BBA 66150

PURIFICATION AND PROPERTIES OF LEAF RIBONUCLEASE FROM SUGAR CANE*

W.-J. TANG AND A. MARETZKI
Experiment Station, Hawaiian Sugar Planters' Association, Honolulu, Hawaii 96804 (U.S.A.)

(Received April 6th, 1970)

SUMMARY

A ribonuclease from mature sugar cane leaves was purified and characterized. The enzyme has a pH optimum of 5.0 and is relatively heat-stable. It does not require the presence of metal ions for its activity. The enzyme is strongly inhibited by Cu^{2+} and Zn^{2+}. It is a phosphotransferase which hydrolyzes yeast RNA to yield 2',3'-cyclic GMP, 2',3'-cyclic AMP and pyrimidine 2',3'-cyclic phosphates, in that order. Very little further hydrolysis of the 2',3'-cyclic mononucleotides was found.

INTRODUCTION

Studies of ribonucleases from various sources have been numerous; however, the ribonucleases characterized from flowering plants are relatively few compared to those from animal systems. The ribonucleases purified from plants so far are all phosphotransferases (cyclizing enzymes) with acid pH optima. These enzymes appear to cleave next to all four bases either at similar or varying rates. Nucleoside 2',3'-cyclic phosphates are obligate primary products resulting from the intramolecular attack by the 2'-OH group at the 3',5'-phosphodiester bond. Divalent ions are not required for activity[1]. The existence of an active ribonuclease in sugar cane leaf tissue is indicated by the difficulties encountered in obtaining intact ribosomal RNA from sugar cane leaf extract. We found the ribonuclease activity per g of tissue in the crude homogenate from leaves of a 6-month-old sugar cane plant to be about twice as great as that obtained from leaves of 20-month-old plants. This could indicate that sugar cane leaf ribonuclease plays a role during the growth and development of this plant.

The study of ribonuclease in leaves as well as in other tissues of sugar cane could determine how this activity may be controlled during the isolation of nucleic acids. In addition, such a study affords a better understanding of the role of ribonuclease in the normal growth of sugar cane and a possible relationship of this enzyme to hormonal systems functioning in sugar cane.

* Published with the approval of the Director as Paper No. 252 in the Journal Series of the Experiment Station, Hawaiian Sugar Planters' Association.

MATERIALS AND METHODS

Materials

Young, fully expanded leaves of sugar cane (*Saccharum officinarum*) of the variety H 50-7209 (about 20 months old) were used. Commercial yeast RNA (Nutritional Biochemical Corporation, Cleveland, Ohio) was purified according to WOODWARD[2]. A fresh solution of purified RNA (15 mg/ml) in dilute NaOH (pH 6) served as the substrate for the ribonuclease assay. Polyclar AT powder (polyvinylpyrrolidone) was obtained from General Aniline and Film Corporation, New York, N.Y. Sephadex G-100-40 (particle size 10–40 μ); 2′,3′-cyclic AMP; 2′,3′-cyclic GMP; 2′,3′-cyclic CMP; and 2′,3′-cyclic UMP were purchased from Sigma Chemical Company, St. Louis, Mo. The homopolynucleotides were obtained from Miles Laboratories, Inc. Elkhart, Ind., and the remaining mononucleotide standards were the products of CalBiochem., Los Angeles, Calif. DEAE-cellulose (Cellex-D) was obtained from Bio-Rad Laboratories, Richmond, Calif. Precoated cellulose thin-layer plates (thickness 0.1 mm) were purchased from Brinkman Instruments, Inc., Westbury, N.Y.

Methods

Ribonuclease determination. The ribonuclease activity was determined by measuring the increase in absorbance at 260 mμ of the acid-soluble hydrolysis products, using purified yeast RNA as the substrate. The reaction mixture contained 0.10 ml of 0.2 M sodium acetate (pH 5.0), 0.10 ml of RNA solution and the enzyme solution; the total volume was adjusted to 0.5 ml with water. After incubation at 37° for 30 min, the reaction was stopped by the addition of 0.12 ml of 0.75% uranyl acetate in 25% $HClO_4$ (ref. 3). The precipitate formed after standing at 2° for 15 min was removed by centrifugation and 0.1 ml of the supernatant was diluted to 3.0 ml for the measurement of absorbance at 260 mμ with a Beckman D.U. spectrophotometer. One unit of ribonuclease activity was defined as the amount of enzyme that caused an increase in absorbance of 0.1 unit per 30 min under the assay conditions described above. Because STOCKS *et al.*[4] have shown that those oligonucleotides that are normally acid-soluble can be coprecipitated by RNA, when the effect of substrate concentration was studied, additional RNA was added just prior to the addition of $HClO_4$ so that each tube had the same concentration of RNA. Deoxyribonuclease activity of the purified ribonuclease was determined in the same manner, using heat-denatured deoxyribonucleic acid as the substrate.

Protein determination. Protein concentration was determined by the method of LOWRY *et al.*[5] using bovine serum albumin (4 times crystallized) as a standard. In purification steps prior to gel filtration, aliquots for protein determination were first adjusted to 5% trichloroacetic acid concentration to precipitate proteins. The precipitate was washed once with 5% trichloroacetic acid and then dissolved in 1 M NaOH before color development.

Chromatographic determination. The following chromatographic solvents were used: Solvent I, $(NH_4)_2SO_4$ (45 g), 1 M sodium acetate (20 ml), water (55 ml) and isopropanol (2 ml)[6]; Solvent II, $(NH_4)_2CO_3$ (9.6 g), water (250 ml), and isopropanol (750 ml)[7]; Solvent III, *n*-propanol–conc. NH_4OH–water (60 : 30 : 10, by vol.)[8].

The monocucleotides released by the enzyme were identified as follows. An aliquot of the enzymatic hydrolysate of yeast RNA was spotted on a precoated thin-

layer cellulose plate and developed in Solvent I or II. After spraying with 0.2% fluorescein in 95% ethanol, the plate was observed under ultraviolet light and the locations of visible spots were compared with spots produced by various nucleotide standards.

Enzymatic hydrolysis of cyclic nucleotides. The incubation mixture contained 200 μmoles of sodium acetate (pH 5.0), 0.3 μmole of nucleoside 2',3'-cyclic phosphate (2',3'-cyclic AMP; 2',3'-cyclic GMP; 2',3'-cyclic CMP or 2',3'-cyclic UMP) and 2 units of purified ribonuclease in a final volume of 0.5 ml. A control without enzyme was prepared for each substrate. After incubation at 37° for 24 h, an aliquot of each sample was applied to Whatman No. 3 paper. The chromatogram was developed in Solvent III (ascending) for 5 h. The spots corresponding to the cyclic phosphates and their hydrolysis products were located under ultraviolet light and eluted with 0.1 M HCl. The ultraviolet spectrum of each eluate was measured with a Beckman D.K. spectrophotometer and the ratio of absorbance at maximum wavelength between the cyclic nucleotide and its corresponding hydrolytic product was calculated to determine the degree of hydrolysis.

Enzymatic degradation of polynucleotides. The 0.5-ml reaction mixture contained 1.5 mg of polynucleotide (yeast RNA, poly A, poly G, poly C, or poly U), 200 μmoles of sodium acetate (pH 5.0) and sugar cane ribonuclease. Aliquots of 0.1 ml were withdrawn from each sample after incubation at 37° for various periods of time. The volume of these aliquots was adjusted to 0.5 ml with water, and 0.12 ml 0.75% uranyl acetate in 25% $HClO_4$ was added. The precipitate was removed by centrifugation and the supernatant was diluted 6-fold before measuring its absorbance at 260 mμ. The degradation of poly U could not be followed by this method since poly U is acid-insoluble. Instead, 1.0 ml of cold absolute ethanol was added to precipitate the undegraded poly U present in the 0.1-ml aliquot[9].

Enzyme purification. Unless otherwise noted, all operations were performed at 4°.

Step 1. Homogenization. Sugar cane leaf tissue (60 g after removal of the midribs) was cut into small pieces and soaked in 8 vol. of ice-cold 0.05 M potassium phosphate buffer (pH 6.7) containing 60 g of Polyclar AT. The tissue was homogenized with an Omni Mixer (Sorvall, Inc., Norwalk, Conn.) set at high speed for four 30-sec periods with cooling between each homogenization. After passing the suspension through three layers of cheesecloth, the residue was again homogenized with 4 vol. of the same buffer. The two extracts were combined and designated "crude homogenate".

Step 2. Acidification. The crude homogenate (pH 6.7) was acidified with 1 M HCl to pH 5.0 and left at 4° overnight. During this period, most of the green-colored material settled. A yellow supernatant was obtained after centrifugation for 15 min at 27 000 × *g*.

Step 3. $(NH_4)_2SO_4$ fractionation. The acidified extract was brought to 50% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate was discarded and the supernatant further saturated to 80% with $(NH_4)_2SO_4$. The precipitated proteins were collected by centrifugation at 27 000 × *g* for 30 min, suspended in a small volume of 0.04 M potassium phosphate buffer (pH 6.7) and dialyzed against 2 l of the same buffer for 3 h. The dialysate was centrifuged at low speed to remove precipitate.

Step 4. Gel filtration. The dialysate supernatant was placed on a column of

Sephadex G-100-40 (2.1 cm × 35 cm) previously equilibrated with 0.04 M potassium phosphate buffer (pH 6.7). The column was eluted at room temperature with the same buffer. Active fractions were pooled and saturated to 80% with $(NH_4)_2SO_4$. The precipitated proteins were collected by centrifugation at 27 000 × *g* for 30 min, dissolved in a small amount of 0.04 M potassium phosphate buffer and dialyzed against 2 l of the same buffer solution for 3 h. Precipitates formed during dialysis were removed by low-speed centrifugation.

Step 5. DEAE-cellulose column chromatography. The dialyzed active fraction from gel filtration was adsorbed on a column of DEAE-cellulose (0.9 cm × 17 cm) preequilibrated with 0.04 M potassium phosphate buffer (pH 6.7). The column was washed with the same buffer followed by a linearly increasing concentration gradient at room temperature. Both the flow rate and the concentration of the eluting buffer were controlled by a programmed gradient pump (Dialagrad Model 190, Instrumentation Specialties Company, Inc., Lincoln, Nebr.). Active fractions were pooled and dialyzed against 2 l of double-distilled water for 3 h. The final dialysate was stored in the freezer and used for characterization studies of the enzyme.

TABLE I

PURIFICATION OF RIBONUCLEASE FROM 20-MONTH-OLD H 50-7209 SUGAR CANE LEAF TISSUE

Step	*Total units*	*Total protein (mg)*	*Recovery (%)*	*Relative purification (specific activity)*
Homogenization	6515	1457	100	1
Acidification	6200	413	95	3.3
50–80% $(NH_4)_2SO_4$ and dialysis	2015	22.68	31	19.8
Sephadex G-100	280*	0.86	4.3	72.2
DEAE-cellulose and dialysis	312*	0.26	5	300

* Due to the difference of assay pH.

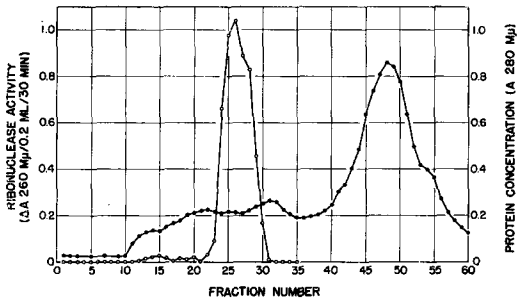
RESULTS

Purification

The results of a typical purification of ribonuclease are summarized in Table I. The data are given for 60 g of fresh leaves. A purification of 300-fold was obtained. Fig. 1 is a representative gel filtration with Sephadex G-100. Most of the ribonuclease activity was contained in Fractions 24–30. The elution profile from DEAE-cellulose column is shown in Fig. 2. A single, sharp, enzymatically active peak was always obtained, and this activity was eluted when the buffer concentration was approx. 0.3 M.

Properties of the ribonuclease

Effect of pH on ribonuclease activity. The activity of sugar cane ribonuclease at different pH's was measured in several buffer systems. Purified enzyme showed an optimal pH of 5.0 (Fig. 3). At pH 4.0 and 6.0 in acetate buffer, 73 and 24%, respectively, of the optimal activity were observed. Crude enzyme extract, however, had its optimal pH at 5.5 and the decrease in activity with increasing pH was less severe than that observed with the purified enzyme (Fig. 4). In citrate and citrate–phosphate



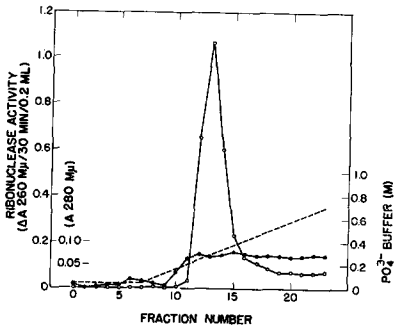


Fig. 1. Fractionation of sugar cane leaf ribonuclease on a column of Sephadex G-100-40 (2.1 cm × 35 cm). The flow rate was 7.5 ml/h and each fraction contained 2.5 ml. Protein was measured by absorption at 280 mμ. ○—○, ribonuclease activity; ●—●, absorbance at 280 mμ.

Fig. 2. Fractionation of sugar cane leaf ribonuclease on a DEAE-cellulose column (0.9 cm × 17 cm). The flow rate was 5 ml/h and each fraction contained 2.5 ml. Protein was measured by the absorbance at 280 mμ. ○—○, ribonuclease activity; ●—●, absorbance at 280 mμ; — — —, concentration of the eluting phosphate buffer.

buffer systems between pH 4 and 6, the change in ribonuclease activity was less than in the case of acetate buffer (Figs. 3 and 4).

Effect of substrate concentration. Purified ribonuclease (2 units) was assayed under the conditions described above but with varying amounts of yeast RNA. A typical Michaelis–Menten curve was obtained (Fig. 5). The K_m value calculated by the method of LINEWEAVER AND BURK[10] is 0.45 mg/ml for purified yeast RNA.

Effect of temperature. Both crude and purified enzymes were relatively heat-stable at their optimal pH. Heating at 50° for 10 min before assay did not cause inactivation of the enzyme, and at 60° approx. 70–80% of the activity was still retained. When the ribonuclease activity was measured at various temperatures (ranging from 0 to 80°) for 30 min at optimal pH, the purified enzyme showed 60° to be optimal

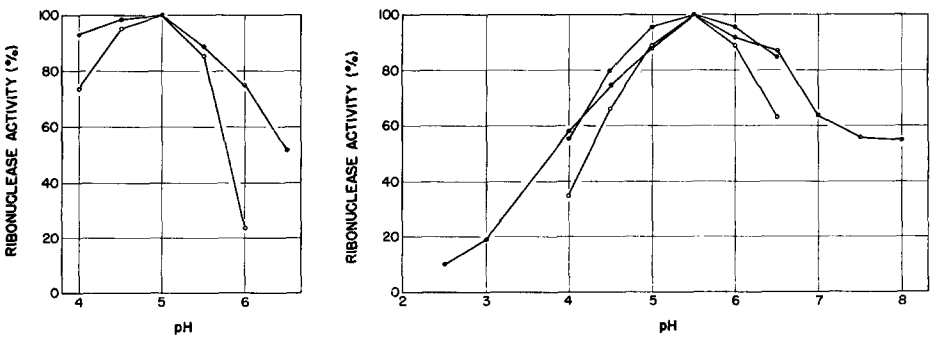
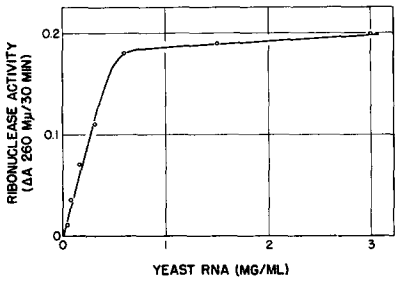


Fig. 3. Effect of pH on the activity of purified sugar cane leaf ribonuclease. The reaction mixture and procedure were as described for the standard assay. ○—○, acetate buffer; ●—●, citrate buffer.

Fig. 4. The effect of pH on the activity of crude sugar cane leaf ribonuclease. The crude homogenate was first dialyzed against double-distilled water for 3 h before assay. The reaction mixture and procedure were as described for the standard assay. ○—○, acetate buffer; ●—●, citrate buffer; ○—○, citrate phosphate buffer.



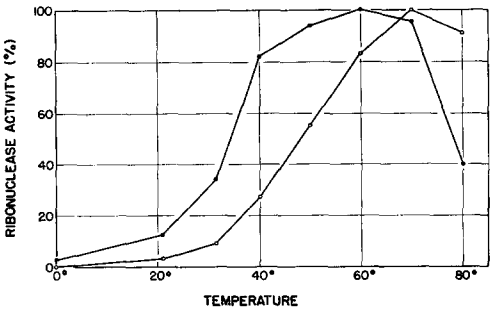


Fig. 5. Dependence of the rate of degradation of RNA on substrate concentration. The conditions for the reaction are described in the text.

Fig. 6. Effect of temperature on the activity of sugar cane leaf ribonuclease. The reaction mixture, as described in the text, was incubated at various temperatures for 30 min before uranyl acetate in $HClO_4$ was added. ●—●, purified enzyme; ○--○, dialyzed crude homogenate.

temperature for maximal activity; maximal activity for the crude homogenate was obtained at 70° (Fig. 6).

Effect of small molecules. Table II shows the effect of various small molecules on the activity of purified ribonuclease at pH 5.0. Zn^{2+} and Cu^{2+} at concentrations higher than 0.1 mM inhibited the enzyme. EDTA and cysteine had no effect, while *p*-chloromercuribenzoic acid at a concentration of 0.1 mM gave more than 80% inhibition.

Product identification. With Solvent I for chromatography, 2',3'-cyclic GMP was found to be the only mononucleotide released from yeast RNA when the incubation period was 30 min. If the hydrolysate was adjusted to pH 1 with HCl for 1 h at room temperature before spotting[11], two spots with higher R_F values were found, and these corresponded to standard 2'- and 3'-GMP. On further incubation, 2',3'-cyclic AMP and some pyrimidine 2',3'-cyclic phosphates could be detected in turn. This observation was verified by developing the thin-layer plate in Solvent II in which all the 2',3'-cyclic mononucleotides have an R_F value higher than 0.25, while the R_F values of non-cyclic mononucleotides are in the range of 0 to 0.05.

TABLE II

EFFECT OF SMALL MOLECULES ON RIBONUCLEASE ACTIVITY IN TERMS OF RELATIVE ENZYME ACTIVITY AT OPTIMAL pH

	Concentration (M)			
	$1 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	$1 \cdot 10^{-2}$
No additions	100			
Zn^{2+}	98.5	62.4	2.4	3.4
Cu^{2+}	98.5	76.0	5.4	5.0
Mg^{2+}	92	94	96	88
Mn^{2+}	111	107	100	80.5
KCl	96	90	102	88
EDTA	97	97	102	107
Cysteine	103	97	102	99
p-Chloromercuribenzoic acid	—	16.5	13.7	—

Specificity of the sugar cane ribonuclease. The enzyme did not degrade native DNA. The rate of degradation of heat-denatured DNA was found to be less than 1% of that of yeast RNA at pH 5.0. When 2',3'-cyclic mononucleotides were used as substrates, only negligible amounts of the noncyclic mononucleotides resulting from enzymatic hydrolysis were found, even after 24 h of incubation. The enzyme hydrolyzed poly A, poly C and poly U but not poly G. At pH 5.0 and low ionic strength, the relative rate of degradation was yeast RNA>poly U>poly A>poly C.

DISCUSSION

Like all the plant ribonucleases studied to date, the sugar cane leaf ribonuclease has an acidic pH optimum of 5.0 and its activity is not dependent upon the presence of metal ions. Divalent ions like Cu^{2+} and Zn^{2+} cause strong inhibition of activity, whereas ryegrass ribonuclease was activated by zinc acetate.[12] The sugar cane ribonuclease is a phosphotransferase and cleaves all internucleotide bonds in ribonucleic acid, giving rise to nucleoside 2',3'-cyclic phosphate but at different rates. 2',3'-cyclic GMP was the first mononucleotide to appear in quantity, followed by 2',3'-cyclic AMP. This observation suggests that the phosphodiester bond adjacent to a purine base is more susceptible to enzymatic attack than that adjacent to a pyrimidine base, especially when that purine base is a guanine. In this respect the sugar cane leaf ribonuclease resembles ryegrass ribonuclease[13]. It also suggests that the sugar cane leaf ribonuclease does not act as an exonuclease, at least at the early stage of degradation.

When homopolynucleotides served as substrates, the relative rate of degradation was not in agreement with the results obtained from yeast RNA. The highly ordered secondary structure of poly G may account for its failure to be degraded by the enzyme. In addition to the presence or absence of the secondary structure of the polymer under the assay conditions[14], different ionic requirements[15] and polymer concentrations[16] for optimal rates may also contribute to the observed sequence of degradation of poly U>poly A>poly C.

The purified ribonuclease showed some degradative activity toward heat-denatured DNA. The increase in acid-soluble materials was less than 1% compared with that found by using RNA. When the much higher molecular weight of the DNA sample is considered, this value may actually be slightly higher.

Unlike the situation with most other phosphotransferases, further hydrolysis of the nucleoside 2',3'-cyclic phosphates to either 2'- or 3'-monophosphates occurred at a negligible rate under the assay conditions; this is similar to the ribonucleases isolated from Avena leaf tissues[17] and *Proteus mirabilis*[18]. Both SHUSTER *et al.*[13] and FREEMAN[12] report the further hydrolysis of ryegrass ribonuclease to 3'-phosphates although at much lower rates than the formation of cyclic nucleotides. The possibility that this hydrolysis may be inhibited by the contaminating nucleotides bound to the ribonuclease cannot be ruled out[1].

Upon differential centrifugation, most the ribonuclease activity present in the homogenate of sugar cane leaves was found in the soluble fraction, while a small amount of activity was retained in the microsomal pellet. The latter may be an artifact of the homogenization procedure. There appears to be only one ribonuclease present in mature sugar cane leaves, since the pH profile of the ribonuclease activity

in crude homogenate showed only one optimum. If additional nucleases are present, their pH profiles must be very similar.

REFERENCES

1 E. A. BARNARD, *Ann. Rev. Biochem.*, 38 (1969) 677.
2 G. E. WOODWARD, *J. Biol. Chem.*, 156 (1944) 143.
3 K. TAKAHASHI, *J. Biochem. Tokyo*, 49 (1961) 1.
4 J. STOCKS, W. DIERICK AND L. VANDENDRIESSCHE, *Arch. Intern. Physiol. Biochim.*, 72 (1964) 647.
5 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
6 C. M. WILSON, *Plant Physiol.*, 43 (1968) 1339.
7 J. STOCKS AND R. VAN PARIJS, *Naturwissenschaften*, 47 (1960) 39.
8 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
9 P. F. SPAHR AND D. SCHLESSINGER, *J. Biol. Chem.*, 238 (1963) PC 2251.
10 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
11 D. M. BROWN, D. I. MAGRATH AND A. R. TODD, *J. Chem. Soc.*, 2708 (1952).
12 K. B. FREEMAN, *Can. J. Biochem.*, 42 (1964) 1099.
13 L. SHUSTER, H. G. KHORANA AND L. A. HEPPEL, *Biochim. Biophys. Acta*, 33 (1959) 452.
14 A. M. MICHELSON, J. MASSOULIÉ AND W. GUSCHLBAUER, *Progr. Nucleic Acid Res. Mol. Biol.*, Vol. 6 (1969).
15 P. F. SPAHR, *J. Biol. Chem.*, 239 (1964) 3716.
16 M. F. SINGER AND G. TOLBERT, *Biochemistry*, 4 (1965) 1319.
17 N. V. WYEN, T. UDVARDY, F. SOLYMOSY, E. MARRÉ AND G. L. FARKAS, *Biochim. Biophys. Acta*, 191 (1969) 588.
18 M. S. CENTER AND F. J. BEHAR, *Biochim. Biophys. Acta*, 151 (1968) 698.