

Purification and immunochemical characterization of Pla I 2, the profilin from *Plantago lanceolata*

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ABSTRACT

Profilins are small actin-binding proteins found in eukaryotes and involved in cell development, cytokinesis, membrane trafficking, and cell motility. From an allergenic point of view, profilins are panallergens usually involved in allergic polysensitization, although they are generally recognized as minor allergens.

The objectives of this study were to identify and characterize the profilin from *Plantago lanceolata* pollen and to investigate the cross-reactivity between profilins from different pollen allergenic sources.

Profilins from *P. lanceolata* (Pla I 2) and palm tree pollen (Pho d 2) were purified by affinity chromatography, deeply characterized and identified by mass spectrometry. Pla I 2 allergenicity was confirmed by immunoblot with serum samples from a patient population sensitized to profilin. Immunoblot inhibition was performed to study IgG reactivity between different pollen profilins. IgE cross-reactivity was demonstrated by ImmunoCAP inhibition.

Pla I 2 is the second *P. lanceolata* allergen included in the IUIS Allergen Nomenclature database. Four peptides from purified Pla I 2 were identified with percentages of homology with other pollen profilins between 73 and 86%. Eighty-six percent (21/24) of the patient population recognized Pla I 2. The allergenic relatedness between Pla I 2, Pho d 2 and six pollen profilins was confirmed, and IgE cross-reactivity of Pla I 2 with rBet v 2 and rPhl p 12 was demonstrated.

Pla I 2 is the profilin from *P. lanceolata*. The demonstrated allergenicity of this protein and its cross-reactivity with other pollen profilins support its use in profilin diagnostic assays.

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1. Introduction

English plantain (*Plantago lanceolata*) is a weedy plant common in warm zones and its pollen is an important cause of asthma, allergic rhinitis and allergic conjunctivitis (Mohapatra et al., 2008; Sousa et al., 2014; Tormo-Molina et al., 2010). Monosensitization to plantain pollen is uncommon and cross-reactivity between *P. lanceolata* and grasses, olive trees, or foods, such as melon, has been described (Asero et al., 2000; García Ortiz et al., 1996). Although this cross-reactivity seems to be caused by allergens different than panallergens, other studies (Sousa et al., 2014) have suggested the relevant role that profilin might play in the allergenicity of this plant. However, plantain allergy has not been deeply studied and until now only one allergen has been described and listed in the

IUIS database as Pla I 1, an Ole e 1-related protein (Calabozo et al., 2001; Castro et al., 2007). Other allergens from this plant, characterized by different studies, are a cytochrome C protein (Matthews et al., 1988), and a calcium-binding protein (Grote et al., 2008).

Profilins are proteins of a molecular weight ranging from 12 to 15 kDa. They are present in all eukaryotic cells where they play a role in many essential cellular processes (Witke, 2004). They have been identified as allergens in the pollen of trees, grasses, and weeds, as well as in latex, and plant foods (Santos and Van Ree, 2011). Profilins are considered minor allergens in most pollen triggers of respiratory allergy in the Mediterranean area, and the prevalence of sensitization to this allergen has been estimated among 5–40% (Hauser et al., 2010). However, the prevalence increases in polysensitized patients where it is considered a clinically relevant food allergen (Asero et al., 2003; Asero et al., 2008). The homology between the amino acid sequences of the described profilins is high, being in general greater than 75%. A large number of studies have demonstrated cross-reactivity involving profilins (Amini et al., 2011; Asero et al., 2004; Asturias et al., 1998; Asturias

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et al., 2005; Barderas et al., 2004; Ebner et al., 1995; Fedorov et al., 1997; Kwaasi et al., 2002; Sirvent et al., 2011; Valenta et al., 1992; van Ree et al., 1992; Wensing et al., 2002). Previous sensitization to profilins from pollens has been described to possibly lead to a higher susceptibility for latter sensitization to profilins from foods and the development of an oral allergy syndrome (Santos and Van Ree, 2011).

In spite of the relevance of this panallergen and its involvement in cross-reactivity, profilin has not been described and identified until now in the weedy plant *P. lanceolata*.

The aim of this study was to identify and characterize the profilin from *P. lanceolata* pollen and to investigate the cross-reactivity between *P. lanceolata* profilin and palm tree pollen profilin, which is the most frequently used profilin in *in vivo* diagnosis, with profilins from other pollen allergenic sources.

2. Material and methods

2.1. Profilin purification and identification

Profilins from *P. lanceolata* and *P. dactylifera* pollen were purified by affinity chromatography in an ÄKTAexplorer system (GE Healthcare, Uppsala, Sweden). Briefly, *P. dactylifera* and *P. lanceolata* freeze-dried extracts were dissolved in 0.01 M phosphate-buffered saline (PBS) to a final concentration of 1 mg/ml and loaded onto a poly-L-proline-sepharose column (Lindberg et al., 1988). The elution of the protein was performed with 6 M urea, 0.01 M PBS. Fractions were then collected and analysed by SDS-PAGE. Those fractions containing a band of about 14 kDa were pooled and concentrated using an Amicon centrifugal device (Millipore, Bedford, USA) with a Nominal Molecular Weight Limit of 3 kDa. Urea was removed by sequential exchange of the urea by glycerol by dialysis. The two purified profilins were analysed by SDS-PAGE and immunoblot with a previously prepared pool of sera from patients sensitized to Phl p 12. The molecular weight (MW) of the proteins was calculated by scanning densitometry using the ImageQuant TL software (GE Healthcare, Uppsala, Sweden).

The identity of profilin from *P. dactylifera* (Pho d 2) and *P. lanceolata* (Pla l 2) was confirmed by liquid chromatography-mass spectrometry/mass spectrometry (LC/MS-MS). The purified proteins were digested with trypsin and obtained fragments were analysed by liquid chromatography with electrospray ion-trap mass spectrometry. In the case of Pla l 2, the obtained peptide sequences were compared with the profilin sequences available in the knowledgebase of UNIPROT (<http://www.uniprot.org/help/uniprotkb>).

Purified protein quantification was carried out by spectrophotometry in a Nanodrop (Thermo Scientific, Waltham, MA, USA) using the specific characteristics of Pho d 2 (MW: 14.3 kDa; ϵ : 18,300 M⁻¹ cm⁻¹).

2.2. Serum samples

Serum samples were obtained from 24 patients allergic to grasses. Patients were recruited at Hospital Universitario de Guadalajara (Spain) from a previously published study approved by the scientific ethics committee of said Hospital (Beitia et al., 2014).

All of them were positive to Phl p 12 as determined by ImmunoCAP (Thermo Fisher, Uppsala, Sweden) with specific IgE (sIgE) values ranging between 0.38 and 49.10 kUA/L. Eight sera from the patient population, with sIgE to Phl p 12 higher than 1.5 kUA/L, were used to prepare a pool. Patient population characteristics are summarised in Table 1.

Table 1

Characteristics of the patient population used in the study.

Number	Sex	Age	Symptoms	Phl p 12 sIgE (kUA/L)
1	M	23	RC, A	14.1
2	M	28	RC, A	0.38
3	F	28	RC, A	7.8
4	F	43	RC, A	2.87
5	F	41	RC, A	0.71
6	M	21	RC, A	1.54
7	M	33	RC, A	0.85
8	M	37	RC, A	0.87
9	M	33	RC, A	1.25
10	M	40	RC, A	1.09
11	M	27	RC, A	49.1
12	F	40	RC, A	0.71
13	M	30	RC, A	0.59
14	M	23	RC, A	5.89
15	F	35	RC, A	1.15
16	M	31	RC, A	0.37
17	M	21	RC	0.75
18	F	24	RC, A	3.81
19	M	37	RC, A	1.24
20	F	20	RC, A	1.93
21	F	30	RC, A	0.6
22	M	32	RC	1.51
23	F	18	RC, A	0.66
24	F	19	RC, A	7.94

∴ Serum samples used for preparing a pool of sera.

Sex: M, male; F, female. Symptoms: RC, rhino conjunctivitis; A, asthma.

2.3. Pla l 2 IgE binding

Three µg of Pla l 2 were processed in 15%T acrylamide:bis-acrylamide gels. After electrophoresis, gels were electrotransferred onto a Trans-Blot® Turbo™ Transfer Pack (Bio-Rad, Hercules, CA, USA). These membranes were incubated overnight with an individual patient's sera (diluted 1/2 in PBS 0.01 M). After washing, the membranes were incubated with anti-human IgE-PO diluted 1:20000 (Southern Biotech, Birmingham, AL, USA). Finally, the membranes were developed with luminol solution (Clarity™ Western ECL Substrate, Bio-Rad) and detected by chemiluminescence (ChemiDoc XRS, Bio-Rad).

The identification of Pla l 2 in the *P. lanceolata* extract was carried out by immunoblot inhibition. Twenty µg of purified Pla l 2 were incubated for 2 h with the pool of sera before being added to the membrane containing 50 µg of the *P. lanceolata* whole extract. The immunoblot was developed as previously described.

2.4. Pollen extracts

Pollen extracts from *Ambrosia artemisiifolia*, *Artemisia vulgaris*, *Betula alba*, *Chenopodium album*, *Cynodon dactylon*, *Helianthus annuus*, *Mercurialis perennis*, *Olea europaea*, *Parietaria judaica*, *Phleum pratense*, *P. dactylifera* and *P. lanceolata* were prepared following in-house manufacturing procedures (Laboratorios LETI SL, Madrid, Spain) under Practice Guidelines as described in a previous study (Morales et al., 2014). Protein profile of each extract was identified by SDS-PAGE under reducing conditions in 15%T acrylamide:bis-acrylamide gels and stained with Coomassie R-250.

2.5. Rabbit polyclonal antibodies

Two New Zealand white rabbits were immunized with 200 µg of purified Pho d 2 in Complete Freund's adjuvant. Three extra doses of 100 µg protein were injected at three-week intervals in Incomplete Freund's adjuvant. After 78 days, the animals were bled and the serum samples collected. Rabbit immunization was performed at the Vivotecnia Research facilities (Madrid, Spain). All the procedures were approved by the Institutional Review Board of

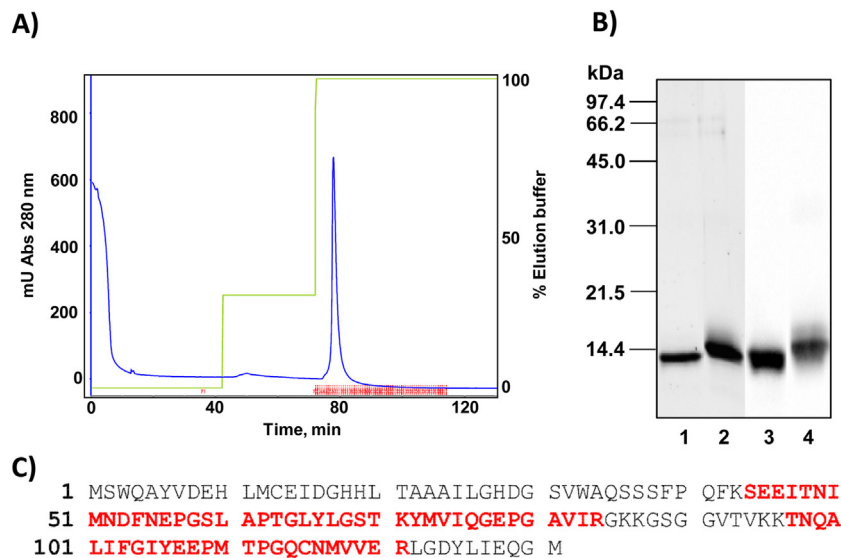


Fig. 1. (A) Chromatographic profile characteristic of the affinity purification of Phod 2 and Pla 12 showing the peak corresponding to the purified proteins (blue line) and the % of elution buffer (green line). (B) Pooled fractions of Phod 2 (1, 3) and Pla 12 (2, 4) were analysed by SDS-PAGE (1, 2) and immunoblot (3, 4). MW marker is shown. (C) LC/MS-MS result for Phod 2. Peptides obtained are shown in bold red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Vivotecnia Research and followed the local ethical rules for animal experimentation.

2.6. Cross-reactivity studies

2.6.1. Immunoblots

The recognition of the different profilins present in the pollen extracts described in Section 2.4 with the polyclonal antibodies anti-Phod 2 was performed by immunoblot. Fifty μ g of each extract were run and electrotransferred onto a membrane. Membranes were blocked with 5% skimmed milk for 1 h and incubated overnight with polyclonal anti-Phod 2 (diluted 1/30,000 in PBS 0.01 M; Tween 0.1%). After washing, the membranes were incubated with anti-rabbit IgG-PO (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted 1:75000. Finally, membranes were developed as described in Section 2.3. *P. dactylifera* pollen extract was used in the solid phase as a positive control.

Positive extracts were selected for immunoblot inhibition assays. In these assays, 20 μ g of purified Phod 2 or Pla 12 were incubated for 2 h with anti-Phod 2 before the addition to the membrane containing 50 μ g of each pollen extract in the solid phase. *P. dactylifera* and *P. lanceolata* extracts were used as positive controls.

2.6.2. ImmunoCAP

ImmunoCAP inhibition assays were performed in the ImmunoCAP 100E system (Thermo Fisher) to investigate the IgE cross-reactivity between Pla 12 and the profilins Bet v 2 and Phl p 12. Different aliquots of the pool of sera (diluted 1/2) were previously incubated with amounts of Pla 12 ranging from 0.6 to 312.5 ng. The same dilution of the pool of sera in 0.01 M PBS without Pla 12 was used as a control. After 2 h of incubation, specific IgE (kUA/L) binding to rBet v 2 (t216) or rPhl p 12 (g212) was determined in all the aliquots.

3. Results

3.1. Profilin purification and identification

Phod 2 and Pla 12 were purified in a single peak (Fig. 1A) in separated processes by affinity chromatography. The proteins presented

the same elution time. The purification degree was confirmed by SDS-PAGE and immunoblot assays (Fig. 1B) where a single band was observed for both proteins which were recognized by the pool of sera from patients sensitized to profilin. The purified Phod 2 and Pla 12 showed a MW of 14.3 and 14.5 kDa, respectively.

The identity of Phod 2 was confirmed by LC/MS-MS, where 3 peptides were identified with a sequence coverage of 50% (Fig. 1C). Four peptides were obtained after trypsin digestion and sequencing of Pla 12 (Supplementary material, Fig. 1). The sequences of the 4 peptides identified in Pla 12 were compared with different pollen profilins, which allowed for the identification of the peptide positions in the protein sequence (Fig. 2). The highest homology was found with profilin from *B. alba* (Bet v 2) (85.7%) followed by Par j 3 (83.3%), the profilin from *P. judaica*. The protein sequence data of Pla 12 reported in this paper appears in the UniProt Knowledgebase (<http://www.uniprot.org/uniprot/>) under the accession number COHJX6. Additionally, profilin from *P. lanceolata* was included in the IUIS (<http://allergen.org/>) as Pla 12.0101.

3.2. *P. lanceolata* profilin IgE binding

Twenty-one out of the 24 serum samples (87.5%) recognized the purified profilin Pla 12 under reducing conditions (Fig. 3). Serum samples from patients 2, 13 and 16 were considered negative. All the negative patients showed the lowest sIgE values for Phl p 12 by ImmunoCAP (≤ 0.6 kUA/L) (Table 1).

The inhibition of the pool of sera with Pla 12 allowed for the identification of the protein in the *P. lanceolata* extract that corresponded with a protein band of approximately 14 kDa (Fig. 4).

3.3. Cross-reactivity studies

3.3.1. Profilin detection in the extracts

The identification of a band of approximately 14 kDa by immunoblot, that could correspond to profilin was observed in all the extracts except *O. europaea*, *A. vulgaris*, *C. album*, and *P. judaica* (Fig. 5A and B) with the anti-Phod 2 antibody. The protein profiles of all the pollen extracts was investigated by SDS-PAGE (Supplementary material, Fig. 2) showing that in some of the extracts, specially

Pla_l_2	-----AILGQDGSVWAQ-----	12
Par_j_3	MSWQAYVDDHLMCDVGDGN--TPASAAIIGHDGSVWAQSANFPQLKPEEVTGIMNDFNEA	58
Hel_a_2	MSWQAYVDEHLMCDIEGTGQ-HLTSAAILGLDGTVWAQSAKFPQFKPEEMKGIKEFDEA	59
Amb_a_8	MSWQTYVDEHLMCDIEGTGQ-HLASAAIFGTDGNVWAKSSSFPEFKPDEINAIKEFSEP	59
Art_v_4	MSWQTYVDDHLMCDIEGTGQ-HLTSAAIFGTDGTVWAKSASFPEFKPNEIDAIKEFNEA	59
Ole_e_2	MSWQAYVDDHLMCDIEGHEDHRLTAAAIIVGHDSVWAQSATFPQFKPEEMNGIMTDFNEP	60
Bet_v_2	MSWQTYVDEHLMCDIDGQASNS-LASAIIVGHDSVWAQSSSFQFKPQEITGIMKDFEFP	59
Mer_a_1	MSWQTYVDDHLMCDIDGQGQHL-AAASIVGHDSIWAQSAFPQLKPEEITGIMKDFDEP	59
Che_a_2	MSWQTYVDDHLMCDIEGN---HLSSAAILGHDGTVWAQSPSPFQLKPEEVSAIMKDFNEP	57
Phl_p_12	MSWQTYVDEHLMCEIEGH---HLASAAILGHDGTVWAQSAFPQFKPEEITGIMKDFDEP	57
Cyn_d_12	MSWQAYVDDHLMCEIEGH---HLTSAAIIGHDGTVWAQSAAFPQFKPEEMANIMKDFDEP	57
Pho_d_2	MSWQAYVDEHLMCEIDGH---HLTAAAILGHDGSVWAQSSSFQFKSEEITNIMNDFNEP	57
	****:***:****::: :*:.* **.:***: ** :* :*: *:::.*	
Pla_l_2	-----GLHLGGAKY-VIAGEPGAIVR-----	31
Par_j_3	GFLAPTGLFLGGTKYMQVIGGESGAVIRGKKGSGGATLKKTGQAIVIGIYDEPMTPGQCNL	118
Hel_a_2	GTLAPTGMFIAGAKYMLVQGEPGAIVIRGKKGAGGICIKKTGQAMIMGIYDEFPVAPGQCNM	119
Amb_a_8	GALAPTGLFLAGAKYMQVIGGEPGAIVIRGKKGAGGICIKKTGQAMVFGIYEFPVNPQGNCM	119
Art_v_4	GQLAPTGLFLGGAKYMQVIGGEAGAVIRGKKGAGGICIKKTGQAMVFGIYDEFPVAPGQCNM	119
Ole_e_2	GHLAPTGLHLGGTKYMQVIGGEAGAVIRGKKGSGGITIKKTGQALVFGIYEFPVTPGQCNM	120
Bet_v_2	GHLAPTGLHLGGIKYMQVIGGEAGAVIRGKKGSGGITIKKTGQALVFGIYEFPVTPGQCNM	119
Mer_a_1	GHLAPTGLYIAGTKYMQVIGGESGAVIRGKKGSGGITIKKTGQALVFGIYEFPVTPGQCNM	119
Che_a_2	GSLAPTGLHLGGTKYMQVIGGEPGDVIRGKKGPGGVTIKKTQALVIGIYGEPMTPGQCNM	117
Phl_p_12	GHLAPTGMFVAGAKYMQVIGGEPGRVIRGKKGAGGITIKKTGQALVVGIYDEPMTPGQCNM	117
Cyn_d_12	GFLAPTGLFLGPTKYMQVIGGEPGAVIRGKKGSGGVTVKKTGQALVIGIYDEPMTPGQCNM	117
Pho_d_2	GSLAPTGLYLGSTKYMQVIGGEPGAVIRGKKGSGGVTVKKTQALVIGIYEFPMTPGQCNM	117
	* *****:..: *****: ** * ***** ** :*** **.:*** ** :*****:	
% Identity		
Pla_l_2	----LGDYLLDQGL	42
Par_j_3	VVERLGDYLLLEQGL	132
Hel_a_2	VVERLGDYLLLEQGM	133
Amb_a_8	VVERLGDYLLDQGM	133
Art_v_4	VVERLGDYLLDQGM	133
Ole_e_2	VVERLGDYLLDQGM	134
Bet_v_2	VVERLGDYLLDQGL	133
Mer_a_1	VVERLGDYLLLEQGM	133
Che_a_2	VVERIGDYLLLEQGM	131
Phl_p_12	VVERLGDYLLLEQGM	131
Cyn_d_12	VIEKLGDYLLLEQGM	131
Pho_d_2	VVERLGDYLLLEQGM	131
	:*:***:	

Fig. 2. Alignment of identified peptides from Pla l 2 with pollen profilin sequences and percentage of identity related to Pla l 2. Peptide positions in profilin sequences are in grey boxes. * = Conserved sequence; : = Conservative change; . = Semi-conservative change. Allergenic sources of included profilins: *P. lanceolata* (Pla l 2), *P. judaica* (Par j 3), *H. annuus* (Hel a 2), *A. artemisiifolia* (Amb a 8), *O. europaea* (Ole e 2), *B. alba* (Bet v 2), *M. annua* (Mer a 1), *C. album* (Che a 2), *P. pratense* (Phl p 12), *C. dactylon* (Cyn d 12), *P. dactylifera* (Pho d 2).

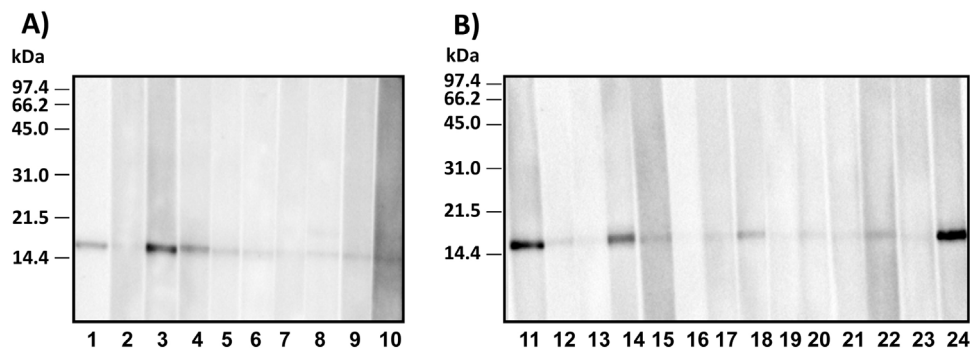


Fig. 3. IgE immunoblot of purified Pla l 2 incubated with individual serum samples. (A) Serum samples 1–10. (B) Serum samples 11–24. The MW marker is indicated on the left.

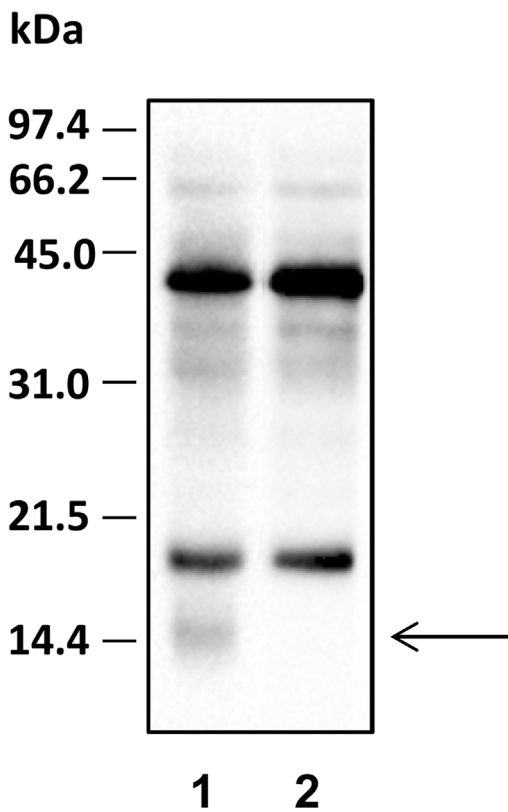


Fig. 4. Immunoblot of 50 µg of *P. lanceolata* extract incubated with the pool of sera (1) and the pool of sera previously incubated with 20 µg of purified Pla I 2.

in *O. europaea*, *A. vulgaris*, *C. album*, and *P. judaica*, no clear protein bands were observed at 14 kDa approximately.

3.3.2. Immunoblot inhibition

Immunoblot inhibition was performed in all the extracts in which a 14-kDa band was identified in the previous step. The incubation of anti-Pho d 2 antibodies with purified Pho d 2 or Pla I 2 completely inhibited the band corresponding to profilin in all the extracts assayed (*A. artemisiifolia*, *B. alba*, *C. dactylon*, *H. annuus*, *M. annua*, *P. pratense*, *P. dactylifera*, and *P. lanceolata*) and with both purified profilins as can be observed in Fig. 6. In the *P. dactylifera* extract, the band corresponding to profilin was partially inhibited by Pla I 2 (Fig. 6C, lines PH).

3.3.3. ImmunoCAP inhibition

A 50% inhibition of IgE binding to rBet v 2 was obtained with 0.51 ng of Pla I 2 (25.7 ng/ml) whereas 0.55 ng (27.5 ng/ml) were necessary for a 50% inhibition of IgE to rPhl p 12 (Fig. 7). The maximum rate of achieved inhibition was 84% for both rBet v 2 and rPhl p 12.

4. Discussion

Taking into consideration the relevance of profilin in multiple sensitizations and its wide presence in pollen allergenic sources, in this study we sought to identify and characterize the profilin from English plantain, a common allergenic source in warm zones and a predominant aeroallergen in the center and southwest regions of Spain. Additionally, we tried to demonstrate the cross-reactivity between different pollen profilins, including this new allergen. The profilin characterized in this study, Pla I 2, is the first panallergen and the second allergen from this allergenic source included in the IUIS.

English plantain has been involved in different studies in cross-reactivity with other plants (García Ortiz et al., 1996) and patients sensitized to *P. lanceolata* are usually sensitized to other pollen sources or to foods such as melon, in which profilin has been described as a major allergen (Rodríguez-Pérez et al., 2003). Moreover, Sousa et al. (2014) identified a band of 13–14 kDa from *P. lanceolata* extract recognized by 55% of a *Plantago* sensitized population, that was described as most likely a profilin. All these data suggest that profilin is an important *P. lanceolata* allergen involved in cross-reactivity reactions and this was the reason for focusing the attention of this study on this allergen.

Another profilin, Pho d 2, was purified in our study in order to optimize the purification process as well as for the production of rabbit polyclonal antibodies. Pho d 2, the profilin from *P. dactylifera* pollen (Asturias et al., 2005), is one of the most studied and characterized profilins. The same method of purification optimized for Pho d 2 allowed for the obtaining of the profilin from *P. lanceolata*, recently denominated Pla I 2. The protein was highly purified, as demonstrated by SDS-PAGE and sequencing results. Pla I 2 presented a MW of 14.5 kDa, as we could confirm by the identification of the protein in the *P. lanceolata* extract. The immunogenicity of this protein, described as an allergen for the first time in this study, was demonstrated by its recognition by 87.5% of the patient population (21 out of 24). This result was expected, taking into consideration that the patients were selected based on their sensitization to Phl p 12 and the high cross-reactivity described for profilins. Negative patients showed the lowest sIgE values for Phl p

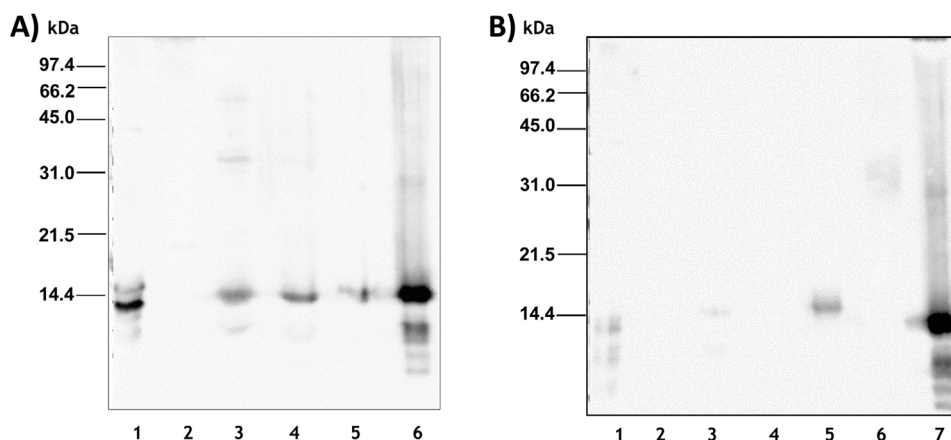


Fig. 5. Immunoblot of pollen extracts incubated with antibodies anti-Pho d 2. (A) 1-*B. alba*, 2-*O. europaea*, 3-*P. pratense*, 4-*C. dactylon*, 5-*H. annuus*, 6-*P. dactylifera*. (B) 1-*M. annua*, 2-*A. vulgaris*, 3-*A. artemisiifolia*, 4-*C. album*, 5-*P. lanceolata*, 6-*P. judaica*, 7-*P. dactylifera*. The MW marker is indicated on the left.

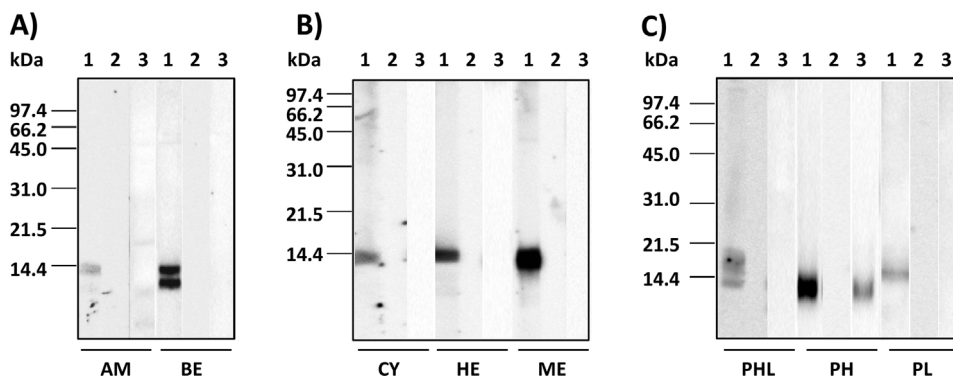


Fig. 6. IgG immunoblot inhibition of pollen extracts incubated with antibodies anti-Pho d 2 (1) or antibodies anti-Pho d 2 previously incubated with 20 μ g of purified Pho d 2 (2) or Pla l 2 (3). (A) AM: *A. artemisiifolia*; BE: *B. alba*. (B) CY: *C. dactylon*; HE: *H. annuus*; ME: *M. annua*. (C) PHL: *P. pratense*; PH: *P. dactylifera*; PL: *P. lanceolata*. The MW marker is indicated on the left.

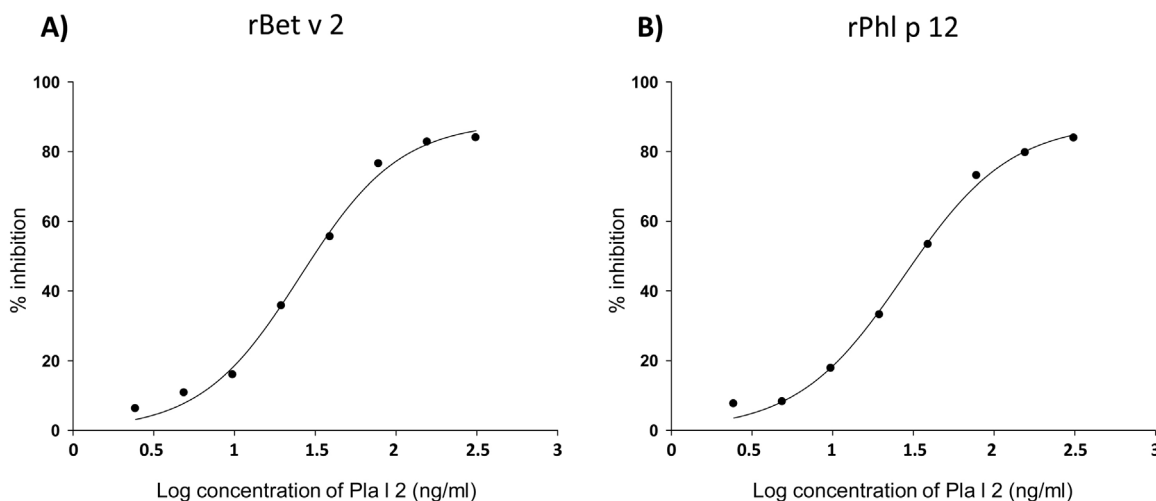


Fig. 7. Inhibition of IgE binding to rBet v 2 (A) and rPhl p 12 (B) with purified Pla l 2 by ImmunoCAP.

12 by ImmunoCAP which could indicate that in these patients Phl p 12 is the primary sensitizer. Differences in the recognition of the two profilins could also be explained by the different techniques used (ImmunoCAP vs. immunoblot). Further studies of skin prick test diagnosis would be necessary for demonstrating the *in vivo* allergenicity of the protein.

The aminoacid analysis of the Pla l 2 peptides revealed that this newly identified profilin presents a high homology with profilins described in other pollen sources (Par j 3, Hel a 2, Amb a 8, Art v 4, Ole e 2, Bet v 2, Mer a 1, Che a 2, Phl p 12, Cyn d 12, and Pho d 2), finding the highest homology with Bet v 2. Although only a partial sequence of Pla l 2 was compared with other profilin sequences, percentages of homology found (73–86%) were very similar to those previously described for other profilins (Hauser et al., 2010; Sirvent et al., 2011).

In order to study the response of an IgG anti-Pho d 2 antibody to different profilins, we selected different pollen allergenic sources (*A. artemisiifolia*, *A. vulgaris*, *B. alba*, *C. album*, *C. dactylon*, *H. annuus*, *M. perennis*, *O. europaea*, *P. judaica*, *P. pratense*, and *P. dactylifera*). The IgG antibodies anti-Pho d 2 allowed for the identification of profilin in 7/11 (63.6%) of the extracts. The absence of a detection band in 4 of the extracts could be explained by a different reactivity of the polyclonal antibodies to the different profilins or a low profilin content in these extracts. Taking into consideration that no bands at the MW of profilin were observed in these extracts when they were analysed by SDS-PAGE, it seems likely that the absence of detection was probably due to the low content in this allergen in

some of the pollen extracts used for the study. More studies with purified profilins would be necessary to elucidate the antigenic equivalence between all these allergens. Different IgG reactivity of polyclonal antisera to profilins has also been described by other authors (Sirvent et al., 2011). In the extracts selected for the inhibition assays, both Pho d 2 and Pla l 2 inhibited the recognition of the different bands which demonstrated the allergenic relatedness between Pla l 2 and Pho d 2 and between Pla l 2 and Pho d 2 with Amb a 8, Bet v 2, Cyn d 12, Hel a 2, Mer a 1, and Phl p 12. Pla l 2 did not completely inhibit the recognition of Pho d 2 by the polyclonal antibody which could reflect the presence of specific epitopes in this profilin. Other studies have also described IgG inhibition with different allergenic sources (Maize, Bermuda grass, ash, olive and birch tree) and using an anti-Bet v 2 sera (Ledesma et al., 1998) although whole extracts were used for the inhibition assays instead of the purified profilin.

Concerning IgE cross-reactivity, there are several papers that have studied the cross-reactivity between profilins from different pollen allergenic sources (Asturias et al., 1998; Asturias et al., 2005; Valenta et al., 1992) and from pollen and plant foods (Ebner et al., 1995; Ledesma et al., 1998; Mas et al., 2014; Sirvent et al., 2011; van Ree et al., 1992).

IgE cross-reactivity of Pla l 2 with the available pollen profilins (rBet v 2 and rPhl p 12) in ImmunoCAP was demonstrated. Percentages of inhibition higher than 80% were obtained in both cases. Similar results have been obtained in other studies with different profilins and different techniques. Sirvent et al. (2011) obtained

IgE inhibition values higher than 70% by ELISA for Sin a 4, Cuc m 2, Che a 2 and Bet v 2 whereas Asturias et al. (2005) obtained similar rates of IgE inhibition between Pho d 2 and Ole e 2 and Mer a 1. A recent study from Wolbing et al. (2016) demonstrates using different *in vitro* techniques the full cross-reactivity between Phl p 12 and Bet v 2. The absence of complete IgE inhibition observed in this study could be explained by the existence of protein-specific IgE epitopes and differences in the IgE binding capacity for different epitopes as modeling studies have indicated (López-Torrejón et al., 2007; Radauer et al., 2006).

In vivo and *in vitro* diagnosis with purified allergens have become a relevant and potent tool for the diagnosis of allergy, especially for polysensitization. For these cases, different authors support the use of a single profilin for the diagnosis of this allergen sensitization (López-Torrejón et al., 2007; Radauer et al., 2006; Santos and van Ree, 2011; Villalta and Asero, 2010). Pollen profilins have been proposed as the best candidates for molecular diagnosis as they are the primary sensitizers and usually higher specific IgE responses are obtained. The identification and characterization of Pla l 2 increases the knowledge about profilins and supports their broad presence among different pollen allergenic sources. Moreover, cross-reactivity data from our study reinforce their role as panallergens and their use as purified allergens for molecular diagnosis.

To sum up, in this study we have purified and characterized the profilin from *P. lanceolata* (Pla l 2) for the first time. The allergenicity of the protein has been demonstrated with a patient population sensitized to profilin by *in vitro* studies. Pla l 2 showed a high sequence homology with other profilins from pollen which is reflected in the cross-reactivity assays performed in this study. Pla l 2 could be used in profilin diagnostic assays.

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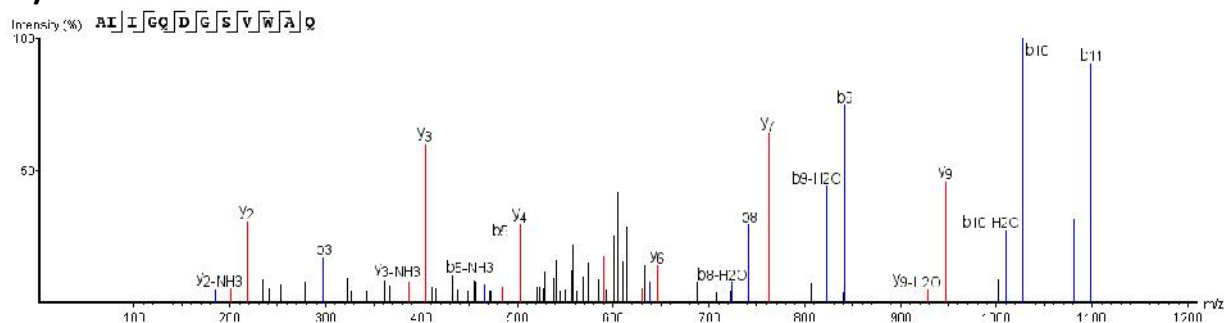
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2017.01.017>.

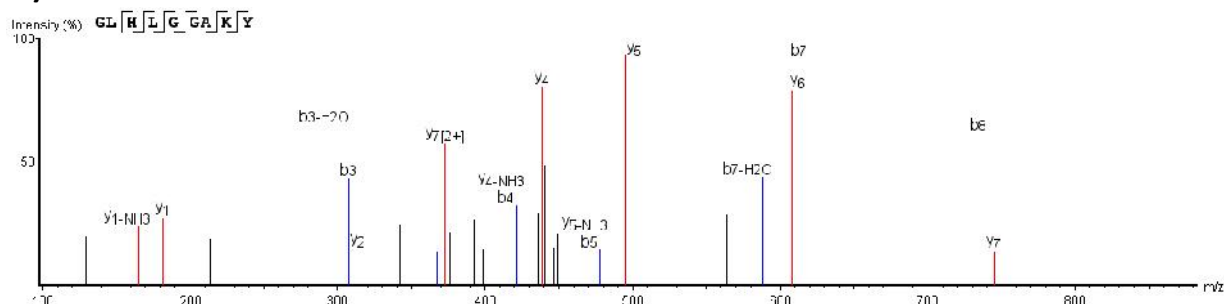
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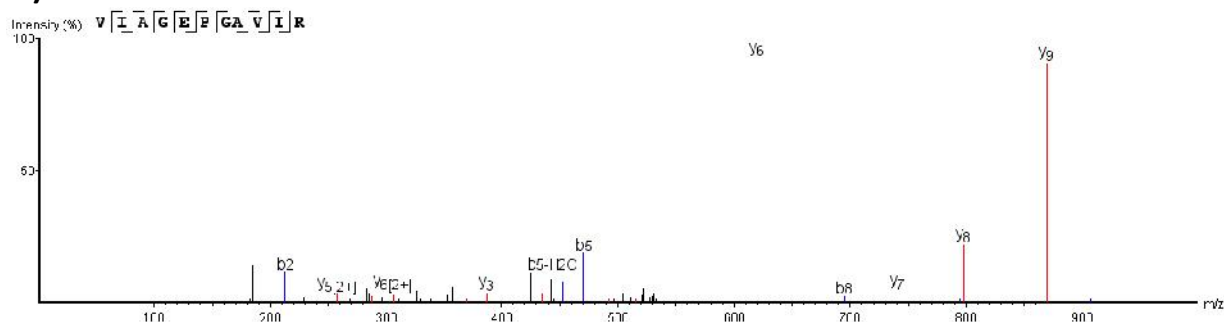
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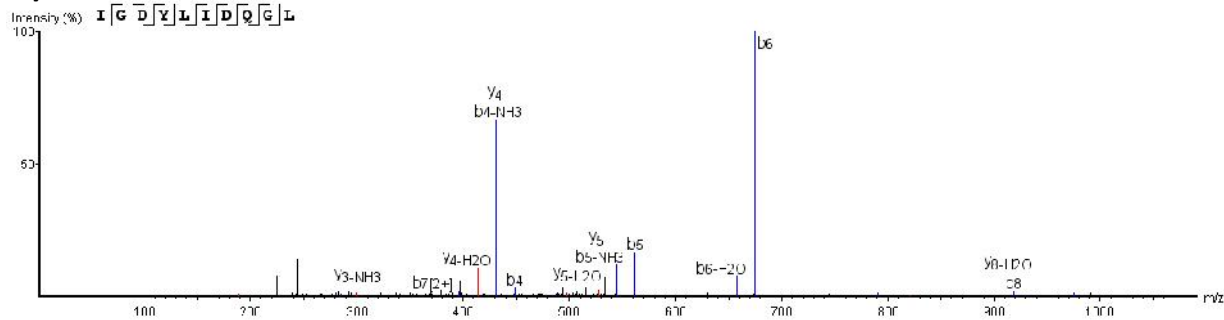


Figure 1. Peptides 1-4 identified for Pla l 2. Position 3 at peptide 1 and positions 1 and 10 at peptide 4 can correspond with both leucine (L) or isoleucine (I) as they can not be differentiated by mass-spectrometry.

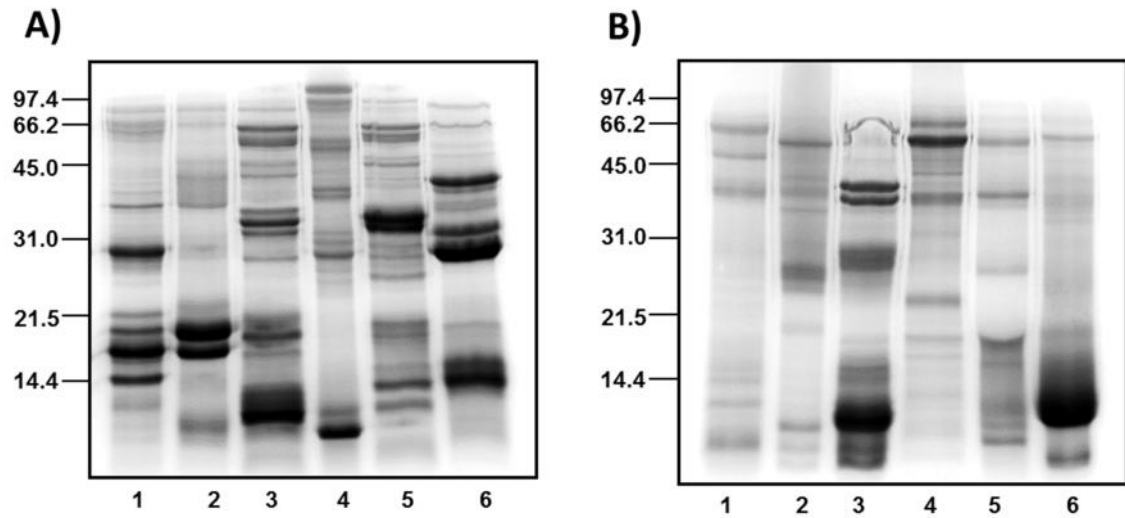


Figure 2. SDS-PAGE of pollen extracts included in the study: A) 1-*B. alba*, 2-*O. europaea*, 3-*P. pratense*, 4-*P. dactylifera*, 5-*C. dactylon*, 6-*H. annuus*. B) 1-*M. annua*, 2-*A. vulgaris*, 3-*A. artemisiifolia*, 4-*C. album*, 5-*P. lanceolata*, 6-*P. judaica*. The MW marker is indicated on the left.