Studies on the Characterization of Ribonucleases

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Ribonucleases with strict hydrolytic specificities for certain internucleotide linkages would be useful for the determination of nucleotide sequences in a polynucleotide chain since they would produce enzymatic digests of ribonucleic acid containing oligonucleotides of partially defined composition. At the present time, two types of endonuclease specificity are known: the pyrimidine specificity of pancreatic RNase (1), and the guanylic acid specificity of RNase T1 (2). However, the choice of starting material for purification of these enzymes has been more or less haphazard, and little systematic effort has been made to search for or to examine a large number of RNases. Such a systematic study would increase the chances of finding an enzyme with new and useful specificity. Furthermore, from the point of view of protein chemistry, it would be desirable to have available enzymes with the same specificity derived from many different organisms. A project of this kind is feasible in the case of RNases because, for the most part, they are remarkably stable, resistant to low pH, and relatively easy to purify.

In this report, we describe the isolation by essentially the same procedure of seven new RNases from microorganisms. Three of the enzymes were found to have a RNase T1 specificity, while the others completely hydrolyzed RNA to mononucleotides.

Possible uses of the enzymes are discussed.

EXPERIMENTAL PROCEDURE

Materials and Methods—All spectrophotometric measurements were made in a Beckman DU spectrophotometer with silica cells and a 1-cm light path and are expressed as absorbance. A Leeds and Northrup meter with microelectrodes was used for pH determinations. For paper chromatography with Whatman No. 3MM paper, a solvent containing 100 g of (NH₄)₂SO₄ per 100 ml of 0.1 m sodium phosphate, pH 7.0, was used (Solvent A).

RNA—High molecular weight RNA was prepared from pressed cakes of bakers' yeast by treatment with 2% sodium dodecyl sulfate (3). The fraction insoluble in m NaCl was then extracted with phenol. A 0.1% solution of this RNA in water at neutral pH was taken to have an absorbance at 260 μg of 24.0.

DNA—DNA from sperm was obtained from Nutritional Biochemicals Corporation, Cleveland.

Oligonucleotides—Compounds terminating in Gp were obtained from RNase T1 digests, and the other oligonucleotides, from pancreatic RNase digests of RNA (4, 5).

Adsorbents—Diethylaminoethyl and carboxymethyl cellulose (6) (0.8 and 0.6 meq per g, respectively) were obtained from Brown Company, Berlin, New Hampshire. Before use, fines were removed by repeated decantation, and the adsorbent was sieved and washed (6). Columns were packed with increasing pressure to 15 p.s.i. with 100 to 230 mesh adsorbent equilibrated with the starting buffer (see below), and were washed until the conductivity and pH of the effluent solution were the same as those of the influent solution. All runs with CM- and DEAE-cellulose were performed at room temperature. Sephadex G-75 (Pharmacia) was freed of fines by repeated settling and decanting, and packing of columns was performed by gravity.

Assay for RNases—In order to cover the pH range of enzymatic activity during the purification of new enzymes, assays were routinely performed at pH 4.5 and 7.0 as follows. To 0.8 ml of the assay solution (2.0 mg of yeast RNA per ml, and 0.125 M in sodium acetate, pH 4.5, or Tris-Cl, pH 7.0) was added 0.2 ml of properly diluted enzyme solution; the mixture was held at 37° for 15 minutes. The reaction was stopped by the addition of 1 ml of 6% HClO₄. After 15 minutes at 0°, the precipitate was removed by centrifugation, 0.5 ml of the supernatant was diluted with 4.5 ml of water, and the absorbance at 260 μg was determined against the proper blank. Several dilutions of the enzyme solution were thus tested at early stages of purification as a check on the linearity of the assay. An increase in absorbance at 260 μg of 1.0 under these conditions was defined as 100 units of enzyme activity.

Source of Starting Material Containing RNases—Four of the enzymes studied here were first observed and described by Nakao and Ogata (7). These authors obtained a large number of inocula of microorganisms from the Institute of Fermentation, Osaka, Japan (IFO), and checked the level of enzymatic activity at various stages of growth after removal of cells or mycelia from the media. The enzymes were then classified according to the degree of degradation of RNA (at pH 5.0 and 7.5) into two groups, i.e., those that produced mononucleotides only, or those that produced both mononucleotides and oligonucleotides.

We obtained the same four inocula listed by IFO numbers in Table I, and RNases were purified from each of these organisms. Two enzymes were isolated from a commercial preparation of lipase B (Rohm and Hass, Bristol, Pennsylvania). The last enzyme was derived from a strain of Bacillus cereus obtained from the American Type Culture Collection (Table I).

Purification of RNases—The isolation and purification of Enzyme I from Bacillus pumilus (Table I) will be given in detail. Two test tubes with 4.5 ml of sterile medium (7), containing (in grams per liter) glucose, 50; peptone, 5; tryptone, 5; beef extract, 2 Dr. T. N. Harris, the Children's Hospital, Philadelphia, Pennsylvania, informed us about the ribonuclease activity present in these preparations (8).
column chromatography on the same adsorbent but with different pH values. The purification of enzymes 3, 6, and 7 began with the acid step as described above. The ammonium sulfate concentration step was omitted in these cases. The purified and dialyzed enzymes were assayed at the pH optimum as described above in the presence of 0.001 M MgCl₂ plus 0.001 M CaCl₂, or 0.001 M EDTA. Enzymes 3 and 4 were also incubated for 10 minutes with 0.001 M EDTA and then assayed in solutions containing each metal alone at 0.005 M or both metals in combination, each at 0.005 M.

**Assay of RNases for Contaminating Enzymes**—Aliquots of the purified RNases were tested for acid phosphatase and nonspecific phosphodiesterase activity (9). The presence of DNase activity was checked by substituting DNA for RNA (weight for weight) in the standard assay at the pH optimum of each RNase.

**Stability of RNases to Phenol Extraction** (10, 11)—The purified solutions (2 ml each) were adjusted to the molarity of the limit buffer used for column chromatography (Table I) and stirred for 5 minutes with an equal volume of phenol saturated with water at room temperature. The phases were separated by centrifugation, and the upper (aqueous) phase was re-extracted with phenol. The aqueous layers and the combined phenol phases were separately lyophilized, taken up in 2 ml of water, and lyophilized again to remove last traces of phenol. The residue from both phases was dissolved in water and assayed for enzymatic activity together with aliquots of the untreated enzyme solutions.

**Analytical Column Chromatography on Sephadex G-75**—This was performed as described previously (12), except that the columns were equilibrated and operated with one solution that was 0.5 N in NaCl and 0.01 M in sodium phosphate, pH 7.0. Samples were applied to the column in a volume of 0.5 ml. The flow rate was between 2.3 and 2.1 ml per hour (decreasing gradually with successive runs), and 2.5-hour fractions were collected. The enzyme concentrations in the effluent were too low to be detected spectrophotometrically or fluorometrically, so that only enzymatic activity was used to locate the enzyme in the fractions. The bovine plasma albumin used as the reference substance was determined spectrophotometrically at 280 μM.

**Enzymatic Hydrolysis of RNA**—Aliquots of the purified enzyme solutions (3, 15, 60, and 300 units in a volume of 0.4 ml), 0.05 m buffer of the optimal pH, and 3 mg of yeast RNA were held at 37°C for 6 hours. The compounds in the digests were then fractionated and identified after mapping as described (4, 13). The amounts of the mono-, di-, and trinucleotides found in digests of RNA with Enzymes 1, 2, and 7 were compared with the corresponding values derived from maps of RNase T₁ digests of the same RNA (4). Similarly, the amounts of mononucleotides found in RNA digests prepared with Enzymes 3, 4, and 5 were compared with the corresponding values derived from alkaline hydrolysates (N KI-KOH for 24 hours at 23°C) of the same RNA.

**Enzymatic Hydrolysis of Oligonucleotides**—GpApCp and ApGpUp were hydrolyzed as above with Enzymes 1, 2, and 7 with 100 units of enzyme per mg of substrate. ApAPuP, GpGPuP, and ApUpGP were similarly hydrolyzed with Enzymes 3, 4, 5, and 6.

**RESULTS**

Seven RNases from various sources (Table I) were purified by a procedure involving acid treatment, concentration with ammonium sulfate (omitted in several cases), and column chroma-
TABLE I

Purification of ribonucleases from various sources

<table>
<thead>
<tr>
<th>Enzyme No., source, and strain</th>
<th>Total initial activity</th>
<th>Assay pH</th>
<th>Specific activity at step*</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>1. Bacillus pumilus, IFO 3098</td>
<td>97,000</td>
<td>7</td>
<td>12</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td>2. Mucor genevensis, IFO 4585</td>
<td>170,000</td>
<td>7</td>
<td>6.2</td>
<td>10.4</td>
<td>79</td>
</tr>
<tr>
<td>3. Bacillus cereus, ATCC 10987</td>
<td>153,000</td>
<td>7</td>
<td>4.2</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>4. Lenzites tenuis, IFO 4940</td>
<td>440,000</td>
<td>7</td>
<td>2.0</td>
<td>4.1</td>
<td>62</td>
</tr>
<tr>
<td>4.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Monascus pilosus, IFO 4480</td>
<td>39,000</td>
<td>4.5</td>
<td>0.4</td>
<td>0.6</td>
<td>5.3</td>
</tr>
<tr>
<td>6. Lipase B</td>
<td>216,000</td>
<td>4.5</td>
<td>56</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>7. Lipase B</td>
<td>36,500</td>
<td>7</td>
<td>9.4</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* See Diagram 1 for sequence of steps: (a) bacterial medium or extract, (b) removal of cells, (c) acid treatment, (d) precipitation with (NH₄)₂SO₄ and dialysis, and (e) column chromatography and dialysis. For definition of units of activity, see the text.
† Precipitation with (NH₄)₂SO₄ was omitted for Enzymes 3, 6, and 7.
‡ After the first column chromatography step, Enzyme 4 was found to contain an early (Enzyme 4.1) and a late (4.2) fraction, with specific activities of 1,060 and 838, respectively.

TABLE II

Column chromatography of ribonucleases

All chromatographic systems utilized columns 2.2 × 75 cm, operated at a flow rate of 100 ml per hour; 20-ml fractions were collected.

<table>
<thead>
<tr>
<th>Chromatographic system and starting buffer*</th>
<th>Limit buffer*</th>
<th>Chamber and fraction of limiting buffer in each</th>
<th>Varigrad gradient volume ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. CM-cellulose, 32 g; 0.02 M sodium acetate, pH 4.6</td>
<td>A, 0.1 M sodium acetate, pH 4.6</td>
<td>2, 0.5 A; 3, A</td>
<td>9 × 500</td>
</tr>
<tr>
<td></td>
<td>B, 0.1 M sodium acetate, pH 5.7</td>
<td>4, 0.2 B; 5, 0.5 B; 6, B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C, 0.1 M sodium phosphate, pH 7.0</td>
<td>7, 0.2 C; 8, 0.5 C; 9, C</td>
<td></td>
</tr>
<tr>
<td>II. CM-cellulose, 32 g; 0.02 M sodium acetate, pH 4.6</td>
<td>A, 0.1 M sodium acetate, pH 4.6</td>
<td>2, A</td>
<td>9 × 500</td>
</tr>
<tr>
<td></td>
<td>B, 0.1 M sodium acetate, pH 5.2</td>
<td>3, B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C, 0.1 M sodium phosphate, pH 7.0</td>
<td>4, 0.2 C; 5, C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D, 0.1 M sodium bicarbonate, pH 8.6</td>
<td>6, 0.2 D; 7, D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E, 0.5 M NaCl</td>
<td>8, D + 0.2 E; 9, D + E</td>
<td></td>
</tr>
<tr>
<td>III. DEAE-cellulose, 30 g; 0.02 M Tris-Cl, pH 8.0</td>
<td>A, 0.1 M Tris-Cl, pH 8.0</td>
<td>2, 0.5 A; 3, A</td>
<td>9 × 500</td>
</tr>
<tr>
<td></td>
<td>B, 0.1 M sodium phosphate, pH 7.0</td>
<td>4, 0.2 B; 5, 0.5 B; 6, B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C, 0.5 M NaCl</td>
<td>7, B + 0.2 C; 8, B + 0.5 C; 9, B + C</td>
<td></td>
</tr>
<tr>
<td>IV. DEAE-cellulose, 30 g; 0.02 M Tris-Cl, pH 8.0</td>
<td>A, 0.02 M sodium phosphate, pH 7.0, and 0.5 M NaCl</td>
<td>2, A</td>
<td>2 × 2000</td>
</tr>
</tbody>
</table>

* See Peterson and Sober (14) for definition of “starting” and “limit” buffers.
† The chambers of the Varigrad contained starting buffer if not indicated in this column. A nine-chamber Varigrad was used throughout except in System IV, in which a two-chamber Varigrad was employed.

The activity at the optimum pH (Table II). The linearity of the assay used was ascertained at the pH optimum for each enzyme, and found to extend over values of absorbance at 260 nm up to 0.6 to 0.8.

The effect of 0.001 M MgCl₂ and CaCl₂ as well as of 0.001 M EDTA on enzyme activity was also determined. Under these conditions Enzymes 1 and 2 were somewhat inhibited by the metals, and Enzyme 2, by EDTA (Table II). More strikingly, assay of Enzymes 3 and 4 in the presence of EDTA resulted in complete inhibition, which could be only partially reversed by addition of a 5-fold excess of either MgCl₂ or CaCl₂, or both metals.
All RNases were free of acid phosphatase and nonspecific phosphodiesterase activity when tested as described by Koerner and Sinsheimer (9). DNase activity was not detectable with DNA in the standard assay with 100 or more RNA units of each enzyme. The RNases were stable to treatment with phenol and were quantitatively extracted into the phenol phase with the exception of Enzymes 3 and 4 (Table III).

Analytical column chromatography on Sephadex G-75 was used to ascertain the homogeneity and approximate molecular weight of the enzymes. Enzymes such as pancreatic RNase, Bacillus subtilis RNase, and bovine plasma albumin were used as standards and were clearly distinguished from each other by their elution volumes (12) of 77, 79, and 47.5 ml, respectively. An inspection of Table III shows that the seven RNases fall roughly into two groups: those with an elution volume of 52 to 58 ml, and those with an elution volume 79 to 80.5 ml. The widths (12) of the enzyme peaks at half-maximal enzymatic activity were between 6 to 7 ml for all samples, including pancreatic RNase (Sigma, type III, Lot R 22 B-70), indicating that the enzymes were quite homogeneous with respect to molecular size. This also applied to the two enzyme fractions obtained from Lenes tenuis (Enzyme 4), listed as Enzymes 4.1 and 4.2 in Table III, which were found to be identical with respect to pH optimum, activation by metals and EDTA, adsorption on CM-cellulose, and enzymatic specificity (see below).

The specificity of the RNases was determined by hydrolyzing yeast RNA and several oligonucleotides with various dilutions (i.e. at different enzyme to substrate ratios) of each enzyme. The products obtained were fractionated by and identified after mapping (4,13), and the amounts of the major compounds agreed (in the case of RNA digests) with the amounts of corresponding compounds obtained on maps of the same RNA hydrolyzed with RNase T1 or by alkali. Enzymes 1, 2, and 7 were thus found to be guanylic acid-specific and to hydrolyze the 5'-linkage of RNA or oligonucleotides, leaving the 3'-phosphate moiety of guanosine, as does RNase T1 (4). This specificity did not change when a 10-fold excess of each enzyme was used, i.e. when 1000 rather 100 units of enzyme per mg of RNA were held at 37° for 6 hours and then mapped.

Enzymes 3, 4, 5, and 6 were similarly found to quantitatively hydrolyze RNA and various oligonucleotides to mononucleoside 3'-phosphates (Table III). Partial digestion of trinucleotides

### Diagram 1

**Summary of purification of ribonucleases**

Bacterial medium (a) (centrifuge or filter)

Discard cells

Supernatant (b)

0.4 N H₂SO₄ at 4° for 16 hours

Neutralize solution with NH₄OH to pH 6 (c)

(II)*

(I)* Saturate with (NH₄)₂SO₄; hold at 4° for 16 hours; filter or centrifuge

Residue

Dialyze, centrifuge

Discard supernatant

Dialyze, centrifuge

Discard residue

Supernatant (d)

Column chromatography (repeat if more than one enzyme peak is found)

Pool enzyme fractions; dialyze; store (e)

* In several instances the ammonium sulfate concentration step was omitted.

### Table III

**Properties of purified ribonucleases**

<table>
<thead>
<tr>
<th>Enzyme No. (Table I)</th>
<th>Adsorption of enzymes on System (Table II)</th>
<th>pH at which activity is Optimal</th>
<th>Within 65% of optimal</th>
<th>Enzyme activity remaining In 0.001 M MgCl₂ + 0.001 M CaCl₂</th>
<th>In 0.001 M EDTA</th>
<th>After phenol extraction Aqueous phase Phenol phase</th>
<th>Elevation volume from Sephadex (ml) Specificity for RNA and oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CM I</td>
<td>7.9</td>
<td>6.8-9.2</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>108</td>
</tr>
<tr>
<td>2</td>
<td>CM I</td>
<td>7.9</td>
<td>6.6-9.0</td>
<td>68</td>
<td>54</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td>CM I</td>
<td>7.9</td>
<td>6.8-8.4</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>CM II†</td>
<td>7.9</td>
<td>6.5-8.4</td>
<td>100</td>
<td>0</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>4.1†</td>
<td>DEAE III</td>
<td>4.5</td>
<td>3.5-5.2</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>110</td>
</tr>
<tr>
<td>4.2†</td>
<td>DEAE III</td>
<td>4.5</td>
<td>3.0-4.6</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td>7</td>
<td>DEAE III, IV†</td>
<td>4.5</td>
<td>3.7-7.3</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>102</td>
</tr>
</tbody>
</table>

* A dash indicates that the mode of action of the enzyme is nonspecific.

† After the first column chromatography step, Enzyme 4 was found to contain an early (4.1) and a late (4.2) fraction (see Table I); these were rechromatographed in Systems I and II, respectively.

‡ Enzymes 6 and 7 were separated from one another with System III, and each was then rechromatographed individually as indicated.
revealed that the latter enzymes hydrolyze such substrates from either end (15).

**DISCUSSION**

It was the purpose of this study to isolate RNases from various sources, preferably by the same general procedure. Since the characterization of enzymes by their properties as proteins requires large amounts of highly purified material, the characterization was restricted to the determination of enzymatic specificity. This permitted the use of small amounts of enzymes which had to be purified only to a point at which they were free of contaminating RNases or phosphodiesterases. Treatment with 0.4 N acid was therefore used early in the procedure to remove acid-labile phosphatases and nonspecific phosphodiesterases. This step also inhibits autolysis of the RNases present. Next, saturation with ammonium sulfate was employed to reduce the volume of the crude enzyme solution. As an alternative, the solution was dialyzed directly and concentrated by adsorption on the column of adsorbent used for column chromatography (Tables II and III). The data obtained (Table III) indicate that the procedure did in fact yield single enzymatic activities. Only Enzyme 7 showed a rather broad pH range, but it resembles RNase T1 (4) in this respect as well as in enzymatic specificity. Both enzymes are derived from Aspergillus.

While this study did not uncover enzymes with new hydrolytic specificity toward RNA, several points may be noted. One of them concerns the ease of isolation of enzymes with an RNase T1 specificity. At present, RNase T1 is prepared from Aspergillus oryzae, which also produces the nonspecific RNase T3 (2). Our Enzymes 1 and 2 are produced by microorganisms that do not elaborate other extracellular RNases. As a result, Enzymes 1 and 2 (with guanylic acid specificity like RNase T3) are free of contaminating RNases in the crude state, and provide a source from which this activity can be more readily purified than RNase T1 from Taka-Diastase.

Another point of interest concerns the small oligonucleotides from partial digests of RNA prepared with Enzymes 3, 4, 5, and 6. The mapping procedure is capable of revealing the presence of mono- to pentanucleotides at a total value for absorbance at 260 mμ of 1.0 or more. However, during degradation, mononucleotides, “core” material (4), and only small amounts of a few intermediate compounds were found, a result similar to that obtained with RNase T2 (15). If these enzymes exhibit exonuclease as well as endonuclease action, then the core material would contain not only RNA degraded from the termini but also larger oligonucleotides. The isolation and characterization of such oligomers would be helpful for the elucidation of nucleotide sequences.

From the point of view of protein structure and function, the comparative approach, in which enzymes of the same specificity but derived from different sources or with different physical or chemical properties are utilized, may help to define the active center by reducing the number of common oligopeptide sequences. The marked difference in the chromatographic behavior of those RNases which are specific for guanylic acid linkages seems to provide such a situation.

Thus, RNase T1 (16), with an isoelectric point at pH 3.0, is more strongly bound on DEAE-cellulose than RNase T3, with an isoelectric point at pH 7.0 (2). Enzymes 6 and 7, derived from another strain of Aspergillus, exhibit the reverse order of elution; i.e., Enzyme 7 (resembling RNase T3), under identical conditions, is eluted much more readily from DEAE-cellulose than Enzyme 6 (resembling RNase T3). Still further deviation is seen with Enzymes 1 and 2, both specific for Gp, which are not bound at all by DEAE-cellulose but are strongly adsorbed on CM-cellulose.

It is also interesting to note that the molecular weights of the isolated RNases fall into two groups. The nonspecific nucleases have molecular weights of about 30,000 to 40,000, while those of the specific enzymes range from 11,000 to 13,000. This is consistent with the molecular weight distribution of previously described RNases. Thus, the nonspecific enzyme, RNase T3, has a molecular weight of 30,000 (15), while the specific RNases, e.g., pancreatic RNase, micrococal nuclelease (17), B. subtilis RNase (11), and RNase T1 (4, 18), are smaller.

**SUMMARY**

Seven ribonucleases from microorganisms were partially purified and characterized with respect to their enzymatic specificity. Three enzymes were found to hydrolyze ribonucleic acid at guanylic acid residues only, while the other four ribonucleases showed no specificity, degrading ribonucleic acid completely to mononucleotides. The protein moieties of the enzymes were found to differ from each other within each group with respect to chromatographic behavior and molecular weights.

**Acknowledgment**—We are indebted to Dr. L. A. Heppel for a critical reading of the manuscript.

**REFERENCES**

Studies on the Characterization of Ribonucleases
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