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Solid-state ^{13}C NMR spectroscopy studies of xylans in the cell wall of *Palmaria palmata* (L. Kuntze, Rhodophyta)

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Abstract

The chemical structure and interactions of the cell wall polysaccharides from the red edible seaweed *Palmaria palmata* were studied by liquid-like magic-angle-spinning (MAS) and cross-polarization MAS (CPMAS) solid-state ^{13}C NMR spectroscopy. The liquid-like MAS and CPMAS ^{13}C NMR spectra of the rehydrated algal powder revealed the presence of β-(1→4)/β-(1→3)-linked D-xylan with chemical shifts close to those observed in the solution ^{13}C NMR spectrum of the polysaccharide. Observation of mix-linked xylan in the liquid-like MAS ^{13}C NMR spectrum indicated that part of this cell wall polysaccharide is loosely held in the alga. The CPMAS NMR spectrum of the dry algal powder alcohol insoluble residue (AIR) showed broad peaks most of which corresponded to the mix-linked xylan. Hydration of AIR induced a marked increase in the signal resolution also in the CPMAS NMR spectra together with a shift of the C-3 and C-4 signals of the (1→3)- and (1→4)-linked xylose, respectively. Such modifications were present in the spectrum of hydrated (1→3)-linked xylan from the green seaweed *Caulerpa taxifolia* and absent in that of (1→4)-linked xylan from *P. palmata*. This result emphasizes the important role of (1→3) linkages on the mix-linked xylan hydration-induced conformational rearrangement. The mix-linked xylan signals were observed in the CPMAS NMR spectrum of hydrated residues obtained after extensive extractions by NaOH or strong chaotropic solutions indicating strong hydrogen bonds or covalent linkages. $T_{1\rho}$ relaxations were measured close or above 10 ms for the mix-linked xylan in the dry and hydrated state in AIR and indicated that the overall xylan chains likely remain rigid. Rehydration of the mix-linked xylan lead to a decrease in the motion of protons bounded to the C-1 and C-4 carbons of the (1→4)-linked xylose supporting the re-organization of the xylan chains under hydration involving junction-zones held by hydrogen bonds between adjacent (1→4)-linked xylose blocks. The CPMAS NMR spectrum of both dry and rehydrated residues obtained after NaOH and HCl extractions demonstrated the presence of cellulose and (1→4)-linked xylans. The structures of the different polysaccharides are discussed in relation to their interactions and putative functions on the cell wall mechanical properties in *P. palmata*.

Keywords: *Palmaria palmata*; Rhodophyta; Xylan; Solid-state NMR; Cell wall

1. Introduction

Cell walls of seaweeds are hydrophilic and soft macromolecular assemblies composed of fibrillar and matrix polysaccharides with minor structural proteins.[1,2] They play important roles in different biological aspects (development, defense) and in the various uses of the algae. In particular, like for other plants, entanglement and various interactions of these complex polymers are involved in the cell cohesion and tissue mechanical properties of seaweeds. In red algae, the fibrillar network is made of low crystalline cellulose, mannan or xylan and represents only about 10% of the cell wall weight.[2] It can also contain minor amounts of sulfated glucans, mannoglycans and complex galactans.[3,4] Most of our current knowledge of red algal cell wall polysaccharides is on the gelling and thickening water-soluble galactans, agars and carrageenans, used in

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various applications.[2,5] Unlike most red seaweed generally studied, *Palmaria palmata* (L. Kuntze, Palmariales) does not produce matrix galactans but instead (1→4)- and (1→3)-linked β-D-xylan together with a minor amount of fibrillar cellulose and β-(1→4)-D-xylan.[6–10] This edible seaweed[11] has biotechnological values[12,13] and its tissue mechanical properties have direct impacts on its texture perception by the consumer and on its ability to be processed. In order to better understand the cell wall contribution to the mechanical properties of the algal tissues, the interactions of the mix-linked matrix xylan have been recently investigated by sequential solvent extractions, fractionation methods and physico–chemical characterizations.[14] These studies revealed that this xylan was partly acidic, contained small amounts of sulfate and phosphate groups and was essentially held in the cell wall by hydrogen bonds. However, in extracts obtained with solutions of increasing concentrations of sodium hydroxide and taken as representative of xylan chains interacting by higher numbers of H-bonds, the mean proportions of (1→3) linkage did not differ while their mean molecular weight decreased. Endo-β-(1→4)-D-xylanase degradation of these extracts revealed that the xylan represents a family of related molecules with slightly different (1→3) linkages distributions, free of contiguous (1→3)-linked xylose and idealized by a repeating pentameric structure made of four contiguous (1→4)-linked xylose for one (1→3) linkage.[15] This study also revealed the presence of a minor amount of an unidentified compound substituting the xylan. Besides these polysaccharides, xylosylated and galactosylated structural proteins were also reported in the alga cell wall and have been proposed to contribute to the organization and mechanical properties of the cell wall through covalent linkage with xylan.[16]

Solid-state ^{13}C NMR spectroscopy has been shown to provide ways to characterize the chemical structure, organizations and interactions of polysaccharides in the wall of higher plants,[17–21] and has already been used for the study of galactan structures and metabolites in red seaweed.[22–24] In this report, the mix-linked xylan structure and interactions in *P. palmata* cell wall were studied further by different solid-state NMR spectroscopy experiments.

2. Experimental

2.1. Materials

Palmaria palmata and fractions were obtained as described in Ref. 14. Briefly, *P. palmata* was collected at La Plage de la Grave, Saint Malo, France, December 1999 and specimen are held in the laboratory (LM1). The fresh algae (equiv 10 g dry wt.) were immersed in a volume of 96% boiling EtOH to reach a final EtOH concentration of 70% taking into account the algae water content. The suspension was boiled for 20 min and recovered by filtration (porosity, 150 μm). The algal material was repeatedly washed at room temperature (rt) with 70% EtOH, 96% EtOH, Me_2CO and 3:2, $CHCl_3$–MeOH until each filtrate was colorless and free of sugars as detected by PheOH–H_2SO_4 test.[25] The final residue was referred to as alcohol insoluble residue (AIR) and was dried overnight at 40 °C under diminished pressure.

AIR (10 g dry wt.) was sequentially extracted using successively 250 mL of 0, 0.5, 1.0, 1.5 and 2 M NaCl solutions containing 0.2% sodium azide (20 °C twice for 24 h). The 2 M NaCl residue was then extracted with 300 mL of 1 M NaOH (twice for 30 min at 20 °C) and with 200 mL of 8 M NaOH (twice for 30 min at 20 °C). Finally, the 8 M NaOH residue was extracted with 300 mL of 0.05 M HCl (thrice for 30 min at 85 °C) to obtain the 0.05 M HCl residue.

AIR (10 g of dry wt.) was sequentially extracted with 1 L of 2 M urea (twice for 60 min at 25 °C), 2 L of 8 M urea in HEPES 50 mM, pH 7.5 (twice for 24 h at 25 °C) and 2 L of 4.5 M guanidium thiocyanate (twice for 24 h at 25 °C) to obtain the 4.5 M guanidium thiocyanate residue.

2.2. Mercerization of cellulose and preparation of birchwood β-(1→4)-D-xylan

Microcrystalline cellulose (Whatman CC31) and β-(1→4)-D-xylan from birchwood (Sigma X-0502) were suspended in 8 M NaOH for 2 days (100 mg per 2 mL). They were rinsed five times with deionized water to eliminate NaOH and freeze-dried.

2.3. Preparation of β-(1→3)-D-xylan from *Caulerpa taxifolia*

β-(1→3)-D-Xylan from *C. taxifolia* was extracted according to the procedure described.[26] Algae were collected at Cap Martin (6 April, 2002; depth, 15 m) and air-dried. Algae were rehydrated and thoroughly washed with deionised water. They were then boiled in 10 vol of 1.25% NaOH for 30 min and extensively washed by deionised water. The same operations were repeated with 1.25% H_2SO_4 and finally the alga was bleached with $NaClO_4$ (1%) for 1 h followed by extensive washings by deionized water before air-drying. Xylan was isolated from these residues by three extractions with 10% NaOH for 1 h at 4 °C with magnetic stirring. The extracts were filtered (porosity 15–40 μm) and the xylan precipitated overnight in 4 vol of 95% EtOH at 4 °C. The precipitate was centrifuged at 25 °C for 10 min at 8000*g* and washed with 33% HOAc then with deionized water. An aqueous suspension of the

precipitate was dialyzed against deionized water until the conductivity of the dialyzate was less than 5 μs cm^{-1} and freeze-dried to obtain insoluble β-(1→3)-xylan.

2.4. Chemical characterization

All data are expressed as means of duplicates.

Dry weight of materials and that of the initial fresh algae was determined after 2 h at 120 °C. Ash content was determined after incineration overnight at 550 °C and 1 h at 900 °C. Sugar composition of dry insoluble algal materials (25 mg freeze-milled) was obtained after pre-hydrolysis in 26 N H_2SO_4 at 25 °C for 30 min followed by hydrolysis with 2 N H_2SO_4 at 100 °C for 2 h.[27] Monosaccharides were reduced to alditols with $NaBH_4$ and acetylated by Ac_2O and *N*-methylimidazole.[28] The acetylated alditols were identified and quantified by gas chromatography using a DB225 column (J&W Scientific, Folsom, CA, temperature 205 °C, carrier gas H_2). Inositol was used as internal standard and sugar-specific weight response factors were obtained from standard monomers.

The nitrogen content of dry fractions (100 mg freeze-milled) was performed using the Kjeldahl method. The protein amount was deduced using a conversion factor of 6.25.

Methylation analysis was carried out using lithium-methylsulfinylmethanide carbanion.[29] Methylated polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (120 °C, 2.5 h) and converted into alditol acetates.[30] The partially methylated alditol acetates were analyzed by GLC on DB-225 fused-silica capillary columns (J&W, USA, 30 m × 0.32 mm i.d.). Identification was based on relative retention times.

2.5. ^{13}C NMR spectroscopy

High-resolution liquid state proton decoupled ^{13}C NMR spectra of 4–10% D_2O solutions were recorded at 60 °C on a Bruker ARX 400 at 100.62 MHz. Chemical shifts were measured from internal Me_2SO assigned to 39.6 ppm.

The solid-state NMR spectra were performed on dry (residual humidity about 10–15%) or rehydrated to saturation with D_2O (100 mg for 250 μL D_2O except for mix-linked xylan which was 150 μL). Spectra were recorded on a Bruker DMX 400 spectrometer operating at a proton frequency of 400.13 MHz and carbon frequency of 100.62 MHz. A triple resonance ^{1}H/X/Y/CPMAS 4 mm probe was used working with high power-level amplifiers for cross-polarization magic-angle-spinning (CPMAS) experiments or with low power-level amplifiers and deuterium lock in the third channel for acquisition in liquid-like conditions. The magic-angle-spinning (MAS) rate was fixed at 5 kHz and each acquisition was recorded at rt (294 ± 1 K).

CPMAS experiments were realized using a 90° proton pulse of 3.5 μs, a contact time of 1 ms at 62.5 kHz and a 4 s recycling time for an acquisition of 17 ms during which dipolar decoupling (TPPM) of 62.5 KHz was applied. Chemical shifts were calibrated with external glycin, assigning the carbonyl carbon at 176.03 ppm. Measurements of the proton rotating-frame relaxation times $T_{1\rho}$ were achieved using a delayed-contact experiment by varying the spin-locking pulse delay (10 points between 500 μs and 50 ms) according to Ref. 31. The radio-frequency power level of the spin-locking pulse was varied between 14.71 and 55.55 KHz corresponding to angular frequencies between 92.4 and 349.1 × 10^3 rad s^{-1}. As the dry material showed low-resolution spectra with large resonances, peaks were decomposed using the Simplex method (dmfit2000[32]). This process allowed to measure relaxation times on the various carbons expected to contribute to each large peak. Proton T_2 relaxation times were obtained using the standard sequence[33] where a delay τ following the 90° proton pulse was varied between 5 and 200 μs.

Liquid-like MAS experiments used a single 90° pulse excitation of 6.5 μs for carbon with proton decoupling (WALTZ16) of 3 KHz during acquisition (1.3 ms) and during the recycling delay (2 s) to build up Nuclear Overhauser Enhancement.[34]

2.6. X-ray diffraction

X-ray diffraction diagrams were recorded using an INEL spectrometer (Artenay, France) working at 40 kV and 30 mA operating in the Debye–Scherrer transmission method. The Cu K_{α_1} ($\lambda = 0.15405$) radiation was selected with a quartz monochromator and detected by a curved position sensitive detector (INEL CPS-120). The dry samples were stored at 40 °C prior recording while the wet samples were obtained by adding, respectively 7, 20 and 50 μL to 20 mg of dry product. Data recording time was 4 h. All diagrams were normalized at the same total area integrated between 3 and 40° (2θ).

3. Results and discussion

3.1. Liquid-like MAS ^{13}C NMR spectroscopy

Liquid-like MAS ^{13}C NMR spectroscopy of rehydrated algal powder, whose composition is given in Table 1, revealed mobile compounds giving signals with chemical shifts close to those of the extracted mix-linked xylan in solution (Fig. 1(A and B), Table 2). Thus, part of the mix-linked xylan is loosely held in the cell wall and could correspond to incompletely assembled newly synthesized polysaccharides, hanging tethered segments and/or partially degraded xylan resulting from physiological con-

Table 1
Chemical composition as % dry weight of the different samples studied

Samples	Neutral sugars				Xylose linkages		Proteins	Ash
	Ara	Xyl	Gal	Glc	1→3	1→4		
P. palmata								
Whole dry alga		34.4	3.0	2.9	19.4	80.6	30.0	3.5
AIR		37.1	2.2	3.6	21.5	78.5	29.2	4.3
NaOH 8 M residue		41.8	3.0	5.6	20.1	79.9	21.0	11.4
HCl residue		14.5	0.2	13.8	1.3	97.7	47.3	4.4
Guanidium thiocyanate 4.5 M residue		19.6	1.7	3.7	19.2	80.8		
Caulerpa xylan	0.2	63.5	0.0	0.3	100			
Birchwood xylan	0.1	84.1	0.1	2.1		100		

ditions of the alga or from its dehydration process. The relatively higher signal intensity for the C-1 of the A1 sugar compared to that on the CPMAS spectrum (Fig. 1(C)) likely originated from impurities and not from a higher proportion of (1→3) linkage as other signals for (1→3)-linked xylose residue (B3, B4, B5) were not affected. Small broad resonances in the 20–40 and 170–180 ppm regions were attributed to aliphatic and carboxylic/carbonyl carbons, assigned to proteins since uronic acids were not detected in cell wall extracts[14] and this alga is poor in lipids.[8] Other sharp signals pointed to the presence of highly mobile molecules, some of which were attributed to floridoside[35] (Fig. 1(B)) while the other remain unassigned. The quantity of loosely held xylan observed by liquid-like MAS ^{13}C NMR spectroscopy could not be precisely determined. However, based on the signals area of floridoside which represent at the most 2–3% of the dry weight of winter collected alga[36] and assuming a similar relaxation process for all carbons, we could estimate this xylan population to roughly 9–14% of the algal powder dry weight. The fast motional averaging of the dipolar interactions between proton and carbon nuclei of these mobile compounds prevented the observation of the sharp signals with the CPMAS NMR acquisition conditions (Fig. 1(C)). The signals in the latter spectrum corresponded to that of the mix-linked xylan since their chemical shifts corresponded well with those observed

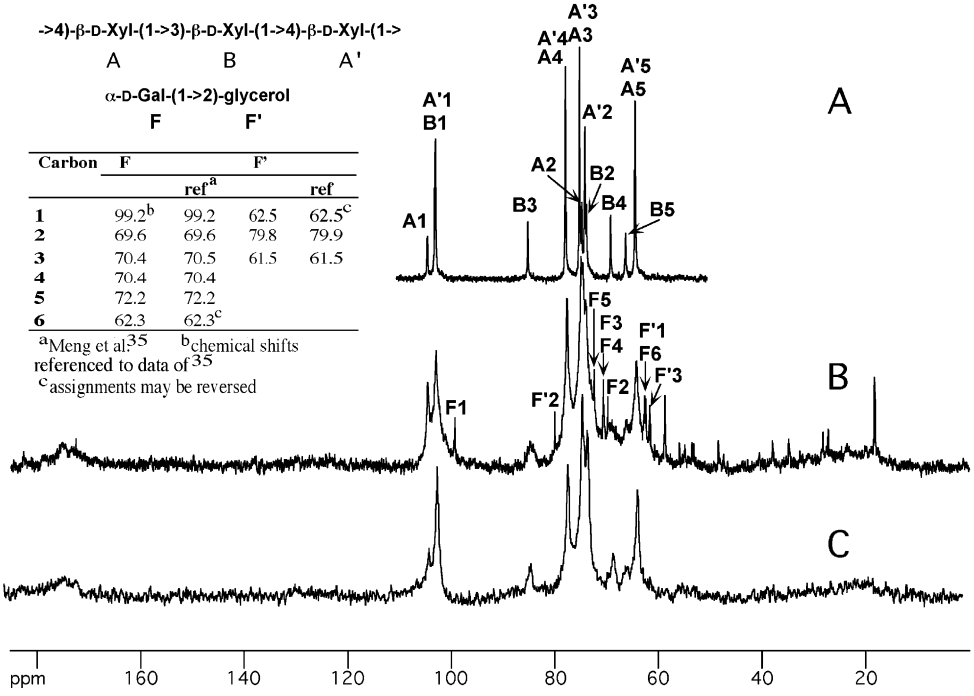

Fig. 1. Chemical structure, nomenclature and ^{13}C NMR spectra of mix-linked xylan from *P. palmata*: (A) solution; (B) liquid-like MAS; and (C) CPMAS spectra of rehydrated *P. palmata* powder. (A): 60 °C, Me_2SO 39.6 ppm, 10,000 scans, recycling: 1.2 s; (B): ns = 20k, lb = 5 Hz; (C): ns = 20k, lb = 10 Hz; the chemical shifts of floridoside carbons are reported in the insert.

Table 2
^{13}C NMR chemical shifts of *P. palmata* and reference xylans

	Unit		C-1	C-2	C-3	C-4	C-5
(1→3)/(1→4)-linked β-D-xylan	A	liquid	103.7	73.8	74.3	77.0	63.6
	B		102.2	72.8	84.4	68.3	65.4
	A′		102.2	73.3	74.3	77.0	63.6
Mix-linked xylan in *Palmaria*	A	solid/wet	103.4	nd	73.7	76.5	63.0
	B		101.7	72.7	83.6	67.8	64.9
	A′		101.7	72.7	73.7	76.5	63.0
(1→3)-Linked β-D-xylan from *C. taxifolia*		solid/dry	105.3	75.1	90.1	69.0	67.0
		solid/wet	104.7	72.9	86.3	68.5	64.9
(1→4)-linked β-D-xylan from birchwood		solid/dry	101.7	74.3	74.3	80.0/82.0 [a]	63.4

[a] Disordered/ordered.[36,37]

on the liquid-like MAS and the solution state ^{13}C NMR spectra (Fig. 1(A and B); Table 2).

Subsequent analyses were made on cell wall enriched material[14] (AIR, yield: 88% of the starting algal dry weight (Table 1)) which was prepared to remove small metabolites and to inhibit residual enzymatic activities. Such treatment did not affect the overall polysaccharide composition (Table 1) although, based on the slight increase in (1→3)-linked xylose proportion, it probably removed some short (1→4)-linked xylan fragments. This small modification had no consequences on the liquid-like MAS ^{13}C NMR spectrum of the hydrated AIR which was very close that of the whole rehydrated alga except for the absence of the sharp signals of the highly mobile small metabolites (data not shown).

3.2. CP-MAS ^{13}C NMR spectroscopy of AIR and model xylans in the dry and hydrated states

The CPMAS ^{13}C NMR spectra of AIR in the dry and rehydrated states are shown on Fig. 2(A) and (B), respectively. The spectrum of the dry sample showed broad unresolved peaks that corresponded to mix-linked xylan (see below) and to proteins (170–180, 45–55, 15–35 ppm) contained in this material (Table 1). Rehydration of AIR markedly improved the resolution of the signals and affected the chemical shifts of broad resonances particularly at around 97, 88 and 82 ppm. This hydration effect was further investigated on extracted mix-linked xylan (Fig. 3(A)). Besides the absence of resonance at around 97 ppm, whose attribution remains unknown in AIR, broad peaks at 86.7 and 81.0 ppm were observed in the spectrum of the dry xylan. These peaks were attributed to C-3 and C-4 for the 3- and the 4-linked β-D-xylose residues, respectively, by comparison with data published for (1→3)-[37] and for (1→4)-linked β-D-xylan.[38] As a means of comparison, the spectrum of (1→3)-linked β-D-xylan from *C. taxifolia* and that of (1→4)-linked β-D-xylan from birchwood were recorded (Fig. 3(B and C); Table 2). In the spectrum of the mix-linked xylan in the dry state (Fig. 3(A)), the C-3 resonance at 86.7 ppm corresponded to

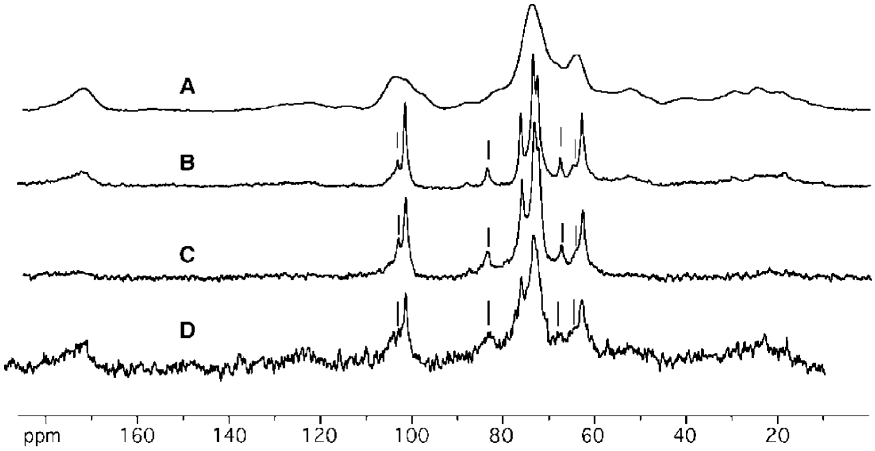

Fig. 2. ^{13}C CPMAS spectra of *P. palmata* AIR: dry (A), rehydrated (B), rehydrated 8 M NaOH extraction residue (C), and rehydrated guanidium thiocyanate 4.5 M extraction residue (D). (A) ns = 5k, lb = 20 Hz; (B) and (C) ns = 20k, lb = 20 Hz; (D) ns = 40k, lb = 20 Hz; marks indicate signals for (1→3)-linked xylose.

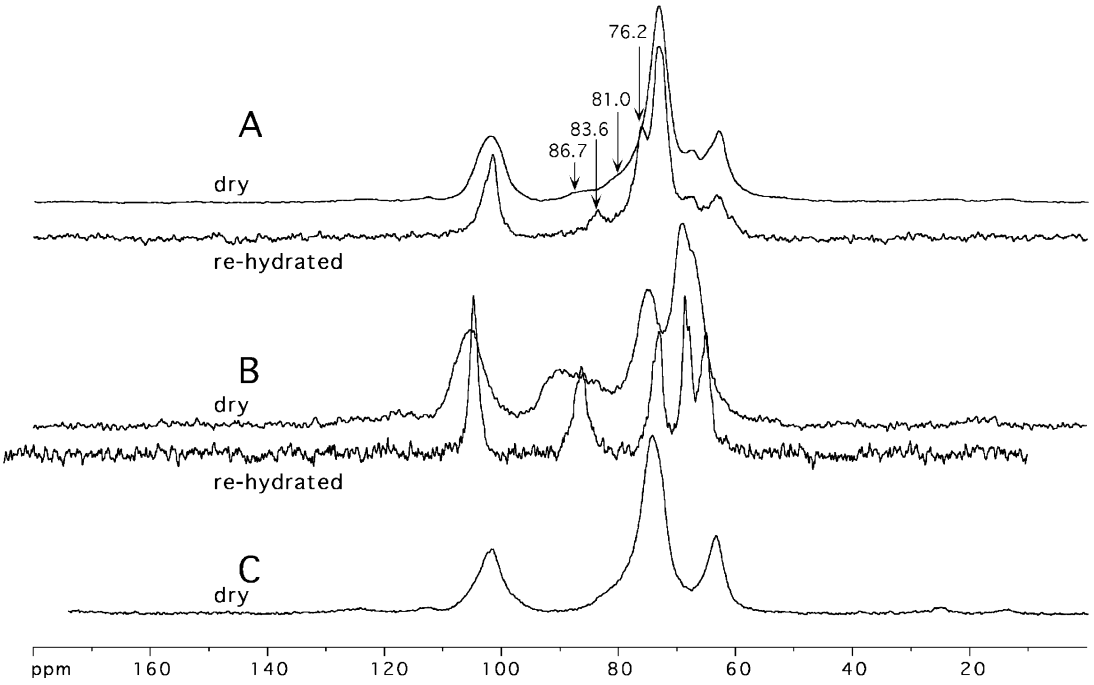

Fig. 3. ^{13}C CPMAS spectra of dry and rehydrated *P. palmata* extracted mix-linked xylan (A), dry and rehydrated (1→3)-linked xylan from *C. taxifolia*, (B), and dry (1→4)-linked xylan from birchwood (C). (A) dry: ns = 5k, lb = 30 Hz; hydrated: ns = 5k, lb = 30 Hz; (B) dry: ns = 2k, lb = 30 Hz; hydrated: ns = 100k, lb = 30 Hz; (C) dry: ns = 5k, lb = 20 Hz; chemical shifts indicated are discussed in the text.

the broad signal around 90 ppm of *Caulerpa* xylan (Fig. 3(B)) while the C-4 resonance at 81 ppm, corresponded to an equivalent broad signal observed on the spectrum of birchwood xylan (Fig. 3(C)) and was close to the 82 ppm signal for C-4 of ordered (1→4)-linked β-D-xylans.[38,39] These broad resonances disappeared in the spectrum of the hydrated sample where new narrow peaks appeared at 83.6 and 76.2 ppm. Such signals chemical shifting and sharpening were observed for the signals of the spectrum of the hydrated *C. taxifolia* (1→3)-linked β-D-xylan and particularly for C-3, which moved to 86.3 ppm (Fig. 3(C)) but not for the (1→4)-linked β-D-xylan (Fig. 4(A and B)). Hydration-induced conformational re-organization has already been reported for several helix-forming polysaccharides, such as (1→3)-linked β-D-glucans, amyloses[40,41] and for (1→3)-linked β-D-xylans.[37] The similar behavior observed for *P. palmata* mix-linked xylan suggests that water induces a helical conformation of the polysaccharide.

Hydration of plant cell wall materials,[20,21] algal galactans[23] or other hydrophilic polysaccharides, such as starch and β-glucans[40,42] has also been reported to increase the CPMAS ^{13}C NMR signal resolution. Such modifications have been interpreted as the result of an increased segmental mobility of loosely organized and/or interacting polymers. As a consequence, the carbons of the more mobile hydrated compounds become invisible in the CPMAS experiment and therefore, the resulting spectrum corresponds to rigid polysaccharides or polysaccharide segments. In order to assess whether mix-linked xylan mobility was affected by hydration, proton $T_{1\rho}$ and T_2 relaxation times were measured on the dry and rehydrated AIR using the CPMAS technique (Tables 3 and 4). To overcome the low resolution of the dry sample spectrum, the signals were decomposed and the relative proportion of the different carbon signals was fixed at 21.5% for the A and B units, and 78.5% for the A′ units (see Table 1). Unfortunately, the resonance of C-3 of the (1→3)-linked xylose (B3) was too weak to allow the measurement of its directly bonded proton relaxation rate and C-2, C-3, C-4 and C-5 in the units A and A′ ((1→4)-linked xylose) could not be distinguished. Table 3 displays the different $T_{1\rho}$ proton relaxation times measured at an angular radio-frequency of 349.1×10^3 rad s^{-1}. $T_{1\rho}$ values between 1.7 and 14.5 ms were observed and agreed with literature data for hydrated cellulose.[43,44] The T_2 relaxation times measured for each proton in the A, A′ and B units are displayed in Table 4. T_2 values showed a large increase under hydration, traducing a water disturbance of the local mobility of the xylose ring protons and argued for a significant perturbation of their local environment likely due to the nearby hydroxyl group engaged in hydrogen bonds with water molecules. On the other hand, rehydration of AIR had few effects on the $T_{1\rho}$ values except for the protons bonded to C-1, C-2 and C-4 of the (1→4)-linked xylose units (A and A′). Moreover, the $T_{1\rho}$ values of the (1→3)-linked xylose (B units)

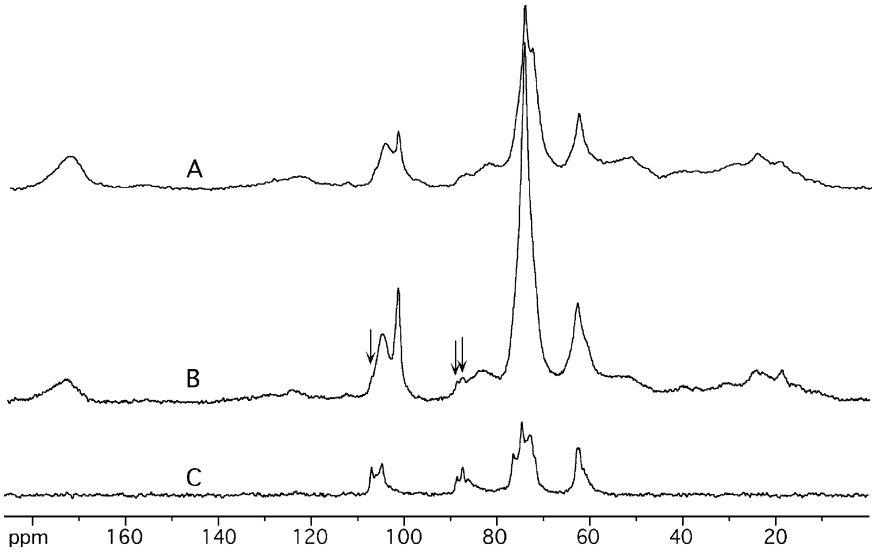

Fig. 4. ^{13}C CPMAS spectra of *P. palmata* insoluble residues after HCl extraction: dry (A), hydrated (B) and cellulose II (C). (A) ns = 2k, lb = 20 Hz; (B) ns = 20k, lb = 20 Hz, (C) ns = 4k, lb = 20 Hz; arrows indicate cellulose II signals.

were very short compared to the values measured for the A and A′ protons. The evolution of the $T_{1\rho}$ values obtained in this study contrasted with published results obtained for other polysaccharides like cellulose or pectins[44] for which the proton $T_{1\rho}$ relaxation times decreased with hydration. In order to clarify our results, we realized $T_{1\rho}$ measurements at different power levels of the spin-locking pulse. Indeed, while the T_2 relaxation times is proportional to the spectral density J of molecular motions at the null frequency, the $T_{1\rho}$ depends on the spectral density for motions in the radio-frequency domain ($J(\omega_1)$ with an angular frequency ω_1) as described in the following expression:

$$\frac{1}{T_{1\rho}} = K\left[\frac{9}{2}J(\omega_1) + 3J(2\omega_0) + \frac{15}{2}J(\omega_0)\right]$$

$$\text{with } J(\omega) = \frac{\tau_c}{1 + \omega^2\tau_c^2}$$

where ω_1 is the angular frequency of the spin-lock field B_1; ω_0 is the Larmor precessional frequency in the B_0 field; τ_c is the correlation time for the rotational motion causing relaxation; K is a constant; J is the spectral density.

In the extremely slow motion domain ($\omega_1^2\tau_c^2 \gg 1$), $1/T_{1\rho}$ decreases with the applied radio-frequency field, whereas it is independent of ω_1 for faster motions ($\omega_1^2\tau_c^2 \ll 1$). Thus, by varying the spin-lock power level we can precise the time scale of the studied motions. Table 5 displays the proton relaxation times $T_{1\rho}$ as a function of ω_1 (angular frequency of the spin-lock field) for the protons linked to C-1, C-3 and C-4 in the dry and rehydrated samples. Whatever the moisture content, the $T_{1\rho}$ values showed a dependency on the angular frequency ω_1 applied. These changes of the $T_{1\rho}$ relaxation times demonstrated that the motions of the mix-linked xylan protons were in the extreme slow domain. In these conditions, the hydration-associated increase of the $T_{1\rho}$ values, observed especially for the protons

Table 3
Proton $T_{1\rho}$ values (in ms) of dry and hydrated AIR (proton spin-lock at $\omega_1 = 349 \times 10^3$ rad s^{-1})

Proton	Residue					
	A dry	A hyd	A′ dry	A′ hyd	B dry	B hyd
1	6.4 [a]	14.2	6.4 [a]	7.1	2.3	2.6
2	5.5 [a]	14.5[a]	5.5 [a]	14.5 [a]	2.1	1.8
3	5.0 [a]	6.7[a]	5.0 [a]	6.7 [a]	nd	6.5
4	8.0 [a]	10.1[a]	8.0 [a]	10.1 [a]	5.4	7.7
5	6.1 [a]	7.3[a]	6.1 [a]	7.3 [a]	3.0	nd

[a] Unresolved peaks; nd: not determined.

Table 4
Proton T_2 values (in μs) of dry and hydrated AIR

Proton	Residue					
	A dry	A hyd	A′ dry	A′ hyd	B dry	B′ hyd
1	10.2 [a]	nd	10.2 [a]	91.4	9.4	75.4
2	8.3 [a]	79.9	8.3 [a]	131.9	9.0	118.3
3	9.7 [a]	76.9	9.7 [a]	50.2	nd	76.1
4	10.0 [a]	88.1 [a]	10.0 [a]	88.1 [a]	nd	116.7
5	9.3 [a]	72.9 [a]	9.3 [a]	72.9 [a]	5.3	122.3

[a] Unresolved peaks between A and A′; nd: not determined.

bounded to C-1, C-2 and C-4 of the (1→4)-linked xylan, argued for a decrease in motions, then with slower τ_c values. Unfortunately, the determination of the motions correlation times τ_c failed because of an insufficient number of points obtained for ω_1.

Concerning the (1→3)-linked xylose (B unit), very short $T_{1\rho}$ values but ω_1 dependent were measured for the B1 proton. However, few differences were noted between the $T_{1\rho}$ relaxation times measured on the dry and rehydrated samples. This behavior was characteristic of (1→3)-linked xylose protons implied in motions near the '$T_{1\rho}$ minimum' for which $\omega_1 = \tau_c$ but faster than protons in the (1→4)-linked xylan segments.

Thus, taken together, the NMR relaxation times measurements and chemical shifts strongly suggest a hydration-induced conformational re-organization of the xylan chains. The ordered conformation is interpreted as a helix stabilized by junction-zones between adjacent xylan chains through hydrogen bonds between (1→4)-linked xylan blocks (A′ units). In such chains, the (1→3)-linked xylose residue would play a key role in controlling the chain interactions and thus allows flexibility for this cell wall matrix polysaccharide.

3.3. Structure of xylans and glucans in residues from sequentially extracted AIR

Solutions of increasing concentration of NaOH or of different strong chaotropic salts extracted most of the mix-linked xylan from AIR.[14] The extraction residues still contained both (1→3)- and (1→4)-linked xylose (Table 1) with close proportions of (1→3) linkages than that of AIR. The CPMAS ^{13}C NMR spectrum of these hydrated residues showed signals confirming the presence of the residual mix-linked xylan (Fig. 2(C and D)).

Treatment of the 8 M NaOH residues with hot dilute acid markedly decreased residual xylan content to only 1.5% of that in the initial AIR content and the resulting material represented 10.1% of the original total AIR dry weight. It contained mainly proteins and a small amount of xylose and glucose shown by methylation analysis to be (1→4)-linked (Table 1). CPMAS ^{13}C NMR spectroscopic analysis of the dry and hydrated material (Fig. 4(A and B)) failed to show the characteristic signals for (1→3)-linked β-D-xylose (A1 and B3, B4 and B5). Instead it showed signals of (1→4)-linked β-D-xylan, cellulose II (C-1: 107.2, C-4: 89.0, 87.7 ppm) and

Table 5
Proton $T_{1\rho}$ values (in ms) of dry and hydrated AIR as a function of the spin-lock radio-frequency (ω_1)

AIR	ω_1 (10^3 rad s^{-1})	(1→4)-Linked xylose						(1→3)-Linked xylose	
		A proton			A′ proton			B proton	
		1	3	4	1	3	4	1	3
Dry	349.1	6.4	5.0	8.0	6.4	5.0	8.0	2.3	nd
	174.5	3.6	2.5	5.9	3.6	2.5	5.9	1.5	nd
	130.9	2.3	1.8	4.3	2.3	1.8	4.3	0.8	nd
	92.4	1.5	1.2	2.2	1.5	1.2	2.2	0.5	nd
Hydrated	349.1	14.2	6.7	10.1	7.1	6.7	10.1	2.6	6.5
	184.8	9.4	4.2	7.0	5.1	4.2	7.0	2.5	3.3
	125.7	4.5	3.1	4.3	2.3	3.1	4.3	1.7	2.4
	92.4	2.6	2.5	2.8	1.7	2.5	2.8	0.9	2.0

amorphous cellulose (C-1: 105.0, C-4: 83.6, C-6: 63.0 ppm). The latter signals were attributed from published data[45,46] and by comparison with the spectrum of laboratory prepared cellulose II (Fig. 4(C)). Cellulose II likely resulted from the mercerization of cellulose I induced by the alkali extractions. Even though glucose is present in the same amount as xylose in the residue, the NMR signals attributed to cellulose II were qualitatively much smaller that those of (1→4)-linked xylan (Fig. 4(A and B)). Although other non-cellulosic glucans can be also present, it is likely that most of the glucose be under the form of amorphous cellulose as the result of mercerization of the initial cellulose I. Such alkaline conversion is known to decrease the crystallinity of cellulose.[45] Additionally, part of the amorphous cellulose can reflect interactions between cellulose and the (1→4)-linked xylan which can contribute to the broad NMR signal at around 81–82 ppm (Fig. 4(A and B)).[38] Hydration did not affect chemical shift and resolution of the signals of these residues but resulted in a small relative increase in intensity of xylan signals. Such xylan may represent a minor distinct population in the algal cell wall xylan but could also result from the acid hydrolysis of (1→3)-linked xylose containing segments in mix-linked xylan. An increase in the organization of cellulose has been reported after its mild acid hydrolysis[47] and previous works on *Rhodymenia* (*Palmaria*) *palmata* indicated that harsh oxidative, acidic and alkaline extractions of the alga modified the fibrillar organization.[48] Other signals at 170 ppm, and in the regions of 130–120 and 50–10 ppm of the CPMAS ^{13}C NMR spectrum of these HCl residues were attributed to residual proteins of low mobility. The observation of rigid proteins suggests the presence of highly cross-linked proteins but their cell wall origin cannot be ascertained. Indeed, the method of samples preparation likely contributed to the cross-linking of different types of proteins, including those of the cytoplasmic compartment. However, recent data showed the presence of tyrosine or methionine rich water insoluble cell wall proteins in *P. palmata*.[16] The observation of cellulose and crystalline xylan is in good agreement with previous reports[7,48,49] and with the X-ray diffraction diagrams obtained on this fraction. These were recorded from the dry and rehydrated material at 100 and 250% water content, the latter corresponding to that used for solid state NMR (Fig. 5). An optimal signal to noise ratio was obtained for 100% water, which corresponded to a probable compromise between the known improvement of the diffraction signature of (1→4)-linked β-D-xylan with water uptake[50] and the classical absorption of X-rays by water. This diagram shows clearly the simultaneous presence of crystalline (1→4)-linked β-D-xylan with characteristic peaks at ($2\theta = 10.6$, 11.9, 18.4 and 21.9°) corresponding to d-spacings (0.83, 0.74, 0.48 and 0.40 nm) and cellulose II with peaks at $2\theta = 12.3$, 20.0 and 22.0° (d-spacings 0.72, 0.44 and 0.40 nm, respectively). The crystallinity of (1→4)-linked β-D-xylan is improved by hydration in agreement with the increased CPMAS ^{13}C NMR signals intensity observed (Fig. 4(B)). Indexing of diagrams was performed following the structural models proposed for (1→4)-linked β-D-xylan[50] and for cellulose II.[51] β-D-Xylan crystalline structures are usually considered as hydrates, and the diagrams recorded on highly hydrated samples evidenced the presence of xylan dihydrate.[50]

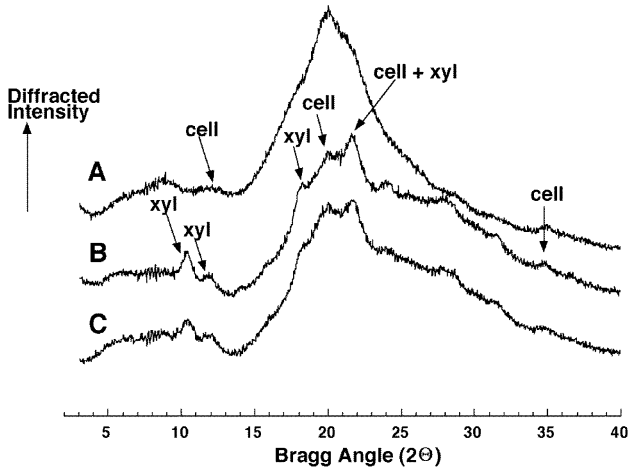

Fig. 5. X-ray diffraction diagrams of *P. palmata* insoluble residues after HCl extraction: dry (A), 100% H_2O (B) and 250% H_2O (C). The characteristic peaks for xylan (xyl) and cellulose II (cell) are shown.

4. Conclusions

Palmaria palmata mix-linked xylan have different levels of interaction in the cell wall spanning from loosely interacting chains observed by liquid-like MAS ^{13}C NMR spectroscopy to tightly bonded chains observed by CPMAS NMR spectroscopy in the NaOH and guanidium thiocyanate extraction residues. CPMAS NMR spectroscopy and relaxation measurements emphasized interactions of *P. palmata* xylans through H-bonds modulated by the presence of (1→3) linkages and water, which would induce an helical conformation. However, the presence of tightly bound mix-linked xylans resisting extraction by strong alkali or chaotropic salt solutions suggests the existence of additional kinds of interactions for these matrix polysaccharides, such as for example through covalent linkages. Whether the small population of residual (1→4)-linked β-D-xylans is part of the mix-linked xylan or represents a specific fibrillar network topologically distinct (different cell wall layer, different cell) or associated with cellulose remains to be established. (1→4)-Linked β-D-xylan is known to form a crystalline assembly and to form hydrogen bonded complexes with cellulose[52,53] of importance in wood fiber mechanical properties.[54] Further

work is required to get more precisely the structure and location of this small xylan fraction as it could serve as interfacial polysaccharides between the mix-linked xylan network and cellulose and play an important role in the alga mechanical properties.

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