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AN ACID RIBONUCLEASE FROM RYE GERM CYTOSOL

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Abstract—Acid ribonuclease from rye germ cytosol was purified 1200-fold. The enzyme is homogeneous on polyacrylamide gel. The optimum pH for ribonuclease activity is 5.8, its MW is 28 500. The enzyme is an endonuclease yielding in the first step of its activity oligonucleotides with a free —OH group in the 3′ position. The end products of RNA hydrolysis are cyclic purine and pyrimidine nucleoside phosphates and the corresponding nucleoside 3′-phosphates. This ribonuclease preferentially attacks sites close to the adenine base and shows a lag in the release of the cytosine base. Specificity tests on natural and synthetic substrates are in good agreement.

INTRODUCTION

In recent years several highly purified ribonucleases have been obtained from higher plants [1–4] and their specificity has been established in detail.

According to Wilson [5] higher plant RNases may be divided into two groups: RNase I and RNase II. The enzymes of both these groups are endonucleases which split all the phosphodiester bonds of the RNA chain. They release 2′,3′-cyclic nucleosides and show a preference for the guanine base. The difference between them consists of a different optimum pH for their activity and a different ability of hydrolysing cyclic nucleotides. Some new enzymes were also detected [3, 6] which do not fit the up-to-date classification of plant RNases according to Wilson.

The present study concerns a RNase which, contrary to other higher plant RNases, has a preference for the adenine base.

RESULTS

Purity of enzyme

A typical purification scheme is shown in Table 1. The purification was *ca* 1200-fold. Acid RNase is completely free of phosphomonoesterase and phosphodiesterase activity assayed on synthetic substrates. Furthermore, the enzyme is inactive towards DNA.

Polyacrylamide gel electrophoresis of the enzyme at pH values of 8.4 and 5, as well as in the presence of sodium dodecylsulfate at pH 7, gave a single band indicating the homogenity of the enzyme.

Enzyme properties

The RNase activity at various pH values was measured in 0.1 M acetate buffer, 0.1 M citrate–phosphate buffer and 0.1 M Tris–HCl buffer. The optimum pH was 5.8. Studies of the influence of temperature on activity showed an optimum activity close to 70°. The effect of a variety of small molecules and ions on acid RNase is shown in Table 2. The data obtained indicate that bivalent metal ions depress enzyme activity by 50–70%. EDTA, urea and KCl have only a slight inhibitory effect. The activity does not change under the influence of PCMB (*p*-chloromercuribenzoate) or dithioerythritol, thus ruling out the necessity for —SH groups in enzymatic catalysis. From the factors modifying amino acid residues in the enzyme molecule, *N*-bromosuccinimide and *N*-ethyl maleimide lowered its activity by 50 and 15% respectively. The polyamines putrescine and cadaverine, in 0.2 mM concentration stimulate enzyme activity, while spermidine in this concentration reduces the activity by 67%. The purine and pyrimidine nucleotides (both 2′,3′-cyclic and the 2′,3′-non-cyclic ones) have no inhibitory effect on the enzyme activity. From the relation between the MW of several marker proteins and the elution volume, the MW of RNase was estimated to be *ca* 28 500. The contents of neutral and amino sugars were 6.5 and 0.3%, respectively. The amino acid composition is shown in Table 3. For reference, the amino acid composition of RNase I from corn endosperm is listed in the same table.

Mode of action

After incubation of highly polymerized wheat germ RNA with the enzyme, successive formation of oligonucleotides decreasing in MW was observed. The results clearly show that RNase is a typical endonuclease.

Structure of termini produced by RNase action

In order to determine the structure of the termini produced by RNase, the reaction product was incubated separately with exonuclease, snake-venom phosphodiesterase which requires a free 3′-OH end

Table 1. Purification of acid RNase from rye germ cytosol

	Volume (ml)	mg protein per ml	Enzyme activity (units/ml)	Specific activity (units/mg)	Purification factor
Crude enzyme	170	30.6	202	6.6	1
105 000 g supernatant	120	21.8	204	9.4	1.4
$(NH_4)_2SO_4$ fractionation	27	8.1	129	16.0	2.4
CM-cellulose	25	1.06	79	75.0	11.3
CM-Sephadex C-50	6	0.25	125	500.0	75.7
Sephadex G-75	9	0.06	240	4000.0	606.0
Phosphocellulose	5	0.034	272	8000.0	1200.0

Table 2. Effect of inhibitors and activators on RNase from rye germ cytosol*

Reagent	Concentration (mM)	Relative activity (%)	Reagent	Concentration (mM)	Relative activity (%)
None	0	100	Urea	3	71
$MnCl_2$	3	42	*N*-Ethyl maleimide	3	85
$MgCl_2$	3	40	*N*-Bromosuccinimide	3	51
$CuCl_2$	3	34	Dithioerythritol	3	80
$FeCl_2$	3	31	PCMB	1	110
$CaCl_2$	3	45	Putrescine†	0.1	135
$CoCl_2$	3	45		0.2	135
$ZnCl_2$	3	28	Cadaverine†	0.1	105
$HgCl_2$	3	28		0.2	132
EDTA	3	92	Spermidine†	0.1	67
KCl†	10	62		0.2	33
	100	28			

* Standard assay conditions. Enzyme (3.5 μg of enzyme protein) was preincubated at 37° for 10 min.
† No preincubation.

Table 3. Amino acid composition of acid RNase from rye germ cytosol

Component	Acid RNase μmol*	Acid RNase Residues	RNase I from corn endosperm (1) Residues
Lysine	0.429	24.5	9
Histidine	0.022	1.28	4
Arginine	0.118	6.86	9
Aspartic acid	0.264	15.3	24
Threonine	0.171	9.9	7
Serine	0.382	22.2	13
Glutamic acid	0.460	26.7	17
Proline	0.202	11.7	15
Glycine	0.420	24.,4	20
Alanine	0.202	11.7	16
Cysteine	0.000	0.0	8
Valine	0.164	9.5	9
Methionine	trace	trace	0
Isoleucine	0.087	5.0	4
Leucine	0.133	7.7	15
Tyrosine	0.000	0.0	10
Phenylalanine	0.062	3.6	11
Tryptophan	trace	trace	6

* The values given are μmol recovered from 0.5 mg of protein.

and produces 5′-mononucleotides, and spleen phosphodiesterase which requires a free 5′-OH end and produces 3′-mononucleotides. The products of RNA hydrolysis obtained under the action of RNase are intensively decomposed by snake-venom phosphodiesterase, whereas spleen phosphodiesterase digests RNA hydrolysis products as effectively as RNA.

Digestion of wheat germ RNA

The base composition of mononucleotide fractions after 1 and 48 hr of digestion is shown in Table 4. The preference for cleavage next to a purine residue after 1 hr digestion is shown by the high purine/pyrimidine

Table 4. Base composition of mononucleotides from wheat germ RNA degradation by acid RNase from rye germ cytosol

Time of enzymatic digestion (hr)	Mol% Ade	Cyt	Gua	Ura	Purine/pyrimidine ratio
1	51	0	26	18	4.2
48	40	28	21	11	1.5

The mononucleotide fractions were hydrolysed to free bases with 70% $HClO_4$ by heating to 100° for 1 hr. After cooling the mixture was diluted with water and centrifuged. The bases were separated by PC on Whatman No. 1 using *iso*-PrOH–HCl–H_2O (65 : 16.7 : 18) as solvent. Each component was eluted with 0.1 N HCl for quantitation.

Table 5. Composition of 2',3'- and 3'-mononucleotides from digestion of wheat germ RNA by acid RNase from rye germ cytosol

Time (hr)	Mol%							
	3'-AMP	2',3'-AMP	3'-GMP	2',3'-GMP	3'-CMP	2',3'-CMP	3'-UMP	2',3'-UMP
1	16	35	6	20	0	0	0	18
48	10	30	1	20	8	20	3	8

The mononucleotide fraction was separated into components by PC on Whatman 3MM using *iso*-PrOH–NH_3–H_2O (7:1:2) as solvent. The separated products were eluted with 0.1 N HCl and their UV spectra examined.

ratio (4.2). Adenine constitutes 51% of the 1 hr mononucleotide fractions. It is followed by guanine (26%),uracil (18%) and cytosine is absent. After 48 hr of hydrolysis the purine/pyrimidine ratio declined. The base composition of the mononucleotide fraction also changes. A relatively high amount of adenine (40%) persists, and a considerable quantity of cytosine (28%), later guanine (21%) and uracil (11%) appears. The absence of cytosine in the mononucleotide fraction after 1 hr of hydrolysis suggests a high resistance of RNase to internucleotide phosphodiester bonds next to cytosine residues. After 48 hr of hydrolysis a high increase in cytosine content is observed. In the mononucleotide fraction (Table 5) after 1 hr of hydrolysis the cytidine-2',3'-cyclic phosphate and cytidine 3'-mononucleotide as well as uridine 3'-mononucleotides are lacking. Cyclic purine and pyrimidine mononucleotides as well as the corresponding 3'-mononucleotides appear after 48 hr of hydrolysis. Acid RNase from rye germ cytosol functions as a phosphotransferasae enzyme.

Degradation of dinucleotides by RNase

Breakdown of dinucleoside monophosphates was studied by determination of the rate of nucleoside formation. All dinucleotides containing adenine are hydrolysed most rapidly (Table 6). Owing to the presence of cytosine in the dinucleotide, there is no breakdown in the first hr of enzyme activity. As the time of digestion advances, a distinctly enhanced hydrolysis of dinucleotides containing cytosine is observed, notwithstanding on which side of the phosphodiester bond the base lies. Dinucleotides containing guanine and uridine are hydrolysed correspondingly slower.

Table 6. Cleavage of dinucleoside monophosphates by acid RNase from rye germ cytosol

Substrate	μmol of nucleosides liberated/ml of substrate		
	1 hr	2 hr	3 hr
GpU	2.0	20.0	35.0
GpC	1.0	25.0	75.0
CpG	0.0	35.0	85.0
CpU	0.0	20.0	60.0
CpA	2.0	45.0	100.0
ApA	25.0	120.0	195.0
ApU	10.0	40.0	66.0
ApG	20.0	35.0	96.0
ApC	20.0	40.0	98.0
UpC	0.0	10.0	55.0
UpG	3.0	10.0	20.0

Dinucleoside monophosphate (1 μmol of each) in 1 ml 0.1 M acetate buffer, pH 5.8, was incubated with 3 μg of enzyme protein at 37°. At given time intervals, aliquots of the digest were taken and loaded on a DEAE-cellulose column (0.5× 10 cm, acetate form). The nucleosides eluted with water were identified and analysed spectrophotometrically after acidification to pH 1.

Table 7. Degradation of nucleoside cyclic phosphates by acid RNase from rye germ cytosol

Substrate	Percentage of cyclic phosphate hydrolysed	
	24 hr	48 hr
2'3'-AMP	45	81
2',3'-GMP	12	36
2',3'-CMP	32	76
2',3'-UMP	39	70

2',3'-Cyclic mononucleotide (100 μg) in 0.2 ml 0.1 M acetate buffer, pH 5.8, was incubated with 30 units of enzyme at 37°. After incubation for 24 and 48 hr, 50 μl aliquots were taken and chromatographed on Whatman 3MM paper with *iso*-PrOH–NH_3–H_2O (7:1:2) as solvent. Spots were detected under UV light and nucleotides were eluted from chromatogram with 4 ml 0.1 M HCl. The spectra of the cyclic nucleotides were used to calculate the extent of hydrolysis of each nucleotide. Enzyme free blanks were run for each nucleotide to determine the amount of non-enzymatic hydrolysis.

Table 8. Hydrolysis of homopolynucleotides and the double stranded complex

Homopolymer	$\Delta A_{260\,nm}$	
	30 min	1 hr
Poly A	0.76	1.1
Poly G	0.00	0.03
Poly C	0.20	0.78
Poly U	0.35	0.64
Poly A:Poly U	0.00	0.00

The reaction mixture contained in a final volume of 0.8 ml: 100 μg of substrate and 20 μg of enzyme protein in 0.1 M acetate buffer, pH 5.8. Incubation was carried out at 37°. At given time intervals, 200 μl aliquots were taken and mixed with 200 μl of cold 12% $HClO_4$ containing 20 mM lanthanum acetate. After 20 min at 0°, the precipitates were removed by centrifugation in the cold at 12 000 g for 30 min. The supernatant (100 μl) was then diluted with 10 vols. of water and the *A* at 260 nm, was measured.

Action of the enzyme on cyclic nucleotides and homopolymers

RNase hydrolyses both cyclic purine and pyrimidine nucleosides to nucleoside 3′-phosphates. The cyclic adenosine nucleoside is cleaved much faster, next come cytidines, uridines and finally guanosine (Table 7). As shown in Table 8, RNase splits all homopolymers in the following order: poly A > poly C > poly U > poly G. The mixture of poly A and poly U [7] in the proportion 1:2 was not attacked by the enzyme.

DISCUSSION

The acid RNase preparation obtained in the present work from rye germ cytosol is an electrophoretically homogeneous protein. The properties of this RNase, such as optimum pH, thermolability, sensitivity to bivalent cations and lack of response in activity to EDTA, are very similar to the features of other higher plant RNases [5].

The specificity of the studied RNase was established by degradation of natural and synthetic substrates. The enzyme splits RNA to form oligonucleotides with a phosphomonoester bond at position 5′. The enzyme is an endonuclease and like other ribonucleases of higher plants (except that of rye [3]) decyclizes 2′,3′-cyclic nucleoside phosphates. The investigated RNase differs, however, from those known to date from higher plants by its preference to attack the sites closest to adenine in RNA. After 1 hr of RNA hydrolysis from wheat germ there appears predominant quantities of adenine nucleoside, whereas cytidine nucleoside is not found. Adenine nucleoside is still prevalent after 48 hr hydrolysis, but large quantities of cytidine nucleoside are also present.

Other authors observed earlier [2–4, 8–13] that higher plant RNases release, by acting on the RNA molecule, in the first place guanine mononucleotide and then adenine mononucleotide. Later investigations confirmed these suppositions on the basis of a strict quantitative analysis of the products of RNA hydrolysis in the presence of highly purified preparations from RNase of oats [2], barley [4] and wheat [3].

Quantitative analysis of products of RNA breakdown performed in the present study indicates that rye germ cytosol RNase first releases adenine nucleotide. This was further confirmed by investigation of the enzyme action on dinucleotides and cyclic nucleotides. Further evidence was supplied by experiments in which the action of the enzyme on synthetic polynucleotides was tested. It was found that the enzyme hydrolyses these compounds in the order poly A > poly C > poly U > poly G. Since degradation of these compounds by the enzyme was studied at pH 5.8, it would seem that their secondary structure has little influence on the rate of hydrolysis [14].

The agreement between the results obtained in experiments with natural and synthetic substrates proved that the enzyme preferentially attacks sites adjacent to the adenine base. It exhibits, like other higher plant RNases, a lag in cytidine mononucleotide release, however, the much greater quantities of cytidine nucleotide released after the lag distinguish this enzyme from other RNases of higher plants.

Since the studied enzyme was isolated from a definite cell compartment—cytosol—it may be useful in the future, and help to determine the physiological role of this enzyme. Acid RNase from rye germ cytosol, like many other enzymes without absolute specificity, may find application in investigations of the structure–function relation as compared with that of other RNases already thoroughly known.

EXPERIMENTAL

Plant material. Rye embryos (*Secale cereale* L.) were commercial prepns from the Swiebodzin Milles, Poland.

Chemicals. Mononucleotides, cyclic mononucleotides, *p*-nitrophenyl phosphate (diNa salt), bis-*p*-nitrophenyl phosphate (Na salt), dinucleoside monophosphates, were Sigma Chem. Co. products. Highly polymerized wheat germ RNA was supplied by Calbiochem.

Enzyme purification. Prepn of crude enzyme and of 105 000 *g* supernatant (cytosol) and $(NH_4)_2SO_4$ fractionation were as described previously [6]. Chromatographic separation was conducted at 0–4°. Column effluents were monitored at 260 nm for protein and the eluates assayed for RNase activity. The final supernatant after $(NH_4)_2SO_4$ fractionation was applied to a 2.5 × 40 cm column of CM-cellulose equilibrated with 0.1 M Na citrate–NaPi buffer, pH 7. The column was eluted with the same buffer at a flow rate of 50 ml/hr. The RNase-rich fractions were dialysed against 0.1 M NaPi buffer, pH 7, lyophilized and dissolved in 10 ml 0.1 M NaPi buffer pH 7. The product was then applied to a 2 × 20 cm column of CM-Sephadex C-50 equilibrated with 0.1 M NaPi buffer pH 7, and eluted with the same buffer, using a flow rate of 12 ml/hr. The active fractions were dialysed against 0.1 M Tris–HCl buffer pH 7.5, lyophilized and dissolved in 2 ml of the same buffer. The enzyme soln was applied to a 1.5 × 60 cm column of Sephadex G-75 equilibrated with 0.1 M Tris–HCl buffer pH 7.5 and eluted with the same buffer. Flow rate was 18 ml/hr. RNase activity was eluted in two peaks. Only the major peak of RNase was further processed. The material was dialysed against 50 mM Tris–HCl buffer pH 7, lyophilized, dissolved in 1 ml of the same buffer and was applied to a 1.5 × 18 cm column of phosphocellulose. The column was equilibrated with 50 mM Tris–HCl buffer pH 7, and eluted with a linear gradient of 0.01–0.3 M NaCl in the same buffer at a flow rate of 30 ml/hr. RNase activity was eluted in the range of 0.1–0.15 M NaCl.

Enzyme assays. RNase was assayed by measuring *A* at 260 nm of the acid-soluble digestion products from yeast RNA according to the method described in ref. [15] with the use of 0.1 M Tris–HCl buffer pH 5.8 instead of NaPi. The enzyme unit was defined as the amount of activity required to increase *A* at 260 nm by 0.1 units in 1 hr. Sp. act. is expressed in units/mg of protein. DNase activity was assayed by the method of ref. [16]. Phosphodiesterase and phosphomonoesterase were assayed on *p*-nitrophenyl phosphate and bis-*p*-nitrophenyl phosphate [17].

Polyacrylamide gel electrophoresis was performed at pH 8.4 [18] and 5 [19]. Electrophoresis in the presence of SDS was performed according to the method of ref. [20] with 10% gel at pH 7. Gels were stained with 0.1% Coomassie Brilliant Blue in 50% MeOH and 7% HOAc and were electrophoretically destained in 7% HOAc and 20% MeOH.

Estimation of MW. The MW was estimated by gel filtration on Sephadex G-75 following the procedure of ref. [21].

Mode of RNA degradation. The products of partial hydrolysis were analysed according to the method of ref. [22] using Sephadex G-50 instead of Sephadex G-100.

Analysis of termini of degradation products. The termini produced by scission of RNA by RNase were analysed according to the method described in ref. [23].

Action of RNase on highly polymerized wheat germ RNA. For examination of the degradation products, 10 mg wheat germ RNA were incubated with the enzyme (10 μg protein) in 0.1 M NaOAc buffer, pH 5.8, at 37° in a total vol. of 4 ml. The incubation for 48 hr was carried out in the presence of a trace of thymol to prevent microbial growth. Aliquots were withdrawn at intervals of 1 and 48 hr. The reaction was terminated by flash heating to 100° and the pH was adjusted to 7.8. The digests were fractionated by chromatography on DEAE-cellulose according to the method of ref. [24]. The mononucleotides obtained by this procedure were characterized by PC on Whatman 3 MM paper using *iso*-PrOH–NH_3–H_2O (7:1:2) as solvent. The separated products were identified by comparison with known standards and by examination of their UV spectra. The base composition of the original RNA and the various mononucleotide fractions was determined by PC on Whatman No. 1 after hydrolysis with $HClO_4$ according to the method of ref. [25]. The concn of each component was determined by measuring *A* at appropriate wavelengths. Absorption spectra were examined to confirm the identification of the components.

Cleavage of dinucleoside monophosphates. The rate of cleavage of the dinucleoside monophosphates was determined at pH 5.8. Each dinucleoside (1 μmol) in 1 ml 0.1 M NaOAc buffer was incubated with 3 μg of enzyme at 37°. At given time intervals, aliquots of the digest were applied to a 0.5 × 15 cm column of DEAE-cellulose (acetate form). The nucleosides eluted with H_2O were identified and analysed spectrophotometrically after acidification to pH 1.

Sugar analysis. The content of neutral sugars was determined according to the procedure of ref. [26]. Amino sugars were analysed after a 16 hr hydrolysis in 2 N HCl at 100° [27].

Amino acid analyses. Amino acids were determined with an automatic amino acid analyser. Samples for analysis were prepared by hydrolysis in glass-distilled 6 N HCl at 110° for 20 hr in evacuated sealed tubes. Spectrophotometric determination of tryptophan content of ribonuclease was carried out as described by in ref. [28].

Protein estimation. Protein was estimated either by *A* at 280 nm or by the method of ref. [29] using trypsin as the standard protein.

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