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PURIFICATION AND PROPERTIES OF A RIBONUCLEASE FROM CORN LEAF TISSUES

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Key word Index—*Zea mays*; Gramineae; corn; ribonuclease; enzyme purification; enzyme characterization.

Abstract—An acid endoribonuclease isolated from corn leaf tissues was purified 530 times. Gel electrophoresis indicated that the enzyme was homogeneous. The enzyme showed an optimum pH at 5.5 and an apparent molecular weight of 32000. Corn RNase attacks natural RNAs and synthetic polyribonucleotides and the relative rate of degradation was poly U > yeast RNA > *E.coli* tRNA > poly A ≫ poly C. Zn^{2+}, Mg^{2+}, Mn^{2+} and EDTA inhibited the enzyme activity. No stimulation by K^+ was observed. Cu^{2+} and heparin had no effect on the activity. The results suggest that the investigated RNase differs from other known corn ribonucleases.

INTRODUCTION

In recent years several highly purified RNA-splitting enzymes have been obtained from different tissues of higher plants and in some cases their properties have been established in detail [1–5]. According to Wilson [6] the enzymes that degrade RNA in corn fall into three general classes: (a) RNase I, a soluble ribonuclease; (b) RNase II, associated to crude microsomes; (c) Nuclease I, associated with large cell particles. RNase I has been obtained from endosperm, while RNase II and Nuclease I have been purified from corn roots. RNases I and II are endonucleases which hydrolyse all the phosphodiester bonds of the RNA chain and show a preference for the guanine base. Nuclease I is a sugar non-specific endonuclease which hydrolyses preferentially adenine bases [7].

This report describes the isolation of a ribonuclease obtained from corn leaf tissue. The properties of this RNase such as chromatographic behaviour, thermolability, optimum pH, sensibility to bivalent cations, EDTA, as well as its capacity to hydrolyse preferentially poly U would indicate that the investigated RNase differs from those known to date from corn ribonucleases.

RESULTS AND DISCUSSION

RNase was purified from 10-day-old corn leaves using ammonium sulphate fractionation, cationic and anionic exchange resins, gel filtration and chromatography on a Cibacron Blue column. The protocol of corn ribonuclease purification indicates that the enzyme is not retained on CM-cellulose at pH 5.5. This fact facilitates a rapid method for the enzyme purification that removed contaminating nucleases and supports the idea that the isolated enzyme differs from similar RNases reported from corn roots [1, 8]. In that respect, Wilson showed that RNase I and II as well as Nuclease I were adsorbed onto CM-cellulose equilibrated in citrate buffer at pH 5.0 and upon shifting the pH to 6.0 the elution of RNase I, with other less adsorbed enzymes, was achieved. He reported the existence in this fraction of an RNase activity eluting at pHs higher than 5.2 and lower than pH 6.0. However, this fraction was not analysed [8]. Hence, the investigated RNase in the present report corresponds to this active fraction which was not retained on CM-cellulose at these pHs.

The results of a typical purification procedure of the corn leaf RNase are summarized in Table 1. The data are given for 1 kg of fresh leaves. A purification of 530-fold was achieved. This is a conservative estimate since it is not possible to determine the activity of one specific nuclease in the crude extract when there are several others present.

A crucial step in the purification was the utilization of affinity chromatography (Fig. 1). It has been proposed that the binding of proteins to the blue chromophore of Blue Sepharose (Cibacron Blue F3GA) is diagnostic for the presence of a 'dinucleotide fold' in their structure [9]. Nevertheless, this seems not to be the case, since although around 3% of the applied protein and 60% of the applied activity are retained on Blue Sepharose, the enzyme is eluted with a low concentration of potassium chloride, suggesting that simple non-specific ionic binding occurs [9].

The purity of the enzyme after Cibacron Blue chromatography was tested by polyacrylamide electrophoresis using the buffer system at pH 8.3 in the presence of SDS [10] or at pH 5.0 [11]. The analysis gave a single band in both electrophoretic systems indicating homogeneity of the enzyme. The apparent M_r was estimated to be 32000. The M_r of the enzyme is at the high end of the range commonly reported for plant RNases; e.g. for barley leaf and barley seed RNases, 25000 and 19000 [2, 12]; the rice bran enzymes, 14500 and 35000 [4]; corn RNase I, 23000; corn RNase II, 17000 [1].

The procedure of Kasai and Grunberg-Manago [13] was used for the determination of the endo- or exonucleolytic activity of the isolated enzyme. Paper chromatography of the hydrolytic products did not reveal the appearance of mononucleotides, after the incubation of [8-^{3}H]polyadenylic acid or [5-^{3}H]polyuridylic acid incubated for 10–60 min (data not shown). This result

Table 1. Purification of RNase

Purification step	Total protein (mg)	Total units	Specific activity (units/mg protein)	% Recovery	Purification factor
Crude extract	3190	33077	10.4	100	1
$(NH_4)_2SO_4$	1548	30431	19.6	92	1.9
CM-cellulose	138	4564	33	13.8	3.2
DEAE-cellulose	50	2875	58	8.6	5.6
Sephadex G-100	8.1	2315	286	7.0	30
Cibacron Blue Sepharose	0.25	1389	5556	4.2	534

*Specific activity of product / Specific activity of crude extract.

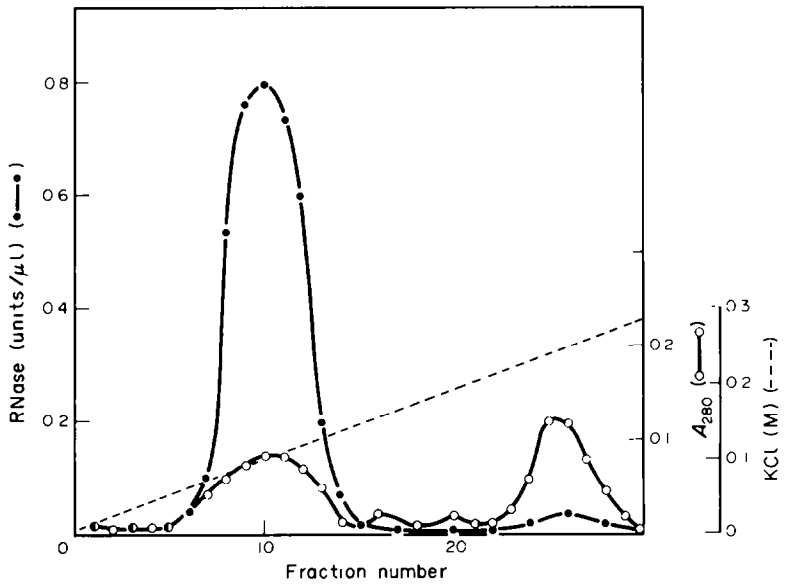

Fig. 1. Cibacron Blue Sepharose column (1 × 25 cm) equilibrated with 10 mM citrate buffer pH 5.5. Around 8 mg of protein (2300 units) were loaded and the column was washed with the equilibrium buffer until A_{280} (○) was null. The RNase activity (●) was eluted with a linear KCl gradient (- - - - - -) between 0 and 300 mM.

suggests that the corn RNase appears to be an endonuclease. RNase from corn leaves as obtained by the present procedure is completely free of phosphodiesterase and phosphatase [14] activity (see Experimental).

The corn leaf RNase showed an acidic pH optimum of 5.5. Activity decreased sharply above and below this value. The pH requirement for optimal activity is 0.5 units higher than most RNases isolated from various plant sources [2, 4, 15, 16]. Concerning corn RNases, Wilson [8] found that RNase I and RNase II extracted from root tissues showed optimal pHs of 5.0 and 6.2, respectively.

The ribonuclease activity was measured at various temperatures, ranging from 10° to 70° at optimal pH. The enzyme showed that the maximal activity is reached at 30–40°. Corn RNase is more thermolabile than has been usually reported for plant RNases [4, 7]. A complete inactivation was attained preincubating the enzyme at 55° for 30 min. As a control, pancreatic RNase A remained at full activity even at 80° under comparable experimental conditions.

To obtain information about the specificity of the enzyme with respect to base composition of the substrate, synthetic homopolymers were assayed at pH 5.5 according to the method outlined in the Experimental. Table 2 indicates that RNase from corn leaves degrades poly U, poly A and poly C but not poly G. The highly ordered secondary structure of poly G may account for its failure to be degraded by the enzyme. The relative rate of degradation was poly U > yeast RNA > *E. coli* tRNA > poly A ≫ – poly C. In this respect, the corn leaf ribonuclease resembles RNase I from barley seeds [12], a sugar cane ribonuclease [17] and a ribonuclease extracted from wheat germ [18]. As a control, pancreatic RNase A was tested showing a preference for pyrimidine phosphodiester linkages (Table 2). Concerning corn RNases, Wilson [7, 19] found that RNases I and II are endonuc-

Table 2. Activity of RNase from corn leaves with various substrates

Substrate	Relative activity (%)* A	B
Yeast RNA	100	100
E. coli tRNA	83	92
Poly U	159	192
Poly A	60	0
Poly C	31	158
Poly G	0	0
Native DNA (calf thymus)	0	13
Denatured DNA (calf thymus)	0	15

A, The enzyme activity was measured as described under Experimental, using 19 ng (0.1 unit) of the corn ribonuclease and 40 μg of substrate.

B, As a control, pancreatic RNase A was tested. The activity was measured in 20 mM sodium citrate at pH 5.5.

* 100% corresponds either to 0.1 units of the corn nucleolytic activity or 1.0 unit of pancreatic RNase activity.

Table 3. The effect of cations, EDTA and heparin on the activity of corn ribonuclease

Substance added*	Concentration	Relative activity† (%)
None	—	100
$MgCl_2$	5 mM	53
	10 mM	43
$MnCl_2$	5 mM	50
	10 mM	40
$ZnCl_2$	5 mM	50
	10 mM	0
$CuCl_2$	30 mM	100
KCl	150 mM	100
NaCl	150 mM	100
EDTA	5 mM	83
	10 mM	52
Heparin‡	200 μg/ml	100

*The enzyme (19 ng) was preincubated at 30° for 30 min with the substance to be tested. The reaction was initiated by adding 40 μg of yeast RNA and the mixture was incubated at 30° for 30 min. The reaction was stopped and the activity measured as described in Experimental.

†100% corresponds to a 0.1 unit of enzyme activity.

‡As a control, pancreatic RNase A activity was tested, being strongly inhibited by heparin.

leases which hydrolyse all the phosphodiester bonds of the RNA chain and show a preference for the guanine base. The digestion of homopolymers only gives an indication of the behaviour of enzyme towards four phosphodiester linkages out of 16. It is possible that the neighbouring sequences or secondary structures themselves could enhance or decrease the velocity of the enzyme reaction. The enzyme is inactive on either native or heat-denatured calf thymus DNA, indicating that it is a sugar-specific nuclease.

The effects of some cations, EDTA and heparin on corn ribonuclease activity are summarized in Table 3. The data obtained indicate that the monovalent cations, Na^+ and K^+ have no effect even at high concentration (150 mM). These results indicate that the response of the corn RNase is different to that in many plant RNases, e.g. a chromatin-associated RNase from wheat leaves is inhibited by 40% either in the presence of 150 mM Na^+ or K^+ [16]; K^+ at 100 mM strongly depressed the activity of a ribonuclease from rye cytosol [20]. On the other hand, the activities of both RNase I and Nuclease I from corn at or near their optimum pH are increased by increasing concentrations of K^+. *Vicia faba* root RNase is another example of an enzyme that is slightly stimulated by K^+ [21].

Much is known about the influence of bivalent cations on the enzymatic activity of nucleases. In general, bivalent cations like Cu^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} in the range of 0.1–10 mM cause strong inhibition of the nucleases [3, 7, 20, 21]. As shown in Table 3, these effects were similar to those previously reported, except that the corn ribonuclease was insensitive to Cu^{2+} even at concentrations up to 30 mM. To the best of our knowledge this feature has not been found in any plant RNase previously reported.

Wilson [8] reported an unusual effect of Mg^{2+} on corn RNase I. The enzyme activity was stimulated at optimal pH and inhibited at pH 5.8. On the other hand, Mg^{2+} tested at 10 mM at optimal pH depresses the present RNase by 50% (Table 3).

As shown in Table 3, EDTA inhibits the corn ribonuclease by nearly 50% at a concentration of 10 mM. The sensitivity of the corn RNase to EDTA is rather unusual since it has been reported that this agent has no inhibitory effect on bivalent cation independent-RNase activities [3, 7, 20–22]. The inhibitory effect of EDTA suggests that the enzyme requires a very small amount of a very tightly bound cation for optimal activity. On the other hand, corn RNases I and II are not inhibited by EDTA [6].

Heparin, a natural occurring polyanion which inhibits many RNase activities [23], has no effect on the studied RNase even at concentrations up to 200 μg/ml (Table 3). Chevrier and Sarhan [3, 16] found that heparin had no significant effect on a wheat soluble RNase but strongly inhibited that wheat nuclear RNase. According to our data, this corn leaf RNase appears to differ from any of the corn RNases previously reported.

EXPERIMENTAL

Plant material. Young, fully expanded 10-day-old leaves from a new-semident variety of corn (*Zea mays*), 'Pinguino', was used. This variety was obtained after six cycles of stratifield mass selection by Universidad Austral de Chile, Valdivia, Chile. Its origin, agronomic characteristics and sowing area are described in detail in ref. [24].

Chemicals. Polyribonucleotides and Cibacron Blue F3GA Sepharose CL-6B were Sigma Chemical Co. products. [8-^{3}H]Polyadenylic acid and [5-^{3}H]polyuridylic acid were from Amersham.

Enzyme purification. Unless otherwise stated, all procedures were carried out at 4°. Leaves (1 kg) were homogenized with

three vols (v/w) of 0.05 M sodium citrate buffer (pH 5.5) containing 50 μg/ml of phenylmethylsulphonyl fluoride. The tissue was homogenized for two 30 sec periods, the homogenate being passed through four layers of cheesecloth and centrifuged at 5000 *g* for 10 min. The supernatant was precipitated between 30 and 80% $(NH_4)_2SO_4$ satn. The pellet was recovered by centrifugation and resuspended in 0.01 M sodium citrate, pH 5.5 (buffer A) and then dialysed against 30 vols of the same buffer with constant stirring for 24 hr, with four changes of buffer. After dialysis the suspension was centrifuged at 3000 *g* for 10 min. The supernatant was applied to a column of CM-cellulose (2.5 × 20 cm) equilibrated with buffer A. The active fraction non-adsorbed on the CM-cellulose was loaded into a DEAE-cellulose column (DE-52; 2.5 × 30 cm) equilibrated with buffer A. The enzyme soln non-adsorbed on DEAE-cellulose was concd by vacuum until the protein concn was enough to get a quantitative $(NH_4)_2SO_4$ precipitation. The pellet, containing nucleic-acid splitting enzymes, was dissolved in buffer A and dialysed against the same buffer. After dialysis, the sample was applied to a Sephadex G-100 column (1.5 × 80 cm). The nuclease activities eluted in two peaks. The excluded fractions showing low specific activity were discarded and lower M_r active-fractions were pooled for additional purification. The dialysed active pool was applied on a Cibacron Blue F3GA Sepharose column (1 × 25 cm) equilibrated with buffer A. The column was washed with the same buffer until A_{280} was null. The RNase activity investigated was eluted with a linear KCl gradient between 0 and 300 mM.

Enzyme assays. RNase activity was assayed by measuring A_{260} nm of the acid-soluble digestion products from either yeast RNA or synthetic homopolynucleotides essentially as described in ref. [25] in 20 mM sodium citrate buffer (pH 5.5) instead of NaPi. Assay of DNase activity using native or denatured calf thymus DNA was done according to Kato and Ikeda [26]. A unit of RNase activity was defined as the amount of enzyme required to increase *A* at 260 nm by 1 unit in 1 min [7]. Phosphodiesterase and phosphatase were assayed on Ca-bis(*p*-nitrophenyl)$_2$ phosphate and β-glycerophosphate respectively [14].

Endo- or exonucleolytic activity. Experiments were performed at 30°, incubating 1 μCi (15 Ci/mmol) of either [8-^{3}H]poly A or [5-^{3}H]poly U with 0.1 units of purified corn RNase at optimal pH in a final vol. of 80 μl. Aliquots were taken at various intervals, and spotted on 3 MM Whatman paper. Descending chromatography was carried out with *n*-PrOH–NH_4OH–H_2O (11:2:7) according to Kasai and Grunberg-Manago [13]. Chromatograms were cut in small squares eluted with 0.1 M HCl and neutralized with NaOH. Radioactivity was measured by adding PPO–POPOP–toluene scintillation mixture.

SDS-polyacrylamide gel electrophoresis was performed with 12% gel at pH 8.3 as described by Laemmli [10]. For the electrophoresis of the nondenatured enzyme, gel of 10% polyacrylamide at pH 5.0 were used according to Reisfeld *et al.* [11]. The M_r was estimated by using the procedure described by Weber and Osborn [27]. Bovine serum albumin, 66 000; ovoalbumin, 45 000; trypsinogen, 24 000; β-lactoglobulin, 18 400; and lysozyme, 14 400 were used as protein markers.

Determination of protein concentration. The protein content was determined according to Lowry [28] using bovine serum albumin as standard.

Thermal stability. Isolated corn RNase was heated to the indicated temp. for 30 min and after cooling for 5 min on ice, the enzyme activity was measured at optimum pH.

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