JOURNAL OF PROTEOMICS 86 (2013) 16–26

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Proteomic and immunological characterization of a new food allergen from hazelnut (*Corylus avellana*)

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ARTICLE INFO

Article history:
Received 4 April 2013
Accepted 1 May 2013
Available online 9 May 2013

Keywords:
11S globulin-like isoallergen
Cor a 9
Food allergy
Hazelnut (*Corylus avellana*)
IgE-binding proteins

ABSTRACT

Hazelnuts (*Corylus avellana*) are one of the most common sources of life-long IgE-mediated food allergies. In this study, we investigated the IgE-reactivity pattern of children with hazelnut allergy (N = 15) from Regione Campania, located in Southern Italy, and addressed proteomic strategies for characterizing IgE-binding proteins. For all of the patients (15/15), the predominant IgE-reactive component was a minor ~55 kDa protein not previously described. Similar to the hazelnut 11S globulin Cor a 9 allergen, the immunoreactive protein consisted of two subunits linked via a disulfide bridge. In contrast to Cor a 9, only the 20.7 kDa alkaline subunit exhibited IgE-affinity. The immunogenic subunit was purified by a two-step chromatographic procedure, but peptide mass fingerprinting was unsuccessful in identifying it, due to the incompleteness of the annotated hazelnut genome. Several tryptic peptides were de novo sequenced by tandem mass spectrometry and showed a high degree of homology with the 11S globulin storage proteins from other seeds, some of which have already been reported as food allergens. The structural characterization suggests that the new putative allergen is a divergent isoform of the hazelnut 11S globulin. These results provide a new platform for developing innovative diagnostic and therapeutic intervention plans.

Biological significance
Over the years, at least five proteins have been reported as potential food hazelnut allergens. The predominance of specific allergens appears to be strictly related to the geographical origin of the allergic subjects. The complex patterns of the IgE-reactivity of hazelnut storage proteins result in a poor diagnostic and prognostic accuracy. In the perspective of a component-resolved "molecular approach" to the hazelnut allergy we investigated the immune-reactivity patterns to hazelnuts of 15 patients (14 in the pediatric age range) from Region Campania, located in Southern Italy. For all the patients the predominant IgE-reactive component was a minor ~55 kDa protein not previously annotated in either protein or genomic databases. The putative allergen was isolated,

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http://dx.doi.org/10.1016/j.jprot.2013.05.001

partially characterized by MS/MS de novo sequencing and appears to be an isoallergen of the hazelnut 11S globulin Cor a 9. Like this latter, the immunoreactive protein consisted of two subunits linked via a disulfide. In contrast to Cor a 9, only the 20.7 kDa alkaline subunit exhibited IgE-affinity, in analogy to 11S allergens from other seeds (pistachio, cashew, soybean). We believe that the application of combined immunochemical and proteomic strategies to characterize the new food allergen could be of interest for the readers of *Journal of Proteomics*. In addition, the results of this study have functional worth in providing a new platform to plan innovative diagnostic and therapeutic intervention approaches to treat hazelnut allergy.

1. Introduction

Hazelnuts (*Corylus avellana*) are one of the most common triggers of IgE-mediated food allergies. In the majority of cases, hazelnut allergy is a life-long condition with symptoms that can vary from mild to severe, including anaphylaxis [1].

The prevalence of this allergy in Western countries is estimated to range from 0.4 to 1.1% [2–4]. Unfortunately, an exact evaluation of the prevalence of hazelnut allergy is hindered at least in part by the lack of availability of standardized allergens for clinical use [5].

Over the years, at least five proteins have been reported as potential hazelnut allergens. The predominance of specific allergens appears to be strictly related to either the geographical origin [1] or the age of the allergic subjects [6].

Indeed, in birch-endemic regions of Northern Europe, hazelnut allergy has been primarily associated with the cross-reactivity of Cor a 1.04 with the aeroallergens Bet v 1 and Bet v 2 (profilin homologues) from *Betula verrucosa*. The pollen-related hazelnut allergy usually induces mild oral allergy syndromes (OAS) [7]. The main non-pollen associated allergen in the United States appears to be an 11S globulin-like protein (Cor a 9), composed of a 30–40 kDa acidic subunit and a 20–25 kDa basic subunit linked via an inter-chain disulfide bridge [8]. In particular, only the acidic chain is immunoreactive. A 9.5 kDa *nonspecific*-lipid transfer protein (*ns*-LTP) called Cor a 8, has been described as the predominant hazelnut allergen in the nonendemic-birch Mediterranean area [1–9]. Because of their stable structure, which is conserved across the species, *ns*-LTPs are the causative agents of severe life-threatening allergies to a variety of plant foods [10]. It has been demonstrated that sensitization to the hazelnut *ns*-LTP can also occur in children from birch-endemic areas [11]. Additional potential hazelnut allergens are the 48 kDa-glycoprotein vicilin (Cor a 11) [12] and 2S albumin (Cor a 14) [13].

Cor a 9 is the predominant allergen in infants, while Cor a 11 is the predominant allergen among adults [14].

The complex patterns of IgE-reactivity may result in a poor diagnostic and prognostic accuracy [15,16]. Because the clinical symptoms of hazelnut allergy seem to be associated with the nature of the allergens, the component-resolved "molecular approach" aimed at the precise identification of allergen(s) is expected to greatly improve both the diagnosis and treatment of food allergies.

In the current study, we investigated the immune-reactivity patterns to hazelnuts in 15 patients (14 in the pediatric age range) from Region Campania, located in Southern Italy, where hazelnuts are widely cultivated. Preliminarily, one and two-dimensional electrophoresis (2DE)–mass spectrometry (MS) was used for analyzing the protein fractions from four commercial hazelnut cultivars, three of which were autochthon from Southern Italy and one of which was from Oregon (USA). Then, crude hazelnut extracts were immunoblotted with the sera of allergic children, and the IgE-binding proteins were characterized by combined proteomic methodologies, including MS/MS de novo sequencing.

2. Material and methods

2.1. Patients

Sera were obtained from hazelnut allergic subjects (N = 15, 80% male), all from Regione Campania (Southern Italy). Diagnosis of IgE-mediated hazelnut allergy was confirmed using skin prick test (SPT) and an oral food challenge. Either an SPT hazelnut solution or fresh hazelnut was applied to the patient's volar forearm. Tests were performed using a 1-mm single peak lancet (ALK, Copenhagen, Denmark), with histamine dihydrochloride (10 mg/ml) and isotonic saline solution (0.9% NaCl) as the positive and negative controls, respectively. Reactions were recorded based on the largest diameter (in millimeters) of the wheal and flare at 15 min. An SPT result was considered "positive" if the wheal was 3 mm or larger, without a reaction to the negative control. The allergy symptoms ranged from urticaria to angioedema and anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table 1. The total serum IgE was quantified with the ImmunoCAP system (Phadia, Uppsala, Sweden). All of the serum samples were stored at −20 °C before being used. Any sensitization was regarded as positive when the total IgE was greater than 0.35 kUA/l.

2.2. Material

Three hazelnut cultivars from Regione Campania (Mortarella, San Giovanni, Tonda) and one from Oregon were utilized in this study. Trifluoroacetic acid (TFA), dithiothreitol (DTT), iodoacetamide, phenylmethanesulfonylfluoride (PMSF), α-cyano-4-hydroxycinnamic acid (4-CHCA), sinapinic acid and phosphate buffer saline (PBS) were provided by Sigma-Aldrich (Milan, Italy). The electrophoresis reagents were from GE-healthcare (Milan, Italy). Ammonium bicarbonate (AMBIC), reagents and HPLC-grade solvents were from Carlo Erba (Milan, Italy) and were used without any further purification. Sequencing-grade modified trypsin was supplied by Promega (Madison, WI, USA). The skin prick test (SPT) solution was from Lofarma (Milan, Italy).

Table 1 – Clinical features of the hazelnut-allergic patients enrolled in the study.

Patient	Sex	Age at diagnosis (months)	Atopic risk	Skin prick test to hazelnut (>3 mm)	Total serum IgE (kU/l)	Symptoms at diagnosis	Concomitant other food allergies	Concomitant allergy to aeroallergens
1	M	18	Yes	Positive	191	Urticaria, angioedema	Yes	Yes
2	F	60	Yes	Positive	94,2	Angioedema, anaphylaxis	Yes	Yes
3	M	24	Yes	Positive	478	Urticaria	Yes	No
4	F	54	Yes	Positive	18,5	Angioedema	Yes	No
5	M	204	No	Positive	75,7	Urticaria	Yes	No
6	M	75	Yes	Positive	355	Urticaria	Yes	Yes
7	F	23	Yes	Positive	878	Angioedema	Yes	No
8	M	6	No	Positive	1417	Urticaria, angioedema, anaphylaxis	Yes	No
9	M	6	Yes	Positive	1378	Urticaria, angioedema	Yes	Yes
10	M	60	Yes	Positive	229	Urticaria	Yes	Yes
11	M	48	Yes	Positive	153	Urticaria	Yes	No
12	M	44	No	Positive	162	Urticaria	Yes	Yes
13	M	36	Yes	Positive	581	Angioedema	Yes	No
14	M	12	Yes	Positive	948	Urticaria	Yes	No
15	M	24	Yes	Positive	564	Urticaria, angioedema	Yes	No

2.3. *Preparation of protein extract*

Shelled raw hazelnuts were ground using an electric grinder to a fine powder, and defatted by stirring twice for 1 h in five volumes (w/v) of diethyl ether. Proteins were extracted into 0.1 M PBS, (pH 7.5; 1/10, w/v) containing protease inhibitor (1 mM PMSF) by stirring overnight at 4 °C. After centrifugation (10,000*g*, 30 min, 4 °C), the supernatant was collected and filtered through a 0.2 mm cellulose acetate filter (Millipore, Darmstadt, Germany). The protein concentration was determined by the Bradford assay. For 2 DE analyses, the hazelnut proteins were reduced with 10 mM DTT in 0.3 M Tris–HCl containing 6 M guanidine/HCl at pH 8.0, for 30 min at 56 °C and alkylated with 55 mM iodoacetamide, dissolved in the same buffer above, for 30 min at room temperature. Low molecular weight compounds were removed from the sample by filtration through a PD-10 desalting column in 5% acetic acid and lyophilized.

2.4. *SDS-PAGE analysis*

The hazelnut extracts and SPT-protein were separated by 1D-SDS-PAGE electrophoresis using 12% polyacrylamide gels. Proteins (12 μg per well) were resuspended in 20 μl of the Laemli buffer (0.125 M Tris–HCl pH 6.8, 5% SDS, 20% glycerol, 5% (w/v) β-mercaptoethanol, 0.02% bromophenol blue) and boiled for 5 min. Non-reducing electrophoresis was carried out in the same condition of denaturant SDS-PAGE, but omitting β-mercaptoethanol. Electrophoresis was carried out using the Tris–glycine–SDS buffer system (25 mM Tris, 192 mM glycine and 0.1% SDS) on a Mini-PROTEAN® Tetra Handcast Systems.

2.5. *Two-dimensional IEF/SDS-PAGE*

Hazelnut extracts (200 μg) were separated by 2DE, as described by O'Farrell [17]. The 1D step was performed using an Ettan IPGphor II system (GE healthcare). Immobilized pH 3–10 l gradient strips (11 cm) were rehydrated overnight with 200 μg of reduced and alkylated crude hazelnut protein extracts previously suspended in 200 μl of IPG strip rehydration buffer (8 M urea; 2% CHAPS; 20 mM DDT; 2% IPG buffer; 0.002% bromophenol blue). The proteins were focused up to 11,000 Vh at a maximum voltage of 6000 V at 20 °C. IPG strips were soaked in the equilibration solution (6 M Urea, 2% w/v SDS, 30% w/v glycerol, 50 mM Tris–HCl, pH 8.8, 0.002% bromophenol blue) for 15 min with 1% DTT (w/v) and 15 min with 2.5% iodoacetamide (w/v) at room temperature. The 2D-SDS-PAGE was carried out using a HOEFER SE 600 series system (Amersham), separating proteins on a 12% hand-cast SDS-polyacrylamide gel (16 × 14 cm) using the Tris–glycine–SDS buffer system at 15 mA/gel for 30 min and then at 30 mA/gel. The protein spots were stained with Coomassie Brilliant Blue (CBB) R-250. The 2-DE protein patterns were recorded as digitalized images using and ImageScanner (Amersham Biosciences) operated by the software Lab-Scan 3.00 (Amersham Biosciences). Spot analyses were performed using the ImageMaster 2D Platinum software 6.0 (Amersham Biosciences). The spots were excised and the proteins were submitted to mass spectrometry analysis.

2.6. *Protein in-gel digestion*

The protein spots were destained by repeated washing with 25 mM AMBIC/acetonitrile (1/1, v/v). The proteins were reduced for 1 h at 57 °C with 10 mM DTT in 25 mM AMBIC and alkylated for 30 min at room temperature with 55 mM iodoacetamide in 25 mM AMBIC. The digestion was carried out overnight at 37 °C with modified proteomic grade trypsin (12.5 ng/μl) in 25 mM AMBIC. The peptides were extracted three times in 5% formic acid/acetonitrile (1/1, v/v) and finally dried in a "speed-vac" centrifuge. Prior to MS analysis, peptide digests were desalted using C_{18} Zip-Tip pre-packed micro-columns (Millipore, Bedford, MA, USA), previously equilibrated with 0.1% TFA and eluted with 50% acetonitrile (v/v) containing 0.1% TFA (v/v).

2.7. *MS analysis*

Spectra were acquired using a Voyager DE Pro mass spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N_2 laser (λ = 337 nm). The instrument was operated at an accelerating voltage of 20 or 25 kV. The mass spectra of the

peptides were acquired in the reflector mode using 4-CHCA (10 mg/ml in 50% acetonitrile/0.1% TFA). The analysis of protein samples was carried out in the linear ion mode using sinapinic acid (10 mg/ml in 50% acetonitrile/0.1% TFA) as the matrix. Typically, 250 laser shots were accumulated for each spectrum. External mass calibration was performed with commercial standard peptide/protein mixtures (Sigma).

*Nano*flow LC–ESI MS/MS analysis was carried out using an Ultimate 3000 HPLC (Dionex, Sunnydale, CA, USA) coupled to a Q-STAR mass spectrometer (Applied BioSystems, Framingham, USA). The eluents were (A) 5% ACN in 0.1% FA and (B) 80% ACN in 0.08% FA. The peptides were loaded into a C_{18} loading cartridge (LC Packings, USA) and separated with a C_{18} PepMap100 column (15 cm length, 75 μm ID, 300 Å [LC Packings]), using a linear gradient of 5–40% B over 60 min at a constant flow rate of 300 nl/min. LC–MS/MS experiments were performed in the information-dependent acquisition (IDA) mode. Precursor ions were selected using the following MS to MS/MS switch criteria: ions greater than m/z 400, charge states 2 to 4, intensity exceeding 15 counts, former target ions were excluded for 30 s and ion tolerance was 50.0 mmu. CID was used to fragment multiple charged ions and nitrogen was used as the collision gas. The raw spectrum files were used to generate text files in mascot generic file format (.mgf), which were submitted to the Mascot ver. 2.3 (http://www.matrixscience.com) and Batch-tag (Protein Prospector, University of California San Francisco, USA) search engines. De novo sequencing of the tryptic peptides was assisted by the Analyst 1.1 software (Applied BioSystems), followed by manual validation of the sequences. De novo generated peptide sequences were used for homology searches using the MS BLAST and MS-Pattern (Protein Prospector) algorithms against the NCBI non-redundant database using standard settings with no taxonomical restriction.

2.8. MS and MS/MS data interpretation

MALDI-TOF spectra in reflectron mode were handled using Data Explorer software version 4.0. Prior to data base searching, mass spectra were baseline corrected and Gaussian smoothed with filter width of 5. Peaks with S/N >15 were selected and deisotoped.

*Nano*LC–MS/MS raw spectrum files were treated using a standard Mascot.dll script to generate text files in mascot generic file format. MS or MS/MS peak-lists were submitted to the Mascot search engine (http://www.matrixscience.com) using the following criteria: database, NCBI or Swiss-Prot; taxonomy, other green plants; type of search, MS or MS/MS ion search; enzyme, trypsin/P; fixed modifications, carbamidomethyl; variable modifications, oxidation on methionine and the *N*-terminal loss of ammonia at Gln; mass values, monoisotopic; parent tolerance, 0.07 Da; ms/ms tolerance, 0.1 Da; and number of maximum missed cleavages, 1. Unassigned MS/MS spectra were manually identified with the aid of the Analyst software (Applied BioSystems).

2.9. Immunoblot for IgE-binding assay and immunoblot inhibition

Both 1DE and 2DE gels were electroblotted onto nitrocellulose paper using a Trans-Blot Cell from BioRad (Bio-Rad Laboratories, Hercules, CA, USA) at 400 mA at 4 °C for 1 h. Membranes were blocked for 1 h at room temperature with (5% w/v) bovine serum albumin (Sigma) in Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C, with the sera of allergic children or control individuals (N = 3). Several combinations of hazelnut protein amounts and serum dilutions (in TBS-T) were tested to minimize the non-specific antibody response. The 2DE blot was immunostained with 200 μg of pooled sera in 40 ml of TBS-T. Immunoblot inhibition experiments were performed by pre-incubating a serum (1 h at room temperature) with 2 μg of the purified ~21 kDa IgE-reactive protein. After washing with TBS-T, monoclonal peroxidase-conjugated mouse anti-human IgE antibody (Sigma) diluted in blocking solution (1/10,000) was applied to the membrane for 1 h at room temperature. The membrane was extensively rinsed with TBS-T (3 × 10 min) and finally with TBS (1 × 10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare) and X-ray film (Kodak, Chalons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times ranging from 0.5 to 10 min.

2.10. Purification of the hazelnut IgE-binding protein

The predominant IgE-binding protein was purified by a sequential two-step procedure. The first step was size-exclusion chromatography on a Superdex 75 column, previously calibrated with a standard protein mixture (Amersham), using 50 mM sodium phosphate buffer (pH 7.5) and 150 mM NaCl as the eluents. The second step of purification was reverse phase (RP)—HPLC using a C_8 Vydac 2.1 mm i.d. column (Hesperia, CA, USA). After 10 min of isocratic elution using 25% solvent B (0.1% TFA in acetonitrile, v/v), a fractionated step was applied: 25–30% B for 5 min, 30–35% B for 30 min, 35–50% B for 60 min, 50–55% B for 15 min at a flow rate of 0.200 ml/min. Solvent A was 0.1% TFA in water (v/v). In both cases, the HPLC chromatograph was an HP 1100 Agilent modular system equipped with diode array detector (Palo Alto, CA, USA). The column effluents were monitored by detection at λ = 220 and 280 nm. The allergen-containing fractions were monitored by Western immunoblotting using pooled sera. IgE-immunoreactive fractions were pooled and concentrated.

3. Results

3.1. SDS-PAGE analysis and immunoblotting

The IgE-reactivity of raw hazelnut proteins was tested by immunoblotting using sera from 15 pediatric patients affected by hazelnut allergy. To rule out the possibility that the immune-reactive patterns were associated with specific hazelnut cultivars, experiments were performed using protein extracts from three cultivars from Southern Italy (Mortarella, San Giovanni and Tonda) and one from Oregon (USA). SDS-PAGE analysis revealed very similar protein profiles regardless of the variety, with no appreciable qualitative and quantitative differences associated with the major storage proteins (Fig. 1). In agreement with previous reports [1,9,11,12,18], immunoblotting experiments were first conducted using serum dilutions of 1:10–1:50. Using this method, all of the most abundant proteins were

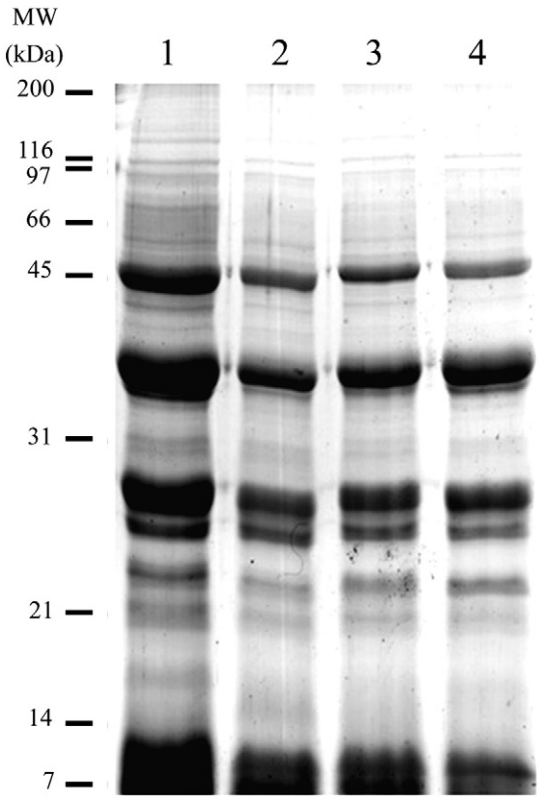

Fig. 1 – CBB R250 stained SDS-PAGE analysis of crude protein extracts from three hazelnut cultivars autochthonous of Regione Campania (lane 2: Mortarella, lane 3: San Giovanni, lane 4: Tonda) and one from Oregon, USA (line 1).

immunostained, exhibiting clear IgE-recognition (not shown). Further tests with several combinations of crude hazelnut protein amounts and serum dilutions allowed for the minimization of the non-specific antibody responses. Finally, an optimal response for the most reactive protein components was obtained by applying 7 μg of the protein extract in the electrophoretic lane and 1:200 dilution of the sera (Fig. 2). Using these conditions, all of the sera showed specific IgE reactivity against a polypeptide of approximately 21 kDa (Fig. 2). A faint additional immunoreactivity to bands at 48 kDa and 9 kDa was also detected for 12 and 7 allergic patients, respectively (Fig. 2). In accordance with the apparent molecular weight of the proteins and the data reported in the literature, the additional immunoreactive proteins were identified as vicilin-like 7S glycoprotein (also known Cor a 9 or 48 kDa glycoprotein) and the *ns*-LTP (also known as Cor a 8) respectively.

The ~21 kDa IgE-binding protein occurred in all the examined cultivars, exhibiting comparable reactivity in all cases (not shown). Importantly, the electrophoretic profile and the pattern of IgE-reactivity did not appreciably changed when the hazelnuts were extracted with a denaturing/reducing buffer containing 7 M urea, 2 M thiourea and 10 mM DTT (not shown), confirming that the prevalent allergens of hazelnuts are saline-soluble proteins.

3.2. *Immunoblot analysis in non-reducing/reducing conditions*

To investigate the possibility that the IgE-reactive band was a subunit of a larger polypeptide, we compared the 1D SDS-PAGE analyses of the hazelnut extracts separated under reducing and non-reducing conditions and the corresponding immunoblot carried out with a pool of sera (Fig. 3). Under the non-reducing conditions (in the absence of β-mercaptoethanol), three IgE-reactive bands with estimated MWs of ~38, 40 and 58 kDa were detected, while the ~21 kDa IgE-reactive band was completely absent. When analyzed by MALDI-TOF MS after tryptic in-gel digestion, several peptides of the higher molecular weight

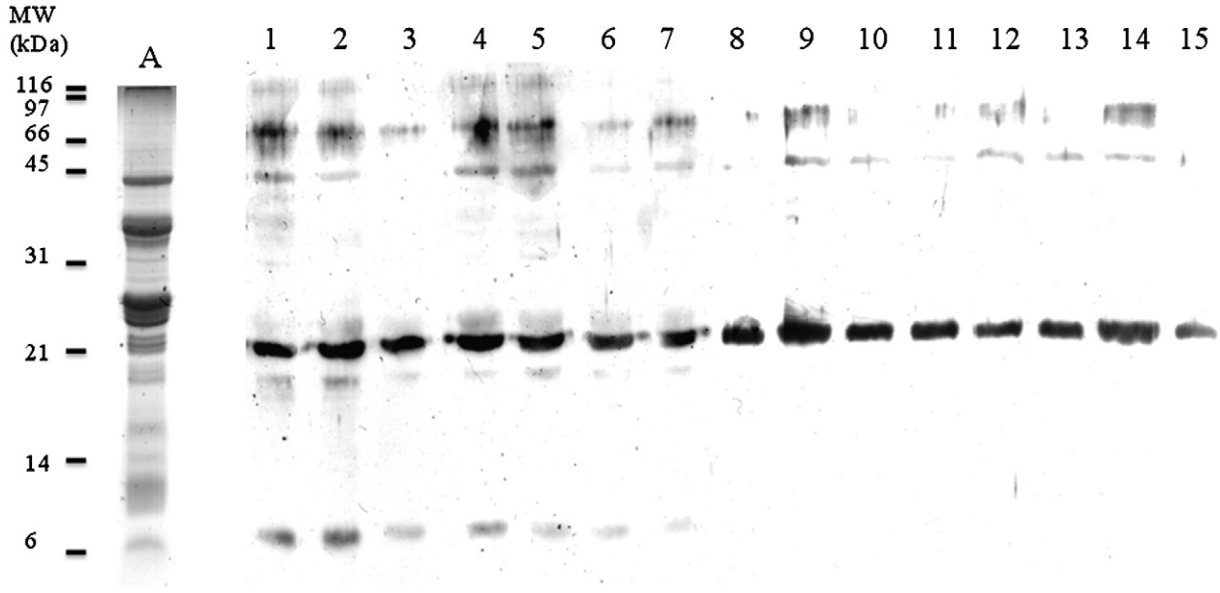

Fig. 2 – ECL-immunoblot of a PBS protein extracts from raw hazelnuts (Cv Mortarella) immunostained with individual sera from 15 hazelnut-allergic subjects. The secondary antibody was HRP-conjugated monoclonal anti-human IgE. All of the sera (15/15) were predominantly IgE-reactive to a ~21 kDa band. Additional faint immunoreactivity to 9 kDa (ns-LTP) and 48 kDa (7S vicilin-like) bands was detected for 7/15 (lanes 1–7) and 12/15 (lanes 1–2, 4–7 and 9–14) of the sera, respectively.

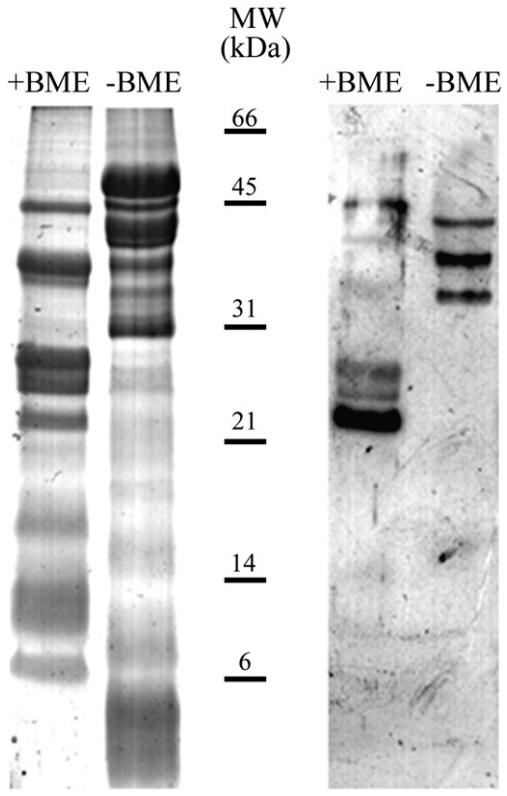

Fig. 3 – Comparison of CBB 250 (left part) and IgE immunodetection (right part) of hazelnut proteins separated under non-reducing (–β-mercaptoethanol, –BME) and reducing (+BME) conditions. CBB R250 Cys-reduced protein bands are assigned in the figure according to the estimated MWs. Immunodetection was performed with a serum pool from hazelnut-allergic patients. Under –BME conditions, three bands in the 35–60 kDa range were IgE-reactive, while only the ~21 kDa chain was recognized after disulfide reduction (+BME).

bands were common to those of the ~21 kDa protein. All together, these data indicated that the putative allergen is a disulfide-linked subunit of a larger protein and shares structural traits with the "canonical" 11S globulin-like protein of hazelnuts [8]. The occurrence of more than a single immunoreactive band could be attributed to a secondary N-terminal trimming subsequent to the proteolytic split of the mature protein, analogous to what has been described for the hazelnut 11S globulin [8]. Different from previous investigations about the immunogenic potential of hazelnut 11S globulin in which the acidic subunit has been described as the immunoreactive ones, we found that the IgE-reactive component was the alkaline subunit of the protein.

3.3. *Two-dimensional electrophoresis and IgE-immunoblotting*

To obtain a more exhaustive description of the hazelnut proteome, proteins were profiled by CBB stained 2DE (Fig. 4A). At least three protein spots in the 8.5 – 9.2 pI range were detected at an estimated mass of ~21 kDa. Immunoblotting analysis against a pool of 15 sera confirmed the immunoreactivity of the protein at ~21 kDa (Fig. 4B). A faint additional IgE reactivity against bands at 48, 25 and 7 kDa was also confirmed, and the immunoreactive spots were in-gel trypsinized and identified by *nano*-LC–MS/MS (Table 2). The 48 kDa band was identified as Cor a 11, the 25 kDa band was the basic chain of Cor a 9 and the 7 kDa band was Cor a 8. The IgE-affinity of the ~21 kDa spots, which quantitatively are much less represented than other storage proteins (Fig. 4A), was clearly the most intense. Both MALDI-TOF MS fingerprinting and *nano*-LC–MS/MS were unsuccessful in definitively identifying the immune-reactive ~21 kDa spots, most likely because of the incomplete database annotation of the hazelnut proteome and genome.

3.4. *Immunoblotting inhibition experiment*

IgE-binding was completely inhibited (Fig. 5) when an aliquot of the pool of 15 sera was pre-incubated with the purified ~21 kDa protein (~5 μg) (see below for the purification details), and no other IgE-reactive bands were detected as a consequence of the signal suppression.

3.5. *Purification of the hazelnut IgE-reactive protein*

The IgE-binding protein was purified by a sequential two-step chromatographic strategy. The first step was size-exclusion chromatography on a Sephadex 75 column. Based on the monitoring of the immunoblot (using sera of an allergic child) in the native chromatographic conditions, the IgE-reactive protein eluted in a broad peak containing proteins with estimated MWs ranging from 140 to 160 kDa, including hazelnut 11S albumin (Cor a 9) and the 48 kDa glycoprotein (Cor a 11) [19]. A complete separation of the IgE-binding protein was successively achieved with a C_8 RP-HPLC step (*not shown*). When the crude hazelnut extracts were reduced with β-mercaptoethanol, a single-step C_8 RP-HPLC analysis was sufficient to purify the ~21 kDa subunit in a sharp peak (Fig. 6). MALDI-TOF MS analysis of the purified subunit showed the occurrence of a cluster of protein isoforms with MWs centered at 20.7 kDa, having a mass difference compatible with a sequential N- or C-terminus cleavage or with other post-synthetic processing events (inset of Fig. 6).

3.6. *Partial de novo sequencing of the IgE-reactive protein*

The peptides arising from the tryptic digestion of the HPLC purified 20.7 kDa IgE-reactive subunit after Cys-alkylation with iodoacetamide were analyzed by MALDI-TOF MS (not showed). Despite the high quality of the spectra, the PMF identification of the protein was not definitive. The subsequent *nano*-HPLC–ESI-Q-TOF MS/MS analysis of the tryptic digest was unsuccessful in providing a definitive protein assignment in both the MS and MS/MS modalities of the proteomic search engines (Mascot and Batch-Web). The de novo sequences of several tryptic peptides which are listed in Table 3, were used for the identification of the protein by homology, using the BLAST algorithm and the MS-Pattern tool of Protein Prospector. The exemplificative MS/MS de novo sequencing of three peptides is shown in Fig. 7. Due to their identical molecular masses, the assignments of Ile or Leu in the sequences are only suggestive, and they have been designated for convenience

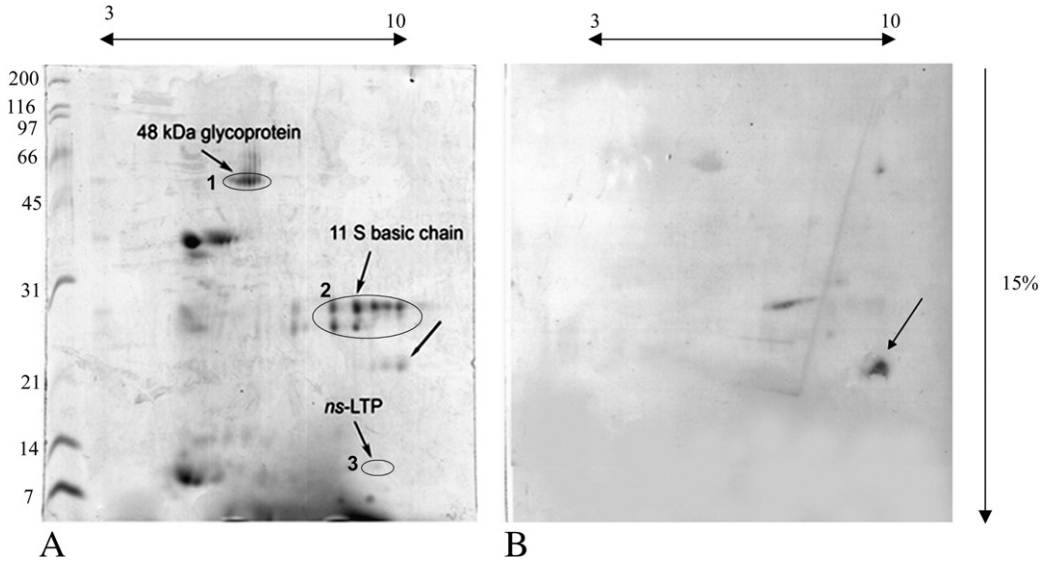

Fig. 4 – 2D IEF/SDS-PAGE of PBS-extracted proteins from Cv Mortarella stained with CBB (A) and corresponding immunoblot analysis with a serum pool (B). Immunoreactive spots were analyzed by MALDI-TOF MS-based peptide mass fingerprinting. The predominant IgE-binding protein was the ~21 kDa/pI ~9.0–9.2 spot. Faint IgE-immunoreactivity was also detected for the 48 kDa-glycoprotein (Cor a 11), the basic chain of 11S globulin albumin-like protein (Cor a 9) and the *ns*-LTP (Cor a 8).

according to the sequence of the top-ranking homologous peptides. The top-scoring proteins were 11S globulin-like proteins from several plant organisms and exhibited significant or high mutual homology. A significant homology degree is also shared by the peptide sequences with the "canonical" hazelnut 11S globulin, as evidenced by the partial sequence reconstruction of Fig. 8. In particular, the N-terminal peptide of the alkaline subunit of the canonical hazelnut 11S globulin, arising from the endo-proteolytic post-translational cleavage of the 56 kDa protein, appears fairly conserved [8]. Altogether, these results suggest that the immunogenic protein is a new, less-represented isoform of hazelnut 11S globulin (isoallergen).

3.7. *Analysis of the hazelnut skin-prick test solution*

The immunoreactive pattern of hazelnut extracts was compared by immunoblotting with that of an SPT solution used for the diagnosis of food allergies. When compared by CBB-stained 1D SDS-PAGE, the pattern of the SPT solution was quite different from that of the hazelnut extracts. This discrepancy can be attributed to the proteolysis of the SPT proteins induced by seed proteases, as well as to probable supplementation of recombinant allergens [15,16] and, more importantly, to the presence of stabilizing and denaturing/reducing agents. Nevertheless, when immunoassayed with the sera of children allergic to hazelnuts, the ~21 kDa protein was practically the unique IgE-reactive component of both the hazelnut extract and SPT solution (Fig. 9).

4. Discussion

Schocker et al. [20] described 5 SPT-positive patients with a history of anaphylaxis, angioedema and OAS to hazelnut whose sera were negative when assayed by immunoblotting with hazelnut extracts, thereby suggesting that in many cases the allergy could be triggered by weakly expressed allergens. Therefore, the allergic response to minor protein components of hazelnut was to be expected to some extent. According to our results, an alkaline 20.7 kDa subunit that has not been previously characterized, was the predominant IgE-reactive polypeptide of hazelnuts for all of the patients from Region Campania that were evaluated in this study. The new putative allergen is a minor component of the storage protein fraction of hazelnuts and shares homology with the alkaline subunit of canonical Cor a 9 (Fig. 8) as well as with several 11S globulin-like isoforms of other plants, as demonstrated by the alignment of the de novo sequenced peptides (Table 2). Similar to Cor a 9, which is composed of two subunits, one acidic (34.4 kDa, pI ~5.8) and one alkaline (22.2 kDa, pI ~9.0) [8], the immunoblotting analysis in non-reducing conditions demonstrated that the 20.7 kDa (pI ~9.0) IgE-binding subunit

Table 2 – *Nano*-HPLC–ESI-Q-TOF MS/MS identification of the IgE-immunoreactive protein spots.

Spots	Protein identification	Score	Peptide matches (unique)	Expected MW/pI	Accession	Allergen
1	48-kDa glycoprotein precursor	293	18 (14)	45072.6./6.05	Q8S4P9	Cor a 11
2	11S globulin-like protein basic chain	642	14 (5)	59127.3/6.5	Q8W1C2	Cor a 9
3	*Non-specific* lipid-transfer protein	48	2 (2)	9476.0/9.30	Q9ATH2	Cor a 8

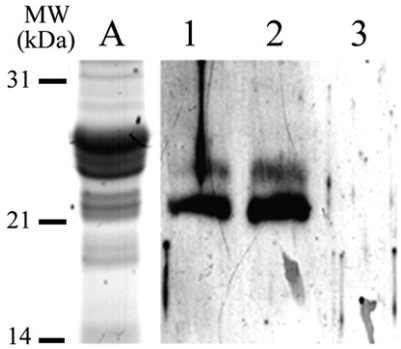

Fig. 5 – Immunoblot inhibition assay of the ~21 kDa IgE-binding protein. Immunodetection of the HPLC purified ~21 kDa subunit (1) and of a crude hazelnut extract with a serum pool (2) is compared with a crude hazelnut extract immunostained with a serum pool previously incubated with the purified ~21 kDa subunit (3).

is engaged in a disulfide bond with one or more protein chains (Fig. 3). Thus, the new putative allergen is the alkaline subunit of an isoform of the 11S like-globulin. The faint immune-reactivity that we observed for the "canonical" alkaline Cor a 9 subunit is most likely due to its homology with the 20.7 kDa IgE-binding subunit. Despite the large number of investigations carried out to identify hazelnut allergens, the new putative allergen has never been previously described, probably because it has never been characterized at either the protein or gene level. The current MS data provide a preliminary sequence of the protein, due to the incompleteness of the hazelnut genomic annotation. One of the most extensive screenings of the hazelnut IgE-binding proteins was performed by Pastorello et al. [1], who recruited 58 individuals allergic to hazelnuts from 3 Northern European centers and 7 from the Northern Italy. In this case, the immune-reactivity patterns were highly heterogeneous with no specific evidence of a ~21 kDa reactive band. Under the same experimental conditions used by these and others [1,9,11,12,18], we also obtained very complex recognition patterns in which all of the major storage proteins of hazelnuts exhibited a certain IgE-affinity. Under optimized blotting conditions (7 μg of proteins and 1:200 dilution of sera), IgE-affinity was essentially limited to the alkaline 20.7 kDa band. The canonical Cor a 9 has been described as the major non-pollen-related hazelnut allergen in the United States. Although, different from our results, the immunogenic region of Cor a 9

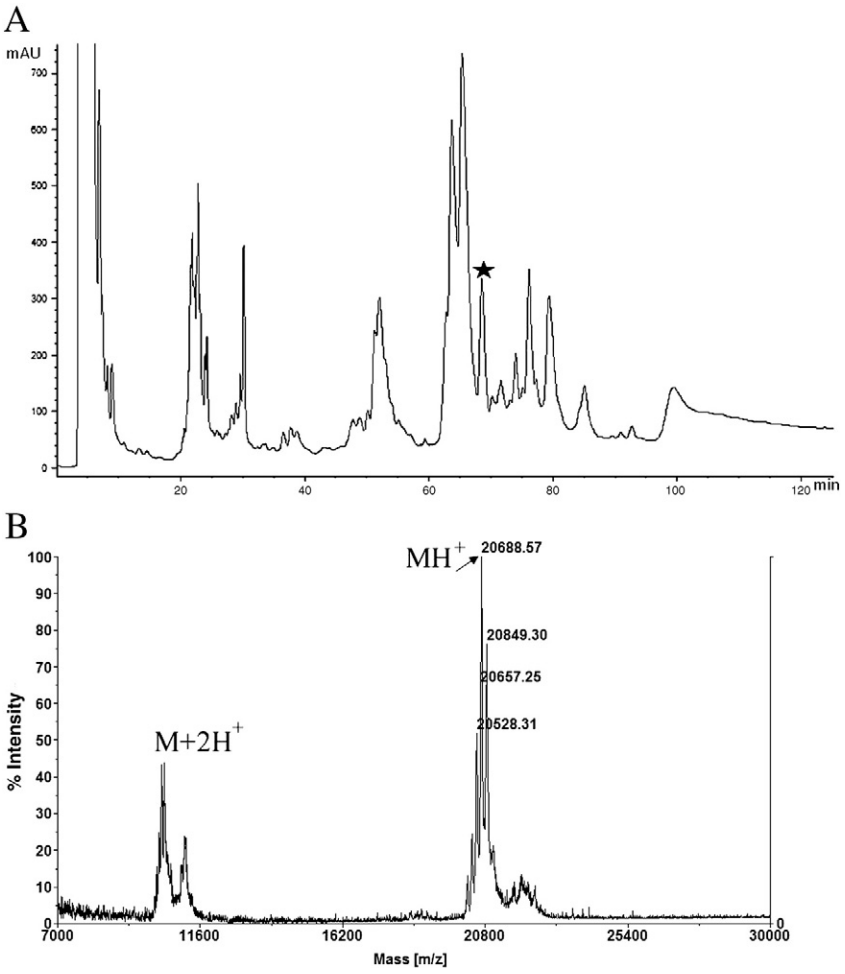

Fig. 6 – C8 RP-HPLC separation of the Cys-reduced hazelnut protein extract (panel A). The ~21 kDa subunit is eluted in a sharp peak labelled with the filled star. The MALDI-MS analysis of the intact subunit exhibited a cluster of signals centered at 20.7 kDa (panel B).

Table 3 – ESI-Q-TOF MS/MS de novo sequenced tryptic peptides of the 20.7 kDa putative hazelnut allergen and their homology with related 11S globulins from other plants. Amino acidic substitutions with respect to the sequenced peptides are highlighted in bold.

	$[M + H]^+$	De novo sequenced peptides	Top-ranking homologue protein	Notes
1	978.51	YIQLSAER	Identical sequence in gi|224062141 54.7 kDa predicted protein *Populus trichocarpa*	
2	1211.5	GVEETFCTLR	GVEETFCTAR in gi|224147821 56.0 kDa Predicted protein Cupin superfamily *Populus trichocarpa*	GLEETFCTLR in 56.3 kDa 11S globulins of *Ficus pumila* and GVEETFCTMR of *Ricinus communis*
3	1342.6	AGSQGFEWVSFK	identical sequence in gi|224126783 55.9 kDa predicted protein *Populus trichocarpa*	ASSDGFEWVSFK gi|156001070 in 53.2 kDa 11S globulin of *Pistacia vera*
4	1386.6	TNDNAQISQLAGR	identical sequence in gi|224147821 56.6 kDa Predicted protein Cupin superfamily *Populus trichocarpa*	TNDNAQISPLAGR in gi|75248608 in 59.1 kDa 11S globulin of *Corylus avellana* TNDNALINTLSGR in gi|122211016 in 52.7 kDa 11S globulin isoform 4 of *Sesamum indicum*
5	1389.8	ALPEDVLLNSYR	ALPEDALLSSTR in gi|34395154 hypothetical 21.9 kDa protein of *Oryza sativa*	
6	1400.7 1453.8	HDGQNLFDWLR ITSLNSLNLPILR	No significant homology ITSLNSLNLPILK in gi|171853010 53.3 11S allergen of *Pistacia vera*	

is the acidic subunit [8]. Interestingly, the allergenic potential of the alkaline 11S subunit has been already described and assessed for other seeds. Helm et al. identified and characterized a 22 kDa alkaline subunit of glycinin, an 11S globulin-like storage protein, as one of the major soybean (*Glycine max*) allergen (Gly m 6) [21,22]. Similarly, a 21 kDa alkaline 11S subunit, a homolog to soybean G2 glycinin, has been described as the primary allergen of both pistachios and cashew nuts [23].

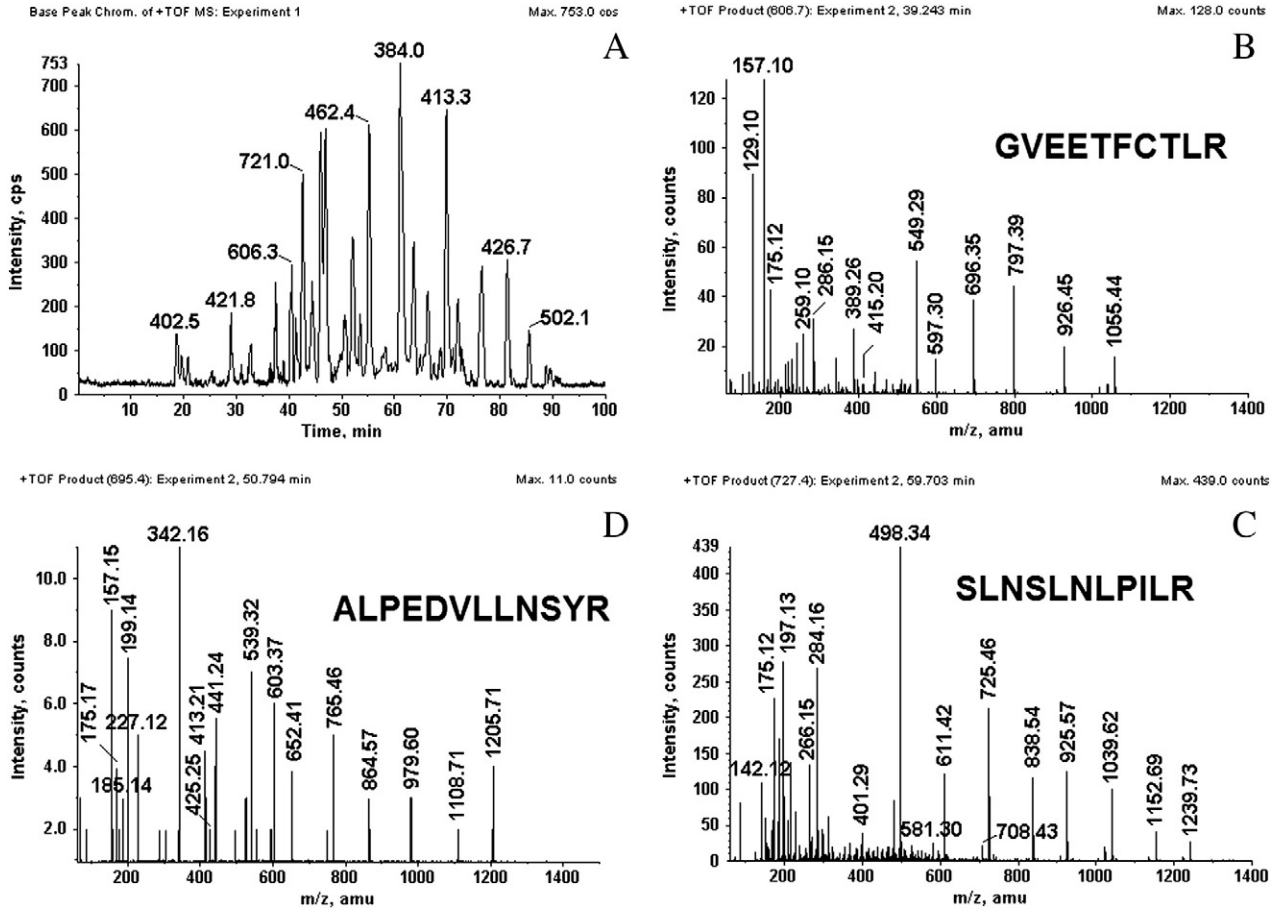

Fig. 7 – *Nano*-HPLC ESI MS separation of the tryptic peptides of the 20.7 putative allergen (A) and MS/MS-based de novo sequencing of selected peptides (B, C and D).

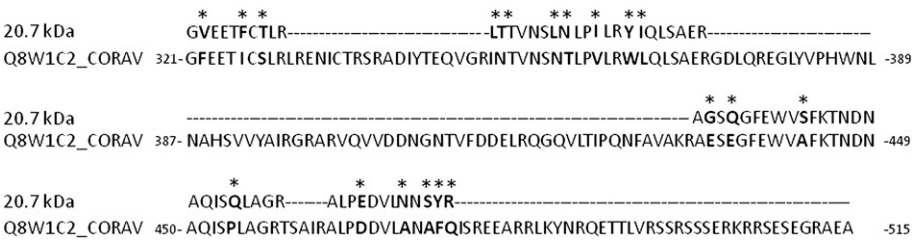

Fig. 8 – Alignment of de novo sequenced peptides of the putative 20.7 kDa allergen with the basic subunit of the canonical hazelnut 11S globulin (Cor a 9, Uniprot entry: Q8W1C2). The amino acid substitutions are highlighted in bold and labelled with an asterisk.

In particular, the allergen of pistachios shares several common peptide sequences with the 20.7 kDa hazelnut subunit (Table 3). Very recently, the basic subunit of the 11S legumin has been identified as the primary IgE binding component of chickpeas [24]. Having enrolled in the study many allergic children with multiple legume and nut allergies and because of its several highly conserved protein domains, the authors suggest that the alkaline subunit of 11S may underlie the IgE-cross-reactivity between nuts and legumes [24]. Recent findings demonstrate that sensitization to Cor a 9 is age-dependent [25]. Cor a 9 is the dominant sensitizing factor in infants (<1 year), while IgE-reactivity to Cor a 9 decreases later in life, even when the hazelnut allergy persists. The age-dependent sensitization to Cor a 9 has been assessed using microarrays (Phadia) with commercially available, purified (Cor a 9) or recombinant (Cor a 1 and Cor a 8) allergens [25]. The possible involvement of the 11S isoform in Cor a 9 sensitization cannot be excluded, as the two 11S isoforms are generally co-purified under ordinary separation conditions [19]. Indeed, we found that the 20.7 kDa subunit is also the dominant IgE-reactive component of the commercial SPT solution by Lofarma, the same used in this study to diagnose hazelnut allergies. However, due to the extensive proteolysis of the SPT solution, observed by MALDI-TOF analysis of the low-molecular-weight fraction (*not shown*), the occurrence of additional immunoreactive small polypeptides (<6 kDa) cannot be ruled out. Moreover, commercial SPT solutions generally have a diversified protein concentration/composition, and, therefore, other SPT extracts might provide different responses [14,15]. A clinical SPT screening for validating the allergenic potential of the purified 20.7 kDa protein and its larger protein precursor is in progress. The protein characterization remains to be completed either at the gene or protein expression levels, for instance, by constructing an opportune probe, based on the sequenced peptides, to clone the corresponding cDNA. The allergenic effects of this new 11S-globulin isoform should also be studied in allergic individuals from other geographical regions. The possible allergenic role of this protein presents a new opportunity for developing a component-resolved diagnostic of hazelnut allergy and evolved therapeutic intervention protocols.

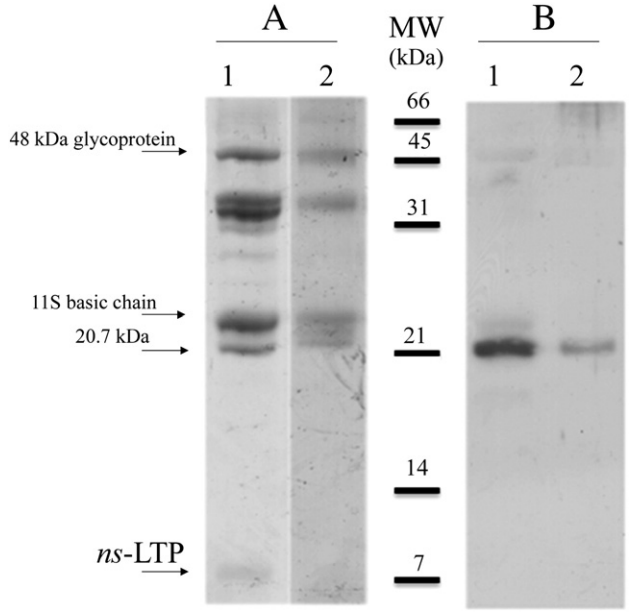

Fig. 9 – SDS-PAGE CBB R250 (A) and immunoblot (B) comparison of PBS hazelnut protein extracts (A1 and B1) and the Lofarma commercial skin prick test solution (A2 and B2).

Acknowledgments

This work was supported by a dedicated grant from the Italian Ministry of Economy and Finance to CNR and ENEA for the project "Innovazione e sviluppo del Mezzogiorno – Conoscenze Integrate per la Sostenibilità ed Innovazione del Made in Italy Agroalimentare" – Legge n.191/2009. The authors thank the American Journal Experts (www.journalexpert.com) for text revision.

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