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Nonaqueous Biocatalysis

Synthesis of Cellulose In Vitro by Using a Cellulase/Surfactant Complex in a Nonaqueous Medium**

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Glycoengineering has recently attracted much attention from biological, medical, and physicochemical standpoints owing to the unique functions of sugar assemblies that essentially act in all living things.[1,2] Although various functional sugars have been synthesized to date, easy-to-make oligosaccharides have only been made in very small quantities, even with large amounts of effort.[3,4] In particular, structural polysaccharides whose regular and strong interactions are expected for such sugar assemblies are still extremely difficult to artificially synthesize.

Cellulose is a typical structural polysaccharide and the main constituent of the cell wall in higher plants. It has a very simple primary structure comprised of β-1,4-linked anhydrous D-glucose. Recently, the self-assembly and hierarchical features of cellulosic molecules have also become central attractions in the biomaterials field.[5] However, the specific intra/intermolecular hydrogen bonds and stereoregular packing of cellulose chain assemblies cause high chemical resistance, poor solubility in common solvents, and high thermal stability without any glass transition or melting points,[6] thereby leading to difficulties with ordinary synthesis methods.

Organic synthesis by cationic ring-opening polymerization of 3,6-di-*O*-benzyl-α-D-glucose-1,2,4-orthopivalate was carried out by Nakatsubo and co-workers; chemically synthesized cellulose with a degree of polymerization (DP) of approximately 20 was obtained with much effort.[7,8] Kobayashi and co-workers have reported the enzymatic synthesis of cellulose from β-D-cellobiosyl fluoride by using cellulase in an acetonitrile/acetate system.[9,10] In this approach, which is simpler than chemical synthesis, pure cellulose was prepared successfully but the DP value only reached approximately 22. This method is excellent, but is associated with two major problems, both of which inevitably stop the chain-elongation reaction. First, cellulase is gradually inactivated by acetonitrile, which is indispensable for this reaction system, and second, the cellulose produced is not at all soluble in this reaction medium.

Herein, we propose a novel approach for the synthesis in vitro of longer-chain cellulose. Our strategy is as follows: 1) utilization of a nonaqueous solvent system comprising lithium chloride in dimethylacetamide (DMAc), which dissolves cellulose to give a homogeneous condensation reaction, 2) enzymatic polymerization with cellulase for precise regio-/stereoregulation, and 3) functional preservation of cellulase in an aprotic solvent by a specific nonionic surfactant, namely dioleyl-*N*-D-glucona-L-glutamate ($2C_{18}\Delta^9GE$).[11] Furthermore, cellobiose, a structural subunit of cellulose, was used in its original (unactivated) form as a starting material.

The synthesis procedure of cellulose from cellobiose with a cellulase/surfactant (CS) complex in nonaqueous media is shown in Scheme 1. Commercial cellulase (50 mg), mainly consisting of 1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4) and 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) from the fungus *Trichoderma viride*, in 50 mM phosphate buffer solution (10 mL) and $2C_{18}\Delta^9GE$ (25 mg) in purified toluene (20 mL) were mixed. The mixture was vigorously homogenized and emulsified in a water-in-oil (W/O) state, followed by immediately freezing with liquid nitrogen and then freeze-drying in its current state for more than 24 h to obtain a CS complex.[12] Cellobiose (2.0 g) and the powder-state CS complex (75 mg) were poured into LiCl/DMAc (3.2 g:40 mL) and incubated at 37 °C for 24 h. The reaction medium appeared transparent by visual inspection during the process. The product was first deposited with chloroform, and the precipitate was thoroughly washed with acetone (three times) to remove the CS complex. Subsequently, the water-soluble portion of the product including cellobiose was thoroughly washed with distilled water. Such purification was repeated according to demand. The synthesized product was obtained as a white powder.

MALDI-TOF mass spectrometry (MS) clearly shows that the synthetic products correspond well to a sugar-linked substance, showing characteristic repeating signals at intervals that approximately correspond to the molecular weight of an anhydrous glucose molecule (Figure 1). Although residual Li ions improved the sample ionization, each MS profile was broadened and shifted to a higher molecular weight. Such anhydrous glucose intervals in the mass profile implied that the partial hydrolysis possibly occurred owing to water that was inevitably generated as the dehydration polycondensation reaction proceeded. The types of component sugars and glycosidic linkage positions were determined by carbohydrate analysis by using a partially methylated

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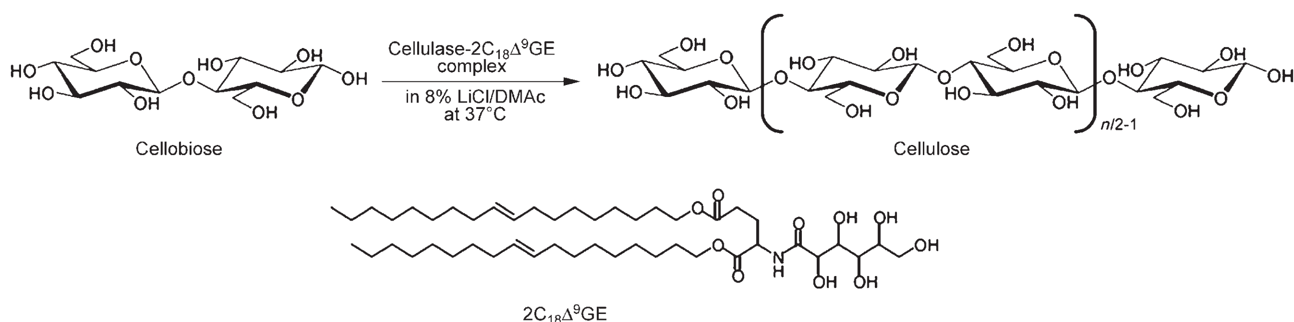

Scheme 1. Schematic representation of the synthesis in vitro of cellulose from cellobiose by using a cellulase/$2C_{18}\Delta^9$GE complex in a nonaqueous LiCl/DMAc solvent system.

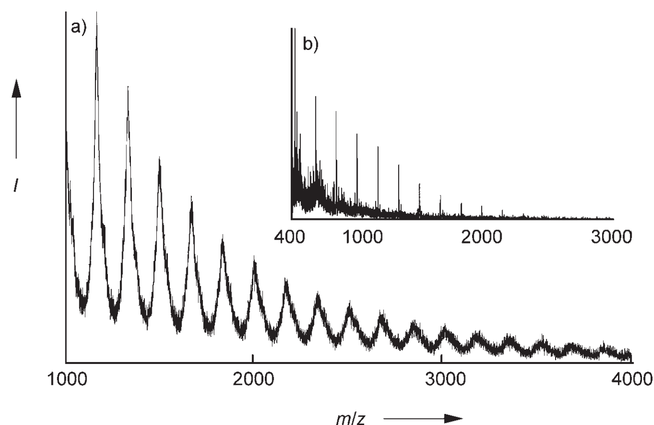

Figure 1. MALDI-TOF mass spectra of a) synthetic cellulose and b) cotton cellulose in its untreated state.

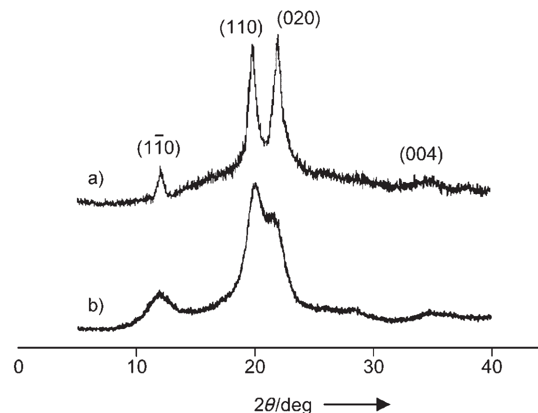

Figure 2. XRD patterns of a) synthetic cellulose and b) mercerized cotton cellulose.

alditol acetate method.[13] The product was found to only have a 1,4 linkage, and no other glucan chains were detected (data not shown). Furthermore, ^{1}H NMR spectroscopic analysis of the acetylated synthetic product revealed that the value of the coupling constant of C1–H (anomeric proton) was approximately 8.8 Hz, as calculated from the $J_{1,2}$ value of C2–H appearing at $\delta = 4.9$ ppm. This constant is comparable with that of 8.0 Hz at $\delta = 4.8$ ppm for commercial cellulose triacetate. This result strongly suggested that the synthetic product has a β-glycosidic linkage, as the $J_{1,2}$ value is approximately 3 Hz for an α-type linkage.[7] Thus, the product is presumably a homopolysaccharide consisting only of a β-1,4-linked anhydrous D-glucose, that is, cellulose.

The X-ray diffraction (XRD) patterns of the synthesized product (Figure 2) revealed a cellulose type-II crystalline structure ($2\theta = 12.2°, 19.9°, 22.0°$) with an antiparallel chain alignment.[14] Mercerized cotton cellulose also showed a cellulose type-II crystalline morphology. Both profiles were perfectly identical in their crystal lattice d spacing, and there is evidence that the enzymatic cellulose synthesis is possible even in nonaqueous media. The synthesized cellulose had a relatively higher crystallinity, apparently resulting in the narrow molecular-weight distribution.

From these results, the enzymatic polymerization of cellobiose (DP 2) to cellulose was successfully achieved by using a CS complex in a nonaqueous LiCl/DMAc solvent system for the first time. Some types of aprotic polar solvents, for example, DMAc and dimethyl sulfoxide, strongly deactivate enzymatic activity and thus were impossible to use for cellulose synthesis.[9] Nevertheless, in the present study, the enzymatic polymerization reaction proceeded by using a CS complex even under extremely severe conditions for enzymes. Therefore, this technique is expected to have a potentially wide range of applications. Furthermore, cellobiose without activation of the anomeric carbon atom at the reducing end was applicable as a starting material. Monomer design is generally an important factor for glycosyntheses,[7–10,15,16] however, in the present system no tedious precursor preparation was necessary.

Figure 3 shows the conversion rates of water-insoluble residues and their DP values. Although the yields were less than 5 % (100 mg of cellulose from 2.0 g of cellobiose), the DP values attained were over 100, representing a great deal of progress for chain elongation during the synthesis of cellulose in vitro. In this study, water-soluble cello-oligomers ($DP < 6$) were not taken into consideration. Thus, the actual yield may be higher. On the other hand, more than half of the added cellobiose was detected in its original form (data not shown). Acetylated synthetic cellulose had a DP value of approximately 60, which was determined by gel permeation chromatography (GPC). This was shorter than that obtained by viscometry; a partial decomposition and/or hydrolysis of synthesized cellulose possibly occurred during the acetylation process. At any rate, the successful synthesis of longer-chain cellulose was confirmed. In this study, commercial crude cellulase was directly used without further purification. In the

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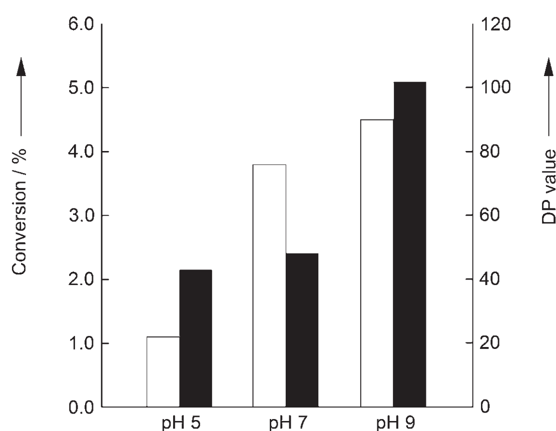

Figure 3. Conversion rates (empty bars) and DP values (filled bars) of the synthetic cellulose that is insoluble in water.

same way, when pure cellobiohydrolase from *Trichoderma viride* was applied to this system, the conversion rate reached up to approximately 20% but the DP values were less than 20.

The pH value of the water phase of the W/O emulsion during preparation of the CS complex greatly affected both the conversion rates and the DP values, even though cellulose synthesis was carried out in a nonaqueous system. Specifically, a higher pH value brought about higher conversion and longer cellulose chains. Such pH-dependent enzymatic activity has been reported in relation to the cryo-preservation of higher-order protein structure and the state of the active center.[12] In the present study, phosphate buffer solution proved effective, even in pH value regions where there is no buffering effect. Similar phenomena have been reported with regard to glycosylation with chitinase,[15,16] but the details remain to be solved and are currently under investigation.

In summary, by using our unique system, effective elongation of cellulose chains during synthesis in vitro became possible for the first time by using a CS complex as a catalyst and nonaqueous LiCl/DMAc as a reaction medium. This novel method of utilizing the substrate specificity of glycohydrolases in organic solvents allows us to precisely control the regio- and stereoselectivity and to design structural polysaccharides that are often difficult to prepare owing to their inherent self-assembling features. The major advantages are summarized as follows: 1) it is possible to make structural sugar chains that are insoluble in water and common organic solvents, 2) aprotic polar solvents, which frequently destroy enzymatic activity, are applicable, 3) cheap enzymes can be utilized and enzyme/surfactant complexes are easy to form by using a simple preparation process, 4) the synthetic products are easily obtained in a one-pot reaction process, and 5) tedious preparation of precursor sugar derivatives as starting materials by complicated chemical synthesis is unnecessary. Therefore, biocatalysts acting in organic solvents would have potentially wide applications in the glycoengineering field.

Experimental Section

XRD profiles were acquired by using Ni-filtered CuKα radiation ($\lambda = 1.5418$ Å) at a voltage of 30 kV and a current of 40 mA in the scanning range from 5–40° at a diffraction angle of 2θ, as determined by an XD-D1 system (Shimadzu Ltd.). The synthetic cellulose was first treated by thorough washing with a nitric acid solution. Cotton cellulose powder (CF1, Whatman Ltd.) was completely mercerized by immersion into 16% NaOH aqueous solution.[17]

The conversion rates were calculated by dividing the amount of water-insoluble synthetic cellulose by the amount of cellobiose supplied to the reaction system. The DP values were obtained by viscometric analysis. Briefly, the intrinsic viscosity $[\eta]$ of the cellulose molecules dissolved in 0.5 M cupriethylenediamine solution was measured by using an Ostwald-type viscometer at 27°C, and the DP values were calculated according to the Mark–Houwink–Sakurada equation: $[\eta] = 5.7\times10^{-3}$ DP. The standard deviations were within 5% for the conversion rates and 3% for the DP values.

Acetylation of synthetic cellulose was carried out according to the previous report.[18] Pyridine (0.5 mL) was added to a solution (5 mL) of synthetic cellulose (25 mg), LiCl (0.4 g), and DMAc (5 mL), and then acetic anhydride (0.7 mL) was added dropwise at 70°C for 8 h. The molecular weight of acetylated cellulose was analyzed in tetrahydrofuran by GPC with a refractive index detector in accordance with the previous report.[7] The flow rate was 1.0 mL min^{-1}. Calibration curves were obtained by using polystyrene standards.

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