Vol. 84, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 29, 1978 Pages 313-321

TWO RIBONUCLEASE H ACTIVITIES FROM RAT LIVER NUCLEI

Yasuko Sawai, Mieko Unno and Kinji Tsukada

Department of Pathological Biochemistry, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, 101, Japan

Received August 1,1978

Summary: Two distinct, Mn^{2+}- and Mg^{2+}-dependent enzymes with ribonuclease H (RNase H) activities, which degrade RNA-DNA hybrid have been purified from rat liver nuclei. These two enzymes were eluted at 0.16 M and 0.37 M of potassium chloride in phosphocellulose chromatography, respectively, and further purified by blue Sepharose. They are distinguished from one another by their ionic requirement, molecular weight, sedimentation coefficient, optimal pH, sensitivity to the -SH reagent and mode of cleavage.

The existence of multiple species of ribonuclease H (RNase H) has been demonstrated in a wide variety of eukaryotic cells and tissues (1-4). The nature of multiplicity as well as the actual cellular localization and the function of enzyme activities remained to be elucidated. In the course of our studies on the purification of RNase H from rat liver nuclei, we noticed that in the presence of Mg^{2+} or Mn^{2+}, fresh nuclear extract showed similar activity to degrade RNA moiety of RNA-DNA hybrid structure, but the partially purified enzyme using Mg^{2+} as an ion had little activity in the presence of Mn^{2+}. These observations suggested to us that Mn^{2+}-dependent RNase H might exist in rat liver nuclei. Same observation was demonstrated in RNase H from calf thymus extract by Büsen and Hausen (4), although they could not succeed in separating two enzyme activities completely.

0006-291X/78/0842-0313$01.00/0

In this report, we describe the isolation of two distinct, Mg^{2+}- and Mn^{2+}-dependent RNase H located in rat liver nuclei, and also the evidences that these enzymes could be distinguished on the basis of differences in sedimentation and chromatographic behavior as well as optimal pH, sensitivity to *p*-chloromercuribenzoate and mode of attack to substrate.

MATERIALS AND METHODS

Male Wistar rats, weighing 120-150 g obtained locally were used.

Unlabeled deoxyribo- and ribonucleoside triphosphates, *p*-chloromercuribenzoate and ApA were the products of the Sigma Chemical Company. Crystalline bovine serum albumin, beef liver catalase, cytochrome C, *E. coli* RNA polymerase, polynucleotide kinase, pancreatic RNase, calf thymus DNA, poly(dT), poly(A) and lactate dehydrogenase were purchased from Boehringer Mannheim GmbH. [^{3}H] UTP (23 Ci/mmol), [^{3}H] ATP (15 Ci/mmol), [^{3}H] TTP (5 Ci/mmol) and [γ-^{32}P] ATP (10 Ci/mmol), were obtained from the Radiochemical Center. Sephadex G-150, phosphocellulose (P11), DEAE-cellulose (DE52) and blue Sepharose CL-6B were obtained from Pharmacia Fine Chemicals. All other compounds were of the highest purity available from commercial sources.

Radioactively labelled DNA-RNA hybrid was synthesized on denatured calf thymus DNA using *E. coli* RNA polymerase and [^{3}H] UTP as described previously (5), and [^{3}H] poly(A) poly(dT) was prepared according to the similar method of Sarngadharan *et al* (6). The preparation of rat liver nuclei was employed by the same methods as described previously (7). 5 g (wet weight) of nuclei were suspended in 20 ml of 0.15 M NaCl containing 5 mM 2-mercaptoethanol, and stirred gently with a magnetic stirrer for 20 min at 0°C. The suspension was centrifuged at 8,000 X *g* for 10 min to obtain nuclear extracts. The nuclear extracts were dialyzed against 5 mM potassium phosphate buffer (pH 7.7), 5 mM 2-mercaptoethanol and 10% glycerol (Buffer A) for 5 hr.

The assay of RNase H was based on the release, into acid soluble fraction of radioactive material from [^{3}H]-RNA hybridized to DNA as described previously (7).

Protein was determined by the method of Lowry *et al* (8).

RESULT

Purification of Mg^{2+}- and Mn^{2+}-dependent RNase H

Dialyzed nuclear extracts (20 ml) were applied to a phosphocellulose column (1.6 X 20 cm) that was equilibrated previously with Buffer A. The column was washed with Buffer A to remove the unadsorbed protein. The adsorbed protein was then eluted with a 200 ml linear gradient of Buffer A containing KCl from 0 to 0.8 M. Fractions

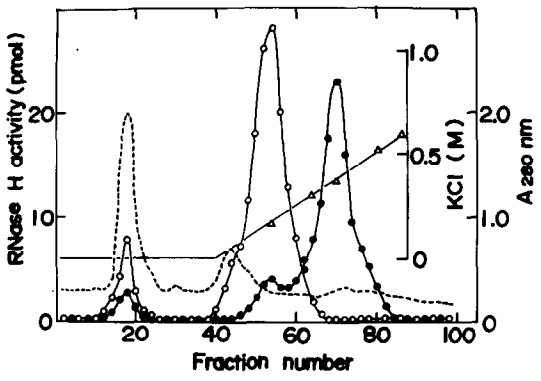


Fig. 1. Phosphocellulose column chromatography of RNase H from rat liver nuclei. The dialyzed nuclear extracts (20 ml) were loaded onto the column which was equilibrated with Buffer A, and eluted as described under "RESULTS". Aliquots (0.02 ml) of each fraction were assayed for RNase H. The reaction mixture contained, in a final vol. of 0.5 ml, 100 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$ or 0.4 mM $MnCl_2$, 2 mM 2-mercaptoethanol, 50 mM NaCl, [^{3}H] RNA-DNA hybrid (10,000 cpm/70 pmol. of [^{3}H]-labeled nucleotide), 50 µg bovine serum albumin. After 15 min incubation at 25°C the reaction was stopped by the addition of 0.1 ml 0.1 M EDTA (pH 7.0), 0.4 mg bovine serum albumin and 0.5 ml cold 10% trichloroacetic acid. The reaction mixtures were centrifuged, the supernatants were collected and counted in Triton X-100/toluene scintillation fluid. ○, Mn^{2+}-dependent RNase H; ●, Mg^{2+}-dependent RNase H; -----, protein as A280; △, potassium chloride concentration.

(3.8 ml) were collected. As shown in Fig. 1, two major peaks of Mn^{2+}- and Mg^{2+}-dependent RNase H activities were eluted at 0.16 M and 0.37 M potassium chloride from phosphocellulose column. A small amount of RNase H activity which was recovered in the flow through and buffer wash fraction was not further examined. Fractions of the two enzyme activities separated on phosphocellulose were pooled and dialyzed separately for 4 hr against Buffer B (20 mM Tris-HCl, pH 7.2, 2 mM 2-mercaptoethanol and 10% glycerol). The dialyzed enzyme fractions were then chromatographed on blue Sepharose columns (1.4 X 6 cm) previously equilibrated with Buffer B. The columns were washed with Buffer B. The adsorbed protein was eluted with 100 ml of linear gradient of Buffer B containing KCl from 0 to 0.8 M. Both Mn^{2+}- and Mg^{2+}-dependent RNase H were eluted at 0.44 M KCl.

The fractions corresponding to RNase H activities were pooled and concentrated with an Amicon Ultrafiltration apparatus with Um-10 membrane, and stored at -80°C in Buffer C (20 mM Tris-HCl, pH 7.7, 2 mM 2-mercaptoethanol and 10% glycerol) containing 0.5 mg/ml of crystalline bovine serum albumin for further analysis. Mg^{2+}- and Mn^{2+}-dependent RNase H were purified about 70 and 50 fold, respectively, with respect to the nuclear extracts. When examined for DNase, RNase, DNA polymerase, alkaline phosphatase and RNA polymerase activities, the most purified preparations were found to be free of them.

Properties of partially purified enzymes.

The concentrated proteins containing RNase H were filtered in a Sephadex G-150 column (1.5 X 48 cm) equilibrated with a 20 mM Tris-HCl buffer (pH 7.5), 2 mM 2-mercaptoethanol, 10% glycerol and 0.5 M KCl, and 1.5 ml of fractions were collected. The RNase H activity appears in a single peak. On the basis of molecular weight markers, we estimated the molecular weight of Mg^{2+}-dependent RNase H as approximately 35,000 and that of Mn^{2+}-dependent RNase H as approximately 150,000. As shown in Table I, the sedimentation coefficients of the two enzymes, estimated in 5 to 20% sucrose gradient in the presence of 0.3 M KCl, are 3.0 S and 5.4 S for Mg^{2+}- and Mn^{2+}-dependent RNase H, respectively. Mg^{2+}-dependent RNase H exhibits maximal activity at pH 9.0, whereas Mn^{2+}-dependent RNase H is most active at pH 8.0.

As shown in Fig. 2, Mg^{2+}-dependent enzyme requires 10 to 15 mM Mg^{2+} for maximal activity, whereas Mn^{2+}-dependent enzyme is maximally active at the Mn^{2+} concentration of 0.4 mM, and has some activity toward Mg^{2+}. Mg^{2+}-dependent RNase H in the absence of 2-mercaptoethanol is inhibited almost completely by *p*-chloromercuribenzoate at 0.2 mM, and this inhibition was reversed by adding 10 mM 2-

Table I

Summary of Properties of Purified RNase H from Rat Liver Nuclei

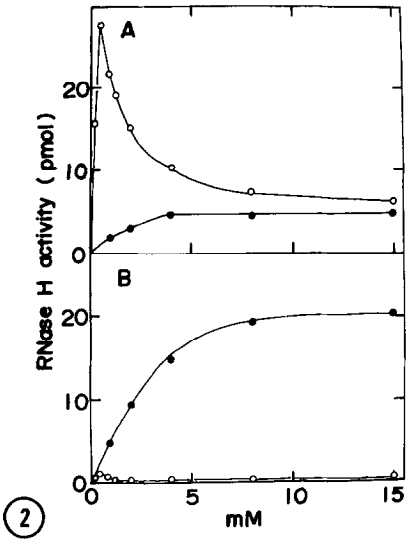
Properties	RNase H Mg^{2+}-dependent	RNase H Mn^{2+}-dependent
Molecular weight (gel filtration)	35,000	150,000
Sedimentation velocity[a]	3.0 S	5.4 S
Conditions for maximal activity		
pH optimum	9.0	8.0
Metal optimum (mM)	10-15	0.4
	RNase H activity (nmol/mg protein)	
Native substrate		
Complete	40	31
Omit 2-mercaptoethanol	6	28
Plus *p*-chloromercuribenzoate (0.2 mM)	<1	27
Plus *p*-chloromercuribenzoate (0.2 mM) Plus 2-mercaptoethanol (10 mM)	39	30
Denatured substrate		
Complete	<1	12

[a]Sedimentation coefficients were estimated in a 5 to 20% (V/V) sucrose gradient containing 0.05 M Tris-HCl (pH 8.0), 0.5 mM EDTA, 3 mM 2-mercaptoethanol and 0.3 M KCl without glycerol. Sedimentation was carried out in a 50.1 rotor at 38,000 rpm for 17 hr. Protein molecular weight markers, bovine liver catalase ($S_{20,w}$=11.3), lactate dehydrogenase ($S_{20,w}$=7) and bovine serum albumin ($S_{20,w}$=4.4) were sedimented simultaneusly in parallel tubes.

mercaptoethanol. *p*-chloromercuribenzoate has no effect on the activity of Mn^{2+}-dependent RNase H (Table I).

Mg^{2+}-dependent RNase H is highly specific for RNA complexed to DNA, whereas Mn^{2+}-dependent RNase H shows the weak degradation of denatured hybrid (Table I).

The reaction products formed after digestion of homopolymeric hybrid, [^{3}H] poly(A) poly(dT) as substrate by RNase H were analyzed



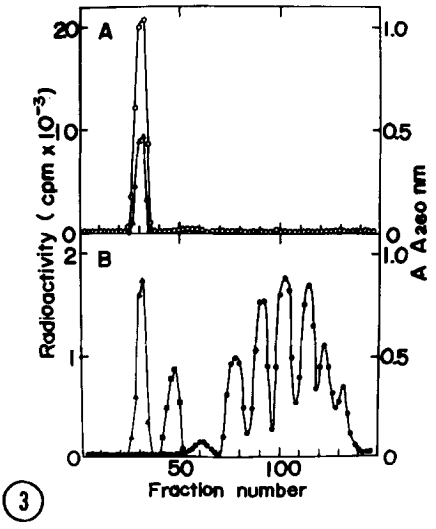


Fig. 2. Influence of the concentration of divalent metal ions on RNase H activity. The reaction was studied under same assay conditions described in Fig. 1 except varying amounts of Mn^{2+} or Mg^{2+} as shown. A; Mn^{2+}-dependent RNase H, 4 µg of protein was used. B; Mg^{2+}-dependent RNase H, 3 µg of protein was used. ●, $MgCl_2$; ○, $MnCl_2$.

Fig. 3. Separation of reaction products on DEAE-cellulose in 8 M urea. The standard reaction mixtures containing [^{3}H] poly(A) poly (dT) (2 X 10^5 cpm) as substrate were incubated with Mn^{2+}-dependent RNase H (A) and Mg^{2+}-dependent RNase H(B) respectively at 37°C until all of the radioactivities were acid soluble. The reactions were stopped by the addition of 0.05 ml of denatured salmon sperm DNA (1 mg per ml) and perchloric acid to final concentration of 0.5 M. After centrifugation, the supernatants containing acid-soluble material were collected, and neutralized with 1 N KOH. The resulting residues in ice were removed by centrifugation. The products of a parallel reaction with 20 nmol of unlabelled poly(A) and poly(dT) were added as carrier. The resulting products, AMP (0.25 µmol in A, 0.5 µmol in B) and [^{32}P] pApA (15,000 cpm) obtained by polynucleotide kinase and [γ-^{32}P] ATP (9) were adjusted to 0.02 M Tris-HCl (pH 7.5), and 8 M urea (Buffer C) and applied on a DEAE-cellulose column (1.0 X 30 cm) equilibrated with Buffer C at room temperature. The adsorbed oligonucleotides were eluted with a 300 ml linear NaCl gradient (0 to 0.45 M) in Buffer C. Fractions of 2 ml were collected, and 1 ml of each was counted in 10 ml of toluene-Triton scintillation fluid. ▲, Absorbance at 260 nm; ■, [^{32}P]-radioactivity; ○, ●, [^{3}H]-radioactivity.

by DEAE-cellulose chromatography in 8 M urea. As shown in Fig. 3, several oligonucleotides of various chain lengths were eluted from the column, when Mg^{2+}-dependent RNase H was used. The product, [^{32}P]

pApA after treatment of ApA and [γ-^{32}P] ATP with polynucleotide kinase (9) was eluted between 40 and 50 tubes from the column. Little radioactivity was detected in the area of AMP and pApA after digestion of [^{3}H] poly(A) poly(dT). Snake venom phosphodiesterase completely digested the oligonucleotides product to 5'-AMP. These results indicated that the oligonucleotides product contained 3'-hydroxyl and 5'-phosphate termini and possessed the structure $(pA)_{3-9}$, and that [^{3}H] poly(A) was endonucleolytically cleavaged by Mg^{2+}-dependent RNase H. On the other hand, breakdown products by Mn^{2+}-dependent RNase H were isolated by the same chromatography. As shown in Fig. 3, only one peak corresponding to 5'-AMP was eluted from the column. Further proof for the identification of the product as 5'-AMP, not 2'(3')-AMP was provided by chromatography on Dowex 1-formate (10).

Discussion

These observations have clearly shown that two distinct RNase H activities; Mg^{2+}- and Mn^{2+}-dependent enzymes are present in rat liver nuclei. The two enzymes are distinguishable by physical and biochemical criteria as shown in Table I. Multiple forms of RNase H have also been demonstrated in other eukaryotic cells (1-4). although the physiological function of its presence in multiple forms is unknown, Büsen and Hausen (4) described the occurrence of three enzymes with RNase H activities in whole extracts form calf thymus, one of these acts optimally in the presence of Mn^{2+} ions, another requires Mg^{2+} ions for optimal activity. A slight separation of these two enzymes was occasionally observed on ion-exchange chromatography. The third enzyme acts in the presence of both Mn^{2+} and Mg^{2+}, and degrades double strand RNA and poly(rA) as well as RNA-DNA hybrids. In rat liver cytosol, two RNase H activities were reported by Sekeris and Roewekamp (1), and also we did separate 3 types of RNase H

activities from the cytosol by phosphocellulose chromatography (unpublished observation).

In our experiments, Mn^{2+}-dependent RNase H activity behaves like an exonuclease which produces a 5'-phosphate mononucleotides, and this enzyme is essentially resistant to 0.2 mM *p*-chloromercuribenzoate, whereas the Mg^{2+}-dependent RNase H cheavage RNA-DNA hybrid endonucleolytically, and shows sensitivity to *p*-chloromercuribenzoate.

The precise function of RNase H activity and the significance of its occurrence in distinct forms in the cell remained still to be clarified. There is some evidence that RNase H may be integrated in the DNA replication in animal cells (7, 11, 12), while other evidence has presented a possible role in the process of transcription (11, 13-15). It may be possible that in DNA replication the role of RNase H is to selectively remove initiating RNA strands to elongate the DNA chain by DNA polymerase and DNA ligase.

Acknowledgements; This work was supported in part by a Grand-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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