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Purification and properties of recombinant β-galactosidase from *Bacillus circulans*

Hiroshi Fujimoto[1], Mariko Miyasato[1], Yoshiyuki Ito[2], Takashi Sasaki[2] and Katsumi Ajisaka[1]

[1]*Meiji Institute of Health Science and* [2]*Meiji Central Research Institute, Naruda Odawara 250, Japan*

A gene encoding β-galactosidase from *Bacillus circulans* which had hydrolysis specificity for the β1-3 linkage was expressed in *Escherichia coli*. The β-galactosidase was purified from crude cell lysates of *E. coli* by column chromatographies on Resource Q and Sephacryl S-200 HR. The enzyme released galactose with high selectivity from oligosaccharides which had terminal β1-3 linked galactose residues. However it did not hydrolyse β1-4 linked galactooligosaccharides. Moreover, Galβ1-3GlcNAc, Galβ1-3GalNAc, and their *p*-nitrophenyl glycosides were regioselectively synthesized in 10–46% yield by the transglycosylation reaction using this enzyme.

Keywords*: β-galactosidase, *Bacillus circulans

***Abbreviations*: GalβpNP, *p*-nitrophenyl β-D-galactopyranoside; GlcNAcβpNP, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide; GalNAcαpNP, *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide**

Introduction

Recently, the carbohydrate chains of glycoproteins or glycolipids have been highlighted as the ligands in molecular recognition. The syntheses of various types of carbohydrate chains have been needed for the elucidation of the function of carbohydrate chains. Above all, sialyl Lewis X (sLe^x) and sialyl Lewis a (sLe^a) have been reported to be ligands in the metastasis of cancer cells [1]. Galβ1-3GlcNAc is a key oligosaccharide of sLe^a. Galβ1-3GlcNAc and Galβ1-3GalNAc are also important constituents of mucin type or complex type glycoproteins.

For the synthesis of the β1-3 galactosyl linkage, the transglycosylation reaction using β-galactosidase from bovine testes has been reported by Hedbys *et al.* [2, 3]. In this reaction, unwanted β1-6 linked isomer was obtained together with β1-3 linked isomer, and was eliminated by hydrolysing with *E. coli* β-galactosidase. This process is not effective since it takes two steps to obtain target disaccharides. Moreover, bovine testes are not easily available, and the purification of this enzyme is cumbersome.

Recently, we have succeeded in the cloning of a gene encoding a new β-galactosidase from *B. circulans* ATCC 31382 [4]. The coding region was 1738 bp and encoded a polypeptide of 586 amino acids with a deduced molecular mass of 66 888.

In the present report, we show the purification and the properties of this enzyme. The enzymatic transglycosylation for the synthesis of β1-3 linked galactosyl oligosaccharides is also presented.

*To whom correspondence should be addressed. Tel: +81(465)37-3661; Fax: +81(465)36-2776; E-mail: RXG01204@niftyserve.or.jp.

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Materials and methods

Galβ1-3GlcNAc, Galβ1-4GlcNAc, Galβ1-6GlcNAc, Galβ1-3GalNAc, GalβpNP, GlcNAcβpNP, and GalNAcαpNP were obtained from Sigma. Galβ1-4GalNAc and Galβ1-6GalNAc were prepared by the procedure of Yoon *et al.* [5]. Other oligosaccharides were purchased from Oxford Glyco Systems and Bio Carb Chemicals. Resource Q, Sephacryl S-200 HR, and Sephadex G-10 were the products of Pharmacia. Precast SDS-polyacrylamide gel and isoelectric focusing gel (pH 3–7) were purchased from TEFCO (Tokyo, Japan).

HPLC

HPLC was performed with Pharmacia P-3500 system with Asahipak NH2P50 column (4.6 × 250 mm) and UV-monitor (215 nm). The flow rate of 70% acetonitrile was 0.8 ml min^{-1}. In the hydrolysis experiment, a Dionex Bio-LC system (CA, USA) was used with a pulsed amperometric detector and a CarboPac PAl column. The flow rate of 50 mM NaOH solution was 0.8 ml min^{-1}.

NMR Spectroscopy

The ^1H and ^{13}C-NMR spectra were measured at 500 and 125 MHz, respectively, on a Varian Unity-500 spectrometer using D_2O as solvent containing a small amount of

acetonitrile (δ2.00 for ^{1}H spectra and 1.27 ppm for ^{13}C spectra) as the internal standard.

Measurement of protein concentration

The amount of protein was quantitated by micro BCA protein assay reagent (Pierce).

β-Galactosidase assays

β-Galactosidase activity was estimated using GalβpNP as substrates. The enzyme solution (0.5 ml) and 0.5 ml of 5 mM GalβpNP solution were added to 1 ml of 0.1 M potassium phosphate buffer (pH 6.0). After incubation of the mixture for an appropriate period at 37 °C, 2 ml of 0.2 M sodium carbonate solution was added. The amount of *p*-nitrophenol liberated was determined by measuring the absorption at 400 nm. One unit of the enzyme was defined as the amount of enzyme which hydrolyses 1 μmol of GalβpNP per minute, under the conditions described above.

Electrophoresis

The fractions obtained at the various purification steps were analysed by SDS-polyacrylamide gel electrophoresis using commercial precast gels of 12% concentration. The gels were run for 90 min at 18 mA in a TEFCO electrophoresis unit. They were then stained for protein using Coomassie brilliant blue G250. For the molecular weight standards (Pharmacia), α-lactalbumin (14 400), soybean trypsin inhibitor (20 100), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000), and phosphorylase b (94 000) were used.

Isoelectric focusing

Isoelectric point was determined by using commercial precast gels of 4% concentration. The gels were run for 60 min at 100 V, 60 min at 200 V and 30 min at 500 V in a TEFCO electrophoresis unit. Then, they were stained for protein using Coomassie brilliant blue G250. For the isoelectric point standards (Sigma), amyloglucosidase (3.6), glucose oxidase (4.2), trypsin inhibitor (4.6), β-lactoglobulin A(5.1), and carbonic anhydrase II (5.9) were used.

Purification of β-galactosidase

Unless otherwise stated, all purification steps were done at 4 °C.

Ion exchange chromatography with Resource Q column

The recombinant *E. coli* cell was incubated as shown by Ito *et al.* [4]. The *E. coli* cells were harvested by centrifugation at $7000 \times \mathbf{g}$ for 10 min after cultivation in 100 ml of LB broth. The cells were suspended in 20 ml of 10 mM potassium phosphate buffer (pH 6.0) and sonicated at 9 kHz for 20 min. The cell-free extract was obtained by centrifugation at $17\,000 \times \mathbf{g}$ for 10 min.

The cell-free extract was put on a Resource Q column (1 ml) equilibrated with 10 mM sodium phosphate buffer (pH 7.4). The column was washed with 10 bed volumes of the initial buffer, and the enzyme was eluted with a linear gradient of sodium chloride (0–0.5 M, 60 ml) in the same buffer. Fractions containing β-galactosidase were pooled and concentrated to 1 ml with Ultrafree CL™ (molecular weight cut off: 30 kDa, Millipore).

Gel permeation chromatography with Sephacryl S-200 HR

The concentrated solution was put on a column (2.6×100 cm) of Sephacryl S-200 HR equilibrated with 10 mM potassium phosphate buffer (pH 6.0) containing 0.2 M sodium chloride. After elution with the same buffer at the flow rate of 0.5 ml min^{-1}, fractions containing β-galactosidase activity were pooled and concentrated to 1 ml with Ultrafree CL™.

Effect of pH

For the determination of optimum pH, the β-galactosidase activity was measured at 37 °C for 10 min at various pH using the glycine buffer (pH 1.17–3.72), acetate buffer (pH 3.32–6.22), and phosphate buffer (pH 5.45–8.43). The effect of pH on the stability of the β-galactosidase was examined by incubating the enzyme at various pH using Britton-Robinson buffer [6] at 25 °C for 20 h. After the incubation, the enzyme activity was measured in the 0.1 M potassium phosphate buffer solution (pH 6.0) at 37 °C for 10 min.

Effect of temperature

For the determination of optimum temperature, the β-galactosidase was incubated at various temperatures for 10 min in 0.1 M potassium phosphate buffer (pH 6.0). The thermal stability of the enzyme was examined by incubation at various temperatures for 30 min in 0.1 M potassium phosphate buffer (pH 6.0). In both cases, the enzyme activity was measured at 37 °C for 10 min after the incubation.

Hydrolysis specificity of β-galactosidase

Galβ1-3GlcNAc and Galβ1-3GalNAc

The assays were performed at 37 °C using 25 μg of Gal-GlcNAc or Gal-GalNAc and β-galactosidase (0.65 mU) in 50 μl of 0.1 M potassium phosphate buffer (pH 6.0). After incubation of various galactosyl disaccharides in the presence of the enzyme, percentage hydrolysis of the disaccharides was calculated from the peak area in HPLC.

Natural β-galactosyloligosaccharides

The structure of galactooligosaccharides 1-6 are shown in Table 2. The assays were performed at 37 °C using 10 μg of galactosyloligosaccharides 1-4 and β-galactosidase (1.3 mU) in 20 μl of 0.1 M potassium phosphate buffer (pH 6.0). After 17 h, the reaction mixture was analysed by HPLC. For oligosaccharides 5 and 6 (Table 2), β-galactosidase of 1.3 and 13 mU was used at the same reaction condition.

Synthesis of disaccharides

Galβ1-3GalNAc

A reaction mixture consisting of 72 mg of GalβpNP, 160 mg of GalNAc, and β-galactosidase (0.13 U) in 0.1 M potassium phosphate buffer (pH 6.0, 1 ml) containing 20% (v/v) of dimethyl formamide (DMF) was incubated at 37 °C. After 8 h, the enzyme was denatured by heating the mixture in boiling water for 5 min. The reaction mixture was then applied to an activated carbon column (1.6 × 40 cm). The products were eluted using a gradient from zero to 40% (v/v) ethanol solution (1 l each) at flow rate of 2 ml per min. The eluent was collected in 20 ml fractions. After concentration of the fractions containing disaccharide, 9.3 mg of Galβ1-3GalNAc was obtained (10.1% yield).

Galβ1-3GlcNAc

A reaction mixture consisting of 72 mg of GalβpNP, 160 mg of GlcNAc, and β-galactosidase (0.13 U) in 0.1 M potassium phosphate buffer (pH 6.0, 1 ml) containing 20% (v/v) of acetonitrile was incubated at 37 °C. After 3 h, the enzyme was denatured by heating the mixture in boiling water for 5 min. After purification using an activated carbon column, 11.2 mg of Galβ1-3GlcNAc was obtained (12.2% yield).

Galβ1-3GalNAcαpNP

A reaction mixture consisting of 15.4 mg of GalβpNP, 35 mg of GalNAcαpNP, and β-galactosidase (0.4 U) in 0.1 M potassium phosphate buffer (pH 6.0, 3 ml) containing 600 µl of DMF was incubated at 37 °C. After 3.5 h, the enzyme was denatured by heating the mixture in boiling water for 5 min. The solution was then applied to a Sephadex G-10 column (2.6 × 100 cm). The product was eluted with water at flow rate of 0.5 ml per min. The eluent was collected in 10 ml fractions. After concentration of the disaccharide fractions, 11.8 mg of Galβ1-3GalNAcαpNP was obtained (45.7% yield).

Galβ1-3GlcNAcβpNP

A reaction mixture consisting of 13.2 mg of GalβpNP, 30 mg of GlcNAcβpNP, and β-galactosidase (0.4 U) in 0.1 M potassium phosphate buffer (pH 6.0, 2.4 ml) containing 480 µl of DMF was incubated at 37 °C. After 4 h, the enzyme was denatured by heating the mixture in boiling water for 5 min. After purification using a Sephadex G-10 column, 8.3 mg of Galβ1-3GlcNAcβpNP was obtained (37.6% yield).

Results and Discussion

Purification of β-galactosidase

The fractions containing β-galactosidase activity were pooled and concentrated with Ultrafree CL™. Then the solution was applied to gel permeation chromatography using Sephacryl S-200 HR column. The fractions containing β-galactosidase activity were used for the analysis and reaction after concentration with Ultrafree CL™.

Table 1. Purification of recombinant β-galactosidase from *Bacillus circulans*.

Step	*Total activity (U)*	*Total protein (mg)*	*Specific activity (U/mg^{-1})*	*Yield (%)*
Crude extract	3.33	2400	1.39	100.0
Resource Q	3.03	926	3.27	91.0
Sephacryl S-200	2.39	466	5.13	71.8

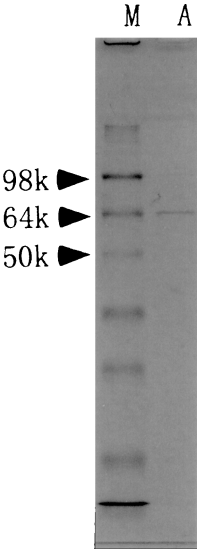

Figure 1. SDS-electrophoresis of the purified β-galactosidase. Lane A, purified β-galactosidase. Lane M, molecular weight standard.

As summarized in Table 1, the enzyme was purified 3.7-fold from the crude enzyme with a yield of 71.8%. The purified β-galactosidase showed a single band on SDS-PAGE as shown in Figure 1. As the productivity of the β-galactosidase in *E. coli* was high enough, the lysate of *E. coli* contained β-galactosidase exclusively. Therefore the SDS-PAGE showed a single band even at only 3.7-fold purification.

Properties of the β-galactosidase

Molecular weight and isoelectric point

The protein band of the purified β-galactosidase on SDS-PAGE showed a molecular weight of 62 000 (Figure 1). When the molecular weight of the β-galactosidase was estimated according to the gel permeation chromatography, an apparent molecular weight of about 67 000 was obtained. From these results, the β-galactosidase was considered to be a monomeric enzyme. The isoelectric point of the enzyme was determined as 4.5 by the isoelectric focusing method.

Effects of pH and temperature

The purified β-galactosidase was stable at a pH of between 5 and 9, and showed a pH optima about 5–6 as

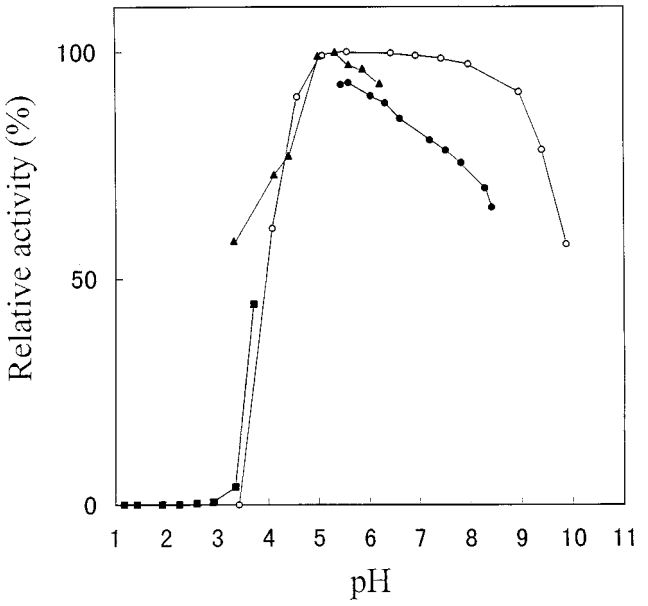

Figure 2. Effects of pH on the enzyme activity and stability. The measurement of enzyme activity was performed in the following buffer: ■--■, glycine buffer (pH 1.17–3.72); ▲—▲, acetate buffer (pH 3.2–6.22), and ●—●, phosphate buffer (pH 5.45–8.43). ○--○ shows pH stability

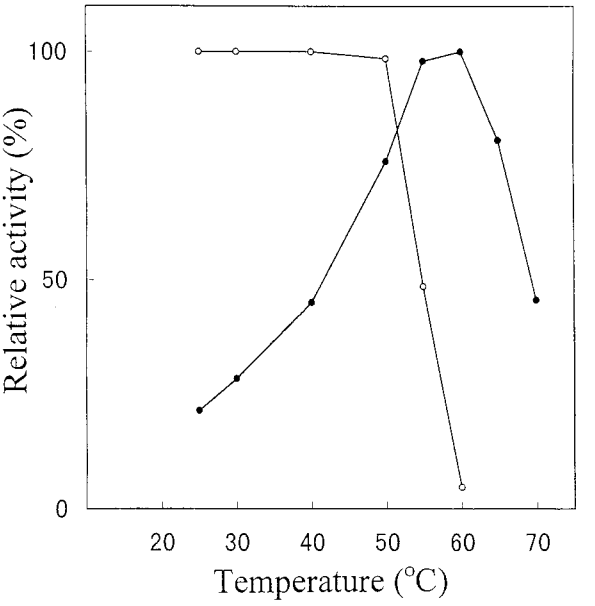

Figure 3. Effects of temperature on the enzyme activity and stability. ●—●, temperature activity; ○--○, temperature stability

demonstrated in Figure 2. The enzyme was stable below 50 °C and the activity was maximum at 60 °C under the standard assay conditions as shown in Figure 3.

Kinetic property

The activity of the purified enzyme was studied as a function of GalβpNP concentrations (0.3–2 mM), at 37 °C and pH 6.0. From the Lineweaver-Burk plot (1/velocity as a function of 1/substrate concentration), K_m for GalβpNP was 0.69 mM and V_{max} was 8.00 μmol min^{-1} mg^{-1}.

The hydrolysis specificity of the β-galactosidase

The substrate specificity of the enzyme was examined using various oligosaccharides. The values were plotted against the incubation time to indicate the susceptibility of each disaccharide to the enzyme (Figure 4a). The results indicated that the enzyme hydrolyses the disaccharides in the following order; Galβ1-3GlcNAc > > Galβ1-6GlcNAc > Galβ1-4GlcNAc. A series of Gal-GalNAc afforded similar results as shown in Figure 4b.

In order to determine more detailed aglycon specificity, six kinds of galactose-containing oligosaccharides from

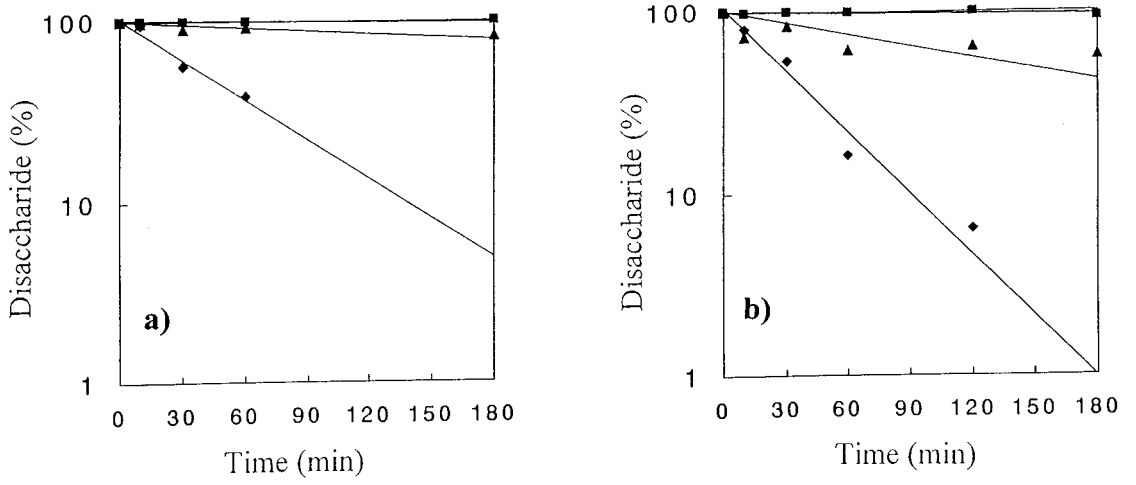

Figure 4. Time courses of the hydrolysis reaction of Gal-GlcNAc and Gal-GalNAc with β-galactosidase. (a) ◆–◆, Galβ1-3GlcNAc; ■–■, Galβ1-4GlcNAc; ▲–▲, Galβ1-6GlcNAc. (b) ◆–◆, Galβ1-3GalNAc; ■–■, Galβ1-4GalNAc; ▲–▲, Galβ1-6GalNAc

Table 2. Hydrolysis activity of β-galactosidase towards various oligosaccharides.

Compound	*Structure*	*Hydrolysis activity*
1	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	++
2	Neu5Acα2-6Galβ1-4GlcNAcβ1-6Galβ1-4Glc 3 Galβ1-3GlcNAcβ1	++
3	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 Neu5Acα2	++
4	Galβ1-3GalNAcβ1-4Galβ1-4Glc 3 Neu5Acα2	++
5	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	+
6	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 Fucα1	–

natural origin were examined. When oligosaccharide was incubated in the presence of 1.3 mU of enzyme for 17 h, galactose was released from oligosaccharides **1–4**. In contrast, oligosaccharides **5** and **6**, which had branched Galβ1-3GlcNAc and Galβ1-4GlcNAc linkage, respectively, did not release galactose as a hydrolysis product by the same condition. However, Galβ1-3GlcNAc linkage of **5** was cleaved when the amount of enzyme was increased by a factor of ten (13 mU). But **6** was not hydrolysed even by overnight incubation with 13 mU of the enzyme. The results were summarized in Table 2.

The regioselective synthesis of β1-3 linked galactooligosaccharides

In previous studies, the regioselectivity in the transglycosylation reaction has been revealed to be strongly related to the specificity in the hydrolysis reaction [7, 8]. Therefore, the transglycosylation products by the present β-galactosidase were expected to be regioselective to β1-3 linkage. GalβpNP and GalNAc were used as a donor and an acceptor, respectively, in the transglycosylation reaction. HPLC of the reaction mixture is demonstrated in Figure 5. The disaccharide fractions, which coincided to the peak at 8.2 min in Figure 5, were collected. Only the signal due to Galβ1-3GalNAc was observed in ^{13}C-NMR spectrum of the concentrated fractions (Figure 6). The yield of Galβ1-3GalNAc was 10.1%, and no other disaccharide was obtained. In

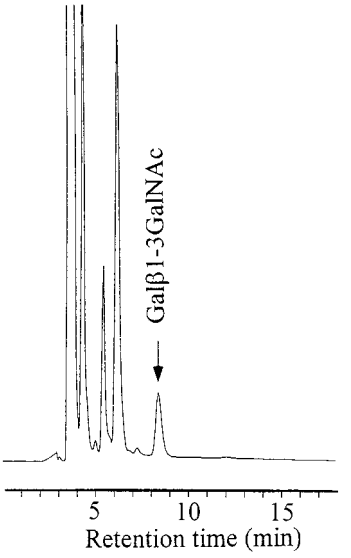

Figure 5. HPLC of the solution in the transglycosylation reaction using GalβpNP as a donor and GalNAc as an acceptor in the presence of β-galactosidase on Asahipak NH2P50. The column was eluted with 70% acetonitrile at a flow rate of 0.8 ml min^{-1}.

the reactions in which GlcNAc, GalNAcαpNP, or GlcNAcβpNP was used as an acceptor, the corresponding β1-3 linked disaccharides were obtained in remarkably high yield of 12.2%, 45.7% and 37.6%, respectively. These

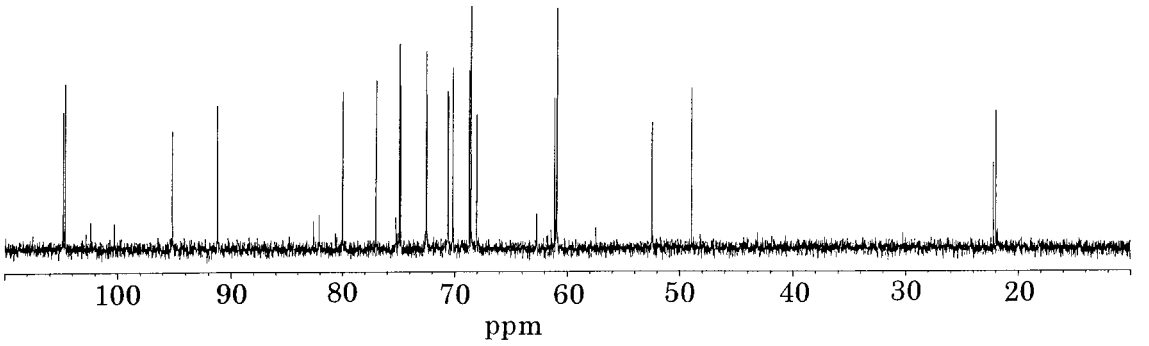

Figure 6. ^{13}C-NMR of the transglycosylation product, Galβ1-3GalNAc.

oligosaccharides were identified by the comparison of the ^{13}C-NMR spectra with those of authentic samples and literature data [5, 9].

The β-galactosidase contained in a commercial culture broth of *B. circulans*, BIOLACTA N5™, is known to hydrolyse β1-4 linkage selectively [10]. It is also well known that this β-galactosidase catalyses a transglycosylation reaction to give a β1-4 transfer product [7, 11]. The β-galactosidase specific for β1-3 linkage was detected in a negligibly small amount in the SDS-PAGE of the culture broth of *B. circulans* ATCC 31382 (data not shown). Therefore, only the β1-4 specificity would have been observed in previous studies.

Recently, Wong–Madden *et al.* [12, 13] reported β-galactosidase from *Xanthomonas manihotis* which showed high specificity towards β1-3 linkages. Although Galβ1-3GalNAc was formed preferentially in the transglycosylation reaction using β-galactosidase from *X. manihotis* with GalβpNP and GalNAc as donor and acceptor, respectively, Galβ1-6GalNAc was also obtained at about half the amount of Galβ1-3GalNAc [14]. Therefore, the β-galactosidase from *X. manihotis* is less preferable for use in the synthesis of Galβ1-3GalNAc.

In summary, β-galactosidase from *B. circulans* in the present study was revealed to be extremely specific for β1-3 linkage of oligosaccharides in glycoconjugates. It means that this enzyme can be a useful tool for the sequence analysis of a variety of glycoconjugates. Moreover this enzyme showed high potency for the regioselective synthesis of Galβ1-3GlcNAc, Galβ1-3GalNAc and their derivatives, which can be used as a component for the synthesis of glycoconjugates.

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References

1 Takada A, Ohmori K, Yoneda T, Tsuyuoka K, Hasegawa A, Kiso M, Kannagi R (1993) *Cancer Res* **53**: 354–61.
2 Hedbys L, Johansson E, Mosbach K, Larsson PO, Gunnarsson A, Svensson S, Lönn H (1989) *Glycoconjugate J* **6**: 161–8.
3 Hedbys L, Johansson E, Mosbach K, Larsson PO (1989) *Carbohydr Res* **186**: 217–23.
4 Ito Y, Sasaki T (1997) *Biosci Biotech Biochem* **61**: 1270–6
5 Yoon J-H, Ajisaka K (1996) *Carbohydr Res* **292**: 153–63.
6 Britton HST, Robinson RA (1931) *J Chem Soc* **458**: 1456–62.
7 Ajisaka K, Fujimoto H, Isomura M (1994) *Carbohydr Res* **259**: 103–15.
8 Fujimoto H, Isomura M, Miyazaki M, Matsuo I, Walton R, Ajisaka K (1997) *Glycoconjugate J* **14**: 75–80.
9 Murata T, Itoh T, Hayakawa Y, Usui T (1996) *J Biochem* **120**: 851–5.
10 Mozaffar Z, Nakanishi K, Matsuno R, Kamikubo T (1984) *Agric Biol Chem* **48**: 3053–61.
11 Usui T, Kubota S, Ohi H (1993) *Carbohydr Res* **244**: 315–23.
12 Wong–Madden ST, Landry D (1995) *Glycobiology* **5**: 19–28.
13 Taron CH, Benner JS, Hornstra LJ, Guthrie EP (1995) *Glycobiology* **5**: 603–10.
14 Fujimoto H (1997) *J Carbohydr Chem* in press.

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