Purification and Characterization of Two Extracellular Ribonucleases from *Rhizopus oligosporus**

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ABSTRACT: Growth of *Rhizopus oligosporus* on a glucose- and peptide-rich medium results in the formation of extracellular ribonuclease activity, but little RNase is detected in mycelial extracts. Two ribonucleases have been isolated and shown to be homogeneous in polyacrylamide gel electrophoresis, ion-exchange chromatography, and (in one case) sedimentation equilibrium. Specific activities are comparable to other pure ribonucleases. The two enzymes are separable by ion-exchange chromatography or polyacrylamide gel electrophoresis, but are similar or identical in amino acid and carbohydrate composition and give indistinguishable tryptic peptide electrophoretic patterns. Each is characterized as a glycoprotein of about 30,000 molecular weight. The nucleases have similar pH profiles with optima at pH 6.9, and both degrade RNA or synthetic polynucleotides to mostly 2′,3′-cyclic phosphates and, eventually, 3′-nucleotides. Hydrolysis of homopolynucleotides or dinucleoside monophosphates shows no absolute base specificity, but indicates that the enzymes prefer substrates containing pyrimidine residues, particularly at the 5′-terminal position, to form a pyrimidine 2′,3′-cyclic phosphate.

The detailed study of the properties of ribonucleases has recently been stimulated by at least two factors. First, structural analysis of RNA has demanded highly specific enzymes for use in controlled degradation and nucleotide sequence analysis. Thus RNase T_1 (Sato and Egami, 1957) and RNase U_2 (Arima *et al.*, 1968), as well as pancreatic RNase, were readily exploited in sequence analysis because of their valuable substrate specificities (see Gilham, 1970). Since additional specificities would be of practical value, workers have attempted to find new and perhaps useful enzymes. Second, the ribonucleases often are small, stable, and easily isolated enzymes. Such characteristics help to make the proteins themselves good materials for the study of enzyme action and structure–function relationships. Comparative studies have been fostered by the considerable information on pancreatic RNase, RNase T_1, and the staphylococcal (micrococcal) nuclease (see Barnard (1969) or Egami and Nakamura (1969)).

As a result of such efforts Barnard (1969) was able to compile characteristics of over 75 RNases from many sources. In this paper we shall report on the characterization of two similar but separable ribonucleases from the culture medium of *Rhizopus oligosporus*. In each case, the enzymes are classified as cyclizing ribonucleases without absolute base specificity, but with some preference for attack at internucleotide bonds involving a pyrimidine 3′-phosphate.

Materials and Methods

Materials. All chemicals used were reagent grade or the equivalent unless otherwise specified. Buffers were prepared from imidazole (Matheson Chemicals, No. IX5), Tris (Trizma Base, Sigma Chemical Co.), and succinic acid (Sigma Chemical Co.). Thionine and Amido Black (aniline blue-black) were purchased from Matheson Chemicals, and materials for polyacrylamide gel electrophoresis from Eastman Organic Chemicals.

Ion-exchange celluloses were all standard grade products of Carl Schleicher and Schuell, and were purified by repeated removal of fines before use. Dowex 1-X2 and Bio-Gel P-2 were obtained from Bio-Rad Laboratories, and Sephadex products were purchased from Pharmacia.

Synthetic polynucleotides and dinucleoside monophosphates were purchased from either Miles Laboratories or Calbiochem. In most cases 1% solutions of the dinucleoside phosphates were passed through a 1 × 30 cm Bio-Gel P-2 column (eluted with water) and lyophilized prior to use. Nucleoside 2′,3′-cyclic phosphate and 3′-nucleotides were purchased from Calbiochem or from the Sigma Chemical Co.

Fraction V bovine serum albumin, five-times-recrystallized bovine pancreatic ribonuclease, and ribonuclease T_1 (Sankyo) were all purchased from Calbiochem. BPN′ subtilisin was a gift of Dr. F. Markland. Triphenyl chloromethyl ketone trypsin and *Escherichia coli* alkaline phosphatase were purchased from the Worthington Biochemical Co.

Assay Procedures. The quantitative assay for ribonuclease was modified from that of Glitz and Dekker (1964). A total of 1.1 ml of buffer (0.1 M imidazole–2 mg/ml of bovine serum albumin, pH 7.0) and 0.9 ml of enzyme (*ca.* 0.5 RNase unit) in water was mixed and brought to 30°. The reaction was started by adding 0.2 ml of RNA (high molecular weight wheat germ RNA (Glitz and Dekker, 1963), at 10 mg/ml and equilibrated at 30°). After thorough mixing, 1.0 ml was transferred to 3.0 ml of cold 10% perchloric acid in a centrifuge tube, mixed, and kept in an ice bath for at least 10 min. Fifteen minutes after removal of the first sample, another 1.0-ml aliquot was added to a second centrifuge tube containing 3.0 ml of cold 10% perchloric acid, mixed, and kept in an ice bath for at least 10 min. Each perchloric acid suspension was centrifuged for 10 min at 8000*g*, and the absorbance of the supernatant was measured at 260 mμ. The absorbance of the initial sample (defined as zero time) was subtracted from that of the final sample (15 min) to give the increase in acid-soluble, ultra-

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[1] Abbreviation used is: BSA, bovine serum albumin.

violet-absorbing materials resulting from enzyme action. An increase in absorbance at 260 mμ of 1.0 was defined as 1 unit of RNase activity. The assay was linear over the range of 0.1–1.2 units.

In studies of the effect of substrate concentration on reaction velocity, the procedure was altered as follows. The volume of the reaction mixture was doubled, and hydrolysis started by adding enzyme. Samples were withdrawn at 0, 5, 10, and 15 min, and initial velocities calculated from the straight-line portions of plots of acid-soluble nucleotide formation as a function of time.

Breakdown of dinucleoside monophosphates was measured by quantitative separation of nucleoside products from other reaction components (substrates, nucleoside cyclic phosphates, and nucleotides) which were retained on small columns of Dowex 1-X2 formate. In a typical reaction mixture 0.140 ml of assay buffer (0.1 M imidazole–2 mg/ml of bovine serum albumin, pH 7.0) and 20–800 μg of dinucleoside monophosphate in 0.160 ml of H_2O were incubated in a 1-ml centrifuge tube maintained at 30 ± 0.05° in a closed water bath. The reaction was started by adding 0.020 ml of enzyme in assay buffer and thoroughly mixing the solution. Immediately after mixing (about 20 sec) a 0.100-ml portion was removed and applied directly to a 0.5 × 7.0 cm Dowex 1-X2 formate column (equilibrated with 0.03 M formic acid adjusted to pH 3.7 with ammonia). The column was immediately eluted with 0.03 M formate (pH 3.7) and a 5.0-ml fraction was collected. At 10 and 20 min (after starting the reaction) additional 0.100-ml portions were taken, applied to separate columns, eluted as described above, and 5.0-ml fractions collected. The absorbance of each fraction at 260 mμ was measured and plotted as a function of time. The recovery of nucleoside (product) in each aliquot applied to a particular column was quantitative and directly proportional to the absorbance at 260 mμ of the 5.0-ml fraction eluted from that particular column. The increase in absorbance at 260 mμ with time was usually linear over 20 min, and was used to measure the initial velocity, v_i, of the reaction. The concentration of nucleoside was calculated using extinction coefficients for the nucleosides at pH 3.7.

If the substrates were nucleoside 2′,3′-cyclic phosphates, the procedure was modified by increasing the incubation times to 45 and 90 min. Samples (0.050 ml) from the reaction mixture were further incubated with 0.01-ml portions of *E. coli* alkaline phosphatase (0.77 mg/ml in assay buffer) for 10 min, and 0.05 ml of these samples applied to the Dowex 1-X2 columns.

Protein was estimated by the procedure of Lowry *et al.* (1951) as described in Cowgill and Pardee (1957), with a BSA standard, Approximate concentrations of protein were estimated by measurement of absorbance at 280 mμ.

Descending paper chromatography of nucleotides resulting from enzymic breakdown of RNA or polynucleotides was done on Whatman No. 3MM paper with an *n*-propyl alcohol–concentrated NH_3 solution–H_2O (6:3:1, v/v) solvent. Products were eluted from the paper with 0.1 M NH_3 solution.

Production of Ribonucleases. A strain of *Rhizopus oligosporus* (from the culture collection of the Department of Bacteriology, University of California, Los Angeles) was maintained on Antibiotic Medium No. 3 (Penassay broth, Difco Laboratories, Detroit, Mich.). An inoculum was prepared by swirling 4 ml of sterile water over the surface of a 4-day-old culture (Antibiotic Medium No. 3 + 1.5% agar) in a 10-cm petri dish. Portions (0.1 ml) of this suspension were added to two-liter portions of sterile Antibiotic Medium No. 3 contained in 6-l. erlenmeyer flasks, and the cultures were incubated at 25° on reciprocal shakers operated at 90 cpm. Nuclease activity in the medium was measured periodically, and when no further increase was apparent (usually after 6–8 days) the culture was filtered through Whatman No. 1 paper.

Ammonium sulfate was added to the filtered medium (step 1) until the solution was 70% saturated (472 g/l. of medium). This solution was kept at 4° for 7–10 days, until it became reasonably clear and a precipitate had settled. Most of the supernatant was then carefully siphoned off and discarded, and the precipitate stored as a suspension until sufficient quantities had been accumulated to permit large-scale purification of RNase.

Purification Procedure. The crude 70% ammonium sulfate precipitate above (step 2) was stored at 4° for up to 6 months with no apparent loss of RNase, thus permitting purification of a relatively large amount of enzyme (*e.g.*, 10^6 units). All steps were carried out at 4°.

The 70% ammonium sulfate suspension (about 10 l.) was centrifuged at 13,700*g* for 20 min, the supernatant discarded, and the precipitate dissolved in about 2 l. of water and dialyzed against at least four 10-l. portions of cold distilled water. The dialyzed material (2600 ml) was brought to 25% saturation by the slow addition of ammonium sulfate (144 g/l. of dialyzed material), and then centrifuged at 13,700*g* for 20 min. The supernatant was decanted and brought to 60% saturation, again by the slow addition of ammonium sulfate (230 g/l. of supernatant). The 60% ammonium sulfate suspension was centrifuged for 20 min at 13,700*g*, and the supernatant was discarded. The precipitate was dissolved in *ca.* 800 ml of water and dialyzed overnight against four 4-l. portions of distilled water (step 3).

Acetone fractionation was carried out on the material of step 3 (825 ml, protein concentration 30.6 mg/ml). Cold acetone (550 ml) was slowly added until the solution was 40% v/v with respect to acetone. The suspension was centrifuged for 30 min at 13,700*g*, and the supernatant decanted and brought to 60% v/v with respect to acetone (by adding 785 ml of acetone, dropwise). This suspension was centrifuged for 30 min at 13,700*g*, the supernatant discarded, and the precipitate dissolved in *ca.* 1200 ml of 0.02 M sodium acetate (pH 4.5) and dialyzed overnight against two 4-l. portions of the same buffer (step 4). The contents of the dialysis bag (1400 ml) were applied to a 4 × 120 cm CM-cellulose column, and the column was washed with 1 l. of starting buffer (0.02 M sodium acetate, pH 4.5) followed by a 6-l. linear salt gradient (0–1.0 M NaCl, in starting buffer) at a flow rate of 1 ml/min. Fractions (10 ml) were collected, and those which contained RNase activities of more than 100 units/ml were combined and dialyzed against 0.02 M Tris-HCl (pH 8.0) (step 5). This material (3840 ml, pH 8.0) was adsorbed on a DEAE-cellulose column (same dimensions as above) and the column was washed with 1 l. of starting buffer (0.02 M Tris-HCl, pH 8.0), followed by a linear sodium chloride gradient (0–0.5 M NaCl, in 2 l. of starting buffer) at a flow rate of 1 ml/min. The RNase activity was fractionated into two major components (steps 6a and 6b, see Figure 1), each of which was separately purified. The first component, eluted from the DEAE column at lower salt concentrations, will be referred to as RNase R_1 and the second component as RNase R_2.

Purification of RNase R_1. Fractions (step 6a) containing RNase R_1 with activity greater than 100 units/ml were combined and dialyzed overnight against two 4-l. portions of 0.02 M sodium succinate (pH 5.0). The contents of the dialysis bag (655 ml, pH 5.0) were applied to a 1.5 × 90 cm phosphocellu-

TABLE I: Purification of *Rhizopus oligosporus* Ribonucleases.

Step	Vol (ml)	RNase Act. (Units/ml)	Protein (mg/ml)	Sp Act. (Units/mg of Protein)	Yield (%)
1. Crude medium	2.3×10^5	6	2.0	3	100
2. Concentrate	2600	500	20.3	22.4	94
3. Ammonium sulfate	825	1,530	50.0	30.6	92
4. Acetone	1400	848	12.5	67.8	86
5. CM-cellulose	3840	202	0.346	583	57
6a DEAE-cellulose I	655	400	0.330	1,210	(19)
7a Phosphocellulose	265	750	0.040	18,750	(14.4)
8a Sephadex G-100	180	1,170	0.057	20,600	(10.6)
9a DEAE-cellulose II	3.65	40,000	1.17	34,200	(10.6)
6b DEAE-cellulose I	950	290	0.340	582	(20)
7b Phosphocellulose	100	2,000	1.5[a]	1,333	(14.5)
8b Sephadex G-100	5.0	32,000	8.0[a]	4,000	(11.6)
9b Sephadex G-200	5.0	24,000	2.4[a]	10,000	(8.7)
10b DEAE-cellulose II	1.86	37,200	1.2	31,000	(5.0)

[a] Estimated from absorbance at 280 mμ.

lose column, and the column was washed with 500 ml of starting buffer (0.02 M sodium succinate, pH 5.0) at a flow rate of 0.75 ml/min. The wash was followed by a 3-l. linear sodium chloride gradient (0–0.4 M NaCl, in starting buffer). Ten-ml fractions were collected, assayed for RNase, and absorbancies at 280 mμ measured. Those fractions with specific activity (RNase units/A_{280}) greater than 10,000 were pooled and dialyzed overnight against water (step 7a). The contents of the dialysis bag (265 ml, 199,000 RNase units) were lyophilized, dissolved in 4–5 ml of water, and applied to a 1.5 × 90 cm Sephadex G-100 column. The column was eluted with water at a rate of 0.2 ml/min and 5.0-ml fractions were collected. Fractions with specific activity (RNase units/A_{280}) greater than 15,000 were combined (180 ml, step 8a), adjusted to pH 8.5 with Tris-HCl buffer, and then applied to a 1.5 × 30 cm DEAE-cellulose column. The column was washed with 100 ml of 0.02 M Tris-HCl (pH 8.5) followed by a 2-l. sodium chloride gradient (0–0.25 M, in 0.02 M Tris-HCl, pH 8.5) at a flow rate of 1 ml/min. Fractions (10 ml) were collected and those with specific activities (RNase units/A_{280}) greater than 21,000 were combined, dialyzed overnight against water, lyophilized, and dissolved in water to give an absorbance (280 mμ) of about 1.9. These samples of pure RNase R_1 (step 9a) were frozen and stored at −10°.

Purification of RNase R_2. Fractions containing RNase R_2 (950 ml, from step 6b) were carried through phosphocellulose chromatography (step 7b) and Sephedex G-100 chromatography (step 8b) as described for RNase R_1 but with less absolute purification. Fractions from step 8b were lyophilized, dissolved in 5 ml of water, and applied to a 4 × 120 cm column of Sephadex G-200 and eluted with water. Fractions with specific activities greater than 10,000 units/A_{280} were pooled and dialyzed against 0.02 M Tris-HCl (pH 8.5). DEAE-cellulose chromatography (at pH 8.5) was then done as described for RNase R_1 (step 9a), and fractions with specific activities (RNase units/A_{280}) greater than 20,000 pooled, dialyzed against water, lyophilized, and redissolved in water (step 10b). Data summarizing the purification are presented in Table I.

Electrophoresis. Polyacrylamide gel electrophoresis at pH 9.5 was done as described by Davis (1964). Electrophoresis at 4.0 mA/tube was carried out at room temperature for 1–2 hr. Electrophoresis was also performed at pH 4.3 in 4 M urea using the method of Martinez *et al.* (1967). In these experiments the dried samples (20–60 μg of protein) were dissolved in 4 M urea and electrophoresed at 4° for 2.5 hr at 2.5 mA/tube. Electrophoresis at pH 8.9 in 8 M urea was done using a modified method of Davis (1964). A 7.5% polyacrylamide gel was used and samples were electrophoresed in 0.01 M Tris-glycine buffer at room temperature for 1–2 hr at 3 mA/tube. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). Staining with Amido Black and destaining were described by Davis (1964).

Sedimentation equilibrium was performed with a Spinco Model E analytical ultracentrifuge equipped with Rayleigh interference optics. A Spinco three-channel cell similar to that described by Yphantis (1964) was used, and protein concentration was varied from 0.2 to 1.0 mg per ml. Centrifugation was at 29,500 rpm and at 20° and photographs were taken at times ranging from 15 to 45 hr after the equilibrium speed had been attained. Half-fringe displacements were read on a Gaertner microcomparator after alignment of the photographic plates as suggested by Yphantis (1964). The data were plotted as the logarithm of the protein concentration in fringe units (log C) *vs.* the square of the distance (in centimeters) of each fringe from the center of the rotor (X^2), and weight-average molecular weights determined from the slope of the best-fit line according to the equation of Van Holde and Baldwin (1958). The partial specific volume was calculated from the amino acid and carbohydrate composition of the protein, as described by Smith *et al.* (1954).

Amino Acid Analysis. Aliquots of about 0.3 mg of protein were pipetted into acid-washed glass hydrolysis tubes and dried by lyophilization, and 1.5 ml of 6 N HCl (reagent grade, three-times distilled) and 1 drop of phenol were added to each hydrolysis vessel and the contents of each frozen, degassed, sealed, and hydrolyzed at 110° for 20, 24, or 72 hr. Aliquots were analyzed by the method of Moore *et al.* (1958) with a Spinco Model 120B automatic amino acid analyzer.

Tryptophan content was measured by the method of Mat-

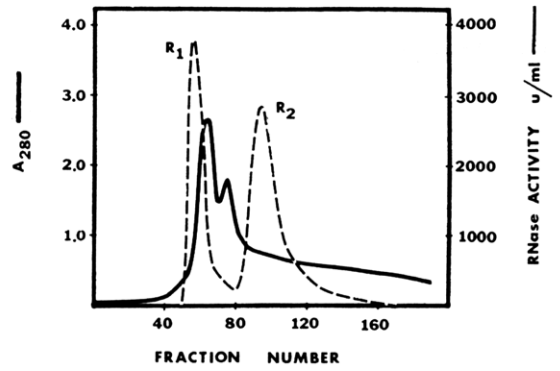

FIGURE 1: DEAE-cellulose chromatography of *Rhizopus oligosporus* ribonucleases at step 6. The dialyzed preparation of step 5 was applied to a 4 × 120 cm column of DEAE-cellulose, and eluted with 1 l. of 0.02 M Tris-HCl (pH 8.0) followed by a 2-l. linear sodium chloride gradient of 0–0.5 M in the same buffer. Fractions (10 ml) were collected at a flow rate of 1.0 ml/min. (Note: only the gradient portion of the elution pattern is plotted.)

subara and Sasaki (1969). A control was run on a hydrolysate of BPN′ subtilisin, and the tryptophan content of nuclease corrected according to the recovery of tryptophan from the BPN′ subtilisin control. Performic acid oxidation for the determination of half-cystine and methionine residues was done as described by Moore (1963).

Carbohydrate analysis was performed by the procedure of Kim *et al.* (1967).

Peptide analysis was performed using proteins which had been reduced and carboxymethylated as described by Hirs (1967) for ribonuclease A. The enzymes were then digested with 2% their weight of triphenyl chloromethyl ketone trypsin for 2 hr at 37°, in 0.1 M ammonium bicarbonate buffer (pH 8.0) and the resulting peptides subjected to electrophoresis for 1 hr at 3000 V (40 V/cm) with a water-cooled flat-bed apparatus and an acetic acid–pyridine–water (10:100:890, v/v) buffer (pH 3.5). Peptides were located by dipping the paper in a solution of 0.2% ninhydrin plus 5% collidine in 95% ethanol.

Results

Formation of the Enzymes. The conditions for growth employed in this work were those found to be optimal for enzyme production after variation of factors including inoculation method, aeration, and temperature. RNase activity in the medium ranged from 3 to 10 units per ml, with an average of about 6 units/ml and a specific activity of 3 units/mg of protein. Significant nuclease levels were not found in sand or alumina-ground mycelial extracts at any stage of growth.

Purification of the Enzymes. Table I summarizes the results of a large-scale preparation. The two enzymes were separated from each other by DEAE-cellulose chromatography at pH 8.0 (purification step 6, illustrated in Figure 1) and then separately purified until apparently homogeneous. Designation of the first peak as RNase R_1 and the second peak as RNase R_2 follows common procedures (Egami and Nakamura, 1969).

Distinct differences in behavior upon ion-exchange chromatography were observed between RNases R_1 and R_2. The R_1 enzyme was eluted from DEAE-cellulose at pH 8.0 (step 6) at lower salt concentrations (about 0.125 M NaCl) than the R_2 enzyme (about 0.20 M NaCl). In phosphocellulose chromatography (steps 7a and 7b), RNase R_2 was eluted at a lower salt

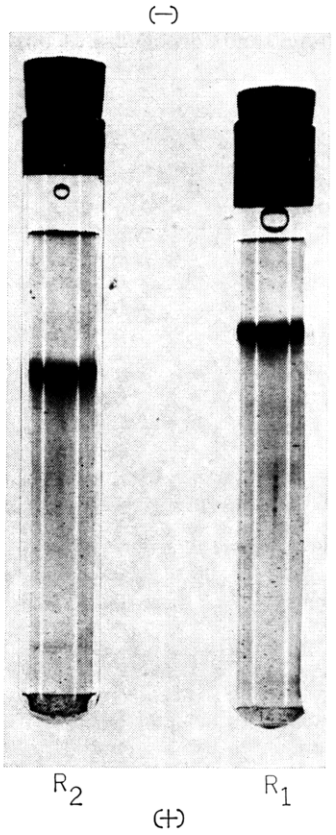

FIGURE 2: Polyacrylamide gel electrophoresis of *Rhizopus* ribonucleases. Electrophoresis in 7.5% gels was performed at pH 8.9 with buffers containing 8 M urea. About 60 μg of each enzyme was used.

concentration (about 0.12 M NaCl) than RNase R_1 (about 0.19 M NaCl). In DEAE-cellulose chromatography at pH 8.5 the R_1 enzyme was again eluted at a lower salt concentration than the R_2 enzyme (0.056 and 0.16 M NaCl, respectively). Finally, RNase R_1 exhibited much sharper activity peaks than

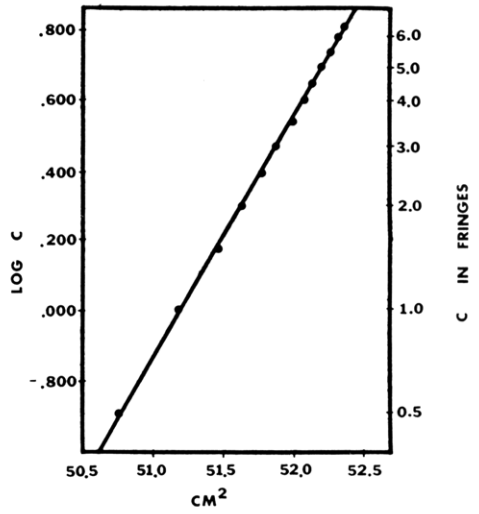

FIGURE 3: Sedimentation equilibrium of RNase R_1. Sedimentation equilibrium was performed in 0.1 M NaCl–0.005 M Tris-HCl (pH 7.0) at 20°. The initial protein concentration was about 0.8 mg/ml, and the time of the run was 43 hr at 29,500 rpm. Data are expressed as a plot of the log of protein concentration in fringes (log *c*) *vs.* the square of the distance from the axis of rotation $(cm)^2$.

TABLE II: Amino Acid and Carbohydrate Compositions of *Rhizopus oligosporus* Ribonucleases.

Component	RNase R_1			RNase R_2		
	Av or Extrapolated (μmole)	Residues		Av or Extrapolated (μmole)	Residues	
		Calcd[a]	Assumed		Calcd[a]	Assumed
Lysine	0.2865	16.4	16	0.3159	17.9	18
Histidine	0.1026	6.1	6	0.0710	4.0	4
Arginine	0.1087	6.4	6	0.0835	4.7	5
Aspartic acid	0.5783	34.3	34	0.5592	31.7	32
Threonine	0.2515[b]	14.9	15	0.2580[b]	14.6	15
Serine	0.4400[b]	26.1	26	0.5280[b]	29.9	30
Glutamic acid	0.3342	19.7	20	0.2814	16.0	16
Proline	0.1824	10.8	11	0.1813	10.3	10
Glycine	0.3575	21.2	21	0.3756	21.3	21
Alanine	0.1559	9.2	9	0.1592	9.0	9
Half cystine	0.1601[c]	9.5	9	0.1346[c]	7.6	8
Valine	0.2840	16.9	17	0.2875	16.3	16
Methionine	0.0337[c]	2.0	2	0.0353[c]	2.0	2
Isoleucine	0.1289	7.6	8	0.1375	7.8	8
Leucine	0.2457	14.6	15	0.3000	17.0	17
Tyrosine	0.2636	15.6	16	0.2682	15.2	15
Phenylalanine	0.1272	7.5	7	0.0954	5.4	5
Tryptophan	0.180[d]	11.0	11	nd	11.0[e]	11
Glucosamine	0.0870	5.2	5	0.0714	4.0	4
Mannose	0.1936	11.5	11	0.1637	9.3	9
Fucose	0.0140	0.8	1	0.0110	0.6	1
Glucose	0.0542	3.2	3	0.0795	4.5	4

[a] Based on methionine (sulfone) as two residues. [b] Values obtained by linear extrapolation to zero time. [c] Values obtained from performate-oxidized samples. [d] Value corrected for incomplete recovery. [e] Assumed value based on physical and spectral similarities to RNase R_1.

RNase R_2 in all ion-exchange chromatography (see, *e.g.*, Figure 1).

Criteria of Purity. The purity of enzyme preparations was estimated by polyacrylamide gel electrophoresis. Electrophoresis of RNase R_1 at step 8a (at pH 4.3 or 9.5) showed one distinct main band and two trace bands. Electrophoresis of RNases R_1 and R_2 at steps 9a and 10b, respectively, as illustrated in Figure 2, indicated that both enzymes were apparently homogenous with no visible trace impurities.

Constant specific activity of fractions from chromatography was considered a second criterion of purity. In the final chromatographic steps, the specific activity across each enzyme peak was constant. In addition, the specific activity of these ribonucleases was equal to or greater than that of other well-characterized enzymes in the same assay procedure: pancreatic ribonuclease and RNase T_1 showed specific activities of 16,600 and 9000 units per mg of protein, respectively.

In the case of RNase R_1, homogeneity in sedimentation equilibrium, as seen in Figure 3, further indicates physical homogeneity. Finally, the ultraviolet absorption spectra were typical of protein, and indicated no contamination by nucleotides.

Molecular Weights. Sedimentation equilibrium experiments with RNase R_1 were done in both 6 N guanidine hydrochloride and in 0.1 M sodium chloride. A partial specific volume of 0.715, calculated from the composition of RNase R_1, was used to estimate the weight-average molecular weight. In each experiment three different protein concentrations were used (about 0.4, 0.6, and 0.8 mg per ml), and in each case a straight-line relationship between log C and the square of the distance from the axis of rotation was found (see Figure 3) indicating the physical homogeneity of the preparation. Molecular weights of 29,800 ± 3.7% and 32,300 ± 3.5% were determined using the sodium chloride and guanidine hydrochloride solvents, respectively.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis has been used to estimate the molecular weights of proteins (Shapiro *et al.*, 1967). Samples of RNase R_1, RNase R_2, and mixed RNase R_1 and R_2 (58 and 60 μg, respectively) were applied to separate gels and electrophoresed at pH 7.0 in 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. In each case the major component migrated 36–38% of the full length of the gel, and the two enzymes were indistinguishable.

Chemical Composition. The amino acid and carbohydrate composition of each enzyme was determined, and the results are presented in Table II. As a second direct comparison, the (uncorrected) percentage composition results obtained from 24-hr hydrolyses of each enzyme are plotted against each other in Figure 4. In such a plot proteins of similar composition give points lying along the theoretical line drawn at 45°.

The reduced and carboxymethylated enzymes were hydrolyzed with trypsin and the resulting peptides submitted to paper electrophoresis. As shown in Figure 5, no clear differences could be seen in the comparison of the two proteins, although an area containing a number of unresolved peptides remained near the origin in each case.

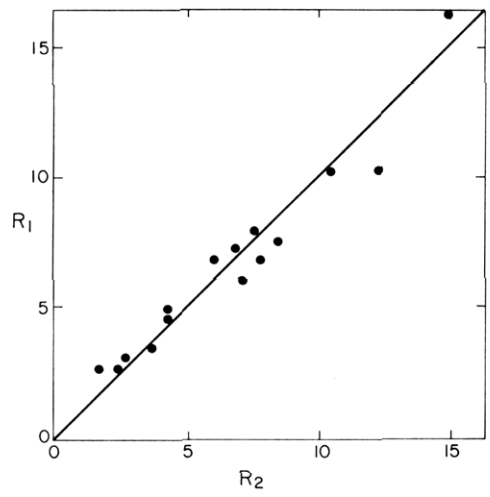

FIGURE 4: Comparative amino acid composition of *Rhizopus* nucleases. Data from identical 24 hydrolyses of each enzyme are plotted as the molar percentage of a component recovered in the analysis. Data for half-cystine, methionine, tryptophan, and ammonia were not included. The line at 45° is theoretical, and was not drawn to correspond to the data.

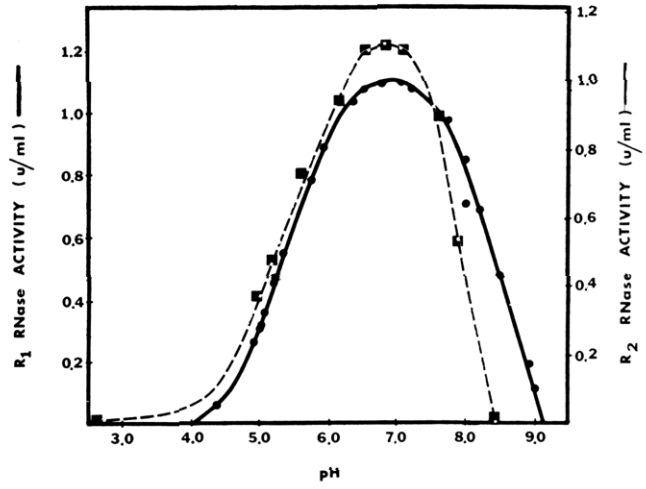

FIGURE 6: pH optima of *R. oligosporus* ribonucleases. The RNase activities were measured in the standard assay, except that the pH of the incubation mixture was adjusted to the desired value and measured before and after each reaction. Equal activities of purified RNases (about 1.1 units) were used, but the data are plotted on slightly different scales to emphasize differences between the enzymes.

pH Optima. The effect of pH on the activity of each enzyme was examined with 1.1 units of ribonuclease in the standard assay procedure. The pH was varied over the range 3.5–9.5, as measured directly in the reaction mixtures at the start and finish of each assay. The results are plotted in Figure 6 with slightly different activity scales for each enzyme to emphasize any differences. Since both enzymes have optima near pH 7, all further studies were carried out at this pH.

Breakdown of high molecular weight substrates was examined with each enzyme and wheat germ high molecular weight RNA. At a relatively low enzyme:substrate ratio (8 units/mg of RNA), only the four nucleoside 2′,3′-cyclic phosphates were detected by paper chromatography after 22-hr digestion.

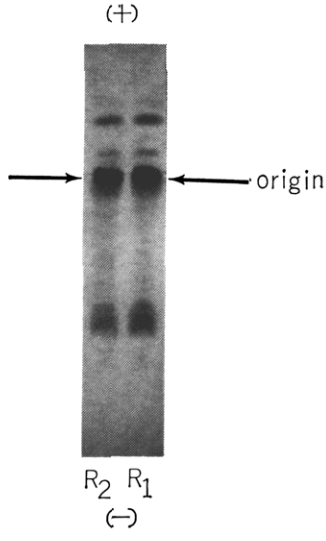

FIGURE 5: Tryptic peptides of *Rhizopus* ribonucleases. Reduced and carboxymethylated enzymes were digested with trypsin and *ca.* 0.5 mg of the digest submitted to electrophoresis on Whatman No. 3MM paper with a pyridine–acetic acid–water (10:100:890, v/v) pH 3.5 buffer. Electrophoresis was at 3000 V (40 V/cm) for 1 hr. Peptides were visualized by dipping the paper in 0.2% ninhydrin plus 5% collidine in 95% ethanol.

Under identical conditions but with higher enzyme levels (80 units/mg of RNA) the four nucleoside 2′,3′-cyclic phosphates and the four 3′-nucleotides were found in approximately equal quantities. No differences between RNases R_1 and R_2 were apparent in these experiments.

The effect of substrate RNA concentration on reaction velocity is illustrated in Figure 7. At RNA concentrations greater than about 0.15 mg/ml (not shown in Figure 7) reaction velocities levelled off and eventually were less than those determined at lower substrate concentrations. Thus the RNA concentration in our assay is not optimal for either enzyme, but the assay results are reproducible and proportional to enzyme concentrations. Values for kinetic constants were calculated by extrapolation of the data in Figure 7 and are included in Table III. Turnover numbers were calculated from values of V_{max} and are based on a molecular weight of 30,000

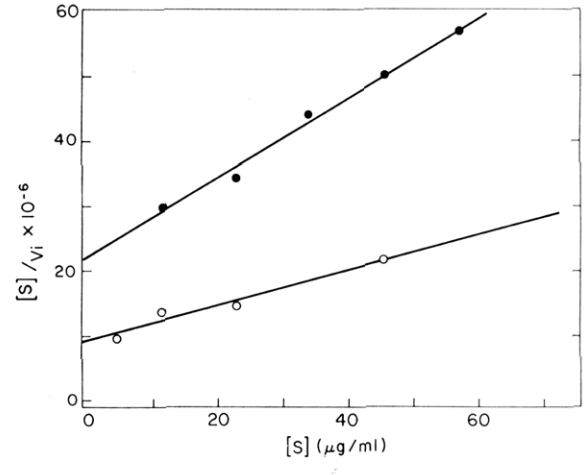

FIGURE 7: Kinetic analysis of RNA hydrolysis by *R. oligosporus* ribonucleases. The data are plotted according to Lineweaver and Burk (1934). The units of $[S]/V_i$ are μg/ml per μg of RNA solubilized per ml of reaction per min per mg of serum albumin equivalent in the Lowry *et al.* (1951) protein determination. Open circles, RNase R_1; closed circles, RNase R_2.

TABLE III: Kinetic Data for *Rhizopus oligosporus* Ribonucleases.

	RNase R_1			RNase R_2		
	K_m			K_m		
Substrate	mg/ml	mM	Turnover No.[a]	mg/ml	mM	Turnover No.[a]
RNA	0.035	0.11[b]	(258×10^2)[b]	0.036	0.11[b]	(104×10^2)[b]
CpU	0.91	1.7	35×10^2	1.14	2.1	26×10^2
UpC	0.98	1.8	14×10^2	0.88	1.6	5×10^2
UpG	0.74	1.3	30×10^2	2.40	5.1	32×10^2
UpA	0.33	0.6	14×10^2			
CpA	0.54	1.0	15×10^2	0.66	1.2	6×10^2
ApC	3.26	7.3	9×10^2	1.35	2.4	5×10^2
ApU	1.91	3.3	4.6×10^2	1.30	2.3	4.4×10^2
GpU	0.63	1.1	2.0×10^2	1.37	2.3	3.2×10^2
ApA	0.80	1.3	0.5×10^2	0.58	1.0	0.4×10^2
G>p	2.90	8.4	1.1	4.65	13.5	2.3
A>p	4.69	13.4	11			
C>0	3.69	12.9	3.7			
U>p	2.10	7.3	3.7			

[a] Calculated from values of V_{max} using a specific activity of 69,000 units/mg of protein as determined in amino acid recovery in acid hydrolysates, and a molecular weight of 30,000. [b] Calculated on the basis of mononucleotide equivalents in RNA, using a value of 325 as the average molecular weight of a nucleotide and 8000 as a typical molar extinction coefficient of a nucleotide in RNA. The turnover numbers are certainly too high, since a variety of acid-soluble oligonucleotides are released which are considered as mononucleotides (and thus bonds cleaved) in the calculations, but are included only for purposes of rough comparison of RNases R_1 and R_2.

and a specific activity of 69,000 units/mg of enzyme, as estimated from recoveries in hydrolysates for amino acid analyses. (The specific activities may also be expressed as 21,000 units/A_{280}, or 34,000 units/mg of serum albumin equivalent in the Lowry *et al.* (1951) protein determination.)

Synthetic homopolynucleotides were treated with RNase R_1, and the approximate rates of nucleoside 2′,3′-cyclic phosphate formation estimated by chromatography of aliquots of the reaction mixtures at various times. Since some oligonucleotides and 3′-nucleotides were also produced in these reactions exact quantitation of the data was not possible, but the relative susceptibility of the polymers to degradation was judged to be: poly(U) > poly(C) ≅ poly(I) > poly(A) > poly(G). Breakdown of poly(U) was about one thousand times as rapid as that of poly(G).

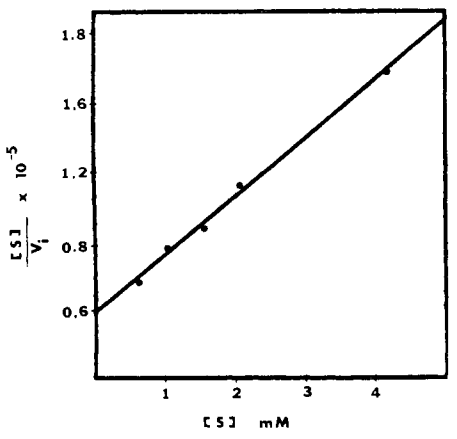

FIGURE 8: Kinetic analysis of a phosphotransferase reaction catalyzed by RNase R_1. The substrate was cytidylyl 3′→5′-adenosine (CpA), and the units of V_i are moles of product/mmoles of enzyme per sec.

Breakdown of dinucleoside monophosphates was examined by determination of rates of nucleoside formation under standardized conditions. Typical data, in which the substrate is CpA and the enzyme RNase R_1, are presented in Figure 8. Values for kinetic constants are summarized in Table III.

Hydrolysis of nucleoside 2′,3′-cyclic phosphates was determined by measurement of nucleoside formation upon treatment of reaction mixtures with *E. coli* alkaline phosphomonoesterase (which does not attack nucleoside cyclic phosphates). Calculated kinetic constants are also included in Table III.

Product inhibition was studied using RNase R_1 hydrolysis of CpA as an example. The results are summarized in Table IV.

Discussion

Purification of Enzymes. The purification scheme resulted in the isolation of two separable ribonuclease activities in homogeneous form. We believe the enzymes to be essentially pure on the basis of homogeneity in electrophoresis in gels

TABLE IV: Competitive Inhibition of RNase R_1.

Substrate	Inhibitor	K_I (mM)
CpA	Cytidine 2′,3′-cyclic phosphate	3.7
CpA	Adenosine 2′,3′-cyclic phosphate	2.6
CpA	Cytidine 3′-phosphate	0.6
CpA	Adenosine 3′-phosphate	0.7

(*e.g.*, Figure 2), specific activities equivalent to other pure ribonucleases, homogeneity in column chromatography, and the demonstration of a single component in sedimentation equilibrium (of RNase R_1 only). Spectra show little or no nucleotide contamination.

The two peaks of nuclease activity of Figure 1 are approximately equal, although the relative quantities of each enzyme did vary somewhat in other preparations. The eventual yield of RNase R_1 is greater than that of RNase R_2 for two reasons: one additional step was needed for the purification of the second activity, and the tendency of RNase R_2 to chromatograph as a broad peak required that the yield be sacrificed for better purification in the final steps.

Comparison of Two Enzymes. These two ribonucleases are clearly very similar in a number of respects including chemical and physical properties as well as specificity. Indeed, there is some problem in detailing exactly how the two nucleases differ.

The enzymes were separated by chromatography on DEAE-cellulose at pH 8.0; the elution of RNase R_2 at a somewhat higher salt concentration suggests a greater net negative charge. In electrophoresis at pH 8.9 RNase R_2 migrates ahead of RNase R_1. Since the two enzymes appear to be of similar molecular weight, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the different rates of migration might similarly be due to a greater effective negative charge on RNase R_2. Phosphocellulose chromatography at pH 5 results in the elution of RNase R_2 at a lower salt concentration than RNase R_1, again suggesting that the R_2 enzyme has a greater negative charge.

The amino acid and carbohydrate analyses of Table II do not show the two enzymes to be clearly different, but rather emphasize their similarity as does the plot of Figure 4. The electrophoretic patterns of tryptic peptides (Figure 5) may contain dissimilar unresolved fragments, but clearly show only a number of apparently identical peptides. Thus it appears that the two enzymes have similar or identical amino acid sequences. Perhaps they differ in some other component such as carbohydrate, but our data do not support this possibility (although an undetermined substituent, such as sialic acid, might differentiate them). Such a situation would be analogous to the bovine pancreatic ribonucleases (Plummer, 1968). The molecular weight of the enzymes is about 30,000 in either normal or dissociating solvents. Thus each appears to consist of a single polypeptide chain, as has been reported for all other ribonucleases. The molecular weights are also similar to those of other nonspecific cyclizing ribonucleases (EC 2.7.7.17).

Studies of enzyme action and specificity similarly emphasize their similarities. Figure 6 shows both enzymes to have pH optima at about 6.9 or 7.0, and to have similar pH profiles; however, RNase R_2 activity does drop off somewhat more rapidly at higher pH values. Since RNase R_2 is a more anionic protein the pH profile may reflect a less effective interaction with RNA in this case.

The breakdown of macromolecular substrates clearly showed that both enzymes produce mainly the four nucleoside 2′,3′-cyclic phosphates, which are slowly hydrolyzed to the 3′-nucleotides. Thus both enzymes appear to be cyclizing ribonucleases or phosphotransferases with no absolute base specificity.

The data of Figure 7 and Table III indicate that RNA is probably the best substrate tested for each enzyme, although the calculations involve major assumptions since neither the substrate nor the products are chemically defined species. In fact the calculated turnover numbers are certainly too large, since acid-soluble oligonucleotides were considered as mononucleotides in the analysis. If the average perchloric acid soluble oligonucleotide initially released in the reaction was, *e.g.*, a decanucleotide, the turnover numbers would be reduced by a factor of ten. More meaningful than the absolute values are a comparison of the turnover numbers, which indicate that RNase R_2 is only about 40% as effective as RNase R_1 under these conditions. Again, it is tempting to suggest that the greater net negative charge of RNase R_2 may be responsible for its lower activity.

The breakdown of synthetic polynucleotides by RNase R_1 suggested that although these nucleases might lack absolute base specificity, there was still likely to be some preference as to cleavage sites. The quantitative interpretation of these data was difficult, however, because of the variety of products formed and the problems of separating effects due to bases or base sequences from those due to the secondary structure of each homopolymer (see Barnard, 1969).

Simple natural substrates for these ribonucleases are the nucleoside 2′,3′-cyclic phosphates and dinucleoside monophosphates. Nuclease action on such substrates has been studied by paper chromatographic or electrophoretic separation of products (*e.g.*, Rushizky and Sober, 1963; Whitfeld and Witzel, 1963), which is tedious and difficult to quantitate, and by spectrophotometric techniques (Witzel and Barnard, 1962; Irie, 1968) which limit useful concentrations because of the high extinction coefficients of the nucleotides. We have used here a technique based on quantitative separation by ion exchange of nucleoside product. The method is somewhat tedious, but does not seriously limit substrate concentrations and is sufficiently simple to permit the determination of one set of kinetic constants with about 5-hr effort. The data of Table III show that these nucleases are strongly influenced by the nature of the nucleosides on each side of the phosphodiester bond cleaved in the phosphotransferase reaction (formation of nucleoside 2′,3′-cyclic phosphates). The best substrates include a pyrimidine at the 5′end (*e.g.*, CpU and UpG), but contain either purine or pyrimidine at the 3′ end (*e.g.*, UpC and UpG). If the substrate has a purine at the 5′ end the reaction is generally less rapid, and if both residues are purines the reaction appears slowest of all. Both RNases are rather similar except for detail. RNase R_2 has lower turnover numbers in these data, as was the case with RNA, with one exception: in substrates containing guanosine in either position RNase R_2 is equivalent to or slightly more active than RNase R_1. This is also true of the hydrolysis of guanosine cyclic phosphate.

As would be predicted from the analysis of polynucleotide degradation products, the nucleoside 2′,3′-cyclic phosphates are poor substrates; values for K_m are relatively large and turnover numbers are small. Large rate differences between the phosphotransferase and hydrolase reactions have been previously noted with other cyclizing ribonucleases (Egami and Nakamura, 1969; Barnard, 1969).

Inhibition of the phosphotransferase reaction by reaction products (Table IV) further explains the accumulation of nucleoside 2′,3′-cyclic phosphate products in the degradation of macromolecular polynucleotides. As might be expected both cyclic and 3′-nucleotides are competitive inhibitors of the phosphotransferase reaction, but the latter show values of K_I about 5-fold smaller than the former.

We conclude that ribonucleases R_1 and R_2 are nonspecific cyclizing ribonucleases (phosphotransferases) which preferentially attack internucleotide bonds to form a pyrimidine 2′,-

3′-cyclic phosphate. The physical characteristics of the enzymes and their glycoprotein nature seem typical of such ribonucleases, but to our knowledge they are the first nonspecific ribonucleases reported to show a preference for pyrimidines (see Barnard, 1969; Egami and Nakamura, 1969).

Acknowledgments

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Structural Properties of 5-Fluorouracil-Containing Transfer Ribonucleic Acids from *Escherichia coli**

Ivan I. Kaiser

ABSTRACT: The structural properties of unfractionated, 5-fluorouracil-containing tRNAs (FU-tRNAs) from *Escherichia coli* B, having 84% of the uracil residues replaced by 5-fluorouracil, have been compared with unfractionated, normal tRNAs. The content of the minor pyrimidines, pseudouridine, ribothymidine, 4-thiouridine, and 5,6-dihydrouridine, have previously been shown to be reduced to essentially the same extent as uridine in the analog-containing tRNA. The effect of these massive replacements on the structure of FU-tRNAs have been examined by disc gel electrophoresis, ultraviolet absorbance–temperature and denaturation spectra, ribonuclease digestions, cochromatography on Sephadex, and cosedimentation on sucrose gradients. The results indicate that fluorouridine replacement of uridine and uridine-related minor base components has only minor effects on the secondary and tertiary properties of tRNA, particularly in the presence of Mg^{2+}. It is suggested that these minor bases are not essential in the formation or stabilization of the three-dimensional structures found in normal tRNAs.

When the uracil analog FU[1] is added to a rapidly growing culture of *Escherichia coli* B, extensive replacement of uracil and structurally related minor components in tRNA occurs. The odd nucleosides replaced include pseudouridine (Andoh and Chargaff, 1965; Lowrie and Bergquist, 1968; Johnson *et al.*, 1969), ribothymidine (Lowrie and

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[1] The following abbreviations are used: FU, 5-fluorouracil; FU-tRNA, tRNA containing FU; N-tRNA, tRNA from normal cells; A_{260} unit, a unit of material which in a volume of 1 ml will have an absorbance of one at 260 mμ when measured in a cell of 1-cm path length.