DNA COVALENTLY LINKED TO CARBOXYMETHYL-CELLULOSE AND ITS APPLICATION IN AFFINITY CHROMATOGRAPHY

Hans POTUZAK and Ulrike WINTERSBERGER
Institut für Krebsforschung der Universität Wien, A-1090 Wien, Austria

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1. Introduction

Affinity chromatography on columns containing DNA has been successfully used in the isolation and purification of DNA dependent polymerases and other DNA-binding proteins as well as for the purification of nucleic acids hybridizable to the column bound DNA [1]. Two drawbacks of DNA column materials used so far have limited their application: firstly, in the most widely used column materials DNA-cellulose [2] and DNA-agarose [3] the nucleic acid is not bound covalently, imposing a restriction of the ionic strength of the buffers used to avoid a loss of DNA; secondly, in those instances where DNA was bound covalently to the matrix, the amount of bound DNA was very low [4,5].

We have therefore been interested in developing a column material containing covalently linked DNA in amounts comparable to those in DNA-agarose. In this paper we describe the preparation of an affinity chromatography material containing up to 3.6 mg DNA per ml bedvolume and its successful application in the purification of DNA dependent DNA polymerases from yeast nuclei and mitochondria. While this work was in progress Arndt-Jovin et al. [6] reported an improved method for the preparation of agarose containing similarly high amounts of covalently attached DNA.

2. Materials and methods

Carboxymethyl-cellulose of the type CM 23 Nr. 45030 was purchased from SERVA-Feinbiochemica GmbH. Heidelberg. Calf thymus DNA, type V No. D-1751 was obtained from Sigma Chemical Company, St. Louis. Yeast DNA polymerases from nuclei and mitochondria were purified up to the DEAE-cellulose step according to published procedures [7,8]. The activity of these enzymes was measured as previously described [8]. One unit of DNA polymerase activity corresponds to an incorporation of 1 nmol of dTMP in 15 min at 35°C. Protein was measured by the method of Lowry et al. [9], DNA by the diphenylamine test [10].

2.1. *Preparation of the column material (standard procedure)*

1 g of carboxymethyl-cellulose (CM-cellulose) is suspended in 50 ml of 0.5 N NaOH and stirred for 30 min. After decantation from the supernatant the material is washed with water until the effluent reaches a pH of 8. The cellulose derivative is suspended in about 100 ml of water and the pH is adjusted to 3.5 by addition of 0.01 N HCl. After filtration under suction the material is washed three times with 5 ml ethanol/ether 1:1 (v/v) each, followed by 20 ml of ether and finally dried for 60 min at 40°C. 20 mg of calf thymus DNA dissolved in 10 ml water are added to the prepared CM-cellulose. The resulting suspension is spread over the surface of a Petri dish (about 10 cm in diameter) and dried slowly over a period of 60 h at 40°C during which the drying process is controlled by periodically covering the dish. The resulting DNA-cellulose is scraped from the surface of the dish, pulverized, suspended in 50 ml of 0.05 M phosphate buffer at pH 7.0 containing 50% glycerol (v/v) and left for 24 h at room temperature. The material is then washed 10 times with 50 ml of water each and stored at 4°C in a 1 M NaCl solution. If the

recommended cellulose is used the resulting column material contains about 15 mg DNA per g of dry CM-cellulose which corresponds to about 2.5 mg per ml bedvolume.

2.2. *Fractionation of DNA polymerases on DNA-cellulose*

All column operations are carried out at 3°C and pH 7.6 in buffer A (0.02 M Tris-HCl, 1 mM EDTA, 0.5 mM 1,4-dithioerythritol, 10% (vol.) glycerol) containing 6 mg/l of the protease inhibitor phenylmethylsulfonylfluoride. After equilibration of the column with buffer A containing 0.1 M NaCl about 1 mg of protein per ml bedvolume is applied (2 vol./h). The column is then washed with 2–3 vol. of the equilibration buffer, 2–3 vol. of buffer A containing 0.2 M NaCl and the enzyme activity is finally eluted with buffer A containing 0.6 M NaCl at a speed of 4 column volumes per h. The column is regenerated in situ by washing with 1 M NaCl and thereafter may be used for several times without any detectable loss in the binding capacity.

3. Results and discussion

3.1. *Preparation of DNA-cellulose*

DNA attached to insoluble matrices by both physical and chemical methods has frequently been employed for the chromatographic purification of DNA binding proteins and nucleic acids. As the materials most widely used until now contain adsorbed but no covalently bound DNA, it was our objective to prepare a column material with covalently linked DNA in reasonable amounts which is suitable for the affinity chromatography of DNA binding enzymes.

In this paper we describe a method for coupling DNA to CM-cellulose. The acid catalyzed reaction is carried out in a suspension of CM-cellulose in an aqueous solution of calf thymus DNA which is slowly evaporated at 40°C. The resulting material is washed with 50% glycerol solution for 24 h followed by deionized water. The washing procedure which is described in detail in the methods section was found suitable to specifically remove any noncovalently attached DNA from the cellulose derivative as it leads to a column material which is stable to desorbing conditions such as elevated temperature, organic solvents and low ionic strength of the buffers used. Both single stranded (denatured) as well as double stranded DNA are coupled with similar efficiency.

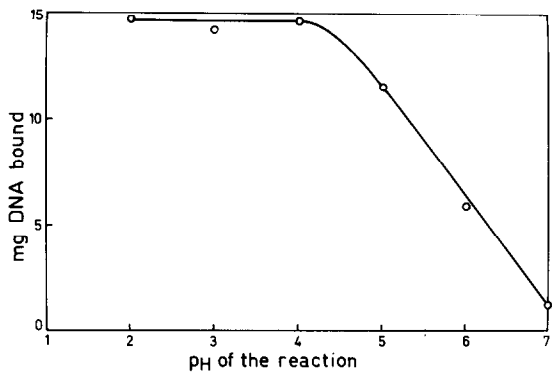

Fig.1. Dependence of the amount of linked DNA/g CM-cellulose on the pH of the reaction (input of 20 mg native calf thymus DNA per 1 g CM-cellulose).

The coupling reaction was found to be strongly dependent on the pH (fig.1). Considering the possibility of a depurination of DNA at low pH and high temperature [11] we decided to use a minimum pH of 3.5 and temperatures not exceeding 40°C as depurination occurs only to a low degree under those conditions. The dependence of the amount of linked DNA on the DNA input is shown in fig.2. We investigated the coupling conditions up to a DNA input of 40 mg/g CM-cellulose and thereby obtained a material containing 21.5 mg (54% of the input)

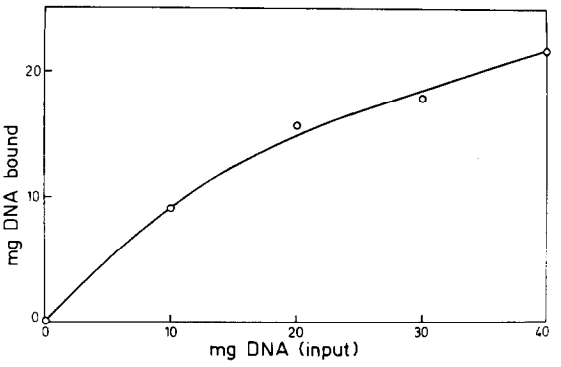

Fig.2. Dependence of the amount of linked DNA/g CM-cellulose on the DNA input (pH 3.5, 1 g CM-cellulose).

covalently attached DNA which corresponds to about 3.6 mg/ml bedvolume. It should be emphazised that the efficiency of coupling is especially high (90–100%) if DNA concentrations not exceeding 10 mg/g CM-cellulose are used. Such conditions are most favourably employed for the linkage of DNA species which are available only in small amounts. Our standard conditions in the case of coupling commercial calf thymus DNA were 20 mg DNA/g CM-cellulose, pH 3.5 and 40°C leading to a column material which contains 15 mg DNA per g CM-cellulose (74% of the input) corresponding to a column of 2.5 mg DNA per ml bedvolume. In order to show that large amounts of DNA are not attached unspecifically to the cellulose derivative a control reaction with unsubstituted cellulose (CF-11) was carried out under the same conditions. In this case less than 3% of the DNA input remained attached to the matrix.

To gain more information about the properties of our DNA-cellulose the following experiments were carried out: when the material containing double-stranded DNA is exposed to a 50% formamide solution at room temperature 43% of the DNA is detached indicating that most of the DNA molecules are linked to the cellulose only by one strand. The DNA-cellulose containing single-stranded calf thymus DNA is stable to temperature up to 70°C and to formamide concentration up to 80% in neutral buffer solutions and therefore seems to be suitable also for nucleic acid hybridization experiments. Exposure to buffers up to pH 9 at moderate temperatures (up to 35°C) does not cause any detectable loss of DNA; about 90% of the DNA is detached, however, when the material is incubated in 0.1 N NaOH at 20°C for one hour. We therefore assume that the coupling of DNA to the cellulose derivative is effected by an esterification to the carboxyl functions of the CM-cellulose with terminal hydroxyl groups of the DNA. Because of the low stability of the material to even mild alkaline conditions a linkage by means of amide functions seems unlikely. An attempt to achieve a coupling by means of the water soluble carbodiimide l-cyclohexyl-3-(2-morpholinoethyl) carboiimide metho-*p*-toluenesulfonate did not lead to any improvement and was therefore not subjected to further investigations.

3.2. *Application of DNA-cellulose for the purification of yeast DNA polymerases*

A column material containing 2.5 mg DNA/ml bedvolume obtained from the reaction of 20 mg denatured calf thymus DNA with 1 g of CM-cellulose was used. In the case of yeast nuclear DNA polymerase an enzyme fraction after DEAE-cellulose chromatography [7] was dialyzed against buffer A (see Materials and methods section) containing 0.1 M NaCl and applied to the DNA-cellulose column which was previously equilibrated with the same buffer. When the column was washed with this buffer about 80% of the protein but no enzyme activity was eluted. Further protein was eluted with buffer A containing 0.2 M NaCl; the active enzyme fraction was recovered

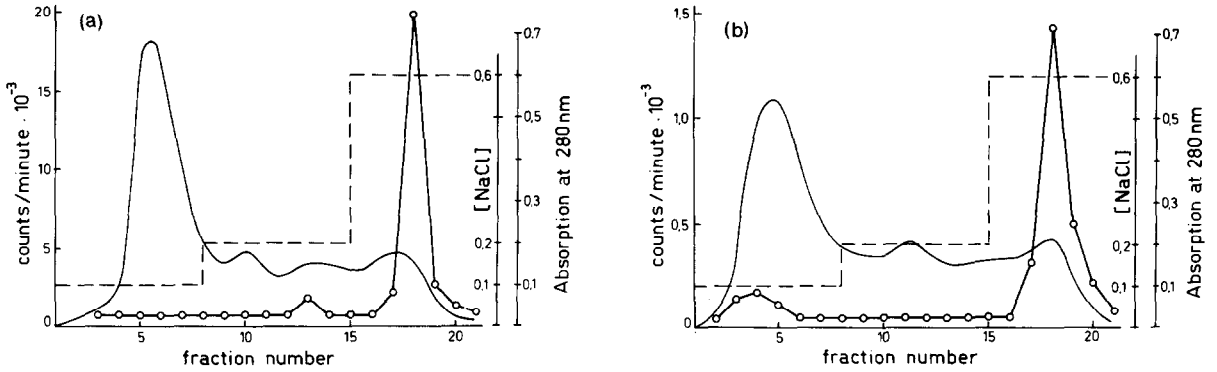

Fig.3. Enzyme fractions (a) 88.0 units (3.3 mg protein) nuclear DNA polymerase A, (b) 8.0 units (1.9 mg protein) mitochondrial DNA polymerase from *Saccharomyces cerevisiae* were applied to the column (0.8 × 6 cm) and eluted as indicated in the method section. Fractions of 1 ml were collected and 10 μl of each were tested for DNA polymerase activity. Continuous line: absorption at 280 nm; broken line: NaCl concentration (M) of the buffers used; (o———o) enzyme activity.

Table 1
Fractionation of the DNA polymerases on DNA-cellulose

Fraction	Total protein	Total activity	Specific activity
	mg	units	units/mg protein
(a) Nuclear DNA polymerase			
Crude enzyme	3.30	88.0	26.8
DNA-cellulose:			
0.1 M NaCl	2.75	0	0
0.2 M NaCl	0.21	18.0	85.7
0.6 M NaCl	0.13	47.8	368
(b) Mitochondrial DNA polymerase			
Crude enzyme	1.90	8.0	4.2
DNA-cellulose:			
0.1 M NaCl	1.50	0.5	0.3
0.2 M NaCl	0.23	0	0
0.6 M NaCl	0.10	4.1	41.0

with buffer A containing 0.6 M NaCl (fig.3a). As indicated in table 1a a more than 10-fold increase of specific enzyme activity combined with a yield of more than 50% was achieved. If a column consisting of CM-cellulose without DNA is run in the same manner enzyme activity is entirely eluted with the first buffer wash. This indicates that separation of DNA polymerase from other proteins is effected by the DNA linked to the matrix and not by ion exchange action of remaining carboxy groups. A mitochondrial DNA polymerase fraction from yeast was also successfully chromatographed on the new DNA column. Again a preparation after the DEAE-cellulose purification step [8] was used for the chromatography on DNA-cellulose which was carried out as described above for the nuclear enzyme (fig.3b). The yield of more than 50% as well as the 10-fold increase of specific activity of this unstable enzyme (table 1b) represents a valuable improvement of a result (10–20% yield, specific activity increase 3–10-fold) achieved in earlier attempts using DNA-agarose (Wintersberger U. and Blutsch, unpublished). After use the DNA-cellulose can be washed in situ with buffer A containing 1 M NaCl. It can be reused several times with equal efficiency and without any loss of DNA.

In conclusion we described an affinity column material containing DNA covalently bound to carboxymethyl-cellulose. The advantages of the new material are: a rapid and simple preparation procedure, a high yield of bound DNA especially at input amounts around 10 mg DNA/g matrix and the possibility to link native as well as denatured DNA to the cellulose. Columns can be used repeatedly and allow high flow rates.

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