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Transglycosylation of cellobiose by partially purified *Trichoderma viride* cellulase

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Abstract

A commercial cellulase from *Trichoderma viride* was fractionated into three fractions, F1, F2, and F3, in order to investigate transglycosylation activities. Among these fractions, F3, which demonstrated highly hydrolytic activity toward *p*-nitrophenyl β-D-glucopyranoside and Avicel, most effectively catalyzed the transglycosylation of cellobiose and converted cellobiose into β-Glc-(1→6)-β-glc-(1→4)-Glc and β-Glc-(1→6)-β-Glc-(1→6)-β-Glc(1→4)-Glc. The F3 fraction contained the enzyme to catalyze β-glucosyl transfer toward only the C-6 position of the sugar acceptor, and thus it is expected to be of use for syntheses of functional oligosaccharides.

Keywords: *Trichoderma viride* cellulase; Transglycosylation; Oligosaccharide synthesis

1. Introduction

The cellulolytic activities of cellulase have been well investigated. The fungus *Trichoderma viride* cellulase [1,2], in particular, has been studied with regard to substrate specificity [3], protein structure [4–6], and kinetics of cellulolysis [7]. It is well known that cellulases exhibit not only cellulolytic activities but also catalysis activities of transglycosylation [8], which become very important in the syntheses of biologically and medically active carbohydrates.

Cellulase is generally characterized by three main components [9,10]: an endo-glucanase [EC 3.2.1.4], an exo-glucan cellobiohydrolase [EC 3.2.1.91], and a β-glucosidase [EC 3.2.1.21]. Regarding the transglycosylation, Barker et al. reported that culture filtrates of *Aspergillus niger* catalyzed β-glucosyl transfer of cellobiose to another cellobiose molecule to form a trisaccharide [11]. Their findings suggested that β-glucosidase catalyzed the transglycosylation, because the reaction synthesized the β-(1→2), β-(1→3), β-(1→4), and β-(1→6) linkages. β-Glucosidases purified from *Fusarium oxysporum* [12] and *Ruminococcus albus* [13] were reported to demonstrate both exo-glucan cellobiohydrolase and transglycosylation activities. Shoemaker and Brown reported finding three endo-glucanases that catalyzed the transglycosylation of cello-oligosaccharides (tetramer through hexamer) [8], though the newly synthesized linkages

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were not clear. Research has widely documented the transglycosylation of cellulase components, although identifying precisely which components catalyze this reaction remains elusive.

In this paper, we have described the transglycosylation reaction of cellobiose by three fractions prepared from *T. viride* cellulase and have determined the structures of the transglycosylated products. The effects of the reaction conditions on the transglycosylation are also described herein.

2. Results and discussion

Preparation of cellulase fractions.—As shown in Fig. 1, three fractions (P1–P3) were obtained from chromatography on Mono Q HR 5/5 of crude cellulase after ammonium sulfate precipitation at 80% saturation. The β-glucosidase activity was mainly associated with the materials in P1 and P2. Both of these fractions also contained traces of activity toward Avicel and carboxymethyl cellulose (CMC). The activity toward Avicel was mainly located in the protein peak of P3. Fractions P1 through P3 were applied to gel-filtration chromatography on a Superdex 75 HR10/30 column. Protein peaks containing β-glucosidase activity from P1 and P2 and fractions containing activity toward Avicel from P3 were collected and lyophilized. We designated the three fractions obtained from gel-filtration of P1, P2, and P3 as F1, F2, and F3, respectively.

Table 1 summarizes the yields and the enzyme activities of F1–F3. Identification of their purities and their molecular weights were estimated by SDS–PAGE. The main bands of F1, F2, and F3 were observed at 76.0, 74.0, and 68.0 kDa, respectively, though a few minor protein bands were contained in all fractions (data not shown).

Transglycosylation reactions of cellobiose.—One of the most important factors to increase the yields of transglycosylation products is an increase of the substrate concentration, which should be as high as possible to shift the equilibrium towards the transglycosylation reaction. In this study, therefore, aqueous 500 mM cellobiose was used for the reaction. Table 2 summarizes the yields of the tranglycosylation products of cellobiose by HPLC analysis after 24-h incubation at 40 °C. Samples from the cellobiose mixtures reacted with the crude cellulase showed the production of trace amounts of gentiobiose and laminaribiose, while F1 and F2, both containing high β-glucosidase activity, were far more effective than the crude cellulase at converting cellobiose into the glucobioses. Total respective yields of the glucobioses by the F1 and F2 were 11.6- and 14.8-fold, both of which were higher than the yield by crude cellulase. The

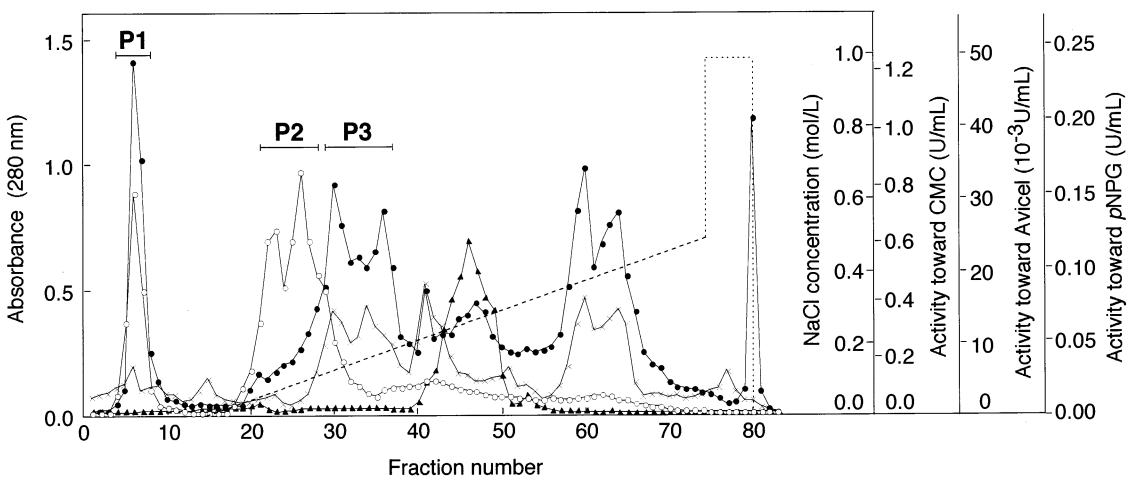

Fig. 1. Ion-exchange chromatography on Mono Q HR 5/5 column of crude cellulase after ammonium sulfate precipitation. (-●-) Absorbance at 280 nm; (-▲-) activity toward CMC; (- × -) activity toward Avicel; (-○-) activity toward *p*NPG; dashed line is indicated concentration of sodium chloride. Fractions (10 mL) were collected.

Table 1
Specific activities of fractions during purifications of *T. viride* cellulase

Steps and fraction	Yield of protein (mg)	Specific activity (U/g protein)		
		CMC	Avicel	*p*NPG
$(NH_4)_2SO_4$ precipitation	300	154	16.2	67.0
Mono Q HR5/5				
P1 (fr. 4–8 in Fig. 1)	28.3	10.6	7.8	113
P2 (fr. 21–28 in Fig. 1)	19.1	61.8	11.3	456
P3 (fr. 29–37 in Fig. 1)	60.8	25.3	17.4	13.0
Superdex 75 HR10/30				
F1	5.1	0.30	42.3	554
F2	2.6	94.4	70.2	2900
F3	25.4	31.3	32.6	280

Table 2
Yield of oligosaccharides through the transglycosylation of cellobiose analyzed by HPLC [a]

Enzyme	Enzyme concentration (mg/mL)	Concentration of saccharide (mM)					
		Glucose	Disaccharide			Trisaccharide	Tetrasaccharide
			Cellobiose	Laminaribiose	Gentiobiose		
Crude cellulase	1	237	325	1.0	5.5	0	0
F1	0.5	324	263	15.5	60.0	0	0
F2	0.5	396	206	25.5	70.5	0	0
F3	0.1	34	480	0	0	2.0	0
	0.5	133	411	0	0	15.3	0
	1	169	292	0	0	70.3	9.0
	2	370	188	0	0	66.0	14.0
	3	511	171	0	0	40.3	6.8

[a] The reactions were carried out in 50 mM sodium acetate buffer (pH 5.0) at 40 °C for 24 h.

study of glucose production by F1 or F2 as a function of cellobiose concentration showed a decrease in the concentration of 2 mM cellobiose (data not shown). This result indicated that F1 and F2, like other β-glucosidases [14–16], catalyzed hydrolysis and transglycosylation reactions simultaneously.

In the case of F3, samples from the cellobiose mixtures showed both trisaccharide and tetrasaccharide formation. The yield of the trisaccharide increased with the enzyme concentration and reached a maximum at 1 mg/mL of the enzyme. At high concentrations (1–3 mg/mL) of the enzyme, a tetrasaccharide was also detected, and the yield of tetrasaccharide was at a maximum at 2 mg/mL of the enzyme. In the HPLC analyses, the tri- and tetrasaccharide were detected as one peak, and formation of glucobioses other than cellobiose, as well as other tri- and tetrasaccharides, was not observed. Based on these findings and the fact that isomers were not observed, it is considered that F3 contained an enzyme catalyzing a high regioselective transglycosylation of cellobiose. F3, therefore, was expected to be a useful catalyst in oligosaccharide syntheses.

Effects of substrate concentration, temperature, and pH on the transglycosylation by F3.— In order to investigate the efficiency of transglycosylation by F3 under various conditions, it was first necessary to follow the reaction progress under varying conditions to obtain the maximum yields of the desired

Table 3
Formation of oligosaccharides by F3 (1 mg/mL) with time at three cellobiose concentrations analyzed by HPLC [a]

Substrate concentration (mM)	Reaction time (h)	Concentration of saccharide (mM)			
		Glucose	Disaccharide	Trisaccharide	Tetrasaccharide
100 (3.42 (w/v)%)	1	11.8	90.1	2.7	0
	2	27.4	76.1	6.8	0
	4	52.8	54.5	12.7	0
	8	103	30.9	12.3	0
250 (8.56 (w/v)%)	4	49.0	215	6.8	0
	8	95.5	178	16.7	0
	16	159	138	22.0	0.13
	24	208	46.1	20.2	0.28
500 (17.1 (w/v)%)	8	53.6	422	32.0	1.6
	16	103	354	56.2	5.2
	24	169	292	70.3	9.0
	32	349	214	60.7	10.3

[a] The enzyme reactions were carried out in 50 mM sodium acetate buffer, pH 5.0 at 40 °C.

compound. As shown in Table 3, the time-course of the enzyme-catalyzed reaction at various cellobiose concentrations was examined by HPLC analysis. At a low initial concentration (100 mM) of substrate, transglycosylation activity was almost negligible. At higher concentrations, appreciable amounts of the tri- and tetrasaccharides were obtained by transglycosylation. The rate of the trisaccharide formation was highly dependent on the initial cellobiose concentration, and the reaction times at which trisaccharide production reached maximum were 4, 16, and 24 h at 100, 250, and 500 mM initial cellobiose concentrations, respectively. This indicates that, when the ratio of remaining cellobiose to initial cellobiose is approximately 0.55–0.60, the yield of the trisaccharide has reached maximum. Productivity of the tetrasaccharide depended on the concentration of the trisaccharide. The formation of the tetrasaccharide began when the concentration of the trisaccharide reached approximately 20 mM. These findings indicate that the trisaccharide serves as a glycosyl acceptor, and that it transfers a glucosyl residue of cellobiose to the trisaccharide to form the tetrasaccharide.

The effects of temperature on the transglycosylation reaction were also examined. Fig. 2 shows the time-courses of trisaccharide and tetrasaccharide production, respectively, from initial cellobiose incubated at 30, 40, 50, or 60 °C. The maximum rate of trisaccharide formation at each temperature was much faster with rising temperature below 60 °C. The maximum yield of both the trisaccharide and the tetrasaccharide was observed at 40 °C. Fig. 3 shows the effects of pH on the transglycosylation reaction. The maximum rates of both tri- and tetrasaccharide production at pH 5.0 were faster than those at pH 4.0 and 6.0.

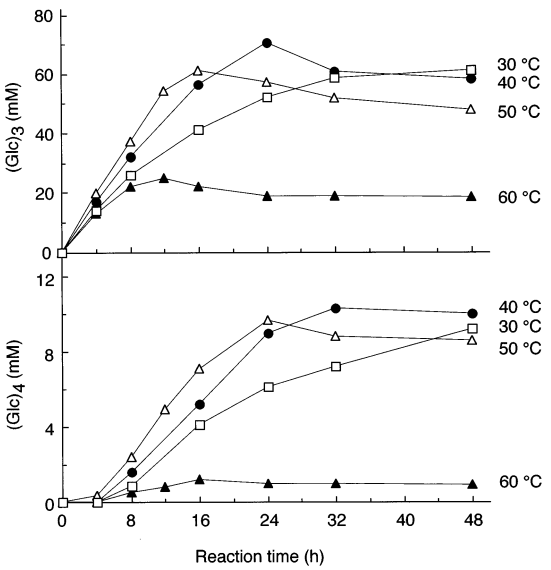

Fig. 2. Temperature dependence of F3-catalyzed trisaccharide $(Glc)_3$ (upper) and tetrasaccharide $(Glc)_4$ (lower) production as a function of time. The enzyme reaction was performed with a 500 mM cellobiose solution at 30, 40, 50, and 60 °C, respectively, in 50 mM sodium acetate buffer, pH 5.0.

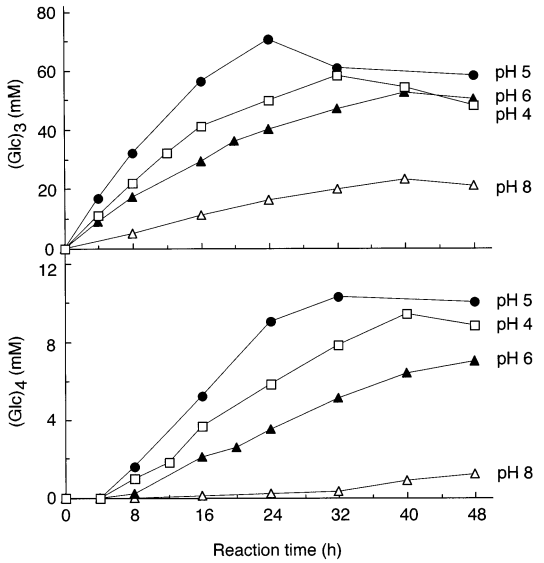

Fig. 3. pH dependence of F3-catalyzed trisaccharide $(Glc)_3$ (upper) and tetrasaccharide $(Glc)_4$ (lower) production as a function of time. The enzyme reaction was performed with a 500 mM cellobiose solution at 40 °C in 50 mM sodium acetate buffer (pH 4.0, 5.0, and 6.0) and 50 mM phosphate buffer (pH 8.0), respectively.

At pH 8.0, transglycosylation activity seemed to be almost negligible. From these results, it is suggested that the optimum reaction conditions of transglycosylation for F3 corresponded to those for the hydrolysis of cellobiose (data not shown).

Structural analyses of the tri- and tetrasaccharides.—In order to investigate the synthetic modes of the oligosaccharides by F3, their structures were determined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Before the assignments of ^{13}C NMR spectra, proton signals were readily assigned from analyses of DQFCOSY and HOHAHA spectra. After the assignments of all proton signals, the corresponding ^{13}C resonances were located in the HSQC spectra. The 1D ^{13}C spectra and the assignments of the oligosaccharides are shown in Fig. 4. In the spectrum of the trisaccharide, three C-6 signals carrying an unsubstituted hydroxyl group were observed at 62.79, 62.93, and 63.53 ppm, which indicated that the C-6 carbons of the non-reducing and reducing end units assume an α-D-glucosyl form and a β-D-glucosyl form, respectively. Substitution of C-6 causes displacement of the signal to a lower field, near 69–72 ppm [17]. Only one signal at 71.48 ppm derived from C-6 of internal residue of the trisaccharide was found in this area. In the spectrum of the tetrasaccharide, two signals at 71.43 and 71.89 ppm derived from C-6 carbons from two internal residues were observed. In the ^{13}C spectrum, chemical shifts of the glycosidic carbon with an α-linkage are in the region of 97.5–101 ppm, and those with a β-D-linkage are downfield in the region of 104–106 ppm [17]. The spectra of both oligosaccharides gave no resonances at 97.5–101 ppm with the exception of the C-1 β-anomeric carbon of the reducing-end unit, while two and three resonances were observed in the β-glycosidic carbon region of the tri- and tetrasaccharide, respectively. These results identified the oligosaccharide structures as β-Glc-(1→6)-β-Glc-(1→4)-Glc and β-Glc-(1→6)-β-Glc-(1→6)-β-Glc-(1→4)-Glc.

Based on the above results, the transglycosylation mechanism of cellobiose using F3 may be recognized as follows. The enzyme acts on the non-reducing end unit of cellobiose to form the glucosyl–enzyme complex, and subsequently the glucosyl residue is specifically transferred to the position-6 of the non-reducing end of another cellobiose molecule, which results in the formation of the trisaccharide. The reaction proceeds, and the concentration of the trisaccharide is increased in the reaction mixtures. When the concentration of the trisaccharide is sufficient, the trisaccharide itself also acts as a glycosyl acceptor. Further, a similar regioselective β-glucosyl transfer occurrs at the non-reducing end of the trisaccharide to produce the tetrasaccharide. These reaction mechanisms were confirmed by results of studying the effect of cellobiose concentration on transglycosylation (Table 3).

An enzymatic approach to oligosaccharide syntheses based on transglycosylation has the advantage of using free sugars as both acceptors and donors. Wider uses of cellulase for the synthesis of oligosaccharide have previously been limited because the predominant formation of the (1→6)-linkage occurs, while the (1→2)-, (1→3)-, and (1→4)-linkages are formed to a lesser extent. Fujimoto et al. [16] explained that the free energy of activation required the hydrolysis of the β-(1→6)-link-

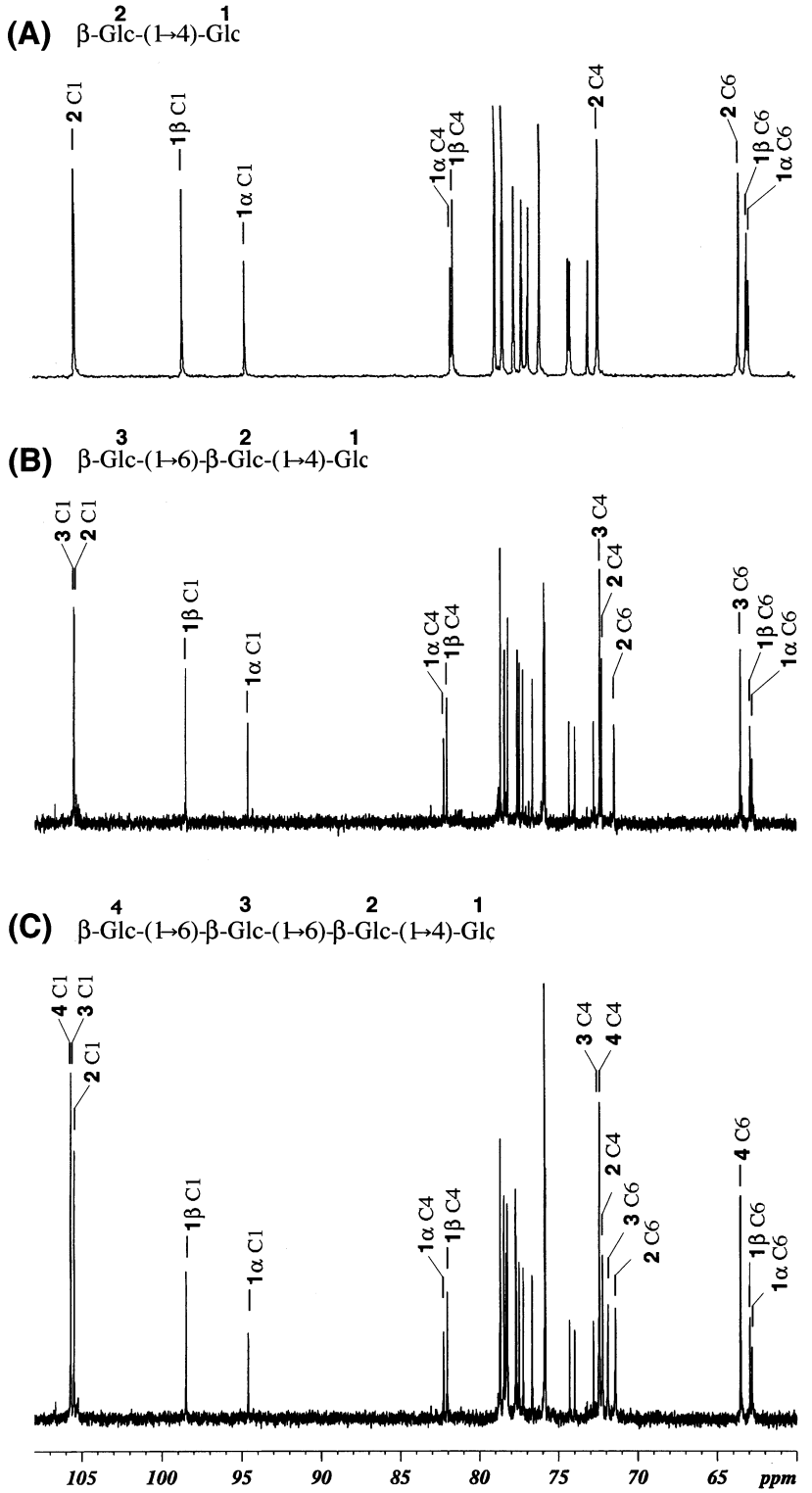

Fig. 4. ^{13}C NMR spectra of (A) cellobiose, (B) trisaccharide, and (C) tetrasaccharide. Chemical shifts are in ppm downfield from internal acetone.

age to be larger than that required for other β-linkages, while the standard free energy of the β-(1 → 6)-linkage is much smaller than that of other linkages; therefore, formation the β-(1 → 6)-linkage preferentially occurs in enzyme-catalysed transglycosylation. In our ex-

periments, β-(1→2)-, β-(1→3)-, and β-(1→4)-linkages were not observed in the reaction products by F3. This indicated that the F3 had high regioselectivity, as well as stereoselectvity towards the hydroxyl group of the sugar acceptor in the transglycosylation.

It was concluded that enzyme fraction partially purified *T. viride* cellulase could not only hydrolyze Avicel and cellobiose, but could also synthesize the oligosaccharides up to tetrasaccharides from cellobiose. Furthermore, an unexpected regioselectivity was observed in the transglycosylation of cellobiose, which could apply to the regioselective synthesis of biologically important carbohydrates.

3. Experimental

Enzyme and chemicals.—*Trichoderma viride* cellulase ONOZUKA R-10 was obtained from Yakult Pharmaceutical Industry Co., Ltd. and was used as the crude cellulase for the present work. Sodium carboxymethylcellulose (CMC, average MW 50,000, DS = 0.48–0.60) was purchased from Kanto Chemicals Ltd. Avicel (50 μm) was purchased from Fluka Chemicals Ltd. *p*-Nitrophenyl β-D-glucopyranoside (*p*-NPG) was purchased from Sigma Chemical Co.

Protein determination.—The protein was determined with Coomassie brilliant blue G250 as described by Bradford [18]. Bovine serum albumin was used as the standard. The absorbance at 280 nm was used for monitoring protein in column effluents.

Determination of molecular mass.—This was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [19]. The molecular mass was estimated using an electrophoresis calibration kit (Pharmacia).

Enzyme assay

(A) Activity toward CMC. A reaction mixture containing 20 μL of enzyme solution and 0.3 mL of 1(w/v)% CMC in 50 mM sodium acetate buffer, pH 5.0 (buffer *A*), was incubated at 40 °C. After 20 min of incubation, the amount of reducing sugars in the mixture was measured by the method of Miller et al. [20] with glucose as a standard. One unit (U) of the activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar per min.

(B) Activity toward Avicel. A reaction mixture containing 50 μL of enzyme solution and 0.95 mL of 1(w/v)% Avicel suspended in buffer *A* was incubated at 30 °C. After 2 h of incubation, the mixture was centrifuged at 3000*g* for 5 min. The amount of reducing sugars in the supernatant was measured by the Nelson [21]–Somogyi [22] method with glucose as a standard. One U of activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar per min.

(C) Activity toward p-NPG (β-Glucosidase activity). A reaction mixture containing 50 μL of enzyme solution and 0.95 mL of 1 mM *p*-NPG in buffer *A* was incubated at 40 °C. After 10 min of incubation, the amount of *p*-nitrophenol in the mixture was measured using a calibration curve at 405 nm. One U of β-glucosidase was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min.

Hydrolysis of cellobiose.—A reaction mixture containing 50 μL of enzyme solution and 0.95 mL of 2 mM cellobiose in buffer *A* was incubated at 40 °C for 30 min. Samples were removed at different intervals, and the amount of glucose in the samples was analyzed by the mutarotase–glucose oxidase method [23].

Partially purified cellulase fractions.—Enzyme purification was carried out at 4 °C unless otherwise stated.

(A) Ammonium sulfate precipitation. ONOZUKA R-10 was saturated with $(NH_4)_2SO_4$ at 80% saturation. The resulting precipitate was collected by centrifuge at 12,000*g* for 20 min, dissolved in 100 mM sodium acetate buffer, pH 5.0. The solution was then desalted and concentrated by ultrafiltration using a Q0100 filter (Advantec Toyo Co.). The enzyme solution was used directly in the following procedure.

(B) Mono Q ion-exchange chromatography. The cellulase (300 mg) from the $(NH_4)_2SO_4$ precipitation was dissolved in buffer *A*. The enzyme solution was applied to a column of Mono Q HR 5/5 (Pharmacia) equilibrated with buffer *A*. The column was washed with buffer *A* and then eluted with a linear gradient of NaCl (0–0.5 M) in buffer *A*. The eluate was

collected in 10-mL fractions. Three re-spective fractions, P1–P3 (see Fig. 1), were pooled and concentrated to low volume (2 mL) using the ultrafiltration already described.

(C) Gel-filtration chromatography. The enzyme solution from step (B) was directly loaded onto a Superdex 75 HR 10/30 column (Pharmacia) equilibrated with buffer *A* and eluted with buffer *A*. The eluate was collected in 1-mL fractions. Fractions containing cellulase activity were collected, concentrated to 2 mL using the ultrafilter, lyophilized, and stored at 4 °C. Three fractions F1, F2, and F3 were obtained from the gel-filtration of P1, P2, and P3, respectively.

Transglycosylation of cellobiose.—The standard procedure of transglycosylation of cellobiose was as follows. A reaction mixture containing 1 mg enzyme and 1 mL of 500 mM cellobiose in buffer *A* was incubated at 40 °C for 24 h. Samples were removed at different intervals, heated at 100 °C for 5 min to inactivate the enzyme, and filtered off. The filtrate was analyzed by HPLC using TSKgel amide-80 column (4.6 × 250 mm, Tosoh Co.). For the fractionation of the products, the large column with the same packing (25 × 300 mm, Tosoh Co.) was used. The products were quantified on the basis of peak area using standards of glucose, cellobiose, cellotriose, and cellotetraose.

Effects of temperature and pH on transglycosylation.—The optimum temperature for transglycosylation was determined by carrying out the standard procedure of transglycosylation at various temperature between 30 and 60 °C. The optimum pH was determined by carrying out the standard procedure at 40 °C over pH region pH 4.0–8.0. Buffer solutions over pH regions 4.0–6.0 and 6.0–8.0 were used 50 mM sodium acetate and 50 mM potassium phosphate, respectively.

NMR spectroscopy.—Before NMR experiments, the oligosaccharides were lyophilized twice from D_2O and then dissolved in 600 μL of D_2O (99.996 atom%, ISOTEC, Inc.). The NMR experiments were carried out on a Bruker Avance 600 spectrometer at 23 °C. DQF-COSY and HOHAHA with spin-lock time of 60 ms were used to assign ^1H signals. HSQC and HMBC were used to assign ^{13}C signals. All NMR experiments were performed according to standard pulse sequences. The ^1H and ^{13}C chemical shifts were referenced to internal DSS, 0.015 ppm, and acetone, 31.55 ppm, respectively.

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