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Note

Rapid separation of single-stranded DNA from double-stranded DNA by reversed-phase high-performance liquid chromatography

J. P. LIAUTARD
Unité 249 I.N.S.E.R.M. et C.R.B.M., Institut de Biologie, Boulevard Henri IV, 34060 Montpellier Cedex (France)

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Separation of single-stranded DNA (ssDNA) from double-stranded DNA (dsDNA) has been achieved by various methods, such as hydroxyapatite chromatography[1], caesium sulphate centrifugation[2], phase partitioning[3] and nitrocellulose chromatography[4]. More recently, charge-transfer chromatography has been successfully applied to this problem[5]. Because these methods are time consuming or the necessary materials are difficult to prepare, we tried to use high-performance liquid chromatography (HPLC) to solve this problem.

Small nucleic acid molecules can be separated by reversed-phase HPLC[6–8]. Recently we have shown that this method can be used to separate large nucleic acids according to their base composition[9]. A study of natural RNA molecules on reversed-phase materials[10] showed that their secondary structure also greatly influences their retention behaviour.

In this paper we show that ssDNA is retained more strongly than dsDNA on an octadecylsilane column. We have taken advantage of this difference in behaviour to separate ssDNA from dsDNA in a single rapid step.

EXPERIMENTAL

Equipment and materials

Chromatography was performed on a Varian 5000 liquid chromatograph equipped with a Varian MCH 10 (octadecylsilane) column (25 × 0.40 cm I.D.) of particle size 10 μm.

Acetonitrile (HPLC grade) was purchased from Touzart et Matignon (France). S_1 nuclease was obtained from Boehringer (F.R.G.).

Methods used

DNA labelling. HeLa cells were labelled with $H_3{}^{32}PO_4$ using a long incubation time (40 h) as described previously[11].

DNA was prepared from a purified chromatin fraction using proteinase K digestion, phenol extraction and ribonuclease treatment as described by Wu *et al.*[12].

S_1 nuclease treatment. DNA (100 μg) was dissolved in buffer D [50 m*M* sodium acetate (pH 4.5)–100 m*M* sodium chloride–2 m*M* zinc sulphate] and was incubated with 10^4 units of S_1 nuclease at 37°C for 30 min.

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DNA denaturation. DNA was dissolved in buffer A [10 m*M* Tris–HCl (pH 7.2)–1 m*M* EDTA–10 m*M* sodium chloride] and incubated in boiling water (95–98°C) for 5–30 min as described in the legends of the figures.

Analytical separation of DNA. The column (particle size 10 μm) was equilibrated with at least 15 ml of 0.1 *M* ammonium acetate (pH 6.6) (solution A). DNA was applied and eluted by increasing solution B [acetonitrile–water (1:1)] from 0 to 40% in 200 min with a flow-rate of 0.2 ml/min, then the column was washed by increasing solution B to 100% in 10 min and continued for 60 min at 100%. The flow-rate was kept at 0.2 ml/min throughout.

Rapid separation of DNA. DNAs was applied to the column equilibrated as described above. Elution was performed by increasing solution B to 50% in 15 min, then from 50 to 100% in 5 min. The flow-rate was 1 ml/min.

Radioactivity measurements. Fractions of 1 ml were collected and the radioactivity of ^{32}P-labelled DNA was measured in solution, using Cerenkov radiation, in an Intertechnique liquid scintillation counter. The efficiency was 30%.

RESULTS AND DISCUSSION

Behaviour of DNA in reversed-phase HPLC

DNA was prepared as described above and applied to a C_{18} column equilibrated with 0.1 *M* ammonium acetate. Elution was performed by increasing the acetonitrile concentration. The results are presented in Fig. 1A (closed circles). A major peak eluted at 110 min with a shoulder lagging behind this peak (from 140 to 250 min). This shoulder was eliminated by treating the DNA with S_1 nuclease under conditions where only ssDNA was degraded (Fig. 1A, open circles). This result suggests that undegraded dsDNA containing no ssDNA elutes as a peak around 110 min under the chromatographic conditions used. The shoulder is probably due to the presence of some ssDNA. One would expect the free bases of this ssDNA to interact with the stationary phase and retard the macromolecule.

In order to confirm this hypothesis, the DNA was denatured by heating for different periods of time. When the DNA was heated for 5 min at 95°C, most of the DNA was denatured but some DNA stretches with a high G + C content remained double-stranded, as about 30% of this DNA could not be digested by S_1 nuclease. This DNA was chromatographed on a C_{18} column (Fig. 1B, closed circles). Under these conditions about 25% of the DNA eluted at 110 min as dsDNA. The remaining 75% eluted as large peak of heterogeneous material after 180 min. This heterogeneity could be due both to the differences in the base composition of the molecules and to the presence of short stretches of dsDNA in the molecule. The base composition of RNA molecules has previously been shown to play an important role in their retention behaviour[9]. The effect of short stretches of dsDNA could be eliminated by totally denaturing the DNA by boiling for 15 min, which leaves less than 3% of the DNA undigested with S_1 nuclease. Its chromatographic behaviour is shown Fig. 1B (open circles). The material eluting at 110 min (dsDNA) is absent and the amount of material eluting at about 210 min was reduced. This shows that the part of peak eluted at about 210 min was ssDNA containing some stretches of dsDNA. A similar phenomenon in which dsDNA stretches have changed the behaviour of ssDNA has been observed during chromatography on hydroxyapatite[1]. The fact that heterogeneity is

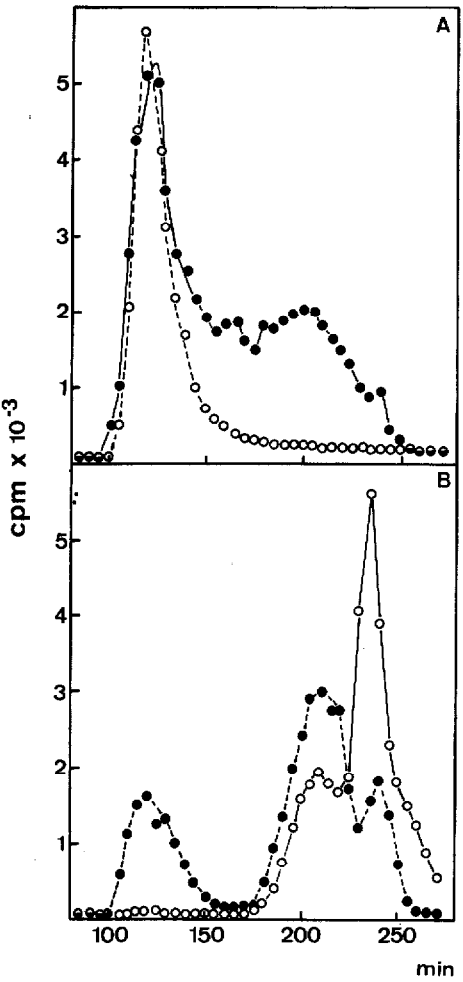

Fig. 1. Behaviour of ssDNA and dsDNA in reversed-phase chromatography. Labelled DNA was prepared as described under Experimental; 50 μg of DNA were applied in each instance. The column was equilibrated with 100 m*M* ammonium acetate (pH 6.6) and elution was performed by increasing the concentration of acetonitrile solution (50% solution in water) from 0 to 40% in 200 min, then from 40 to 100% in 10 min and continuing at 100% for 60 min. The flow-rate was 0.2 ml/min and the chromatography was performed at room temperature. Fractions of 1 ml were collected and ^{32}P radioactivity counted. (A) ●, crude DNA; ○, DNA after treatment with S_1 nuclease (contains only dsDNA). (B) ●, DNA partially denatured by incubation for 5 min at 95°C; ○, DNA totally denatured by incubation for 15 min at 98°C (contains only ssDNA).

not eliminated completely by denaturation suggests that the base composition of the ssDNA molecules can affect their retention.

Rapid separation of ssDNA from dsDNA

dsDNA (prepared by S_1 nuclease treatment) and ssDNA (prepared by boiling for 15 min and cooling with ice) were mixed and chromatographed on an octadecylsilane column. Fig. 2 shows that these two species could be completely separated in 15 min.

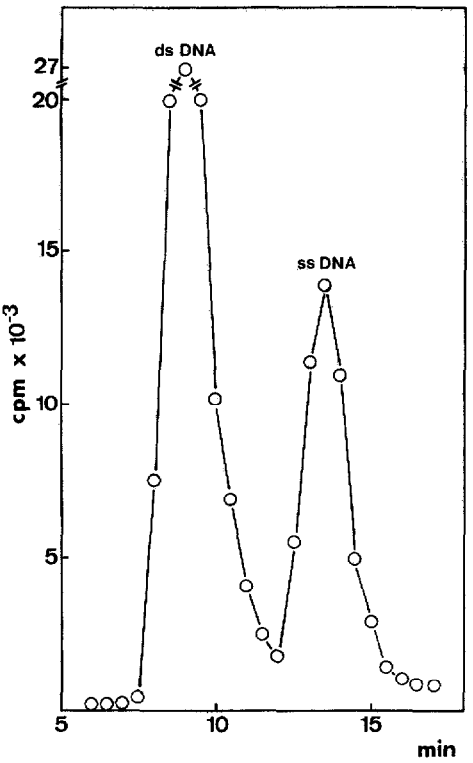

Fig. 2. Rapid separation of ssDNA from dsDNA. A 100-μg amount of labelled DNA prepared as described under Experimental was treated with S_1 nuclease (dsDNA) and mixed with 50 μg of DNA denatured by incubation for 15 min at 98°C (ssDNA). This mixture was applied to the octadecylsilane column equilibrated with 100 m*M* ammonium acetate (pH 6.6) and eluted by increasing the concentration of acetonitrile solution (50% in water) from 0 to 50% in 15 min, then from 50 to 100% in 5 min. The flow-rate was 1 ml/min. dsDNA eluted at 9 min and ssDNA at 13.5 min.

CONCLUSION

ssDNA and dsDNA are retained on an octadecylsilane column used in reversed-phase chromatography. They are eluted by increasing the concentration of acetonitrile. dsDNA elutes first as a sharp peak and ssDNA elutes as a wide peak at higher acetonitrile concentrations. We have used these properties to separate ssDNA from dsDNA in a few minutes.

The separation is both faster and easier than the other methods used to separate dsDNA from ssDNA, such as hydroxyapatite chromatography[1], nitrocellulose chromatography[4], caesium sulphate centrifugation[2] or phase partitioning[3].

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