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Regioselective syntheses of new tri- and tetrasaccharides from β-glucobioses by *Trichoderma viride* β-glucosidase and their structural analyses by NMR spectroscopy

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Abstract

A new β-glucosidase was partially purified from *Trichoderma viride* cellulase complex. This β-glucosidase hydrolyzed β-(1→2)-, β-(1→3)-, β-(1→4)-, and β-(1→6)-linked glucobioses and catalyzed a transglycosylation reaction of cellobiose to give regioselectively β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (yield: 18.8%) and β-D-Glc-(1→6)-β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (3.7%). Furthermore, the enzyme converted laminarabiose and gentiobiose into β-D-Glc-(1→6)-β-D-Glc-(1→3)-D-Glc (15.3%) and β-D-Glc-(1→6)-β-D-Glc-(1→6)-D-Glc (20.2%), respectively. The structures of the products were determined by ^{1}H and ^{13}C NMR spectroscopy. This high regio- and stereoselectivity demonstrated by the enzyme could be applied for oligosaccharide synthesis in general.

Keywords: *Trichoderma viride* β-glucosidase; Transglycosylation reaction; Two-dimensional NMR spectroscopy; Oligosaccharide synthesis

1. Introduction

Cellulase is generally characterized by three components [1,2]: an endoglucanase [EC 3.2.1.4], an exo-glucan cellobiohydrolase [EC 3.2.1.91], and a β-glucosidase [EC 3.2.1.21]. Of these enzymes, the endoglucanase and the exo-glucan cellobiohydrolase act synergistically to hydrolyze crystalline cellulose to form cellooligosaccharides, while the β-glucosidase hydrolyzes the cellooligosaccharide to glucose [1,2]. Because this suggested that β-glucosidase had no direct role in cellulolysis, the catalytic properties of β-glucosidase had not been well studied in comparison with the other cellulase components.

Recently, with increasing interest in oligosaccharide components that serve as information-rich molecules responsible for biological recognition, attention has turned to β-glucosidase because β-glucosidase-catalyzed transglycosylation can be used as a new method of oligosaccharide synthesis. The transglycosylation by β-glucosidase generally proceeds with substrate specificity and stereoselectivity to the acceptor molecules and has the advantage of using free sugars as both acceptor and donor [3–6]. However, wider use of β-glucosidase has been limited, because

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most of the β-glucosidases have no regioselectivities for the hydroxyl linkages to the sugar acceptor. Thus, in many cases, the products of the transglycosylation are obtained as mixtures of regioisomers [5,6]. In order to use 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 β-glucosidase from *Trichoderma viride* cellulase complex [7–9] 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.

2. Experimental

Materials.—*T. viride* cellulase ONOZUKA R-10 was purchased from Yakult Pharmaceutical Industry Co. Ltd., Japan. *p*-Nitrophenyl β-glucopyranoside (*p*NP-Glc), sophorose, laminarabiose, 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. [10], using bovine serum albumin as standard. Elemental analysis was carried out with a Hewlett–Packard model 185 analyzer. HPLC was carried out using a Shimadzu LC10-VP apparatus with a TSK gel amide-80 column (Tosoh, Co., Japan). The ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded on a Bruker Advance 600 spectrometer or MSL-400 spectrometer at 23 °C. In recording one-dimensional ^{1}H NMR spectra, the WEFT sequence [11] 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. Positions of the glycosyl linkages were determined by rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) experiments with a spin lock time of 300 ms. All the NMR experiments were performed according to standard pulse sequences. The structural analysis of the oligosaccharides was carried out by observing the marked downfield shifts of ^{1}H and ^{13}C resonances [12,13] and the type of residual rotating frame nuclear Overhauser and exchange (ROE) connectives from newly bounded glucose residues.

β-Glucosidase activity.—Enzyme solution (50 μL) was incubated with 1 mM *p*NP-Glc (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.—ONOZUKA R-10 powdered cellulase (2 g, with 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 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 (Fractions 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 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 procedure described above. Fractions that could produce trisaccharide (Fractions 16–29, data not shown) were collected and lyophilized to give 16.6 mg of β-glucosidase (0.141 units/mg protein). The enzyme was used in the following experiments without further purification.

Measurement of molecular weight.—SDS–PAGE was carried out according to the method of Laemmli [15] using 12.5% gel. From the calibration curve obtained by using a molecular marker kit (Pharmacia), the molecular weight was estimated as ca. 68.0 kDa.

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 and 80 °C. A 50 mM sample of 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 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.—A 2 mM sample of 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].

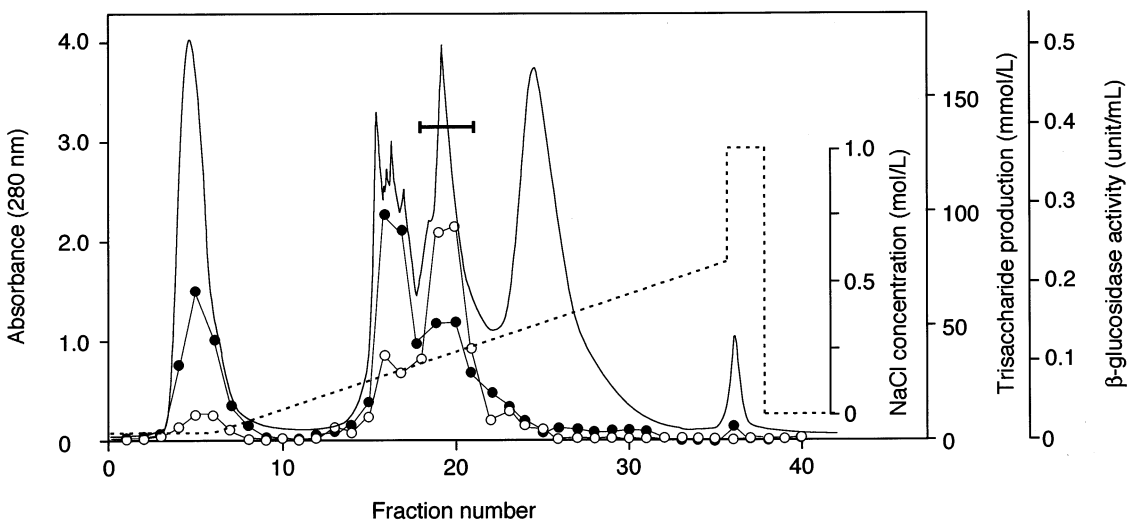
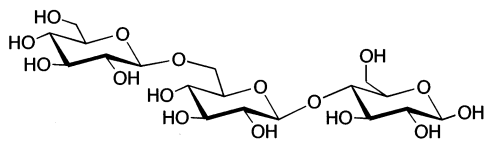


Fig. 1. Purification of β-glucosidase on a Hi Load Q HP column. β-Glucosidase activity (●) and trisaccharide production (○) were measured. The solid and broken lines represent absorbance at 280 nm and NaCl concentration, respectively.

Syntheses of β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (**1**) *and β-D-Glc-(1→6)-β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc* (**2**) *by transglycosylation of cellobiose.*—A solution (1 mL) of buffer A containing cellobiose (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 the resulting suspension was filtered. The filtrate was applied onto a Bio-Gel P-2 column (Bio Rad Labs, 2.5 × 120 cm). The column was eluted with water. The sugar content of the fraction was estimated by the Nelson–Somogyi [16,17] method. Fractions containing trisaccharide and tetrasaccharide were pooled and lyophilized to give **1** (32.2 mg, 18.8% yield) and **2** (6.4 mg, 3.7%), respectively. On the other hand, no oligomers were formed on the reaction without the β-glucosidase. Anal. Calcd for $C_{18}H_{32}O_{16}$ (**1**): C, 42.86; H, 6.39. Found: C, 42.73; H, 6.38. Anal. Calcd for $C_{24}H_{42}O_{21}$ (**2**): C, 43.24; H, 6.35. Found: C, 43.30; H, 6.34.



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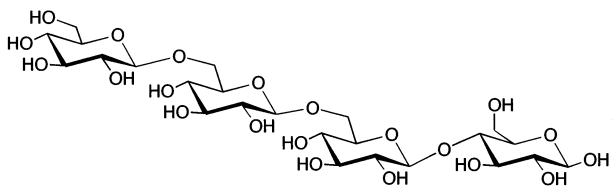

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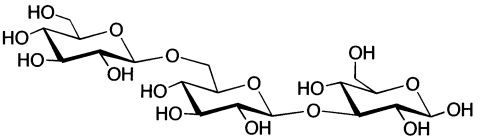
Synthesis of β-D-Glc-(1→6)-β-D-Glc-(1→3)-D-Glc (**3**) *by transglycosylation of laminarabiose.*—A solution (1 mL) of buffer A containing laminarabiose (171 mg) and the purified β-glucosidase (0.141 units) was incubated at 40 °C for 24 h. After the transglycosylation reaction was stopped by heating at 100 °C for 5 min, the resulting suspension was filtered. By purification of the filtrate with Bio-Gel P-2 column chromatography similar to the purification of **1**, 26.1 mg of **3** was obtained (15.3% yield). Anal. Calcd for $C_{18}H_{32}O_{16}$ (**3**): C, 42.86; H, 6.39. Found: C, 42.69; H, 6.37.



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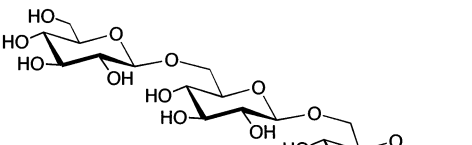
Synthesis of β-D-Glc-(1→6)-β-D-Glc-(1→6)-D-Glc (**4**) *by transglycosylation of gentiobiose.*—A solution (1 mL) of buffer A containing gentiobiose (171 mg) and the purified β-glucosidase (0.141 units) was incubated at 40 °C for 24 h. After the transglycosylation reaction was stopped by heating at 100 °C for 5 min, the resulting suspension was filtered. The filtrate was applied to a Bio-Gel P-2 column and chromatographed similarly to **1** to give 34.6 mg of **4** (20.2%). Anal. Calcd for $C_{18}H_{32}O_{16}$ (**4**): C, 42.86; H, 6.39. Found: C, 42.89; H, 6.34.



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3. Results and discussion

Purification of β-glucosidase and its hydrolytic properties.—For the purification of the β-glucosidase having transglycosylation ability from the *T. viride* cellulase complex, Hi Load Q HP column chromatography was performed. The elution of the cellulase after the ammonium sulfate precipitation is shown in Fig. 1. Three peaks of β-glucosidase activity associated with the protein peaks were observed. Of these, the β-glucosidase fraction that eluted at the end of the salt gradient and was shown to have transglycosylation activity was further 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). This β-glucosidase fraction had high transglycosylation ability, as described later in this paper.

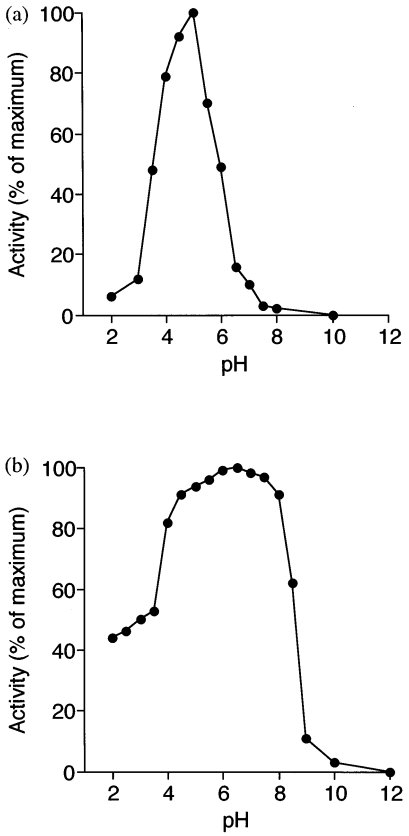

Fig. 2. Effect of pH on *T. viride* β-glucosidase. (a) Optimum pH for the hydrolysis of cellobiose. (b) Effect of pH on the stability of *T. viride* β-glucosidase.

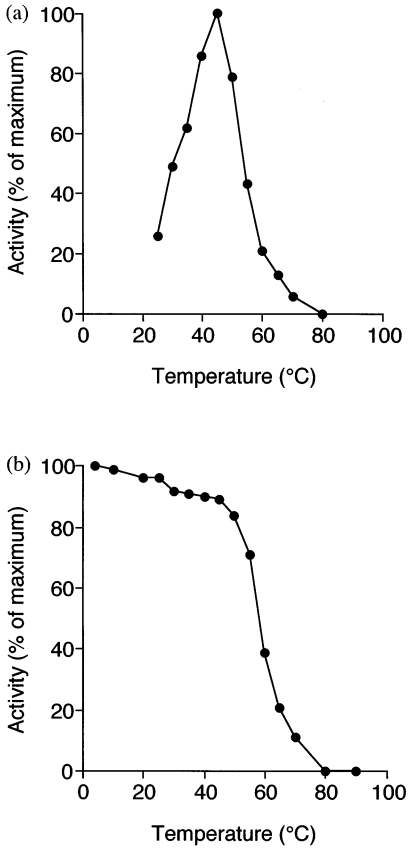

Fig. 3. Effect of temperature on *T. viride* β-glucosidase. (a) Optimum temperature for hydrolysis of cellobiose. (b) Thermal stability of *T. viride* β-glucosidase.

In the examination of the effect of pH on hydrolysis of cellobiose, as shown in Fig. 2, 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. In the experiment measuring the effect of temperature, as shown in Fig. 3, the optimum temperature for cellobiose hydrolysis of the enzyme was observed to be 45 °C. The purified enzyme was sensitive to temperatures above 50 °C at pH 5.0. The optimum pH and temperature for hydrolysis of cellobiose by purified β-glucosidase corresponded to those by cellulase before the purification (data not shown). 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 [6], 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).

Syntheses of oligosaccharides **1–4** *by transglycosylation of β-glucobioses using the purified β-glucosidase.*—We tried to perform the transglycosylation reaction with cellobiose, laminarabiose, gentiobiose, or sophorose as substrates using the purified β-glucosidase. Fig. 4 shows the elution profile of transglyco-

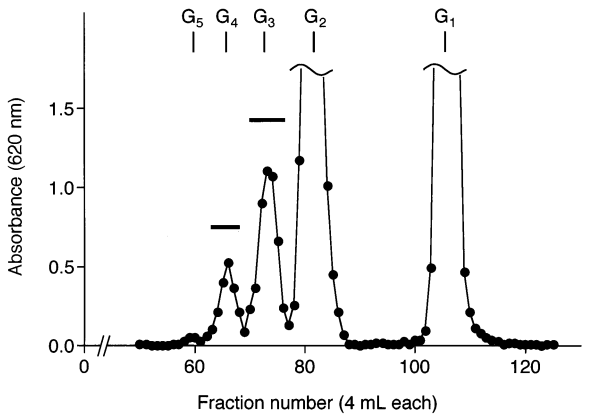

Fig. 4. Gel-chromatographic separation of transglycosylation products by the action of β-glucosidase on cellobiose. G_1 and G_2–G_5 indicate the elution positions of glucose and the series of cellooligosaccharides, respectively.

Table 1
^{1}H chemical shifts (ppm) of the saccharides in D_2O at 23 °C

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

[a] Numbers indicate the position of glucose residues from the reducing-end unit of the oligosaccharides.

sylation reaction products of cellobiose by the purified β-glucosidase from Bio-Gel P-2. Fractions G_1–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 tri- (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 ^{1}H and ^{13}C NMR spectroscopy (Tables 1 and 2, respectively) as β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (**1**) and β-D-Glc-(1→6)-β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (**2**), respectively. There were no signals derived from the other tri- and tetrasaccharides in the NMR analysis. This result indicated that the D-glucose residue at the nonreducing end of cellobiose was regioselectively transferred to O-6 of D-glucose residue at the nonreducing end of another cellobiose to produce trisaccharide **1**. Furthermore, the β-glucosidase regioselectively catalyzed the transferring β-D-glucose residue of cellobiose to O-6 of the nonreducing terminus of product **1** to synthesize tetrasaccharide **2**.

A quite similar regioselectivity was observed in the transglycosylation reaction that used laminarabiose or gentiobiose as a substrate instead of cellobiose. Trisaccharides were synthesized from laminarabiose 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 laminarabiose and gentiobiose also were regioselectively converted into the trisaccharides β-D-Glc-(1→6)-β-D-Glc-(1→3)-D-Glc (**3**) and β-D-Glc-(1→6)-β-D-Glc-(1→6)-D-Glc (**4**), respectively. Production of no other regioisomers from laminarabiose and gentiobiose was observed by HPLC analysis.

On the other hand, when sophorose was used as a substrate for the transglycosylation reaction, a trisaccharide was not obtained. In the products from sophorose, small amounts of gentiobiose and cellobiose were detected, which indicated that the β-glucosidase could

Table 2
^{13}C chemical shifts (ppm) of the saccharides in D_2O at 23 °C

Residue [a]	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (**1**)						
1α	94.63	73.97	74.34	82.28	72.79	62.79
1β	98.51	76.65	77.48	82.07	77.26	62.93
2	105.49	75.87	78.43	72.28	77.61	71.48
3	105.50	75.94	78.22	72.41	78.69	63.53
β-D-Glc-(1→6)-β-D-Glc-(1→6)-β-D-Glc-(1→4)-D-Glc (**2**)						
1α	94.60	74.00	74.32	82.27	72.81	62.81
1β	98.49	76.69	77.25	82.04	77.51	62.94
2	105.50	75.91	78.46	72.23	77.73	71.43
3	105.70	75.85	78.33	72.45	77.70	71.89
4	105.72	75.91	78.24	72.43	78.71	63.53
β-D-Glc-(1→6)-β-D-Glc-(1→3)-D-Glc (**3**)						
1α	94.68	73.46	85.10	70.35	72.86	62.78
1β	98.51	75.21	87.32	70.42	77.68	69.91
2	105.50	75.53	78.20	71.90	77.79	71.38
3	105.31	75.62	78.12	71.95	78.42	63.42
β-D-Glc-(1→6)-β-D-Glc-(1→6)-D-Glc (**4**)						
1α	94.62	73.26	74.40	71.76	72.76	71.26
1β	98.48	76.65	77.80	71.87	77.94	71.06
2	105.50	75.12	77.86	71.96	77.76	71.42
3	105.51	75.46	78.19	72.30	78.60	63.49

[a] Numbers indicate the position of glucose residues from the reducing-end unit of the oligosaccharides.

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 the transglycosylation reaction, although the enzyme could hydrolyze all the β-glucobioses.

Regarding the mechanism of action with synthetic and natural substrates of glycosidases, *Escherichia coli* β-galactosidase [18–20] has been studied quite extensively. Research has shown that the β-galactosidase has two subsites for binding lactose (β-D-Gal-(1→4)-D-Glc), one for the galactose residue and the other for the glucose residue [18–20]. By the time the β-galactosidase has caused breakage of the glycosidic bond of lactose, glucose has diffused away and the enzyme is in the 'galactosyl' form. In the intermolecular transglycosylation of lactose, the enzyme converts lactose into allolactose (β-D-Gal-(1→6)-D-Glc). In the transglycosylation of lactose by the β-galactosidase, Huber et al. [19] suggested that the glucose site of the 'galactosyl' form of the galactosidase becomes highly specific for binding glucose after the glycosidic bond of lactose is broken, and it is best to bind glucose more tightly at the C-6 hydroxyl group, not the other hydroxyl group, because it is in close contact with the active galactose in the galactosyl form of the enzyme. Further, Huber and Gaunt [21] suggested that definite specificities for binding of the transglycosylation products were found at the acceptor site (glucose site) of the β-galactosidase. With respect to β-glucosidase-catalyzed transglycosylation, Gopalan et al. [22] 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, laminarabiose, gentiobiose, or product **1** to bring the C-6 hydroxyl group of the nonreducing end of the acceptor close to the C-1 hydroxyl group of glucose within the donor binding-site in order to produce **1**–**4**, respectively. To clearly elucidate the regioselectiviy 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 [3,5,7], higher plants [4,6], and animals [22] 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.

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