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POLYGUANYLIC ACID-INHIBITED RIBONUCLEASE OF *KLEBSIELLA*

I. PURIFICATION AND GENERAL PROPERTIES

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SUMMARY

A ribonuclease (ribonucleate nucleotido-2'-transferase (cyclizing), EC 2.7.7.17) was purified 540-fold from *Klebsiella* sp. and its properties were investigated. The enzyme is an endonuclease able to hydrolyze yeast RNA completely to the 2':3'-cyclic phosphate derivatives of AMP, CMP, UMP and GMP. The 3'-phosphates can be found as minor products of the hydrolysis. The enzyme has a pH optimum in the neutral range, is moderately heat stable, has a molecular weight of 24 000, and has a K_m of 32 μg yeast RNA per ml. Polyguanylic acid was found to be a potent inhibitor of this enzyme.

INTRODUCTION

In an attempt to find a nuclease specific for adenylic acid (AMP) or deoxyadenylic acid (dAMP) residues, a *Klebsiella* sp. microorganism was isolated on a medium containing either polyadenylic acid or dAMP as the sole energy and nitrogen source. A ribonuclease (ribonucleate nucleotido-2'-transferase (cyclizing), EC 2.7.7.17) was purified 540-fold from this microorganism, and its properties were investigated. Although this enzyme can hydrolyze yeast RNA completely, it is unable to attack polyguanylic acid (poly(G)). Because of this, and because attempts in this laboratory to grow bacteria on a medium containing poly(G) as a sole energy and nitrogen source have been unsuccessful, it was suspected that poly(G) may be an inhibitor of some bacterial nucleases. Such was found to be true in the case of *Klebsiella* endonuclease. This paper deals with the purification and general properties of the *Klebsiella* enzyme, while the following paper will report in detail its interactions with synthetic nucleic acid polymers.

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MATERIALS AND METHODS

Microorganism

A *Klebsiella* sp. microorganism was isolated from soil by growth on agar plates containing either poly(A) or dAMP as the sole carbon and nitrogen source.

Chemicals

RNA was prepared from baker's yeast by the method of Crestfield *et al.*[1]. *Escherichia coli* alkaline phosphatase (orthophosphonic monoester phosphohydrolase, EC 3.1.3.1) and bovine serum albumin were bought from the Sigma Chemical Co. (St. Louis, Mo.); calf thymus DNA, snake venom phosphodiesterase (onthophosphonic diester phosphohydrolase, EC 3.1.4.1), and bovine spleen phosphodiesterase were purchased from the Worthington Biochemical Corp. (Freehold, N.J.). *E. coli* ribosomal and tRNA were obtained from the Miles Laboratories (Elkhart, Ind.). Poly(G) was the product of the Sigma Chemical Co. or the Miles Laboratories. Sephadex was bought from Pharmacia Fine Chemicals (Piscataway, N.J.), and CM-cellulose, DEAE-cellulose, and cellulose phosphate from Reeve Angel (Clifton, N.J.).

Culture conditions

The *Klebsiella* sp. was inoculated from agar plates into flasks containing 100 ml of medium whose composition per l was as follows: KH_2PO_4, 0.5 g; $K_2HPO_4 \cdot 3H_2O$, 1.2 g; $MgSO_4 \cdot 4H_2O$, 0.27 g; $Fe_2(SO_4)_3 \cdot nH_2O$, 2.5 mg; $MnCl_2 \cdot 4H_2O$, 20 mg; and yeast extract, 0.5 g. In addition, each l contained either Sigma Type IV degraded DNA, 1 g, and D(−)-ribose, 10 g; or sodium succinate, 2 g, and 5'-AMP, 0.1 g. The flasks were incubated at 30 °C 16 h in an NBS gyrorotatory shaker. The contents of each flask were then transferred to 1 l of fresh medium for an additional 16-h incubation period, after which the cells were harvested by use of a Sharples cream separator and frozen at −20 °C until used.

Purification

Two procedures were developed. The one resulting in the greatest purity is given in detail, while the alternate procedure which produced a greater yield is presented briefly. All manipulations were performed at 5 °C.

Standard procedure

Crude extract. The bacterial paste (8.85 g wet wt) was suspended in 30 ml of 0.1 M sodium phosphate buffer (pH 8.0), and the cells were disrupted in a French pressure cell at 20 000 lb/inch^2. After centrifugation at 20 000 × *g* for 20 min, the supernatant solution was decanted and diluted to 50 ml with buffer.

$(NH_4)_2SO_4$ fractionation. The enzyme solution was brought to 30% saturation with $(NH_4)_2SO_4$ while the pH was maintained at 8.0 by dropwise addition of 1 M NaOH. The mixture was allowed to stand 20 min and then was centrifuged at 20 000 × *g* for 15 min. Enough solid $(NH_4)_2SO_4$ was added to the supernatant solution to achieve 55% satn. After centrifugation, the 31–55% $(NH_4)_2SO_4$ precipitate was suspended in 20 ml of 0.1 M sodium phosphate buffer (pH 8.0).

Ethanol precipitation. To the 20-ml $(NH_4)_2SO_4$ fraction, an equal volume of absolute ethanol was added dropwise with stirring over a 20-min period. The mixture

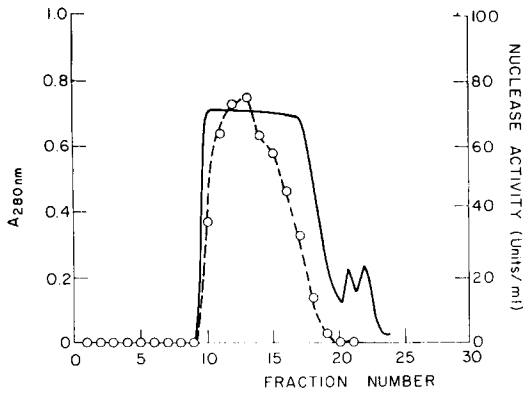

Fig. 1. Cellulose phosphate chromatography of *Klebsiella* nuclease (standard procedure). Column was equilibrated with 0.01 M KCl buffer (pH 6.8). See text for details of sample application and elution. ———, absorbance at 280 nm; ○---○, nuclease activity.

was allowed to stand 20 min and then was centrifuged 10 min at 20 000 × *g*. The precipitate was suspended in 10 ml of 0.01 M sodium phosphate buffer (pH 6.8).

Cellulose phosphate chromatography. A 1.5 cm × 42.5 cm cellulose phosphate column (Whatman P11) was equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). 9 ml of the ethanol fraction were applied to the column and eluted with equilibrating buffer (Fig. 1). Fractions 4 ml in volume were collected. Tubes 10–18 (32.2 ml) were pooled for further purification.

DEAE-cellulose chromatography. A 1.5 cm × 42 cm DEAE-cellulose column (Whatman DE52) was equilibrated with 0.1 M Tris–HCl buffer (pH 7.0). After 30 ml

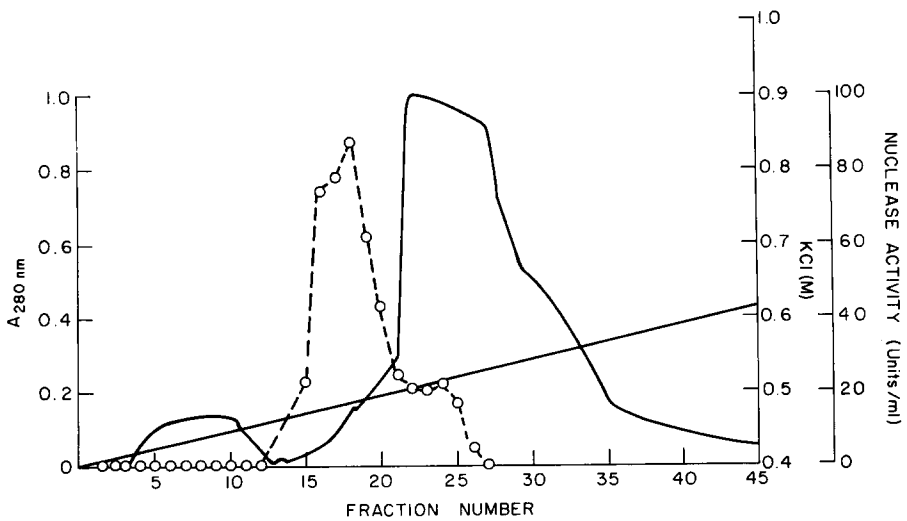

Fig. 2. DEAE-cellulose chromatography of *Klebsiella* nuclease (standard procedure). Column was equilibrated with 0.1 M Tris–HCl buffer (pH 7.0). After sample application, the enzyme was eluted with a 0.4–1.0 M KCl gradient. See text for details. ———, absorbance at 280 nm; ○---○, nuclease activity.

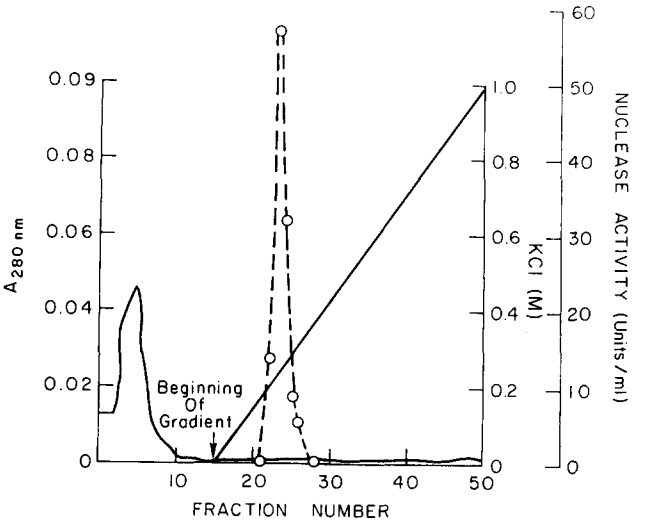

Fig. 3. CM-cellulose chromatography of *Klebsiella* nuclease (standard procedure). Column was equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). After sample application, column was washed with equilibrating buffer, and enzyme was then eluted with a 0–1.0 M KCl gradient. See text for details ———, absorbance at 280 nm; ○---○, nuclease activity.

of the enzyme solution obtained in the previous step were applied to the column, the enzyme was eluted with a 500-ml linear KCl gradient (0.4–1.0 M) in equilibrating buffer (Fig. 2). The eluant was collected in 4-ml fractions. Tubes 15–20 (20.8 ml) which contained the nuclease activity were pooled; and the pooled enzyme was dialyzed for 3 h against 100 vol. of 0.01 M sodium phosphate buffer (pH 6.8).

CM-cellulose chromatography. A 1.5 cm × 20 cm carboxymethyl cellulose column (Whatman CM-52) was equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). 10 ml of the dialyzed enzyme were placed on the column, which was then washed with 40 ml of equilibrating buffer. A 150-ml linear gradient (0–1.0 M KCl) in equilibrating buffer was used to elute the enzyme (Fig. 3). Fractions 4 ml in volume

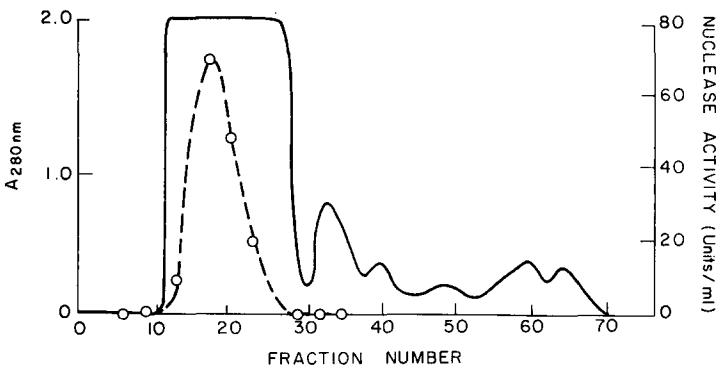

Fig. 4. Sephadex G-25 chromatgoraphy of *Klebsiella* nuclease (alternate procedure). Column was equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.05 M KCl and 0.2 mM dithiothreitol. See text for details. ———, absorbance at 280 nm; ○---○, nuclease activity.

were collected, and activity was found in Tubes 22–25 (15.4 ml). This step was repeated with the remaining dialyzed enzyme preparation.

Alternate procedure

Cells were disrupted as described above in 0.1 M Tris–HCl buffer (pH 7.5). After clarification by centrifugation, enough 2% protamine sulfate solution was added to the supernatant solution to give a 1:20 protamine to protein ratio. The solution was again clarified by centrifugation, and an $(NH_4)_2SO_4$ fractionation was done. That fraction precipitating between 25 and 50% satn was collected, dissolved in buffer (0.1 M Tris–HCl buffer (pH 7.5)), applied to a Sephadex G-25 column (40 cm × 2.5 cm), and eluted with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.05 M KCl and

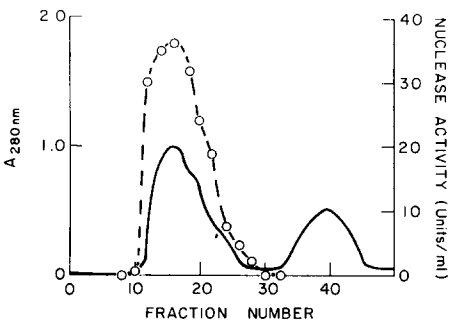

Fig. 5. DEAE-cellulose chromatography of *Klebsiella* nuclease (alternate procedure). Column was equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.05 M KCl and 0.2 mM dithiothreitol. See text for details. ———, absorbance at 280 nm; ○---○, nuclease activity.

0.2 mM dithiothreitol (Fig. 4). Fractions 13–25 (52 ml), which contained the nuclease activity, were pooled; and 46 ml were applied to a DEAE-cellulose column (20 cm × 2.5 cm) and eluted with the same solution used in the Sephadex step (Fig. 5). Nuclease activity was found in Fractions 12–24 (56 ml), which were pooled, heated 10 min at 55 °C, clarified by centrifugation, and dialyzed 2 h against 200 vol. of 0.02 M sodium phosphate buffer (pH 6.8) containing 0.2 mM dithiothreitol. 18 ml fractions of the dialysate were chromatographed on CM-cellulose columns as described in the standard preparation, and the elution pattern was essentially the same as that shown in Fig. 3.

Ribonuclease assay

The reaction mixture contained 100 μmoles Tris buffer (pH 7.5), 500 μg bovine serum albumin, 250 μg yeast RNA, and an appropriate amount of enzyme in a total volume of 1 ml. After incubation at 37 °C for 15 min, 1 ml of 12% $HClO_4$ was added. The mixture was kept on ice for 10 min and was then centrifuged at 50 000 × *g* 5 min. The increase in supernatant solution absorbance at 260 nm was measured in a Gilford Model 2400 spectrophotometer. One unit of enzyme activity is defined as that amount of enzyme which produces an increase in absorbance of 1.0 under the conditions described. The bovine serum albumin was added as an enzyme stabilizer and as a co-precipitant for unhydrolyzed RNA. The assay was linear with time for as long

as 1 h. In a 15-min assay, results were linear with enzyme levels up to 2.4 units per tube.

Deoxyribonuclease (deoxyribonuleate digonucleotidohydrolase, EC 3.1.4.5) assay

Conditions and procedures were the same as for the ribonuclease assay except that the substrate was 250 μg of calf thymus DNA. DNA was denatured by heating 10 min at 100 °C followed by rapid cooling.

Protein determination

Protein was measured by the biuret[2] and Lowry[3] methods.

Nuclease digestion of yeast RNA

Analysis of products. Yeast RNA was incubated with purified nuclease for varying periods at 37 °C. Each digestion was terminated by the addition of an equal volume of water-saturated phenol. This mixture was shaken 10 min, centrifuged, and the phenol layer was discarded. The aqueous layer was extracted three times with diethyl ether[4] and then chromatographed in at least two solvent systems.

Separation of nucleotides and oligonucleotides. Ascending chromatography on Whatman No. 3MM paper utilized the following solvent solutions[5]: isobutyric acid–NH_4OH–water (66:1:33, by vol.) adjusted to pH 3.7 (Solvent 1); ethanol–1.0 M ammonium acetate (pH 7.5) (7:3, by vol.) (Solvent 2); 0.1 M sodium phosphate (pH 6.8)–$(NH_4)_2SO_4$–*n*-propanol (100:60:2, v/w/v) (Solvent 3). Ultraviolet-absorbing areas on each chromatogram were eluted with water and the absorption spectra determined using a Cary Model 14. Mononucleotides were identified by their R_F values and ultraviolet absorption spectra. Those compounds identified as oligonucleotides after passage through two paper chromatographic systems were subjected to high-voltage electrophoresis as previously described[6]. The nucleotide composition and sequences of these compounds were determined as outlined by Levy and Goldman[6] using alkaline hydrolysis and treatment with *E. coli* alkaline phosphatase[7], snake venom phosphodiesterase[8], and bovine spleen phosphodiesterase[9].

Determination of molecular weight

The molecular weight of the enzyme was determined by the Sephadex gel-filtration method of Andrews[10]. The column was calibrated with bovine serum albumin (M_r 60 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000), and cytochrome *c* (M_r 12 500).

Phosphodiesterase and alkaline phosphatase assays

Alkaline phosphatase was measured by the method of Takeda and Tsugita[11] at pH 8.0 in 0.5 M Tris–HCl buffer. Phosphodiesterase was measured by the method of Anraku[12] at pH 7.5 and 8.9. In each assay, 3 units of nuclease were incubated in the reaction system for 1 h.

RESULTS

Enzyme purification

Typical purifications are summarized in Tables I and II. The alternate proce-

TABLE I

SUMMARY OF ENZYME PURIFICATION (STANDARD PROCEDURE)

Step	*Volume (ml)*	*Protein (mg)*	*Enzyme activity*	*Specific activity*	*Recovery (%)*
Crude	50	481.36	3422	7.1	—
$(NH_4)_2SO_4$	10	175.9	3444	19.58	100.6
Ethanol	9	24.4	1740.6	71.37	50.8
Cellulose phosphate	30	6.5	1624.0	248.7	47.0
DEAE-cellulose	20	2.8	1524.0	502.8	44.5
Dialysis	20		1240		36
CM-cellulose	30.8	0.208	798	3837	23

dure gives a 320-fold purification with a relatively high recovery (41%) while the standard procedure produces a higher purification with a lower recovery.

In the standard procedure, the ethanol precipitation step requires that the $(NH_4)_2SO_4$ fraction be diluted so that its volume is at least 40% of the starting volume of the crude extract. If this is not done, all activity is lost. This may be necessary to dilute out an inhibitor which may otherwise precipitate with the enzyme in the alcohol fraction.

Alkaline phosphatase and phosphodiesterase activities are not detectable in the purified enzyme preparation.

Molecular weight

After gel filtration of protein standards and *Klebsiella* nuclease on a Sephadex G-75 column, the molecular weight of the nuclease was obtained from a plot of K_{av} *vs* log M_r as suggested by Laurent and Killander[13], and was found to be 24 000 (Fig. 6). When crude preparations of the enzyme were chromatographed on Sephadex G-100 or G-150, nuclease activity was always found in the void volume. Thus the crude enzyme was thought to have a high molecular weight. When, however, the bulk of the contaminating nucleic acids was removed, this phenomenon did not occur. Therefore, it is probable that the crude enzyme is bound to nucleic acid.

General properties

As shown in Fig. 7, the pH optimum is different in Tris–HCl and phosphate

TABLE II

SUMMARY OF ENZYME PURIFICATION (ALTERNATE PROCEDURE)

Step	*Volume (ml)*	*Protein (mg)*	*Enzyme activity*	*Specific activity*	*Recovery (%)*
Crude	40	1200	2400	2.0	—
Protamine sulfate	40	1000	2360	2.35	98
$(NH_4)_2SO_4$	15	630	2340	3.7	94
Sephadex G-25	52	520	2080	4.0	80
DEAE-cellulose	56	105	1260	11.8	54
Heat	56	61.6	1120	18.2	47
CM-cellulose	45	1.568	996	641.0	41.5

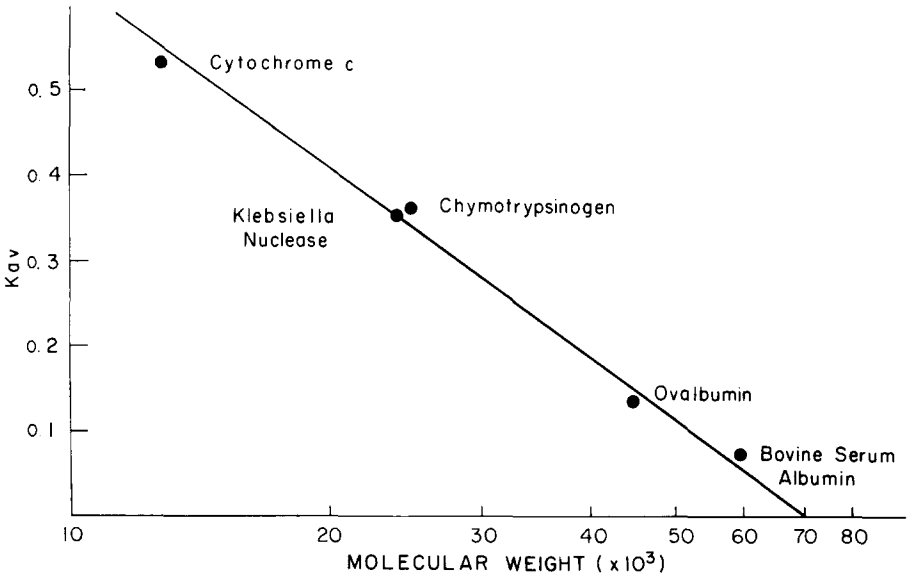

Fig. 6. Molecular weight determination by gel filtration on sephadex G-75 column. A 2.5 cm × 35 cm column equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) was used. The K_{av} values of standards were plotted against the logs of the molecular weights and the molecular weight of the nuclease was obtained from this plot after determining its K_{av}.

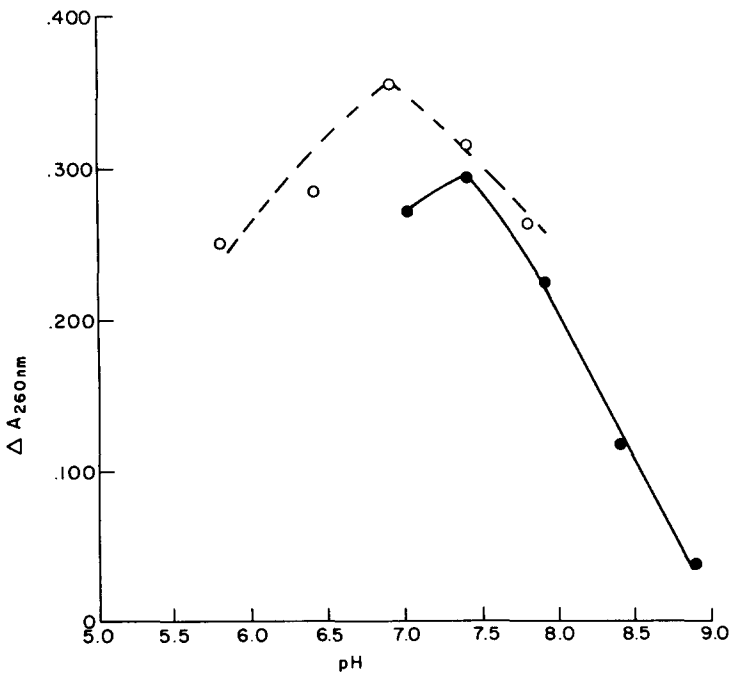

Fig. 7. pH Curve of *Klebsiella* nuclease. Each reaction contained 0.3 unit of enzyme. See text for assay procedure. ○---○, 0.1 M sodium phosphate buffer; ●—●, 0.1 M Tris–HCl buffer. pH was varied as indicated.

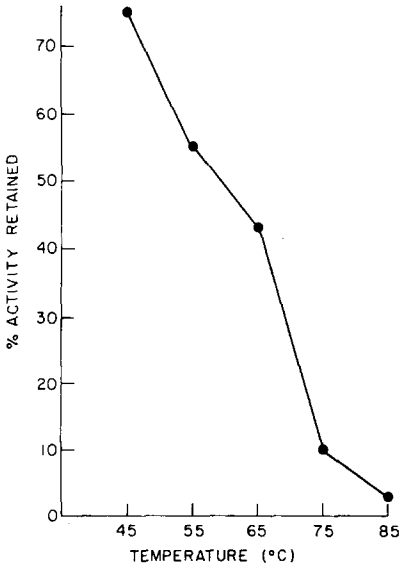

Fig. 8. Heat inactivation of *Klebsiella* nuclease. Enzyme aliquots were heated for 10 min at indicated temperatures and then assayed for residual nuclease activity.

buffers, the optimum being at 7.0 in phosphate and 7.5 in Tris–HCl buffer. In addition, total activity is higher when assayed in the phosphate system at pH 7.0.

In Fig. 8 can be seen results of heat stability experiments. Enzyme was in-

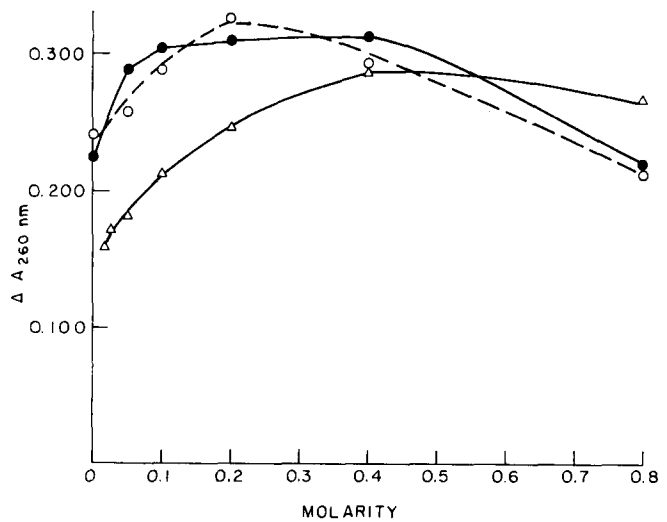

Fig. 9. Effect of ionic strength on *Klebsiella* nuclease activity. 0.22 unit of enzyme were used per assay. Concentration of Tris–HCl buffer (pH 7.5) (△—△), varied as indicated. Salt concentration was varied by addition of NaCl (●—●), or KCl (○---○), as indicated in figure, with buffer concentration being maintained at 0.1 M.

TABLE III

EFFECT OF DIVALENT CATIONS ON NUCLEASE ACTIVITY

Assays were performed as described in Materials and Methods, using 0.6 unit of enzyme per ml. Divalent ions were present in 0.2 mM concn.

Addition	*Relative activity (%)*
0	100
Cd^{2+}	70
Ca^{2+}	100
Co^{2+}	82
Cu^{2+}	75
Fe^{2+}	71
Mg^{2+}	103
Mn^{2+}	86
Ni^{2+}	66
Zn^{2+}	15

cubated 10 min at pH 6.8 at the specified temperature and residual activity was determined. Almost all activity was lost after incubation at 75 °C.

The effects of change in ionic strength on enzyme activity are shown in Fig. 9. Increasing Tris–HCl buffer concentration up to 0.4 M or salt (KCl or NaCl) concentration up to 0.2 M results in stimulation of enzyme activity.

None of the divalent ions tested at 0.1 mM concentration was stimulatory. The most marked effects were produced by Cd^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}, all of which inhibited nuclease activity (Table III).

The sulfhydryl reagents *N*-ethylmaleimide, glutathione (reduced and oxidised), iodoacetamide, iodoacetate, and dithiothreitol had no effect on enzyme activity at a concentration of 0.2 mM. This experiment was performed in the absence of added

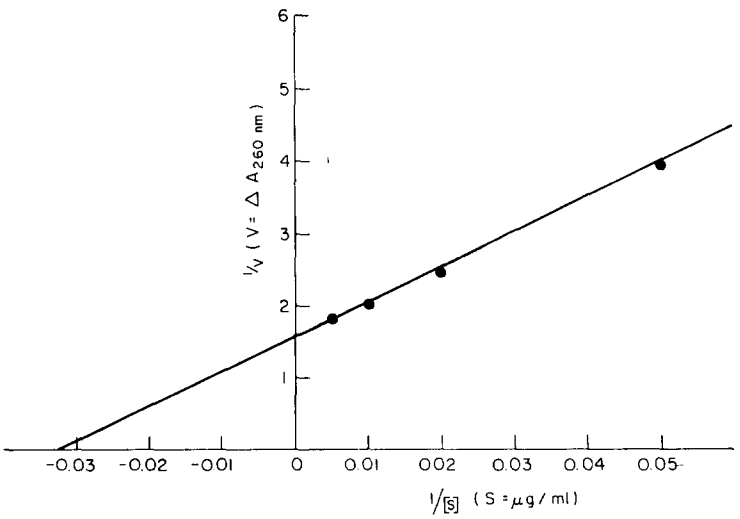

Fig. 10. Lineweaver–Burk plot of *Klebsiella* nuclease activity with yeast RNA substrate. Each assay contained 0.6 unit of enzyme, and substrate concentration was varied as indicated.

TABLE IV

RELATIVE ACTIVITIES OF *Klebsiella* NUCLEASE AGAINST NUCLEIC ACID SUBSTRATES

Assays were performed as described in Materials and Methods, with 0.6 unit of enzyme per ml and 250 μg/ml of the indicated substrate.

Substrate	*Relative activity (%)*
Yeast RNA	100
rRNA	80
tRNA	85
DNA (native)	0
DNA (denatured)	0

bovine serum albumin and no serum albumin was present in the enzyme preparation. No striking effects were noted.

Purified enzyme stored for 1 month at −20 °C in a solution containing 1% bovine serum albumin retained 91% of its activity while a sample stored without albumin retained only 31% of its activity. Assays were usually done with an enzyme preparation containing 1% bovine serum albumin.

Digestion of RNA

Enzyme saturation occurs at a relatively low substrate level (about 200 μg/ml) with yeast RNA as the substrate. A plot of $1/v$ *vs* $1/[S]$ is shown in Fig. 10. The K_m for RNA from this graph is 32 μg/ml. Poly(G) at a concentration as low as 5 μmoles per l of polynucleotide phosphorus completely inhibited the digestion of yeast RNA.

Yeast RNA, *E. coli* RNA (16 S + 23 S), *E. coli* tRNA, and calf thymus DNA (native and denatured) were tested as substrates for the purified nuclease. Results are listed in Table IV. Yeast RNA was slightly superior to tRNA and rRNA as a substrate for the nuclease. Neither native nor denatured DNA could serve as a substrate.

When 2.5 mg of RNA in 1 ml of 0.01 M Tris–HCl buffer (pH 7.5) were incubated for 16 h at 37 °C in the presence of 5.5 units of purified nuclease, the substrate was completely digested to mononucleotides, 2′:3′-cyclic compounds predominating

TABLE V

MONONUCLEOTIDE COMPOSITION OF COMPLETE DIGEST OF YEAST RNA

2.5 mg of yeast RNA were incubated with 40 μmoles Tris–HCl buffer (pH 7.0) and 5.5 units of nuclease in a 1-ml volume. After 16 h, the digest was treated with phenol and diethyl ether as described in Materials and Methods and put through chromatographic Solvent systems 1 and 2. Compounds were identified by R_F values and ultraviolet absorption spectra and quantitated by ultraviolet absorbance. Percentages indicate the fraction of each compound present as 2′:3′- or 3′-mononucleotide. There were no products other than mononucleotides.

Product	*2′:3′ (%)*	*3′ (%)*
AMP	86	14
CMP	92	8
UMP	91	9
GMP	81	19

(Table V). After a 4-h digestion a number of oligonucleotides were found and those identified were ApGp, UpAp, ApAp, UpCp, and ApGpGp. No pattern of specificity was thus apparent.

DISCUSSION

Klebsiella ribonuclease has been purified 540-fold. At this stage of purification the preparation contains no nucleosidase or nucleotidase activity, since even after prolonged hydrolysis nucleotides are the only products. Further, no deoxyribonuclease, alkaline phosphatase, or phosphodiesterase activities were detectable. The enzyme, in short term incubations, digests yeast RNA to a mixture of oligonucleotides and 2′:3′-AMP, CMP, UMP, and GMP. Small amounts of 3′-mononucleotides may also be detected. On the basis of the digestion products, the enzyme is a cyclicizing endonuclease. There appears to be no nucleotide residue specificity. The nuclease is able to degrade yeast RNA completely to mononucleotides in a prolonged digestion. This property suggests that the enzyme might be of value in determination of RNA base ratios and hyperchromic effects. The enzyme has a molecular weight of approximately 24 000, a pH optimum in the neutral range, and no detectable divalent ion requirement. One of the divalent ions tested, Zn^{2+}, produced significant inhibition at a concentration of 0.2 mM.

To our knowledge, inhibition of nuclease activity by poly(G) has not previously been reported. Preliminary experiments suggest that this property may be a general characteristic of bacterial endonucleases[14]. The type of inhibition demonstrated by poly(G) may be related to the mechanism by which organisms such as bacteria are able to regulate nuclease activity.

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