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Efficient large-scale purification of restriction fragments by solute-displacement ion-exchange HPLC

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ABSTRACT

Extreme overloading of HPLC columns with sample can create a condition of binding site saturation causing competition and displacement among solutes during column elution. This has been termed solute-displacement chromatography (SD-HPLC). We present an example of this phenomenon for the preparative fractionation and purification of restriction fragments of almost identical size (1337 and 1388 bp) which cannot be resolved by agarose gel electrophoresis. Standard analytical ion-exchange HPLC chromatography failed to separate these fragments from each other and from an unexpectedly early eluting pUC-derived vector fragment of 2.7 kbp. We demonstrate that by intentional overloading of the small (4.6 x 35 mm) non-porous TSK-DEAE HPLC column, hundreds of micrograms of DNA restriction fragments could be resolved and purified in a single HPLC run of less than 30 minutes.

INTRODUCTION

Purification of restriction fragments of DNA is typically accomplished by separation of fragments by size using agarose gel electrophoresis followed by elution of the desired DNA fragment from gel slices (1–2). This standard method is limited to small amounts of restriction enzyme fragmented DNA, typically a few micrograms of plasmid DNA. Yields range from 20 to 90 percent depending on fragment size, agarose percentage and method of DNA separation from the gel material (1–3).

Over the years many different methods and resins have been developed for the purification of plasmid DNA (2, 4–7) and for the separation of DNA fragments created by restriction nuclease digestions (7–14). Very recently a high-performance ion-exchange chromatography method has been introduced (15) that will separate PCR* products and restriction fragments on small, non-porous DEAE HPLC resin. This high-resolution analytical chromatography method that primarily separates by size is also limited to a few micrograms of restriction enzyme digested DNA and it fails to separate DNA fragments longer than a few thousand base pairs. However, we obtain essentially quantitative DNA recovery by ethanol precipitation if the eluent is concentrated by lyophilization or by ultrafiltration, e.g. in Centricon-10 devices (unpublished results).

This paper describes a novel extension of this ion-exchange HPLC methodology by intentional overloading of the ion-exchange capacity of the column. It is analogous to published methods for separation of protein species during reversed-phase HPLC (16–17). At loading levels where ion-exchange capability of the resin becomes limiting, solute-solute displacement phenomena increase the separating power of the chromatography for the interacting compounds. This condition has been named Solute-Displacement chromatography (SD-HPLC). It allows purification of restriction fragments at several hundred micrograms per run on HPLC columns whose analytical capacity is rated at only a few micrograms of DNA (15). Preparative separation is even accomplished for DNA fragments that cannot be separated under analytical HPLC conditions. This paper presents a method to enable separation in preparative amounts of restriction fragments that co-migrate in agarose gels and that co-elute under conditions of analytical ion-exchange chromatography.

MATERIALS AND METHODS

Plasmid no. 4 digested by Eco RI and Pst I was used in this study as an example substrate for the preparative purification by SD-HPLC of restriction fragments which do not separate under standard DEAE-NPR HPLC conditions. This plasmid is a 6.3 kbp derivative of pUC9 with as insert the 3.6 kbp Pst I–Pst I fragment of cosmid no. 6 (Accession number X60429) (18) containing histone H3.III genomic sequences of *Arabidopsis thaliana*. Plasmid DNA (4.4 mg) was prepared from a 1 liter overnight culture by preparative alkaline cell lysis and purified by CsCl density gradient centrifugation (2). Plasmid DNA (2 mg) was digested to completion for 2 hours at 37°C with 2000 units each of Eco RI and Pst I (Promega) in 1 ml 50 mM NaCl, 90 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ into 4 fragments: 2.7 kbp pUC9 vector and 873, 1388 and 1337 bp insert sequences. The latter two fragments co-electrophorese in 1 % agarose gel electrophoresis in TBE (1). Samples of the restriction digest were injected directly onto the HPLC column. Small samples were diluted with TE8 (10 mM Tris–HCl, 1 mM EDTA, pH 8) to a final volume of 20 µl.

The DEAE non-porous TSK gel resin (0.15 meq per ml) ion-exchange column consisted of a 0.5 cm×4.6 mm ID, 5 micron, DEAE-NPR guard column (0.012 meq) (TosoHaas) and a

3.5 cm×4.6 mm ID, 2.5 micron, DEAE-NPR analytical column (0.087 meq) (TosoHaas). The column was equilibrated at 250 mM NaCl in 25 mM Tris−HCl, pH 9.0, and developed at a flow rate of 1 ml/min by a gradient for 0.5 min from 250 to 500 mM NaCl and for 22 min from 500 to 610 mM NaCl in 25 mM Tris−HCl, pH 9.0. This gradient was created by an ISCO 2360 gradient programmer and 2350 HPLC pump. DNA elution was monitored by absorbance at 260 nm with an ISCO V^4 detector and HPLC cell. The complete solvent flow path consisted of corrosion-resistant steel or PEEK. Fractions of 0.3 ml were collected. DNA was prepared by addition of 0.6 ml cold absolute ethanol to each fraction, storage at −20°C for 1 hour, centrifugation for 15 min at 13,000 *g* in the cold room, decanting and air drying. DNA was solubilized in TE8 and analyzed by 1 % agarose gel electrophoresis (1). DNA fragment elution profiles were determined by semi-quantitative densitometry (19) of Polaroid negatives of ethidium bromide stained gels. Elution of 1337 bp and 1388 bp fragments was evaluated by fragmentation of the latter fragment by Eco RV. Molecular weight markers (pGEM3 from Promega) were used to confirm predicted restriction fragment sizes.

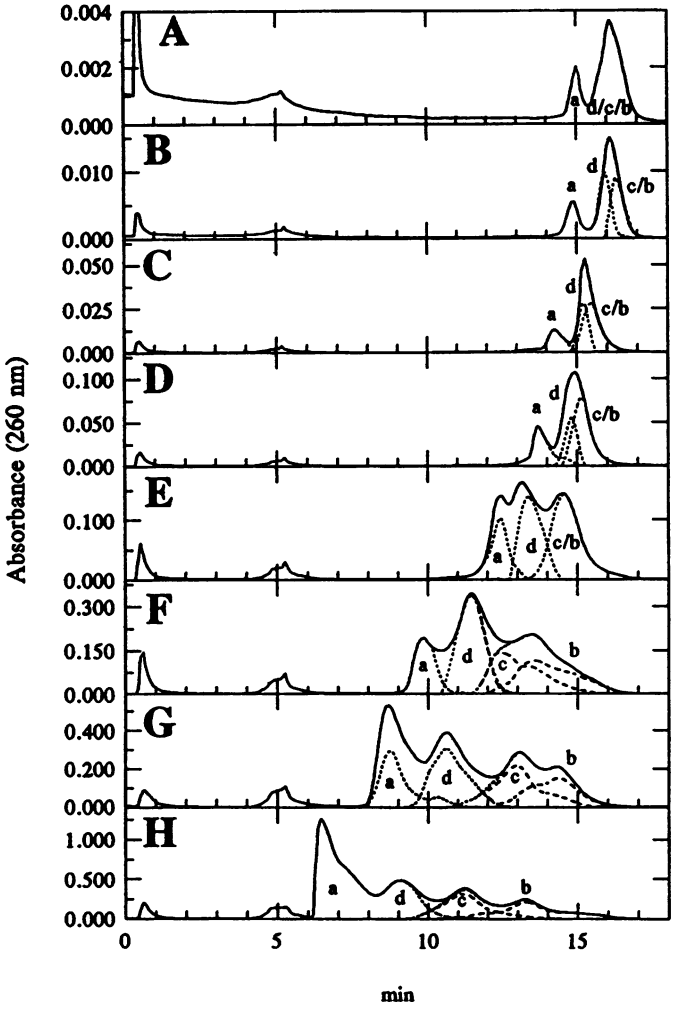

Figure 1. Ion-exchange DEAE-NPR chromatography, monitored by absorbance at 260 nm, of 1 μg (**A**), 3 μg (**B**), 10 μg (**C**), 30 μg (**D**), 100 μg (**E**), 250 μg (**F**), 400 μg (**G**) and 750 μg (**H**) of plasmid no. 4 DNA digested by Pst I and Eco RI. Elution of the four limit digest products 873 bp (**a**), 1337 bp (**b**), 1388 bp (**c**) and 2.7 kbp (**d**) was determined by agarose gel electrophoresis of column fractions (dotted lines) (Fig. 2), and by electrophoresis of selected column fractions after digestion with Eco RV (broken lines) (Fig. 3).

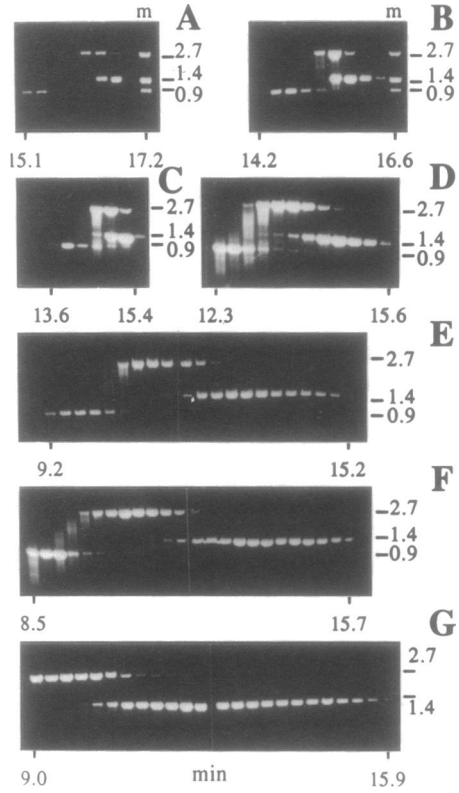

Figure 2. Agarose gel electrophoresis of fractionated 3 μg (**A**), 10 μg (**B**), 30 μg (**C**), 100 μg (**D**), 250 μg (**E**), 400 μg (**F**) and 750 μg (**G**) of plasmid no. 4 DNA digested by Pst I and Eco RI (Fig. 1B−1H). The time of elution of the first and last fraction analyzed is indicated for each section. **m** indicates marker lanes with 0.5 μg of total digest. Fragment size is shown. For gels **A** and **B** all DNA collected by precipitation of the 0.3 ml fraction was loaded. Fractional loading on gel **C** was 1/3, on **D** 1/10, on **E** and **F** 1/20 and on **G** 1/30.

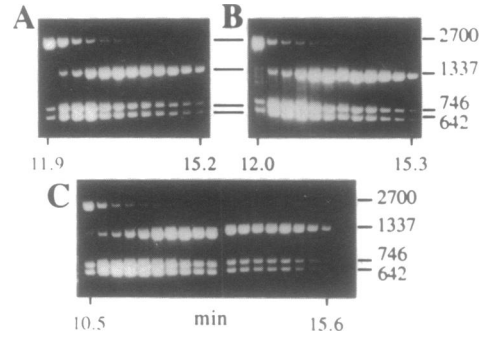

Figure 3. Agarose gel electrophoresis of fractionated 250 μg (**A**), 400 μg (**B**) and 750 μg (**C**) of plasmid no. 4 DNA digested by Pst I and Eco RI (Fig. 1F−1H). The time of elution of the first and last fraction analyzed is indicated for each section. Fragment size is shown. For gels **A** and **B** 1/10 and for gel **C** 1/15 part of the DNA collected by precipitation of the 0.3 ml fraction was digested with Eco RV and loaded. Only the 1388 bp DNA is cut by Eco RV into the 642 bp and 746 bp fragments.

RESULTS AND DISCUSSION

Analytical ion-exchange chromatography on DEAE-NPR HPLC columns of Eco RI−Pst I digested plasmid no. 4 (see Materials and Methods) failed to separate the largest 2.7 kbp restriction fragment from 1337 bp and 1388 bp products (Fig. 1A−B). This was contrary to expectation. Restriction fragments of pBR322 (15) and pGEM3 (results not shown) eluted primarily according to size with near base-line separation between pGEM3 molecular weight marker restriction fragments of 1605 bp and 2645 bp (results not shown). Agarose gel analysis revealed the abnormally early elution of the 2.7 kbp pUC9 vector fragment, ahead of and overlapping with, the unresolved mixture of the 1337 bp and 1388 bp fragments (Fig. 2A).

When we exceeded the reported column capacity of a few μg of DNA for complete analytical resolution of DNA fragments (15) with samples of 10 μg (Fig. 1C, 2B) or 30 μg (Fig. 1D, 2C) digested plasmid DNA, column resolving power decreased as expected. However, the two-peak elution profile changed into a three-peak pattern when 100 μg digested DNA was loaded (Fig. 1E). Gel electrophoresis confirmed increased separation between the earlier eluting 2.7 kbp fragment (d) and the 1388/1337 bp fragments (c/b) (Fig. 2D). Increasing sample load to 250 μg (Fig. 1F, 2E, 3A), 400 μg (Fig. 1G, 2F, 3B) or 750 μg (Fig. 1H, 2G, 3C) further increased fragment separation. The partial separation between the co-electrophoresing 1337 bp (b) and 1388 bp (c) fragments (Fig. 2) was demonstrated by gel analysis after the 1388 bp fragment was cut by Eco RV (Fig. 3).

The combination of increased column resolving power and high sample load under these conditions of extreme column overload is ideal for the preparation of large amounts of restrictions fragments. A single 30 min HPLC run will purify hundreds of micrograms of restriction fragments, even ones that cannot be separated by gel electrophoresis.

The basis of the increased separation, observed at extreme column overload, appears to be the interaction between solutes with slightly different column absorption characteristics under conditions that column binding sites have become limiting. This is distinct from analytical separation conditions where column binding sites are always present in large excess and applies equally to ion-exchange and reversed-phase chromatography modes (16−17). Increased resolving power is obtained because similar but distinct solutes are more effective in competition for, and displacement from, limiting binding sites than simple salts like NaCl. In the method described the NaCl gradient is required to drive the solute-solute displacement phenomenon. The high-performance column characteristics are likely essential to obtain and retain the resolved elution profile (16).

One of the characteristics expected for effective chromatographic resolution is base-line separation between components. Inherently, this is not true for SD-HPLC. When distinct solutes displace each other, both must be present. Thus SD-HPLC always will elute solutes with significant mixing of the components in some intermediate fractions. Monitoring absorbance can detect the resolution of distinct components (Fig. 1), but additional methods will usually be required to measure the extent of mixing (Fig. 2 and 3). In some cases, absorbance differences between solutes and multi-wavelength detection systems can be combined to measure directly the extent of overlap (17).

As shown, large amounts of purified DNA fragments can be obtained. The amount of overlap is minimized by increased column loading (Fig. 1) because this increases column saturation, solute-solute displacement effects and resolving power. The upper limit of column capacity for SD-HPLC, i.e. the failure to bind the solutes of interest at all under column loading conditions, was not reached in this study. To attain solute-displacement separation conditions with generally limited amounts of DNA fragments to be separated, it is typically advantageous to use a column with a small binding capacity. Using the DEAE-NPR guard-column alone (0.012 meq binding capacity) may enable SD-HPLC of 10 μg to 75 μg digested DNA, sample amounts too low to cause solute-displacement effects on the complete system (0.1 meq capacity) used in this study. However, one should expect reduced resolving power on such a short column. Longer, microbore column configurations with the same amount of ion-exchange resin would be preferable. The maximum column size to reach solute-displacement effects at a known sample size is not immediately obvious, especially if non-equimolar ratios of solutes are present. Fractionation of restriction digests with inherently equimolar amounts of distinct solutes may be a type of sample for which SD-HPLC can be readily employed. Preparative separation of similarly sized PCR products could also be possible by SD-HPLC chromatography.

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