

Molecular Cloning and Expression of Cucumisin (Cuc m 1), a Subtilisin-like Protease of *Cucumis melo* in *Escherichia coli*

Mojtaba Sankian¹, Farideh Talebi¹, Maliheh Moghadam¹, Fatemeh Vahedi²,
Farahzad Jabbari Azad³ and Abol-Reza Varasteh^{1,4}

ABSTRACT

Background: Oral allergy syndrome resulted from plant-derived foods is frequent among adults. Allergy to melon (*cucumis melo*) is one of the most frequent fruit allergies in Iran. Three different major allergens have been found in *Cucumis melo* that Cuc m 1 (cucumisin) has been identified as the major allergen of melon. Cucumisin is an alkaline serine protease that it is found as a 78 kDa protein in precursor form. The aim of this study was production of recombinant Cuc m 1 in *Escherichia coli* (*E. coli*) cells and characterization of its allergenicity property.

Methods: Production of recombinant Cuc m 1 was carried out by cDNA cloning technique into the pET32b(+) vector using specific primers designed based on cucumisin nucleotide sequence available in Genbank database, cucumisin encoding gene and directional cloning method. Cloned plasmid into *E. coli* TOP10 was transformed into *E. coli* BL21 and expression of the protein was induced by IPTG. The recombinant protein was purified via Ni-NTA affinity chromatography using histidine tag in recombinant protein. IgE binding of this protein was assessed by IgE-immunoblotting, ELISA and inhibition ELISA.

Results: The directional cloning was resulted in expression of a fusion Cuc m 1. Immunoblotting with sera of patients allergic to melon showed strong reactivity with purified protein band. Inhibition assays demonstrated that purified rCuc m 1 could be the same with natural form of Cuc m 1 in total extract.

Conclusions: In the present study, we have provided a functional recombinant cucumisin allergen, rCuc m 1 with 86 kDa, which may be used as a standard allergen for clinical diagnosis and study of allergy to melon.

KEY WORDS

allergy, cloning, Cucumisin, *Cucumis melo*, subtilisin-like proteases

INTRODUCTION

It is estimated that true food allergies occur in 2-5% of the population and the prevalence of food allergy continues to increase worldwide.¹⁻³

Food allergy is defined as an immune-mediated adverse reaction to foods causing symptoms affecting the skin, gastrointestinal (GI) tract, or respiratory system. The reactions can be immunoglobulin E (IgE)-mediated and non IgE-mediated processes.^{4,5}

An associated food allergy condition often described as oral allergy syndrome.⁶ This allergy usually associated with hypersensitivity to pollens and cross-reactivity between pollen and food allergens.⁷ The symptoms of oral allergy or pollen-food allergy syndrome may differ from moderate-to-severe atopic dermatitis to food-induced wheezing and asthma.^{8,9}

However the number of deaths associated with food allergy has been reported very low, but some reactions can suffer the atopic subjects due to sever

¹Bu-Ali Research Institute, Immunology Research Center, ³Department of Immunology and Allergy, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, ²Razi Vaccine and Serum Research Institute and ⁴Varastegan Institute for Medical Sciences, Mashhad, Iran.

MS and FT contributed equally in this work.

Correspondence: Abol-Reza Varasteh, Bu-Ali Research Institute,

Immunology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Ferdowsi Square, Bu-Ali Square, Mashhad, 9196773117, Iran.

Email: varasteha@mums.ac.ir

Received 20 February 2010. Accepted for publication 24 August 2010.

©2011 Japanese Society of Allergology

problems.¹⁰

The most common food allergens include cow milk, eggs, peanuts, tree nuts, soy, in the pediatric population. The most common food allergies reported by adults are allergies to fruits and vegetables such as wheat; peanuts, tree nuts, fish, and shellfish.^{2,11}

Melon or Common Melon (*Cucumis melo* spp.) belongs to Cucurbitaceae family which may result in allergy symptoms in sensitized individuals.¹² The melon (*Kharbozeh* in Persian language) is grown anywhere in Iran and there are many varieties of it.¹³ As the melon is a favorite fruit in Iran and regards to high rate of food allergy to it (unpublished data), research has been focused to production of recombinant allergens of melon to aid further studies.

Several IgE-binding proteins between 13 and 60 kDa have been detected in Melon extract with pooled sera from patients with Melon allergy.¹⁴ A 13 kDa was the main reactive protein band was detected and identified as a profilin.¹⁵ A lipid transfer protein has also been detected.¹⁶ Cuc m 1, also known as cucumisin, a plant alkaline serine protease with caseinolytic activity can denature the casein of milk.¹⁷ This protein is resistance to alkaline pH and heat, with optimal pH around 10 and optimal temperature of about 70°C. A tetra amino acid sequence around the reactive serine residue of cucumisin is identical to that of subtilisin, a *Bacillus subtilis* serine protease.^{18,19} These two last proteins are highly resistant to pepsin digestion and are heat-stable, making them potentially potent allergens.^{15,16}

The molecular weight of native form of cucumisin from the juice of melons has been reported 67 kDa.¹⁸⁻²⁰ This 67-kDa enzyme due to autolysis produces a 14-kDa polypeptide and a 54-kDa protease which are resistant to further processing therefore thought to be identical to the 54-kDa cucumisin that has been previously reported. The protease (caseinolytic) activity will remain after autolysis.^{21,22}

The sequence of cucumisin has been deduced and defined as a 2552-bp nucleotide sequence that codes for a 78-kD precursor protein consisting of four functional domains: a 22-amino acid signal peptide, an 88-amino acid NH-terminal pro-sequence, a 54-kDa protease domain with 505 residues, which is the active enzyme domain of the 67-kDa native cucumisin, and a 14-kDa COOH-terminal polypeptide with 116 residues, which is product of autolysis of the 67-kDa native cucumisin.^{19,20,22-24} The 67-kDa native cucumisin is found in juice of melon, naturally.^{18,25}

The aim of this study was the cloning the cDNA sequence and expression of cucumisin, Cuc m 1. In addition, expressions of Cuc m 1 in *E. coli*, its purification and evaluation of its allergenicity were aimed.

Table 1 Clinical characteristics, prick tests result and total IgE of allergic patients to melon

Number	Age (Year)	Sex	Prick test result (mm ²) [†]	Total IgE (IU/ml)
1	43	F	36	>100
2	24	F	25	>100
3	19	F	25	>100
4	11	M	25	>100
5	25	M	82	>100
6	27	F	64	>100
7	22	M	36	>100
8	18	M	9	>100

[†] Surface area of wheal.

Prick test was performed with total melon extract.

METHODS

SUBJECTS AND SERA

Sera from a group of 8 individuals sensitized to melon, were selected by allergy specialists according to case histories and skin prick test from recruited people to Immunology and Allergy Department, Ghaem Hospital, Mashhad, Iran (Table 1).

By mixing equal volume aliquots of the individual sera, a serum pool was prepared and kept in -80°C. The skin prick test was done using total melon extract based on the guideline of the subcommittee on skin tests of the *European Academy of Allergology and Clinical Immunology* after acquiring permission and informed written consent from each patient.²⁶ The study was approved by the local ethic committee. The sera from 8 healthy subjects without any signs of allergy and negative skin prick test were collected to use as negative control in assays.

Total IgE levels were assessed with a commercially available ELISA kit according to the manufacturer's instructions (Total IgE IRMA kit, Radim, Italy).

TOTAL EXTRACT

The total extract was prepared as described previously with minor modifications.²⁷ In brief, after washing the ripe fruits of melon, the inner part of the pulp was isolated and homogenized using a homogenizer. The extracting buffer, phosphate buffer 100 mM, pH 8 containing 2% (w/v) polyvinyl pyrrolidone and 10 mM ethylene diaminetetracetic acid (EDTA), was added to homogenized mix in 1 : 10 w/v ratio. The slurry was mixed on magnetic stirrer for 2 h and then subjected to centrifuge (15000 g for 30 min) at 4°C. The supernatant was separated and dialyzed against phosphate buffer (100 mM, pH 7.4) for 24 h in 4°C. The extract was freeze-dried and kept in -80°C until use.

RNA EXTRACTION FROM FRUIT OF MELON

One gram of upper layer of melon was freeze-dried in liq-

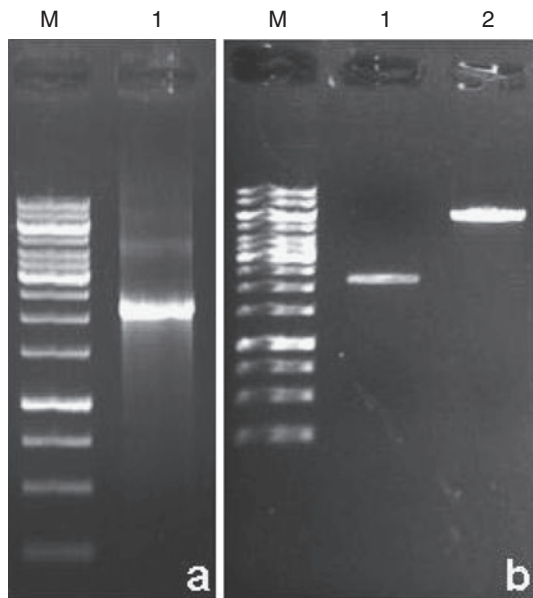


Fig. 1 Gel agarose electrophoresis, M: PCR marker (1 kb DNA Ladder, Fermentas: fragment sizes from up to down, 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2000, 1500, 1000, 750, 500 and 250 bp). **a:** PCR-amplification of cDNA encoding Cuc m 1. PCR product of 2193-bp Cuc m 1 is seen in lane 1. **b:** Lane 1, the 2193-bp Cuc m 1 PCR product was treated by *Xho* I and *Not* I restriction enzyme and purified. Lane 2, treated plasmid with *Xho* I and *Not* I was purified.

uid nitrogen and then finely grounded. Total RNA was extracted using guanidine thiocyanate as Chomczynski method.²⁸

PCR-AMPLIFICATION OF cDNA ENCODING Cuc m 1

Following the synthesis of cDNA from RNA using a first-strand cDNA synthesis Kit Fermentas with an oligo (dT) 18 as primer, according to the manufacturer's instructions, amplification of Cuc m 1 specific cDNA fragments were carried out using specific primers designed according to the genebank deposition sequences using gene Runner software (<http://www.generunner.com>).

(Sense primer: 5'- ATTAGCGGCCGCAATGTCTTC TTCTCTAATCTTCAA-3' with incorporated *Not* I site, Anti-sense primer: 5'- ATACTCGAGAACTAGACTTG TGATGGTTATAGG -3' with incorporated *Xho* I site).

CLONING

The predicted 2193-bp PCR product was purified from low melting agarose gel using DNA extraction Kit (QIA quick Gel Extraction Kit, QIAGEN, Hilden, Germany) and was cloned into the pET32b(+) vector, a fusion expression system with *E. coli* thioredoxin (Novagen, Madison, WI, USA), which expresses a recombinant fusion protein containing a His-tag at the

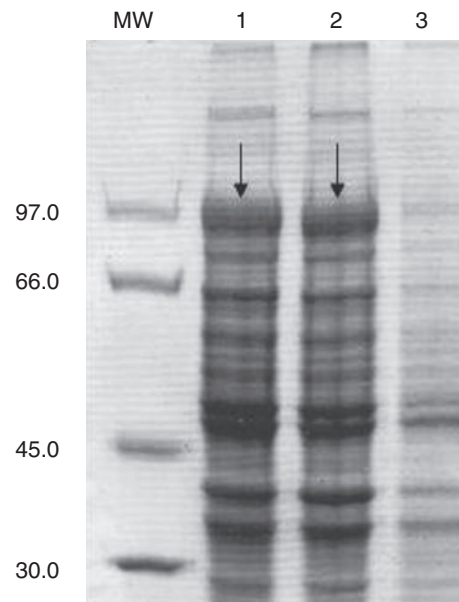


Fig. 2 Coomassie blue staining of SDS-PAGE (10%) gel for analyzing of Cuc m 1 expression in bacterial lysate. Lane MW: molecular weight marker, size in kDa, Lane 1 and 2: insoluble phase of bacterial lysate with constructed plasmid after IPTG induction. Arrows show the 86-kDa rCuc m 1. Lane 3: soluble phase of bacterial lysate with constructed plasmid after IPTG induction. The expressed protein was not seen in soluble phase.

C-terminal end. The construct was used to transform TOP10 *E. coli* cells. Appropriate colonies were selected and analyzed on ampicillin agar. The fidelity of the cloned product was verified by PCR reaction using specific primers of Cuc m 1 and restriction enzyme digestion with *Xho* I and *Not* I. The purified plasmid was subjected to sequencing (SeqLab, Göttingen, Germany).

EXPRESSION OF Cuc m 1 IN *E. coli* AND PURIFICATION

The constructed plasmid was transformed into competent *E. coli* BL21 cells (Invitrogen, Carlsbad, CA, USA). Then, transformed cells were incubated on LB agar containing ampicillin, overnight. Protein synthesis was induced with 1 mM IPTG (isopropyl β -D-thiogalactoside) in 5-ml culture for 8 h at 37°C followed by cell collection using centrifugation (3000 g, 20 min, 4°C). The pellets of bacterial cultures from before and after induction were resuspended in lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl and 2 mM imidazole, pH 8) and subjected to three freeze-thaw cycles using liquid nitrogen. The production of rCuc m 1 in supernatant and pellet fractions of cell lysate was analyzed by SDS/PAGE.

Metal affinity chromatography using Ni-NTA agarose (Invitrogen) was performed to purify recombi-

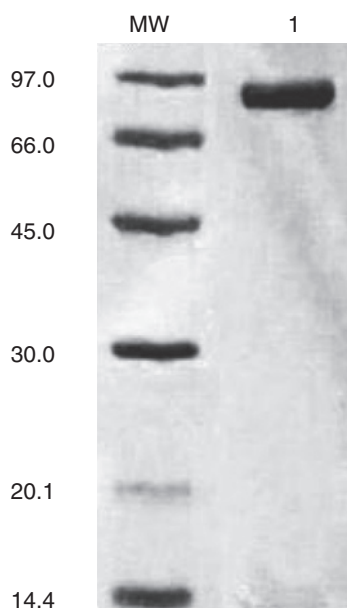


Fig. 3 SDS/PAGE of purified expressed proteins using metal affinity chromatography with Commassi blue staining. Lane MW: molecular weight marker, size in kDa. The purified rCuc m 1 is seen as a 86-kDa band.

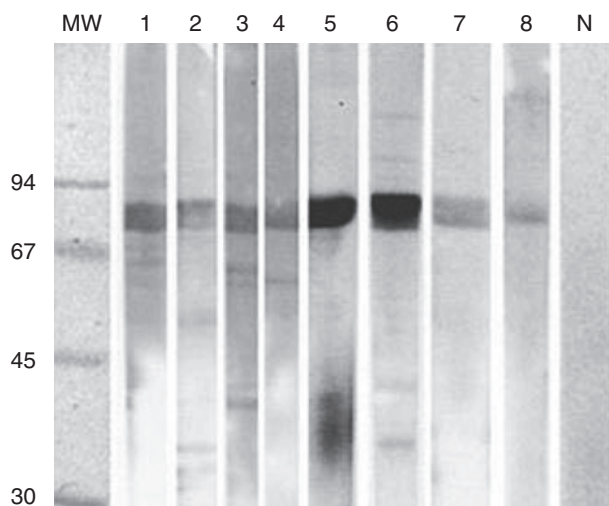


Fig. 4 Immunoblotting of purified recombinant Cuc m 1 protein with allergic subjects sera. The 86-kDa band is seen with reactivity in lanes 1-8. MW, protein marker, size in kDa. N, is negative control using non-allergic serum.

nant Cuc m 1 (rCuc m 1) from insoluble phase as described previously.²⁹ The purified protein was analyzed on SDS-PAGE electrophoresis.

IgE-IMMUNOBLOTTING

To evaluate the allergenicity of rCuc m 1, it was subjected to reducing 12% (w/v) SDS-PAGE and electroblotting onto polyvinylidene difluoride (PVDF)

Table 2 The result of specific ELISA with eight sera of allergic patients with their pooled serum as positive control and negative pooled serum

Number	ELISA result with total melon extract	ELISA result with rCuc m 1
1	0.81	0.98
2	0.71	0.86
3	1.05	0.68
4	0.56	0.74
5	1.00	0.98
6	0.93	1.00
7	1.04	0.86
8	0.71	0.50
Positive pooled serum	0.82	0.80
Negative pooled serum	0.12	0.05

The OD was measured in 450 nm in all experiments.

membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). The membranes were exposed for 3 h at room temperature to the diluted sera (1 : 5 in PBS) of allergic patients to melon followed by blocking with 2% BSA. The pooled serum of non-allergic patients was used as negative control. The reactivity between rCuc m 1 and specific IgE in sera were appeared by using anti-human IgE biotin-conjugated goat antibody (Sigma-Aldrich, St. Louis, MO, USA) (1 : 1000 diluted in BSA 1%) and horseradish peroxidase-streptavidin (1 : 20000 diluted). These specific reactivities were documented by G-BOX Chemi-Doc (Syngene, Cambridge, UK).

SPECIFIC ENZYME-LINKED IMMUNOSORBENT (ELISA) AND INHIBITION ASSAYS

To assess the specific IgE reactivity, the pool serum was examined by ELISA and inhibition ELISA as described previously.²⁷ Each well was coated with 1 µg/well of total melon extract or purified rCuc m 1. Optical density (OD₄₅₀) more than four times the median values of the negative control were considered to be positive.

ELISA inhibition was performed with 100 µl of the positive pooled serum pre-incubated individually with 100 µl (400 µg/ml) of total extract of melon and purified rCuc m 1 for 2 hours at room temperature. These adsorbed sera were then added to the ELISA plates that had been coated with rCuc m 1 or total extract of melon, respectively, at a similar concentration (1 µg/well). The ELISA procedure thereafter was followed as normal ELISA for measurement of specific IgE. The percentage of inhibition was calculated using this formula: (OD without inhibitor- OD of inhibitor/OD without inhibitor) × 100. ELISA assessments were performed in triplicate to achieve more accuracy. Inhibition with BSA was used as negative control.

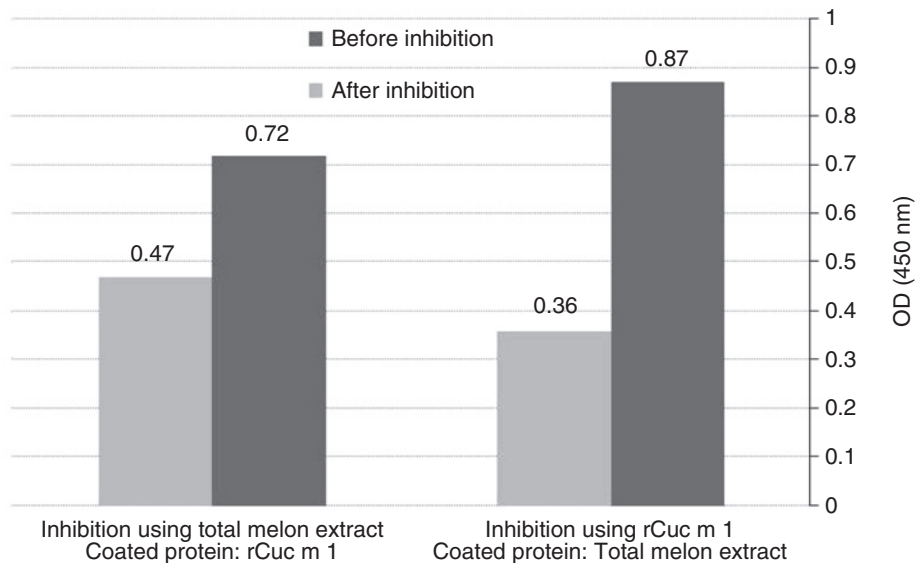


Fig. 5 Inhibition of IgE-binding by ELISA was found after pre-incubation of sera with rCuc m 1 or total melon extract. Negative BSA control was not shown.

RESULTS

CLONING AND SEQUENCING OF cDNA ENCODING Cuc m 1

cDNAs encoding a partial sequence of Cuc m 1 were deduced from RNA of *Cucumis melo* by specific amplification with specific primers. The PCR products showed a size of 2193 bp (Fig. 1a). The PCR product and pET32b(+) plasmid were purified following restriction enzyme digestion using *Xho* I and *Not* I. The purified products were analyzed on agarose gel (Fig. 1b).

EXPRESSION AND CHARACTERIZATION OF rCuc m 1

The cDNA coding for the Cuc m 1 allergen was expressed in the *E. coli* as described in the experimental section. The expressed protein band were detected by SDS/PAGE after induction with IPTG in 86 kDa size in insoluble phase of cells (Fig. 2). The highest expression level was observed at 24 hours after induction with 0.1 molar IPTG under shaking incubation in 37°C.

PURIFICATION AND ALLERGENICITY OF rCuc m 1

Purification of expressed proteins was done with metal affinity chromatography successfully. SDS/PAGE of purified proteins showed the protein in expected size (Fig. 3). In immunoblotting with allergic sera reactivity with purified rCuc m 1 was found (Fig. 4).

SPECIFIC IgE ELISA AND INHIBITION ASSAYS

The specific IgE values against total melon extract and purified rCuc m 1 were determined with 8 individual patients' sera (Table 2). All of these patients had significantly elevated specific IgE levels to melon total extract with OD values at 450 nm four times greater than the negative controls.

Pre-incubation of pooled serum with rCuc m 1 revealed inhibition of IgE binding to natural form of Cuc m 1 in total melon extract (58.62%). The inhibition of IgE binding to rCuc m 1 after pre-incubation with total melon extract was found lower (34.73%) (Fig. 5).

DISCUSSION

Melon has been known as a fruit to cause of oral allergy.^{18,27,30,31} At least 3 different allergens of melon has been identified that the cucumisin has been considered as major allergen of melon. Cucumisin is a subtilisin-like protease of *Cucumis melo* known as Cuc m 1. Cucumisin that has been expressed as a 78 kDa-precursor and it is shown that autolysis generates smaller N-terminal fragments. The remained 67 kDa-fragment as mature form of cucumisin has allergenicity effect and is considered as an active allergen.¹⁹

In this study, we cloned and expressed successfully cucumisin gene. The expressed protein identified as a 86-kDa protein, more heavy than natural cucumisin (67 kDa) regards to production as a fusion protein with histidine residues at C-terminal. Immunoblotting of this protein with sera of allergic subjects to extract of melon showed specific reactivity and confirmed functionality of newly expressed rCuc

m 1.

Previously, at least 10 allergens of melon with different sizes from 10 to 80 kDa were identified that four of them has been known as major allergens of melon in sizes of 14, 36, 54, 67 kDa.¹⁸ Except than 14-kDa protein, 3 others had similar N-terminal, confirmed as products of a lysis process of a precursor that was named cucumisin. Native cucumisin (67-kDa cucumisin), mature cucumisin (54-kDa cucumisin) and NH₂-end cucumisin (36-kDa cucumisin) are known as Cuc m 1.01, Cuc m 1.02 and Cuc m 1.03 allergens, respectively.¹⁸ They showed a few sera of allergic subjects, positive IgE reactivity to 67-kDa cucumisin. Here we found positive IgE reactivity in all 8 sera of allergic subjects who had been showed positive prick test with melon extract.¹⁸ This difference between these two evaluations may be related to different folding and conformational shape of recombinant Cuc m 1.

To express of recombinant proteins prokaryotic systems are used widely. *E. coli* as a prokaryotic expressing system is growing very fast in non-complex and non-expensive culture media.³² Using of this system to expressing of allergen with high molecular weight is not common. The highest molecular weight of recombinant allergen expressed in *E. coli* is the 64-kDa allergen of *Arachis hypogaea*, Ara h 1.³³

Specific IgE reactivity of allergic sera to total melon extract and rCuc m 1 in immunoblotting and inhibition assays confirmed a strong cross reactivity between natural form of Cuc m 1 allergen in total extract and rCuc m 1 that shows presence of tags at the ends recombinant protein did not have effect on the specific reactivity of rCuc m 1 with IgE. The intervention of tags in recombinant proteins with their activity has not been showed previously.^{29,34-36}

In this study Cuc m 1 was cloned and expressed in *E. coli*. rCuc m 1 was purified and its allergenicity was confirmed. Indeed, we successfully expressed and purified a heavy allergen, Cuc m 1, in *E. coli* cells with allergenicity property.

ACKNOWLEDGEMENTS

Financial support from The Research Council of Mashhad University of Medical Sciences (MUMS), Mashhad, Iran is acknowledged.

REFERENCES

1. Benhamou AH, Eigenmann PA. Atopic dermatitis and food allergies. *Rev Med Suisse* 2007;**3**:1038-43.
2. Lee LA, Burks AW. Food allergies: prevalence, molecular characterization, and treatment/prevention strategies. *Annu Rev Nutr* 2006;**26**:539-65.
3. Gotua M, Lomidze N, Dolidze N, Gotua T. IgE-mediated food hypersensitivity disorders. *Georgian Med News* 2008;**157**:39-44.
4. Hallett R, Teuber SS. Food allergies and sensitivities. *Nutr Clin Care* 2004;**7**:122-9.
5. Tatachar P, Kumar S. Food-induced anaphylaxis and oral allergy syndrome. *Pediatr Rev* 2008;**29**:e23-7.
6. Schafer T, Breuer K. Epidemiology of food allergies. *Hautarzt* 2003;**54**:112-20 (in German).
7. Malandain H. Allergies associated with both food and pollen. *Eur Ann Allergy Clin Immunol* 2003;**35**:253-6.
8. Benhamou AH, Eigenmann PA. Atopic dermatitis and food allergies. *Rev Med Suisse* 2007;**3**:1038-43.
9. Czarnecka-Operacz M, Jenerowicz D, Silny W. Oral allergy syndrome in patients with airborne pollen allergy treated with specific immunotherapy. *Acta Dermatovenol Croat* 2008;**16**:19-24.
10. Pumphrey RS. Fatal anaphylaxis in the UK, 1992-2001. *Novartis Found Symp* 2004;**257**:116-28;discussion 128-32, 157-60, 276-85.
11. Vigi V, Fanaro S. Food allergies in early childhood. 1. General concepts, etiopathogenesis, and main clinical features. *Minerva Pediatr* 2000;**52**:215-25.
12. Wehner T. Watermelon. *Vegetables*. New York: Springer, 2007;381-418.
13. Fisher WB, Boyle JA, Gershevitch I. *The Cambridge History of Iran*. Cambridge University Press, 1968;502.
14. Roby D, Gadelle A, Toppan A. Chitin oligosaccharides as elicitors of chitinase activity in melon plants. *Biochem Biophys Res Commun* 1987;**143**:885-92.
15. Rodriguez-Perez R, Crespo JF, Rodriguez J, Salcedo G. Profilin is a relevant melon allergen susceptible to pepsin digestion in patients with oral allergy syndrome. *J Allergy Clin Immunol* 2003;**111**:634-9.
16. Asero R, Mistrello G, Roncarolo D et al. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000;**122**:20-32.
17. Uchikoba T, Yonezawa H, Kaneda M. Cleavage specificity of cucumisin, a plant serine protease. *J Biochem* 1995;**117**:1126-30.
18. Cuesta-Herranz J, Pastor C, Figueredo E et al. Identification of Cucumisin (Cuc m 1), a subtilisin-like endopeptidase, as the major allergen of melon fruit. *Clin Exp Allergy* 2003;**33**:827-33.
19. Yamagata H, Masuzawa T, Nagaoka Y, Ohnishi T, Iwasaki T. Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. *J Biol Chem* 1994;**269**:32725-31.
20. Uchikoba T, Yonezawa H, Kaneda M. Cucumisin like protease from the sarcocarp of *Benincasa hispida* var. Ryukyu. *Phytochemistry* 1998;**49**:2215-9.
21. Yamagata H, Yonesu K, Hirata A, Aizono Y. TGTCACA motif is a novel cis-regulatory enhancer element involved in fruit-specific expression of the cucumisin gene. *J Biol Chem* 2002;**277**:11582-90.
22. Arima K, Yonezawa H, Uchikoba T, Shimada M, Kaneda M. Cleavage specificity of cucumisin, a serine protease, with synthetic substrates. *Phytochemistry* 2000;**54**:451-4.
23. Arima K, Uchikoba T, Yonezawa H, Shimada M, Kaneda M. Cucumisin-like protease from the latex of *Euphorbia supina*. *Phytochemistry* 2000;**53**:639-44.
24. Kaneda M, Ohmine H, Yonezawa H, Tominaga N. Amino acid sequence around the reactive serine of cucumisin from melon fruit. *J Biochem* 1984;**95**:825-9.
25. Uchikoba T, Kaneda M. Milk-clotting activity of cucumisin, a plant serine protease from melon fruit. *Appl Biochem Biotechnol* 1996;**56**:325-30.
26. Malling HJ. Skin prick testing and the use of histamine references. *Allergy* 1984;**39**:596-601.
27. Sankian M, Varasteh A, Pazouki N, Mahmoudi M. Sequence homology: a poor predictive value for profilins

- cross-reactivity. *Clin Mol Allergy* 2005;**3**:13.
28. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156-9.
29. Varasteh AR, Moghadam M, Vahedi F, Kermani T, Sankian M. Cloning and expression of the allergen Cro s 2 profilin from saffron (*Crocus sativus*). *Allergol Int* 2009; **58**:429-35.
30. Lopez-Torrejon G, Diaz-Perales A, Rodriguez J *et al.* An experimental and modeling-based approach to locate IgE epitopes of plant profilin allergens. *J Allergy Clin Immunol* 2007;**119**:1481-8.
31. Asakura K, Honma T, Yamazaki N, Ishikawa T. [Relationships between oral allergy syndrome and sensitization to pollen antigen, especially to mugwort]. *Arerugi* 2006;**55**: 1321-6(in Japanese).
32. Aruffo A. Expression cloning systems. *Curr Opin Biotechnol* 1991;**2**:735-41.
33. Burks AW, Cockrell G, Stanley JS, Helm RM, Bannon GA. Recombinant peanut allergen Ara h I expression and IgE binding in patients with peanut hypersensitivity. *J Clin Invest* 1995;**96**:1715-21.
34. Reschly EJ, Sorci-Thomas MG, Davidson WS, Meredith SC, Reardon CA, Getz GS. Apolipoprotein A-I alpha-helices 7 and 8 modulate high density lipoprotein subclass distribution. *J Biol Chem* 2002;**277**:9645-54.
35. Luo Y, Huang X, McKeehan WL. High yield, purity and activity of soluble recombinant *Bacteroides thetaiotaomicron* GST-heparinase I from *Escherichia coli*. *Arch Biochem Biophys* 2007;**460**:17-24.
36. Candi E, Melino G, Lahm A *et al.* Transglutaminase 1 mutations in lamellar ichthyosis. Loss of activity due to failure of activation by proteolytic processing. *J Biol Chem* 1998;**273**:13693-702.