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New ribosome-inactivating proteins with polynucleotide:adenosine glycosidase and antiviral activities from *Basella rubra* L. and *Bougainvillea spectabilis* Willd.

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Abstract. New single-chain (type 1) ribosome-inactivating proteins (RIPs) were isolated from the seeds of *Basella rubra* L. (two proteins) and from the leaves of *Bougainvillea spectabilis* Willd. (one protein). These RIPs inhibit protein synthesis both in a cell-free system, with an IC_{50} (concentration causing 50% inhibition) in the 10^{-10} M range, and by various cell lines, with IC_{50}s in the 10^{-8}–10^{-6} M range. All three RIPs released adenine not only from rat liver ribosomes but also from *Escherichia coli* rRNA, polyadenylic acid, herring sperm DNA, and artichoke mottled crinkle virus (AMCV) genomic RNA, thus being polynucleotide:adenosine glycosidases. The proteins from *Basella rubra* had toxicity to mice similar to that of most type 1 RIPs (Barbieri et al., 1993, Biochim Biophys Acta 1154: 237–282) with an LD_{50} (concentration that is 50% lethal) $\leq 8\ mg \cdot kg^{-1}$ body weight, whilst the RIP from *Bougainvillea spectabilis* had an $LD_{50} > 32\ mg \cdot kg^{-1}$. The N-terminal sequence of the two RIPs from *Basella rubra* had 80–93% identity, whereas it differed from the sequence of the RIP from *Bougainvillea spectabilis*. When tested with antibodies against various RIPs, the RIPs from *Basella* gave some cross-reactivity with sera against dianthin 32, and weak cross-reactivity with momordin I and momorcochin-S, whilst the RIP from *Bougainvillea* did not cross-react with any antiserum tested. An RIP from *Basella rubra* and one from *Bougainvillea spectabilis* were tested for antiviral activity, and both inhibited infection of *Nicotiana benthamiana* by AMCV.

Key words: Antiviral protein – *Basella* – *Bougainvillea* – Polynucleotide:adenosine glycosidase – Ribosome-inactivating protein

Introduction

It has been known for a long time that extracts from many plant tissues possess antiviral activity (reviewed by Verma et al. 1995), which in several cases is due to proteins identified as inhibitors of protein synthesis, called ribosome-inactivating proteins (RIPs; reviewed by Barbieri et al. 1993). Pokeweed antiviral protein (PAP), the first antiviral protein to be identified as an RIP (reviewed by Irvin 1995), and, consistently, several other RIPs tested possess antiviral activity against plant viruses, and also against animal viruses, HIV included (reviewed by Battelli and Stirpe 1995).

All RIPs, with either a single chain (type 1) or two chains (type 2), enzymatically release adenine from a single nucleotide in a precise position (A_{4324} in the case of rat liver ribosomes) in a universally conserved GAGA tetraloop of the major rRNA (Endo and Tsurugi 1987; reviewed by Barbieri et al. 1993). Depurinated ribosomes are unable to increase the length of the nascent peptide chain. Since this enzymatic activity was common to all RIPs, it was assumed to be the cause of both their inhibition of protein synthesis and of their toxicity. Also, the antiviral activity of these proteins was commonly attributed to the inactivation of ribosomes, inhibition of protein synthesis of the host cell and consequent arrest of viral replication. However, experimental evidence from various authors (Li et al. 1991; Barbieri et al. 1992; Ling et al. 1994; Roncuzzi and Gasperi-Campani 1996) indicated different additional substrate(s) for the enzymatic activity of RIPs. It was shown that at least some RIPs act on RNA species other than ribosomal, including viral RNAs, and on polyadenylic acid [poly (A)], and that all RIPs tested (more than 40) depurinated DNA

Abbreviations: AMCV = artichoke mottled crinkle virus; hsDNA = DNA from herring sperm; IC_{50} = concentration causing 50% inhibition; PAP = pokeweed antiviral protein; PAP-S = pokeweed antiviral protein from seeds; RIP = ribosome-inactivating protein

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(Barbieri et al. 1994, 1996, 1997; Olivieri et al. 1996; Stirpe et al. 1996). Thus, RIPs can be more appropriately called polynucleotide:adenosine glycosidases, and this activity may have a role in the antiviral activity besides the inactivation of the host cell ribosomes.

The antiviral activity of plant extracts has been a clue to the identification of new RIPs, and indeed dianthins (from *Dianthus caryophyllus*, a member of the Caryophyllaceae) were identified in this way (Stirpe et al. 1981), and inhibitors of viral infection from the roots of *Mirabilis jalapa* (Nyctaginaceae; Kubo et al. 1990) and from the leaves of *Clerodendrum inerme* (Prasad et al. 1995) turned out to be RIPs (Habuka et al. 1990; Olivieri et al. 1996). Infection of tobacco plants by tobacco mosaic virus was prevented by leaf extracts from *Basella alba* (Basellaceae, belonging to the order Caryophyllales as does the family Caryophyllaceae) and from *Bougainvillea spectabilis* Willd. (Nyctaginacea; Murthy et al. 1981; Verma and Dwivedi 1984). At least in the former case, prevention of the infection was attributed to one (or more) protein factor(s) (Ushasri et al. 1982).

Since it has turned out that RIPs analyzed so far actually differ in their stability and activity, and also in their substrate specificity and antiviral activity, the study of further RIPs will help to clarify the properties and to understand the roles of these proteins in plants. In this study, we report the purification of proteins with properties of a single-chain RIP from seeds of *Basella rubra* and from leaves of *Bougainvillea spectabilis*. The RIP from *Bougainvillea spectabilis* is probably the least-toxic RIP known, thus making it particularly suitable for practical applications. The antiviral acitivity of the proteins and their ability to depurinate nucleic acids are described.

Materials and methods

Materials. *Basella rubra* L. seeds were purchased from Florsilva, Idice, BO, Italy, and *Bougainvillea spectabilis* Willd. leaves were from the Botanic Garden of the University of Bologna. Pokeweed antiviral protein from seeds (PAP-S) was purified as described by Barbieri et al. (1987).

L-[^{14}C]Leucine and L-[^{3}H]leucine were from Amersham International (Amersham, UK). Materials for chromatography, including calibrating substances, and electrophoresis markers were from Pharmacia (Uppsala, Sweden); adenine and tRNA from Sigma (St. Louis, Mo., USA). Both poly(A) and rRNA from *Escherichia coli* (16S + 23S, MW 1.75×10^6) were from Boehringer (Mannheim, Germany). Cell culture medium and supplements and all other chemicals were as in previous work (Parente et al. 1993). Rabbit antisera to various RIPs were a gift from Dr. P. Strocchi (Dipartimento di Farmacologia, Bologna). All other reagents were of analytical or molecular biology grade and, when possible, RNase-free. Milli-Q water (Waters-Millipore, Milford, Mass, USA) was used where applicable.

The DNA from herring sperm (hsDNA; Sigma) was mechanically sheared and made RNA-free by treatment with DNase-free RNase A (Boehringer) for 2.5 h at 37 °C. The DNA was then repeatedly precipitated in ethanol to remove the enzyme. Genomic RNA (m ssRNA positive + one small satellite, MW 1.49×10^6) from artichoke mottled crinkle virus (AMCV) was prepared by phenol extraction and ethanol precipitation from purified virus isolates.

Rat liver ribosomes were prepared essentially as described elsewhere (Arias et al. 1992) in RNase-free conditions. Their concentration was determined by the absorbance at 260 nm (A_{260}) according to Montanaro et al. (1978), assuming that 12.5 absorbance units $\cdot$ ml^{-1} were equivalent to 1 mg $\cdot$ ml^{-1} and that 1 mg contained 250 pmol of ribosomes. Ribosomes were stored in aliquots at −80 °C.

Purification of ribosome-inactivating proteins. *Basella rubra* seeds (100 g) were homogenised with an Ultraturrax apparatus (IKA, Staufen, Germany) in cold phosphate-buffered saline (PBS; 0.14 M NaCl, 5 mM sodium phosphate buffer, pH 7.5; 8 ml $\cdot$ g^{-1} seeds), and *Bougainvillea spectabilis* leaves (1400 g) were ground in a mortar with liquid nitrogen and homogenised with an Ultraturrax apparatus in PBS (4 ml $\cdot$ g^{-1} leaves). The slurries were extracted overnight at 4 °C with magnetic stirring, filtered through cheesecloth and, in the case of *Basella*, clarified by centrifugation at 10 000 $\cdot$ *g* for 30 min at 4 °C. The crude extract from *Basella* and the slurry from *Bougainvillea* were adjusted to pH 4.0 with glacial acetic acid and centrifuged as above. The supernatants were applied to an S-Sepharose Fast Flow column (Pharmacia; 15 cm long, 5 cm i.d. and 12 cm long, 18 cm i.d. for *Basella* and *Bougainvillea*, respectively) equilibrated with 10 mM sodium acetate (pH 4.5). The column was washed with 1 vol. of the equilibration buffer and extensively with 5 mM sodium phosphate buffer (pH 7.0), and bound protein was eluted with 1 M NaCl in the same buffer. Fractions inhibiting cell-free protein synthesis were pooled, dialysed exhaustively against water at 4 °C, adjusted to 5 mM phosphate buffer (pH 7.0) containing 100 mM NaCl in the case of *Basella*, and applied to a CM-Sepharose Fast Flow column (Pharmacia; 30 cm long, 1.6 cm i.d.) equilibrated with the same buffer. The column was washed with the equilibration buffer until the A_{280} was lowered to base line and was eluted with an NaCl linear gradient in the same buffer (100–280 mM and 0–200 mM for *Basella* and *Bougainvillea* respectively; total volume 800 ml). Fractions inhibiting protein synthesis were pooled, dialysed extensively against water at 4 °C, and stored frozen at −80 °C.

Analytical methods. Purity and molecular mass were determined by SDS-PAGE: gels were analysed with an Epson GT8000 densitometer, utilising a Gel Image program (Pharmacia). Purity was checked also by reverse-phase HPLC on a Vydac C4 column (Hesperia, Calif., USA) as described previously (Parente et al. 1993). The isoelectric point was determined with a Phast System (Pharmacia) with the gels provided by the manufacturer. The $A^{1\%}_{280}$ of the purified proteins was determined using aqueous solutions of freeze-dried samples.

Protein concentration and amino acid sequences, the latter of proteins further purified by reverse-phase HPLC, were determined as described previously (Parente et al. 1993).

The cross-reactivity of RIPs with antisera to other RIPs was measured with an enzyme-linked immunosorbent assay (ELISA) as described previously (Parente et al. 1993).

Cell cultures. The cell lines used, namely mouse 3T3 (fibroblasts), and human HeLa (carcinoma), NB100 (neuroblastoma) and BeWo (chorion carcinoma) were maintained as monolayer cultures in RPMI 1640 medium (Moore et al. 1966) supplemented with antibiotics and 10% foetal calf serum, in a humidified atmosphere containing 5% CO_2, at 37 °C. Subcultures were obtained by trypsin treatment of confluent cultures. The human JM cell line (monocyte-derived) was grown in suspension and treated with phorbolmyristate acetate to induce adhesion as described by Bolognesi et al. (1995).

Effect on protein synthesis. The effect of RIPs on protein synthesis in a cell-free system (a rabbit reticulocyte lysate) was studied essentially as described by Parente et al. (1993). Reaction mixtures

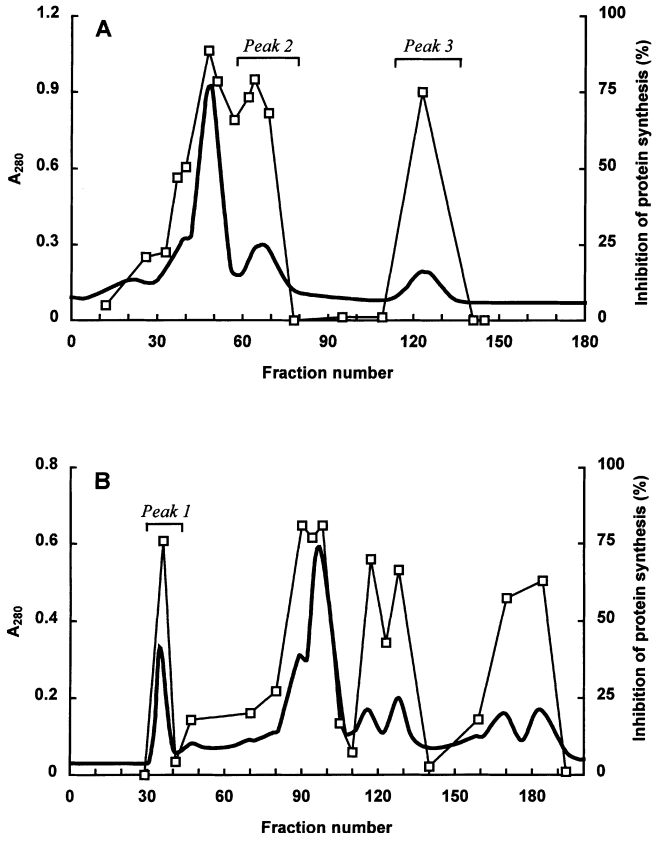

Fig. 1A,B. Ion-exchange chromatography of extracts. The extracts of *Basella rubra* seeds (**A**) and *Bougainvillea spectabilis* leaves (**B**) were applied to a CM-Sepharose column eluted as described in *Materials and methods*. The absorbance at 280 nm (A_{280}) was recorded (*thick line*) and the inhibitory activity on protein synthesis of selected fractions (*thin line*) was measured. Fractions pooled are indicated by the *horizontal bars*

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), 3300 Bq of L-[^{14}C]leucine, and 25 µl of rabbit reticulocyte lysate. Incubation was at 28 °C for 5 min.

Protein synthesis by various cell lines was assayed as described previously (Ferreras et al. 1993). Cells (10^5 per well) were incubated with RIPs for 18 h, followed by a 2-h pulse with L-[4,5-^{3}H]leucine (4625 Bq in 250 µl).

The concentration causing 50% inhibition (IC_{50}) was calculated by linear-regression analysis.

Determination of polynucleotide:adenosine glycosidase activity. Enzymatic activity was determined by measuring adenine released from various substrates by HPLC (Zamboni et al. 1989), essentially following the procedure of McCann et al. (1985) as described by Barbieri et al. (1996). Reactions were run for 40 min at 30 °C in a final volume of 50 µl containing 50 mM sodium acetate (pH 4.0), 100 mM KCl, in the presence of increasing concentrations of RIP and the indicated amount of polynucleotide substrates. When ribosomes were used as substrate the reaction mixture contained 7 mM magnesium acetate, 100 mM ammonium chloride, 20 mM Tris-HCl buffer (pH 7.8), 1 mM dithiothreitol. A standard curve of adenine was run with each experiment.

Determination of bases other than adenine was performed as described (Barbieri et al. 1996).

Virus assays. Virus inoculum containing purified AMCV at 0.04 $\mu g \cdot ml^{-1}$ and various concentrations of RIPs in 50 mM sodium phosphate buffer (pH 7.0) were rubbed on basal opposite leaves of *Nicotiana benthamiana* plants using carborundum (600 mesh) as an abrasive. Each treatment was replicated eight times and randomised on the leaves of control plants. Leaves of control plants were rubbed with virus alone or RIP alone in 50 mM sodium phosphate buffer (pH 7.0). The plants were placed in a growth chamber under light-dark cycles of 16 h of light and 6 h of darkness at 22 °C and 60% relative humidity. Plants were scored as having positive symptoms of infection when apical leaves showed necrotic lesions.

Toxicity to animals. Scalar doses of the purified proteins were injected intraperitoneally to groups of four male and four female Swiss mice. The ratio between doses was two, and the animals were observed up to 16 d after treatment. The mice had free access to food and water and the national guidelines for the care and use of laboratory animals were followed throughout the experiments.

Results

Purification and general properties. The extracts from the seeds of *Basella rubra* and the leaves of *Bougainvillea spectabilis* inhibited protein synthesis by a rabbit

Table 1. Purification of RIPs from the seeds of *Basella rubra* and from the leaves of *Bougainvillea spectabilis*

Source	Preparation[a]	Total protein (mg)	IC_{50}[b] ($ng \cdot ml^{-1}$)	Specific activity[c] (10^3 U $\cdot$ mg^{-1})	Total activity[c] (10^6 U)	Yield (%)
Basella	Crude extract	8699	162	6.2	53.6	(100)
	Acidified extract	4212	87	11.5	48.4	90
	S-Sepharose eluate	213.2	8.0	124.8	26.6	50
	CM-Sepharose eluate					
	peak 2 (RIP 2)	13.0	1.70	588.2	7.7	14
	peak 3 (RIP 3)	10.4	1.66	602.4	6.3	12
Bougainvillea	Acidified extract	3454	871	1.15	3.97	(100)
	S-Sepharose eluate	300	100	10.0	3.00	75
	CM-Sepharose eluate					
	peak 1 (RIP 1)	3.5	10.5	95.5	0.33	8
	other active peaks	26.3	–	–	0.99	25

[a]Results refer to 100 g of starting material
[b]IC_{50} is the amount of protein which inhibits protein synthesis by 50% in a rabbit reticulocyte lysate system
[c]One unit (U) is the amount of protein causing 50% inhibition of cell-free protein synthesis in 1 ml

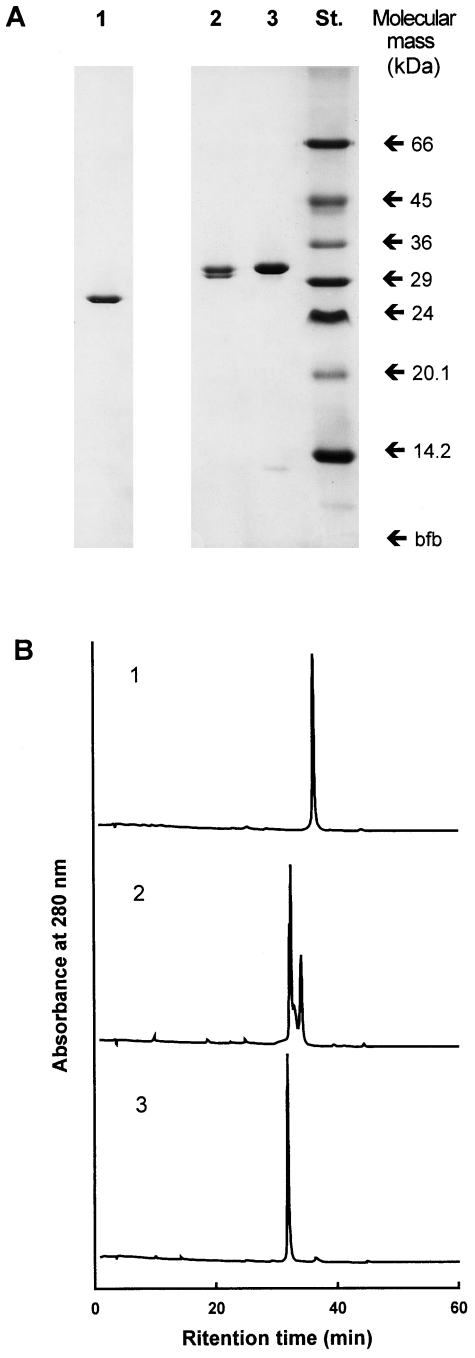

Fig. 2A,B. Ribosome-inactivating proteins were subjected to SDS-PAGE (**A**) and reverse-phase chromatography (**B**) as described in *Materials and methods. 1*, *Bougainvillea spectabilis* RIP 1; *2*, *Basella rubra* RIP 2; *3*, *Basella rubra* RIP 3

reticulocyte lysate. With the standard procedure used in the purification of RIPs, the inhibitory activity could be resolved into several protein peaks eluted from a CM-Sepharose column (Fig. 1). The fractions of peaks 2 and 3 from *Basella* and of peak 1 of *Bougainvillea* had the highest specific inhibitory activity on cell-free protein synthesis and gave the highest yield after purification (Table 1), and therefore were pooled and used for further experiments.

The proteins of peak 3 from *Basella* and of peak 1 from *Bougainvillea* appeared to be 98 ± 1% homogeneous by reverse-phase HPLC analysis, and on SDS-PAGE gave a single protein band with mobility corresponding to M_r 31 200 (*Basella* peak 3) and 26 200 (*Bougainvillea* peak 1; Fig. 2), respectively. The fractions from peak 2 of *Basella* were eluted as two peaks (2a and 2b) from reverse-phase HPLC (data not shown), and gave two very close bands (M_r 30 600 and 31 200) on SDS-PAGE (Fig. 2) which, however, could not be separated by various non-denaturing chromatographic techniques. In view of the close similarity of their amino acid sequences (see below), they were considered as isoforms, and the proteins contained in the peaks examined will hereafter be referred to as *Basella* RIP 2 (2a + 2b) and 3, and *Bougainvillea* RIP 1.

The N-terminal amino acid sequences of the proteins (Table 2) showed that *Basella* RIP 2a had 24 out of 30 residues identical to those of the *Basella* RIPs 2b and 3, the latter differing from each other by 2 out of 30 residues. The proteins from *Basella* had only six to seven residues identical to those of the sequence of *Bougainvillea* RIP 1. An RIP from *Mirabilis jalapa* (Habuka et al. 1989), a plant belonging to the family Nyctaginaceae as does *Bougainvillea*, had eight to nine residues identical to those of the amino acid sequence of *Basella* RIP, and eight residues identical to those of the sequence of *Bougainvillea* RIP 1.

The pI of both RIPs from *Basella* was greater than 9.0 and that of the RIP from *Bougainvillea* was 9.0 (data not shown). The $A_{280}^{1\%}$ values of the purified RIPs were 7.89, 6.92 and 8.72 for *Basella* RIPs 2 and 3, and *Bougainvillea* RIP 1, respectively.

When tested with sera against six other RIPs (saporin-S6 and dianthin 32 from the Caryophyllaceae, root PAP (PAP-R) from the Phytolaccaceae, momordin I, momorcochin-S and trichokirin from the Cucurbitaceae; reviewed by Barbieri et al. 1993), *Basella* RIP 2 gave a partial cross-reactivity (6%, compared with the relevant antigen) only with serum against dianthin 32, whereas *Basella* RIP 3 gave a stronger cross-reactivity

Table 2. N-terminal amino acid sequence[a]

	10[b]										20										30
Basella rubra RIP 2a	A	D	L	Y	W	D	L	R	S	T	T	H	E	K	Y	T	G	F	I	G	G	I	R	D	N	L	K	D	S	T
Basella rubra RIP 2b	A	D	L	Y	W	D	L	R	T	T	T	H	D	K	Y	T	S	F	I	G	G	I	R	N	K	L	K	A	S	T
Basella rubra RIP 3	D	D	L	Y	W	D	L	R	S	T	T	H	D	K	Y	T	S	F	I	G	G	I	R	N	K	L	K	A	S	T
Bougainvillea spectabilis RIP 1			Y	N	T	V	S	F	N	L	G	E	A	Y	E	Y	P	T	F	I	Q	D	L	R	N	E	L	A	K	G	T	P
MAP[c]	A	P	T	L	E	T	I	A	S	L	D	L	N	N	P	T	T	Y	L	S	F	I	T	N	I	R	T	K	V	A	D	K	T	E

[a]Alignment is for residues 15, 18, 23 which are highly conserved in aminoterminal sequences of RIPs
[b]Residue numbers refer to the sequences of *Basella rubra* proteins
[c]MAP refers to *Mirabilis* antiviral protein from *Mirabilis jalapa*. Sequence is from Habuka et al. (1989)

Table 3. Inhibition of protein synthesis by cell lines caused by *Basella rubra* and *Bougainvillea spectabilis* RIPs[a]

Cell line	Origin	IC_{50}[b] (nM)		
		Basella RIP 2	*Basella* RIP 3	*Bougainvillea* RIP 1
BeWo	Chorion carcinoma	63.7 ± 15.6	43.8 ± 9.2	950 ± 16
HeLa	Carcinoma	166 ± 24	315 ± 25	>3300
JM	Monocyte-derived	16.6 ± 3.7	9.3 ± 0	1218 ± 484
NB100	Neuroblastoma	169 ± 87	110 ± 75	665 ± 0
3T3	Fibroblasts	353 ± 5.7	700 ± 369	>3300

[a]Results are mean values ± SD of two experiments performed in triplicate. Incorporation of [^{3}H]leucine by control cells was (dpm ± SD): BeWo 18 995 ± 7332, HeLa 24 082 ± 6367, JM 8555 ± 824, NB100 12 607 ± 3694, and 3T3 4317 ± 2652
[b]IC_{50}, concentration of RIPs inhibiting protein synthesis by 50% compared with controls

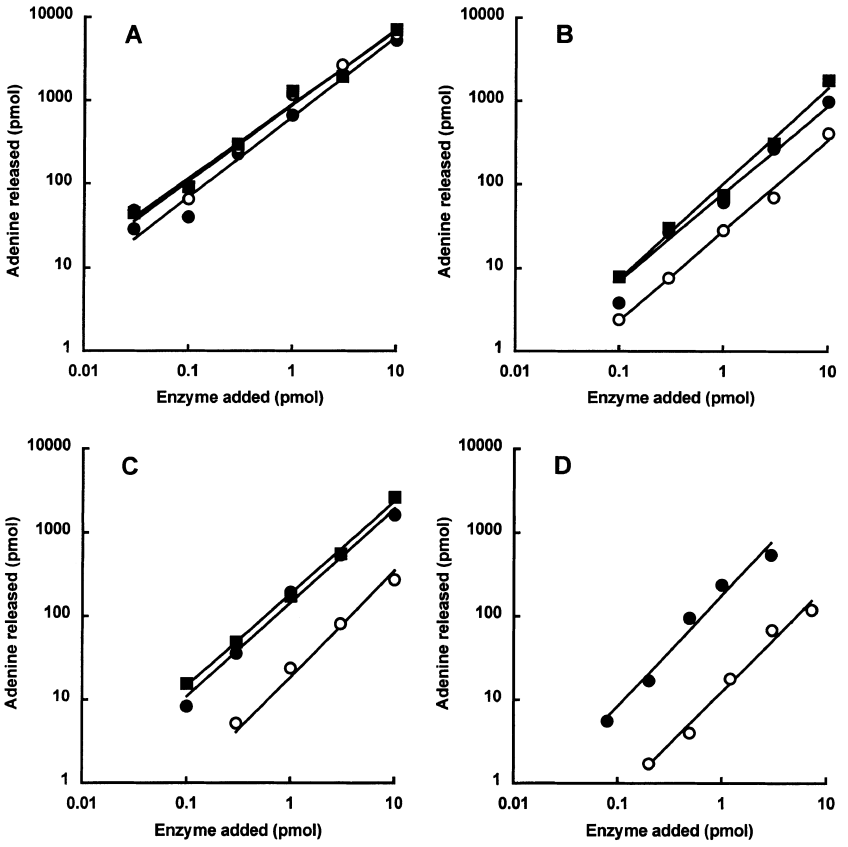

Fig. 3A–D. Polynucleotide:adenosine glycosidase activity of the RIPs assayed with various substrates. The substrates were: **A** hsDNA, **B** poly(A), **C** rRNA, **D** AMCV RNA. The RIPs used were *Basella rubra* RIP 2 (●), *Basella rubra* RIP 3 (■) and *Bougainvillea spectabilis* RIP 1 (○). Reaction mixtures contained 20 μg of substrate (in the case of hsDNA, poly(A) and *E. coli* rRNA) or 2.5 μg (AMCV genomic RNA). Controls were run with complete assay components except RIPs (less than 6 pmol of adenine was spontaneously released from all substrates)

Table 4. Polynucleotide-adenosine glycosidase activity of RIPs from *Basella rubra* and *Bougainvillea spectabilis*

Substrate	Adenine released[a]					
	Basella RIP 2		*Basella* RIP 3		*Bougainvillea* RIP 1	
	pmol	pmol · (pmol substrate)$^{-1}$	pmol	pmol · (pmol substrate)$^{-1}$	pmol	pmol · (pmol substrate)$^{-1}$
Rat liver ribosomes	31.2	0.78	20.8	0.52	32.4	0.81
hsDNA	636	n.a.[b]	900	n.a.[b]	877	n.a.[b]
poly(A)	78.3	n.a.[b]	102	n.a.[b]	28.0	n.a.[b]
E. coli rRNA	145	12.7	185	16.2	18.7	1.64
AMCV RNA	178	106	n.d.[c]	n.d.[c]	18.1	10.8

[a]Reactions were run with 40 pmol of rat liver ribosomes or 20 μg of hsDNA, poly(A) or rRNA or 2.5 μg of AMCV RNA and 1 pmol of RIPs (10 pmol in the experiments with rat liver ribosomes). In the absence of RIPs (controls), 1.8 (ribosomes), 5.5 (hsDNA), 3.1 (poly(A)), 1.5 (rRNA) and 0 (AMCV RNA) pmol of adenine were released
[b]*n.a.*, not applicable
[c]*n.d.*, not determined

(35%) with the antiserum against dianthin 32 and a weak cross-reaction (1.5% in both cases) with sera against momordin I and momorcochin-S (data not shown). *Bougainvillea* RIP 1 gave no reaction with any antiserum tested.

Effects on protein synthesis. The RIPs purified from *Basella* and from *Bougainvillea* inhibited protein synthesis in a rabbit reticulocyte lysate, with IC_{50} values of 5.48×10^{-11} M (*Basella rubra* RIP 2), 5.35×10^{-11} M (*Basella* RIP 3), and 4.01×10^{-10} M (*Bougainvillea* RIP 1; calculated from Table 1).

The same RIPs inhibited protein synthesis by various cell lines, although at much higher concentrations than those effective on cell-free protein synthesis (Table 3), a characteristic property of type 1 RIPs. Furthermore, the effect varied greatly from one cell line to another, the IC_{50} ranging by more than two orders of magnitude. Amongst tested cell lines, 3T3 fibroblasts and HeLa cells seemed the most resistant, and JM cells, tested after the acquisition of adherence, appeared the most sensitive, to *Basella* RIPs 2 and 3. The latter proteins had similar cytotoxicity, which was higher than the toxicity of *Bougainvillea* RIP 1 to all cell lines tested.

Polynucleotide:adenosine glycosidase activity. *Basella* RIPs 2 and 3 and *Bougainvillea* RIP 1 released adenine from hsDNA, poly(A), *Escherichia coli* rRNA and genomic AMCV RNA in a concentration-dependent manner (Fig. 3). The enzymatic activities of the three RIPs, as well as the number of depurination sites of examined substrates, were compared (Table 4). Among polynucleotides, DNA appeared to be the best substrate, and *Basella* RIPs 2 and 3 were more active than *Bougainvillea* RIP 1 on poly(A), rRNA and viral RNA. Several adenine residues per mole of rRNA or AMCV RNA (the only substrates with a defined molecular mass) were released. With ribosomes as substrate a higher concentration of RIPs was used, to allow the reaction to proceed to exhaustion: the number of adenine residues released was never greater than one per ribosome. No adenine was released from whole AMCV by any RIP (data not shown).

No bases other than adenine were released from DNA by *Basella* RIP 2 and *Bougainvillea* RIP 1 (data not shown).

Antiviral activity. The more abundant *Basella* RIP 2 and *Bougainvillea* RIP 1 were tested, and both prevented systemic infection of *N. benthamiana* plants by AMCV (Table 5). *Basella* RIP 2 had the highest activity, comparable to that of PAP-S, assayed as positive control.

Toxicity to animals. The purified RIP had a toxicity similar to that of other type 1 RIPs. The LD_{50} (concentration that is 50% lethal) values appeared to be around 8 $mg \cdot kg^{-1}$ for the two RIPs from *Basella* and above 32 $mg \cdot kg^{-1}$ for *Bougainvillea* RIP 1 (Table 6).

Table 5. Antiviral activity of RIPs: prevention of infection by AMCV of *Nicotiana benthamiana* plants, as indicated by the ratio of infected to treated plants

	RIP concentration ($\mu g \cdot ml^{-1}$)	
	100	1
Controls	13/15	15/15
Basella rubra RIP 2	0/8	1/8
Bougainvillea spectabilis RIP 1	1/8	8/8
PAP-S	0/8	1/8

Table 6. Toxicity to mice of RIPs

RIP	Dose ($mg \cdot kg^{-1}$)	Mortality (dead/treated mice)
Basella rubra RIP 2	4	0/6
	8	3/6
	16	5/6
Basella rubra RIP 3	4	0/6
	8	5/6
Bougainvillea spectabilis RIP 1	8	0/6
	16	0/6
	32	0/6

Discussion

The proteins isolated from *Basella rubra* and *Bougainvillea spectabilis* have the properties of type 1 RIPs, in that they (i) are single-chain proteins with a molecular mass of 30 kDa, approximately, and an isoelectric point in the alkaline region, (ii) inhibit protein synthesis in a cell-free system, but have much less effect on protein synthesis by whole cells and consequently have a relatively low toxicity to animals, (iii) have *N*-glycosidase activity, and (iv) have antiviral activity.

The various points deserve some comments. *Basella rubra* RIP 3 and *Bougainvillea spectabilis* RIP 1 were homogeneous, whereas reverse-phase HPLC and SDS-PAGE showed that *Basella rubra* RIP 2 was a mixture of two similar proteins, presumably isoforms. Different forms of RIP, considered isoforms, have been found in several plants (reviewed by Barbieri et al. 1993). The N-terminal sequences of the proteins isolated from *Basella* had 80–93% identical residues, and the substitutions in the remaining residues were conservative. Both RIPs had only 20–23% identity with the RIP from *Bougainvillea*, which in turn had only 27% identity with the RIP from *Mirabilis jalapa*, another plant from the Nyctaginaceae. This is somewhat surprising, in view of the similarities often observed among RIPs from plants belonging to the same family (see for instance Bolognesi et al. 1995).

The fibroblast-derived 3T3 cell line and HeLa cells showed a low sensitivity to these RIPs, consistent with results obtained with other RIPs; the JM line was very sensitive to *Basella* RIPs 2 and 3, as it has often been observed with other macrophagic cells (Barbieri et al. 1993). These differences might be ascribed to differences in (i) binding mechanisms and penetration into different cells, (ii) intracellular routing of the RIPs, or (iii)

resistance or/and degradation. Although not reported so far, a different sensitivity of ribosomes of various cells to a given RIP cannot completely be excluded.

The RIPs released a single adenine residue only from rat liver ribosomes, and acted also on purified rRNA from *E. coli*, from viral RNA, on poly(A) and on hsDNA, thus being polynucleotide:adenosine glycosidases. As observed with other RIPs, no bases other than adenine were released, and more than one mole of adenine was released per mole of rRNA or AMCV RNA, indicating that these RIPs do not require a specific base sequence to act. This confirms that the property of acting on substrates other than ribosomes is not limited to saporins (Barbieri et al. 1994, 1996), but is common to several RIPs (Marchant and Hartley 1995; Orita et al. 1996; Stirpe et al. 1996; Barbieri et al. 1997).

Consistent with previous reports on other RIPs (reviewed by Barbieri et al. 1993; Battelli and Stirpe 1995), the RIPs had no effect on the whole AMCV, and still prevented systemic infection of the plants, presumably by inhibiting viral replication at the site of infection.

The RIPs from *Basella rubra* were more active than *Bougainvillea spectabilis* RIP 1 in that they (i) had a stronger inhibitory effect on protein synthesis in both a cell-free system and in cells, with IC_{50}s comparable to those of some isoforms of saporin and PAP (Barbieri et al. 1993), the most potent RIPs known; (ii) had a higher glycosidase activity than *Bougainvillea* RIP 1 on all substrates but DNA; and (iii) had a higher antiviral activity against AMCV. On the other hand, *Bougainvillea* RIP 1 appears to be one of the least toxic RIPs known (Barbieri et al. 1993). This makes this RIP particularly suitable for practical applications in agriculture, for the transfection of plants to confer on them resistance to viruses (Lodge et al. 1993; Hong et al. 1996; Lam et al. 1996), or in medicine, for the preparation of conjugates with antibodies (''immunotoxins'') or other carriers selectively toxic to a given type of target cell, currently under study as therapeutic agents (reviewed by Ramakrishan 1993). Moreover, the RIPs from both *Basella rubra* and *Bougainvillea spectabilis* had little or no cross-reactivity with sera against several RIPs from other taxonomically related or unrelated plants, a useful property for circumventing the immune response after repeated administration of immunotoxins.

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