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Chromatographic behaviour and purification of linear lambda phage and plasmid DNA molecules on 2-hydroxyethyl methacrylate–ethylene dimethacrylate-based supports

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Abstract

The HEMA-BIO 1000 support, which is based on a copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate, was used for separation of lambda DNA and its fragments and plasmid pBR322 DNA. The separation of fragments greater than 6.6 kbp was demonstrated according to the slalom chromatography mechanism on column for size-exclusion chromatography in the case of linear lambda DNA fragments. The influence of particle size of column packing, mobile phase rate, and KCl concentration in mobile phase is discussed. The purification of plasmid DNA pBR322 using size-exclusion chromatography was more rapid compared to gel electrophoresis. The presence of salts in the eluate is not disadvantageous. DNA can be recovered from the eluate by ethanol precipitation. Plasmid DNA pBR322 isolated in this way was suitable for different biological applications (cleavage with restrictases, electrotransformation into bacterial cells).

Keywords: Stationary phases, LC; Slalom chromatography; DNA; Hydroxyethyl methacrylate–ethylene dimethacrylate

1. Introduction

Large-scale processing of gene vectors, such as plasmid DNAs and lambda phage DNA, is very important in modern biotechnology [1]. Lambda phage cloning vector (cloning capacity from 8 to 22 kilo base pairs (kbp)) was improved as an important vector system for cloning of both genomic and cDNA. DNA of very high purity is required for some applications (gene therapy, DNA vaccination, cloning purposes), and has to be free of impurities [2] as mentioned below.

The procedures for the large-scale isolation of plasmid and lambda DNA involve a phenol–chloroform extraction procedure and centrifugation through a CsCl gradient [3]. This method requires the use of toxic chemicals (phenol, chloroform, CsCl) and is time-consuming. Downstream processing is used for the elimination of cellular components in the bacterial cell lysates such as bacterial proteins, lipids, liposaccharides, chromosomal DNA fragments, low- and high-molecular-mass RNAs and their fragments, DNA plasmid variants, and cell debris. The majority of plasmid DNA molecules isolated from bacterial cells are negatively supercoiled (sc). A fraction of plasmid DNA molecules also exists in a relaxed or open circular (oc) form (with no coiling of double helix). Other variants, such as linear, denatured or dimeric, can be also present in bacterial lysates.

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High-scale preparations of pure phage or plasmid DNAs are thus routinely required. A widely used method for the purification of plasmid and/or fragmented DNA is polyacrylamide and agarose gel electrophoresis [4]. However, fragments recovered from gel are contaminated by soluble impurities which can inhibit DNA processing enzymes [5]. Therefore further purification steps are required.

For the above-mentioned reasons, alternative purification techniques have been studied. Various chromatographic techniques such as ion-exchange [6–8], size-exclusion [6,9,10], reversed-phase [11], normal-phase (adsorption) [12], hydrophobic-interaction [13], and affinity [14,15] have been used. Size-exclusion chromatography (SEC), for instance, has been useful as a practical preparative procedure substituting for the CsCl density gradient; in addition the dialysis step necessary to remove the CsCl makes this process too long. The separation of DNA fragments was markedly improved by the introduction of small-size, non-porous ion-exchanger particles [6,16]; DNA fragments interact only with functional groups on the surface of the carrier and do not permeate the carrier pores in this case. A new chromatographic mode for size-dependent DNA fractionation was discovered independently by Boyes et al. [17] and Hirabayashi and Kasai [18], and was utilised by other authors [19–23]. Nucleic acids can also be isolated from bacterial lysates by adsorption to silica carriers in the presence of chaotropic salts [24,25] or to magnetic anion exchange (DEAE) particles using batch mode [26].

The aim of our work was to study the chromatographic behaviour of lambda DNA fragments and whole lambda DNA using HEMA-BIO 1000 (a copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate) based sorbents. The high-performance size-exclusion chromatographic purification of pBR322 plasmid DNA was also studied.

2. Materials and methods

2.1. Chemicals

Agarose was purchased from Lachema (Brno, Czech Republic), while RNase A and DNase I immobilized on magnetic bead cellulose (particle size 63–135 μm) were obtained from the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic (Prague, Czech Republic). They were prepared according to published procedures [27,28]. A mixture of lambda/*Hin*dIII DNA fragments (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6, 0.1 kbp) was from Sigma (St. Louis, MO, USA). The mixture was pre-heated (65 °C/10 min) and then cooled to prevent binding of fragments with cohesive ends. Without heating, this mixture also contained the 27.5-kbp fragment due to the annealing of 23.1- and 4.4-kbp fragments via cohesive ends. Restriction nuclease *Eco*RI and *Bam*HI were from Bio-Tech (Prague, Czech Republic). HEMA-BIO 1000 columns (250×8 and 500×8 mm, particle size 10 μm; and 250×8 mm, particle size 7 μm), and HEMA-BIO 1000 DEAE (diethylaminoethyl; 250×4 mm, particle size 10 μm) were used (Tessek, Prague, Czech Republic). The other chemicals and solvents were of analytical grade and from commercial sources.

2.2. Equipment

Agarose gels were run in a gel electrophoresis apparatus from Bio-Rad (Richmond, USA). Spectrophotometric measurements were carried out on a DMS 100S Varian Techtron spectrophotometer (Mulgrave, Australia). Chromatography was performed on an LCP 4100 Chromatograph (Ecom, Prague, Czech Republic) equipped with a UV–Vis LCD 2563 detector (Laboratorní přístroje, Prague, Czech Republic). The samples were injected with a 10-μl sampling loop injector (Rheodyne, Berkeley, USA). Magnetic particles were separated using a Dynal MPC-M magnetic particle concentrator (Dynal, Oslo, Norway). The exponential decay pulser and pulse controller were home-made and their arrangement was published previously [29].

2.3. Isolation of lambda DNA and pBR322 DNA

Lambda DNA (48.5 kbp) was isolated from *Escherichia coli* JRS154 cells (lambda cI857S7) [3]. The purification of lambda DNA includes three consecutive steps: obtaining a cellular lysate, isolating lambda particles, and extracting phage DNA. The lysis was induced by adding chloroform (1:1, v/v) as the S7 mutation of phage gene was used. Briefly, the

cell debris was cleared by centrifugation, and bacterial chromosomal DNA and high-molecular-mass RNA were digested with DNase I and/or RNase A immobilized on magnetic cellulose particles. The bacteriophage DNA was protected from digestion because it was packed in phage heads. Bacteriophage particles were precipitated with poly(ethylene glycol) (PEG 8000) and a high concentration of NaCl. The phage DNA was released from the phage particles by extracting with phenol (pH 8.0) and the aqueous layer was extracted twice (1:1) with a mixture of chloroform–isoamyl alcohol (24:1). The lambda DNA obtained in the final aqueous layer was precipitated with 1/10 vol. of 3 *M* sodium acetate (pH 6.0) and 2 vol. of ice-cold ethanol. The isolated lambda DNA was dissolved in TE buffer (10 m*M* Tris–HCl and 1 m*M* EDTA, pH 7.8). The plasmid DNA pBR322 (4322 bp) was isolated from *E. coli* DH5α(pBR322) cells by alkaline lysis [3]. The isolated plasmid DNA was dissolved in TE buffer.

2.4. Gel electrophoresis and chromatography

Gel electrophoresis was carried out in 0.8% agarose for 16 h at 1.5 V/cm using TBE (45 m*M* Tris–borate, 1 m*M* EDTA, pH 8.0) as buffer. The gels were stained with ethidium bromide (0.5 μg/ml). Chromatographic separation was carried out with 20 m*M* Tris–HCl, pH 7.8, 1 m*M* EDTA buffer (mobile phase I) and 20 m*M* potassium phosphate, pH 6.9, 1 m*M* EDTA (mobile phase II). The mobile phases I and II at concentrations from 0.00 to 0.25 *M* KCl or from 1.0 to 1.1 *M* KCl were used in SEC and ion-exchange chromatography (IEC), respectively. The flow-rate of mobile phase varied from 0.2 to 0.8 ml/min. The mobile phase was degassed before use. The measurements were made at 254 nm. The DNA eluted from the column was recovered after ethanol precipitation. The identity of DNA fragments was estimated using agarose gel electrophoresis and UV spectrophotometry. The spectra of DNA and RNA fractions were measured in TE buffer. The absorbance ratio $A_{260\ nm}/A_{280\ nm}$ was used as a test of nucleic acid purity [30]. In slalom chromatography [19], the relative retention time (RRT) of a DNA fragment is given by the equation:

$$RRT = \frac{t_R}{t_{NR}} \quad (1)$$

where t_R is the retention time of DNA fragment and t_{NR} is the retention time of a high-molecular-mass compound eluting in the interstitial volume. In IEC the retention factors k' were used.

The resolution $R_{i,j}$ between two adjacent DNA fragments is defined by the equation:

$$R_{i,j} = \frac{t_j - t_i}{[0.5(Y_i + Y_j)]} \quad (2)$$

where t_i and t_j are the retention times, and Y_i and Y_j the peak widths of the two DNA fragments *i* and *j*, respectively. Peak asymmetry A/B was calculated according to Refs. [31,32]. Briefly, peak asymmetry was expressed as the ratio of the distance from the peak apex to the back side of the chromatography curve (distance A) and the distance from the peak apex to the front side of that (distance B) in the horizontal line at 10% of the peak height.

2.5. Electrotransformation

Electrotransformation was carried out at 14 kV/cm with a time of ~4 ms according to the procedure published previously [29]. Ampicillin resistant transformants were recovered in 2% ($8 \cdot 10^5$) of surviving cells after plating on L-agar supplemented with ampicillin (50 μg/ml) for pBR322 plasmid in three independent experiments.

3. Results and discussion

3.1. Slalom chromatography and SEC

Large amounts of DNA restriction fragments of high purity are needed for molecular hybridization and other molecular diagnostic methods. The HEMA-BIO chromatographic support used in this study possesses interesting properties, such as chemical stability, hydrophilicity and biocompatibility [33,34]. In this study, the HEMA-BIO 1000 carriers were used for lambda DNA fragment separation. Lambda DNA (48.5 kbp) and DNA fragments larger than 9.5 kbp (or 6.6 kbp, see below) were not eluted in interstitial volume. However, the order of their elution was opposite to that of SEC. The DNA molecules were separated according to their size in

agreement with the principle of slalom chromatography [18,19]. A typical chromatogram of lambda/*Hin*dIII fragments on a HEMA-BIO 1000 column is shown in Fig. 1. Electrophoretic analysis of aliquots from chromatographic elution profiles showed that fraction F_1 contained fragments ≤9.5 kbp, fraction F_2 contained 27.5-kbp fragments, and fraction F_3 contained intact lambda DNA (48.5 kbp).

HEMA-BIO based polymers are formed by agglomerates of submicroscopic highly crosslinked particles (nodules) and are produced with controlled pore dimensions [33]. Most pores of typical macroporous sorbents intended for SEC are no larger than ~100 nm [35]. DNA fragments larger than 1000 bp form random coils which are very flexible and therefore it is difficult to define their size in terms of the molecule volume. The size of a molecule naturally depends on the velocity of mobile phase. For example, a 1000-bp fragment will be stretched to a maximum of 340 nm [6]. The limit of resolution is reached when the radius of gyration of a linear double-stranded DNA molecule exceeds the gel pore size. It is possible that the separated DNA fragments (2.0–9.5 kbp) were co-eluted in a column interstitial volume because they were larger than the pores present in the gel matrix. From this point of view the sieving nature of the gel matrix becomes irrelevant for the separation of the fragments of interest. Large DNA fragments are fibrous (see below) and thus they migrate end-on through the matrix with the remainder of the molecule following in a snake-like fashion (often referred to as reptation). This manner of molecule movement is equal to the gel electrophoresis of large DNA molecules [36]. In agarose gel electrophoresis, the sieving properties are lost when the DNA exceeds ~30–40 kbp in size. The separation of DNA molecules is achieved by a reorientation of molecules using periodically changing direction of the electric field. The mechanism of the reorientation of DNA molecules was introduced to the theory of slalom chromatography by Ref. [20].

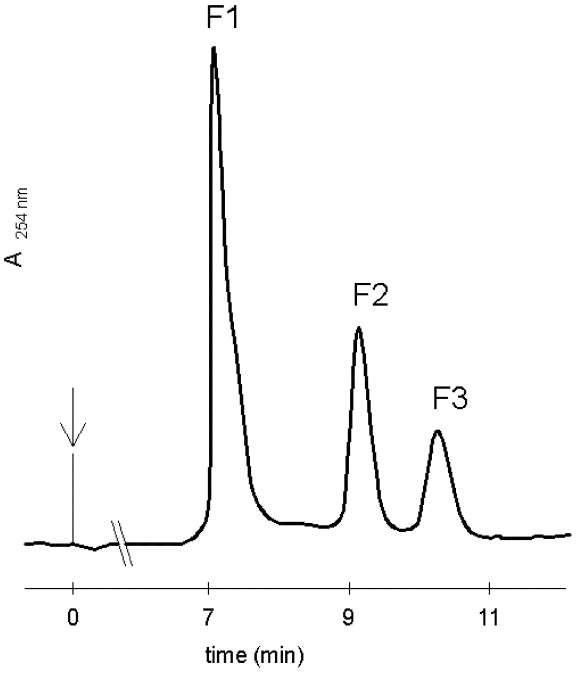

Fig. 1. Slalom chromatography of lambda DNA and lambda DNA/*Hin*dIII restriction fragments. Conditions: sample size 10 μl; column HEMA-BIO 1000, 250×8 mm; particle size 10 μm; lambda DNA/*Hin*dIII (0.5 μg); mobile phase: 20 m*M* potassium phosphate, pH 6.9, 1 m*M* EDTA, 0.2 *M* KCl; flow rate 0.6 ml/min; F_1, F_2, F_3, peak fractions (aliquots from chromatographic elution profile).

Separation depends on the particle size of the column packing and the flow rate, and not on the pore size of packing or the chemical nature in slalom chromatography. Therefore the influence of the particle size of the column (and also column length) on the separation process was studied. It was shown that a better resolution of fragments was achieved on an 500×8-mm than on a 250×4-mm column. Partial separation of a 9.5-kbp fragment from unretained fragments was achieved on a 500×8-mm column. The fragments of interest were better separated on a column packed with 10-μm than with 7-μm particles in the case of 250×8-mm columns (results not shown). This result is in agreement with Ref. [19]. The column packed with 5-μm particles was more suitable for the separation of smaller fragments (less than 20 kbp), while larger fragments were better separated on the column packed with 9-μm particles (with the same pore size) [19]. The influence of flow rate is discussed in the next paragraph.

The mechanism of slalom chromatography can be explained as the result of the hydrodynamic principle [18–20]. Another chromatographic technique based on the hydrodynamic principle used for separation of large polymers or particles is hydrodynamic chromatography (HDC). This technique is based on the

Table 1
Slalom chromatography of lambda DNA/*Hin*dIII restriction fragments: influence of flow rate

Flow rate (ml/min)	Peak	Fragment (kbp)	RRT	R_{ij}	A/B
0.8	1	<6.6	1.00	$R_{34}=2.71$	–
	2	9.4	1.01		–
	3	27.5	1.38		0.79
	4	48.5	1.56		0.81
0.6	1	<6.6	1.00	$R_{34}=2.70$	–
	2	9.4	1.03		–
	3	27.5	1.28		0.91
	4	48.5	1.48		0.85
0.4	1	<6.6	1.00	$R_{34}=2.67$	–
	2	9.4	1.04		–
	3	27.5	1.23		1.00
	4	48.5	1.37		1.00
0.2	1	<6.6	1.00	–	–
	2	9.4	1.06		–
	3	27.4	1.13		–
	4	48.5	1.18		–

Conditions: column HEMA-BIO 1000, 500×8 mm; particle size 10 μm; sample size 10 μl; mobile phase 20 m*M* potassium phosphate, pH 6.9, 1 m*M* EDTA, 0.15 *M* KCl; UV detection at 254 nm. A/B, peak asymmetry; R_{ij}, peak resolution; RRT, relative retention time.

separation of molecules in laminar flow, which occurs in the interstitial spaces created between non-porous particles in the chromatographic column [37–39]. A comparison of both separation techniques is proposed for this reason. However, the elution order in HDC is opposite to slalom chromatography, and is the same as in SEC. In the case of packed-column HDC the relative molecule velocity decreases with increasing solvent flow velocity [39], and retention factor values depend considerably on the ionic strength of the mobile phase [37]. Thus both the above-mentioned parameters were monitored with the aim of studying their influence on the ''hydrodynamic'' character of separation. The dependence of DNA fragment separation on the flow-rate in slalom chromatography is given in Table 1. The results obtained in this study corresponded to those presented in Ref. [20], which used a column packed with 4-μm diameter particles. The actual length of DNA fragments depends on the velocity of the mobile phase in the space between the support particles. Small DNA fragments are in the form of random coil and are eluted at low flow rates with less retardation. Large double-stranded DNA fragments are fibrous, are stretched by increasing flow rate and hence are more retarded. For this reason, the relationship between flow rate and molecule size is not linear. Lower flow rates are usually preferable to obtain good resolution in chromatographic modes. In slalom chromatography, better resolution has been obtained at higher flow rates [21]. However, the influence of flow rate on peak resolution was not too significant and remained constant in our experiments (Table 1). It was obviously affected by the applied range of flow rates.

The influence of KCl concentrations in mobile phase on the separation of lambda/*Hin*dIII fragments was also studied. The addition of salt was not of great influence on the retention time values (Table 2) in contrast to the literature [19]. This effect can be attributed to extended hydrophobic interactions between DNA and non-polar packing in the case of packing developed for reversed-phase liquid chromatography. Large DNA molecules tend to bind to

Table 2
Relative retention times (RRT) of lambda DNA and lambda DNA/*Hin*dIII restriction fragments

Fragment (kbp)	RRT											
	Mobile phase I/KCl (*M*)						Mobile phase II/KCl (*M*)					
	0.00	0.05	0.10	0.15	0.20	0.25	0.00	0.05	0.10	0.15	0.20	0.25
≤9.4	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
23.1	1.28	1.25	1.22	1.28	1.28	1.29	1.27	1.28	1.28	1.28	1.27	1.26
27.5	1.29	1.29	1.28	1.28	1.28	1.27	1.28	1.27	1.28	1.28	1.28	1.28
48.5	1.86	1.85	1.82	1.85	1.84	1.78	1.81	1.82	1.86	1.86	1.85	1.88

Conditions: column HEMA-BIO 1000, 250×8 mm; particle size 10 μm; mobile phase I: 20 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.8; mobile phase II: 20 m*M* potassium phosphate, 1 m*M* EDTA, pH 6.9; flow rate 0.6 ml/min.

highly hydrophobic supports [40]. No such great salt effect was observed for packings designed for SEC such as TSK-G3000SW (silica based) or Asahipak GS-310 (polymer based) [19].

These results are in agreement with the results in Ref. [19] that the slalom chromatography separation mechanism does not depend on the type of chromatographic support used. This was shown by the use of quite different packing types with hydrophilic properties, which are quite different from the packing types used in the literature [18–23].

3.2. Purification of lambda and plasmid DNAs

In chromatographic purification of lambda DNA, co-elution with RNA fragments degraded by immobilized RNase A was observed (results not shown). In this case different mechanisms of nucleic acid separation were applied (slalom mechanism for lambda DNA and SEC for RNA fragments).

Plasmid pBR322 DNA was purified without loss of its biological activity. Using SEC, plasmid DNA was eluted in the column interstitial volume. As high-molecular-mass RNA was also co-eluted in the interstitial volume, it was necessary to cleave it to small fragments using RNase A. The enzyme immobilized on magnetic cellulose particles was used with success. The immobilized enzyme can be easily removed from the reaction mixture by a powerful magnet and the laborious removal of free enzyme is avoided. The total RNA content in the sample depends on preliminary operations before chromatography. RNA reduction can be achieved, for example by precipitation with 4 *M* LiCl [41]. As the added salt has a small molecular mass, it is eluted at the end of analysis. The possible presence of salt in sample fractions is not disadvantageous because DNA molecules can be recovered by ethanol precipitation. The recovered DNA molecules were of course free of contamination originated from agarose gel. No ethidium bromide for DNA detection was required. The purity of plasmid DNA was quite good as was shown by gel electrophoresis (Fig. 2). It was found that the relative standard deviation, s_r, of the peak height was 3.58% after repeated injection ($n = 14$) of an equal amount of DNA on the column. The purified pBR322 plasmid was cleavable with restriction nucleases *Eco*RI and *Bam*HI, which demonstrated the purity of the plasmid DNA.

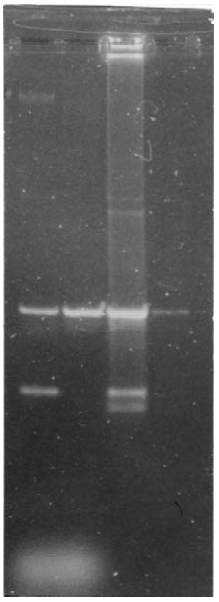

Fig. 2. Electrophoretic gel analysis of RNA removal in plasmid DNA pBR322 preparation. Conditions: 0.8% agarose gel, TBE (45 m*M* Tris–borate, 1 m*M* EDTA, pH 8.0) buffer. Lanes: (1) control plasmid DNA pBR322 without chromatographic purification; (2) plasmid DNA pBR322 linearised by *Eco*RI restrictase; (3) plasmid DNA pBR322 purified by HEMA-BIO 1000 column (250×8 mm, particle size 10 μm), top of peak; (4) plasmid DNA pBR322 purified by HEMA-BIO 1000 column (250×8 mm, particle size 10 μm), foot of peak.

The biological activity of purified plasmid DNAs was also tested by their electrotransformation into bacterial cells. It was shown that the electrotransformed cells expressed the ampicillin resistance carried by plasmids which provides evidence that plasmid DNA is not changed during migration through the chromatographic column and is biologically active. A comparison of the electrophoretic behaviour of the original plasmid DNA and the plasmid DNA isolated from electrotransformants was carried out using agarose gel electrophoresis. Plasmid DNAs linearised by *Eco*RI showed the same migration mobilities.

3.3. Ion-exchange chromatography

Separation of nucleic acids by IEC is mainly based

Table 3
Ion-exchange chromatography of lambda DNA and lambda DNA/*Hin*dIII restriction fragments

Mobile phase	Concentration KCl (*M*)	*k′*/fragment (kbp)							
		2.0	2.3	4.4	6.6	9.4	23.1	27.5	48.5
II A	1.0	0.00	0.05	–	0.30	0.51	–	0.88	0.83
	1.1	0.00	0.04	0.07	0.18	0.67	0.82	0.84	0.80
II B	1.0	0.00	0.05	0.05	0.33	0.61	0.89	0.96	0.83
	1.1	0.00	0.00	0.00	0.04	0.08	0.77	0.83	0.73

Conditions: column HEMA-BIO 1000 DEAE, 250×4 mm; particle size 10 μm; flow rate 0.6 ml/min; mobile phase II A: 20 m*M* potassium phosphate, pH 6.9, 1 m*M* EDTA; mobile phase II B: mobile phase II A with 6 *M* urea; UV detection at 254 nm.

on electrostatic interactions between the phosphate groups of the backbone and the positively charged groups of ion exchanger. The pores of the ion exchanger have two functions: (a) they enlarge the interaction surface of the carrier, and (b) they allow the DNA fragments to permeate into particles according to the size-exclusion effect. According to the literature [6], the double-stranded DNA fragments larger than several hundred bp can be separated from each other with difficulty as DNA fragments cannot permeate the pores of the ion exchanger. In contrast to this, the separation of large fragments (9.4–23.1 kbp) was reached in our experiments. Phosphate buffers exhibit superior resolution with NaCl or KCl as eluting salts [42,43]. The HEMA-BIO 1000 DEAE support was inefficient for separation of fragments smaller than 4.4 kbp, as poor resolution was obtained. The best separation of 6.6–23.1 kbp lambda/*Hin*dIII fragments was reached using a 1.0–1.1 *M* KCl concentration in the given mobile phase at a flow rate of 0.60 ml/min (Table 3). The resolution R_{ij} between the fragments of 6.6 and 27.5 kbp for 1.0 *M* KCl concentration in the given mobile phase was calculated as $R_{ij} = 1.93$.

DNA was adsorbed on column support at lower KCl concentrations. This adsorption allows the capture, subsequent purification, and volume reduction during the isolation and purification processes coupled with the production of DNA vectors.

According to Ref. [43], the addition of urea to mobile phase improved DNA fragment separations. Urea is included in the mobile phase as a denaturant to ensure that the separated DNA strands remain apart and migrate through the column as linear molecules. However, under normal chromatographic conditions the addition of 4–6 *M* urea does not denature the double-helical structure of DNA restriction fragments, which are always in the double-stranded conformation structure [43]. Urea addition also eliminates interactions between DNA and support. For this reason the separated DNA fragments have to be eluted early (at lower *k′* values). Hecker and Riesner [43] estimated a reduction of the retention times in the separation of DNA fragments of 65–616-bp lengths. In our case this phenomenon was observed for fragments in the range from 6.6 to 27.5 kbp (Table 3). The addition of urea also contributed to the prevention of cross-contamination of the samples by nucleases. Chromatography under denaturing conditions was used for the analysis of shorter nucleic acid fragments of typically 50–100 nucleotides in size that differed in single or multiple bases [44].

Acknowledgements

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