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Purification of Chicken Liver Ribonucleases by Affinity Chromatography with UMP-Sepharose (Nucleosides and Nucleotides. LII)[1)]

KAZUNOBU MIURA,* YASUHIKO INOUE, YUKARI HASHIMOTO, ATSUKO INOUE, and TOHRU UEDA

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12,Nishi-6, Kita-ku, Sapporo 060, Japan

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A procedure for the preparation of chicken liver ribonucleases (RNases) is described, involving affinity chromatography on Sepharose coupled with 5′-amino-5′-deoxyuridine 2′(3′)-phosphate.

Two kinds of RNases having acidic pH optima were obtained. One RNase exhibited preferential nucleolytic activity toward poly C rather than poly U and the other was specific for poly U. The former RNase may be identical to that reported by Levy *et al.*[2)] and the latter resembles acid RNase detected in several mammalian tissues.

Keywords——chicken liver RNase; poly C-selective RNase; poly U-selective RNase; UMP-Sepharose; affinity chromatography

Base-specific ribonucleases (RNase T_1, U_2 and A) have been used for the sequence analysis of ribonucleic acids (RNAs). While RNases T_1 and U_2 can specifically recognize guanylate and adenylate, respectively, RNase A cleaves both cytidylate and uridylate. RNases specific to either uridylate or cytidylate should be useful for the sequence analysis of RNAs. Recently, Levy *et al.* have purified from chicken liver an RNase (RNase CL) having preferential affinity to cytidylate.[2)] This enzyme was used for the sequence analysis of RNAs.[3)]

In the course of our studies on chemical modifications of tRNAs and 5S rRNA this cytidylate-specific RNase seemed to be useful. We attempted, therefore, to purify RNase CL from chicken liver by means of affinity chromatography. 5′-(*p*-Aminophenylphosphoryl)-uridine 2′(3′)-phosphate-coupled Sepharose has been reported as a strong specific adsorbent of pancreatic RNase A.[4)]

In this paper we report the preparation of 5′-amino-5′-deoxyuridine 2′(3′)-phosphate-coupled Sepharose (UMP-Sepharose) and its utilization as an affinity adsorbent for the purification of RNases from chicken liver. We obtained two kinds of RNases, of which one was identical with RNase CL, and the other had a different base-specificity.

Materials and Methods

Materials——Chicken livers used were from White Leghorn chickens. Homopolyribonucleotides (poly C, poly U, poly A and poly I) were from Yamasa Shoyu Co., Ltd. RNase A, bovine serum albumin and yeast RNA were from Sigma Chemical Co. DEAE-Sepharose, CM-Sephadex, Blue-agarose, Sephacryl S-200 and Sepharose 4B were purchased from Pharmacia Fine Chemical Inc. Yeast RNA was purified as described by Crestfield *et al.*[5)]

Synthesis of 5′-Amino-5′-deoxyuridine 2′(3′)-phosphate——5′-Azido-5′-deoxyuridine[6)] (1.6 g, 6 mmol) was added to a solution (100 ml) of anhydrous dimethylformamide containing polyphosphoric acid (29.4 mmol as H_3PO_4) and tri-*n*-butylamine (2.8 ml, 11.8 mmol), and the mixture was kept for 3 h at 130 °C.[7)] The mixture was concentrated *in vacuo* and the residue was dissolved in water and subjected to ether extraction to remove excess tri-*n*-butylamine. The aqueous layer was evaporated and the residue was dissolved in water (200 ml). The solution was loaded on a DEAE-Sephadex A-25 column (2.6 × 40 cm, HCO_3^- form) and the product, 5′-azido-5′-deoxyuridine 2′,3′-cyclic phos-

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phate, was eluted with a linear gradient of 0—0.1 M triethylammonium bicarbonate (pH 8.0, 1 l of H_2O and 1 l of 0.1 M triethylammonium bicarbonate). The combined fraction containing the product was evaporated and the residue was dissolved in 0.2 N NaOH. This solution was kept at 100 °C for 2 h to afford 5′-azido-5′-deoxyuridine 2′(3′)-phosphate. The reduction of the azido group to an amino group was carried out in H_2S–saturated 50% pyridine in water (50 ml) at room temperature overnight with stirring.[8] Finally, the product was purified by DEAE-Sephadex A-25 column chromatography (column size; 1.9 × 36 cm, elution; a linear gradient of 0—0.3 M triethylammonium bicarbonate). The yield was 19000 optical density (OD) units (260 nm). Paper electrophoresis (0.05 M triethylammonium bicarbonate (pH 8.0), 700 V, 60 min); $R_{UMP}=0.8$. Ninhydrin test; positive (violet).

Coupling of 5′-Amino-5′-deoxyuridine 2′(3′)-phosphate to Sepharose 4B——The nucleotide (3000 OD units at 260 nm) was added to a suspension of BrCN–activated Sepharose 4B in 0.1 M $NaHCO_3$ and the mixture was kept at 4 °C for 24 h. The resin was packed in a column and washed extensively with 0.1 M $NaHCO_3$. The column was equilibrated with 0.5 M ethanolamine–HCl (pH 7.5), kept at room temperature for 4—5 h, washed with 2 M NaCl (300 ml) and water (500 ml), and then equilibrated with 50 mM acetate buffer (pH 4.5). The quantity of nucleotide coupled to Sepharose was estimated as 0.02 mmol of UMP per 1 ml of the resin by measurement of ultraviolet (UV) absorbance of the alkaline hydrolysate of UMP-Sepharose.

Preparation of RNases from Chicken Livers——The preparation was carried out in a cold room. Minced chicken livers were homogenized in cold 0.25 N H_2SO_4 in a Waring blender (1 g of liver in 1 ml of 0.25 N H_2SO_4). After centrifugation at 8000 rpm for 30 min, the supernatant was neutralized and centrifuged at 110000 *g* for 1 h in a Beckman SW 27 rotor. A highspeed supernatant was dialyzed extensively against 20 mM Tris-HCl (pH 7.5)–1 mM ethylenediaminetetra acetic acid (EDTA) and subjected to DEAE-Sepharose column chromatography. Elution was performed with a linear gradient of NaCl (0—1.0 M) in 20 mM Tris-HCl (pH 7.5)–1 mM EDTA. Two peaks showing RNase activity were obtained and appropriate fractions were combined and dialyzed against 10 mM phosphate buffer (pH 6.0).

The two RNase fractions (RNase CL-I and RNase CL-II) were subjected to CM-Sephadex column chromatography. Elution was performed with a linear gradient of NaCl or KCl in buffer. The combined RNase fraction was dialyzed and concentrated by adsorption-elution on carboxymethyl (CM)-cellulose.

Further column chromatography of the RNase CL-I preparation was performed on Sephacryl S-200. Elution was performed with 0.5 M NaCl–50 mM Tris-HCl (pH 7.2). The RNase CL-II preparation was subjected to Blue-agarose column chromatography. Elution was performed with a linear gradient of NaCl (0—0.6 M) in 10 mM Tris-HCl (pH 6.5).

The RNase CL-I and RNase CL-II fractions were each dialyzed against 50 mM acetate buffer (pH 4.8) and then loaded on a UMP-Sepharose column (0.9 × 12 cm). The column was washed with acetate buffer, and elution was performed with a linear gradient of NaCl (0—1.0 M). The fractions containing RNase were combined and concentrated by means of a Mini-module concentrater. Final preparations of RNases were stored at −20 °C.

Assay of RNase Activity[9]——An appropriate volume of enzyme preparation was added to an assay mixture containing 10 mM KH_2PO_4 (pH 6.5), 0.2% gelatin and a substrate (yeast RNA or homopolyribonucleotides; final concentration, 0.02%). The mixture was incubated at 37 °C for 15 min, then cold 1 N HCl–75% ethanol was added and the whole was kept in ice for 30 min. Then, the activity was estimated by measurement of the UV absorbance at 260 nm of the supernatant obtained by centrifugation of the mixture at 3000 rpm for 20 min. When poly C was used as a substate, the UV absorbance was measured at 280 nm.

Results and Discussion

Preparation of UMP-Sepharose

5′-Amino-5′-deoxyuridine 2′(3′)-phosphate was synthesized by 2′,3′-cyclic phosphorylation[7] of 5′-azido-5′-deoxyuridine followed by hydrolysis and subsequent reduction of the azido group with H_2S–pyridine.[8] The coupling of the nucleotide to Sepharose was carried out by a conventional method using BrCN–activated Sepharose 4B. The quantity of nucleotide coupled to Sepharose 4B was 20 μmol per 1 ml of Sepharose. The advantage of 5′-amino-5′-deoxyuridine 2′(3′)-phosphate-coupled Sepharose (UMP-Sepharose) is the stability of the linkage against phosphodiesterases or nucleases which might be present in the crude RNase preparation. UMP-Sepharose does not have a phosphodiester linkage between the 5′-position and the Sepharose.

The capability of specific adsorption was tested with pancreatic RNase A and bovine serum albumin. As expected, RNase A was strongly adsorbed but albumin was not retarded in the UMP-Sepharose column (data not shown). Thus, affinity chromatography using UMP-Sepharose should be useful for the purification of other RNases which have affinity to 2′(3′)-

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uridylate.

Preparation of RNases from Chicken Livers

The homogenization of chicken livers was performed in 0.25 N H_2SO_4 and the homogenate was centrifuged at 8000 rpm for 30 min. The supernatant was neutralized and centrifuged at 110000 *g*. This crude extract was subjected to DEAE-Sepharose column

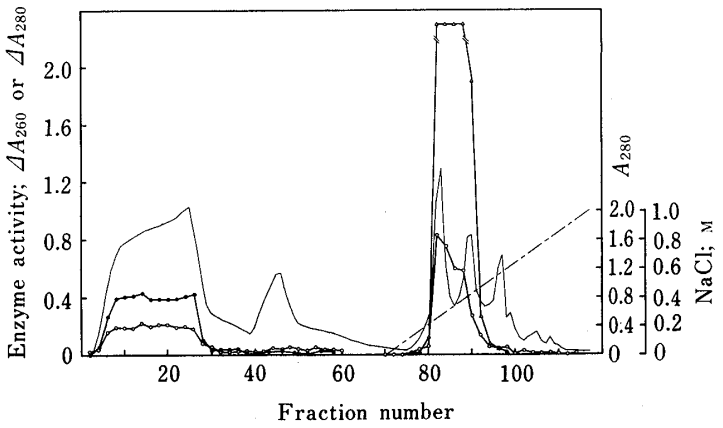

Fig. 1. Chromatography on DEAE-Sepharose of the H_2SO_4 Extract of Chicken Livers

High-speed supernatant (400 ml) was loaded on a 2.6×21 cm column of DEAE-Sepharose equilibrated with 20 mM Tris-HCl (pH 7.5)–1 mM EDTA. The column was washed with the same buffer, then linear gradient elution was started at tube No. 70 (20 ml/tube). The continuous line indicates the absorption at 280 nm. Open circles, closed circles and triangles indicate the activities for the hydrolysis of yeast RNA, poly C and poly U, respectively. Fractions No. 5—28 and 81—92 were combined as RNase CL-I fraction and RNase CL-II fraction, respectively.

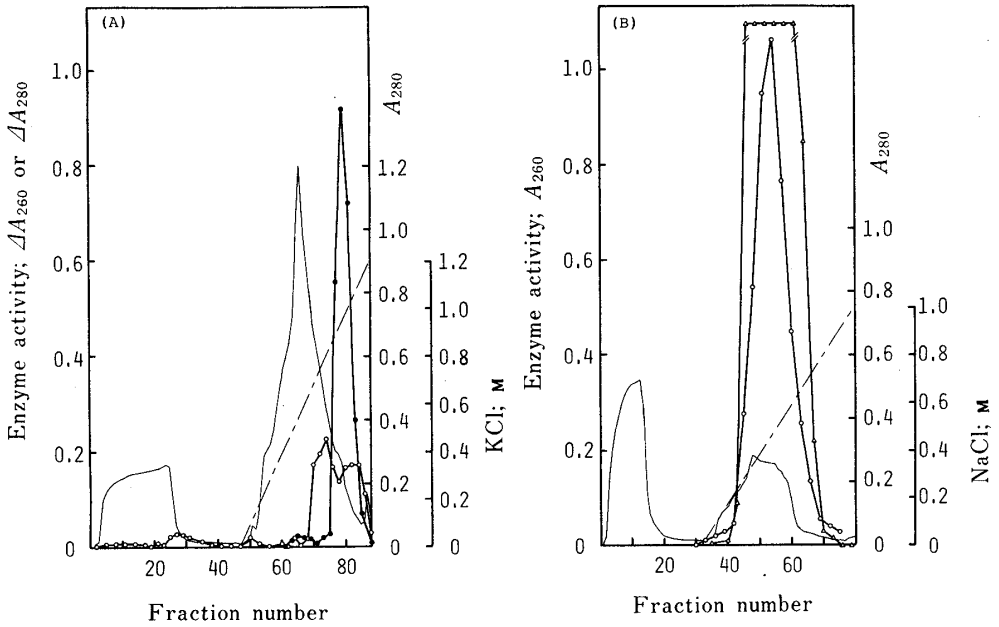

Fig. 2. Chromatography on CM-Sephadex of RNases CL-I and CL-II

(A) RNase CL-I (480 ml) was loaded on a CM-Sephadex column (1.8×32 cm) equilibrated with 10 mM KH_2PO_4 (pH 6.0) and elution was performed with a linear gradient of KCl (one fraction; 20 ml). (B) RNase CL-II (200 ml) was applied to a CM-Sephadex column (1.8×32 cm) equilibrated with 50 mM sodium acetate (pH 5.3) and elution was performed with a linear gradient of NaCl (one fraction; 20 ml). The continuous line indicates the absorption at 280 nm. Open circles, closed circles and triangles indicate the activities for the hydrolysis of yeast RNA, poly C and poly U, respectively.

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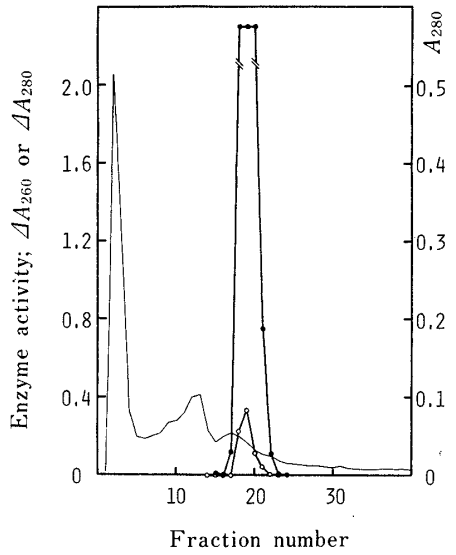

Fig. 3. Gel Filtration on Sephacryl S-200 of RNase CL-I

The column (2.6×123 cm) was equilibrated with 0.5 M NaCl–50 mM Tris-HCl (pH 7.2) and elution was performed with the same solution (one fraction; 20 ml). The continuous line indicates the absorption at 280 nm. Open circles and closed circles indicate the activities for the hydrolysis of yeast RNA and poly C, respectively.

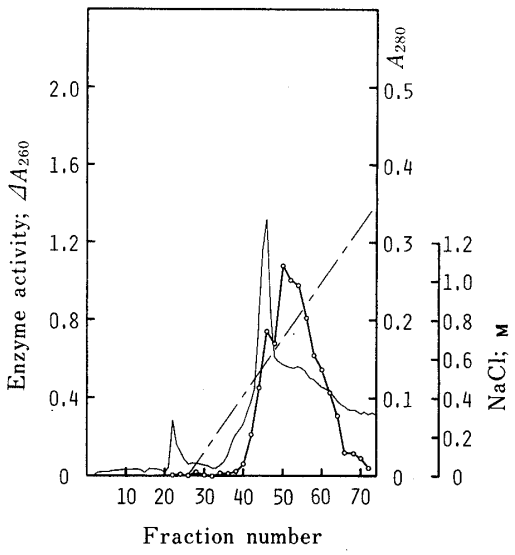

Fig. 4. Blue-Agarose Column Chromatography of RNase CL-II

The column (1.5×8 cm) was equilibrated with 10 mM Tris-HCl (pH 6.7) and elution was performed with a linear gradient of NaCl (one fraction; 4 ml). The continuous line and open circles indicate the absorption at 280 nm and the activity for the hydrolysis of yeast RNA, respectively.

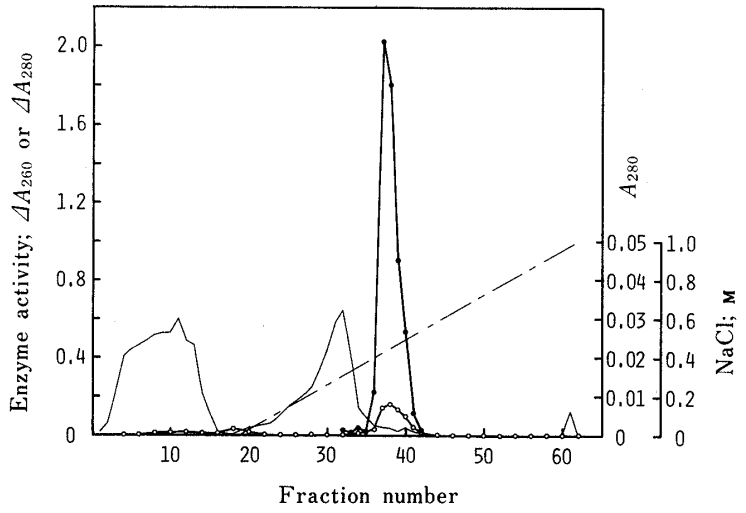

Fig. 5. Affinity Chromatography on UMP-Sepharose of RNase CL-I

RNase CL-I (116 ml) was loaded on a column (0.6×12 cm) equilibrated with 50 mM sodium acetate buffer (pH 4.8). The column was washed with the same acetate buffer, then RNase was eluted with a linear gradient of NaCl in the buffer (pH 4.8). Fractions of 20 ml per tube were collected during loading and washing, and the linear gradient eluate was collected in fractions of 4 ml per tube. The continuous line indicates the absorption at 280 nm. Open circles and closed circles indicate the activities for the hydrolysis of yeast RNA and poly C, respectively.

chromatography. As shown in Fig. 1, the RNase activity was separated into two fractions; one appeared in the passed-through fraction and the other in the linear gardient eluate. These two RNase activities could be distinguished by assay using poly C and poly U as substrates. RNase CL-I cleaved poly C preferentially and poly U slowly, while RNase CL-II hydrolyzed poly U selectively and did not hydrolyze poly C (Fig. 1).

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TABLE I. Purification of RNase CL-I[a]

Step	Protein (mg)	Activity[b] (unit)	Specific activity (unit/mg)	Fold	Recovery (%)
1. H_2SO_4 extract	2511	12470	5	1	100
2. DEAE-Sepharose	835	11020	13	2.6	88
3. CM-Sephadex	67	12450	186	37	99
4. Sephacryl S-200	14	9340	658	132	37
5. UMP-Sepharose	0.48	2500	5208	1041	20

a) The purification was started from 200 g of chicken livers.
b) Activity was determined by assay using poly C as a substrate. One unit corresponds to the activity hydrolyzing one OD unit of a substrate at 37 °C for 15 min.

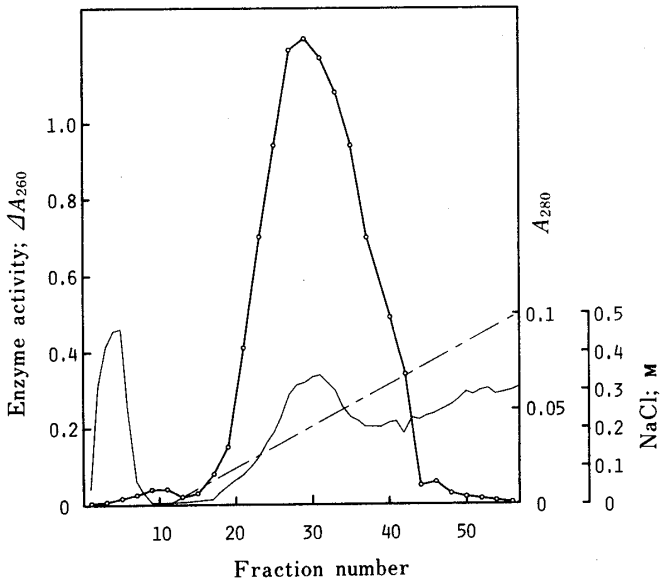

Fig. 6. Affinity Chromatography on UMP-Sepharose of RNase CL-II

RNase CL-II (95 ml) was loaded on a column (0.6 × 12 cm) equilibrated with 10 mM sodium acetate buffer (pH 4.3). Elution was performed as described in the legend to Fig. 5. The continuous line and open circles indicate the absorption at 280 nm and the activity for the hydrolysis of yeast RNA, respectively.

Further purification by CM-Sephadex column chromatography was carried out for both RNases, and subsequently, by Sephacryl S-200 gel filtration for RNase CL-I and by Blue-agarose column chromatography for RNase CL-II (Figs. 2, 3 and 4).

Finally, RNase Cl-I and Cl-II were each subjected to the affinity chromatography. Dialyzed RNase CL-I preparation was applied to a UMP-Sepharose columm and the column was washed extensively with acetate buffer. RNase activity was completely retained in the UMP-Sepharose column and was eluted with a linear gradient of NaCl in acetate buffer (Fig. 5). The combined peak fraction was dialyzed and concentrated to yield the final RNase CL-I preparation. The specific activity of RNase CL-I was increased 1000-fold base on the first tissue extract (Table I). RNase CL-II was similarly adsorbed on UMP-Sepharose and eluted with the salt solution (Fig. 6). As shown in Table II, RNase CL-II was highly purified by the affinity chromatography. The purity of the RNases was examined by polyarylamide gel electrophoresis. RNase CL-I preparation showed a single band and RNase CL-II preparation showed one major band having a molecular weight of about 40000 daltons in addition to several minor bands (data not shown).

Properties of RNase CL-I and RNase CL-II

Some properties of RNase CL-I and RNase CL-II were investigated. The optimal pHs for RNases CL-I and CL-II were 6.5 and 6.0, respectively. The molecular weights were estimated by gel filtration (Sephadex G-50 for RNase CL-I and Sephadex G-100 for RNase

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TABLE II. Purification of RNase CL-II[a]

	Step	Protein (mg)	Activity[b] (unit)	Specific activity (unit/mg)	Fold	Recovery (%)
1.	H_2SO_4 extract	2511	118430	47	1	100
2.	DEAE-Sepharose	358	135500	378	8	114
3.	CM-Sephadex	83	84180	1009	21	72
4.	Blue-agarose	22	74200	3451	73	63
5.	UMP-Sepharose	3	36100	10939	853	35

a) The purification was started from 200 g of chicken livers.
b) Activity was determined by assay using poly U as a substrate. One unit corresponds to the activity hydrolyzing one OD unit of a substrate at 37 °C for 15 min.

TABLE III. Effects of Cations on RNase CL-I and RNase CL-II

Cation	mM	RNase activity (%)[a]	
		RNase CL-I	RNase CL-II
None		100	100
NaCl	10	132	81
	100	111	8
	200	78	7
KCl	10	113	87
	100	112	9
	200	106	6
Mg^{2+}	0.05	136	96
	0.5	150	71
	1.0	127	59
	5.0	97	34
Mn^{2+}	0.05	125	79
	0.5	110	56
	1.0	108	43
	5.0	39	22
Ca^{2+}	0.05	132	98
	0.5	133	81
	1.0	119	70
	5.0	93	38
EDTA	0.05	100	109
	1.0	96	117
	5.0	96	143

a) Activity was determined by assay using yeast RNA as a substrate.

TABLE IV. Rates of Hydrolysis of Various RNAs by RNase CL-I and RNase CL-II

Substrate	Rates of hydrolysis	
	RNase CL-I	RNase CL-II
Yeast RNA	100	100
Poly C	1090	0
Poly U	301	475
Poly A	0	6.5
Poly I	7	2.2

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CL-II) to be 13000 and 41000 for RNase CL-I and RNase CL-II, respectively.

The effects of cations on both RNases are summarized in Table III. RNase CL-II was significantly affected by various cations, and consequently EDTA apparently enhanced the activity of RNase CL-II. However, such inhibitory effects were not observed with RNase CL-I, and the activity was rather enhanced by appropriate amounts of cations.

The base-specificity was investigated by using homopolyribonucleotides as substrates and the results are summarized in Table IV. RNase CL-I showed preferential activity toward poly C and the rate of hydrolysis of poly U was 1/3 of that of poly C. RNase CL-II had strong activity toward poly U but did not hydrolyze poly C. Both RNases showed very low activity for hydrolyses of poly A and poly I.

Analysis of the hydrolyzate of poly U with RNase CL-II showed that the products were a mixture of uridine 2′,3′-cyclic phosphate and short oligonucleotides with terminal 2′,3′-cyclic phosphate.

These two RNase preparations did not show deoxyribonuclease, diesterase or nucleotidase activities since they did not cleave heat-denatured deoxyribonucleic acid, or 5′- or 3′-nucleotides at all.

The properties of RNase CL-I are quite similar to those reported for RNase CL[2] and these enzymes may be indentical. RNases similar to RNase CL-II have been found as acid RNases in several mammalian tissues[10] but there are some differences in various properties, such as the hydrolysis rates of substrates and the molecular weight. Recently, Uchida *et al.* have reported the isolation from hen oviducts of a similar RNase which exhibits nucleolytic activity hydrolyzing poly U and poly I at similar rates.[11] Their enzyme seems to be different from the present one.

Further characterization of RNase CL-II and use of the enzyme for sequencing of RNAs are in progress.

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References

1) Part LI: T. Ueda, S. Shuto, and H. Inoue, *Nucleosides and Nucleotides*, **3**, 173 (1984).
2) C. C. Levy and T. P. Karpetsky, *J. Biol. Chem.*, **255**, 2153 (1980).
3) M. S. Boguski, P. A. Hieter, and C. C. Levy, *J. Biol. Chem.*, **255**, 2160 (1980).
4) M. Wilchek and M. Gorecki, *Eur. J. Biochem.*, **11**, 491 (1969).
5) A. M. Crestfield, K. C. Smith, and F. W. Allen, *J. Biol. Chem.*, **216**, 185 (1955).
6) T. Hata, I. Yamamoto, and M. Sekine, *Chem. Lett.*, **1975**, 977.
7) T. Ueda and I. Kawai, *Chem. Pharm. Bull.*, **18**, 2303 (1970).
8) T. Adachi, Y. Yamada, I. Inoue, and M. Saneyoshi, *Synthesis*, **1977**, 45.
9) J. A. Roth, "Methods in Cancer Research," Vol. III, ed. by H. Busch, Academic Press, New York, 1967, pp. 153—242.
10) H. Sierakowska and D. Shugar, "Progress in Nucleic Acids Research and Molecular Biology," Vol. 20, ed. by W. E. Cohn, Academic Press, New York, 1977, pp. 59—130, and the references therein.
11) T. Uchida, S. Kanetani, and K. Suzuki, *Seikagaku*, **53**, 645 (1981).

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