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Affinity chromatography of bovine pancreatic and hog spleen deoxyribonucleases

Covalent coupling of DNA to a cellulose support and the use of a DNA cellulose matrix as the substrate for the purification of DNA-polymerase has been described by LITMAN[1] and GILHAM[2]. Recently the covalent attachment of nucleic acids to agarose has also been reported by POONIAN *et al.*[3]. The very efficient binding of DNA-polymerase to the DNA-Sepharose matrix was also described by them[3].

Affinity chromatography of bovine pancreatic deoxyribonuclease (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5.) and hog spleen deoxyribonuclease (deoxyribonucleate 3'-nucleotidohydrolase, EC 3.1.4.6.) have been studied under various experimental conditions. Very efficient binding of both enzymes to single-stranded calf thymus DNA-Sepharose matrixes was observed. The binding of hog spleen deoxyribonuclease to a polynucleotide inhibitor–Sepharose matrix appeared to be irreversible under the experimental conditions used.

Experimental

Materials. Calf thymus DNA, bovine pancreatic and hog spleen deoxyribonuclease were obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A. Sepharose 4B is a product of Pharmacia, Uppsala, Sweden and cyanogen bromide was purchased from Eastman Kodak Co., Rochester, N. Y., U.S.A. A polydeoxyribonucleotide inhibitor of spleen acid deoxyribonuclease was the gift of Dr. M. J. PITOUT of the South African Medical Research Council, Pretoria.

Sepharose activation and DNA-coupling reaction. The method described by POONIAN *et al.*[3] for Sepharose activation was used with certain modifications. The most important modification was that the temperature was kept between 5° and 10°. Cessation of the fall of the pH below 11 and the disappearance of cyanogen bromide were observed after about 100 min at this temperature compared to 8 to 12 min reported by CUATRECACAS[4]. The activated Sepharose 4B, finally washed with 0.05 *M* potassium phosphate (pH 8.0), was added to the single-stranded (heat-denatured) calf thymus DNA or polydeoxyribonucleotide ligand in a volume of 0.05 *M* potassium phosphate (pH 8.0) similar to that of the packed Sepharose 4B used in the reaction. The mixture was stirred very gently at 4° for 48 h. Single-stranded DNA was obtained by keeping a solution of DNA in 0.05 *M* potassium phosphate (pH 8.0) at 100° for 30 min. No annealing or reformation of the double-stranded structure seemed to occur within 24 h when this DNA solution was kept at 4°. This was confirmed by the absence of any decrease in the absorbancy observed after maximal thermal hyperchromicity at 260 nm. The mixture was then washed with the above-mentioned buffer as well as with a 0.5 *M* potassium phosphate buffer (pH 8.0) by stirring gently for periods of 4 to 6 h until no more nucleic acids appeared. The amount of DNA coupled was obtained from the difference between the input and the wash at 260 nm.

Assay of bovine pancreatic deoxyribonuclease (DNase I). DNase I activity was determined by the hyperchromicity assay of KUNITZ[5] as modified by PRICE *et al.*[6] in 0.05 *M* Tris–HCl buffer (pH 7.5) and 5 m*M* $MgCl_2$ or $MnCl_2$ at 25° and

expressed in absorbance increase at 260 nm. A Perkin-Elmer Model 26 spectrophotometer was used in these studies.

Assay of hog spleen acid deoxyribonuclease (DNase II). A method similar to that used for DNase I was used except that the activity was determined in 0.05 *M* sodium acetate buffer (pH 4.9) in the absence of any divalent cation and in the presence of 0.1 *M* NaCl.

Affinity chromatography of DNase I. The DNA–Sepharose slurry was washed and equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$ or $MnCl_2$ and a column (0.8 cm × 26 cm) was packed. About 1.0 mg of DNase I dissolved in 0.3 ml of 0.05 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$ was applied to the column which was then developed with this buffer. In some cases 1.0 mg of ovalbumin was applied with the DNase I. The protein concentration in the effluent was determined spectrophotometrically at 280 nm. Control experiments were performed on Sepharose 4B columns of similar dimensions. The observed elution volumes of DNase I and ovalbumin were compared. All experiments were performed at 4° to 6° at a flow-rate of 5 ml/h.

Affinity chromatography of DNase II. The procedure used was essentially similar to that described for DNase I except that the buffer system used throughout these experiments was 0.05 *M* sodium acetate (pH 4.9) containing 0.1 *M* NaCl.

Results and discussion

Single-stranded calf thymus DNA coupled effectively to the Sepharose 4B derived matrix activated with cyanogen bromide. Approximately 48% of 11 mg single-stranded DNA per 40 ml 0.05 *M* potassium phosphate buffer (pH 8.0) was covalently coupled to 40 ml packed Sepharose 4B. About 40% of the polydeoxyribonucleotide DNase II inhibitor was coupled under similar experimental conditions.

Some of the results obtained by affinity chromatography of DNase I and II are illustrated in Figs. 1 and 2. The elution volume of ovalbumin was 18 to 20 ml in 0.05 *M* potassium phosphate buffer (pH 8.0) in the presence of DNase I as well as in 0.05 *M* sodium acetate buffer (pH 4.9) in the presence of DNase II on both Sepharose 4B and DNA–Sepharose columns, indicating the absence of any interaction of DNA with ovalbumin. The elution volumes of DNase I and II on Sepharose 4B columns under the respective chromatographic conditions on columns with dimensions of 0.8 cm × 26 cm were 49 to 52 ml. No DNase I or II activity was eluted on DNA-Sepharose columns with 0.05 *M* phosphate or acetate buffers, respectively, indicating enzyme–substrate interactions. Both enzymes were eluted in 18 to 20 ml after the application of the appropriate buffer containing 0.3 *M* NaCl. Higher concentrations of NaCl eluted no further enzymatic activity. When using a salt concentration gradient both enzymes were eluted at a total salt concentration of approximately 0.3 to 0.35 *M*. Elution with buffers of a lower pH in the case of DNase I and a higher pH in the case of DNase II, did not give better results.

No enzymatic activity could be eluted from the polydeoxyribonucleotide inhibitor–Sepharose columns at salt concentrations up to 2 *M* in the presence of EDTA and the absence of divalent cations at as much as 2 pH units from the pH optimum of DNase II.

The capacity by which single-stranded DNA–Sepharose columns can adsorb

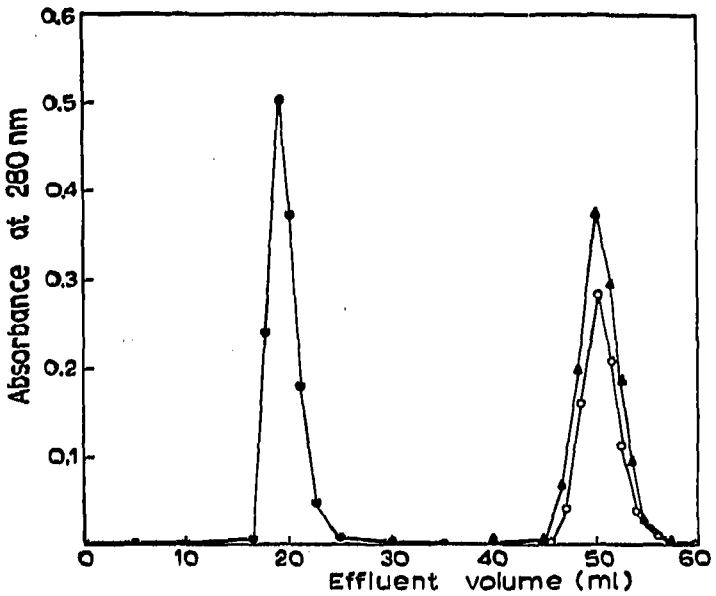

Fig. 1. Chromatographic pattern obtained for ovalbumin (●), bovine pancreatic (▲) and hog spleen deoxyribonuclease (○) on Sepharose 4B columns (0.8 × 26 cm) in 0.05 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$ in the case of experiments with ovalbumin and DNase I and 0.05 *M* sodium acetate buffer (pH 4.9) containing 0.1 *M* NaCl in the case of experiments with ovalbumin and DNase II. 1 mg of each of these proteins was applied to the columns. Protein concentration in the effluent was determined spectrophotometrically at 280 nm. The deoxyribonuclease peaks were identified by using the assay methods for DNase I and II referred to in the text.

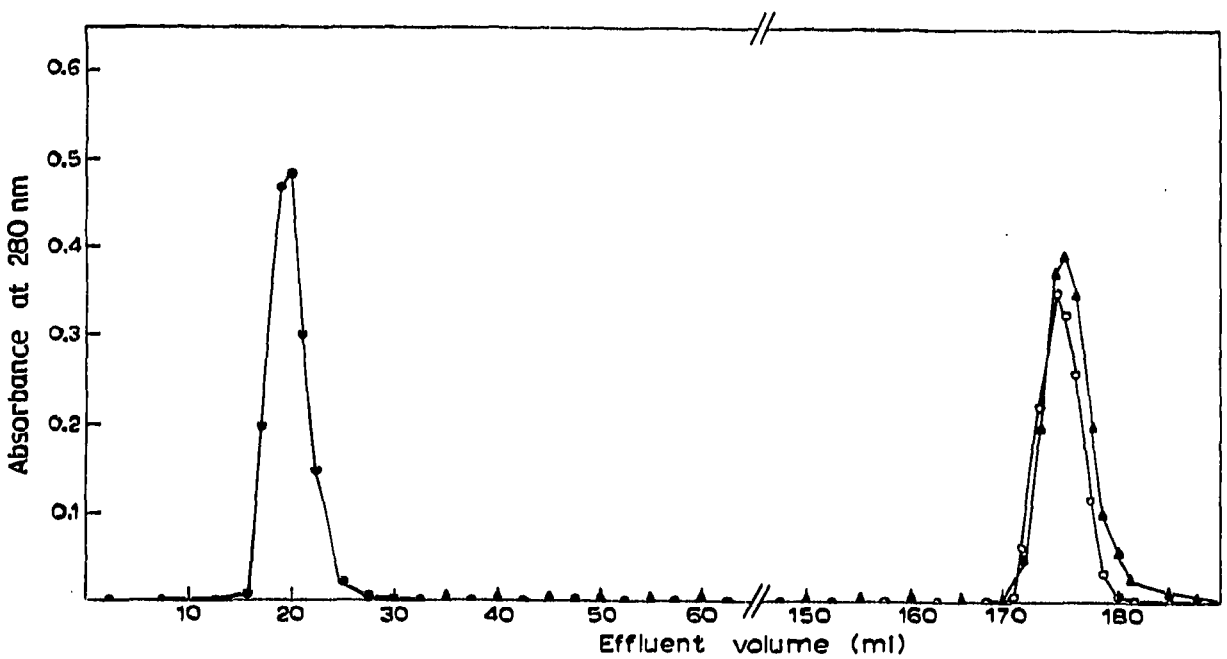

Fig. 2. Chromatographic pattern obtained for ovalbumin (●), bovine pancreatic deoxyribonuclease (▲) and hog spleen deoxyribonuclease (○) on single-stranded DNA–Sepharose 4B columns (0.8 × 26 cm) in 0.05 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$ in the case of experiments with ovalbumin plus DNase I and 0.05 *M* sodium acetate buffer (pH 4.9) containing 0.1 *M* NaCl in the case of experiments with ovalbumin plus DNase II. 1 mg of each protein was applied to the columns. Protein concentration in the effluent was determined spectrophotometrically at 280 nm. Corrections had to be made for nucleotides eluted with the deoxyribonucleases. The deoxyribonuclease peaks were identified by using the assay methods referred to in the text. After 140 ml had been eluted the NaCl concentration of the developing buffers was increased to 0.3 *M*.

the deoxyribonucleases seemed to be much higher than that of the DNA–Sepharose columns for HeLa DNA-polymerase reported by POONIAN *et al.*[3]. In the presence of 25 μM β-butyrolactone, a strong carcinogen and activator of DNase I (see ref. 7), the affinity of DNase I for the DNA–Sepharose matrix increased. This was verified by the fact that a salt concentration of 0.45 M was necessary to elute the enzyme from the column. This effect as well as the fact that other proteins such as ovalbumin, bovine serum albumin and alcohol dehydrogenase (also applied to single-stranded DNA–Sepharose columns in some experiments) eluted in 18 to 20 ml (equivalent to the void volume) indicated the specificity of binding of both deoxyribonucleases to the single-stranded DNA–Sepharose matrix. The single-stranded DNA–Sepharose columns have also been found to possess good flow-rate properties. Finally, it may be concluded that single-stranded DNA–Sepharose columns should be useful for the isolation and purification of bovine pancreatic and hog spleen deoxyribonuclease by affinity chromatography.

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