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PURIFICATION OF RIBONUCLEASE T_1 ON POROUS GLASS AFFINITY ADSORBENTS

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SUMMARY

Purification of ribonuclease T_1 has been achieved using a porous glass–2′,(3′)-GMP affinity adsorbent. This adsorbent exhibits specific affinity for ribonuclease T_1 and is almost devoid of non-specific ion-exchange properties.

INTRODUCTION

Ribonuclease (RNase) A[1] and tobacco RNase[2,3] have been purified on Sepharose-based affinity adsorbents. The tendency of agarose gel beads to distort limits the use of agarose-based adsorbents for large-scale work. In addition, agarose is susceptible to microbial attack and cannot be regenerated from adsorbents for re-use. Porous glass beads have been used as an alternative insoluble matrix for affinity chromatography adsorbents[4,5]. They are resistant to distortion and microbial attack and can easily be regenerated for re-use by treating adsorbents with boiling nitric acid.

This paper reports the preparation of several porous glass derivatives and describes their use in the purification of RNase T_1.

MATERIALS AND METHODS

Sources of materials were as follows: Taka diastase (Parke & Davis, London, Great Britain); highly polymerized yeast RNA, calf thymus DNA, Corning CPG 10 (2000 Å pore diameter) 200-mesh glass beads (BDH, Poole, Great Britain); 3-aminopropyltriethoxysilane (Ralph Emanuel, Wembley, Great Britain); guanosine 2′,(3′)-monophosphoric acid (2′,(3′)-GMP) (Sigma, St. Louis, Mo., U.S.A.).

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Preparation of 5'-(4-aminophenylphosphoryl)-guanosine 2',(3')-monophosphate (APP-2',(3')-GMP)

APP-2',(3')-GMP was synthesized from guanosine 2',3'-cyclic monophosphate and 4-nitrophenylphosphate as described elsewhere[3].

Preparation of 1,5-dihydroxynaphthalene–glass (DHN–glass)

Porous glass beads were cleaned and reacted with 3-aminopropyltriethoxysilane in dry acetone[4]. The resulting aminoalkyl glass beads were converted to aminoaryl glass, diazotized[6], and added to a solution of 1,5-dihydroxynaphthalene (5 mg/ml) in ice-cold ethanolic sodium acetate (1 g glass per 20 ml). The mixture was stirred gently for 1 h at 4° after which the DHN–glass (Fig. 1) was collected by filtration. Unreacted dihydroxynaphthalene was removed by washing the product with ethanol. Finally the DHN–glass was washed with ether and air dried. Preparations were found to contain 90 to 110 μmoles dihydroxynaphthalene per gram of glass.

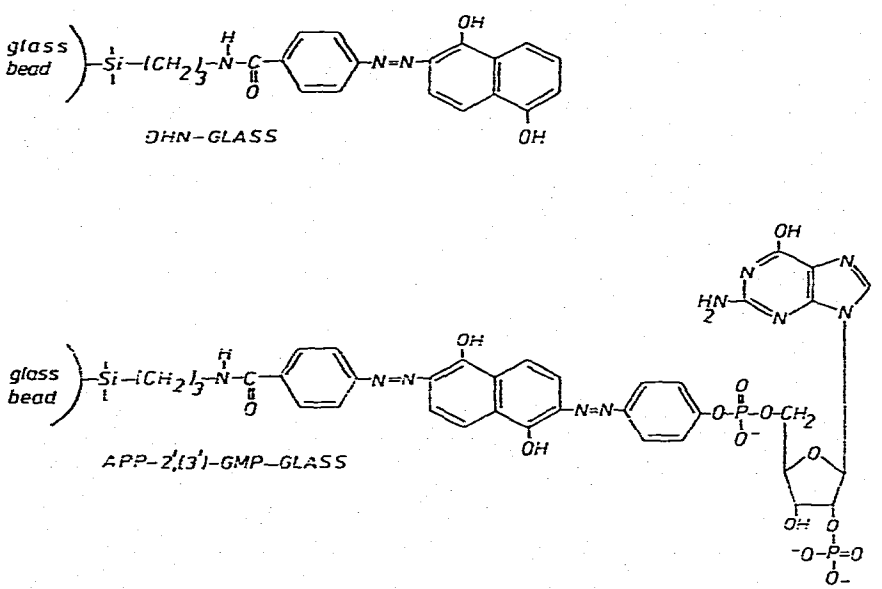

Fig. 1. Structures of porous glass derivatives.

Preparation of APP-2',(3')-GMP–glass

APP-2',(3')-GMP (200 μmoles) was dissolved in 5 ml of ice-cold 200 m*M* hydrochloric acid and diazotized by addition of 50 mg of sodium nitrite. After 10 min the solution was poured into a stirred suspension of DHN–glass (1 g) in 20 ml of ice-cold 500 m*M* pH 9.0 bicarbonate buffer. The reaction mixture was stirred for 1 h at 4°, after which the product was collected by filtration. The product was exhaustively washed, first with 200 m*M* ammonium acetate buffer pH 5.4 containing 500 m*M* KCl, and then with 200 m*M* Tris–HCl buffer pH 9.5 containing 500 m*M* KCl. This washing procedure was found to remove ionically bound material from the porous glass. Finally, the APP-2',(3')-GMP–glass (Fig. 1) was washed with distilled water

and stored at 4°. Preparations were found to contain 3–8 μmoles 2',(3')-GMP per gram of glass.

Preparation of RNA–glass and DNA–glass

Highly polymerized yeast RNA and heat-denatured calf thymus DNA were attached to aminoalkyl glass using glutaraldehyde as the coupling reagent[4]. Aminoalkyl glass was treated with 1% (v/v) ice-cold glutaraldehyde at 4° for 30 min. The treated glass was washed with cold distilled water and then packed into a small column (5 × 0.7 cm). Solutions of nucleic acid in 50 m*M* phosphate buffer pH 7.5 (1 mg/ml) were pumped over the treated glass for 24 h, at 4° using a peristaltic pump. The pumping ensures complete access of the nucleic acids to all surfaces of the porous beads during the coupling reaction. After coupling, derivatives were washed as described above to remove ionically bound material.

Preparations of RNA–glass contained 7.5 to 8.5 mg of RNA per gram of glass. DNA–glass preparations contained 10 to 12 mg of heat-denatured DNA per gram of glass. Control preparations made by omitting the glutaraldehyde treatment contained less than 0.5 mg of nucleic acid per gram of glass.

Determination of ligand content of derivatives

Derivatives were washed with ethanol and ether and air dried. Weighed samples of the dried derivatives were used to determine ligand content. Equal weights of aminoaryl glass were used as controls. Samples of derivatives were treated with orcinol–ferric chloride reagent[7] to determine GMP or RNA content, or with diphenylamine reagent[8] to determine DNA content.

The dihydroxynaphthalene content of DHN–glass was determined by treating a weighed sample of the derivative with a solution of sodium dithionite. The dithionite reduces the azo linkage binding the DHN to the glass and releases the DHN, which can then be measured spectrophotometrically.

Preparation of RNase T_1

RNase T_1 was partially purified from Taka diastase by extracting 2 g of the latter with 20 ml of 50 m*M* ammonium acetate buffer pH 5.4 containing 50 m*M* KCl (acetate–KCl buffer). Extracts were clarified by centrifugation and applied to a 4 × 100 cm column of Sephadex G-100, equilibrated and eluted with acetate–KCl buffer. Separation of RNase T_1 activity from RNase T_2 activity was achieved in this way. Fractions containing RNase T_1 activity were collected and used for affinity chromatography without further treatment.

RNase T_1 activity was assayed[9] using highly polymerized yeast RNA as substrate.

Column operation

Columns were made from small (0.7 cm I.D.) filter tubes containing sintered glass discs. These were packed to a height of 5 cm with glass derivatives suspended in acetate–KCl buffer. Solutions were pumped through the columns at a flow-rate of 30 ml/h using a peristaltic pump. Column effluents were monitored at 254 nm using an LKB Uvicord. Fractions were collected on an LKB Mini-rac fraction collector.

Elution of columns was carried out using the procedures described elsewhere for Sepharose–APP-2',(3')-GMP derivatives[3].

RESULTS AND DISCUSSION

APP-2',(3')-GMP–glass

RNase T_1 was completely adsorbed onto columns of APP-2',(3')-GMP–glass beads from solutions of Sephadex G-100-purified material. The contaminating proteins in the Sephadex preparations were not adsorbed by the glass derivative and passed through columns unretarded (Fig. 2). Elution of the bound RNase could be achieved either by changing the pH of the elution buffer (Fig. 2a), or by specific elution with substrate (Fig. 2b), or inhibitor (Fig. 2c). Elution of the bound RNase could not be achieved with an ionic strength gradient from 50 m*M* KCl up to 1 *M* KCl[3].

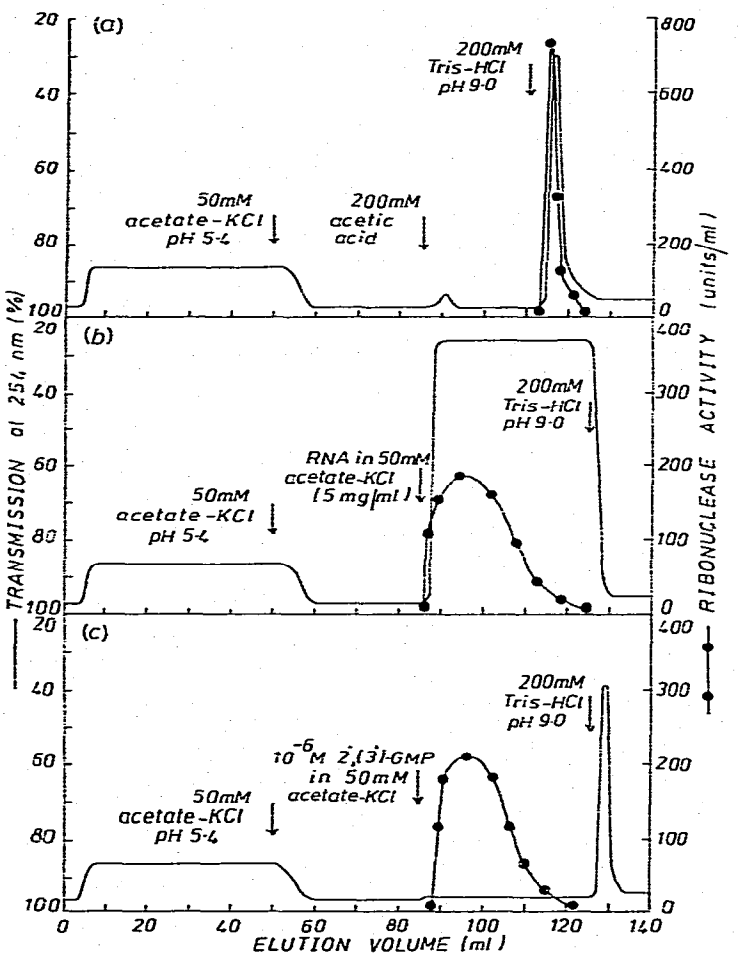

Fig. 2. Chromatography of Sephadex G-100-purified RNase T_1 on APP-2',(3')-GMP–glass. 50 ml of Sephadex effluent were applied to a 5-cm column of adsorbent and eluted as shown. (a) RNase was eluted by raising the pH of the eluting buffer to 9.0. (b) RNase was eluted by washing the column with a solution of RNA in 50 m*M* acetate–KCl buffer. (c) RNase was eluted by washing the column with 10^{-6} *M* 2',(3')-GMP in 50 m*M* acetate–KCl buffer.

Of the elution systems used, 10^{-6} *M* 2′,(3′)-GMP was the most satisfactory as this did not elute any of the non-specifically bound proteins.

DHN–glass, RNA–glass and DNA–glass

RNase T_1 was not adsorbed onto DHN–glass from Sephadex G-100-purified material. This result confirms that the APP-2′,(3′)-GMP–glass derivative functions as a specific affinity adsorbent for RNase T_1, rather than as a simple ion exchanger.

Neither RNA–glass nor DNA–glass bound RNase T_1. DNA–Sepharose has been used as an affinity adsorbent for *E. coli* RNase[10] and heat-denatured DNA does inhibit RNase T_1[11]. Evidently, the affinity of RNase T_1 for single stranded DNA is not sufficient to allow the latter to be used for affinity chromatography.

Purification of RNase T_1 on APP-2′,(3′)-GMP–glass

Purification of RNase T_1 on APP-2′,(3′)-GMP–glass beads yielded a product that appeared homogeneous on polyacrylamide disc gel electrophoresis[12,*]. The porous glass based adsorbent appeared to be at least as effective as similar Sepharose-based adsorbents. It also has the advantage that column flow-rate remained constant, even after prolonged use. The glass derivative was very stable under conditions used for RNase T_1 purification. However, very slight loss of ligand occurs at high pH, a problem also encountered with Sepharose-based adsorbents[13].

The porous glass adsorbents described here were primarily developed to aid the isolation of RNases from large volumes of waste liquor produced during plant protein manufacture[14]. In these liquors, RNase is present, and active, after most other proteins have been precipitated by heat treatment. However, the large volumes involved make isolation of the RNase by most conventional techniques very difficult. In preliminary experiments, columns of APP-2′,(3′)-GMP–glass were successfully used to remove the RNase from several litres of waste liquor. Under the conditions employed in these experiments, Sepharose-based adsorbents quickly lost their flow properties. Porous glass adsorbents do seem to have significant advantages over Sepharose derivatives for large-scale work. APP-2′,(3′)-GMP–glass adsorbents have been used continuously under non-alkaline conditions for several months without detectable loss of RNase T_1 binding capacity. Such adsorbents have been stored dry for up to two years and then successfully used for RNase T_1 purification.

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* Polyacrylamide disc gel electrophoresis was performed on a two-layer system[15]. The buffer system used was 0.1 *M* Tris–0.004 *M* EDTA–0.015 *M* boric acid, pH 8.9. After electrophoresis, protein bands were visualized by staining with Coomassie brilliant blue[16].

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