methodology

A simple, nonenzymatic method for desialylating polysialylated ganglio-N-tetraose series gangliosides to produce GM1

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Abstract Dowex-50W-H$^+$ was used to catalyze the highly selective desialylation of polysialylated ganglio-N-tetraose series gangliosides to yield primarily GM1. High performance thin-layer chromatographic analysis of recovered lipid indicated that 60–70% of the recovered ganglioside was GM1. Identification of the major product as GM1 was confirmed by proton NMR spectra and lack of sialic acid release by *Vibrio cholerae* sialidase.—Schengrund, C-L., and P. Kováč. **A simple, nonenzymatic method for desialylating polysialylated ganglio-N-tetraose series gangliosides to produce GM1. *J. Lipid Res.* 1999.** 40: **160–163.**

Supplementary key words ganglioside GM1 • Dowex-H$^+$ • sialic acid

The ganglioside GM1 [ganglioside abbreviations are those defined by Svennerholm (1)] has been used in numerous studies. For example, experiments were done to determine *a*) its effect on neurite outgrowth (2–4), *b*) the mechanism by which it alters cell division (5), *c*) its ability to function as a receptor for certain bacterial toxins such as cholera and the heat labile enterotoxin of *E. coli* (6) or viruses such as simian rotavirus (7), and *d*) its ability to enhance recovery from specific neuronal lesions (8, 9). Between 1991 and January 1998, over 250 papers were published in which ganglioside GM1 was mentioned in either the title or abstract.

In vivo studies using GM1 can require multiple grams (8), and in vitro studies, in which ganglioside derivatives such as neoganglioproteins (10) or neogangliodendrimers (11) are prepared, may require hundreds of mgs. The cited studies highlight the need for a simple procedure to obtain quantities of GM1. This paper describes the use of Dowex-H$^+$, a cation exchange resin known to catalyze the nonspecific hydrolysis of glycosidic bonds in polysaccharides (12, 13), to promote the highly selective conversion of polysialylated $GgOse_4$ gangliosides to the monosialoganglioside GM1.

METHODS

Gangliosides were isolated from bovine brain gray matter (14), saponified to degrade susceptible lipid contaminants and, after removal of salt by gel permeation chromatography, freeze-dried. Ganglioside standards were purchased from Matreya Inc. (Pleasant Gap, PA); high performance silica gel thin-layer chromatography (HPTLC, silica gel 60) plates were from VWR (Bridgeport, NJ); DEAE-Sephadex A-25 was from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden); and *V. cholera* sialidase (VCN) was from Calbiochem (LaJolla, CA).

Dowex-50W (hydrogen form, dry mesh 200–400, 4% cross linked), purchased from Sigma Chemical Co. (St. Louis, MO), was purified as follows. After suspension of the Dowex in a 5% solution of potassium carbonate for 15–30 min, it was filtered, washed with water, and put in 1 m HCl for 1 h at room temperature. It was then filtered and exhaustively washed with water to remove excess acid prior to washing with anhydrous methanol. After it was air dried for several hours, it was dried overnight in a vacuum oven with the temperature between 35° and 40°C.

For hydrolysis, 100 mg of bovine brain gangliosides and either an equal or 1/3 weight of dry Dowex-H$^+$ were taken up in 25 ml of water and heated in an oil bath at 80°C with gentle stirring. After 60 min. [for ganglioside-Dowex-H$^+$ 1:1 (w.w)] or 120 min [for ganglioside-Dowex-H$^+$ 1:3 (w/w)], the mixture was filtered through a cotton plug and the filtrate was freeze-dried. The weight of lipid plus free sialic acid recovered in three samples was 96% ± 3.4% of the initial sample of crude bovine brain gangliosides. Glycosphingolipid composition of the dried material was determined by thin-layer chromatography with either chloroform–methanol–0.3% $CaCl_2 \cdot 2H_20$ 60:35:8 (by volume) or chloroform–methanol–0.2% $CaCl_2 \cdot 2H_20$ 50:40:10 (by volume) (15) as the solvent. Resorcinol (16) was used to identify sialic acid-containing compounds; lipids were visualized by charring plates sprayed with 5% sulfuric acid in 95% ethanol. Plates were

Abbreviations: GM1, Galβ1-3GalNAcβ1-4[sialic acidα2-8]Galβ1-4Glcβ1-ceramide or II^3 sialic acid-$GgOse_4$Cer. Sialic acid is used instead of a specific abbreviation for neuraminic acid as both N-acetyl- and N-glycolyl-neuraminic acid are present in bovine brain gangliosides.

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scanned using a Microtek ScanMaker with Adobe Photoshop software. NIH image ppc60 software was used to integrate and analyze the density of the bands.

Monosialogangliosides were isolated from the Dowex-H^+-treated bovine brain gangliosides by chromatography on DEAE-Sephadex G-25. Lipids were eluted using a step gradient of methanol–chloroform–sodium acetate of increasing polarity (17). Individual glycosphingolipids were isolated by column chromatography on silica gel. Prior to use, impurities associated with the silica gel were removed by adding the gel to approximately two volumes of methanol–concentrated ammonium hydroxide 1:1 (by volume) and while stirring, heating it at 45–50°C for 30–60 min. The silica gel was then allowed to settle (about 10 min) and the supernatant, which contained fines, was decanted. Residual methanol and ammonium hydroxide and additional fines were removed by suspending the gel several times in two to three volumes of boiling water. After each addition of water the silica gel was dispersed by stirring, allowed to settle for about 10 min, and the supernatant was decanted. This procedure was repeated until the water was essentially clear. The gel was then dispersed in an equal volume of hot water and filtered through a large, coarse, sintered glass funnel. It was washed with hot and then with cold water, followed by methanol. It was air dried first, then at 40°C for several hours, and then overnight at 120°C. The initial drying at a low temperature served to remove residual methanol that at higher temperatures might be oxidized and contaminate the silica gel. Elution of the lipids applied to the silica gel column was accomplished using a step gradient of chloroform–methanol–water of increasing polarity (17).

In addition to mobility on HPTLC, identification of the monosialoganglioside as GM1 was accomplished as follows. As the sialic acid residue of GM1 is not susceptible to *Vibrio cholerae* sialidase (18), the effect of the enzyme on the monosialoganglioside fraction obtained by column chromatography on DEAE-Sephadex A-25 was determined. Ganglioside (1 mg) was incubated with 0.02 units of sialidase in 1 ml of 0.01 M Tris acetate buffer, pH 6.5, containing 4 mM $CaCl_2$ (19). After 1 h, samples were either taken for analysis of free sialic acid using the thiobarbituric acid procedure (20) or dialyzed to remove salts and free sialic acid, prior to lyophilization. Lipid composition of the recovered material was determined by HPTLC. One-dimensional proton NMR spectra were obtained for the putative GM1 after its purification by column chromatography on silica gel. Two mg lipid was dissolved in 800 μl of $(CD_3)_2SO:D_2O$ 49:1 (by volume) (21) and the spectra were obtained at 300 MHz. Optical rotation of the purified GM1 was measured using a Perkin Elmer 341 Polarimeter.

RESULTS AND DISCUSSION

Use of a 1:1 w/w ratio of Dowex-H^+ to ganglioside and a temperature of 80°C resulted in conversion of the majority of di- and trisialogangliosides to GM1 and some asialo-GM1 (**Fig. 1**). Comparable results were obtained when a 1:3 w/w ratio of Dowex-H^+ to ganglioside was used with the time of heating at 80°C increased from 1 to 2 h. The enhanced intensity of the GM1 band seen in the lanes showing the lipid patterns obtained upon thin-layer chromatography of the Dowex-H^+-treated samples (Fig. 1) indicates its increase relative to the other gangliosides. Densitometric analysis of both resorcinol-stained and sulfuric acid-charred samples indicated that the relative amount of GM1 was comparable in samples treated either way. No change was seen in controls that were heated at 80°C in

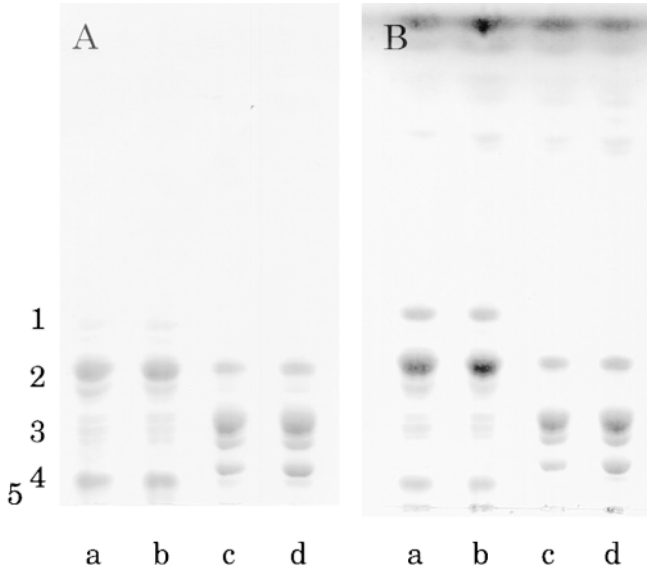

Fig. 1. HPTLC chromatographs of the lipid mixture obtained after treatment of bovine brain gangliosides with Dowex-H^+. Plates were developed in chloroform–methanol–0.3% $CaCl_2 \cdot 2H_20$ 60:35:8 (by volume) and visualized with A: resorcinol spray, or B: 5% sulfuric acid in 95% ethanol. Samples shown are bovine brain gangliosides incubated at 80°C with *a*) an equivalent weight of Dowex-H^+ for 1 h; *b*) a one-third weight of Dowex-H^+ for 2 h; *c*) no Dowex-H^+ for 2 h. Lane *d*) shows the pattern obtained for bovine brain gangliosides not incubated at 80°C. The numbers on the side indicate 1) asialo-GM1; 2) GM1; 3) GD1a; 4) GT1b; and 5) sialic acid. Note the presence of asialo-GM1 in lanes 1 and 2 of panel B and the presence of resorcinol-positive free sialic acid in lanes 1 and 2 of panel A.

the absence of Dowex-H^+ compared with untreated samples (**Table 1**). Despite the noticeable increase in asialo-GM1, the weight of resorcinol-negative lipids increased by only about 6% in Dowex-H^+-treated samples. The selective hydrolysis of sialyl residues obtained under these conditions is in contrast to the more usual use of cation exchange resins to catalyze the nonspecific hydrolysis of sugars from polysaccharides (12, 13).

HPTLC analysis of the monosialoganglioside fraction obtained after chromatography of the Dowex-H^+-treated gangliosides on DEAE-Sephadex A-25 showed the presence of a single resorcinol-positive band with a mobility similar to that of known GM1. However, the band appeared to have a slight tail, suggesting that it might not be absolutely pure. Purification of the predominant monosialoganglioside by column chromatography on silica gel resulted in a product that gave a single band with the same mobility as GM1 upon HPTLC. When the plate was overloaded with sample, a single broader band was obtained. No faster or slower moving components were seen when components on the plate were visualized using either resorcinol or sulfuric acid charring. Fractions eluted from the silica gel column just after those containing apparently pure GM1 contained a minor component that migrated just below GM1 upon HPTLC. It appeared to be the contaminant seen in the monosialoganglioside fraction obtained by chromatography on DEAE-Sephadex.

TABLE 1. Ganglioside composition of Dowex-50W-H^+-treated samples and controls

	Percent Ganglioside Visualized on HPTLC with									
	Resorcinol					Sulfuric Acid				
Treatment	GM1	NI[a]	GD1a	GD1b	GT1b	GM1	NI	GD1a	GD1b	GT1b
Dowex, 1:1	60	18	6.8	9.3	5.7	68	16	5.2	7.4	3.7
Dowex, 1:3	65	15	8.2	7.9	4.7	70	13	7.1	5.9	3.8
Control, 80°C	17	2.3	50	13	18	18	2	49	13	18
Untreated	16	2.8	47	13	22	18	2	45	12	23

Treatments of the bovine brain gangliosides were as follows: Dowex, 1:1, equivalent weights of Dowex-50W-H^+ and bovine brain gangliosides were incubated at 80°C for 1 h. Dowex, 1:3, three times more bovine brain gangliosides by weight were incubated with Dowex-50W-H^+ at 80°C for 2 h. Control, 80°C, bovine brain gangliosides were incubated in water at 80°C for 2 h.

[a] The resorcinol-positive band was not identified.

The following observations confirmed that the major product produced by Dowex-H^+ treatment of the bovine brain ganglioside mixture was GM1. Analysis of sialidase susceptible sialic acid residues associated with the monosialoganglioside fraction, isolated from the Dowex-H^+-treated gangliosides by chromatography on DEAE-Sephadex, indicated that about 1% of the sialic acid was hydrolyzed in an hour while about 12% of that associated with the untreated mixed bovine brain gangliosides was cleaved. In contrast to GM1 in which the sialic acid residue is linked α2–3 to the internal galactose, the sialic acid linked α2–3 to the terminal galactose of GM1b (15) is accessible to sialidase (18). Thus, the low level of sialic acid released from the GM1 fraction relative to bovine brain gangliosides indicated that the monosialoganglioside was predominantly GM1. This was confirmed by the ^1H NMR spectrum obtained for Dowex-H^+ generated GM1 that had been purified by chromatography on a silica gel column. Chemical shifts and coupling constants obtained at room temperature for the anomeric protons of the galactosaminyl, internal galactosyl, external galactosyl and glucosyl residues of the core tetrasaccharide were δ 4.84 ($J_{1,2}$ 8.5 Hz), 4.27 ($J_{1,2}$ 7.4 Hz), 4.21 ($J_{1,2}$ 6.6 Hz), and 4.14 ($J_{1,2}$ 7.7 Hz), respectively. These results are in good agreement with those of Koerner et al (21). The $[\alpha]_D$ + 8.2° (c. 1, $CHCl_3$:CH_3OH:H_2O, 5:5:1 by volume) obtained for the isolated GM1 compares well with that reported for synthetic GM1 {$[\alpha]_D$ + 7.2° ($CHCl_3$:CH_3OH:H_2O, 5:5:1)} (22).

Use of a 5:1 w/w ratio of Dowex-H^+ to bovine brain gangliosides coupled with heating for 1 h at 100°C resulted in conversion of essentially all of the bovine brain $GgOse_4$ gangliosides to predominantly asialo-GM1. Loss of sialic acid was indicated by the observation that the product was resorcinol-negative and did not adhere to DEAE-Sephadex A-25, as evidenced by its recovery in the methanol–chloroform–water fraction. The comigration of the major product with standard asialo-GM1 upon HPTLC indicated that it was asialo-GM1. The second asialo product formed was classified as a trihexosylceramide as it was less polar than asialo-GM1 but more polar than lactosylceramide on HPTLC.

This procedure has several advantages over the use of other acidic or enzymatic methods. *1*) It is suitable for the large scale production of GM1. It was equally effective when either 3.75 or 100 mg of bovine brain gangliosides was used with proportional amounts of Dowex-H^+ and water. *2*) The reaction is terminated by removal of Dowex-H^+ thereby negating the need to neutralize acid prior to isolation of the GM1 or analysis of the lipids by TLC. *3*) There is no contamination by protein as there would be if an enzyme were used. *4*) The reaction is relatively quick (60–120 min). *5*) The degree of sialic acid hydrolysis can be regulated by adjusting the ratio of Dowex-H^+ to ganglioside and/or the temperature. *6*) The cost is less than it would be for the enzymatic reaction.

This work was supported in part by grant NS35653 awarded by the National Institute of Neurological Disorders and Stroke. The authors thank Drs. Christine Jurasinski and Martin Garraffo for scans of the thin-layer plates.

Manuscript received 13 July 1998 and in revised form 1 September 1998.

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