A Convenient Preparation Method of Cello-oligosaccharides by High Performance Liquid Chromatography

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高速液体クロマトグラフィーによるセロオリゴ糖の簡便な調製法

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Abstract

A convenient isolation method of cello-oligosaccharides was presented. The cello-oligosaccharide mixture was prepared by partial hydrolysis of Whatman CF-11 cellulose powder with 72% sulfuric acid for 1 hr at 5℃ followed by extraction with 1 : 1 (v/v) mixture of acetonitrile and water. It was found that the high performance liquid chromatography on a new silica gel column, chemically modified with polyamine resin, was very effective not only for analytical point of view but also for isolation of cello-oligosaccharides up to degree of polymerization of 8.

要　　旨

セロオリゴ糖の簡便な調製法を開発した。セルロースパウダー（Whatman CF-11）を72%硫酸で5℃，1時間部分加水分解し，生じたセロオリゴ糖をアセトニトリル-水1：1(v/v)の混液で回収した。シリカの表面にポリアミン樹脂を化学結合させた新しいアミン結合型担体を用いた高速液体クロマトグラフィーを行った結果，8量体までのセロオリゴ糖の分離・分析のみならず，調製が円滑に行えることがわかった。

1. Introduction

Cello-oligosaccharides have been proved to be the useful model compounds for structural investigations of cellulose[1,2]. In our course of the systematic survey work on the conformation-function relationships of cellulose derivatives, there is a strong and growing

demand for preparation of cello-oligosaccharides.

Several methods have been described for the preparation of free cello-oligosaccharides : acetolysis followed by deacetylation[3], hydrolysis with fuming hydrochloric acid[4], 85% phosphoric acid[5], trifluoroacetic acid[6], 70% sulfuric acid[7], conc. sulfuric acid - conc. hydrochloric acid[8], or enzyme[9]. Among these methods, the partial hydrolysis with conc. sulfuric acid seems to be most adequate as a convenient preparation method of cello-oligosaccharides because cellulose is soluble in conc. sulfuric acid, and 72% sulfuric acid is the well known reagent for the first stage hydrolysis of cellulose[10].

Previously, separation of the cello-oligosaccharides has been accomplished by chromatography on columns of charcoal-celite[4], polyacrylamide gels[8,11], ion-exchange resin[12], and silica gels[12,13]. These methods are satisfactory for separation of cello-oligosaccharides but has an inevitable drawback of time-consuming tedious procedures. The recent high performance liquid chromatography (HPLC) technique was applied for rapid separation of cello-oligosaccharides by using amino-bonded columns[6], but the use was limited to analytical use because amine was gradually leaked from the column, and thus deteriorated the column and eventually shortened life-time of the column[14,15]. The recent progress in preparation of immobile phase of the HPLC column enabled as to produce a new stable column for separation of oligosaccharides in which the chemical bonding was formed between polyamine resin and silica gels. The columns of this type are now commercially available as YMC PA series columns.

In this paper, we present a convenient preparation method of cello-oligosaccharides by partially hydrolyzing Whatmann CF-11 with 72% sulfuric acid followed by HPLC on a newly packed YMC PA-43 column.

2. Experimental

2.1 General

Melting points were measured with a Yanaco Micro Melting Point Apparatus (Yanagimoto, Kyoto) and reported as measured. Optical rotations were determined with a JASCO DIP-181 digital polarimeter at 25℃.

2.2 Partial hydrolysis of cellulose

Five grams of dried cellulose powder (Whatman CF-11) was solubilized in 40 ml of 72% sulfuric acid solution by stirring with glass rod at 5℃ and kept at this temperature for various period of time. The partial hydrolysate was diluted to 500 ml with ice-cold water, neutralized with barium hydroxide-barium carbonate, filtered and evaporated to dryness below 40℃. Deionization with Dowex 50X8 (H^+ form) and Dowex 1X8 (acetate form) columns connected in tandem was sometimes neccessary to remove trace amount of barium sulfate. The white powder obtained was extracted with a 1 : 1 (v/v) mixture of acetonitrile and water to remove high-molecular weight materials and to prevent the formation of precipitate during subsequent HPLC analysis. The cello-oligosaccharide mixture soluble in the aqueous acetonitrile solution was recovered by centrifugation and evaporation.

2.3 High performance liquid chromatography

HPLC was carried out on a Waters Assoc. (Milford, Madison, U.S.A.) instrument with a model M-600 multisolvent delivery system, Model U6K sample injector, and Model R401 differential refractometer. Chromatograms were recorded and integrated with a Waters M740 Data Module. The columns used were (a) YMC PA-03 (4.6×250 mm, 5 μm) and (b) YMC PA-43 (20×250 mm, 5 μm), both being packed by Yamamura Chem. Lab., Kyoto. Both columns were used under ambient temperature. The column (a) was used only for analytical HPLC using various mix ratios of acetonitrile and water, while the column (b) was used for semi-preparative HPLC using a 1 : 1 (v/v) mixture of acetonitrile and water. Samples of less than 20 μl were injected into the column (a) and eluted at a flow rate of 0.1-2.0 ml/min, while a set of 2 ml and 6.0 ml/min was used in the case of the column (b). The pressure of the column (b) was in the range from 3.6 MPa to 4.1 MPa. The individual cello-oligosaccharides eluted from the column (b) were pooled, evaporated to dryness. Purity of the isolated cello-oligosaccharides was checked by analytical HPLC.

2.4 Determination of degree of polymerization

Degree of polymerization (D.P.) of the isolated oligosaccharides was determined from the plots of log(retention time, t_R) against D.P., and from ^{1}H-nuclear magnetic reasonance (NMR) spectroscopic analysis. The NMR spectra were obtained on a Varian XL-200 NMR spectrometer (200 MHz) in deuterium oxide at 80℃. Chemical shifts in p.p.m. are given as the values relative to that of internal sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate.

3. Results and Discussion

3.1 Preparation of cello-oligosaccharide mixture

Time course of the conversion of cellulose to water-soluble cello-oligosaccharides was analyzed by partial hydrolysis of cellulose with 72% sulfuric acid. Fig. 1 shows the data obtained by using liquid to solid cellulose ratio of 8 : 1 (v/w). Cellulose was completely solubilized within 3 hr at 5℃. The amount of the water-soluble cello-oligosaccharides increased with increasing incubation time to attain 85% after 48 hr incubation. The amount of cello-oligosaccharides soluble in 1 : 1 (v/v) acetonitrile and water was about 50% of that soluble in water within 3 hr incubation, but became higher with increasing incubation time to attain 77% after 4 hr incubation.

3.2 Influence of the acetonitrile-water composition on separaton of cello-oligosaccharides

Prior to separation of the individual cello-oligosaccharides, the applicability of a newly packed chemically bonded amino column (a) was analyzed by using a cello-oligosaccharide mixture obtained by partial hydrolysis for 1 hr. Fig. 2 shows the change in the capacity factor (k′) with variation in the mixing ratio of acetonitrile and water ranging from 40 : 60 to 75 : 25 (v/v). The capacity ratio became higher for oligosaccharides having higher D.P., *i.e.*, higher hydroxyl groups, confirming the previous results that the total number of hydroxyl group and their distribution on the molecule is

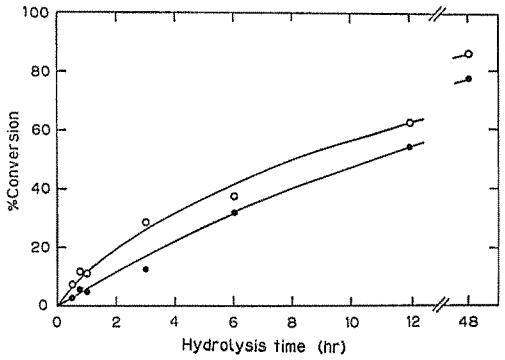

Fig. 1 Conversion rate of Whatman CF-11 cellulose powder into cello-oligosaccharides. The amount of oligosaccharides soluble in water (—○—) and 1 : 1 (v/v) mixture of acetonitrile and water (—●—).

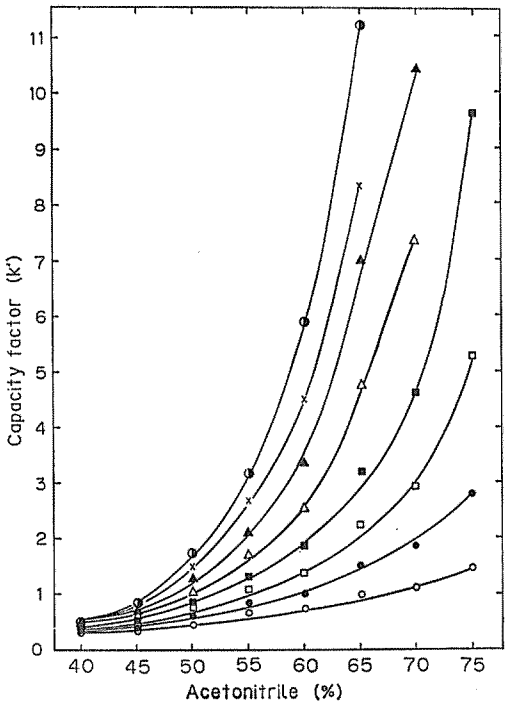

Fig. 2 Capacity factor (k') as a function of the acetonitrile composition : glucose (○), cellobiose (●), cellotriose (□), cellotetraose (■), cellopentaose (△), cellohexaose (▲), cellohexaose (×), and cellooctaose (◑).

important for separation[16]. With this system baseline-separation of cello-oligosaccharides up to D. P. 8 could be achieved at acetonitrile compositions higher than 50%. Acetonitrile composition higher than 65% produced the high capacity factors but needed longer time for operation ; elution time of cellohexaose was 26 min at flow rate of 1 ml/min. Use of high proportion of acetonitrile had also a drawback of the low solubility of cello-oligosaccharides. Another factor important for separation of cello-oligosaccharides is the pressure applied to the column. Fig. 3 shows the dependence of the pressure on the flow rate. Addition of water increased the pressure. The pressure lower than 14 MPa was found to be critical for insuring a good separation. Based on these results, an eluting solvent of 1 : 1 (v/v) acetonitrile-water and a flow rate of 1 ml/min were chosen for determination of the distribution profile of cello-oligosaccharides. Under these conditions, separation of cellooctaose through glucose were achieved within an analysis time of 8 min.

3.3 Optimization of hydrolysis time for preparation of cello-oligosaccharides

Fig. 4 shows the change in oligosaccharide composition with variation in the duration of hydrolysis time. A homologous series of cello-oligosaccharides up to D. P. 8 could be well separated within a hydrolysis time of 1.5 hr. A hydrolysis time less than 45 min resulted in high proportion of water-soluble cello-oligosaccharides ; however, their yield was unfortunately low (<10%). A hydrolysis time more than 2 hr produced predominant amount of monomeric glucose. Therefore, a hydrolysis time of 1 hr was chosen for determination of the distribution profile of cello-oligosaccharides.

3.4 Separation of cello-oligosaccharides and their properties

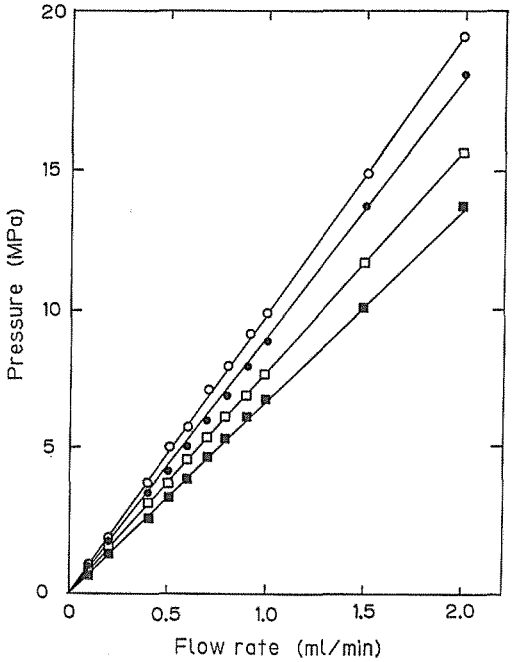

Fig. 3 Relationships between flow rate and pressure of the analytical column (YMC PA-03) at acetonitrile : water mixing ratios of 45 : 55 (○), 55 : 45 (●), 65 : 35 (□), and 75 : 25 (■).

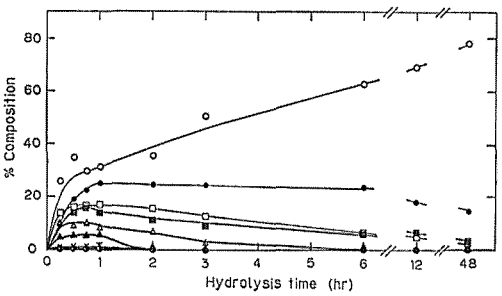

Fig. 4 Relationships between oligosaccharide distribution profile and duration of hydrolysis time : glucose (○), cellobiose (●), cellotriose (□), cellotetraose (■), cellopentaose (△), cellohexaose (▲), celloheptaose (×), and cellooctaose (◑).

About 300 mg of the cello-oligosaccharide mixture obtained by partial hydrolysis for 1 hr was solubilized in 2 ml of 1 : 1 (v/v) acetonitrile-water and applied on the semi-preparative scale column. As shown in Fig. 5, the cello-oligosaccharides having D. P. of 2-8 were well separated. About 70 mg of cellobiose, 30 mg of cellotetraose, 15 mg of cellopentaose, 7 mg of cellohexaose, 1 mg of celloheptaose and 0.5 mg of cellooctaose could be isolated by a single run (50 min). Each cello-oligosaccharide was purified by rechromatography.

The D. P. values of the isolated cello-oligosaccharides were determined by the following two methods. First, log t_R was plotted against D. P. which showed a good linear relationships as shown in Fig. 6. The retention times of cello-oligosaccharides became longer with an increasing ratio of acetonitrile to water. Secondly, the values of D. P. was determined by ^{1}H-NMR measurement. The ^{1}H-NMR spectra of the anomeric regions of the isolated cello-oligosaccharides were shown in Fig. 7. The chemical shifts of the anomeric protons are easily assigned[17] as listed in Table 1. The anomeric protons of the reducing end glucose appeared as doublets at 5.22 p. p. m. (H-1$_\alpha$) and 4.65 p. p. m. (H-1$_\beta$), while that of the non-reducing end residue at 4.50-4.51 p. p. m. (H-1$_n$). The signals at 4.52-4.53 p. p. m. (H-1$_i$, doublet) due to the inter-sugar anomeric-proton reasonances first appeared in cellotriose and became larger with increasing D. P. Based on these results, the D. P. values could be determined by integrating these differnt sets of signals.

Table 1 also summarized the properties of the isolated cello-oligosaccharides from cellobiose through cellohexaose. The solubility of the cello-oligosaccharides in 1 : 1 (v/v) mixture of acetonitrile and water was about 1/7-1/20 of those in water. The values of

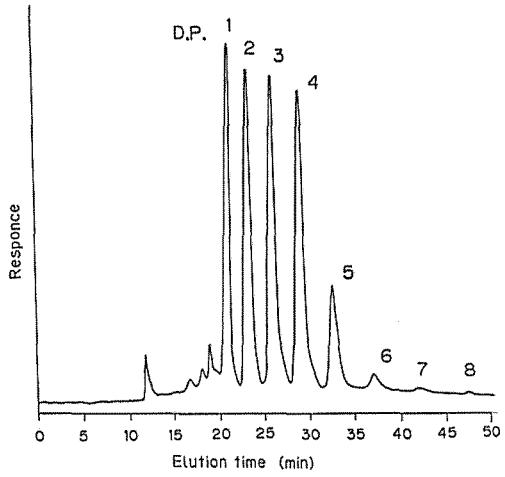

Fig. 5 Separation of cello-oligosaccharides on a semipreparative column (YMC PA-43) with 1 : 1 (v/v) acetonitrile and water as eluent. 1 to 8 represent the degree of polymerization.

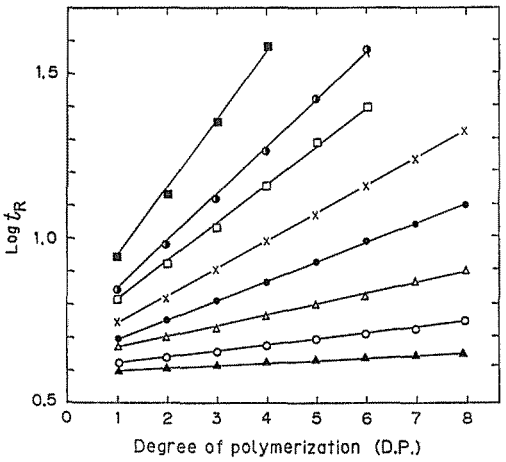

Fig. 6 Relationships between log (retention time, t_R) and degree of polymerization at acetonitrile : water mixing ratios of 40 : 60 (▲), 45 : 55 (○), 50 : 50 (△), 55 : 45 (●), 60 : 40 (×), 65 : 35 (□), 70 : 30 (◑), and 75 : 25 (■).

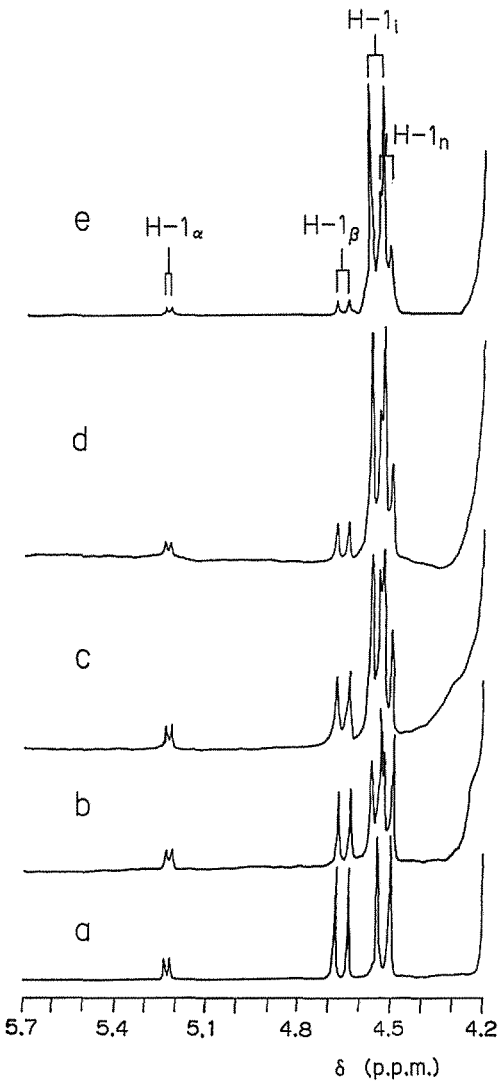

Fig. 7 ^{1}H-NMR spectra of cello-oligosaccharides at anomeric region in deuterium oxide : cellobiose (a), cellotriose (b), cellotetraose (c), cellopentaose (d), and cellohexaose (e) ; (H-1$_\alpha$, H-1$_\beta$), H-1$_n$, and H-1$_i$ represent anomeric protons of the reducing end, non-reducing end, and intermediate glucose residues, respectively.

Table. 1. Characteristics of cello-oligosaccharides

Oligomers	Melting points (℃)		$[\alpha]_D$ (H_2O)		Solubility (g/l) in 1 : 1 (v/v) aceto-nitrile-water at 30℃	^{1}H-NMR data (p.p.m.)			
	Obs.	Lit.[3,18]	Obs.	Lit.[3,18]		H-1$_\alpha$	H-1$_\beta$	H-1$_i$	H-1$_n$
cellobiose	242-244 (decomp.)	225-230 (decomp.)	+37.0°	+34.6° ~36.0°	32.9	5.22 (3.77)[a] [0.4][b]	4.65 (7.92) [0.6]		4.51 (7.79) [1.1]
cellotriose	201-205 (decomp.)	201-209 (decomp.)	+21.4°	+21.6° ~23.6°	23.6	5.22 (3.58) [0.4]	4.65 (7.87) [0.6]	4.52 (7.76) [1.1]	4.50 (7.76) [1.0]
cellotetraose	251-254 (decomp.)	252-255 (decomp.)	+16.9°	+16.5° ~17.3°	12.7	5.22 (3.88) [0.4]	4.65 (7.90) [0.6]	4.52 (7.84) [2.0]	4.51 (7.78) [1.0]
cellopentaose	266-270 (decomp.)	264-268 (decomp.)	+13.0°	+11° ~13.8°	2.2	5.22 (3.88) [0.4]	4.65 (7.90) [0.6]	4.53 (7.84) [3.4]	4.51 (7.78) [1.1]
cellohexaose	——[c]	275-278 (decomp.)	+10.4°	+10°	1.3	5.22 (3.80) [0.4]	4.65 (7.89) [0.6]	4.52 (7.90) [4.2]	4.51 (7.80) [0.9]

a) Coupling constant ($J_{1,2}$, Hz) ; b) Molar ratio ; c) Difficult to measure.

melting point and specific rotation of the isolated oligosaccharides were in good accordance with those of reported in the literatures[3,18] except in the decomposition temperature of cellobiose.

In conclusion, the present results indicate that the partial hydrolysis with 72% sulfuric acid followed by HPLC provide a convenient and powerful cello-oligosaccharide preparation suitable for any routine laboratory use.

Acknowledgement

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4. References

1) Chu, S.S.C. and Jeffrey, G.A. : The refinement of the crystal structures of β-D-glucose and cellobiose. Acta Cryst. **B24.** 830 - 838, 1968

2) Pérez, S. and Brisse, F. : The crystal and molecular structure of a trisaccharide, β-cellotriose undecaacetate : 1,2,3,6-tetra-*O*-acetyl-4-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]-β-D-glucopyranose. Acta Cryst. **B33.** 2578-2584, 1977

3) Wolfrom, M.L. and Dacons, J.C. : The polymer-homologous series of oligosaccharides from cellulose. J. Am. Chem. Soc. **74.** 5331 - 5333, 1952

4) Miller, G.L., Dean, J. and Blum, R. : A study of methods for preparing oligosaccharides from cellulose. Arch. Biochem. Biophys. **91.** 21 - 26, 1960

5) Beélik, A. and Hamilton, J.K. : The ultraviolet irradiation of model compounds related to cellulose. J. Org. Chem. **26.** 5074 - 5080, 1961

6) Wing, R.E. and Freer, S.N. : Use of trifluoroacetic acid to prepare cellodextrins. Carbohydr.

Polym. **4.** 323 - 333, 1984
7) Sandhu, J.S., Fraser D.R. and Kennedy, J.F. : Simple procedures for addition of a ^{3}H label to cellulose oligo- and polysaccharides and for esterification of the products with [^{14}C] nicotinic acid. Carbohydr. Res. **103.** 181 - 185, 1982
8) Sasaki, T., Tanaka, T. and Kainuma, K. : A simple method for preparation of cellooligosaccharides. Nippon Shokuhin Kogyo Gakkaishi. **26.** 538 - 541, 1979
9) Reese, E.T. and Mandels, M. : Enzymic hydrolysis of cellulose and its derivatives. Methods In Carbohydr. Chem. **III.** 139 - 143, 1963
10) Saeman, J.F., Moore, W.E., Mitchell, R.L. and Millett, M.A. : Techniques for the determination of pulp constituents by quantitative paper chromatography. Tappi. **37.** 336-343, 1954
11) Brown, W. : The separation of cellodextrins by gel permeation chromatography. J.Chromatog. **52.** 273 - 284, 1970
12) Huebner, A., Ladisch, M.R. and Tsao, G.T. : Preparation of cellodextrins : An engineering approach. Biotechnol. Bioeng. **20.** 1669-1677, 1978
13) Streamer, M., Eriksson, K.-E. and Pettersson, B. : Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. Eur. J. Biochem. **59.** 607 - 613, 1975
14) Aitzetmüller, K.: Sugar analysis by high-performance liquid chromatography using silica columns. J. Chromatog. **156.** 354 - 358, 1978
15) Wheals, B.B. and White, P.C. : *In situ* modification of silica with amines and its use in separating sugars by high-performance liquid chromatography. J.Chromatog. **176.** 421 - 426, 1979
16) D'Amboise, M., Noël D. and Hanai, T. : Characterization of bonded-amine packing for liquid chromatography and high sensitivity determination of carbohydrates. Carbohydr. Res. **79.** 1 - 10, 1980
17) Usui, T., Yokoyama, M., Yamada, N., Matsuda, K., Tuzimura, K., Sugiyama, H. and Seto, S. : Proton magnetic resonance spectra of D-gluco-oligosaccharides and D-glucans. Carbohydr. Res. **33.** 105 - 116, 1974
18) Ihnat, M. and Goring, D.A.I. : Shape of the cellodextrins in aqueous solution at 25℃. Can. J. Chem. **45.** 2353 - 2361, 1967