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PARTIAL PURIFICATION AND CHARACTERIZATION OF TWO *NICOTIANA TABACUM* LEAF RIBONUCLEASES

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Abstract—Two enzymes with similar properties that degrade RNA but not DNA have been partially purified from tobacco leaves. They differ in sub-cellular localization and in ability to hydrolyse ribonucleoside 2′,3′-cyclic phosphates.

INTRODUCTION

STUDIES on the ribonucleases (RNases), in tobacco leaf extracts treated with phenol[1] showed that at least two enzymes were present. The pH optima of these two RNases suggested that they were similar to those reported previously.[2,3] Recently,[4] it has been reported that infected or mechanically damaged tobacco leaves contain two RNases, one a relatively guanine-specific endoribonuclease and the other a relatively adenine-specific endoribonuclease. Earlier work[5] suggested that tobacco leaves damaged by being infiltrated with water contained only one RNase that apparently did not correspond to the RNases found in healthy, undamaged leaves.

A purification procedure has been developed that resolves tobacco RNase preparations into several fractions with apparently distinct activities. Two of these are relatively guanine-specific endoribonucleases that resemble the enzymes reported earlier.[2,3] These enzymes have now been purified and some of their properties are reported here. The other enzymes have been partly characterized and their properties will be reported elsewhere.

RESULTS AND DISCUSSION

Purification of crude enzyme preparations

The dialysed crude leaf extract prepared as described in the Experimental was applied in 20 ml portions to a 4 × 90 cm column of Sephadex G100 equilibrated and eluted with buffer A. The column effluent was monitored for absorbance at 254 nm and RNase activity.

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Two distinct peaks of RNase activity were detectable. The higher MW fraction eluted immediately after the void volume of the column and was contaminated with phosphodiesterase and phosphomonoesterase activities. The lower MW fraction was eluted between 200 and 500 ml after the void volume. This fraction contained phosphomonoesterase but was free from phosphodiesterase. It was collected and dialysed against 10 mM Na citrate buffer, pH 4·8 (buffer B), and the precipitate that formed was removed by centrifugation.

Chromatography on carboxymethyl Sephadex

The soluble material obtained by the above procedure was applied to a 3 × 15 cm column of Sephadex CM25 equilibrated with buffer B. Most of the coloured material in the extract was not adsorbed and passed through the column unretarded. The column was then washed with 500 ml buffer B followed by a linear gradient from 10 mM Na citrate pH 4·8 (500 ml) to 50 mM Na citrate pH 5·8 (500 ml). Finally the column was washed with 300 ml of 50 mM Tris-HCl buffer, pH 8·0, containing 100 mM NaCl. Four distinct RNase containing fractions were detectable. One fraction (Peak A) was present in the material not adsorbed by the column. Two fractions (Peaks B and C) were eluted by the citrate gradient, and a final fraction (Peak D) was eluted by Tris-NaCl. The enzymes dealt with in this paper are those eluted by citrate. Peak B (RNase 1) was eluted between 220 and 480 ml after the start of the gradient. Peak C (RNase 2) was eluted between 490 and 650 ml after the start of the gradient. The fractions constituting each peak were combined, concentrated and dialysed against buffer A.

TABLE 1. PURIFICATION OF *Nicotiana tabacum* RIBONUCLEASES

Purification stage	Total RNase (units)	Total protein (mg)	Sp. act. (U/mg protein)	Fold purification	Recovery (%)
Crude extract	56200	2760	20·4	1	100
Non-diffusible fraction	44200	276	160	7·8	78
Low MW fraction from G100	38700	53	729	35·7	68
Citrate-dialysed G 100 fraction	29400	20·4	1440	70·7	52
CM-Sephadex					
Peak 1	9350	0·16	58400	3730	20
Peak 2	2100	0·68	3100	635	
Sephadex G75					
RNase 1	8450	0·13	61100	4070	18
RNase 2	1830	0·44	4200	905	

Sephadex G75 chromatography

Each concentrated enzyme solution was applied to a 3 × 100 cm column of Sephadex G75 equilibrated with buffer A. The column was calibrated for MW determination using a series of proteins of known MW as markers.[6] After elution from the column, RNase fractions were combined, dialysed against distilled water and stored at −20°. Estimates of the MW of the two enzymes gave values of 19700 for RNase 1 and 21000 for RNase 2. The

[6] ANDREWS, P. (1964) *Biochem. J.* **91**, 222.

results of a typical purification from 1 kg of leaves are summarized in Table 1. RNase 1 was purified about 4000-fold and RNase 2 was purified 1000-fold, assuming that RNase 2 accounted for 22·5% of the total activity of the crude leaf extract.

Effect of pH and temperature on enzyme activity

Determination of the pH optima of the two enzymes in a range of citrate-phosphate buffers gave values of 5·1 for RNase 1 and 5·8–6·0 for RNase 2. These figures agree well with those obtained earlier,[2,3] and are typical of plant RNases. The relatively guanine-specific RNase obtained from infected or damaged tobacco leaves[4] had a pH optimum of 5·5. This enzyme corresponds to the low MW fraction from Sephadex G100 obtained here. Further purification of this fraction has resolved the activity into the two enzymes described here.

Both enzymes were heat stable, RNase 1 showing only 5% loss of activity after 10 min at 100°. Under identical conditions, RNase 2 lost about 20% of its activity.

Substrate specificity

Both enzymes degraded RNA to acid-soluble products but neither had any detectable effect on double- or single-stranded DNA. With highly polymerized yeast RNA as the substrate, RNase 1 had a K_m of 0·34 mg/ml and RNase 2 had a K_m of 1·2 mg/ml. Both had the two-stage action typical of plant RNases. PC analysis of the hydrolysis products showed that the first products were the 2′,3′-cyclic ribonucleotides, G > p appearing first followed by A > p, U > p and finally C > p. The second stage of the enzyme action was a hydrolysis of the cyclic nucleotides and here, a previously reported difference in action[7] was confirmed. RNase 1 caused hydrolysis of G > p and A > p to give the 3′-monophosphates. No significant enzymic hydrolysis of the pyrimidine cyclic nucleotides could be detected, even after prolonged incubation. In contrast, RNase 2 hydrolysed both purine and pyrimidine cyclic nucleotides, but exhibited a marked preference for the purine nucleotides. Incubation of purified enzymes with cyclic nucleotides as described in the Experimental resulted in RNase 1 hydrolysing 54% G > p, 38% A > p, 2% U > p and 3% C > p in 24 hr. Under similar conditions RNase 2 hydrolysed 47% G > p, 33% A > p, 13% U > p and 9% C > p.

The appearance of G > p as the first detectable product of RNA hydrolysis by both enzymes suggests that both are endo-nucleases. This was confirmed by separating the products of partial RNA hydrolysis on a column of Sephadex G75.[8]

Inhibition of RNases

As described previously,[1] reagents such as phenol, bentonite and diethyl pyrocarbonate have been widely used as RNase inhibitors. Many leaf RNases are inhibited by metal ions, especially by zinc and copper.[9] Few results are available about inhibition of RNases by compounds present in leaves. Polyamines were used to inhibit RNase during extraction

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[8] BIRNBOIM, H. (1966) *Biochim. Biophys. Acta* **119**, 198.
[9] ANFINSEN, C. B. and WHITE, F. H., Jr., (1961) *Enzymes* **5**, 95.

of microsomal RNA from pea roots.[10] RNA hydrolysis by an *Avena* RNase was inhibited by the end products of hydrolysis.[11] DNA inhibited a RNase from *Phaseolus*.[12]

A number of substances normally present in leaves were examined for their ability to inhibit tobacco RNase. Both of the enzymes were inhibited by heat-denatured DNA but not by native DNA. Inhibition was about 50% for both RNase 1 and RNase 2 when DNA and RNA were present in the incubation mixture in the same concentration.

The purine nucleotides (both 2',3'-cyclic and the 2',(3')-non-cyclic) caused inhibition of both enzymes. At a concentration of 1 mM 2',(3')-GMP inhibited both enzymes by about 75%; 2',(3')-AMP by about 50%; 2',3'-GMP by about 60% and 2',3'-AMP by about 40%. The 5'-nucleotides had no effect at this concentration.

The pyrimidine nucleotides had no inhibitory effect on RNase 1, but at 1 mM 2',(3')-UMP inhibited RNase 2 by 15%; 2',(3')-CMP by 11%; 2',3'-UMP by 10% and 2',3'-CMP by 8%. Again the 5'-nucleotides had no effect. The inhibitory properties of 2',(3')-GMP have been used to develop an affinity chromatographic method for the purification of tobacco RNase.[13,14]

Polyamines are potent inhibitors, as might be expected from their ability to form stable complexes with nucleic acids.[15] At a concentration of 0·5 mg/ml, spermine completely inhibited both enzymes. Putrescine and cadaverine were slightly less effective at the same concentration giving *ca* 60 and *ca* 50% inhibition respectively.

Both enzymes were inhibited by a large number of divalent metal ions, copper, zinc and mercury being the most effective. Stimulation of activity could not be demonstrated by any divalent metal ion and the presence of EDTA or citrate in incubation mixtures usually resulted in slight stimulation of activity.

The monovalent ion Ag^+ was a potent inhibitor, 10^{-4} M Ag causing 100% inhibition, as did 10^{-4} M Hg^{2+} and Cu^{2+}. Zn^{2+} was needed at 5×10^{-4} M to achieve complete inhibition. NaCl and KCl stimulated the activity of RNase 1 slightly but had no effect on RNase 2. At higher concentrations than 100 mM, KCl inhibited both enzymes.[14]

Table 2 summarizes the properties of the two RNases.

Intracellular distribution

Identification of the sub-cellular location of the two RNases described might give some clue as to the role of these enzymes *in vivo*. The difficulties involved in obtaining an unequivocal answer about the true location of enzymes are well known and in most cases the results obtained can, at best, only provide a suggestion of the *in vivo* situation. Where attempts have been made to locate plant RNases, group 1 RNases appear to be present in the soluble fraction and group 2 RNases appear to be microsomal.[7,16] Both of these designations could easily be erroneous. The group 1 RNases may be present in sphero-

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[11] WYEN, N. V., UDVARDY, J., SOLYMOSY, F., MARRE, E. and FARKAS, G. L. (1969) *Biochim. Biophys. Acta* **191**, 588.
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[13] JERVIS, L. (1972) *Biochem. J.* **127**, 29P.
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[15] BAWDEN, F. C. and PIRIE, N. W. (1959) *J. G. Microbiol.* **21**, 438.
[16] WILSON, C. M. (1968) *Plant Physiol.* **43**, 1332.

TABLE 2. SUMMARY OF THE PROPERTIES OF *Nicotiana tabacum* RIBONUCLEASES

Enzyme	MW	pH optimum	Substrates hydrolysed	Base specificity	Inhibitors	Sub-cellular localization
RNase 1	19700	5·1	RNA G>p; A>p	G>A>U>C	Denatured DNA 2′(3′)GMP, G>p 2′(3′)AMP, A>p Spermine Cadaverine Putrescine Zn^{2+}, Cu^{2+}, Hg^{2+}, Ag^{+} Tetrametaphosphate	Soluble
RNase 2	21000	5·8–6·0	RNA G>p; A>p U>p; C>p	G>A>U>C	Denatured DNA 2′(3′)GMP, AMP, UMP, CMP G>p, A>p, U>p, C>p Spermine Cadaverine Putrescine Zn^{2+}, Cu^{2+}, Hg^{2+}, Ag^{+} Tetrametaphosphate	Microsomal

somes *in vivo*.[17–20] The group 2 RNases may be adsorbed onto microsomes during cell disruption,[21,22] and when a high ionic strength buffer is used to extract leaves, this group of RNases is frequently found in the soluble fraction.

When tobacco leaves were macerated in 400 mM sucrose and the resulting extract fractionated by differential centrifugation, RNase activity was found in all fractions obtained. CM-Sephadex chromatography of RNase released from microsomes revealed that the main enzyme present was RNase 2, some RNase 1 also being present. The results obtained with the post-microsomal supernatant were rather less clear but RNase 1 was by far the most abundant component. This result indicates a probable microsomal location for RNase 2 and that RNase 1 may be a soluble enzyme, thus confirming previous work.[7]

As can be seen from Table 1, the ratio of RNase 1 to RNase 2 is *ca* 5 when the whole plants are used for enzyme isolation. Preliminary results indicate that this ratio is very variable and depends on the age of leaves and their physiological state. Old leaves have a high ratio whereas young leaves have approximately equal amounts of the two enzymes.

EXPERIMENTAL

Plant material. Tobacco plants (*Nicotiana tabacum* var. Java) were grown under greenhouse conditions and harvested when 1·5–2 m high.

Chemical reagents. Highly polymerized yeast RNA was obtained from B.D.H. Ltd., as were calf thymus DNA, soya bean trypsin inhibitor, sperm whale myoglobin, cytochrome-*c*, α-chymotrypsinogen, *p*-nitrophenylphosphate and bis-*p*-nitrophenylphosphate. Nucleotides were from Boehringer.

Preparation of crude tobacco ribonuclease. 1 kg of tobacco leaves was frozen at −20° in a polythene bag. The frozen leaves were crushed to a powder and added to 2 l. of 50 mM pH 6 Na citrate buffer. The suspension was macerated for 1 min in a blender and the resulting slurry was filtered through 2 layers of muslin. The filtrate was adjusted to pH 4 with HOAC and allowed to stand at 4° for 1 hr before centrifugation. The ppt. was free from RNase activity and was discarded. The supernatant was adjusted to 85% saturation with $(NH_4)_2SO_4$ and the pH was raised to 5·5 with NH_4OH. After 12 hr at 4°, the precipitated material was collected and dissolved

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[18] MATILLE, PH. (1968) *Z. Pflanzenphysiol.* **58**, 365.
[19] MATILLE, PH., BALZ, J. P. and SEMADENI, E. (1965) *Z. Naturforsch.* **20b**, 693.
[20] MATILLE, PH. and SPICHIGER, J. (1968) *Z. Pflanzenphysiol.* **58**, 277.

in 40 ml 50 mM pH 5·4 ammonium acetate containing 50 mM KCl (buffer A). Insoluble material was removed and the crude enzyme solution was dialysed against 3 changes of buffer A. After clarification by centrifugation the non-diffusible fraction was stored at −20° until required.

Enzyme assays. Ribonuclease was assayed at pH 5·5 by incubating 0·1 ml of RNA soln (1 mg/ml) with 0·1 ml pH 5·5 NH_4 acetate buffer (100 mM), 0·2 ml H_2O and 0·1 ml of enzyme soln at 37°. After incubation the reaction was stopped by addition of 0·1 ml 1 M HCl. The tubes were cooled for 1 hr and diluted with 1·9 ml 200 mM HCl. After centrifugation at 2000 rpm for 40 min, the supernatant solns were decanted into 1 cm quartz cells and the absorbance at 260 nm was measured on an Optica CF 4R spectrophotometer. The enzyme unit was defined as the amount needed to cause an increase of 0·1 units of absorbance at 260 nm in 1 hr.

Phosphomonoesterase was assayed using *p*-nitrophenylphosphate as substrate,[23] and phosphodiesterase was assayed using bis-*p*-nitrophenylphosphate as substrate at pH 9·8 and 5·8.[24]

Protein estimation. Protein was estimated either by absorbance at 280 nm or by the method of Lowry *et al.*[25] using bovine serum albumin as the standard protein. Crude leaf extracts contain considerable amounts of material that interfere with protein estimation. This interference was minimized by precipitating protein from crude preparations with Cl_3C COOH (TCA). The protein precipitated by 5% TCA was dissolved in 1 M NaOH before estimation.

Identification of hydrolysis products. Highly polymerized yeast RNA (5 mg) in 1 ml 100 mM citrate buffer (pH 5·1 or 5·8) was incubated with 10 units of RNase at 37°. After incubation for various intervals, 0·1 ml aliquots were taken and chromatographed on Whatman 3 MM paper using propan-1-ol conc. NH_3–H_2O (6:3:1) as solvent. The separated products were identified by comparison with known standards and by examination of their UV absorbance spectra.

Hydrolysis of cyclic nucleotides. Solns of the four main 2′,3′-cyclic ribonucleotides (5 mM) in 100 mM Na citrate buffer (pH 5·1 or 5·8) were incubated with 150 units of RNase at 37°. After incubation, the products were separated as described above. Spots were detected under UV light and nucleotides were eluted from chromatograms with 100 mM HCl. The spectra of the cyclic nucleotides was used to calculate the extent of hydrolysis of each. Enzyme-free blanks were run for each nucleotide to determine the amount of non-enzymic hydrolysis occurring.

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