Ebulitins: a new family of type 1 ribosome-inactivating proteins (rRNA *N*-glycosidases) from leaves of *Sambucus ebulus* L. that coexist with the type 2 ribosome-inactivating protein ebulin l

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Received 18 January 1995

Abstract A new family of single chain (type 1) ribosome-inactivating proteins (RIPs), that we have named ebulitins, have been found in mature leaves of *Sambucus ebulus* L., a caprifoliaceae plant also known to contain a non-toxic two chain (type 2) RIP named ebulin l in its leaves. Ebulitins are basic proteins of M_r 32,000, 29,000 and 29,000 for ebulitins α, β and γ, respectively. The simultaneous presence of different basic type 1 and acidic type 2 RIPs in the same plant and in the same tissue is described here for the first time and opens a new door in research into RIPs.

Key words: Ribosome-inactivating protein; rRNA *N*-glycosidase; Protein synthesis inhibition; Ebulitin; Ebulin l; *Sambucus ebulus* L.

1. Introduction

Ribosome-inactivating proteins (RIPs) are *N*-glycosidases that depurinate large rRNA from all kinds of sensitive ribosomes [1]. RIPs are classified as single chain (type 1; an active A chain) RIPs; two chain (type 2; an active A chain linked to a lectin B chain) RIPs, and four chain (type 4; two couples, A-B, held together by non-covalent forces) RIPs [1, 2]. Concerning toxicity, type 2 and type 4 may be classified as toxic and non-toxic [2]. Toxic RIPs are the type 2 RIPs ricin [3, 4], abrin [3] and related proteins [1] and the type 4 RIP *Viscum album* four-chain agglutinin [2]. Non-toxic RIPs are the type 2 RIPs ebulin l [5] and nigrin b [6] and the type 4 RIPs *Ricinus communis* four-chain agglutinin [2] and *Abrus precatorius* four-chain agglutinin [2].

RIPs inhibit protein synthesis by mammalian, plant, protozoal, fungal and bacterial ribosomes [1,7–12]. Very recently, it has been reported that saporin L1, an RIP from *Saponaria officinalis* L., displays depurinating activity on viral RNA and herring sperm DNA [13], which could account for the antiviral activity generally assigned to RIPs [14]. However, despite the antiviral role assigned to RIPs, the precise physiological role played by RIPs in plants is as yet unknown and requires much more research.

The simultaneous presence of a lectin and a type 1 RIP has been already reported for *Trichosanthes kirilowii* [15]. In this work we report for the first time that mature leaves of *Sambucus ebulus* L. contain a new family of basic type 1 RIPs, that we have called ebulitins, that coexist with the acidic non-toxic type 2 RIP ebulin l and a lectin [5]. Since both ebulitins and ebulin l display the same enzymic activity (both are 28 S rRNA *N*-glycosidases), the simultaneous presence of both kinds of different proteins opens a new door in research into RIPs.

2. Materials and methods

2.1. Materials

All chemicals, biochemicals and radioactive materials were of the highest purity available and were obtained as in previous reports [5,16]. Mature leaves from *Sambucus ebulus* L. were collected from Cobos de Cerrato (Palencia, Spain) in the summer. Ebulin l was prepared from mature leaves as described elsewhere [5].

2.2. Isolation of RIPs

Essentially, the new RIPs were isolated by a general protocol used for the preparation of basic RIPs [17]. The chromatographic steps included S-Sepharose Fast Flow, CM-Sepharose Fast Flow and Superdex 75 HiLoad. With α-ebulitin, purification was achieved by a final Blue-Sepharose CL-6B chromatography carried out as described elsewhere [18].

2.3. Cell-free translation systems and polypeptide synthesis

Rabbit reticulocyte lysates were prepared as described elsewhere [19]. Rat liver and plant cell-free translation systems (wheat germ, *Vicia sativa* and *Cucumis melo*) were prepared as described previously [16,20,21]. The cell-free extracts were filtered through Sephadex G-25 to remove low M_r compounds and stored in small aliquots under liquid N_2 until use. Translation was coded by endogenous messengers and in all cases the reaction mixtures were optimized for concentrations of ions and the $30,000 \times g$ supernatant [16,20,21]. Control reaction mixtures without inhibitor were carried out separately.

2.4. 28 S rRNA N-glycosidase activity of ebulitins

100 μl of rabbit reticulocyte lysate was incubated with 10 μg of either ebulitins or ebulin l for 15 min at 37°C under the same conditions as described elsewhere [2]. The RNA was extracted by phenolization and ethanol precipitation and then electrophoresis of RNA was carried out in 5% acrylamide gels 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 [2]. The gels were photographed after staining for 20 min with 0.5 μg/ml of ethidium bromide.

2.5. Analysis of amino acid composition

Protein was hydrolysed with 100 μl of 5.7 M HCl containing 0.05% (v/v) 2-mercaptoethanol in evacuated and sealed tubes at 110°C for 24 h. Analysis was performed as indicated elsewhere [16].

2.6. Other assays

SDS-polyacrylamide gel electrophoresis of proteins was carried out as has been reported previously [16].

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SSDI 0014-5793(95)00130-1

3. Results and discussion

Upon affinity chromatography using acid-treated Sepharose 6B the acidic protein fraction obtained by saline extraction of mature leaves of *S. ebulus* yielded a protein preparation containing the non-toxic type 2 RIP ebulin l, whose enzymic A chain had an M_r of 26,000 [5]. We found that the basic protein counterpart unbound to the affinity chromatography column retained a large amount of protein synthesis inhibitory activity (data not shown). Therefore, we subjected this basic protein extract to a general protocol to purify basic RIPs [17]. In our case, this included several chromatographic steps through S-Sepharose Fast Flow, CM-Sepharose Fast Flow, Superdex 75 HiLoad, and finally (in the case of α-ebulitin), Blue-Sepharose CL-6B. As shown in Fig. 1, ionic exchange chromatography resolved three protein synthesis inhibitory activities. Subsequent chromatographies yielded single chain proteins which proved to be homogeneous upon SDS-polycrylamide gel electrophoresis, despite the presence of a thiol-reducing agent such as 2-mercaptoethanol and migrated at M_r values of 32,000, 29,000 and 29,000, in contrast to the M_r of the ebulin A chain (Fig. 2). We named these proteins ebulitins α, β and γ, respectively, according to the order of elution from the CM-Sepharose Fast Flow column. The corresponding yields were 1.3, 3.5 and 5.7 mg per kg of fresh plant tissue, respectively.

All three ebulitins strongly inhibit protein synthesis in the mammalian cell-free systems assayed (Fig. 3). However, rabbit reticulocyte lysates were nearly 50-fold more sensitive to ebulitins than the rat liver cell-free system. By contrast, none of the plant cell-free systems assayed here, namely wheat germ, *Vicia sativa* and *Cucumis melo* was sensitive to ebulitins (Fig. 3). The different behaviour of mammalian ribosomes towards ebulitins contrasts with ebulin l [5] and nigrin b [6].

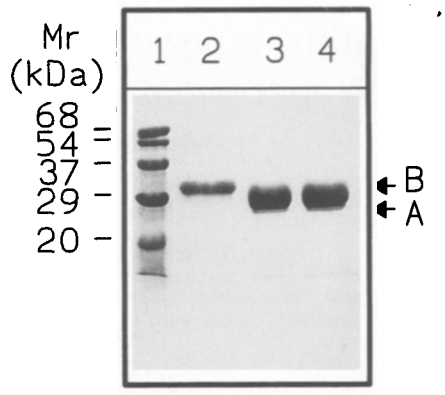

Fig. 2. Polyacrylamide gel electrophoresis of ebulitins. α-Ebulitin (lane 2), β-ebulitin (lane 3) and γ-ebulitin (lane 4) were subjected to SDS-PAGE as indicated in section 3. Thereafter, gels were stained with Coomassie blue for visual assessment. Numbers on the left indicate the corresponding molecular masses of standards (lane 1) in kilodaltons. The arrows on the right indicate the positions at which migrate the A and B chains of ebulin l.

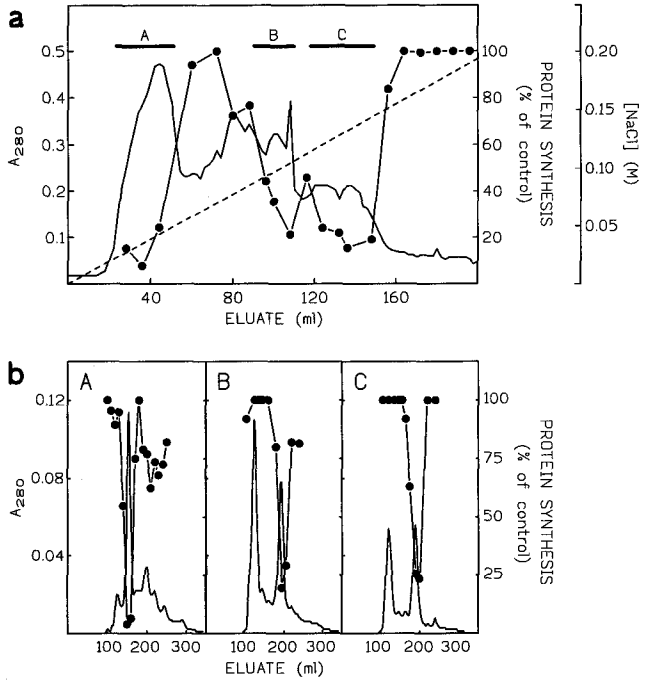

Fig. 1. Purification of type 1 RIPs from *Sambucus ebulus* leaves by chromatography through CM-Sepharose FF (a) and Superdex 75 HiLoad (b). Purification was performed from 500 g of *Sambucus ebulus* leaves. For experimental procedures, see section 2. —, absorbance at 280 nm; ●—●, salt gradient; ●, effects of a dilution of 1:5,000 of the eluate on protein synthesis in rabbit reticulocyte lysate. Horizontal bars in panel (a) indicate the fractions that were collected and subjected to Superdex 75 chromatography (panel b). Protein peaks showing inhibitory activity in panel b were collected and observed to contain ebulitins α (A), β (B) and γ (C). α-Ebulitin showed protein contamination that was removed by Blue-Sepharose CL-6B chromatography.

Attempts to obtain the N-terminal amino acid sequence were unsuccessful thus indicating that they were blocked. Therefore, to compare the ebulitins among one another, we analyzed the amino acid composition of each. As illustrated in Table 1, ebulitins β and γ contain roughly the same amino acid composition. By contrast, ebulitin α exhibits a quite different pattern. All functional and structural data suggest that ebulin l and ebulitins are in fact different proteins but with the same mechanism of action on mammalian protein synthesis and that all are apparently inactive against plant ribosomes.

The molecular mechanism through which ebulitins inhibit protein synthesis is consistent with the general mechanism displayed by common RIPs [1]. As illustrated in Fig. 4, treatment of rabbit ribosomes with ebulitins such as ebulin l led to the

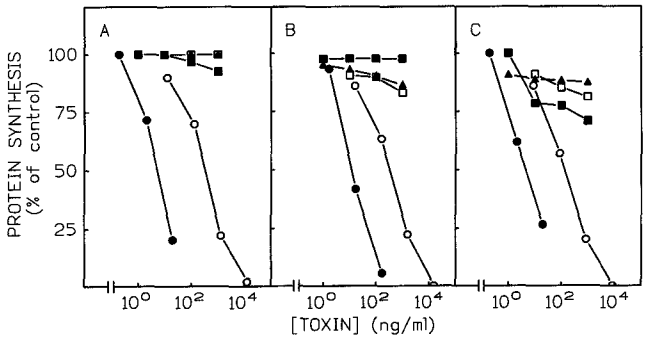

Fig. 3. Effects of ebulitins on protein synthesis carried out by mammalian and plant cell-free translation systems. Varying concentrations of α-ebulitin (A), β-ebulitin (B) and γ-ebulitin (C) were added to translation mixtures of each in vitro system, as indicated in section 2. Protein synthesis is expressed as the percentage of label incorporated into proteins referred to controls run in the absence of ebulitin. Symbols: ●, rabbit reticulocytes lysate; ○, rat liver; ■, *Vicia sativa* germ; □, *C. melo* germ; ▲, wheat germ.

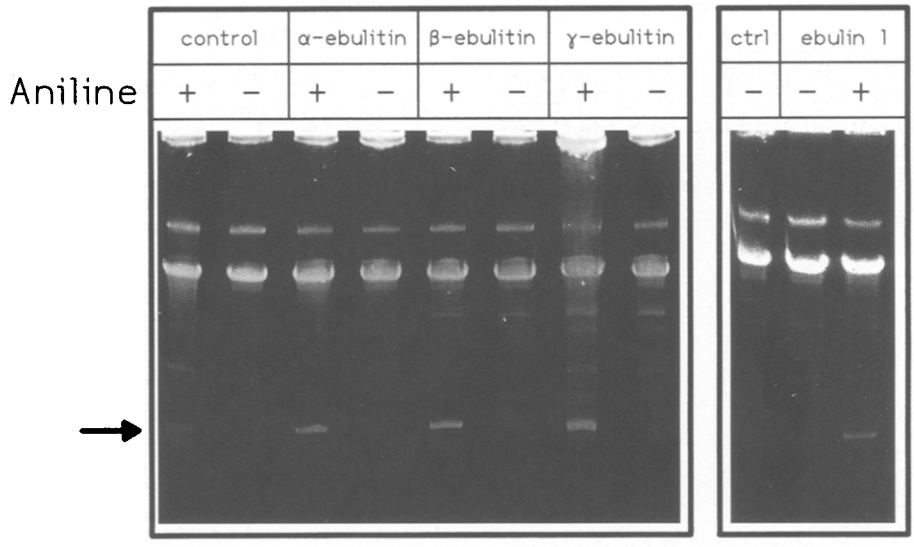

Fig. 4. rRNA *N*-glycosidase activity of ebulitins on rabbit ribosomes. The 28 S rRNA *N*-glycosidase activities of α-, β- and γ-ebulitin and ebulin l were assayed by incubation of 100 μl of rabbit reticulocite lysate with 10 μg of RIP for 15 min at 37°C. rRNA depurination was analyzed as indicated in section 2. Arrow indicates the RIP diagnostic RNA fragment split by acid aniline treatment.

depurination of the rRNA, which upon isolation and treatment with acid aniline allowed the release of the RNA fragment diagnostic for RIPs.

Our results indicate for the first time that *S. ebulus*: (i) contains acid and basic RIPs and a lectin; (ii) contains type 1 and type 2 RIPs in the same tissue and, (iii) that ebulin and ebulitins are very different proteins but act on mammalian ribosomes through the same mechanism. Since ebulin l contains D-galactose lectin activity in its B chain whereas ebulitins lack such activity, it would appear that ebulin l and ebulitins would not exert exactly the same biological functions in the plant. Further studies, i.e. on the subcellular location of both kinds of proteins and on their potential antiviral activity, should help us to clarify the biological role played by both ebulin l and ebulitins from *S. ebulus*.

Table 1
Amino acid compositions of ebulitins

Amino acid	α-Ebulitin	β-Ebulitin	γ-Ebulitin
Cys	0	6	6
Asp	35	36	31
Thr	27	31	33
Ser	21	15	16
Glu	36	21	22
Pro	14	15	15
Gly	24	17	18
Ala	21	21	22
Val	23	12	9
Met	5	2	4
Ile	14	15	18
Leu	28	19	18
Tyr	7	9	7
Phe	10	6	8
Lys	9	9	6
His	4	8	9
Arg	16	19	20
Trp*	–	–	–

Composition is expressed as the rounded-off number of residues per mol of protein based on an M_r of 32,000, 29,000 and 29,000 for α-, β- and γ-ebulitin, respectively.
*Trp not determined.

Acknowledgements: This work was supported by a grant from CICYT (BIO91-0231). L. Citores holds a predoctoral fellowship from the Ministerio de Educación y Ciencia. We thank Mr. N. Skinner for correcting the English version of the manuscript and J.E. Basterrechea for his technical support.

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