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EXOCELLULAR RIBONUCLEASE FROM *STREPTOMYCES AUREOFACIENS*

I. ISOLATION AND PURIFICATION

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SUMMARY

1. The ribonuclease from the cultural medium of *Streptomyces aureofaciens* BM-K, a chlorotetracycline-producing strain, was purified to a chromatographically and disc-electrophoretically homogeneous state. The enzyme was purified 1000-fold with a yield of 13% with the aid of ammonium sulfate fractionation and by chromatography on DEAE-Sephadex A-25 and DEAE-cellulose.

2. The purified enzyme was found to be free of deoxyribonuclease, non-specific phosphodiesterase and monophosphatase activity.

3. The amino acid composition of *Streptomyces aureofaciens* ribonuclease was determined.

4. The isoelectric point was found to be around pH 4.3.

INTRODUCTION

Several enzymes, proteases[1–3], amylases[4], and different nucleolytic enzymes[5–8], have been found in grown cultural media of Streptomyces. Similarly many other Actinomycetes also produce exocellular nucleolytic enzymes[9–11]. The specficity of exocellular ribonucleases from *Streptomyces erythreus*[5], *Streptomyces albogriceolus*[7] and *Actinomyces aureoverticillatus*[12] is similar to that of ribonuclease T_1 (EC 2.7.7.26, ribonucleate guaninenucleotido-2′-transferase, cyclizing) isolated by EGAMI[13] from *Aspergillus oryzae*.

In our preceding paper[8] biosynthesis of an exocellular ribonuclease during the fermentation of *Streptomyces aureofaciens*, a strain producing the antibiotic chlortetracycline, has been reported.

The present study deals with the isolation, purification, and some properties of this ribonuclease.

MATERIALS AND METHODS

Organism

For the isolation of ribonuclease a high-productive strain of *S. aureofaciens* BM-K producing about 3000 μg of chlortetracycline per ml was used.

Medium and growth conditions

These were described in our preceding paper[8].

Assay of enzyme activity

Ribonuclease activity was determined as described by TAKAHASHI[14] in 25 mM sodium phosphate buffer (pH 7.0). Commercial yeast RNA (Cambrian Chemicals LTD, London) purified by ethanol precipitation, dialyzed and lyophilized was used as the substrate.

Phosphomonoesterase activity was determined with *p*-nitrophenyl phosphate as substrate according to the method of JONES[15] in sodium acetate buffer (pH 5.0), in sodium phosphate buffer (pH 7.0) and in Tris–HCl buffer (pH 8.2). The reaction mixture was incubated for 2 h at 37°.

Phosphodiesterase activity was determined by the method of SINSHEIMER AND KOERNER[16]. Incubation time was 5 h at 37°.

Deoxyribonuclease activity was assayed by measuring at 260 mμ the acid-soluble material liberated from DNA. The reaction mixture contained in 1 ml:3 mg DNA (*ex* herring sperm, Calbiochem), 0.3 mg enzyme protein, 0.1 M Tris–HCl buffer (pH 7, 7.5 and 8.8, respectively) and 3.10^{-3} M $MgCl_2$. Incubation time was 5 h at 37°.

Carbohydrate content of purified ribonuclease was estimated by the method of DUBOIS *et al.*[17].

Protein content was estimated by measuring the absorbance at 280 mμ and by the method of LOWRY *et al.*[18] using human plasma albumin (Mann. Res. Lab. Inc. New York) as standard.

Acrylamide gel electrophoresis

Electrophoresis was performed by the method of DAVIS[19] in 7.5% polyacrylamide gel in a Tris–glycine buffer (pH 8.7) and by the method of REISFELT *et al.*[20] in 10% polyacrylamide gel in β-alanine–acetic acid buffer (pH 3.0). Staining according to the method of WILSON[21] for detection of ribonuclease was also used.

The localization of ribonuclease activity was confirmed by the standard ribonuclease assay after elution of ribonuclease from gel slices with 2.0 ml of 0.1 M sodium phosphate buffer (pH 7.0) for 18 h at 4°.

Amino acid analysis

Enzyme samples were dissolved in constant boiling HCl and hydrolyzed in evacuated sealed tubes at 110° for 24 and 72 h, respectively. Amino acid analyses were performed in an automatic amino acid analyzer[22].

Cystine and methionine were estimated as cysteic acid and methionine sulfon, respectively, after performic acid oxidation of ribonuclease[23].

Paper electrophoresis

300 μg enzyme protein was applied to Whatman No. 1 filterpaper (4 cm × 30

cm). Electrophoresis was carried out in 0.05 μ glycine–HCl buffer (pH 2.25), 0.05 μ sodium acetate buffers (pH 3.6 and 5.6) and 0.05 μ sodium phosphate buffer (pH 7.0) and was run at 10 V/cm for 5 h at 4°. Protein was stained with 0.5% solution of amido black in 7.5% acetic acid for 1 h. Destaining was carried out using 7.5% acetic acid. The non-stained paper after electrophoresis was cut off by 1-cm width, eluted with 2 ml of redistilled water for 3 h and ribonuclease was determined by the standard assay procedure.

RESULTS AND DISCUSSION

Isolation and purification of the enzyme

Step 1: heat treatment

The supernatant obtained after centrifugation of the cultivation broth of *S. aureofaciens* (25 h of fermentation) was adjusted to pH 1.5–2 with sulfuric acid and heated at 80° for 2 min. The resulting precipitate was removed by centrifugation.

Step 2: $(NH_4)_2SO_4$ precipitation

After adjusting the acid supernatant to pH 4.0 with NH_4OH, the protein was precipitated with solid $(NH_4)_2SO_4$ to 90% saturation. After 24 h at 4° the solution

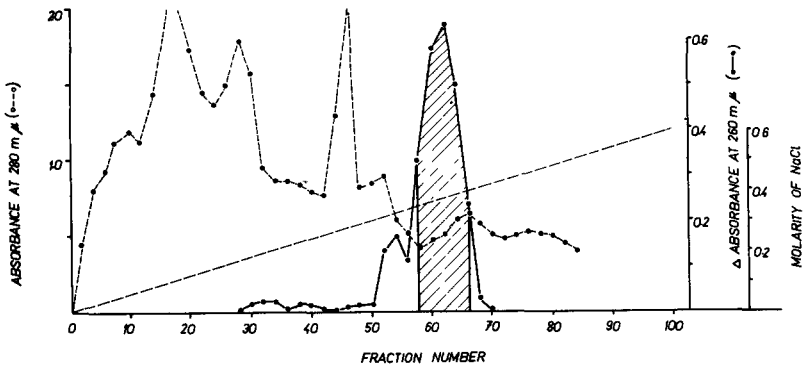

Fig. 1. First chromatography of ribonuclease on a DEAE-Sephadex A-25 column. Column: 3 cm × 35 cm (equilibrated with 0.1 M phosphate buffer pH 6.7). Load: about 450 mg of protein after ammonium sulfate fractionation. Elution: gradient (from 0 to 0.6 M) NaCl in 0.1 M phosphate buffer (pH 6.7). Elution rate: 60 ml per h. Fractions: 20 ml.

was filtered and the precipitate was dissolved in a minimal vol. of 0.01 M sodium phosphate buffer (pH 6.7) and dialyzed for 24 h against the same buffer at 4°.

Step 3: chromatography on DEAE-Sephadex A-25

The dialyzed enzyme solution was applied to a DEAE-Sephadex A-25 column (3 cm × 35 cm) equilibrated with 0.1 M sodium phosphate buffer (pH 6.7). Elution was carried out with a linear NaCl gradient (from 0.0 to 0.6 M) in the same buffer. 20-ml fractions were collected at 20 min intervals. Each fraction was assayed for protein at 280 mμ and enzyme activity (Fig. 1).

Step 4: chromatography on DEAE-cellulose

Combined active fractions were dialyzed against distilled water and chromatographed on DEAE-cellulose column (3 cm × 30 cm) equilibrated with 0.01 M

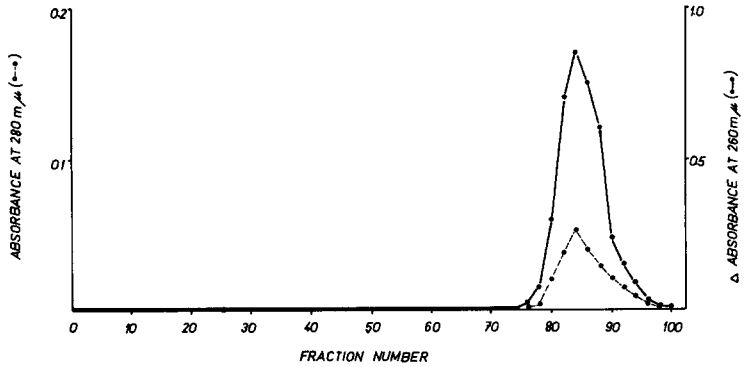

Fig. 2. Chromatography of ribonuclease on a DEAE-cellulose column (Step 6). Column: 1 cm × 30 cm (equilibrated with 0.01 M phosphate buffer pH 6.7). Load: about 2 mg of protein. Elution. gradient (from 0 to 0.3 M) NaCl in 0.01 M phosphate buffer (pH 6.7). Elution rate: 20 ml per h. Fractions: 6.5 ml.

phosphate buffer (pH 6.7). The enzyme was eluted with the same buffer with linear gradient NaCl (from 0.0 to 0.4 M).

Step 5: concentration on DEAE-Sephadex A-25

Combined and dialyzed active fractions were applied to a 1 cm × 3 cm column of DEAE-Sephadex A-25 equilibrated with 0.1 M phosphate buffer (pH 6.7). The enzyme was eluted with 0.5 M NaCl in the same buffer. Fractions of 1 ml were collected and those containing the ribonuclease activity were pooled.

Steps 5 and 6: rechromatography on DEAE-Sephadex A-25 and DEAE-Cellulose

After the dialysis of the enzyme solution against water, rechromatography on DEAE-Sephadex A-25 and DEAE-cellulose columns (1 cm × 30 cm) was carried out under conditions identical to those employed in Steps 3 and 4. The elution pattern of Step 6 is shown in Fig. 2. Ribonuclease was eluted as a single peak with respect to the enzyme activity as well as to the protein.

The results of the purification procedures of this enzyme are summarized in Table I.

TABLE I

PURIFICATION OF RIBONUCLEASE FROM *S. aureofaciens*

	Total protein (g)	*Total units (× 10⁻³)*	*Specific activity (units per mg protein)*	*Activity yield (%)*
Filtrate	18.8	957	50.7	100
Heat-treatment	15.9	746	46.9	78
$(NH_4)_2 SO_4$ fractionation	1.5	588	392.0	61
First chromatography on DEAE-Sephadex A-25	0.096	497	5 180	52
First chromatography on DEAE-cellulose	0.021	382.8	17 880	40
Second chromatography on DEAE-Sephadex A-25	0.0059	267.9	45 230	27.9
Second chromatography on DEAE-cellulose	0.0018	132.0	72 160	13.8

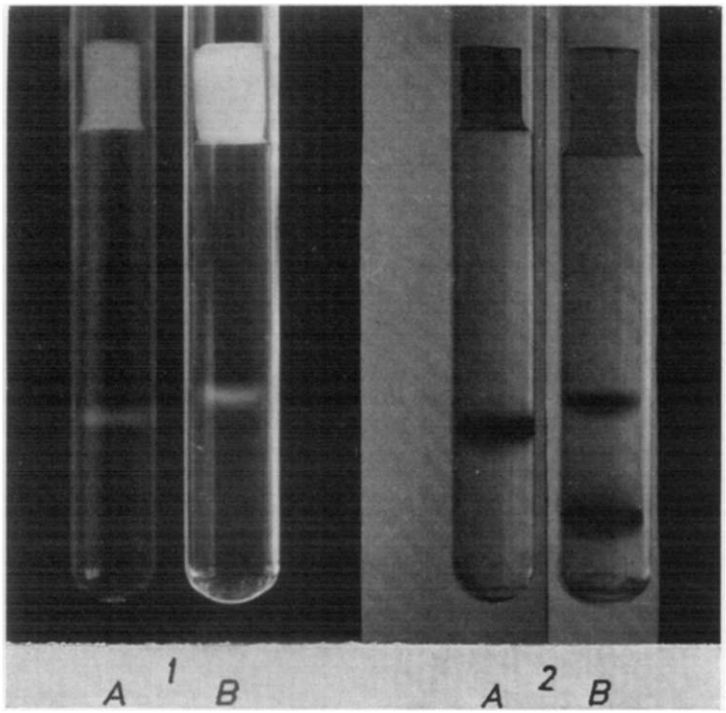

Fig. 3. Disc electrophoresis of purified *S. aureofaciens* ribonuclease. No. 1, specific detection of ribonuclease[21]. No. 2, staining with amido black. A, electrophoresis in alanine–acetic acid buffer (pH 3.0), B, electrophoresis in Tris–glycine buffer (pH 8.7). No. 2B, the lower band was formed by the bromophebol blue.

Purity of the enzyme

The purified ribonuclease preparation in chromatography on DEAE-cellulose and by disc-electrophoresis was shown to be homogeneous. Only one band was seen after polyacrylamide gel electrophoresis at pH 8.7 and pH 3.0 after staining the protein with Amido black and by specific detection of ribonuclease[21] (Fig. 3). Ribonuclease was eluted from non-stained gel columns and its activity corresponded with the location of protein.

Ribonuclease was found to contain 0.8–1.9% of sugar in glucose equivalent and a small amount of pigment impurities. Ribonuclease T_1 was reported to contain 0 to 0.5% of sugar[13], and the sugar content of guanyl-ribonuclease from *Actinomyces aureoverticillatus* was 3%[12].

The purified *S. aureofaciens* ribonuclease did not release any acid-soluble deoxyribonucleotide from DNA during 5 h of incubation. It did not show any non-specific phosphodiesterase activity when incubated with bis (*p*-nitrophenyl) phosphate, and it was free of acid and alkaline phosphomonoesterase activity.

Amino acid composition

Amino acid composition of the purified *S. aureofaciens* ribonuclease is represented in Table II. The values represent the averages of three separate analyses of

24-h and 72-h hydrolysates of native ribonuclease and 24-h hydrolysates of performic acid oxidized enzyme for half-cystine and methionine determination. The values for aspartic and glutamic acids, threonine and serine were corrected for destruction by extrapolation to zero time. The values for isoleucine and valine were corrected for incomplete liberation by calculating the averages of 72-h hydrolysates only.

S. aureofaciens ribonuclease is characterized by a relatively high content of acidic amino acids, and by a relatively low content of basic and neutral ones. In comparison with ribonuclease T_1[24] *S. aureofaciens* ribonuclease contains considerably lower amounts of serine, tyrosine, glycine and aspartic acid. The content of the basic

TABLE II

AMINO ACID COMPOSITION OF *S. aureofaciens* RIBONUCLEASE IN COMPARISON WITH RIBONUCLEASE T_1[24]

The values for aspartic acid, threonine, serine and glutamic acid were obtained by extrapolation to zero time. The values for isoleucine and valine are the averages of 72-h hydrolysates only.

Amino acid	*Amino acid per 100 g of protein (g)*	
	S. aureofaciens ribonuclease	*Ribonuclease T_1*
Lysine	4.89	1.36
Histidine	2.76	4.18
Arginine	5.17	1.54
Aspartic acid	12.67	18.0
Threonine	5.81	6.49
Serine	6.51	16.51
Glutamic acid	15.30	13.44
Proline	4.04	4.57
Glycine	4.19	8.26
Alanine	5.49	5.57
Half-cystine	4.67	4.06
Valine	4.93	7.84
Methionine	2.09	0
Isoleucine	3.69	2.04
Leucine	5.55	3.52
Tyrosine	4.74	13.43
Phenylalanine	3.67	5.69

amino acids, lysine and arginine, is higher than that of ribonuclease T_1. In contrast to ribonuclease T_1 which contains no methionine, *S. aureofaciens* ribonuclease does contain methionine.

Isoelectric point

The acidic character of *S. aureofaciens* ribonuclease was confirmed by its electrophoretic mobility. After paper electrophoresis only one band of protein by amido black was stained. The ribonuclease activity corresponded with protein localization. From the mobilities at different pH the isoelectric point was determined

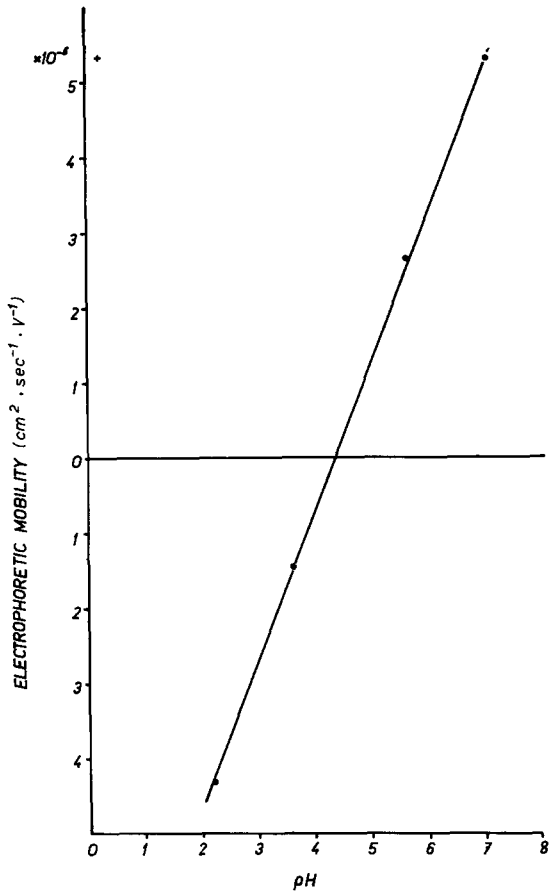

Fig. 4. Electrophoretic mobility of *S. aureofaciens* ribonuclease.

to lie around pH 4.3 (Fig. 4). The acidity of this ribonuclease is not so high as that of ribonuclease T_1, which has an isoelectric point around pH 2.9[24].

REFERENCES

1 E. D. KAVERZNEVA AND JU. A. RASSULIN, *Biokhimiya*, 29 (1964) 1042.
2 N. NOMOTO AND J. NARAHASHI, *J. Biochem.*, 46 (1959) 653.
3 Ľ. POLÍVKA AND J. ZELINKA, *Biológia*, (1971) 26 (1971) 245.
4 E. HOSTINOVÁ AND J. ZELINKA, *Biológia*, 24 (1969) 653.
5 K. TANAKA AND G. L. CANTONI, *Biochim. Biophys. Acta*, 72 (1963) 641.
6 M. YONEDA, *J. Biochem.*, 55 (1964) 475.
7 M. YONEDA, *J. Biochem.*, 55 (1964) 469.
8 E. ZELINKOVÁ AND J. ZELINKA, *Biológia*, 24 (1969) 456.
9 R. I. TATARSKAYA, A. I. KORENYAKO, I. A KUROEDOVA, N. M. ABROSIMOVA-AMELYANCHIK, V. D. AKSELROD AND A. A. BAEV, *Izv. Akad. Nauk SSSR, Ser. biol.*, 5 (1966) 653.
10 A. A. BASKAKOVA, A. V. BALDINA AND A. M. BEZBORODOV, *Mikrobiologiya*, 36 (1967) 19.
11 A. M. BEZBORODOV, A. A. BASKAKOVA AND A. V. BALDINA, *Mikrobiologiya*, 36 (1967) 36.
12 R. I. TATARSKAYA, N. M. ABROSIMOVA-AMELYANCHIK, V. D. AKSELROD, A. I. KORENYAKO, N. JA. NIEDRA AND A. A. BAEV, *Biokhimiya*, 31 (1966) 1017.

13 F. EGAMI, *J. Sci. Ind. Res.*, 25 (1966) 442.
14 K. TAKAHASHI, *J. Biochem*, 49 (1961) 1.
15 TH. C. JONES, *Mol. Gen. Genetics*, 105 (1969) 91.
16 R. L. SINSHEIMER AND J. F KOERNER, *J. Biol. Chem.*, 198 (1952) 293.
17 M. DUBOIS, K A GILLES, J. K. HAMILTON, P A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
18 O. H. LOWRY, N. J ROSENBROUGH, A. L. FARR AND R. J. RANDALL, *J Biol. Chem.*, 193 (1951) 265.
19 B. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
20 R. A. REISFELD, U. J. LEWIS AND D. E. WILLIAMS, *Nature*, 195 (1962) 281.
21 C. W. WILSON, *Anal. Biochem*, 31 (1969) 506.
22 D. H. SPACKMAN, W. H. STEIN, S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
23 S. MOORE, *J. Biol. Chem.*, 238 (1963) 235.
24 K TAKAHASHI, *J. Biochem.*, 51 (1962) 95.