

## **Analysis by HPLC of Distributive Activities and the Synthetic (Back) Reaction of Pancreatic-Type Ribonucleases**

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### **1. Introduction**

#### ***1.1. Distributive vs Processive Cleavage of Polymeric Substrates***

Nucleic-acid cleavage can be processive, with the enzyme moving from one site to the next in the polymer before dissociating from the substrate, or distributive, with partially cleaved substrates released to the medium after the initial reaction. Kinetics indicate that either mechanism can be seen in reactions with polymerases, helicases, or nucleases (*1*). However, many factors affect these kinetics, including the strength of the enzyme–substrate binding, which can be altered by the salt concentration of the buffer, and intermediates in the formation of the complex or the release of the products that may be poorly characterized. The distinction between processivity and distributivity is based primarily on the ratio of rate constants for cleavage and dissociation. Unlike the distinction between endo- and exo-nucleases (i.e., those cleaving within or from one end of the nucleic acid), which will not change with reaction conditions, although in some cases enzymes show only a preference from one or the other activity, assay conditions can blur the distinction between processivity and distributivity.

An example of the analysis of a processive reaction, that of DNA repair by bacterial glycosylases at low salt concentrations, is presented in Chapter 1. Here we present HPLC-based methods to analyze the distributive cleavage of RNA by bovine pancreatic ribonuclease (RNase A), and other enzymes of this family.

#### ***1.2. The Enzymatic Mechanism of Pancreatic Ribonucleases***

Bovine pancreatic ribonuclease A (RNase A) and related enzymes catalyze the breakdown of their natural substrate, RNA, in two steps. The first step in the cleavage of the 3'-5' phosphodiester bonds of RNA is a transphosphorylation reaction from the 5' position of one nucleotide to the 2' position of an adjacent pyrimidine nucleotide to form two polynucleotides, one ending in a 2',3'-cyclic phosphate and another with a free 5'-OH end (**Fig. 1A**). These RNases have some degree of base specificity, cleaving polyadenylic acid (poly(A)) poorly in comparison to polycytidylic acid (poly(C)) or

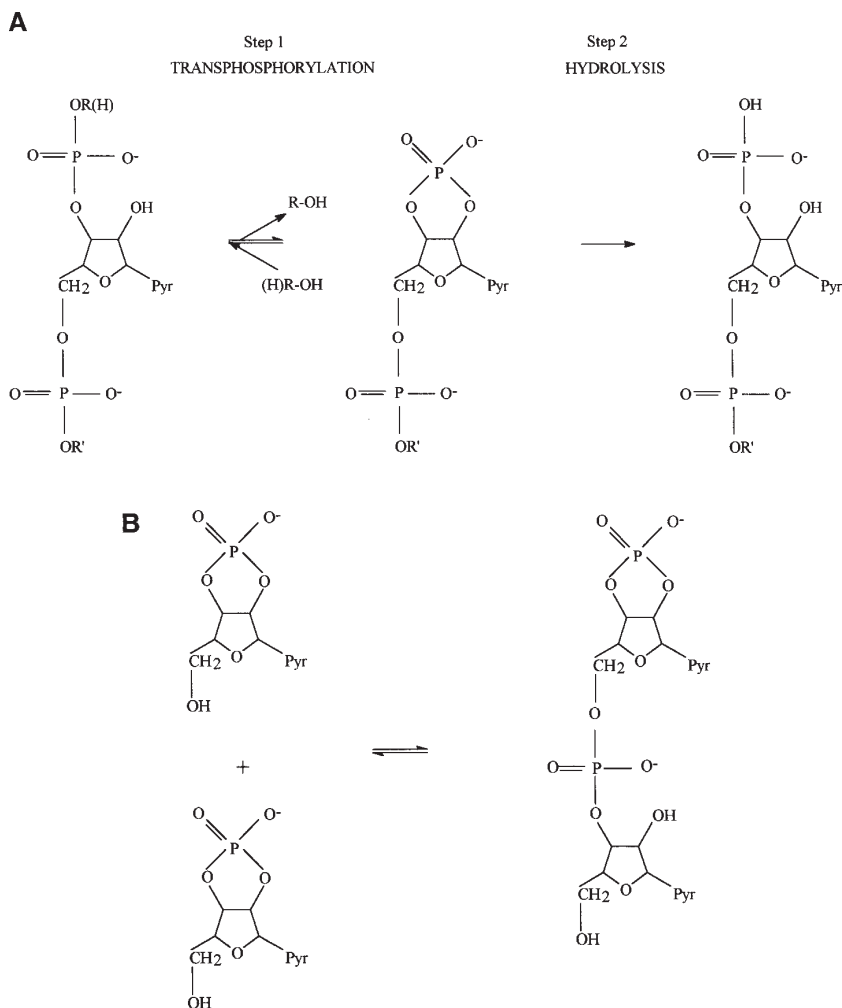


Fig. 1. **(A)** The reaction catalyzed by RNase A. The reaction for the complete breakdown of RNA takes place in two steps: transphosphorylation and hydrolysis. The pyrimidine 2',3'-cyclic phosphate intermediates are released to the medium as true products of the reaction. The hydrolysis of these cyclic compounds only begins when the transphosphorylation reaction is nearly finished. **(B)** The RNase A synthetic (back) reaction from cytidine 2',3'-cyclic phosphate (C>p) to cytidyl 3',5'-cytidine 2',3'-cyclic phosphate (CpC>p).

polyuridylic acid (poly(U)) (2). Thus the RNA cleavage will yield purine-core oligonucleotides ending with a pyrimidine 2',3'-cyclic phosphate nucleotide ((Pur)<sub>n</sub>Pyr>p). In the second step of the enzyme reaction, the terminal 2',3'-cyclic phosphodiester of the core oligonucleotides is hydrolyzed to a 3'-nucleotide (3) (**Fig. 1A**). The transphosphorylation reaction needs a dinucleoside monophosphate as the minimum size for the substrate, but the hydrolysis reaction can take place with a 2',3'-cyclic pyrimidine mononucleotide.

### 1.3. Cleavage of Polymeric Substrates

Characterizing the RNA breakdown kinetically is complicated by the structural complexity of the RNA molecule, the difficulty of monitoring a very fast reaction, and the fact that most products of the reaction are also substrates, although with different specificity constants (4). In **Subheadings 3.1.** and **3.2.** we describe assays to study different aspects of the processes catalyzed by RNase A (4–6) and the eosinophil cationic protein (ECP) (7), using methods based on the separation of the reaction products by HPLC. The pattern of product formation from poly(C) or poly(U) to oligonucleotides of different size,  $(\text{Cp})_n\text{C}>\text{p}$  or  $(\text{Up})_n\text{U}>\text{p}$  respectively, indicates the endo- or exonuclease preference of these “nonspecific” enzymes. The pattern of poly(C) cleavage, for example, indicates that RNase A does not act in a random fashion. Rather, it preferentially binds longer-substrate molecules and cleaves phosphodiester bonds 6–7 residues from the end of the chain (5). In contrast, ECP cleaves predominantly in an exonuclease-like manner (7).

### 1.4. Characterizing the Synthetic (Back) Reaction

At sufficiently high concentrations of cytidine 2',3'-cyclic phosphate ( $\text{C}>\text{p}$ ) as substrate, RNase A generates the hydrolysis product 3'-CMP, or the synthesis product cytidyl 3',5'-cytidine 2',3'-cyclic phosphate ( $\text{CpC}>\text{p}$ ) (see **Fig. 1B**). The hydrolysis reaction must be considered formally as a special case of the transphosphorylation back reaction in which the R group of the R-OH substrate is H (see **Fig. 1A**). We show in **Subheading 3.3.** how the products of the RNase A reaction of  $\text{C}>\text{p}$  at concentrations above 10 mM (pH 5.5), 3'-CMP and  $\text{CpC}>\text{p}$ , can be separated and quantified by means of anion-exchange HPLC. This method was used to characterize the kinetics of both reactions that take place in the same assay mixture (4,8,9).

The methods described here, developed for RNase A, can be applied to different RNases by adjusting the enzyme and substrate concentrations and the assay conditions (pH, ionic strength, temperature, and reaction time).

## 2. Materials

### 2.1. Equipment

1. Apparatus: An HPLC system with two pumps (or one pump with a gradient mixer system), a liquid chromatography injector, a UV absorbance detector, and a computer for both control of the system and data processing. We have used the following systems: Amersham Pharmacia Biotech, Waters Corp. (Mildford, MA), and Varian Associates (Sunnyvale, CA).
2. Chromatography columns: reversed-phase HPLC column, Nova Pak  $\text{C}_{18}$ , 4  $\mu\text{m}$ , 3.9  $\times$  150 mm (Waters Corp.), anion-exchange column, Nucleosil 10 SB, 300 mm  $\times$  4 mm I.D., and Vydac-310 SB precolumn stationary phase (Macherey, Nagel and Co., Düren, Germany).
3. Use distilled water treated with a MilliQ water purification system (Millipore Corp.) for the preparation of all solutions.
4. HPLC solvents: acetonitrile 240/farUV HPLC grade (Scharlau Chemie [Barcelona, Spain] or Carlo Erba [Milano, Italy]), ammonium acetate GR (Merck) and acetic acid (analytical grade) (Carlo Erba).
5. Substrates and enzymes: poly(C),  $\text{C}>\text{p}$  ( $\epsilon_{254} = 7214 \text{ M}^{-1}/\text{cm}$ ), 3'-CMP, 2'-CMP, pepsin from porcine stomach mucosa and bovine pancreatic RNase A (Type XII-A) ( $\epsilon_{277.5} = 9800 \text{ M}^{-1}/\text{cm}$  [10]) from Sigma. All other reagents are of analytical grade.

## 2.2. Solutions

1. Poly(C) solution: 5 mg/mL in 10 mM HEPES, pH 7.5, with KOH (or 10 mM Tris-HCl, pH 7.5).
2. C>p solution: between 10 and 40 mM in 0.2 M sodium acetate, pH 5.5.
3. Dissolve RNase A (or the enzyme that is analyzed) in the same buffer as the substrate, and adjust the enzyme concentration according to its activity.
4. Pepsin solution: 3.6 mg/mL in 0.2 M H<sub>3</sub>PO<sub>4</sub> in 0.1 M HCl.
5. Solvent A: 10% ammonium acetate (w/v) in water, degassed before use, to which 1% acetonitrile (v/v) is added.
6. Solvent B: 10% ammonium acetate (w/v) in water, degassed before use, to which 11% acetonitrile (v/v) is added.
7. 0.1 M ammonium acetate, pH 5.5, and 0.6 M ammonium acetate, pH 5.5, solutions are degassed before use.

## 3. Methods

### 3.1. Analysis of the Digestion Products of Poly(C) by Ribonuclease A (Fig. 2)

1. Combine 50  $\mu$ L of poly(C) solution with 10  $\mu$ L of 30 nM RNase A at 25°C. Stop the reaction by applying samples at different time intervals depending on the enzyme activity (between 0 and 45 min in this case) directly to the HPLC.
2. HPLC procedure:
  - a. Nova Pak C<sub>18</sub> column conditions: flow-rate: 1 mL/min, pressure: 1000–2000 psi (68–136 atm). Fix the maximum pressure limit to 6000 psi (408 atm) to avoid damaging the column. Store the column in 100% acetonitrile, and wash for 20 min with 1% acetonitrile in HPLC-grade water before use.
  - b. Separation procedure: Wash the column for 15 min with solvent A. Inject 20  $\mu$ L of the reaction mixture onto the column. Wash for 10 min, and then elute with a 50-min linear gradient from 100% solvent A to 10% solvent A plus 90% solvent B.
  - c. After each run, wash the system for 5 min with water containing 1% acetonitrile and for 10 min with 100% acetonitrile.
  - d. Before reequilibrating with solvent A, wash 5 min with water containing 1% acetonitrile. Slight differences in the retention times of oligonucleotides can be produced, depending on the equilibration time of the column. The intermediate washes with water are important to avoid contact between a highly concentrated saline solution and a concentrated organic solvent, which can produce some precipitates with the subsequent clogging of the column and a sharp increase in the pressure of the system.
  - e. Monitor the absorbance at 260 nm of the eluate to detect and quantify product. Previously, obtain the number of integration counts per absorbance unit using a standard solution of nucleotide.
  - f. Identification of the products: at the initial conditions (**Fig. 2**,  $t = 0$ ), although poly(C) is not electrophoretically homogeneous (according to the information provided by Sigma), all high-molecular-mass components elute as a single peak, and no oligonucleotides or other small-molecular-mass contaminants are present in the sample. The elution position of the small oligonucleotides (**Fig. 3**) is deduced from the pattern of the poly(C) digestion by RNase A after a long incubation time (100 min) when no high molecular mass poly(C) is left, the pattern found by McFarland and Borer (*II*) for the chemical hydrolysis of polynucleotides, and the MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass-spectrometry analysis of the individual peaks, except for the two mononucleotide species (3'-CMP and C>p) which yield

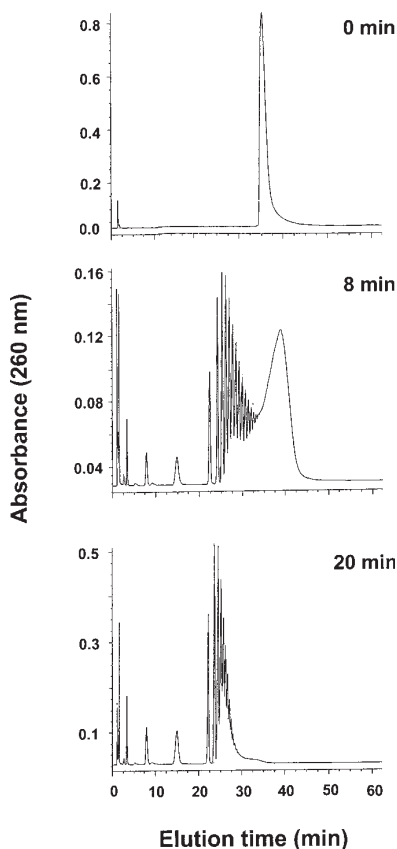


Fig. 2. Analysis by reversed-phase HPLC column (Nova-Pak C<sub>18</sub> column) of products obtained from poly(C) digestion by RNase A at different time intervals. In each case, 50  $\mu$ L of a 5 mg/mL poly(C) solution in 10 mM HEPES-KOH, pH 7.5, is digested with 10  $\mu$ L of 30 nM RNase A at 25°C. At the initial conditions ( $t = 0$  min) poly(C) elute as a single peak and small molecular mass contaminants are not observed. Poly(C) fraction diminished with the increase of the reaction time ( $t = 8$  and 20 min) with the subsequent formation of oligonucleotides.

unclear spectra. The two mononucleotide products 3'-CMP and C>p elute sequentially, but very close in the first peak, and oligomers of increasing size elute with increasing retention times (Fig. 3). Oligonucleotides up to nine residues can be separated with a good resolution.

### 3. Quantification of results:

- a. Determine the relative formation of mononucleotides and low-mol-mass oligonucleotides (up to nine nucleotides) by integration of the 260-nm peak area and, to normalize the relation between the peak area and the quantity of oligonucleotides, divide the area of each peak by the number of nucleotide residues. As the system cannot resolve nucleotides >10 bases, for the calculation of the relative distribution of each oligonucleotide, the area corresponding to all oligonucleotides with  $n > 9$  can be considered as a high-mol-mass product together with the undigested substrate fraction (9).

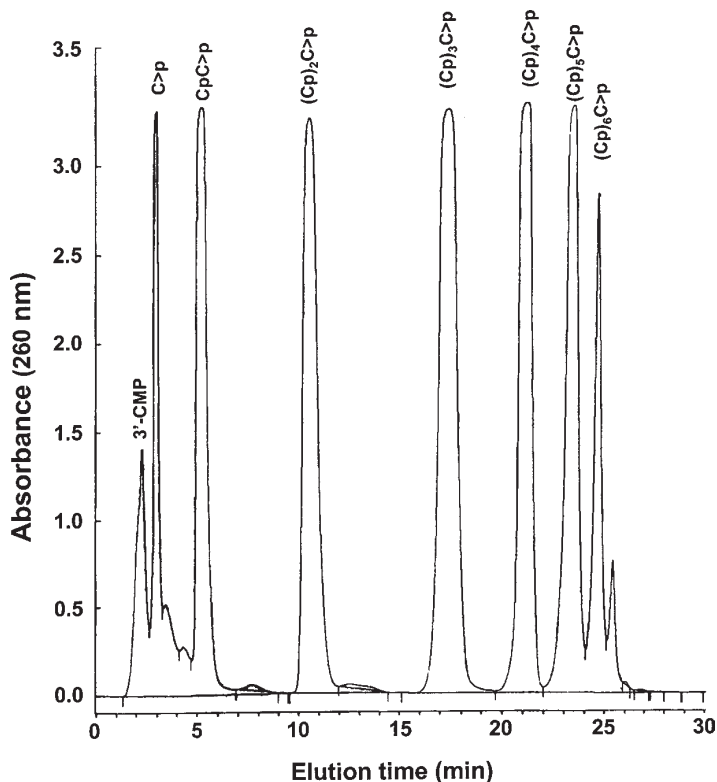


Fig. 3. Elution profile on a reversed-phase HPLC column (Nova-Pak  $C_{18}$  column) of oligocytidylic acids  $(Cp)_n C > p$  ( $n = 0-6$ ) from poly(C) digestion. 200  $\mu L$  of 10 mg/mL poly(C), in 10 mM HEPES-KOH, pH 7.5, are digested with 15  $\mu L$  of 0.5  $\mu M$  RNase A at 25°C for 16 min.

- b. To quantify the relative amount of oligonucleotides up to seven residues, divide the 260-nm peak area by the extinction coefficient at 260 nm ( $\epsilon_{260}$ ): 15,175  $M^{-1}/cm$  for  $CpC > p$ , 20,745  $M^{-1}/cm$  for  $(Cp)_2 C > p$ , 24,282  $M^{-1}/cm$  for  $(Cp)_3 C > p$ , 28,683  $M^{-1}/cm$  for  $(Cp)_4 C > p$ , 37,711  $M^{-1}/cm$  for  $(Cp)_5 C > p$ , and 42,428  $M^{-1}/cm$  for  $(Cp)_6 C > p$ .

### 3.2. Analysis of Product Formation from Oligocytidylic Acids $(Cp)_n C > p$ by Ribonuclease A (Fig. 4)

The method described in **Subheading 3.1.** can also be used for the large-scale preparation of oligonucleotides of the general structure  $(Cp)_n C > p$  or  $(Up)_n U > p$ , in which  $n$  ranges from 0–6. These oligonucleotides are good substrates for assessing the role of the noncatalytic binding subsites, adjacent to the active site, in the catalytic process.

1. Preparation of oligocytidylic acids: Digest 500  $\mu L$  of poly(C) solution with 50  $\mu L$  of 7  $\mu M$  RNase A at 25°C for 5 min. Separate oligonucleotides of different length according to the method described in **Subheading 3.1.** Collect the fractions that correspond to tetra, penta, hexa, and heptacytidylic acids, pool with the corresponding fractions of several chromatographic runs, freeze-dry, and keep at  $-20^\circ C$  until use.

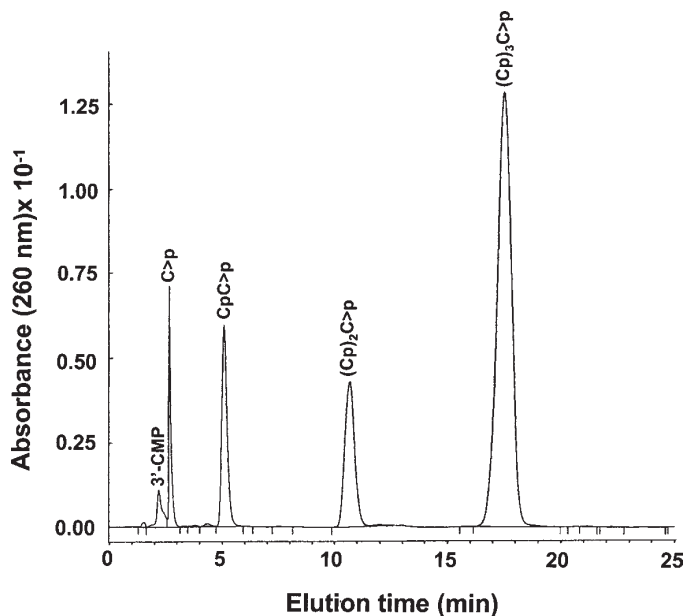


Fig. 4. HPLC separation by reversed-phase HPLC (Nova-Pak  $C_{18}$  column) of the products obtained from  $(Cp)_3C>p$  digestion by RNase A. 100  $\mu$ L of  $(Cp)_3C>p$  solution (0.3 U of A at 260 nm) in 10 mM HEPES-KOH, pH 7.5, are digested with 10  $\mu$ L of 0.1 nM RNase A at 25°C for 15 min.

2. Digestion of oligonucleotides: Use the general reaction conditions described for poly(C) digestion. Specific characteristics are related to the substrate and enzyme concentrations. Use substrate solutions with an approximate  $A_{260}$  of 0.3; the enzyme concentration depends both on the substrate and the enzyme species used. **Figure 4** is an example, which shows the specific conditions applied to the digestion of  $(Cp)_3C>p$  by RNase A.
3. Separate the reaction products by the HPLC procedure described in **Subheading 3.1**.
4. Calculate the amount of each oligonucleotide product by integration of the area of the peaks corresponding to the absorbance at 260 nm divided by the corresponding extinction coefficient ( $\epsilon_{260}$ ). The number of integration counts per AU, using a standard solution of nucleotide, should be obtained beforehand.

### 3.3. Analysis of the Hydrolysis (3'-CMP Formation) and Synthesis ( $CpC>p$ Formation) Reactions by RNase A Using $C>p$ as Substrate (Fig. 5)

The synthetic reaction is only measurable at substrate concentrations above 10 mM. At lower substrate concentrations, only the hydrolysis reaction is observed.

1. In a typical assay the reaction mixture contains 30  $\mu$ L substrate solution and 5  $\mu$ L RNase A.
2. Quenching procedure: Incubate the assay mixture (35  $\mu$ L) at 25°C for the desired time. Quench the reaction by the addition of 25  $\mu$ L 0.2 M  $H_3PO_4$  in 0.1 M HCl; the final pH of the reaction is 2.0. Immediately add 5  $\mu$ L of the pepsin solution and incubate at 25°C for 15 min. Pepsin cleaves the Phe120–Asp121 bond in RNase A, and irreversible inactivation takes place. The mixture can be analyzed immediately or stored at –20°C. Storage

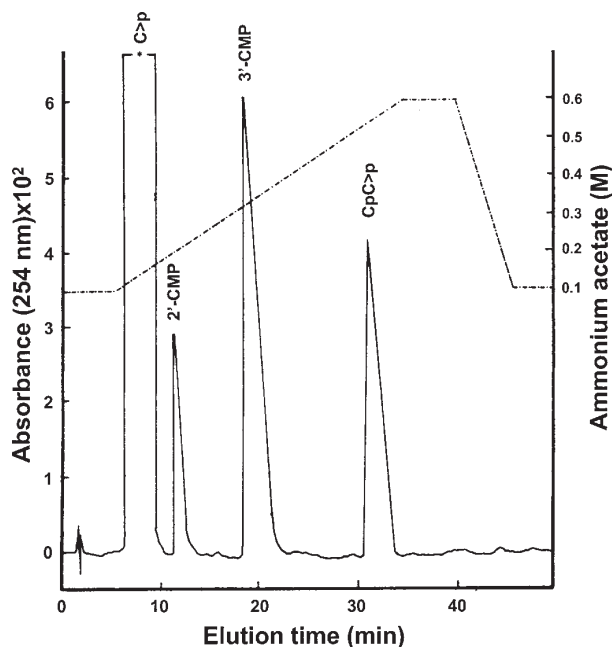


Fig. 5. HPLC separation by anion-exchange chromatography (Nucleosil 10 SB) of the products of the simultaneous hydrolysis and synthesis activities of RNase A. In this case, a reaction mixture containing 40 mM C>p and 0.6  $\mu$ M RNase A in 0.2 M sodium acetate, pH 5.5, is incubated at 25°C for 5 min, and the reaction is stopped by the quenching procedure described in the text. Product separation is carried out with a gradient from 0.1–0.6 M ammonium acetate, pH 5.5.

of the samples for several months at  $-20^{\circ}\text{C}$  does not affect the reproducibility of the results if the correct controls are included.

3. Increase the pH of the sample to 5.5 by the addition of 4  $\mu$ L 2 M NaOH before injecting the sample onto the HPLC column.
4. HPLC procedure:
  - a. Nucleosil 10 SB anion-exchange column and Vydac-310 SB precolumn stationary phase conditions: flow-rate: 1 mL/min, pressure: 500–600 psi (34–40 atm). Fix the maximum pressure limit to 3000 psi (204 atm) to avoid problems in the event of collapse of the column. The precolumn prevents the clogging of the HPLC column with protein (pepsin or digested RNase). Change the precolumn after approx 20 runs. Store the column in 20% methanol and wash the column for 20 min with MilliQ-water before use.
  - b. Separation procedure: wash the column with 0.1 M ammonium acetate, pH 5.5 for 30 min. Inject 20  $\mu$ L of the sample onto the column. Elute with a linear-salt gradient from 0.1 to 0.6 ammonium acetate, pH 5.5.
  - c. Monitor and quantify product elution from the absorbance at 254 nm.
  - d. Identification of the products: **Fig. 5** shows a typical chromatogram. Although the hydrolytic action of RNase A on C>p produces only 3'-CMP, a small amount of 2'-CMP appeared in the chromatogram. Both nucleotide monophosphates are produced by direct hydrolysis of C>p in the acid quench. The peaks corresponding to C>p, 2'-CMP, and 3'-CMP can be confirmed by the use of the respective standards. C>p, the sub-



strate of the reaction, elutes with a retention time of 10 min, the 2'-CMP a product of the acid treatment elutes at 12 min; the 3'-CMP (the product of the hydrolysis reaction) at 16 min, and CpCp (the product of the synthesis reaction) at 34 min. The effect of the acid in the formation of 2'-CMP and 3'-CMP is evaluated by a blank containing all the components of the assay except RNase A. It must be considered that the chemical hydrolysis yields equal amounts of 2'-CMP and 3'-CMP; thus, in order to quantify the amount of 3'-CMP formed by RNase A, an amount of 3'-CMP equal to the amount of 2'-CMP is subtracted from the total amount of 3'-CMP.

- e. Quantification of the results: Determine the amount of each nucleotide from the integration of the peak areas divided by the corresponding extinction coefficient,  $\epsilon_{254}$ , which are  $7214 \text{ M}^{-1}/\text{cm}$  for C>p,  $6686 \text{ M}^{-1}/\text{cm}$  for 3'-CMP, and  $14,400 \text{ M}^{-1}/\text{cm}$  for Cp >p. The number of integration counts per AU from standard solutions of C>p or 3'-CMP should be determined beforehand.

#### 4. Notes

1. The method described for poly(C) digestion analysis (**Subheading 3.1.**) has been applied to poly(U) digestion with similar results, although the digestion pattern depends on the specificity of the enzyme (7).
2. In the digestion of oligocytidylic acids  $(\text{Cp})_n\text{C}>\text{p}$  by RNase A (**Subheading 3.2.**) the enzyme concentrations must be extremely low (in the nanomolar range). To avoid denaturation, prepare a concentrated solution of the enzyme and dilute further immediately before the assay. It is also advisable to distribute the substrate in Eppendorf tubes, each one with the exact volume for an individual assay, and keep them frozen. They are thawed immediately before use, equilibrated to  $25^\circ\text{C}$ , and then the enzyme solution is added.
3. In the quenching procedure described in **Subheading 3.3.** the final pH (2.0) is critical. Below this pH, too much acid hydrolysis to 2'-CMP and 3'-CMP occurs. At higher pH levels, residual RNase activity can falsify the results.

#### Acknowledgments

We thank M. Moussaoui (Departament de Bioquímica i Biologia Molecular, UAB) for technical support, S. Bartolomé (Laboratori d'Anàlisi i Fotodocumentació, Departament de Bioquímica i Biologia Molecular, UAB) for the processing of the figures, and F. Canals (Servei de Seqüenciació de Proteïnes, IBF, UAB) for assessment in MALDI-TOF measurements. This work was supported by Grant PB96-1172-C02-01 from the Dirección General de Enseñanza Superior of the Ministerio de Educación y Cultura, Spain, and 1996SGR-00082 from Comissió Interdepartamental de Recerca i Tecnologia of the Generalitat de Catalunya. We thank the Fundació M. F. de Roviralta (Barcelona, Spain) for grants for the purchase equipment.

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<http://www.springer.com/978-0-89603-679-6>

Nuclease Methods and Protocols

Schein, C.H. (Ed.)

2001, XVII, 525 p., Hardcover

ISBN: 978-0-89603-679-6

A product of Humana Press