



Differential sensitivity of D-galactose-binding lectins from fruits of dwarf elder (*Sambucus ebulus* L.) to a simulated gastric fluid

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ABSTRACT

Some lectins from *Sambucus* spp. share amino acid sequences with the pollen Sam n1 allergen. The lectins ebulin f and SELfd from the early stages of growth were isolated and subjected to analysis by MALDI-TOF mass spectrometry, tryptic peptide fingerprinting, molecular characterization and pepsin digestibility. The molecular mass (33.214) and other structural features of the Sam n1 allergen fit best with a monomeric lectin like SELlm (Mr 34.2 kDa) found in shoots of dwarf elder. Ebulin f toxicity to mice was higher intraperitoneally than orally at the same dose (5 mg/kg body weight). In contrast SELfd at the same dose lacks of apparent toxicity. Ebulin f, but not SELfd, undergoes extensive pepsin proteolysis, which could explain the differences in toxicity. The present study supports our hypothesis that the Sam n1 allergen could be a sequence-related monomeric lectin like SELlm present in shoots of *Sambucus ebulus* rather than ebulin.

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1. Introduction

Different reports in recent years have suggested a close relationship between the presence of allergens in pollen and foods derived from plants and the allergic response (Ballmer-Weber, 2011; Vieths, Scheurer, & Ballmer-Weber, 2002). Astwood, Leach, and Fuchs (1996) studied the digestive stability of a major group of food allergens and non-allergic enzymes using a simulated gastric fluid containing acidic pepsin. They found that allergenic proteins were more stable than non-allergenic ones and concluded that digestibility could be a good means to distinguish them. However, other authors have reported that low digestibility is not an absolute criterion for food protein allergens since allergens from the same plant, for instance of various allergens contained in peanut (*Arachis hypogaea*) (Koppelman, Hefle, Taylor, & de Jong, 2010), display different stability patterns towards pepsin digestion. Consequently, resistance to pepsin digestion would be insufficient to define the potential allergenicity of a protein (Koppelman et al., 2010).

Among the pollen allergens that may trigger type I allergy is Sam n1, isolated from elderberry (*Sambucus nigra* L.), which has been proposed as a type 2 ribosome-inactivating protein (Förster-Waldl et al., 2003). Since elderberry is fairly ubiquitously

distributed, allergenicity derived from exposure to its pollen driven by air during blossoming and allergy to elderberry products and derivatives used as food and in medicine could be of interest not only from a general point of view of allergies but also from a food allergy point of view. Berries from *S. nigra* and *Sambucus ebulus*, also known as dwarf elder, have been consumed since the Neolithic. In fact, remains in France and Italy more than 5000 years old have been associated with their consumption as food and for medicine (Martin, Jacomet, & Thiebault, 2008). In modern times, all parts of elder have been used for food or food complements. *Sambucus* spp. contains a variety of compounds that display pharmacological activities such as flavonoids, vitamins and lectins (Girbes, Ferreras, Arias, & Stirpe, 2004; Kaack & Austed, 1998; Veberic, Jakopic, Stampar, & Schmitzer, 2009; Vlachoianis, Cameron, & Chrubasik, 2010). Antiviral, anti-inflammatory and healing activities have been described in *Sambucus* spp., and the responsible molecules have been identified (Roschek, Fink, McMichael, Li, & Alberte, 2009; Sunter et al., 2010).

The lectins are proteins with sugar-binding ability and usually sugar specificity and cell-agglutinating proteins ubiquitous and found in all kinds of organisms (Sharon, 2008). Many lectins are glycosylated and display allergenicity through both the protein and the sugar chain moieties (van Ree, 2002; van Ree et al., 2000). *Sambucus* lectins with protein synthesis inhibitory activity have been detected and isolated from *S. nigra*, *Sambucus racemosa* and *Sambucus mexicana* (Girbes et al., 2004). Nigrin isoforms from bark (nigrin b), fruits (nigrin f) and seeds (nigrin s) have been

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isolated from *S. nigra* (Citores et al., 1996, 1994). Ebulin isoforms from fruits (ebulin f), leaves (ebulin l), and rhizomes (ebulins r1 and r2) have been isolated from *S. ebulus* (Citores et al., 1997, 1998; Girbes et al., 1993; Pascal et al., 2001). Nigrins and ebulins have two chains: namely the A chain, with N-glycosidase activity (E.C.3.2.2.22) on 28S rRNA, which is responsible for the irreversible inactivation of ribosomes, and a B chain, with D-galactose-specific lectin activity. Therefore, they are known as type 2 (two chains) ribosome-inactivating proteins, or RIPs (Girbes et al., 2004; Ng, Wong, & Wang, 2010). Together with type 2 RIPs *Sambucus* spp. contain lectins without translational inhibitory activity of the B-B type with only D-galactose-specific activity (Girbes et al., 2004). The bark of *S. nigra* contains SNA I (four chains) and SNA II (single-chain) and the fruits contain SNA IV (single chain) (Citores et al., 1996). *S. ebulus* contains two chain lectins named SELId in leaves (Citores et al., 1997), SELIm in shoots (Citores et al., 2008) and SELfd in fruits (Citores et al., 1998).

Since ebulin from leaves shares amino acid sequences with Sam n1 isolated from the flowers and berries of *S. nigra* (Förster-Waldl et al., 2003), we speculated that both proteins might be members of a broad family of structurally related proteins (*Sambucus* lectins) bearing the Sam n1 allergen. We surmised that most probably all *Sambucus* related proteins would have nearly the same allergenic properties. As a consequence, in a first stage to approach that issue we isolated both lectins ebulin f and SELfd from fruits collected in the early stages of formation, which are traditionally considered to be toxic, and performed structural and stability analyses. The aim of the present work was: to gain insights into the structure of fruit form of ebulin (ebulin f) and the related lectin SELfd, both present in green fruits of dwarf elder and that could contribute to its toxicity; to ascertain the sensitivity to pepsin.

2. Materials and methods

2.1. Materials

All common chemicals and biochemicals were of the highest purity available. All chromatographic supports were purchased from Pharmacia Ibérica (Madrid, Spain). Acid-treated Sepharose 6B was prepared by treatment of Sepharose 6B beads with 0.1 N HCl at 50 °C for 3 h and extensive washing with Helix Millipore water (Girbes et al., 1993). Green fruits of dwarf elder (*S. ebulus* L.) were harvested from Barruelo del Valle (Valladolid, Spain) in early July and stored frozen at –20 °C. The average weight of the fruits was approximately of 6.5 mg/fruit from the same bush. Porcine pepsin (P-7000; specific activity of 800–2500 U/mg) was purchased from Sigma (St. Louis, MO, USA). The already prepared acrylamide (37.5%) – bis-acrylamide (0.8%) mixture was obtained from Amresco (Solon Ind., Solon, Ohio, USA).

2.2. Isolation of D-galactose-binding proteins from the green fruits of dwarf elder

200 g of green dwarf elder were ground in a blender for 2 min to obtain a finely cut material. The minced material was extracted overnight with 800 ml of extraction buffer (280 mM NaCl containing 5 mM sodium phosphate – pH 7.5). The extract was strained through cheesecloth and the fluid was then centrifuged at 7500g for 30 min at 4 °C, after which the supernatant was centrifuged again at the same speed for 30 min and removed and stored at 4 °C until use. At this stage, the extract contained a material with a plastic mucilaginous consistence that adhered to all surfaces and was quite difficult to remove. In order to reduce its presence in the protein extract and avoid dirtying the chromatography system, the extracts were filtered through a two layers of common

laboratory filter paper. This simple procedure efficiently removed this contaminant material and improved the isolation procedure considerably. The filtered extracts were chromatographed through acid-treated Sepharose 6B (AT-Sepharose 6B) to obtain D-galactose-binding proteins, which included SELfd and ebulin f, as reported previously (Citores et al., 1998). 650 ml of extract was applied to a XK50 (5 × 15 cm) column (GE-Pharmacia) containing 200 ml of freshly prepared AT-Sepharose 6B equilibrated with 0.28 M NaCl and 5 mM Na-phosphate (pH 7.5) buffer. Then, the column was washed with the same buffer until the A_{280} reached values lower than 0.1 and the protein fraction not retained by the AT-Sepharose 6B column was discarded. The retained protein fraction was further eluted with the same buffer, now containing 0.2 M lactose. Fractions of 10 ml were collected and those containing proteins were pooled and concentrated with an Amicon system using an Y10 membrane down to 5 ml. This protein solution was applied to a Superdex 75 (26/60; GE) column equilibrated with 400 mM NaCl and 5 mM Na-phosphate (pH 7.5) buffer. Then, the column was eluted with the same buffer at 2.5 ml/min. Fractions of 2.5 ml were taken and their A_{280} was measured and those containing proteins were pooled. Finally, all fractions containing the separated lectins were dialyzed against water and finally concentrated with an Amicon system (Y10 membrane) to afford concentrations of 2.5–4.0 mg/ml, divided in aliquots of 0.1 ml, and stored frozen at –20 °C. The purity of both proteins was assessed by SDS–PAGE.

2.3. Red blood cell agglutination

The agglutination assay was performed in round bottom 96-well plates. O⁺ human red blood cells at 1% in 0.14 M NaCl containing 5 mM sodium phosphate (pH 7.5) buffer and serial dilutions amounts of the desired lectin in a final volume of 0.1 ml were incubated to agglutinate by incubation either at 22 °C or 4 °C for at least 60 min.

2.4. Molecular mass determinations

One microlitre of sample (3.8 µg/µl) was spotted onto the sample target plate and allowed to air-dry at room temperature. Then, 0.5 µl of a saturated solution of sinapinic acid (Sigma) in 30% acetonitrile and 0.3% Trifluoroacetic acid (TFA) were added to the dried protein spots and again allowed to air-dry at room temperature. Then, analyses in the MALDI-TOF/TOF mass spectrometer operating in positive linear mode, with an accelerating voltage of 20,000 V, were performed. Mass spectra were obtained between 20,000 and 150,000 laser shot intensity (30 Shots/Sub-Spectrum for 1800 Total Shots/Spectrum). An external mass spectrum calibration was performed using BSA as standard.

2.5. Protein identification: MS analyses and database search

The samples were reduced with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate for 30 min at 37 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, samples were digested with 1:20 sequencing-grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5), overnight at 37 °C. After digestion, 1 µl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.5 µl of a 3 mg/ml of α -cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and again allowed to air-dry at room temperature. MALDI-TOF MS analyses were performed with a 4800 Proteomics Analyser MALDI-TOF/TOF mass spectrometer (Applied Biosystems MDS Sciex, Toronto, Canada) operated in positive reflector mode, with

an accelerating voltage of 20,000 V. Mass spectra were obtained (Nd:YAG laser at 355 nm, 40 Shots/Sub-Spectrum for 800 Total Shots/Spectrum) by reflector positive mode. All mass spectra were internally calibrated using peptides from the auto-digestion of trypsin. Peptide mass fingerprints observed with a Signal to Noise greater than 20 were collected and represented as a list of monoisotopic molecular weights. Proteins identified ambiguously by peptide mass fingerprinting, were subjected to MS/MS sequencing analyses. Thus, from the MS spectra suitable precursors were selected for MS/MS analyses with CID in (air was used) 1 kV ion reflector mode and precursor mass; windows ± 2 Da. For protein identification, the non-redundant NCBI Viridiplantae (Green Plants) database (1,716,557 sequences) was searched using a licensed version of MASCOT 2.3 through the Global Protein Server v3.6 from Applied Biosystems. The search parameters were: oxidised methionine as a variable modification; carbamidomethyl cysteine as a fixed modification; peptide mass tolerance 150 ppm (MS/MS); 1 missed trypsin cleavage site; peptide charge state +1; MS/MS fragment tolerance 0.4 Da.

2.6. Toxicity to mice

Lectins at the concentrations indicated in the text were administered either through the intraperitoneal (IP) route or by oral gavage (OG). Proteins dissolved in 0.1 ml of water up to the required concentrations were used. Owing to the clarity of the results for the IP administration – ebulin f at 5 mg/kg body weight and SELfd at 5 mg/kg body weight – only nine animals were used. For the OG administration of ebulin f at 5 mg/kg, fourteen mice were used to obtain a clear result. Treatments and experiments were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the University of Valladolid (File Number 41–2011).

2.7. Pepsin digestion assay conditions

The reaction mixtures of 90 μ l contained 0.3 mg of pepsin (800–2500 U/mg; Sigma Ref. P 7000), 0.066 N HCl, 30 mM NaCl herein called simulated gastric fluid (SGF) and the test protein. Incubations were carried out at 37 °C for the times indicated in the legends of the figures. Digestion was stopped at appropriate times by mixing with 30 μ l of 200 mM NaHCO₃ (pH 11.0). From these mixtures 18 μ l were taken and mixed with 6 μ l of four-times concentrated electrophoresis buffer containing 40% glycerol, 20% SDS, 0.33 M Tris (pH 6.8), and 0.05% bromophenol blue. The samples were boiled for 5 min immediately after collection for denaturing and used immediately for SDS–PAGE analysis. Potential pepsin auto-digestion was tested in reaction mixtures lacking lectins which, were incubated for 60 min at 37 °C. Protein stability at low pH was tested by SGF without pepsin, and incubating the test protein for 60 min, with no observed change at all.

2.8. SDS–PAGE

The analysis of proteins by SDS–PAGE (Ferrerías et al., 2011) was performed with the Mighty-Small II minigel (70 $\times$ 80 $\times$ 0.75 mm) system (Hoefer), using 4% stacking gel and 15% polyacrylamide separation slab gels. The samples were incubated for 5 min at 100 °C in the loading buffer (62.5 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue). A volume of 18 μ l of sample buffer containing the protein sample was loaded into each well and electrophoresis was carried out at 20 °C and 25 mA per gel, using a buffer containing 25 mM Tris–HCl (pH 8.3), 192 mM glycine, and 0.1% (w/v) SDS. Gels were then stained overnight with a solution containing 1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, and 10% (v/v) acetic acid and

further destained with a solution containing 5% (v/v) methanol and 7% (v/v) acetic acid.

3. Results

Previous work indicated that the green fruits of dwarf elder (*S. ebulus*) collected in September contain two lectins, namely SELfd and ebulin f in a ratio of 9.0 (Citores et al., 1998). In the present work we collected the fruits in April. As shown in Fig. 1, the proteins present in the green fruits only display two peaks in contrast with fruits collected in September which also contain a polymerized form of both proteins with an apparent relative mass (M_r) close to 250 kDa and which have been implicated in developmental functions (Citores et al., 1998). The first peak of Fig. 1A accounted for the SELfd lectin and the second one for ebulin f. SDS–PAGE analysis of the peaks of Fig. 1B confirmed the purity of the preparations and the apparent M_r already reported for these proteins of 56 kDa for ebulin f and 68 kDa for SELfd (Citores et al., 1998). A typical preparation of D-galactose-binding proteins from green fruits of *S. ebulus* collected in April yielded 4.6 mg of SELfd

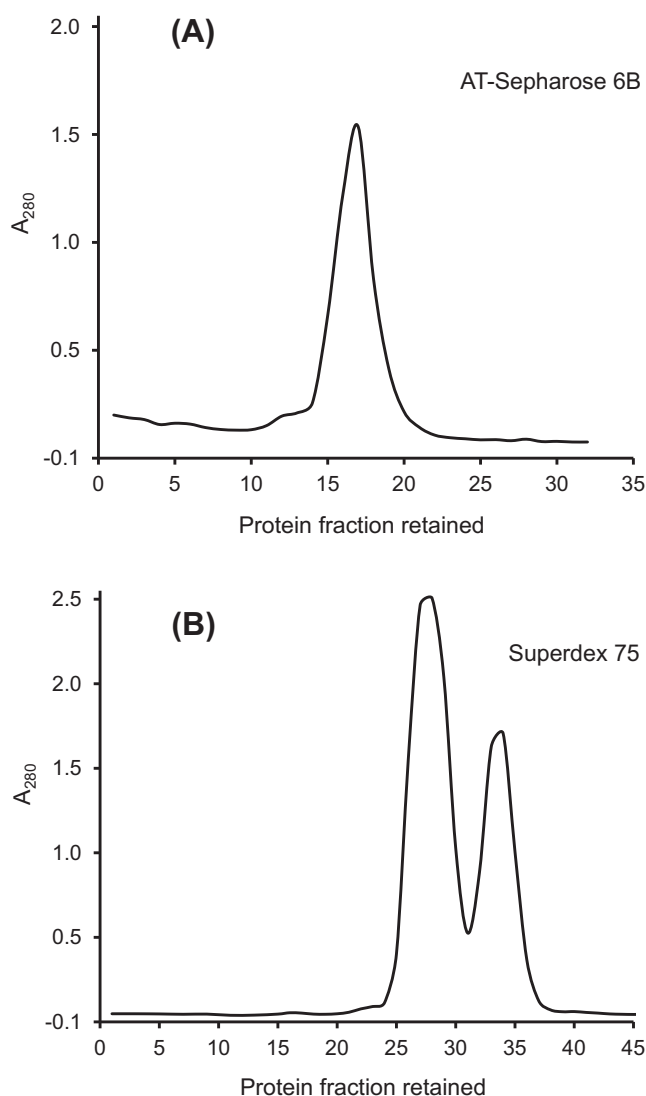


Fig. 1. Chromatography patterns of *Sambucus ebulus* L. fruit lectins. Mixed D-galactose-binding lectins were obtained by affinity chromatography in AT-Sepharose 6B (A) and further separated by Superdex 75 chromatography (B) in a highly purified form.

and 4.3 mg of ebulin f per 100 g of fruits with a SELfd to ebulin f ratio close to 1.0.

It has been reported that ricin binds to a dense D-galactose matrix generated by acid treatment of Sepharose 6B like that used in the present work with the same efficiency at 20 °C as at 0 °C, in contrast to the leaf forms of ebulin and the dimeric lectin, which bind at 0 °C but not at 20 °C (Citores et al., 2008). We wished to see whether this temperature dependence of the interaction of the lectin on the dense D-galactose matrix also operated with intact red blood cells and with the fruit forms of both lectins. As shown in Fig. 2, at 25 °C ebulin f triggers full agglutination at 0.022 mg/ml and SELfd at 0.1780 mg/ml. In contrast, at 4 °C ebulin f did it at 0.006 mg/L and SELfd at 0.022 mg/ml. This temperature dependence is in agreement with the data obtained in the analysis of the interaction of the leaf and the AT-Sepharose 6B (Citores et al., 2008).

To determine the exact value of the Mr we used MALDI-TOF mass spectrometry analysis. As shown in Fig. 3 SELfd has a mass of 68,678 *m/z* and ebulin f a mass of 58,904 *m/z*. To gain further insight into the structural features of both lectins, we determined the tryptic fingerprint of both proteins by MALDI-TOF mass spectrometry (Fig. 4). As expected, the fingerprints were completely different. The following tryptic peptides were sequenced in ebulin f: 2628.31 (ESDVSLQEVIIIPATGNPNQQR), 1539.88 (SLVLIQMVPEAAR), 1588.85 (VATGTVEVNGLPVLR), 876.39 (DGLCVDVR) and 1116.70 (ITGIAILLFR). In SELfd the 1884.33 tryptic peptide has the sequence EIIIFPATGNPNQQR. These sequences were identical to protein sequences from NCBI BLAST data bank, those from ebulin f to ebulin l present in dwarf elder leaves (ebulin: gi/13171073) and those from SELfd to SNAIm from black elder leaves (gi/26793998). The molecular mass (33,214 *m/z*) and other structural features of the Sam n1 allergen fit best with a monomeric lectin found previously in dwarf elder leaves namely SELIm (Citores et al., 2008) which is clearly different of ebulin f (58,904 *m/z*) and SELfd (68,678 *m/z*).

There are no data on ebulin toxicity in mice. Therefore, we undertook assays of toxicity of ebulin f and SELfd in this species. As shown in Fig. 5, intraperitoneal administration (IP) of 5 mg/kg body weight of ebulin f to 7 female mice killed all of them 3–5 days after administration. The LD₅₀ was calculated to be 2.8 mg/kg body weight. This is in contrast with nigrin b, which has an IP LD₅₀ of 12 mg/kg body weight (Batelli et al., 1997). We also studied the oral toxicity of ebulin f at 5 mg/kg body weight. As also shown in

Fig. 5, ebulin f already started to kill the animals at 3 days after the administration, leaving alive nearly 50% of animals after 14 days. Owing to the relevance of that result, fourteen female mice were used to ensure the result of the toxicity especially in relation with nigrin and ricin toxicities, whose meaning will be discussed later. In contrast to ebulin f, the IP administration of SELfd at the same dose did not show apparent toxicity, or changes in the body weight and no animal died during the study that lasted for 14 days. The lack of acute toxicity of SELfd at the same dose than ebulin f was expected because this lectin has no antiribosomal activity (Citores et al., 1998).

The different *in vivo* toxicity dependent on the route of administration of ebulin f prompted us to study the sensitivity of both lectins to acidic pepsin with a view to exploring the possibility that a reduction of ebulin f effectiveness through a reduction in the concentration of protein after passage through the stomach could account for the reduction in the effect. In keeping with this line of reasoning it has been suggested that the differences in toxicity seen with ricin administered through the intraperitoneal route and *per os* could be due to a potential gastric inactivation of the toxin (Griffiths, 2011; He, McMahon, Henderson, Griffey, & Cheng, 2010). On the other hand, the *in vitro* sensitivity of protein substrate to pepsin at pH 1.5–2.0 has been considered a good procedure for assessing the potential allergenicity of ricin (Astwood et al., 1996). On these grounds we wished to see whether pepsin at pH 2 in HCl media could affect the integrity of both lectins. As shown in Fig. 6, the incubation of ebulin f with pepsin triggered a time-dependent degradation of the lectin that was significant after 30 min and almost complete after 90 min. In contrast to ebulin f SELfd did not show sensitivity to pepsin at all, at least at 90 min after incubation. As a control of pepsin activity under the same conditions, bovine serum albumin (BSA) was used and, as also shown in Fig. 6, it was already completely hydrolyzed at 30 min.

4. Discussion

Ebulin from dwarf elder leaves has been related to the allergen Sam n1, isolated from pollen of *S. nigra* through alignment of Sam n1 tryptic peptides with the published amino acid sequence of ebulin l (Förster-Waldl et al., 2003).

The results indicated that fruits collected in early April contained much more ebulin f than those collected in September in

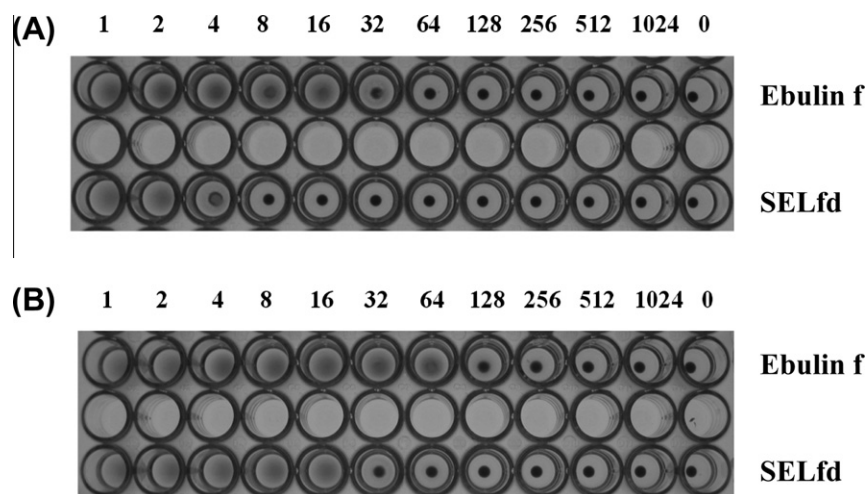


Fig. 2. Hemoagglutinating activity of ebulin f and SELfd lectins. Human O⁺ red blood cells in a round-bottomed microtitre plate for 60 min either at 22 °C (A) or 4 °C (B). Concentrations of protein were: 0.35 mg/ml of either ebulin f or SELfd in well 1 and a series of consecutive twofold dilutions with buffer in the other wells (numbers on the panels). The non-agglutinated cells slides down and concentrated in the bottom of well (wells marked with 0 does not contain lectin).

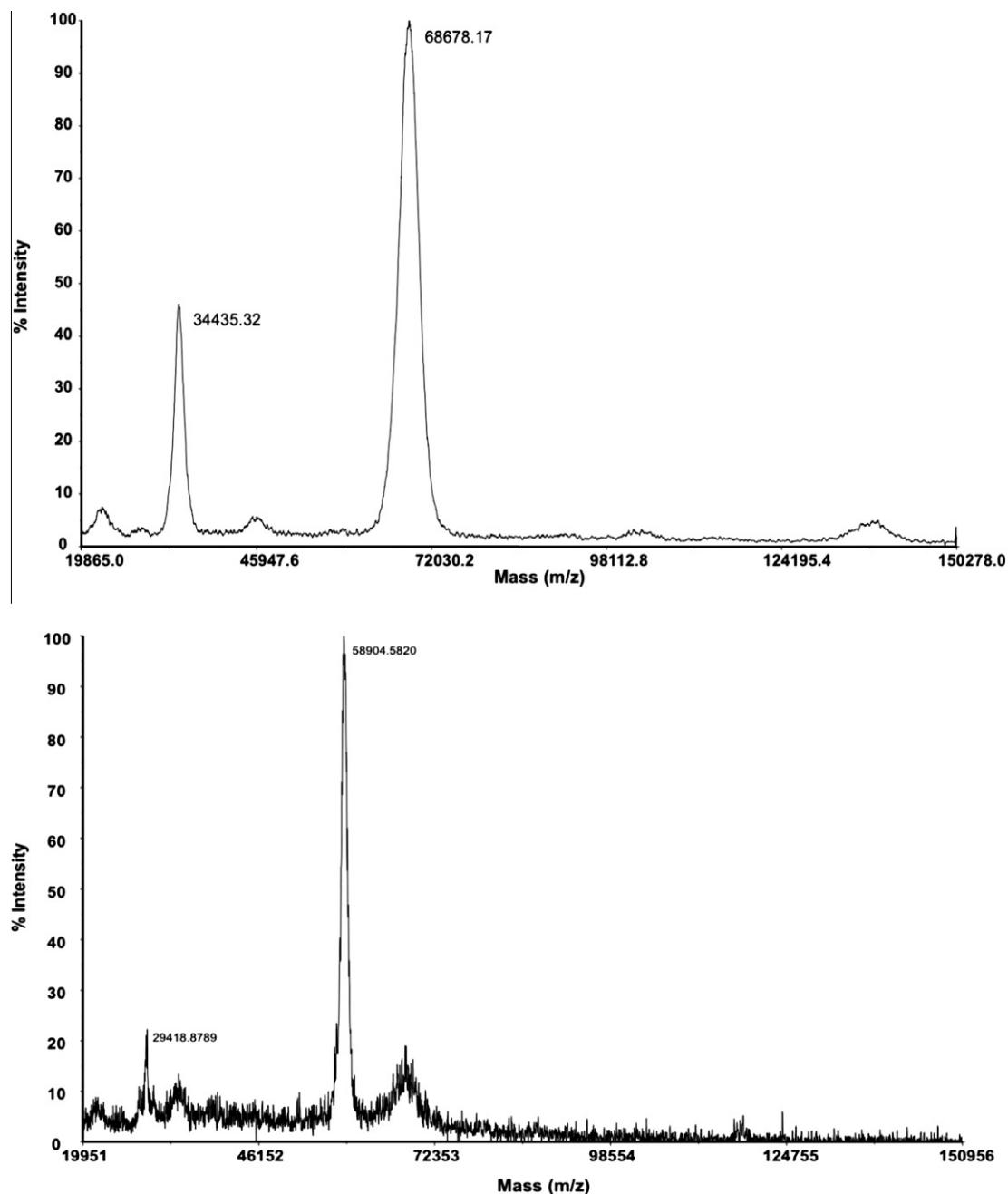


Fig. 3. MALDI-TOF spectra of ebulin f and SELfd. MALDI-TOF/TOF mass spectrometry analysis (positive linear mode and an accelerating voltage of 20,000 V) showing the exact molecular mass of SELfd (upper panel) and ebulin f (lower panel). An external mass spectrum calibration was performed using BSA as standard.

similar environmental areas and temperatures. The ratios of purified ebulin f to SELfd were 0.9 in July and 0.1 in September. This is consistent with the hypothesis that part of the allergy attributed to the *S. nigra* allergen Sam n1 could be related to the presence of ebulin in blossoms of *S. ebulus* and most probably in green fruits of *S. ebulus* as well. In this line, in a preliminary approach we detected ebulin in the flowers of *S. ebulus* bushes studied in March–April (data not shown).

Mass spectrometry analysis of ebulin f and SELfd size revealed values of 58,904 and 68,678, respectively. These values fit in well with the apparent Mr values reported previously for these proteins by SDS–PAGE (apparent Mr of 56,000 for ebulin f and 68,000 for SELfd). Tryptic peptide fingerprinting of both proteins confirms that they are different proteins. The sequence analysis of several

tryptic peptides revealed that these proteins share sequences with protein sequences reported previously in the NCBI BLAST search (ebulin: gi/13171073; SELfd: gi/26793998).

The similarity of the antiribosomal activities of ricin and ebulin, both very close to 0.1 nM, and the extreme toxicity of ricin administered through the intraperitoneal route prompted us to investigate the effects of ebulin. As reported recently (He et al., 2010), ricin has much more toxicity when injected into mice through the IP route (LD₅₀: 0.023 mg/kg body weight) than when administered orally (LD₅₀: 29 mg/kg body weight). Therefore, for ricin the oral to IP administration ratio is 1260. For ebulin f we found LD₅₀ values close to 3 mg/kg body weight and 5 mg/ml for IP and oral administration respectively, with an oral versus IP ratio of 1.6. Taking into account the toxicity of ricin our results with ebulin f were

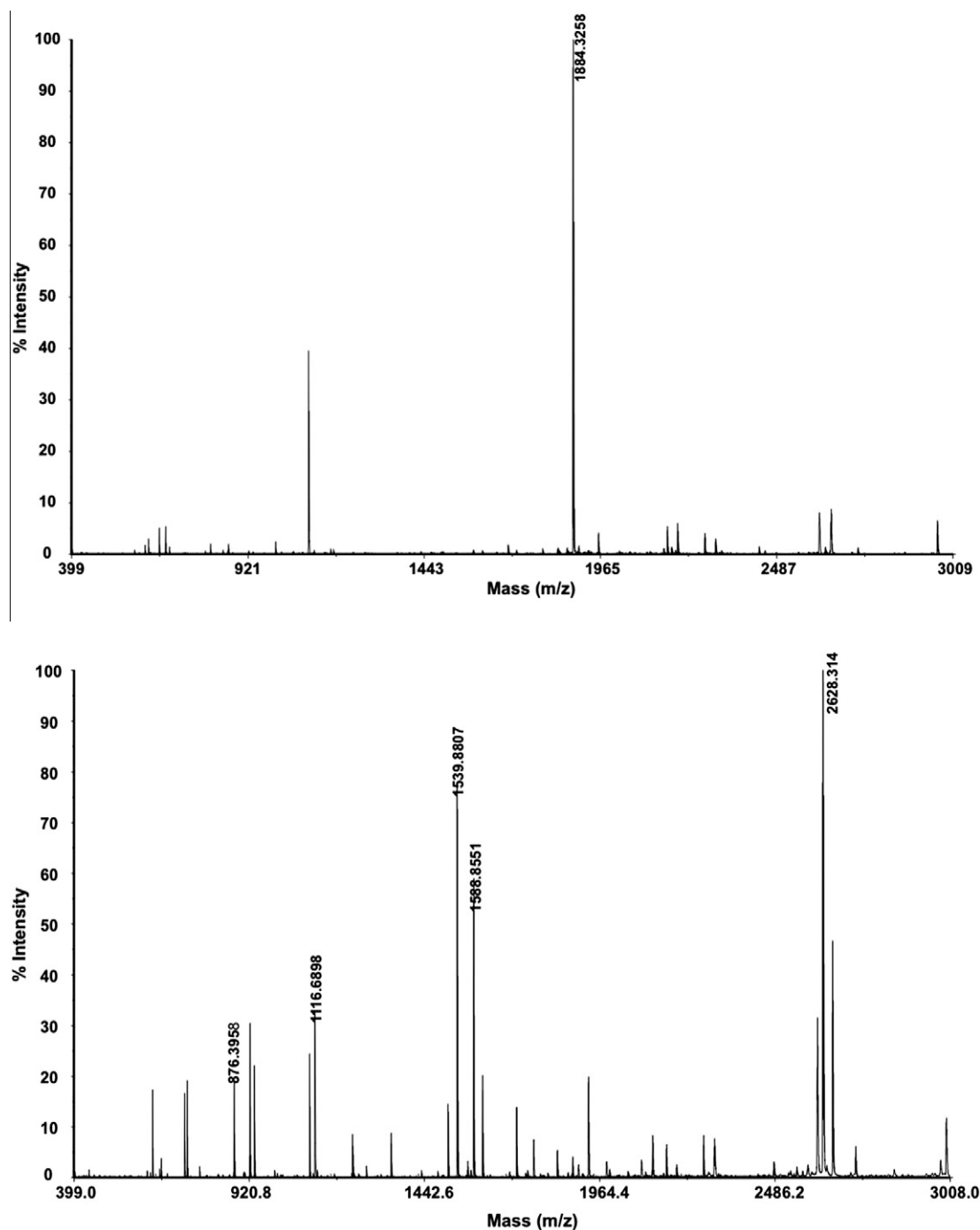


Fig. 4. MALDI-TOF spectra of the tryptic digests of SELfd and ebulin f. The masses displayed represent the $[M + H]^+$ ions of the peptides. Peptides 2628.31, 1539.88, 1588.85, 876.39 and 1116.70 from ebulin f and 1884.33 from SELfd were further sequenced by MS/MS analysis.

surprising and unexpected since ebulin f oral toxicity is at least sixfold higher than that reported for ricin in mice (He et al., 2010). This raised the question of whether ebulin f should be considered a highly poisonous toxin when administered orally, especially because of the relatively high concentration present in green fruits of dwarf elder. This should be taken into account, as it regards its potential use in large amounts as a bioterror weapon is concerned.

The differences between the IP and oral ebulin f toxicities led us to investigate whether gastric passage would inactivate ebulin f, as has been suggested for ricin (He et al., 2010). The results indicate that ebulin f was in fact sensitive to the action of pepsin in acidic conditions but not so much so as BSA.

Explanation of plant lectin toxicity has been reviewed recently (Nasi, Picariello, & Ferranti, 2009). In order to explain the oral toxicity of ebulin f it must be assumed that before it is hydrolyzed some molecules bind to and damage the gastric mucosa and perhaps enter the blood stream to trigger toxicity, leading to death. Furthermore, a toxic action of some products of ebulin f degradation that would also trigger toxicity through the gastric wall or/and the intestinal wall, although improbable, cannot be ruled out. Therefore, it is conceivable that the reduction in oral toxicity when compared to the IP toxicity of ebulin f could be attributed to the reduction in the bioavailability of ebulin f by degradation in the stomach. Since the triggering of an almost complete

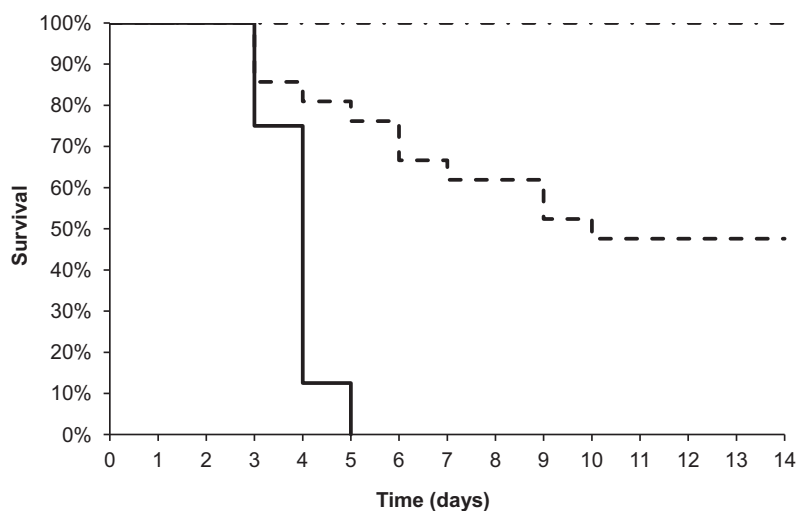
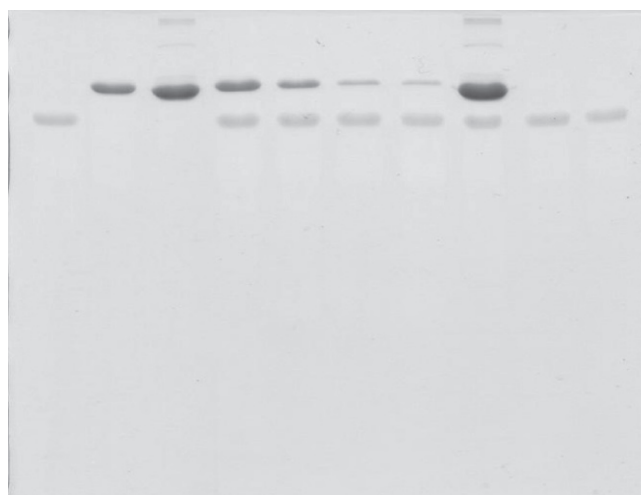


Fig. 5. Toxicity to mice of ebulin f and SELfd. The lectins were administered to female mice either i.p. (ebulin f and SELfd) or orally (ebulin f) at the dose of 5 mg/kg bodyweight. The percentage of survivors is plotted versus the time. Continuous line: i.p. ebulin f; dotted line: i.p. SELfd; dash-dotted line: oral ebulin f.

(a) 1 2 3 4 5 6 7 8 9 10



(b) 1 2 3 4 5 6 7 8 9 10

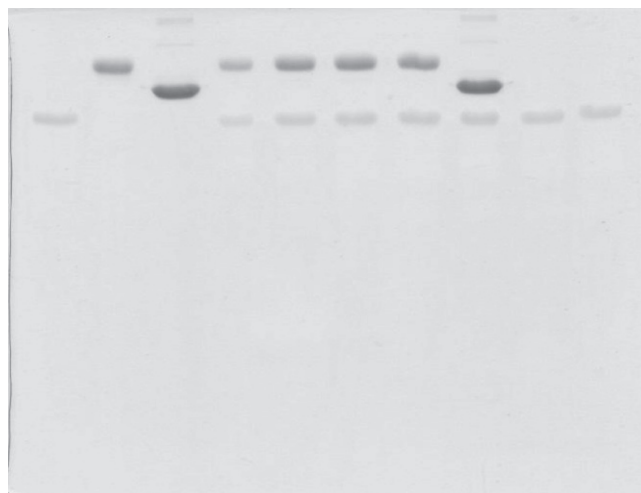


Fig. 6. SDS-PAGE profiles of the ebulin and SELfd digestion by pepsin. The pepsin to substrate protein ratios were 6.4 and 3.4 for ebulin f and SELfd respectively. The amounts of protein per well were 28 μ g pepsin (both panels, lanes 1, 4–10), 4.5 μ g ebulin f (panel a, lanes 2, 4–7), 8.4 μ g SELfd (panel b, lanes 2, 4–7) and 4.5 μ g BSA (both panels, lanes 3, 8–10). Incubations in both panels were carried on at 37 °C for 0 (lanes 1–3, 4, 8), 15 (lane 5), 30 (lane 6, 9) and 60 (lane 7, 10) min.

in vitro degradation of ebulin f in a gastric simulated fluid took at least 60 min, there must be a window in which ebulin f could trigger its toxic effects before being destroyed. In contrast to ebulin f, SELfd was resistant to acidic pepsin and was not toxic, at least as far as mortality is concerned, up to 12 days after IP injection. To date we do not know the reason for the difference in sensitivity to pepsin with ebulin f. SELfd, in contrast to ebulin f, is highly glycosylated, and this opens the possibility that the glycan chains linked to the polypeptide chains would be involved in SELfd resistance to pepsin digestion. In fact, it has been reported that glycoproteins show resistance to pepsin digestion (Wilson, Wu, Peluso, & Nock, 2002).

There are some clues which prompt us to speculate that SELlm and related *Sambucus* lectins would have the nature of the Sam n1 allergen: SELlm is glycosylated (Citores et al., 2008), like Sam n1 allergen (Förster-Waldl et al., 2003); the molecular masses of both proteins are very close (Citores et al., 2008; Förster-Waldl et al., 2003); SELlm contains tryptic peptides with the same or a high amino acid similarity with the Sam n1 allergen (Citores et al., 2008; Förster-Waldl et al., 2003).

The fact that some tryptic peptides isolated from Sam n1 and sequenced showed a well match with the A chain of ebulin f, would merely be a consequence of the procedure of allergen isolation by chromatography under reducing conditions afforded by 4 mM DTT like those used to isolated the Sam n1 allergen (Förster-Waldl et al., 2003). The proteins therein isolated could in fact be a mixture containing free A and B chains from ebulin f and free B chains from SELfd, which in gel filtration move as a group of proteins with an apparent Mr close to 30 kDa owing to the presence of DTT. Therefore, tryptic digestion of Sam n1 would afford a mixture of tryptic peptides with sequences sharing good amino acid homology with B chain of ebulin f (Girbes et al., 1993). The molecular mass of Sam n1 also deserves comments. The allergen was isolated from the pollen of elderberry and purified by gel filtration chromatography as a reduced form (Förster-Waldl et al., 2003). In that work, the approximate molecular mass for Sam n1 allergen by SDS–PAGE was 30 kDa and the mass by MS was 33.2 kDa. This is consistent with the idea of a protein structure composed of two identical subunits linked by a disulphide bond that would readily become reduced under conditions of isolation to yield the free subunits. SELfd meets this idea, since it has two identical subunits, each with a molecular mass of 34.3 kDa linked by a disulphide bond. Alternatively, our data and data of Sam n1 allergen fit well also with a monomeric lectin like SELlm isolated from shoots of *S. ebulus* which share a very good amino acid sequence homology with SELfd (Citores et al., 2008). Furthermore, SELlm and SELfd, contrary to ebulin f, are glycosylated, like the Sam n1 allergen (Förster-Waldl et al., 2003). It is conceivable that pollen would contain a monomeric lectin slightly shorter than Sam n1 allergen. In fact, small differences in size and sequence among the *Sambucus* forms of the same protein, depending on the part of the plant, used as source of the protein have been reported previously (Girbes et al., 2004).

To date there are no data available regarding the potential allergenicity of dwarf elder extracts nor of purified ebulin f and SELfd. This is a question that needs to be answered from the food safety and allergenic points of view. Our hypothesis is that the *Sambucus* proteins that share very large amino acid sequences with one another and also with the Sam n1 allergen would constitute a new family of allergens, the Sam n1 isolated from elderberry pollen being the first one disclosed. Nevertheless, a potential cross-reactivity of the pollen Sam n1 allergen with foods has not yet been explored and therefore cannot be excluded. In fact it has been reported that the birch allergen Bet v1 and Bet v6 from birch pollen are related to allergens from different plant foods considered as defence-related proteins (Vieths et al., 2002), a role proposed to be

played by class I chitinases (Wagner & Breiteneder, 2002), lectins (Peumans & Van Damme, 1995) and ribosome-inactivating proteins (Girbes et al., 2004).

5. Conclusions

Unripe *Sambucus* fruits show a yet undefined toxicity especially those from dwarf elder. They contain ebulin f a type 2 ribosome inactivating protein and the related lectin SELfd. It was reported that the Sam n1 allergen present in elderberry pollen could be also a type 2 RIP. One of the novelties of the present research is that the amino acid sequence homology between the dwarf elder lectins and Sam n1 allergen and the molecular features of the allergen fit better with a monomeric lectin related with ebulin like SELlm present in dwarf elder shoots. Our results support our hypothesis that members of the *Sambucus* lectin family would contain the features of the Sam n1 allergen and therefore they would also be considered allergenic. Another noteworthy conclusion is ebulin f is toxic to mice less orally than intraperitoneally as usual with type 2 RIPs which could be explained by its sensitivity to pepsin. The oral toxicity of ebulin f is important because it is more toxic (LD₅₀ 5.0 mg/kg body weight) than oral ricin (LD₅₀ 29.0 mg/kg body weight). Hence ebulin f must be taken into account and studied because its potential use as poison both in foods and elder medicinal preparations and as a potential bioterrorism element. Further work will address whether these lectins in a purified form are able to trigger allergenic responses in mice. In addition, the gastrointestinal fate of these lectins and the intestinal transit, binding to and uptake by the intestinal mucosa of SELfd will be approached.

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