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Chemical Structure of a Novel Xylogalactan Isolated from Commercially Cultured Seagrape, *Caulerpa lentillifera*

Yukihiro Tamaki[1,2], Teruko Konishi[1], Masakuni Tako[1*]

[1]Department of Subtropical Bioscience and Biotechnology, University of the Ryukyus, Okinawa, Japan
[2]Molecular Bioscience Research Center, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan
Email: *tako@agr.u-ryukyu.ac.jp

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Abstract

A novel xylogalactan was isolated from green seaweed named Seagrape, *Caulerpa lentillifera*, which is commercially cultured in Okinawa, Japan. D-galactose (molar ratio, 2.7) and D-xylose (1.0) were identified by HPAEC analysis. The optical rotation was estimated to be +0.005˚ at 25˚C, but decreased to −0.001˚ at 50˚C, indicating α- and β-linkages co-involved. D-galactose and D-xylose were also identified from infrared spectrum that was the first to report. The spectrum indicated that the xylogalactan was involved in both α- (small peak) and β-(large) glycosides. Well resolved ^{13}C-NMR spectrum was obtained and assigned to be 1,4-linked α-D-galactose (large) and 1,3-linked β-D-xylose (small). All ring ^{13}C-NMR (C-2-C-6) were assigned and some C-6 of 1,4-linked α-D-galactose residues were estimated to be a branching sugar. From ^{1}H-NMR spectrum, 1,4-linked α-D-galactose, terminal and 1,3-linked β-D-xylose were assigned. Methylation analysis was used to identify 2,3,4-tri-*O*-methyl-D-Xyl*p* (terminal; 0.6 mol), 2,4-di-*O*-methyl-D-Xyl*p* (1→3-linked; 2.4), 2,3,6-tri-*O*-methyl-D-Gal*p* (1→4-linked; 3.2), 2-mono-methyl-D-Xyl*p* (1→3,4-linked; 0.3), and 2,3-di-*O*-methyl-D-Gal*p* (1→4,6)-linked; 1.0). The structure of a novel xylogalactan was branching trisaccharide side-chains, β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→), at C-6 of 1,4-linked α-D-Galactoses main-chain.

Keywords

Novel Xylogalactan, NMR Analysis, Methylation Analysis, Chemical Structure, *Caulerpa lentillifera*

1. Introduction

In the course of the investigation of polysaccharides, we isolated many industrially useful polysaccharides, such as agar [1], methyl agar [2], κ-carrageenan [3], ι-carrageenan [4], fucoidan [5]-[8], alginate [8] [9], galactomannan [10]-[12], pectin [13]-[15], and rhamnan sulfate [16] [17] from the subtropical biomasses grown in Okinawa Islands, Japan. Specifically, a novel fucoidan, which was substituted with an acetyl group from commercially cultured *Cladosiphon okamuranus*, was identified [5] and patented [18]. The acetyl fucoidan exhibits some biological activities, such as antitumor [19] and immune-enhancing abilities [20]. An over-sulfated acetyl fucoidan, the sulfate content of which was 32.8%, showed a significant antitumor activity *in vitro* [19].

Caulerpa lentillifera, an edible green seaweed, named Seagrape, is widespread in the natural environment of Southeast Asia. In Okinawa Islands, Japan, the green algae have been commercially cultivated since 1980. The production of the seaweed is reported to be about 400 t in 2022. We previously isolated β-1,3-linked xylan in 24% KOH solution from the seaweed [21]. The xylan might be extracted from cell wall because it was not soluble in aqueous solution.

Although, polysaccharide [22] and xylogalactomannan [23] were reported, but not xylogalactan from *C. lentillifera*. We report here chemical structure of a novel xylogalactan from the seaweed.

2. Materials and Methods

2.1. Materials

Caulerpa lentillifera was gifted by Seeds Company, Ginoza Village, Okinawa, Japan. The algae were washed with tap water and air dried at 40˚C for 48 h before being ground into powder. The powder (20 g) was soaked in ethanol overnight and soaked again in acetone to remove lipids, then dried *in vacuo*.

The defatted powder (2 g) was stirred in water to extract polysaccharide at room temperature for 2 h, and filtered through filter aid (Celite 545, Nakarai, Japan). Ethanol (2 vols) was added to the filtrate and the polysaccharide was dried *in vacuo*. The crude polysaccharide was dissolved in distilled water at room temperature and the solution was passed through the filter aid. Then, the filtrate was precipitated by adding 2 volumes of ethanol and the resulting solid was dried *in vacuo*. The semi-purified polysaccharide was dissolved in distilled water and deionized by passing through a cation exchange column composed of Amberlite 120A H^+ (Organo, Japan). After neutralization with 0.1 M NaOH, the solution was subsequently lyophilized [6] [7].

2.2. Chemical Component Analysis

The total carbohydrate contents were determined with the phenol-sulfuric acid method [24] using D-galactose as standard. The purified polysaccharide (70 mg) was dissolved in distilled water (20 mL) and sulfuric acid was added to reach a final concentration of 1.0 M. The mixture was subsequently heated to 100˚C for 3

h. The hydrolysate was neutralized with $BaCO_3$.

2.3. High-Performance Anion Exchange Chromatography Coupled with a Pulse Amperometric Detector (HPAEC-PAD)

The monosaccharides in the hydrolysate of the polysaccharide were identified using a HPAEC (DX-500, Dionex Co., CA, USA), fit with a Carbopack PA1 column and a pulsed amperometric detector. The column was eluted at flow rate of 1 mL/min at 35˚C with 10 mM NaOH.

2.4. Infrared Spectrum (FT-IR) and Optical Rotation of the Polysaccharide

The FT-IR of the polysaccharide was recorded in KBr discs using a spectrophotometer (FTS-3000; Bio-Rad Laboratories Inc., CA, U.S.A.) in transmittance mode from 4000 to 400 cm^{-1}.

The optical rotation was measured at 589 nm using a polarimeter (P-1010; JASCO Inc., Tokyo, Japan) at room temperature. The polysaccharide solution (0.2%) was prepared in distilled water.

2.5. Methylation Analysis

Methylation of the polysaccharide was carried out as described by Cicanue and Kerek [25]. The methylated polysaccharide was extracted with $CHCl_3$. The extracted methylated polysaccharide was hydrolyzed with 2 m TFA (2 mL) at 120˚C for 2 h. The hydrolysate was dissolved in 1 M NH_4OH (0.2 mL). DMSO (1 mL) containing 20 mg of $NaBH_4$ was added and the mixture was incubated at 40˚C for 90 min. Subsequently, acetic anhydride (0.2 mL) was added to the mixture. Anhydrous 1-methylimidazole (0.2 mL) and acetic anhydride (1 mL) were then added, and the reaction mixture was incubated at ambient temperature for 10 min. After extraction with chloroform and washing with water, partially methylated alditol acetates were obtained.

The partially methylated alditol acetates of the polysaccharide were analyzed using a gas chromatograph (GC-14A; Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector using a capillary column (DB-1: 40 m × 0.25 mm, J&W Scientific Inc., CA, U.S.A.). The injector and detector temperatures were 210˚C and 270˚C, respectively. After injection, the oven temperature was maintained at 150˚C for 5 min, and then raised at 5 ˚C/min to 250˚C. This temperature was maintained for 5 min. The identities of the peaks were confirmed using GC-MS (GCMS-QP 1000EX; Shimadzu., Kyoto, Japan).

2.6. ^{1}H- and ^{13}C-Nulear Magnetic Resonance (NMR) Spectroscopy

^{1}H- and ^{13}C-NMR spectra were recorded on a*α*500 FT-NMR spectrometer (JEOL Ltd, Japan) at 500.00 and 125.65 MHz, respectively. The polysaccharide (2%, W/V) was dissolved in D_2O and recorded at 60˚C. The ^{1}H-and ^{13}C-NMR chemical shifts were expressed in parts per million (ppm) relative to sodium 3-(trimethylsilyl)

propionic-2,2,3,3-d acid (TSP, 0.00 ppm), which was used as an internal standard.

3. Results

3.1. Seaweed

The green seaweed resembles bunches of grapes that was why named as Seagrape. Each grape is spherical with 2 - 4 mm in diameter. It is increasingly popular as dietary food due to containing minerals and some nutrients. The seaweeds are eaten fresh as a salad. In Okinawa Prefecture, some Companies are being cultivated and are reported to be 400 t production in 2022.

3.2. Chemical Components of the Polysaccharide

The yield of purified polysaccharide was estimated to be 3.7% based on the dried weight of algae. The polysaccharide was 91.3% (W/W) carbohydrates.

An anion exchanged high-performance liquid chromatogram of the hydrolysate of the polysaccharide (Figure 1) showed that peaks 1 and 2 were D-galactose and D-xylose in the molar ratio of 2.7:1.0. The result indicates that the polysaccharide isolated from *C. lentillifera* is a xylogalactan.

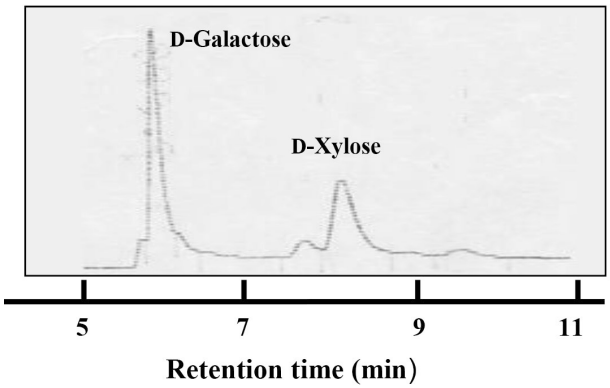

Figure 1. Liquid chromatogram of hydrolysate of the polysaccharide from *C. lentillifera*.

3.3. Optical Rotation and FTIR of the Polysaccharide

The optical rotation of the xylogalactan (0.2% in water) at 25˚C showed a value of +0.005˚, but it decreased a little in −0.001˚ at 50˚C, indicating both α and β-linkages were co-involved.

The FTIR spectrum of the xylogalactan is presented in Figure 2. The major absorption at approximately 3400 cm^{-1} was attributed to the stretching of hydroxyl groups. Absorption at 2900 cm^{-1} resulted from C-H stretching of C-H groups. Absorption at 1628 cm^{-1} resulted from bound water. There were two absorptions at 1216 (small) and 1020 (large) cm^{-1} which were caused from pyranose form [26]. The both absorptions might be derived from D-xylose and D-galactose residues, which were suggested from the molar ratio (Figure 2). Such identification is the first to report in the polymer molecules. Characteristic absorption at 905 (small)

and 814 (large) cm^{-1} were observed indicating that β- and α-configuration of the sugar units were involved [26].

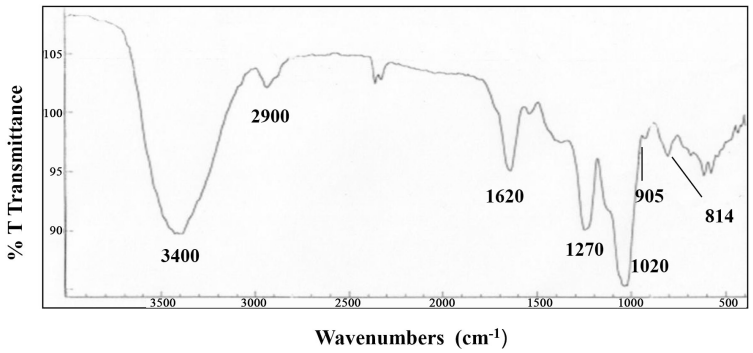

Figure 2. Infrared spectrum of xylogalactan isolated from *C. lentillifera* at 4000 - 400 cm^{-1}.

3.4. ^{13}C- and ^{1}H-NMR Spectra of Xylogalactan

The ^{13}C-NMR spectrum is presented in **Figure 3(a)**. Well characterized spectrum was obtained. From published papers [27]-[34], the signal at 105.94 ppm (X1) was assigned as anomeric carbon of 1,3-linked β-D-xylose. The ring carbon signals (63 - 81 ppm) of the residue were to be C-2, 76.16; C-3, 80.80; C-4, 65.54 and C-5, 63.86 ppm. The signal at 102.56 was assigned as anomeric carbon of 1,4-linked α-D-galactose residue. The ring-carbon signals (63 - 81 ppm) of the residue were also characterized C-2, 74.90; C-3, 75.20; C-4, 80.18; C-5, 77.25; C-6, 63.54 and C'-6, 69.18 ppm, the signal at the latter suggested that side-chain substitutes at C-6 of D-galactose residue. The signal at 106.27 ppm (X') might be attributed from non-reducing end of D-xylose residue. The signals are indicated in **Figure 3(a)** and are presented in **Table 1**.

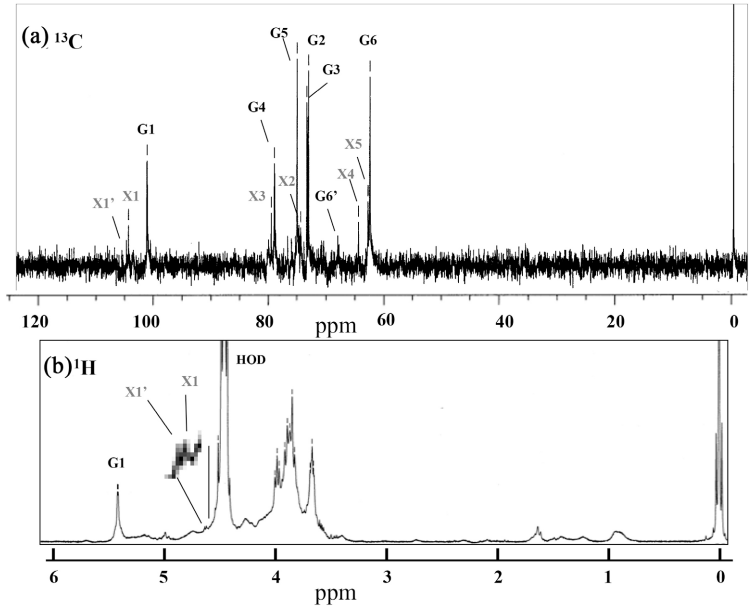

Figure 3. ^{13}C- and ^{1}H-NMR spectra of xyloglactan from *C. lentillifera* in D_2O at 60˚C.

The ^{1}H spectrum of the xylogalactan is presented in Figure 3(b). Three chemical signals were observed in the anomeric region (δ 5.5 - 4.5) at G1 (D-galactose) 5.397, X'1 (D-xylose) 4.631 and X1 (D-xylose) 4.626 ppm. From published papers [26]-[34], signal G (5.397 ppm) was assigned to be α-1,4-linked α-D-galactose. One of double signals X'1 was to be non-reducing end of β-D-xylose and another one was 1,3-linked β-D-xylose. The ring proton signals (3.5-4.1) ppm were overlapped, so it was difficult to do assignment. Such similar ^{1}H-NMR spectrum has been reported [35]. The signals are presented in Table 1.

Table 1. ^{13}C-and ^{1}H-NMR chemical shifts for the xylogalactan isolated from *C. lentillifera*.

Mode of linkage	C/H-1	C/H-2	C/H-3	C/H-4	C/H-5	C/H-6
β-D-Xylose-(1→	106.27/4.637					
→3)-β-D-Xylose-(1→	105.94/4.626	76.16/-	80.80/-	65.54/-	63.86/-	
→4)-α-D-Galactose-(1→	102.56/5.397	74.90/-	75.20/-	80.18/-	77.25/-	63.54/-
→4,6) α-D-Galactose-(1→						69.18/-

3.5. Methylation Analysis

The gas chromatogram of xylogalactan is shown in Figure 4. From publishing papers [36]-[38], peak number (1) was 2,3,4-tri-*O*-methyl-D-xyl*p* (terminal; relative molar ratio, 0.6), (2) 2,4-di-*O*-methyl-D-Xyl*p* (1→3-linked; 2.4), (3) 2,3,6-tri-*O*-methyl-D-Gal*p* (1→4-linked; 3.2), (4) 2-mono-*O*-mtheyl-D-Xyl*p* (1→3,4-linked; 0.3) and (5) 2,3-di-*O*-methyl-D-Gal*p* (1→4,6-linked; 1.0). The results are summarized in Table 2.

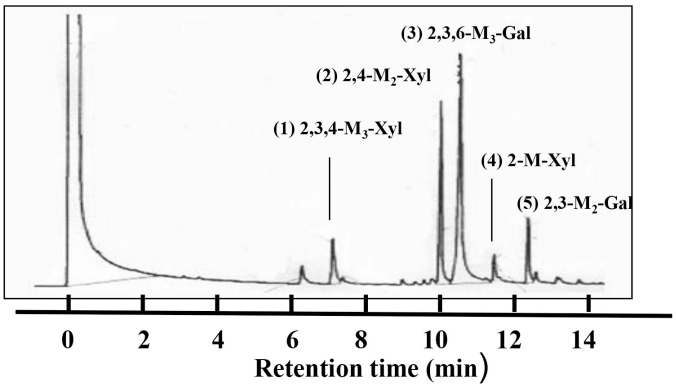

Figure 4. Gas chromatogram of methylalditol acetates of xylogalactan isolated from *C. lentillifera*.

Table 2. Methylation analysis of xylogalactan.

No. peak	Methylated sugars	Molar ratio	Mode of linkage
(1)	2,3,4-tri-*O*-methyl-D-Xylopyranose	0.6	D-Xylp-β-(1→
(2)	2,4-di-*O*-methyl-D-Xylopyranose	2.4	→3)-D-Xylp-β-(1→
(3)	2,3,6-tri-*O*-methyl-D-Galactopyranose	3.2	→4)-D-Galp-α-(1→
(4)	2-mono-*O*-methyl-D-Xylopyranose	0.3	→3,4)-D-Xylp-β-(1→
(5)	2,3-di-*O*-methyl-D-Galactopyranose	1.0	→4,6)-D-Galp-α-(1→

4. Discussion

Maeda *et al*. isolated a polysaccharide from *Caulerpa lentillifera*, which was cultivated in Okinawa, Japan, that contained D-galactose (44.2%), D-xylose (49.2), D-glucose (2.2), and uronic acid (4.3). The molecular mass was about 100 kDa [22]. The polysaccharide showed a strong immunoenhancing activity. Sun *et al*. reported xylogalactomannan from the seaweed cultivated in Dalian, China [23].

The study presents an investigation of xylogalactan isolated from *Caulerpa lentillifera*, which was commercially cultured in Okinawa, Japan. From HPAEC, D-galactose and D-xylose were identified with a molar ratio of 2.7:1.0. Sugar component of the polysaccharide was also identified by FT-IR spectrum, which was the first to report. Optical rotation and infrared spectrum indicated that the xylogalactan was co-involved in α- and β-glycosides. ^{13}C- and ^{1}H-NMR analysis indicated that D-galactose and D-xylose residues were involved in α-1,4- and β-1,3-glycoside linkages. From methylation analysis, terminal D-xylose (0.6 mol), 1,3-linked D-xylose (2.4), 1,4-linked D-galactose (3.2), 1,3,4-linked D-xylose (0.3), and 1,4,6-linked D-galactose (1.0) were identified. The mode of linkages of the both sugar residues was in agreement with that of NMR analysis.

We conclude that the xylogalactan consists of 1,4-linked α-D-galactan backbone substituted with tri-saccharide (α-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→) side-**chains at** C-6 of D-galactose residues, as shown in **Figure 5**. Such structure of xylogalactan is not reported yet.

As mentioned above, *C. letillifera* involved some soluble polysaccharides [22] [23] [35]. Indeed, we sometimes isolated polysaccharides involving D-glucose and/or D-mannose in addition to D-xylose and D-galactose from the seaweeds. Such results might be caused by cultivating site and/or season.

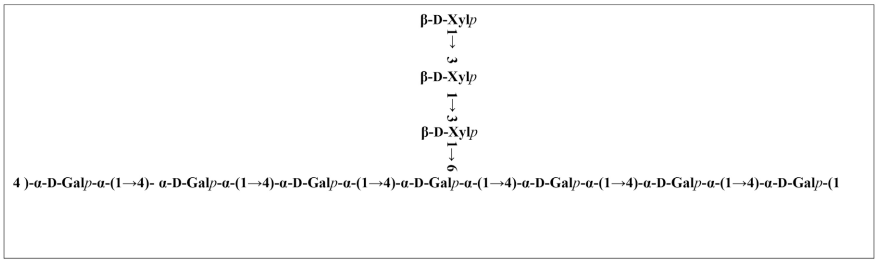

Figure 5. Chemical structure of xylogalactan isolated from *Caulerpa lentillifera.*

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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