

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Key Words

Pla a 1 · Planetree · *Platanus acerifolia* · Two-site sandwich ELISA

Abstract

Background: *Platanus acerifolia* is an important cause of pollinosis in Western European cities. Pla a 1, a nonglycosylated 18-kDa protein with a prevalence of 80%, is a major allergen in *P. acerifolia* pollen extracts. Our aim was to develop a Pla a 1-specific ELISA to quantify this protein in allergenic extracts and preparations for clinical use. **Methods:** Pla a 1 was purified by cation exchange at pH 7.0, gel filtration, and anion exchange chromatography at pH 10.0. Monoclonal (mAb) and polyclonal antibodies were obtained by immunizing mice and rabbits with nPla a 1. One (5C1) of the 13 mAb obtained was used as capture antibody at 5 µg/ml and biotin-labeled specific polyclonal antiserum at 0.63 µg/ml served for detection. **Results:** The prevalence of Pla a 1-specific IgE to purified Pla a 1 among 47 *P. acerifolia*-allergic patients was 79%. The Pla a 1-ELISA developed has a linear range of 3–25 ng/ml, high sensitivity with a detection limit of 0.5 ng/ml and is highly specific as none of the 24 pollen, mite, mold, and plant food extracts tested gave positive

results. The assay could quantify Pla a 1-like proteins in other planetree pollen extracts. A good correlation was obtained between Pla a 1 content of 11 *P. acerifolia* pollen extracts (average content 0.69% of the total protein) and their IgE-binding activity. **Conclusions:** The described two-site sandwich ELISA to measure Pla a 1 is useful for standardization of planetree pollen extracts intended for clinical use.

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Introduction

Platanus acerifolia (London planetree) is one of the most frequently observed urban tree in Western European cities due to its resistance to diseases and urban pollution. In recent years, an increasing prevalence of positive skin reactions to *Platanus* species has been reported [1, 2] which are now considered as some of the most important pollens producing pollinosis in Spain [3]. Moreover, a strong association has been observed between *P. acerifolia* pollinosis and food allergy [4–6]. Several allergens have been described in *P. acerifolia* pollen extract but only two of them, Pla a 1, a nonglycosylated protein of 18 kDa that belongs to a new class of allergens related

to invertase inhibitors, and Pla a 2, a polygalacturonase of 43 kDa, are considered major allergens. Pla a 1 and Pla a 2 inhibit 60 and 52% of the total IgE-binding capacity of the *P. acerifolia* pollen extract, respectively, and have a prevalence of about 80% among planetree pollen-allergic patients [7–9].

Standardization of the allergenic extracts is essential to guarantee their diagnostic and therapeutic quality. But a significant inconvenience with the current standardization protocols is that only an overall allergenic potency is determined on the basis of reactivity with IgE antibodies of pooled serum samples from allergic patients, and extracts with similar potency may have differences in major allergen ratios [10]. However, mAb-based assays allow quantifying specific allergen levels with the sensitivity, specificity and reproducibility required. In the present study we describe a two-site sandwich ELISA, based on a mAb and a specific rabbit polyclonal antibody against purified Pla a 1, to quantify this allergen in *P. acerifolia* pollen extracts. This assay could be a valuable tool to improve standardization of allergenic products intended for clinical use.

Materials and Methods

Patient Sera

Sera from 47 Spanish allergic patients with monosensitization to London planetree pollen or with additional sensitizations to other allergenic sources, such as olive, grass, wall pellitory, and cypress pollens, mites, and cats, were used in this study. All patients showed immediate hypersensitivity to *P. acerifolia* according to a clinical history of seasonal or perennial rhinitis, asthma, or both, and positive skin reaction to a biologically standardized commercial *Platanus acerifolia* extract (Bial-Aristegui, Bilbao, Spain).

Pollen Extracts and Pla a 1 Purification

P. acerifolia (IberPolen, Málaga, Spain), *P. occidentalis*, *P. orientalis*, and *P. racemosa* (Biopol, Spokane, Wash., USA) pollen crude extracts were prepared as previously described [11]. Natural Pla a 1 (nPla a 1) was purified by cation exchange at pH 7.0, and gel filtration through an SX200 column, as previously described [7], but an anion exchange chromatography at pH 10.0 was used as the final step. Briefly, fractions containing Pla a 1 from the SX200 column were pooled, buffer exchanged to 20 mM ethanolamine pH 10.0 and loaded in a monoQ 4.6/100 PE column fitted in a Smart System (Amersham Biosciences, Uppsala, Sweden). Bound proteins were eluted with a 0–150 mM NaCl gradient and nPla a 1 was found as a single peak at 120 mM NaCl. Recombinant Pla a 1 (rPla a 1) was expressed in *Escherichia coli* and purified as previously reported [8]. Briefly, cells expressing rPla a 1 were harvested by centrifugation, and the pellet, resuspended in 0.05 M Tris-HCl, pH 8.0, 5 mM dithiothreitol, 0.1% Triton X-100, was lysed by lysozyme treatment (100 µg/ml, 30 min, 37°C) and mild sonication. After centrifugation at 15,000 g for 15 min, sediment was resuspended

into 2 M urea, 0.2% Triton X-100 and centrifuged under the same conditions. Supernatant was twice diluted with 20 mM phosphate buffer and equilibrated to pH 6.5. rPla a 1 was purified in a single step by cation exchange chromatography in the conditions used for nPla a 1. Protein concentration was determined by a modification of the Lowry assay [12].

Enzyme Allergosorbent Test and Enzyme Allergosorbent Test Inhibition

Specific IgE levels were quantified by an enzyme allergosorbent test (EAST; Hytec-specific IgE EIA; Hycor Biomedical Inc., Kassel, Germany) as described by the manufacturer. *P. acerifolia* extract and nPla a 1 were coupled to CNBr-activated paper disks [13] at 400 and 50 µg/ml, respectively. The IgE-binding activity of *P. acerifolia* pollen extract was evaluated by EAST inhibition using a serum pool from London planetree pollen-sensitized patients. Relative allergenic activity of the extracts was estimated on the basis of the 50% inhibition EAST values and expressed in arbitrary allergenic units.

Production and Purification of Pla a 1-Specific mAbs and Polyclonal Antiserum

Pla a 1-specific mAbs were prepared essentially as described [14]. Briefly, spleen cells obtained from BALB/c mice immunized with purified nPla a 1 were fused with Sp2/O myeloma cells in the presence of polyethylene glycol as described by Köhler and Milstein [15]. Positive hybridomas were identified by ELISA with use of nPla a 1-coated plates and then subcloned by limiting dilution. Isotype detection was done by IsoStrip kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Polyclonal serum was obtained by immunizing one female New Zealand rabbit with 200 µg of purified nPla a 1 emulsified in complete Freund adjuvant (Difco, Detroit, Mich., USA). The boosts were carried out at 2-week intervals and serum collected 10 days after the last injection was tested and stored at –80°C. For antibody purification, hybridoma culture supernatants or rabbit anti-Pla a 1 serum were purified by affinity chromatography with protein G-Sepharose (Amersham Biosciences) following the manufacturer's instructions. The purified rabbit IgG was labeled with biotin by a protein biotinylation kit (Amersham Biosciences).

Electrophoresis and Immunoblotting

P. acerifolia extracts or purified allergens were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gels under reducing conditions [16] and stained with Coomassie. Separated polypeptides were electrotransferred onto polyvinylidene difluoride membranes (Amersham Biosciences) and blocked with 5% skimmed milk and 0.1% Tween 20 in PBS (PBS-T). Then, blots were incubated with 6 ml of PBS-T containing mAb 5C1 (0.25 µg/ml), rabbit anti-Pla a 1 serum (diluted 1:500,000), or a pool of sera (diluted 1:4) from *P. acerifolia*-allergic patients. After washing, membranes were incubated with 1:10,000 horseradish peroxidase-conjugated anti-mouse IgG (Sigma, St. Louis, Mo., USA), anti-rabbit immunoglobulins, or anti-human IgE (both from Dako, Copenhagen, Denmark) in PBS-T, respectively. Finally, the blot was washed and bound antibodies were detected by the ECL-plus Western blotting detection system (Amersham Biosciences).

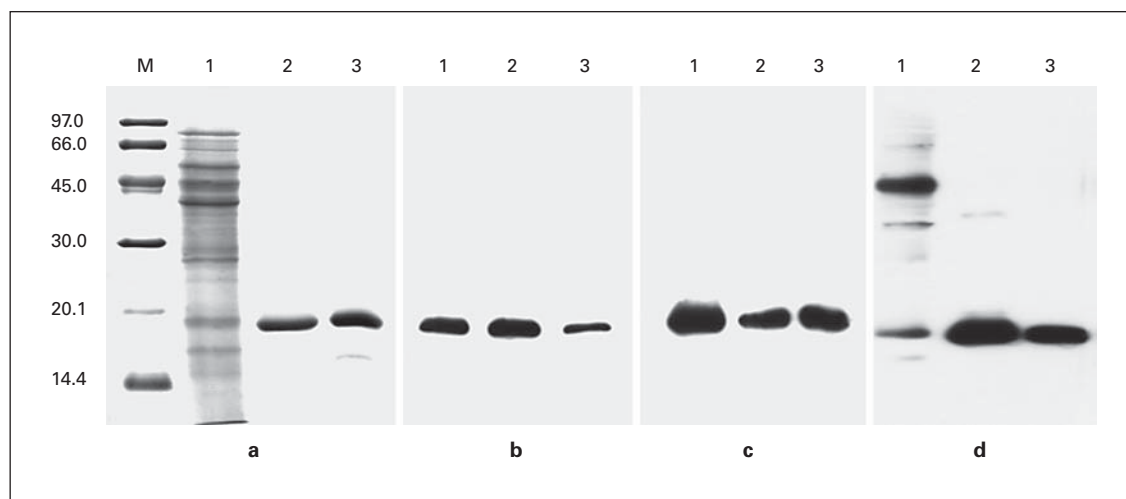


Fig. 1. Coomassie-stained SDS-PAGE (**a**) and Western blotting immunostained with mAb 5C1 (**b**), with rabbit anti-Pla a 1 serum (**c**), or with pool of sera from *Platanus*-allergic patients (**d**) of *P. acerifolia* extract (lane 1), nPla a 1 (lane 2), and rPla a 1 (lane 3).

Table 1. Specific IgE levels to *P. acerifolia* pollen extract and purified Pla a 1 in patients allergic to planetree (n = 47)

EAST class	<i>P. acerifolia</i> extract	Pla a 1
0	0	2
1	1	8
2	15	13
3	9	8
4	19	13
5	3	3
Positivity (%)	98	79

Data show number of patients with the indicated EAST class. EAST class >1 was considered positive.

Quantification of Pla a 1 by a Two-Site Sandwich ELISA

Microwell plates (Greiner, Frickenhausen, Germany) were coated with 100 μ l of mAb 5C1 at 5 μ g/ml in PBS overnight at room temperature. After blocking with 200 μ l of PBS containing 1% BSA and 0.05% Tween 20 (PBS-BT), they were incubated with 100 μ l/well of sample diluted in blocking buffer for 1 h at 37°C. A standard curve was obtained using dilutions from 0.4 to 200 ng/ml of purified nPla a 1. After washing with 200 μ l of PBS containing 0.05% Tween 20, 100 μ l of biotin-labeled anti-Pla a 1 polyclonal antibodies (0.625 μ g/ml in PBS-BT) was added, and the plates were incubated for 1 h at 37°C. Following further washes, bound antibodies were detected by incubation with 100 μ l/well of streptavidin-peroxidase (0.25 μ g/ml in PBS-BT) (Sigma) for 1 h at 37°C. Plates were finally washed and developed by incubation at room temperature in the dark with 200 μ l of freshly prepared enzyme

substrate *o*-phenylenediamine (Sigma). After 30 min, the reaction was stopped by adding 50 μ l of 3 M H₂SO₄ and the optical density was measured at 492 nm. Crude extracts were assayed as dilution series in triplicate, and concentrations were interpolated from the linear portion of the standard curve. A standard curve ranging from 0.4 to 200 ng/ml of Pla a 1 was run per day for 10 days to evaluate inter-assay variations. The detection limit was calculated as the amount of Pla a 1 which corresponded to the median plus 3.33-fold the standard deviation obtained after 30 measurements of the zero standard. In order to determine intra-assay variations, four Pla a 1 concentrations corresponding to the lineal portion of the standard curve (3.12, 6.25, 12.5 and 25 ng/ml) were incubated 20 times during the same day.

Results

Purification and Clinical Relevance of Pla a 1

nPla a 1 and rPla a 1 were purified from an extract of *P. acerifolia* pollen or recombinant *E. coli*, respectively, by chromatography in native conditions avoiding any denaturing manipulation. A purity degree higher than 95% was achieved in both preparations displaying a major band at 18 kDa (fig. 1) which is in agreement with molecular mass derived for the Pla a 1 sequence [8].

In order to confirm the allergenic significance of the purified Pla a 1, the titre of specific IgE to Pla a 1 was determined (table 1). Thirty seven out of 47 tested patients (79%) had specific IgE to Pla a 1, those higher than EAST class 1 being considered positive.

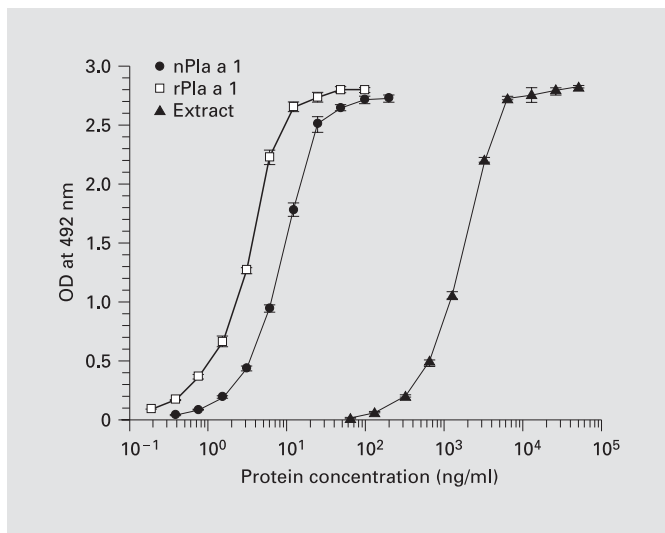


Fig. 2. Dose-response curves in two-site sandwich ELISA of natural Pla a 1, recombinant Pla a 1 and *P. acerifolia* pollen extract.

Pla a 1 ELISA

After fusion of spleen cells from BALB/c mice immunized with purified Pla a 1 and Sp2/0 myeloma cells, thirteen hybridomas secreting mAbs anti Pla a 1 were obtained. mAbs were assayed in pairs to capture and to detect Pla a 1 in solution, but no combination was successful. Five of the thirteen mAbs were able to capture the allergen when biotin-labeled polyclonal antibodies were used as tracer. Among them, 5C1 was chosen as coating mAb in combination with biotin-labeled rabbit anti-Pla a 1 immunoglobulins as detector since this combination yielded the highest sensitivity with Pla a 1 standard curve. 5C1 mAb was an IgG1 subclass antibody with κ light chains and detected an 18-kDa band in the natural and recombinant purified Pla a 1 but also in the *P. acerifolia* pollen extract. The rabbit anti-Pla a 1 serum showed the same recognition pattern (fig. 1).

Pla a 1 standard dose-response curve was done between 0.4 and 200 ng/ml. It revealed a sigmoid shape when plotted on a semi-logarithmic scale, being linear in the range from 3.125 to 25 ng/ml with a coefficient of regression of 0.995 (fig. 2). In this range the intra-assay coefficients of variation (CVs) were 10, 5, 6, and 3% at 3.12, 6.25, 12.5 and 25 ng/ml, respectively. The inter-assay CVs were 9, 12, 10 and 8% at the same concentrations. The practical working range was from 1.6 to 200 ng/ml since these concentrations the CVs were <20%. The calculated detection limit was 0.5 ng/ml. The assay was applied to rPla a 1 and the resulting standard curve was

parallel to the one obtained with the natural allergen but shifted to the left 2.5 times since 3 ng/ml of rPla a 1 gave the same absorbance than nPla a 1 at 7.5 ng/ml ($OD_{492} = 1.5$) (fig. 2). Interference of the skin prick diagnostic solution components, such as glycerol (50%) and phenol (0.05%), on the Pla a 1 ELISA were tested. No effect of these components was detected since the sensitivity of the assay allowed dilutions of 1/500.

The specificity of the two-site sandwich ELISA was demonstrated with extracts of different origins: tree pollen extracts (*Olea europaea*, *Betula verrucosa*, *Pinus radiata* and *Cupressus arizonica*), grass pollen (*Lolium perenne*, *Cynodon dactylon*, *Dactylis glomerata*), other important allergenic weeds included in different taxonomic families (*Parietaria judaica*, *Artemisia vulgaris*, *Ambrosia trifida*, *Plantago lanceolata*, *Helianthus annuus*), and non-plant extracts (*Alternaria alternata* and *Dermatophagoides pteronyssinus*). Apple, peach, tomato and potato peel, tobacco leaf, hazelnut, peanut, chickpea, lettuce and maize extracts were also tested. All extracts rose to absorbances under the detection limit of 0.5 ng/ml when 10,000-fold higher concentration of extracts were used (50 μ g/ml).

Pla a 1 in Planetree Pollen Extracts and Correlation with IgE-Binding Activity

Proteins sharing epitopes for mAb 5C1 and Pla a 1-specific rabbit antiserum were found in other species of *Platanus*. Pla a 1-like proteins content of *P. occidentalis*, *P. orientalis*, and *P. racemosa* were 0.52, 0.43, and 0.65% of the total protein, respectively. Immunoblotting experiments using 5C1, Pla a 1-specific rabbit antiserum, and serum pool from planetree-allergic patients showed that detected proteins in these *Platanus* species have the same molecular mass as Pla a 1 (fig. 3). Eleven *P. acerifolia* pollen extracts were analyzed by specific Pla a 1 ELISA by interpolation in the linear portion of the standard curve. Pla a 1 concentrations ranged between 0.10 and 2.02% of the total protein (average = 0.69, SE = 0.19). IgE-binding activity of these extracts was analyzed by EAST inhibition and results were compared with their corresponding Pla a 1 content values (fig. 4). A very good correlation ($R = 0.96$, $p < 0.001$) was obtained, suggesting that the *P. acerifolia* extract is highly representative of the total IgE-binding activity of the whole pollen extract. According to these results, an average Pla a 1 content at maintenance doses of specific immunotherapy with *P. acerifolia* pollen extracts was estimated at 0.49 μ g/ml.

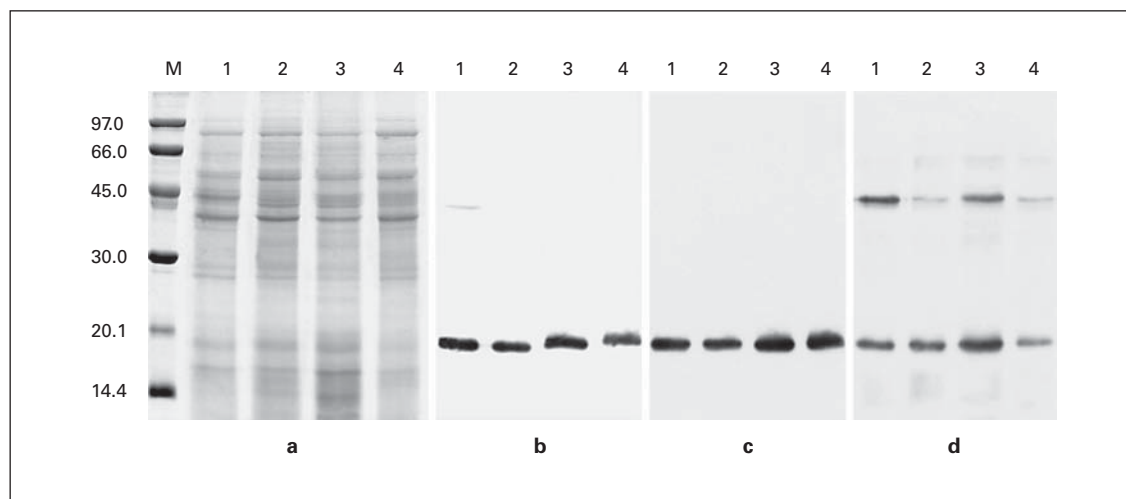


Fig. 3. Coomassie-stained SDS-PAGE (**a**) and immunoblots incubated with mAb 5C1 (**b**), with rabbit anti-Pla a 1 serum (**c**), or with pool of Pla a 1-reactive sera (**d**) of pollen extracts from *P. acerifolia* (lane 1), *P. occidentalis* (lane 2), *P. orientalis* (lane 3), and *P. racemosa* (lane 4).

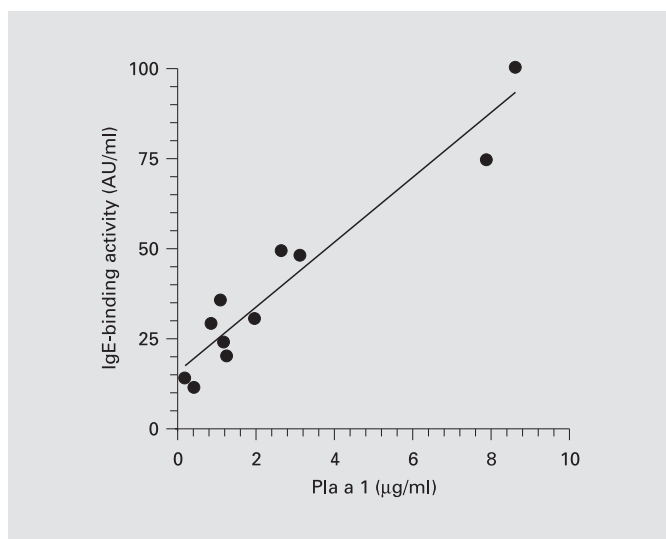


Fig. 4. Correlation between IgE-binding activity values determined by EAST inhibition and Pla a 1 content measured by the two-site enzyme-linked immunosorbent in 11 *P. acerifolia* pollen extracts ($R = 0.96$; $p < 0.001$).

Discussion

The importance of *P. acerifolia* pollen in allergy has increased over the last years. Although depending on the studied geographical area, its pollen is one of the most frequently found in pollen counts [3, 5], and a high per-

centage of patients with seasonal pollinosis are sensitive to it [1]. The quantification of major allergens has become an important goal for the standardization of allergen products intended for clinical use [17]. The determination of Pla a 1 content in *P. acerifolia* extracts is very useful since it is the major allergen in London planetree pollen, even though its relative amount in extracts is very low [7]. Pla a 1 recognition by the specific IgE from the studied population was very high (79%), a value very close to the 83% previously reported [7, 8].

The used of mAbs and polyclonal antibodies in immunoassays allows allergenic quantitation with the required sensitivity, reproducibility and specificity which are the main criteria for a good immunoassay. For this reason, nPla a 1 purified under non-denaturing conditions was used to immunize mice and to produce anti-Pla a 1 mAbs. In spite of the Pla a 1 high-affinity mAbs found, they did not recognized Pla a 1 in solution when mAb were used to capture and trace in all possible combination pairs, therefore these mAbs might detect overlapping epitopes in the protein. The combination of 5C1 mAb as capture and biotin-labeled anti-Pla a 1 polyclonal antibody as tracer was applied in a two-site sandwich ELISA to quantify Pla a 1. Both mAbs and polyclonal antibodies were highly specific as the immunoblotting analysis of the complete extract demonstrated. This combination was chosen since it yielded the Pla a 1 standard curve with the highest sensitivity (0.5 ng/ml) to be used for the routine measurement of Pla a 1 content in production batches of

pollen extracts and prick test solutions. Purified Pla a 1 had a dose-response curve parallel to that of crude *P. acerifolia* pollen extracts, suggesting that the assay measured the same molecule in the different preparations and that the allergens had not been altered during the purification process. The assay reproducibility was proved by the low intra- and interassay CV to be less than 10 and 12%, respectively. Natural and recombinant Pla a 1 also showed parallel curves, but the recombinant Pla a 1 was 2.5-fold more reactive in ELISA than its natural counterpart. This fact could be attributed to partial misfolding of the recombinant protein in *E. coli* cytoplasm when foreign proteins are overproduced [18] increasing antibody affinity. The Pla a 1 content in planetree pollen extract could be underestimated 2.5-fold by using rPla a 1 as reference material.

The described assay is highly specific for *Platanus* genera pollen since it is able to detect Pla a 1-like proteins in other species of planetrees present in different geographical areas such as *P. orientalis* (oriental plane) present in Southeast Europe and Southwest Asia; *P. occidentalis* (American sycamore) found in Eastern North America, and *P. racemosa* (Californian sycamore) present in Western North America. Nevertheless, for a more accurate determination of these Pla a 1-like proteins, corresponding purified proteins should be used as standards in the dose-response curve. Significant cross-reactivity to grasses has previously been observed by ELISA inhibition studies with sera from *P. acerifolia*-allergic patients [19]. In our case, the ELISA was applied to pollen extracts of different origins and negative results were obtained even when grass pollen extracts were analyzed. Cross-reactivity among *P. acerifolia* pollen and plant-derived foods has also been described [4–6]. Oral allergy syndrome in these patients may have been caused by primary respiratory sensitization to *P. acerifolia* [4]. The assay was used to test food plant extracts commonly associated with *P. acerifolia* pollinosis, but no Pla a 1-like protein was detected. This finding suggests that the reported cross-reactivity was not due to Pla a 1 or else that the epitope recognized by 5C1 was not present in these cross-reactive proteins.

A highly significant correlation was obtained between the content of Pla a 1 in *P. acerifolia* pollen extracts determined by ELISA and IgE-binding activity (fig. 4), which confirms that Pla a 1 is an important allergen in planetree pollen extracts, demonstrating that the present immunoassay could be useful for standardization. There is evidence for specific immunotherapy with ragweed, grass, cat, and mite extracts that the optimal maintenance

dose for major allergens is 5–20 µg per injection [20, 21]. Nevertheless, this range should be broadened as higher and lower reactive allergens were included, such since Cup a 1 and Art v 1, with an allergen content of 0.7 and 45 µg/ml, respectively, in maintenance doses of specific immunotherapy with *C. arizonica* and *A. vulgaris* extracts [22, 23]. Pla a 1 could be included among the highest reactive allergens since it is responsible for 60% IgE-binding activity of *P. acerifolia* whole extract [8], but only an average of 0.49 µg/ml of Pla a 1 is present in maintenance doses of specific immunotherapy with this pollen extract vaccine.

In conclusion, this paper describes for the first time an ELISA procedure for measurement Pla a 1 in *P. acerifolia* pollen extracts that could be useful for quality control of preparations intended for clinical use.

Acknowledgments

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