Isolation and characterization of a new non-toxic two-chain ribosome-inactivating protein from fruits of elder (Sambucus nigra L.)

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Abstract

*Sambucus* (Caprifoliaceae) species contain nigrin b and ebulin I, which are two-chain ribosome-inactivating proteins (RIPs) belonging to a new type of RIPs which are non-toxic to mice and cultured human cells. In this work the presence in fruits of elder (*S. nigra* L.) of a new non-toxic type 2 RIP (nigrin f) that co-exists with a lectin known as SNA IV is described. Nigrin f strongly inhibited protein synthesis in mammalian, but not in plant, ribosomes, promoting the depurination of sensitive ribosomes and thus allowing the release of the RIP diagnostic RNA fragment. Nigrin f is composed of two dissimilar subunits linked by disulphide bridges with apparent Mr values of 31 600 and 26 300. The N-terminal amino acid sequence revealed close homology of the catalytic A chain with type 1 RIPs, especially those from Cucurbitaceae, and the B chain with several lectins previously isolated from *Sambucus* species. Nigrin f was not toxic to mice when injected intraperitoneally up to 2 mg kg⁻¹. In addition, NHC human cells were also insensitive to nigrin f up to 60 µg ml⁻¹. Anti-nigrin b rabbit polyclonal antibodies reacted with nigrin f, indicating that nigrin b and nigrin f are proteins with similar structures.

Key words: *Sambucus nigra*, elder fruits, nigrin f, ribosome-inactivating protein, characterization.

Introduction

Some plants contain protein translational inhibitors collectively named ribosome-inactivating proteins (RIPs); for a recent review see Barbieri et al., 1993). These proteins display a N-glycosidase activity on large ribosomal RNA of mammalian (Endo and Tsurugi, 1987), plant (Taylor and Irvin, 1990; Prestle et al., 1992b; Iglesias et al., 1993b; Bonness et al., 1994), yeast (Stirpe et al., 1988), and bacterial (Hartley et al., 1991; Prestle et al., 1992a; Iglesias et al., 1993a; Girbès et al., 1993a; Ferreras et al., 1994a, b) sensitive ribosomes, thus promoting an irreversible impairment of protein synthesis. The molecular action of plant RIPs involves the hydrolysis of one (Barbieri et al., 1993) or more (Iglesias et al., 1993b; Barbieri et al., 1992, 1994) adenines from the rRNA.

The classification of RIPs includes at least two categories: types 1 and 2 (Barbieri et al., 1993). Type 1 RIPs consist of a single enzymic polypeptide chain. Type 2 RIPs contain two polypeptide chains linked by disulphide bonds, one being the enzymic chain, which is equivalent to a type 1 RIP, and the other a D-galactose and D-galactosamine-binding lectin. Recently, a new RIP with an unusual molecular structure and specificity for Neu5Ac(a-2,6)-Gal/GalNAc has been described (van Damme et al., 1996a). Four- and eight-chain RIPs are special type 2 RIPs that are composed of either two or four dimers, each one being composed of two polypeptide chains linked by disulphide bonds (Barbieri et al., 1993; van Damme et al., 1996a). Type 2 RIPs, such as ricin and visumcin, are able to associate to give tetramers (Barbieri et al., 1993). Type 2 RIPs can also be divided...
into two categories: the toxic and non-toxic RIPs (Citores et al., 1993). Type 2 RIPs are very infrequent and since the isolation of ricin in 1888 by Stillmark, only a few toxic type 2 RIPs (ricin, abrin, modeccin, viscumin, volkensin, and Eranthis hyemalis lectin) have been isolated (Barbieri et al., 1993; Kumar et al., 1993). Non-toxic type 2 RIPs are even rarer and only ebulin 1 (Girbès et al., 1993c) and nigrin b (Girbès et al., 1993b) have been isolated to date. Furthermore, the presence of a seed isoform of nigrin has been reported in elder seeds (Citores et al., 1994). Toxic type 2 four-chain RIPs are Ricinus communis four-chain agglutinin and Abrus precatorius four-chain agglutinin and non-toxic type 2 four-chain RIP is the Viscum album four-chain agglutinin (Citores et al., 1993).

Although the biological role of RIPs in plant producers is presently unknown, based on the extracellular location of the pokeweed antiviral protein from Phytolaccac americana they have been proposed as antiviral agents (Taylor and Irvin, 1990; Bonness et al., 1994; Ready et al., 1986; Taylor et al., 1994). In this hypothesis homologous RIPs would enter the virus-infected cell, thus triggering ribosome inactivation and therefore cell death (Bonness et al., 1994; Taylor et al., 1994). RIPs display antiviral action on both animal and plant viruses containing either DNA or RNA as their genome (Kumar et al., 1993; Foá-Tomasi et al., 1982; McGrath et al., 1989; Zarling et al., 1990; Lee-Huang et al., 1990, 1991a, b; Lodge et al., 1993) in good agreement with Irvin’s hypothesis. As alternative, and probably working together, the direct enzymic actions of some RIPs on TMV genome RNA and herring sperm DNA have been recently described (Barbieri et al., 1994; Girbès et al., 1996). Moreover, the application of mediators of systemic acquired resistance promote the expression of two type 1 RIPs named beetins in sugar beet (Girbès et al., 1996), thus supporting the hypothesis of antiviral function of RIPs.

Some RIPs share amino acid sequence homology with topoisomerase II from Drosophila (Lee-Huang et al., 1994). This led to the finding that some RIPs display topoisomerase activity that is exerted at rather high RIP concentrations (Lee-Huang et al., 1994).

Due to their use as a toxic moiety in immunotoxins for cancer (Olins et al., 1989; Brinkmann and Pastan, 1994) and AIDS (Zarling et al., 1990; Till et al., 1989) treatment, interest in RIPs is increasing rapidly. Moreover, it has been shown that some RIPs have a direct inhibitory action on HIV-1 replication (McGrath et al., 1989; Zarling et al., 1990; Lee-Huang et al., 1990, 1991a, b).

The presence has recently been described of a novel family of non-toxic type 2 (Girbès et al., 1993b, c; Citores et al., 1994) and type 1 RIPs (de Benito et al., 1995) in Sambucus spp. It was observed then that the B chain of these type 2 RIPs shares strong amino acid sequence homology with some lectins isolated from Sambucus spp. (Girbès et al., 1993b, c). In particular, it has been reported that elder (Sambucus nigra L.) fruits contain a monomeric lectin named SNA IV that is able to dimerize and oligomerize (Mach et al., 1991).

Very recently, SNA I has been found to be also a type 2 RIP (van Damme et al., 1996a). Furthermore, SNA I and nigrin b have been cloned and their structure elucidated (van Damme et al., 1996a, b).

This work reports that S. nigra fruits contain a new non-toxic type 2 RIP that has been named nigrin f. This new RIP coexists with SNA IV (Mach et al., 1991) and is both functionally and structurally related to the type 2 RIPs ebulin 1 (Girbès et al., 1993c), nigrin b (Girbès et al., 1993b) and SNA I (van Damme et al., 1996a).

Materials and methods

Materials

All chemicals and biochemicals were of the highest purity available. Their sources are described elsewhere (Girbès et al., 1993b, c; Citores et al., 1994). Sepharose 6B and Sephadex G-25 were purchased from Pharmacia Ibérica (Madrid, Spain). DEAE-cellulose was obtained from Sigma (St Louis, MO, USA). 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 Milli-Q water (Girbès et al., 1993c). Immobilon membranes were purchased from Millipore Ibérica (Madrid, Spain). Reagents for immunology were purchased from Sigma (St Louis, MO, USA). L-[3H]valine (sp. act. 32 Ci mmol⁻¹) was purchased from Amersham Ibérica (Madrid, Spain). [35S]Translabel (sp. act. 1164 Ci mmol⁻¹) was obtained from ICN through Nuclear Ibérica (Madrid, Spain). Pokeweed antiviral protein from seeds (PAP-S) was a generous gift from Professor F Stirpe (Dipartimento di Patologia Sperimentale, Universita di Bologna, Bologna, Italy). Green and mature fruits of S. nigra were harvested from Cobos de Cerrato (Palencia, Spain) in September.

Purification of nigrin f

100 g of either green (4 mm average diameter) or mature fruits (5 mm average diameter) were cut into small pieces and then ground in a blender and extracted overnight with 500 ml of extraction buffer (280 mM NaCl containing 5 mM sodium phosphate (pH 7.5). With this procedure elder seeds remained intact, thus avoiding contamination of fruit extracts with seed proteins. The extract was filtered through cheesecloth and the fluid was then centrifuged at 25,900 × g for 45 min at 0°C. The resulting clear crude protein extract was filtered again through cheesecloth to remove the fat layer that had developed. This extract was subjected to affinity chromatography on acid-treated Sepharose 6B (AT-Sepharose 6B) pre-equilibrated with extraction buffer at 4°C. The protein was applied to the column (10 x 5 cm) and the unretained protein fraction was discarded. The column was then washed with the same buffer until absorbance reached the baseline value. The bound protein was desorbed by elution with 0.2 M lactose in the extraction buffer and dialysed against 5 mM sodium phosphate (pH 7.5). Thereafter, the protein solution was applied to a DEAE-cellulose column (5.6 x 2.6 cm) equilibrated with the same buffer and fitted to an FPLC (Pharmacia) apparatus. After washing with the same buffer, the column was eluted with the same buffer, the column was eluted with the same buffer, followed by a linear gradient of NaCl (50-200 mM).

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buffer this time containing a gradient of 200 ml of 0–0.3 M NaCl. This chromatographic step resolved two small protein peaks at the start of the gradient and a major protein peak spanning almost the whole gradient. Fractions containing the protein peaks were pooled, dialysed against Milli Q-purified water and finally freeze-dried.

Analysis of proteins by SDS-polyacrylamide gel electrophoresis and detection of glycan chains

Analyses of proteins by SDS–polyacrylamide gel electrophoresis were carried out as described by Laemmli (1970) using 15% acrylamide gels and the Mighty-Small II system from Hoefer (San Francisco, CA, USA; technical bulletin No. 124). The standards used were trypsin inhibitor (M, 20 100), carbonic anhydrase (M, 29 000), alcohol dehydrogenase (M, 37 000), glutamate dehydrogenase (M, 54 000) and bovine serum albumin (M, 68 000). The presence of glycan chains was studied using the Glycan Detection Kit from Boehringer (Mannheim, Germany) using transferrin (M, 79 500) as standard.

Protein synthesis

Polypeptide synthesis was carried out in cell free translation systems derived from rabbit reticulocyte lysates, rat liver, wheat germ, Vicia sativa germ, and Cucumis sativus germ following current procedures (Girbes et al., 1993b, c). The cell-free extracts were filtered through Sephadex G-25 (8 x 2.6 cm) to remove low M, compounds and stored under liquid N2 until use and were thawed only once. In all cases translations were carried out encoded by endogenous messengers since under such conditions ribosomal sensitivity to RIPs has been shown to be maximal (Arias et al., 1993).

Protein synthesis in NHC cells was carried out using [35S]Translabel (sp. act. 1164 Ci mmol−1) as described elsewhere (Girbes et al., 1993c).

Analysis of 28 S rRNA N-glycosidase activity

100 μl of rabbit reticulocyte lysate containing 20 mM TRIS–HCl (pH 7.8), 50 mM KCl, 1 mM MgCl2, and 5 mM dithiothreitol were incubated with 5 μg of nigrin f or known RIPs for 30 min at 37°C. Reactions were stopped by the addition of 500 μl of 0.5% SDS containing 50 mM TRIS–HCl (pH 7.6). RNA was extracted by phenolization and ethanol precipitation. Aniline treatment was carried out as follows: RNA was dissolved in 20 μl of water and incubated with 1 vol. of 2 M aniline (pH 4.5) at 4°C in the darkness for 10 min. The reaction was stopped by dilution with 400 μl of water, and the aniline was removed by two extractions with 1 vol of ether. RNA was recovered by ethanol-precipitation. Electrophoresis of RNA was carried out in 5% acrylamide gels at 15 mA for 110 min as described elsewhere (de Benito et al., 1995). The gels were photographed after staining for 20 min with 0.5 μg ml−1 of ethidium bromide.

NH2-terminal amino acid sequence analysis

Nigrin f was subjected to SDS–PAGE in the presence of 2-mercaptoethanol to dissociate the A and B chains. Following this, proteins were electroblotted on to PVDF membranes and detected by staining with 0.2% Coomassie blue R-250 (w/v) in methanol:water:acetic acid (50:40:10, by vol.), for 0.5–2 min. The membranes were then washed in methanol:water:acetic acid (48:47:5, by vol.), and the well-defined protein bands were cut out of the PVDF and applied to a KNAUER sequencer with an on-line PTH amino acid analyser according to manufacturer’s instructions.

Enzyme-Linked Immunosorbent Assay for nigrin f and related proteins

ELISA was performed essentially using the method outlined in Pérez-Maceda et al. (1991). Detection was performed with anti-nigrin b rabbit polyclonal antibodies (67 pg per well) as the first antibody, obtained as described elsewhere (Harlow and Lane, 1988) and partially purified by chromatography through protein-A Sepharose 4B (Pharmacia-LKB), as described (Harlow and Lane, 1988). Alkaline phosphatase-linked goat anti-rabbit antibodies (Sigma, St Louis, MO, USA) were used as the second antibody. Substrate treatment and colour development were carried out as indicated elsewhere (Pérez-Maceda et al., 1991). Absorbance was measured at 405 nm in an ELISA reader (LP400 from Pasteur Diagnostics). Measurements were made in quadruplicate.

Red blood cells agglutination

Red blood cell agglutination was carried out at room temperature in microtitre plates containing 100 μl of 5 mM sodium phosphate (pH 7.5), 0.14 M NaCl and 0.5% of red blood cells.

Results

Isolation of nigrin f and SNA III from green and mature fruits of S. nigra

The rationale of our search in elder fruits came from preliminary experiments that indicated the presence of translational inhibitory activity in crude extracts of green elder fruits (Table 1). From our own data (Girbes et al., 1993b, c), it was concluded that these could contain a new type 2 RIP. To isolate this possible inhibitor, crude protein extracts were chromatographed by an affinity process as reported previously (Girbes et al., 1993c). As shown in Fig. 1a, elder fruit protein extracts contained α-galactose-binding proteins that bind to acid-treated Sepharose 6B and can be detached by 0.2 M lactose, in agreement with the results of Mach et al. (1991). These protein extracts strongly inhibited protein synthesis (Table 1). Attempts to resolve the α-galactose and N-acetylglactosamine-binding proteins by exclusion chromatography were unsuccessful due to the presence of a major single-chain protein revealed as the previously reported lectin SNA IV (Mach et al., 1991). A similar problem has been found for the isolation of ebulin I from leaves of S. ebulis. In this case, the RIP was retained by the Superdex 75 matrix, thus eluting as a protein with an apparent M, of around 30 000. With nigrin f and SNA IV this prevents protein resolution by size exclusion chromatography. The strategy was therefore changed and ion exchange chromatography tried. As shown in Fig. 1b, a salt gradient (0–0.3 M NaCl in buffer phosphate) in DEAE-cellulose afforded the resolution of two small protein peaks at the start of the gradient and a major protein peak that spanned almost the whole gradient. As reported below, the first two peaks (nigrin f) strongly
Table 1. Purification of nigrin f from green and mature Sambucus nigra fruits

The protein was purified as described in Materials and methods. Inhibition of protein synthesis was performed using a rabbit reticulocyte cell-free system. One unit (U) is the amount of inhibitory protein that reduces 1 ml translation reaction mixture by 50%.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ng ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Specific activity (&lt;i&gt;x&lt;/i&gt; 10&lt;sup&gt;-3&lt;/sup&gt; U mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Total activity (&lt;i&gt;x&lt;/i&gt; 10&lt;sup&gt;-6&lt;/sup&gt; U)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green fruits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>2640</td>
<td>58</td>
<td>17.2</td>
<td>45.5</td>
<td>100</td>
</tr>
<tr>
<td>AT-Sepharose</td>
<td>90</td>
<td>26</td>
<td>38.5</td>
<td>3.4</td>
<td>7.5</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>1.3</td>
<td>1.8</td>
<td>556</td>
<td>0.7</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Mature fruits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>2930</td>
<td>190</td>
<td>5.3</td>
<td>15.4</td>
<td>100</td>
</tr>
<tr>
<td>AT-Sepharose</td>
<td>13</td>
<td>145</td>
<td>6.9</td>
<td>0.9</td>
<td>5.8</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>0.12</td>
<td>1.9</td>
<td>526</td>
<td>0.06</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Fig. 1. Isolation of nigrin f from green fruits of <i>Sambucus nigra</i> L. Nigrin f was isolated as described in Materials and methods. (a) Affinity chromatography of crude protein extract from green fruits of <i>S. nigra</i>. The arrow indicates addition of 0.2 M lactose. (b) DEAE-cellulose-FPLC of protein from the affinity chromatography step. Horizontal bars indicate fractions that were pooled and subjected to further studies.  

Inhibited protein synthesis in a rabbit reticulocyte lysate while the third peak (SNA IV) proved to be largely inactive as a translation inhibitor.

**Molecular structure analysis**

As shown in Fig. 2, SDS-PAGE analyses of these peaks in the absence of 2-mercaptoethanol (2-ME) revealed that the first protein peak contains a homogeneous protein that migrates with an apparent <i>M</i><sub>r</sub> of 58 000. This was named nigrin f and, as shown in this paper, it is a new type 2 RIP. The second protein peak contained the same protein although it was contaminated with a small amount of the protein from peak three. Peak three contained a homogeneous protein that migrates at an apparent <i>M</i><sub>r</sub> of 30 000 and that, on the grounds of previously published properties and our own results, is the SNA IV lectin reported earlier (Mach et al., 1991).

SDS-PAGE analyses of the protein peaks in the presence of 2-ME (to break disulphide bridges) revealed that nigrin f has four proteins: two major ones with apparent <i>M</i><sub>r</sub> values of 31 600 and 26 200, and another two minor ones with apparent <i>M</i><sub>r</sub> values of 30 000 and 28 200. SNA IV is a polypeptide chain with apparent <i>M</i><sub>r</sub> of 30 000 accompanied by a very minor and closely related band exhibiting faster migration properties. Isoelectrofocusing analysis revealed that nigrin f has two significant isoforms one of them major and other one almost negligible (data not shown). These data are consistent with a major structure with a <i>M</i><sub>r</sub> of 58 000 (<i>M</i><sub>r</sub> 26 200 plus <i>M</i><sub>r</sub> 31 600) and a minor structure with a <i>M</i><sub>r</sub> of 58 000 (<i>M</i><sub>r</sub> 28 200 plus <i>M</i><sub>r</sub> 30 000). By analogy with ebulin I and nigrin b, and as confirmed by N-terminal amino acid sequence analysis (see below), the protein of <i>M</i><sub>r</sub> 26 300 would be a catalytic A chain while the protein of <i>M</i><sub>r</sub> 31 600 would be a lectin moiety able to bind to p-galactose residues.

As shown in Fig. 2b, the B chains of nigrin b-the type 2 RIP from elder bark- and nigrin f are both highly glycosylated proteins, as revealed by the glycan detection assay. Additionally, SNA IV is also strongly glycosylated, in agreement with Mach et al. (1991).
Effect of elder fruit maturation on protein content

Mature fruits of elder displayed a similar pattern of proteins as green fruits but with much less protein. The steps and yields of the isolation procedure of nigrin f and SNA IV from mature fruits are also outlined in Table 1. The total content of inhibitory activity, much of which is lost during the isolation of nigrin f, is striking. This is probably due to the presence of inhibitory compounds such as RNases, polyphenols, pigments, etc., and of type 1 RIPs named nigritins (manuscript in preparation) which are similar to those described recently in the leaves of dwarf elder (Sambucus ebulus L.) (de Benito et al., 1995). Additionally, there is a strong reduction in the amounts of nigrin f and SNA IV contents (10-fold) in mature fruits with respect to the green fruits.

Inhibition of protein synthesis

To define nigrin f as an RIP, its effects on translation in vitro were analysed. As shown in Fig. 3, nigrin f strongly inhibited protein synthesis carried out by rabbit reticulocyte lysates and rat liver cell-free systems while it had no effect on plant cell-free systems derived from wheat, V. sativa and C. sativus germs. Its inhibitory potency is well defined by its IC50 (concentration of protein that promotes a 50% inhibition of protein synthesis). Nigrin f IC50 were 1.8 and 3.7 ng ml⁻¹ for rabbit reticulocyte lysates and rat liver cell-free systems, respectively. These values are lower than those reported for ebulin I (Girbès et al., 1993c) and nigrin b (Girbès et al., 1993b), such differences being highly reproducible among the different batches of nigrin f. The effects of nigrin f on protein synthesis carried out by NHC cells of human origin were also analysed. The results (data not shown) indicated that up to a concentration of 60 μg ml⁻¹, nigrin f was inactive on such cells in contrast to the highly toxic type 2 RIP ricin (Olsnes and Pihl, 1982). Additionally, and to confirm the data on cultured cells, up to 1.56 mg kg⁻¹ of body weight of nigrin f to mice was injected with no apparent toxic effects over 20 d. However, derangement of non-vital processes cannot be excluded in such de visu analyses.

N-glycosidase activity

In order to confirm the inhibitory activity of nigrin f on mammalian ribosomes, the mechanism of action of the protein was investigated. Accordingly, the N-glycosidase activity characteristic of RIP action (Endo and Tsurugi, 1987) was analysed on rabbit reticulocyte ribosomes. As shown in Fig. 4, treatment of rabbit reticulocyte lysates with nigrin f for 30 min, which as shown in Fig. 3 inactivates ribosomes, promoted the depurination of ribosomes, which upon rRNA isolation and subsequent treatment with acid aniline splits the RNA fragment diagnostic of RIP action (Endo and Tsurugi, 1987). Such inactivation occurs through depurination of the 28 S rRNA at the highly conserved adenine responsible for the interaction of elongation factors with the ribosome, in both eukaryotic (Brigotti et al., 1989) and prokaryotic organisms (Iglesias et al., 1993b). The same result was obtained with pokeweed antiviral protein (PAP-S), a type 1 RIP.
Agglutination of human red blood cells

Both nigrin f and SNA IV promote the agglutination of human red blood cells at quite low concentrations. Total agglutination by nigrin f of red cells from type O blood was achieved at 58 \( \mu \text{g ml}^{-1} \) and from type AB blood at 116 \( \mu \text{g ml}^{-1} \). SNA IV isolated by this procedure agglutinated red cells from type O blood at 88 \( \mu \text{g ml}^{-1} \). These values lie within the range of those reported for SNA II (Kaku et al., 1990) and the lectin described in elder seeds, also named SNA III by Peumans et al. (1991). Both of these are N-acetylgalactosamine-specific lectins and their agglutination capacities are much lower than those reported for SNA I which is a sialic acid-specific lectin (Broekaert et al., 1984).

N-terminal amino acid sequence of nigrin f

In order to compare nigrin f with known proteins, in particular the SNA IV from elder fruits (Mach et al., 1991), and the RIP SNA I from elder bark (van Damme et al., 1996a), the N-terminal end of nigrin f was sequenced. As shown in Fig. 5, the nigrin f-A chain shares strong amino-acid sequence homology with the A chain of both ebulin 1 (Girbés et al., 1993c), nigrin b (Girbés et al., 1993b) and SNA I (van Damme et al., 1996a), and also with several type 1 RIPs such as trichosanthin (Collins et al., 1990), TAP 29 (Lee-Huang et al., 1991a), bryodin-S (Montecucchi et al., 1989), luffin a (Funatsu et al., 1988), all from Cucurbitaceae, and gelonin (Montecucchi et al., 1989) and ricin-A chain (Lamb et al., 1985), from Euphorbiaceae and PAP (Kung et al., 1990) and an internal region of topoisomerase II from Drosophila (Lee-Huang et al., 1994). The nigrin f B-chain also shares strong amino acid sequence homology with the B-chains of nigrin b (Girbés et al., 1993b), ebulin 1 (Girbés et al., 1993c), SNA I (van Damme et al., 1996a) and ricin (Araki and Funatsu, 1987) and also with the lectins SNA IV (Mach et al., 1991) and SNA II (Kaku et al., 1990). These results clearly indicated that nigrin f isolated here is a protein other than SNA IV.

Immunological cross-reactivity of nigrin f with anti-nigrin b rabbit polyclonal antibodies

As shown in Fig. 6, ELISA analysis of non-toxic type 2 RIPs revealed that anti-nigrin b rabbit polyclonal antibodies at a dilution of 1/3500 strongly reacted with nigrin f. By contrast, these antibodies showed only a weak reaction with several lectins isolated from S. nigra such as SNA II (Kaku et al., 1990) and IV (Mach et al., 1991) and the RIP SNA I (van Damme et al., 1996a).

Discussion

A recent paper dealing with the finding of a new lectin reported that elder fruits contain a new lectin, named SNA IV, that tends to form di- and oligomeric aggregates (Mach et al., 1991). Here, the presence of SNA IV in elder fruits has been confirmed, but have found among the dimeric aggregates of SNA IV a novel non-toxic type 2 RIP of the ebulin 1 (Girbés et al., 1993c) and nigrin b
which is the case of RIPs in general. Nonetheless, it has structurally related lectins in these genera. The presence of equivalent non-toxic type 2 RIPs or to the best of our knowledge no studies have reported potent anti-HIV-1 effects (McGrath et al., 1993c). The small changes in the amino acid sequence of nigrin f with respect to nigrin b indicate that all of them derive from a common ancestor, also probably related to the lectins SNA II (Kaku et al., 1990) and SNA IV (Table 1). This is also consistent with a storage role for both proteins. However, it remains unclear whether nigrin f might trigger plant cell maturation or whether such a reduction would merely be a consequence of maturation. On the other hand, these results also indicate that anti-nigrin b rabbit polyclonal antibodies react in Western blot analysis with SNA f and SNA II (data not shown), thus pointing to a close structural correlation between the elder RIP and the elder lectins. Further work will address the potential antiviral properties of nigrin f on the basis of its primary structure and activity on RNA.

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References


de Benito FM, Citores L, Iglesias R, Ferreas JM, Soriano F, Arias FJ, Méndez E, Girbes T. 1995. Ebulitins: a new family of type 1 ribosome-inactivating proteins (rRNA N-glycosidases) from leaves of Sambucus ebulus L. that has been suggested that SNA I, a sialic acid and galactose-binding lectin with RIP activity from elder bark probably related to the structure of nigrin f B-chain, as found with other lectins isolated from elder (Mach et al., 1993; Kaku et al., 1990), is located in the protein bodies of phloem parenchyma of bark (Nsima-Lubaki and Peumans, 1986). On these grounds, the involvement of SNA I in protein storage processes was suggested. These results indicate that maturation of dwarf elder fruits leads to a strong reduction in the content of both nigrin f and SNA IV (Table 1). This is also consistent with a storage role for both proteins. However, it remains unclear whether nigrin f might trigger plant cell maturation or whether such a reduction would merely be a consequence of maturation. On the other hand, these results also indicate that anti-nigrin b rabbit polyclonal antibodies react in Western blot analysis with SNA f and SNA II (data not shown), thus pointing to a close structural correlation between the elder RIP and the elder lectins. Further work will address the potential antiviral properties of nigrin f on the basis of its primary structure and activity on RNA.

Fig. 6. Immunological cross-reactivity of rugrin f with anti-nigrin b rabbit polyclonal antibodies. Immunological cross-reaction with anti-nigrin b was studied by ELISA, as described in Materials and methods. Symbols: (●) nigrin b; (○) nigrin f; (▲) SNA f; (△) SNA II; (□) SNA IV. The points represent the means from four sets of measurements and bars indicate the standard error of the mean.

(Girbes et al., 1993b) family. This compound has been named nigrin f.

Nigrin f contains two different polypeptide chains that, in view of all the data on protein synthesis, electrophoretic analysis, amino acid sequence and red blood cell agglutinating activity, are clearly the catalytic A chain (Mr, 26 200) and the lectin B chain (Mr, 31 600), characteristic of common type 2 RIPs.

Nigrin f fulfils all requirements to be classified as a non-toxic type 2 RIP that strongly resembles, but is clearly different from the other two non-toxic type 2 RIPs reported to date: nigrin b (Girbes et al., 1993b) and ebulin I (Girbes et al., 1993c). The small changes in the amino acid sequence of nigrin f with respect to nigrin b and ebulin I indicate that all of them derive from a common ancestor, also probably related to the lectins SNA II (Kaku et al., 1990) and SNA IV (Mach et al., 1991). It is very interesting to note the close correlation between the nigrin f A-chain and type 1 RIPs of Cucurbitaceae (a genus very close to the Caprifoliaceae) and the ricin A-chain (Euphorbiaceae), which display potent anti-HIV-1 effects (McGrath et al., 1989; Lee-Huang et al., 1990, 1991a, b; Olson et al., 1991). However, to the best of our knowledge no studies have reported the presence of equivalent non-toxic type 2 RIPs or structurally related lectins in these genera.

Nothing is known of the biological role of nigrin f, which is the case of RIPs in general. Nonetheless, it has...


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