Production of the Positional Isomers of α -Galactobiose by the Reverse Reaction of α -Galactosidase from *Candida guilliermondii* H-404

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Time course of the positional isomers of α -galactobiose in α -linked galactooligosaccharide (α -GOS A) synthesized from galactose by the reverse reaction of α -galactosidase from *Candida guilliermondii* H-404 was analyzed by using *p*-aminobenzoic ethyl ester (ABEE)-conversion method. α -Galactobioses in α -GOS A consisted of α -1,6-, 1,3-, and 1,2-galactobioses. α -1,6-Galactobiose was the main product from an early reaction stage. α -1,6-, 1,3-, and 1,2-Galactobioses were prepared from α -GOS A by an activated carbon chromatography.

Oligosaccharides having α -galactosidic linkages, α -linked galactooligosaccharides such as raffinose and stachyose, are widely distributed in plants.¹⁾ These oligosaccharides have attracted attention as a strong bifidus growth factor²⁾ and are utilized in various foods. In animals, most α -D-galactosidic linkages occur at the nonreducing termini of oligosaccharides linked to cell surface lipids,³⁾ proteins,⁴⁾ and unidentified glycoconjugates.⁵⁾ Some of those α -D-galactosidic linkages have been reported to be important in various biological systems.⁶⁻⁸⁾ In particular, it is reported that α -galactobioses or oligosaccharides containing α -galactobioses have various physiological functions: α -1,4-Galactobiose is an inhibitor of Siga-like toxin of pathogenic

Escherichia coli O-157: H7, also called Verotoxin.⁹⁾ Trisaccharide containing α -1,3-galactobiose neutralizes Toxin A of *Clostridium difficile*, the causative agent in almost all cases of pseudomembranous colitis and antibiotic associated diarrhea.¹⁰⁾ α -1,3-Galactobiose could be used as an antagonist to depress hyperacute rejection during xenotransplantation which is actively being pursued as a solution for the shortage of organs for human recipients.¹¹⁾ α -1,6-Galactobiose is an important disaccharide sequence in the glycosylphosphatidylinositol (GPI) anchor of *Trypanosoma brucei*.¹²⁾ α -1,2-Galactobiose is a structural unit of a GPI anchor for attaching variable surface glycoproteins of *T. brucei*¹³⁾ and certain proteins of *Trypanosoma cruzi*¹⁴⁾ onto their cell surface.

No effective preparation methods for α -1,2-, 1,3-, 1,4-, and 1,6-galactobioses, however, have been developed. *p*-Nitrophenyl α -1,3-galactobioside was synthesized from *p*-nitrophenyl α -D-galactoside by the self-transfer reaction of α -galactosidase from coffee bean¹⁵⁾ or *Penicillium funiculosum*.¹⁶⁾ This method requires expensive *p*-nitrophenyl α -D-galactoside as a substrate and deprotection of *p*-

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Abbreviations: α -GOS A, α -linked galactooligosaccharide synthesized by the reverse reaction of α -galactosidase of *Candida guilliermondii* H-404 from galactose; GPI, glycosylphosphatidylinositol; ABEE, *p*-aminobenzoic ethyl ester; HPLC, high-pressure liquid chromatography.

nitrophenyl group to prepare α -1,3-galactobiose. In previous papers,^{17,18)} we have reported that α -linked galactooligosaccharide (α -GOS A) containing α -1,6-, 1,3-, and 1,2-galactobioses were formed from galactose by the reverse reaction of α -galactosidase from *Candida guilliermondii* H-404.

No rapid, facile, and sensitive analysis methods of the positional isomers of α -galactobiose have been developed. *p*-Aminobenzoic ethyl ester (ABEE)-conversion method is a simple and highly sensitive analysis method of monosaccharide in the pmol order.¹⁹⁾ This method is popularly utilized to analyze the composition of aldose, hexosamine, and sialic acid residues of glycoproteins, glycolipids, and oligosaccharides.

In this paper, we describe the analysis of four positional isomers of α -galactobiose (α -1,6, 1,2, 1,3, and 1,4) by ABEE-conversion method, analysis of the production course of α -galactobioses formed from galactose by the reverse reaction of α -galactosidase from *C. guilliermondii* H-404 by ABEE-conversion method, and practical preparation of α -1,6-, 1,3-, and 1,2-galactobioses from α -GOS A by an activated carbon chromatography.

MATERIALS AND METHODS

Enzyme preparation. The heat-treated cells of *C. guilliermondii* H-404 were prepared as described in our previous paper.¹⁷⁾ The heat-treated cells were used for the preparation of α -GOS A as an enzyme preparation (about 150 U/g wet cells). The Cell-bound α -galactosidase from *C. guilliermondii* H-404 was solubilized, purified as described in our previous paper,²⁰⁾ and used for analysis.

Assay of enzyme activity. α -Galactosidase and β -galactosidase activities were assayed by a spectrophotometric measurement of the release of *p*-nitrophenol from corresponding *p*-nitrophenyl synthetic glycosides as described in our previous paper.¹⁷⁾ One unit of enzyme activity was defined as the amount releasing 1.0 μ mol of *p*-nitrophenol per min.

Materials. An ABEE monosaccharide analysis kit (containing an ABEE reagent solution) was purchased from Seikagaku Corp. (Tokyo, Japan).

α -1,3- and 1,4-Galactobioses were purchased from Sigma-Aldrich Japan (Tokyo, Japan). α -1,2-Galactobiose was purchased from Funakoshi (Tokyo, Japan). α -1,6-Galactobiose was isolated from α -GOS A by a preparative HPLC with an Asahipak NH2P-50 column (21.5 $\times$ 300 mm) (Showa Denko: Tokyo, Japan) as described in our previous paper.²¹⁾ Oligosaccharides larger than disaccharide in α -GOS A were prepared by a column (2.6 $\times$ 100 cm) of Bio-Gel P-2 (Bio-Rad: Hercules, California) as described in our previous paper.¹⁷⁾ All other chemicals used were obtained from commercial sources.

High-pressure liquid chromatography (HPLC).

Ligand-exchange chromatographic analysis (method 1) was done using an LC-6A pump, RID-6A differential refractive index detector, and C-R4A data processor (all from Shimadzu: Kyoto, Japan) under the following conditions: column, ULTRON PS-80C (8.0 $\times$ 300 mm) (Chromato Packings Center: Kyoto, Japan); column temperature, 80°C; mobile phase, water; flow rate, 0.6 mL/min.

ABEE-conversion method. An oligosaccharide was converted to an ABEE-converted oligosaccharide by reductive amination according to the method of Yasuno *et al.*²²⁾ with slight modifications. An ABEE reagent solution (40 μ L) was added to a saccharide solution (10 μ L). The mixture was vortexed and then incubated at 80°C for 1 h. The mixture was cooled to room temperature, before distilled water (0.2 mL) and an equal volume of chloroform were added. After vigorous vortexing, the mixture was centrifuged for 3 min, and the upper aqueous layer was subjected to an HPLC analysis. ABEE-converted oligosaccharide analysis (method 2) was done using an LC-6A pump, SPD-6A UV detector, and C-R4A data processor (all from Shimadzu) under the following conditions: column, Honepak C18 (4.6 $\times$ 75 mm) (Seikagaku Corp.); column temperature, 30°C; mobile phase, 0.1 M ammonium acetate buffer (pH 4.0)/acetonitrile (88/12 (v/v)); flow rate, 1.0 mL/min. ABEE-converted oligosaccharides were detected at 305 nm.

Analysis. Methylation analysis was done as described in our previous paper.¹⁷⁾ Analysis of non-

reducing sugar was done by the alkali treatment method. A mixture of an equal volume of 1% saccharides (reverse reaction products) and 0.2 M NaOH was heated at 100°C for 30 min to decompose the reducing saccharides in the sample (Under these conditions, melibiose, a reducing saccharide, was completely decomposed, but trehalose, a nonreducing saccharide, was not.). The solution was analyzed by HPLC (method 1) as described in our previous paper.¹⁷⁾

Enzymatic hydrolysis of oligosaccharides. The reaction mixture (100 μ L) containing oligosaccharides (0.25 mg) and purified α -galactosidase from *C. guilliermondii* H-404 (0.15 U) in 20 mM acetate buffer (pH 4.5) was incubated for 20 h at 40°C. The reaction was stopped by boiling for 10 min, and then analyzed by HPLC (method 1).

Preparation of α -1,6, 1,3, and 1,2-galactobioses from α -GOS A. α -GOS A (10 g) was produced from galactose (40 g) as described in our previous paper¹⁸⁾ under the following reaction conditions: 58% (w/v) galactose, 53 U/g galactose, pH 4.5, 50°C, 90 h. α -GOS A (10 g) was dissolved in H₂O (40 mL). The α -GOS A solution was put on an activated carbon column (5.0 $\times$ 46 cm). The column was washed with distilled water (8 L), and eluted first with 0% (3 L)–10% (3 L) ethanol gradient, and then 10% ethanol (3 L) into 100 mL fractions. Each fraction was collected, concentrated using a rotary evaporator, and analyzed by HPLC (method 1). The fractions containing disaccharide were analyzed by ABEE-conversion method. The fractions containing α -1,6-galactobiose were collected and lyophilized to give a white powder (4.0 g). The fractions containing α -1,2- and 1,3-galactobioses were collected separately, concentrated using a rotary evaporator, and purified by rechromatography of an activated carbon column (1/10 scale). The fractions containing α -1,3- and 1,2-galactobioses were collected separately, concentrated using a rotary evaporator, and lyophilized to give white powders (1.0 g and 0.5 g, respectively).

RESULTS AND DISCUSSION

Analysis of the positional isomers of α -galactobiose by ABEE-conversion method.

Analysis of four ABEE-converted positional isomers of α -galactobiose (α -1,6, 1,2, 1,3, and 1,4) was examined by using the HPLC conditions as described in the paper.¹⁹⁾ However, ABEE-converted α -1,2- and 1,4-galactobioses were not separated under the condition with borate buffer (pH 9.0) as a solvent. ABEE-converted α -1,6- and 1,4-galactobioses were not separated under the condition with trifluoroacetic acid solution as a solvent.

Effect of solvent's pH on separation of ABEE-converted galactobioses was examined by using 0.1 M ammonium acetate buffer which was easy to use because of its volatility. ABEE-converted α -1,6- and 1,4-galactobioses were not separated by replacement of borate buffer (pH 9.0) by ammonium acetate buffer (pH 9.0). However, separation of ABEE-converted α -1,6- and 1,4-galactobioses were improved with decreasing pH of the buffer. By using 0.1 M ammonium acetate buffer (pH 4.0), ABEE-converted α -1,6- and 1,4-galactobioses were separated roughly. As a result of optimization of analytical conditions such as acetonitrile concentration and temperature, it was found that four ABEE-converted positional isomers of α -galactobiose (α -1,6, 1,2, 1,3, and 1,4) were able to be separated by reverse-phase HPLC under the conditions described in MATERIALS AND METHODS (method 2). ABEE-converted oligosaccharides larger than disaccharide produced by the reverse reaction of α -galactosidase hardly overlapped with ABEE-converted α -galactobioses. The relationship between the peak areas and the amounts of ABEE-converted α -galactobioses was linear in the range of 1×10^{-4} to 1% (w/v). The detection sensitivities of four positional isomers of α -galactobiose were nearly equal in the analytical condition. The chromatogram of ABEE-converted α -GOS A used for the preparation of α -galactobioses was shown in Fig. 1. All ABEE-converted α -galactobioses (peaks 1, 2, 3, and 4) were identified by comparison with authentic specimens. ABEE-conversion

Fig. 1. The chromatogram of α -GOS A by ABEE-conversion method.

Analytical conditions are described in MATERIALS AND METHODS (method 2). Peaks of 1, 2, 3, and 4: ABEE-converted α -1,2-galactobiose, α -1,3-galactobiose, α -1,4-galactobiose, and α -1,6-galactobiose, respectively. Asterisk peaks are the ABEE-converted oligosaccharides larger than disaccharide.

method was able to analyze the positional isomers of α -galactobiose produced by the reverse reaction of α -galactosidase from *C. guilliermondii* H-404 from galactose.

Reverse reaction of α -galactosidase of *C. guilliermondii* H-404 from galactose.

C. guilliermondii H-404 produced intracellularly α -galactosidase with a small amount of β -galactosidase which affected the reverse reaction of α -galactosidase from galactose. The β -galactosidase in intact cells was inactivated by heat treatment with no damage to the α -galactosidase. Therefore, the heat-treated cells can be used as a α -galactosidase preparation to synthesize α -GOS A by the reverse reaction from galactose.¹⁷⁾ The course of reverse reaction from galactose by the cell-bound α -galactosidase was examined by analyzing the saccharide compositions in the reaction mixture (Fig. 2A). Disaccharide was the major product. The yield of α -GOS A (disaccharide and oligosaccharides larger than disaccharide) reached a near equilibrium value (approximately 27%) at 110 h.

The course of synthesis of the main reverse reaction product, α -galactobioses was examined by ABEE-conversion method (Fig. 2B). α -1,6-Galactobiose was rapidly synthesized from an early reaction stage, and increased with the elapse of time. α -1,3- and 1,2-Galactobioses were synthesized in a nearly equal yield at an early reaction stage. α -1,3-Galactobiose increased gradually up to the equilibrium, and the yield near the equilibrium was about twice that of α -1,2-galactobiose. There was a trace amount (<1%) of α -1,4-galactobiose from an early reaction stage.

To elucidate the factors controlling the α -galactobiose composition, the substrate specificity for hydrolysis against α -galactobioses was examined. Hydrolysis rates of α -galactobioses by α -galactosidase from *C. guilliermondii* H-404 were shown in Fig. 3. α -1,6- and 1,2-Galactobioses were hydrolyzed quite rapidly. α -1,3-Galactobiose was hydrolyzed more slowly than α -1,6- and 1,2-galactobioses. α -1,4-Galactobiose was not hydrolyzed.

From these results, it was supposed that α -1,4-galactobiose would be hardly synthesized and α -

Fig. 2. Reverse reaction of α -galactosidase of *C. guilliermondii* H-404 from galactose.

A reaction mixture (5 mL) containing 58% (w/v) galactose and 30 U/mL of cell-bound enzyme in 20 mM acetate buffer (pH 4.5) was incubated at 50°C. Samples (0.1 mL) were taken at various time intervals from the reaction mixture and heated at 100°C for 10 min to inactivate the enzyme. Water (5.9 mL) was added to the reaction mixture. Then the reaction mixtures were centrifuged to remove cell debris and analyzed by HPLC (method 1) (A) and ABEE-conversion method (method 2) (B). (A): $\circ$, galactose; $\bullet$, α -galactobiose; $\square$, α -galactotriose; $\blacktriangle$, oligosaccharides larger than trisaccharide. (B): $\circ$, α -1,6-galactobiose; $\bullet$, α -1,3-galactobiose; $\diamond$, α -1,2-galactobiose.

Fig. 3. Courses of hydrolysis of α -galactobioses by α -galactosidase from *C. guilliermondii* H-404.

The reaction mixture (300 μ L) containing 0.1% α -galactobioses (composed of α -1,6-, 1,2-, 1,3-, and 1,4-galactobioses equally) and 0.025 U/mL purified α -galactosidase from *C. guilliermondii* H-404 in 20 mM acetate buffer (pH 4.5) was incubated at 40°C. The reaction mixtures were analyzed by ABEE-conversion method (method 2) at various time intervals. $\circ$, α -1,6-galactobiose; $\bullet$, α -1,3-galactobiose; $\diamond$, α -1,2-galactobiose; $\blacklozenge$, α -1,4-galactobiose.

1,6- and 1,2-galactobioses would be synthesized more readily than α -1,3-galactobiose. However, the order of α -galactobioses synthesized actually by the reverse reaction was α -1,6- $\gg$ α -1,3- $>$ α -1,2-galactobiose. The reasons for this discrepancy may be explained as follows. The factors controlling the composition of the reverse reaction product appear to have a relationship with the substrate

and action specificities of the enzyme. Namely, as reported in the previous paper,²¹⁾ α -galactosidase of *C. guilliermondii* H-404 has a strong transfer activity, and produces α -1,6-galactosyl (main product) and α -1,3-galactosyl (minor product) transfer products in a self-transfer reaction using melibiose as a substrate. Therefore, α -1,6- and 1,2-galactobioses mainly synthesized by the reverse reaction may act as a good substrate for hydrolysis or transgalactosylation. The composition of the final product is likely to reflect the results of reverse, hydrolytic, and transfer reactions.

Preparation of α -1,6-, 1,3-, and 1,2-galactobioses from α -GOS A.

α -GOS A consisted of 79% disaccharide and 21% oligosaccharides larger than disaccharide. Disaccharide consisted of α -1,6- (68%), 1,3- (21%), and 1,2-galactobioses (11%) with a trace amount ($<1\%$) of α -1,4-galactobiose. Nonreducing sugar (probably α -1,1-galactobiose) was detected with a trace amount ($<1\%$). α -GOS A was separated by an activated carbon column chromatography (Fig. 4). α -1,6-Galactobiose (peak A) was eluted with distilled water. α -1,3- (peak B) and 1,2-Galactobioses (peak C) were eluted with 0–10% ethanol gradient in this order. Subsequently trisaccharide (α -galactotriose) was eluted.

Fig. 4. Activated carbon column chromatogram of α -GOS A.

The conditions are described in MATERIALS AND METHODS. The fractions were collected, concentrated using a rotary evaporator, and analyzed by HPLC (method 1). Solid line, ethanol concentration; ●, α -galactobiose; △, α -galactotriose.

Each fraction of α -1,3- (peak B) and 1,2-galactobiose (peak C) was collected and concentrated. The concentrated fractions of α -1,3- and 1,2-galactobioses contained small amounts (about 10%) of α -1,6- and 1,3-galactobioses, respectively. Consequently, these fractions were purified by rechromatography of an activated carbon column (1/10 scale). These α -galactobioses were completely hydrolyzed by α -galactosidase to galactose. The structures of α -galactobioses prepared were identified by methylation analysis and confirmed by comparison with authentic saccharides with ABEE-conversion method. Nonreducing sugar was not detected in these α -galactobiose preparations.

ABEE-conversion method was able to analyze the positional isomers of α -galactobiose produced by the reverse reaction of α -galactosidase from galactose. α -1,6-, 1,3-, and 1,2-Galactobioses were separated into three fractions from α -GOS A by an activated carbon chromatography. It has been noticed that glycosidases from different origins catalyze the synthesis of different linkages. This method will be useful for screening of the α -galactosidases that catalyze the synthesis of α -1,3-, 1,4-, or 1,2-galactobiose predominantly from galactose by the reverse reaction.

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Candida guilliermondii H-404 株の
 α -ガラクトシダーゼの縮合反応を用いた
 α -ガラクトビオースの調製

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α -ガラクトビオースはさまざまな生理機能が報告されている。 α -ガラクトビオースの結合位置異性体を同時に高感度で分析する方法を、アミノ安息香酸エチルエステル標識化法 (ABEE 標識化法) を用いて確立した。*Candida guilliermondii* H-404 株の α -ガラクトシダーゼの縮合反応を用いて、ガラクトースから α -ガラクトビオースを主体とする α -結合ガラクトオリゴ糖 (α -GOS A) を効率よく合成できる。ABEE 標識化法を用いて、 α -ガラクトビオースの合成の経時変化を調べた。その結果、反応初期から、主生成物は α -1,6-ガラクトビオースで、その他、 α -1,3-と1,2-ガラクトビオースが生成した。この α -GOS A から、活性炭クロマトグラフィーを用いて、 α -1,6-, 1,3-, 1,2-ガラクトビオースを調製した。