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Ribosome-inactivating proteins from plant cells in culture

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1. Ribosome-inactivating proteins were found in high amounts in one line of cells of *Phytolacca americana* (pokeweed) cultured *in vitro* and, in less quantity, in lines of *Saponaria officinalis* (soapwort) and of *Zea mays* (corn) cells. 2. The main ribosome-inactivating protein from pokeweed cells was purified to homogeneity. It is a protein with M_r 29000 and basic pI, similar to the 'pokeweed antiviral protein' (PAP), a ribosome-inactivating protein from pokeweed leaves. We propose to call the pokeweed antiviral protein isolated from pokeweed cells PAP-C. 3. PAP-C inactivates ribosomes in a less-than-equimolar ratio, thus inhibiting protein synthesis by a rabbit reticulocyte lysate with an IC_{50} (concentration causing 50% inhibition) of 0.067 nM (2 ng/ml), and modifies rRNA in a manner apparently identical to that of ricin and other ribosome-inactivating proteins. It inhibits protein synthesis by intact cells with an IC_{50} of 0.7–3.4 μM, and is toxic to mice with an LD_{50} of 0.95 mg/kg.

INTRODUCTION

Single-chain ribosome-inactivating proteins (RIPs type 1, as opposed to two-chain RIPs type 2, reviewed in refs. [1–4]), so far isolated only from plants, catalytically inactivate the 60S subunit of eukaryotic ribosomes, thus arresting protein synthesis. The mechanism of action of RIPs has recently been elucidated: they have an unusual *N*-glycosidase activity and cleave the glycosidic bond of adenine-4324 of 28S rRNA [5–8], rendering the latter cleavable by aniline.

RIPs are frequent in nature and are present in several parts of many plants belonging to unrelated families, sometimes in unusually high concentrations [9–11].

The aim of the present research was to ascertain whether RIPs could be produced by cultured plant cells *in vitro*. Cells from four RIP-producing plants, namely *Phytolacca americana* (pokeweed), *Saponaria officinalis* (soapwort), *Dianthus caryophyllus* (carnation) and *Zea mays* (corn) were examined. It was observed that an RIP analogous to the 'pokeweed antiviral protein' (PAP, reviewed in ref. [12]) was present in more than one form and in sufficient quantities for purification, in one of two lines of pokeweed cells examined. A much lower amount of RIPs was found in another line of pokeweed cells and in lines of soapwort and corn cells, whereas a line of carnation cells did not produce any RIP. Addition of phytohormones to the cultures strongly influenced their RIP activity, although in a quite different manner with pokeweed and soapwort cells.

The main form of RIP produced by pokeweed cells was purified and partially characterized. This RIP, that we propose to call pokeweed antiviral protein from cell cultures (PAP-C), appears to be very similar, but not identical to, one of the forms of PAP purified from pokeweed leaves.

EXPERIMENTAL PROCEDURES

Materials

L-[^{14}C]Leucine and L-[^{14}C]phenylalanine were from Amersham International, Bucks., U.K. Materials for chromatography were from Pharmacia AB, Uppsala, Sweden. Poly(U) and tRNA were from Sigma Chemical Co., St. Louis, MO, U.S.A. Phenylalanine-tRNA was prepared as described by Hultin & Näslund [13]. All other chemicals were as in previous work [14]. PAP and PAP II were a gift from Dr. J. D. Irvin, San Marcos, TX, U.S.A. and were further purified as described below. PAP-S was prepared as described by Barbieri *et al.* [15]. Cloned ricin A-chain and α-sarcin were kindly given by Dr. J. D. Robertus, Austin, TX, U.S.A.

Plant cell cultures

Cell cultures, prepared from leaf tissue, were grown either as calli or in suspension. Calli were grown at 28 °C for 3–4 weeks in the dark on a solid Gamborg's B5 medium [16] containing naphthalene acetic acid (1 mg/l), kinetin (1 mg/l), 2,4-dichlorophenoxyacetic acid (0.2 mg/l) and Difco agar (7 g/l). To avoid trace contamination of phytohormones from the inoculum, callus cultures were subcultured three times on solid Gamborg's B5 medium supplemented with the appropriate hormone before evaluating the effects of light and hormones. To prepare suspension cultures, approx. 1 g of callus tissue was suspended and inoculated into 350 ml flasks with 50 ml of Gamborg's B5 medium supplemented with naphthalene acetic acid (1 mg/l),

Abbreviations used: RIP(s), ribosome-inactivating protein(s); PAP, pokeweed antiviral protein; PAP II, second form of pokeweed antiviral protein, obtained from pokeweed summer leaves; PAP-C, pokeweed antiviral protein from cell cultures; PAP-S, pokeweed antiviral protein from seeds; IC_{50}, concentration giving 50% inhibition.

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kinetin (1 mg/l) and 2,4-dichlorophenoxyacetic acid (0.2 mg/l). The cultures were incubated at 28 °C in the dark, with constant agitation (120 rev./min) for 20 days.

Preparation of plant cell extracts and purification of RIP

All operations were performed in a closed fume cupboard to avoid allergy to plant materials. Plant cells grown in suspension cultures were collected by centrifugation at 1500 *g* for 15 min and calli were detached from the solid medium by scraping. Cells were used immediately or were stored at −20 °C. Cells were homogenized twice with 5 vol. (5 ml when the material was 1 g or less) of ice-cold acetone. The resulting powder was collected on filter paper on a Büchner funnel and dried in the air. Yield was approx. 3–5% of cell wet weight. The dried powder was stirred overnight at 4 °C with enough phosphate-buffered saline (0.14 M-NaCl containing 5 mM-sodium phosphate buffer, pH 7.5) to obtain a liquid slurry (10–100 ml/g of powder). The suspension was centrifuged at 10000 *g* for 45 min at 2 °C, and the sediment was extracted again with phosphate-buffered saline; the combined supernatants are called 'crude extract'.

RIPs from extracts of *P. americana* cells were purified essentially by the procedure of Barbieri *et al.* [15] by chromatography on S-Sepharose, gel filtration on Sephacryl S-200 (instead of on Sephadex G-50) and chromatography on CM-Sepharose, to which a final chromatography on a Cibachrome Blue-Sepharose column was added [17,18]. The active protein obtained is called pokeweed antiviral protein from cells (PAP-C).

Analytical methods

M_r values were determined by the polyacrylamide-gel-electrophoresis method of Laemmli [19] and by gel filtration through a column (95 cm × 1.6 cm) of Sephacryl S-200, as described previously [14].

The isoelectric point and the amino acid, amino sugar and neutral sugar compositions of the protein were determined as described by Falasca *et al.* [20]. *N*-Terminal sequence was determined by the method described by Lappi *et al.* [21]. Protein was determined by the method of Lowry *et al.* [22], with bovine serum albumin (Sigma) as a standard, or spectrophotometrically [23].

Protein synthesis was measured as described previously [14]: (i) with a rabbit reticulocyte lysate; (ii) as poly(U)-directed phenylalanine polymerization, with rabbit reticulocyte ribosomes; and (iii) with cultured human cell lines *in vitro* (TG, oviduct carcinoma; JAR, chorion-carcinoma; NB 100, neuroblastoma; HeLa; and fibroblasts).

The modification of rRNA induced by PAP-C was detected according to Endo *et al.* [5], as described by Stirpe *et al.* [8].

Toxicity experiments

The toxicity of the purified protein was evaluated in Swiss female mice weighing 27–32 g, fed *ad libitum*. The protein, dissolved in 0.9% NaCl, was injected intraperitoneally at six different dose levels ranging from 20 to 0.31 mg/kg of body weight, with a ratio between doses of 2, to groups of six animals for each dose. An acute and an acute-delayed LD_{50} were calculated at 48 h and 14 days, respectively, by the linear-regression method. From dead animals the following organs were removed and were fixed in 3.7% formaldehyde in saline and embedded in paraffin: heart; intestine; kidney; liver; lung; pancreas; spleen. Sections were cut and stained with haematoxylin-eosin for light microscopy.

RESULTS

Effects of plant cell extracts on cell-free protein synthesis

The presence of RIPs in cell extracts was detected from their inhibitory activity on protein synthesis by a rabbit reticulocyte lysate (Table 1). A strong activity was obtained from pokeweed cell line no. 42, whereas pokeweed cell line no. 39 and lines from other RIP-producing plants had much less activity (*S. officinalis* and *Z. mays*) or were inactive (*D. caryophyllus*).

No significant differences were observed in the specific activity of extracts from pokeweed cell line no. 42 grown in suspension and harvested at various times after the inoculum, or from cells grown as calli (results not shown). No significant activity was detected in the culture media in which pokeweed or soapwort cells were grown in suspension.

The effect of factors affecting cell growth on the inhibitory activity was studied (Table 2). In the case of pokeweed cells maximum activity was obtained under conditions of light, and in the presence of a mixture of hormones ensuring optimal growth. In the case of soapwort cells, maximum activity was obtained from cells grown in the light in the presence of benzyladenine or kinetin, and in the darkness without any addition or in the presence of benzyladenine.

The inhibitory activity of cell extracts appeared due to a basic protein, since it was retained by an S-Sepharose column and could be eluted with 1 M-NaCl at pH 7.5. On polyacrylamide-gel electrophoresis these eluates showed a protein band with mobility corresponding to M_r 30000 (results not shown). These properties are consistent with those of RIPs [4].

Table 1. Inhibitory activity of crude extracts from plant cell lines on protein synthesis by a rabbit reticulocyte lysate

Reaction mixtures for protein synthesis 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-aminoacids (minus leucine), 89 nCi of L-[^{14}C]leucine, and 25 μl of a rabbit reticulocyte lysate prepared as described by Allen & Schweet [24]. Incubation was at 28 °C for 5 min. The reaction was arrested with 1 ml of 0.1 M-KOH, and the radioactivity incorporated into protein was measured [25]. One unit is defined as the amount of protein necessary to inhibit protein synthesis by 50% in 1 ml of rabbit reticulocyte lysate reaction mixture.

Cell lines	Specific activity (units/mg of protein)
Dianthus caryophyllus	Inactive
Phytolacca americana no. 42	20000, 38500, 50000
Phytolacca americana no. 39	Inactive, 200
Saponaria officinalis	1400, 3030, Inactive, 690
Zea mays	148

Table 2. Effect of phytohormones and light on the growth and on RIP activity of callus cultures from *Phytolacca americana* and *Saponaria officinalis*

Culture conditions are described in the text. One unit of activity is defined as in the legend to Table 1. Growth was evaluated by visual inspection and scored from − to +++++.

Cell lines +conditions	Light		Darkness	
	Growth	RIP activity (units/mg of protein)	Growth	RIP activity (units/mg of protein)
Phytolacca americana (42*) hormones				
None	−	11628	−	3774
Benzyladenine (1 mg/l)	+	15385	++	7092
Kinetin (1 mg/l)	+	11111	++	23809
Naphthaleneacetic acid (1 mg/l)	−	2242	−	500
2,4-Dichlorophenoxyacetic acid (1 mg/l)	+	2475	+	5650
Kinetin (1 mg/l)+naphthaleneacetic acid (1 mg/l)+2,4-dichlorophenoxyacetic acid (0.2 mg/l)	+++	50000	+++	33333
Saponaria officinalis (79*) hormones				
None	−	3155	−	5525
Benzyladenine (1 mg/l)	+++++	5917	+++	6060
Kinetin (1 mg/l)	+	6098	+	3205
Naphthaleneacetic acid (1 mg/l)	+++	1297	+++	700
2,4-Dichlorophenoxyacetic acid (1 mg/l)	+++	1064	+++	1127
Kinetin (1 mg/l)+naphthaleneacetic acid (1mg/l)+2,4-dichlorophenoxyacetic acid (0.2 mg/l)	+++	690	+++	309

* No. of the Farmitalia Carlo Erba collection.

Table 3. Purification of pokeweed antiviral protein from cells

The purification procedure is described in the text and the ion-exchange chromatography is shown in Fig. 1. The starting material was 0.94 kg (wet weight) of *Phytolacca americana* cells (no. 42 of the Farmitalia Carlo Erba collection). Conditions for cell-free protein synthesis assay to determine RIP activity are described in the legend to Table 1. One unit of activity is defined as in the legend to Table 1.

	Protein (g)	IC_{50} (ng/ml)	Specific activity ($10^{-3}\times$ units/mg of protein)	Total activity ($10^{-6}\times$ units)	Yield (%)
Crude extract	7.510	26.0	38.5	289	(100)
Crude acidified	7.140	19.4	51.5	368	127
S-Sepharose	2.380	6.5	153.8	366	127
Sephacryl S-200	0.910	6.2	162.3	148	51
CM-Sepharose	0.225	2.2	444.4	100	35
Cibachrome Blue-Sepharose	0.164	2.0	490.2	80	28

Purification of pokeweed antiviral protein from cells

From the active extract of pokeweed cells an RIP was purified as summarized in Table 3. After acidification the total activity recovered was 30% higher than that of the crude extract. A similar phenomenon was observed during the purification of PAP-S (PAP isolated from seeds) [26] and of other RIPs [15], and could be due to the removal of an inhibitor or to a modification of the RIPs. At least three active protein peaks were separated by chromatography on the CM-Sepharose column (Fig. 1). The most abundant one was further purified on Cibachrome Blue-Sepharose, was characterized, and is referred to as pokeweed antiviral protein from cell culture (PAP-C).

Physico-chemical properties of PAP-C

The M_r, pI and amino acid composition of PAP-C are shown in Table 4. The protein has a similar M_r to that for the previously identified PAP. The amino acid

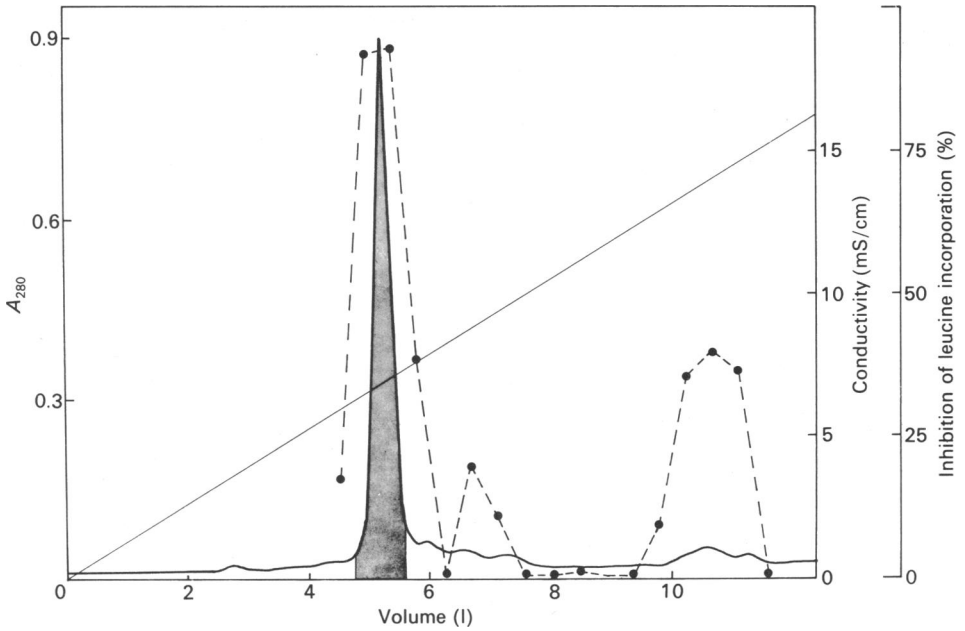

Fig. 1. Purification of pokeweed antiviral protein from cells (PAP-C) by using CM-Sepharose chromatography

Pooled active fractions from S-Sepharose after gel filtration were loaded on to a CM-Sepharose Fast Flow column (5 cm × 30 cm) equilibrated with 5 mM-sodium phosphate buffer, pH 7. The column was sequentially eluted with the equilibration buffer until the A_{280} was practically zero, and then with a 0–300 mM-NaCl linear gradient in the same buffer (total volume 20 litres) at 20 °C, at the rate of 1.2 litres/h. A_{280} (thick line) and conductivity (thin line) were recorded. Fractions of 450 ml were collected and tested for protein-synthesis inhibition activity in the lysate system, as described in the legend to Table 1 at a 1:20000 dilution (broken line). Shaded area indicates active fractions that were collected for further purification.

Table 4. Physico-chemical properties of pokeweed antiviral protein from cells (PAP-C) compared with pokeweed antiviral protein from leaves (PAP)

Values for amino acids are averages of determinations obtained after hydrolysis for 24, 48 and 72 h. Values for serine and threonine were obtained by extrapolation to zero time hydrolysis. Other experimental conditions are described in the text. n.d., not determined.

Property	PAP-C	PAP
M_r		
By gel filtration	22000	n.d.
By gel electrophoresis	29000	29000
pI	⩾ 9.5	⩾ 9.5
Amino acid composition (mol/mol)*		
Lys	17.6	19.1
His	1.7	2.6
Arg	9.8	10.8
Asx	30.7	28.8
Thr	16.9	17.0
Ser	15.1	16.7
Glx	21.4	21.5
Pro	12.2	11.1
Gly	12.7	14.0
Ala	13.0	14.7
½ Cys	3.8	2.2
Val	17.8	16.7
Met	3.3	2.3
Ile	13.9	13.5
Leu	20.2	20.8
Tyr	7.8	7.3
Phe	7.4	6.9
Trp	n.d.	n.d.

* An M_r of 29000 has been assumed.

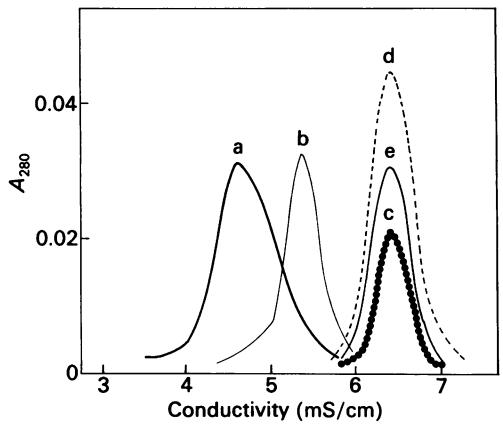

Fig. 2 Chromatographic behaviour of pokeweed antiviral proteins on Cibachrome Blue-Sepharose

Proteins [PAP-S, 10 mg a; PAP II, 10 mg b, PAP, 5 mg c, PAP-C, 10 mg d, PAP, 2.5 mg + PAP-C, 5 mg e] dissolved at 5 mg/ml in 10 mM-Tris/HCl, pH 8.0, were applied separately onto a Cibachrome Blue-Sepharose column (5 cm × 20 cm) equilibrated with the same buffer. The column was washed with 1 vol. of the equilibration buffer and was eluted with a 0–200 mM-NaCl linear gradient in the same buffer (total gradient volume 4 litres), at 25 °C, at the rate of 235 ml/h. A_{280} and conductivity at 25 °C were recorded.

composition shows only slight differences compared with PAP, analysed at the same time. Like other forms of PAP, PAP-C contains only traces of glucose. The partial *N*-terminal sequence of PAP-C (Table 5) is identical to that of PAP, and shows numerous homologies with the sequences of the other forms of PAP and of dodecandrin,

Table 5. Sequence homology between pokeweed antiviral proteins

The protein sequences were aligned to maximize similarities between the proteins.

	5	10	15	20	25	30	35	40
PAP-C	V N T I I Y N V G S T T I S K Y A T F L N D L R N E A - K D P S L K X Y G I P M? L P N T?							
PAP*	V N T I I Y N V G S T T I S K Y A T F L N D L R N E A - K D P S L							
PAP II*	N - I V F D V E N A T P E T Y S N F L T S L R - E A V K D K L T							
PAP-S*	I N T I T E D A G H A T I N K Y A T F M E S L X N E - - K D							
Dodecandrin†	V N T I I Y N V G S T T I S N Y A T F M D N L R N E A - K D P S L							

* From Bjorn *et al.* [27]; † from Ready *et al.* [28].

the RIP isolated from the leaves of another *Phytolaccacea*, *Phytolacca dodecandra* [28]. On chromatography on Blue Sepharose, PAP and PAP-C were eluted at the same ionic strength, and upon co-chromatography they appeared as a single symmetrical peak (Fig. 2). Similarly, PAP-C and PAP co-migrated in electrophoresis, whilst PAP II and PAP-S showed a slightly slower migration (Fig. 3).

The $A^{0.1\%}_{280,1\,cm}$ of PAP-C in 0.14 M-NaCl containing 5 mM-sodium phosphate buffer, pH 7.5, was 0.8.

Biological properties of PAP-C

Effect on ribosomal RNA. After incubation with PAP-C, the reticulocyte rRNA could be cleaved by aniline with the production of a fragment indistinguishable in size from that produced by ricin (Fig. 4).

Cell-free systems. PAP-C was a potent inhibitor of protein synthesis by a rabbit reticulocyte lysate, with an IC_{50} of 0.067 nM (2.0 ng/ml) (Table 3), as well as of poly(U)-directed polyphenylalanine synthesis by purified reticulocyte ribosomes (Fig. 5). The concentration of ribosomes in the lysate assay was 31.3 nM, as determined from the ribosomes recovered by centrifugation of the lysate and measured as described by Montanaro *et al.* [29]. Thus one molecule of PAP-C inactivated 244 ribosomes in 5 min (assuming a complete recovery of totally active ribosomes).

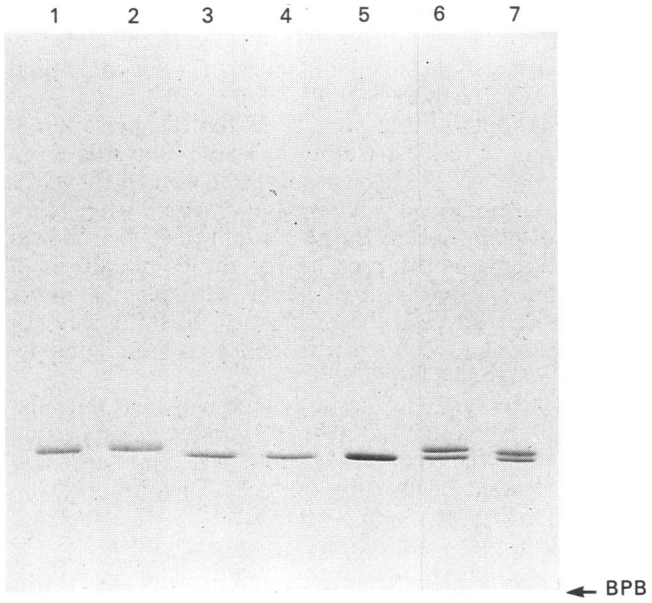

Fig. 3. Gradient electrophoresis of the known forms of pokeweed antiviral proteins

Electrophoresis was performed as described by Laemmli [19] in a gradient of polyacrylamide (7–18%). Migration was from top to bottom and the Bromophenol Blue front is indicated (BPB). The gel was loaded with 2 μg of each protein. Lane 1, PAP-S; lane 2, PAP II; lane 3, PAP; lane 4, PAP-C; lane 5, PAP-C + PAP; lane 6, PAP-C + PAP II; lane 7, PAP-C + PAP-S.

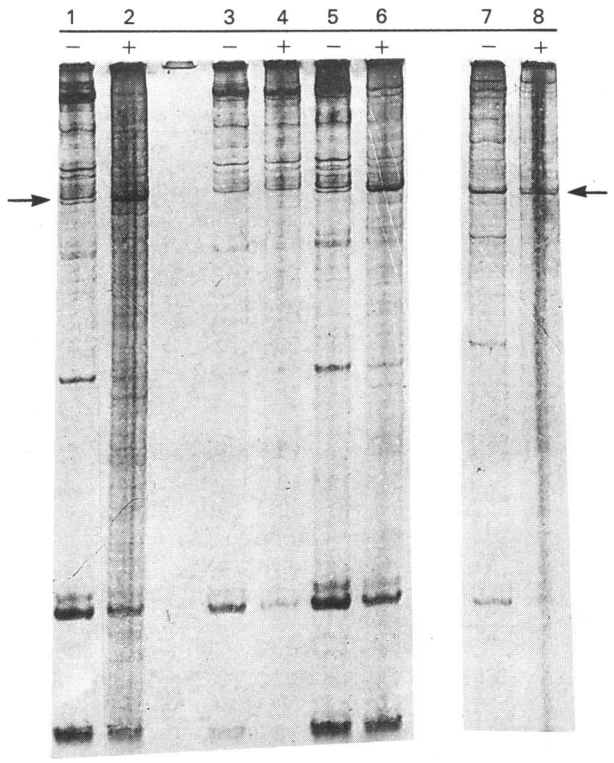

Fig. 4. Gel electrophoretic analysis of rRNA from reticulocyte lysates inactivated by RIPs

The RIPs were present at a concentration of 1 μg/ml as follows: lanes 1 and 2, PAP-C; lanes 3 and 4, none (controls); lanes 5 and 6, ricin A-chain; lanes 7 and 8 α-sarcin. Samples in the lanes designated with a (−) were not treated, and those designated with a (+) were treated with aniline. The arrows indicate the rRNA fragments cleaved by aniline from RIPs-treated ribosomes or by α-sarcin.

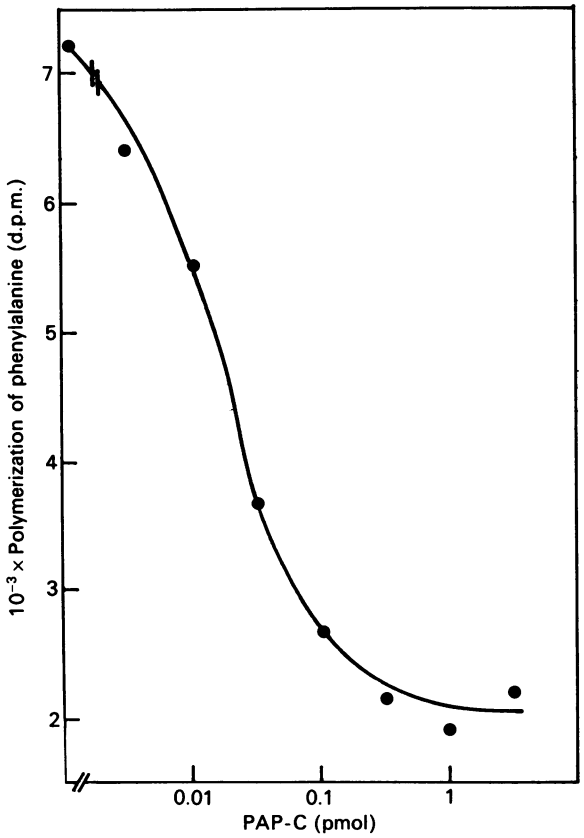

Fig. 5. Effect of PAP-C on phenylalanine polymerization by purified reticulocyte ribosomes

Assays were done with duplicate samples containing in a final volume of 0.125 ml: 80 mM-Tris/HCl buffer, pH 7.4, 120 mM-KCl, 7 mM-magnesium acetate, 2 mM-GTP, 100 μg of poly(U), 125 μg (as protein) of 'pH 5 supernatant', 25 pmol of [^{14}C]phenylalanyl-tRNA and 20 pmol of reticulocyte ribosomes calculated as described in ref. [29]. After incubation at 30 °C for 30 min, 125 μl of 10% (w/v) trichloroacetic acid was added, and the acid-insoluble radioactivity was determined as described by Montanaro *et al.* [29]. Control values were 7194 d.p.m.

Animal cells. Protein synthesis by intact cells was also inhibited by PAP-C, although at concentrations three orders of magnitude higher than those effective on cell-free systems, and with some differences from one cell line to another (Table 6). Except in the case of fibroblasts, the effects of PAP-C were comparable with those of PAP-S, assayed at the same time.

Toxicity. Most mice injected with lethal doses of PAP-C died within 48 h of injection, but some surviving after this time continued to lose weight and died within 2 weeks. The LD_{50} values calculated from the number of deaths occurring within 48 h and 14 days were 1.89 mg/kg and 0.95 mg/kg, respectively.

Dead mice showed gross signs of fatty degeneration and necrosis in the liver and, occasionally, of inflammation of the bowel accompanied by a moderate ascites. Histologically, massive liver necrosis was observed, especially of the parenchymal cells; caryorexis and caryolysis were present, and all other nuclei had condensed chromatin granules near the nuclear membrane. Scattered focal necrosis of the red pulp of the spleen was observed; evident lesions were absent in the other organs examined.

Animals which survived doses around the LD_{50} regained weight within 2 weeks, and at that time only the liver showed histological alterations, with residues of necrotic tissue and vacuoles in the cytoplasm of the hepatocytes.

Table 6. Effect of PAP-C on protein synthesis by various human cell lines compared with that of PAP-S

Experimental conditions are described in the text.

Cell type	IC_{50} (μM) PAP-C	PAP-S
TG	1.1	2.0
Fibroblasts	0.9	0.2
JAR	1.6	3.3
HeLa	3.4	3.4
NB 100	0.7	0.5

DISCUSSION

The cell cultures examined in present research derived from plants containing comparable levels of RIPs in their leaves (pokeweed, carnation, and soapwort) or seeds (corn). It was therefore surprising to observe that a high RIP activity was present in only one of the two lines derived from pokeweed, whereas much less or no activity at all was detectable in the other lines.

The non-producing lines are apparently as viable as the producing line. This suggests that a high production of RIP is not essential for the life of plant cells and may reflect variations between cell lines or even individual plants in the production of RIPs.

It seems that cultural conditions that ensure optimal production of RIPs are not the same for cells from different plant species, as it is suggested also by the effect of phytohormones on pokeweed and soapwort cells. In the case of pokeweed cells, the amount of RIP produced was maximum in the presence of the combination of factors that ensured optimal cell growth, whereas in the case of soapwort cells the production of RIPs was not strictly related to cell growth, and was stimulated by benzyladenine and kinetine.

No protein-synthetic inhibitory activity was detected in the growth medium of RIP-producing cells, suggesting that RIPs may be constitutive proteins of plant cells and are not secreted or shed by the cells. This differs from other proteins, such as soya-bean lectin, which is released in the culture medium of soya-bean cells [30].

Pokeweed cells cultured *in vitro* produced at least three protein-synthesis inhibiting proteins, which are presumably different forms of the same RIP as observed in leaves and seeds of pokeweed and of other plants [26,31,32]. The most abundant protein (PAP-C) was purified; it appears homogeneous by several criteria, has all the properties of an RIP, and seems very similar, although not identical, to the other previously known RIPs purified from pokeweed [26,31,32] and particularly to PAP [31].

Since pokeweed is a common weed, and the yield of

the various forms of PAP is good, it would be uneconomical to purify PAP from cultured cells rather than from leaves or seeds. However, cell cultures are available throughout the year and, compared with seeds, are a better source of mRNA required for cloning. Moreover, the possibility of producing RIPs from plant cell cultures would be valuable in the case of RIPs present in plants that are difficult to obtain or to grow.

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