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# Applied slalom chromatography
# Improved DNA separation by the use of columns developed for reversed-phase chromatography

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**Abstract**

Improved resolution in slalom chromatography, a novel size-fractionation method discovered recently for relatively large DNA molecules (>5 kbp), was obtained by using columns generally employed for reversed-phase chromatography: i.e., two types of Capcell-Pak (methyl or phenyl-derivatized 5-$\mu$m microbeads), and five types of Hypersil-3 packings (trimethylsilyl, dimethyloctyl, cyanopropyl, octadecyl or phenyl-derivatized 3-$\mu$m microbeads). The resolution of 5–15-kbp DNA was significantly improved by employing these columns, though the separation characteristics differed. When Capcell-Pak columns were used with a normal low-salt eluting solvent (10 m*M* sodium phosphate, pH 6.8, 1 m*M* EDTA), chromatograms were obtained for $\lambda$/*Hin*dIII fragments (a mixture of 0.1, 0.5, 2.0, 2.3, 4.4, 6.6, 9.4 and 23.1-kbp fragments) similar to those obtained previously with Asahipak GS-310 5-$\mu$m size-exclusion packings. However, when up to 0.2 *M* NaCl was added to the solvent, the DNA was increasingly retarded, particularly the 4.4, 6.6 and 9.4-kbp fragments, resulting in improved resolution in the low to middle molecular-mass range. The effect of salt was more significant with Capcell-Pak Phe than C1, although various features characteristic of slalom chromatography were preserved with both columns; i.e., dependency on DNA size, flow-rate, and temperature. This suggests that a mixed mode of separation, that is, slalom mode and hydrophobic-interaction mode, was operating. Although all of the Hypersil-3 packings showed significant adsorption of $\lambda$/*Hin*dIII fragments under low-salt conditions, the fragments could be eluted with satisfactory yield and resolution by adding acetonitrile (>5%) to the solvent. Notably, these Hypersil-3 packings allowed resolution of a 4.4-kbp $\lambda$/*Hin*dIII fragment from the flow-through fraction for the first time, possibly due to their small particle size. Thus, various packing materials developed for high-performance liquid chromatography proved to be applicable for slalom chromatography, though the eluting conditions still need to be refined. The results support the concept that slalom chromatography is based on a hydrodynamic phenomenon.

## 1. Introduction

Slalom chromatography was discovered in 1988 by two independent groups [1,2] as a novel size-fractionation method for relatively large DNA molecules (>5 kbp) (for a recent review, see Ref. [3]). A particular feature of this chromatography is that the separation occurs via a hydrodynamic phenomenon rather than an equilibrium one [4,5]: that is, in slalom chromatography, larger DNA molecules are eluted much later than smaller ones, and the degree of DNA retardation is significantly affected by various hydrodynamic factors, e.g., particle size

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of packings, flow-rate and solvent temperature, whereas chemical factors, such as the chemical nature and pore size of packings, and solvent hydrophobicity, do not have a critical effect.

The method is also important as an emerging technique for treating large DNA molecules for both analytical and preparative purposes [6]. Conventional steady-state and pulsed-field gel electrophoresis have various drawbacks as regards speed, reproducibility, automation, purity of the preparation (possible impurities derived from the agarose gel), etc. In contrast, slalom chromatography can be performed by simply introducing DNA solutions in a conventional high-performance liquid chromatographic (HPLC) system, where rapid, sensitive and reproducible DNA separation can be automated, and no extraction of DNA from the gel is required.

In previous experiments, commercially available packed columns for size-exclusion chromatography were mainly used: for example, TSK-G2000SW [7] and Asahipak GS-310 [8]. However, both porous and non-porous packings originally developed for cation-exchange chromatography [9] also proved applicable [4,5]. It therefore seemed worthwhile to examine a wider range of packing materials. Here, we report results obtained on columns developed for reversed-phase chromatography. Though such packings have been extensively used for the separation of various bio-materials including DNA in a hydrophobic-interaction mode [10–14], their application to slalom chromatography has not previously been attempted. Two types of microbeads (Capcell-Pak and Hypersil-3) were chosen for this purpose, with the aim of realizing a mixed mode of separation, i.e., slalom mode and hydrophobic-interaction mode, and also expanding the separation range to smaller DNA molecules. The results should also improve our understanding of the actual separation mechanism in slalom chromatography [3,5].

## 2. Experimental

Capcell-Pak columns (250 × 4.6 mm I.D.), C1 (total carbon content including that used for silicon coating, 4.5%) and Phe (8.1%) were generous gifts from Shiseido (Tokyo, Japan). A series of Hypersil-3 packings, Phe (phenyl, 5.0%), SAS (trimethylsilyl, 2.6%), CPS (cyanopropyl, 4.0%), MOS (dimethyloctyl, 7.0%) and ODS (octadecyl, 10%) were obtained from Shandon (UK), and were purchased from and packed into columns (250 × 6 mm I.D.) by Senshu Chemicals (Tokyo, Japan).

Wild-type λ DNA (48.5 kbp), a mixture of λ/*Hin*dIII fragments (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.5 and 0.12 kbp), and restriction endonucleases *Apa*I, *Xho*I and *Kpn*I were purchased from Nippon Gene (Toyama, Japan). All other chemicals were of analytical grade, obtained from Wako Pure Chemicals (Tokyo, Japan). Various-sized DNA fragments (10–40 kbp) were prepared by digestion with the above restriction endonucleases as described previously [4,5].

Chromatography was performed essentially as described previously [7,8] by using a Tosoh CCPD dual pump and a Tosoh UV-8011 detector coupled to a Shimadzu C-R4A integrator. The DNA solution was pre-heated at 65°C for 5 min and then cooled on ice until injection to prevent binding via *cos* sites. This heat-treated DNA was injected through a Rheodyne 20-μl injector. When necessary, columns were heat-controlled (10–60°C) by placing them, together with the solvent reservoir, in a water bath. DNA retardation under different conditions was compared in terms of relative retention time (RRT), as defined by the following equation:

$$RRT = t/t_0 \quad (1)$$

where $t_0$ is the retention time corresponding to the void fraction from each column. Experimentally, it was determined by elution of the 2.3-kbp λ/*Hin*dIII fragment.

## 3. Results

### 3.1. Capcell-Pak C1

In order to investigate the feasibility of slalom/hydrophobic mixed-mode chromatography, we first examined the use of Capcell-Pak C1 (carbon content including that used for silicon coating,

4.5%), because large DNA molecules have a tendency to bind to highly hydrophobic resins [10–14]. In addition, the Capcell-Pak packings consist of silicon-coated fine microbeads 5-$\mu$m in diameter that are chemically inert and stable even under alkaline conditions. Resistance to alkali is more favorable for DNA separation, because DNA is in general acid-insoluble, and thus, usually dissolved in weak basic solvents such as Tris-HCl buffer, pH 7.5–8.0.

Chromatograms obtained on the Capcell-Pak C1 column are shown in Fig. 1, where $\lambda$/*Hin*dIII fragments were eluted under normal conditions using PE buffer (10 m*M* sodium phosphate, pH 6.8, 1 m*M* EDTA) as the eluting solvent. As expected from previous studies, the packing, having 5-$\mu$m particle diameter, allowed separation of the 6.6-kbp fragment from the flow-through fraction at a relatively fast flow-rate (e.g., >1.0 ml/min). The chromatograms obtained were very similar to those obtained on size-exclusion columns packed with Asahipak GS-310 and GS-510 5-$\mu$m particles [4,5]. Four peaks representing the 4.4, 6.6, 9.4 and 23.1-kbp $\lambda$/*Hin*dIII fragments were eluted in that order, and retardation of the latter three fragments, i.e., 6.6, 9.4 and 23.1 kbp, increased when a higher flow-rate was applied. These observations indicate that the separation achieved on the Capcell-Pak C1 column is based on the slalom mode, and not on the hydrophobic-interaction mode. Moreover, DNA retardation was not decreased by the addition of acetonitrile to the eluting solvent up to 20% (v/v) (data not shown).

The separation profiles of $\lambda$/*Hin*dIII fragments changed dramatically when NaCl was added to the normal solvent system up to 0.2 *M*. DNA resolution in the low to middle molecular-mass range, e.g. between 4.4, 6.6 and 9.4-kbp, improved markedly. This improvement is due to much greater retardation of the smaller fragments (4.4–9.4 kbp) (Fig. 2). DNA recovery was satisfactory (>50% of the starting DNA) and almost the same when NaCl up to 0.15 *M* was included in the solvent, but became poor at 0.2 *M* NaCl (ca. 50% of the recovery at 0.15 *M*) (Fig. 1). It should be noted that no such dramatic salt effect was observed with various size-exclusion packings, such as Asahipak GS-310 and TSK-G3000SW [4,5], and thus, the effect can possibly be attributed to enhanced hydrophobic interaction between DNA and the packings, although the reason why the effect is less for larger DNA molecules is not clear.

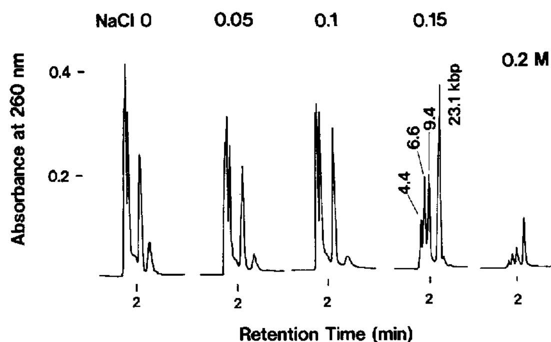


Fig. 1. Separation profiles of $\lambda$/*Hin*dIII fragments on a Capcell-Pak C1 column. NaCl was added to PE buffer (10 m*M* Na–Pi, pH 6.8, 1 m*M* EDTA) to give a concentration of 0, 0.05, 0.1, 0.15 *M* or 0.2 *M* (from left to right). The flow-rate is 1.0 ml/min.

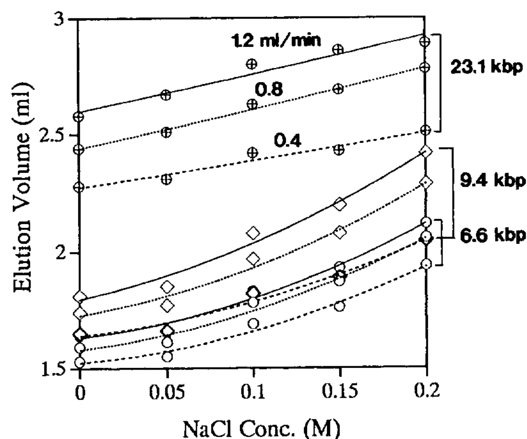


Fig. 2. Relationship between NaCl concentration and elution volumes of three $\lambda$/*Hin*dIII fragments. Elution volumes of the 6.6, 9.4 and 23.1-kbp fragments at flow-rates of 0.4, 0.8 and 1.2 ml/min were plotted versus NaCl concentration. Note that the effect of salt on retardation of DNA fragments is more significant for the smaller fragments, 6.6 and 9.4 kbp, than for the largest, 23.1 kbp.

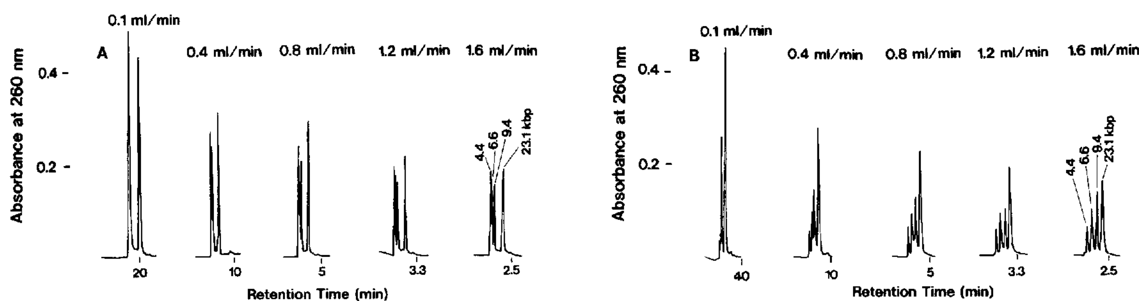


Fig. 3. Separation profiles of λ/*Hin*dIII fragments on a Capcell-Pak Phe column in the absence (A) and presence (B) of 0.1 *M* NaCl at various flow-rates from 0.1 to 1.6 ml/min. Note that resolution in the low to middle molecular-mass range (4.4–9.4 kbp) is significantly improved by the addition of salt.

### 3.2. Capcell-Pak Phe

Another Capcell-Pak packing, Capcell-Pak Phe has the same particle diameter of 5 μm, but a significantly higher carbon content (8.1%, including that used for silicon coating). However, like Capcell-Pak C1, the Phe column was also found to be useful for slalom chromatography (Fig. 3A). Slalom chromatograms obtained in the absence of salt are closely similar to those obtained on Capcell-Pak C1 (Fig. 1), and also to those obtained previously under the same conditions on Asahipak GS-310 and GS-510 columns, both of which are packed with 5-μm size-exclusion packings [4,5]. The Capcell-Pak Phe column also showed size and flow-rate dependency under the normal low-salt conditions, suggesting that the separation is based on the slalom mode.

Retardation of DNA was enhanced, as in the case of the C1 column, by the addition of salt. The chromatograms obtained in the presence of 0.1 *M* NaCl at various flow-rates (0.1–1.6 ml/min) are shown in Fig. 3B, and the effect of flow-rate on DNA retardation, in either the presence or absence of 0.1 *M* NaCl, is plotted in Fig. 4. Again, the effect of salt on the Phe column was more evident for smaller DNA fragments (4.4–9.4 kbp) than for the larger one (23.1 kbp), resulting in improved separation in the former region. However, when compared at the same concentration (e.g., 0.1 *M* NaCl), the effect of salt was greater in the Phe column than

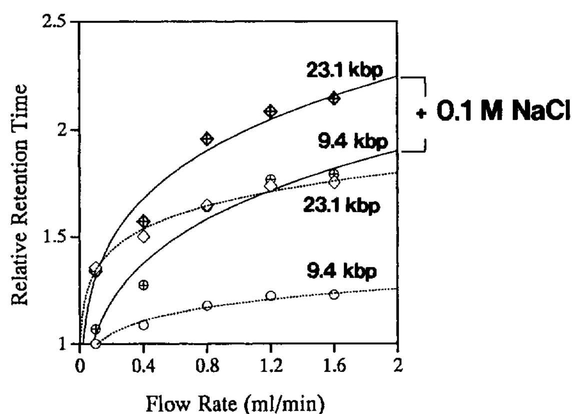


Fig. 4. Relationship between flow-rate and relative retention time in the case of Capcell-Pak Phe. DNA retardation was compared in terms of the relative retention times of 9.4 and 23.1-kbp λ/*Hin*dIII fragments in the presence and absence of 0.1 *M* NaCl.

in the C1 column (compare Fig. 1 with Fig. 3B). This supports the idea that the improved separation is due to significantly enhanced hydrophobic interaction. However, too strong a hydrophobic interaction between packing materials and DNA seemed to make the separation based on a mixed mode impractical, because individual peaks grow much broader in the presence of 0.2 *M* NaCl (Fig. 1). We found that λ/*Hin*dIII fragments were no longer recovered from octadecyl-packing columns, for example, TSK-ODS 80 (carbon content 15%) and ODS–Hypersil-3 (carbon content 10%; described below) (data not shown).

### 3.3. Hypersil-3 packings

It is of particular interest to utilize the smallest possible packing materials for applied slalom chromatography, since previous experiments demonstrated that packings having particles of 5-, 9-, 13- and 19-μm diameter resolved DNA fragments larger than 6, 9, 13 and 17 kbp, respectively [5]. This observation implies that the use of 3-μm particles might allow resolution of even smaller fragments, for instance, 4 kbp. Five Hypersil-3 packings having 3-μm diameter particles (i.e., those derivatized with trimethylsilyl, cyanopropyl, phenyl, dimethyloctyl or octadecyl groups) were used. The packed columns showed relatively low backpressure for 3-μm packings when eluted with a normal low-salt buffer (ca. 100 $kgf/cm^2$, at a flow-rate of 1.0 ml/min; column size, 250 × 6 mm I.D.).

However, none of the λ/*Hin*dIII fragments was recovered from the five Hypersil columns under the normal conditions (data not shown). We assumed that poor recovery of the fragments is due to excessively strong or effective hydrophobic interaction between the Hypersil-3 packings and DNA, although Capcell-Pak columns gave much more satisfactory results under the same conditions (see above). In order to examine this possibility, we added acetonitrile to the normal low-salt buffer up to 20% (v/v) to block possible hydrophobic interaction. When more than 5% acetonitrile was added, λ/*Hin*dIII fragments, including the largest 23.1-kbp fragment, were eluted in a satisfactory yield except in the case of ODS–Hypersil-3 (in Fig. 5, only the result with Phe–Hypersil-3 is presented). On the other hand, 2% acetonitrile was only half as effective on DNA recovery. It should be noted that DNA recovery has been reported to increase on a size-exclusion column (Asahipak-GS510) [4], when increasing concentrations of NaCl were added. In the present case, however, the addition of organic solvent was necessary to eliminate undesirable hydrophobic interaction between DNA and packings. Thus, appropriate conditions still need to be defined for this applied slalom chromatography.

λ/*Hin*dIII separation on Hypersil-3 was found

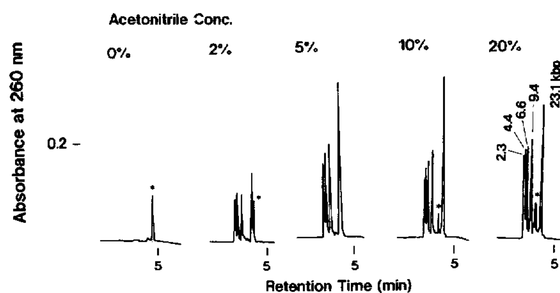


Fig. 5. Separation profiles of λ/*Hin*dIII fragments on a Phe–Hypersil-3 column in the presence of various concentrations of acetonitrile (0–20%, v/v). The flow-rate was 1.2 ml/min. Peaks with an asterisk (*) denote an unknown impurity (not DNA). In the presence of 5% acetonitrile or more, all of the fragments were eluted with satisfactory yield (at least 50% of the starting quantity) and resolution. This is the first time that the small 4.4-kbp fragment has been resolved from the flow-through fraction by slalom chromatography.

to be excellent in terms of peak resolution, particularly in the low to middle molecular-mass range: i.e., DNA fragments of 4.4, 6.6 and 9.4 kbp were more completely separated from one another than was the case with other columns, packed with larger packings. In fact, the Hypersil columns for the first time resolved a 4.4-kbp λ/*Hin*dIII fragment from the flow-through fraction; this had not been achieved in any of the previous experiments.

Despite the significantly different carbon contents of the four Hypersil-3 packings, all of them showed almost the same retardations of λ/*Hin*dIII fragments in the presence of 10% (v/v) acetonitrile (Table 1). This fact suggests that hydrophobic interaction is no longer significant when a hydrophobic solvent, such as acetonitrile, is included in the eluting solvent, and the slalom mode becomes predominant.

### 3.4. Detailed analysis of separation with Phe–Hypersil-3

Because it is important to analyze in more detail the separation characteristics on 3-μm packing columns in order to understand the separation mechanism, further analyses were performed on one of the Hypersil columns, Phe–

Table 1
Comparison of relative retention times of λ/*Hin*dIII fragments eluted from variously derivatized Hypersil-3 columns[a]

| Fragment size (kbp) | Flow-rate (ml/min) | 20°C | | | | 40°C | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SAS[b] | CPS[c] | MOS[d] | Phe[e] | SAS[b] | CPS[c] | MOS[d] | Phe[e] |
| 2.3 | 0.4 | 6.31 (1.00) | 6.31 (1.00) | 6.03 (1.00) | 6.29 (1.00) | 6.14 (1.00) | 6.12 (1.00) | 5.93 (1.00) | 6.19 (1.00) |
| | 0.8 | 3.22 (1.00) | 3.27 (1.00) | 3.07 (1.00) | 3.24 (1.00) | 3.17 (1.00) | 3.19 (1.00) | 3.07 (1.00) | 3.18 (1.00) |
| | 1.2 | 2.17 (1.00) | 2.17 (1.00) | 2.08 (1.00) | 2.17 (1.00) | 2.17 (1.00) | 2.19 (1.00) | 2.05 (1.00) | 2.17 (1.00) |
| 4.4 | 0.4 | 6.31 (1.00) | 6.31 (1.00) | 6.03 (1.00) | 6.29 (1.00) | 6.14 (1.00) | 6.19 (1.00) | 5.93 (1.00) | 6.19 (1.00) |
| | 0.8 | 3.30 (1.02) | 3.27 (1.00) | 3.13 (1.03) | 3.24 (1.00) | 3.17 (1.00) | 3.19 (1.00) | 3.07 (1.00) | 3.18 (1.00) |
| | 1.2 | 2.52 (1.04) | 2.23 (1.03) | 2.16 (1.04) | 2.23 (1.03) | 2.17 (1.00) | 2.17 (1.00) | 2.05 (1.00) | 2.18 (1.01) |
| 6.6 | 0.4 | 6.66 (1.06) | 6.59 (1.05) | 6.34 (1.05) | 6.61 (1.05) | 6.33 (1.03) | 6.34 (1.03) | 6.14 (1.04) | 6.34 (1.02) |
| | 0.8 | 3.55 (1.10) | 3.52 (1.07) | 3.38 (1.10) | 3.49 (1.08) | 3.36 (1.06) | 3.39 (1.06) | 3.28 (1.07) | 3.37 (1.06) |
| | 1.2 | 2.46 (1.13) | 2.43 (1.12) | 2.35 (1.13) | 2.43 (1.12) | 2.34 (1.08) | 2.36 (1.08) | 2.28 (1.11) | 2.36 (1.09) |
| 9.4 | 0.4 | 7.43 (1.18) | 7.35 (1.16) | 7.08 (1.18) | 7.41 (1.18) | 6.90 (1.12) | 6.97 (1.13) | 6.77 (1.14) | 6.93 (1.12) |
| | 0.8 | 4.08 (1.27) | 3.99 (1.22) | 3.86 (1.26) | 3.99 (1.23) | 3.78 (1.19) | 3.82 (1.20) | 3.72 (1.21) | 3.80 (1.19) |
| | 1.2 | 2.82 (1.30) | 2.78 (1.28) | 2.70 (1.30) | 2.80 (1.29) | 2.66 (1.22) | 2.68 (1.23) | 2.61 (1.23) | 2.68 (1.23) |
| 23.1 | 0.4 | 9.52 (1.51) | 9.35 (1.48) | 9.29 (1.54) | 9.56 (1.52) | 8.92 (1.45) | 8.97 (1.45) | 8.93 (1.51) | 9.07 (1.47) |
| | 0.8 | 5.16 (1.60) | 5.03 (1.54) | 6.52 (1.59) | 5.18 (1.60) | 4.83 (1.53) | 4.84 (1.52) | 4.87 (1.59) | 4.93 (1.55) |
| | 1.2 | 3.60 (1.66) | 3.51 (1.62) | 3.55 (1.71) | 3.64 (1.67) | 3.39 (1.56) | 3.39 (1.55) | 3.42 (1.67) | 3.47 (1.60) |

[a] Retention times are expressed in minutes. Relative retention times calculated as described in the Experimental section are indicated in parentheses. DNA elution was performed with 10% (v/v) acetonitrile, 10 m*M* sodium phosphate, pH 6.8, 1 m*M* EDTA.
[b] Trimethylsilyl (carbon content, 3.6%).
[c] Cyanopropyl (4.0%).
[d] Dimethyloctyl (7.0%).
[e] Phenyl (5.0%).

Hypersil-3. Only selected results of this systematic examination are shown in Fig. 6, illustrating the effects on DNA retardation (in terms of elution volume here) of DNA fragment size (2.3–38.4 kbp), flow-rate (0.2–1.2 ml/min), and temperature (20–60°C). The results showed

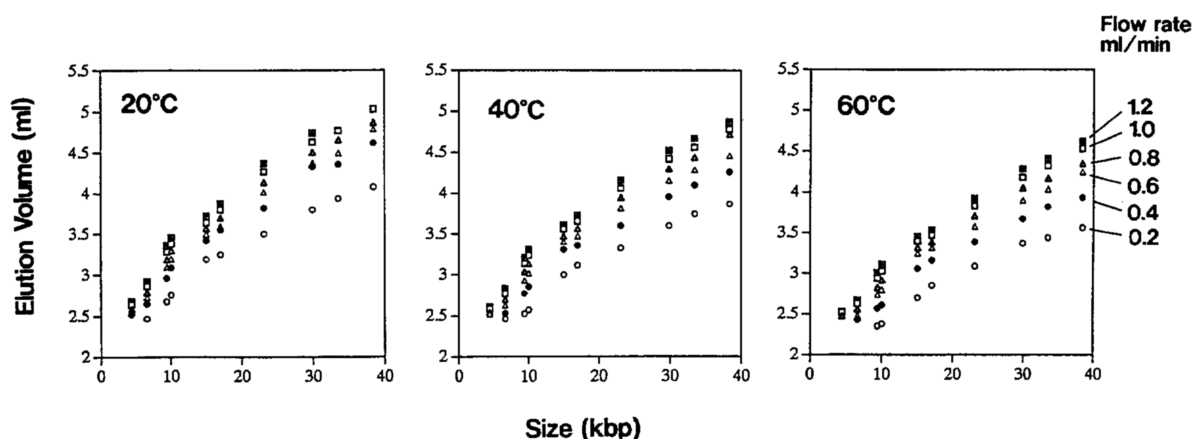


Fig. 6. Relationship between elution volume and DNA size (in kbp) on a Phe–Hypersil-3 column. Systematic analyses were made on the column using various-sized DNA fragments (2–38 kbp) at different temperatures (10, 20, 30, 40, 50, and 60°C) and flow-rates (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 ml/min). For the sake of simplicity only selected data are included in the figure.

clearly that the separation is based on a slalom mode: i.e., DNA molecules were size-fractionated in the opposite order of elution from that expected for size-exclusion chromatography, in a flow-rate and temperature-dependent manner. Temperature affects various physicochemical factors, such as solvent viscosity, DNA flexibility, and packing ratio, and thus results in altered retardation of DNA molecules.

In a previous study, the presence of a characteristic high-resolution zone of each column has been demonstrated. Empirically, such zones have been shown to become narrower and shift to a lower-molecular region [5]. Consistent with this prediction, the high-resolution zone for the 3-$\mu$m packing, which shows the steepest slope in Fig. 6a, is relatively narrow and shifted left (i.e., 5–10 kbp), compared with those observed previously for larger packings, i.e., 9–17 kbp, 15–30, 23–40 kbp and 35–50 kbp, for 5-$\mu$m (Asahipak GS-310), 9-$\mu$m (Asahipak GS-310), 13-$\mu$m (Asahipak GS-310) and 19-$\mu$m (Asahipak GS-310), respectively [5]. It should be emphasized that the DNA lengths in micrometer corresponding to such high-resolution zones are roughly comparable to the radii of the packing particles used, assuming that the DNA molecules are extended to their maximum lengths (i.e., 0.34 nm/bp). This finding should provide a key to elucidating in detail the separation mechanism of slalom chromatography.

## 4. Discussion

In the present study, it was shown that slalom chromatography can be conducted with various microbeads originally developed for conventional HPLC, e.g., size-exclusion [4,5], cation-exchange [5], and reversed-phase chromatographies (present results), though chromatographic conditions have to be appropriately set up for individual applications. As demonstrated with the Capcell-Pak columns, modified slalom chromatography resulted in improved DNA separation, possibly through the involvement of two completely different separation modes, i.e., slalom mode and hydrophobic-interaction mode. Even so, DNA fragments were separated in the order expected for the slalom mode [5].

It is not clear why different results were obtained for two columns with the same derivatization, i.e., Capcell-Pak Phe and Phe–Hypersil-3. The significantly different carbon contents of Capcell-Pak Phe (8.1%) and Phe–Hypersil-3 (5.0%) may be the reason. Stronger DNA retardation with Capcell-Pak Phe than with C1 seems to be associated with the stronger hydrophobicity of the former packing than the latter. However, all of the Hypersil-3 packings, differing in carbon content (2.6–10%), required the addition of more than 5% acetonitrile to the eluting solvent for adequate DNA recovery. This may suggest that either special features of the silicon coating of Capcell-Pak packings or the small 3-$\mu$m packing size of Hypersil packings accounted for the different results, though further study will be needed to confirm this. The use of 3-$\mu$m Capcell-Pak type silicon-coated packings may resolve the question.

In conclusion, several columns developed for reversed-phase chromatography were found to be useful for slalom chromatography. The results are important from a practical viewpoint, because a number of packing materials have been industrially developed for reversed-phase chromatography, some of which show excellent physicochemical performance. The chemical nature of the packing materials was shown to have little effect on slalom chromatography, at least in its basic principle. Overall, the results support the concept that this chromatography is based on a hydrodynamic phenomenon. The result of a detailed study of the separation mechanism in slalom chromatography will be presented elsewhere.

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