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Preparation of Fluorescence-Labeled GM1 and Sphingomyelin by the Reverse Hydrolysis Reaction of Sphingolipid Ceramide *N*-Deacylase as Substrates for Assay of Sphingolipid-Degrading Enzymes and for Detection of Sphingolipid-Binding Proteins[1]

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Sphingolipid ceramide *N*-deacylase is an enzyme capable of hydrolyzing the *N*-acyl linkages of ceramides of various sphingolipids. Recently, it was found that the enzyme catalyzes the reverse hydrolysis reaction in which free fatty acids are condensed to lyso-sphingolipids to produce sphingolipids. This paper describes a simple method for the synthesis of fluorescence-labeled sphingolipids utilizing the condensation reaction of the enzyme. *N*-TFAc-aminododecanoic acids were efficiently condensed by the enzyme to the lyso-forms of GM1 and sphingomyelin in glycine buffer (pH 10). The reaction products, *N*-TFAc-amino-GM1 and sphingomyelin, were obtained with overall yields of 60%. The purified products were identified to be ω-amino-GM1 and ω-amino-sphingomyelin, respectively, by TLC and FAB-MS or ESI-LC/MS analysis after removal of the *N*-TFAc by mild alkaline treatment. NBD-labeled GM1 and sphingomyelin were prepared from ω-amino-GM1 and ω-amino-sphingomyelin by coupling with 4-fluoro-NBD. These fluorescence-labeled substrates, C12-NBD-GM1 and C12-NBD-sphingomyelin, were hydrolyzed by endoglycoceramidase and sphingomyelinase, respectively, to produce NBD-dodecanoyl-sphingosines, but were resistant to hydrolysis by sphingolipid ceramide *N*-deacylase. C12-NBD-sphingomyelin was found to be a better substrate than the commercially available C6-NBD-sphingomyelin for the assay of sphingomyelinase from various sources. We also describe a new method to detect GM1-binding proteins using fluorescence-labeled GM1.

Key words: endoglycoceramidase, fluorescence-labeled sphingolipids, glycosphingolipid, sphingomyelin, sphingolipid ceramide *N*-deacylase.

Simons and Ikonen reported that glycosphingolipids (GSLs) and sphingomyelin (SM), both of which are synthesized from the hydrophobic molecule ceramide, are enriched with cholesterol and GPI-anchor proteins to form microdomains on the plasma membrane of vertebrates (*1*). GSLs and SM may play important roles in cell-cell interaction and recognition, and act as possible modulators of various cellular activities (*2, 3*). GM1 ganglioside, ganglio-tetraose GSL having one sialic acid residue, was found to modulate the function of various membrane proteins including enzymes (*4*), ion channels (*5*), receptors (*6*), and cell adhesion molecules (*7*). GM1 have also been reported to enhance neurite outgrowth and neuron survival, and so clinical applications of GM1 for neurological disorders were undertaken (*8-10*). Ceramide, sphingosine, and sphingosine-1-phosphate, metabolites of SM and GSLs, have emerged as novel lipid biomodulators of intracellular signal transduction pathways (*2, 3*).

Sphingolipid ceramide *N*-deacylase (SCDase) hydrolyzes GSLs and SM to produce fatty acids and lyso-sphingolipids (*11*), while endoglycoceramidase (EGCase) (*12*) and sphingomyelinase (SMase) (*13*) specifically hydrolyze GSLs and SM, respectively, to produce ceramides. Interestingly, SCDase efficiently condenses free fatty acids to the lyso-forms of sphingolipids (*14*). We have reported the synthesis of ^{14}C-ceramide (*15*) and ^{14}C-GSLs (*16*) using a unique condensation reaction involving SCDase. These radioactive sphingolipids may assist not only in elucidating

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Abbreviations: BSA, bovine serum albumin; CBB-R, Coomassie Brilliant Blue-R; CTB, cholera toxin B subunit; DMβCD, 2,6-*O*-dimethyl-β-cyclodextrin; C12-NBD-GM1, NBD-dodecanoylsphingosine-containing GM1; C6-NBD-SM, NBD-hexanoylsphingosine-containing sphingomyelin; C12-NBD-SM, NBD-dodecanoylsphingosine-containing sphingomyelin, EGCase II, endoglycoceramidase isoform II; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1, 1'ceramide; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PAGE, polyacrylamide gel electrophoresis; SCDase, sphingolipid ceramide *N*-deacylase; SM, sphingomyelin; SMase, sphingomyelinase; TBS, Tris-buffered saline; TFAc, trifluoroacetyl.

the metabolism and functions of sphingolipids but also in characterizing sphingolipid-degrading enzymes. However, handling radioactive substances requires special facilities. Thus, sensitive, but non-radioactive, sphingolipid derivatives are required.

This paper shows a simple method for the preparation of fluorescent GM1 and SM involving the unique condensation reaction of SCDase, and describes the actions of several sphingolipid-degrading enzymes on these fluorescent substrates. We also report a new method for the detection of GM1-binding proteins using fluorescence-labeled GM1.

MATERIALS AND METHODS

Materials—ω-Aminododecanoic acid and 4-fluoro-NBD were obtained from Wako Pure Chemical (Osaka) and Dojindo Laboratories (Kumamoto), respectively. C6-NBD-ceramide was purchased from Molecular Probe (USA). Triton X-100 was purchased from Sigma (USA), precoated Silica Gel 60 TLC plates (No. 1.05721) were from Merck (Germany), and Sep-Pak Plus C18 and Sep-Pak Plus Silica were from Waters (USA). SMase was obtained from Funakoshi (Tokyo). ^{14}C-SM (labeled at the choline residue) was obtained from ARC (USA). C18-^{14}C-GM1 was prepared by the previously described method (*16*).

Preparation of SCDase and EGCase and Definition of Enzyme Unit—SCDase was purified from the culture supernatant of *Pseudomonas* sp. TK4 as described in Ref. *11*. An isoform of endoglycoceramidase, EGCase II, was purified from the culture supernatant of *Rhodococcus* sp. M-777 as previously described (*12*). Both enzymes can also be purchased from Takara Shuzo (Shiga). One unit (U) of SCDase or EGCase was defined as the amount capable of hydrolyzing 1 μmol GM1 per min under the conditions described in Refs. *11* and *12*, respectively.

Preparation of the Lyso-Forms of GM1 and SM—To prepare lyso-GM1 and lyso-SM in large scale, a SCDase-producing bacterium was used instead of the purified SCDase. The method for the preparation of lyso-SM using *Shewanella alga* NS-589 has been reported (*17*). For the preparation of lyso-GM1, another SCDase-producing bacterium, *Pseudomonas* sp. TK4, which produces much more SCDase than NS-589, but also produces SMase, was used. Briefly, for the preparation of lyso-GM1, TK4 strain cells were cultured at 25°C in 200 ml of medium (0.4% tryptone, 0.25% NaCl, 0.05% NH_4Cl, 0.05% K_2HPO_4, 0.05% DMβCD, pH 7.2) containing GM1 at a concentration of 0.5 mg/ml. After 3 days, 90% of the GM1 in the medium was hydrolyzed to produce lyso-GM1. After removal of the bacterial cells by centrifugation (7,000×*g* for 15 min), lyso-GM1 in the culture supernatant was adsorbed on a reverse phase preparative C18 column (35×70 mm; Waters, USA), and eluted from the column with 100 ml of methanol and 300 ml of chloroform/methanol (2:1, v/v). The fractions containing lyso-GM1 were evaporated and the residue was dissolved in 10 ml of chloroform/methanol (9:1, v/v), and applied to a Silica Gel column (35×130 mm; Merck, Germany) equilibrated with the same solvent. After washing with 600 ml of the same solvent to remove DMβCD, lyso-GM1 was eluted with 800 ml of chloroform/methanol (2:1, v/v). The fractions containing lyso-GM1 were evaporated and the residue was dissolved in 10 ml of chloroform/methanol/distilled water (30:60:8, v/v) and loaded onto a DEAE-Sephadex A25 column (24×150 mm) equilibrated with the same solvent. After washing with 250 ml of the same solvent, lyso-GM1 was eluted from the column with a sodium acetate gradient from 0 to 65 mM in 500 ml of the same solvent. The fractions containing lyso-GM1 were evaporated, the residue was dissolved in 10 ml of chloroform/methanol/distilled water (5:4:1, v/v), and the sample was applied to a Silica Gel column (24×700 mm). Lyso-GM1 was eluted from the column with the same solvent. After removal of the solvent, the purified lyso-GM1 was suspended in distilled water and lyophilized.

Preparation of N-TFAc-Aminododecanoic Acid—*N*-Trifluoroacetylation of ω-aminododecanoic acid was carried out according to the method described by Kamio *et al.* (*18*).

Determination of Extent of ω-Amino-Sphingolipids Synthesis—The reaction mixture was dried, and the residue was dissolved in 10 μl of chloroform/methanol (2:1, v/v) and applied to a TLC plate developed with chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v). The reaction products were visualized with orcinol-H_2SO_4 and quantified with a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm. The extent of reaction was calculated as follows: reaction efficiency (%) = (peak area for *N*-TFAc-amino-GM1 generated) × 100/(peak area for lyso-GM1 remaining + peak area for *N*-TFAc-amino-GM1 generated).

Preparation of ω-Amino-GM1 and SM—Lyso-GM1 or lyso-SM (2 μmol) was incubated with 4 μmol of *N*-TFAc-aminododecanoic acid in the presence of 10 mU of SCDase at 37°C for 19 h in 10 ml of 25 mM glycine-NaOH buffer (pH 10). The yields of ω-amino-GM1 and ω-amino-SM were 95 and 90%, respectively. After incubation, each reaction mixture was applied to a Sep-Pak Plus C18 cartridge equilibrated with distilled water. The cartridge

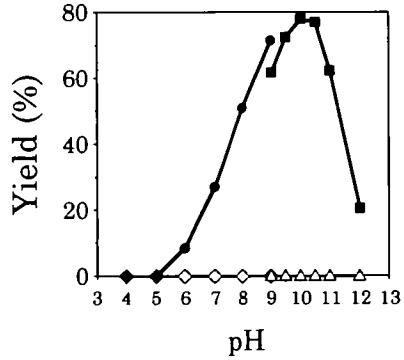

Fig. 1. **Effects of pH and blocking with TFAc on the synthesis of GM1 containing ω-aminododecanoic acid by SCDase.** 10 nmol of lyso-GM1 was incubated with 20 nmol of ω-aminododecanoic acid or *N*-TFAc-aminododecanoic acid at 37°C for 20 h with 100 μU of SCDase in 40 μl of 25 mM GTA buffer or glycine-NaOH buffer containing 0.025% Triton X-100 at the indicated pH. After incubation, the yield was determined by the method described in "MATERIALS AND METHODS." ●, lyso-GM1 with *N*-TFAc-aminododecanoic acid in GTA buffer in the presence of SCDase; ■, lyso-GM1 with *N*-TFAc-aminododecaoic acid in glycine-NaOH buffer in the presence of SCDase; ◇, lyso-GM1 with ω-aminododecanoic acid in GTA buffer in the presence of SCDase; △, lyso-GM1 with ω-aminododecanoic acid in glycine-NaOH buffer in the presence of SCDase. Values are averages of duplicate determinations from a typical experiment.

were then washed with 50 ml of distilled water, and *N*-TFAc-amino-GM1 or *N*-TFAc-amino-SM was eluted with 2 ml of methanol and 10 ml of chloroform/methanol (2:1, v/v). The eluates were combined, dried under N_2 gas, dissolved in 1 ml of chloroform/methanol/distilled water (60:35:5, v/v), and subjected to HPLC on a normal phase column (AQUASIL SS-1251, 4.6×250 mm, Senshu Pak., Tokyo), and eluted with the same solvent. The fractions containing *N*-TFAc-amino-GM1 or *N*-TFAc-amino-SM were pooled, dried under N_2 gas, and dissolved in 1 ml of methanol/distilled water (1:2, v/v). To removed the *N*-TFAc group, 10 μl of sodium methoxide (28% in methanol) was added. The mixture was stirred at room temperature for 4 h, neutralized with acetic acid, dried under N_2 gas, dissolved in 1 ml of distilled water, and applied to a Sep-Pak Plus C18 cartridge. The cartridge was washed with 50 ml of distilled water, and ω-amino-GM1 or ω-amino-SM was eluted with 2 ml of methanol and chloroform/methanol (2:1, v/v). In a typical experiment, 1.0 μmol of ω-amino-GM1 and 1.2 μmol of ω-amino-SM were obtained from 2 μmol of lyso-GM1 and lyso-SM, respectively.

Preparation of NBD-Labeled Sphingolipids—ω-Amino-GM1 and ω-amino-SM were labeled with NBD as follows: 700 μl of methanol containing 1 μmol of ω-amino-sphingolipid, 200 μl of ethanol containing 10 μmol of 4-fluoro-NBD, and 100 μl of 10% triethylamine were mixed and incubated at 60°C for 2 min. After incubation, the reaction mixtures were dried under N_2 gas, dissolved in chloroform/methanol/acetic acid (90:10:1, v/v), and applied to a Sep-Pak Plus Silica cartridge. After washing the cartridge with 100 ml of the same solvent, NBD-labeled sphingolipids were eluted with 2 ml of chloroform/methanol (2:1, v/v) and 50 ml of methanol.

Mass Spectrometry—ω-Amino-SM and C12-NBD-SM were analyzed by FAB-MS with a JMS LX-2000 (JEOL Ltd., Tokyo) using a positive-ion mode and 3-nitrobenzyl alcohol as a matrix. ω-Amino-GM1 and C12-NBD-GM1 were analyzed by ESI-LC/MS with an AP1-III (Perkin-Elmer Sciex, Canada). The samples were introduced in 0.1% formic acid/acetonitrile (1:1, v/v) as a solvent at a flow rate of 2 μl/min. The mass spectrometer was operated in the negative mode (the ion-spray voltage set at −4,000 V and the orifice voltage at −80 V) or the positive mode (the ion-spray voltage set at 5,000 V and the orifice voltage at 90 V).

Assay for Sphingolipid-Degrading Enzymes—The substrate specificities of three sphingolipid-degrading enzymes (EGCase II, SCDase and SMase) were examined using fluorescence- or radio-labeled substrates. One hundred picomoles of fluorescence-labeled substrates (C12-NBD-GM1, NBD-lyso-GM1, C12-NBD-SM, C6-NBD-SM, or NBD-lyso-SM) or radio-labeled substrate (C18-^{14}C-GM1

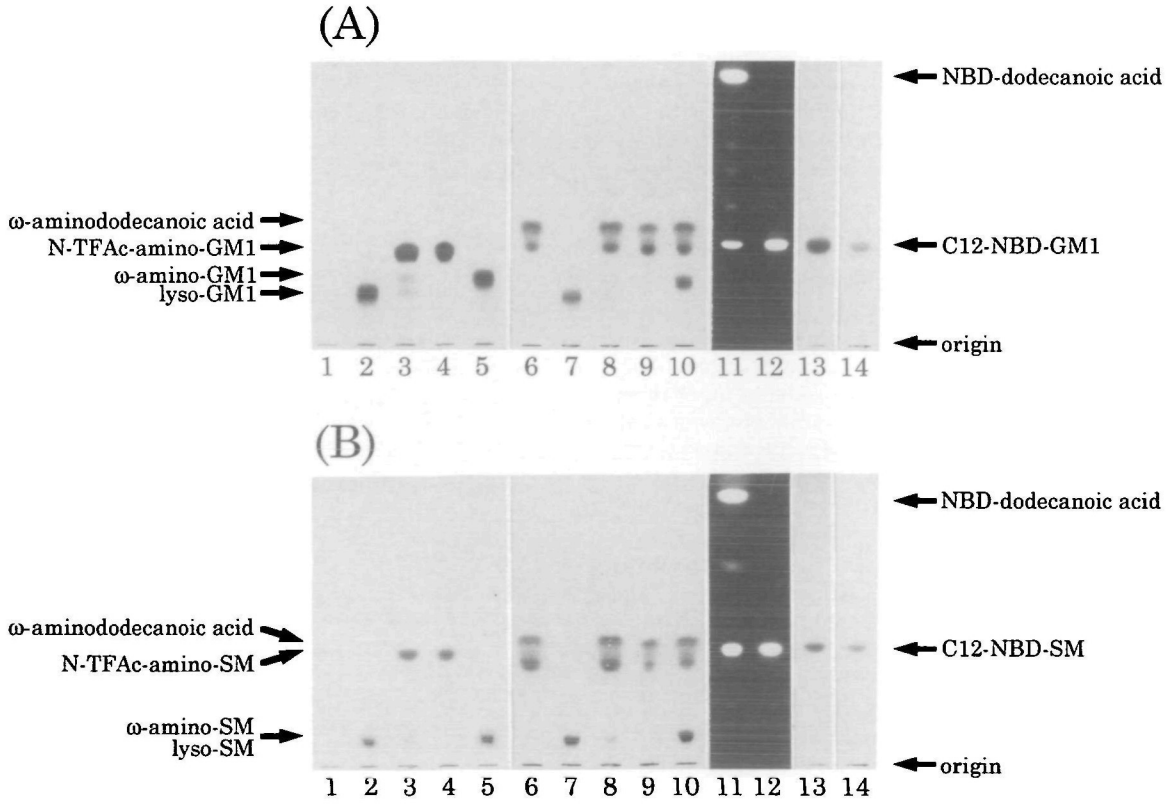

Fig. 2. **TLC showing the preparation of C12-NBD-GM1 (A) and C12-NBD-SM (B).** 2 μmol of lyso-GM1 or lyso-SM was incubated with 4 μmol of *N*-TFAc-aminododecanoic acid in the presence of 15 mU of SCDase at 37°C for 19 h in 10 ml of 25 mM glycine-NaOH buffer (pH 10). After incubation, *N*-TFAc-amino-sphingolipids were subjected to purification, de-blocking and NBD-labeling as described in "MATERIALS AND METHODS." Lanes 1 and 6, ω-aminododecanoic acid standard; lanes 2 and 7, lyso-GM1 (A) and lyso-SM (B) standards, respectively; lanes 3 and 8, reaction products after incubation with SCDase; lanes 4 and 9, reaction products after purification by HPLC; lanes 5 and 10, reaction products after de-blocking with sodium methoxide; lane 11, reaction products after labeling with 4-fluoro-NBD; lanes 12, 13 and 14, purified C12-NBD-sphingolipid preparations. Samples in lanes 1-5 and 13 were visualized with CBB-R (*25*), those in lanes 6-10 and 14 with ninhydrin and those in lanes 11 and 12 with a transilluminator.

or ^{14}C-SM) was incubated with an appropriate amount of various enzymes at 37°C for the indicated times under the following conditions: (i) 10 mM sodium acetate buffer (pH 5.0) containing 0.2% Triton X-100 for EGCase, (ii) 25 mM sodium phosphate buffer (pH 6.0) containing 0.1% Triton X-100 for SCDase, and (iii) 25 mM sodium phosphate buffer (pH 7.0) containing 0.2% Triton X-100 for SMase. After incubation, the solvent was evaporated and the residue was dried, dissolved in 10 μl of chloroform/methanol (2:1) and analyzed by TLC using chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v) as the developing solvent. Degradation products and remaining substrates were separated by TLC and quantified with a Shimadzu CS-9300 chromatoscanner (excitation 470 nm, emission 525 nm) for fluorescence-labeled substrates or with a BAS1500 imaging analyzer (Fuji Film, Tokyo) for radio-labeled substrates. The extent of the reaction was calculated as follows: reaction (%) = (peak area for degradation product produced) × 100/(peak area for substrate remaining + peak area for degradation product produced).

Preparation of Crude SMase from Mouse Brain and Kidney—The whole brain and kidney from a mouse were homogenized in 5 ml and 2 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.2% of Triton X-100, respectively. After centrifugation at 15,000 rpm for 20 min, the supernatants were used for enzyme assay as a crude SMase preparation.

Detection of Cholera Toxin B Subunit (CTB) Using C12-NBD-GM1 on SDS-PAGE—Ten micrograms of CTB was incubated at 37°C for 1 h with 2 nmol of C12-NBD-GM1 in TBS containing 0.025% Triton X-100. The reaction mixture was applied to a 10% polyacrylamide gel containing 0.1% SDS. SDS-PAGE was performed by the method of Laemmli (*19*) under non-reducing conditions. The complex of CTB with C12-NBD-GM1 was detected using a transilluminator at 302 nm (TMD-20, UVP, USA). Proteins were visualized with CBB-R.

RESULTS

We previously reported that ^{14}C-palmitic acid and ^{14}C-stearic acid are efficiently condensed to lyso-GM1 to generate ^{14}C-GM1 in 25 mM phosphate buffer (pH 7.0) in the presence of SCDase (*16*). However, under these conditions ω-aminododecanoic acid was hardly condensed to lyso-GM1 by SCDase (Fig. 1). Once the ω-amino group was blocked with TFAc and the reaction pH was made alkaline, the reaction proceeded efficiently showing an optimum pH of 10 (Fig. 1).

Figure 2 shows TLCs of the preparation of C12-NBD-GM1 (A) and C12-NBD-SM (B). When *N*-TFAc-aminododecanoic acids were incubated with the lyso-forms of sphingolipids in the presence of SCDase at pH 10, the new spots appeared on TLCs after staining with CBB-R but not with ninhydrin (Fig. 2, A and B, lanes 3 and 8). These spots were most likely to be *N*-TFAc-amino-sphingolipids. The *N*-TFAc-amino-sphingolipids were purified by HPLC using a normal phase silica column (Fig. 2, A and B, lanes 4 and 9). After alkaline treatment, the spots disappeared and new ninhydrin-positive spots corresponding to each ω-amino-sphingolipid were generated (Fig. 2, A and B, lanes 5 and 10). Doublet bands on the TLC plate after staining with ninhydrin seemed to be ω-aminododecanoic acids (Fig. 2, A and B, lanes 6, 8, 9, and 10). The contaminating ω-aminododecanoic acids were converted to NBD-dodecanoic acid by treatment with NBD-fluoride (Fig. 2, A and B, lane 11), and removed from the ω-amino-sphingolipids by passage through a Sep-Pak Plus Silica cartridge (Fig. 2, A and B, lane 12). The final preparations of C12-NBD-GM1 and C12-NBD-SM showed one band each on TLC when observed under a transilluminator (Fig. 2, A and B, lane 12) or stained with CBB-R (Fig. 2, A and B, lane 13). The final preparation contained no ω-aminododecanoic acids (Fig. 2, A and B, lane 14). A faint yellow, not red, band was observed at the positions corresponding to C12-NBD-GM1 and C12-NBD-SM (Fig. 2, A and B, lane 14). These bands could be visualized by NBD itself, since they were observed before staining with ninhydrin. In a typical experiment, 0.9 and 1.1 μmol of purified C12-NBD-GM1 and C12-NBD-SM, respectively, were obtained from the reaction using 2 μmol of each lyso-sphingolipid and 4 μmol of *N*-TFAc-aminododecanoic acid. The scheme for the preparation of fluorescence-labeled GM1 and SM is shown in Fig. 3.

The ω-amino-GM1 and C12-NBD-GM1 prepared were subjected to ESI-LC/MS using positive and negative ion modes, respectively, while ω-amino-SM and C12-NBD-SM were subjected to FAB-MS using positive ion mode. As shown in Fig. 4A, the characteristic pseudomolecular ions $[M+H]^+$ and $[M+2H]^{2+}$ were found at m/z 1,479 and 740, which correspond to ω-amino GM1, the ceramide portion of which is composed of ω-aminododecanoic acid

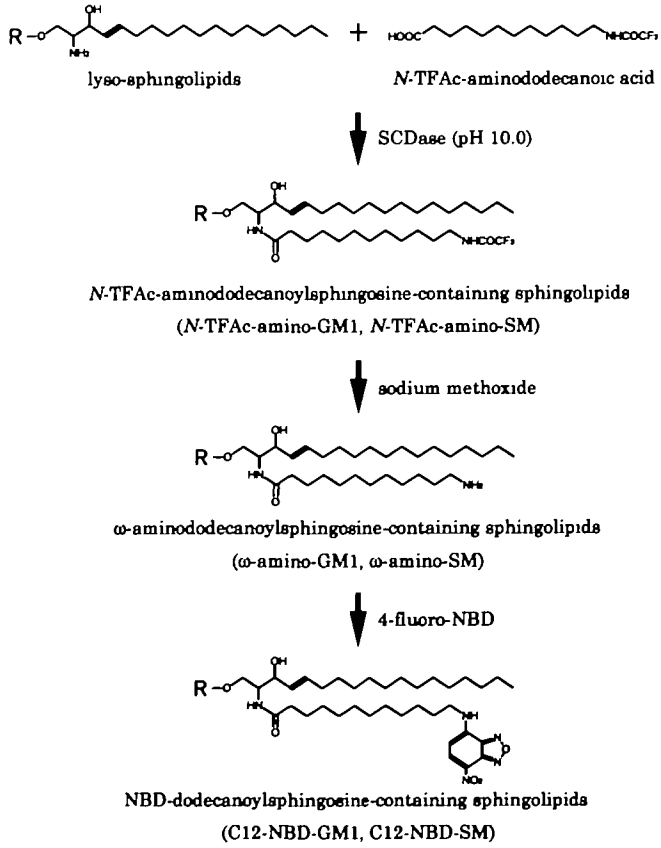

Fig. 3. **Scheme for the preparation of C12-NBD-GM1 and SM.** R are Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc and choline phosphate for GM1 and SM derivatives, respectively.

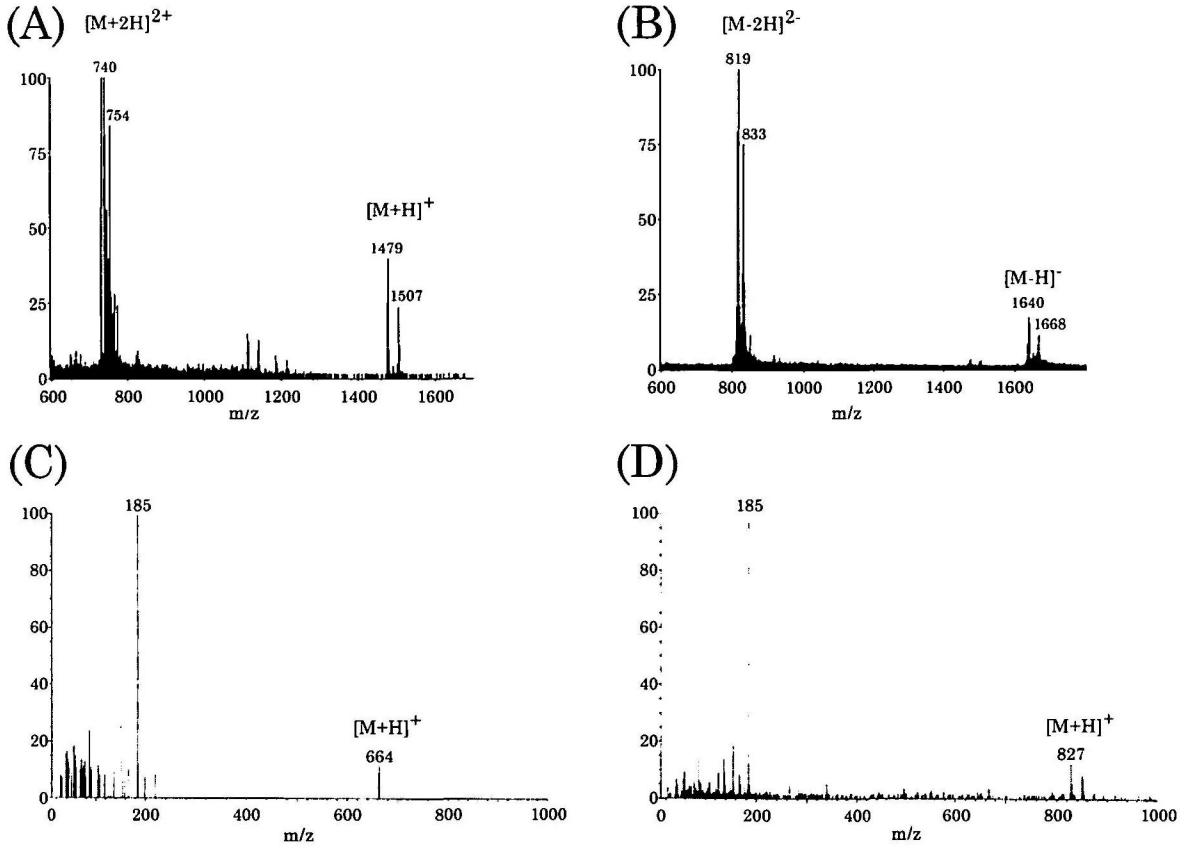

Fig. 4. **Mass spectrometry analyses of ω-amino-GM1 (A), C12-NBD-GM1(B), ω-amino-SM (C), and C12-NBD-SM (D).** (A), (B), ESI-LC/MS analyses using positive and negative ion modes, respectively. (C), (D), FAB-MS analyses using positive ion mode. Details are described in "MATERIALS AND METHODS."

and d18:1 sphingoid base. $[M+H]^+$ and $[M+2H]^{2+}$, corresponding to ω-amino GM1 containing a d20:1 sphingoid base, were also observed at *m/z* 1,507 and 754, respectively. For NBD-labeled GM1, $[M-H]^-$ were found at *m/z* 1,640 and 1,668, corresponding to C12-NBD-GM1 containing d18:1 and d20:1 sphingoid bases, respectively (Fig. 4B). The corresponding $[M-2H]^{2-}$ ions were also observed at *m/z* 819 and 833, respectively. Using FAB-MS in positive ion mode, $[M+H]^+$ was found at *m/z* 664 for ω-amino-SM (Fig. 4C) and *m/z* 827 for C12-NBD-SM (Fig. 4D), indicating that both SM-derivatives contain a d18:1 sphingoid base. A fragment ion was also observed at *m/z* 185, which corresponds to choline phosphate (Fig. 4, C and D).

We previously reported a sensitive method for the assay of EGCase II using [^{14}C]GSLs (*16*). In this study, an alternative method for the assay of EGCase using fluorescence-labeled GM1 instead of radio-labeled GM1 was designed. This method was also applied to the assays of SCDase and SMase using fluorescence-labeled SM. The method consists of TLC separation of the fluorescent products released from the parent sphingolipids by the action of the enzymes, followed by quantification of both fluorescent substances with a TLC-chromatoscanner with a fluorescence detector. Figure 5 shows the results of digestion of C12-NBD-sphingolipids by three different enzymes. C12-NBD-GM1 was hydrolyzed by EGCase II and SCDase to produce NBD-dodecanoylsphingosine and NBD-dodecanoic acid, respectively (Fig. 5, lanes 5 and 6), whereas C12-NBD-GM1 was completely resistant to SMase (Fig. 5, lane 7). C12-NBD-SM was digested by SCDase and SMase to produce NBD-dodecanoic acid and NBD-dodecanoylsphingosine, respectively (Fig. 5, lanes 9 and 10). C12-NBD-SM was not hydrolyzed by EGCase II (Fig. 5, lane 8).

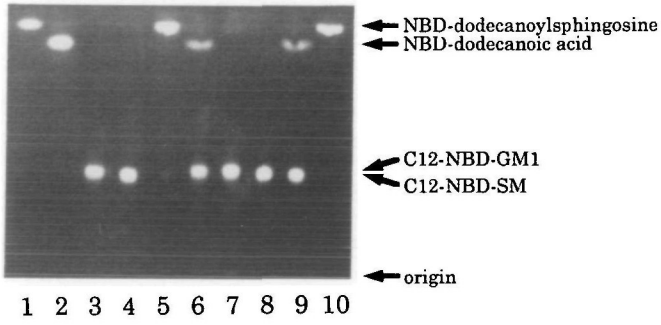

Fig. 5. **Actions of various sphingolipid-degrading enzymes on C12-NBD-GM1 and SM.** 100 pmol of C12-NBD-GM1 or C12-NBD-SM was incubated with 1 mU of EGCase II, SCDase, and SMase in an appropriate buffer at 37°C for 24 h as described in "MATERIALS AND METHODS." Lane 1, C12-NBD-dodecanoylsphingosine standard; lane 2, NBD-dodecanoic acid standard; lane 3, C12-NBD-GM1 standard; lane 4, C12-NBD-SM standard; lane 5, C12-NBD-GM1 with EGCase II; lane 6, C12-NBD-GM1 with SCDase; lane 7, C12-NBD-GM1 with SMase; lane 8, C12-NBD-SM with EGCase II; lane 9, C12-NBD-SM with SCDase; lane 10, C12-NBD-SM with SMase.

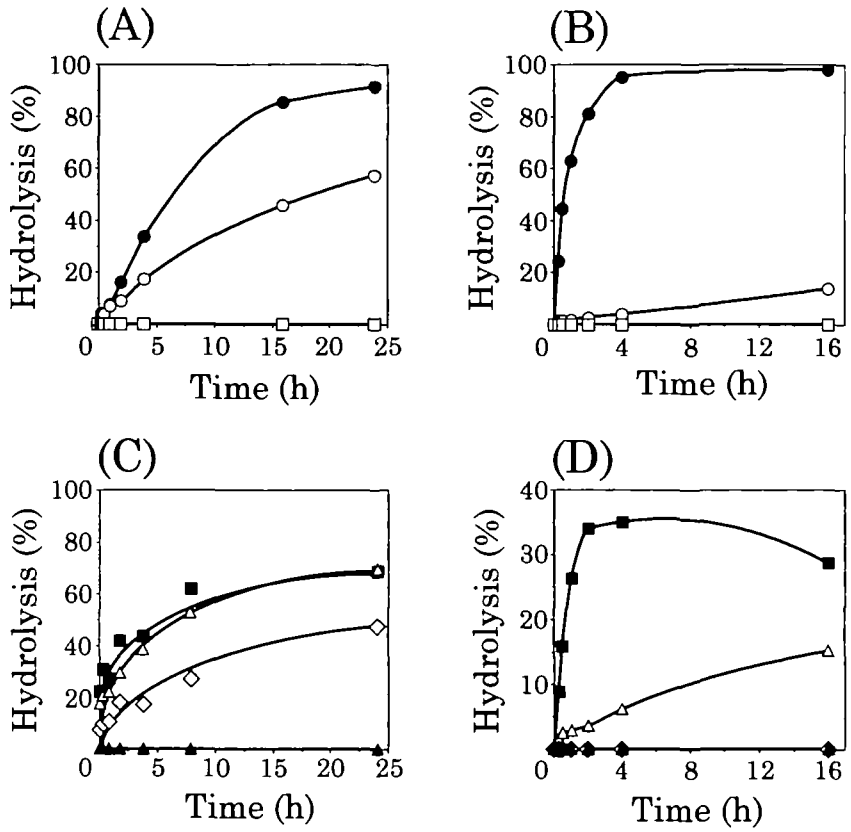

Fig. 6. **Time courses for the hydrolysis of various sphingolipid derivatives by EGCase II (A), SCDase (B, D), and SMase (C).** 100 pmol of GM1 derivatives were incubated with 85 μU of EGCase (A) or 80 μU of SCDase (B) at 37°C for the indicated times. 100 pmol of SM-derivatives were incubated with 40 μU of SMase (C) or 320 μU of SCDase (D) at 37°C for the indicated times. ○, C12-NBD-GM1; □, NBD-lyso-GM1; ●, C18-^{14}C-GM1. △, C12-NBD-SM; ◇, C6-NBD-SM; ▲, NBD-lyso-SM; ■, ^{14}C-SM. Values are means of triplicate determinations from a typical experiment.

The sensitivity of the present method using fluorescent substrates is comparable to the previous method using radio-labeled substrates, *i.e.*, 500 fmol of NBD-products released from NBD-sphingolipids by the action of enzyme can be detected when 100 pmol of NBD-sphingolipids are used for the assay.

Time courses for the hydrolysis of various sphingolipids by EGCase II, SCDase and SMase are shown in Fig. 6. C12-NBD-GM1 was found to be a preferential substrate for EGCase II (Fig. 6A), while this substrate was almost resistant to SCDase (Fig. 6B). C12-NBD-SM was digested by SCDase, but more slowly than ^{14}C-SM (Fig. 6D). On the other hand, the rate of hydrolysis of C12-NBD-SM by SMase from *Bacillus sereus* was comparable to that of ^{14}C-SM (Fig. 6C). It seems remarkable that C12-NBD-SM was hydrolyzed by SMase faster than C6-NBD-SM (Fig. 6C), although the latter commercially available substrate is preferentially used for SMase assay. NBD-lyso-GM1 and NBD-lyso-SM, in which NBD is directly coupled to the amino groups of the sphingoid moieties, were completely resistant to hydrolysis by these three enzymes (Fig. 6, A-D).

The susceptibility of C12-NBD-SM and C6-NBD-SM was examined using crude SMases from mouse brain (Fig. 7A) and kidney (Fig. 7B). The results indicate that C12-NBD-SM is a better substrate for SMases not only from bacteria but also from mammalian tissues.

The structures of the fluorescence-labeled sphingolipids used and the points of actions of EGCase II, SCDase and SMase are shown in Fig. 8.

We also applied fluorescence-labeled GM1 to the detection of GM1-binding proteins. C12-NBD-GM1/protein complexes were separated from C12-NBD-GM1 on SDS-PAGE and both fluorescent substances were detected with a transilluminator. For example, the complex of C12-NBD-GM1 with CTB, a well-known GM1-binding protein (*20*), migrated much more slowly in polyacrylamide gels containing SDS under non-reducing conditions (Fig. 9, A and B, lane 1) compared with free C12-NBD-GM1 (Fig. 9A, lane 7) or CTB (Fig. 9B, lane 2). However, the mobilities of

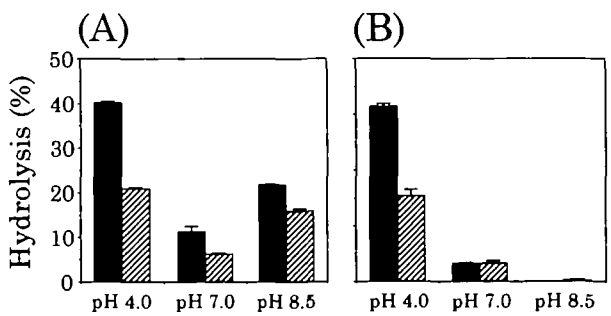

Fig. 7. **Assay for crude SMases from mouse brain (A) and kidney (B) using C6-NBD-SM and C12-NBD-SM.** 100 pmol of C12-NBD-SM (black bars) or C6-NBD-SM (hatched bars) was incubated at 37°C for 2 h with crude enzymes from mouse tissues in 20 μl of 50 mM sodium acetate buffer (pH 4.0), sodium phosphate buffer (pH 7.0) or glycine-NaOH buffer (pH 8.5). All reaction mixtures contained 0.2% Triton X-100. The hydrolysis of NBD-SM was determined by the method described in "MATERIALS AND METHODS." Values are means±SD of triplicate determinations from a typical experiment.

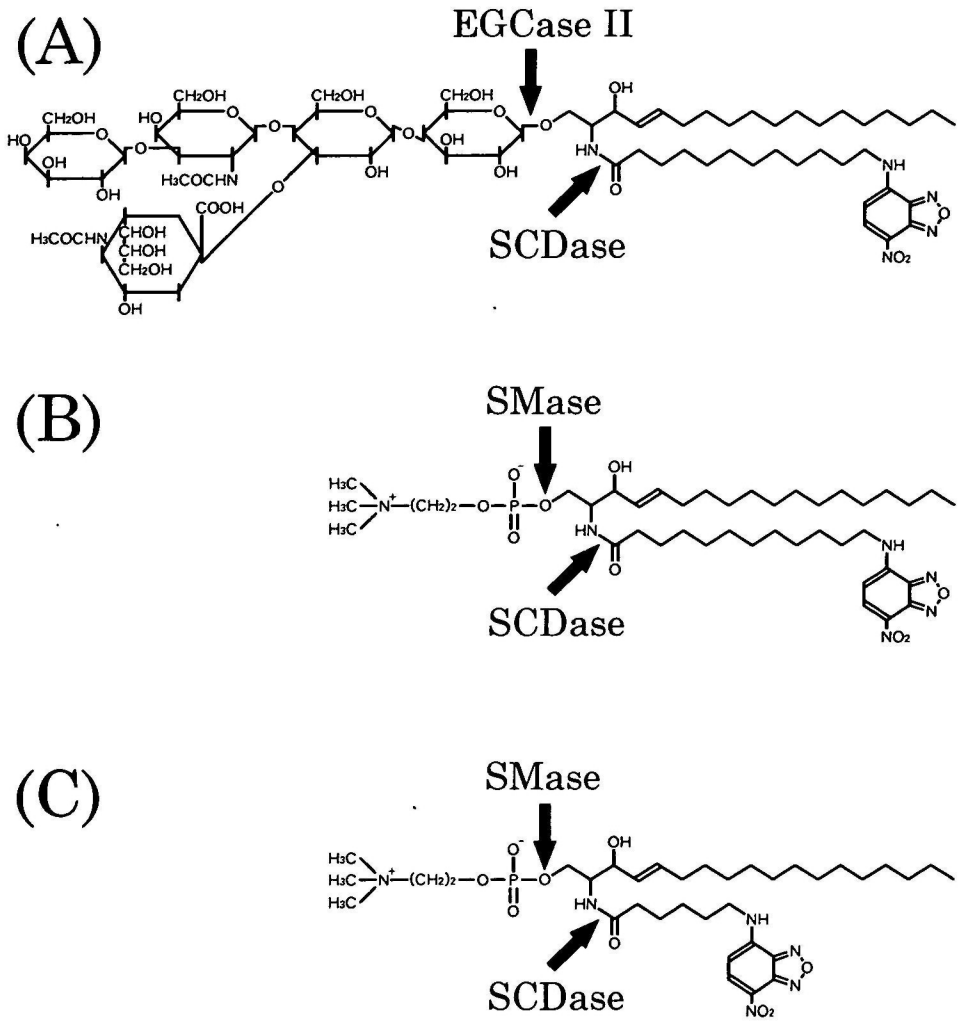

Fig. 8. **Structures of NBD-labeled sphingolipids and points of action of EGCase II, SCDase and SMase.** (A) C12-NBD-GM1, (B) C12-NBD-SM, (C) C6-NBD-SM.

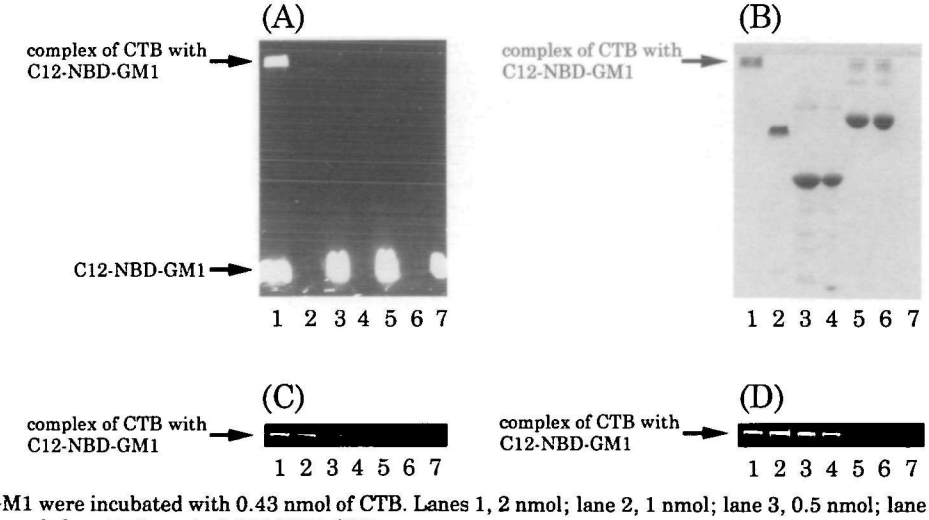

Fig. 9. **Detection of GM1-binding proteins using C12-NBD-GM1.** CTB were incubated with C12-NBD-GM1 in TBS (pH 7.4) containing 0.025% Triton X-100 at 37°C for 1 h. After incubation, the mixtures were applied to SDS-PAGE under non-reducing conditions, followed by visualization with a transilluminator for (A), (C), and (D) or CBB-R for (B). For (A) and (B), lane 1, CTB+C12-NBD-GM1; lane 2, CTB; lane 3, carbonic anhydrase+C12-NBD-GM1; lane 4, carbonic anhydrase; lane 5, BSA+C12-NBD-GM1; lane 6, BSA; lane 7, C12-NBD-GM1. (C), different amounts of CTB were incubated with 2 nmol of C12-NBD-GM1. Lanes 1, 0.86 nmol; lane 2, 0.43 nmol; lane 3, 0.17 nmol; lane 4, 0.086 nmol; lane 5, 0.043 nmol; lane 6, 0.017 nmol; lane 7, 0.009 nmol of CTB. (D), different amounts of C12-NBD-GM1 were incubated with 0.43 nmol of CTB. Lanes 1, 2 nmol; lane 2, 1 nmol; lane 3, 0.5 nmol; lane 4, 0.2 nmol; lane 5, 0.1 nmol; lane 6, 0.05 nmol; lane 7, 0 nmol of C12-NBD-GM1.

BSA and carbonic anhydrase, neither of which forms complexes with GM1, were the same both with and without C12-NBD-GM1 (Fig. 9, A and B, lanes 3, 4, 5, and 6). This result indicates that the non-specific binding of proteins with NBD-GM1 was eliminated under the conditions used. The CTB-GM1 complex could be detected when at least 0.043 nmol of CTB was included in the reaction mixture with 2 nmol of C12-NBD-GM1 (Fig. 9C), and at least 0.2

nmol of C12-NBD-GM1 was needed to detect 0.43 nmol of CTB (Fig. 9D). We also detected the complex of C12-NBD-SM with lysenin, a hemolytic protein derived from earthworms that specifically binds to SM (*21*), using C12-NBD-SM (data not shown), suggesting that this method is useful for screening various sphingolipid-binding proteins.

DISCUSSION

The preparation of fluorescence-labeled GM1 and SM seems so far to be restricted by synthetic chemical procedures (*18, 22*). The present method using the reverse hydrolysis reaction of SCDase is preferred at present. Since the enzyme is very specific for the amino group of sphingoid bases in various sphingolipids, no by-products are generated during the reaction. The reverse hydrolysis reaction of SCDase is possible in various GSLs including polysialogangliosides; therefore, the present method can be applied to the preparation of various fluorescent gangliosides.

Recently, the catalytic products of SM and GSLs have emerged as novel biomodulators of intracellular signal transduction (*2, 3*). To elucidate the significance of sphingolipids and their metabolites in cellular activities, a sensitive probe and assay method are needed. Furthermore, the search for proteins that bind specifically to GSLs and SM is a timely and important task. To date, radiolabeled substrates have been used most frequently to measure the activity of sphingolipid-degrading enzymes. The method using fluorescence-labeled substrates described in this paper may be an alternative for sphingolipid-degrading enzymes and sphingolipid-binding proteins.

We have prepared C12-NBD-ceramide using the reverse hydrolysis reaction of SCDase (*23*). C12-NBD-ceramide was hydrolyzed much faster than radio-labeled ceramides by alkaline and neutral, but not acid, ceramidases, indicating that the coupling of NBD to the ω-amino group of the fatty acid increases the susceptibility of ceramides to alkaline and neutral ceramidases and decreases their susceptibility to acid ceramidases (*24*). In this paper, we compare the susceptibility of C12-NBD-GM1 and SM with ^{14}C-labeled substrates to various sphingolipid-degrading enzymes. The attachment of NBD did not increase the susceptibility of the substrates to enzymes examined, although C12-NBD-GM1 and C12-NBD-SM were found to be good substrates for EGCase and SMase, respectively, but not for SCDase. It is noteworthy that C12-NBD-SM is a better substrate for the assay of SMases from different sources compared to commercially available C6-NBD-SM.

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