A Novel Sulfatide, GlcCer-I$_6$ Sulfate, from the Ascidian Ciona intestinalis

So Yamada$^1$, Yuki Matsumuro$^1$, Takashi Inoue$^1$, Tomonori Kitamura$^2$, Saki Itonori$^2$, Mutsumi Sugita$^2$ and Masahiro Ito$^{1,*}$

$^1$Department of Bioscience and Bioinformatics, College of Information Science and Engineering, Ritsumeikan University (1-1-1 Nohigashi, Kusatsu, Shiga 525-8577, JAPAN)
$^2$Department of Chemistry, Faculty of Liberal Arts and Education, Shiga University (2-5-1 Hiratsu, Ohtsu, Shiga 520-0802, JAPAN)

Abstract: A novel sulfated glycosphingolipid, SGL-1, was isolated from the ascidian Ciona intestinalis, prepared from chloroform/methanol extracts and fractionated successively on DEAE Sephadex-A25, Florisil and Iatrobeads column chromatographies. Chemical structural analysis was performed using methylation analysis, gas-liquid chromatography, combined gas-liquid chromatography-mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and $^1$H-NMR spectroscopy. This chemical structure is presented as GlcCer I$_6$-Sulfate. The ceramide moiety was specified by t16:0, t17:0, br,t17:0, t18:0 and br,t18:0 as sphingoids, and 2-hydroxy, saturated fatty acids as represented by docosanoic and tetracosanoic acids.

Key words: chemical structure, glycosphingolipid, sulfatide, chordate, sulfotransferase

1 INTRODUCTION

Glycosphingolipids (GSLs) consist of a hydrophilic carbohydrate chain and a non-polar ceramide moiety, and are a member of membrane components. GSLs play important roles in a wide variety of cell functions, for instance cell-cell recognition, cell-cell interaction, cell growth and differentiation and apoptosis, etc$^1$-$^7$. Many chemical structural variations of GSLs were reported in mammals, other vertebrates and invertebrates$^8$-$^{10}$. Sulfatides were once thought to be the relatively small number of only two sulfatides, SM$_4$s and LacCer I$_3$-sulfate but is used recently as a generic term for sulfosphingolipids. Sulfatides are a major component of the myelin sheath and are also located at high concentrations in the kidneys and are found to be primarily localized in the peripheral nerves$^{11,12}$.

Acidic GSLs that have sulfate esters on their carbohydrate residues are termed sulfatides, which formerly meant galactosylsulfatide (SM$_4$s, GalCer I$_3$-sulfate) but is used recently as a generic term for sulfosphingolipids. Sulfatides are a major component of the myelin sheathe and are also located at high concentrations in the kidneys and are found to be primarily localized in the peripheral nerves$^{11,12}$.

Sulfatides were once thought to be the relatively small number of only two sulfatides, SM$_4$s and lactosylsulfatide (SM$_3$, LacCer I$_3$-sulfate), compared to neutral GSLs and gangliosides$^{13}$. However, their various chemical structures, which are mono- and bis-sulfated GSLs as well as a sulfated ganglioside and a sulfated GlcA-containing GSL, have been isolated and identified since the 1980s$^{14-17}$. Sulfate ester-binding carbohydrate residues were found in only HSO$_3$-3Gal, HSO$_3$-3Glc, HSO$_3$-3GlcNAc, HSO$_3$-6GlcNAc and HSO$_3$-3GlcA in GSLs$^{18}$, and mono-sulfate has only ever been found in two types, SM$_4$s and SM$_4$s-Glc (GlcCer I$_3$-sulfate)$^{13,19}$. One of the important targets in the post-genome sequencing era is to discover the common and various roles of GSLs in multicellular organisms. That is, it is to discover the relationship between the diversity of cell-surface GSLs and the construction of cellular societies. However, the chemical structures of GSL are various and variable from the history of carbohydrate research, and alteration of the gene expression of glycosyltransferase and lipid synthetase affects not only glycolipids that are directly biosynthesized, but also broadly the expression patterns of the glycolipid system. Ascidian is a good model organism for understanding vertebrates and their development because it belongs to a chordate and its cell lineage can be traced$^{20,21}$. Furthermore, a large number of genome-related data have been accumulated, including genome sequences and gene expression patterns$^{22-25}$. However, very few studies have
been directed toward the ascidian acidic GSLs.

This article describes the isolation and characterization of a novel sulfatide (SGL-1, GlcCer 1\(^\text{st}\)-sulfate) from the ascidian Ciona intestinalis. The binding power between the carbohydrate chain and ceramide of SGL-1 was analyzed. Furthermore, 56 genes of sulfotransferase were extracted using a keyword search in the GTOP database\(^ {26}\), and 6 homologous genes of cerebroside 3'-sulfotransferase (CST) were predicted using a blastp search\(^ {27}\) for the C. intestinalis genome sequence\(^ {22}\). The mRNA expression patterns of these genes were searched in the Ghost database\(^ {28}\).

### 2 EXPERIMENTAL

#### 2.1 Extraction of acidic GSLs from C. intestinalis

The ascidian C. intestinalis used in this study, 5.5 kg wet w.t., was gathered at Tosa Port around the Usa Marine Biological Institute in Japan, boiled for 2 h, suspended in acetone over night and dried for a half day in the shade. The obtained sample (221 g dry w.t.) was extracted three times with 1 liter of chloroform/methanol (2:1, v/v), and then extracted twice further with 0.5 liters of chloroform/methanol (1:1, v/v). The extracts were combined and concentrated using an evaporator at 40°C, and became 25 g dry w.t. The obtained dry extracts were subjected to mild alkaline hydrolysis to remove acyl- and alkenyl- glycerolipids and to obtain sphingolipids. The obtained sphingolipids of the alkali-stable product were applied to a DEAE-Sephadex A-25 column (Amersham Pharmacia Biotech) (column size 3.4 cm \(\times\) 230 mm) and eluted with 3 vol. of chloroform/methanol/water (30:60:8, v/v/v) for the extraction of neutral and zwitterionic sphingolipid fractions. Furthermore, the fractions of different acidic sphingolipids of ionic strength were eluted with 5 vol. of 0.05 M, and 3 vol. of 0.15 M and 0.45 M ammonium acetate in methanol, respectively. The obtained acidic sphingolipid fraction was acetylated by adding pyridine/acetic anhydride (2:3, v/v) and reaction at 20°C for 18 h. The acetylated acidic sphingolipid fraction was applied to a Florisil column (magnesium silicate; Nacalai Tesque) (column size 1.8 cm \(\times\) 100 cm) to remove acylphospholipids, and was eluted with 3 vol. of 1,2-dichloroethane/methanol (3:1, v/v) to obtain an acetylated acidic GSL fraction. This acetylated acidic GSL fraction was de-acetylated by reaction in 0.5 M potassium hydroxide in methanol at 37°C for 12 h.

#### 2.2 Isolation of SGL-1 from C. intestinalis

The acidic GSL fraction obtained by two chromatographies was applied to a column of Iatrobeads (silica gel 6RS-8060; Mitsubishi Kagaku Iatron, Inc., Tokyo; column size 1.5 cm \(\times\) 60 cm) by a two-step linear gradient method from chloroform/methanol/water (80:20:1, v/v; 120 ml) to chloroform/methanol/water (50:50:5, v/v; 150 ml). This fraction was collected into test tubes by the above column, checked by thin-layer chromatography (TLC), and each molecular weight was measured by direct application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

#### 2.3 TLC

The elution was monitored by TLC plates of silica gel 60 (Merck, Germany) and a solvent system of chloroform/methanol/water (60:40:10, v/v/v). The GSLs were visualized by spraying with orcinol/\(\text{H}_2\text{SO}_4\) for sugars, 5% \(\text{H}_2\text{SO}_4\)/ethanol reagent for organic substances and ninhydrin reagent for free amino groups and by heating. Detection was performed with Dittmer-Lester reagent for phosphorus, azure-A reagent for sulfate and resorcinol reagent for sialic acid.

#### 2.4 GLC and GLC-MS

GLC was performed on a GC-18A instrument (Shimadzu, Japan) equipped with a chemically bonded capillary column of Hi Cap-CBP5 (column size 0.22 mm \(\times\) 25 m). GLC-MS was performed on a GLC-MS instrument (Shimadzu, Japan) equipped with the same column as above.

#### 2.5 Sugar and ceramide components

To determine the components of fatty acids and sugars in SGL-1, the purified SGL-1 (100 \(\mu\)L) was methanolysed in thick glass test tubes (16 mm \(\times\) 125 mm with Teflon-lined screw caps; Pyrex, Iwaki Glass Co., Japan) with 200 \(\mu\)L of 1 M anhydrous methanolic \(\text{HCl}\), using a microwave oven method (35), and cooled at room temperature. The fatty acid methyl esters products were extracted three times with 0.5 ml n-hexane, and then analyzed using a GLC programmed at 4°C/min from 170°C to 240°C. The methanolic phase remaining was neutralized with sliver carbonate, and then evaporated to dryness under a nitrogen stream. The residue containing methylglycosides was trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1, v/v/v). The aliquot of the residues was analyzed by GLC at 2°C/min from 140°C to 230°C. Sphingolipids prepared from glycolipids by methanalysis with 1 M aqueous methanolic \(\text{HCl}\) at 70°C for 18 h were converted into O-trimethylsilyl (nitrogen-free) derivatives and then analyzed by GLC with a temperature program of 2°C/min from 210°C to 230°C.

The partially methylated alditol acetates were resolved by GLC programmed at 4°C/min from 140°C to 230°C and GLC-MS programmed at 80°C (2 min) – 160°C (20°C/min) – 240°C (4°C/min).

#### 2.6 \(^{1}H\)-NMR spectroscopy

NMR spectra were obtained using a 400-MHz A-400
spectrometer (JEOL, Ltd., Japan) at 60°C. The samples were dissolved in 0.60 mL of [2H6]DMSO containing 2% 2H2O and 0.05% v/v tetramethylsilane (TMS) (Wako Pure Chemical Industries, Ltd. Japan) in a sampling tube (φ5 mm Kusano Science Co., Japan), and chemical shifts were referenced to the solvent signal, 0.00 ppm in TMS, as the internal standard.

2.7 Matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS analysis was performed using a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer, operating in the negative linear mode. Ionization was performed with a 337-nm pulsed N2 laser. The MS spectra were calibrated externally using a standard peptide calibration mixture containing 1 mg/mL each of bradykinin, fragment 1-5 (Arg-Pro-Pro-Gly-Phe, 573.31 Mass units; Sigma Chemical Co.) and angiotensin I (1296.68 Mass Units; Wako Pure Chem. Industries, Ltd. Japan). Coumarin 120 (7-amino-4-methyl-coumarin; Sigma Chemical Co.) was used as the matrix. The GSLs were prepared as 1mg/mL solution in chloroform/methanol (2:1, v/v). They were separately put on a plate for MALDI-TOF MS in the order of SGL (2 µL) and matrix (0.5 µL) solutions and were crystallized by natural drying. The target plate was then introduced into MALDI-TOF MS, and the analysis of MALDI-TOF MS was conducted on target GSLs.

2.8 De-sulfuration of sulfatide

The de-sulfuration of sulfatides was performed as follows: To one mg sulfatide was added 0.5 mL dioxane, and this was reacted at 100°C for 20 min. The reactants were dissolved in 1 mL chloroform/methanol (2:1, v/v), added to 0.2 mL water and stirred using a vortex.

3 RESULTS

3.1 DEAE-sephadex A-25 column chromatography

The obtained dry extracts (4.4 g) were divided into one non-adsorption fraction and three adsorption fractions by DEAE-Sephadex A-25 column chromatography. The amounts of the three adsorption fractions were 248.9 mg, 1032 mg and 47.5 mg in fractions eluted with 0.05 M, 0.15 M and 0.45 M ammonium acetate in methanol, respectively.

The positive reaction with orcinol/H2SO4 and azure-A reagents was only shown in the adsorption fraction eluted with 0.15 M ammonium acetate in methanol. This fraction showed a negative reaction to other chemical reagents, Dittmer-Lester, ninhydrin and resorcinol reagents. The other adsorption fractions showed a negative reaction to all chemical reagents (Fig. 1). Therefore, we estimated that only sulfatides were included as acidic GSL in C. intestinalis, and we next purified a sulfatide-containing acidic GSL fraction, adsorption fraction eluted with 0.15 M ammonium acetate, in methanol using DEAE-Sephadex A-25 column chromatography.

3.2 Florisil column chromatography

The sulfatide-containing acidic GSL fraction, 103.2 mg, was acetylated. This acetylated fraction next purified by Florisil column chromatography and was divided into four fractions (Fig. 2). The amounts of the obtained fractions were 1.3 mg in 1,2-dichloroethane(DCE)/acetone (1:1, v/v), 14.5 mg in DCE/acetone (3:1, v/v), 55.0 mg in DCE/methanol/water (2:8:1, v/v/v) and 5.2 mg in chloroform/methanol/water (6:4:1, v/v/v). Each fraction was deacetylated and was returned to an original state.

Bands of the same Rf value, which showed positive reactions to both orcinol/H2SO4 and azure-A reagents, were detected in the DCE/acetone (1:1, v/v and DCE/acetone (3:1, v/v) elution fractions (Fig. 2, Lanes 1 and 2). Therefore, the mixture of these two fractions, a sulfatide-containing acidic GSL fraction, was then purified. On the other hand, in the DCE/acetone (1:1, v/v) elution fraction, a positive-reaction band was detected by only azure-A reagent, and this was deduced to be sulfo-lipid except sulfatides (Fig. 2, Lane 3). As GSLs may be not included in this fraction from TLC analysis results, this fraction will be reported in another article.

3.3 Iatrobeads column chromatography

The sulfatide, called SGL-1, (4.4 mg), was purified from

**Fig. 1** TLC after DEAE Sephadex A-25 Column Chromatography. TLC was performed using chloroform/methanol/water (6:4:1, v/v/v) and visualized by orcinol/H2SO4 (A) and azure-A (B) staining. Lane T, total lipid extract fraction; Lanes 1, 2 and 3, fractions eluted with 0.05 M, 0.15 M and 0.45 M ammonium acetate in methanol by DEAE Sephadex A-25 column chromatography, respectively.
15.8 mg of the sulfatide-containing acidic GSL fraction by Iatrobeads column chromatography (JH). TLC analysis of SGL-1 showed positive reactions with orcinol/H2SO4 and azure-A reagents (JH, Lane 2), and negative reactions with Dittmer-Lester, ninhydrin and resorcinol reagents (data not shown).

SGL-1 was characterized by molecular masses of 841.2, 855.2, 869.2, 883.2, 897.2 and 911.3 in negative ion linear mode using MALDI-TOF MS (JH).

To determine the sugar component of SGL-1, compositional analysis of SGL-1 was conducted by methanolysis using GLC. The composition of SGL-1 was determined to be Glc.

To determine the sugar linkage and sulfated site, SGL-1 was prepared by partially methylated alditol acetates for analysis of the substitution sites by GLC. Methylation analysis of SGL-1 showed the presence of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol (1, 6 Glc). The characteristic fragmentation ions of 1,6Glc, [M-117]=233m/z, [M-161]=189 m/z and [M-189]=161 m/z, were detected by GLC-MS analysis.

To determine the anomeric configuration, SGL-1 was subjected to 1H-NMR spectroscopy. In the anomeric region of the spectrum for SGL-1, anomeric proton resonances were observed at 4.15 ppm (J1,2 7.7 Hz). This result suggests that the chemical structure of SGL-1 was HSO3-6Glcβ1-Cer, GlcCer I6-sulfate.

### 3.6 Ceramide analysis
Characterization of the ceramide moiety was performed by GLC-MS analysis of fatty acids and sphingoids. However, for sphingoid analysis, methanolysis of SGL-1 afforded only small amounts of sphingoids, most likely owing to the attachment of SGL-1, which may interfere with acidic cleavage. When SGL-1 was de-sulfated by solvolysis, five sphingoids, t16:0, t17:0, brt17:0, t18:0 and brt18:0, were detected and analyzed by GLC-MS. The main fatty acids of SGL-1 were 22h:0, 20h:0 and 16:0 (Tables 1 and 2).

### 4 DISCUSSION
The mono-sulfated cerebrosides were reported to be GalCer I3-sulfate (SM4s) and GlcCer I3-sulfate (SM4s-Glc)(12,13).

Fig. 2 TLC after Florisil Column Chromatography. TLC was performed in the same way as above. Lane T, fraction eluted with 0.15 M ammonium acetate in methanol by DEAE Sephadex A-25 column chromatography; Lane 1, fraction eluted with DCE/acetone (1:1, v/v); Lane 2, fraction eluted with DCE/acetone (3:1, v/v); Lane 3, fraction eluted with DCE/methanol/water (2:8:1, v/v/v); Lane 4, fraction eluted with chloroform/methanol/water (6:4:1, v/v/v).

Fig. 3 TLC of the Purified SGL-1 from C. intestinalis. TLC was performed in the same way as above. Lane 1, mixed fraction (DCE/acetone (1:1, v/v) and DCE/acetone (3:1, v/v) elution fractions); Lane 2, purified SGL-1 fraction.

Fig. 4 MALDI-TOF MS Analyses of C. intestinalis SGL-1. SGL-1 obtained after Iatrobeads column separation were monitored as [M-H]- by MALDI-TOF MS with Coumarin 120 as a matrix in the negative-ion mode.
In this study, ceramic acid analysis of SGL-1 was performed to de-sulfate SGL-1 because methanolation of SGL-1 afforded only small amounts of sphingoids. We thought that the sulfate ester with a formal negative charge on its carbohydrate residue would disturb cutting between the carbohydrate chain and ceramide; furthermore, the binding power of the sulfated position on the Glc residue might be different. Therefore, the peak area of Glc from Glc 6-sulfate, Glc 3-sulfate and Glc was analyzed using GLC, and it was found at a ratio of 0.45:0.92:1.00. Similarly, the peak area of Glc from SGL-1 and GlcCer was analyzed. The peak area of Glc from SGL-1 showed a much lower value than that from GlcCer. (SM4s-Glc sugar was not analyzed because it is not commercially available.) This result suggests that our inference above will be correct.

On the other hand, a specific sulfotransferase must participate to transform from GlcCer to GlcCer 16-sulfate. CST is specifically the only sulfotransferase for GSL as far as we know. GalCer was the best acceptor for CST. LacCer, GalAAG, and GalDG were also good acceptors. GlcCer, Gg3Cer, Gg4Cer, Glb4Cer, and nLc4Cer did serve as acceptors although the relative activities were low.

To find sulfotransferase of SGL-1 from C. intestinalis genome, "sulfotransferase" was used for a keyword search in the GTOP database (http://spock.genes.nig.ac.jp/~genome/gtop.html). As a result of the database search, 56 genes annotated as sulfotransferase were found, and six homologous genes of CST (DDBJ/GenBank/EBI accession number: D88667) using a blastp search in the C. intestinalis genome sequence and were included in 56 genes annotated as sulfotransferase. In addition, 56 genes were analyzed a sequence alignment and a phylogenetic tree using clustalW and were classified into six groups including one CTS homologous gene group. Next, all 56 genes were searched using the Ghost database to examine the expression patterns of mRNA (data not shown).

Glycosphingolipids have been characterized in a range of animal phyla including arthropods, and have been shown to participate in important functions such as cellular development. Comparison of protostome and deuterostome glycolipids shows dramatic structural differences even between species with closely related glycolipid expression. In particular, the structures of acidic glycolipids are unique. Gangliosides and sulfatides have been widely distributed within echinoderms and deuterostomes. For example, although gangliosides and sulfatides have not been detected in protostomia, other acidic glycosphingolipids containing uronic acid or inositol phosphate have been characterized. In the post-genome era, one of the important targets is to understand molecular function and to discover the roles that are common to all multicellular
organisms in GSLs. The chemical structures of GSL are various and variable, and it has been pointed out that alteration of the distribution of glycosyltransferase affects not only GSLs that are directly biosynthesized, but also broadly the distributions of GSLs. On the other hand, a large number of genomic-related data have recently been accumulated, including genome sequences, EST sequences, DNA chip data, mRNA in situ hybridizations, expression patterns of proteins and protein-protein interactions. In the glycoscience field, genetic research is becoming mainstream. However, the substrate specificity of a glycosyltransferase cannot be identified if actual glycan structures are not analyzed. Therefore, direct chemical structural analysis of GSLs is essential. A combination approach to chemical structures and reverse genetic research from genome-related information might be a shortcut to understanding the molecular function of GSLs and to discovering the roles of GSLs that are common to all multicellular organisms.

ACKNOWLEDGMENTS

We are grateful to Dr. Takahito Nishikata of Konan University (Faculty of Science and Engineering) for his critical comments. We are also grateful to Mr. Zenzo Imoto of Kochi University (Usa Marine Biological Institute) for the supply of C. intestinalis. This research was supported in part by a Grant-in-Aid for Young Scientists B and a High-Tech Research Center Project for Private Universities matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M.I.

References


Fig. 5 Phylogenetic Tree of 56 Predicted Homologous Genes of CST. The phylogenetic tree was visualized by the unrooted N-J method. Each gene ID was used with an accession number of JGI Ciona V1.0. The bold letters indicate the CST homologous gene.

