

Identification of Cucumisin (Cuc m 1), a subtilisin-like endopeptidase, as the major allergen of melon fruit

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Summary

Background Allergenic components in melon extracts have not been described in spite of the fact that melon (*Cucumis melo*) is a frequent allergy-eliciting fruit. The aim of this study was to evaluate allergenic components in melon extract and to report the identification of cucumisin as a major melon allergen.

Materials and methods Sera from 35 patients allergic to melon were selected on the basis of clinical symptoms, skin prick tests and oral challenge test. Allergenic components were detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting. Molecular characterization of IgE-binding bands was performed by N-terminal amino acid sequencing.

Results More than 10 IgE-binding bands, between 10 and 80 kDa, were identified in melon extract. Out of them, four IgE-binding bands were major allergens: 14 kDa, 36 kDa, 54 kDa and 67 kDa. These major allergens, except 14 kDa band, showed the same N-terminal sequence: T-T-R-S-W-D-F-L. Research conducted with protein databases identified this N-terminal sequence as cucumisin, an alkaline serine protease, which shares structural homology with microbial subtilisin. The molecular mass of the identified bands corresponds with different molecular forms of cucumisin produced during the processing or degradation of the enzyme: 67 kDa native cucumisin, 54 kDa mature cucumisin and 36 kDa NH₂-terminal cucumisin fragment.

Conclusion Cucumisin (Cuc m 1) and several N-terminal cucumisin fragments are the major allergens of melon. The ubiquitous distribution of this protein family (cucumisin-like proteases) in many plant species and its high structural similarity suggest its potential role as a new panallergen in plant foods.

Keywords allergen, cucumisin, food allergy, melon, serine protease

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Introduction

Allergy to plant-derived foods is a frequent cause of food allergy. Plant-derived foods have been explained by cross-reactive IgE to pollen [1] and, thus, allergy to plant-derived foods has also been linked to particular pollens, for instance, apple to birch [2], banana/melon to ragweed [3]; hazelnut to hazel [4]; celery to birch/mugwort [5]; tomato/melon/watermelon to grass [6]; fennel/celery to birch [7]; kiwi to grass [8] or birch [9]; melon to plantago/grass [10], etc. Some of these associations have been demonstrated to be due to cross-reactivity between homologous structures shared between pollens and fruits, such as Bet v 1 [11], profilin [12], etc. Nonetheless, allergy to fruits is not yet fully clear. Although several food proteins have been identified as major allergens and characterized at molecular level [i.e. 13–17], there are still many allergens to be investigated in food

allergy. We consider that molecular characterization of food allergens will allow a better understanding of food allergies and help to explain cross-reactivities between vegetables and pollens or between vegetables and other ones.

This is the case of melon (*Cucumis melo*), a fruit belonging to *Cucurbitaceae* family, which includes several warm season vegetables such as watermelon, squash, cucumber and pumpkin. Muskmelon has been reported as a frequent cause of fruit allergy, both in some areas from the USA [3] and the European Union [18]. However, little is known with regard to components of melon extracts [10, 19] and major allergens have neither been identified nor characterized.

The aim of this study was to identify and characterize the major allergens of melon fruit.

Materials and methods

Patient sera

Thirty-five individual sera and a serum pool from patients allergic to melon, recruited in a birch- and ragweed-free area,

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were used in this study. According to their clinical history all patients showed immediate hypersensitivity to melon fruit, positive response to skin prick test to fresh melon pulp and oral challenge test. Clinical data of melon-allergic patients are shown in Table 1. In addition, sera from five melon-allergic patients were used as a positive serum pool (patients 2, 3, 8, 13 and 17) and sera from five subjects not sensitized to fruits were used as negative controls.

Production of allergenic extracts

Melon extracts were prepared from 'Piel de Sapo' melon (*In-odorus* variety). It was purchased at a local market and 2 kg were used for pulp extraction. Melons were peeled, liquidized and then processed. Extraction was started by homogenization with magnetic stirring (2000 rpm) at 4 °C for 30 min. A total of 10 mM/L potassium phosphate buffer, pH 7, containing 2% suspended solid polyvinylpyrrolidone (PVPP) and 2 mmol/L ethylene-diaminetetraacetic acid disodium salt (EDTA) were used as a solubilizing agent. The extract was then clarified by

centrifugation (26 000 g) for 30 min at 4 °C. The pellet was discarded, and the supernatant containing the solubilized proteins was dialysed against distilled water by diffusion in Spectra/Por tubing (Spectrum Laboratories Inc., Breda, the Netherlands) of 3.5 kDa at 4 °C overnight. The dialysed extract was sterilized by filtration through a 0.22-µm pore diameter membrane (Millipore, Bedford, MA, USA) and frozen at -80 °C. Finally the material was lyophilized and stored at -20 °C in desecator until needed. A method described by Martínez et al. [20] based on ion-exchange column chromatography for the enrichment of fruit-allergenic extracts was used. For *in vitro* experiments, the protein concentration of all allergenic extracts was adjusted to 1 mg/mL.

In vivo tests

Skin tests to fresh melon pulp were performed in duplicate by the skin prick tests (SPT), following the technique described by Dreborg et al. [21]. The ALK-LANCET needle (ALK-LANCET, ALK-Abelló, Hørsholm, Denmark) was used for

Table 1. Clinical features of melon-allergic patients

Patient	Age	Sex	Age onset	Symptoms	Pollinosis	P	Lo	Se	Cy	Ch	Ta	Sa	O	C	Be	A	Ar	Pa	Pl	Ph	Fr	Po	Ul
1	28	F	19	OAS, C	R	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-	-
2	7	M	2	OAS	R, A	+	+	+	+	+	-	-	+	-	+	-	-	-	+	+	+	+	-
3	25	M	7	OAS	R, A	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
4	59	F	56	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
5	20	F	16	OAS	R, A	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-	-	+
6	24	M	9	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	22	F	22	OAS	R, A	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
8	23	M	20	OAS	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	13	M	7	OAS	R	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	+	+
10	32	F	18	OAS, CU	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	25	F	23	OAS	R	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+
12	33	F	23	OAS	R, A	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+
13	27	M	13	OAS, CU, C	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	38	M	14	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
15	31	F	24	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
16	22	M	10	OAS	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	13	M	9	OAS	R	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
18	28	F	18	OAS, C	R	+	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	+
19	24	F	22	OAS	R, A	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+
20	28	F	28	OAS	R	+	+	+	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+
21	32	F	27	OAS	R	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
22	12	F	7	OAS, C	R, A	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
23	28	M	19	OAS	R	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
24	29	F	20	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	22	F	18	OAS	R	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
26	24	F	19	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
27	22	M	20	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
28	31	M	25	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	29	M	23	OAS, R	R	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+
30	23	F	13	OAS	R, A	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-
31	28	M	25	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	27	M	22	OAS	R	+	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+
33	29	F	9	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	53	M	18	OAS, GIS	R, A	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+
35	21	F	19	OAS	R, A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

OAS, oral allergy syndrome; M, male; F, female; R, rhinitis; A, asthma; GIS, gastrointestinal symptoms; P, phleum; Lo, lolium; Se, secalle; Cy, cynodon; Ch, chenopodium; Ta, taraxacum; Sa, salsola; O, olea; C, corylus; Be, betula; A, ambrosia; Ar, artemisia; Pa, parietaria; Pl, plantago; Ph, phragmites; Fr, fraxinus; Po, populus; Ul, ulmus.

skin tests, which were performed according to EAACI guidelines [22]. SPTs were also carried out with a battery of commercially available pollens. Histamine phosphate at 10 mg/mL and normal saline solution were used as positive and negative controls, respectively. A weal with a diameter of 3 mm larger than negative control was considered a positive reaction.

Oral challenge tests were carried out with fresh melon pulp, following the open method previously described [18]. Briefly, each dose was chewed and then swallowed. The melon doses were gradually increased until a positive response was obtained, or until the entire melon portion (about 200 g) was eaten without symptoms. The *in vivo* tests with fresh melon pulp were carried out from March to December, which is the melon season in Spain.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [23] utilizing a Mini-Protein II cell (Bio-Rad, Richmond CA, USA). Polyacrylamide concentrations of 15% and 5% were used for separating and stacking gels, respectively. The samples were mixed with 0.1 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (w/v) glycerol, 10% (w/v) 2 β -mercaptoethanol and 0.005% (w/v) bromophenol blue. The samples were denatured by heating at 100 °C for 5 min. An amount of 50 μ g protein was applied per lane. Electrophoresis was run at 20 mA constant current until the tracking dye bromophenol blue reached the bottom of the gel, using as buffer of electrodes 0.025 M (w/v) Tris-HCl, pH 8.3, 0.192 M glycine and 0.1% SDS. Gels were stained with Coomassie brilliant blue (Sigma Chemical Co., St Louis, MO, USA), or used for blotting as described below.

Immunoblot analysis

Protein bands separated by SDS-PAGE were transferred by semidry blotting onto nitrocellulose sheets by the method of Towbin et al. [24], in transfer buffer (0.048 M Tris-HCl, 0.039 M glycine, 20% methanol and 0.036% SDS). Blotted bands were visualized by soaking the membranes for a few minutes in Ponceau S (0.1% in 1% acetic acid). Membranes were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and 3% skimmed milk powder (M-PBS-T) for 2 h at room temperature (20 °C), and incubated overnight at 4 °C with the patients' sera diluted 1 : 5 in 20 mM Tris-buffered saline pH 7.4 containing 1% Tween-20 (TBS-T) and 3% bovine serum albumin (TBS-T-BSA). After washing five times with PBS-T, blots were incubated for 3 h at room temperature with anti-human IgE peroxidase-conjugate antibody (The Binding Site, Birmingham, UK) diluted 1 : 1000 in TBS-T containing 5% fetal calf serum. After five washes in PBS-T, the protein bands were visualized by chemiluminescence with ECL (Amersham International, Buckinghamshire, UK), following the instructions given by the manufacturer.

For inhibition experiments, serum pool (1 : 30 dilution) was incubated with 1 mg of melon extract overnight at 4 °C.

Sequence analysis

Melon extract from *Piel de Sapo* variety was used for sequence analysis of protein bands (Edman degradation). When the gels were used for blotting and sequencing experiments several

modifications [25] were introduced: (i) diacrylpiperazine was used instead of *N,N'*-methylene bisacrylamide as cross-linker, at the same concentration; (ii) the thickness was increased from 1.5 mm to 3 mm; (iii) gels were cast 1 day in advance and kept overnight at 4 °C, to allow a complete polymerization; (iv) thioglycolic acid was added to the upper buffer chamber to a 0.002% (v/v) final concentration; (v) pre-electrophoresis for 30–60 min at 10 mA. In order to make sure that the immunostained proteins correspond to the sequenced ones a SDS-PAGE gel with three lanes was carried out: lane 1 (50 μ g of extract), lane 2 (mw markers) and lane 3 (50 μ g of extract), thus lane 2 was in the middle. The gel was cut lengthways the lane 2 (markers) producing two identical gel halves containing half of the marker's lane and one sample (extract) lane (1 or 3). One half of the gel was transferred to polyvinylidene difluoride (PVDF) membrane for sequencing, and the other to nitrocellulose membrane for blotting. In this way, and using the markers as references, by re-joining the two membranes, the identity (correspondence) of each protein band (sequence-blotting) can be established.

PVDF membranes (ProBlott) with CAPS buffer (3-cyclohexylamino-1-propanesulphonic acid) were used for transferring the proteins, following the manufacturer's recommendations (Applied Biosystems). Proteins were stained with Coomassie blue, cut out with a razor and sequenced in a Procise sequencer (Applied Biosystem). Eight N-terminal amino acid residues were determined for each band. The N-terminal sequences obtained were analysed by Scan Prosite Program (ExPASy; <http://www.expasy.ch>) to identify the proteins in the databases.

Results

SDS-PAGE

SDS-PAGE of melon extract showed multiple protein bands with mw ranging from 10 to 80 kDa. The most intense bands were observed at about 67, 54, 49, 45, 43, 40, 26, 14 and 10 kDa, both under reduced and non-reduced conditions (Fig. 1).

Immunoblotting

More than 10 allergenic components could be identified in ME. IgE immunoblotting obtained with patient sera demonstrated a broad spectrum of IgE-binding bands from approximately 10–80 kDa.

Incubation with serum pool revealed six stained bands with an apparent molecular weight of 67, 54, 49, 36, 26 and 14 kDa (Fig. 1) which coincided with the bands most frequently found when using individual sera (Fig. 2, Table 1). Four IgE binding bands were recognized by more than 50% of patient sera: 67 kDa, 54 kDa, 36 kDa and 14 kDa. It should be stressed that the 36 kDa was the most frequent IgE-binding band, which was detected by 100% of the patient sera. Control immunoblot experiments with a serum pool from non-atopic patients did not show IgE-binding bands in melon extract.

Identification of proteins

The 67, 54, 49, 36, 26 and 14 kDa bands were characterized by analysing the N-terminal sequence of the allergenic proteins blotted on PVDF membranes. Results showed that all of the major bands, except the one at 14 kDa, shared the same

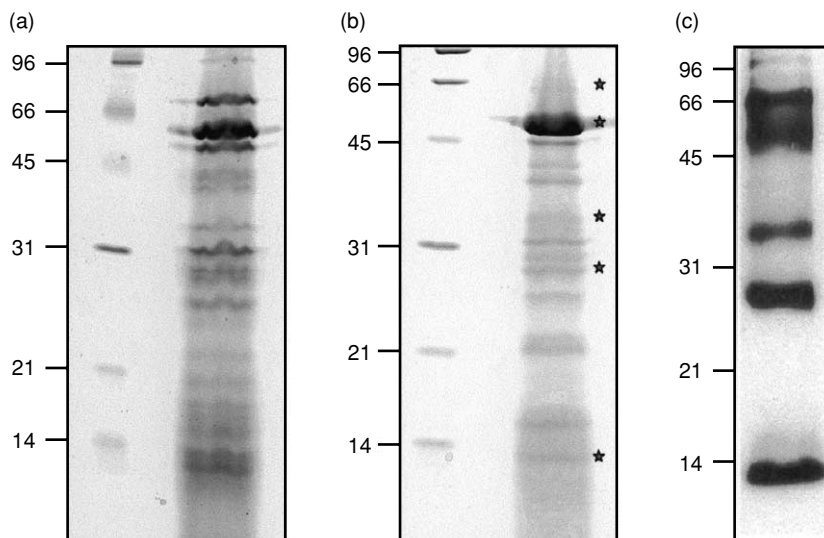


Fig. 1. SDS-PAGE analyses of melon extract under non-reducing (a) and reducing conditions (b). Immunoblotting of a reduced sample (c) revealed with a serum pool of melon allergic patients. The N-terminal sequence was determined for the bands marked with an asterisk. Figures on the left indicate the position of molecular markers in kDa.

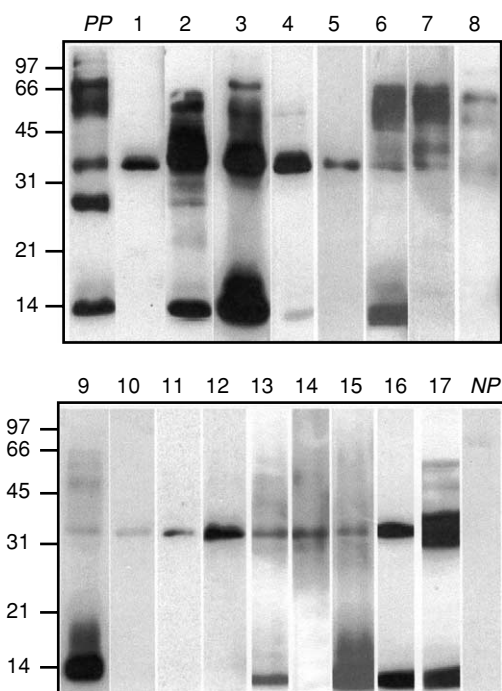


Fig. 2. IgE-binding bands in melon extract by Immunoblotting. Lane PP represents serum pool from several melon-allergic patients. Lanes 1 through 17 represent serum from 17 patients. Lane NP represents a negative control of serum pool from non-atopic patients.

N-terminal sequence: T-T-R-S-W-D-F-L. Research conducted with protein databases identified this N-terminal sequence as cucumisin (PIR:A55800), a protein which was originally described in 1994. Cucumisin is an alkaline serine protease from melon fruits of about 67 kDa, which shares structural homology with microbial subtilisin [26] and it has been recently classified within clan C (S8C) of serine proteases, according to evolutionary markers [27]. This 67-kDa enzyme shows limited autolysis to produce a 54-kDa protease and a 14-kDa polypeptide

(Fig. 3). These two bands of 67 and 54 kDa, closely correspond with those intensively and frequently stained in the immunoblots revealed with the sera of patients (Figs 1 and 2). Bands with lower mw (49, 36 and 26 kDa) and with the same N-terminal sequence correspond to progressive degradation fragments of cucumisin. The band at about 14 kDa yields a mixture of heterogeneous amino acids without any identifiable sequence. The high frequency of patient sera that recognized the band of about 36 kDa in the immunoblots is remarkable. It is conceivable that this fragment of the molecule retains some of the most allergenic epitopes and thus, this region, which is the N-terminal half of the cucumisin, deserves a deeper study.

Proteins with similar domain architecture to cucumisin

Interestingly, plant serine proteases are ubiquitous enzymes and cucumisin shares sequence identity with other plant serine proteases, such as that from soybean [28], tomato [29], latex [30], rice [31], barley [32], alnus glutinosa [33], etc., some of them having been named cucumisin-like protease.

In order to check whether the structure of cucumisin, and specifically the N-terminal half of the molecule (corresponding to the 36 kDa fragment), was shared by other proteins, a comparative analysis of conserved motifs was performed using DART software (www.ncbi.nlm.nih.gov).

Figure 4 shows the domain architecture of cucumisin molecule and a diagram of several representative proteins with similar domain arrangement along the molecule. The presence of two types of domains (Peptidase S8, PA domain) which are shared by numerous proteins belonging to different families of plants is apparent. Whether this structural similarity implies sharing IgE-reactive epitopes is currently under investigation. Nonetheless, we can anticipate an example of this possible cross-reactivity (Fig. 5) between melon and pumpkin (a taxonomic-related fruit) or between melon and tomato (a taxonomic-non-related plant food), in which bands with similar molecular weight of that of cucumisin were detected both in melon, pumpkin and tomato extracts and all of them were inhibited by melon extract.

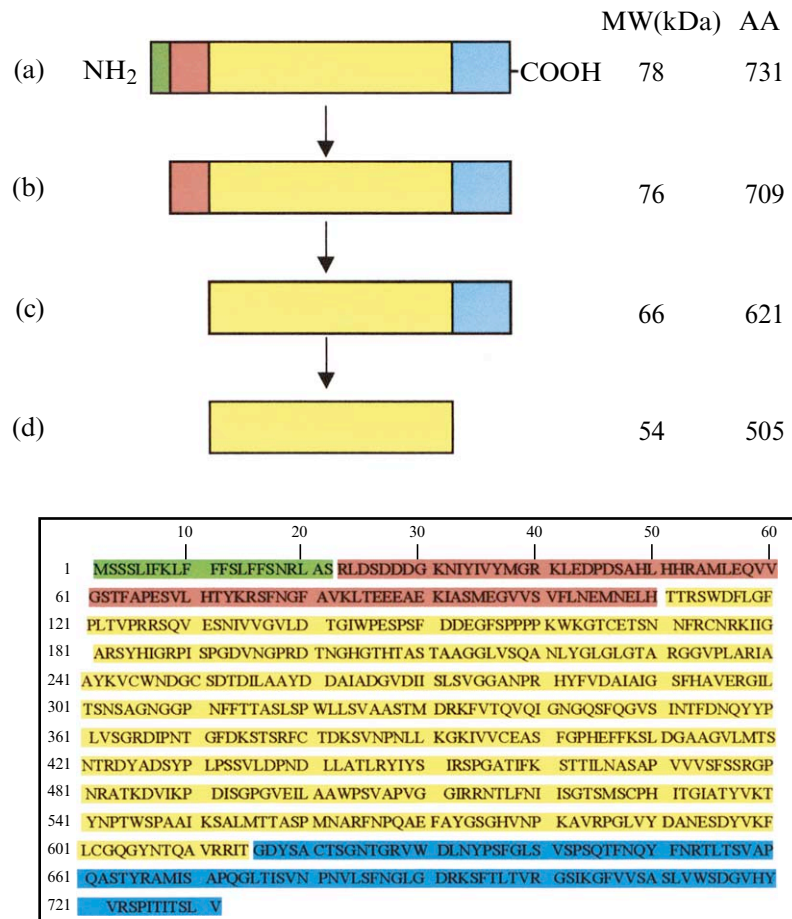


Fig. 3. Diagram of the processing pathway of cucumisin precursor, from prepro-cucumisin (a) to pro-cucumisin (b), native protein (c) and eventually to mature active cucumisin (d; see Discussion for details). The amino acid sequence of cucumisin is shown in the lower part, with the same colour code as shown above.

Discussion

In this study we have identified three out of four major IgE-binding bands from melon which were found to share the same N-terminal sequence corresponding to cucumisin. The presence of any protein or contaminant other than cucumisin in the bands was ruled out for two reasons. First, the chromatograms of the PTH of each identified residue were very neat, even without traces of other amino acids. So, a putative contaminant should be below the limit of detection of the sequencer (< 5 pmol). Second, if a contaminant were responsible for the immunoreactivity, it should be present in the five sequenced bands corresponding to cucumisin, with multiple molecular masses, which was highly unlikely.

A 36-kDa band was the most frequent IgE-binding band (100% of the sera), showing the highest reactivity with the sera of the melon-allergic patients and indicating that this N-terminal fragment of cucumisin is highly allergenic (Fig. 2). In addition, other bands corresponding to native (67 kDa) and mature (54 kDa) cucumisin were also found to be major allergens, although they were recognized with lower frequency. The higher allergenic activity of cucumisin N-terminal-fragment (36 kDa) than that of mature or native cucumisin probably corresponded to conformational changes in tertiary structure of cucumisin molecule as they shared the same primary structure (amino acid sequence).

Taken together, these data evidenced that several molecular forms of cucumisin are major allergens in melon. Cucumisin has been named Cuc m 1 [34]. This serine protease is present in the melon extract in several molecular forms originated during the process of maturation and subsequently as degradation fragments and, similarly to Hev b 6 [34], they might be named Cuc m 1.01 (67 kDa), Cuc m 1.02 (54 kDa) and Cuc m 1.03 (36 kDa).

Cucumisin accounts for more than 10% of total fruit protein [26]. It was fully sequenced and cloned in 1994 and it was found to be a glycosylated and thermostable alkaline serine protease homologous to subtilisin [26]. Examination of the primary structure of cucumisin (Fig. 3) revealed that it is synthesized as a prepro-cucumisin precursor (79 kDa polypeptide) and then processed to a pro-cucumisin form (76 kDa) [26]. The N-terminal pro-sequence is at that time removed and the 67 kDa native cucumisin is produced. Finally, a 14-kDa fragment from the C-terminus of the 67 kDa molecule is released by the autolytic activity of the enzyme and the 54 kDa form of the mature active cucumisin is produced.

Cucumisin is the first plant protease of the subtilisin family for which the complete primary structure has been determined. The discovery of cucumisin as a new member of the subtilisin family raises intriguing questions regarding the evolutionary descent of the plant subtilisin-like serine protease. Furthermore, the identification of several other proteins in plants

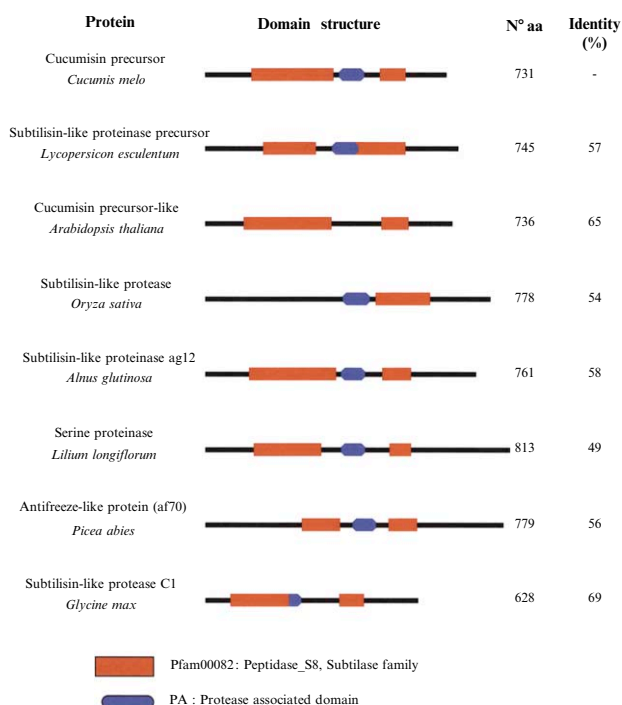


Fig. 4. Domain architecture of cucumisin molecule and a diagram of several representative proteins with similar domain arrangement along the molecule. The presence of two types of domain (Peptidase S8, PA domain) which are shared by numerous proteins belonging to different families of plants is apparent.

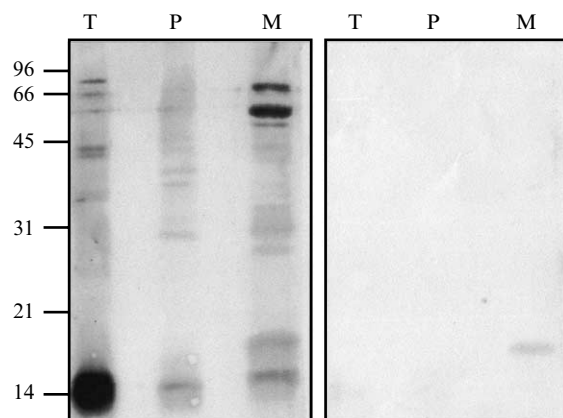


Fig. 5. IgE-binding bands in melon (M), pumpkin (P) and tomato (T) extracts by immunoblotting (left panel) and inhibition immunoblotting by melon extract pre-adsorption (right panel).

(Fig. 4), such as soybean, tomato, latex, rice, barley, *Alnus glutinosa*, etc., that have structural homology to cucumisin (cucumisin-like proteases) suggests its widespread and ubiquitous occurrence in plants. Additionally, to the best of our knowledge several authors have found an association between allergy to melon and latex [35] or melon and tomato [36], but the protein responsible for such cross-reactivity has not been evaluated, and it could be linked to cross-reactivity to cucumisin-like proteases from unrelated vegetables. Carbohydrate cross-reactive determinant (CCD) should be bear in mind on evaluating this possibility. Nonetheless, we are addressing several studies to clarify this question and we can anticipate the

existence of cross-reactivity between bands from melon, pumpkin and tomato, which are examples of cucumisin like endopeptidase from taxonomic-related fruit (pumpkin) and taxonomic-non-related plant foods (tomato).

In summary, cucumisin and several N-terminal cucumisin fragments are major allergens of melon. The ubiquitous distribution of this protein family (cucumisin-like proteases) in many plant species, its high structural similarity and the inhibition data suggest its potential role as a new panallergen in plant foods.

Acknowledgements

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