Purification of Soybean DNA-Dependent RNA Polymerase I on a Column of Plasmid pHFK 206 Covalently Attached to Agarose

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The plasmid pHFK 206 consisted of plasmid pBR 322 and an 1.3×10^{-6} D insert, a cloned segment of a soybean rRNA repeating unit with a preferential binding region for soybean RNA polymerase I, was coupled to cyanogen bromide activated agarose (Sepharose 4B) and used for affinity chromatography of RNA polymerases.

It could be shown by elution profiles and sodium dodecylsulphate/polyacrylamide slab gels that highly purified RNA polymerase from *Escherichia coli* MRE 600 bound to pHFK 206-Sepharose as holoenzyme with an apparently full complement of σ-subunit. With initially purified soybean RNA polymerase I from chromatin of 2,4-D treated hypocotyls it was demonstrated that pHFK 206-Sepharose could be used as an affinity chromatography method for purification of soybean RNA polymerase I in high degrees and economy of time.

Introduction

The reaction of cyanogen bromide with agarose [1] results in a reactive product to which protein, nuclei acids or other biopolymers can be coupled via primary amino groups or similar nucleophilic groups. For example these adsorbents prepared of cyanogen bromide (CNBr)-activated agarose (Sepharose) and specific coupled ligands were used for purifying specific antibodies which are subsequently radiolabelled for immunoassay [2]. Other methods have been described for immobilizing DNA on CNBr-activated Sepharose such as for chromatography of chromatin proteins [3] but also for purification of enzymes which bind DNA [4]. Arndt-Jovin and co-workers [4] have used the covalent attachment of DNA to agarose for affinity chromatography of *Escherichia coli* DNA polymerase I and RNA polymerase from crude extracts or after initial purification steps with the results of high yields and degrees of purification.

During the usual purification procedure of RNA polymerase I from higher plants with several cellulose column chromatography steps the enzymes were often unstable by virtue of protein degradation during the long lasting purification procedure [5] and because of the complex subunit structure of these enzymes [6]. Unlike bacterial RNA polymerase which consists of only four to five subunits the eukaryotic nuclear RNA polymerases are multisubunit enzymes with ten to fifteen distinct polypeptide chains whose removal affects the activity of the catalytic properties of the enzyme [7]. In this communication we attempted to use the plasmid pHFK 206, containing a soybean rDNA insert with a preferential binding region for soybean RNA polymerase I, coupled to CNBr-activated Sepharose 4B, for purification of pre-purified soybean RNA polymerase I.

Materials and Methods

The plasmid pHFK 206 was obtained by *in vitro* recombination of Hind III fragments of total soybean DNA [8] with pBR 322 and cloning in *Escherichia coli* SK 1590. Transformed colonies were screened for hybridization with iodinated 18S and 25S rRNA and the clone pHFK 206 carrying the plasmid pBR 322 and an 1.3×10^{-6} D insert of soybean rDNA identified and characterized according to Friedrich [9]. DNA protection experiments showed that the cloned insert contains a binding region for soybean RNA polymerase I [9].

Soybean RNA polymerase I was isolated from 2,4-dichlorophenoxy-acetic acid (2,4-D) treated

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hypocotyls and initially purified from chromatin and characterized by using Agarose A – 1.5 m gel filtration as described by Guilfoyle *et al.* [10]. RNA polymerase from *Escherichia coli* MRE 600 was purchased from Boehringer (Mannheim, FRG). Soybean RNA polymerase activity was assayed as in [11] with the following modifications. The standard assay mixture contained 40 mmol l^{-1} Tris. HCl (pH 7.9), 50 mmol l^{-1} $(NH_4)_2SO_4$, 25% glycerol (v/v), 0.4 mmol l^{-1} ATP, CTP and GTP, 0.04 mmol l^{-1} 5′-^{3}H UTP (296 KBq), 0.1 mmol l^{-1} dithiothreitol, 100 µg/ml of heat denatured salmon sperm DNA, 6 mmol l^{-1} $MgCl_2$ and 100 µl of the enzyme. The mixture was incubated at 32 °C for 30 min. The assay conditions for *E. coli* RNA polymerase were the same as for the soybean enzyme with the exceptions that 8 mmol l^{-1} $MgCl_2$, 75 mmol l^{-1} KCl instead of $(NH_4)_2SO_4$ and 50 µl of the enzyme were present. The incubation took place at 35 °C for 30 min.

For coupling the plasmid pHFK 206 to CNBr-activated Sepharose and for affinity chromatography of RNA polymerase the methods described by Pharmacia (Uppsala, Sweden) and Arndt-Jovin *et al.* [4] with several modifications were used. 1 g freeze-dried CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was swollen in 1 mmol l^{-1} HCl for 15 min and washed in aqua dest. and 10 mmol l^{-1} K_3PO_4 (pH 8.2). The cake of filtered, activated Sepharose was taken up in 5 ml of 10 mmol l^{-1} K_3PO_4, added to 1 mg of plasmid pHFK 206 in a Corex reagent tube and stirred gently at room temperature overnight. The coupling procedure was terminated by addition of 10 ml 1 mol l^{-1} ammonium acetate (pH 7.0) for 2 h. For column chromatography the DNA-Sepharose product was equilibrated with binding buffer containing 40 mmol l^{-1} Tris. HCl (pH 7.9), 75 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} dithiothreitol, 5% glycerol (v/v) and 8 mmol l^{-1} $MgCl_2$, and washed with 40 ml until no absorbance at 260 nm was released. For purification of soybean RNA polymerase I 1 ml of the enzyme after agarose gel filtration was diluted with binding buffer (1 : 1) and applied to a 3.5 ml bed volume pHFK 206-Sepharose column. For affinity chromatography of *E. coli* RNA polymerase 20 µl of the enzyme (1 unit) was diluted with 200 µl binding buffer and applied to the column. The first and second running-through of the applied enzyme material was collected and loaded on the column again. Then the column was washed with 2 ml of binding buffer and eluted subsequently with 10 ml of a linear NaCl gradient between 0.05 – 1 mol l^{-1} (dissolved in binding buffer without $MgCl_2$) at a flow rate of 5 bed vol./h. The volume of each fraction was 0.5 ml. After the gradient, the column was washed with buffer containing 2.5 mmol l^{-1} NaCl until no more absorbance at 280 nm was eluted.

The NaCl concentration of the fractions were measured with a Zeiss refractometer and the polymerase activity was determined as described above. The fractions containing polymerase activity were combined (indicated in the figures) and subjected to sodium dodecylsulphate/polyacrylamide slab gels [12, 13]. The gel included a stacking gel of 2.1% acrylamide and a running gel of 8.75% acrylamide. Transmission scans at white light of gels stained with Coomassie brilliant blue were carried out with a Quick Scan R + D (Helena Lab.).

Results and Discussion

Recently DNA protection experiments showed that a cloned segment of a soybean rRNA repeating unit with a mol. wt. of 1.3×10^{-6} D which is an insert of the plasmid pHFK 206 contains a binding region for soybean RNA polymerase [9]. It is assumed that this binding region represents a specific recognition sequence for RNA polymerase I. The optimum metal ion concentrations and the ionic strength for template-binding of the polymerase were determined as 8 mmol l^{-1} of $MgCl_2$ and 75 mmol l^{-1} of KCl. Under these ion conditions we attempted to use the binding capacity of soybean RNA polymerase I to plasmid pHFK 206 in order to establish an affinity chromatography for purification of this enzyme from crude extracts.

Thus the intact plasmid pHFK 206 was coupled to cyanogen bromide activated Sepharose 4B and packed into a column with a final bed volume of 3.5 ml.

In a preliminary experiment highly purified RNA polymerase from *Escherichia coli* MRE 600 was applied to the column. Fig. 1A shows the elution profile of the enzyme with a first peak of activity eluted before application of a gradient indicating some over-loading of the column. The main peak of polymerase activity eluted at a NaCl concentration of 0.15 mol l^{-1}. The transmission scans of dodecyl-

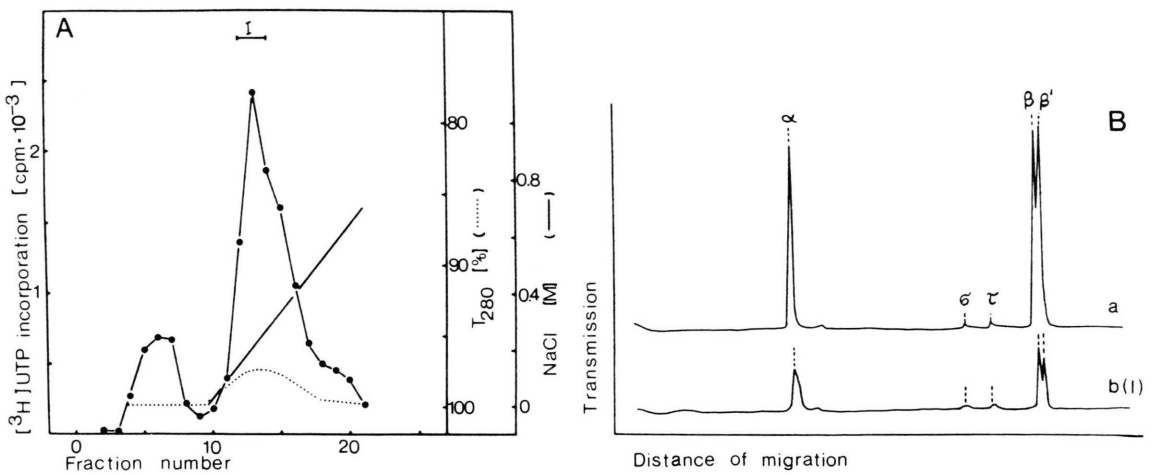

Fig. 1. Affinity chromatography of *Escherichia coli* RNA polymerase on a DNA-Sepharose column. Plasmid pHFK 206 containing a soybean rDNA insert was coupled to CNBr-activated Sepharose 4B. The column with a bed volume of 3.5 ml was eluted with a linear gradient between 0.05–1 M; the fraction size was 0.5 ml. A) ●—● RNA polymerase activity; ····· transmission at 280 nm (protein); —— NaCl concentration. B) Transmission scans at white light of dodecylsulfate-polyacrylamide slab gels of (a) RNA polymerase before chromatography and (b) of combined fractions of peak I from the DNA-Sepharose column stained with Coomassie brilliant blue. 40 µl of each extract was subjected to electrophoresis. The protein bands have mol. wts. of 39000 (α), 95000 (σ), 110000 (τ), 150000 (β), and 160000 (β′).

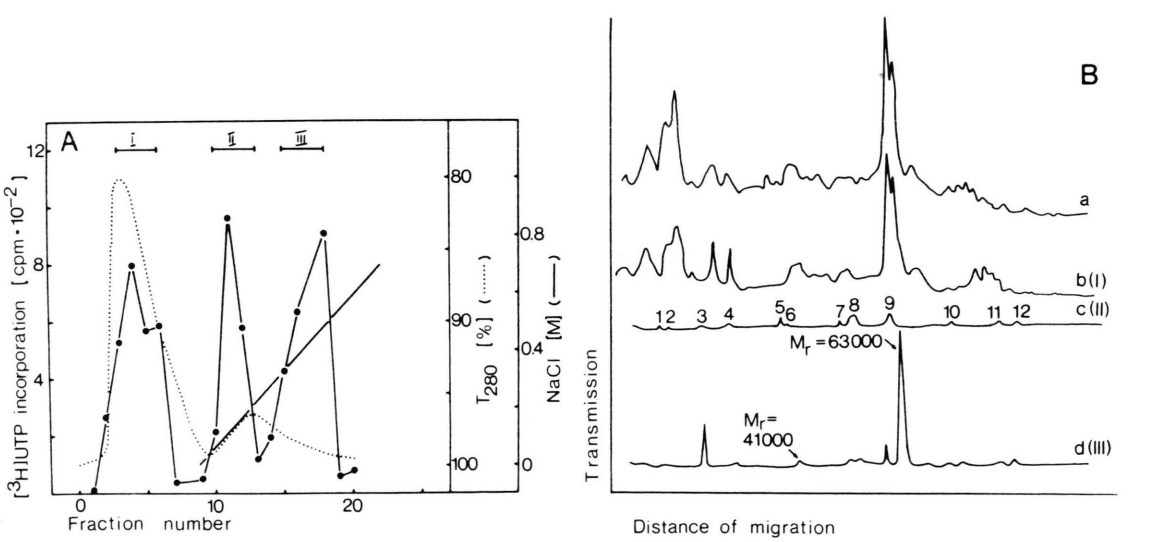

Fig. 2. Affinity chromatography of soybean RNA polymerase I on a DNA-Sepharose column. The enzyme extract initially purified from chromatin by using Agarose gel filtration was applied to a Sepharose column with covalent attached plasmid pHFK 206 containing a soybean rDNA insert. The column with a bed volume of 3.5 ml was eluted with a linear NaCl gradient; the fraction size was 0.5 ml. A) ●—● RNA polymerase activity; ····· transmission at 280 nm (protein); —— NaCl concentration. B) Transmission scans of dodecylsulfate-polyacrylamide slab gels of (a) polymerase extract before chromatography; (b) combined fractions of non-binding activity of peak I; (c) combined fractions of peak II, the protein bands (1–12) have mol. wts. of 29000, 30000, 32000, 34000, 38000, 39000, 48000, 50000, 60000, 97000, 135000, and 180000; (d) combined fractions of peak III from DNA-Sepharose column stained with Coomassie brilliant blue. 20 µl of extract in a), b), and 40 µl of extract in c), d) were subjected to electrophoresis.

sulfate-acrylamide gels of the pooled fractions of the peak (I) show that in comparison to the enzyme before chromatography the DNA-Sepharose bound enzyme consists of the same subunit structure with the presumed species $\beta\beta'\alpha_2\tau\sigma$ (Fig. 1 B). This is an indication that the *E. coli* RNA polymerase was bound to the DNA-Sepharose as holoenzyme with an apparently full complement of σ-subunit.

As a result of affinity chromatography of soybean RNA polymerase I on the soybean rDNA-Sepharose column the elution profile shown in Fig. 2 A was obtained. The RNA polymerase initially purified from chromatin by using Agarose gel filtration eluted in a first peak of activity together with most of the proteins before application of a gradient indicating, as in the case of the *E. coli* enzyme, some over-loading of the column. Upon application of a linear gradient two distinct peaks of activity eluted at NaCl concentrations of 0.1 mol l^{-1} and 0.45 mol l^{-1}, respectively. It should be pointed out that a NaCl gradient without $MgCl_2$ was a prerequisite for elution of the DNA-Sepharose bound enzyme in distinct peaks and could not be replaced by a KCl gradient with 8 mmol l^{-1} of $MgCl_2$. The active fractions of the peaks were combined and analyzed electrophoretically. The transmission scans in Fig. 2 B show that the crude polymerase extract before chromatography with its 27 protein bands nearly corresponds with the profile obtained by the non-bound enzyme material of peak I. However, electrophoresis of the enzyme eluted subsequently after application of the NaCl gradient (peak II) demonstrated substantial purification. According to Guilfoyle *et al.* [10], it is assumed that most of the twelve protein bands represent components of the soybean RNA polymerase I. The active fractions of peak III eluted last gave two additional bands with mol. wts. of 63 000 and 41 000, which were also obtained in the crude enzyme extract (arrows in Fig. 2 B).

Considering the results presented here we can summarize that *E. coli* RNA polymerase as well as soybean RNA polymerase I bound to plasmid pHFK 206, containing a soybean rDNA insert, coupled to CNBr-activated Sepharose. Thus, it was possible especially shown for soybean RNA polymerase I from an initially purified extract to use pHFK 206-Sepharose as an affinity chromatography method resulting in high degrees of purification and economy of time which is important to avoid protein degradation.

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