Constitutive and inducible type 1 ribosome-inactivating proteins (RIPs) in elderberry (*Sambucus nigra* L.)

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Abstract Two novel highly basic type 1 (single chain) ribosome-inactivating proteins (RIPs) with *N*-glycosidase activity have been found in elderberries (the fruits of *Sambucus nigra* L.). Mass spectrometry of these RIPs, which we named nigritins f1 and f2, gave M_r values of 24 095 and 23 565, respectively. Both proteins strongly inhibited protein synthesis in rabbit reticulocyte lysates but were inactive against plant ribosomes. Both nigritins have a similar topological activity on pBlueScript SK$^+$ DNA as that displayed by dianthin 30. Nigritin f1 is a constitutive RIP since it is present in both green and mature intact elderberries at nearly the same proportion with respect to total fruit protein. By contrast, nigritin f2 is inducible and only appeared in mature intact elderberries. Elderberries also contain two isoforms of a basic nigrin equivalent to the recently found basic nigrin b in elder bark (De Benito et al., FEBS Letters 413 (1997) 85–91). Our results indicate that probably not all plant RIPs exert the same biological function and that this may be determined by the physiological state of the tissue.

Key words: Nigritin; Ribosome-inactivating protein; Polynucleotide:adenine glycosidase; Protein synthesis inhibition; Elderberry; *Sambucus nigra*

1. Introduction

An increasing but still small number of plants contain proteins that irreversibly inhibit protein synthesis in mammals and fungi, some of them also in plants and bacteria which act at ribosomal level and are thus known as ribosome-inactivating proteins [1,2]. Current classification of plant ribosome-inactivating proteins (RIPs) is performed on the basis of RIP structure [1]. Type 1 (single chain) RIPs are enzymes (*N*-glycosidases) that depurinate the large rRNA [1]. Type 2 (two chains) RIPs are enzymes containing a catalytic chain (A chain) equivalent to a type 1 RIP and a sugar-binding (usually D-galactose) chain (B chain), both linked through a disulphide bond. Four-chain and even eight-chain RIPs have also been described and these are functionally equivalent to pairs of type 2 RIPs [1,3–5].

In recent years the molecular mechanism of the depurination activity of RIPs at ribosomal level has been elucidated [1]. Moreover, it has been reported that RIPs also promote the depurination of non-ribosomal nucleic acids such as poly(A), genomic viral RNA and even DNA [6–8]. RIP-triggered depurination renders the nucleic acid polyphosphate backbone more sensitive to acid aniline. In the case of the large rRNA of mammalian ribosomes this allows the release of an RNA fragment considered diagnostic of RIP action [1].

To date, the biological role played by RIPs in plants is unknown. Nonetheless, a sound function as antiviral agents has been hypothesized since they inhibit in vitro viral infection [1,2]. This is further confirmed by the observations that: (i) RIPs can inhibit the ribosomes of their plants [9]; (ii) foreign RIPs expressed in transgenic tobacco plants prevent viral infection [10]; and (iii) both viral infection and the molecular mediators of viral infection such as H_2O_2 and salicylic acid induce the expression of the type 1 RIPs beetins in sugar beet [7].

Interest in RIPs is steadily growing. First, they are increasingly being used as the toxic moieties of immunotoxins (antibody-mediated targeting of cellular toxins) in experimental cancer therapy [11]. Second, RIPs act as anti-HIV-1 agents [12] through some yet unknown mechanism which could involve inhibitory action against the viral integrase [13].

In contrast to the generally low toxicity to animal cells and intact animals of type 1 RIPs, type 2 RIPs such as ricin, abrin and other toxins are extremely toxic [1]. This is due to lectin moiety, which allows the toxin to bind to and enter the animal cell, thus inhibiting protein synthesis and promoting cell death [1]. In the last few years, a number of novel type 2 RIPs (i.e. nigrins and ebulins) have been isolated from *Sambucus* species [14,15]. These share the property that they are not so toxic as ricin and ricin-related toxins and have therefore been called non-toxic type 2 RIPs.

Elder (*Sambucus nigra* L.) bark contains a complex mixture of type 2 RIPs; namely nigrin b [15], basic nigrin b [16], SNA I [4], SNA I′ [5] and SNLRP [17]. Elder fruits contain nigrin f [18] and nigrin s [19]. By contrast, the dwarf elder (*Sambucus ebulus* L.) contains the type 2 RIPs ebulin l in leaves [14], ebulins r1 and r2 in rhizomes [20] and ebulin f in fruits [21]. In addition, dwarf elder leaves contain a family of type 1 RIPs named ebulitins [22]. A recent report has indicated that ebulin and an ebulin structure-related lectin can be polymerized through disulfide bonds in green dwarf elder fruits, which could act as a storage mechanism for these proteins [21].

With the exception of basic nigrin b [16], all type 2 RIPs

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Abbreviations: IC_{50}, concentration of RIP that gives 50% of inhibiton of translation in rabbit reticulocyte lysates; Nigritins f1 and f2, basic type 1 RIPs present in elderberry; rRNA, ribosomal ribonucleic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; RIP(s), ribosome-inactivating protein(s)

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isolated from *Sambucus* species to date bind D-galactose and derivatives. We have found that upon removal of D-galactose-binding proteins from a saline extract of elderberries by affinity chromatography the unretained fraction strongly inhibits protein synthesis. We undertook the present investigation with the aim of identifying the translational inhibitors present in such fraction and report that elderberry contains a constitutive type 1 RIP (nigritin f1) and a ripening-dependent, thus inducible, type 1 RIP (nigritin f2), both RIPs displaying a strong activity on pBlueScript SK$^+$ DNA. In addition, it contains two isoforms of basic nigrin and two highly active RNases.

2. Materials and methods

2.1. Materials

Chemicals, biochemicals and chromatographic media were of the highest purity available and were obtained from the sources described elsewhere [14]. 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 [14]. L-[^{3}H]valine (sp. act. 32 Ci/mmol) was purchased from Amersham Ibérica (Madrid, Spain). Both green and mature elderberries (*S. nigra* L.) were collected in May and July–August, respectively, from Cobos de Cerrato (Palencia, Spain), frozen, and stored at −20°C.

2.2. Isolation of nigritins from elderberries

500 g of elderberries were cut into small pieces and then ground in a blender and extracted overnight with 4000 ml of extraction buffer (280 mM NaCl containing 5 mM sodium phosphate (pH 7.5)). The extract was processed at 4°C by affinity chromatography through acid-treated Sepharose 6B to remove all D-galactose-binding protein material. The unretained protein fraction was further resolved by several chromatographic steps as described for the isolation of ebulitins, the basic type 1 RIPs present in dwarf elder leaves [22]. The procedure included salt gradient ion-exchange chromatography with CM-Sepharose and Superdex 75 [22]. Finally all peak protein fractions were chromatographed through a Mono S column (salt gradient 0–0.3 M NaCl containing 5 mM sodium phosphate (pH 6.7)), yielding homogeneous proteins as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All protein fractions obtained after the chromatographic steps were assayed for translational inhibitory activity.

2.3. Polypeptide synthesis in cell-free systems

The corresponding cell-free translation systems and polypeptide synthesis encoded by endogenous messengers were prepared according to current procedures described in the respective references. The following cell free translation systems were used: rabbit reticulocyte lysates [23], rat liver [24], wheat germ [25], *Vicia sativa* [26] and *Cucumis sativus* [26]. All cell-free extracts were filtered through Sephadex G-25 (8×2.6 cm) to remove low-M_r compounds and stored under liquid N_2 until use and were thawed only once. In all cases control reactions were carried out in parallel without inhibitor.

2.4. Activity of nigritins on nucleic acids

The polynucleotide:adenosine glycosidase activity of nigritins on rabbit reticulocyte ribosomes was determined as follows. 100 μl of rabbit reticulocyte lysate containing 20 mM Tris-HCl (pH 7.8), 50 mM KCl, 1 mM $MgCl_2$ and 5 mM dithiothreitol were incubated with 10 μg of either nigritin f1 or nigritin f2 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). rRNA depurination was assessed as described elsewhere [14].

The activity of nigritins on DNA was measured essentially as described elsewhere [16], but using 1 μg of pBlueScript SK$^+$ DNA as substrate. Incubation was for 1 h at 30°C.

2.5. Standard molecular procedures

The following analytical procedures were performed as described in the corresponding references: analyses of proteins by SDS-PAGE [27], detection of glycan chains in proteins [16], analyses of N-terminal amino acid sequences [24], amino acid composition [24], isoelectric focusing [16] and matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis [20]. 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) and for glycan detection transferrin (M_r 79 500) was used. RNase activity was assayed as indicated elsewhere [28]

3. Results and discussion

Either the green or mature elderberry protein fraction unretained by acid-treated Sepharose 6B affinity chromatography was subjected to salt gradient CM-Sepharose ion-exchange chromatography. As shown in Fig. 1A, the protein fraction of mature elderberries gave three zones of protein synthesis inhibition active in a reticulocyte lysate, indicated as the CM1, CM2 and CM3 fractions. In contrast, the fraction from green elderberries gave only two zones, corresponding to the CM1 and CM3 of mature elderberries (Fig. 1B). Further purification by Superdex 75 chromatography of the CM1 and CM2 fractions resolved the inhibitory translational activity into three protein peaks, S1 and S2 from CM1 from both mature and green elderberries, and S3 from CM2, only from mature elderberries. As shown in Fig. 2, a final salt gradient Mono-S ion-exchange chromatographic step of peaks S2 and S3 from mature elderberries yielded several pure proteins as judged by SDS-PAGE, two of which (indicated by the shadowed areas in the lower panels of Fig. 2) are strongly inhibitory proteins which we named nigritins f1 (derived from S2 fraction) and f2 (derived from S3 fraction). Nigritin f1 is present in both green and mature intact fruits (Fig. 1A), while nigritin f2 is present only in mature intact fruits (Fig. 1B), since the fractions corresponding to CM2 of green elderberries were not inhibitory (data not shown). The yields of the current preparations were 0.4 and 0.3 mg per 100 g of fruits for nigritin f1 and f2, respectively. Peak CM3 contained two very active RNases that can be separated by ion exchange chromatography (data not shown) and will be characterized in a further study. We named the RNases contained in peak CM3 RNases f1 and f2 and preliminary experiments indicated that both of them are poly(C)-specific RNases (data not shown).

As illustrated in Fig. 3, SDS-PAGE analysis of the S1 frac-

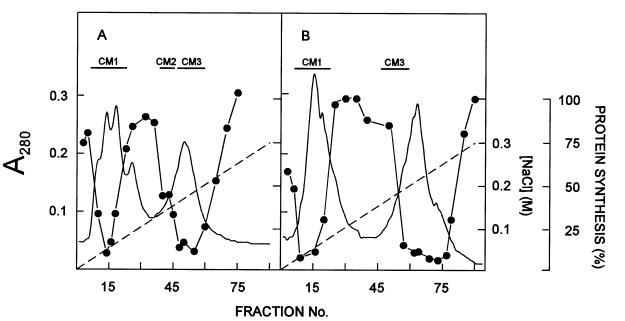

Fig. 1. CM-Sepharose FF chromatography of the protein fraction without affinity for D-galactose from green and mature elderberries. Salt gradient (– – –) CM Sepharose chromatography of the protein fraction unretained by acid-treated Sepharose 6B from either mature (A) or green (B) elderberries was carried out as described in Section 2.2. Translational inhibitory activity (●) was followed using rabbit reticulocyte lysates. Fractions with inhibitory activity (referred to as CM1, CM2 and CM3) were pooled and CM1 and CM2 were subjected to further purification.

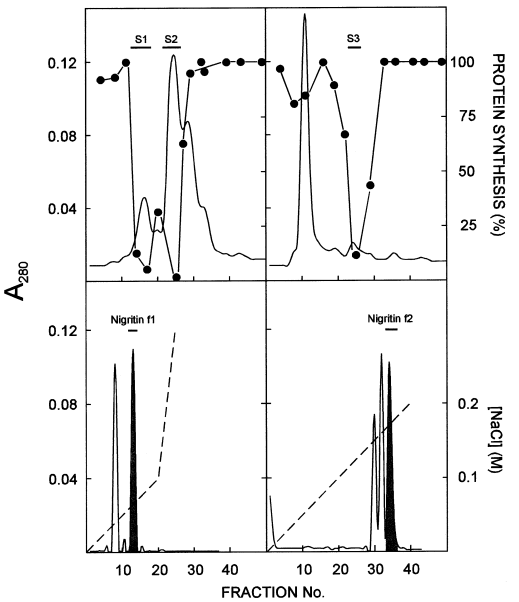

Fig. 2. Purification of the CM1 and CM2 protein fractions from CM Sepharose chromatography of mature elderberry extracts by Superdex 75 chromatography and Mono S. Protein fractions CM1 and CM2 from CM Sepharose chromatography of mature elderberry extracts were chromatographed through Superdex 75 (top) as indicated in Section 2.2 and the inhibitory activity was followed as in Fig. 1. Fractions with translational inhibitory activity were pooled (S2 and S3) and subjected to salt gradient Mono S chromatography (bottom). Only the peaks indicated with shadowed area strongly inhibited protein synthesis and contained homogeneous proteins that were named elderberry nigritin f1 (bottom left) and nigritin f2 (bottom right).

tion indicated that it contains a mixture of two isoforms of a type 2 RIP, being most probably the equivalent of the recently found basic nigrin b [16]. All our attemps to isolate such type 2 RIP isoforms from S1 fraction by chromatography yielded only fractions enriched in one or another RIP but always cross-contaminated. SDS-PAGE analysis in the absence or presence of 2-mercaptoethanol as reductant indicated that nigritins f1 and f2 are monomeric proteins (Fig. 3a). The reduction with 2-mercaptoethanol induces changes in the apparent molecular weight of both nigritins, thus they seem to contain intrachain disulphide bonds but the effect is more pronounced in nigritin f2. On the other hand, and as shown in Fig. 3a, nigritin f1 is strongly glycosylated but nigritin f2 is not. Mass spectrometry analysis of nigritins gave M_r values of 24 095 and 23 565 for nigritin f1 and f2, respectively (Fig. 3b). Doubly charged signals at 11 989 and 11 783 of corresponding nigritins are also displayed in the spectra. As shown in Fig. 3a, RNases f1 and f2 have M_r values of 26 000.

As shown in Fig. 4, nigritin f1 and nigritin f2 from mature elderberries strongly inhibited protein synthesis in rabbit reticulocyte lysates. However, both nigritins were less active on the rat liver cell-free system than on the rabbit reticulocyte lysate system, as reported for other RIPs [24], including those isolated from *Sambucus* [14,15]. In contrast to mammalian ribosomes, nigritins did not affect the wheat germ-derived cell-free translation system (the *Cucumis sativus* and *Vicia sativa* cell-free systems were also unaffected by nigritins; data not shown). The lack of effect on plant ribosomes is a characteristic of all RIPs isolated from *Sambucus* [14,15,22]. This property could be useful for the construction of transgenic plants carrying genes coding for RIPs in order to increase the resistance of plants to pathogens, as reported for *Phytolacca* antiviral protein [10].

As shown in Fig. 5a, the molecular mechanism of action of both nigritins is the release of an adenine from the 28 S rRNA, which upon treatment with acid aniline releases an RNA fragment that is diagnostic of RIP action against mammalian ribosomes [1,7]. Therefore, these proteins could be considered as polynucleotide:adenosine glycosidases, as pointed out recently by Stirpe and coworkers [6–8]. Both nigritins also acted on double-stranded DNA as reported for some other RIPs. As also shown in Fig. 5b, nigritins trigger the conversion of supercoiled pBlueScript SK^+ DNA forms to both relaxed and linear forms. This new topological activity on DNA, that was irreversible even in the presence of high concentrations of ethidium bromide (data not shown), is in agreement with the general depurination activity of RIPs [8], and could explain the antiviral action reported for some RIPs

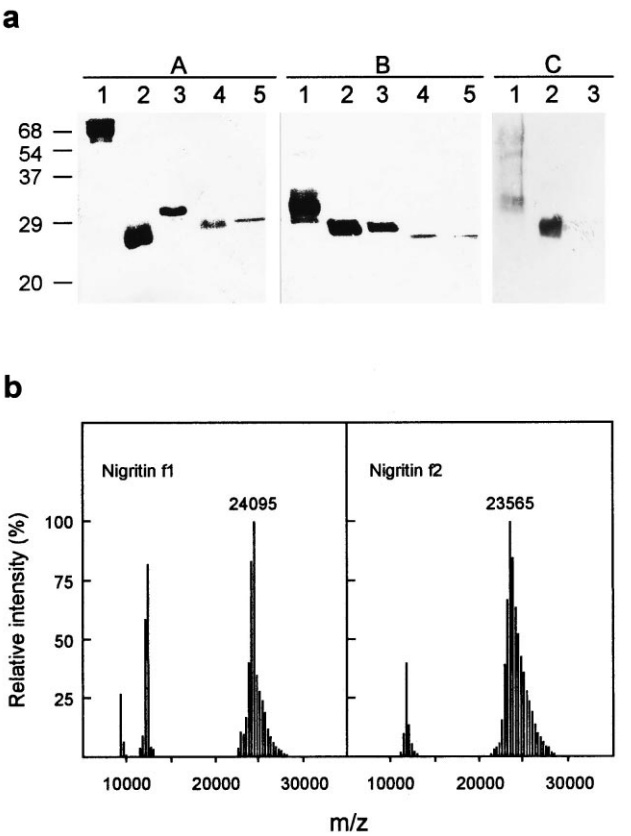

Fig. 3. Analysis of nigritins f1 and f2 by SDS-PAGE and MALDI-TOF/MS. Panel a: SDS-polyacrylamide gel electrophoresis. Protein fractions (7 μg per well) were electrophoresed in the absence (A) or the presence (B) of 2-mercaptoethanol (2ME) and then stained with Coomassie blue, as indicated in Section 2.5. The numbers on the left indicate the corresponding apparent M_r values of the standards (in kDa). Additionally, 3 μg of protein per well (C) were electrophoresed in the presence of 2-mercaptoethanol, transferred to an Immobilon-P membrane and stained for glycan detection as indicated in Section 2.5. Lane 1: S1 fraction containing a mixture of basic nigrins f; lane 2: nigritin f1; lane 3: nigritin f2; lane 4: RNase f1; lane 5: RNase f2. The numbers on the left indicate the corresponding apparent M_r values of the standard proteins (kDa). Panel b: Matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF/MS) spectra were obtained as indicated in Section 2.5 in the presence of 2-mercaptoethanol (2ME). A smooth background has been removed.

[29]. As a control, we used dianthin 30, the RIP from *Dianthus caryophyllus* [1], which has been reported among the RIPs to have the strongest topological activity on DNA [29]. Notably, nigritins seem to be at least as active as dianthin 30 and much more active than the recently described basic nigrin b [16]. In contrast basic nigrin b inhibits protein synthesis at much lower concentration than nigritins (Fig. 4 and [16]).

To compare nigritins with other RIPs we analyzed the N-terminal amino acid sequence. We found NH_2-AQVLD-SYQFVSSXTPTYXXN for nigritin f1 and NH_2-VQXLGXFXAPXSPPQXGGTSFPD for nigritin f2. Neither sequence seems to be related to the other and we did not find any sequence homology with other known RIPs nor other proteins. Concerning the amino acid composition, both nigritins differ strongly in their content of Asp, Thr, Gly, Ile, Phe, Lys and Arg (data not shown). In particular, nigritin f2 is very rich in Pro, Gly and Cys (data not shown). It would be interesting to investigate whether there might be any kind of relationship between this nigritin and glycine-rich proteins inducible in old tissue but by mechanical wounding [30].

Investigation by ELISA with anti-nigrin b, anti-ebulin l and anti-ebulitin lα of the immunological correlation between elderberry nigritins and other *Sambucus* RIPs indicates that neither nigritin f1 nor f2 are related with ebulin l, nigrin b and ebulitin, despite the glycosylation state of nigritin f1 and some of the other elder RIPs [16,20,22]. This is very interesting since, thus, nigritins cannot be considered as elderberry isoforms of the ebulitins found in dwarf elder leaves [22], rather they are new RIPs. As found in the case of nigrin f, a neutral non-toxic type 2 RIP found in elderberry [18,29], the amount of protein, and consequently of RIP, relative to the total weight of elderberries decreases with ripening (data not shown). Nonetheless, the ratio of nigritin f1 to total protein in elderberries remains more or less constant, nigritin f2 only appearing upon ripening.

Preliminary experiments indicated that most of the translational inhibitory activity without affinity for D-galactose in both green and mature elderberries is present in the skin (around 100-fold more inhibitory activity than in flesh; data not shown). Since the skin is a natural first barrier to pathogens, our data add some support to the hypothesis that RIPs could act as antipathogenic agents [1,7,9]. In contrast to these basic proteins, the neutral D-galactose-binding type 2 RIP nigrin f is mainly present in the flesh (data not shown). This suggests that not all RIPs play the same role in the plant and that the effects of RIPs are complex and most probably pleiotropic. Experiments are in progress to ascertain the potential antiviral activity of nigritins and their precise localization in the elderberry skin.

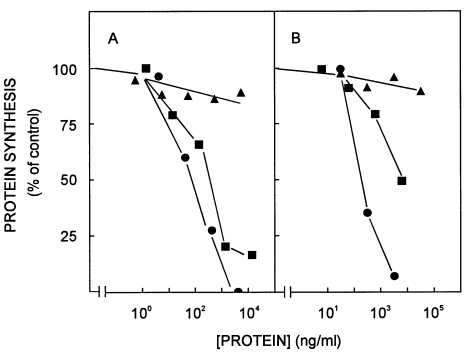

Fig. 4. Effects of elderberry nigritins on protein synthesis carried out by eukaryotic cell-free translation systems. Protein synthesis inhibition by elderberry nigritins f1 (panel A) and f2 (panel B) was assayed in rabbit reticulocyte lysates (●), rat liver (■) and wheat germ (▲) cell-free translation systems, as indicated in Section 2. Controls were run in the absence of translational inhibitor.

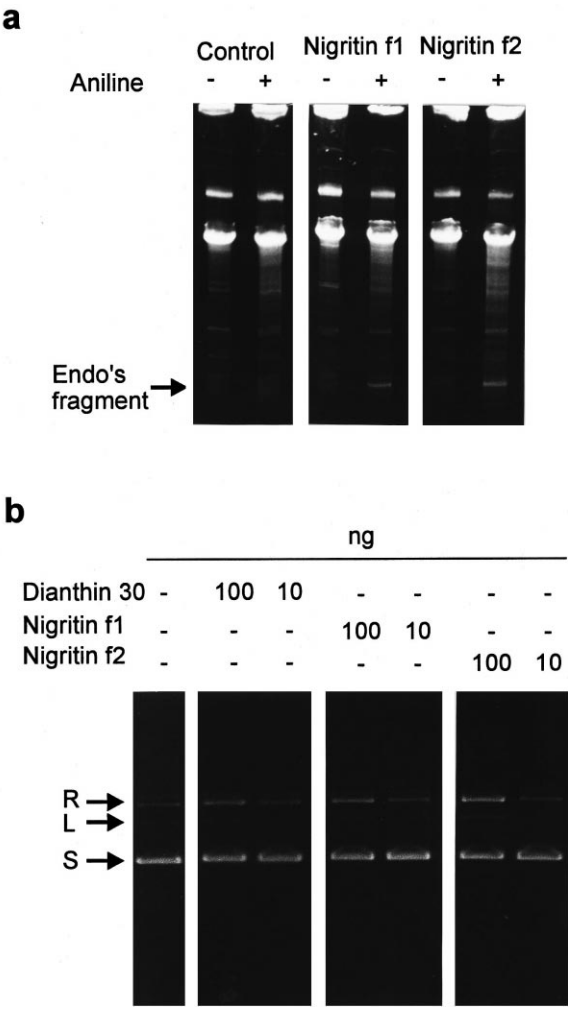

Fig. 5. Effects of elderberry nigritins on nucleic acids. a: *N*-glycosidase activity was assayed as indicated in Section 2.4. Each lane contains 3 μg of RNA. The arrow indicates the RNA fragment released as a consequence of RIP action upon acid aniline treatment. b: Topological activities on DNA were assayed as indicated in Section 2.4. Each lane contains 1 μg of pBlueScript SK^+ DNA. The arrows indicate the topological form of DNA: S, supercoiled; R, relaxed; L, linear.

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