Base Specificity and Primary Structure of Poly U-preferential Ribonuclease from Chicken Liver†

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The primary structure and base specificity of chicken liver RNase CL₁ which has been reported by Miura et al. [Chem. Pharm. Bull., 32, 4053–4060 (1984)] as poly U-preferential RNase, were extensively studied. The sequence study of this enzyme and comparison of the amino acid sequence of the enzyme with homologous RNases from oyster and Drosophila melanogaster suggested that RNase CL₁ consists of three peptides with 17, 19, and 163 amino acid residues. The amino acid sequence of these three peptides were identified. The two small peptides are joined to the large peptide by disulfide bridges. The amino acid sequence of RNase CL₁ had 62 (31.2%) and 63 residues (31.6%) identical with oyster RNase and D. melanogaster RNase, respectively, and belongs to the RNase T₁ family RNase.

Reassessment of the base specificity of RNase CL₁ found that it is guanylic acid, then uridylic acid-preferential, and not poly U preferential.

Key words: chicken liver; poly U; ribonuclease; primary structure; base specificity

While there are several classes of RNases in living things, pyrimidine base specific RNases with molecular masses of approximately 14 kDa (RNase A family) have been found as major RNase only in vertebrates.1–3) One of the typical RNase of the pyrimidine base specific group is bovine pancreatic RNase A, which prefers cytidylic acid to uridylic acid.4,5) Levy and Karpetzky, however reported the existence of an RNase in chicken liver that could distinguish between cytidylic acid and uridylic acid.6,7) They reported that this poly C-preferential RNase cleaves poly C about 16 fold faster than poly U. On the other hand, Miura et al. purified two RNases from chicken liver and recharacterized them as poly C-preferential and poly U-preferential RNases, respectively.8,9) They showed that both U-preferential RNase hydrolyzes poly U but not poly C, although hydrolysis of a small amount of poly A was observed.

Hayano et al. purified these RNases, analyzed the primary structure of the poly C-preferential RNase (RNase CL₂) and concluded that it was a unique member of the RNase A family10) in terms of the location of the disulfide bridges. Believing that it would also of great interest to characterize "the poly U-preferential" RNase (RNase CL₁), in this paper we studied its primary structure and re-assessed the base preference of this enzyme. The structure identified and base specificity indicated that it is a member of the RNase T₁ family (base non-specific RNase with molecular mass of about 24 kDa).

Materials and Methods

Reagents. Yeast RNA was purchased from Marlin Bio (Tokyo). 3′-Mononucleotides and 2′,3′-cyclic mononucleotides were obtained from Sigma (St. Louis, MO). Molecular mass maker proteins were obtained from Oriental Yeast (Tokyo). The reagents for polycracylamide gel electrophoresis (SDS-PAGE) were purchased from Wako Pure Chemicals (Osaka). A Capcellpak C-18 column for reversed-phase HPLC was obtained from Shimadzu (Tokyo). Superdex 75HR10/30 was a product of Pharmacia LKB Japan (Tokyo).

Enzymes. RNase CL₁ was prepared by the method of Hayano et al.60) Lysylendopeptidase was a product of Seikagaku Kogyo (Tokyo).

Enzyme assay. (a) Estimation of RNase activity by measuring acid-soluble nucleotides with RNA and homopolyribonucleotides as substrates: Enzymatic activity towards RNA and homopolyribonucleotides was measured by following the increase in acid-soluble nucleotides after digestion of substrates at pH 5.0 and 37 C, as described in our previous paper.39) (b) Estimation of RNase activity by measuring hyperchromicity upon cleavage of polynucleotides: The rates of cleavage of RNA and four homopolynucleotides were monitored by measuring hyperchromicity upon cleavage of the (nucleotidyl) linkage in 0.1 M acetic buffer (pH 6.0) at 22 C. The wavelengths used were 300, 260, 250, and 270 nm for RNA, poly A, poly I, poly U, and poly C, respectively. The substrate concentration was 30 μg/ml. The rates are expressed as percentages of maximum hyperchromicity. (c) Dinucleoside phosphates as substrates: The rates of hydrolysis of dinucleoside phosphates were measured by the method of Imaizawa et al.93 and Wittel Barnard.94 Changes in absorbance were monitored with a Shimadzu UV200 spectrophotometer.

Protein concentration. Protein concentrations were measured spectrophotometrically assuming the absorbancy of 0.1% protein solution at 280 nm to be 1.0.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was done in 12% polyacrylamide gel by the method of Laemmli.100 The gel was stained with Coomassie Brilliant Blue.

Amino acid analysis. Amino acid analysis was done with an AccQTag amino acid analysis system (Millipore, Tokyo, Japan). Tryptophan contents were measured spectrophotometrically.11)

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Abbreviations: PTH-, phenylthiohydantoin-; RNase CL₁ and RNase CL₂, poly U- and poly C-preferential RNase from chicken liver, respectively; RCM RNase CL₁, reduced and S-carboxymethylated RNase CL₁.
Hexosamine and neutral sugar. Hexosamine contents were measured with the AccQtag amino acid analysis system after hydrolysis in sulfuric acid at 100°C for 5 h. Neutral sugar content was measured by the phenol-sulfuric acid method of Dubois et al.13

Preparation of RCM RNase CL₁. Reduction and S-carboxymethylation of RNase CL₁ were done as described by Crestfield et al.14 Reduced and carboxymethylated RNase CL₁ (RCM RNase CL₁) was separated from the excess reagents by gel filtration on FPLC Superdex 75HR 10.30 equilibrated with 50 mM trimethylamine-acetate buffer (pH 8.0).

Digestion of peptides with proteases. The protein was digested with mercaptoethanol

without with

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Fig. 1. SDS-PAGE of Purified RNase CL₁ and Reduced and Carboxymethylated RNase CL₁.

The details of the experimental conditions are described in Materials and Methods. Lanes 1, RNase CL₁; lane 3. RCM RNase CL₁; lanes 2 and 4, molecular mass marker proteins. With and without mercaptoethanol indicated slab electrophoresis was performed in the presence and in the absence of mercaptoethanol.

lysylendopeptidase in 100 mM trimethylamine-acetate buffer (pH 8.0). Protease was added at an enzyme-substrate ratio of 1:200 (w/w). The digestion was done at 37°C for 5 h.

BrCN cleavage of RCM RNase CL₁. BrCN cleavage of RCM RNase CL₁ was done by the method of Gross-Witkop.14 The cleavage products were dried in vacuo, then separated according to their solubility in 50 mM trimethylamine-acetate buffer (pH 8.0). The soluble fraction was separated by reversed-phase HPLC on a Capcellpak C18 column (4 x 250 mm) equilibrated with 50 mM trimethylamine-acetate buffer (pH 8.0) with a gradient of acetonitrile (0-40%v) in 128 min.

Results

Primary structure of RNase CL₁

The N-terminal sequence of RNase CL₁ was measured by Edman degradation to identify the primary structure of RNase CL₁. The results showed the presence of three

Fig. 2. Fractionation of RCM RNase CL₁ by FPLC.

Fractionation of RCM RNase CL₁ by FPLC on a Superdex 75HR 10.30 column equilibrated with 50 mM trimethylamine-acetate buffer (pH 8.0). The column was eluted with the same buffer at a flow rate of 1 ml/min. One ml fractions were collected. The last two large peaks are due to the excess reagents.

Fig. 3. Amino Acid Sequence of RCM RNase CL₁.

The alignment of peptides is summarized, →, amino acid sequences found by Edman degradation. K and BR indicate the peptide obtained by lysylendopeptidase digestion and BrCN cleavage of RCM RNase CL₁, respectively. All peptides were numbered from the N-terminus to the C-terminus in the primary structure. RCM RNase CL₁ pep-N and pep-C were numbered using primes and double primes, respectively, e.g., n and ″n″. The lines with vertical bars on both ends indicate the peptides whose position was estimated from their amino acid composition. The site of the carbohydrate junction is indicated by CHO.
peptides (Table III). Reduced and carboxymethylated RNase CL₁ (RCM RNase CL₁) was electrophoresed by SDS–PAGE. RCM RNase CL₁ gave a protein with molecular mass of 26 kDa, slightly smaller than that of the native enzyme (31 kDa) (Fig. 1). Gel filtration of RCM RNase CL₁ on Superdex 75 (Fig. 2) yielded two fractions, a large protein fraction (protein fraction) and a peptide fraction (RCM RNase CL₁ pep-C). The peptide fraction (RCM RNase CL₁ pep-C) was further purified by HPLC under the same conditions as in Fig. 4. The results of Edman degradation indicated that the peptide consisted of 19 amino acid residues, as shown in Fig. 3 and Table I. The N-terminal sequence of the protein fraction obtained from the Superdex was analyzed by Edman degradation. It showed the presence of two sequences shown in Table I, but after exhaustive dialysis against distilled water (RCM RNase CL₁ pro), the N-terminal sequence (DCQ····) was identified up to the 35th residue as shown in Fig. 3. By subtracting these two sequences of RCM RNase CL₁ pro and RCM RNase CL₁ pep-C from the N-terminal sequence of native RNase CL₁, or the sequence of RCM RNase CL₁ pro from the sequence of protein fraction of the Superdex column, we were able to estimate the residual the 3rd sequence (RCM RNase CL₁ pep-N) as EWSKLYLHHWPVTV. RCM RNase CL₁ was digested with lysylendopeptidase, and then separated by HPLC on a Capcellpak C18 column into 16 fractions, as shown in Fig. 4. The sequences and amino acid compositions of the 16 fractions are shown in Fig. 3 and Table II, respectively. The sequences of K3 and K4 were included in the N-terminal sequence of RCM RNase CL₁ pro. The sequences of the C-terminal of K5 and K6 were included in that of K7. The sequence of K10 is also included in that of K9. The sequence of K16 was the same as that of the peptide from RCM RNase CL₁ pep-C. The sequences of K1 and K2 were included in the N-terminal sequence of RCM RNase CL₁ pro. The sequence of the C-terminal of K5 and K6 were included in that of K7. The sequence of K10 is also included in that of K9. The sequence of K16 was the same as that of the peptide from RCM RNase CL₁ pep-C.

### Table I. N-Terminal Amino Acid Residues of RNase CL₁, Protein Fraction Obtained by Gel-filtration (Fig. 2), RCM RNase CL₁ pep-C and RCM RNase CL₁ pro

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Table IIa. Amino Acid Composition of the Peptides Obtained by Lysylendopeptidase Digestion of RCM RNase Cl

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The figures in parentheses were obtained from the sequence.
* About 0.3-0.5 residues of hexosamine were included in the serine residue peak. —, not determined.

Yield, yield of each peptide calculated from RCM RNase Cl.

Table IIb. Amino Acid Composition of Peptides Obtained by Lysylendopeptidase Digestion of RCM RNase Cl

| Asp  | 1.3 (1) | 0.3 (0) | 0.3 (0) | 2.3 (2) |
| Glu  | 2.2 (2) | 7.2 (7) | 3.7 (3) | 3.5 (3) |
| CM-Cys | 1.7 (2) | 0.6 (1) | 0.7 (1) |
| Ser  | 0.8 (1) | 0.4 (0) | 0.8 (1) | 2.4 (2) |
| Gly  | 2.5 (2) | 0.8 (1) | 2.5 (2) | 0.2 (0) |
| His  | 1.2 (1) |
| Arg  | 1.8 (2) | 1.6 (2) | 1.6 (2) | 1.0 (1) |
| Thr  | 3.1 (3) | 0.3 (0) | 1.4 (1) |
| Ala  | 0.9 (1) | 0.8 (1) | 2.2 (2) | 0.7 (1) |
| Pro  | 1.6 (2) | 1.2 (1) | 1.5 (2) |
| Tyr  | 1.2 (1) | 2.7 (3) |
| Val  | 0.8 (1) | 2.2 (2) |
| Met  | 1.1 (1) |
| Ile  | 2.0 (2) | 1.3 (1) | 1.8 (2) |
| Leu  | 0.7 (1) | 2.0 (2) | 1.7 (2) | 1.7 (2) |
| Phe  | 1.4 (1) | 2.4 (2) |
| Lys  | 2.1 (2) | 1.0 (1) | 1.2 (1) | 1.3 (1) |
| Trp  | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |

The figures in parentheses were obtained from the sequence.
* About 0.3-0.5 mol of hexosamine was included in the serine residue peak. —, not determined.

Yield, yield of each peptide calculated from RCM RNase Cl.

Sequence of K1 and K2 were included in the amino acid sequence of the 3rd peptide (RCM RNase Cl pep-N).

To identify the alignment of these lysylendopeptidase peptides further, the RCM RNase Cl fraction was cleaved with BrCN. After elimination of excess reagent, the portion soluble in 50 mm triethylamine-acetate buffer (pH 8.0) was fractionated by HPLC on a Capcellpak C-18 column equilibrated with triethylamine acetate buffer (pH 8.0). The column was eluted with a linear gradient of acetonitrile (0-50%) at a flow rate of 3 ml/min. Three ml fractions were collected.

Fractionation of lysylendopeptidase digest of RCM RNase Cl by reversed-phase HPLC.

Fig. 4. Fractionation of Lysylendopeptidase Digest of RCM RNase Cl by Reversed-phase HPLC.

Fractionation of lysylendopeptidase digest of RCM RNase Cl by reversed phase HPLC on a Cap cellpak C-18 column equilibrated with triethylamine acetate buffer (pH 8.0). The column was eluted with a linear gradient of acetonitrile (0-50%) at a flow rate of 3 ml/min. Three ml fractions were collected.
Table III. Amino Acid Compositions of the Peptides Obtained by BrCN Cleavage of RCM Nase CL₁

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>BR₁*</th>
<th>BR₂*</th>
<th>BR₃</th>
<th>BR₄</th>
<th>BR₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>4.8</td>
<td>3.4</td>
<td>1.3</td>
<td>1.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Glu</td>
<td>4.2</td>
<td>5.4</td>
<td>2.2</td>
<td>11.2</td>
<td>26.3</td>
</tr>
<tr>
<td>CM-Cys</td>
<td>1.5</td>
<td>0.8</td>
<td>2.9</td>
<td>3.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>0.5</td>
<td>4.2</td>
<td>1.4</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Gly</td>
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<td>1.2</td>
<td>3.4</td>
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</tr>
<tr>
<td>His</td>
<td>1.4</td>
<td>2.9</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Arg</td>
<td>1.1</td>
<td>2.6</td>
<td>0.4</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Thr</td>
<td>2.4</td>
<td>3.4</td>
<td>1.2</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Ala</td>
<td>0.6</td>
<td>1.7</td>
<td>1.1</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Pro</td>
<td>3.3</td>
<td>2.2</td>
<td>2.1</td>
<td>3.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Val</td>
<td>1.1</td>
<td>1.7</td>
<td>2.4</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Met</td>
<td>1.2</td>
<td>0.7</td>
<td>1.1</td>
<td>1.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Ile</td>
<td>1.5</td>
<td>1.6</td>
<td>1.3</td>
<td>3.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Leu</td>
<td>2.0</td>
<td>4.5</td>
<td>3.4</td>
<td>4.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Phe</td>
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<td>2.3</td>
<td>1.4</td>
<td>2.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Lys</td>
<td>1.6</td>
<td>2.3</td>
<td>2.2</td>
<td>4.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Total (37) | (48) | (24) | (51) | (19) |
Yield (%)   | 35   | 54   | 45   | 38   | 32   |
Sequence 1-37 | 41.88 | 89-112 | 113-163 | 1"-19" |

The values in parenthesis are those obtained from the sequence.

The hexosamine peak was included in the serine peak. —, not determined.

Yield, yield of each peptide calculated from RCM Nase CL₁.

Fig. 5. Fractionation of 50 mm Tris-Hydroxymethyl-Acetate Buffer (pH 8.0) Soluble Fraction of BrCN Cleavage Products of RCM Nase CL₁ on a Capcellpak C18 Column. The reaction conditions are described in detail in Fig. 4. Fractionation was done on a Capcellpak C-18 column, as described in Fig. 4, except for the use of a final gradient of acetonitrile (40%).

Fig. 6. Comparison of the Amino Acid Composition of RCM Nase CL₁ with That of Typical Rnase T₂ Family Relatives. Rh. Rnase Rh from Rhizopus niveus; T₂, Rnase T₂ from Aspergillus oryzae; M, Rnase M from A. salina; Trv, Rnase Try from Trichoderma viride; Le₂, Rnase Le₂ from Lentinus edulis; Phyb, Rnase Phyb from Physarum polycephalum; L.E, Rnase Le from Lycoperdon exscentrum; MC, Rnase MC from bitter gourd (Momordica charantia); S₂, S-rNase from Nicotiana alata; Oy, Rnase Oy from oyster; Dm, Rnase from D. melanogaster; CL₁, Rnase CL₁.

The numbers at the top of the matrix represent Rnase Rh numbering.
these peptides are produced from the native enzyme as a result of processing by certain proteases and still attached to the protein moiety by disulfide bridges. They were released from RCM RNase CL₁ pro by reduction and carboxymethylation. The N-terminal amino acid sequencing of native RNase showed the presence of three sequences, for K₃ and K₁₆, and one estimated by subtraction of the N-terminal sequence. The last sequence was partially confirmed by lysylendopeptidase digestion. There were one or two unidentified amino acid residues in the amino acid sequences of K₄, K₅, K₁₆, BR₁, and BR₂. Based on the evidence described, we concluded that these positions are occupied by Asn residues. The evidence is that (i) RNase CL₁ is a glycoprotein (Table IV), (ii) the sequence coincides with the consensus sequence for N-glycoside junction in protein, and (iii) these peptides, contain one or two more Asp residues as expected from the sequence. Thus, as described above, the entire sequence of the RNase CL₁ preparation was identified. The amino acid composition of the RNase CL₁ preparation is shown in Table IV. Its composition is very similar to that calculated from the sequence thus far known.

The sequence found indicated that RNase CL₁ has no structural similarity with the RNase CL₂ previously reported,⁴⁶ and higher similarity with oyster RNase¹⁵ (62 amino acid residues) and Drosophila RNase (63 amino acid residues).¹⁶

### Base specificity of RNase CL₁

The base specificity of RNase CL₁ was investigated with homopolynucleotides as substrates by measuring acid-soluble nucleotides formed by incubation with RNase CL₁. The results in Table V show that RNase CL₁ is poly U preferential, as reported by Miura et al.⁵ However, when we measured enzymatic activity by hyperchromicity induced upon incubation of RNA and homopolynucleotides with the enzyme, RNase CL₁ seemed to be poly I, then poly U preferential (Table V). These differences may be attributable to differences in solubility of the oligonucleotides depending on their bases. That is, oligouridylic acid is the most soluble of these oligohomonucleotides, and thus a marked increase in acid soluble oligouridylic acid was observed. The initial velocities of cleavage of several dinucleoside phosphates, UpYs (where Y is A, G, U, or C) were measured (Table VI). The results showed that the rate of hydrolysis was greatest when Y = G. We then examined the rates of hydrolysis of XpGs. The results indicated that GpG was hydrolyzed most rapidly, indicating the guanylic acid preference of RNase CL₁. The results were very similar to those obtained by measuring hyperchromicity.

### Discussion

We compared the sequence of RNase CL₁ with that of the typical RNases of RNase T₂ family as shown in Fig. 6. The sequences around the presumed cleavage sites indicated that 4 or so amino acid residues between the N-terminal peptide (RCM RNase CL₁ pep-N) and the protein moiety (RCM RNase CL₁ pro) are probably missing, but that no amino acid residues, or no more than one, are missing from the C-terminal part (RCM RNase CL₁ pep-C) and the protein moiety (RCM RNase CL₁ pro). The previous studies on RNase Rh¹⁷ (the only one RNase of the RNase T₂ family RNases the three-dimensional structure of which was analyzed¹⁸,¹⁹) showed that (i) His46 and His109 play a major role as a general acid and base catalysts, respectively,²⁰ (ii) His104 is a phosphate binding site,²¹ (iii) Glu105 plays a role in activation by polarizing...
the P=O bond or stabilizing the pentacovalent intermediate,28) (iv) Lys108 also has some role in the activation, possibly by stabilizing the intermediate,29) and that (v) the B1 site (major binding site for the base of nucleotide which is 5'-side of scissile phosphodiester bond) is composed of Tyr57, Trp49, and Asp51.30) The base moity stacks with the Tyr57 and Trp49, and Asp51 forms hydrogen bonds with the adenine base of the substrate. These amino acid residues are concentrated in two common segments of the RNase T2 family RNases, as shown in Fig. 6. All of these amino acid residues except for Tyr57 (RNase Rh numbering) are conserved in RNase CL1. Tyr57 is deleted from RNase CL1. Fungal RNases, which have Asp51, are mostly adenylic acid preferential.17-21) RNases which have amino acid residues other than Asp at the 51st position are guanylic acid-preferential.30)

However, RNase CL1 has Asp51 but is guanylic acid and then uridylic acid preferential, instead of being adenylic acid preferential as observed in the cases of fungal RNases such as RNase Rh and RNase M.17-21) The absence of Tyr57 in RNase CL1 may perturb the role of Asp51. As for the B2 site (base binding site adjacent to the 3'-side of the B1 base), Nakamura et al. showed that the cytosine of RNase Rh-d(ApC) complex stacks with Phe101, and interacts with the side chain of Pro92, Asn94, and Gin95 and that Gin32 may be the lid of the base binding site*1 by X-ray crystallography. In RNase CL1, Phe101 is conserved but the others are not necessarily conserved.

Among RNase T2 family RNases, most fungal RNases contain 10 half-cystine residues. Since RNase CL1 has no free cysteine so far detected with 5,5'-dithiobis(2-nitrobenzoic acid) (unpublished data), RNase CL1 has four of half cystine residues in common with those of the fungal RNases. The locations of eight half-cystine residues of RNase CL1 are the same as those of most plant RNases (although some of them have an odd number of half-cystine residues)23-25) and other animal RNases.15,16) In addition to these finding, the N-terminal part of the RNase CL1 molecule is more similar to those of animal RNases and of plant RNases than to fungal RNases.

The results of this study indicated that, although the acid-soluble method is very useful for estimation of base specificity from a diagnostic viewpoint, it is not useful for comparing of the rates of hydrolysis of different polynucleotides. The base preference of RNase CL1, which is different from those of fungal RNase and plant RNase is probably due to the lack of Tyr57 and the different sequence around the B2 site. The precise relation between base preference and amino acid sequence will be clarified by further protein engineering studies.

The results of this study showed that RNase T2 family RNases, have been demonstrated to range from viruses, bacteria, and plants to lower animals such as the oyster, then to amphibians (bullfrog)31) and even to birds. It is very interesting in terms of the molecular evolution of RNases that RNase T2 RNases are the major RNase in plants and lower animals such as the oyster, but that, in vertebrates such as bullfrog and chicken we observed RNase A family RNases (pyrimidine specific RNases)20,31) in addition to RNase T2 family RNase (base non-specific RNase).

References

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