methods

Oxidation and base-catalyzed elimination of the saccharide portion of GSLs having very different polarities

Brian C. Yowler,* Stephanie Ann Stoehr,* and Cara-Lynne Schengrund[1],*

Department of Biochemistry and Molecular Biology,* The Pennsylvania State University College of Medicine, Hershey, PA 17033

Abstract **Glycosphingolipids (GSLs), present in cell membranes, participate in a variety of biological functions. Although their exact role(s) may not be understood, it has been shown that 1) embryos lacking glucosylceramide synthase activity do not develop normally, 2) GSLs can affect neuritogenesis, and 3) they can function as receptors for some pathogens. To study the role of the saccharide portion of a GSL in any of these functions, it is necessary to either isolate it from the intact GSL or synthesize it. Because syntheses are more complex, modifications were made to the oxidation/elimination procedure previously described for the isolation of the saccharide portion of GM1 and GD1a to enable it to be used with GSLs of varying polarity. The key is to use a mixture of GSLs that differ in polarity. This appears to eliminate problems encountered when purified GSLs such as sulfatide or GT1b are used.** — Yowler, B. C., S. A. Stoehr, and C-L. Schengrund. **Oxidation and base-catalyzed elimination of the saccharide portion of GSLs having very different polarities. *J. Lipid Res.* 2001.** 42: **659–662.**

Supplementary key words glycosphingolipids • saccharide isolation • gangliosides • sulfatide

Glycosphingolipids (GSLs) have a number of different functions. The fact that they may have a role in embryonic development was indicated by the observation that embryos lacking glucosylceramide synthase activity failed to develop normally (1). GSLs have also been shown to affect both neuritogenesis (2) and cell proliferation (3) and to serve as receptors for certain bacterial toxins (4) and viruses (5). To carry out studies to determine the role of the carbohydrate portion of a GSL, it is necessary to have a simple method for obtaining it. An example of the need for such a procedure is provided by studies carried out to determine the portion of GM1 [nomenclature according to Svennerholm (6)] recognized by the pentavalent binding subunit of cholera toxin [as reviewed in (7)]. To determine whether the oligosaccharide, the ceramide, or both portions of GM1 were recognized by the toxin, it was necessary to isolate the oligosaccharide portion of GM1 from the ceramide. Although two different procedures were used to accomplish this, the results indicated that as long as the oligosaccharide portion of GM1 was presented as a multivalent ligand, the ceramide portion was not needed for binding (8, 9).

In previous work (10), we described a simple method for isolating the saccharide portion of GM1 and GD1a. The gangliosides were treated with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) to oxidize the C(3)-OH of the sphingenine to a ketone (11), then with triethylamine to catalyze the elimination of the oligosaccharide (10). Although the procedure was effective for the isolation of the oligosaccharide portions of GM1 and GD1a, it was ineffective for more polar purified GSLs such as ganglioside GT1b, as well as with less polar purified GSLs such as sulfatide. GT1b was not acted on by DDQ, presumably because it failed to form inverted micelles (12). Sulfatide, on the other hand, was lost in the procedure described for removal of the DDQ. Therefore, the technique was modified so that it could be used to isolate the saccharide portion of both less polar and more polar GSLs.

METHODS

Isolation of GSLs

Gangliosides were isolated from bovine brain gray matter and sulfatide from bovine brain white matter by extracting the tissue in 10 volumes of chloroform–methanol 2:1 (v/v) (13). Insoluble material was removed by filtration, and the extract was dialyzed against water for 48 h at 4°C. Most of the gangliosides and some uncharged GSLs and phospholipids were recovered in the aqueous layer, whereas sulfatide was isolated from the chloroform phase. Purified mixed brain gangliosides were obtained by 1) exposing lipids recovered in the aqueous phase to methanolic NaOH in order to hydrolyze glycerophospholipids, 2) dialyzing them against water to remove salt, and 3) chromatographing them on DEAE-Sephadex to separate sialylated from neutral GSLs (14). Purified sulfatide was obtained by chromatography of

Abbreviations: GSLs, glycosphingolipids; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; HPTLC, high performance thin-layer chromatography; HPLC, high-performance liquid chromatography.

[1] To whom correspondence should be addressed.

lipids recovered in the chloroform phase on a silica gel 60 column with chloroform–methanol–water 65:25:4 (v/v/v) as the solvent. Silica gel 60 high performance thin-layer chromatography (HPTLC) plates were purchased from VWR (Bridgeport, NJ). DDQ (Aldrich Chemical Co., Milwaukee, WI) was recrystallized from benzene and chloroform prior to use.

HPTLC

HPTLC was used to determine the relative amount of each GSL and each oligosaccharide. Chloroform–isopropanol–50-mM KCl 2:13.4:4.6 (v/v/v) was used to separate gangliosides, chloroform–methanol–water 65:25:4 (v/v/v) to separate sulfatides from gangliosides, and acetonitrile–isopropanol–50-mM KCl 2:13.4:4.6 (v/v/v) to separate individual oligosaccharides present in the mixture obtained after the elimination procedure. Resorcinol was used to visualize sialic acid-containing components (15), and 5% H_2SO_4 in ethanol to visualize all sugar-containing compounds. In samples for which the relative amount of each component was determined, the bands were scanned with a Hewlett Packard ScanJet 6300C scanner and Adobe Illustrator 8.0 software. Band density was determined with NIH Image 1.62 software. Since the density of resorcinol-positive bands was linear over the range of concentration of sialic acid found in 2–10 μg of GD1a (~2–10 nmol sialic acid), care was taken not to exceed that range.

Isolation of saccharides from GSLs

DDQ was used to oxidize the C(3)-OH of the sphingenine base to a ketone (11). In brief, 80 mg of bovine brain gangliosides were combined with 20 mg of sulfatide in 50 ml of chloroform–methanol 2:1 (v/v) containing 3 gm of Triton X-100 and evaporated to dryness on a flash evaporator. The residue was taken up in 50 ml of anhydrous toluene containing 1.8 g of recrystallized DDQ. After approximately 40 h at 37°C, the sample was evaporated to dryness on a rotary evaporator. The bulk of excess DDQ was removed by extracting the residue with acetone. Residual DDQ was subsequently removed by chromatography on a Bio-Gel P-2 (Bio-Rad, Hercules, CA) column (25 × 1.2 cm) using pyridine-acetate (25 mM, pH 7.0) as the eluent. The sample was applied in 0.5 ml of water, and oxidized and unreacted GSLs were recovered in the void volume (~5–7ml), and DDQ was eluted in the included volume (~10–14ml). HPTLC was used to monitor recovery of oxidized and unoxidized GSLs. Oxidation of the C(3)-OH was indicated by an increase in lipid mobility upon HPTLC (**Fig. 1A** and **B**).

Oxidized GSLs were taken up in 16 ml of methanol–water 1:1 (v/v) and dispersed by sonication. Triethylamine (1.5 ml) was added and the elimination reaction allowed to proceed at 50°C for 90 min (10). At the end of the reaction, solvent was removed by evaporation and samples were dissolved in 10 ml of water. Saccharides were separated from ceramide and unreacted GSLs by centrifugation in a Centriplus-30 concentrator (Amicon, Inc., Beverly, MA) @ 5,000 *g* for 30 min. Retentate was washed with 1 ml of water three times. The filtrate, containing free saccharides, was collected and lyophilized. Recovery of saccharides was verified by HPTLC. Individual saccharides were isolated from the mixture by chromatography on a Bio-Gel P-4 column (85 × 3 cm), maintained at 55°C (16), with pyridine-acetate (0.1 M, pH 5.5) as the eluent. Samples containing 20 mg or less of saccharides were applied to the column in 1 ml of water, and 2 ml fractions were collected. Isolated saccharides were identified by their mobility upon HPTLC.

Characterization of the isolated saccharides

To determine whether sugar components within saccharides, eliminated from a mixture of bovine brain gangliosides, were modified by the isolation procedure, their mobility upon high performance liquid chromatography (HPLC) was compared with that of known standards. Gal, glc, and galNAc were obtained by hydrolyzing the mixed oligosaccharides (250 μg) in 0.5 ml of 2-M trifluoroacetic acid at 100°C. Five h later the sample was frozen and dried in a vacuum desiccator. The dried sample was then taken up in 400 μl of water and filtered through a 0.2-μm Rainin microfilterfuge. 6-O-Methylgalactose was added as an internal standard, and the sugar composition determined using a Dionex HPLC (Sunnyvale, CA) with a Dionex CarboPac PA1 column (4 × 250 mm). Sugars were eluted using 16 mM NaOH, and 0.4 M NaOH was added to the eluent prior to its passage through a pulsed amperometric detector. Free sialic acid was obtained by hydrolyzing the oligosaccharides (250 μg) in 262 μl of 0.1N H_2SO_4 at 80°C for 1 h and then filtered as described above. Sialic acid content was determined by HPLC as described above, except that it was eluted using a linear gradient that went from 50% NaOH (200 mM), 10% sodium acetate (1 M), and 40% H_2O to 50% NaOH (200 mM), 25% sodium acetate (1 M), and 25% H_2O over 12 min.

Intactness of the chemically isolated saccharides was determined by comparing their mobilities on HPTLC to those of saccharides obtained using ceramide glycanase (17) to catalyze their release from mixed bovine brain gangliosides. The enzymatic procedure followed was essentially that described by Li et al. (17), with samples exposed to the enzyme for 6 h at 37°C. Because the aqueous suspension in which the saccharides were recovered contained salt, the sample was desalted on a Bio-Gel P-2 column as described for the separation of DDQ-oxidized GSLs. Saccharides were recovered in the void and partially included volume (~5–9 ml). HPTLC analysis was performed using acetonitrile–isopropanol–50-mM KCl 2:13.4:4.6 (v/v/v) as the solvent.

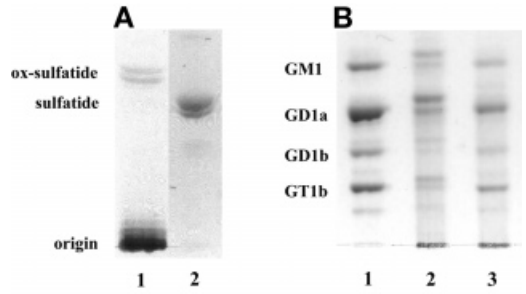

Fig. 1. High performance thin-layer chromatography (HPTLC) of oxidized glycosphingolipids. A: Sulfatides were separated using chloroform–methanol–water 65:25:4 (v/v/v), and bands visualized using 5% H_2SO_4 in ethanol. Note the increase in mobility of the oxidized sulfatide (lane 1) compared with sulfatide alone (lane 2). The more polar gangliosides included in the oxidation reaction remained at or near the origin (lane 1). B: Gangliosides were separated using chloroform–isopropanol–50-mM KCl 2:13.4:4.6 (v/v/v) and bands visualized using resorcinol (15). Lane 1) original ganglioside mixture, lane 2) mixed bovine brain gangliosides following oxidation by 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), and lane 3) centricon-30 retentate following triethylamine elimination of the mixture of DDQ-treated bovine brain gangliosides. Note in lane 3 the loss of the oxidized bands seen in lane 2.

RESULTS AND DISCUSSION

The key to using this method to isolate the saccharide portion from either very polar (e.g., GT1b) or less polar

TABLE 1. Percentages of individual gangliosides in the mixture of glycosphingolipids (GSLs) used and of the corresponding oligosaccharides recovered following oxidation and elimination

Ganglioside	% Ganglioside	% Oligosaccharide
GM1	15.6 ± 0.7	17.7 ± 1.2
GD1a	44.4 ± 0.8	40.4 ± 0.8
GD1b	13.6 ± 0.8	17.6 ± 0.8
GT1b	22.1 ± 0.4	19.9 ± 1.0
GQ	4.3 ± 0.2	4.4 ± 0.4

Values were calculated from data obtained by densitometric analyses of GSLs and oligosaccharides separated by high performance thin-layer chromatography and visualized using resorcinol. Values were not corrected for the number of sialic acid residues per molecule. Percentages were calculated as (density of band/density of all GSL or oligosaccharide bands in the sample) × 100. Standard deviations are for n = 3.

(e.g., sulfatides) GSLs is to include them in a mixture of GSLs of differing polarity. When pure GT1b was used, approximately 2 mg/100 mg starting material, or a 2.7% yield, was obtained. This result agrees with previous observations that led Sonnino, Nicolini, and Chigorno (12) to postulate that, owing to their high charge, the GT1b molecules repel each other, thereby inhibiting formation of the inverted micelles needed for the oxidation step. We found that when sulfatide alone was used, the products of the oxidation reaction partitioned into the acetone washes used to remove DDQ. However, when a mixture of GSLs of differing polarities was used, each was oxidized, as shown by the appearance of corresponding bands of higher mobility on HPTLC (Fig. 1A and B). The mixture of GSLs presumably allowed the formation of inverted micelles, thereby exposing the C3-hydroxy of sphingosine residues to the DDQ. Comparison of the intensity of resorcinol-positive bands obtained for mixed bovine brain gangliosides after oxidation with DDQ indicated that ~60% of GM1 and ~65% of GD1a, GD1b, and GT1b were oxidized. However, as indicated in Fig. 1A, all of the sulfatide appeared to be oxidized by the DDQ.

The average percent yield of ganglioside oligosaccharide, obtained in five separate experiments with 20 mgs of mixed bovine brain gangliosides being used in each, was 35 ± 4.7%. Yields were calculated using the molecular weight for GD1a having a ceramide moiety composed of C18 sphingosine and stearic acid. If the yield is calculated based on the fact that ~64% of the gangliosides were oxidized, the average yield becomes about 55%. Densitometric analyses of GSLs and their oxidized products on HPTLC indicated that the percentages of individual gangliosides used were similar to the percentages of oligosaccharides recovered (**Table 1**). The yield of saccharide obtained by this method is dependent on the efficiency of the oxidation step. The observation that only unoxidized gangliosides were present in the retentate following the elimination reaction (Fig. 1B) indicated that saccharides were eliminated from all oxidized GSLs. The lack of sulfatide after the elimination step confirmed that all of it was oxidized by the DDQ.

Gel permeation chromatography on Bio-Gel P-4 at 55°C resulted in separation of the tri-, di-, and mono-sialylated

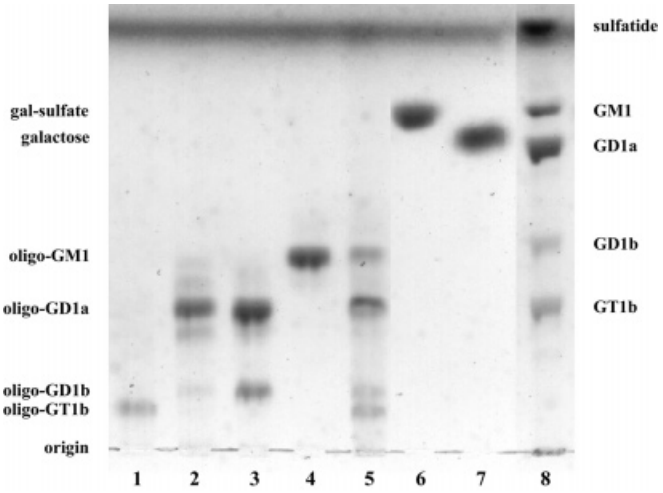

Fig. 2. HPTLC showing the relative mobilities of isolated saccharides and intact glycosphingolipids (GSLs). Bio-Gel P-4-purified oligosaccharide from lane 1) GT1b, lanes 2 and 3) disialogangliosides, and lane 4) GM1. Lane 5) oligosaccharides eliminated from bovine brain gangliosides, lane 6) galactose, lane 7) galactose-3-sulfate, and lane 8) bovine brain gangliosides plus sulfatide. The plate was developed in acetonitrile–2-propanol–50-mM KCl 2:13.4:4.6 (v/v/v), and bands visualized using 5% H_2SO_4 in ethanol. Labels for the isolated saccharides are on the left side of the figure, those for intact gangliosides are on the right.

oligosaccharides from GM1 (fractions 94–99), GD1a, GD1b, and GT1b (fractions 64–71, **Fig. 2**). This method is also effective for separating galactose-3-sulfate from the saccharides isolated from bovine brain gangliosides. Although the disialylated oligosaccharides (fractions 74–85) were separated from the mono- and tri- sialylated ones, they were not separated from each other. The fact that galactose-3-sulfate migrated further than galactose (Fig. 2) reflects the use of acetonitrile–isopropanol–50-mM KCl 2:13.4:4.6 (v/v/v) to develop the plate. Galactose migrated ahead of galactose-3-sulfate when n-butanol–acetic acid–water 2:1:1 (v/v/v) was the solvent (data not shown). However, the latter solvent was not as effective at separating saccharides isolated from gangliosides.

The observation that saccharides cleaved from mixed bovine brain gangliosides using ceramide glycanase (17) had the same mobility upon HPTLC as those obtained by oxidation and elimination (**Fig. 3**) indicated that the saccharides were probably not modified by the methods used. HPLC analysis indicated that the neutral sugar components of the mixed saccharides eliminated from bovine brain gangliosides had the same relative mobilities as galactose, glucose, and galactosamine, compared with the internal standard, 6-O-methylgalactose. Sugars were also present in the relative molar ratio of 2.07:1.12:1.00, close to the predicted 2:1:1. In addition, sialic acid obtained from the mixture of isolated saccharides had the same mobility as standard N-acetylneuraminic acid. Combined, these results indicated that the saccharides were not altered by the oxidation and elimination reactions.

In conclusion, the procedure described 1) eliminates

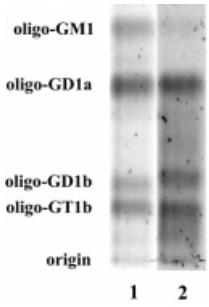

Fig. 3. HPTLC of a mixture of oligosaccharides obtained by lane 1) DDQ oxidation and base-catalyzed elimination of mixed bovine brain gangliosides, and lane 2) cleavage of mixed bovine brain gangliosides by ceramide glycanase. The plate was developed in acetonitrile–2-propanol–50-mM KCl 2:13.4:4.6 (v/v/v), and bands visualized using resorcinol (15).

lack of oxidation of the C3-OH owing to failure of the lipid to form inverted micelles, 2) can be applied to a variety of GSLs, 3) can be adjusted for use with a few to hundreds of mg of sample, and 4) is more economical to use than the enzyme when working with large quantities of GSLs.

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