Isolation and partial characterization of a novel and uncommon two-chain 64-kDa ribosome-inactivating protein from the bark of elder (*Sambucus nigra* L.)

Fernando M. de Benito[a], Lucía Citores[a], Rosario Iglesias[a], J. Miguel Ferreras[a], Emilio Camafeita[b], Enrique Méndez[b], Tomás Girbés[a,*]

[a]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, E-47005 Valladolid, Spain*
[b]*Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Canto Blanco, Madrid, Spain*

Received 6 June 1997; revised version received 3 July 1997

Abstract A novel, strongly basic, two-chain ribosome-inactivating protein (RIP) with an apparent M_r of 64 000 by SDS-PAGE and 63469 by mass spectrometry analysis, that we have named basic nigrin b, has been found in the bark of elder (*Sambucus nigra* L.). The new protein does not agglutinate red blood cells, even at high concentrations and displays an unusually and extremely high activity towards animal ribosomes (IC_{50} of 18 pg/ml for translation by rabbit reticulocyte lysates). However, it is inactive against plant and HeLa cells protein synthesis. Our functional and structural data are consistent with a heterodimeric structure for basic nigrin b of the type A-B*, B* being a truncated lectin lacking functional binding domains equivalent to the B (lectin) chain of the type 2 RIP SNA I and nigrin b present also in elder bark.

Key words: Ribosome-inactivating proteins; rRNA *N*-glycosidase; Protein synthesis inhibition; Basic nigrin b; Nigrin b; *Sambucus nigra* L.

1. Introduction

Ribosome-inactivating proteins (RIPs) may be considered as antipathogenic proteins present in a number of plants. RIPs are translation inhibitors that act by arresting protein synthesis by eukaryotic and, in some cases, prokaryotic ribosomes [1,2]. The molecular mechanism by which these proteins inactivate ribosomes is the single depurination of the large rRNA which, upon treatment with acid aniline, releases the so-called RIP diagnostic fragment [1–3]. In some cases, RIPs promote multidepurination of rRNA [4,5]. Some RIPs act on nucleic acid other than rRNA such as salmon sperm DNA [6,7] and genomic viral RNA [6–8], and even on poly(A) [6,7].

According to Barbieri et al. [1], RIPs may be classified in functional terms into two categories: type 1 and type 2 RIPs. Type 1 RIPs are single-chain proteins with *N*-glycosidase activity and are the most widely distributed proteins [1]. Type 2 RIPs are two-chain proteins linked by disulphide bonds with an A chain, functionally identical to a type 1 RIP, and a B chain which is a lectin, usually specific for D-galactose and derivatives [1], although an exception which also binds sialic acid has also been described [8].

Very recently it has been reported that viral infection and the molecular mediators of viral infection such as H_2O_2 and salicylic acid trigger the expression of two single-chain RIPs called beetins [8]. These data, together with those showing a direct effect of PAP on *Phytolacca americana* L. ribosomes [10] and the preventive effects of a number of RIPs on tobacco mosaic virus propagation [10,11], support the antiviral hypothesis of RIPs. However, pleiotropic effects of RIPs cannot be ruled out.

In recent few years, the species *Sambucus* has been found to contain several RIPs. From *Sambucus ebulus* L., the non-toxic type 2 RIP ebulin [12] and the family of type 1 RIPs ebulitins [13] have been isolated and partially characterized. From *Sambucus nigra* L., the non-toxic type 2 RIPs nigrin b, nigrin s, and nigrin f have been isolated [14–17]. The non-toxic expression refers to the fact that these two-chain RIPs are several orders or magnitude less toxic to animals than ricin [18]. The lectin SNA I has been recognized as a type 2 RIP in *S. nigra* [9]. Furthermore, the gene sequence coding for nigrin b (also called SNA V) has also been published [19]. We have continued study of *Sambucus* RIPs and have found a novel type of RIP that we have named basic nigrin b, the compound having unusual functional properties. The simultaneous occurrence in elder bark of two-chain RIPs structural and functionally different (i.e. nigrin b, basic nigrin b and SNA I) is reported for the first time. We believe that our observations are interesting since basic nigrin b lacks the red blood agglutination ability characteristic of all type 2 RIPs known to date.

2. Materials and methods

2.1. Materials

All chemicals, biochemicals, chromatographic supports and radioactive compounds were of the highest purity available and were obtained as described previously [12,14]. pGEM4Z was purchased from Promega (Madison, WI, USA). Tobacco mosaic virus (TMV) RNA was obtained from tobacco (*Nicotiana tabacum*) leaves field-infected with TMV and purified by high speed centrifugation and phenolization as described elsewhere [20]. One-year old elder bark was obtained from a bark pool from not less than 12 elder trees grown in Cobos de Cerrato (Palencia, Spain) in September–October. Nigrin b and ebulin l were prepared as described elsewhere [12,14].

2.2. Isolation of basic nigrin b

Two hundred g of elder bark were ground in a blender and extracted as described elsewhere [14]. The crude extract was subjected to affinity chromatography on acid-treated Sepharose 6B (AT-Sepharose) as described previously in order to remove nigrin b [14]. Puri-

*Corresponding author. Fax (34) (83) 423 082.

Abbreviations: IC_{50}, concentration of inhibitory protein that gives 50% of inhibition in the rabbit reticulocyte lysate translation system; Nigrin bb, basic nigrin from bark; PAGE, polyacrylamide gel electrophoresis; RIP(s), ribosome-inactivating protein(s); TMV, Tobacco mosaic virus

PII S0014-5793(97)00882-X

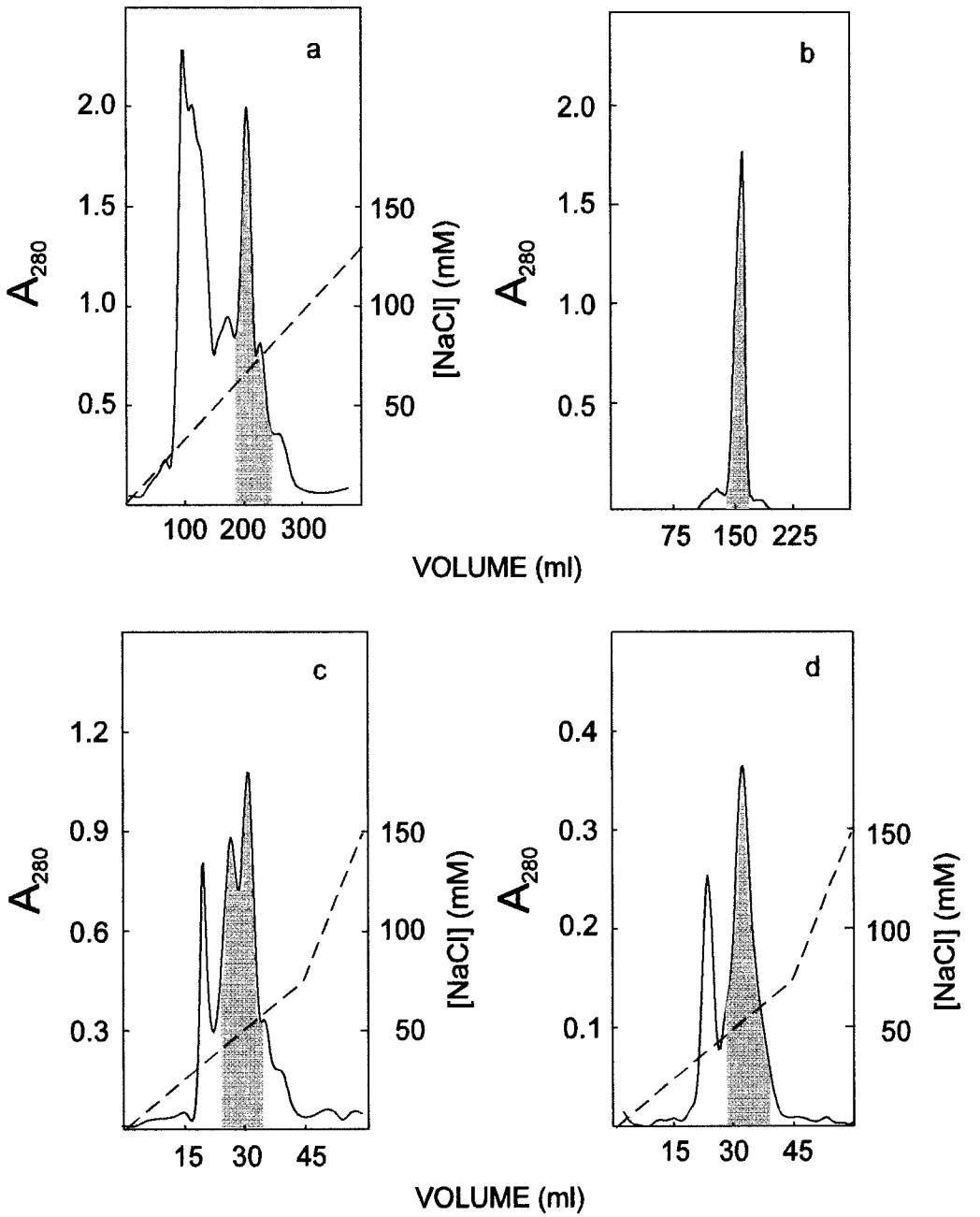
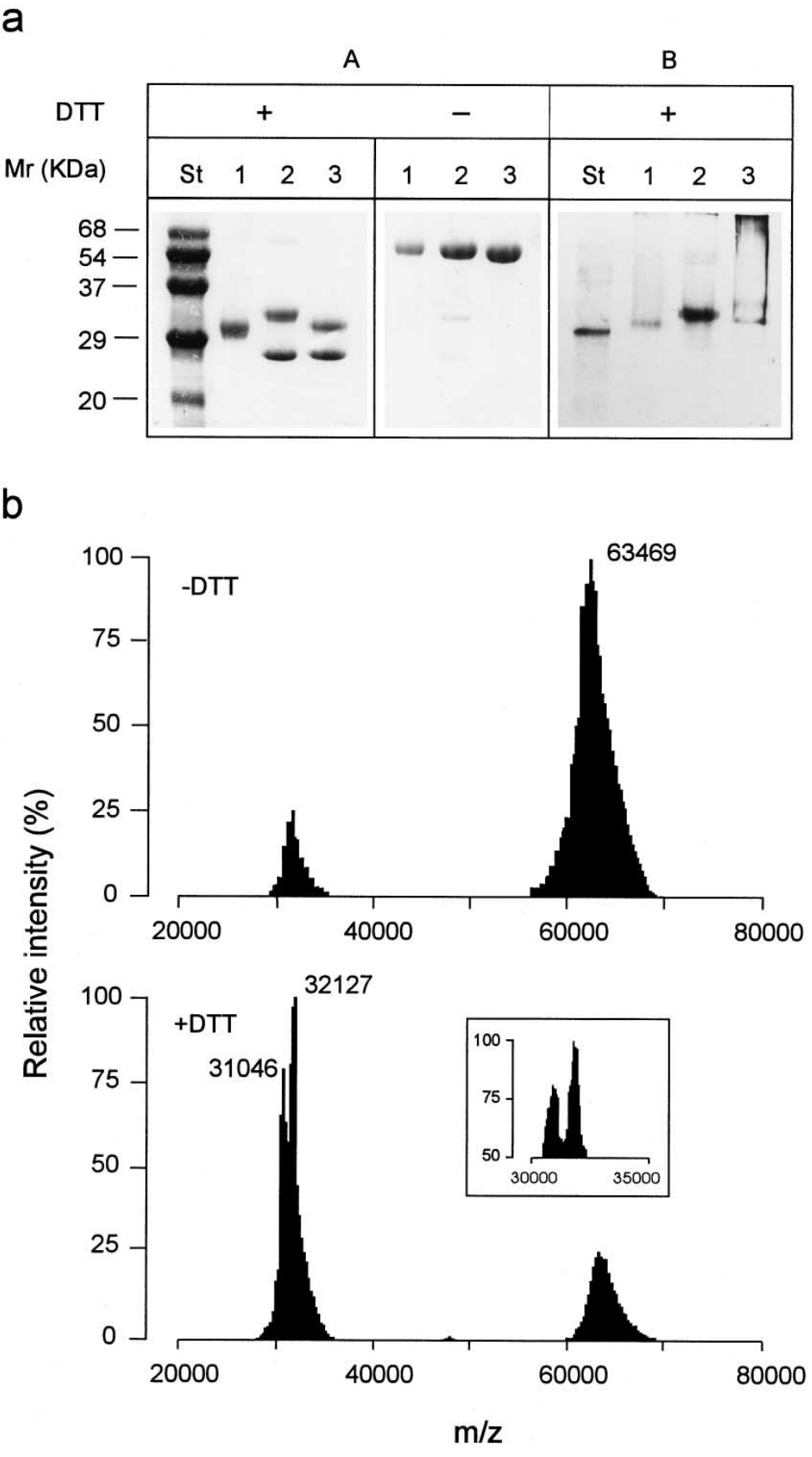


Fig. 1. Purification of basic nigrin b from *Sambucus nigra* bark by chromatography through CM-Sepharose FF (a), Superdex 75 HiLoad (b), first MonoS (s) and second MonoS (d). For experimental procedures, see Section 2. Solid line, A_{280} elution profile; dashed line, salt gradient. Fractions were assayed for protein synthesis inhibition on a rabbit reticulocytes lysate protein synthesis system. Those fractions that showed to contain the major inhibitory activity (shaded peeks) were pooled and subjected to further purification.

fication of basic nigrin b was performed by a general protocol used for the preparation of other RIPs [13,21]. This included a first chromatography on SP-Sepharose Fast Flow [13], followed by chromatography on CM-Sepharose Fast Flow, Superdex 75 HiLoad and MonoS. CM-Sepharose chromatography was developed in a column of 5×2.6 cm with a 0–0.3 M NaCl linear gradient in 5 mM sodium phosphate (pH 6.7; total volume 800 ml). This chromatographic step yields three peaks of protein synthesis inhibition activity, the second

→

Fig. 2. Analysis of basic nigrin b by SDS-PAGE and MALDITOF/MS. (a) Proteins were electrophoresed in the absence or presence of dithiothreitol (DTT). Thereafter, two gels were stained with Coomassie blue (A) and the third gel was used to detect glycan chains (B) as indicated in Section 2. Lanes contained 8 (A) or 3.5 (B) μg of protein. St, standards; 1, basic nigrin b; 2, nigrin b; 3, ebulin l. Numbers on the left indicate the corresponding molecular masses of the standards in kDa. (b) Matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF/MS) spectrum was obtained as indicated in Section 2 in the absence or presence of dithiothreitol (DTT). A smooth background has been subtracted. Inset shows details of the region *m/z* 30000–35000.


a
A
B
DTT
+
–
+
Mr (KDa)
St 1 2 3 1 2 3 St 1 2 3
68
54
37
29
20
b
-DTT
63469
+DTT
32127
31046
Relative intensity (%)
100
75
50
25
0
20000
40000
60000
80000
30000
35000
m/z

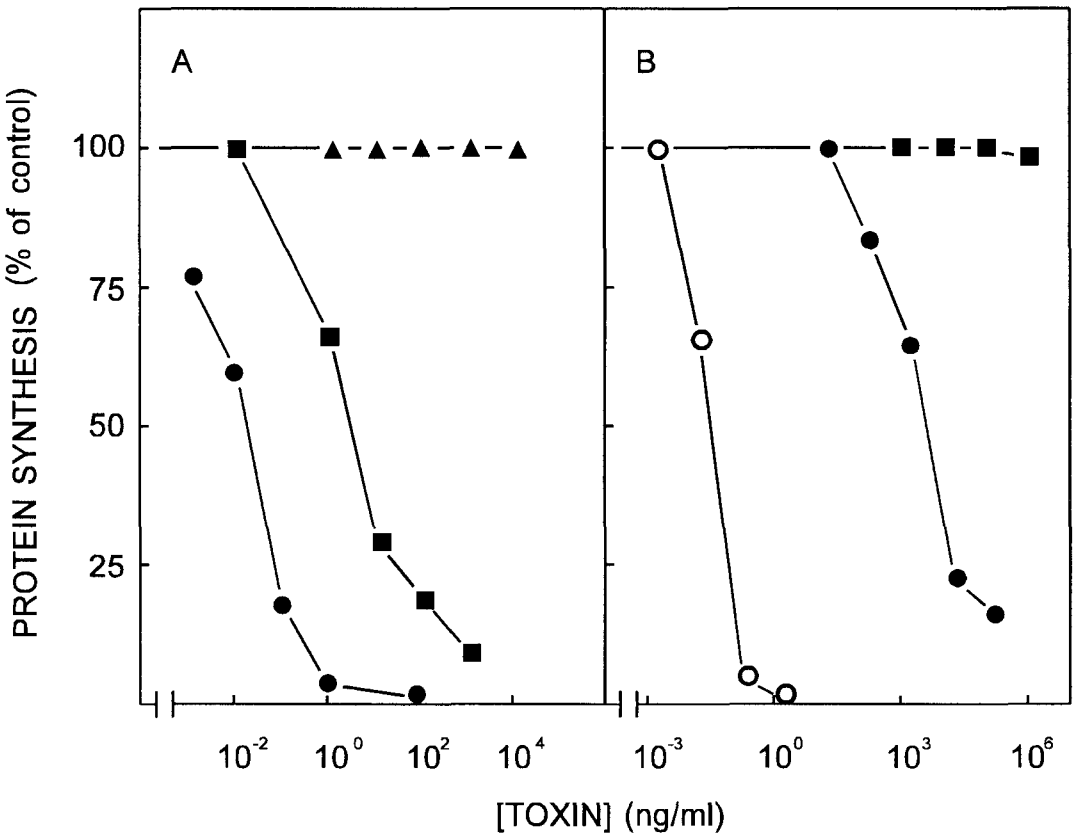


Fig. 3. Effect of basic nigrin b on protein synthesis carried out by cell-free and intact cell translation systems. Experimental details were as indicated in Section 2. Protein synthesis inhibition by basic nigrin b was assayed in cell-free systems (A) derived from rabbit retyculocytes (●), rat liver (■) and *Triticum aestivum* germ (▲). Panel (B) shows the effect of basic nigrin b (■) on HeLa cells compared with nigrin b (●) and ricin (○). Controls were run in the absence of inhibitor and values of 100% were given.

one being most prominent. This protein peak was concentrated and subjected to Superdex 75 HiLoad chromatography to yield a single peak of inhibition (228.5 mg) that was dialyzed and further purified by chromatography through a Mono-S column (in aliquots of 3.5 mg) with a sodium chloride gradient in 5 mM sodium phosphate (pH 6.7). Fractions containing the highest protein synthesis inhibition activity were pooled, dialyzed and subjected to further MonoS chromatography. This afforded a homogeneous protein preparation. The purified protein was collected, dialyzed against MilliQ-water and freeze-dried.

2.3. Preparation of cell-free translation systems and polypeptide synthesis

Animal-derived and plant-derived cell-free translation systems were prepared according to standard published procedures [22,23]. The cell-free extracts were filtered through a column (8×2.5 cm) of Sephadex G-25 to remove low M_r compounds that could interfere with translation and were stored in small aliquots under liquid N_2 until their use, being thawed only once.

2.4. Protein synthesis in HeLa cells

HeLa cells were maintained with RMPI 1640 complete medium supplemented with 10% foetal calf serum, glutamine and antibiotics. For protein synthesis analysis, HeLa cells were seeded on 24-well plates and processed as described elsewhere [18], using 1 μCi/ml of [^{35}S] in vitro cell labelling mix (Promix L [^{35}S] from Amersham; sp. act. 1000 Ci/mmol). After 2 h of incubation at 37°C in a CO_2 incubator, protein synthesis was stopped by the addition of 1 ml of 5% trichloroacetic acid and the radioactivity incorporated into proteins was assessed as described elsewhere [18].

2.5. N-Glycosidase activities of basic nigrin b on 28 S rRNA and on TMV genomic RNA

Either 100 μl of rabbit reticulocyte lysate or 5 μg of purified genomic TMV RNA in 25 μl were incubated with variable amounts of either basic nigrin b or nigrin b for 15 min at 37°C under the same conditions as described elsewhere [12]. The RNA was extracted by phenolization and ethanol precipitation and then RNA electrophoresis was carried out in gels of 5% acrylamide at 21 mA for 50 min using a buffer containing 89 mM Tris-HCl (pH 8.3), 89 mM boric acid and 2.5 mM EDTA [12]. The gels were photographed after staining for 20 min with ethidium bromide (0.5 μg/ml).

2.6. DNA topoisomerase activity

Two hundred ng of pGEM4Z were incubated in a reaction mixture that contained in 10 μl: 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 15 μg/ml bovine serum albumin. After incubation for 1 h at 30°C, the different forms of DNA were analyzed by electrophoresis in 0.8% agarose gels with 89 mM Tris-HCl (pH 8.3), 89 mM boric acid and 2.5 mM EDTA buffer at 60 V for 2 h. DNA bands were then photographed after staining for 30 min with ethidium bromide (0.5 μg/ml).

2.7. Mass spectrometry analysis of basic nigrin b

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 on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Bruker Instruments, Billerica, MA) [24]. The experimental conditions used ensure the non-covalent interactions of sample proteins in solution. Molecular weights of protein peaks were assigned using cytochrome c and bovine serum albumin as external standards.

2.8. Preparation of tryptic peptides and analysis of N-terminal amino-acid sequences

Tryptic peptides were prepared as described elsewhere [25]. The

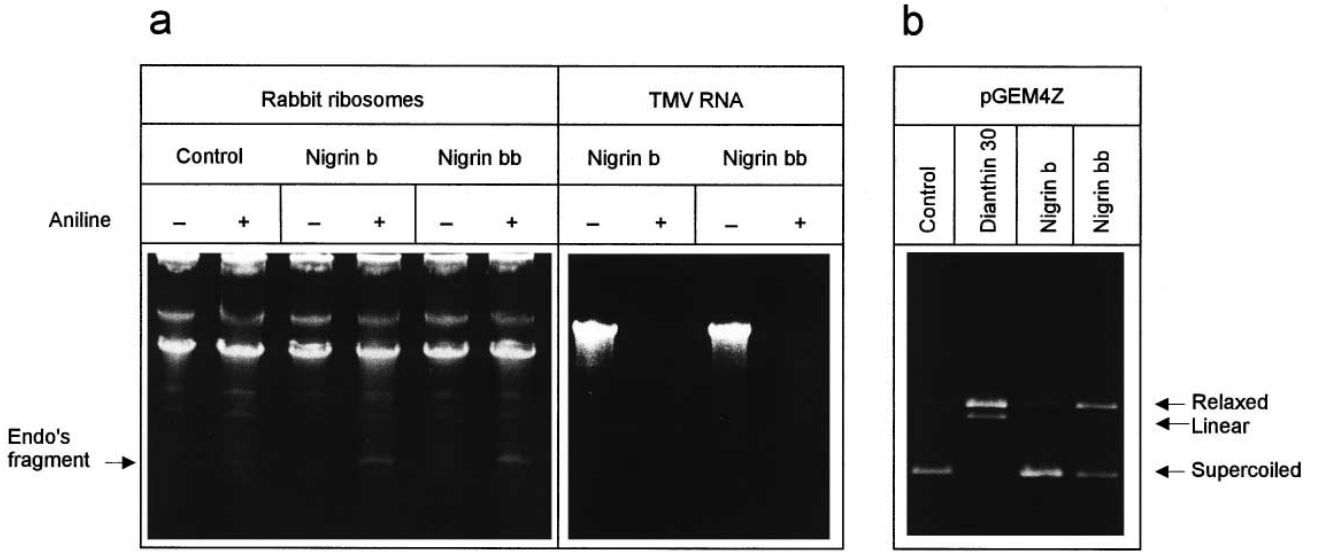


Fig. 4. *N*-glycosidase activity of basic nigrin b on rabbit ribosomes and Tobacco Mosaic Virus RNA and topoisomerase activity on DNA. (a) RNA *N*-glycosidase activity was assayed as indicated in Section 2 using either 50 μg/ml (rabbit ribosomes) or 24 μg/ml (TMV RNA) of toxin. Each lane contained 1.5 μg of RNA. The arrow on the left indicates the RNA fragment released from the rabbit 28 S rRNA as a consequence of RIP action upon acid aniline treatment. (b) Topoisomerase was assayed on pGEM4Z as indicated in Section 2 with either 10 μg (Dianthin 30) or 1000 μg (nigrin b and basic nigrin b) of toxin. The arrows on the right indicate the position of the relaxed, linear and supercoiled forms of the plasmid.

analysis of N-terminal amino-acid sequences of some peptides was performed as described elsewhere [25].

2.9. Other assays

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins and the detection of glycan chains in proteins were carried out as has been described previously [25]. Isoelectric focusing (IEF) was carried out using a Fast System and pH 3–10 gradient gels (Pharmacia Ibérica, Madrid, Spain)

3. Results and discussion

Acid-treated Sepharose 6B chromatography of a saline crude extract of elder bark yielded a retained D-galactose-binding protein fraction and an unbound protein fraction [12]. The unbound protein fraction displayed strong inhibitory activity on translation carried out by rabbit reticulocyte lysates (data not shown). Such protein fraction was subjected to a current protocol of RIP purification, including SP-Sepharose Fast Flow, CM-Sepharose Fast Flow, Superdex 75 Hi-Load and two Mono-S chromatographic steps. As shown in Fig. 1, CM-Sepharose chromatography resolved several protein peaks. The second peak displayed a strong inhibitory protein synthesis activity on a rabbit reticulocyte lysate. After all the chromatographic steps, this peak was seen to contain a protein with an apparent M_r of 64 000, which was found to be homogeneous in SDS-polyacrylamide gel electrophoresis in the absence of reducing agent (Fig. 2aA). Incubation of this protein with 2-mercaptoethanol but not with dithiothreitol promoted its precipitation. Therefore we used dithiothreitol as reducing agent. SDS-PAGE in the presence of dithiothreitol yielded a protein band with an apparent M_r of 32 000 (Fig. 2aA). The protein, which we call basic nigrin b since the isoelectric point is higher than 10 measured by IEF (data not shown), contains sugar chains (Fig. 2aB). Analysis of basic nigrin b by mass spectrometry clearly indicates that it is a heterodimer whose subunits have M_r of 31 046 and 32 127 (Fig. 2b). Unfortunately, we have been unable to resolve and isolate both subunits by any known conventional chromatographic or electrophoretic procedures.

We further studied the effects of basic nigrin b on protein synthesis carried out by cell-free systems from both mammals and plants. As shown in Fig. 3A, the protein was inactive on

N b b peptide 1		**DG**- - - - - - - - - -**GQQS SQQ**...	
SNLRP 2	28	LAK**DGN**- - PVQLLSC**GQQS SQQ**WTF	50
SNA I	31	HYI**DGN**- - PVQLRPC**GNE CNQL**WTF	53
SNA I′	30	NGDEKDTTPVQLSSC**GEQS NQQ**WTF	54
N b	29	YDT**DGT**- - PLQWP - C**GTQR NQR**WTF	51
N b b peptide 2		**AMI YK**...	
SNLRP 2	72	NY**AMI YN**CDT	82
SNA I	69	SSSV**MI YN**DCT	79
SNA I′	70	SSSV**MI YN**CKV	80
N b	69	LNNGSIFNCST	82
N b b peptide 3		**N IHAASQGWI VY**...	
SNLRP 2	119	LQK**N IHAASQGWI V**GNV	135
SNA I	117	LEN**N IHAARQGWT V**GDV	133
SNA I′	118	LEK**NVHAARQGWI V**GNV	134
N b	120	LED**N IYAASQGWT VT**NV	136
N b b peptide 4		**TEQMWR**...	
SNLRP 2	167	NRR**EQMWA**LYG	177
SNA I	165	NRV**QQEWA**LYG	175
SNA I′	168	SKV**DQKWA**LYG	179
N b	169	TSL**QQQWA**LYG	179

Fig. 5. Amino-acid sequence comparison of basic nigrin b and the B chain of several type 2 RIPs. Tryptic peptides were obtained and sequenced for the N-terminal region as indicated in Section 2. Identical amino acids are bolded and the numbers indicate the position of the amino acids in the corresponding proteins, points indicate undetermined sequences.

the plant systems. By contrast, it was strongly active on both rabbit reticulocyte lysates and rat liver cell-free systems. The IC_{50} for rabbit reticulocyte lysates was 18 pg/ml, which to our knowledge is the lowest value found for an inhibitor of translation. Unlike its activity on cell-free systems, basic nigrin b had no inhibitory action at all against protein synthesis in HeLa cells (Fig. 3B) as compared with ricin and nigrin b [18]. In support of this lack of toxicity on intact cells is the fact that the intraperitoneal injection of 40 mg/kg body weight of basic nigrin b was not toxic to mice.

Concerning its mechanism of action, basic nigrin b was able to trigger the single depurination of rabbit reticulocyte ribosomes, which upon treatment with acid aniline yielded the RIP diagnostic RNA fragment (Fig. 4a). Furthermore, it was able to multidepurinate genomic TMV RNA, which after acid aniline treatment underwent complete degradation (Fig. 4a) thus suggesting that this RNA could be a multitarget for the RIP. Additionally, as may be seen in Fig. 4b, basic nigrin b promoted the conversion of supercoiled pGEM4Z DNA into both relaxed-circle and linear DNA. This indicated that basic nigrin b also displays the topoisomerase activity found in some RIPs, which seems to be involved in an important way in their anti-HIV-1 action [26].

Surprisingly, basic nigrin b was completely unable to agglutinate red blood cells, even at very high concentrations (0.42 mg/ml). This contrasts with other type 2 RIPs present in *Sambucus*, such as nigrin b [14] and SNAI in elder bark [6], nigrin s in elder seeds [15], nigrin f in elder fruits [16], and ebulin 1 in dwarf elder leaves [12]. It also contrasts with all known data on the toxic type 2 RIPs (i.e. ricin, abrin, etc [1]). To compare with other type 2 RIPs from *Sambucus* we studied the N-terminal amino-acid analysis. Unfortunately, the protein was blocked in the N-terminus. Therefore we prepared tryptic peptides and analyzed their N-terminus. As shown in Fig. 5, three peptides analyzed fit well with amino-acid sequences of the B chain of several type 2 RIPs from *Sambucus*, namely nigrin b [14,19], SNA I [9] and SNA I′ [27]. Therefore, both functional and structural data supports our proposal that basic nigrin b has a heterodimeric structure of the type A-S-S-B*, B* being an altered B (lectin) chain unable to trigger red blood cell agglutination.

Very recently a similar protein, a type 2 RIP called SNLRP, has been isolated from the bark of elder [28]. SNLRP has an IC_{50} of approximately 0.5 μg/ml in rabbit reticulocyte lysates which contrasts with the IC_{50} value of 18 pg/ml of basic nigrin b in the same cell-free translation system. This large difference in the IC_{50} on rabbit reticulocyte lysates cannot be accounted by the variability in the activity and sensitivity to RIPs of such cell-free system. In fact, the IC_{50} values reported by different laboratories using the same cell-free system and the same RIPs fall very closely [1,29]. The IC_{50} of nigrin bb is the lowest reported to date for a RIP on cell-free translation systems. On the other hand, our peptide sequences (in particular peptide 1) indicate that SNLRP2 is more related to SNA I and SNA I′ than to nigrin bb. Taken together, the data on enzymic activity and on amino-acid sequence, support the idea that they are actually different type 2 RIPs. During revision of this article, Battelli et al. Reported that SNLRP inhibits protein synthesis by a cell-free system and, at much higher concentration, by cells [30].

Finally, we found that the content in elder bark protein was season-dependent (data not shown). The yield of basic nigrin b was 180 mg from 200 g of elder bark collected in September–October. This, together with its very high activity on mammalian ribosomes, makes basic nigrin b a good candidate for use as a toxic moiety of immunotoxins (i.e. for cancer therapy). Additionally, the simultaneous presence of basic and neutral, non-toxic two-chain RIPs with different biological activities in the same plant opens a new door in RIP research.

Acknowledgements: This work was supported by grants from CICYT (BIO95-0610; PTR94-0062) and from Consejería de Sanidad y Bienestar Social (Junta de Castilla y León). We wish to thank N. Skinner (University of Salamanca, Spain) for correcting the English version of the manuscript. The technical assistance of J.E. Basterrechea is greatly appreciated.

References

[1] Barbieri, L., Battelli, M.G. and Stirpe, F. (1993) Biochim. Biophys. Acta 1154, 237–282.
[2] Girbés, T., Ferreras, J.M., Iglesias, R., Citores, L., de Torre, C., Carbajales, M.L., Jiménez, P., de Benito, F.M. and Muñoz, R. (1996) Cell. Mol. Biol. 42, 461–471.
[3] Endo, Y. and Tsurugi, K. (1987) J. Biol. Chem. 262, 8128–8130.
[4] Barbieri, L., Ferreras, J.M., Barraco, A., Ricci, P. and Stirpe, F. (1992) Biochem. J. 286, 1–4.
[5] Iglesias, R., Arias, F.J., Rojo, M.A., Escarmis, C., Ferreras, J.M. and Girbés, T. (1993) FEBS Lett. 325, 291–294.
[6] Barbieri, L., Gorini, P., Valbonesi, P., Castiglioni, P. and Stirpe, F. (1994) Nature 372, 624.
[7] Barbieri, L., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi, A. and Stirpe, F. (1997) Nucleic Acids Res. 25, 518–522.
[8] Girbés, T., de Torre, C., Iglesias, R., Ferreras, J.M. and Méndez, E. (1996) Nature 379, 777–778.
[9] Van Damme, E.J.M., Barre, A., Rougé, P., Van Leuven, F. and Peumans, W.J. (1996) Eur. J. Biochem. 235, 128–137.
[10] Taylor, B. and Irvin, J. (1990) FEBS Lett. 273, 144–146.
[11] Bonness, M., Ready, M.P., Irvin, J.D. and Mabry, T. (1994) Plant J. 5, 173–183.
[12] Girbés, T., Citores, L., Iglesias, R., Ferreras, J.M., Muñoz, R., Rojo, M.A., Arias, F.J., García, J.R., Méndez, E. and Calonge, M. (1993) J. Biol. Chem. 268, 18195–18199.
[13] de Benito, F.M., Citores, L., Iglesias, R., Ferreras, J.M., Soriano, F., Arias, F.J., Méndez, E. and Girbés, T. (1995) FEBS Lett. 360, 299–302.
[14] Girbés, T., Citores, L., Ferreras, J.M., Rojo, M.A., Iglesias, I., Muñoz, R., Arias, F.J., Calonge, M., García, J.R. and Méndez, E. (1993) Plant Mol. Biol. 22, 1181–1186.
[15] Citores, L., Iglesias, R., Muñoz, R., Ferreras, J.M., Jiménez, P. and Girbés, T. (1994) J. Exp. Bot. 45, 513–516.
[16] Girbés, T., Citores, L., de Benito, F.M., Iglesias, R. and Ferreras, J.M. (1996) Biochem. J. 315, 343–344.
[17] Citores, L., de Benito, F.M., Iglesias, R., Ferreras, J.M., Jiménez, P., Argüeso, P., Farias, G., Méndez, E. and Girbés, T. (1996) J. Exp. Bot. 47, 1577–1585.
[18] Citores, L., Muñoz, R., de Benito, F.M., Iglesias, R., Ferreras, J.M. and Girbés, T. (1996) Cell. Mol. Biol. 42, 473–476.
[19] Van Damme, E.J.M., Barre, A., Rougé, P., Van Leuven, F. and Peumans, W.J. (1996) Eur. J. Biochem. 237, 505–513.
[20] Marcus, A., Efron, D. and Weeks, D.P. (1974) Methods Enzymol. 30, 749–754.
[21] Barbieri, L., Stoppa, C. and Bolognesi, A. (1987) J. Chromatogr. 408, 235–243.
[22] Merino, M.J., Ferreras, J.M., Muñoz, R., Iglesias, R. and Girbés, T. (1990) J. Exp. Bot. 41, 67–70.
[23] Arias, F.J., Rojo, M.A., Ferreras, J.M., Iglesias, R., Muñoz, R., Rocher, A., Méndez, E., Barbieri, L. and Girbés, T. (1992) Planta 186, 532–540.
[24] Burlingame, A.L., Boyd, R.K. and Gaskell, S.J. (1994) Anal. Chem. 66, 634R–683R.
[25] Rojo, M.A., Arias, F.J., Iglesias, R., Ferreras, J.M., Muñoz, R., Escarmis, C., Soriano, F., López-Fando, J., Méndez, E. and Girbés, T. (1994) Planta 194, 328–338.

[26] Lee-Huang, S., Kung, H.F., Huang, P.L., Bourinbaiar, A.S., Huang, P.L., Tsai, W.P., Chen, A.Y. and Huang, H.I. (1994) Proc. Natl. Acad. Sci. USA 91, 12208–12212.
[27] Van Damme, E.J.M., Roy, S., Barre, A., Citores, L., Mostafapous, K., Rougé, P., Van Leuven, F., Girbés, T., Goldstein, I.J. and Peumans, W.J. (1997) Eur. J. Biochem. (in press).
[28] Van Damme, E.J.M., Barre, A., Rougé, P., Van Leuven, F. and Peumans, W.J. (1997) J. Biol. Chem. 272, 8353–8360.
[29] Stirpe, F. and Barbieri, L. (1986) FEBS Lett. 195, 1–8.
[30] Battelli, M.G., Barbieri, L., Bolognesi, A., Buonamici, L., Valbonesi, P., Polito, L., Van Damme, E.J., Peumans, W.J. and Stirpe, F. (1997) FEBS Lett. 408, 355–359.