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PARTIAL PURIFICATION AND CHARACTERIZATION OF A RIBONUCLEASE FROM *PETUNIA HYBRIDA*

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Abstract—A crude extract from leaves of *Petunia hybrida* contains three ribonucleases (RNases) as revealed by polyacrylamide gel electrophoresis (PAGE). One of these was isolated and purified *ca* 200-fold. The pH optimum lies between 5.4 and 6.0. The MW was estimated to be *ca* 26000. The isoelectric point is 3.9–4.0. The RNase is an endonuclease, which cleaves dinucleotide phosphates to 2',3'-cyclic nucleotides, but cyclic nucleotides could not be further hydrolysed to the corresponding 3'-nucleotides using incubation periods six times longer than necessary for cleavage of dinucleotide phosphates. Studies with homopolymers have shown a preference for the hydrolysis of poly (U).

INTRODUCTION

RNase preparations have been obtained from only a few plant species, although many have been characterized from micro-organisms [1, 2]. To obtain a better insight into the physiological role of RNases in plants, it seemed desirable to investigate the enzymes of a well characterized model plant. For this reason we chose *Petunia hybrida* [3–5], which is also a popular subject for genetic modification [6–8].

RESULTS

Enzyme purification

The purification of the enzyme is summarized in Table 1. The *P. hybrida* RNase obtained by this procedure was completely free of phosphomonoesterase and phosphodiesterase activities, assayed on synthetic substrates. Furthermore, the enzyme was inactive on calf thymus DNA. The absence of the other RNases present in a crude enzyme extract was shown by PAGE. Only 1, instead of 3 bands, was visible, using 5–30 units of the Sephadex G-75 pool, and 10 units of the crude extract, respectively.

Effect of pH *on reaction rate*

The pH activity curve measured in 0.03 M sodium acetate buffer (pH 4.2–5) and sodium cacodylate buffer (pH 5–7) showed a pH optimum at pH 6. Half maximal activity was at pH 5.4 and 6.2. No detectable shift of the pH optima was obtained with crude enzyme extract and the purified preparation. The pH optimum of the crude extract showed only a broader peak. When the assays were carried out with 0.1 M buffer, a lower value of pH 5.4 was found.

Effects of various agents on reaction rate

Enzyme assays in the presence of different cations demonstrated a strong inhibitory effect of Zn^{2+} (10% of

Table 1. Summary of the purification of *Petunia hybrida* ribonuclease

	Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Recovery (%)
I.	Crude extract	12900	127000	10	1	100
II.	60–80% $(NH_4)_2SO_4$ precipitate	350	14600	42	4	11.5
III.	Heat denaturation	119	13000	108	11	10.2
IV.	Concentrated DEAE-Sephacel pool	5.8	3410	587	59	2.7
V.	Sephadex G-75 pool*	0.54	1180	2170	218	1.1

*Calculated from the recoveries of protein and RNase activity from 3 ml of the Amicon concentrate applied to the Sephadex G-75 column.

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control activity remaining), while Ca^{2+}, Mg^{2+} and NH_4^+ showed a lower effect (80–90% of the control activity remaining). EDTA does not significantly affect the enzyme activity.

Influence of temperature on stability of enzyme

The purified enzyme preparation was diluted 10-fold with 0.05 M sodium cacodylate buffer and incubated in the presence of bovine serum albumin (1 mg/ml) at various temperatures for 10 min. Then the solution was cooled on ice and the precipitated material removed by centrifugation. The RNase activity of the supernatant was determined in a standard enzyme assay. The ribonuclease is relatively stable up to 60°. Raising the temperature to 70° caused a significant loss of activity (only *ca* 10% of the control activity remaining).

Isoelectric point of enzyme

In the isofocusing experiments we obtained one ribonuclease band with the purified enzyme preparation. The isoelectric point was estimated to be *ca* pH 4.

MW

The MW was determined by gel filtration on Sephadex G-75. The enzyme eluted near the marker protein α-chymotrypsinogen indicating a MW of *ca* 26 000.

Mode of action

As indicated by gel filtration on Sephacryl S-200 of RNA hydrolysates, the enzyme is an endonuclease which caused a time-dependent shift in MW of the substrate. The products liberated by the enzyme were 2′,3′-cyclic nucleotides as determined by cleavage of dinucleotide phosphates. A significant base specificity concerning the cleavage of dinucleotide phosphates could not be detected. Only CpA would not serve as a substrate. A further degradation of 2′,3′-nucleotides was not observed using incubation periods six times longer than necessary for cleavage of dinucleotide phosphates. Comparing the rate of hydrolysis of homopolymers (Table 2), we obtained a preference for the hydrolysis of poly (U) at pH 6. At the higher pH value (7), we found essentially the same order of degradation (poly (U) > poly (I) > poly (A) > poly (C)) with a somewhat lower specificity for poly (U) compared with the other values obtained at pH 6.

DISCUSSION

The *P. hybrida* RNase was purified *ca* 200-fold. Taking into account the purification from the other RNase activities, the final value should have been even higher. Many properties of the enzyme are similar to those observed with RNases from other plant sources [1]. In some properties we found quite interesting differences. The enzyme from *P. hybrida* shows a preference for the hydrolysis of poly (U) compared with both purine homopolymers. On the contrary, most plant RNases show the following order of degradation: poly (I) ≥ poly (A) ≫ poly (U) ≫ poly (C) or a slightly different one: poly (U) being more degraded than poly (A) [9]. However, in the latter case, incubation of the homopolymers was at pH 5.6, whereby poly (A) degradation could have been impaired by the helical protonated structure, which forms at pH values of *ca* 5 [10]. There are, in fact, two RNases from *Vicia faba* that [11] prefer poly (U) and poly (C) as substrates, respectively, but these enzymes have a cationic nature, which is accompanied by their inability to cleave purine homopolymers. An RNase from cucumber seedlings [12] showed a preference for the release of uridylic acid from RNA, but poly (U) degradation was not investigated and the enzyme has a quite unusual MW of 12 500. A further important result is that the *P. hybrida* nuclease cannot decyclize 2′,3′-cyclic mononucleotides. This quite unusual property was found only with wheat germ RNase [9], which has a rather low MW of 9000 compared to other MWs reported from plants. In contrast, the RNase from *P. hybrida* has a MW of *ca* 26 000.

Table 2. Hydrolysis of homopolymers by *Petunia hybrida* ribonuclease

	Enzyme activity (units/ml)			
pH	poly (I)	poly (A)	poly (U)	poly (C)
6.0	69.6	21.7	942	7.76
7.0	2.07	0.437	5.9	0.084

The assays were incubated for 30 min. The activity towards each homopolymer was evaluated by recording the acid-soluble products absorbing at 260 nm.

EXPERIMENTAL

Leaves of *P. hybrida* 'cyanidin type' were used [13].

Enzyme assays. The RNase assay used was the same as that described in ref. [14]. Yeast–RNA (0.5 ml) at a concn of 2 mg/ml was incubated in 0.03 M Na cacodylate buffer (pH 6) with an aliquot of enzyme up to 0.1 ml, in a total vol. of 1.5 ml. One unit of enzyme is defined as the amount which catalyses, in 1 min, the formation of lanthanum acid-soluble material with an A_{260} of 1 per ml of incubation mixture. The assay for DNase activity was the same as that described for RNase except that calf thymus DNA was used as substrate. Phosphodiesterase and phosphomonoesterase activities were determined as described in ref. [15] using *p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phosphate and thymidine 5′-*p*-nitrophenyl phosphate as substrates.

Determination of protein. The protein concn was measured according to ref. [16] or by the biuret method [17].

MW determination. The MW of the partially purified RNase was determined by gel filtration according to ref. [18] on a Sephadex G-75 column (1.6 × 88 cm). γ-Globulin, ovalbumin, BSA, chymotrypsinogen and cytochrome *c* were used as marker proteins.

Determination of endonucleolytic or exonucleolytic type of enzyme action. The method of ref. [19] was used. High MW RNA (Serva, Heidelberg) was incubated in a standard assay with 0.5 units of the *P. hybrida* RNase to produce partial digests in order to determine stages in degradation. After an incubation period of 10, 40 and 140 min, the mixture was treated at 100° for 5 min to inactivate the enzyme. The solns were fractionated on a Sephacryl S-200 column (0.9 × 26 cm) using 3′-AMP as marker.

Degradation of polynucleotides, dinucleotide phosphates and 2′,3′-cyclic nucleotides. The rate of hydrolysis of polynucleotides was measured by incubating up to 40 μl aliquots of the enzyme soln in 0.05 M Na cacodylate buffer (pH 6) or in 0.01 M Na-Pi buffer (pH 7) containing 0.1 M KCl in a final vol. of 0.6 ml with 0.2 mg of the homopolymer. The reaction was stopped by the addition of 0.6 ml lanthanum nitrate–HCl reagent. The 3000 *g* for 10 min supernatant was used for measuring *A* at 260 nm. Five

different dinucleotide monophosphates (UpA, ApU, CpA, ApA and ApC) were incubated with 0.3 units of the RNase for 1–4 hr at a concn of 1 mg/ml in 0.05 M Na cacodylate buffer (pH 6) in a total vol. of 100 μl. The 2',3'-nucleotides were incubated under the same conditions for up to 24 hr. The resulting products were separated by cellulose-TLC according to ref. [20].

PAGE and isoelectric focusing. Electrophoresis was performed in 5% acrylamide stacking gel and 10% migration gel in the buffer system of ref. [21]. Electrophoresis was conducted at 4 mA per tube (dimensions 10 × 0.6 cm) for 2 hr. Electrofocusing was done in an LKB-Multiphor apparatus using LKB-Ampholine Pag plates with a pH gradient from 3.5 to 9.5. The pH of the plates was measured directly with micro-glass electrodes for isofocusing from DESAGA (Heidelberg). About 2 units of the purified RNase preparation was used for one run after dialysis against 0.05 M Tris–HCl (pH 7.5) containing 1 mM 2-mercaptoethanol (2-ME). The method of detection of RNase in polyacrylamide gels has been presented in ref. [22]. The stained gels were scanned in a Joyce–Loebl densitometer.

Enzyme purification. Step I. Preparation of crude enzyme extract: Leaves (2 kg) were homogenized in 4 l. of extraction buffer (0.05 M Tris–HCl, pH 7.5; 0.5 M KCl; 1 mM 2-ME). The homogenate was stirred for 3 hr at 4° and the insoluble material was removed by centrifugation at 27 000 g in 30 min.

Step II. $(NH_4)_2SO_4$ *precipitation*: The supernatant 6.1 l. was brought to 60% satn with solid $(NH_4)_2SO_4$. The soln was stirred for 30 min, and the ppt. removed and discarded after centrifugation at 30 000 *g* for 30 min. The resulting soln was brought to 80% satn and stirred again for 30 min. The ppt. was collected by centrifugation at 30 000 *g* in 30 min and dissolved in 500 ml 0.05 M Tris–HCl buffer (pH 7.5) containing 1 mM 2-ME.

Step III. Heat denaturation: The temp. of the 60–80% $(NH_4)_2SO_4$ fraction was raised to 55° and maintained at this temp. for 30 min. The flask was then cooled to below 10°. The ppt. was removed by centrifugation at 30 000 *g* for 30 min. The supernatant was exhaustively dialysed against 0.02 M Tris–HCl (pH 7) containing 1 M 2-ME.

Step IV. Chromatography on DEAE-Sephacel: The enzyme soln (478 ml) was loaded on a DEAE-Sephacel column (2.6 × 40 cm) equilibrated with 0.2 M Tris–HCl (pH 7); 1 mM 2-ME. The column was washed with 2 vols of the same buffer and subsequently eluted with a linear gradient of 1 l. 0–0.4 ml NaCl in the same buffer. Fractions (10 ml) were collected at a flow rate of 7 ml per hr. The RNase eluted between 0.16 and 0.22 M NaCl. Fractions 63–67 were pooled and concd to a vol. of 3.7 ml with an Amicon ultrafiltration cell equipped with PM10 filter.

Step V. Sephadex G-75 gel filtration: To a 3 ml aliquot of the concd DEAE-Sephacel pool was added sucrose (1% w/v). The soln was applied to a Sephadex G-75 column (2.6 × 40 cm) equilibrated with 0.05 M Tris–HCl (pH 7.5) containing 0.1 M KCl and 1 mM 2-ME. The RNase activity was eluted with a flow rate of 2.5 cm per hr. Fractions 56–61 were pooled (12 ml) and all subsequent work was carried out with this prepn.

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