

Watermelon Profilin: Characterization of a Major Allergen as a Model for Plant-Derived Food Profilins

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Key Words

Food allergy · Profilin · Watermelon

Abstract

Background: Plant profilins have been reported as minor allergens. They are a well-known pan-allergen family responsible for cross-reactivity between plant-derived foods and pollens. Watermelon profilin has been reported to be a major allergen in watermelon (*Citrullus lanatus*). The aim of this study was to characterize recombinant watermelon profilin, confirming its reactivity for diagnostic purposes and the development of immunotherapy. **Methods:** Native profilin was purified from watermelon extract by affinity chromatography using poly-L-proline. Recombinant His-tagged profilin was produced in *Pichia pastoris* yeast using pPICZαA vector and purified by metal chelate affinity chromatography. ELISA and immunoblot were carried out with sera from 17 watermelon-allergic patients. Biological activity was tested by the basophil activation test. **Results:** Native profilin and recombinant profilin were purified and identified by mass spectrometry. Both show similar IgE reactivity in vitro and are biologically active. **Conclusions:** Similarities were found in the IgE-binding patterns and biological activity of recombinant profilin and native profilin. Recombinant profilin may be a powerful tool for specific diagnosis.

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Introduction

Watermelon is a fruit commonly eaten in Mediterranean countries. In spite of its low protein content, some reports have found patients sensitized to this fruit by using specific IgE and positive skin tests, and others have found watermelon to elicit symptoms [1, 2]. Three major allergens have been described in watermelon, with oral allergy syndrome as the main manifestation, i.e. malate dehydrogenase, triosephosphate isomerase and profilin, with frequencies of sensitization among watermelon-allergic patients of 96, 78 and 56%, respectively [3].

Profilins are a family of proteins with a molecular mass of 12–15 kDa. These monomeric actin-binding proteins play a role in the function of the cytoskeleton by sequestering actin monomers and preventing actin filament polymerization [4].

Profilins are among the most frequent allergens involved in fruit and vegetable allergy. Profilin was first described as a minor allergen in birch pollen and named Bet v 2 [5]. Profilins are highly conserved proteins showing high identity in their amino acid sequences and tertiary structures [6] and have been identified as allergens

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in many vegetable sources, such as grasses, weeds, trees and fruits, as reviewed elsewhere [7–9]. Furthermore, several studies have demonstrated that profilin is an extremely cross-reactive plant allergen, and sensitized patients usually react to a broad range of pollen and food sources [10–12], caused by cross-reactive IgE epitopes [13]. Therefore, profilins are considered pan-allergens [5, 13, 14].

It has been suggested that profilins provoke only mild symptoms, such as oral allergy syndrome, which are confined exclusively to the oropharynx. This is probably due to the fact that profilin is unaffected by human saliva [3, 15, 16], though it loses its IgE-binding capability when it comes in contact with gastric fluids. In spite of this, the clinical relevance of profilins is still under debate [13, 17, 18]. Therefore, the purification and characterization of profilin in plant-derived food is an extremely important step towards the correct allergy diagnosis.

In this study, we report a high-yield method to obtain native watermelon profilin. A procedure is proposed for cloning and purifying the recombinant form so as to compare it to its native counterpart and evaluate the comparative benefits of using recombinant profilins over native forms in diagnostics and immunotherapy.

Patients and Methods

Patient Sera

Individual sera from 17 patients and a serum pool from watermelon-allergic patients with specific IgE to watermelon profilin were used in this study; all of the patients also suffered from pollen allergy. Watermelon allergy was diagnosed on the basis of a history of symptoms developing upon eating watermelon, with positive prick-to-prick testing to fresh watermelon pulp, following the technique described by Dreborg and Foucard [19], according to European Academy of Allergology and Clinical Immunology guidelines [20] using the ALK-Lancet needle (ALK-Lancet, ALK, Denmark). Histamine phosphate at 10 mg/ml and normal saline solution were used as positive and negative controls, respectively. A wheal with a diameter 3 mm larger than the negative control was considered a positive reaction. Diagnosis was confirmed by open oral challenge to watermelon pulp following the previously reported method [21]. Specific IgE to watermelon profilin was determined by ELISA and immunoblot techniques. In addition, sera from 5 nonatopic patients were used as a negative control in this study. The study was approved by the Hospital Ethics Committee, and all patients gave their written consent to participate in the study. Characteristics of the patients are shown in table 1.

Preparation of Watermelon Extracts

Watermelon extracts were prepared from *Citrullus lanatus* var. *lanatus*, which is the most widely consumed variety in Spain.

Table 1. Characteristics of patients whose sera were used in this study

No.	Age years	Sex	Symptoms of watermelon allergy	Water-melon skin test ^a , mm ²	Pollen allergy	Pollen allergy symptoms
1	20	M	OAS	19	yes	R, A
2	21	M	OAS	23	yes	R, A
3	23	F	OAS	36	yes	R
4	5	M	OAS	20	yes	R, A
5	32	F	OAS	18	yes	R, A
6	33	F	OAS	13	yes	R, A
7	32	F	OAS	27	yes	R
8	27	M	OAS	27	yes	R, A
9	29	M	OAS	19	yes	R, A
10	7	M	OAS	15	yes	R, A
11	15	M	OAS	32	yes	R, A
12	22	F	OAS	24	yes	R, A
13	20	F	OAS	28	yes	R, A
14	29	F	OAS	20	yes	R, A
15	29	M	OAS	68	yes	R
16	26	F	OAS	28	yes	R, A
17	24	F	OAS	38	yes	R, A

OAS = Oral allergy symptoms; R = rhinitis; A = asthma.
^a Wheal area.

Watermelons were peeled, liquidized and then processed as previously described [3]. Seeds were not included in the extract, since these are not usually ingested.

Isolation of Native Profilin

Native watermelon profilin was purified by affinity chromatography from watermelon extract applied to a poly-L-proline-Sepharose column, taking advantage of its high-affinity binding capability to this matrix. Watermelon extract was dialyzed against 0.1 M KCl, 0.1 M glycine, 10 mM Tris-HCl, pH 7.8, and 0.5 mM dithiothreitol, the same buffer (buffer A) with which the column was equilibrated. Profilin was eluted with 8 M urea in buffer A and dialyzed against ammonium bicarbonate, pH 7.6, as previously described, in order to eliminate the excess urea [22].

Isolation of cDNA Encoding Watermelon Profilin

Frozen watermelon tissue was ground to powder and total RNA was extracted using the RNA Plant Kit (Clontech) according to the manufacturer's instructions. RNA was reverse transcribed with the Reverse Transcription System (Promega) with 1 µg of total RNA. cDNA was amplified using Pfu polymerase (Takara) and gene-specific primers (forward: 5'-GCT GAA GCT ATG TCG TGG CAG (A/G)CG TA(C/T) GTC GAT G-3'; reverse: 5'-TTC AAT CCC CCC GAG GCC CTG CTC GAT AAG GT-3') selected based on published tomato and melon profilin sequences.

Using pGEM-T Easy containing profilin as a template, constructs for the expression vector pPicZαA were prepared by PCR with a sense primer (5'-CTC GAG AAA AGA GAG GCT GAA

GCT ATG TCG TGG CA-3') in-frame with the α -factor present in plasmid pPICZ α A insertion including an *Xho* I restriction site. Antisense primer (5'-TCT AGA TCT CCC TTC AAT CCC CCC GAG GCC CT-3') allows fusion with the c-myc epitope and His tag (6 $\times$ His) from the expression vector, which facilitates purification of recombinant proteins and contains an *Xba* I restriction site. The excised profilin gene was cloned into the expression vector pPICZ α A, which had been digested with the same restriction endonucleases. The profilin gene was cloned in-frame with the *Saccharomyces cerevisiae* α -factor pre-pro-peptide signal sequence under the control of the methanol-inducible alcohol oxidase 1 promoter.

Transformation, Expression and Purification of Recombinant Watermelon Profilin

Transformation was performed with the EasySelect *Pichia* Expression Kit (Invitrogen) following the instructions provided by the manufacturer. Every 24 h, a 1-ml sample was removed and centrifuged for 1 min at maximum speed in a microcentrifuge, and the supernatant was removed and stored at -20°C . Aliquots of samples obtained at different growing times (0, 24, 48, 72, 96 h) were analyzed by SDS-PAGE to select the optimum conditions for the large-scale production of recombinant profilin, which was performed using the colony that rendered the greatest yield in the small-scale experiments. The optimal time for recombinant profilin expression was determined to be 48 h. The expressed profilin was filtered through a membrane with a pore diameter of 0.22 μm (Millipore, Bedford, Mass., USA) and purified by metal chelate affinity in a HiTrap Chelating HP Column (GE Healthcare) according to the manufacturer's instructions.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to Laemmli [23] using the Hoefer SE 600 electrophoresis system (GE Healthcare). Polyacrylamide concentrations of 14% (w/v) and 5% (w/v) were used for separating and stacking gels, respectively. Samples were mixed with 0.1 M Tris, pH 6.8, containing 4% (w/v) SDS, 20% (w/v) glycerol, 10% (w/v) 2- β -mercaptoethanol and 0.02% (w/v) bromophenol blue. The samples were denatured by heating at 95°C for 5 min. Then, 50 μg of protein extract was applied per lane. Electrophoresis was carried out in 0.025 M (w/v) Tris-HCl, pH 8.3, 0.192 M (w/v) glycine and 0.1% (w/v) SDS. To ensure proper protein separation and visualization, the gels were stained with Coomassie brilliant blue (Sigma Chemical Co., St. Louis, Mo., USA) or used for immunoblotting as described below.

Protein Identification and Characterization by Mass Spectrometry

Identification of proteins by mass spectrometry (MS) and/or tandem MS (MS-MS) was performed, as previously described [3, 24, 25], in the proteomics service of the Universidad Complutense de Madrid, a member of the ProteoRed Network. Briefly, selected bands were cut out from the gels with a sterile scalpel and subjected to in-gel trypsin digestion according to Shevchenko et al. [26], with some modifications. The in-gel digests were purified as described by Gobom et al. [25].

Immunoblot Analysis and Inhibition Assays

Protein bands separated by SDS-PAGE were transferred by semi-dry blotting onto nitrocellulose sheets by the method used

by Towbin et al. [27]. Membranes were blocked with PBS containing 0.1% Tween-20 (PBS-T), 3% skimmed milk powder and 3% bovine serum albumin (BSA) for 2 h at room temperature and incubated overnight at 4°C with sera diluted 1:10 in 50 mM Tris-buffered saline, 150 mM NaCl, 5 mM EDTA and 0.05% Triton X-100, pH 8.8. After washing them 5 times with PBS-T, the blots were incubated for 1 h at room temperature with goat antihuman IgE antibody conjugated with horseradish peroxidase (Nordic, CultiK) diluted 1:5,000 in PBS containing 0.05% Tween-20 and 1.5% skimmed milk powder. Finally, after 5 washings with PBS-T, the protein bands were visualized by enhanced chemiluminescence (GE Healthcare), following the instructions provided by the manufacturer. To perform immunoblotting inhibition assays, the serum pool was incubated overnight with 50 $\mu\text{g}/\text{ml}$ purified protein (native and recombinant) at 4°C .

ELISA and Inhibition ELISA

For ELISA and inhibition ELISA assays, 96-well flat-bottom plates were used (Immunolon 4HBX, Thermo). The plates were coated in duplicate overnight at 4°C with 20 μg per well of watermelon extract or 2 μg per well of native or recombinant profilin diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). The plates were blocked with PBS containing 0.05% Tween-20 and 2% BSA for 1 h at room temperature. After blocking, the plates were washed 3 times with PBS containing 0.05% Tween-20. The plates were then incubated for 2 h at room temperature with patients' sera diluted 1:10 in blocking buffer. The plates were washed again and incubated with the secondary antibody (goat antihuman IgE-PO, Nordic, CultiK) diluted 1:5,000 with 2% BSA and 0.05% PBS-T for 1 h at room temperature. IgE reactivity was detected by adding tetramethylbenzidine (Chemicon) and then measuring the optical density (OD) at 620 nm. Sera from 5 nonatopic patients were used as negative controls. IgE reactivity was considered positive when OD 650 nm was $\geq$ OD 650 nm of the negative control $\times$ 2.

For inhibition ELISA, sera from allergic patients were preincubated for 2 h at 37°C with 10-fold dilutions from 10 to 0.00001 μg of the inhibitor (purified native profilin and recombinant profilin) per milliliter of serum.

Basotest Assays

Basophil activation tests were performed with the Basotest test kit (Orpegen Pharma), which allows quantitative determination of basophil degranulation in heparinized human whole blood following incubation with purified allergens. The evaluation of basophil degranulation was performed by flow cytometry, following the manufacturer's instructions.

Cells were sensitized with purified native profilin and recombinant profilin in allergen concentrations ranging from 0.001 to 50 $\mu\text{g}/\text{ml}$ of heparinized whole blood.

Statistical Analysis

Statistical analysis was performed with SPSS software. Descriptive statistics included the frequency of positive results (expressed as a percentage) with the 95% confidence interval for qualitative variables and mean $\pm$ SEM for quantitative variables. Paired correlations were calculated using the Pearson test. Values were considered significant at $p < 0.05$.

<i>Citrullus lanatus</i>	MSWQAYVDDHLMCEIEGNHLTSAAIIGQDGSVWAKSENFPQLKPEEITGILNDFNEPGTL	60
Cuc m 2	-----D-D-R-A-L-----SQ-AT-A-R-AA-K-DQ-----	
Mal d 4.0201	-----SA-----HN-----Q-AT-----V-M-----S-	
Pru p 4.02	-----SA-----H-----Q-AT-----V-M-----S-	
Phl p 12	-----YE-----SH-S-L-H-T-Q-TA-F-----MK-D-H-	
Fra a 4	---T-----SA-----Q-AT-----V-VR-D-----	
Bet v 2	---T---E---D-D-QASN-LAS--V-H-----Q-SS---F-Q---MK-E---	
<i>Citrullus lanatus</i>	APTGLYIGGSKYMIQGEPGAIVIRGKKGGVTVKKTALALVIGIYDEPMTPGQCNIIVE	120
Cuc m 2	---FL---T-----A-----S-I---NQ-I-----L-----	
Mal d 4.0201	---L---T-----V-----STM---L-----V---	
Pru p 4.02	---L---T-----S-T---L-----	
Phl p 12	---HL---T-----A-I---GQ-IF-----L---I---	
Fra a 4	---L---T-----T---L-----	
Bet v 2	H-----HL---I-----A-----S-I-I---GQ---F---E---V-----V	
<i>Citrullus lanatus</i>	RLGDYLIEQGL	131
Cuc m 2	-----D---	
Mal d 4.0201	-----	
Pru p 4.02	-----V---	
Phl p 12	-----VK---	
Fra a 4	-----V---	
Bet v 2	-----D---	
	Identity (%)	Positive (%)
	78	90
	86	96
	89	96
	77	90
	88	96
	72	85
		Gaps (%)
		0
		0
		0
		0
		0
		1

Fig. 1. Alignment of amino acid sequences of different profilins deduced from cDNA encoding for fruits (accession numbers: *Citrullus lanatus*, AY730591; *Cucumis melo* Cuc m 2, CAD92666; *Fragaria ananassa* Fra a 4, P0C0Y3; *Prunus dulcis*, *P. persica*, ACE80973, *Malus domestica* Mal d 4.0201, Q9XF41) and pollens (accession numbers: *Betula verrucosa* Bet v 2, M64179; *Phleum pratense* Phl p 12, O24650). Only differences with respect to watermelon profilin (*C. lanatus*) are shown.

Results

Cloning of Recombinant Profilin

The cloned profilin sequence length was 393 bp, encoding a 131-amino-acid protein. The sequence has been deposited in GenBank (accession number GI: 52352488).

Figure 1 shows the sequence of watermelon profilin and the alignment of amino acid sequences of profilins from different plant sources. The high homology between plant-derived food and pollen profilins is remarkable.

Native Profilin Purification and Identification

Profilin was purified to homogeneity using the method described above based on affinity to poly-L-proline-Sepharose [22]. The 14-kDa band was identified by MS-MS analysis according to the sequence of internal peptides obtained, i.e. LGDYLVEQGL and YMVIQGEPGAIVIR. Searches of protein databases led to the identification of the 14-kDa protein peptide sequence as

watermelon profilin. This result was confirmed by an inhibition blot in which watermelon extract was incubated with patients' sera preinhibited with recombinant or native profilin. The purification yield from watermelon pulp was approximately 0.15%, with approximately 141 µg of native profilin recovered from 100 g of watermelon pulp. The IgE reactivity of the purified profilin was maintained throughout the whole process, as confirmed by immunoblots performed with a serum pool from allergic patients (fig. 2).

Purification of Recombinant Profilin

Two milligrams of purified recombinant profilin were obtained per liter of buffered glycerol-complex medium inoculated. Three bands were observed in both SDS-PAGE and immunoblot at 16.8, 17 and 17.2 kDa (analyzed by MS-MS), and all were identified as profilin (data not shown). Furthermore, immunoblots were performed with individual sera and a serum pool, and sera from the 17 patients recognized the 3 bands of the recombinant

Fig. 2. SDS-PAGE (lane 1) and immunoblot with sera from watermelon-allergic patients (lane 2) and immunoblot with polyclonal anti-profilin antibody (lane 3) of native watermelon profilin (**a**) and recombinant profilin (**b**). Immunoblots were incubated with a serum pool from watermelon-allergic patients diluted 1:10 in blocking buffer.

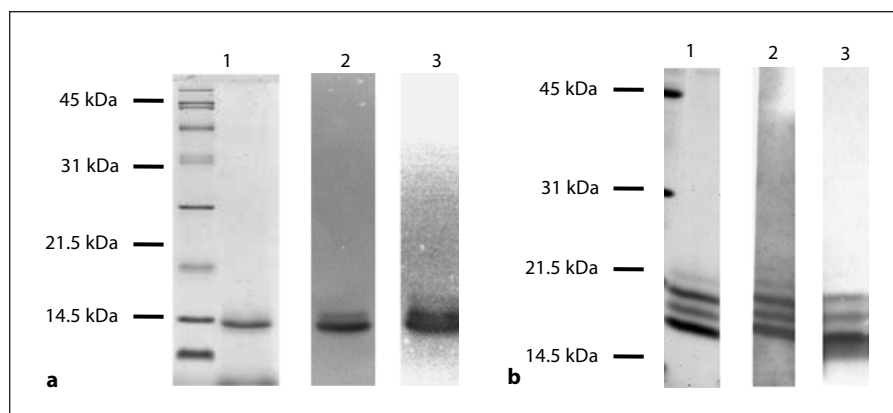
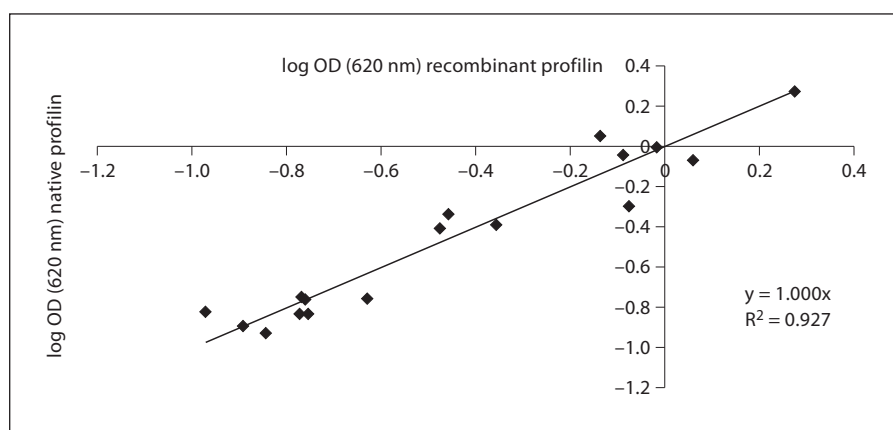


Fig. 3. Correlation between levels of specific IgE to native and recombinant profilin ($R^2 = 0.927$).



purified profilin. These bands were also recognized by specific anti-profilin antibody produced following the technique described by Anton et al. [31, 32] (fig. 2). These results support the MS-MS identification.

Immunodetection

To evaluate the profilin sensitization of patients, ELISA experiments were performed on 17 patients' sera with native and recombinant profilins. A high correlation coefficient ($n = 17$; $R^2 = 0.927$, $p < 0.05$) was observed between levels of specific IgE to native and recombinant profilin (fig. 3), suggesting that recombinant profilin may be an efficient tool that could replace native profilin in in vitro tests in watermelon-allergic profilin-sensitized patients.

An inhibition ELISA assay was carried out by preincubating a pool of sera from patients with watermelon allergy with different concentrations of purified profilins, both native and recombinant (fig. 4a). The results obtained by inhibiting sera with recombinant profilin are

similar to the results reported with native profilin inhibition, indicating that IgE reactivity to native and recombinant profilins was similar. Additionally, homologous ELISA inhibition with purified native and recombinant profilin was performed (fig. 4b), showing their ability to inhibit each other. As inhibition control for ELISA experiments, sera were inhibited with the same watermelon extract, and complete inhibition was observed (fig. 5).

Control immunoblot and ELISA experiments with a serum pool from 5 nonatopic patients did not show IgE-binding bands for profilin.

Basotest Assays

To confirm the ability of purified native and recombinant profilin to induce a response at the cellular level, basophil activation tests were performed using increasing concentrations of native and recombinant profilins in order to activate basophils. Allergen concentrations ranged from 0.001 to 50 $\mu\text{g}/\text{ml}$ of whole blood from an allergic patient with strong IgE binding to profilins. An

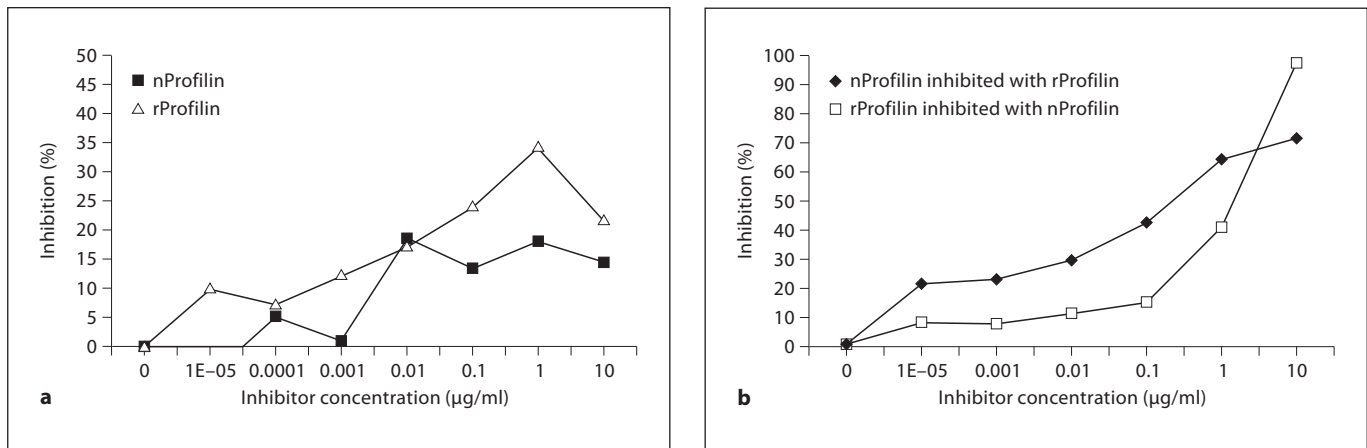


Fig. 4. **a** ELISA inhibition assay. Watermelon extract was used as the solid phase, and a serum pool was preincubated with increasing concentrations of native profilin (nProfilin) and recombinant profilin (rProfilin). All tests were performed twice. **b** ELISA inhibition assay with native and recombinant profilin, incubated with a serum pool inhibited with increasing concentrations of recombinant and native profilin, respectively. All assays were performed twice.

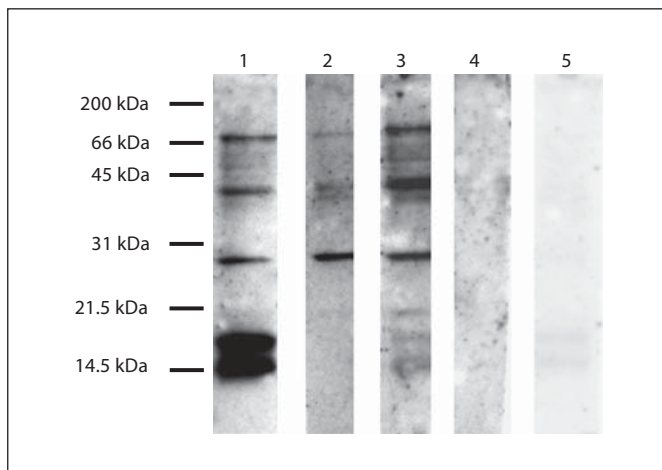


Fig. 5. Lane 1: immunoreactivity of a serum pool to watermelon extract. Lanes 2–4: immunoblots of watermelon extract with a serum pool inhibited with native profilin (lane 2), recombinant profilin (lane 3) and watermelon (lane 4). Lane 5: immunoblot with serum from a nonatopic patient used as a negative control.

activation response was observed in a dose-dependent manner for both profilins. Activation ranged from 61.42% for 0.001 µg/ml native profilin to 73.84% for 50 µg/ml, with values of 32.22 and 2.94% for the positive and negative controls, respectively. Using the recombinant form of profilin, basophil activation ranged from 54.34 to 88.01% for recombinant profilin concentrations of 0.001–50 µg/

ml. Positive and negative controls were 54.97 and 2.11%, respectively. No activation response was observed in blood from a nonatopic patient used as a negative control, since the values obtained were lower than the negative control at all concentrations tested.

Discussion

Profilin has been found to be a minor but important allergen in many pollen and some plant food extracts, with IgE reactivity of about 20% [13, 14, 33]. Nonetheless, it has been found to be a major allergen in other fruits, such as muskmelon [34], orange [35] or watermelon, with a rate of profilin-sensitized patients of 56% [3].

In this study, we report the isolation of native watermelon profilin with a high yield and also characterization of watermelon profilin [3]. Many profilins from plant-derived foods have been characterized to date (e.g. orange, peach, apple, peanut). However, as can be seen in the case of *Phaseolus vulgaris* [36], whose purification yield ranges from 1 to 3 µg/mg of the total protein loaded in the affinity column, the results obtained were not as useful as those obtained for watermelon profilin, for which we obtained 10.6 µg of profilin per milligram of total protein loaded. This is most probably due to the lower profilin concentration in those fruits compared to watermelon.

We consider it important to report this particular characterization because watermelon profilin is such a

major allergen in watermelon-allergic patients, having the highest reported rate of sensitization among patients with plant-derived food allergies who are also allergic to profilin.

Although watermelon profilin has no post-translational modifications, a eukaryotic system, *Pichia pastoris*, was selected to produce the recombinant form because it allows for high expression levels and easy purification through secretion to the extracellular medium. This system offers purification that is superior to *Escherichia coli* expression, where recombinant proteins are not secreted to the extracellular medium and thus have to be purified from a lysate. Frequent misfolding of target proteins has been observed when proteins are expressed in a bacterial system. Furthermore, formation of insoluble aggregates, such as inclusion bodies and oligomerization, which can lead to a biologically inactive protein [37], can occur in expression in bacterial systems. In this case, the *P. pastoris* expression vector pPICZ α A allowed expression of the recombinants as His-tagged fusion proteins. Although some studies suggest that use of native profilin from several plant sources makes for better diagnosis, at least in vitro, than the recombinant form [13, 38], our study shows that the recombinant form of profilin may be very helpful in diagnosing sensitization to watermelon, even if the recombinant profilin is only a single isoform among many [39].

Patients included in this study were all sensitized to watermelon profilin and pollen [2]. The positive basophil activation test for native and recombinant profilin demonstrated the biological activity of this allergen, confirming that specific IgE to profilin is able to elicit basophil degranulation in patients allergic to watermelon.

The clinical relevance of both native and recombinant profilin in watermelon allergy has been demonstrated, and there is a possibility of using recombinant profilin for clinical purposes over its native counterpart, which is not standardized.

The high sequence similarity among profilins explains the existence of sensitization to profilins from different plant sources, and changes found in sequences may explain the existence of different clinical patterns. Thus, it has been reported that the high specificity of IgE in directing itself to different epitopes may influence the clinical manifestation of profilin sensitization [38]. Comparing the sequences that make up the principal epitopes in Cuc m 2 [40] with the corresponding sequences in watermelon shows 75% identity (positive = 85%), which may explain the differences found between patients allergic to melon and those allergic to watermelon. Epitopes were

found on the surface of the protein in both watermelon and melon profilins.

Different studies have demonstrated that the pattern of IgE binding to linear epitopes is related to the clinical manifestation and evolution of the process, such as cow's milk casein allergy [41], wheat allergy [42] or peanut allergy [43]. Linear epitopes in plant profilin may be elucidated by microarray techniques. This may explain the different symptoms caused by a plant-derived food or groups of plant-derived foods among profilin-sensitized patients, a discrepancy that cannot be explained by the complete allergen molecule sensitization hypothesis.

We can conclude that recombinant watermelon profilin shows a similar pattern of IgE binding and biological activity to native profilin. Regarding clinical practice, recombinant profilin may be a useful tool for specific diagnosis. The findings described in this study will help to develop innovative techniques to characterize the clinical features of pollen- and plant-derived food allergy caused by profilin sensitization. In addition, these results will allow relation of epitopes recognized by patients' sera with clinical patterns, and this represents an important improvement in patients' quality of life.

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