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Molecular Cloning, Characterization, and Expression of Cuc m 2, a Major Allergen in *Cucumis melo*

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Abstract

Background: Several studies reported the clinical features of IgE-mediated hypersensitivity after ingestion of melon. Melon allergy is a common IgE-mediated fruit allergy in Iran. This prompted us to investigate immunochemical and molecular properties of the major allergen in melon fruit, to compare the IgE-binding capacity of the natural protein with the recombinant allergen, and to determine cross-reactivity of the major allergen with closely-related allergens from other plants displaying clinical cross-reactivity with melon.

Methods: Identification and molecular characterization of the major melon allergen were performed using IgE immunoblotting, allergen-specific ELISA, affinity-based purifications, cross-inhibition assays, cloning, and expression of the allergen in *Escherichia coli.*

Results: Melon profilin was identified and isolated as a major IgE-binding component and designated as Cuc m 2. Sequencing corresponding cDNA revealed an open reading frame of 363 bp coding for 131 amino acid residues and two fragments of 171 bp and 383 bps for the 5'and 3' UTRs, respectively. Significant cross-reactivity was found between melon profilin and *Cynodon dactylon*, tomato, peach, and grape profilins in cross-inhibition assays. Although the highest degree of amino acid identity was revealed with watermelon profilin, there was no significant cross-reactivity between melon and watermelon profilins.

Conclusion: Melon profilin is the major IgE-binding component in melon extract, and the recombinant and natural forms exhibited similar IgE-binding capacities. A part of the fruit-fruit and pollen-fruit cross-reactions could be explained by the presence of this conserved protein; however, sequence homology provides insufficient information to predict IgE cross-reactivity of profilins.

Keywords: Cross-reactivity, Fruit allergy, Melon, Profilin, Recombinant allergen

Introduction

Allergy to melon (*Cucumis melo*) is one of the most frequent fruit allergies in Iran (1). Melon hypersensitivity has been confirmed by means of double-blind, placebo-controlled food challenges (DBPCFCs) (2). Oral allergy syndrome (OAS) is the most common clinical feature associated with melon allergy and rarely affects other organs (3). IgE-mediated reaction to melon has been reported mainly in patients with allergies to pollens (4).

Pollen-food allergy syndromes, inducing oral symptoms, are mostly due to the cross-reactivity of IgE antibodies to ubiquitous cross-reactive plant allergens, particularly Bet v 1 homologous allergens (5), and the actin-binding protein profilin (6). Profilin could also play an important role in the patient-allergic reaction to different allergic sources, because it is shared by a high number of animal and plant species, mainly in pollens and foods (7). In addition, it is well-

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established that IgE of food- and tree pollen-hypersensitive individuals cross-react with Bet v 2 homologous proteins (profilin) from apple, pear, carrot, celery, and potato (8). According to the published data (9), IgE of ragweed pollen-allergic individuals cross-reacted with Cucurbitaceous family members including watermelon, melon, and cucumber, but potentially cross-reactive allergens have not been clearly identified. Recently, profilin was identified as a cross-reacting melon allergen recognized by IgE antibodies of patients with allergies to zucchini, cucumber, and watermelon (10, 11). In addition to profilin, a 67 kDa subtilisin-like protease and a 16 kDa pathogenesis-related protein belonging to the PR-1 family were also reported as allergic components of melon (12, 13). In the present study, we investigated immunochemical and molecular properties of protein allergens, especially profilin, in *Cucumis melo var. reticulatus*. We present the complete coding sequence of melon profilin. The respective recombinant protein was expressed in *Escherichia coli (E. coli)* and the allergic features of this protein were investigated in detail in comparison with its natural counterpart. Furthermore, we studied cross-reactions between melon profilin and other plant profilins.

Material and Methods

Patient's sera

Patients who complained of clinical symptoms after ingestion of melon were recruited at the Department of Immunology and Allergy of Ghaem Hospital Mashhad, Iran. Twenty-two subjects (8 male, 14 female; mean age 33±8 years) with positive clinical histories of immediate-type reactions to melon fruit were included in the study. The diagnoses were confirmed by positive skin tests with melon extract. Each subject responded to a standardized questionnaire and donated a blood sample for further immunological studies after giving informed consent according to our institution's internal review board approval. Control subjects (*N=15*) with no histories of allergic diseases and negative skin prick tests to melon were also selected.

Skin prick test

The skin prick tests (SPTs) were performed according to the guidelines of the Subcommittee on Skin Tests of the European Academy of Allergology and Clinical Immunology (14). The SPTs were performed on the volar side of the forearm using melon extracts prepared from different parts of melon (as described below) at protein concentrations of 0.001, 0.01, 0.1 and 1 mg/ml. Diluent (50% glycerol in the extraction buffer) and histamine dihydrochloride solution at 10 mg/ml were used as negative and positive controls, respectively. The allergen concentration eliciting a wheal equal in size to a wheal produced by positive controls was defined as 1 unit. We used these allergen concentrations to perform SPTs for each allergen. A mean wheal area of more than 3 mm that developed within 15 min after puncture was considered as a positive response.

Fruit protein extraction crude extracts

After washing the fruits, the seeds were aseptically removed and then the peel, pulp, and the loose layer of melon that covered the inner part of the pulp were isolated. These were homogenized in a blender and then extracted in 100 mM phosphate buffer, pH 8.2 (1/10w/v) containing 2%(w/v) polyvinyl polypyrrolidine (PVP), 10 mM ethylenediaminetetraacetic acid (EDTA). Diethyldithiocarbamate (DIECA) was added to 10 mM to prevent degradation of allergens, and thiomersal 0.02% (w/v) was added as a preservative. The slurry was centrifuged at 15,000 x g for 30 min at 4 °C and the supernatant was extensively dialyzed against 100 mM phosphate buffer, pH 7.4 at 4 °C for 72 h and freeze-dried (12B). Aliquots of the lyophilized samples were reconstituted in distilled water and glycerinated for skin testing. Watermelon, cantaloupe, peach, tomato, and grape extracts were prepared as described for the melon extract. Allergen

extract of banana was prepared as described previously by Moller et al (15).

Allergen-enriched preparation from inner layer of melon

The inner layer extract was fractionated in the ranges of 0% to 30% (I), 30% to 60% (II), and 60% to 90% (III) saturation in $(NH_4)_2SO$. After being dissolved in phosphate-buffered saline (PBS) each precipitated fraction was dialyzed separately (molecular weight cut-off limit 6 kDa). The allergenic activities of the fractions were determined by enzyme linked immunosorbent assay (ELISA). The most active fraction was fraction II. This fraction was collected and used for measurement of specific IgE against the melon inner layer by ELISA. The protein concentrations in both the crude and allergen-enriched preparations were determined using the Bradford method (16).

Pollen protein extracts

Pollens from *Cynodon dactylon* and *Poa pratensis* were obtained from commercially available pollens (Sigma, St. Louis, MO, USA). The extracts were prepared as described previously (17). Pollens were extracted by stirring for 1 h at 4 ℃ with 50 mM ammonium bicarbonate, pH 8.5, at a 1/10 (w/v) ratio containing 1 mM phenylmethylsulfonyl fluoride. Soluble components were separated by centrifugation at 22,000 x g for 20 min at 10 ℃. The supernatants were dialyzed against distilled water, filtered through 0.22-µm pore size membranes, and lyophilized. The lyophilized extracts were stored at -20 ℃.

Total and specific IgE

Total IgE: Measurement of total IgE was performed with the IgE total kit (Radim, Roma, Italy) according to the to the manufacturer's protocol.

Measurement of melon allergen–specific IgE in sera: The wells of ELISA assay plates (Nalge Nunc International, Roskilde, Denmark) were coated with 100 µl of allergenic fraction II at a concentration of 100 µg/ml in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) at 4 °C for 16 h. After blocking with 150 µl of 2% bovine serum albumin (BSA) in PBS at 37 °C for 30 min, the plates were incubated with 100 µl of each subject's serum for 3 h at room temperature (RT) followed by incubation with a goat biotinylated anti-human IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1/1,000 in PBS containing 1% BSA for 2 h. The wells were then incubated for 1 h with horseradish peroxidase-labeled streptavidin (Sigma, St. Louis, MO, USA) diluted 1/1,000 in PBS containing 1% BSA. Each incubation step was followed by 5 washes with PBS containing 0.05% Tween 20 (PBS-T). Enzyme reactions were performed using tetramethyl benzidine (TMB)/H_2O_2 as the substrate. The reactions were stopped by adding 50 µl of 3 M HCl after 30 min at RT in the dark. Absorbance was determined at 450 nm with an ELISA reader. Results were expressed as optical density (OD) units. Based on the mean value of 15 control sera (<0.15 OD units), OD values of greater than 0.3 were considered positive.

Measurement of recombinant Cuc m 2-specific IgE in sera: In addition to the immunoblotting, the reactivity of each patient's IgE to recombinant Cuc m 2 (rCuc m 2) was also detected by ELISA. The ELISA was performed as described for measurement of melon allergen-specific IgE except that the wells of the microtiter plates were coated with 100 µl of rCuc m 2 at a concentration of 0.5µg/ml in the carbonate/bicarbonate coating buffer at 4 °C for 16 h.

ELISA Inhibition experiments

To assess relatedness of rCuc m 2 to profilins in other fruits and pollens, ELISA inhibition was performed as follows: 100 µl of pooled sera from five from subjects showing IgE antibodies to rCuc m 2 were pre-incubated with 100 µl of different concentrations of extracts from pollens, fruits, purified rCuc m 2, and BSA by shaking for 2 hours at RT. The mixtures consisting of the sera and inhibitor or sera with BSA were then added to flat-bottomed microtiter plates that had been coated with rCuc m 2 (0.5 µg/ml). The ELISA

procedure thereafter was the same as described for measurement of melon allergen-specific IgE.

Polyacrylamide Gel Electrophoresis and immunoblotting analysis

Analytic electrophoresis was performed according to Laemmli in 15% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (18). Protein bands were visualized by Coomassie brilliant blue and silver staining. The molecular masses of protein bands were estimated with Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, Rochester, NY, USA) by comparison with protein markers of known molecular weights (LMW Electrophoresis Calibration Kit, Pharmacia Biotech, Uppsala, Sweden). Proteins were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA, U.S.A.), essentially by the method of Towbin *et al* (19). Immunodetection was carried out on PVDF after treatment with methanol for 15 sec and blocking with Superblock at 4 °C for 16 h. Membranes were probed with individual sera from melon-allergic subjects (diluted 1/5 in PBS containing 1/10 v/v blocking buffer) or with sera from non-allergic subjects for 4 h, or overnight for IgE immunoblot of total extract, at RT. Membranes were then washed 4 times for 5 min with PBS-T and incubated for 2 h with a goat biotinylated anti-human IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1/1,000 in PBS containing blocking buffer (1/10 v/v). After washing, blots were incubated for 1 h with horseradish peroxidase-labeled streptavidin diluted 1/40,000 (Sigma, St. Louis, MO, USA). The peroxidase reaction was developed with Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, Il, USA) for 5 min, and IgE-binding proteins were detected by ECL-hyperfilm (Amersham Pharmacia Biotech, Uppsala, Sweden) after exposure for 5 min. Alternatively, replica-blocked membranes were incubated for 2 h with rabbit antisera against Cuc m 2 (1/500 dilution) or saffron pollen profilin (1/1,000, kindly provide by F. Shirazi, Bu-ali research institute, Mashhad, Iran) to detect profilin in fruit extracts, purified natural, and recombinant Cuc m 2. Membranes were then treated with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) at 1/20,000 for 1 h. The peroxidase reaction was developed with Super Signal West Pico Chemiluminescence substrate as described above.

Purification of natural profilin from melon

The lyophilized allergen-enriched extract from the melon inner layer was re-dissolved in 0.02 M sodium acetate pH 4.6 and subjected to cation exchange chromatography on a MonoS HR5/5 column of a FPLC system (Pharmacia Biotech, Uppsala, Sweden). Elution was performed with 0.1, 0.3, and 0.5 M NaCl in the 0.02 M acetate buffer pH 4.6. The absorbance at 280 nm was measured. After SDS-PAGE, the fractions containing protein bands in the 14-16 kDa range were pooled, dialyzed, and loaded on a poly-L-proline (PLP) -Sepharose 4B column. The PLP was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The column was washed with 10 volumes of PBS and then washed with 4 volumes of 2 moles/L urea in PBS. Finally, the profilin was eluted with PBS containing 6 M urea (20). The protein-containing fractions were immediately desalted on a 25 G sephadex column. Fractions were lyophilized and re-dissolved in PBS. Proteins were analyzed by reducing SDS-PAGE according to Laemmli (18). Afterwards, immunostaining was performed as described using rabbit antisera against saffron pollen profilin.

Isolation of RNA, first–strand cDNA synthesis, and amplification of melon profilin cDNA by Polymerase chain reaction

Total RNA was extracted by means of the guanidine thiocyanate extraction method (21) from 1 g of fine powder of melon inner layer

that had been pulverized under liquid nitrogen.

First-strand cDNA was synthesized from 2 µg of total RNA using a first-strand cDNA synthesis Kit (Fermentas, Lithuania) with an oligo (dT)18 as primer. For cDNA amplification, two degenerate oligo nucleotide primers: Pro1, 5'-ATGTCGTGGCARRCBTAYGT-3' and Pro2, 5'-ATRADCCYTGNTCDATNAGRTART-3' were designed according to the known N- and C-terminal amino acid sequences of different plant profilins. After amplification of profilin cDNA, PCR products were cleaned using a Gene Clean II kit (Q–Biogen, Illkirch, France) and sub-cloned into the vector pTZ57R/T with the TA cloning kit (Fermentas, Lithuania). Clones carrying inserts were characterized by restriction analysis and sequenced (MWG Biotech AG, Ebersberg, Germany). Homology searches were performed using BLASTN for gene sequence alignment.

Analysis of predicted protein sequence

The predicted molecular mass and isoelectric point were determined by the Gene Runner program v 3.05 (Hastings software). The deduced protein sequence of Cuc m 2 was next subjected to a BLAST similarity search (22). Multiple sequence alignment was performed by BioEdit software (23). The deduced amino acid sequences of profilins were obtained from the NCBI Protein Database.

5′ and 3′ RACE (rapid amplification of cDNA end)

The unknown 3' and 5' ends of melon profilin cDNA were amplified using 3'- and 5'-RACE methods according to the manufacturer's instructions (GeneRacer kit, Invitrogen, San Diego, CA, USA). Briefly, GeneRacerTM Oligo dT was ligated using SuperscriptTM II RT and GeneRacer Oligo dT primer in first-strand cDNA synthesis. A GeneRacerTM RNA oligo-tail was ligated to the 5' end of the purified cDNA. These reactions created first-strand cDNA with known priming sites at the 5' and 3' ends. Then, the 5' UTR of the cDNA was amplified with a GeneRace 5' primer and a gene-specific primer (5'-UTR)
5'-GTCTTGACCGATAATGGCCGCAG-3'
which were designed according to the results obtained from sequencing of melon profilin cDNA. The 3' end of the cDNA was amplified by PCR using a gene-specific primer (Pro 1) and a GeneRaceTM 3' primer. After amplification of 5' and 3' end cDNAs, the PCR products were cleaned using Gene Clean II kitTM (Q-Biogen, Illkirch, France) and sub-cloned into the vector pTZ57R/T with the TA cloning kit (Fermentas, Lithuania). Clones carrying inserts were characterized by restriction analysis and sequenced**.** All the amplifications for T/A cloning were performed with Pfu DNA polymerase and addition of the A-tail with Taq polymerase.

Expression and purification of recombinant melon profilin

The Cuc m 2 coding region was amplified with Pfu DNA polymerase (Fermentas, Lithuania). The 5' primer (5'-TCACATATGTCGTGGCAAGTTTACGTC G-3') mimics the first six codons and introduces an *NdeI* restriction site (underlined). The 3' primer (5'-AAGCTCGAGGCCCTGATCAATAAGAT AATC-3') mimics the last seven codons, excluding the stop translation codon, and introduces an *Xho I* restriction site (underlined). After PCR amplification, the 400-bp product was ligated into pET21b+ (Novagen, Madison WI, USA). The fidelity of the cloned product was verified by sequencing. The resulting pET21b+Cuc m 2 construct was transformed into the BL21 (DE3) strain of *E coli*. *E. coli* cells carrying pET21b+Cuc m 2 were cultured in 5 ml of lysogeny broth (LB) medium containing 100 µg/ml ampicillin with shaking at 37 °C overnight. The preculture (1 ml) was inoculated into 100 ml of LB medium containing 100 µg/ml ampicillin in a 250 ml Erlenmeyer flask and incubated with shaking at 37 °C. After reaching an absorbance of 0.5-0.6 OD, isopropyl-1-thio-ß-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM. After 6 h of growth

at 30 °C, the cell pellet was collected by centrifugation at4000 g for 20 min) and dissolved in 30 ml of binding buffer (10 mM imidazole, 0.4 M NaCl, 0.1 M KCl, 10% glycerol, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.6). Suspended bacteria were disrupted with three freeze-thaw cycles in liquid nitrogen. Insoluble material was removed by centrifugation (10,000 g, 30 min). The supernatant of the disrupted cells was bound to 5 ml of Ni-NTA superflow resin (Invitrogen) in a falcon tube for 1 h at RT with gentle shaking. The resin was loaded into a column for purification. Then, the column was washed with 10 volumes of binding buffer and 5 volumes of washing buffer (20 mM imidazole, 0.4 M NaCl, 10% glycerol, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.6). The (His)6-tagged recombinant Cuc m 2 was eluted with 5 volumes of elution buffer (200 mM imidazole, 0.4 M NaCl, 0.1 KCl, 10% glycerol , pH 7.6). The purified protein was extensively dialyzed against phosphate buffer at 4 °C for 72 h, subjected to reducing SDS-PAGE, and electroblotted onto PVDF membrane. Immunodetection was carried out as described above, using sera from 22 melon-allergic subjects with melon extract-positive skin prick tests and pooled sera from three control subjects.

Production of polyclonal Antibody against rCuc m 2 in rabbits and immunoaffinity purification of nCuc m 2

The immunization schedule and methods of immunization via the multiple-injection technique were performed according to the guidelines of Babiker, et al (24). Two rabbits were injected with recombinant melon profilin (1 mg/ml) in 0.01 M phosphate buffer (pH 7.5) containing 0.85% NaCl (PBS) emulsified with 1 ml of Freund's complete adjuvant. First booster injection of immunogen (1.0 mg/ml) in PBS and 1.0 ml of Freund's incomplete adjuvant were used for each rabbit. The antisera were collected and stored at -20 °C. The natural Cuc m 2 was purified by immunoaffinity chromatography. In the first step, rabbit antibody was purified from the antisera by affinity chromatography on a protein A column. The purified antibody was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to manufacturer's instructions. The freeze-dried allergen-enriched melon extract was dissolved in PBS (1/10, w/v) and applied onto an immunoaffinity column. The column was washed with PBS until the absorbance of the 280 nm flow-through was less than 0.01. The bound fraction was eluted with 0.1 M glycine-HCl pH 2.8 and fractions were immediately neutralized with a 10% volume of 1 M Tris, pH 8.0. Protein-containing fractions were pooled, dialyzed against PBS, and stored at -20 °C until use. In addition, this protein solution was analyzed by reducing SDS-PAGE according to Laemmli (18). Immunoblotting was performed with the Cuc m 2 allergic-patients' sera and rabbit anti-saffron pollen profilin.

Results

Clinical data, specific IgE levels and SPT responses

Twenty-two subjects suffering from melon allergy were included in our study. A summary of their clinical histories and the results of skin prick tests and *in vitro* assays are presented in Table I. The clinical histories of the subjects included the following manifestations upon ingestion of melon: OAS (95%), rhinoconjunctivitis (68%), rhinitis (22%), urticaria (18%), gastrointestinal symptoms such as nausea, stomach pain, vomiting, and diarrhea (9%), skin itching (45%), and dyspenia (13%). Skin prick tests with the extract of melon inner layer were positive in 22 out of 37of subjects. Skin testing with the extracts of melon peel and pulp were positive in one and three subjects, respectively. Three subjects were skin prick test positive to both the pulp and inner layer. According to the questionnaire, melon-allergic subjects also complained of allergic reactions to grape (50%), tomato (25%), zucchini (20%), cantaloupe (8%), watermelon (8%), and kiwi (12%). The subjects with

melon allergy showed significantly higher ($P<0.05$) total IgE values (*N=22*, median 411±223 IU/ml) than the non-atopic control subjects (*N=15*, median 60±35 IU/ml). Sera from 18 melon-allergic subjects showed increased IgE reactivity to melon extract and 13 of these sera displayed IgE reactivity to both rCuc m 2 and melon extract in ELISA (Table 1).

Table 1. Clinical data, specific IgE levels, and SPT responses of patients suffering from allergenic reaction after ingestion of melon.

No	*Age (years)*	*Sex*	*Symptoms**	*Allergy to other fruits*	*Melon Specific IgE (OD)*	*rCuc m2 Specific IgE (OD)*	**SPT with melon extracts**		
							Peel	Pulp	Inner
1	29	F	R	Grape, kiwi	0.7	0.6	-	-	5
2	28	M	RC, OAS, SI	Tomato, grape, peach, zucchini, cantaloupe,	0.68	0.95	-	-	8
3	46	M	R, OAS, C, D	ND	0.46	0.55	-	-	5
4	39	F	RC, OAS, U, SI	Grape, tomato	0.4	-	-	-	3
5	30	M	RC, SI, OAS, U	Grape	0.91	0.83	-	5	8
6	27	F	R, OAS	Fig, grape, zucchini	0.4	-	-	-	3
7	34	M	RC, OAS, C	Tomato	0.5	-	-	-	6
8	21	M	R, OAS	Zucchini, grape, watermelon	0.64	-	-	-	8
9	30	F	RC, OAS, C	Kiwi	0.98	1.39	-	-	5
10	44	M	RC, OAS, SI, G	Cantaloupe, kiwi	0.9	1.1	3	6	5
11	43	F	RC, OAS, SI, C	Walnut, spice	0.6	0.75	-	-	8
12	26	F	RC, OAS, SI	Peach, tomato	0.74	0.55	-	-	11
13	39	F	RC, OAS, U, SI, D	Grape, garlic	0.45	-	-	-	10
14	24	F	RC, OAS, SI, C	Grape	ND	ND	-	-	4
15	52	M	RC, OAS, D, U, G	Grape, tomato, zucchini, cantaloupe	0.7	0.65	-	-	12
16	28	F	RC, OAS, SI, C	Tomato	0.62	0.89	-	5	10
17	37	M	RC, OAS	Grape	0.3	-	-	-	3
18	45	F	OAS	Zucchini, watermelon	0.3	-	-	-	4
19	30	F	R, OAS	Grape	0.3	-	-	-	4
20	24	F	RC, OAS	-	1.1	1.43	-	-	5
21	30	F	RC, OAS, SI, C	Tomato, grape	0.72	1.2	-	-	10
22	27	F	R, OAS	Grape	0.64	0.93	-	-	5

**C*, cough; *D*, dyspnea; *R*, rhinitis; *RC*, rhinoconjunctivitis; *G*, *g*astrointestinal symptoms; *SI*, skin itching; *U*, urticaria; *OAS*, oral allergy syndrome (OAS; defined as the onset of immediate oral itching with or without angioedema of the lips and oral mucosa); *ND*, not determined

Identification of IgE-binding melon proteins

Immunoblot analysis of melon extract was carried out with the sera from all the melon-allergic subjects and control sera from three non-allergic subjects. Sera from 13 of 22 allergic subjects recognized a protein band at about 15 kDa. IgE binding to other components of 6, 16, 27-32, and about 58 kDa were also observed in 3, 4, 6, and 11 sera, respectively. No proteins reacted with control serum of non-allergic subjects (Fig. 1).

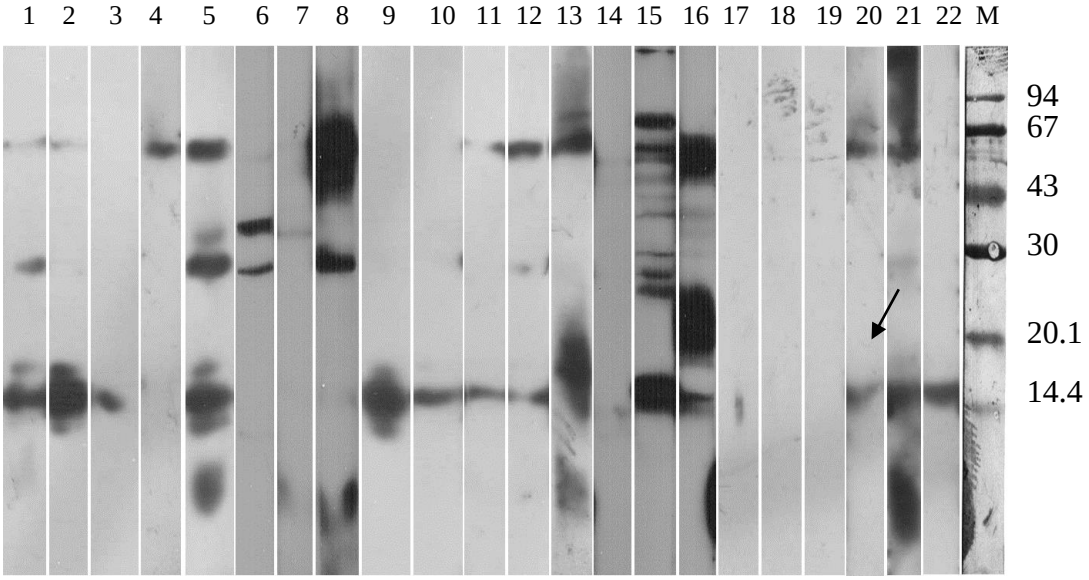

Fig. 1. IgE-immunoblot of melon extract proteins incubated with sera of 22 melon-allergic patients. Melon extract proteins were separated by SDS-PAGE (15%) and electro-transferred onto PVDF membranes; Lane M, molecular weight markers.

Purification of natural melon profilin

By PLP-affinity chromatography, a protein component with an apparent molecular weight of 15 kDa was isolated from the 15 kDa enriched fraction of MonoS HR5/5 column. This band was detected by both silver staining and immunoblotting using rabbit anti-saffron pollen profilin antibody (Fig. 2). The same band was shown in IgE immunostaining with a pool of four sera (serum Nos. 9, 10, 11, and 22) that had IgE binding only to the 15 kDa protein component of melon extract (not shown). Immunoaffinity purification of natural Cuc m 2 with rabbit anti-rCuc m 2 polyclonal antibodies showed two main bands with approximate molecular weights of 15 and 58 kDa by SDS-PAGE analysis and immunoblotting using pooled sera from the subjects whose IgE recognized only the 15 kDa protein component of melon extract (Fig. 2).

Amplification of the melon profilin cDNA and sequence analysis

PCR amplification of the melon cDNA using the degenerate 5' primer (*Pro1)* and the 3'-primer (*Pro2)* resulted in a single 396 bp fragment that was cloned into pTZ57R/T. Four of these clones were sequenced.

Asn116 (NMIV). Amplification of 3' end of the melon profilin cDNA produced a 779 bp product (AY292387) that overlapped with 396 bp of the coding region. Amplification of the 5’ end of the melon profilin cDNA produced a 262 bp product (AY879598) that overlapped with 87 bp of the coding region (Fig. 3). Sequence similarity searches using the protein-protein BLAST program revealed that Cuc m 2 is over 80% identical with other plant profilins (Fig. 4). Pairwise alignments indicated the highest homology (89% identity and 96% similarity) with profilin from

Citrullus lanatus profilin (watermelon), another member of the Cucurbitaceae family. Melon (*Cucumis melo*) profilin was submitted and accepted (Apr 2003) as Cuc m 2 by the International Union of Immunological Society (IUIS) allergen nomenclature subcommittee (http://www.allergen.org).

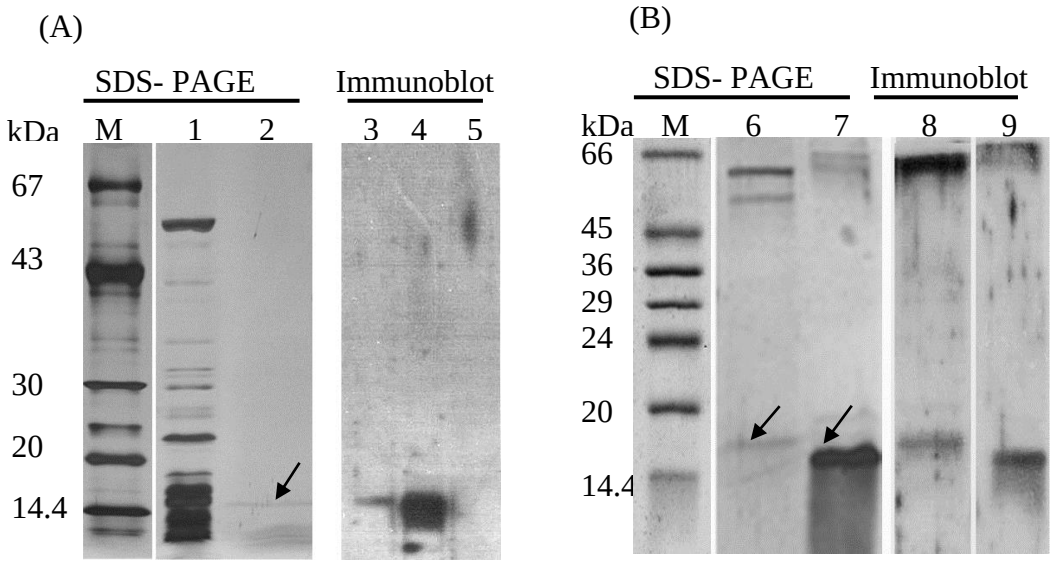

Fig. 2. Purification of natural melon profilin from melon extract. (A) SDS–PAGE and immunoblot analysis of purified nCuc m 2 on PLP-Sepharose column: the 15 kDa enriched fraction of Mono S column (lane 1) and purified nCuc m 2 (lane 2) stained with Silver nitrate; immunoblotting of the fraction of Mono S column (lane 3) and PLP-purified profilin (lane 4) with anti-saffron pollen profilin antibody and non-immunized rabbit serum (lane 5). (B) SDS–PAGE and immunoblot analysis of purified nCuc m 2 on profilin immunoaffinity column; Coomassie blue staining of nCuc m 2 (lane 6) and rCuc m 2 (lane 7); immunoblotting of nCuc m 2 (lane 8) and rCuc m 2 (lane 9) with polyclonal rabbit antibody against saffron pollen profilin. Lane M: Molecular Mass Markers (kDa).

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1   ggacactgacatggactgaaggagtagaaaatagctcatgtggacagaaatcaaaacttcaatcccaacc
71  cattaaacgattgacggaaagaaaaaagaaaaaaaaaaccaaaaccctagaagagatttgagcctgaag
141 aagaaacaagaaggaagaggaagaagccgggaaagatgtcgtggcaagtttacgtcgatgaacatttgat
                                    M  S  W  Q  V  Y  V  D  E  H  L  M

211 gtgcgagattgagggcaatcacctcacttctgcggccattatcggtcaagacggcagcgtttgggctcaa
      C  E  I  E  G  N  H  L  T  S  A  A  I  I  G  Q  D  G  S  V  W  A  Q

281 agccaaaatttccctcagctcaagcctgaagaagttgctggcatcgtgggggacctcgcggaccctggga
     S  Q  N  F  P  Q  L  K  P  E  E  V  A  G  I  V  G  D  L  A  D  P  G

351 cgcttgctccaactggtttgtacattggtggtacaaaatatatggtaatccaaggggagcctggagctgt
    T  L  A  P  T  G  L  Y  I  G  G  T  K  Y  M  V  I  Q  G  E  P  G  A  V

421 tattcgtgggaagaagggcccaggtggggttactgttaagaagactggtatggctttagtcattggtatc
      I  R  G  K  K  G  P  G  G  V  T  V  K  K  T  G  M  A  L  V  I  G  I

491 tatgatgaaccaatgactcctggtcaatgcaatatgattgttgaaaggcttggggattatcttattgatc
     Y  D  E  P  M  T  P  G  Q  C  N  M  I  V  E  R  L  G  D  Y  L  I  D

561 agggcctctaaatatcgggaagccatgtattgtttgtacaagtctatggactcttgaatggaaatgggtt
    Q  G  L  *
631 tgatgcttgaggagtgatgatgcttatgtttctcgttattagtgttgtctctttcacttttttttctattt
701 tttccctttgaaatattgggggtggttttcaagtgaggtccatgttgtagcatcaaatatggatatggta
771 aaccaatcagcataaatttataattactcttctctatttttatctgttgatttttgcaccgggctcctgta
841 taacatgagcagttatagtctctcgtaacccaaatgtggggatgtttggaaatttagtcgatcttgttca
911 acataagttccttttctttttctcctcaaaaaaaaaaaaaaaaaa
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Fig. 3. Nucleotide sequence of complete Cuc m 2 cDNA and its deduced amino acid sequence. The estimated molecular mass is 13.94 kDa, and no potential N-linked glycosylation sites were identified. Asterisk indicates the stop codon.

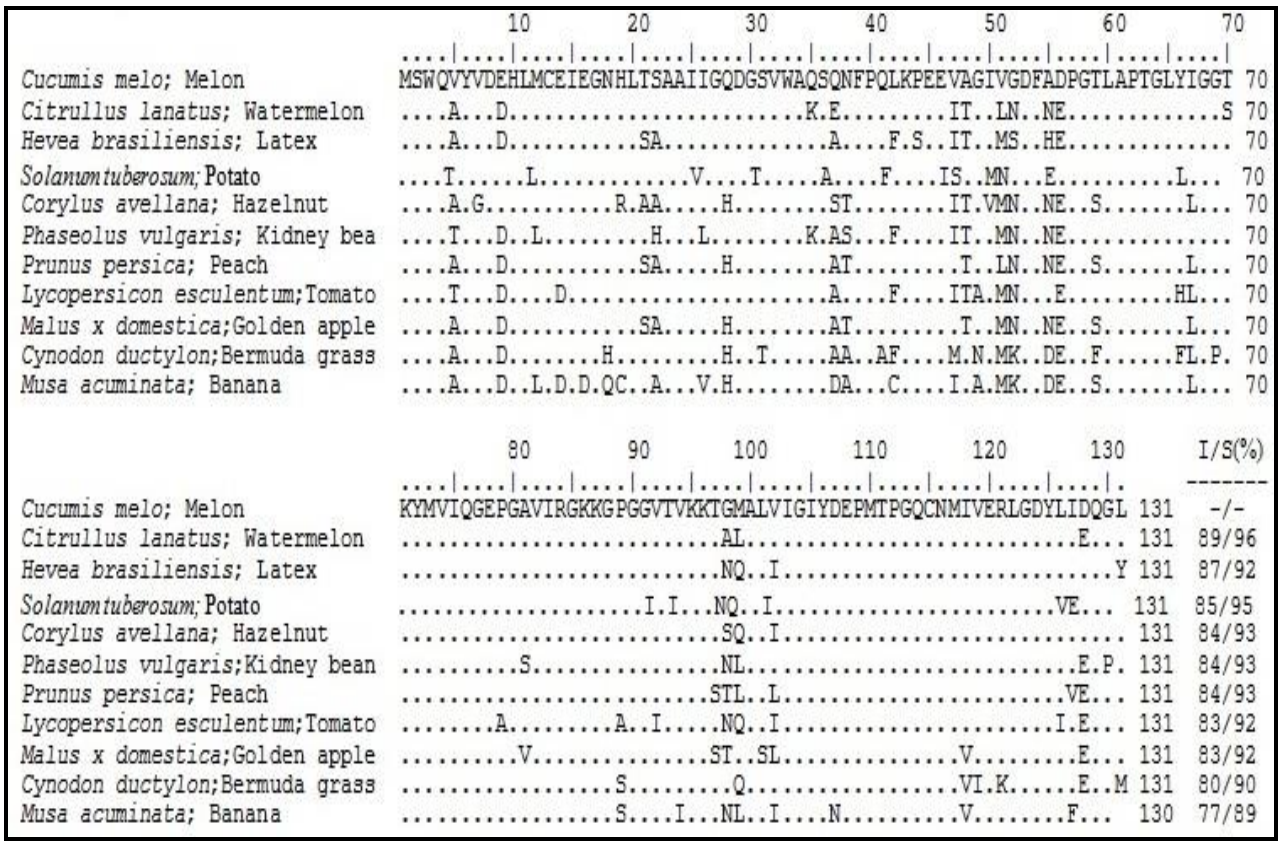

Fig. 4. Comparison of amino acid sequence deduced from Cuc m 2 cDNA (AY271295) with other plant profilins from *Citrullus lanatus* (AAU43733.1), *Hevea brasiliensis* (CAB51914.1), *Solanum tuberosum* (ABB16985.1), *Corylus avellana* (AAK01236.1), *Phaseolus vulgaris* (CAA57508.1), *Prunus persica* (CAD37202.1), *Lycopersicon esculentum* (CAD10377.1), *Cynodon dactylon* (CAA69670.1), and *Musa acuminata* (AAK54834.1), *Malus x domestica* (CAD46560.1). Profilin sources indicated at the right. I/S: Identity/Similarity.

Expression and purification of Cuc m 2 protein: The coding sequence of Cuc m 2 cDNA (AY271295) was cloned into pET 21b+. The C-terminal His-tagged protein was expressed in *E. coli* BL21-DE3 cells. Recombinant proteins were in insoluble inclusion bodies, which were made soluble by changing the incubation temperature from 37 to 28 °C. The yield of Cuc m 2 produced using the described conditions (cell density of 0.6, 0.4 mM IPTG, and overnight expression) was 40-50% of the total protein. The soluble material was purified by Ni-NTA affinity chromatography. After elution with a lysis buffer containing 200 mM imidazole, Cuc m 2 showed a single band with an apparent molecular mass of 14.7 kDa (Fig. 5). The yield of purified rCuc m 2 was about 50 mg/L of the bacterial culture. Purity of more than 98% was achieved as documented using the SDS-PAGE gels.

Immunological characterization of recombinant Cuc m 2: Purified rCuc m 2 and natural Cuc m 2 were analyzed by immunological assays. Recombinant and natural melon profilin were recognized by rabbit antiserum obtained against saffron pollen profilin in immunostaining (Fig. 2). Specific IgE binding to rCuc m 2 was also demonstrated by ELISA and immunoblotting. Sera from 13 of 22 subjects (59%) showed IgE reactivity to rCuc m 2 in both ELISA and immunoblot analyses (Table 1 and Fig. 6) and to a 15 kDa protein band in melon extract by immunoblotting (Fig. 1). rCuc m 2 was more convenient and provided more reproducible results than melon extracts in immunoblotting analyses and ELISAs.

Inhibition experiments

Inhibition of IgE binding to rCuc m 2 by nCuc m2, rCuc m 2, and extracts of pollens, peach, tomato, grape,banana, watermelon, cantaloupe, and melon are represented in Fig. 8. ELISA inhibition assays showed complete inhibition of IgE reactivity to the solid phase rCuc m 2 after preincubation with rCuc m 2 and nCuc m 2. Extracts of *Cynodon dactylon* pollen, melon, peach, tomato, and grape significantly inhibited specific IgE binding to rCuc m 2 (Fig. 7). Surprisingly, melon extract reduced IgE binding to the solid phase rCuc m 2 less than peach, tomato, *Cynodon dactylon* pollen, or grape extracts. Preincubation of the pooled serum with *Poa pratensis*, cantaloupe, watermelon, and banana resulted in low inhibition of rCuc m 2-specific IgE.

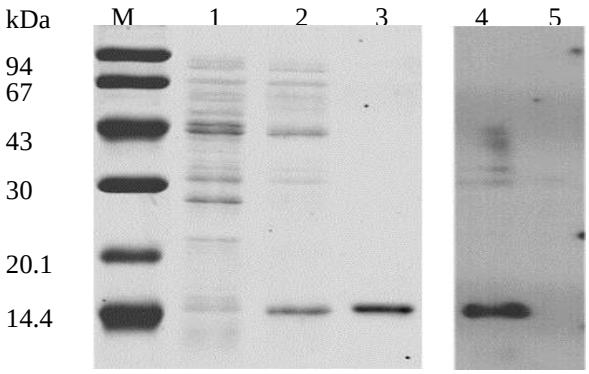

Fig. 5. SDS-PAGE and immunoblotting of Cuc m 2 expressed in *E. coli*. (A) Non-induced bacteria (lane 1), Total protein extracts of the transformed *E. coli* culture induced with 0.4 mM IPTG (lane 2), metal affinity-purified rCuc m 2 (lane 3) and Molecular mass markers (M). (B) Immunoblotting of purified rCuc m2, melon extract (lane 4), and *E. coli* containing no plasmid (lane 5), using polyclonal rabbit antibody against saffron pollen profilin.

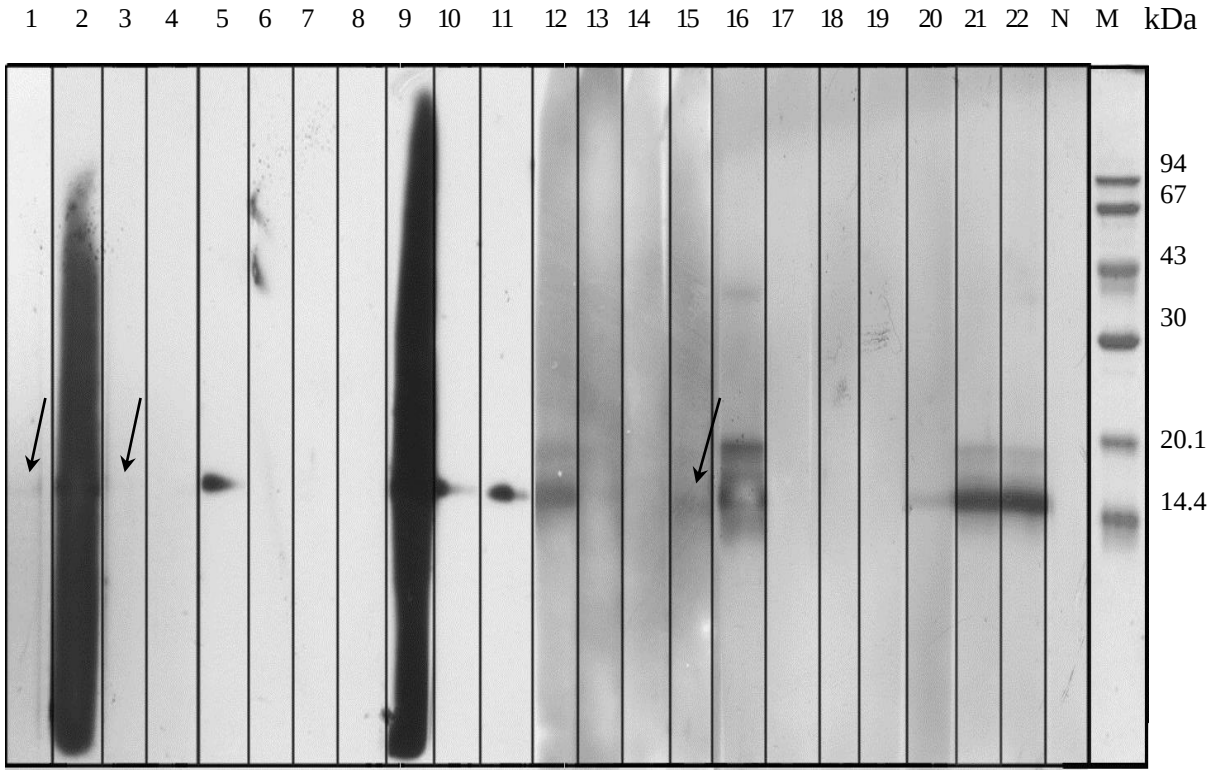

Fig. 6. Immunoblotting of recombinant Cuc m 2 with sera from 22 melon-allergic subjects with melon extract-positive skin prick tests (1-22), Lane N indicates reactivity of a pooled serum from three non-allergic control subjects. Lane M: Molecular Mass Markers.

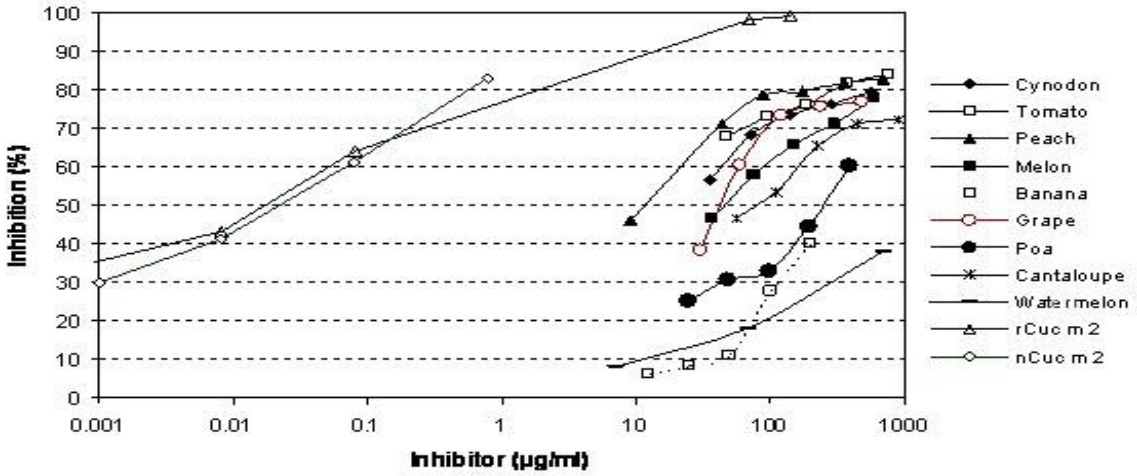

Fig. 7. Inhibition of binding of IgE antibodies in sensitized serum to immobilized rCuc m 2. Inhibition was assayed by a competitive ELISA method. The pooled serum (1:5 dilution) was pre-incubated for 1 h with an equal volume of various concentrations of each extract, nCuc m 2, rCuc m 2, and BSA before addition to the plate coated with rCuc m 2 (0.5 µg/ml). Sample concentrations are expressed as those in the preincubation mixture.

Discussion

Several studies reported the clinical features of IgE-mediated hypersensitivity after ingestion of melon (1, 2, 25). Immunoblot, ELISA, and radioallergosorbent test (RAST) inhibition experiments have confirmed the occurrence of melon allergy mainly by means of conservative cross-reactive allergens (4, 26). Recently, a 13 kDa protein component has been identified as an allergen in melon extract that showed immunoreactivity with rabbit antiserum against sunflower pollen profilin. This finding was confirmed by immunoblotting inhibition with Bet v 2 (birch profilin) (10). Melon allergy is a common IgE-mediated fruit allergy in Iran (1). Sequence analysis revealed that our PCR products correspond to a 393-bp open reading frame (AY271295) that encodes Cuc m 2, a 131-amino-acid polypeptide with an average molecular mass of 13.94 kDa and a theoretical isoelectric point value of 4.46 (Fig. 3). There are no potential N-glycosylation (N-X-S/T) sites in the predicted amino acid sequence but the NetNglyc program predicts two N-glycosylation sites at Asn18 (NHLT). Using skin prick tests and melon specific-IgE immunoassays, we have confirmed that allergy to melon is IgE-mediated. This study has revealed for the first time that the part of the melon located between the pulp and seeds is more allergenic than the pulp. Furthermore, removing the inner part of the melon significantly reduced the allergenicity of the fruit for most of the melon-allergic subjects. Localization of allergens also has been reported in Rosaceae fruits in which allergens accumulate in the peel, and up to 40% of patients in the Rosaceae fruit-allergic population tolerated the ingestion of pulp but not the whole fruit (27). Consistent with other studies, oral allergy syndrome (OAS) was the most common clinical feature associated with melon allergy in this study, but another common manifestation in these subjects was rhinoconjunctivitis (68%). This result is significantly higher than was observed in another study (2). Immunoblot analysis of allergenic melon extract fraction indicated a 15 kDa IgE-binding component in more than 50% of the melon-allergic subjects' sera. In addition, a 15 kDa protein component was purified by PLP that showed reactivity with rabbit anti-saffron pollen profilin and pooled sera from melon-allergic subjects. Presence of a 15 kDa allergen with reactivity to PLP and rabbit anti-saffron profilin antibody and oral allergy syndrome after consumption of melon

in subjects with pollinosis leads us to conclude that profilin is the most common cause of allergenic reactions to melon in our study. Recently, profilin was identified as a cross-reacting melon allergen recognized by IgE antibodies of patients with allergies to zucchini, cucumber, and watermelon (10, 11).

Attempts to purify natural profilin from fruit and vegetables have not been successful, probably due to low levels of the allergen in the crude extracts, as well as its tendency to associate with other components (10). We overcame this problem by using the enriched extract from the inner part of melon, which is the most allergenic part as shown by our SPT results. However, the Immunoaffinity method was more efficient than PLP chromatography in purification of melon profilin. The fact that most of the available profilin in *Phaseolus vulgaris* is highly phosphorylated, which has lesser affinity for PLP than the unphosphorylated form (29), suggests that melon profilin tyrosine phosphorylation may be involved in inhibiting the interactions between melon profilin and PLP. Melon profilin shows a high degree of sequence homology with *Phaseolus vulgaris* profilin (84% identity and 93% similarity).

Rodriguez-Perez et al also failed to isolate melon profilin by means of subjecting the melon extract (or gel-filtration fractions derived from it) to either chromatographic (affinity chromatography on PLP columns or reverse-phase HPLC) or semi-preparative electrophoretic fractionation in their first attempt (10). However, they isolated a putative profilin from melon pulp PBS extract by affinity chromatography on a PLP-Sepharose column in their next study without further explanation. In addition to the 15.5 kDa band, we obtained a 56 kDa protein in immunoaffinity purification that could be a tetrameric form of natural melon profilin. Immunoblot analysis of immunoaffinity-purified melon profilin revealed a ~31 kDa IgE-binding component that could be a dimeric form of nCuc m 2. The possibility of formation of profilin tetramers and dimers, either under physiologic conditions or as a result of food processing conditions, might enhance the immunogenic properties of this molecule. Psaradellis et al also reported a predominant band for monomeric ZmPRO1 profilin (approximately 14.8 kDa) and a higher molecular weight protein (60 kDa) that was resistant to reducing agents (30). In the next step, melon profilin cDNA was cloned, sequenced, and expressed in *Escherichia coli*. The coding region of the cDNA of melon profilin revealed a 393 open reading frame, coding for a 131 amino acid length protein with a predicted pI of 4.46 and apparent molecular weight of 13.95 kDa. Despite the expression of rCuc m 2 with six additional C-terminal histidine residues, the purified recombinant Cuc m 2 revealed an apparent molecular weight slightly less than the purified natural Cuc m 2 (14.7 kDa and 15-16 kDa, respectively). This may be due to post-translational modifications, such as phosphorylation and/or amidation of the Cuc m 2 protein. There are also two putative N-glycosylation sites in the amino acid sequence of Cuc m 2 based on NetNglyc program prediction; however, there has been no experimental evidence for glycosylation of plant profilin. Thirteen sera showed reactivity with melon extract as well as rCuc m 2, but nine sera indicated no reactivity with rCuc m 2 in ELISA, despite their reactivity with melon extract. This may be due to the presence of other IgE-binding components that have been detected by immunoblotting analysis of melon extract. In addition to profilin, a 67 kDa subtilisin-like protease and a 16 kDa pathogenesis-related protein belonging to the PR-1 family also have been reported as allergic melon components (12, 13). In Immunoblot analysis and ELISA, rCuc m 2 was recognized by all of the sera (13/13) that showed IgE reactivity to the 15 kDa component of melon extract. This implies that the recombinant melon profilin retains most of the immunological properties of the natural component, and could be used as a diagnostic tool. The deduced amino acid sequences showed high similarity with other plant profilins (89-77%) recently described as

allergens. The highest sequence homology was found with watermelon profilin (89% identity and 96% similarity) and latex profilin (89% identity and 96% similarity). This may explain previous reports on the clinical cross-reactivity between latex and melon (15, 30). Surprisingly, only 76% sequence identity was found with melon profilin (see accession number AJ565931) recently reported after our data submission to GenBank. This sequence indicates 98.5% identity with peach Pru p 4 and only 78% identity with the watermelon profilin, which is another closely related member of the Cucurbitaceae family. Unfortunately, there is no information about the variety or cultivar of the melon corresponding to this isoform of Cuc m 2.

Alignment of amino acid sequences of Cuc m 2 and watermelon showed up to 89% identity. However, only two of 22 melon-allergic individuals had histories of watermelon allergies (Table 1). The lack of cross-reactivity between melon and watermelon has also been observed in inhibition experiments. The profilin sequence of cantaloupe, the other fruit belonging to Cucurbitaceae, is not available; however, it seems that there is only a low level of cross-reactivity between these two profilins according to ELISA inhibition experiments and clinical data. Interestingly, extracts of peach, tomato, grape, and *Cynodon dactylon* pollen inhibit IgE binding to Cuc m 2 nearly as effectively as melon extract, whereas the homology of their profilins with rCuc m 2 is much lower than the homology of the watermelon profilin with rCuc m 2. Our study indicated that high homology between two profilins does not necessarily result in their cross-reactivity. The occurrence of differences in the IgE epitopes of plant profilins from different species and from the same taxonomical family was also reported (31). An alternative explanation for the observed cross-reactivity might be lower profilin content in watermelon than in melon extract. Other studies also reported significant differences in the inhibition potency of the closely-related profilins, despite using recombinant profilins (32). In summary, we have demonstrated that profilin is the major allergenic component in melon fruit. Fruit-fruit and pollen-fruit cross-reactivity could be explained by the presence of these cross-reactive profilin polypeptides. Because the level of homology between different profilin molecules does not correlate with experimental and clinical cross-reactivity, further studies are required to clarify this observation.

Note: The nucleotide sequences for the coding region, 3' UTR, and 5'UTR of *Cuc m 2* genes were deposited in the GenBank database by M. Sankian, AR Varasteh, N. Pazouki, and M. Mahmoudi (8 Apr 2003) under GenBank Accession Numbers of AY271295, AY292387, and AY879598, respectively. The amino acid sequence of this protein can be accessed through the NCBI Protein Database, Accession Number AAP13533.2.

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