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RIBONUCLEASES IN *NICOTIANA TABACUM* LEAF EXTRACTS TREATED WITH PHENOL

L. JERVIS* and N. W. PIRIE
Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ

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Abstract—When tobacco leaf extracts are treated with phenol, *ca* 20% of the ribonuclease (RNase) activity survives and can be measured when the phenol is removed. After purification, the resistant RNase is inactivated by phenol; this suggests that tobacco leaves contain material that protects the RNase. Phenol-resistant RNase may be one of the TMV-RNA inactivating systems present in phenol extracts of tobacco leaves.

INTRODUCTION

INTACT viruses are probably the agents that usually transmit infection between hosts although the spread of infection between cells may be due to free nucleic acid. Isolation of infective nucleic acid from leaf tissue is difficult because of its extreme sensitivity to inactivation.[1] Bawden and Pirie[2,3] tried to correlate the susceptibility of leaves to infection with the amounts of various inactivating agents present in leaves. One of these agents may be a leaf RNase that resists the phenol treatment used in nucleic acid isolation. This RNase fraction is probably an artefact but it has some intrinsic interest because nucleic acids are so often extracted in the presence of phenol. Some properties of this fraction seem worth recording.

RESULTS AND DISCUSSION

Ion-exchange chromatography of phenol-treated RNase

Chromatography of low MW tobacco leaf RNases on carboxymethyl Sephadex showed four distinct activities.[4] When phenol-treated tobacco RNases were chromatographed under similar conditions, only two peaks of activity were detectable. These were eluted with citrate buffer and corresponded to RNase 1 and RNase 2 described earlier.[4] We could not detect RNase activity in the material not adsorbed by CM-Sephadex, nor in the material eluted by Tris-HCl buffer at pH 8·0. The two RNase fractions that survived phenol treatment had pH optima of 5·1 and 5·8–6·0; they did not act on DNA and had apparent K_m values of 0·33 and 1·15 mg/ml respectively with RNA as substrate. Both were completely inhibited by 10^{-3} M Zn^{2+} and by 5×10^{-4} M Cu^{2+}. The first product of RNA hydrolysis

* Present address: Biology Department, Paisley College of Technology, High Street, Paisley PA1 2BE, Renfrewshire, Scotland.

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detectable by PC was G > p with both enzymes, indicating that both were relatively guanine-specific endo-ribonucleases. The endo-nuclease nature of both enzymes was confirmed by analysis of the products of partial RNA hydrolysis by gel filtration.[5]

TABLE 1. PURIFICATION OF PHENOL-TREATED *Nicotiana tabacum* RIBONUCLEASE

Purification stage	Total RNase (units)	Total protein (mg)	S. act. (U/mg protein)	Fold purification protein	Recovery (%)
Crude extract	56160	2760	20·4	1	100
Phenol-treated concentrate (non-diffusible fraction)	5200	150	34·6	1·7	9·2
Sephadex G100 fraction	6930	8·85	783	38	12
CM-Sephadex					
Peak 1	2780	1·1	2530	123	4·9
Peak 2	3000	0·9	3760	184	5·3
Sephadex G75					
RNase 1	2660	0·7	3800	186	4·7
RNase 2	2880	0·5	5760	282	5·1

In all these properties, the phenol resistant RNases resembled RNases 1 and 2 isolated from leaf extracts not treated with phenol. We therefore concluded that the phenol-resistant fraction of tobacco leaf RNase was a mixture of two enzymes and that, in spite of the rather drastic treatment they had received, no significant changes in properties had occurred.

TABLE 2. EFFECT OF PURIFICATION ON THE RESISTANCE OF *Nicotiana tabacum* LEAF RNASE TO TREATMENT WITH PHENOL

Purification stage	RNase surviving phenol treatment (%)	Purification stage	RNase surviving phenol treatment (%)
Crude extract	12·4	CM-Sephadex	
Dialysed concentrate	16	Peak 1	6·6
Low MW RNase G100 fraction	13·3	Peak 2	7·2

Tobacco leaf ribonuclease was purified as described in the accompanying paper. At each stage a sample of the RNase was treated with phenol and the total amount of enzyme surviving the treatment was assayed at pH 5·5 after removal of the phenol by ether extraction and dialysis.

Purification and stability

The results of a typical purification from 1 kg of tobacco leaves (Table 1) show that, after chromatography on CM-Sephadex, the two components were present in roughly equal amounts. This contrasts with the state in leaf extracts not treated with phenol where there is 5 times as much RNase 1 as RNase 2, and suggests that RNase 2 is more resistant to phenol treatment than RNase 1. However, when samples of both enzymes, extensively purified from phenol-treated extracts, were treated again with phenol, both lost over 95% of their activity. This indicates that resistance to phenol is not a property of the enzymes, rather, resistance must be conferred by a component(s) of leaf extracts. Also, RNase 2 probably combines more readily with the component(s) than RNase 1, hence its apparently greater stability. The loss of resistance to phenol with increasing purification demonstrates

[5] BIRNBOIM, H. (1966) *Biochim. Biophys. Acta* **119**, 198.

the removal of the protecting component(s) from the RNase. Resistance to phenol decreases sharply after chromatography on CM-Sephadex (Table 2). By adding the material not adsorbed by CM-Sephadex to RNases purified with this ion-exchanger some resistance to phenol is restored (Table 3).

TABLE 3. RESTORATION OF PHENOL RESISTANCE BY ADDITION OF LEAF COMPONENTS TO PURIFIED RIBONUCLEASE

Enzyme	Additions	Ribonuclease activity (units) Before phenol	After phenol	Enzyme	Additions	Ribonuclease activity (units) Before phenol	After phenol
RNase 1	None	124	4·7	RNase 2	None	147	5·5
	Fraction 1	124	2·7		Fraction 1	147	1·2
	Fraction 2	124	19		Fraction 2	147	·18
	Fraction 3	124	35		Fraction 3	147	38

The amounts of RNase shown were treated with 1 ml of each non-RNase fraction for 1 hr at room temp. before shaking with 2 ml 90% phenol. Phenol was removed by ether extraction and dialysis and residual RNase was assayed. Fraction 1—diffusible material from concentrated crude extract; Fraction 2—material eluting from G100 before RNase; Fraction 3—material not adsorbed by CM-Sephadex. RNase activity was not detectable in Fractions 1–3.

Identity of factors conferring resistance to phenol

As stated above, tobacco RNases themselves are irreversibly denatured by phenol in contrast to some other RNases.[6–9] During leaf extraction RNases could combine with some leaf component(s) that protect the enzymes from denaturation in phenol. Combination with RNA and DNA almost certainly occurs;[10] proteins having opposite overall charges to the RNase could combine and exert a protective effect; polysaccharides may also be involved. Table 4 shows the extent to which various substances added to purified RNases can protect these enzymes from phenol denaturation. Both DNA and RNA exerted a significant protective effect but soya bean trypsin inhibitor (SBTI) had no effect. When RNase was treated with phenol in the presence of either DNA or RNA as well as SBTI, resistance to denaturation was increased even though SBTI alone had no effect.

TABLE 4. MATERIAL CONFERRING RESISTANCE TO PHENOL

Material added	Ribonuclease activity (units) % Before phenol	After phenol	Protection
None	6500	488	7·5
RNA	6500	858	13·2
Soya bean trypsin inhibitor	6500	429	6·6
DNA	6500	620	9·5
RNA + SBTI	6500	1391	21·0
DNA + SBTI	6500	1660	25·0

5 ml RNase solution was incubated with solutions of the added materials (10 mg in 5 ml H_2O) for 5 min at room temp. and then 2 ml 90% PhOH was added. After shaking, PhOH was removed by ether extraction and residual RNase activity was measured. Values quoted are average of three experiments.

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The increased protection afforded by the protein-nucleic acid mixture offers a possible explanation for the apparently greater resistance of RNase 2 than RNase 1 when crude leaf extracts are treated with phenol. In cell extracts, RNase 2 is found in the microsomal fraction whereas RNase 1 is a soluble enzyme.[4] If RNase 2 is closely associated with a nucleo-protein complex it would probably survive phenol treatment better than RNase 1. However, this explanation would not account for the phenol stability of partly purified RNase. The gradual loss of resistance to phenol during purification suggests slow dissociation of a relatively stable complex, such as might be formed between RNase and RNA breakdown products. As with many other enzymes, the presence of substrate or substrate analogues appears to stabilize the RNases to treatment with denaturing agents. The purine and pyrimidine 2',3'-cyclic nucleotides are primary hydrolysis products of both enzymes and the purine nucleotides, in particular, are potent inhibitors of tobacco RNase.[4,11,12] During leaf extraction, RNase could complex with RNA, DNA or RNA breakdown products, and such complexes might well survive phenol treatment better than free enzymes. We could not detect RNase-nucleic acid complexes in leaf extracts, although such complexes can be detected when pancreatic RNase is chromatographed on dextran gels in the presence of RNA.[13] RNase-nucleotide complexes may be present but we have no evidence that this is so, or that such complexes are responsible for the increased resistance to phenol. Identification of the factor(s) responsible for conferring resistance has not yet been achieved.

RNase activity in the presence of phenol

Breakdown of RNA molecules during extraction from leaves is unlikely, as long as the aqueous extract is saturated with phenol. Tobacco ribonuclease was incubated at pH 5·4 with various amounts of phenol. After incubation, enzyme action was stopped by the addition of HCl, phenol was removed by ether extraction and the amount of acid-soluble hydrolysis products was determined. Little inhibition of tobacco RNase activity occurs until the solution is 25% saturated with phenol. After this there is a rapid loss of activity until, at 50% saturation, activity is undetectable. When phenol is removed from aqueous RNase-RNA mixtures, residual RNase degrades the RNA. Precipitation of RNA with ethanol before phenol removal does not overcome this problem as co-precipitation of RNA and RNase occurs.[14]

Diethyl pyrocarbonate (DEP) has often been used as a nuclease inhibitor[15–18] and it causes denaturation of pancreatic RNase,[19] an enzyme known to survive phenol treatment.[6–9] DEP has been used to inhibit plant RNases during polysome preparation.[20–22] Samples of phenol-treated tobacco RNase at various stages of purification were incubated

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with 0·5% DEP in water for 5 min at 37°. After this treatment all RNase activity was destroyed, so addition of small amounts of DEP to leaf extracts might avoid problems caused by phenol resistant RNases. However, DEP is known to modify nucleic acids[23–25] and so is an unsuitable reagent when their biological activities are being studied.

TABLE 5. EXTRACTION OF PHENOL-RESISTANT RNASE FROM LEAVES OF DIFFERENT AGES

Extraction procedure	Ribonuclease activity (units) Upper leaves	Middle leaves	Lower leaves
Buffer alone	319	426	310
Buffer/phenol (50:50)	56	61	54
Buffer followed by phenol			
(a) phenol added before fibre removed	35	63	66
(b) phenol added after fibre removed	16	52	62

2 g fresh leaf tissue was extracted under the conditions shown. The buffer was 50 mM pH 6 Na citrate. After centrifugation, PhOH was removed by Et_2O extraction and RNase activity was measured.

Amount of resistant RNase in leaves of different ages

The infectivity of extracts from tobacco leaves infected with TMV and all finally treated with phenol, depends on the conditions of extraction. In the presence of air, extracts from lower leaves are more infective when the addition of phenol is delayed till after pulping is complete than when phenol is present during pulping. With upper leaves, the sequence is immaterial. Bawden and Pirie[2] concluded that a difference in the extent of fixation of TMV: RNA to leaf fibre was the main reason for this difference in infectivity, but suggested that differences in the amount of phenol resistant RNases could also be involved. The proportion of phenol resistant RNase extracted from tobacco leaves is unaffected by leaf age and also the extraction procedure (Table 5). However, the extraction of RNA is affected (Table 6). The effects observed with TMV infected leaves are, therefore, more likely to be due to differences in nucleic acid extraction than to differences in extraction of phenol resistant RNase.

Diffusibility of phenol resistant RNase

The observation[26] that diffusates from concentrated, phenol treated extracts from uninfected tobacco leaves inactivated TMV: RNA, that, after further fractionation, inactivating material was retained on dialysis, and that these retained preparations contained RNase, led Bawden and Pirie to suggest that RNase aggregates during purification, or that its passage through dialysis membrane is facilitated by other substances.

The first possibility can be excluded; only a small fraction of the total RNase activity of crude leaf extracts is diffusible and there is no marked change during fractionation (Table 7). Gel filtration studies likewise gave no evidence for aggregation. Leaf extracts

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vary in composition and the turgor imposed on the dialysis tubing during the preparation of diffusates by Bawden and Pirie[26] was uncontrolled; it obviously decreases during purification because large amounts of small MW material are removed. This material does not appear to facilitate the passage of RNase through a strained membrane (Table 7) but whether phenol can do so remains unestablished. The small amount of enzyme present in diffusates makes assay by a chemical method difficult and measurement by the inactivation of TMV:RNA is imprecise; but TMV:RNA preparations are so often freed of phenol by dialysis that it would be interesting to know if phenol does facilitate the diffusion of RNase.

TABLE 6. EXTRACTION OF NUCLEIC ACIDS FROM LEAVES OF DIFFERENT AGES

Extraction procedure	Total nucleic acid (E_{260} units) Upper leaves	Middle leaves	Lower leaves
Buffer/phenol (50:50)	61	14	8
Buffer followed by phenol			
(a) Phenol added before fibre removed	63	39	25
(b) Phenol added after fibre removed	58	30	25

2 g fresh leaf tissue extracted as described. Buffer was 50 mM Na citrate containing 1% Na dodecyl sulphate. The nucleic acids were precipitated from the aq. phase with 2 vol. EtOH at −20°. The precipitates were washed 2× with EtOH, 2× with Et_2O, redissolved in citrate buffer and reprecipitated with EtOH before estimation by UV absorbance at 260 nm.

The phenol resistant fraction of tobacco leaf RNase would almost certainly be a factor involved in the inactivation of TMV:RNA but the extent to which it is involved is difficult to assess. Other leaf components are certainly involved in inactivation and several of these have been discussed by Bawden and Pirie.[1–3] The phenol resistant RNase fraction appears to be an artefact due to interaction of the enzymes with other, so far unidentified, leaf components.

TABLE 7. DIFFUSIBILITY OF TOBACCO RNASE

Purification stage	Ribonuclease activity (units) Diffusible	Indiffusible
Concentrated extract	47	16700
Phenol-treated concentrate	24	1600
G100 fraction	18	1300
CM-Sephadex		
Peak 1	Not detectable	430
Peak 2	Not detectable	304

Samples of ribonuclease were dialysed in 6 mm Visking dialysis tubing against three changes of dist. H_2O over 48 hr. The diffusates were concentrated before assay.

EXPERIMENTAL

Plant material. Leaves from mature *Nicotiana tabacum* var. Java (usually 1·5 m high and forming flower buds) grown in a glasshouse were used.

Extraction. Intact leaves were frozen at −15° in about 5 vol. H_2O and kept frozen for at least 24 hr; after thawing they were left at 4° for a further 24 hr. The limp mass of leaf was pressed lightly without rubbing. To

each l. of the pale brown extract, 2 ml conc. NH_3 soln was added. The ppt. settled quickly and, after decanting the supernatant, was compacted by centrifugation. The ppt. contained 25% of the RNase. This was recovered by suspending the ppt. and adding enough $(COOH)_2$ soln to bring the suspension to pH 3, centrifuging and adjusting the pH of the extract to 8 with NH_3. A crystalline deposit of Mg and Mn salts separated out in a few hr and was centrifuged off. The soln was then added to the original supernatant and conc. *in vacuo*. It was convenient to continue until the soln had 0·25 of the initial wt of the leaves because it then had such a density that, when mixed with PhOH, the PhOH floated. 2–3 ml of 90% PhOH were added to each 100 ml conc. extract. enough to ppt. much of the protein but not to form a PhOH phase. The supernatant after centrifugation was adjusted to pH 8·5 and shaken with an equal vol. of PhOH. The PhOH layer was separated and the aq. layer re-extracted with PhOH. After a third extraction, most of the RNase activity and little of the salts and sugars of the leaf extract were in the PhOH layers. This initial separation of enzyme from material of small MW is useful when enzyme assays are being made at early stages of purification: it is often of no importance when the whole preparation is to be purified by gel filtration. Because RNase movement into the PhOH layer was incomplete, bulk preparations were therefore made without the separation. Either the combined PhOH extractions, or the H_2O:phenol emulsion, were distilled *in vacuo*. The gum obtained after removal of PhOH was dissolved in a wt of H_2O equal to 2–5% of the initial wt of the leaves, adjusted to pH 8·5, mixed with 2 vol. EtOH, and left overnight at −20°. Less than 1% of the RNase activity remained in the supernatant and was discarded. The precipitated material was dissolved in 50 mM pH 5·4 NH_4 acetate containing 50 mM KCl and dialysed against the same buffer overnight to remove residual EtOH. The soluble material remaining after dialysis was purified on Sephadex G100, CM-Sephadex and Sephadex G75 as described elsewhere.[4]

Extraction of nucleic acids. 2 g of fresh leaf tissue was ground in a Ten-Broeck all-glass homogenizer with 4 ml Na citrate buffer (50 mM pH 6·0 + 1% Na dodecyl sulphate). PhOH was added as a deproteinizing agent at various times (Table 6). The pulped material was centrifuged at 2000 *g* for 20 min to aid phase separation. The aq. phase was removed and the PhOH phase was re-extracted with a further portion of buffer. The combined aq. phases were added to 2 vol. of cold (−20°) EtOH and stored at −20° for 24 hr to ensure complete precipitation. The flocculent ppt. was collected, washed 2 × with EtOH, 2 × with Et_2O and then dissolved in 5 ml buffer and re-precipitated with EtOH. The resulting material was dissolved in citrate buffer and the nucleic acid in it was measured by absorbance at 260 nm. The material had an absorbance maximum at 260 nm and a minimum at 234 nm. The ratio of E_{max} to E_{min} was usually greater than 2 except when old leaves were used.

Measurement of phenol-resistant RNase in leaves. Measurements of the amount of PhOH-resistant RNase in leaves were carried out by extracting 2 g samples of fresh leaf tissue as described above. Instead of separating PhOH and aq. phases, PhOH was removed from fibre-free extracts by ether extraction and dialysis. The RNase contents of the extracts were then determined.

Enzyme assays. Ribonuclease was assayed as described in the accompanying paper[4] using highly polymerized yeast RNA as substrate.

Protein estimation. Protein was estimated by the method of Lowry *et al.*[27] using bovine serum albumin as the standard protein.

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