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NOTES

Gel Filtration Fractionation of Cellulase from *Trichoderma viride* and Its Application to the Synthesis of the Branched Polysaccharide

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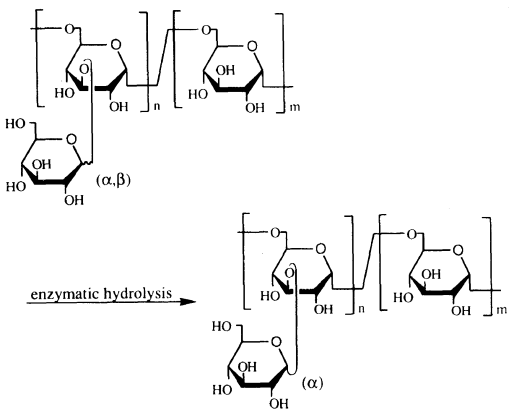
The synthetic methods for the branched polysaccharides have been investigated in two ways. One is the addition of the branching units to the linear polysaccharides with the chemical glycosylation,[1,2] and the other one is the polymerization or copolymerization of disaccharide derivatives.[3,4] Since there are some problems in the synthesis of stereoregular branched polysaccharides, such as stereoselectivity of the chemical glycosylations and the polymerizability of disaccharide monomers, the synthesis of branched polysaccharide is still difficult. We have recently described a novel synthetic method, which includes the enzymatic removal of unnecessary branching units after the glycosylation reaction of the polysaccharides, for stereoregular branched polysaccharides.[5] The fractionation of cellulase prepared from *Trichoderma viride* with gel filtration column and the activity of each fraction toward the synthetic branched polysaccharide are now reported.



EXPERIMENTAL

General Methods

^{13}C NMR spectra of the polysaccharides were recorded in deuterium oxide using sodium 4,4-dimethyl-4-silapentanesulfonate as external reference at room temperature by means of a JEOL EX-270 spectrometer. Optical rotations were recorded with a JASCO Model 370 polarimeter at room temperature in water using 0.5-dm cell. Gel permeation chromatography was run on 1% solution of the polymer in water by means of Shimadzu liquid chromatograph (Model LC-9A, column: Asahipak GS-510 (Asahi Chemical Industry Co., Ltd.)). The number-average molecular weights of polymers were determined by GPC using standard pullulan as reference.

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Materials

Synthetic branched polysaccharide (GBGLN), which is (1→6)-α-D-glucopyranan having both α-D-glucopyranose and β-D-glucopyranose branches was obtained as described previously.[5] *p*-Nitrophenyl β-D-glucopyranoside, microcrystalline cellulose, CM-cellulose, and curdlan were purchased from Sigma Chemical Co., Asahi Chemical Industry Co., Tokyo Kasei Kogyo Co., Ltd., and Wako Pure Chemical Industries, Ltd., respectively. Cellulase was purchased from Yakult Co., Ltd., Tokyo ("Onozuka" 3S).

Gel Filtration Chromatography

Gel filtration chromatography was carried out on Sephadex G-75 (Pharmacia Fine Chemicals Co.). The column was equilibrated in 0.05 M sodium acetate buffer (pH 4.8). The sample was applied to the column in a cold room at 6°C and fractionated. The eluted fractions were used for the analysis of enzyme activity toward several substrates. Protein was detected at 280 nm.

Enzyme Assay

The reaction mixture was composed of 1 ml of 0.2% solution of GBGLN in 50 mM sodium acetate buffer (pH 4.8) and 0.2 ml of each fraction including enzyme. After the incubation by continuous shaking at 40°C for 20 min, the liberated glucose was analyzed by the glucose oxidase method.[6] The unit of the enzyme activity was defined as the amount of enzyme needed to liberate 0.01 μg of glucose per min under the assay condition.

Complete removal of β-D-glucopyranosyl units from GBGLN was attempted by the long-time incubation with the fraction of gel filtration. The reaction mixture consisted of 1 ml of 3% GBGLN solution in 50 mM sodium acetate buffer (pH 4.8) and 1 ml of the enzyme solution (fraction nos. 81 and 169). The mixture was incubated in the shaking water bath at 40°C for 2 days. After incubation, the reaction was terminated by the addition of 2 ml of ice-cold 25% trichloroacetic acid, and the mixture was maintained at 0°C for 30 min. The precipitated enzyme was removed by centrifugation and the supernatant was neutralized by 0.5 *N* sodium hydroxide solution. After dialysis of the polysaccharide solution with distilled water for 3 days, the polysaccharide was obtained by freeze-drying from water. The enzyme-treated polysaccharides were characterized by optical rotation, ^{13}C NMR spectroscopy, and gel permeation chromatography.

p-Nitrophenyl β-D-glucopyranoside was used for the substrate of β-glucosidase. The β-glucosidase-assay was performed by the addition of 0.2 ml of enzyme solution to 1 ml of 5 mM *p*-nitrophenyl β-D-glucopyranoside in 50 mM sodium acetate buffer (pH 4.8) and incubated at 40°C for 20 min. The amount of *p*-nitrophenol liberated was determined from the absorbance of its alkaline solution at 400 nm.[7] The unit of enzyme activity was defined as the amount of enzyme which liberated 10 μmol of *p*-nitrophenol per min under the assay conditions.

The glucosidase activity toward CM-cellulose was measured in a mixture containing 2 ml of 1% CM-cellulose solution in 50 mM sodium acetate buffer (pH 4.8) and 0.2 ml of the enzyme solution. After incubation at 40°C for 10 min, 1 ml of the reaction mixture was taken and analyzed for reducing sugars by the dinitrosalicylic acid method.[8,9] The unit of enzyme activity was defined as the amount of enzyme needed to liberate the reducing sugars equivalent to 0.1 μg of glucose per min under the assay conditions.

The reaction mixture for the glucosidase activity measurement toward the microcrystalline cellulose consisted of 2 ml of 1% microcrystalline cellulose suspension in 50 mM sodium acetate buffer (pH 4.8) and 0.2 ml of enzyme solution. The mixture was incubated with shaking at 40°C for 3 h. After centrifugation, 1 ml of reaction solution was taken and analyzed for reducing sugar by the dini-

trosalicylic acid method. The unit of enzyme activity was defined as the amount of enzyme needed to liberate the reducing sugars equivalent to 5 μg of glucose per min under the assay conditions.

The activity toward curdlan was measured by the addition of 0.2 ml enzyme solution to 2 ml of 2% curdlan suspension in 50 mM sodium acetate buffer (pH 4.8). The mixture was incubated with shaking at 40°C for 3 h. After centrifugation, 1 ml of supernatant was analyzed for reducing sugar by the dini-

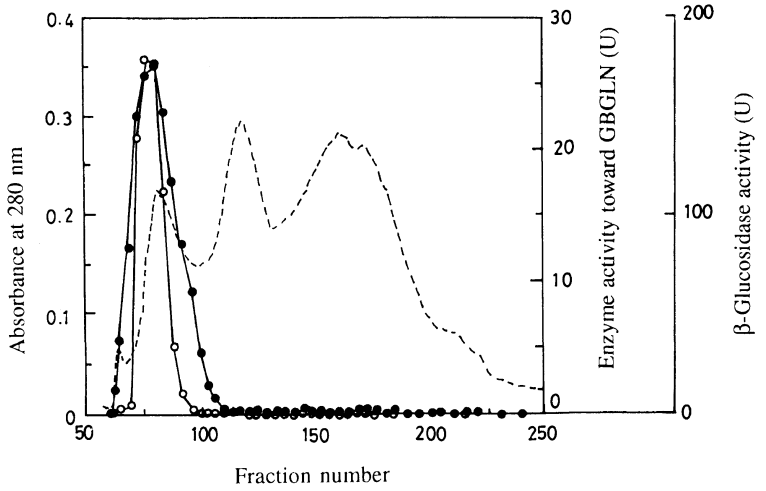

Figure 1. Protein elution profile at 280 nm (---) and fractionation of β-glucosidase activity (○) and the activity toward GBGLN (●) with gel filtration chromatography on Sephadex G-75.

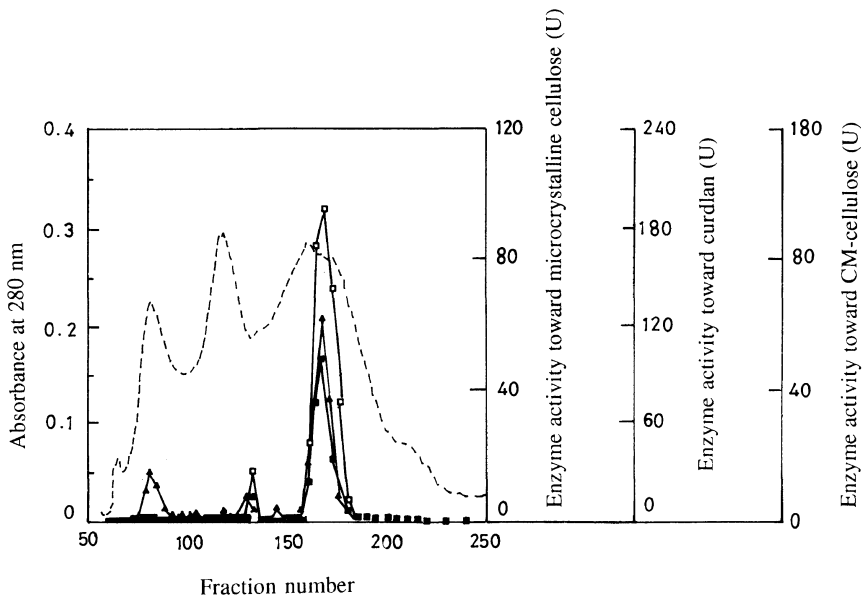

Figure 2. Protein elution profile at 280 nm (---) and fractionation of glucanase (□, microcrystalline cellulose; ▲, CM-cellulose; ■, curdlan as substrates) activities with gel filtration chromatography on Sephadex G-75.

trosalicylic acid method. The unit of enzyme activity was defined as the amount of enzyme needed to liberate the reducing sugars equivalent to 1 μg of glucose per min under the assay conditions.

RESULTS AND DISCUSSION

Cellulase prepared from *Trichoderma viride* is generally classified according to the enzyme activity into three types, *i.e.*, 1,4-β-D-glucan glucanohydrolase (endoglucanase), 1,4-β-D-glucan cellobiohydrolase (exoglucanase), and β-D-glucoside glucohydrase (β-glucosidase).[10,11] Several investigators have separated the cellulase into 4,[12-14] 6,[15] or 10[16] enzymatically active components. However, the exact fractionation system has not been established yet, because of the complex cellulase composition which depends on the microorganisms and the culture conditions.

Figure 1 shows the fractionation of cellulase by chromatography on a column of Sephadex G-75 and the enzyme activity toward *p*-nitrophenyl β-D-glucopyranoside which is the substrate of β-glucosidase and the synthetic branched polysaccharide, that is, (1→6)-α-D-glucopyranan having both α-D-glucopyranose and β-D-glucopyranose branches (abbreviated as GBGLN). The β-glucosidase activity and the activity toward GBGLN were mainly associated with high molecular weight region and showed almost the same patterns, indicating that the β-D-glucopyranosyl unit of the synthetic branched polysaccharide was removed by the β-glucosidase. Gong *et al.* reported that the molecular weight of the β-glucosidase from *Trichoderma viride* was 76000 and it was higher than those of endoglucanase and exoglucanase.[17]

The enzyme activities toward microcrystalline cellulose, carboxymethyl cellulose (CM-cellulose), and curdlan were found in the low molecular weight region (Figure 2). Fraction no. 169 showed the highest enzyme activity toward microcrystalline cellulose, CM-cellulose, and curdlan, while this enzyme did not hydrolyze GBGLN and *p*-nitrophenyl β-D-glucopyranoside. Bergham *et al.* reported that endoglucanase and exoglucanase prepared from *Trichoderma viride* were not active toward *p*-nitrophenyl β-D-glucopyranoside.[18,19] Therefore, fraction no. 169 was assumed to be identified as endoglucanase and/or exoglucanase.

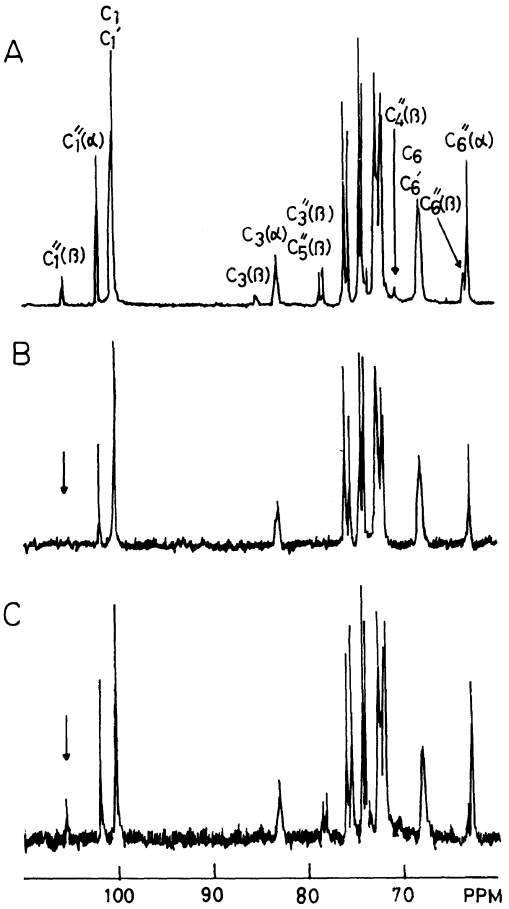

Figure 3. ^{13}C NMR spectra of enzyme-treated branched polysaccharides. A, non-treated GBGLN; B, GBGLN treated with fraction no. 81; C, GBGLN treated with fraction no. 169. Peaks are assignable to carbons on three kinds of sugar residues as follows: C_n (n=1—6), main chain unit with branching; C'_n, main chain unit without branching; C''_n, branching sugar unit.

Figure 3 shows the ^{13}C NMR spectra of GBGLN and enzyme-treated GBGLN. After

Table I. Enzymatic treatment of branched polysaccharide (GBGLN)

Fraction no. of cellulase	$[\alpha]_D$[a] (deg)	$\bar{M}_n$[b] ($\times 10^4$)	Degree of branching[c]	Anomeric ratio[d] ($\alpha:\beta$)
Non-treated	+178.9	1.8	0.40	83:17
81	+188.8	1.6	0.32	100: 0
169	+179.4	1.8	0.43	80:20

[a] Measured in water (*c* 0.5).
[b] Determined by GPC.
[c] Mole fraction of glucosylated residues in main chain calculated by ^{13}C NMR.
[d] Anomeric ratio of the branching units calculated by ^{13}C NMR.

enzymatic reaction with fraction no. 81, the peak at 105.5 ppm, which was assigned to the anomeric carbon of the β-D-glucopyranosyl branching unit, completely disappeared, indicating that the β-D-glucopyranosyl unit of branched polysaccharide was removed by enzymatic hydrolysis catalyzed by β-glucosidase. On the other hand, ^{13}C NMR spectrum of GBGLN treated with fraction no. 169 was almost the same as that of non-treated GBGLN, indicating that the enzyme, which was quite active toward microcrystalline cellulose, CM-cellulose, and curdlan, did not hydrolyze the β-D-glucopyranosyl unit of branched polysaccharide. The treatment of GBGLN with fraction no. 81 resulted in a decrease of 20% in the degree of branching as shown in Table I, indicating that only β-D-glucopyranosyl branching unit was removed by β-glucosidase. Moreover, the comparison of number-average molecular weights of non-treated GBGLN and GBGLN treated with fraction no. 81 indicated that the main chain as well as α-D-glucopyranose branch was not hydrolyzed by β-glucosidase. Therefore, it was concluded that the β-glucosidase, which was the different component from the active enzyme for cellulose and curdlan, prepared from *Trichoderma viride* removed only β-D-glucopyranosyl unit of the branched polysaccharide to give a stereoselectively branched polysaccharide having only α-D-glucopyranosyl units.

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