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Fucosyl-GM1a, an Endoglycoceramidase-resistant Ganglioside of Porcine Brain

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The use of bovine brain has been prohibited in many countries because of the world-wide prevalence of mad cow disease, and thus porcine brain is expected to be a new source for the preparation of gangliosides. Here, we report the presence of a ganglioside in porcine brain which is strongly resistant to hydrolysis by endoglycoceramidase, an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. Five major gangliosides (designated PBG-1, 2, 3, 4, 5) were extracted from porcine brain by Folch's partition, followed by mild alkaline hydrolysis and PBA column chromatography. We found that PBG-2, but not the others, was strongly resistant to hydrolysis by the enzyme. After the purification of PBG-2 with Q-Sepharose, Silica gel 60 and Prosep-PB chromatographies, the structure of PBG-2 was determined by GC, GC-MS, FAB-MS and NMR spectroscopy as Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer (fucosyl-GM1a). The ceramide was mainly composed of C18:0 and C20:0 fatty acids and d18:1 and d20:1 sphingoid bases. The apparent kcat/Km for fucosyl-GM1a was found to be 30 times lower than that for GM1a, indicating that terminal fucosylation makes GM1a resistant to hydrolysis by the enzyme. This report indicates the usefulness of endoglycoceramidase to prepare fucosyl-GM1a from porcine brain.

Key words: endoglycoceramidase, fucosyl-GM1a, ganglioside, glycosphingolipid, porcine brain.

Abbreviations: BBG, bovine brain ganglioside; CBB, coomassie brilliant blue; EGCase, endoglycoceramidase; FAB-MS, fast-atom bombardment mass spectrometry; Fuc-GM1a, fucosyl-GM1a; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; GSL, glycosphingolipid; NMR, nuclear magnetic resonance; PBG, porcine brain ganglioside; rEGCase II, recombinant endoglycoceramidase II; TLC, thin-layer chromatography. Nomenclature system of gangliosides is followed by that of Svennerhorm (*24*).

INTRODUCTION

Glycosphingolipids (GSLs), amphipathic compounds composed of sugar and lipid (ceramide) moieties, are characteristic components of the plasma membranes of vertebrates. The heterogeneity of the sugar moiety has led to the generation of more than 300 different species of GSLs. The ceramide portion of the GSL molecule is embedded in the fluid phase of the plasma membrane, and a sugar chain faces the external environment, possibly causing some GSLs to be receptors for microbes and their toxins as well as modulators of cell–cell interactions (*1*). Notably, gangliosides, GSLs containing one or more sialic acids in a molecule, were considered to function in the nervous system (*2*).

Endoglycoceramidase (EGCase; EC 3.2.1.123) is a GSL-specific enzyme that catalyzes the hydrolysis of the linkage between oligosaccharides and ceramides of various GSLs (*3*) and is known to be useful in the structural analysis of both neutral and acidic GSLs (*4, 5*). EGCase is thought to hydrolyze all GSLs except for cerebrosides and sulfatides. Three isoforms of EGCase (EGCase I, II, III), each with a different molecular weight, pI and substrate specificity, were found in various sources (*6*). EGCases I and II were capable of hydrolyzing the GlcCer linkage of ganglio-, lacto-, and globo-type GSLs and that of ganglio-, and lacto-type GSLs, respectively. In contrast, EGCase III specifically hydrolyzes the GalCer linkage of gala- and neogala-type GSLs, which are completely resistant to hydrolysis by EGCases I and II (*6*).

Although gangliosides are usually prepared from bovine brain, a new source is required because of the world-wide prevalence of mad cow disease. During the course of isolating gangliosides from porcine brain

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instead of bovine brain, we found a ganglioside (PBG-2) completely resistant to hydrolysis by EGCase II. We determined the structure of PBG-2: Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer (fucosyl-GM1a; Fuc-GM1a). The ceramide portion of PBG-2 was almost the same as bovine brain GM1a, i.e. composed of C18:0 and C20:0 fatty acids and d18:1 and d20:1 sphingoid bases. The apparent kcat/Km for Fuc-GM1a was found to be 30 times lower than that for GM1a, indicating that terminal fucosylation made GM1a resistant to hydrolysis by the enzyme. Based on this observation, we developed a new method to isolate Fuc-GM1a from porcine brain with high yield.

MATERIALS AND METHODS

Materials——GM1a from bovine brain was purchased form Wako Chemical (Osaka, Japan). Fuc-GM1a was prepared from bovine thyroid (*7*). Recombinant EGCase II (rEGCase II), expressed in *Rhodococcus erythropolis* or *Escherichia coli*, was purified according to the method described in (*8*) or purchased from Takara Bio Inc. (Otsu, Shiga, Japan). Standard Fuc, Gal, Glc, GalNAc, and NeuAc for gas chromatography (GC) were purchased from Sigma (MO, USA). Phenyl Boronate Agarose (PBA-60) and Prosep-PB were obtained from Amicon (KY, USA) and Millipore (MA, USA), respectively. Q-Sepharose gel and Silica gel 60 were purchased from Amersham Bioscience (NJ, USA), and Merck (Darmstadt, Germany). Pre-coated Silica gel 60 TLC plates were purchased from Merck. Sep-Pak Plus Silica was obtained from Waters (CT, USA). All other reagents were of the highest quality available.

Preparation of porcine brain gangliosides (PBGs)——Crude gangliosides were prepared from porcine brain by the same method used for the isolation of gangliosides from bovine brain (*9*) but with some modifications. The porcine brains (about 2 kg, wet weight) were homogenized with acetone, left overnight at room temperature, and then filtered with filter paper. The acetone powder was subjected to Folch's partition with chloroform/methanol/water (8/4/3, v/v/v) and then the upper phase was evaporated to dry. The residue was dissolved in chloroform/methanol (2/1, v/v) and then subjected to mild alkaline hydrolysis. After neutralization with 0.2 M acetic acid, the GSL fraction was applied to a PBA-60 column which was equilibrated with chloroform/methanol (8/2, v/v) and then eluted with the same solvent. PBGs were eluted with chloroform/methanol/H_2O (5/5/1, v/v/v) and used for subsequent experiments.

Thin-layer chromatography (TLC)——GSLs were applied to glass TLC plates pre-coated with Silica gel 60, which were developed with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v/v) (solvent I) (*10*). GSLs on the TLC plates were visualized by spraying with orcinol–H_2SO_4, resorcinol, or Coomassie Brilliant Blue (CBB) reagent (*11*).

Assays for EGCase II activity and protein——The activity of EGCase II was examined using GM1a as the substrate as described in (*6, 12*). Briefly, the reaction mixture contained 10 nmol of substrate and an appropriate amount of enzyme in 20 μl of 50 mM acetate buffer, pH 5.5, containing 0.2% Triton X-100. Following incubation at 37°C for predetermined periods, the reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was evaporated with a Speed Vac concentrator (Savant Instruments, Inc., USA), dissolved in 10 μl of 50% methanol, and applied to TLC plates which were then developed with chloroform/methanol/0.02% $CaCl_2$ (2/3/1, v/v/v) (solvent II). GSLs and oligosaccharides were visualized by spraying the TLC plates with orcinol-H_2SO_4 reagent and scanning them with a Shimadzu CS-9300 chromatoscanner (Shimadzu, Kyoto, Japan) with the reflectance mode set at 540 nm. The extent of hydrolysis was calculated as follows: hydrolysis (%) = (DR for oligosaccharide released) × 100/(DR for remaining substrate + DR for oligosaccharide released); where DR = densitometric response at 540 nm. One unit (U) of the EGCase was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of GM1a/min under the conditions described above. A value of 10^{-3} U of enzyme was expressed as 1 milliunit (mU). Protein content was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard.

Hydrolysis of PBG with EGCase II——Twenty nanomoles of PBG was treated with rEGCase II in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.2% Triton X-100. Following incubation at 37°C for predetermined periods, the reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was evaporated with a Speed Vac concentrator, dissolved in 10 μl of chloroform/methanol (2/1, v/v), and applied to TLC plates which were then developed with solvent II.

Purification of EGCase II-resistant PBG-2——Crude PBGs (2.5 g) were incubated at 37°C for 20 h with 3.7 U of EGCase II. The reaction mixture was dried with a Rotatory Evaporator, dissolved in 25 ml of chloroform/methanol/water (30/60/8, v/v/v) and then loaded on a Q-Sepharose column (volume, 24 ml; 1 cm × 30 cm) which was equilibrated with the same solvent. After being washed with 120 ml of the same solvent, PBGs were eluted from the column using a linear gradient of 240 ml each of the same solvent and the same solvent containing 4 M sodium acetate (*13*). The eluate containing PBGs was then applied to a Silica gel 60 column (2.4 cm × 18 cm) equilibrated with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v/v). PBGs were eluted with the same solvent. Desalting was achieved with a Prosep-PB column (volume 1 ml) which was equilibrated with chloroform/methanol (8/2, v/v). After being washed with 10 ml of the same solvent, PBG-2 was eluted from the column with chloroform/methanol/H_2O (5/5/1, v/v/v). An aliquot of each fraction was applied to a TLC plate which was developed with solvent I. The TLC plate was stained with orcinol-H_2SO_4 reagent and CBB reagent.

Analyses of fatty acid, sphingoid base and sugar composition by GC——The composition of fatty acids, sphingoid bases and sugars of PBG-2 was determined by the method described in (*14, 15*). Briefly, fatty acid methyl esters and methyl glycosides were obtained by methanolysis of PBG-2 with 1.0 M anhydrous methanolic

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HCl at 80°C for 16–22 h. Fatty acid methyl esters were extracted with hexane, and the methyl glycosides and sphingoid bases remaining in the methanolic phase were concentrated, *N*-acetylated, and trimethylsilylated. The composition of fatty acids, sphingoid bases, and sugars was then determined separately by GC on a Shimadzu GC-14A equipped with a fused silica capillary column (CBP-1, 0.25 mm × 30 m, J. & W. Scientific, USA).

Sugar linkage analysis——Sugar linkages of PBG-2 were determined by the method described in (*16*). Briefly, 15-30 nmol of PBG-2 was partially methylated with NaOH in DMSO and CH_3I. The permethylated PBG-2 was subjected to hydrolysis with 2 M trifluoroacetic acid, before undergoing reduction with $NaBD_4$. The products were acetylated with a mixture of acetic anhydride-pyridine (1:1, v/v) at 121°C for 3 h. The partially methylated alditol acetates thus obtained were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GCMS-QP 5000 spectrometer.

Fast atom bombardment mass spectrometry (FAB-MS)——Approximately 10 nmol of PBG-2 was mixed with triethylene glycol and hexamethylphosphoric triamide as a matrix and then subjected to FAB-MS using a Jeol SX102A mass spectrometer in the negative ion mode (*17*).

Nuclear magnetic resonance (NMR) spectroscopy——^1H NMR spectra of PBG-2 (3 mg) in DMSO-d_6 were obtained on a Varian Unity-500 spectrometer (*18*). The frequency was 500 MHz, and the spectral width was 7.5 kHz. The operation was performed at 30°C in DMSO.

Hydrolysis of GM1a and PBG-2 with sialidase ——Ten nanomoles of GM1a and PBG-2 were treated with *Arthrobacter ureafaciens* sialidase in 20 μl of 100 mM sodium acetate buffer, pH 5.5, containing 3 mM sodium cholate. Following incubation at 37°C for predetermined periods, the reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was evaporated with a Speed Vac concentrator, dissolved in 10 μl of chloroform/methanol (2/1, v/v), and applied to TLC plates which were then developed with solvent I (*19*).

RESULTS

Isolation and EGCase treatment of PBGs——Five major GSLs were isolated from porcine brain and tentatively designated PBG-1, 2, 3, 4 and 5 (Fig. 1A, lane 1). These five GSLs were stained with orcinol-H_2SO_4 (Fig. 1A, lane 1) as well as resorcinol–HCl reagents (Fig. 1B). All the PBGs except PBG-2 were efficiently hydrolyzed by a recombinant EGCase II (rEGCase II), while surprisingly, PBG-2 seems to be completely resistant to hydrolysis by the enzyme under the conditions used (Fig. 1A, lane 2). EGCase II is known to hydrolyze the β1,1-glucosidic linkage between oligosaccharides and ceramides of various GSLs (*6*). Actually, all bovine brain gangliosides (BBGs) (Fig. 1A, lane 3; GM1a, GD1a, GD1b and GT1b from the top) were hydrolyzed by the enzyme to produce oligosaccharides under the same conditions (Fig. 1A, lane 4). Fig. 2A shows the time course for hydrolysis of PBG-1, 2, 3, 4 and 5 by rEGCase II. Under these conditions, PBG-1 (possibly GM1a) was hydrolyzed most rapidly followed by PBG-3 (possibly GD1a), PBG-5 (possibly GT1b), and PBG-4 (possibly GD1b) in this order. It should be emphasized that PBG-2 was strongly resistant to the enzyme with less than 5% hydrolyzed even after a prolonged incubation (Fig. 2A) or with an increasing amount of the enzyme (Fig. 2B).

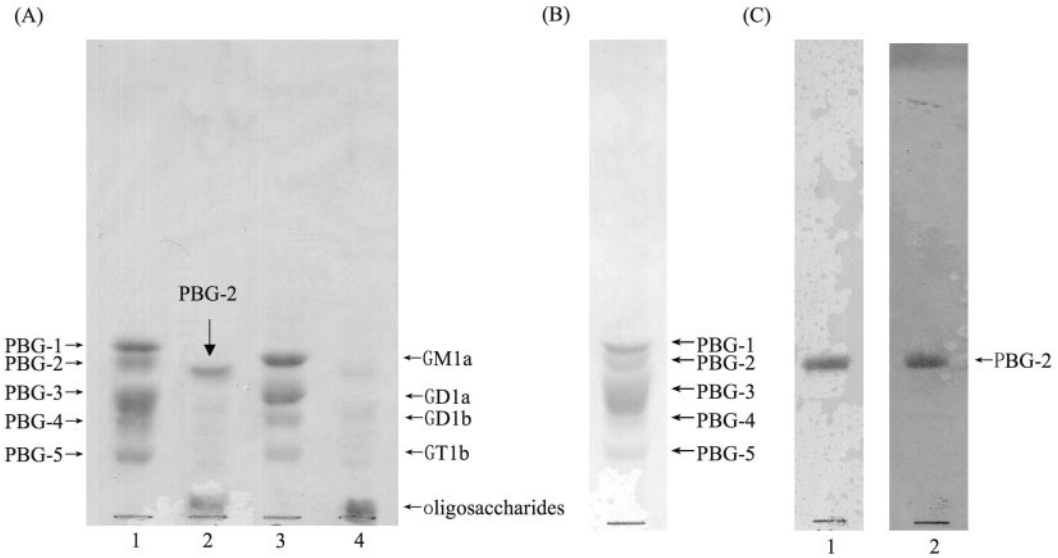

Fig. 1. **TLC showing the PBGs and BBGs before and after treatment with rEGCase II and an EGCase-resistant PBG-2.** (A) TLC showing total PBG (lane 1), PBGs digested with rEGCase II (lane 2), total BBG (lane 3), and BBGs digested with rEGCase II (lane 4) after staining with orcinol–H_2SO_4 reagent. Ten micrograms of either total PBG or total BBG was treated with 14 mU of rEGCase II for 20 h. The products of the digestion were applied to a TLC plate which was developed with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v/v). (B) TLC showing total PBG after staining with resorcinol reagent. (C) TLC showing the purified PBG-2 after staining with orcinol-H_2SO_4 reagent (lane 1) and CBB (lane 2). In (B), (C), TLC was developed with solvent I. Details are described in Materials And Methods.

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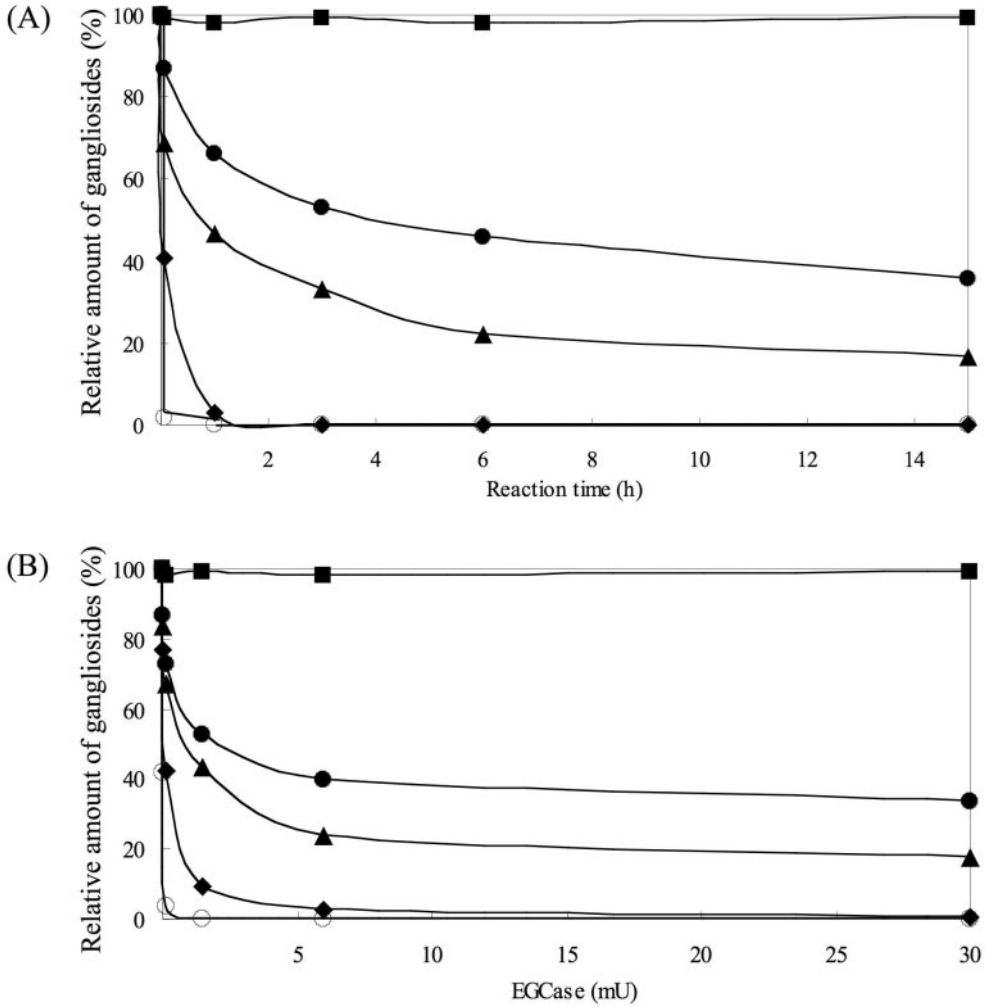

Fig. 2. **Hydrolysis of PBGs by EGCase II.** (A) Time-dependent hydrolysis of total PBG by EGCase II. Twenty micrograms of total PBG were incubated with 30 mU of EGCase II for the periods indicated. (B) EGCase concentration-dependent hydrolysis of total PBG. Twenty micrograms of total PBG were incubated with increasing amounts of EGCase II for 16 h. After treatment with the enzyme, digestion products were analysed by TLC as described in Materials And Methods. ○, PBG-1 (possibly GM1a); ■, PBG-2; ◆, PBG-3 (possibly GD1a); ●, PBG-4 (possibly GD1b); ▲, PBG-5 (possibly GT1b).

Structural analyses of PBG-2——The purified PBG-2, which gave an apparent single band when stained with orcinol–H_2SO_4 (Fig. 1C, lane 1) and CBB (Fig. 1C, lane 2), was subjected to GC after methanolysis. As shown in Table 1, the carbohydrate moiety of PBG-2 was composed of glucose, galactose, fucose, *N*-acetylgalactosamine and *N*-acetylneuraminic acid, the molar ratio of which was approximately 1:2:1:1:1. Methylation analysis of PBG-2 showed the presence of 2-substituted hexopyranose, 4-substituted hexopyranose, 3,4-substituted hexopyranose, 3-substituted *N*-acetyl-hexopyranosamine, unsubstituted deoxy-hexopyranose, and unsubstituted neuraminic acid (data not shown). GC analysis also showed that the ceramide of PBG-2 was composed of C18:0 and C20:0 fatty acids, the percentage of which was 89.8% and 10.2%, respectively (Table 1). PBG-2 was also composed of d18:1 and d20:1 sphingoid bases, the percentage of which was 63.4% and 36.6%, respectively (Table 1).

The parental molecular ions, $[M-H]^-$, of PBG-2 were observed on FAB-MS in the negative ion mode at *m/z* 1690 and 1718, which corresponded to Fuc-GM1a containing a ceramide composed of d18:1 sphingosine and C18:0 fatty acid, and that containing a ceramide composed of d18:1 shingosine and C20:0 fatty acid or d20:1 sphingosine and C18:0 fatty acid, respectively (Fig. 3). Concomitantly, fragment ions, $[M-H]^-$, were generated from PBG-2 containing d18:1 sphingosine and C18:0 fatty acid at *m/z* 1544, 1382, 1091, 888, 726 and 564 which corresponded to GM1a (Fuc-GM1a – deoxyhexose), GM2 (GM1a – hexose), asialo-GM2 (GM2 – *N*-acetylneuraminic acid), LacCer (asialo-GM2 – *N*-acetylhexosamine), GlcCer (LacCer – hexose) and ceramide (GlcCer – hexose). In the same manner, fragment ions, $[M-H]^-$, at *m/z* 1572, 1410, 1119, 916, 754 and 592 were generated from PBG-2 containing d20:1 sphingosine and C18:0 fatty acid or d18:1 sphingosine and C20:0 fatty acid.

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Table 1. **Chemical composition of the purified PBG-2.**

Component	Composition
Monosaccharide	Molar ratio
Fuc	1.18
Gal	2.02
Glc	1.00
GalNAc	1.44
NeuAc	1.06
Fatty acid	(%)
18:0	89.8
20:0	10.2
Sphingoid base	(%)
d18:1	63.4
d20:1	36.6

The composition of sugar, fatty acid and sphingoid base was analysed by GC as described in Materials And Methods.

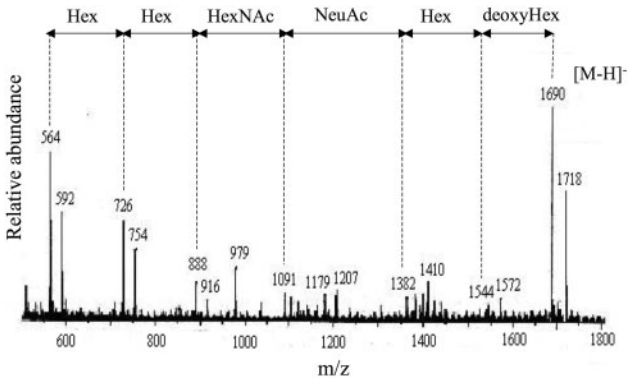

Fig. 3. **FAB-MS spectra of the purified PBG-2.** FAB-MS was performed in the negative ion mode using triethylene glycol and hexamethylphosphoric triamide as the matrix as described in Materials And Methods**.**

The ^{1}H NMR spectrum of the PBG-2 clearly exhibited the presence of four β-anomeric signals at 4.42 ppm ($J = 7.8$ Hz), 4.70 ppm ($J = 8.2$ Hz), 4.23 ppm ($J = 8.2$ Hz) and 4.14 ppm ($J = 7.0$ Hz), and one α-anomeric signal at 5.14 ppm ($J = 3.7$ Hz). These chemical shifts and J values of PBG-2 virtually corresponded to those of authentic Fuc-GM1a (*18*) (Table 2).

Taken together, PBG-2 was identified as Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer (Fuc-GM1a). The ceramide portion was composed of C18:0 and C20:0 fatty acids and d18:1 and d20:1 sphingoid bases. In our knowledge, this is the first report demonstrating the presence of Fuc-GM1a in the porcine brain.

Kinetics of EGCase II for PBG-2 and GM1a——Fig. 4A shows the time course for hydrolysis of PBG-2, bovine thyroid Fuc-GM1a, and bovine brain GM1a by rEGCase II. It should be noted that GM1a was hydrolyzed rapidly by rEGCase II with a plateau reached within 30 min at which more than 70% of the substrate was hydrolyzed, while PBG-2 and authentic Fuc-GM1a were hydrolyzed very slowly with less than 20% hydrolysis even after 180 min (Fig. 4A). Fig. 4B shows Lineweaver–Burk plots for the relationship between the initial reaction velocity of rEGCase II and concentrations of two substrates (GM1a and PBG-2). The apparent Km and Vmax values for GM1a were calculated from the Lineweaver–Burk plots as 2.01 mM and 4.63 U/mg, and those for PBG-2, as 8.55 mM and 0.67 U/mg, respectively. The kcat and kcat/Km of the enzyme for GM1a were calculated as 3.9 S^{-1} and 1.9 $mM^{-1}S^{-1}$, and those for PBG-2, as 0.56 S^{-1} and 0.065 $mM^{-1}S^{-1}$, respectively,

Table 2. **^{1}H chemical shifts and coupling constants of PBG-2.**

Sugar component	Chemical shift, δ (ppm)		J value (Hz)	
	PBG-2	Fuc-GM1[a]	PBG-2	Fuc-GM1[a]
Terminal Fuc(p)	5.14	5.11	3.7	3.5
2-linked Gal(p)	4.42	4.48	7.8	7.9
3-linked Gal(p)NAc	4.70	4.72	8.2	8.0
3,4-linked Gal(p)	4.23	4.27	8.2	7.8
4-linked Glc(p)	4.14	4.20	7.0	7.8

[a]The values of Fuc-GM1a were referred from the literature (*18*). p represents pyranose.

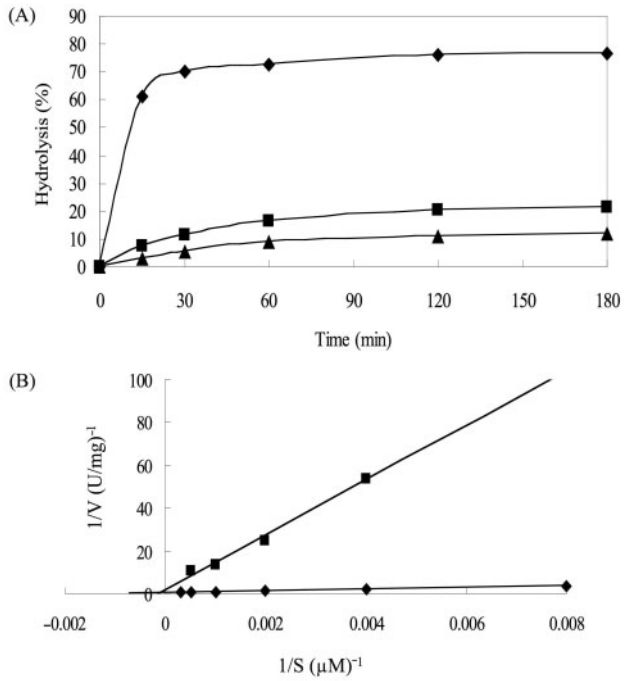

Fig. 4. **Kinetics of EGCase II for PBG-2 and GM1a.** (A) Time course for the hydrolysis of PBG-2, bovine thyroid Fuc-GM1a and bovine brain GM1a by rEGCase II. Each of the gangliosides (10 μg) was incubated with 0.4 mU of rEGCase II in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.2% Triton X-100. After incubation at 37°C for the periods indicated, the hydrolysis of substrates was analysed by TLC as described under Materials And Methods. ◆, authentic GM1a; ■, authentic Fuc-GM1a; ◆, PBG-2. (B) Lineweaver–Burk plots for the action of rEGCase II on PBG-2 and GM1a. The hydrolysis of increasing amounts of substrate was determined using 0.01 mU and 0.4 mU of rEGCase II for GM1a and PBG-2, respectively. After incubation at 37°C for 60 min, the extent of the hydrolysis of the substrates was analysed by TLC as described in Materials And Methods. ◆, GM1a; ■, PBG-2**.**

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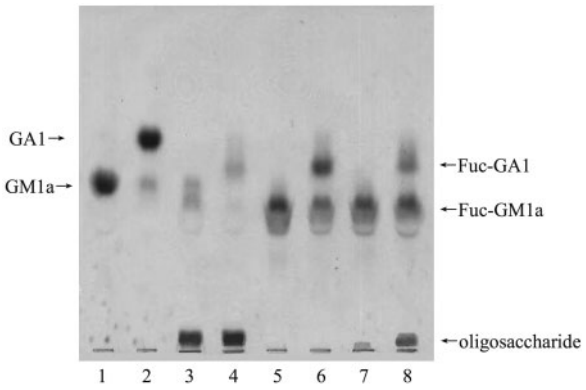

Fig. 5. **Effects of fucosyl and sialyl residues on hydrolysis of GSLs by rEGCase II and sialidase.** GM1a (lanes 1–4) and PBG-2 (lanes 5–8) were hydrolyzed by rEGCase II, sialidase or both. Lane 1, GM1a without enzyme; lane 2, GM1a with sialidase; lane 3, GM1a with rEGCase II, lane 4, GM1a with rEGCase II + sialidase; lane 5, PBG-2 without enzyme; lane 6, PBG-2 with sialidase; lane 7, PBG-2 with rEGCase II, lane 8, PBG-2 with rEGCase II + sialidase. GSLs were treated with 10 mU of *Arthrobacter* sialidase, 0.4 mU of rEGCase II, or 10 mU of *Arthrobacter* sialidase + 0.4 mU of rEGCase II at 37°C for 14 h. The extent to which the substrates were hydrolyzed was analysed by TLC as described in Materials And Methods.

indicating the kcat/Km value for PBG-2 was 30 times lower than that for GM1a. These results clearly indicated that PBG-2 was much more resistant to hydrolysis by EGCase II than was GM1a.

Effects of fucosyl and sialyl residues on hydrolysis of a GSL by EGCase II and sialidase——To determine whether the fucosylation of GM1a affected the susceptibility of the substrate to not only EGCase II but also sialidase, GM1a and PBG-2 were treated with *Arthrobacter ureafaciens* sialidase. As shown in Fig. 5, the hydrolysis of GM1a by the sialidase occurred much faster than that of Fuc-GM1a (lane 1, 2 *vs*. lane 5, 6), indicating that the terminal fucosylation of GM1a affects the susceptibility of the substrate to the sialidase. Interestingly, the removal of sialic acid from Fuc-GM1a caused the substrate to be hydrolyzed much faster by rEGCase II (lane 7 *vs*. lane 8). However, the rate of hydrolysis of GM1a by rEGCase II was almost the same as that of asialo-GM1 under the conditions used suggesting sialic acid *per se* little affected the susceptibility of the substrate to rEGCase II (lane 3 *vs*. lane 4). These results may indicate that the terminal α-fucosyl residue strongly affects the susceptibility of the ganglioside substrate to the enzyme if an internal α-sialic acid residue co-exists in the molecule.

DISCUSSION

The minimum structural requirement for the hydrolysis of GSLs by EGCase II was thought to be LacCer (Galβ1-4Glcβ1-1'Cer). The enzyme cannot hydrolyze GlcCer, GalCer, sulfatides, gala-/neogala-series GSLs, or sphingomyelin (*6*). In this study, we found a EGCase II-resistant GSL (PBG-2) in porcine, but not bovine, brain and identified it as Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer (Fuc-GM1a) by GC, GC-MS, FAB-MS and NMR spectroscopy. It is worth noting that the structure of the ceramide of PBG-2 was almost the same as that of bovine GM1a. However, the kcat/Km of the enzyme for PBG-2 was about 30 times less than that for GM1a (Fig. 4), indicating that the terminal α-fucosylation of GM1a made the substrate resistant to hydrolysis by the enzyme. This tendency was also observed for the hydrolysis of an α-sialic acid residue of the ganglioside by *Arthrobacter* sialidase, i.e. the hydrolysis of Fuc-GM1a by the sialidase was much slower than that of GM1a (Fig. 5). In this context, it is interesting that the terminal fucosylation of neolactotetraosylceramide resulted in a decrease in the susceptibility of the substrate to endo-β-galactosidase (*21*). Furthermore, it was revealed that in Fuc-GM1a, but not GM1a, an inner sialic acid residue could also affect the susceptibility of the substrate to EGCase II, because removal of the sialic acid residue from Fuc-GM1a by *Arthrobacter* sialidase accelerated the hydrolysis of the substrate by rEGCase II although the rate of hydrolysis of GM1a was almost the same as that of asialo-GM1 under the conditions used (Fig. 5). Taken together, it was hypothesized that the co-existence of a terminal fucosyl residue and inner sialic acid residue made it difficult for the substrate to enter the substrate-binding cleft of EGCase II. However, the precise mechanism of enzyme-substrate interaction will not be elucidated until the X-ray crystal structure of EGCase II is completely solved.

Fuc-GM1a was found to aberrantly express on the small cell lung cancer carcinoma (SCLC) and thus thought to be a possible target for the immunotherapy of SCLC (*22*). Vaccination of patients with SCLC with Fuc-GM1a conjugated to keyhole limpet hemocyanin was performed and found to be effective to induce an IgM antibody response against Fuc-GM1a and tumour cells expressing Fuc-GM1a (*23*). Fuc-GM1a, the ceramide of which was mainly composed of C22:0 fatty acid and d18:1 sphingoid base, was previously prepared from bovine thyroids (*7*). However, alternative source for Fuc-GM1a was required to avoid the possible contamination of mad cow disease-derived prion. We found that Fuc-GM1a was quite difficult to prepare from porcine brain because it was present in very small amounts and behaved similarly to GM1a when subjected to various chromatographies. However, we found in this study that Fuc-GM1a remained intact after the exhaustive digestion of crude PBGs with EGCase II while GM1a and other gangliosides were almost completely hydrolyzed, resulting in a drastic increase in the proportion of Fuc-GM1a in the crude ganglioside sample. By using EGCase II treatment and column chromatography as described in Materials And Methods, 27 mg of pure Fuc-GM1a was obtained from 370 g of acetone powders which was prepared from a total of 20 porcine brains (about 2 kg). By employing the method with EGCase II as described in this paper, porcine brain can be used as a source of Fuc-GM1a instead of bovine thyroids. Furthermore, EGCase II could be used to eliminate the contaminating GM1a in a Fuc-GM1a preparation which is prepared from porcine brain by conventional methods. Collectively, the use of

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EGCase II is effective for preparation of Fuc-GM1a from porcine brain as well as elimination of ganglioides such as GM1a contaminating the Fuc-GM1a preparation.

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