Studies on Extracellular Ribonucleases of *Ustilago spheerogena*

CHARACTERIZATION OF SUBSTRATE SPECIFICITY WITH SPECIAL REFERENCE TO PURINE-SPECIFIC RIBONUCLEASES

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1. Ribonuclease U_1 splits only the phosphodiester bonds of guanosine 3'-phosphates in RNA. It may be regarded as a guanyloribonuclease [ribonuclease (guanine nucleotide)-2'-transferase (cyclizing), EC 2.7.7.26] similar to ribonuclease T_1 (Egami, Takahashi & Uchida, 1964). It seems to be identical with the extracellular ribonuclease described by Glitz & Dekker (1963, 1964a,b). 2. Ribonucleases U_2 and U_3 are novel enzymes with a strict specificity. They split the internucleotide bonds between purine 3'-nucleotides and 5'-hydroxy groups of adjacent nucleotides in RNA with the intermediary formation of purine nucleoside 2',3'-(cyclic)-phosphates, which are slowly hydrolysed to purine 3'-nucleotides. So they may be classified as 'puryloribonucleases [ribonuclease (purine nucleotide)-2'-transferase (cyclizing)]'. Double-stranded RNA is scarcely split by ribonucleases U_2 and U_3 . 3. Ribonuclease U_4 has no absolute base specificity, and produces the mononucleotides 3'-adenylate, 3'-guanylate, 3'-cytidylate and 3'-uridylylate from RNA.

In the preceding paper (Arima, Uchida & Egami, 1968) the isolation of four ribonucleases, designated ribonucleases U_1 , U_2 , U_3 and U_4 , was reported from culture media of *Ustilago spheerogena*.

In the present paper the mode of action and substrate specificities of these ribonucleases are considered. These enzymes provide new materials for enzyme chemistry and RNA research, and we hope that ribonucleases U_2 and U_3 , novel ribonucleases specific for purine nucleotides, will be of value in the analysis of nucleotide sequences in RNA.

MATERIALS AND METHODS

Materials. The four extracellular ribonucleases from *Ustilago spheerogena* were prepared as described in the preceding paper (Arima *et al.* 1968).

Alkaline phosphomonoesterase from *Escherichia coli*, which was used for removal of the terminal 3'-phosphate, was a commercial product purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

High- and low-molecular-weight yeast RNA were prepared from pressed cakes of baker's yeast by treatment with detergent (Crestfield, Smith & Allen, 1955). The fraction insoluble in M -NaCl was then extracted with phenol. The U:C:G:A base composition was 2.7:2.0:2.8:2.5.

Double-stranded cytoplasmic-polyhedrosis-virus RNA from silk worm (Miura, Fujii, Sakaki, Kawase & Fuke, 1967) was kindly given by Dr K. Miura.

Methods. All spectrophotometric measurements were

made with an Ito spectrophotometer (Hakusan, Bunkyo, Tokyo, Japan), with silica cells of 1 cm. light-path.

For paper chromatography with Toyo Roshi no. 51A paper, three solvents were used: solvent 1 [butan-1-ol-isobutyric acid-water-aq. 25% (w/v) NH_3 (30:15:10:1, by vol.), descending]; solvent 2 [isobutyric acid-0.5 N - NH_3 (5:3, v/v), ascending]; solvent 3 [saturated $(NH_4)_2SO_4$ -propan-2-ol-0.1 M -citrate buffer, pH 6.0 (40:1:9, by vol.), ascending]. After chromatography, the paper was dried at room temperature and photographed on print paper Konicopy EM by u.v. light (Manasuru lamp) through a special filter (Institute of Physical and Chemical Research, Tokyo, Japan). For quantitative analysis, the isolated spots on the chromatogram were located under u.v. light, cut out and eluted in 3 ml. of 0.01 N -HCl for 48 hr. with occasional stirring. Each eluate was identified by its R_F value and u.v.-absorption spectrum measured in a Hitachi type ESP-2U automatic recording spectrophotometer.

Assay for ribonucleases. To each 0.1 ml. of the assay solution (containing 1.0 mg. of yeast RNA, 0.4 μ mole of EDTA and 10 μ moles of tris-HCl buffer, pH 7.5, for ribonucleases U_1 and U_4 , or sodium acetate buffer, pH 4.5, for ribonucleases U_2 and U_3) was added 0.1 ml. of appropriately diluted enzyme solution; the mixture was maintained at 37° for 22 hr.

3'-Terminal nucleotide analysis of ribonuclease-digestion products. Details are given in the legend to Fig. 2.

Mapping procedure for the ribonuclease digests of yeast RNA. To determine the mode of enzymic action, mapping of the digestion products of yeast RNA with the ribonucleases was carried out, by two-dimensional paper chromatography, with solvent 2 in the first dimension and with solvent 3 in the second dimension.

Fig. 1. Digestion of yeast RNA with ribonucleases U₁ (a), U₂ (b), U₃ (c) and U₄ (d). The reaction mixture (0.2 ml.) contained 1.0 mg. of yeast RNA, 0.4 μ mole of EDTA, 10 μ moles of tris-HCl buffer, pH 7.5, for ribonucleases U₁ and U₄, or 10 μ moles of sodium acetate buffer, pH 4.5, for ribonucleases U₂ and U₃, and enzymes. The mixture was incubated at 37° for 22 hr. Degradation was determined by measuring acid-soluble digestion products from yeast RNA (○, low-molecular-weight yeast RNA, and ●, high-molecular-weight yeast RNA, were used as substrates) expressed as percentage of that obtained by alkali digestion.

RESULTS

Digestion of yeast RNA with the ribonucleases.

The degree of digestion is presented as a function of enzyme concentration in Fig. 1. The reaction mixture was incubated at 37° for 22 hr. and diluted with 0.8 ml. of water; 0.25 ml. of uranyl reagent was then added. After the precipitate had been centrifuged off, 0.2 ml. of the supernatant was diluted with 5.0 ml. of water and E_{260} was determined against the blank containing no enzyme. The percentage degradation was presented as the ratio of E_{260} of acid-soluble products to that obtained by alkaline hydrolysis of the RNA used. Complete digestion was obtained by using 0.156, 0.625, 0.625 and 2.5 units of ribonucleases U₁, U₂, U₃ and U₄ respectively. After exhaustive digestion with ribonucleases U₁, U₂ and U₃, 55, 11 and 12% of substrates remained as resistant core respectively. With ribonuclease U₄ no resistant core remained.

3'-Terminal nucleotide analysis of ribonuclease-digestion products. High-molecular-weight yeast RNA was exhaustively digested with ribonucleases U₁, U₂, U₃ and U₄, and mononucleotides and terminal

Fig. 2. Paper chromatogram of mononucleotides and terminal groups of ribonuclease-digestion products. The reaction mixture (0.2 ml.) contained 1.0 mg. of high-molecular-weight yeast RNA, 0.4 μ mole of EDTA, 10 μ moles of tris-HCl buffer, pH 7.5, for ribonucleases U₁ and U₄, or 10 μ moles of sodium acetate buffer, pH 4.5, for ribonucleases U₂ and U₃, and ribonucleases U₁, U₂, U₃ and U₄, 0.156, 0.625, 0.625 and 2.5 units respectively. After incubation at 37° for 22 hr., the mixture was treated with 0.1 N-HCl for 6 hr. at room temperature and then neutralized with N-KOH and adjusted to pH 8.2 with 0.2 M-tris-HCl buffer. The mixture was incubated again with alkaline phosphatase (enzyme/RNA ratio 1:250, by wt.) at 37° for 18 hr. and then incubated with 0.3 N-KOH at 37° for 18 hr. The incubation mixture was neutralized again with HClO₄. Samples of the incubation mixture were spotted on paper and chromatographed with solvent 1. Nucleosides were identified by their R_F values and u.v.-absorption spectra. 1, With ribonuclease U₁; 2, with ribonuclease U₂; 3, with ribonuclease U₃; 4, with ribonuclease U₄; 5, with no enzyme.

nal nucleotides were determined by paper chromatography with solvent 1 and estimated as described in the Materials and Methods section. The paper chromatogram is shown in Fig. 2. Each spot was cut out, eluted, identified and determined (Table 1). Ribonuclease U₁ attacked only 3'-guanylate bonds and 80.5% of the 3'-guanylate bonds in yeast RNA were cleaved, whereas ribonuclease U₂ (and U₃) attacked only 3'-purine nucleotide bonds and 100% (and 85.9%) of 3'-guanylate bonds and 98.7% (and 99.0%) of adenylate bonds in RNA were cleaved. Unlike ribonucleases U₁, U₂ and U₃,

Table 1. *Determination of 3'-terminal residues of digestion products as nucleosides*

The isolated spots on the paper chromatogram (Fig. 2) were cut out, eluted with 3 ml. of 0.01 N-HCl, and estimated by measuring E_{260} . The amount of each nucleoside was calculated as a percentage of that obtained by alkali digestion.

Ribonuclease ...	U ₁	U ₂	U ₃	U ₄
3'-Terminal U estimated as uridine				
Total uridylyl residues	0	0	0	86.9
3'-Terminal C estimated as cytidine				
Total cytidylyl residues	0	0	0	100.0
3'-Terminal G estimated as guanosine				
Total guanylyl residues	80.5	100.0	85.9	95.2
3'-Terminal A estimated as adenosine				
Total adenylyl residues	0	98.7	99.0	100.0

ribonuclease U₄ attacked all nucleotide bonds in RNA. Therefore they are considered as guanine-specific, purine-specific, purine-specific and non-specific ribonucleases respectively.

Digestion of yeast RNA with ribonucleases U₂ and U₃. The reaction mixture (0.2 ml.; see the Materials and Methods section) contained 0.005 and 0.0025 unit of ribonucleases U₂ and U₃ respectively. The time-course of the formation of mononucleotides and terminal nucleotides was followed and they were determined as described above (Fig. 3).

In this quantitative analysis of the digestion products, it was shown that ribonucleases U₂ and U₃ have a preference for 3'-adenylate bonds in yeast RNA. Thus when 58% (and 39%) of the adenylate bonds in RNA were attacked by ribonuclease U₂ (and U₃), only 24% (and 7%) of the 3'-guanylate bonds were attacked.

Digestion of double-stranded RNA with ribonucleases U₂ and U₃. Cytoplasmic-polyhedrosis-virus RNA was incubated with ribonucleases U₂ and U₃. The incubation mixture contained 10 μ moles of sodium acetate buffer, pH 4.5, 0.4 μ mole of EDTA, approx. 400 μ g. of cytoplasmic-polyhedrosis-virus RNA and 20 (or 10) units of ribonuclease U₂ (or ribonuclease U₃). The mixture was incubated at 37° for 22 hr. and was then spotted on paper and chromatographed with solvent 2. All material remained at the origin, indicating that neither mononucleotides nor oligonucleotides appeared.

Mode of action of the ribonucleases on yeast RNA. Two reaction mixtures (0.2 ml.; see the Materials and Methods section) of each of the four ribonucleases were incubated at 37° for 22 hr. After incubation, one of the two reaction mixtures was applied to paper and chromatographed with solvent 2 in the first dimension and with solvent 3 in the second dimension (Fig. 4). Another reaction mixture was also chromatographed as above after treatment with 0.1 N-perchloric acid for 6 hr. at room temperature, to cleave the 2',3'-(cyclic)-phosphate bonds, and

Fig. 3. Partial digestion of yeast RNA with ribonucleases U₂ (a) and U₃ (b). The reaction mixture (0.2 ml.) contained 1.0 mg. of low-molecular-weight RNA, 0.4 μ mole of EDTA, 10 μ moles of sodium acetate buffer, pH 4.5, and 0.005 unit of ribonuclease U₂ or 0.0025 unit of ribonuclease U₃. After the reaction at 37°, the mononucleotides and 3'-terminal nucleotides were determined as indicated in Fig. 2 and Table 1. ○, Adenosine; ●, guanosine; ■, pyrimidine nucleosides.

neutralization with n-potassium hydroxide. Spots that were diminished on the acid-treated digests were identified as nucleoside 2',3'-(cyclic)-phosphates. Nucleoside 2'(3')-phosphates and nucleoside 2',3'-(cyclic)-phosphates were determined by the R_F values and typical absorption spectra. The spot of guanylate was identified by its typical

Fig. 4. Two-dimensional paper chromatograms of the RNA digests with ribonucleases U_1 (a), U_2 (b), U_3 (c) and U_4 (d). The reaction mixture (0.2 ml.) contained 1.0 ml. of high-molecular-weight yeast RNA, 0.4 μ mole of EDTA, 10 μ moles of tris-HCl buffer, pH 7.5, for ribonucleases U_1 and U_4 , or sodium acetate buffer, pH 4.5, for ribonucleases U_2 and U_3 , and ribonucleases U_1 , U_2 , U_3 and U_4 of 0.156, 0.625, 0.625 and 2.5 units respectively. After the reaction mixtures had been incubated at 37° for 24 hr., samples of these reaction mixtures were spotted on papers and chromatographed separately with solvent 2 in the first dimension and with solvent 3 in the second dimension. Spots: 1, 2',3'-(cyclic)-adenylate; 2, 3'-adenylate; 3, 2'- or 5'-adenylate; 4, 2',3'-(cyclic)-guanylate; 5, 3'-guanylate; 6, 3'-cytidylate; 7, 3'-uridylate.

fluorescence revealed by u.v. irradiation after treatment with hydrogen chloride gas.

In the exhaustive digestion products with ribonucleases U_2 and U_3 , 2',3'-(cyclic)-adenylate, 3'-adenylate, 2',3'-(cyclic)-guanylate and 3'-guanylate could be detected and no pyrimidine mononucleotides were found (Figs. 4b and 4c). Small oligonucleotides appeared between the mononucleotide region and the origin. Small oligonucleotides also appeared in the limited digestion of RNA. Thus ribonucleases U_2 and U_3 attacked yeast RNA as endonucleases. In the region of adenylate three spots were observed (Figs. 4b and 4c). Two of them were identified as 2',3'-(cyclic)-adenylate and 3'-adenylate respectively. The third one remains to be identified but is probably 2'- or 5'-adenylate. 2',3'-(cyclic)-Adenylate, 3'-adenylate and the un-

known component estimated as adenylate were in the proportions 12:5:1.

With ribonuclease U_1 only 3'-guanylate appeared as a mononucleotide in the map (Fig. 4a) and the main product was 2',3'-(cyclic)-guanylate. Small oligonucleotides also appeared if the incubation time was shortened.

Ribonuclease U_4 seems to be an exonuclease because the products of exhaustive digestion by the enzyme were four mononucleotides and no oligonucleotide appeared in the map (Fig. 4d).

DISCUSSION

During this research we recognized the probable existence of purine-specific ribonucleases as minor components. Since then our efforts have been concentrated on isolating them. Though we have not yet isolated them in a homogeneous state, they have been partially purified and it has been demonstrated that these enzymes, designated ribonucleases U_2 and U_3 , are indeed purine-specific ribonucleases. They do not attack at all pyrimidine nucleotide phosphodiester bonds. We believe that they will be of value for the analysis of nucleotide sequences in RNA.

Like ribonucleases T_1 and T_2 (Miura *et al.* 1967), neither ribonuclease U_2 nor U_3 attacks the double-stranded cytoplasmic-polyhedrosis-virus RNA from silk worm. They may therefore be further used to differentiate double-stranded RNA from others or even double-stranded segments in RNA from other segments.

Ribonucleases U_2 and U_3 attack adenylate and guanylate bonds in RNA with a preference for the former, but since they are not homogeneous the possibility that both or one is adenine-specific has not been excluded.

The mode of action of these enzymes is the same as that of other typical ribonucleases such as pancreatic ribonucleases (ribonuclease I-A) or ribonuclease T_1 (Egami, Takahashi & Uchida, 1964). They split RNA to 3'-nucleotides with the intermediary formation of nucleoside 2',3'-(cyclic)-phosphates. The first step, transphosphorylation, is reversible and faster than the second step, hydrolysis. So it is expected that these enzymes will be useful for the synthesis of certain oligonucleotides, such as adenylyl-(3'-5')-uridine, adenylyl-(3'-5')-adenylyl-(3'-5')-cytidine 3'-phosphate, adenylyl-(3'-5')-adenylyl-(3'-5')-adenosine 3'-phosphate etc., by the reverse reaction of the first step.

Ribonuclease U_1 , the main component, has been shown to be a guanyloribonuclease. As mentioned in the preceding paper (Arima *et al.* 1968) it is highly probable that it is the extracellular ribonuclease described by Glitz & Dekker (1963, 1964a,b), though some slight differences have been noted.

The studies on ribonuclease U₄ are as yet only preliminary. The products of exhaustive digestion of RNA by the enzyme are four 3'-mononucleotides and no small oligonucleotides could be detected during RNA digestion. This suggests the possibility that it might be a non-specific exonuclease that produces 3'-nucleotides.

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