Preparation of Laminari-oligosaccharides by Acetolysis of Curdlan

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カードランの加酢分解による ラミナリオリゴマーの調製

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Abstract

A simple method for preparation of homologous series of laminari-oligosaccharides was presented. Curdlan, a kind of (1→3)-β-D-glucan, was subjected to direct acetolysis, followed by saponification to give a mixture of laminari-oligosaccarides. Acetolysis for 1 hr at 60℃ was found to be an optimum for the preparation of a mixture of laminari-oligosaccharides having degree of polymerization 2 to 7. Each oligosaccharide was separated by a size exclusion chromatography on Toyopearl HW 40 S and its properties were characterized.

要　旨

β-(1→3) 結合したラミナリオリゴマーの簡便な調製法を開発した。(1→3)-β-D-グルカンの一種であるカードランを種々の条件下で加酢分解後，ケン化し水可溶性のラミナリオリゴマーの調製を試みた結果，60℃で1時間の加酢分解が最適であることがわかった。得られたラミナリオリゴマーはToyopearl HW 40 Sを用いた立体排除クロマトグラフィーにより7量体まで分離・精製することができた。また，得られた各オリゴマーの化学的な性質について検討した。

1. Introduction

In plant cell walls three types of β-D-glucans are known to be widely distributed, *i. e.*, cellulose, (1→3)-β-D-glucans (callose), and linear β-D-glucans containing both 1-3 and 1-4 linkages[1,2]. Of these β-D-glucans with no detectable branching are peculiar in that they are present as a component of special walls, *e. g.*, sieve plates[3,4], pollen tubes[5], and cotton seed hairs[2]. Compression wood cell walls also contain a similar β-D-glucan, Laricinan[6]. Deposition of (1→3)-β-D-glucans was also stimula-

ted by wounding or by infection by microorganisms[7]. Analogous (1→3)-β-D-glucans were also isolated from the bacterium *Alcaligenes faecalis* (curdlan)[8] and the unicellular algae *Euglena gracilis* (paramylon)[9]. The conformation of the (1→3)-β-D-glucans was unique in their triple-stranded helix[10,11] and some of them have been interested in their high antitumor activity[12,13].

Recently, we are interested in the effects of CO_2 laser beam on polysaccharides and found that in the cases of cellulose and starch various kinds of water-soluble oligosaccharides were produced by irradiation of the laser beam[14-16]. The structure of these oligosaccharides could be successfully characterized by using homologous series of cello- and malto-oligosaccharides as standards. In order to extend this line of investigation, we nextly intended to characterize the effects of CO_2 laser beam on (1→3)-β-D-glucans, because of their unique distribution, conformation and biological activity. For this purpose, (1→3)-β-D-linked oligomers which were conventionally designated as laminari-oligosaccharides were required as the authentic standard.

Previously, laminari-oligosaccharides have been prepared from (1→3)-β-D-glucans by partial acid hydrolysis[3,17-19], enzymatic hydrolysis[20], and enzymatic transglycosylation[21]. Laminaribiose was also prepared by optimized acetolysis of pachyman[22]. Koizumi *et al.*[23] analyzed the distribution profiles of laminari-oligosaccharides prepared by acid hydrolysis or acetolysis of the formolyzed curdlan and reporterd that the proportions of higher oligomers in the acetolysate are higher than those in the acid hydrolysate. Since curdlan is hardly soluble in water, partial acid hydrolysis is not adequate for preparation of a large amount of laminari-oligosaccharides. Previously, we showed that direct acetolysis of ivory nut mannan gave a mixture of manno-oligosaccharides in a high yield[24]. In order to obtain a large amount of laminari-oligosaccharies, the time-consuming formolysis prior to acetolysis seems not to be necessary.

We now report a simple method for the preparation of laminari-oligosaccharides by direct acetolysis of curdlan followed by size exclusion chromatography on Toyopearl HW 40 S.

2. Experimental

2.1. General

Curdlan, which was produced by *Alcaligenes faecalis* var. myxogenes 10 C 3 K, was purchased from Wako Pure Chemical Industries, Ltd. The degree of polymerization (D.P.) of curdlan has been reported to be 455[25]. Gas liquid chromatography (G.L.C.) was carried out on a Shimadzu GC-15 AF_{SC} gas chromatograph equipped with flame ionization detectors. Shimadzu Chromatopac C-R 6 A was used as an integrated recorder. Separation was performed on a column of SP-2330 (30 m x 0.28 mm) with temperature program from 150℃ to 220℃ at 1.5℃/min. Gas liquid chromatography-mass spectrometry (G.L.C.-M.S.) was carried out on a Shimadzu QP-1000 system using a column (25 m × 0.22 ㎜) of CP-Sil 88 at 215℃.

The proportion and purity of the laminari-oligosaccharides were analyzed by analytical size exclusion chromatography on a column (7.6 mm × 50 cm) of Asahipak GS-220 at 60℃ and a flow rate of 0.6 ml/min using distilled water as an eluent. The purity and identification of the isolated laminari-oligosaccharides were also carried out by reversed phase chromatography on an amino-bonded silica gel column (44.6 mm × 25 cm) of YMC PA-03 at room temperature and a flow rate of 0.6 ml/min using a 1 : 1 (v/v) mixture of acetonitrile and water as an eluent. Both chromatographic separations were carried out by using JASCO 880-PU intelligent HPLC pump, Shodex RI SE-51 differential refractometer, and Waters M-740 data Module.

Nuclear magnetic resonance (N. M. R.) spectra were obtained on a Varian XL-200 N. M. R. spectrometer (200 MHz for ^{1}H and 50.3 MHz for ^{13}C in deuterium oxide). The ^{1}H-N. M. R. spectra were obtained at 80℃ and chemical shifts in p. p. m. for anomeric protons were given with acetone (29.80 p. p. m.) as an internal standard. The ^{13}C-N. M. R. spectra were obtained at 25℃ with complete proton-decoupling and chemical shifts in p. p. m. were given with dioxane (67.40 p. p. m.) as an internal standard.

The other materials, instruments and experimental conditions not specified were the same as described previously[24,26].

2. 2. Acetolysis of curdlan

One gram of dried curdlan was added to mixtures of glacial acetic acid (3.8 ml), acetic anhydride (3.8 ml) and sulfuric acid (0.8 ml) previously kept at four different temperatures (40℃, 50℃, 60℃, and 70℃). After reaction under vigorous stirring for 15 min to 3 hr, the reaction mixture was poured into ice-water. The acetolysate was extracted with chloroform after neutralization with sodium carbonate and saponified with 0.1 N potassium hydroxide in toluene-methanol (1 : 3, v/v) as described previously[21]. The water-soluble laminari-oligosaccharides were obtained by repeated extraction with distilled water followed by deionization with Dowex 50 × 8 (H^{+} form) and Dowex 1 × 8 (acetate form) resin columns jointed in tandem. Distribution profile of laminari-oligosaccharides was analyzed by size exclusion chromatography (S. E. C.).

2. 3. Isolation of laminari-oligosaccharides

The water-soluble laminari-oligosaccharide mixture was extracted with 80% aqueous ethanol and the extract was evaporated to dryness. About 300 mg of 80% aqueous ethanol-soluble laminari-oligosaccharides was dissolved in 5 ml of distilled water and applied on a column (4.0 × 92 cm) of Toyopearl HW 40 S (TOSOH), then eluted with distilled water at a flow rate of 3 ml/min (Kusano Kagakukikai KP-7 pump). The eluting position of each oligosaccharide was monitored by Waters Model R 401 refractometer coupled with Shimadzu Chromatopac C-R 6 A integrated recorder and Advantec Toyo Model SF-139 peak collector which was actuated at 200 drops (8.5 ml) per one fraction. Each isolated oligomer was recovered by evaporation and pur-

ified by repeated chromatography on the same column. Purity of the isolated oligomers was checked by analytical S.E.C. on Asahipak GS-220 and reverse-phase chromatography on YMC PA-03.

2.4. Methylation analysis

Each isolated oligosaccharides was methylated by the modified method of Hakomori[27] followed by the method of Kuhn[28]. The fully methylated oligosaccharides were hydrolyzed with 3 N trifluoroacetic acid for 3 hr at 100℃. The partially methylated monosaccharides were converted into alditol acetates and analyzed by G.L.C. and G.L.C.-M.S.

2.5. Determination of degree of polymerization

Degree of polymerization (D.P., n) of the isolated laminari-oligosaccharides was determined from methylation analysis, ^{1}H-NMR spectroscopic analysis, thin layer chromatographic (T.L.C.) analysis, and molecular optical rotation ([M]) analysis.

3. Results and Discussion

3.1. Preparation of laminari-oligosaccharide mixture by aetolysis of curdlan

Percent conversion of curdlan to water-soluble laminari-oligosaccharides was analyzed by acetolysis followed by saponification. Figure 1 shows the dependence of conversion rate on reaction times (within 3 hr) and temperatures (40℃, 50℃, 60℃, and 70℃) of acetolysis. The conversion rate increased with raising the acetolysis temperature. A remarkable difference was observed between the conversion rates given at 40℃ and 50℃. The reason of this difference was ascribed to insolubility of curdlan in the reaction medium; the periods needed to solubilize curdlan completely were more than 3 hr at 40℃, 2 hr at 50℃, 1 hr at 60℃, and within 15 min at 70℃, respectively.

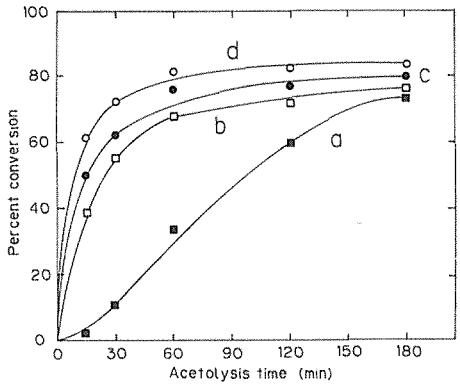

Fig. 1 Conversion rate of curdlan into laminari-oligosacchrides by direct acetolysis followed by saponification. The amount of oligosaccharides soluble in water at acetolysis at (a) 40℃ (■), (b) 50℃ (□), (c) 60℃ (●), and (d) 70℃ (○).

3.2. Influence of acetolysis temperature and time on composition of laminari-oligosaccharides

Figure 2 shows the dependence of composition of laminari-oligosaccharides on acetolysis time and temperature. At 40℃ and 50℃, laminari-oligosaccharides having D.P. 2 to 7 together with glucose were detected during the whole acetolysis time (3 hr). At 40℃, the oligosaccharide formation reached plateau after reaction for 1 hr.

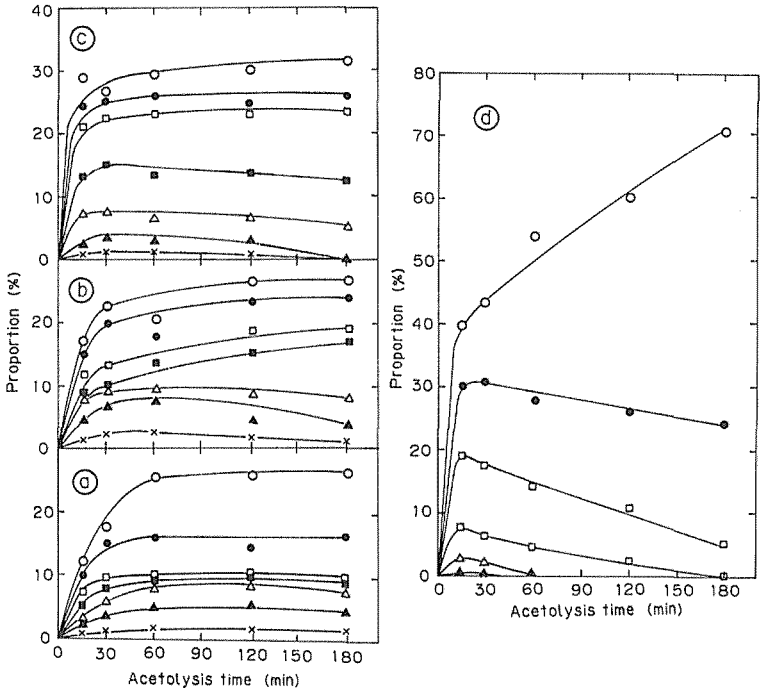

Fig. 2 Effects of direct acetolysis time and temperature on oligosaccharide distribution profile : glucose (○), laminaribiose (●), laminaritriose (□), laminaritetraose (■), laminaripentaose (△), laminarihexaose (▲), and laminariheptaose (×). Acetolysis temperatures at (a) 40℃, (b) 50℃, (c) 60℃, and (d) 70℃.

At 50℃, however, the proportion of pentaose to heptaose had a maximum at 60 min. At 60℃, however, the proportion of tetraose to heptaose had a maximum at 30 min, but neither hexaose nor heptaose could be detected after reaction for 3 hr. At 70℃, the proportion of oligosaccharides attained a maximum at 15 min. At this temperature, no heptaose was detected and proportion of glucose predominated. Based on the results presented above and those shown in Fig. 1, the optimal condition for production of laminari-oligosaccharides was concluded to be direct acetolysis for 1 hr at 60℃. The S. E. C. elution profile given at this acetolysis condition is shown in Fig. 3.

3. 3. Separation of laminari-oligosaccharides

Dried curdlan was subjected to acetolysis for 1 hr at 60℃, saponified, extracted with distilled water, and deionized to give a mixture of laminari-oligosaccharides in a yield of 76%. Since this mixture of oligosaccharides contained higher molecular weight components which seriously disturbed the clear cut separation of laminaribiose to laminariheptaose by S. E. C., the mixture was extracted with 80% aqueous ethanol to give 80% ethanol-soluble oligosaccharides in a yield of 82% of the water-soluble materials. About 300 mg of this mixture of laminari-oligosaccharides was ap-

plied on a column of Toyopearl HW 40 S which was eluted with distilled water. A typical elution profile shown in Fig. 4 indicates that the laminari-oligosaccharides having D. P. 2-7 were well separated within 5 hr. About 83 mg of laminaribiose, 81 mg of laminaritriose, 52 mg of laminaritetraose, 26 mg of laminaripentaose, 12 mg of laminarihexaose, and 6 mg of laminariheptaose were separated by a single chromatography as amorphous powder. Each oligosaccharide was purified by rechromatography. Previously, Toyopearl HW 40 S gel was successfully used to prepare homologous series of malto-[29], isomalto-[30], and manno-oligosaccharides[24]. The present study demonstrates that this gel can also be useful for separation of homologous series of laminari-oligosaccharides.

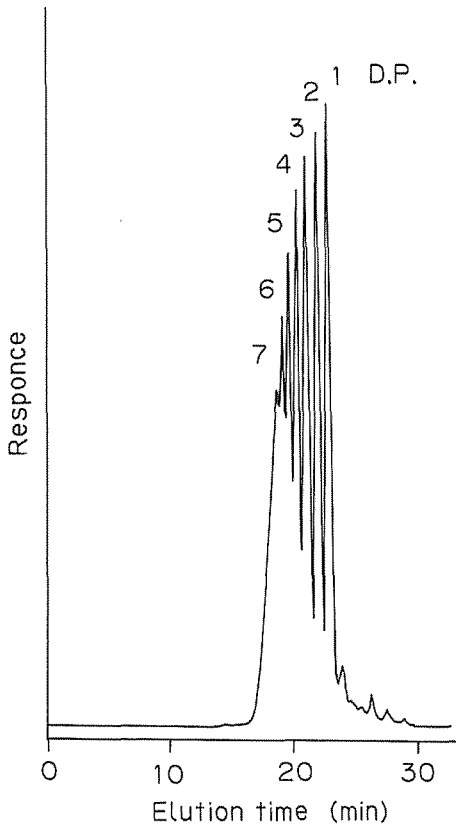

Fig. 3 Elution profile of size exclusion chromatography on Asahipak GS-220 of the mixture of laminari-oligosaccharides obtained by direct acetolysis at 60℃ for 1 hr. The numbers (1 to 7) represent the degree of polymerization.

3.4. Characterization of the isolated laminari-oligosaccharides

The glycosidic linkage analysis of the isolated oligosaccharides was carried out by methylation analysis. The results are shown in Table 1. The all isolated oligosaccharides gave two kinds of partially methylated glucoses, *i.e.*, 2, 3, 4, 6-tetra-*O*-methyl-D-glucose and 2, 4, 6-tri-*O*-

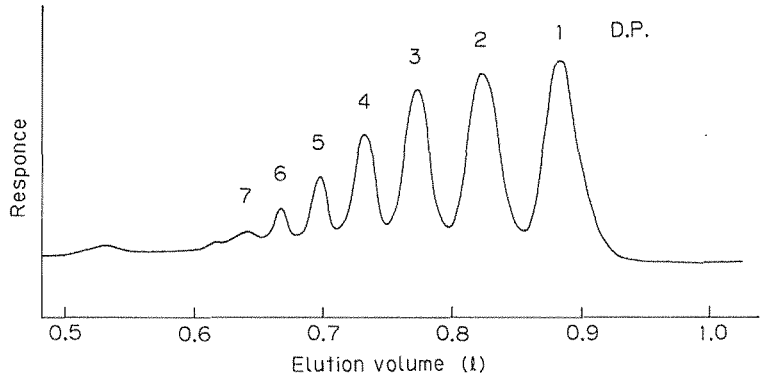

Fig. 4 Elution profile of laminari-oligosaccharides on Toyopearl HW40S. The numbers (1 to 7) represent the degree of polymerization.

Table 1. Properties and methylation analysis of the isolated laminari-oligosaccharides

Oligosaccharides	$[\alpha]_D^{25}$ (c 1.0, H_2O)	Partially methylated alditol acetates[a]		D. P.
		2, 3, 4, 6-Glc[b]	2, 4, 6-Glc[c]	
Laminaribiose	+19.5°	1.0	0.9	2
Laminaritriose	+ 2.9°	1.0	1.8	3
Laminaritetraose	− 3.0°	1.0	2.7	4
Laminaripentaose	− 7.7°	1.0	4.2	5
Lominarihexaose	−10.5°	1.0	4.9	6
Laminariheptaose	−12.3°	1.0	6.2	7

[a]Values are given as molar ratios.
[b]2, 3, 4, 6-Glc=2, 3, 4, 6-tetra-*O*-methyl-D-glucitol
[c]2, 4, 6-Glc=2, 4, 6-tri-*O*-methyl-D-glucitol

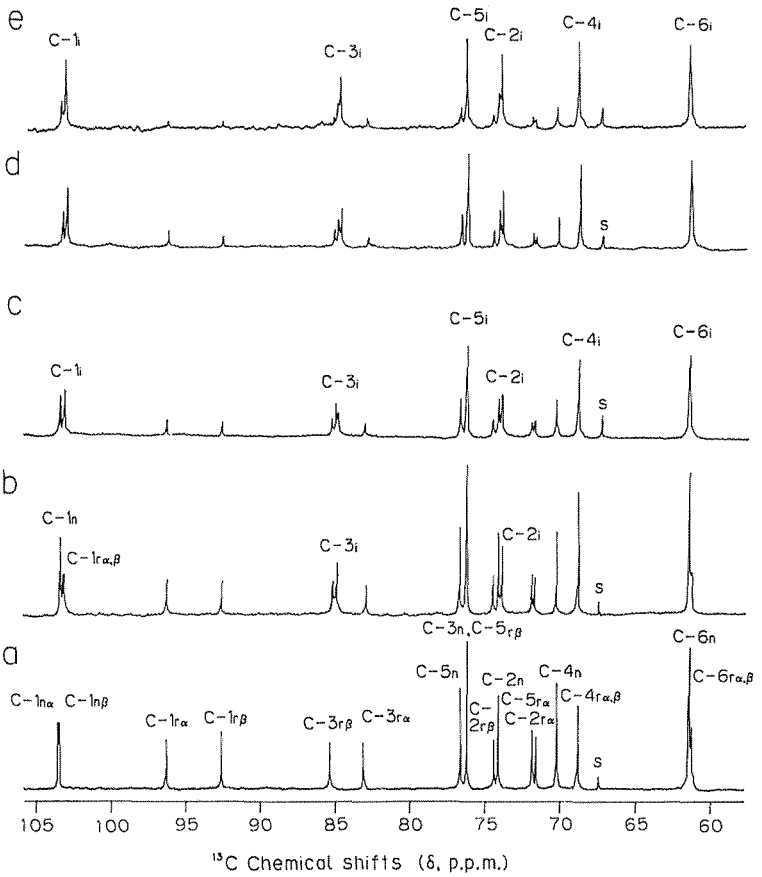

Fig. 5 ^{13}C-N. M. R. spectra of the laminari-oligosaccharides at 25℃ in deuterium oxide: laminaribiose (a), laminaritriose (b), laminaritetraose (c), laminaripentaose (d), and laminariheptaose (e). Symbols: dioxane (s), reducing end residue (r), internal residues (i), and non-reducing end residue (n).

Table 2. ^{13}C-N. M. R. Data for laminari-oligosaccharides (chemical shifts, in p. p. m.)

			Laminari-oligosaccharides			
			Laminari-biose	Laminari-triose	Laminari-tetraose	Laminari-pentaose
Reducing end residue	$C\text{-}1_r$	α	96.50	96.51	96.53	96.52
		β	92.82	92.84	92.84	92.85
	$C\text{-}2_r$	α	71.81	71.88	71.89	71.90
		β	74.59	74.66	74.67	74.67
	$C\text{-}3_r$	α	83.38	83.19	83.24	83.14
		β	85.61	85.40	85.46	85.46
	$C\text{-}4_r$	α	68.99	68.96	68.97	68.94
		β	68.99	68.96	68.97	68.94
	$C\text{-}5_r$	α	72.03	72.04	72.06	72.06
		β	76.39	76.40	76.85	76.45
	$C\text{-}6_r$	α	61.57[a]	61.56[a]	61.56	61.53
		β	61.44[a]	61.44[a, b]	61.56	61.53
Internal residue	$C\text{-}1_i$			103.46 (α) 103.37 (β)	103.38	103.37
	$C\text{-}2_i$			74.05	74.06	74.13
	$C\text{-}3_i$			85.20	85.23, 85.07	85.12, 84.94
	$C\text{-}4_i$			68.96	68.97	68.94
	$C\text{-}5_i$			76.40	76.43	76.45
	$C\text{-}6_i$			61.57	61.56	61.53
Non-reducing end residue	$C\text{-}1_n$		103.76 (α) 103.67 (β)	103.66	103.69	103.66
	$C\text{-}2_n$		74.30	74.28	74.30	74.29
	$C\text{-}3_n$		76.39	76.40	76.43	76.45
	$C\text{-}4_n$		70.42	70.42	70.43	70.42
	$C\text{-}5_n$		76.82	76.82	76.85	76.84
	$C\text{-}6_n$		61.57	61.56	61.56	61.53

[a]Assignments may be interchanged.
[b]signals appeared as a shoulder.

methyl-D-glucose, indicating that glucose residues are linked each other by (1→3)-glucopyranosidic linkages. The D. P. values of the isolated oligosaccharides were estimated based on the molar ratios of these partially methylated glucoses as listed in Table 1. The structure of the isolated oligosaccharides was confirmed by the ^{13}C-N. M. R. spectroscopic analyses as shown in Fig. 5 and Table 2.

The configuration of glucosidic linkages was analyzed by ^{1}H-N. M. R. spectroscopic measurements. The ^{1}H-N. M. R. spectra of the anomeric regions of the isolated laminari-oligosaccharides are shown in Fig. 6, and the assignments of the signals were listed in Table 3. In the case of the oligosaccharides having D. P. higher than 4, the chemical shifts of the anomeric protons of the reducing end, the glucose residue adjacent to the reducing end, internal, and non-reducing end glucose residues were assigned as signals appeared at 5.26 p. p. m. ($H\text{-}1_\alpha$), 4.77 p. p. m. ($H\text{-}1_\beta$), 4.76 p. p. m. ($H\text{-}1_{i\alpha}$), 4.68 p. p. m. ($H\text{-}1_{i\beta}$), 4.79 p. p. m. ($H\text{-}1_i$), and 4.74 p. p. m. ($H\text{-}1_n$), respectively. The signals due to the intermediate glucose residues became larger with increase of D. P. Based on the large coupling constants ($J_{1,2}$, 7-8 Hz), D-glucoses

Table 2. ^{13}C-N. M. R. Data for laminari-oligosaccharides (chemical shifts, in p. p. m.) (continued)

			Laminari-oligosaccharides	
			Laminarihexaose	Laminariheptaose
Reducing end residue	$C\text{-}1_r$	α	96.52	96.52
		β	92.86	92.86
	$C\text{-}2_r$	α	71.91	71.90
		β	74.63	74.66
	$C\text{-}3_r$	α	83.16	83.16
		β	85.39	85.40
	$C\text{-}4_r$	α	68.95	68.95
		β	68.95	68.95
	$C\text{-}5_r$	α	72.06	72.04
		β	76.46	76.46
	$C\text{-}6_r$	α	61.54	61.56
		β	61.54	61.56
Internal residue	$C\text{-}1_i$		103.38	103.37
	$C\text{-}2_i$		74.13	74.13
	$C\text{-}3_i$		85.12, 84.96	85.12, 84.96
	$C\text{-}4_i$		68.95	68.95
	$C\text{-}5_i$		76.46	76.46
	$C\text{-}6_i$		61.54	61.56
Non-reducing end residue	$C\text{-}1_n$		103.66	103.67
	$C\text{-}2_n$		74.29	74.28
	$C\text{-}3_n$		76.46	76.46
	$C\text{-}4_n$		70.42	70.42
	$C\text{-}5_n$		76.84	76.86
	$C\text{-}6_n$		61.54	61.56

were concluded to be linked each other by β-glycosidic linkages.

The D. P. values of the isolated laminari-oligosaccharides were also estimated by T. L. C. and optical rotation measurement. Figure 7 shows the linear D. P. dependence of the values of $R_f/(1-R_f)$ using two different irrigants, indicating that the isolated oligosaccharides were homologous. Similary, molecular rotation values of the isolated oligosaccharides ($[M]_n$) were plotted against (n-1)/n, where n equals D. P. Figure 8 showed a good linear relationship, confirming that the present isolated oligosaccharides were homologous. Based on the results presented above, the isolated oligosaccharides were concluded to be a homologous series of (1→3)-β-D-linked oligosaccharides having D. P. 2 to 7.

As we have reported previously, acetolysis followed by saponification permits effective preparation of manno-oligosaccharides from ivory nut mannan[2]. The present results further extended the applicability of this method to prepare laminari-oligosaccharides from curdlan.

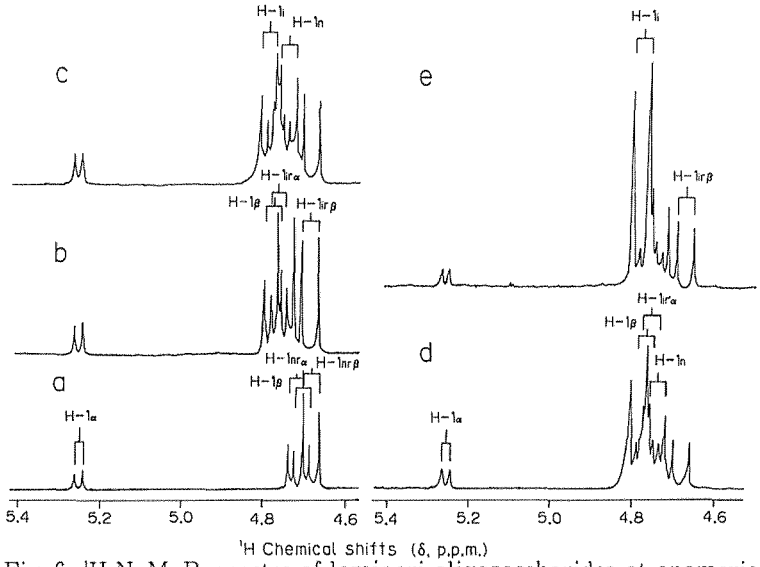

Fig. 6 ¹H-N. M. R. spectra of laminari-oligosaccharides at anomeric region at 80℃ in deuterium oxide: laminaribiose (a), laminaritriose (b), laminaritetraose (c), laminaripentaose (d), and laminariheptaose (e). Symbols: anomeric protons of the reducing end (H-1_α, H-1_β), internal and adjacent to the reducing end (H-$1_{ir\alpha}$, H-$1_{ir\beta}$), internal (H-1_i), non-reducing and adjacent to the reducing end (H-$1_{nr\alpha}$, H-$1_{nr\beta}$), and non-reducing end (H-1_n) glucose residues.

Table 3. ¹H-N. M. R. Data for laminari-oligosaccharides (chemical shifts, in p. p. m.)

Oligosaccharides	H-1_r		H-1_{ir}		H-1_i	H-1_n	
	H-$1_{r\alpha}$	H-$1_{r\beta}$	H-$1_{ir\alpha}$	H-$1_{ir\beta}$		H-$1_{nr\alpha}$	H-$1_{nr\beta}$
Laminaribiose	5.26 (3.8)[a] [0.4][b]	4.72 (7.3) [0.6]				4.70 (7.7) [0.4]	4.68 (7.9) [0.6]
Laminaritriose	5.26 (3.8) [0.4]	4.77 (7.9) [0.6]	4.75 (7.8) [0.3]	4.68 (7.9) [0.7]		4.76 (7.7) [1.1]	
Laminaritetraose	5.26 (3.8) [0.4]	4.77 (7.9) [0.6]	4.75 (7.8) [0.4]	4.68 (7.9) [0.6]	4.78 (8.0) [1.1]	4.74 (7.7) [1.1]	
Laminaripentaose	5.26 (3.9) [0.3]	4.77 (7.9) [1.2]	4.76 (7.8) [0.3]	4.68 (8.0) [0.8]	4.79 (7.9) [1.8]	4.74 (7.7) [0.8]	
Laminarihexaose	5.26 (3.7) [0.3]	4.77 (7.8) [0.8]	4.76 (7.8) [0.3]	4.68 (7.9) [0.9]	4.79 (7.9) [3.2]	4.74 (7.6) [0.9]	
Laminariheptaose	5.26 (3.7) [0.4]	4.77 (7.9) [0.7]	4.76 (7.8) [0.3]	4.68 (7.9) [0.8]	4.79 (7.9) [4.3]	4.74 (7.6) [0.8]	

[a]Coupling constant ($J_{1,2}$, Hz).
[b]Molar ratio.

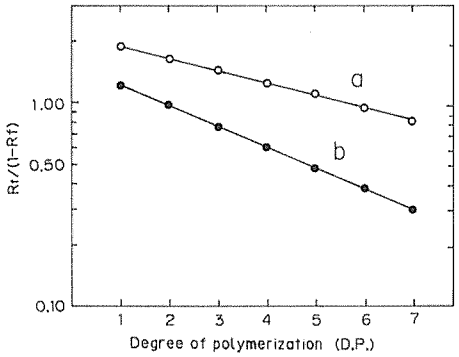

Fig. 7 Relation between $R_f/(1-R_f)$ and degree of polymerization of laminari-oligosacchrides: The R_f values were obtained by T. L. C. using (a) 1-butanol-2-propanol-water (3 : 12 : 4, v/v) (o) and (b) 1-butanol-ethanol-water (5 : 3 : 2, v/v) (●).

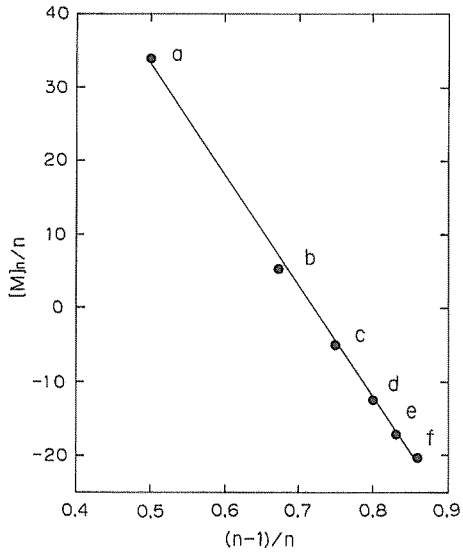

Fig. 8 Relation between $[M]_n/n$ and (n-1)/n, where [M] and n were molar optical rotation and degree of polymerization, respectively: laminaribiose (a), laminaritriose (b), laminaritetraose (c), laminaripentaose (d), laminarihexaose (e), and laminariheptaose (f).

Acknowledgement

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