Purification and partial characterization of another form of the antiviral protein from the seeds of *Phytolacca americana* L. (pokeweed)

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1. The pokeweed antiviral protein, previously identified in two forms (PAP and PAP II) in the leaves of *Phytolacca americana* (pokeweed) [Obrig, Irvin & Hardesty (1973) *Arch. Biochem. Biophys.* **155**, 278–289; Irvin, Kelly & Robertus (1980) *Arch. Biochem. Biophys.* **200**, 418–425] is a protein that prevents replication of several viruses and inactivates ribosomes, thus inhibiting protein synthesis. 2. PAP is present in several forms in the seeds of pokeweed. One of them, which we propose to call 'pokeweed antiviral protein from seeds' (PAP-S) was purified in high yield (180 mg per 100 g of seeds) by chromatography on CM-cellulose, has mol.wt. 30 000, and is similar to, but not identical with, PAP and PAP II. 3. PAP-S inhibits protein synthesis in a rabbit reticulocyte lysate with an ID_{50} (concentration giving 50% inhibition) of 1.1 ng/ml (3.6×10^{-11} M), but has much less effect on protein synthesis by whole cells, with an ID_{50} of 1 mg/ml (3.3×10^{-5} M), and inhibits replication of herpes simplex virus type 1.

The pokeweed antiviral protein ('PAP') (Obrig *et al.*, 1973; Irvin, 1975) and the related form PAP II (Irvin *et al.*, 1980) were purified from the leaves of *Phytolacca americana* (pokeweed). These proteins inhibit the replication of plant and animal viruses (Wyatt & Shepherd, 1969; Ussery *et al.*, 1977; Aron & Irvin, 1980; Grasso *et al.*, 1980; Irvin *et al.*, 1980) and act by inactivating the 60S ribosomal subunit, thus inhibiting protein synthesis (Obrig *et al.*, 1973). Hence PAP and PAP II act in an apparently identical manner to the A chains of ricin and abrin (Olsnes & Pihl, 1977) and other single-chain proteins isolated from various plant materials [tritin from wheat germ (Roberts & Stewart, 1979; Coleman & Roberts, 1981), *Momordica charantia* (bitter melon) inhibitor, from the seeds of the same plant (Barbieri *et al.*, 1980), gelonin, from *Gelonium multiflorum* seeds (Stirpe *et al.*, 1980), dianthins, from *Dianthus caryophyllus* (carnation) leaves (Stirpe *et al.*, 1981)].

A crude extract of *P. americana* seeds had a strong inhibitory effect on protein synthesis in a cell-free system, which suggested the presence of PAP or of a related protein (Gasperi-Campani *et al.*, 1977). The present paper describes the purification

from *P. americana* seeds of another form of PAP, similar to, but not identical with, PAP and PAP II, which we propose to call 'pokeweed antiviral protein from seeds' ('PAP-S').

Experimental

Materials

Pokeweed seeds, collected in Bologna, were washed out of the berries and were stored at room temperature in the presence of silica gel. CM-cellulose (CM 52) was obtained from Whatman, Maidstone, Kent, U.K., and Bio-Gel P-100 from Bio-Rad Laboratories, Richmond, CA, U.S.A. Chemicals for protein synthesis were purchased from the same sources as in previous work (Gasperi-Campani *et al.*, 1977). Protein markers for molecular-weight determinations were purchased from Boehringer Mannheim, Mannheim, Germany (markers for polyacrylamide-gel electrophoresis) or from Pharmacia Fine Chemicals, Uppsala, Sweden (markers for gel filtration). Ricin and abrin (abrin C) were prepared as described by Nicolson *et al.* (1974) and by Wei *et al.* (1974) respectively, and their A and B chains were dissociated by reduction as described by Olsnes & Pihl (1972).

Anti-PAP rabbit serum prepared as described previously (Irvin *et al.*, 1980) was kindly given by Dr. J. D. Robertus, The Clayton Foundation Biochemical Institute, Austin, TX, U.S.A.

Abbreviation used: PAP, pokeweed antiviral protein; SDS, sodium dodecyl sulphate.

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Polyacrylamide-gel electrophoresis and determination of molecular weight and of isoelectric point

Proteins were analysed by SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Molecular weights were determined by SDS/polyacrylamide-gel electrophoresis as described by Weber & Osborn (1969) with the following markers (molecular weights are given in parentheses): bovine serum albumin (68 000), aprotinin (52 000) and B (35 000) chains, ricin A (32 000) and B (34 000) chains and soya-bean trypsin inhibitor (21 500). Molecular weight of the major fraction of PAP-S (see below) was determined also by gel filtration through a column (100 cm $\times$ 2 cm) of Bio-Gel P-100, equilibrated with 0.3 M-NaCl containing 5 mM-sodium phosphate buffer, pH 7.2, and eluted at the rate of 14 ml/h at room temperature. The following standards were used: bovine serum albumin (67 000 mol.wt.), ovalbumin (43 000), chymotrypsinogen A (25 000) and ribonuclease A (13 700).

The isoelectric point was determined by electrofocusing 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.

Amino acid analysis

Samples of PAP-S were hydrolysed at 110°C with 6 M-HCl for 24 h in sealed evacuated tubes. Triplicate samples containing 2 nmol of protein were separated and quantified on a Durrum amino acid analyser.

Protein synthesis

Protein synthesis was determined with a lysate of rabbit reticulocytes as described previously (Stirpe *et al.*, 1981) or with HeLa cells; details are given in the legends to appropriate Tables and Figures.

Toxicity experiments

The toxicity of purified PAP-S was evaluated in female Swiss mice, weighing 25 g. Animals received food and water *ad libitum*. The protein, dissolved in 0.9% NaCl, was injected intraperitoneally at doses ranging from 1 to 50 mg/kg of body wt., with a ratio between doses of 1.778. LD₅₀ was evaluated by the method of Spearman–Kärber as described by Finney (1964).

Virus multiplication

African-green-monkey kidney cells (Vero cells) monolayers at 1×10^5 – 2×10^5 cells per plate were infected with herpes simplex virus type 1, strain KOS, at a multiplicity of 10 plaque-forming units per cell in the presence or in the absence of PAP-S. Infected cells were incubated at 37°C and virus yields were determined at 24 h post infection as described by Aron & Irvin (1980).

Other determinations

Sugar analysis was performed by gas chromatography as described by Dunstan *et al.* (1974) with D-mannitol as a standard.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as a standard, or spectrophotometrically (Kalb & Bernlohr, 1977).

Radioactivity was measured as described by Gasperi-Campani *et al.* (1977).

Results

Purification of pokeweed anti-viral protein from seeds

Pokeweed seeds (200 g) were ground in a blender for 10 min with 1.5 litres of 0.2 M-NaCl/5 mM-sodium phosphate buffer, pH 7.2. The homogenate was left overnight at 2–4°C on a magnetic stirrer, and was then centrifuged at 7000 g for 30 min at 2°C. A pink supernatant was separated from the sediment and from a floating layer of solidified fat, and was filtered through filter paper (Rapid A; Cartiera Galvani, Cordenons, Italy). The filtrate was dialysed for 48 h against 10 litres of 5 mM-sodium phosphate buffer, pH 6.5, with two changes of buffer. Any precipitate formed during dialysis was centrifuged off and discarded. The dialysed supernatant from 50 g of seeds (315 ml) was filtered through filter paper and was applied to a column (17 cm $\times$ 4 cm) of CM-cellulose (CM 52), previously equilibrated with buffer. The column was washed with 350 ml of buffer and the retained material was eluted with a 3-litre linear gradient of 0–0.3 M-NaCl in the same buffer. The

Fig. 1. Purification of the pokeweed antiviral protein from seeds

The dialysed extract (315 ml) from 50 g of pokeweed seeds was chromatographed on a column of CM-cellulose (CM 52) as described in the text. Fractions (19 ml) were eluted with a 0–0.3 M-NaCl gradient (●) and their A_{280} was monitored (—). The inhibitory activity on protein synthesis (○) was assayed in the lysate system described in the text with 50 μ l of 1:300 000 dilution of the fractions. Active fractions were pooled as indicated by the horizontal bars.

Table 1. *Purification of the pokeweed antiviral protein from seeds*

Experimental conditions are described in the text. Peaks are identified as in Fig. 1. A unit of activity is defined as the amount giving 50% inhibition of protein synthesis in the reticulocyte lysate system. Data refer to 100g of seeds.

Preparation	Total protein (mg)	$10^{-6} \times$ Total activity (units)	$10^{-5} \times$ Specific activity (units/mg of protein)	Recovery (%)
Crude extract	7182	172	0.24	(100)
Dialysed extract	4724	606	1.28	352
CM-cellulose peaks				
a	98	8.4	0.86	5
b	86	13.6	1.59	8
c	180	72.0	4.00	42
d	174	20.4	1.18	12
e	94	13.4	1.43	8
f	134	27.8	3.57	16

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Fig. 2. *Polyacrylamide-gel electrophoresis of the pokeweed antiviral proteins*

Proteins were analysed by SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Stained gels were scanned at 610nm with a Zeiss PM 6 spectrophotometer equipped with a gel scanner. Gels contained: (a) 9 μ g each of PAP and PAP-S; (b) 9 μ g each of PAP II and PAP-S; (c) 12 μ g each of PAP and PAP II.

chromatography was performed at room temperature (23–27°C).

Diluted samples of the fractions were added to the lysate system for protein synthesis, and several peaks of inhibitory activity were observed (Fig. 1).

Table 2. *Molecular weight and isoelectric point of pokeweed antiviral protein from seeds*
Peaks are identified as in Fig. 1.

CM-cellulose peak	Molecular weight	Isoelectric point
a	31000	8.30
b	31000	8.30, 8.45
c	31000	8.45
d	30000	8.70
e	31000	8.65, 8.75
f	30000	8.80

Peak c had the highest specific inhibitory activity and was studied in detail as PAP-S. The yield of inhibitory activity is given in Table 1. The activity recovered after dialysis of the extract was more than 100%, a phenomenon previously observed during the purification of similar proteins (Barbieri *et al.*, 1980; Stirpe *et al.*, 1981).

On polyacrylamide-gel electrophoresis all peaks with inhibitory activity on protein synthesis (Fig. 1) showed a single protein band with a molecular weight near to 30000. The band of peak c (PAP-S) was distinct from that of PAP, but coincided with that of PAP II (Fig. 2).

Peak c was eluted as a single peak from the Bio-Gel column, with apparent mol.wt. 28600.

On isoelectric focusing, material under peaks b and e showed two bands, and other peaks showed a single band, all with isoelectric points above 8. The molecular weights and isoelectric points of the various peaks are reported in Table 2. These data suggest that peak b may contain a mixture of the proteins of peaks a and c.

The amino acid composition of PAP-S (Table 3) is similar to those previously reported for PAP and PAP II (Irvin *et al.*, 1980). If any difference can be deduced, PAP-S seems closer to PAP than to PAP II.

Table 3. *Amino acid composition of pokeweed antiviral protein from seeds*

Data refer to peak c of Fig. 1. Experimental conditions are described in the text.

Amino acid	Composition (mol/mol of protein)
Asx	34.3
Thr	13.9
Ser	15.0
Glx	29.0
Pro	12.1
Gly	17.9
Ala	16.4
Val	15.6
Cys	2.2
Met	5.9
Ile	19.6
Leu	26.6
Tyr	10.3
Phe	6.8
Lys	23.6
His	2.2
Arg	13.6

Fig. 3. *Effect of pokeweed antiviral protein from seeds on protein synthesis by HeLa cells*

Each test was performed with 10^5 HeLa cells grown in 1 ml of RPMI medium containing 10% human AB serum in each well of 16 mm multiwell trays. Cells were incubated for 18 h at 37°C in a humidified atmosphere of air/CO₂ (19:1) in the RPMI medium without serum and containing the appropriate amount of PAP-S. The medium was then removed and was replaced with 1 ml of RPMI medium minus leucine but containing L-[¹⁴C]leucine (0.25 µCi/well). After incubation for 2 h the medium was removed and replaced with 1 ml of 0.1 M-KOH. Protein was precipitated by adding 1 ml of 20% (w/v) trichloroacetic acid, and treated as described by Barbieri *et al.* (1980). Data are mean values for triplicate samples ± S.E.M. (vertical bars).

No sugar was detected, even in traces, by gas-chromatographic analysis.

Immunological characterization

Serum anti-PAP, which does not cross-react with PAP II (Irvin *et al.*, 1980) gave a weak reaction with PAP-S. Further, the inhibitory activity of PAP-S on protein synthesis (see below) was inhibited by an amount of anti-PAP serum five times higher than that necessary to neutralize PAP (results not shown).

Effects on protein synthesis and toxicity

Protein synthesis by a rabbit reticulocyte lysate was strongly inhibited by PAP-S, with an ID₅₀ (concentration giving 50% inhibition) ranging from 1.09 to 2.5 ng/ml (3.6×10^{11} – 8.3×10^{11} M) in different preparations. The shape of the inhibition curve was very similar to those reported previously for similar proteins (see, for instance, Barbieri *et al.*, 1980). A significant effect of PAP-S on protein synthesis by HeLa cells was observed only at a concentration of 1 mg/ml (3.3×10^{-5} M) (Fig. 3).

The toxicity of PAP-S for mice is reported in Table 4. Dead animals showed histological signs of fatty degeneration and necrosis in the liver (results not shown).

Table 4. *Toxicity of pokeweed antiviral protein*

Seven scalar doses of PAP-S (peak c of Fig. 1) were administered to groups of six mice per dose. Other details are described in the Experimental section.

Time after administration (days)	LD ₅₀ (mg/kg)	95% Confidence limits (mg/kg)
2	6.4	3.8–10.8
3	4.2	2.5–9.7
4	3.8	2.3–6.3
7	3.2	2.0–5.0
10	2.6	1.7–4.0

Table 5. *Inhibition of herpes simplex virus type 1 multiplication by pokeweed antiviral protein from seeds*

Experimental conditions are described in the text. Results are presented as percentage virus yields from infected cells in the absence of PAP; 100% is equal to 1.4×10^8 plaque-forming units/ml.

Concentration of pokeweed antiviral protein from seeds (µM)	Virus yield (%)
0	(100)
0.1	81
0.3	59
1.0	37
3.0	32

Inhibition of virus multiplication

The yield of herpes simplex virus type 1 in Vero cells infected in the presence of PAP-S was decreased in a dose-related manner, a 50% decrease in virus yield being caused by a concentration below 10^{-7} M (Table 5).

Discussion

The seeds of *P. americana* contain several forms of a protein that inhibits protein synthesis and can be purified with a good yield by a simple chromatographic procedure. The most abundant of these forms at least, PAP-S, has biological properties similar to those already known for PAP and PAP II, previously isolated from the leaves of the same plant, in that: (i) it is a powerful inhibitor of protein synthesis in a cell-free system, with little effect on intact cells, and (ii) it inhibits the replication of herpes simplex virus type 1.

Proteins similar to PAP have been purified from various plant materials (see the introduction) and have been compared with the A-chains of ricin and related toxins (Olsnes & Pihl, 1977): all of them inhibit the replication of tobacco-mosaic virus (Stevens *et al.*, 1981) and inactivate ribosomes by acting enzymically in a still-unknown manner. Previous and present results demonstrate that these proteins may be present in different parts of the same plant, sometimes in different forms.

In contrast with *M. charantia* inhibitor, gelonin, dianthins and the A-chains of ricin and abrin at least, PAP-S does not contain detectable amounts of sugars. In view of the similarity between these proteins, this suggests that sugars, when present, may not be necessary for the biological activity of the proteins. This is consistent with the previous observation that demannosylated gelonin inhibits protein synthesis as effectively as the native protein (Stirpe *et al.*, 1980).

Gelonin was conjugated to 'carriers' such as concanavalin A (Stirpe *et al.*, 1980) or a monoclonal antibody (Thorpe *et al.*, 1981) to form cytotoxic complexes that hopefully may be used for experimental therapy. It will be useful for this purpose to have several active proteins to overcome the immune reactions that would presumably occur on prolonged administration of these materials to animals. The protein purified from the seeds of pokeweed could be advantageously used for this purpose, since it is very potent, and can be prepared easily and in relatively large quantities from fairly common material.

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References

- Aron, G. M. & Irvin, J. D. (1980) *Antimicrob. Agents Chemother.* **17**, 1032–1033
- Barbieri, L., Zamboni, M., Montanaro, L., Sperti, S. & Stirpe, F. (1980) *Biochem. J.* **186**, 443–452
- Coleman, W. H. & Roberts, W. K. (1981) *Biochim. Biophys. Acta* **654**, 57–66
- Dunstan, D. R., Grant, A. M. S., Marshall, R. D. & Neuberger, A. (1974) *Proc. R. Soc. London Ser. B* **186**, 297–316
- Finney, D. J. (1964) *Statistical Methods in Biological Assay*, pp. 524–530, Griffin, London
- Gasperri-Campani, A., Barbieri, L., Lorenzoni, E. & Stirpe, F. (1977) *FEBS Lett.* **76**, 173–176
- Grasso, S., Jones, P. & White, R. F. (1980) *Phytopathol. Z.* **98**, 53–58
- Irvin, J. D. (1975) *Arch. Biochem. Biophys.* **169**, 522–528
- Irvin, J. D., Kelly, T. & Robertus, J. D. (1980) *Arch. Biochem. Biophys.* **200**, 418–425
- Kalb, V. F., Jr. & Bernlohr, R. W. (1977) *Anal. Biochem.* **82**, 362–371
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nicolson, G. L., Blaustein, J. & Etzler, M. E. (1974) *Biochemistry* **13**, 196–204
- Obrig, T. G., Irvin, J. D. & Hardesty, B. (1973) *Arch. Biochem. Biophys.* **155**, 278–289
- Olsnes, S. & Pihl, A. (1972) *FEBS Lett.* **28**, 48–50
- Olsnes, S. & Pihl, A. (1977) in *Receptors and Recognition*, series B, vol. 1 (Cuatrecasas, P., ed.), pp. 129–173, Chapman and Hall, London
- Roberts, W. K. & Stewart, T. S. (1979) *Biochemistry* **18**, 2615–2621
- Stevens, W. A., Spurdon, C., Onyon, L. J. & Stirpe, F. (1981) *Experientia* **37**, 257–259
- Stirpe, F., Olsnes, S. & Pihl, A. (1980) *J. Biol. Chem.* **255**, 6947–6953
- Stirpe, F., Williams, D. G., Onyon, L. J., Legg, R. F. & Stevens, W. A. (1981) *Biochem. J.* **195**, 399–405
- Thorpe, P. E., Brown, A. N. F., Ross, W. C. J., Cumber, A. J., Detre, S. I., Edwards, D. C., Davies, A. J. S. & Stirpe, F. (1981) *Eur. J. Biochem.* **116**, 447–454
- Ussery, M. A., Irvin, J. D. & Hardesty, B. (1977) *Ann. N.Y. Acad. Sci.* **284**, 431–440
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wei, C.-H., Hartman, F. C., Pfuderer, P. & Yang, W.-K. (1974) *J. Biol. Chem.* **249**, 3061–3067
- Wyatt, S. D. & Shepherd, R. J. (1969) *Phytopathology* **59**, 1787–1794