Spleen Acid Ribonuclease (1)

GIORGIO BERNARDI
CENTER FOR RESEARCH IN MACROMOLECULES
STRASBOURG, FRANCE

The preparation of a purified spleen acid ribonuclease has been described by Maver and Greco (2). A very highly purified preparation has been obtained by Bernardi and Bernardi (1) as a by-product of acid deoxyribonuclease during the purification of this latter enzyme.

ASSAY

Reaction

The ribonuclease activity is assayed by measuring the liberation of acid-soluble oligonucleotides from soluble RNA, which is an excellent substrate for acid ribonuclease.

Reagents for the Acid Ribonuclease Assay

1. The reaction mixture (total volume 1.25 ml) contains

   a. 0.8 μmoles of sRNA-P†
      187.5 μmoles of acetate buffer, pH 5.0
      12.5 μmoles of EDTA
   b. Enzyme—if necessary this is diluted with 0.15 M acetate buffer +

† Yeast-soluble RNA from General Biochemicals, Chagrin Falls, Ohio, was used in the original work.

0.01 M EDTA, pH 5.0, containing 0.05% Armour bovine serum albumin

2. 12% Perchloric acid

Reagents for the Basic Ribonuclease Assay

1. The reaction mixture (total volume 1.25 ml) contains

   a. 0.8 μmoles of sRNA-P†
      125 μmoles of phosphate buffer, pH 7.2
      12.5 μmoles of $MgCl_2$

   b. Enzyme—if necessary this is diluted with 0.1 M phosphate buffer, pH 7.2 + 0.01 M $MgCl_2$, containing 0.05% Armour bovine serum albumin

2. 12% Perchloric acid

Procedure

The procedure described for acid deoxyribonuclease (p. 103) is used, except that all volumes are halved. Activity units are defined as for acid deoxyribonuclease (p. 103), except that the activity is not divided by two.

CHROMATOGRAPHIC PURIFICATION

The starting material is crude spleen nuclease II (see p. 105). The chromatographic purification is patterned on Procedure C for spleen acid deoxyribonuclease and summarized in Table 1.

STEP 1. DEAE-SEPHADEX A-50 (FIG. 1). This step has been described on p. 113. Two ribonuclease activities are not retained by the column equilibrated with 0.05 M phosphate buffer, pH 6.8. The first peak shows a ratio of acid to basic activity equal to about 40. The second peak shows a ratio equal to about 10. These ribonuclease activities were not studied further.‡

A third ribonuclease activity peak is retained by the column and eluted by 0.5 M phosphate buffer, pH 6.8; the ratio of acid to basic activity is over 500. This fraction is processed further. For the sake of convenience, acid ribonuclease-rich fractions from several chromato-

† Yeast-soluble RNA from General Biochemicals, Chagrin Falls, Ohio, was used in the original work.

‡ Their behavior on hydroxyapatite is shown in Fig. 4 (Spleen acid deoxyribonuclease, p. 114).

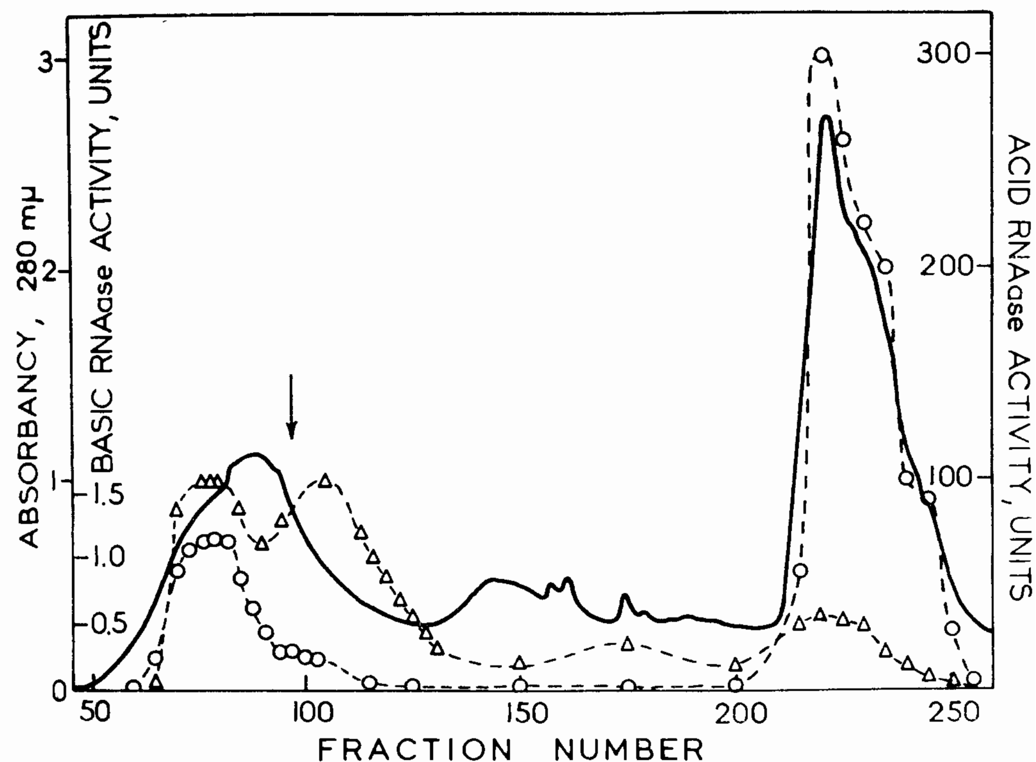

Fig. 1. Chromatography on DEAE-Sephadex A-50 of crude spleen nuclease II (see also p. 112) (Step 1). 330 ml of preparation HS 9 (OD_{260} = 10.3: OD_{280} = 6.9) were loaded on a 8- × 80-cm column of DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer, pH 6.8. This buffer was also used to elute the first protein peak. 0.5 M phosphate buffer, pH 6.8, was loaded at the fraction indicated by the arrow. 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Triangles indicate the basic ribonuclease activity (left-hand inner scale). Acid deoxyribonuclease, cytochrome c, acid phosphomonoesterase, and phosphodiesterase were also assayed; the results are shown on pp. 112 and 238.

TABLE 1. *Chromatographic Purification of Spleen Acid Ribonuclease*[a]

	Volume (ml)	Total OD_{280}	Total Units	Specific Activity
Crude spleen nuclease II				12.5[b]
Step 1. DEAE-S	70	1820	87,000	48
Step 2. Sephadex G-100	200	680	74,000	109
Step 3. Hydroxyapatite	120	80.5	43,300	538
Step 4. CM-Sephadex	183	19	41,000	2160
Step 5. Hydroxyapatite	5.1	6.2	15,000	2410

[a] All values reported refer to the fractions that were processed further or to the final product. The side fractions of the activity peaks were processed separately.
[b] This is the value obtained for preparation HS 11.

graphic purifications of crude spleen nuclease II preparations are precipitated with $(NH_4)_2SO_4$ (0.8 saturation at 20 C; the solutions are made 0.001 M with EDTA). The precipitate is collected by centrifugation and dissolved in a small volume of water.

STEP 2. SEPHADEX G-100 (FIG. 2). The acid ribonuclease solution from the previous step is loaded on a Sephadex G-100 column equilibrated with 0.05 M phosphate buffer, pH 6.8. A complete separation of an acid phosphomonoesterase and acid ribonuclease is obtained by eluting with 0.05 M phosphate buffer, pH 6.8.

STEP 3. HYDROXYAPATITE (FIG. 3). The active fraction from Sephadex G-100 is loaded on a hydroxyapatite column equilibrated with 0.1 M phosphate buffer, pH 6.8, which is also used to wash the column to elute some inactive material. A molarity gradient (0.05 to 0.5) of phosphate buffer, pH 6.8, elutes the ribonuclease activity at a molarity of about 0.12. The active fractions are concentrated to a small volume by freeze-drying and loaded on a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer, pH 6.0.

STEP 4. CM-SEPHADEX C-50 (FIG. 4). The ribonuclease fraction from Sephadex G-25 is loaded on a CM-Sephadex C-50 column, equilibrated with 0.05 M phosphate buffer, pH 6.0. Elution is carried out with a 0.05–0.4 M phosphate buffer gradient, pH 6.0. Acid ribonuclease is eluted after an inactive peak at a molarity of 0.15.

STEP 5. HYDROXYAPATITE (FIG. 5). The central fractions from the activity-rich peak are pooled and run on a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer.

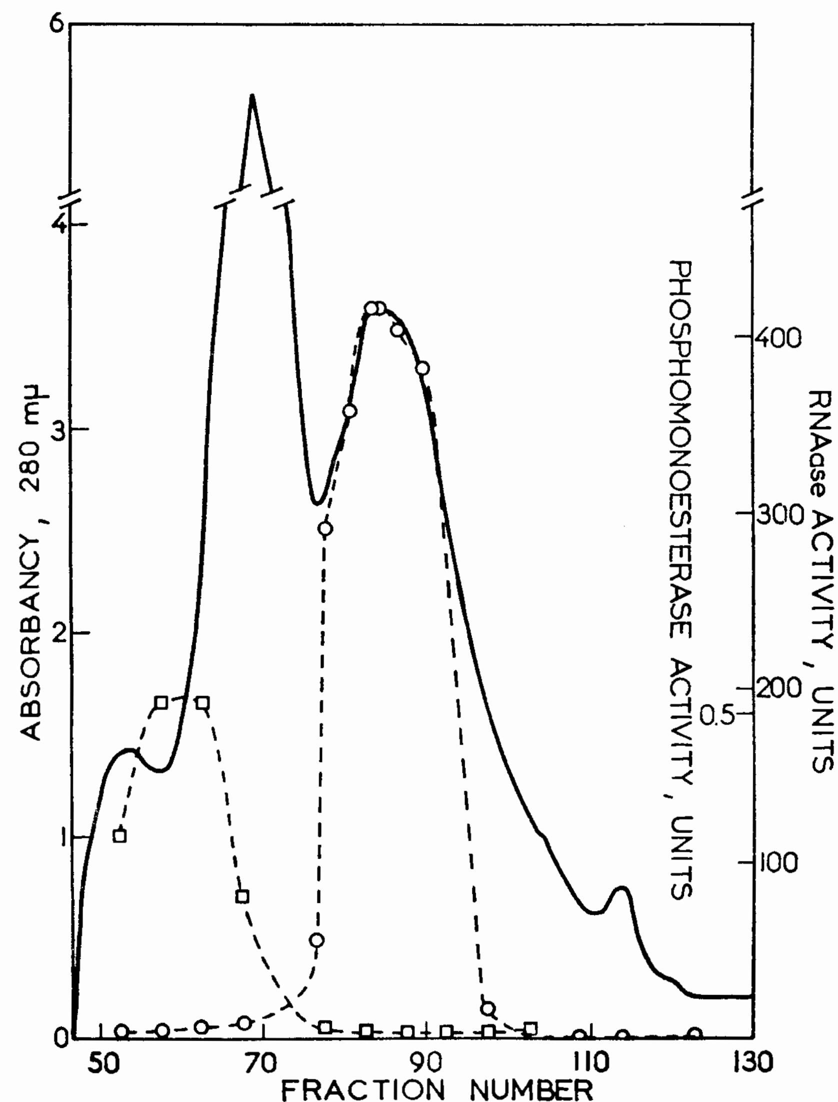

Fig. 2. Gel filtration on Sephadex G-100 of an acid ribonuclease–rich fraction obtained from DEAE-Sephadex. 70 ml ($OD_{280} = 26.00$; $OD_{260} = 15.80$) were loaded on a 4- × 90-cm column of Sephadex G-100 equilibrated with 0.05 M phosphate buffer, pH 6.8. The same buffer was used for the elution. 10-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Squares indicate the phosphomonoesterase activity (right-hand inner scale). Fractions 77–96 were processed further.

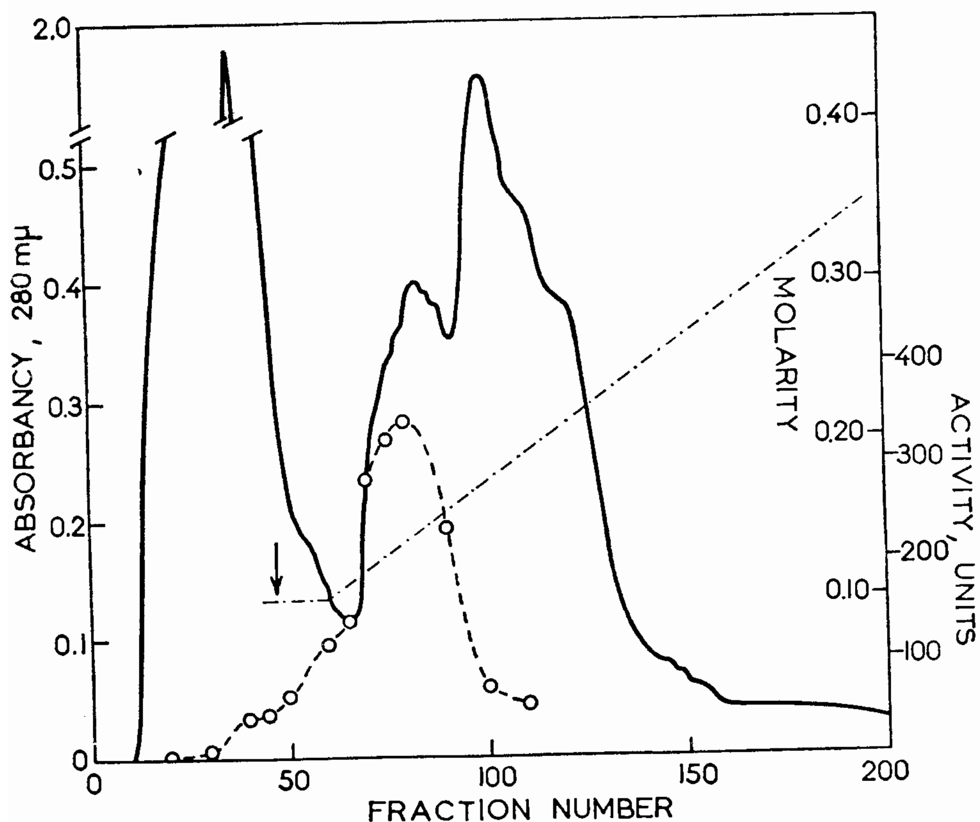

Fig. 3. Chromatography on hydroxyapatite of acid ribonuclease fractions from Sephadex G-100. 200 ml ($OD_{280} = 3.40$; $OD_{260} = 2.78$) were loaded on a 2- × 40-ml column of hydroxyapatite. Elution was carried out with a molarity gradient (0.1–0.5) of phosphate buffer, pH 6.8; this was started at the fraction indicated with an arrow (right-hand inner scale). 10-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand outer scale). No phosphomonoesterase activity was detected in fractions 20–120. Fractions 60–90 were processed further.

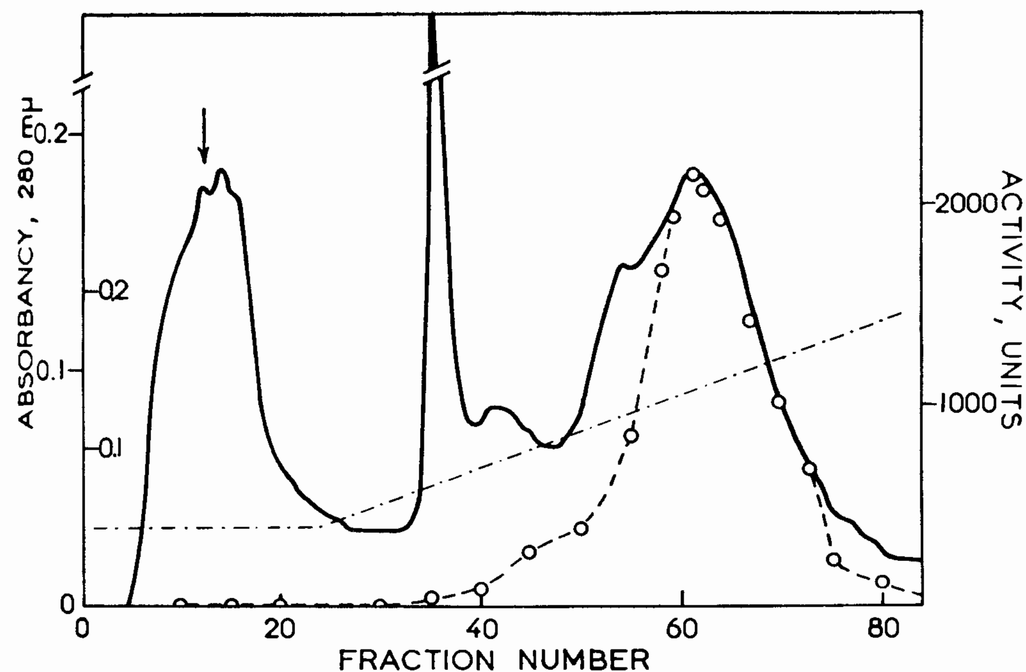

Fig. 4. Chromatography on CM-Sephadex C-50 of acid ribonuclease fractions from hydroxyapatite. 120 ml ($OD_{280} = 0.67$; $OD_{260} = 0.40$) are loaded on a 2- × 40-cm column of CM-Sephadex C-50 equilibrated with 0.05 M phosphate buffer, pH 6.0. Elution is carried out with a molarity gradient (0.05–0.4 M) of phosphate buffer. This was started at the fraction indicated by the arrow (left-hand inner scale). 10-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Fractions 58–72 were processed further.

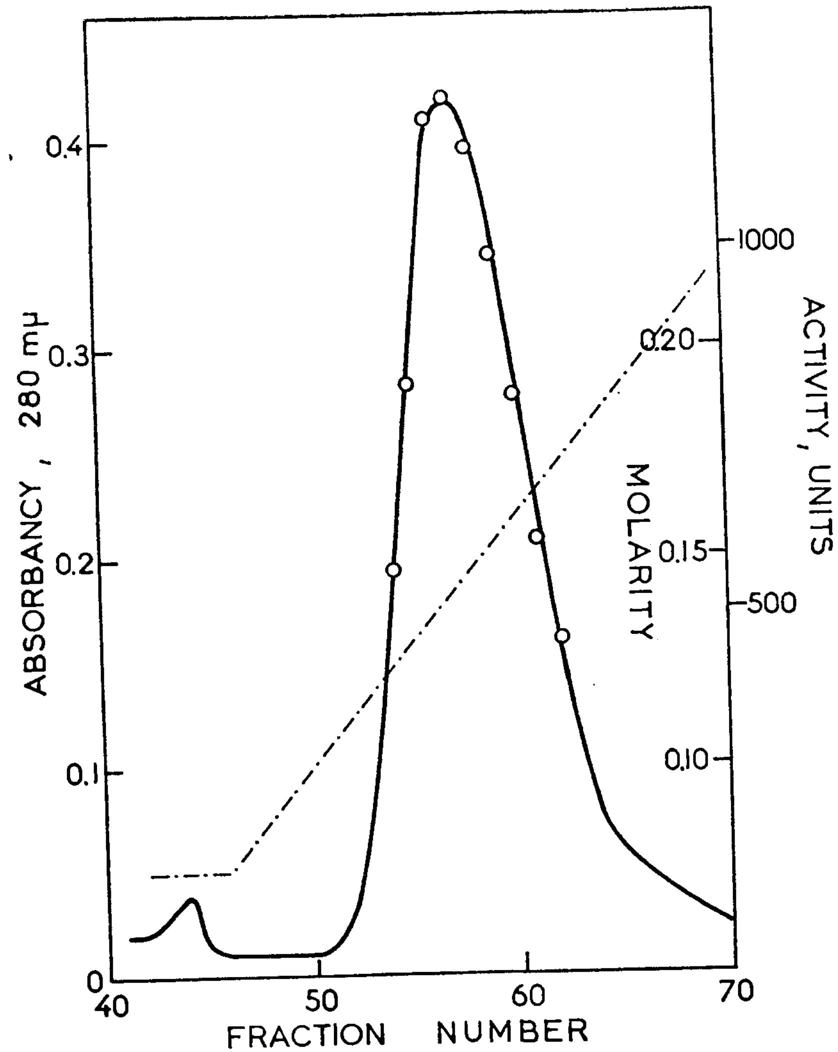

Fig. 5. Chromatography on hydroxyapatite of acid ribonuclease fractions from CM-Sephadex. 183 ml ($OD_{260} = 0.104$; $OD_{280} = 0.064$) are loaded on a 1- × 13-cm hydroxyapatite column equilibrated with 0.05 M phosphate buffer, pH 6.8. A molarity gradient (0.005–0.3) of phosphate buffer was used to elute acid ribonuclease (right-hand inner scale). 4.6-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand outer scale). Fractions 53–60 were processed further.

The ribonuclease activity is then loaded on a hydroxyapatite column equilibrated with 0.05 M phosphate buffer and eluted with a 0.05–0.3 M molarity gradient. The ribonuclease activity is eluted at 0.12 M phosphate; the central fractions of the peak show a constant specific activity. They are run through a Sephadex G-25 column equilibrated with 0.004 M acetate buffer, pH 5.0, and then they are concentrated by freeze-drying to an $OD_{280} = 1.2$. The concentrated enzyme solution referred to as the final product in Table 1 is then frozen and stored at −60 C. Alternatively, the enzyme may be freeze-dried and stored at 60 C without any loss in activity.

Properties of the Enzyme

Spleen acid ribonuclease as obtained by this method is completely free of the following activities: phosphodiesterase as assayed on bis(*p*-nitrophenyl) phosphate, basic ribonuclease, and acid deoxyribonuclease. Its pH optimum is close to 5.3.

It is highly active on yeast-soluble RNA and on RNA "core," the water undialyzable RNA oligonucleotides resistant to pancreatic ribonuclease. Among synthetic polyribonucleotides, polyadenylic and polyuridylic acid are attacked, whereas polycytidylic acid is highly resistant.

The enzyme is heat labile; incubation at 55 C for 20 min causes a 50% loss in activity.

REFERENCES

1. BERNARDI, A., and G. BERNARDI. In preparation.
2. MAVER, M. E., and A. E. GRECO. 1962. J. Biol. Chem. *211:* 907.