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Enzymatic synthesis of cellulose II-like substance via cellulolytic enzyme-mediated transglycosylation in an aqueous medium

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ABSTRACT

The enzymatic synthesis of cellulose-like substance via a non-biosynthetic pathway has been achieved by transglycosylation in an aqueous system of the corresponding substrate, cellotriose for cellulolytic enzyme *endo*-acting endoglucanase I (EG I) from *Hypocrea jecorina*. A significant amount of water-insoluble product precipitated out from the reaction system. MALDI-TOF mass analysis showed that the resulting precipitate had a degree of polymerization (DP) of up to 16 from cellotriose. Solid-state ^{13}C NMR spectrum of the resulting water-insoluble product revealed that all carbon resonance lines were assigned to two kinds of anhydroglucose residues in the corresponding structure of cellulose II. X-ray diffraction (XRD) measurement as well as ^{13}C NMR analysis showed that the crystal structure corresponds to cellulose II with a high degree of crystallinity. We propose the multiple oligomers form highly crystalline cellulose II as a result of self-assembly via oligomer–oligomer interaction when they precipitate.

1. Introduction

Cellulose, which is the most ubiquitous and abundant biopolymer on earth, possesses a linear chain of β-(1→4)-glycosidic linked D-glucose units. This homo-polysaccharide serves as a crucial structural component in many life forms throughout the natural world.[1] Despite its relative chemical simplicity, the physical and morphological structures of native cellulose in higher plants are complex and heterogeneous.[2,3] Furthermore, cellulose chains are intimately associated with other polysaccharides and lignin in plant cell walls, resulting in even more complex morphologies.[4] Recently, the self-assembly and hierarchical features of the cellulose molecule have attracted considerable interest in the field of biomaterials.[5,6] The specific intra/intermolecular hydrogen bonds and stereoregular packing of cellulose chain assembly result in significant chemical resistance and poor solubility in commonly used solvents.[7] Hence, standard synthetic methods are unsuitable for producing the biopolymer.

So far, in vitro syntheses of cellulose using cellulose synthase (EC 2.4.1.12) as a catalyst have been reported.[8–13] These synthetic products from uridine diphosphate glucose always result in the formation of cellulose II, in which all the glycan chains adopt an anti-parallel arrangement.[14,15] Cellulose II is normally obtained by mercerization of cellulose I,[16] in which the cellulose chains adopt a parallel arrangement, or regeneration from cellulose I dissolved in solvent. The anomalous cellulose II has been also produced by microbes.[16,17] Recently, the use of a glycosyl hydrolase as catalyst for the selective synthesis of complicated polysaccharides has become a hot topic in glycotechnology.[18] The first in vitro synthesis of cellulose was reported as an enzymatic polymerization using β-cellobiosyl fluoride as substrate in an acetonitrile/acetate system.[19] This synthetic cellulose displayed the characteristic structure of cellulose II. The polymerization reaction of α-cellobiosyl fluoride catalyzed by a mutant cellulase has also been investigated.[20] As an alternative approach, the in vitro synthesis of cellulose I by means of a partially purified cellulase-catalyzed polymerization of β-cellobiosyl fluoride has also been reported.[21] However, introduction of fluoride onto anomeric center of cellobiose requires laborious synthetic process including protection and deprotection of the hydroxyl group. In contrast, the use of cellobiose or cellotriose as a substrate is attractive for the synthesis, because it is easy to obtain their oligomers by partial acid hydrolysis of cellulose. Egusa et al. have recently reported the enzymatic polymerization of cellobiose to longer-chain cellulose.[22,23] This synthesis was successfully achieved by using a cellulase/surfactant complex in a non-aqueous medium. Hiraishi et al. prepared cellooligosaccharide with an average DP of 9 by

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cellodextrin phosphorylase and the product showed a cellulose II crystalline structure.[24]

The present paper describes the enzymatic synthesis of cellotriose to cellulose II-like substance via a non-biosynthetic pathway through EG I-mediated transglycosylation in an aqueous medium. The enzyme reaction was performed by using non-modified substrate and enzyme in an aqueous system without special techniques. The resulting cellulose substance was then characterized by MALDI-TOF mass spectrometry, solid-state ^{13}C NMR spectroscopy, and XRD analyses.

2. Results

2.1. EG I-mediated transglycosylation with cellobiose and cellotriose

In the present study, EG I from *Hypocrea jecorina* was used to catalyze a transglycosylation reaction with either cellobiose or cellotriose as substrate. When cellobiose was incubated with a large amount of EG I, water-soluble oligomers (G_3~G_6) were detected by HPAEC-PAD analysis (data not shown) but no water-insoluble product was obtained. The reaction solution using cellobiose as an initial substrate at 16 h was further analyzed by MALDI-TOF mass spectrometry, which showed a DP value of 7 had been achieved (Fig. 1A). Three main peaks with *m/z* values of 689, 851, and 1013 were observed as molecular ions corresponding to $[M+Na]^+$ species of $(Hex)_4$, $(Hex)_5$, and $(Hex)_6$, respectively. A minor peak was also present with *m/z* value of 1175 as molecular ion corresponding to $(Hex)_7$. MALDI-TOF mass analysis clearly shows that the synthetic products correspond to a sugar-linked substance with repeated signals at intervals corresponding to the molecular weight of an anhydrous glucose molecule. By contrast, when the reaction was performed using cellotriose as substrate, the initial homogenous solution gradually became turbid and eventually formed a precipitate. The time required for the turbidity to reach a maximum was 1 h (data not shown). However, the aqueous suspension became completely clear with prolonged reaction time. The precipitate generated after 1 h was analyzed by MALDI-TOF mass spectrometry and the results are shown in Figure 1B. A series of peaks with a wide range of *m/z* values were observed corresponding to molecular ion $[M+Na]^+$ species of $(Hex)_{4-16}$ (i.e., up to DP 16). The precipitate produced by the EG I-mediated reaction was subsequently identified as β-(1→4)-glucan (see later results). In addition, we also analyzed the change in composition of the water-soluble fraction of the reaction mix by HPAEC-PAD (Fig. 2). The concentration of cellotetraose reached a maximum value after approximately 1 h but then the level rapidly decreased with extended reaction time. Furthermore, this sharp decrease in the concentration of cellotetraose was closely correlated with the rapid utilization of cellotriose substrate. The sequential generation of cellopentaose and cellohexaose was then observed, although the concentration of these two molecules gradually decreased with extended reaction. It is notable that cellooligomers of DP >7, which are only slightly soluble in water, were not detected under the present reaction conditions.

2.2. Preparation of cellulose-like substance

The EG I-mediated transglycosylation using cellotriose as the initial substrate gave a maximum yield of water-insoluble product after a reaction time of 1 h. When the enzyme was incubated for 1 h with cellotriose (100 mg) the initially homogenous solution gradually became turbid and eventually formed a gel-like precipitate, which was centrifuged and washed three times with 50% methanol to afford 30 mg of product. The structure of the resulting precipitate was determined by MALDI-TOF mass spectrometry and solid-state ^{13}C NMR spectroscopy. MALDI-TOF mass analysis revealed a broad distribution of products that is, up to DP 16 (Fig. 1B). The CP/MAS solid-state NMR spectrum of the precipitate was compatible with that of the structure of cellulose II, which was biosynthesized as a ^{13}C-enriched cellulose by *Acetobacter xylinum*.[25–27] From the earlier data, each signal was easily assigned to the corresponding carbon atom of the glucose unit (Fig. 3). Thus, the spectrum enabled us to assign all carbon resonance lines of two kinds of anhydroglucose residues in the structure of cellulose II as previously reported.[26] The results of the ^{13}C resonance assignment of the two-anhydroglucose residues A and B in cellulose II are listed in Table 1. These data clearly indicate that glycosidic bond formation catalyzed by EG I occurs in a regio- and stereoselective manner between glucose units during the polymerization to afford

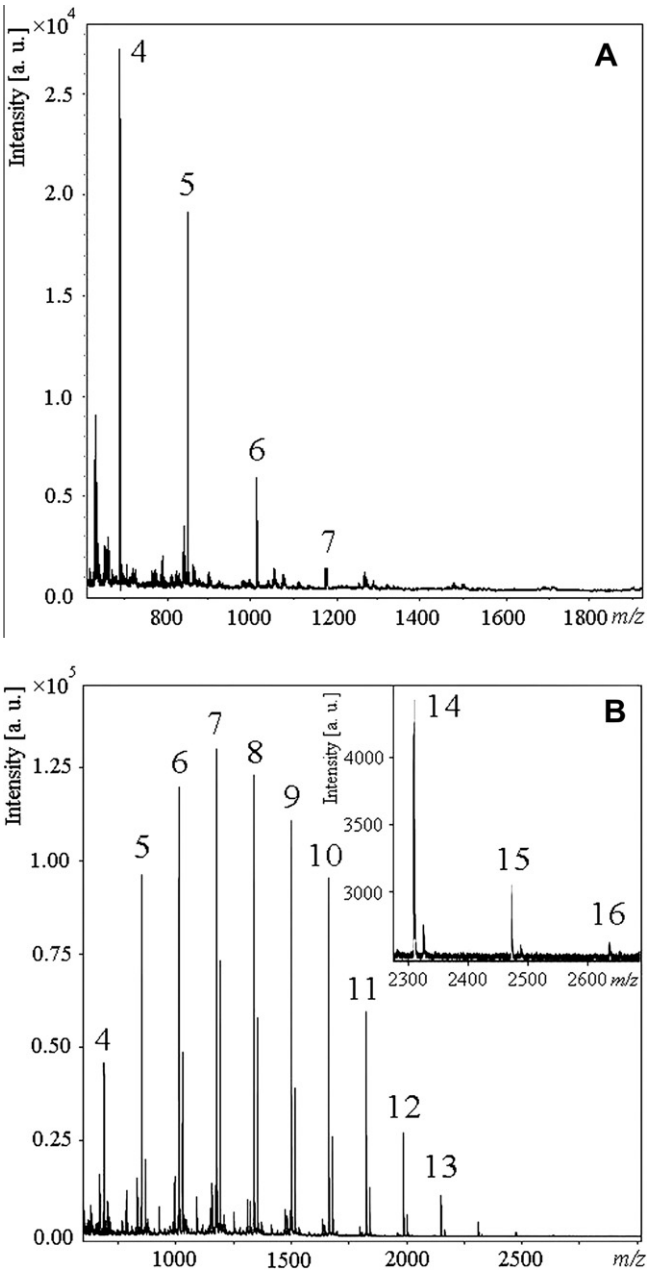


Figure 1. An EG I-mediated reaction was carried out using cellobiose or cellotriose as starting material. MALDI-TOF mass spectra of the reaction products are shown: (A) cellooligosaccharides from cellobiose and (B) a cellulose-like substance from cellotriose.

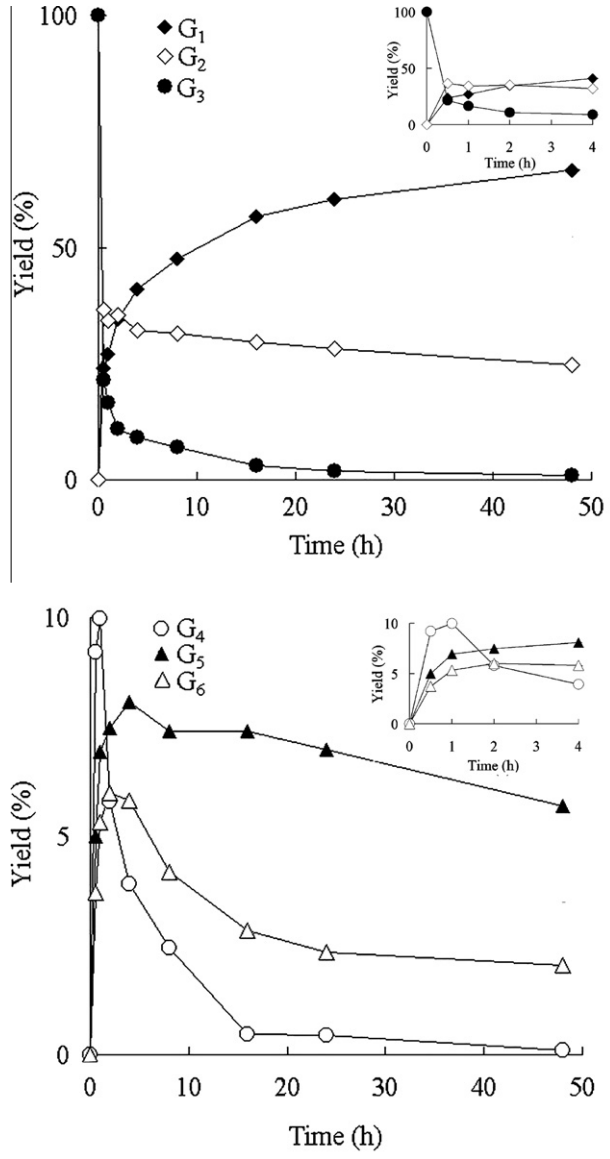


Figure 2. Time-course of products of the EG I-mediated reaction using cellotriose as substrate. Reaction conditions are described in Section 4. The water-soluble fraction of the reaction mixture was analyzed by HPAEC-PAD. G_1 and G_2–G_6 represent glucose and a series of cellooligosaccharides (DP 2∼6), respectively.

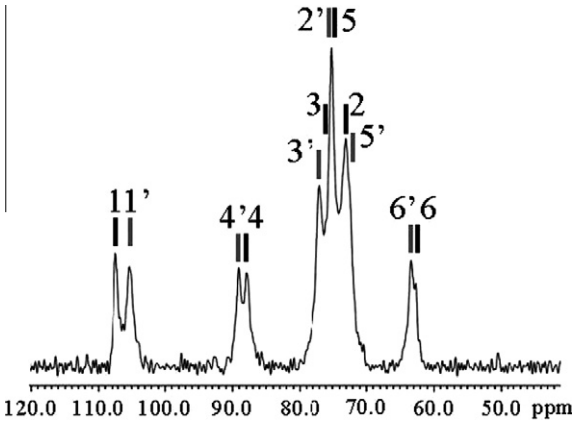


Figure 3. 1D CP/MAS ^{13}C NMR spectrum of the cellulose-like substance obtained by EG I-mediated transglycosylation using cellotriose as substrate.

Table 1
^{13}C Chemical shifts of residues A and B in the structure of cellulose II

	^{13}C Chemical shifts/ppm					
	C1	C2	C3	C4	C5	C6
Residue A	107.4	73.4	76.0	87.9	74.4	62.7
Residue B	105.3	75.2	77.0	89.1	72.6	63.4
^{13}C Chemical shift difference	2.1	−1.8	−1.0	−1.2	1.8	−0.7

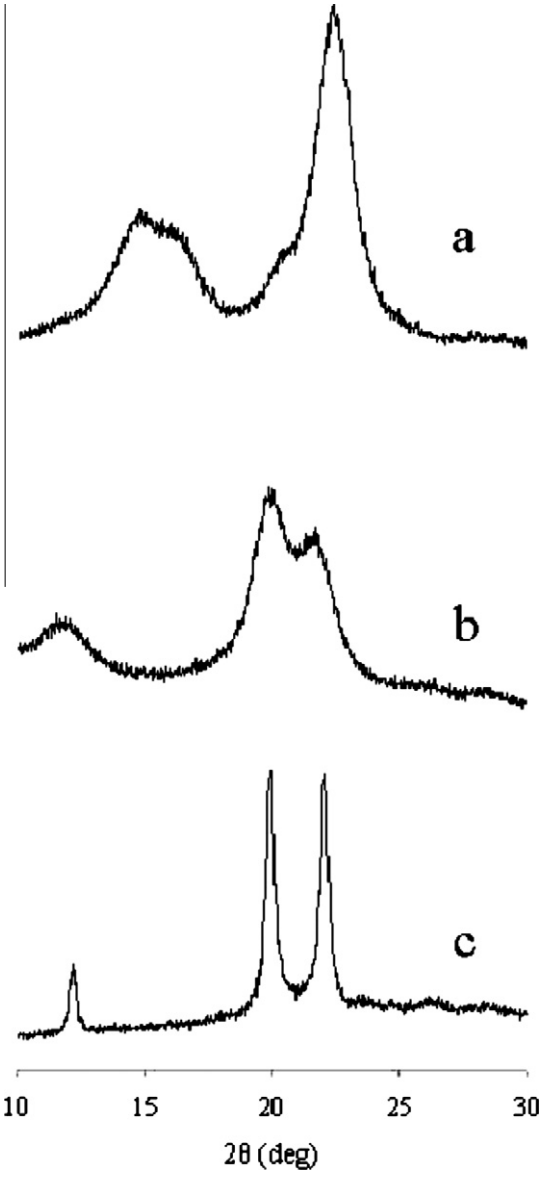


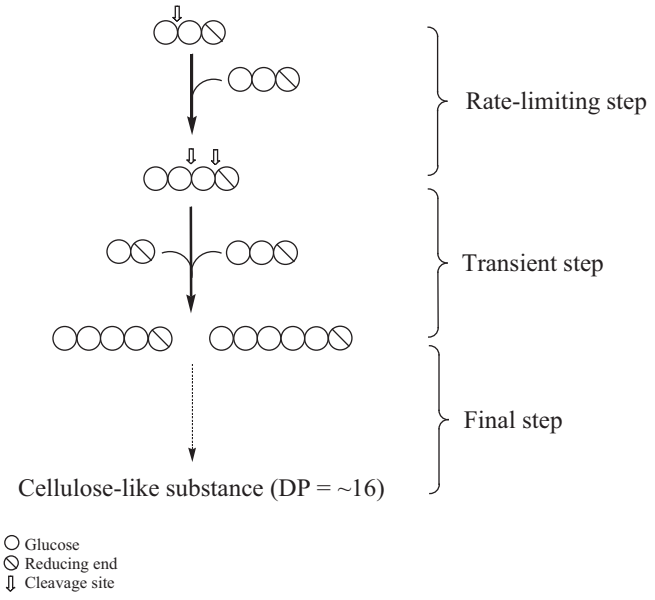
Figure 4. XRD patterns of (a) avicel, (b) mercerized cellulose and (c) cellulose-like substance.

a stereoregular cellulose product with β-(1→4) linkages. XRD analysis gave sharp peaks at $2\theta = 12.22$, 19.96 and 22.08, which are characteristic of type II cellulose (Fig. 4). The sharp peaks also indicate that the precipitate adopts a highly ordered structure with a high level of crystallinity.

3. Discussion

We have already reported that an *endo*-glycosidase from *H. jecorina* (EG I), which is a type of cellulase, catalyzes two types of

reactions, condensation and transglycosylation.[28,29] In the present study, we demonstrate an alternative approach for the in vitro synthesis of a cellulose-like substance through the enzyme-mediated transglycosylation via a non-biosynthetic route. Interestingly, the reaction of EG I with cellotriose led to the synthesis of a more extended cellooligomer that is, up to DP 16. A significant amount of water-insoluble product precipitated out from the reaction system. The results shown in Figure 2 suggest that this gradual clearing of the reaction mixture is caused by depletion of soluble lower oligomers, which are involved in the elongation reaction, and a steady hydrolysis of insoluble transglycosylation products. An outline of the chain-elongation reaction from cellotriose is shown in Scheme 1. Thus, cellotetraose formation through transfer of a glucosyl residue to cellotriose is the rate-limiting step in the overall process of transglycosylation. The accumulation of a sufficient amount of tetramer then initiates the formation of pentamer and hexamer in sequence. Biely et al. have reported that bond cleavage of [1-^{3}H]cellotriose by EG I tends to occur at the glucosidic linkage at the non-reducing end of the glucose unit to generate [1-^{3}H]cellobiose as the major product.[30] These findings suggest that the glucose unit at the non-reducing end of cellotriose, which serves as both donor and acceptor, is transferred to another cellotriose molecule to generate cellotetraose. Consequently, the sugar chain-elongation reaction from the tetrasaccharide proceeds in sequence to produce the pentamer and hexamer, which in turn act as chain carriers in a series of reactions to form heptamer or above. Finally, when higher oligomers of DP >7 are produced by transglycosylation, most of them precipitate out from the reaction system. Indeed, the hexamer displayed only about 0.4% solubility in the medium. EG I is well known to possess a more open substrate binding groove at the active site.[31] It makes possible to accommodate the resulting longer chain cellooligosaccharides. The resulting water-insoluble product was then analyzed by solid-state ^{13}C NMR. The spectrum resembled that of cellulose II, which consists of the corresponding monomers joined exclusively in a β-(1→4)-linked chain with stringent regio-/stereo selection.[25,26] Various cellulose allomorphs have been identified by their characteristic XRD patterns.[32–35] The results from XRD analysis of the water insoluble product were also consistent with a cellulose II structure displaying a high degree of crystallinity. Our results suggest that once the higher oligomers with a DP of up to 16 are produced in solution, multiple oligomeric chains spontaneously unite to form a highly ordered cellulose II-like structure as a result of self-assembly via oligomer–oligomer interactions during precipitation. Cellulose II is easily obtained by mercerization of native cellulose.[36] Kobayashi and co-workers have reported the synthesis of cellulose II having a DP of up to 22 via a transglycosylation reaction catalyzed by cellulase using β-cellobiosyl fluoride as substrate.[19] In another report, the successful synthesis of longer-chain cellulose II has also been achieved by using a cellulose/surfactant complex in non-aqueous media, possibly through reverse hydrolysis.[22,23] Hiraishi et al. have reported an enzymatic synthesis of oligosaccharides with an average DP of 9 using cellodextrin phosphorylase, which quickly forms a highly ordered cellulose II structure regardless of the length of the oligomer chains.[24]



Scheme 1. Schematic outline for EG I-mediated formation of a cellulose-like substance from cellotriose in an aqueous system.

In conclusion, one-pot synthesis of cellulose II-like substance was achieved by means of an *endo*-glucanase, cellulase-catalyzed polymerization of non-modified cellotriose in an aqueous system. The enzyme produced a significant amount of higher cellulose oligosaccharides with a DP of up to 16. In addition, once produced in a higher oligomer form, the oligosaccharides immediately assemble to adopt a cellulose II with a high level of crystallinity. The present findings may assist in determining the shape of crystals and molecular organization of higher-ordered structures as well as the mechanism for the in vivo biosynthesis of native cellulose.

4. Experimental

4.1. Materials

Cellobiose and cellotriose were kindly provided from Yaizu Suisan Kagaku Industry Co., Ltd (Shizuoka, Japan). Avicel PH-101 was a gift from Asahi Kasei Chemical Industry Co., Ltd (Tokyo, Japan). Mercerized cellulose was prepared by immersion of Avicel PH-101 into 18% NaOH aqueous solution for 5 days at 20 °C. Cellulase (GODO TCD-H3, crude enzyme) from *H. jecorina* was a kind gift from Godo Shusei Co., Ltd (Tokyo, Japan). EG I was purified by β-lactosylamidine-based affinity chromatography and ion exchange chromatography on a Mono P column (GE Healthcare, Piscataway, NJ) starting from the crude enzyme as described previously.[37] *p*-Nitrophenyl β-lactoside (Lac-β-*p*NP) was prepared by our previously described methods.[28,29] All other commonly used chemicals were obtained from commercial sources.

4.2. Analytical methods

MALDI-TOF mass spectra were obtained using on an AutoFlex (Bruker Daltonics, Bremen, Germany). The spectra were measured in the positive reflection mode with 10 mg/mL 2,5-dihydroxybenzoic acid in H_2O/ethanol 70:30 as the matrix solution. The sample solutions were mixed with the matrix solution (1:4 v/v) and then a 1 μL droplet was applied to a stainless target plate and dried at room temperature. A mass calibration procedure was employed prior to the analysis of samples using peptide calibration standard II (Bruker Daltonics). The solid-state ^{13}C NMR experiments were carried out on a Chemagnetics CMX Infinity 400 MHz (9.05 T) wide-bore magnet spectrometer operating at room temperature. A double resonance magic-angle spinning (MAS) probe equipped with a 4-mm spinning module was used. The sample was restricted to approximately 20 mg in the rotor to increase the ratio frequency field homogeneity. Spectra were acquired with cross-polarization MAS (CP/MAS) techniques using a contact time of 100 μs for artificial cellulose samples. The recycle delay was set to 4.0 s. The total number of acquisitions collected for artificial

cellulose was 50,000. Proton decoupling fields of ca. 83 kHz were employed in these experiments together with a spinning speed of 12.0 kHz. Spectra were calibrated through the methyl carbon resonance of hexamethylbenzene as an external reference at 17.35 ppm. High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis was carried out using a DIONEX DX-500 system (Dionex, Sunnyvale, CA) fitted with a PAD (ED-40) and a CarboPac PA1 column (4.0 × 250 mm). The column was pre-equilibrated in 60% (v/v) deionized water (eluent A) and 40% (v/v) 0.2 M NaOH (eluent B). Bound substance was then eluted using the following gradient: 0–40 min, linear decrease in ratio of eluent A from 60% (v/v) to 45% (v/v) with increase of eluent C (0.1 M NaOH, 1.0 M sodium acetate) from 0% (v/v) to 15% (v/v) with eluent B remaining constant at 40% (v/v); 40–50 min, 100% (v/v) eluent C; 50–60 min, 60% (v/v) eluent A and 40% (v/v) eluent B. The flow rate was 1.0 mL/min. XRD were obtained with Cu Kα from a powder XRD generator (JDX3530; Japan Electronic Organization Co. Ltd, Tokyo Japan) operating at 30 kV and 30 mA.

4.3. Enzyme assay

EG I activity was colorimetrically determined with Lac-β-*p*NP as a substrate according to our previous method.[37] A mixture containing 25 μL of 10 mM substrates Lac-β-*p*NP in 50 mM acetate buffer (pH 4.0, 50 μL volume) and an appropriate amount of enzyme (25 μL) was incubated in a 96-well microplate for 20 min at 40 °C. One-tenth of the reaction mixture was removed at 5-min intervals and immediately transferred to a microplate containing 190 μL of 1 M Na_2CO_3 to stop the reaction. The amount of *p*NP liberated was determined by measuring the absorbance at 405 nm using a microplate reader (Ultrospec Visible Plate Reader II 96, GE Healthcare Bio-Sciences, Little Chalfont, UK). One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*NP per min.

4.4. Analysis of EG I-mediated transglycosylation

Analysis of the time course of transglycosylation and characterization of the resulting products was performed by four different methods as outlined below.

4.4.1. HPAEC-PAD

To a solution (200 μL) of cellobiose (110 mg) or cellotriose (100 mg) in 50 mM acetate buffer (pH 4.0) was added 200 μL of enzyme solution (0.2 U) in the same buffer. The mixture was then incubated at 50 °C. The amount of transglycosylation product as a function of time was examined on the 0.1 mL scale. Aliquots of 5 μL were taken and mixed with 145 μL of H_2O. The reaction was then immediately quenched by heating in a boiling water bath for 15 min and the mixture was clarified by filtration through a 0.45 μm filter unit. The reaction mixture (water soluble part) was subsequently analyzed by HPAEC-PAD.

4.4.2. MALDI-TOF mass spectrometry, solid-state ^{13}C NMR spectroscopy, and XRD analysis

Synthetic artificial cellulose was directly analyzed by MALDI-TOF mass spectrometry, solid-state ^{13}C NMR spectroscopy, and XRD analysis without derivatization.

4.5. In vitro preparation of cellulose-like substances

For the preparation of cellulose, a solution (200 μL) of cellotriose (100 mg) in 50 mM acetate buffer (pH 4.0) was added to 200 μL of enzyme solution (0.2 U) in the same buffer and the mixture was maintained at 50 °C. After 1 h, the resulting insoluble substance was isolated by centrifugation and washed three times with 50% methanol to afford 30 mg of substance, which was directly applied to MALDI-TOF mass spectrometry, solid-state ^{13}C NMR spectroscopy, and XRD analysis.

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