Application of Regioselectively Substituted Methylcelluloses To Characterize the Reaction Mechanism of Cellulase

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ABSTRACT: 6-*O*-Methylcellulose (6MC) and 2,3-di-*O*-methylcellulose (23MC), having every structural unit of them regioselectivery substituted, and ununiformly 2,3-di-*O*-methylated cellulose (23MCU), having a trace amount of unsubstituted glucose units (U) in the same molecular chain, were used to investigate the structure of the scission point in enzymatic degradation with *Trichoderma viride* cellulase. For 6MC, the glycosidic bond between two adjacent substituted units could be cleaved to give oligomers with a degree of polymerization of ca. 8, while 23MC was not degraded. The characteristics of hydrolysis changed by substituted positions of substituents even though the same methyl groups were used. In addition, 23MC inhibited the hydrolysis of (carboxymethyl)cellulose. Interestingly, even in such unfavorable circumstances, 23MCU which was almost composed of 23MC was hydrolyzed, and further the initial velocity of this reaction was significantly high. Moreover, the amount of reducing ends produced corresponded to the amount of U, suggesting that only the linkages between U and 2,3-di-*O*-methylated units were cleaved because the cellulase could not cleave glycosidic bonds between two adjacent substituted 2,3-di-*O*-methylated units. By analyzing the kinetics of the cellulase reactions on the above cellulose model compounds, we were able to estimate the effect of the substituted regions on the hydrolysis in the cellulose derivatives. Especially, 2,3-di-*O*-methylated regions competitively inhibited the hydrolysis of U in other molecular chains. However, they activated the scission of U in the same molecular chain.

Introduction

Biological degradation of various polymers has been studied to develop a new ecological waste system. Cellulose and cellulose derivatives have also been studied as biodegradable polymers that have been utilized in many industrial fields. In some studies, cellulase enzyme assay was used as a model for biodegradation instead of treatment by microorganisms or soil burial.[1] Thus, the reaction mechanism of cellulase has been extensively investigated.[2] In particular, there has been a focus on deriving a correlation between the introduction of substituents and degradation for some cellulose derivatives.[3−9] In the case of six types of cellulose derivatives ((carboxymethyl)-, (carboxyethyl)-, (sulfoethyl)-, (cyanoethyl)-, and methylcellulose and cellulose sulfate) having varying values of degree of substitution (DS) from 0.23 to 1.9, an increase in DS for cellulose derivatives interfered with the enzymatic hydrolysis, and the unsubstituted residues in the polymers were considered as the main sections for cleavage.[3] For example, in (carboxymethyl)cellulose (CMCU), having some unsubstituted glucose units (U), the scission of the main chain was reported to occur between two or more adjacent unsubstituted units.[4−6] The assessment of the substitution patterns for the enzymatically hydrolyzed monomer and dimer fractions in (hydroxyethyl)cellulose revealed that the hydrolysis could also occur at the units having a 6- or 2-substituted unit as an aglycone.[7−9] Thus, at present it is assumed essential for cellulose derivatives to have an unsubstituted unit in order to undergo enzymatic degradation, namely, every unit having at least one substituent showed a confirmed resistance to biodegradation.

However, these studies did not completely clarify the substitution patterns for both neighboring units of the cleaved glycosidic bonds. The substrates used so far were not homogeneously substituted within a glucose unit, and the structure of the adjacent neighboring unit to the hydrolyzed oligomer could not be confirmed by investigating the produced oligomer fractions. In addition, because of its very low reactivity, it was difficult to detect the hydrolytic reaction itself; therefore, low rates of reactivity may have gone undetected.

The evaluation of cellulase activity, particularly *endo*-type activity has been studied widely using CMCU.[10] However, it cannot give the correct value because the cellulase activity has been analyzed without regard to substituted and unsubstituted regions, and so it also ignores the effect of the substituted regions on the hydrolytic reaction.

In this paper, we have used regioselectively and uniformly substituted methylcelluloses to investigate the structure of the scission point more clearly and evaluate regioselective effects of substituents on cellulose hydrolysis. Furthermore, the kinetics of the depolymerization by the crude cellulase from *Trichoderma viride* was interpreted.

Experimental Section

Materials. The water-soluble cellulose derivatives 6-methylcellulose (6MC), 2,3-di-*O*-methylcellulose (23MC), ununiformly 2,3-di-*O*-methylated cellulose (23MCU), and sodium CMCU, shown in Figure 1, were used as substrates for the enzymatic reactions. 6MC and 23MCU were synthesized by the methods previously reported.[11,12] CMCU was used as a soluble, degradable cellulose analog.

6MC had a uniform structure that means every structural unit of a polymer was uniform. 23MCU contained 3.3 wt % U randomly distributed along the main chain. They were determined by gas chromatographic (GC) analysis of the alditol acetates from acid hydrolyzates.[11,12] CMCU with a nominal DS of 0.7 was purchased from Wako Pure Chem. Ind. Ltd., and the content of U in the CMCU was 27 wt % as determined by the glucose oxidase method.[13] 23MC was prepared from 23MCU by elimination of the fraction containing U. The procedure for the elimination of U was as follows: A 23MCU solution (1.0 mg/mL in 50 mM sodium acetate buffer, pH 5.0) was incubated at 37 °C for 96 h with 1.0 wt % Meicelase (CEPB-5081 Meiji Seika Kaisya, Ltd.; *T. viride* cellulase) to the amount of substrate. The hydrolyzed product was frac-

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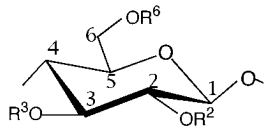

	R^2	R^3	R^6	
6MC	H	H	CH_3	(100%)
23MC	CH_3	CH_3	H	(100%)
23MCU	CH_3	CH_3	H	(96.7%)
	H	H	H	(3.3%)
CMCU	CH_2COONa			(23.3%)
	H			(76.7%)
	(nonregioselective)			

Figure 1. Chemical structures of regioselectively substituted cellulose derivatives: 6MC, 6-*O*-methylcellulose; 23MC, 2,3-di-*O*-methylcellulose; 23MCU, heterogeneously substituted cellulose having a trace amount of unsubstituted units in the 23MC molecular chain; and CMCU, (carboxymethyl)cellulose.

Table 1. Conditions of the Enzymatic Reaction

substrate	substrate (g/L)	enzyme (mg/L)
6MC	0.45−1.8	11.2
23MCU	0.80−4.0	10.0
CMCU	0.20−10	16.08
CMCU + 23MC	CMCU: 0.20−10 23MC: 0.6	16.08

tionated, and those fractions having a MW of 10 000 and above, as identified by size exclusion chromatography (SEC), were used to obtain the 23MC sample. The 23MC thus obtained was identified by ^{13}C-NMR.[14]

Meicelase, a crude cellulase originating from *T. viride*, was used for all experiments. The activity of this enzyme at pH 5.0 and 50 °C to Avicel (Asahi Chem. Ind. Co.) and CMCU was 0.36 and 0.47 unit/mg, respectively, determined by reducing sugar-end analysis.[15]

Determination of Final Products by Enzymatic Reaction. 6MC, 23MC, and 23MCU were enzymatically treated in the following way. The substrates were completely dissolved at a concentration of 0.98 mg/mL in 50 mM sodium acetate buffer at pH 5.0, and then 1.0 wt % cellulase to the amount of substrate was added. The reaction mixtures were incubated at 37 °C for desired periods from 24 to 96 h. After incubation, the solutions were placed in a boiling water bath for 10 min to inactivate the enzyme. The substrates were then precipitated by heating. They were redissolved by cooling in ice water for 3 h. The enzymatically treated products were then subjected to both SEC and reducing sugar-end analyses.

Determination of Kinetic Parameters. 6MC, 23MCU, and a mixture of CMCU and 23MC were also used as substrates in determining kinetic parameters. These substrates and the enzyme were dissolved in 50 mM sodium acetate buffer at pH 5.0. Concentrations of the substrates and the enzyme for a series of four reactions are listed in Table 1. The reaction mixtures were incubated at 27 °C and sampled after various periods of time ranging from 0.5 to 4 min following the addition of the enzyme solution. The samples were treated as described above to achieve enzyme inactivation and subsequent dissolution of the precipitated substrates. The dissolved solutions were analyzed by SEC methods. The initial velocity for the reaction was determined by calculating the increase in the amount of end groups in the substrates on the basis of the number-average molecular weight (M_n). This SEC method was more sensitive than a direct analysis of the end groups by the Somogi−Nelson method,[15] especially in the initial stage of the reaction. In 6MC which was uniformly substituted along the molecular chain, the concentration of the substrate was estimated to equal the number of glycosidic bonds in all the units. In the case of 23MCU, CMCU, and the mixture of CMCU and 23MC, each substrate concentration was taken as the number of glycosidic bonds attached to unsubstituted units.

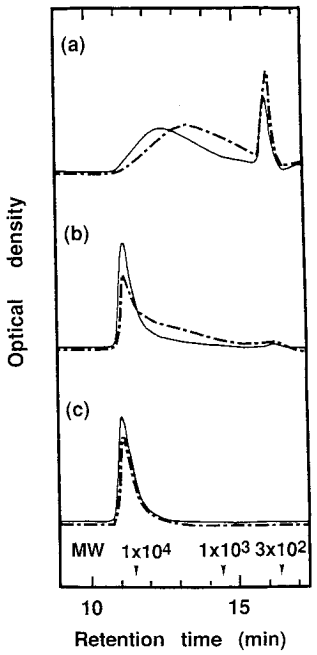

Figure 2. Size exclusion chromatograms of 6MC (a), 23MCU (b), and 23MC (c) before and after cellulase treatment. Solid and broken lines are untreated and after 90 h of treatment, respectively.

General Analysis. SEC analyses were carried out using a HPLC system (Tosoh Co.). A TSKgel G2500 PWXL column (7.8 mm i.d., 30 cm) was used as the HPLC column to prepare the 23MC and measure the treated samples. Kinetic parameters were determined using a TSKgel G3000 PWXL (7.8 mm i.d., 30 cm) column. A 50 mM sodium acetate buffer at pH 5.0 was used as the eluent at a flow rate of 0.5 mL/min. The elution patterns were monitored using a differential refractometer. All experimental data were calculated using a CP-8000 integrator.

Results and Discussion

Characterization of the Cellulase Reaction. Figure 2 shows the changes in the SEC patterns both before and after hydrolysis for 90 h. Cellulose derivatives have been reported to be cleaved only at the glycosidic linkages adjacent to U, and thus cellulose derivatives having no U have not been considered as depolymerized by the enzymatic hydrolysis.[6] The two results that 23MCU (trace b) was depolymerized and that 23MC (trace c) was intact before and after the cellulase treatment support the above expectations. However, in the case of 6MC (trace a), the distribution of molecular weights is shifted to the region of lower molecular weights. In this pattern, the sharp peak at a retention time of about 16 min seems to be due to dimer fractions including contaminants originating from the untreated substrate. This suggested that the main products were not monomeric sugars and dimers but oligomers. The weight-average degree of polymerization (DP) for these oligomers was ca. 8. In other words, the results of the degradation of 6MC indicate clearly that cellulase can even cleave the glycosidic bonds between two substituted glucose units in a row because 6MC dose not have unsubstituted units. This does not agree with the assumption that the cleavage reaction requires unsubstituted units. Thus, for the first time it has been proved that cellulose derivatives in which every unit has been substituted can still be cleaved. In addition, the enzyme predominantly participating in this reaction must be an *endo*-type component in a crude mixture of *endo*- and *exo*-cellulase.

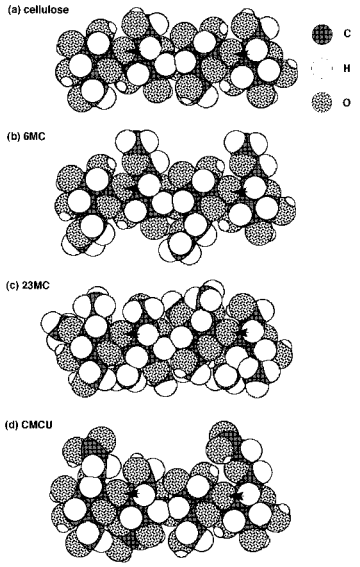

Figure 3. Space-filling models of cellulose (a), 6MC (b), 23MC (c), and CMCU (d). Arrows show glycosidic oxygens.

The above results also provide some interesting insights into the possible reaction mechanism of the cellulase and the resulting structure for analogs of the substrates, respectively. The molecular structures for 6MC and 23MC using space-filling atomic models are shown in Figure 3. The catalytic mechanism of cleavage for the β-1,4 linkage by cellulase can be explained by an analogy to the hydrolysis of lysozyme.[16] Glycosidic linkages are cleaved by a mechanism of acid catalysis with participation by the carboxyl group of glutamic acid (Glu) and aspartic acid (Asp) residues in the enzyme. Considering intramolecular hydrogen bonds,[17] the β-1,4 bonding oxygen in 6MC appears to be exposed to the aqueous media. The hydrophilic environment around the oxygen is similar to that of the cellulose homopolymer, namely, the methyl substitution at the C-6 positions may not have a profound influence on the hydrophilicity of the main chain. Thus, the glycosidic bonding oxygen can be attacked by the amino acid residues such as Glu and Asp in the active center of the cellulase. On the other hand, the oxygens in 23MC are covered by the hydrophobic methyl groups at the C-2 and C-3 positions. Therefore, the hydrophobic atmosphere is expected to inhibit the approach by the catalytic charged amino acid residues of the cellulase. The substituents in 6MC are further away from the main chain when compared with those in 23MC. We can assume that the hydrophobic inhibition in 6MC will have less effect on the enzymatic hydrolysis than that in 23MC. Of course, this is based on the premise that the enzymatic specificity in both derivatives is not so very different.

After 24 and 96 h of cellulase treatment, the extents of hydrolysis for 6MC were 10.3% and 10.5%, respectively. Thus, the extent of hydrolysis reached a plateau at about 10%. This phenomenon is thought to depend on decrease of DP of substrate.[18] The maximum extent of hydrolysis for 23MCU was 3.2%. As this value agreed with the amount of U, it indicated that scission of the glycosidic linkages by the cellulase occurred only between 2,3-di-*O*-substituted and nonsubstituted units instead of cleavage occurring between two 2,3-di-*O*-substituted units.

Kinetic Studies. Figure 4 shows double-reciprocal plots for the relationship between the initial velocities (v) and the concentrations of substrate ([S]) for 6MC (a), 23MCU (b), and CMCU (c) when each initial concentration of enzyme ($[E]_0$) was 1.0 g/L. Here, as mentioned in the Experimental Section, the concentration of the substrate ([S]) was assumed to equal the amount of unsubstituted units (U) except for the case of 6MC. In 6MC, the concentration was calculated as the number of 6-*O*-methylated units. In order to estimate the binding property of the cellulase toward 23MC, the catalytic reactivity was investigated on a mixture of CMCU and 23MC (CMCU + 23MC, Figure 4c). We discuss the enzyme kinetics on the assumption that a component which hydrolyzes both 23MCU and CMCU in crude cellulase is identical. Therefore we determined that the purificated cellulase having high activity for CMCU also hydrolyzed 23MCU effectively.[19]

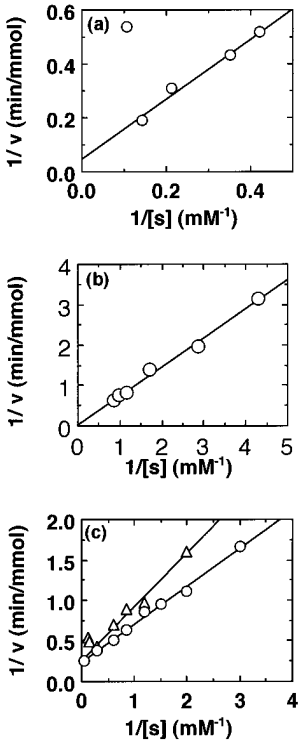

Figure 4. Double-reciprocal plots of initial velocity versus substrate concentration for *T. viride* cellulase hydrolysis of 6MC (a), 23MCU (b), CMCU (c, ○), and CMCU + 23MC (c, △). The concentration of the substrate ([S]) for b and c was assumed to be the number of glycosidic bonds attached to unsubstituted units.

Table 2. Kinetic Parameters for Some Substrates of the Cellulase from *T. viride*

	6MC	23MCU	CMCU	CMCU + 23MC
K_m[a]	22	0.72	1.8	2.5
$V_{max}/[E]_0$[b]	20	24	3.8	3.8
$V_{max}/[E]_0K_m$[c]	0.9	33.7	2.1	1.5
K_i[d]				8.0

[a] K_m is Michaelis constant (mM). [b] $V_{max}/[E]_0$ is maximum velocity/g weight of cellulase (mmol min^{-1} g^{-1}). [c] $V_{max}/[E]_0K_m$ corresponds to catalytic coefficiency (mmol min^{-1} g^{-1} mM^{-1}). $[E]_0$ is initial concentration of enzyme. [d] K_i is inhibition constant of 23MC (mM).

For all substrates, the relationship between $1/v$ and 1/[S] shows an approximate linearity. The kinetic parameters Michaelis constant (K_m, mM) and maximum velocity (V_{max}, mmol min^{-1}) were calculated from the Michaelis−Menten equation. Comparison of the two plots in Figure 4c indicates that 23MC performed competitively as an inhibitor of the hydrolysis of CMCU because these plots followed eq 1 representing competi-

tive inhibition. Therefore, the inhibition constant (K_i, mM) for 23MC was calculated from eq 1:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} \quad (1)$$

where v and [S] are initial velocity (mmol min^{-1}) and concentration of substrate (mM), respectively. [I] (mM) is the concentration of the 23MC inhibitor. The K_m value, the enzymatic activity/1.0 g of the enzyme ($V_{max}/[E]_0$), the catalytic coefficiency ($V_{max}/[E]_0K_m$), and the K_i value are shown in Table 2.

Interestingly, the $V_{max}/[E]_0K_m$ value for 23MCU was high relatively. We expected that hydrolysis of 23MCU was inhibited by 23MC regions (96.7 wt %) in 23MCU because 23MC inhibited hydrolytic reaction of CMCU (Figure 4c). Therefore, the value showing the catalytic coefficiency was thought to be low. However, the reactivity of 23MCU was high competitively. In addition, the K_m and V_{max} for 23MCU calculated from inhibition eq 1 should be negative values and not agree with the results shown in Table 2. These data indicate that 23MC regions in a 23MCU molecule did not act as inhibitor. In other words, the effect of the 2,3-substituted units in 23MCU is different from that of 23MC in the mixture of CMCU and 23MC with regard to the inhibition phenomenon. The existence type of 23MC regions and U seemed to be responsible for the variation of the effect of 2,3-methylated units.

The behavior of the cellulase toward 23MC units can be explained in the following way. When 2,3-substituted units and U are in separate molecules, the 23MC units do not change $V_{max}/[E]_0$ for U but act as competitive inhibitors. This means that 23MC units and U compete for binding in the active site of the enzyme. However, when they are in the same polymer molecule as 23MCU, then the $V_{max}/[E]_0$ for U is very high. This means that it is a better substrate for the enzyme. A reason for this may be that the interaction between 23MC regions and cellulase is engaged favorably in order to relatively increase the concentration of the enzyme in the vicinity of U.

Most cellulases have two interactive domains with the substrate: a catalytic domain and a noncatalytic cellulose-binding domain.[20] The cellulose-binding domain was reported to act for mainly insoluble cellulose.[21] The functional amino acid residues were expected to be aromatic residues.[22] On the other hand, the catalytic domain has six or seven subsites for binding to sugars.[23,24] Tryptophan residues in the subsites were reported to mediate the interaction of the cellulase with cellulose.[25] Hydrophobicity of amino acid residues concerned with cellulase−substrate interaction cannot be disregarded. For 23MCU, the hydrophobicity is thought to increase compared to that of nonsubstituted cellulose by methyl groups. It is necessary to investigate the influence of the increasing hydrophobicity for a substrate in terms of cellulase−substrate interaction and clarify which domain interacts with the 23MC regions.

Conclusions

The cleavage of β-glycosidic linkages by cellulase was investigated using a kinetic study on regioselectively substituted methylcelluloses. The following three conclusions can be made based on these results.

1. Glycosidic linkages between two adjacent 6-*O*-methylated units could be cleaved. The hydrolyzed products were not monomeric sugars but rather oligomers having a DP of ca. 8. The enzyme participating in this reaction was considered to be an *endo*-type cellulase. The cellulase activity for the glycosidic linkages between two adjacent substituted 6-*O*-methylated units was about 30% of that for the bonds between unsubstituted units.

2. Glycosidic linkages between two continuous 2,3-di-*O*-methylated units could not be cleaved. However, these units could bind to the cellulase, when the substrate constant was 8.0 (mM). When 23MC units existed in the same molecule of the substrate, then the cellulase activity was activated by them. On the other hand, the presence of 23MC units in other molecules inhibited the cellulase activity.

3. Cellulase activity for unsubstituted units in water-soluble cellulose was very susceptible to a condition of substituents. Therefore, the investigation of effect of substituents is necessary for a measurement of cellulase activity by using CMCU.

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