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ISOLATION AND SOME PROPERTIES OF RIBONUCLEASE FROM *FUSARIUM MONILIFORME*

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SUMMARY

1. Two ribonucleases were found in the dried culture filtrate of *Fusarium moniliforme*. The major one, tentatively named ribonuclease F_1, was purified 1500–2000 fold. The most highly purified preparation possessed a specific activity of 60% of that of ribonuclease T_1.

2. Ribonuclease F_1 splits internucleotide bonds between 3'-guanylic acid and the 5'-hydroxyl group of the adjacent nucleotide with intermediary formation of guanosine 2',3'-cyclic phosphate.

3. The optimal activity of ribonuclease F_1 was found at about pH 7. Ribonuclease F_1 was inhibited by Zn^{2+} and Hg^{2+} and activated by histidine and EDTA. The molecular weight was estimated to be about 11 000. The isoelectric point was found around pH 4.

INTRODUCTION

Many ribonucleases have been described so far, *e.g.* ribonuclease T_1 of *Aspergillus orizae*[1], ribonuclease IA of bovine pancreas[2], ribonuclease N_1 of *Neurospora crassa*[3] and ribonuclease U_1 of *Ustilago shaerogena*[4].

The present paper reports the isolation and properties of a ribonuclease from the dried culture filtrate of *Fusarium moniliforme*.

MATERIALS AND METHODS

Materials

Dried powder of the culture filtrate of *F. moniliforme*, prepared as follows, was kindly supplied by Toyo Brewry Co., Ohito, Shizuoka, Japan. The culture filtrate was concentrated under reduced pressure at below 30° and 7 vols. of 95% cold ethanol were added. The precipitate was collected by centrifugation and dried *in vacuo*. The preparation is available as "Toyocellase" from the company and is called the crude enzyme in this paper. Guanosine 2',3'-cyclic phosphate was synthesized as described by SMITH *et al.*[5]. Ribonuclease T_1 was a kind gift of Dr. T. Uchida of

Tokyo University. Yeast RNA (Toyo Spinning Co., Osaka, Japan) and alkaline phosphomonoesterase of *Escherichia coli* (Worthington Co., Freehold, New Jersey) were commercial preparations.

Determination of enzyme activity and protein content

The enzyme activity was assayed by the hydrolysis of yeast RNA as described by TAKAHASHI[6]. The protein content was determined by the absorbance at 280 nm. The enzyme unit and the specific activity were calculated as described by TAKAHASHI[6].

Identification of reaction products

The mononucleotides, 3'-terminal nucleotides and 2',3'-cyclic mononucleotides were identified by paper chromatography on Toyo No. 51A filter paper (Toyo Roshi Co., Tokyo, Japan) as described by UCHIDA AND EGAMI[7]. The hydrolysis of 2',3'-cyclic guanylic acid by the enzyme was followed as described in the legend of Fig. 7.

Estimation of molecular weight

The molecular weight was estimated by gel filtration on a Sephadex G-75 column (1.9 cm × 75 cm), according to the method of ANDREWS[8]. Erabutoxin a, crystalline neurotoxin from a sea snake (mol. wt. 6700)[9], ribonuclease T_1 (mol. wt. 11 000)[10] and trypsin (mol. wt. 24 000)[11] were used as marker proteins.

Estimation of isoelectric point

The isoelectric point was determined by paper electrophoreses on Toyo No. 51A paper (2 cm × 40 cm) in four buffer solutions with ionic strength of 0.05. The buffers were as follows, disodium citrate–HCl buffer at pH 3, sodium acetate buffer at pH 4 and 5 and sodium potassium phosphate buffer at pH 6. Glucose was run with the sample as calibration for electroosmosis. After 5 h at 11 V/cm at 4°, each strip of paper was cut into two halves (1 cm × 40 cm). One was used to detect glucose by ammoniacal silver nitrate spray[12], the other was cut into pieces of 5 mm length and each piece was extracted with 1 ml of distilled water and ribonuclease activity determined.

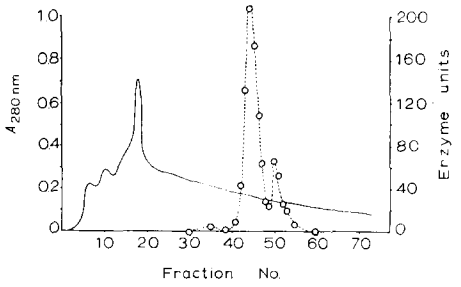
RESULTS

DEAE-cellulose chromatography of the crude enzyme

As a preliminary experiment, the crude enzyme (500 mg) was extracted with 5 ml of distilled water and the extract adjusted to pH 2.0 with 2 M HCl. The precipitate was removed by centrifugation (3000 rev./min, for 15 min). The supernatant was neutralized with 2 M NaOH, dialyzed against distilled water, adjusted to 0.005 M Na_2HPO_4 with 0.2 M Na_2HPO_4 and loaded onto a DEAE-cellulose column. The elution pattern is shown in Fig. 1. Ribonuclease activity appeared in two peaks which were tentatively named ribonuclease F_1 (major peak) and ribonuclease F_2 (minor peak).

Purification

Step 1. Batchwise treatment with DEAE-Sephadex A-25. The crude enzyme



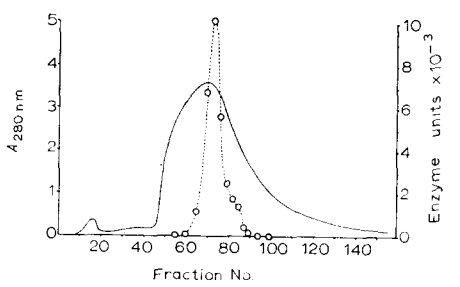


Fig. 1. DEAE-cellulose column chromatography of the crude enzyme extract. Heat-treated extract (5 ml) was loaded onto a DEAE-cellulose column (1.2 cm × 30 cm). The gradient elution was made between 0.05 M Na_2HPO_4 in a constant volume mixing chamber (600 ml) and 0.25 M NaH_2PO_4 containing 0.25 M NaCl. Fractions of 10 ml were collected. ———, $A_{280\,nm}$; ○---○, enzyme units.

Fig. 2. DEAE-Sephadex A-25 column chromatography. The protein solution (135 ml, total $A_{280\,nm} = 3.71 \cdot 10^3$, total activity $= 2.34 \cdot 10^5$ units) was applied to a DEAE-Sephadex A-25 column (1.4 cm × 40 cm). The gradient elution was carried out as described in the legend of Fig. 1. Fractions of 10 ml were collected. ———, $A_{280\,nm}$; ○---○, enzyme units.

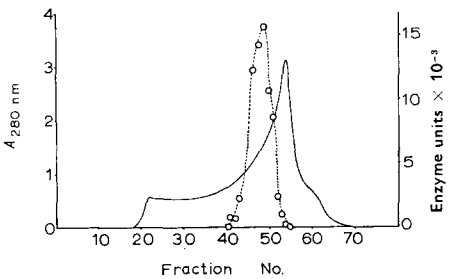
(500 g) was extracted with 5 l of distilled water. The extract was adjusted to pH 5.5 with 2 M NaOH. DEAE-Sephadex A-25 (180 g, dry weight), which had been washed with 0.1 M NaOH containing 0.5 M NaCl and with distilled water, was added to the solution. After 30 min at room temperature, the DEAE-Sephadex was collected by filtration and washed with 0.066 M sodium-potassium phosphate buffer containing 0.06 M NaCl, pH 4.6 (500–1000 ml, 4 times). The ribonuclease was eluted from the Sephadex with 0.25 M NaH_2PO_4 containing 0.25 M NaCl (100 ml, 10 times). The eluate was diluted with 3 vols. of water and DEAE-Sephadex (20 g, dry weight) was added. The DEAE-Sephadex was collected, washed with 0.066 M sodium potassium phosphate buffer containing 0.06 M NaCl, pH 4.6 (200–400 ml, 4 times) and eluted with 0.25 M NaH_2PO_4 containing 0.25 M NaCl (60 ml, 15 times). The combined eluate was dialysed against 0.05 M acetic acid and freeze–dried.

Step 2. DEAE-Sephadex A-25 column chroma'ography. The dried powder was dissolved in 0.04 M sodium–potassium phosphate buffer (pH 7.0, 135 ml) and chromatographed on a DEAE-Sephadex A-25 column (Fig. 2). The activity was eluted as a peak with a shoulder. Active fractions were combined and freeze–dried. The residue was desalted on a Sephadex G-25 column (2.8 cm × 41 cm) with distilled water and freeze–dried again.

Step 3. Gel filtration with Sephadex G-75. The dried powder was dissolved in 8 ml of 0.01 M sodium-potassium phosphate buffer (pH 6.2) and gel-filtered on a Sephadex G-75 column (Fig. 3). The ribonuclease activity was eluted earlier than the major proteins. The active fractions were collected and freeze–dried.

Step 4. DEAE-cellulose column chromatography. The dried powder containing phosphate was dissolved in 12 ml of water and chromatographed on a DEAE-cellulose column (Fig. 4). As shown in Fig. 4, the ribonuclease activity appeared in two peaks, ribonuclease F_1 (major) and ribonuclease F_2 (minor). The F_1 fractions were freeze–dried.

Step 5. Gel filtration through a Sephadex G-50. The dried F_1 preparation was dissolved in 5 ml of water and applied to a Sephadex G-50 column (Fig. 5). The



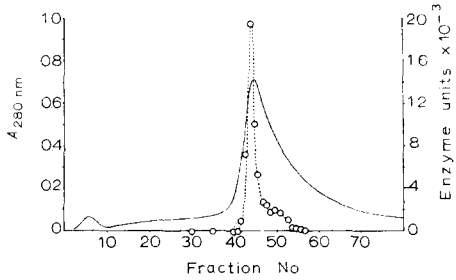


Fig. 3. Gel filtration on Sephadex G-75. The enzyme solution (8 ml, total $A_{280\ nm} = 6.76 \cdot 10^2$, total activity $= 8.85 \cdot 10^4$ units) was applied to a Sephadex G-75 column (2.8 cm × 120 cm) and eluted with 0.01 M sodium potassium phosphate buffer (pH 6.2). Fractions of 10 ml were collected. ———, $A_{280\ nm}$; ○---○, enzyme units.

Fig. 4. DEAE-Cellulose column chromatography. The enzyme solution (12 ml, total $A_{280\ nm} = 1.45 \cdot 10^2$, total activity $= 9.04 \cdot 10^4$ units) was applied to a DEAE-cellulose column (1.4 cm × 40 cm). The gradient elution was carried out as described in the legend of Fig. 1. Fractions of 10 ml were collected. ———, $A_{280\ nm}$; ○---○, enzyme units.

maximal elution points of protein and the activity were coincident. The elution pattern of the protein was, however, broader than that of the activity.

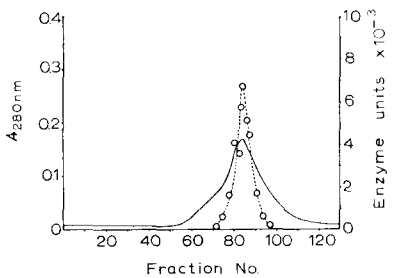


Fig. 5. Gel filtration on Sephadex G-50. The enzyme solution (5 ml, total $A_{280\ nm} = 4.5 \cdot 10$, total activity $= 5.17 \cdot 10^4$ units) was applied to a Sephadex G-50 column (2.8 cm × 287 cm). The elution was carried out under the same conditions as described in the legend of Fig. 3. Fractions of 10 ml were collected. ———, $A_{280\ nm}$; ○---○, enzyme units.

The results of purification are summarized in Table I.

TABLE I

RESULTS OF PURIFICATION AFTER EACH STEP

Step	*Total* $A_{280\ nm}$	*Total activity*	*Specific activity*	*Yield (%)*
Water extraction	$1.84 \cdot 10^5$	$3.87 \cdot 10^5$	0.21	100
Batchwise DEAE-Sephadex treatment	$3.71 \cdot 10^3$	$2.34 \cdot 10^5$	6.3	60
DEAE-Sephadex A-25 chromatography	$6.76 \cdot 10^2$	$8.85 \cdot 10^4$	13	23
Sephadex G-75 chromatography	$1.45 \cdot 10^2$	$9.04 \cdot 10^4$	63	23
DEAE-cellulose chromatography	$4.5 \cdot 10$	$5.17 \cdot 10^4$	120	13
Sephadex G-50 chromatography	$1.6 \cdot 10$	$4.5 \cdot 10^4$	300	12
(peak)	—	—	400	—

pH optimum of ribonuclease F_1

The pH–activity curve is shown in Fig. 6. The optimal activity was found at around pH 7.

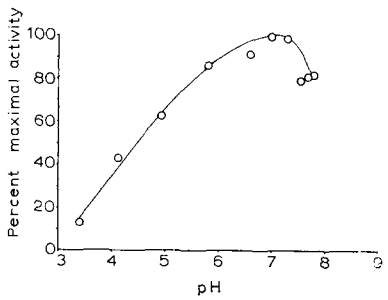


Fig. 6. pH–activity curve of ribonuclease F_1. The enzyme activity was measured in citrate–phosphate buffers (0.05 M). The activity was expressed as percentage of maximal activity.

Effect of some chemicals on the ribonuclease F_1 activity

The results are summarized in Table II. The results with ribonuclease T_1 (ref. 1) are also shown for comparison.

TABLE II

FACTORS AFFECTING ACTIVITY

Reagent	*Concentration (− log M)*	*Activity (%)*	
		Ribonuclease F_1	*Ribonuclease T_1*
—	—	100	100
p-Chloromercuribenzoate	3	100	
Hg^{2+}	4	90	
	3	58	0
Mg^{2+}	2	97	
	1	90	60
Zn^{2+}	3	27	0
Histidine	3	160	150
	2	150	82
EDTA	2	120	125–150

The mode of action of ribonuclease F_1

Base specificity of ribonuclease F_1. The reaction with yeast RNA proceeded rapidly at the early stages and stopped at about 30% hydrolysis in 10 h. The results suggest that the ribonuclease F_1 has some base specificity.

2′- and 3′-guanylic acids were detected in the acid soluble digest after paper chromatography by the R_F values and the fluorescence under ultraviolet light. Guanosine only was detected by the 3′-terminal nucleotide analysis. These results indicate that ribonuclease F_1 splits phosphodiester bonds of 3′-guanylic acid residues in RNA to produce 3′-guanylic acid and oligonucleotides terminating with 3′-guanylic acid.

Hydrolysis of guanosine 2′,3′-cyclic phosphate. When ribonuclease F_1 digest of yeast RNA was analyzed by paper chromatography without acid treatment, 2′,3′-

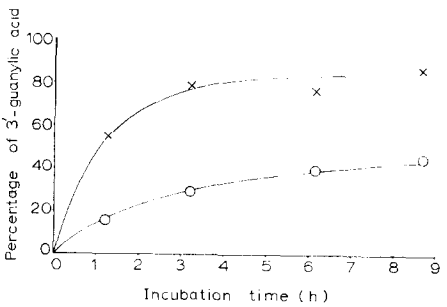


Fig. 7. Time course of 2′,3′-cyclic guanylate hydrolysis with ribonuclease F_1 and ribonuclease T_1. The reaction mixture, consisting of 0.6 μmole of 2′,3′-guanylate in 0.1 ml of Tris–HCl buffer (pH 7.5) and 0.025 ml of ribonuclease F_1 solution (15 units) or ribonuclease T_1 (11 units), was incubated at 37°. After intervals, 10 μl of the solution was subjected to paper electrophoresis on Toyo No. 51A paper for 90 min at 13 V/cm in 0.1 M ammonium bicarbonate buffer, pH 8.0. After runs, the spots of 3′-guanylate and 2′,3′-cyclic guanylate were located under an ultraviolet lamp, cut out and eluted with 0.01 M HCl. The absorbance of each eluate was measured at 257 nm. ×—×, ribonuclease F_1; ○—○, ribonuclease T_1.

cyclic guanylic acid corresponding to 2–3% of the total absorbance of the acid soluble products was detected. As shown in Fig. 7, ribonuclease F_1 hydrolyzed 2′,3′-cyclic guanylic acid into 3′-guanylic acid completely. The lower curve in this figure shows the result with ribonuclease T_1. From these results, it is concluded that ribonuclease F_1 hydrolyzes phosphodiester bonds with the formation of 3′-phosphates with 2′,3′-cyclic phosphates as intermediates.

Some other properties of ribonuclease F_1

Molecular weight. Ribonuclease F_1 was eluted at about the same volume as ribonuclease T_1 from the Sephadex G-75 column. The mol. wt. was estimated to be about 11 000.

Isoelectric point. The isoelectric point was determined by paper electrophoreses at various pH values. At pH 5.0 and 6.0, ribonuclease F_1 migrated to the anode, at pH 3.0 it migrated to the cathode. It remained at the origin with glucose at pH 4.0. These results show that the isoelectric point of the enzyme lies around pH 4.

DISCUSSION

Ribonuclease F_1 belonging to guanyloribonuclease (ribonucleate–guanine nucleotide-2′-transferase (cyclizing), EC 2.7.7.26), was purified 1200–2000 fold from the culture filtrate of *F. moniliforme*. It resembles ribonuclease T_1, ribonuclease N_1 and ribonuclease U_1 in base specificity, optimal pH and molecular weight. *F. moniliforme* was taxonomically classified as Deuteromycetes in Eumycophyta, and *A. orizae* and *N. crassa* as Ascomycetes and *U. shaerogena* as Basidomycetes. T_1 type ribonucleases seem to be found in culture media of fungi belonging to Eumycophyta. The most highly purified preparation obtained so far possessed a specific activity of $4 \cdot 10^2$ (Table I), this value being smaller than that of ribonuclease T_1, $7 \cdot 10^2$.

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