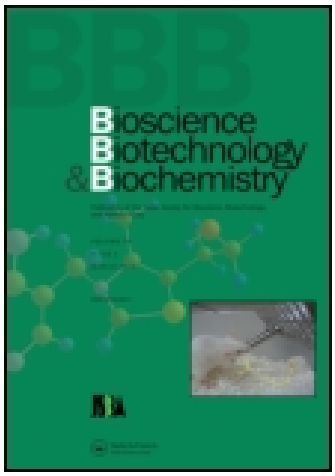

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
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Published online: 12 Jun 2014.

To cite this article: Hiroyuki Hashimoto, Chie Katayama, Masaru Goto, Tatsuyuki Okinaga & Sumio Kitahata (1995) Transgalactosylation Catalyzed by α-Galactosidase from Candida guilliermondii H-404, Bioscience, Biotechnology, and Biochemistry, 59:4, 619-623, DOI: 10.1271/bbb.59.619

To link to this article: http://dx.doi.org/10.1271/bbb.59.619

Biosci. Biotech. Biochem., **59** (4), 619–623, 1995

Transgalactosylation Catalyzed by α-Galactosidase from *Candida guilliermondii* H-404

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The thermostable α-galactosidase from *Candida guilliermondii* H-404 synthesized self-transfer products in the absence of a suitable acceptor. The main self-transfer product, using melibiose as a donor substrate, was *O*-α-D-galactosyl-(1,6)-*O*-α-D-galactosyl-(1,6)-D-glucose. This enzyme had a wide acceptor specificity. D-Glucose, D-galactose, maltose, maltitol, and 1,4-butandiol were the most effective acceptors in the transgalactosylation catalyzed by this enzyme. The enzyme could also transfer α-galactosyl residues to pentoses (L-arabinose, D-xylose, and D-ribose) and methyl pentoses (D-fucose and L-rhamnose). The main transfer products to lactose, maltose, and sucrose as acceptors were identified as *O*-α-D-galactosyl-(1,6)-*O*-β-D-galactosyl-(1,4)-D-glucose, *O*-α-D-galactosyl-(1,6)-*O*-α-D-glucosyl-(1,4)-D-glucose, and *O*-α-D-galactosyl-(1,6)-*O*-α-D-glucosyl-(1,2)-β-D-fructoside (raffinose), respectively.

Oligosaccharides having α-galactosidic linkages (α-linked galactooligosaccharides) have attracted attention as strong bifidus growth factors[1–2] and been used in various foods. In animals, some of the α-D-galactosidic linkages have been reported to be important in various biological systems.[3–5]

α-Linked galactooligosaccharides can be synthesized by the transgalactosylation or the reverse reaction of α-galactosidase.[6] Few studies, however, have been done on the synthesis of α-linked galactooligosaccharides by use of the transgalactosylation of α-galactosidase, for lack of available and inexpensive donor substrates. In our previous paper,[7] we reported that *Candida guilliermondii* H-404 α-galactosidase synthesized α-linked galactooligosaccharides effectively by the reverse reaction from lactose hydrolyzates and those saccharides were available as donor substrates for the transgalactosylation catalyzed by this α-galactosidase.

In this study, we examined the transgalactosylation catalyzed by *Candida guilliermondii* H-404 α-galactosidase from the view-points of the self-transfer reaction using melibiose as a donor substrate, the details of acceptor specificities, the effects of the concentration of the acceptor on the production of the transfer products, and the structures of several oligosaccharides synthesized by the transgalactosylation in the presence of several disaccharides as acceptors.

Materials and Methods

Enzyme preparation. The soluble α-galactosidase from *Candida guilliermondii* H-404 was prepared by autolysis of intact cells. The intact cells were prepared as described in our previous paper.[7] The intact cells were washed with distilled water and the washed cells were left to autolyze at 40 C for 22 h. The autolyzate was centrifuged and the supernatant obtained was dialyzed against 20 mM acetate buffer (pH 4.5). The dialyzed enzyme solution was incubated at 60 C for 6 h. The heat-treated soluble enzyme was used as the enzyme preparation. This enzyme preparation did not attack the compounds used as acceptors in this paper. Purified α-galactosidase from *Candida guilliermondii* H-404 was prepared as described in our previous paper.[8]

Materials. F kit (glucose/sucrose) was purchased from Boehringer Mannheim (Tokyo, Japan). All chemicals used were obtained from commercial sources.

Assay of enzyme activity. The α-galactosidase and other glycosidase activities (β-galactosidase, α-glucosidase, and β-glucosidase) were assayed by spectrophotometric measurement of the release of *p*-nitrophenol from the corresponding *p*-nitrophenyl synthetic glycosides as described in our previous paper.[7] One unit of enzyme activity was defined as the amount releasing 1.0 μmol of *p*-nitrophenol per min. The sucrose-splitting acitivity was assayed by measurement of the release of glucose from sucrose. The reaction mixture (450 μl), containing 0.26 M sucrose and enzyme in 20 mM acetate buffer (pH 4.5), was incubated at 40 C for 10 min. The reaction was stopped by heating at 100 C for 10 min. Glucose in the reaction mixture was measured by the F kit. One unit of enzyme activity was defined as the amount releasing 1.0 μmol of glucose from sucrose per min.

High pressure liquid chromatography (HPLC). HPLC analysis was done by use of an LC-6A pump, RID-6A differential refractive index detector, and C-R3A data processor (all from Shimadzu). Preparative HPLC was done under these conditions: column, Asahipak NH2P-50 (21.5 × 300 mm) (Showa Denko); column temperature, 30 C; mobile phase, acetonitrile–water (70 : 30, (v/v)); flow rate, 4.0 ml/min.

Acceptor specificities. The reaction mixture (100 μl) containing 0.3 M substrate (melibiose or raffinose), acceptor (alcohols except sugar alcohols: 10% (v/v), saccharides and sugar alcohols: 0.6 M), and *Candida guilliermondii* H-404 enzyme (0.5 U/ml) in 20 mM acetate buffer (pH 4.5) was incubated at 40 C for 15 h. The reaction was stopped by heating at 100 C for 10 min. The reaction mixtures were analyzed by HPLC under the conditions given in Fig. 1. The transfer ratio to acceptor was defined as (amount of galactose in transfer products to acceptor/amount of galactose in total transfer products) × 100 (%). The amount of galactose in transfer products to acceptor and in total transfer products (self-transfer

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products and transfer products to acceptor) was calculated from the amounts of transfer products by HPLC.

Enzymatic hydrolysis of transfer product. The reaction mixture (500 μl) containing the transfer product (0.25%) and purified α-galactosidase from *Candida guilliermondii* H-404 (1.5 U/ml) in 20 mM acetate buffer (pH 4.5) was incubated for 24 h at 40°C. The reaction was stopped by heating at 100°C for 10 min. The reaction products were analyzed by HPLC (conditions as in Fig. 1 and Fig. 2).

Methylation analysis. Methylation analysis was done as described in our previous paper.[7]

Nuclear magnetic resonance (NMR). ^{13}C-NMR spectra were recorded on a Bruker AC300 spectrometer operating at 300 MHz in D_2O at 25°C. Chemical shifts were measured with sodium 4,4-dimethyl-4-silapentane sulfonate (DSS) as an internal standard.

Results and Discussion

Various glycosidase activities in soluble enzyme preparations from Candida guilliermondii H-404

The cell-bound enzymes of *Candida guilliermondii* H-404 were solubilized easily by autolysis. α-Galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and sucrose-splitting activities in the soluble enzyme were 76, 0.13, 0.035, 9.5, and 1600 U/ml, respectively. The β-galactosidase, β-glucosidase, and sucrose-splitting activities were inactivated completely by heat-treatment. The α-galactosidase and α-glucosidase activities were not affected by heat-treatment. But the activity of α-glucosidase was over 2200 times less than that of α-galactosidase. Therefore the heat-treated soluble enzyme was used as an α-galactosidase preparation without further purification to synthesize the α-linked galactooligosaccharides by transgalactosylating activity.

Self-transfer reaction of α-galactosidase from Candida guilliermondii H-404

The term "self-transfer reaction" is used when a substrate acts as both galactosyl donor and acceptor. The α-galactosidase from *Candida guilliermondii* H-404 catalyzes a self-transfer reaction.[8] The self-transfer reaction by this enzyme was done with melibiose as a donor substrate. As shown in Fig. 1(a), three self-transfer products (M1, M2, and M3) were produced. The main product was M2. The course of the self-transfer reaction using melibiose as a donor substrate is shown in Fig. 2. The yield of M2 reached a maximum value at 3 h, and then gradually decreased. As the reaction proceeded, the yields of M1 and M3 increased gradually. The yields of M1, M2, and M3 at 3 h were 4, 29, and 6%, respectively. The amounts of glucose and galactose correspond to the overall rate of degradation and the rate of hydrolytic reaction of melibiose, respectively. The ratio of transfer reaction calculated from the difference in the amounts of galactose and glucose at 3 h was 86%. The self-transfer products M1, M2, and M3 were isolated from the reaction mixture at 3 h by preparative HPLC as described in Materials and Methods.

Structure analysis of the self-transfer products

The self-transfer products M1 and M2 were completely hydrolyzed to galactose and glucose in the molar ratio of 2:1 through melibiose by α-galactosidase. The structures of M1 and M2 were confirmed by the methylation analysis. Methylation analysis of M1 gave 2,3,4,6-tetra-*O*-methyl-galactitol acetate, 2,4,6-tri-*O*-methyl-galactitol acetate, and 1,2,3,4-tetra-*O*-methyl-glucitol acetate in a nearly equivalent molar ratio. Methylation analysis of M2 gave 2,3,4,6-tetra-*O*-methyl-galactitol acetate, 2,3,4-tri-*O*-methyl-galactitol acetate, and 1,2,3,4-tetra-*O*-methyl-glucitol acetate in a nearly equivalent molar ratio. The results indicated that galactose residues of M1 and M2 were linked to the C3-OH and the C6-OH of D-galactose of melibiose, respectively. From these results, the structures of M1 and M2 were identified as *O*-α-D-galactosyl-(1,3)-*O*-α-D-galactosyl-(1,6)-

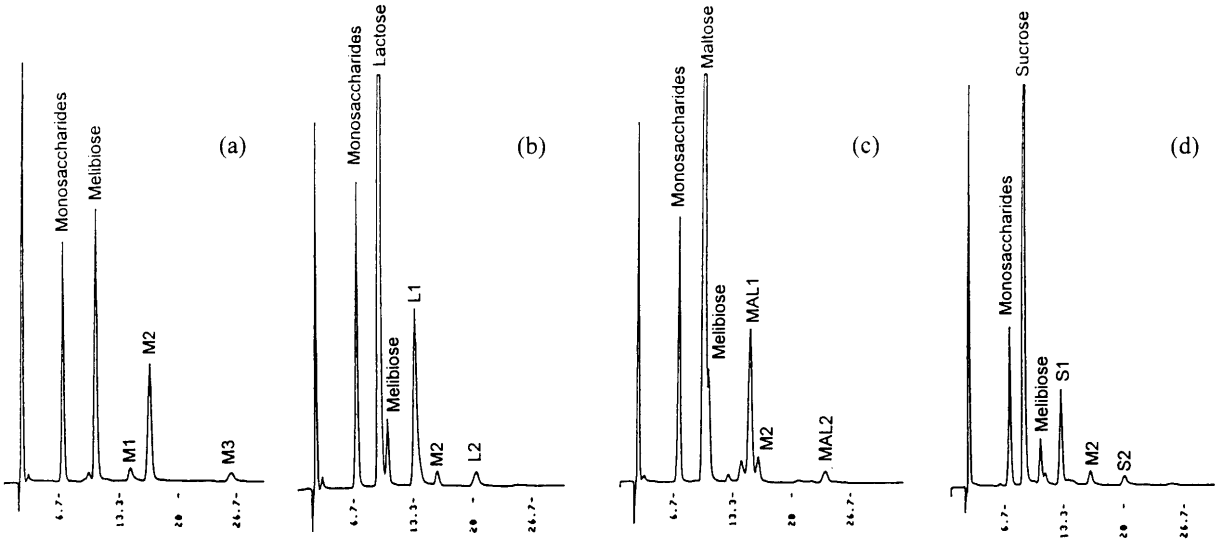

Fig. 1. High Pressure Liquid Chromatograms of Self-transfer Products (a) and Transfer Products to Lactose (b), Maltose (c), and Sucrose (d) by α-Galactosidase from *Candida guilliermondii* H-404.

(a): The reaction mixture (10 ml), containing 1 M melibiose and 12 U/ml α-galactosidase from *Candida guilliermondii* H-404 in 20 mM acetate buffer (pH 4.5), was incubated at 40°C for 3 h. (b), (c), and (d): The reaction mixture (10 ml), containing 0.29 M melibiose, 0.58 M disaccharide, and 8 U/ml α-galactosidase from *Candida guilliermondii* H-404 in 20 mM acetate buffer (pH 4.5), was incubated at 40°C. The reaction times were 4, 3, and 5 h for lactose, maltose, and sucrose, respectively. The reaction mixtures were analyzed by HPLC.

Chromatographic conditions: column, Asahipak NH2P-50 (4.6 × 250 mm) (Showa Denko: Tokyo, Japan); column temperature, 30°C; mobile phase, acetonitrile–water (70:30, (v/v)); flow rate, 1.0 ml/min.

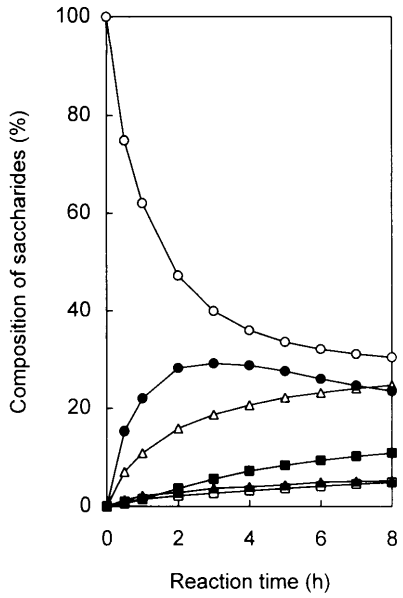

Fig. 2. Courses of the Self-transfer Reaction by α-Galactosidase from *Candida guilliermondii* H-404 Using Melibiose as a Donor Substrate.

The reaction was done under the conditions as described in Fig. 1(a). The reaction mixtures were analyzed by HPLC at intervals. Chromatographic conditions for glucose and galactose: column, Shim-peak SCR-101C (7.9 × 300 mm) X2 (Shimadzu: Kyoto, Japan); column temperature, 80 C; mobile phase, water; flow rate, 0.6 ml/min. For the others (melibiose, M1, M2, and M3): as in Fig. 1. (■), M3; (●), M2; (▲), M1; (○), melibiose; (△), glucose; (□), galactose.

D-glucose and *O*-α-D-galactosyl-(1,6)-*O*-α-D-galactosyl-(1,6)-D-glucose, respectively. The ratio of α-(1,3)- and α-(1,6)-galactosyl transfer action by the enzyme was estimated to be 1 : 7 from the yields of M1 and M2. The transfer product M3 was completely hydrolyzed to galactose and glucose (molar ratio of 3 : 1), through the saccharide M2 by α-galactosidase. From this result and the ratio of M1 and M2, the saccharide M3 seems to be *O*-α-D-galactosyl-(1,6)-M2.

Acceptor specificities

The acceptor specificities of α-galactosidase from *Candida guilliermondii* H-404 were examined with several saccharides and alcohols as acceptors (Table).

(*1*) *Saccharides.* Of the monosaccharides tested, D-galactose and D-glucose were the most effective acceptors. The efficiency as acceptor of aldohexoses (D-galactose, D-glucose, D-allose, D-mannose, and D-talose) was higher than that of ketohexoses (D-fructose and L-sorbose). The amino sugars were also effective as acceptors (their transfer ratios were between 25 and 75%). Aldopentoses (L-arabinose, D-xylose, and D-ribose) and methyl pentoses (D-fucose and L-rhamnose) acted as acceptors. Of the oligosaccharides tested, maltose was the most effective acceptor. The efficiency as acceptors decreased in the following order: glucose (monosaccharide) ≈ maltose(disaccharide) > maltotriose(trisaccharide) > maltotetraose (tetrasaccharide).

(*2*) *Alcohols.* Of the sugar alcohols tested, maltitol was the most effective acceptor. D-Xylitol and D-sorbitol were effective as acceptors (their transfer ratios were between 50 and 75%). D-Mannitol and dulcitol also acted as acceptors, but their transfer ratios were less than 25%. Of the polyhydric alcohols tested, 1,4-butanediol was the most effective acceptor. Of the monohydric alcohols tested, isobutanol and 1-propanol were the most effective acceptors (their transfer ratios were between 50 and 75%). *tert*-Butanol gave no detectable amounts of transfer products under the conditions used.

Table Transgalactosylation of α-Galactosidase from *Candida guilliermondii* H-404 to Various Saccharides and Alcohols

Acceptors (Saccharides)	Transfer ratio to acceptor	Acceptors (Alcohols)	Transfer ratio to acceptor
L-Arabinose	+	Maltitol	+ + + +
D-Xylose	+	D-Xylitol	+ + +
D-Ribose	+	D-Sorbitol	+ + +
D-Arabinose	±	D-Mannitol	+
		Dulcitol	+
D-Fucose	+ +		
L-Rhamnose	+	*myo*-Inositol	+
L-Fucose	±		
		Glycerol	+ + +
D-Glucose	+ + + +		
D-Galactose	+ + + +	1,4-Butanediol	+ + + +
D-Allose	+ + +	1,3-Propanediol	+ + +
D-Mannose	+ + +	1,4-Cyclohexanediol	+ + +
D-Talose	+ + +	1,3-Butanediol	+ + +
D-Fructose	+ +	1,2-Butanediol	+ + +
L-Sorbose	+ +	1,2-Propanediol	+ + +
2-Deoxy-D-glucose	+ +	1,2-Ethanediol	+ + +
		2,3-Butanediol	+ + +
D-Galactosamine	+ + +		
D-Glucosamine	+ + +	Isobutanol	+ + +
GalNAc	+ + +	1-Propanol	+ + +
GlcNAc	+ +	Ethanol	+ +
		Methanol	+ +
Maltose	+ + + +	1-Butanol	+ +
Lactose	+ + +	2-Butanol	+ +
Trehalose	+ + +	2-Propanol	+
Cellobiose	+ + +	1-Pentanol	+
Sucrose	+ + +	*tert*-Butanol	—
Palatinose	+ + +		
Maltotriose	+ + +		
Maltotetraose	+ +		

+ + + + , the transfer ration to accepter is more than 75%; + + +, 75–50%; + +, 50–25%; +, 25–1%; ±, below 1%; —, not detected transfer product to acceptor. When glucose was used as an acceptor, raffinose was used as a donor substrate.

Several reports have so far been published on the acceptor specificity of α-galactosidases, such as those from *Pycnoporus*,[9] coffee bean,[10] the seeds of white clover,[11] germinating *Vicia sativa* seeds,[12] sweet almond,[13] and *Pseudomonas fluorescens* H-601.[14] There are no reports saying that D-xylose acts as an acceptor of these enzymes. D-Fructose is not an acceptor for these enzymes except *Pycnoporus* and *Pseudomonas*. α-Galactosidase from *Candida guilliermondii* H-404, however, had a broader acceptor specificity than these α-galactosidases, and D-fructose and D-xylose were also effective acceptors of this enzyme.

Transgalactosylation to disaccharides by α-galactosidase from Candida guilliermondii H-404

The transgalactosylation of α-galactosidase from *Candida guilliermondii* H-404 using melibiose as a donor substrate and disaccharide (lactose, maltose, or sucrose) as an

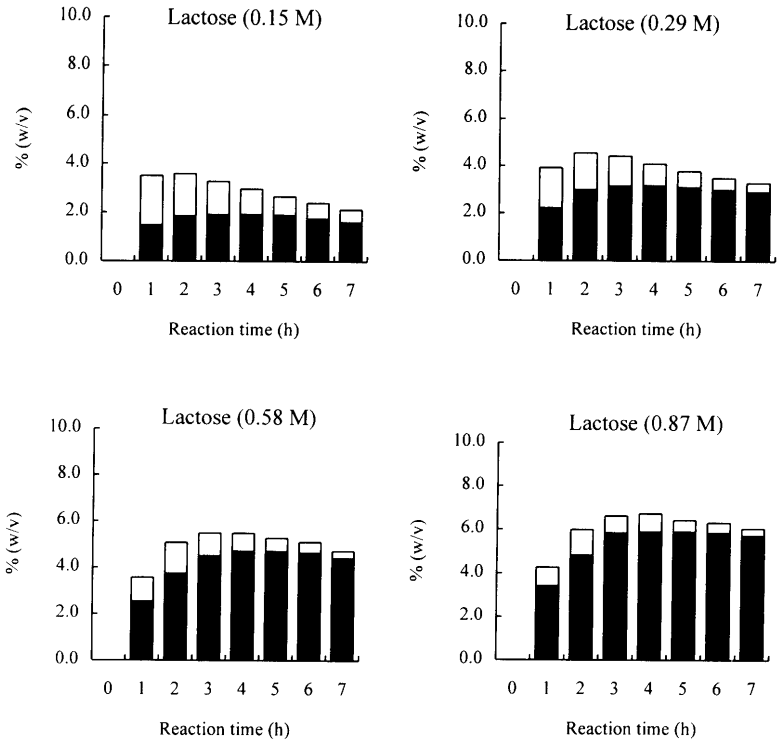

Fig. 3. Courses of Transgalactosylation to Lactose by α-Galactosidase from *Candida guilliermondii* H-404.

The reaction conditions were identical to those in Fig. 1(b). The reaction mixtures were analyzed by HPLC (conditions as in Fig. 1) at intervals. Closed box, L1 (the main transfer product to lactose); Open box, M2 (the self-transfer product).

acceptor was investigated. In the presence of lactose as an acceptor, the transfer products to lactose (L1 and L2) and the self-transfer product (M2) were produced (Fig. 1(b)). The yield of the main product L1 was 16%. In the presence of maltose as an acceptor, the transfer products to maltose (MAL1 and MAL2) and the self-transfer product (M2) were produced (Fig. 1(c)). The yield of the main transfer product MAL1 was 14%. In the presence of sucrose as an acceptor, the transfer products to sucrose (S1 and S2) and the slef-transfer product (M2) were produced (Fig. 1(d)). The yield of the main transfer product S1 was 12%. The yields of the minor transfer products L2, MAL2, and S2 were about 2%. The transfer products L1, L2, MAL1, MAL2, S1, and S2 were isolated from the reaction mixture by preparative HPLC as described in Materials and Methods.

Effects of acceptor concentration on transgalactosylation by α-galactosidase from Candida guilliermondii H-404

The effects of acceptor concentration on transgalactosylation by α-galactosidase from *Candida guilliermondii* H-404 were examined with melibiose as a donor substrate and lactose as an acceptor. The concentrations of the main transfer product to lactose (L1) and the self-transfer product (M2) in the reaction mixtures were measured by HPLC (Fig. 3). As the concentration of lactose increased, the production of L1 increased, while that of M2 decreased. At all lactose concentrations, the yield of L1 reached a maximum value at 4 h. At 0.15, 0.29, 0.58, and 0.87 M lactose concentrations, the ratios of L1 to the transfer products (L1 and M2) at 4 h were 65, 77, 85, and 87%, respectively. These results indicated that the acceptor concentration affected the ratio of transgalactosylation to acceptor, but even a high acceptor concentration could not completely suppress the self-transfer reaction.

Structure analysis of the transfer products to disaccharides

The saccharides L1, MAL1, and S1 were completely hydrolyzed by α-galactosidase to galactose and lactose, galactose and maltose, and galactose and sucrose in the molar ratio of 1 : 1, respectively. Taking account of the enzymatic analysis, the structure of L1 was confirmed to be *O*-α-D-galactosyl-(1,6)-*O*-β-D-galactosyl-(1,4)-D-glucose by the ^{13}C-NMR analysis: ^{13}C-NMR data (D_2O) of L1: 103.5 (C-1′), 98.7 (C-1″), 96.1 (C-1β), 92.3 (C-1α), 71.5 (C-2′), 68.8 (C-2″), 74.2 (C-2β), 70.3 (C-2α), 72.9 (C-3′), 69.7 (C-3″), 74.8 (C-3β), 71.5 (C-3α), 69.0 (C-4′), 69.7 (C-4″), 79.9 (C-4β), 79.7 (C-4α), 73.5 (C-5′), 71.2 (C-5″), 75.0 (C-5β), 71.9 (C-5α), 66.8 (C-6′), 61.6 (C-6″), 60.4 (C-6β), 60.6 (C-6α). The structure of MAL1 was confirmed by the methylation analysis, which gave 2,3,4,6-tetra-*O*-methyl-galactitol acetate, 2,3,4-tri-*O*-methyl-glucitol acetate, and 1,2,3,6-tetra-*O*-methyl-glucitol acetate in a nearly equivalent molar ratio. These results indicated that the galactose residue of MAL1 was linked to the C6-OH of non-reducing terminal D-glucose of maltose. From these results, the structure of MAL1 was assigned to *O*-α-D-galactosyl-(1,6)-*O*-α-D-glucosyl-(1,4)-D-glucose. On ^{13}C-NMR analysis, the structure of S1 was assigned to be *O*-α-D-galactosyl-(1,6)-*O*-α-D-glucosyl-(1,2)-β-D-fructoside (raffinose). All the main transfer products from melibiose as a substrate and disaccharides as acceptors were α-(1,6)-galactosyl transfer products (trisaccharides). Generally, α-galactosidases preferentially transfer galactosyl residues to the primary alcoholic groups of acceptor sugars.[6] These results indicated that α-galactosidase from *Candida guilliermondii* H-404 catalyzed predominantly α-galactosyl transfer action to the primary alcoholic goups as other enzymes did. The saccharides L2, MAL2, and S2

were hydrolyzed by α-galactosidase to galactose and the corresponding acceptor (lactose, maltose, and sucrose, respectively) in the molar ratio of 2 : 1 through L1, MAL1, and S1, respectively. From these results, the saccharides L2, MAL2, and S2 seem to be tetrasaccharides, and *O*-α-D-galactosyl-L1, *O*-α-D-galactosyl-MAL1, and *O*-α-D-galactosyl-S1, respectively.

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