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AN EXORIBONUCLEASE FROM SACCHAROMYCES CEREVISIAE: EFFECT OF MODIFICATIONS OF 5' END GROUPS ON THE HYDROLYSIS OF SUBSTRATES TO 5' MONONUCLEOTIDES[1]

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Summary: Using poly(A) as a substrate, an exoribonuclease has been purified from the high-salt wash of ribosomes of Saccharomyces cerevisiae. The product of the reaction of the exoribonuclease is 5' AMP. Hydrolysis of $[^3H](pA)_3[^{14}C](pA)_n$ shows that both labels are released at the same rate, suggesting that the enzyme acts in a processive manner. Removal of the terminal phosphate of poly(A) with alkaline phosphatase reduces the rate of hydrolysis by 80%. Treatment of the terminally dephosphorylated poly(A) with polynucleotide kinase restores the activity. Two 5' capped mRNA's have been tested and they are hydrolyzed slowly, if at all, by the enzyme. In contrast, phage T4 mRNA, ribosomal RNA, and encephalomyocarditis viral RNA are hydrolyzed at greater than 50% of the rate of poly(A).

A large number of ribonucleases of yeast have been described. Ohtaka et al. (1) isolated a ribonuclease from yeast autolysates which yielded 3' mononucleotides as the final products. Nakao et al. (2) and Lee et al. (3) partially purified two ribonucleases from yeast which hydrolyzed polyribonucleotides to di- and trioligoribonucleotides with 5' phosphate end groups. Dauber (4) described two ribonucleases from S. cerevisiae which were endonucleases with oligoribonucleotides containing 3' phosphate end groups as degradation products. A ribonuclease which yielded 3' mononucleotides as products was isolated from yeast during the stationary phase of growth (5). Two ribonucleases H from S. cerevisiae which specifically degrade RNA in RNA-DNA hybrid structures have been highly purified and studied (6, 7).

Fractionation of extracts of S. cerevisiae by DEAE-cellulose and hydroxylapatite chromatography has shown the presence of four peaks of exoribonuclease activity when poly(A) is used as a substrate (Stevens, unpublished results) and 5' AMP is the product with all the enzymes. In this paper I describe some of the properties of one of the enzymes, in particular the effect of modifications of 5' end groups on the hydrolysis of substrates.

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MATERIALS AND METHODS

The exoribonuclease was purified from the high-salt wash of ribosomes of S. cerevisiae by DEAE-cellulose and hydroxylapatite chromatography. [The details of the procedure will be reported elsewhere (Stevens, unpublished)]. The procedure results in a 200-fold purification of the enzyme. No endoribonuclease activity is detectable in the purified enzyme using the assay of Tsiapalis et al. (8). Exonucleolytic activity on DNA is also not detectable. A slight contamination with phosphatase activity (10% of the 5' AMP is found as adenosine) is detectable when 20 times as much enzyme is used as that used in the experiments reported here.

The enzyme was assayed by measurement of the conversion of labeled polyribonucleotides to acid-soluble radioactivity. The reaction mixtures (0.15 ml) contained: labeled polyribonucleotide; 1.5 mM $MgCl_2$; 33 mM sodium glycinate buffer, pH 9.5; 100 µg bovine serum albumin; 50 mM NH_4Cl and enzyme. After incubating at 37° for 30 min, the reaction was stopped by the addition of 0.1 ml 7% $HClO_4$ and 200 µg bovine serum albumin. After sitting in ice for 10 min, the acid-insoluble material was sedimented by centrifugation for 10 min at 3000 X g. An aliquot of the supernatant solution was counted with aqueous scintillation fluid (4 g of BBOT per liter of solvent containing toluene: methyl cellosolve, 2:1) in an Intertechnique scintillation counter. {BBOT is 2,5-bis[2-(5-tert-butyl-benzoxazolyl)] thiophene.} Reaction mixtures lacking enzyme were used as controls.

For examination of the product of the reaction, reaction mixtures as described above were set up using 0.15 µmol [^{3}H]poly(A) and amounts of enzyme that catalyzed a 20 and 80% hydrolysis of the poly(A). The acid-soluble supernatant solutions obtained were neutralized with KOH, and the $KClO_4$ was removed by centrifugation. Aliquots of the supernatant solutions were applied to sheets of Whatman 3MM paper and chromatographed with two solvent systems. The first was isopropanol:NH_4OH:0.1 M boric acid, 70:10:30, and was used to separate 5' AMP from 3' AMP. The second was N-propanol:NH_4OH:H_2O, 55:10:35, and was used to look for possible oligonucleotide intermediates. After the chromatography the labeled material was identified by counting of strips.

[^{3}H]Poly(A) was prepared using polynucleotide phosphorylase Type 15 (Micrococcus luteus) from P-L Biochemicals, Inc. The procedure of Singer and O'Brien (9) was used. The final product had a specific activity of 8 X 10^5 cpm/µmol. [^{3}H](pA)$_3$[^{14}C](pA)$_n$ was prepared using [^{3}H](pA)$_3$, obtained by hydrolysis of [^{3}H]poly(A) with an Azotobacter nuclease (11), as a primer with primer-dependent polynucleotide phosphorylase (P-L Biochemicals, Inc.), according to the procedure of Klee and Singer (10). The product was isolated by phenol extraction and extensive dialysis. It was then passed through a Sephadex G-75 column to collect a product of more definite size. The final product had an average chain length of 150, and its specific activity was as follows: ^{14}C, 7.5 X 10^5 cpm/µmol; ^{3}H, 2 X 10^5 cpm/µmol.

The reaction mixture for the dephosphorylation of [^{3}H]poly(A) contained 3.85 µmol poly(A); 0.17 M Tris buffer, pH 8.0; 2 mM $MgCl_2$; and 500 µg (15 units) of E. coli alkaline phosphatase in a final volume of 1.2 ml. The mixture was incubated for 2 h at 37° and then extracted twice using an equal volume of phenol. The final aqueous layer was dialyzed for 18 h at 0° against 10 mM Tris buffer, pH 7.8.

The reaction mixture to incorporate phosphate at the 5' terminus of poly(A) contained 1.1 µmol dephosphorylated poly(A); 50 mM Tris buffer, pH 7.5; 10 mM dithiothreitol; 2.5 mM $MgCl_2$; 40 µM [γ-^{32}P]ATP (8 X 10^5 cpm); and 1.15 µg (17.5 units) of polynucleotide kinase in a final volume of 0.6 ml. The reaction was assayed by measuring the incorporation of ^{32}P into acid-insoluble material and stopped after 2 h at 37° by the addition of an equal volume of phenol. After two phenol extractions, the aqueous layer was dialyzed for 48 h at 0° against 10 mM Tris buffer, pH 7.8. The final product had incorporated one 5' phosphate per 200 nucleotides.

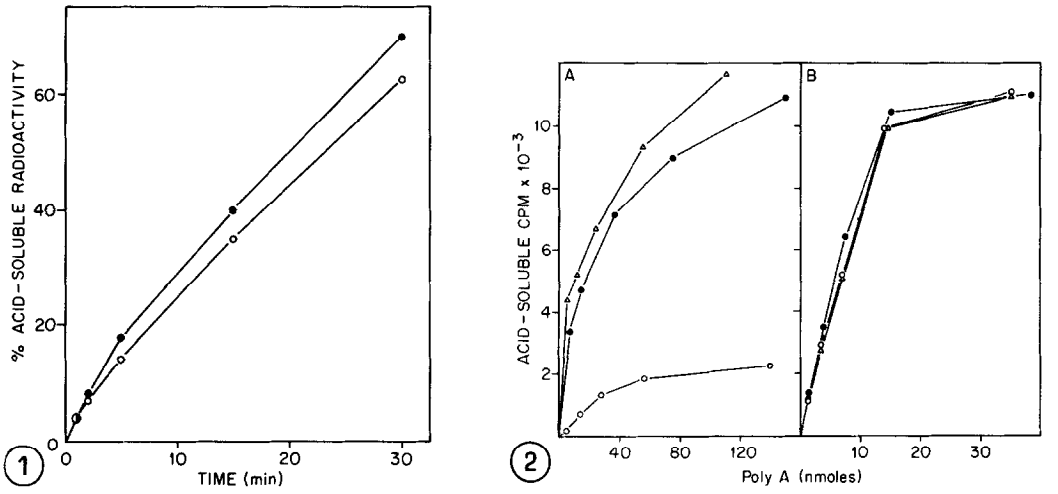

Fig. 1. Hydrolysis of $[^3H](pA)_3[^{14}C](pA)_n$ by the exoribonuclease. 0.018 μmol $[^3H](pA)_3[^{14}C](pA)_n$ was used as the substrate with 1.24 μg of exoribonuclease in reaction mixtures as described in Materials and Methods. The incubation time was varied as shown and acid-soluble radioactivity was measured. ^{14}C (●——●); ^3H (O——O).

Fig. 2. Hydrolysis of untreated poly(A), dephosphorylated poly(A), and dephosphorylated poly(A) treated with polynucleotide kinase by the exoribonuclease (A) and by snake venom phosphodiesterase (B). The reaction mixtures and assay for exoribonuclease are as described in Materials and Methods with the amount of each poly(A) sample as shown on the abscissa, and 0.62 μg of exoribonuclease. The reaction mixtures of venom phosphodiesterase contained the amount of each poly(A) sample as shown on the abscissa; 33 mM Tris buffer, pH 8.4; 1.5 mM $MgCl_2$; 100 μg of bovine serum albumin; and 0.56 μg of snake venom phosphodiesterase in a final volume of 0.15 ml. After incubating for 30 min at 37°, the assay was carried out as described for the exoribonuclease. Untreated poly(A) (●——●); dephosphorylated poly(A) (O——O); dephosphorylated poly(A) treated with polynucleotide kinase (Δ——Δ).

The globin mRNA and [^{3}H]encephalomyocarditis viral RNA (EMC RNA) were the gifts of Dr. Melvin Stulberg. The globin mRNA was a 9S material which was isolated from rabbit reticulocytes by affinity chromatography and gradient centrifugation. The EMC RNA was a 39S material which was obtained from EMC virus by the procedure of Boime and Aviv (12). It had a specific activity of 3 X 10^7 cpm/μmol. The liver polysomal RNA was the gift of Dr. Kai-Lin Lee. It was a 10-14S material isolated from liver polysomes by affinity chromatography and gradient centrifugation. It was determined to be capped by finding that its translation was inhibited by 7-methyl guanosine using the procedure of Weber et al. (13). The [^{3}H]phage T4 mRNA was a 5-min sample prepared according to the procedure of Bolle et al. (14). It had a specific activity of 7.5 X 10^5 cpm/μmol and contained unlabeled ribosomal RNA and tRNA of E. coli.

E. coli alkaline phosphatase and snake venom phosphodiesterase were obtained from Worthington Biochemicals Corporation. Polynucleotide kinase was from P-L Biochemicals, Inc. Ribosomal RNA from E. coli was obtained from Miles Laboratories, Inc.

Protein was determined by UV absorbance at 280 nm.

The concentration of all polyribonucleotides is expressed as μmol of nucleotide.

RESULTS AND DISCUSSION

The general properties of the exoribonuclease are as follows: Mg^{++} or Mn^{++} is required for the reaction. Mg^{++} stimulates optimally at 1.5 mM. Mn^{++} stimulates optimally at 0.3 mM and at about two-thirds the rate with Mg^{++}. The enzyme has a broad pH optimum between 7 and 10. NH_4Cl stimulates the reaction at pH 9.5 about 100%. The optimal concentration is 50 mM.

5' AMP was identified as the only product of the hydrolysis of poly(A) by chromatography as described above. No oligonucleotide intermediates were detectable using the solvent N-propanol:NH_4OH:H_2O.

In the early experiments of Nossal and Singer (15) with ribonuclease II of E. coli, the hydrolysis of terminally labeled polyribonucleotides was measured; if the terminal label was hydrolyzed at the same rate as that of the molecule as a whole, the results suggested that the hydrolysis was processive. With the exoribonuclease described here, the hydrolysis of $[^3H](pA)_3[^{14}C](pA)_n$ was assayed. The results (Fig. 1) show that the ^{14}C and ^3H labels are released at about the same rate, suggesting that the enzyme acts in a processive manner.

It was observed that alkaline phosphatase inhibited the degradation of poly(A). Further study of the inhibition showed that it was due to the action of alkaline phosphatase on poly(A); after treatment of poly(A) with alkaline phosphatase, its effectiveness as a substrate was reduced. Treatment of the dephosphorylated polymer with polynucleotide kinase restored its substrate activity. These observations indicate that it is the removal and reincorporation of 5' phosphate end groups of the poly(A) that affects the reaction rate. Figure 2A shows substrate concentration curves with untreated poly(A), dephosphorylated poly(A), and the dephosphorylated poly(A) treated with polynucleotide kinase. Upon dephosphorylation, 80% of the substrate activity is lost. The polymer with new 5' phosphate end groups is a slightly better substrate than untreated poly(A) which may be because it has more 5' phosphate termini. For comparison, the hydrolysis of the three polymers was also tested with snake venom phosphodiesterase, which is an exonuclease degrading from the 3' end. The hydrolysis of short oligonucleotides (chain length 2 to 5) by venom phosphodiesterase is favored 5–20-fold by the presence of a 5' phosphate group (16, 17). Longer chains might not be affected by the nature of the 5' terminus and that this is true is shown by the results in Fig. 2B. No difference was found between the three types of long poly(A) substrates.

Since the exoribonuclease was affected by 5' phosphate termini on poly(A), the effect of 5' capping on mRNA was tested. The results using two RNA's with 5' caps are shown in Table I. Each RNA tested was compared with poly(A) at a similar concentration.

Table I. Hydrolysis of different RNA's by the exoribonuclease

Globin mRNA, liver polysomal mRNA, and E. coli ribosomal RNA were not labeled, and their hydrolysis was assayed by measuring acid-soluble absorbancy at 260 nm. The reaction mixtures are described in Materials and Methods with 0.62 μg of exoribonuclease being used for globin mRNA and liver polysomal mRNA and 1.24 μg for E. coli ribosomal RNA. 0.2 ml of the final acid-soluble fraction was diluted to 1 ml and read at 260 nm. Reaction mixtures lacking enzyme were used as controls. Phage T4 mRNA and EMC RNA were ^{3}H-labeled and their hydrolysis was measured as described in Materials and Methods. 0.62 μg of enzyme was used for T4 mRNA and 1.24 μg for EMC RNA.

RNA	Amount (nmol)	Hydrolysis (%)
Globin mRNA	43	<1
Poly(A)	38	24
Liver polysomal mRNA	126	1
Poly(A)	115	12
T4 mRNA	80	8
Poly(A)	77	15
E. coli ribosomal RNA	150	10
Poly(A)	150	14
EMC RNA	27	45
Poly(A)	30	54

The results show that the two capped RNA's (globin and liver polysomal mRNA) are hydrolyzed very slowly by the enzyme. The limit of detection of hydrolysis was about 1%. In contrast, the other RNA's tested (5-min phage T4 mRNA, E. coli ribosomal RNA, and EMC RNA) were all hydrolyzed at greater than 50% of the rate with polyA.

These results suggest that the enzyme may degrade polyribonucleotides from the 5' end. If so, the 5' cap of mRNA and the loss of a 5' phosphate from poly(A) might be expected to inhibit the hydrolysis. However, it is possible that the enzyme hydrolyzes from the 3' end but can also bind to a suitable 5' end, resulting in inhibition of the hydrolysis. In either case, it is an interesting enzyme to consider for the hydrolysis of mRNA. Experiments are in progress, using oligoribonucleotides as substrates, to determine the direction of hydrolysis. The hydrolysis of oligoribonucleotides requires 10–20 times more enzyme than

poly(A), however, and the enzyme may require further purification. Preliminary studies show that $(pA)_4$ is hydrolyzed at least 50 times faster than $(Ap)_3A$.

Studies of Furuichi et al. (18) and Shimotohno et al. (19) have shown that the 5' cap of mRNA protects it from hydrolysis in wheat germ extracts. They suggest that this finding indicates the presence of an enzyme degrading from the 5' end.

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