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A MICROSOMAL EXORIBONUCLEASE FROM RAT LIVER

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Summary

A exoribonuclease has been purified from the microsomes of rat liver. The enzyme had an apparent molecular weight of 80 000—83 000 and produced, via a processive mechanism, 5'-AMP as the only product from poly(A). The degradation was found to proceed in the 3' to 5' direction. The relative rates of breakdown of synthetic polynucleotides by the enzyme under standard conditions were in the order poly(A) ≒ poly(U) > poly(C). In addition to Mg^{2+}, K^+ was required for maximum activity. The enzymic activity was inhibited by *p*-chloromercuribenzoate and poly(G), but not by a rate liver RNAase inhibitor.

The effect of spermine on the brakdown of synthetic polynucleotides by the enzyme has been studied. In the absence of K^+, the breakdown of poly(C) was stimulated and that of poly(A) was stimulated slightly. However, the breakdown of poly(U) was inhibited slightly by spermine.

Introduction

Escherichia coli RNAase II, which liberates ribonucleoside 5'-phosphate, is known to be implicated in the breakdown of mRNA [1—7]. If mRNA of eukaryotic cells is degraded in a manner similar to mRNA degradation in *E. coli*, it may be thought that mRNA is inactivated first by an endonuclease attack and then the degraded mRNA is hydrolyzed by a 5'-exonuclease [8]. In this respect, Kwan [9] has recently purified an 5'-exonuclease from the cytoplasm of HeLa cells. Other exonucleases have been purified from the nuclei of Ehrlich ascites, rat liver and HeLa cells [10,11]. Since the degradation of mRNA probably occurs in the cytoplasm, we have purified 5'-exonuclease from the microsomes of rat liver and examined the properties of this enzyme.

Abbreviation: Pipes, 1,4-piperazine-*N*,*N*'-bis(2-ethanesulfonic acid).

Materials and Methods

Preparation of rat liver microsomes and RNAase inhibitor. Male Wistar rats (200—250 g) were starved for 16 h and exsanguinated without anesthesia. After the livers were perfused with isotonic saline, they were removed quickly, rinsed in Buffer A (200 mM sucrose, 5 mM magnesium acetate, 100 mM NH_4Cl, 6 mM 2-mercaptoethanol and 20 mM Tris-HCl, pH 7.5), and homogenized in 2.5 vols. of the same buffer by an Ultra-Turrax homogenizer. The homogenate was centrifuged for 10 min at 17 000 × *g* and the supernatant fraction collected. Microsomes were prepared by centrifuging the supernatant fraction for 60 min at 100 000 × *g*. The pellet was suspended in Buffer A and the suspension was centrifuged for 10 min at 20 000 × *g* to remove the precipitate. RNAase inhibitor was purified about 1800-fold from the 100 000 × *g* supernatant fraction according to the method of Gribnau et al. [12].

Enzyme assays. The reaction mixture (0.2 ml) for the assay of RNAase activity contained 100 μg poly(A) (Boehringer Mannheim GmbH), 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 1 mM magnesium acetate, 0.5 mM EDTA and enzyme. After incubation of the reaction mixture at 37°C for 30 min, the reaction was terminated by the addition of 0.2 ml 5% $HClO_4$ containing 0.25% uranyl acetate. The mixture was centrifuged, after being cooled in an ice-bath for 30 min, and the resulting supernatant fraction was diluted with 4 vols. H_2O. The acid-soluble nucleotides were measured at 260 nm. One unit of RNAase activity was defined as the amount of enzyme which caused an increase in absorbance at 260 nm of 1.0.

Thin-layer chromatography of hydrolyzed products. Enzymic hydrolysis of a polynucleotide was stopped by heating at 90°C for 1 min. After the reaction mixture (0.2 ml) was concentrated to 20 μl by rotary evaporation, it was applied to a polyethyleneimine-cellulose thin-layer plate (20 × 20 cm, Merck) together with standards. The plate was developed with saturated $(NH_4)_2SO_4$/1 M sodium acetate/isopropanol (80 : 18 : 2, v/v) for 16 h at room temperature. The spots were observed under ultraviolet light.

Estimation of molecular weight of the exoribonuclease. The molecular weight of the exoribonuclease was estimated by gel filtration according to the procedure of Andrews [13]. The exoribonuclease was applied to a Sephadex G-150 column (4.2 × 91 cm) equilibrated and eluted with Buffer B (10% glycerol, 10 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate and 50 mM 2-mercaptoethanol) containing 0.1 M KCl (8-ml fractions) and the exoribonuclease activity was measured. The markers used were collagenase from *Clostridium histolycum*, bovine serum albumin, chymotrypsinogen A, and bovine pancreatic RNAase A. All markers were purchased from Boehringer Mannheim GmbH. SDS-polyacrylamide discontinuous gel electrophoresis was performed by the procedure of Weber and Osborn [14].

Purification of rat liver microsomal exoribonuclease. All purification steps were carried out at 4°C. To the microsomal suspension, 10% Triton X-100 was added to a final concentration of 0.5%. This solution, after being dialyzed against three changes of 5 l Buffer A, was used for the enzyme purification.

Streptomycin treatment and $(NH_4)_2SO_4$ fractionation. Nucleic acids were removed by adding 0.05 ml 10% streptomycin sulfate to each ml of the

dialyzed microsomal fraction. After the precipitate was removed by centrifugation at 20 000 × *g* for 10 min, 0.13 μmol $CdCl_2$/mg protein was added. The precipitate was removed by centrifugation at 20 000 × *g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid; protein precipitated between 25 and 50% saturation of $(NH_4)_2SO_4$ was collected, dissolved in Buffer B and dialyzed against Buffer B.

DEAE-Sephadex A-25 column chromatography (1). This fraction was applied to a column of DEAE-Sephadex A-25 (9 × 7 cm) previously equilibrated with Buffer B. The column was washed with 1 l Buffer B and the enzyme was eluted with 1 l Buffer B/0.2 M KCl (15-ml fractions; flow rate 3 ml/min). The active fractions were concentrated by ultrafiltration.

Sephadex G-200 gel filtration. The enzyme solution was applied to a column of Sephadex G-200 (2.6 × 91 cm), equilibrated with Buffer B/0.1 M KCl. The enzyme was eluted with the same buffer (5-ml fractions; flow rate 0.5 ml/min). The active fractions were concentrated by ultrafiltration and dialyzed against Buffer B/30 mM KCl.

DEAE-Sephadex A-25 column chromatography (2). The enzyme solution was applied to a column of DEAE-Sephadex A-25 (3.7 × 11 cm), equilibrated with Buffer B/30 mM KCl. The column was washed with the same buffer followed by a linear gradient of 0.03—0.35 M KCl in Buffer B. The active fractions were concentrated and dialyzed against Buffer B/30 mM potassium phosphate (pH 7.5).

Hydroxyapatite column chromatography The enzyme solution was applied to a column of hydroxyapatite (3 × 7 cm), equilibrated with Buffer B/30 mM potassium phosphate (pH 7.5). The column was washed with the same buffer and eluted with a linear gradient of 0.03—0.3 M potassium phosphate (pH 7.5) in Buffer B (5-ml fractions). The active fractions were concentrated and dialyzed against Buffer C (10% glycerol, 20 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol).

Results

A typical purification procedure of exoribonuclease is summarized in Table I. The enzyme had been purified 100-fold at this stage. One major and four minor bands were observed by polyacrylamide gel electrophoresis.

TABLE I

PURIFICATION OF RAT LIVER MICROSOMAL EXORIBONUCLEASE

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Microsomes	825	23 045	14 025	0.61
2. Ammonium sulfate	102	2 629	7 917	3.01
3. DEAE-Sephadex A-25 (1)	12	441	3 514	7.97
4. Sephadex G-200	27	193	2 525	13.1
5. DEAE-Sephadex A-25 (2)	5	90	1 838	20.4
6. Hydroxyapatite	2.4	14	874	62.4

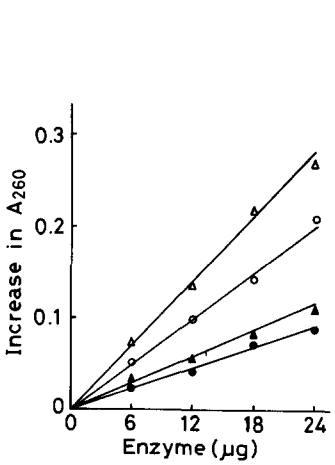
Exonucleolytic cleavage

The hydrolyzed product of poly(A) by the enzyme from rat liver microsomes was identified by a polyethyleneimine-cellulose thin-layer chromatography. The enzyme produced 5′-AMP as the only product. In addition, the amount of acid-soluble nucleotides was nearly equal to the amount of 5′-AMP formed. These results indicate that our purified enzyme is a 5′-exonuclease.

To show that a 3′-OH terminus is required for enzymic activity, the enzyme was assayed with yeast RNA as substrate, in the presence or absence of alkaline phosphatase. The addition of alkaline phosphatase into the reaction mixture stimulated the breakdown of yeast RNA by both our enzyme and the polynucleotide phosphorylase of *Micrococcus luteus* (Fig. 1). The results indicate that the degradation proceeds in the 3′ to 5′ direction.

Processive mechanism

It has been reported that RNA is degraded via a processive mechanism by *E. coli* RNAase II [15] and polynucleotide phosphorylase [16]. The enzymes remain complexed with their polynucleotide substrates during degradation until an individual polynucleotide molecule is completely degraded before the enzyme starts degrading another molecule of substrate. In order to elucidate whether or not the 5′-exonuclease described here degrades poly(A) via a processive mechanism, the average size of the residual polymer substrate was



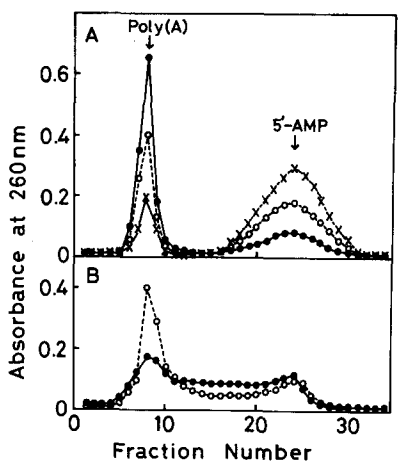


Fig. 1. Effect of alkaline phosphatase on the activities of 5′-exonuclease and polynucleotide phosphorylase with yeast RNA as substrate. The assays for polynucleotide phosphorylase activity were carried out as described previously [24]. ●———●, 5′-exonuclease; ○———○, 5′-exonuclease plus 2 μg alkaline phosphatase ([1], grade I, Boehringer Mannheim GmbH); ▲———▲, *Micrococcus luteus* polynucleotide phosphorylase (Boehringer Mannheim GmbH); △———△, polynucleotide phosphorylase plus 2 μg alkaline phosphatase.

Fig. 2. Sephadex G-100 chromatography of hydrolyzed products of poly(A) by 5′-exonuclease, venom phosphodiesterase or RNAase M. The reaction mixture (0.2 ml) was layered on a Sephadex G-100 column (1 X 33 cm), and the column was developted with 0.1 M NH_4HCO_3. Fractions of 1.5 ml were collected. A. 5′-exonuclease. ●———●, 19% degradation of poly(A); ○------○, 39% degradation of poly(A); X———X, 70% degradation of poly(A). B. ●———●, 23% degradation of poly(A) by venom phosphodiessterase; ○------○, 23% degradation of poly(A) by RNAase M.

investigated by gel filtration on Sephadex G-100 during the course of poly(A) digestion by rat liver 5′-exonuclease, venom phosphodiesterase or RNAase M (Fig. 2). Regardless of the extent of degradation, 5′-exonuclease digests of poly(A) contained only poly(A) of the same size as the original substrate and AMP. In contrast, venom phosphodiesterase or RNAase M digests of poly(A) contained polynucleotides of intermediate length, as shown by the significant absorbance in the fractions between the original substrate and AMP. These results indicate that 5′-exonuclease degrades poly(A) via a processive mechanism.

Molecular weight

The molecular weight of 5′-exonuclease was 83 000, as estimated by gel filtration and 80 000 as estimated by SDS-polyacrylamide disc gel electrophoresis, if one assumes that the major peak was 5′-exonuclease.

Substrate specificity and ion requirement

Fig. 3 shows the relative rates of hydrolysis of different synthetic polynucleotides and yeast RNA under standard conditions (0.1 M KCl and 1 mM magnesium acetate). The relative rates of breakdown of substrates were in the order poly(A) ≒ poly(U) > poly(C) > yeast RNA. Calf thymus DNA, heat-denatured calf thymus DNA, double-stranded RNA [poly(A) + poly(U)] and poly(G) were not degraded by the enzyme under our experimental conditions.

Mg^{2+} was essential for the enzymic activity, the optimal concentration being 1—2 mM. The further addition of Mg^{2+} gradually inhibited the enzymic activity (data not shown). The enzymic activity varied greatly in its K^+ requirement depending on the substrate used (Fig. 4A). When poly(C) was used as substrate, the optimal concentration of K^+ was 25 mM, while it was 100 mM using poly(A) as substrate. When poly(U) was used as substrate, the enzymic activity was not affected by K^+ significantly. Recently it has been reported that poly-

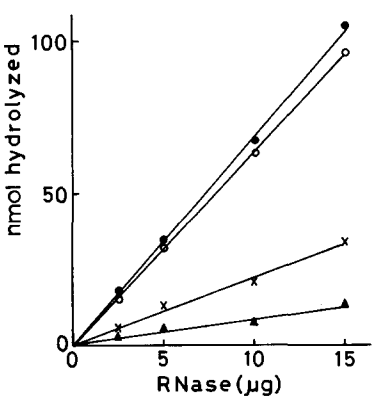


Fig. 3. Activity of 5′-exonuclease against various substrates. The assays were carried out under standard conditions, except that substrate was changed as specified in the figure. ●, poly(A); ○, poly(U); ×, poly(C); ▲, yeast RNA. The products of hydrolyses were calculated according to the following molecular extinction coefficients: 5′-AMP, 14 200; 5′-CMP, 6200; 5′-UMP, 10 000; 5′-GMP, 11 800. In the case of yeast RNA, it is postulated that RNA contains equal amounts of each nucleotide.

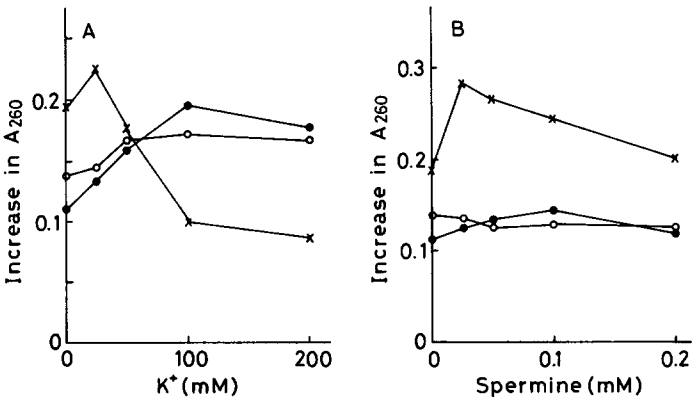


Fig. 4. Effect of K^+ (A) and spermine (B) on the activity of 5′-exoribonuclease. The assays were carried out under standard conditions, except that the concentration of K^+ (A) or spermine (B) was changed as specified in the figure. ●, poly(A) and 0.2 unit of 5′-exoribonuclease; ○, poly(U) and 0.2 unit of 5′-exoribonuclease; X, poly(C) and 1 unit of 5′-exoribonuclease.

amines can stimulate the activity of RNAases and alter their base specificity [17—26]. Therefore, the effect of spermine on 5′-exoribonuclease activity using various substrates was examined. In the absence of K^+, the breakdown of poly(C) was stimulated by spermine, and that of poly(A) was stimulated slightly by spermine (Fig. 4B). However, the breakdown of poly(U) was inhibited slightly by spermine (Fig. 4B). In the presence of 100 mM K^+, spermine did not influence the enzymic activity significantly.

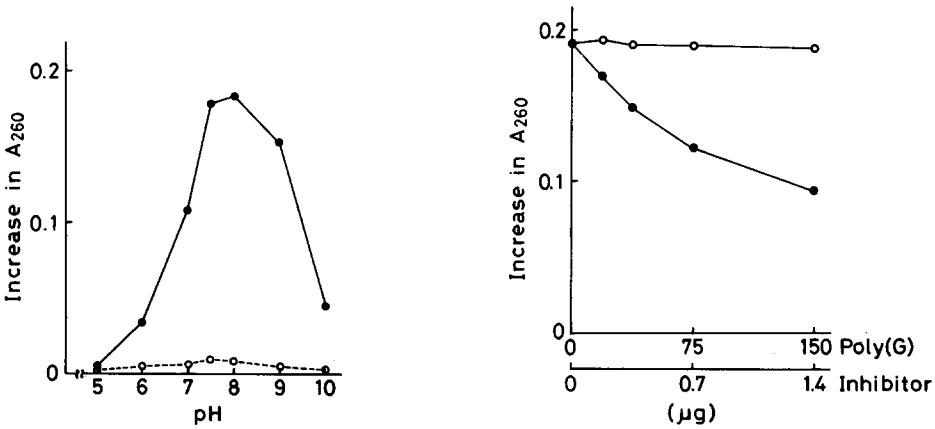


Fig. 5. Effect of pH and *p*-chloromercuribenzoate on the activity of 5′-exoribonuclease. The assays were carried out under standard conditions, except that the pH was changed as specified in the figure. The buffers used were 50 mM potassium acetate buffer (pH 5.0), 50 mM Pipes-NaOH buffer (pH 6.0 and 7.0), 50 mM Tris-HCl buffer (pH 7.5, 8.0 and 9.0) and 50 mM glycine-NaOH buffer (pH 10.0). In the assays, 0.2 unit of 5′-exoribonuclease was used. ●———●, no *p*-chloromercuribenzoate; ○- - - - -○, 0.1 mM *p*-chloromercuribenzoate.

Fig. 6. Effect of poly(G) or rat liver RNAase inhibitor on the activity of 5′-exoribonuclease. The assays were carried out under standard conditions, except that poly(G) (●) or rat liver RNAase inhibitor (○) was added to the reaction mixture as specified in the figure. In the assays, 0.2 unit of 5′-exoribonuclease was used.

Other properties

The enzymic activity was inhibited by *p*-chloromercuribenzoate and the optimal pH was 7.5—8 (Fig. 5). Although poly(G) could inhibit the enzymic activity, rat liver RNAase inhibitor did not inhibit the enzyme (Fig. 6).

Discussion

The data presented show that rat liver 5′-exoribonuclease has properties similar to *E. coli* RNAase II in respect to degradation mechanism (processive mechanism) [15], substrate specificity [27], molecular weight [28] and Mg^{2+} requirement [27]. However, the stimulation of the enzymic activity by K^+ and polyamines was less than the stimulation of *E. coli* RNAase II by these cations [23].

The properties of rat liver 5′-exoribonuclease were also similar to a cytoplasmic 5′-exoribonuclease from HeLa cells, except that the molecular weight of the former seems to be smaller than that of the latter [9].

The rat liver 5′-exoribonuclease was found in both the 100 000 × *g* supernatant and microsome fractions, but not in the mitochondrial fraction (data not shown). Since the 5′-exoribonuclease activity per mg protein in the microsomal fraction was 5 times higher than that in the 100 000 × *g* supernatant fraction, we have first purified 5′-exoribonuclease from the microsomal fraction. It is of interest to learn whether the 5′-exoribonuclease from the microsomal fraction is the same enzyme as that from the 100 000 × *g* supernatant fraction.

It is known that the half life of mRNA in eukaryotes is much longer than that in prokaryotes. Although our *E. coli* RNAase II [23] and rat liver 5′-exoribonuclease are not homogeneous, the specific activity of the *E. coli* RNAase II was roughly 20—50 times higher than that of the rat liver 5′-exoribonuclease, when poly(A) was used as a substrate. A similar relationship seems to be observed between 5′-exoribonuclease from HeLa cells [9] and *E. coli* RNAase II purified by Gupta et al. [28]. The specific activity of 5′-exoribonuclease may be related to the stability of mRNA.

The degradation mechanism of globin mRNA was studied in eukaryotic cells (Gotoh, S., personal communication). Globin mRNA was found to be degraded into small molecules directly without any intermediates. This suggests that eukaryotic mRNA is also degraded processively.

When one considers the properties and location of rat liver 5′-exoribonuclease, there is a possibility that the enzyme may be implicated in the breakdown of rat liver mRNA. Further studies are necessary to elucidate the above points.

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References

1 Sphar, P.F. and Schlessinger, D. (1963) J. Biol. Chem. 238, PC 2251—PC 2253
2 Sphar, P.F. (1964) J. Biol. Chem. 239, 3716—3726
3 Singer, M.F. and Tolbert, G. (1964) Science 145, 593—595
4 Futai, M., Anraku, Y. and Mizuno, D. (1966) Biochim. Biophys. Acta 119, 373—384
5 Holmes, R.K. and Singer, M.F. (1971) Biochem. Biophys. Res. Commun. 44, 837—843
6 Bothvell, A.L.M. and Apirion, D. (1971) Biochem. Biophys. Res. Commun. 44, 844—851
7 Lenette, E.T., Gorelic, L. and Apirion, D. (1971) Proc. Natl. Acad. Sci. U.S. 68, 3140—3144
8 Datta, A.K. and Niyogi, S.K. (1976) Prog. Nucleic Acid Res. Mol. Biol. 17, 271—308
9 Kwan, C.N. (1977) Biochim. Biophys. Acta 479, 322—331
10 Lazarus, H.M. and Spron, M. (1967) Proc. Natl. Acad. Sci. U.S. 57, 1386—1393
11 Kwan, C.N., Gotoh, S. and Schlessinger, D. (1974) Biochim. Biophys. Acta 349, 428—441
12 Grinau, A.A.M., Schoemakers, J.G.G. and Bloemendal, H. (1969) Arch. Biochem. Biophys. 130, 48—52
13 Andrews, P. (1964) Biochem. J. 91, 222—233
14 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
15 Nossal, N.G. and Singer, M.F. (1968) J. Biol. Chem. 243, 913—922
16 Klee, C.B. and Singer, M.F. (1968) J. Biol. Chem. 243, 923—927
17 Levy, C.C., Mitch, W.E. and Schmukler, M. (1973) J. Biol. Chem. 248, 5712—5719
18 Schmukler, M., Jewett, F.B. and Levy, C.C. (1975) J. Biol. Chem. 250, 2206—2212
19 Igarashi, K., Kumagai, H., Watanabe, Y., Toyoda, N. and Hirose, S. (1975) Biochem. Biophys. Res. Commun. 67, 1070—1077
20 Yanagawa, H., Ogawa, Y. and Egami, F. (1976) J. Biochem. (Tokyo) 80, 891—893
21 Akagi, K., Murai, K., Hirao, N. and Yamanaka, M. (1976) Biochim. Biophys. Acta 442, 368—378
22 Frank, J.F. and Levy, C.C. (1976) J. Biol. Chem. 251, 5745—5751
23 Kumagai, H., Igarashi, K., Yoshikawa, M. and Hirose, S. (1977) J. Biochem. (Tokyo) 81, 381—388
24 Igarashi, K., Kumagai, H., Oguchi, H. and Hirose, S. (1977) J. Biochem. (Tokyo) 81, 389—394
25 Igarashi, K., Watanabe, Y., Kumagai, H., Ishizaki, N. and Hirose, S. (1977) J. Biochem. (Tokyo) 81, 579—585
26 Hirose, S., Kumagai, H., Yoshikawa, M., Mikami, T. and Igarashi, K. (1977) J. Biochem. (Tokyo) 82, 1605—1612
27 Singer, M.F. and Tolbert, G. (1965) Biochemistry 4, 1319—1330
28 Gupta, R.S., Kasai, T. and Schlessinger, D. (1977) J. Biol. Chem. 252, 8945—8949
29 Irie, M. (1967) J. Biochem. (Tokyo) 62, 509—518