Note

# Enzymatic preparation of *O*-β-D-galactopyranosyl-(1,6)-2-amino-2-deoxy-D-gluconic acid by transgalactosylation catalyzed by *Aspergillus oryzae* β-D-galactosidase

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**要旨**：β-ガラクトシダーゼによるガラクトース残基転移反応は種々のガラクトオリゴ糖合成に用いられる有用な実験系である。本研究では、黄麹カビ（*Aspergillus oryzae*）由来のβ-ガラクトシダーゼによるガラクトース残基転移反応を利用して、グルコサミン酸を含有したガラクトオリゴ糖の生産を試みた。ガラクトース残基供与体である乳糖とグルコサミン酸を共に10%含有する酢酸緩衝液（pH 4.6）にβ-ガラクトシダーゼを添加して、37℃にて50分間インキュベートしたところ、薄層クロマトグラフィーでニンヒドリン反応陽性の新たなスポット（GI）が確認された。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 解析の結果、GI は新規ガラクトオリゴ糖 *O*-β-D-galactopyranosyl-(1,6)-2-amino-2-deoxy-D-gluconic acid であることが示された。

キーワード：アミノ糖、β-ガラクトシダーゼ、糖転移反応、乳糖、グルコサミン酸

***Summary***

β-D-Galactosidase is well known to catalyze not only hydrolysis of lactose into glucose and galactose but also transgalactosylation to various acceptors. The transgalactosylation reaction is useful for synthesis of novel galacto-oligosaccharides. In this study, a new aminosugar-containing oligosaccharide was prepared by transgalactosylation catalyzed by *Aspergillus oryzae* β-D-galactosidase using lactose as a donor of D-galactose residue and D-glucosaminic acid as an acceptor. After incubation at 37°C for 50 min, a nascent ninhydrin-positive spot (GI) located in the lower portion of D-glucosaminic acid on the TLC plate was detected in the reaction mixture (pH 4.6) containing lactose, D-glucosaminic acid and β-D-galactosidase. Carbon-13 nuclear magnetic resonance analysis of GI purified by charcoal column chromatography suggested that GI was identified as a new galacto-oligosaccharide *O*-β-D-galactopyranosyl-(1,6)-2-amino-2-deoxy-D-gluconic acid.

***Key words***: aminosugar, β-D-galactosidase, transgalactosylation, lactose, D-glucosaminic acid

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It is well known that certain oligosaccharides promote selective growth of Bifidobacteria in human gut as the bifidus factor. In particular, various aminosugar-containing oligosaccharides are available for powerful bifidus factor[1]. Transglycosylation reactions catalyzed by 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 acceptors, and produces β-D-galactosides including various galacto-oligosaccharides[3)-8)]. In this study, a new aminosugar-containing galacto-oligosaccharide was prepared by *Aspergillus oryzae* β-D-galactosidase using lactose as a donor of D-galactose residue and D-glucosaminic acid (2-amino-2-deoxy-D-gluconic acid, GA) as an acceptor. Although GA, also a kind of α-amino acid, is a component of bacterial lipopolysaccharides, it is used as a sweetener, condiments, and a chiral synthon[9].

The reaction mixture containing 10% lactose (Wako, Osaka, Japan), 10% GA (Tokyo Chemical Industry, Tokyo, Japan) and 0.2 units/ml β-D-galactosidase (Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) in 0.4 M acetic acid/sodium acetate buffer (pH 4.6) was incubated at 37°C for 50 min, and the reaction was stopped by heating at 100°C for 5 min. The reaction mixtures without lactose or β-D-galactosidase were used as negative controls. These experiments were performed in duplicate for each reaction. Transgalactosylation products were detected by thin layer chromatography (TLC). TLC was performed using precoated silica gel 60 plate Art. 5553 (Merck Millipore, Billerica, MA, USA) in an ethyl acetate/acetic acid/water (2:1:1) solvent system, and aminosugars were detected by ninhydrin reagent[10]. While a nascent ninhydrin-positive spot (GI) located in the lower portion of GA on the TLC plate was detected in the reaction mixture containing lactose, GA and β-D-galactosidase, it was not found in the reaction mixtures without lactose or β-D-galactosidase (Fig. 1). For the isolation of GI, the reaction mixture containing GI (10 mL) was adsorbed on a charcoal (Wako) column (1 cm in diameter and 40 cm in length)[4]. After washing with water, the column was eluted with a linear gradient of 0-2.5% ethanol. All fractions were monitored by ninhydrin reagent and 5% sulfuric acid-methanol reagent on TLC plates, and fractions containing purified GI were collected and freeze-dried.

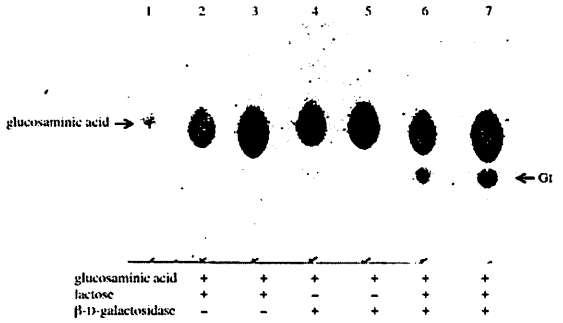


**Fig. 1.** Transgalactosylation by β-D-galactosidase using lactose as a donor of D-galactose residue and GA as an acceptor. The reaction mixture containing 10% lactose, 10% GA and 0.2 units/ml β-D-galactosidase in 0.4 M acetic acid/sodium acetate buffer (pH 4.6) was incubated at 37°C for 50 min. Aminosugars were detected by ninhydrin reagent on a TLC plate. Lane 1 shows a standard GA.

Purity of GI was examined by TLC and gas-liquid chromatography (GLC). GLC was performed with a 163 gas chromatograph equipped with a flame ionization detector (Hitachi, Ibaraki, Japan) using trimethylsilylated sugar samples[11]. GLC conditions were as follows: column; 2% Dexsil 300GC (3 mm in diameter and 50 cm in length), carrier gas ($N_2$) flow rate; 40 ml/min, column temperature; from 150°C to 300°C at a rate of 10°C/min, and injection temperature; 350°C. As shown in Fig. 2, purification of GI was almost successful.

GI was hydrolyzed into galactose and glucosaminic acid by *Escherichia coli* β-D-galactosidase (Grade VI; Sigma-Aldrich, St. Louis, MO, USA) (data not shown), suggesting that GI is composed of galactose and glucosaminic acid joined by a β-galactosidic linkage.

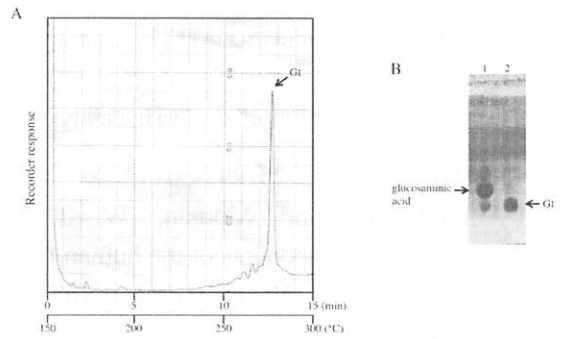


**Fig. 2.** Purification of GI. Purification of GI was performed by charcoal column chromatography. (A) GLC analysis. Trimethylsilylated GI was subjected to GLC. (B) TLC analysis. Reaction mixture of transgalactosylation (lane 1) and purified GI (lane 2) were subjected to TLC, and aminosugars were visualized by ninhydrin reaction.

Structural elucidation of GI was carried out by Carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectral analysis. Dried sugar samples (GI or D-glucosaminic acid) 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 chemical shifts of each sugar unit in GI are given in Table 1.

**Table 1.** $^{13}$C-NMR chemical shifts for GI

| Chemical Shift (ppm) | β-Gal(1- | -6)GA |
|---|---|---|
| 175.3 | | C-1 |
| 105.9 | C-1 | |
| 77.8 | C-5 | |
| 75.2 | C-3 | |
| 75.1 | | C-5 |
| 73.8 | C-2 | |
| 73.5 | | C-3 |
| 72.2 | | C-6 |
| 71.3 | C-4 | |
| 69.8 | | C-4 |
| 63.7 | C-6 | |
| 60.9 | | C-2 |

The spectrum of GI was interpreted with reference to the previous report[12] and also compared with that of D-glucosaminic acid shown in Table 2. The signal at 105.9 ppm of D-galactose residue in GI suggested β-configuration of the galactose anomeric carbon. This result agreed with hydrolysis of GI by β-D-galactosidase. The signal at 72.2 ppm of GI, which was shifted 6.8 ppm downfield from the non-linked C-6 carbon of D-glucosaminic acid, was assigned to the linkage position on C-6 of D-glucosaminic acid residue. These results suggested that GI was identified as a new oligosaccharide, *O*-β-D-galactopyranosyl-(1,6)-2-amino-2-deoxy-D-gluconic acid (Fig. 3).

**Table 2.** $^{13}$C-NMR chemical shifts for GA

| Chemical Shift (ppm) | GA |
|---|---|
| 175.3 | C-1 |
| 75.4 | C-5 |
| 73.4 | C-3 |
| 69.9 | C-4 |
| 65.4 | C-6 |
| 61.0 | C-2 |

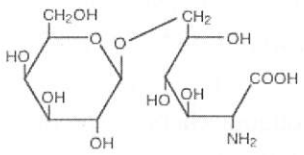


**Fig. 3.** Chemical structure of GI

In this paper, a new aminosugar-containing galacto-oligosaccharide, *O*-β-D-galactopyranosyl-(1,6)-2-amino-2-deoxy-D-gluconic acid was prepared by transgalactosylation catalyzed by Aspergillus oryzae β-D-galactosidase using lactose and D-glucosaminic acid. As is mentioned above, certain galacto-oligosaccharides and aminosugar-containing oligosaccharides play an important role as the bifidus factor. Studies on effective utilization of this oligosaccharide should be considered in the future.

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