A RAPID AND SIMPLE METHOD FOR THE PURIFICATION OF RAT LIVER RNase INHIBITOR

C. Gagnon and G. de Lamirande

Laboratoires de Recherche, Institut du Cancer de Montréal Hôpital Notre-Dame and Département de Biochimie, Université de Montréal, Montréal, Québec, Canada.

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SUMMARY

A rapid and simple method for the purification of rat liver alkaline RNase inhibitor from a 105,000 g supernatant is reported. It involves protein precipitations by $(NH_4)2SO_4$ and chromatography on carboxymethyl cellulose-RNase column. The purification procedure gives a 1020-fold increase in specific activity with a yield of 32%. This purified inhibitor can be stored for 5 weeks without any loss in activity.

INTRODUCTION

Ribonuclease inhibitors have been found in mammalian, plant and bacterial cells (1-7). In rat liver this inhibitor prevents the degradation of the rapidly labeled nuclear RNA (8), polysomes (10-11) and polyribosome-like particles (12).

Although methods for the isolation and purification of the inhibitor have been described, they are tedious and time consuming (5,6,13-15). The present report deals with a rapid and simple method of isolation giving a recovery of 32% and 1020 fold purification from a 105,000 g supernatant. The effect of salt on the stability and the activity of the inhibitor are also reported.

MATERIALS AND METHODS

Highly polymerized yeast RNA was purchased from Calbiochem,

pancreatic ribonuclease A type XIIA, gelatin (300 bloom), and bovine serum albumin from Sigma Chemical Corp., EDTA (disodium salt) from J.T.Baker and carboxymethyl cellulose-RNase-A, batch R5, from Miles Seravac Laboratories.

The activity of pancreatic RNase and of the inhibitor was determined by a method based on those of Gribnau et al (14) and of Shortman (6). The reaction mixture contained 0.4 ml of buffer (0.5 M Tris-HCl pH 7.8 and 0.04M EDTA), 0.2 ml of RNase (1 ng/ml of a 0.1% gelatin solution), 0.2 ml of phosphate buffer containing the inhibitor or of buffer alone (0.05M phosphate pH 6.0, 0.001M EDTA and 0.01M β-mercaptoethanol) and 0.4 ml of a solution of RNA at 4 mg/ml. After a 30 min. incubation at 37°C, an aliquot of 0.5 ml is acidified with 2.5 ml of cold 4% $HClO_4$ and the precipitate is centrifuged at 20,000g for 10 min. The absorbance of the supernatant at 260 nm is taken as a measure of the RNA breakdown. Appropriate controls without RNase or RNA were run concurrently. This method is sensitive enough to detect the activity of 0.166 ng/ml of RNase.

The unit of inhibitor is defined as the amount necessary to produce a 50% inhibition of 1 ng of RNase.

Protein concentration was estimated by the phenol procedure of Lowry et al (16) using crystalline bovine serum albumin as standard.

RESULTS

A typical ribonuclease inhibitor purification experiment is described in detail below. Table I lists the protein concentration, the activity, the specific activity, the recovery and the purification at each step of the procedure. All operations were carried out at 0-4°C.

Table I. Purification of alkaline RNase inhibitor from rat liver 105,000 g supernatant.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Over-all recovery (%)	Purification (-fold)
Supernatant	3,390	50,000	14.8	100	1
Amm. sulf. 35-60%	1,870	45,000	24.1	90	1.63
CM-RNase column	1.06	16,030	15,120	32	1,020

Preparation of the supernatant. Wistar male rats of an average weight of 250 g were sacrificed by decapitation. The livers were excised, chilled on ice, blotted on filter paper and then minced in a cold plexiglass squeezer. The liver pulp was homogenized in a Potter-Elvehjem homogenizer (9 strokes) to give a 33% homogenate in the following solution: 0.35 M sucrose in 0.05M Tris-HCl buffer pH 7.6 containing 0.025M KCl, 0.01M Mg $(CH_3COO)_2$ and 0.01M β-mercaptoethanol. This homogenate was centrifuged for 2 hours at 105,000 g to obtain the supernatant from which the lipid layer on top was discarded.

Step 1. Ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ was gradually added to the supernatant to obtain a 35% saturation. The mixture, after 30 min. equilibration, was centrifuged for 10 min. at 20,000 g. The precipitate, which contained 2-4% of the total inhibitor activity, was discarded. The degree of saturation of the clear supernatant was brought to 60% by addition of $(NH_4)_2SO_4$. After centrifugation the supernatant, which did not contain any inhibitor activity, was discarded and the precipitate

was dissolved in the phosphate buffer described above. This step gives a purification factor of 1.63 and a recovery of 90%.

Step 2. CM-RNase chromatography. Carboxymethyl-cellulose-pancreatic RNase A (2 g) was rehydrated and washed six times with water and three times with the phosphate buffer. It was then poured into Whatman column (type PC 1020) with flow adaptors.

The precipitate obtained between 35% and 60% saturation was dissolved in phosphate buffer (same volume as the 105,000 g supernatant fraction) and applied directly to the CM-RNase column. The column was washed first with phosphate buffer (about 75 ml) and then with 0.2 M NaCl in the same buffer (about 40 ml). The inhibitor is eluted with 2 M NaCl in phosphate buffer (4 ml/hr). This step raises the purification factor to 1020 with a recovery of 32% as shown in Table I.

Stability of the purified inhibitor. Since the RNase inhibitor is very labile as found by many investigators (5,6,13-15), various substances have been tested to increase its stability during storage. Results are summarized in Table II. The inhibitor solution in 2 M NaCl-phosphate buffer obtained from the column was diluted ten times in buffer with or without the stabilizing substance to be tested. The samples were kept at -20°C for 5 weeks. The control without any addition retained only 15% of its original activity after 5 week storage as shown in Table II. Similar result was obtained when glycine was used as stabilizing agent. However albumin and $(NH_4)_2SO_4$ proved to be excellent stabilizers since 100% of the original activity was preserved. It might be added that the dilution is not a factor in the instability of the inhibitor since undiluted samples kept at -20°C for five weeks retained only 17% of the original activity as compared to 15% for the diluted samples.

Table II. The stabilizing effect of various substances on the RNase inhibitor.

Treatment	Activity (%) after 5 week storage
Control	15
Albumin 1 mg/ml	100
Ammonium sulfate 74%	100
Glycine 1 M	15

Since some of the substances tested as stabilizers have been shown to decrease the activity of RNase when present at high concentration (17,18), inhibitor activity might have been over estimated. Fig.1 shows that NaCl, $(NH_4)_2SO_4$ or glycine had no effect on the RNase inhibitor activity when their final concentration in the incubation mixture was lower than 0.1 M. Albumin at a final concentration up to 1.0 mg/ml was without effect on the inhibitor activity. Since the samples, in Table II, were diluted 300 times for their determination, none of the stabilizers could have caused an over estimation of the RNase inhibitor activity.

DISCUSSION

The purification of rat liver RNase inhibitor from a supernatant fraction has been achieved in less than 24 hours with a 1020 fold increase in specific activity and a yield of 32%. This rapid purification procedure is reproducible and eliminates dialysis or desalting of solutions, concentration by lyophilisation, DEAE and G-100 chromatographies necessary in previously published methods (5,6,13-15). The ammonium sulfate precipitation is essential since direct passage of the original liver supernatant through the

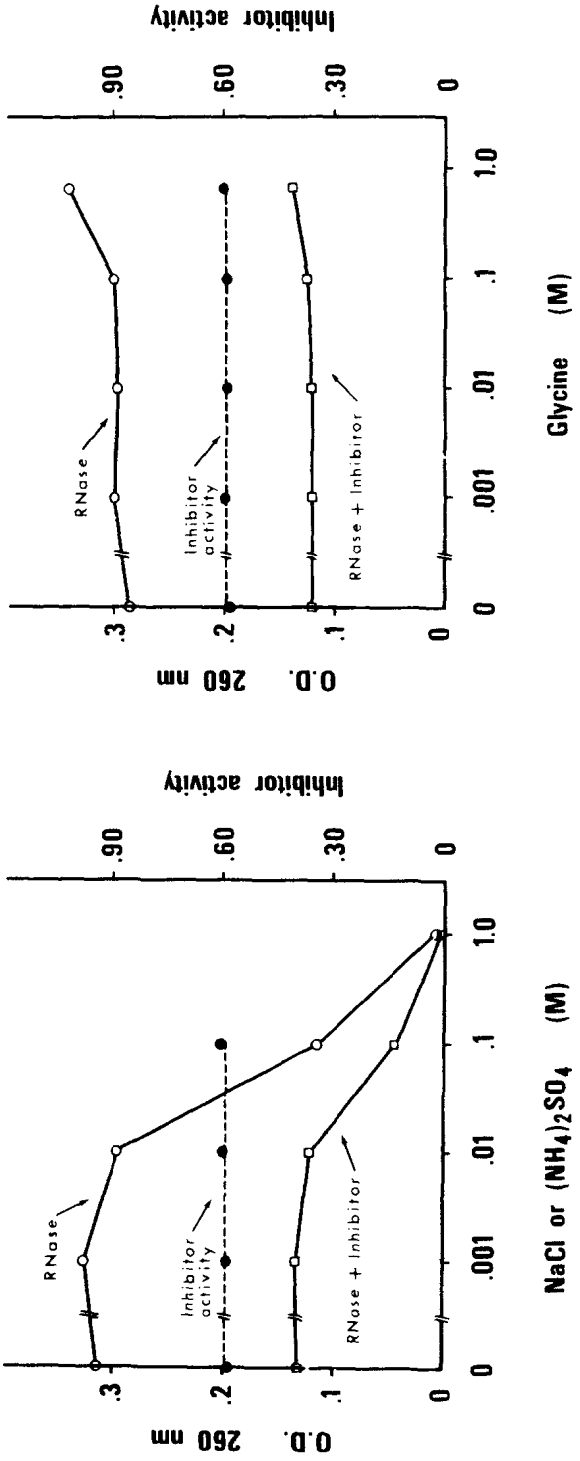

Fig.1: Effect of NaCl, $(NH_4)2SO_4$ and glycine on the RNase-Inhibitor system. The inhibitor activity is defined as the ratio of RNase activity minus the (RNase + inhibitor) activity over the RNase activity.

CM-RNase column gives a recovery of only 10% of the original activity.

The loss of activity observed at the CM-RNase step was partly due to non-adsorption on the column and to instability of the inhibitor during the adsorption and the washings at low salt concentration. The 2M NaCl used as eluant stabilizes the inhibitor which can be kept for at least 5 weeks at 4°C with only a 20% loss of activity. For a 100% preservation of activity the addition of ammonium sulfate at 74% saturation or of albumin at 1 mg/ml at -20°C is necessary.

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