Journal of Chromatography, 467 (1989) 299–303
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 319

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High-performance hydroxyapatite chromatography of nucleic acids

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(First received October 17th, 1988; revised manuscript received January 16th, 1989)

Hydroxyapatite chromatography (HAC) was developed by Tiselius *et al.*[1] and is widely used for the separation of nucleic acids[2–19]. High-performance HAC has recently been developed since smaller particles with mechanical stability could be supplied reproducibly. Although high-performance HAC has been used for the separation of proteins[20–27] and glycosides[28], the separation of nucleic acids has been limited to only total DNA and tRNA[29,30]. More recently, a new high-performance HAC column has become commercially available under the trade-name TSKgel HA-1000, which has 5-μm particles with a large pore size of 1000 Å[25]. In this work we examined the separation of various types of nucleic acids such as whole DNA, plasmids, phage RNA, tRNA and oligonucleotides on TSKgel HA-1000.

EXPERIMENTAL

All chromatographic separations were performed at 25°C with a high-performance liquid chromatograph consisting of a CCPM pump (Tosoh, Tokyo, Japan), a Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.), a UV-8000 detector operated at 260 nm and an FBR-2 recorder (Tosoh).

Samples of 2 μg of calf thymus DNA, one before and one after heat denaturation, were separated using a 30-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) containing 0.01 m*M* calcium chloride. The sample was denatured by incubation at 100°C for 5 min. A plasmid mixture containing 12 μg of M13mp8 and 11 μg of pBR322 and a 16-μg sample of MS2 phage RNA were separated with a 30-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8). A mixture of oligodeoxyadenylic acids containing 375 milliabsorbance units (260 nm) of $pd(A)_{40-60}$ and 15 milliabsorbance units (260 nm), of $pd(A)_{20}$ was separated with a 240-min linear gradient from 2 to 100 m*M* sodium phosphate (pH 6.8). The number of oligodeoxyadenylic acids was calculated by the injection of a pure sample of $pd(A)_{20}$. A 100-μg sample of tRNA from *E. coli* and Baker's yeast were separated with a 120-min linear gradient from 10 to 400 m*M* sodium phosphate (pH 6.8).

All columns except that used for the separation of oligodeoxyadenylic acids were

0021-9673/89/$03.50

75 × 7.5 mm I.D. with a guard column (10 × 6 or 10 × 4.6 mm I.D.). The oligodeoxyadenylic acid sample was separated on a glass column (75 × 8 mm I.D.). The dead volume between the pump and the column was 2.0 ml and the column volume was 2.8 ml. The concentration of sodium phosphate in the eluate was calculated from the linear gradient in the separation system considered with the dead volume and the column volume. The flow-rate was 1.0 ml/min. The recovery of nucleic acids was determined by measuring the absorbance of eluate at 260 nm.

Calf thymus DNA, *E. coli* and baker's yeast tRNA were purchased from Sigma (St. Louis, MO, U.S.A.). Plasmid M13mp8 was obtained from New England Biolabs. (Beverly, MA, U.S.A.). Plasmid pBR322 was prepared according to Tsurimoto and Matsubara[31]. MS2 phage RNA was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). The oligodeoxyadenylic acids $pd(A)_{20}$ and $pd(A)_{40-60}$ were obtained from Pharmacia (Piscataway, NJ, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows the separation of calf thymus DNA before and after denaturation. The sample before heat denaturation (*i.e.*, double-stranded DNA) eluted at a sodium phosphate concentration of 216 m*M* at 18 min. On the other hand, the heat-denatured sample (*i.e.*, single-stranded DNA) eluted at sodium phosphate concentration of 134 m*M* at 13 min, while a trace amount of native DNA was eluted at 18 min. Accordingly, the double- and single-stranded linear DNAs could be completely separated within 20 min. The addition of calcium chloride to the eluent did not affect the separation but it increased the lifetime of the column. The recovery of DNA was more than 88%. We examined other DNAs obtained from herring sperm and salmon testis. Native and denatured DNA in these samples could also be separated well at the same concentration of sodium phosphate as the DNA samples in Fig. 1 (data not shown).

Fig. 2 shows the separation of plasmid DNAs. The circular and single-stranded plasmid M13mp8, with 7229 bases, was eluted at a sodium phospate concentration of 150 m*M* in the eluent at 14 min. On the other hand, the circular and double-stranded

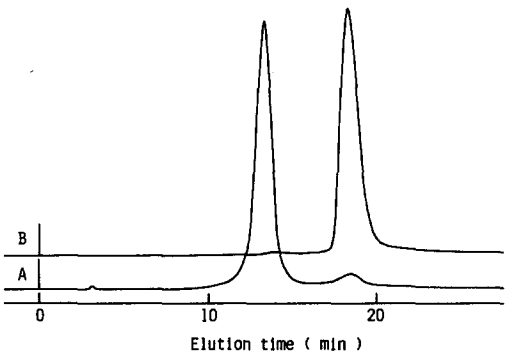

Fig. 1. Separation of calf thymus DNA by high-performance HAC before and after heat denaturation. 2 μg of calf thymus DNA (A) after and (B) before heat denaturation were separated with a 30-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) containing 0.01 m*M* calcium chloride at a flow-rate of 1.0 ml/min.

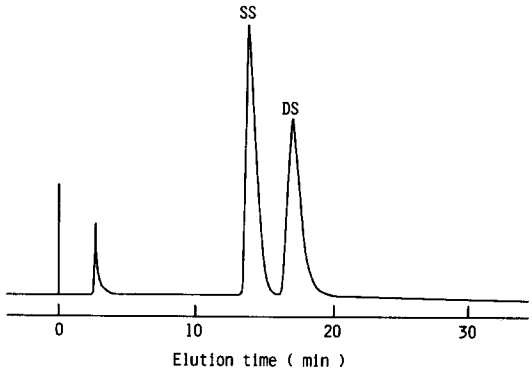

Fig. 2. Separation of single-, and double-stranded plasmid DNA by high-performance HAC. The plasmid mixture of 12 μg of M13mp8 and 11 μg of pBR322 was separated with a 30-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) at a flow-rate of 1.0 ml/min.

plasmid pBR322, with 4362 base pairs, was eluted at a sodium phosphate concentration of 216 m*M* at 18 min. The total recovery of the plasmid DNAs was 90%. It was found that single- and double-stranded DNA could not only be separated in linear DNA but also in circular plasmid DNA. Hence the sodium phosphate concentration for elution was independent of the size of DNA in relatively high-molecular-weight DNA samples.

We also examined linear DNA fragments of λ DNA-Hind III digest and φX-174 RF DNA-Hae III digest. The DNA fragments, however, could not be separated with higher resolution by HAC than by ion-exchange chromatography (IEC) on the non-porous resin TSKgel DEAE-NPR[32] (data not shown).

Fig. 3 shows the separation of the oligodeoxyadenylic acids $pd(A)_{20}$ and $pd(A)_{40-60}$. These samples were found to contain many contaminant oligonucleotides of $pd(A)_{<39}$ and $pd(A)_{>60}$, and were eluted at a fairly low concentration of sodium phosphate as the oligodeoxyadenylic acids were single-stranded DNA of low molecular weight. The chromatogram showed high resolution, and the oligodeoxyadenylic acids could be separated up to at least the 70-mer. This chromatographic pattern was similar to that obtained by IEC on TSKgel DEAE-NPR[33], although HAC took much longer for separation. The total recovery of the oligonucleotides was 96%.

Fig. 4 shows the separation of MS2 phage RNA, which has a relatively high molecular weight (3569 bases) and was eluted at a sodium phosphate concentration of

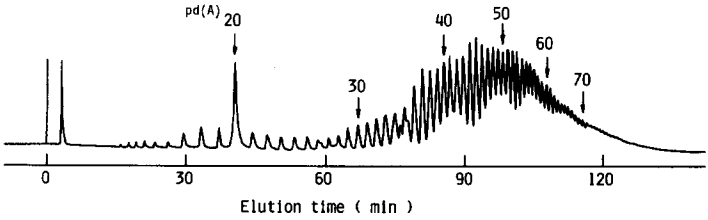

Fig. 3. Separation of oligodeoxyadenylic acid by high-performance HAC. The mixture of 15 milliabsorbance units (260 nm) of $pd(A)_{20}$ and 375 milliabsorbance units (260 nm) of $pd(A)_{40-60}$ was separated with a 240-min linear gradient from 2 to 100 m*M* sodium phosphate (pH 6.8) at a flow-rate of 1.0 ml/min.

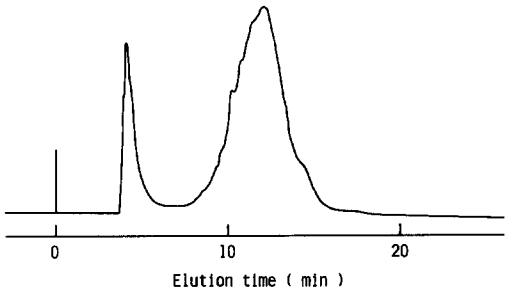

Fig. 4. Separation of MS2 phage RNA by high-performance HAC. 16 μg of MS2 phage RNA was separated with a 30-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) at a flow-rate of 1.0 ml/min.

134 m*M* at 13 min. It had the same retention time as single-stranded DNA prepared from calf thymus, but it eluted as a broad peak. This may reflect the different conformation derived from the secondary and tertiary structure of RNA[2]. The total recovery of MS2 phage RNA was 95%.

Fig. 5 shows the separation of tRNAs from *E. coli* and baker's yeast. The tRNAs were eluted earlier than MS2 phage RNA at a concentration of sodium phosphate between 65 and 140 m*M* in the eluent as the tRNAs have a lower molecular weight of *ca.* 76 bases. Many peaks were observed, which suggests the multiplicity of tRNA and also the presence of secondary and tertiary structure as indicated by Kawasaki *et al.*[29]. The total recoveries of the tRNA were 91 and 96%, respectively.

As demonstrated above, various types of nucleic acids could be separated quantitatively by high-performance HAC on TSKgel HA-1000. In the case of DNA, single- and double-stranded DNA were separated completely, not only in linear DNA but also in circular plasmid DNA, within 20 min. The sodium phosphate concentration needed for elution was independent of the steric structure and molecular weight for relatively large DNA. Hydroxyapatite could discriminate the rigid helical structure of double-stranded DNA from the flexible single-stranded DNA. On the other hand, relatively small double-stranded DNA, such as DNA fragments, seemed to be separated according to the size whereas the resolution with HAC was lower than that with IEC. However, single-stranded DNAs consisting of oligodeoxynucleotides were separated according to size with the same resolution in both HAC and IEC.

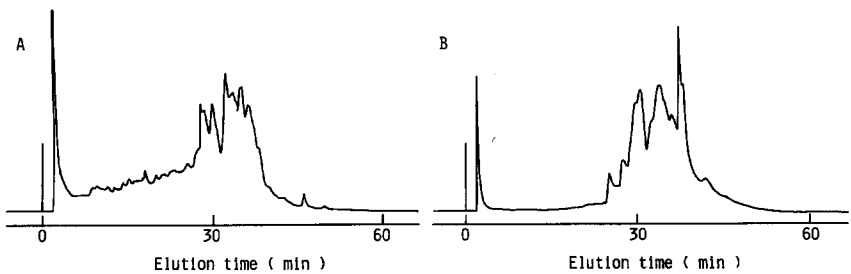

Fig. 5. Separation of tRNA by high-performance HAC. 100 μg of tRNA from (A) *E. coli* and (B) baker's yeast were separated with a 120-min linear gradient from 10 to 400 m*M* sodium phosphate (pH 6.8) at a flow-rate of 1.0 ml/min.

Large RNA such as MS2 phage RNA was eluted at the same concentration of sodium phosphate as single-stranded DNA, although it eluted as a broad peak. It is also suggested that the secondary and tertiary structure of phage RNA give rise to different interactions with hydroxyapatite. tRNAs could also be separated successfully into a number of peaks. tRNA is known to exist in a clover-leaf conformation in spite of having only *ca.* 76 bases. Therefore, differences in the secondary and tertiary structures and the base sequence may be resonsible for interactions of tRNAs with hydroxyapatite.

In conclusion, high-performance HAC on TSKgel HA-1000 should be a very useful tool for the separation of various nucleic acids. Nucleic acids can be separated effectively according to their conformation and molecular weight.

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