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RAT SERUM RIBONUCLEASE

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SUMMARY

Rat serum ribonuclease has been purified about 6000-fold. The optimal pH of this enzyme was 7–8. The purified enzyme was rather heat labile at pH 7.6. The K_m of this enzyme is 30% higher than that of the crystalline bovine pancreatic ribonuclease (polyribonucleotide 2-oligonucleotide-transferase, EC 2.7.7.16). No stimulatory or inhibitory effect on this enzyme was observed with EDTA, KCl, $MgCl_2$, *p*-chloromercuribenzoic acid, or mercaptoethanol.

INTRODUCTION

The presence of serum ribonuclease in several animals has been reported[1–6]. The characteristics of this enzyme remained to be elucidated, although some similarity exists in the properties of serum ribonuclease and of the crystalline pancreatic enzyme[6] (EC 2.7.7.16). In this paper we present the results of extensive purification and some properties of rat serum ribonuclease, and discuss some differences from bovine pancreatic ribonuclease.

MATERIALS AND METHODS

Materials

Yeast RNA and pancreatic ribonuclease (polyribonucleotide 2-oligonucleotide-transferase, EC 2.7.7.16) were from Worthington Biochemical Corporation. DEAE-cellulose (Brown Co.) was prepared by the method of PETERSON AND SOBER[7], and Amberlite CG-50 Type II was obtained from the Rohm and Haas Co., Philadelphia. *p*-Chloromercuribenzoic acid was a product of Wako Pure Chemical Ind. All other compounds were of the highest purity available from commercial sources.

Preparation of serum

Wistar rats weighing about 100 g, obtained locally, received food and water *ad libitum* at all times. Blood samples were removed by heart puncture under ether anesthesia, and serum was prepared by centrifugation.

Purification of RNA used as substrate for ribonuclease assay

Yeast RNA was dissolved in water, KOH added to pH 7.0, and additional water to give a 10% RNA solution, which was then precipitated with 1 vol. acid–ethanol[8], washed twice with acid–ethanol and 3 times with 0.2% $HClO_4$. The precipitate was dissolved in water, KOH added to pH 7.0, and sufficient water to make a 1% solution.

Assay of ribonuclease activity

In a final volume of 0.5 ml, the incubation mixture contained 0.1 ml of 0.2 M Tris–HCl buffer (pH 7.6), 0.2 ml of 1% purified RNA and enzyme preparation. In some cases, several reagents were added. The assay was run in duplicate when possible. The RNA was added last, and the incubation was carried out at 37° for 20 min. Then 0.5 ml of precipitating agent (1 M HCl in 76% ethanol) was added and the mixture shaken well. The soluble fraction was separated by centrifugation. One ml of the clear supernatant was added to 3.0 ml of water in a test tube. The diluted solution was mixed well, and $A_{260\ m\mu}$ was determined. Under the conditions of the assay, the reaction rate was proportional to the amount of enzyme, and the reaction rates were linear with time during the periods employed.

A unit of activity was defined as an $A_{260\ m\mu}$ increment of 1.0 at 20 min. 0.02 ml of original serum shows 2 units of ribonuclease activity in this assay condition. 0.01 μg of pancreatic ribonuclease has almost the same activity as 0.02 ml of serum. Specific activity was expressed as units of ribonuclease activity per mg of protein.

Protein was determined by the method of LOWRY *et al.*[9].

RESULTS

Purification of ribonuclease from rat serum

All steps were carried out in the cold room at 4°.

Heat treatment

10 ml of 0.1 M citrate–phosphate buffer (pH 3.0), was added to 30 ml of rat serum and the mixture was heated at 60° for 5 min with stirring in a water bath, then cooled in ice water, and centrifuged at 10 000 × g for 10 min. Although this step achieves little enzyme purification on a protein basis, the subsequent step is much more efficient after this condition has been effected.

Dialysis

The supernatant was dialyzed against cold distilled water for 3 h with three changes of the outer fluid. The resulting precipitate was discarded by centrifugation.

Amberlite CG-50

The dialyzed solution (about 40 ml) was applied to an Amberlite CG-50 Type II column (2 cm × 15 cm), which had been equilibrated with 0.1 M phosphate buffer (pH 6.7). The elution was carried out using 250 ml of 0.1 M of phosphate buffer (pH 6.7), in the mixing vessel and 1 l of 1 M NaCl in the reservoir, and 5-ml fractions were collected. The ribonuclease activity was thereby eluted in dissociation with the main protein peak, as shown in Fig. 1.

DEAE-cellulose treatment

The fractions containing the enzyme activity (tubes No. 38–65) were combined. The combined solution was lyophilized and dialyzed against cold distilled water for

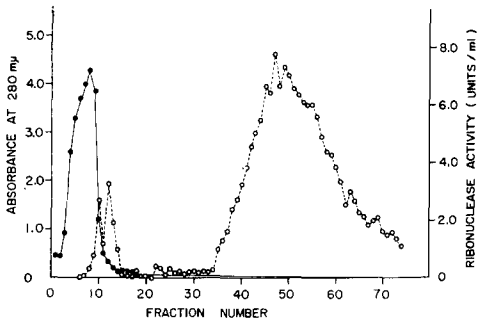


Fig. 1. Elution pattern of rat serum ribonuclease from Amberlite CG-50 column. ●—●, $A_{280\ m\mu}$; ○- - - - -○, activity of ribonuclease. Experimental conditions were described in the text.

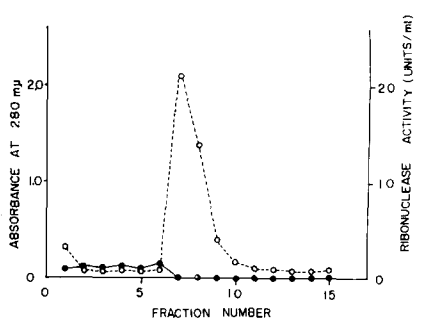


Fig. 2. DEAE-cellulose column chromatography of rat serum ribonuclease. ●—●, $A_{280\ m\mu}$; ○- - - - -○, activity of ribonuclease. Experimental conditions were described in the text.

3 h with three changes of the outer fluid. The solution was applied to DEAE-cellulose column (1 cm × 3 cm), which had been equilibrated with 0.01 M phosphate buffer (pH 6.7). The elution was carried out using 150 ml of 0.01 M phosphate buffer (pH 6.7), in the mixing chamber and 200 ml of 1 M NaCl in the reservoir, and 5-ml fractions were collected. The enzyme activity was eluted in the tubes No. 6–10, as shown in Fig. 2. As can be seen from the figure, Fractions 6–10 did not contain sufficient protein to be detectable by $A_{280\ m\mu}$. The fractions containing the enzyme were combined, then dialyzed against 0.01 M phosphate buffer (pH 6.7), and used as the purified enzyme.

Summary of purification

Table I summarizes the results of the purification experiment starting from 30 ml of rat serum. As is shown, the final preparation represented a 6000-fold purification, and the yield was 10% of the original total activity.

TABLE I

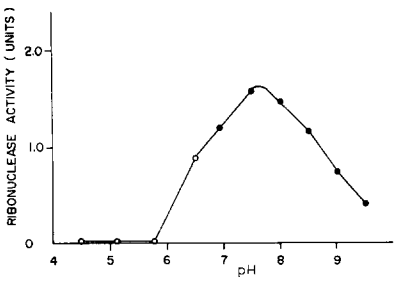
PURIFICATION OF RAT SERUM RIBONUCLEASE

Step	*Total vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*
Serum	30	1800.00	1762	0.98	100
Heat treatment	40	1020.00	988	0.96	58
Dialysis	40	780.00	776	1.00	44
Amberlite CG-50	120	1.84	658	357.00	37
DEAE-cellulose	10	0.03	174	5800.00	10

Properties of purified ribonuclease from rat serum

Optimum pH

The pH optimum for the purified rat serum ribonuclease was found to be 7.5. Activity at pH 6.0 or lower was not found (Fig. 3).



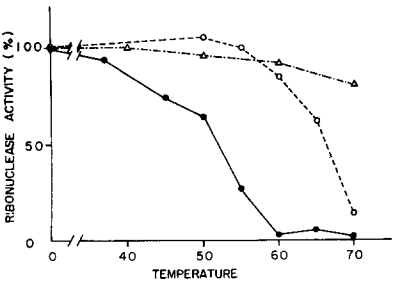


Fig. 3. pH-activity curve of the most purified enzyme preparation. Standard assay conditions with 0.1 ml of the purified enzyme were used at various pH values as indicated. ○, acetate buffer; ●, Tris–HCl buffer.

Fig. 4. Thermal stability of original rat serum preparation, the most highly purified rat serum ribonuclease and bovine pancreatic ribonuclease. The enzyme activity was assayed after 5-min exposure in 0.05 M Tris–HCl buffer (pH 7.6) at the indicated temperatures. Assay conditions were described in the text. △—·—·—△, crystalline pancreatic ribonuclease; ●—●, purified rat serum ribonuclease; ○— — —○, original rat serum ribonuclease.

Effect of substrate concentration on enzyme activity

The purified enzyme preparation was compared to the pancreatic enzyme in its behavior towards different substrate concentrations. The results, plotted according to Lineweaver–Burk, show that the K_m value is consistently 1/3 higher for the purified serum ribonuclease than for crystalline bovine pancreatic ribonuclease. These results are in agreement with previously reported results[6].

Heat stability

Fig. 4 shows the results of an experiment in which purified enzyme, original serum preparation and pancreatic ribonuclease were heated in 0.05 M Tris–HCl buffer (pH 7.6), for 5 min at various temperatures.

TABLE II

EFFECT OF ACTIVATOR AND INHIBITOR ON RAT SERUM RIBONUCLEASE

The most highly purified enzyme preparation (0.1 ml) was employed to assay the activity. Assay conditions were described in the text.

Addition	*Concn. (mM)*	*Ribonuclease activity (units)*
None	0	1.4
EDTA	3.3	1.5
	17.0	1.1
p-Chloromercuribenzoic acid	0.23	1.4
KCl	1.7	1.7
	8.3	1.6
$MgCl_2$	1.7	1.4
	8.3	1.2
Mercaptoethanol	3.1	1.4
	15.0	1.3
Rat liver ribonuclease inhibitor*	5 μg	0.2
	10 μg	0.1

* Rat liver ribonuclease inhibitor was purified by the method of MORIYAMA *et al.*[10].

Heating the original serum preparation at 55° had little or no effect upon ribonuclease activity; however, purified enzyme was destroyed after 5 min at 60°. The activity in original serum was destroyed only after heating at 70° for 5 min. In the same assay conditions, however about 10–20% of the activity: of crystalline pancreas ribonuclease was destroyed.

Activitor and inhibitor

As shown in Table II no stimulatory or inhibitory effects on the activity of the purified enzyme preparation were observed by adding a metal chelating agent; EDTA, KCl, $MgCl_2$, *p*-chloromercuribenzoic acid or mercaptoethanol. The purified ribonuclease from rat serum was inhibited in the presence of partially purified ribonuclease inhibitor from rat liver supernatant[10].

DISCUSSION

The experimental results shown above have confirmed the previous observation that rat serum has high ribonuclease activity[6]. Serum ribonuclease has been shown to be similar to crystalline pancreatic ribonuclease in various respects[6]. Although the enzyme described in this paper cannot be distinguished from the bovine pancreatic enzyme completely, it seems that the former enzyme differs from the latter in several points. Heat stability at pH 7.6 of both enzymes is not the same. The kinetic data show that the K_m of the serum ribonuclease is consistently higher than that of pancreatic enzyme, and pancreatic enzyme is inhibited by *p*-chloromercuribenzoic acid, $MgCl_2$ or Macaloid[11], whereas the serum enzyme is not inhibited by them under similar conditions. Further characterization, of course, including substrate specificity of serum ribonuclease, must be studied to distinguish from pancreatic ribonuclease or others.

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