Release of Oligosaccharides from Various Glycosphingolipids by Endo-
β-galactosidase*

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The hydrolyzability of various glycosphingolipids catalyzed by endo-β-galactosidase of Escherichia freundii has been studied. The lacto-N-glycosylceramide series having the common structure R → GlcNAcβ1 → 3Galβ1 → 4Glc (or GlcNAc) were hydrolyzed at Galβ1 → 4Glc (or GlcNAc) linkages. β-Galactosyl linkages in "globo series" or "ganglio series" of glycolipids were not hydrolyzed. Oligosaccharides liberated from glycolipids were isolated, and their structures were identified by mass spectrometry. The kinetics of hydrolysis of various substrates by endo-β-galactosidase and of H2- and H3-glycolipids were studied. The following findings were of particular interest: 1) sialosyl substitution at the terminal galactosyl residue of lacto-N-neotetraosyl structure of paragloboside greatly enhances the hydrolyzability of the internal Galβ1 → 4Glc linkage, thus producing a high yield of sialosyltetrascarachide (AcNeu2 → 3Galβ1 → 4GlcNAcαβ1 → 3Galα1 → 4Glc); 2) the galβ1 → 4GlcNAc linkage located in the middle of the repeating Galβ1 → 4GlcNAc (or Glc) unit seen in H2- or Aα-glycolipid, (R → Galβ1 → 4GlcNAcαβ1 → 3Galβ1 → 4GlcNAcαβ1 → 3Galα1 → 4GlcNAcαβ1 → 3Galβ1 → 4Glc → Cer) was preferentially hydrolyzed relative to the Galβ1 → 4Glc linkage directly attached to ceramide, and 3) the branched structure as is found in H2-glycolipid greatly reduced the hydrolyzability. The H2-glycolipid was hydrolyzed at a higher concentration of enzyme, resulting in the liberation of a branched nonasaccharide which was, in turn, degraded into a branched heptasaccharide and a disaccharide.

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RESULTS

Hydrolysis of Paragloboside and Its Substituted Analogues by Endo-β-Galactosidase—Fig. 2 shows the thin layer chromatogram of glycolipids incubated with or without endo-β-galactosidase. Sialosylparagloboside, H1-glycolipid and Aα-glycolipid were hydrolyzed to Cer(Hex)α and an oligosaccharide, respectively. Similarly, paragloboside, α-galactosylparagloboside, β-galactosylparagloboside, and lacto-N-triacylglyceride were hydrolyzed to Cer(Hex)α and a corresponding oligosaccharide. In contrast, GM1, GM2, asialo GM1, asialo GM2, globoside, Forssman hapten glycolipid, and Cer(Hex)α were not hydrolyzed by this enzyme.

Oligosaccharides were isolated from the aqueous layer after partition by chloroform/methanol/water, reduced by NaBH4, and applied to paper chromatography. Each oligosaccharide alcohol showed a single peak corresponding to the position of oligosaccharide which shows the reasonable Rf value as expected from the structure of the starting glycolipid. No monosaccharide or intermediate peak was observed (Fig. 3, A to F).

To identify the products further, oligosaccharides isolated from enzymatic hydrolysate of lacto-N-triacylglyceride and sialosylparagloboside were reduced, permethylated, purified on thin layer chromatography and analyzed by mass spectrometry.

Endo-β-N-acetylglucosaminidases which specifically hydrolyze the internal glycosidic linkage in glycoproteins are becoming powerful tools for determining the structure and function of the carbohydrate moiety of glycoproteins (1-8). However, an endoglycosidase that can hydrolyze the carbohydrate moiety of glycolipids has not been described yet.

We have shown that endo-β-galactosidase from Escherichia freundii can hydrolyze a glycoprotein from ovarian cyst mucin and oligosaccharides from human milk (9, 10); although this enzyme was originally found as a keratan sulfate-degrading enzyme (11, 12).

Recently, we have found that this endo-β-galactosidase can hydrolyze glycolipids endoglycosidically (see Footnote 6 of Ref. 10). This paper describes the hydrolyzability of various glycosphingolipids including blood group A- and H-active glycolipids by the endo-β-galactosidase and compares the substrate susceptibility. In addition, the specificity of the enzyme has been determined by structural characterization of hydrolysis products.

The abbreviations used are Cer(Hex), ceramide monohexoside, Glc → Cer; Cer(Hex)α, ceramide trihexoside, Galα1 → 4Galβ1 → 4Glc → Cer; globoside, GalNAcβ1 → 3Galα1 → 4Glcβ1 → 4Glc → Cer; forssman hapten glycolipid, GalNAcα1 → 3GalNAcβ1 → 3Galβ1 → 4Galβ1 → 4Glc → Cer; paragloboside, Galβ1 → 4GlcNAcαβ1 → 3Galβ1 → 4Galβ1 → 4Glc → Cer; sialoxylparagloboside, AcNeu2 → 3Galβ1 → 4GlcNAcαβ1 → 3Galβ1 → 4Glc → Cer; a-galactosylparagloboside, Galβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Cer; globoside, forssman hapten glycolipid, and Cer(Hex)α were hydrolyzed to Cer(Hex)α and an oligosaccharide. In contrast, GM1, GM2, asialo GM1, asialo GM2, globoside, Forssman hapten glycolipid, and Cer(Hex)α were not hydrolyzed by this enzyme.
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The above results indicate that paragloboside and related glycolipids are hydrolyzed by endo-β-galactosidase as follows:

\[ R \rightarrow \text{GlcNAcβ1} \rightarrow 3\text{Galβ1} \rightarrow 4\text{Glc} \rightarrow \text{Cer} \]

The mass spectrum of each permethylated oligosaccharide alcohol indicates the structure shown in Fig. 4. The relative effect of the substituent group on the hydrolyzability is shown in a separate paragraph (see below).

**Hydrolysis of H₂-Glycolipid and A°-Glycolipid—**Endo-β-galactosidase can hydrolyze H₂-glycolipid and A°-glycolipid as shown in Fig. 5. Using the enzyme concentration of “Condition 1” (see “Materials and Methods”), lacto-N-triisosylceramide and the major slow migrating oligosaccharide were produced within a short period of incubation (see Fig. 5, Columns 3 and 8). The major oligosaccharide released from H₂-glycolipid under “Condition 1” (Fig. 3G; Fig. 5, Column 3) was isolated, reduced, and permethylated. The mass spectrum of this oligosaccharide derivative indicated that the structure was Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galol (Fig. 4C).

However, with a higher concentration of enzyme (“Condition 2”), H₂-glycolipid and A°-glycolipid were hydrolyzed to Cer(Hex) and two oligosaccharides, respectively (see Fig. 5, Columns 4 and 7). Paper chromatography of these oligosaccharides derived from H₂-glycolipid indicated the presence of Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galol and GlcNAcβ1 → 3Galol (Fig. 4C).

These results indicate that H₂-glycolipid or A°-glycolipid can be hydrolyzed by endo-β-galactosidase as follows:

\[ R \rightarrow \text{Fucβ1} \rightarrow 2\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 3\text{Galβ1} \rightarrow 4\text{Glc} \rightarrow \text{Ceramide} \]

mainly under “Condition 1”

\[ R \rightarrow \text{Fucβ1} \rightarrow 2\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 3\text{Gal} + \text{Glc} \rightarrow \text{Ceramide} \]

(R: H, GalNAcα1 → 3)

**Hydrolysis of H₂-Glycolipid—**In contrast to H₂-glycolipid, H₂-glycolipid was hydrolyzed to Cer(Hex) and oligosaccharide only under “Condition 2” (see “Materials and Methods”). Using a lower concentration of enzyme (such as under “Condition 1”), hydrolysis of H₂-glycolipid was scarcely observed.

Thin layer chromatography (Fig. 6) and paper chromatography of released oligosaccharides (Fig. 3H) show that only an oligosaccharide corresponding to a nonasaccharide and
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Cer(Hex) were produced initially, whereas oligosaccharides corresponding to a heptasaccharide and a disaccharide (GlcNAcβ1→3Gal) were released later as well. No lacto-N-triosylceramide was detected at any time. In addition, the amount of nonasaccharide released from H₂-glycolipid appeared to be maximum after 4 h and then decrease without reaching 100% hydrolysis (Fig. 7C). Furthermore, the amount of released heptasaccharide was almost equal to that of disaccharide during hydrolysis.

The structure of nonasaccharide was confirmed by mass spectrometry after reduction and permethylation (Fig. 4D). The minor oligosaccharide component, which migrates slightly faster than nonasaccharide on thin layer chromatography after methylation, showed m/e 189, 393, 638, and 204. The results strongly suggest the presence of heptasaccharide since branched reducing terminal galactitol gives m/e 204.

These results indicate that H₂-glycolipid is hydrolyzed essentially as follows:

\[
\begin{align*}
\text{Fucα1} & \rightarrow 2\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \\
\text{Galβ1} & \rightarrow 4\text{GlcNAcβ1} \rightarrow 3\text{Galβ1} \rightarrow 4\text{Glc} \rightarrow \text{Cer}
\end{align*}
\]

Relative Rate of Hydrolysis of the Various Glycosphingolipids—Release of oligosaccharide from various glycosphingolipids was measured and shown in Fig. 7. The paragloboside group (Fig. 7A) with sialosyl substitution at the terminal galactosyl residue greatly enhanced the enzyme susceptibility. Other substitutions to the paragloboside structure slightly affected the hydrolysis rate. Since the release of Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Gal from H₂-glycolipid (Fig. 7B) is much faster than that from H₁-glycolipid (Fig. 7A), the enzyme can hydrolyze the β-Gal → 4 bond in H₂-glycolipid which is located at the distance of 4 sugar residues from the ceramide moiety than that which is linked directly to the Glc → Cer moiety. The release of GlcNAcβ1 → 3Gal from H₁-glycolipid (Fig. 7B) was slower than that from lacto-N-triosylceramide (Fig. 7A), and appears to have a lag time. This fact suggests that the release of GlcNAcβ1 → 3Gal from H₁-
glycolipid does not occur until Fucα1 \(\rightarrow\) 2Galβ1 \(\rightarrow\) 4Galβ1 \(\rightarrow\) 3Gal is liberated.

H₂-glycolipid was hydrolyzed very slowly (Fig. 7C), possibly due to the presence of a branched galactose residue which is hardly hydrolyzed and in some manner restricts the enzyme access to the more internal galactosidic bond.

**DISCUSSION**

Of the various glycolipid substrates tested, the endo-β-galactosidase of *Escherichia freundii* hydrolyzes only the “lactoglycosylceramide” series which has the common structure R \(\rightarrow\) GlcNAcβ1 \(\rightarrow\) 3Galβ1 \(\rightarrow\) 4Glc (or GlcNAc). The linkage of Galβ1 \(\rightarrow\) 4Glc (or GlcNAc) is the susceptible structure. In contrast, the enzyme is totally incapable of hydrolyzing β-galactosyl structures involved in “ganglioglycosylceramide” series which has R \(\rightarrow\) Galβ1 \(\rightarrow\) 3GlcNAcβ1 \(\rightarrow\) 4Galβ1 \(\rightarrow\) 4Glc, or “globo-glycosylceramide” series which has R \(\rightarrow\) Galα1 \(\rightarrow\) 4Galβ1 \(\rightarrow\) 4Glc.

Comparative hydrolyzability of various lactoglycosyl series glycolipids by this enzyme indicate the following interesting features.

1. Sialosyl substitution at the terminal Gal residue of lacto-N-neotetraosyl residue greatly enhances the hydrolyzability of the internal Galβ1 \(\rightarrow\) 4Glc linkage, thus producing a high yield of sialosyltetrasaccharide (AcNeuα2 \(\rightarrow\) 3Galβ1 \(\rightarrow\) 4GlcNAcβ1 \(\rightarrow\) 3Galβ1 \(\rightarrow\) 4Glc \(\rightarrow\) Cer), indicating that the middle βGal1 \(\rightarrow\) 4 residue was the most susceptible followed by the βGal1 \(\rightarrow\) 3 residue linked to Glc \(\rightarrow\) Cer. The external R \(\rightarrow\) Galβ1 \(\rightarrow\) 4 residue was not susceptible in any tested glycolipids of the lactoglycosylceramide series, as far as it is not β-N-acetylgalactosaminyl group. Interestingly, the external R \(\rightarrow\) Galα1 \(\rightarrow\) 4GlcNAc linkage of carrier carbohydrate chains of blood group determinant (type 2 chain) was found to be hydrolyzed by an endo-β-galactosidase of *Diplococcus pneumoniae* recently described by Takanashi and Kobata [33].

In this study, we found that this enzyme can hydrolyze A- and H-active glycolipids. However, the intact AB-active glycoprotein was not hydrolyzed previously (10). This discrepancy should be studied further. Because a high concentration of enzyme was necessary to hydrolyze H₂-glycolipid, it is possible that the AB blood group glycoprotein could be hydrolyzed with a large amount of enzyme.

Endo-β-galactosidase is capable of hydrolyzing various substrates such as keratan sulfate, glycoproteins from mucins, and oligosaccharides from human milk (9, 10). It is now apparent that the enzyme is also capable of hydrolyzing various glycosphingolipids of the “lacto series.” Since this class of glycolipid is the carrier of antigenic determinants of blood group ABH, Lewis (17, 18, 34), Ti (17, 35, 36), P (37), and p (38) and probably for tumor-associated antigens (24, 39, 40), the enzyme will be useful to analyze and modify the antigenicity of membrane antigens in cellular extracts and on cell surfaces in situ. It is particularly noteworthy that the enzyme is capable of hydrolyzing the internal β-galactosyl linkage without removal of nonreducing sialosyl termini; thus, direct modification of cell surface antigens is possible by applying the enzyme on cell surfaces. An obvious change of the antigenicity of human erythrocytes has been observed by treating erythrocytes with this enzyme (41). The enzyme has therefore great potential as a tool for structural analysis and functional modification of cell surface glycoconjugates.

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**Experimental Method**

**Release of Glycolipids from Human Glycolipids**

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**Materials and Methods**

The following glycolipids were prepared in our laboratory: Octyl-β-D-galactopyranoside (13), β-D-galactosyl(1-β-D-galactopyranosyl) β-D-galactopyranoside (15) and β-D-galactosyl(1-β-D-galactopyranosyl) β-D-galactopyranosyl(1-β-D-galactopyranosyl) β-D-galactopyranoside (17).

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**Fig. 1**

*Fig. 1* shows the positions of the major glycolipids that were detected by thin-layer chromatography. The positions are designated by the letters A, B, C, and D. The letters represent the following glycolipids: A = galactose, B = galactose-galactose, C = galactose-galactose-galactose, and D = galactose-galactose-galactose-galactose-galactose-galactose.
Fig. 4. Mass spectra of oligosaccharides from glycosphingolipids as their reduced and permethylated derivatives.  A. GlcNAcβ1-3Galβ1 from lacto-N-triosylceramide. The mass spectrum of the same structure has been reported (32). B. AsNε2-3
Galβ1-4GlcNAcβ1-3Galβ1 from sialylparagloboside. C. Fucα1-2Galβ1-4GlcNAcβ1-3
Galβ1 from H₂-glycolipid. D. Branched nonasaccharide from H₃-glycolipid.
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