# Structural and Morphological Diversity of (1→3)-β-D-Glucans Synthesized *in Vitro* by Enzymes from *Saprolegnia monoïca*. Comparison with a Corresponding *in Vitro* Product from Blackberry (*Rubus fruticosus*)†

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ABSTRACT: Detergent extracts of microsomal fractions from *Saprolegnia monoïca* and blackberry (*Rubus fruticosus*) cells were incubated with UDP-glucose to yield *in vitro* (1→3)-β-D-glucans. The insoluble products were analyzed by conventional and cryo transmission electron microscopy, X-ray diffraction, and $^{13}C$ CP/MAS NMR, and their molecular weights were determined by light scattering experiments. All the products were microfibrillar, but for the detergent extracts from *S. monoïca*, important morphological differences were observed when the pH of the synthesizing medium was modified. At pH 6, the product had a weight average degree of polymerization ($\overline{DP}_w$) exceeding 20 000 and consisted of endless ribbon-like microfibrils. The microfibrils obtained at pH 9 had a length of only 200−300 nm, and their $\overline{DP}_w$ was ∼5000. Of all the *in vitro* (1→3)-β-D-glucans, the one from *R. fruticosus* had the shortest length and the smallest $\overline{DP}_w$. Crystallographic and spectroscopic data showed that the three *in vitro* samples consisted of triple helices of (1→3)-β-D-glucans and contained substantial amounts of water molecules in their structure, the shortest microfibrils being more hydrated. In addition, the long microfibrils from *S. monoïca* synthesized at pH 6 were more resistant toward the action of an endo-(1→3)-β-D-glucanase than the shorter ones obtained at pH 9. These results are discussed in terms of molecular biosynthetic mechanisms of fungal and plant (1→3)-β-D-glucans, and in relation with the possible existence of several (1→3)-β-D-glucan synthases in a given organism. The interpretation and discussion of these observations integrate the current knowledge of the structure and function of (1→3)-β-D-glucans.

(1→3)-β-D-Glucans are abundant polysaccharides synthesized by plants, fungi, yeasts, some algae, and bacteria, mainly as structural or storage material, but also for specialized functions such as, for instance, plant cell division, defense responses of the plant cell to microbial attack, etc. (*1*). The name (1→3)-β-D-glucan is in fact a generic name that encompasses a number of glucans containing either exclusively β-(1→3) linkages or β-(1→3) linkages together with a variable proportion of β-(1→4) or β-(1→6) glycosidic bonds. Depending on their origin, these polysaccharides are known under a number of common appellations, namely, callose, Curdlan, scleroglucan, pachyman, lentinan, laricinan, laminaran, paramylon, etc. (*1*). Several of these glucans are attracting strong interest as they not only lead to the formation of potentially useful hydrogels (*2*) but also present specific immunomodulating, antitumor, antiviral, anticoagulatory, and wound healing activities (*3*, *4*).

The biological activities of (1→3)-β-D-glucans appear to be related to several factors: a high molecular weight, a highly ordered structure, and the presence of glucosyl side units connected to the main chain by β-(1→6) linkages (*3*, *5*). The ordered structure of (1→3)-β-D-glucans is in itself quite remarkable and has been investigated in great detail for Curdlan, a linear (1→3)-β-D-glucan produced by a mutant of *Alcaligenes faecalis* (*2*). Depending on the preparation conditions, three allomorphs of Curdlan have been identified by X-ray fiber diffraction methods (*6*−*10*) and $^{13}C$ cross-polarization/magic-angle-spinning nuclear magnetic resonance (CP/MAS NMR)[1] spectroscopy (*11*−*13*). The anhydrous allomorph is obtained after the samples had been subjected to vacuum heat annealing. It is characterized by the intertwining of three glucan chains having a right-handed six-fold conformation, a fiber repeat of 0.58 nm, and a $P6_3$ space group (*8*−*10*). If the annealing is performed under hydrothermal conditions, a crystalline hydrate containing two molecules of water per glucosyl residue is obtained (*14*).

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; cmc, critical micelle concentration; CP/MAS NMR, cross-polarization/magic-angle-spinning nuclear magnetic resonance; DMSO, dimethyl sulfoxide; $\overline{DP}_n$, number average degree of polymerization; $\overline{DP}_w$, weight average degree of polymerization; TEM, transmission electron microscopy.

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This second allomorph, which is usually observed in fairly crystalline specimens, also consists of triple helices, but due to a loss of symmetry brought by the water molecules, a *P*1 space group and a fiber repeat of 1.8 nm are adopted. This allomorph occurs in nature in paramylon granules, a storage form of glucose in the alga *Euglena gracilis*. So far, paramylon is the most crystalline form of known (1→3)-β-D-glucans (*15*, *16*). A third allomorph, sometimes called "swollen Curdlan" by some authors (*13*) or "form I" by others (*17*), is obtained by dialyzing alkaline solutions of Curdlan against distilled water (*8*, *10*, *13*, *17*, *18*). This hydrated allomorph displays a fiber repeat of ∼2.3 nm. The study of its fiber diagram points toward a $7_1$ or $6_1$ helical conformation for the glucan chains. It is not clear at the moment whether this allomorph also consists of rather loose intertwined triple helices with approximately two water molecules per glucosyl residue (*10*, *13*, *19*), or is made of single helices with ∼20 molecules of water per glucosyl unit (*8*, *17*). Interestingly, (1→3)-β-D-glucans having the characteristics of swollen Curdlan seem to be quite potent in terms of biological activity (*5*).

The diversity of solid-state structures of (1→3)-β-D-glucans is reflected by the variety of the morphologies that are adopted by these polysaccharides. In the native state, microfibrillar networks are observed in the cell walls of yeasts, pollen tubes, and fungi (*20*−*24*), whereas compact and highly crystalline structures are characteristic of the intracellular paramylon granules (*15*, *16*, *25*). After dissolution in aqueous NaOH or dimethyl sulfoxide (DMSO) and reprecipitation, and depending on the molecular weight of the original sample and the preparation conditions, Curdlan yields a number of morphologies, ranging from endless microfibrils (*15*) to spindle-shaped fibrils of various lengths (*26*, *27*) or even to micron-sized platelet crystals (*28*). Relatively large amounts of microfibrillar (1→3)-β-D-glucans have also been obtained during *in vitro* synthesis experiments in the presence of UDP-glucose, using detergent extracts of microsomal fractions or membrane preparations from plants or fungi (*29*−*33*). These microfibrils correspond to linear (1→3)-β-D-glucans and have morphologies resembling those observed in some Curdlan samples with a high degree of polymerization (*28*). They consist of short spindle-like elements connected through their tips to form longer microfibrillar strings. In one case, much shorter elements have also been observed (*34*). It is worth mentioning that besides morphological observations made by transmission electron microscopy (TEM), there is almost no physicochemical characterization of *in vitro* (1→3)-β-D-glucans. Also, to our knowledge, the products synthesized *in vitro* by cell-free extracts have not been tested for their immunomodulating behavior.

Even though the molecular mechanisms responsible for the synthesis of (1→3)-β-D-glucans remain unclear, significant progress has recently been made on the characterization of (1→3)-β-D-glucan synthases. Indeed, the development of genetic and molecular biology approaches has allowed the identification of a number of genes homologous to the yeast *fks1* gene (*35*) believed to encode the catalytic subunit of the (1→3)-β-D-glucan synthase from *Saccharomyces cerevisiae*. For instance, plant homologues to *fks1* have been identified in *Arabidopsis thaliana* (*36*, *37*), *Gossypium hirsutum* (*38*), and *Nicotiana alata* (*39*). It seems that a given plant species usually contains several homologues of *fks1*, and that at least some of these homologues may correspond to different isoforms of the (1→3)-β-D-glucan synthase catalytic subunit (an update of the genes identified so far is available at http://cellwall.stanford.edu). The existence of several isoforms having specific functions in the plant cell has recently been proposed for the (1→3)-β-D-glucan synthase of *A. thaliana* (*36*). Biochemical approaches also suggest the existence of several forms of (1→3)-β-D-glucan synthase. For instance, the developmentally expressed enzyme from pollen tubes of *N. alata* is active *in vitro* in the absence of $Ca^{2+}$ and is activated by proteases (*40*) and some detergents (*41*), whereas the (1→3)-β-D-glucan synthases from other plant species are usually calcium-dependent (*30*, *42*−*45*). However, there are currently no *in vitro* experiments that clearly demonstrate the existence of several isoforms in a given plant species. It is only in the Oomycete *Saprolegnia monoïca* that the existence of two types of (1→3)-β-D-glucan synthase activities has been confirmed *in vitro* (*46*). Nevertheless, as for most *in vitro* products from plant enzymes, no physicochemical characterization of the *in vitro* (1→3)-β-D-glucans from *S. monoïca* is available.

This work is dedicated to gaining further knowledge about the structure and morphology of *in vitro* (1→3)-β-D-glucans, using cells of *S. monoïca* and blackberry (*Rubus fruticosus*) as a source of enzymes. We have taken advantage of the biochemical studies that have demonstrated the possibility of synthesizing *in vitro* amounts of (1→3)-β-D-glucans compatible with the use of physicochemical techniques that require milligrams of polymer (*30*, *31*). The other objective of our work was to show that, depending on the assay conditions, enzymes from a given organism can yield linear (1→3)-β-D-glucans that differ from one another in many respects. The two (1→3)-β-D-glucan synthase activities from *S. monoïca* were used for these investigations because they are currently the only known activities that can be turned on separately under specific pHs (*46*). Interestingly, the *in vitro* products of these enzymes differed also from the *in vitro* polysaccharide of a solubilized (1→3)-β-D-glucan synthase from *R. fruticosus* membranes. These differences are revealed by a series of chemical and physicochemical analyses that describe the macromolecular properties of these products. The properties of the *in vitro* polysaccharides are discussed not only in terms of biosynthetic processes of crystalline homopolymers of glucose but also in relation with the possible existence of several isoforms of (1→3)-β-D-glucan synthases. The current knowledge of the structure and function of (1→3)-β-D-glucans is also integrated in the discussion.

## EXPERIMENTAL PROCEDURES

*Strains and Conditions of Culture*. The strain *S. monoïca* Pringsheim 53−967 Dick was obtained from the Centraal Bureau voor Schimmel Culture (CBS, Baarn, The Netherlands). Liquid cultures were grown for 3 days in Petri dishes (14 cm diameter) containing 100 mL of the medium of Machlis (*47*) inoculated with ∼30 mycelial disks (4 mm diameter) cut from the margins of 4-day-old colonies.

Cells from *R. fruticosus* (*48*) were maintained as suspension cultures in Heller medium (*49*). They were grown at 23 °C under agitation on a rotary shaker (120 oscillations/min) and successive 12 h night and day periods. The cells

were harvested in exponential phase after being cultured for 12−16 days.

*Isolation of Membrane Fractions from S. monoïca and R. fruticosus Cells.* In the case of *S. monoïca*, mycelia grown for 3 days were harvested by filtration through filter paper. They were then mixed at 4 °C in extraction buffer [10 mM Tris-HCl (pH 7.4)] with a Waring Blendor homogenizer for two 60 s periods at maximum speed. Membrane-bound enzymes were isolated by differential centrifugation as described by Fèvre and Rougier (*50*). Pelleted membranes were resuspended in a minimum volume of extraction buffer containing 10% (v/v) glycerol. The protein content was determined by the Bradford dye binding assay (*51*), and the suspensions were diluted (extraction buffer containing 10% glycerol) to a protein concentration of 4 mg/mL. CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} was added to a final concentration of 0.5% (w/v), corresponding to the critical micelle concentration (cmc). After 45 min at 4 °C under continuous stirring, the suspensions were centrifuged at 48000*g* for 1 h. The supernatants were used as the solubilized enzyme preparations. The (1→3)-*β*-D-glucan synthase activities extracted under these conditions were stable for 16−24 h when the fractions were kept at 4 °C.

*R. fruticosus* cells were washed thoroughly at 4 °C with distilled water and resuspended in extraction buffer [1 g of cells in 1 mL of 100 mM Mops/NaOH buffer (pH 6.8) containing 2 mM EDTA and 2 mM EGTA]. They were disrupted at 4 °C using a French pressure cell (SLM-Aminco Instruments, Thermo Spectronic, Rochester, NY) at 80 MPa. The microsomal fractions were isolated by differential centrifugation following the protocol described previously (*52*). CHAPS was used at the cmc to extract the membrane-bound proteins under the conditions previously described (*30*). After detergent extraction, the preparations were centrifuged at 150000*g* for 1 h and the supernatants used as the enzyme source. The (1→3)-*β*-D-glucan synthase from *R. fruticosus* showed a constant activity after several hours when the fractions were kept at 4 °C.

*In Vitro Synthesis of (1→3)-β-D-Glucans.* For large-scale synthesis of (1→3)-*β*-D-glucans from *S. monoïca*, the solubilized enzyme preparations (15 mL) were incubated in a total volume of 55.5 mL of a reaction mixture containing 25 mM Pipes-Tris buffer (pH 6 or 9), 0.4 mM DTT, 10 mM cellobiose, and 1 mM UDP-glucose (final concentrations). The enzymatic synthesis was terminated by centrifugation at 5000*g* for 10 min, after various incubation times at 25 °C ranging from 30 min to 5 h.

To determine the yield of (1→3)-*β*-D-glucan synthesis, CHAPS-solubilized (1→3)-*β*-D-glucan synthases (200 *μ*L) were assayed in a total volume of 740 *μ*L containing 25 mM Pipes-Tris buffer (pH 6 or 9), 0.4 mM DTT, 10 mM cellobiose, 1 mM UDP-glucose, and 1.9 *μ*M UDP-D-[U-$^{14}$C]glucose (300 mCi/mmol) (final concentrations). Assays were performed in triplicate at 25 °C. The reaction was stopped after 5 h by addition of 1.5 mL of absolute ethanol, and the product was precipitated overnight at −20 °C. The insoluble material was pelleted by centrifugation at 5000*g* for 10 min, and washed three times with 1 mL of distilled water. The synthase products were finally collected by centrifugation and resuspended in 200 *μ*L of 100 mM acetate buffer (pH 5) containing either 0.5 unit of laminarinase (Sigma) or 40 units of barley endo-(1→3)-*β*-D-glucanase (*53*). An enzymatic unit was defined as the amount of enzyme releasing 5.5 *μ*mol of reducing sugar/min in the case of laminarinase, or 1 *μ*mol/min for the barley endo-(1→3)-*β*-D-glucanase. Controls were performed under the same conditions, but without addition of hydrolytic enzymes. The reaction mixtures were incubated for 16 h at 37 °C, and the enzymes were inactivated by the addition of 2 volumes of absolute ethanol. Hydrolysates were filtered through glass fiber filters (Millipore, APFA). They were successively washed with 4 mL of water and 4 mL of absolute ethanol. The glucosyl content remaining in the ethanol insoluble glucans retained on the filters was measured by scintillation counting, using 4 mL of scintillation cocktail (Amersham Biosciences). The extent of hydrolysis was calculated for each experiment by comparison with the corresponding control where no hydrolytic enzyme was used.

Large-scale synthesis of (1→3)-*β*-D-glucan from *R. fruticosus* was performed using CHAPS extracts. The enzyme preparations (15 mL) were incubated in a 60 mL mixture (total volume) containing 100 mM Mops/NaOH buffer (pH 6.8), 20 mM cellobiose, 8 mM $CaCl_2$, and 1 mM UDP-glucose. The presence of $Ca^{2+}$ cations in the reaction mixture was required for (1→3)-*β*-D-glucan synthase activity, as recently reported (*54*).

*Isolation and Purification of the Glucan Synthase Products*. The glucan synthase products from the large-scale synthesis reactions performed with enzymes from *S. monoïca* and *R. fruticosus* were collected by centrifugation at 5000*g* for 10 min. The pellets were washed twice with distilled water. Lipids and membrane-bound proteins were extracted with chloroform and methanol (2:1, v/v) at 4 °C. The products collected by centrifugation were washed with distilled water (centrifugation at 5000*g* for 10 min). The pellets were resuspended in a solution of sodium chlorite (1.7%) in 75 mM sodium acetate buffer (pH 4.5). After these suspensions had been heated for 30 min at 65 °C, the products were washed several times with distilled water and stored at 4 °C as aqueous suspensions in 0.01% (w/v) $NaN_3$.

*Liquid $^{13}$C NMR Spectroscopy of (1→3)-β-D-Glucans.* The purified *in vitro* (1→3)-*β*-D-glucans were freeze-dried and subsequently dissolved in $(CD_3)_2SO$ at a concentration of 10 mg/mL. $^{13}$C NMR spectra were recorded with a Bruker AC 300 spectrometer operated at 295 K and 75.5 MHz. The central peak of the $(CD_3)_2SO$ multiplet (39.5 ppm) was used as a reference.

*Solid-State $^{13}$C CP/MAS NMR Spectroscopy.* The suspensions containing the purified *in vitro* (1→3)-*β*-D-glucans were either dried under vacuum or analyzed in a never-dried state. In this latter case, the suspensions were centrifuged at 3000*g* for 15 min and the pellets were immediately introduced into airtight $ZrO_2$ rotors. Care was taken to check that the sample weight did not change during the NMR experiment, thus ensuring that the samples kept their initial hydration.

The NMR spectra were recorded with a Bruker Avance spectrometer operated at a $^{13}$C frequency of 100 MHz, using the combined techniques of proton dipolar decoupling (DD), magic angle spinning (MAS), and cross-polarization (CP). The cross-polarization transfer was achieved using a ramped amplitude sequence (RAMP-CP) for an optimized total contact time of 2 ms (*55*). The spinning speed was set at 6

Table 1: Enzymatic Sensitivity of the *in Vitro* (1→3)-β-D-Glucans from *S. monoïca* toward Glycoside Hydrolases[a]

| | insoluble polysaccharide[b] (nmol of glucose incorporated) | | % hydrolysis[c] | |
|---|---|---|---|---|
| hydrolytic enzyme | *S. monoïca*, pH 6 | *S. monoïca*, pH 9 | *S. monoïca*, pH 6 | *S. monoïca*, pH 9 |
| laminarinase | 11.6 ± 0.5 | 12.5 ± 0.5 | 91 ± 4 | 91 ± 4 |
| endo-(1→3)-β-D-glucanase | 92.3 ± 2.1 | 36.8 ± 1.5 | 31 ± 2 | 73 ± 4 |
| control (no enzyme) | 134.2 ± 1.9 | 139.3 ± 5.3 | 0 ± 1 | 0 ± 4 |

[a] *In vitro* synthesis experiments were performed using 200 μL of CHAPS extracts. The *in vitro* products were then submitted to enzymatic hydrolysis as described in Experimental Procedures. Average values were obtained from three different experiments. [b] Part of the *in vitro* product was resistant to the action of the hydrolytic enzymes and collected on the glass fiber filters. [c] The percentage of hydrolysis was deduced from the counting of the nondigested insoluble product with respect to that of the control sample.

kHz for all samples. A sweep width of 50 000 Hz and a recycled delay of 4 s were selected. A typical number of 10 000 scans were acquired for each spectrum. Chemical shifts were referred to tetramethylsilane after external calibration with the carbonyl signal of glycine at 176.03 ppm.

*X-ray Diffraction Analysis*. Freeze-dried *in vitro* products were hydrated for 48 h in a desiccator where a relative humidity of 95% was maintained with a saturated aqueous solution of $Na_2HPO_4 \cdot 12H_2O$. The hydrated samples were then inserted into thin-walled capillaries and sealed. They were positioned in a Wharus flat film X-ray camera mounted on a Philips 1720 X-ray generator operated at 30 kV and 20 mA with Ni-filtered Cu Kα radiation. Calcite was used for calibration.

*Transmission Electron Microscopy*. TEM observations were performed using a Philips CM200 CRYO microscope operated at 80 kV. All images were recorded on Kodak SO163 films. Purified (1→3)-β-D-glucan specimens were negatively stained with 2% uranyl acetate. In some experiments, *in vitro* synthesis was immediately followed by cryo-TEM observations. A Leica EM CPC quench-freezing device was used to prepare cryo-TEM specimens. Drops of detergent extracts, before and after *in vitro* synthesis for 30 min, were deposited on lacey carbon films supported by 300 mesh copper grids. After the excess of liquid had been blotted with filter paper, the grids were immediately plunged into liquid ethane cooled to −170 °C with liquid nitrogen. The excess of ethane was blotted away with filter paper, and the grids were then mounted on a Gatan 626 cryoholder, transferred into the microscope, and observed under low-dose conditions at 20000× using an underfocus of 1−3 μm.

*Determination of Molecular Weights from Static Light Scattering Experiments.* The purified and freeze-dried *in vitro* (1→3)-β-D-glucans were dissolved in DMSO. The solutions were filtered directly into light scattering cells through 0.2 μm nylon membranes (Pall Gelman Laboratory). The concentration of the solutions for each sample was chosen so that the experiment could be carried out under dilute regime conditions. Static light scattering (SLS) experiments were performed with a spectrometer equipped with an argon ion laser (Spectra Physics, model 2020, $\lambda = 488$ nm) and fitted with a variable-angle detection system operated by a stepping motor (ALV, Langen-Germany Instruments). The sample temperature was set at 25 ± 0.1 °C, and the scattered intensity was measured through a band-pass filter (488 nm) and a 200 μm pinhole with a photomultiplier tube (ALV).

In the SLS experiments, the excess of scattered intensity $I(q)$ from the sample was measured with respect to that of the solvent. The scattering wave vector $q$ is defined in relation to the scattering angle $\theta$, according to the following equation:

$$q = \frac{4\pi n}{\lambda}\sin\frac{\theta}{2} \quad (1)$$

where $n$ is the refractive index of the solvent (here $n = 1.4795$) and $\lambda$ is the incident wavelength of light in a vacuum. During the experiments, $\theta$ varied from 20 to 150°, corresponding to $q$ values within the range of $6 \times 10^{-4}$ to $3.2 \times 10^{-3}$ Å$^{-1}$. The absolute scattering intensities $I$ (cm$^{-1}$), i.e., the excess Rayleigh ratios, were deduced by using a toluene sample for which the Rayleigh ratio is well-known. The scattering constant $K$ is

$$K = 4\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2 / N_A \lambda^4 \quad (2)$$

where $\mathrm{d}n/\mathrm{d}c = 0.043$ cm$^3$/g (*56*) and $N_A$ is Avogadro's number. The radius of gyration ($R_G$), the second virial coefficient ($A_2$), and the weight average molecular weight ($\overline{M_w}$) were deduced following the Zimm plot method (*57*).

## RESULTS

*Biochemical and Chemical Characterization.* Table 1 presents the sensitivity toward (1→3)-β-D-glucanases of the *in vitro* (1→3)-β-D-glucans synthesized at pH 6 and 9 by the enzymes from *S. monoïca*. The measurement of the radioactivity retained on glass fiber filters before and after enzymatic hydrolysis of the two products showed that both samples were very sensitive to the action of laminarinase. Indeed, more than 90% of the *in vitro* products was hydrolyzed by this enzyme (Table 1). The extent of hydrolysis with the barley endo-β-(1→3)-D-glucanase (*53*) was much lower (30%) for the *in vitro* product synthesized at pH 6 than for the polysaccharide obtained at pH 9 (70% hydrolysis). Altogether, these data demonstrate that the *in vitro* products were newly synthesized and did not arise from the fungus cell wall. They also suggest that the *in vitro* polysaccharides correspond to (1→3)-β-D-glucans. In addition, the results presented in Table 1 indicate that the products synthesized at the two pHs are different, since one of them is more resistant than the other one to the action of the barley endo-β-(1→3)-D-glucanase.

Approximately 50 mg of purified *in vitro* products could be obtained at each pH. These substantial quantities allowed us to perform a number of morphological and structural characterizations on each sample. As the purified *in vitro* products were readily soluble in DMSO, they could be characterized by liquid-state $^{13}$C NMR spectroscopy. This characterization is exemplified in Figure 1, which shows a

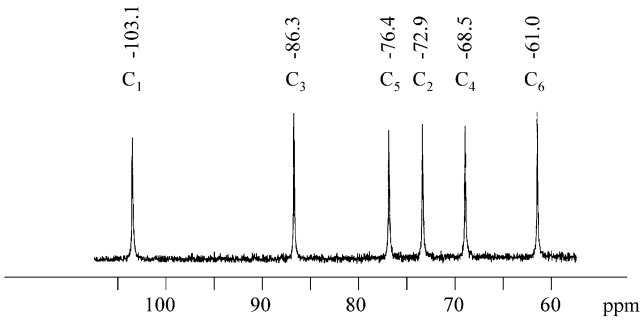


FIGURE 1: $^{13}C$ NMR spectrum of the purified *in vitro* (1→3)-*β*-D-glucan synthesized at pH 6 by the CHAPS extract from *S. monoïca*. The chemical shifts were measured at 75.5 MHz in $(CD_3)_2SO$ at 295 K, by reference to the central peak of the DMSO multiplet (39.5 ppm).

spectrum of the *in vitro* sample synthesized at pH 6. This spectrum contains six sharp peaks at 61.0, 68.5, 72.9, 76.4, 86.3, and 103.1 ppm. It is characteristic of a strictly linear (1→3)-*β*-D-glucan (*58*, *59*). Spectra identical to the one in Figure 1 were obtained for the *in vitro* product synthesized at pH 9 by the enzyme from *S. monoïca*, as well as for the *in vitro* product of the enzyme from *R. fruticosus* (data not shown). These results unequivocally demonstrate that the three polysaccharides synthesized *in vitro*, and analyzed as described in the following sections, are chemically identical and consist of strictly linear chains of glucose units linked to one another by *β*-(1→3) glycosidic bonds.

*Ultrastructural Characterization*. Cryo-TEM observations of the CHAPS extracts from *S. monoïca* revealed that these preparations consisted of vesicles having various shapes ranging from spherical to oblong, and diameters varying from 10 to 500 nm (image not shown). When these extracts were incubated with UDP-glucose, microfibrillar precipitates were readily observed after incubation for a few minutes (Figure 2). Depending on the pH of the reaction mixture, the *in vitro* products had different morphologies. Figure 2a corresponds to an enzyme preparation incubated at pH 6 for 30 min in the presence of 1 mM UDP-glucose. In addition to a few leftover vesicles, this sample contains endless microfibrillar filaments 10−30 nm in width, consisting mostly of straight segments connected to one another by rather sharp kinks. A close observation of Figure 2a reveals that the filaments are composed of loosely organized packets of subfilaments whose individual width approaches the resolution of the corresponding defocused electron micrograph, i.e., ∼3 nm. The micrograph shown in Figure 2b was recorded from a preparation identical to that used in Figure 2a, but where the pH of the synthetic reaction was set at 9. The morphology of the product is quite different from the one shown in Figure 2a. It still consists of an abundant number of filaments, but in this case, the elements are much shorter since they present a length on the order of 200−300 nm. As in Figure 2a, each individual filament appears to be composed of a number of subfilaments having a width of ∼3 nm. A sample of the *in vitro* product of the solubilized enzyme from *R. fruticosus* was also examined by cryo-TEM (image not shown). It was similar to the sample shown in Figure 2b; it consisted of filaments of a comparable width, but somewhat shorter.

The various *in vitro* (1→3)-*β*-D-glucans were also observed by TEM after purification and negative staining (Figure 3). Figure 3a corresponds to the *in vitro* (1→3)-*β*-D-glucan

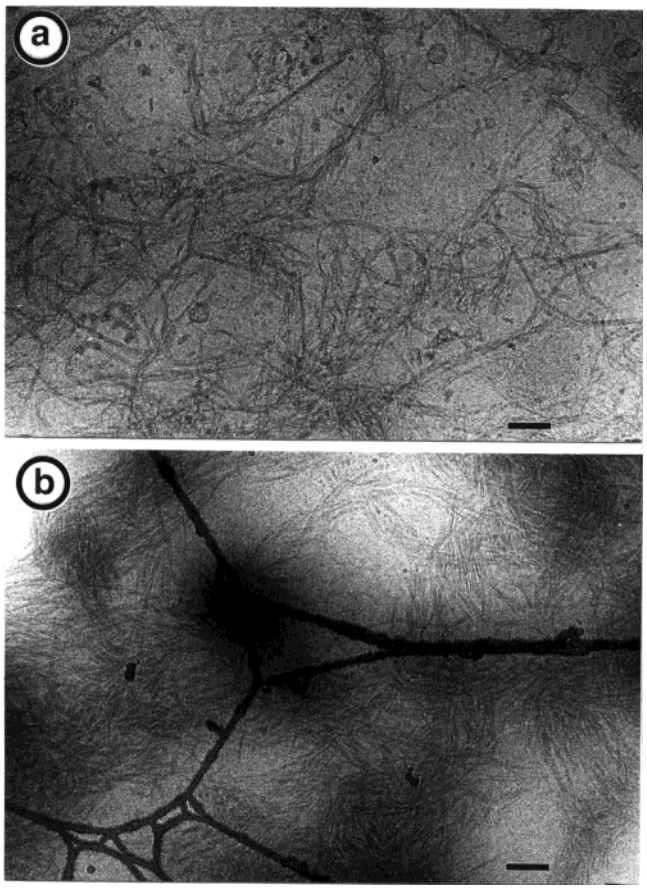


FIGURE 2: Cryo-TEM images of the *in vitro* (1→3)-*β*-D-glucans synthesized at pH 6 (a) and pH 9 (b) by CHAPS extracts from *S. monoïca*. The observations were performed after incubation of the enzyme preparations for 30 min in the presence of 1 mM UDP-glucose. The scale bars are 0.1 *μ*m long.

prepared at pH 6 using the enzyme from *S. monoïca*. As expected from the structure shown in Figure 2a, this sample contains endless ribbon-like filaments that consist of straight domains separated by sharp kinks in a crankshaft manner. In addition, some negative staining was observed within a number of filaments, clearly showing that these are composed of subfilaments (Figure 3a). A measurement of the lateral size of these substructures indicates that they are of no more than 3 nm in width.

Figure 3b corresponds to the same type of preparation as in Figure 3a, but where the pH of the synthetic mixture was set at 9. In Figure 3b, in agreement with Figure 2b, the sample consists essentially of short filaments organized as bundles of subelements as thin as those visible in Figure 3a. Figure 3c corresponds to the *in vitro* (1→3)-*β*-D-glucan synthesized by the enzyme from *R. fruticosus*. This sample has the same gross structure and ultrastructure as the one shown in Figure 3b; however, the elements appear to be somewhat shorter, and their substructure seems to be slightly less organized. The morphology of the product synthesized at pH 6 by the enzyme from *S. monoïca* (Figures 2a and 3a) was not altered when this (1→3)-*β*-D-glucan was incubated for 16 h at pH 9 (data not shown). This indicates that the morphology of the product synthesized at pH 9 is not a result of alkaline degradation of longer microfibrils.

*Diffraction and Spectroscopic Characterization*. X-ray powder diffraction diagrams of the three hydrated *in vitro* samples, together with that of paramylon, are shown in Figure

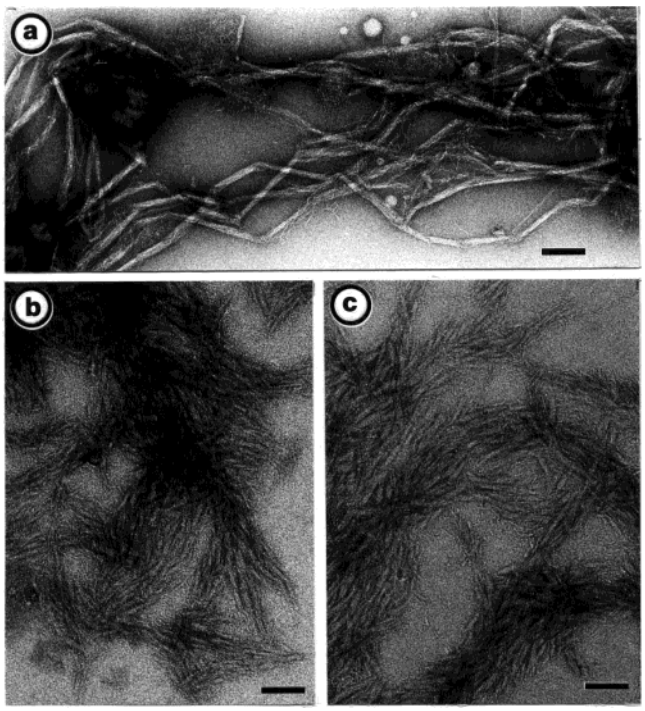


FIGURE 3: TEM images of the purified *in vitro* (1→3)-*β*-D-glucans synthesized by the enzymes from *S. monoïca* at pH 6 (a) and pH 9 (b), and by the glucan synthase from *R. fruticosus* (c). All images were recorded after negative staining of the samples with 2% uranyl acetate. The scale bars are 0.1 *μ*m long.

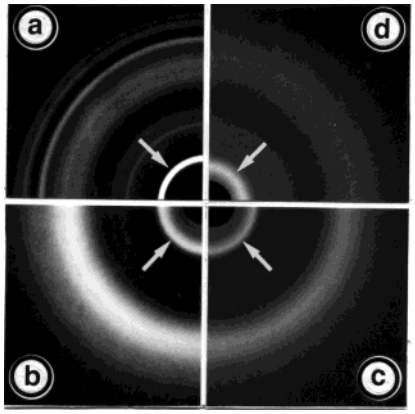


FIGURE 4: X-ray powder diagrams recorded under hydrated conditions of the paramylon standard (a), of the *in vitro* (1→3)-*β*-D-glucans synthesized by the enzymes from *S. monoïca* at pH 6 (b) and pH 9 (c), and by the glucan synthase from *R. fruticosus* (d). The arrows indicate the position of the inner rings near 1.35 nm for the diagrams shown in panels a−c and near 1.5 nm for the one in panel d.

4. As opposed to paramylon that displays a high crystallinity denoted by a number of sharp diffraction rings (Figure 4a), the three *in vitro* products were only weakly crystalline. Nevertheless, the diffraction diagrams of these three samples display a sharp inner ring centered at $d = 1.35$ nm for the two *in vitro* products from *S. monoïca* (arrows in panels b and c of Figure 4). This ring presents a higher *d* spacing (1.51 nm) in the case of the *in vitro* (1→3)-*β*-D-glucan synthesized by the enzyme from *R. fruticosus* (arrow in Figure 4d). Beside these features, the three diagrams in panels b−d of Figure 4 contain some diffuse diffraction rings at ∼0.4−0.5 nm, the reflections in Figure 4b being somewhat sharper than those in the two other samples. Interestingly,

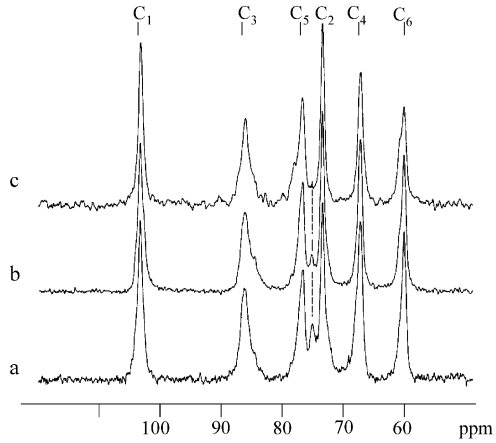


FIGURE 5: $^{13}$C CP/MAS NMR spectra recorded under hydrated conditions of the *in vitro* (1→3)-*β*-D-glucans synthesized by the enzymes from *R. fruticosus* (a) and *S. monoïca* at pH 9 (b) and pH 6 (c). The dashed vertical line shows an additional resonance signal near 76.0 ppm attributed to C5.

the strong inner ring near 1.35 nm observed in panels b and c of Figure 4 is observed at the same *d* spacing as in the spectrum of paramylon (arrow in Figure 4a). The position of this ring for paramylon (Figure 4a) is in good agreement with the data reported in the literature for this polymer (*60*).

The differences occurring in the diffraction data of the *in vitro* samples from *R. fruticosus* and *S. monoïca* are also detected in the corresponding solid-state $^{13}$C NMR spectra (Figure 5). The spectrum in Figure 5a corresponds to the *in vitro* product synthesized by the enzyme from *R. fruticosus*, whereas spectra b and c of Figure 5 were obtained from the *in vitro* (1→3)-*β*-D-glucans synthesized at pH 9 and 6, respectively, by the enzymes from *S. monoïca*. The three spectra look nearly identical and display the classical chemical shifts (Table 2) for carbon atoms of crystalline (1→3)-*β*-D-glucans (*12*). The resonance signals for C1 (103.6 ppm), C2 (74.2 ppm), C3 (86.8 ppm), C4 (68.1 ppm), C5 (77.4 ppm), and C6 (61.1 ppm) are indeed clearly visible on the spectra presented in Figure 5. In addition to these common features, an additional resonance signal near 76.0 ppm is observed with variable intensities in the three spectra (Figure 5, dashed vertical line). This resonance peak displays a substantial intensity in spectrum a of Figure 5; it becomes weaker in spectrum b of Figure 5, and it is almost absent in spectrum c of Figure 5. It is interesting to notice that such a resonance signal is totally absent in the spectra of hydrated paramylon and annealed Curdlan (*12*; Table 2).

*Molecular Weight Determination.* Figure 6 shows a typical Zimm plot corresponding to a DMSO solution of the *in vitro* (1→3)-*β*-D-glucan synthesized at pH 6 by the enzyme from *S. monoïca*. The three values $\overline{M_w}$, $R_G$, and $A_2$ can be deduced after adequate extrapolations from this plot (*57*). Plots of a similar quality were also obtained with the product from *S. monoïca* synthesized at pH 9 and from the *in vitro* (1→3)-*β*-D-glucan from *R. fruticosus* (data not shown). Table 3 summarizes the macromolecular characteristics of the three samples. The (1→3)-*β*-D-glucan synthesized at pH 6 by the enzyme from *S. monoïca* presents the highest $\overline{DP_w}$. The value obtained with this sample (20 000) is roughly 5 times higher than the one corresponding to the *in vitro* product synthesized at pH 9. The $\overline{DP_w}$ of this last product is ∼2 times higher than the one obtained with the sample from *R.*

Table 2: $^{13}C$ Chemical Shifts (parts per million) of the *in Vitro* (1→3)-β-D-Glucans Compared with Those of Paramylon and Curdlan[a]

| | *in vitro* products | | | | | |
|---|---|---|---|---|---|---|
| | *S. monoïca* | | | | | |
| | pH 6 | pH 9 | *R. fruticosus* | hydrated paramylon[b] | annealed Curdlan[b] | Curdlan gel[b] |
| C1 | 103.6 | 103.6 | 103.6 | 103.3 | 103.5 | 104 |
| C3 | 86.8 | 86.8 | 86.8 | 86.7 | 86.5 | 87.5 |
| C2 | 74.2 | 74.2 | 74.2 | 74.0 | 74.3 | 73 |
| C4 | 68.1 | 68.1 | 68.1 | 67.9 | 68.3 | 69.1 |
| C5 (major peak) | 77.4 | 77.4 | 77.4 | 78.7/77.4 | 77.5 | absent |
| C5 (minor peak) | 76.0 | 76.0 | 76.0 | absent | absent | 75.9 |
| C6 | 61.1 | 61.1 | 61.1 | 61.5 | 61.6 | 61.4 |

[a] Tetramethylsilane was used as a reference to compare the position of the resonance signals from the different samples. [b] Taken from Saito et al. (*12*).

Table 3: Macromolecular Characteristics of the *in Vitro* (1→3)-β-D-Glucans[a]

| origin of the *in vitro* (1→3)-β-D-glucan | $\overline{M_w}$ (g/mol) | $R_G$ (nm) | $A_2$ (cm$^3$ mol g$^{-2}$) | $\overline{DP_w}$ (=$\overline{M_w}$/162) |
|---|---|---|---|---|
| *R. fruticosus* | $(3.38 \pm 0.2) \times 10^5$ | $43 \pm 5$ | $(4.13 \pm 0.5) \times 10^{-4}$ | 2086 |
| *S. monoïca* (pH 9) | $(7.4 \pm 0.7) \times 10^5$ | $55.6 \pm 5$ | $(1.80 \pm 0.5) \times 10^{-4}$ | 4568 |
| *S. monoïca* (pH 6) | $(3.36 \pm 0.17) \times 10^6$ | $70 \pm 5$ | $(1.82 \pm 0.5) \times 10^{-4}$ | 20741 |

[a] $\overline{M_w}$ is the weight average molecular weight, $R_G$ the radius of gyration, $A_2$ the second virial coefficient, and $\overline{DP_w}$ the weight average degree of polymerization (162 was used as the molecular weight of one glucosyl unit).

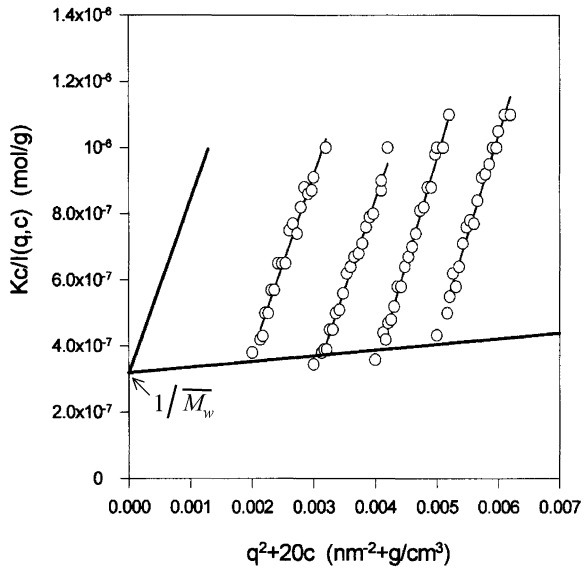


FIGURE 6: Typical Zimm plot of a DMSO solution of the *in vitro* (1→3)-β-D-glucan synthesized at pH 6 by the enzyme from *S. monoïca*. The weight average molecular weight ($\overline{M_w}$) was obtained by extrapolation to both zero concentration and zero wave vector (thick black lines).

*fruticosus* ($\overline{DP_w} \sim 2\ 000$). The radii of gyrations of the three samples follow the same trend, the one corresponding to the sample from *S. monoïca* synthesized at pH 6 being the highest and the one obtained with the product from *R. fruticosus* being the smallest. As for $A_2$, the second virial coefficient, it is nearly the same for both samples from *S. monoïca*, and substantially higher for the sample from *R. fruticosus*. For all three samples, positive small values were calculated for $A_2$.

## DISCUSSION

In the world of polysaccharides, the various morphologies adopted by linear (1→3)-β-D-glucans are quite unique since microfibrils are persistently observed not only in the native state (*20*−*23*, *61*) but also after dissolution and recrystallization (*8*, *15*, *62*). Known exceptions are the platelet crystals resulting from the crystallization of very low molecular weight Curdlan (*28*) and the paramylon granules that can be considered as wrapped-around endless microfibrils (*7*, *16*, *25*). It is believed that the systematic microfibrillar structure of recrystallized (1→3)-β-D-glucans is due to the arrangement of the (1→3)-β-D-glucan chains into triple helices (*10*, *14*). This unique organization leads to a reinforced supramolecular arrangement that is too stiff to allow the chains to fold or to adopt any of the "flexible" structures that are common to most recrystallized polymers and biopolymers. The extended chain microfibrillar morphology is therefore anticipated from recrystallization experiments with (1→3)-β-D-glucans, and the length of the resulting microfibrils is expected to be directly related to the molecular weight of the polysaccharides. In agreement with this concept, microfibrils of increasing lengths were observed when Curdlans of increasing molecular weight were recrystallized (*28*). The occurrence of Curdlan crystals of the platelet type, resulting from the crystallization of molecules for which $\overline{DP_n} = 49$, is consistent with a hexagonal parallel packing of triple helices too short to form microfibrils, but where the molecular lengths correspond more or less to the thickness of the platelets (*28*).

From this background, it seems logical that any *in vitro* (1→3)-β-D-glucan should occur as microfibrils if products with significant molecular weights are biosynthesized, and if no constraint restricts the elongation of the growing chains. Beyond this statement, it remains to be determined whether the tuning of the *in vitro* synthesis would be able to provide different sizes of microfibrils and, if so, what the implication for the morphogenesis of (1→3)-β-D-glucans would be. Another subject of interest is to know whether structures other than triple helices, and likely not fibrillar, could be obtained under specific biosynthetic conditions. The results presented in this paper give partial answers to these questions.

The most striking discovery made in this study is the contrast observed in the morphology of the *in vitro* products synthesized at pH 6 and 9 by the same detergent extracts from *S. monoïca* (Figures 2 and 3). Of both samples, the one obtained at pH 6 had the most unusual appearance since it consisted of endless filaments, as opposed to the *in vitro* product grown at pH 9, which was made of much shorter microfibrils. To our knowledge, microfibrils of (1→3)-β-D-glucan similar to those shown in Figures 2a and 3a have been observed only in yeast protoplasts (*20*, *61*), but not during *in vitro* synthesis experiments. If the morphology of the product synthesized at pH 6 is quite new for *in vitro* (1→3)-β-D-glucans, the one polysaccharide synthesized at pH 9 corresponds more to those observed for the (1→3)-β-D-glucans synthesized by the enzyme from *R. fruticosus* (Figure 3c) or from other plants (*30*, *31*) or fungi (*29*).

If we assume that in our *in vitro* products the observed structures were made of extended chains, the measured $\overline{DP}_w$ values are consistent with the microfibrillar lengths of the three *in vitro* (1→3)-β-D-glucans. Indeed, the *in vitro* product synthesized at pH 6 by the enzyme from *S. monoïca* corresponds to the longest microfibrils and to the highest $\overline{DP}_w$. Conversely, the shortest microfibrils observed in our *in vitro* preparations, i.e., the ones synthesized by the enzyme from *R. fruticosus*, correspond to the product with the lowest $\overline{DP}_w$. In the classical triple-helical structure of (1→3)-β-D-glucans, the rise per monomer unit is on the order of 0.3 nm (*10*, *14*). Thus, if the samples were monodisperse and if the triple helices were disrupted by DMSO when the light scattering experiments were performed, the length of an extended chain of (1→3)-β-D-glucan would be $\sim 20000 \times 0.3 \times 10^{-3}$ μm (6 μm) for the sample from *S. monoïca* synthesized at pH 6. On the same basis, it would be $4500 \times 0.3 \times 10^{-3}$ μm (1.5 μm) for the product synthesized at pH 9 and $2000 \times 0.3 \times 10^{-3}$ (0.6 μm) for the *in vitro* polysaccharide from *R. fruticosus*. If the triple helices were not disrupted during the molecular weight measurements, the length of the extended triple helices would be 3 times smaller, i.e., 2 μm for the sample from *S. monoïca* synthesized at pH 6, 0.5 μm for the one grown at pH 9, and 0.2 μm for the *in vitro* (1→3)-β-D-glucan from *R. fruticosus*. The lengths of the microfibrils shown in Figures 2 and 3 are in good agreement with these latter dimensions; it is therefore likely that the molecular weights given in Table 3 correspond to extended triple helices. However, the possibility that the $\overline{DP}_w$ shown in Table 3 actually corresponds to single helices cannot be ruled out. This would suggest that the apparent length of the microfibrils (Figures 2 and 3) should be smaller than expected when the products are observed by TEM. Besides these uncertainties which cannot be lifted off, a further discrepancy could be due to the existence of some polydispersity in the molecular weights of the samples. Indeed, the length of the chains, and therefore that of the microfibrils, should be related to the value of $\overline{DP}_n$ (which was not measured) and not to the value of $\overline{DP}_w$ that is deduced from light scattering experiments.

In contrast with their morphological differences, the *in vitro* products from *S. monoïca* synthesized at pH 6 and 9 presented similar structural details denoted by nearly identical X-ray diffraction patterns (Figure 4) and $^{13}C$ CP/MAS NMR spectra (Figure 5). In both samples, the occurrence of the strong X-ray ring near $d = 1.35$ nm indicates that the crystalline parts of the products are organized in the standard hexagonal crystalline (1→3)-β-D-glucan hydrate form, with triple helices 1.55 nm apart (*10*, *14*). This occurrence is supported by the analysis and comparison of the $^{13}C$ NMR spectra of our samples with those of annealed Curdlan or paramylon hydrate (*12*; Table 2). Despite the fact that our spectra exhibit peaks slightly broader than those of annealed Curdlan or paramylon hydrate, the chemical shifts for each carbon atom in our samples (Figure 5) have roughly the same values as those of the well-characterized (1→3)-β-D-glucan standards (*12*; Table 2). However, in the spectra shown in Figure 5, the additional small resonance signal near 76 ppm (dashed vertical line) is not present in the spectrum of either paramylon hydrate or annealed Curdlan, but it is observed in the spectra resulting from the so-called swollen Curdlan or "type I" (1→3)-β-D-glucans (Table 2). This resonance peak is barely observed in the *in vitro* product synthesized at pH 6 by the enzyme from *S. monoïca*, but starts to be clearly visible in the sample grown at pH 9. Its occurrence indicates that a minor part of the sample must adopt the same molecular organization as the swollen Curdlan. As aforementioned, this swollen structure is considered by some authors (*7*, *17*) to be made of (1→3)-β-D-glucan single helices in the middle of large amounts of crystallization water. In agreement with other authors (*10*, *13*), we rather believe that such structures correspond more to swollen triple helices with packing distances exceeding those of the standard (1→3)-β-D-glucan hydrate. Remarkably, the *in vitro* (1→3)-β-D-glucan synthesized by the enzyme from *R. fruticosus* presents a stronger peak near 76 ppm (Figure 5a). When analyzed by X-ray diffraction, it displayed an inner diffraction ring at $d = 1.51$ nm, which clearly indicates that the crystalline part of the sample corresponds to the swollen Curdlan type of organization (*10*, *17*). If we adopt the concept of swollen triple helices with hexagonal packing for such samples, an interhelical distance of 1.7 nm has to be considered for the diffracting part of the specimen.

The perfection of the lateral organization of the three *in vitro* (1→3)-β-D-glucans that have been characterized in this work appears to depend on their molecular weight. Indeed, the comparison between the samples from *S. monoïca* synthesized at pH 6 and 9 indicates that the *in vitro* product with the lowest molecular weight is much more sensitive to the action of the barley endo-(1→3)-β-D-glucanase, and therefore less organized, than the one synthesized at pH 6. Also, the occurrence, for the product synthesized at pH 9, of a resonance signal near 76.0 ppm in the $^{13}C$ CP/MAS NMR spectra is an indication that the sample is characterized by an excess of intracrystalline hydration, and therefore by a relatively loose lateral packing of the chains within the crystalline domains. The intensity of this resonance signal seems to be directly related to the molecular weight of the sample. Of particular interest is the case of the *in vitro* (1→3)-β-D-glucan from *R. fruticosus* which exhibits the lowest molecular weight, the strongest NMR resonance signal near 76.0 ppm, and an X-ray diffraction diagram that clearly indicates that the packing of the chains in the diffracting part of the sample is loose by comparison with the products from *S. monoïca*.

The difference between the two morphologies observed for the *in vitro* (1→3)-β-D-glucans synthesized at pH 6 and

9 by the enzymes from *S. monoïca* may be explained in several ways. A logical explanation relies on the presence of two (1→3)-*β*-D-glucan synthases, as proposed by Billon-Grand et al. (*46*). One of them would be turned on at pH 6 and the other at pH 9. It has been demonstrated that the enzyme working at pH 9 is stimulated *in vitro* by cations such as $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$, whereas the enzyme with a pH optimum of 6 is inhibited by these cations (*46*). Also, in the cytosol of fungal hyphae, the $Ca^{2+}$ concentration is known to be higher at the elongating apex than in the nongrowing parts of the hyphae (*63*, *64*). From these observations, and in agreement with the data of Girard and Fèvre (*65*), it has been proposed that the enzyme working at pH 9 would be more specifically located in the growing apical part of the hyphae, whereas the enzyme with a pH optimum of 6 would have a subapical localization (*46*). This hypothesis is in keeping with a recent report that shows that (1→3)-*β*-D-glucans are present in all regions of both elongating and nonelongating hyphae of the Oomycete *Achlya bisexualis*, a close relative of *S. monoïca* (*66*). Also, it has been shown that in *Achlya* the elongating apex lacks microfibrillar components such as cellulose (*67*). Since (1→3)-*β*-D-glucan is uniformly distributed in the wall of the hyphae (*66*), it has been suggested that, in the elongating apical zone, this polysaccharide is synthesized before cellulose (*67*). From these observations, and if we assume that the lack of well-organized microfibrillar polymers in the elongating apex is a common feature in all organisms having tip growth, one can speculate that a (1→3)-*β*-D-glucan with a rather loose organization, like the one synthesized at pH 9 by the enzyme from *S. monoïca*, will be preferentially located in the growing zone, i.e., at the apex. Conversely, a more tightly packed polysaccharide, like the one synthesized at pH 6 by the enzyme from *S. monoïca*, would be more characteristic of the nongrowing mature or subapical regions. However, care must be taken when making such speculations since the data obtained during *in vitro* experiments cannot necessarily be extrapolated to situations occurring *in vivo*.

Interestingly, the enzyme from *S. monoïca* working at pH 9 can be compared with most of the plant (1→3)-*β*-D-glucan synthases studied so far. Indeed, these enzymes can be activated *in vitro* by cations such as $Ca^{2+}$, and they synthesize products with very similar properties (*30*−*33*, *43*). In contrast, the enzyme from *S. monoïca* with a pH optimum of 6 shares a number of biochemical properties with the developmentally expressed (1→3)-*β*-D-glucan synthase from pollen tubes of *N. alata* (*40*). Indeed, both enzymes are not cation-dependent and can be activated *in vitro* by partial proteolysis (*40*, *46*, *68*). It is likely that in higher plants, as in *S. monoïca*, several (1→3)-*β*-D-glucan synthases being involved in different specific functions coexist in a given species. Even though this hypothesis has not been demonstrated using biochemical approaches, it is well-supported by the recent identification in a specific organism, for instance, in *A. thaliana*, of a number of genes that potentially encode several (1→3)-*β*-D-glucan synthases (*36*, *37*; http://cellwall.stanford.edu).

From the morphologies and the $\overline{DP_w}$ observed in our work, it can be hypothesized that the enzyme working at pH 6 would be highly processive in the sense that the glucose units would be inserted continuously from UDP-glucose into the growing (1→3)-*β*-D-glucan chains, with little or no disruption of the polymerization reaction. The other enzyme, working at pH 9, would be less processive and therefore would release the growing chain after the polymerization of a smaller number of monomers. Another interpretation of the results presented in this work would be the occurrence of a unique (1→3)-*β*-D-glucan synthase exhibiting a higher processivity at pH 6 than at pH 9. This could be explained by a weaker interaction of the enzyme with the product during polymerization at pH 9. In this case, the release of the newly synthesized polysaccharide would occur faster than in the reaction at pH 6, leading to the synthesis of (1→3)-*β*-D-glucan chains with a lower degree of polymerization and, consequently, to the synthesis of microfibrils of a different morphology. Even though this hypothesis cannot be ruled out, the recent important progress made by genetic approaches (*36*, *37*; http://cellwall.stanford.edu) rather supports the existence of several isoforms for the (1→3)-*β*-D-glucan synthase, as described in detail above.

Despite their structural and morphological differences, the samples described in our work all had in common a fibrillar morphology indicating an extended chain structure and the biosynthesis of fairly high molecular weight products. Since (1→3)-*β*-D-glucan is insoluble in the synthesizing medium, the biogenesis of (1→3)-*β*-D-glucan microfibrils must correspond to a precise and continuous mechanism based on successive events: (i) the introduction of the sugar donor into the catalytic site of the glucan synthase, (ii) the transfer of the glucose units to the growing chain, and (iii) the extrusion and precipitation of the growing chains into the medium. As for other polysaccharides such as cellulose or chitin, the biogenesis of (1→3)-*β*-D-glucan microfibrils must result from a coordinated action of a number of glucan synthases working cooperatively so that multichain microfibrils are being spun. In the case of (1→3)-*β*-D-glucan synthesized from detergent extracts, it is likely that the synthases and/or their catalytic subunits are organized as triplets within a given micelle or vesicle, since the microfibrils consist of triple helices made of parallel glucan chains. The idea that (1→3)-*β*-D-glucan synthases consist of multimeric complexes containing several catalytic subunits is supported by enzyme kinetics (*30*). Indeed, such kinetic experiments have shown the existence of an allosteric effect, more precisely of a positive homotropic cooperativity for the substrate UDP-glucose, during *in vitro* synthesis of (1→3)-*β*-D-glucan by detergent extracts from *A. thaliana* (*30*). Another feature of the biogenesis of polysaccharide microfibrils is that the enzyme catalytic sites should not be buried by the growing chains, as their entrapment would lead to a rapid blocking of the synthesis. The chain elongation must therefore be concomitant with a dynamic effect where the catalytic sites need to move backward to stay abreast of the tips of the growing microfibrils.

As opposed to the biosynthesis of (1→3)-*β*-D-glucan by synthases organized into micelles or vesicles, an attempt to biosynthesize this glucan from isolated soluble enzymes [mutants of an endo-(1→3)-*β*-D-glucanase] has recently been reported (*69*). Unlike the fibrillar morphology reported in this work, only a low-molecular weight material that consisted of platelet crystals could be synthesized by these soluble enzymes (*69*). Thus, it is likely that the soluble isolated enzymes, as opposed to synthases organized into

micelles or vesicles, became quickly buried in the growing product, and therefore were not able to undergo the movement required to spin microfibrils in the synthesizing medium. Quite remarkably, related studies on the biogenesis of cellulose and chitin have also shown that isolated synthesizing enzymes were able to yield only low-molecular weight platelet-like or spherulitic crystals (*70*−*72*). In addition, as shown in this work for (1→3)-*β*-D-glucans, cellulose or chitin microfibrils were only obtained from membrane extracts that likely contain a number of synthases (*52*, *73*). It remains to be determined whether the association of isolated synthesizing enzymes into micelles or vesicles would lead to the production of microfibrils.

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