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Effects of DNA topology, temperature and solvent viscosity on DNA retardation in slalom chromatography

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Abstract

Slalom chromatography is a unique size-fractionation method applicable to large DNA molecules [>5 kilobase pairs (kbp)]. The method was first developed by using columns packed with microbeads (diameter, <20 μm) used for high-performance liquid chromatography and by applying a relatively fast flow-rate (>0.3 ml/min). Previous studies suggested that the separation is attributed to a hydrodynamic rather than to an equilibrium phenomenon (J. Hirabayashi and K. Kasai, Anal. Biochem. 178 (1989) 336; J. Hirabayashi, N. Itoh, K. Noguchi and K. Kasai, Biochemistry, 29 (1990) 9515). In the present report, the results of a systematic study on the effects of DNA topology, temperature, and solvent viscosity on DNA retardation are described. Firstly, the behaviour of circular (super-coiled) and linearized forms of charomid DNAs (20–42 kbp) was studied. Circular-form DNA molecules were found to be fractionated size-dependently similarly to linear forms in a flow-rate dependent manner. However, the extent of retardation of the circular form DNA was apparently less than that of the corresponding linear forms. Circular DNAs showed almost the same retardation (e.g., 42 kbp) as DNA fragments (e.g., 20 kbp) having approximately half of the size of the former. This observation indicates that DNA retardation is basically related to physical length, not to mass. Secondly, to study the effect of temperature with special reference to solvent viscosity, we carried out chromatographic analysis at various temperatures ranging from 6 to 65°C in both the absence and presence of sucrose (10 or 20%, w/v). The results showed that it is the solvent viscosity that determines the extent of retardation. Taken together, all of physicochemical parameters that define hydrodynamic properties, i.e., particle size, flow-rate and solvent viscosity, proved to be critical in slalom chromatography as well as the potential physical length of the DNA, thus supporting the concept that slalom chromatography is based on a hydrodynamic principle.

Keywords: Slalom chromatography; Temperature effects; Viscosity; Retention mechanisms; DNA

1. Introduction

Slalom chromatography is a unique method for size-fractionation of DNAs that was developed by two independent groups [1,2]. In this method, relatively large DNA molecules [>5 kilobase pairs (kbp)] are size-fractionated in the order opposite to that expected for gel-permeation chromatography, where larger DNAs are eluted faster than smaller ones; though the method was first devised by using gel permeation columns (i.e., Asahipak GS-310 and 510). Subsequent studies [3,4] showed that none of (i) chemical nature (silica or synthetic polymer) of packings, (ii) pore size of packings, or (iii) ionic

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strength (or hydrophobicity) of the solvent are important for separation in slalom chromatography. Instead, the importance of the presence of narrow open spaces constructed by packaging microbeads (<10 μm) developed for high-performance liquid chromatography (HPLC) and application of a relatively fast flow (>0.3 ml/min) were emphasized. Slalom chromatography proved to be attainable by use of various types of columns developed for HPLC, e.g., those for gel-permeation chromatography [3,4], porous and non-porous cation-exchange chromatography [4] and reversed-phase chromatography [5]. So far, some physicochemical factors closely related to hydrodynamics have been shown to have a critical effect on the separation, i.e.,(i) DNA size, (ii) flow-rate, (iii) particle size of packing material and (iv) temperature. A proposed mechanism of slalom chromatography is as follows: application of a fast flow-rate makes a steep flow-rate gradient in the narrow open spaces in the column, which effectively extends DNA molecules. However, since larger DNAs have a longer reorientation time, they have more difficulty in passing through the particles, and thus, this results in larger retardation (reviewed in [6,7]). More recently, a theory to explain size-dependent separation of large DNAs in slalom chromatography has been proposed along with the above principle [8].

However, further experiments are necessary to establish the concept that slalom chromatography is based on a hydrodynamic principle rather than an equilibrium one. Firstly, the actual meaning of ''size'' must be defined, i.e., whether it means ''mass'' or ''length'' of DNA molecules. Secondly, the observed effect of temperature remains to be elucidated more definitely, since temperature affects various physicochemical parameters, such as packing ratio of columns, molecular dynamics of DNA, and solvent viscosity. To address the first topic, we compared the elution of DNAs having the same molecular weights but different topologies (i.e., linear and circular forms). Analysis was performed with Asahipak GS-310 columns packed with particles of various sizes (5–19 μm in diameter). As to the second topic, we analyzed retardation of λ phage restriction fragments (15.0, 23.1, and 33.5 kbp) in both the absence and presence of sucrose (10 and 20%, w/v) at various temperatures (6–65°C). The data were normalized as a function of solvent viscosity. The results clearly showed that the retardation occurred on the basis of DNA length, not mass, and that solvent viscosity is a direct parameter that determines the retardation as well as flow-rate and particle size. These findings unambiguously support the previously proposed concept that slalom chromatography is based on a hydrodynamic principle.

2. Materials and methods

2.1. Materials

Wild-type λ phage DNA (48.5 kbp), λ/*Hin*dIII fragments (a mixture of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56, and 0.13 kbp), charomid 9–20 (19.7 kbp), 9–28 (27.9 kbp), 9–36 (36.0 kbp), and 9–42 (42.2 kbp) [9], and restriction endonucleases *Apa*I, *Xho*I and *Eco*RI were purchased from Nippon Gene (Toyama, Japan). Circular forms of charomid DNAs (>95%, super-coiled) were prepared by a conventional alkali-SDS (sodium dodecyl sulfate) method [10]. Linear forms of charomid DNAs were prepared by *Eco*RI digestion (37°C, 16 h) at a unique cleavage site for this enzyme. Preparation of λ phage DNA fragments was performed by restriction endonuclease digestion as described previously [11]. Asahipak GS-310 columns (250 mm×7.6; M_r exclusion size determined for pullulan, $4 \cdot 10^4$) packed with different particles (average diameters: 5.0, 9.0, 13.1, and 19.1 μm) were products of Asahi Chemical. All chemicals were of analytical grade and obtained from Wako (Tokyo, Japan).

2.2. Chromatography

A conventional HPLC system composed of a Tosoh CCPD pump and UV-8011 detector equipped with a Shimadzu C-R4A data processor was used throughout the experiments. A 10 μl volume of solution of DNA dissolved in PE buffer (10 m*M* sodium phosphate, pH 7.0, containing 1 m*M* EDTA) was applied to a given column through a Rheodyne 7125 manual injector (sample loop volume: 10 μl). The columns were equilibrated with PE buffer, and DNA elution was monitored by absorbance at 260 nm. For analysis of the effect of temperature, a

column of Asahipak GS-310 packed with 9 μm particles was immersed in a water bath (6–65°C). Unless otherwise mentioned, analysis was performed at room temperature (23±1°C). Solvent viscosity (η) was regarded as that of pure water and calculated by the following equation:

$$\log \eta = -1.64779 + 262.37/(T - 133.98) \quad (1)$$

where T is absolute temperature (K). Viscosities of sucrose-containing solutions are cited from Ref. [12].

As in previous studies, DNA retardation was defined in terms of relative retention time (RRT) given by the following equation:

$$\mathrm{RRT} = t_R / t_{R,0} \quad (2)$$

where t_R is the retention time of a particular DNA molecule and $t_{R,0}$ is that corresponding to flow-through fraction.

3. Results

3.1. Elution of linear and circular terms of charomid DNA

Both linear and circular forms of charomid DNAs having different sizes (approximately 20, 28, 36, and 42 kbp) were prepared, and applied to Asahipak GS-310 columns packed with various sizes of packing particles (5, 9, 13 and 19 μm in diameter). Extents of their retardations were compared in terms of RRT. Examples of elution profiles of circular (>95%, super-coiled) and linear charomid DNAs are shown in Fig. 1A (20 kbp) and in Fig. 1B (28 kbp). In this experiment, both circular and linear forms were co-injected into a column of Asahipak GS-310 (particle size, 9 μm) and were eluted at a flow rate of 0.6 ml/min. In each case, circular (C) forms were eluted much faster than linear (L) forms; i.e., in terms of RRT 0.98 (20 kbp, C), 1.25 (20 kbp, L), 1.05 (28 kbp, C), 1.56 (28 kbp, L). However, circular forms were also size-fractionated, as were linear forms, in a size and flow-rate-dependent manner (Fig. 2). We also examined the effect of particle size of the packings by using 5, 9, 13, and 19 μm particles of Asahipak GS-310. Apparently, retardation of each circular DNA became larger when smaller packings were used (Table 1). The use of larger packings (e.g., 13, 19 μm), however, had little practical merit, because most of the circular DNAs were not fully resolved from the flow-through fraction.

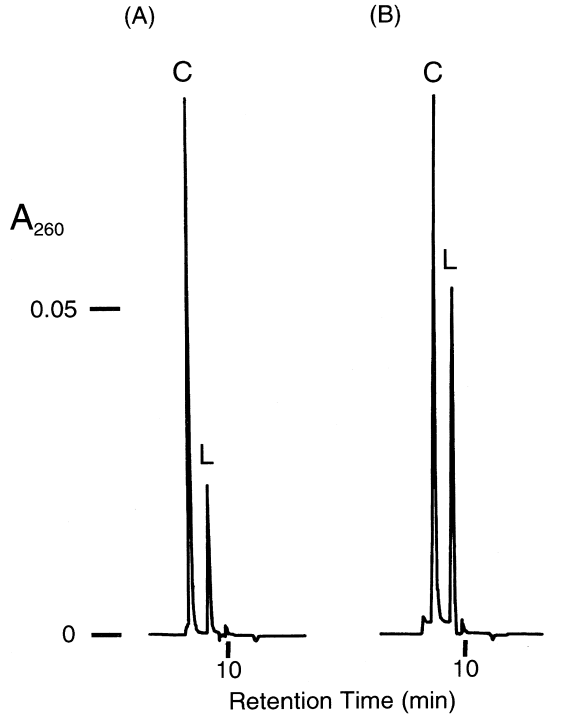

Fig. 1. Elution profiles of super-coiled and linearized charomid DNAs. (A) charomid 9–20 (20 kbp). (B) charomid 9–28 (28 kbp). In both cases, a mixture of super-coiled circular (C) and linearized (L) charomid DNAs (0.8 and 0.4 μg, respectively) was applied to an Asahipak GS-310 column (250 mm×7.6; particle size in diameter, 9 μm), and elution profile was monitored by absorbance at 260 nm. Flow-rate was 0.6 ml/min.

Fig. 2 also implies that 42 kbp circular DNA behaves like 20 kbp linear DNA with respect to retardation i.e., RRT 1.27 (42 kbp, C) and 1.25 (20 kbp, L) at a flow rate of 0.6 ml/min. Therefore, it is reasonable to speculate that the extent of retardation of a circular form is comparable to that of a linear form having half of the molecular size of the former. To confirm this, we compared the elution profiles of four circular charomid DNAs with those of linear DNA fragments, i.e., 10, 15, 17, 20, and 23 kbp, derived from λ phage DNA [4]. Flow-rate dependency is shown in Fig. 3. Evidently, the obtained curves were very similar for the following pairs: 10 kbp (L)

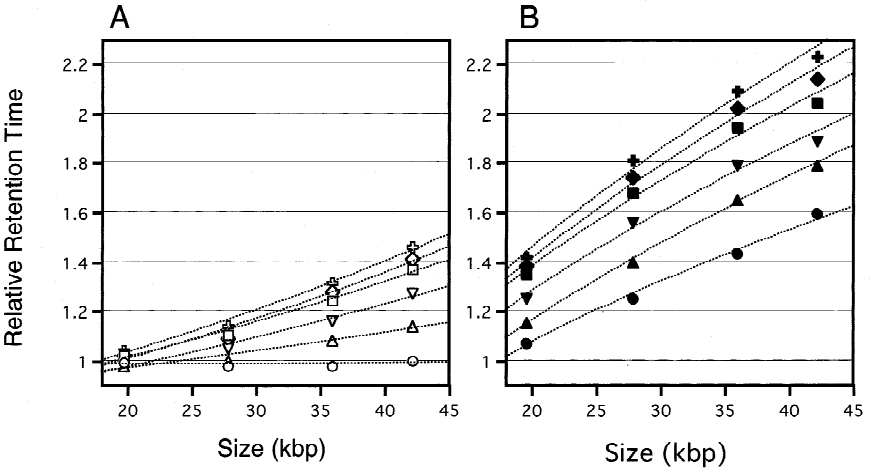

Fig. 2. Comparison of size dependency between super-coiled circular(A) and linearized charomid DNAs (B). Analysis was performed with an Asahipak GS-310 column (250 mm×7.6; particle size, 9 μm). Open and closed represent data for circular and linear DNAs, respectively. Relative retention times (RRT) determined as described under Materials and methods at various flow-rates (0.15–1.5 ml/min), and were plotted against DNA size in kbp. Flow rates were as follows; 0.15 ml/min(○, ●), 0.3 ml/min (△, ▲), 0.6 ml/min (▽, ▼), 0.9 ml/min (□, ■), 1.2 ml/min (◇,♦) and 1.5 ml/min (✣, ✚).

and 20 kbp (C), 17 kbp (L) and 36 kbp (C), 20 kbp (L) and 42 kbp (C). This observation strongly suggests that DNA separation in slalom chromatography is based on extended ''length'', not on molecular ''mass''.

Fig. 3 also suggests a difference in molecular flexibility between circular and linear forms. At a relatively low flow-rate (e.g., 0.3 ml/min), 36 kbp circular DNA was eluted slightly but significantly

Table 1
Relative retention times (RRTs) obtained for circular forms of charomid DNA

Particle size of packing (μm)	Flow-rate (ml/min)	RRT Charomid			
		9–20	9–28	9–36	9–42
5	0.3	1.02	1.13	1.25	1.42
	0.6	1.07	1.23	1.38	1.55
	0.9	1.12	1.02	1.47	1.64
9	0.3	0.98	1.00	1.08	1.14
	0.6	0.98	1.05	1.16	1.27
	0.9	1.02	1.10	1.24	1.37
13	0.3	0.99	0.98	0.99	1.01
	0.6	0.99	1.01	1.04	1.10
	0.9	1.05	1.03	1.10	1.18
19	0.3	0.99	0.99	1.00	1.02
	0.6	0.99	1.00	1.02	1.04
	0.9	0.99	1.00	1.03	1.06

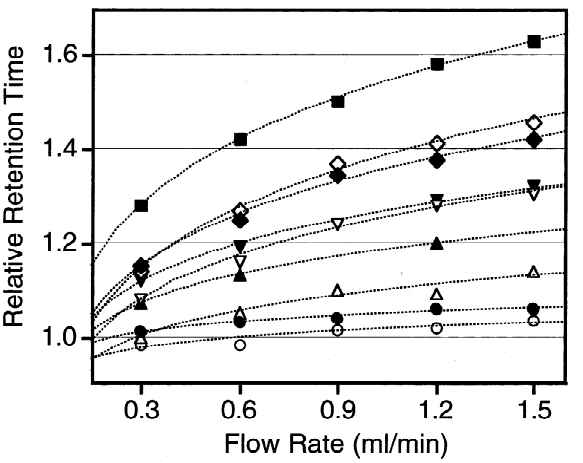

Fig. 3. Effect of flow-rate on retardation of super-coiled circular charomid DNAs and restriction fragments derived from λ phage DNA. Data for the the latter were taken from Ref. [4]. Analysis was performed with an Asahipak GS-310 column (250 mm×7.6; particle size, 9 μm). Open and closed symbols represent data for circular and linear DNAs, respectively. DNA sizes were as follows: 19.7 kbp (circular, ○), 10.1 kbp (linear, ●) , 27.9 kbp (circular, △), 15.0 kbp (linear, ▲) 36.0 kbp (circular, ▽), 17.1 kbp (linear, ▼), 42.2 kbp (circular, ◇) 19.7 kbp (linear, ♦), and 23.1 kbp (linear, ■).

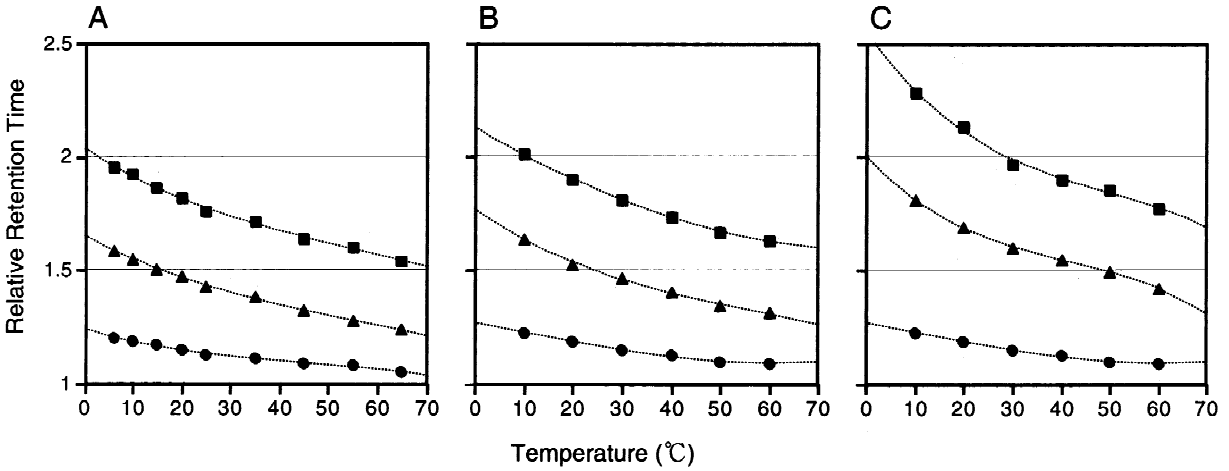

Fig. 4. Effect of temperature and addition of sucrose. Restriction fragments, 15.0 kbp (●), 23.1 kbp (▲) and 33.5 kbp (■), derived from λ phage DNA were applied to an Asahipak GS-310 column (250 mm×4.6, packed with 9 μm particles) at a flow rate of 0.6 ml/min at various temperatures ranging 6–65°C either in the absence (A) or presence of sucrose (w/v; 10% (B), 20% (C)). Relative retention times (RRTs) obtained for the three fragments were plotted as a function of temperature.

earlier (RRT=1.08) than the 17 kbp linear fragment (RRT=1.15), whereas at a faster flow-rate (e.g., 0.9 ml/min) they were eluted at the same position (RRT=1.24 both). As an opposite case, both 42 kbp circular and 20 kbp linear charomid DNAs showed almost the same retardation (RRT=1.14 and 1.15, respectively) at 0.3 ml/min, whereas the former eluted significantly later (RRT=1.41) than the latter (RRT=1.38) at 1.2 ml/min. These observations indicate that super-coiled circular DNAs take a relatively rigid conformation compared with linear forms having half of the molecular size of the former, probably because the former are stabilized by super-coil formation.

3.2. Effect of temperature and solvent viscosity

In our previous study, we observed that retardation of λ/*Hin*dIII fragments became larger when the temperature was lowered from 45 to 15°C [4]. In the present work, three restriction fragments of 23.1 (λ/*Hin*dIII), 15.0, and 33.5 kbp (λ/*Xho*I) were prepared, and their elutions from an Asahipak GS-310 column packed with 9 μm packings were compared over a wider range of temperature (6–65°C) at a fixed flow rate of 0.6 ml/min. As a result, we confirmed that lowering of the temperature significantly enhanced the extent of retardation of each DNA (Fig. 4A; also see chromatograms in Fig. 5C–E). Apparently, the effect of temperature became more prominent at lower temperatures (<20°C). RRT values determined for the three fragments at the various temperatures are summarized in Table 2.

Since temperature affects many physicochemical factors, such as packing ratio, molecular dynamics, and solvent viscosity, the principal factor that defines

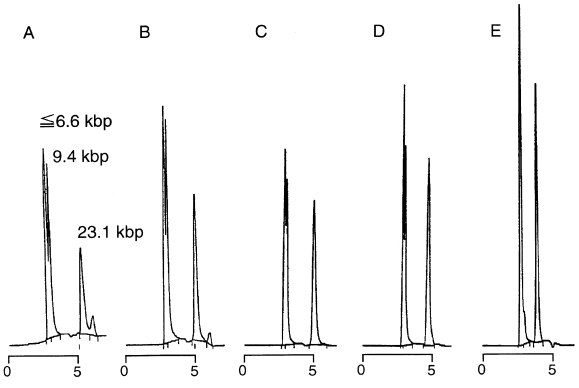

Fig. 5. Elution profiles of DNA fragments under various conditions. In each analysis, 1 μg of λ/*Hin*dIII fragments (mixture) was applied to an Asahipak GS-310 column (250 mm×4.6, packed with 9 μm particles) at a flow rate of 0.6 ml/min. Temperature and sucrose concentration (%, w/v) were varied as follows: (A) 20°C, 20%; (B) 30°C, 20%; (C) 6°C, 0%; (D) 20°C, 0%; (E) 55°C, 0%.

Table 2
Effects of temperature on the relative retention time (RRT) of DNA fragments[a]

Temperature (°C)	Solvent viscosity (cP)	RRT DNA fragment		
		15.0 kbp	23.1 kbp	33.5 kbp
6	1.45	1.20	1.59	1.95
10	1.31	1.19	1.55	1.92
15	1.14	1.17	1.50	1.86
20	1.00	1.15	1.47	1.82
25	0.89	1.13	1.43	1.76
35	0.72	1.11	1.38	1.71
45	0.60	1.09	1.32	1.64
55	0.51	1.08	1.28	1.60
65	0.43	1.05	1.24	1.54

[a] Three restriction fragments (15.0, 23.1 and 33.5 kbp) were eluted from an Asahipak GS-310 column (250 mm×4.6) packed with 9 μm particles at a flow rate of 0.6 ml/min at the indicated temperatures, and DNA retardation was evaluated in terms of RRT calculated as described under Materials and methods.

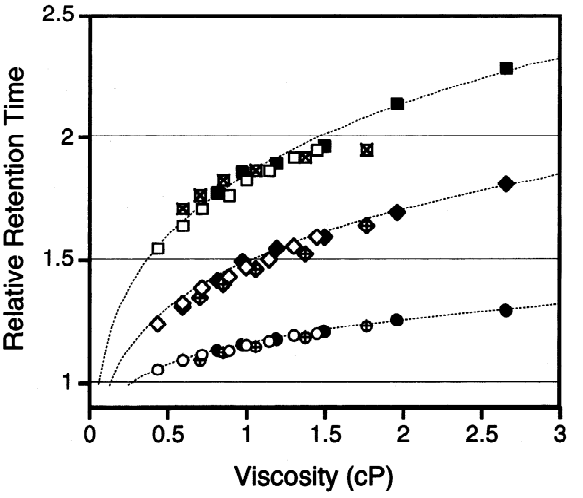

Fig. 6. Relationship between solvent viscosity and relative retention time (RRT). Data from Tables 2 and 3 (Fig. 4) were plotted as a function of solvent viscosity. Symbols are for the three restriction fragments, 15.0 kbp (○, ⊕, ●), 23.1 kbp (◇, ⟡, ♦) and 33.5 kbp (□, ⊠, ■) in the absence (○, ◇. □) or in the presence sucrose (10%; ⊕, ⟡, ⊠; 20%; ●, ♦, ■).

temperature-related DNA retardation in slalom chromatography must be evaluated. For this purpose, we added sucrose to the standard elution buffer (PE) to modify the viscosity up to 20% (w/v), and then compared DNA retardation in terms of RRT at various temperatures (10–60°C). As a result, significantly enhanced retardation was observed in the presence of sucrose even at the same temperature (e.g., compare chromatograms D (20°C, 0% sucrose) with A (20°C, 20% sucrose) in Fig. 5). *T* versus RRT plots are shown in Fig. 4. For example, RRT of 33.5 kbp fragment was 1.82 in the absence of sucrose (Fig. 4A) at 20°C, whereas it increased to 1.90 (Fig. 4B) and 2.13 (Fig. 4C) in the presence of 10 and 20% sucrose, respectively. Both in the absence and presence of sucrose, the nature of slalom chromatography did not change: i.e., retardation was dependent

Table 3
Effects of addition of sucrose on the relative times (RRTs) of DNA fragments[a]

Temperature (°C)	RRT							
	10% (w/v) Sucrose				20% (w/v) Sucrose			
	Solvent viscosity (cP)	15.0 kbp	23.1 kbp	33.5 kbp	Solvent viscosity (cP)	15.0 kbp	23.1 kbp	33.5 kbp
10	1.77	1.23	1.64	2.01	2.65cP	1.29	1.81	2.28
20	1.38	1.19	1.53	1.90	1.96	1.25	1.69	2.13
30	1.06	1.15	1.46	1.81	1.50	1.21	1.60	1.96
40	0.85	1.12	1.40	1.74	1.19	1.17	1.55	1.89
50	0.71	1.09	1.34	1.67	0.97	1.15	1.49	1.85
60	0.60	1.09	1.31	1.63	0.81	1.29	1.42	1.77

[a] Three restriction fragments (15.0, 23.1 and 33.5 kbp) were eluted from an Asahipak GS-310 column (250 mm×4.6) packed with 9 μm particles at a flow rate of 0.6 ml/min at the indicated temperatures in the presence of sucrose (10 or 20%, w/v), and relative times (RRTs) were calculated as described under Materials and methods.

on DNA size and flow-rate. RRT values determined for the three fragments at various temperatures in the presence of sucrose are summarized in Table 3.

These data were normalized on the basis of solvent viscosity (η) in Fig. 6. A clear correlation was observed between η and RRT. This means that if the solvent viscosities are the same, RRT values are essentially the same regardless of temperature. For example, RRT values of 23.1 kbp λ/*Hin*dIII fragment were 1.79 at 6°C in the absence of sucrose ($\eta = 1.50$) and 1.77 at 30°C in the presence of 20% sucrose ($\eta = 1.45$), respectively. Therefore, the larger retardation (RRT) at lower temperature *(T)* is attributed, at least as a first approximation, to the increased solvent viscosity (η).

4. Discussion

The present results confirmed the separation principle of slalom chromatography. Firstly, by a comparative analysis of linear and circular forms of DNA, we proved that DNA retardation (RRT) is defined by the ''length'', not by the ''mass'', of each DNA molecule. Circular forms were retarded to only half of the extent in comparison with linear forms of the same size. Notably, the elutions of circular forms was slightly but significantly less dependent on flow-rate than their corresponding linear forms having half of the molecular size of the former (Fig. 3). In this context, we also prepared relaxed forms of the four charomid DNAs (9–20, 9–28, 9–36 and 9–42) by using topoisomerase, and tried to determine their RRT values. However, none of them except for the smallest charomid DNA (9–20) could be recovered in satisfactory yields. Since relaxed forms are known to have extremely rigid structure, and thus, have the lowest electrophoretic mobility among topoisomers [11], they seemed to have sequestered in the narrow open spaces in the column.

Another important finding is that DNA retardation is a direct function of solvent viscosity. The greater the solvent viscosity (given either by lowering temperature or increasing the sucrose concentration), the greater the DNA retardation. We also confirmed that addition of glycerol instead of sucrose gave the same results (data not shown). The present results show that the effect of temperature is primarily explained in terms of solvent viscosity, though it may have some effects on other physicochemical parameters as well.

In view of the results obtained thus far, we can conclude that DNA retardation is defined primarily by DNA length and is also affected by the following three hydrodynamic parameters: (i) particle size, (ii) flow-rate, and (iii) solvent viscosity. The hydrodynamic nature of fluid is defined by the well-known Reynolds number: $Re = lv/\eta$, where l and v are width of the flow path and flow-rate, respectively. Our previous and present experiments indicate that all of these essential hydrodynamic parameters contribute to the realization of slalom chromatography. However, its precise mechanism is still inexplicable because of the extremely complex nature of the field where the retardation takes place. A simple model that has been recently proposed may explain some of the data [8]. Apparently, such studies should contribute to both understanding this new mode of chromatography based on hydrodynamics and expansion of its application to the separation and analysis of other macromolecules.

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References

[1] J. Hirabayashi, K. Kasai, Nucleic Acids Symp. Ser. 20 (1988) 57.
[2] B.E. Boyes, D.G. Walker, P.L. McGeer, Anal. Biochem. 170 (1988) 127.
[3] J. Hirabayashi, K. Kasai, Anal. Biochem. 178 (1989) 336.
[4] J. Hirabayashi, N. Itoh, K. Noguchi, K. Kasai, Biochemistry 29 (1990) 9515.
[5] J. Hirabayashi, K. Kasai, J. Chromatogr. A 722 (1996) 135.
[6] J. Hirabayashi, K. Kasai, in: T.T. Ngo (Ed.), Molecular Interactions in Bioseparations, Plenum Press, New York, 1993, p. 69.
[7] J. Hirabayashi, K. Kasai, in: H.Y. Aboul-Einen (Ed.), Analytical and Preparative Separation Methods of Biomacromolecules, Marcel Dekker, New York, 1999, p. 415.

[8] E. Peyrin, Y.C. Guillaume, A. Villet, A. Favier, Anal. Chem. 72 (2000) 853.
[9] I. Saito, G.R. Stark, Proc. Natl. Acad. Sci. USA. 83 (1986) 8664.
[10] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning Cold Spring Harbor, Laboratory Press, New York, 1989.
[11] R. Bowater, F. Aboul-Ela, D.M. Lilley, Methods Enzymol. 212 (1992) 105.
[12] Japanese Chemical Society, Japanese Chemical Handbook, Maruzem, Tokyo, version 2, 1975.