

Type I allergy to elderberry (*Sambucus nigra*) is elicited by a 33.2 kDa allergen with significant homology to ribosomal inactivating proteins

E. Förster-Waldl*, M. Marchetti†, I. Schöll‡, M. Focke‡, C. Radauer‡, T. Kinaciyan§, I. Nentwich¶, S. Jäger||, E. R. Schmid†, G. Boltz-Nitulescu‡, O. Scheiner‡ and E. Jensen-Jarolim‡

*Department of Pediatrics and Juvenile Medicine, University of Vienna Medical School, Vienna, Austria, †Institute of Analytical Chemistry, University of Vienna, Vienna, Austria, ‡Department of Pathophysiology, University of Vienna Medical School, Vienna, Austria, §Department of Dermatology, University of Vienna Medical School, Vienna, Austria, ¶Institute of Clinical Immunology and Allergology, Masaryk University, Brno, Czech Republic and ||Department of Otolaryngology, University of Vienna Medical School, Vienna, Austria

Summary

Background Patients suffering from allergic rhinoconjunctivitis and dyspnoea during summer may exhibit these symptoms after contact with flowers or dietary products of the elderberry tree *Sambucus nigra*.

Objective Patients with a history of summer hayfever were tested in a routine setting for sensitization to elderberry. Nine patients having allergic symptoms due to elderberry and specific sensitization were investigated in detail. We studied the responsible allergens in extracts from elderberry pollen, flowers and berries, and investigated cross-reactivity with allergens from birch, grass and mugwort.

Methods Sera from patients were tested for IgE reactivity to elderberry proteins by one-dimensional (1D) and 2D electrophoresis/immunoblotting. Inhibition studies with defined allergens and elderberry-specific antibodies were used to evaluate cross-reactivity. The main elderberry allergen was purified by gel filtration and reversed-phase HPLC, and subjected to mass spectrometry. The in-gel-digested allergen was analysed by the MS/MS sequence analysis and peptide mapping. The N-terminal sequence of the predominant allergen was analysed.

Results 0.6% of 3668 randomly tested patients showed positive skin prick test and/or RAST to elderberry. IgE in patients' sera detected a predominant allergen of 33.2 kDa in extracts from elderberry pollen, flowers and berries, with an isoelectric point at pH 7.0. Pre-incubation of sera with extracts from birch, mugwort or grass pollen rendered insignificant or no inhibition of IgE binding to blotted elderberry proteins. Specific mouse antisera reacted exclusively with proteins from elderberry. N-terminal sequence analysis, as well as MS/MS spectrometry of the purified elderberry allergen, indicated homology with ribosomal inactivating proteins (RIPs).

Conclusion We present evidence that the elderberry plant *S. nigra* harbours allergenic potency. Independent methodologies argue for a significant homology of the predominant 33.2 kDa elderberry allergen with homology to RIPs. We conclude that this protein is a candidate for a major elderberry allergen with designation Sam n 1.

Keywords berries, elderberry flower, IgE, pollen, ribosomal inactivating protein (RIP), *Sambucus nigra*, type I allergy

Submitted 9 December 2002; revised 5 April 2003; accepted 25 July 2003

Introduction

The elderberry tree (*Sambucus nigra*) grows ubiquitously in regions of moderate climate and blossoms during the months of May to July. Some of its proteins, mainly from bark and from berries, have been widely studied because of their lectin activity [1] and for their use in analytical medicine [2–4]. Type I allergic reactions, after inhalative contact with pollen of the

elderberry plant, have been suspected by clinical allergologists during the last years. However, the description of elderberry allergy and analysis of the composition of elderberry allergens have not yet been achieved.

The elderberry plant belongs to the botanic family of Sambucaceae, previously Caprifoliaceae, which is unrelated to plant families already described to harbour allergenic potency, such as the Betulaceae, Rosaceae, Poaceae and Compositae. The fact that only little is known about the frequency of allergic reactions to elderberry might be attributable to its flowering season, which completely overlaps with the season of allergy to grass and weed pollen [5] [<http://www.pollenwarndienst.at/>]. Moreover, patients with

Correspondence: Prof. Dr Erika Jensen-Jarolim, Department of Pathophysiology, AKH, University of Vienna Medical School, Waehringer, Guertel 18-20, A-1090 Vienna, Austria.

E-mail: erika.jensen-jarolim@akh-wien.ac.at

summer pollinosis are often not sensitized to a single allergenic plant, but appear to be polysensitized. It thus seems likely that diagnosis of allergy against elderberry might be overseen or underestimated.

Contact with compounds of the elderberry plant does not only occur via inhalation of pollen, as the products of the elderberry tree are widely used in foods (juices) and in herbal medicine (teas) [http://www.herbmed.org/Herbs/Herb64.htm]. Elderberry extracts are traditionally used as household remedies, e.g. for the treatment of common colds postulating anti-infectious activity, although their mechanism of action was unclear for a long time. Only recently, proteins from the bark and the berries of elderberry trees were isolated and identified as ribosomal inactivating proteins (RIPs) [1]. Interestingly, RIPs display anti-infectious activity and, thus, are upcoming candidates for the therapy of viral infectious diseases of plants [6] or of humans [7, 8]. Furthermore, RIPs are currently tested for therapy of malignancies due to their anti-proliferative activity [9].

Our study demonstrates that patients may develop rhinoconjunctivitis and asthmatic symptoms after inhalation and ingestion of allergens from the elderberry plant. The aim of our work was to identify the allergens responsible for IgE reactivity in elderberry compounds, and to reveal possible cross-reactions with other allergenic plants flowering during the elderberry season. A detailed description of this novel allergy and definition of the elderberry allergens should help to diagnose the patients affected with pollinosis, and eventually notify them of possible immediate-type reactions after inhalation, ingestion or contacts during novel therapeutic regimens with elderberry compounds.

Materials and methods

Patients

Nine patients reporting symptoms consistent with type I allergy such as rhinoconjunctivitis and/or dyspnoea after inhalative or dietary contact with elderberry blossoms or dietary products (juice from flowers) were tested by skin prick test (SPT), and/or specific IgE antibodies were determined using the CAP-FEIA System (Pharmacia, Uppsala, Sweden) in order to diagnose specific allergy against elderberry. SPT was performed according to a standard procedure on the volar side of the forearm using an SQ elderberry pollen extract from ALK-Abello (Epipharm, Linz, Austria). The positive reference was histaminhydrochloride (10 mg/mL) [10].

Furthermore, data of 3668 elderberry SPT performed in a routine setting of diagnosis for type I allergic sensitization were evaluated, retrospectively, in order to estimate the frequency of elderberry sensitization.

Pollen counts

The overall exposition to elderberry pollen in the Vienna region was evaluated by pollen counting. As sampler a continuous, volumetric apparatus of Hirst type (Burkard) was used, with a flow rate at 10 L/min using vaseline as adhesive. Microscopic evaluation was performed daily with a

compound light microscope using a magnification of $\times 400$. The results are given in daily mean of pollen grains per cubic metre of air (corresponding to a surface area of at least 48 mm²). The annual period lasts from January 1 to September 30 (at least covering the start of pollination of *Alnus* and *Corylus* until the end of the *Artemisia* and *Ambrosia* period). The daily recording period was chosen from 00:00 to 24:00 hours. This method was approved by the EAACI Subcommittee 'Aerobiology and environmental aspects of inhalant allergens' (1995).

Extracts of elderberry pollen, flowers and berries

Flowers and berries from the elderberry plant were collected in the Vienna woods and in Upper Austria. Elderberry pollen was purchased from Allergon (Angelholm, Sweden). Protein extracts were obtained by grinding the flowers, berries or pollen in liquid nitrogen, and by overnight shaking at 4 °C in a potassium phosphate buffer, pH 7.2, described before [11]. After centrifugation at 40 000 *g* for 60 min at 4 °C, supernatants were extensively dialysed against ddH₂O with an MW-cut off of 8 kDa (Spectra/Por[®] CE, Spectrum Industries, Houston, TX, USA), lyophilized and stored at -20 °C. The protein concentration of the extracts was determined automatically in a Vitros 250 apparatus (Johnson & Johnson, Hallein, Austria) by the method of Bradford [12].

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to the protocol from Laemmli [13]. Briefly, 20 µL of elderberry extracts (pollen, flower, berry; 1 mg/mL) were heated to 95 °C for 5 min in a denaturing Laemmli buffer before loading them onto lanes of SDS-PAGE gels (15%) and were run in a vertical gel slab apparatus (Biometra, Göttingen, Germany). Protein masses were estimated by Coomassie brilliant blue staining of the gel and comparison with molecular weight markers (RPN 756 or RPN 800, Amersham Pharmacia Biotech, Buckinghamshire, UK). Electric-powered transfer of proteins [14] was performed onto nitrocellulose with 0.2 µm pore size (Protran[®], Schleicher&Schuell, Dassel, Germany) for subsequent Western blotting with human Ig or onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) for subsequent sequencing of the N-terminus of the elderberry allergen.

For IgE immunoblotting, unspecific binding of serum proteins to nitrocellulose strips was blocked by pre-incubation in a buffer of 50 mmol/L sodium phosphate (pH 7.5), 0.5% BSA and 0.5% Tween 20. Sera from patients were diluted 1:10 in blocking buffer, and blot strips were incubated at 4 °C. The binding of patients IgE to the blotted elderberry proteins was specifically detected by ¹²⁵I-rabbit anti-human IgE (CAP RAST system, Pharmacia-Diagnostics, Vienna, Austria), used 1:20 in blocking buffer.

Sera of elderberry-immunized mice (see below) were pooled at equal volumes and applied for immunoblotting in a 1:100 dilution. Bound murine antibodies were detected with ¹²⁵I-labelled sheep anti-mouse Ig antibody (Amersham Pharmacia Biotech). To visualize specific antibody binding (either human IgE or mouse Ig), blot strips were exposed to radiography films (Kodak Biomax MS, Rochester, NY, USA) at -70 °C for 3-5 days.

For inhibition experiments, sera were pre-incubated with excess amounts (50 µg) of extracts from birch (*Betula verrucosa*), grass (*Phleum pratense*), mugwort (*Artemisia vulgaris*) or elderberry pollen (*S. nigra*) (Allergen) or with 2 µg of recombinant allergens (rBet v1a, rPhl p1, rPhl p2 and rPhl p5), purchased from Biomay Prod. & Handelsges.m.b.H. (Vienna, Austria), overnight at 4 °C. Strips with blotted elderberry proteins were then incubated with these sera at 4 °C. The detection of patients' IgE binding was performed as described above.

Mouse immunization with elderberry extract

Three Balb/c mice were immunized intraperitoneally with 1 mg elderberry pollen extract using Al(OH)₃ as adjuvant, on days 0 and 14. Blood was drawn from the tail vein before and 3 weeks after the last immunization.

Two-dimensional (2D) gel electrophoresis

2D gel electrophoresis was performed according to protocols from Görg [15]. The first dimension, an immobilized linear pH gradient (IPG) strip from pH 3–10 (Immobiline DryStrip, 7 cm, Amersham Biosciences, Vienna, Austria), was rehydrated for 12 h in 125 µL of 8 M urea, 2% CHAPS, 2% IPG buffer pH 3–10 (Amersham Biosciences) and 7 mg DTT. The sample was applied during rehydration by dissolving the residue of 100 µL vacuum-dried protein extract in the rehydration solution. Isoelectric focusing was run at 20 °C for 11 h (0–200 V for 1 min, 200–500 V for 1 h, 500–3500 V for 1 h, 3500 V const. for 8 h). Before transferring the IPG strip onto the second dimension, the strip was equilibrated for 15 min in a buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 100 mg DTT/10 mL and traces of bromophenol blue. After equilibration, 1 cm of the strip was cut at the basic end to fit it into the 2D well of the 10% NuPAGE Bis-Tris gel, which was run with an MES running buffer (Invitrogen, Carlsbad, CA, USA). For molecular weight comparison, 10 µL SeeBlue[®] Plus2 pre-stained protein standards (Invitrogen) were applied to the second dimension. Immunoblotting of 2D-electrophoretic gels onto nitrocellulose sheets was performed as described above for 1D gels.

Amino acid sequencing of the N-terminus of the major allergen

For N-terminal amino acid sequencing, samples were separated by 1D or 2D SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore). The membrane was stained with 0.1% (w/v) Coomassie brilliant blue R 250 in 50% methanol. For localization of the antigenic protein, strips from the PVDF blots were tested with serum IgE. The bands on the PVDF membrane, which bound IgE, were excised and subjected to sequence analysis using a Procise 491 protein sequencing system (Applied Biosystems, Foster City, CA, USA). Protein samples were loaded directly onto the hydrophobic part of a biphasic sequencer column and subjected to Edman degradation using the manufacturer's Routine 3.0 chemistry.

Protein purification

The protein extract from elderberry pollen was loaded onto a Sephadex G50 chromatography column (Amersham Biosciences), equilibrated in 5 mM NaH₂PO₄, 5 mM Na₂HPO₄ (pH 7.4), 3% (v/v) glycerol, 4 mM DTT and 0.02% NaN₃. The protein concentration of fractions collected in minute intervals was determined by UV spectroscopy using an extinction coefficient of 0.785 for a 1 mg/mL solution at 280 nm, calculated according to Gill and von Hippel [16]. SDS-PAGE and Coomassie staining confirmed the purity and size of eluted proteins.

The sample containing the main allergen of approximately 33 kDa was applied to RP-HPLC for further purification. The chromatographic system consisted of a C4 reversed-phase column. A UV detector measured the protein elution at 280 nm. As mobile phase A, 1% TFA in H₂O was used, and the mobile phase B was 1% TFA/90% isopropanol. The protein eluting from the column with a single peak after 75 min was subsequently applied to mass spectrometric experiments.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

For exact molecular mass determination, the major allergen was applied to MALDI-TOF-MS. The mass spectrometer, a Kompact MALDI IV (Shimadzu Biotech, Kratos Analytical, Manchester, UK), was equipped with an N₂-laser (337 nm, pulse width 3 ns) and a 0.7 m flight tube. The positive ion mass spectrum, representing the average of 100 consecutive laser shots, was recorded in the linear mode with 24 kV extraction voltages. The purified protein sample was prepared with sinapic acid (saturated solution in acetonitrile–0.1% trifluoroacetic acid (TFA), 1/1, v/v) using the dried-droplet method [17]. One microlitre of matrix solution was deposited on a stainless-steel target and mixed on-target with 1 µL of the protein sample. The sample was allowed to dry at room temperature. Mass calibration was performed externally using the doubly and singly charged molecular ions of bovine serum albumin.

In-gel digestion, peptide map and sequence analysis

In-gel digestion was performed according to a protocol suitable for MS [18]. The Coomassie-stained protein bands were excised from the gel, cut into small cubes and washed three times with water for 5 min. Gel pieces were shrunk with acetonitrile and dried in a Speed Vac concentrator (Univapo 150H, Uniequip, Martinsried, Germany). Sulfhydryl groups of the protein were reduced with 10 mM DTT in 50 mM NH₄HCO₃ (pH 7.7) at 56 °C for 45 min and carboxymethylated with 55 mM iodoacetamide solution in 50 mM NH₄HCO₃ (pH 7.7) for 30 min in the dark. Gel pieces were washed three times with 50 mM NH₄HCO₃ (pH 7.7), shrunk with acetonitrile and vacuum dried again. Samples were reswollen in 20 µL 50 mM NH₄HCO₃ (pH 7.7) containing 12.5 ng trypsin (modified, sequencing grade, from bovine pancreas, Roche, Vienna, Austria) per 5 µL at room temperature for 15 min. Supernatants were discarded and replaced with 50 mM NH₄HCO₃ (pH 7.7), and then gel pieces were incubated overnight at 37 °C. The next day, the

Table 1. Characterization of patients with clinical and serological reactivity to elderberry

Patients	History of pollinosis*	Total IgE (kU/L)	Symptoms†	SPT‡	IB§	Contact¶
1	Grass	606	RC, A	n.d.	+	Inhalative
2	Grass	4080	RC, A	++	+	Inhalative
3	Grass	n.d.	RC	n.d.	+	Inhalative
4	Grass, weed, conifers	193	RC, A	++	+	Inhalative
5	Grass	178	RC	++	+	Inhalative
6	Grass, birch	90	RC	+	+	Inhalative
7	Birch, grass, weed	54	RC	+	+	Inhalative
8	Birch, grass, weed	267	RC, A	++	+	Inhalative
9	Grass, weed	n.d.	RC, W	n.d.	+	Inhalative and ingestion

*Type I allergy was diagnosed by a positive case history, skin prick test (SPT) and RAST. †RC: rhinoconjunctivitis; A: asthma; W: wheezing after contact with *Sambucus nigra*. ‡SPT performed with a commercial elderberry pollen extract. §Immunoblots revealed IgE binding to the 33.2 kDa elderberry allergen. ¶Exposition route to elderberry allergens. n.d., not determined.

supernatant was removed and saved, and the tryptic peptides were extracted from the gel by incubating the gel three times with acetonitrile–0.1% TFA (1 : 1, v : v). The extracts and the previously removed supernatant were combined, vacuum dried and Zip-Tip C₁₈ (Millipore, Vienna, Austria) purified. Positive ion masses of the tryptic digest were generated on an AXIMA-CFR (Shimadzu Kratos Analytical) instrument, equipped with a nitrogen laser emitting at 337 nm. Mass spectra were recorded in the linear mode with the extraction voltage set to 20 kV. For improved resolution, the time-delayed extraction technique was applied. Every mass spectrum represents the average of 200 consecutive laser shots. α -Cyano-4-hydroxy cinnamic acid (6 mg/mL in acetone) was used as the matrix. One microlitre of the Zip-Tip C₁₈ purified peptide solution was applied on a pre-crystallized matrix spot [17]. For sequence analysis of some selected peptides, a nano-electrospray ionization quadrupole reflectron mass spectrometer (QStarTM Pulsar Plus, Sciex, Canada) was used. Doubly and triply charged positive ions were chosen as precursor ions for the MS/MS experiments.

Database search

Peptide mass fingerprinting was performed using the on-line protein identification tool Mascot[®] (Matrix Science Ltd, London, UK). Sequence alignment was analysed by submitting the data generated by Edman degradation and MS/MS experiments to BLAST[®], a search tool provided by the National Centre for Biotechnology Inf. (NCBI, Bethesda, MD, USA).

Results

Patients' characteristics

Patients' type I allergic symptoms and laboratory findings are detailed in Table 1. All the patients tested had reported rhinoconjunctivitis and/or dyspnoea after contact with elderberry blossoms; in addition, patient 9 reported hoarseness and upper-airway obstruction after drinking elderberry juice. The patients were examined serologically (CAP-FEIA)

and by SPT for specific sensitization. All the patients suffered from additional allergies, mostly to grass pollen and weeds.

On retrospective analysis of 3668 elderberry SPT from an out-patient allergy clinic, positive type I skin reactions were found in 0.654% (in total 24 patients)

Pollen counts

The peak value of *S. nigra* pollen in long-term average in the Vienna region was 40/m³ (years 1976–2002), with peak values from mid-May until mid-June. In the year 1992, the peak value even reached 167/m³. This represents at least a quarter of the amount of grass pollen in the same region (111/m³ in the year 2001 and 188/m³ in the year 2002).

Patients' IgE binding to blotted elderberry extracts

Coomassie staining of SDS-PAGE-separated extracts from elderberry pollen, flowers and berries (Fig. 1, lanes C) shows that a relatively broad range of proteins is embodied in the extracts. Testing patients' sera for their IgE-binding profile in immunoblots revealed that all nine patients exhibited IgE-binding predominantly directed towards an allergen at approximately 33 kDa, present in pollen, flower and berry extracts. IgE bindings of three representative sera are shown in Fig. 1.

The identity of the 33 kDa allergen in pollen, berries and flowers was demonstrated by testing a monospecific serum-pool from mice immunized with elderberry pollen extract on all elderberry extracts (data not shown).

Cross-reactivity

To study cross-reactivity with pollen allergens from non-related plants, patients' sera were pre-incubated with pollen extracts or recombinant allergens. Only partial inhibition of specific IgE binding to an elderberry blot was achieved by pre-incubation with extracts from birch (*B. verrucosa*) and mugwort pollen (*A. vulgaris*) (Fig. 2, panel a). Pre-incubation with Bet v 1 had no effect (data not shown). Inhibition of IgE binding to an elderberry blot was not seen either after pre-incubation with grass (*P. pratense*) pollen extract (Fig. 2, panel a), nor by pre-incubation with the recombinant major

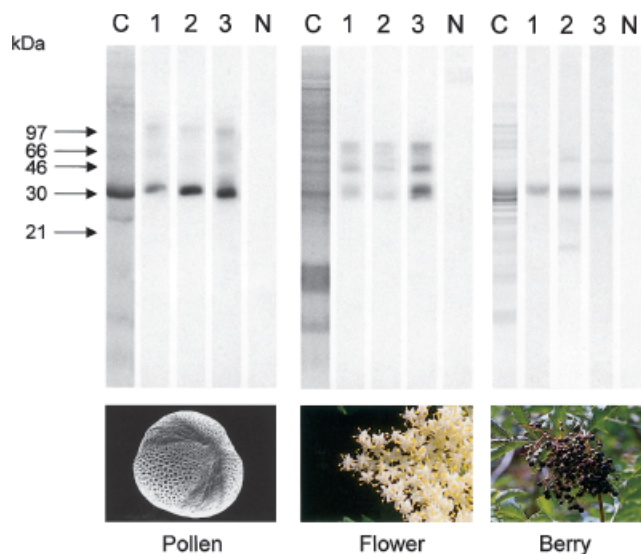


Fig. 1. Analysis of protein extracts from elderberry pollen, flowers or berries by SDS-PAGE and immunoblotting. Lanes C: Coomassie staining of the gels. Lanes 1–3: IgE binding of representative patients' sera in immunoblot experiments. Lane N: serum of a non-allergic volunteer. Sera were diluted 1 : 10 and bound IgE was detected by ^{125}I -labelled anti-human IgE antibody. The autoradiograms are shown. The bottom panels show a scanning electron microscopy picture of the *Sambucus nigra* pollen, and photographs of elderberry blossoms and berries used for extract preparations.

allergens (Phl p1, Phl p2, Phl p5) (data not shown). Inhibition with elderberry pollen extract itself was taken as a positive control (lane E, panel a). The possible cross-reactivities were further investigated with mouse anti-elderberry sera. Elderberry-specific mouse sera contained Ig reacting with the 33 kDa and a 25 kDa elderberry antigen, and not with birch, grass or mugwort pollen allergens (Fig. 2b).

2D gel electrophoresis

The physicochemical properties of the 33 kDa elderberry pollen allergen were further analysed by 2D electrophoresis (Fig. 3). Coomassie protein staining of 2D-electrophoretic gels revealed a number of spots scattered between pI 5 and pI 7 at an approximate molecular mass of 33 kDa (data not shown). The most prominent binding of human IgE occurred

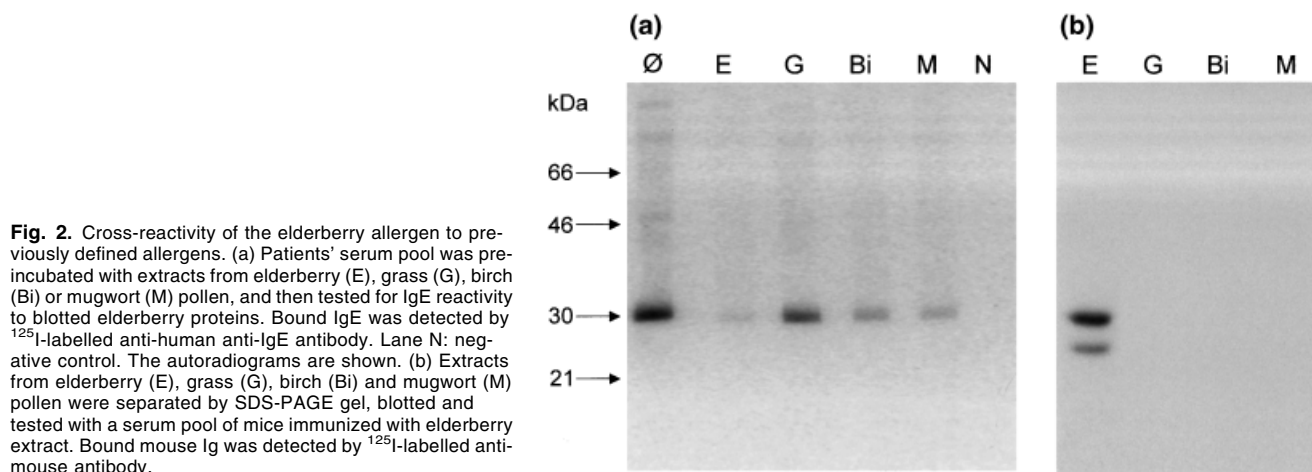
to a protein spot at pI 7, with fuzzy shadings presumably indicating glycosylation of the protein (Fig. 3).

N-terminal sequencing

1D- and 2D-separated elderberry extracts were blotted onto PVDF membranes and Coomassie stained. Subsequent Edman degradation of the 33.2 kDa allergen yielded the N-terminal amino acid sequence RDYPFTSRISGGD. Database search for homologous sequences using BLAST revealed, beside others, a protein that is a partial peptide of the N-terminus of the β -chain of Ebulin-1, a non-toxic type 2 ribosome-inactivating protein (GenBank database entry gi/409349/gb/AAB27855) from the plant *Sambucus ebulus*, belonging to the elderberry family of Sambucaceae. As can be seen from Table 2, seven of the 13 assigned amino acids are identical.

Purification and MS of the elderberry allergen

In a first step of purification, the protein was applied to gel filtration. One protein fraction obtained after gel filtration was highly enriched in the 33 kDa allergen, as demonstrated by Coomassie staining and IgE immunoblotting (data not shown). The 33 kDa protein fraction was further applied on RP-HPLC, and a single major peak of protein was eluted from the column after 75 min. Coomassie staining of an SDS-PAGE gel confirmed the purity of this protein (data not shown). In MS on a linear MALDI-TOF-MS, the molecular mass of the allergen was determined to be 33 214 Da ($\pm 0.1\%$, Fig. 4). In-gel digestion with trypsin of the 33.2 kDa protein and subsequent MALDI-TOF-MS analysis (Fig. 5) followed by nano-ESI MS/MS analysis was performed. Selected tryptic peptides exhibited a sequence identity with RIPs, which were recently cloned from the fruit and the bark of the elderberry tree [19, 20]. Notably, sequencing of the 986.38 Da peptide rendered a partial sequence (DYPFTS), which was identical to the N-terminus obtained by Edman degradation homologous to a RIP from *S. ebulus* (see above). Furthermore, database searches with the partial amino acid sequences of the peptides with the protonated molecular weights of 804.54 Da (QSDVSLR), 933.50 Da (LCVDV), 986.38 Da (DYPFTS), 1608.27 Da (QWTFDKDGDV), 1704.93 Da (ALYGD) and 1957.70 Da (TGPNPQQW)



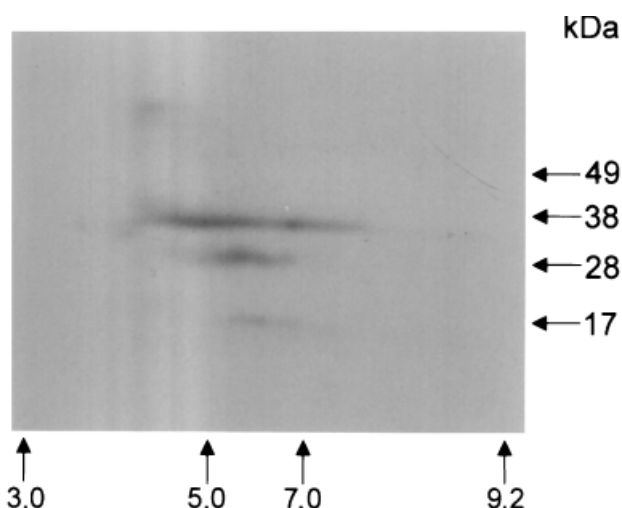


Fig. 3. Elderberry extract was separated by 2D electrophoresis for subsequent immunoblotting. Electrophoretic separation in the first dimension was carried out in a pH gradient (pH 3–10), and in the second dimension in a 15% SDS-PAGE gel. The blot was tested with a serum pool composed of equal volumes of patients' sera (diluted 1 : 10). Bound IgE was detected by ^{125}I -labelled anti-human anti-IgE antibody.

showed significant homologies with RIPs originating from *S. nigra* (Table 2).

Discussion

The list of elicitors of type I allergy is continuously increasing [11, 21–25]. We report on type I allergy to proteins of *S. nigra*, a ubiquitous elderberry tree that blossoms over a period of

approximately 2 months during the early summer season. We provide evidence that exposition to elderberry pollen is of relevance as the peak value pollen counts of *S. nigra* pollen reach as much as a quarter of peak values of grass pollen counts in the Vienna region. Furthermore, 0.6% of patients diagnosed with type I allergic symptoms in the early summer season exhibited positive SPTs and/or positive RAST assays against elderberry. Besides inhalative contact with elderberry pollen, patients may also be exposed to this allergen via the oral route, as elderberry flowers and fruits have been used in plant remedies and in foods since centuries. The clinical history of one of our patients underlines this importance, as she developed upper-airway obstruction when drinking elderberry juice.

In the present study, we intended to characterize the molecules causative for type I allergy to elderberry. Strikingly, out of numerous proteins of the extracts, one predominant distinct protein from elderberry pollen, flowers and berries could be defined as allergenic. We suggest that this compound is also responsible for the clinical symptoms. Few allergenic plants display a comparable profile containing a single major allergen: e.g. Betulaceae contain Bet v 1, which is relevant for IgE binding of more than 95% of the birch pollen-allergic patients, and for 60% of them, represents the only relevant IgE ligand [26]. In this context, numerous therapeutic approaches have been suggested on this single allergen, including hypoallergenic isoforms, mutants or mimotopes [27–29]. It is tempting to speculate that the prominent 33.2 kDa elderberry allergen could be a worthy tool for immunotherapy, given that an ample number of patients make the development of such a vaccine reliable. In this respect, the epidemiological data for the overall impact of this allergen are clearly missing, maybe due to the fact that a number of important allergenic plants flower during the

Table 2. Alignment of the 33.2 kDa elderberry allergen partial sequences with previously published RIPs and RIP homologues from *Sambucus nigra* or *S. ebulus*

Name	Data base Entr.	aa 35–46	345–349	371–380	557–561	619–625	632–640
<i>S. nigra</i> RIP precursor -	gi 1754981 gb A -	-DYPVS S FNLDGA	LCVDV	RWTFDSDDTI	ALYGD	ASN V SLR	ATGN P NQ QW
<i>S. nigra</i> RIP precursor	gi 1929497 gb A -	PNYP S VS L KMAGA	LCVDV	QWTFRTDGTI	ALYGD	QSD V SLR	PTGN L NQ QW
<i>S. nigra</i> agglutinin I prec.	gi 2130006 pir -	PVYP S VS F NLTGA	LCVDV	LWTFRTDGTI	ALYGD	QRD V SLR	PTGN P NQ QW
<i>S. nigra</i> RI P	gi 15625503 gb -	-DYPVS S FNLDGA	LCVDV	RWTFDSDDTI	ALYGD	ASN V SLR	ATGN P NQ QW
<i>S. nigra</i> le ctin	gi 15625501 gb -	-DYPVS S FNLDGA	LCVDV	RWTFYTD D TI	ALFGD	ESD V SLR	YHGD P NQ QW
<i>S. ebulus</i> RIP precursor	gi 13171073 emb -	-DYPVS S FNLAGA	LCVDV	QWTFYNDKTI	ALFDD	ESD V SLQ	ATGN P NQ QW
<i>S. ebulus</i> type 2 RIP N-terminal / partial peptide	gi/409349/gb/	-PAP F TRRIVGXD					
nano-ESI MS/MS		-DYP F TS	LCVDV	QWTFDKDGDV	ALYGD	QSD V SLR	-TGN P NQ QW
N-termin al sequen cing		RDYP F TSRISGGD					
Consensus		* . . : :	*****	*** . * :	** : . *	:***:	* : ****

Amino acid (aa) homologies of MS/MS sequenced tryptic peptides of the 33.2 kDa allergen with *S. nigra* RIPs are highlighted in red and aa homologies of its N-terminus with an *S. ebulus* type 2 RIP are indicated in blue. The consensus points out identities with an asterisk (*), high-grade familiarity with a colon (:) and simple familiarity with a dot (.).

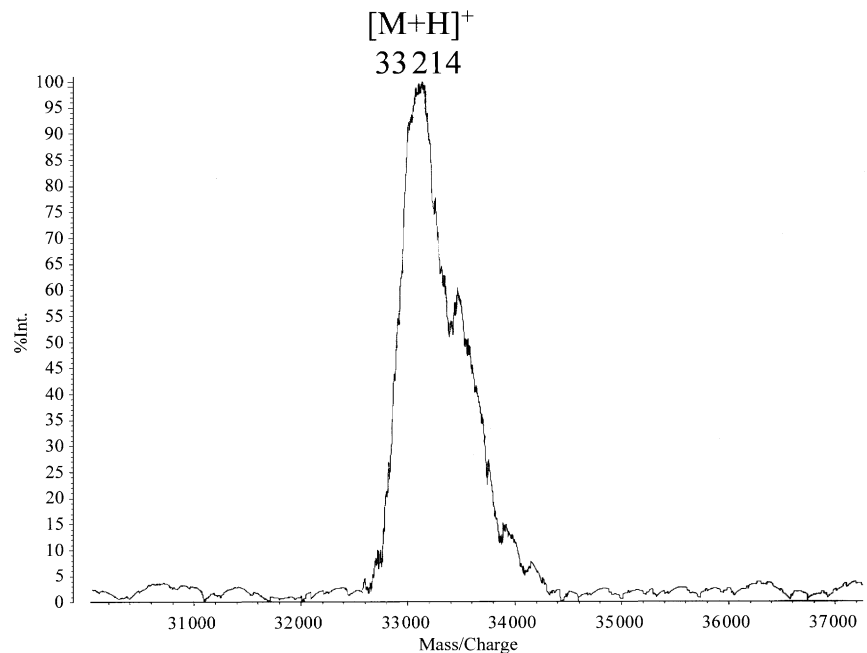


Fig. 4. Positive ion mass spectrum obtained on a linear MALDI-TOF-MS showing the exact molecular mass of the predominant elderberry allergen at 33 214 Da ($\pm 0.1\%$ mass accuracy).

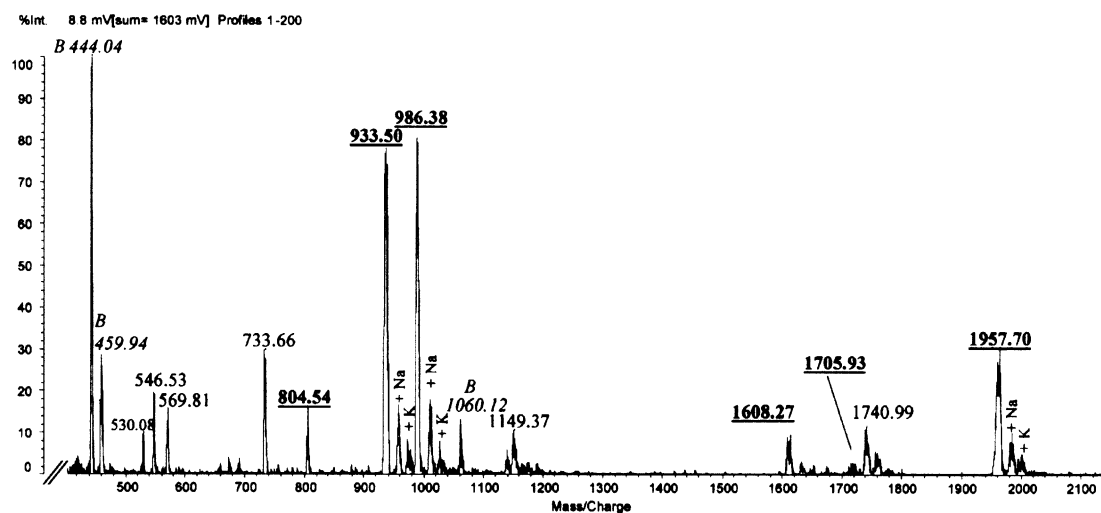


Fig. 5. MALDI-IDE-TOF mass spectrum of an in-gel digest of the 33.2 kDa protein. Ions with *m/z* values italicized and marked with 'B' are present in the blank gel or derived from the background, whereas *m/z* values highlighted in bold and underlined were further sequenced with nano-ESI MS/MS; the displayed masses represent the $[M+H]^+$ ions of the peptides.

elderberry season, camouflaging elderberry allergy. Once the diagnosis of allergy to grass or weed is established, the relevance of additional sensitization might be underestimated.

Cross-reactivity between allergens has already been demonstrated between a large number of plants and fruits from botanically related or unrelated sources [30–32]. These phenomena have been explained by the occurrence of phylogenetically conserved proteins in distinct plant species, which harbour similar T cell and B cell epitopes.

As in all our patients, besides elderberry allergy, grass pollen and other inhalative allergies had also been diagnosed clinically and serologically, we postulated cross-reactivity to elderberry allergens at the beginning of our investigations.

However, only partial inhibition of IgE binding to elderberry proteins with birch or mugwort pollen, and no inhibition with grass pollen allergens, could be demonstrated. Furthermore, elderberry-specific mouse Ig detected no cross-reactive compounds in either of the extracts, indicating that the 33.2 kDa elderberry allergen represents a novel allergen, Sam n1. These data were further strengthened as (1) sequence alignment of the N-terminus of the 33.2 kDa elderberry allergen did not reveal homology with any defined allergens, but with a RIP previously found in the plant *S. ebulus*, and (2) MS and sequence analysis of tryptic fragments rendered significant sequence homologies with RIPs previously detected in the bark and fruits of the *S. nigra* plant [19, 20].

These peptides were mapped throughout the C-terminal part of various selected elderberry RIPs, which is highly conserved within this plant protein family.

We conclude that the 33.2 kDa elderberry allergen represents a RIP, and this protein is a candidate for the major *S. nigra* allergen, Sam n 1. Conversely, we present evidence that RIPs harbour allergenic properties. This observation might be of immediate importance, because due to their anti-mitogenic and anti-proliferative activity, the RIPs family has gained continuous interest as possible anti-viral and anti-cancer therapeutics [6, 9].

Acknowledgements

E. Förster-Waldl was supported by the Charlotte-Bühler scholarship H176 of the Austrian National Science Fund (FWF). This work was mainly supported by Grant SFB F018 #8 of the FWF and in part by Grant P14181 of the FWF.

We are indebted to the team of the Allergieambulatorium Rennweg, Vienna, for providing retrospective data of the Skin Prick Tests, and we thank Dr Heidemarie Halbritter, Institute of Botany, University of Vienna, Austria, for providing the scanning electron microscopy picture of the *S. nigra* pollen. We thank Ing. Magdolna Vermes for excellent technical assistance.

References

- Van Damme EJ, Peumans WJ, Barre A, Rouge P. Plant lectins. A composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. In: Critical reviews in plant sciences. Boca Raton, FL, 1998; 575–692.
- Baum LG, Derbin K, Perillo NL, Wu T, Pang M, Uittenbogaart C. Characterization of terminal sialic acid linkages on human thymocytes. Correlation between lectin-binding phenotype and sialyltransferase expression. *J Biol Chem* 1996; 271:10793–9.
- Chintalacharuvu SR, Emancipator SN. The glycosylation of IgA produced by murine B cells is altered by Th2 cytokines. *J Immunol* 1997; 159:2327–33.
- Tong HH, James M, Grants I, Liu X, Shi G, DeMaria TF. Comparison of structural changes of cell surface carbohydrates in the eustachian tube epithelium of chinchillas infected with a *Streptococcus pneumoniae* neuraminidase-deficient mutant or its isogenic parent strain. *Microb Pathogen* 2001; 31:309–17.
- Durham S. ABC of allergies. Summer hay fever. *BMJ* 1998; 316:843–5.
- Chen Y, Peumans WJ, Van Damme EJ. The *Sambucus nigra* type-2 ribosome-inactivating protein SNA-I' exhibits in planta antiviral activity in transgenic tobacco. *FEBS Lett* 2002; 516:27–30.
- Au TK, Collins RA, Lam TL, Ng TB, Fong WP, Wan DC. The plant ribosome inactivating proteins luffin and saporin are potent inhibitors of HIV-1 integrase. *FEBS Lett* 2000; 471:169–72.
- Wang YX, Jacob J, Wingfield PT et al. Anti-HIV and anti-tumor protein MAP30, a 30 kDa single-strand type-I RIP, shares similar secondary structure and beta-sheet topology with the A chain of ricin, a type-II RIP. *Protein Sci* 2000; 9:138–44.
- Citores L, Miguel Ferreras J, Munoz R, Benítez J, Jimenez P, Girbes T. Targeting cancer cells with transferrin conjugates containing the non-toxic type 2 ribosome-inactivating proteins nigrin b or ebulin I. *Cancer Lett* 2002; 184:29–35.
- Sibbald B, Barnes G, Durham SR. Skin prick testing in general practice: a pilot study. *J Adv Nurs* 1997; 26:537–42.
- Jensen-Jarolim E, Gerstmayer G, Kraft D, Scheiner O, Ebner H, Ebner C. Serological characterization of allergens in poppy seeds. *Clin Exp Allergy* 1999; 29:1075–9.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976; 72:248–54.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotechnology* 1992; 24:145–9.
- Görg A, Postel W, Gunther S. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1988; 9:531–46.
- Gill SC, von Hippel PH. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 1989; 182:319–26.
- Kusmann M, Roepstorff P. Sample preparation techniques for peptides and proteins analyzed by MALDI-MS. *Methods Mol Biol* 2000; 146:405–24.
- Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996; 68:850–8.
- Van Damme EJ, Roy S, Barre A, Rouge P, Van Leuven F, Peumans WJ. The major elderberry (*Sambucus nigra*) fruit protein is a lectin derived from a truncated type 2 ribosome-inactivating protein. *Plant J* 1997; 12:1251–60.
- Van Damme EJ, Barre A, Rouge P, Van Leuven F, Peumans WJ. Isolation and molecular cloning of a novel type 2 ribosome-inactivating protein with an inactive B chain from elderberry (*Sambucus nigra*) bark. *J Biol Chem* 1997; 272:8353–60.
- Rodriguez M, Vega F, Garcia MT et al. Hypersensitivity to latex, chestnut, and banana. *Ann Allergy* 1993; 70:31–4.
- Gall H, Kalveram KJ, Forck G, Sterry W. Kiwi fruit allergy: a new birch pollen-associated food allergy. *J Allergy Clin Immunol* 1994; 94:70–6.
- Blanco C, Carrillo T, Castillo R, Quirarte J, Cuevas M. Avocado hypersensitivity. *Allergy* 1994; 49:454–9.
- Jensen-Jarolim E, Leitner A, Hirschwehr R et al. Characterization of allergens in Apiaceae spices: anise, fennel, coriander and cumin. *Clin Exp Allergy* 1997; 27:299–306.
- Reider N, Sepp N, Fritsch P, Weinlich G, Jensen-Jarolim E. Anaphylaxis to camomile: clinical features and allergen cross-reactivity. *Clin Exp Allergy* 2000; 30:1436–43.
- Jarolim E, Rumpold H, Endler AT et al. IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 1989; 44:385–95.
- Ferreira F, Hirtenlehner K, Jilek A et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996; 183:599–609.
- Breitenbach M, Ferreira F, Jilek A et al. Biological and immunological importance of Bet v 1 isoforms. *Adv Exp Med Biol* 1996; 409:117–26.
- Ganglberger E, Sponer B, Schöll I et al. IgE mimotopes are safe for therapy of type I allergy when presented as monovalent fusion proteins. *FASEB J* 2001; 15:254–56.
- Valenta R, Duchene M, Ebner C et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; 175:377–85.
- Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 2000; 106:27–36.
- Mikkola JH, Alenius H, Kalkkinen N, Turjanmaa K, Palosuo T, Reunala T. Hevein-like protein domains as a possible cause for allergen cross-reactivity between latex and banana. *J Allergy Clin Immunol* 1998; 102:1005–12.