Biospecific fractionation matrices for sequence specific endonucleases

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ABSTRACT

Fractionation of several type II specific restriction endonucleases was achieved by separation on two novel biospecific matrices. The matrices are pyran, a copolymer of divinyl ether of maleic anhydride, and Cibacron Blue F3GA, a blue dye commonly used for the calibration of molecular sieves. Both compounds are insolubilized by coupling to sepharose through a cyanogen bromide linkage and in their soluble form inhibit the restriction endonucleases which we have tested. These affinity matrices can be used to obtain restriction endonucleases from crude extracts after removal of nucleic acids. They have also proven to have a high capacity when used as subsequent steps in enzyme purification. Their additional advantage is the rapid development time and reusability of columns packed with the two matrices.

INTRODUCTION

Site specific DNA endonucleases are used in several aspects of molecular biology. Since their discovery (1), a variety of enzymes have been purified from numerous strains (2). The major task in the purification of these enzymes is to remove contaminating enzymes and proteins which also use DNA as a substrate. In general, these enzymes appear to be stable; however, a number of fractionation steps are routinely required to obtain enzymes at a purity level useful for such structural studies as DNA sequencing.

Our recent interest has been to purify type II restriction endonucleases to homogeneity in order to study details of their interactions with naturally occurring DNAs and synthetic oligonucleotides containing the catalytic palindromic sites. We explored the possibility of developing matrices which could be used to purify these enzymes by biospecific affinity chromatography in conjunction with commonly used fractionation procedures. Specifically, we considered using two matrices, pyran-

sepharose, which we had previously used to purify DNA polymerases (3), and blue CNBr-sepharose as methods of purification. The pyran copolymer (Fig. 1) is a divinyl ether of maleic anhydride and is hydrolyzed in solution to its corresponding polycarboxylic acid which mimics the phosphodiester backbone in nucleic acids (4). Cibacron Blue F3GA (Fig. 1), by contrast, is a blue dye which appears to have biospecific affinity for nucleotide-requiring enzymes (5). The two affinity matrices pyran-sepharose and blue-CNBr-sepharose appear to be useful as a general method for the purification of restriction endonucleases. A preliminary report on this work has been made (6).

MATERIALS AND METHODS

Phage lambda DNA is purified from CsCl banded phage particles isolated from *E. coli* lysogenic for Cl857 SAM 7 (7). Agarose, redistilled phenol, ϕ X174 and Adeno type 2 DNAs were obtained from Behtesda Research Laboratories, Rockville, Maryland.

Cell growth: Cells were grown by inoculating the specific medium with a 2% overnight culture. After growing for 16 hrs, cells were harvested by centrifugation at 8,000 x g, washed with

Figure 1. (A) Proposed structure for coupled Cibacron blue 3FGA to cyanogen bromide activated sepharose. (B) Structure of pyran copolymer and suggested cyclic derivative obtained upon reacting pyran with hydrazine activated sepharose.

0.9% saline and frozen at -90°C . Growth media and temperature conditions for the different strains were as follows: Bacillus amyloliquefaciens and Bacillus globiggi were grown on brain heart infusion (Difco), 37 g/l at 37° ; Haemophilus influenzae R_f was also grown on brain heart infusion supplemented with 2 $\mu\text{g/ml}$ of NAD and 5 $\mu\text{g/ml}$ of Hemin; Providencia stuartii 164 was grown on tryptone (15 g/l), yeast extract (10 g/l) and NaCl (5 g/l) at 37° ; Xanthomonas badrii was grown on nutrient broth (8 g/l) at 32° .

Analysis of DNA endonuclease activities and digestion products by gel electrophoresis: Assays were performed in 25 μl volumes in either optimal buffers based on our findings or values published by other laboratories (details for each specific enzyme are indicated in the legends). Selection of the specific DNAs was based on obtaining a characteristic and recognizable fragmentation pattern. Incubations to locate activity were at 37°C for 1 hr or for 16 hrs to determine absence of contaminants. Reactions were stopped by the addition of 5 μl of reaction stop solution. For routine assays, gels at a concentration of 1.0-1.4% agarose were prepared and electrophoresed in Tris (48.4 g/l), sodium acetate (16.4 g/l), and EDTA (7.4 g/l); adjusted to pH 7.8 with acetic acid; and run for 3 hrs at 6 volts/cm. Tube polyacrylamide (4%) gels were run for 2 hrs at 4 mamp/gel in Tris-borate EDTA buffer (8). Gels were stained with ethidium bromide, and DNA visualized with short wavelength UV light source. Photographs were obtained by the use of a red filter (Kodak 23A) and Polaroid type 57 film.

Unit determination for type II restriction endonuclease: Unit determination was performed with serial dilution of enzyme using a fixed amount of substrate. A unit of enzyme is defined as the minimum amount of enzyme which will totally degrade 1 μg of lambda or other DNA in 60 min at 37° . Definitions such as pmoles of phosphodiester bonds cleaved under specified conditions and activity based on synthetic oligonucleotides containing specific palindromes were considered as substrates. The deciding factors in our selection of the definition parameters were the difficulty of obtaining synthetic oligonucleotides as well as the widely current usage of the enzymes to generate end

point products with DNA substrates.

Preparation of pyran-sepharose and blue CNBr-sepharose:

(a) CNBr-activated Sepharose In order to activate the sepharose 4B (Pharmacia), 100 g was suspended in 100 ml of distilled water and 100 mg of finely divided solid cyanogen bromide (CNBr) was added over 5 min. The pH was adjusted and maintained at 11 by addition of 8 N NaOH with stirring. After 10 min at 20°C, 200 ml of cold distilled water was added. The suspension was washed and the activated sepharose was used routinely within a few hours.

(b) Pyran-sepharose Pyran was coupled to activated sepharose as described previously (3). Specifically, the pH was adjusted to 10 and hydrazine was added at a concentration of 2 mM/ml sepharose. The reaction was allowed to proceed overnight at 4°C for 16 hrs. Hydrazine-activated sepharose was washed with 0.1 M sodium acetate pH 5.0 containing 10 mM MgCl₂. Pyran was added, at 3 mg/ml of hydrazine sepharose and reacted at 45°C for 16 hrs. Coupled pyran-sepharose with a characteristic off-white color was washed with 2 M NaCl to remove free polymer. To verify that the chemical reactions are successful, the matrix is first evaluated by its ability to bind avian myeloblastosis virus reverse transcriptase (3).

(c) Blue-CNBr-sepharose Cyanogen bromide-activated sepharose was washed with excess 1 mM HCl, and Cibacron Blue F3GA was coupled at pH 8.3 in 0.1 M NaHCO₃ and 0.5 M NaCl at 4°C for 16 hrs. If necessary, pH was maintained at 8.3 with 0.1 N NaOH. The ratio of starting blue dye to swelled activated sepharose was 1 g to 10 ml. To block the remaining active groups, 1 M Tris HCl pH 9.0 was added and incubated for 1 hr at 22°C. Excess dye was removed by several washes of acetate (Na⁺) pH 4.0 buffer containing 0.5 M NaCl and a final 7 M urea wash to remove traces of unreacted blue dye.

Storage of matrices Both affinity matrices were stored at 4°C in the presence of high NaCl and 2 mM azide and will remain stable for several years.

RESULTS

Preparation of bacterial crude extracts prior to affinity chromatography fractionation A typical preparation utilizes 15 g

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of prewashed bacterial cells suspended in 35 ml of Buffer A (20 mM Tris-HCl pH 7.2, 1 mM Na₂ EDTA, 7 mM β -mercaptoethanol and 10% glycerol). Cells were at least 80% lysed by sonication using 30 second pulses for 8-10 min with intermittent off cycles. The temperature of the slurry was maintained at 10° through the lysing, and all procedures to follow were carried out at 4° unless otherwise specified. The cell extract was then separated by two sequential centrifugation steps: the first at 10,000 x g for 15 min and the second for 100,000 x g for 2 hrs. The supernatant (approx. 40 ml), referred to as crude extract, is recovered and pellets discarded. Nucleic acids are then removed by one of several procedures, e.g., batch elution from DEAE-cellulose under conditions where the enzyme was recovered, or by precipitation with streptomycin sulfate (9) and PEG-6000 (10). Removal of nucleic acid was found to be a prerequisite to enhancing the capacity of the matrix to bind enzyme. The supernatant after the removal of nucleic acid is submitted to fractionation by affinity matrices as described in the legends.

Purification of restriction endonucleases from unfractionated bacterial extracts Pyran-sepharose and blue-CNBr-sepharose are effective in the separation of restriction endonucleases from nucleic acid free bacterial extracts. We have chosen three enzymes, Hinf 1, Pst 1, and Xba 1, obtained from three different genera. For the initial experiments, a 1.5 ml column each of blue-CNBr-sepharose and pyran-sepharose was equilibrated with Buffer A. Columns were developed with a 20 ml salt gradient 0-0.8 M in Buffer A. As shown in Figure 2A, the restriction endonuclease Hinf 1 can be purified by a single pass on a blue-CNBr-sepharose. On visual examination of digestion patterns using excess enzyme in overnight assays, Hinf 1 obtained appeared to be free of interfering nucleases. In the case of Pst 1 and Xba 1, sequential chromatography over blue-CNBr-sepharose and pyran-sepharose was needed to yield contaminant-free enzyme as shown in Figures 2B and 2C. Salt concentration at the peak of enzyme activity for these and other enzymes tested is summarized in Table 1.

Usage of pyran-sepharose and Blue-CNBr-Sephacrose in further fractionating bacterial extracts In contrast to obtaining enzymes

Figure 2. Analysis of restriction endonuclease activity after fractionation on blue CNBr-sepharose and pyran-sepharose. (A) *Hinf* I activity from crude extracts after fractionation on blue-CNBr-sepharose. Reactions were carried out in 7 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 7 mM 2-mercaptoethanol, 1 μ g of ϕ X174 DNA and enzyme. Reaction products were displayed on 4% polyacrylamide gels using Tris-borate buffer (8). (B) *Pst* I activity from extracts after sequential fractionation on blue-CNBr-sepharose and pyran-sepharose. Reactions were carried out and displayed as described above using 1 μ g of λ DNA. Polyacrylamide 4% was employed to separate the small DNA fragments. (C) *Xba* I activity after sequential fractionation by blue-CNBr-sepharose and pyran-sepharose. Reactions were carried out in 6 mM Tris-HCl pH 7.4, 6 mM MgCl₂, 100 mM NaCl and 5 mM Mercaptoethanol using Adeno 2 DNA. Reaction products were displayed on 1% agarose using Tris-acetate buffer. Select fractions were free of exonuclease activities. Those containing contaminating activities displayed a light smear. All assays were carried out at 37 $^{\circ}$ for 16 hours to determine levels of exonuclease contamination. Recovery of enzyme units could not be calculated due to interfering activities in the starting crude extracts.

TABLE I

Salt Concentration at Peak of Endonuclease Activity

<u>Organism</u>	<u>Enzyme</u>	Peak of elution (Molar KCl)	
		<u>Pyran-Sephadex</u>	<u>Blue-CNBr-Sephadex</u>
Bacillus amyloliquefaciens	Bam H1	0.20	0.45
Bacillus globigii	Bgl 1	0.25	--
Haemophilus influenzae R _f	Hinf 1	--	0.30
Providencia stuartii 164	Pst 1	0.50	0.30
Xanthomanus badrii	Xba 1	0.20	0.25

free from contaminating nucleases, purification of restriction endonucleases to homogeneity has been a relatively difficult task. As part of our effort in pursuing structural studies on Bam H1 (11) and Bgl 1 (12), we have been involved in purifying these two enzymes to homogeneity. We have investigated the feasibility of the two matrices in such purification schemes. Both Bam H1 and Bgl 1 bound to pyran-sephadex (Figure 3) and to blue-CNBr-sephadex (data not shown). In both cases, 2,000-6,000 units were bound by 1 ml swelled pyran-sephadex and blue-CNBr-sephadex.

DISCUSSION

A variety of biological materials have been fractionated by affinity chromatography. This approach takes advantage of biospecific interactions between molecules not offered by conventional fractionation methods. A major use of affinity chromatography has been in the purification of enzymes. With substrates, cofactors or biospecific macromolecules known to have an inhibitory effect, enzymes can be concentrated selectively and then eluted by gradients containing substrates or salt. The advantages offered by such a procedure are speed of purification and potential protection against denaturation during fractionation procedures.

The affinity matrices we investigated in some detail are blue-CNBr-sephadex and pyran-sephadex. The capacity for both

Figure 3. Fractionation of purified Bam HI and Bgl I on pyran-sepharose. Pooled samples of Bam HI and Bgl I which had previously been fractionated by phosphocellulose and hydroxylapatite were submitted to fractionation on pyran-sepharose. (A) Bam HI (pH 7.5), 7 mM MgCl_2 , 7 mM 2-mercaptoethanol and 1 μg of DNA in a total incubation mixture of 50 μl . (B) Bgl I digest of λ DNA. Reaction mixture contained 20 mM Tris-HCl (pH 7.4), 7 mM MgCl_2 , 7 mM 2-mercaptoethanol, 100 $\mu\text{g}/\text{ml}$ gelatin. All assays were carried out at 37°C for 15 minutes. DNA digestion products for both enzymes were displayed on 1.4% agarose gels as described in the text.

matrices is satisfactory although they vary ten-fold when partially purified enzyme is further fractionated on the matrices. Thus, 2,000-6,000 units of enzyme binding per gram of swelled affinity matrix is the obtained capacity as opposed to 200-600 units per gram for the crude starting material. Cibacron Blue

F3GA has also been covalently coupled by the triazine method (14) and has been used extensively to purify a variety of nucleotide-binding enzymes. Several reports (13,14) show kinases and dehydrogenases to bind to this matrix and to be eluted with substrates. These observations strongly suggest the binding to be affinity in nature (13). The blue-CNBr-sepharose we describe in this paper, unlike that coupled by the triazine method (14), is coupled through the amino group of the blue chromophore (Fig. 1A). Currently available commercial preparations were not as effective for restriction endonuclease purification. It is not clear at this time whether the difference is due to coupling site or potential difference in the amount of coupled dye to sepharose.

The exact linkage between pyran and sepharose has not been investigated, however, the proposed linkage (3) is shown in Fig. 1B. We previously have reported pyran-sepharose to be a bio-specific affinity matrix for the purification of DNA polymerases (3). Since pyran mimics the phosphodiester backbone of nucleic acid, it is not surprising that it can be used to fractionate restriction endonucleases. A recent report has also described an affinity fractionation of restriction endonuclease using heparin-sepharose (15). In our hands heparin-sepharose proved to be ineffective when relatively pure samples of Bam HI preparations were submitted to it. The method we have described appears to be readily applicable to a variety of restriction endonucleases (Table I). The stability and rapid development time clearly offer distinct advantages to be exploited in purifying restriction endonucleases.

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