Identification of a biological inactive complex form of pokeweed antiviral protein

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Abstract Pokeweed antiviral protein (PAP) inactivates both eukaryotic and prokaryotic ribosomes via a specific depurination of rRNA. The sensitivity of pokeweed ribosomes to PAP implies the existence of a mechanism to protect the plant. Using monoclonal antibodies specific to PAP, a protein complex (PAPi) which contained PAP was identified in leaf extract. In this complex, the enzymatic activity of the toxin was strongly inhibited. This protein complex had a p*I* lower than that of PAP and was separated from free PAP by a preparative native gel electrophoresis. PAPi had an apparent molecular mass of 57 kDa and was dissociated by heating for 5 min at 80°C or by treatment by alkaline or acidic pH or by 7 M urea. The other components involved in the complex remain unknown.

Key words: *Phytolacca americana*; Pokeweed antiviral protein; Ribosome inactivating protein; Toxin inhibitor

1. Introduction

Pokeweed antiviral Protein (PAP) is a type I ribosome inactivating protein (RIP) isolated from the leaves of the plant *Phytolacca americana* (pokeweed). RIPs are RNA *N*-glycosidases that catalyze the removal of a single adenine residue (adenine-4327) from a conserved loop of the 28S rRNA of eukaryotic ribosomes [1]. This depurination prevents the binding of the elongation factor EF-2 and therefore inhibits translation. This activity makes RIPs powerful inhibitors of protein synthesis.

Other isoforms of PAP have been identified in *P. americana*, PAP-S in seeds [2] and PAP II in summer leaves [3]. Although PAP seems to be constitutively synthesized, PAP II is found in leaves later in the growing season.

PAP has been shown to inhibit infection by many different animal and plant viruses [4,5]. This property has been used to develop transgenic tobacco resistant to viral infection [6]. Ready et al. [7] showed that PAP was localized in the cell wall matrix. These authors suggested that the toxin could protect the plant from virus infection by penetrating into the cell together with the viruses and thus preventing viral replication by inhibiting protein biosynthesis. Recent evidence has indicated that internalization of PAP may be virus induced [8]. Moreover, Bonness et al. [9] demonstrated that pokeweed ribosomes were sensitive to PAP. Local suicide may be particularly effective against mechanically transmitted viruses which require local wounding in order to enter cells. Since PAP has been shown to be effective on its own ribosomes, the toxin should be either spatially separated in a different cell compartment or temporally inhibited until it reaches the cell wall. The cDNA of PAP contains sequences encoding N- and C-terminal peptides which are absent in the mature protein [10], but these peptides do not inhibit enzymatic activity of recombinant PAP (Radom, personal communication). It can be thought that they could be involved in the targeting of PAP to the cell wall.

This paper investigates the mechanism involved in the plant protection against its own toxin. Previously monoclonal antibodies (mAb) highly specific to PAP were produced and characterized [11]. These mAbs led to the identification of a minor form of PAP, present as a complex in a crude pokeweed leaf extract and showed that the toxin involved in this complex was strongly inhibited.

2. Materials and methods

2.1. Removal of free PAP from leaf extract by native preparative electrophoresis

Frozen spring leaves (100 g) were pulverized in liquid nitrogen and homogenized in 300 ml of extraction buffer (3 mM DTT, 1 mM PMSF, 2 mM EDTA, 25 mM Tris-HCl, pH 7.5). After 20 min centrifugation at 20 000×*g* (GSA type rotor, Sorvall) the supernatant was fractioned by ammonium sulfate between 40 and 100% of saturation. The 100% pellet was resuspended in 25 ml of buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.2 mM EDTA, 2 mM DTT and desalted by gel filtration on a 2.5×60 cm Sephadex G10 column (Pharmacia) equilibrated with the same buffer. Then, proteins were concentrated by ultrafiltration to a final volume of 10 ml (AMICON cell, cut-off 10 kDa). This extract, called E40, was adjusted to 62.5 mM Tris-HCl, pH 6.8, and 10% glycerol (sample buffer) and then loaded onto a non-denaturing polyacrylamide preparative gel electrophoresis prepared as follows. The glass column of the Bio-Rad model 491 'Prep Cell System' (internal diameter: 57 mm) was filled with 30 ml of a solution containing 4.5% acrylamide, 0.012% bis-acrylamide, 125 mM Tris-HCl, pH 6.8. Polymerization was catalysed with ammonium persulfate and TEMED. Electrophoresis was performed at 10 W constant power in the migration buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3). Elution was performed at a flow rate of 0.5 ml/min and 3 ml fractions were collected. The presence of free PAP and complexed PAP was then checked in eluted fractions by ND-PAGE and Western blotting. The obtained fraction was called the PE extract.

2.2. Double sandwich ELISA

Microplates were coated for 2 h at 37°C with 100 μl of anti-PAP purified polyclonal antibodies (3 μg/ml) in a 50 mM carbonate buffer, pH 9.6. After washing, wells were saturated with 1% PBS-BSA for 2 h at 37°C. Subsequently, 100 μl of diluted PE extract were incubated for 1 h at room temperature under gentle shaking. Then, wells were incubated for 1 h at room temperature with mAb 7.1E5 (0.3 μg/ml) [11] mixed with diluted anti-mouse peroxidase-labeled antibodies (1:2000,

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Abbreviations: mAb, monoclonal antibody; E40, pokeweed leaf extract after ammonium sulfate fractionation; PE extract, pokeweed leaf extract after preparative native gel electrophoresis; ND-PAGE, non-denaturing polyacrylamide gel electrophoresis

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Biosys). Enzymatic activity was measured by incubation for 10 min with a commercial solution of 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 as substrates. The reaction was stopped by the addition of 50 μl of 2 N HCl and absorbance was measured at 450 nm with a microplate reader.

2.3. Analytical electrophoresis and immunoblotting

Proteins were separated under denaturing conditions by SDS-PAGE according to Laemmli [12] or under non-denaturing conditions according to Blackhear [13]. Briefly, the native gel electrophoresis consisted of a separating gel at 11% acrylamide, pH 8.8, and a stacking gel at 4.5% acrylamide, pH 6.8, without SDS. The sample buffer was the Laemmli sample buffer, but it contained neither SDS nor β-mercaptoethanol. After migration, gels were transferred to a PVDF membrane using a Bio-Rad trans-blot apparatus in 25 mM Tris-HCl, 192 mM glycine, 0.02% SDS and 10% methanol. Immunoblotting analysis was performed as previously described [11].

2.4. In vitro translation assay

Different amounts of PE extract and purified PAP, native or heat treated for 5 min at 80°C (2 μl each) were added to the translation mixture containing 4 μl of rabbit reticulocyte lysate (Promega), 0.2 μl of a mixture of the different amino acids (minus methionine) at a concentration of 1 mM, 0.2 μl of L-[^{35}S]methionine (3.10^8 Bq/ml, DuPont), 5.2 μl of H_2O. The reaction was started by the addition of 0.2 μl of a stock solution of Brome mosaic virus (BMV) RNA at 0.5 mg/ml. After incubation (1 h at 30°C), 2 μl aliquots of each assay were analyzed by SDS-PAGE and the gel was then autoradiographed. Densitometric analysis of the band at 35 kDa encoded by BMV RNA was performed with the Bio-Rad 'gel-Doc' system.

3. Results

3.1. Detection of the complexed PAP by non-denaturing electrophoresis

Proteins from a pokeweed leaf extract (E40) were separated by non-denaturing gel electrophoresis including a stacking gel at pH 6.8 and a separating gel at pH 8.8. At pH 6.8, PAP was protonated and migrated toward the cathode and not into the gel. Nevertheless, Western blotting analysis using a mixture of different monoclonal antibodies specific to PAP revealed an immunoreactive component (Fig. 1, lane 2). This band was not detected when the sample was heated for 5 min at 80°C prior to loading onto the electrophoresis gel (lane 1). As expected, no band was detected in the control lane where purified PAP was loaded (lane 3) indicating that electrophoresis conditions did not allow its migration into the gel.

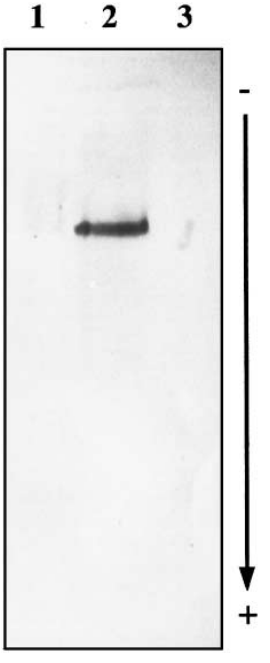

Fig. 1. Analysis of proteins from E40 extract (50 μg for each lane) by Western blotting after ND-PAGE. Proteins were detected with a mixture of mAbs (1.3B5, 2.5H2, 6.1C8 and 7.1E5) as described in Section 2. Lane 1: E40 extract heated for 5 min at 80°C prior to electrophoresis; lane 2: non-heated E40 extract; lane 3: 5 μg of purified PAP as control.

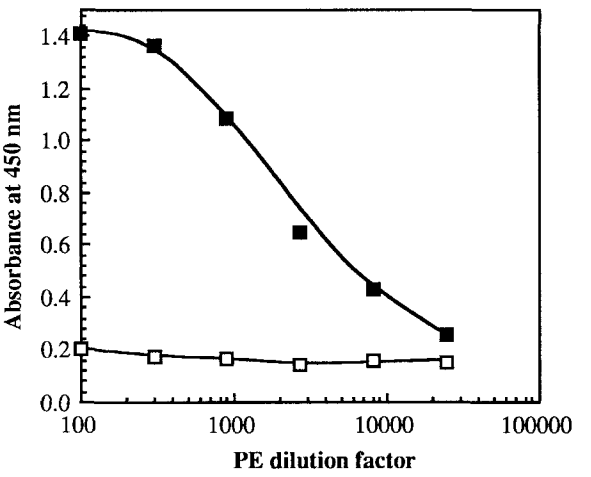

Fig. 2. Detection and quantification of PAP in PE extract by double sandwich ELISA. Wells were coated with purified anti-PAP polyclonal antibody (3 μg/ml) and saturated with 1% PBS-BSA. PE extract was then incubated at different dilutions before (□) or after (■) heat denaturation for 5 min at 80°C. The ELISA protocol is described in Section 2. The absorbance value corresponding to 1:900 and 1:2700 dilution factor were used for estimating the concentration of PAP in the PE extract with reference to a standard curve established with heat-treated purified PAP. Each value was the average of three assays with the same PE extract.

These results indicate that the leaf extract contains another protein or a protein complex detected by the anti-PAP mAbs. The identified component was called PAPi. In order to perform preliminary characterization, an extract containing PAPi but devoid of free PAP was prepared. As indicated above, pH 6.8 of the stacking gel electrophoresis could separate both PAP and PAPi entities. Thus, a simple non-denaturing preparative gel electrophoresis consisting of a 4.5% polyacrylamide gel at pH 6.8 was developed. Non-denaturing gel electrophoresis and Western blotting analysis performed on the eluted fraction (PE extract), confirmed the presence of PAPi (data not shown).

3.2. ELISA detection and quantification of PAP in PE extract

A sandwich ELISA had been previously developed in our laboratory to quantify PAP in plant extracts (unpublished results). In this assay the antigen was trapped between a coated polyclonal antibodies and the mAb 7.1E5 [11] which was known to react with a recombinant peptide including the 63 C-terminal amino acids of mature PAP [14]. Fig. 2 shows the ELISA response versus increasing dilutions of either native or heat-treated PE extract. No PAP could be detected in the untreated sample while the same sample, heated for 5 min at 80°C, gave a strong ELISA response. Using a standard curve established with heat-treated purified PAP as reference, PAP concentration in the heat-treated PE extract was estimated at 12 μg/ml. These results indicated that the mAb

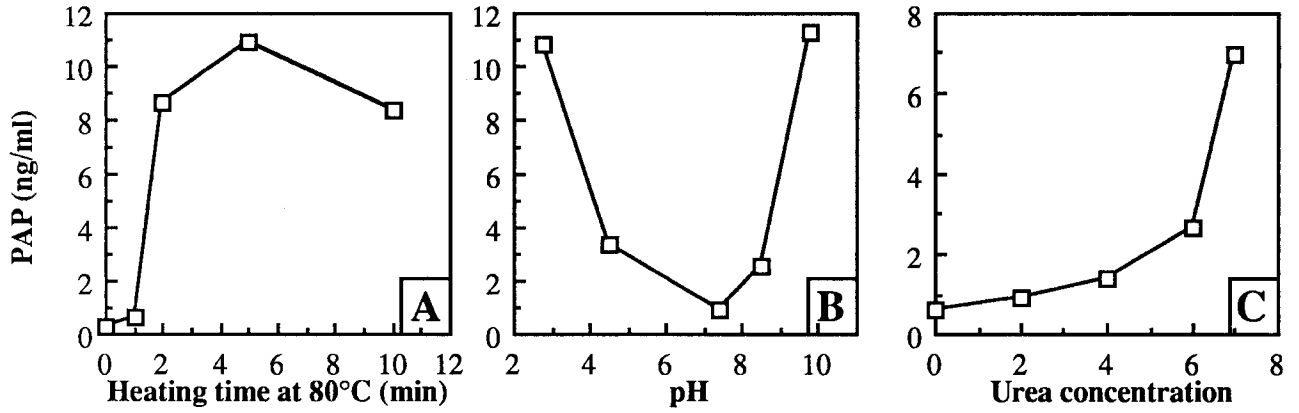

Fig. 3. Effect of different treatments on the stability of PAPi. A: Effect of temperature: PE extract was heated at 80 °C for increasing incubation times and then diluted in PBS and tested by ELISA. B: Effect of pH: A PE extract fraction was equilibrated with various buffers with a pH ranging from 2.8 to 9.6. After 1 h incubation, samples were re-equilibrated at pH 7.4 by dilution in PBS buffer and then tested by ELISA. C: Effect of urea: PE extracts were incubated with increasing concentrations of urea ranging from 2 to 7 M for 2 h at room temperature. Then samples were diluted with PBS and were immediately tested by the sandwich ELISA as described in Section 2. PAP was quantified as in Fig. 2. The amounts of PAP were calculated on the basis of a PAP standard curve. Each value is the average of three assays with the same PE extract.

7.1E5 epitope was not accessible in PAPi. Thus, PAPi could be a complex involving PAP and an unknown protein. This double sandwich ELISA proved to be a very reliable criterium for the determination of PAPi integrity since PAP was detected only after dissociation of the complex.

3.3. Preliminary characterization of the PAPi complex

The stability of the complex in relation to different denaturing agents such as temperature, pH and urea was investigated. The effect of the different treatments was evaluated by the quantification of the resulting PAP released from the complex and thus was monitored by the double sandwich ELISA technique.

3.3.1. Effect of temperature. The PE extract was incubated for various periods of time at 80°C and then tested by ELISA. Fig. 3A shows that a maximal quantity of PAP was released from the complex after a treatment of 5 min.

3.3.2. Effect of pH. The equilibration of the PE extract in buffers at different pHs ranging from 2.8 to 9.5 showed that PAPi was stable between pH 6 and 8, but was dissociated at alkaline or acidic pHs (Fig. 3B).

3.3.3. Effect of urea. PE fractions were equilibrated with increasing concentrations of urea ranging from 2 to 7 M and incubated for 2 h at room temperature. Then, the fractions were diluted in PBS buffer and tested by ELISA. Fig. 3C shows the effect of urea concentration on the PAPi complex.

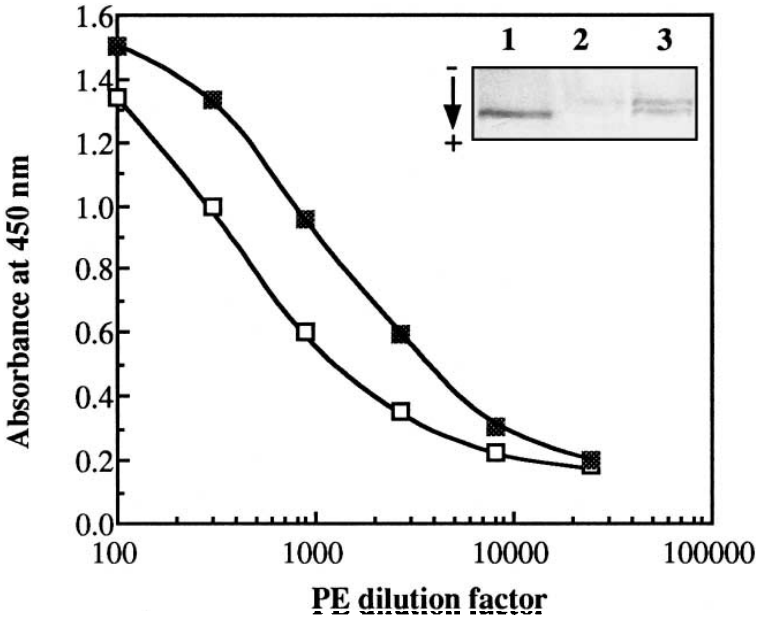

Fig. 4. Refolding of PAPi after denaturation by 7 M urea. Proteins from PE extract were equilibrated with 7 M urea as described in Fig. 3C and assayed by ELISA at various dilutions (■). Samples were then diluted 20-fold in the refolding buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8), incubated under gentle shaking overnight at 4°C and tested by sandwich ELISA at various dilutions (□). Inset: The same samples were concentrated 20-fold by ultrafiltration (Amicon, cut-off 10 kDa) and analyzed by Western blotting after ND-PAGE with 1:1000 dilution of mAb 1.3B5 and 6.1C8. Lane 1: native PE extract; lane 2: PE extract denatured by 7 M urea; lane 3: PE extract after refolding.

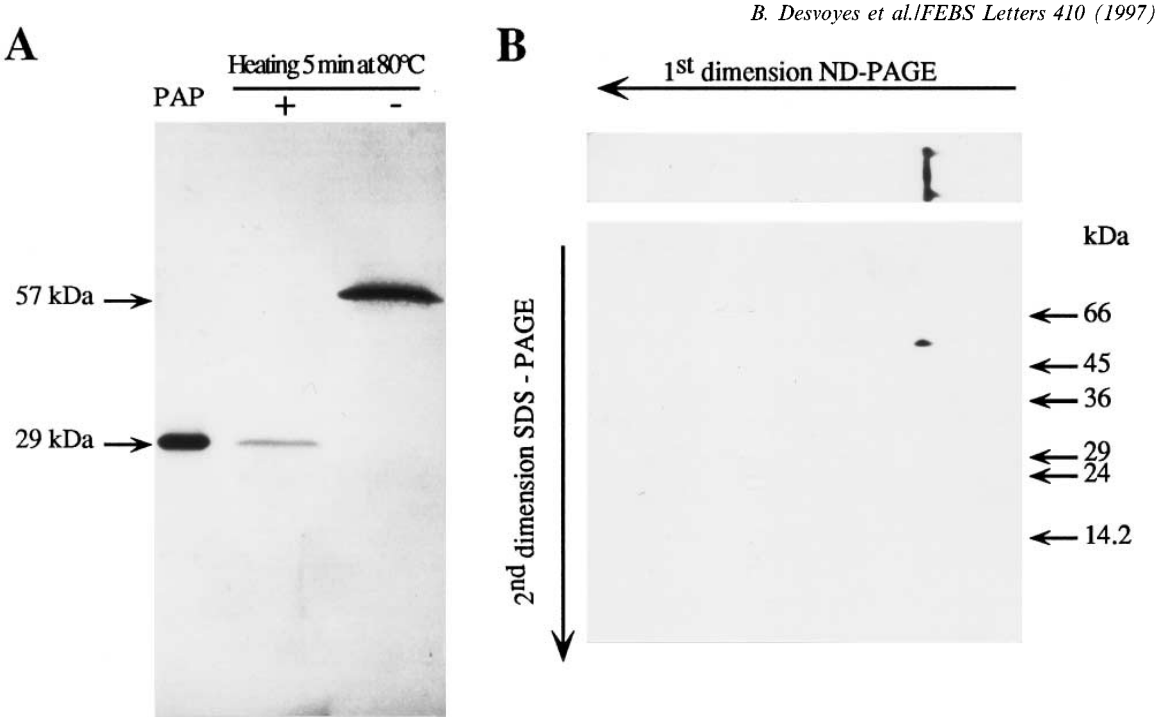

Fig. 5. A: Molecular mass determination of PAPi by Western blotting analysis. PE extract proteins (20 μg) were separated by SDS-PAGE (12.5% acrylamide) and transferred to a PVDF membrane. Immunodetection was performed with a mixture of mAb 1.3B5 and 6.1C8 (dilution factor: 1:1000). Lane 1: 0.5 μg of purified PAP; lane 2: PE extract heated for 5 min at 80°C prior to electrophoresis; lane 3: unheated PE extract. B: Two-dimensional electrophoresis analysis of PE extract. PE extract was first run on a ND-PAGE and the corresponding lane of polyacrylamide was cut and loaded on the top of a SDS-polyacrylamide gel. Then the resulting gel was transferred for Western-blotting analysis using the mAb 1.3B5 and 6.1C8 at 1:1000 dilution.

Concentrations below 4 M had no effect on its stability while PAP was progressively released from the PAPi complex at concentrations above 4 M. The maximal release was observed at 7 M urea. Purified PAP treated under the different conditions (temperature, pH and urea) and assayed by ELISA reacted in the same way as native PAP (data not shown).

Renaturation of the PAPi complex after urea treatment was performed by dilution of the denatured proteins in a refolding buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8). Analysis of the denatured and renatured proteins performed by ELISA showed that the two curves exhibited similar features indicating that the quantity of PAP in both samples could be compared (Fig. 4). Free PAP concentrations were estimated at 11.5 μg/ml and 4.5 μg/ml in the denatured and renatured samples respectively indicating that the complex was refolded at 60%. The Western blot (inset Fig. 4) confirmed these results. PAPi was not revealed when the sample was incubated in 7 M urea (lane 2), indicating the complex dissociation. After refolding, two neighboring bands were detected (lane 3). These two bands indicate that PAPi was probably refolded into two different spatial conformations.

Table 1
Biological activity of PAPi on in vitro protein synthesis in a rabbit reticulocyte system

PAPng/ml[a]	Rate of translation (%)			
	Complexed PAP		Purified PAP	
	(nh)	(h)	(nh)	(h)
50	26	< 5	< 5	< 5
20	34	8	< 5	< 5
10	63	15	9	18
5	nt	nt	11	42

Equivalent amounts of purified PAP and complexed PAP from PE extract either heated for 5 min at 80°C (h) or not (nh) were added to the translation mixture as described in Section 2. Results were normalized to controls obtained in the absence of PAP and PAPi (100%) and in the absence of BMV-RNA template (0%). Values represent the average from duplicate sample. These results were reproduced 5 times with different preparations of PE extract and the nh/h ratio continued to range from 3 to 4.
[a]Amounts of PAP were determined by ELISA on heat-treated PE extract and heat-treated PAP.
nt: non-tested.

3.4. Estimation of the molecular mass of the PAPi complex

The molecular mass of PAPi was estimated by SDS-PAGE according to Laemmli [12] and Western blotting, except that the sample was not heated prior to electrophoresis in order to avoid the dissociation of subunits. As shown in Fig. 5A, lane 3, the PAPi complex was resistant to SDS and had an apparent molecular mass of 57 kDa. After heat treatment (Fig. 5A, lane 2) the complex was dissociated and PAP was detected by monoclonal antibodies at 29 kDa in the same way as the control performed with purified PAP (lane 1). A two-dimensional electrophoresis was performed in order to confirm that the 57 kDa protein and those detected after ND-PAGE were indeed the same. In the first dimension, proteins from PE extract were separated by ND-PAGE and in the second by SDS-PAGE. The proteins were then analyzed by Western blotting using a mixture of mAbs. Fig. 5B shows a single spot at 57 kDa indicating that the band detected initially after ND-PAGE corresponded to the band detected by SDS-PAGE. These experiments showed that the complex was resistant to a 2% concentration of SDS as well as to β-mercaptoethanol, thus indicating that no disulfide bond was involved in the quaternary structure.

3.5. Comparison of the PAPi biological activity before and after denaturation

The biological activity of PAPi was investigated with an in vitro translation system. The PAP contained in the PAPi fraction was first quantified by ELISA after its release from the complex by heat treatment. Table 1 compares the residual rate of translation obtained in the presence of equivalent concentrations of PAPi and purified PAP, either native or heat treated. In all cases the translation rate was reduced by a factor around 4 when the PE extract was heated prior to its addition to the translation mixture. Moreover, when PAPi was dissociated the translation rates were comparable to those obtained with the same quantities of heat treated purified PAP. PAP is known to be a temperature resistant protein [15]. However, the activity of native and heat treated purified PAP was compared. Table 1 shows that the treatment slightly decreased the enzymatic activity of the toxin indicating that a minor fraction of the toxin was denatured. This would suggest that the inhibition of the biological activity of PAP in PAPi could have been underestimated. These results clearly indicate that the biological activity of the toxin is strongly reduced in PAPi and that its thermal denaturation induced a release of enzymatically active PAP.

4. Discussion

Bound ribosomes synthesize the toxin which is then targeted to the cell wall through the endoplasmic reticulum and the golgi apparatus. Nevertheless, it could be thought that a few PAP molecules could escape this pathway and, considering the high toxicity of PAP, kill the cell. Bonness et al. [9] have already suggested the existence of a protective element able to take over the free PAP molecules in the cytoplasm, but were unsuccessful in finding it. Since these authors worked with a PAP free cell line, they could not definitively exclude the existence of such a protective element because its expression could be correlated with that of PAP. Our experiments attempted to find this inhibitor in pokeweed leaf cells. The hypothesis was that if such an inhibitor existed it would be found in plant extract as a complex with PAP. Then, the p*I* of the complex would be different and probably lower than that of PAP which is 8.1 [16]. Thus, a native PAGE was developed under pH conditions that did not allow free PAP to migrate into the gel. Western blotting analysis using specific mAb did detect a minor form of PAP that proved to be involved in a complex showing a lower p*I*. These analyses were performed with a mixture of mAbs in order to avoid any problem of detection due to hidden PAP epitopes in PAPi. Further Western blotting analysis after SDS-PAGE and ELISA showed that this complex was dissociated by different chemical or physical treatments to release free PAP. The urea treatment appeared reversible. The possibility of refolding PAPi represented an additional argument to demonstrate that PAP was associated by non-covalent interactions with another uncharacterized component. The ELISA emphasized the role of the C-terminus domain of PAP in the complex. mAb 7.1E5 whose epitope had been identified in the C-terminal domain was not able to bind to PAP in the complex. It can be concluded that the mAb 7.1E5 epitope is hidden in the complex because the association of PAP and its partner implies at least a part of this domain.

Hur et al. [17] have recently described and characterized PAP mutants in a yeast expression system. Mutants with a deletion of 25 amino acyl residues at the C-terminus were enzymatically active in vitro, but non-toxic to yeast in vivo. These results suggest that PAP cytotoxicity is not due solely to its enzymatic activity. Moreover, the C-terminal extremity of PAP was shown to be homologous to the consensus sequence for a prokaryotic lipid binding site which could facilitate the passage across membranes. Chaudhary et al. [18] have shown that removal of the C-terminal sequence of *Pseudomonas aeruginosa* exotoxin A strongly reduced its toxicity without affecting its enzymatic activity. This evidence strongly supports the physiological importance of this domain and gives relevance to our data.

It has been demonstrated that the RIP activity of the native PAPi complex was strongly reduced. PAP molecules involved in the complex were inhibited, but they could be released as enzymatically active molecules by heat treatment. Nevertheless, complexed PAP represented only a minor part of the toxin in comparison with the high amount of PAP present in the cell.

The apparent molecular mass of PAPi was estimated at 57 kDa. So far, the other component involved in this complex has not been characterized. PAPi could also result from a dimerization of PAP, although experiments of spontaneous dimerization of purified PAP have still not been successful. A dimeric form of α-sarcin, a RIP with a ribonuclease activity, has been identified in a purified toxin preparation [19]. Recently, these dimers have been shown to be inactive and it has been suggested that a dimer formation could play a biological role in preventing the cytotoxicity of α-sarcin [20]. Nevertheless, the existence of an unknown protein associated to PAP in a heterodimer cannot be excluded. Experiments should be oriented now to complete the biochemical characterization of PAPi. Further purification of PAPi is being undertaken and involves an affinity chromatography step. The purification should be able to identify the protein composition of the complex.

The physiological relevance of these data, now, needs to be proven in vivo in order to be able to propose this complex as a mechanism developed by pokeweed to protect itself from the toxicity of this toxin.

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References

[1] Endo, Y. and Tsurugi, K. (1987) J. Biol. Chem. 262, 8128–8130.
[2] Barbieri, L., Aron, G.M., Irvin, J.D. and Stirpe, F. (1982) Biochem. J. 203, 55–59.
[3] Irvin, J.D., Kelly, T. and Robertus, J.D. (1980) Arch. Biochem. Biophys. 200, 418–425.
[4] Aron, G.M. and Irvin, J.D. (1980) Antimicrob. Agents Chemother. 17, 1032–1033.
[5] Chen, Z.C., White, R.F., Antoniw, J.F. and Lin, Q. (1991) Plant Pathol. 40, 612–620.
[6] Lodge, J.K., Kaniewski, W.K. and Tumer, N.E. (1993) Proc. Natl. Acad. Sci. USA 90, 7089–7093.
[7] Ready, M.P., Brown, D.T. and Robertus, J.D. (1986) Proc. Natl. Acad. Sci. USA 83, 5053–5056.
[8] Irvin, J.D. and Uckun, F.M. (1992) Pharmacol. Ther. 55, 279–302.

[9] Bonness, M.S., Ready, M.P., Irvin, J.D. and Mabry, T.J. (1994) Plant J. 5, 173–183.
[10] Lin, Q., Chen, Z.C., Antoniw, J.F. and White, R.F. (1991) Plant Mol. Biol. 17, 609–614.
[11] Desvoyes, B., Dulieu, P., Poyet, J.L. and Adami, P. (1995) Hybridoma 14, 571–575.
[12] Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
[13] P.J. Blackhear, in: W.B. Jakoby (ed.), Methods in Enzymology, Vol. 104, Academic Press, New York, 1984, pp. 237–255.
[14] B. Desvoyes, Thesis No. 538, University of Franche-Comté, France, 1996.
[15] Barbieri, L. and Stirpe, F. (1982) Cancer Sur. 1, 489–520.
[16] Irvin, J.D. (1983) Pharmacol. Ther. 21, 371–387.
[17] Hur, Y., Hwang, D.J., Zoubenko, O., Coetzer, C., Uckun, F.M. and Tumer, N.E. (1995) Proc. Natl. Acad. Sci. USA 92, 8448–8452.
[18] Chaudhary, V.K., Jinno, Y., FitzGerald, D. and Pastan, I. (1990) Proc. Natl. Acad. Sci. USA 87, 308–312.
[19] Sacco, G., Drickamer, K. and Wool, I.G. (1983) J. Biol. Chem. 258, 5811–5818.
[20] Cheung, J.I., Wang, Y.R. and Lin, A. (1996) FEBS Lett. 386, 60–64.