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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DNA RESTRICTION FRAGMENTS

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SUMMARY

High-performance liquid chromatography on Nucleogen-DEAE 4000-10 has been applied to several problems of the isolation of DNA restriction fragments. Large amounts of DNA fragments of high purity are necessary for biophysical studies and for molecular hybridization in basic research, as well as in medical diagnosis. The influence of various parameters, such as buffer, pH, eluting salt, gradient slope, flow-rate and the addition of urea on the resolution of fragments by high-performance liquid chromatography were studied on an analytical scale, and the optimal conditions were then used for the large-scale preparation of milligram amounts. The best resolution of fragments between 25 and 1500 base pairs was obtained with a linear gradient from 500 m*M* to 1200 m*M* sodium chloride in 6 *M* urea –30 m*M* sodium phosphate (pH 6.0). Quantitative data are given for the purity and recovery of the sample, and the capacity and lifetime of the column. The following applications of high-performance liquid chromatography of restriction fragments are described: preparation of 2 mg of fragments, separation of 1 mg of DNA insert from 7 mg of its plasmid vector, and analysis of DNA–RNA hybrids.

INTRODUCTION

Large amounts of DNA fragments of high purity are required for biophysical studies and for molecular hybridization. Molecular hybridization as a routine procedure will in the future gain particular importance for medical diagnosis. The preparation of DNA restriction fragments by the classical method, *i.e.* gel electrophoresis, is time-consuming. The high resolution of gel electrophoresis is very favourable; the low capacity and the low yield of recovery, however, are major disadvantages. To circumvent these problems, several chromatographic procedures using gel permeation[1], ion-exchange[2] and reversed-phase chromatography[3] have been developed. According to these reports, gel chromatography on TSK-Gel SW is limited to DNA fragments with fewer than 300 base pairs (bp); the resolution of ion-exchange chromatography on TSK-DEAE-5PW decreases with DNA fragments over 350 bp. The disadvantages of reversed-phase chromatography on RPC-5 are the very long elution times and the bleeding of the stationary phase.

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This paper describes the application of the novel high-performance liquid chromatography (HPLC) anion exchanger Nucleogen-DEAE to the analysis and large-scale preparation of DNA restriction fragments. The combination of anion-exchange chromatography and large pore size offers high resolution, high capacity, and short separation times, *e.g.* 7 mg of a restriction fragment were isolated within 2 h.

MATERIALS AND METHODS

Chemicals and buffers

All chemicals and solvents were of analytical reagent grade from commercial sources. Buffers were made from high-purity water (Milli-Q-System, Millipore, Neu-Isenburg, F.R.G.) and filtered through a 0.8-μm filter. The eluents for HPLC were degassed *in vacuo* before use. All restriction endonucleases were obtained from Boehringer-Mannheim (Mannheim, F.R.G.) and the polyethylene glycol 6000 from Roth (Karlsruhe, F.R.G.).

DNA restriction fragments

For plasmid preparation, cleared lysates of *E. coli* cells were prepared according to the method of Clewell and Helinsky[4]. Further preparation of supercoiled plasmid DNA was carried out as previously described[5]. The following *E. coli* strains with plasmids were used: *E. coli* RRI with pSP 64[6]; *E. coli* RRI with pRH 101. Plasmid pRH 101 is derived from pVH 51[7] and carries the duplicate of the cDNA of potato spindle tuber viroid (PSTV). The cDNA of PSTV was taken from the plasmid pAV 401[8] and inserted in pVH 51 (R. Gilz, personal communication). The plasmid DNA was digested with restriction endonucleases in accordance with the guidelines of the manufacturer. In preparative digests, 1 m*M* spermidine was added to the buffer in order to achieve a higher activity and specifity of the restriction enzymes (A. Pingoud, personal communication). The concentration of the DNA was 100 μg/ml, that of the restriction enzymes 20–100 U/ml. A quarter of the total amount of enzyme was added at the following intervals: the beginning of the digestion, after 1 h, 2 h and 3 h. The reaction was terminated after 6 h by adjusting the solution to 10 m*M* EDTA; the DNA was precipitated with two volumes of ethanol. The precipitate was dissolved in 100 m*M* sodium chloride–10 m*M* Tris (pH 7.0)–1 m*M* EDTA, extracted twice with phenol–chloroform (1:1), and precipitated with ethanol. The DNA was dissolved in a small volume of 10 m*M* Tris (pH 7.0)–1 m*M* EDTA and the concentration of the nucleic acids was determined spectrophotometrically.

Sample preparation

For analytical chromatography, 0.2 μg–0.5 μg of DNA per fragment in a volume of 10–500 μl was injected. For the preparative separations up to 7 mg of DNA in a volume of 2.5–7.5 ml were adjusted to the initial buffer conditions and injected in 2.5-ml portions before the gradient was started.

HPLC equipment

HPLC was performed on a LC 850 liquid chromatograph, equipped with a variable-wavelength UV detector and a thermostatted column compartment (DuPont, Bad Nauheim, F.R.G.). Samples were injected with a loop injector (Rheodyne, Berkeley, CA, U.S.A.) and a sample loop with a 2.5-ml volume.

HPLC columns

Nucleogen-DEAE 4000-10 columns, 125 × 10 mm I.D. and 125 × 6 mm I.D., were used (Macherey-Nagel, Düren, F.R.G.). Nucleogen-DEAE 4000-10 is a silica gel-based weak anion exchanger with a hydrophilic surface. The pore size of the silica gel is 4000 Å, and the particle size is 10 μm. Before use the columns were washed with two column volumes of water, two volumes of low-salt buffer, and two volumes of high-salt buffer, and then equilibrated with two volumes of low-salt buffer. After use, the columns were washed with four volumes of water and stored after equilibration with methanol or acetonitrile.

Desalting

All fractions of a single peak were combined. For precipitation, solid polyethylene glycol was added and dissolved up to a final concentration of 10% (w/v), and then the DNA was allowed to precipitate on ice for 2 h or longer.

Gel electrophoresis

Polyacrylamide (5%) slab gels were prepared as described previously[9]. The gels were stained with silver, as described for proteins[10]. This method is extremely sensitive, allowing the detection of amounts of nucleic acid as low as 500 pg.

RESULTS AND DISCUSSION

An overview of the application of Nucleogen-DEAE to several separation

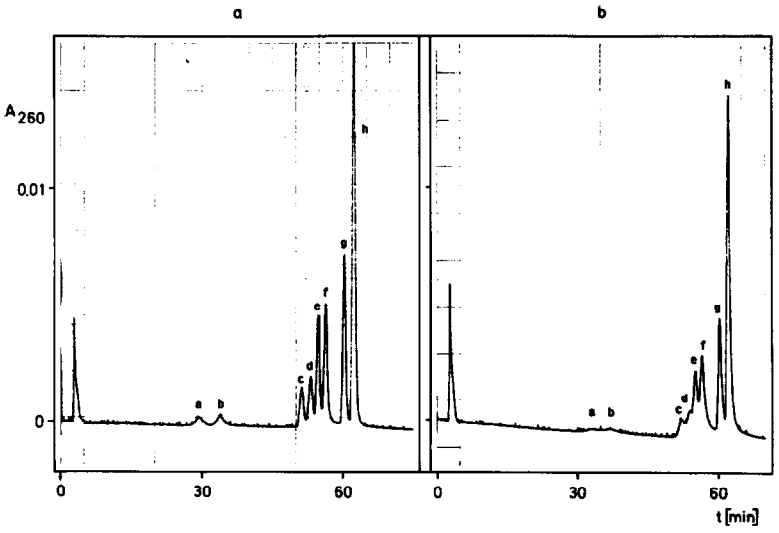

Fig. 1. Influence of the buffer on the resolution of DNA fragments. Analytical chromatogram of a set of restriction fragments from plasmid pSP 64. Cleavage was carried out with the restriction enzyme Hinf I. The total amount of DNA was 5 μg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate 1 ml/min; pressure, 66 bar; temperature, 22°C; linear gradient, 650 m*M* to 1200 m*M* sodium chloride in 110 min (5 m*M*/min). (a) In 6 *M* urea–30 m*M* sodium phosphate (pH 6.0); (b) in 6 m*M* urea–50 m*M* sodium cacodylate (pH 6.0). The lengths of the restriction fragments were: 71 bp (a); 76 bp (b); 182 bp (c); 229 bp (d); 355 bp (e); 398 bp (f); 575 bp (g); 1230 bp (h).

problems of DNA or RNA has been given before[11]. In this work, we focus on the details of the purification of DNA restriction fragments. Examples from our research work on viroid complementary DNA are given. Furthermore, a set of restriction fragments of different length was used to determine the optimum conditions for resolution and purification.

Influence of different chromatographic parameters

Choice of buffer and pH. The pH range of Nucleogen-DEAE columns is limited to pH 3.0–7.5; at higher pH a slight dissolution of the silica gel matrix cannot be avoided. To protect DNA from depurination, the pH should be higher than 4.0.

The suitability of several buffers used in biochemistry was tested. These buffers were phosphate, acetate, cacodylate, and Tris. Fig. 1 shows the chromatogram of restriction fragments; samples from the same solution were fractionated in phosphate (Fig. 1a) or in cacodylate buffer (Fig. 1b) under otherwise identical conditions. All fragments are baseline-separated in phosphate buffer, but the resolution is diminished with cacodylate buffer. Tris and acetate buffers led to a more drastic loss of resolution (data not shown). As a consequence, all further experiments were carried out in phosphate buffer. Varying the pH of the phosphate buffer resulted in a slight increase in resolution from pH 7.0 to pH 5.5. However, this increase is not essential, and the whole range between pH 5.5 and pH 7.0 may be used.

Effect of different salts. Sodium chloride, potassium chloride, ammonium sulphate and sodium chlorate were tested as eluting salts. With sodium chloride the elution is shifted to lower ionic strength compared with potassium chloride (*cf.* Fig. 2). The separation is somewhat better by elution with potassium chloride, one has, however, to take into account that, in the case of potassium chloride, the chromatogram is spread over a broader range of ionic strength. The resolution is decreased

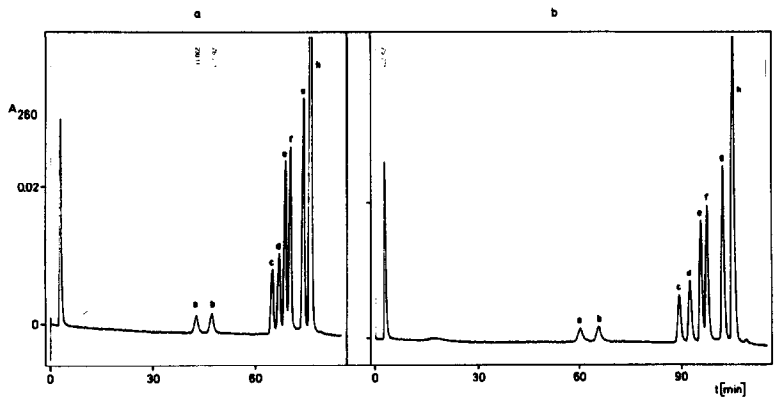

Fig. 2. Influence of the eluting salt on the resolution of DNA fragments. Analytical chromatogram of the same sample as in Fig. 1. The total amount of DNA was 5 μg. (a) Column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 67 bar; temperature, 23°C; linear gradient, 500 m*M* to 1200 m*M* sodium chloride in 140 min (5 m*M*/min) in 6 *M* urea–30 m*M* sodium phosphate (pH 6.6). (b) Column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 62 bar; temperature, 23°C; linear gradient, 500 m*M* to 1200 m*M* potassium chloride in 140 min (5 m*M*/min) in 6 *M* urea–30 m*M* potassium phosphate (pH 6.6).

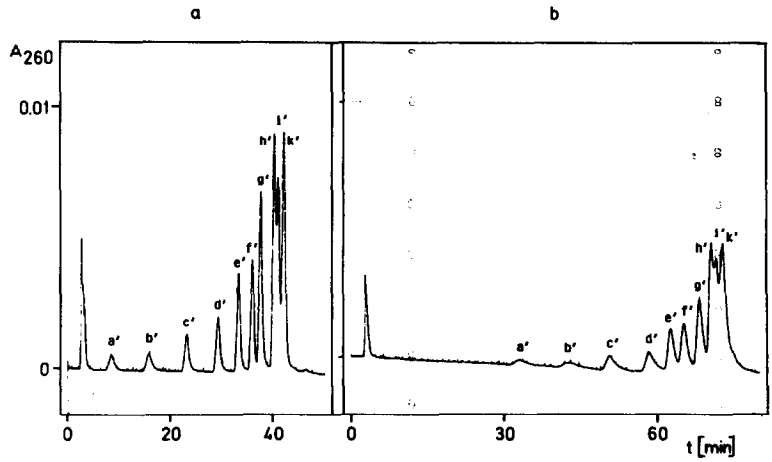

Fig. 3. Influence of urea on the resolution of DNA fragments. Analytical chromatogram of a set of restriction fragments from plasmid pSP 64. Cleavage was carried out with the restriction enzyme Hae III. The total amount of DNA was 5 μg. (a) Column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 65 bar; temperature, 23°C; linear gradient, 700 m*M* to 1200 m*M* sodium chloride in 100 min (5 m*M*/min) in 6 *M* urea–30 m*M* sodium phosphate (pH 6.0). (b) Column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 45 bar; temperature, 23°C; linear gradient, 700 m*M* to 1200 m*M* sodium chloride in 100 min (5 m*M*/min) in 30 m*M* sodium phosphate (pH 6.0). The lengths of the restriction fragments were: 65 bp (a'); 69 bp (b'); 78 bp (c'); 126 bp (d'); 182 bp (e'); 269 bp (f'); 426 bp (g'); 490 bp (h'); 537 bp (i'); 616 bp (k').

drastically with ammonium sulphate and sodium chlorate, which are therefore not recommended for use in this HPLC procedure (data not shown).

Effect of urea. As shown in Fig. 3, a set of restriction fragments was separated in the presence (a) and absence (b) of 6 *M* urea. Addition of urea clearly lowers the ionic strength of elution and increases the resolution remarkably. As it is known that urea is a denaturant for nucleic acid double strands, it should be emphasized that, under the conditions used, the fragments are always in the double-strand conformation. This is true even at a temperature of 45°C, and identical chromatograms were obtained at 20°C and 45°C.

Gradient slope and flow-rate. It is obvious that a higher flow-rate and a shallower gradient slope will increase the peak volume and therefore decrease the detection limit. In Fig. 4, chromatograms obtained at different gradient slopes (10 m*M*/min; 5 m*M*/min; 2.5 m*M*/min) but at constant flow-rate are shown. The absolute amounts of DNA fragments in the chromatograms were the same. The steepest gradient resulted in the highest peak amplitudes and nearly the same resolution compared with the shallower gradients. Therefore, conditions of steep gradients (10 m*M*/min) are most appropriate for fast analysis, *i.e.* they give all the information needed in a very short time. For preparative applications, however, shallower gradients lead in some cases to a better baseline separation (*cf.* peaks f' and g') and facilitate collection of the different peaks without cross-contamination.

The effect of an increase in the flow-rate was similar to that caused by a decrease in the gradient slope, but the flow-rate is limited by the increasing pressure. For our preparation of milligram amounts of restriction fragments, flow-rates of 2–3 ml/min and gradients of 1–2 m*M*/min were used.

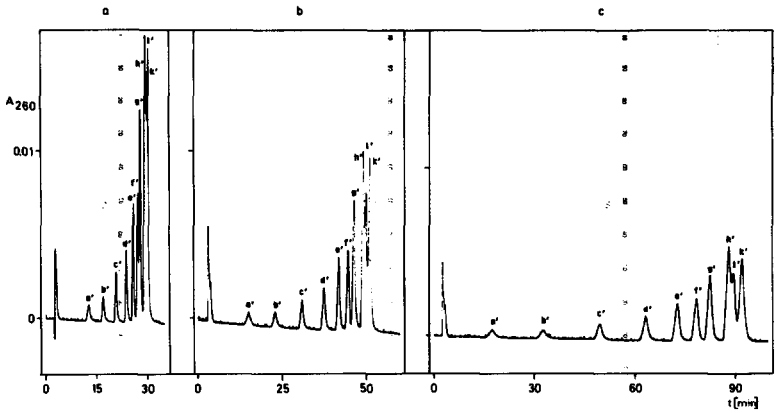

Fig. 4. Influence of the gradient slope on the resolution of DNA fragments. Analytical chromatogram of the same sample as in Fig. 3. The total amount of DNA was 5 μg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 69 bar; temperature, 22°C. (a) Linear gradient from 700 m*M* to 1200 m*M* sodium chloride in 50 min (10 m*M*/min); (b) linear gradient from 700 m*M* to 1200 m*M* sodium chloride in 100 min (5 m*M*/min); (c) linear gradient from 700 m*M* to 1200 m*M* sodium chloride in 200 min (2.5 m*M*/min), in 6 *M* urea–50 m*M* sodium phosphate (pH 5.5).

Capacity, purity and recovery

The high capacity of ion exchangers was also exploited in this work. A 125 × 10 mm I.D. column was used for analytical amounts of DNA as well as for amounts a thousand times greater, typically 5 mg. Although the capacity of the Nucleogen-DEAE material is even larger, it is not advisable to adsorb more than the

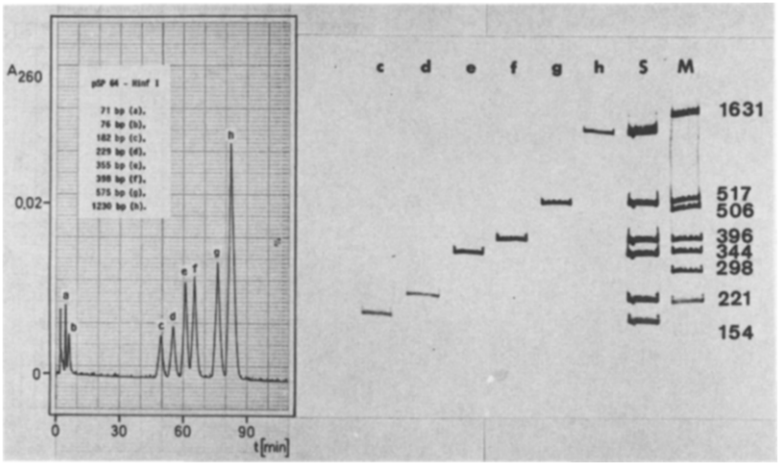

Fig. 5. Purity of DNA fragments, obtained by HPLC and analysed by gel electrophoresis. (a) Chromatogram of the same sample as in Fig. 1. The total amount of DNA was 25 μg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 68 bar; temperature, 23°C; linear gradient, 750 m*M* to 1200 m*M* sodium chloride in 225 min (2 m*M*/min) in 6 *M* urea–50 m*M* sodium phosphate (pH 6.0). (b) Polyacrylamide gel (5%) electrophoresis of the fractions from the HPLC in Fig. 5a. Slots c–h correspond to peaks c–h of Fig. 5a, S is the unfractioned sample, M is a marker (pBR 322, Hinf I digested). The lengths are in base pairs.

amounts mentioned above, because the resolution decreases when the column is loaded close to its total capacity. A further advantage is that the capacity is limited only by the total amount and not by the volume of the sample; even a very dilute sample may be adsorbed by application from a big sample loop.

In order to demonstrate the purity of the different fractions, a set of restriction fragments from the plasmid pSP 64 was fractionated, and the single peaks were analysed by gel electrophoresis (*cf.* Fig. 5a and b). From the silver-stained polyacrylamide gel in Fig. 5b it is obvious that the DNA fragments are completely separated and that no cross-contamination can be detected. The DNA fragments could be cleaved with restriction enzymes and ligated with T4-DNA ligase, in order to show that no enzyme-inhibiting compounds were eluted from the column.

The recovery of DNA from the columns was determined by injecting a known amount of restriction fragments, collecting all peaks, and determining the amount of DNA recovered spectrophotometrically. The yield was higher than 95%, and no decrease was observed after permanent use of the column over more than a year and a half.

Desalting the sample by polyethylene glycol precipitation (*cf.* Materials and Methods) is a simple and fast one-step procedure. It allows the quantitative recovery of the DNA, even from concentrations as low as 0.25 μg/ml.

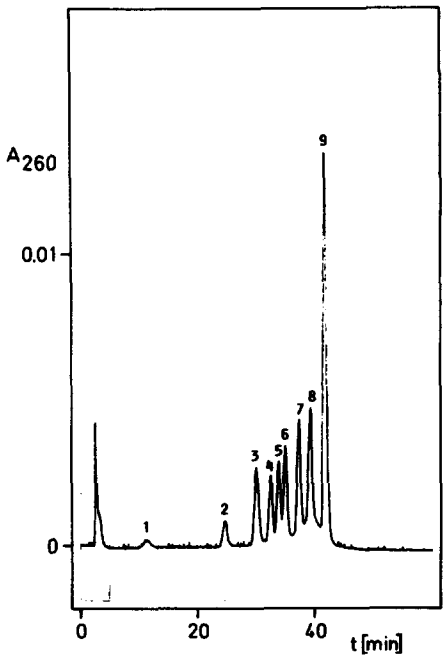

Fig. 6. HPLC of a set of restriction fragments from plasmid pBR 322. Cleavage was carried out with the restriction enzyme Hinf I. The total amount of DNA was 10 μg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 68 bar; temperature, 23°C; linear gradient, 700 m*M* to 1200 m*M* sodium chloride in 100 min (5 m*M*/min) in 6 *M* urea–30 m*M* sodium phosphate (pH 6.0). The lengths of the restriction fragments were: 75 bp (1); 154 bp (2); 220/221 bp (3); 298 bp (4); 344 bp (5); 396 bp (6); 506 bp (7); 517 bp (8); 1631 bp (9).

Application to different separation problems

From the experiments described so far, the following standard combination of chromatographic parameters for optimum separation of DNA fragments from 25 to 1500 bp has been derived: linear gradient from 500 m*M* to 1200 m*M* sodium chloride in 6 *M* urea–30 m*M* sodium phosphate (pH 6.0). These conditions can be routinely applied with only minor adjustment to nearly every separation of DNA fragments. In the following, several different examples of separations with this HPLC technique are given. The main strategy in adapting this technique to new problems of separation

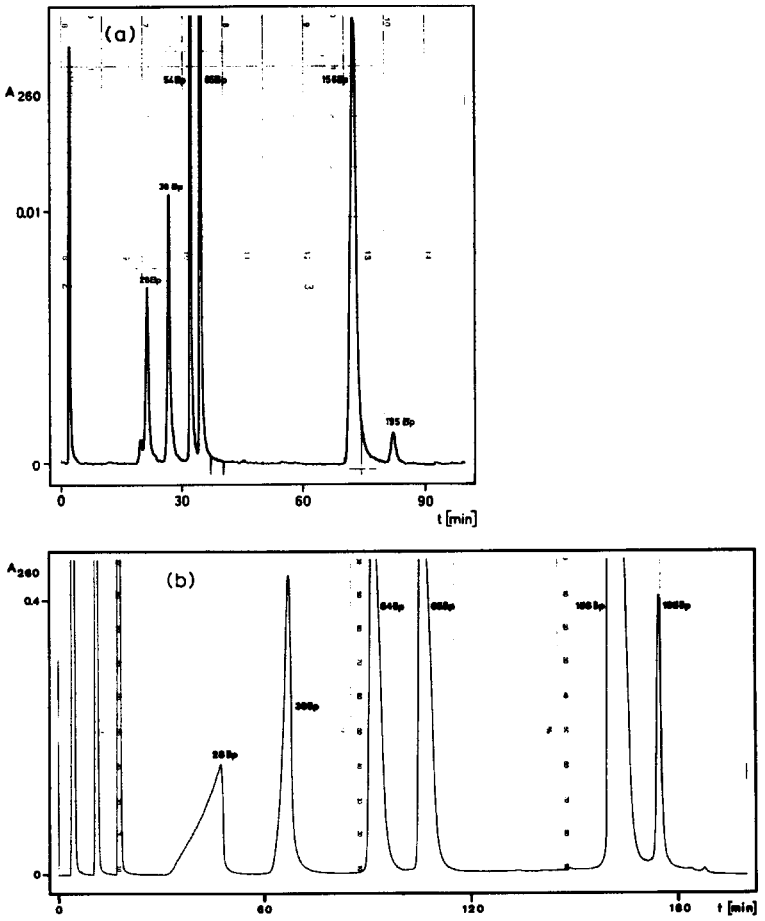

Fig. 7. HPLC of a set of restriction fragments from the cDNA of PSTV. Cleavage was carried out with the restriction enzymes Alu I, Hae III, and Cfo I. In the analytical chromatogram (a) the total amount of DNA was 10 μg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 10 mm I.D.); flow-rate, 2 ml/min; pressure, 27 bar; temperature, 22°C; linear gradient, 500 m*M* to 1200 m*M* potassium chloride in 70 min (10 m*M*/min) in 6 *M* urea, 20 m*M* potassium phosphate (pH 6.7). In the preparative chromatogram (b) the total amount of DNA was 2 mg. Conditions: Nucleogen-DEAE 4000-10 (125 × 10 mm I.D.); flow-rate, 2 ml/min; pressure, 28 bar; temperature, 23°C; linear gradient, 500 m*M* to 1200 m*M* potassium chloride in 350 min (2 m*M*/min) in 6 *M* urea, 20 m*M* potassium phosphate (pH 6.7). The highly asymmetric peak of 28 bp is due to an instrumental error.

is to carry out first analytical chromatography with only small amounts of nucleic acid under the described standard conditions.

Restriction fragments from pBR 322. Fig. 6 shows the chromatogram of a set of restriction fragments from the plasmid pBR 322, obtained under the standard conditions described above. It is remarkable that the fragments with 506 and 517 bp are separated. Whereas the 506-bp fragment is eluted as expected, the 517-bp fragment is eluted at a ionic strength significantly higher than expected. The 517-bp fragment contains a region of 51 bp with 86% AT. The extraordinary chromatographic behaviour of this fragment could be caused by pecularities of the direct interaction of the AT stretch with the chromatographic resin. However, structural influence by a uniform bending of the AT double strand seems more likely, as no base pair specifities have been observed with the Nucleogen material, but on the other hand, structural specifities are known for the different forms of plasmids[5].

cDNA of the potato spindle tuber viroid (PSTV). Viroids are the causative agents of several diseases of higher plants; they consist of a protein-free, circular, single-stranded RNA molecule of *ca.* 350 bases[12]. The sequence of viroids has been cloned in plasmids, and the cDNA of viroids is used for many studies on the molecular biology of viroids. The simultaneous cleavage of the cDNA of PSTV with three different restriction enzymes resulted in DNA fragments of 28, 38, 54, 65, and 156 bp length. In the analytical chromatogram (Fig. 7a) as well as in the preparative chromatogram (Fig. 7b), baseline separation was achieved. In order to accomplish optimum separation in the preparative scale of as much as 2 mg of DNA as well, the

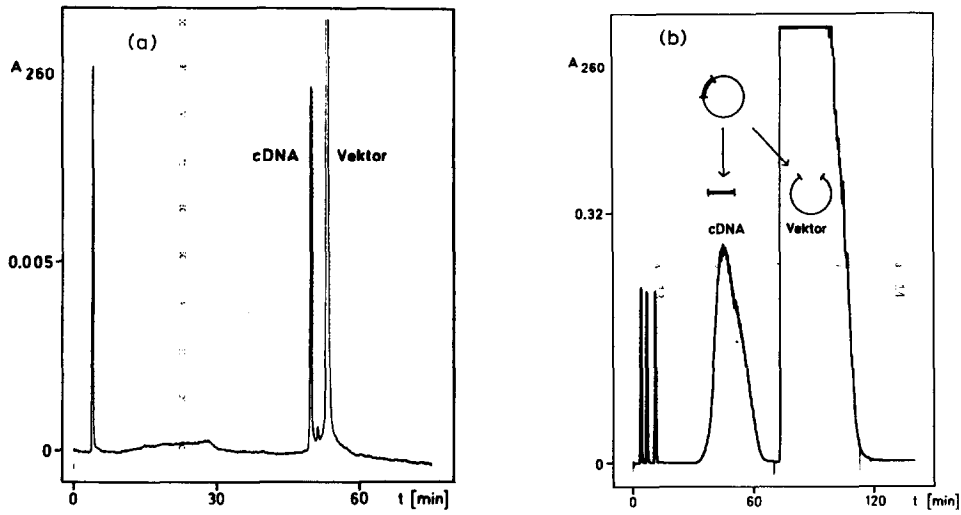

Fig. 8. Separation of a DNA insert from its plasmid vector by HPLC. The cleavage of plasmid pRH 101 with the restriction enzyme Bam HI resulted in the cDNA of PSTV (358 bp) and the plasmid vector pVH 27 (3739 bp). In the analytical chromatogram (a) the total amount of DNA was 5 μg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 10 mm I.D.); flow-rate, 2 ml/min; pressure, 27 bar; temperature, 23°C; linear gradient, 500 m*M* to 1200 m*M* potassium chloride in 70 min (10 m*M*/min) in 6 *M* urea–20 m*M* potassium phosphate (pH 6.7). In the preparative chromatogram (b) the total amount of DNA was 7 mg. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 10 mm I.D.); flow-rate, 2.25 ml/min; pressure, 24 bar; temperature, 23°C; linear gradient, 840 m*M* to 1200 m*M* potassium chloride in 360 min (1 m*M*/min) in 6 *M* urea–20 m*M* potassium phosphate (pH 6.7).

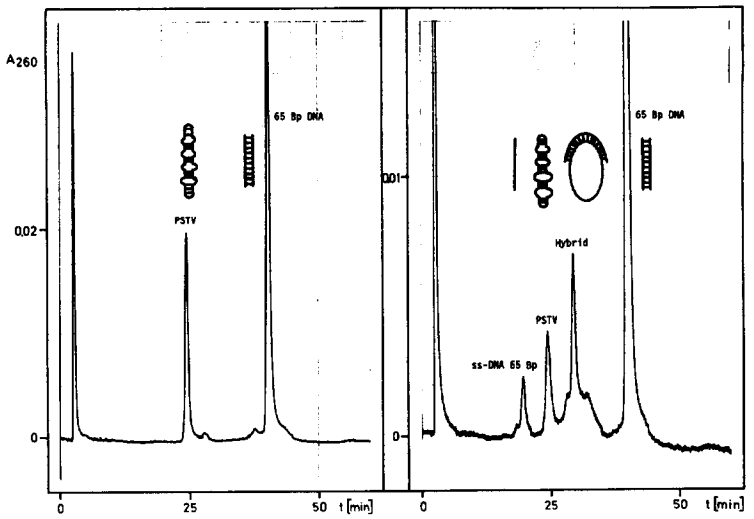

Fig. 9. HPLC of DNA–RNA hybrids. Chromatographic analysis of a hybridization of PSTV with a 65 bp restriction fragment from the PSTV-cDNA: (a) 1 μg of PSTV, 2 μg of 65 bp DNA; (b) hybridization of 1 μg of PSTV with 4 μg of 65 bp DNA. Conditions: column, Nucleogen-DEAE 4000-10 (125 × 6 mm I.D.); flow-rate, 1 ml/min; pressure, 48 bar; temperature, 24°C; linear gradient, 500 m*M* to 1200 m*M* potassium chloride in 70 min (10 m*M*/min) in 6 *M* urea–25 m*M* potassium phosphate (pH 6.6).

gradient slope was lowered from 10 m*M*/min to 2 m*M*/min. The reasons for this have been discussed above. The peak 195 bp was caused by incomplete digestion. The three peaks at the beginning of the chromatogram were due to the three-fold injection of the sample.

The HPLC technique described in this work is also very useful for separating a cloned DNA insert from its plasmid vector. The DNA insert was released from its vector by a restriction enzyme, and 5 μg of the mixture were analysed (Fig. 8a). The preparative fractionation (Fig. 8b) yielded 1 mg of DNA insert from 7 mg of plasmid in less than 2 h.

RNA–DNA hybrids. Incubation of a single-stranded RNA with complementary double-stranded DNA under appropriate conditions leads to the formation of the RNA–DNA hybrid double strand, and the release of the non-complementary DNA strand and the original compounds. Fig. 9 shows the analysis of such an experiment before hybridization (a) and after hybridization (b). The extent of hybridization may be estimated quantitatively from this chromatogram.

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