Enzymatic synthesis of gentiooligosaccharides by transglycosylation with β-glycosidases from *Penicillium multicolor*

Yoshinori Fujimoto,[a] Takeshi Hattori,[b] Shuji Uno,[b] Takeomi Murata[b] and Taichi Usui[a,b,*]

[a]Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, Ohya 836, Suruga ward, Shizuoka 422-8529, Japan

[b]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University Ohya 836, Suruga ward, Shizuoka 422-8529, Japan

*Correspondence author. Tel./fax: +81 54 238 4873; e-mail: actusui@agr.shizuoka.ac.jp

Abstract—A crude enzyme preparation from *Penicillium multicolor* efficiently produced mainly gentiotriose to gentiopentaose (d.p. 3–5) by transglycosylation using a high concentration of gentiobiose as the substrate. The resulting gentiotriose was examined in a gustatory sensation test using human volunteers, and was determined to have one-fifth of the bitterness of gentiobiose. The crude enzyme preparation was analyzed by chromatography to determine the enzyme responsible for formation of the gentiooligosaccharides. The transglycosylation was shown to take place in two stages by a combination of β-glucosidase and β-(1→6)-glucanase. In the initial stage, which was the rate-limiting step in the overall process, β-glucosidase produced mainly gentiotriose from gentiobiose. In the second step, β-(1→6)-glucanase acted on the resulting gentiotriose, which served as both donor and acceptor, to produce a series of gentiooligosaccharides (d.p. 4–9) by transglycosylation.

Keywords: Gentiooligosaccharide; Enzymatic synthesis; Transglycosylation; *Penicillium multicolor*; Bitterness sugar

1. Introduction

There is a growing appreciation of the potential of biological oligosaccharides. Gentiobiose is well known as a representative sugar possessing a bitter taste. This bitterness makes gentiobiose useful as a taste improver for certain beverages.[1] However, there have been only a limited number of reports of free gentiobiose as a oligosaccharin in xylem sap[2] and in ripening tomato fruit.[3] In general, gentiooligosaccharides are not prevalent as free molecules. For example, β-(1→6)-linkages occur frequently in the glucans of yeast and filamentous-fungal cell walls.[4-7] Gentiooligosaccharides (d.p. 2–3) have also been noted as prebiotic substances showing bifidogenetic activity.[8] Such characteristics suggest the further potential of these gentiooligosaccharides in food and biology.

Although gentiooligosaccharides have been obtained from partial hydrolysis of the lichen polysaccharide pustulan,[9] this procedure gives only low yields of the desired higher gentiooligosaccharides (d.p. >3). Chemical methods for obtaining gentiooligosaccharides have been also developed,[10] but they involve various elaborate procedures for protection, glycosylation, and deprotection. In general, enzyme-catalyzed synthesis of oligosaccharides is a useful method because it allows the formation of well-defined oligosaccharides selectively in the absence of any protecting groups.[11,12] From a practical viewpoint, the use of glycosidases is especially attractive for oligosaccharide synthesis, because these enzymes are generally more available and less expensive than glycosyltransferases, and do not require expensive sugar nucleotide donors. Thus, glycosidases usually hydrolyze glycosidic bonds, but they can be used for glycoside formation.

Our purpose is to develop an efficient synthetic method to obtain gentiooligosaccharides in sufficient amounts and to discover other functions of these oligosaccharides. The present paper describes the facile enzymatic conversion of gentiobiose into a series of gentiooligosaccharides by transglycosylation mediated by a combination of β-glucosidase and β-(1→6)-glucanase from *P. multicolor*. In addition, we have elucidated the mechanism underlying formation of the gentiooligosaccharides.

2. Results

2.1 Gentiooligosaccharide production (d.p. 3–5) by crude enzyme preparation

A crude enzyme preparation from *P. multicolor* IAM7153 was used directly without purification for the synthesis of gentiooligosaccharides. The enzyme reaction was carried out at a high substrate concentration of gentiobiose (15.9 mmol). Chromatographic separation was carried out on a column of a charcoal-Celite with a linear gradient of ethanol as shown in Fig. 1. The chromatogram showed six fractions (G_2 to G_7), numbered according to their order of elution; the four main fractions (G_2 to G_5) were separated and analyzed. Fraction G_2 was recovered as gentiobiose (2.8 g), and this excess of unreacted gentiobiose was reused for the synthesis. Fraction G_3 was recovered as target gentiotriose in a yield of 10.4% based on the gentiobiose added. The G_4 and G_5 fractions were further purified on a Bio-gel P-2 column to separate them into two fractions (G_4; $G_{4\text{-}1}$ and $G_{4\text{-}2}$ and G_5; $G_{5\text{-}1}$ and $G_{5\text{-}2}$). In this case, $G_{4\text{-}1}$ and $G_{5\text{-}1}$ were obtained as gentiotetraose and gentiopentaose in yields of 2.7 and 0.8%, respectively, based on the gentiobiose added. Fraction $G_{4\text{-}2}$ was obtained as the unwanted isomer of gentiotriose, 4'-*O*-glucosyl-β-gentiobiose.[13] The structures of these fractions were elucidated by ^{1}H and ^{13}C NMR analyses in D_2O, coupled with published data.[9,10,13] In addition, ESIMS analysis of $G_{4\text{-}1}$, $G_{4\text{-}2}$ and $G_{5\text{-}1}$ showed molecular ions at m/z 689.2109, 527.1581 and 851.2627, respectively, arising from the $[M + Na]^+$ ions. In summary, the crude enzyme preparation was shown to produce gentiotriose as a main product plus gentiotetraose and gentiopentaose by transglycosylation from a starting substance of gentiobiose.

2.2 Bitterness of gentiotriose

The degree of bitterness of the gentiotriose prepared above was quantified. Because gentiotriose is expected to have a degree of bitterness similar to that of gentiobiose, in the present study gentiobiose was used as a standard in order to evaluate the bitterness of the target gentiotriose according to the method of Nakamura *et al.*[14] Six human volunteers

performed the sensory evaluation of the gentiotriose relative to gentiobiose. A concentration of gentiobiose of 0.3% (w/v) was defined arbitrarily as a bitterness of 100. In this case, the relative intensity of gentiotriose relative to gentiobiose was determined to be 18.9, about a one-fifth of the bitterness of gentiobiose.

2.3 Comparison of transglycosylation by partially purified β-glucosidase with β-(1→6)-glucanase

To determine the enzyme responsible for the gentiooligosaccharide production mentioned above, the crude enzyme preparation was applied to a column of DEAE-Sepharose Fast Flow. This chromatography step served to separate completely a peak showing β-glucosidase activity (F-1) with assays A and B from a peak showing β-(1→6)-glucanase activity (F-2) with assay C, as shown in Fig. 2. These two partially purified enzyme fractions were used to analyze the mode of transglycosylation as follows. Transglycosylation of gentiobiose after 27 h by the crude enzyme was first compared with that by the partially purified β-glucosidase by HPLC analysis. The reaction with the crude enzyme gave gentiotriose and gentiotetraose, plus the gentiotriose isomer, 4'-*O*-glucosyl-β-gentiobiose. When gentiotriose was used as the substrate instead of gentiobiose, the crude enzyme produced gentiotetraose and gentiopentaose in appreciable amounts over the entire course of the reaction (Fig. 3a). In contrast, the reaction with partially purified β-glucosidase gave mainly gentiotriose plus its isomer, and little gentiotetraose. With gentiotriose, the partially purified β-glucosidase formed a little gentiotetraose, but no gentiopentaose (Fig. 3b). These results suggested that an enzyme other than β-glucosidase also participated in the transglycosylation process.

Next, we examined the participation of β-(1→6)-glucanase in the present enzyme system. In this case, the amount of product as a function of time was analyzed by HPAEC-PAD instead of HPLC because this method is more suitable for detecting higher oligosaccharides. When gentiobiose was used as the initial substrate, the enzyme did not act on it under the present conditions. In contrast, it carried out transglycosylation when gentiotriose was used as a substrate. Gentiopentaose was first produced and its formation obeyed first-order kinetics within 2 h (Fig. 4a). Once the maximum production was reached, the concentration of the pentaose varied little during the entire course of reaction. Much more

pentaose than tetraose was found in the initial stages of the reaction, but the relationship between this yield was reversed in the latter stages of the reaction. In the subsequent reaction, hexaose to nonaose oligomers were produced and gradually increased in amount over time. In summary, it was shown that the partially purified β-(1→6)-glucanase, which was completely devoid of β-glucosidase, produced a series of oligomers (d.p. 4–9) from a starting substance of gentiotriose (2 g) (Fig. 4b).

2.4 Gentiooligosaccharide synthesis by partially purified β-(1→6)-glucanase-mediated transglycosylation

The partially purified β-(1→6)-glucanase preparation was used for the synthesis of gentioligosaccharides. The reaction was carried out at a high substrate concentration of gentiotriose (0.5 g/mL). The reaction mixture was fractionated into G_2 to G_9 fractions, numbered according to their order of elution, by chromatography on a charcoal-Celite column (Fig. 5). Fraction G_2 contained gentiobiose as a degradation product and G_3 was recovered as unreacted gentiotriose. The G_4 to G_7 fractions, corresponding to the d.p. 4 to 9 gentiooligosaccharides, were separated from each other in one step as transglycosylation products. Fractions G_8 and G_9 were further purified by a Bio-Gel P-4 column to give gentiooctaose and gentiononaose, respectively.

A series of six gentiooligosaccharides was obtained in a total yields of 31.4% (w/w) based on the gentiotriose added. Gentiotetraose, gentiopentaose and gentiohexaose were mainly obtained in yields of 7.8, 11.0 and 5.7%, respectively. No isomers other than the β-(1→6)-linked isomers were detected during the reaction. The reaction shows the regioselective synthesis of gentiooligosaccharides by β-(1→6)-glucanase-mediated transglycosylation. The structures of G_4– G_9 were evaluated by ^{1}H NMR analysis in D_2O, coupled with published data.[9,10,13] Characteristic signals were commonly observed in the region of δ 4.2–5.2 at low field as shown in Fig. 6. In the spectrum of fraction G_4, corresponding to gentiotetraose, the anomeric signals at peaks a (δ 5.22, d, $J_{1,2}$ 3.7 Hz) and b (δ 4.65, d, $J_{1,2}$ 7.9 Hz) were assigned to H-1α and H-1β, respectively. Peak c at δ 4.49~4.54 was observed as overlapping signals due to H-1', H-1", and H-1"'. Signals at peaks d (δ 4.20, d) and e (δ 4.15, d) were assigned to H-6β, H-6', and H-6", and H-6α, respectively.

These characteristic signals of tetraose resonated at the same positions in the spectra of the corresponding gentiooligosaccharides (d.p. 5–9). The ^{1}H NMR spectra also showed that the d.p. of each gentiooligosaccharide was easily determined from the integration data of the anomeric signal. For example, the d.p. of G_5 was observed to be 5, because the relative intensities of peak a + b and peak c were 1:4.

The structures of these gentiooligosaccharides were further confirmed by ^{13}C NMR analysis in D_2O solution, coupled with reference to published data (Table 1).[9, 10,13] Each peak could be assigned to the corresponding carbon atom of an oligosaccharide having a β-(1→6) linkage. The spectrum showed signals of carbon atoms from internal glucose units as well as those of the reducing and nonreducing glucose end units in the gentiooligosaccharides. No signals derived from other linkages were detected. In addition, ESIMS analysis of G_4–G_9 showed molecular ions at m/z 689.2109, 851.2627, 1013.3252, 1175.3712, 1337.4239, and 1499.4748, respectively, arising from the $[M + Na]^+$ ions. These results indicate that the resulting oligosaccharides consist exclusively of stereoregular oligosaccharides with a β-(1→6) unit (gentiooligosaccharide).

3. Discussion

A crude enzyme preparation from *P. multicolor* was shown to catalyze the transglycosylation of gentiobiose to gentiotriose, gentiotetraose, gentiopentaose, and probably hexaose and heptaose as well, from a high concentration of gentiobiose. In this case, gentiotriose was separated in one step from a charcoal-Celite column, whereas the fractions containing gentiotetraose and gentiopentaose were further purified by chromatography using a Bio-Gel P-2 column. As a result, the target gentiotriose was obtained in mmol amounts as a main product together with gentiotetraose and gentiopentaose. The unreacted gentiobiose (> 50%) could be recovered by straightforward chromatography and reused. The reaction led to the predominant synthesis of gentiotriose in sufficient amounts. One of the purposes of this study was to quantify the degree of the perceived bitterness of gentiotriose. The purified gentiotriose was examined for bitterness in a gustatory sensation test using human volunteers and determined to have one-fifth of the bitterness of gentiobiose. The degree of bitterness is

thought to be related to the structure of anomeric center of glycosides.[15] The synthetic product β-D-glucosyl saccharin is intensely bitter and devoid of sweetness.[16] In maltooligosaccharides, the sweetness is well known to decrease with increasing d.p.[17] This relationship might also hold for the bitterness of gentiooligosaccharides. It must be kept in mind that in the present study, the test of bitterness used a standard solution of 0.3% gentiobiose at 20˚C, because gustatory sensation is known to be greatly influenced by experimental conditions (temperature, concentration etc).

To examine the mode of action of transglycosylation, the crude enzyme preparation was applied to DEAE-Sepharose Fast Flow column. This chromatography served to separate completely a peak showing β-glucosidase activity from a peak showing β-(1→6)-glucanase activity. These two partially purified enzymes relate to a process for producing higher gentioligosaccharides, as shown by HPLC analysis. Thus, when partially purified β-glucosidase acted on gentiobiose, it was capable of transferring a glucosyl unit to gentiobiose itself to produce gentiotriose as the main product. However, this enzyme had little effect on gentiotriose. On the contrary, the partially purified β-(1→6)-glucanase acted on gentiotriose, but had little effect on gentiobiose. Thus, this enzyme produced a series of gentiooligosaccharides (d.p. 4–9) from gentiotriose. The reaction led to the preferential synthesis of pentaose over tetraose in the initial stages of the reaction, but the retention reversed in the latter stages. This observation indicates that the enzyme predominantly transfers a gentiobiosyl unit to gentiotriose acceptor molecules to form pentaose. Thus, as the pentaose builds up, the formation of tetraose to heptaose gentiooligosaccharides increases with time. As a result, the chain-elongation reaction from gentiotriose proceeds in sequence to produce tetraose, hexaose and heptaose, which may act as chain carriers in a series of reactions for the formation of octaose, nonaose etc. The enzyme reaction was in fact carried out on a synthetic scale. A series of gentiotetraose to gentiononaose oligosaccharides from a starting substrate of gentiotriose was prepared in a total yield of 18.5% based on the gentiobiose added. This one-pot reaction ensured the regioselectivity of glycosylation to readily afford gentiooligosaccharides.

Such well-defined oligosaccharides possessing the specified d.p. would be useful not only as substrates for glucanase and glucosyltarnsferase, but also as signal molecules called

elicitors in plants.[18-20] Generally, polysaccharide hydrolases such as amylase,[21,22] amylomaltase,[23] cellulose,[24] and lysozyme[25,26] exhibit high regioselectivity of the acceptor and this selectivity is much more predictable than that of glycosidase.[11,12] From these results, it is evident that the formation of gentiooligosaccharides is carried out by a combination of at least two kinds of β-glucosidase and β-(1→6)-glucanase in the crude enzyme preparation. Thus, oligosaccharide production is achieved in two stages by transglycosylation from gentiobiose as an initial substrate as shown in Scheme 1. The first step is the formation of gentiotriose through β-glucosidase-mediated transglycosylation. In the second step, β-(1→6)-glucanase acts on the resulting gentiotriose, which serves as both the donor and the acceptor. The enzyme transfers mainly a gentiobiosyl unit to the gentiotriose and an excess of the starting gentiobiose to produce gentiopentaose and gentiotetraose, respectively. As a result, the resulting tetraose and pentaose, which act as chain carriers in a series of reactions for formation of hexaose to heptaose, produce a series of gentiooligosaccharides. In this case, the formation of triose is a rate-limiting step in the overall process of transglycosylation because, as the triose is formed, the formation of tetraose and pentaose is initiated by β-(1→6)-glucanase.

In conclusion, we have developed a novel enzymatic synthetic method for obtaining a series of gentiooligosaccharides from a starting substrate of gentiobiose. This transglycosylation was achieved in two stages through a combination of β-glucosidase and endo-β-(1→6)-glucanase in the enzyme system from *P. multicolor*. This method also provides a facile way to obtain gentiooligosaccharide with a specified d.p. To our knowledge, this is the first report of such a transglycosylation reaction by these enzymes. In addition, gentiotriose, of which the provision of a sufficient supply has become feasible, was determined to have one-fifth of the bitterness of gentiobiose.

4. Experimental

4.1 General Methods

A crude enzyme preparation from *Penicillium multicolor* IAM7153 (Institute of Molecular

and Cellular Biosciences, The University of Tokyo) was kindly supplied by Amano Enzyme, Inc. (Gifu, Japan). Gentiobiose and *p*-nitophenyl β-glucopyranoside were purchased from Sigma ChemicalCo. (St. Louis, MO, USA). The charcoal-Celite column for the separation of transglycosylation products was prepared as follows: equal parts by weight of dry charcoal (Wako Pure Chemical Industries, Ltd. (Osaka, Japan)) and Celite (Kanto Chemical CO. Inc. (Tokyo, Japan)) were slurried in water and packed into a glass column. Gentiooligosaccharides (d.p. 3–5) were prepared as authentic samples by partial hydrolysis of pustulan (Calibiochem).[7] Glucose assay kits were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Protein assay kits were purchased from Bio-Rad Laboratories (Richmond, CA. U.S.A.). All other chemicals were obtained from commercial sources.

4.2 Enzyme assay

4.2.1. β-Glucosidase activity

β-Glucosidase activity was assayed by two methods. ***Assay A.*** A mixture containing 4 mM gentiobiose in 40 μL of 50 mM sodium acetate buffer (pH 5.5) and an appropriate amount of enzyme in a total volume of 10 μL were incubated in a 96-well microplate for 30 min at room temperature. The reaction mixtures were then incubated for 30 min at 40˚C with 150 μL of Glucose-oxidase peroxidase's reagent and measured photometrically at 492 nm in a microplate reader (Ultrospec Visible Plate Reader II 96, GE Healthcare Bio-Sciences KK, England).[27-29] One unit of activity was defined as the amount of enzyme releasing 1 μmol of glucose per min. ***Assay B.*** This assay was similar to assay A except that the substrate was *p*-nitrophenyl β-D-glucopyranoside (0.5 mM). The reaction was stopped by adding 0.1M Na_2CO_3 (50 μL), and the amount of *p*-nitrophenyl liberated was determined spectrophotometrically at 420 nm. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenyl per min.

4.2.2. β-(1→6)-Glucanase activity

β-(1→6)-Glucanase was assayed (***Assay C***) as follows: A mixture containing 180 μL of substrate (0.5% pustulan) in 50 mM sodium acetate buffer (pH 5.5) and 20 μL of an

appropriate amount of enzyme was incubated for 30 min at 40 °C. The reducing sugars produced were determined directly by Somogyi-Nelson's method.[30, 31]

4.3 Analytical methods

HPLC analysis were performed with a JASCO PU-980 Intelligent HPLC pump, JASCO AS-950 Intelligent Sampler, Alltech Evaporative Light Scattering Detector, and JASCO LCSS-905 HPLC System Station under the following conditions; column, Mightsil Si60 (ϕ 4.6 × 250 mm); column temperature, 70°C; mobile phase, acetonitrile–water (8:2, v/v); flow rate, 1 mL/min. HPAEC analysis was carried out with a DIONEX DX-500 system (Dionex, CA) using a PAD (ED-40) with a CarboPac PA1 column (ϕ 4.0 × 250 mm), and the following gradient: 0–40 min, ratio of eluent A was decreased down to 45% (v/v) from 60% (v/v), ratio of eluent B was increased up to 15% (v/v) from 0% (v/v), and 40% (v/v) eluent C; 40–50 min, 100% (v/v) eluent B; 50–60 min, 60% (v/v) eluent A and 40% (v/v) eluent C. Eluents A, B, and C were deionized water, sodium hydroxide (100 mM) containing sodium acetate (1M), and sodium hydroxide (200 mM), respectively; the flow rate was 1 mL/min. The ^{1}H and ^{13}C NMR spectra of each sample in D_2O were recorded on a JEOL JNM-LA 500 spectrometer at 25˚C. Chemical shifts were expressed in δ relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an external standard. ESIMS spectra were recorded on an JMS-T100LC mass spectrometer. The spectrum of each sample in methanol (1 mg/mL) was injected directly into a methanol flow.

4.4 Enzymatic synthesis of gentiooligosaccharides (d.p. 3–5)

To a solution of gentiobiose (5.43 g, 15.9 mmol) in 27.5 mL of 50 mM sodium acetate buffer was added the crude enzyme preparation from *P. multicolor* IAM7153 (5.8 U by assay A) in 0.1 M sodium acetate buffer (2.5 mL, pH 5.5). The mixture was incubated at 30 °C for 25 h. The reaction mixture was terminated by heating in boiling water for 5 min. The solution was loaded onto a charcoal-Celite column (ϕ4.5 × 50 cm) equilibrated with water. The column was washed with 1.5 L of water (flow rate, 6 mL/min) and eluted with a linear

gradient of 10 (5 L) to 40% (5 L) ethanol. The elution was monitored at 485 nm (carbohydrate content determined by the phenol–H_2SO_4 method). Fractions of 50 mL were collected. The eluate showed four main peaks G_2 (tubes 10-16), G_3 (22-32), G_4 (40-52), and G_5 (56-64), which were concentrated and lyophilized. Fraction G_2 was recovered as gentiobiose (2807 mg) as unreacted substrate and fraction G_3 gave gentiotriose (564 mg). Fractions G_4 and G_5 were further purified by Bio-Gel P-2 column (ϕ 5.0 × 80 cm, 10 mL/fraction). Both the fractions were separated into respective two fractions (G_4; $G_{4\text{-}1}$ (tubes 98–111) and $G_{4\text{-}2}$ (tubes 114–125) and G_5; $G_{5\text{-}1}$ (tubes 91–100) and $G_{5\text{-}2}$ (tubes 102–111). Each fraction was concentrated and lyophilized. Fractions $G_{4\text{-}1}$ and $G_{5\text{-}1}$ were recovered as gentiotetraose (149 mg) and gentiopentaose (44 mg), respectively. Fraction $G_{4\text{-}2}$ was recovered as 4'-*O*-glucosyl-β-gentiobiose (154 mg).

4.5 Gustatory sensation study

The gustatory sensation study was carried out according to the method of Nakamura *et al*.[14] Six human panelists performed the sensory evaluation of gentiotriose relative to gentiobiose as a bitterness standard. Each samples was dissolved in water to make an appropriate concentration corresponding to 0.3% (w/w) gentiobiose solutions with respect to the intensity of bitterness. The panelists were asked to taste the gentiobiose solution and estimate the maximum intensity relative to that of the sample solutions. The bitterness relative to gentiobiose was calculated according to the following formula; $(\sum A/B)$/(the number of tests) × (the number of panelists), where A is the concentration (%) of the gentiobiose solution, and B is the concentration (%) of the gentiotriose solution with the same intensity of bitterness as the gentiobiose solution.

4.6 Chromatographic separation of β-glucosidase and β-(1→6)-glucanase from *P. multicolor*

To a solution (2.5 mL) of the crude enzyme preparation from *P. multicolor* IAM7153 (0.3 g) was added solid ammonium sulfate to give 40% saturation. The resulting insoluble material

was removed by centrifugation. The supernatant was brought to 80% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation, dissolved in deionized water, and dialyzed. The solution was applied directly to a DEAE Sepharose Fast Flow column (ϕ 1.6 × 10 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.5) and eluted with the same buffer (100 mL), a linear gradient of 100% 20 mM sodium acetate buffer (pH 5.5) to 100% 20 mM sodium acetate buffer (pH 5.5) containing 0.5 M NaCl (600 mL) and 20 mM sodium acetate buffer (pH 5.5) containing 0.5 M NaCl (100 mL). A peak (tubes 27–37) containing β-glucosidase activity (by Assays A and B) was eluted at the beginning. The eluates were combined and concentrated to low volume (0.5 mL) by using an Amicon Doaflo unit equipped with a PM-10 membrane (total activity 0.9 U). A peak (tubes 51–53) containing β-(1→6)-glucanase activity (by Assay C) emerged from the column considerably behind the peak with β-glucosidase activity. The eluates were combined and concentrated to low volume (0.7 mL) by using an Amicon Doaflo unit (total activity 4.0 U)

4.7 Analysis of transglycosylation

Analysis of the time course of transglycosylation was done by the two methods of HPLC and HPAEC-PAD.

4.7.1. HPLC.

To a solution (50 μL) of gentiobiose (34 mg) or gentiotriose (50 mg) in water was added each enzyme solution (50 μL; 0.2 U by assay A) in 0.1M sodium acetate buffer (pH 5.5). The mixture was incubated at 30 °C. The amount of transfer products as a function of time was examined on the 0.1 mL scale. Samples (10 μL) were taken at intervals at during the incubation, diluted with 90 μL of water, inactivated by heating in a boiling water bath for 5 min. and then analyzed by HPLC.

4.7.2. HPAEC-PAD.

The enzyme reaction was the same as described above, except that the samples were examined by HPAEC-PAD.

4.8 Enzymatic synthesis of gentiooligosaccharides from gentiotriose utilizing β-(1→6)-glucanase

To a solution of 3.6 mL of gentiotriose (2 g) in sodium acetate buffer, (pH 5.5) was added 0.4 mL of partially purified β-(1→6)-glucanase from *P. multicolor* (280 mU by assay C) After the solution was incubated for 24 h at 30 °C, it was inactivated by boiling for 5 min and applied to a charcoal-Celite column (ϕ 4.5 × 50 cm) equilibrated with water. The column was washed with 1.5 L of water and eluted with a linear gradient of 5% (5 L) to 40% (5 L) ethanol (flow rate, 7 mL/min; a fraction size 50 ml/tube). The elution was monitored at 485 nm, with carbohydrate content, determined by the phenol-H_2SO_4 method. The chromatogram showed eight peaks (G_2: tubes 36–45, G_3: tubes 65–81, G_4: tubes 99–109, G_5: tubes 119–131, G_6: tubes138–149, G_7: tubes 153–163, G_8: tubes 167–173, and G_9: 179–183). Each peak was collected, concentrated, and lyophilized. G_2 and G_3 contained gentiobiose (226 mg) and gentiotriose (642 mg), respectively. G_4, G_5, G_6, G_7, G_8, and G_9 gave gentiotetraose (157 mg), gentiopentaose (221 mg), gentiohexaose (114 mg) and gentioheptaose (79 mg), gentiooctaose (33 mg) and gentiononaose (25 mg), respectively.

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Figure Legends

Fig. 1. Chromatographic separation of transglycosylation products formed by the action of crude enzyme preparation on gentiobiose. Chromatography was carried out on a column (ϕ 4.5 × 50 cm) of charcoal-Celite. The elution positions of G2 ~ G7 correspond to a d.p. 2–7 of gentiooligosaccharides, respectively. Dotted line, ethanol concentration (%).

Fig. 2. Chromatography of the crude enzyme preparation from P. multicolor on a DEAE-Sepharose Fast Flow column (ϕ 1.6 × 10 cm). Filled triangle, Assay A; open circle, Assay B; filled square, Assay C; dotted line, NaCl concentration (M).

Fig. 3. L.C. analysis of transglycosylation products formed by the action of crude enzyme (a) and partially purified β-glucosidase (b) on gentiobiose or gentiotriose. The reaction mixture was analyzed after 27 h as described in Experimental. The retention times of G_2 ~ G_5 correspond to gentiooligosaccharides with d.p. 2–5 of, respectively.

Fig. 4. HPAEC-PAD analysis of transglycosylation products formed by the action of partially purified β-(1→6)-glucanase on gentiotriose. Analytical conditions are described in Experimental. a) Time course of the formation of gentiooligosaccharides. Filled triangle, gentiotetraose; open circle, gentiopentaose; open triangle, gentiohexaose; filled circle, gentioheptaose; filled square, gentiooctaose; open square, gentiononaose. b) Elution pattern after 24 h.

Fig. 5. Chromatographic separation of transglycosylation products formed by the action of partially purified β-(1→6)-glucanase on gentiotriose. Chromatography was carried out on a column (ϕ 4.5 × 50 cm) of charcoal-Celite. The elution positions of G_2–G_9 correspond to gentiooligosaccharides with d.p. 2–9, respectively. Dotted line, ethanol concentration (%).

Fig. 6. ^{1}H-NMR spectra of G_4 ~ G_9 as transglycosylation products in D_2O solution.

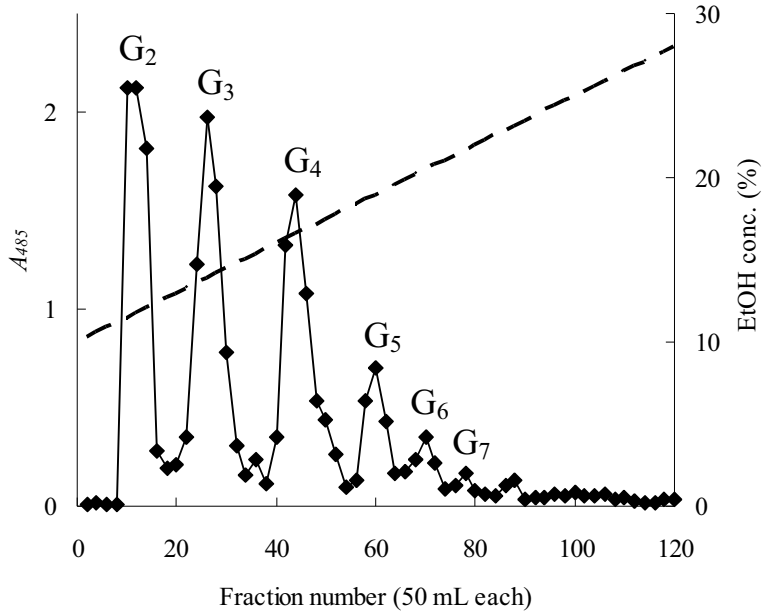


Fig. 1.

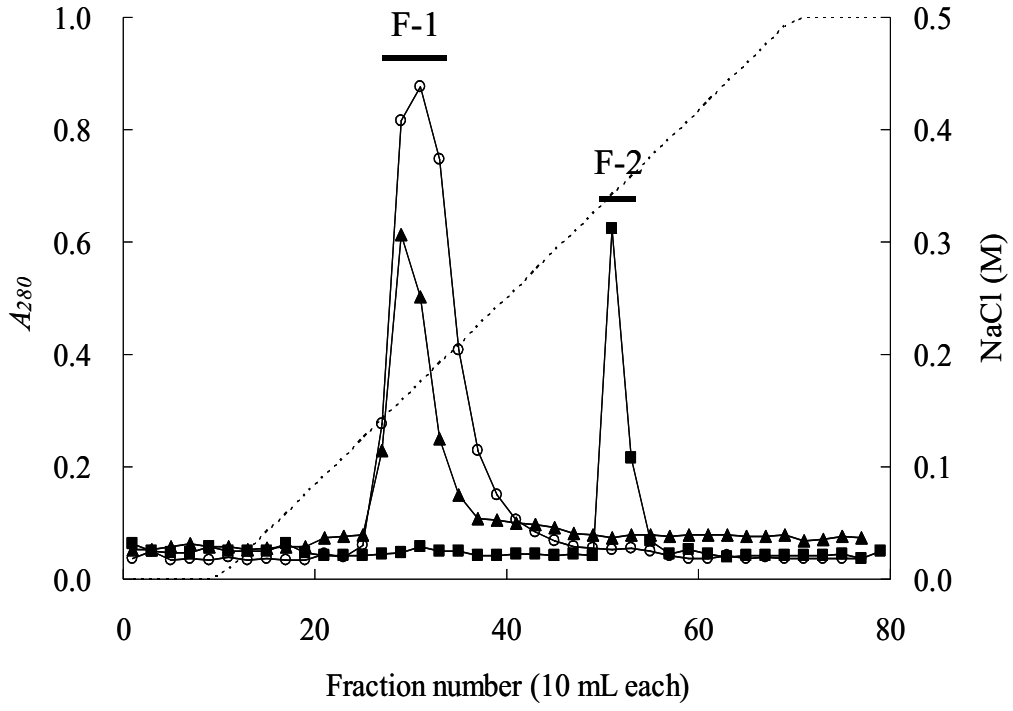

F-1
F-2
1.0
0.8
0.6
0.4
0.2
0.0
0.5
0.4
0.3
0.2
0.1
0.0
A280
NaCl (M)
0
20
40
60
80
Fraction number (10 mL each)

Fig. 2.

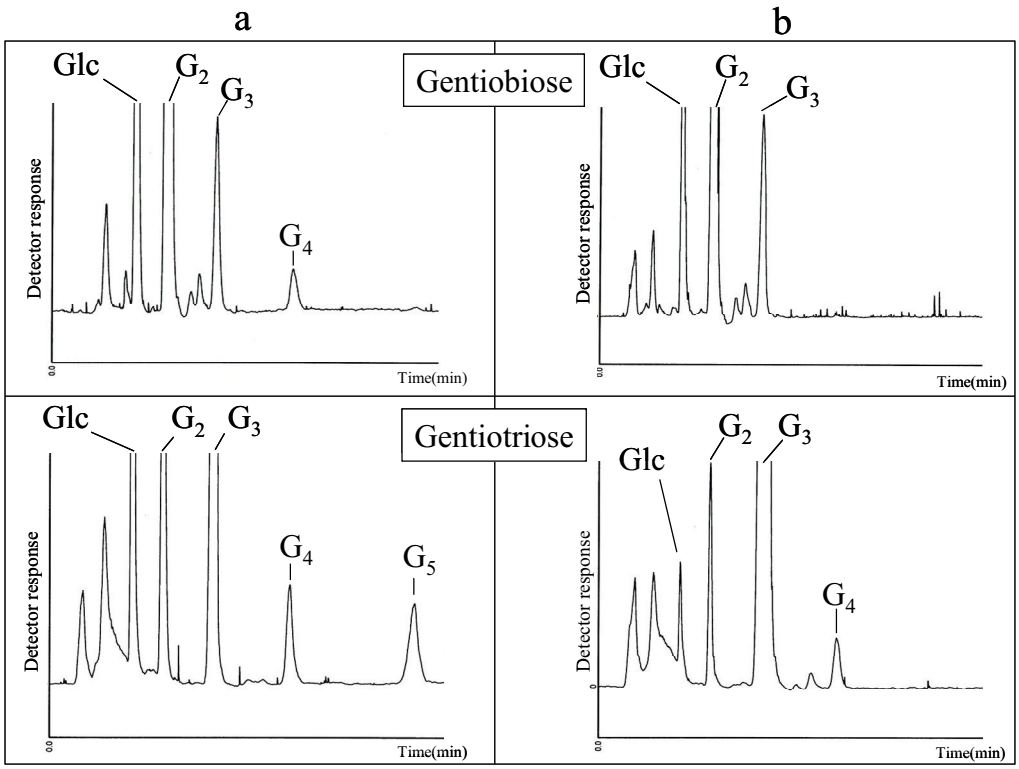

a
b
Glc
G2
G3
G4
Gentiobiose
Detector response
Time(min)
Glc
G2
G3
Detector response
Time(min)
Glc
G2
G3
G4
G5
Gentiotriose
Detector response
Time(min)
G2
G3
Glc
G4
Detector response
Time(min)

Fig. 3.

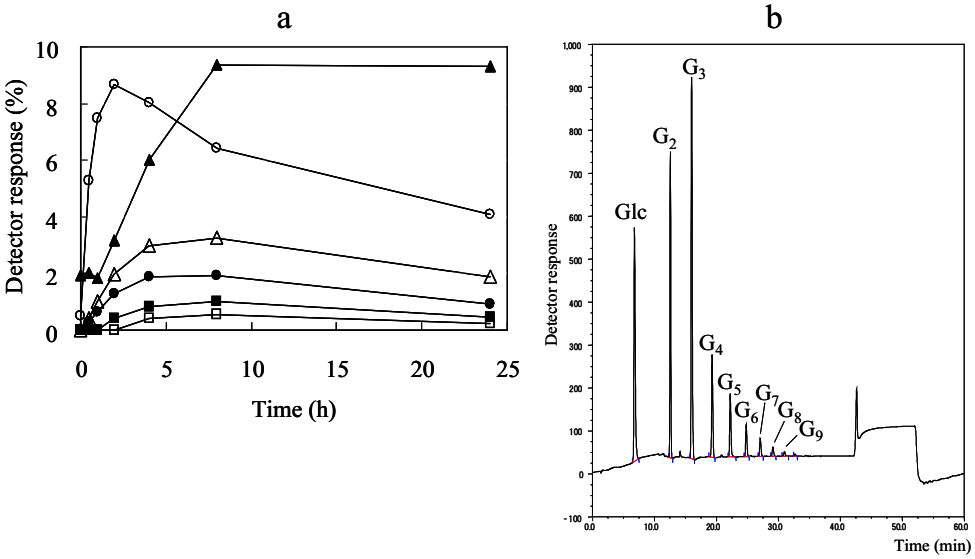

a
b
Detector response (%)
Time (h)
Detector response
Time (min)
Glc
G2
G3
G4
G5
G6
G7
G8
G9

Fig. 4.

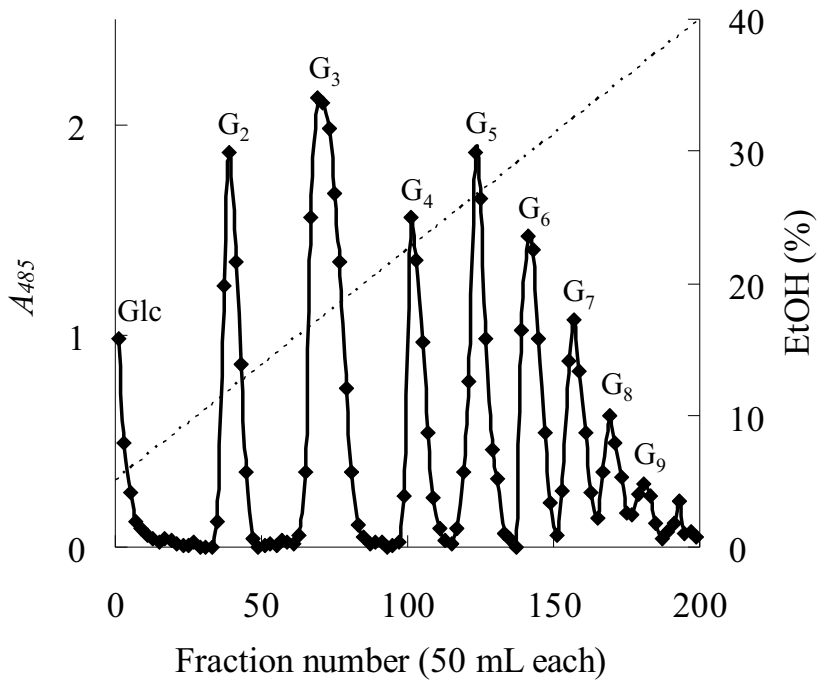

Glc
G2
G3
G4
G5
G6
G7
G8
G9
A485
EtOH (%)
Fraction number (50 mL each)
0
1
2
0
10
20
30
40
0
50
100
150
200

Fig. 5.

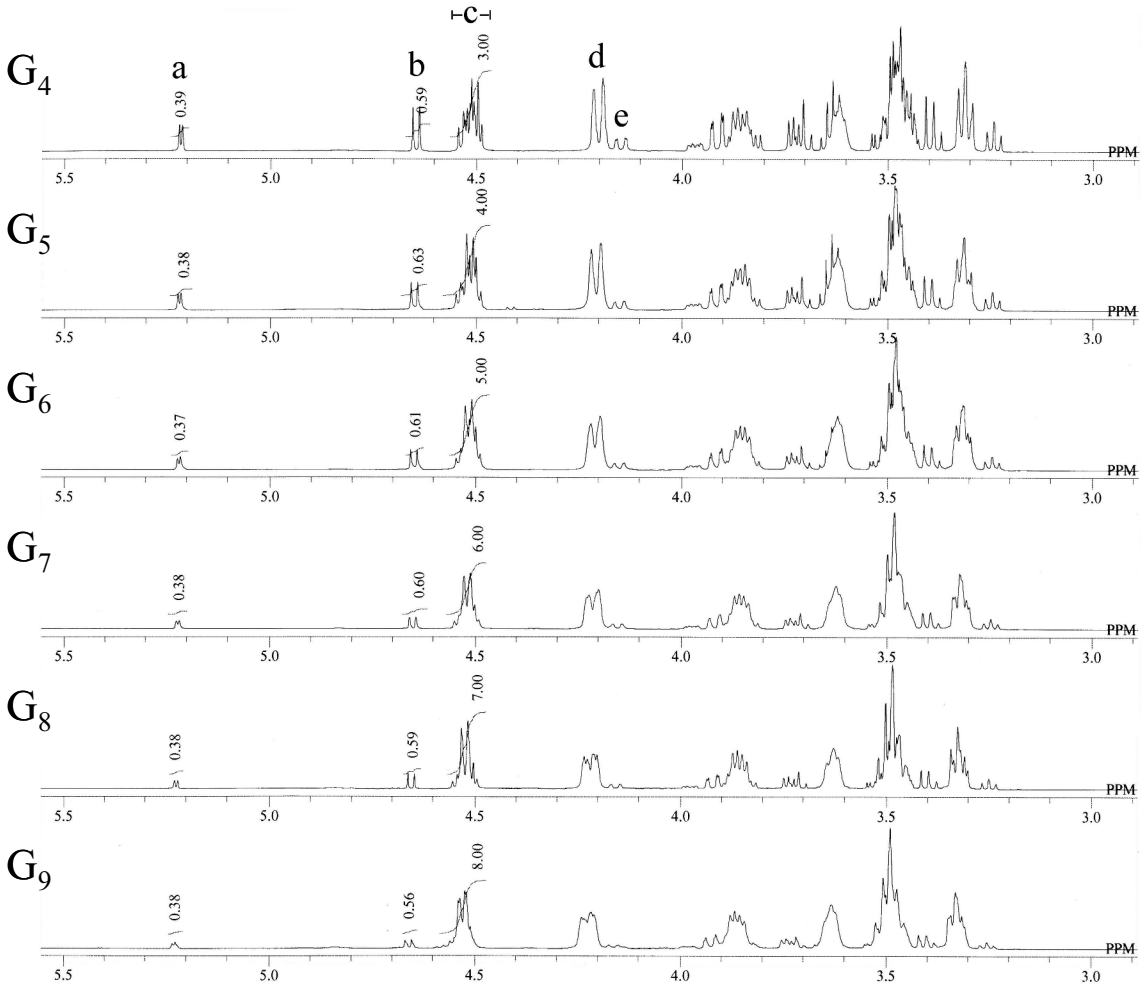
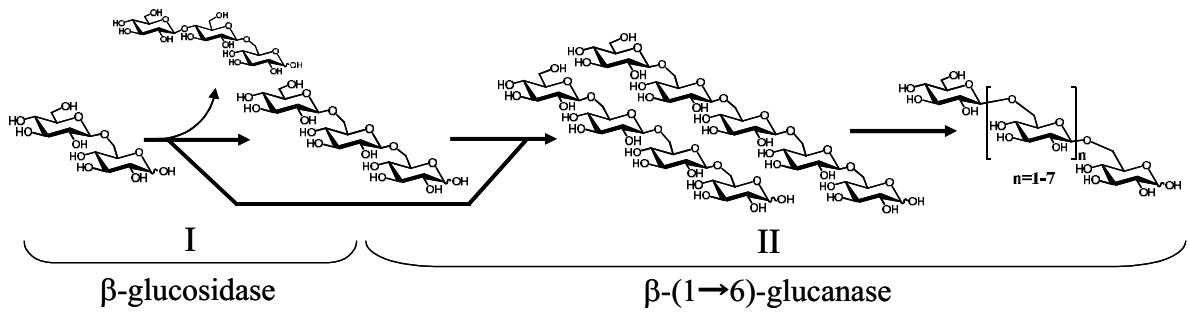

G4
a
b
c
d
e
0.39
0.59
3.00
G5
0.38
0.63
4.00
G6
0.37
0.61
5.00
G7
0.38
0.60
6.00
G8
0.38
0.59
7.00
G9
0.38
0.56
8.00
PPM
5.5
5.0
4.5
4.0
3.5
3.0

Fig. 6.



Scheme 1. Proposed mechanism of the formation of gentiooligosaccharides from gentiobiose in two stages by glycosidases-mediated transglycosylation.

Compounds[a]		Chemical shifts (δ)					
		C-1	C-2	C-3	C-4	C-5	C-6
G_4	Iα[b]	94.93	74.22	75.46	72.22	73.21	71.56
	Iβ	98.77	76.83	78.46	72.32	77.64	71.78
	II	105.53	75.83	78.36	72.22	77.77	71.56
	III	105.78	75.83	78.36	72.22	77.77	71.38
	IV	105.70	75.88	78.46	72.43	78.72	63.53
G_5	Iα	94.92	74.21	75.46	72.22	73.21	71.54
	Iβ	98.76	76.82	78.46	72.31	77.63	71.76
	II	105.53	75.83	78.37	72.22	77.73	71.54
	III	105.81	75.83	78.37	72.22	77.73	71.54
	IV	105.81	75.83	78.37	72.22	77.73	71.44
	V	105.71	75.87	78.46	72.41	78.71	63.53
G_6	Iα	94.92	74.21	75.46	72.24	73.22	71.54
	Iβ	98.76	76.82	78.46	72.31	77.63	71.76
	II	105.50	75.83	78.37	72.24	77.73	71.54
	III, IV	105.82	75.83	78.37	72.24	77.73	71.54
	V	105.82	75.83	78.37	72.24	77.73	71.41
	VI	105.70	75.87	78.46	72.42	78.71	63.56
G_7	Iα	94.92	74.21	75.46	72.25	73.21	71.61
	Iβ	98.76	76.83	78.46	72.31	77.63	71.77
	II	105.50	75.84	78.37	72.25	77.73	71.61
	III-V	105.81	75.84	78.37	72.25	77.73	71.61
	VI	105.81	75.84	78.37	72.25	77.73	71.41
	VII	105.70	75.87	78.46	72.42	78.71	63.53
G_8	Iα	94.93	74.21	75.46	72.25	73.21	71.61
	Iβ	98.77	76.83	78.46	72.32	77.64	71.78
	II	105.50	75.84	78.37	72.25	77.71	71.61
	III-VI	105.81	75.84	78.37	72.25	77.71	71.61
	VII	105.81	75.84	78.37	72.25	77.71	71.42
	VIII	105.70	75.88	78.46	72.42	78.72	63.53
G_9	Iα	94.92	74.21	75.46	72.26	73.21	71.60
	Iβ	98.77	76.83	78.46	72.31	77.63	71.77
	II	105.52	75.84	78.37	72.26	77.71	71.60
	III-VII	105.81	75.84	78.37	72.26	77.71	71.60
	VIII	105.81	75.84	78.37	72.26	77.71	71.42
	IX	105.70	75.88	78.46	72.42	78.72	63.53

[a]G_4 to G_9 correspond to gentiooligosaccharides with d. p. 4-9, respectively.

[b]Roman numeral indicates the position of glucose residue from reducing-end of gentiooligosaccharides.

Table 1. ^{13}C-chemical shifts of compounds G_4 to G_9 in D_2O solution.