DETECTION AND ESTIMATION OF VERY LOW RIBONUCLEASE ACTIVITIES IN BIOLOGICAL FLUIDS

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1. Introduction

Numerous RNA hydrolysing enzymes (RNAses) with manifold fragmentation patterns exist in many tissues. The ubiquitous presence of RNAses sometimes hinders severely the purification, preparation and characterisation of RNA or RNP particles. The different methods described for detection of RNAse activity are based on chemical analysis or radioactivity measurements; either on released acid soluble nucleotides or on the residual acid insoluble RNA after the enzymatic reaction [1–3]; a more refined detection of hydrolysis products can be obtained by polyacrylamide gel electrophoresis [4].

In the present study, we have worked out a new procedure to detect the presence and to estimate small quantities of RNAse activity. Iodine-labelled RNA with high specific activity, bound by 5′ terminal phosphate to beaded agarose and thereby insolubilized, was incubated with the sample to be tested for RNAse activity. With this method the presence of RNAse activities at a concentration down to 10^{-8} μg/ml expressed as pancreatic RNAse (EC 3.1.4.22) in the incubated sample, could be detected.

2. Materials and methods

2.1. *Extraction of the RNA*

Solid transplantable plasma cell tumor RPC_5, grown on Balb/C mice were used. RNA was extracted from plasma cell tumors by phenol at 0°C [5]. The extracted RNA, precipitated first with 2.5 vol ethanol, was dissolved in water and reprecipated with 1.5 M NaCl. RNA was dissolved in water.

2.2. *RNA labelling in vitro with iodine-125*

In vitro iodination with ^{125}I was found to be a simple and useful technique for the preparation of radioactive RNA. ^{125}I labelled RNA was prepared according to the procedure of Commerford [6] modified by Getz et al. [7]. (Specific activity of the ^{125}I-labelled RNA was 33 000 cpm/μg).

2.3. *Preparation of ECD-Agarose*

We used the method described by Porath et al. [8] for the preparation of epichloridrine desulphated agarose (ECD-agarose) with Sepharose 2-B (Pharmacia).

2.4. *Attachment of the ^{125}I-labelled RNA to ECD-Agarose*

The attachment of the RNA to ECD-agarose is influenced by factors, particularly pH and RNA concentration [9], ^{125}I-labelled RNA was bound to ECD-agarose following the technique of Wagner et al. [10]. According to these authors, the binding of nucleic acid to agarose occurs through the coupling of the terminal 5′ phosphate moiety of the ribonucleic acid to an hydroxyl group of the agarose. The work to test this hypothesis will be described in detail elsewhere [9] 10 ml of ECD-agarose in 10 ml of water were first activated with 2 g of CNBr. The pH was immediately adjusted to and maintained at pH 11 by

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titration with NaOH and then the gel was rapidly washed with a solution containing 40 mM MES* buffer pH 6 and 80% formamide. 300 μg of ^{125}I-labelled RNA in 2.0 ml of water were added to the activated ECD-agarose in the same MES-formamide solution and incubated for 14 h at 4°C. After this reaction, the gel was washed respectively with 100 ml of 1 mM phosphate buffer pH = 7 (90% formamide; 10 mM EDTA; 0.2% NLS); 100 ml of 10 mM Tris-HCl buffer pH = 7 (25% formamide, 700,mM NaCl; 10 mM EDTA) and 100 ml of water. Under these conditions, 23 μg of RNA per ml of ECD-agarose could be bound. The RNA–agarose can be stored several weeks at 4°C in phenol saturated water, or in MES–formamide solution.

2.5. *Assay of ribonuclease activity*

Ribonuclease activity was determined as follows. The reaction mixture contained 10 mM Tris-HCl pH 7.6, a fixed amount of ^{125}I-labelled RNA–agarose with a known amount of bound RNA and 0.1 ml aliquot of the enzyme solution at various predetermined concentrations, bringing the total sample volume to 1 ml. After incubation at 37°C for 30 min with slight stirring, the mixture was filtered on a 'combustocone, Packard', and washed with respectively 10 ml of 90% formamide solution, 10 ml of 25% formamide solution and 10 ml of bi-distilled water. The agarose fraction was then diluted in 2 ml of water and allowed to settle in a 1 ml disposable plastic syringe. The volume of gel was measured and the agarose was suspended in 2 ml of distilled water in a Packard vial. The sample was then mixed with 5 ml Insta-gel (Packard) by vigorous agitation. The radioactivity was measured by liquid scintillation spectrometry (Intertechnique ABAC/SL 40).

All the glass-ware and buffer solutions had been previously sterilized. Polystyrene plastic tubes were used. These tubes offer the advantage over glass tubes of providing a surface to which the agarose does not adhere.

3. Results and discussion

We consider the following enzymatic reaction:

RNAse + RNA∿Aga $\rightleftharpoons$ RNAse + rna∿aga + (RNA–rna)

RNA∿Aga: ribonucleic acid bound to agarose

rna∿aga: ribonucleic acid remaining bound after enzymic reaction.

RNA rna: ribonucleic acid break-down products released after enzymatic reaction.

The effect of the RNA–agarose as a substrate in the enzymatic reaction was studied. At various substrate concentrations for one given enzyme concentration a linear increase was obtained by the graphic representation of Lineweaver and Burk [11]. According to these results the Michaelis-Menten equation could be written as follows:

$$\frac{v}{V_{max}} = K\,\frac{(\text{RNA–Aga})}{1 + K\,(\text{RNA–Aga})} \qquad (1)$$

3.1. *Time course of hydrolysis*

Hydrolysis of RNA–agarose in the presence of RNAse was found to be time-dependent (fig.1). For a substrate concentration on 23 μg ^{125}I-labelled RNA

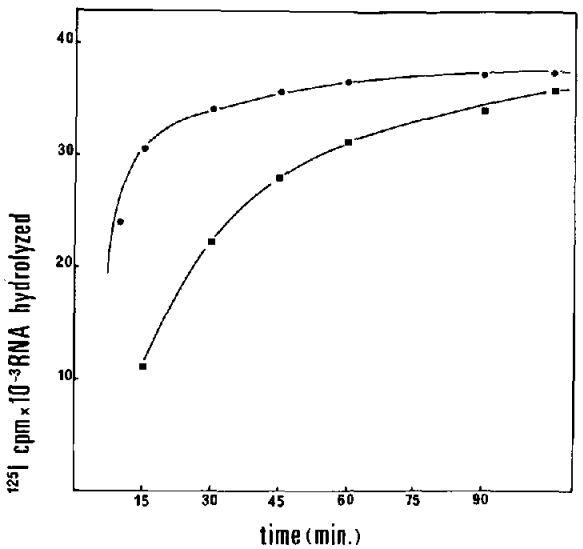

Fig.1. Time course of hydrolysis. 0.5 ml of RNA–agarose at a concentration of 23 μg of ^{125}I-labelled RNA per ml were incubated for different time periods with 2 different RNAse concentrations. (●–●) 10^{-2} μg RNAse/ml of sample. (■–■) 10^{-5} μg RNAse/ml of sample. The enzyme was ribonuclease A (Worthington Biochemicals).

* *Abbreviations:* MES 2 [*N*-Morpholino] ethanesulfonic acid; NLS *N*-Lauroyl sarcosine.

per ml of agarose, the hydrolysis by the RNAse at a concentration of 10^{-2} mg/ml proceeded as follows: after a short linear increase, the process of hydrolysis tended progressively towards a plateau which could be reached after nearly one hour; but in the presence of an RNAse concentration of 10^{-5} mg/ml, the time course of hydrolysis was different: it increased quite linearly for about 45 min and tended towards a plateau after 90 min.

3.2. *Effect of enzyme concentration*

The rate of hydrolysis of RNA was proportional to the RNAse concentration. It can be deduced from equation (1)

$$\frac{(\text{RNA}-\text{ma})}{(\text{RNA}\backsim\text{Aga})} = V_{\max}\frac{K}{1+K(\text{RNA}\backsim\text{Aga})} \qquad (2)$$

Fig.2a shows the effect of different enzyme concentrations on the hydrolysis of RNA–agarose at a concentration of 23 μg/ml of agarose. At a lower substrate concentration (6 μg of RNA/ml of agarose), the test became useful between 10^{-5} and 10^{-8} μg/ml of enzyme (see fig.2b).

For the detection of even lower enzyme concentrations, the RNA bound on the agarose would be decreased to 1 or 2 μg/ml; this is possible because the ^{125}I-labelled RNA can have a very high specific activity.

4. Applications

Different commercial preparations of enzymes, subcellular fractions or tissue extracts are sometimes contaminated by ribonucleases. As an application of our method, we tested the RNAse activity of different protein fraction as shown in table 1. We used ^{125}I-labelled RNA agarose (23 μg of RNA/ml of agarose) as substrate for the assay, in the same conditions as described in Materials and methods.

Using the graph on fig.2a, the different levels of RNAse activity in the tested sample could be estimated, expressed as pancreatic RNAse equivalent weight. By measuring the RNA remaining bound after the reaction, and calculating by difference the hydrolyzed RNA, the values obtained for the ratio, hydrolyzed RNA/total RNA, were calculated. These values, compared with those read on fig.2a for 0.5 ml of RNA–agarose, allowed an estimation of the RNAse concentration in the tested samples, by a choice of the curve which fits best with the determined value of hydrolyzed RNA/total RNA. By extrapolation, it is possible to estimate more accurately the RNAse concentration of samples with hydrolyzed RNA/total RNA lying between two curves.

In table 1, an RNAse activity is observed in the samples A and B. Only the buffer differed; in the sample A, the buffer was stored for two weeks at 4°C, which may have allowed some bacterial growth. In the sample B and C, where the buffer used was fresh and sterilized, a lower RNAse contamination was observed: for the sample of pronase (B), about 10^{-6} μg/ml expressed in pancreatic RNAse equivalent weight and less than 10^{-7} μg/ml of RNAse for the sample of proteinase K (C).

We have also tested a subcellular fraction of free

Fig.2. Effect of enzyme concentration. Various volumes of RNA–agarose as substrate (fig.2a and 2b with respectively 23 and 6 μg of ^{125}I-labeled RNA per ml of agarose) were incubated 30 min. with different RNAse A concentrations. (●—●)10^{-4} μg RNAse/ml. (◆—◆) 10^{-5} μg RNAse/ml. (■—■) 10^{-6} μg RNAse/ml. (▲—▲) 10^{-7} μg RNAse/ml. (○—○) 10^{-8} μg RNAse/ml.

Table 1

The reaction mixture contained 0.1 ml of the different protein sample: A and B Pronase grade B lot. 53.702 from Calbiochem, C, Proteinase K (fungal) lot. 4161359 from Merck; and D total protein of same free cytoplasmic ribonucleoprotein particles (dRNP) prepared as described elsewhere [12] are incubated 30 min at 37°C with 0.5 ml of ^{125}I-labelled RNA–agarose (23 μg of RNA fixed per ml of agarose) and 0,4 ml of 10 mM Tris/HCl pH 7,6 freshly prepared and sterilized. These different samples are compared with a standard sample incubated without protein fraction in sterile conditions. This standard sample contains 113 050 cpm of total RNA. The different operations (washing and counting) were as described in Materials and methods.

Fractions		Quantity of protein added	Hydrolyzed RNA	Hydrolyzed RNA / Total RNA	Estimation on contaminating RNAse μg/ml of reaction mixture[b]
(A)	Pronase in Tris/ HCl buffer stored 2 weeks at 4°C	0.10 μg	58 076[a] cpm	0.49	10^{-5}
(B)	Pronase in Tris/HCl fresh and sterilized buffer	0.10 μg	8667[a] cpm	0.08	10^{-6}
(C)	Proteinase K in Tris/HCl fresh and sterilized buffer	0.50 μg	1548[a] cpm	0.013	10^{-7}
(D)	Total protein from a dRNP fraction in Tris/HCl fresh and sterilized buffer	0.25 μg	13 254[a] cpm	0.11	10^{-6}

[a] Average of three experiments.
[b] Expressed in pancreatic RNAse equivalent weight (see fig. 2a)

cytoplasmic ribonucleoprotein particles (D). In this fraction prepared as described elsewhere [12], a sample of 0.25 μg contains about 10^{-6} μg of pancreatic RNAse equivalent weight.

5. Conclusion

The method we have developed allows the detection of pancreatic RNAse (EC 3.1.4.22) at a concentration as small as 10^{-8} μg/ml. The method is fast (see Materials and methods, 2.5.) and can be applied to the detection of RNAse activities in biological extracts, or contained in enzyme preparations. In the case of an unidentified RNAse activity, the quantity of enzyme can of course not be known but only its activity, but this activity can be expressed in pancreatic RNAse equivalent weight as we have done in table 1.

Each break in the RNA strand catalysed by the enzyme, results in a loss of RNA from the insoluble matrix. Thus RNA need not be degraded down to acid soluble products in order to allow the detection of a nuclease activity. In contrast to the classic methods, this fact confers on the described method a much higher sensitivity.

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References

[1] Kalnitsky, G., Hummel, J. P., Resnick, H., Carter, J. R., Barnett, L. B. and Dierks, C. (1969) Ann. N.Y. Acad. Sci. 81, 542.
[2] Kunitz, M. (1946) J. Biol. Chem. 164, 563.

[3] Kempf, J., Zahnd, J. P. and Mandel, P. (1970) Eur. J. Biochem. 17, 124.
[4] Loening, U. E. (1967) Biochem. J. 102, 251.
[5] Kempf, J. and Mandel, P. (1966) Bull. Soc. Chim. Biol. 48, 211.
[6] Commerford, S. L. (1971) Biochemistry 10, 1993.
[7] Getz, M. J., Altenburg, L. C. and Saunders, G. F. (1972) Biochim. Biophys. Acta 287, 485.
[8] Porath, J., Janson, J. C. and Laas T. (1971) J. Chromatogr. 60, 167.
[9] Egly, J. M. and Kempf J. in preparation for J. Chromatogr.
[10] Wagner, A. F., Bugianesi, R. L. and Smen, T. Y. (1971) Biochem. Biophys. Res. Commun. 45, 184.
[11] Lineweaver, M. and Burk, D. L. (1934) J. Am. Chem. Soc. 56, 658.
[12] Kempf, J., Egly, J. M., Stricker, Ch., Schmitt, M. and Mandel, P. (1972) FEBS Lett. 26, 130.