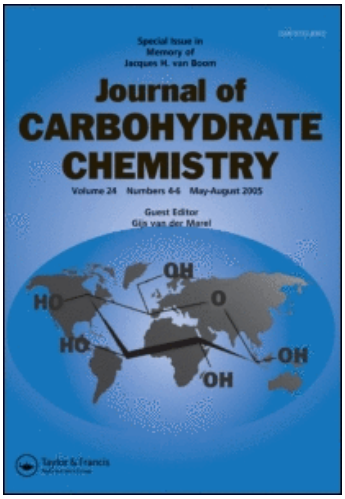

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ENZYMATIC SYNTHESES OF *N*-ACETYLLACTOSAMINE AND *N*-ACETYLALLOLACTOSAMINE BY THE USE OF β-D-GALACTOSIDASES

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ABSTRACT

A β-D-galactosidase from *Bacillus circulans* induced β-D-galactopyranosyl transfer from lactose predominantly to a secondary (OH-4) rather than the primary hydroxyl group (OH-6) of 2-acetamido-2-deoxy-D-glucopyranose. 4-*O*-β-D-Galactopyranosyl-2-acetamido-2-deoxy-D-glucopyranose (*N*-acetyllactosamine) was thus readily synthesized on a gram scale and conveniently isolated by chromatography on a column of charcoal-Celite. On the other hand, the glycosyl transfer to the 6-position predominantly was efficiently induced to give 6-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylallolactosamine) by consecutive use of β-D-galactosidases from *Kluyveromyces lactis* and *B. circulans*. These enzyme reactions were efficient enough to allow the one-pot preparation of the desired disaccharides.

INTRODUCTION

N-Acetyllactosamine (1) is well known as a representative core structure in oligosaccharide components of glycoproteins and glycolipids.[1,2] Compound 1 was isolated from porcine gastric mucin as a growth factor for *Lactobacillus bifidus var.*

pennysylvanicus by Tomarelli *et al.*[3] It was also found by Kuhn *et al.*[4] in a partial hydrolyzate of higher oligosaccharides obtained from human milk and later in the free form from bovine colostrum by Saito *et al.*[5] Zilliken *et al.* reported the first enzymatic synthesis of 1 from lactose and *N*-acetylglucosamine (GlcNAc) in the presence of extracts from *Lactobacillus bifidus*,[6] although the yield was low from the standpoint of practical use. Organic chemical methods for obtaining 1 have been developed,[7,8] but are characterized by variously elaborate protection and deprotection procedures. There is at present great interest in developing synthetic routes to oligosaccharides located on cell surfaces as components of glycoconjugates. An assumption that an enzymatic approach to the synthesis of disaccharide 1 might be the basis for its large scale preparation, promoted us to investigate such an enzymatic preparation.

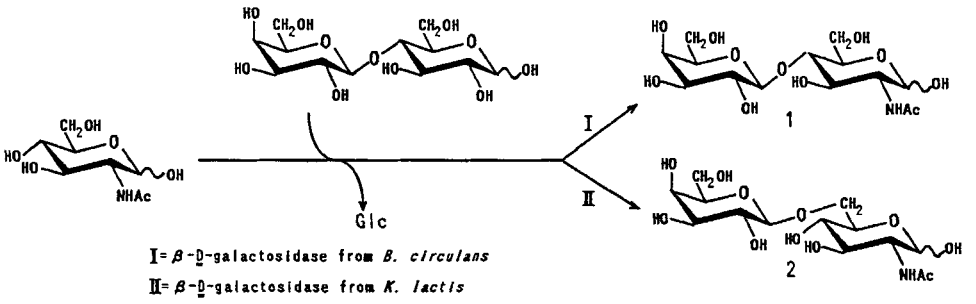
An approach to a preparative-scale synthesis of 1 by D-galactopyranosyl transfer from UDP-D-galactose to the hydroxyl group at 4-position of GlcNAc by the use of 2-acetamido-2-deoxy-D-glucopyranose 4-β-D-galactosyltransferase (EC 2.4.1.90) was first carried out by Wong *et al.*[9] Although this method is certainly elegant, the wide use of the above synthetase has been restricted since it is not readily available and requires cofactors for inducing the reaction. From a practical viewpoint, the use of glycosidases, which are commercially available and much less expensive than glycosyltransferases, is very attractive in the synthesis of an oligosaccharide involved in glycoconjugates utilizing the transglycosylation reaction.[10-14] However, use of glycosidase in synthesis has been limited due to the preponderant formation of the (1→6) linkage over of the (1→2), (1→3), and (1→4) linkages.[14,15] On the other hand, Kuhn *et al.* reported that *N*-acetylallolactosamine (2) could be synthesized by both enzymatic and chemical approaches,[16] although its yield was low from the standpoint of its preparative-scale synthesis. We now report an efficient syntheses of 1, utilizing the transglycosylation reaction through β-D-galactosidase (EC 3.2.1.23) from *Bacillus circulans*,

and of 2 by the consecutive reactions in terms of β-D-galactosidase from *Kluyveromyces lactis*, and that from *B. circulans*; the latter was used in order to hydrolyze the small amount of 1 concomitantly produced.

RESULTS AND DISCUSSION

Enzymatic synthesis of 1. The present study shows that the commercially available β-D-galactosidase from *B. circulans* can be used to produce the disaccharide 1 with the desired β-(1→4) linkage. 1 was predominantly synthesized through a route I as in Scheme I. The cheap sugars, lactose and GlcNAc, were used as substrates.

Scheme I



Effects of pH, temperature and enzyme concentration on 1. The effect of pH on the production-efficiency of 1 was not significantly observed from the results shown in Fig. 1A, but the formation of undesirable product 2 could be minimized by performing the reaction at pH 5.0. Prolonging the incubation period was found to bring about the conspicuous decrease in the production of 1. The effect of temperature on the production efficiency of 1 was also examined; the results thus obtained are summarized in Fig. 1B. Reaction times for maximal production 1 were 25 h at 30 °C, 10 h at 40 °C, and 5 h at 50 °C, respectively. As the temperature was raised, the ratio 1/1+2 became smaller as time progressed. Thus, reaction at a lower temperature minimized the formation of undesirable 2. The course of the reaction at 60 °C, on the other hand, afforded a

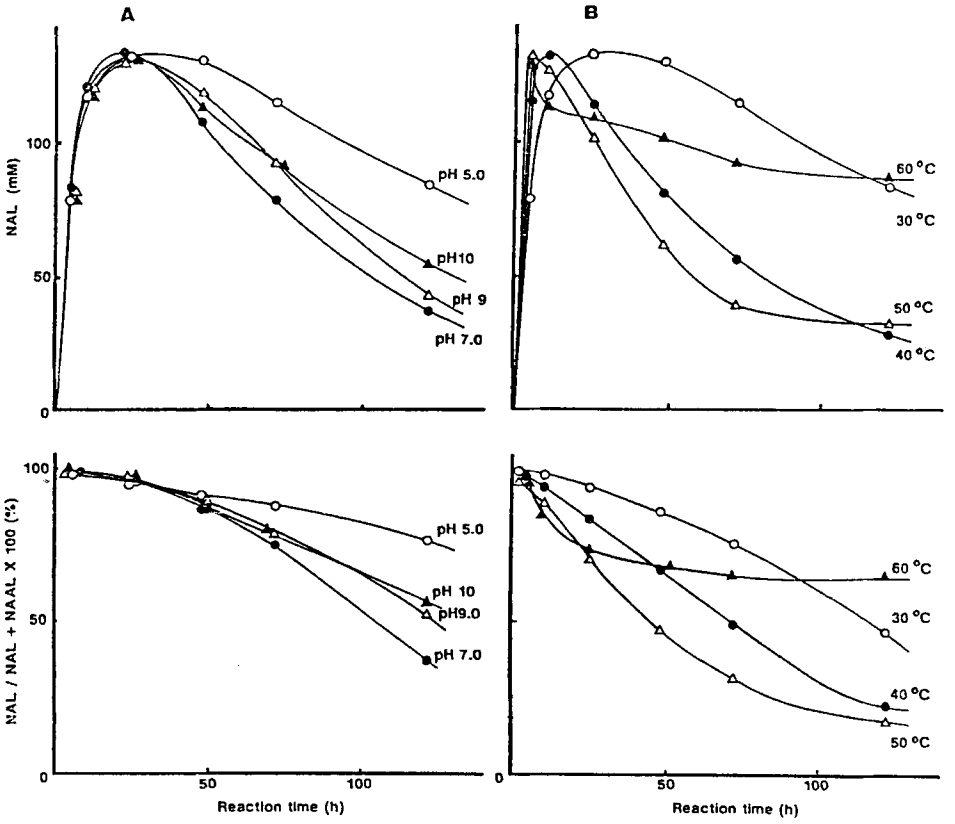


Fig. 1. Effects of pH (A) and temperature (B) on β-D-galactosidase-mediated 1 production. A; lactose (180 mg) and GlcNAc (220 mg) in 1 ml of different pH buffers were incubated with β-D-galactosidase (0.15 U) at 30°C and samples were taken during the incubation for analysis by HPLC. Sodium acetate buffer (50 mM, pH 5.0) sodium phosphate buffer (50 mM, pH 7.0 and 9.0) and sodium borate buffer (50 mM, pH 10.0) were used. The upper figure shows the amount of 1 as a function of time and lower the ratio of 1/1+2 production by HPLC analysis. B; conditions were the same as those in (A). The substrate were incubated in 50 mM sodium acetate buffer (pH 5.0) at different temperatures.

different profile compared with those performed at lower temperatures. Thus, once 1 and 2 formation reached their maximum, their amounts produced remained almost unaltered during the subsequent reaction. It is suggested that the inactivation of the enzyme by heat might occur during the

course of the reaction. The rate of transglycosylation was also largely dependent on the enzyme concentration under the same conditions (data not shown). With rising enzyme concentrations, the maximum rate of 1 production increased, but a significant decrease in its ratio to 2 was observed. As a result, we adopted the best condition taking into account the efficiency of the preparation of 1 on the transglycosylation as mentioned in Experimental section.

Enzymatic synthesis of 2. The transglycosylation in terms of β-D-galactosidase obtained from *K. lactis* gave 2 predominantly over its isomer 1. The latter was selectively removed from the resulting mixture by treatment with β-D-galactosidase from *B. circulans*.

Selective removal of by-product on 2 production. As mentioned above, the small portion of undesirable 1 produced by the transglycosylation, which contained 2 and 1 in the molar production of 8 : 1, was selectively removed by treatment with the *B. circulans* β-D-galactosidase. Thus, reaction mixture from lactose and GlcNAc with the *K. lactis* β-D-galactosidase was firstly applied to a charcoal-Celite column in order to obtain a fraction containing transglycosylation products 1 and 2 as in Experimental section. When the fraction was incubated at a 0.5% concentration, 1 was hydrolyzed completely after 3.5 h, whereas <5% of 2 was hydrolyzed during the entire course of reaction. In the separate experiment, the relative rate of hydrolysis of 2 compared with 1 (100) is 2.0, a 50-fold difference. Thus, 1 should be a considerably better substrate than 2 under hydrolytic conditions, thereby allowing the selective removal of 1 from the isomeric mixture. When the hydrolysis was complete, it was necessary to lower the pH before heating because of instability of 1 in solution at pH 7.0 at a higher temperature.[10]

CONCLUSION

The present study demonstrated that the transferase activity of the specific β-D-galactosidase was useful for the large-scale syntheses of 1 and 2. The enzymatic process is

simple and the yield is sufficiently high to make the method practical. Furthermore, the one-pot preparation of Gal-GlcNAc from cheap sugars (lactose and GlcNAc) and enzymes (commercially available β-D-galactosidases) is also possible.

EXPERIMENTAL

Materials. Commercially available β-D-galactosidases, Biolacta (Daiwa Kasei Co., Ltd.) prepared from the culture filtrates of *B. circulans* and Godo YNL (Godo Shusei Co., Ltd.) from *K. lactis*, were directly used for the enzymatic syntheses without further purification. 1 And 2 as authentic samples were purchased from Sigma. All other chemicals were obtained from commercial sources. The charcoal-Celite column for the separation of transglycosylation products was prepared as follows: equal parts by weight of dry charcoal and Celite were slurried in water and packed into a glass column (4.0 × 120 cm).

Preparation of 1. To a solution of lactose (18 g, 50 mM) and GlcNAc (22 g, 99 mM) in 50 mM sodium acetate buffer (100 mL, pH 5.0) was added β-D-galactosidase (15 U). The mixture was incubated at 30 °C for 24 h and terminated by heating at 95 °C for 10 min. When the reaction solution was concentrated to about half of the volume, the unreacted GlcNAc was crystallized. The mixture was filtered through a glass filter and the crystals washed with a small amount of methanol. The combined filtrate and washings were directly applied to a charcoal-Celite column as in Fig. 2A. The column was first eluted with water (480 mL) and then with a linear gradient of 0 (5 L)-30% (5 L) ethanol. The elution was monitored by measuring the absorbance at 210 nm (characteristic absorption of *N*-acetyl group) and at 485 nm (carbohydrate content, determined by the phenol-sulfuric acid method). The chromatogram shows that peak F-a (fraction numbers 47-67) is contaminated by lactose judging from the overlapping of the absorption at 485 nm. The fraction F-a was concentrated to a small volume (100 mL), which was then subjected to rechromatography as above (Fig. 2B). The sharp fraction F-a' (fraction numbers 43-65) eluted out with 10% aqueous ethanol and

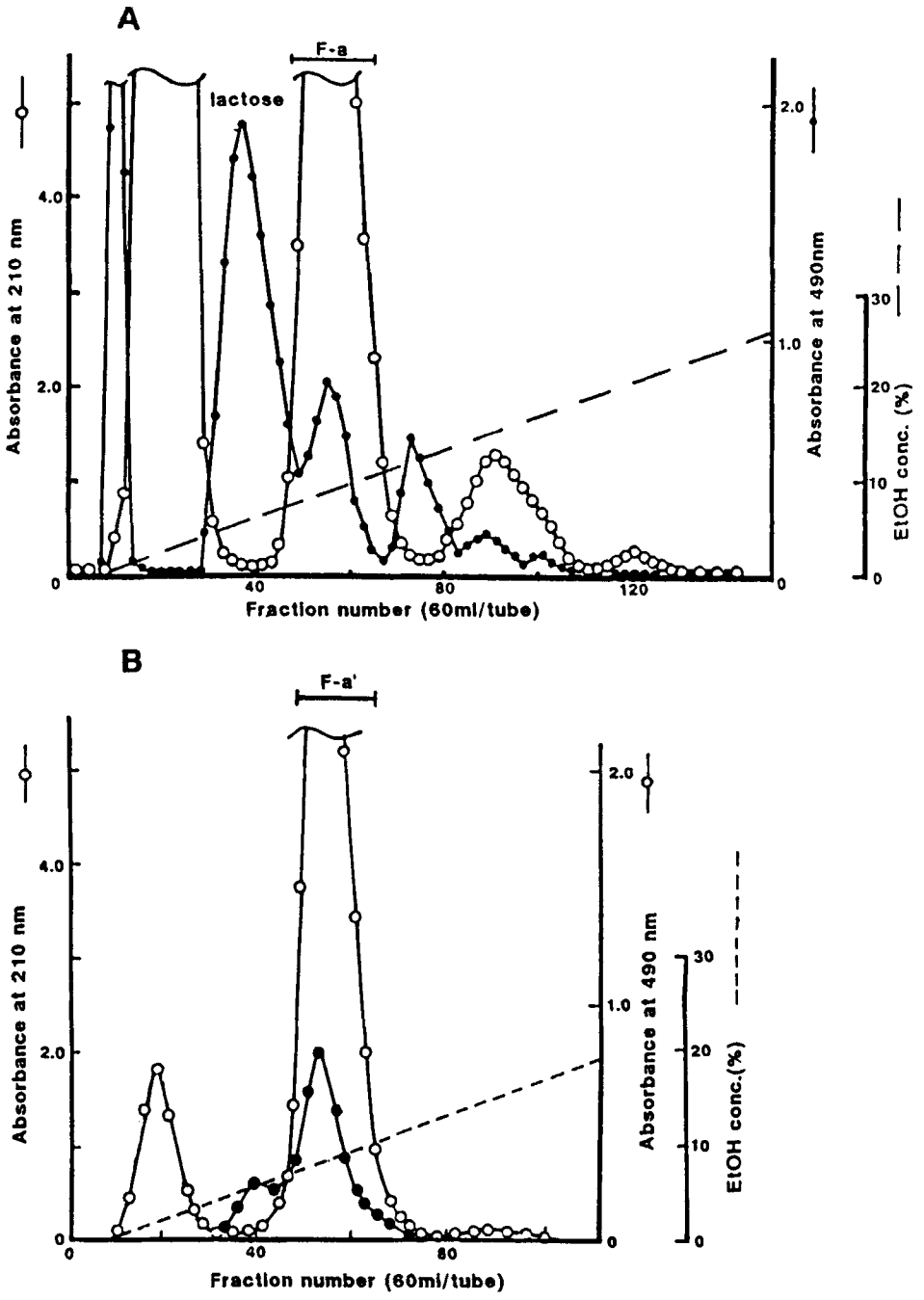


Fig. 2. Charcoal-Celite chromatography of transglycosylation products obtained by treating a solution of lactose and GlcNAc with *B. circulans* β-D-galactosidase (A) and the rechromatography of fraction F-a (B). Chromatography was carried out on a column (4.0 × 120 cm) of charcoal-Celite at room temperature.

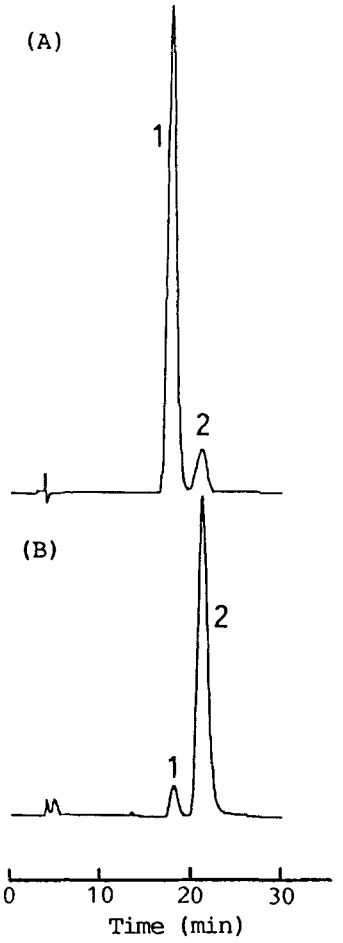


Fig. 3. Separation patterns of F-a' (A) and that of F-b' (B) on HPLC. The elution patterns were obtained by monitoring an absorbance at 210 nm.

was concentrated to dryness. The HPLC analysis of the residue (F-a') showed two separate components (F-a'-1 and F-a'-2) in a molar proportion of 10 : 1, which were chromatographically superimposable with authentic samples of 1 and its isomer 2, respectively (Fig 3A). The former fraction (F-a'-1), after concentration to dryness followed by crystallization from methanol, gave 1 (5.1 g, 23.2% yield based on GlcNAc used),

which gave a single peak on HPLC, identical with authentic 1; $[\alpha]_D^{25}$ +27.0 (*c* 1.0 water); ^{1}H-NMR (400 MHz, D_2O) δ 2.059 (s, 3H, N-COC<u>H</u>$_3$), 5.214 (d, J=2.2 Hz, H-1α), 4.731 (d, J=7.3 Hz, H-1β), 4.479 (d, 1H, J=7.3 Hz, H-1'), MS (m/z), 384. The ^{13}C NMR spectrum of F-a'-1 could be superimposed on that of 1 reported.[5] The data are given in Table I.

Anal. Calcd for $C_{14}H_{25}N_1O_{11}\cdot H_2O$: C, 41.90; H, 6.78; N, 3.49. Found; C, 41.94; H, 7.11; N, 3.19.

Preparation of 2. To a solution of lactose (18 g) and GlcNAc (22 g) in 0.1M sodium phosphate buffer (100 mL, pH 6.5) was added β-<u>D</u>-galactosidase from *K. lactis* (30 U/mL). After the mixture was incubated at 30 °C for 17 h, the reaction was terminated by heating at 95 °C for 10 min. The resulting insoluble material was centrifuged off. The supernatant was directly applied to a charcoal-Celite column and worked up as above. After washing the column with water (5 L), the absorbates were eluted by a linear gradient of 0 (5 L)-30% (5 L) ethanol as in Fig. 4. F-b fraction, which was eluted at about 15% ethanol concentration (fraction number 130-180), was collected, concentrated and lyophilized to give 4.6 g of 2 together with a small amount of 1. This was further treated with a specific β-<u>D</u>-galactosidase in order to remove the undesirable 1. Thus, F-b (4.6 g) was dissolved in 920 mL of 20 mM sodium phosphate buffer (pH 6.0) containing the β-<u>D</u>-galactosidase from *B. circulans* (25 U/mL) and the reaction was allowed to proceed at 40 °C until the contaminant 1 was no longer detected by HPLC; the required time for this reaction was 3.5 h as a criterion. The reaction was terminated by heating at 95 °C for 15 min after adjusting the pH to 3.5 with 0.3 N HCl. The mixture was again adjusted to pH 6.5 with 0.2 N NaOH and concentrated to about a half volume. The concentrate was loaded onto a charcoal-Celite column and worked up as above. F-b' fraction was eluted as a sharp peak (tube numbers 133-177) at 15% of ethanol, combined, concentrated to dryness and the residue was lyophilized to give 2 (3.9 g, 17.7% yield based on GlcNAc used). On HPLC analysis, fraction F-b' showed only a single peak, identical with authentic 2. The ^{1}H- and ^{13}C-NMR

TABLE I

Carbon-13 chemical shifts of fractions F-a'-1 and F-b' in D_2O solution

Chemical shifts in ppm downfield from internal DSS.

F-a'-1; Galβ1→4GlcNAc F-b'; Galβ1→6GlcNAc

II I II I

		C-1	C-2	C-3	C-4	C-5	C-6	CH_3 ($NHCOCH_3$)	C=O ($NHCOCH_3$)
F-a'-1	Iα	93.269	56.437	72.000	81.546	72.969	62.710	24.689	177.120
	β	97.603	58.924	75.200	81.108	77.560	62.819	24.981	177.394
	IIα	105.668	73.701	75.237	71.287	78.072	63.770		
	β	105.613	73.701	75.237	71.287	78.072	63.770		
F-b'	Iα	93.585	56.790	73.283	72.582	73.341	71.298	24.695	177.406
	β	97.701	59.301	76.494	72.422	77.588	71.298	24.958	177.216
	II	106.020	73.458	75.385	71.356	77.837	63.708		

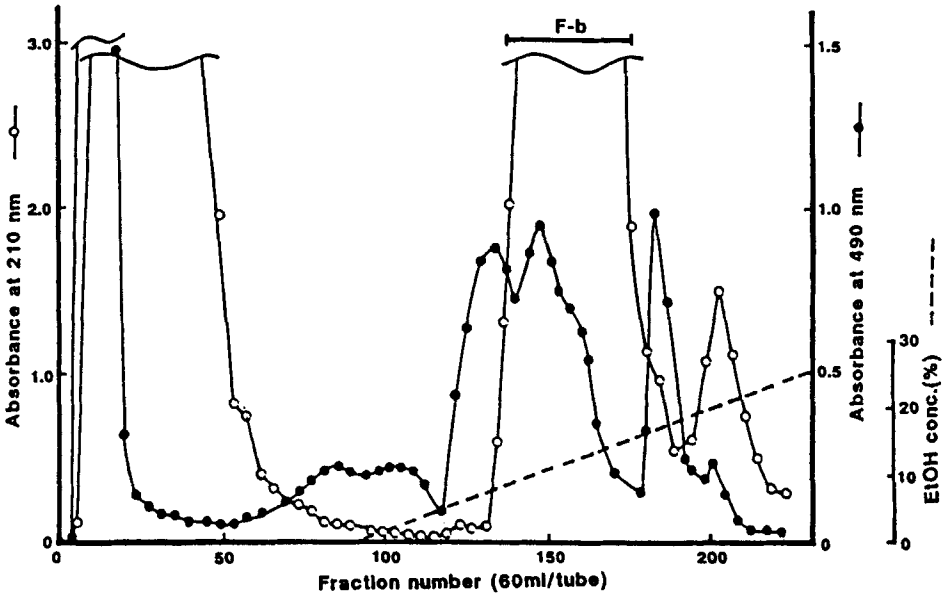


Fig. 4. Charcoal-Celite chromatography of reaction mixture obtained by treating a solution of lactose and GlcNAc with *K. lactis* β-D-galactosidase. Conditions were the same as those for Fig. 2.

spectra were superimposable on those of authentic 2; $[\alpha]_D^{25}$ +29.5 (*c* 1.0, water); ^{1}H NMR (400 MHz, D_2O) δ 2.055 (s, 3H, N-COC<u>H</u>$_3$), 4.439 (d, 1H, J=7.3 Hz, H-1'), 4.455 (d, J=7.0 Hz, H-1'β), 5.205 (d, J=3.3 Hz, H-1α), MS (m/z), 384. The carbon resonances were assigned by comparing the spectrum with data for the corresponding reference sugars of 1, *N,N'*-diacetylchitobiose and β-1,6 linked disaccharide of GlcNAc,[5,17,18] as in Table I.

Anal. Calcd for $C_{14}H_{25}N_1O_{11}\cdot H_2O$: C,41.90; H, 6.78; N, 3.49. Found; C, 41.90; H, 6.59 ; N, 3.21.

Enzyme assays. β-D-Galactosidase activity was assayed as follows. The mixture containing 2 mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) in 0.9 mL of 50 mM sodium phosphate buffer (pH 7.0) and an appropriate amount of enzyme in a total volume of 1.0 mL was incubated at 30 °C for 10 min. The enzymatic 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 Shimadzu LC-6A liquid chromatograph equipped with a SPD-6A ultraviolet detector. Elution was effected with water/acetonitrile (1:3, v/v). The flow rate was 1.0 mL/min at a pressure of 60 kg/cm^2. ^{13}C and ^{1}H-NMR were determined with a JEOL GSX-400 spectrometer operating at 100.0 MHz in the pulsed Fourier-transform mode with computer proton decoupling and at 400 MHz, respectively. Chemical shifts are expressed in ppm relative to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as an internal standard. The FAB MS spectra of oligosaccharides were recorded with a JEOL DX-303 HF mass spectrometer, operating at the full accelerating potential (3 kV) and coupled to a JEOL DA-500 mass data system. The sample in distilled water was added to the glycerol matrix. The molecular weight of the sample was estimated from the m/z value of the quasimolecule-ion $(M+H)^+$ peak. Specific rotations were determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken Corp., Ltd.). Elemental analyses were performed using a Perkin-Elmer 240C apparatus.

Enzyme hydrolysis. The relative rates of attack of *B. circulans* β-D-galactosidase on 1 and 2 were determined by incubating 0.5% of the substrate in 50 mM phosphate buffer (pH 7.0) with 0.05 U enzyme per mL. Thus, the amounts of GlcNAc formed from the initial substrate during the reaction (3, 6, 9, 12, and 15 min) were determined by HPLC. The reaction was linear from 3 to 15 min. The rate of attack on 1 was arbitrarily set at 100.

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