*Review Letter*

# Ribosome-inactivating proteins up to date

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Received 14 October 1985

Ribosome-inactivating proteins (RIPs) from plants inactivate eukaryotic ribosomes, as far as studied by rendering their 60 S subunit unable to bind elongation factor 2. These proteins seem widely distributed and possibly ubiquitous in plants. They are either type 1, those consisting of a single polypeptide chain, or type 2 (ricin and related toxins), those consisting of two chains, one of which is a galactose-binding lectin. The literature on RIPs from 1982 has been reviewed with respect to (i) the chemical and biological properties of RIPs, (ii) their use for the preparation of immunotoxins and (iii) new perspectives.

*Ribosome-inactivating protein* *Toxin* *Immunotoxin*

## 1. INTRODUCTION

Ribosome-inactivating proteins from plants, reviewed in [1], inactivate eukaryotic ribosomes, as far as studied by rendering their 60 S subunit unable to bind the elongation factor 2. These proteins seem to be widely distributed and possibly ubiquitous in the plant kingdom. Apart from their intrinsic properties, RIPs are attracting considerable interest for their possible use in the construction of 'immunotoxins', namely conjugates with antibodies capable of selectively directing them toward specific cell targets (review in [2]).

## 2. NOMENCLATURE

A provisional nomenclature of RIPs has been proposed [3] pending elucidation of the mechanism of action of these proteins which will allow the adoption of a more appropriate terminology.

We proposed [1] to designate those RIPs existing in nature as single-chain proteins as type 1 and those consisting of an A (active) chain with RIP properties covalently linked to a B (binding) chain with lectin properties as type 2; the latter RIPs can enter cells more easily and are potent toxins (reviewed in [4]).

The term 'hemitoxins' for type 1 RIPs [5] meaning literally 'half-toxins', although elegant, may be misleading, giving the impression that type 1 RIPs may be half-molecules like the subunits of the toxins, whereas they are complete, single-chain proteins.

## 3. GENERAL PROPERTIES

Up to date lists of RIPs type 1 and 2, with their main characteristics, are given in tables 1 and 2, respectively. Type 1 RIPs are single-chain proteins of $M_r$ ~30000 and strongly basic (p*I* often $\geqslant$9.5); most of them are glycoproteins. Type 2 RIPs consist of a ribosome-inactivating A chain of $M_r$ ~30000, p*I* between 4.8 and 8, linked by a single disulphide bond to a heavier B chain which binds sugars with the configuration of D-galactose.

A survey [31] revealed the presence of proteins with the characteristics of type 1 RIPs in most plant materials examined, including seeds, roots, leaves and latices, thus confirming the wide

*Abbreviations:* $ID_{50}$, concentration causing 50% inhibition; PAP, pokeweed antiviral protein (prepared from seeds, PAP-S); RIP(s), ribosome-inactivating protein(s)

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/86/$3.50 

Table 1

Ribosome-inactivating type 1 proteins

| Source | Name | Yield (mg/100 g) | $M_r$ | Sugar content (%) | Inhibition of protein synthesis | | Toxicity to mice ($LD_{50}$) (mg/kg) | Refs |
|---|---|---|---|---|---|---|---|---|
| | | | | | Cell-free[a] ($ID_{50}$) (nM) | Cells[b] ($ID_{50}$) (nM) | | |
| Caryophyllaceae | | | | | | | | |
| *Agrostemma githago* (corn cockle) seeds | agrostin 2 | 8.4 | 30600 | 6.68 | 0.6 | | 1 | [6] |
| | agrostin 5 | 34.2 | 29500 | 6.87 | 0.47 | 9200 | 1 | |
| | agrostin 6 | 18.4 | 29600 | 7.17 | 0.57 | 7800 | 1 | |
| *Dianthus caryophyllus* (carnation) leaves | dianthin 30 | 2 | 29500 | 1.56 | 0.3 | 18000[cd] | | [7] |
| | dianthin 32 | 2 | 31700 | 2.34 | 0.12 | 14000[cd] | 30 | |
| *Saponaria officinalis* (carnation) seeds | saporin 5 | 63 | 29500 | | 0.041 | | | [6] |
| | saporin 6 | 414 | 29500 | absent | 0.037 | 2300 | 4.0 | |
| | saporin 9 | 115 | 29500 | absent | 0.037 | 5400 | 1.7 | |
| Cucurbitaceae | | | | | | | | |
| *Luffa cylindrica* seeds[e] | luffin | 51 | 26000 | | 0.002 | | | [8] |
| *Momordica charantia* (bitter gourd) seeds[e] | momordin | 150–180 | 31000 | 1.74 | 0.06 | 32000 | 4.3 | [9] |
| Euphorbiaceae | | | | | | | | |
| *Gelonium multiflorum* seeds[e] | gelonin | 250–300 | 30000 | 2.34 | 0.4 | 34000 | 40 | [10] |
| *Hura crepitans* (sandbox tree) latex | | 146 | 28000 | 40 | 0.17 | 140 | | [6] |
| Gramineae | | | | | | | | |
| *Hordeum vulgare* (barley) seeds | | 139[f] | 30000 | | 2.13[g] | | | [11] |
| seeds | | 3–4 | 31000 | absent | 0.83 | | | [12] |
| *Secale cereale* (rye) seeds | | 200[f] | 30000 | | 4.0[g] | | | [11] |
| *Triticum aestivum* (wheat) seeds | | 32[f] | 30000 | | 1.87[g] | | | [11] |
| germ | tritin | 3 | 30000 | | 2.3 | | | [13, 14] |
| *Zea mays* (corn) seeds | | 35[f] | 23000 | | 2.13[g] | | | [11] |

Table 1 (continued)

| Source | Name | Yield (mg/100 g) | $M_r$ | Sugar content (%) | Inhibition of protein synthesis | | Toxicity to mice ($LD_{50}$) (mg/kg) | Refs |
|---|---|---|---|---|---|---|---|---|
| | | | | | Cell-free[a] ($ID_{50}$) (nM) | Cells[b] ($ID_{50}$) (nM) | | |
| Liliaceae | | | | | | | | |
| *Asparagus officinalis* (asparagus) seeds | peak 2 | 24 | 32500 | 1.42 | 0.43 | 3100 | | [6] |
| | peak 3 | 8 | 32500 | 1.2 | 0.37 | 3100 | | |
| | peak 5 | 18 | 32500 | 1.32 | 0.17 | 3100 | | |
| Phytolaccaceae | | | | | | | | |
| *Phytolacca americana* (pokeweed) leaves | PAP | 9.2 | 29000 | absent | 0.24 | | | [15] |
| summer leaves | PAP II | 3.6 | 30000 | absent | 0.25 | | | [16] |
| seeds | PAP-S | 100–180 | 31000 | absent | 0.037 | 33000 | 2.6 | [17] |
| *Phytolacca dodecandra* leaves | dodecandrin | 3.4 | 29000 | | 0.043 | | | [18] |

[a] Determined on a rabbit reticulocyte lysate, unless stated otherwise
[b] Determined on HeLa cells, unless stated otherwise; 18 h incubation
[c] EUE cells
[d] F. Stirpe and L. Barbieri (unpublished)
[e] Decorticated seeds
[f] These figures could not be confirmed in our laboratory
[g] Determined on an Ehrlich ascites cell lysate

distribution of RIPs [32–34]. The concentrations of RIPs in seeds vary considerably, ranging from less than 1 to over 100 mg per 100 g. The higher levels were found in the seeds of Caryophyllaceae, Cucurbitaceae, Euphorbiaceae and Phytolaccaceae, although distribution studies were too limited to allow any generalisation.

In a few instances more than one form of RIP has been purified from the same plant; they differ only slightly from each other and can be considered as 'isoforms' of the same protein.

In addition to ricin, abrin, modeccin and viscumin which have been reviewed previously [4] a new toxin, volkensin [29], has been purified from the roots of *Adenia volkensii*, a Passifloracea from Kenya. This toxin is also present in the seeds of the same plant. Volkensin is very similar to the other toxins, particularly to modeccin, the toxin of another Passifloracea, *A. digitata*, although being more toxic than the latter, especially to rats ($LD_{50}$ 60 ng/kg body wt) [30].

Different RIPs cross-react with specific antisera only when they are produced by the same plant or by plants belonging to the same family [11,18,30,35–37]. Partial amino acid sequences showed similarities between RIPs from the same, or from different, unrelated plants and even between type 1 RIPs and the A chain of ricin and modeccin [37–40], suggesting a common genetic origin of these proteins.

## 4. EFFECTS OF RIPs ON PROTEIN SYNTHESIS

### 4.1. *Cell-free systems*

The mechanism of action of RIPs is still unclear.

Table 2

Ribosome-inactivating type 2 proteins (toxins)

| Source | Name | Yield (mg/100 g) | $M_r$ | Sugar content (%) | Inhibition of protein synthesis | | Toxicity to mice ($LD_{50}$) ($\mu$g/kg) | Refs |
|---|---|---|---|---|---|---|---|---|
| | | | | | Cell-free[a] ($ID_{50}$) (nM) | Cells[b] ($ID_{50}$) (nM) | | |
| Euphorbiaceae | | | | | | | | |
| *Ricinus communis* (castor bean) seeds | ricin | 120 | 62057 | 4.5 | 84 | 0.0011[c] | 2.6 | [19–21] |
| | A chain | | 30625 | 2.6 | 0.1 | | | |
| | B chain | | 31432 | 6.4 | | | | |
| Leguminosae | | | | | | | | |
| *Abrus precatorius* (jequirity beans) seeds | abrin | 75 | 65000 | 7.4 | 88 | 0.0037[c] | 0.56 | [22] |
| | A chain | | 30000 | absent | 0.5 | | | |
| | B chain | | 36000 | 7.4 | | | | |
| Loranthaceae | | | | | | | | |
| *Viscum album* (mistletoe) leaves | viscumin | 6.8 | 60000 | 9.7 | 43.3 | 0.008 | 2.4 | [23–25] |
| | A chain | | 29000 | | 3.5[d] | | | |
| | B chain | | 32000 | | | | | |
| Passifloraceae | | | | | | | | |
| *Adenia digitata* roots | modeccin | 20–180 | 63000 | 2.66 | 45 | 0.0003[c] | 2.3 | [26–28] |
| | A chain | | 28000 | | 2.3 | | | |
| | B chain | | 31000 | | | | | |
| *Adenia volkensii* (Kilyambiti) roots | volkensin | 37.5 | 62000 | 5.74 | 84 | 0.0123 | 1.4 | [29,30] |
| | A chain | | 29000 | | 0.37 | | | |
| | B chain | | 36000 | | | | | |

[a] Determined on a rabbit reticulocyte lysate
[b] Determined on HeLa cells; 18 h incubation
[c] F. Stirpe and L. Barbieri (unpublished)
[d] Reduced toxin

They inactivate mammalian 80 S ribosomes in a less than equimolar ratio, and thus a possible catalytic (enzymic) activity can be postulated, but the nature of this activity is unknown. A proteinase activity associated with gelonin has been described but it seems unrelated to the effect on ribosomes [41].

Equally unknown is the nature of the change(s) caused by RIPs in the 60 S subunit of susceptible ribosomes. The extent of ribosomal inactivation depends upon the system used, being greater in a crude reticulocyte lysate than in a poly(U)-directed system containing ribosomes purified from the same lysate [6,7]. A ribosome-associated protein

and a protein present in the reticulocyte lysate seem necessary, under certain conditions, for the action of tritin [42] and of PAP [43], respectively.

Among protozoa, ribosomes from *Tetrahymena pyriformis* [44] and *Acanthamoeba castellani* [45] are insensitive to ricin, although the latter are inactivated by PAP, with an $ID_{50}$ (concentration causing 50% inhibition) of 0.2–0.4 nM (C.L. Villemez, P.L. Carlo and J.D. Irvin, personal communication).

Studies of the effects of RIPs on plant systems showed lower and more variable activities on ribosomes from plants (especially their own species) than on those from animals, ranging from zero to an $ID_{50}$ of 10 nM [11,46–48].

Ribosomes from *Escherichia coli* are unaffected by RIPs; amongst archaebacteria, ribosomes from *Sulfolobus sulfataricus* are unaffected, whereas those from *Thermoplasma acidophilum* are inactivated by dianthin 32 and gelonin, with $ID_{50}$ of 170 and 100 nM, respectively ([49]; and P. Cammarano, personal communication).

### 4.2. *Intact cells*

A feature of type 1 RIPs is their relatively low toxicity to most intact cells (table 1) with the exception of macrophages ($ID_{50} \simeq 10$ nM), as compared with toxins of the ricin type (table 2), due to the absence of a binding B subunit, with consequent poor entry into cells. This was confirmed by the marked cytotoxic effects which are observed when the entry into the cytoplasm was enhanced. This was obtained when RIPs were conjugated with molecules capable of binding to cell membranes (concanavalin A [10], neoglycoproteins [50], mannose 6-phosphate [51], antibodies, see below), or included into structures such as liposomes [52], reconstituted Sendai virus envelopes [53] or erythrocyte ghosts [54] which could be fused with cells (table 3).

The results of a limited study of the effects of RIPs on plant cells in culture were somewhat puzzling. Thus the growth of carrot cells was inhibited by PAP, but was stimulated by ricin and, to a lesser extent, by gelonin. Furthermore gelonin, PAP-S and ricin significantly stimulated the growth of rice cells [48].

Table 3

Cytotoxicity of ribosome-inactivating type 1 proteins attached to or included in carriers

| Carrier | Ribosome-inactivating protein | $ID_{50}$ (nM) | Refs |
|---|---|---|---|
| Concanavalin A | gelonin | 20 | [10] |
| Glycosylated bovine serum albumin | gelonin | 80 | [50] |
| Mannose 6-phosphate | gelonin | 500 | [51] |
| Liposomes | gelonin | 0.01 | [52] |
| Reconstituted Sendai viral envelopes | PAP-S | 0.017 | [53] |
| Erythrocyte ghosts | gelonin | 0.06 | [54] |
| | momordin | 0.03 | |
| | PAP-S | 0.06 | |
| | saporin 6 | 0.002 | |

## 5. IMMUNOTOXINS

Various toxins or their A chains have been conjugated to antibodies, mainly monoclonal, to form immunotoxins specifically toxic to the target cells of the antibody (reviewed in [55]).

After type 1 RIPs became known, their use was proposed as an alternative to the A chains of the toxins [10], the forseeable advantages being their easier and safer preparation, their stability, the lack of contaminating toxins and the availability of a large number of different proteins, with the consequent possibility of circumventing the immune response during long-term in vivo treatment.

The main characteristics of immunotoxins made with type 1 RIPs are summarized in table 4.

Hopefully immunotoxins will be useful in the chemotherapy of cancer and parasitic diseases; they have already been used to remove T-lymphocytes from bone marrow heterografts, thus preventing the graft-versus-host reaction [63].

An unexpected and disturbing observation was that immunotoxins made with saporin 6 and an anti-Thyl.1 antibody or its $F(ab')_2$ fragment were 4–8-fold more toxic to mice than free saporin 6 [62]. This higher toxicity is probably due to the longer persistence of the immunotoxins in the bloodstream, where they remain for some hours, whereas free saporin is quickly excreted through the kidney (unpublished).

Table 4
Immunotoxins made with ribosome-inactivating type 1 proteins

| Ribosome-inactivating protein | Antibody against | Target cells | $ID_{50}$ (nM) | References |
|---|---|---|---|---|
| Gelonin | Thyl.1 | T-lymphocytes | 0.001–0.4 | [56] |
| | | AKR lymphomas | 100–1300 | |
| | | T-lymphoblasts | 100 | |
| | Thyl.2 | EL4 | 3 | [57] |
| | Burkitt's associated glycolipid | Ramos (EL cells) | 0.8 | [58] |
| PAP | L-1210 | L-1210 | 3 | [59] |
| | human B cells | B-ALL | 400 | [5] |
| | Thyl.1 | AKR SL3 | 3–30 | [60,61] |
| | transferrin receptor | human breast tumour (MCF-7) | 0.7 | [39] |
| Saporin 6 | Thyl.1 | T-lymphocytes | 0.1 | [62] |
| | | AKR lymphomas | 0.003–0.03 | |

## 6. EFFECTS OF RIPs ON THE IMMUNE SYSTEM

Macrophages are more sensitive to RIPs than other cells [64]. In view of the role of macrophages in the immune system, the effects of PAP and momordin [64] and subsequently of gelonin [65] on various forms of the immune response were studied. These RIPs all exerted a strong inhibitory effect on the production of antibody-secreting cells in response to the administration of a T-dependent antigen, with no (PAP and momordin) or less effect (gelonin) on the antibody formation against a T-independent antigen. Other effects were investigated separately: PAP-S and momordin significantly delayed the rejection of skin allografts in mice, gelonin depressed the response to *Listeria monocytogenes* and allowed the growth of L-1210 leukaemic cells in incompatible mice. All these effects occurred only if the RIPs were administered to mice some time before, and not together with, or after, the antigens. This indicates that the RIPs must act at a very early stage of the immunity-forming process, affecting a step which is already accomplished when RIPs given together with antigen hit their target(s). This would be consistent with the notion of an immunosuppressive effect mediated through the action of RIPs on macrophages. An apparently enhanced cytotoxic activity of macrophages from mice given PAP-S or momordin [64], which was however not observed in animals receiving gelonin [65], could possibly be due to the toxic action of substances released in vitro by damaged or dying macrophages.

## 7. PERSPECTIVES

Two main unanswered questions on the biological role of RIPs emerge from the existing literature, namely, (i) the nature of their mechanism of action and (ii) the role of these proteins in the physiology of the plants. Obviously, the two questions are related, but unfortunately the numerous efforts of several laboratories have given only vague clues concerning the mode of action of RIPs. Our views on the matter can be summarized as follows.

(i) The fact that no cofactors, donors or acceptors are required for the inactivation of ribosomes by RIPs seems to exclude any type of transfer reaction, such as alkylation, phosphorylation, etc.

(ii) Since no detectable modification of the ribosomal protein or RNA have been reported, the change(s) caused by RIPs in ribosomes are likely to be small. However, the complete arrest of protein synthesis induced by RIPs suggests that the change(s) must occur in a functionally important region in the 60 S ribosomal subunit, probably at,

or near to, the binding site for elongation factor 2. (iii) There must be differences in the specificity or some other characteristics of RIPs, as indicated by the different effect of any given RIP on ribosomes from different plants [48], and by the inactivation of *Ac. castellanii* by PAP but not by ricin ([45] and see above).

RIPs can enter into cells when naturally associated with a carrier (the B chain of a toxin), or when artificially linked to a suitable vector, such as an antibody. Once inside a cell they are highly toxic: it was estimated that one molecule of ricin is sufficient to kill a cell [66].

All RIPs examined so far have antiviral activity against plant [67] or animal viruses [68], but probably they are not natural antiviral agents since crude plant extracts with antiviral and RIP activity do not act against viruses on their own species [67,69].

It has been suggested that RIPs may act as regulators of protein synthesis in plants [63], or that they may eliminate homologous ribosomes when they are altered for any reason [3]. On the other hand, the stimulating effect of RIPs on the growth of some plant cells in culture [48] makes one wonder whether they may play a role in cell multiplication.

The role of RIPs in nature will be revealed when the mechanism of their enzymic action becomes known. Whatever this may be, the wide distribution and abundance of RIPs are suggestive of an important function of these proteins in plants, and one wonders whether equivalent proteins may exist in the animal kingdom.

## ADDENDUM

It has recently been reported that ricin A-chain and PAP, at concentrations of 100 nM or higher, hydrolyze 5 S or 5.8 S rRNA [70].

## ACKNOWLEDGEMENTS

We thank P. Cammarano and C.L. Villemez for permission to quote their unpublished results. The work performed in the authors' laboratory was supported by the Consiglio Nazionale delle Ricerche, Roma, within the Progetti finalizzati 'Controllo della Crescita Neoplastica' and 'Oncologia', by the Ministero della Pubblica Istruzione, Roma, and by the Pallotti's Legacy for Cancer Research.

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