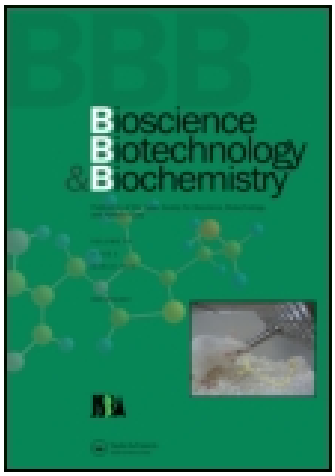

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Takeomi Murata[a], Satoru Akimoto[a], Miki Horimoto[a] & Taichi Usui[a]
[a] Faculty of Agriculture, Department of Applied Biological Chemistry, Shizuoka University, 836 Ohya, Shizuoka 422, Japan
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Galactosyl Transfer onto *p*-Nitrophenyl β-D-Glucoside Using β-D-Galactosidase from *Bacillus circulans*

Takeomi MURATA, Satoru AKIMOTO, Miki HORIMOTO, and Taichi USUI*

Faculty of Agriculture, Department of Applied Biological Chemistry, Shizuoka University, 836 Ohya, Shizuoka 422, Japan
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β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* and its isomers (β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p*) were synthesized from lactose and β-D-Glc-$OC_6H_4NO_2$-*p*, using transglycosylation by the β-D-galactosidase from *Bacillus circulans*. This reaction was efficient enough for us to do a one-pot preparation of galactosyl-glucoside from lactose. The order of the production of the transfer products was (1→4)≫(1→3)>(1→6) in the initial stage of the reaction, and the same relationship was observed for the hydrolytic rate toward the three galactosyl-glucosides. The production of (1→4)- and (1→3)-linkages greatly decreased during the subsequent reaction and much more of the (1→6)- than of the (1→4)- and (1→3)-transfer products was found in the later stage of the reaction.

Key words: β-D-galactosidase; galactosyl-glucoside; enzymic synthesis; transglycosylation; regioselectivity

Significant attentions have been focused on the application of glycosidase-catalyzed transglycosylation for practical synthesis of oligosaccharides involved in glycoconjugates.[1] The enzymic synthesis has become more practical by the use of several glycosidases available in sufficient quantity, because glycosidases do have some regioselectivity for the hydroxyl linkage to the acceptor. Some researchers have reported that the regioselectivity can be manipulated to some extent by using the following techniques: careful selection of enzymes,[2–5] organic co-solvent system,[6,7] inclusion complex of acceptor glycoside with cyclodextrin,[8,9] and the configuration of the glycosidic linkage of acceptor.[10,11] We have already developed a practical route for the synthesis of LacNAc and its glycoside by transglycosylation, using the β-D-galactosidase from *Bacillus circulans*. This paper extends our regioselective galactosylation methodology by the enzyme to sugar other than GlcNAc.

This paper details a convenient synthesis of *p*-nitrophenyl galactosyl-glucoside by using the *B. circulans* β-D-galactosidase-catalyzed transfer and hydrolytic reaction with *p*-nitrophenyl β-D-glucoside acceptor and the regioselectivity of its transglycosylation.

Materials and Methods

Materials. Commercially available β-D-galactosidase (EC 3.2.1.23), Biolacta (Daiwa Kasei Co., Ltd., Osaka, Japan) from *B. circulans* was directly used for the enzymic synthesis. The crude enzyme was further purified by the previously developed method to analyze exactly the regioselectivity of the enzyme on the galactosyl transfer reaction.[6] β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (*p*-nitrophenyl β-lacto-*N*-bioside I, **5**), β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (*p*-nitrophenyl *N*-acetyl-β-lactosaminide, **4**), and β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (*p*-nitrophenyl *N*-acetyl-β-isolactosaminide, **6**) as authentic samples and substrates for hydrolytic reactions were prepared by our method.[2,7] 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 sodium phosphate 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 2 ml of 0.1 M Na_2CO_3, and then the liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmol of *o*-nitrophenyl β-D-galactopyranoside per min.

Analytical method. HPLC was done with a YMC-packed column type AQ-312 (ODS) (φ6 × 150 mm) in a Hitachi 6000-series liquid chromatograph with an L-4000 ultraviolet detector. Elution of the column was done with H_2O–MeOH of 88:12. The flow rate was 0.8 ml/min at 40°C. ^{13}C- and ^1H-NMR spectra were recorded on a JEOL JNM-EX 270 spectrometer. Chemical shifts are expressed in ppm relative to sodium 3-(trimethylsilyl)-propionate as an external standard. FAB-MS analyses were done in the positive ion mode using a JEOL JMS DX-303HF mass spectrometer, coupled to a JEOL DA-5000 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 was used. A sample of 1 μl in distilled water was put onto a probe tip and mixed with 1 μl of glycerol as a matrix. Mass calibration was done using Ultramark. Specific rotation was measured with a digital polarimeter DIP-1000 apparatus (JASCO Corp. Ltd., Tokyo, Japan).

p-Nitrophenyl β-lactoside (**1**) *and its isomers* (*β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-p*, **2** *and β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-p*, **3**). Commercially available crude β-D-galactosidase was used directly for the preparation of galactosyl-glucosides. Thus, following our previously developed method,[8] we prepared compounds **1**, **2**, and **3** using the galactosyl transfer reaction of *B. circulans* β-D-galactosidase. These compounds were readily synthesized on a mmol scale. To a solution (12 ml) of lactose (2.4 g) and *p*-nitrophenyl β-D-glucoside (2.0 g) in 20 mM phosphate buffer (pH 7.0) containing 20% MeCN was added β-D-galactosidase from *B. circulans* (20 U). The molar ratio of the donor and acceptor was 1:1 and the total substrate concentration was 36.7%. After being incubated for 6 h at 40°C, the reaction mixture was heated for 10 min at 95°C and centrifuged. The supernatant was directly put onto a Toyopearl HW-40S column (φ5 × 100 cm) as in Fig. 1. The eluate was monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group) and at 485 nm (phenol–sulfuric acid method). The chromatogram showed four peaks (F-1, tubes 117–122; F-2, tubes 133–142; F-3, tubes 152–160; F-4, tubes 161–168) as transglycosylation products displaying coincident absorbance at 300 nm and 485 nm. Fractions F-1, F-3, and F-4, after concentration to dryness followed by crystallization from MeOH, gave compounds **1** (270.4 mg), **2** (119.5 mg), and **3** (75.7 mg), respectively.

† To whom correspondence should be addressed.

Abbreviations: FAB-MS, fast atom bombardment-mass spectrometry, HPLC, high pressure liquid chromatography; MeCN, acetonitrile; MeOH, methanol; NMR, nuclear magnetic resonance.

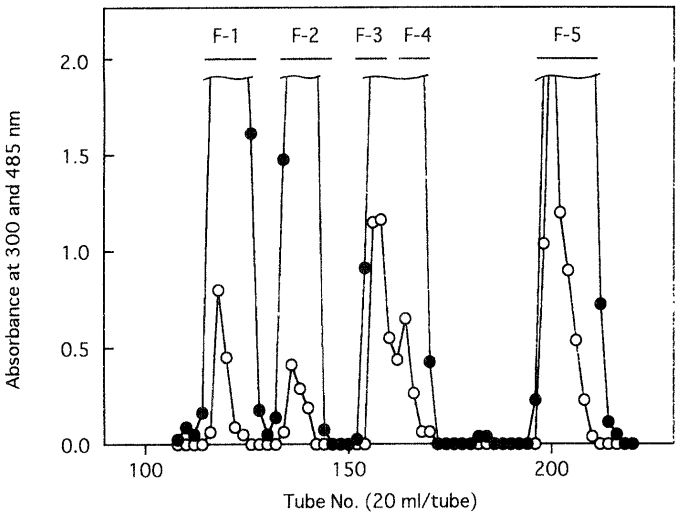

Fig. 1. Toyopearl HW-40S Chromatographic Separation of Transglycosylation Products Formed from Lactose and β-D-Glc-$OC_6H_4NO_2$-*p* by the *B. circulans* β-D-Galactosidase.

(●), absorbance at 300 nm; (○), absorbance at 485 nm.

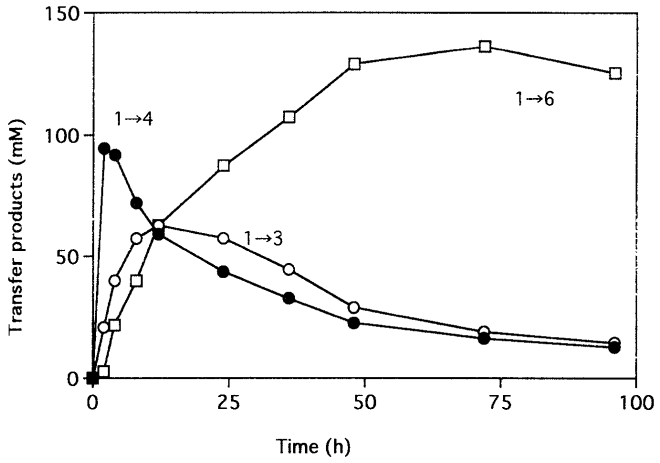

Fig. 2. Course of *B. circulans* β-D-Galactosidase-mediated Transglycosylation from Lactose and β-D-Glc-$OC_6H_4NO_2$-*p*.

The amounts of **1** (●), **2** (○), and **3** (□) as a function of time were examined on the 0.3-ml scale as described in the Materials and Methods section, and samples were analyzed by HPLC during incubation.

Compound **1** and its isomers **2** and **3** were obtained in a total yield of 15.1% based on the *p*-nitrophenyl β-D-glucoside added and in a ratio of 58:26:16. Another fraction F-2 was combined, concentrated, and lyophilized to yield 43.8 mg. It was assumed to be trisaccharide glycoside as transfer product, because its FAB-MS spectrum showed *m/z* 626 corresponding to the molecular ion (Hex-Hex-Hex-$OC_6H_4NO_2$-*p*). Fraction F-5 (tubes 195–213) contained *p*-nitrophenyl β-D-glucoside used acceptor substrate. All physical data for **1** and **2** were identical to those of β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p* reported previously.[8] These synthetic compounds were used as authentic samples for analyzing the regioselectivity of *B. circulans* β-D-galactosidase.

Hydrolytic reaction of B. circulans β-D-galactosidase on p-nitrophenyl galactosyl-glucosides. The relative rates of attack of β-D-galactosidase on *p*-nitrophenyl galactosyl-glucosides were measured by incubating a mixture (1 ml) containing 0.4 mM of substrates in 50 mM sodium phosphate buffer (pH 6.0) with 0.5 U of the enzyme at 40°C. Samples (100 μl) were taken out at 5-min intervals during the reaction (0, 5, 10, and 15 min). After inactivation of each sample by adding 200 μl of 1 M acetic acid, the liberated *p*-nitrophenyl glycosides were measured by HPLC and spectrophotometry at 300 nm. The reaction was linear from 5 to 15 min. The rate of attack on **1** (or **4**) was arbitrarily set at 100.

Table The Hydrolytic Rates of *B. circulans* β-D-Galactosidase on *p*-Nitrophenyl Galactosyl-glycosides

Compounds	Relative rate (%)
β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**1**)	100[a]
β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p* (**2**)	12
β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p* (**3**)	3
β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**4**)	100[a]
β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**5**)	4
β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**6**)	0

[a] The hydrolytic rate on **1** (or **4**) was arbitrarily set at 100.

Results and Discussion

Characterization of compound 3

The positive ion mode FAB-MS spectrum shows a molecular ion at *m/z* 464 ($[M+H]^+$). It indicated that compound **3** is a *p*-nitrophenyl disaccharide consisting of Hex-Hex-$OC_6H_4NO_2$-*p*. ^{1}H- and ^{13}C-NMR were used for further study of the structure of compound **3**. ^{1}H-NMR (D_2O): δ 8.25 (d, 2H, *J*=9.2 Hz, *m*-Ph), 7.27 (d, 2H, *J*=9.2 Hz, *o*-Ph), 5.27 (d, 1H, *J*=7.6 Hz, H-1), 4.41 (d, 1H, *J*=6.9 Hz, H-1′). The carbon resonances were assigned by the C–H shift-correlation map and by comparing the spectrum with the data for compounds **1** and **2**.[8] ^{13}C-NMR (D_2O), δ 164.45 (Ph carbon attached to the phenolic oxygen), 145.44 (*p*-Ph), 128.95 (*m*-Ph), 119.41 (*o*-Ph) 105.89 (C-1′) 103.00 (C-1), 78.22, (C-5′), 78.13 (C-5), 77.93 (C-3), 75.51 (C-3′), 75.57 (C-2), 73.57 (C-2′), 72.07 (C-4), 71.45 (C-4′), 71.14 (C-6), 63.76 (C-6′). These results indicated that compound **3** is a *p*-nitrophenyl disaccharide β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p*: $[\alpha]_D^{25}$ −106.4° (*c* 1, H_2O); mp 137–139°.

Course of the transglycosylation reaction on p-nitrophenyl β-D-lactoside production

The purified enzyme was used in this study, since it can be assumed with certainly that all of the transfer reactions observed with the respective substrate were catalyzed by a single enzyme. Figure 2 is a transglycosylation profile of the reaction of *B. circulans* β-D-galactosidase with lactose and *p*-nitrophenyl β-D-glucoside in phosphate buffer (pH 7.0). The amounts of **1**, **2**, and **3** as a function of time were examined on the 0.3-ml scale. Samples (10 μl) were taken out at intervals during the incubation, inactivated by adding 20 μl of 1 M CH_3COOH and then were diluted with 4 vol. of water for analysis by HPLC. The transglycosylation reaction led to the preferential synthesis of **1** in the initial stage of the reaction. When the production of **1** reached a maximum at 4 h, **1**, **2**, and **3** were obtained in the ratio of 79:17:4 and in 21.6% overall yield (based on the donor). No (1→2)-transfer product was detected during the reaction. However, once formation of **1** reached its maximum, the amount markedly decreased during the subsequent reaction. On the other hand, the formation of **2** was a little slower than that of **1** and the time for its maximum product was 12 h. At that time, the molar ratios of **1**, **2**, and **3** were almost equal. When the production of **3** reached a maximum at 70 h, **1**, **2**, and **3** were obtained in the ratio of 9:12:79 and in 31.1% overall yield (based on the donor). This reaction makes it possible to selectively synthesize **1** and its isomer **3** by controlling the reaction time. It also indicates

that the order of formation of these disaccharides in the initial stage of the reaction was (1→4)≫(1→3)>(1→6), and the same relationship was also observed for the hydrolytic rate as in Table. In a separate experiment, the relative rate of hydrolysis of **2** and **3** compared with **1** (100) was 12 and 3, 8–33-fold differences. Compound **1** should be a much better substrate than **3** under these hydrolytic conditions. Thus, this order of the hydrolytic rate of (1→4)≫(1→3)>(1→6) corresponded to that of their transglycosylation. We have already reported that the rate of the formation of β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**4**, *p*-nitrophenyl *N*-acetyl-β-lactosaminide) through the enzymic transglycosylation from lactose to *p*-nitrophenyl *N*-acetyl-β-glucosaminide is much faster than that of β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**5**, *p*-nitrophenyl *N*-acetyl-β-D-isolactosaminide), but no (1→3)-transfer product, β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**6**, *p*-nitrophenyl β-lacto-*N*-bioside I) was detected during the reaction.[7] The same relationship among these disaccharides was also observed for the hydrolytic rate. Namely, the relative rate of hydrolysis of **5** compared with **4** (100) was 4, a 25-fold difference, and the (1→3)-linked isomer **6** was not hydrolyzed by this enzyme under these conditions. It indicates that the order of formation of transfer product closely corresponds to that of the hydrolytic rate.

Regioselectivity of galactosyl-disaccharide formation

The positions of enzymic galactosylation in all galactosyl-glucoside products, identified by HPLC, are depicted by arrows in Fig. 3. In two cases, each number shows the percentage of galactosylation, based on the time at which the (1→4)-linked disaccharide production reached its maximum. When *p*-nitrophenyl β-D-glucoside was acceptor, the enzyme galactosylated O-4 of the acceptor preferentially to O-3 and reacted only weakly at the O-6. No (1→2)-transfer product was detected during the reaction. The regioselectivity is lower than that of β-D-GlcNAc-$OC_6H_4NO_2$-*p* acceptor,[7] because (1→3)-linked product is formed other than (1→4)- and (1→6)-linked ones. Yanahira *et al.* have reported the formation of β-D-Gal-(1→2)-D-Glc from lactose and glucose employing by transglycosylation of *B. circulans* β-D-galactosidase.[12] The presence of the aglycon moiety in the glycoside acceptor may hinder galactosylation to its neighboring hydroxyl group OH-2. In the case that *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide is used an acceptor, galactosylation occurs overwhelmingly at O-4 and reacts only weakly at the O-6. No (1→3)-transfer product was detected during the reaction. We have recently reported that, when 3-acetamido-3-deoxy-D-glucose (Glc3NAc) was the acceptor, the enzyme transferred Gal not only to O-6 of Glc3NAc, but also to the β-anomeric position (O-1). No galactosylation to O-2 and O-4 of the acceptor (which are adjacent to the 3-acetamido group) was observed during the reaction.[6] These results indicate that the existence of substituent group (*N*-acetyl group and *p*-nitrophenyl group) in the acceptor hinders galactosylation to its neighboring hydroxyl group. In contrast, enzymes from bovine and porcine testes induced β-D-galactosyl transfer to OH-3 of β-D-GlcNAc-$OC_6H_4NO_2$-*p* and β-D-GalNAc-$OC_6H_4NO_2$-*p*.[2]

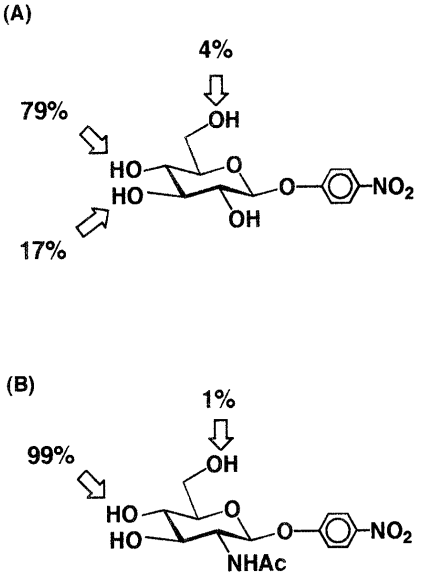

Fig. 3. Percentages of *p*-Nitrophenyl Galactosyl-glucosides Formed by *B. circulans* β-D-Glactosidase Transglycosylation.

(A) β-D-Glc-$OC_6H_4NO_2$-*p* and (B) β-D-GlcNAc-$OC_6H_4NO_2$-*p* were used as acceptors.

* The numbers show the function of a given transglycosylation compared with the total.

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References

1) Y. Ichikawa, G. C. Look, and C.-H. Wong, *Anal. Biochem.*, **202**, 215–238 (1992).
2) T. Murata, T. Itoh, Y. Hayakawa, and T. Usui, *J. Biochem.*, **200**, 851–855 (1996).
3) K. Sakai, R. Katsumi, H. Ohi, T. Usui, and Y. Ishido, *J. Carbohydr. Chem.*, **11**, 553–565 (1992).
4) L. Hedbys, E. Johansson, K. Mosbach, and P. Larsson, *Carbohydr. Res.*, **186**, 217–223 (1989).
5) L. Hedbys, E. Johansson, K. Mosbach, P. Larsson, A. Gunnarssion, S. Svennsson, and H. Lönn, *Glycoconjugate J.*, **6**, 161–168 (1989).
6) T. Usui, S. Morimoto, Y. Hayakawa, M. Kawaguchi, T. Murata, Y. Matahira, and Y. Nishida, *Carbohydr. Res.*, **285**, 29–39 (1996).
7) T. Usui, S. Kubota, and H. Ohi, *Carbohydr. Res.*, **244**, 315–323 (1993).
8) Y. Matahira, A. Tashiro, T. Sato, H. Kawagishi, and T. Usui, *Glycoconjugate J.*, **12**, 664–671 (1995).
9) T. Murata, A. Tashiro, T. Itoh, and T. Usui, *Biochim. Biophys. Acta*, in press. (1997).
10) K. G. I. Nilsson, *Carbohydr. Res.*, **167**, 95–103 (1987).
11) D. H. G. Crout, O. W. Howarth, S. Singh, B. E. P. Swoboda, P. Critchley, and W. T. Gibson, *J. Chem. Soc., Chem. Commun.*, **1991**, 1550–1551.
12) S. Yanahira, T. Kobayashi, T. Suguri, M. Nakakoshi, S. Miura, H. Ishikawa, and I. Nakajima, *Biosci. Biotech. Biochem.*, **56**, 1021–1026 (1995).