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Convenient Synthesis of β-(1→3)-Galactosyl Disaccharide α-Glycoside and Its Analogs as Mimic Units of Mucin-Type Carbohydrate[1]

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β-Galactosidase from porcine testes induced regioselective transglycosylation from lactose to the 3-position of 2-acetamido glycosides. When α-D-GalNAc-$OC_6H_4NO_2$-*p* was used as an acceptor, the enzyme synthesized mainly β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* with its (1→6) linked isomer. The use of an inclusion complex of the glycoside acceptor with β-CD increased the efficiency of transglycosylation by increasing the solubility of the acceptor. In the same way, the use of β-D-GalNAc-$OC_6H_4NO_2$-*p* as acceptor led to the preferential synthesis of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* over that of its (1→6) linked isomer. β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* was also synthesized with β-D-GlcNAc-$OC_6H_4NO_2$-*p* acceptor by the consecutive use of β-D-galactosidases from porcine testes and *Bacillus circulans*. These enzyme reactions are efficient enough to allow the one-pot preparation of the desired disaccharide glycosides.

Key words: endo-α-*N*-actylgalactosaminidase, β-D-galactosidase, β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, mucin-type oligosaccharide, transglycosylation.

The *O*-linked oligosaccharide components of glycoprotein are of interest because of their numerous roles in biological events such as cell-cell recognition (*1*). The common structure at the carbohydrate core region of mucin is a 3-*O*-β-D-galactosyl-*N*-acetylgalactosamine unit α-glycosidically linked to serine or threonine. An organic chemical method for obtaining β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* has been developed (*2*), but it involves various elaborate procedures for protection, glycosylation, and deprotection. From a practical viewpoint, the use of glycosidase is an attractive alternative for synthesis of such a disaccharide glycoside. On the other hand, Hedbys *et al.* have synthesized reducing disaccharides β-D-Gal-(1→3)-D-GalNAc (*3*) and β-D-Gal-(1→3)-D-GlcNAc (*4*) by transglycosylation utilizing β-D-galactosidase from bovine testes. Our interest was directed to an enzymic approach involving a 3-*O*-β-D-galactosyl-*N*-acetylgalactosamine unit α-glycosidically linked to *p*-nitrophenol as a mimic unit of mucin-type carbohydrate. This compound would be useful as an exogenous substrate for endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase, EC 3.2.1.97) (*5*), as a probe for lectin and as a model compound of *O*-linked glycan. Thus, the object of the present investigation is to develop a system for selective transfer of a galactosyl residue to the C-3 position of an α-glycoside acceptor, based on this approach.

The present paper describes a preparative synthetic method for β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, which is a mimic unit of mucin type I, and its analogs, by means of porcine testes β-D-galactosidase-catalyzed transglycosylation using an inclusion complex of *p*-nitrophenyl glycoside acceptor with β-CD.

MATERIALS AND METHODS

Materials—β-D-Galactosidase [EC 3.2.1.23] from *Bacillus circulans* was obtained from Daiwa Kasei (Osaka). Endo-α-GalNAc-ase from *Alcaligenes* sp. was purchased from Seikagaku (Tokyo). β-D-Gal-(1→4)-α-D-GlcNAc-$OC_6H_4NO_2$-*p* was synthesized by our method (*6*). Nagstatin, an inhibitor of *N*-acetyl-β-D-hexosaminidase (β-NAHase) (*7*), was obtained from Meiji Seika (Tokyo). 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 determined spectrophotometrically at 420 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmol of *o*-nitrophenyl β-D-galactopyranoside per min.

Partially Purified β-D-Galactosidase from Porcine Testes—A crude preparation of β-D-galactosidase was prepared by precipitation with ammonium sulfate from an extract of porcine testes by the same method as previously used with bovine testes (*3*). The crude β-D-galactosidase was used without further purification for the syntheses of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*. For synthesis of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, the crude enzyme

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Abbreviations: endo-α-GalNAc-ase, endo-α-*N*-acetylgalactosaminidase; α-NAHase, *N*-acetyl-α-D-hexosaminidase; β-NAHase, *N*-acetyl-β-D-hexosaminidase; β-CD, β-cyclodextrin.

preparation was purified by removal of *N*-acetyl-α-D-hexosaminidase (α-NAHase) by column chromatography. The crude enzyme (1 g) was dissolved in 60 ml of 20 mM citrate phosphate buffer (pH 4.5) and charged onto a column (φ3.2×26 cm) of CM-Sepharose Fast Flow equilibrated with the same buffer. The column was washed with 200 ml of the same buffer. The enzyme was eluted with a linear gradient of concentration of NaCl from 0 (500 ml) to 1.0 M (500 ml) and the eluate was collected in 20-ml fractions. The fractions containing β-D-galactosidase activity (tubes 34-42) were eluted at about 0.5 M NaCl. Eluates of the corresponding enzyme fractions were combined and concentrated to small volume (1.5 ml) using an Amicon Diaflo unit. The concentrated enzyme solution was loaded onto a column (φ2.6×60 cm) of HiLoad 26/60 Sephacryl S-200 equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and 0.02% NaN_3. Elution was performed with the same buffer at a flow rate of 1.5 ml/min, and fractions of 2.5 ml were collected. The elution pattern showed two β-D-galactosidase peaks (F-1: tubes 32-38 and F-2: 44-56) and one α-NAHase peak (tubes 52-62). The fractions consisting the F-1 peak were completely devoid of α-NAHase, and these was combined and used for synthesis of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (total activity: 0.12 U; specific activity: 0.044 U/mg).

Analytical Method—HPLC was performed with a YMC-packed column type AQ-312 (ODS) (φ6×150 mm) and TSK-GEL G-Oligo-PW (φ7.8×300 mm) in a Hitachi 6000-series liquid chromatograph equipped with an L-4000 ultraviolet detector. Elution of the former column was effected with 88 : 12 H_2O-CH_3OH, and that of the latter with H_2O. 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 (TPS) as an external standard. FAB-MS analyses were carried out in the positive ion mode using a JEOL JMS DX-303HF mass spectrometer, coupled to a JEOL DA-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1,000 was employed. A sample of 1 μl in distilled water was loaded onto a probe tip and mixed with 1 μl of glycerol as a matrix. Mass calibration was performed using Ultramark. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken).

Preparation of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-p (A-1) and Its Positional Analog β-D-Gal-(1→6)-α-D-GalNAc-$OC_6H_4NO_2$-p (A-2)—α-D-GalNAc-$OC_6H_4NO_2$-*p* (120 mg) and β-CD (398 mg) were first dissolved in 18 ml of 20 mM sodium acetate buffer (pH 5.5), then lactose (2.48 g) was added, followed by partially purified β-D-galactosidase from porcine testes (0.12 U). The molar ratio of the donor to acceptor was about 20 : 1, and the total substrate concentration was about 14%. The mixture was incubated for 50 h at 40°C and the reaction was terminated by adding 8 ml of 1 M acetic acid followed by heating at 95°C for 10 min. Insoluble material was removed by centrifugation, and the supernatant was loaded onto a Toyopearl HW-40S column (φ4.5×90 cm) equilibrated with 25% MeOH in aqueous solution, and the effluent solution was monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group) and 485 nm (carbohydrate content, determined by phenol-sulfuric acid method). As shown in Fig. 1, the chromatogram contained two peaks

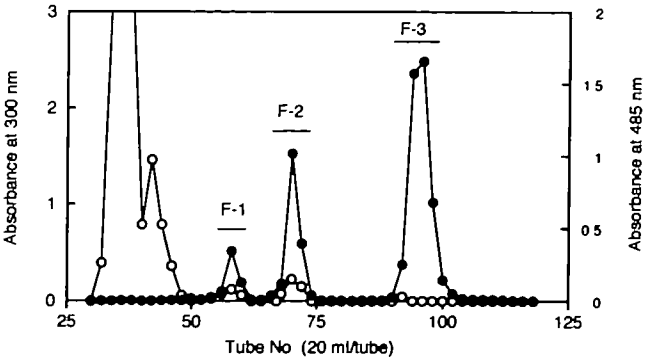

Fig. 1. **Chromatographic separation of transglycosylation products formed from lactose and α-D-GalNAc-$OC_6H_4NO_2$-*p* by porcine testes β-D-galactosidase.** ●, absorbance at 300 nm; ○, absorbance at 485 nm.

(F-1, tubes 56-61; and F-2, tubes 66-74) displaying coincident absorbance at 300 and 485 nm. These peaks were presumed to contain transfer products. Fraction F-2, after lyophilization to dryness followed by crystallization from ethanol, gave compound A-1 (27.2 mg). The eluates corresponding to F-1 were combined, concentrated and lyophilized to afford compound A-2 (8.8 mg). F-3 (tubes 93-104) contained α-D-GalNAc-$OC_6H_4NO_2$-*p* (64 mg) used as the acceptor.

The physical data for compound A-1 were identical to those of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* reported previously (*2*). NMR data (D_2O) of A-1: ^1H, δ 8.25 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.26 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.80 (d, 1H, <*J* 1 Hz, H-1), 4.53 (d, 1H, *J* 7.6 Hz, H-1′), 1.92 (s, 3H, NAc); ^{13}C, δ 177.52 (C=O of Ac), 164.02 (Ph carbon attached to the phenolic oxygen), 145.12 (*p*-Ph), 128.79 (*m*-Ph), 119.39 (*o*-Ph), 107.64 (C-1′), 98.60 (C-1), 79.31 (C-3), 77.86 (C-5′), 75.29 (C-3′), 74.77 (C-5), 73.39 (C-2′), 71.37 (C-4,4′), 63.83 (C-6′), 63.74 (C-6), 50.10 (C-2), 24.73 (Me of Ac).

A-2 had: $[\alpha]_D^{25}$+135.4°(c 0.5, H_2O); m.p. 207° (from ethanol); and *m/z* 505 $(M+H)^+$. NMR data (Me_2SO-d_6): ^1H, δ 8.15 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.22 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.53 (d, 1H, *J* 3.5 Hz, H-1), 4.09 (d, 1H, *J* 7.6 Hz, H-1′), 1.84 (s, 3H, NAc); ^{13}C, δ 163.09 (Ph carbon attached to the phenolic oxygen), 143.31 (*p*-Ph), 127.06 (*m*-Ph), 118.51 (*o*-Ph), 104.30 (C-1′), 97.70 (C-1), 76.03 (C-5′), 74.00 (C-3′), 72.10 (C-5), 71.59 (C-2′), 69.37 (C-4′), 69.37 (C-4), 69.02 (C-6′), 67.91 (C-3), 61.73 (C-6), 49.76 (C-2), 22.94 (Me of Ac).

Preparation of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-p (B-1) and β-D-Gal-(1→6)-β-D-GalNAc-$OC_6H_4NO_2$-p (B-2)—β-D-GalNAc-$OC_6H_4NO_2$-*p* (66 mg) and β-CD (219 mg) were first dissolved in 12.5 ml of 20 mM acetate buffer (pH 5.5), then lactose (2.79 g) and Nagstatin (10 mM) were added, followed by the crude β-D-galactosidase (1.0 U) prepared by precipitation with ammonium sulfate from porcine testes. The molar ratio of the donor to acceptor was about 10 : 1, and the total substrate concentration was about 23%. The mixture was incubated for 12 h at 40°C and the reaction was terminated by heating at 95°C for 10 min. The resulting insoluble material was removed by centrifugation, and the supernatant was extracted with diethyl ether in order to remove *p*-nitrophenol liberated during the reaction. The aqueous phase was concentrated to small

volume (20 ml) and loaded onto a Toyopearl HW-40S column as described above. The chromatogram showed two main peaks (F-1, tubes 67-75; and F-2, tubes 84-98) for which the absorbance at 300 nm coincided with that at 485 nm (data not shown). F-1 and F-2 were each concentrated and lyophilized to afford B-2 (2.8 mg) and B-1 (12.2 mg), respectively. F-3 peak (tubes 94-108), which showed absorbance only at 300 nm, contained β-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* used as the acceptor.

NMR data (DMSO-d_6) of B-1: ^{1}H, δ 8.28 (d, 2H, *J* 8.9 Hz, *m*-Ph), 7.23 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.33 (d, 1H, *J* 8.6 Hz, H-1), 4.51 (d, 1H, *J* 7.3 Hz, H-1′), 2.00 (s, 3H, NAc); ^{13}C, δ 171.79 (C=O of Ac), 163.79 (Ph carbon attached to the phenolic oxygen), 143.38 (*p*-Ph), 127.35 (*m*-Ph), 118.18 (*o*-Ph), 106.38 (C-1′), 99.89 (C-1), 80.39 (C-3), 77.38 (C-5′), 76.98 (C-5), 74.77 (C-3′), 72.22 (C-2′), 69.65 (C-4), 68.61 (C-4′), 62.07 (C-6′), 61.85 (C-6), 52.27 (C-2), 21.65 (Me of Ac). Other physical data for B-1 were identical to those of β-D-Gal-(1→3)-β-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* reported previously (*2*).

B-2 had: $[\alpha]_D^{25}$ +7.66° (c 0.5, H$_2$0); and *m/z* 505 (M+H)$^+$. NMR data (D$_2$O): ^{1}H, δ 8.28 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.28 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.31 (d, 1H, *J* 8.6 Hz, H-1), 4.48 (d, 1H, *J* 7.2 Hz, H-1′), 2.05 (s, 3H, NAc); ^{13}C, δ 177.93 (C=O of Ac), 164.65 (Ph carbon attached to the phenolic oxygen), 145.44 (*p*-Ph), 128.93 (*m*-Ph), 119.47 (*o*-Ph), 106.16 (C-1′), 101.875 (C-1), 77.99 (C-5′), 77.32 (C-5), 75.51 (C-3′), 73.60 (C-2′), 73.22 (C-6), 71.84 (C-4), 71.42 (C-4′), 70.51 (C-3), 63.83 (C-6′), 54.91 (C-2), 24.96 (Me of Ac).

Preparation of β-D-Gal-(1→3)-β-D-GlcNAc-OC$_6$H$_4$NO$_2$-p (B-3)—To a solution β-D-GlcNAc-OC$_6$H$_4$NO$_2$-*p* (1.0 g) and β-CD (3.3 g) in 50 ml of 20 mM acetate buffer (pH 5.5) were added lactose (21.0 g) and Nagstatin (0.1 mM), followed by the crude β-D-galactosidase from porcine testes (5 U). The molar ratio of the donor to acceptor was about 20 : 1, and the total substrate concentration was about 44%. The mixture was incubated for 50 h at 40°C and the reaction was terminated by heating at 95°C for 10 min. The resulting insoluble material was removed by centrifugation, and one half of the supernatant was loaded onto Chromatorex-ODS DM 1020T column (φ3×50 cm) equilibrated with 20% MeOH in aqueous solution to remove the *p*-nitrophenol liberated during the reaction. The column was eluted with same solution. The eluate fractions (600 ml) showing coincident absorbance at 300 and 485 nm were combined and concentrated to a small volume (15 ml). The remaining half of the supernatant was similarly processed. The two batches of concentrated solution were combined and further applied to the Toyopearl HW-40S column described above. The elution conditions were the same as in Fig. 1. The eluate (20 ml fraction) showed two main peaks (F-1, tubes 50-57; and F-2, tubes 67-79) with coincident absorbance at 485 and 300 nm (data not shown). The latter peak was concentrated and lyophilized (133 mg), then treated with β-D-galactosidase from *B. circulans* in order to hydrolyze selectively β-D-Gal-(1→4)-β-D-GlcNAc-OC$_6$H$_4$-NO$_2$-*p* (B-4) in the product mixture. Thus, F-2 (133 mg) was dissolved in 66.5 ml of 20 mM sodium phosphate buffer (pH 7.0) containing the β-D-galactosidase (40 U) and incubated at 40°C until the contaminant B-4 was no longer detected by HPLC; the required time for this reaction was 4 h on the average. The reaction was terminated by heating at 95°C for 10 min and the resulting insoluble material was centrifuged off. The supernatant was concentrated to a small volume (20 ml) and loaded onto the same column of Toyopearl HW-40S. F-2′ fraction was eluted as a sharp peak (tubes 63-75). After concentration to dryness followed by crystallization from ethanol, this fraction gave compound B-3 in a yield of 75 mg. F-1 was concentrated to afford β-D-Gal-(1→6)-β-D-GlcNAc-OC$_6$H$_4$NO$_2$-*p* (B-5, 40 mg) as reported previously (*6*). F-3 peak, showing absorbance only at 300 nm (tubes 85-100), contained β-D-GlcNAc-OC$_6$H$_4$NO$_2$-*p* used as an acceptor.

NMR data (D$_2$O) of B-3: ^{1}H, δ 8.22 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.18 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.34 (d, 1H, *J* 8.6 Hz, H-1), 4.47 (d, 1H, *J* 7.6 Hz, H-1′); ^{13}C, δ 177.79 (C=O of Ac), 164.47 (Ph carbon attached to the phenolic oxygen), 145.51 (*p*-Ph), 128.91 (*m*-Ph), 119.37 (*o*-Ph), 106.34 (C-1′), 101.18 (C-1), 84.64 (C-3), 78.63 (C-5′), 78.15 (C-5), 75.33 (C-3′), 73.51 (C-2′), 71.36 (C-4), 71.23 (C-4′), 63.86 (C-6′), 63.29 (C-6), 57.16 (C-2), 24.98 (Me of Ac). Other physical data for compound B-3 were identical to those of β-D-Gal-(1→3)-β-D-GlcNAc-OC$_6$H$_4$NO$_2$-*p* (*8*).

Enzyme Assay of Endo-α-GalNAc-ase from Alcaligenes sp.—The standard endo-α-GalNAc-ase assay was carried out as follows. A reaction mixture (1 ml) containing substrate (0.4 mM) and 0.19 mU of endo-α-GalNAc-ase in 20 mM citrate buffer (pH 6.0) was incubated at 40°C and samples (100 μl) were taken at 2-min intervals during the reaction. After inactivation of each sample by adding 200 μl of 1 M acetic acid, the liberated *p*-nitrophenol was determined by HPLC and spectrophotometry at 405 nm.

RESULTS AND DISCUSSION

Enzymic Synthesis of β-D-Gal-(1→3)-α-D-GalNAc-OC$_6$H$_4$NO$_2$-p (A-1)—The present paper describes a preparative synthetic method for A-1, a mimic unit of mucin type I carbohydrate, utilizing porcine testes β-D-galactosidase-catalyzed transglycosylation. The enzyme used in this case was completely devoid of α-NAHase activity, which degrades the acceptor substrate α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* (vide infra). On the other hand, α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* is only sparsingly soluble (0.1%) in aqueous medium. In general, the efficiency of the transglycosylation process is enhanced by the presence of a minimal amount of water and an excess of substrate (*9*). The problem of low solubility was partially solved by using β-CD, which is thought to form an inclusion complex with a *p*-nitrophenyl group, as reported in our previous study (*10*). Thus, the solubility of α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* was enhanced 6.5-fold by the presence of β-CD. When α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* was dissolved in amounts equimolar with β-CD, β-(1→3) and β-(1→6)-galactosyl disaccharide glycosides were observed by HPLC, in 22.1% total yield (based on the acceptor added) and in a ratio of 3 : 1. The maximum production of transfer products in the presence of β-CD was 2.5-fold higher (4.3 mM) than in its absence. This resulted in a significant improvement of the yield. Moreover, unreacted α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* acceptor, which is quite expensive, could be recovered by straightforward column chromatography and reutilized for synthesis.

Figure 2 shows a transglycosylation profile of the reaction with lactose and α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* in the absence and presence of β-CD. The maximum production of com-

pound A-1 in the presence of β-CD was observed after 24 h, at which time it predominated 3-fold over A-2, and its concentration varied little during the subsequent reaction. On the other hand, the formation of A-2 was much slower, and maximum production was reached at ~50 h. In this way, the increased solubility of the acceptor by its formation of an inclusion complex with β-CD facilitated the production of the desired product A-1. We previously reported that the use of an organic co-solvent (50-60% Me_2SO, acetonitrile *etc.*) in transfer reactions utilizing glycosidases not only ensured a sufficient solubility of *p*-nitrophenyl glycosides, but also resulted in high yields of desired compounds (*11-15*). However, this concept was not adopted in the present reaction system because of the instability of the enzyme in organic co-solvent systems.

Enzymic Synthesis of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-p (B-1)—Crude β-D-galactosidase preparation from the extract of porcine testes was used directly for the preparation of B-1. Close attention was paid to the reaction system, because contaminant β-NAHase activity degrades β-D-GalNAc-$OC_6H_4NO_2$-*p*, the acceptor substrate, leading to loss of the transglycosylation. This problem was solved by using Nagstatin, a strong inhibitor of β-NAHase (7). When an inclusion complex of β-D-GalNAc-$OC_6H_4NO_2$-*p* with β-CD was used as the acceptor instead of α-D-GalNAc-$OC_6H_4NO_2$-*p*, two transfer products B-1 and B-2 were observed by HPLC in 15.3% total yield (based on the acceptor) and in a ratio of 5 : 1. These values are based on a time of 12 h for maximum yield of the desired β-(1→3)-linked compound B-1. Figure 3 shows a transglycosylation profile of the reaction with lactose and β-D-GalNAc-$OC_6H_4NO_2$-*p*. In this case, the transfer reaction led initially to the preferential formation of B-1 to B-2. However, once formation of B-1 reached its maximum, the amount decreased markedly during the subsequent reaction. B-2 formation was much slower, and the time for maximum production was ~240 h. At that time, its concentration surpassed that of B-1 in the latter stage of reaction. Thus, much more (1→3)-linked transfer product than the (1→6)-linked product was formed in the initial stage of reaction. This result was in marked contrast to that with the α-D-GalNAc-$OC_6H_4NO_2$-*p* as an acceptor.

Enzymic Synthesis of β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-p (B-3)—The crude enzyme preparation was also used for the synthesis of B-3 with Nagstatin. When an inclusion complex of β-D-GlcNAc-$OC_6H_4NO_2$-*p* with β-CD was used as the acceptor, β-(1→3)-(B-3), β-(1→4)-(B-4), and β-(1→6)-(B-5) disaccharide glycosides were observed by HPLC, in 20.9% total yield (based on the acceptor) in the ratio of 48 : 20 : 32. These values are based on a time of 50 h for maximum production of the desired β-(1→3) compound (B-3) (Fig. 4). The ratio of the three transfer compounds varied little during the entire course of reaction: the (1→4)-linked transfer product was formed in significant amounts along with the (1→3)-linked and (1→6)-linked products. The regioselectivity is much lower than when the *N*-acetyl galactosaminide was used as acceptor. These transfer products were first separated into two fractions (F-1 and F-2) by chromatography on a Toyopearl HW-40S column. F-1 contained only compound B-5. The purification of F-2 into the desired compound B-3 was somewhat cumbersome, because this fraction was heavily contaminated by B-4. The B-4 was therefore selectively removed by hydrolytic treatment with *B. circulans* β-

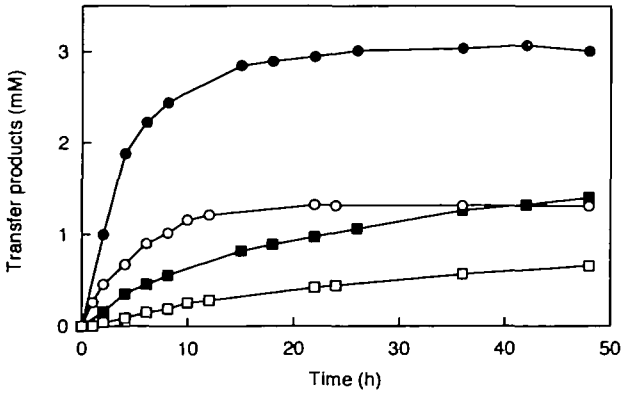

Fig. 2. **Time course of porcine testes β-D-galactosidase-mediated isomer formation of A-1 and A-2 in the presence (closed symbols) and absence (open symbols) of β-CD.** The amounts of A-1 (β1→3, ●, ○) and A-2 (β1→6, ■, □) products as a function of time were examined on the 0.5-ml scale as described in the "MATERIALS AND METHODS" section, and samples were analyzed by HPLC during incubation.

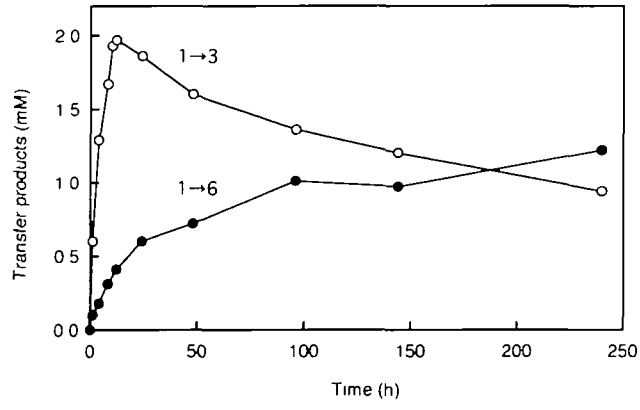

Fig. 3. **Time course of porcine testes β-D-galactosidase-mediated isomer formation of B-1 and B-2 in the presence of β-CD.** The amounts of B-1 (β1→3, ○) and B-2 (β1→6, ●) products as a function of time were examined on the 0.5-ml scale as in Fig. 2.

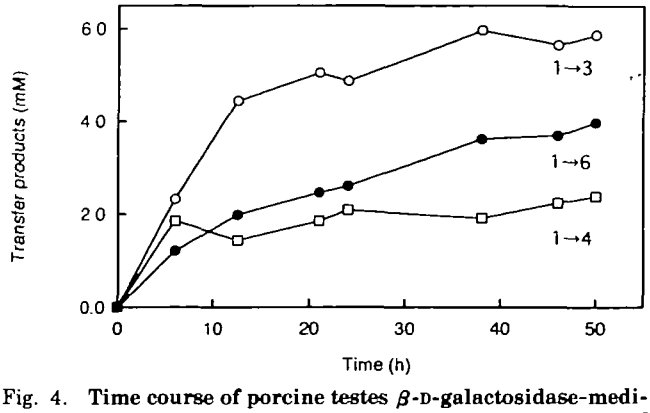

Fig. 4. **Time course of porcine testes β-D-galactosidase-mediated isomer formation of B-3, B-4, and B-5 in the presence of β-CD.** The amounts of B-3 (β1→3, ○), B-4 (β1→4, □), and B-5 (β1→6, ●) products as a function of time were examined on the 0.5-ml scale as in Fig. 2.

D-galactosidase, which does not hydrolyze B-3. When the lyophilized fraction was incubated at a 0.2% concentration with the enzyme, B-4 was hydrolyzed completely after 4 h. The hydrolysate was then rechromatographed on Toyopearl HW-40S, enabling the selective removal of B-4 from the F-2 fraction.

Substrate Specificity and Kinetics of Endo-α-GalNAc-ase—DiCioccio *et al.* have reported that endo-α-GalNAc-ase from *Clostridium perfringens*, which cleaves the endo-*O*-acetylgalactosaminidic linkage from serinyl or threoninyl oligosaccharide, hydrolyzes compound A-1 into β-D-Gal-(1→3)-D-GalNAc and *p*-nitrophenol, that is, it can bypass a block of the disaccharide (*5*). In the present study, the substrate specificity of commercially available endo-α-GalNAc-ase from *Alcaligenes* sp. (*16*) was investigated using a series of synthetic *p*-nitrophenyl galactosyl-disaccharide glycosides (A-1, A-2, B-1, B-2, B-3, B-4, and B-5). The enzyme products were detected by HPLC using a TSK-GEL G-Oligo-PW column. The enzyme was capable of liberating a reducing disaccharide only from A-1. This indicates that its specificity for the substrate is very high.

By measuring the amount of *p*-nitrophenol as described in "MATERIALS AND METHODS," the initial velocities (v) were obtained directly from the initial slopes of the time-course plots (2, 4, 6, and 8 min) of the reaction. Six different substrate concentrations (0.05–0.8 mM) were used per experiment. The kinetic parameters for A-1 of the enzyme assayed were calculated by the method of least squares with use of a $[S]/v$–$[S]$ plot. The Michaelis constant (K_m) and k_0/K_m were 0.041 mM and 2,030 $mM^{-1} \cdot min^{-1}$, respectively. This indicates that A-1 is a very sensitive substrate for the endo-α-GalNAc-ase from *Alcaligenes* sp. DiCioccio *et al.* have reported that the K_m value of A-1 for endo-α-GalNAc-ase from *C. perfringens* was 0.5 mM (*5*). The K_m value for this enzyme is much greater than that from *Alcaligenes* sp. Fan *et al.* have also reported that the activity of *Diplococcus pneumoniae* enzyme on DNS-Ser-GalNAc-Gal and DNS-Thr-GalNAc-Gal was lower than that of the *Alcaligenes* sp. enzyme (*17*). This indicates that the reactivity of endo-α-GalNAc-ases using these substrates depends greatly on the enzyme source.

In conclusion, β-D-galactosidase from porcine testes catalyzes formation of the β-(1→3)-linked galactosyl disaccharide glycosides in a similar manner to that from bovine testes. The enzymic process for obtaining the desired compound A-1 is simple and the yield is sufficiently high to make the method practicable. From a practical viewpoint, a well-defined and homogenous substrate might facilitate the screening of new sources for endo-α-GalNAc-ase. Nitrophenyl glycoside can not only serve as an enzyme substrate, it can also be reduced to aminated glycosides. The amino function may be derivatized for reactions with electrophiles (*18*). The approach described above can be further extended to the synthesis of type 1 core (β-D-GlcNAc-(1→3)-β-D-Gal-(1→3)-D-GalNAc) and type 2 core (β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-D-GalNAc) trisaccharides at the carbohydrate core region of mucin.

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