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Polymorphism of curdlan and (1 → 3)-β-D-glucans synthesized in vitro: A ^{13}C CP-MAS and X-ray diffraction analysis

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Abstract

The polymorphism of three different forms of curdlan and four (1 → 3)-β-D-glucans synthesized in vitro was investigated by ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy and X-ray powder diffraction. Dried samples of curdlan presented a disordered state whereas two distinct hydrated crystalline structures were evidenced for the polymer after hydrothermal treatment or swelling in water at room temperature. The samples synthesized in vitro by detergent extracts of plasma membranes from *Rubus fruticosus* and *Saprolegnia monoica* and by a mutated barley (1 → 3)-β-D-glucan endohydrolase exhibited a structural heterogeneity that can be explained in the light of the results obtained on standard samples. A 76-ppm resonance signal corresponding to carbon five was identified by CP/MAS NMR spectroscopy. The relative importance of this peak was shown to be linked to the proportion of the different crystalline allomorphs in a given sample. This peak can be considered as a new marker of the degree of organization of (1 → 3)-β-D-glucans. The observed polymorphism provides further detailed information on the conformation of the different (1 → 3)-β-D-glucan allomorphs.

Keywords: (1 → 3)-β-D-Glucans; Curdlan; Triple helix; Single helix; Biopolymers; Biosynthesis of polysaccharides; CP/MAS NMR spectroscopy; X-ray diffraction

1. Introduction

(1 → 3)-β-D-Glucans are distributed in a wide range of living organisms such as plants, fungi, yeasts, and some bacteria (Stone & Clarke, 1992). They not only occur as structural or storage material, but are also involved in more specialized functions (Stone & Clarke, 1992). For example, plant (1 → 3)-β-D-glucans play an important role in cell division and defense responses against various kinds of stresses like microbial attacks and mechanical stresses (Stone & Clarke, 1992). Research on (1 → 3)-β-D-glucans has been quite active over the years mainly because of their biological activities such as immunomodulation and anti-tumoral activity, and the corresponding potential applications in the biomedical field (Ooi & Liu, 2000). Such biological activities seem to be dependent on specific features of the (1 → 3)-β-D-glucan molecules, especially their conformation, molecular weight, and the presence or absence of branches, which usually consist of β-glucans linked to the main chain through β-(1 → 6) linkages (Bohn & BeMiller, 1995). The structure of these polysaccharides has been investigated either in solution, as gels or crystalline solids, with the objective of unravelling their conformation in detail.

One of the most studied (1 → 3)-β-D-glucans is curdlan, which is a strictly linear bacterial polysaccharide (Harada, Masada, Fujimori, & Maeda, 1966). The crystal structure

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and conformational properties of this polymer have been investigated by several groups, using X-ray diffraction (Chuah, Sarko, Deslandes, & Marchessault, 1983; Deslandes, Marchessault, & Sarko, 1980; Fulton & Atkins, 1980; Kasai & Harada, 1980; Marchessault & Deslandes, 1979; Marchessault, Deslandes, Ogawa, & Sudararajan, 1976; Okuyama et al., 1991; Takeda, Yasuoka, Kasai, & Harada, 1978) and high-resolution solid-state ^{13}C NMR spectroscopy (Fyfe et al., 1984; Saito, Ohki, & Sasaki, 1977; Saito, Tabeta, & Harada, 1981; Saito, Yokoi, & Yoshida, 1989; Saito et al., 1991; Saito, Yoshioka, Yokoi, & Yamada, 1990; Stipanovic & Giammatteo, 1987). The results obtained have allowed the proposition of several crystal models, which are briefly presented in Table 1. Three crystalline allomorphs – one anhydrous and two hydrated – have been identified depending on the crystallization and hydration conditions (Marchessault et al., 1976). When fibers spun from a curdlan spinning dope are annealed, either dry or after a hydrothermal treatment, they become substantially crystalline, with crystallites consisting of parallel molecules of (1 → 3)-β-D-glucan intertwined into triple helices (Deslandes et al., 1980). Hydratation simply expands the hexagonal unit cell, permitting the water molecules to enter into the intertriplex space (Chuah et al., 1983). The third allomorph, called hereafter hydrated curdlan, is usually obtained by neutralizing alkaline solutions of the spray-dried polymer. This yields gels that can be stretched in the form of poorly crystalline fibers containing a large number of intra-crystalline water molecules (Fulton & Atkins, 1980; Takeda et al., 1978). Two conflicting structures have been proposed for this allomorph (Table 1). In one of them it is believed that the curdlan molecules occur as highly hydrated single helices (Okuyama et al., 1991; Saito et al., 1989), whereas in the other the fibers would consist of loose intertwined triple helices (Fulton & Atkins, 1980; Stipanovic & Giammatteo, 1987). Importantly, controversial data were also obtained for the biological activity of (1 → 3)-β-D-glucans in relation to the debated helical structures. In particular, some results suggest a more potent biological activity of the single versus triple helices (Aketagawa, Tanaka, Tamura, Shibata, & Saito, 1993; Nagi et al., 1993; Ohno, Hashimoto, Adachi, & Yadomae, 1996; Saito et al., 1991; Suzuki, Ohno, Saito, & Yadomae, 1992), whereas others suggest the opposite (Falch, Espevik, Ryan, & Stokke, 2000; Kojima, Tabeta, Itoh, & Yanaki, 1986; Mueller et al., 2000).

From these observations, it seems important to develop more structural and conformational studies on linear (1 → 3)-β-D-glucans to establish a link between the structure of these polymers and their biological activity. We have undertaken such detailed structural investigations by taking advantage of the possibility to synthesize in vitro (1 → 3)-β-D-glucans with specific properties, using either detergent-extracted (1 → 3)-β-D-glucan synthases from the Oomycete *Saprolegnia monoica* and from blackberry (*Rubus fruticosus*) cell suspension cultures (Pelosi et al., 2003) or a mutated (1 → 3)-β-D-glucan endohydrolase from barley (*Hordeum vulgare*) (Hrmova et al., 2002). The polymers synthesized in vitro by these enzymes represent interesting starting materials for comparative structural and conformational analyses of (1 → 3)-β-D-glucans. Indeed, they not only exhibit various morphologies, ranging from long kinked microfibrils to short spindle-like elements or even thin lamellar hexagonal crystals, but they are also characterized by different degrees of crystallinity and polymerization ($\overline{DP}$) and susceptibility to hydration (Hrmova et al., 2002; Pelosi et al., 2003). A detailed analysis is required to understand the observed morphological differences. In the present work, which is based on X-ray diffraction and solid-state ^{13}C NMR spectroscopy analyses, we have obtained detailed structural information on different allomorphs of curdlan taken as a reference sample and compared these structural data with those obtained on the different (1 → 3)-β-D-glucans synthesized in vitro. We took advantage of the structural heterogeneity of the biosynthesized samples to gain further insight into the conformational transition of (1 → 3)-β-D-glucans and to estimate the amount of each allomorph in a given sample.

2. Materials and methods

2.1. Preparation of samples

A spray-dried curdlan ($\overline{DPn} = 540$) powder from *A. faecalis* was obtained from Takada Chemical Industries (Osaka, Japan). Annealed curdlan was prepared following a hydrothermal treatment in which a 10% (w/v) aqueous suspension of curdlan contained in a sealed glass tube

Table 1
Literature data on the crystal structure of curdlan

Curdlan	Hydrated	Annealed dry	Annealed hydrated
Helical conformation and helix multiplicity	7/1 triplex (Fulton & Atkins, 1980; Stipanovic & Giammatteo, 1987) or single 6/1 helix (Okuyama et al., 1991; Saito et al., 1989)	6/1 triplex (Deslandes et al., 1980; Marchessault et al., 1976)	6/1 triplex (Chuah et al., 1983; Marchessault et al., 1976; Saito et al., 1989)
Space group	Hexagonal (Fulton & Atkins, 1980) or orthorhombic (Okuyama et al., 1991)	Hexagonal $P6_3$ (Deslandes et al., 1980; Marchessault et al., 1976)	Triclinic P1 (Chuah et al., 1983; Marchessault et al., 1976)
Water molecules/ glucosyl residue	Two (Fulton & Atkins, 1980) or 20 (Okuyama et al., 1991)	None (Deslandes et al., 1980; Marchessault et al., 1976)	One (Chuah et al., 1983; Marchessault et al., 1976)

was heated at 120 °C for 1 h in an autoclave and then cooled to room temperature under tap water (Saito et al., 1989). Another (1 → 3)-β-D-glucan was synthesized in vitro by a mutated barley (1 → 3)-β-D-glucan endohydrolase as described previously (Hrmova et al., 2002). (1 → 3)-β-D-Glucans synthesized in vitro by detergent (Chaps)-extracted (1 → 3)-β-D-glucan synthases from *S. monoica* and *R. fruticosus* were prepared as reported earlier (Lai Kee Him, Pelosi, Chanzy, Putaux, & Bulone, 2001; Pelosi et al., 2003) and kept either as aqueous suspensions or freeze-dried.

2.2. ^{13}C solid-state CP/MAS NMR spectroscopy

Curdlan was analyzed either in a dried or hydrated state, before or after a hydrothermal treatment. The hydrated curdlan samples were either soaked in an excess of water for 48 h or kept in a 95% relative humidity atmosphere obtained in a desiccator containing a saturated aqueous solution of $Na_2HPO_4{\cdot}12H_2O$. (1 → 3)-β-D-Glucans synthesized in vitro were analyzed in a never-dried state, after removal of the excess of water by centrifuging the suspensions at 3000*g* for 15 min. The samples were then introduced into airtight sealed 4-mm BL type ZrO_2 rotors. ^{13}C NMR spectra (100 MHz) in the solid state were recorded with a Bruker Avance spectrometer equipped with a 4-mm BL type probe. The spectra were acquired at room temperature under a 80-kHz proton dipolar decoupling field, matched cross-polarization (CP) fields of 80 kHz, a proton 90° pulse of 2.5 μs and magic angle spinning (MAS) at a spinning speed of 6 kHz. The cross-polarization transfer was achieved using a ramped amplitude sequence (RAMP-CP) for an optimized total contact time of 2 ms (Peersen, Wu, Kustanovich, & Smith, 1993). The sweep width was of 50,000 Hz to avoid baseline distortion with 2994 TD points, and the Fourier transformation was achieved without apodization over 8k points. The repetition time was 4 s and an average number of 10,000 scans was acquired for each spectrum. The ^{13}C chemical shifts were determined relative to the carbon chemical shift of the glycine carboxyl group (176.03 ppm) and the resonance peaks were assigned according to Saito et al. (1989). The intensity of the resonance signals was evaluated by integrating the peaks obtained in three of the spectra corresponding to the in vitro products. A possible overlap of the resonances was accounted for by using spectral deconvolution with lorentzian lines. The T1 measurements were achieved using the pulse sequence proposed by Torchia (1978).

2.3. X-ray diffraction analysis

Samples were inserted in thin wall capillaries (0.5 mm outer diameter), which were sealed in the case of hydrated specimens. These capillaries were positioned in a vacuum Wharus flat film X-ray camera mounted on a Philips 1720 X-ray generator operated at 30 kV and 20 mA with Ni-filtered CuKα radiation. The resulting X-ray diffraction diagrams were recorded on Kodak DEF-5 films. They were calibrated using calcite powder as a reference.

3. Results

3.1. Re-investigation of curdlan structure in the solid state

The solid state structure of curdlan (dry, swollen in water, and hydrothermally annealed) was first re-investigated by solid state ^{13}C CP/MAS NMR spectroscopy. The spectra obtained are presented in Fig. 1 and the corresponding values of the ^{13}C chemical shifts are listed in Table 2. For the dried sample, only five broad peaks appear (Fig. 1A) and illustrate the poor organization and the disorder of the curdlan chains, in agreement with published results on linear and branched (1 → 3)-β-D-glucans analyzed in similar conditions (Fyfe et al., 1984; Saito et al., 1989, 1990; Stipanovic & Giammatteo, 1987). The spectra obtained from curdlan swollen in water (hydrated curdlan) (Fig. 1B) and from hydrothermally annealed curdlan (Fig. 1C) have a better resolution than those available in the literature (Saito et al., 1989, 1990; Stipanovic & Giammatteo, 1987) and reveal new structural details. In particular, in Fig. 1B, the signals corresponding to C1 and C5 near 104 and 76 ppm are split into rather sharp doublets while those of C2, C4, and C6 near 73, 69, and 61 ppm occur as singlets (Table 2). A substantially broader resonance signal near 86 ppm is assigned to C3. The spectrum recorded from hydrothermally annealed curdlan (Fig. 1C) shares common features with the one presented in Fig. 1B. It consists of four relatively narrow signals for C1, C2, C4, and C6, and of a rather broader peak for C3. The signal corresponding to C5 occurs near 77 ppm in Fig. 1C and it is not clear whether the new and much smaller peak near 76 ppm, marked X in Fig. 1, arises from C5 or C2. Except for C3 and C6, the other resonances are shifted by about 1 ppm for the annealed sample compared to the hydrated one. Interestingly, the most important difference is observed for C5, which yields a signal centered at 77.3 ppm for the hydrothermally annealed curdlan and at 75.5 ppm for the hydrated curdlan (Table 2). This difference of almost 2 ppm is most probably related to the exposure of C5 to water in the triple helical structure (Saito et al., 1989). Based on our observations, the ^{13}C NMR patterns of the curdlan types are found to be sufficiently different to illustrate the structural variations between these two polymorphs, as proposed in the literature (Saito et al., 1989; Stipanovic & Giammatteo, 1987). The structure of hydrothermally annealed curdlan and other crystalline linear (1 → 3)-β-D-glucans such as paramylon appears to be relatively well known (Saito et al., 1989; Stipanovic & Giammatteo, 1987) and consisting of a triple-helical structure, whereas the one of hydrated curdlan is still debated. It is not clear, using solid state CP/MAS spectroscopy data, whether this sample consists of highly hydrated single

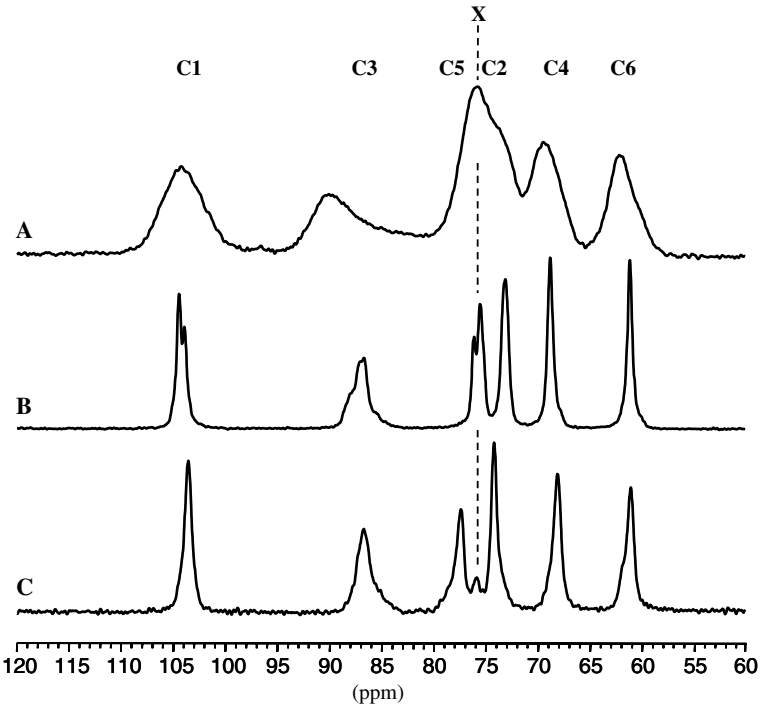

Fig. 1. One hundred megahertz ^{13}C NMR spectra recorded in the solid state. (A) Anhydrous curdlan. (B) Curdlan swollen in water (hydrated). (C) Hydrothermally annealed curdlan. The indexation of the carbon atoms is derived from Saito et al. (1989). The vertical dotted line marked X corresponds to a resonance signal near 76 ppm that refers to the hydrated curdlan phase.

Table 2
^{13}C-chemical shift (in ppm, obtained by reference to TMS) and *d*-spacing values (nm) of the inner ring of the powder diffraction diagrams obtained from the various linear (1 → 3)-β-D-glucans analyzed as shown in Figs. 1–4

Samples analyzed	C1	C2	C3	C4	C5	X[a]	C6	Inner ring *d*-spacing values (nm)
Dried curdlan	104.2	ND[b]	90.0	69.4	75.7	–	62.1	ND[b]
Hydrated curdlan	104.4[c]	73.0	86.6	68.7	76.1	–	61.1	1.54
	103.9				75.5			
Hydrothermally annealed curdlan	103.5	74.1	86.7	68.0	77.3	75.8	61.0	1.36
(1 → 3)-β-D-Glucan synthesized in vitro at pH 6 by a detergent-solubilized synthase from *S. monoica*	103.5	74.2	86.6	68.0	78.7	–	61.1	1.36
					77.4			
(1 → 3)-β-D-Glucan synthesized in vitro at pH 9 by a detergent-solubilized synthase from *S. monoica*	103.6	74.2	86.7	68.1	77.4	76.0	61.1	1.36
			85.1					
(1 → 3)-β-D-Glucan synthesized in vitro by a detergent-solubilized synthase from *R. fruticosus*	103.6	74.2	86.9	68.1	77.4	75.8	61.1	1.54
(1 → 3)-β-D-Glucan synthesized in vitro by a mutated glucan endohydrolase from barley	103.6	74.1	86.7	68.2	78.8	76.1	61.9	1.36[d]
					77.4		61.1	1.54

[a] X corresponds to the resonance of small intensity near 76 ppm.
[b] ND, not determined.
[c] In case of multiplets, the most intense resonance is underlined.
[d] This diagram contains two diffraction rings; the reflection of the most intense is underlined.

helices (Saito et al., 1989) or loose intertwined hydrated triple helices (Stipanovic & Giammatteo, 1987).

In order to address this question and to complete the previous results, we have performed X-ray diffraction analyses of curdlan in dry, hydrated or hydrothermally annealed conditions (Fig. 2). Paramylon, which can be considered as a reference for highly crystalline (1 → 3)-β-D-glucan (Booy, Chanzy, & Boudet, 1980; Marchessault & Deslandes, 1979) was used as a standard (Fig. 2A). The diagram corresponding to anhydrous curdlan is rather poor with a weak inner ring (Fig. 2B). This result confirms the lack of organization of this polysaccharide observed by solid state NMR spectroscopy (Fig. 1A), and illustrates one more time the importance of water molecules in the structure of (1 → 3)-β-D-glucans. Thus, samples were hydrated to increase their crystallinity. The diagram recorded from curdlan hydrated in a 95% relative humidity atmosphere presented a strong inner ring calibrated at

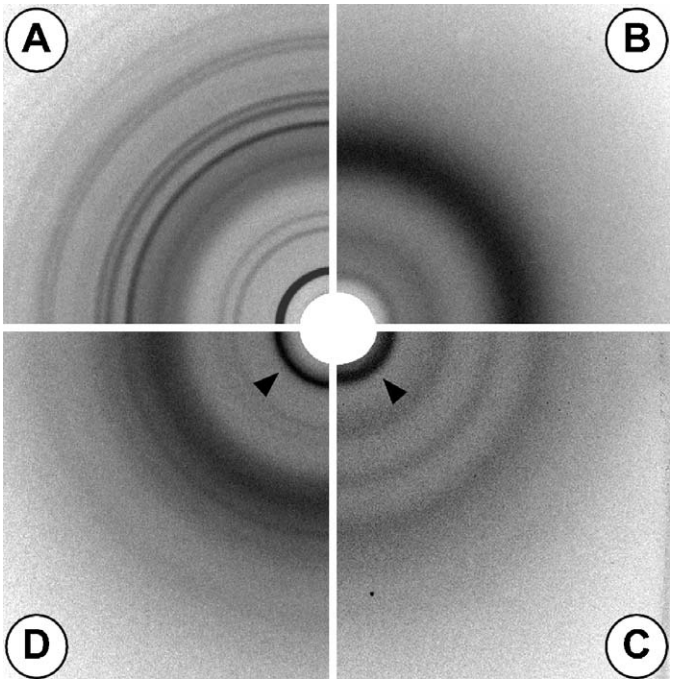

Fig. 2. X-ray powder diffraction diagrams of (1 → 3)-β-D-glucan reference samples. (A) Never dried paramylon. (B) Anhydrous curdlan. (C) Hydrated curdlan. (D) Hydrothermally annealed curdlan. The diagrams in A, C, and D were recorded under hydrated conditions. Arrowheads in the spectra indicate the inner diffraction rings that are characteristic of the samples analyzed.

1.54 nm (Fig. 2C and Table 2). The latter was previously observed by Fulton and Atkins (1980) and Okuyama et al. (1991). Because of the low crystallinity of the sample, the occurrence of the 1.54-nm spacing is not well understood and remains much debated. In particular, the hypotheses available in the literature suggest the existence of two different types of helical conformation and helix multiplicity, i.e., a sixfold repeat single helix (Okuyama et al., 1991) or a sevenfold repeat triplex (Fulton & Atkins, 1980) (Table 1). The diagram corresponding to hydrothermally annealed curdlan (Fig. 2D) is of a better quality and shows a strong inner ring measured at 1.36 nm (Table 2). This value, which corresponds to the 100 *d*-spacing, suggests the occurrence of a triplex of parallel (1 → 3)-β-D-glucan molecules which adopt a sixfold repeat in a hexagonal packing (Chuah et al., 1983).

3.2. Solid-state structural characterization of (1 → 3)-β-D-glucans synthesized in vitro

Figs. 3A–C show spectra obtained from (1 → 3)-β-D-glucans synthesized in vitro using cell-free enzyme preparations as described by Pelosi et al. (2003). The spectra exhibit six resonances corresponding to the six carbon atoms of (1 → 3)-β-linked glucosyl residues and are very similar to the spectrum of the hydrothermally annealed curdlan (Fig. 1C). As observed previously, a small resonance signal near 76 ppm (marked X) is present with a variable intensity in the spectra in Figs. 3A–C. This 76-ppm signal is smaller in Fig. 3B than in Fig. 3A and even smaller in Fig. 3C where it is almost absent. In the latter, the resonance peaks assigned to C3, C5, and C6 show some broadening, which indicates the presence of multiplets that are only partly resolved. The spectrum presented in Fig. 3D was obtained from the (1 → 3)-β-D-glucan synthesized in vitro by the mutated barley (1 → 3)-β-D-glucan endohydrolase prepared as described by Hrmova et al. (2002). In this spectrum, the splitting of the C3, C5, and C6 resonances is more pronounced. As for the other polysaccharides synthesized in vitro, the minor peak near 76 ppm is detected (Fig. 3D).

From these results, it seems that all the polysaccharides synthesized in vitro and analyzed in this work present the same overall spectral features as hydrothermally annealed curdlan. This suggests a similar triple-helical structure for these samples. Interestingly, only the intensity of the minor signal at 76 ppm varied among the different spectra, despite strong differences in the physico-chemical and biochemical properties of these polysaccharides (Hrmova et al., 2002; Pelosi et al., 2003). Its assignment and its signification were therefore investigated in greater detail. From the data in the literature (Saito et al., 1989; Stipanovic & Giammatteo, 1987), and from the spectrum in Fig. 1B, the small resonance at 76 ppm could be assigned to the presence of a phase similar to the one of hydrated curdlan. We have evaluated the relative importance of each peak by integration of deconvoluted lines as described in Section 2. Table 3 gives quantitative information on the intensity of the carbon signals from the (1 → 3)-β-D-glucans synthesized in vitro by the Chaps-extracted enzymes from *S. monoica* and *R. fruticosus*. The results show that the resonance signal designated as X near 76 ppm can be unambiguously attributed to C5 and not to C2. Indeed, the intensity of this small resonance always increases at the detriment of the C5 signal near 77.4 ppm, whereas the intensity of the peak assigned to C2 is unaffected (Table 3). In addition, the sum of the contribution of the C5 signal and that of the peak near 76 ppm is always equal to one, within experimental errors (Table 3). The relaxation times of each carbon atom were also measured (Table 3). The results show two types of relaxation times. The long ones are of 5 s and above for C1, C2, C3, C4, and the C5 major contribution near 77.4 ppm, whereas the short ones are of 1.7 s and below for the C5 minor contribution near 76 ppm. The shortest relaxation times (0.5 s and below) of the C6 are related to the documented structural mobility of this carbon in D-glucans (Chuah et al., 1983; Teeäär & Lippmaa, 1984). A small relaxation time (1.7 s) is observed for the minor resonance at 76 ppm indicating that this signal arises from a fraction having a greater mobility than the rest of the sample. This observation further supports the assignment of the 76-ppm signal to a less ordered and more mobile part of the sample. Such a hydrated-like structure is in agreement with the substantial hydration of the phase and its low crystallinity (Fulton & Atkins, 1980; Kasai &

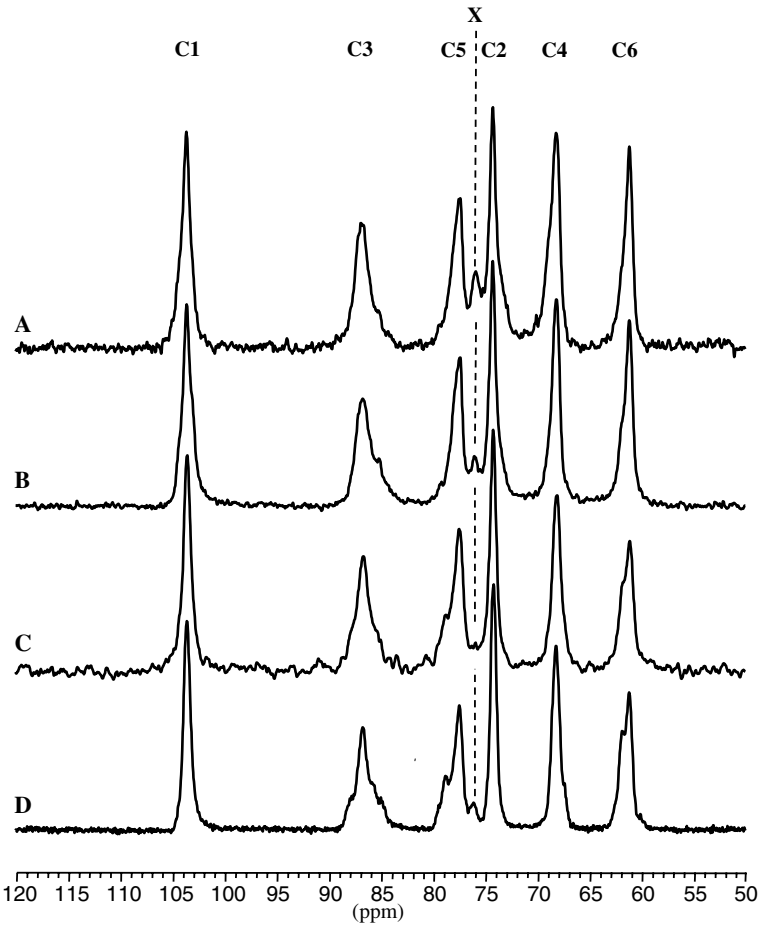

Fig. 3. One hundred megahertz ^{13}C NMR spectra recorded in the solid state from never-dried in vitro (1 → 3)-β-D-glucans. (A) Sample synthesized by cell-free enzyme preparations from *R. fruticosus*. (B) As in A, but synthesized by enzymes from *S. monoica* at pH 9. (C) As in (B), but synthesized at pH 6. (D) Sample synthesized by a mutated (1 → 3)-β-D-glucan endohydrolase from barley. As in Fig. 1, the vertical dotted line marked X corresponds to a resonance near 76 ppm that refers to the hydrated curdlan phase.

Table 3
^{13}C measurements and corresponding integration areas obtained by deconvolution after solid-state ^{13}C NMR analysis of in vitro (1 → 3)-β-D-glucans synthesized by detergent-extracted enzymes

Samples analyzed	C1	C2	C3	C4	C5	X[a]	C6
(1 → 3)-β-D-Glucan, *R. fruticosus* (hydrated)							
Area	1.00[b]	1.06	0.96	1.05	0.73	0.30	0.88
^{13}C T1	7.1	10.4	5.3	5.9	5.7	1.2	0.3
(1 → 3)-β-D-Glucan, *S. monoica*, pH 9 (hydrated)							
Area	1.00	1.08	0.96	0.91	0.83	0.21	0.92
^{13}C T1	8.8	10.2	8.1	6.5	5.0	1.7	0.4
(1 → 3)-β-D-Glucan, *S. monoica*, pH 6 (hydrated)							
Area	1.00	0.99	0.99	0.94	0.92	0.06	0.90
^{13}C T1	11.5	13.6	10.7	6.5	6.0	ND[c]	0.5

The relaxation times (in s) of each carbon atom are indicated.
[a] X corresponds to the resonance of small intensity near 76 ppm.
[b] All the areas are scaled with respect to the C1 contribution.
[c] ND, not determined.

Harada, 1980; Okuyama et al., 1991). By integration of the NMR signal, this phase is estimated to account for at least 6–30% of the total samples synthesized in vitro by Chaps-extracted (1 → 3)-β-D-glucan synthases. However, one has to remember that CP/MAS data are quantitative only for rigid molecules, the efficiency of the cross-polarization process decreasing with increasing mobility. Thus, the integration of the resonance at 76 ppm corresponds to a lower estimation of the real percentage of this phase.

As for curdlan, each sample synthesized in vitro was analyzed by X-ray diffraction after swelling in 95% of humidity. The powder diffraction diagrams obtained are presented in Fig. 4. All of them have in common a strong inner ring calibrated at a *d*-spacing ranging from 1.36 to 1.54 nm. Both samples prepared from *S. monoica* enzyme extracts yield X-ray diagrams of a poor quality, the (1 → 3)-β-D-glucan synthesized at pH 6 (Fig. 4B) being slightly more crystalline than the sample synthesized at pH 9 (Fig. 4C). In the two diagrams, the inner ring was measured at 1.36 nm (Table 2) indicating that the major diffracting part of this sample is mostly of the hydrothermally annealed curdlan type, i.e., a triplex of right-handed, sixfold chains organized in a hexagonal unit cell (Chuah et al., 1983). The diagram obtained from the polysaccharide synthesized in vitro by the enzyme from *R. fruticosus* is poorer than the others (Fig. 4D). Interestingly, it was

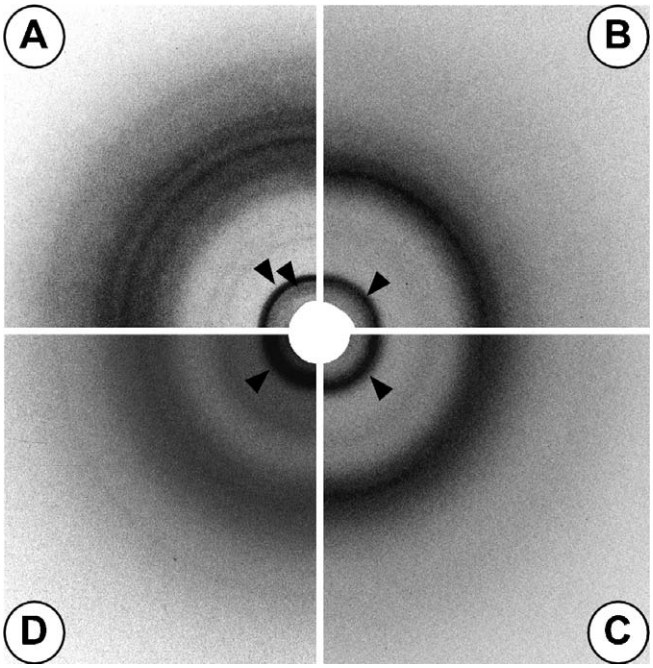

Fig. 4. X-ray powder diffraction diagrams of in vitro synthesized (1 → 3)-β-D-glucans. (A) Sample synthesized by a mutated (1 → 3)-β-D-glucan hydrolase from barley. (B) Sample synthesized at pH 6 by enzyme preparations from *S. monoica*. (C) As in (B), but synthesized at pH 9. (D) As in (C), but synthesized by enzyme preparations from *R. fruticosus*. Arrowheads in the four spectra indicate the position of the inner diffraction rings that are characteristic of the samples analyzed.

characterized by a strong inner ring centered at a *d*-spacing of 1.54 nm (Table 2) as for hydrated curdlan. The diagram recorded from the (1 → 3)-β-D-glucan synthesized by the mutated glucan endohydrolase from barley displays several diffraction rings indicating a relatively high crystallinity (Fig. 4A) compared to the other polysaccharides synthesized in vitro. Its inner diffraction ring is in fact a doublet, which consists of a strong ring calibrated at 1.36 nm and a weaker one at 1.54 nm (Table 2). From these results it can be concluded that the (1 → 3)-β-D-glucans synthesized in vitro present several types of crystalline structures, in agreement with the morphological diversity observed previously for these polysaccharides (Hrmova et al., 2002).

4. Discussion

The NMR data presented in this work show that the hydrothermally annealed curdlan and the (1 → 3)-β-D-glucans synthesized in vitro are characterized by nearly identical ^{13}C NMR spectra. In agreement with the data in the literature, these results suggest a similar structure for these polysaccharides, which consist of triplex of parallel (1 → 3)-β-D-glucan molecules organized in a hexagonal packing. The main difference between these samples is the relative intensity of the 76-ppm signal, which is stronger for the (1 → 3)-β-D-glucan synthesized by cell-free extracts from *R. fruticosus* with at least 30% of the crystallographic phase similar to the one of hydrated curdlan. The appearance of this new signal at 76 ppm in solid-state ^{13}C CP/MAS NMR spectra can be considered as a marker of the coexistence of two allomorphs.

The X-ray diffraction diagram of the annealed allomorph of curdlan is characterized by an inner diffraction ring at 1.36 nm, as observed for the samples prepared from *S. monoica*. However, the X-ray diffraction diagram corresponding to the (1 → 3)-β-D-glucan synthesized by the enzyme from *R. fruticosus* shows that the inner diffraction ring was no longer at 1.36 nm as expected, but at 1.54 nm as measured for hydrated curdlan (Table 2). Interestingly, this observation indicates that the crystalline structure of this sample is dominated by a packing of (1 → 3)-β-D-glucan helices which corresponds to the hydrated curdlan-type phase. This type of controversial data was observed recently for the conversion of cellulose I to cellulose II during mercerization (Dinand, Vignon, Chanzy, & Heux, 2002). X-ray diffraction is sensitive to the packing of the chains, whereas solid state ^{13}C NMR spectroscopy is related to their conformation. Therefore, if the hexagonal packing of the triple helices is disturbed by the progressive introduction of disorder, the specific diffraction ring at 1.36 nm disappears at the expense of a disordered packing with a typical ring at 1.54 nm. This is particularly clear for the polysaccharide produced by the mutated (1 → 3)-β-D-glucan endohydrolase from barley in which two distinct ordered and disordered morphologies coexist. In this particular case, the two diffraction rings coexist in the X-ray diffraction diagram.

The properties of the (1 → 3)-β-D-glucans analyzed in hydrated conditions in this work are summarized in Table 4. As mentioned in Sections 1 and 3, there is a conflicting view on the crystalline structure of the curdlan hydrated crystalline phase (allomorph 1), which is considered by some authors as being made of (1 → 3)-β-D-glucan single helices, while others propose the occurrence of intertwined triple helices (Fulton & Atkins, 1980; Kasai & Harada, 1980; Okuyama et al., 1991; Saito et al., 1989; Stipanovic & Giammatteo, 1987). It is important to recall that the (1 → 3)-β-D-glucans synthesized by detergent-extracted synthases occur as microfibrils (Bulone, Fincher, & Stone, 1995; Lai Kee Him et al., 2001; Pelosi et al., 2003). Since the polysaccharide synthesized at pH 9 by the enzyme from *S. monoica* and the one from *R. fruticosus* are produced in a nearly microfibrillar form (spindle-like morphology) under very similar conditions (Pelosi et al., 2003) and exhibit very similar NMR spectra, it is hard to conceive that one sample – the one from *R. fruticosus* – would correspond to single-helical chains, whereas the other – from *S. monoica* – would occur as triple-helical chains. In addition, in the case of *S. monoica*, the microfibrils are very long when the in vitro synthesis is performed at pH 6 ($\overline{DPw}$ of the order of 21,000), whereas shorter ones are observed at pH 9 ($\overline{DPw}$ of 4 600) (Pelosi et al., 2003). The microfibrils synthesized by the enzyme from *R. fruticosus* are the shortest ($\overline{DPw}$ of 2100) between the three polysaccharides synthesized by (1 → 3)-β-D-glucan synthases

Table 4
Summary of the measurements performed on the curdlan allomorphs and the (1 → 3)-β-D-glucans synthesized in vitro by detergent-solubilized synthases

Samples analyzed	$\overline{DPn}$[a] or $\overline{DPw}$[b]	Area of the 76-ppm ^{13}C-chemical shift[c]		Major inner ring (*d*-spacing in nm)
		Allomorph 1 (%)	Allomorph 2 (%)	
Hydrated curdlan (allomorph 1)	540	100	0	1.54
(1 → 3)-β-D-Glucan synthesized in vitro by a detergent-solubilized synthase from *R. fruticosus*	2100	~30	~70	1.54
(1 → 3)-β-D-Glucan synthesized in vitro at pH 9 by a detergent-solubilized synthase from *S. monoica*	4600	~20	~80	1.36
Hydrothermally annealed curdlan (allomorph 2)	ND[d]	~20	~80	1.36
(1 → 3)-β-D-Glucan synthesized in vitro at pH 6 by a detergent-solubilized synthase from *S. monoica*	21,000	~0	~100	1.36

[a] Data from Harada et al. (1966).
[b] Data from Pelosi et al. (2003).
[c] All the areas are scaled with respect to the C1 contribution.
[d] ND, not determined.

(Pelosi et al., 2003). The main difference between these samples is the amount of hydrated-like structure, which is characterized by the appearance of the 76-ppm NMR signal. There is therefore a close relationship between the $\overline{DP}$ of the (1 → 3)-β-D-glucans and the percentage of the crystallographic phase similar to the one of hydrated curdlan in the corresponding samples (Table 4). Only the sample synthesized in vitro by the enzyme from *R. fruticosus*, which exhibits the shortest $\overline{DP}$ and the shortest microfibrils (Pelosi et al., 2003), presents an inner diffraction ring near 1.54 nm. Based on these observations, we propose that our in vitro microfibrillar (1 → 3)-β-D-glucans all consist of triple helices that are more or less tightly associated in a hexagonal packing depending on the degree of polymerization, the shorter chains being less tightly bound and therefore more susceptible to inter-chain hydration especially at the microfibril ends. This phenomenon could account for the higher content of hydrated curdlan type phase (allomorph 1) detected in the shortest microfibrils (Table 4). From these observations, it can be suggested that the spray-dried curdlan of a $\overline{DPn}$ of 540 used in hydrated condition in this study is organized in swollen triple helices, where the glucan chain conformation is slightly distorted in the hexagonal packing, as proposed by Fulton and Atkins (1980) and Stipanovic and Giammatteo (1987). Our results are also in agreement with the hypothesis of Young, Dong, and Jacobs (2000) in which the use of fluorescence resonance energy transfer spectroscopy suggested that a partially opened triple helix rather than a single helix is formed upon treatment of the (1 → 3)-β-D-glucan triple helices with NaOH.

As opposed to the previous samples, the one produced by the mutated (1 → 3)-β-D-glucan endohydrolase from barley has a $\overline{DPn}$ of no more than 30–40 (Hrmova et al., 2002). The latter has a very peculiar morphology since it consists essentially of lamellar crystals made of short chains of (1 → 3)-β-D-glucan crystallized parallel to one another in a hexagonal system (Hrmova et al., 2002). These crystals yielded sharp electron diffraction diagrams (Hrmova et al., 2002) and there was a perfect match between the information resulting from these diagrams and the X-ray diagram shown in Fig. 4A. In addition to the lamellar crystals, a small amount of the sample was observed as tiny microfibrils located at the edge of the lamellar crystals (Hrmova et al., 2002). It is likely that these minor microfibrils are responsible for the weak diffraction ring near 1.54 nm (Fig. 4A) and the small resonance near 76 ppm (Fig. 3D).

5. Conclusion

The initial goal of this study was to correlate the structural characteristics of a variety of linear (1 → 3)-β-D-glucans synthesized in vitro with those of the better known curdlan prepared in different conditions. The results shown in this paper indicate that, by controlling the biosynthesis conditions, it is possible to obtain in hydrated conditions polysaccharides formed by the two types of curdlan allomorphs. In addition, the hydrated and hydrothermally annealed phases of curdlan are probably organized in triple helices where the (1 → 3)-β-D-glucan chain conformations were more or less distorted in a hexagonal packing. Further, we have identified by CP/MAS NMR spectroscopy a resonance signal corresponding to C5 as a new marker of the hydrated-type phase of curdlan. Besides the crystallographic interpretation of these differences, the presence of this NMR signal is clearly related to the degree of organization of the synthesized products. These results are of particular significance since (1 → 3)-β-D-glucans have therapeutic properties that seem to depend on a number of structural parameters, such as their conformation, helical organization, state of hydration and molecular weight (Bohn & BeMiller, 1995). The results presented here also show that in vitro syntheses can yield a range of (1 → 3)-β-D-glucans that should be most useful to identify the parameters that are crucial for the biological activities of these polysaccharides.

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