BBA 66310

EXOCELLULAR RIBONUCLEASE FROM *STREPTOMYCES AUREOFACIENS*

II. PROPERTIES AND SPECIFICITY

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(Received December 10th, 1970)

SUMMARY

1. Properties and specificity of exocellular ribonuclease from *Streptomyces aureofaciens* BM-K, a strain producing the antibiotic chlortetracycline, were studied. The enzyme had maximal activity at pH 7.0. The optimal temperature has been found to be around 45°.

2. Ribonuclease was inhibited by divalent cations Cu^{2+} and Zn^{2+} and by increasing ionic strength.

3. The enzyme was relatively heat-stable, the highest stability was observed at pH 7.0. At acidic pH values, the enzyme retained relatively high activity; at basic pH values (about 12) the loss of the activity was nearly 100%.

4. The ribonuclease hydrolyzed yeast RNA to the only mononucleotide, guanosine 3′-phosphate, forming guanosine 2′,3′-cyclic phosphate as intermediate, and to the oligonucleotides with terminal guanosine 3′-phosphate.

5. Polyguanylic and polyinosinic acids, but not polyadenylic, polyuridylic and polycytidylic acids, were split by the low ribonuclease activity.

6. In excess the ribonuclease hydrolyzed polyadenylic acid, as well as polyguanylic and polyinosinic acids, partially hydrolyzed polyuridylic acids.

7. From these results it was concluded that *Streptomyces aureofaciens* ribonuclease was an endonuclease specific for guanosine 3′-phosphate, similar to the ribonuclease T_1 (ribonucleate 2′-guaninenucleotido-2′-transferase (cyclising, EC 2.7.7.26)).

INTRODUCTION

In our previous paper[1], the isolation and purification of the exocellular ribonuclease produced by *Streptomyces aureofaciens* BM-K, a strain producing the antibiotic chlortetracycline, were reported.

The present paper reports studies on some properties, kinetics of RNA hy-

drolysis and specificity of the enzyme. It has been found that the enzyme is a ribonuclease possessing a specificity similar to that of ribonuclease T_1[2].

MATERIALS AND METHODS

Ribonuclease was isolated and purified from cultural medium of *Streptomyces aureofaciens* BM-K, producer of the antibiotic chlortetracycline, by the procedure described in our previous paper[1]. *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) was a product of the Worthington Biochemical Co. Commercial yeast RNA was obtained from Cambrian Chemicals Ltd., London. High-molecular-weight yeast RNA was prepared according to the method of CRESTFIELD *et al.*[3]. Yeast transfer RNA was kindly provided by Dr. A. D. Mirzabekov, Institute of Molecular Biology, Academy of Sciences USSR, Moscow. Poly A, poly G, poly C, poly U and poly I were purchased from Calbiochem.

Determination of ribonuclease activity was described in our preceding paper[1].

Hydrolysis of RNA

At arbitrary time intervals aliquots of the reaction mixture (3 mg RNA in 1 ml 25 mM sodium phosphate buffer (pH 7.0), 5 units of enzyme) were pipetted and the absorbance of acid-soluble products of RNA hydrolysis was measured at 260 mμ.

Paper chromatography

For paper chromatography of digestion products, Whatman paper No. 1 and two solvent systems were used: Solvent system 1, 0.5 M isobutyric acid–NH_4OH (100:60, by vol.), descending; Solvent system 2, ethanol–1 *M* ammonium acetate buffer (pH 7.5, 70:30, by vol.), descending.

Identification of digestion products

The spots of mononucleotides were located on the chromatogram under ultraviolet light, cut out and eluted in 3 ml 0.1 M HCl for 24 h. Each spot was identified by aid of standard mononucleotide solutions and by ultraviolet absorption spectra of the eluate measured in a Beckman automatic recording spectrophotometer, Model DB.

Identification of cyclic nucleotides

Identification of cyclic nucleotides was carried out by determination of R_F values in Solvent system 2 and by measuring the ultraviolet absorption spectra, comparing the non-hydrolyzed eluate of cyclic nucleotide spot with the eluate after hydrolysis with 0.1 M HCl.

Digestion of transfer RNA with ribonuclease

The reaction mixture containing 1 mg of transfer-RNA and 10 units of ribonuclease in 80 μl 50 mM sodium phosphate buffer (pH 7.0) was incubated at 37° for 20 h. The products of enzymatic hydrolysis, without enzymatic reaction stopping and after stopping the reaction with 5% trichloroacetic acid, were separated by one-dimensional descending chromatography in Solvent systems 1 and 2, successively. After chromatography the paper contact print was obtained in ultraviolet light.

Digestion of homopolynucleotides

The reaction mixture contained in 0.2 ml: 2 mg each of poly G, poly I, poly A, poly C and poly U, 10 μmoles sodium phosphate buffer (pH 7.0) and 3000 units of ribonuclease. In a parallel experiment the reaction mixture contained 1 mg of homopolynucleotide and 10 units of ribonuclease. After 5 and 20 h of incubation at 37°, the digestion products were separated by paper chromatography with both solvents, successively.

Analysis of terminal nucleotides

Enzymatic hydrolysis was carried out for 24 h at 37°. 0.3 ml of the reaction mixture contained 1 mg transfer RNA, 12 units of ribonuclease and 30 μmoles Tris–HCl buffer (pH 7.05). Splitting of 2',3'-cyclic nucleoside phosphates into 2'- or 3'-phosphates was carried out with formic acid for 3 h at room temperature. Formic acid was removed by evaporation *in vacuo*. For dephosphorylation of mononucleotides and of terminal nucleotides of oligonucleotides in ribonuclease digest, *E. coli* alkaline phosphatase was used. Incubation was carried out for 1 h at 37°. The hydrolysate was heated at 100° for 1 h to stop the enzymatic reaction. Water was evaporated *in vacuo*, and the digest was hydrolyzed with 0.3 M KOH at 37° for 18 h. The hydrolysate was applied on Whatman filter paper No. 1 and chromatographed with use of *n*-butanol saturated with water. In this solvent, nucleosides were separated from the mixture of nucleotides. After development, the spots of nucleosides were cut out and eluted with 3 ml of 0.1 M HCl for 3 h. Nucleosides were identified with the aid of standard nucleoside solutions and by measuring the ultraviolet absorption spectra.

RESULTS AND DISCUSSION

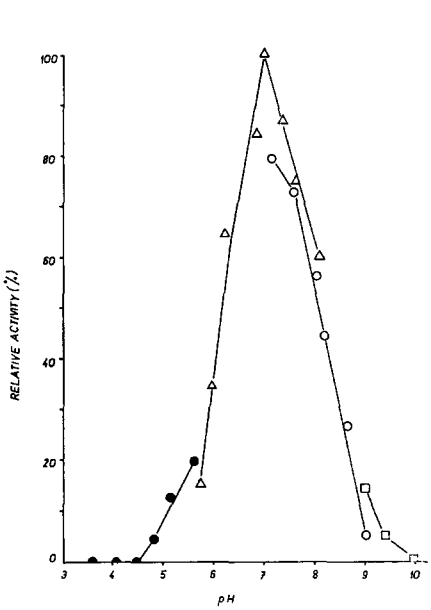
pH and temperature optimum

The effect of pH on the rate of hydrolysis of commercial yeast RNA by *S. aureofaciens* ribonuclease is shown in Fig. 1. The activity decreased sharply on either side of the pH optimum (7.0), with 50% of maximal activity remaining at pH 6.2 and pH 8.0. The optimal temperature was found to be about 45° (Fig. 2). Fig. 3 illustrates the strict proportionality between enzyme concentration and its activity.

Stability

Ribonuclease stored frozen for several months at neutral pH value retained almost full activity. On lyophilization about 20% inactivation was observed. In contrast to the ribonuclease T_1, in 50 mM HCl (pH 1.35) at room temperature (24°) the loss of ribonuclease activity was about 70% during 24 h. In 50 mM NaOH (pH 12.2) the enzyme retained only 8% of its activity. In 50 mM sodium phosphate buffer (pH 7.0) the enzyme suffered only 10% inactivation.

To study the temperature inactivation of the enzyme at different pH, ribonuclease solutions in different buffers were incubated for 5 min at different temperatures. After adjusting pH to 7.0, enzymatic activity was measured by the standard assay procedure. It was found that the ribonuclease was most stable at pH 7.0. Heating of the enzyme at 100° at pH 7.0 eliminated only 30% of its activity; at pH 12.2 the loss of the activity was nearly 100%.



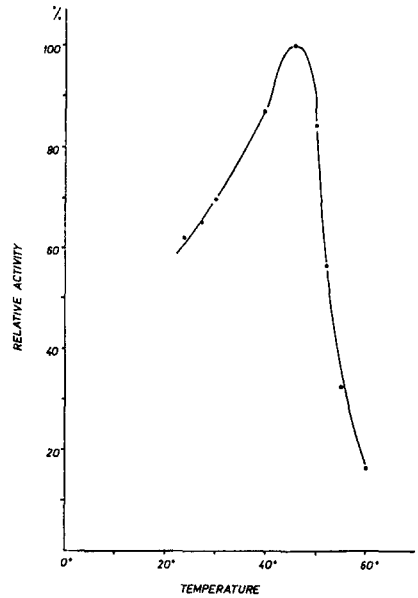


Fig. 1. Effect of pH on the activity of *S. aureofaciens* ribonuclease. Enzyme activity was measured by the standard assay in 50 mM sodium acetate buffer (●), 25 mM sodium phosphate buffer (△), 50 mM Tris–HCl buffer (○) and 50 mM glycine buffer (□).

Fig. 2. Effect of temperature on the ribonuclease activity. Standard reaction mixture (1 ml) contained 0.35 μg enzyme. 3 mg RNA in 25 mM sodium phosphate buffer (pH 7.0).

The rate of heat inactivation at different pH's is shown in Fig. 4. At acid pH values the inactivation of enzyme was more rapid than at pH 7.0.

The effect of divalent cations

The effect of divalent cations and some other reagents upon activity of ribonuclease is presented in Table I. Tris–HCl buffer (pH 7.2) was used in these experi-

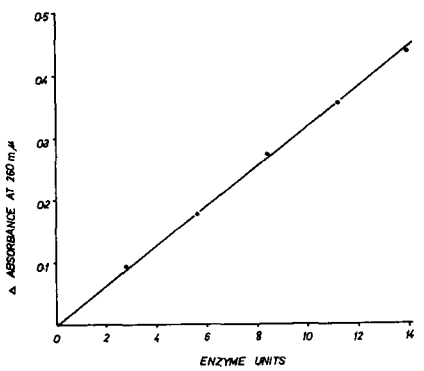


Fig. 3. Influence of ribonuclease concentration on activity. Enzyme activity was determined by standard assay procedure with 3 mg RNA in 25 mM sodium phosphate buffer (pH 7.0).

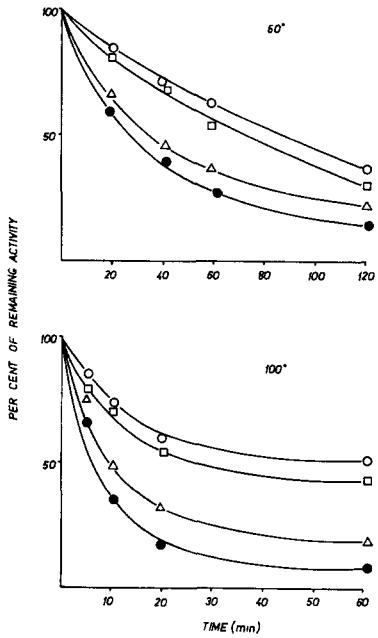


Fig. 4. Rate of heat inactivation of the ribonuclease. Ribonuclease (23 μg of protein per ml of the reaction mixture) was heated at 60° and 100° in 50 mM sodium phosphate–citric acid buffers, pH 2.2 (●), pH 3.4 (△), pH 5.4 (□) and pH 7.0 (○). Samples of 0.2 ml were withdrawn at appropriate time intervals, diluted with 0.1 M sodium phosphate buffer (pH 7.0), and enzyme activity by standard procedure was assayed.

TABLE I

EFFECT OF SOME REAGENTS ON THE ACTIVITY OF RIBONUCLEASE

The reaction mixture contained in 1 ml: 3 mg RNA, 50 μmoles of Tris–HCl buffer (pH 7.2), 5 units of ribonuclease and 1 μmole of reagent.

Reagent	*Activity remaining (%)*
None	100.0
NaCl	99.1
KCl	98.7
$MgCl_2$	96.4
Magnesium acetate	95.2
$MnSO_4$	89.0
$Ba(NO_3)_2$	82.8
$FeSo_4$	81.5
$CoCl_2$	79.8
$ZnSO_4$	47.6
$CuSO_4$	45.0
ICH_2COOH	100.4
EDTA	103.6
Histidine	100.0
Glycine	103.5

ments because some assayed salts of divalent cations reacted with sodium phosphate buffer (pH 7.0) forming insoluble precipitate. It was found that ribonuclease did not require any divalent cation for its activity. The enzyme was slightly inhibited by some divalent cations, Zn^{2+} and Cu^{2+} having the largest influence in this respect. Several ribonucleases have been found to be inhibited by Cu^{2+} and Zn^{2+}, pancreatic ribonuclease[4], ribonuclease T_1[2] and T_2[5], ribonuclease from *Aspergillus clavatus*[6] and exocellular ribonuclease from *Streptomyces albogriceolus*[7]. It is probable that the inhibitory effect of these cations on the ribonuclease activity is due, to some extent, to the formation of the metal–RNA complexes[8,9] when RNA is used as substrate in the assay. However TAKAHASHI *et al.*[4], using benzyl cytidine 3'-phosphate as substrate, observed the inhibition of bovine pancreatic ribonuclease by Cu^{2+}, Zn^{2+} and to a lesser extent by Hg^{2+} due to the formation of enzyme–metal complexes.

The effect of ionic strength

Ribonuclease activity was measured in various concentrations of sodium phosphate buffer (pH 6.7 and 7.0). The maximal activity was dependent not only on the pH of the buffer but also on the concentration of the buffer used (Fig. 5A). It was found that the enzyme was most active at concentrations of the sodium phosphate buffer of 10 mM (at pH 7.0) and 25 mM (at pH 6.7). The activity gradually decreased with increasing buffer concentration. Fig. 5B illustrates the inhibition of

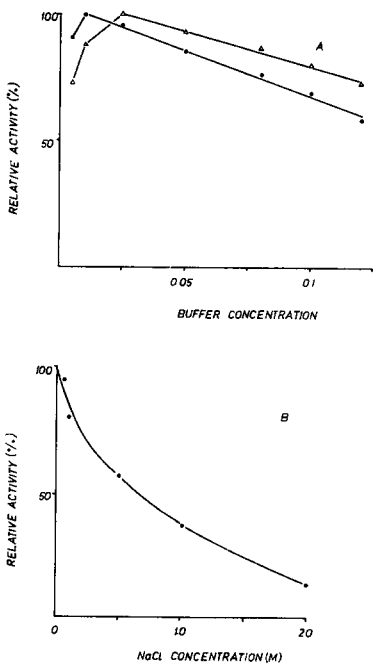


Fig. 5. Effect of ionic strength on the ribonuclease activity. A. The effect of the buffer concentration. The enzyme activity was assayed by the standard procedure in sodium phosphate buffer, pH 6.7 (△) and pH 7.0 (○). B. The effect of NaCl concentration. Enzyme assay was performed in 50 mM Tris–HCl buffer (pH 7.2) with various NaCl concentrations.

the ribonuclease activity in the presence of various concentrations of NaCl. KCl influenced the enzyme activity by the same manner.

High concentrations of NaCl depressed the ribonuclease activity of *Aspergillus oryzae*[10], *Actinomyces aureoverticillatus*[11] and rat liver[12]. On the other hand stimulation of the activity with increasing ionic strength was observed with pancreatic ribonuclease[13], with the ribonuclease from *Aspergillus saitoi*[14] and with that from human skeletal muscle[15]. EDELHOCH AND COLEMAN[13] assumed that the actual effect of neutral salts on the activity of pancreatic ribonuclease was an effect of ionic strength. IRIE[16], in his study on the effect of salts of univalent cations on the pancreatic ribonuclease activity, has concluded that the inhibitory effect of these salts was probably due to the binding of anions with the protonated group of ribonuclease basic protein. In the case of acidic protein of *S. aureofaciens* ribonuclease, this hypothesis does not explain the inhibitory effect of NaCl and other neutral salts on the enzymatic activity.

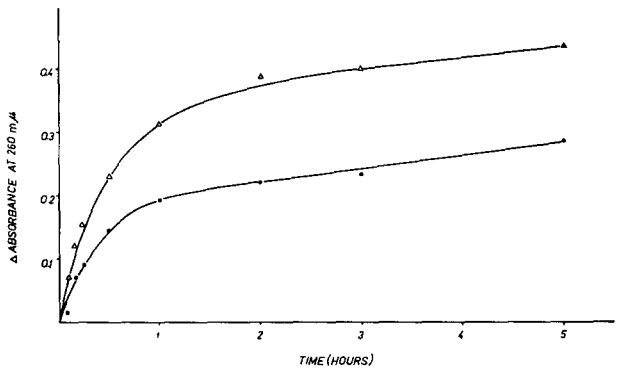


Fig. 6. Time-course of RNA hydrolysis by *S. aureofaciens* ribonuclease. Commercial yeast RNA (△) and high-molecular-weight yeast RNA (●) were used as substrates. The assay is described in MATERIALS AND METHODS. The reaction mixture contained 20 units of ribonuclease.

Time-course of the hydrolysis

The time-course of the hydrolysis of commercial and high-molecular-weight yeast RNA with *S. aureofaciens* ribonuclease is shown in Fig. 6. Low-molecular-weight RNA seemed to be more sensitive to ribonuclease action than high-molecular-weight RNA was. The increase of acid-soluble products of hydrolysis was linear for approx. 15 min. The initial velocity for the hydrolysis of commercial and high-molecular-weight yeast RNA was found to be 0.017 and 0.010 optical units per min, respectively.

Michaelis constant

Fig. 7 shows the Lineweaver–Burk plot from which $K_m = 0.679$ mg/ml and $v_{max} = 55.5 \cdot 10^{-2}$ optical units per 15 min for commercial yeast RNA, and 0.819 mg/ml and $40.5 \cdot 10^{-2}$ optical units per 15 min for high-molecular-weight RNA were calculated. A K_m value for ribonuclease isolated from lymphosarcoma P 1798 has been found to be 0.8 mg yeast RNA per ml[17], similar to that of *S. aureofaciens* ribo-

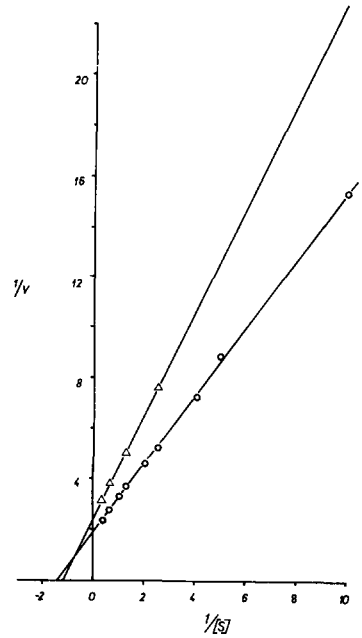


Fig. 7. LINEWEAVER–BURK plot for the purified *S. aureofaciens* ribonuclease. S = mg yeast RNA per ml, v = $A_{260\ m\mu}$ per 15 min. Low-molecular-weight commercial RNA (○), high-molecular-weight RNA (△). Enzymatic reactions were performed in 25 mM sodium phosphate buffer (pH 7.0) with 20 units of the enzyme.

nuclease. For ribonuclease from tobacco leaves[18] and for pancreatic ribonuclease[13], higher K_m values have been determined (1.2 and 1.25 mg yeast RNA per ml, respectively). A K_m of 0.098 mg RNA per ml for ribonuclease from Pleospora has been reported[19].

Specificity

The chromatography of the products resulting from the action of *S. aureofaciens* ribonuclease on transfer RNA (Fig. 8) indicates that it is an endonuclease splitting the ester bond between the guanosine-3′-phosphate and the –OH group at the 5′-position of ribose of the adjoining nucleotide, as guanosine monophosphate is produced practically exclusively as mononucleotide. As with ribonuclease T_1, the guanosine-2′, 3′-cyclic phosphate is first formed and then hydrolyzed with the enzyme to give guanosine-3′-phosphate (Fig. 8A). After trichloroacetic acid treatment, the spot of guanosine-2′,3′-cyclic phosphate on the chromatogram nearly disappeared, as the cyclic phosphodiester linkage by this treatment had been opened (Fig. 8B). The analysis of terminal nucleotides of the ribonuclease digest confirmed this specificity because the only nucleoside found was guanosine. The ability of the enzyme to degrade poly G, but not poly A, poly U or poly C, also suggests that it can split only secondary phosphate ester bonds of guanosine-3′-phosphate, thus showing its resemblance to ribonuclease T_1[2] and to guanyloribonuclease from *Actinomyces aureoverticillatus*[11]. Using 10 units of ribonuclease, poly A, poly C and poly U did not

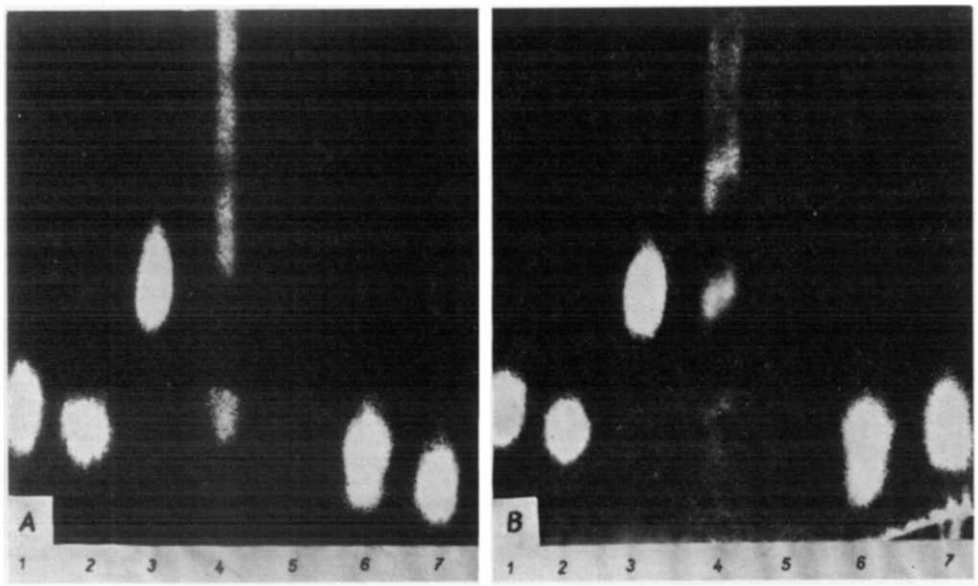


Fig. 8. Paper chromatograms of the RNA digest with *S. aureofaciens* ribonuclease. Reaction mixture containing 1 mg tRNA, 50 mM sodium phosphate buffer (pH 7.0) and 10 units of ribonuclease was incubated 20 h at 37°. Control sample without the enzyme was incubated under the same conditions. A. The RNA digest was applied on the filter paper without stopping the enzymatic reaction. B. The enzymatic reaction was stopped with 5% trichloroacetic acid. 1, 3'-UMP; 2, 2',3'-cyclic GMP; 3, 3'-GMP; 4, RNA digest; 5, control sample; 6, 3'-CMP; 7, 3'-AMP.

appear to be attacked even after incubation for 20 h, the test samples remaining at the origin of the chromatograms. It was found that poly I was also cleaved by *S. aureofaciens* ribonuclease.

Using large amounts of ribonuclease, poly A as well as poly G and poly I were hydrolyzed while poly U was partially hydrolyzed. Depolymerization of poly A, poly U and poly C by ribonuclease T_1 was also reported[20]. BEERS[21] and IMURA *et al.*[22] observed that pyrimidine-specific pancreatic ribonuclease hydrolyzed the phosphodiester linkage in polyadenylic and polyinosinic acids.

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