An enzyme releasing lacto-N-biose from oligosaccharides
(glycosidase/type 1 sugar chain/streptomyces/pyridylamination)

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Communicated by Christian B. Anfinsen, May 27, 1992

ABSTRACT α-L-Fucosidase (α-L-fucoside fucohydrolase; EC 3.2.1.51) preparations from Streptomyces sp. 142 were found to contain an enzyme specific for lacto-N-biosidic (Galβ1-3GlcNAcβ1-3Glcβ1-4Glc) linkages (type 1 structure) in oligosaccharides. The enzyme preparation, which was eluted after α-fucosidase from a CM-Sepharose column, contained some α-fucosidase activity but was free from other glycosidases and proteases. Substrate specificity studies with oligosaccharides labeled with 2-aminopyridine showed that the enzyme specifically hydrolyzed lacto-N-tetraose (Galβ1-3GlcNAcβ1-3Glcβ1-4Glc) but did not hydrolyze lacto-N-neotetraose (Galβ1-4GlcNAcβ1-3Glcβ1-4Glc), lacto-N-triose, sialyl lacto-N-tetraose, lacto-N-fucopentaose I, II, or III, asialo-GM1 tetrasaccharide, or poly-N-acetyllactosamine. Structural analysis of the enzyme digestion of the N-acetyllactosamine type of triantennary sugar chain with type 1 structure showed that lacto-N-biose (Galβ1-3GlcNAc) and the N-acetyllactosamine type of biantennary sugar chain were produced. Thus, this enzyme was tentatively named lacto-N-biosidase, because it hydrolyzes oligosaccharides containing a type 1 structure at the nonreducing terminus and produces lacto-N-biose.

Oligosaccharides of glycosphingolipids and glycoproteins change in various biological events including cancer and cell differentiation. Most of the cancer-associated oligosaccharide antigens have structures related to Leα, Leβ, and blood group ABH (O) antigens (1–3). Recently, a structure related to sialyl-Leα was found to be an integral part of the ligand for E-selectin (ELAM1) and P-selectin (GMP140/PADGEM/CD62), which mediate the binding of leukocytes to endothelial cells in the initial stage of an inflammatory response (4). These oligosaccharide structures have been divided into two groups: type 1, with Galβ1-3GlcNAcβ1- and type 2, with Galβ1-4GlcNAcβ1-. The N-acetyllactosamine type of sugar chains from several glycoproteins also have the type 1 structure (5–8). Methylation analysis, mass spectrometry, and NMR have been used to distinguish type 1 and type 2 structures, but large amounts of oligosaccharide are needed for these analyses. Another analytical method is β-galactosidase digestion, which requires little oligosaccharide. Diplococcal β-galactosidase specifically cleaves β1-4 galactosyl linkages but not β1-3 or β1-6 galactosyl linkages (9). Thus one can conclude that oligosaccharides hydrolyzed by this enzyme have type 2 structure and that oligosaccharides not hydrolyzed by this enzyme do not have type 2 structure; however, type 1 structure cannot be identified directly with this enzyme.

Here, we describe an enzyme specific for the type 1 structure, produced by Streptomyces sp. 142, which also produces α-L-fucosidase (α-L-fucoside fucohydrolase; EC 3.2.1.51) that specifically hydrolyzes terminal α1-3 and α1-4 fucosidic linkages in oligosaccharides (10). This enzyme activity was found in the course of the preparation of α-fucosidase. This enzyme, lacto-N-biosidase, hydrolyzed oligosaccharides with type 1 structure at the nonreducing terminus to release lacto-N-biose (Galβ1-3GlcNAc). This enzyme may be useful to identify type 1 structures in glycoconjugates.

MATERIALS AND METHODS

Oligosaccharides and Enzymes. All oligosaccharides used here were labeled with 2-aminopyridine by the method of Kondo et al. (11). The pyridylamino (PA) derivatives of oligosaccharides a, b, d, e, and g–m (see Table 1) were products of Takara Shuzo. Oligosaccharide f was obtained from BioCarb (Lund, Sweden) and labeled with 2-aminopyridine with a Palstation apparatus (Takara Shuzo). The structures of PA-oligosaccharides a–j are shown in Table 1, and those of PA-oligosaccharides k–m are shown below.

![Chemical structure of PA-oligosaccharides](image)

To prepare PA-poly(N-acetyllactosamine) [(3Galβ1-4GlcNAcβ1-3Glcβ1-4Glcβ1-3Glc)–PA], keratan sulfate from bovine cornea (Seikagaku Kogyo, Tokyo) was desulfated and labeled with 2-aminopyridine. After the labeling reaction, the PA-oligosaccharide was purified by HPLC with an amino-silica column (YM pack PA-23 column, 10 × 250 mm). PA-oligosaccharide e was prepared from oligosaccharide b by digestion with β-galactosidase from bovine testes. The amount of each purified PA-oligosaccharide was measured by gas chromatography (12). β-Galactosidase from Diplococcus pneumoniae, which specifically hydrolyzes the Galβ1-4GlcNAc linkage (9), and β-galactosidase from bovine testes, which has a wide substrate specificity (13), were purchased from Boehringer Mannheim.

Microorganism and Culture. Streptomyces sp. strain 142, isolated from a soil sample, was used. This strain can be obtained from the Fermentation Research Institute, Tsukuba, Japan. This strain produced lacto-N-biosidase as well as...

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Abbreviation: PA, pyridylamination.
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Table 1. Substrate specificity of lacto-N-biosidase

<table>
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<th>Oligosaccharide</th>
<th>Relative activity, *</th>
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<tr>
<td>GlcNAcβ1-3Glcα1-4Glc-PA</td>
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<td>Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA</td>
<td>0</td>
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<tr>
<td>a-Fucosidase 2d</td>
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</tr>
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<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA</td>
<td>2</td>
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<tr>
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<tr>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA</td>
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*Conditions used for enzymatic hydrolysis are described in *Materials and Methods.*

The enzyme preparation used here contained some α,1,3/4-fucosidase activity, so l-fucose was added to the reaction mixture to a final concentration of 0.1 M to inhibit α-fucosidase. The incubation time was 20 min and the amount of the enzyme used was 0.24 microunits. The enzyme did not hydrolyze the substrate under these conditions, and the relative activity was calculated to be 2% or less from the incubation time, the amount of the enzyme used, and the detection limit for PA-oligosaccharides by HPLC (0.05 pmol).

α-fucosidase when grown on a medium containing l-fucose as the sole carbon source as described (10).

**Lacto-N-biosidase Assay.** Lacto-N-biosidase was assayed by incubation for 20 min with a PA-oligosaccharide at the concentration of 2 μM at 37°C in 10 μM of 0.1 M potassium phosphate buffer at pH 6.0. Then 40 μl of 1% trifluoroacetic acid was added to the reaction mixture to stop the reaction. A portion containing 10 pmol of PA-oligosaccharides was then analyzed by HPLC with an amino-silica column or a C18-silica column, and the labeled product was detected by fluorescence under the conditions described below. The degree of hydrolysis was calculated from the peak areas of the substrate and this product in comparison with the peak area of the corresponding standard PA-oligosaccharide. One unit of activity was defined as the amount of enzyme needed to release 1 μmol of product per min under the conditions described above. In the standard assay, PA-oligosaccharide a was used as the substrate.

α-Fucosidase Assay. α-Fucosidase was assayed with PA-lacto-N-fucopentaose III as the substrate, and the reaction mixture was assayed by HPLC with an amino-silica column as described elsewhere (10).

**Other Glucosidase Assays.** Other exoglycosidase activities were assayed with the corresponding p-nitrophénylglycosides as the substrates. Here, 20 μl of purified lacto-N-biosidase (8 millimoles/ml) was incubated for 16 h at 37°C in a final volume of 0.1 ml containing 0.3 M potassium phosphate buffer at pH 6.0 and 100 mmol of p-nitrophénylglycoside. Then 0.2 ml of 1 M sodium carbonate was added to the reaction mixture to stop the reaction, and the absorbance of the reaction mixture was measured at 405 nm. One unit of activity was defined as the amount of enzyme needed to release 1 μmol of p-nitrophényl per min under the assay conditions described above.

PA-oligosaccharides were also used as substrates in assays done to detect contaminating exo- and endoglycosidase activities. In this case, 50 microunits of enzyme was incubated for 16 h at 37°C in a final volume of 10 μl of 100 mM potassium phosphate buffer at pH 6.0 and 2 μM PA-oligosaccharide b-f, j, or l. After the incubation, a portion of the reaction mixture containing 10 pmol of PA-oligosaccharide was analyzed by HPLC with an amino-silica column under the conditions described below.

**Protease Assay.** Protease activity was assayed with the oxidized B chain of insulin as the substrate, and the reaction mixture was analyzed by HPLC with a C18-silica column (10). The final enzyme preparation (80 microunits) was incubated for 16 h at 37°C.

**Lacto-N-biosidase Digestion of PA-oligosaccharide i.** PA-oligosaccharide i (about 100 nmol) mixed with lacto-N-biosidase (total of 0.6 microunits) prepared as described below was digested for 60 h at 37°C in a final volume of 360 μl containing 0.1 M potassium phosphate buffer at pH 6.0. The reaction was monitored by HPLC with a C18-silica column under the conditions described below. The reaction mixture was lyophilized and pyridylaminated by the method of Kondo et al. (11). PA-oligosaccharides were then purified by HPLC with an amino-silica column under the conditions described below. Fractions 1 and 2 (see Fig. 4A) were desalted by Sephadex G-15 chromatography, and portions containing 20 pmol of both fractions 1 and 2 were digested with β-galactosidase as described below. The structures of the oligosaccharides in fractions 1 and 2 were confirmed by mass spectrometry and 1H NMR measurements as described below.

**β-Galactosidase Digestion of PA-Oligosaccharides.** In four different reaction mixtures, fraction 1 or 2 was digested with one of the two β-galactosidases (100 millimoles) at 37°C. Incubation was 24 h for fraction 1 and 1 h for fraction 2. Digestion with β-galactosidase from bovine testes was done in 0.1 M citrate/phosphate buffer at pH 4.8, and digestion with diplococcal β-galactosidase was done in 0.1 M sodium acetate buffer at pH 6.0.

**Mass Spectrometry.** Fractions 1 and 2 were analyzed by ion-spray mass spectrometry with a Perkin–Elmer Sciex API III apparatus. Fractions 1 and 2 (200 pmol each) were analyzed in the positive mode.

**1H NMR Measurements.** Fractions 1 and 2 and authentic PA-oligosaccharides were dissolved in 99.9% H2O, lyophlized, and dissolved again in 99.9% H2O (Aldrich). After this cycle was repeated a total of two times, samples (40 nmol) were dissolved in 99.96% H2O (Aldrich) at a concentration of 100 μM. NMR measurements were made on a Bruker AM-400 spectrometer (Bruker, Rheinstetten, F.R.G.) operating at 400 MHz in the Fourier transform mode. The spectra were measured at 35°C.

**HPLC.** PA-oligosaccharides were analyzed by HPLC with a Shimadzu (Kyoto) LC-6A chromatograph with two kinds of columns. Reversed-phase HPLC was done with a C18-silica column (Palpak type R column, 4.6 × 250 mm; Takara Shuzo). PA-oligosaccharides were eluted at a flow rate of 1.0 ml/min at 40°C with 0.05% trifluoroacetic acid containing 0.2% 1-butanol. PA-oligosaccharides were detected by fluorescence with excitation and emission wavelengths of 320 and 400 nm, respectively. Size-fractionation HPLC was done with an amino-silica column (Palpak type N column, 4.6 × 250 mm (Takara Shuzo) or, for preparation, an Asahipak NH2-P50 column, 6.0 × 150 mm (Asahi Chemical Industry, Tokyo)). PA-oligosaccharides were eluted at a flow rate of 1.0 ml/min at 40°C with solvents A and B. Solvent A was 0.2
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Fig. 1. Third CM-Sepharose CL-6B column. The chromatographic conditions are described under Materials and Methods. The arrow indicates when elution with the NaCl gradient was started. The eluate was monitored by absorbance at 280 nm (solid line), and fractions were assayed for α-fucosidase activity (○) and lacto-N-biosidase activity (●). The bar marks the fractions pooled for further study.

M triethylamine acetate, pH 7.3/acetonitrile, 10:90 (vol/vol). Solvent B was 0.2 M triethylamine acetate, pH 7.3/acetonitrile, 50:50 (vol/vol). The column was equilibrated with solvent A. After injection of the sample, the ratio of solvent B to solvent A was constant for 15 min. The proportion of solvent B was changed to 20% at 15 min after injection of the sample, and then this proportion was increased in a linear gradient to 90% in 30 min. PA-oligosaccharides were detected by fluorescence with excitation and emission wavelengths of 310 and 380 nm, respectively.

Preparation of Lacto-N-biosidase. Lacto-N-biosidase activity was found in the course of the preparation of α-fucosidase. Thus the strain of Streptomyces sp., the culture conditions, and the initial two steps of the purification (extraction of enzyme, first CM-Sepharose CL-6B chromatography step, and second CM-Sepharose CL-6B chromatography step) were those for α-fucosidase, and only fractions with α-fucosidase activity were collected and pooled, since that was the enzyme we were interested in at first (10). Unless otherwise stated, the procedures described below were done at 4°C. Cell-free extract was obtained from 5 liters of culture broth. Fractions with α-fucosidase activity from the second CM-Sepharose CL-6B column were pooled and concentrated to 13 ml. The concentrated enzyme solution was dialyzed against 300 volumes of 10 mM potassium phosphate at pH 6.0. The dialyzed enzyme solution was chromatographed again on CM-Sepharose CL-6B (2.6 × 17 cm) equilibrated with 50 mM potassium phosphate at pH 6.0; the column was washed at a flow rate of 60 ml/h with two bed volumes of the initial buffer followed by a linear gradient of NaCl (0–0.3 M, 600 ml) in the initial buffer (fraction size, 3 ml). Fractions with lacto-N-biosidase activity (see Fig. 1) were pooled and concentrated to 5 ml, and 0.1% Brij 58 was added to stabilize the enzyme activity. This enzyme preparation was used in the studies described here.

RESULTS AND DISCUSSION

Lacto-N-biosidase Preparation. The activity of the lacto-N-biosidase preparation from the CM-Sepharose CL-6B column (Fig. 1) toward PA-oligosaccharide a was 107 milliunits/mg. This enzyme preparation was assayed for α-fucosidase, α-mannosidase, α-galactosidase, β-galactosidase, and β-N-acetylglucosaminidase activities toward synthetic p-nitrophenyglucoside substrates, but such activity was not found. There was no sialidase, β-galactosidase, α-1,2-fucosidase, β-N-acetylhexosaminidase, or endoglycosidase activity to-

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)

Fig. 2. Effects of pH on lacto-N-biosidase activity and stability. (a) Enzyme activity was assayed in 0.1 M buffers of various pHs. (b) Enzyme was treated with 0.5 M buffers of various pHs at 4°C for 16 h, and the remaining activity was assayed under the standard conditions as described in Materials and Methods. ○, Sodium citrate (adjusted to the desired pH with HCl); ●, sodium citrate (citric acid); △, potassium phosphate; ▲, glycine (NaOH).

![Graph 4](image4)

Fig. 3. HPLC of the enzyme reaction mixture. The conditions used for enzymatic hydrolysis are as described in Materials and Methods. (A and B) Elution profiles on the a-amino-silica column. (C and D) Elution profiles on the C8-silica column. Substrates used were PA-oligosaccharide a (A), PA-oligosaccharide b (B), PA-oligosaccharide i (C), and PA-oligosaccharide j (D). PA-oligosaccharide a was incubated with 0.2 micromolars of lacto-N-biosidase for 20 min. PA-oligosaccharide i was incubated with 1.5 micromolars of the enzyme for 20 min. PA-oligosaccharides b and j were incubated separately with 50 micromolars of the enzyme for 16 h. Arrows 1–6 indicate the elution positions of the standard PA-derivatives: 1, PA-lactose; 2, PA-oligosaccharide a; 3, PA-oligosaccharide b; 4, PA-oligosaccharide k; 5, PA-oligosaccharide j; 6, PA-oligosaccharide l.
ward PA-oligosaccharides b-f, j, and l; however, some α-1,3/4-fucosidase activity toward PA-lacto-N-fucopentaoses II and III (PA-oligosaccharides g and h) was found. Purified α-1,3/4-fucosidase did not hydrolyze PA-oligosaccharide a. There was no protease activity toward the substrate (the oxidized B chain of insulin) according to the HPLC results, which showed no peak other than the peak of the substrate.

General Properties. The enzyme activity was maximum at about pH 5.5 (Fig. 2A), and the enzyme was stable in the pH range of 4.0–7.0 when kept at 4°C for 16 h (Fig. 2B). The activity was maximum at 50°C under the standard assay conditions. The enzyme retained 80% activity when kept at 50°C for 30 min.

Substrate Specificity of Lacto-N-biosidase. Lacto-N-biosidase activity toward 1 mM p-nitrophenyl-β-lacto-N-bioside was 684 milliunits/mg, but this enzyme did not hydrolyze p-nitrophenyl-β-lactoside or p-nitrophenyl-β-cellobioside (data not shown). Lacto-N-biosidase readily hydrolyzed PA-oligosaccharides a and i, which had type 1 structure (Fig. 3 and Table 1). The elution position of the labeled reaction product of PA-oligosaccharide a was the same as that of PA-lactose on HPLC with an amino-silica column (Fig. 3A), and the elution position of the labeled reaction product of PA-oligosaccharide i was the same as that of PA-oligosaccharide k on HPLC with a C18-silica column (Fig. 3C) or with an amino-silica column (data not shown). The hydrolysis of PA-oligosaccharide i was slower than that of PA-oligosaccharide a (Table 1). The enzyme did not hydrolyze PA-oligosaccharides b and j, which contained only the type 2 structure (Fig. 3 B and D and Table 1). PA-oligosaccharides c, d, e, and f were completely resistant to hydrolysis by the enzyme (Table 1).

![Figure 4](image-url)

**Fig. 4.** Structural analysis of lacto-N-biosidase digests of PA-oligosaccharide i by HPLC with β-galactosidase digestion. (A–E) Elution profiles with the amino-silica column. Fractions 1 and 2 were prepared as described in Materials and Methods. (A) Pyridylaminated digests of PA-oligosaccharide i with lacto-N-biosidase. (B) Fraction 1 digested with β-galactosidase from bovine testes. (C) Fraction 1 digested with diplococcal β-galactosidase. (D) Fraction 2 digested with β-galactosidase from bovine testes. (E) Fraction 2 digested with diplococcal β-galactosidase. Arrows 1–4 indicate the elution position of the standard PA-oligosaccharides: 1, Galβ1-4GlcNAc-PA; 2, Galβ1-3GlcNAc-PA, 3, GlcNAc-PA; 4, PA-oligosaccharide m.

![Figure 5](image-url)

**Fig. 5.** Mass spectra of fractions 1 and 2 from PA-oligosaccharide i after lacto-N-biosidase digestion. (A) Fraction 1. (B) Fraction 2. Fractions 1 and 2 were prepared as described in Materials and Methods, and 200 pmol of each was analyzed in the positive mode. The ion-spray voltage was 4500 V, and the orifice voltage was 70 V for fraction 1 and 100 V for fraction 2.
was cleaved by the enzyme reaction. The fractions were separated by HPLC with the preparative amino-silica column and then digested with two β-galactosidases as described in Materials and Methods. By digestion with β-galactosidase from bovine testes, with its wide substrate specificity, fraction 1 was converted into a smaller saccharide with an elution position coincident with that of standard PA-GlcNAC (Fig. 4B). Fraction 1 was completely resistant to digestion with diplococcal β-galactosidase, which specifically hydrolyzes Galβ1-4GlcNAC linkages (Fig. 4C). These findings show that fraction 1 was Galβ-GlcNAC-PA and that its linkage was not β1,4. By digestion with both β-galactosidases, fraction 2 was converted into a smaller oligosaccharide with an elution position coincident with that of PA-oligosaccharide m (Fig. 4D and E). The same results were obtained by HPLC with a C18-silica column (data not shown). Thus, fraction 2 might be PA-oligosaccharide k. The structures of fractions 1 and 2 were checked by mass spectrometry (Fig. 5) and 1H NMR spectroscopy (Fig. 6). For fraction 1 (Fig. 5A), the molecular ion (M + H)+ was observed at m/z 462, indicating that this was the PA-derivative of the disaccharide hexose-N-acetylatedhexosamine-PA. For fraction 2 (Fig. 5B), the molecular ion (M + H)+ was observed at m/z 1720, indicating that this was the PA-derivative of the nonasaccharide (hexose)3(N-acetylatedhexosamine)6-PA. Fig. 6 shows the 1H NMR spectra of standard Galβ1-4GlcNAC-PA (A) and Galβ1-3GlcNAC-PA (B). The chemical shifts for the anomic protons and the acetyl groups of Galβ1-3GlcNAC-PA were about the same as those of Galβ1-4GlcNAC-PA, but the 1H NMR spectrum of fraction 1 (Fig. 6 C and D) had signals at 3.5–4.0 ppm that were identical to those of Galβ1-3GlcNAC-PA (Fig. 6B). Signals at 4.1–4.2 ppm, characteristic of Galβ1-3GlcNAC-PA, were also observed in the 1H NMR spectrum of fraction 1. These results show that fraction 1 was Galβ1-3GlcNAC-PA. The chemical shifts for fraction 2 and those for PA-oligosaccharide k (16) were in good agreement. Thus, fraction 2 was PA-oligosaccharide k. This enzyme therefore hydrolyzed PA-oligosaccharide 1 to produce Galβ1-3GlcNAC and PA-oligosaccharide k as shown below.

![Chemical structures](image)

This enzyme was tentatively named lacto-N-biosidase. It specifically hydrolyzed oligosaccharides with type 1 structure at the nonreducing terminus and produced lacto-N-biose (Galβ1-3GlcNAC). Lacto-N-biosidase may be useful in identifying type 1 structures in glycoconjugates and to distinguish type 1 oligosaccharides from type 2 oligosaccharides.

We thank Drs. K. Hiromi and H. Iwamoto for the 1H NMR spectroscopic analysis and J. Suzuki-Sawada for the mass spectrometric analysis.