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Structure of β-1,3-Xylooligosaccharides Generated from *Caulerpa racemosa* var. *laete-virens* β-1,3-Xylan by the Action of β-1,3-Xylanase

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Recently we reported the molecular cloning and characterization of a novel β-1,3-xylanase from the marine bacterium *Vibrio* sp. AX-4 [Kiyohara *et al.* (2005) *Biochem. J.* 388, 949–957]. We report here the structural analysis of oligosaccharides generated from β-1,3-xylan of a siphonous green alga, *Caulerpa racemosa* var. *laete-virens*, by the action of β-1,3-xylanase. The enzyme degraded the polysaccharide producing oligosaccharides with different R_fs on TLC (EX2–EX5). Sugar component, linkage, and MALDI-TOF-MS analyses revealed that EX2 and EX3 were Xyl-1,3-Xyl and Xyl-1,3-Xyl-1,3-Xyl, respectively. On the other hand, EX4 was a mixture of Glc-1,3-Xyl-1,3-Xyl, Xyl-1,4-Xyl-1,3-Xyl and Xyl-1,3-Xyl-1,4-Xyl, while EX5 was a mixture of tetra-saccharides containing 3-substitued Glc in addition to the same components of EX4. Branching was not likely present in EXOs prepared from the polysaccharide by the enzyme. These results strongly suggest that the *C. racemosa* β-1,3-xylan is a linear heteropolysaccharide containing 1,3-Glc and 1,4-Xyl both of which are thought to be located within a β-1,3-Xyl chain and linked via covalent bonds. This report indicates the usefulness of the enzyme for the structural analysis of β-1,3-xylan.

Key words: cell wall, β-1,3-xylan, β-1,3-xylanase, β-1,3-xylooligosaccharide.

Abbreviations: AXO(s), β-1,3-xylooligosaccharide(s) prepared by TFA; DP(s), degree(s) of polymerization; EXO(s), β-1,3-xylooligosaccharide(s) prepared by β-1,3-xylanase; Glc, D-glucose; MALDI-TOF-MS, matrix-assisted laser desorption ionization/time of flight/mass spectrometry; TFA, trifluoroacetic acid; XO(s), β-1,3-xylooligosaccharide(s); XYL4, β-1,3-xylanase from *Vibrio* sp. AX-4; Xyl, D-xylose.

β-1,3-Xylan is a polysaccharide composed of β-1,3-linked D-xylose (Xyl). This polysaccharide was first found in the siphonous green seaweed *Caulerpa filiformis* as a water-insoluble and alkaline-soluble component of cell walls (*1*). It was then found in cell walls of some red and green algae, such as *Porphyra*, *Bangia*, *Caulerpa*, and *Bryopsis* spp. (*2*, *3*). However, it has not been detected in cell walls of land plants, which contain β-1,4-xylan instead.

There have been several studies on the structure of β-1,3-xylan derived from different algal sources. Iriki *et al*. reported that xylans from *Bryopsis maxima*, *Caulerpa anceps*, *Halimeda cuneata*, and *Chlorodesmis formosana* contained not only β-1,3-xylan but also up to 10% D-glucose (Glc) (*2*). Mackie and Percival also reported that β-1,3-xylan from *Caulerpa filiformis* contained Glc, however, it was thoroughly removed by extraction with hot water and thus Glc seems to be non-covalently attached to a β-1,3-xylan chain (*1*). Interestingly, Turvey and Williams reported that the β-1,3-xylan from *C. filiformis* and *C. racemosa* was composed of not only linear β-1,3-linked Xyl but also branched β-1,4-linked Xyl (*4*). In contrast, Fukushi *et al*. indicated that the β-1,3-xylan from *B. maxima* was a homopolysaccharide consisting of only β-1,3-linked Xyl without side chains (*5*, *6*). Yamagaki *et al*. also reported that the β-1,3-xylan from *Caulerpa brachypus* was a linear homopolysaccharide composed of at least 25 Xyl residues using matrix-assisted laser desorption ionization/time of flight/mass spectrometry (MALDI-TOF-MS) and NMR (*7*, *8*). Collectively, the structure of β-1,3-xylan is still ambiguous.

Recently, we reported the molecular cloning and characterization of a novel β-1,3-xylanase (XYL4) from a marine bacterium *Vibrio* sp. AX4 (*9*). The enzyme was found to hydrolyze the internal β-1,3-xylosidic linkages of β-1,3-xylan generating β-1,3-xylooligosaccharides (XOs) with various degrees of polymerization (DPs) (*9*). In this study, we performed a structural analysis of XOs derived from the β-1,3-xylan of *C. racemosa* var. *laete-virens* by the actions of XYL4. Here, we report the novel structural units of β-1,3-xylan and show the usefulness of XYL4 as a tool for the structural analysis of β-1,3-xylan.

MATERIALS AND METHODS

Materials—Precoated Silica Gel 60 TLC plates were purchased from Merck (Germany). The ion-exchange resins Dowex 1x8 and Dowex 50x8 were purchased from Dow Chemical (USA). All other reagents were of the highest purity available. The β-1,3-xylanase gene was cloned from the genomic library of *Vibrio* sp. AX4 and expressed in

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Escherichia coli BL21(λDE3)pLysS and the recombinant XYL4 was purified as described in a previous report (*9*) and then used for experiments.

Preparation of β-1,3-Xylan—β-1,3-Xylan was prepared by the method of Iriki *et al*. (*2*) from a green alga, *C. racemosa* var. *laete-virens*. Briefly, the seaweed was collected along the coast of Kagoshima City, Kagoshima Prefecture, Japan (31°34′ N, 130°33′ E) in April 1990. Thalli of the seaweed were dried, and ground with a mill. The powder was soaked in water, and washed until the chromatophore was removed. The suspension was then filtered through a cloth sack. The residue obtained was treated with 1% HCl at room temperature for 45 min with stirring, and washed as above. The residue was treated successively with 1.25% NaOH and 1.25% H_2SO_4 with stirring at 100°C under a stream of N_2 gas for 1 h, respectively. The residue was filtered through a cloth sack and re-suspended in de-ionized water, then bleached with 1% sodium chlorite with stirring. The bleached residue was thoroughly washed with de-ionized water and the crude fiber obtained was subjected to extraction with 10% NaOH with stirring at room temperature. Two volumes of ethanol was added to the extract and the mixture was centrifuged at 6,000 rpm for 20 min. The precipitated β-1,3-xylan was neutralized with 1% HCl in ethanol followed by washes with ethanol and diethylether, and then air dried.

Preparation of β-1,3-Xylooligosaccharides Using XYL4—Enzymatic hydrolysis of β-1,3-xylan with XYL4 was carried out as follows: a reaction mixture consisting of 1 g of β-1,3-xylan and 500 μg of purified recombinant XYL4 in 20 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl was incubated at 37°C until the insoluble β-1,3-xylan particles were completely hydrolyzed (approximately 48 h). Next, 2 volumes of ethanol was added and the mixture was left at –20°C for 30 min. The mixture was then centrifuged and the supernatant was concentrated with a rotary evaporator to remove the ethanol, and the concentrated hydrolysate was dissolved in 10 ml of de-ionized water. The hydrolysate was desalted with Dowex 50×8 (H^+ form, 100 ml) and Dowex 1×8 (CO_3^{2-} form, 100 ml). The resultant EXOs were concentrated with a rotary evaporator and then lyophilized.

Preparation of β-1,3-Xylooligosaccharides by Trifluoroacetic Acid (TFA)—Partial hydrolysis of β-1,3-xylan with TFA was carried out as follows: a reaction mixture consisting of 1 g of β-1,3-xylan in 20 ml of 1 N TFA was heated at 70°C for 3 h. After removal of the residual insoluble β-1,3-xylan by centrifugation and subsequent filtration with quartz wool, the hydrolysate was neutralized by adding Dowex 1×8 (CO_3^{2-} form, 100 ml) and then the hydrolysate was desalted with Dowex 50×8 (H^+ form, 100 ml). The resultants were concentrated with a rotary evaporator and lyophilized.

Quantification of Sugars—The total concentration of sugar was measured by the phenol–sulfuric acid method (*10*) and quantified using D-xylose as the standard.

Separation of XOs with Different DPs—The separation of XOs with different DPs was performed by TLC. The lyophilized XOs were dissolved in de-ionized water and diluted to 75 mg/ml, then spotted on a Silica Gel 60 TLC plate (20 cm × 20 cm). The TLC plate was developed with a solvent system comprising 1-butanol/acetic acid/de-ionized water (10:5:1, by volume). After development, a portion of the plate was cut, sprayed with diphenylamine/aniline/phosphoric acid reagent (*11*), and baked at 100°C for 10 min to detect XOs. Each XO, corresponding to xylobiose (X2) to xylopentaose (X5), was scraped from the TLC plate, dried by lyophilization, and extracted from the TLC-derived silica using de-ionized water. Each extract was centrifuged and the supernatant obtained was concentrated.

TLC Analysis of the Oligosaccharides after Digestion with XYL4—The XOs digested with XYL4 were analyzed as follows: a reaction mixture containing 2 mM of each XO and 10 μg of XYL4 in 10 μl of 10 mM sodium phosphate buffer, pH 7.5, was incubated at 37°C for 12 h. Two volumes of ethanol was then added, the reaction mixture was centrifuged, and the supernatant was evaporated with a Speed Vac concentrator. The dried material was dissolved in de-ionized water and spotted on a Silica Gel 60 TLC plate. The solvent system and the method used to detect sugars were the same as described above.

Analysis of Sugar Composition—The neutral sugars in β-1,3-xylan and XOs were determined according to Oades's method with minor modifications (*12*). Briefly, the β-1,3-xylan (1 mg) or XO (500 μg) was hydrolyzed at 100°C for 2 h in 1 ml of 2 N HCl in an evacuated, sealed tube. Then the hydrolysate was neutralized by passage through a Dowex 1×8 (CO_3^{2-} form, 5 ml) column and lyophilized. To the hydrolysate dissolved in 2 ml of distilled water, 10 mg of sodium borohydride and 200 μg of *myo*-inositol (internal standard) were added, and then the mixture was incubated at room temperature for 30 min. After reduction with sodium borohydride, the hydrolysate was passed through a Dowex 50×8 (H^+ form, 2 ml) column to remove sodium ion. The effluent were dried under vacuum, then evaporated with methanol several times. The residue was dissolved in 300 μl of acetic anhydride/pyridine (1:1, by volume) in an evacuated, sealed tube and incubated at 100°C for 10 min for the acetylation of alditols. The residual acetic anhydride/pyridine was then removed under a stream of N_2 gas. The resultant alditol acetates were analyzed by gas chromatography (GC) on a Shimadzu GC-14A (Shimadzu, Japan) equipped with a fused silica capillary column (ULBON HR-SS-10, ϕ0.25 mm × 25 m, Shinwa Chemical Industries, Japan). The temperature of the column was maintained at 200°C during the analysis.

Analysis of Sugar Linkages—Sugar linkages of XOs were determined by the method of Ciucanu and Kerek (*13*). Briefly, the XOs (15–30 nmol) were methylated with NaOH in DMSO and CH_3I. The permethylated XOs were subjected to hydrolysis with 2 M TFA before undergoing reduction with $NaBD_4$. The products were acetylated with acetic anhydride/pyridine (1:1, by volume) at 121°C for 3 h. The partially methylated alditol acetates thus obtained were analyzed by GC-mass spectrometry (GC-MS) using a Shimadzu GCMS-QP 5000 (Shimadzu, Japan) equipped with a fused silica capillary column (DB-1, ϕ0.25 mm × 30 m, J. & W. Scientific, USA). The column temperature was maintained at 50°C for 3 min, then increased to 180°C at 40°C/min and subsequently to 276°C at 4°C/min.

MALDI-TOF-MS of XOs—The MALDI-TOF-MS of XOs was conducted with a Voyager-DE mass spectrometer

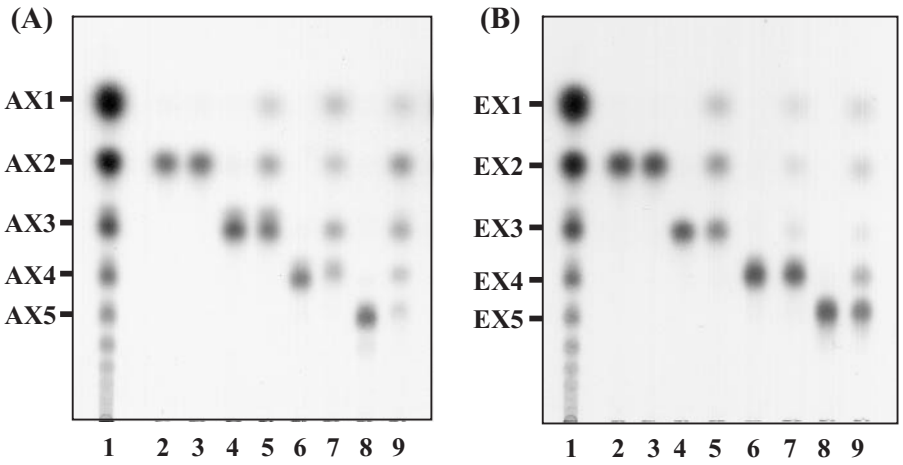

Fig. 1. **Mode of action of XYL4 on XOs prepared by different methods.** The patterns for the hydrolysis of XOs by XYL4 were analyzed by TLC. The reaction mixture containing 2 mM substrate and 10 μg of recombinant XYL4 in 10 μl of 10 mM sodium phosphate buffer, pH 7.5, was incubated at 37°C for 12 h. (A) AXOs were used as substrate. Lane 1, marker AXOs (25 μg); lanes 2 and 3, AX2; lanes 4 and 5, AX3; lanes 6 and 7, AX4; lanes 8 and 9, AX5 were used as substrate. Lanes 2, 4, 6 and 8 were negative controls (de-ionized water was used instead of enzyme). Lanes 3, 5, 7 and 9 were AXOs after treatment with XYL4. (B) EXOs were used as substrate. Lane 1, marker AXOs (25 μg); lanes 2 and 3, EX2; lanes 4 and 5, EX3; lanes 6 and 7, EX4; lanes 8 and 9, EX5 were used as substrate. Lanes 2, 4, 6 and 8 were negative controls (de-ionized water was used instead of enzyme). Lanes 3, 5, 7 and 9 were EXOs after treatment with XYL4.

(PE Biosystems, USA) using 2,5-dihydrobenzoic acid (DHB) as a matrix. The sample was mixed with the matrix on an occupied sample plate, and then dried completely. The analysis was conducted in the positive polarity mode with an accelerating voltage of 20 kV.

RESULTS

Mode by Which XYL4 Acts on XOs Prepared by Different Methods—β-1,3-Xylan, prepared from the green alga *C. racemosa* var. *laete-virens*, was hydrolyzed by XYL4 or TFA to obtain XOs with various DPs. In this study, the XOs prepared with XYL4 and TFA were tentatively designated EXOs and AXOs, respectively. Interestingly, the mode of action of XYL4 on AXOs was somewhat different from that on EXOs (Fig. 1). AX4 and AX5 were efficiently hydrolyzed by XYL4 and mainly converted into AX1 and AX3, and AX2 and AX3, respectively. In contrast, EX4 and EX5 were little hydrolyzed by XYL4. These results strongly suggest that EX4 and EX5 are somewhat different in structure from AX4 and AX5. It is noteworthy that AX2 and EX2 were not hydrolyzed by the enzyme at all.

Analysis of the Sugar Composition of XOs—To reveal the structure of XOs, the sugar composition of the oligosaccharides was analyzed as described in "MATERIALS AND METHODS." In addition to Xyl, a small amount of Glc was found in the β-1,3-xylan used in this study, with the molar ratio of Xyl/Glc being 16.7 (Table 1), indicating that the *C. racemosa* β-1,3-xylan is not a homopolysaccharide composed of Xyl, but contains approximately 6% Glc. Expectedly, AX3, AX4 and AX5 also contained approximately the same proportion of Glc as β-1,3-xylan (6.3–9.0%). These results may indicate that Glc is distributed uniformly in a β-1,3-xylan chain and TFA hydrolyzes β-1,3-xylosidic linkages randomly, generating Glc-containing XOs in a uniform ratio. On the other hand, the Glc content of EX4 and EX5 was extremely high (17.5–38.5%), which possibly due to the specificity of XYL4, *i.e.* the presence of Glc could make the oligosaccharide resistant to hydrolysis by the enzyme (Fig. 1). It is noteworthy that no Glc was detected in EX2 or EX3.

Table 1. **Sugar composition of β-1,3-xylan and its oligosaccharides prepared by hydrolysis with TFA or XYL4.** Sugar composition was analyzed by GC as described in "MATERIALS AND METHODS." The ratio was calculated from the peak area of GC. "All Xyl" indicates that all sugar components are Xyl, *i.e.* Glc was not detected.

	Xyl/Glc ratio	Glc content (%)
β-1,3-Xylan	16.7	6.0
XOs prepared by TFA (AXOs)		
AX2	64.4	1.6
AX3	15.9	6.3
AX4	14.0	7.1
AX5	11.1	9.0
XOs prepared by XYL4 (EXOs)		
EX2	All Xyl	0.0
EX3	All Xyl	0.0
EX4	2.6	38.5
EX5	5.7	17.5

Analysis of Sugar Linkages of EXOs—Methylation analysis of EX2 and EX3 revealed the presence of 1,5-di-(*O*-acetyl)-2,3,4-tri-(*O*-methyl)-xylitol (unsubstituted Xyl, 1-Xyl) and 1,3,5-tri-(*O*-acetyl)-2,4-di-(*O*-methyl)-xylitol (3-substituted Xyl, 1,3-Xyl), with a molar ratio of 1.00:0.99 for EX2 and 1.00:2.17 for EX3 (Table 2). The molar ratio of internal sugars to the terminal sugar at the non-reducing end (IS/NRE) was found to be 0.99 for EX2 and 2.17 for EX3. These data clearly indicate that EX2 and EX3 are Xyl-1,3-Xyl and Xyl-1,3-Xyl-1,3-Xyl, respectively. On the other hand, EX4 gave two components, 1,4,5-tri-(*O*-acetyl)-2,3-di-(*O*-methyl)-xylitol (4-substituted

Xyl, 1,4-Xyl) and 1,5-di-(*O*-acetyl)-2,3,4,6-tetra-(*O*-methyl)-glucitol (unsubstituted Glc, 1-Glc), in addition to 1-Xyl and 1,3-Xyl. The IS/NRE was approximately 1:2 for EX4 (Table 2), suggesting that EX4 is a mixture of tri-saccharides containing Glc and Xyl at the non-reducing end. Collectively, EX4 was thought to be a mixture of Glc-1,3-Xyl-1,3-Xyl, Xyl-1,4-Xyl-1,3-Xyl and Xyl-1,3-Xyl-1,4-Xyl. The ratio of 1-Glc to 1-Xyl (approximately 4:1) suggests that the ratio of the oligosaccharides containing Glc at the non-reducing end to those containing Xyl at the non-reducing end is approximately 4:1. Judging from the IS/NRE (Table 2), EX5 seems to be a mixture of tetra-saccharides, which contain internal 1,3-linked Glc in addition to the same components of EX4. The presence of 1-Glc and 1,3-Glc indicated that Glc is present not only at the non-reducing end but also inside of the chain in EX5. Branching Xyl or Glc was not found in EX2–EX5.

MALDI-TOF-MS of EXOs—When subjected to MALDI-TOF-MS, pseudomolecular ions of all EXOs were observed as $[M+Na]^+$. The pseudomolecular ions for EX2 and EX3 were found at m/z 305 and m/z 437 (Fig. 2, A and B), respectively, indicating EX2 and EX3 to be xylobiose, $(Xyl)_2$, and xylotriose, $(Xyl)_3$, respectively. The pseudomolecular ions at m/z 437 and 467 for EX4 showed the presence of tri-saccharides composed of three Xyl, $(Xyl)_3$, and one Glc and two Xyl, $Glc(Xyl)_2$, respectively, indicating that EX4 was a mixture of tri-saccharides (Fig. 2C). On the other hand, two main peaks of EX5 at m/z 569 and 599 (Fig. 2D) indicate the presence of tetra-saccharides composed of four Xyl, $(Xyl)_4$, and one Glc and three Xyl, $Glc(Xyl)_3$, indicating that at least two different tetra-saccharides were included in EX5. These results were well consistent with those obtained in the GC-MS analyses (Table 2). The putative structures of EX2, EX3, and EX4 are shown in Fig. 3.

Table 2. **Sugar linkage analysis of EXOs.** Sugar linkages of XOs were analyzed by GC-MS as described in "MATERIALS AND METHODS." The ratio was calculated from the peak area of GC. ND indicates "not detected." IS/NRE indicates the ratio of internal sugars (IS) to the terminal sugars at the non-reducing end (NRE).

	1-Xyl	1,3-Xyl	1,4-Xyl	1-Glc	1,3-Glc	IS/NRE
EX2	1.00	0.99	ND	ND	ND	0.99
EX3	1.00	2.17	ND	ND	ND	2.17
EX4	1.00	8.18	0.64	3.66	ND	1.89
EX5	1.00	2.76	0.61	0.27	0.36	2.94

DISCUSSION

Although several reports have indicated the presence of Glc in β-1,3-xylan (*1*–*3*), the Glc was found to be removed by gel-filtration chromatography under alkaline conditions, or successive extraction with hot water (*5*). These results suggest that β-1,3-xylan is a homopolysaccharide composed of Xyl (*5*, *7*) and the Glc moiety seems to be non-covalently attached to a β-1,3-xylan chain. Using β-1,3-xylanase XYL4, however, we clearly showed the presence of 1-Glc in EX4 and 1-Glc and 1,3-Glc in EX5 (Table 2 and Fig. 2, C and D), indicating that Glc is covalently linked to a β-1,3-xylan chain prepared from *Caulerpa racemosa* var. *laete-virens* by the method of Iriki *et al*. (*2*). It is noteworthy that no branching Glc or Xyl was found in EX4 and EX5. To our knowledge, this is the first report

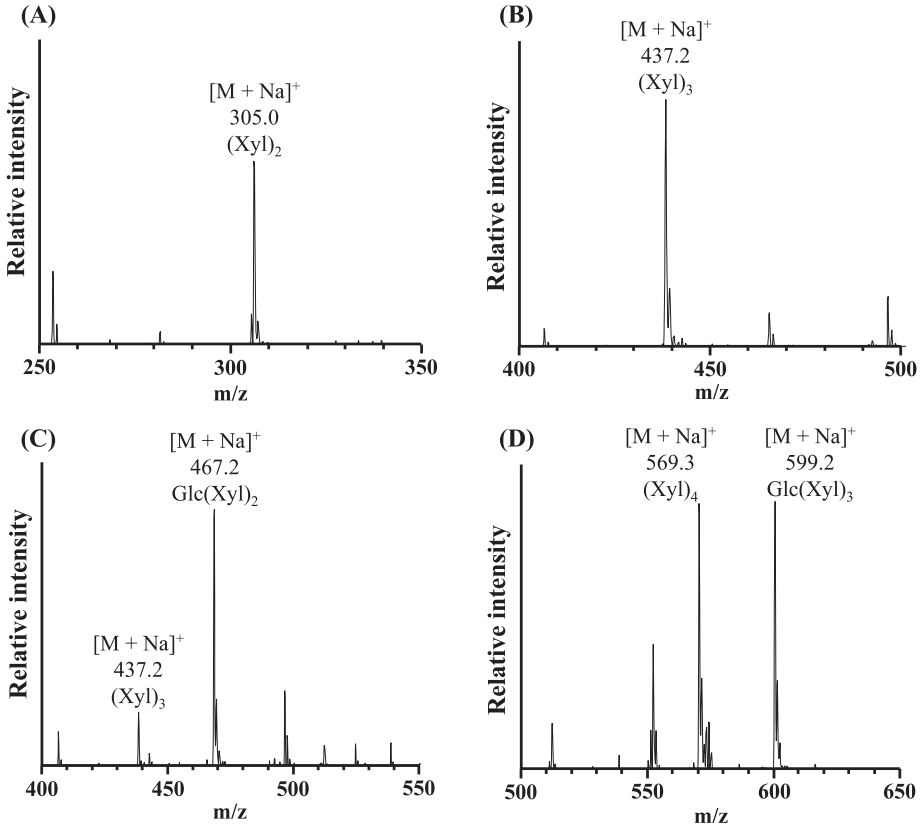

Fig. 2. **MALDI-TOF-MS spectra of EXOs.** The analysis was performed by MALDI-TOF-MS in the positive-ion mode as described in "MATERIALS AND METHODS." (A) EX2, (B) EX3, (C) EX4 and (D) EX5. XOs were observed as pseudomolecular ions (sodium adduct, $[M+Na]^+$).

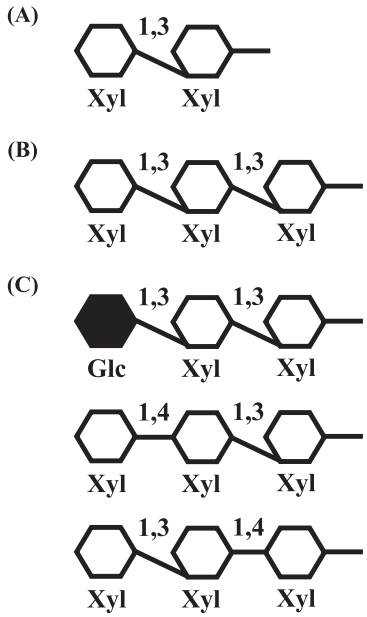

Fig. 3. **Putative structures of EXOs.** The structures of EXOs are shown as schematic representations. Putative structure of (A) EX2, (B) EX3, and (C) EX4. Open and closed hexagons indicate Xyl and Glc, respectively. Numbers over bars linking hexagons indicate the linkage forms.

describing an analysis of oligosaccharides obtained from β-1,3-xylan with β-1,3-xylanase.

AX4 and AX5 prepared by partial hydrolysis with TFA contained Glc in almost the same ratio as the β-1,3-xylan used (7.1–9.0% *vs* 6%, Table 1). On the other hand, the EX4 and EX5 prepared with XYL4 contain Glc at a much higher ratio than the β-1,3-xylan used (17.5–38.5% *vs*. 6%, Table 1). This discrepancy may stem from that TFA randomly hydrolyzed the glycosidic linkages in β-1,3-xylan whereas XYL4 specifically hydrolyzed the internal β-1,3-xylosidic linkage of β-1,3-xylan. The aberrant sugar (Glc) and linkage (β-1,4) detected in EX4 and EX5 were thought to be present in the β-1,3-xylan of *C. racemosa* var. *laete-virens* and could interfere with the actions of the enzyme. As a result, oligosaccharides with high content of Glc were generated after exhaustive digestion of β-1,3-xylan with the enzyme. In other words, β-1,3-xylan could be completely converted into di- and tri-xylooligosaccharides by the enzyme if β-1,3-xylan was a homopolysaccharide without aberrant sugar and/or linkage.

The sugar linkage analysis and MALDI-TOF-MS showed that EX3 was a Xyl-1,3Xyl-1,3Xyl and EX4 was a mixture of Glc-1,3Xyl-1,3Xyl, Xyl-1,4-Xyl-1,3Xyl and Xyl-1,3-Xyl-1,4-Xyl. As shown in Fig. 1, the R_f of EX4 on TLC was clearly slower than that of EX3 though both are trisaccharides, suggesting that the R_fs of hetero-oligosaccharides containing Glc or 1,4-Xyl were somewhat slower than those of β-1,3-xylooligosaccharides.

In summary, β-1,3-xylan from *C. racemosa* var. *laete-virens* is a linear heteropolysaccharide containing 1,3-Glc and 1,4-Xyl both of which are thought to be located within a β-1,3-Xyl chain and linked via covalent bonds. Branching is not likely present in EXOs prepared from the polysaccharide by XYL4. This study provides new insight into the structure of β-1,3-xylan, and proves that XYL4 is a useful tool for the structural analysis of the polysaccharide.

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