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A pyrimidine-guanine sequence-specific ribonuclease from *Rana catesbeiana* (bullfrog) oocytes

You-Di Liao
Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, China

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ABSTRACT

A pyrimidine-guanine sequence-specific ribonuclease (RC-RNase) was purified from *Rana catesbeiana* (bullfrog) oocytes by sequential phosphocellulose, Sephadex G75, heparin Sepharose CL 6B and CM-Sepharose CL 6B column chromatography. The purified enzyme with molecular weight of 13,000 daltons gave a single band on SDS-polyacrylamide gel. One CNBr-cleaved fragment has a sequence of NVLSTTRFQLNT/TRTSITPR, which is identical to residues 59–79 of a sialic acid binding lectin from *R. catesbeina* eggs, and is 71% homologous to residues 60–80 of an RNase from *R. catesbeina* liver. The RC-RNase preferentially cleaved RNA at pyrimidine residues with a 3′ flanking guanine under various conditions. The sequence specificity of RC-RNase was further confirmed with dinucleotide as substrates, which were analyzed by thin layer chromatography after enzyme digestion. The values of *k*cat/km for pCpG, pUpG and pUpU were 2.66×10^7 $M^{-1}s^{-1}$, 2.50×10^7 $M^{-1}s^{-1}$ and 2.44×10^6 $M^{-1}s^{-1}$ respectively, however, those for other phosphorylated dinucleotides were less than 2% of pCpG and pUpG. As compared to single strand RNA, double strand RNA was relatively resistant to RC-RNase. Besides poly (A) and poly (G), most of synthetic homo- and heteropolynucleotides were also susceptible to RC-RNase. The RC-RNase was stable in the acidic (pH 2) and alkaline (pH 12) condition, but could be inactivated by heating to 80°C for 15 min. No divalent cation was required for its activity. Furthermore, the enzyme activity could be enhanced by 2 M urea, and inhibited to 50% by 0.12 M NaCl or 0.02% SDS.

INTRODUCTION

In the mature oocytes of Xenopus, nuclei are arrested at the first meiotic prophase until fertilization. During the growing stages, the oocytes synthesize and accumulate large quantity of mRNA and ribosomes, which are considered to be a store of protein synthesizing machinery for early development of the embryo (1, 2). The cell extracts of full-grown oocytes of *Xenopus laevis* were highly active for *in vitro* RNA synthesis (3). This *in vitro* system has been widely employed to study the mechanism of transcription (4, 5, 6). The establishment of a similar system using oocyte extracts of *Rana catesbeiana* (bullfrog) was not successful as expected. The main reason was due to a predominant ribonuclease activity in the extract of growing or full-grown oocytes from the bullfrog. However, eggs derived from RNase-abundant oocytes were able to develop into embryos. The role of RNase on RNA metabolism during oogenesis and embryogenesis is unclear. This report describe the purification and characterization of an RNase from bullfrog oocytes. This *R. catesbeiana* (bullfrog) RNase, designated as RC-RNase, was shown to specifically cleave 3′ site of pyrimidine residues with a 3′ flanking guanine of single strand RNA.

MATERIALS AND METHODS

Materials

Mature native *Rana catesbeiana* (400–600 grams per frog) were normally kept between 20°C and 25°C. [α-^{32}P] GTP and [γ-^{32}P] ATP were purchased from Amersham (UK); adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytosine triphosphate (CTP), uridine triphosphate (UTP), dinucleotides, synthetic polynucleotide, α-amanitin, yeast tRNA and total RNA from Sigma. Hydroxyethylpiperazine ethane sulfonic acid (Hepes), dithiothreitol (DTT), 16S and 23S rRNA of *E. coli*, and phage MS2 RNA from Boehringer Mannheim Biochemicals (W. Germany). EDTA, KCl, ammonium sulfate, perchloric acid, uranium acetate, and thin layer plates (PEI-cellulose F) were the products of E. Merck. Ribonuclease T_1, U_2, Phy M, *B. cereus*, CL_3, dephosphorylated yeast 5S rRNA in RNA sequencing kit and T_4 polynucleotide kinase were obtained from Bethesda Research Laboratories (Maryland). Phosphocellulose was from Whatman. Heparin Sepharose CL 6B, Sephadex G75 and CM Sepharose CL 6B were from Pharmacia-LKB. PVDF membrane (Problott) for electroblotting was from Applied Biosystem Inc. (Foster City, CA). The double strand RNAs with length of 5 kilobase and 0.5 kilobase of a T_4 virus from *Trichomonas vaginalis* was a gift of Dr. J.H. Tai of this Institute.

Preparation of oocyte extracts

All operations were carried out at 4°C. Ovaries (60–100 g per frog) were excised from mature female *R. catesbeiana*, and cut into approximate 4 cm^3 pieces. They were washed with a digestion buffer (5 mM Hepes, pH 7.9, 50 mM NaCl, 25 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$ and 1 mM $CaCl_2$), and incubated with 0.15% collagenase type II (Sigma) in the same buffer for 4 to 6 hrs at room temperature till most of the oocytes were released. Oocytes were washed ten times by decantation with digestion buffer and twice with extraction buffer (30 mM Tris-HCl, pH 7.9, 90 mM KCl, 2 mM EGTA, 1 mM DTT and

10 mM β-glycerophosphate), and suspended in extraction buffer. One ml of extraction buffer was layered onto the top, and centrifuged for 45 min in a Beckman SW41 rotor at 150,000 g. The oocyte extracts (Fraction I), located between the fat layer and the yolk was removed with a 2.5 ml syringe and stored at −70°C in small aliquots (7).

Purification of the RC-RNase from *R. catesbeiana* oocyte extracts

The oocyte extracts from *R. catesbiana* (Fraction I) was dialyzed against PC buffer (20 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT and 5% glycerol) containing 0.09 M KCl, and then loaded onto a phosphocellulose column (2.5×20 cm). The column was washed with PC buffer containing 0.09 M KCl, and eluted with a 400 ml linear salt gradient of 0.09 to 0.4 M KCl. Each fraction (9 ml) was collected and assayed for RNase activity by thin layer chromatography (TLC). Active fractions (Fraction II) were combined for further purification.

Fraction II was precipitated by addition of solid ammonium sulfate to 90% saturation with continuously stirring for 30 min. The pellets were centrifuged at 15,000 g for 20 min and re-dissolved in 1 M KCl in PC buffer. After dialysis against the same buffer, the sample was loaded on a Sephadex G75 column (2.5×60 cm) and eluted with the same buffer. Fractions (5 ml each) were collected and assayed for RNase activity. The active fractions were pooled (Fraction III).

Fraction III was dialyzed against PC buffer containing 0.06 M KCl, loaded on a heparin Sepharose CL 6B column (1.5×15 cm), and eluted with a 100 ml linear salt gradient of 0.06 to 0.4 M KCl in PC buffer. Fractions of 1.5 ml were collected and assayed as described. The active fractions were combined (Fraction IV).

The dialyzed Fraction IV was loaded onto a CM-Sepharose column (1.0×10 cm), washed with PC buffer containing 0.09 M KCl and then eluted stepwise with 0.5 M KCl in PC buffer. Fractions of 1.5 ml were collected and assayed as described. The active fractions were pooled (Fraction V).

The elution profiles of proteins from each column chromatography were also monitored by the absorbance at 280 nm with a Gilson UV-112 monitor. The protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (8). The KCl concentration of each eluate was determined by conductivity measurement. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (9).

Protein sequence analysis

RNase (770 pmoles) was treated (24 hrs, room temperature) with a 100×molar equivalent of CNBr in 70% formic acid (100 µl). Reaction was stopped by adding distilled water and evaporated to dryness in a Sped Vac concentration for several times. The chemically cleaved RNase was separated by SDS-PAGE according to Laemmli (9) on a 15% gel. Resulting fragments were transferred onto a ProBlott membrane with a semi-dry blotting aparatus with high pH buffer according to Chang *et al.* (10). Protein bands were visualized by Coomassie blue dye staining and excised from the membrane for sequence analysis. Automated cycles of Edman degradation were performed on an Applied Biosystems Inc. gas/liquid phase Model 470A/900A sequencer equipped with an on-line Model 120A phenythiohydantoin-amino acid analyzer according to Hewick *et al.* (11).

Assays of RC-RNase activity

Three methods used to measure RC-RNase activity are described as follows:

1. Two µg of dinucleotide CpG or UpG were incubated with a serial dilution of RNase in 50 mM Tris-HCl, pH 8.0 for 15 min at 37°C. The reaction mixtures were spotted on a PEI-cellulose F thin layer plate, and developed in 0.8 M LiCl-0.8 M acetic acid or 0.25 M LiCl-1.0 M acetic acid. The nucleotides were visualized by UV illumination at 254 nm (12). The method was used to determine the active fractions of column eluates. It takes only one hour to complete the whole process including incubation, development and UV visualization.
2. 5′ end of dinucleotides were phosphorylated with either cold ATP or γ-^{32}P-ATP by T_4 polynucleotide kinase, and then purified by thin layer chromatography developed in 0.25 M LiCl-1.0 M acetic acid. Small aliquots of ^{32}P labelled dinucleotide (20,000 cpm) mixed with various amounts of corresponding phosphorylated dinucleotide were incubated with RC-RNase in 50 mM Tris-HCl, pH 8.0, for 10 min at 37°C. The digested products were spotted on TLC plate and developed in 0.25 M LiCl-1.0 M acetic acid. The relative amounts of products and substrates were quantified by phosphor image (Molecular Dyanamics) (13).
3. Phenol extracted yeast total RNA (120 µg) or other synthetic polynucleotides were incubated together with the appropriate amounts of enzyme in 50 mM Tris-HCl, pH 8.0, in a final volume of 50 µl for 10 min at 37°C. At the end of incubation, 200 µl of 7% perchloric acid and 0.1% uranyl acetate were added.

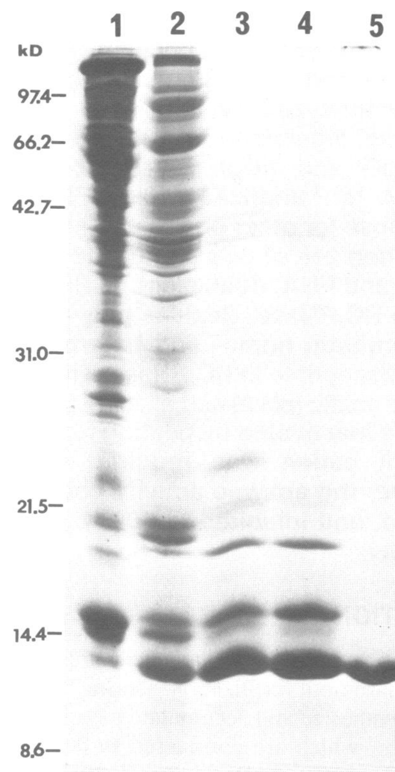


Fig. 1. SDS-PAGE analysis of proteins during purification. Samples were electrophoresed on a 15% SDS-PAGE using the discontinuous pH system of Laemmli (9). The gel was stained with Coomassie Brilliant Blue R. Lane 1, crude extracts; lane 2, phosphocellulose column eluate; lane 3, G-75 column eluate; lane 4, heparin column eluate and lane 5, CM Sepharose column eluate. Molecular weight markers are shown at the left.

The mixture was chilled on ice for 30 min. After centrifugation for 20 min in an Eppendorf centrifuge, 200 μl of the supernatant were diluted to 0.8 ml with water and the absorbance at 260 nm was measured. One unit of enzyme activity is defined as the amount of enzyme producing 1 A_{260}acid-soluble material under standard assay condition as described above (14). This method was used to quantify RNase activity for enzyme recovery and general characterization.

RNA sequencing

Dephosphorylated yeast 5S rRNA was 5′ end-labelled by [γ-^{32}P] ATP and T_4 polynucleotide kinase. The labelled 5S rRNA was separated by 8% polyacrylamide gel electrophoresis and recovered from the gel by electroelution. The purified 5′ end-labelled 5S rRNA (2 ng) and carrier tRNA (50 ng) were digested with RNase T_1 (specific for G), RNase Phy M (specific for A and U), RNase U_2 (specific for A), RNase *B. cereus* (specific for C and U), RNase CL_3 (specific for C), or RC-RNase in the appropriate buffer as described in the kits, or in 0.05 M sodium bicarbonate/carbonate, pH 9.2 (15). The digestion of end-labelled 5S rRNA with RC-RNase was carried out in 50 mM Tris-HCl, pH 8.0, for 5 min at 37°C except when indicated otherwise. All limit-digested 5S rRNAs were electrophoretically separated on a 7 M urea-10% polyacrylamide gel and visualized by autoradiography.

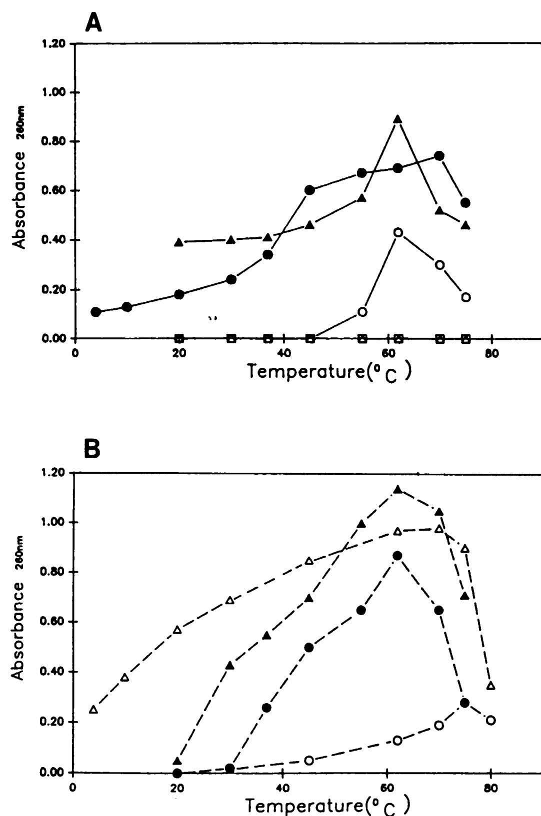
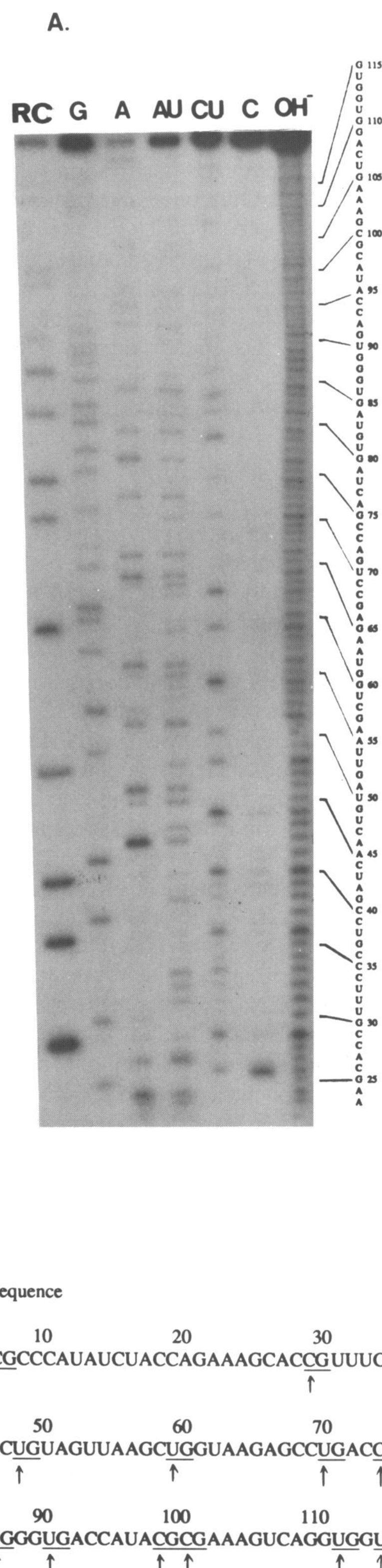


Fig. 2. Temperature dependence of cleavage of synthetic polymers by RC-RNase. A. 120 μg of yeast total RNA and four kinds of homopolymers were incubated with 1.5 ng RC-RNase in 50 mM Tris-HCl, pH 8.0 for 10 min at the temperature as indicated. The acid-soluble ribonucleotides were measured at 260 nm. △—△: poly (A), ○—○: poly (C), □—□: poly (G), ▲—▲: poly (U), ●—●: yeast total RNA. B. 120 μg of heteropolymers were incubated with RC-RNase as described above. ○- - -○: poly (G-U), ●- - -●: poly (C-U), △- - -△: poly (I-U), ▲- - -▲: poly (C-I).

A.



B.

Yeast 5S RNA Sequence

5′ pppGGUUGCGCCCAUAUCUACCAGAAAGCACCGUUUCCCGUCC (10, 20, 30, 40)

GAUCAACUGUAGUUAAGCUGGUAAGAGCCUGACCGACUAG (50, 60, 70, 80)

UGUAGUGGGUGACCAUACGCGAAAGUCAGGUGGUGCAAUCU 3′ (90, 100, 110, 120)

Fig. 3. Sequence analysis of RC-RNase cleavage sites using yeast 5′ labelled 5S rRNA as substrate. A. 5′ end labelled 5S rRNA was partially digested with commercial RNase and RC-RNase as described in Materials and Methods. G: RNase T_1, A: RNase U_2, AU: RNase Phy M, CU: RNase *B. cereus*, C: RNase CL_3, OH^-: 0.05 M sodium bicarbonate-carbonate, pH 9.2, RC: RC-RNase. B. The complete yeast 5S rRNA sequence was shown and the pyrimidine-guanine sequences was underlined. Arrows indicate the cleavage sites of RC-RNase.

RESULTS

Purification of RC-RNase

The RNase was purified to 133 fold through a series of chromatography including phosphocellulose, Sephadex-G75, heparin Sepharose CL 6B and CM-Sepharose CL 6B column. The pooled fractions from each purification step was analyzed by SDS-PAGE (Fig. 1). Only one band with molecular weight of 13,000 daltons was observed on the gel after these purification steps. The purification procedure is summarized in Table 1. The overall yield was approximately 11%, i.e. approximate one milligram of purified RC-RNase could be obtained from four mature female bullfrogs. The specific activity of the purified RC-RNase was 209 unit/μg, which is slightly higher than that of bovine pancreatic RNase A (172 unit/μg) using the same reaction condition.

Substrate specificity

1. *Single strand RNA*: 50 μg of single strand RNAs, such as *E. coli* 16S, 23S rRNA, phage MS2 RNA, yeast tRNA and total RNA, could be completely digested by 0.15 ng of RC-RNase within 15 min at 37°C in 50 mM Tris-HCl, pH 8.0 (data not shown).
2. *Double strand RNA*: The *Trichomonas vaginatis* T_4 virus contains two double strand RNA fragments, 5 kilobase and 0.5 kilobases. The larger fragment was poorly digested by 15 ng (3 unit) RC-RNase under standard reaction condition. However, the digestion of the large fragment could occur by raising the temperature to 60°C or by using excess amount of RC-RNase (150 ng, 30 units) at 37°C. By comparison, 150 ng RC-RNase could not cleave the small fragment at 37°C, but completely cleave this fragment at 60°C. If 0.5 M NaCl was included in the reaction mixture, both fragments were resistant to RC-RNase even in the amount of 150 ng at standard assay condition. These results indicate that RC-RNase preferentially cleaves single strand RNA rather than double strand RNA.
3. *Synthetic polymers*: The susceptibility of synthetic homopolynucleotides and heteropolynucleotides to RC-RNase was analyzed in 50 mM Tris-HCl, pH 8.0 at different temperatures. As shown in Fig. 2A, the degradation of poly (U) was comparable to that of yeast total RNA between 30°C and 75°C. The degradation of poly (C) was less than that of poly (U) between 45°C and 75°C. There is no degradation was observed for poly (C) below 45°C (Fig. 2A). Poly (A) and poly (G) were not degraded by RC-RNase at all even in excess RC-RNase and extended incubation. With regard to heteropolynucleotides, polycytidylic-uridylic acid (poly (C-U)) and polycytidylic-inosinic acid (poly (C-I)) were degraded by RC-RNase in similar manner but with different threshold temperatures, i.e. 30°C and 20°C, respectively. Polyinosinic-uridylic acid (poly (I-U)) was the most susceptible substrate, 25% of the polynucleotides was degraded at 4°C compared to that of optimal temperature (60°C). Polyguanylic-uridylic acid (poly (G-U)) was the most resistant heteropolynucleotides tested. The optimal temperature was shifted to 75°C from 60°C (Fig. 2B). These results suggest that the relative susceptibility of each polynucleotide varies with the temperature which may affect the secondary structure of the polynucleotide, e.g. formation or breakage of double strand.

Sequence specificity

^{32}P-labelled 5S rRNA, tRNA and VA-RNA were synthesized *in vitro* by *Xenopus laevis* oocyte extracts (3) and partially digested with RC-RNase, several RNA fragments with different intensity were generated (data not shown). The results suggest that RC-RNase preferentially cleave these RNAs at several specific sites.

The sequence specificity of the RC-RNase was further analyzed by comparing its digestion pattern of 5′ end labelled yeast 5S rRNA with those cleaved by RNase T_1, RNase U_2, RNase Phy M, RNase *B. cereus* and RNase CL_3. The digests were electrophoretically analyzed on a 7 M urea-10% polyacrylamide gel. The sequence of 5S rRNA was shown on the right side of Fig. 3A. The specific cleavage sites of RC-RNase were indicated in Fig. 3B. All the cleavage sites were located at pyrimidines with a 3′ flanking guanine.

In order to confirm the sequence specificity, all possible combination of dinucleotides were tested for their sensitivity to RC-RNase for 30 min at 37°C in 50 mM Tris-HCl, pH 8.0. Only CpG and UpG (2 μg, each) were completely cleaved by 15 ng of RC-RNase. Other ribodinucleotides were not cleaved. When excess RC-RNase was used (150 ng), only UpU was cleaved. To know the effect of 5′ phosphorylation on the sensitivity of dinucleotide to RC-RNase, a series of titration experiments were performed. It was found that 1 ng of RC-RNase could completely cleave 2 μg pCpG but 15 ng of RC-RNase was required for cleavage of 2 μg CpG. These results indicated that phosphorylated dinucleotides were more sensitive substrates for RNase assay. Values of *k*cat/Km for phosphorylated dinucleotides were determined according to their relative radioactivity of substrates and products on TLC plate. The *k*cat/km values of pCpG, pUpG and pUpU were 2.66×10^7 $M^{-1}s^{-1}$, 2.50×10^7 $M^{-1}s^{-1}$ and 2.44×10^6 $M^{-1}s^{-1}$ respectively, but those for other CpN′ and UpN′ were less than 2% of pCpG and pUpG. All pApN′ and pGpN′ were not cleaved under the same condition. To examine the exact cleavage site of CpG and UpG, the digests along with suspected products were separated by thin layer chromatography with two different development systems. During the first 30 min digestion, CpG was cleaved into 2′,3′ cyclic CMP and guanosine intermediates by 15 ng RC-RNase. However, the 2′,3′ cyclic CMP was further converted into 3′CMP when the reaction was incubated at 37°C overnight (Fig. 4 A and B). UpG was also cleaved into 2′,3′ cyclic UMP and guanosine, then into 3′UMP and guanosine under the conditions described above (Fig. 4 C and D). We therefore conclude that the linkage between Cp and G, or Up and G was the cleavage site of RC-RNase upon CpG and UpG. As shown in Fig. 4 C and D, the product of poly U was found to be 3′UMP.

The base specificity of several RNases is dependent on the reaction condition, such as concentration of urea and pH values. It seems necessary to check whether the sequence specificity of RC-RNase is affected by these factors. The sequence specificity of RC-RNase was not affected by the presence of EDTA to 50 mM (Fig. 5 lanes 2 and 3), or NaCl to 100 mM (lanes 4−6), or by the wide range of pH (pH 3 to pH 9) in 50 mM acetate or 50 mM Tris-HCl buffer (lanes 7−13). The sequence specificity was also not altered in the temperature range 0°C to 60°C (lanes 14−17).

General properties

1. *Protein sequence analysis*. The purified RNase has a modified N-terminus which does not yield any sequence upon Edman degradtion. Consequently, the enzyme was fragmented with CNBr for internal sequence analysis. A single band, which migrated faster than untreated sample on SDS gel, was observed

Table I. Purification of RC-RNase

Step	Fraction	Volume	Total protein[a]	Total activity[b]	Specific activity	Yield
		ml	mg	unit	unit/mg	%
1. Crude extracts	I	61	508.0	799,500	1,573	100
2. Phosphocellulose	II	47.5	20.0	601,200	30,060	75.2
3. Sephadex G-75 gel filtration	III	36	3.0	428,400	142,800	53.6
4. Heparin Sepharose CL 6B chromatography	IV	10.2	0.73	140,600	192,600	17.6
5. CM Sepharose CL 6B	V	6.5	0.43	90,000	209,300	11.3

[a] Protein concentration was measured by Bradford's method (8).
[b] One enzyme unit is defined as the amount of enzyme producing 1 A_{260} acid-soluble material under standard assay condition as described in the text.

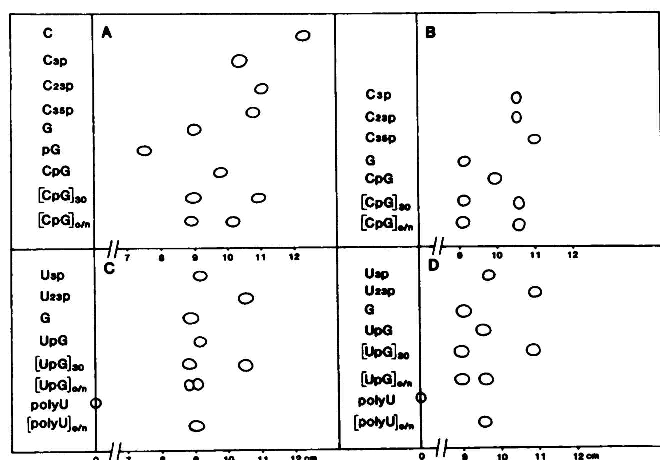


Fig. 4. Sequence analysis of RC-RNase cleavage sites by using dinucleotides as substrates. Dinucleotides, CpG and UpG, and poly (U) (2 μg of each) were individually incubated with 15 ng of RC-RNase in 4 μl of 50 mM Tris-HCl, pH 8.0 at 37°C for 30 min or overnight. The digested products and several suspected products were spotted on a PEI-cellulose F TLC plate. The air dried plates were developed in 0.25 M LiCl-1.0 M acetic acid (panel A and C), or 0.8 M LiCl-0.8 M acetic acid (panel B and D). The products were visualized by UV illumination at 254 nm. Panel A and B are digestion products of CpG and its related standard compounds; panel C and D are digestion products of UpG and poly (U), and their related standard compound. The symbols used are: C, cytidine; C_3p, 3'CMP; $C_{23}p$, 2',3' cyclic CMP; $C_{35}p$, 3',5' cyclic CMP; G, guanosine; pG, 5'GMP; CpG, cytidylyl (3' → 5') guanosine; U_3p, 3'UMP; $U_{23}p$, 2',3' cyclic UMP; UpG, uridylyl (3' → 5') guanosine; $[CpG]_{30}$ and $[UpG]_{30}$, 30 min digestion of CpG and UpG by RC-RNase; $[CpG]_{o/n}$, $[UpG]_{o/n}$ and $[poly (U)]_{o/n}$, overnight digestion of CpG, UpG and poly (U) by RC-RNase, respectively.

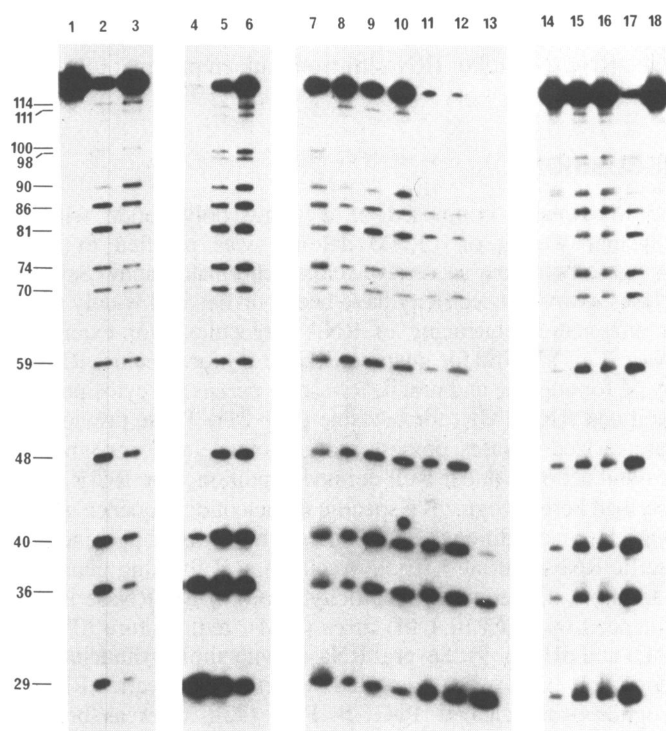


Fig. 5. Sequence specificity of RC-RNase under various conditions. Two ng of 5' end labelled 5S rRNA including 50 ng of yeast tRNA was incubated with 0.15 ng of RC-RNase at standard assay condition in 50 mM Tris-HCl, pH 8.0, for 15 min at 37°C except indicated otherwise. The partial digested 5S rRNA was separated by 7 M urea-10% polyacrylamide gel electrophoresis and analyzed by autoradiography. Lanes 1 and 18: no RNase. Lanes 2 and 3, digested by 0.15 ng of RC-RNase with 10 and 50 mM EDTA; lanes 4−6, 0.75 ng of RC-RNase with 20, 50 and 100 mM NaCl; lanes 7−10, 0.15 ng of RC-RNase in 50 mM acetate buffer, pH 3, 4, 5 and 6; lanes 11−13, 0.15 ng of RC-RNase in 50 mM Tris HCl buffer, pH 7, 8 and 9; lanes 14−17, 0.15 ng of RC-RNase in 50 mM Tris-HCl, pH 8.0, at 0°C, 25°C, 37°C and 60°C, respectively.

and collected for protein sequencing. The peptide has a sequence of: NVLSTTRFQLNT/TRTSITPR. The protein sequence is identical to residues 59−79 of a sialic acid binding lectin from *R. catesbeiana* eggs (16), and has 71% identical sequence with residues 60−80 of an RNase from *R. catesbeiana* liver (17). We could not obtain any signal from cycle 13. Upon aligning with the sequence of lectin from *R. catesbeiana* eggs and RNase from *R. catesbeiana* liver, a conserved cysteine residue was possibly located at that position.

2. *The effects of pH on RC-RNase activity.* Under standard reaction condition, 120 μg of yeast total RNA was incubated with 3 ng of RC-RNase for 10 min at 37°C at pH values from 3.5 to 9.5 using 50 mM acetate (pH 3.5−6.5) or 50 mM Tris-HCl buffer (pH 6.5−9.5). The optimal pH for the enzyme activity was between pH 7.5 and 8.0. The RC-RNase was still active after preincubation in acidic (pH 2) or alkaline (pH 12) condition for 15 min (data not shown).

3. *The effects of temperature on RC-RNase activity.* As shown in Fig. 2, the optimal temperature of RC-RNase activity was

60°C for RNA substrates except poly (G-U) which had optimal temperature of 75°C. However, the threshold temperature for RNase reaction varied with the substrates used. Yeast total RNA and poly (I-U) could be hydrolyzed at 4°C, poly (G-U) at 20°C, poly (C-U) at 30°C and poly (C) at 45°C. The RC-RNase remained active when it was heated at 75°C for 15 min. The critical temperature for heat inactivation was 80°C.

4. *The effects of divalent cations, NaCl, urea, SDS, RNasin on RC-RNase activity.* No divalent cation (Mg^{+2}, Zn^{+2} or Mn^{+2}) was required for RC-RNase activity and EDTA at the concentration of 100 mM did not inhibit enzyme activity (data not shown). NaCl was not required for RC-RNase activity, but it caused 50% inhibition at the concentration of 0.12 M. The optimal concentration of urea for the enzyme activity was 2 M. Urea may prevent the formation of double strand RNA as described above and facilitate the single strand RNA being more accessible to RC-RNase. Trace amount of SDS (0.02%) inhibited 50% of the RC-RNase activity. The RC-RNase was not inhibited by the RNase inhibitor (RNasin) from human placenta (data not shown).

DISCUSSION

The RC-RNase, composed of a single polypeptide with a molecular weight of 13,000 daltons, was purified to near homogeneity by four successive column chromatography. Several RNases with site-specificity have been purified and widely used for enzymatic sequencing of RNA molecules. For example, RNase T_1 is specific for guanine, RNase U_2 for adenine, RNase Phy M for adenine and uracil, RNase *B. cereus* for cytosine and uracil and RNase CL_3 for cytosine (18–21). These previously characterized RNases possess monoribonucleotide recognition sites and act only under well defined condition. The RC-RNase described here recognizes a specific dinucleotide sequence under a variety of conditions. RC-RNase is not only a pyrimidine-specific RNase but also strictly requires a 3′ flanking guanine. In addition, the sequence specificity (PyG) of RC-RNase is not influenced by NaCl (0.1 M), urea (7 M), temperature (0°C–60°C) and pH (3–9). Several RNases with similar dinucleotide recognition site were reported. Turtle pancreatic RNase recognizes and cleaves PyG > PyA (22), whereas bovine pancreatic RNase A, bovine seminal RNase, horse pancreatic RNase and whale pancreatic RNase recognize and cleave PyA > PyG (23). Although these RNases are pyrimidine-guanine or pyrimidine-adenine specific, their sequence specificities are less strict than that of RC-RNase. The 3′ flanking base recognized by RC-RNase is invariably guanine, while that of turtle and mammalian RNases are guanine and adenine. Thus, RC-RNase could be a valuable enzyme for RNA sequencing due to its uniform cleavage of all the pyrimidine-guanine sites. Since approximate one eighth of the base sequence on RNA can be recognized and cleaved by the RC-RNase, partial digests of end-labelled RNA may be useful size marker for RNA in denaturing gels.

An RNase has been isolated from *R. catesbeiana* (bullfrog) liver (24). This liver RNase could hydrolyze poly (C) and produce 2′, 3′ cyclic CMP as intermediate and 3′CMP as final product. Although RC-RNase and the liver RNase share similar properties of molecular weight, optimal pH, thermostability and divalent cation requirement, their substrate specificities differ (24). The base and sequence specificity of liver RNase was not well characterized. The liver RNase was composed of a single polypeptide with molecular weight 13,500 daltons and 111 amino acids. The amino acid sequence of liver RNase has been determined (17) and is highly homologous to those of lectins isolated from *R. catesbeiana* and *R. japonica* eggs, 70.2% and 64.8%, respectively (16, 25). Those egg lectins could agglutinate transformed animal cells and might have ribonuclease activity (25, 26). In addition, their sequences are also homologous to that of bovine and human pancreatic ribonuclease, bovine and human angiogenin (27, 28). Angiogenin, a blood vessel-inducing protein from HT-29 human colon adenocarcinoma cells, is highly homologous to that of pancreatic ribonuclease and was reported to catalyze the limited cleavage of 28S and 18S rRNA and produce fragments with 100–500 nucleotides in length. Although the complete sequence of RC-RNase was not determined yet, 20 amino acids has been sequenced and found to be identical to residues 59–79 of the sialic acid binding lectin from *R. catesbeina* egg. Due to the highly homologous between the RC-RNase and egg lectin, it will be of great interest to see whether RC-RNase plays certain roles in agglutination and angiogenesis.

During the purification process, an inhibitor, which forms a complex with RC-RNase, was found in the flowthrough of phosphocellulose column chromatography. The complexed form of RC-RNase is termed latent RNase. The RNase activity of latent RNase could be released by treatment with 1 mM p-hydroxy-mercuribenzoic acid, 0.25 N sulfuric acid or 60°C for 15 min. In general, the latent form accounts for 20% of total RNase. The inhibitor has also been purified to homogeneity by affinity column chromatography in this laboratory (manuscript in preparation). According to the hypothesis proposed by Blackburn *et al.* (29) and Feldman *et al.* (30), the increase of RNA in actively dividing cells is preceded by a rise in the concentration of the RNase inhibitor. Alternatively, the decrease of protein synthesis and increase of catabolic activity in nondividing cells may be associated with lower levels of the inhibitor and elevated RNase activity. Since RNase activities exist in a large quantity in bullfrog liver, oocyte, egg and serum, it is possible that the regulation of RNase activity was under the control of RNase inhibitor instead of RNase turnover and *de novo* synthesis.

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