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

Raquel Moya [a], Virginia Rubio [a], Juan Mª. Beitia [b], Jerónimo Carnés [a,*], M. Angeles López-Matas [a]

[a] R&D Department, Laboratorios LETI, S.L.U., C/del Sol 5, Tres Cantos, 28760, Madrid, Spain
[b] Allergy Service, Hospital de Guadalajara, Guadalajara, Spain

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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. lancelolata* (Pla l 2) and palm tree pollen (Pho d 2) were purified by affinity chromatography, deeply characterized and identified by mass spectrometry. Pla l 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 l 2 is the second *P. lanceolata* allergen included in the IUIS Allergen Nomenclature database. Four peptides from purified Pla l 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 l 2. The allergenic relatedness between Pla l 2, Pho d 2 and six pollen profilins was confirmed, and IgE cross-reactivity of Pla l 2 with rBet v 2 and rPhl p 12 was demonstrated.

Pla l 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.

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 us 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 l 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

* Corresponding author at: R&D Department, Laboratorios LETI, S.L., Calle del Sol No. 5, Tres Cantos, 28760, Spain.
E-mail address: jcarnes@leti.com (J. Carnés).

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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