Activities associated with the presence of ribosome-inactivating proteins increase in senescent and stressed leaves

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Abstract The ribosome-inactivating proteins (RIPs) from *Hura crepitans* and *Phytolacca americana* release adenine from herring sperm DNA. Leaf extracts from these plants show the same enzymatic activities as the RIPs. The translation inhibitory activity and the activity on DNA are both increased in the leaves of both plants during senescence or when subjected to heat or osmotic stress. It is proposed that a physiological role of RIPs could be to intervene in the death of plant cells.

Key words: Ribosome-inactivating protein; Polynucleotide:adenosine nucleosidase; Leaf senescence; Plant stress

1. Introduction

Some plant proteins were provisionally called ribosome-inactivating proteins (RIPs) because they damage ribosomes acting in an enzymatic manner (for review see [1]). They include type 1 RIPs, single-chain proteins, and fewer type 2 RIPs, consisting of an active A chain linked to a B chain with the properties of a galactose-specific lectin (ricin and related toxins, and some less toxic proteins [2–4]).

The mechanism of action of RIPs on ribosomes was elucidated by Endo's group (reviewed in [5]) who found that the A chain of ricin is an *N*-glycosidase which releases adenine from a precise position in rRNA (A_{4324} in the case of rat liver ribosomes), a property shared by all other RIPs tested, of either type (reviewed in [1]). More recently it was observed that some RIPs released more than one adenine per ribosome [6], saporin-L1, a RIP from the leaves of *Saponaria officinalis* [7], being particularly active. Subsequently it was found that (i) this RIP released many, probably all, adenine residues not only from rRNA, but from all other tested RNAs, from poly(A) and from herring sperm DNA [8], and (ii) that other RIPs of either type tested released adenine from the same DNA (unpublished results). Thus some RIPs can be considered polynucleotide:adenosine nucleosidases.

It was reported recently that a RIP is expressed in barley leaves subjected to the action of methyl jasmonate or during senescence [9]. The present investigation was undertaken to ascertain whether senescence or other stimuli changed the level of RIPs in leaves normally containing them.

Ribosome-inactivating activity is present in the leaves and several tissues of *Phytolacca americana* (pokeweed antiviral protein, PAP, reviewed in [10]), in the latex [11] and in the leaves (unpublished results) of *Hura crepitans*. We report now that RIPs purified from, and extracts from the leaves of, these plants release adenine from herring sperm DNA, and the level of both translation inhibitory and DNA deadenylating activities increased in senescent and stressed leaves.

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Abbreviations: RIP, ribosome-inactivating protein; PAP, pokeweed antiviral protein.

2. Materials and methods

2.1. Materials

All reagents were of the best quality available, when possible RNase-free, and were from the same sources as in [12]. Herring sperm DNA (from Sigma, St. Louis, MO) was mechanically sheared and extensively treated with RNase A. Ribosome-inactivating proteins were purified from the latex of *H. crepitans* [11] and from the leaves of *P. americana* (PAP isoform 2, separated from PAP by Blue Sepharose chromatography as performed in the preparation of PAP-C [13]). PAP and PAP II were generous gifts from J.D. Irvin.

Leaves from *P. americana* and *Vitis vinifera* were taken from plants grown in the garden of our Department; and those from *H. crepitans* from a plant grown in a pot (kept inside during winter) in the same garden; leaves from *Rhus toxicodendron* were from a plant in the Botanical Garden of the University of Bologna. Leaves were collected from October through July. Senescent and green leaves were collected as much as possible at the same time.

2.2. Treatment of leaves and preparation of extracts

For stress experiments *H. crepitans* and *P. americana* leaves were removed from the plants and were rapidly bound to glass refrigerators in which water at 3°C, 23°C or 45°C was circulating. The stem of the leaves was immersed in distilled water at room temperature (23°C). The leaves were kept for 24 h in these conditions, under continuous light provided by fluorescent tubes. Osmotic stress was obtained by keeping the leaf stem in 1 M sorbitol for 24 h. Control leaves were kept in distilled water.

Leaves were ground with an Ultra-Turrax apparatus with 9 or 19 volumes of phosphate-buffered saline (PBS, 0.14 M NaCl containing 5 mM sodium phosphate buffer, pH 7.4). The extracts were left for 8–12 h at 4°C on a magnetic stirrer, then were strained through cheesecloth and centrifuged at $30000 \times g$ for 30 min, and the supernatants were stored at −20°C until use.

For the determination of enzymatic activities the supernatants were filtered through Sephadex G25 superfine to eliminate small molecular weight molecules, and protein was determined by the method of Kalb and Bernlohr [14].

2.3. Determination of enzyme activities

The inhibitory effect on protein synthesis was determined with a reticulocyte lysate system as described previously [12], with the details given in the legend to Table 1.

The release of adenine from DNA by the RIPs and by the leaf extracts was determined by HPLC [15] essentially following the procedure of McCann et al. [16] with a Kontron high-pressure liquid chromatograph equipped with a model SFM 25 spectrophotofluorimeter and a model 450 MT data system for chromatography control and data analysis. The column, a reverse-phase Spherisorb C_{18} (5 μm particle size, 25 cm × 0.46 cm), was equilibrated in 20 mM Na-tetraborate/phosphoric acid buffer, pH 7.7, containing 16% fluorescence grade methanol and was eluted with a linear gradient (10 ml) 16–32% of methanol in the same buffer. The flow rate was 1 ml/min. The spectrophotofluorimeter was set at 315 nm excitation and

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415 nm emission. Controls were run without RIP, and a standard curve of adenine was run with each experiment. Assay conditions were optimized in preliminary experiments (results not shown) and details are given in the legend to the appropriate Tables and Figures.

3. Results

Ribosome-inactivating proteins purified from both *H. crepitans* and *P. americana*, at very low concentrations, released adenine from DNA (Fig. 1).

The extracts from the leaves of *H. crepitans* and *P. americana* strongly inhibited protein synthesis by a rabbit reticulocyte lysate, consistent with the presence of RIPs. The same extracts brought about a release of adenine from DNA (Table 1 and Fig. 1 inset). Both activities increased in senescent leaves. A similar enhancement of both activities was observed when *H. crepitans* leaves were subjected to heat, or to an osmotic stress, but not when they were exposed to cold, whereas the activities of *P. americana* leaves were moderately increased in all these conditions (Table 2).

The extracts from *V. vinifera* and *R. toxicodendron*, either green or senescent, did not inhibit protein synthesis or brought about release of adenine from DNA (results not shown).

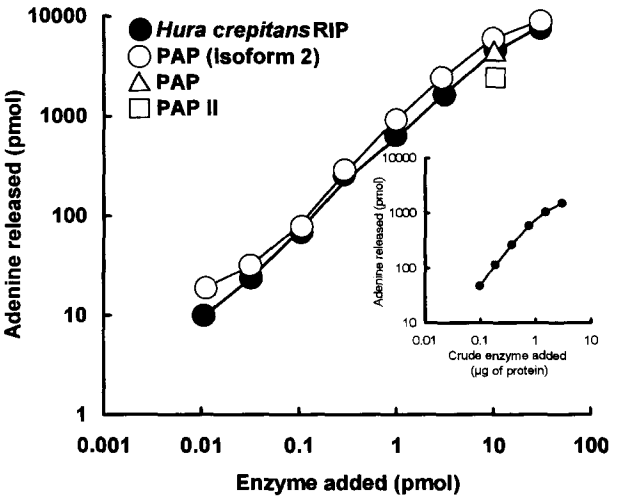

Fig. 1. Dose-response curve of purified ribosome-inactivating proteins and of *Hura crepitans* leaf extract on herring sperm DNA. Experimental conditions were as described in the legend to Table 1. Reaction mixtures contained, in a final volume of 50 μl: 50 mM Na acetate buffer, pH 4.0; 160 mM KCl; 20 μg of mechanically sheared DNA from herring sperm and the indicated amount of purified RIP (main panel) or Sephadex-filtered leaf extract (2.5 μg protein) (inset). Incubation was for 40 min at 30°C. Samples were processed and adenine was determined by HPLC as described in section 2.

4. Discussion

The RIPs purified from *H. crepitans* and *P. americana* release adenine from DNA as saporins do [8]. Leaf extracts from both plants not only inhibit protein synthesis, but also release adenine from DNA. It seems reasonable to assume that the effect on DNA of semi-purified extracts is due to the RIP contained in the leaves, consistently with the release of adenine from DNA by saporins [8], PAP and *H. crepitans* RIP (present results) and other RIPs (Barbieri et al., unpublished results). In the leaves from the same plants both the translation inhibitory and the DNA deadenylating activities increase during senescence or under stressing conditions. The changes were all in the same direction, although being not statistically significant in each condition, due to the high variability within each group of leaves. Presumably this was because the leaves were collected at different times from plants in the open field, and consequently exposed to the effect of environmental changes and natural stresses. The variations of the specific activity were often smaller than those of the activity per g of tissue. This is particularly evident in the case of heat stressed leaves, and may be due to loss of water. However, the increases of the specific activity indicate that the increments are not entirely apparent due to loss of water, whilst the increases per wet weight suggest that they are not apparent due to selective loss of some other proteins. It should be noted that the changes of the translation inhibitory activity were generally higher than those of the nucleosidase

Table 1
Enzymatic activities of extracts from leaves of RIP-containing plants

	Activity per g of tissue				Activity per mg of protein			
	Inhibition of translation		Nucleosidase activity		Inhibition of translation		Nucleosidase activity	
	U	%	U	%	U	%	U	%
Hura crepitans leaves								
Green (3)	5,638 ± 2,072	(100)	590.7 ± 221	(100)	1,139 ± 414	(100)	119.8 ± 45.3	(100)
Senescent (5)	12,961 ± 2,863	230	930.2 ± 139	157	3,213 ± 781	282	227.5 ± 39.3	190
Phytolacca americana leaves								
Green (6)	24,248 ± 3,012	(100)	5,195 ± 910	(100)	815.5 ± 256	(100)	126.5 ± 18.2	(100)
Senescent (4)	34,301 ± 3,351	141	6,289 ± 386	121	1,894 ± 413	232	339.1 ± 46.9	268

For the inhibition of protein synthesis reaction mixtures contained, in a final volume of 62.5 μl: 10 mM Tris-HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 3 μg of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of L-[^{14}C]leucine, various amounts of Sephadex-filtered leaf extract and 25 μl of rabbit reticulocyte lysate. Incubation was for 5 min at 28°C. Samples were treated as described in [12]. One unit of inhibitory activity is defined as the amount of protein necessary to inhibit translation by 50% in 1 ml of reaction mixture. For the experiments on DNA reaction mixtures contained, in a final volume of 50 μl: 50 mM Na acetate buffer, pH 4.0; 160 mM KCl; 20 μg of mechanically sheared DNA from herring sperm and Sephadex-filtered leaf extract (2.5 μg protein). Incubation was for 40 min at 30°C. Samples were processed and adenine was determined by HPLC as described in section 2. Other experimental conditions are described in the text. One unit of polynucleotide:adenosine nucleosidase activity is defined as the amount of enzyme that releases 1 mol per min of adenine from herring sperm DNA at 30°C. All results are mean ± S.E.M., and the numbers of experiments are given in parentheses.

Table 2
Effect of stress on enzymatic activities by extracts from *Hura crepitans* leaves

	Activity per g of tissue				Activity per mg of protein			
	Inhibition of translation		Nucleosidase activity		Inhibition of translation		Nucleosidase activity	
	U	%	U	%	U	%	U	%
Hura crepitans leaves								
21–23°C (3)	12,413 ± 3,807	(100)	1,057 ± 312	(100)	1,082 ± 357	(100)	113.4 ± 47.4	(100)
3°C (3)	14,423 ± 2,613	116	1,229 ± 413	116	1,229 ± 368	113	108.4 ± 48.8	95.5
45°C (3)	33,384 ± 10,217	269	3,607 ± 1,178	341	1,932 ± 596	178	207.3 ± 66.7	183
H_2O (3)	16,497 ± 5,834	(100)	1,309 ± 299	(100)	865.6 ± 214	(100)	73.3 ± 4.5	(100)
Sorbitol (4)	60,835 ± 26,581	369	2,177 ± 202	166	2,943 ± 1,106	340	117.5 ± 24.2	160
Phytolacca americana leaves								
23–25°C (4)	49,192 ± 22,988	(100)	3,427 ± 535	(100)	2,540 ± 1,369	(100)	146.8 ± 52	(100)
3°C (5)	85,530 ± 50.969	173	6,693 ± 701	195	3,151 ± 2,075	125	171.2 ± 25.7	117
45°C (4)	63,209 ± 27,016	128	5,823 ± 523	170	2,929 ± 1,719	115	175.6 ± 35.9	120
H_2O (3)	7,040 ± 1,004	(100)	5,060 ± 1,260	(100)	142.7 ± 14.1	(100)	101.5 ± 20.2	(100)
Sorbitol (3)	35,429 ± 5,002	503	10,844 ± 1,516	214	343.3 ± 39.4	240	104.8 ± 11.2	103

Experimental conditions are described in the text. Activities were measured as described in the legend to Table 1. All results are mean ± S.E.M., and the numbers of experiments are given in parentheses.

activity. The latter is a more accurate measure of the variation, being a direct determination of the enzymatic activity. The inhibition of protein synthesis is an indirect measure, based on the damage to ribosomes, and is probably amplified, since the damage of a single ribosome would arrest protein synthesis by the whole polysome.

The present results are consistent with previously reported observations: (i) the release of adenine from DNA by saporins [8] and other RIPs (Barbieri et al., unpublished results), (ii) the higher level of dianthins in old than in young carnation leaves [17], (iii) the appearance of a RIP in barley leaves senescent or treated with methyl jasmonate [9], and (iv) the higher level of trichosanthin in *Trychosanthes kirilowii* cell cultures infected by micro-organisms [18].

The effect of RIPs on DNA and the variations of their activity in different conditions lead us to reconsider the possible role of RIPs in plants. This is still not clear, although the hypotheses were formulated that RIPs could be defences against pests and predators, antiviral agents, regulators of protein synthesis, storage proteins. It was suggested also that RIPs may arrest the metabolism of endosperm storage cells at seed maturity [9]: this is consistent with the sudden appearance of RIP activity in the seeds of *Saponaria officinalis* toward the end of their maturation [7]. It was reported also that in senescent barley leaves a pro-form of a RIP is transformed into the active form, and that ribosomes become sensitive to their own RIP, by which normally they are not inactivated [19]. The appearance or increase of these enzymes in seeds at maturation, in senescence or in stress conditions strongly suggests that a function of RIPs could be to arrest the vital functions of cells bound to die. It is conceivable that RIPs are expressed [9], or are activated [19] or, if segregated from cytoplasm [20,21], come into contact with their substrate [22] when cells must die undergoing apoptosis in senescence, viral infection, and possibly other conditions, such as the well-known hypersensitivity of plants after viral infections [23].

A defensive role of RIPs was suggested on the basis of their antiviral activity. A direct effect of RIPs on viruses was excluded [24] and other observations (reviewed in [1]), and it was postulated that PAP could re-enter infected cells and inactivate their ribosomes, thus killing the cells and preventing viral spread [20,25]. This mechanism would not operate if ribosomes were resistant to their own RIP, such as those from wheat germ, [26] or carnation [17]. If the action of RIPs on RNA and on DNA observed in vitro [8] (and unpublished results) occurs also in vivo, this would suggest a direct effect of RIPs on viral or virally induced nucleic acids. The observation that trichosanthin inhibits HIV replication at doses not affecting cellular protein or DNA synthesis [27] would support this notion. Also, it would be possible that RIPs kill their own cells by acting on DNA. The latter would be a highly efficient mechanism, few alterations or even a single change in a critical DNA region being sufficient to kill a cell.

Finally, possibly the effect on DNA could contribute to the pathogenesis of the apoptosis induced in animal cells by ricin and abrin [28] and saporin (unpublished results from our laboratory).

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