Properties of the ribosome-inactivating proteins gelonin, *Momordica charantia* inhibitor, and dianthins

Anna FALASCA,* Anna GASPERI-CAMPANI,† Ada ABBONDANZA,† Luigi BARBIERI† and Fiorenzo STIRPE†

*Istituto di Chimica Biologica dell'Università, and †Istituto di Patologia generale dell'Università di Bologna, I-40126 Bologna, Italy

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The amino acid and sugar compositions of four ribosome-inactivating proteins (gelonin, *Momordica charantia* inhibitor, dianthin 30 and dianthin 32) were determined. The proteins are all basic glycoproteins ($pI > 8$) containing mannose (more abundant in gelonin), glucose, xylose, fucose (absent from gelonin) and glucosamine. The ribosome-inactivating properties of the proteins examined are not modified by pretreatment with *N*-ethylmaleimide. Precipitating and inactivating antibodies can be raised against ribosome-inactivating proteins; a weak cross-reaction was observed only between dianthin 30 and dianthin 32.

The 'inhibitor' from the seeds of *Momordica charantia* (bitter gourd) (Barbieri *et al.*, 1980), gelonin, from the seeds of *Gelonium multiflorum* (Stirpe *et al.*, 1980), and dianthin 30 and dianthin 32 from the leaves of *Dianthus caryophyllus* (carnation) (Stirpe *et al.*, 1981) are proteins that inhibit protein synthesis by cell-free systems with little effect on intact cells, and prevent local lesions by tobacco-mosaic virus (Stevens *et al.*, 1981; Stirpe *et al.*, 1981). These proteins are similar to the pokeweed antiviral proteins purified from the leaves (PAP, Obrig *et al.*, 1973; PAP II, Irvin *et al.*, 1980) and from the seeds (PAP-S, Barbieri *et al.*, 1982) of *Phytolacca americana* (pokeweed), and to tritin, from wheat germ (Roberts & Stewart, 1979; Coleman & Roberts, 1981), and like these act in an apparently identical manner by inactivating eukaryotic ribosomes, on which they act in a less than equimolar ratio. All together, the ribosome-inactivating proteins are reminiscent of the A-chains of ricin and abrin (reviewed by Olsnes & Pihl, 1977) and, like the A-chains, could be rendered toxic to cells by conjugation to suitable carriers capable of binding to the cell surface. Cytotoxic complexes were obtained by conjugating gelonin to concanavalin A (Stirpe *et al.*, 1980) and to a monoclonal antibody (Thorpe *et al.*, 1981) or PAP to a monoclonal antibody (Masuho *et al.*, 1982).

In view of this and of other possible applications, the main properties of the *M. charantia* inhibitor, of gelonin and of dianthins were studied. Their isoelectric points, amino acid compositions and sugar contents were determined in the present experi-

ments. It was ascertained also that proteins purified from different plants are immunologically distinct from each other, whereas a cross-reaction was observed between dianthin 30 and dianthin 32, which both are purified from carnation leaves.

Experimental

Proteins and antisera

M. charantia inhibitor (Barbieri *et al.*, 1980), gelonin (Stirpe *et al.*, 1980) and dianthins (Stirpe *et al.*, 1981) were purified as described in the respective references.

For the production of antisera, New Zealand White rabbits were immunized by subcutaneous injections of the proteins (0.5 mg/rabbit), emulsified with complete Freund's adjuvant for the first dose and with incomplete adjuvant for the subsequent injections, given 3 and 6 weeks after the first one. Rabbits were bled 2 weeks after the last injection. Sera were dialysed overnight against 0.14 M-NaCl containing 5 mM-sodium phosphate buffer, pH 7.2, and were stored frozen at -25°C .

Chemical analyses

The amino acid and amino sugar compositions of the proteins were determined in samples extensively dialysed against distilled water. Protein samples were hydrolysed in sealed tubes under N_2 at 110°C with 6 M-HCl for 24, 48 and 72 h, and were analysed with an amino acid analyser (Multichrom B 4235; Beckman, Munich, West Germany). The assay for cysteine was performed after oxidation of the

proteins with performic acid (Moore, 1963). A separate hydrolysis with toluene-*p*-sulphonic acid was performed for determination of tryptophan and hexosamine (Allen & Neuberger, 1975).

The neutral sugar composition was analysed by g.l.c. as described by Dunstan *et al.* (1974), with D-mannitol as a standard.

Isoelectric point was determined by isoelectric focusing on Ampholine polyacrylamide-gel plates (LKB, Stockholm, Sweden) at a pH range of 3.5–9.5, according to the instructions supplied by the manufacturer.

Treatment with N-ethylmaleimide

Ribosome-inactivating proteins (17 μ M) were incubated at 37°C for 30 min in 20 mM-sodium phosphate buffer, pH 7.4, with or without a 10- or 100-fold molar excess of *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO, U.S.A.). The proteins were then dialysed overnight at 4°C against 500 vol. of 20 mM-sodium phosphate, pH 7.4.

Protein synthesis

Protein synthesis was determined with a lysate of rabbit reticulocytes (Allen & Schweet, 1962) with

the techniques and chemicals described by Barbieri *et al.* (1982).

Immunodiffusion tests

Double immunodiffusion was carried out in agarose gel [1% (w/v) in 0.14 M-NaCl containing 20 mM-sodium phosphate buffer, pH 7.2], cast to a depth of 4 mm on glass slides. Wells of 8 mm diameter were cut in the gel for antigen and wells of 11 mm for antiserum. Diffusion was allowed to take place for 24 h in humidity chambers at room temperature (approx. 20°C).

Results and discussion

The main characteristics of the ribosome-inactivating proteins examined with present experiments can be summarized as follows.

All proteins examined by isoelectric focusing gave a single band with a basic isoelectric point (Table 1), consistent with their chromatographic behaviour (the proteins bind to CM-cellulose at pH 6.5).

A comparison of the amino acid compositions

Table 1. *Isoelectric points and amino acid compositions of ribosome-inactivating proteins*
Experimental conditions are described in the text.

	Mol.wt. pI	Composition (mol/mol of protein)			
		<i>Momordica charantia</i> inhibitor	Gelonin	Dianthin 32	Dianthin 30
	...	31 000*	30 000	31 700	29 500
	...	8.60	8.15	8.55	8.65
Amino acid					
Lys		11.4	20.9	22.3	17.3
His		4.7	2.2	1.4	0.8
Arg		12.6	12.2	11.2	11.0
Asx		23.6	22.1	26.3	27.9
Thr†		16.2	14.9	13.8	10.6
Ser†		16.8	17.0	12.6	9.9
Glx		24.8	24.8	21.0	23.7
Pro		9.0	7.9	7.1	5.3
Gly		15.8	14.8	15.4	15.9
Ala		24.6	16.9	20.5	21.9
Half-Cys‡		2.2	3.3	5.1	5.0
Val		13.7	16.1	17.9	17.8
Met		7.0	4.5	11.0	8.9
Ile		13.8	13.9	14.2	12.0
Leu		21.2	19.4	21.2	20.8
Tyr†		13.4	8.2	10.8	6.6
Phe		8.4	11.9	10.8	12.6
Trp§		1.0	0.6	1.0	0.8

* Redetermined by L. Barbieri (unpublished work). The value given previously (23 000; Barbieri *et al.*, 1980) was found to be incorrect.

† Values obtained from the hydrolysis at 24, 48 and 72 h were extrapolated to zero time. Error was less than 1%.

‡ Determined as cysteic acid.

§ Determined as described by Allen & Neuberger (1975).

(Table 1) shows some difference in the contents of lysine, which is lower in the *M. charantia* inhibitor, and of cystine and methionine, which are more abundant in dianthins. As could be expected, the amino acid composition of dianthins 30 and 32 is very similar. The amino acid composition of gelonin is similar to that of PAP-S (Barbieri *et al.*, 1982). From the amino acid analysis it appears that the ribosome-inactivating proteins bear some thiol groups. These can be used conveniently for linking proteins together, and, since one of the possible uses of these proteins is the preparation of cytotoxic conjugates with antibodies or other proteins, it is useful to know whether reactive thiol groups are involved in the ribosome-damaging activity. When ribosome-inactivating proteins were incubated in the presence of a molar excess of *N*-ethylmaleimide to block free thiol groups, no significant variation in their activity on cell-free protein synthesis was observed (Fig. 1), as expected from previous observations with gelonin (Stirpe *et al.*, 1980).

The sugar contents are shown in Table 2. All proteins contain mannose, in which gelonin is especially rich, glucose, xylose, fucose (absent in gelonin) and glucosamine, the content of which is lower in dianthin 30 than in other proteins. It should be pointed out that dianthin 30 contains twice as much xylose as does dianthin 32. This rules out the possibility, considered previously (Stirpe *et al.*, 1981), that dianthin 30 could be derived from dianthin 32.

Immune sera against the ribosome-inactivating proteins could be obtained from rabbits. The immunoprecipitation test (Fig. 2) showed that gelonin and dianthin 30 gave a single band of precipitation with the respective antiserum, whereas *M. charantia* inhibitor and dianthin 32 gave two bands. The precipitation was specific, except for the two dianthins, which showed some cross-reactivity. The antisera also abolished the inhibitory effect on protein synthesis of the respective ribosome-inactivating proteins (Table 3), again without significant cross-reactivity between the proteins. This strongly suggests that the functionally active sites of the proteins are immunologically different from one protein to another. Alternatively, they may not coincide with an antibody-binding site, and in this case the loss of inhibitory activity in the presence of the antiserum could be the consequence of conformational changes following the binding of the proteins to the respective antibody.

As a whole, these results show that the ribosome-inactivating proteins purified from different plants are all basic proteins, have a different amino acid composition, and are immunologically distinct from each other. The last observation may be of practical significance, should these proteins be administered to animals either as such or in the form

Fig. 1. Activity of ribosome-inactivating proteins after treatment with *N*-ethylmaleimide

Protein synthesis was measured with a lysate of rabbit reticulocytes as described in the text. *M. charantia* inhibitor (a) or gelonin (b) was added after prior incubation, as described in the text and in Table 3, with buffer (○) or with a 10-fold (●) or 100-fold (△) molar excess of *N*-ethylmaleimide. Control values were 1692 and 1750 d.p.m. respectively for (a) and (b). Results are the means of duplicate experiments.

of conjugates selectively toxic to a given type of cells (Thorpe *et al.*, 1981; Masuho *et al.*, 1982).

The four proteins examined here are glycoproteins. Their different carbohydrate compositions suggest that a specific sugar component is not required for their action on ribosomes, consistent with (i) the lack of detectable sugars in PAP-S (Barbieri *et al.*, 1982) and in the A-chain of abrin (Olsnes, 1978), and (ii) the inhibitory activity of demannosylated gelonin (Stirpe *et al.*, 1980). On the other hand, it cannot be excluded that sugars may

Table 2. *Sugar content of ribosome-inactivating proteins*

Experimental conditions are described in the text. Results are means of at least two determinations on two separate samples of each protein; the s.e.m. was <0.09.

Sugar	Total neutral sugar (%) ...	Content (residues/molecule)			
		<i>Momordica charantia</i> inhibitor	Gelonin	Dianthin 32	Dianthin 30
		1.74	4.46	2.34	1.56
Fucose		0.9	Traces	0.7	Traces
Glucose		0.8	Traces	1.5	1.0
Mannose		1.3	6.9	2.1	0.4
Xylose		0.5	1.1	0.4	1.1
Glucosamine		2.0	1.6	1.4	Traces
Galactosamine		Absent	Absent	Absent	Absent

Fig. 2. *Immunodiffusion of ribosome-inactivating proteins against antisera*

The following rabbit antisera (100 µl) were in the centre well: (a) anti-gelonin; (b) anti-*M. charantia* inhibitor; (c) anti-dianthin 30; (d) anti-dianthin 32. Antigens (50 µg) were: gelonin in well G, *M. charantia* inhibitor in well M, dianthin 30 in well D30 and dianthin 32 in well D32.

have a role in the uptake of ribosome-inactivating proteins by some cells. It should be recalled, in this respect, that gelonin conjugated to pentamannose

6-phosphate acquired toxicity for human normal lymphoid (F-265) and leukaemic (K-562) cells (Forbes *et al.*, 1981).

Table 3. *Effect of antisera on the protein-synthetic inhibitory activity of ribosome-inactivating proteins*

The reaction mixture contained, in a final volume of 125 μ l: 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, 6 μ g of creatine kinase (Boehringer, Mannheim, West Germany), 0.05 mM-amino acids (minus leucine), 0.19 μ Ci of L-[14 C]leucine, 50 μ l of rabbit reticulocyte lysate and, when appropriate, the indicated amount of ribosome-inactivating proteins and 2.5 μ l of antiserum. Incubation was at 27°C for 5 min. The radioactivity incorporated into protein was measured in 25 μ l samples. These results are accurate within $\pm 10\%$.

Ribosome-inactivating protein	Antiserum against	Protein synthesis (% of control)					
		None	Normal rabbit serum	<i>M. charantia</i> inhibitor	Gelonin	Dianthin 32	Dianthin 30
None (control)	...	100*					
<i>M. charantia</i> inhibitor (10 ng/ml)		19.8		100	18.8	38.1	22.9
Gelonin (50 ng/ml)		16.3	13	34.8	67.9	42.2	18.7
Dianthin 32 (25 ng/ml)		7.8		11.3	10.3	69.1	Not tested
Dianthin 30 (50 ng/ml)		13.4		17.4	12.8	Not tested	100

* 1006 d.p.m. incorporated.

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