

Purification of Antiviral Proteins with Ribosome-Inactivating Properties from Plants

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A generally applicable highly effective purification method for antiviral plant proteins, selective for those with ribosome-inactivating properties, was developed. It involved affinity chromatography on Cibacron Blue Sepharose of the extracted plant sap after acid-ethanol precipitation and finally cation exchange chromatography on Mono S (Pharmacia). After the second chromatography step electrophoretically pure proteins from the four plant species, *Chenopodium amaranticolor*, *Dianthus barbatus*, *Phytolacca americana* and *Spinacia oleracea*, were obtained. These proteins inhibited infection of test plants with alfalfa mosaic virus and *in vitro* translation of TMV-RNA. The apparent molecular weights of the purified proteins were determined by SDS polyacrylamide gel electrophoresis as: 29 kDa for *C. amaranticolor*, 33 kDa for *D. barbatus*, 29 kDa for *P. americana* and 36 kDa for *S. oleracea*. Comparisons of the proteins by indirect ELISA and by Western blotting with polyclonal antisera from rabbits revealed that all four proteins were serologically related but not identical. In addition all four proteins were found to be glycosylated.

Introduction

Antiviral proteins have been shown to be present in several plant species many of which belong to the Caryophyllales [1–5]. For most of these proteins it has been demonstrated that they inhibit *in vitro* translation systems in a catalytical manner [6] and it is generally accepted that they inhibit virus infections of plants due to this function when they are applied during mechanical inoculations. The exact mechanism by which these proteins inhibit the translational system was first described for the functionally related ricin A-chain [7, 8]. This protein acts as a RNA N-glycosidase, cleaving the N-glycosidic bond between an adenine and the ribose at one defined position of the 28 S rRNA from rat liver ribosomes, which renders the 60 S subunit inactive. The same enzymatic cleavage occurs according to [9] with 11 single chain ribosome inactivating proteins (Type 1 RIP's) at exactly the same location.

In order to study these proteins we have developed a highly efficient and selective purification method. To achieve this we exploited two properties all known RIP's have in common: 1. their affinity for rRNA which should enable the proteins to bind to Cibacron Blue Sepharose [10–13]; 2. their highly basic nature which should permit separation on cation exchangers. The method was developed for *Dianthus barbatus* and thereafter proved to be applicable, with only minor variations, for three additional plant species: *Chenopodium amaranticolor*, *Phytolacca americana* and *Spinacia oleracea*. Purified proteins from *C. amaranticolor* (CAP), *D. barbatus* (DBP), *S. oleracea* (SOP) and *P. americana* (PAP) were used for the immunization of rabbits to produce antisera that could be applied for detection and comparison of the proteins.

Materials and Methods

Material

Chenopodium amaranticolor Coste and Reyn, *Dianthus barbatus* L. and *Spinacia oleracea* L. were grown in the greenhouse. *Phytolacca americana* L.

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was obtained from the Botanical Garden of the University of Braunschweig. Bioassays for antiviral activity were performed on primary leaves of *Phaseolus vulgaris* var. Saxa, using alfalfa mosaic virus (AIMV, DSM PV-0040* purified from *Nicotiana tabacum* var. White Burley according to [14]. The *in vitro* translation system from rabbit reticulocytes was obtained from Boehringer, Mannheim. Translation grade [³⁵S]methionine was purchased from Amersham-Buchler. Chromatography was carried out on a FPLC system from Pharmacia. Cibacron Blue Sepharose (Reactive Blue 2 Sepharose CL-6B) was obtained from SIGMA. Protein concentrations were determined with bicinchoninic acid according to [15]. The following biotinylated lectins were obtained from Kem-En-Tec, Copenhagen, DK: *Ulex europaeus* agglutinin, UEA; wheat germ agglutinin, WGA; *Phaseolus vulgaris* agglutinin, PHA; *Pisum sativum* agglutinin, PSA; *Solanum tuberosum* agglutinin, STA; soy bean agglutinin, SBA; *Dolichus biflorus* agglutinin, DBA and Concanavalin A, ConA. Streptavidin-alkaline phosphatase complexes were purchased from DAKOPATTS, Hamburg, F.R.G. The glycan detection kit was purchased from Boehringer, Mannheim, F.R.G.

Purification of RIP's

RIP's were purified from homogenized leaves after acidic ethanol precipitation according to [16]. The remaining protein material from the pellet was dialyzed against binding buffer (10 mM Tris, pH 7.5, 0.2 M lactose, 1% (v/v) 2-mercaptoethanol) and circulated three times through a 8 ml Cibacron Blue Sepharose column equilibrated with the same buffer. Thereafter the column was connected in the reverse flow direction to a FPLC unit, washed with 10 mM Tris, pH 7.5 and eluted with a NaCl gradient from 0–1 M, at a flow rate of 0.5 ml/min. Peak fractions, according to absorption at 280 nm, were pooled, dialyzed against 20 mM potassium phosphate buffer, pH 5.5, and applied to a cation exchange column (Mono-S HR 5/5, Pharmacia) equilibrated with the same buffer. The column was washed with the same buffer and bound material was eluted at a flow rate of 1.0 ml/

min with a linear 0–1 M NaCl gradient in this buffer. The eluent was collected in 0.5 ml fractions and measured as described above. Peak fractions were pooled, dialyzed against 0.1 M NaPO₄/KPO₄ buffer, pH 7.0 or double distilled water and stored at –80 °C.

Gel electrophoresis

Electrophoretic analyses of proteins were performed on 12.5% polyacrylamide gels (2.7% cross-linker) using the buffer system of Laemmli [17]. After separation the proteins were stained with silver according to [18]. To test for glycosylation of proteins with the glycan detection kit according to [19], the carbohydrate moieties of the proteins were oxidized with metaperiodate and the hapten digoxigenin coupled to the newly formed aldehyde groups *via* an attached hydrazide group as described by the vendor.

Western blotting

Proteins, separated by SDS-PAGE as described above, were transferred to nitrocellulose as described by Towbin *et al.* [20]. Probing of blotted proteins with antibodies and lectins was carried out as described by Adam *et al.* [21]. The detection of digoxigenin-labelled glycoproteins on nitrocellulose was carried out according the instructions of the vendor.

Bioassays for antiviral activity

Primary leaves of *P. vulgaris* var. "Saxa" were inoculated either with virus and inhibitor as test or with virus alone as control. Virus as well as protein fractions were dialyzed overnight against inoculation buffer (0.1 M NaPO₄/KPO₄, pH 7.0) and then mixed 10 min prior to inoculation to give final concentrations of 8 µg/ml for the virus and 2 µg/ml for the protein. Plant leaves were inoculated with a flat brush and Celite as abrasive. Usually 5–7 test plants were inoculated for one sample, using one primary leaf for the mixture of virus and protein and the opposite leaf for the control with virus only. Local lesions were counted three days after infection. The biological activity was calculated as % inhibition according to the following formula:

$$100 - ((LL \text{ on test leaf} \cdot 100) / LL \text{ on control leaf}).$$

* DSM numbers refer to the Catalogue of Plant Viruses ed. 3 from DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Antibody production

Polyclonal antisera against purified proteins were obtained from rabbits after intramuscular injection of 250 µg protein emulsified in 1 ml of Freund's complete adjuvants, followed by two booster injections of the same amount of protein in 1 ml of Freund's incomplete adjuvants, two and four weeks later. First bleedings were taken three weeks after the first injection and then weekly after five weeks. The antibodies were purified by affinity chromatography on a protein A superose column (Pharmacia) as described by Harlow and Lane [22].

ELISA tests

Indirect ELISA tests using plate-trapped antigen (PTA-ELISA) were performed as described by Jaegle and Regenmortel [23]. Briefly, the purified proteins were diluted in sodium carbonate buffer, pH 9.6 to a final concentration of 4 µg/ml and used to coat ELISA microtiter plates with 200 µl per well for 4 h at 37 °C. Such coated plates were then incubated with serial dilutions of the different antisera in all possible combinations overnight at 4 °C. In a last step a goat *anti*-rabbit alkaline phosphatase conjugate at a dilution of 1:1000 was used to detect and quantify the bound IgG. All buffers used for the ELISA tests were according to Clark and Adams [24].

Results

Development of the purification procedure

The purification method was developed with the inhibitor from *D. barbatus* and later adapted for the inhibitors from *P. americana*, *C. amaranticolor* and *S. oleracea*. As a first clarification step either acid ethanol precipitation [16, 25] or ammonium sulfate fractionation [26] were used. We have tested both methods and found the acid ethanol precipitation more suitable because it reduced the contamination with colored plant material. For the affinity chromatography step on Cibacron Blue Sepharose the buffer described by Sperti *et al.* [12] was chosen, because it has been successfully employed for the separation of the ricin subunits. An example of the first purifications, where a linear 0–1 M NaCl gradient was used to develop the affinity column, is shown in Fig. 1a. Bound material eluted in three peaks at salt concentra-

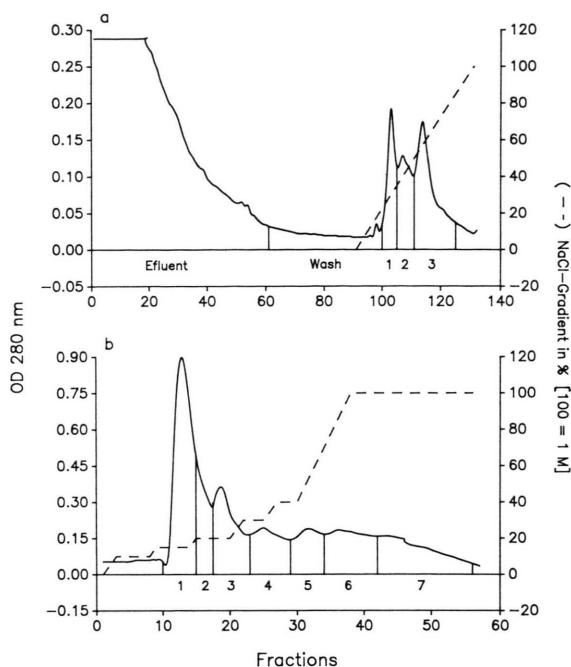


Fig. 1. Cibacron Blue Sepharose affinity chromatography. Proteins from *Dianthus barbatus* obtained after acid-ethanol precipitation were circulated three times through a 8 ml Cibacron Blue Sepharose column, using 10 mM Tris buffer, pH 7.5, with 0.2 M lactose and 1% (v/v) 2-mercaptoethanol. Columns were connected in reverse flow to the FPLC unit, washed with 10 mM Tris, pH 7.5, and eluted with a linear NaCl gradient (Fig. 1a) or a step gradient (Fig. 1b) at flow rates of 0.5 ml/min. Fractions pooled are indicated at the bottom.

tions from 150 mM to 600 mM. Aliquots of the three peaks were examined with SDS-PAGE for their corresponding protein pattern (Fig. 2a). All peaks contained two main proteins with molecular weights of 30 and 33 kDa. The first peak contained mainly the 33 kDa protein, the second peak about the same amount of both proteins and the third peak mainly the 30 kDa protein. In bioassays all three peaks inhibited virus infections, however, the main biological activity with 68% inhibition was associated with the first peak (Table I). From the electrophoretic analysis and the bioassay data we concluded that possibly only the 33 kDa protein was active and that the remaining biological activity in the other two peaks was due to contamination with it. In order to get a better separation we introduced steps at 100, 150, 200, 300 and 400 mM NaCl in the gradient (Fig. 1b). This modification

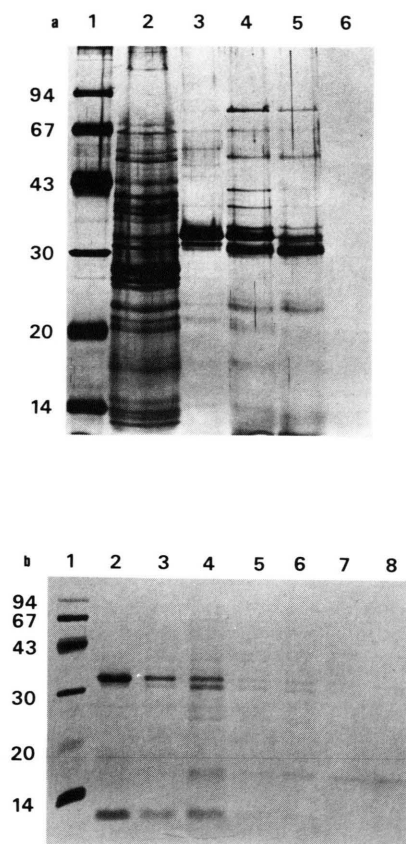


Fig. 2. SDS protein gel electrophoretic analyses of fractions from Cibacron blue affinity chromatography. 10 μ l of each fraction were mixed 1:1 with double concentrated sample buffer, heated for 5 min to 95 °C, cooled on ice and applied to a 12.5% polyacrylamide gel. Separated proteins were stained with silver.

a. Pooled fractions from Fig. 1a. Lane 1: molecular weight marker proteins, the molecular weights are given in kDa on the left; lane 2: effluent; lane 3: pool 1; lane 4: pool 2; lane 5: pool 3; lane 6: wash.

b. Pooled fractions from Fig. 1b. Lane 1: molecular weight marker proteins; lane 2: pool 1; lane 3: pool 2; lane 4: pool 3; lane 5: pool 4; lane 6: pool 5; lane 7: pool 6; lane 8: pool 7.

resulted in an improvement of the resolution as shown by the elution profile (Fig. 1b) and the accompanying electrophoretic protein pattern (Fig. 2b). The first peak in Fig. 1b contained mainly the 33 kDa protein and a second protein migrating below 14 kDa. The other peaks now contained only small amounts of the 33 kDa protein, indicating an improvement of the chromatographic separation. This was further substantiated by the bioassay data summarized in Table I. The

Table I. Antiviral activity of DBP at different stages of purification. The antiviral activity was determined with the bioassay system AIMV – *P. vulgaris*, using 5–7 test plants per sample. The virus concentration was 8 μ g/ml and each fraction was tested at 2 μ g/ml final protein concentration. Inhibition is calculated from the reduction of local lesions in the presence of protein compared to virus alone.

Cibacron Blue Sepharose separation*		Mono-S separation** of peak 1 from Fig. 1b	
Pool	% Inhibition	Pool	% Inhibition
	Fig. 1a		Fig. 1b
Effluent	28	Effluent	19
Wash	0	Wash	0
Peak 1	68	Peak 1	97
		Peak 2	19
Peak 2	35		
Peak 3	29		
Peak 4	–		
Peak 5	–		
Peak 6	–		
Peak 7	–		

* Pools correspond to the separation profile shown in Fig. 1.

** Pools correspond to the separation profile shown in Fig. 3.

*** Not tested.

biological activity in the first peak increased to 86% and concomitantly decreased in the following peaks.

To remove further contaminations in the 33 kDa fraction, a second chromatography step was performed with the cation exchanger MONO-S. Bound material was eluted with a linear 0–1 M NaCl gradient as one main peak and a trailing shoulder, marked as 2 in Fig. 3. The electrophoretic analysis of the two pooled fractions revealed that the first peak contained only the 33 kDa protein (Fig. 4). This protein inhibited local lesion formation in bioassays by 97% (Table I).

When the 33 kDa protein from *D. barbatus* (DBP) was tested for its ability to inhibit the *in vitro* translation of TMV-RNA in a rabbit reticulocyte system, 350 ng/ml DBP was sufficient to inhibit the incorporation of [³⁵S]Met by 99% (Table II). This demonstrated that the material we have purified with a two step chromatographic procedure behaved like other RIP's with respect to its antiviral activity and inhibition of translation.

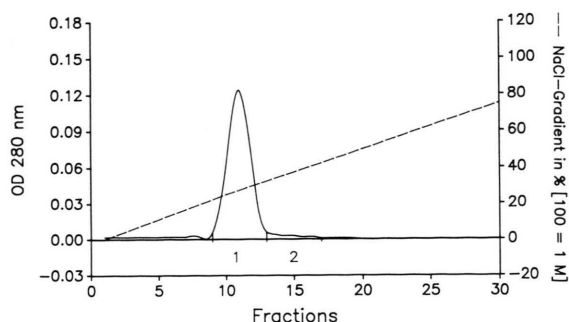


Fig. 3. Cation exchange chromatography of pool 1 from Fig. 1b. Pooled fractions, dialyzed overnight against 20 mM phosphate buffer, pH 5.5, were applied to a MONO-S HR 5/5 column. Bound proteins were eluted with a linear NaCl gradient in the same buffer at a flow rate of 1 ml/min.

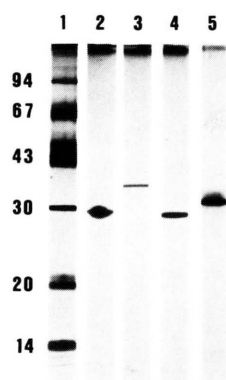


Fig. 4. SDS protein gel electrophoresis of active RIP's after cation exchange chromatography. After heat denaturation, as described in Fig. 2, proteins from biologically active peaks of MONO-S chromatographies were separated on 12.5% polyacrylamide gels. Proteins were stained with silver. Lane 1: molecular weight marker proteins; lane 2: PAP; lane 3: SOP; lane 4: CAP; lane 5: DBP.

Adaptation to other RIP's

The same purification method was applied to isolate the ribosome-inactivating proteins from *C. amaranticolor*, CAP, *P. americana*, PAP, and *S. oleracea*, SOP. It was possible to isolate from all three species electrophoretically pure proteins (Fig. 4) that inhibited viral infections and *in vitro* translation (Table II). Moreover, the molecular weights of the obtained proteins (Fig. 4) were in good accordance with previously published values [4, 27]. The only modifications to optimize the pro-

Table II. Properties of the different antiviral proteins. (1) Values given are the NaCl concentrations at protein peaks. (2) Antiviral activity expressed as inhibition of the number of AIMV local lesions on *P. vulgaris* in the presence of 2 µg/ml purified protein. (3) Inhibition of incorporation of [³⁵S]methionine by a rabbit reticulocyte lysate *in vitro* translation system, using TMV-RNA as template. Protein concentrations given are the final concentrations in the assay mix.

Protein	Elution from Mono-S (1)	Antiviral activity (2)	Inhibition of translation (3)
CAP	40 mM	90%	100% at 300 ng/ml
DBP	130 mM	97%	99% at 350 ng/ml
PAP	50 mM	100%	100% at 6 ng/ml
SOP	90 mM	92%	95% at 350 ng/ml

cedure for the different plant species were variations of the salt gradient for the cation exchange column. The results are summarized in Table II together with the corresponding data of bioassays and *in vitro* translation tests.

Serological comparisons of different RIP's

In order to compare the different RIP's we have produced antisera against the purified proteins DBP, PAP and SOP in rabbits. Sera obtained were first tested on Western blots of their homologous antigen to ascertain their specificity. In all cases these antisera allowed the detection of the respective homologous protein bands (Fig. 5). Even when applied to Western blots obtained from crude plant sap the antisera detected one major protein, migrating like the purified antigen and sometimes minor protein bands that could be polymeric forms of the proteins according to their molecular weights (results not shown). In addition the antibodies neutralized the inhibition of *in vitro* translation by their corresponding proteins [28]. These results further substantiated the high purity of the proteins obtained with our new purification method.

The antisera were also used to examine the serological relationships between the four different RIP's in indirect PTA-ELISA tests. The results are summarized in Table III. When comparing the results from both tests, a serological relatedness between all four proteins was obvious, however, the grouping differed, depending on the type of test. In the ELISA tests the homologous reactions were dominant above the heterologous ones, whereas

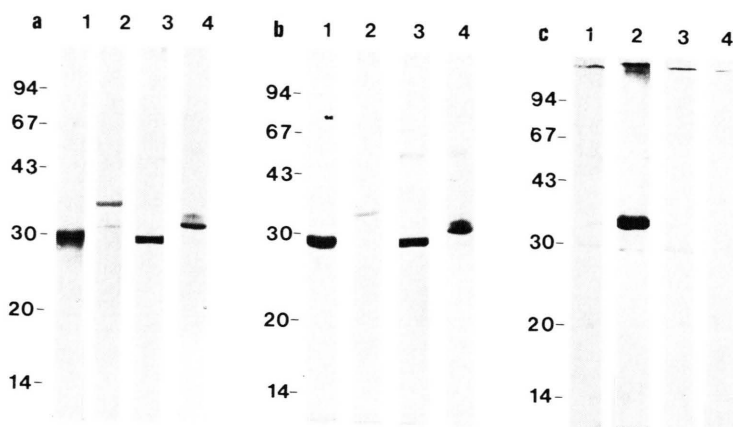


Fig. 5. Serological comparison of purified RIP's with different RIP antisera. Equivalent amounts of purified proteins were separated on 12.5% polyacrylamide gels as described in Fig. 2. Proteins were electroblotted to nitrocellulose and after a blocking step treated with different antisera. Lane 1: PAP; lane 2: SOP; lane 3: CAP; lane 4: DBP. Molecular weights in kDa are given at the left. a: Section treated with *anti*-PAP antiserum; b: section treated with *anti*-DBP antiserum; c: section treated with *anti*-SOP antiserum.

on Western blots the differences were not so obvious except in case of SOP, which reacted only very weakly with the heterologous antisera (Fig. 5). This could indicate that common serological determinants are not exposed in native proteins but become accessible after denaturation during electrophoresis and blotting. After denaturation PAP, DBP and CAP appeared to be more closely related to each other than to SOP (Fig. 5). In ELISA under non-denaturing conditions, DBP, SOP and CAP seemed to form a group of more closely related proteins while PAP was more distantly related (Table III).

Table III. Serological comparison of CAP, DBP, PAP and SOP.

Antisera	Strength* of colour reaction in indirect PTA-ELISA**			
	Antigens			
	CAP	DBP	PAP	SOP
<i>anti</i> -DBP	++	+++	+	+
<i>anti</i> -SOP	+-	+-	+-	+++
<i>anti</i> -PAP	+	+	+++	+-

* Colour reactions were rated as: +++ strong; ++ medium; + weak; +- barely visible; - not visible.

** PTA-ELISA = plate-trapped antigen ELISA with 2 µg/ml purified protein and 200 µl per well.

Examination for glycosylation

Western blots of all purified proteins, after oxidation and reaction with digoxigenin-hydrazide, were probed with *anti*-digoxigenin antibodies coupled with alkaline phosphatase. In addition, un-

modified DBP and PAP was tested with 8 different biotinylated lectins (UEA, WGA, PHA, PSA, STA, SBA, DBA, ConA). As shown in Fig. 6, CAP, DBP, PAP and SOP reacted positively in the general test for glycosylation. Of the four proteins PAP reacted most weakly, but consistently in different experiments using different preparations. This result was corroborated by the lectin assays, where PAP reacted with DBA and PHA, and DBP reacted with ConA and WGA. This substantiated

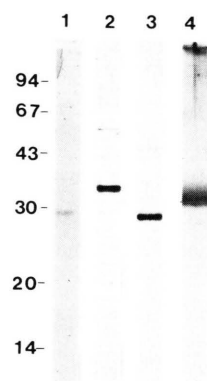


Fig. 6. Test for glycosylation of purified RIP's. Equivalent amounts proteins from biologically active peaks of MONO-S chromatographies were oxidized with meta-periodate and coupled with digoxigenin containing a hydrazide group. After heat denaturation as described in Fig. 2 the modified proteins were separated on 12.5% polyacrylamide gels. Proteins were then electroblotted to nitrocellulose and after a blocking step treated with *anti*-digoxigenin antibodies coupled with alkaline phosphatase. Bound antibodies were detected with the insoluble enzyme substrate naphthol AS-MX phosphate - fast red TR. Lane 1: PAP; lane 2: SOP; lane 3: CAP; lane 4: DBP. Molecular weights in kDa are given at the left.

the general glycosylation tests and verified that PAP is a glycoprotein like the other three RIPs, although its carbohydrate content seems to be much less than with CAP, DBP and SOP. According to the type of lectins that reacted positively with PAP its carbohydrate moiety should contain α -D-N-acetyl-galactoseamine, whereas DBP contains N-acetyl-glucoseamine together with α -D-mannose and/or α -D-glucose.

Discussion

We developed a highly selective, fast and convenient purification procedure for plant proteins that have antiviral activity due to their ability to inhibit the translational apparatus of eukaryotic cells. The method is superior to previously published ones [4, 5, 13, 25, 26, 29] with respect to speed and its general suitability for the purification of RIPs from several different sources. Though the affinity step has been used previously [11–13], this paper describes for the first time that this step can be used as a first selective step with crude preparations. Purification by cation exchange media has been applied to RIP's in most of the published procedures [4, 5, 25–28]. The advantage of using MONO-S columns, rather than cellulose- or Sephadex-based cation exchange media, is their high binding capacity (10 mg protein) and flow rates that can be achieved without losing resolution. The purification procedure described in this paper can be finished easily in two days when the dialysis steps are replaced by gel chromatography.

All purified proteins inhibited infections of test plants with plant viruses, when applied together with the virus and inhibited *in vitro* translation in an eukaryotic system. That the proteins are true RIP's was substantiated by results of Prestle *et al.* [30] who have tested their ability to deadenylate the 28 S rRNA. In addition we have previously demonstrated that the inactivation of the *in vitro* translation is irreversible, follows an enzyme kinetic and can be neutralized by addition of specific antibodies to the *in vitro* system [28]. These results exclude the possibility of nucleolytic inactivation of the template RNA and are in agreement with those published previously for other RIP's [31–33]. The differences in their specific activities (Table II) appeared to be protein specific and have been reported also by others [4].

The results concerning the molecular weights of the proteins, their basic nature and their glycosylation are in good agreement with previously published reports by others [25, 26, 34] with one exception. Our glycosylation tests revealed that PAP is a glycoprotein, in contrast to the data reported by [25] based on sugar analysis of purified PAP. This discrepancy is probably due to the low carbohydrate content of PAP which allowed a positive result only by the improved sensitivity of the glycan detection kit. Our results about glycosylation of PAP are further confirmed by a recent publication describing the glycosylation of PAP-S after computer analysis and gas chromatographic detection of sugars [35]. The lectin-binding tests with DBP revealed the presence of mannose and/or glucose as well as GlcNAc, which is in accordance with results published for the probably closely related dianthins from *D. caryophyllus* in [5] and [31].

A serological relationship between different antiviral proteins with the same activities has already been demonstrated by [4], using double gel diffusion techniques. The results obtained in these previous studies compare well with ours obtained with indirect ELISA, where the strongly glycosylated proteins CAP, DBP and SOP were found to be more closely related to one another than to PAP. Whether this is due to the larger carbohydrate contents in common for these three proteins remains to be determined. The different results obtained by us when comparing the proteins with antisera after electrophoretic separation and transfer to nitrocellulose were surprising at first, because the SOP antiserum revealed only slight cross reactions with the other three proteins that showed strong cross reactions to each other. The explanation for this discrepancy could be the modification of secondary and tertiary structures during the denaturation process, leading either to the exposure of hidden epitopes, or the presence of common non-contiguous epitopes that are lost during denaturation.

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