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Structural characterization of β-D-(1→3, 1→6)-linked glucans using NMR spectroscopy

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Abstract

Nondestructive structural analysis of a series of β-D-(1→3, 1→6)-linked glucans (laminaran, curdlan, yeast glucan, scleroglucan, etc.) was performed using two-dimensional NMR spectroscopy. The relative ratios of H-1 at different AGUs provided the information about DP_n and DB. The α-, and β-anomeric protons on reducing terminals were assigned at 5.02 ~ 5.03 ppm (*J* 3.6 ~ 3.7 Hz), and 4.42 ~ 4.43 ppm (*J* 7.6 ~ 7.9 Hz), respectively, whereas the H-1 protons of internal AGUs and β-(1→6)-branched AGUs appeared at 4.56 ~ 4.59 ppm (*J* 7.6 ~ 7.8 Hz), and 4.26 ~ 4.28 ppm (*J* 7.6 ~ 10.6 Hz), respectively, in a mixed solvent of 6:1 Me_2SO-d_6–D_2O at 80 °C. In the solvent, the OH peaks were eliminated from the spectra allowing the H-1 protons to appear clearly. In addition, the nonreducing terminal H-1 and H-1 at the AGU next to reducing terminal could be assigned at 4.45 ~ 4.46 ppm (*J* 7.8 ~ 7.9 Hz), and 4.51 ~ 4.53 ppm (*J* 7.8 Hz), respectively. The DP_n of the laminaran was 33 (polydispersity 1.12) and the DB was 0.07. The number of glucosyl units in the side chain of laminaran is more than one. The DP_n and DB of the water-insoluble yeast glucan were 228 and 0.003, respectively. However the DP_n of water soluble yeast glucan phosphate and curdlan was changed upon the number of freeze-drying processes and the content of water in the mixed solvent, respectively. And the DB of those were calculated as 0.02 and 0, respectively. The DB of scleroglucan was precisely calculated as 0.33, compared with the previously reported data. The H-1s at different AGUs of the various β-D-(1→3, 1→6)-linked glucans having different DB can be exactly assigned by their chemical shifts in the mixed solvent system. This NMR analysis can be effectively used to determine the DP and DB of polysaccharides in a simple and non-destructive manner.

Keywords: β-D-(1→3, 1→6)-linked glucan; NMR spectroscopy; Scleroglucan; Glucan phosphate

1. Introduction

β-D-(1→3, 1→6)-linked glucans have the ability of enhancing and stimulating the immune system of humans [1–5] and are thus called biological response modifiers (BRMs). A variety of β-glucans having different structures have originated from various sources and by different extraction processes [5,6]. β-glucans from microorganisms generally have β-D-(1→3)-linked anhydro D-glucose units (AGUs) as a backbone and periodic β-D-(1→

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6)-linked side chains. But some glucans consist of a linear β-D-(1→3) linkage without branching. So far, numerous studies [7–9] have revealed that the molecular weight (MW) and water solubility of β-glucans, which depend on the degree of branching (DB) and the chemical modification, were the critical properties in determining their clinical usage as BRMs. The most effective MW of clinically useful glucans ranges from 100 to 200 kDa with the DB ranging from 0.2 to 0.5 [5].

The molecular structure of β-glucans, including degree of polymerization (DP) and DB, has been analyzed by various destructive and nondestructive methods. The MW and polydispersity can be nondestructively determined by the laser light scattering method [7,10], which is extremely sensitive to macromolecular aggregation.

Glycosidic linkages and DB of β-glucans have been analyzed by methylation analysis, specific enzymatic digestion and ^{13}C NMR analysis. Up to now, the enzymatic digestion method has not been widely used compared with other biological polymers such as DNA or proteins, because all β-glucans cannot be digested enzymatically, and the isolation procedure after digestion is difficult [4,11,12]. ^{13}C NMR analysis can approximate glycosidic linkages and DB, but it suffers from the low signal-to-noise ratio (S/N ratio) and the large amount of sample required for analysis [13]. Methylation analysis has been used to determine glycosidic linkages, but it requires extensive time and careful pretreatment [14,15]. Besides, these analytical procedures often provide inconsistent results for the same β-D-glucan samples [5,16]. Read et al. [17] performed the destructive structural analysis of water-soluble laminaran consisting of two groups (G-series and M-series) by electrospray-ionisation–mass spectrometry. However, this method has a limitation in MW and can be applied to oligo-glucans whose DP is smaller than 30.

Due to the complexity of the NMR spectrum and the molecular aggregation problem, the molecular structure of the larger β-D-(1→3)-linked glucans could not have been thoroughly assigned by ^{1}H NMR spectroscopy [7]. The anomeric protons of several disaccharides, including a β-D-(1→3) linkage, could be assigned by ^{1}H and ^{13}C NMR analysis [18]. More recently, Ensley et al. [19] showed that the internal protons of the water-insoluble yeast glucan could be assigned by 2D NMR spectroscopy, providing conclusive evidence for β-(1→3) linkages. Some anomeric protons of bacteria-induced polysaccharides, β-glucans having different linkages, could be observed by ^{1}H NMR spectroscopy [20]. However, quantitative analysis has not been conducted by ^{1}H NMR spectroscopy. Gidley [21] first investigated the possibility of measuring the DP and DB of polysaccharides having a complex ^{1}H NMR spectrum. But, the various H-1s at different AGUs were not assigned, and the DP of the oligosaccharide could be calculated only because of the limited resolution of the polysaccharides.

So far, the full assignments of the resolvable minor peaks of different AGUs of β-glucans have not been achieved. The identification of these minor peaks is essential to determine the DB and DP from the ^{1}H NMR spectra. The minor peaks include the resonances from the reducing terminal H-1 (RT), the nonreducing terminal H-1 (NRT), H-1 of the second AGU next to the reducing terminus (SRT), H-1 of the branched single or terminal AGU in the side chain (TSC), and H-1 of all the AGUs except the terminal AGU in the side chain (SC) when there are more than one AGU in the side chain.

Here, we report the determination of the structural features of a series of β-D-(1→3, 1→6)-linked glucans having the activities of BRMs by using ^{1}H NMR spectroscopy including the complete assignment of all H-1s at different AGUs. We also describe a simple and nondestructive method for the determination of the DP_n and DB of β-D-(1→3, 1→6)-linked glucans up to the MW of pachyman (DP_n 540) using ^{1}H NMR spectroscopy.

2. Materials and methods

Materials.—Table 1 summarizes trivial names, physical properties and sources of isolation for a variety of β-D-(1→3, 1→6)-linked glucans examined in this study. Laminara-

Table 1
Structural characteristics and solubility of standard compounds and β-D-(1→3, 1→6)-linked glucans examined in this study

Trivial name	Linear or branched	Solubility in water	Solubility in DMSO	Solubility in alkali	Polydispersity	Sources
Gentiobiose	linear	soluble	soluble	soluble	–	–
Laminarabiose	linear	soluble	soluble	soluble	–	–
Laminara-pentaose	linear	soluble	soluble	soluble	–	–
Laminara-heptaose	linear	soluble	soluble	soluble	–	–
Laminaran	branched	soluble	soluble	soluble	1.12 [a]	*Laminaria digita*
Pustulan	linear	soluble	soluble	soluble	[b]	*Umbiliaria papullosa*
Yeast glucan	branched	insoluble	soluble	soluble	1.12 [c]	*Saccharomyces cerevisiae*
Glucan phosphate	branched	soluble	soluble	soluble	1.67–6.2 [d]	*Saccharomyces cerevisiae*
Curdlan	linear	insoluble	soluble	soluble	[b]	*Alcaligenes faecalis*
Pachyman	branched	insoluble	partial soluble	partial soluble	[b]	*Poria cocos*
Scleroglucan	branched	soluble	soluble	soluble	1.83 [e]	*Sclerotium glucanicum*

[a] Result from our measurement.
[b] Unknown value.
[c] See Ref. [22].
[d] See Refs. [7,24].
[e] See Ref. [25].

biose, laminarapentaose and laminaraheptaose were purchased from Seikagaku Co. (Tokyo, Japan), gentiobiose and laminaran from Sigma Chemical Co. (MO, USA), and pustulan and pachyman from Calbiochem Co. (CA, USA). The lyophilized yeast glucans and scleroglucan (Fig. 1) were prepared by following the previous reports [7,8,22–25].

Measurement of MW and polydispersity of laminaran.—High-performance size-exclusion chromatography–Multiangle laser light scattering–Refractive index system (HPSEC–MALLS–RI) was used in determining the absolute DP_w and polydispersity of laminaran. The system consisted of a pump (P2000, Spectra System, San Jose, CA), an injection valve (model 7021, Rheodyne, Cotati, CA), a guard column (TSK PWH, Tosoh Corp., Tokyo, Japan), one SEC column (TSK Gel 3000PW, 7.8 × 600 mm, Tosoh Corp., Japan), Multiangle laser light-scattering (Dawn DSP-F, Wyatt Technology, Santa Barbara, USA), and RI (Shodex SE71, Tokyo, Japan) detectors. Columns were maintained at 50 °C, and the detector was maintained at 35 °C. The mobile phase (0.15 M $NaNO_3$ with 0.02% sodium azide) was filtered first through 0.2-μm, and then through 0.1-μm cellulose acetate filters. Flow rate of the mobile phase was 0.5 mL/min, and the sample injection volume was 500 μL. Output voltages of RI and LS at the 18 angles were collected and used to calculate MW and polydispersity using Astra 4.50 software. The MW and polydispersity of yeast glucan, yeast glucan phosphate, and scleroglucan were used with the previously reported data [7,8,22–25]. However, those of curdlan and pachyman were not reported by using the MALLS system and could not also be determined by our MALLS system.

Reduction procedure.—Laminarapentaose and laminaraheptaose were reduced in 1 M NH_4OH solution with $NaBH_4$ (3 mg/mL). This allowed the reducing terminal unit to be changed into glucitol. The reduced products

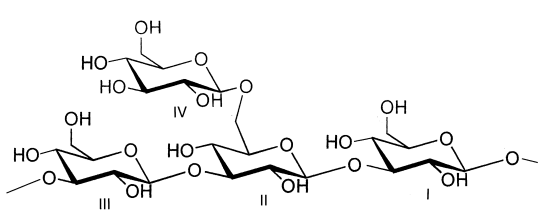

Fig. 1. Primary structure model of scleroglucan.

were recovered by a precipitation process with ethanol and by centrifuging at 3000 rpm for 10 min. Finally, the boric acid, a byproduct produced by neutralization, was removed by repeated co-distillation with methanol. The reduced products were dissolved in a mixed solvent system of 6:1 Me_2SO-d_6–D_2O for NMR analysis by stirring at 50 ~ 60 °C, and the residual $NaBH_4$ was removed by centrifugation.

NMR spectroscopy.—All NMR experiments were carried out on a Bruker FT-NMR spectrometer DMX 600 resonating at 600.13 MHz for H. The samples were dissolved in Me_2SO-d_6 or in a mixed solvent of 6:1 Me_2SO-d_6–D_2O. The concentration of the samples was 5 mg of glucan/mL for ^1H experiments and 20 mg of glucan/mL for ^{13}C experiments. All spectra were taken at 80 °C. Chemical shifts were referenced internally to tetramethylsilane (TMS, 0 ppm) for ^1H and to Me_2SO-d_6 (39.5 ppm) for ^{13}C. For ^1H, 32K complex data points were acquired, and 128 FIDs were averaged with a 30° observe pulse. The repetition delay was 5 s. The FIDs were zero-filled to 64K complex data points and were apodized by an exponential function before Fourier-transform. The spectra were baseline corrected for peak area integration. For ^{13}C experiments, 64K complex data points were acquired, and 2048 ~ 4096 scans were averaged with a 30° observe pulse. The repetition delay was 5 s. Two-dimensional magnitude correlation spectroscopy (COSY) and heteronuclear multiple quantum correlation spectroscopy (HMQC) experiments were performed to assign the ^1H and ^{13}C resonances [26]. For COSY experiments, 2048 (t_2) × 256 (t_1) complex data points were acquired, and 16 scans were averaged for each FID with the relaxation delay of 1.5 s. The FIDs were zero-filled to 1024 for t_1 and were apodized by a squared sinebell function for both dimensions. For HMQC, 2048 (t_2) × 256 (t_1) complex data points were acquired with a relaxation delay of 2 s, and 64 ~ 128 scans were averaged for each FID. The FIDs were zero-filled to 1024 for t_1 and were apodized by a 90° phase-shifted squared sinebell function for both dimensions.

Choice of NMR solvents.—It is well known that the conformational changes in the three-dimensional structure can occur in aqueous systems for some of the β-glucans. Dimethyl sulfoxide (Me_2SO-d_6) has been employed to prevent the aggregation of molecules (the increased immobility of molecules) for ^{13}C NMR analysis of β-(1→3,1→6)-D-glucan [25]. Me_2SO-d_6 is also known to provide a better resolution of the signals than aqueous solvents such as D_2O or NaOD [2,25]. However, the appearance of OH peaks that sometimes overlap with anomeric proton peaks makes the quantitative analysis of the spectra difficult. To remove the OH peaks, we introduced mixed solvents of Me_2SO-d_6 and D_2O. In addition, this solvent system increases the partial molecular mobility of polysaccharides and separates peaks of NRT and β-anomeric proton in the 1D NMR spectrum.

3. Results and discussion

Assignments of ^1H NMR signals in the backbone with standard materials.—The assignments of H-2, H-3, H-4, H-5 and H-6 protons of internal backbone AGUs were trivial since each proton of different AGUs resonates at the same frequency. They were easily assigned from COSY and HMQC spectra and the previously reported assignment [19]. The assignments of these protons, unless related to the assignment of H-1 protons, will not be discussed here.

For gentiobiose (Fig. 2(A)), α-H-1 and β-H-1 of the RT were located at 4.96 ppm (d, *J* 3.7 Hz) and 4.34 ppm (d, *J* 7.9 Hz), respectively. H-1 of the second AGU next to the reducing terminal (SRT) appeared as two doublets at 4.23 ~ 4.26 ppm due to the two anomeric forms of the RT. Coupling constants [27], correlation to the H-2 proton in the COSY spectrum, correlation to the C-1 carbon in the HMQC spectrum and their peak integration values confirmed the assignments. The anomeric proton peaks of laminarabiose were similarly assigned (Fig. 2(B), Table 2).

For laminarapentaose and laminaraheptaose, six H-1 resonances appeared as doublets in 4.41 ~ 5.04 ppm. α- and β-H-1

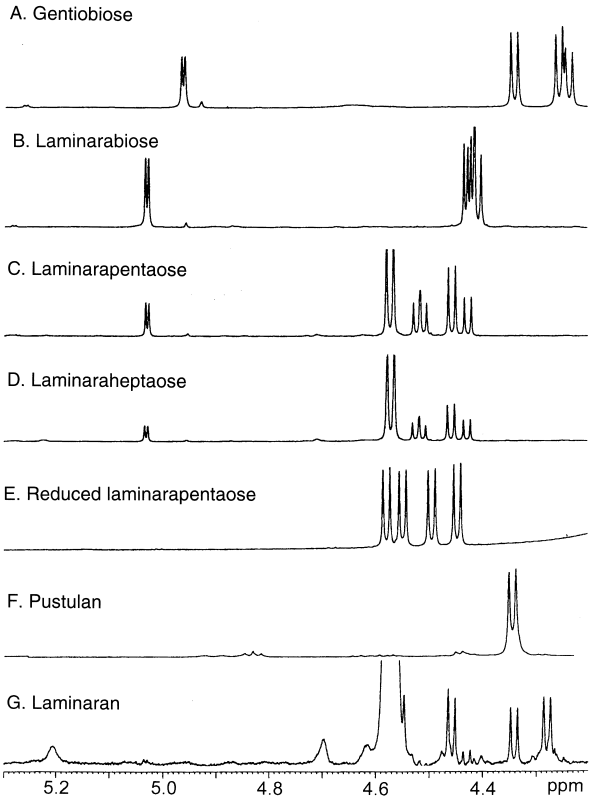

Fig. 2. ^{1}H NMR spectrum of standard materials in a mixed solvent (6:1 Me_2SO-d_6–D_2O) at 80 °C.

protons of RT were assigned to 5.03 ppm (d, *J* 3.6 Hz), and 4.43 ppm (d, *J* 7.7 Hz), respectively. The couplings to H-2, peak integration values and correlation to C-1 (91.46 ppm for α-C-1, and 96.10 ppm for β-C-1) confirmed the assignments (Figs. 3 and 4). The strongest peak resonating at 4.57 ppm (d, *J* 7.8 Hz) was assigned to the internal H-1 at the backbone chain (BC). The triplet-like peak at 4.50 ~ 4.53 ppm is actually two doublets from SRT. They were split into two peaks for the same reason as the cases of laminarabiose and gentiobiose discussed above. The assignment was confirmed by the spectrum of the reduced laminarapentaose (Fig. 2(E)), where the two doublets of the second AGU were merged into a single doublet (4.49 ppm, d, *J* 7.8 Hz). The remaining doublet (4.45 ppm, d, *J* 7.8 Hz) was assigned to the H-1 of NRT. To assign the OH peaks, Me_2SO-d_6 was used as solvent. In the COSY spectrum of laminaraheptaose (Fig. 4), the signals of 6.19 and 6.47 ppm were coupled to α-H-1 and β-H-1 of RT and thus were assigned to α-OH-1 and β-OH-1 of RT, respectively [28]. The hydroxy OH-2, OH-4 and OH-6, peaks, were also observed, and they were assigned from the correlation to their corresponding non-exchangeable proton peaks (Fig. 4). The assignments of these signals were in good agreement with the data previously reported, with a slight difference in the chemical shifts due to temperature and solvent [19,27–30].

For the reduced laminarapentaose, which have no anomeric protons, all H-1 peaks of the individual AGUs were separated, and their area corresponded to a single proton. Based on the chemical shifts, 4.45 ppm (d, *J* 7.8 Hz) was assigned to the H-1 of NRT, 4.49 ppm (d, *J* 7.8 Hz) to the H-1 of SRT, and 4.57 ppm (d, *J* 7.8 Hz) and 4.58 ppm (d, *J* 7.8 Hz) to the internal H-1. For the reduced laminaraheptaose (spectrum not shown), the internal H-1 showed up as two doublets (4.57 and 4.56 ppm). Their peak area ratio was 3:1, showing that 4.57 ppm corresponded to H-1 of 4th, 5th and 6th AGUs, and 4.56 ppm to that of the 3rd AGU.

Assignments of ^{1}H NMR signals in the side chain with laminaran and pustulan.—For the assignment of the H-1s in the side chain, laminaran extracted from *Laminaria digita* was used as a good standard material in this work. Laminaran has β-D-(1→6)-linked side chains and a β-(1→3)-linked backbone [31,32]. ^{13}C NMR analysis of the main chain has been reported previously [7,8]. The laminaran consists of two different terminal units: one terminated with a mannitol unit (M-series) and the other terminated with a reducing glucosyl unit (G-series) present in about a 3:1 ratio [17,32,33]. Since laminaran is polydispersed with an average DP_n of 20 ~ 30, all molecules do not have identical DPs. Moreover, the number of the glucosyl units in the side chain is also heterogeneous [17].

As shown in Fig. 2(G) and Table 2, the H-1 peaks of the backbone AGUs (RT, NRT, SRT and internal AGUs) were easily assigned by comparing the chemical shifts of oligolaminarans and by COSY and HMQC spectra. The H-1 of SRT in the M-series (next to mannitol) appeared at 4.47 ppm (d, *J* 7.8 Hz), overlapped with the NRT H-1 (4.46 ppm).

The β-(1→6)-linked H-1 of the side chain appeared at 4.28 ppm (d, *J* 7.7 Hz) and 4.34

ppm (d, J 7.7 Hz) exhibiting correlation to the C-1 (105.34 ppm). Their chemical shifts corresponded to those of β-(1→6)-linked H-1 of gentiobiose and pustulan. The signal at 4.28 ppm appeared for all β-D-(1→3,1→6)-glucans. On the other hand, the peak at 4.34 ppm appeared only for laminaran that had more than one glycosyl units in the side chains [17]. Based on these observations and their chemical shifts, the peak at 4.28 ppm was assigned to H-1 of the branched single or terminal AGU in the side chain (TSC), and 4.34 ppm to H-1 of all the side chain AGUs except the terminal AGU in the side chain (SC).

The content of SC in the side chain was calculated as 29% (±1%) from the peak area relative to β-(1→6)-linked H-1 from the branching point of the backbone in the side chain. However, this content did not indicate the number of glucosyl units in the side chain. On the other hand, the peak integral difference of SC and TSC as well as signals at δ 4.47 and anomeric region provided the conclusive evidence for the heterogeneity of molecules consisting of laminaran.

Determination of DP_n and DB of standard materials.—From the peak integrals of the assigned H-1s, DP_n and DB of β-glucans can be calculated by using the following equations:

$$DP_n = \text{all H-1 peak area/RT H-1 peak area, or} \quad (1a)$$

$$= \text{all H-1 peak area/NRT H-1 peak area} \quad (1b)$$

$$DB = \text{TSC H-1 peak area/all H-1 peak area in backbone} \quad (2)$$

For the laminarapentaose and laminaraheptaose, the calculated DP_n from Eqs. (1a) and (1b) gave the identical values of 5 and 7, respectively (Table 3). In addition, the DP_n of laminaraheptaose could be also calculated from the clearly resolvable ^{13}C resonances of RT, NRT, SRT and internal C-1s, although the S/N ratio was lower than that of ^1H NMR spectrum (Fig. 3). Their ^{13}C peak area ratio was approximately 1:1:1:4, resulting in the

Table 2
^1H NMR chemical shifts (ppm) and coupling constants (Hz) in parenthesis of anomeric protons at different AGUs of standard compounds and β-D-(1→3, 1→6)-linked glucans in mixed solvents (6:1 Me_2SO-d_6–D_2O) at 80 °C

Compound	RT		SRT	BC	NRT	TSC	SC
	α	β					
Gentiobiose	4.96(3.7)	4.34(7.9)	4.25(7.9) 4.23(7.9)	–	–	–	–
Laminarabiose	5.03(3.7)	4.39(7.8)	4.42(7.9) 4.23(7.9)	–	–	–	–
Laminarapentaose	5.03(3.6)	4.43(7.8)	4.51(7.8) 4.50(7.8)	4.57(7.8)	4.46(7.8)	–	–
Reduced laminarapentaose	[a]	[a]	4.49(7.8)	4.58(7.8) 4.55(7.8)	4.45(7.8)	–	–
Laminaraheptaose	5.03(3.6)	4.43(7.6)	4.52(7.8) 4.50(7.8)	4.57(7.8)	4.46(7.8)	–	–
Reduced laminaraheptaose	[a]	[a]	4.49(7.8)	4.56(7.8) 4.57(7.8)	4.47(7.8)	–	–
Laminaran	5.03(3.6)	4.43(7.7)	4.52(7.8) 4.51(7.8)	4.57(7.8)	4.46([a])	4.28(7.7)	4.34(7.7)
Yeast glucan	5.02(3.6)	4.42(7.8)	4.53 [a]	4.57(7.6)	4.46(7.8)	4.26(10.6)	–
Glucan phosphate	5.02([a])	4.42([a])	4.53 [a]	4.57(7.6)	4.46(7.8)	4.27(7.6)	–
Curdlan	5.02(3.7)	4.42(7.8)	4.52 [a]	4.57(7.6)	4.46(7.8)	4.27(7.8)	–
Pachyman	5.03(4.0)	4.42([a])	4.51 [a]	4.57(7.7)	4.45(7.9)	4.27(7.8)	–
Scleroglucan	5.02([a])	4.42([a])	completely overlapped	4.59~4.56	4.45([a])	4.28(7.7)	–
Pustulan	[a]	[a]	[a]	4.32(7.8)	[a]	[a]	[a]

[a] Undetectable value; slightly overlapped value with that of internal H-1 at main chain unit. RT: the reducing terminal H-1, NRT: the nonreducing terminal H-1, SRT: H-1 of the second AGU next to the reducing terminus, TSC: H-1 of the branched single or terminal AGU in the side chain, SC: H-1 of all the AGUs except the terminal AGU in the side chain when there are more than one AGUs in the side chain.

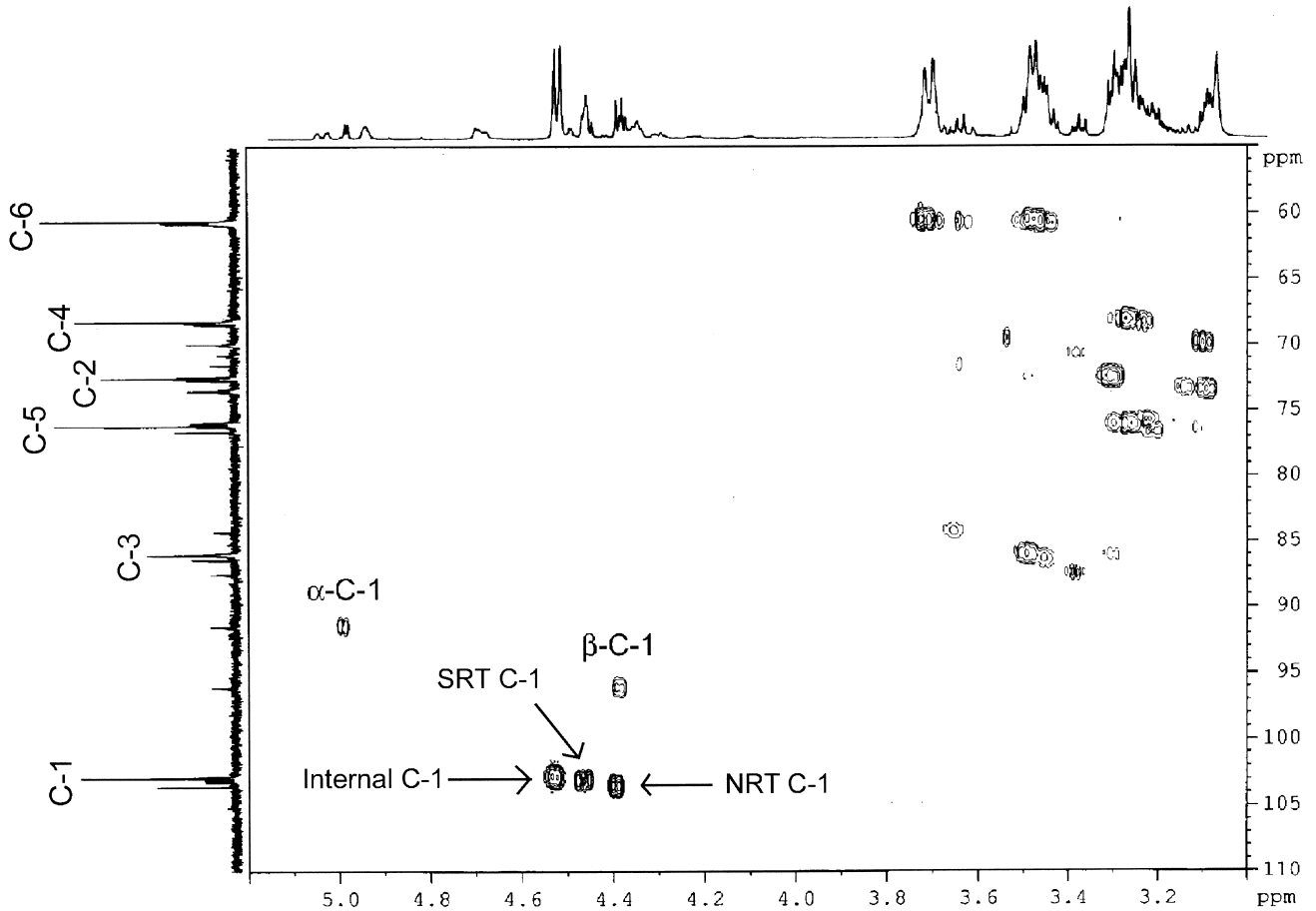

Fig. 3. $^{1}H-^{13}C$ NMR spectrum of laminaraheptaose in Me_2SO-d_6 at 80 °C.

same DP_n of 7 as was the case of the ^{1}H NMR analysis. However, ^{13}C NMR analysis was possible only for the oligosaccharide.

Using Eq. (1b), the DP_n of laminaran was calculated to be 33. This value was not significantly different from the absolute DP_w 37 measured by HPSEC–MALLS–RI. Considering the measured polydispersity of 1.12 for the system, which represented the ratio of DP_w/DP_n, the two measurements agreed perfectly. Using Eq. (2), the DB of laminaran was determined to be 0.07, similar to the previously reported value of 0.06 by EMSI analysis [17]. The ratios of the M-series and G-series could not be calculated because of the signal overlapping among some of the H-1 peaks.

Structural analysis of yeast glucan.—The chemical shifts of water-insoluble yeast glucan were assigned based on the chemical shifts of laminaran and laminaraoligomers and were confirmed by HMQC and DQF-COSY spectra (Fig. 5(A) and Table 2). For all macromolecules, the H-1 resonance of SRT overlapped with that of internal AGUs as was the case for laminaran. A recent study [22] showed that the polydispersity of yeast glucan was 1.12 and was relatively constant when analyzed by the MALLS system. The weight average MW was known to be 3.5×10^4, which was equivalent to DP_n 219. However, the DB has been ignored by most of the researchers because of relatively small amounts. Our NMR analysis revealed that the DP_n of yeast glucan was 218 showing a good agreement with the result analyzed by MALLS. The DB was measured to be 0.003 and the α:β ratio, 1:1.02 (Table 3). The obtained α:β ratio and DB as small as 0.003 prove the power of the ^{1}H NMR analysis. It has not been possible to measure these values from the other methods, so far.

Water solubility of the β-D-(1→3)-linked glucans is one of the most important features to exhibit effective activities, and it depends on the DB. Chemical modifications such as

phosphorylation and sulfation have been used to increase the solubility [5,7–9]. In contrast to water-insoluble yeast glucan, its phosphorylated form is clinically useful [33]. The ^{1}H NMR analysis of the yeast glucan phosphate revealed that the DP_n was 210. The previously reported values varied from 137 ~ 450 [7,24,33] because of the molecular aggregation of the yeast glucan phosphates during purification steps. Our result was similar to the DP_n value of untreated yeast glucan (DP_n 218). However, our value cannot represent DP_n of all macromolecules including a molecular aggregated portion, since it was considered undetected by our system. The DB of yeast glucan phosphate was 0.02. This discrepancy in DB values between water-insoluble and water-soluble yeast glucans resulted from the initial extraction process of the yeast glucans. Similar results have been observed previously showing the variety of DB values from the same source (unpublished data).

Lowman et al. [23] have pointed out that the noncovalently bound phosphate retained within the center of the helix of yeast glucan phosphate might change the pH of the solvent system. Although we did not check the pH of the solvent after the sample was dissolved, the chemical shifts of the yeast glucan phosphate were the same as those of the yeast glucan.

Structural analysis of curdlan and pachyman.—Both curdlan and pachyman are insoluble in water. Pachyman is known to have the physicochemical characteristics and structure in the solid state similar to those of curdlan, but it has a higher DP (Fig. 5 and Table 3)

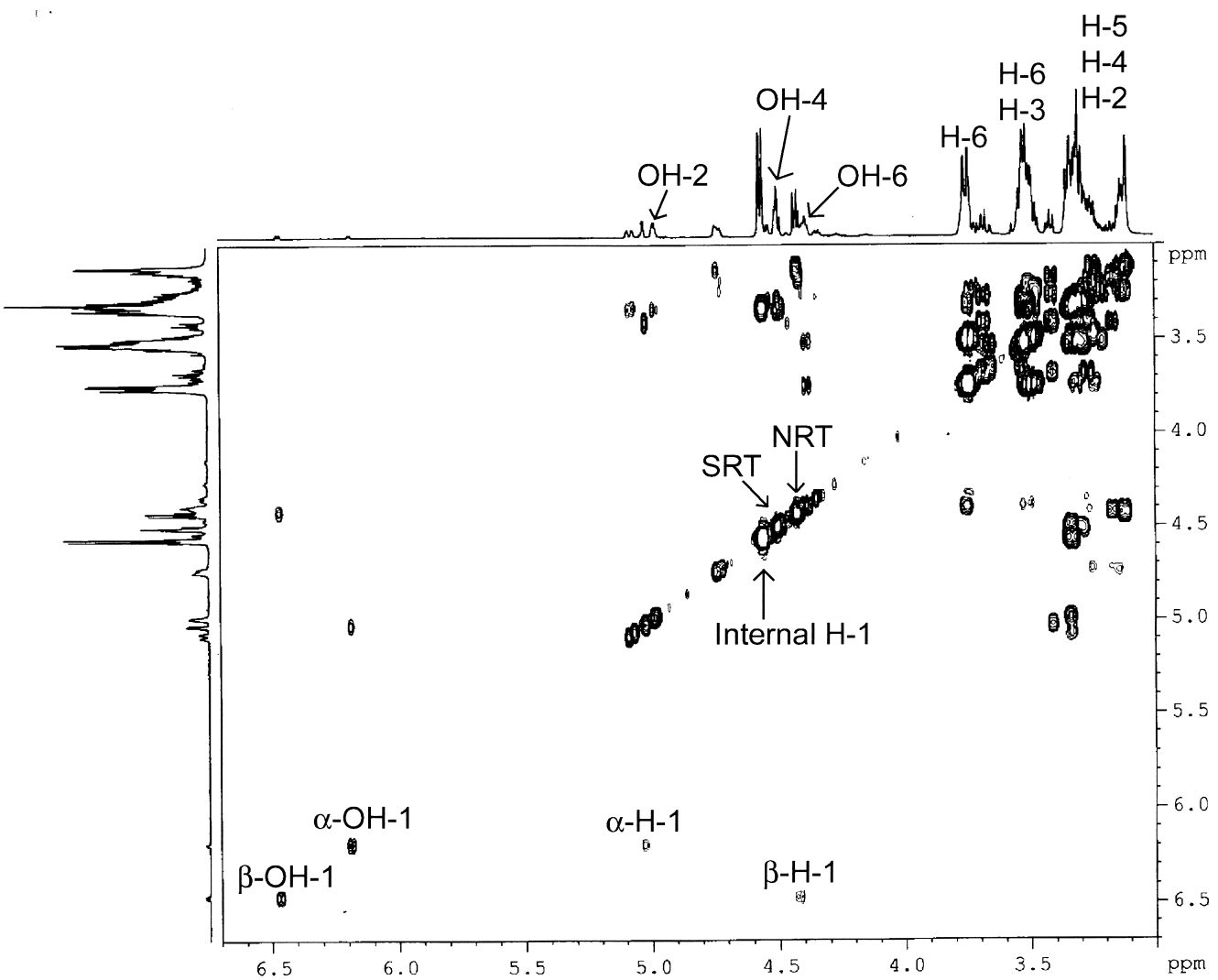

Fig. 4. ^{1}H–^{1}H NMR spectrum of laminaraheptaose in Me_2SO-d_6 at 80 °C.

Table 3
The calculated value for the characteristics of various β-glucans by using the relative ratio of anomeric protons at different AGUs in mixed solvent at 80 °C

Compound	α:β ratio	DB		DP	
		Our study	Other study	Our study	Other study
Gentiobiose	1:1.46	0	0	2	2 [a]
Laminarabiose	1:1.01	0	0	2	2 [a]
Laminarapentaose	1:1.09	0	0	5	5 [a]
Laminaraheptaose	1:1.03	0	0	7	7 [a]
Laminaran	[b]	0.07	0.05 [c]	33	20 ~ 30 [c]
Yeast glucan	1:1.02	0.003	[b]	218	220 [d]
Glucan phosphate	[b]	0.02	0.04 [e]	210	137 ~ 450 [e,f]
Curdlan	1:1.25	0	0 [g]	225	< 540 [g]
Pachyman	[b]	0.003	0.015 ~ 0.02 [h]	540	255 ~ 690 [i]
Scleroglucan	[b]	0.33	0.33 [j]		9700 [j]

[a] Value given by manufacturer.
[b] Undetectable values. Error is below 1%.
[c] Ref. [17].
[d] Ref. [22].
[e] Ref. [7].
[f] Ref. [24].
[g] Ref. [35].
[h] Ref. [5].
[i] Ref. [36].
[j] Ref. [25].

[34,35] and few, if any, branching side chains.

In the mixed solvent of 6:1 Me_2SO-d_6–D_2O, curdlan gave a DP_n value of 163. By increasing the Me_2SO-d_6 content in the solvent to a 9:1 ratio, however, the DP_n increased to 225. This discrepancy might result from the changes of solubility and conformation due to both the water content and the polydispersity intrinsic to curdlan [36,37], although it dissolved optically in the solvents with different Me_2SO-d_6 concentration. The ratio of α- and β-H-1 and the DB of curdlan were calculated to be 1:1.25 and 0, respectively.

The DP_n and DB of pachyman were calculated to be 540 and 0.003, respectively. The DP_n value of pachyman was the largest one among the β-D-glucans measured by NMR analysis reported in our study.

Structural analysis of scleroglucan.—As shown in Fig. 5(E), the peak integral ratio between internal backbone H-1 and side chain H-1 was 3:1, which results in the DB value of 0.33 by Eq. (2) (Table 3), showing a good agreement with the previously reported data [25]. We were not able to obtain the DP_n and the α:β ratio because the H-1 peaks of RT and NRT were too small to be used in quantitative

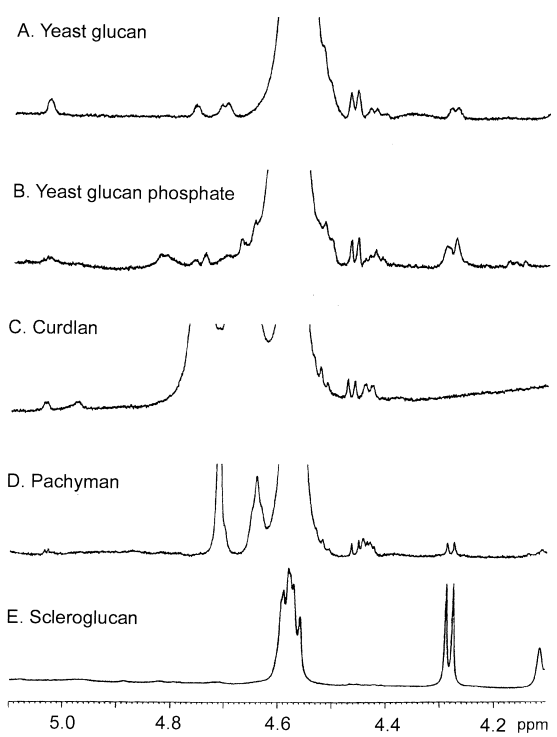

Fig. 5. ^1H NMR spectrum of β-D-(1→3, 1→6)-linked glucans in a mixed solvent (6:1 Me_2SO-d_6–D_2O) at 80 °C.

Table 4
^{13}C NMR chemical shifts of a series of β-D-(1→3, 1→6)-linked glucans in Me_2SO-d_6 at 80 °C

Compound	Unit	C-1	C-2	C-3	C-4	C-5	C-6
Laminaraheptaose		102.81	72.39	86.01	68.16	75.94	60.59
	α [a]	91.46					
	β [a]	96.10					
Laminaran		105.34	76.04	87.36	71.03	78.50	63.63
Yeast glucan		104.62	74.23	88.09	69.93	78.05	62.46
Glucan phosphate		104.73	74.63	87.90	70.23	78.15	62.95
Scleroglucan	I A [b]	–	74.14	88.22	70.01	78.05	62.56
	II B [b]	104.60	74.14	88.00	69.98	76.48	70.01
	III C [b]	–	74.33	88.37	70.31	78.05	62.46
	IV D [b]	–	75.29	78.37	71.94	77.87	62.83

[a] α and β = the configuration of reducing terminal C-1 units.
[b] I, II, III and IV stand for glucose units (Fig. 1).

analysis, although their presence was observable in the spectrum (4.3 ~ 5.1 ppm, Fig. 5(E)). Pretus et al. [25] reported that the number average molecular mass and polydispersity of scleroglucan studied in this work were 850,000 (DP 9700) and 1.83, respectively. Numerous studies [38–42] reported that the β-glucans existed predominantly in single-stranded conformation in Me_2SO-d_6, NaOH solution, and even in mixed solvent containing water (below 50%), regardless of the linearity. The internal H-1 resonances of scleroglucan appeared as three doublets at 4.56 ~ 4.58 ppm, and that of β-D-(1→6)-linked side chain H-1 appeared as a doublet at 4.28 ppm. The NMR result agreed with the primary structure of scleroglucan previously reported, the repeating trimers with one branching point out of three [37,41]. Our NMR result further proved that the number of glucose units in the side chain is one, since the peak integral ratio of internal H-1 and side chain H-1 was exactly 3:1, which fits the trimer structure. The simplicity of the internal H-1 peak (three doublets) confirmed that the polymer existed as a random coil of single strand rather than other forms of multiple strands.

The ^{13}C spectrum of scleroglucan also showed four resolved peaks of C-1 with slightly different chemical shifts (104.49 ~ 104.73 ppm), as shown in Table 4. The clearly resolvable resonances of C-2, C-3 and C-5 provided the approximate DB values to be 0.32 ~ 0.36, which agreed with that obtained by ^1H NMR analysis (DB 0.33).

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