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Structural studies of the capsular polysaccharide of a non-*neoformans Cryptococcus* species identified as *C. laurentii*, which was reclassified as *Cryptococcus flavescens*, from a patient with AIDS

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Abstract—*Cryptococcus flavescens*, a strain originally identified as *C. laurentii*, was isolated from the cerebrospinal fluid of an AIDS patient, and the soluble capsular polysaccharide of the yeast was investigated. Glucuronoxylomannan (GXM) was obtained from *C. flavescens* under conditions similar to those used to obtain *C. neoformans* polysaccharide. However, the GXM differed from *C. neoformans* polysaccharide in the decreased *O*-acetyl group content. The structure of GXM was determined by methylation analysis, partial acid hydrolysis, NMR analyses, and controlled Smith degradation. These analyses indicated that GXM has the following structure: an α-(1 → 3)-D-mannan backbone with side chains of β-D-glucuronic acid residues bound to the C-2 position of the mannose residue. The C-6 position of the mannose is substituted with D-man-β-(1 → 4)-D-xyl-β-(1 → disaccharide. Furthermore, the existence of side chains containing more than two xylose residues was suggested. This mannosylxylose side chain is a novel structure in polysaccharides of *C. neoformans* and other *Cryptococcus* species.

Keywords: *Cryptococcus laurentii*; *Cryptococcus flavescens*; Polysaccharide; Capsule; Structure

1. Introduction

The capsulated yeast *Cryptococcus neoformans* causes life-threatening cryptococcosis infection. Recently, the incidence of opportunistic infections due to non-*neoformans Cryptococcus* species, *C. albidus* and *C. laurentii*, has increased and they can be considered emerging fungal infections.[1–3] *C. neoformans* virulence factors include the polysaccharide capsule, growth ability at 37 °C, and melanin synthesis. We have described the ability of *C. albidus*, *C. laurentii*, and *C. curvatus* strains to synthesize melanin,[4] although these strains oxidize L-DOPA at a slower rate than does *C. neoformans*. A study of serological relationships revealed that *C. albidus* and *C. curvatus* cross-react with factor sera prepared using *C. neoformans* serotypes, whereas *C. laurentii* cells do not.[5] This suggests that the chemical structure of *C. laurentii* capsular polysaccharide might differ from the capsular polysaccharides of *C. neoformans* serotypes.[6] Carbohydrates located on the surface of microorganisms are responsible for interactions with other cells, such as recognition by immune cells or adherence to target cells. These carbohydrates may play an important role in disease progression. Therefore, a comparison of the surface polysaccharide structural features of *C. laurentii* clinical isolates and *C. neoformans* serotypes could provide useful information about the role of carbohydrates as virulence factors. This study describes a novel structure of extracellular polysaccharide from *C. laurentii*, which was reclassified as *Cryptococcus flavescens*, isolated from the cerebrospinal fluid of a patient with AIDS.

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2. Results

2.1. Capsule formation

The capsule was observed with an India ink preparation using cells grown on YM agar for two days at 27 °C. The thickness of the capsule varied within cells (Fig. 1).

2.2. Fractionation of the extracellular polysaccharide

The protease-treated polysaccharide was separated into six fractions (F-1–F-6) on a DEAE-Toyopearl 650 M column (Fig. 2). Fraction F-4 was eluted at the same NaCl concentration (about 0.15 M) as the major fraction of *C. neoformans.*[7] Fraction F-4 was subjected to chemical analysis.

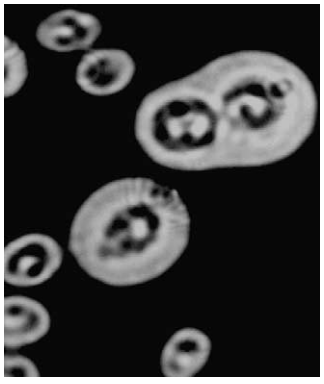

Figure 1. Extracellular polysaccharide from strain CBS8645.

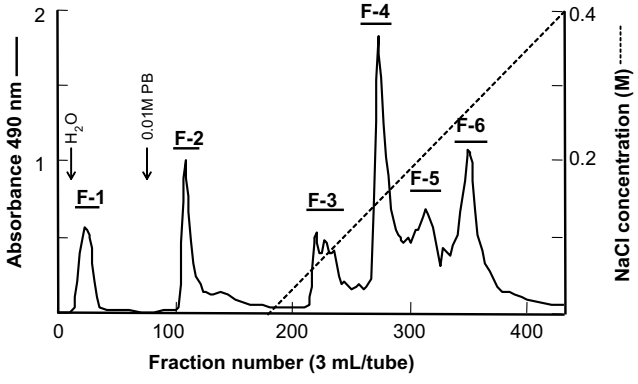

Figure 2. Ion-exchange chromatography of the polysaccharide from strain CBS8645 on DEAE-Toyopearl 650 M.

Table 1. Sugar composition of polysaccharides

Component sugar	Molar ratio[a]	
	F-4	Carboxyl reduced F-4
Xylose	0.8	1.1
Mannose	4.0	4.0
Glucose	Trace	0.7
Glucuronic acid	+	–

[a]The alditol acetates were identified by their retention times relative to xylitol pentaacetate.

The signal at 2.18 ppm in the NMR spectra of F-4 showed the presence of an *O*-acetyl group. The content was approximately 7%, from the integrated value. This is less than in *C. neoformans* serotypes A, D, and B, which contain 8–17%.[8]

2.3. Sugar composition of the polysaccharide

Table 1 lists the fraction F-4 sugar components and molar ratios. Glucuronic acid was measured as glucose after carboxyl reduction of fraction F-4. The hydrolysates of F-4 contained mannose, xylose, and glucuronic acid with molar ratios of about 4:1:0.7. Fraction F-3 contained galactose in addition to the components of F-4.

2.4. Methylation analyses of polysaccharides

Methylation analysis of F-4 and carboxyl-reduced F-4 polysaccharide revealed the presence of 2,3,4,6-tetra-*O*-methyl-glucitol and mannitol, 2,3-di-*O*-methyl-xylitol, 2,4,6-tri-*O*-methyl-mannitol, 2,4-di-*O*-methyl-mannitol, and 4-*O*-methyl-mannitol (Table 2). The identification of 2,3-di-*O*-methyl-xylitol was confirmed by reducing methylated sugars with $NaBD_4$ and analyzing the reduced sugars with gas chromatography–mass spectrometry (GLC–MS). Although the prominent peak at 118 m/z was observed, the peak at 117 m/z was absent. This suggests that the internal xylose residues were substituted at C-4.

Table 2. Methylation analysis of polysaccharides[a]

Methylated residue	Molar ratio				Structural units deduced
	F-4	Carboxyl reduced F-4	A1-1	Carboxyl reduced A1-1	
Xyl-2,3,4	Trace	Trace	0.26	0.47	Xyl*p*-(1→
Glc and/or Man-2,3,4,6	0.99	1.62	1.15	1.40	Glc*p* and/or Man*p*-(1→
Xyl-2,3	2.40	2.46	1.00	1.00	→4)-Xyl*p*-(1→
Man-2,4,6	3.00	3.00	3.10	2.97	→3)-Man*p*-(1→
Man-2,4	1.23	0.54	0.88	0.29	→3,6)-Man*p*-(1→
Man-4	0.63	1.98	0.70	0.43	→2,3,6)-Man*p*-(1→

[a]The methylated alditol acetates were identified by their retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

After reduction, 2,3,4,6-tetra-*O*-methyl-glucitol and mannitol were increased, because glucuronic acid was converted to glucose. However, more derivatives of 2,4-di-*O*-methyl-mannitol were observed in fraction F-4 than in the reduced fraction. We speculate that the glucuronic acid linkage at the C-2 of mannose was released by β-elimination due to repeated base treatment with methylsulfinyl sodium during methylation.[9]

2.5. Analyses of oligosaccharides

Neutral and acidic oligosaccharides were obtained by hydrolysis of fraction F-4 (Fig. 3). Under condition A, fractions N1-1 and N1-2 were obtained. N1-1 contained xylose and mannose in a ratio of 1:3.6. N1-2 was further hydrolyzed with 0.5 M TFA, at 95 °C, for 5 h to the monosaccharide level, and was found to be composed of mannose and xylose in a 1:1 ratio. The mode of linkage of N1-2 was determined by NMR spectroscopy (Fig. 4, Table 3). The signals at 5.15–5.16 ($J_{1,2}$ 3.4 Hz) and 4.56–4.58 ($J_{1,2}$ 7.8 Hz) were identified as the α and β configurations of xylose, respectively. The signal at 4.74 ppm was identified as β-D-mannose. The chemical shift

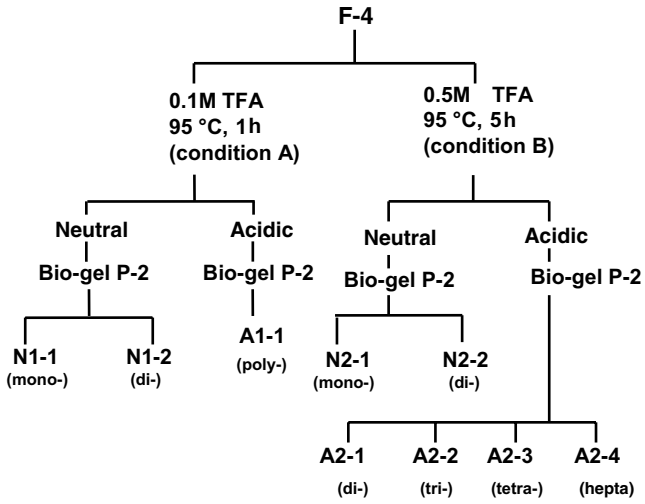

Figure 3. Preparation of the oligosaccharide by partial acid hydrolysis from F-4 polysaccharide.

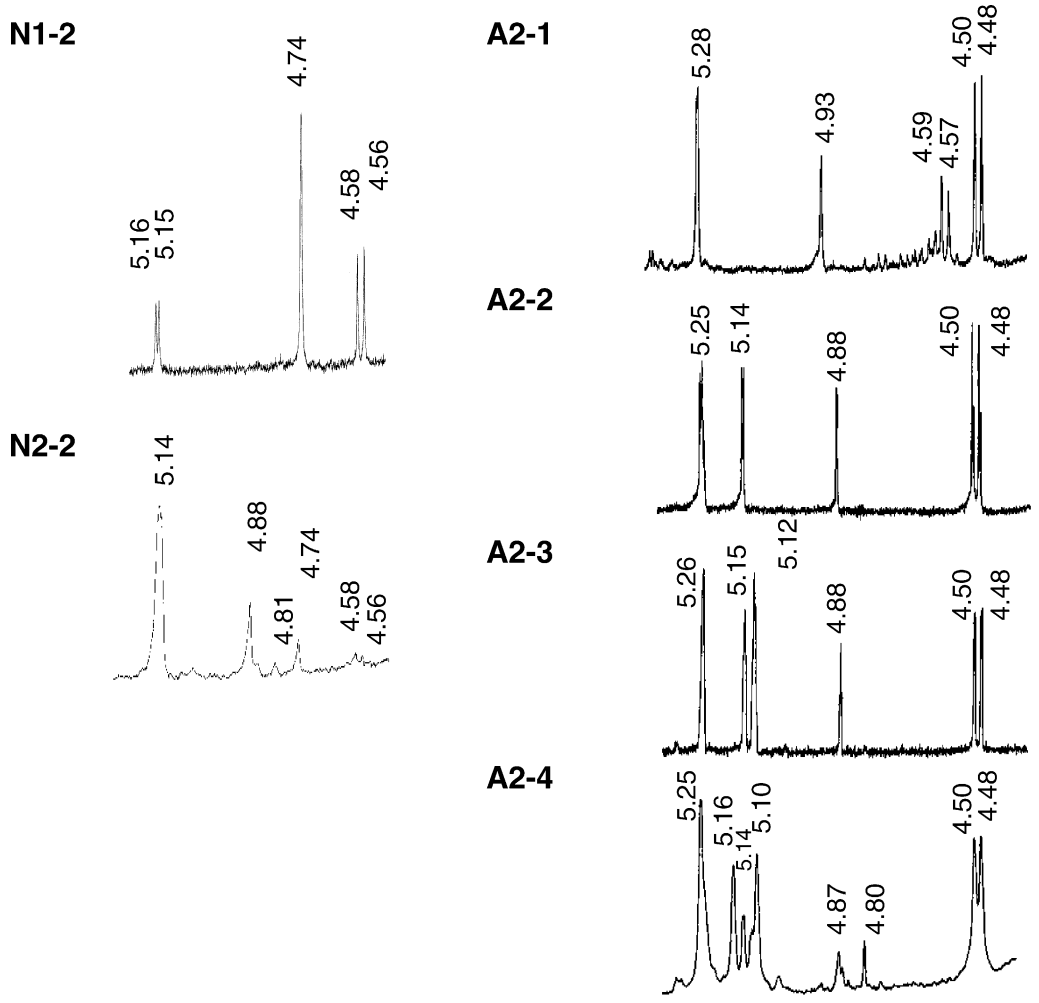

Figure 4. ^{1}H NMR spectra (H-1 region) of the acidic oligosaccharides and the neutral disaccharides obtained by partial acid hydrolysis.

Table 3. The structures and chemical shifts of oligosaccharides obtained by acid hydrolysis

Fraction	Sugar residue						Chemical shift of the component sugars ppm ($J_{1,2}$)					
	A	B	C	D	E	F	A	B	C	D	E	F
N1-2	β-Man-(1→4)-Xyl						4.74 (<1)	4.56, 4.58 (7.8) 5.15, 5.16 (3.4)				
N2-2	β-Man-(1→3)-Man (main) and β-Man-(1→4)-Xyl						5.14 (<1) 4.74 (<1)	4.88 (<1) 51.4 (<1) 4.56, 4.58 (7.8) 5.14 (<1)				
A2-1	β-GlcA-(1→2)-Man						4.48, 4.50 (7.8)	5.28 (1.5) 4.93 (<1)				
A2-2	β-GlcA-(1→2)-α-Man-(1→3)-Man						4.48, 4.50 (7.8)	5.25 (2.0)	5.14 (2.0) 4.88 (<1)			
A2-3	β-GlcA-(1→2)-α-Man-(1→3)-α-Man-(1→3)-Man						4.48, 4.50 (7.8)	5.26 (1.2)	5.12 (1.7)	5.15 (1.5) 4.88 (<1)		
A2-4	β-GlcA-(1→2)-α-Man-(1→3)-α-Man-(1→3)-α-Man-(1→3)-Man 2) ↑ β-GlcA-(1						4.48, 4.50 (7.6)	5.25 (<1)	5.16 (<1)	5.10 (<1)	5.25 (<1) 4.48, 4.50 (7.6)	5.14 (1.2) 4.87 (<1)

reported by Suzuki et al. in mannooligosaccharides[10] supports this identification.

The phase-sensitive NOESY spectrum showed a correlation peak of Man H-1/Xyl H-4 (4.74/3.78 ppm). Methylation analysis of N1-2 revealed the presence of 2,3,4,6-tetra-*O*-methyl-mannitol and 2,3-di-*O*-methyl-xylitol. Fraction N1-2 was found to be the disaccharide: D-Man-β-(1→4)-D-Xyl.

Partial acid hydrolysis under condition A produced fraction A1-1, which might be a polysaccharide after the loss of fractions N1-1 and N1-2. Fractions N1-1 and N1-2 could originate from side chains. Methylation analysis of reduced A1-1 indicated that the amount of nonreducing terminal xylose was increased to a detectable level, whereas 4-*O*-substituted xylose and the nonreducing terminal of mannose decreased. The amounts of 3,6- and 2,3,6-substituted mannose also decreased (Table 2). This revealed the presence of mannosylxylose side chains in fraction F-4.

Partial acid hydrolysis under condition B produced acidic oligosaccharides A2-1, A2-2, A2-3, and A2-4. The modes of linkage in the fractions were determined by ^{1}H NMR spectroscopy (Fig. 4, Table 3) with reference to our previous data.[7,11,12] Fraction A2-1 was identified as a disaccharide, fraction A2-2 as a trisaccharide, and fraction A2-3 as a tetrasaccharide containing a glucuronic acid residue as a nonreducing terminus. The gel filtration profile indicated that A2-4 was not a pentasaccharide. The ^{1}H NMR spectrum of A2-3 suggested that it is a heptasaccharide with two glucuronic acid residues. Methylation analyses of the polysaccharides suggested that the major polysaccharide of *C. laurentii* contains an α-(1→3)-linked mannan backbone. The ^{1}H NMR spectrum of the controlled Smith degradation product of F-4 contained a signal at 5.13 ppm that corresponded to the anomeric proton of mannose residues. It was assumed that the backbone contained two mannose residues with glucuronic acid side chains, and not a glucuronic acid residue flanked by two mannose residues.

Fraction N2-2, which was obtained by partial hydrolysis with 0.5 M TFA at 95 °C for 5 h, was identified as mannobiose with α-(1→3)-linkages derived from the mannan backbone (Fig. 4, Table 3).

3. Discussion

This study investigated the chemical structure of soluble capsular polysaccharide from *C. laurentii* CBS8645, a yeast recently reclassified as *C. flavescens*,[13] which was isolated from the cerebrospinal fluid of an AIDS patient. Chemical analyses suggested that the polysaccharide contains an α-(1→3)-linked mannan backbone with glucuronic acid side chains at C-2. This structure is

common in reported *C. neoformans* serotypes.[14,15] The polysaccharide fraction investigated here was eluted from DEAE-Toyopearl column with 0.15 M NaCl under the same conditions as described for the major fraction of *C. neoformans*.[7] Although the oligosaccharides containing glucuronic acids were very similar to those derived from *C. neoformans* polysaccharides, striking differences were observed. The results of methylation analysis suggested that the mannose residue substituted at C-2 with glucuronic acid has also the mannosylxylose substituent at C-6 position. The oligosaccharide side chain containing xylose and mannose was characteristic for this polysaccharide. Furthermore, the number of *O*-acetyl groups, which are detected in *C. neoformans* and *Trichosporon asahii* at C-6 of mannose in the backbone, was decreased more in *C. flavescens* than in *C. neoformans* serotypes A, B, and D. Since the *O*-acetyl group contributes to serological reactivity,[7,12,16] the differences described in this study could explain the lack of reactivity of *C. flavescens* to anti-*C. neoformans* sera.

Suggested partial structures of the extracellular polysaccharide from strain CBS8645 are shown in Fig. 5. The major parts of the polysaccharide were determined directly using oligosaccharides. In the methylation analyses of F-4 and reduced F-4 polysaccharide, there were fewer hexose derivatives from the nonreducing end than expected. Although cross-linking could not be ruled out, this possibility is unlikely in a yeast polysaccharide.

The amounts of C-4 substituted xylose in F-4 and reduced F-4 suggest the existence of two or more xylose residues in the side chains. The number could vary with the side chain. In A1-1 polysaccharide obtained after partial acid hydrolysis under condition A, the ratio of nonreducing terminal mannose and $(1\rightarrow4)$-linked xylose was about 1:1, suggesting that the side chain is composed of equal numbers of mannose and xylose residues after the hydrolysis.

F-4 polysaccharide had an α-$(1\rightarrow3)$-mannan backbone substituted at C-6, or at C-2 and C-6. Since glucuronic acid was linked to C-2 of a mannose residue, xylose might be linked to C-6. However, not every mannose residue in the backbone with a side chain would have a glucuronic acid residue, because some had only a side chain composed of xylose and mannose.

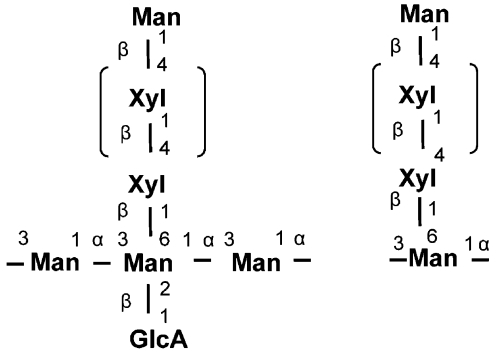

Figure 5. Suggested partial structures of extracellular polysaccharide from strain CBS8645 (Man = mannose; Xyl = xylose; GlcA = glucuronic acid).

Perry and Webb[17] and Matulová et al.[18] investigated the chemical structures of polysaccharides from *C. laurentii*. Perry and Webb reported glucuronoxylomannan and Matulová et al. examined another fraction, galactoglucoxylomannan. The structure reported here is similar to the structure of glucuronoxylomannan from *C. laurentii* NRRL Y-1401, except for the finding of a nonreducing terminal mannose residue in the side chains. The strain investigated in this study was isolated from a clinical specimen identified as *C. flavescens*.[13]

We are interested in the role of surface polysaccharide in interactions with host animal cells, and the importance of structural characteristics in these interactions. Here, we report on a side chain with a novel structure. In *C. flavescens*, a nonreducing terminal xylose is masked by mannose, whereas polysaccharides from *C. neoformans* serotypes contain side chains of xylose residues that are not masked. This difference might affect the adherence of yeast cells to other cells or recognition by other cells. However, the relationship between the structural difference and pathogenicity is not known.

4. Experimental

4.1. Strains used

C. laurentii strain CBS8645 isolated from the cerebrospinal fluid of the patient with AIDS[19] was used in the study. Recently, analysis of the sequences of the internal transcribed spacer regions and the 28S rRNA gene showed that *C. laurentii* is a genetically heterogeneous species.[20] Strain CBS8645 has been reclassified as *C. flavescens*.[13]

4.2. Extraction of extracellular polysaccharide

The strain was cultured in yeast nitrogen base broth (Difco Laboratories, Detroit, MI, USA) containing 2% glucose, 1% casamino acids, and 100 μg/mL streptomycin at 27 °C for 5 days with shaking. Cells were separated by centrifugation and the culture supernatant was concentrated. After dialysis, 9 vol of EtOH and 1/10 vol of 10% AcONa solution were added. The collected precipitate was dissolved, dialyzed against water, and lyophilized. Crude polysaccharide was treated with Actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) at 40 °C for 24 h, to degrade protein.

Purification of polysaccharide by ion-exchange column chromatography—The deproteinized polysaccharide was purified by DEAE-Toyopearl 650 M (Tosoh Co., Tokyo, Japan) column chromatography. Unbound material was eluted with water at a flow rate of 3.5 mL/min, and bound polysaccharides were eluted with 0.01 M sodium phosphate buffer (PB), pH 7.4, and then eluted with a linear gradient of 0–0.5 M NaCl in PB. The total carbohydrate content of the effluents was determined using the phenol–H_2SO_4 method.

4.3. Qualitative and quantitative analysis of the constituent carbohydrate of the polysaccharide

The polysaccharide was hydrolyzed in 0.5 M H_2SO_4 at 100 °C for 18 h. The hydrolysate was neutralized with $BaCO_3$ and analyzed by TLC on a silica gel with *n*-butanol–EtOH–water (40:11:19 v/v). The hydrolysate was reduced with $NaBH_4$ and then acetylated with Ac_2O–pyridine (1:1 v/v). Alditol acetate was analyzed using a gas chromatograph GC-7A (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a flame-ionization detector in a glass column containing 3% ECNSS-S on a Gas Chrom Q at 190 °C. Nitrogen was used as the carrier gas at 40 mL/min. To identify uronic acid, the carbohydrate was converted into its corresponding carboxyl-reduced product and analyzed by gas chromatography (GLC) as described for alditol acetate.

4.4. NMR analysis

^{1}H NMR spectra were recorded with a GSX-400 NMR spectrometer (JEOL, Tokyo, Japan) in 99.9% D_2O, at 90 °C; sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) was the external standard. Two-dimensional (2D) NMR spectra were recorded with a JNM-LA500 NMR spectrometer (JEOL).

4.5. Carboxyl reduction

The acidic sugars were converted into corresponding neutral sugars according to the method of Taylor et al.[21] Polysaccharide carboxyl groups were reduced by treatment with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride maintained at pH 4.7, followed by $NaBH_4$.

4.6. Methylation analysis

Polysaccharides were methylated using the method of Hakomori,[22] as described by Hellerqvist et al.[23] The methylation procedure was repeated three times to complete the reaction. The methylated polysaccharides were hydrolyzed in 0.25 M H_2SO_4 at 100 °C for 12 h. The partially methylated alditol acetate was analyzed by GLC and GLC-MS with a JMS-700 (JEOL) using an HP-5 column (30 m × 0.32 mm, Agilent). Mass spectra (75 eV) were recorded using an ion-source temperature of 190 °C, an accelerating voltage of 10 kV, and an ionizing current of 300 μA.

4.7. Partial acid hydrolysis

Polysaccharide was hydrolyzed under the following two conditions: (A) 0.1 M trifluoroacetic acid (TFA) for 1 h at 95 °C or (B) 0.5 M TFA for 5 h at 95 °C. In condition A, the partially hydrolyzed products were separated into neutral and acidic fractions by chromatography on a AG1-X2 column eluted with water and then with 10% formic acid.

4.8. Bio-Gel P-2 gel-filtration chromatography

The partially hydrolyzed products were applied to a 130 × 2.5-cm Bio-Gel P-2 column equilibrated with a water–pyridine–AcOH (500:5:2 v/v) solvent system and eluted at a flow rate of 6 mL/h; 2.0 mL fractions were collected.

4.9. Controlled Smith degradation

Polysaccharide was oxidized with 0.02 M sodium metaperiodate for 120 h. The oxidized solution was dialyzed against water, reduced with sodium borohydride, and then hydrolyzed with 0.5 M HCl at room temperature for 30 h.

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