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Analysis of reducing end-groups produced by enzymatic scission of glycosidic linkages in *O*-methylcellulose ☆

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Abstract

O-Methylcellulose (ds 1.8) was subjected to hydrolysis by cellulase and the portion that was enzymatically cleaved was analyzed. End-group analysis of the enzymatic hydrolyzate gave direct evidence for scission at the glycosidic linkages of 6-*O*-methyl-, 2-*O*-methyl-, and also unsubstituted glucose residues. The rates of cleavage of glycosidic linkages was: unsubstituted glucose residues > 6-*O*-methylglucose residues > 2-*O*-methylglucose residues.

Keywords: Glycosidic linkages; Enzymatic scission; Methylcellulose, *O*-; Cellulase

1. Introduction

There is increasing interest in the microbial degradation of synthetic polymers and on synthesis of biodegradable polymers. As cellulose is a biodegradable natural polymer, its use as a basis for semisynthetic polymers may be regarded as an approach to biodegradable polymers. Certain water-soluble cellulose derivatives of low ds (degree of substitution), such as *O*-(carboxymethyl)cellulose [1], *O*-(hydroxyethyl)cellulose [2], and *O*-methylcellulose [3], are known to be partially degraded by cellulase, a cellulolytic enzyme. However, there is no direct information as to whether or not cellulase can cleave a glycosidic linkage that has a partially substituted glucose residue as the glycon.

☆ Part I of the series, Enzymatic Hydrolysis of *O*-Methylcellulose.

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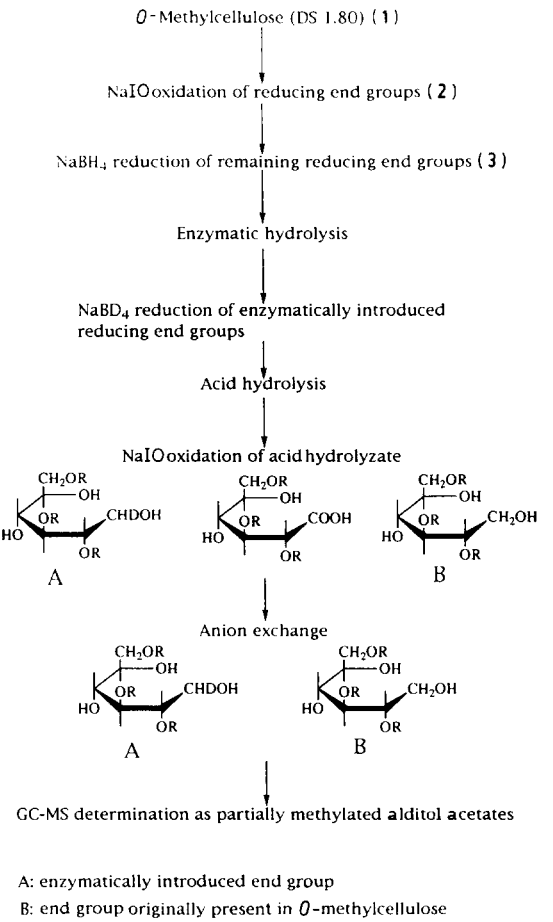

Fig. 1. Scheme for the analysis of enzymatically produced reducing end-groups.

In the present study, *O*-methylcellulose (**1**) was subjected to treatment by cellulase to furnish direct information about the position of glycosyl cleavage. The experimental scheme (Fig. 1) consists of two principal steps: (*1*) conversion of reducing end-groups originally present in *O*-methylcellulose (**1**) into aldonic acid end-groups, and (*2*) labeling of enzymatically produced reducing end-groups by sodium borodeuteride reduction.

2. Results and discussion

In order to detect reducing end-groups enzymatically produced, these had to be distinguished from those originally present in *O*-methylcellulose (**1**). For this purpose, prior to enzyme treatment, **1** was treated with iodine to oxidize those original reducing-

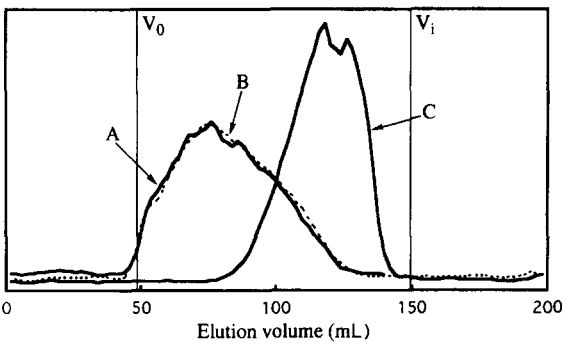

Fig. 2. Gel-filtration chromatogram of **1**, **3**, and the enzymatic hydrolyzate of **3**. A, **1**; B, **3**; C, enzymatic hydrolyzate of **3** (enzyme: Cellulase Nagase, V_0: 49 mL; V_i: 150 mL).

end groups into aldonate end-groups. The oxidized methylcellulose (**2**) was then reduced with $NaBH_4$ in order to convert the reducing end-groups still remaining into glucitol end-groups (Fig. 1). This product (**3**) was then subjected to enzymatic hydrolysis with four kinds of cellulases of different origins. After enzymatic treatment of **3**, the new reducing end-groups produced by enzymatic hydrolysis were reduced with $NaBD_4$ to give deuterated glucitol end-groups. Although neutral sugars present as stabilizers in cellulase preparations seemed to interfere with the sugar analysis of enzymatically hydrolyzed **1**, most of these neutral sugars were readily removed from enzyme preparations by simple gel-filtration (Sephadex G-50) as described in the Experimental section.

Molecular-weight distributions of **1**, **3**, and an enzymatic hydrolyzate of **3** are shown in Fig. 2. The molecular-weight distribution of **3** was the same as that of **1**, indicating that oxidation by iodine affected only the reducing end-groups of the cellulose chain. On the other hand, the cellulase treatment caused a significant depolymerization of **3**. After cellulase treatment, high molecular-weight fractions disappeared and low-molecular weight fractions in a narrow distribution were observed. This result suggests that enzymatically cleavable linkages are rather uniformly distributed in the molecule of *O*-methylcellulose.

Fig. 3 is a gas chromatogram of glucitol derivatives produced from reducing end-groups in the enzymatic hydrolyzate of **3**. Unsubstituted glucitol hexaacetate was the major component. Quite remarkable is the presence of 6-*O*-methylglucitol pentaacetate. The mass spectrum (MS) of the peak of unsubstituted glucitol hexaacetate (data not shown) and that of 6-*O*-methylglucitol pentaacetate (Fig. 4) were recorded by gas chromatography--mass spectroscopy (GC–MS). The results confirmed that more than 95% of these peaks were the deuterated forms (compare peaks at m/z 362 and 361, or peaks at 218 and 217), indicating that most of the unsubstituted glucitol and 6-*O*-methylglucitol can be attributed to enzymatically produced end-groups. This provided evidence that cellulase can cleave glycosidic linkages of 6-*O*-methylglucose residues as well as those of unsubstituted glucose residues.

Enzymatic scission of the glycosidic linkage of 2-*O*-methylglucose residues was also evidenced by GC–MS with selected ion-monitoring at m/z 118, which is one of the

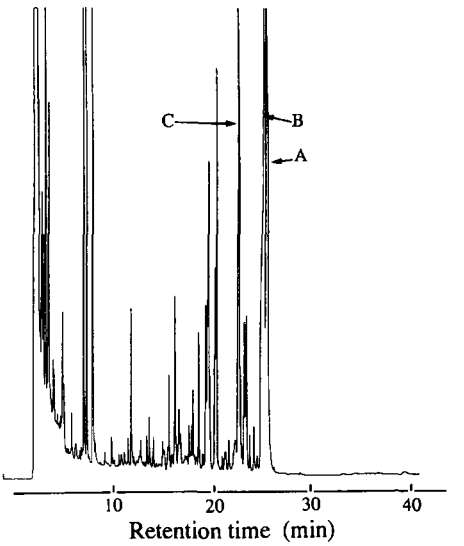

Fig. 3. Chromatogram of partially methylated alditol acetates derived from enzymatically produced reducing end-groups. A, glucitol hexaacetate; B, *myo*-inositol hexaacetate; C, 6-*O*-methylglucitol pentaacetate.

characteristic fragmentation-peaks of the deuterated form of 2-*O*-methylglucitol pentaacetate. Although selected ion-monitoring at m/z 118 showed a clear peak at the position corresponding to 2-*O*-methylglucose pentaacetate (Fig. 5), selected ion-monitoring at m/z 117, corresponding to its protio form, did not show any significant peak at this position. When Meicelase, Celluclast, or Liftase A40 were used as the cellulase preparation, selected ion-monitoring at m/z 118 showed another peak at a position corresponding to 2,6-di-*O*-methylglucitol tetraacetate, suggesting that a part of the 2,6-di-*O*-methylglucose residues in *O*-methylcellulose was also enzymatically cleaved at its glycosidic linkage.

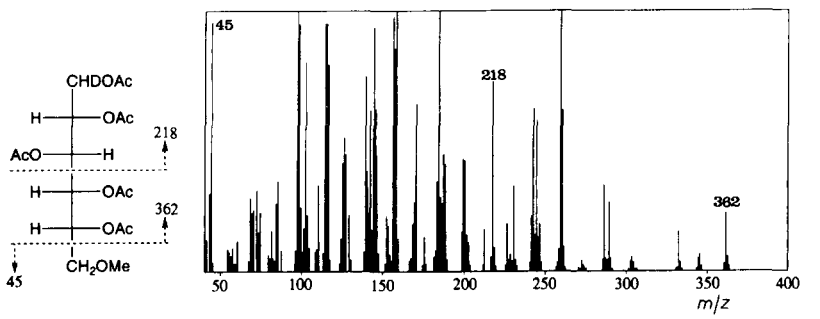

Fig. 4. Mass spectrum of 6-*O*-methylglucitol pentaacetate derived from reducing end-groups of the enzymatic hydrolyzate of **3**.

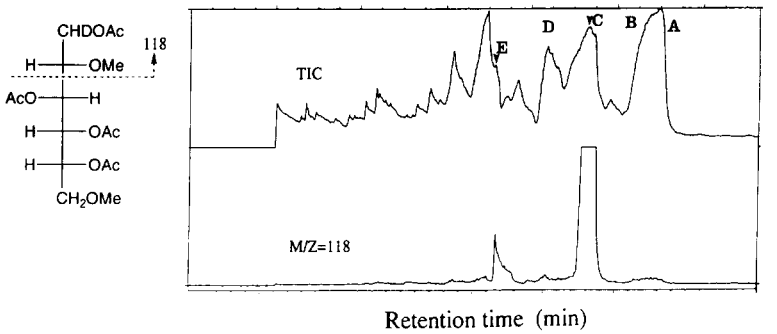

Fig. 5. Selected ion-monitoring at m/z 118. TIC, total ion chromatogram: A, glucitol hexaacetate; B, *myo*-inositol hexaacetate; C, 2-*O*-methylglucitol pentaacetate; D, 6-*O*-methylglucitol pentaacetate; E, 2,6-di-*O*-methylglucitol tetraacetate.

Quantitative analysis of reducing end-groups in enzymatically treated **3** are given in Table 1 in comparison with the sugar composition of the original **1** and remaining reducing end-groups in **2**. As described previously, enzymes used in this experiment were purified by gel-filtration. By this purification, as shown in Fig. 3, no interfereing peak overlapped with the two peaks measured, peak C (6-*O*-methylglucitol pentaacetate) and peak A (glucitol hexaacetate). However, many unknown small peaks appeared, along with those of the expected partially methylated glucitol acetates, in the region of retention times of 20 min or below. Quantitative determination of peaks in this region could not therefore be done. The amounts of remaining reducing end-groups in **2** are smaller than those in enzymatically treated **3**, indicating that most of reducing end-groups found in enzymatically treated **3** may be regarded as being produced by enzymatic scission. This observation is in good accord with results just described in that most of the glucitol end-groups in enzymatically cleaved **3** were deuterated. As shown in Table 1, the rates of cleavage of glycosidic linkages depends on the enzyme preparation. Although these cleavage rates may be improved by modification of the reaction conditions, quite interesting is the ratio between the unsubstituted glucose end-group and

Table 1
Analysis of reducing end-groups enzymatically introduced in comparison with structural units of **1** and with reducing end-groups of **2**

	Enzyme	Partially methylated sugars [a]							
		S_{236}	S_{26}	S_{36}	S_{23}	S_6	S_2	S_3	U
Enzymatically produced end-group	Meicelase		+			17.1	+	−	26.2
	Celluclast		+			6.54	+	−	17.0
	Liftase A40		+			7.22	+	−	17.2
	CellulaseNagase		−			13.6	+	−	10.9
Structural unit of **1**		246	208	65.8	55.8	110	98.4	12.0	40.0
Remaining reducing end group in **2** ($\times 10^{-3}$)				125	50.2	91.8	97.6	29.8	13.3

[a] +, present; −, absent; mg per 1 g of sample.

the 6-*O*-methylglucose end-group. Among the four enzymes used, Cellulase Nagase produced the least amount of unsubstituted glucose end-groups, whereas it produced a much higher amount of 6-*O*-methylglucose end-groups than did Celluclast or Liftase A40. This result suggests that an enzyme of unique function is present in the cellulase preparation, and it is capable of cleaving the glucosidic linkage of a 6-*O*-methylglucose residue, and that Cellulase Nagase is rich in this unique enzyme.

The rates of cleavage of 2-*O*-methyl- and 2,6-di-*O*-methyl-glucose residues could not be determined because of overlapping with unknown peaks on the gas chromatogram. Although the glucosidic linkage of unsubstituted glucose residue is more readily cleaved by cellulases than those of substituted glucose residues, it is clear that *O*-methyl substitution at the 6- and 2-positions does not completely inhibit enzymatic scission at glucosidic linkages of methylated glucose residues.

Table 2 summarizes the types of reducing end-groups detected in enzymatically treated **3** with various cellulases and their detection methods. These results, as well as those in Table 1, suggest that Cellulase Nagase is slightly different from the other enzymes.

3. Conclusion

Cellulases of four different origins cleaved glycosidic linkages of 6-*O*- and 2-*O*-methylglucose residues in *O*-methylcellulose, albeit at a lower rate than that of unsubstituted glucose residues.

4. Experimental

Materials.—*O*-Methylcellulose: Methylcellulose (**1**) commercially available from Wako Pure Chemical Industries was used. The ds (degree of substitution) was 1.8. Cellulases: Cellulases of four different origins (Meicelase – *Trichoderma viride* origin, Cellulase Nagase – *Aspergillus niger* origin, Celluclast – *Trichoderma reesei* origin, and Liftase A40 – *Trichoderma longibrachiatum* origin) were used. To remove neutral sugar contaminants in cellulase preparations, the solutions were subjected to gel-filtration chromatography (Sephadex G-50 medium, 35 × 220 mm), and high-molecular weight fractions (Meicelase 70–100 mL; Cellulase Nagase, Liftase A40, and Celluclast 100–130 mL of eluate) were used as cellulase preparations.

Preparation of oxidized and reduced methylcellulose (**3**).—Oxidized methylcellulose (**2**) was obtained by a stepwise addition of 33 mL of 0.1 M NaOH and 22 mL of 0.05 M iodine solutions to an aqueous solution of 2.0 g of **1** according to conditions for the oxidation of 2,3-*O*-isopropylidene-D-lyxofuranose [4]. The mixture was dialyzed in a cellulose tube against distilled water and then reduced by $NaBH_4$. Reduced **2** (**3**) was recovered by dialysis and subsequent lyophilization.

Enzymatic hydrolysis of **3** *and determination of enzymatically produced reducing end-groups.*—Compound **3** (0.50 g) was subjected to treatment by cellulase under agitation as a solution in 80 mL of 50 mM acetate buffer for 115 h at or below 37°C.

Table 2
Detection of partially methylated glucoses produced as reducing end-groups in enzymatically treated **3** by various methods [a]

	Detection method	S_{236}	S_{26}	S_{36}	S_{23}	S_6	S_2	S_3	U
Meicelase	Method 1 Method 2 Method 3	– – –	○ – –	– – –	– – –	○ ○ ○	○ ○ –	– – –	○ ○ ○
Celluclast	Method 1 Method 2 Method 3	– – –	○ – –	– – –	– – –	○ ○ ○	○ ○ –	– – –	○ ○ ○
LiftaseA 40	Method 1 Method 2 Method 3	– – –	○ – –	– – –	– – –	○ ○ ○	○ ○ –	– – –	○ ○ ○
CellulaseNagase	Method 1 Method 2 Method 3	– – –	× – –	– – –	– – –	○ ○ ○	○ ○ –	– – –	○ ○ ○

[a] Method 1: GC–MS with selected ion-monitoring. Method 2: scanning GC–MS with analysis of individual spectra. Method 3: GC with FID detection. ○: detected, X: detected, –: not evaluated

The pH of the buffer was adjusted to the optimum pH for each cellulase (5.0 for Celluclast and Meicelase, 5.5 for Liftase A40, and 4.5 for Cellulase Nagase). The concentrated cellulase hydrolyzate was reduced with 0.1 g of $NaBD_4$ in 22 mL of D_2O. As all acidic components must be transformed into their sodium salts by evaporation of acetic acid from the acetate buffer-solution, the posibility of the formation of lactones from aldonate end-groups and subsequent reduction to alditols may be ruled out. The reduced enzymatic hydrolyzate was concentrated and subjected to a swelling treatment in 72% H_2SO_4, followed by acid hydrolysis in 60 mL of 4% H_2SO_4 at or below 120°C for 1 h and neutralized with an aqueous saturated $Ba(OH)_2$ solution. The whole acid hydrolyzate (corresponding to 0.50 g of **3**) was oxidized with iodine as described in the previous section, and then passed through an anion-exchange column (Dowex 2-X8, acetate form, 80 mL) to remove aldonates produced by the iodine oxidation of aldoses. The first 200 mL of eluate (neutral fraction) from the anion-exchange column was subjected to analyses by GC and GC–MS as alditol acetates [5] to determine deuterated glucitols derived from enzymatically produced reducing end-groups. Columns used for GC and GC–MS analyses were OV-101, 50 m (GC) and 25 m (GC–MS) capillary column. The column temperature was raised from 150 to 280°C at a rate of 4°C/min. *myo*-Inositol was used as the internal standard for the quantitative determination of constituent sugars in original **1** and **2**, and of reducing end-groups in **3** and enzymatically treated **3**. An FID detector was used for GC.

Gel-filtration chromatography.—**1**, **3**, and enzymatically hydrolyzed **3** were subjected to gel-filtration chromatography (Sepharose CL-4B, 1.6 × 82 cm, eluted with 0.1 M aqueous LiCl), and sugars were determined by the phenol–H_2SO_4 method [6].

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