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Chemical characteristics of insoluble glucans from the cell wall of the marine green alga *Ulva lactuca* (L.) Thuret

M. Lahaye [a,*], D. Jegou [a], A. Buleon [b]

[a] *Institut National de la Recherche Agronomique, Laboratoire de Biochimie et Technologie des Glucides, B.P. 527, 44026 Nantes, France*

[b] *Institut National de la Recherche Agronomique, Laboratoire de Physico-Chimie des Macromolécules, B.P. 527, 44026 Nantes, France*

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Abstract

The chemical composition and structure of the glucan in the alkali-insoluble cell-wall polysaccharides from the marine green seaweed *Ulva lactuca* are reported. The α-cellulose is composed (in mol%) of glucose (72.9), xylose (9.7), rhamnose (4.6), uronic acid (4.3), and sulfate (8.5). The last three components do not appear to be part of the glucan as they were partially removed by methylmorpholine *N*-oxide treatment. Enzymatic hydrolysis with two commercial glucanases containing preparations generated short oligosaccharides and sugars that were identified by chemical analyses and NMR spectroscopy as glucose, xylose, glucitol, cellobiose, and a new trisaccharide identified as β-D-xylopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose. The amorphous-like cellulose X-ray diffraction diagram obtained from the alkali-insoluble and enzyme resistant polymers was interpreted in the light of the presence of xylose-containing glucan in the insoluble cell-wall polysaccharides from *U. lactuca*.

Key words: Cellulose; Xyloglucan; Cell wall; *Ulva lactuca*; Chlorophyceae; Algae

1. Introduction

Marine green algae belonging to the Ulvaceae family represent an important and underexploited biomass. At times, these seaweeds proliferate along European coastlines in response to eutrophication of seawaters [1] and cause economic and ecological problems. The collected beach-cast biomass is generally dumped, al-

* Corresponding author.

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though part of it is used in the making of compost [2]. *Ulva* and *Enteromorpha* species which belong to this algal familly, have traditionally been consumed in Asia and have recently been authorized for human consumption in France as vegetables. Among the nutritional benefits of these edible seaweeds is the high percentage of dietary fibre [3] which are composed of polysaccharides resistant to endogeneous human digestive enzymes [4]. These food polymers may have protective roles against some pathologies prevailing in Western countries [4], and the consumption of green seaweeds as vegetables could contribute to an increase in the presently low dietary fibre intake in the West. This biomass may also be used for the production of colloids, since water-soluble polysaccharides from species of *Ulva* form gels in the presence of both boric and calcium ions [5,6].

The chemical composition and the ultrastructural organisation of the insoluble polysaccharides from ulvals have been little studied although they can form up to 33% (dry weight) of *U. lactuca* as insoluble dietary fibres [3], and can be used for applications such as paper making [7]. The insoluble residue obtained after alkaline extraction (4 M KOH) of *U. lactuca* was reported [8] to be composed of glucose and xylose, and was not modified further by treatment with chlorite. Such a glucan failed to give a cellulose-I type X-ray diffractogram [8], but the presence of this type of crystalline cellulose was later described [9] after the algal cell walls had been extensively hydrolysed with sulfuric acid (1.2 M, 100°C, 24 h). However, on isolated and chemically treated cell walls from two closely related species, *Enteromorpha intestinalis* and *U. rigida*, X-ray diffractograms resembling that of cellulose-II or amorphous cellulose were obtained [10,11]. Furthermore, endo-(1 → 3)-β-D-glucanase hydrolysis of the α-cellulose from *E. intestinalis* generated laminaribiose, indicative of the presence of β-(1 → 3)-D-glucan in the cell wall of these algae [10].

In the present report, the insoluble glucans from the cell wall of *Ulva lactuca* were investigated by chemical, enzymatic, NMR spectroscopy, and X-ray diffraction methods in order to establish the xylose location, the presence of β-(1 → 3)-D-glucan chains, and to see whether cellulose-I microfibrils could be obtained by enzymatic enrichment of the α-cellulose fraction.

2. Materials and methods

Alga.—Ulva lactuca (L.) Thuret was collected in March 1992 at Pointe St. Gildas (Loire Atlantique, France) and was used immediately.

Preparation of the material.—The seaweed (150 g, wet weight, 85% moisture) was rinsed with tap water, suspended in sodium acetate (0.1 M, pH 4.0, 1 L) containing pepsin (205 mg, Merck, Darmstadt), and minced in a blender. The slurry was left for 1 h at 45°C and for 30 min in a boiling water-bath, passed through a nylon net to recover the insoluble residues, and the liquid fraction was centrifuged for 30 min at 13 300 *g*. The combined centrifugation pellet and insoluble residues were re-extracted in deionized water (250 mL) for 30 min in a boiling water-bath and the insoluble material recovered as above. This aqueous extraction

was repeated once more and the water-insoluble residues were dehydrated by solvent exchange (95% EtOH, acetone, and diethyl ether), and dried in vacuo at 40°C over P_2O_5 (5.8% yield from the starting wet weight). This fraction is referred to in the text as A. These residues (5 g) were extracted twice for 1 h in 1 M and 4 M KOH solutions (100 and 50 mL) containing $NaBH_4$ (0.3% w/v) [12]. The insoluble residues were recovered by centrifugation (5 min, 13300*g*). The final insoluble residues were resuspended in deionized water, dialyzed extensively against deionized water, recovered by centrifugation (15 min, 13300*g*), dehydrated by solvent exchange as above, and dried in vacuo at 40°C over P_2O_5 (1.3% yield from the starting wet weight). This fraction is referred to in the text as B.

Enzymatic digestion of the insoluble material.—Fraction B in 5 mM sodium acetate, pH 4.0 (1% w/v) was digested with Finizym (Novo Industrie, Bagsvard, Denmark; 1 mg of protein per 100 mg of substrate) added at 0 and 24 h (for further details, see Ref. [13]). The suspension was filtered through a sintered glass filter (G4, porosity 5–15 μm) and the insoluble enzyme-resistant fraction was dehydrated and dried as above (55.8% on the initial sugar basis of B). This resistant fraction was then treated in a similar way with Celluclast (Novo) and yielded a resistant fraction (44.4% on the basis of the initial sugar content of the Finizym resistant fraction). Two soluble fractions, SF and SC, were obtained with 36.7 and 45.5% yields (on the basis of the initial sugar content of B and the Finizym resistant faction, respectively) after Finizym and Celluclast treatments, respectively. The two commercial enzyme preparations were extensively dialyzed against deionized water before use.

Treatment of fraction B with methylmorpholine N-oxide (MMNO).—The procedure used followed that described [14]. Fraction B (0.1 g) with MMNO (2.5 g) was heated under Ar for 30 min at 100°C. After cooling to room temperature, Me_2SO (10 mL) was added with stirring. The solution was poured into deionized water (10 mL) and the floculated material was recovered by centrifugation (13500*g* for 10 min), and dehydrated by solvent exchange as above. The supernatant was dialyzed extensively against deionized water and freeze-dried. Determination of the yields of the soluble (fraction S) and insoluble (fraction I) fractions was impossible because of the incomplete removal of residual reagents.

Anion exchange chromatography.—Anion exchange chromatography was carried out on a DEAE-Sepharose CL-6B column (17 × 2 cm; Pharmacia, Uppsala, Sweden) eluted with deionized water and a 0 → 1 M NaCl gradient at room temperature.

Gel filtration chromatography.—Gel filtration chromatography was carried out on a BioGel P2 column (96 × 2.6 cm; BioRad, Richmond, CA, USA) eluted with deionized water at 30°C and at a flow rate of 27 mL h^{-1}. Elution was monitored by differential refractometry. Fractions were pooled and freeze-dried.

Chemical analyses.—Neutral sugars were quantified by gas chromatography (GC) [15]. Water insoluble polysaccharides were subjected to pre-hydrolysis with H_2SO_4 (13 M, 30 min, 20°C) prior to hydrolysis with M H_2SO_4 (2 h, 100°C); water soluble fractions were hydrolysed with M H_2SO_4 (2 h, 100°C). Alditol acetates were chromatographed on a DB 225 fused-silica capillary column (J&W Scientific) running isothermally at 210°C and with H_2 as the carrier gas.

Methylation, reduction, and acetylation of the oligosaccharides were carried out following the methods of Blakeney and Stone [16] and Harris et al. [17] as described [18]. Identification of methylated alditol acetates was by comparison with standards and by a GC coupled to a mass spectrometer (R10-10C, Delsi–Nermag) operating in the EI mode at 70 eV, with a transfer chamber temperature at 250°C. The GC was equipped with the same capillary column used for the alditol acetates analysis except that a temperature gradient was used (175°C for 15 min, then 175 → 210°C at 15°C min^{-1}, and then isothermal at 210°C for 13 min).

Uronic acids were quantified by colorimetry from the sulfuric acid hydrolysate recovered from the neutral sugar analysis [19,20].

Total sugar content in the anion-exchange chromatography fractions was determined by colorimetry [21].

Sulfate content was determined by high pressure liquid chromatography [6] on a Nucleosil 10 Anion II column (Macherey–Nagel, Duren, Germany) eluted with 2 mM potassium hydrogenophthalate (pH 5.7) at 2 mL min^{-1}.

Nitrogen content of the fractions was measured according to the micro-Kjeldahl method and converted to proteins (N × 6.25). Protein content in the dialyzed enzyme preparations was measured according to the method of Lowry [22].

Ash was measured gravimetrically after 16 h incineration at 550°C followed by 2 h at 900°C.

Nuclear magnetic resonance spectroscopy.—^{13}C and ^{1}H NMR spectra were recorded at 30°C for solutions in D_2O using a Bruker AM-500 spectrometer. Hydroxyl protons were exchanged with deuterium by repeated freeze-drying (3 times) of the oligosaccharides in D_2O. Spectral conditions: ^{13}C NMR: 125.75 MHz, 60° pulse, recycling time of 1 s, and acquisition of 8K data points. ^{1}H NMR: 500.12 MHz, 90° pulse, and recycling time of 2.7 s. Chemical shifts were measured in ppm from Me_2SO (H-6) assigned at 43.5 ppm for ^{13}C and 2.6 ppm for ^{1}H.

X-ray diffractometry.—Diffraction diagrams were recorded using a Inel X-ray generator operating at 40 kV and 30 mA and a curve position sensitive detector (Inel CPS 120). Cu $K\alpha$ radiation (0.15405 nm) was selected using a quartz monochromator. The samples (100 mg dry matter) were sealed between two sheets of aluminum foil to prevent any significant change in the water content during the measurement. All samples were studied in the dry state and after equilibration at room temperature over satd $BaCl_2$ ($a_w = 0.90$).

3. Results

The chemical composition of the insoluble fraction A obtained by aqueous extraction of *Ulva lactuca* was similar to that of the insoluble dietary fraction obtained from this algae [3]. It was composed of 40.5% protein, 36.1% sugar (neutral sugars and uronic acids), and 9.4% ash including 3.9% sulfate. Glucose was the major sugar (56.9 mol%, Table 1), followed by xylose (11.0 mol%), rhamnose (8.9 mol%), and uronic acid (6.9 mol%). After extraction of A with 1.0 and 4.0 M KOH, an insoluble fraction B was recovered in 1.3% yield based on the

Table 1
Molar percentages of neutral sugars, uronic acids, and sulfate in fractions from *Ulva lactuca*

	Fraction [a]							
	A	B	S	I	RF	SF	RC	SC
Rhamnose	8.9	4.6	22.3	3.5	4.2	9.3	4.6	3.0
Arabinose			0.8			1.6		
Xylose	11.0	9.7	3.6	10.8	5.8	10.8	1.0	7.5
Mannose			0.6		0.7	5.8		
Galactose			0.6		0.3	1.6		
Glucose	56.9	72.9	28.5	78.9	64.5	65.1	38.5	86.5
Sulfate	16.3	8.5	29.0	4.4	nd [b]	nd	nd	nd
Uronic	6.9	4.3	14.6	2.4	24.5	5.8	55.9	3.0

[a] A and B refer to the insoluble residues from *Ulva lactuca* after hot-water extraction and alkali treatement, respectively; S and I refer to the soluble and insoluble fractions, respectively, recovered after MMNO treatment of fraction B; RF and SF refer to the resistant and soluble fractions recovered after Finizym treatment of fraction B, respectively; RC and SC refer to the resistant and soluble fractions recovered after Celluclast treatment of fraction RF, respectively.
[b] nd, Not determined.

starting algal wet weight (8.5% dry weight basis). It was composed of 56.6% sugar (neutral sugars and uronic acids), 11.3% protein, and, although it was extensively washed, 26.3% ash including 2.0% sulfate. The glucose proportion increased from 56.9 in fraction A to 72.9 mol% in fraction B, whereas that of rhamnose and uronic acid decreased (Table 1). The chemical composition and structure of the alkali-soluble polysaccharides will be reported elsewhere. Attempts to separate the xylose containing polysaccharides from the glucan by MMNO (a solvent of cellulose [14]), were unsuccesful since its content in fraction I (10.8 mol%) after such treatment was close to that measured before treatment (fraction B, 9.7 mol%, Table 1). Nevertheless, MMNO treatment indicated that rhamnose, sulfate, and uronic acid were probably not part of the insoluble glucan fraction as they were recovered in the MMNO soluble fraction S (Table 1). At this point, xylose appeared to be an inherent constituent of the glucan. In order to corroborate this, fraction B was hydrolyzed by Finizym which is a commercial enzyme preparation containing β-(1 $\rightarrow$ 4)-xylanase (77 nKat mg^{-1} protein with 1 nKat = 10^{-9} Katal = conversion of 10^{-9} mol of substrate per second), microcrystalline cellulase (14 nKat mg^{-1} protein), carboxymethyl cellulase (10.6 nKat mg^{-1} protein), laminarinase and lichenanase (8.7 and 4.3 nKat mg^{-1} protein, respectively) activities and very little glycosidase activities (0.08, 0.05, and 0.25 nKat mg^{-1} protein for glucosidase, xylosidase, and cellobiosidase, respectively; Bonnin, personal communication). Such treatment generated soluble (SF) and resistant (RF) fractions, the sugar compositions of which are given in Table 1. The soluble fraction was essentially composed of glucose, but also contained xylose and rhamnose. The resistant fraction was essentially composed of glucose and uronic acid. Fraction SF, treated with anion-exchange chromatography, allowed the recovery of a neutral fraction in 63.0% yield on the basis of the total sugar applied and determined by colorimetry. This fraction was chromatographed through BioGel P2

into one peak eluted at the void volume and peaks F1, F2, F3, and F4 eluted at K_{av} values of 0.86, 0.81, 0.77, and 0.68, respectively (Fig. 1). ^{13}C NMR analysis of the void volume eluted material established that this fraction was not composed of orderly structured poly- and/or oligo-saccharide since no resolved resonance was obtained. However, the ^{13}C NMR spectrum of the other fractions revealed that F1 was composed of glucose with some xylose monomers, F2 was glucitol most probably originating from reducing ends of glucan residues modified to alditol by $NaBH_4$ treatment during the alkali extractions, F3 was composed of cellobiose (Table 2), and F4 could not be readily identified. From chemical and methylation analyses, the latter oligosaccharide was determined to be composed of 2.7 glucose residues per xylose residue, and that xylose was at the nonreducing end, whereas the glucose residues were (1 → 4)-linked. The intensity of the four ^{1}H NMR anomeric signals of F4 (Fig. 2) indicated that it was a trisaccharide, and the chemical shifts of three of the signals were in agreement with those published [23] for glucose residues at the reducing end and internal position in cellotriose. The downfield resonance at 4.43 ppm ($J_{1,2}$ 7.6 Hz) was attributed to the anomeric proton of a β-xylose residue by deduction from the ^{13}C NMR spectrum (Fig. 3) on which all the resonances were attributed by comparison with chemical shifts published for cellotriose [24] (Table 2) and β-D-xylose [25]. Thus, the following structure is proposed for this xyloglucan trisaccharide:

β-D-xylopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose

The resistant fraction RF was subjected to enzymatic hydrolysis by Celluclast known to possess activity toward carboxymethyl cellulose (60.5 nkat mg^{-1} protein), lichenan (30.3 nKat mg^{-1} protein), filter paper (10 nKat mg^{-1} protein), and

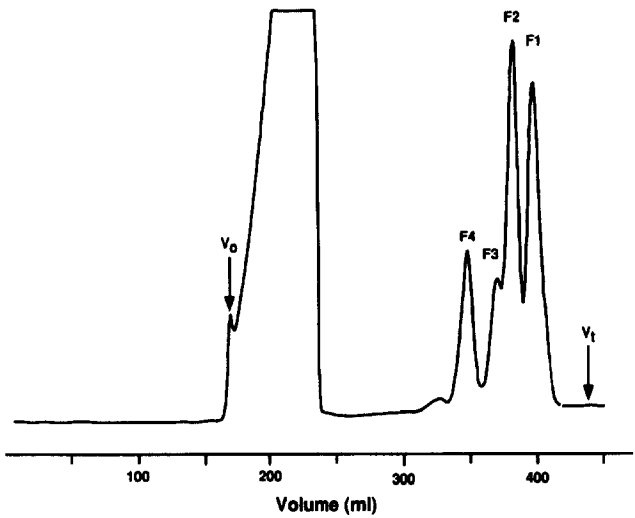

Fig. 1. Gel filtration chromatogram of the neutral oligosaccharides recovered after Finizym hydrolysis of *Ulva lactuca* α-cellulose: V_0 and V_t refer to the void and total volume of the column, respectively. Detection was by differential refractometry.

β-(1 → 4)-xylane (12 nKat mg^{-1} protein) among other activities [26]. Soluble (SC) and resistant (RC) fractions were obtained, the sugar compositions of which are given in Table 1. SC was mostly composed of glucose, whereas RC was essentially composed of uronic acid and glucose, and was not studied further. Subjected to anion-exchange chromatography, SC yielded 68% of neutral oligosaccharides on the basis of the total injected sugar and determined by colorimetry. The oligosaccharides were chromatographed on BioGel P2; one fraction eluted at the void volume and two fractions eluted at K_{av} values of 0.86 and 0.77, respectively. These were identified by ^{13}C NMR spectroscopy as containing mostly glucose, with some xylose monomers and cellobiose, respectively (data not shown). The peak eluting at

Table 2
^{13}C NMR chemical shifts of oligosaccharides recovered from the BioGel P2 gel chromatography of the neutral Finizym hydrolysate of *Ulva lactuca* α-cellulose

Sample	Residue [a]	Carbon [b]						Ref [c]
		1	2	3	4	5	6	
Glucose	α	92.9	72.5	73.8	70.6	72.3	61.6	27
	β	96.7	75.1	76.7	70.6	76.8	61.7	27
F1	α	92.8	72.2	73.6	70.5	72.2	61.6	
	β	96.6	74.9	76.6	70.5	76.6	61.6	
Xylose	α	93.1	72.5	73.9	70.4	61.9		27
	β	97.5	75.1	76.8	70.2	66.1		27
F1	α	*92.8*	*72.2*	*73.6*	*70.5*	*61.6*		
	β	97.4	*74.6*	*76.6*	70.0	65.9		
Glucitol		63.2	73.7	70.4	72.0	71.9	63.6	27 [d]
F2		63.3	73.6	70.5	72.1	72.0	63.6	
Cellobiose	α	92.4	71.8	71.9	79.2	70.7	60.6	24 [e]
	β	96.3	74.6	74.9	79.2	75.3	60.7	24 [e]
	nr	103.0	73.8	76.2	70.1	76.5	61.3	24 [e]
F3	α	92.2	71.9	71.9	79.4	70.6	60.7	
	β	96.3	74.6	75.0	79.4	75.4	60.7	
	nr	103.0	73.7	76.2	70.1	76.5	61.2	
Cellotriose	α	92.4	71.8	71.9	79.3	70.7	60.5	24 [e]
	β	96.3	74.6	74.8	79.1	75.4	60.4	24 [e]
	int	102.9	73.5	74.6	79.0	75.4	60.5	24 [e]
	nr	103.1	73.7	76.1	70.0	76.5	61.2	24 [e]
F4	α	92.4	71.8	72.0	79.3	70.7	60.5	
	β	96.4	74.5	74.9	79.1	75.5	60.6	
	int	102.9	73.7	74.5	78.7	75.5	60.5	
	nr	103.9	73.7	76.3	69.8	65.8		
Xylotriose	α	92.5	71.9	71.5	76.9	59.4		25 [f]
β-(1 → 4)	β	97.0	74.5	74.5	76.9	63.5		25 [f]
	int	102.2	73.3	74.2	76.9	63.5		25 [f]
	nr	102.4	73.3	76.2	69.7	65.8		25 [f]

[a] Internal (int), nonreducing (nr), and reducing sugar under the α or β configuration.
[b] Figures in italic are overlapping peaks.
[c] Reference.
[d] A correction of −0.6 ppm, [e] −2.2 ppm, and [f] −0.3 ppm was given to the reported chemical shifts.

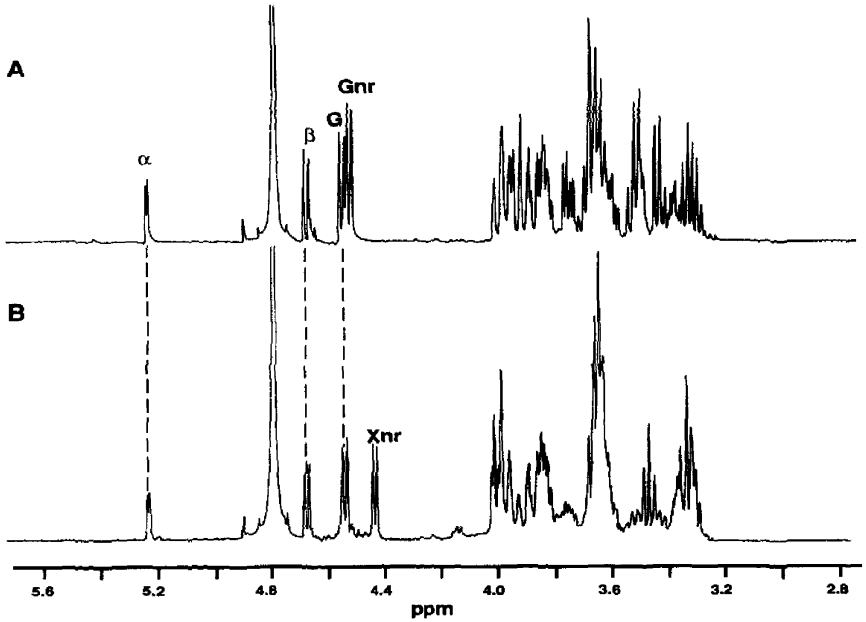

Fig. 2. ^{1}H NMR spectrum of cellotriose (A) and the oligosaccharide eluted as fraction F4 from BioGel P2 (B): α, β, G, Gnr, and Xnr refer to the anomeric proton α, β, to the internal glucose, and to the nonreducing glucose and xylose units, respectively.

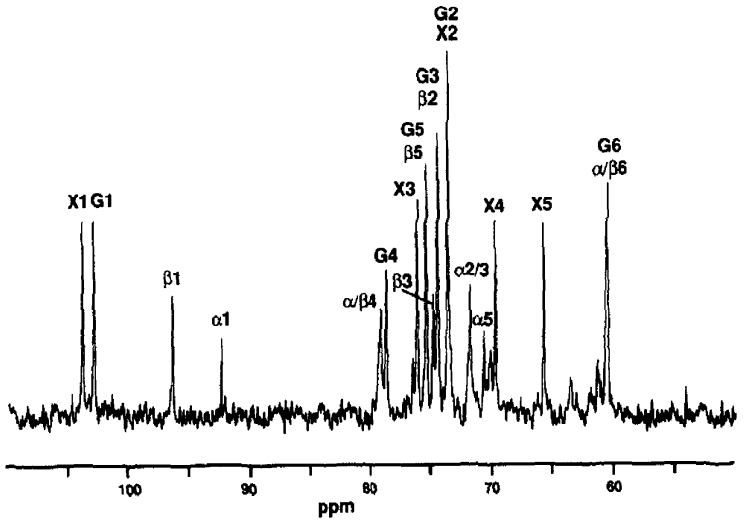

Fig. 3. ^{13}C NMR spectrum of the oligosaccharide eluted as fraction F4 from BioGel P2: G and X refer to carbon of the glucose and xylose units, respectively; α and β to the carbon of the reducing glucose unit in the α and β configuration, respectively.

the void volume failed to give resolved ^{13}C NMR signals, thus, no orderly, structured poly- and/or oligo-saccharide was present in this fraction.

X-ray diffraction analysis of the α-cellulose and the cellulose resistant fraction after hydrolysis by Finizym yielded poor crystalline diagrams, similar to that published [11] from *U. rigida* cellulose. Only two broad peaks (intermolecular spacings: 0.44 and 0.25 nm) were clearly visible as described earlier by Cronshaw et al. [8] for *U. lactuca* after alkaline extraction. These reflections could be attributed to cellulose-II, but in that case, a third band (intermolecular spacing 0.40 nm) should be present with an intensity comparable to that of the 0.44 nm band. Therefore, it is not possible to conclude that cellulose-I or -II are present. No significant change was observed in the diffraction diagram after hydrolysis by Finizym. The high content of uronic acid in this resistant fraction may mask or perturb the cellulose organisation, the fibrils may be of low cristallinity and/or the crystals may be too small.

4. Discussion

Xylose has been reported in the α-cellulose from the cell wall of all ulvals studied [8–10], and β-(1 → 3)-glucan has also been detected in *E. intestinalis* [10]. From chemical degradation and X-ray diffraction analyses, Dennis and Preston [9] postulated that the cellulose microfibrils in *U. lactuca* occurred as cellulose-I and were wrapped by xylan or mixed xylan–glucan polymers. In the present study, to test the presence of xylan, xylose-containing glucan, or β-(1 → 3)-glucan in the α-cellulose from *U. lactuca*, the alkali-insoluble cell-wall residues were digested by a commercial enzyme preparation (Finizym) containing β-(1 → 4)-xylanase, β-(1 → 3)-glucanase, and cellulase activities. The oligosaccharides expected were xylobiose, cellooligomers with some containing xylose and/or laminaribiose, but only glucose, glucitol, cellobiose, and a xyloglucan trisaccharide were found. Thus, this enzymatic hydrolysis clearly demonstrates the linkage of β-D-xylose to β-(1 → 4)-glucan, and suggests that β-(1 → 4)-D-xylan and β-(1 → 3)-D-glucan chains were absent from the α-cellulose of *U. lactuca*. Since the linkage at the nonreducing end of the xylose residue of the xyloglucan trisaccharide is unknown, and since glucitol was also obtained in the hydrolysate, the xylose residue may either be at the nonreducing end and/or randomly dispersed in short cellulose-like glucan chains. The existence of a cellulose core surrounded by mixed xylan–glucan chains [9] was confirmed by the Finizym hydrolysis results and by the chemical composition of the resistant fraction obtained. This fraction was enriched in glucose that was hydrolysed by a cellulase rich commercial enzymatic preparation (Celluclast) to essentially glucose and cellobiose. However, the X-ray diffractogram obtained from the α-cellulose and from the Finizym resistant fraction were not that of cellulose-I, but that of an amorphous cellulose with very weak bands similar to that seen for cellulose-II. The cellulose chains packed in the α-cellulose may be affected by the presence of the mixed xylan–glucan chains, whereas in the enzyme resistant fraction enriched in cellulose, the crystallinity may be too low, the crystals may be

too small (cellulose chains of too low a molecular weight after enzyme hydrolysis) to be detected by X-ray diffraction analysis and/or perturbed by the high concentration of uronic acids. The latter acidic sugars are apparently not linked to the glucan since they were solubilized by MMNO, and may in fact, form a polyuronan that is partly insoluble in strong alkali and chlorite, but may be completely destroyed by strong acid treatment. A β-(1 → 4)-glucuronan has been isolated from these cell walls (Ray et al., [28]).

Thus, the cellulose microfibrils in the α-cellulose from *U. lactuca* are associated with xyloglucan, whose structure differs markedly from that of higher-plants where the xylose residue is in the α configuration and is (1 → 6)-linked to the cellulose backbone [29]. The nature of the associations between the cellulose, xyloglucan, polyuronide, and the water-soluble sulfated xylorhamnoglucuronan that form the cell wall of *U. lactuca* remains to be established.

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