

Identification of Major Allergens in Watermelon

Carlos Pastor^{a, c, d} Javier Cuesta-Herranz^b Barbara Cases^a
Marina Pérez-Gordo^a Elena Figueredo^b Manuel de las Heras^b
Fernando Vivanco^{a, d}

^aDepartamento de Inmunología and ^bServicio de Alergia, Fundación Jiménez Díaz, ^cSIC Ingenieros SLL and
^dUniversidad Complutense, Madrid, Spain

Key Words

Malate dehydrogenase · Triose phosphate isomerase ·
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Abstract

Background: Watermelon is a worldwide consumed Cucurbitaceae fruit that can elicit allergic reactions. However, the major allergens of watermelon are not known. The aim of this study is to identify and characterize major allergens in watermelon. **Methods:** Twenty-three patients allergic to watermelon took part in the study. The diagnosis was based on a history of symptoms and positive skin prick-prick tests to watermelon, confirmed by positive open oral challenge testing to watermelon pulp. Allergenic components were detected by SDS-PAGE and immunoblotting. Molecular characterization of IgE-binding bands was performed by N-terminal amino acid sequencing and mass spectrometry. Allergens were purified combining several chromatographic steps. **Results:** Several IgE binding bands (8–120 kDa) were detected in watermelon extract. Three major allergens were identified as malate dehydrogenase (36 kDa), triose phosphate isomerase (28 kDa) and profilin (13 kDa). Purified allergens individually inhibited IgE binding to the whole watermelon extract. **Conclusions:** All in all these results indicate that malate dehydrogenase, triose phosphate isomerase and profilin are major allergens involved in watermelon allergy.

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Introduction

In the last few years, food allergy has increased in developed countries, particularly allergies to plant-derived foods [1, 2], and the phenomenon is now recognized as a worldwide problem [3, 4]. Several food proteins have been identified and characterized as allergens in peanut, peach, kiwi, melon and others [5, 6]. Nonetheless, there are several plant-derived foods whose allergens have not been identified or characterized to date, including watermelon.

Watermelon (*Citrullus lanatus*) is a fruit belonging to the Cucurbitaceae family, and the name itself gives an idea of its high water content (over 90%). The fruit originated in Africa and later on spread to the rest of the world, being known in both North and South America, probably as a result of its introduction by the Spaniards. In Europe, it is greatly consumed in Mediterranean areas, particularly in Spain, Italy and Greece, which are the main producers.

In 1970, Anderson et al. [7] reported the association between food-induced oropharyngeal symptoms and ragweed pollinosis in patients from the United States. Since then, only 2 manuscripts centered on watermelon allergy have been published. In 1987, Enberg et al. [8] demonstrated cross-reactivity between watermelon and

C.P. and J.C.-H. contributed equally to this work.

ragweed pollen, and 1 year later, Enberg et al. [9] described the presence of several IgE-binding proteins in immune-isoelectrofocusing tests. In this sense, Jordan-Wagner et al. [10] found watermelon-allergic patients to display IgE reactivities at about 15 kDa, and Asero et al. [11] found a high prevalence of watermelon-induced oral allergy symptoms in patients sensitized to profilin. Although there have been important advances in the characterization of food allergens, none of the watermelon allergens have been characterized to date, with the exception of profilin, cloned and sequenced by our group, which subsequently included the sequence in the database with accession No. GI:52352488.

The aim of this study was to identify and characterize major allergens in watermelon.

Materials and Methods

Patient Population

Twenty-three patients allergic to watermelon took part in the study. The diagnosis of watermelon allergy was based on a history of symptoms elicited by eating watermelon, with positive skin prick-prick testing to fresh watermelon pulp and performed in duplicate using the ALK-Lancet needle (ALK-Lancet; ALK, Horsholm, Denmark) following the technique described by Dreborg et al. [12] according to EAACI guidelines [13]. 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. Results were confirmed by open oral challenge to watermelon pulp following the previously reported method [14].

For the immunoblot assays, individual sera and a serum pool prepared by mixing each of the 23 individual sera from the patients allergic to watermelon were used in the study. In addition, sera from 2 nonatopic patients were used as negative controls.

The study was approved by the Hospital Ethics Committee and all patients gave written consent to participate in the study.

Production of Allergenic Extracts

Watermelon extracts were prepared from watermelon (*Citrus lanatus* var. *lanatus*), which is the most widely consumed variety in Spain. The fruit was purchased at a local market, and 2 kg were used for pulp extraction. Firstly, the watermelons were peeled, liquidized and then processed as described below. Extraction was started by homogenization with magnetic stirring (2,000 rpm) at 4°C for 30 min. A formulation of 10 mmol/l phosphate-buffered saline (PBS), pH 7, containing 2% suspended solid polyvinylpyrrolidone and 2 mmol/l ethylene-diaminetetraacetic acid disodium salt was used as antioxidant and solubilizing agent. The extract was then clarified by centrifugation at 13,000 rpm (26,000 g) for 30 min at 4°C. The pellet was discarded and the supernatant containing the solubilized proteins was dialyzed against distilled water by diffusion in Spectra/Por tubing (Spectrum Laboratories Inc., Breda, The Netherlands) of 3.5 kDa at 4°C overnight. The dialyzed extract was sterilized by filtration through a 0.22-μm pore diameter membrane (Millipore, Bed-

ford, Mass., USA) and frozen at -80°C. A method based on ion-exchange column chromatography for the enrichment of fruit-allergenic extracts was used, as described by Martínez et al. [15]. For in vitro experiments, the protein concentration of all allergenic extracts was adjusted to 1 mg/ml. Determination of the protein concentration was performed according to the method of Bradford [16].

SDS-PAGE

SDS-PAGE was carried out according to Laemmli [17] using the Hoefer SE 600 electrophoresis system (Amersham Bioscience, Uppsala, Sweden). Polyacrylamide concentrations of 14% (w/v) and 5% (w/v) were used for separating and stacking gels, respectively. The 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 100°C for 5 min. An amount of 50 μg protein was applied per lane. Electrophoresis was run at 25 mA constant current until the tracking dye bromophenol blue reached the bottom of the gel, using as electrode buffer 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 blotting as described below.

Immunoblot Analysis and Inhibition Assays

Protein bands separated by SDS-PAGE were transferred by semidry blotting onto nitrocellulose sheets by the method of Towbin et al. [18], in transfer buffer [0.048 M (w/v) Tris-HCl, 0.039 M (w/v) glycine, 20% (v/v) methanol and 0.036% (w/v) SDS] for 2 h at 70 mA. Blotted bands were visualized by soaking the membranes for a few minutes in Ponceau S (0.1% in 1% acetic acid) and marked with a pencil before destaining with water. Membranes were blocked with PBS containing 0.1% Tween 20 (PBS-T), 3% skimmed milk powder and 3% bovine serum albumin for 2 h at room temperature, and incubated overnight at 4°C with the patient sera diluted 1:10 in 20 mM Tris-buffered saline pH 7.5 containing 0.1% Tween 20 and 3% bovine serum albumin. After washing 5 times with PBS-T, blots were incubated for 1 h at room temperature with goat antihuman IgE (Serotec) diluted 1:2,500 in PBS containing 0.05% Tween 20 and 1.5% skimmed milk powder. Finally, washed 5 times with PBS-T, blots were incubated for 1 h at room temperature with rabbit antigoat IgG peroxidase (Nordic, Cultek, Madrid, Spain) diluted 1:10,000 in PBS containing 0.05% Tween 20 and 1.5% skimmed milk powder. After 5 washes in PBS-T, the protein bands were visualized by chemiluminescence with ECL (Amersham Bioscience), following the instructions given by the manufacturer. To perform immunoblotting inhibition assays, 1 ml of serum pool was previously incubated with 15 μg of each purified protein at 4°C overnight.

Sequence Analysis

Watermelon extract was used for sequence analysis of protein bands (Edman sequencing). When the gels, under reducing conditions, were used for blotting and sequencing experiments, several previously described modifications [19] were introduced: (1) gels were cast 1 day in advance and kept overnight at 4°C, to allow complete polymerization; (2) thioglycolic acid was added to the upper buffer chamber to 0.002% (v/v) final concentration; (3) pre-electrophoresis was carried out for 30–60 min at 10 mA.

ProBlott membranes with CAPS buffer [3-(cyclohexylamino)-1-propanesulfonic acid] were used for transferring the proteins, following the recommendations of the manufacturer (Applied Biosystems, Foster City, Calif., USA). Proteins were stained with Coomassie Blue, cut out with a razor and sequenced in a Procise sequencer (Applied Biosystems). Eight N-terminal amino acid residues were determined for each band. The obtained N-terminal sequences were analyzed by the BLAST program to identify the proteins in the data bases.

Protein Identification and Characterization by Mass Spectrometry

Identification of protein by mass spectrometry (MS) and/or MS/MS was performed as previously described [20, 21]. 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. [22], with some modifications. The in-gel digests were purified as described by Gobom et al. [23].

Peptides were analyzed using a Voyager-DE STR BioSpectrometry Workstation instrument (Applied Biosystems), and positively charged ions were analyzed in reflector mode, using delayed extraction with the following settings: acceleration voltage 20 kV, grid 72%, delay time 300 ns, 100 shots/spectrum and mass range 800–3,500 Da. Spectra were calibrated using trypsin autolysis products, resulting in a mass accuracy of <50 ppm. The spectra were analyzed using Data Explorer software (Applied Biosystems). For those proteins whose identification was not possible by matrix-assisted laser desorption/ionization-MS, further analysis by MS/MS was carried out [22]. The following parameters were used: monoisotopic mass accuracy <50 ppm, 1 missed cleavage, allowed modifications carbamidomethylation of cysteine (complete) and methionine oxidation and pyroglutamic acid formation (partial).

Protein identification was performed by searching a non-redundant protein sequence database (NCBI) using the Mascot program (<http://www.matrixscience.com>).

Protein Purification

Proteins were purified by different chromatography steps in an FPLC system (AKTA Purifier; Amersham Bioscience). Protein identification in the extracts was followed by SDS-PAGE, blotting and MS. Briefly, watermelon extract, dialyzed in 50 mM Tris pH 7.4, was loaded onto a HiPrep 16/10 DEAE FF column (Amersham Bioscience). The bound proteins were eluted with a linear salt gradient from 0 to 1 M NaCl, obtaining 2 fractions: fraction A, containing malate dehydrogenase (MDH) and triose phosphate isomerase (TPI), and fraction B, in which profilin was almost purified. Fraction A was loaded in a Superdex 75 10/300 GL column (Amersham Bioscience) equilibrated in PBS. In this step, we obtained another 2 fractions: one containing TPI and a second containing MDH. The profilin fraction was purified using a Mono Q 5/50 GL column (Amersham Bioscience) equilibrated in 20 mM Tris pH 7.9, and eluted with a linear salt gradient from 0 to 1 M NaCl. Two additional chromatography steps were necessary to obtain purified MDH and TPI. First, an ionic exchange step was carried out with a Mono Q 5/50 GL column (Amersham Bioscience) under the conditions previously described. Finally, hydrophobic interaction chromatography was performed in a Source 15PHE 4.6/100 PE column (Amersham Bioscience) equilibrated in 20 mM Tris, 1 M (NH₄)₂SO₄, with a linear gradient 100–0% (NH₄)₂SO₄.

Table 1. Clinical features of watermelon-allergic patients

No.	Age years	Sex	Watermelon skin test ^a , mm ²	Pollen allergy symptoms
1	7	female	17	rhinitis
2	20	male	19	rhinitis; asthma
3	21	male	23	rhinitis; asthma
4	23	female	36	rhinitis
5	26	female	30	rhinitis; asthma
6	39	male	27	rhinitis; asthma
7	5	male	20	rhinitis; asthma
8	32	female	18	rhinitis; asthma
9	33	female	13	rhinitis; asthma
10	20	male	32	rhinitis
11	32	female	27	rhinitis
12	27	male	27	rhinitis; asthma
13	29	male	19	rhinitis; asthma
14	7	male	15	rhinitis; asthma
15	38	male	23	rhinitis; asthma
16	15	male	32	rhinitis; asthma
17	22	female	24	rhinitis; asthma
18	20	female	28	rhinitis; asthma
19	29	female	20	rhinitis; asthma
20	29	male	68	rhinitis
21	18	male	43	rhinitis; asthma
22	53	male	21	rhinitis; asthma
23	26	female	28	rhinitis; asthma

All patients had oral allergy symptoms and pollen allergy.

^a Wheal area.

Analysis of Stability of Watermelon Extract to in vitro Digestion

The watermelon extract (525 µg of protein) was dissolved in 250 µl of prewarmed 0.7% (v/v) HCl with 30 mmol/l NaCl, pH 1.2, and in the same solution containing 0.32% (w/v) pepsin A (Sigma). Digestion was performed with continuous shaking for 30 min at 37°C. Aliquots of these solutions (corresponding to 25 µg of watermelon extract) were withdrawn at 5, 10 and 15 min, stopped by neutralization with 1 M Tris and redissolved in sample buffer and analyzed by SDS-PAGE and immunoblotting using pooled sera from patients with watermelon allergy [24].

Results

Clinical Features of Watermelon-Allergic Patients

The mean age of the participants in this study is 24.8 years (range 5–53 years); 56.5% were males and 43.5% females. All experienced oral allergy symptoms on eating watermelon, without systemic allergic reactions. Characteristics of the watermelon-allergic patients are reported in table 1.

Immunoblotting

SDS-PAGE of watermelon extract showed multiple protein bands with an apparent molecular weight (MW) ranging from 8 to 120 kDa. IgE immunoblotting obtained with patient sera demonstrated a broad spectrum of IgE-binding bands from approximately 12 to 70 kDa (fig. 1). Incubation with serum pool revealed three high intensity bands with an apparent MW of 36, 28 and 13 kDa, which were coincident with the bands most frequently found when using individual sera. Three IgE-binding bands were recognized by more than 50% of patient sera: 36, 28 and 13 kDa (fig. 2). In addition, a group of bands with MW >36 kDa was also detected, which was present in some of the individual patients. Control immunoblot experiments with a serum pool from nonatopic patients did not show IgE-binding bands in watermelon extract.

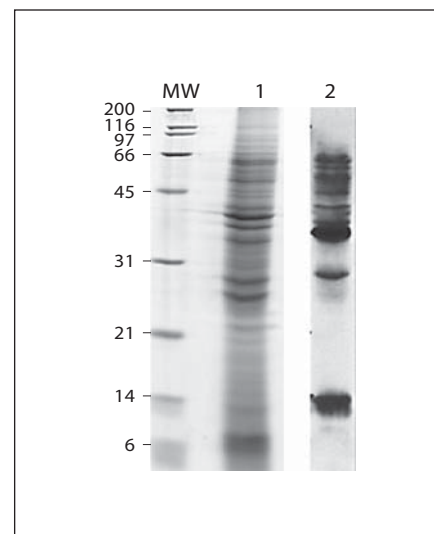


Fig. 1. Lane 1: SDS-PAGE analysis of watermelon extract under reducing conditions. Lane 2: immunoblotting of a reduced sample revealed with a serum pool of watermelon allergic patients.

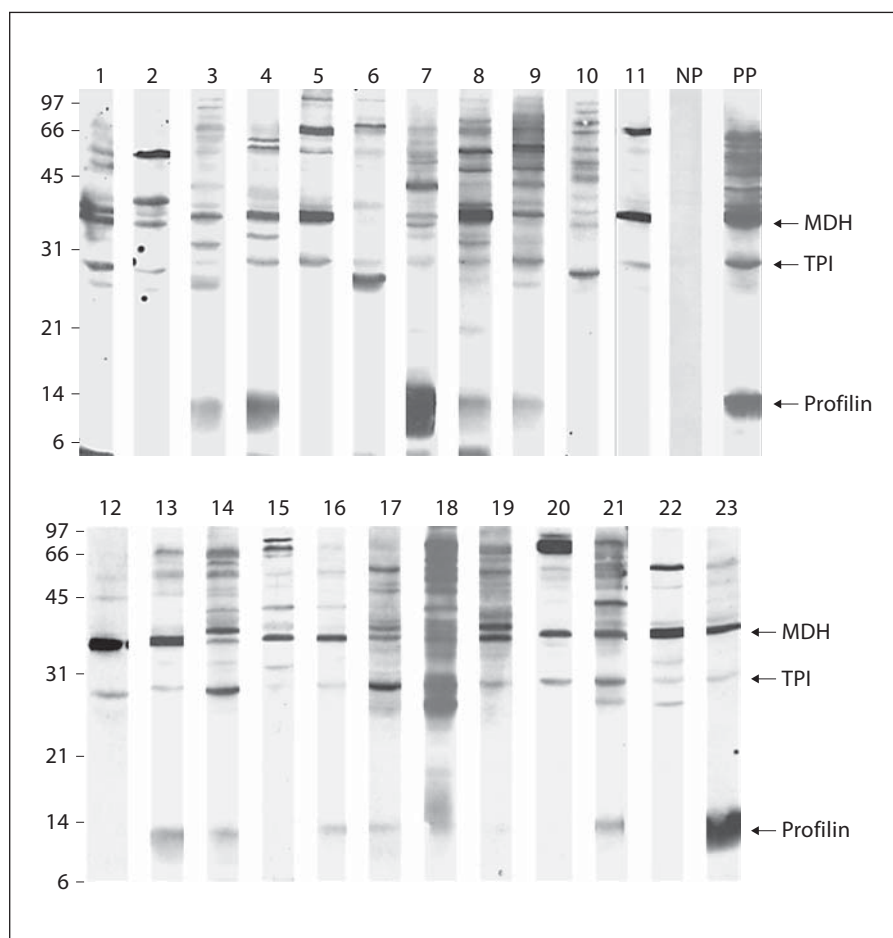


Fig. 2. IgE-binding bands in watermelon extract by immunoblotting. Lanes 1–23 represent serum from 23 patients. Lane NP represents a negative control of serum pool from nonatopic patients. Lane PP represents serum pool from several watermelon-allergic patients.

Identification of Proteins

IgE-binding bands were characterized by analyzing the N-terminal sequence of the allergenic proteins blotted onto membranes, and by MS from the extract and the purified proteins. The results showed the following N-terminal sequence: 36 kDa protein (ATESVPE), 28 kDa protein (no sequence) and 13 kDa protein (no sequence). To identify the 28- and 13-kDa proteins, we performed MS/MS and obtained the sequence of several internal peptides with the following sequences: VIACVGETLEQR and FFVGGNWK for 28-kDa protein; LGDYLVEQGL and YMVIQGEPGAIVIR for 13-kDa protein. Research conducted with protein databases identified the 36-kDa protein N-terminal sequence as mitochondrial malate dehydrogenase (GI:126896), which was confirmed by MS/MS analysis, and 13-kDa protein peptides sequence as watermelon profilin (GI:52352489). The 28-kDa protein peptides sequence yielded a high match with triose phosphate isomerase from related species such as *Oryza sativa*, *Zea mays*, *Spinacea oleracea* and many others, due to the fact that watermelon TPI has not yet been sequenced.

Allergen Purification

Figure 3 provides a schematic representation of the chromatographic steps and SDS-PAGE analysis as well as immunoblotting of protein purified after chromatography steps and immunoblotting with serum pool. TPI and profilin were purified to homogeneity (fig. 3), but the MDH fraction had a 40-kDa contaminant protein without IgE-binding capacity.

IgE Immunoblot Inhibition

To demonstrate that the ability of IgE binding by the purified proteins was the same as the binding found with the watermelon extract, immunoblot inhibition assays were performed with preincubating pooled sera from patients allergic to watermelon (fig. 4). IgE binding to profilin, TPI and MDH was almost completely inhibited by using 15 µg of each purified protein in 1 ml of pooled sera. No inhibitory effect was detected in the control experiment with a 40-kDa watermelon-purified protein described in the MDH purification.

Susceptibility of Watermelon Protein Extract to Pepsin

The effect of treatment with pepsin on watermelon extract is depicted in figure 5. As is shown in figure 5a, all protein bands present in the extract (left lane) were digested to low MW fragments, and only the bands corresponding to pepsin can be detected after 5, 10 and 15 min

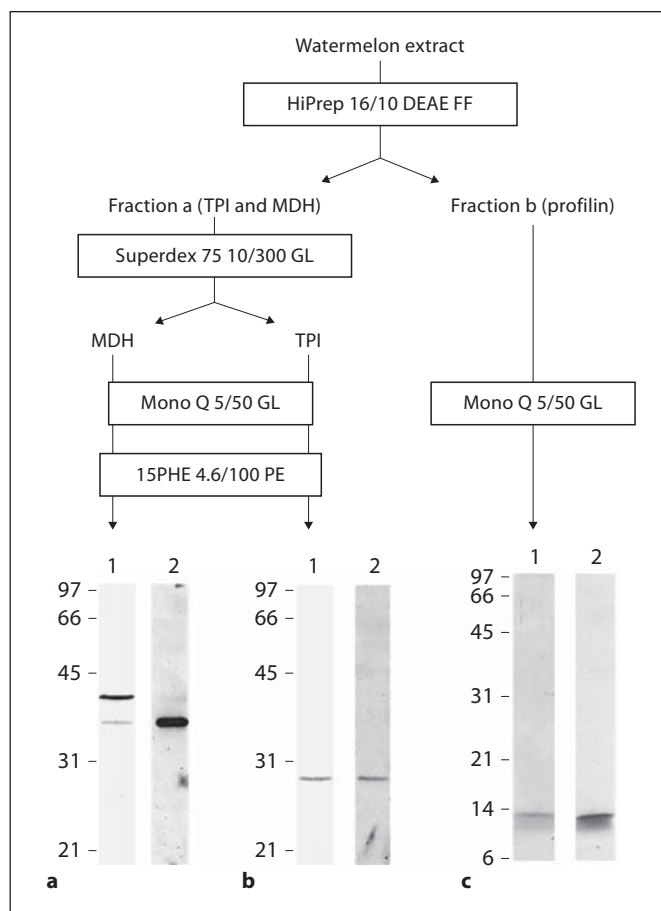


Fig. 3. Purification scheme of watermelon allergens. **a** MDH, **b** TPI, **c** profilin. Lane 1: SDS-PAGE analysis of purified protein under reducing conditions. Lane 2: Immunoblot analysis of purified protein under reducing conditions.

lanes. Similar results were recorded in immunoblotting (fig. 5b), with disappearance of the IgE-binding bands corresponding to MDH, TPI and profilin – thus indicating the susceptibility of the 3 proteins to pepsin.

Discussion

Results of this manuscript describe for the first time 3 major allergens (profilin, MDH and TPI) involved in watermelon-allergic reactions.

Allergy to plant-derived foods is a frequent condition usually associated to pollen-allergic patients [25, 26]. In Mediterranean areas (such as Spain), allergy to some plant-derived foods (such as peach [25] and kiwi [27]) in non-pollen-allergic patients has been reported in up to

Fig. 4. IgE immunoblot inhibition. Inhibition of IgE binding in watermelon extract after preincubation of pool patient serum with 15 μ g of the different allergens. Pooled patient serum without (lane 1) and with inhibitor (lane 2): inhibited with MDH (**a**); inhibited with TPI (**b**); inhibited with profilin (**c**).

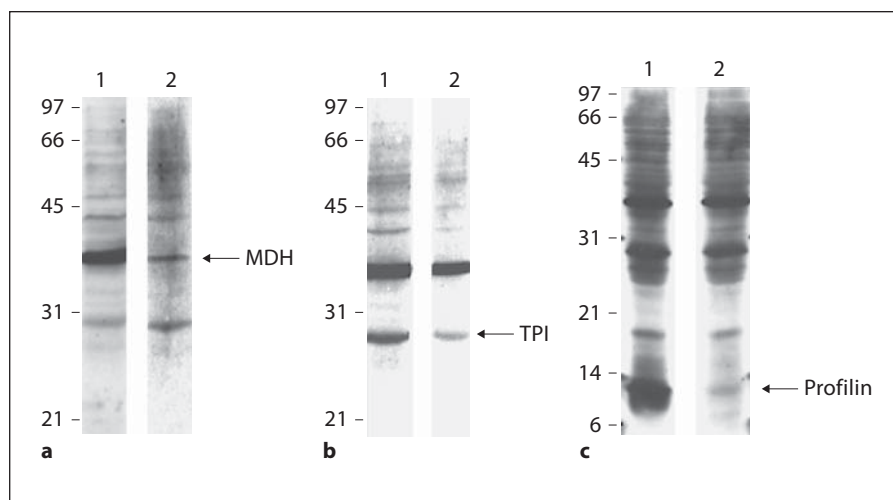
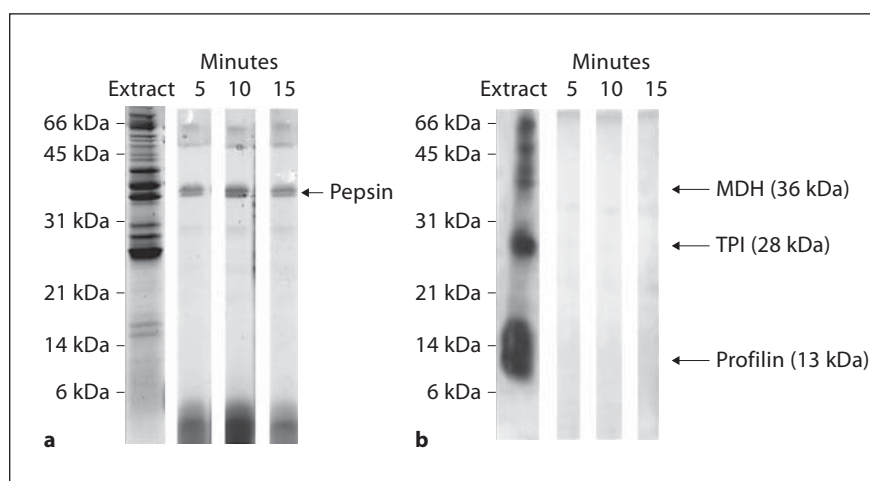


Fig. 5. Effect of pepsin on watermelon protein extract. **a** Coomassie staining. **b** Immunoblotting with pooled sera from patients with watermelon allergy.



20–30% of individuals, and this subgroup of patients (non-pollen-allergic patients) is prone to suffer systemic reactions [25, 27]. Results from this study, in which watermelon-allergic patients were evaluated, showed that watermelon allergy happened among pollen-allergic patients (100%), and in accordance with the study published by Asero et al. [11], oral allergy symptoms was the main manifestation of watermelon allergy. Although the number of patients evaluated in this report did not allow us to exclude the existence of infrequent systemic reactions, we have never found systemic reactions as a manifestation of watermelon allergy. In this sense, it is tempting to speculate that mild allergy symptoms (oral allergy symptoms) in watermelon allergy could be linked to the fact that watermelon allergens are quickly degraded in the gastrointestinal tract after ingestion (fig. 5). It would be interesting to evaluate in future reports whether the

link between pollen and watermelon allergy was due to cross-reactivity or to an additional expression of atopy (cosensitization). At molecular level, we identified for the first time 3 major allergens in watermelon extract: TPI, MDH and profilin. These 3 proteins have been previously isolated and characterized as allergens from other sources. MDH, which was recognized in 96% of the patients, has been described for the first time as an allergen in plant-derived food, since it was previously only reported as an allergen in yeast [28]. TPI, which was recognized in 78% of the patients, has been reported as an allergen in latex [29], wheat protein [30] and lately also in lychee [31]. Finally, profilin was identified as the third major allergen in watermelon which was recognized in 56% of the patients. Watermelon profilin was cloned and sequenced by our group (data not shown), and included in the databank (accession No. GI:52352488). Profilin, a

ubiquitous panallergen, is a conserved protein in eukaryotes and is known to be responsible for many of the cross-reactivities between pollen and plant-derived food extracts [32, 33]. However, despite high sequence similarity among profilins, it has been reported that the fine specificity of IgE directed to variable epitopes may influence the clinical manifestation of profilin sensitization [34, 35].

TPI, MDH and profilin, described as major allergens in this work, are highly conserved in plant-derived foods, with homologies that range from 85% (soybean) to 66% (cucumber) for MDH, and are 89% (peach) and 88% (melon) for profilin – the latter of both described as allergens. We could not identify watermelon TPI directly from the peptides sequence, because watermelon TPI has not been previously sequenced. However, homologies between TPI

from other plant-derived foods are very high. In the future, the cloning of DNA will allow us to know the sequence of this protein and to evaluate homologies with TPI from other plant-derived foods. Although the study was not designed to evaluate allergy to lychee or wheat, we reviewed the clinical history of the patients, and none of them reported symptoms to these foods.

In summary, we have identified MDH, TPI and profilin as major allergens involved in watermelon allergy.

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