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Characterization of the Cleavage of β-Glucosidic Linkage by *Trichoderma viride* Cellulase Using Regioselectively Substituted Methylcelluloses

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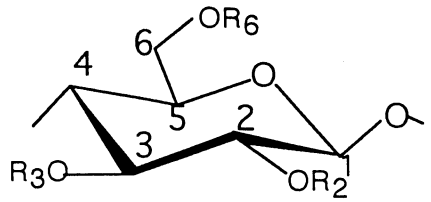
Regioselectively substituted methylcelluloses having a uniform structure, 6-*O*-methylcellulose, was degraded by the cellulase of *Trichoderma viride* while 2,3-di-*O*-methylcellulose(**2**) was not. In contrast, a substrate having a trace amount of unsubstituted units in compound **2** was depolymerized by the cellulase, suggesting that glucosidic bonds between non-substituted and 2,3-di-*O*-substituted units were selectively cleaved.

Cellulose and cellulose derivatives are known to be degraded by cellulase. The enzymatic degradation is considered as an ecologically important system that extends a possibility to apply to biodegradable materials and therefore has been studied extensively. While the glucosidic linkages of cellulose are completely cleaved to yield glucoses, in the enzymatic hydrolysis of the cellulose derivatives such as methylcellulose, carboxymethylcellulose and hydroxyethylcellulose, it has been so far reported that the chain scission is assumed to mainly occur between two or more adjacent unsubstituted anhydroglucose (AHG) units, and can also occur at a residue having a 6- or 2-substituted residue as an aglycone.[1-3] Those studies were primarily based on the investigation of the structure of the produced AHG units by the enzymatic degradation. Namely, as compounds obtained by the treatment were glucoses and oligomers all having at least an unsubstituted AHG unit, it is assumed to be essential to have an unsubstituted AHG unit in the hydrolysis by cellulase. However, this is not an enough evidence for the assumption. Because the substrates thus far used were not homogeneously substituted within an AHG unit, the structure of the neighboring AHG unit to the hydrolyzed oligomer was uncertain. In addition, because of the low yield of the scission between the unsubstituted and substituted units in heterogeneously substituted cellulose derivatives, it was difficult to detect the reaction itself. Therefore, it is necessary to have a substrate of cellulose derivatives having a uniform structure for investigating the structure of the scission point clearly and easily.

Recently, Kondo *et al.* synthesized regioselectively substituted methylcelluloses having a uniform structure.[4,5] These polymers enable us to evaluate the ability of cellulase-degradation with homogeneous cellulose derivatives. In this communication, by the examination of the enzymatic degradation of the cellulosic homopolymers we wish to show that the glucosidic linkage between the adjacent AHG units thus particularly substituted as well as that among unsubstituted AHG units can be cleaved by the cellulase of *Trichoderma viride.*

Methylcelluloses, MC3000P (Daiichi Kogyo Seiyaku. Co.) and MC 400CP (Wako Chem. Co.) were employed as commercial methylcellulose substrates. 6-*O*-Methylcellulose (**1**) and a substrate (**3**) having a trace amount of unsubstituted units in compound **2** were synthesized according to the previous papers.[4,5]



	R_2	R_3	R_6
1 : H	H	CH_3	
2 : CH_3	CH_3	H	

Substrates **1** had no unsubstituted AHG unit and the unsubstituted unit of **3** was 3%, determined by gas chromatographic analysis of alditol acetates of the acid-hydrolyzates. All substrates were analyzed by ^{13}C-NMR for identification. Meicelase (CEPB-5081:Meiji Seika) originated from *Trichoderma viride* was used as a cellulase. The concentration for a substrate was 0.29 mg/ml for **1** and 0.98 mg/ml for others in 50 mM sodium acetate beffer (pH 5.0) and 1.0% of the cellulase to the amount of the substrate was added. The solution was incubated at 37 °C. A reaction mixture was sampled after a certain period of the incubation. The extracted sample was measured by a size exclusion chromatography (SEC) with a HPLC system (Tosoh Co.) to observe the change of distribution of the molecular weight. The column for the SEC was TSK gel G2500 PWXL (Tosoh; 7.8 mm ID x 30 cm) and the eluent was 50 mM sodium acetate buffer (pH 5.0).The degraded products were also provided for the determination of the produced reducing sugar-end by Somogyi-Nelson method[6] with 30% dimethyl sulfoxide as solvent for dissolving all fractions from high to low molecular weight products.

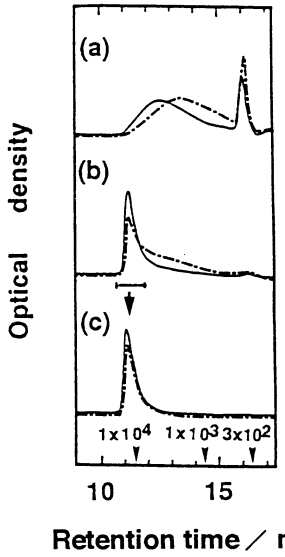


Fig. 1. Size exclusion chromatogram of methylcellulose subtrates before and after cellulase treatment. (a)**1**, (b) **3** and (c) **2**. Solid and broken lines are untreated and after 90 hours' treatment, respectively.
Numbers in the figure show the Mw corresponding to the retention time. The arrow in (b) indicates the fractionated portion for (c).

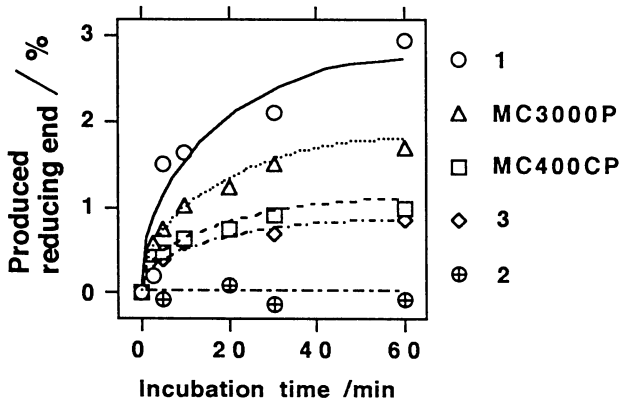


Fig. 2. Changes of reducing ends produced from various methylcelluloses by the cellulase treatment.

Produced reducing end (%) = Number of produced reducing ends X 100 / Number of glucosidic bonds of untreated substrate

The patterns of SEC in Fig. 1 clearly show the changes of distribution of molecular weights and decrease of the average molecular weights of **1** and **3** after 90 h cellulase treatment (broken line in (a) and (b), respectively). The increase of reducing ends produced from **1** and **3** also supports the depolymerization by the enzymatic hydrolysis (Fig.2). After 24 and 96 h cellulase treatment, the amounts of the reducing ends produced in substrate **1** were 3.5% and 5.1%, respectively. The enzymatic hydrolysis of **1** further proceeded slowly. As already

mentioned, compound **1** had a uniform structure and no unsubstituted AHG unit. Thus, these results indicate that the glucosidic linkage between the two 6-*O*-substituted AHG units can be cleaved. The enzymatic hydrolyzates were not monomeric sugars, but oligomers. The degree of polymerization of the oligomers was approximately 8 on the basis of Mw. This suggests that endo-type cellulase predominantly hydrolyzed the substrate **1**, and that exo-type cellulase and β-glucosidase considered to have specificities to the terminal residue did not work because of the hindrance of the methyl substituent at the 6-position hydroxyl of an AHG unit. The peak at about 16 min of the retention time was assumed to be dimer fractions including contaminants from the untreated substrate. In addition, an enzymatic reactivity to **1** was higher than that to commercial methylcelluloses as shown in Fig. 2.

Furthermore, in the SEC pattern (b) of Fig. 1 for substrate **3**, the low molecular weight fraction increased to reach 1.6% with an increase of the produced reducing ends after 96 h treatment. However, there appeared to still remain non-degraded high molecular weight fraction that was over $1x10^4$ in Mw. As compound **3** was synthesized under a homogeneous reaction condition,[5] the unsubstituted AHG units corresponding to 3% was assumed to distribute at random along a cellulosic chain. Therefore, the scission of glucosidic linkages by the cellulase could be considered that it occurred between the 2,3-di-*O*-substituted and non-substituted AHG units in stead of the cleavage between the two substituted units. To confirm the postulation, as shown in Fig. 1 (c), the apparently intact fraction with high molecular weight was fractionated repeatedly and purified to give ca. 2 in the Mw/Mn value. The obtained fraction was provided for ^{13}C-NMR measurement. Figure 3 shows the ^{13}C-NMR spectra in a region of C-1 carbon of **3** and the fractionated product. Generally the presence of an ether substituent at the C-2 position causes an upfield shift of the C-1 resonance relative to that of glucose.[7] In the spectrum of **3**, a main signal was 101.1 ppm due to C-1 carbon of 2,3-di-*O*-substituted AHG unit of **2**. Besides, there appeared two signals of 102.5 and 101.8 ppm due to C-1 carbon of unsubstituted AHG and non-identified one, respectively. After the fractionation, the two signals disappeared and only a main signal corresponding to the same one of **3** appeared. Consequently, the fractionated compound was identified as completely methylated fraction at the C-2 and C-3 positions in **2**. As shown in Fig. 1 (c) and Fig. 2, the substrate **2** was intact before and after the cellulase treatment, suggesting that the cellulase has no activity against **2**. These results show that while the cleavage of the glucosidic bond between the two AHG units in compound **2** was completely inhibited by the substitution at the C-2 and C-3 positions, the scission of the bond occurred when the adjacent AHG unit to the 2,3-substituted unit was unsubstituted.

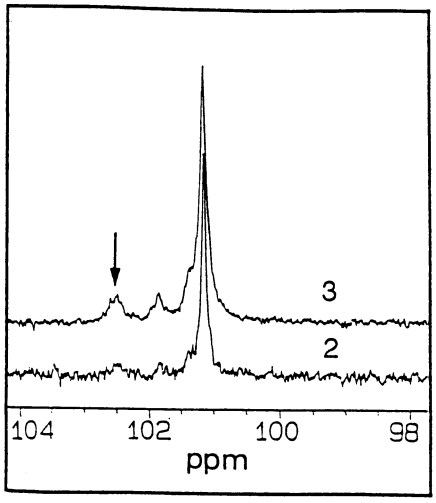


Fig. 3. ^{13}C-NMR spectra with a range of C-1 carbon of the AHG unit of compound **3** and **2**.
The arrow indicates C-1 carbon signal of the unsubstituted AHG unit.

There seems to be some inhibiting factors on the subtrate **2**. An effect of the molecular structure is among them. The catalytic mechanism of cleavage of glycosyl oxygen by cellulase has been explained as an analogy of hydrolysis by lysozyme[8]: It split glucosidic linkages by a mechanism of an acid catalysis with participation of carboxyl group of glutamic acid residue (Glu) and aspartic acid residue (Asp). Considering intramolecular hydrogen bonds in **1** and **2**,[9] the glucosidic oxygens in **1** appear to be exposed to the aqueous media. On the

other hand, the oxygens in **2** are covered in a hydrophobic atmosphere by the substitution at the C-2 and C-3 positions. Therefore, the hydrophobic atmosphere in **2** may inhibit the approach of amino acids such as Glu and Asp in the active center of cellulase to the glycosyl oxygen. In addition, Legler and Bause reported that there are two or three binding subsites adjacent to the catalytic site towards the nonreducing end.[10] In substrate **2**, there may not have a binding subsite because of its hydrophobicity. On the contrary, the 6-*O*-methyl substituent in **1** is rather at a distance from the main chain. Thus, we assume that the hydrophobic inhibition in **1** has less influence than that in **2** and endo-type cellulase can hydrolyze the glucosidic bonds between the two 6-*O*-substituted AHG units. Consequently, here it is found that cellulose derivatives can be hydrolyzed when the primary structure of the AHG unit is controlled by regioselective substitution.

The kinetics of the reaction and identification of the active cellulase for each of the substrates **1** and **3** by purification of the crude *Trichoderma viride* cellulase are now under investigation. The effect of the primary and secondary structures of the substrate polymers to the cellulase degradation should be also investigated.

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