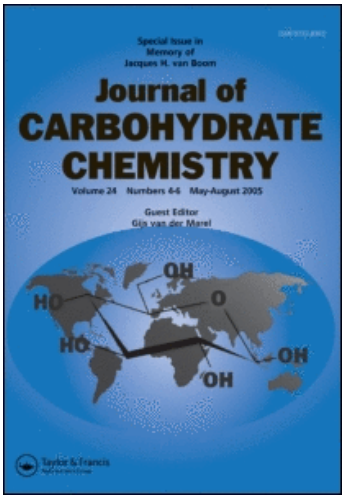

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REGIOSELECTIVE SYNTHESES OF NEW TRI- AND TETRASACCHARIDES BY TRANSGLYCOSYLATION OF TRICHODERMA VIRIDE β-GLUCOSIDASE

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ABSTRACT

A new β-glucosidase, which was partially purified from *Trichoderma viride* cellulase, catalyzed a transglycosylation reaction of cellobiose to give β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc*p* **1** and β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc*p* **2**, regioselectively. Furthermore, the enzyme converted laminaribiose and gentiobiose into β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-D-Glc*p* **3** and β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-D-Glc*p* **4**, respectively. Selective β-(1→6) transglycosylation was achieved.

INTRODUCTION

β-Glucosidase [EC 3.2.1.21], which is a component of cellulase system [EC 3.2.1.4], is well known to hydrolyze cellooligosaccharides to glucose units.[1] Recently, with increasing interests in oligosaccharide components as information-rich molecules responsible for biological recognition, much attention has been paid to β-glucosidases because β-glucosidase-catalyzed transglycosylation can be applied to a new method of oligosaccharide synthesis. The transglycosylation by a β-glucosidase generally proceeds with substrate specificity and stereoselectivity to acceptor molecules and has the advantage of using unprotected sugars as both acceptor and

donor.[2-5] However, wider use of β-glucosidases has been limited, because most of the β-glucosidases show no regioselectivities for hydroxyl groups to the sugar acceptors. Thus, in many cases, the products of the transglycosylation are obtained as mixtures of regio-isomers.[4,5] In order to use a β-glucosidase as a catalyst for oligosaccharide synthesis at a practical level, both high regio- and stereoselectivity are necessary for the β-glucosidase.

In this study, we report on the purification of a β-glucosidase from *Trichoderma viride* (*T. viride*) cellulase[6,7] and oligosaccharide syntheses from β-glucobioses effected by purified β-glucosidase. We also carried out a structural analysis of the products to elucidate the regioselectivity of the β-glucosidase, which is also described herein.

RESULTS AND DISCUSSION

For the purification of β-glucosidase having transglycosylation activity from *T. viride* cellulase, Hi Load Q HP column chromatography was performed. The elution of the cellulase after the ammonium sulfate precipitation is shown in Figure 1. Three peaks of β-glucosidase activity associated with the protein peaks were observed. Of these, the

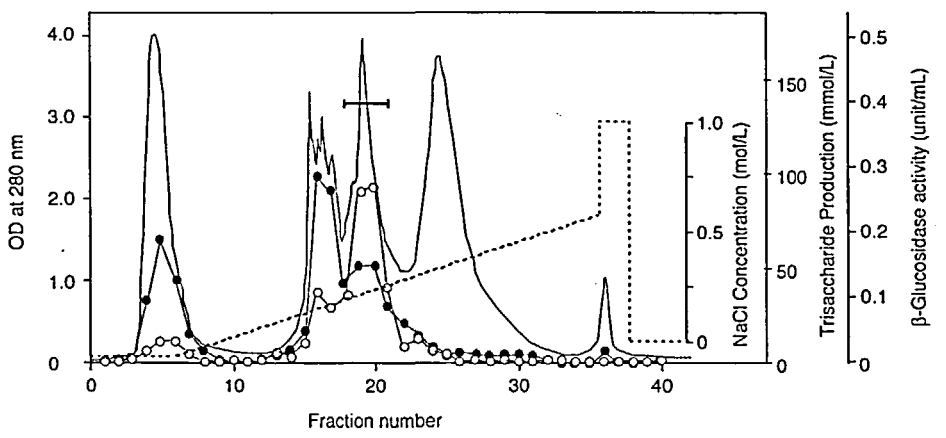


Figure 1. Purification of β-glucosidase on a column Hi LoadQ HP. β-glucosidase activity (●) and trisaccharide production (○) were measured. The solid and broken lines represent absorbance at 280 nm and NaCl concentration, respectively.

β-glucosidase fraction containing transglycosylation activity at the end of the salt gradient was fractionated. After ultrafiltration, the enzyme solution was applied to a Superdex 75 HR10/30 column. In the gel-filtration chromatography, one symmetrical peak of β-glucosidase activity associated with the protein peak appeared at the position where the other proteins were not eluted (data not shown). The β-glucosidase fraction (β-glucosidase activity: 0.141 unit/mg-protein) had high transglycosylation activity, as described later in this paper. As shown in Figure 2, the purified β-glucosidase was homogeneous when judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denatured conditions. The molecular mass of the purified β-glucosidase was approximately 68,000 dalton.

In the examination of the effects of pH and temperature on hydrolysis of cellobiose, as shown in Figure 3, the maximum activity was observed at pH 5.0 in sodium acetate buffer. The purified enzyme was stable in the range pH 4.0-8.0. The optimum temperature for cellobiose hydrolysis of the enzyme was observed at 45 °C. The purified enzyme was sensitive to temperatures above 50 °C at pH 5.0. In order to investigate the substrate specificity of the purified β-glucosidase in the hydrolysis reaction, the rate of the hydrolysis of the disaccharides was measured. The enzyme, like other β-glucosidases,[5] hydrolyzes all the disaccharides, and the hydrolysis rate is in this order: β-(1→3)-, β-(1→4)-, β-(1→2)-, and β-(1→6)-linked disaccharide (data not shown).

In order to investigate the effects of pH and temperature on the transglycosylation ability of the purified β-glucosidase, trisaccharide synthesized from cellobiose by the purified β-glucosidase under various conditions were estimated by HPLC analysis, which is shown in Figure 4. The formation of trisaccharide was dependent on pH, and the maximum rate of the trisaccharide formation was observed at pH 5.0 at 40 °C. The most suitable pH for maximum production of trisaccharide was 5.0. The rate of trisaccharide formation increased with rising temperatures below 60 °C. Reaction at 40 °C for 24 h was best suited for production of the trisaccharide. These results indicated that the optimum conditions for transglycosylation activity corresponded to those for cellobiose-hydrolyzing activity.

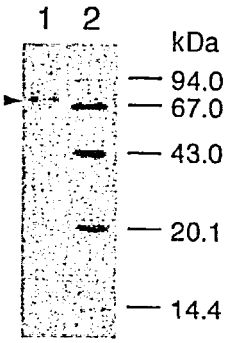


Figure 2. SDS-PAGE of β-glucosidase. Lane 1, standard protein markers in order of increasing molecular mass; phosphorylase b, albumin, ovalbumin, trypsin inhibitor, α-lactalbumin (10 μg); lane 2, β-glucosidase from *T. viride* (10 μg)

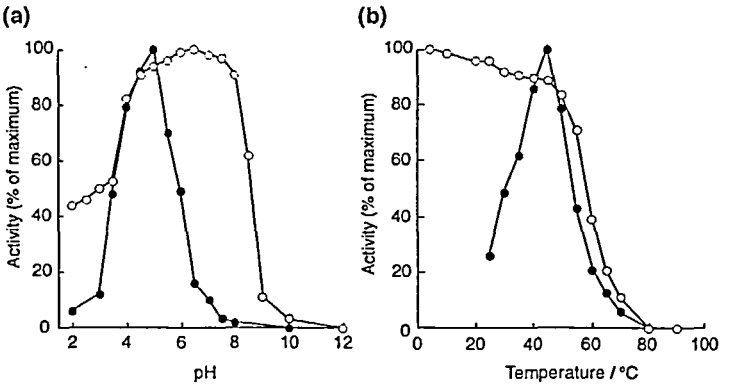


Figure 3. (a) Effect of pH on activity (●) and stability (○) of the *T. viride* β-glucosidase. (b) Effect of temperature on activity (●) and thermal stability (○) of the β-glucosidase.

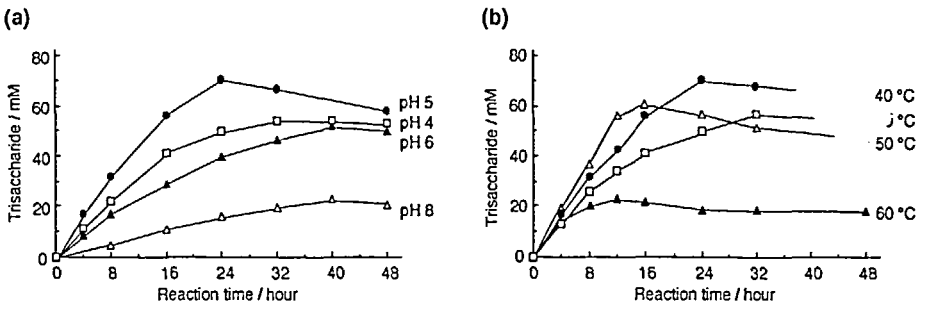


Figure 4. (a) Effect of pH on trisaccharide production from cellobiose by the *T. viride* β-glucosidase. (b) Temperature dependence of the trisaccharide production from cellobiose by the *T. viride* β-glucosidase.

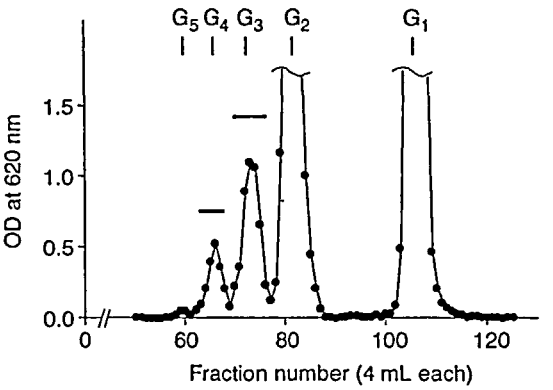


Figure 5. Gel-chromatographic separation of transglycosylation products by the action of β-glucosidase on cellobiose. The positions of G_1 and G_2-G_5 indicate the elution volumes of the standard glucose and the series of cellooligosaccharides from cellobiose through cellopentaose, respectively.

We undertook the transglycosylation reaction with cellobiose, laminaribiose, gentiobiose, or sophorose as substrates using the purified β-glucosidase. Figure 5 shows the elution profile of transglycosylation reaction products of cellobiose by the purified β-glucosidase from Bio-Gel P-2. Fraction numbers from G_1 through G_5 appeared at the same filtration volume compared with standard glucose and cellooligosaccharides from cellobiose through cellopentaose. The chromatogram showed two remarkable high peaks in the trisaccharide (G_3) and tetrasaccharide (G_4) regions. After the fractions corresponding to the tri- and tetrasaccharide elution were collected and lyophilized, the trisaccharide and tetrasaccharide were identified by ^1H and ^{13}C NMR spectroscopy (Tables 1 and 2, respectively) as β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc*p* (**1**) and β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc*p* (**2**), respectively (Scheme 1). There were no signals derived from the other tri- and tetrasaccharides in the NMR analysis. The fractions corresponding to the disaccharide elution contained only cellobiose, which was confirmed by HPLC analysis. This result indicated that D-glucose residue at the non-reducing end of cellobiose was regioselectively transferred to the *O*-6 of D-glucose residue at the non-reducing end of another cellobiose to produce trisaccharide **1**. Furthermore, the β-glucosidase regioselectively catalyzed transferring the β-D-glucose residue of cellobiose to *O*-6 of the non-reducing terminus of product **1** to synthesize tetrasaccharide **2**.

Table 1. ^{1}H NMR chemical shifts[a] of the saccharides (1-4)

Compound	Residue[b]	Proton						
		H-1	H-2	H-3	H-4	H-5	H-6a[c]	H-6b[c]
1	1α	5.226	3.584	3.834	3.620[d]	3.955	3.870	3.968
	1β	4.667	3.290	3.641	3.656[d]	3.655	3.815	3.955
	2	4.518	3.334	3.522	3.508	3.672	3.882[d]	4.224[d]
	3	4.537	3.329	3.517	3.405	3.468	3.736	3.924
2	1α	5.228	3.587	3.829	3.617[d]	3.954	3.871	3.965
	1β	4.667	3.294	3.638	3.647[d]	3.656	3.813	3.957
	2	4.520	3.338	3.526	3.509	3.672	3.877[d]	4.227[d]
	3	4.520	3.342	3.486	3.484	3.687	3.856[d]	4.230[d]
	4	4.555	3.331	3.519	3.405	3.462	3.740	3.928
3	1α	5.225	3.621	3.823[d]	3.521	3.871	3.816	3.899
	1β	4.664	3.353	3.704[d]	3.509	3.533	3.815	3.911
	2	4.713	3.423	3.568	3.513	3.679	3.889[d]	4.226[d]
	3	4.549	3.334	3.510	3.406	3.466	3.733	3.926
4	1α	5.220	3.531	3.689	3.490	3.878	3.966[d]	4.130[d]
	1β	4.666	3.236	3.482	3.462	3.615	3.839[d]	4.198[d]
	2	4.516	3.340	3.489	3.486	3.619	3.855[d]	4.223[d]
	3	4.516	3.327	3.501	3.396	3.458	3.734	3.919

a. In ppm relative to a signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using acetone at δ 2.225) in D_2O at 23 °C. b. A number indicates the position of a glucose residue from the reducing-end unit of oligosaccharides. c. Chemical shifts for H-6a and H-6b can be interchanged. d. Chemical shifts of glycosidically linked position.

In the transglycosylation reaction that used laminaribiose or gentiobiose as a substrate instead of cellobiose, a quite similar regioselectivity was observed. Trisaccharides were synthesized from laminaribiose and gentiobiose by the β-glucosidase-catalyzed transglycosylation, although no formation of tetrasaccharides was observed in HPLC analysis (data not shown). As summarized in Tables 1 and 2, ^{1}H and ^{13}C NMR spectroscopic analysis revealed that laminaribiose and gentiobiose also were regioselectively converted into trisaccharide β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-D-

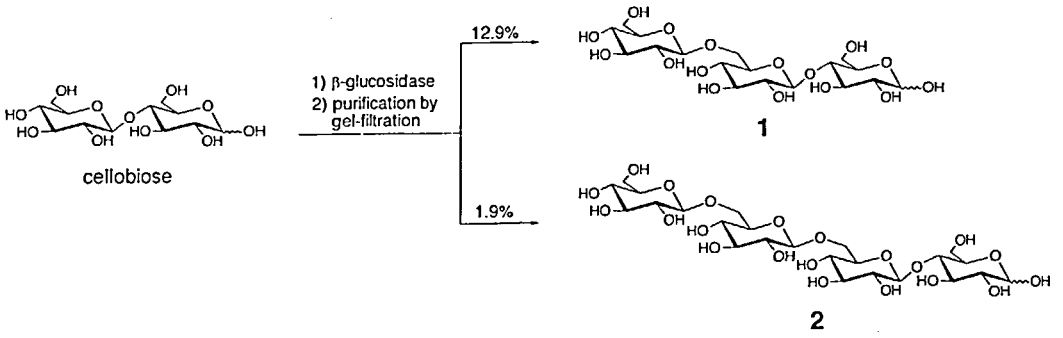
Table 2. ^{13}C NMR chemical shifts[a] of the saccharides (1-4)

Compound	Residue[b]	Carbon					
		C-1	C-2	C-3	C-4	C-5	C-6
1	1α	94.63	73.97	74.34	82.28[c]	72.79	62.79
	1β	98.51	76.65	77.48	82.07[c]	77.26	62.93
	2	105.49	75.87	78.43	72.28	77.61	71.48[c]
	3	105.50	75.94	78.22	72.41	78.69	63.53
2	1α	94.60	74.00	74.32	82.27[c]	72.81	62.81
	1β	98.49	76.69	77.25	82.04[c]	77.51	62.94
	2	105.50	75.91	78.46	72.23	77.73	71.43[c]
	3	105.70	75.85	78.33	72.45	77.70	71.89[c]
	4	105.72	75.91	78.24	72.43	78.71	63.53
3	1α	94.68	73.46	85.10[c]	70.35	72.86	62.78
	1β	98.51	75.21	87.32[c]	70.42	77.68	62.91
	2	105.50	75.53	78.20	71.90	77.79	71.38[c]
	3	105.31	75.62	78.12	71.95	78.42	63.42
4	1α	94.62	73.26	74.40	71.76	72.76	71.26[c]
	1β	98.48	76.65	77.80	71.87	77.94	71.06[c]
	2	105.50	75.12	77.86	71.96	77.76	71.42[c]
	3	105.51	75.46	78.19	72.30	78.60	63.49

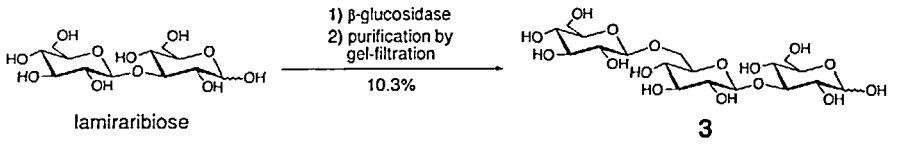
a. In ppm relative to a signal of internal acetone, 31.55 ppm in D_2O at 23 °C. b. A number indicates position of glucose residue from reducing-end unit of oligosaccharides. c. Chemical shifts of glycosidically linked position.

Glc*p* (3) and β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-D-Glc*p* (4), respectively (Schemes 2 and 3). Productions of no other regio-isomers from laminaribiose and gentiobiose were observed by HPLC analysis, respectively.

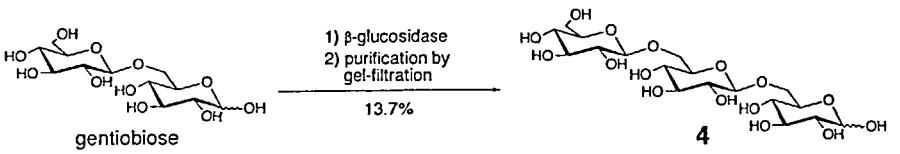
In comparison, when sophorose was used as a substrate for the transglycosylation reaction, trisaccharide was not obtained. In the transglycosylation products from sophorose, small amounts of cellobiose and gentiobiose were detected. This indicated



Scheme 1



Scheme 2



Scheme 3

that glucose from sophorose was transferred to glucose, a hydrolysis product from sophorose, resulting in the formation of cellobiose and gentiobiose. Although the purified β-glucosidase catalyzed not only β-(1→4)-glucosyl transfer but also β-(1→6)-glucosyl transfer to glucose, the β-glucosidase could not recognize sophorose as a glycosyl acceptor in the transglycosylation. From these results of the transglycosylation of four β-glucobioses, we concluded that the purified β-glucosidase has a strict specificity for acceptors in transglycosylation reaction, although the enzyme could hydrolyze all the β-glucobioses.

In general, disaccharides hydrolyzable by one glycosidase should be formed by the transglycosylation of the glycosidase.[5,8] In addition, the order of hydrolytic activity of the glycosidase toward disaccharides generally corresponds to that of transglycosylation activity toward the disaccharides.[9] In our experiment, the hydrolysis rate of the disaccharides by the purified *T. viride* β-glucosidase was in this order: β-(1→3)-, β-(1→4)-, β-(1→2)-, and β-(1→6)-linked disaccharide. In contrast, β-(1→2)-, β-(1→3)-, and β-(1→4)-linkages were not observed in the newly bounded glycosidic linkages of the transglycosylation products **1**, **2**, **3**, or **4** which were synthesized by the action of the purified *T. viride* β-glucosidase. From these results, the transglycosylation by the purified *T. viride* β-glucosidase was also confirmed regioselective.

Regarding the mechanism of β-glucosidase-catalyzed transglycosylation, Gopalan et al.[10] suggested that the presence of two binding subsites for acceptor and donor molecules within the catalytic center of the mammalian cytosolic liver β-glucosidase and that the structure of the acceptor-binding subsite have a direct effect on regioselectivity in the transglycosylation. In this experiment, since it is not clear that such sugar binding-sites in the catalytic center of the purified *T. viride* β-glucosidase exist, interpretation of the results is not possible. If the transglycosylation reaction in this experiment proceeds within such acceptor and donor subsites, the acceptor site has no affinity for sophorose and it binds cellobiose, laminaribiose, gentiobiose, or product **1** to bring the C-6 hydroxyl group of the non-reducing end of the acceptor close to the C-1 hydroxyl group of glucose within the donor binding-site in order to produce **1**, **2**, **3**, or **4**, respectively. To clearly elucidate the regioselectivity of this β-glucosidase, structural analysis of the catalytic region in β-glucosidase is necessary, although such an analysis will be difficult to achieve.

Many researchers have reported that β-glucosidases originating from a wide variety of microorganisms,[2,4,6,8] higher plants,[3,5] and animals[10] catalyze transglycosylation reactions. Whereas transglycosylation reactions with these enzymes result in the formation of several isomers, in our experiments, only β-(1→6)-linkages were formed in the transglycosylation products **1-4**. We therefore conclude that the β-glucosidase obtained from *T. viride* cellulase in our experiment has precise regioselectivity as well as stereoselectivity toward the hydroxyl group of

the sugar acceptor in the transglycosylation. Thus, this enzyme can be used for the regio- and stereoselective synthesis of oligosaccharides in an aqueous buffer solution.

EXPERIMENTAL

Materials. *T. viride* cellulase ONOZUKA R-10 was purchased from Yakult Pharmaceutical Industry Co. Ltd., Japan. *p*-Nitrophenyl β-D-glucopyranoside (*p*NP β-D-Glc*p*), sophorose, laminaribiose, and gentiobiose were purchased from Sigma Chemical Co., USA. The series of cellooligosaccharides from cellobiose through cellopentaose were purchased from Seikagaku Co., Japan.

Analytical Methods. Protein concentration was determined spectrophotometrically at 280 nm or by the method of Lowry et al.,[11] using bovine serum albumin as standard. Elemental analysis was carried out with a 185 analyzer (Hewlett-Packard Ltd., USA). HPLC was carried out using a LC10-VP apparatus (Shimadzu Co., Japan) with TSK gel amide-80 column (Tosoh, Co., Japan). The ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded on an Avance 600 spectrometer (Bruker Co., Germany) or an MSL-400 spectrometer (Bruker) at 23 °C. In recording one-dimensional ^{1}H NMR spectra, the water-eliminated FT sequence[12] was used. The ^{1}H and ^{13}C chemical shifts were referenced to internal 4,4-dimethyl-4-silapentane sodium sulfonate (DSS), 0.015 ppm, and acetone, 31.55 ppm, respectively. Double-quantum filter ^{1}H, ^{1}H-correlated spectroscopy (DQF-COSY) and total correlation spectroscopy (TOCSY) with a spin-lock time of 60 ms were used to assign ^{1}H resonances. Heteronuclear single quantum coherence (HSQC) and ^{1}H-decoupled multiple-bond heteronuclear multiple quantum coherence (HMBC) were used to assign ^{13}C resonances. Position of glycosyl-linkage was determined by rotating frame nuclear overhauser and exchange spectroscopy (ROESY) experiment with a spin lock time of 300 ms. All the NMR experiments were performed according to standard pulse sequence. The structural analysis of the oligosaccharides was carried out by observing the marked down-field shifts of ^{1}H and ^{13}C resonances,[13] and the type of residual rotating frame nuclear overhauser and exchange (ROE) connectives from newly bounded glucose residue.

β-Glucosidase activity. 50 μL of enzyme solution was incubated with 1 mM *p*NP β-D-Glc*p* (950 μL) in 50 mM acetate buffer, pH 5.0, (buffer *A*) at 40 °C for 10 min. The reaction was stopped by heating the solution at 100 °C for 5 min and then filtering it. The amount of released *p*-nitrophenol in the filtrate was determined by absorbance at 405 nm. One unit of β-glucosidase corresponds to the amount of enzyme that produces 1 μmol of *p*-nitrophenol per min.

Cellobiose hydrolysis. The standard procedure for cellobiose hydrolysis was as follows: 50 μL of enzyme solution (0.1 unit) and 2 mM cellobiose solution (950 μL) dissolved in buffer *A* was incubated at 40 °C for 20 min. The reaction mixture was heated at 100 °C for 5 min and filtered. The amount of glucose in the filtrate was measured by the mutarotase-glucose oxidase method.[14]

Partial purification of β-glucosidase with transglycosylation ability from cellulase. Powder (2g) of cellulase ONOZUKA R-10 (β-glucosidase activity; 0.051 units/mg-protein) was dissolved in buffer *A*. To this solution was added solid $(NH_4)_2SO_4$ to give 80 % saturation. The precipitate formed was collected by centrifugation and desalted by ultrafiltration using a Q0100 filter (Advantec Toyo Co., Japan). The enzyme solution (266 mg protein, 0.121 units/mg-protein) was applied to a Hi Load Q HP column (Pharmacia LKB Biotechnology Co., Sweden) equilibrated with buffer *A* and then eluted with linear gradient of NaCl (0-550 mM) in the buffer *A*. Fractions (25 mL each) were collected, and 50 μL of each fraction was incubated with 500 mM cellobiose (950 μL) in buffer *A* for 12 h at 40 °C. The reaction was stopped at 100 °C for 5 min. The products of the reaction mixture were quantified by HPLC on the basis of peak areas using standard glucose and cellooligosaccharides (degree of polymerization: 2-5). Fractions that could produce trisaccharide (Fraction number 18–21 in Fig. 1) were collected and concentrated to ca. 3 mL by ultrafiltration. The enzyme solution (45.6 mg-protein, 0.057 units/mg-protein) was directly applied to a Superdex 75 HR10/30 column (Pharmacia), eluted with buffer *A* with a flow rate of 1 mL/min. Fractions (1 mL of each) were collected, and the trisaccharide productions of these fractions were estimated according to the procedure described above. Fractions that could produce trisaccharide (Fraction number 16–29, data not shown) were collected and lyophilized to give 16.6 mg β-glucosidase (0.141 units/mg-protein). The enzyme was used in the experiments without further purification.

Measurement of molecular weight. SDS-PAGE was carried out according to Laemmli,[15] using 12.5 % gel. In order to estimate the molecular weight of the purified β-glucosidase, the calibration curve obtained by using molecular marker kit (Pharmacia) was used.

Effect of pH and temperature on cellobiose hydrolysis. The optimum pH for hydrolysis of cellobiose was measured by the standard procedure using 50 mM acetate buffer (pH 3.0-6.0) or 50 mM phosphate buffer (pH 6.0-8.0) in spite of the presence of buffer *A*. The optimum temperature for the hydrolysis of cellobiose was determined by carrying out the standard procedure at various temperatures between 25 °C and 80 °C. Fifty mM glycine buffer (pH 2.0-3.0), acetate buffer (pH 3.0-6.0), and phosphate buffer (pH 6.0-9.0) was used to measure pH stability. The enzyme solutions at the various pH buffers were kept at 4 °C. After 24 h, the remaining activity toward cellobiose of the enzyme solution was measured according to the standard procedure. The thermal stability was also measured. The enzyme solutions in buffer *A* were incubated at various temperatures between 4 °C and 90 °C for 30 min. The remaining activity toward cellobiose of the incubated enzyme solution was measured according to the standard procedure.

Substrate specificity. Two mM disaccharide solution (950 μL) in buffer *A* was mixed with 50 μL of enzyme solution (0.1 unit) and incubated at 40 °C. An aliquot (100 μL) was withdrawn at appropriate time intervals. The aliquot was heated at 100 °C for 5 min and then filtered off. The amount of glucose in the filtrate was analyzed by the mutarotase-glucose oxidase method.[14]

Effect of pH and temperature on the transglycosylation of cellobiose. The optimum pH for the trisaccharide production was determined as follows. A solution (1 mL) of acetate buffer (pH 4.0-6.0) or phosphate buffer (pH 8.0) containing cellobiose (0.5 mmol, 171 mg) and the purified β-glucosidase (0.141 units) was incubated at 40 °C. Samples were removed at different intervals, heated at 100 °C for 5 min, and filtered off. The amount of trisaccharide in the filtrate was analyzed by HPLC. The optimum temperature was determined as follows. A solution (1 mL) of buffer *A* containing cellobiose (0.5 mmol, 171 mg) and the purified β-glucosidase (0.141 units) was incubated at various temperature between 30 and 60 °C. Samples were removed at different intervals, heated at 100 °C for 5 min, and filtered off. The amount of trisaccharide in the filtrate was analyzed by HPLC.

β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (1) and **β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (2)**. A solution (1 mL) of buffer *A* containing cellobiose (0.5 mmol, 171 mg) and the purified β-glucosidase (0.141 units) was incubated at 40 °C. After 24 h, the enzyme was denatured by heating at 100 °C for 5 min and filtering it off. The filtrate was applied onto a Bio-Gel P-2 column (Bio Rad Labs., USA, 2.5 x 120 cm). The column was eluted with water. The sugar content of the fraction was estimated by the Nelson[16]-Somogyi[17] method. Fractions containing trisaccharide and tetrasaccharide were pooled and lyophilized to give 1 (32.2 mg, 12.8 % yield) and 2 (6.4 mg, 1.9 %), respectively. On the other hand, no oligomers were formed in the reaction without the β-glucosidase.

Anal. Calcd for 1 $C_{18}H_{32}O_{16}$ (504.4): C, 42.86; H, 6.39. Found (for 1): C, 42.73; H, 6.38.

Anal. Calcd for 2 $C_{24}H_{42}O_{21}$ (666.6): C, 43.24; H, 6.35. Found (for 2): C, 43.30; H, 6.34.

β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose (3). A solution (1 mL) of buffer *A* containing laminaribiose (171 mg, 0.5 mmol) and the purified β-glucosidase (0.141 units) was incubated at 40 °C for 24 h. After the transglycosylation reaction was stopped by heating at 100 °C for 5 min, the mixture was filtered off. By the purification with Bio-Gel P-2 column chromatography similar to 1 purification, 3 (26.1 mg, 10.3 %) was obtained.

Anal. Calcd $C_{18}H_{32}O_{16}$ (504.4): C, 42.86; H, 6.39. Found: C, 42.69; H, 6.37.

β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-D-glucopyranose (4). A solution (1 mL) of buffer *A* containing gentiobiose (171 mg, 0.5 mmol) and the purified β-glucosidase (0.141 units) was incubated at 40 °C for 24 h. After the transglycosylation reaction was stopped by heating at 100 °C for 5 min, the mixture was filtered off. By the purification with Bio-Gel P-2 column chromatography similar to 1 purification, 4 (34.6 mg, 13.7 %) was obtained.

Anal. Calcd for $C_{18}H_{32}O_{16}$ (504.4): C, 42.86; H, 6.39. Found: C, 42.89; H, 6.34.

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