A NOVEL NUCLEASE FROM MITOCHONDRIA OF RAT LIVER

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SUMMARY: Alkaline RNase partially purified from rat liver mitochondria hydrolyzes both RNA and denatured DNA. The behaviors of RNase activity of the nuclease are closely similar to those of the DNase activity. The nuclease has a pH optimum between 9.0 and 9.5, and the activity is absolutely dependent on Mg^{2+} and reversibly inhibited by p-hydroxymercuribenzoate.

INTRODUCTION

In rat liver, three types of RNase activity are present in different distributions; acid RNase (pH optimum 5-5.5) is localized in lysosomes, neutral RNase (pH optimum 7-8) has a diffuse distribution, and alkaline RNase (pH optimum 9-9.5) is localized in mitochondria (1-6). Alkaline RNase is far more labile than the other two RNases, and shows activation by Mg^{2+}, inhibition by EDTA and susceptibility to inhibition by monovalent cations (1,4). There is no report on the purification and further characterizations of alkaline RNase. We have partially purified alkaline RNase from mitochondria of rat liver and found that the enzyme is a novel nuclease hydrolyzing both RNA and denatured DNA.

MATERIALS AND METHODS

Materials. Yeast RNA from Sigma (type VI) was purified by the method of Wojnal and Roth (7). Calf thymus DNA (Sigma type V) was denatured by heating at 95° for 10 min. Bovine pancreatic RNase was obtained from Worthington. Sephadex G-75 and G-25 were purchased from Pharmacia, phosphocellulose (P 11) from Whatman, and hydroxyapatite from Seikagaku-Kogyo (Tokyo). All other reagents were of analytical grade.

Preparation of Mitochondria. Livers from Wistar male rats were homogenized in 6 volumes of 0.25 M sucrose containing 1 mM EDTA in a glass-Teflon homogenizer. The homogenate was centrifuged at 1,000 x g for 5 min. The supernatant solution was centrifuged at 8,000 x g for 20 min. The resulting pellet was suspended in 3 volumes of 0.25 M sucrose containing 1 mM EDTA and the suspension was centrifuged at 8,000 x g for 20 min. The washing was repeated three times. The operations described above were carried out at 0°-4°. The mitochondrial fraction was stored at -70°.

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Purification of Nuclease. The frozen mitochondria (9.6 g by wet weight) were ground in a mortar with levigated alumina (29 g) at -5° to -10°. The following operations were carried out at 0°-4°. To the mortar was added 150 ml of Buffer A (0.05 M Tris-HCl, pH 7.5, 0.5 mM dithiothreitol and 0.1 mM EDTA) containing 0.5 M KCl. After mixing for 20 min, the mixture was centrifuged at 16,000 x g for 20 min. The mitochondrial extract was fractionated with ammonium sulfate (30% to 50% saturation). The resulting precipitate was dissolved in 10 mM KPi, pH 7.5 containing 0.5 mM dithiothreitol and 0.1 mM EDTA (Buffer B). The solution which had been dialyzed against Buffer B for 18 hr was applied to a phosphocellulose column (2.6 x 12 cm) previously equilibrated with Buffer B, was washed with Buffer B containing 0.05 M KCl, and was eluted with a linear gradient of KCl concentration (0.05 M to 0.5 M in Buffer B). The active fractions were concentrated with ammonium sulfate of 70% saturation. The precipitate was dissolved in Buffer A containing 0.2 M KCl. The solution was applied to a Sephadex G-75 column (1.5 x 30 cm) previously equilibrated with Buffer A containing 0.2 M KCl, and eluted with the same buffer. The active fractions were passed through a Sephadex G-25 column previously equilibrated with Buffer B. The protein fraction was applied to a hydroxyapatite column (1 x 3 cm) and eluted with a linear gradient of KPi concentration (10 mM to 0.2 M). The purified enzyme solution was stored at 0°. Before use, the enzyme solution was passed through a Sephadex G-25 column previously equilibrated with 0.02 M Tris-HCl, pH7.5.

Assay of Nuclease. Reaction mixture for RNase activity (0.5 ml) contained 25 μmoles of Tris-HCl, pH 9.0, 0.5 μmole of $MgCl_2$, 1 μmole of dithiothreitol, 1 mg of RNA, 0.1 mg of bovine serum albumin and enzyme. The mixture was incubated at 37° for 20 min and then chilled in ice bath. To the reaction mixture 0.5 ml of cold acid-ethanol (76% ethanol in 1 N HCl) was added. After 20 min at 0°, the precipitate was removed by centrifugation at 5,000 x g for 10 min at 4°. The supernatant was diluted with 3 to 5 ml of water and the absorbance was measured at 260 nm. DNase activity was measured by the method described above except for the use of 0.25 mg of denatured DNA instead of RNA and the use of 3 ml of 5% perchloric acid instead of acid ethanol and water. One unit of activity was defined as the amount giving an increase in absorbance of 1.0 per min in the reaction mixture.

Product Analysis. RNA (9 mg) was incubated at 27° for 18 hr with the purified enzyme (50 μg) in the reaction mixture scaled up to 10-fold. The product analysis was performed according to the method of Bishayee and Maitra (8). The reaction products were applied to a DEAE-cellulose column (1 x 27 cm) and eluted with a linear gradient between 150 ml of 0.02 M Tris-HCl, pH 7.5 containing 7 M urea and 150 ml of 0.02 M Tris-HCl, pH 7.5 containing 7 M urea and 0.4 M NaCl. Fractions of 1.7 ml were collected and the absorbance at 260 nm was measured.

RESULTS

From the mitochondrial extract, alkaline RNase activity was purified 400-fold with a yield of 6%. Since the purified enzyme hydrolyzed not only RNA but also denatured DNA at the similar rates (Table 1), nuclease is preferable to RNase as enzyme name. Table 1 shows the properties of the nuclease. Both activities of RNase and DNase absolutely required Mg^{2+} which was partly replaced by Mn^{2+}. The activities were inhibited by p-hydroxymercuribenzoate and the inhibition was removed by the addition of excess dithiothreitol. N-Ethylmaleimide also inhibited the activities (data not shown). These results

Table 1. Catalytic Properties of Nuclease from Mitochondria

Reaction mixture[a]	RNase activity (unit)	DNase activity (unit)
Complete	0.40	0.33
minus Mg^{2+}	0.03	0.03
minus Mg^{2+}, plus Mn^{2+} (1 mM)	0.20	0.19
minus dithiothreitol	0.36	0.32
minus dithiothreitol, plus pHMB[b] (0.5 mM)	0.04	0.02
minus dithiothreitol, plus pHMB, plus dithiothreitol (2 mM)	0.43	0.33
plus natural inhibitor[c] (2 units)	0.38	0.32
plus natural inhibitor (4 units)	0.37	0.32
minus denatured DNA, plus native DNA (0.25 mg)	—	< 0.01

[a] The reaction mixture contained 3 μg of the purified enzyme.

[b] pHMB=p-hydroxymercuribenzoate.

[c] Natural inhibitor of neutral RNase was partially purified from 10^5 x g supernatant solution of rat liver homogenate (4). One unit of the inhibitor was defined as the amount inhibiting 50% of the activity of 5 ng of pancreatic RNase in the assay mixture described in MATERIALS AND METHODS.

indicate that the nuclease contains SH group(s) essential to the activity. Native DNA was not attacked by the nuclease. Neither RNase activity nor DNase activity was inhibited by natural inhibitor of neutral RNase, even when the nuclease activity was measured at pH 8.5*.

The pH-dependency profile of RNase activity shows a resemblance to that of DNase activity (Fig. 1). For both activities, pH optima were at 9.0 to 9.5.

*Bartholeyns et al. (4,9) reported that natural inhibitor is effective for alkaline RNase in the large granule fraction of rat liver at pH 8.5 but not at pH 9.1 and that the difference is attributed to the destruction of the inhibitor at alkaline pH (10). When dithiothreitol was added to the reaction mixture (see MATERIALS AND METHODS), natural inhibitor was fully active at alkaline pH (cf. 10). Therefore, the inhibition of alkaline RNase activity at pH 8.5 is probably due to the presence of neutral RNase in the large granule fraction of rat liver (6,9).

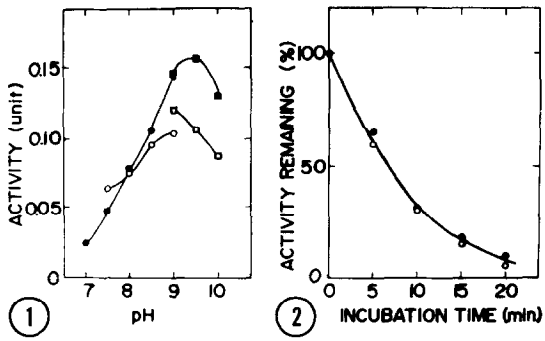

Fig. 1. Effect of pH on nuclease activity. The reaction mixture contained 1.2 μg of the purified enzyme, and the buffer used were 0.05 M Tris-HCl (pH 7-9) (●,○) and 0.05 M Tris-glycine (pH 9-10) (■,□). (●,■), RNase activity; (○,□), DNase activity.

Fig. 2. Heat inactivation of nuclease. The purified nuclease (40 μg) was heated at 47.5° in 1 ml of 0.05 M Tris-HCl, pH 7.5 containing 0.5 mM dithiothreitol and 0.1 mM EDTA. Aliquots of 0.1 ml were withdrawn at the indicated times and quickly chilled in ice bath for the assay of RNase activity (●) and DNase activity (○).

The activities of RNase and DNase decreased identically by the incubation at 47.5° with half-lives of 6 min (Fig. 2). Two activities similarly responded to changes in concentration of Mg^{2+}, KCl, Pi and spermidine (Fig. 3). The optimum concentration of Mg^{2+} was 0.2-0.5 mM for RNase activity and 0.2 mM for DNase activity. Apparent Ki values of RNase activity for KCl, Pi and spermidine were 60 mM, 20 mM and 1 mM, respectively. Similar values were obtained for DNase activity.

The reaction products formed after exhaustive digestion of RNA by the nuclease were analyzed by DEAE-cellulose chromatography in 7 M urea. The products were mainly 4-8 nucleotides in length and about 15% of the total products were mono-, di-, and tri-nucleotides (data not shown). These results indicate that RNA was endonucleolytically cleavaged by the nuclease.

DISCUSSION

Mitochondria of rat liver contain polyadenylase (pH optimum 7.5), alkaline DNase (pH optimum 7.6) and alkaline RNase (pH optimum 9.1)(5). The alkaline RNase purified from mitochondria of rat liver hydrolyzed denatured DNA as well as RNA. Furthermore, closely similar behaviors of RNase activity and DNase

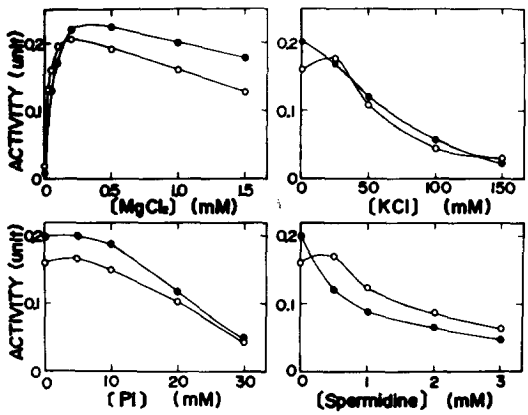

Fig. 3. Effects of Mg^{2+}, KCl, Pi and spermidine on nuclease activity. The reaction mixture contained 1.4 μg of the purified enzyme. (●), RNase activity; (○), DNase activity.

activity were found not only in catalytic nature but also in column chromatography of Sephadex G-75 and hydroxyapatite (data not shown). Therefore, the alkaline RNase seems to be a nuclease which endonucleolytically hydrolyzes both RNA and denatured DNA in a single protein. It is noteworthy that the nuclease is a SH-enzyme requiring Mg^{2+} for the activity. Previously, Curtis et al. (11, 12) reported that a nuclease partially purified from mitochondrial fraction has a pH optimum at 6.6 to 6.8 for DNase activity and a pH optimum at 6.8 to 7.0 for RNase activity. As discussed by Bartholeyns et al. (4), the nuclease preparation probably includes at least two different enzymes.

The physiological role of the alkaline nuclease and the regulation of the activity in mitochondria remain unknown. Tsukada et al. (13,14) indicated that the level of natural inhibitor of RNase in rat liver or in serum increases after partial hepatectomy. These results suggest that the changes in neutral RNase activity in liver or in serum are regulated by the changes in level of its inhibitor. The natural inhibitor was ineffective for the alkaline nuclease (Table 1), and the level of alkaline RNase activity measured in liver homogenate (4) did not change after partial hepatectomy (unpublished results).

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