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Cellulose in the cell wall of the siphonous green alga, *Bryopsis maxima**

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ABSTRACT

The cell wall microfibrils of *Bryopsis maxima*, a member of the order Siphonales which have been referred to as "noncellulosic plants", contain <10% of D-glucan and >90% of (1→3)-β-D-xylan. A combination of enzymic analysis and ^{1}H-n.m.r. spectroscopy confirmed that the D-glucan was cellulose and that the algal cell-wall microfibril seems to be composed not of a D-gluco-D-xylan but mainly of a (1→3)-β-D-xylan with small proportions of cellulose, which seemed to be present in an amorphous state in contrast to the trihelical strands of the (1→3)-β-D-xylan.

INTRODUCTION

The cell-wall polysaccharides of the microfibrils, obtained after successive treatment of siphonous green algae (Siphonales) with dilute hot acid and alkali, are composed of xylan or mannan[2,3], and these algae are referred to as "noncellulosic plants". These characteristics of the algae have been used for taxonomy and for the phylogenetic evaluation of these algae[4,5].

In early structural investigations[6,7] of the D-xylan isolated from the cell wall of various siphonous green algae, (1→3) linkages were found to be preponderant. In addition, about 10% of D-glucose was always found in the hydrolyzates of the crude cell-wall D-xylan. Methylation analysis[6] of the D-glucan isolated from *Caulerpa filiformis* suggested a laminaran-type structure. It has not been established whether D-glucose arose from a D-gluco-D-xylan or from separate D-xylan and D-glucan.

In our previous studies[1], a pure and homogeneous (1→3)-β-D-xylan was isolated from the cell-wall microfibrils of *Bryopsis maxima*[8], and its chemical structure was determined. During the purification of the D-xylan, a polysaccharide fraction referred to as the D-glucose-rich fraction (GRF), was obtained by successive hot-water treatments of the crude, alkali-soluble polysaccharide. We describe herein the chemical properties of the D-glucose-rich fraction in order to ascertain the existence of the D-glucan in cell-wall polysaccharides.

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RESULTS AND DISCUSSION

Hot-water treatment of crude cell-wall polysaccharides gave turbid supernatant extracts that were insoluble in water but readily soluble in dilute alkali and dimethyl sulfoxide. The molar ratio of D-glucose and D-xylose in the first hot-water supernatant extract of Fraction 1 was 1.27:1; this material was used for the investigation of GRF. Every D-glucose-rich fraction obtained exhibited a negative specific rotation or a υ_{max} value at 890 cm^{-1} (so called type-2b) in the i.r. spectrum, which suggested a β-D configuration[9]. The fractions did not stain with iodine, but gave a blue color with the zinc chloride–iodine–potassium iodide reagent[10] and Lugol–sulfuric acid reagent[10], and a pink color with the calcium chloride–iodine reagent[10]. These results suggested that the fractions not only consisted of D-xylan but also of cellulose.

Toyopearl HW-65 gel-filtration chromatography under dilute alkaline conditions (0.5M NaOH) of Fraction I gave three peaks (Fig. 1, A, B, and C) showing a glucose-to-xylose ratio of 0.94:1, 1.86:1, and 2.67:1, respectively. Rechromatography of Peak C under the same conditions increased the ratio to 3.46:1. However, the molecular sizes of the D-xylan and D-glucan seem so close that it was not possible to separate them further. The apparent molecular weight of ~18 000 was based on a preliminary comparison of the elution volume of the highest peak, as compared to a series of "Shodex P-82" (commercial products of pullulan manufactured as molecular-weight markers by Showa Denkoh Co. Ltd.).

GRF was treated with paraformaldehyde, and the products were separated on Toyopearl HW-65 gel by elution with dimethyl sulfoxide[1]. A material was eluted in the same region as that eluted with dilute alkaline solution, which suggests that, unlike the

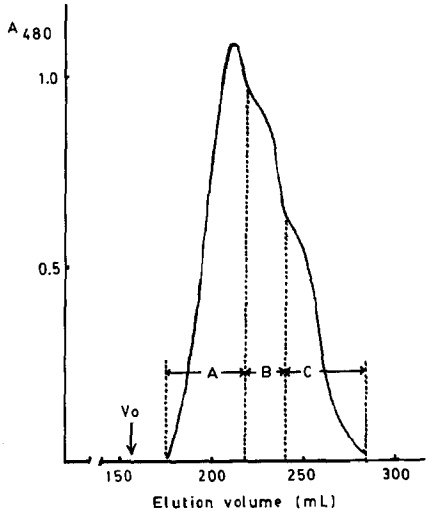

Fig. 1. Elution profile of Toyopearl gel-filtration chromatography of Fraction I.

(1→3)-β-D-xylan described in the previous report[1], the polysaccharide chains of GRF are not aggregated through intermolecular hydrogen-bonds.

Treatment of an alkaline solution of GRF with sodium borohydride at room temperature for 20 h, followed by acid hydrolysis and reverse-phase h.p.l.c. gave four peaks, two minor ones identified as xylitol and glucitol, and two major ones identified as D-xylose and D-glucose. The d.p. of the cell-wall glucan was estimated as ~110 from the peak area ratio of glucitol-to-D-glucose. After 98 h of periodate oxidation, GRF consumed 0.72 mol of periodate per anhydrohexose unit; the detection of formaldehyde with a chromotropic acid reagent[11] indicated that the reducing end of the periodate-oxidized polysaccharide was D-glucose and not D-xylose.

G.l.c. of the trimethylsilyl oxime derivatives derived from the complete hydrolyzate of the products of Smith degradation yielded three peaks. The first one was the glycolaldehyde oxime derivative (*T* 4.0) resulting from the oxidation of terminal reducing residues of (1→3)-β-D-xylan and the (1→4)-linked D-glucose units. The second peak was identified as the D-xylose derivative (*T* 7.4) resulting from nonoxidized (1→3)-linked D-xylose units. The third peak (*T* 12.4) was identified as the erythritol derivative and its probable source was the (1→4)-linked D-glucose units.

G.l.c. of the partially methylated alditol acetates derived from permethylated GRF gave four peaks, which were identified by g.l.c.–m.s. and their retention times on g.l.c. Two of the peaks coincided with those of 1,5-di-*O*-acetyl-2,3,4,-tri-*O*-methyl- and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-xylitol from (1→3)-β-D-xylan[1]. The remaining two peaks corresponded to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- and 1,4,5-tri-*O*-ace-

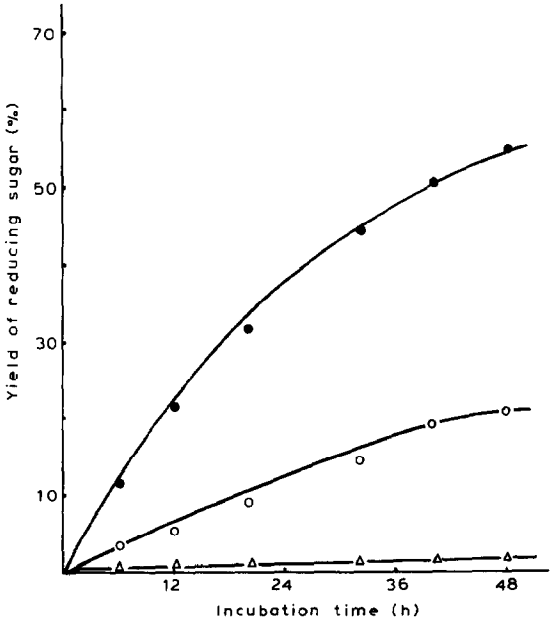

Fig. 2. Action of cellulosic enzyme on: cellulose (—●—), GRF, (—o—), and (1→3)-β-D-xylan (—△—).

tyl-2,3,6-tri-*O*-methyl-D-glucitol, respectively, indicating that the (1→4)-linked-D-glucan was separate from the xylan in the cell wall. The d.p. values, calculated from the ratio of terminal, nonreducing groups to chain residues, were 123 for the D-xylan and 106 for the D-glucan.

D-Glucose and cello-oligosaccharides were detected by t.l.c. of the enzymic hydrolyzate of GRF and cellulose by a partially purified cellulase preparation. D-Xylose or xylo-oligosaccharides from the (1→3)-β-D-xylan were not detected. The enzyme preparation used in this experiment is inactive on pure (1→3)-β-D-xylan. Significantly more reducing sugar was liberated from cellulose than from GRF (Fig. 2). Toyopearl gel-filtration chromatography of the enzyme-reaction mixture showed a single peak

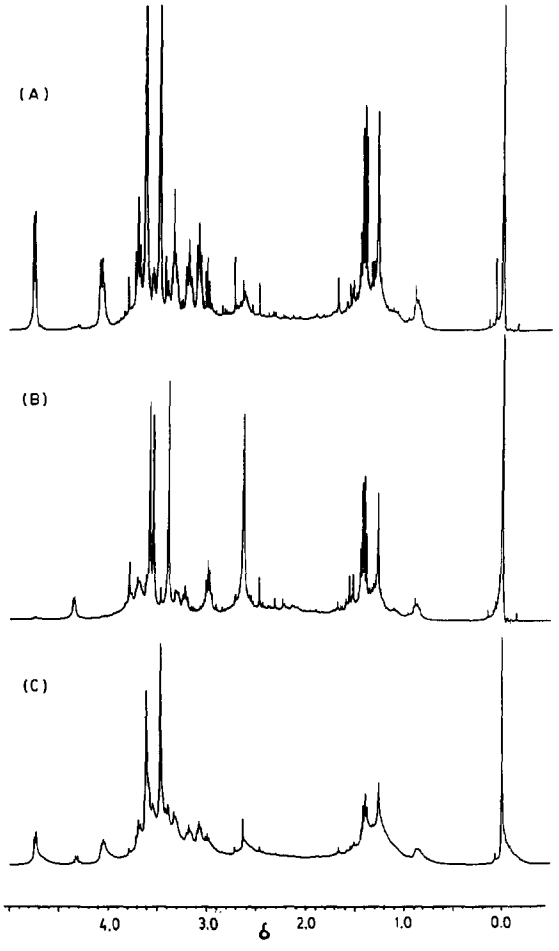

Fig. 3. ^{1}H-N.m.r. spectra for a solution in (^{2}H)chloroform of: Permethylated (1→3)-β-D-xylan (A), cellulose (B), and GRF (C).

corresponding to an apparent mol. wt. of ~16000, indicating a (1→3)-β-D-xylan as only D-xylose was detected in its hydrolyzate.

Acetolysis of GRF gave crystalline octaacetylcellobiose, m.p. and mixed m.p. 222°, and i.r. and ^{1}H-n.m.r. spectra identical to those of a pure preparation.

The ^{1}H-n.m.r. spectrum of permethylated cellulose in (^{2}H)chloroform (Fig. 3A) showed a doublet at δ 4.35 ($J_{1,2}$ 7.3 Hz) which was assigned, on the basis of the H-1 values for methylated cellobiose, to H-1 of a β-D-glucosyl residue. The ^{1}H-n.m.r. spectrum of permethylated (1→3)-β-D-xylan in (^{2}H)chloroform (Fig. 3B) showed a signal for the anomeric protons at δ 4.73 ($J_{1,2}$ 7.6 Hz), which indicated the β-D-anomeric configuration of the xylosyl residues in this polysaccharide. The ^{1}H-n.m.r. spectrum of a 1:1, (w/w) mixture of the methylated derivatives of cellulose and *Bryopsis* cell-wall xylan in (^{2}H)chloroform showed signals at δ 4.35 and 4.73, in the ratio of 51:49, for the anomeric protons of the linear (1→4)-β-D-glucan and (1→3)-β-D-xylan. The ^{1}H-n.m.r. spectrum of permethylated GRF (Fig. 3C) was similar to that obtained from a mixture of permethylated cellulose and (1→3)-β-D-xylan.

The cell-wall xylan and cellulose could not be separated by Toyopearl gel-filtration chromatography, but the acetylated GRF and D-xylan were separated through gel-permeation chromatography in oxolan. Their respective apparent mol. wts. of 32000 and 26600 were in agreement with the results previously obtained. The acid hydrolyzates of the two separate peaks contained D-glucose or D-xylose, respectively.

In our previous study[1], it was shown that the (1→3)-β-D-xylan molecules were rigidly associated through intermolecular hydrogen bonds to form stable three-strand

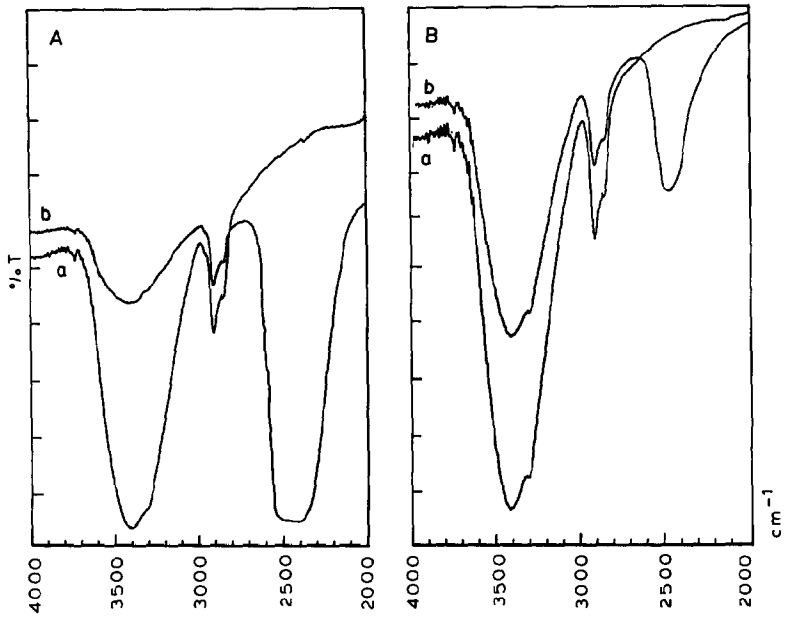

Fig. 4. I.r. spectra of: GRF (A) and (1→3)-β-D-xylan (B), before (a) and after (b) deuteration.

helices. The degrees of intermolecular hydrogen bonding in the (1→3)-β-D-xylan and GRF were compared by determing the rate of hydrogen–deuterium exchange by i.r. spectroscopy (Fig. 4). When the (1→3)-β-D-xylan was exposed to D_2O for 4 h, the exchange reaction progressed slowly and the i.r. spectrum still showed distinct OH stretching bands, indicating an ordered or highly crystalline state with multiple intermolecular hydrogen-bonds. The deutero exchange reaction proceeded rapidly in GRF, the OH stretching bands disappeared, and new OD stretching bands appeared within 30 min, indicating an amorphous structure[12]. This may explain the ready suspension of GRF in the supernatant liquid by such moderate treatments as hot-water agitation.

In conclusion, the cell wall of *B. maxima* consists of a mixture containing a large proportion of linear (1→3)-β-D-xylan and cellulose. The presence of a laminaran-type D-glucan in *Caulerpa filiformis*[6] seems to be the result of contamination by a reserve polysaccharide[13]. Thus, the present study furnishes chemical evidence of the occurrence of cellulose in the siphonous green algae for the first time and, therefore, *B. maxima* cannot be called a "noncellulosic plant".

EXPERIMENTAL

General methods. — Optical rotations were measured with a Jasco model DIP-181 polarimeter. I.r. spectra were recorded with a Jasco IR-700 infrared spectrophotometer on KBr disks for free sugars and liquid films in dichloromethane for the methylated sugars. T.l.c. was performed on microcrystalline cellulose (Avicel SF) plates in 6:4:3 (v/v) butanol–pyridine–water; the spots were detected with an acetone–$AgNO_3$ reagent[14]. Alditol trifluoroacetates were analyzed by g.l.c. using a Jeol 20K-FP gas chromatograph equipped with an FID, in a packed glass column (1m × 2mm, i.d.) containing 1.5% of QF-1 (fluorinated silicone) on Gas Chrom Q at an isotherm of 110°, and N_2 as the carrier gas[15]. L.c. was performed in a Toyo model 803D chromatograph and a Toyo RI-8 differential refractometer. Reversed phase elution was performed on a column (3 mm, i.d. x 30 cm) of Lichrosorb-NH_2 (Merck, 5-μm particle size) at a flow rate of 0.4 mL/min. with aqueous 70% acetonitrile. Peak areas on the chromatograms were determined by planimetry. The sugars were identified on the basis of a comparison of retention times with those of authentic sugars. Evaporations were conducted under reduced pressure at temperatures not exceeding 45°, unless otherwise stated. Acid hydrolysis of a polysaccharide was usually achieved by heating with 2M H_2SO_4 for 2 h at 100°. In the case of complete hydrolysis of the GRF, 72% conc. H_2SO_4 was added to the sample in a Teflon-lined, screw-capped test tube, and the content well mixed[16]. After 1 h at room temperature, distilled water was added to dilute H_2SO_4 to a 2M concentration and the sample was hydrolyzed for 2 h in a boiling-water bath. The hydrolyzate was analyzed by g.l.c., h.p.l.c., and t.l.c.

Materials. — *Bryopsis maxima* was collected in May, 1981, at Kazusaokitsu, Chiba Prefecture. Cell-wall microfibrils were obtained in the form of residual fibrous material after successive heatings of air dried alga in 1.25% NaOH (10 vols.) and 1.25%

H_2SO_4 (10 vols.) for 30 min at 100°. Then crude cell-wall polysaccharide was precipitated by the addition of 80% of ethanol to the ice-cooled 10% NaOH extract[8].

Fractionation of GRF from crude cell-wall polysaccharide. — Crude cell-wall polysaccharide (2 g) was suspended in water (10 vols.) and heated to reflux with mechanical stirring in a boiling-water bath for 2 h. After settling at room temperature, the turbid supernatant suspension was removed, and replaced with a nearly equal volume of water, and the same procedure was repeated five more times. The supernatant suspensions obtained (Fractions I to V) were separately dialyzed and lyophilized.

Periodate oxidation. — Samples (10 mg) were suspended in 0.1M acetate buffer (2 mL, pH 5.6) and a 0.1M $NaIO_4$ solution (2 mL) was added to make the final concentration 0.05M. Oxidation was continued at 4° in the dark with continuous stirring. Portions (20 μL) of the mixture were removed at time intervals and diluted with water to 10 mL. The amount of periodate consumed per anhydrous sugar residue was determined[17] from the extinction of the resulting solution at 223 nm. Cellobiose and potato amylose were also oxidized under the same conditions as controls. After the periodate consumption had reached a constant value, an aliquot of the reaction mixture was diluted with water, the excess periodate was reduced with dilute H_2SO_4 and $Na_2As_2O_3$, and formaldehyde was determined at A_{570} after addition of the chromotropic acid reagent[11].

Smith degradation[18]. — After the periodate oxidation was completed, the mixture of oxidation was reduced with $NaBH_4$, acidified with acetic acid, dialyzed, and concentrated. The reduced polyhydroxy compound was hydrolyzed with 0.25M HCl for 1 h at 80°. Portions (2 mL) of the hydrolyzate were treated with hydroxylamine hydrochloride, per(trimethylsilyl)ated in pyridine, and analyzed by g.l.c. using a capillary column of OV-17 at a temperature of 140°.

Permethylation of polysaccharides. — Samples (~5 mg) of GRF, (1→3)-β-D-xylan, and cellulose were methylated three times by Hakomori's method[19]. Extent of permethylation was monitored by i.r. spectroscopy for OH bands and methoxy content determination[20].

The ^{1}H-n.m.r. spectra of the permethylated polysaccharides were recorded with a Jeol FX-90Q spectrometer, operated at 90 MHz in the pulsed, F.t. mode, at ambient temperature for solutions in (^{2}H)chloroform at a concentration of 1%. The chemical shifts are expressed downfield from the signal of internal tetramethylsilane.

The acid hydrolyzates of permethylated samples were converted to partially methylated alditol acetates and analyzed by g.l.c. using a packed-glass column (1 m x 2 mm, i.d.) containing 3% ECNSS-M on Gaschrom Q (80–100 mesh) with an isothermal column temperature of 180°. The peaks on the chromatogram were identified by their retention times (*T*), relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, in comparison with reported values[21]. The e.i.–m.s. of partially methylated alditol acetates were recorded with a Hitachi M-80A mass spectrometer and a g.l.c.-inlet system. Packed columns of 3% OV-17 (1 m x 2 mm, i.d.) were used at an oven temperature of 190°. The mass spectra were recorded at an inlet temperature of 230°, an ionizing potential of 20eV, an ionizing current of 110 μA, and an ion-source temperature of 270°. Identification of fragmentation patterns was made by comparing major mass-spectral ions with data in the literature[16].

Enzymic hydrolysis. — A commercial, crude cellulase preparation (marketed by Hoechst, Japan) produced from *Aspergillus niger* was used; one unit of enzyme liberated 1 mg of D-glucose from cellulose powder per h at 37° and pH 5.2. Successive purifications with 50–80% $(NH_4)_2SO_4$ precipitation, and column chromatography on DEAE-Sephadex, Amberlite IRC-50, and hydroxyl-apatite, gave[22] partially purified preparations of F-II which were treated with the enzyme as follows. Enzyme powder (0.06 unit per mg of substrate) was incubated with a suspension of GRF (50 mg) in 50mM sodium acetate buffer (pH 5.2, 30 mL) for 48 h at 37° in the presence of toluene. The enzyme activity was assayed against cellulose powder (Whatman), as well as *B. maxima* (1→3)-β-D-xylan. After the reaction had been stopped by heating for 5 min in a boiling-water bath, the reducing sugars were identified by t.l.c. or determined by the Somogyi–Nelson method[23].

After the amounts of reducing sugar produced had reached a maximum, the enzymic reaction mixture was deproteinized with trichloroacetic acid, the precipitate was removed by centrifugation, and the supernatant solution was dialyzed against running tap water for 3 days and then lyophilized. The white residual powder was dissolved in 0.5M NaOH and eluted from a Toyopearl HW-65 column with dilute alkaline solution. The elution profile of the polysaccharide was monitored by the phenol–H_2SO_4 method[24], and the sugar composition of the hydrolyzate determined, after neutralization with HCl and dialysis, by g.l.c. or h.p.l.c.

Gel-permeation chromatography of peracetylated GRF. — GRF was peracetylated with acetic anhydride and pyridine, and the peracetylated sample was eluted through a TSK gel G-3000H column (7.8 mm i.d. x 60 cm) with oxolan. The peaks were collected separately and hydrolyzed, and the sugar components detected by g.l.c. as alditol trifluoroacetyl derivatives.

Identification of acetolysis products of GRF. — GRF was mixed with cooled acetic anhydride–conc. H_2SO_4 and the mixture maintained for 96 h at 30°. The acetolysis products were extracted with chloroform and they crystallized by the addition of methanol.

Determination of hydrogen–deuterium exchange in cell-wall polysaccharides. — *Bryopsis* (1→3)-β-D-xylan and GRF, were each suspended in D_2O, and the disappearance of the OH stretching bands was measured by i.r. spectrophotometry[25]. A matched pair of CaF_2 liquid cells of 0.05-mm path length was used, one cell being always filled with D_2O and used as the reference cell, and the other being the sample cell. Frequent short scans of the spectrum starting from 4000 to 2000 cm^{-1} was performed after appropriate periods of exchange, and the cells were removed from the beams in the spectrophotometer between the scans to minimize heating effects.

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