

Purification and characterization of the main allergen of *Plantago lanceolata* pollen, Pla 1 1

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Summary

Background English plantain (*Plantago lanceolata*) pollen is an important cause of pollinosis in the temperate regions of North America, Australia and Europe. However, very little is known about its allergen composition.

Objective The aim of this study was to identify plantain allergens, and to isolate and characterize a major allergen.

Methods Allergens were identified by immunoblotting with individual allergic patients' sera. Isolation of the major allergen was achieved by sequential reverse-phase and size-exclusion HPLC. Allergenic characterization was performed by ELISA and immunoblotting after SDS-PAGE with sera from plantain-allergic patients. N-terminal amino acid sequence was established by Edman degradation.

Results Allergograms showed that 13 out of the 14 sera assayed had IgE to a group of proteins with a molecular weight in the range of 16–20 kD, that turned out to be different isoforms or variants of the major allergen Pla 1 1. Eighteen amino acid residues from the N-terminal end of one of the isoforms, and 10 of three others, were sequenced, and a partial sequence identity with Ole e 1 was found. Prevalence of specific IgE to purified Pla 1 1 in plantain allergic patients was 86%, and represents about 80% of the total IgE-binding capacity of the plantain extract.

Conclusions The most relevant allergen from *P.lanceolata* pollen, Pla 1 1, has been purified and characterized. This contributes to a greater knowledge of the allergen composition of this important weed, and clears the way for the standardization of plantain allergen products in terms of major allergen content.

Keywords: *Plantago lanceolata*, plantain, pollen allergy, allergen, Pla 1 1, amino acid sequence

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Introduction

The genus *Plantago* belongs to the family *Plantaginaceae* and comprises about 250 species. One of the most common species is *Plantago lanceolata* (English plantain or ribwort), which is distributed in the temperate zones of Europe, Australia and North America [1–4]. *P. lanceolata* pollen has been associated with hay fever since the beginning of this century [5–7], and this weed has been considered as one of the most important dicotyledons that cause allergic diseases [8].

In the past years, several studies in different countries have shown the clinical importance of this species. The highest incidence of allergy to *Plantago lanceolata* has been described in Australia [9] and in the Mediterranean area [10]. For instance, Bousquet et al. [11] examined patients with pollinosis and found that 36% of them were sensitized to English plantain in Montpellier (France). In England, two different studies showed that more than 20% of patients with seasonal respiratory allergy gave positive skin reactions to plantain [12,13]. Likewise, a high prevalence of allergy to this pollen has been reported in different cities of Spain [14–16]. Nevertheless, the true role of *P. lanceolata* pollen in the aetiology of pollinosis is

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unclear, because sensitization to plantain alone is unusual. Patients sensitized to plantain are also sensitive to other plants that pollinate in the same season, especially grass pollens [13–17].

Despite the above-mentioned publications, few data on the characterization of the major plantain allergens have been reported, and only a few studies dealing with the identification of *P. lanceolata* allergens have been published so far [13,17,18].

In this article, a study on the allergen composition of *P. lanceolata* pollen is described. Furthermore, the major allergen (Pla 1 1) has been purified, and data on its biochemical features as well as its allergenic significance are presented and discussed.

Materials and methods

Source materials and preparation of the extract

Plantago lanceolata pollen was obtained from Allergen Pollens (Madrid, Spain). The pollen was extracted with 50 mM ammonium bicarbonate, pH 8.5, at a 10% (w/v) ratio by stirring for 60 min at room temperature. Soluble components were separated by centrifugation at 22 000 g for 20 min at 10 °C. The supernatant was dialysed against distilled water, filtered through a 0.22-µm pore size

membrane and lyophilized. Protein concentration was measured by the Lowry method [19], using bovine serum albumin as a standard. The lyophilized extract was stored at 4 °C until use.

Human sera

Sera from 14 individuals allergic to plantain pollen were acquired from PlasmaLab International (Everett, WA, USA). These sera were of RAST class 3 and 4 to *P. lanceolata* (Pharmacia). They were used for immunoblot assays and, for some experiments, a serum pool was made by mixing equal volume aliquots of the individual sera. Sera from eight additional patients with hypersensitivity to *P. lanceolata*, as demonstrated by clinical history, positive skin reaction and presence of serum specific IgE (RAST class 2–4), were used to study the frequency of Pla 1 1 recognition by specific IgE. All the serum samples were stored at –80 °C.

Polyacrylamide gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed with an 8–25% acrylamide gradient in a high-molarity Tris buffer system [20], under non-reducing conditions.

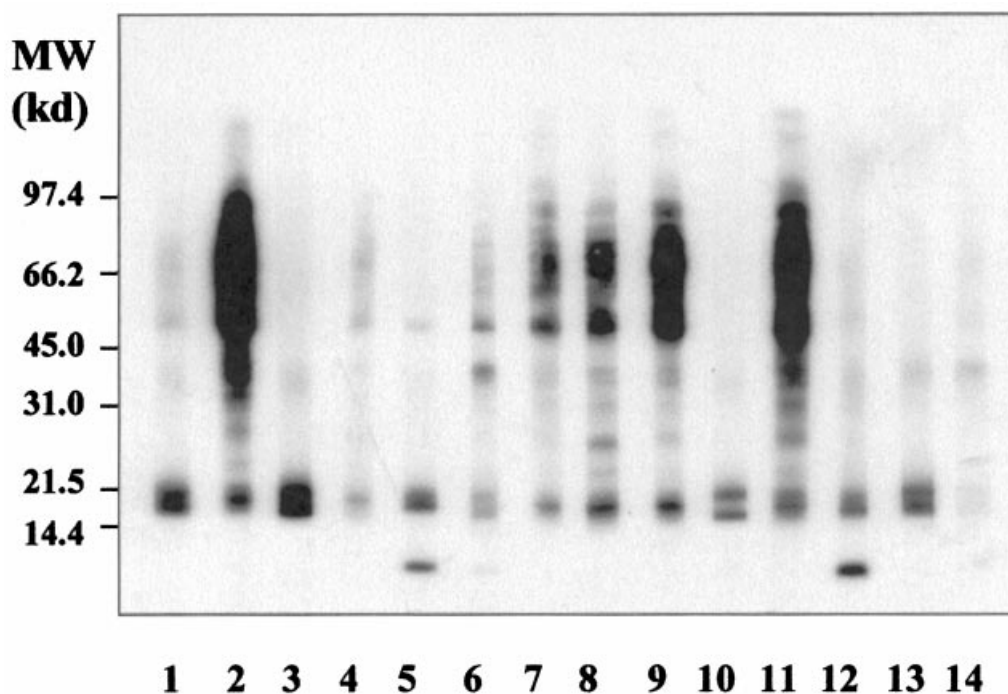


Fig. 1. Immunodetection of IgE-binding polypeptides in PLE with individual sera (diluted 1:5) from plantain allergic patients (lanes 1–14). PLE (400 µg per lane) was subjected to SDS-PAGE under non-reducing conditions. Separated polypeptides were transferred to nitrocellulose membranes and processed as described in Materials and Methods.

Low-range SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular weight markers. Protein bands were visualized with Coomassie Brilliant Blue.

Electrotransfer of the separated polypeptides onto nitrocellulose sheets was carried out according to the method described by Towbin *et al.* [21]. For immunodetection of allergenic polypeptides, the nitrocellulose membrane was incubated overnight at room temperature with either individual sera or with a pool of sera from 14 individuals. The membrane was thoroughly washed and a solution of rabbit antihuman IgE polyclonal antibodies conjugated with peroxidase (Dako, Glostrup, Denmark) was added. After 2 h of shaking at room temperature, the nitrocellulose sheet was incubated with Super Signal^R Substrate Western Blotting (Pierce, Rockford, IL, USA) following the manufacturer's instructions. IgE binding proteins were detected by autoradiography after exposure for 20 s.

Purification of Pla 1 allergen

The lyophilized *Plantago lanceolata* extract was dissolved

in 0.1% trifluoroacetic acid in water, and chromatographed in a short alkyl chain-bonded silica column (Vydac C4, 25 × 1.0 cm, 10 µm particle size, 30 nm pore size). Elution was performed with a 20–50% gradient of acetonitrile in 0.1% trifluoroacetic acid, at a constant flow rate of 1 mL/min. Fractions of 1 mL were collected, and the protein concentration was measured by Waddell's method [22]. Fractions containing IgE-binding proteins were identified by immunoblotting after SDS-PAGE. Subsequently, these fractions were lyophilized and dissolved in 100 mM Na₂SO₄, 20 mM NaH₂PO₄ buffer, pH 6.8, and applied to a size-exclusion HPLC column (Beckman Spherogel TSKG 3000 SW, 30 × 2.1 cm, 13 µm particle size, 25 nm pore size), using the same solvent as the mobile phase at a constant flow rate of 1 mL/min. Fractions obtained were analysed by the same techniques as before, and those containing plantain allergen Pla 1 were dialysed against distilled water, using dialysis tubing with a molecular weight cut-off of 3500 (SpectraPor, Houston, TX, USA), and lyophilized. The chromatographic homogeneity of the Pla 1 preparation was tested on an

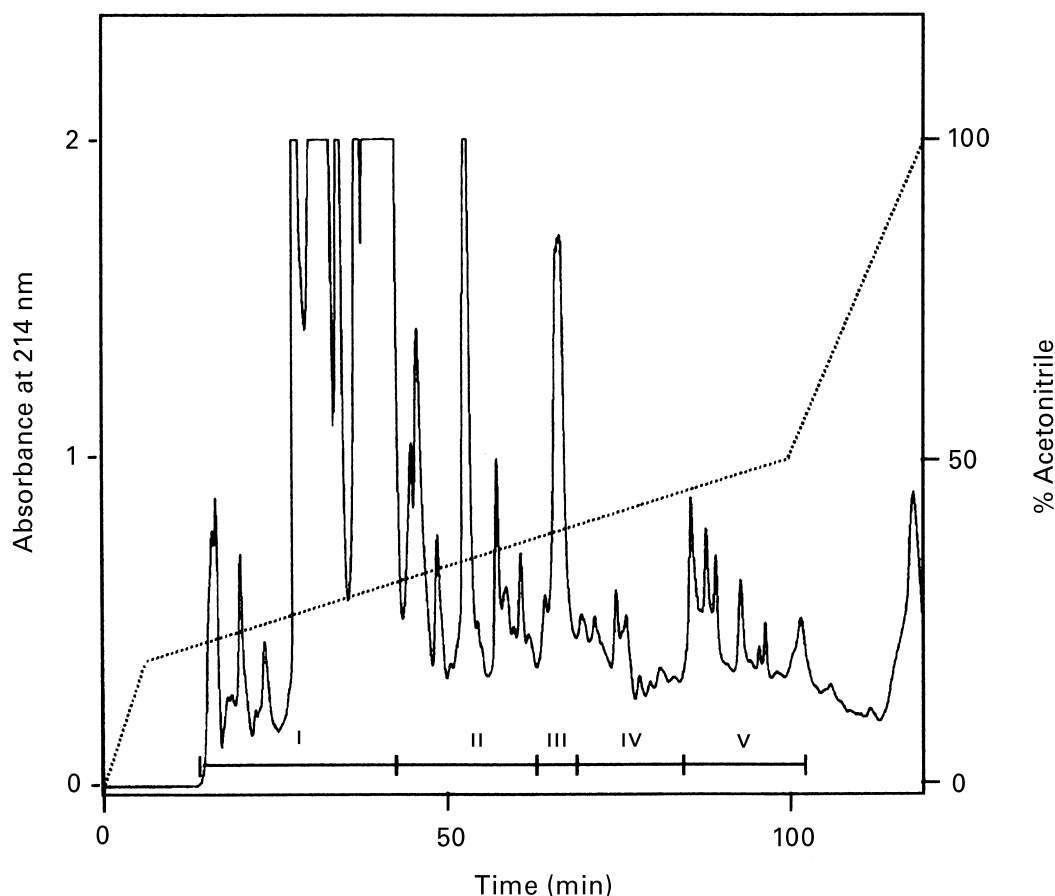


Fig. 2. Elution profile of PLE on reverse-phase HPLC monitored at 214 nm. The lyophilized extract was dissolved in 0.1% trifluoroacetic acid in water and applied to a Vydac C4 column. The dashed line depicts the acetonitrile gradient shape.

analytical size-exclusion Beckman Ultraspherogel SEC 2000 column using the same phosphate buffer as described above.

Immunoassays

Detection of specific human IgE for *Plantago lanceolata* pollen extract (PLE) and for the purified allergen was performed by ELISA. Either PLE (at a 100- μ g/mL protein concentration in PBS) or purified protein (at 1 μ g/mL) was adsorbed to polystyrene microtiter wells (Costar, Corning, NY, USA) and, after blocking with 1% BSA in PBS, wells were sequentially incubated with individual sera from patients (2 h at room temperature) and rabbit antihuman IgE antibodies conjugated with peroxidase (Dako). After 1 h at room temperature, a solution of *ortho*-phenyldiamine (OPD, Dako) was added to each well and left to react for 30 min at room temperature in the dark. The reaction was stopped with 2N HCl and the absorbance measured at 490 nm with a reference filter at 650 nm in an automatic microplate reader.

The allergenic activity of purified Pla 1.1 preparations

was evaluated by competition ELISA experiments that were carried out as before, except that the allergosorbent was made to react with a mixture of 50 μ L of patients' serum pool (diluted 1 : 2 with PBS) and 50 μ L of serial dilutions of the samples.

N-terminal sequence analysis

N-terminal amino acid sequences of Pla 1.1 isoforms were determined on samples electrophoresed and electroblotted onto PVDF (polyvinylidene difluoride) membranes (Millipore, Bedford, MA, USA) using an Applied Biosystems Model 477 A gas-phase sequenator (Foster City, CA, USA).

Similarity searches were performed in protein and DNA sequence databases (Gen Bank, EMBL, PIR, NBR, NBR3D and Swiss Prot) using the FASTA3 program.

Results

Identification of *Plantago lanceolata* allergens

Allergens of PLE were identified using immunoblotting

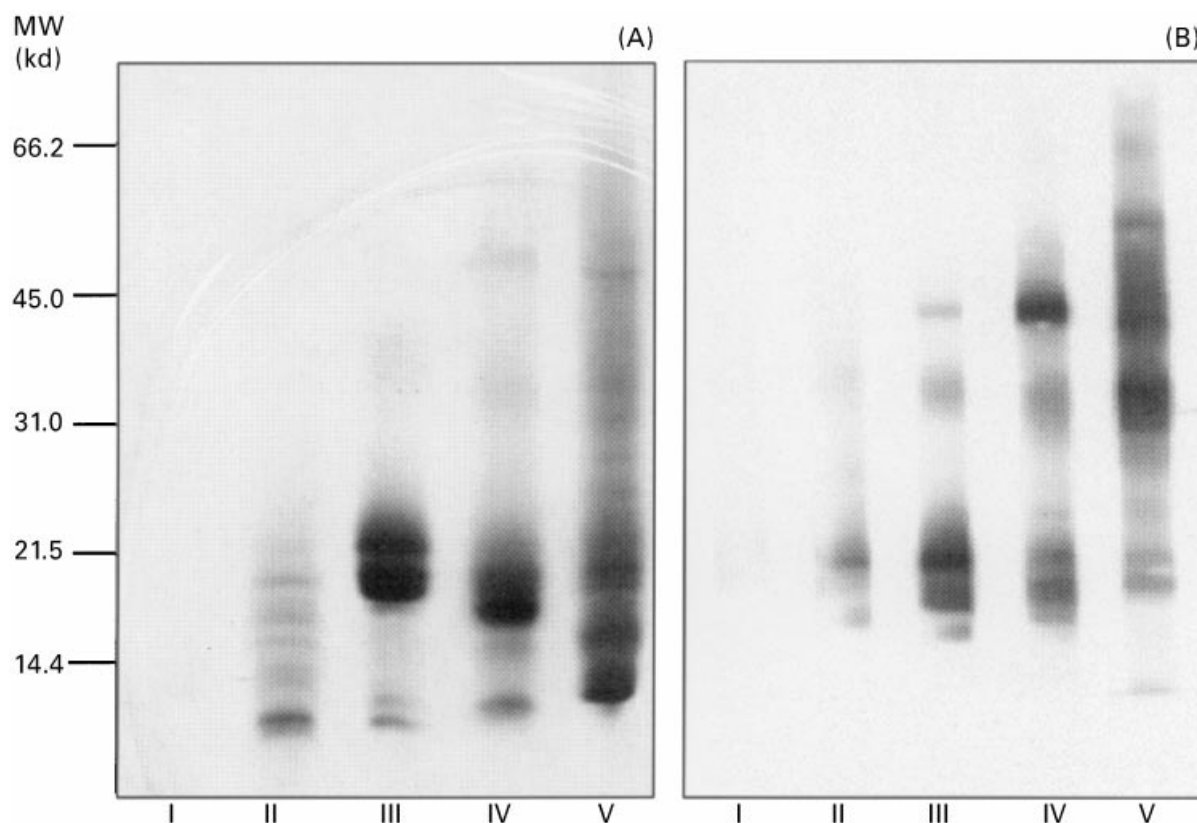


Fig. 3. a. SDS-PAGE analysis of fractions I–V obtained from reverse-phase HPLC (Fig. 2). These fractions were lyophilized and re-dissolved in water. 30 μ g of each fraction was applied per lane. Proteins were stained with Coomassie Blue. b. Immunodetection of IgE-binding polypeptides in fractions from reverse-phase HPLC. Samples were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane that was processed as described in Materials and Methods.

after SDS-PAGE (Fig. 1). Allergenic components were distributed in a wide range of molecular weights. A group of proteins with a molecular weight between 16 and 20 kd reacted with almost all the sera (13 of 14) assayed, and other allergenic polypeptides (molecular weight 36, 50 and 66 kd) were detected with more than 50% of sera.

Purification and biochemical characterization of Pla 1

In order to purify the 16–20 kd allergens two successive chromatographic separations were performed. In the first purification step, PLE was chromatographed on a reverse-phase Vydac C4 column. The chromatographic profile is illustrated in Fig. 2. Major peaks eluting at the initial phase of the acetonitrile gradient corresponded to pigments present in the extract and did not contain any protein, as was evidenced from the results of the analysis by SDS-PAGE of the collected fractions (Fraction I, Fig. 3, a). This technique also showed that proteins in the 16–20 kd molecular weight range were particularly enriched in fraction III. Besides, these proteins exhibited a high IgE-binding activity when analysed by immunodetection with a serum pool from *P. lanceolata*-sensitive patients (Fig. 3, b).

Thus, we selected this fraction as the starting material for the following purification step.

The second chromatographic process consisted of size-exclusion HPLC (Fig. 4). The main peak was collected and analysed as above. Four bands of proteins with molecular weights of 16–20 kd were visualized by SDS-PAGE (Fig. 5, a) and all of them showed IgE-binding activity by immunoblotting (Fig. 5, b). Coomassie Blue staining of SDS-PAGE disclosed the presence of a very weak protein band corresponding to a molecular weight of 32–36 kd; however, the intensity of this band was stronger in the immunodetection experiment, suggesting that it might be a dimeric form of the allergen with enhanced IgE-binding capacity.

The group of isolated proteins mostly eluted as a single peak when chromatographed on an analytical size-exclusion chromatography (data not shown). The molecular weight of Pla 1 under non-denaturing conditions, calculated from the calibration curve in this experiment, was 22 kd. A small peak, accounting for 5% of the area of the major peak, was detected at a retention time that corresponded to a molecular weight of 46 kd, thus supporting the idea that the protein(s) is partially a dimer.

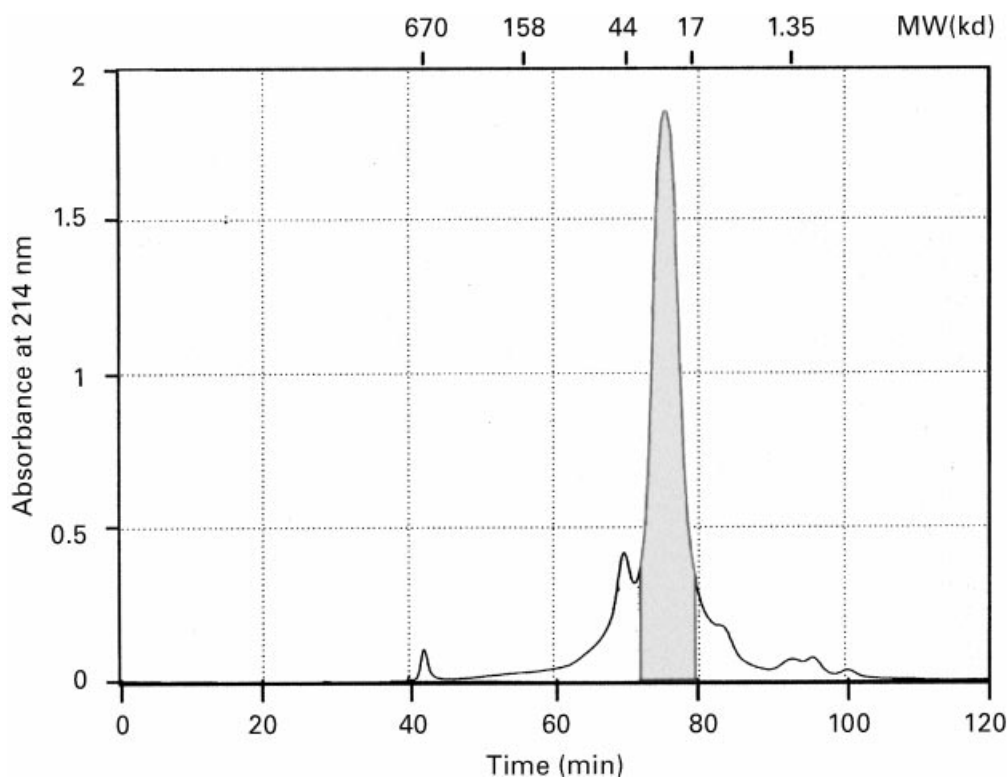


Fig. 4. Size-exclusion HPLC of fraction III from reverse-phase chromatography (Fig. 2). Lyophilized fraction III was dissolved in 100 mM Na₂SO₄, 20 mM NaH₂PO₄, pH 6.8, injected onto a Spherogel TSKG 3000 SW column, and eluted with the same solvent. The shadowed area indicates the collected fractions containing Pla 1. The retention times of the standard proteins used to calibrate the column are indicated at the top of the figure.

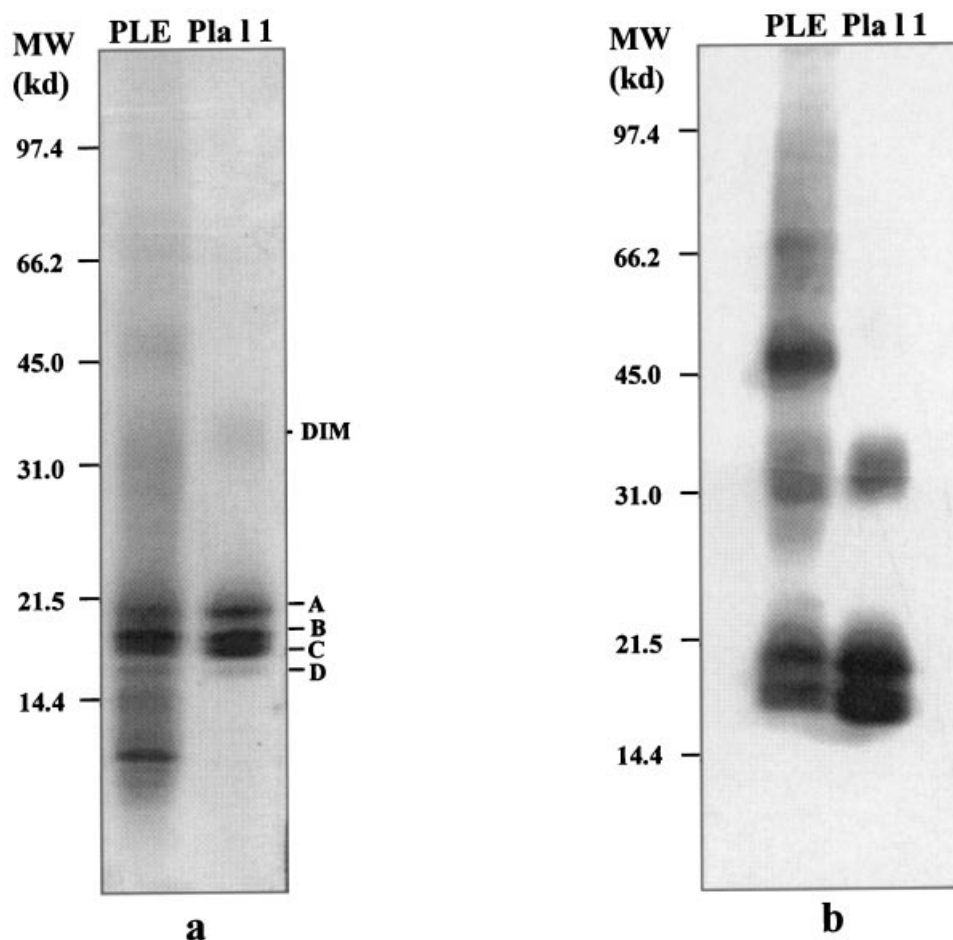


Fig. 5. a. SDS-PAGE Coomassie Blue stained pattern of PLE (300 μ g) and Pla 1 1 eluted from size-exclusion HPLC (7 μ g). Letters on the right identify the protein bands that were subjected to N-terminal sequencing. b. Immunoblotting of the same samples with patients' serum pool, carried out as described in Materials and Methods.

The N-terminal amino acid sequence of the four proteins that could be separated by SDS-PAGE in the range of 16–20 kD (Fig. 5, a) was established by Edman degradation on samples electrotransferred onto PVDF membranes. The sequence of 18 amino acid residues was determined for the band **A**#, and 10 residues for the rest (Fig. 6). The sequences were identical for all of them, except for positions 7 and 10 of band **D**, where amino acid changes were found. Moreover, in the case of protein band **D**, amino acid sequence polymorphism was detected at positions 1, 2 and 6. These results point out that the different proteins sequenced are isoforms or variants of the same protein. We will refer to this group of proteins as Pla 1 1 further on.

In addition, the N-terminal amino acid sequence obtained for the protein band at 32–36 kD (**DIM**, Fig. 6),

that perfectly matched sequences from **A–D**, confirmed the hypothesis that it is a dimeric form of Pla 1 1.

On the other hand, the amino acid sequence established for Pla 1 1 showed a 50% identity with the major olive allergen Ole e 1 for the sequence stretch between positions 9 and 16.

Pla 1 1 also has a considerable charge heterogeneity, yielding a number of bands in a wide pH range, from 5 to 9, when analysed by isoelectric focusing (data not shown). As one of the possible causes for this charge heterogeneity, the presence of carbohydrate prosthetic groups linked to the polypeptide chain was investigated using an enzymatic deglycosylation commercial kit containing NANase II, O-Glycosidase and PNGase F (BioRad). Bovine fetuin was used as a positive control. In order to check the efficiency of the deglycosylation reaction, the samples were run on SDS-PAGE and no shift in the electrophoretic mobility of Pla 1 1 was observed. Therefore, this proves that Pla 1 1 does not bear the

#The sequence of the component **A** was submitted to the SWISS-PROT Data Bank and appears with the accession No. P82242.

Pla l 1	5	10	15
A	T Q T S H P A K F H V E G E V Y X N		
B	T Q T S H P A K F H		
C	T Q T S H P A K F H		
D	T Q T S H P T K F Y E K A		
DIM	T Q T S H P X K F H		

Fig. 6. N-terminal amino acid sequence of Pla l 1 isoallergens. Sequencing was performed on samples blotted onto PVDF membranes after SDS-PAGE. Letters as in Figure 5a identify samples.

N-glycan structures that can be cleaved by the glycosydases used.

Allergenic significance of Pla l 1

The capacity of isolated Pla l 1 to bind specific human IgE after the purification process was evaluated by a direct ELISA in which the purified allergen was adsorbed to the plate. Nineteen out of the 22 sera analysed (86.4%) had specific IgE to Pla l 1 (Table 1), and a very good correlation between the absorbance values obtained with the whole extract and the purified allergen was observed.

Furthermore, the allergenic activity of Pla l 1 was estimated by a competition ELISA. Pla l 1 could strongly inhibit the binding of specific IgE to the whole extract, up to 80%, as shown in Fig. 7. This demonstrated that most of the IgE-binding activity of the extract is contributed by Pla l 1.

Discussion

Recently, sensitization to *P. lanceolata* pollen has been shown to represent an important cause of pollinosis in many temperate regions. However, simultaneous pollination with grasses and the low incidence of patients monosensitized to this pollen has probably led to the tendency of underestimating its clinical importance. In turn, very little attention has been paid to the study of its allergen composition, and only a few reports on this subject have been published [13,17,18]. The allergens we have identified in this work using 14 individual sera essentially match those reported by Mehta et al. [13]. The highest IgE-binding capacity was associated with a low molecular weight complex allergen, Pla l 1, consisting of at least four different allergen components.

Here we describe the isolation of this major allergen in a

high degree of purity and immunoreactive state, achieved by using a two-step chromatographic method.

Pla l 1 showed heterogeneity in mass and charge, giving rise to multiple protein bands in SDS-PAGE and IEF. Nevertheless, it is worth mentioning here that the large differences observed in the apparent molecular weight of the different isoforms might be at least partially due to conformational dissimilarities when disulphide bridges are not disrupted, since no reducing agent was included in the electrophoresis sample buffer. Many pollen allergens have been characterized as glycoproteins, and sugar moieties have been shown to be at least partially responsible for their heterogeneity [23,24]. Although glycosylation of Pla l 1 cannot be ruled out yet, the presence of glycosylated substitutions could not be demonstrated, and thus other post-translational modifications or sequence polymorphisms

Table 1 Binding of specific human IgE to PLE and purified allergen Pla l 1.

Serum No.	PLE	Pla l 1
1	2.795	2.374
2	1.407	1.097
3	1.625	1.482
4	0.428	0.276
5	1.517	1.356
6	0.844	0.782
7	0.426	0.333
8	0.542	0.259
9	0.889	0.812
10	1.359	1.290
11	2.294	2.208
12	1.354	1.049
13	0.968	0.682
14	0.404	<i>0.143</i>
15	0.974	1.169
16	0.470	<i>0.057</i>
17	1.441	1.481
18	0.263	0.360
19	0.326	<i>0.036</i>
20	0.295	0.265
21	0.375	0.268
22	1.892	1.468
NC	0.036	0.031

Figures represent values of absorbance at 490 nm obtained in an ELISA screening. Allergosorbent wells were prepared with either PLE or Pla l 1, and sequentially incubated with individual sera from patients sensitized to *P. lanceolata* and rabbit anti-human IgE as described in Material and Methods. NC: negative control. Values higher than five-fold the negative control were considered positive. Values that failed this condition are indicated in italic type in the Pla l 1 column.

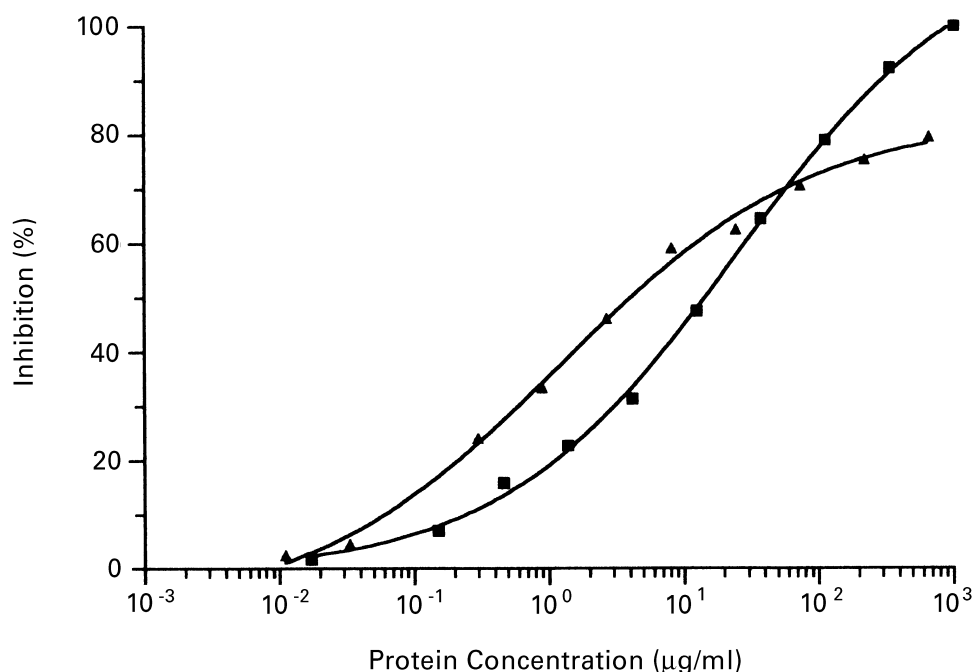


Fig. 7. Competition ELISA curves. The binding of specific human IgE to PLE was inhibited using Pla 1 1 (▲) or the same PLE (■) as a control.

might be the cause of Pla 1 1 heterogeneity. Whichever the cause is, the modifications do not seem to significantly affect the hydrophilic properties of the protein, as Pla 1 1 eluted as a single peak in reverse-phase HPLC. Obviously, a more detailed knowledge of the structure of Pla 1 1 isoforms will be needed to disclose the origin of the heterogeneity. Studies aimed at the elucidation of this question are being carried out using two different approaches: on the one hand, through the separation of isoforms by ion exchange HPLC and, on the other hand, by isolation of the mRNA and synthesis of the cDNA encoding the allergen using specific primers derived from the N-terminal amino acid sequence here described.

The molecular weight of Pla 1 1 determined by SDS-PAGE is in accordance with those previously reported by other authors. Thus, Dreborg *et al.* [18] identified a 15 kD allergen as the component responsible for most IgE binding activity of the extract. Years later, Mehta *et al.* [13] analysed extracts of *P. lanceolata* pollen by SDS-PAGE immunoblotting and observed that the main allergens had a molecular weight between 16 000 and 20 000 *d*. Also in agreement with their results, we detected other proteins in the pollen extract that reacted with IgE from patients with seasonal respiratory allergy. However, some of these proteins could be structurally related to Pla 1 1. In fact, we detected a Coomassie Blue faintly stained band corresponding to 32–36 kD when purified Pla 1 1 was analysed by SDS-PAGE. This protein, that could correspond to the

small 46 kD peak observed in size-exclusion HPLC, produced a heavily stained band in immunoblot with a patient's serum pool. From the results of N-terminal amino acid sequencing, it can be concluded that this protein band arises from the dimerization of Pla 1 1 molecules. Association of proteins into multimeric forms with enhanced affinity for IgE is not an unusual feature of pollen allergens, as it has also been found, for instance, for Par j 1 and Ole e 1 [25–27].

Regarding the allergenic activity, Pla 1 1 retained the IgE-binding capacity after the purification process, and the data reported in this article prove that this protein is the most clinically relevant allergen from *Plantago lanceolata*. Most sera from allergic patients assayed have specific IgE to the purified allergen and in several cases the response with Pla 1 1 was even higher than with the whole extract. Likewise, this allergen inhibited by up to 80% the allergenic activity of the plantain extract, which suggests that the allergens not related to Pla 1 1 in the extract are remarkably less important from a clinical point of view.

As mentioned previously, the plantain pollen season coincides with the grass pollen season and monosensitized patients allergic to plantain are extremely infrequent. These facts may have led to the general thinking that plantain has a certain degree of cross-reactivity with grasses, despite the results obtained by Mehta *et al.* [13], who did not find common IgE-reacting epitopes in grass and plantain pollen extracts by RAST inhibition. The N-terminal amino acid

sequence we have determined for Pla l 1, which is not similar to any known protein sequence of grass pollen allergens, indicates that the cross-reactivity with grasses, if any, should be ascribed to other plantain allergens. On the other hand, the sequence similarity of Pla l 1 with Ole e 1 here reported discloses an unexpected source of potential cross-allergenicity that should be further investigated.

In summary, this work describes the identification of plantain allergens and the isolation and characterization of the most important allergen. This polymorphic allergen, which consists of a complex of at least four isoforms and which tends to form dimers, has been named Pla l 1 according to the recommendations of the Allergy Nomenclature Subcommittee of the International Union of the Immunological Societies [28]. The availability of purified Pla l 1 will enable us to carry out some studies aimed at a more detailed characterization of its biochemical and allergenic properties. Moreover, it will allow us to produce monoclonal antibodies and develop a method for quantifying this allergen that could be used for standardization, in terms of Pla l 1 content, of plantain extracts for diagnosis and treatment of allergic patients.

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