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Purification of genomic DNA by short monolithic columns

Dubravko Forcic[a,*], Karmen Branovic-Cakanic[a], Jelena Ivancic[a], Renata Jug[a], Milos Barut[b], Ales Strancar[b]

[a] *Institute of Immunology Inc., Rockefellerova 10, 10000 Zagreb, Croatia*
[b] *BIA Separations d.o.o., Teslova 30, 1000 Ljubljana, Slovenia*

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Abstract

The isolation and purification of nucleic acids is essential for many procedures in molecular biology. After showing that bacterial and eukaryotic genomic DNA can be specifically bound to the CIM DEAE monolithic column, this characteristic was exploited in development of a simple and fast chromatographic procedure for isolation and purification of genomic DNA from cell lysates that does not include the usage of toxic organic solutions. The purity and the quality of the isolate as well as the duration of the procedure was similar to other chromatographic methods used today for isolation of genomic DNA, but the initial sample volume was not restricted.
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Keywords: Genomic DNA; Monolithic column; Purification

1. Introduction

Genomic DNA constitutes the total genetic information of an organism. The nature of genomic DNA, size and number of chromosomes varies among different organisms. In contrast to bacteria, whose DNA is in direct contact with the cytoplasm, the DNA in an eukaryotic cell is sequestered in the nucleus, which occupies about 10% of the total cell volume [1]. Eukaryotic cells additionally contain genomic DNA in the mitochondria and plants and lower eukaryotes, in the chloroplasts. Understanding the organization and function of the genome requires many molecular studies and first of all an understanding of its primary structure.

The isolation and purification of nucleic acids is essential for many procedures of molecular biology. In recent years, many different protocols and methods have been developed for isolation and purification of genomic DNA from prokaryotic and eukaryotic cells. Some of these procedures are based on phenol extraction and alcohol precipitation [2], while others are based on chromatographic separation, as is the case with most commercial kits. The choice of a method depends on the degree of purity of the genomic DNA required for the analysis to be performed.

Chromatographic purification, especially the introduction of ion exchange chromatography and affinity chromatography has enabled the production of highly purified macromolecules. The crucial factor for a successful and fast separation of macromolecules is a reduced mass transport resistance within large open-end pores/channels as described by various authors like, e.g. Tennikova and co-workers [3,4] for high performance membrane chromatography on porous monolithic disks or Teeters et al. [5] and Endres et al. [6] on stacked-membrane columns based on polyethersulfone.

Monoliths are considered as a novel generation of stationary phases introduced in the past 15 years [7–11]. As opposed to individual particles packed into chromatographic columns, monolithic supports are cast as continuous homogeneous phases. They represent an approach that provides high rates of mass transfer at lower pressure [12]. Mass transfer in monoliths is mainly based on convection and that is the basis for naming one particular type of these supports as Convective Interaction Media® (CIM) [13].

In this article, an application of CIM® monolithic columns for the purification of genomic DNA from eukaryotic and prokaryotic cells is described.

* Corresponding author. Tel.: +385 1 4684500; fax: +385 1 4684303.
E-mail address: dforcic@imz.hr (D. Forcic).

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2. Experimental

2.1. Chemicals

Luria–Bertani (LB) medium was from Institute of Immunology Inc. (Zagreb, Croatia). Tris(hydroximethyl)-methylamine, ethylene–diamine tetraacetic acid (EDTA), isopropanol, boric acid, sodium acetate and ethidium bromide were purchased from Sigma (St. Louis, MO, USA). Ethanol and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were obtained from Amresco (Solon, OH, USA). RNase A, lysozyme, proteinase K, Tween-20, Triton X-100, guanidine·HCl, $MgCl_2$, sucrose, λ DNA-*Hin*dIII digested size marker and KiloBase DNA size marker were obtained from Amersham Biosciences (Uppsala, Sweden). Qiagen Blood & Cell Culture DNA kit was from Qiagen (Hilden, Germany).

2.2. Instrumentation

All column chromatography experiments were performed using ÄKTA purifier (Amersham Biosciences, Uppsala, Sweden).

Electrophoresis of genomic DNA was carried out using the electrophoretic unit HE 33 (Hoefer, San Francisco, CA, USA). Agarose gels were UV illuminated and photographed using Kodak digital science Image Station 440 CF (Eastman Kodak, Rochester, NY, USA).

Spectrophotometric determination of genomic DNA purity and yield was performed by BioPhotometer (Eppendorf, Hamburg, Germany).

2.3. Bacterial and mammalian cells

Escherichia coli DH5α cell culture (Life Technologies, Paisley, UK) was grown overnight at 37 °C in LB medium. Pellet from the appropriate volume was obtained by centrifugation at $4000 \times g$ for 10 min and was resuspended in an appropriate volume of lysis buffer.

The MRC-5 (human lung fibroblasts), obtained from the European Collection of Animal Cell Culture (ECACC, catalog no. 84113001), were grown in MEM-H supplemented with 10% fetal calf serum. Cells that have grown in a monolayer were harvested using 0.25% trypsin and centrifuged at $1500 \times g$ for 10 min at 4 °C. The pellet was washed and resuspended in an appropriate volume of cold phosphate-buffered saline (PBS).

2.4. Lysis of bacterial and mammalian cells

Preparation of the bacterial or mammalian cells lysate was carried out with buffers and by procedures suggested in Qiagen Genomic DNA Handbook 08/2001 with some modifications as indicated.

Bacterial pellet was resuspended in an appropriate volume (0.5–1 ml/1 ml bacterial culture) of lysis solution (50 mM TrisCl, pH 8.0; 50 mM EDTA pH 8.0; 0.5% Tween-20; 0.5% Triton X-100 and 200 μg/ml RNase A). Lysozyme (2 mg/1 ml bacterial culture) and proteinase K (final concentration of 100 μg/ml) were added in the homogeneous suspension and the solution was incubated at 37 °C for 30 min. Solution of 3 M guanidine·HCl and 20% Tween-20 was added to the lysate (in volume of 0.3 volume of lysate) and the lysate was incubated at 50 °C for 30 min. Lysate was mixed with an equal volume of chromatographic binding buffer and it was applied on the monolithic disk.

Suspension of MRC-5 cells was mixed with one volume of lysis buffer (1.28 M sucrose; 40 mM TrisCl, pH 7.5; 20 mM $MgCl_2$; 4% Triton X-100) and three volumes of ice-cold distilled water and incubated on ice for 10 min. Cell lysate was centrifuged at 4 °C for 15 min at $1300 \times g$ and supernatant was discarded. Pellet was resuspended in 0.25–2 ml of ice-cold lysis buffer and three volumes of ice-cold distilled water, centrifuged at 4 °C for 15 min at $1300 \times g$. Supernatant was discarded and pellet was resuspended in digestion buffer (800 mM guanidine·HCl; 30 mM TrisCl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween-20; 0.5% Triton X-100). Proteinase K was added to the final concentration of 100 μg/ml and the solution was incubated at 50 °C for 30 min. Lysate was mixed with an equal volume of chromatographic binding buffer and applied on the monolithic column.

As a positive control, the genomic DNA purified by a commercial Qiagen Blood & Cell Culture DNA Genomic-tip 20/G Kit was used. Maximal amount of DNA that can be purified with these columns is 20 μg.

2.5. Chromatographic media and buffers

CIM® monolithic columns bearing weak (diethylaminoethyl, DEAE) anion group made of highly porous poly(glycidyl methacrylate–co-ethylene dimethacrylate) were from BIA Separations, Ljubljana, Slovenia. CIM® monolithic columns consist of a 3 mm × 12 mm i.d. disk shaped highly porous matrix that is seated in a non-porous self sealing fitting ring. The bed volume of one disk is 0.34 ml, and the porosity is 62%. The disk shaped matrix is inserted in commercially available housing (BIA Separations) and connected to an HPLC system.

The binding buffer used for the separation of both purified genomic DNA and cell lysates on CIM® DEAE was 25 mM MOPS, 0.5 M NaCl, pH 7.0, containing 15% (v/v) isopropanol. As the elution buffer, 25 mM MOPS, 1.5 M NaCl, pH 7.0, containing 15% (v/v) isopropanol, was used.

2.6. Purification of genomic DNA using monolithic disk

CIM® DEAE disk monolithic column was placed in an appropriate housing connected to the HPLC system. Equilibration of monolithic column was carried out by 50 column volumes of binding buffer (25 mM MOPS, 0.5 M NaCl, pH 7.0, containing 15% isopropanol) at a flow rate of 4 ml/min.

During the chromatography absorbance at 280 and 260 nm were monitored at flow rate of 3 ml/min.

Genomic DNA was applied on a CIM® DEAE monolithic column. Depending on the sample volume, application was carried out by using sample loop or HPLC pump. Non-bound substances were washed out by binding buffer at a flow rate of 3 ml/min. Elution was undertaken by using step-wise gradients (at 50 and 100% buffer B, each step lasted for 2 min). Non-bound macromolecules, fractions collected during washing step as well as fractions eluted by increasing of salt concentration were collected and further analyzed.

Regeneration of CIM® DEAE disk monolithic column was carried out after each run by washing with 10 column volumes of 2 M NaCl followed by 10 column volumes of 0.5 M NaOH at a flow rate of 4 ml/min.

2.7. Ethanol precipitation

Sodium acetate (3 M, 0.1 volume), pH 4.6 and 2.5 volumes of 95% ethanol were added to each sample. After 10 min incubation on ice, samples were centrifuged at maximum speed for 20 min and supernatant was discarded. Pellets were washed by adding 400 μl of 70% ethanol and were centrifuged for 10 min at maximum speed. All traces of alcohol were carefully removed by pipetting; pellets were dried and resuspended in an appropriate volume (minimally 50 μl) of 10 mM TrisCl, pH 8.0.

2.8. Analytical gel analysis

To analyze the results of purification procedure, agarose gel electrophoresis was carried out by using a horizontal 0.7% agarose slab gels in 89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3. The electrophoretic separation was performed at a constant voltage at room temperature for 45 min. Genomic DNA was stained with ethidium bromide solution included in the gel (1 μg of ethidium bromide was added to 1 ml of agarose gel).

3. Results and discussion

Results of our previous research on viral RNA [14] and plasmid DNA purifications [15] have shown that nucleic acids can be successfully bound to CIM® DEAE monolithic columns. Based on these results and on the fact that the pores in monolithic columns are large enough to harbour nanoparticles [4,14–16], we wanted to investigate whether genomic DNA, both bacterial and eukaryotic, could be specifically bound, isolated and purified from complex solutions by chromatography on CIM® DEAE columns. Such a method would enable a simple and fast DNA isolation without the usage of toxic organic solvents like phenol or chlorophorm.

In order to find out whether genomic DNA could be bound to CIM® DEAE monolithic column, we applied a sample of bacterial genomic DNA purified with Qiagen Blood & Cell culture DNA Kit from a lysate prepared from 1.2 ml of overnight culture (approximately 4.5×10^9 of cells; approximately 20 μg of DNA). The chromatographic profile is presented in Fig. 1 and it shows two peaks, the first is in the flow through (FT) fraction and the second was obtained during the elution step (E). During the run, fractions were collected and afterwards were precipitated with ethanol in order to provide genomic DNA free of substances from chromatographic buffers. Namely, in the presence of high (0.1–0.5 M) concentration of monovalent cations, ethanol induces a structural transition in nucleic acid molecules which causes them to aggregate and precipitate from solution [17]. Unlike DNA, most salts and small organic molecules (including isopropanol) are soluble in 70% ethanol, and therefore, ethanol precipitation and washing of the pellet will effectively desalt DNA.

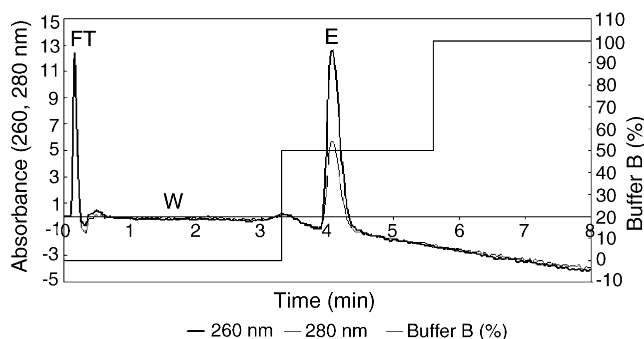

Fig. 1. Profile obtained during chromatography of purified bacterial genomic DNA on CIM® DEAE disk. During the run flow through (FT), washing (W) and elution (E) fractions were collected. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; flow rate: 3 ml/min.

Fig. 2 shows agarose gel with the precipitates of fractions collected during chromatographic run presented in Fig. 1. Genomic DNA is present only in the peak obtained during elution (Fig. 2, lane E) and not in the flow through or washing fractions (Fig. 2, lanes FT and W).

In order to find out whether eukaryotic genomic DNA could also be successfully bound to CIM® DEAE monolithic

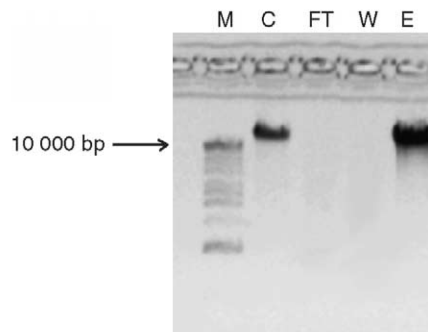

Fig. 2. Ethidium bromide-stained agarose gel with the fractions collected during chromatography of purified bacterial genomic DNA on CIM® DEAE disk (Fig. 1): M, KiloBase DNA size marker; C, positive control (bacterial genomic DNA isolated by QIAGEN Blood & Cell culture DNA Kit); FT, W, E, fractions collected during the flow through, washing and elution step, respectively; bp, base pairs.

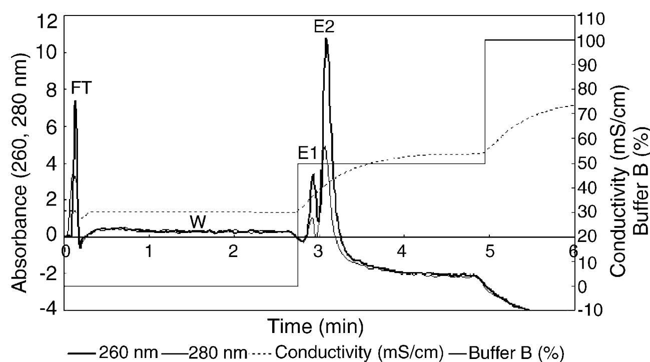

Fig. 3. Profile obtained during chromatography of purified eukaryotic genomic DNA on CIM® DEAE disk. During the run flow through (FT), washing (W) and two elution (E1 and E2) fractions were collected. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% isopropanol (v/v); elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; flow rate: 3 ml/min.

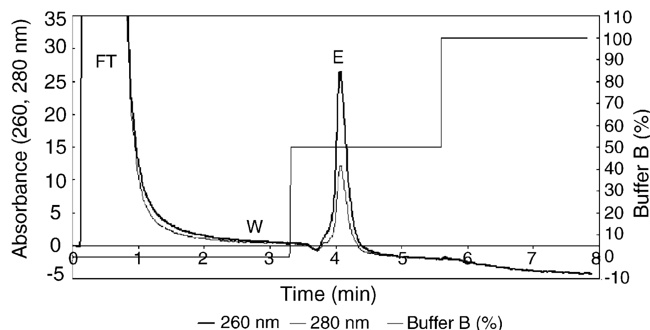

Fig. 5. Profile obtained during chromatography of bacterial cell lysate on CIM® DEAE disk. During the run flow through (FT), washing (W) and elution (E) fractions were collected. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; flow rate: 3 ml/min.

column, we applied a sample of DNA isolated with Qiagen Blood & Cell culture DNA Kit from 0.5×10^6 MRC-5 cells (approximately 20 μg of DNA). The obtained chromatogram is presented in Fig. 3 and it contains three peaks: one is in the flow through fraction and two were obtained during elution step (Fig. 3). Presence of genomic DNA in the precipitates of collected fractions was determined by agarose gel electrophoresis (Fig. 4). All genomic DNA was bound to the column as evidenced by the absence of DNA in lanes corresponding to the flow through and washing fractions (Fig. 4, lanes FT and W). After the wash, the concentration of salt in the chromatographic buffer was raised, DNA was eluted and it was present in both elution peaks (Fig. 4, lanes E1 and E2). Although only one stepwise gradient was used (50% buffer B), two well separated peaks were observed. Possible explanation could be in technical limitation of HPLC system. Making of stepwise gradient was too slow and practically, linear gradient was obtained. This was the reason why two peaks were eluted in a narrow time period. The analytical methods we used during this research (agarose gel electrophoresis, polymerase chain reaction (PCR) and spectrophotometric measurements) did not show any differences in DNA molecules form the two elution peaks, and therefore, we can only speculate that they result from DNA fragments of different conformation. During all chromatographic runs of samples containing eukaryotic DNA on CIM® DEAE columns, we always obtained two elution peaks of genomic DNA. After we have established that the DNA purity in both peaks is the same, in following experiments, we have collected the two peaks as a single elution fraction.

After we have shown that genomic DNA from both bacterial and mammalian cells can be efficiently bound to CIM® DEAE disks, we were interested in testing whether genomic DNA could be specifically bound, isolated and purified directly from cell lysates by using these chromatographic columns. A bacterial lysate prepared from 1.2 ml of overnight culture was applied. The run is presented in Fig. 5 and gel electrophoresis of precipitated chromatographic fractions in Fig. 6. Bacterial DNA was bound to the column and was eluted in a single peak, which is in agreement with the results presented in Figs. 1 and 2.

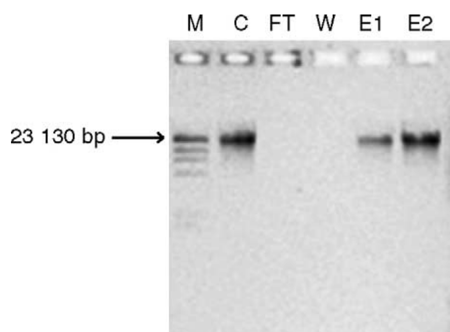

Fig. 4. Ethidium bromide-stained agarose gel with the fractions collected during chromatography of purified eukaryotic genomic DNA on CIM® DEAE disk (Fig. 3): M, λ DNA-*Hind*III digested size marker; C, positive control (eukaryotic genomic DNA isolated by Qiagen Blood & Cell culture DNA Kit); FT, W, fractions collected during the flow through and washing step, respectively; E1, E2, the first and the second peak obtained during the elution, respectively.

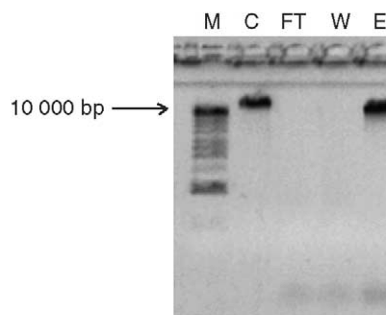

Fig. 6. Ethidium bromide-stained agarose gel with the fractions collected during chromatography of bacterial cell lysate on CIM® DEAE disk (Fig. 5): M, KiloBase DNA size marker; C, positive control (bacterial genomic DNA isolated by Qiagen Blood & Cell culture DNA Kit); FT, W, E, fractions collected during the flow through, washing and elution step, respectively.

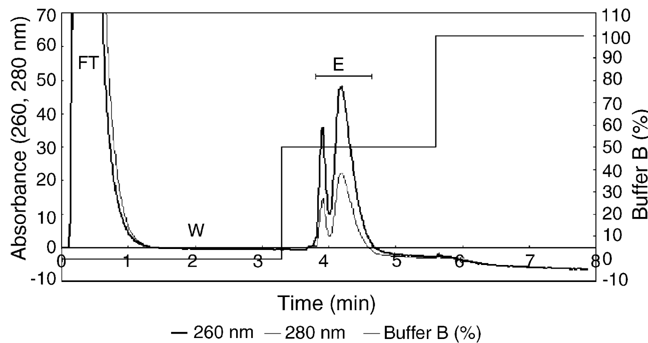

Fig. 7. Profile obtained during chromatography of eukaryotic cell lysate on CIM® DEAE disk. During the run flow through (FT), washing (W) and elution (E) fractions were collected. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; flow rate: 3 ml/min.

The profile obtained after application of lysate (containing eukaryotic genomic DNA) prepared from 0.5×10^6 MRC-5 cells is presented in Fig. 7 and as expected, it shows three peaks: the first is in the flow through fraction and it does not contain DNA (as shown by gel electrophoresis in Fig. 8, lane FT) and the following two peaks, collected as a single chromatographic sample, are in the elution fraction and contain genomic DNA (Fig. 8, lane E).

Our next interest was to investigate whether we can achieve the up-scale of applied volume. We applied various sample volumes to the same CIM® DEAE column. The highest number of MRC-5 cells from which the lysate was prepared was 1.06×10^8 and we did not detect the presence of genomic DNA in the flow through or washing fractions. Likewise, the largest volume of overnight bacterial cell culture used to prepare the lysate was 30 ml, and DNA was also detected only in the fraction collected during elution. The chromatographic profiles were in concordance with the profiles obtained after application of smaller sample volumes (data not shown). Neither of the samples caused column clogging and our opinion is that much larger volumes could be used but this remains to be confirmed.

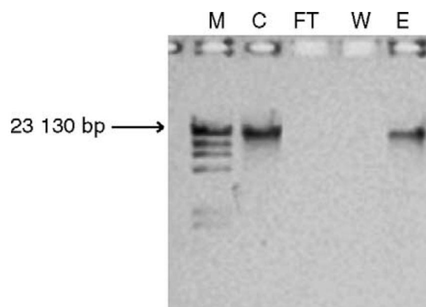

Fig. 8. Ethidium bromide-stained agarose gel with the fractions collected during chromatography of eukaryotic cell lysate on CIM® DEAE disk (Fig. 7): M, λ DNA-*Hin*dIII digested size marker; C, positive control (eukaryotic genomic DNA isolated by Qiagen Blood & Cell culture DNA Kit); FT, W, E, fractions collected during the flow through, washing and elution step, respectively.

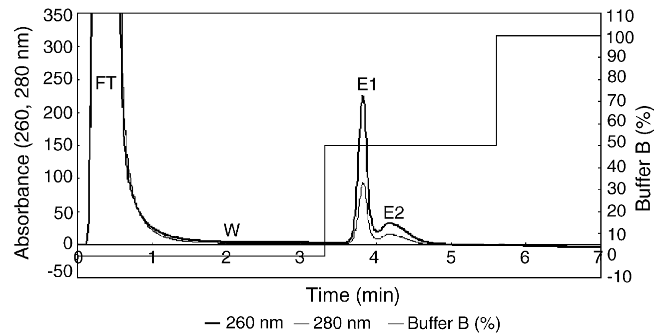

Fig. 9. Profile obtained during chromatography of bacterial cell lysate not treated with RNase on CIM® DEAE disk. During the run flow through (FT), washing (W) and two elution (E1 and E2) fractions were collected. Binding buffer: 25 mM MOPS, 0.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; elution buffer: 25 mM MOPS, 1.5 M NaCl, pH 7.0, 15% (v/v) isopropanol; flow rate: 3 ml/min.

One of the most common impurities in DNA samples after the isolation procedure are RNA remnants. In majority of protocols for DNA isolation, RNA is removed by addition of RNases, but in order to validate the process, it is preferable to omit the usage of all enzymes, even the usage of lysozyme in DNA isolation from bacteria [18].

During preparations of bacterial or eukaryotic cell lysates, we treated the samples with RNase and no RNA was detected in any of the fractions. As we have shown in our previous paper, by using chromatography on CIM® DEAE column it is possible to separate RNA from plasmid DNA in a single step, without the usage of RNases [15]. In order to show that genomic DNA can also be separated from RNA and that it is not necessary to conduct enzymatic RNA digestion, we applied a cell lysate from 1.2 ml of overnight bacterial culture on CIM® DEAE column. The chromatographic profile is presented in Fig. 9 and is shows two elution peaks, first contains RNA and the second contains genomic DNA, as is confirmed by agarose gel electrophoresis (Fig. 10). This separation pro-

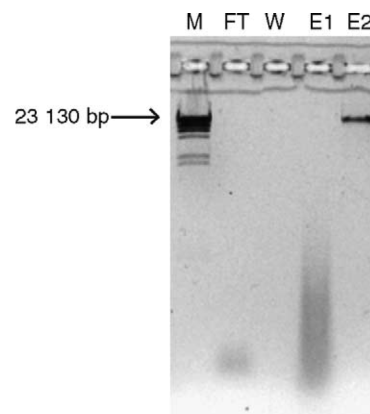

Fig. 10. Ethidium bromide-stained agarose gel with the fractions collected during chromatography of bacterial cell lysate not treated with RNase on CIM® DEAE disk (Fig. 9): M, λ DNA-*Hin*dIII digested size marker; FT, W, fractions collected during the flow through and washing step, respectively; E1, E2, the first and the second peak obtained during the elution, respectively.

file is in agreement with our previous results of separation of RNA from plasmid DNA. Namely, those results showed that during chromatography on CIM® DEAE column, RNA is eluted in a single peak prior to plasmid DNA [15]. The same results were obtained for separation of eukaryotic DNA and RNA (data not shown).

After each chromatographic separation and ethanol precipitation of the collected fractions, the purity and concentration of obtained genomic DNA were examined by agarose gel electrophoresis and by spectrophotometric measurements. These two methods are complementary because the measurement of absorbance on 260 nm does not discriminate between DNA and RNA [2], and therefore, should be used for determination of DNA concentrations only after the absence of RNA is confirmed by agarose gel electrophoresis. The absence of proteins from genomic DNA samples was determined spectrophotometrically. A_{260}/A_{280} was always between 1.7 and 1.9 for both bacterial and eukaryotic DNA samples. The purity and the quantity of isolated DNA were comparable to those obtained by other chromatographic methods used nowadays for isolation of genomic DNA (for instance, Qiagen Blood & Cell culture DNA Kit) (data not shown).

Furthermore, the integrity of genomic DNA isolated by chromatography on CIM® DEAE columns was satisfactory. Besides visual examination on agarose gels, the integrity was also monitored by PCR and by restriction fragment length polymorphism (data not shown).

Regeneration of monolithic columns was already tested and results show the possibility of consecutive usage of the same disk when regeneration procedure was included after each run [19]. In our work with genomic DNA, just one disk was used for all purifications of bacterial DNA and another for all purifications of eukaryotic DNA. After approximately 15 runs on each disk, we did not observe any change in separation results. Sample volume that can be applied on monoliths is not restricted, and various volumes also did not affect the chromatographic separations. The duration of the procedure is substantially shorter when compared to classical DNA isolation protocols (protocols that do not include chromatographic steps) and is comparable to the duration of DNA purification with other commercially available columns.

In order to obtain as intact genomic DNA molecules as possible, we monitored the pressure inside the HPLC system and adjusted the flow velocity so that the pressure does not exceed 20 bar. Generally, we used flow velocities between 3 and 4 ml/min, but depending on the technical characteristics of a HPLC system, even faster separations can be achieved.

4. Conclusion

In this paper, we have shown that both bacterial and eukaryotic genomic DNA can be successfully bound to CIM® DEAE monolithic column and we have developed a simple and fast chromatographic procedure for isolation and purification of genomic DNA from cell lysates that does not include the usage of toxic organic solutions.

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