Original Article

PCR-based cloning and immunological characterization of *Parietaria judaica* pollen profilin

J. A. Asturias[1], I. Ibarrola[1], J. L. Eseverri[2], M. Carmen Arilla[1], R. González-Rioja[1], and A. Martínez[1]

[1]Bial-Arístegui, Research and Development Department, Bilbao and [2]Servicio de Alergología, Hospital Materno-Infantil "Vall d´Hebrón", Barcelona, Spain

Summary.Profilin has been described as an allergen present in pollen of trees, grasses and weeds. Since *Parietaria judaica* profilin has a molecular mass similar to other *Parietaria* allergens (Par j 1 and Par j 2) in the 14-10 kDa range, it is difficult to assess the prevalence of profilin by immunoblotting or to obtain sufficient amounts of purified native profilin for investigation and diagnosis. The aim of this study was to identify *P. judaica* profilin by PCR-based cDNA cloning and to elucidate its allergenic characteristics. Two cDNA clones encoding *P. judaica* pollen profilin were isolated by polymerase chain reaction (PCR) amplification using degenerate primers. Sequencing of both clones (Par j 3.0101 and Par j 3.0102) demonstrated a high amino acid sequence homology. Immunodetection of *P. judaica* pollen after isoelectrofocusing and incubation with rabbit antiserum against profilin indicated the existence of at least 2 isoforms. Expression in *Escherichia coli* BL21 (DE3) was carried out using a vector based in the T7 expression system, and the recombinant allergen was isolated by affinity chromatography on poly-(L-proline)-Sepharose. Cross-reactivity has been found between recombinant *P. judaica* pollen profilin and profilins from other botanical unrelated plants.

Keywords: profilin, pan-allergen, cloning, bacterial expression, recombinant allergen, IgE-binding

Introduction

Up to 40% of the human population in developed countries suffers from IgE-mediated atopic diseases in any of their different manifestations such as rhinitis, conjunctivitis, urticaria, angioedema, or asthma [1]. *Parietaria judaica* pollen is the main cause of allergy in the Mediterranean area, and other temperate climates of Central and Eastern Europe, Australia and California, with a prevalence of 60-80% in Italy and Greece, and 25-40% in Spain and Southern France [2].

P. judaica pollen contains at least nine allergens, and two of them, named Par j 1 and Par j 2, have been cloned and sequenced showing prevalences of 95% and 82% among *P. judaica* allergic patients [3,4]. Profilins are ubiquitous cytosolic actin-binding proteins implicated in the signal transduction cascade to cytoskeletal rearrangements [5]. They have been identified as allergens in pollen of several species of trees, grasses, and weeds and in many fruits and vegetables. The widespread IgE cross-reactivity towards a broad range of profilins has led to the designation of profilins as pan-allergens [6].

Major allergens of this pollen (Par j 1 and Par j 2) have a molecular mass similar to profilin in the 10-14 kDa range, and for this reason it was difficult to assess the prevalence of profilin by immunoblotting using crude extracts. Since separation of proteins with the same molecular mass is difficult and time-consuming, we decided to produce *P. judaica* pollen profilin by recombinant methods. The advent of molecular biology and its application to the study of allergens have been a great advance for the characterization of the most common allergens. Recombinant products are pure, well defined, and reproducible compounds which will help to develop of new diagnostic procedures and therapeutic strategies [7], and to facilitate the elucidation of specific amino acid residues involved in recognition by B- and T-cells [8]. In this study, we describe the cloning,

expression and purification of the panallergen profilin from *P. judaica* pollen, and the immunological characterization of the recombinant allergens.

Material and methods

Chromatographic purification of *P. judaica* pollen profilin

P. judaica pollen (Mediterráneo Polen, Jaén, Spain) were extracted as previously described [9] and stored lyophilized at -20ºC. The first step in the profilin purification was accomplished by affinity chromatography in PLP-Sepharose column, following the modified method of Lindberg previously described [10]. Quantification of profilin content was performed using double-sandwich ELISA as previously described [11].

Patient sera and experimental antisera

Thirty-three serum samples were selected from a sera collection of patients with clinical history of *P. judaica* allergy, and skin prick test reactivity to *P. judaica* pollen extract. Sera were chosen based on specific IgE to *P. judaica* pollen extract class > 3 (quantified by EAST, Hytec-specific IgE EIA; Hycor Biomedical Inc., Kassel, Germany). Profilin specific rabbit antiserum was prepared as reported in Asturias et al [12].

Cloning of *P. judaica* pollen profilin cDNA

Poly(A^+) mRNA was isolated from 100 mg of *P. judaica* pollen using the Quick Prep MicroRNA Purification Kit (Amersham Biosciences, Uppsala, Sweden). Cloning by RT-PCR, using degenerate oligodeoxynucleotide primers (Sigma-Genosys, Cambridge, UK), were performed under previously reported conditions [13]. Primers were: PF, 5´-AGAGAATTCCATATGT-CGTGGCA(A/G)(A/G)CGTACGT (for the N-terminus) and PR1, 5´-AGAAAGCTT(C/T)TACA(G/T)GCC(C/T)TGTTC(G/A/T)A(G/T/C)(G/A/C)AGGTA (for the *C-terminus*). *Eco*RI, *NdeI*, and *Hind*III restriction sites, respectively, are underlined. The predominant band was isolated from 2% agarose gels (GeneClean, Bio101, La Jolla, CA) and, after cutting with *Nde*I and *Hin*dIII, cloned into the expression vector pKN172 [14]. Nucleotide sequences were determined on an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA) and analyzed using the GCG Program Package (Genetics Computer Group Inc., Madison, WI).

Production of recombinant profilin

E. coli BL21 (DE3) [15] cells containing the pKN172-derived recombinant plasmids were grown in LB with 200 μg/ml ampicillin. IPTG at final concentration of 0.6 mM was added to the culture when they reached an OD_{600} of 0.6, and incubation continued for another 3 h. Cells were harvested by centrifugation, and the pellet, resuspended in 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, was lysed by lysozyme treatment (100 mg/ml, 15 min, 37ºC) and mild sonication. Insoluble material was removed by centrifugation and profilin in the supernatant was purified by PLP-Sepharose chromatography as above described.

Electrophoretic techniques

Proteins were analyzed by SDS-PAGE under reducing conditions [16] and visualized by Coomassie Blue R250 staining. Separated protein bands were electrophoretically transferred onto polyvinylene difluoride (PVDF) [17] and blocked for 1 h at room temperature with 0.1% Tween-20 in Tris-buffered saline (TBS). Membranes were incubated overnight at 4ºC with allergic patients sera (diluted 1:4 or 1:6 in 0.1% Tween-20 in TBS) followed by anti-human IgE-horseradish peroxidase conjugate incubation. Bound immunoglobulins were visualized by chemiluminescence as recommended by the manufacturer (ECL-Plus; Amersham Biosciences).

IEF was performed on Isogel Agarose plates (FMC BioProducts, Rockland, MN), pH 3 to 10, following the manufacture recommendations and separated protein bands were transferred onto PVDF. After blocking for 1 h with 8.8% defatted dry milk in TBS, membranes were incubated at 37ºC for 60 min with rabbit serum against profilin (diluted 1:1000). IgG-peroxidase conjugate were detected by the addition of 0.06% w/v of 4-chloro-1-naphtol solution (Bio-Rad Laboratories, Richmond, CA) and 0.01% H_2O_2 in TBS.

Results

Low profilin content in *P. judaica* pollen extracts

The presence of profilin in crude pollen extract of *P. judaica* was determined by immunoblotting using rabbit antiserum against profilin (Fig. 1A). Low content of profilin was detected when it was compared with pollen extracts from date palm tree, olive tree, and Bermuda grass. The yield of purified profilin from *P. judaica* pollen extract by affinity chromatography on PLP-Sepharose column was extremely low (0.004% of total protein). Molecular mass of the isolated natural Par j 3, estimated by Coomassie Brillant Blue-stained SDS-PAGE, was 14.5 kDa (Fig. 1A). Profilin content of *P. judaica* pollen extract, estimated by monoclonal antibody-based-sandwich ELISA, was 0.003-0.002% of

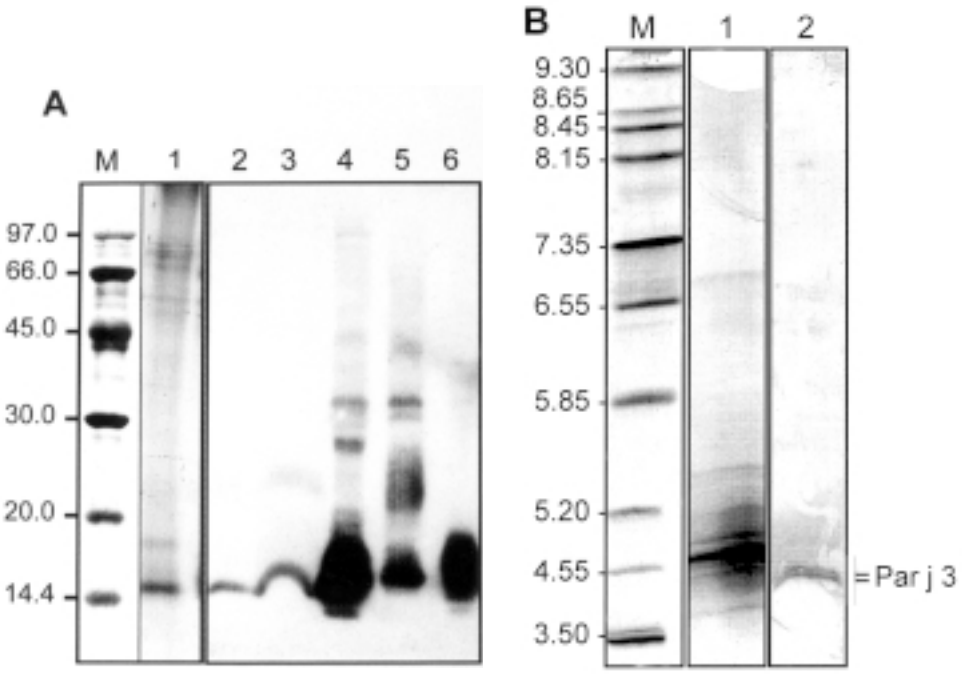

Figure 1. Specific detection of *P. judaica* profilin. **A)** Immunoblot of pollen extract of *P. judaica*, (lane 2 and 3), date palm (lane 4), Bermuda grass (lane 5), and olive tree (lane 6) (20 µg/lane, except lane 3 where 80 µg/lane was loaded) incubated with rabbit anti-serum against profilin. A sample of purified natural profilin from *P. judaica* was stained with Coomassie (lane 1) and molecular mass markers (lane M) were stained with Amido Black B. **B)** Coomassie stained-IEF of *P. judaica* extract (lane 1) and immunoblotting after incubation with profilin antiserum (lane 2).

total protein. Specific immunodetection of profilin after isoelectrofocusing analysis of *P. judaica* pollen extract revealed at least 2 isoforms of slightly different pI values that reacted with specific antiserum against profilin (Fig. 1B).

Amplification, cloning and sequencing of cDNA coding for *P. judaica* profilins

Amplification of *P. judaica* pollen cDNA produced a main fragment of approximately 400 bp. After cloning of this PCR product in the pKN172 expression vector, two clones were sequenced, corresponding to full-length profilin sequences. *P. judaica* pollen profilins were denominated Par j 3.0101 and Par j 3.0102 in accordance with the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee. Par j 3.0101 encoded a polypeptide of 132 amino acids with a predicted molecular mass of 13.9 kDa and a pI value of 4.55. Par j 3.0102 encoded a polypeptide of 131 amino acids with a predicted molecular mass of 13.8 kDa and a pI value of 4.43. High homology (97% amino acid identity) was found between both translated sequences (Fig. 2). There were twelve nucleotide changes in the sequence and one in frame-deletion of three nucleotides, but only four amino acid conversions were resulted of these changes. Two of the four amino acids changes found in Par j 3.0102 (Ala^{58}→Gly, Leu^{132}→Met) are conservative, while others are a non-conservative change (Pro^{21}→Leu) and a deletion of a positively charged residue (Arg^{85}) located in the motif (A/V)^{82}VIRGKKG(T/S/A)GGIT(V/I)KKT^{97}. Comparison of the Par j 3.0101 deduced amino

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                      10        20        30        40        50        60
Par j 3.0101 MSWQAYVDDHLMCDVGDGNTPASAAIIGHDGSVWAQSANFPQLKPEEVTGIMNDFNEAGF
             ||||||||||||||||||| |||||||||||||||||||||||||||||||||||.||
Par j 3.0102 MSWQAYVDDHLMCDVGDGNTLASAAIIGHDGSVWAQSANFPQLKPEEVTGIMNDFNEGGF

                      70        80        90       100       110       120
Par j 3.0101 LAPTGLFLGGTKYMVIQGESGAVIRGKKGSGGATLKKTGQAIVIGIYDEPMTPGQCNLVV
             ||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Par j 3.0102 LAPTGLFLGGTKYMVIQGESGAVI-GKKGSGGATLKKTGQAIVIGIYDEPMTPGQCNLVV

                     130
Par j 3.0101 ERLGDYLLEQGL
             ||||||||||.
Par j 3.0102 ERLGDYLLEQGM
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Figure 2. Comparison of deduced amino acid sequences of Par j 3.0101 and Par j 3.0102. Identical (|) and conserved (.) residues are marked. The nucleotide sequences reported in this paper have been submitted to the GenBank under accession numbers Y15208 (Par j 3.0101) and Y15209 (Par j 3.0102).

Table I. Amino acid identity (AA-ID) and similarity (AA-SIM) comparison of Par j 3.0101 with profilins from different plants and vegetable foods. AC: Genbank accession number.

PROFILIN SOURCE / ALLERGEN	AC	AA (n)	AA-ID (%)	AA-SIM (%)
Apple	AF1294272	131	81.0	90.9
Olive tree pollen / Ole e 2	O24170	134	79.8	88.8
Corylus avellana / Cor a 2	AF327623	131	79.5	91.6
Bermuda grass pollen / Cyn d 12	O04725	131	78.8	89.3
Mercury pollen / Mer a 1	O49894	133	78.2	90.9
Maize	O22655	131	78.0	89.4
Ricinus comunis pollen	AF092547	131	78.0	87.8
Rubber tree / Hev b 8	Y15042	131	77.8	90.8
Soybean / Gly m 3	O65809	131	77.8	87.0
Wheat	049233	141	76.3	87.0
Birch pollen / Bet v 2	P25816	133	75.9	88.7
Timothy grass pollen / Phl p 12	Y09457	131	75.0	88.6
Sunflower pollen / Hel a 2	Y15210	133	72.2	86.4

acid sequence using the BLAST program showed high homology with other plant profilins. The highest homology was found with profilin from apple and olive tree pollen (81% and 79.8% identical amino acids, respectively) and the lowest with that from sunflower pollen (Table I). Comparison of *P. judaica* pollen profilin with human profilin showed an amino acid identity of 34% with 45% similar amino acids.

Expression, purification, and immunological characterization of pellitory recombinant profilins

The expression of full-length profilin region was carried out using the T7 system in which the gene of interest is expressed under the control of f10 promoter which is specifically activated by T7 RNA polymerase produced from the *lac* promoter. A non-fusion form of profilin was expressed in *E. coli* BL21 (DE3). The soluble material was chromatographed over a PLP-Sepharose affinity column. After elution with 6 M urea in PBS, recombinant *P. judaica* profilins showed a single band with an apparent molecular mass of 15.6 kDa (rPar j 3.0101) and 14.3 kDa (rPar j 3.0102), as determined by Coomassie Brillant Blue-stained SDS-PAGE (Fig. 3A).

Sera from allergic patients to *Parietaria* pollen were tested for IgE reactivity to crude pollen extracts and purified recombinant profilins after SDS-PAGE (data not shown). The frequency of the IgE binding to pellitory recombinant profilins was 18% (6 out of 33) for rPar j 3.0101, and 6% (2 sera with low IgE reactivity out of 33) for rPar j 3.0102.

In order to test cross-reactivity of rPar j 3 against other plant profilins, immunoblotting experiments were performed by incubating both *Parietaria* recombinant profilins with profilin-reactive sera (Fig. 3B). Par j 3.0101 and Par j 3.0102 were recognized by profilin-reactive sera from sunflower and date palm-allergic patients, but a specific serum against Par j 3.0101 did not recognize Par j 3.0102 isoform and other profilins, such as that from date palm (Fig. 3B).

Discussion

P. judaica pollen contains at least nine allergens but only two of them, Par j 1 and Par j 2, have been studied in detail [3,4,18], although allergy to *P. judaica* pollen is very common in Mediterranean countries, reaching a prevalence of 80% in Italy and Greece [2]. Profilin from plants was first isolated from birch pollen and described as an allergen due to its ability to bind specific IgE from birch pollen-allergic patients. Because of high sequence identity between profilins, and consequently a comparable immunogenicity, profilins have been described as pan-allergens of various plant species [6]. *P. judaica* pollen has a very low content of profilin (Fig. 1) and therefore, the preparative isolation of this allergen from pollen is time-consuming and thus expensive. If no post-translational modification occurs, the cloning, production and purification of recombinant allergen in *E. coli* is an economical alternative and may provide sufficient amount of highly purified proteins for diagnostics and therapeutics.

In the present study, we have chosen the latter approach to identify the profilin-encoding sequence from *P. judaica* pollen. Isoelectrofocusing detected several profilin isoforms that reacted with rabbit antiserum against profilin. Two clones with slight differences except a residue deletion were obtained. This deletion

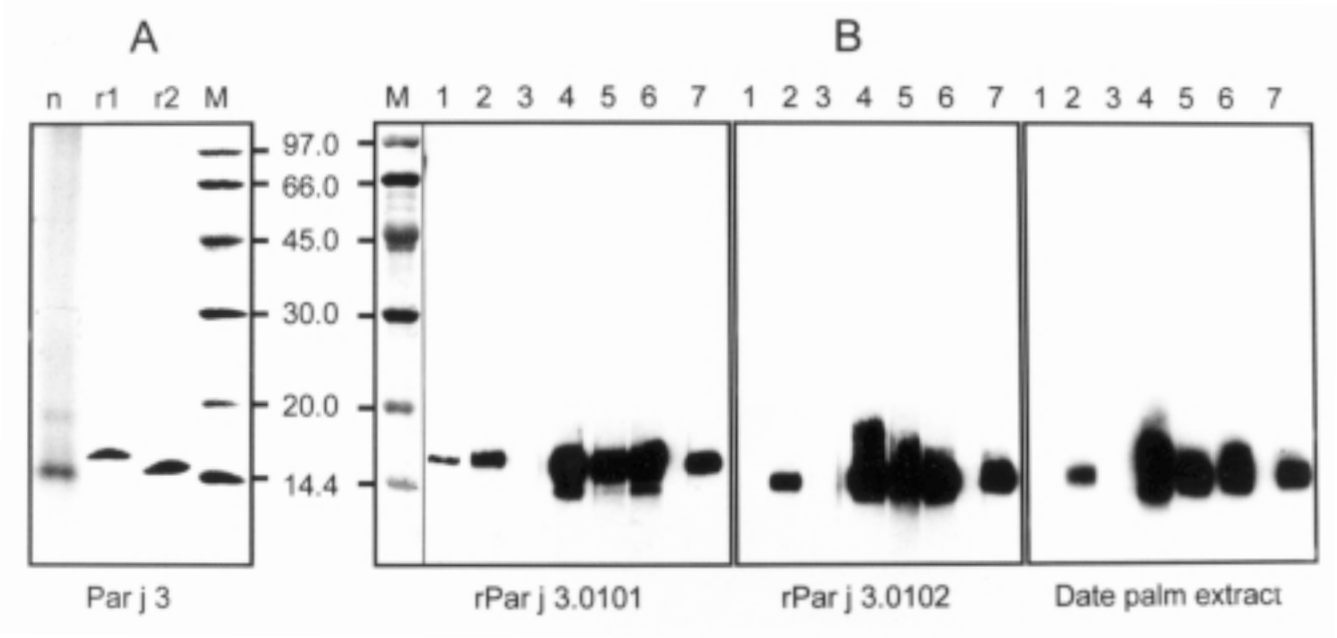

Figure 3. Immunological characterization of *P. judaica* recombinant profilins. **(A)** Coomassie-stained SDS-PAGE of purified nPar j 3 (lane n), rPar j 3.0101 (lane r1) and rPar j 3.0102 (lane r2). **(B)**. Proteins transferred onto PVDF-membranes were probed with profilin-reacting sera from patient allergic to pollen of *P. judaica* (lane 1), date palm (lane 2), and sunflower (lanes 4-6), with a pool of sera from healthy individuals (lane 3), and with specific rabbit antiserum against profilin (lane 7). Molecular mass markers (lane M) were stained with Amido Black B.

mapped in the β-strand 5 according to the crystal structure of birch pollen profilin [19], and is located in a region highly conserved in all the plant profilins sequenced, and described to be involved in binding of phosphatidylinositol-4,5-bisphosphate. Although the frequency of nucleotide exchanges is much higher than the expected error rate of the Taq polymerase ($1/10^{-5}$ nucleotides per cycle) [20] and the number of silent exchanges and deletion of a single triplet is far from the statistical probability, the possibility of PCR introduced mutation cannot be completely ruled out. Nevertheless, profilin polymorphism has been described in several plants, i.e. *Phleum pratense* [21], olive tree [22], soybean [23], maize [24] and wheat [25], but not in birch [26], or *Cynodon* [13]. The ocurrence of profilin isoforms could be a cellular mechanism to assure funcionality of this important protein involved in signal transduction between the outside of the cell and action cytoskeleton [5].

Immunological characterization showed 18% and 6% of allergic patients´ sera with specific IgE-reactivity to rPar j 3-0101 and rPar j 3.0102, respectively. These values are much lower than those found with recombinant profilins from soybean (69%) [23], or *Mercurialis annua* (59%) [27], but similar that those found with recombinant profilins from peanut (13%) [28] and birch (20%) [6]. We suppose that recombinant *Parietaria* profilins, prepared in our laboratory, are only two from the pool of various profilin isoforms detected herein by isoelectrofocusing, and therefore, the prevalence of the whole range of natural *Parietaria* profilin isoforms should be higher. Cross-inhibition experiments with profilin support the assumption that *P. judaica* profilins share epitopes with profilins from different botanical origins such as sunflower, and date palm tree pollens.

The use of well-characterized protein fragments with T-cell epitopes for specific immunotherapy has been recently proposed as a possible new tool instead of using natural allergen extracts [29]. The identification of common epitopes among plant profilins needs to be addressed in order to develop a putative immunotherapy based on profilin pan-allergenic epitopes which could be used in cases of profilin-allergy.

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Juan A. Asturias

Bial-ARISTEGUI,
R&D Department;
Alameda Urquijo, 27;
48008-Bilbao; Spain.
Tel: +34 944 438 000.
Fax: +34 944 438 016.
E-mail: juan.asturias@bial.com