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A DNA–Acrylamide Gel Column for Analyzing Proteins That Bind to DNA, I. DNA Polymerase*

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Abstract. A new procedure is described for purifying proteins that specifically bind to DNA. DNA is entrapped in polyacrylamide gel particles which can then be used in standard column chromatographic procedures. The method was developed using *Escherichia coli* DNA polymerase as the test material. The crude enzyme was applied at low ionic strength and eluted at high ionic strength with a 200-fold increase in specific activity on a single passage through the column. The method is versatile and simple and is not restricted to DNA-protein systems. Any macromolecule can be entrapped in the gel particles; these can interact with other large or small molecules in the liquid phase. The gel is stable at elevated temperatures and can therefore be used in hybridization experiments.

The binding of DNA-specific proteins to DNA provides the basis for a highly efficient and relatively simple procedure for isolating and purifying such proteins. Several methods have been described[1–4] for immobilizing DNA on solid matrices which are then employed in standard column chromatographic procedures. We present here a new method in which the immobile matrix is composed of polyacrylamide gel particles. Aside from being versatile and simple, the method has the main advantages that no chemical steps are needed to fix the DNA in the matrix and the same column can be used repeatedly. The gel is made by dissolving DNA in the monomer solution before polymerization. The pore size of the gel particle is such that the DNA remains trapped, unless it is attacked enzymatically. So far, we have used only native DNA in the gel, but there is no reason why denatured DNA, RNA, or in fact any other macromolecule cannot be used since the pore size may be varied at will; therefore, even small molecules can be entrapped in the particles. The gel is stable at elevated temperatures, so that it is also possible to carry out hybridization experiments using this method. If the molecules that one seeks to purify are too large to enter the gel particle, this procedure can still be used, for there appears to be enough immobilized DNA protruding from the surface to be able to interact efficiently with the molecules in the liquid phase.

We report here the details of this new procedure using as a test material a crude fraction of DNA polymerase previously described.[5] One passage through the DNA–gel column increased the specific activity about 200 times. The physical characterization of this polymerase will be described in detail elsewhere.

Materials and Methods. Polyacrylamide gels were polymerized from Cyanogum 41, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine, all purchased from the E. C. Apparatus Co., Philadelphia, Pa. *Escherichia coli* DNA was isolated using freshly grown cells.[6] Gel filtration was carried out with Sephadex G-100 and G-200 purchased from Pharmacia Co. Deoxynucleoside-5'-triphosphates, labeled and unlabeled, were obtained from Schwarz BioResearch, Orangeburg, N.Y. Agarose was obtained from Bio-Rad Laboratories. DNA polymerase was isolated from *E. coli* cells purchased from Grain Processing, Inc., Muscateen, Iowa. In all experiments described here, the polymerase applied to the DNA-gel column was the ammonium sulfate precipitate (step 4).[5]

Preparation of DNA–acrylamide gel: 60 mg of *E. coli* DNA is dissolved in 100 ml of 0.05 M Tris, pH 7.8, containing 10 g of Cyanogum 41, a commercial brand of acrylamide monomer that contains 97% acrylamide and 3% *N,N'*-methylenebisacrylamide. The DNA monomer solution is cooled to 15°C and 0.8 ml of *N,N,N',N'*-tetramethylethylenediamine is added. This mixture is added to 100 ml of 1% (w/w) agarose solution at a temperature of 50°C. After thorough mixing (requiring 30–60 sec) the temperature drops to about 33°C and 0.13 g of ammonium persulfate in 1 ml of water is added, again with thorough mixing. The solution becomes turbid in about 2 min and begins to set soon thereafter. After a minimum of 30 min, the gel is cut into 0.5-cm cubes and pressed through a 75-mesh stainless steel screen. The wire mesh of a hypodermic Millipore holder is convenient for this purpose. The gel is washed by suspending it in an equal volume of buffer (0.05 M Tris, pH 7.8) and centrifuging for 5–10 sec at 800 rpm. The supernatant is decanted and the process is repeated six times. The total volume of gel remaining after the washing procedure is about 400 ml.

Packing the column: A column 1.5 × 30 cm is convenient in size (Pharmacia, 15/30). A thick slurry of about 100 ml total volume, containing about 50 ml of gravity-settled gel and 50 ml of supernatant buffer, is poured into the column to the desired height and is packed while washing with buffer under a constant pressure head of about 60 cm until the column contents no longer contract. If the head is not used, there is a tendency toward uneven packing, which results in channeling. The volume of gel in the column is about 85% of the volume obtained by gravity settling in a cylinder. About 1 hr is required to pack the column. The head is then removed and the column is attached at its bottom outlet to a peristaltic pump. The column is ready for use when the effluent absorbance at 260 nm is about 0.005.

Flow rate: We have used a peristaltic pump to obtain a flow rate of 12 ml/hr. Slower flow-rates can be used, but a rate of 20 ml/hr will cause packing and a subsequent slowing down of the column.

Amount and stability of DNA in the column: There are 2.2 mg of DNA in a bed volume of 39 ml. This corresponds to 40% of the DNA put into the polymerizing solution; the remainder is lost in the fines and washes. Very little DNA is washed out with use; we have re-used the same column as many as ten times. After the column is stripped with a high concentration of salt, and washed with 0.05 M Tris, it is ready for re-use. If the column remains idle for several days, the first 10 ml of effluent has an absorbance (260 nm) of approximately 0.04. However, when a column was stored for 6 months after repeated use, a significant amount of the DNA had leached out, apparently because of previous exposure to nucleases. Since DNA polymerase will bind to DNA in the absence of Mg^{2+}, all fractionations were carried out without Mg^{2+}, to limit the action of nucleases.

Sample application: The sample is applied as in ordinary gel filtration, making certain that the sample layer is flat, by using a sample applicator (Pharmacia) or a filter paper disc. In the experiments discussed here, no sucrose was added to increase the density since the protein concentration was high (12–35 mg/ml). Before applying the polymerase sample, it was dialyzed against 0.05 M Tris, pH 7.8, to remove ammonium sulfate. Sample sizes of 0.1–0.6 ml are convenient, although volumes as high as 5 ml can be used if the sample is dilute. The lower limit of protein applicable is determined

by the sensitivity of the assay. For the DNA polymerase fraction used here, the upper limit for total protein applied was 20 mg with the 1.5 × 26 cm column.

Excluded volume: For a 1.5 cm diameter column with a height of 9 cm, the peak of the excluded volume (determined with Blue Dextran) occurred at 38% of the bed volume; for a height of 11 cm or 22 cm, at 42%.

Pore size: The pore size of the gel matrix was only estimated. Both bovine serum albumin (mol wt 69,000) and polyuridylic acid (mol wt 150,000, pH 7.8) were retarded. These data, together with the fact that similar commercial acrylamide gel beads (BioGel) have an upper exclusion limit of about 200,000, suggest that the largest molecules that can penetrate into the gel have a molecular weight of about 200,000.

Effect of the polymerizing agents on DNA: A polymerizing solution containing *E. coli* DNA prepared in our laboratory was prepared as described above except that the monomer and agarose were omitted. After allowing the solution to stand for one-half hour it was diluted 1:7 with 0.05 M Tris, pH 7.8 and sedimented in the Spinco Model E ultracentrifuge in neutral and alkaline (0.1 N NaOH) solutions. Molecular weights were calculated according to the equations of Studier.[7] The molecular weight of the untreated DNA was 28×10^6 in neutral solution and 9×10^6 in alkaline solution. This corresponds to less than one nick per polynucleotide strand; for the reagent-treated DNA, the molecular weights were 28×10^6 and 3×10^6, respectively. This corresponds to about three additional nicks per strand. We do not know where the nicks occur or what type they are. However, this amount of nicking probably represents a maximum, if it is assumed that the reagents preferentially attack the monomer (it is present during polymerization at a mass ratio of 200:1 with respect to DNA). The treated DNA was further examined by determining its melting characteristics. The melting point in 0.05 M Tris, pH 7.8, was 78°C and the hyperchromic effect was 37%. The untreated control behaved identically. Therefore, no denaturation of the DNA had occurred.

Results. Effect of ionic strength of the eluting solution: In all of the experiments with DNA polymerase, the ammonium sulfate fraction (step 4, ref. 5), containing about 5 mg protein was dissolved in about 0.5 ml of 0.05 M Tris, pH 7.8. After the sample was applied, the column was washed with the same buffer until four bed volumes of eluate were collected. The polymerase could be eluted with KCl with a molarity of 0.2 or greater. The elution pattern with 0.4 M KCl–0.05 M Tris, pH 7.8, is shown in Fig. 1. Two points are noteworthy. (1) The bulk of the polymerase activity appears in a single peak, when eluted with a fixed concentration of KCl of 0.2 M or higher. However, when 0.6 M KCl was applied after 0.3 M KCl, a second peak containing about an equal amount of activity appeared. With 0.1 M KCl, the polymerase activity is eluted gradually rather than as a peak. (2) The amount of activity recovered in the single peak decreased with ionic strength. All activity was recovered when the polymerase was eluted with 0.6 M KCl; 90% was recovered with 0.4 M KCl; 20% was recovered with 0.2 M KCl. A salt gradient from 0.1–1.0 M KCl gave no peaks of activity. The results are consistent with interpretation that activity is spread on the column if the ionic strength of the eluent is not high enough. Throughout this work, we compromised by using 0.4 M KCl, in order to minimize possible dissociating effects of the KCl.

Conductivity measurements were made on the eluates at all concentrations of KCl to establish that the polymerase activity was in fact being eluted by the salt. A typical conductivity curve is shown in Fig. 1 for the 0.4 M KCl elution, wherein it is seen that activity is eluted as the salt concentration increases.

Yield of polymerase and specific activity: Use of 0.4 M KCl for elution leads

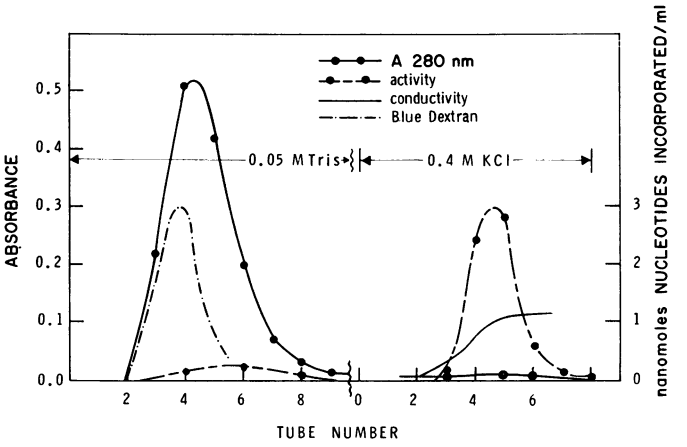

FIG. 1. DNA-acrylamide gel chromatography. 5.2 mg of DNA polymerase (step 4, ref. 5) dissolved in 0.3 ml of 0.05 M Tris buffer, pH 7.8, was applied to a 1.5 × 15 cm column (row 1, Table 1). The column was washed with 90 ml of the same buffer (30 fractions, 3 ml/tube), then the polymerase was eluted with 0.4 M KCl. The solid line on the left is absorbance (280 nm) and the dotted line is absorbance (650 nm) for Blue Dextran. The dashed line on the right represents polymerase activity; some activity appears on the left. The solid sigmoid line on the right is the conductivity (arbitrary units). Polymerase activity was assayed by adding 0.1 ml from each tube to 0.4 ml of a mixture containing 3 μg native calf thymus DNA, 3 nmol of each of the four deoxynucleoside-5'-triphosphates and 1.6 μmol each of $MgCl_2$ and mercaptoethanol, in 0.05 M Tris, pH 7.8. The mixture was incubated for 20 min at 37°C, then chilled in ice; 0.1 ml of 0.07% bovine serum albumin was added, then 1 ml of 10% trichloroacetic acid. The mixture was filtered through HA Millipore filters, dried on planchets, and counted.

to essentially total recovery of protein and about 90% recovery of the activity applied to the column. In a typical case, 15 mg of ammonium sulfate fraction was applied to the column and 0.08 mg of protein was recovered in the active fractions. The specific activity increased from 10 nmol of nucleotides incorporated per mg of protein to 2000–3000 nmol/mg in the pooled purified fractions. The specific activity was based on protein content estimated from the absorbance of the pooled fractions. The over-all yield is 4–5 mg of active material from 1 kg of cell paste.

Elution of DNA polymerase from gel containing no DNA: To test the sieving and adsorption characteristics of the gel matrix, DNA polymerase was applied to a column that contained no DNA in the gel. The elution pattern is shown in Fig. 2. Some of the activity appears in the void volume (indicated by the Blue Dextran peak) but a significant amount is included in the gel particles and is eluted later, as was to be expected from the pore size estimated above. There is also evidence of nonspecific adsorption since some activity appears beyond the total bed volume. In practice, it is advisable to wash the column with four bed volumes of the 0.05 M Tris buffer before increasing the ionic strength to elute the protein(s) of interest.

Column capacity and recovery of polymerase activity: Data for several DNA columns are contained in Table 1. The total recovery of polymerase activity was usually about 90%, but in some cases it dropped to the vicinity of

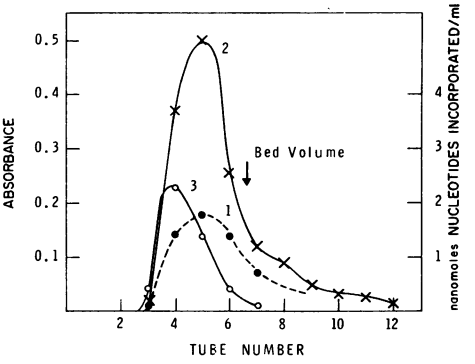

FIG. 2. Chromatography of DNA polymerase on an acrylamide gel column containing no DNA. 0.05 ml, containing 0.5 mg of DNA polymerase, was applied to a 0.9 × 6 cm column. The column was eluted with 0.05 M Tris, pH 7.8. Each fraction contained 0.62 ml. *Curve 1* shows the absorbance at 280 nm. Recovery of protein was quantitative. *Curve 2* is the polymerase activity. *Curve 3* is the absorbance of Blue Dextran at 650 nm.

60% although total protein recovery remained at 100%. This is not related to the DNA column itself, but rather to the stability of the particular polymerase preparation that was applied to the column.

The capacity of the DNA column depends on the DNA used (Table 1). Evidently, the more 3′-OH ends present in the DNA, the higher its binding capacity for DNA polymerase. Commercial *E. coli* DNA, which was heterogeneous in sedimentation and contained much low molecular weight material, had a very high binding capacity; upon reprecipitation, the higher molecular weight material was selected and the binding capacity dropped. High molecular weight DNA with few single-strand breaks, prepared in our laboratory, had the lowest capacity.

Two artifacts can affect the apparent capacity of the DNA column. The first artifact is DNA (or other polynucleotide) contamination in the protein solution applied to the column; if soluble polymerase–DNA complexes are

TABLE 1. *Binding capacity of DNA columns.*

DNA source	No. termini per 10^6 daltons*	Dimensions of column	Vol of gel bed (ml)	Amount DNA in column (mg)	% Activity bound†	Protein applied‡ (total mg/total ml)
Commercial§	≥20	1.5 cm × 15 cm	26	1.5	100	5.2/0.30
"	"	" "	"	"	94	14.0/0.50
"	"	" "	"	"	64	22.0/0.64
Reprecipitated commercial**	1.25	1.5 cm × 22 cm	39	2.2	70	5.1/1.0
"		" "	"	"	49	10.0/1.0
Laboratory††	0.11	" "	"	"	38	14.5/1.0

* Termini, defined as free 3′-OH groups occurring at both ends and internal breaks, were measured by sedimentation under neutral and alkaline conditions; the Studier equation[7] was used to calculate molecular weights.

† Assay conditions given in Fig. 1. Bound activity is that eluted with 0.4 M KCl; total recoverable activity also includes that obtained in the initial 0.05 M Tris wash.

‡ Protein concentration estimated from absorbance. The specific activity of the applied polymerase ranged from 4 to 10 nmol nucleotides incorporated/mg protein/20 min.[5] The specific activity of the eluted polymerase was in the range 1000–3000 nmol/mg/20 min. Protein recovery was always 100%.

§ All DNA samples were prepared from *E. coli*. The commercial sample was obtained from General Biochemicals, Inc. (lot no. 676137).

** Commercial sample dissolved in 0.05 M Tris, pH 7.8 at a concentration of 0.35 mg/ml, then made 0.1 N in NaCl and precipitated with 1.1 vol of 100% ethanol.

†† Prepared from *E. coli* K12 in our laboratory.[6]

formed they will, for the most part, be washed out with the 0.05 M Tris solution. However, if one is willing to sacrifice part of the yield, whole lysates of *E. coli* can be applied directly to the column. A lysate prepared by osmotically shocking 10^{12} *E. coli* spheroplasts contains more than enough free polymerase to saturate a 1.5 × 20 cm column. The second artifact is channeling in the gel bed. The column must be carefully packed as described to avoid this. However, the effect can easily be checked using Blue Dextran.

Sedimentation constant and molecular weight of eluted polymerase: The $s_{20,w}$ value for DNA polymerase eluted with 0.4 M KCl is 4.6 as determined in the analytical ultracentrifuge, corresponding to a molecular weight of 82,000. There was a distinctly faster sedimenting component which was not included in the calculation. Sedimentation equilibrium corroborated the heterogeneity and the molecular weight range determined by sedimentation velocity. That these sedimenting components in fact carry active polymerase was confirmed by sucrose gradient sedimentation and assay of the polymerase activity itself. This technique revealed a number of active species, some of them still smaller than $s_{20,w}$ of 4.6. These studies, together with a detailed analysis of the polymerase purified by the DNA column method, will appear elsewhere.

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[1] Bautz, E. K. F., and B. D. Hall, *Proc. Nat. Acad. Sci. USA*, **48**, 400 (1962).
[2] Bolton, E. T., and B. J. McCarthy, *Proc. Nat. Acad. Sci. USA*, **48**, 1390 (1962).
[3] Litman, R., *J. Biol. Chem.*, **243**, 6222 (1968).
[4] Alberts, B., F. J. Amodio, M. Jenkins, E. Gutman, and F. Ferris, *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 289 (1968).
[5] Cavalieri, L. F., and E. Carroll, *Proc. Nat. Acad. Sci. USA*, **59**, 951 (1968).
[6] Cavalieri, L. F., and B. H. Rosenberg, *Biophys. J.*, **1**, 301 (1961).
[7] Studier, W., *J. Mol. Biol.*, **11**, 385 (1965).