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Purification and Properties of Acid Ribonucleases in Human Serum and Leukocytes[1]

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ABSTRACT

Acid RNase was purified from normal human serum about 2400-fold by chromatography on phosphocellulose and Sephadex G-75 and rechromatography on Sephadex G-75. Assayed with yeast RNA as substrate, the enzyme showed the maximal activity at about pH 6.5 with sodium phosphate buffer. The reaction was activated by Na^+, K^+, and spermine, but it was not affected greatly by Mg^{2+}, Co^{2+}, and EDTA. Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} inhibited the reaction. Among the synthetic substrates examined, the enzyme preferentially hydrolyzed pyrimidine nucleotides, with a higher affinity for polycytidylate than for polyuridylate. The enzyme was thermolabile, but it stabilized with bovine plasma albumin. The molecular weight was approximately 15,000, estimated by gel filtration on Sephadex G-75, and its isoelectric pH was above 11.0.

From normal human leukocytes, acid RNase was purified about 400-fold by the same procedure described previously except that rechromatography on Sephadex G-75 was omitted. The properties of leukocytic RNase were found to be similar to those of serum acid RNase, but the latter enzyme differed in substrate specificity substantially from leukocytic RNase, preferring polyuridylate to polycytidylate. This evidence shows that serum RNase is not of leukocytic origin under normal physiological conditions.

INTRODUCTION

There have been many reports that serum alkaline RNase activity is increased in various diseases (7, 9, 11, 14, 15, 17, 20, 30) and that the major factors associated with its elevation are considered to be a negative nitrogen balance (3, 23, 24) or impairment of renal function (9, 14, 19, 20). According to the report of Houck and Berman (14), alkaline RNase activity in serum positively correlated with the number of leukocytes, but the origin of the enzyme in serum has been a matter of dispute, and it has been both claimed (12–14) and denied (11, 22) that it arises from WBC.

In addition to alkaline RNase, acid RNase has been also shown to exist in human serum (22). However, few studies on the enzyme have been made, and little valuable information is available. The properties of the enzyme are still unclarified, and even its change of activity under physiological as well as pathological conditions is poorly understood.

In considering any pathogenetic meanings for these serum RNases, it appears important to analyze their properties with purified preparations and to know the origin of the enzymes. Therefore, we recently purified and characterized serum alkaline RNases and found that they existed in at least 5 multiple forms (1). This observation then prompted us to isolate acid RNase from normal serum and leukocytes that may be the probable origin of serum RNases. In the accompanying paper (2), purification procedures and properties of acid RNases from leukemic serum and leukocytes will be described.

MATERIALS AND METHODS

Chemicals. Yeast RNA was purchased from Sigma Chemical Co., St. Louis, Mo. Synthetic polynucleotides were purchased from Miles Laboratories Inc., Elkhart, Ind. Crystalline bovine plasma albumin, Fraction V, was obtained from Armour Pharmaceutical Co., Kankakee, Ill., and ovalbumin, myoglobin and cytochrome *c* were obtained from Schwarz/Mann, Orangeburg, N. Y. Calcium bis(*p*-nitrophenyl) phosphate and *p*-nitrophenyl phosphate were products of Daiichi Pure Chemical Co., Tokyo, Japan. [^{32}P]DNA was prepared from *Escherichia coli* as described previously (28). Phosphocellulose was purchased from Brown Co., Berlin, N. H. Sephadex G-75 was purchased from Pharmacia, Uppsala, Sweden. Seamless cellulose tubing (pore size, 25 Å) was purchased from Visking Co., Chicago, Ill., and anampholyte preparation, pH 9 to 11, was purchased from LKB-Produkter AB, Bromma, Sweden.

Preparations of Serum and Leukocytes. Human serum and leukocytes were obtained by venipuncture from fasting healthy volunteers. Leukocytes were isolated by the method of Christlieb *et al.* (8) from venous blood.

Substrate. The substrate solution for acid RNase was prepared by dissolving 3 g of yeast RNA with 30 ml of 1 M sodium phosphate buffer (pH 6.5) and dialyzed against 2 liters of 0.05 M sodium phosphate buffer (pH 6.5) at 4° for 78 hr with 3 changes of dialysis medium. The substrate was finally adjusted to 0.5% solution with the same buffer. This extensive dialysis of RNA was essential to reduce the absorbance of control mixture without enzyme below 0.05 at 260 nm in the following enzyme assay. The substrate solution for alkaline RNase was prepared as described previously (1).

Enzyme Assay. The standard assay of acid and alkaline RNases was performed in reaction mixture (1.05 ml) containing 1 ml of 0.5% yeast RNA and 0.05 ml of sample. After incubation at 37° for 30 min, the reaction was stopped by the addition of 0.2 ml of 3% perchloric acid containing 0.5% uranium acetate, and then the reaction vessel was kept on ice for 10 min. The cloudy reaction mixture was centrifuged at 2000 × *g* for 5 min at room temperature. A 0.2-ml aliquot

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of the resulting supernatant was diluted with 3 ml of distilled water, and the absorbance of acid-soluble nucleotides was measured at 260 nm with a Beckman Model DU spectrophotometer. One unit of acid and alkaline RNase activities was defined as an $A_{260\ nm}$ increment of 0.01 under the conditions described previously. RNase assay with synthetic polymers as substrate was done by the method of Zimmerman *et al.* (29).

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (16), with bovine plasma albumin as a standard.

Molecular Weight Determination. The molecular weight of the purified enzyme was estimated by using a column of Sephadex G-75 (2.5 x 100 cm) in a reverse-flow procedure with 0.02 M Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl. The flow rate was 25 ml/hr, and 5-ml fractions were collected. As molecular weight standards 5 mg each of ovalbumin (M.W. 45,000), myoglobin (M.W. 17,800), and cytochrome *c* (M.W. 12,400), were used.

Isoelectric Focusing. Purified enzyme (2 ml) was dialyzed against 1 liter of 1% glycine solution for 12 hr. The dialyzed fraction was subjected to electrophoresis in a 110-ml LKB electrofocusing column, as described by Vesterberg and Svensson (26). Carrier ampholytes, pH 9 to 11, were used to establish the pH gradient. The electrophoresis was run at 700 V for 50 hr at 4°. Fractions of 2 ml were collected, and their RNase activities and pH's were measured.

Assay of Other Enzymes. Acid and alkaline phosphatase activities were determined with *p*-nitrophenyl phosphate as substrate under the experimental conditions used by Bernardi (4) for measuring acid phosphatase and those described by Bessey *et al.* (5) for measuring alkaline phosphatase, respectively. Phosphodiesterase activity was measured by the method of Burton and Petersen (6). DNase activities were measured in the acidic condition reported previously (28) or in the modified alkaline condition in which acetate buffer was replaced with 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ without EDTA.

RESULTS

Purification of Serum Acid RNase

The following operations were carried out at 0–4° unless otherwise specified. The purification data are summarized in Table 1.

Phosphocellulose Chromatography. Human serum (100 ml) was dialyzed overnight in ice against 20 volumes of 0.01 M sodium phosphate buffer (pH 6.7) and centrifuged at 3000 × *g* for 15 min to remove the precipitates formed during dialysis. The supernatant was applied to a column (1.5 x 30 cm) of phosphocellulose, equilibrated previously with 0.01 M sodium phosphate buffer (pH 6.7). After the column was washed with 200 ml of the equilibrating buffer, 800 ml (400 ml each) of a linear gradient of elution from 0.1 to 0.5 M NaCl in the same buffer were applied. The flow rate was 35 ml/hr, and 10-ml fractions were collected. Alkaline RNase activity was eluted as RNases 1, 2, and 3, as previously reported (1), with about 0.33, 0.40, and 0.44 M NaCl, respectively; these RNases held the major part of RNase activity in normal serum. On the other hand acid RNase activity was eluted as the single peak with about 0.34 M NaCl (Chart 1). The active fractions (46 to 54) of acid RNase were combined and dialyzed overnight in ice against 20 volumes of 0.01 M sodium phosphate buffer (pH 6.7). For the concentration of the enzyme, the sample was added to a column (0.8 x 15 cm) of phosphocellulose, which had been equilibrated with the same buffer. The column was washed with 30 ml of the buffer and then eluted with 1 M NaCl in the equilibrating buffer. The flow rate was 15 ml/hr, and 1-ml fractions were collected.

Table 1
Summary of isolation procedures of acid RNases from human serum and leukocytes

Procedure step	Volume (ml)	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	% of recovery
Serum	100	91,500	8,030	11.4	100
Phosphocellulose	90	34,700	48.7	713	37.9
Sephadex G-75	69	19,000	0.984	19,300	20.8
Rechromatography on Sephadex G-75	45	8,080	0.293	27,600	8.8
Leukocyte extract	5	8,880	54.0	164	100
Phosphocellulose	42	5,480	22.1	248	61.7
Sephadex G-75	43	4,380	0.064	68,400	49.3

Sephadex G-75 Chromatography. The active concentrated fractions (3 ml) were adjusted to 5 ml with 0.02 M Tris-HCl buffer (pH 8.5) and added to a column (2.5 x 100 cm) of Sephadex G-75, which had been equilibrated with 0.5 M NaCl in 0.02 M Tris-HCl buffer (pH 8.5). The flow rate was 25 ml/hr, and fractions of 5 ml were collected.

Rechromatography of Sephadex G-75. The acid RNase fractions (65 to 78) in the previous step were combined, dialyzed, and concentrated to 5 ml with the same method on phosphocellulose. For further removal of acid RNase from overlapping alkaline enzyme, the concentrated sample was rechromatographed on Sephadex G-75, and fractions (66 to 74) that contained RNase activity of >10 units/ml were pooled.

The acid RNase from serum was purified about 2400-fold with a recovery of 8.8% (Table 1). The enzyme preparation obtained in this step was concentrated by phosphocellulose chromatography as described previously, dialyzed against 2 liters of distilled water, and then used for all subsequent studies.

Purification of Leukocytic RNase

A leukocytes mass that was isolated from 50 ml of blood was suspended in 5 ml of distilled water and homogenized in ice for 5 min with a Teflon homogenizer. The homogenate was dialyzed against 1 liter of 0.01 M sodium phosphate buffer (pH 6.7) and centrifuged at 3000 × *g* for 15 min. The supernatant was applied to a column (1.2 x 10 cm) of phosphocellulose by the same method as that used for serum RNase. After the column was washed with 30 ml of 0.01 M sodium phosphate buffer (pH 6.7), 240 ml (120 ml each) of a linear gradient from 0.2 to 0.9 M NaCl in the same

buffer were applied. Three-ml fractions were collected. Acid RNase activity alone was eluted with approximately 0.44 M NaCl, and the activity was detected also at an alkaline condition of pH 8.5 as a smaller peak (Chart 2). The active fractions (21 to 34) were combined, dialyzed, and concentrated to 5 ml by phosphocellulose. The concentrated sample was applied to the column (2.5 x 100 cm) of Sephadex G-75 as described in the purification of serum RNase, and the active fractions (64 to 73) were pooled. The leukocytic RNase was purified about 420-fold with 49% recovery (Table 1). The obtained enzyme was similarly concentrated by phosphocellulose, dialyzed against 2 liters of distilled water, and then used for all subsequent studies.

Properties of the Enzymes

The properties of acid RNases purified from serum and leukocytes were studied.

Optimal pH. The RNase activity at various pH values was measured in 0.05 M Tris-HCl buffer (for pH 7.5 to 9.0), 0.05 M sodium phosphate buffer (for pH 5.7 to 8.0), and 0.05 M acetate buffer (for pH 3.6 to 5.6). The pH optima of both RNases were around 6.5 with sodium phosphate buffer (Table 2).

Molecular Weight. The activities of both enzymes were eluted as a single peak on Sephadex G-75. By comparison of the relationship between the elution volumes and the

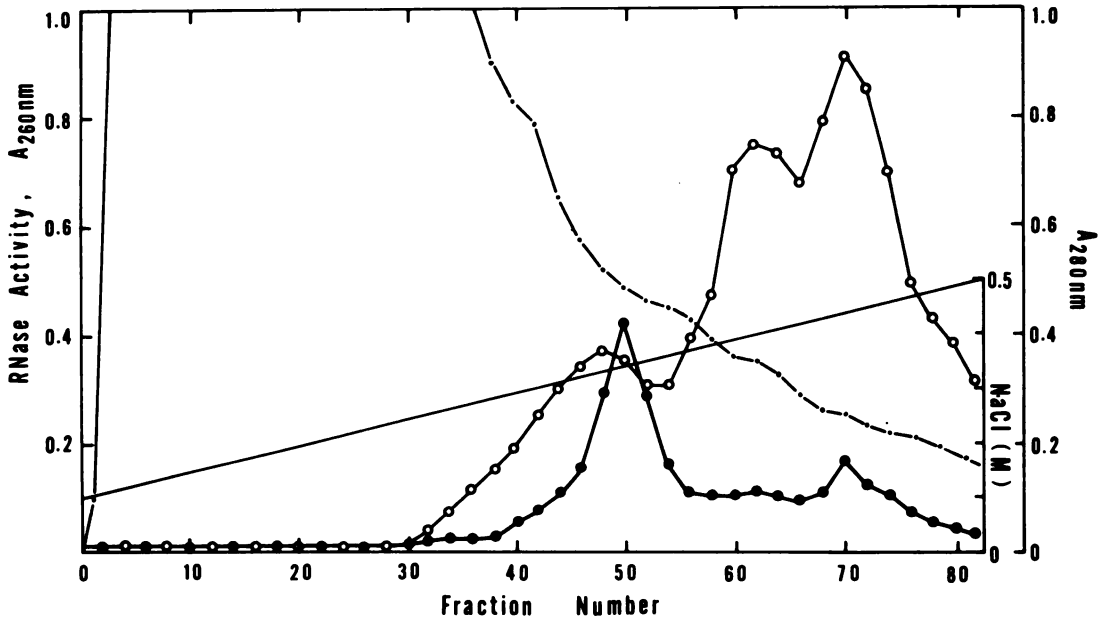

Chart 1. Phosphocellulose chromatography of human serum RNases. ●, RNase activity at pH 6.5; ○, RNase activity at pH 8.5; -·-·-, $A_{280\ nm}$; ——, linear gradient of NaCl.

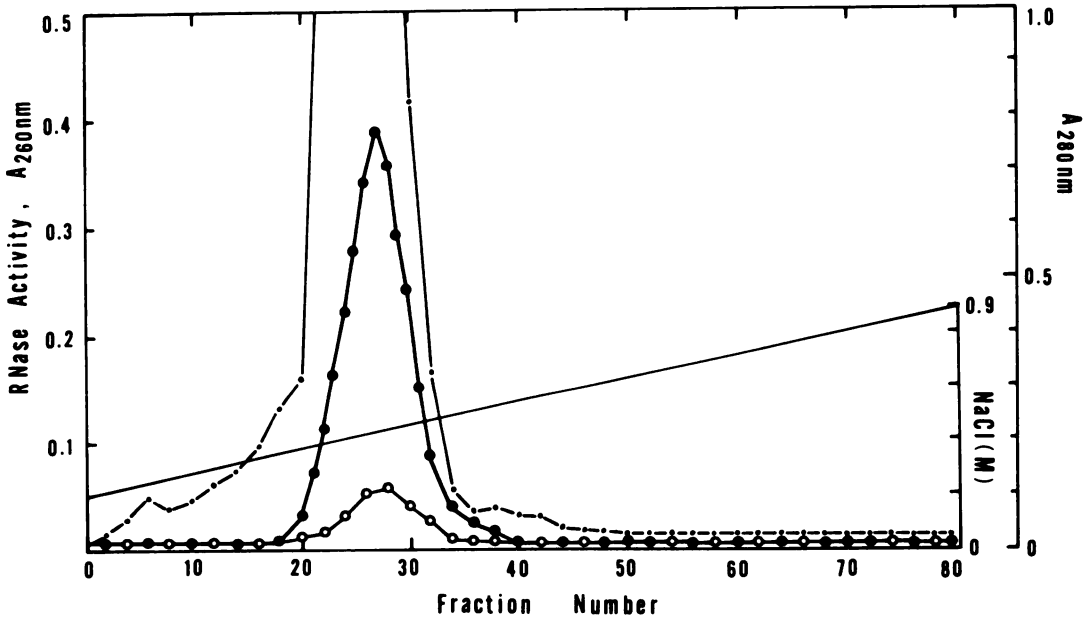

Chart 2. Phosphocellulose chromatography of human leukocytic RNase. ●, RNase activity at pH 6.5; ○, RNase activity at pH 8.5; -·-·-, $A_{280\ nm}$; ——, linear gradient of NaCl.

Table 2
Properties of acid RNases of human serum and leukocytes

	Optimal pH	M.W.	% of heat stability[a]			Isoelectric point	Effects of metal ions and chemicals										
			0.01 mg/ml protein	0.05 mg/ml protein	1.0 mg/ml protein[b]		None[c]	Na^{+d}	K^{+d}	Mg^{2+e}	Co^{2+e}	Ca^{2+e}	Fe^{2+e}	Zn^{2+e}	Cu^{2+e}	EDTA[e]	Spermine[e]
Serum RNase	acid	6.6	15,000	71	99	>11.0	100	125	118	108	99	52	50	27	7	114	142
Leukocytic RNase	acid	6.5	15,000	39	100	>11.0	100	133	129	107	100	50	45	28	12	116	147

[a] Remaining RNase activity (65°; 10 min). Control (which received no heat treatment) activity was taken as 100%.
[b] The protein concentration of each enzyme was adjusted to 1 mg/ml with bovine plasma albumin.
[c] Standard test activity in the absence of ions or chemicals was taken as 100.
[d] Fifty mM.
[e] Five mM.

logarithms of the molecular weights of standard proteins (ovalbumin, myoglobin, and cytochrome *c*) and the elution volumes of the final preparations, it was estimated that both enzymes had a molecular weight of about 15,000 (Table 2).

Stability. The stability of serum RNase has been reported to be variable, depending on the protein concentrations in the preparations (1, 27). For comparison of the stability of the enyzme at the same protein concentration, the samples were adjusted to 1 mg/ml by adding bovine plasma albumin. The remaining activities of each enzyme after heating at 65° for 10 min are presented in Table 2. The results indicate that both enzymes had similar stability under these conditions.

Isoelectric Point. Both enzymes are strongly basic proteins that were run at a pH above 11.0 on isoelectric focusing; thus definite isoelectric pH's could not be determined by this method (Table 2).

Activation and Inhibition. The activity of RNases has been known to vary with the presence of ionic species and chemicals in the reaction medium (1, 10, 18, 25). Hydrolyses of RNA by these isolated RNases at optimal pH values were activated by Na^+, K^+, and spermine. Mg^{2+}, Co^{2+}, and EDTA had little effect on the velocity of the reaction. Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} inhibited the reaction. Thus, both enzymes showed similar properties for these ions and chemicals (Table 2).

Substrate Preference. The acid RNase purified from serum degraded poly(C)[3] more than it did poly(U) to acid-soluble nucleotides and did not hydrolyze polyadenylate, polyguanylate, and poly inosinate, whereas the enzyme purified from leukocytes preferentially hydrolyzed poly(U) rather than poly(C) and did not hydrolyze other polymers. The poly(U)/poly(C) ratios of these 2 enzymes were 0.21 and 27, respectively; thus they were clearly different in substrate specificity (Chart 3).

Absence of Other Nucleolytic Enzyme Activities. None of the final preparations containing 30 units of each RNase showed any activities toward *p*-nitrophenyl phosphate at pH 5.4 or 7.4 for 2 hr and toward calcium bis(*p*-nitrophenyl) phosphate at pH 8.9 for 2 hr. Nor did these preparations depolymerize DNA at pH 5.4 or 7.5 for 1 hr, when assayed as described in "Materials and Methods."

DISCUSSION

Acid RNases have been highly purified and characterized from either serum or leukocytes in a normal human. These enzymes are extremely basic proteins with isoelectric pH above 11.0. Further comparative analyses have indicated that both RNases exhibited nearly identical properties in various aspects. *e.g.*, optimal pH, thermostability, effects of ions or chemicals, and molecular weight. However, it is of particular interest that they differed in substrate specificity.

Acid RNases in human serum and leukocytes have been reported. Ressler *et al.* (22) detected serum acid RNase activity, which was observed as the band behind γ-globulin by electrophoresis and of which maximal activity was observed at pH 5.2, but other properties were not described. Recently, Reddi (21) prepared acid RNase from the super-

[3] The abbreviations used are: poly(C), polycytidylate; poly(U), polyuridylate.

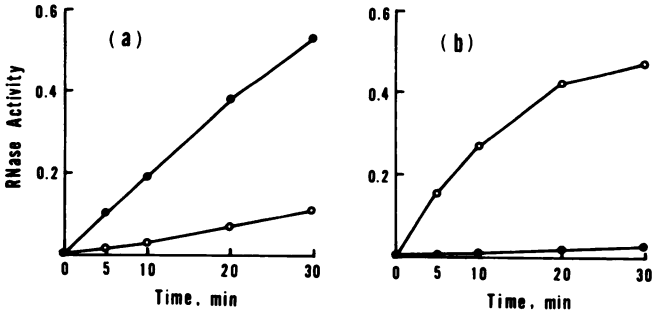

Chart 3. Time course of hydrolysis of synthetic polymers by serum acid RNase (*a*) and leukocytic acid RNase (*b*). Four units of serum RNase and 12 units of leukocytic enzyme were added to a reaction mixture (0.3 ml) containing 0.16 μmol phosphorus of poly(C) or poly(U) and 10 μmol of sodium phosphate buffer, pH 6.5. After incubation at 37°, for the indicated times, the reaction was stopped by the addition of 0.1 ml of 1% bovine plasma albumin in 0.15 M NaCl and 0.6 ml of 4% perchloric acid. After standing for 10 min at 0°, the precipitates were removed by centrifugation at 2000 × *g* for 5 min. The total supernatant was diluted with 2 ml of distilled water, and the absorbance was measured. ●, poly(C) at 278 nm; ○, poly(U) at 260 nm.

natant of serum by 7% HCl_4 precipitation and reported it as an acid RNase since its optimal pH, assayed with poly(C) as substrate, was 6.5. However, an additional separation of the same supernatant by phosphocellulose chromatography showed 3 active fractions of the serum RNases with an optimal pH of 8.5, determined with yeast RNA as substrate. These fractions are compatible with previously reported serum alkaline RNases I, II, and III (1), which are essentially different from the serum acid RNase obtained in this study. Therefore, this is the first report describing the purification of serum acid RNase of humans.

Regarding acid RNase in human leukocytes, Sznajd and Naskalski (25) purified the enzyme with optimal activity at pH 6.8 and with a molecular weight of 15,500. This enzyme hydrolyzes poly(C) but not polyadenylate. The main products of yeast RNA hydrolyzed with this enzyme are the mononucleotides poly(C) and poly(U). The substrate specificity for poly(U) was not examined. However, another study (11) has shown that crude leukocytic RNase preferentially hydrolyzes poly(U). The acid RNase purified from leukocytes in this work is similar in its properties to these RNases, but in leukocytes there was no evidence of alkaline RNase which forms the major part of RNase in normal serum.

Earlier works (12–14) suggest that leukocytes are the primary source of serum RNase. These hypotheses, however, have not yet been proven clearly, probably due to scant investigations conducted with serum and leukocytic RNases simultaneously. In this study we purified the acid RNases of these 2 different sources, one from serum and the other from leukocytes, both of which had very similar properties except that the former preferred poly(C) to poly(U) as substrate, whereas the latter degraded poly(U) to a much greater extent than it did poly(C). The present results revealing a substrate specificity of serum acid RNase different from that of leukocytic enzyme and a lack of alkaline RNase in leukocytes may suggest that either serum alkaline RNase or acid RNase is not derived from leukocytes in normal subjects.

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