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Note

Rapid purification of a ribonuclease from bovine seminal plasma by DNA-affinity chromatography

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A variety of affinity adsorbents have been developed in order to purify ribonucleases. Some of these utilize mononucleotide compounds as ligands. They can be classified into three broad groups depending on the coupling site of the mononucleotide to the matrix, *viz.*, (a) phosphate[1–4], (b) base[5,6] or (c) sugar[7–11]. So far, there has been only one report[12] of the use of DNA-Sepharose column chromatography —to isolate the ribonucleases RNAase II, III, H and polynucleotide phosphorylase from *Escherichia coli*.

In this paper we report the isolation to homogeneity, and in good yields, of a ribonuclease from bovine seminal plasma, based on DNA-affinity chromatography.

EXPERIMENTAL

Semen

Semen was obtained from Bharatiya Agro Industries (Pune, India) or India Detonators (Hyderabad, India). Seminal plasma was obtained from bovine semen by removal of spermatozoa by centrifugation at 600 *g* for 20 min in the cold, followed by centrifugation at 14,000 *g* for 30 min in the cold to remove any debris.

Isolation of bull sperm DNA

DNA was isolated from bull sperm by the method of Borenfreund *et al.*[13], as modified by Kumar[14].

Preparation of DNA-cellulose

DNA-Cellulose was prepared according to Litman[15], using bull sperm DNA.

Isolation of ribonuclease from bovine seminal plasma

Bovine seminal plasma was dialysed against 10 m*M* Tris–HCl buffer (pH 7.4) containing 1 m*M* EDTA and 0.15 *M* NaCl. Any precipitated matter after dialysis was removed by centrifugation at 600 *g* for 20 min in the cold. Usually, 5 ml of this seminal plasma was diluted to 100 ml in the above buffer, and loaded on a DNA-cellulose column previously equilibrated with the same buffer. The column was washed using the buffer until the washings showed no absorbance at 280 nm. It was then eluted either by applying a gradient from 0.15 to 1.5 *M* NaCl in the Tris–HCl

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buffer, or with the same buffer containing 1.5 *M* NaCl. At this stage, in most preparations, the eluted protein gave a single band on polyacrylamide gel electrophoresis (PAGE). However, occasionally some preparations gave more than one band on PAGE, and were further purified by gel filtration on a Bio-Gel P-60 column (110 × 1.5 cm). The preparations were resolved into four peaks. The second, major peak showed RNAase activity and gave a single band on PAGE.

Nucleolytic activity

The nucleolytic activity of the RNAase was measured by the method of Kalnitsky *et al.*[16], except that poly rU was used as the substrate instead of RNA.

The nuclease activity was also measured by the method of Scheit *et al.*[17], except that β-mercaptoethanol was not included in the assay buffer. ^{3}H-Labelled *E. coli* RNA was isolated according to the method of Reddy *et al.*[18], and used as the substrate in this assay.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out according to the method of Reisfeld *et al.*[19].

Analytical ultracentrifugation

The analytical ultracentrifugation runs were done at 60,000 rpm in an AnD rotor in a Beckman Spinco (Model E) ultracentrifuge equipped with Schlieren optics and an RTIC unit. The sedimentation coefficient of the RNAase was determined at

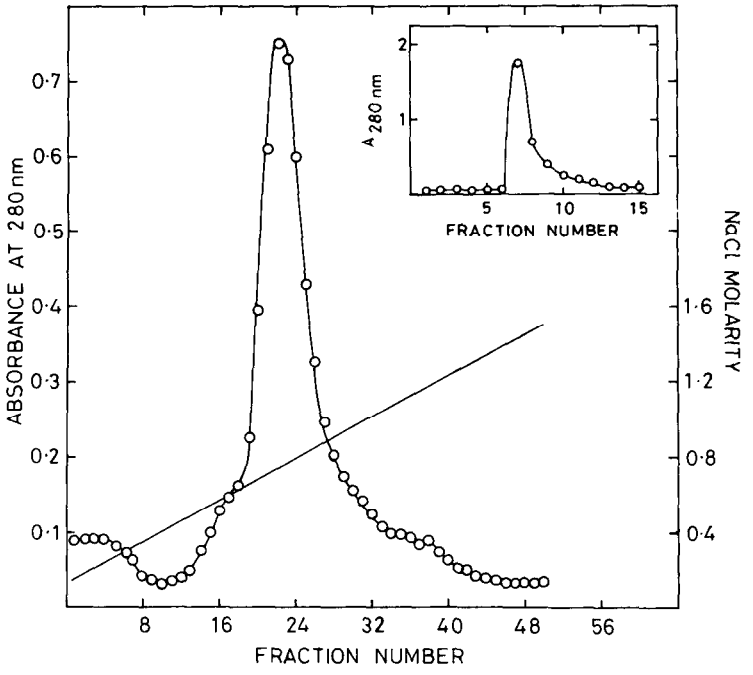

Fig. 1. DNA-cellulose chromatogram of RNAase. Protein loaded: 375 mg. Eluent: gradient from 0.15 to 1.5 *M* NaCl. Inset shows the elution of RNAase from a DNA-cellulose column directly by 1.5 *M* NaCl.

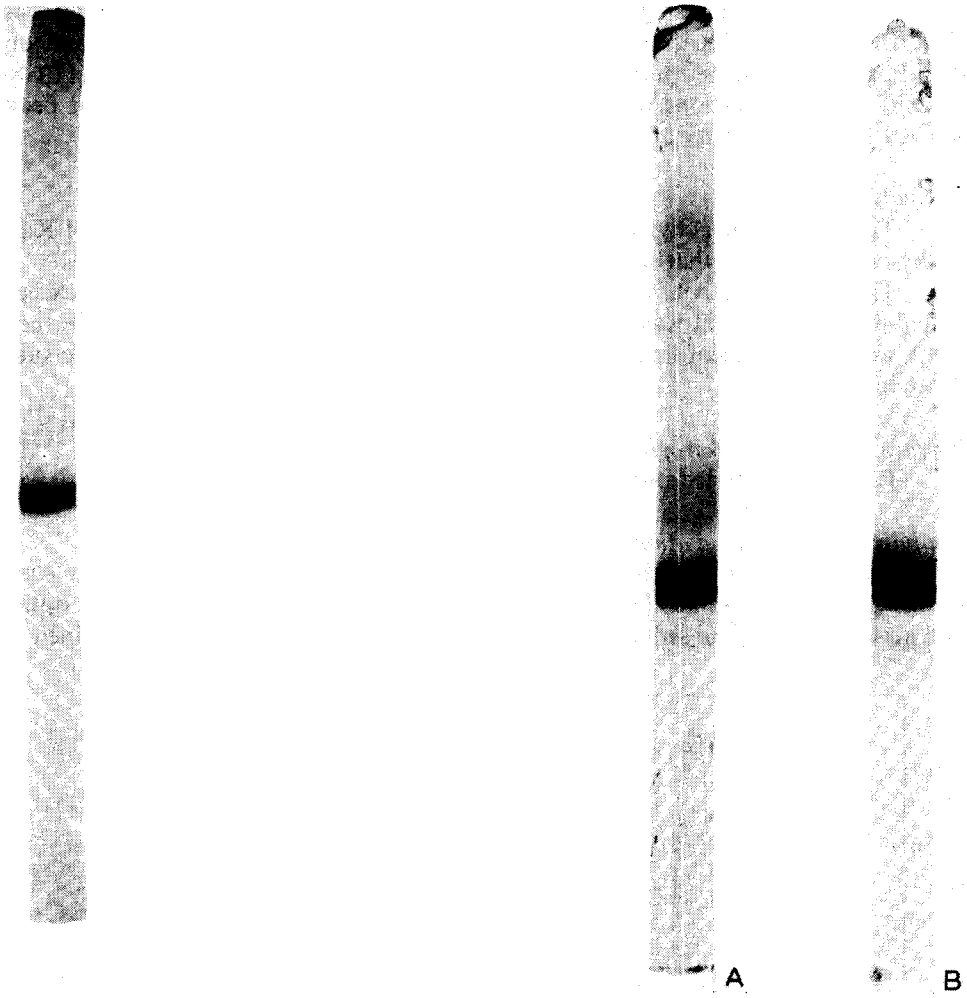

Fig. 2. Polyacrylamide gel electrophoretic pattern of RNAase. 100 μg of RNAase were loaded on the ge

Fig. 3. Polyacrylamide gel electrophoretic pattern of: A, RNAase preparations, before loading on Bio-Gel P-60; B, purified RNAase (peak 2 of Fig. 4) after passing through Bio-Gel P-60. 100 μg of protein were loaded in each case.

a concentration of either 0.5% or 1.0%. The apparent sedimentation coefficient was corrected for the temperature and viscosity of the solvent.

High-performance liquid chromatography (HPLC)

HPLC was performed on a Nucleosil 10 C_{18} column equilibrated with 0.1 *M* NaH_2PO_4, pH 2.8 (solvent A). 200 μg of the protein in 20 μl were loaded on the column and eluted using a logarithmic gradient (0–61.5%) of solvent B in solvent A, where solvent B was prepared by mixing 400 ml of 0.1 *M* NaH_2PO_4 and 600 ml of acetonitrile.

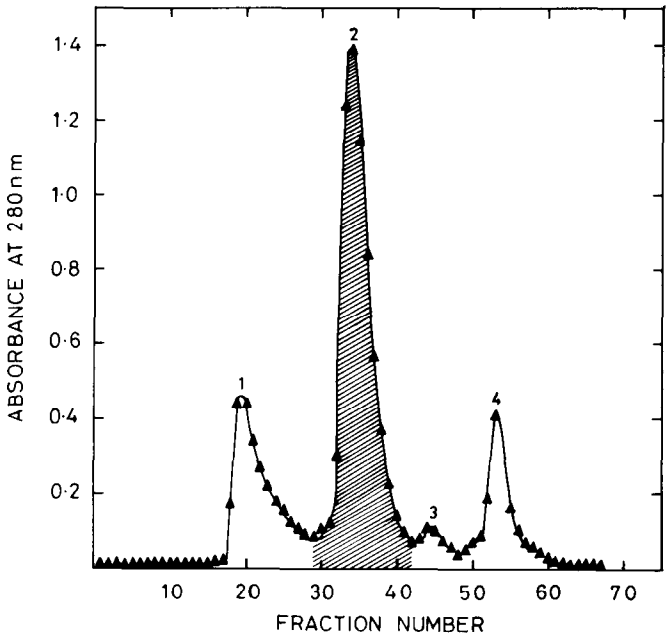

Fig. 4. Bio-Gel P-60 chromatogram of protein after DNA-cellulose chromatography. Peak 2 (shaded) showed RNAase activity. 3-ml fractions were collected with a flow-rate of 12 ml/h.

RESULTS

RNAase from bovine seminal plasma has been isolated by essentially a single-step DNA-cellulose chromatographic procedure (Fig. 1). The yield varied between 1.0 and 2.5% of the total seminal plasma proteins. The RNAase gave a single band on PAGE (Fig. 2). In some of the isolations the RNAase eluted upon DNA-cellulose chromatography gave more than one band on PAGE (Fig. 3A). These preparations were further purified on a Bio-Gel P-60 gel filtration column. The elution pattern of the Bio-Gel chromatography is shown in Fig. 4. Only the second peak had RNAase activity and gave a single band on PAGE (Fig. 3B) which was identical to the band in Fig. 3A. The RNAase gave a single symmetric peak on sedimentation analysis (Fig. 5). The sedimentation coefficient, $S_{20,w}$, of the RNAase at 0.5% (w/v) concentration was found to be 2.37. HPLC of the enzyme on a Nucleosil 10 C_{18} column gave a single peak (Fig. 6), the retention time of which was 20 min. The specific activity of the enzyme increased from 20.2 units/mg in seminal plasma to 862.5 units/mg in the purified RNAase, a 42-fold purification of the enzyme.

DISCUSSION

DNA-affinity chromatography is being increasingly used for selective binding of specific proteins. RNAases are known to have affinity for DNA[20,21]. Weatherford *et al.*[12] used single-stranded DNA-agarose for the isolation of RNAase II, III, H and polynucleotide phosphorylase from *E. coli*. We have used DNA-cellulose for the isolation of ribonuclease from bovine seminal plasma.

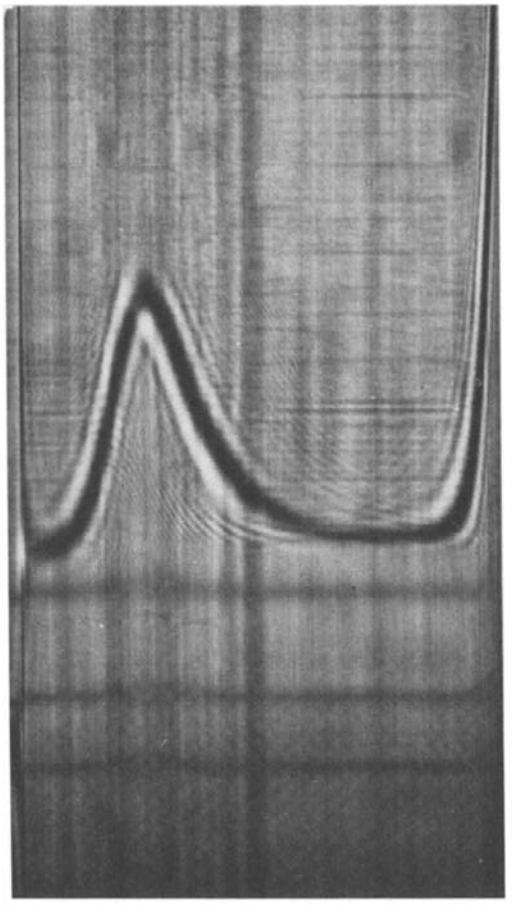

Fig. 5. Sedimentation pattern of the RNAase (1%, w/v). The photograph was taken 85 min after reaching maximum speed. Sedimentation proceeds from left to right.

The preparation of DNA-cellulose is simple and good flow-rates can be achieved. However, the amount of DNA that binds to cellulose may vary with the preparation of DNA. Smaller fragments of DNA bind weakly to cellulose. Moreover, slow leaching of the bound DNA may also occur. The DNA preparation needs to be thoroughly deproteinized before its adsorption to cellulose in order to prevent non-specific adsorption of other proteins to DNA-cellulose. In general, homogeneous preparations of the ribonuclease were obtained by DNA-cellulose chromatography, but some batches of RNAase were heterogeneous after such chromatography, possibly due to insufficient deproteinization of DNA. Bio-Gel P-60 chromatography of such DNA-cellulose eluted preparations gave four peaks. The second peak (50–60% of the total protein loaded) showed RNAase activity, and gave a single band on PAGE. In such preparations the yield was reduced from 2–2.5% to 1–1.5% of the total seminal plasma proteins. Other peaks from Bio-Gel P-60 chromatography did not show any RNAase activity.

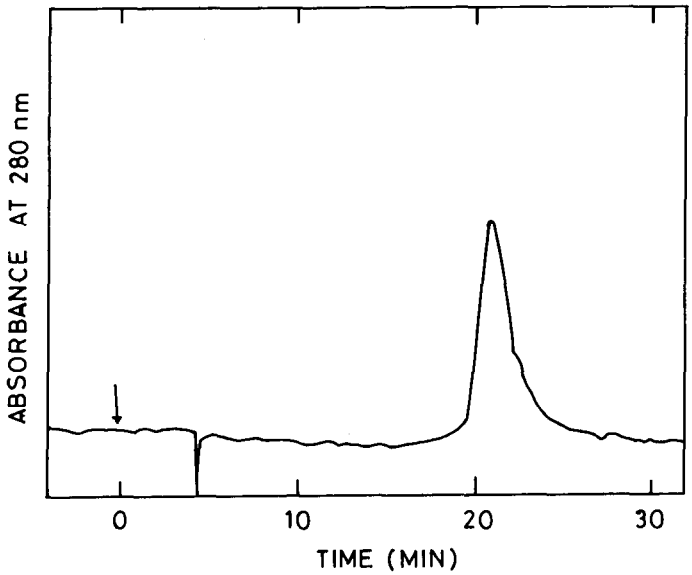

Fig. 6. HPLC of the RNAase on a Nucleosil 10 C_{18} column.

D'Alessio *et al.*[22] reported the isolation of a ribonuclease from bovine seminal plasma using conventional methods such as acid and heat extraction, ammonium sulphate fractionation, SE-Sephadex chromatography and Cellex-P chromatography. Their method resulted in a yield of RNAase of about 1.5% of the total seminal plasma proteins. The recovery of the RNAase was about 50%, indicating that the total RNAase content of seminal plasma may be about 3%. Reddy *et al.*[18] also reported the isolation of a RNAase from bovine seminal plasma, with yields of about 0.2% of the total protein. Our method gives a yield of about 2.5%. It is, therefore, obvious that the DNA-affinity chromatographic technique gives appreciably higher yields of pure RNAase when compared with the methods reported by Reddy *et al.*[18] and D'Alessio *et al.*[22]. In addition, the method is certainly superior in terms of simplicity, number of steps and the total time taken for the purification of RNAase. At this stage, we do not know whether the RNAase purified by DNA-cellulose chromatography is either the same as one of the enzymes reported by the previous workers or completely different. Further work in this direction is in progress.

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