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Preparation of a Molecular Library of Branched β -Glucan Oligosaccharides Derived from Laminarin

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Abstract: To study the structure of β -glucans, we developed a separation method and molecular library of β -glucan oligosaccharides. The oligosaccharides were prepared by partial acid hydrolysis from laminarin, which is a β -glucan of *Laminaria digitata*. They were labeled with the 2-aminopyridine fluorophore and separated to homogeneity by size-fractionation and reversed phase high-performance liquid chromatography (HPLC). Branching structures of all isomeric oligosaccharides from trimers to pentamers were determined, and a two-dimensional (2D)-HPLC map of the β -glucan oligosaccharides was made based on the data. Next, structural analysis of the longer β -glucan oligosaccharide was performed using the 2D-HPLC map. A branched decamer oligosaccharide was isolated from the β -glucan and cleaved to smaller oligosaccharides by partial acid hydrolysis. The structure of the longer oligosaccharide was successfully elucidated from the fragment structures determined by the 2D-HPLC map. The molecular library and the 2D-HPLC map described in this study will be useful for the structural analysis of β -glucans.

Key words: β -glucan, oligosaccharides, fluorescence, molecular library, 2D-HPLC map

INTRODUCTION

Beta (β)-glucans are widely distributed in the cell walls of plants, fungi, and bacteria. Some are utilized as anti-cancer drugs or as components of functional foods because they have immune-stimulating activity.^{1,2)} Among the β -glucan receptors that have been identified on human immune cells, Dectin-1 possesses a C-type lectin domain.^{3,4,5)} Structural information concerning the binding sites of β -glucans to Dectin-1 is limited.^{6,7)} One of the major reasons is the difficulty of fine structural analysis of branched β -glucans.

Laminarin from *Laminaria digitata* is a β -1,3-glucan containing approximately 25 glucosyl residues with several mono- β -1,6-glucosyl branches.^{8,9,10,11)} Laminarin is a good ligand for Dectin-1.^{6,7,12)} The branched structure of β -glucan is essential for the high affinity binding to Dectin-1. The estimated minimum size is approximately 10-mer.

Eighty-nine isomeric structures have been theoretically predicted for β -1,3-glucan oligosaccharides with mono- β -1,6-glucosyl branches consisting of 10 glucose residues. As the isomers have quite similar structures, it is very difficult to isolate them using chromatography.

To overcome this difficulty, we developed a method that combines fragmentation and high-performance liquid chromatography (HPLC) mapping of β -glucan oligosaccharides. β -glucanases are also a powerful tool for the structural analysis of β -glucans. However, their use in structural analyses requires accurate knowledge of substrate specificity. The molecular library prepared in this study could be useful to analyze the fine substrate specificity of β -glucanases.

MATERIALS AND METHODS

Materials. Curdlan was purchased from Wako Pure Chemical Industries (Osaka, Japan). Laminarin of *Laminaria digitata* was from Sigma-Aldrich (St. Louis, MO, USA). The Glc β 1-3Glc-pyridylaminated (PA) and Glc β 1-6Glc-PA disaccharide standards were prepared from laminaribiose and gentiobiose, respectively, which were purchased from Sigma-Aldrich.

Preparation of PA-oligosaccharides. The β -glucans were dissolved or suspended in 0.5 M HCl and heated in boiling water for 5 to 20 min. After neutralizing with aqueous ammonia, the solution was lyophilized. The hydrolyzed frag-

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Abbreviations: 2D-HPLC map, two-dimensional HPLC map; DP, degree of polymerization; LMO, laminarioligosaccharides; LMO3, laminaritriose; LMO4, laminaritetraose; LMO5, laminaripentaose; LMO6, laminarihexaose; LMO7, laminariheptaose; LMO8, laminarioctaose; PA-, pyridylamino.

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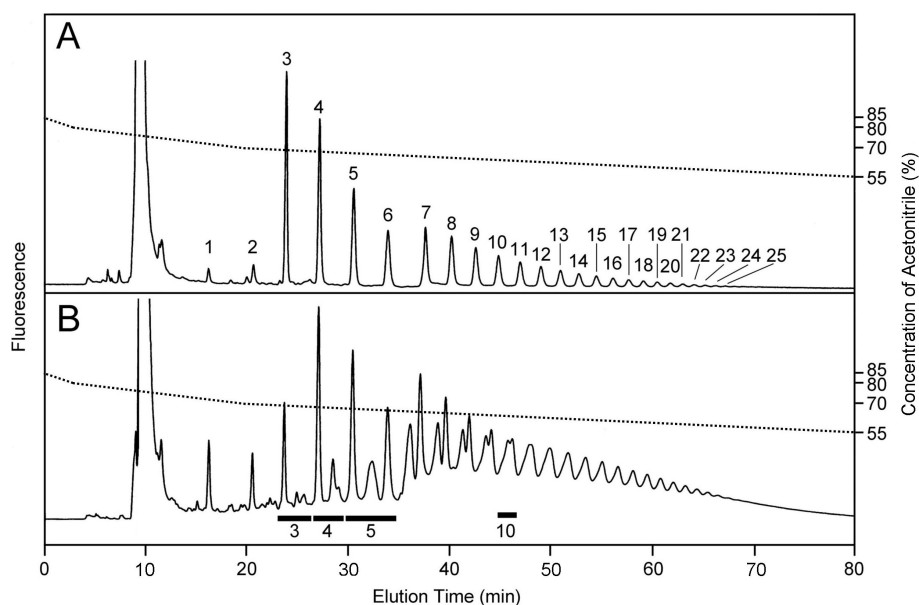


Fig. 1. Separation of PA- β -glucan oligosaccharides on normal phase HPLC.

(A) PA-laminarioligosaccharides derived from curdlan. Peak-top numbers indicate DP of Glc. (B) PA- β -oligosaccharides derived from laminarin. Fractions indicated by bars were collected. Fraction numbers indicate DP of oligosaccharides contained in the fraction. Dotted lines indicate the concentration of acetonitrile.

ments were labeled with 2-aminopyridine as previously described.¹³ Excess reagents were removed with phenol-chloroform extraction and Sep-Pak C18 cartridge (Waters Corp., Milford, MA, USA) as previously described.^{14/15}

Normal phase HPLC of PA-oligosaccharides. Size-fractionation HPLC was performed as previously described with slight modification.¹⁶ Two conditions (A and B) were used in this study. Condition A was for separation of hydrolysis products of polysaccharides, curdlan and laminarin. Condition B was used for the analysis of the hydrolysis product of PA-oligosaccharides. Briefly, for analysis condition A, a TSK gel Amide 80 column (0.46×25 cm; Tosoh Corporation, Tokyo, Japan) was used at a flow rate of 0.8 mL/min. The column was equilibrated with 50 mM ammonium formate, pH 4.4, containing 85 % acetonitrile. After a sample was injected, the acetonitrile concentration was decreased linearly from 85 to 80 % in the first 3 min, from 80 to 70 % in the next 17 min and then from 70 to 55 % in the next 60 min. For the analysis condition B, a TSK gel Amide 80 column (0.46×7.5 cm, Tosoh) was used at a flow rate of 0.5 mL/min. The column was equilibrated with 50 mM ammonium formate, pH 4.4, containing 80 % acetonitrile. After the sample was injected, the acetonitrile concentration was decreased linearly from 80 to 65 % over the first 5 min, from 65 to 55 % over the second 5 min and then from 55 to 30 % over the next 25 min. The molecular size of each PA-oligosaccharide was expressed in terms of the glucose units based on the elution times of PA-LMO, which were prepared from curdlan by partial hydrolysis. The elution times of PA-LMO and the number of glucose residues were related with an approximate curve of a quintic function. The glucose units of PA-oligosaccharides were calculated from the quintic function as a degree of glucose residues.¹⁶

Reversed phase HPLC of PA-oligosaccharides. Reversed phase HPLC was also performed as previously described

with slight modification.¹⁶ Briefly, a Cosmosil 5C18-P column (0.2×25 cm; Nacalai Tesque, Inc., Kyoto, Japan) was used at a flow rate of 0.2 mL/min. The column was equilibrated with 50 mM ammonium acetate, pH 5.0. After a sample was injected, the acetonitrile concentration increased linearly from 0 to 10 % in 60 min. The PA-glycans were detected at an excitation wavelength of 315 nm and an emission wavelength of 400 nm. The retention time of each PA-oligosaccharide was given in terms of the glucose units based on the elution times of the PA-LMO.

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. PA-oligosaccharide separated by HPLC was lyophilized to remove volatile salts in the buffer, and approximately 10 pmol of sample were co-crystallized with 1 mg/mL of 2,5-dihydroxybenzoic acid in acetonitrile on an AnchorChip target plate (Bruker Daltonics Inc., Billerica, MA, USA) according to the manufacturer's protocol. MALDI-TOF mass spectra were recorded using an Autoflex II (Bruker Daltonics) in reflector mode.

Limited acid-hydrolysis of PA-oligosaccharides. The isolated PA-oligosaccharides were partially hydrolyzed with 1 M trifluoroacetic acid at 100 °C for 30 min.¹⁷ After complete evaporation of the trifluoroacetic acid, the hydrolyzed fragments were analyzed sequentially by normal phase and reversed phase HPLC.

RESULTS AND DISCUSSION

Preparation of standard PA-LMO.

Curdlan, a linear polymer with a Glc β 1-3Glc sequence, was partially hydrolyzed and labeled with 2-aminopyridine. The mixture of PA-LMO was separated by normal phase HPLC using condition A as described above. Monomer to 25-mer forms were well separated under this condition

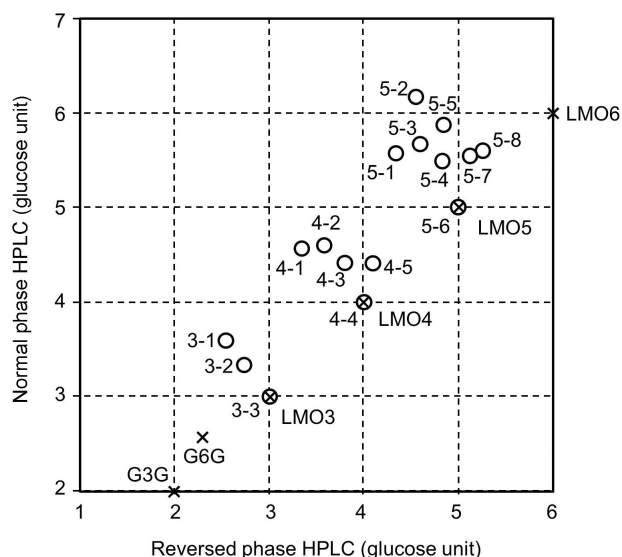


Fig. 2. 2D-HPLC map of PA- β -oligosaccharides.

The elution times on normal phase and reversed phase HPLCs were converted to glucose units and are displayed as a two-dimensional scatter plot. The cross marks indicate the positions of standard PA-disaccharides and PA-LMO prepared from curdlan. The circles indicate the positions of PA- β -oligosaccharides derived from laminarin. Proposed structures of the glycans are 3-1, Glc β 1-3(Glc β 1-6)Glc-PA; 3-2, Glc β 1-6Glc β 1-3Glc-PA; 3-3, Glc β 1-3Glc β 1-3Glc-PA; 4-1, Glc β 1-6Glc β 1-3(Glc β 1-6)Glc-PA; 4-2, Glc β 1-3Glc β 1-3(Glc β 1-6)Glc-PA; 4-3, Glc β 1-3(Glc β 1-6)Glc β 1-3Glc-PA; 4-4, Glc β 1-3Glc β 1-3Glc β 1-3Glc-PA; 4-5, Glc β 1-6Glc β 1-3Glc β 1-3Glc-PA; 5-1, Glc β 1-3(Glc β 1-6)Glc β 1-3(Glc β 1-6)Glc-PA; 5-2, Glc β 1-6Glc β 1-3Glc β 1-3(Glc β 1-6)Glc-PA; 5-3, Glc β 1-3Glc β 1-3Glc β 1-3(Glc β 1-6)Glc-PA; 5-4, Glc β 1-3Glc β 1-3(Glc β 1-6)Glc β 1-3Glc-PA; 5-5, Glc β 1-6Glc β 1-3(Glc β 1-6)Glc β 1-3Glc-PA; 5-6, Glc β 1-3Glc β 1-3Glc β 1-3Glc β 1-3Glc-PA; 5-7, Glc β 1-6Glc β 1-3Glc β 1-3Glc β 1-3Glc-PA; 5-8, Glc β 1-3(Glc β 1-6)Glc β 1-3Glc β 1-3Glc-PA.

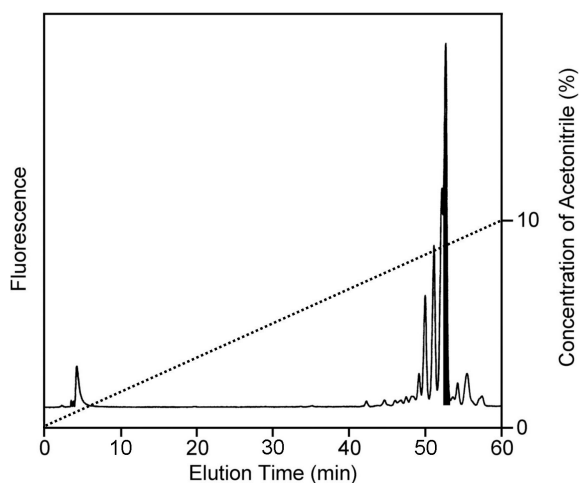


Fig. 3. Separation of PA-branched decamer derived from laminarin.

Fraction 10 in Fig. 1B was chromatographed on reversed phase HPLC. The darkened peak was collected as a major decamer PA- β -oligosaccharide for structure analysis. Dotted line indicates the concentration of acetonitrile.

(Fig. 1A). The elution times of separated PA-oligosaccharides were converted to glucose units by comparison with the elution times of the PA-LMO standard to reduce errors between the experiments.

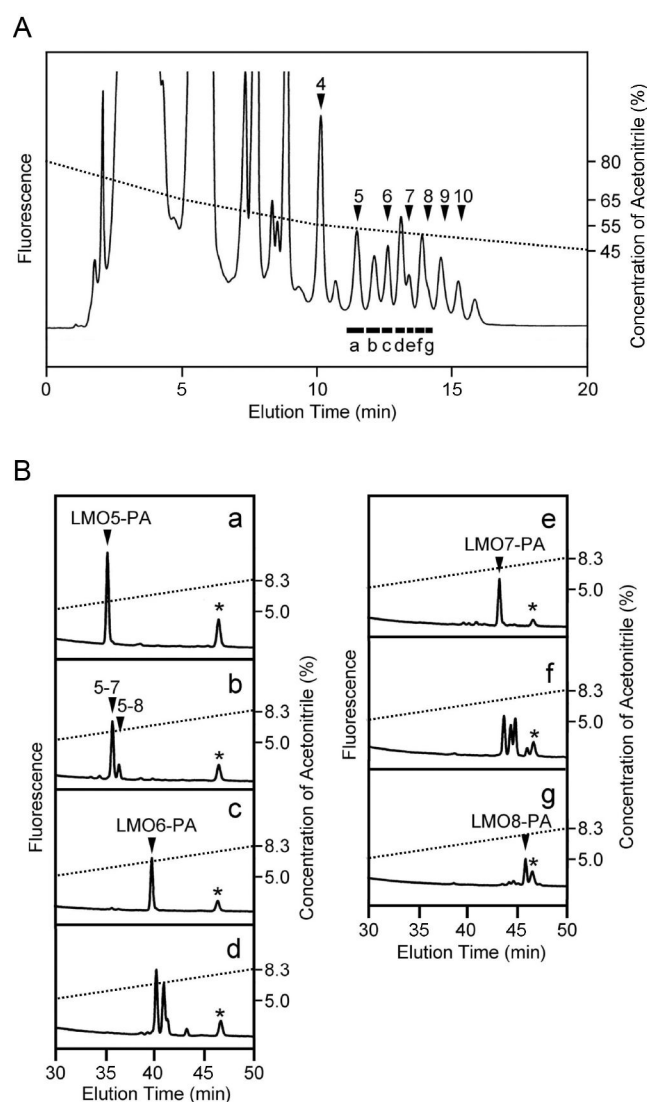


Fig. 4. Separation of limited hydrolysates of the PA-decamer.

(A) Normal phase HPLC using condition B. Numbered arrow heads indicate the elution positions of PA-LMO with corresponding DP. Fractions a to g were collected. (B) Fractions a to g were further separated using reversed phase HPLC. Asterisks indicate non-oligosaccharide peaks, arrow heads indicate the elution positions of PA- β -oligosaccharides, and dotted lines indicate the concentration of acetonitrile.

Isolation of PA-oligosaccharides from *L. digitata* laminarin.

PA-oligosaccharides were prepared from the laminarin of *L. digitata* in the same way as described above for curdlan. The mixture of the PA-oligosaccharides was separated with normal phase HPLC (Fig. 1B). The branched isomers consisting of glucans containing β 1-6Glc were eluted later than linear PA-LMO. Peaks containing trimer, tetramer and pentamer were collected as fractions 3, 4, and 5, respectively (Fig. 1B). Three, five and eight isomers of PA-oligosaccharides were isolated from the fractions of trimer, tetramer and pentamer, respectively, by further separation on reversed phase HPLC. The elution positions of each isolated PA-oligosaccharide were depicted together as a two dimensional (2D)-HPLC map (Fig. 2).

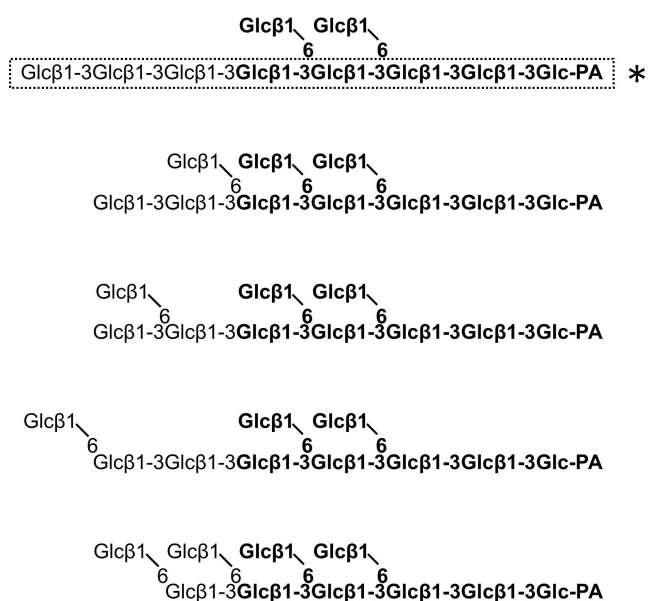
Table 1. Isomeric analysis of tri-, terta- and pentamer PA- β -oligosaccharides derived from laminarin.

Tri	Reducing end fragments		Structure
	3G* ¹	6G	
3-1	+* ²	+	3(6)G* ³
3-2	+	-	63G
3-3	+	-	33G

Tetra	Reducing end fragments					Structure
	3G	6G	3(6)G	63G	33G	
4-1	+	+	+	+	-	63(6)G
4-2	+	+	+	-	+	33(6)G
4-3	+	-	-	+	+	3(6)3G
4-4	+	-	-	-	+	333G
4-5	+	-	-	-	+	633G

Penta	Reducing end fragments										Structure
	3G	6G	3(6)G	63G	33G	63(6)G	33(6)G	3(6)3G	633G	333G	
5-1	+	+	+	+	+	+	+	+	-	-	3(6)3(6)G
5-2	+	+	+	-	+	-	+	-	+	-	633(6)G
5-3	+	+	+	-	+	-	+	-	-	+	333(6)G
5-4	+	-	-	+	+	-	-	+	-	+	33(6)3G
5-5	+	-	-	+	+	-	-	+	+	-	63(6)3G
5-6	+	-	-	-	+	-	-	-	-	+	3333G
5-7	+	-	-	-	+	-	-	-	-	+	6333G
5-8	+	-	-	-	+	-	-	-	+	+	3(6)33G

*¹ 3, 6 and G represent β -1,3 linked Glc, β -1,6 linked Glc and Glc-PA, respectively. *² Detected fragments are indicated as +, and deduced structures are listed in the rightmost column. *³ Parentheses represent branched residue.

**Fig. 5.** Candidate structures of the PA-decamer.

Five candidates were proposed from pentamer analysis of the hydrolysates. The common structure is shown in bold. The box with dotted line shows PA-LMO8 detected in the fraction g (Fig. 4B). Asterisk indicates the proposed structure of the decamer.

Structural determination of the PA-oligosaccharides.

Three trimer PA-oligosaccharides designated 3-1, 3-2, and 3-3 were cleaved by limited acid hydrolysis, followed by separation on normal phase and reversed phase HPLCs. Because the treatment for 3-1 gave rise to both laminaribiosyl-PA and gentiobiosyl-PA as its reducing end fragments,

the structure of 3-1 was determined to be Glc β 1-3(Glc β 1-6)Glc-PA (Table 1). Since the elution positions of 3-3 corresponded with those of PA-LMO3, the structure of 3-3 was determined to be Glc β 1-3Glc β 1-3Glc-PA (Fig. 2). The reducing end fragment of 3-2 was only Glc β 1-3Glc-PA and did not coincide with PA-LMO3 on the 2D-HPLC map. Thus, the structure of 3-2 could be proposed as Glc β 1-6Glc β 1-3Glc-PA.

Structures of the five tetramers (4-1 to 4-5) and eight pentamers (5-1 to 5-8) were analyzed in the same way as the trimers. Deconvoluted structures from their reducing end fragments are listed in Table 1. All isomeric structures that were theoretically predicted for the β -1,3-glucan oligosaccharides with mono- β -1,6-glucosyl branches consisting of three to five glucoses were produced and detected.

Structural analysis of a decamer PA-oligosaccharide.

Next, structural analysis for a longer PA-oligosaccharide was done using the 2D-HPLC map. A major decamer fragment was prepared with normal phase HPLC (Fig. 1B) and reversed phase HPLC (Fig. 3). We could not determine whether the decamer was homogenous. However, we carried out the following experiment with the aim of determining the structure of a major decamer. The limited hydrolysis gave rise to three isomers of the pentamer from its reducing end. They corresponded to LMO5-PA, Glc β 1-6Glc β 1-3Glc β 1-3Glc β 1-3Glc-PA (5-7), and Glc β 1-3(Glc β 1-6)Glc β 1-3Glc β 1-3Glc-PA (5-8), respectively, in HPLC (Fig. 4B, a and b). As a result, the Glc β 1-3(Glc β 1-6)Glc β 1-3(Glc β 1-6)Glc β 1-3Glc β 1-3Glc-PA-branched heptamer

could be proposed as a reducing end structure, with five candidate decamer structures (Fig. 5). Furthermore, since LMO6-PA, LMO7-PA, and LMO8-PA were also detected as the reducing end fragments (Fig. 4B, c, e, and g), the structure of the decamer could be proposed as Glc β 1-3Glc β 1-3Glc β 1-3Glc β 1-3(Glc β 1-6)Glc β 1-3(Glc β 1-6)Glc β 1-3Glc β 1-3Glc-PA.

In this study, we successfully prepared a molecular library and 2D-HPLC map of branched β -glucan oligosaccharides from *L. digitata*. These contained isomers up to pentamer derived from the β -1,3-glucan oligosaccharides with mono- β -1,6-glucosyl branches. These could be powerful tools for isomer analysis of longer and branched β -oligosaccharides and will be used for the fine structure analysis of the physiologically active domain of β -glucan (e.g., the Dectin-1 ligand).

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