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Regioselectivity of β-D-galactosyl-disaccharide formation using the β-D-galactosidase from *Bacillus circulans*

Taichi Usui [a,*], Shigenori Morimoto [a], Yukie Hayakawa [a], Mitsuaki Kawaguchi [a], Takeomi Murata [a], Yoshiharu Matahira [b], Yoshiro Nishida [c]

[a] *Department of Applied Biochemistry, Faculty of Agriculture, Shizuoka University, Ohya 836, Shizuoka 422, Japan*

[b] *United Graduate School of Agricultural Science, Gifu University (Shizuoka University), Ohya 836, Shizuoka 422, Japan*

[c] *Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Tsutumidori-Amamiyamachi 1-1, Sendai 981, Japan*

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Abstract

β-D-Galactosidase from *Bacillus circulans* catalyzed the transfer of galactose from lactose predominantly to the OH-4 position of, respectively, GlcNAc and GalNAc to afford β-D-Gal-(1 → 4)-D-GlcNAc and β-D-Gal-(1 → 4)-D-GalNAc. Thus, preponderant formation of (1 → 4)-linkages occurs and (1 → 6)-linkages are formed to a lesser extent, but no (1 → 3)- or (1 → 1)-linkages are formed. When 3-acetamido-3-deoxy-D-glucose (Glc3NAc, *N*-acetylkanosamine) was used as an acceptor, the enzyme catalyzed the β-D-galactosyl transfer to, respectively, the β-anomeric position (OH-1) and OH-6 of this sugar to afford β,β-D-Gal-(1 ↔ 1)-D-Glc3NAc and β-D-Gal-(1 → 6)-D-Glc3NAc. In contrast, with methyl β-D-glucoside and methyl β-D-galactoside as acceptors, the enzyme induced the formation of (1 → 3)-linked disaccharide glycoside other than (1 → 4)- and (1 → 6)-linked ones. This demonstrates that the regioselectivity of β-D-galactosyl transfer onto GlcNAc, GalNAc, and Glc3NAc acceptors as catalyzed by the enzyme is strongly determined by the presence of the *N*-acetyl group. © 1996 Elsevier Science Ltd.

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* Corresponding author.

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1. Introduction

Carbohydrate molecules represent a particularly challenging target for regioselective glycosylation by either chemical or enzymic methods because of their multiple hydroxyl groups. From a practical viewpoint, transglycosylation by glycosidases is attractive for oligosaccharide synthesis [1–8], because glycosidases do exhibit some regioselectivity for the hydroxyl linkage to the acceptor, and their selectivity may vary with different enzymes [9]. For example, the β-D-galactosidase from *Kluyveromyces lactis* affords β-D-Gal-(1 → 6)-D-GlcNAc (*N*-acetylisolactosamine, IsoLacNAc) as the major transglycosylation product between lactose and GlcNAc [3], whereas the β-D-galactosidase from bovine testes produced the β-(1 → 3)-linked disaccharide [6]. Thus, careful selection of the enzyme can sometimes permit formation of the desired linkage. We have already reported that β-D-galactosidase of *B. circulans* induced preponderant β-D-galactosyl transfer from lactose to the secondary (OH-4) rather than the primary hydroxyl group (OH-6) of GlcNAc and allowed the regioselective synthesis of β-D-Gal-(1 → 4)-D-GlcNAc (*N*-acetyllactosamine, LacNAc) on a gram scale, in 23% yield based on the use of GlcNAc [4]. The present paper extends our regioselective galactosylation methodology by the enzyme to sugars other than GlcNAc.

This paper details the regioselectivity of the *B. circulans* β-D-galactosidase-catalyzed formation of galactosyl-disaccharides with the respective acceptors: methyl β-D-glucoside, methyl β-D-galactoside, GlcNAc, and GalNAc.

2. Experimental

Materials.—Commercially available β-D-galactosidases, Biolacta (Daiwa Kasei Co., Ltd., Osaka) from *B. circulans* and Godo YNL (Godo Shusei Co., Ltd., Tokyo) from *Kluyveromyces lactis*, were used as enzyme sources. LacNAc [4], IsoLacNAc [4], β-D-Gal-(1 → 4)-D-GalNAc [10], β-D-Gal-(1 → 6)-D-GalNAc [10], β-D-Gal-(1 → 4)-β-D-Glc-OMe [11], and β-D-Gal-(1 → 3)-β-D-Glc-OMe [11] were prepared by our methods. 3-Acetamido-3-deoxy-D-glucose (Glc3NAc) was prepared from 3-azido-3-deoxy-1,2:5,6-di-*O*-isopropyridene-α-D-glucofuranose according to the reported method [12]. The charcoal–Celite column for the preparation of transglycosylation products was prepared as follows: equal parts by weight of dry charcoal (activated powder: Wako Pure Chemical Industries, Ltd., Osaka) and Celite (No. 545: Wako Pure Chemical Industries, Ltd., Osaka) were slurried in water and packed into a glass column. All other chemicals were obtained from commercial sources.

Enzyme assay.—β-D-Galactosidase activity was assayed as follows. A mixture containing 2 mM *o*-nitrophenyl β-D-galactopyranoside in 0.9 mL of 50 mM Na_3PO_4 buffer (pH 6.0) and an appropriate amount of enzyme in a total volume of 0.1 mL was incubated for 10 min at 30 °C. The reaction was stopped by adding 0.1 M Na_2CO_3 (2 mL), and then the liberated *o*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of activity was defined as the amount of the enzyme releasing 1 μmol of *o*-nitrophenol per min.

Analytical methods.—HPLC was performed with an Asahipak packed column NH2P-

50 (4.6 × 250 mm) in a Hitachi 6000-series liquid chromatograph equipped with a L-4000 UV detector and L-3350 RI monitor. Elution was effected with 25:75 H_2O–MeCN. The flow rate was 1.0 mL/min at a pressure of 60 kg/cm^2. ^{13}C and ^1H NMR spectra were determined with a JEOL JNM-EX 270 spectrometer operating at, respectively, 68 MHz in the pulsed Fourier-transform mode with complete proton decoupling and 270 MHz. Chemical shifts are expressed in ppm relative to sodium 4,4-dimethyl-4-silapropanoate (TPS) as the internal standard. NMR signal assignments were made using carbon–proton shift correlation spectroscopy (CH–COSY). FABMS analyses were carried out in the positive- and negative-ion mode using a JEOL JMS SX-102 mass spectrometer, coupled to a JEOL DA-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 was employed. The molecular weight of the sample was estimated from the m/z value of the quasi-molecular-ion $[M + 1]^+$ peak. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken Corp., Ltd.).

Preparation of β-Gal-D-(1 → 6)-β-D-Glc-OMe.—To a solution of lactose (1.5 g) and methyl β-D-glucoside (1.6 g) in 50 mM phosphate buffer (4.3 mL, pH 6.0) was added β-D-galactosidase (2.6 U) from *B. circulans*. The mixture was incubated for 20 h at 40 °C and terminated by heating for 10 min at 95 °C. The resulting insoluble material was centrifuged off. The supernatant was directly applied to a charcoal–Celite column (2.2 × 90 cm). The column was first eluted with water (300 mL) and then with a linear gradient of 0 (2 L)–25% (2 L) ethanol. The elution was monitored at 490 nm, with carbohydrate content, determined by the phenol–H_2SO_4 method. The eluate (20-mL fractions) showed two main peaks (F-1: tubes 96–120 and F-2: 131–155) which were presumed to contain transfer products. The former peak was collected, concentrated, lyophilized (95 mg), and further treated with the same enzyme in order to hydrolyze selectively the unwanted β-D-Gal-(1 → 4)-β-D-Glc-OMe in the product mixture [the relative rate of hydrolysis of the (1 → 6)-linked disaccharide with the (1 → 4)-linked one (100) is 2.5, a 40-fold difference]. F-1 was dissolved in 2 mL of the same buffer containing the same enzyme (0.3 U), and the reaction was allowed to proceed at 40 °C until β-D-Gal-(1 → 4)-β-D-Glc-OMe was no longer detected by HPLC; the time required for this reaction was 1 h. The reaction was terminated by heating for 15 min at 95 °C after adjusting the pH to 3.5 with 0.3 M HCl. The mixture was again adjusted to pH 6.5 with 0.2 M NaOH, and the solution was rechromatographed on the same column to afford a sharp peak (F-1′: 130–148) under the same elution conditions. The elution corresponding to F-1′ was combined, concentrated, and lyophilized to afford β-D-Gal-(1 → 6)-β-D-Glc-OMe (37 mg). F-2 was combined and concentrated to afford β-D-Gal-(1 → 3)-β-D-Glc-OMe (50 mg, crystallized from ethanol) as reported previously [11].

F-1′ had: $[\alpha]_D^{25}$ + 19.6° (*c* 1, H_2O) and m/z 357 $[M + H]^+$. NMR data (D_2O): ^1H, δ 4.402 (d, 1 H, *J* 7.6 Hz, H-1′), 4.344 (d, 1 H, *J* 7.9 Hz, H-1), and 3.526 (s, 3 H, OMe); ^{13}C, 106.14 (C-1, C-1′), 78.44 (C-5′), 77.90 (C-3), 77.66 (C-5), 75.81 (C-2), 75.45 (C-3′), 73.51 (C-2′), 72.15 (C-4), 71.41 (C-4′), 71.21 (C-6), 63.77 (C-6′), and 60.20 (OMe).

Preparation of β-D-Gal-(1 → 3)-β-D-Gal-OMe and β-D-Gal-(1 → 4)-β-D-Gal-OMe.—To a solution of lactose (1.5 g) and methyl β-D-galactoside (1.6 g) in 50 mM phosphate buffer (4.3 mL, pH 6.0) was added β-D-galactosidase (2.6 U) from *B.*

circulans. The mixture was incubated for 20 h at 40 °C and the reaction was terminated by heating for 10 min. The resulting insoluble material was centrifuged off and the supernatant was applied to the same carbon–Celite column already mentioned. Elution conditions were also the same. The eluates showed two main peaks (F-1: 98–118 and F-2: 126–146) as transfer products. F-1, after centrifugation to dryness followed by crystallization from ethanol, gave β-D-Gal-(1 → 4)-β-D-Gal-OMe (47 mg). F-2 was combined, concentrated, and lyophilized to afford β-D-Gal-(1 → 3)-β-D-Gal-OMe (78 mg).

F-1 had: $[\alpha]_D^{25}$ +10.69° (*c* 1, H_2O); mp 195–200 °C (from ethanol); and *m/z* $[M + H]^+$ 357. NMR data (D_2O): ^1H, δ 4.585 (d, 1 H, *J* 7.3, H-1′) and 4.359 (d, 1 H, *J* 7.9 Hz, H-1); ^{13}C, 107.02 (C-1), 106.50 (C-1′), 79.93 (C-4), 77.88 (C-5′), 77.02 (C-5), 76.01 (C-3), 75.54 (C-3′), 74.20 (C-2′), 74.03 (C-2), 71.40 (C-4′), 63.79 (C-6′), 63.29 (C-6), and 59.95 (OMe).

F-2 had: $[\alpha]_D^{25}$ +37.7° (*c* 0.07, H_2O); mp 217 °C (from ethanol); and *m/z* $[M + H]^+$ 357. NMR data (D_2O): ^1H, δ 4.571 (d, 1 H, *J* 7.6 Hz, H-1′) and 4.328 (d, 1 H, *J* 7.9 Hz, H-1). ^{13}C, 107.15 (C-1), 106.25 (C-1′), 85.25 (C-3), 77.84 (C-5′), 77.56 (C-5), 75.31 (C-3′), 73.84 (C-2′), 72.63 (C-2), 71.38 (C-4′), 71.25 (C-4), 63.74 (C-6, C-6′), and 59.93 (OMe).

Preparation of β-D-Gal-(1 → 6)-β-D-Gal-OMe.—To a solution of lactose (0.25 g) and methyl β-D-galactoside (0.27 g) in 50 mM phosphate buffer containing 0.1 mM $MnCl_2$ and 0.1 mM KCl (0.7 mL, pH 6.5) was added β-D-galactosidase (1.5 U) from *K. lactis*. The mixture was incubated for 2 h at 40 °C and the reaction was terminated by heating for 10 min. The resulting insoluble material was centrifuged off and the supernatant was applied to a carbon–Celite column (1.1 × 50 cm). The column was first eluted with water (45 mL) and then with a linear gradient of 0 (0.28 L)–25% (0.28 L) ethanol. F-1, which was eluted at ~ 15% ethanol concn (tubes 29–41), was collected, concentrated, and lyophilized (0.21 g). The fraction was further treated with *B. circulans* β-D-galactosidase in order to hydrolyze selectively the contaminated lactose in the product mixture. F-1 was dissolved in 25 mL of the same buffer containing the enzyme (7 U), and the reaction was allowed to proceed for 1 h at 40 °C until lactose was no longer detected by HPLC. The solution was rechromatographed on the same column to afford a sharp peak (F-1′: 33–41) under the same conditions. The eluates corresponding to F-1′ were combined, concentrated, and lyophilized to afford β-D-Gal-(1 → 6)-β-D-Gal-OMe (83 mg).

F-1′ had: $[\alpha]_D^{25}$ +11.41° (*c* 1, H_2O) and *m/z* 357 $[M + H]^+$. NMR data (D_2O): ^1H, δ 4.449 (d, 1 H, *J* 7.6 Hz, H-1′), 4.323 (d, 1 H, *J* 7.9 Hz, H-1), and 3.562 (s, 3 H, OMe); ^{13}C, 106.61 (C-1), 106.07 (C-1′), 77.95 (C-5′), 76.60 (C-5), 75.49 (C-3′), 75.42 (C-3), 73.53 (C-2′), 73.42 (C-2), 71.77 (C-6), 71.48 (C-4′), 71.41 (C-4), 63.77 (C-6′), and 60.13 (OMe).

Preparation of β-D-Gal-D-Glc3NAc.—To a solution of lactose (300 mg) and 3-acetamido-3-deoxy-D-glucose (Glc3NAc, 192 mg) in 100 mM phosphate buffer (0.8 mL, pH 5.0) was added *B. circulans* β-D-galactosidase (9.3 U). The mixture was incubated for 8.5 h at 30 °C and the reaction was terminated by heating for 10 min. The resulting insoluble material was centrifuged off and the supernatant was applied to a charcoal–Celite column (1.1 × 50 cm). The column was first eluted with water (300 mL) and then

with a linear gradient of 0 (400 mL)–25% (400 mL) ethanol. The elution was monitored by measuring the absorbance at 210 nm (*N*-acetyl group) and at 490 nm. The chromatogram showed two peaks (F-1: 50–56 and F-2: 60–72) of transglycosylation products for which the absorbance at 210 nm coincides with that at 490 nm. F-1 and F-2 were each combined, concentrated, and lyophilized to give yields of 54.2 and 71.2 mg, respectively. Each fraction was dissolved in 2 mL of 75% MeCN and one-fourth of this was resolved by HPLC. F-1 was separated as one main peak (F-1′) with a minor component (Glc3NAc). The remaining aliquots were similarly processed. The eluates corresponding to the F-1′ peak were combined, concentrated, and lyophilized to give a yield of 3.7 mg. All physical data for F-1′ were identical to those of β,β-D-Gal-(1 ↔ 1)-β-D-Glc3NAc reported previously [13]. F-2 was separated as one main peak with two minor peaks due to F-1 and Glc3NAc. The remainder of F-2 was similarly treated. The eluates corresponding to the F-2-b peak were combined, concentrated, and lyophilized to afford β-D-Gal-(1 → 6)-β-D-Glc3NAc (9.2 mg).

β-D-Gal-(1 → 6)-β-D-Glc3NAc had: $[\alpha]_D^{25}$ +45.36° (*c* 0.4, H_2O) and *m/z* 383. NMR data (D_2O): ^1H, δ 5.241 (d, 1 H, *J* 3.6 Hz, H-1α), 4.730 (d, 1 H, *J* 7.9 Hz, H-1β), 4.435 (d, 1 H, *J* 7.6 Hz, H-1′β), 4.417 (d, 1 H, *J* 7.9 Hz, H-1′α), 2.059 (s, 3 H, Ac due to the α anomer), and 2.053 (s, 3 H, Ac due to the β anomer); ^{13}C, 177.97 (C = O of Ac due to the α anomer), 177.82 (C = O of Ac due to the β anomer), 106.14 (C-1′), 99.33 (C-1β), 94.41 (C-1α), 78.85 (C-5β), 78.00 (C-5′), 75.49 (C-3′), 75.09 (C-2β), 73.57 (C-2′), 72.60 (C-2α), 71.46 (C-5α, C-6αβ), 71.34 (C-4′), 70.76 (C-4β), 70.55 (C-4α), 63.81 (C-6′), 59.91 (C-3β), 56.55 (C-3α), 25.05 (Me of Ac due to the β anomer), and 25.00 (Me of Ac due to the α anomer).

Purification of β-D-galactosidase from B. circulans.—The β-D-galactosidase of *B. circulans* was purified to apparent homogeneity by affinity chromatography sequentially on Butyl-Toyopearl 650M, pAP-S-Gal-Sepharose 4B, and by Sepharose 6 HR 10/30 gel filtration. Enzyme purification was carried out at 4 °C unless otherwise stated.

Step 1: Hydrophobic chromatography. Crude β-D-galactosidase (295 U, 150 mg) was dissolved in 50 mM phosphate buffer (pH 6.0) containing 30% $(NH_4)_2SO_4$ (buffer A). After the insoluble material had been centrifuged off, the supernatant was applied to a Butyl-Toyopearl 650M column (3.2 × 10 cm) equilibrated with buffer A and the column was washed with 600 mL of the same buffer (flow rate 84 mL/h). The column was eluted with a linear gradient of 2 (400 mL)–0 (400 mL) M $(NH_4)_2SO_4$ and the eluate was collected in 10 mL fractions. The active enzyme was eluted at about 0.8 M $(NH_4)_2SO_4$ (fraction number 105–120). Eluates of this fraction were combined and concentrated to low volume (5 mL) using an Amicon Diaflo unit equipped with a PM-10 membrane operating at 50 psi pressure (total activity 138 U: specific activity 5.1 U/mg).

Step 2: Affinity chromatography. The enzyme solution from step 1 was directly loaded onto a pAP-S-Gal-Sepharose 4B affinity column (1.2 × 5 cm) equilibrated with 50 mM phosphate buffer. pAP-S-Gal-Sepharose 4B affinity adsorbent was prepared by coupling *p*-aminophenyl 1-thio-β-D-galactopyranoside with formyl-Sepharose 4B according to the method of Ito et al. [14]. The column was sequentially washed with the equilibrated buffer (100 mL) and the same buffer containing 0.5 M NaCl (buffer B). When the column was changed to buffer B containing 0.2 M lactose, most of the

β-D-galactosidase activity emerged from the column. The eluates were combined and concentrated to low volume (2 mL) using Amicon Diaflo unit (total activity 75 U: specific activity 10.3 U/mg).

Step 3: Gel filtration. The enzyme solution for step 2 was applied to a column of Superose 6 HR 10/30 (10 × 300 mm) equilibrated with 50 mM phosphate buffer (pH 6.5) containing 0.15 M NaCl. The eluate was collected in 0.5-mL fractions. The elution pattern showed two enzyme peaks: a major peak with a high level of enzyme activity emerged from the column a little behind a minor peak with a low level of enzyme activity. Eluates of this major peak were combined, lyophilized, and then stored at 4 °C (total activity 50 U: specific activity 12.0 U/mg). As a result, the enzyme fraction gave a single protein band on SDS-polyacrylamide gel electrophoresis (data not shown).

3. Results and discussion

Commercially available crude β-D-galactosidases were directly used for the preparation of galactosyl-disaccharides without purification. Thus, following our previously developed methodology [1,4], we prepared β-D-Gal-(1 → 6)-β-D-Glc-OMe, β-D-Gal-(1 → 3)-β-D-Gal-OMe, and β-D-Gal-(1 → 4)-β-D-Gal-OMe utilizing the galactosyl transfer reaction of *B. circulans* β-D-galactosidase. These desired compounds were readily synthesized on a mmol scale and conveniently isolated by chromatography on a column of charcoal–Celite. β-D-Gal-(1 → 6)-β-D-Gal-OMe was also efficiently synthesized by consecutive use of β-galactosidases from *K. lactis* and *B. circulans*. Thus, the *K. lactis* enzyme induced predominantly the (1 → 6)-transfer product over its (1 → 4)-isomer. The latter was selectively removed from the resulting mixture by treatment with *B. circulans* β-D-galactosidase. These synthetic compounds were used as authentic samples for elucidating the regioselectivity of *B. circulans* β-D-galactosidase.

To know exactly the regioselectivity of *B. circulans* β-D-galactosidase on the galactosyl transfer reaction, the commercially available crude enzyme was separated into two enzyme fractions by successive chromatography on Butyl-Toyopearl 650M, pAP-S-Gal-Sepharose 4B, and Superose 6 HR 10/30. The major enzyme fraction, which gave a single protein band on SDS-polyacrylamide gel electrophoresis, was used in the present study. The molecular weight was estimated to be 205 and 247 Kd, as judged by SDS-polyacrylamide gel electrophoresis and gel filtration on Superose 6 HR 10/30, respectively. Mozaffar et al. have reported that β-D-galactosidases from the same enzyme source were purified and separated into two different enzyme forms [15]. Their molecular weights were shown to be 160 and 240 Kd, as judged by gel filtration.

Time-course of transglycosylation reaction on LacNAc production.—Fig. 1a is a transglycosylation profile of the reaction of *B. circulans* β-D-galactosidase with lactose and GlcNAc in acetate buffer (pH 6.0). The amounts of LacNAc and IsoLacNAc production as a function of time were examined on the 0.3-mL scale. The samples (10 μL) were taken at intervals during the incubation, inactivated by adding 20 μL of 1 M CH_3COOH and then were diluted with 4 vol of 50% acetonitrile for analysis by HPLC. In this case, only two transfer products were observed by HPLC. The transglycosylation reaction led to the preferential synthesis of LacNAc over IsoLacNAc in the initial stage

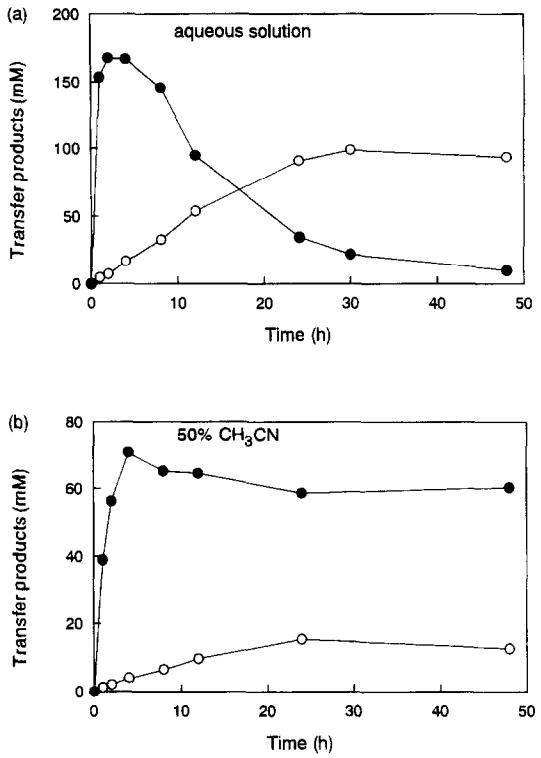

Fig. 1. Time-course of LacNAc and IsoLacNAc formation by the action of β-D-galactosidase from *B. circulans*. (a) The enzyme reaction was performed with lactose (66 mg), GlcNAc (54 mg), and β-D-galactosidase (0.5 U) at 40 °C in 0.3 mL of 50 mM acetate buffer (pH 6.0). The amounts of LacNAc (●) and IsoLacNAc (○) produced as a function of time were examined and samples were analyzed by HPLC during the incubation. (b) Lactose (45 mg), GlcNAc (55 mg), and β-D-galactosidase (0.3 U) were dissolved in 0.5 mL of the same buffer containing 50% CH_3CN. Other conditions were the same as those in (a).

of the reaction. When production of LacNAc reached a maximum at 2 h, LacNAc and IsoLacNAc were obtained in the molar ratio of 20:1 and in 38.4% overall yield (based on the donor). However, once formation of LacNAc reached its maximum, the amount decreased markedly during the subsequent reaction. On the other hand, IsoLacNAc formation was much slower and the time for maximum production was ~ 30 h. At that time, the molar ratio of IsoLacNAc and LacNAc was 5:1. Thus, much more of the (1 → 4)- than of the (1 → 6)-transfer product was found in the initial stage of the reaction, but the relation between this yield was reversed in the later stage of the reaction. The present reaction makes it possible to selectively synthesize LacNAc and its isomer IsoLacNAc by controlling the incubation time in the reaction system.

The solvent effect at 50% acetonitrile on β-D-galactosidase-mediated transglycosylation was investigated with respect to the production of LacNAc and IsoLacNAc as a function of time. LacNAc predominated over IsoLacNAc during the entire course of the reaction, as shown in Fig. 1b. Thus, the maximal production of LacNAc was reached at

5 h and its concentration then varied little during the subsequent reaction. The enzyme led to the regioselective production of (1 → 4)-linked LacNAc, regardless of the reaction time. This indicates that the formation of the (1 → 6)-linkage is unfavored with an increasing concentration of organic co-solvent (0–50%, acetonitrile).

Time-course of transglycosylation reaction on β-D-Gal-(1 → 4)-D-GalNAc production.—When GalNAc was used as acceptor instead of GlcNAc, two disaccharide products were observed by HPLC. Thus, galactosyl transfer occurred preferentially at O-4 of the sugar moiety in the initial stage of the reaction. When the production of β-D-Gal-(1 → 4)-D-GalNAc reached a maximum at 15 h, β-D-Gal-(1 → 4)-D-GalNAc and β-D-Gal-(1 → 6)-D-GalNAc were obtained in a molar ratio of 3:1 and in 23.8% overall yield (based on the donor). Once the formation of β-D-Gal-(1 → 4)-D-GalNAc reached its maximum, the amount decreased gradually during the subsequent reaction (data not shown). In contrast, the formation of β-D-Gal-(1 → 6)-D-GalNAc was much slower during the entire course of the reaction and the time for its maximal production was ~ 50 h. The relation between the yields of (1 → 4)- and (1 → 6)-transfer products was reversed in the latter stage of the reaction at 35 h. This was also the case for the formation of LacNAc and IsoLacNAc with the GlcNAc acceptor already mentioned.

Acceptor effects on β-D-galactosidase-mediated galactosyl-disaccharide formation.—The regioselectivity of the β-D-galactosidase-catalyzed formation of galactosyl-disaccharide was greatly changed by using methyl β-glycosides instead of the corresponding 2-acetamido sugars. When methyl β-D-glucoside was the acceptor, three disaccharide products: β-D-Gal-(1 → 4)-β-D-Glc-OMe, β-D-Gal-(1 → 3)-β-D-Glc-OMe, and β-D-Gal-(1 → 6)-β-D-Glc-OMe, were observed by HPLC, in the ratio of 45:37:18 and in 50.7% overall yield based on the donor added. These values are based on the time for maximum production of the (1 → 4)-linked product after 4 h. In this case, it should be noted that the (1 → 3)-transfer product was formed in appreciable amounts along with the (1 → 4)-transfer in the initial stage of the reaction, as shown in Fig. 2. However,

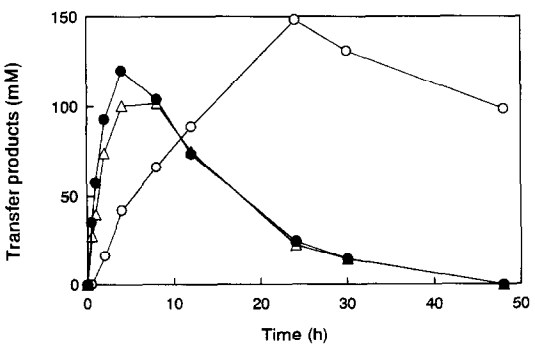

Fig. 2. Time-course of β-D-galactosidase-mediated isomer formation from lactose and methyl β-D-glucoside. The enzyme reaction was performed with lactose (56 mg), methyl β-D-glucoside (64 mg), and β-D-galactosidase (0.5 U) at 40 °C in 0.3 mL of 50 mM acetate buffer (pH 6.0). Other conditions were the same as those in Fig. 1a. β-D-Gal-(1 → 4)-β-D-Glc-OMe (●), β-D-Gal-(1 → 3)-β-D-Glc-OMe (△), and β-D-Gal-(1 → 6)-β-D-Glc-OMe (○) were formed.

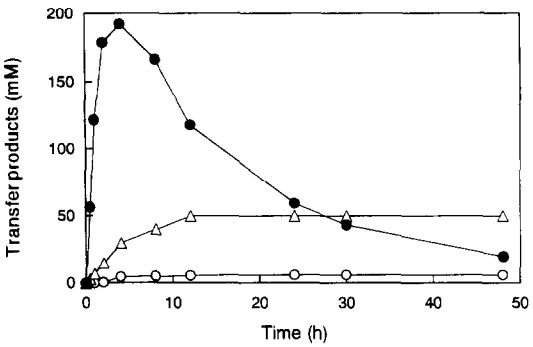

Fig. 3. Time-course of β-D-galactosidase-mediated isomer formation from lactose and methyl β-D-galactoside. Conditions were the same as those in Fig. 2 except for the acceptor. β-D-Gal-(1 → 4)-β-D-Gal-OMe (●), β-D-Gal-(1 → 3)-β-D-Gal-OMe (△), and β-D-Gal-(1 → 6)-β-Glc-D-OMe (○) were formed.

once the formation of the (1 → 4)- and (1 → 3)-linked products reached its maximum, their amounts decreased gradually with time. Formation of the (1 → 6)-linkage was much slower, and the time for the maximal production was ~ 24 h. Its concentration surpassed that of the (1 → 3)- and (1 → 4)-transfer products in the later stage of the reaction. As a result, this regioselectivity was markedly different from that with the GlcNAc acceptor. With methyl β-D-galactoside as the acceptor, a strikingly different result was obtained. Three products: β-D-Gal-(1 → 4)-β-D-Gal-OMe, β-D-Gal-(1 → 3)-β-D-Gal-OMe, and β-D-Gal-(1 → 6)-β-D-Gal-OMe, were observed in the ratio of 83:14:3 and in 39.2% overall yield (based on the donor), on the basis of the time at which the (1 → 4)-linked product reached its maximum at 4 h. In this case, galactosyl transfer favored O-4 of the acceptor rather than O-3, and occurred to a lesser extent at O-6, as shown in Fig. 3. Formation of the (1 → 6)-linkage was not favored during the entire course of the reaction. An interesting result from these studies was the capacity of the enzyme to catalyze the formation of (1 → 3)-linked disaccharide, when a methyl β-glycoside was acceptor instead of its corresponding 2-acetamido analogue.

Regioselectivity of galactosyl-disaccharide formation.—The positions of enzymic galactosylation in all galactosyl-disaccharide products, determined by HPLC, are depicted by arrows in Fig. 4. In all cases, this shows the percentage of galactosylation, based on the time at which the (1 → 4)-linked disaccharide production reached its maximum. In the case of the GlcNAc acceptor, galactosylation occurs overwhelmingly at O-4. Replacement of GalNAc by GlcNAc does not alter the direction of galactosylation, but the regioselectivity at O-4 is not as large as that observed for the GlcNAc. With both the acceptors, no (1 → 3)- and (1 → 1)-transfer products were detected during the reaction. In the case of the methyl β-D-glucoside acceptor, it may be seen that the regioselectivity is much lower than that of GlcNAc: about four-fifths of the galactosylation occurs at O-3 and O-4 and 18% at O-6. Replacement of methyl β-D-galactoside by methyl β-D-glucoside does not alter the direction of galactosylation, but influences the regioselectivity. Thus, the enzyme galactosylates O-4 of the acceptor preferentially to O-3 and reacts only weakly at the O-6. With both of the methyl β-glycoside acceptors,

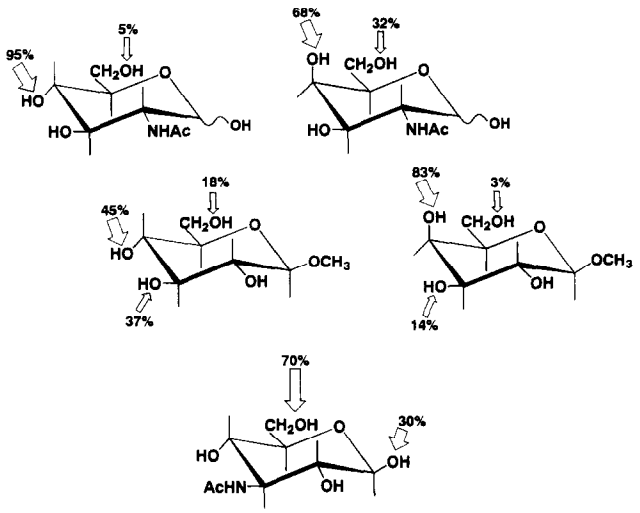

Fig. 4. Structures of carbohydrates used as targets for β-D-galactosidase-catalyzed galactosylation. Arrows indicate the positions of galactosylation. Percentages above the arrows are of the formation of a given transglycosylation compared with the total.

it should be noted that the (1 → 3)-linked product is formed rather than the (1 → 4)- and (1 → 6)-linked ones. These results suggest that the presence of the 2-acetamido group in GlcNAc and GalNAc may hinder galactosylation at its neighboring hydroxyl groups (OH-3 and OH-1). In order to confirm the hypotheses, we tested a reaction with the Glc3NAc acceptor. With the enzyme, two disaccharide products were observed in 13.3% overall yield (based on the donor). The enzyme not only transferred Gal to O-6 of Glc3NAc, but also to the β-anomeric position (O-1). Thus, β-D-Gal-(1 → 6)-D-Glc3NAc and β,β-D-Gal-(1 ↔ 1)-D-Glc3NAc were formed in the molar ratio of 2.3:1. The relation between the molar ratio varied little during the entire course of the reaction (data not shown). In this case, no galactosylation to O-2 and O-4 of the acceptor (which are adjacent to the 3-acetamido group) was observed during the reaction, as expected. Nishida et al. have reported that the galactosyltransferase from bovine milk, which is regiospecific at the OH-4 position of GlcNAc, catalyzes the transfer of galactose from UDP-galactose to the β-anomeric position of Glc3NAc to afford a β,β-D-Gal-(1 ↔ 1)-D-Glc3NAc [13]. This regioselectivity toward a Glc3NAc acceptor, whose β-anomeric stereochemistry along C-1 to C-4 is superposable on that of C-4 to C-1 of GlcNAc, was attributed to an *N*-acetyl binding locus in the active site of both the enzyme and the GlcNAc acceptor. In the present study, there seems to be a similar tendency for allowing the *B. circulans* β-D-galactosidase to transfer galactose to the β-anomeric hydroxyl group of Glc3NAc. These results suggest that the regioselectivity of the β-D-galactosyl transfer to GlcNAc, GalNAc, and Glc3NAc acceptors as catalyzed by the enzyme is strongly determined by the presence of *N*-acetyl group.

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