Journal of Chromatography, 539 (1991) 373–381
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 877

Purification of DNA and group separation of normal and modified DNA components by size-exclusion chromatography

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ABSTRACT

DNAs and their normal or modified constituents are separated in one chromatographic run on a TSK HW 55F column with a 0.1 *M* ammonium formate (pH 6.5) volatile solvent. A group separation between 2′-deoxyribonucleotides, 2′-deoxyribonucleosides and nucleobases was achieved. A good resolution of photoinduced decomposition products of (pyrimidine) nucleobases was obtained on a TSK G 1000 PW column. Separation of nucleic acid components is thought to be governed by mechanisms involving both size exclusion, weak hydrophobic interactions and ionic repulsion between the matrix and the charged molecules.

INTRODUCTION

Purification of chemically modified DNA is required prior to analysis for a specific type of base damage. Ultracentrifugation [1], electrophoresis [2] and liquid chromatography on various supports [3] are techniques generally used to obtain pure DNA. Once purified, DNA may be submitted to acidic hydrolysis [4] or enzymatic digestion [5]. Whatever the hydrolytic method choosen, a reliable measurement of a specific modified nucleoside is possible only after simple monitoring of the completeness of DNA hydrolysis. Subsequently, the separation of the biopolymer from the released low-molecular-mass components is generally achieved by precipitation of the macromolecule using ethanol–salt mixtures [6].

When analysed with a Fractogel TSK HW 55F column eluted with 0.1 *M* ammonium formate (pH 6.5), DNAs (with molecular masses of above 10^5 dalton) are excluded in the void volume (V_0). With this volatile solvent, the excluded DNA may be digested enzymatically directly in the chromatographic eluate, adding only a concentrated nuclease buffer and metallic cofactors. The enzymatic hydrolysate may be analysed with the same chromatographic system.

We achieved a separation of the finally remaining unhydrolysed DNA from its digestion products (2′-deoxyribonucleotides or nucleosides) in a single chromatographic run. Whereas DNAs are excluded according to the well known size-exclusion mechanism, Fractogel TSK HW 55F and TSK G 1000 PW columns may be used for

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the separation of DNA components and pyrimidine photoproducts in the partition mode.

EXPERIMENTAL

Chemicals

Ammonium formate and calcium chloride were obtained from Eastman Kodak (Rochester, NY, U.S.A.) and Merck (Darmstadt, Germany), respectively. Alkaline phophatase, micrococcal nuclease, nuclease P_1, RNAse A and $tARN^{Met}$ were provided by Boehringer (Mannheim, Germany). 2′-Deoxyribonucleosides were purchased from Genofit (Geneva, Switzerland) and thymidine 5′-monophosphate (Na salt) and nucleobases from Sigma (St. Louis, MO, U.S.A.). 8-Oxo-7,8-dihydro-2′deoxyguanosine was synthesized from 2′-deoxyguanosine according to Lin *et al.* [7] and 2′-deoxyadeosine N-1-oxide according to Mouret *et al.* [8].

Synthesis of the two *cis* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine 5′-monophosphate was adapted from Rajagopalan *et al.* [9] with the following modifications suggested by Berger [10]. Briefly, thymidine 5′-monophosphate (Na salt) (1 m*M*) was dissolved in water (10 ml, room temperature), pure bromine (0.1 ml) was added and the solution was stirred for 30 min at room temperature. The excess of bromine was eliminated by a continuous flow of air throughout the solution. Substitution of bromine was carried out by overnigh treatment with NH_4HCO_3 (0.1 g per 50 ml) (37°C).

After quantitative dephosphorylation with alkaline phosphatase (1 h, 37°C, 5 units, pH 8.5, 0.2 *M*, in NH_4HCO_3), thymidine glycol diastereoisomers were purified and characterized according to Cadet *et al.* [11]. The *cis-syn*-cyclobutadithymine (Thy< >Thy) and 5-hydroxy-6-4′-(5′-methylpyrimidin-2′-one)-5,6-dihydrothymine were generated by far-UV irradiation of thymine in frozen aqueous solution [12].

Biopolymers

The following deoxyribonucleic acids were used: *E. coli*, salmon testes, phage X 174, phage lambda hind III fragments, calf thymus $tARN^{Met}$ and poly-dA (Boehringer, Mannheim, Germany).

Purification and enzymatic digestion of DNA

Dry seeds of *Lactuca sativa* (500 mg) precooled in dry nitrogen were disrupted by mechanical grinding in a porcelain mortar and suspended in a ready-made lysis buffer (10 ml) (Applied Biosystems, Foster City, CA, U.S.A.). After thawing for at least 45 min, the lysate was centrifuged at 5000 *g* for 30 min at room temperature in order to eliminate the cellulosic residues. The brownish supernatant was deproteinized by reaction with proteinase K (100 μg/ml, 2 h, 65°C) with occasional shaking. The DNA and RNA pool was precipitated overnight at −20°C by adding 0.5 volume of 7.5 *M* ammonium acetate and 1 volume of 2-propanol in ten siliconized Eppendorf tubes (2 ml). The major part of the hydrolysed peptides and phenolic compounds was eliminated by centrifugation at 10^4 *g* for 1 h at 0°C. The resulting brownish precipitate was rinsed with 70% ethanol. Without excessive drying, the pellets were resuspended in distilled water. Prepurification was carried out on a small bed of Fractogel HW 55F (1 cm high, 1 cm diameter) equilibrated with distilled water before RNAse treatment [13].

Digestions of DNA into 5′-nucleotides or 3′-nucleotides were achieved with nuclease PI [14] and with a mixture of micrococcal nuclease and spleen phosphodiesterase [15], respectively.

Size-exclusion chromatography (SEC)

Fractogel HW 55F was packed in a Superformance glass column (300 mm × 10 or 16 mm I.D., as specified) (Merck). The columns were packed at a flow-rate of 2 ml/min until the level of gel remained constant in the prefilling column. Molecular weight operating ranges for HW 55F are between 10^3 and 10^5 dalton.

High-performance liquid chromatography (HPLC)

A stainless-steel column (600 × 7.5 mm I.D.) prefilled with TSK G 1000 PW was supplied by LKB (Bromma, Sweden). The chromatographic solvents [0.1 *M* ammonium formate (pH 6.5) or water] were degassed by filtration on a Millipore filter (HAWP, 0.45 μm; Millipore, Bedford, MA, U.S.A.). Samples were injected into the chromatographic system with a Rheodyne Model 7125 injector equipped with a 0.5-ml loop. Delivery of the solvents was achieved using a Merck L 6200 pump (fast size-exclusion chromatography) or a Waters Assoc. Model 501 pump with a flow-rate of 1.0 ml/min.

RESULTS

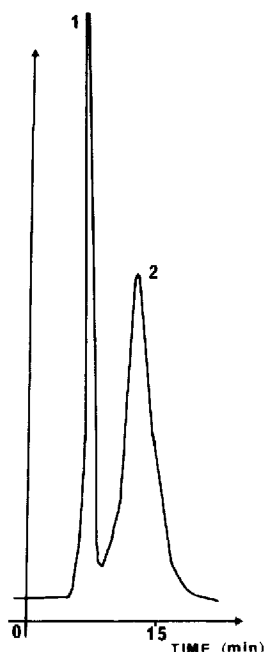
Chromatographic purification of nucleic acids and polynucleotides on the Fractogel HW 55F column

DNAs of different masses (> 10^5 dalton) are excluded in the void volume (V_0) and easily separated from RNA hydrolysate or smaller molecules (10^2–10^3 dalton), which are eluted with higher retention times (Fig. 1). An interesting application is the purification of nucleic acids extracted from dry seeds of *Lactuca sativa*, provided that the detergent is eliminated before the chromatographic run as it coelutes as a macromolecule with DNA in the void volume [16]. Further, Fractogel HW 55F is useful for removing polymerized phenolic compounds [17].

Separation of nucleic acids components

Enzymatic digestion of DNA is generally preferred to acidic hydrolysis because it may provide some insight into the stereochemical structure of the hydroxyl-mediated oxidative adducts [5]. As expected, unhydrolysed DNA is excluded in the void volume before nucleotides, for which the capacity factors (k') remain almost identical regardless of the position of the remaining phosphate (3′- or 5′-).

The nucleotide pool may be dephosphorylated and the resulting 2′-deoxyribonucleosides are further separated on the same column (Fig. 2, Table I). 2′-Deoxycytidine and thymidine partially overlap on Fractogel HW 55F (Fig. 2, Table I) and on TSK G 1000 PW (Table I). Acidic hydrolysis gives rise to nucleobases with concomitant degradation of the polynucleotide chain [18]. A complete separation of the nucleobases was only achieved by liquid chromatography on a TSK G 1000 PW column (Fig. 5, Table I). It should be noted that a faster separation is obtained with a Fractogel HW 55F that permits the separation only of adenine and guanine.



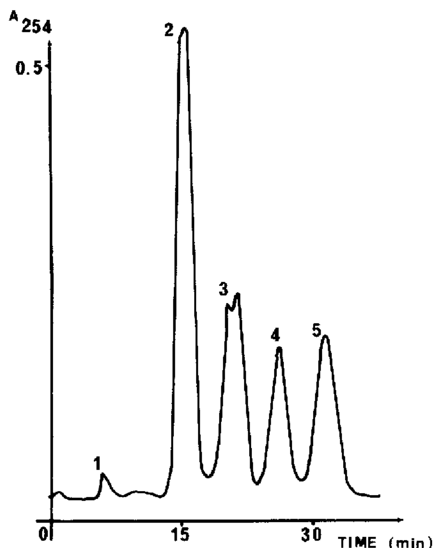


Fig. 1. SEC of (1) DNA and (2) RNA (from *Lactuca sativa*). Column, Fractogel HW 55F (30 × 1 cm I.D.); eluent, 0.1 *M* ammonium formate (pH 6.5); flow-rate, 1.0 ml/min; detection, 254 nm.

Fig. 2. SEC of Salmon testes (1) DNA and into (2) P_1 hydrolysate, (3) 2′-deoxycytidine and thymidine, (4) 2′-deoxyguanosine and (5) 2′-deoxyadenosine. Chromatographic conditions as in Fig. 1.

It is worth noting that TSK G 1000 PW easily discriminates nucleobases from the corresponding 2′-deoxyribonucleosides (Table I).

Separation of radiation- and photoinduced decomposition products of DNA components

Radiation-induced decomposition of nucleosides. The four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine have long been recognized as the most important hydroxyl radical-mediated decomposition products of thymidine [11]. These compounds may be produced by gamma irradiation of DNA in aqueous solutions [19] or by reactive species arising from the reduction of hydrogen peroxide by transition metals (Fe^{2+}, etc.) (Fenton reaction) [20].

Thymidine glycols, which coeluted as a single peak, have a lower retention time than thymidine (Fig. 3, Table I). Another important hydroxyl radical oxidation product of 2′-deoxyguanosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine [21], coelutes with the unmodified nucleoside.

Conversely, 2′-deoxyadenosine N^1-oxide, obtained from 2′-deoxyadenosine in hydrogen peroxide oxidation from a non-Fenton radical pathway [8], was completely separated from its parent compound (Fig. 4).

Photoinduced decomposition products. cis-syn-Cyclobutadipyrimidines, pyrimidine (6–4) pyrimidone photoadducts and 5,6-dihydro-5-(α-thyminyl)thymine, the

TABLE I

CAPACITY FACTORS (*k*′) OF NORMAL AND MODIFIED NUCLEIC ACID CONSTITUENTS

Compound	*k*′	
	HW 55F	TSK G 1000 PW
$tRNA^{Met}$ and phenolic compounds	0.47	–
Deoxyribonucleoside 3′-monophosphates	1.66	–
Deoxyribonucleoside 5′-monophosphates	1.36	–
2′-Deoxyadenosine	4.21	15.10
2′-Deoxycytidine	2.42	4.50
2′-Deoxyguanosine	3.30	16.75
Thymidine	2.40	6.25
(+)*trans*(5*R*,6*R*),( )*trans*(5*S*,6*S*), (+)*cis*(5*S*,6*R*),*cis*(−)(5*R*,6*S*)-5,6-Dihydroxy-5,6-dihydrothymidine	1.46	–
(−)*cis*(5*R*,6*S*)- and (+)*cis*(5S,6*R*)-5,6-Dihydroxy-5,6-dihydrothymidine-5′-monophosphate	1.50	–
Adenine	2.61	16.03
Cytosine	2.42	4.25
Guanine	2.31	14.50
Uracil	2.40	7.00
Thymine	2.41	7.00
2′-Deoxyadenosine N^1-oxide	2.01	–
8-Oxo-7,8-dihydro-2′-deoxyadenosine	3.35	–
cis-5,6-Dihydroxy-5,6-dihydrothymine	1.60	3.25
5-Hydroxy-6-4′-(5′-methylpyrimidin-2′-one)-5,6-dihydrothymine	–	8.50
5,6-Dihydro-5-(α-thyminyl)thymine	–	9.00
5-Hydroxy-5-methylbarbituric acid	1.55	–
5,6-Dihydrothymine	1.55	–
cis-syn-Thy< >Thy, Thy< >Ura, Ura< >Ura	1.21	15.50

so-called "spore photoproduct", constitute the three main classes of DNA pyrimidine dimeric far-ultraviolet photoproducts [22].

The three *cis-syn* isomers of DNA cyclobutadipyrimidine involving thymine dimers (Thy< >Thy), thymine–uracil dimer (Thy< >Ura) or uracil dimers (Ura< >Ura) are eluted in a single peak on a TSK G 1000 PW column (Fig. 5, Table I).

The other dimeric products of thymine, *i.e.*, 5-hydroxy-6-4′-(5′-methylpyridine-2′-one)-5,6-dihydrothymine and 5,6-dihydro-5-(α-thyminyl)thymine, which have one of the two pyrimidines still unsaturated, exhibit higher retention times (Fig. 5, Table I).

DISCUSSION

SEC is generally carried out for purification and/or separation of DNAs of different size [23–28]. Fractogel HW 55F allows the separation of biopolymers in the range 10^3–10^5 dalton (SEC mechanism) and the separation of low-molecular-mass compounds ($<10^3$ dalton) in the partition mode. All biopolymers ($>10^5$ dalton) are eluted in a single peak (void volume, V_0).

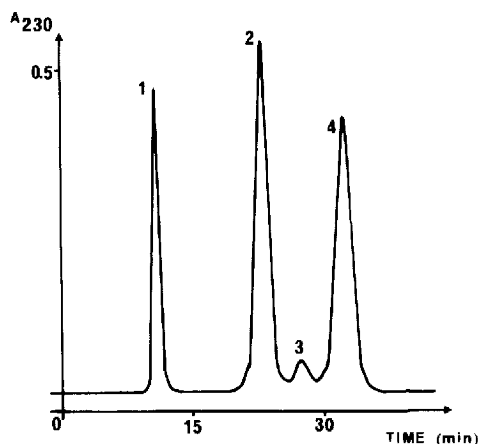


Fig. 3. SEC of (1) phage λ hind III fragments, (2) (+)*cis*(5*S*,6*R*)- and (−)*cis*(5*R*,6*S*)-diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine 5′-monophosphate, (3) (+)*cis*(5*S*,6*R*)-, (−)*cis*(5*R*,6*S*)-, (−)*trans*-(5*S*,6*S*)- and (+)*trans*(5*R*,6*R*)-diastereoisomers of 5,6-dihydroxy-5-6-dihydrothymidine and (4) thymidine. Chromatographic conditions as in Fig. 1, except for the column (30 × 1.6 cm I.D.).

Liquid chromatography in the partition mode with TSK G 1000 PW is limited to low-molecular-mass products (up to 10^3 dalton). Fast SEC on Fractogel HW 55F was particularly useful for the clean-up of DNA to remove undesirable polymerized phenolic compounds which always coprecipitated with the nucleic pool from the crude cell lysate.

The group separation of the nucleic acid components [29–32] has received attention in the past. However, it should be noted that only a few papers have reported

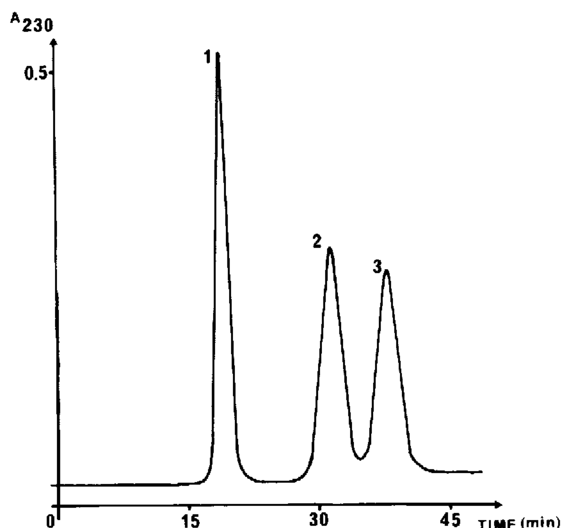


Fig. 4. SEC of (1) 2′-deoxyadenosine N^1-oxide, (2) 2′-deoxyadenosine and (3) adenine. Chromatographic conditions as in Fig. 2.

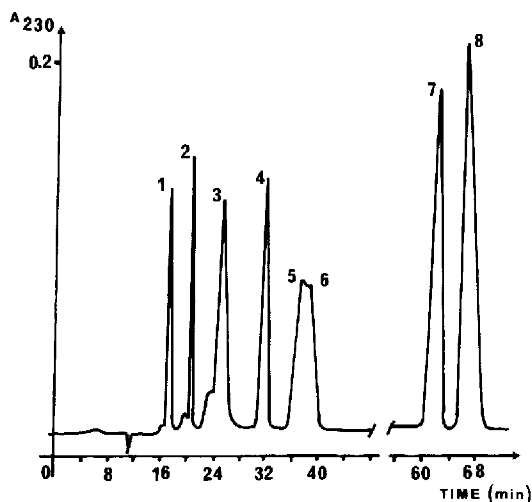


Fig. 5. HPLC of photoinduced decomposition products of nucleobases. Column, TSK G 1000 P (30 × 0.75 cm I.D.); eluent, water; flow-rate, 1.0 ml/min; detection, 230 nm. (1) *cis*-5,6-dihydroxy-5,6-dihydrothymine; (2) cytosine; (3) *cys-syn*-Thy< >Thy, *cis-syn*-Ura< >Thy, *cis-syn*-Ura< >Ura; (4) uracil + thymine; (5) 5-hydroxy-6-4-(5'-methylpyrimidin-2'-one)-5,6-dihydrothymine; (6) 5-dihydro-5-(α-thyminyl)thymine; (7) guanine; (8) adenine.

chromatographic systems that permit the separation of DNA from its digestion products [33–35].

The matrix of these gels can be roughly described as a copolymer of polyacrylamide and poly(vinyl alcohol) the hydrophilicity of which can be attributed to hydroxyl groups (0.5–1 mequiv./ml) and to carboxylic groups (0.001–0.02 mequiv./ml) for TSK HW 55F according to the supplier's information.

In a previous study [29], the chromatographic behaviour of purine and pyrimidine compounds was explained in terms of charge effects. With a gel of the same type, Aoyagi *et al.* [36] assumed that the elution order of DNA compounds is governed by hydrophobic properties. In agreement with Wada [37] and, more recently, with Noguchi *et al.* [38], the fast elution properties of deoxyribonucleotides can be explained by a repulsive interaction between the carboxylic groups of the matrix and the free anionic groups of 2'-deoxyribonucleotides.

The chromatographic behaviour of 2'-deoxyadenosine N^1-oxide may be accounted for in terms of "ion-repulsion" interactions, taking into account the apparent negative charge in the oxygen atom of the N-oxide: $N^+ \rightarrow O^-$ [39]. Weak hydrophobic interactions exist between the polyvinyl structure of the gel matrix and the aromatic ring of purine and pyrimidine compounds [37]. This explains the higher retention of purine nucleobases with respect to the corresponding pyrimidine components (Table I).

Further confirmation of the involvement of weak hydrophobic interactions in the retention mechanism of purine and pyrimidine components was provided by the chromatographic behaviour of the three *cis-syn*-cylobutadipyrimidines involving Thy< >Thy, Thy< >Ura and Ura< >Ura, which exhibit similar capacity factors on the TSK G 1000 PW column (Fig. 5, Table I). This contrasts with the efficient

separation of these compounds on an octadecylsilyl silica gel column in the reversed-phase mode [40], for which the order of elution Ura< >Ura, Ura< >Thy and Thy< >Thy is directly related to the presence of one or two methyl groups. This confirms the lack of any strong hydrophobic interactions of the TSK G 1000 PW matrix with the methyl groups of Thy< >Ura and Thy< >Thy.

Solvophobicity of the (6–4) thymine photoadduct and "spore photoproduct" may explain their longer retention times. This is due to the interactions of the unsaturated pyrimidine ring of the photoproducts with the hydrophobic part of the matrix.

CONCLUSION

SEC is an efficient method for purifying DNAs with molecular masses above those limiting the exclusion limit of the fractionation range (*i.e.*, $>10^5$ dalton for TSK HW 55F). TSK HW 55F gel columns used with a volatile solvent (0.1 *M* ammonium formate, pH 6.5) may separate DNAs from their constituents in a single chromatographic run.

Low-molecular-mass nucleic acid components can be separated on Fractogel TSK HW 55F and on TSK G 1000 PW in a non-exclusion mode. The separation is governed by a balance between "ionic repulsion" interactions which are predominant for anionic compounds and weak hydrophobic affinities developed through aromatic solutes and the polyvinyl structure of the matrix. An interesting application of the TSK G 1000 PW column in the partition mode is the ability to separate pyrimidine photoproducts by classes. This will allow the separation of groups of particular compounds from a complex mixture which will be further resolved by using another appropriate chromatographic system, such as reversed-phase HPLC [12,40].

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Centre National d'Études Spatiales (89/130J) and l'Oréal.

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