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Analysis and purification of DNA restriction fragments by high-performance liquid chromatography

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ABSTRACT

The purification and analysis of restriction fragments play a very important role in molecular biology but the traditional assay methods of DNA fragments, based on gel electrophoresis and caesium chloride gradient centrifugation, are time-consuming and difficult to quantify. High-performance liquid chromatography provides an alternative method which allows the direct quantitation of picogram amounts of eluents in short times. In the present work we report the separation of different restriction fragments, the purification of some fragments and the relationship between the length of double-stranded DNA fragments and peak areas.

INTRODUCTION

DNA restriction fragments are the products of digestion of larger DNA with restriction endonucleases that cleave phosphodiester bonds of specific nucleotide sequences. The analysis and purification of these fragments are very important in molecular biology and several conventional methods based on centrifugation and gel electrophoresis are known [1]. However, in recent years high-performance liquid chromatography (HPLC) has been shown to be a useful alternative [2–5]. In comparison with gel electrophoresis, HPLC provides shorter separation times (within 30 min), nearly quantitative recovery, detection in the picogram range, direct quantitation of eluates and the possibility of automizing the overall procedure.

As a part of our interest in the application of HPLC in the study of a wide variety of biological molecules, we extended the use of an anion-exchange column [6] (Gen-Pak FAX) in resolving fragments obtained from double-stranded plasmid 11.3, Φx174, after digestion with restriction endonucleases (*Pst*I, *Eco*RI and *Hae*III), and a 1-kilobase (kb) ladder DNA.

In the course of the investigation a direct relationship between peak areas and molecular size of the fragments was established.

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EXPERIMENTAL

Materials

Plasmid 11.3 [6.0 kilobase pairs (kbp)], coding for a poly A binding protein, its fragments generated by digestion with *Pst*I (f1 = 0.6 kb, f2 = 1.4 kb and f3 = 4.0 kb) and with *Eco*RI (f4 = 2.9 kb and f5 = 3.1 kb) were obtained from P. Vezzoni (Instituto Tecnologie Biomediche Avanzate, CNR, Milan, Italy).

Φx174 DNA, digested with *Hae*III, and the 1-kb DNA ladder were supplied by J. Malyszko (Farmitalia, Milan, Italy). T4 DNA ligase was from Boehringer Mannheim Biochemica (Mannheim, Germany).

Apparatus

The HPLC system consisted of an automated gradient controller, two Model 510 pumps, equipped with a Model U6K universal injector (Waters Assoc., Milford, MA, USA). For detection of the peaks a Model Lambda Max 480 UV detector (Waters) connected to a CR3A integrator (Shimadzu, Kyoto, Japan) or an HP 1040A photodiode array detector (Hewlett-Packard, Waldbronn, Germany) was used. Peak collection and reinjection was done by means of a Model 232 automatic sample processor and injector with a Model 401 dilutor (Gilson Medical Electronics, Villiers le Bel, France) equipped with a Rheodyne 7010 injector.

Chromatographic conditions

All HPLC runs were performed on Waters Gen-Pak FAX columns (100 × 4.6 mm I.D.). Eluent A was 25 m*M* sodium phosphate (pH 7.0) and eluent B was the same buffer containing 1 *M* sodium chloride. Phosphate buffer was made using the monobasic salt and the pH was adjusted using sodium hydroxide. Eluents were filtered through a 0.45-μm membrane. The gradient profile was 40 to 70% eluent B in 20 min (curve 5). Flow-rate was 0.8 ml/min. The peaks were detected at 260 nm or by means a photodiode array detector.

Electrophoresis

Gel electrophoresis was carried out on 0.7% agarose [7] using 0.08 *M* Tris–phosphate, pH 8.0, 2 m*M* EDTA. Capillary electrophoresis was performed using an Applied Biosystems Model 270A and SepraGene 500 buffer in the laboratory of Dr. G. Sabbatini (Applied Biosystems, Milan, Italy) according to the manufacturer's protocol.

Calibration graphs

Fragments f1, f2 and f3 were collected by means of a Model 232 automatic collector and increasing amounts of each fragment (0.1–2.5 μg) were injected.

RESULTS AND DISCUSSION

The Gen-Pak FAX column is a DEAE anion-exchange column on methacrylate matrix of 2.5 μm particle size optimized for DNA restriction fragment separation. Using this column it is possible to separate the 0.6-, 1.4- and 4.0-kb fragments generated by digestion of the 11.3 plasmid with *Pst*I endonuclease (Fig. 1). Baseline

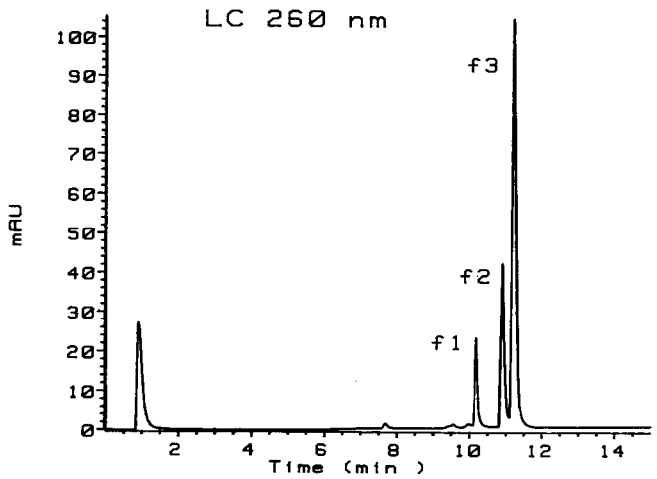

Fig. 1. Typical chromatogram of plasmid 11.3 (0.5 μg) digested with *PstI* endonuclease. See text for chromatographic conditions.

resolution of these relatively small restriction fragments (1.2 and 2.6 kb difference) was achieved. The purity of the collected fractions was checked by HPLC and gel electrophoresis. As shown in Fig. 2, exact coincidence between the UV spectra of the fragments was confirmed, as may be expected. Amounts of up to 15 μg of plasmid were fractionated in a single run with 85% recovery. This means that the approach has an important semi-preparative value. Moreover, the three fragments have been recombined by means of T4 DNA ligase to yield the original plasmid, shown on gel

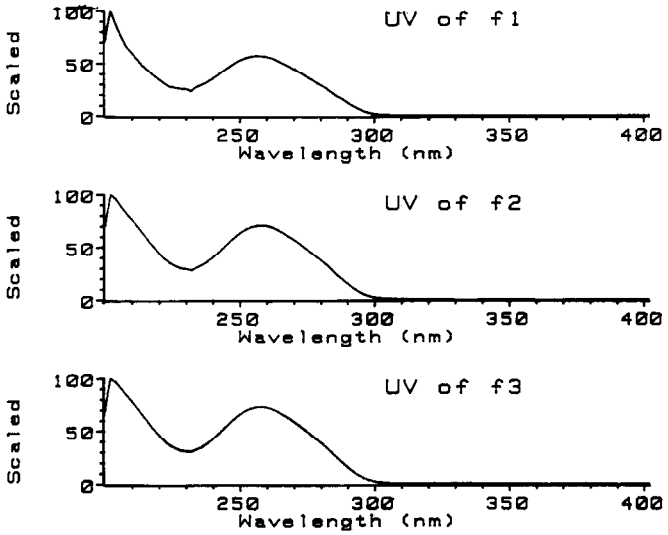

Fig. 2. UV spectra of restriction fragments f1, f2 and f3.

TABLE I

COMPARISON BETWEEN GEL ELECTROPHORESIS AND HPLC KILOBASEPAIR VALUES

Fragment	Electrophoresis (kbp)	HPLC (kbp)	Difference (%)
f1	0.6	0.62	+ 3.3
f2	1.4	1.41	+ 0.9
f3	4.0	3.97	− 0.8

electrophoresis and HPLC analysis, thus indicating that the chromatographic procedure is valuable for the preparation of intact fragments.

Rectilinear responses between peak areas and amounts injected were obtained from four replicate injections of f1, f2 and f3 in the range 0.1–2.5 μg, as indicated by

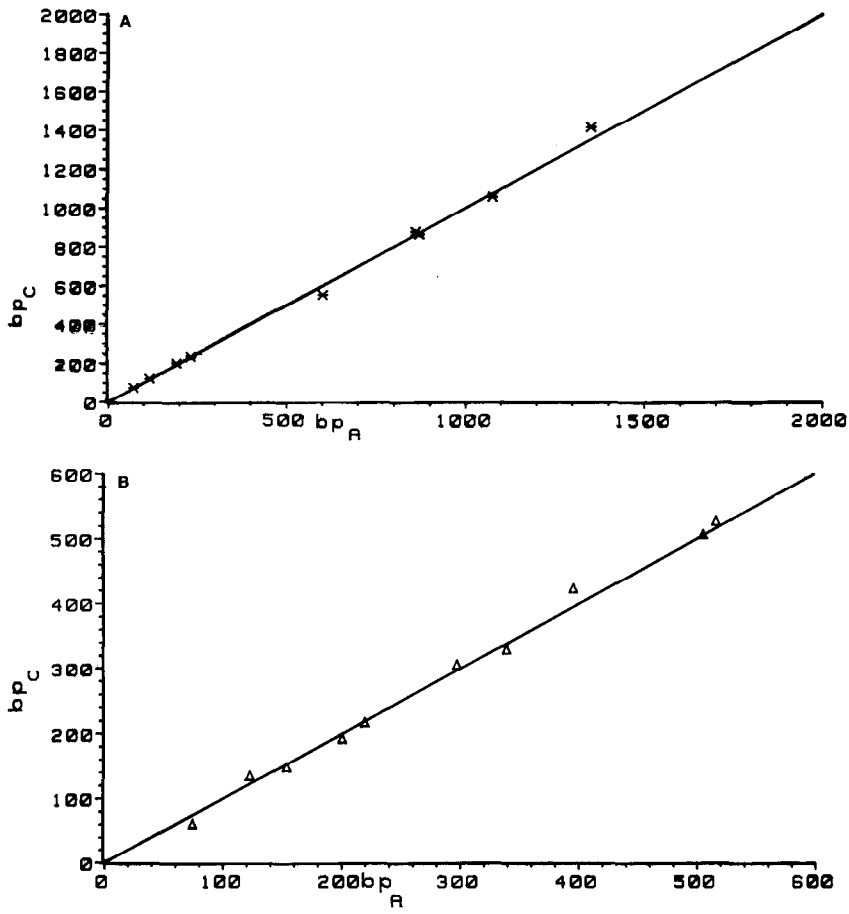

Fig. 3. Comparison between the number of actual (bp_A) and HPLC calculated (bp_C) basepairs for Φx174 DNA digested with *Hae*III (A) and the 1-kb DNA ladder previously isolated by HPLC (fragments from 75 to 517 bp) (B). (A) $bp_C = 1.03\ bp_A + 4.3$; $r = 0.997$. (B) $bp_C = 0.98\ bp_A + 3.2$; $r = 0.998$.

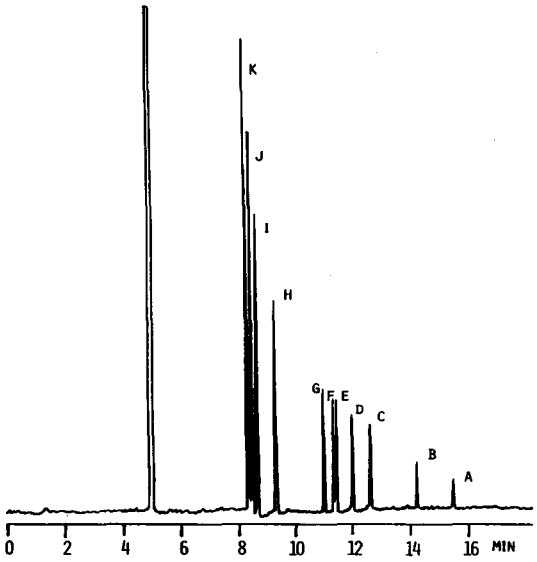

Fig. 4. Typical capillary electropherogram of Φx174 digested with *Hae*III endonuclease (fragments A–K); detection, 260 nm.

the equation $y = 1388\ x + 11.2$ ($r = 0.995$), where y represents the peak area and x the amount injected.

Since exhaustive restriction digestion of the 11.3 plasmid yielded equimolar mixture of f1, f2 and f3, these were present in the same molar concentration. Thus, the mass of the fragment in each peak was proportional to the length of the fragment in that peak. This means that the size of each fragment can be obtained by the equation: $x + ax + bx = K$; where x is the kb number of f1, a is (peak area of f2)/(peak area of

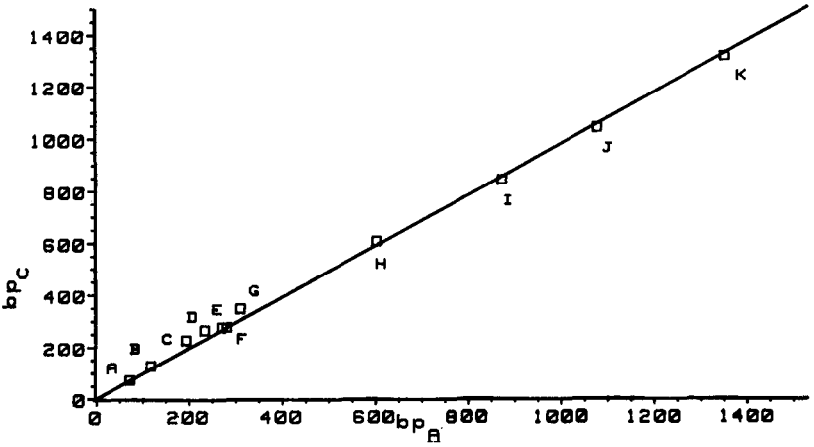

Fig. 5. Comparison between the number of actual and capillary electropherogram calculated basepairs for *Hae*III restriction fragments of Φx174 (fragments: A–K). $bp_C = 1.02\ bp_A + 2.1$; $r = 0.996$.

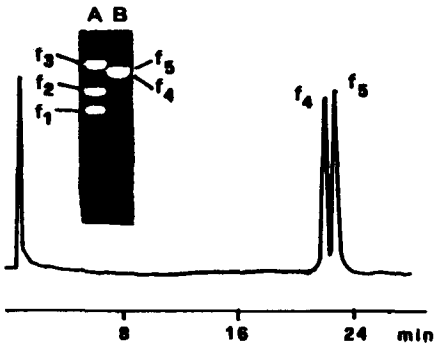

Fig. 6. Comparison of HPLC and slab gel electrophoresis (B) of restriction fragments f4 (2.9 kb) and f5 (3.1 kb) from plasmid 11.3 digested with *Eco*RI. In (A) the electrophoretic separation of restriction fragments f1, f2 and f3 (see Fig. 1) is also shown. See text for HPLC and electrophoresis conditions.

f1); b is (peak area of f3)/(peak area of f1) and K is the length of plasmid 11.3 (6.0 kbp).

Chromatographic data yielded the values of a and b that made it possible to calculate the kilobases of fragments f1 (x), f2 (ax) and f3 (bx). The values of kilobase pairs obtained by this procedure were compared with those from the conventional electrophoretic approach, as shown in Table I.

To verify the validity of this approach, Φx174 DNA (5386 bp), after digestion with *Hae*III, and the 1-kb DNA ladder were chromatographed and the lengths of the fragments evaluated as above. The results shown in Fig. 3A and B indicate that the discrepancy between the actual and the calculated basepair values is less than 3%.

Using this approach the length of restriction fragments can also be determined from capillary electrophoresis data, as confirmed by the analysis of the fragments from Φx174 digested with *Hae*III. In comparison with the described HPLC procedure, capillary electrophoresis [8] yielded a better peak separation (Fig. 4). Moreover, the difference between the calculated and actual number of bases is comparable to that found by the HPLC method (Fig. 5).

Finally, as further evidence of the ability of the Gen-Pak FAX column in resolving DNA fragments, plasmid 11.3 was digested with *Eco*RI endonuclease to yield two fragments (2.9 and 3.1 kb), which cannot be separated by electrophoresis (Fig. 6). HPLC allowed their preparative separation, as well as the evaluation of their length and purity.

In conclusion, the described procedure allows the semipreparative isolation of intact DNA fragments suitable for yielding the original plasmid and for eventual automated DNA sequencing after a single dialysis step. Furthermore, the proposed method for determination of the length of the restriction fragments represents an innovative alternative that can be applied both to HPLC and capillary electrophoresis data.

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