In vitro synthesis of a crystalline (1 → 3,1 → 4)-β-D-glucan by a mutated (1 → 3,1 → 4)-β-D-glucanase from *Bacillus*

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Oligo- and poly-saccharides have a large number of important biological functions, and they occur in natural composite materials, such as plant cell walls, where they self-assemble during biosynthesis in a poorly understood manner. They can also be used for the formation of artificial composite materials with industrial applications. Fundamental and applied research in biology and nanobiotechnology would benefit from the possibility of synthesizing tailor-made oligo-/poly-saccharides. In the present paper, we demonstrate that such syntheses are possible using genetically modified glycoside hydrolases, i.e. glycosynthases. The ability of the endoglycosynthase derived from *Bacillus* (1 → 3,1 → 4)-β-D-glucanase to catalyse self-condensation of sugar donors was exploited for the *in vitro* synthesis of a regular polysaccharide. The specificity of the enzyme allowed the polymerization of α-laminaribiosyl fluoride via the formation of (1 → 4)-β-linkages to yield a new linear crystalline (1 → 3,1 → 4)-β-D-glucan with a repeating 4βG3βG unit. MS and methylation analyses indicated that the *in vitro* product consisted of a mixture of oligosaccharides, the one having a degree of poly-

merization of 12 being the most abundant. Morphological characterization revealed that the (1 → 3,1 → 4)-β-D-glucan forms spherulites which are composed of platelet crystals. X-ray and electron diffraction analyses allowed the proposition of a putative crystallographic structure which corresponds to a monoclinic unit cell with $a = 0.834$ nm, $b = 0.825$ nm, $c = 2.04$ nm and $\gamma = 90.5^\circ$. The dimensions of the ab plane are similar to those of cellulose I_β, but the length of the c -axis is nearly twice that of cellulose I. It is proposed that four glucose residues are present in an extended conformation along the c -axis of the unit cell. The data presented show that glycosynthases represent promising enzymic systems for the synthesis of novel polysaccharides with specific and controlled structures, and for the analysis *in vitro* of the mechanisms of polymerization and crystallization of polysaccharides.

Key words: carbohydrate, (1 → 3,1 → 4)-β-D-glucan, glycosynthase, polysaccharide synthesis, polysaccharide structure.

INTRODUCTION

Arabinoxylans and (1 → 3,1 → 4)-β-D-glucans are the major non-cellulosic cell wall components of the higher plants belonging to the Poaceae family, whereas xyloglucans, rhamnogalacturonans and glucomannans are present in lower amounts [1]. The occurrence of (1 → 3,1 → 4)-β-D-glucans appears to be limited to grasses [2], although a related polymer designated as lichenan is also found in certain lichens, such as *Cetraria islandica* [3]. These β-glucans are linear polymers and their degree of polymerization (DP) can be as high as 1200 [3]. They differ from one another by the proportion of (1 → 3) and (1 → 4) linkages, and therefore by the length of the mixed linked segments. The amount of (1 → 3) linkages usually does not exceed 25–30 %, which indicates that the molecules consist essentially of (1 → 3)-linked cello-oligosaccharides of a degree of polymerization of 2–4 [3–5].

The (1 → 3,1 → 4)-β-D-glucans found in the walls of cereals and some grasses are the substrates of (1 → 3,1 → 4)-β-D-glucanases, which are specific to these plant species [6]. The (1 → 3,1 → 4)-β-D-glucanases play a key role in the degradation of walls in the endosperm of germinating cereal grains [7]. Apart from cereals and grasses, a number of bacteria and fungi produce

(1 → 3,1 → 4)-β-D-glucanases, which degrade the polysaccharides present in their environment to provide a source of energy to the micro-organisms. The amino acid sequence and three-dimensional structure of the plant and microbial enzymes do not have a high degree of similarity. The plant enzymes belong to glycoside hydrolase family 17, with a (β/α)₈ barrel structure, whereas the microbial enzymes are members of family 16, with a jellyroll β-sandwich structure [8–10]. Amongst the bacterial enzymes, those from *Bacillus* species are well characterized and share high similarities, both at the protein and DNA levels (for a review, see [11]).

The hydrolytic mechanism of bacterial (1 → 3,1 → 4)-β-D-glucanases is retaining and involves a double displacement reaction assisted by a general acid/base catalysis [12]. In specific *in vitro* conditions, retaining glycosidases are also able to catalyse transglycosylation reactions to form new glycosidic bonds. This can be achieved by direct reversal of the hydrolytic reaction in the presence of a large excess of substrates (thermodynamically controlled synthesis) or by using activated donors (kinetically controlled transglycosylation) such as aryl glycosides, glycosyl fluorides and oligosaccharides as glycosyl donors [13,14]. Taking advantage of this transglycosylation activity, wild-type endoglycosidases can synthesize regular polysaccharides with high

Abbreviations used: DP, degree of polymerization; E134A, Glu¹³⁴ → Ala mutant; MALDI-TOF MS, matrix-assisted laser-desorption ionization-time-of-flight MS; SAED, selected area electron diffraction; SEM, scanning electron microscopy; TEM, transmission electron microscopy; WAXD, wide-angle X-ray diffraction.

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regio- and stereo-selectivity [15]. For instance, artificial cellulose synthesis was achieved by cellulase-catalysed polycondensation of β -cellobiosyl fluoride in acetonitrile/acetate buffer [16]. Other oligo-/poly-saccharides, such as xylan, chitin, a hybrid cellulose-xylan oligosaccharide, amylose, and (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -glucosaccharides, have also been obtained by this methodology [17–21]. However, in general, the yields of *in vitro* products hardly exceed 40 %, even in the presence of organic co-solvents.

A recent approach, which consists of using mutants of the retaining glycoside hydrolases that are depleted in the catalytic nucleophile residue, allows the synthesis of oligosaccharides with yields as high as 90 %. Such mutants are referred to as glycosynthases. Even though they were initially developed on an *exo*- β -glucosidase [22], their use was successfully extended to endoglycosidases [23]. A clear advantage of the glycosynthase reactions over transglycosylations catalysed by wild-type enzymes is that the product formed is not hydrolysed by the hydrolytically inactive mutant enzyme. Glycosynthases represent new tools for the synthesis of novel oligo-/poly-saccharides with specific and controlled structures (for reviews, see [24–26]). They are also an alternative to natural synthases for the characterization of the mechanisms of polymerization and, eventually, crystallization of polysaccharides. Indeed, glycan synthases are membrane-bound glycosyltransferases, usually organized as multiprotein complexes which are difficult to isolate in an active form and, consequently, difficult to study *in vitro*.

The glycosynthase derived from *Bacillus* (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -D-glucanase has been produced and characterized in one of our laboratories [23,27]. It was shown to catalyse efficiently condensation reactions of donor and acceptor saccharides of different degrees of polymerization. In the present work, we have exploited the donor self-condensation ability of this glycosynthase to synthesize *in vitro* a new (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -D-glucan with a regular structure that consists of glucosyl units linked by alternating (1 $\rightarrow$ 3)- β and (1 $\rightarrow$ 4)- β linkages. The *in vitro* product was characterized by NMR and methylation analysis. Its morphology was analysed by light microscopy, SEM (scanning electron microscopy) and TEM (transmission electron microscopy). Interestingly, it presented an unknown crystalline structure which was partially characterized and resolved by X-ray and electron diffraction analyses. In the present paper, the properties of the glycosynthase product are discussed in terms of biosynthetic processes of homopolymers of glucose.

EXPERIMENTAL

Preparation of the glycosynthase

The E134A (Glu¹³⁴ $\rightarrow$ Ala) mutant of the (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -D-glucanase from *Bacillus licheniformis* was expressed and purified essentially as reported in [28], with some modifications to achieve a larger scale production. *Escherichia coli* BL21-SI cells harbouring the mutated pD6-2 (E134A) plasmid were grown at 37 °C in a 8-litre bioreactor (Applikon 187) containing 4 litres of 2YT medium (64 g of bactotryptone, 40 g of yeast extract and 20 g NaCl) in sodium phosphate buffer (40 mM, pH 7.0), and ampicillin (100 μ g $\cdot$ ml⁻¹). The temperature was kept at 37 °C, and the pH maintained at 7.0 for 21 h. After centrifugation of the extracellular medium at 13 400 g for 10 min, the secreted protein was concentrated by tangential ultrafiltration (PrepScale TTF; Millipore). The protein extract was enriched by lowering the pH to 5.0 to remove insoluble *E. coli* proteins, and dialysed against sodium acetate buffer (5 mM, pH 5.5). The enzyme was purified by two chromatographic steps. The first step was a cation-exchange chromatography on HiLoad™ SP-Sepharose

Figure 1 ¹³C-NMR spectrum of the *in vitro* glycosynthase product

The chemical shifts were measured at 75.5 MHz in [²H]DMSO at 295 K. δ (p.p.m.): 103.2, 102.2 (C-1^{I,II}), 86.8 (C-3^I), 80.0 (C-4^{II}), 76.0, 74.6, 74.1, 73.2, 71.9 (C-2^{I,II}, C-3^{II}, C-5^{I,II}), 68.0 (C-4^I), 60.5, 60.0 (C-6^{I,II}).

(Amersham Biosciences) in sodium acetate buffer (5 mM) at pH 5.5. Proteins were eluted with a linear gradient of NaCl (0–0.25 M) in the same buffer, dialysed and freeze-dried. The second step was a gel-filtration chromatography on a XK-26 Superdex 75 column in sodium acetate buffer (20 mM, pH 5.8), calcium chloride (10 mM) and sodium chloride (100 mM). Protein fractions with a purity higher than 95 % as judged by SDS/PAGE were collected, dialysed against Mops (2 mM) at pH 7.0 and water, and finally freeze-dried for storage. Enzyme concentration was determined by UV spectrophotometry using an ϵ_{280} of $3.55 \cdot 10^5$ M⁻¹ $\cdot$ cm⁻¹ [29].

In vitro synthesis using the E134A glycosynthase

α -Laminaribiosyl fluoride was prepared as previously reported by reaction of laminaribiose peracetate with hydrogen fluoride in pyridine (70 %), followed by de-O-acetylation with sodium methoxide in methanol [23].

A reaction mixture consisting of phosphate buffer (100 mM, pH 7.2), α -laminaribiosyl fluoride (52 mg, 0.15 mmol), the E134A (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -D-glucanase from *B. licheniformis* (1 mg) and CaCl₂ (0.1 mM) was incubated at 35 °C. A gel was formed during the reaction. After 3 days, no donor was left as determined by measuring the fluoride anion released by a selective fluoride electrode. The reaction mixture was centrifuged at 13 400 g for 10 min, and the precipitate was thoroughly washed with water and freeze-dried to yield (4 β G3 β G)_n as a white powder (42 mg, 80 %). In a second experiment, the reaction was performed under the same conditions, except that the final reaction mixture was dialysed (3000 kDa cut-off membrane) against water.

¹³C-NMR spectroscopy

The *in vitro* product was freeze-dried and subsequently dissolved in [²H]DMSO at a concentration of 10 mg/ml. ¹³C-NMR spectra were recorded with a Bruker AC 300 spectrometer operated at 295 K and 75.5 MHz. The central peak of the [²H]DMSO multiplet (39.5 p.p.m.) was used as a reference. δ (p.p.m.): 103.2, 102.2 (C-1^{I,II}), 86.8 (C-3^I), 80.0 (C-4^{II}), 76.0, 74.6, 74.1, 73.2, 71.9 (C-2^{I,II}, C-3^{II}, C-5^{I,II}), 68.0 (C-4^I), 60.5, 60.0 (C-6^{I,II}) (Figure 1).

Methylation analysis

The glycosynthase product (1 mg) recovered after an *in vitro* synthesis performed in the conditions described above was

Scheme 1 Polycondensation reaction catalysed by the E134A (1 → 3,1 → 4)- β -D-glucanase (glycosynthase) from *Bacillus licheniformis*

permethylated and analysed for linkage type, following the alditol acetate method of Harris et al. [30]. In this procedure, the sample is successively methylated in the presence of methyl iodide, hydrolysed with trifluoroacetic acid to release the methylated monosaccharides, reduced with an excess of sodium borodeuteride, and acetylated. The alditol acetates were separated and analysed by GLC using a 30 m $\times$ 0.53 mm SP 2380 macrobore column (Supelco), with a temperature program increasing from 165 to 225 °C at a rate of 2.5 °C $\cdot$ min⁻¹. The derivatives corresponding to the GLC peaks were identified by electron-impact MS at an ionizing potential of 70 eV, using a Nermag R10-10C mass spectrometer connected to a Delsi Di 700 GC fitted with a capillary SP 2380 column.

Light microscopy

The *in vitro* product recovered after dialysis was observed using a Zeiss Axioplan 2 light microscope fitted with a polarization system equipped with a colour-sensitive plate of λ .

Electron microscopy

For SEM experiments, the freeze-dried *in vitro* product was fixed on a stub using conductive glue. It was then coated with a gold/palladium alloy by ion-sputtering and observed at an accelerating voltage of 10 kV and a working distance of 16 mm, using a JEOL JSM-6100 microscope. For TEM, the dialysed sample was dispersed briefly by sonication. The resulting suspension was deposited onto carbon-coated copper grids, and the sample was either negatively stained with 2% (w/v) uranyl acetate or shadowed with a tungsten/tantalum alloy. The observations were performed using a Philips CM200 CRYO electron microscope at an accelerating voltage of 80 kV, and SAED (selected area electron diffraction) experiments were carried out at 200 kV. The diffraction diagrams were recorded using a non-stained sample over an area 1 μ m in diameter. Gold was evaporated under vacuum on the TEM grids for calibration of SAED. Images and SAED diagrams were recorded on Eastman Kodak SO163 films.

WAXD (wide-angle X-ray diffraction)

One part of the freeze-dried *in vitro* sample was hydrated for 4 days at room temperature (25 °C) in a desiccator where a relative humidity of 95% was maintained with a saturated aqueous solution of Na₂HPO₄. Both the hydrated and non-hydrated *in vitro* products were then packed in thin-walled X-ray glass capillaries of 1 mm in diameter and sealed. They were mounted in a flat Wharfedale X-ray camera equipped with a 0.25 mm pinhole collimator. The samples were subjected to Ni-filtered Cu-K α radiations generated at 30 kV and 20 mA by a Philips 1720 generator. The X-ray diffraction diagrams were recorded at room temperature on Kodak DEF-5 films. Calibration was performed by mixing the sample with a small amount of calcite (CaCO₃) which gives a strong diffraction (100) at a *d*-spacing of 0.306 nm.

RESULTS

In vitro synthesis of the glycosynthase product

The self-condensation of α -laminaribiosyl fluoride catalysed by the E134A glycosynthase (Scheme 1) was performed at 35 °C and pH 7.2, i.e. in the conditions previously found to be optimal on the model transglycosylation reaction between α -laminaribiosyl fluoride and 4-methylumbelliferyl β -cellobioside as acceptor [23,27]. After 3 days, the reaction product formed a gel that was either recovered by centrifugation at 13400 *g* for 10 min and freeze-dried, or directly dialysed against water.

Chemical characterization of the glycosynthase product

Methylation analysis of the product synthesized *in vitro* by the mutated (1 → 3,1 → 4)- β -D-glucanase from *Bacillus* yielded three derivatives that were identified by electron-impact MS as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (50%), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (42%) and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (8%) (results not shown). This indicates that the reaction product consisted of glucose units linked by (1 → 3) and (1 → 4) linkages. The ratio between the sum of the major derivatives and the alditol acetate corresponding to the non-reducing end of the chains (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol) indicates that the glycosynthase product had a DP of 12. However, MALDI-TOF MS (matrix-assisted laser-desorption/ionization-time-of-flight MS) analysis of the *in vitro* product showed that it was actually composed of a mixture of oligosaccharides with DPs ranging from 6 to 12 (results not shown).

The *in vitro* product was characterized further by ¹³C-NMR spectroscopy (Figure 1). The results clearly confirm those obtained by methylation analysis and indicate that the glycosynthase forms (1 → 4)- β glycosidic bonds between laminaribiosyl units (δ 80.0 p.p.m., C-4^H), thus synthesizing a strictly linear polysaccharide that consists of alternating (1 → 3)- β and (1 → 4)- β glucosyl units.

Morphological characterization of the glycosynthase product

Light microscopy observations revealed that the *in vitro* product essentially consists of spherulites of a diameter ranging from 5 to 50 μ m (Figure 2). Under crossed Nicols with a colour-sensitive plate of λ , the spherulites showed a negative bi-refringency with a radial refractive index smaller than the tangential one. SEM examination confirmed the spherical morphology observed by light microscopy and revealed that the surface of the spherulites is not perfectly smooth, but is essentially porous (Figure 3). In some instances, several spherulites merged to form larger particles of a diameter as high as 100 μ m (Figures 2A and 3A). TEM observations at the edge of individual spherulites revealed the presence of platelets, which probably correspond to the elementary structures of the spherulites (Figure 4). The thickness of the platelets was estimated from shadowed samples (Figure 4C). It varied from one platelet to another in the range

Figure 2 Polarized light microscopy observation of the *in vitro* glycosynthase product at two different magnifications

1.5–11 nm. The average thickness of the platelet elements was 7.3 ± 3.5 nm.

Structural analysis by WAXD and SAED

In order to characterize further the platelet-like structures shown in Figure 4, the *in vitro* sample was analysed by WAXD (Figure 5) and SAED (Figure 6). WAXD analysis did not show any clear difference between hydrated and non-hydrated preparations of the glycosynthase product (results not shown). Consequently, the diagrams from both samples were analysed equally, assuming no hydrated structure for the *in vitro* product.

The X-ray diffraction diagram contains two strong diffraction rings whose *d*-spacings are calculated as 0.587 and 0.417 nm. Less intense reflections are found at the corresponding *d*-spacings of 1.021, 0.773, 0.371 and 0.327 nm (Figure 5). SAED

Figure 3 SEM micrographs of the *in vitro* glycosynthase product observed at two different magnifications

Figure 4 TEM images of the platelet-like structures observed at the edge of the spherulites shown in Figures 2 and 3

A) and **(B)** Observation at two different magnifications of the negatively stained (2% uranyl acetate) platelets. **(C)** As in **(A)** and **(B)**, but after shadowing with a tungsten/tantalum alloy.

Figure 5 X-ray diffraction powder diagram of the *in vitro* glycosynthase product

The arrows indicate the position of the diffraction rings corresponding to *d*-spacings of 1.021, 0.773, 0.587, 0.417 and 0.327 nm. The rings at 1.021, 0.773, 0.587 and 0.417 nm were indexed as 002, 101, $\bar{1}\bar{1}0/110$ (merged as a single reflection) and 200 respectively. The arrowhead points to the ring arising from calcite which was used for calibration.

Figure 6 SAED diagram taken from the platelet crystals shown in Figure 4

The diffraction signals on the diagram corresponding to the *d*-spacings of 0.589, 0.584 and 0.415 nm are indexed as $\bar{1}\bar{1}0$, 110 and 200 respectively.

diagrams obtained from spherulites that were partially dissociated by sonication show three different reflections corresponding to *d*-spacings of 0.589, 0.584 and 0.415 nm (Figure 6). The difference between the *d*-spacings of 0.589 and 0.584 nm is significant, indicating that these two reflections merge to give one strong signal at a *d*-spacing of 0.587 nm on the WAXD diagram (Figure 5). Also, the reflection observed at 0.417 nm on the WAXD diagram is a single reflection corresponding to that of 0.415 nm on the SAED diagram. However, the other weaker reflections observed by WAXD are not visible on the SAED diagram shown in Figure 6. The diffraction patterns in Figures 5 and 6 support the hypothesis that the elementary structure of the crystals corresponding to the glycosynthase product is a platelet such as those shown in Figure 4.

In order to determine the unit cell and space group of the platelet crystals, it is necessary to obtain diffraction diagrams from single crystals or at least from well-oriented crystals. This was not possible in the present study because the bulky spherulites that are composed of a large number of platelets were never adequate for such diffraction experiments. Moreover, trials to obtain recrystallized single crystals from solution for SAED were not successful. For these reasons, attempts were made to deduce the structure of the *in vitro* product from the WAXD powder diagram and the polycrystalline SAED diagram shown in Figures 5 and 6. Although the structure described below is preliminary, it is consistent with the data presented in this section.

When surveying the structures of known polysaccharide platelets, like for instance those corresponding to linear (1 → 3)-β-D-glucans [31,32], one sees that the plane of the platelet corresponds to the *ab* plane of the unit cell, and that the *c*-axis which coincides with the molecular axis is normal to the platelet. Although this is not necessarily always the case, we deduced the structure of the glycosynthase product on this assumption. In the SAED diagram shown in Figure 6, the signals at 0.589 and 0.584 nm can be indexed as $\bar{1}\bar{1}0$ and 110 respectively, whereas the reflection at 0.415 nm is indexed as 200. It is noteworthy that the SAED diagram contains no detectable 020 diffraction signal. Since this diagram clearly indicates that no other *hk*0 diffraction is found at an angle lower than $\bar{1}\bar{1}0$, the reflections at 1.021 and 0.773 nm observed on the WAXD diagram (Figure 5) were indexed as 002 and 101 respectively. Although more detailed diffraction data are required to assign the reflections measured at 0.371 and 0.327 nm on the WAXD diagram, the other four diffractions could be consistently indexed, as for the SAED diagram. Fitting the *d*-spacings to the *hkl* indices with a least-squares algorithm yields the following unit cell parameters: $a = 0.834 \pm 0.002$ nm, $b = 0.825 \pm 0.004$ nm, $c = 2.04 \pm 0.003$ nm, $\gamma = 90.5 \pm 0.2^\circ$. The unit cell is probably monoclinic, although it is also close to an orthorhombic system.

DISCUSSION

(1 → 3,1 → 4)-β-D-Glucans that are found in Nature usually contain 25–30% of (1 → 3)-β linked glucosyl units, the rest of the molecule consisting of (1 → 4)-β linked glucosyl moieties [3]. As a consequence, the structure of these polysaccharides essentially consists of cello-oligosaccharides, such as cellotriose and cellotetraose, linked to one another by single (1 → 3)-β glycosidic bonds. In the present study, the use of a mutated (1 → 3,1 → 4)-β-D-endoglucanase from *Bacillus* has allowed the synthesis of a saccharide in which the glucose residues are alternately linked by (1 → 3)-β and (1 → 4)-β linkages. Such a (1 → 3,1 → 4)-β-D-glucan is, to our knowledge, unique in terms of regularity of the structure. The glycosynthase methodology provides a novel tool for the regio- and stereo-selective synthesis of oligo-/poly-saccharides. Two different synthetic applications can be envisaged for endoglycosynthases: oligosaccharide synthesis by single coupling of donor and acceptor saccharides (condensation reaction where donor self-condensation and product elongation should be avoided [27]), and polysaccharide synthesis by polycondensation of the saccharide donor in the absence of any other acceptor. In the present study, we have applied this second strategy to the *in vitro* synthesis of a polysaccharide with a well-defined structure.

The chemical structure of the glycosynthase product reported in the present paper was clearly demonstrated by ^{13}C -NMR spectroscopy (Figure 1). Indeed, the spectrum obtained from the

in vitro product was characteristic of a polysaccharide that is a polymer of the repeating $4\beta\text{G}3\beta\text{G}$ unit. This regular structure is a consequence of the specificity of the enzyme which is able to accept α -laminaribiosyl fluoride both as a donor and as an acceptor to catalyse the regioselective formation of $(1 \rightarrow 4)\text{-}\beta$ glycosidic bonds. The *DP* (average *DP*) of the *in vitro* product was estimated from the methylation analysis to be about 12. However, MALDI-TOF MS analysis rather showed a mixture of oligosaccharides with *DP*s ranging from 6 to 12 (results not shown). Methylation analysis does not give any information about the polydispersity of the polymer analysed, but it allows the estimation of a *DP*. Therefore, from the MS and methylation analyses, we can conclude that the glycosynthase used generates a mixture of oligosaccharides, the most abundant in the preparation having a *DP* of about 12.

As discussed later, four glucose residues are supposed to be present in a unit cell along the *c*-axis (approx. 2 nm). Therefore one would expect to observe platelets of 2–3 nm for the chains with a *DP* of 6, whereas platelets of 6 nm thickness would correspond to the molecules with a *DP* of 12. The observed thickness of the platelets, as estimated by TEM after shadowing (Figure 4), is close to the values that can be deduced from the *DP*s obtained by methylation and MS analyses. However, the relatively wide distribution of the estimated thickness values does not rule out the possibility that some platelets actually consist of the folded-chain type of crystals in which single molecular chains in the platelet fold back into the crystallite.

Light microscopy and TEM experiments revealed that the glycosynthase product essentially corresponds to low-molecular-mass polysaccharides that form spherulites composed of lamellar elements, i.e. platelet crystals. Interestingly, it seems that soluble isolated enzymes can yield *in vitro* only a low-molecular-mass material that consists of platelet or spherulitic crystals. Indeed, such features were also reported for the *in vitro* products of two other endoglycosynthases that derived from a barley $(1 \rightarrow 3)\text{-}\beta$ -D-glucan endohydrolase [32], and from a cellulase from *Humicola insolens* [33]. Likewise, low-molecular-mass products were obtained from wild-type transglycosylation reactions used for related studies on cellulose and chitin biogenesis [34–36]. It appears that only enzymes organized in complex supramolecular structures, such as the membrane-bound cellulose and $(1 \rightarrow 3)\text{-}\beta$ -D-glucan synthases, yield *in vitro* high-molecular-mass polysaccharides with a microfibrillar morphology [37,38]. However, it remains to be determined whether or not the association of isolated enzymes, such as glycosynthases, into complex supramolecular structures would lead to the synthesis of microfibrillar high-molecular-mass polymers.

From the crystallographic analysis reported in the present paper, we can propose a monoclinic unit cell for the glycosynthase product, with the following parameters: $a = 0.834 \pm 0.002$ nm, $b = 0.825 \pm 0.004$ nm, $c = 2.04 \pm 0.003$ nm, $\gamma = 90.5 \pm 0.2^\circ$. Interestingly, the dimensions of the *ab* plane of the *in vitro* crystals seem similar to those of cellulose I_β for which the dimensions of the unit cell are $a = 0.778$ nm, $b = 0.820$ nm, $c = 1.034$ nm, $\gamma = 96.5^\circ$ [36]. Also, as for cellulose I_β [39,40], the equatorial 200 diffraction signal of the glycosynthase product has a high intensity, whereas the 020 reflection is not detectable on the SAED diagram. A helical conformation, such as the one observed for $(1 \rightarrow 3)\text{-}\beta$ -D-glucans [41,42], is unlikely. Indeed, the difference in intensity between the 200 and 020 reflections indicates that the atomic density in the crystal is more concentrated on the (200) lattice plane, as for cellulose I [39,40], strongly suggesting that each molecule of $(1 \rightarrow 3,1 \rightarrow 4)\text{-}\beta$ -D-glucan has a flat ribbon-like cross-section on its *ab* plane. In addition, the length of the

b-axis is nearly identical in crystals of cellulose I_β and the *in vitro* $(1 \rightarrow 3,1 \rightarrow 4)\text{-}\beta$ -D-glucan, suggesting a similar packing on the *bc* plane for both crystals. Thus, by analogy with the situation in cellulose I, a two-chain unit cell can be proposed for the crystal corresponding to the glycosynthase product, and intermolecular hydrogen bonds may also occur in the *bc* plane between O_3 and O_6 in the crystal.

The length of the *c*-axis proposed in the present study for the glycosynthase product (2.04 nm) is nearly twice that of cellulose I (1.034 nm) [39]. A cross-sectional view similar to that of cellulose I allows an extended conformation along the *c*-axis for the glycosynthase product, with four glucose residues in a unit cell. Constructing a $4\beta\text{G}3\beta\text{G}$ molecule by linking the cellobiose units of cellulose I with a $(1 \rightarrow 3)\text{-}\beta$ -linkage gives a *c*-axis length of about 1.94 nm corresponding to four glucose residues. Indeed, since the length of a cellobiose unit and the distance between O_3 and O_4 along the *c*-axis in cellulose I are 1.034 nm and 0.064 nm respectively [39], the length of the *c*-axis for the glycosynthase product can be calculated as follows: $(2 \times 1.034 - 2 \times 0.064) = 1.94$ nm. The difference between the observed and the calculated *c*-axis length is, at most, 0.1 nm for the glycosynthase product, which corresponds to 0.025 nm per glucose residue, i.e. to only one-sixth of the length of a typical C–C single bond (0.15 nm). Thus the hypothesis that four glucose residues are found in the unit cell along the *c*-axis seems correct. In many aspects, the molecular conformation in the crystals corresponding to the glycosynthase product is likely to be similar to that of cellulose I crystals.

The present study provides new structural information on an alternating $(1 \rightarrow 3,1 \rightarrow 4)\text{-}\beta$ -D-glucan synthesized *in vitro* by the *Bacillus* $(1 \rightarrow 3,1 \rightarrow 4)\text{-}\beta$ -D-glycosynthase. It also raises several questions and opens new perspectives. For instance, the molecular directionality of the glucan chains within the unit cell, i.e. the parallel or antiparallel organization of the chains, remains to be determined. Also, the organization of the platelet crystals within the observed spherulites needs to be investigated in detail. A finer crystallographic structure than the one proposed in the present paper will require the isolation of individual crystals by re-crystallization of the polymer from solution. Such studies are underway in our laboratory.

In conclusion, glycosynthases appear to be valuable tools to analyse *in vitro* the mechanisms of polymerization and crystallization of polysaccharides, by comparison with synthases isolated from natural membranes. The latter enzymes are indeed more difficult to study because of their complex organization and instability after extraction from the membrane environment. Glycosynthases also represent promising enzymic systems for the synthesis of novel polysaccharides with specific and controlled structures. In addition, glycosynthases are potentially useful enzymes for the preparation of new biomaterials. Indeed, glycosynthase reactions performed in the presence of other polymers should lead to the formation of composite materials owing to self-assembly of the different polymers present in the preparation during *in vitro* synthesis. Furthermore, the high yields of the glycosynthase reactions should allow a detailed analysis, for instance by using NMR techniques, of the intermolecular interactions within the newly generated biomaterials.

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