Assessment of *In Vitro* Binding of Isolated Pectic Domains to Cellulose by Adsorption Isotherms, Electron Microscopy, and X-ray Diffraction Methods

Agata Zykwinska, Cédric Gaillard, Alain Buléon, Bruno Pontoire, Catherine Garnier, Jean-François Thibault, and Marie-Christine Ralet*

INRA, Unité de Recherche Biopolymères, Interactions, Assemblages, BP 71627, 44316 Nantes Cedex 03, France

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Isolated pectic domains representative of the pectic backbone and the neutral sugar side chains were tested for their ability to interact with cellulose in comparison to the well-known binding of xyloglucan. Pectic side chains displayed a significant *in vitro* binding capacity to cellulose, whereas pectic backbone domains exhibited only slight adsorption to cellulose microfibrils. To support the binding results, electron microscopy and X-ray diffraction were applied. Celluloses from bacteria and sugar beet cell walls were used as substrates for the precipitation of isolated pectic domains or xyloglucan by acetone vapor diffusion. Pectic side chains grew attached to the cellulose surfaces, whereas pectic backbone domains were observed separately from cellulose microfibrils. Xyloglucan seeded with cellulose provoked a decrease of microfibrils entanglement, but no clear cross-links between neighboring microfibrils were observed. These results led to the elucidation of the pectic domains responsible for binding with cellulose microfibrils.

1. Introduction

All plant cells are encapsulated in a wall mainly composed of proteins and polysaccharides, including cellulose, hemicelluloses, and pectins. These polymers collectively determine the shape and mechanical properties of the cell.

Cellulose is composed of (1→4)-linked *β*-D-Glcp residues that form linear chains of parallel alignment, which are tightly linked by hydrogen bonds to form microfibrils. Cellulose is considered as a semicrystalline polymer composed of highly organized crystalline domains in the microfibril core and less organized amorphous regions at the microfibril surface.[1] It is likely that crystalline and amorphous domains are linked together and arranged periodically along microfibrils.[2] The degree of crystallinity varies with respect to cellulose origin, from highly crystalline (bacteria or tunicin) to less crystalline (primary cell wall). Cellulose microfibrils are thought to be cross-linked by xyloglucan, the most abundant hemicellulosic polysaccharide in some primary cell walls of dicotyledons.[3] Xyloglucan is composed of a cellulose-like backbone, branched at *O*-6 by α-D-Xyl*p* residues, which can be further substituted at *O*-2 by *β*-D-Gal*p* residues.[4] Some of the Gal*p* residues may be substituted at *O*-2 by α-D-Fuc*p* residues.

Pectins constitute a highly complex and heterogeneous group of polysaccharides due to the presence of distinctive covalently linked domains that differ in their molecular organization and crystallinity. According to the most cited pectin model,[5] the pectin backbone is mainly composed of two structural domains: homogalacturonan (HG) and type I rhamnogalacturonan (RG I). HG constitutes a linear chain composed of (1→4)-linked α-D-GalA*p* units that are often methyl-esterified at *O*-6 and sometimes acetyl-esterified at *O*-2 or *O*-3.[6] RG I contains a backbone of the repeating disaccharide unit: (1→2)-α-l-Rha*p*-(1→4)-α-D-GalA*p*[7] that is predominantly substituted at *O*-4 of Rha*p* residues by neutral sugar side chains, among which arabinan and galactan are the most abundant.[8] Arabinan side chains are composed of (1→5)-α-l-Ara*f* residues, which can be branched by α-l-Ara*f* units attached at *O*-2 and/or *O*-3, whereas galactan side chains are composed of (1→4)-linked *β*-D-Gal*p* units. A type II rhamnogalacturonan (RG II), a complex polysaccharide composed of GalA, Rha, Gal, and some unusual sugars, may also constitute a part of the pectic molecule.[9] RG II, although present as a quantitatively minor pectic subunit, is thought to play a key role in the cell wall architecture.[10] It is noteworthy that an alternative model for the molecular structure of pectin, in which the RG I backbone is substituted by side chains composed not only of neutral sugars but also of HG regions, was recently put forward.[11]

The way in which these polysaccharides are organized within the cell wall is still controversial, and several different models were elaborated ("sticky network", "multicoat", "stratified" models).[12] All those models suggest that cellulose microfibrils and xyloglucan create a network, which is supposed to be completely independent from the one that is formed by pectins. Indeed, pectins or pectic networks are thought to fill the interstices within the cellulose/xyloglucan network. However, we have recently demonstrated that pectins are also able to bind to cellulose microfibrils.[13] This finding allowed us to offer the hypothesis of noncovalent interactions between pectins, presumably their neutral sugar side chains, and cellulose.

In the present work, a multidisciplinary approach combining binding assays, microscopic observations, and X-ray diffraction analyses was developed. The different structural pectic domains were isolated, and their association with two types of cellulose (microcrystalline bacterial cellulose (MCBC) and primary cell wall (PCW) cellulose) was studied. To elucidate which pectic domains are able to bind to cellulose and how this binding can be modulated by the cellulose origin (e.g., crystallinity), two

* To whom correspondence should be addressed. E-mail: ralet@nantes.inra.fr; fax 33-2-40-67-50-84.

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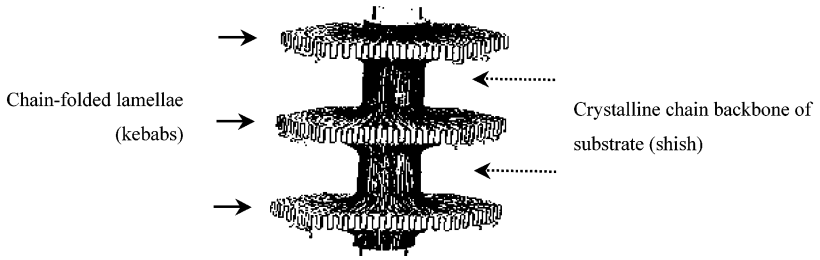

Figure 1. Model of "shish-kebab" morphology.[40]

approaches were developed: (1) binding assays and (2) induced precipitation experiments, in which the different biopolymers were forced to precipitate in the presence of cellulose. Indeed, the oriented growth of crystals of low molar mass polysaccharides (guest) on highly crystalline native cellulose microfibrils (host) was previously reported.[14,15] Polymer chains that crystallize may in some conditions orient themselves with their chain axes parallel to the crystalline substrate surface. This specific adsorption directs the growing lamellae to stand perpendicular to the substrate surface, which results in a so-called "shish-kebab" morphology (Figure 1). This peculiar morphology is obtained when both guest and host crystals have very similar molecular conformations leading to possible epitaxial associations.[14,15] In the case of different molecular conformations, a row nucleation mechanism rather than an epitaxial growth seems responsible for the observed "shish-kebab" morphologies.[16] In the present work, binding assays, performed in parallel with induced precipitation experiments, were implemented to study pectic domains/cellulose associations.

Materials and Methods

Cell Wall Material, Cellulose, and Noncellulosic Polysaccharides. Sugar beet cell wall material (SB-CWM) was prepared from fresh sugar beet pulp (sugar factory in Cagny, France), as described elsewhere.[13]

PCW-cellulose was prepared from SB-CWM, as described by Heux et al.[17] A suspension of 50 g of SB-CWM in 1.5 L of 0.1 M HCl was maintained for 30 min at 85 °C and filtered through a nylon cloth. The extraction was carried out three times. The residue was washed abundantly with distilled water and submitted to an alkaline extraction (0.5 M NaOH, 1.5 L for 30 min at 80 °C). This step was performed three times. The residue was washed abundantly with distilled water, dried by solvent exchange (ethanol, acetone), and left overnight at 40 °C. PCW-cellulose was suspended in distilled water (6 g/L) and mixed for 15 min at 25 °C in a Warring Blender (22 400 rpm). The sample was then homogenized by 10 passes through an APV Gaulin homogenizer operating at 1000 bar. Because of increasing viscosity during homogenization steps, PCW-cellulose was progressively diluted to reach the final concentration of 2 g/L.

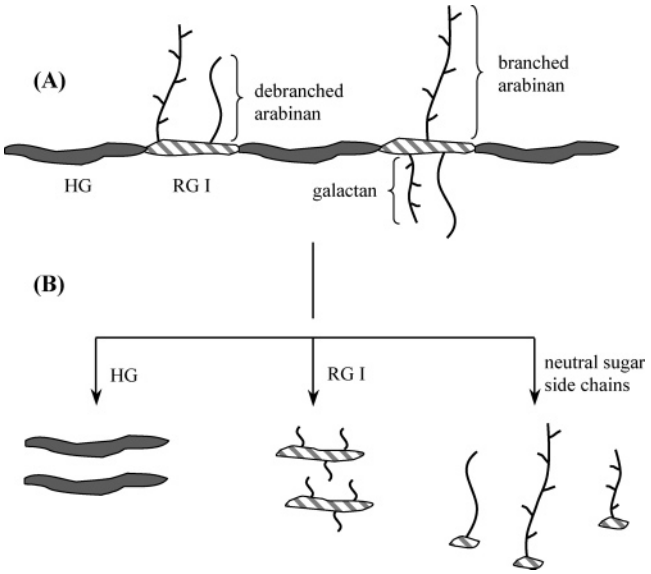

Figure 2. Schematic representation of the conventional pectin structure[5] (A) and the isolation procedure of the different pectic domains (B).

MCBC was obtained from bacteria cellulose synthesized by *Gluconacetobacter xylinus* (ATCC 53524) cultured in Hestrin−Schramm medium,[18] containing 2% of glucose. Bacterial cellulose was hydrolyzed with 2.5 M HCl for 1 h at 100 °C. The hydrolysis was carried out two times. MCBC was dialyzed against distilled water, and the final concentration was determined at 10 g/L.

The different pectic domains were recovered as illustrated on Figure 2. HG was prepared from citrus pectin (Unipectine RS/CA). An aqueous solution of 12 g of pectin (1 L) was adjusted to pH 12 with 1 M NaOH. The deesterification step was carried out for 24 h at 4 °C. The solution was then adjusted to pH 4.5 with 0.1 M HCl, and the pectate was obtained after precipitation with 3 volumes of 95% (w/v) ethanol. After one night at 4 °C, precipitates were carefully rinsed with 60% (w/v) ethanol, 95% (w/v) ethanol, and acetone and dried overnight at 40 °C. HG was obtained after 0.1 M HCl hydrolysis at 80 °C for 72 h and put into Na^+ form by adjusting pH to 7 with 0.1 M NaOH.[19]

RG I was prepared from SB-CWM. A suspension of 100 g of SB-CWM in 3 L of 0.1 M NaOH was stirred at 90 °C for 2 h. The suspension was adjusted to pH 5 with 6 M HCl and filtered through G3 sintered glass. The supernatant was concentrated under vacuum at 40 °C and precipitated with 5 volumes of 95% (w/v) ethanol. After one night at 4 °C, the precipitate was carefully rinsed with 80% (w/v) ethanol, solubilized in water, and freeze-dried. The sample was then resolubilized in 0.5 M HCl (200 mL) and stirred at 80 °C for 2 h.[20] The solution was adjusted to pH 4 with 2 M Na-acetate buffer, dialyzed, concentrated under vacuum at 40 °C, and freeze-dried.

Branched and debranched arabinans (from sugar beet) and galactan (from potato) were purchased from Megazyme (Ireland).

Xyloglucan was extracted from a powder of tamarind seeds from *Tamarindus indica* (Dainippon, Pharmaceutical Co., Japan). The powder was boiled with citric acid (2 g/L) for 40 min and centrifuged, and the clear supernatant was concentrated under vacuum at 40 °C. Xyloglucan was obtained by precipitation with 1 volume of 95% (w/v) ethanol. After 1 night at 4 °C, the precipitate was treated by solvent exchange (ethanol, acetone) and dried overnight at 40 °C.

Analytical. The individual neutral sugars were analyzed as their alditol acetate derivatives by gas−liquid chromatography[21] after hydrolysis by 2 M H_2SO_4 at 100 °C for 2 h for arabinans and galactans, 3 h for xyloglucan, and 6 h for HG and RG I. Longer hydrolysis times were applied to HG and RG I samples to provide a good estimation of their Rha content.[22] MCBC and PCW-cellulose were first prehydrolyzed by 72% (w/v) H_2SO_4 for 1h 30 min at 25 °C. Inositol was used as an internal standard.

Uronic acid (as GalA) and total neutral sugars (as Ara or Gal) were determined colorimetrically by the automated *m*-hydroxybiphenyl and orcinol methods, respectively.[23,24]

Table 1. Sugar Composition of Microcrystalline Bacterial Cellulose (MCBC), Primary Cell Wall Cellulose (PCW-cellulose), Homogalacturonan (HG), Rhamnogalacturonan I (RG I), Branched Arabinan, Debranched Arabinan, Galactan, and Xyloglucan from *Tamarindus indica*

	neutral sugars and galacturonic acid (mg/g)							
	GalA	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
MCBC	10	0	0	3	0	0	0	986
PCW-cellulose[a]	17	0	0	2	26	18	4	925
HG	980	0	0	0	0	0	0	0
RG I	502	175	0	12	0	0	266	17
branched arabinan[a]	98	37	6	681	2	4	138	4
debranched arabinan[a]	114	45	2	518	0	0	181	0
galactan[a]	182	24	5	80	3	0	608	3
xyloglucan[a]	nd[b]	0	0	16	266	0	152	457

[a] Values from Zykwinska et al.[13] [b] nd: not determined.

Binding Assays. Binding assays were performed as described elsewhere.[13] Briefly, solutions of the different pectic domains were prepared at different concentrations, and aliquots were mixed with a known amount of cellulose. After incubation under continuous head-over-tail mixing at 40 °C for 6 h, the blends were centrifuged, and the supernatants were tested for their total neutral sugar or GalA contents by colorimetric assays. The amount of adsorbed matter was calculated from the difference in sugar content measured for isolated pectic domain solutions and blends supernatants, taking into account the amount of sugars released by cellulose blanks. Binding assays were performed in triplicate. The average and the corresponding error of measurement were calculated. Because of limited amounts of MCBC available and experimental difficulties, binding assays were only performed with PCW-cellulose.

Induced Precipitation Experiments. Aqueous solution (4 mL) of HG, RG I or isolated pectic side chains at 5 mg/mL, and xyloglucan at 2 mg/mL was left on a tripod placed into a jar. Acetone (10 mL) was left at the bottom of the jar. Precipitation of the different pectic domains or xyloglucan took place by acetone vapor diffusion during 4 days. Each day, 10 mL of acetone was added. The same procedure was reproduced and 2 mL of MCBC cellulose suspension at 0.5 mg/mL or 2 mL of PCW-cellulose suspension at 1 mg/mL were added to each pectic or xyloglucan solution. Acetone volume and time of precipitation were kept constant as described for pectic domains and xyloglucan blanks. MCBC and PCW-cellulose suspensions were also prepared as described above to give cellulose blanks. The "decorated" cellulose fibers as well as blanks were washed by successive centrifugation in a mixture of water/acetone (25/75, v/v) (3 times) followed by washing in 100% (w/v) ethanol (3 times).

Transmission Electron Microscopy (TEM). Few drops of each sample in ethanol were deposited on carbon-coated copper grids and air-dried before electron microscopy investigation. Observations were performed using either a JEOL JEM-100S transmission electron microscope (JEOL Ltd., Tokyo, Japan) or a JEOL JEM-1230 TEM equipped with Gatan cryo sample holder for low temperature observations. The microscopes were operating at an acceleration voltage of 80 kV using low dose exposure conditions.

Wide-Angle X-ray Scattering (WAXS). Measurements were performed using a spectrometer equipped with a XRG 3000 generator (Inel Orléans, France) working at 40 kV and 30 mA, a quartz monochromator ($\lambda = 0.15405$ nm), and a curve position sensitive detector (Inel CPS 120). Diffraction diagrams of dried samples were obtained in a transmission mode between 3 and 40° (2θ) within 2 h. All diagrams were normalized to the same total integrated area in the range between 10 and 40°.

The degree of crystallinity of MCBC was determined by the method of Wakelin et al.[25] using crystalline and amorphous standards, an acid-hydrolyzed tunicin cellulose and water-reprecipitated cellulose from sulfuric acid solutions, respectively. The lateral crystal size was established from the half peak width of 200 ($2\theta \approx 22.7°$) band, using the Scherrer equation:

$$D_{hkl} = K\lambda/\beta \cos\theta$$

where, D_{hkl} is the average length of the diffracting domains normal to the family of planes (hkl); K is a constant usually taken as 0.9 for cellulose;[26] λ is the wavelength, and β is the half peak width. To determine half peak widths, the WAXS diagrams were decomposed using the Peakfit software (Jandel Scientific) after subtraction of the amorphous standard spectrum. Fitted values obtained with Pearson 7 and Gaussian−Lorentzian profiles were then compared. Determination coefficients R^2 were higher than 0.98, with exception of HG, where R^2 was 0.93. The PCW-cellulose crystallinity was determined after subtraction of the amorphous standard spectrum with a factor corresponding to the amount of amorphous regions.

Results

Chemical characteristics of the isolated pectic domains (HG, RG I, branched arabinan, debranched arabinan and galactan), xyloglucan, and celluloses used in this study are presented in Table 1.

Binding Assays. The binding capacity of the different pectic domains (HG, RG I and isolated neutral sugar side chains) to PCW-cellulose was expressed by binding isotherms where the mass of bound material per mass of cellulose (q_e) was plotted versus the concentration of free material remaining in solution at equilibrium concentration (C_e). The capacity of isolated pectic domains to bind to PCW-cellulose showed large variations, as presented in Figure 3. Debranched arabinan side chains displayed the highest binding capacity to PCW-cellulose microfibrils with a maximum of ~15.5 μg per mg of cellulose reached above 750 μg/mL of debranched arabinan. Galactan side chains exhibited an important binding capacity and a maximum of adsorbed matter of ~14.5 μg per mg of cellulose was measured for 1000 μg/mL of galactan. The extent of galactan binding increased with increasing concentration, as observed for debranched arabinan but no apparent plateau value was obtained in the range of concentrations used. The branched arabinan side chains displayed reduced binding capacity to PCW-cellulose microfibrils, as only ~7 μg per mg of cellulose was bound for concentrations, above 750 μg/mL of branched arabinan. Isolated backbone domains (HG and RG I) displayed a very weak binding capacity to PCW-cellulose. For concentrations below 200 μg/mL, the binding capacity is close to zero and increases only slightly to reach a maximum of ~1.7 μg per mg of cellulose microfibrils above 500 μg/mL of HG and at 1000 μg/mL of RG I.

Induced Precipitation Experiments. To study the influence of the presence of cellulose microfibrils onto isolated pectic

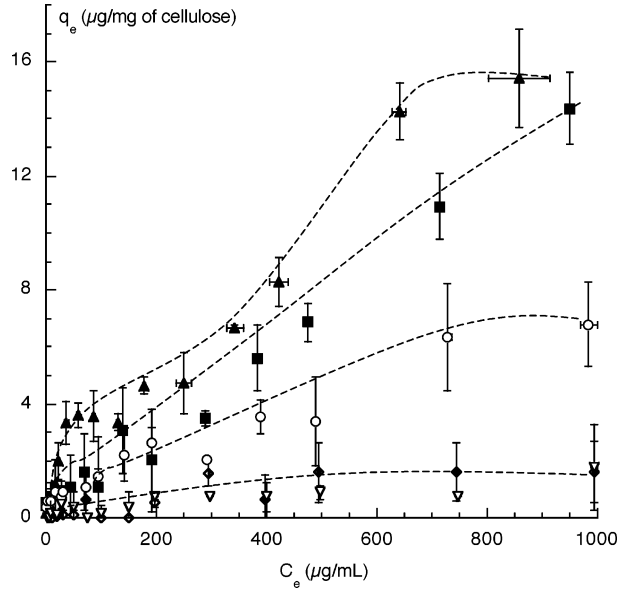

Figure 3. Adsorption isotherms (μg/mg of cellulose) of isolated pectic domains: debranched arabinan (▲), galactan (■), branched arabinan (○), HG (●), RG I (▽) to PCW-cellulose.

domains crystal growth, induced precipitation experiments were performed. Pectic domains were forced to precipitate in the presence of bacterial or primary cell wall celluloses, displaying different morphologies and crystallinities. The effect of cellulose crystallinity modification induced by pectic domains nucleation was also investigated.

Cellulose Blanks. Diffractograms of MCBC and PCW-cellulose blanks are presented in Figure 4A. MCBC is a highly crystalline cellulose with five principal reflections: (1−10) at $2\theta \approx 14.5°$, (110) at $2\theta \approx 16.9°$, (102) at $2\theta \approx 20.4°$, (200) at $2\theta \approx 22.7°$ and (004) at $2\theta \approx 34.5°$. The degree of crystallinity determined by the method of Wakelin et al.[25] was very high (90%). A microfibril lateral size of 13.4 nm was estimated from the (200) scattering peak width ($2\theta \approx 22.7°$). Contrary to MCBC, PCW-cellulose is quite amorphous as it contains only about 40% of ordered zones, as roughly estimated after subtraction of the amorphous standard spectrum multiplied by a factor corresponding to the amount of amorphous regions in the PCW-cellulose diagram. The degree of crystallinity of PCW-cellulose corresponds well to previously published results.[17]

Electron micrographs of MCBC and PCW-cellulose blanks are presented in Figure 4B and 4C, respectively. Treatment of bacterial cellulose in acid conditions yielded a stable suspension of separated MCBC microfibrils. PCW-cellulose was present as a stable mixture of individual but also bundled cellulose microfibrils of apparent random orientation.

Pectic Domain-Induced Precipitation. When aqueous solutions of isolated pectic domains precipitated without cellulose added, the nucleation by acetone vapor diffusion was a long process and precipitates started to be visible after about 3 days, except for debranched arabinan, which precipitated more rapidly (∼1 day).

When the induced precipitation of isolated pectic domains was initiated with cellulose microfibrils, the nucleation process was more rapid: precipitates were visible after 2 days and appeared more abundant.

Pectic Side Chain-Induced Precipitation. The debranched arabinan side chains used in this study were composed mostly of Ara (518 mg/g) with minor amounts of Gal, Rha, and GalA (Table 1). Diffractograms obtained for debranched arabinan blank as well as for debranched arabinan seeded with PCW-

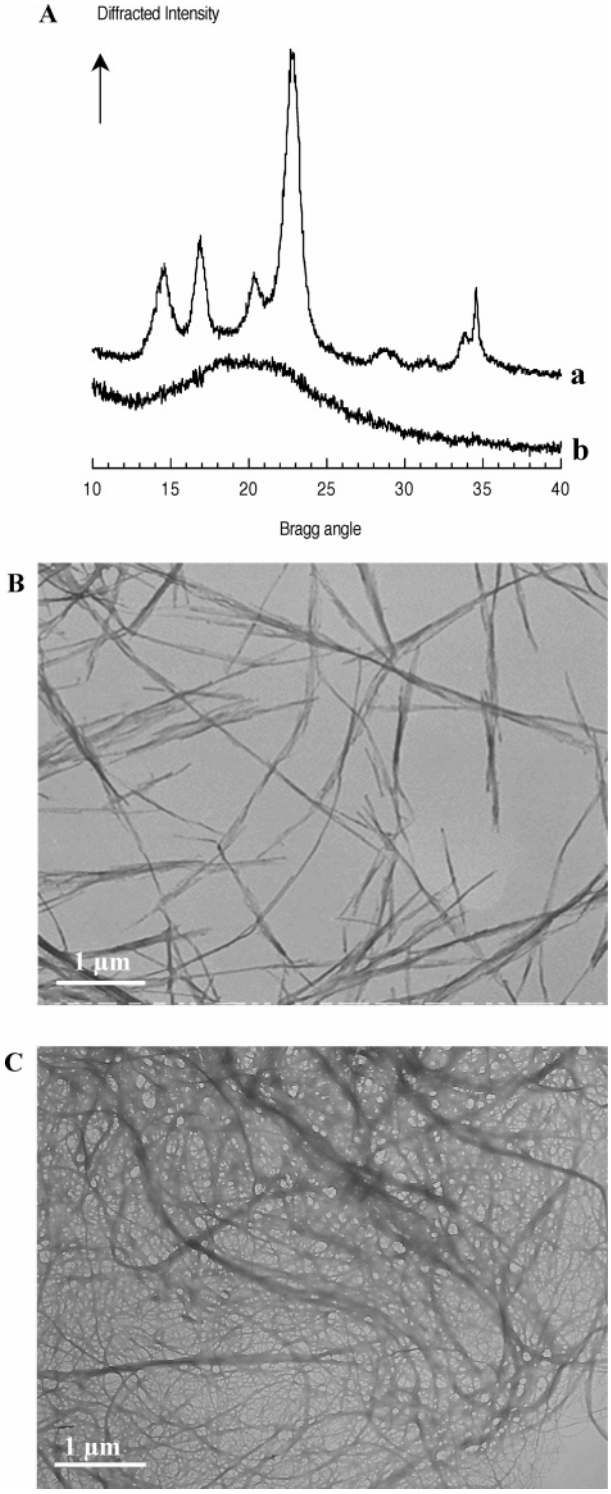

Figure 4. (A) X-ray diffractograms of (a) MCBC and (b) PCW-cellulose; TEM micrographs of (B) MCBC and (C) PCW-cellulose blanks.

cellulose and MCBC are presented in Figure 5A. Debranched arabinan is a semicrystalline polymer. Indeed, four main reflections could be distinguished from debranched arabinan diffractogram (Figure 5Aa). The first at $2\theta \approx 17.4°$ (011) was the strongest one, and the other three were less intense. These findings are in good agreement with results reported by Janaswamy and Chandrasekaran[27] who indexed the reflections of debranched arabinan as a monoclinic unit cell with the dimensions $a = 0.544$, $b = 0.639$, c (chain axis) $= 0.868$ nm, and $\gamma = 99.6°$. The same reflections were also found in

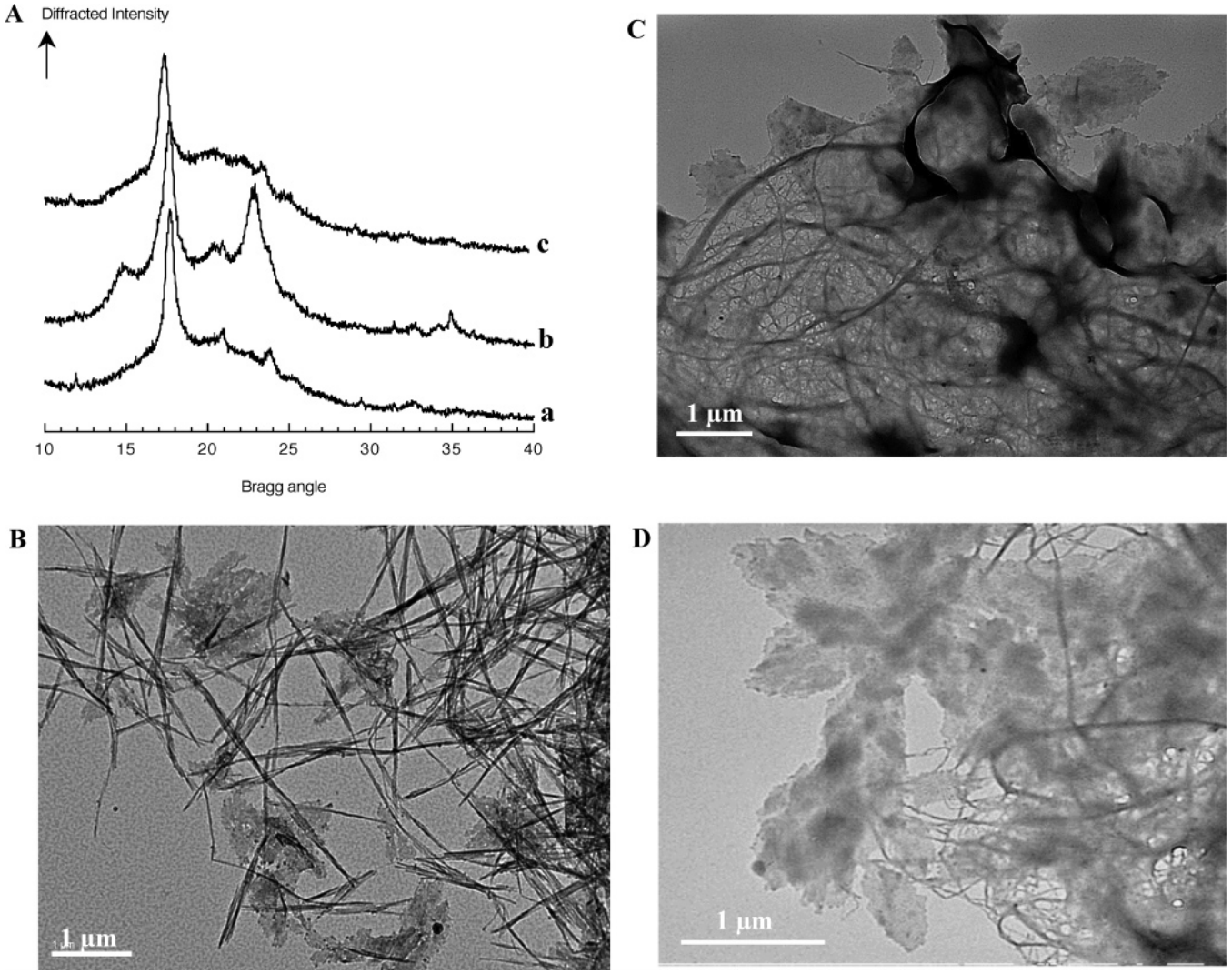

Figure 5. (A) X-ray diffractograms of (a) debranched arabinan, (b) debranched arabinan/MCBC, (c) debranched arabinan/PCW-cellulose; TEM micrographs of (B) debranched arabinan/MCBC and (C and D) debranched arabinan/PCW-cellulose.

debranched arabinan/MCBC and debranched arabinan/PCW-cellulose diffractograms shown in Figure 5Ab and 5Ac, respectively. No significant change of microfibril lateral size (12.4 nm) was observed when debranched arabinan crystallized with MCBC, as determined from the (200) scattering peak width ($2\theta \approx 22.7°$). The 2-fold helical conformation adopted by debranched arabinan with a pitch of 0.868 nm,[27] although close to that of cellulose, which adopts a 2-fold helical conformation with a repeat of 1.036 nm,[28] does probably not allow surface complementarities good enough to form a so-called "shish-kebab" morphology. Indeed, a typical area representative of the whole sample of debranched arabinan side chains crystals grown on MCBC and PCW-cellulose microfibrils is shown in Figure 5B and 5C, respectively. Debranched arabinan formed heterogeneous crystals on cellulose surface, and no free crystals were present after successive washings. Although no typical "shish-kebab" morphology was obtained, some semioriented edge-on crystals were observed in some areas of lower polymer density as shown in Figure 5D. This morphology might result from the slight incompatibility between debranched arabinan and cellulose conformations that induces only a partial transmission of the molecular orientation from PCW-cellulose microfibrils to the debranched arabinan crystals. The semioriented crystal growth was only induced by PCW-cellulose.

The branched arabinan side chains were composed of 681 mg/g of Ara with remaining amounts of Rha, Gal, and GalA (Table 1). Contrary to debranched arabinan, a semicrystalline polymer, the branched arabinan is completely amorphous. The possibility of crystallization of branched arabinan chains is most likely inhibited by the presence of branches on arabinan backbone. The amorphous character was well visualized by X-ray analysis. The diffractograms of branched arabinan blank and branched arabinan seeded with celluloses shown in Figure 6A revealed no Bragg reflections, in contrast to debranched arabinan samples. Branched arabinan seeded with MCBC did not induce any changes in a lateral size of cellulose microfibrils (13 nm). When precipitated with MCBC and PCW-cellulose, the branched arabinan was deposited onto cellulose microfibrils as droplets without any particular shape and size, and no free precipitates were observed. Figure 6B presents the branched arabinan/PCW-cellulose sample, and a similar morphology was observed for branched arabinan/MCBC composite.

The galactan side chains were mainly composed of Gal (608 mg/g) with some residual Ara, Rha, and GalA (Table 1). Galactan is a semicrystalline polymer, since two main Bragg reflections, at $2\theta \approx 14.0°$ and $2\theta \approx 20.9°$, were observed on X-ray diffractogram presented in Figure 7Aa. These reflections could also be distinguished in the galactan/PCW-cellulose diffractogram and were less visible in the galactan/MCBC diffractogram shown in Figure 7Ab and 7Ac, respectively. The microfibril lateral size determined from galactan/MCBC diffractogram was only slightly changed (12.9 nm), in comparison

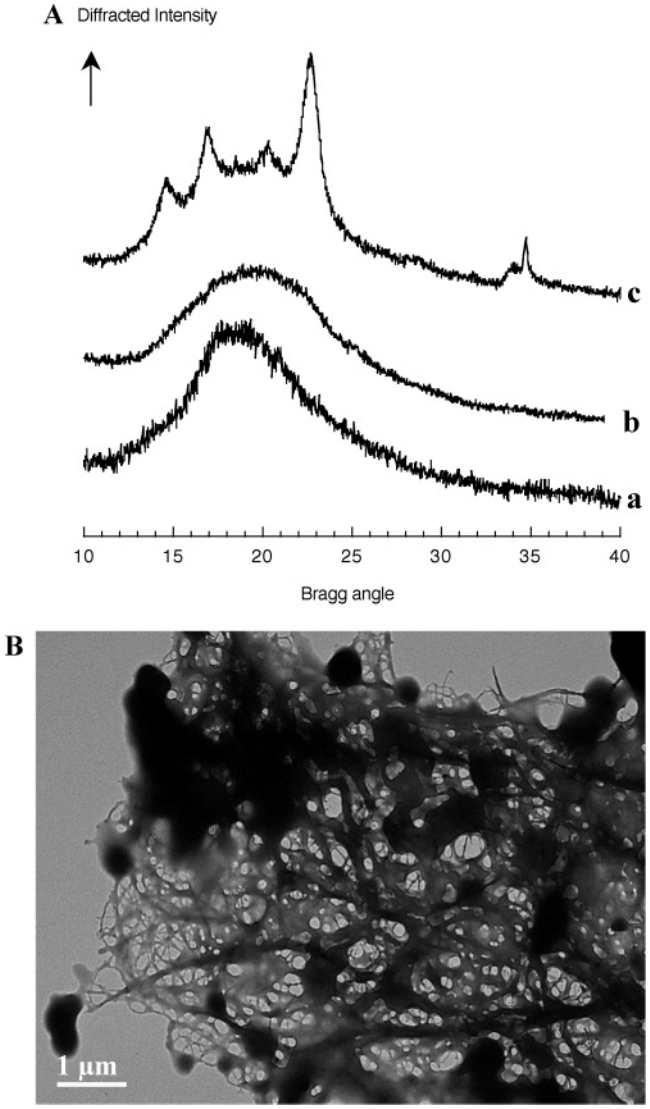

Figure 6. (A) X-ray diffractograms of (a) branched arabinan, (b) branched arabinan/PCW-cellulose, (c) branched arabinan/MCBC; (B) TEM micrograph of branched arabinan/PCW-cellulose.

with MCBC microfibril lateral size (13.4 nm). As far as we know, the three-dimensional structure of galactan has not been resolved yet.

The area representative of the whole sample of galactan grown onto MCBC and PCW-cellulose microfibrils, is presented in Figure 7B and 7C, respectively. Galactan precipitated in a similar way on cellulose surface as debranched arabinan does, by forming a homogeneous deposit onto cellulose microfibrils, and no free precipitates were present. However, no semioriented edge-on crystals were observed onto the cellulose surface. It can be noticed that for the same concentration of galactan, the amount of precipitate on PCW-cellulose was higher than on MCBC.

Pectic Backbone-Induced Precipitation. HG was composed of GalA residues (980 mg/g) (Table 1). RG I contained 175 mg/g of Rha and 502 mg/g of GalA with some residual galactan side chains (266 mg/g). Some Ara (12 mg/g) and Glc (17 mg/g) were also detected (Table 1). Pectic backbone domains are semicrystalline polymers as revealed by X-ray diffraction. The spectrum of HG blank shown in Figure 8A revealed two principal Bragg reflections, at $2\theta \approx 12.6°$ and $2\theta \approx 20.4°$. The reflection at $2\theta \approx 12.6°$ is clearly visible in HG/MCBC diffractogram. RG I is much less crystalline with two weak main

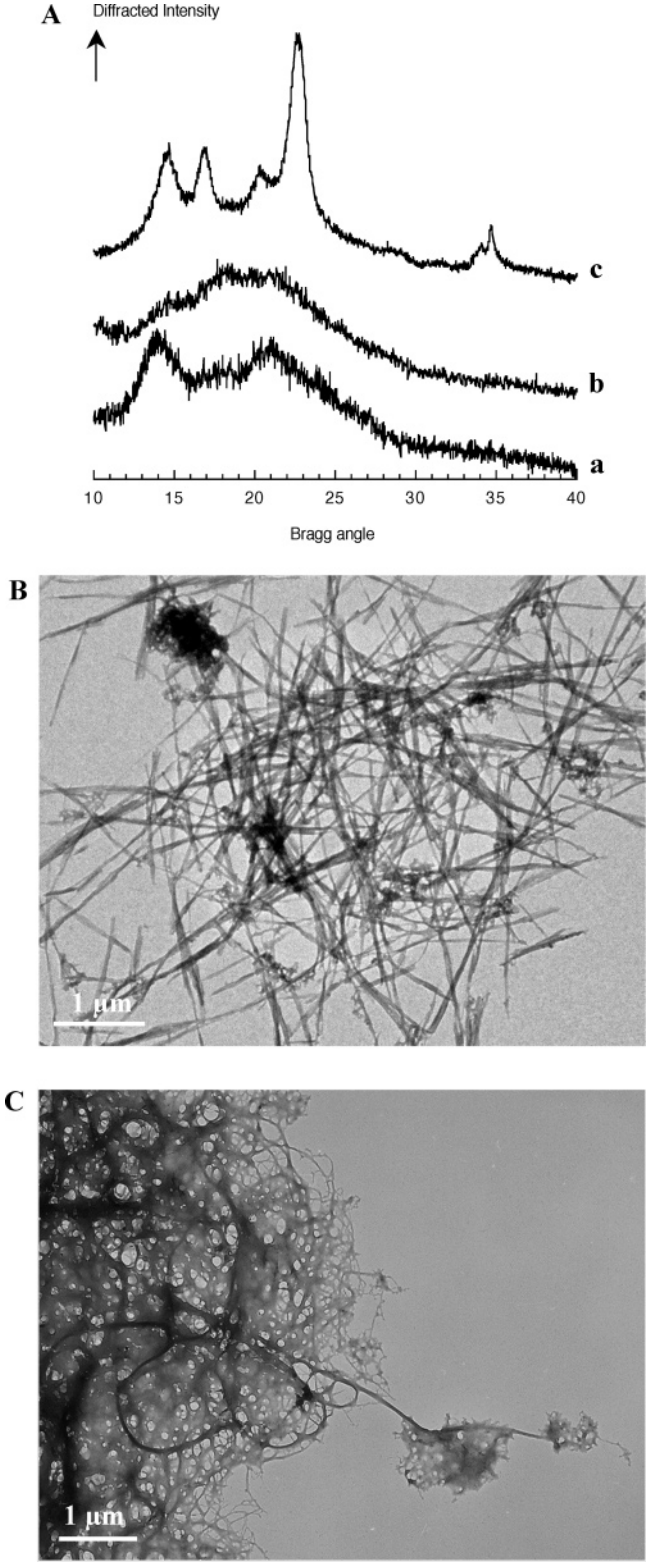

Figure 7. (A) X-ray diffractograms of (a) galactan, (b) galactan/PCW-cellulose, (c) galactan/MCBC; TEM micrographs of (B) galactan/MCBC and (C) galactan/PCW-cellulose.

reflections at $2\theta \approx 14.3°$ and $2\theta \approx 20.8°$ (Figure 8B). Walkinshaw and Arnott[29] determined the conformation of Na^+ pectate gels from fiber diffraction experiments, as a 3-fold helical one with a chain periodicity of 1.3 nm. When considering the molecular conformations of HG and cellulose, a good match between them seems to be unlikely. Few molecular data available for RG I indicate that the presence of Rha residues produces only slight changes in backbone conformation.[30]

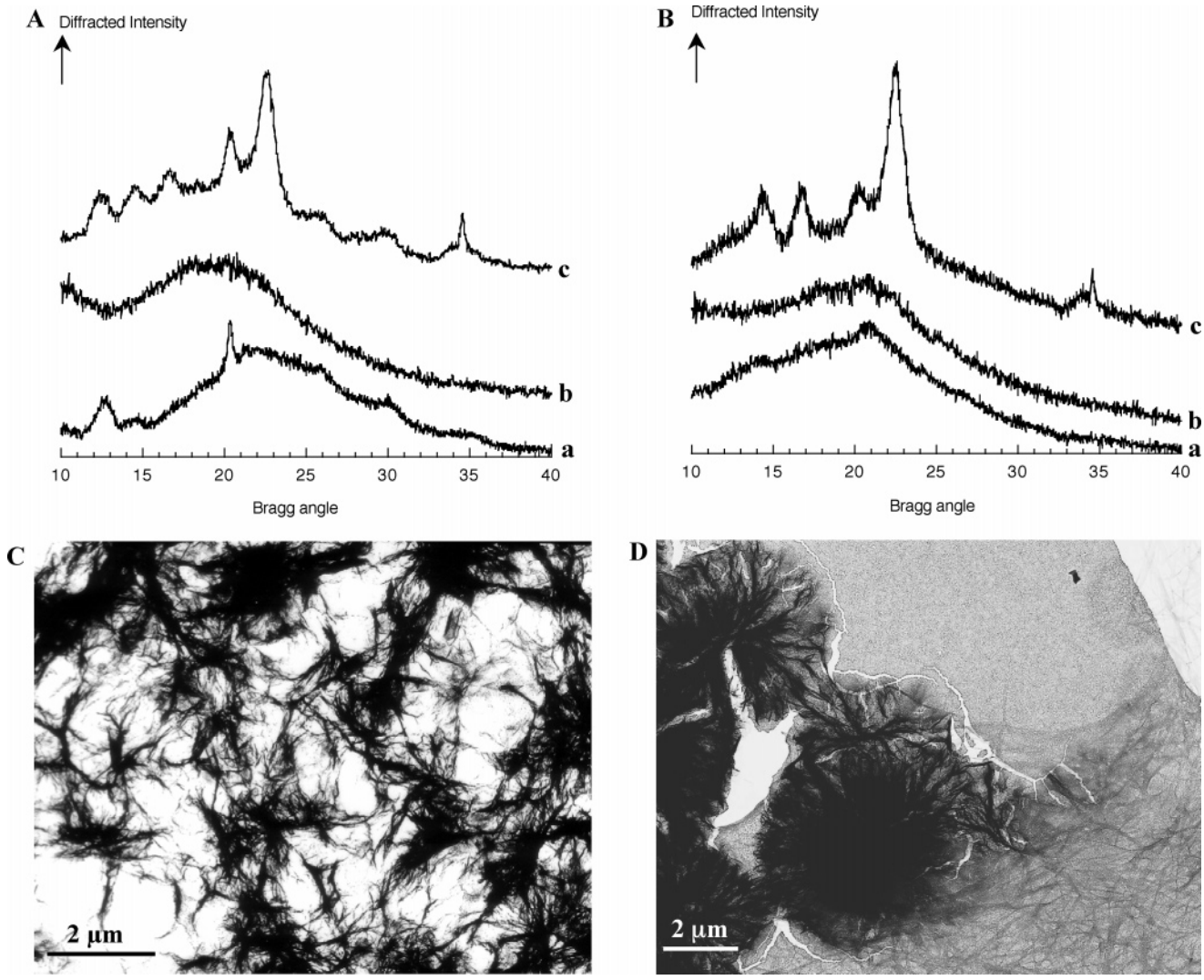

Figure 8. (A) X-ray diffractograms of (a) HG, (b) HG/PCW-cellulose, (c) HG/MCBC; (B) X-ray diffractograms of (a) RG I, (b) RG I/PCW-cellulose, (c) RG I/MCBC; TEM micrographs of (C) HG/MCBC and (D) RG I/MCBC.

Conformational data seem to be confirmed by microscopic observation. Pectic HG and RG I domains seeded with cellulose microfibrils crystallized separately from cellulose, contrary to neutral sugar side chains. HG crystallized as thin interconnected needles in the presence of MCBC (Figure 8C), while the presence of PCW-cellulose caused the formation of a thick gel difficult to observe. PCW-cellulose contains some ashes, and the presence of calcium ions cannot be precluded. These ions may be at the origin of a gel formation.[31] RG I seeded with both celluloses formed spherulites of several micrometers, composed of crystalline lamellae intercalated by amorphous phases (Figure 8D). HG and RG I crystals were found completely isolated from MCBC and PCW-cellulose microfibrils, and only few of them were observed in their vicinity, as shown in Figure 8D for the RG I/MCBC sample. None of those pectic backbone domains induced significant MCBC microfibrils lateral size changes, since lateral sizes of 14.5 and 12.8 nm were calculated for HG/MCBC and RG I/MCBC samples, respectively.

Xyloglucan-Induced Precipitation. Nonfucosylated tamarind xyloglucan was composed of Glc (457 mg/g), Xyl (266 mg/g), and Gal (152 mg/g), with some minor amounts of Ara residues (Table 1). Xyloglucan is an amorphous polymer, as it can be seen from the X-ray diffractograms presented in Figure 9A. No Bragg reflections could be distinguished from xyloglucan blank (Figure 9Aa). Only one MCBC reflection at $2\theta \approx 20.4°$ was visible on the xyloglucan/MCBC diffractogram (Figure 9Ac), while others were completely covered by xyloglucan. X-ray fiber diffraction analysis performed on tamarind xyloglucan films by Taylor and Atkins[32] indicated some molecular orientation of xyloglucan but no lateral crystallinity, which is most likely due to xyloglucan side chains that prevent crystallization. It was determined that xyloglucan can adopt a helical conformation with a pitch of 2.06 nm, twice that of cellulose, corresponding perfectly to the regular cellulose ribbon conformation.[32]

When seeded with cellulose microfibrils, xyloglucan was deposited on both celluloses. However, the cellulose origin seems to play an important role. Xyloglucan in the presence of MCBC microfibrils formed a gelatinous deposit that completely covered the cellulose surface. When seeded with PCW-cellulose, xyloglucan precipitated onto cellulose microfibrils and provoked morphological changes, as shown in Figure 9B, compared to the PCW-cellulose blank in Figure 4C. Cellulose microfibrils exhibited lower entanglement, but no clear cross-links between neighboring microfibrils could be distinguished.

Discussion

In the present work, isolated pectic domains representative of the entire pectic molecule were used to study the interactions between pectins and cellulose. A complementary *in vitro*

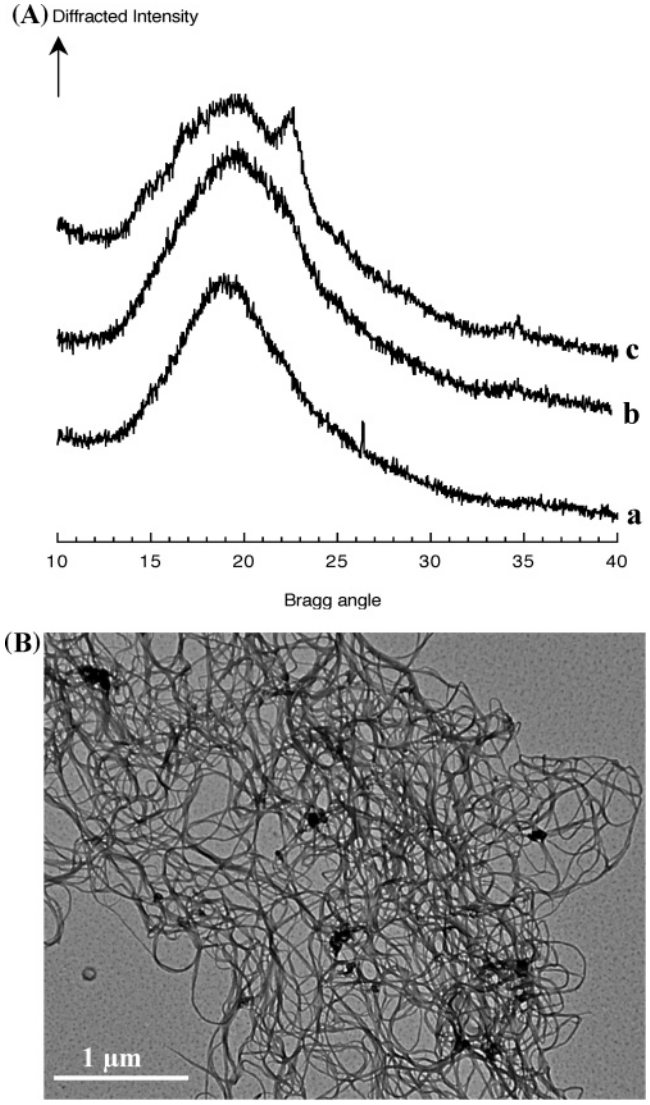

Figure 9. (A) X-ray diffractograms of (a) xyloglucan, (b) xyloglucan/PCW-cellulose, (c) xyloglucan/MCBC; (B) TEM micrograph of xyloglucan/PCW-cellulose.

approach using binding assays together with X-ray diffraction and electron microscopy was applied to figure out which pectic domains are responsible for binding with cellulose.

The results of binding assays performed with isolated pectic domains and PCW-cellulose confirmed our previous findings.[13] Debranched arabinan and galactan exhibited one of the most important bindings to PCW-cellulose microfibrils. It appeared that the isolated neutral sugar side chains displayed higher binding capacities to PCW-cellulose, when compared to Avicel microcrystalline cellulose. Debranching arabinan chains led to an increased binding capacity that could be due to a higher degree of alignment of arabinan backbone with cellulose microfibrils. On the contrary, branched arabinan side chains displayed a limited adsorption to PCW-cellulose, probably because of the steric hindrance of arabinan branching. Contrary to pectic arabinan and galactan side chains, HG and RG I exhibited only weak adsorption to PCW-cellulose, close to the experimental error. These findings are in agreement with our previous results, which revealed that no binding could be observed between commercial citrus pectins, mainly composed of HG regions, and Avicel cellulose.[13]

Pectins/cellulose associations are however weaker than xyloglucan/cellulose associations. Indeed, the weight ratio of xyloglucan adsorbed *in vitro* to PCW-cellulose (3.3%, w/w) revealed that cellulose microfibrils are able to adsorb about 2 times more xyloglucan than debranched arabinan or galactan.[13] This important binding capacity is probably due to good surface complementarities, as both xyloglucan and cellulose adopt a similar ribbon conformation.[33] To establish if a good match between isolated pectin domains and cellulose is also possible, induced precipitation experiments were performed. The influence of cellulose microfibrils on precipitate growth was studied using bacterial and primary cell wall celluloses, which differ in their morphology and crystallinity. The effect of pectic domain precipitation on the cellulose structural characteristics was also investigated. It appeared that arabinan and galactan side chains were exclusively nucleated by cellulose microfibrils, and no free precipitates were present after successive washings. All of these three side chains formed a homogeneous deposit onto MCBC and PCW-cellulose microfibrils. Debranched arabinans, the most crystalline side chains, were found not only deposited onto the cellulose microfibril surface but also organized in semioriented edge-on crystals, the growth of which was only induced by PCW-cellulose. This morphology, although different from the typical "shish-kebab", suggests that debranched arabinan and cellulose conformations are most likely compatible. On the contrary, more amorphous branched arabinan and galactan side chains did not display any induced growth onto the cellulose surface. However, X-ray analysis showed that galactan was more structured than branched arabinan. It is worth mentioning that more galactan precipitated onto PCW-cellulose microfibrils compared to MCBC, which suggests a greater surface area of the PCW-cellulose than the MCBC.

It appears that cellulose arrangement and/or microfibril orientation were not affected by the pectic side chain growth, as observed in electron micrographs. That was also confirmed by X-ray diffractograms, where side chain/cellulose composite spectra were the exact superposition of the individual components. Moreover, the degree of MCBC crystallinity was not affected by the presence of side chains, since microfibril lateral size of cellulose used for nucleation of pectic side chains showed no significant variations with comparison to the MCBC blank. Additionally, no cross-links between neighboring microfibrils can be distinguished. Contrary to neutral sugar side chains, the HG and RG I backbone domains crystallized separately from cellulose microfibrils and independently of cellulose origin. This observation suggests that a good match between pectic backbone domains and cellulose is very unlikely. Indeed, these findings are in good concordance with binding assays and clearly highlight that only pectic neutral sugar side chains are responsible for the adsorption onto cellulose microfibrils.

The "artificial" composites created by both approaches reveal that the amount of pectins and xyloglucan bound onto cellulose under *in vitro* conditions is very low. This is probably because the adsorption is limited only to the cellulose surfaces, and no polysaccharides could be entrapped within the microfibrils, as supposed during biosynthesis. This fact may explain why there is no crystallinity change when cellulose is used as a substrate for induced precipitation of pectic domains. However, an important level of incorporation (~38%, w/w) was reported when bacterial cellulose was synthesized in the presence of xyloglucan[34] that caused a marked decrease in the cellulose crystallinity, which varied from 80−85% to 50−55%. This significant decrease of cellulose crystallinity was most likely due to the fact that xyloglucan was entrapped within cellulose microfibrils during their synthesis. It is noteworthy that primary wall cellulose exhibits a crystallinity degree (40−45%) lower

than cellulose/xyloglucan composite (50−55%). It may suggest that cell wall components other than xyloglucan are responsible for reducing crystallinity further.[34]

The pectin/cellulose interactions were also suggested by some other approaches. Alkaline extractions of increasing severity performed on sugar beet and potato cell walls that are abundant, respectively, in arabinan- and galactan-rich pectins, and cellulose revealed the presence of different pectic populations.[35] One pectic fraction that was suggested to be loosely bound to cellulose microfibrils was rich in HG and RG I with some neutral sugar side chains and was easily extractible. The second one was considerably richer in neutral sugar side chains and was supposed to tightly bind to cellulose, as it withstands extraction in harsher alkaline conditions. The presence of pectic populations with different mobility was also suggested by solid-state ^{13}C NMR measurements. Some minor populations of pectic arabinan and galactan side chains were determined with restricted mobility due most likely to close association with cellulose microfibrils.[35] Sugar beet and potato cell walls, and some other types (e. g. celery, onion, apple), are characterized by a very low xyloglucan content. The limited amount of xyloglucan is most likely insufficient to provide either complete coating of cellulose surface or tethering of cellulose microfibrils, as already suggested.[36,37] In those cell walls particularly poor in xyloglucan, pectins rich in arabinan or galactan side chains that are able to bind to cellulose microfibrils could be the main polymers that are able to maintain the cell wall structure by providing a continuum between microfibrils. Indeed, pectins may act as a "glue" that holds the cellulose microfibrils together.

In conclusion, a complementary approach including binding assays, electron microscopy, and X-ray diffraction methods clearly emphasizes the possibility of associations between pectins and cellulose. The use of isolated pectic domains representative of the entire pectin gave a unique opportunity to confirm that neutral sugar side chains are directly involved in cellulose binding. The associations between pectins and cellulose could be mediated through noncovalent bondings (hydrogen bondings and/or van der Walls interactions), as already suggested for xyloglucan/cellulose associations.[38,39] To understand the impact of these pectin/cellulose associations, it is necessary to produce composites based on bacterial cellulose synthesized in arabinan- and galactan-rich pectin media.

Abbreviations

CWM, cell wall material; HG, homogalacturonan; MCBC, microcrystalline bacterial cellulose; PCW-cellulose, primary cell wall cellulose; RG I, rhamnogalacturonan I; RG II, rhamnogalacturonan II; SB-CWM, sugar beet cell wall material.

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