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Preparation of homogenous oligosaccharide chains from glycosphingolipids

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Abstract After the discovery of glycosphingolipid (GSL) glycan detaching enzymes, Rhodococcal endoglycoceramidase (EGCase) and leech ceramide glycanase (CGase), the method for enzymatically releasing glycans from GSLs has become the method of choice for preparing intact ceramide-free oligosaccharide chains from GSLs. This paper describes (1) the preparation of the intact oligosaccharides from GM1 (II^3NeuAcGgOse$_4$Cer) and GbOse$_4$Cer as examples to show the use of CGase to prepare intact glycan chains from GSLs, and (2) the specificity and detergent requirements of Rhodococcal EGCases for the release of glycan chains from different GSLs.

Keywords Ceramide glycanase · Englycoceramidases · Glycosphingolipids

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Introduction

Since glycosphingolipids (GSLs) have been shown to play many important and intriguing biological functions, the preparation of intact glycan chains from GSLs has been the longstanding interest for investigators working on GSLs. Chemical methods based on the oxidation of the double bond of sphingosine by ozonolysis [1] or osmium-catalyzed periodate oxidation [2], followed by alkaline treatment to release intact glycan chains from GSLs have been reported. However, due to the inherent difficulties and complications associated with the chemical methods, they have not been widely used. The discovery of the GSL glycan detaching enzymes, Rhodococcal endoglycoceramidase (EGCase) [3] and leech ceramide glycanase (CGase) [4], has facilitated the preparations of intact glycan chains from GSLs. Three molecular species of EGCase (EGCase I, II and III) were found in the culture supernatant of *Rhodococcus sp*. M-750 [5]. Among them, the EGCases II [6] and III [7] have been cloned and expressed in *Escherichia coli*. Very recently, EGCase I has also been cloned and characterized [8]. This paper describes the procedures for preparing glycan chains from GM1 (II^3NeuAcGgOse$_4$Cer), GbOse$_4$Cer using leech CGase [9] and also the specificities and detergent requirements for Rhodococcal EGCases [5–8]. It is hoped that they will serve as a guide for the preparation of glycan chains from GSLs.

Methods

For enzymatic liberation of the intact glycan chain from a GSL, one should consider: (1) specificity and purity of the

enzyme, (2) purity of the GSL substrate, and (3) the procedure for enzymatic release of the glycan chain from a GSL and subsequent purification of the released glycan chain. The preparation and specificities of Rhodococcal EGCases [5–8] and leech CGase [9] have been described previously. The method for the preparation of GSLs and their TLC analyses can be found in this special issue (see the article by Sonnino and Prinetti). Furthermore, a wide variety of GSLs are commercially available. Therefore, (3) will be briefly discussed in the following section.

Procedure for enzymatic release of the glycan chain from a GSL and subsequent purification of the released glycan

To facilitate the isolation of the released glycan chain from a given GSL, a "purification-friendly" incubation condition should be used for enzymatic release of the glycan chain. Traditionally, a detergent, such as Triton X-100 [5–8] or sodium cholate [9] is required to stimulate/assist the enzymatic release of glycan chains from GSL substrates. Since a detergent often interferes with the isolation/purification of the released glycan chains, it is preferable to devise a detergent-free condition for releasing the glycan chain from a GSL substrate, if possible. It is also advisable to use a volatile buffer to avoid the inclusion of inorganic salts in the incubation mixture. We found that organic solvents, such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, ethylacetate and methyl cellosolve (2-methoxyethanol) in a concentration between 5% and 15% can replace sodium cholate to stimulate/facilitate the release of glycan chains from GSLs by CGase. Tetrahydrofuran and ethylacetate can be removed by simple evaporation, whereas the removal of dimethylsulfoxide and dimethylformamide requires the use of a high vacuum pump, such as lyophilzation. Another advantage of including an organic solvent in the incubation mixture is that organic solvents can inhibit the microbial growth during a prolonged incubation. We have also replaced the sodium acetate buffer with a volatile ammonium acetate buffer. The first step for purifying the released glycan chain after the enzymatic incubation is to subject the incubation mixture to Folch partitioning [10]. By this step, ceramides will be partitioned to the organic phase and the released glycan to the aqueous phase. A pure glycan preparation can be subsequently obtained by passing the aqueous phase through a gel filtration column, such as Bio-Gel P-2 column, previously equilibrated with water.

Thin-layer chromatography

Glycan chains released from GSLs can be readily analyzed by normal phase TLC using a silica gel 60-precoated TLC plate (Merck, Darmstadt, Germany) and 1-butanol/acetic acid/H_2O (1:1:1, *v*/*v*/*v*) or chloroform/methanol/0.2% $CaCl_2$ (2/3/1, *v*/*v*/*v*) as the developing solvent. Glycoconjugates on the plate are revealed by the diphenylamine-aniline-phosphoric acid reagent [11] or orcinol-H_2SO_4 reagent [12].

Mass spectrometry

A 4700 Proteomics Analyzer (Applied Biosystems, Forster City, CA) in positive ion mode can be used to analyze or validate the structures of GSL substrates, as well as that of the released glycan chains. This Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry as a TOF-TOF reflector mass spectrometer is capable of high-resolution analysis up to 20,000 (full-width at half maximum). All spectra are obtained at 20 kV accelerating voltage and positive ion reflector mode. Samples are dissolved in nanopure water to achieve the concentration of 1 μg/μl. 2,5-Dihydroxybezonic acid (DHBA) from Sigma (St. Louis, MO) is used as matrix without further purification. The matrix solution made with 50% HPLC grade acetonitrile and 50% nanopure water to give 10 mg/ml of DHBA is found to produce consistent results. After pre-mixing the sample solution and the matrix solution at 1:1 ratio, 1 μl of the mixture is deposited onto one well of the mass spectrometry sample plate. The sample is allowed to air dry prior to analysis. Most intense ions are observed as $[M+Na]^+$ along with $[M+K]^+$ and multiple sodiated intact ions.

Preparation of $II^3Neu5AcGgOse_4$ (O-GM1) from GM1 and $GbOse_4$ (O-Gb4) from $GbOse_4Cer$ using leech CGase

GM1 or $GbOse_4Cer$, 1 mg in powder form, is placed in a 1.5 ml screw cap glass vial, mixed with 850 μl of 20 mM ammonium acetate buffer, pH 5.5, 50 μl of ethylacetate, and 100 μl (containing 3.3 units) of CGase [9]. After briefly sonicated in a sonication bath, the mixture is placed in a 37°C incubator. To monitor the release of O-GM1 or O-Gb4, 1 μl-aliquot of the reaction mixture is analyzed by TLC at different time intervals, using a 2 μl-capacity Eppendorf micropipette to apply the sample onto a TLC plate. This method can also be used to monitor the fractions eluted from a gel filtration column. As shown in Fig. 1, GM1 is completely hydrolyzed after 24 h of incubation under the incubation condition described above. However, under the same condition, about 90% of $GbOse_4Cer$ is hydrolyzed after 78 h of incubation. At the end of incubation, each reaction mixture is subjected to Folch partitioning [10] by adding 4 ml of chloroform/methanol (2:1, *v*/*v*). After vortexing and centrifugation, the upper phase that contains the released oligosaccharide is evaporated to dryness, dissolved in 100 μl water and applied onto

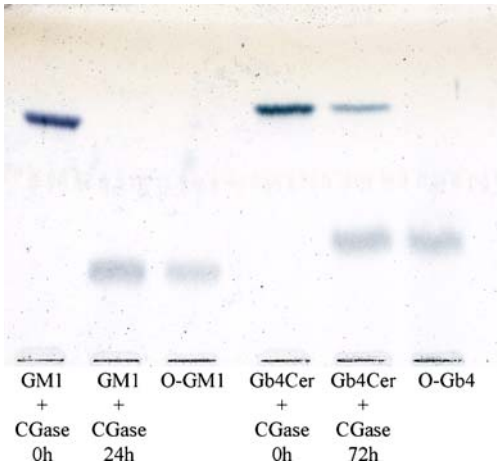

Fig. 1 TLC-analysis showing the liberation of O-GM1 from GM1 and O-Gb4 from $GbOse_4Cer$. Detailed conditions are described in the text

a Bio-Gel P-2 column (200–400 mesh, 0.8×20 cm). The column is eluted with water at 3 ml/h and 0.25 ml/fraction is collected. Figure 2 shows the typical elution profile for O-GM1 (A) and O-Gb4 (B). For O-GM1, fractions 9–12 are pooled, whereas for O-Gb4, fractions 10–12 are pooled. These fractions are lyophilized to obtain pure O-GM1 and O-Gb4. The recovery is usually around 50% of the calculated theoretical yield. The structures of the two GSL substrates, GM1 and $GbOse_4Cer$, and their corresponding released glycan products, O-GM1 and O-Gb4, are validated by MALDI, as shown in Fig. 3 and Fig. 4, respectively.

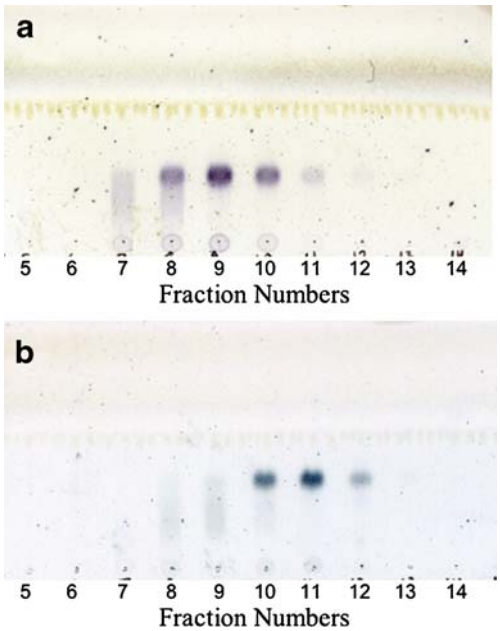

Fig. 2 TLC-analysis showing the purification of O-GM1 liberated from GM1 (**a**) and O-Gb4 liberated from $GbOse_4Cer$ (**b**) by Bio-Gel P-2 filtration. Detailed conditions are described in the text

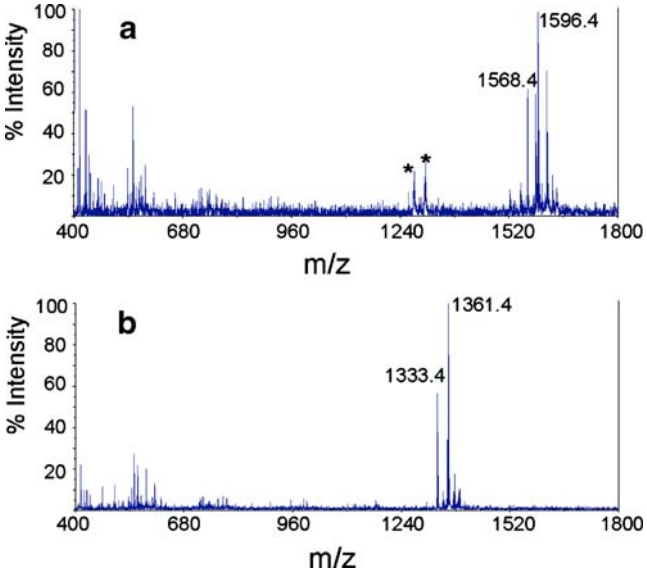

Fig. 3 MALDI analyses of GM1 (**a**) and $GbOse_4Cer$ (**b**). GM1 gives the sodiated ion $[M+Na]^+$ at *m/z* 1,568.4. The detection of the 28 Da heavier ion at *m/z* 1,596.4 indicates the long chain base heterogeneity of GM1. The similar heterogeneity is also observed in $GbOse_4Cer$, where the ion at *m/z* 1,333.4 is $[M+Na]^+$ and that at *m/z* 1,361.4 is $[M+C_2H_4+Na]^+$. The *asterisked peaks* are metastable fragment ions

Specificity and detergent requirement of Rhodococcal EGCases

As shown in Table 1, both Rhodococcal EGCases I and II efficiently hydrolyze ganglio-series GSLs. It is intriguing

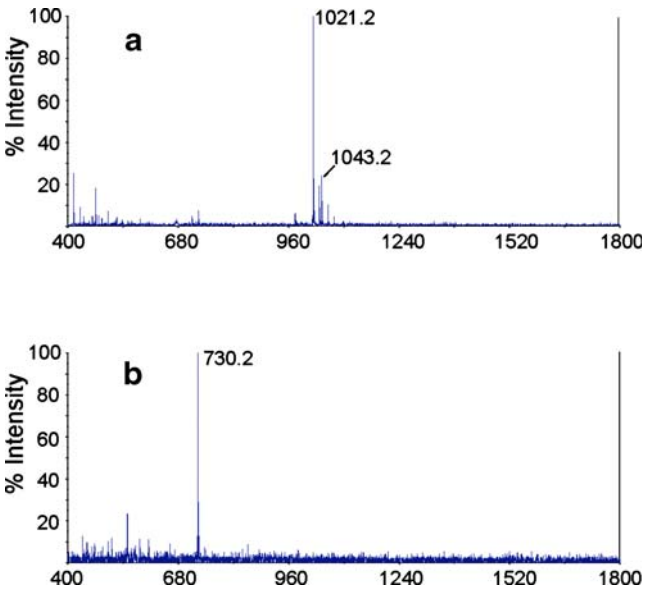

Fig. 4 MALDI analyses of O-GM1 prepared from GM1 (**a**) and O-Gb4 prepared from $GbOse_4Cer$ (**b**). O-GM1 yields the sodiated ion $[M+Na]^+$ at *m/z* 1,021.2, along with minor ions by potassium and multiple sodium attachments, such as $[M+K]^+$ at *m/z* 1,037.2 and $[M+2Na-H]^+$ at *m/z* 1,043.2. In O-Gb4 (**b**), the ion at *m/z* 730.2 represents $[M+Na]^+$

Table 1 Hydrolysis of various GSLs by EGCases*

Substrate		Extent of hydrolysis (%)		
Class and name	Structure	EGCase I	EGCase II	EGCase III
Ganglio series				
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer	100	100	0
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1′Cer	86.3	73.7	nd
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1′Cer	100	100	nd
GM1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer	100	100	0
Fucosyl-GM1	Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer	100	19	nd
Globo series				
$GbOse_3Cer$	Galα1-4Galβ1-4Glcβ1-1′Cer	72.9	23.7	0
$GbOse_4Cer$	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer	33.9	7.2	nd
$GbOse_5Cer$	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer	25.2	6.5	0
Gala series				
TGC	Galβ1−6Galβ1−6Galβ1-1′Cer	0	0	65.9
Sulfatide	HSO_3-3Galβ1-1′Cer	0	0	0
Sphingomyelin	Choline phosphate-′Cer	0	0	0

*One milli unit of the enzyme is incubated with 2 nmol of substrate in 20 μl of 20 mM sodium acetate buffer pH 5.5 containing 0.1% Triton X-100 at 37°C for 12 h; one unit of enzyme is defined as the amount that hydrolyzes 1 μmol of GM1/min under the aforementioned conditions with the exception that incubation time is 30 min

nd Not determined

that fucosyl-GM1 is resistant to EGCase II [13], but not to EGCase I. It should be pointed out that this unique specificity has been used to prepare fucosyl-GM1 from porcine brain [13]. The glycan chain from fucosyl-GM1 in turn can be prepared by using leech ceramide glycanase or EGCase I. The obvious difference in the specificities between EGCases I and II is that EGCase I hydrolyzes globo-series-GSLs much faster than EGCase II. Among EGCase I, II and III (EGALC), EGCase III stands alone to hydrolyze 6-gala-series GSLs. EGCase III, on the other hand, is not able to hydrolyze GM1, GD1a, $GbOse_3Cer$ and $GbOse_5Cer$. All Rhodococcal EGCases, as well as leech CGase, are not able to hydrolyze sphingomyelin. The substrate specificity of leech CGase is similar to that of Rhodococcal EGCases I.

For maximal hydrolysis of GSLs by Rhodococcal EGCases, a detergent such as Triton X-100 is required at a final concentration of 0.1–0.3% of the reaction mixture. However, the hydrolysis of water soluble GSLs such as GM1 does not require the presence of a detergent. For example, 2 nmol of GM1 is completely hydrolyzed by 1 mU of EGCases I or II in 50 μl of sodium acetate buffer, pH 5.5, in the absence of detergents after overnight incubation at 37°C. For the hydrolysis of GM1, the addition of 5% tetrahydrofuran instead of Triton X-100 increases the reaction rate of EGCase I by two fold compared to that in the absence of a detergent. On the other hand, tetrahydrofuran is ineffective in facilitating the hydrolysis of $GbOse_4Cer$ by EGCase I. DMSO, ethyl acetate, and methyl cellosolve are also not effective in stimulating the hydrolysis of GM1 or $GbOse_4Cer$ by EGCase I in the absence of a detergent. To prepare oligosaccharides from 1 mg of GM1 and $GbOse_4Cer$ using EGCase I, the following condition is recommended. One milligram of each of GSL is incubated at 37°C for 16–48 h with 20–50 mU of EGCase I in 1 ml of 20 mM sodium acetate buffer pH 5.5 containing 0.2–0.4% Triton X-100. After incubation, Triton X-100 could be removed with Bio-Beads SM2 Adsorbent (BioRad, catalog 152-3920). However, complete removal of Triton X-100 from the reaction mixture is not possible even using the SM2 beads.

Conclusion

With the availability of CGase and EGCases, it is now quite feasible to prepare homogeneous intact glycan chains from GSLs. Free glycans generated from GSLs can be converted into neoglycolipid probes [14] to form a microarray platform for analyzing glycan-protein interactions [15]. Each free glycan can also be converted into a neoglycolipid [14] to study the effect of the lipophilic portion on the biological properties of the sugar chain. For example, O-GM2 ($II^3Neu5AcGgOse_3$) has been converted into dipalmitoylphosphatidylethanolamine-$II^3Neu5AcGgOse_3$ to study the enzymatic hydrolysis of GalNAc from this neoglycolipid [16]. Free glycan chains from GSLs can also be used to prepare other types of immobilized carbohydrate chip or microarrys [17, 18] to identify/study their specific binding proteins.

Before preparing the glycan chain from a GSL in a large scale, it is advisable to make incubations in a small scale first to establish the optimal condition for detaching the glycan chain. We routinely use a PCR tube (600 μl capacity) to incubate 10 μg of a GSL substrate with 10 μl of buffer containing appropriate amounts of enzyme, organic solvent or detergent. To check the extent of hydrolysis, 1 μl of the incubation mixture is withdrawn and analyzed by TLC at different time intervals. Before placing the tube in a 37°C incubator, we also wrap the entire PCR tube with tin foil to prevent the evaporation and condensation of water at the top of the tube during incubation.

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