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Structural studies of a methyl galacturonosyl-methoxyxylan isolated from the stem of *Lagenaria siceraria* (Lau)

Kaushik Ghosh, Krishnendu Chandra, Sadhan K. Roy, Subhas Mondal, Debabrata Maiti, Debsankar Das, Arnab K. Ojha and Syed S. Islam*

Department of Chemistry and Chemical Technology, Vidyasagar University, Midnapore 721 102, West Bengal, India

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Abstract—A water-soluble polysaccharide was isolated from the aqueous extract of the stem of *Lagenaria siceraria*. The polysaccharide was found to be constituted of methyl D-galacturonate, 2-*O*-methyl-D-xylose, and D-xylose in a ratio of 1:1:1. On the basis of total acid hydrolysis, methylation analysis, periodate oxidation, NMR studies (^{1}H, ^{13}C, 2D-COSY, TOCSY, NOESY, HSQC, and HMBC), and MALDI-TOF MS analysis, the structure of the repeating unit of the polysaccharide is determined as

A **B** **C**

→4)-α-D-Gal*p*A6Me-(1→3)-2-*O*-Me-β-D-Xyl*p*-(1→2)-β-D-Xyl*p*-(1→

Keywords: *Lagenaria siceraria*; Polysaccharide; Structure; NMR spectroscopy; MALDI-TOF MS analysis

1. Introduction

Different plant[1,2] and mushroom[3,4] polysaccharides have drawn the attention of chemists and immunobiologists for their immunomodulatory and anti-tumor properties. Hence, the search for new immunomodulatory antitumor materials has become a matter of great interest. It has been observed that different plant polysaccharides like neutral xyloglucans and the glucuronic acid containing arabinogalactans or 4-*O*-methylglucuronoxylans are involved in stimulating macrophage functions.[5] Glucoxylan and mannoglucoxylan from higher basidiomycetes *Hericium erinaceus*[6] and *Plaurotus pulmonarius*[7] show strong inhibition of tumors.

The plant *Lagenaria siceraria* (Lau) is a herb, which is a tendril climber and grows throughout the year. Its leaves, the stem, mesocarp, and endocarp of young fruit (pepo) are edible. Analysis[8,9] of the edible portion of the pepo (fruits) showed the presence of moisture (96.3%), carbohydrate (2.9%), protein (0.2%), fat (0.1% from an ether extract), and minerals (0.5%), including calcium and phosphorous. It was found to contain vitamin C and vitamin A along with pectin (21% based on dry wt.). The leaves[10] contain moisture (87.9%), carbohydrate (6.1%), protein (2.3%), fat (0.7%), fiber (1.3%), and minerals (1.7%). No reports relating to a polysaccharide isolated from the stem of *L. siceraria* are in the literature. A watersoluble polysaccharide was isolated from a hot aqueous extract of stem bodies of *L. siceraria* with a view to study the structural as well as immunological properties of this macromolecule. We are reporting herein the detailed structural studies of this polysaccharide.

2. Results and discussion

A polysaccharide was isolated from stem bodies of *L. siceraria* (1.0 kg) by boiling with distilled water. The whole extract was cooled at 4 °C overnight and centrifuged. The supernatant was precipitated in ethanol

* Corresponding author. Tel.: +91 03222 276558x437; +91 9932629971 (M); fax: +91 03222 275329; e-mail: sirajul_1999@yahoo.com

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(1:5, v/v). The precipitated polysaccharide was collected through centrifugation, washed with ethanol, and freeze-dried. Then, it was dissolved in minimum volume of water and dialyzed through a dialysis tubing cellulose membrane for 10 h to remove low-molecular-weight materials. The aqueous solution was then freeze-dried, yielding 1.57 g of crude polysaccharide. The water-soluble polysaccharide was fractionated through Sepharose 6B in aqueous medium in seven lots taking 30 mg at one time, and a pure polysaccharide was obtained by monitoring the fraction through a spectrophotometer at 490 nm using the phenol–sulfuric acid method.[11]

The pure polysaccharide has $[\alpha]_D^{25}$ +105.38 (*c* 0.084, water). The molecular weight of the polysaccharide was found to be $\sim 1.67 \times 10^5$ Da. Paper chromatographic analysis of the hydrolyzed product showed the presence of D-galacturonic acid, and D-xylose with another slow-moving spot nearer to D-xylose (R_f, 0.964). GLC analysis of the alditol acetates of the sugars showed only the presence of D-xylose, but the carboxy-methyl-reduced polysaccharide on hydrolysis, followed by GLC examination of the corresponding alditol acetates, showed the presence of D-galactose and D-xylose. GLC–MS analysis of the alditol acetates of the hydrolyzed polysaccharides showed the presence of 2-(or 4)-*O*-methyl D-xylose (*m*/*z* 85, 99, 115, **117**, 127, 189, 201). The absolute configurations of the sugar units were determined by the method of Gerwig et al.,[12] and methylation studies,[13] followed by GLC–MS analysis, revealed the presence of 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-xylitol and 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-D-xylitol in a molar ratio of 1:1. These results indicate the presence of (1→3)- and (1→2)-linked D-xylopyranosyl moieties in the polysaccharide. The methylated reduced[14] polysaccharide showed peaks corresponding to 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-D-xylitol, 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-D-xylitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-galactitol in a molar ratio of 1:1:1. The appearance of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-galactitol indicates the presence of (1→4)-linked D-galacturonic acid in the polysaccharide. Periodate-oxidized-reduced material of the polysaccharide, upon hydrolysis showed the absence of D-xylose, which indicates that the (1→2)-linked D-xylose moiety was consumed during oxidation. A part of the periodate-oxidized polysaccharide on hydrolysis showed the absence of D-galactouronic acid in the paper chromatographic examination,[15] indicating that it has been destroyed during oxidation since D-galacturonic acid is present as a (1→4)-linked residue in the repeating unit of the polysaccharide.

The NMR experiment was carried out at 70 °C for better quality of 2D couplings. The 500 MHz ^{1}H NMR spectrum of the polysaccharide (Fig. 1) showed three anomeric proton signals at δ 5.63, 4.99, and 4.99 in a molar ratio of 1:1:1. The sugar residues were designated as **A**, **B**, and **C** according to their decreasing anomeric chemical shifts (Table 1). In the ^{13}C NMR (125 MHz) spectrum (Fig. 2, Table 1) three anomeric carbon signals appeared at δ 101.06 (**A**) and 105.32 (**B**, **C**). Further δ 53.81 and 61.81 were assigned for carbomethoxy carbon and *O*-methyl carbon, respectively. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC, and HMBC (Fig. 3) NMR experiments.

The spin system of residue **A**, which consisted of only five protons with a relatively high chemical shift of the H-5 signal (δ 4.84) and weak coupling between H-3, H-4, and H-5, indicated that residue **A** is of D-Gal*p*A. The anomeric signal for moiety **A** at δ 5.63 (unresolved) and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz indicated that D-galacturonosyl residue is α linked. The C-1 signal of residue **A** at δ

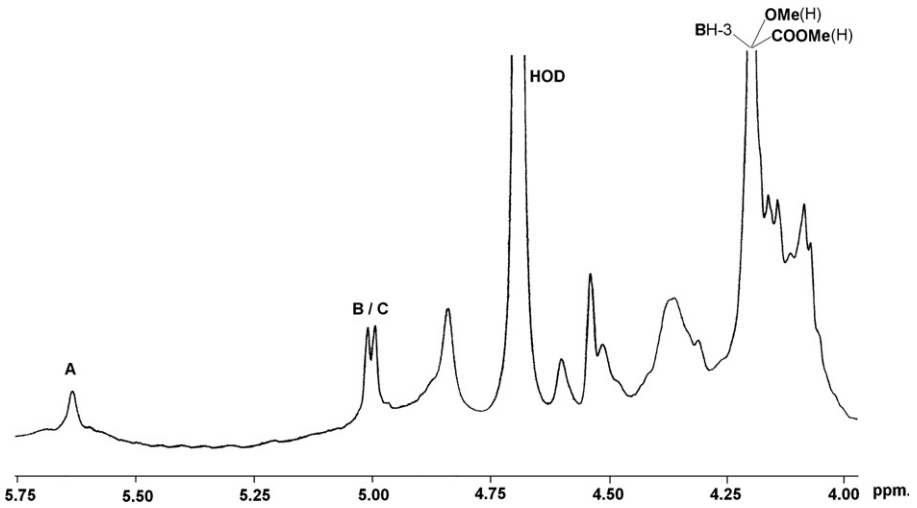

Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 70 °C) of the polysaccharide, methyl galacturonosyl-methoxyxylan, isolated from the stem of *Lagenaria siceraria*.

Table 1. ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the polysaccharide methyl galacturonosyl-methoxyxylan, isolated from stem of *Lagenaria siceraria* recorded in D_2O at 70 °C

Sugar residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	C-6	2-*O*-Me	COOMe
→4)-α-D-Gal*p*A6Me -(1→	5.63	4.27	4.36	4.54	4.84			4.19
A	101.06	69.1	71.5	79.6	72.9	171.6		53.81
→3)-2-*O*-Me-β-D-Xyl*p*-(1→	4.99	4.086	4.19	4.31	4.51[c], 4.14[d]		4.19	
B	105.32	75.51	85.07	69.1	68.0		61.81	
→2)-β-D-Xyl*p*-(1→	4.99	4.07	4.16	4.32	4.54[c], 4.11[d]			
C	105.32	79.6	74.37	70.9	68.0			

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.69.
[b] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 70 °C.
[c,d] Interchangeable.

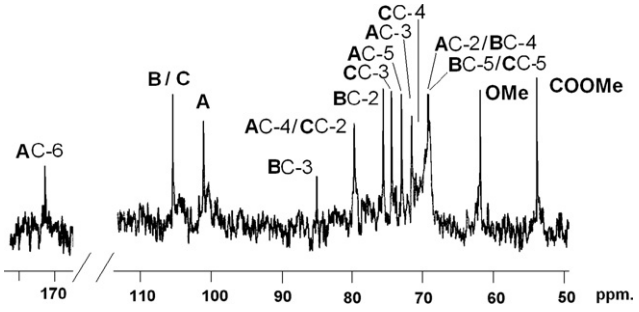

Figure 2. ^{13}C NMR (125 MHz, D_2O, 70 °C) spectrum of polysaccharide methyl galacturonosyl-methoxyxylan, isolated from stem of *Lagenaria siceraria*.

101.06 was confirmed by the appearance of cross peak **A**C-1, **B**H-3 in HMBC experiment (Fig. 3, Table 2). The C-4 peak of residue **A** at δ 79.6 showed a downfield shift compared to that of standard methyl glycosides[16,17] due to the effect of glycosylation. The carbon signals of residue **A** were observed at δ 69.1, 71.5, 72.9, and 171.6 corresponding to C-2, C-3, C-5, and C-6 (ester carbonyl), respectively. The carboxyl group in galacturonic acid is present as the methyl ester. The presence of a carboxymethyl group in residue **A** is confirmed by the appearance of intra-residual coupling between the ester carbonyl carbon (δ 171.6) and the carboxymethyl proton (δ 4.19) in the HMBC experiment (Fig. 3,

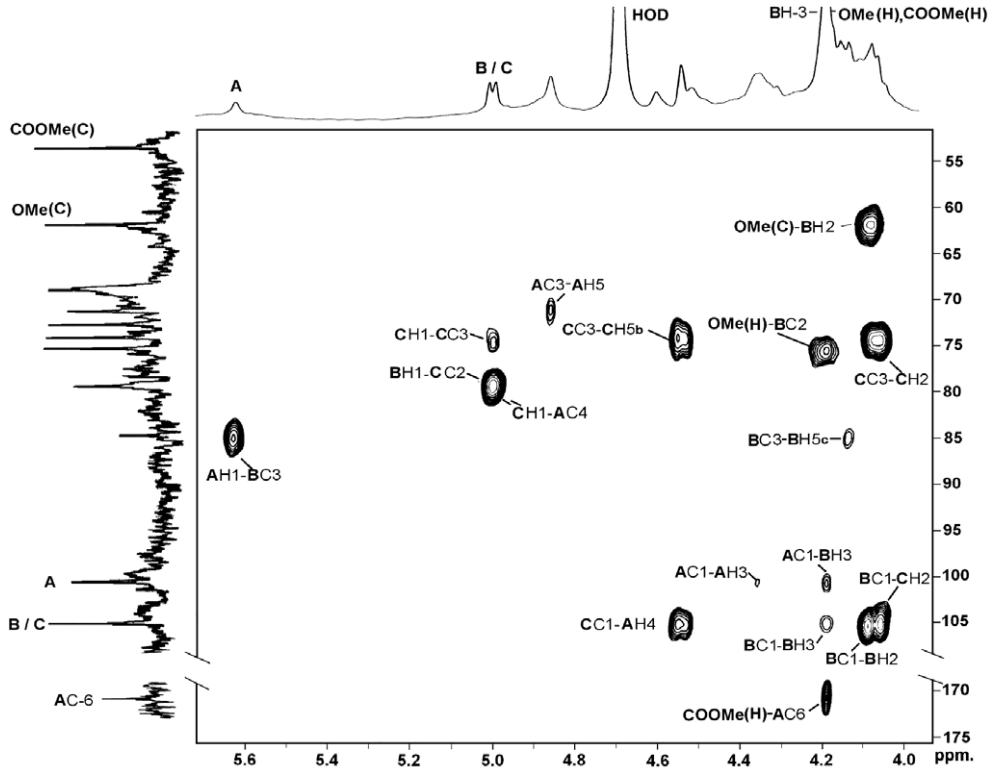

Figure 3. HMBC spectrum of polysaccharide methyl galacturonosyl-methoxyxylan, isolated from stem of *Lagenaria siceraria*. The delay time in the HMBC experiment was 80 ms.

Table 2. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide methyl galacturonosyl-methoxyxylan, isolated from stem of *Lagenaria siceraria.*

Residue	Sugar linkage	H-1/C-1 (δ_H/δ_C)	Observed connectivities		
			δ_H/δ_C	Residue	Atom
A	→4)-α-D-Gal*p*A6Me-(1→	5.63	85.07	**B**	C-3
		101.06	4.19	**B**	H-3
B	→3)-2-*O*-Me-β-D-Xyl*p*-(1→	4.99	79.6	**C**	C-2
		105.32	4.07	**C**	H-2
			4.19	**B**	H-3
C	→2)-β-D-Xyl*p*-(1→	4.99	79.6	**A**	C-4
			74.37	**C**	C-3
		105.32	4.54	**A**	H-4
		COOMe (δ_H)	δ_C		
A	→4)-α-D-Gal*p*A6Me-(1→	4.19	171.60	**A**	C-6
		2-*O*-Me (δ_H/δ_C)	δ_H/δ_C		
B	→3)-2-*O*-Me-β-D-Xyl*p*-(1→	4.19	75.51	**B**	C-2
		61.81	4.086	**B**	H-2

Table 2). These indicate that residue **A** is the methyl ester of a (1→4)-linked α-D-galacturonic acid.

Residue **B** was assigned to D-Xyl*p* as it showed two H-5 (δ 4.54 and 4.14) signals. The anomeric chemical shift for moiety **B** at δ 4.99 ($J_{H\text{-}1,H\text{-}2} > 7.8$ Hz) and $J_{H\text{-}1,C\text{-}1} \sim 160$ Hz indicated that D-xylopyranosyl residue is β-linked. The appearance of intra-residual coupling between the methoxy carbon (δ 61.81) and H-2 atom (δ 4.19) in the HMBC experiment (Fig. 3, Table 2) indicates that the methoxy group is linked at the C-2 position of residue **B**. The anomeric carbon signal of residue **B** at δ 105.32 was confirmed by the presence of a cross peak **B**C-1, **C**H-2 in the HMBC experiment (Fig. 3, Table 2). Another intraresidual cross coupling **B**C-1, **B**H-2 was also observed. The downfield shift of C-3 (δ 85.07) indicates that residue **B** is a 3-linked β-D-xylose moiety. The carbon signals of residue **B** were at δ 75.51, 69.1, and 68.0 corresponding to C-2, C-4, and C-5, respectively. These results indicate that residue **B** is a (1→3)-linked 2-*O*-methyl-β-D-xylopyranose.

Residue **C** has an anomeric chemical shift at δ 4.99 and a large coupling constant $J_{H\text{-}1,H\text{-}2}$ (>7.8 Hz), indicating a β-linked residue. The appearance of two H-5 signals indicates the presence of D-xylopyranosyl moiety (residue **C**). The anomeric carbon signal of residue **C** at δ 105.32 was confirmed by the presence of a cross peak **C**C-1 **A**H-4 in the HMBC experiment (Fig. 3, Table 2). The downfield shift of the C-2 (δ 79.6) signal indicates that residue **C** is a 2-linked β-D-xylose moiety. The above discussion indicates that for two D-xylopyranosyl moieties, although differently linked, the anomeric proton and carbon resonances appear in the same position in both ^{1}H and ^{13}C. If it is assumed that the coupling **B**C-1 **C**H-2 is not an inter-residual, it would be an intra-residual coupling, **C**C-1,**C**H-2. In that case the existence of two H-2 protons for two xylopyranosyl moieties are observed. Hence, (1→3)-linked 2-O-methyl-D-xylose and (1→2)-linked D-xylose, as evidenced from above analysis, are present. The carbon signals of residue **C** were observed at δ 74.37, 70.9, and 68.0 corresponding to C-3, C-4, and C-5, respectively. These results indicated that residue **C** is (1→2)-linked β-D-xylopyranose.

The sequence of the glycosyl residues was determined on the basis of a NOESY experiment, followed by confirmation with HMBC (Table 2, Fig. 3). Residue **B** has a NOE contact from H-1 to H-2 of residue **C** and **C** has NOE contact from H-1 to H-4 of residue **A**, appearing in the same line of β-xylosyl moieties. Residue **A** has a NOE contact from H-1 to H-3 of residue **B.** A long-range HMBC experiment was carried out to confirm the NOESY connectivities. From the HMBC experiment, the cross-peaks of both anomeric proton and carbon of each of the sugar moieties were examined, and intra- and inter-residual connectivities were observed (Table 2). Cross-peaks were observed between H-1 (δ 5.63) of residue **A** and C-3 (δ 85.07) of residue **B** (**A**H-1, **B**C-3), C-1 of residue **A** (δ 101.06), and H-3 of residue **B** (**A**C-1, **B**H-3), with other intra-residual coupling between C-1 (δ 101.06) of residue **A** with its own H-3 atom (**A**C-1, **A**H-3). The cross-peaks between H-1 (δ 4.99) of residue **B** and C-2 (δ 79.6) of residue **C** (**B**H-1, **C**C-2), C-1 (δ 105.32) of residue **B**, and H-2 of residue **C** (**B**C-1, **C**H-2), were observed. Intra-residual coupling between C-1 of residue **B** with its own H-3 atom (**B**C-1, **B**H-3) was observed. Similarly, the cross peaks between H-1 (δ 4.99) of residue **C** and C-4 (δ 79.6) of residue **A** (**C**H-1, **A**C-4), C-1 (δ 105.32) of residue **C**, and H-4 (δ 4.54) of residue **A** (**C**C-1, **A**H-4) were observed with other intra-residual coupling

(**C**H-1, **C**C-3) between, H-1 (δ 4.99) of residue **C** with its own C-3 (δ 74.37) position. Other intra-residual interactions (**A**C-3, **A**H-5) and (**B**C-3, **B**H-5c) were observed. Inter-residual coupling between the carboxymethyl proton (δ 4.19) and the ester carbonyl carbon (δ 171.6) of residue **A** was observed. Inter-residual coupling between methoxyl proton (δ 4.19) and C-2 atom of residue **B** (OCH_3 (H), **B**C-2) and between the methoxy carbon (δ 61.81) and H-2 atom of residue **B** (OCH_3 (C), **B**H-2) were also observed.

Thus, the appearance of HMBC cross-peaks and NOESY connectivities support a trisaccharide repeating unit in the present polysaccharide, isolated from stem of *L. siceraria*. The repeating unit is established as the following:

A **B** **C**

→4)-α-D-Gal*p*A6Me-(1→3)-2-*O*-Me-β-D-Xyl*p*-(1→2)-β-D-Xyl*p*-(1→

MALDI-TOF MS analysis of this polysaccharide was carried out. The nomenclature of fragments shown in Figure 4 was described according to Doman and Costello.[18] The ion peak at *m*/*z* 959.0 is obtained for ($P_2Q_2R_2 + Na^+$), and the other peaks at *m*/*z* 914.2, 900.0, 854.0, 724.4, 709.4, 695.3 were observed due to (1,5), (0,2), (3,5), (1,5), (2,4), (2,5) cleavage of different sugar residues of the repeating unit of the polysaccharide (Fig. 4a).

The fragments at *m*/*z* 959.0, 769.0, 658.0, and 621.4 were solely due to the breaking of glycosidic linkages at C_6, C_5, Z_4, and B_4 fragments. The higher molecular-ion fragments ranging from *m*/*z* 1105.0 to 4703.4 were also observed and are shown in Figure 4b. These fragments are observed due to the different types of breaking at different positions of the sugar moieties present in the polysaccharide. From these cross-linked fragmentations, branching information[19] was also obtained and these mass fragments support the above structure.

3. Experimental

3.1. Isolation and purification of the polysaccharide

The stem of the *L. siceraria* (Lau) (1.0 kg) was collected from the local market and washed with water. It was crushed and boiled in 250 mL of distilled water for 4 h. The whole mixture was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 4 °C.The supernatant was collected and precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitated material (polysaccharide) was washed with ethanol four times and then freeze-dried. The freeze-dried material was dissolved in 30 mL of distilled water and dialyzed through dialysis tubing of cellulose membrane (Sigma–Aldrich, retaining MW > 12,400) against distilled water for 10 h to remove low molecular weight materials. The aqueous solution was then collected from the dialysis bag and freeze-dried. Thus crude polysaccharide was obtained (1.57 g).

The crude polysaccharide (30 mg) was purified by gel-permeation chromatography on a column (90 × 2.1 cm) of Sepharose 6B with water as the eluant (0.4 mL min^{-1}) using a Redifrac fraction collector. A total of 95 test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm with the phenol–sulfuric acid reagent[11] using a Shimadzu UV–vis spectrophotometer, model 1601. One homogeneous fraction (test tubes 28–58) was collected and freeze-dried (yield 20 mg). The purification process was carried out in seven lots, and the polysaccharide fraction was again purified and collected (yield 140 mg).

3.2. Monosaccharide analysis

3.2.1. Alditol acetate analysis. The polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3CO_2H (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3CO_2H. It was then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditols) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give the alditol acetates. These were then analyzed by GLC using column (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C. Gas-liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on a Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 m). The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C. Quantitation was carried out from the peak area using response factors from standard monosaccharides.

3.2.2. Preparation of the carboxymethyl reduced polysaccharide.[20] The polysaccharide (1.0 mg) was dissolved

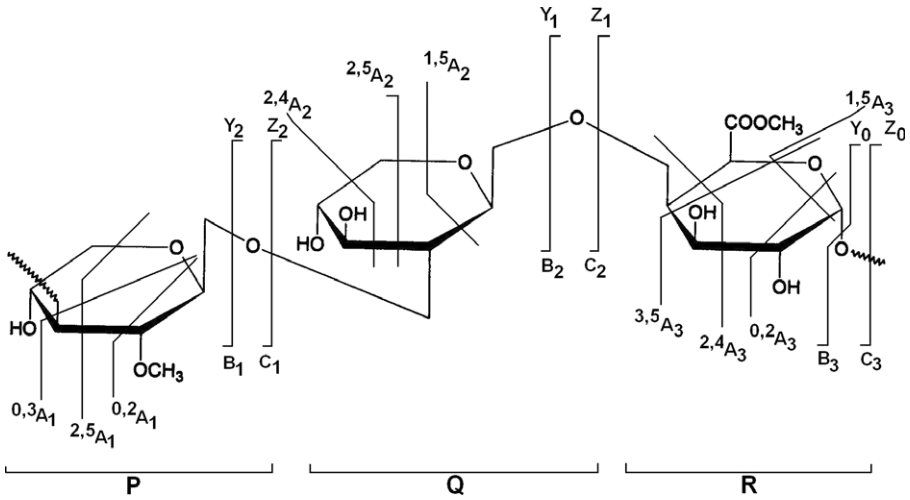

Figure 4. Fragmentation patterns (MALDI-TOF MS) of different mass fragments of methyl galacturonosyl-methoxyxylan, isolated from stem of *Lagenaria siceraria*.

in 1 M imidazole–hydrochloric acid buffer, pH 7.0 (200 μL/mg) and cooled on ice. $NaBH_4$ (40 mg) was then added, and the reaction mixture was maintained on ice for at least 1 h. The excess $NaBH_4$ was decomposed by adding HOAc (100 μL/40 mg $NaBH_4$) slowly to the cooled sample. An equal volume of redistilled water was then added, and the reduced polysaccharide was precipitated by adding 3–4 vols of 95% (v/v) EtOH (2 mL). The sample was reprecipitated two more times with 95% EtOH and freeze-dried. The carboxymethyl-reduced polysaccharide was hydrolyzed with 2 M CF_3CO_2H for 18 h at 100 °C, and after the usual treatment, the sugars were analyzed by GLC.

3.3. Methylation analysis

The polysaccharide (4.0 mg) was methylated using the method of Ciucanu and Kerek.[13] The methylated products were isolated by partitioning between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing the products was washed with 3×3 mL of water and dried. The methylated products were then hydrolyzed with 90% HCO_2H (1 mL) at 100 °C for 1 h, reduced with $NaBH_4$, acetylated with 1:1 Ac_2O–pyridine, and analyzed by GLC (using columns A and B) and GLC–MS (using an HP-5 fused silica capillary column) and the same temperature program as indicated above. Another portion of the methylated product (2.0 mg) was dissolved in dry THF (2 mL) and refluxed with $LiAlH_4$[14] (40 mg) for 5 h and kept overnight at room temperature. The excess of the reductant was decomposed by dropwise addition of EtOAc and aq THF. The inorganic materials were filtered off. The filtrate was evaporated to dryness giving the carboxy-reduced permethylated product. The product was hydrolyzed with HCO_2H as before, and the alditol acetates of the reduced, methylated sugars were prepared in the usual way and analyzed by GLC and GLC–MS.

3.4. Periodate oxidation

The polysaccharide (5 mg) was oxidized with 0.1 M $NaIO_4$ (2 mL) at 27 °C in the dark for 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with HOAc. The resulting material was obtained by co-distillation with MeOH. The periodate-reduced material was divided into two portions. One portion was hydrolyzed with 2 M CF_3CO_2H for 18 h, and the alditol acetates were prepared as usual. Another portion was methylated by the method of Ciucanu and Kerek,[13] and the alditol acetate of this methylated product was prepared. The alditol acetates were analyzed by GLC using columns A and B. Another portion of the periodate-oxidized, LAH-reduced polysaccharide was mixed with 0.5 M CF_3CO_2H and allowed to stand for 48 h at room temperature. The acid was removed, and the hydrolyzate was analyzed by GLC (as alditol acetates).

3.5. Absolute configuration of monosaccharides

The procedure used was based on the method of Gerwig et al.[12] The polysaccharide (1.0 mg) was hydrolyzed with CF_3CO_2H, and then the acid was removed. A solution of 250 μL of 0.625 M HCl in *R*-(+)-2-butanol was added and heated at 80 °C for 16 h. Then the

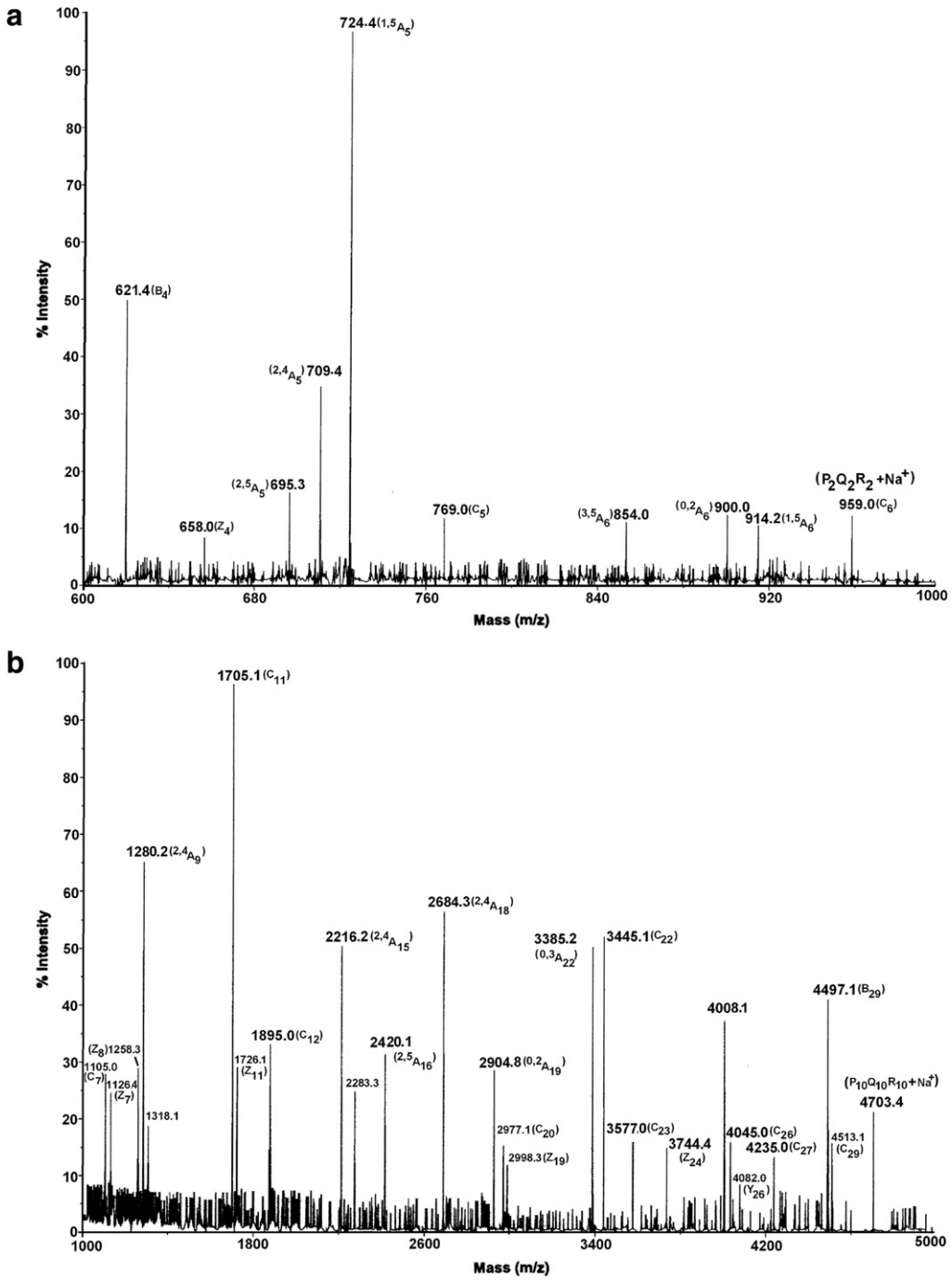

Figure 4a and 4b. MALDI-TOF mass spectrum of polysaccharide methyl galacturonosyl-methoxyxylan, isolated from stem of *Lagenaria siceraria*. The sample was prepared using DHB (10 mg/mL) matrix dissolved in 2:1 acetonitrile–water containing 1% TFA.

reactants were evaporated and *O*-TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The products were analyzed by GLC using an SPB-1 capillary column (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-trimethylsilyl-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

3.6. Optical rotation

Optical rotation was measured on a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C.

3.7. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann Nos. 1 and 3 MM sheets. Solvent

systems used were (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase), and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[15]

3.8. Determination of molecular weight

The molecular weight of polysaccharide was determined by a gel-chromatographic technique. Standard dextrans[21] T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of polysaccharide was then plotted in the same graph, and the molecular weight of the polysaccharide was determined.

3.9. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days and then deuterium-exchanged[22] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. With a Bruker Avance DPX-500 spectrometer,^1H, TOCSY, DQF-COSY, NOESY, and HMBC NMR spectra were recorded in D_2O at 70 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.69) using the WEFT pulse sequence.[23] The 2D-DQF-COSY experiment was carried out using standard Bruker software at 70 °C. The TOCSY experiment was recorded at a mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 200 ms. The ^{13}C NMR spectrum of polysaccharide solution in D_2O was recorded at 70 °C using acetone as the internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

3.10. MALDI-TOF mass spectrometry

3.10.1. Preparation of DHB matrix. 2,5-Dihydroxybenzoic acid (DHB, 2 mg) was dissolved in 200 μL of the matrix solvent (2:1 acetonitrile–water, v/v) and 1% sodium trifluoroacetate (TFA) was added to it. TFA and acetonitrile percentage was optimized using different concentrations of 0.5–3.5% and 20–80%, respectively, using the same instrumental settings.

3.10.2. MALDI-TOF MS analysis.[24,25] The polysaccharide (1.0 mg) was dissolved in 200 μL Milli Q deionized water. Two μL of the sample solution was taken in a vial where DHB matrix (2 μL) was added and centrifuged. One μL of the solution mixture was transferred to the MALDI sample plate for analysis. MALDI-TOF MS was performed on a Voyager-DE PRO (Applied Biosystems) mass spectrometer, equipped with a nitrogen laser operating at 337 nm (laser power 25–30 Jules and accelerating voltage 20 kV). The instrument was calibrated with myoglobin (Sigma) prior to analysis. Mass spectra were recorded in the reflector mode and in positive-ion detection using DHB (10 mg/mL) as matrix.

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