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Note

The chemical structure of the D-xylan from the main cell-wall constituents of *Bryopsis maxima**

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Siphonous green algae (Siphonales) do not contain cellulose in the cell wall but a skeletal glycan constituted of xylan or mannan[2–4]. The structure of the xylan[5] was previously studied on the product obtained by ethanol precipitation from an alkali-solubilized fibrous material of *Caulerpa filiformis*. This had been isolated by extraction with dilute acid and alkali and by chlorite bleaching. The product was essentially resistant to periodate oxidation, and after hydrolysis of the permethylated products, 2,3,4-tri-, 2,4-di-, and a mono-*O*-methyl-D-xylose were obtained in a molar ratio of 2:96:1. In a subsequent methylation analysis of the *Caulerpa filiformis* xylan[6], the mono-*O*-methyl-D-xylose was confirmed as 2-*O*-methyl-D-xylose (2.0%), in addition to 2,4-di- (97.0%) and 2,3,4-tri-*O*-methyl-D-xylose (2.1%). Similar results for a xylan from *Caulerpa racemosa* indicated that the D-xylan from this alga has also a branched structure[6]. Furthermore, the D-xylan contained about 10% of glucose residues which were traced to a laminaran-like glucan; however, the glucose residues could be largely removed by prolonged extraction with water.

On the other hand, the comparative biochemical studies of cell wall of siphonous green algae indicated that the polysaccharides of microfibrils from *Bryopsis, Caulerpa, Halimeda*, and *Chlorodesmis* were xylans[3], and those from *Codium,* and *Derbesia* mannans[4,5]. The chemical structures of the xylans were almost identical and similar to the structure of the xylan of *Caulerpa filiformis*. However, the D-glucose residues of the last-named could not be removed by the same treatments. The cell wall xylan from these algae being resistant to periodate oxidation, the main linkage is expected to be (1→3), with the possibility of another linkage and branching. As the isolation of xylan in the previous works[4,5] had been solely carried out by fractional precipitation with ethanol, the homogeneity of the

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samples remained unresolved. In the present work, a chromatographically and electrophoretically homogeneous pure xylan was isolated, as the major component of the fractions of fibrous materials from *Bryopsis maxima*, by gel-filtration chromatography under dilute alkaline conditions or by successive hot-water treatments[1]. The chemical structure of this xylan is reported herein.

EXPERIMENTAL

General methods. — Specific rotation were measured at 20° with a Jasco Dip-181 digital polarimeter. I.r. spectra were recorded with a Jasco IR-G infrared spectrometer on KBr discs for free sugars and on solutions in dichloromethane for the methylated sugars. N.m.r. spectra were recorded with a Jeol FX90Q spectrometer with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard for solutions in D_2O and with tetramethylsilane as internal standard for solutions in (^{2}H)chloroform. All evaporations were conducted under diminished pressure at a bath temperature <40°. Total sugars were estimated by the phenol–H_2SO_4 reagent[7] and reducing sugars with the Somogyi–Nelson reagent[8].

Viscosity measurements. — The viscosity of a 0.45M alkaline solution of xylan at various concentrations was measured with an Ostwald-type viscometer (No. 1) at 30 ±0.5°. In parallel, the specific viscosities of Dextran T-70 (M_r 62 400, determined from sedimentation equilibrium data), T-40 (40 700), and T-10 (11 900) at various concentrations were measured under the same conditions. The intrinsic viscosities $[\eta]$ of each concentration of the sample were calculated, and the mol. wt. of xylan was obtained from the equation $[\eta]_{sp} = KM^a$, where the constants were calculated from the viscosity data of dextrans as K $1.03 \cdot 10^{-4}$ and 0.89, respectively. The viscosity of the paraformaldehyde-treated xylan was also measured for a solution in dimethyl sulfoxide and the results were compared with the aforementioned ones.

Isolation and purification of cell wall xylan. — After successive treatments of the algae with hot dilute acid and alkaline solution, the crude xylan of the cell wall of *Bryopsis maxima* was obtained by ethanol precipitation of the cold alkaline extract of the residual fibrous materials. The xylan was further fractionated from the concomitant glucan by stirring of a hot aqueous solution and gel-filtration chromatography on Toyopearl gel under alkaline conditions as reported previously[1].

Periodate oxidation. — Xylan (10 mg) was suspended into 0.1M acetate buffer (pH 5.0, 2 mL) and $NaIO_4$ was added to a final concentration of 0.05M. At time intervals, aliquots of the solution were removed and the absorbance was measured[9] at 223 nm. The amount of periodate consumed per xylosyl residue was estimated on the basis of a standard curve for absorbance differences from corresponding molar concentration of periodate and iodic acid. Cellulose powder for column chromatography (Whatman) was also oxidized under the same conditions as control.

After the periodate consumption had reached a maximal value, the oxidized compound was reduced with $NaBH_4$, the solution was acidified, and the reduced polyhydroxy compound was hydrolyzed with 0.5M H_2SO_4 for 2 h at 100°.

In order to convert the aldehyde to oxime, an aliquot of the hydrolyzate was treated with hydroxylamine hydrochloride. The mixture was dried *in vacuo*, treated with chlorotrimethylsilane in pyridine, and then analyzed[10] by g.l.c. in a capillary column of OV-17 at 140°. Each peak was identified either by comparison of the relative retention time to that of a standard, or by cochromatography with a standard compound.

Permethylation analysis. — Xylan (~5 mg) was suspended into dimethyl sulfoxide and methylated three times by the Hakomori method[11]. Proof of complete permethylation was obtained by i.r. spectroscopy for the OH bands and determination of the methoxyl group content[12].

The methylated xylan was dissolved in 72% H_2SO_4 under cooling with an ice bath, and the solution was then diluted to a final concentration of 8% H_2SO_4 and heated in a boiling bath for 4 h. In order to ascertain a possible demethylation during the course of hydrolysis, 2,3,4,6-tetra-*O*-methyl-D-glucose was treated in the same way. The acid hydrolyzate was reduced with $NaBH_4$ and the solution was acidified to pH 3.5 with Dowex 50 (H^+) cation-exchange resin and concentrated to dryness, and the residue was acetylated. The resulting partially methylated alditol acetates were analyzed by gas chromatography in a packed glass column (1 m × 2 mm i.d.) containing 3% ECNSS-M on Gaschrom Q (80–100 mesh) with an isothermal column temperature of 180°. The chromatogram peaks were identified by their retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and comparison with the reported values[13].

E.i.m.s. of the partially methylated xylitol acetates were recorded with an Hitachi M-80 mass spectrometer using a g.l.c. inlet system, a packed column of 3% OV-17 (1 m × 2 mm, i.d.) with the oven temperature at 190°, an inlet temperature of 230°, an ionizing potential of 20 eV, and ionizing current of 110 μA, and an ion-source temperature of 270°. The methylated sugars were identified by comparison of the retention times and fragmentation patterns with those of authentic standards, or by comparison of the major mass-spectral ions with data in the literature[13].

H.p.l.c. of paraformaldehyde-treated xylan. — A mixture of xylan (0.1 g) and dimethyl sulfoxide (10 mL), in a vessel with a Teflon-lined screw cap, was de-aerated, and then paraformaldehyde (0.8 g) was added and heated to 130° for 1 h[14]. On an aliquot of the resulting clear solution, paraformaldehyde (0.5 g) and dimethyl sulfoxide (5 mL) were added, the mixture was heated again under the same conditions, and the treatment was repeated a third time. Each solution was analyzed with a Toyo 803 h.p.l.c. apparatus. The eluate was colorimetrically monitored by the phenol–H_2SO_4 reagent at 480 nm. The Toyopearl gel column was prepared by the dynamic packing method[15]. A thick slurry of de-aerated Toyopearl HW-65 gel (SF) in dimethyl sulfoxide was filled into the reservoir and it was displaced into the column (4 × 350 mm) by dimethyl sulfoxide under pressure. The

packed column was eluted with dimethyl sulfoxide at a flow rate of 0.2 mL/min. The elution profiles for each of the paraformaldehyde-treated xylan solutions were compared and the apparent molecular weights were estimated by comparison with those of the paraformaldehyde-treated molecular-weight markers (pullulan) that were eluted under the same conditions. After elution with dimethyl sulfoxide, the column was thoroughly rinsed with 0.5M NaOH.

RESULTS AND DISCUSSION

Cell wall xylan was isolated from *Bryopsis maxima* as a white powder in a yield of 84% of the dry weight of its fibrous materials. It was completely soluble in dilute alkaline solution. It was less soluble in dimethyl sulfoxide at room temperature but gave a clear solution on heating at 140° in a sealed tube. The u.v. spectrum of the solution showed the absence of proteins and nucleic acid; $[\alpha]_D^{24}$ −25.1° (*c* 0.75, 0.5M NaOH); ν_{max}^{KBr} 890 cm^{-1} (β-D configuration).

Anal. Calc. for $C_5H_8O_4$: C, 45.45; H, 6.06. Found: C, 45.22; H, 6.03.

On glass-fibre paper electrophoresis, xylan moved as a single spot, and on gel filtration chromatography (Toyopearl HW-65) under alkaline conditions, it emerged as a sharp, symmetric single peak indicating its probable homogeneity; M_r 48 000 (tentative approximation value).

After complete hydrolysis of purified xylan with M sulfuric acid, D-xylose was detected as the sole monosaccharide, but no D-glucose either by g.l.c. or l.c. Recrystallization of the hydrolyzate from ethanol–ether gave D-xylose, m.p. 138–141°, $[\alpha]_D^{24}$ +18.3°; the derived 4-nitrophenylhydrazone had m.p. and mixed m.p. 158–159°.

Periodate oxidation of cellulose, used as control, consumed 0.93 mol of periodate per glucosyl residue for 80 h, when the oxidation seemed to reach a maximum. Within the same time of oxidation, *Bryopsis* xylan consumed 0.027 mol of periodate per xylosyl residue. If the xylan is assumed to be a D-(1→3)-linked linear polymer, 3 mol of periodate would be consumed by both end terminals, and a d.p. or molecular weight of 111 or ~14 600, respectively, was calculated.

After Smith degradation, the gas–liquid chromatogram showed two peaks, one corresponding to per-*O*-trimethylsilylated glycolaldehyde oxime (R_T 4.8) derived from both end terminals, and the other to per-*O*-trimethylsilylated D-xylose oxime (R_T 9.0), derived from the nonoxidized xylose residues. These results were consistent with the periodate data and suggested that the xylan was composed from either 3-*O*-substituted or 2,4-di-*O*-substituted units that were resistant to periodate oxidation.

After three methylations, the xylan showed no i.r. absorption band for OH groups. The methoxyl content (38.5%) closely agreed with the theoretical value (38.8%) for di-*O*-methyl-D-pentosyl residues. G.l.c. showed no evidence in the hydrolyzate of completely methylated xylan, of unmethylated or monomethylated xylitol derivatives indicating incomplete methylation. The peaks were identified by

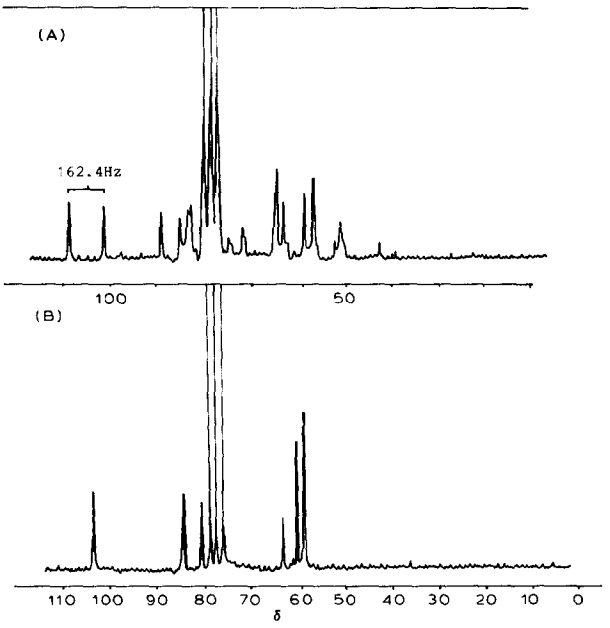

Fig. 1. ^{13}C-n.m.r. spectra of permethylated β-D-(1→3)-xylan: (A) Spectrum of nuclear Overhauser effect, and (B) complete decoupling.

mass spectrometry (Table I). These data confirmed that the xylan is a linear polymer with d.p. of about 120 consisting solely of 1,3-linked D-xylosyl residues.

In the ^{13}C-n.m.r. spectra of the permethylated xylan (Fig. 1), the coupling constant ($J_{C,H}$ 162.4 Hz) and the n.O.e. at the signal of the anomeric carbon at δ 103.2 indicated a β-D configuration. The ^{1}H-n.m.r. spectrum supported this conclusion, showing $J_{1,2}$ 7.4 Hz. The double-resonance spectroscopy for the anomeric proton (signal at δ 4.74) changed the signal at δ 3.31 (H-2) into a doublet ($J_{2,3}$ 8.2 Hz), indicating diaxial protons and a $^4C_1$(D) chair conformation.

TABLE I

G.L.C.–M.S. OF ALDITOL ACETATES DERIVED FROM PERMETHYLATED *Bryopsis* XYLAN

Peak	*T*[a]	*Molar ratio*	*Main mass fragments* (m/z) *and intensity (%)*	*Methylated sugar*
1	0.63	1	87(40), 101(86), 117(100), 161(11)	2,3,4-Me_3-Xyl
2	0.94	118	45(25), 85(41), 86(30), 99(47), 117(100), 118(44), 127(44), 129(30) 158(30), 173(41), 189(14), 233(27)	2,4-Me_2-Xyl

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (1.00).

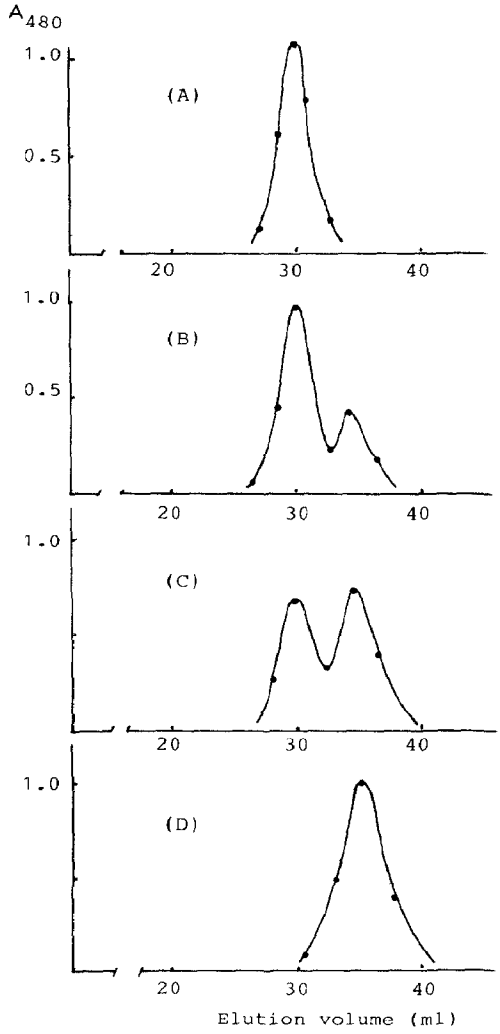

Fig. 2. Elution profiles through Toyopearl gel-filtration chromatograph of *Bryopsis* xylan: (A) Under dilute alkaline condition, and (B) one-, (C) two-, and (D) three-times paraformaldehyde-treated xylan eluted with dimethyl sulfoxide.

In the elution profile of Toyopearl gel filtration chromatography of purified xylan, (Fig. 2), the sharp and symmetric single peak eluted with dilute alkaline solution suggested homogeneity. However, dimethyl sulfoxide elution of the paraformaldehyde-treated xylan gave two peaks, the main one corresponding to the original, and a new minor peak having an apparent lower molecular weight distribution corresponding to ~16 000. With repeated paraformaldehyde treatment the minor, new peak increased and the original peak finally disappeared.

The intrinsic viscosity of the xylan suggested an average molecular weight of 51 8uu. The viscosity of the paraformaldehyde-treated xylan in dimethyl sulfoxide solution indicated a greater decrease of intrinsic viscosity than observed in dilute alkaline solution, suggesting that the ordered structure of the xylan molecules was partially dissociated in dimethyl sulfoxide.

The fractionations by gel-filtration chromatography under alkaline conditions or by successive hot-water treatments showed that the cell wall polysaccharides of *Bryopsis maxima* consists of xylan in majority and less glucan. The structure of the xylan was similar to that of other microfibril or skeletal polysaccharides, such as cellulose, chitin etc., and different from the previously reported branched structure of *Caulerpa* xylan[5,6]. Most of the naturally occurring xylans are usually present as a complex polysaccharide or having substituted residues, such as arabinoxylan or 4-*O*-methylglucuronoxylan. Among the homoxylans, a branched xylan, such as groundnut xylan, is seldom observed[16], whereas the linear xylans, such as esparco grass[17] or guar-seed husk[18] xylans, are rare in Nature. Thus, the linear cell wall β-D-(1→3)-xylan from *Bryopsis maxima* belongs to this rare class.

As the xylan was repeatedly dehydrated during purification, it became insoluble in water and less soluble in dimethyl sulfoxide. Such alteration of the solubility may be attributed to molecular aggregation, most probably through intermolecular hydrogen bonds to form a rigid conformation. Such a conformation of xylan was suggested by comparison of the elution profiles in gel-filtration chromatography under dilute alkaline solution and dimethyl sulfoxide solution. Examination of the dimethyl sulfoxide solution of xylan treated once with paraformaldehyde suggested a partial dissociation of the single polymer chain, and complete dissociation after several treatments. This conclusion was supported by determination of the intrinsic viscosity of the paraformaldehyde-treated xylan in dimethyl sulfoxide solution. Chemical end-group analysis indicated that β-D-(1→3)-xylan takes, in dilute alkaline solution, an ordered structure probably consisting of three xylan chains forming a helical conformation, whereas in dimethyl sulfoxide solution it exists as a random coil.

On the basis of X-ray diffraction, i.r. absorption, and model building. Atkins *et al.*[19] suggested that β-D-(1→3)-xylan from *Penicillum dumetosus* cell wall consists of three intertwined chains forming a three-strand helix in aqueous solution. This proposal is confirmed for other species by the present studies.

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