

Gel Permeation Chromatography of Oligosaccharides Using TOYOPEARL HW40

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Separation of oligosaccharides has been studied with TOYOPEARL HW40 (Fractogel TSK HW40), which was developed as a separation material for aqueous gel permeation chromatography of water-soluble oligomers and substances of low molecular weight. Dependence of the elution volume on temperature and molecular structure of an oligosaccharide, as well as the optimum injection volume, was examined. Chromatographic separation efficiency of this new material was compared with those of Sephadex G series; TOYOPEARL HW40 was found to be more efficient for the separation of oligosaccharides.

1. INTRODUCTION

The separation of oligosaccharides by use of gel permeation chromatography¹⁻⁷⁾, liquid-liquid partition chromatography⁸⁾ and ion exchange chromatography⁹⁾ has been described. The partition chromatography by use of TSK-GEL LS-170 STARCH is effective for separation of oligosaccharides upto 9 monomer units¹⁰⁾, but, it requires the use of undesirable organic solvent. Anion exchange chromatography with a borate buffer eluent, possible to separate monosaccharides, is difficult to separate oligosaccharides consisting of homologs.

Gel permeation chromatography, which separates molecules according to their molecular size with a sole and simple eluent (usually distilled water), is excellent for the separation of oligosaccharides. Dextran gel (Sephadex) and Polyacrylamide gel (Bio Gel P) have been used as a packing material for the separation of water soluble polymers. Dextran gel is not likely to be a useful support for the separation of carbohydrates, because it splits off carbohydrates and it is attacked by bacteria. Polyacrylamide gel (Bio Gel P) has been a most extensively used packing material for the separation of oligosaccharides¹⁻⁷⁾, for example separating glucose oligomers upto 15 glucose units⁷⁾. However, it takes about 7 to 11 hours to finish one measurement.

In this study, Toyopearl HW40 was packed into a column using slurry packing technique and examined for optimum operating condition. Separation efficiencies of Toyopearl HW40 series and Sephadex series for oligosaccharides were compared. The effects of operational temperature upon resolution and other elution behavior were studied with oligosaccharides prepared by hydrolysis of dextran and β -cyclodextrin.

Table 1 Characteristics of separation materials used in this study

	Toyopearl HW40 series			Sephadex series			
	HW40S	HW40F	HW40C	G-10	G-15	G-25 superfine	G-25 fine
Particle diameter hydrated (μm)	20-40	30-60	50-100	60-170*	60-180*	20-70*	30-140*
Water Regain (Wr)	3~4	3~4	3~4	1.0	1.5	2.5	2.5
Exclusion limit							
Globular protein	10,000	10,000	10,000	700	1,500	1,000-5,000	1,000-5,000
Dextran	7,000	7,000	7,000	700	1,500	100-5,000	100-5,000

note) * Wet particle size was approximated by multiplication of dry particle size, which was taken from the manufacturer's technical information, with the factor $(1 + \text{Wr} \times d)^{1/3}$ where Wr is the water regain (ml/dry gram) of the gel and d is the density of the dry gel (about 1.64 g/ml for dextran gels).

2. EXPERIMENTAL

[1] Materials

Characteristics of separation materials used in this study are listed in **Table 1**. Toyopearl HW40 series gels are supplied by Toyo Soda Mfg. Co., Ltd. (Tokyo, Japan). They are the same materials as Fractogel TSK HW40 available from E. Merck (Darmstadt, West Germany). Sephadex series gels are obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Before they were packed in the column, Sephadex series gels had been hydrated for one day at room temperature, and Toyopearl HW40 series gels had been fractionated by settling in order to remove small particle.

Hydrolyzed dextran and hydrolyzed β -cyclodextrin were prepared by partial acid hydrolysis of dextran T-40 obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and β -cyclodextrin obtained from Wako Chemicals (Tokyo, Japan), respectively. Dextrose and polyethyleneglycol are obtained from Wako Chemicals (Tokyo, Japan).

[2] Column packing method

Fig. 1 shows the flow scheme for the packing procedure. Glass columns (2.2 cm ID \times 60 cm L and 2.2 cm ID \times 90 cm L) were obtained from Amicon Co. Massachusetts, USA. Toyopearl HW40S, HW40F, and HW40C were packed into columns using the slurry packing technique¹¹⁻¹⁵⁾ with a peristaltic pump Model SJ-1211H (Atto Co. Tokyo, Japan) at a constant flow rate.

Sephadex G-25 superfine, G-25 fine, G-15 and G-10 were packed into column as follows. Two columns were connected as **Fig. 1**. They were filled with gel slurry, and

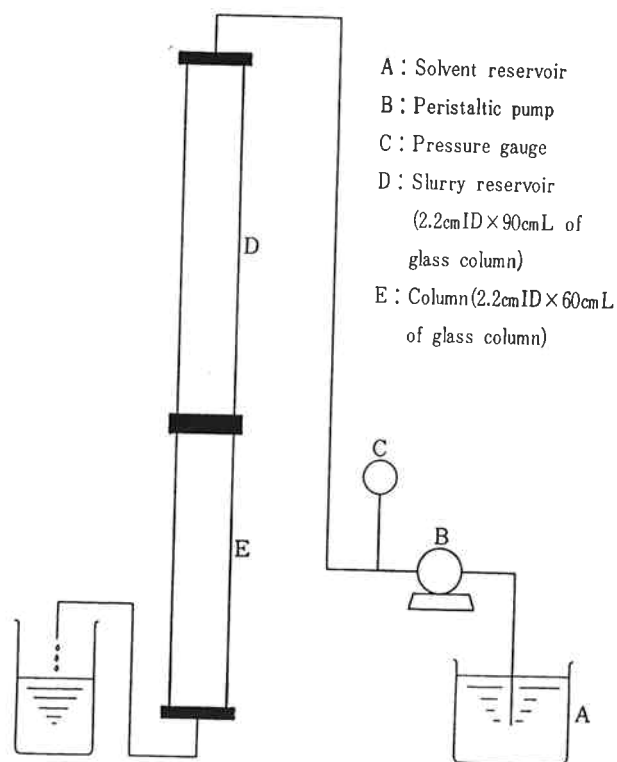


Fig. 1 Flow scheme for column packing with peristaltic pump.

300 ml of the eluent was made to flow through the columns under hydrostatic pressure. After that, the columns were filled with the packing solvent, and 200 ml of the eluent was made to flow with a peristaltic pump at a constant flow rate.

Fig. 2 shows packing flow rate versus number of theoretical plate of ethyleneglycol and pressure drop of HW40 series. There is optimum packing flow rate range for each grade, that is, 1.5~2.5 ml/min. for HW40S, 3.0~4.0 ml/min. for HW40F and 6.0~8.0 ml/min. for HW40C respectively. Optimum packing flow rate decreases, when the gel particle size becomes small. From this experimental result, optimum numerical operating parameters of the column packing techniques are shown in Table 2.

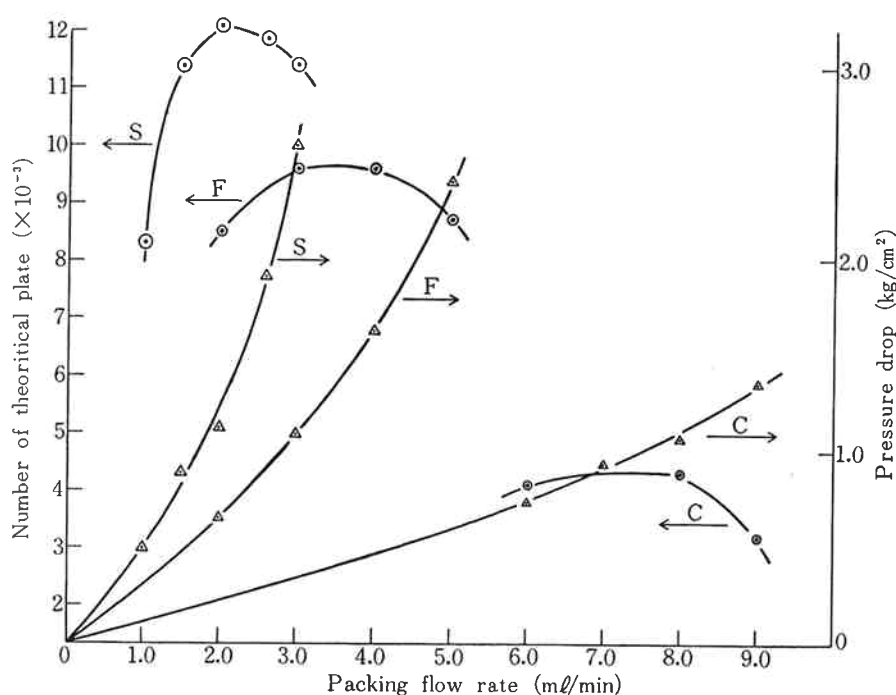


Fig. 2 Packing flow rate versus number of theoretical plate and pressure drop of Toyopearl HW40 series.

Table 2 Operating parameter of column packing techniques

	Toyopearl			Sephadex			
	Hw40S	HW40F	G-10	G-15	G-25 fine	G-25 superfine	
Concentration of slurry (%(V/V))	50	50	50	50	50	50	
Solvent velocity (with a perislaltic pump) (ml/min.)	2.5	4.0	16.8	6.0	5.2	1.2	
Total elution volume (ml)	500	500	500	500	500	500	
Final pressure (kg/cm ²)	1.9	1.5	1.1	0.4	0.4	0.1	
Theoretical plate (TP/one column)	10,000,11,000	9,000	2,400	3,300	3,700	5,900	

[3] Apparatus

Fig. 3 shows the flow scheme for the separation and analysis system. Deionized water was used as the eluent. A peristaltic pump Model SJ-1211H was used to pump water through the column at a fixed rate. The samples of the oligosaccharide and polyethyleneglycol aqueous solution were injected through a Rheodyne Model 7010 sample injector (Reodyne, Inc. California, USA). The detection was carried out with a differential refractometer RI-8 (Toyo Soda Mfg. Co., Ltd.)

3. RESULTS AND DISCUSSION

[1] Number of theoretical plates of columns

As shown in Table 3, the number of theoretical plates of Sephadex G series becomes lower in order of Sephadex G-25 superfine, G-25 fine, G-15 and G-10. The same phenomenon is observed for Toyopearl HW40 series. This is compatible with general consequence that column efficiency becomes lower in order of increase in gel particle size.

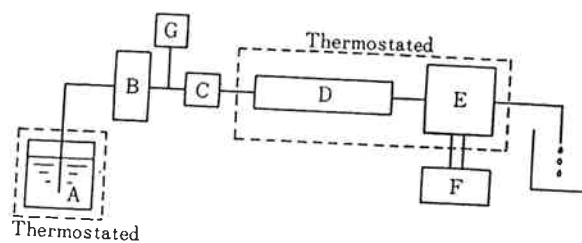
Table 3 Operating parameter of column evaluation and oligosaccharides separation

	Column evaluation	Oligosaccharides separation
Sample	1% (W/V) water solution of Ethyleneglycol	6% (W/V) water solution of hydrolyzed dextran and 6% (W/V) water solution of hydrolyzed β -cyclodextrin
Injection volume	1,000 $\mu\ell$	1,000 $\mu\ell$
Mobile phase	deionized water	deionized water
Flow rate	1.0 ml/min.	0.2 ml/min., 0.5 ml/min. and 1.0 ml/min.
Detector	RI	RI

Toyopearl HW40S indicates higher value of theoretical plate than Sephadex G25 superfine, a corresponding grade of Toyopearl HW40S. This result is compatible with the fact that Toyopearl HW40S (20-40 μ ; swollen in water) has smaller particle size and sharper particle size distribution than Sephadex G-25 superfine (20~70 μ ; swollen in water).

[2] Optimum operating condition under Toyopearl HW40S packed column

In general, resolution of chromatogram becomes better in order of decrease in sample injection volume and/or sample concentration. Fig. 4 shows relationship between number of theoretical plate of dextrose and sample volume, when 15 mg, 30 mg and 60 mg of dextrose were injected to 22 mm ID \times 60 cm L column packed with Toyopearl HW40S, at the flow rate of 1.0 ml/min. Number of theoretical plate is kept high value in the range of 200 $\mu\ell$ to 1,000 $\mu\ell$, but it becomes lower below 200 $\mu\ell$ or above 1,000 $\mu\ell$. It is reasonable to consider that, if the sample is injected above 1,000 $\mu\ell$, large injection volume causes peak broadening, while, if the



- A; Solvent reservoir (thermostated to the same temperature as that of column system.)
- B; Pump
- C; Sample injector
- D; Column system (thermostated to operating temperature required.)
- E; Detector
- F; Strip chart recorder
- G; Pressure-gauge

Fig. 3 Flow scheme for separation and analysis system.

sample is injected below $100 \mu\ell$, "viscous fingering" causes peak broadening.

From these result, measuring condition of oligosaccharides was determined as listed in Table 3.

[3] Calibration curve of Toyopearl HW 40S

Fig. 5 shows relationship between molecular weight and elution volume for oligosaccharides on semi-logarithmic graph paper. Hydrolyzed dextran T-40 homologs elutes faster than hydrolyzed β -cyclodextrin at the same degree of polymerization above dimers. As shown in Fig. 6, hydrolyzed dextran T-40 is composed of D-glucose units linked together by α -1, 6'-glucosidic bond to linear chains, while hydrolyzed β -cyclodextrin by β -1, 4'-glucosidic bond to linear chains. From these results, we conclude that molecular dimension in water solution and interaction with the gel depend on the type of oligomer (e.g. the

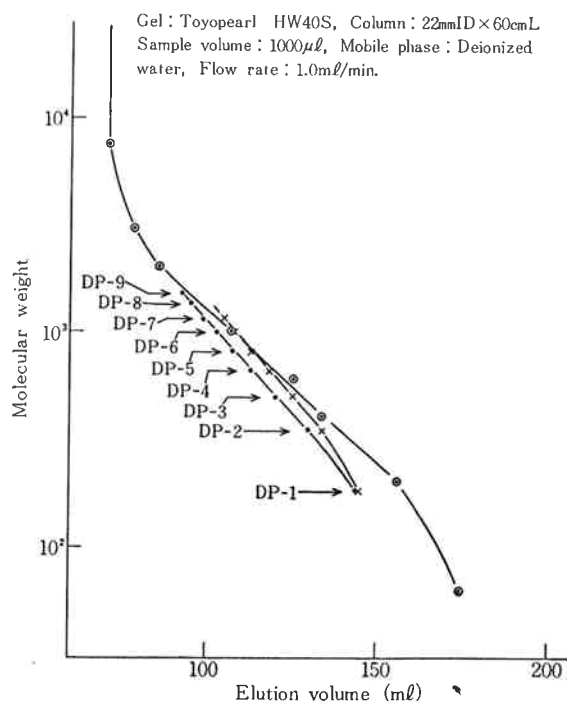


Fig. 5 Semi-log plot of molecular weight versus elution volume of series of hydrolyzed dextran T-40 (— · —), hydrolyzed β -cyclodextrin (— × —) and polyethylene glycol (— ● —).

existence of a primary hydroxyl group, the flexibility of the chain, etc.) It supports thermodynamic approach in the work of A. Heyraud and M. Rinaudo²⁾.

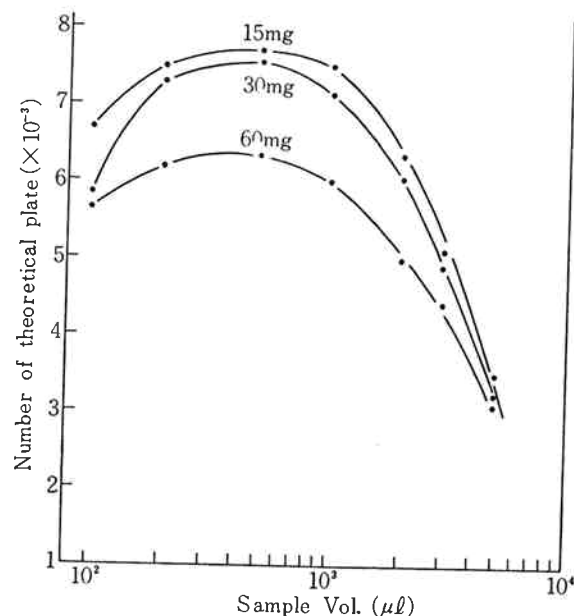


Fig. 4 Relationship between number of theoretical plate and sample volume
Gel: Toyopearl HW40S, Column: 22 mmID \times 60 cmL Flow rate: 1.0 ml/min. Mobile phase: Deionized water

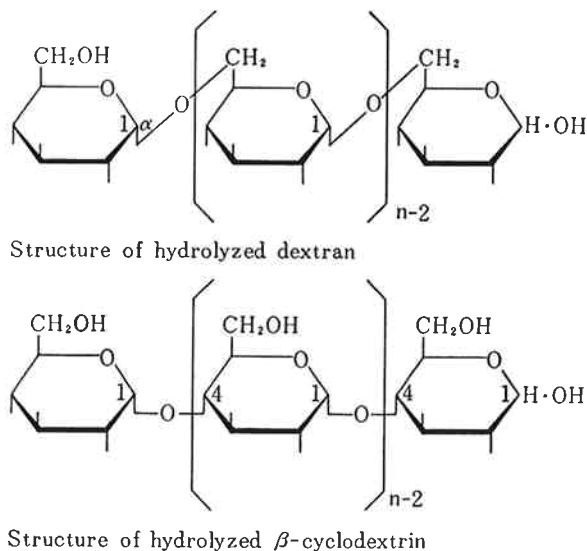


Fig. 6 Structure of oligosaccharides

On the other hand, it is generally supposed that polyethyleneglycol, which does not consist of ring like monomer, may occupy larger dimension in water solution than oligosaccharides in the same molecular weight. But in our experiment, elution volume of polyethyleneglycol is smaller than that of oligosaccharides so that it seemingly occupies smaller dimension than oligosaccharide does. There may be another type of interaction with polyethyleneglycol and gels, different from oligosaccharides.

[4] Comparison between HW40 series and Sephadex G-series based on oligosaccharides measurement

Fig. 7 shows the elution curves of the hydrolyzed dextran measured with Sephadex G-25 superfine and Toyopearl HW40S at the same flow rate of 1.0 ml/min. The oligomers up to 9 glucose units were resolved with Toyopearl HW40S, while 8 units with Sephadex G-25 superfine. Fig. 8 shows the elution curve of the hydrolyzed dextran measured with Sephadex G-25 superfine at flow rates of 0.2 ml/min., 0.5 ml/min. and 1.0 ml/min. The resolution of these chromatograms becomes better in order of decrease in flow rate. Similar separation efficiency is observed between Toyopearl HW40S at a flow rate of 1.0 ml/min. (Fig. 7 (A)) and Sephadex G-25 superfine at that of 0.5 ml/min. (Fig. 8 (B)). From this result, Toyopearl HW40S can reduce the separation time from 6.5 hours to less than 2.5 hours compared with Sephadex G-25 superfine, to obtain nearly equal resolution of the hydrolyzed dextran.

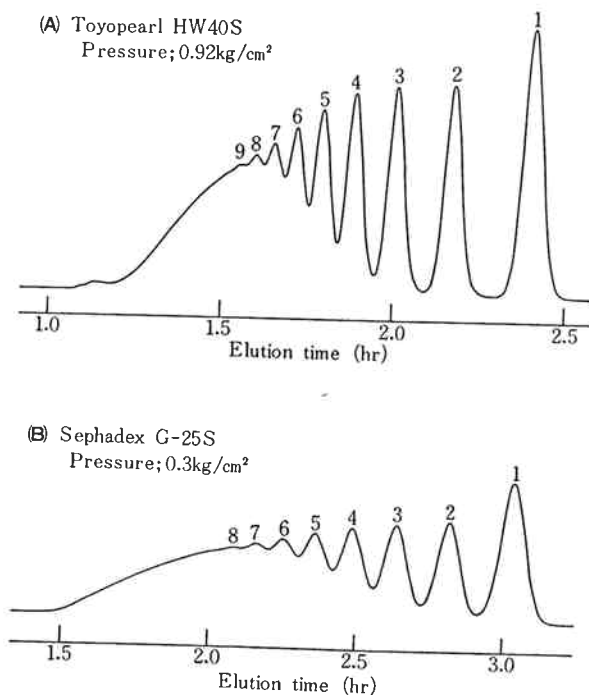


Fig. 7 Elution curve of hydrolyzed dextran measured with Sephadex G-25 superfine and Toyopearl HW-40S at a flow rate of 1.0 ml/min. Conditions; Column; Amicon GF22/60
Sample; Hydrolyzed dextrans
Sample concentration; 6%
Sample volume; 1000 μ l
Mobile phase; Deionized water
Flow rate; 1.0 ml/min.

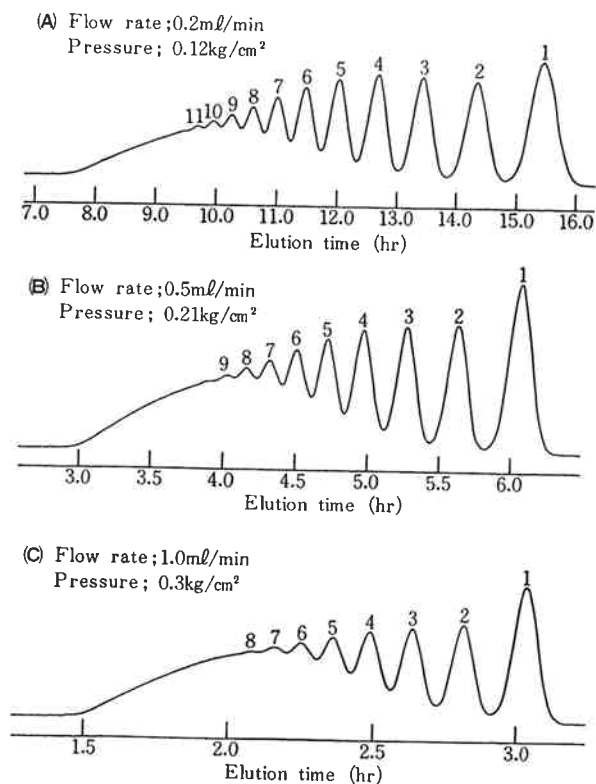


Fig. 8 Elution curve of hydrolyzed dextran measured with Sephadex G-25 superfine at flow rates of 0.2 ml/min., 0.5 ml/min., and 1.0 ml/min. Conditions; Same as Fig. 7 except flow rate and pressure.

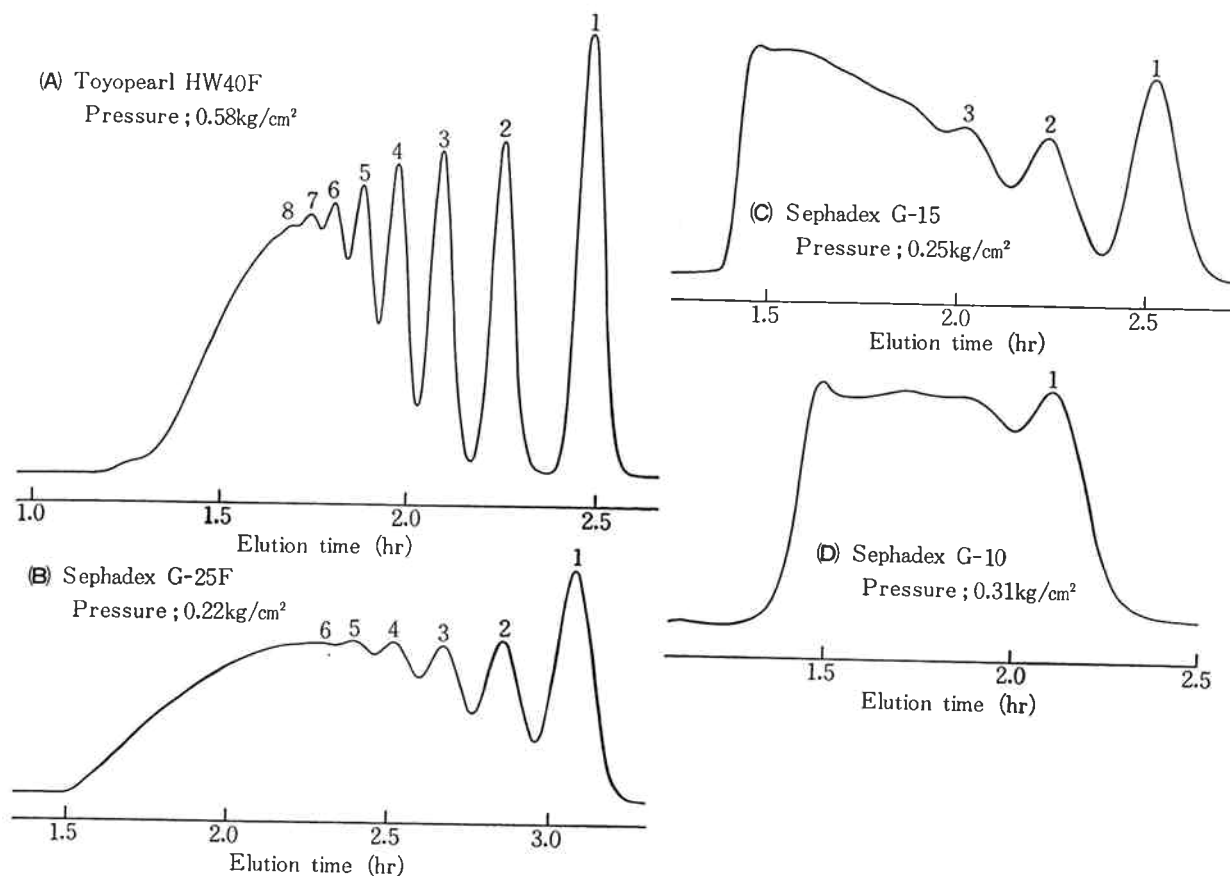
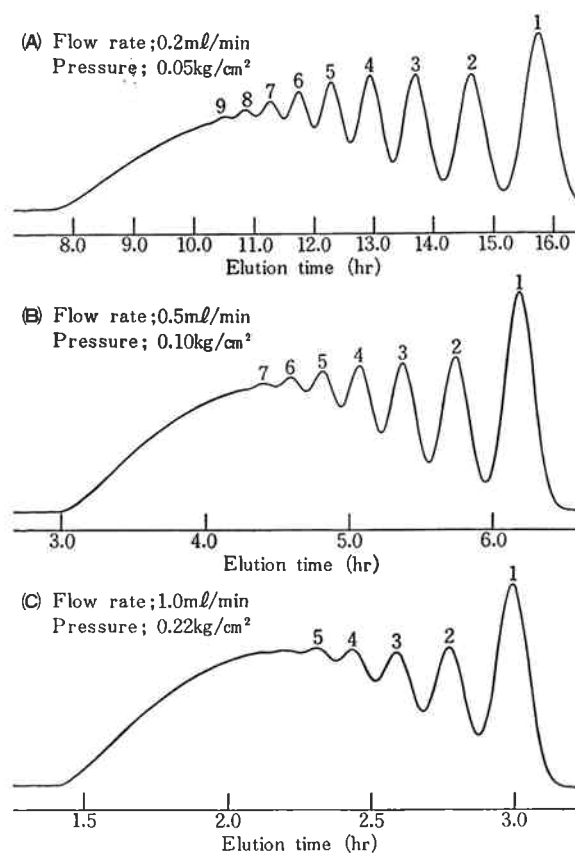


Fig. 9 Elution curve of hydrolyzed dextran measured with Sephadex G-10, Sephadex G-15, Sephadex G-25 fine and Toyopearl HW40F at a flow rate of 1.0 ml/min. Conditions; Same as Fig. 7 except pressure.

Fig. 9 shows the elution curves of the hydrolyzed dextran measured with Sephadex G-10, G-15, and G-25 fine and Toyopearl HW40F at a flow rate of 1.0 ml/min. The oligomers up to 8 glucose units were resolved with Toyopearl HW40F, while 6 units with Sephadex G-25 fine, 3 units with Sephadex G-15 and 1 unit with Sephadex G-10 respectively. In the similar manner as superfine grade, it can be indicated from **Fig. 10 (B)** and **Fig. 9 (A)** that Toyopearl HW40F can reduce the separation time from 6.5 hours to 2.6 hours compared with Sephadex G-25 fine,

Fig. 10 Elution curve of hydrolyzed dextran measured with Sephadex G-25 fine at flow rates of 0.2 ml/min., 0.5 ml/min., and 1.0 ml/min. Conditions; Same as Fig. 7 except flow rate and pressure.



to obtain nearly equal resolution of the hydrolyzed dextran.

[5] Oligosaccharides measurement with Toyopearl HW40S at several temperature

Fig. 11 shows that pressure drop of two 22 cm ID×60 cm L columns in series, packed with Toyopearl HW40S, becomes lower at higher operating temperature. As the operating temperature is elevated from 23°C to 60°C, the pressure drop of this column decreases from 2.75 kg/cm² to 1.12 kg/cm². Generally, separation of oligomers would be much improved if oligomers were chromatographed at an elevated temperature, because solute molecules within gel phase diffuse faster.

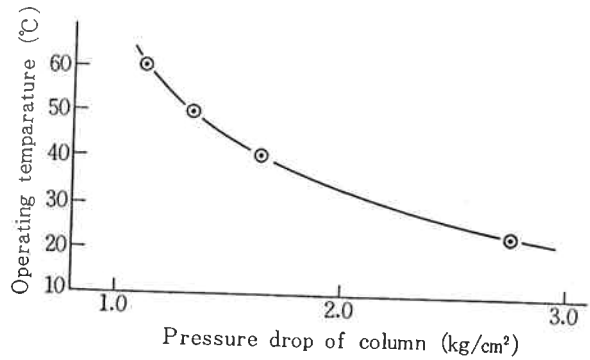


Fig. 11 Relationship between pressure drop of column, packed with Toyopearl HW40S and operating temperature

Fig. 12 and Fig. 13 show chromatograms of oligosaccharides measured at several temperatures. In Fig. 12 oligomers up to 13 glucose units were resolved at 60°C, while only 12 units at 23°C. This demonstrates the effectiveness of increasing measuring temperature to improve the resolution. Oligomers up to 13 glucose units were separated in 11 hr with two 1 m columns in series which were packed with Bio Gel P-2-400 mesh⁷⁾. In this study, nearly equal resolution can be obtained in only 5 hr. Then, improvement of separation of oligosaccharides can be obtained

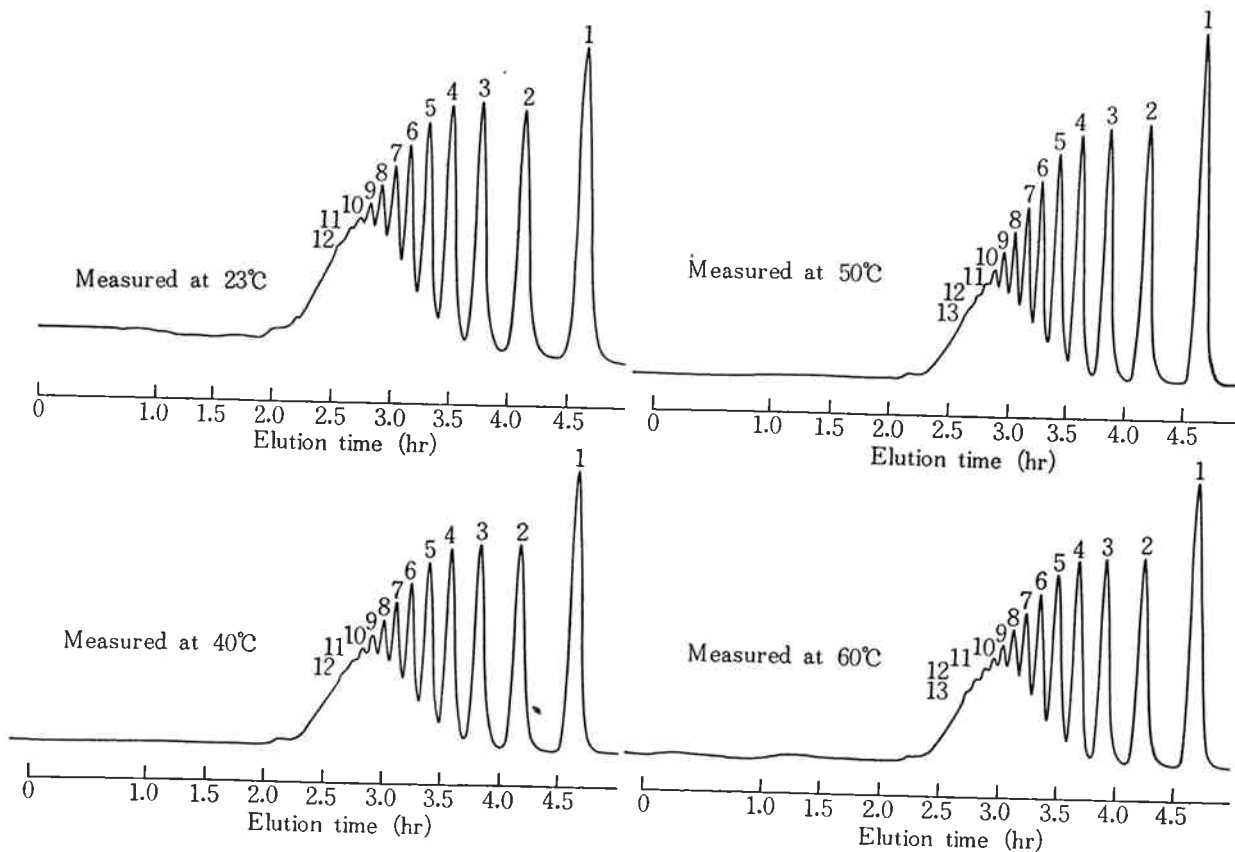


Fig. 12 Separation of hydrolyzed dextran T-40
Gel : Toyopearl HW40S, Column, two 22 mmID×60 cmL Columns in series
Flow Rate : 1.0 ml/min. (15.8 cm/hr), Sample Vol. : 500 μl 5.0 wt %

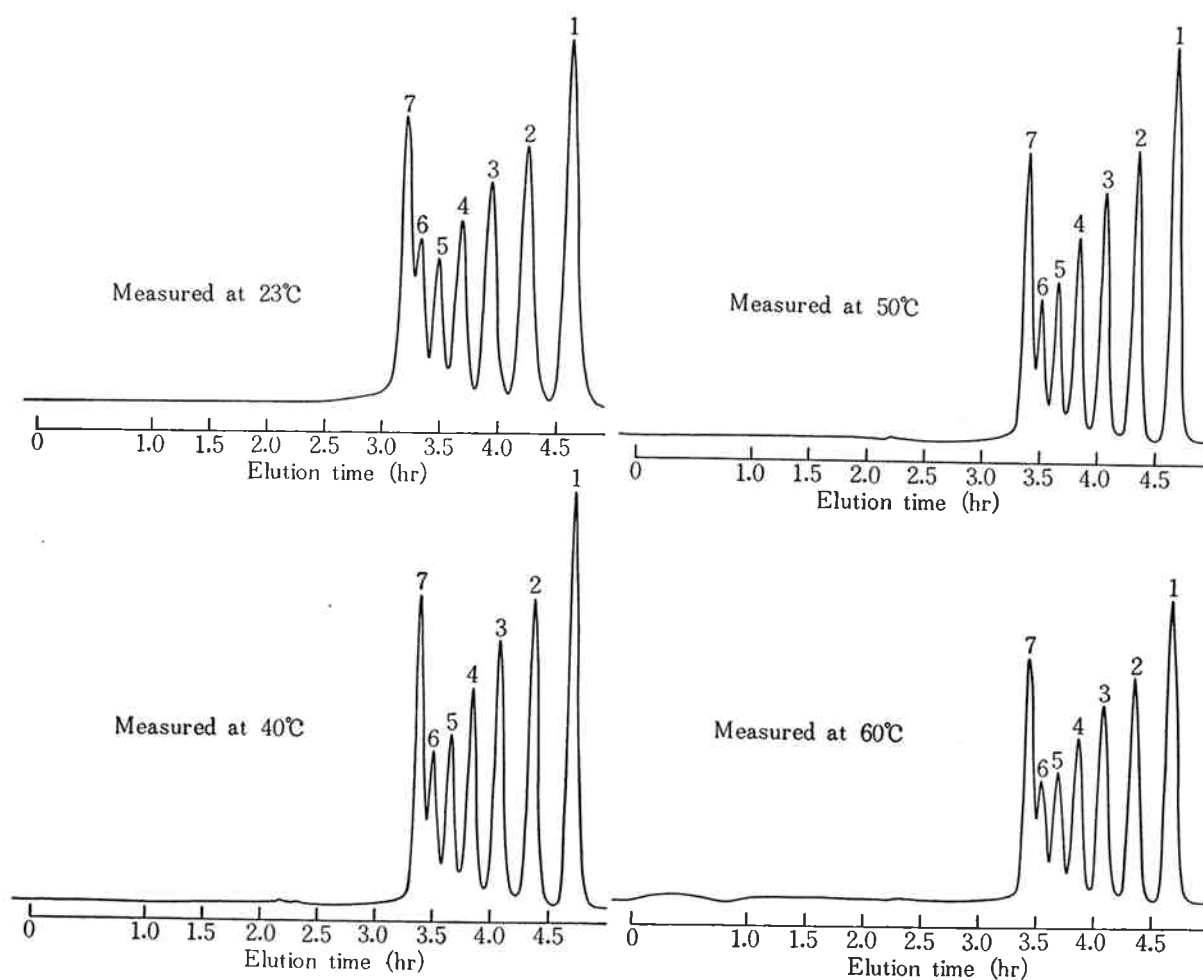


Fig. 13 Separation of hydrolyzed β -cyclodextrin (Measured at the same condition as Fig. 12)

by use of Toyopearl HW40S instead of conventional separation materials.

Fig. 14 shows linear relationship between elution volume and measuring temperature in the case of hydrolyzed dextrans. Elution volume becomes larger at elevated temperature. Feature of this relationship is prominent at higher molecular weight fractions. Our observation is in good accordance with the temperature dependence of the elution behavior of maldextrins^{2,7)} and cellodextrins^{2,6)} on polyacrylamide gel.

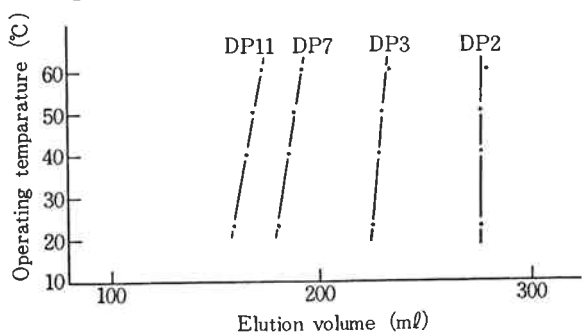


Fig. 14 Relationship between elution volume and operating temperature in measurement of hydrolyzed dextrans

4. CONCLUSION

The results indicate that excellent separation of oligosaccharides can be obtained using Toyopearl HW40.

We conclude the following.

- [1] High resolution column (10,000 TP/F) can be obtained.

- [2] Injection volume is optimum at the range from 200 μl to 1,000 μl when 15 mg~60 mg of saccharide is separated with 22 mm ID of column at the flow rate of 1.0 ml/min. (15.8 cm/hr)
- [3] Oligosaccharides differ from one another in the relationship between molecular weight and elution volume; (1-6') oligomer (hydrolyzed dextran T-40) elutes faster than (1-4') oligomer (hydrolyzed β -cyclodextrin) at the same molecular weight.
- [4] The separation time can be reduced to less than two fifth and one fifth compared with Sephadex G-25 superfine and Sephadex G-25 fine respectively, to obtain nearly equal resolution of the oligosaccharide.
- [5] Elution volume of oligosaccharide becomes larger and resolution of that become better, at elevated temperature.

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