HIGH PURIFICATION OF THE RNASE INHIBITOR FROM RAT LIVER BY AFFINITY CHROMATOGRAPHY

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SUMMARY

A highly purified RNase inhibitor from rat liver can be obtained by chromatography on columns loaded with carboxymethyl cellulose-RNase. Polyacrylamide gel electrophoresis reveals that by this procedure a selective removal of non-inhibitor proteins has been achieved.

As a consequence this factor is now available for the preparation of undegraded polyribosomes and ribosomal subunits.

INTRODUCTION

Bont, Rezelman and Bloemendal (1965) described a polysome stabilizing factor present in rat liver cell sap which appeared to be identical with the RNase inhibitor discovered by Roth (1956). Crude rat liver cell sap, therefore, is not only a source of soluble enzymes but a very useful additive for the isolation of undegraded polyribosomes (Blobel and Potter, 1966) and ribosomal subunits (Gribnau, 1970). The advantage, however, of a highly purified preparation above a complex protein mixture like the 100,000xg supernate is obvious. Recently we described a 3,000-4,000 fold purification of the inhibitor isolated from rat liver 100,000xg supernate (Gribnau, Schoenmakers and Bloemendal, 1969). The purification included ammonium sulphate precipitation, chromatography on DEAE-Sephadex and gel filtration on Sephadex G-100. Although highly active preparations were obtained the polyacrylamide gel electrophoretic patterns still were rather complex (Gribnau *et al.*,1969). The present communication deals with a final purification step which is based on the property of the inhibitor to bind specifically to ribonuclease. This was achieved by chromato-

graphy on a column loaded with pancreatic RNase covalently linked to CM-cellulose according to the method of Mitz and Summaria (1961).

MATERIALS AND METHODS

As starting material the active eluate emerging from a Sephadex G-100 column was used. The isolation of this material has been described previously (Gribnau et al., 1969). The eluates from several columns were combined and desalted by passage over a column of Sephadex G-25 (1.5 x 25 cm), equilibrated in a medium consisting of 0.05 M sodium phosphate buffer pH 6.0, 10^{-3}M DTT and 10^{-3}M EDTA.

The desalted eluate was directly loaded onto a column of 1 gram of CM-RNase (1.0 x 3.5 cm) equilibrated in the same medium. After washing with the equilibration medium the inhibitor activity was eluted with 0.9 M NaCl in the same medium. A flow rate of as low as 5 ml per hour was maintained during the whole chromatographic procedure.

CM-RNase was a commercial product of Seravac Laboratories Ltd. (Enzite RNase, batch R3).

Estimation of RNase inhibitor activity has been described elsewhere (Gribnau et al., 1969).

Polyacrylamide gel electrophoresis was performed on cylindrical polyacrylamide gels as described by Bloemendal (1963). A 10% separating gel was used and electrophoresis was carried out at pH 8.9 in Tris-EDTA-boric acid (L.K.B. high resolution buffer). Routinely 50-100 µg of protein dissolved in 10 µl of the electrophoresis buffer were layered on top of the gels with the aid of sucrose. Electrophoresis was carried out at 5 mA per tube. Runs were terminated when the tracking dye reached a position of 0.5 cm from the lower end of the gel.

The gels were stained for 45 minutes in a solution containing 0.5% Amido Black B in 7% acetic acid and 50% methanol. Destaining was carried out by electrophoresis perpendicular to the length of the gels (25 mA per gel) in 2% acetic acid and was accomplished within 30 minutes.

RESULTS AND DISCUSSION

The concentrated and desalted eluate from the Sephadex

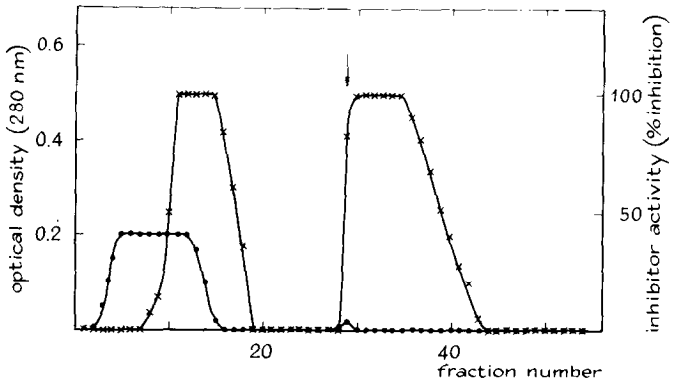

Fig. 1.

Chromatography on a CM-RNase column of the Sephadex G-100 RNase inhibitor fraction.

Several combined 70% ammonium sulphate precipitates of Sephadex G-100 RNase inhibitor fractions (approximately 40,000 units) were, after desalting on Sephadex G-25, applied onto the CM-RNase column, equilibrated in 0.05 M sodium phosphate buffer pH 6.0, 10^{-3}M DTT and 10^{-3}M EDTA.

After washing with the same medium, elution was started with 0.9 M NaCl in the medium (arrow). Fractions of 1.0 ml were collected.

Of each fraction 10 µl was tested in the standard RNase inhibitor assay (Gribnau et al, 1969).

o——o absorbance at 280 nm

x——x RNase inhibitor activity (per cent inhibition)

G-100 column was loaded onto the CM-RNase column. About 40,000 inhibitor units were applied.

A typical elution pattern of the RNase inhibitor activity emerging from the CM-RNase column is shown in fig. 1.

Based on the absorbance at 280 nm practically all protein emerged at the void volume of the column. In the fractions obtained after elution with 0.9 M NaCl no absorbance at 280 nm could be detected. However, in these fractions high inhibitor activity was observed (fractions 29-40, approximately 8,000 units) which means that an extremely high degree of purification has been accomplished. Part of the inhibitor activity did not adsorb onto the column although a slight retardation was observed with regard to the bulk of protein.

The existence of two different types of RNase inhibitor, as might be deduced from the elution pattern, could be excluded as rechromatography of the first protein-inhibitor peak on another CM-RNase column resulted again in a similar

distribution of free and bound inhibitor. Moreover, this distribution appeared to be independent on the elution rate.

Apparently the column used could only bind approximately 7,000-8,000 inhibitor units which is less than theoretically might be expected, taken into consideration the amount of RNase bound to the column. Presumably a considerable percentage of the total RNase molecules, covalently bound to CM-cellulose, are located at the inner site of the matrix and are not accessible to complex formation with the RNase inhibitor.

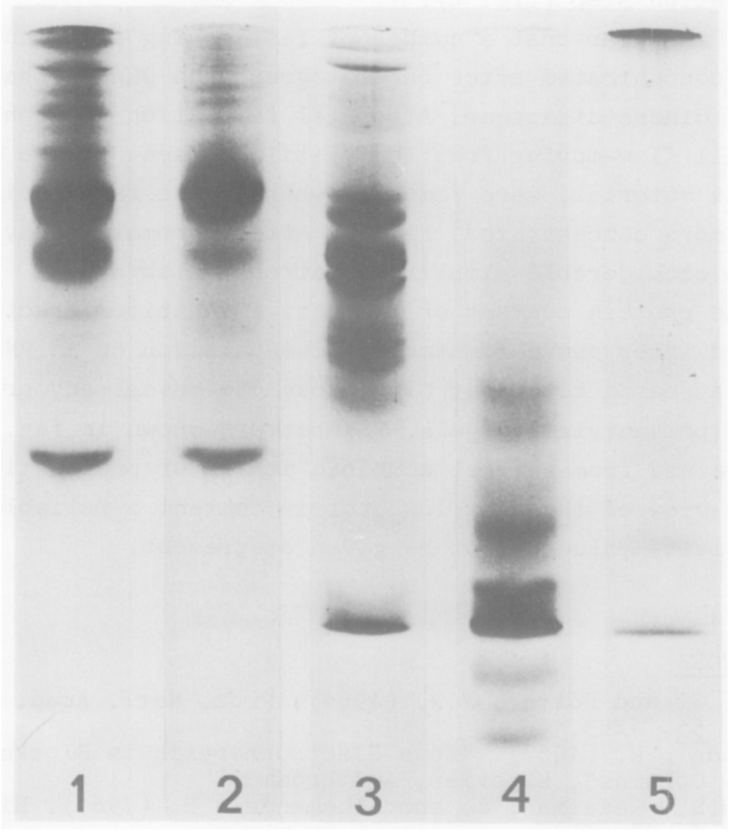

Fig. 2.

Polyacrylamide gel electrophoretic patterns of RNase inhibitor fractions obtained after each purification step.

Polyacrylamide gel electrophoresis was performed in 10% gels at pH 8.9 as described in the Methods section.

gel 1: 100,000xg supernatant fraction from rat liver
gel 2: 35-65% ammonium sulphate fraction
gel 3: DEAE-Sephadex fraction
gel 4: Sephadex G-100 fraction
gel 5: CM-RNase inhibitor fraction

As complex formation between RNase and RNase inhibitor is dependent on pH the chromatographic procedure was also carried out at other pH values. Maximal binding of the inhibitor on the polymer-bound RNase was observed at pH 6.0. At pH 7.5 no inhibitor could be bound onto the column, despite the fact that maximal complex formation was observed at this pH in the standard RNase inhibitor assay.

An impression of the increase in purity of the inhibitor can be deduced by comparison of the polyacrylamide gel electrophoretic patterns of the fractions obtained after each purification step (fig. 2).

It is clear that a number of fast-moving components became concentrated after chromatography on DEAE-Sephadex, whereas others disappear. After gel filtration on Sephadex G-100 all slow-moving fractions, still present in the DEAE-Sephadex material, were removed, whereas the fast-moving bands became more concentrated. Finally after chromatography on CM-RNase a considerable simplified pattern is obtained.

The protein content of the active inhibitor fraction obtained after one chromatographic separation on an RNase column appeared to be not high enough to reveal any protein band on polyacrylamide gels. The pattern shown in fig. 2,5 was obtained from several combined inhibitor preparations.

In view of the very low protein content a reliable specific activity value cannot be given at present.

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