Journal of Chromatography, 600 (1992) 229–233
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 050

Purification of DNA-derived deoxynucleotides from leukocytes involving nuclease elution of an ion-exchange column

Amr Nour Al-Deen☆, Douglas J. Cecchini☆☆ and Roger W. Giese*

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, and Barnett Institute of Chemical Analysis, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (USA)

(First received September 13th, 1991; revised manuscript received January 17th, 1992)

ABSTRACT

A method has been developed in which the DNA of leukocytes (as the buffy coat from blood) is isolated in the form of its constituent deoxynucleotides. The steps in this method are as follows: (1) lyse the leukocytes with sodium dodecyl sulfate (SDS) and enzymatically digest the proteins and RNA, (2) remove the SDS on a non-polar adsorbent (Bio-Beads SM-4) and then trap the DNA on a quaternary amine silica cartridge, (3) wash the column with 1 *M* NaCl–buffer, (4) digest the DNA on the column with staphylococcal nuclease and (5) elute the digested DNA with 0.5 *M* NaCl–buffer and digest it further with bovine spleen phosphodiesterase II to deoxynucleotide-3′-monophosphates. From a 40-μl sample of butty coat was obtained 126 ± 14 μg (two experiments, eight sample total) of deoxynucleotides. Reversed-phase high-performance liquid chromatography, which removed the added enzymes, showed only peaks for deoxynucleotides. For comparison, the amount of deoxynucleotides obtained from the leukocytes by an automated phenol extraction procedure was 101 ± 5.4 μg (one experiment in triplicate).

INTRODUCTION

Several methods are available for extracting DNA from biological samples. Typically these methods start with cell lysis followed by the addition of proteases and ribonucleases to digest the corresponding components of the sample. The DNA can then be isolated by a variety of techniques including aqueous–organic partitioning [1,2], ion-exchange chromatography [3,4] size-exclusion chromatography [5,6], ethanol precipitation [7,8] or centrifugation [9]. Aside from practical considerations, a given method for isolating DNA may be selected depending on the nature of the DNA (*e.g.* chromosomal or plasmid), how pure the DNA needs to be, and the degree to which the DNA must be isolated in an intact *versus* fragmented form.

One purpose for isolating DNA from a biological sample is for the determination of DNA adducts. The latter refers to the chemical damage, typically covalent, that DNA can undergo upon exposure to toxic chemical or physical conditions. Good correlations have generally been observed between the ability of genotoxic agents to cause cancer or mutations, and their capacity to form DNA adducts [10]

We are interested in the determination of DNA adducts by capillary electrophoresis and also mass spectrometry. For some of these studies it is appropriate to isolate the DNA from a biological sample as deoxynucleotides. Here we report a procedure for this purpose which is applied to a sample of leukocytes (buffy coat) obtained from human blood. In one of the steps, DNA bound to an anion exchanger is digestively eluted with a nuclease.

☆ Present address: Department of Pathology, University of Texas, Galveston, TX 77550-2774, USA.
☆☆ Present address: Massachusetts Public Health Biologic Laboratories, Jamaica Plain, MA 02130, USA

MATERIALS AND METHODS

Chemicals and reagents

Leukocyte-enriched blood (Leukopack) was kindly provided by Children's Hospital, Harvard Medical School (Boston, MA, USA). Wild-type staphylococcal nuclease (E.C. 3.1.4.7) was isolated in our laboratory by a previously described procedure [11]. An engineered strain of *Escherichia coli* carrying the expression plasmid PFOG405 was utilized. The bacterium was kindly provided by David Shortle (Johns Hopkins University, Baltimore, MD, USA). Highly polymerized calf thymus DNA type I, ribonuclease A type III-A, proteinase-K type XI, sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (St. Louis, MO, USA). Bovine spleen phosphodiesterase type II (E.C. 3.1.4.18) was from Worthington (Freehold, NJ, USA). Bio-Beads SM-4 were from Bio-Rad (Rockville Center, NY, USA). Bakerbond quaternary amine silica (40 μm, Bulk Prep LC Packing 7043-00, "QAE-silica") was from J. T. Baker (Phillipsburg, NJ, USA).

High-performance liquid chromatography (HPLC)

The HPLC system comprised a Series 4 LC pump from Perkin-Elmer (Norwalk, CT, USA), an Ultra-Sphere-5 ODS 150 × 4.6 mm column (Beckman, San Ramon, CA, USA), a 9060 Polychrom diode array detector (Varian, Walnut Creek, CA, USA), and a Cl-10B Integrator (LDC Milton Roy, Bloomfield, CT, USA). Solvent A was 0.1 *M* potassium dihydrogenphosphate (pH 4.6 inherently), and solvent B was acetonitrile. The deoxynucleoside-3'-monophosphates from the enzymatic digests were separated using a gradient of 0 to 6% B in 9 min followed by 6–35% B in 21 min at a flow-rate of 1 ml/min. Absorbance units full scale was 0.1 and the detection wavelength was 260 or 215 nm.

DNA extraction–digestion procedure

Buffy coat. Leukocyte-enriched blood, obtained at Children's Hospital from a Baxter-Fenwal (Deerfield, IL) CS-3000 plateletpheresis apparatus, was centrifuged at 200 *g* for 30 min at 4°C. The plasma was aspirated and the buffy coat was aliquoted using a Pasteur pipette into plastic tubes and stored at −20°C until further use.

Buffy coat (40-μl samples, two experiments: n = 2 and 6 samples) was thawed and added to 0.5 ml of 10 m*M* Tris–HCl buffer, pH 7.4, 1% SDS, 0.1 m*M* EDTA (TE buffer) in a borosilicate glass tube. To test recovery, a second set of buffy coat samples were run in parallel which were each spiked with 56 μg of calf thymus DNA in 40 μl of water. After each tube was sonicated (water bath sonicator) for 5 min at room temperature, 12 μl of ribonuclease A (10 μg/μl in water) was added. The tube was incubated at 37°C for 1 h in a shaking water bath. The addition of ribonuclease A and 1 h incubation was repeated. Proteinase K (15 μl of 2.5 μg/μl in water) was added and a similar incubation was done for 0.5 h. This proteolytic step was repeated twice more.

Bio-Beads SM-4 (0.5 g) were dry-packed into a Supelclean solid-phase extraction tube (3 ml size, 6.4 × 0.9 cm, catalogue No. 5-7024) and washed under gravity flow with approximately 3 ml each (fill column to the top) of water and then TE buffer. The residual solvent was forced out with a brief stream of nitrogen, and the bottom of the column was capped.

The entire sample was added to the extraction tube (for SDS removal). After the top was capped, the tube was placed on a rocking plate for 10 min at room temperature. The tube was drained (both caps off) and eluted with 3 × 0.5 ml of TE buffer, followed by a brief nitrogen flow through the column at the end. The SDS-free eluents were combined, giving a 2-ml sample which was placed in a boiling water bath for 30 min and then in an ice bath for 15 min, yielding a single-stranded DNA solution.

QAE-Silica (0.5 g) was dry-packed into an extraction tube and washed as above (including intermittent nitrogen flows) with approximately 3 ml each of hexane, methanol, water and TE buffer, followed at the end by nitrogen. The single-stranded DNA sample was applied and rocked in this tube for 2–18 h at room temperature (equivalent results were obtained throughout this period). The QAE-silica was drained and washed with 12 ml of 10 m*M* Tris–HCl, pH 8.8, 1 *M* NaCl, followed by 6 ml of the same buffer containing 0.5 *M* NaCl. Residual buffer was forced out with a brief stream of nitrogen.

To the bottom-capped tube was added 1 ml of 10 m*M* Tris–HCl, pH 8.8, 0.5 *M* NaCl, 10 m*M* $CaCl_2$, and 25 μl of a 1 μg/μl solution of staphylococcal

nuclease in water. After incubating on a rocking plate for 2 h at room temperature, the tube was drained and eluted with 3 × 1 ml of 10 m*M* Tris–HCl, pH 8.8, 0.5 *M* NaCl, followed by nitrogen. The A_{260} values of the fractions were recorded. The pH was adjusted to 5.8 by the addition of 200 μl of 1 *M* sodium acetate, pH 5.8. Each fraction was treated with 20 μl (0.2 Units) of bovine spleen phosphodiesterase II, followed by a 1-h incubation at 37°C in a shaking water bath. A 30-μl aliquot was then subjected to HPLC.

Automated DNA extraction. We are grateful to James Swenberg and Vernon Walker, previously at the Chemical Industry Institute of Toxicology (CIIT), for performing DNA extractions (for comparison purposes) on three 40-μl samples of buffy coat that we provided. They used a Model 340A nucleic acid extractor (Applied Biosystems, Foster City, CA, USA) for this purpose.

RESULTS AND DISCUSSION

Procedure

Our method for the isolation of nucleotide-3′-monophosphates from leukocytes is summarized in Fig. 1. The leuckocytes are first lysed in 1% SDS and the proteins and RNA are enzymatically digested. The SDS is then removed by hydrophobic adsorption onto Bio-Beads SM-4, a polystyrene–divinylbenzene packing.

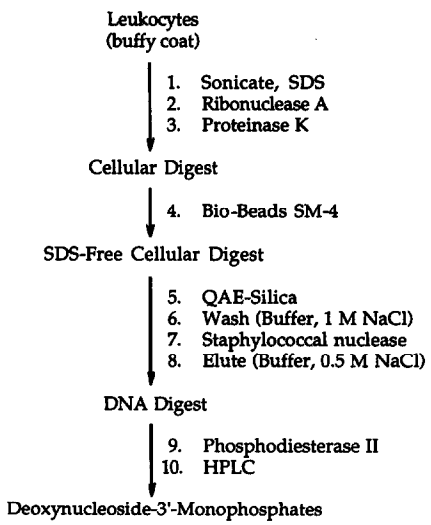

Fig. 1. Scheme for isolating DNA-derived deoxynucleotides from leukocytes.

The SDS-free solution containing the DNA is applied to a cartridge column packed with quaternary amine-silica (QAE-silica). Based on preliminary experiments with calf thymus DNA (data not shown), better recoveries are obtained when the DNA is heat denatured prior to adsorption on this anion exchanger. Contaminants are removed by washing with buffer containing 1 *M* NaCl. The DNA, due to its high molecular weight and polyanionic structure, does not elute from the anion exchanger under these conditions.

In order to prepare the column for the next step, it is washed with a lower-ionic-strength buffer (0.5 *M* NaCl) also containing a small amount of calcium chloride (calcium is essential for nuclease activity). The DNA is then digested on the column with staphylococcal nuclease, giving a mixture of 3′-deoxynucleotides and small oligonucleotides [12]. This low-molecular-weight material readily elutes from the QAE-silica with buffer containing 0.5 *M* NaCl. Prior to HPLC, the final step, any oligonucleotides remaining in the eluate are digested with phosphodiesterase II [12].

Application to buffy coat leukocytes

When deoxynucleotide-3′-monophosphates are isolated from a 40-μl aliquot of buffy coat leukocytes by the above method, the final sample (3.0 ml) gives 1.24 ± 0.13 A_{260} units/ml (mean ± S.D., data from two runs, total of 8 samples). One absorbance unit of DNA at 260 nm corresponds to approximately 50 μg of DNA per ml [13]. We determined that nuclease digestion causes one A_{260} unit/ml of heated DNA to increase up to 1.47 A_{260} units/ml (hyperchromic shift). Relying on this latter value, 126 ± 14 μg of nucleotides are obtained per sample of buffy coat. The A_{260}/A_{280} for the sample is 2.30. For comparison, a sample of calf thymus DNA was similarly digested, also giving an A_{260}/A_{280} of 2.30. Completion of DNA digestion is evidenced by the absence of significant HPLC dinucleotide peaks as we have demonstrated before [12], and equivalent results in this respect are obtained for calf thymus DNA digested directly or via the new procedure.

To estimate recovery, we spiked each of six buffy coat aliquots with 56 μg of calf-thymus DNA. The spiked samples give 183 ± 13 μg of nucleotides

(1.80 ± 0.14 A_{260} units/ml). This indicates a 100% recovery of the added calf thymus DNA.

For comparison, we extracted DNA from identical samples of buffy coat by an automated phenol extraction procedure (Applied Biosystems nucleic acid extractor). After comparable enzymatic digestion of the isolated DNA in 1.0 ml of buffer, the yield is 2.98 ± 0.16 A_{260} units/ml (n = 3) or 101 ± 5.4 μg of deoxynucleotides. This is 20% lower than the apparent quantitative yield of 126 μg (see above) by our method. The automated procedure has been reported by the manufacturer to give 37 ± 3 μg of DNA per ml of blood starting from 6 ml of blood [14]. If one assumes that the blood which was extracted contained 45 μg/ml of DNA, a typical value [2], then the yield of 37 μg corresponds to an 83% recovery. This is consistent with our observation that the automated method appears to recover 80% of the DNA from the buffy coat samples. The value of A_{260}/A_{280} for the deoxynucleotides obtained after the phenol extraction procedure (followed by the exhaustive enzymatic digestion to deoxynucleotides) is 2.31.

Subjecting the deoxynucleotides from leukocytes to reversed phase HPLC gives the chromatograms shown in Fig. 2a (detection at 260 nm) and Fig. 2b (215 nm). The four major deoxynucleotides elute, as seen, in the following order: deoxycytidine-, deoxyguanosine-, thymidine- and deoxyadenosine-3′-monophosphate. The identity of the peaks was confirmed as before [12]. The early-eluting salt peaks are also present in a method blank in which the initial sample is 40 μl of water instead of buffy coat (data not shown). The two minor peaks which elute after deoxyadenosine-3′-monophosphate are apparently trace amounts of residual dinucleotides. The latter have been investigated in more detail in a related study [12]. Enzymatic digestion of the DNA obtained by the automated phenol extraction procedure gives identical chromatograms (data not shown). Ribonucleotides are absent: their retention times are approximately one-half those of the corresponding deoxyribonucleotides by reversed-phase HPLC (*e.g.* ref. 15).

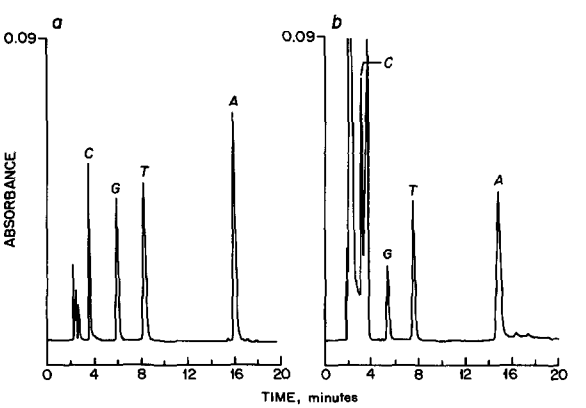

Fig. 2. Reversed-phase HPLC chromatograms of deoxynucleoside-3′-monophosphates obtained from leukocytes. A 30-μl aliquot from the final 3.0-ml sample was injected. Based on calibration with external standards, approximately 2 nmol of each of the four major deoxynucleotides were injected. The HPLC solvent is a gradient of acetonitrile in 0.1 *M* potassium phosphate, pH 4.5: 0–6% acetonitrile in 9 min followed by 6–35% acetonitrile in 21 min. The wavelength of detection is (a) 260 nm or (b) 215 nm.

CONCLUSIONS

We have established a convenient, low-cost, high-yield method for extracting DNA-derived deoxynucleotides from leukocytes. An interesting step in the procedure is the digestive elution of the DNA from a quaternary amine silica packing. Potentially the method will be useful for the isolation of trace amounts of DNA adducts from leukocytes, and from other cells as well.

ACKNOWLEDGEMENT

This work was funded by the Office of Health and Environmental Research Division, Department of Energy, as Grant DE-FG02-90ER60964. Publication number 522 from the Barnett Institute.

REFERENCES

1 J. Marmur, *J. Mol. Biol.*, 3 (1961) 208.
2 S. Gustafson, J. A. Proper, E. J. W. Bowie and S. S. Sommer, *Anal. Biochem.*, 165 (1987) 294.
3 S. E. Murphy, *Carcinogenesis*, 10 (1989) 1435.
4 K. J. Reddy, T. Kuwabara and L. A. Sherman, *Anal. Biochem.*, 168 (1988) 324.
5 J. A. White, R. A. W. Stott and J. A. Matthews, *Nucleic Acids Res.*, 16 (1988) 2727.
6 A. B. Livore, P. Grubb, C. W. Magill and J. A. Magill, *Nucleic Acids Res.*, 16 (1988) 776.
7 D. D. L. Bowtell, *Anal. Biochem.*, 162 (1987) 463.
8 M. Jeanpierre, *Nucleic Acids Res.*, 22 (1987) 9611.
9 R. K. Yacoob and G. R. Zealey, *Nucleic Acids Res.*, 16 (1988) 1639.

10 A. M. Jeffery, in D. Grunberger and S. Goff (Editors), *Mechanisms of Cell Transformation by Carcinogenic Agents*, Pergamon, Oxford, New York, 1987, p. 33.
11 E. H. Serpersu, D. Shortle and A. S. Mildvan, *Biochemistry*, 25 (1986) 68.
12 D. J. Cecchini, K. L. Guan and R. W. Giese, *J. Chromatogr.*, 444 (1988) 97.
13 P. Cuatrecasas, S. Fuchs and C. B. Anfinsen, *J. Biol. Chem.*, 242 (1967) 1541.
14 *Nucleic Acid Research News, No. 6*, Applied Biosystems, Foster City, CA, 1987.
15 H. Martinex-Valdez, R. M. Kothari, H. V. Hershey and M. W. Taylor, *J. Chromatogr.*, 247 (1982) 307.