Production of *N*-acetyllactosamine using transgalactosylation activity of *Aspergillus oryzae* in Czapek-Dox liquid medium

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要旨：ガラクトオリゴ糖はヒト消化管内でビフィズス菌の増殖を促進する活性を有し、プレバイオティクスとして利用されている。β-ガラクトシダーゼはガラクトース残基転移反応を触媒することから、ガラクトオリゴ糖合成に用いられてきた。本研究では、β-ガラクトシダーゼを産生する黄麹カビ（*Aspergillus oryzae*）を用いて、代表的な含窒素ガラクトオリゴ糖である *N*-アセチルラクトサミン（4-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine）の生産を試みた。ガラクトース供与体である乳糖を 10%含有する Czapek-Dox 液体培地 100 mL に *A. oryzae* を接種して 26℃にて 7 日間振盪培養した後、ガラクトース受容体である *N*-アセチルグルコサミンを 10 g 添加してさらに 26℃にて 7 日間振盪培養を行った。培養液中の糖をガスクロマトグラフィーで分析した結果、二種類のオリゴ糖の生成が確認できた。これらを活性炭カラムクロマトグラフィーで単離した後の ^{13}C-NMR 等での構造解析の結果、生成した二種類のオリゴ糖は、*N*-アセチルラクトサミン及び *N*-アセチルアロラクトサミン (6-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine)と決定された。

キーワード：*N*-アセチルラクトサミン、β-ガラクトシダーゼ、糖転移反応、乳糖、*N*-アセチルグルコサミン

Summary

Galacto-oligosaccharides (GOSs) can promote the growth of bifidobacteria in human gastrointestinal tract and are used as effective prebiotic oligosaccharides in the food industry. Because β-D-galactosidase catalyzes transgalactosylation to various acceptors, it has been used for GOSs production. In this study, *Aspergillus oryzae*, which produces β-D-galactosidase, was used in order to synthesize *N*-acetyllactosamine (LacNAc, 4-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine) as a typical nitrogen-containing GOS. *A. oryzae* was inoculated into 100 mL Czapek-Dox liquid medium containing 10% lactose as a galactose donor and incubated in a shaking incubator at 26°C for 7 days. Next, *N*-acetylglucosamine (10 g) as a galactose acceptor was added into the medium and incubated at 26°C for 7 more days. Two oligosaccharides in the culture medium were detected by gas-liquid chromatography. After isolating these oligosaccharides by activated charcoal column chromatography, structural analyses including ^{13}C-NMR were performed in order to identify them. Consequently, these two oligosaccharides were determined to be LacNAc and *allo*-LacNAc (6-*O*-β-D-galacto-pyranosyl-*N*-acetyl-D-glucosamine).

Key words: *N*-acetyllactosamine, β-D-galactosidase, transgalactosylation, lactose, *N*-acetylglucosamine

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Introduction

It has been well known that numerous oligosaccharides obtained from human milk can promote the selective growth of bifidobacteria in human gastrointestinal tract, and these oligosaccharides are inclusively called as bifidus factors[1]. In particular, *N*-acetyllactosamine (LacNAc, 4-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine), one of the nitrogen-containing galacto-oligosaccharides (GOSs), has a powerful bifidus growth-promoting activity[1]. On the other hand, transglycosylation reactions catalyzed by various glycosidases have been used to obtain a large variety of oligosaccharides[2]. β-D-Galactosidase (EC 3. 2. 1. 23) catalyzes not only hydrolysis of lactose into glucose and galactose but also transgalactosylation to various saccharides as acceptors, resulting in production of a large variety of GOSs[3)-9)]. Since LacNAc is an effective prebiotic GOS, enzymatic syntheses of it have been carried out using various microorganisms and their β-D-galactosidase[3, 10-12]. In this study, *Aspergillus oryzae*, which produces β-D-galactosidase, was used in order to synthesize LacNAc.

Materials and Methods

1. Reagents and strain

Chemicals and reagents were purchased from Wako (Osaka, Japan). *A. oryzae* (IFO 4177) was provided by Institute for Fermentation, Osaka (Osaka, Japan).

2. Cultivation of *A. oryzae* for production of LacNAc

A. oryzae in a slant culture was inoculated using a platinum loop into a sterilized plain bread cube (10 mm x 10 mm x 10 mm), incubated at 26°C for 3 days. The moldy bread cube was added into 100 mL Czapek-Dox liquid medium (pH 7.0) containing 10% lactose as a galactose donor in a 300 mL Sakaguchi flask and incubated in a shaking incubator at 26°C and 180 rpm for 7 days. Next, 10 g *N*-acetylglucosamine (GlcNAc) as a galactose acceptor was added into the medium and incubated at 26°C for 7 more days. The culture medium was subjected to filtration using a Buchner funnel, and water was added until the final volume was 100 mL.

3. Detection and purification of GOSs

GOSs in the culture medium of *A. oryzae* were analyzed by gas-liquid chromatography (GLC). GLC was performed with a model 163 gas chromatograph equipped with a flame ionization detector (Hitachi, Ibaraki, Japan) using trimethylsilylated sugar samples[13]. GLC conditions were as follows: column; 2% Dexsil 300GC (3 mm in diameter and 50 cm in length), flow rate of carrier gas (N_2): 40 ml/min, column temperature: from 150˚C to 300˚C at a rate of 10˚C/min, and injection temperature: 350˚C. Two peaks of GOSs (DI and DII) were detected by GLC analysis (see Fig. 1). To purify of these GOSs, the culture filtrate (100 mL) was adsorbed on a charcoal column (5 cm in diameter and 40 cm in length)[4]. After washing with 13.5 L of water, the column was eluted with 14.5 L of 5% ethanol. GOSs in each fraction (500 mL) were monitored by GLC and freeze-dried. Purified GOSs were also analyzed by GLC.

4. Carbon-13 nuclear magnetic resonance (^{13}C-NMR)

Structural elucidations of the GOSs were carried out by ^{13}C-NMR analysis[4]. Dried GOSs samples were dissolved in D_2O, and their proton-decoupled ^{13}C-NMR spectra were measured using a FX100 instrument (JEOL, Tokyo, Japan) operated at 25 MHz. The chemical shifts obtained were referenced to an external standard of 2, 2, 3, 3-tetradeuterio-4, 4-dimethyl-4-silapentanoate. The spectrum of each GOS (DI and DII) was interpreted with reference to the previous report[14].

5. Methylation analysis

Methylation of DI reduced with $NaBH_4$ was performed by the Hakomori's method[15]. The methylated sample was injected into a JEOL DX

300 gas chromatography-mass spectrometer attached with a fused silica capillary column (Supelco Inc., Bellefonte, PA, USA; 0.33 mm in internal diameter and 60 m in length; phase SPB-1). Analysis conditions were as follows: carrier gas: He, injection temperature: 250˚C, column temperature: from 150˚C to 250˚C at a rate of 5˚C/min, ionization voltage of EI mode: 25 eV[4].

Results and Discussion

In order to produce LacNAc, *A. oryzae* was inoculated into 100 mL Czapek-Dox liquid medium containing 10% lactose and incubated in a shaking incubator at 26°C for 7 days. Next, 10 g of GlcNAc was added into the medium, and incubated at 26°C for 7 more days. Two peaks (DI and DII) were detected in the culture medium of *A. oryzae* by GLC analysis (Fig. 1).

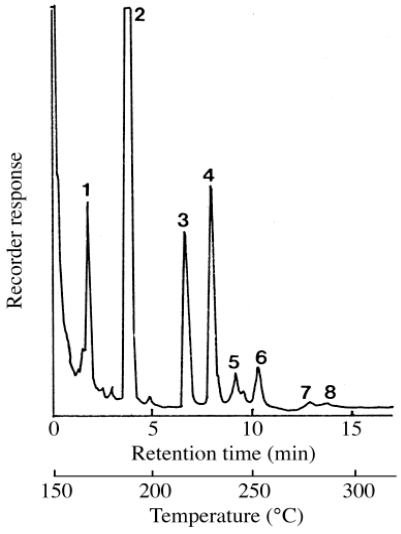

Fig. 1. Typical GLC chromatogram of the saccharides in the culture medium of *A. oryzae*. Peaks 1: galactose, 2: GlcNAc, 3 and 4: lactose, 5: DI, 6: DII, 7 and 8: trisaccharides.

As these peaks did not appear in the *A. oryzae* culture containing lactose but not GlcNAc (data not shown), it was thought that they are the peaks of GlcNAc-containing GOSs. As shown in Fig. 2, both DI and DII were purified by charcoal column chromatography. As shown in Fig. 2, there are two peaks in the GLC chromatogram of purified DI. Because reduction of DI with $NaBH_4$ generated a single peak in the GLC chromatogram, the two peaks of DI in the GLC chromatogram should be derived from its anomers (data not shown).

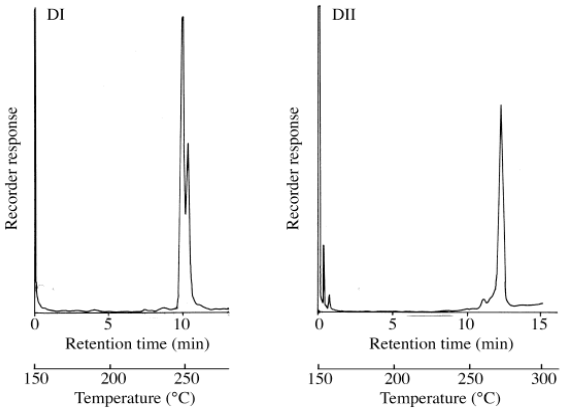

Fig. 2. GLC chromatograms of purified DI and DII.

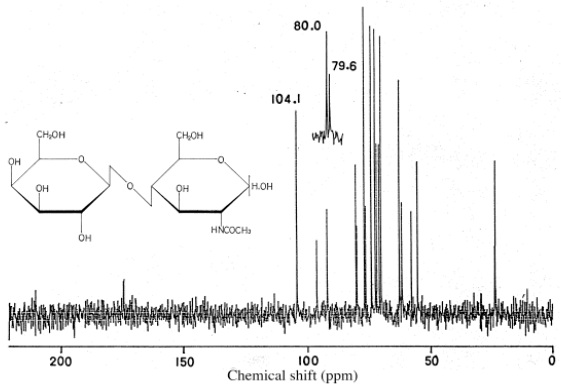

Fig. 3. ^{13}C-NMR spectrum of DI

Table 1. ^{13}C-NMR chemical shifts for DI

Chemical shift (ppm)	β-Gal*p*(1→)	→4)α-GlcNAc	→4)β-GlcNAc
175.9			HNC=O
175.6		HNC=O	
104.1	1		
96.0			1
91.7		1	
80.0		4	
79.6			4
76.5	5		
76.0			5
73.7	3		3
72.1	2		
71.4		5	
70.4		3	
69.7	4		
62.2	6		
61.2		6	6
57.4			2
54.9		2	
24.9			$COCH_3$
24.6		$COCH_3$	

From the culture medium, 145 mg of purified DI, 50 mg of purified DII and 272 mg of their mixture were obtained. DI and DII were hydrolyzed into galactose and GlcNAc (1:1 ratio) by *Escherichia coli* β-D-galactosidase (Grade VI; Sigma-Aldrich, St. Louis, MO, USA), suggesting that both DI and DII are composed of galactose and GlcNAc joined by a β-galactosidic linkage (data not shown).

Structural elucidations of these two GOSs were performed by ^{13}C-NMR analysis and methylation analysis. The ^{13}C-NMR spectrum of DI (Fig. 3) and assignments of the chemical shifts of each sugar unit in DI are shown (Table 1). The spectrum of DI was interpreted with reference to the previous report[14]. The existence of β-configuration for the galactose anomeric carbon was decided by the signal at 104.1 ppm. The signals at 79.6 and 80.0 ppm indicated that 1-4 galactosidic linkage occurred in the GlcNAc portion. These results suggested that DI is identified as LacNAc (4-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine). On the other hand, the ^{13}C-NMR spectrum of DII (Fig. 4) and assignments of the chemical shifts of each sugar unit in DII are shown (Table 2).

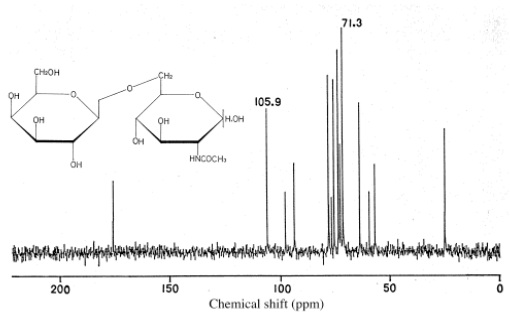

Fig. 4. ^{13}C-NMR spectrum of DII

The spectrum of DII was also interpreted with reference to the previous report[14]. The existence of β-configuration for the galactose anomeric carbon was determined by the signal at 105.9 ppm. The signals at 71.3 ppm showed that 1-6 galactosidic linkage arose in the GlcNAc portion.

Table 2. ^{13}C-NMR chemical shifts for DII

Chemical shift (ppm)	β-Gal*p*(1→)	→6)α-GlcNAc	→6)β-GlcNAc
177.4			HN<u>C</u>=O
177.1		HN<u>C</u>=O	
105.9	1		
97.6			1
93.5		1	
77.8			5
77.5	5		
76.5			3
75.4	3		
73.4	2		
73.2		3, 5	
72.5		4	4
71.3	4	6	6
63.7	6		
59.3			2
56.7		2	
24.9			CO<u>C</u>H$_3$
24.6		CO<u>C</u>H$_3$	

These results suggested that DII is identified as *allo*-LacNAc (6-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine).

Next, in order to confirm the chemical structure of DI, methylation analysis was carried out using DI reduced with $NaBH_4$. After methylation, methylated DI alditol was subjected to gas chromatography-mass spectrometry. The results are shown in Fig. 5. Characteristic fragment peaks [m/z 466 (M^+-45), 276, 219, 187, 174 and 130] showed that DI alditol is *N*-acetyl-lactosaminitol. Taken together, ^{13}C-NMR and methylation analyses revealed that the oligosaccharide DI should be LacNAc.

As mentioned above, LacNAc was generated in the culture medium of *A. oryzae*. Because *A. oryzae* preferentially consumed GlcNAc, little LacNAc was generated when *A. oryzae* was inoculated in the culture medium containing both lactose and GlcNAc (data not shown). Therefore, GlcNAc was added after 7 days of starting culture in this study. Incubation conditions including the timing of GlcNAc addition should be improved in the future. These findings presented in this paper may be useful for production of LacNAc.

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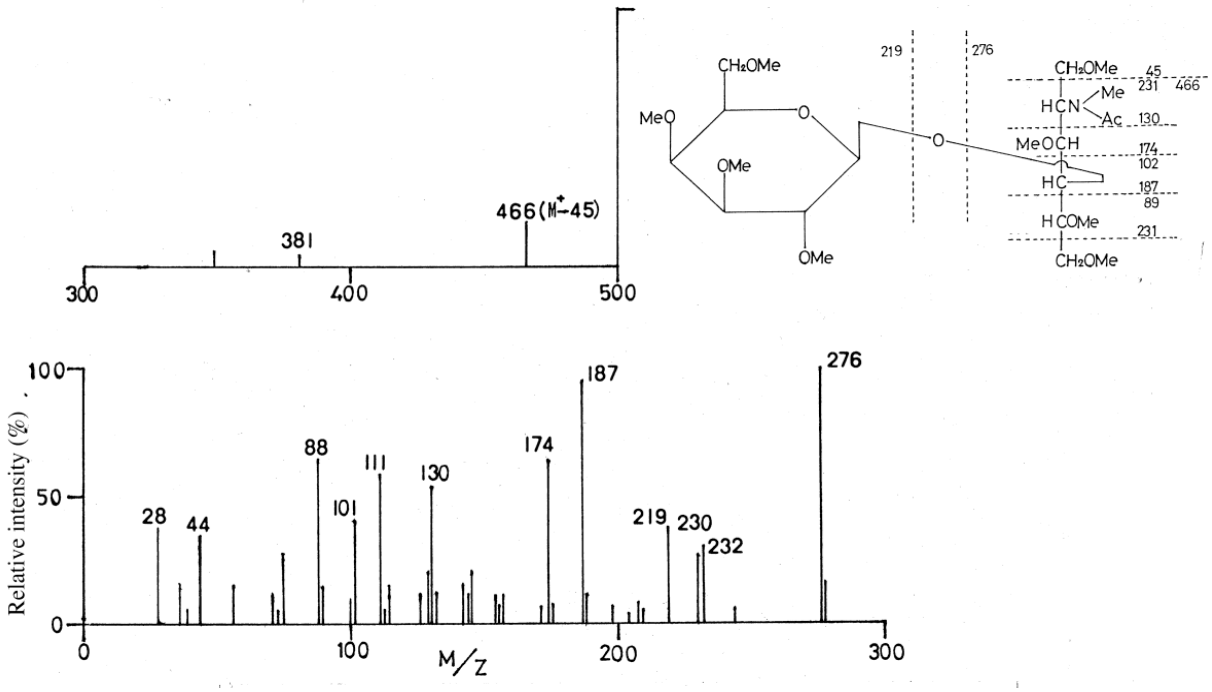

Fig. 5. Mass spectrum of permethylated oligosaccharidiol of DI and the assignment of its major fragment ion peaks. The relative intensities of the peaks were indicated as the percentages of intensity of the base peak (m/z 276).

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