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Transient occurrence of an ebulin-related D-galactose-lectin in shoots of *Sambucus ebulus* L. ☆

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Abstract

Young shoots of *Sambucus ebulus* L. contain a monomeric D-galactose binding lectin (SELlm), which disappears upon shoot development, and was previously undetected since it co-purifies with the non-toxic type 2 ribosome-inactivating protein ebulin l and the dimeric lectin SELld. Molecular cloning of cDNA coding for SELlm and mass spectrometry analysis revealed a protein with a molecular mass of 34,239 Da, which displays 80%, 77% and 45% of amino acid sequence identity with the ebulin l-B chain, SELld and ricin-B chain, respectively. Furthermore, the cloned precursor, with respect to the ebulin l precursor is truncated and contains the signal peptide, a piece of the A chain, a piece of the connecting peptide and the B chain. Further processing yields the lectin protein, which contains only the B chain. Despite the fact that SELlm displays the same D-galactose-binding sites than ricin, it was found that the lectin has different binding properties to D-galactose-containing matrix than ricin. Notably, and unlike ricin, the binding of SELlm and other *Sambucus* lectins to such matrix was maximum in range of 0–10 °C and abolished at 20 °C.

Keywords: *Sambucus ebulus* L.; Dwarf elder; Lectin; SELlm; Ricin; Ebulin; Ribosome-inactivating protein (RIP)

1. Introduction

Lectins are a heterogeneous group of sugar-binding proteins found in microorganisms, plants, algae, fungi and animals (Sharon and Lis, 2004). They differ with respect to their biochemical and physicochemical properties, interaction with sugar and oligosaccharides, molecular structure and biological activity. Based on structural analysis, plant lectins can be grouped into families of structurally and evolutionary-related proteins (Van Damme et al., 1998, 2004). One of these families is the type 2 ribosome-inactivating proteins (RIPs), a group of lectins which share a large structural homology, characterized by displaying two separated activities: lectin activity in one chain and inhibitory activity of protein synthesis in the other. RIPs, in general, are protein synthesis inhibitors that trigger the catalytic inactivation of mammalian, plant, yeast and bacterial sensitive ribosomes (Barbieri et al., 1993; Girbes et al., 1993a; Iglesias et al., 1993; Stirpe, 2004). The mechanism of the action of RIPs is the release of an adenine from the rRNA loop responsible for the interaction of elongation factor 2 in eukaryotes and G in prokaryotes (Girbes et al., 2004). RIPs have been classified into two categories: types 1 and 2. Type 1 RIPs consist of one polypeptide chain with the enzymatic activity. The type 2 RIPs consist of two polypeptide chains, an A chain and a B chain, linked by a disulfide bridge. The A chain is similar to the type 1 enzymic polypeptide chain. The B-chain, a carbohydrate-binding lectin, binds to glycoproteins and glycolipids with terminal galactose from the cell surface facilitating delivery of the toxin to the cytosol, where the A chain inactivates the ribosomes

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank nucleotide database under accession number AM491634.

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leading to cell death. Type 2 RIPs such as ricin have been considered as highly toxic to most mammalian cells in culture and also to animals (Stirpe et al., 2007; Hartley and Lord, 2004; Olsnes, 2004). Among plants, the genus *Sambucus* seems to be paradigmatic since it contains a complex mixture of lectins and ribosome-inactivating proteins with yet an unexplained biological role (Ferreras et al., 2000; Girbes et al., 2004). *S. nigra, S. ebulus, S. racemosa* and *S. sieboldiana* contain monomeric, dimeric and tetrameric lectins and also a kind of type 2 RIPs with the special feature of being non-toxic to whole cells and animals, namely nigrins (Girbes et al., 1993b, 1996; Citores et al., 1994), ebulins (Girbes et al., 1993c; Citores et al., 1997) and sieboldin (Rojo et al., 1997). Their lack of toxicity has been attributed to defective B-chains with reduced affinity for galactosides (Pascal et al., 2001). From a structural and functional point of view these B chains are closely related to lectins from the *Sambucus* species.

Recently, it was found that depending on the leaf development dwarf elder leaves contain variable amounts of ebulin l and SELld (Rojo et al., 2003). Now, we report on a new ebulin l-related monomeric D-galactose-binding lectin that we named SELlm which is only present in the very young shoots of *S. ebulus* together with ebulin-l and the dimeric lectin SELld. The molecular characterization and the cloning of the gene coding for the lectin has been carried out.

2. Results and discussion

S. ebulus young shoots contain a RIP (ebulin l), a dimeric lectin (SELld) and a new monomeric lectin with affinity for D-galactose that we named SELlm (Fig. 1, lane 1). This new lectin rapidly disappears upon shoot development, in contrast to ebulin l and SELld. As the shoot starts to grow, SELlm disappears and ebulin l which is very abundant in shoots, gradually decrease until leaf senescence when it is undetectable and SELld reaches the highest concentration (Rojo et al., 2003). Occurrence of SELlm appears to be restricted to young shoots, raising the question about its function.

A pool of all the three proteins was isolated from young shoots by affinity chromatography through AT(acid-treated)-Sepharose 6B and then, they were separated by gel filtration chromatography. As shown in Fig. 2a, Sephacryl S-100 HR chromatography resolved these D-galactose binding proteins into two peaks. Peak I eluted first and contained SELld, and the peak II contained a mixture of ebulin l and SELlm, which were separated in two peaks by a further Q-Sepharose chromatography with a linear gradient of 0–300 mM NaCl in 5 mM sodium phosphate (pH 7.5) (peaks I and II of Fig. 2b). SDS–PAGE analysis of the protein content from the Q-Sepharose column revealed the presence of homogeneous proteins with apparent M_r of 34 kDa and 56 kDa, respectively (Fig. 2c). In the presence of 2-mercaptoethanol, the values were 34 kDa for SELlm and 30 kDa and 26 kDa for the two chains of ebulin. The precise molecular mass of SELlm was assessed by MALDI-TOF mass spectrometry analysis giving a value of 34,239.75 Da (Fig. 2e), indicating that the lectin exists as a monomeric protein. SELld is a homodimeric lectin with an apparent M_r of 68 kDa with two identical subunits of 34 kDa. Similar to SELld, SELlm was found to be highly glycosylated compared to ebulin l (Fig. 2d). Nonetheless, as shown below, SELlm and SELld are different proteins.

From an immunological point of view ebulin l-B chain, SELld and SELlm are also different proteins. ELISA analysis revealed that anti-SELlm rabbit polyclonal antibodies very poorly recognize both ebulin l and SELld (data not shown). This suggests that ebulin l and SELld have different immunogenic epitopes.

SELlm promoted human red blood cell agglutination at a minimal concentration of 200 μg/ml (data not shown). The lectin had a relatively low specific agglutination activity as compared with the structurally related lectins ebulin l and SELld, which in the same conditions agglutinated at concentrations as low as 12.5 μg/ml and 22.5 μg/ml, respec-

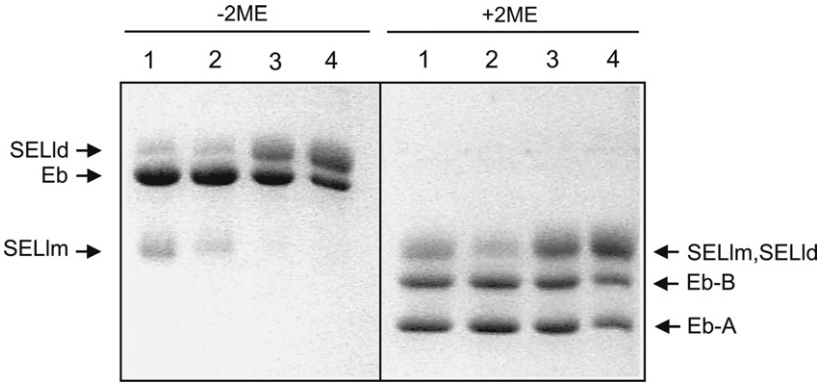

Fig. 1. Protein content of D-galactose-binding material from (1): shoot tips and young leaves less than 2 mm wide; (2): young leaves less than 1 cm wide; (3) leaves 1–3 cm wide and (4) mature leaves more than 3 cm wide of *S. ebulus* L. Total proteins eluted from AT-Sepharose 6B were analysed by SDS–PAGE in the presence (+) and the absence (−) of 2-mercaptoethanol (2ME). Eb, ebulin l; Eb-A, ebulin l-A chain; Eb-B, ebulin l-B chain. Note that in the presence of 2ME SELlm shows the same mobility as SELld chains (34 kDa).

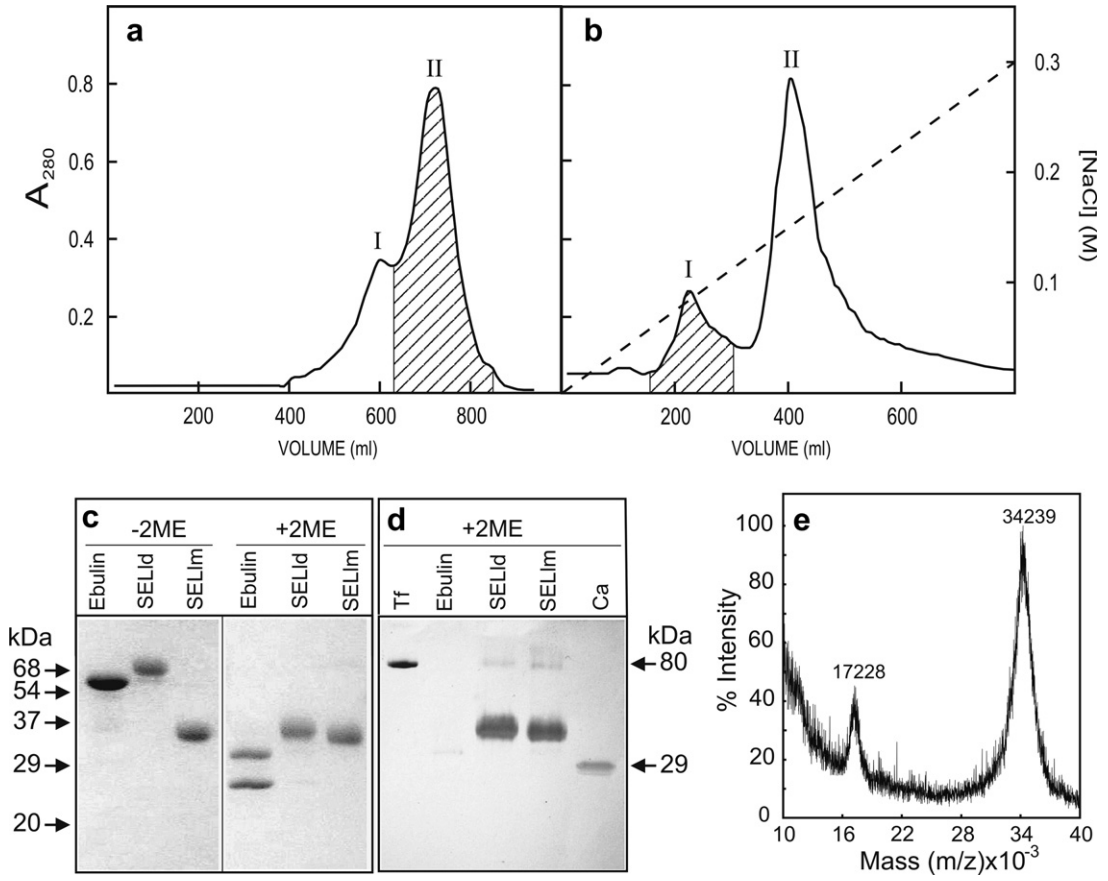

Fig. 2. Purification of SELlm from *S. ebulus* young shoots. D-galactose-binding proteins isolated by affinity chromatography onto AT-Sepharose 6B were purified by Sephacryl S-100 HR chromatography (panel a) as described in materials and methods. Peak II eluted from Sephacryl S-100 chromatography (shaded area) was purified by Q-Sepharose chromatography (panel b) developed with a 0–0.3 M NaCl linear gradient in 5 mM sodium phosphate (pH 7.5) (dashed line), obtaining two peaks (I and II). The fractions corresponding to peak I in panel a (SELld), peak I in panel b (shaded area, SELlm) and peak II in panel b (ebulin l) were pooled, dialyzed and freeze-dried. The proteins were analyzed by SDS–PAGE in 15% gels either in the absence or the presence of 2-mercaptoethanol and then stained with Coomassie brilliant blue (panel c) or blottet onto Immobilon membranes and then treated for glycan detection (panel d). The arrows indicate the corresponding size of the standards in kDa. Tf: transferrin. Ca: carbonic anhydrase. (e) MALDI-TOF mass spectrum obtained from SELlm. Molecular weights of protein peaks were assigned using cytochrome *c* and bovine serum albumin as external standards.

tively. Inhibition of the hemagglutinating activity with several glycoconjugates suggested that SELlm requires terminal Gal/GalNAc residues for the interaction.

To determine the amino acid sequence of the new protein, we performed the molecular cloning of the gene. Four specific primers were designed from the dimeric lectin SNAld gene (GenBank accession no. AF249281) based on homology profiles among *Sambucus* lectins. Total RNA from *S. ebulus* young shoots was used to synthesize the cDNA that served as template in PCR amplifications. The fragments corresponding to the lectin gene ends presented a homologous overlapping region and the analysis of the full-length 1169 bp cDNA sequence revealed an open reading frame of 978 bp encoding a 326-amino acid precursor. Such sequence was identified as the sequence of SELlm since the N-terminal amino acid sequence and some tryptic peptides obtained from highly purified SELlm were found in the reading frame (Fig. 3). According to the rules for protein processing of Von Heijne (Nielsen et al., 1997), a signal peptide cleavage site is predicted producing a putative signal peptide of 25 residues. Furthermore, the N-terminal amino acid sequence of SELlm indicates a further post-translational processing of 42 amino acids yielding a polypeptide of 259 amino acids with a molecular mass of 28,517 Da which is lower than the molecular mass of 34,239 Da determined by mass spectrometry. We suggest that glycosylation of SELlm could account for the difference of 5722 Da between the molecular mass assessed by mass spectrometry and that deduced from cDNA. SELlm contains 6 putative *N*-glycosylation sites which could link polysaccharide chains as revealed with the glycan detection procedure (Fig. 2d). The putative glycosylation sites at positions 73, 94, 134, 179, 222 and 233 are well exposed and, therefore, accessible for glycosylation (data not shown).

Analysis of the deduced amino acid sequence (Fig. 3) indicated that the precursors of SELlm and ebulin l display a striking sequence identity in the signal peptide (76%). In addition, the SELlm precursor has an 86% sequence homology with the first 22 amino acid residues of the ebulin l A-chain and 71% with the linker region between the A

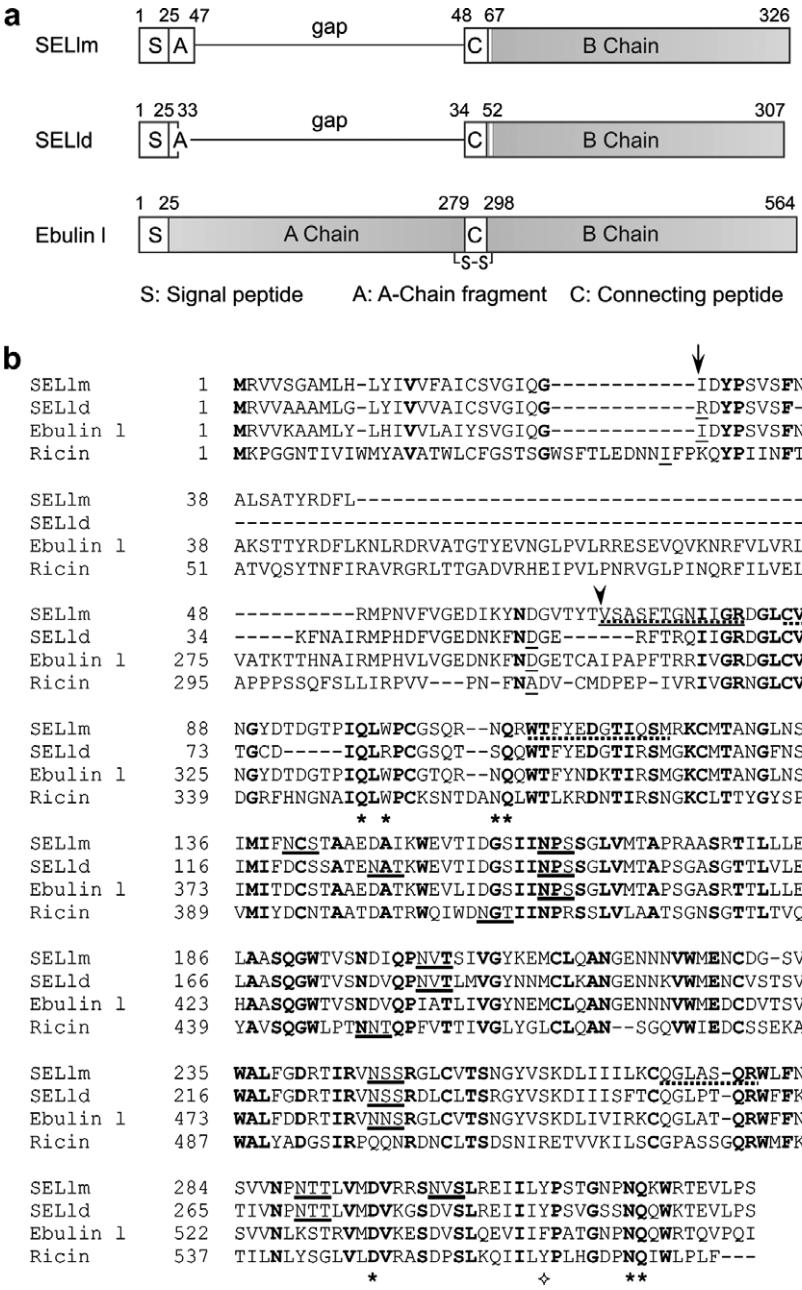

Fig. 3. (a) Graphical representation of the sequences of the precursors encoding SELlm, SELld and ebulin l. Numbers refer to the position of corresponding amino acid residues of the precursor sequence. The shaded areas represent the mature polypeptides after the post-translational processing. (b) Deduced amino acid sequences of the SELlm lectin and alignment with SELld (GenBank accession no. AAM94880) and comparable segments (signal peptide, part of A chain, connecting peptide and B chain) of ebulin l (CAC33178) and ricin (CAA26939). Gaps have been introduced to optimize the identity between sequences. Identical amino acids residues are indicated in bold face type. The putative cleavage site of signal peptide is marked with an arrow. The N-terminal sequence of SELlm isolated from young shoots is underlined with a thin line and its start point marked with and arrowhead. Dotted lines indicate the amino acid sequence determined from the masses obtained by MALDI-TOF analysis of tryptic peptides. Key residues of the sugars-binding sites are marked with asterisks and the Tyr in 2γ sub-domain is marked with a white star. *N*-glycosylation sites are underlined with a gross line. Numbering on the left refers to precursors while numbering on the right refers to the B chains of mature SELlm, SELld, ebulin l and ricin.

and B chains of ebulin l. Besides, SELlm and ebulin l B-chain share a strong homology (80%). The SELlm precursor is converted into SELlm mature protein, through a processing mechanism where, the signal peptide, the small part of A chain, the connecting peptide and six residues of the N-terminal amino acid sequence of the B chain are lost (Fig. 3a).

Our data suggest that SELlm could be encoded by a truncated type 2 RIP gene which lost a substantial part coding for the A chain. A similar truncation has also been described previously in *S. nigra* L and *S. ebulus* L. (Van Damme et al., 1997; Rojo et al., 2003). It has been proposed that type 2 RIPs might have evolved by the gene fusion of a type 1 RIP and a lectin (Ready et al., 1984).

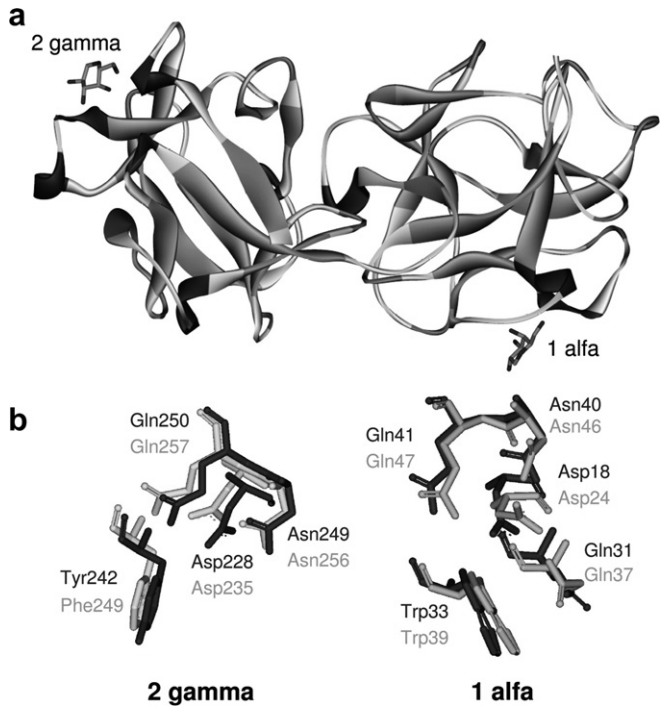

Fig. 4. (a) Ribbon drawing of the D-galactose-bound three-dimensional structure model of SELlm showing the D-galactose molecules interacting with the 1 alpha and 2 gamma carbohydrate-binding sites in the protein. (b) Comparison of the 2 gamma and 1 alfa carbohydrate binding sites of SELlm (black) and ebulin (grey). The key residues are indicated.

However, the finding of several truncated lectins structurally related in *Sambucus* suggests that these lectins evolved from type 2 RIP genes which suffered different processing pathways thus leading to a family of different, but closely related, proteins.

This view is supported by the amino acid sequence identities of SELlm with other related proteins: SELld (GenBank accession no. AAM94880), 77%; SSAlm (BAD93343), 82%; SNAIVf (AAC15885), 79%; nigrin b-B chain (P33183), 80%; and ricin B-chain (CAA26939), 45%.

The SELlm three-dimensional structure (3D) was predicted by comparative modelling using the ebulin l B-chain crystal structure as template (Pascal et al., 2001). The SELlm 3D model obtained was almost identical to that of ebulin l B-chain. SELlm consists of two structurally equivalent domains and each domain comprises three subdomains (1α, 1β and 1γ for domain 1 and 2α, 2β and 2γ for domain 2) (Fig. 4a). The Cys5 in the B chain of ebulin l, able to form an inter-chain disulphide bridge with the A chain, is absent in SELlm, but the polypeptide chain maintains the four intra-chain disulphide bridges. According to the modelling, SELlm possesses two carbohydrate binding sites (1α and 2γ). As shown in Fig. 4b, the D-galactose-binding 1α subdomain of SELlm (black drawing) displays an almost identical 3D arrangement to the ebulin l B-chain (grey drawing). Also all the key amino acid residues forming the pocket responsible of the carbohydrate binding of SELlm in the subdomain 2γ are conserved in the B-chain of ebulin l except Tyr242 replaced by Phe249 in ebulin l B-chain (Fig. 3). The equivalent residue of ricin (Tyr248) in 2γ subdomain is apparently essential for its D-galactose-binding activity as reported elsewhere (de Sousa et al., 1999; Sphyris et al., 1995). Despite the presence of the Tyr242 in SELlm, the carbohydrate-binding ability of SELlm towards D-galactose seems to be reduced (see below) and indicates that most probably, other residues besides such tyrosyl residue are necessary to make a completely functional 2γ subdomain D-galactose-binding site.

To reveal potential differences between the ricin and the *S. ebulus* lectins and ebulin l concerning the binding to and the elution from polysaccharide matrix, we studied the effects of temperature on the interaction of all these pro-

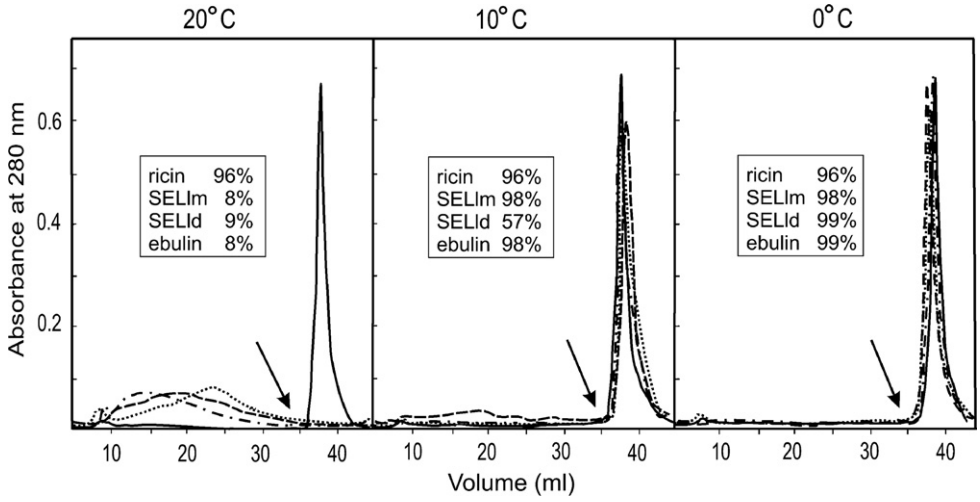

Fig. 5. Binding of ricin (solid line), SELlm (dash-dot line), SELld (dashed line) and ebulin l (dotted line) to AT-Sepharose 6B affinity column at 0, 10 and 20 °C. Approximately 0.5 mg of each protein was loaded on the corresponding column. After washing, bound proteins were eluted with 0.2 M lactose (arrow). The background 280 nm absorbance of lactose was subtracted from the total signal. Insets: the bound protein was collected and the amount of protein determined by spectrophotometry and expressed as percentage of total loaded proteins.

teins with AT-Sepharose 6B. The acid pre-treatment uncovers a dense matrix of D-galactose residues in the Sepharose 6B beads. As shown in Fig. 5, ricin was bound to AT-Sepharose 6B equally well at 0, 10 and 20 °C, while notably ebulin l, SELlm and SELld were bound efficiently to the matrix at 0 and 10 °C but not at 20 °C. The difference was more pronounced in the case of SELld since at 10 °C only 57% of the loaded protein was bound to the matrix. This seems an essential difference between the *Sambucus* proteins and the ricin which could be related to the low cell toxicity of ebulin l as compared with ricin. The pathway followed by both ricin and ebulin l is determined by the features of the corresponding B chain (Svinth et al., 1998). The temperature-dependent differences may reflect a differential interaction of the two RIPs with glycosylated receptors of the plasma membrane as has been hypothesized for the closely related RIP nigrin b (Citores et al., 2003; Battelli et al., 2004).

All the tissues of *S. ebulus* examined to date contain different type 2 RIPs and related lectins (Citores et al., 1997, 1998; Girbes et al., 1993c). In addition, *S. ebulus* leaves also contain highly basic type 1 (single chain) RIPs (de Benito et al., 1995). Although the biological role of the plant lectins is yet unknown, the involvement of lectins in plant defence and protein storage has been proposed (Peumans and Van Damme, 1995; Van Damme et al., 2002). Giving that the occurrence of SELlm, SELld and ebulin l in the developing leaves strongly differs, these proteins might play an important role in the development. A protective role against plant pathogens in early growth stages cannot be ruled out. It has been suggested that abundant type 2 RIPs from *S. nigra* bark (SNAI and SNAV) can behave as storage proteins that can be used as specific defence proteins if the plant is attacked by insects or higher animals (Van Damme et al., 1997). The presence in the same tissue of related proteins with different biological activities suggests a specialized expression pattern of their genes. Future work will address the potential antipathogen abilities of ebulin l, SELlm and SELld and the nature of the control of their expression in plant.

3. Experimental

3.1. Purification of SELlm from Sambucus ebulus L. shoots

Young shoots of *S. ebulus* L. (1.5 kg) were harvested in Cobos de Cerrato, Palencia (Spain) at the end of the spring. The shoots were extracted overnight with eight volumes of extraction buffer (5 mM sodium phosphate (pH 7.5) containing 280 mM NaCl). That crude extract was then centrifugated at 25,900*g* for 45 min at 0 °C and the supernatant was chromatographed through AT-Sepharose 6B at 0 °C to obtain the D-galactose-binding proteins as described elsewhere for the isolation of ebulin l and SELld (Girbes et al., 1993c). The protein fraction retained by the column was further chromatographed through Sephacryl S-100 HR (5 × 100 cm) equilibrated with extraction buffer, giving two peaks. Peak I contained the SELld and the peak II contained ebulin l and SELlm. The fractions corresponding to the peak II were pooled and dialyzed extensively against 5 mM sodium phosphate (pH 7.5). This fraction was applied to Pharmacia Q-Sepharose column equilibrated with dialysis buffer and the proteins were eluted with a linear gradient of 0–300 mM NaCl in 5 mM sodium phosphate (pH 7.5). Fractions corresponding to each protein peak were pooled, dialyzed against water and finally freeze-dried.

3.2. Isolation of D-galactose-binding proteins present in leaves of S. ebulus L.

Twenty-five grams of shoot tips, young leaves less than 2 mm wide, young leaves less than 1 cm wide, leaves 1–3 cm wide and mature leaves more than 3 cm wide, from *S. ebulus* L. were collected in June from Cobos de Cerrato, Palencia (Spain). The crude extract obtained as described above was applied to a column containing AT-Sepharose 6B equilibrated with 0.28 M NaCl in 5 mM Na-phosphate (pH 7.5) at 0 °C. Unbound protein was washed out from the column by elution with the same solution. Bound protein was released by the addition of 0.2 M D-lactose. The eluted protein was concentrated with Amicon YM10 membrane (Millipore Ibérica, Madrid, Spain) to a protein concentration of 0.5 mg/ml.

3.3. cDNA cloning

Molecular cloning of the cDNA coding for SELlm was carried out as described previously for ebulin l using the 3′RACE technique (Pascal et al., 2001). cDNA was synthesized using total RNA from young shoots of *S. ebulus* L. as a template, and the MuLV reverse-transcriptase (Perkin–Elmer) in the presence of an oligo-dT primer. The 5′ end fragment was amplified by PCR using the cDNA as a template in the presence of the sense primer A (5′-GC AAG CAT AGC AAG ATG AGA GTG-3′) and the antisense primer B (5′-TGA CAC AGT CCA ACC CTG GCT AGC-3′), while in the amplification of the 3′ end the sense primer C (5′-GGT CGA GAT GGA TTG TGT GTG GAC GT-3′) and antisense primer D (5′-TTA AAG GCA AGG ACA AGT GCC C-3′) were used. All the primers were synthesised based on the cDNA sequence of the dimeric lectin SNAld from the leaves of *S. nigra* L. (GenBank accession no. AF249281). All PCR products were cloned into the pCR2.1 vector using the Original Kit TA Cloning (Invitrogen) and then transformed into *E. coli* strain XL1-Blue (Stratagene). DNA sequencing in both directions was performed automatically.

3.4. Mass spectrometry

Freeze-dried protein was dissolved in 0.1% trifluoroacetic acid to give 30 pmol/μl of protein. Thereafter, samples were mixed with a saturated solution of sinapinic acid

and analyzed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) using a spectrometer (Voyager-DE STR, Biospectrometric Work station, Applied Biosystems).

3.5. Binding of SELlm, SELld, ebulin l and ricin to immobilized acid treated-Sepharose 6B

SELlm, SELld, ebulin l and ricin were assayed for their binding to AT-Sepharose 6B at 0, 10 or 20 °C. 1 ml of each protein (0.5 mg/ml) was loaded onto separated 3 ml AT-Sepharose 6B columns, which were maintained at the indicated temperatures. Each column was then washed with 10 volumes of PBS and after with PBS containing 0.2 M lactose to allow the elution of the bound protein. The amount of protein was determined by spectrophotometry (Kalb and Bernlohr, 1977).

3.6. Other analytical methods

Analysis of proteins by SDS–PAGE on 15% acrylamide gels was carried out as described by Laemmli (1970). The standards for SDS–PAGE were trypsin inhibitor (M_r 20,100), carbonic anhydrase (M_r 29,000), alcohol dehydrogenase (M_r 37,000), glutamate dehydrogenase (M_r 54,000) and bovine serum albumin (M_r 68,000). The detection of glycan chains in proteins was performed with the Glycan Detection Kit from Boehringer as indicated elsewhere (Ferreras et al., 1993). Preparation of anti-SELlm polyclonal antibodies raised in rabbits was carried out as described elsewhere (Citores et al., 1998). Amino-terminal and tryptic peptides automatic sequence analysis were performed by the services of the Universitat Pompeu Fabra (Spain). Red blood cell agglutination was carried out at room temperature on microtiter plates containing 100 μl of 5 mM sodium phosphate (pH 7.5), 0.14 M NaCl and 0.5% of red blood cells. For agglutination inhibition studies, the protein was preincubated at room temperature for 30 min with increasing amounts of sugar.

3.7. Molecular modelling

Molecular modelling of SELlm was carried out using the program SWISS-MODEL (Schwede et al., 2003). Docking of D-galactose in the carbohydrate-binding sites of SELlm was performed with PatchDock (Schneidman-Duhovny et al., 2005).

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