Xylanase-Catalyzed Synthesis of a Novel Polysaccharide Having a Glucose-Xylose Repeating Unit, a Cellulose-Xylan Hybrid Polymer

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Constructing a hybrid structure from different kinds of monosaccharide units will lead to the creation of a novel class of high-performance polysaccharide derivatives. The direct incorporation of a monosaccharide unit into a natural polysaccharide backbone can, however, hardly be achieved by using the *in vivo* biosynthetic system. Only an *in vitro* approach enables us to construct the hybrid type structure, provided that the regio- and stereoselectivity of each glycosylating process is perfectly controlled. Recently, much attention has been paid to the *in vitro* synthesis of oligo- and polysaccharides catalyzed by a glycanase, the hydrolysis enzyme of polysaccharides.[1,2] Abiogenic preparation of cellulose,[3,4] xylan,[5] and chitin[6] was demonstrated by using β-cellobiosyl fluoride, β-xylobiosyl fluoride, and a chitobiose oxazoline, respectively, as monomers. These reactions proceed with complete regio- and stereoselectivity, giving rise to polysaccharides having a $\beta(1\rightarrow4)$ glycosidic bond. It has also been found that a glycanase can promote the selective polymerization of a modified cellobiosyl monomer to give a nonnatural cellulose derivative, showing an unexpectedly wide substrate specificity of glycanases.[7] These findings prompted us to prepare nonnatural hybrid type polysaccharides by using enzyme catalysis. The present paper describes the first synthesis of a cellulose-xylan hybrid polymer **2** by the enzymatic polycondensation of a new substrate monomer, β-xylopyranosyl-glucopyranosyl fluoride **1**, catalyzed by a xylanase, a hydrolytic enzyme of xylan (Scheme 1).

The new monomer **1** was prepared as follows.[8] Peracetylated xylopyranosyl bromide was coupled with benzyl 2,3,6-tri-*O*-benzyl β-D-glucopyranoside under the condition of the Koenigs−Knorr reaction to construct a disaccharide moiety.[9] The benzyl group of the resulting disaccharide derivative was removed by hydrogenolysis and the anomeric hydroxy group was converted to the fluorine atom via acetylation, bromination, fluorination, and deacetylation.[10−12] The β-orientation of the anomeric fluorine atom was designed for the formation of a reactive intermediate having an α-orientation, leading to a $\beta(1\rightarrow4)$ glycosidic bond formation via the double-displacement mechanism at the active site of the glycanase.[3,13,14]

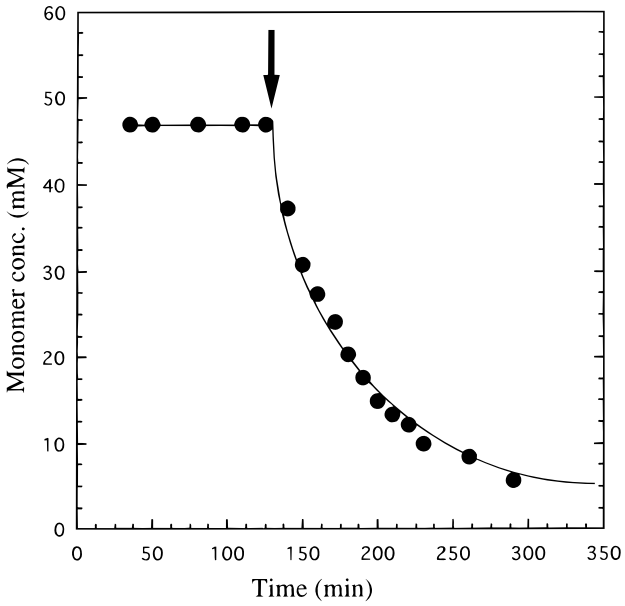

Figure 1. Xylanase-catalyzed hydrolysis (C−F bond cleavage) of β-xylopyranosyl-glucopyranosyl fluoride **1** in acetate buffer (pH 5.0). The arrow shows the addition of xylanase (*Trichoderma viride*).

Scheme 1

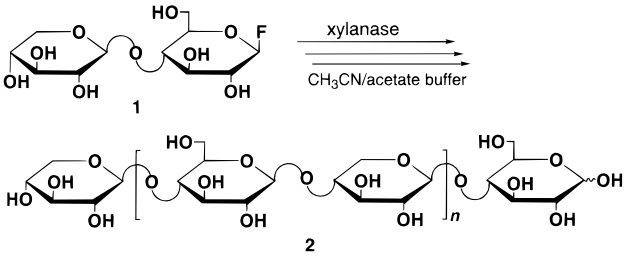

Figure 1 shows the hydrolytic behavior of **1** catalyzed by a xylanase (*Trichoderma viride*) in an acetate buffer (pH 5.0). The concentration change of **1** was followed by means of ^{19}F NMR spectroscopy.[15] Without the enzyme nothing happened. The addition of the enzyme (shown by the arrow), however, caused a rapid hydrolysis (from C−F bond to C−OH bond). These results indicate that the catalytic site of the xylanase can recognize the nonnatural substrate **1** to afford an enzyme−substrate complex that was attacked by a water molecule.

When the substrate **1** was treated with a catalytic amount of the xylanase in a mixed solvent of acetonitrile−acetate buffer (pH 5.0) (5:1) at 30 °C, the polymerization of **1** occurred smoothly

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(15) ^{19}F NMR of **1** (D_2O, C_6F_6 as external standard): 18.6 ppm (*dd*, $J_{1,F}$ = 53.2 Hz, $J_{2,F}$ = 13.9 Hz).

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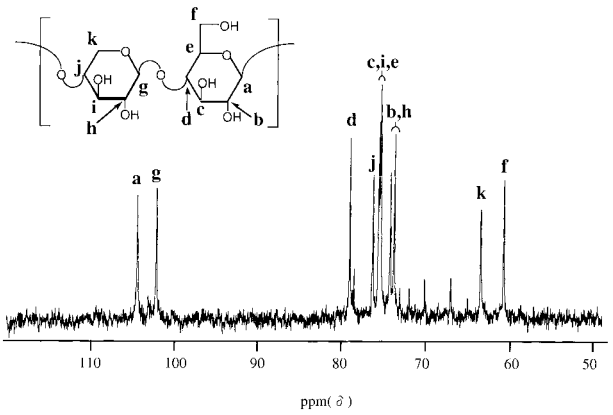

Figure 2. ^{13}C NMR spectrum of polysaccharide **2** having a xylose-gluocse repeating unit in the main chain.

to afford a white precipitate. The resulting precipitate was washed with water to remove the enzyme and low molecular weight oligomers, giving rise to the water-insoluble material.[16]

Figure 2 shows the ^{13}C NMR spectrum of the water-insoluble product **2** in 1 N NaOD/D_2O. The signals at 104.4 and 102.1 ppm are ascribable to the anomeric carbon atoms of the glucose unit and the xylose unit, respectively. The signals derived from the C4 of the glucose unit and the xylose unit were observed at 78.9 and 76.2 ppm, respectively.

These data clearly show that the regio- and stereochemistry of the glycosylating process between each disaccharide monomer are controlled perfectly, affording the $\beta(1 \rightarrow 4)$ glycosidic bond. The signals due to the methine carbon C3 (δ 85 ppm) ascribed to a $\beta(1 \rightarrow 3)$ glycosidic bond[17] could not be detected, indicating the regioselective formation of the $\beta(1 \rightarrow 4)$ linkage.

To determine the molecular weight, the product **2** was converted to the corresponding peracetylated derivative with $(CH_3CO)_2O$/pyridine-LiCl/*N*,*N*′-dimethylacetamide. The MALDI-TOF mass spectroscopy of the acetylated product shows the peaks due to each polysaccharide where polymers with the degree of polymerization (n + 1) up to 12 (24 saccharide units) were detected. The *m/z* width between peaks exactly corresponds to the molecular weight of the peracetylated disaccharide unit (504) consisting of xylose and glucose, indicating that the enzymatic polycondensation of **1** proceeds by the disaccharide unit. The transglycosylation of **1** at the glycosidic bond between the xylose moiety and the glucose moiety did not take place; no cleavage of the *O*-glycosidic bond of the disaccharide monomer **1** was observed. All of these data clearly show that the resulting polysaccharides **2** have an alternating structure of a glucose unit and a xylose unit in the main chain, a cellulose-xylan hybrid polymer. Consequently, the nonreducing monosaccharide unit of the resulting proudct **2** is a xylose unit. The existence of the xylose unit at the nonreducing end of **2** can be supported by the appearance of the small NMR signals derived from the C4 and C5 of the xylose unit at 70.0 and 66.9 ppm, respectively (Figure 2).[18]

The sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of the commercially available xylanase mixture employed for the polymerization showed many protein bands. To identify the enzyme fraction that promotes the polymerization, the xylanase mixture was purified by gel filtration chromatography, affording an active fraction that showed a single band (21.5 KDa) on the SDS−PAGE.[19] The amino acid analysis of the purified enzyme fraction clearly indicated that the amino acid sequence of the peptide fragment (from residue number 34 to 53) of the enzyme is identical with that of an *endo*-xylanase (EC 3.2.1.8) from *Trichoderma reesei*.[20] These results show that the *endo*-xylanase is a very efficient catalyst for the polycondensation of the new disaccharide monomer **1**, where both the glycosyl donor site and the glycosyl acceptor site of the *endo*-xylanase can recognize the xylose-glucose moiety, enabling the polycondensation to proceed in a completely regio- and stereoselctive manner.[21]

In conclusion, we have demonstrated for the first time that a nonnatural disaccharide monomer can be recognized by a glycanase and utilized as a useful glycosyl donor.[22] These findings will open a new method for the synthesis of various nonnatural polysaccharides in glycotechnology in the future. Further studies on the chemical and physical properties of **2** are now in progress.

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(16) A typical polymerization was as follows. The monomer **1** (5.0 mg, 0.016 mmol) and the xylanase (15 μg, 0.25 U) in 0.24 mL of a mixed solvent of acetonitrile−acetate buffer (3:1, pH 5.0) were kept standing under air at 30 °C for 2 h. The reaction mixture was then heated at 100 °C for 10 min. The reaction precipitate was washed with 0.05 mL of water two times to give the water-insoluble material, 2.9 mg, which corresponds to 58% yield determined by the phenol/sulfuric acid method.[19]

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(20) Amino acid analysis was carried out after tyrosine digestion. The amino acid sequence from the residue number 34 to 53 is as follows: Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser Tyr Trp Asn - - - Gly.

(21) It is to be noted that the purified cellulase fraction responsible for the cellulose synthesis[3b] did not show the catalytic activity for the present polymerization under similar reaction conditions.

(22) Other disaccharide monomers, e.g. β-glucopyranosyl-xylopyranosyl fluoride, also polymerize enzymatically, which shows that the combination of a disaccharide fluoride and a glycanase provides a powerful method for synthesis of nonnatural polysaccharides.