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Enzymatic Synthesis of ^{14}C-Glycosphingolipids by Reverse Hydrolysis Reaction of Sphingolipid Ceramide *N*-Deacylase: Detection of Endoglycoceramidase Activity in a Seaflower[1]

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This paper describes the synthesis of ^{14}C-labeled glycosphingolipids using the reverse hydrolysis reaction (condensation) of sphingolipid ceramide *N*-deacylase. It was found that 50-70% of ^{14}C-fatty acids were incorporated into various lyso-glycosphingolipids when a mixture of lyso-glycosphingolipids and fatty acids was incubated at 37°C with 1 mU of the enzyme for 20 h in 1 ml of 25 mM phosphate buffer, pH 6.0-7.0, containing 0-0.1% Triton X-100. The optimum concentration of lyso-glycosphingolipids was 100-400 μM depending on the species of lyso-form when [^{14}C]stearic acid was used at the concentration of 100 μM. Free ^{14}C-fatty acids and lyso-glycosphingolipids were separated from the synthesized ^{14}C-glycosphingolipids by using a Sep-Pak Plus Silica and a Sep-Pak CM or a QMA cartridge, respectively. After treatment of ^{14}C-glycosphingolipids with endoglycoceramidase or sphingolipid ceramide *N*-deacylase, digestion products were clearly separated from the parent glycosphingolipids on TLC and determined using an image analyzer with a sensitivity 100 times higher than that using non-radiolabeled substrates. Using this method, we found endoglycoceramidase activity in a seaflower, *Condylactis* sp., for the first time.

Key words: endoglycoceramidase, ^{14}C-glycosphingolipids, reverse hydrolysis reaction, sphingolipid ceramide *N*-deacylase.

Glycosphingolipids (GSLs) may play an important role in cell-cell interaction and recognition (*1*). To elucidate the function and metabolism of GSLs, radiolabeled GSLs are needed. Such derivatives are also useful for detecting novel GSL-degrading enzymes and examining their specificities. Several chemical procedures have been reported for the synthesis of radiolabeled GSLs (*2, 3*). Despite numerous improvements in the past decade (*4*), these methods are somewhat troublesome, time-consuming, and sometimes give a low yield, especially for GSLs containing sialic acids (gangliosides).

Sphingolipid ceramide *N*-deacylase (SCDase) catalyzes the hydrolysis of *N*-acyl linkages between fatty acids and sphingosine bases in ceramides of GSLs and sphingomyelin (*5*). Recently this enzyme was also found to catalyze the reverse reaction (condensation), depending on the reaction conditions (*6*). The reverse hydrolysis reaction was successfully applied to the preparation of ^{14}C-ceramides (*7*).

Endoglycoceramidase (EGCase; EC 3.2.1.123) is capable of hydrolyzing the glycosidic linkage between oligosaccharide and ceramide of various GSLs (*8*). Three isoforms of this enzyme (EGCase I, II, III) having different specificities have been purified from the culture supernatant of *Rhodococcus* sp. (*9*). Similar enzymes, called ceramide glycanases, have also been found in leech (*10*), earthworm (*11*), and hard-shelled clam (*12*).

This report describes the optimum conditions for enzymatic synthesis of various ^{14}C-GSLs by SCDase and simple procedures for purification of the synthesized ^{14}C-GSLs. We also report a sensitive assay method for EGCase and SCDase, which was successfully applied to detect EGCase activity in a seaflower for the first time.

MATERIALS AND METHODS

Materials—1-^{14}C-labeled lauric acid (55 mCi/mmol), palmitic acid (50 mCi/mmol), and stearic acid (55 mCi/mmol) were purchased from American Radiolabeled Chemicals, and thin-layer chromatography (TLC) plates were from Merck (Germany). Sep-Pak Silica, C18, CM, and QMA cartridges were obtained from Waters and lyso-GalCer (psychosine) was from Sigma. ^{14}C-Ceramide was prepared as described previously (*7*). Other reagents were of the highest grade available.

Preparation of SCDase and EGCase and Definition of an Enzyme Unit—SCDase was prepared from the culture fluid

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Abbreviations: Cer, ceramide; EGCase, endoglycoceramidase; GalCer, Galβ1,1Cer; Gb4Cer, GalNAcβ1,3Galα1,4Galβ1,4Glcβ1,1Cer; GM1a, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1,1Cer; GSL, glycosphingolipid; SCDase, sphingolipid ceramide *N*-deacylase; TLC, thin-layer chromatography.

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of *Pseudomonas* sp. TK4 as described (*5*). An isoform of endoglycoceramidase, EGCase II, was purified from the culture fluid of *Rhodococcus* sp. M-777 as described in Ref. *9* or purchased from Takara Shuzo, Kyoto. One unit (U) of SCDase or EGCase was defined as the amount capable of hydrolyzing 1 μmol of GM1a per min under the conditions described in Ref. *5* or *9*. 10^{-6} U of enzyme was expressed as 1 μU in this study.

Preparation of Lyso-GSLs—Lyso-GSLs were prepared by digestion of GSLs with SCDase as described in Ref. *5*. In this study, however, *Pseudomonas* sp. TK4 was used as the microbial biocatalyst instead of the SCDase preparation. The method was essentially the same as that for the preparation of lyso-sphingomyelin (sphingosylphosphorylcholine) using *Shewanella alga* NS-589 (*13*). The method using a bacterium is suitable for preparation of not only lyso-sphingomyelin but also lyso-GSLs on a large scale. Briefly, strain TK4 was cultured at 25°C for 3 days in a medium (0.5% tryptone, 0.2% NaCl, 0.08% 2,6-*O*-dimethyl-β-cyclodextrin) containing various GSLs (GM1a, sulfatide, or Gb4Cer) at a concentration of 0.5 mg/ml. The lyso-GSLs in the culture supernatant were adsorbed on a reverse phase preparative C18 column, then eluted from the column with 3 ml of methanol and 10 ml of chloroform/methanol (2:1, v/v, solvent A). The lyso-GSLs were purified by HPLC using a silica column which was developed with chloroform/methanol/water (60:35:5, v/v).

Examination of Various Factors in ^{14}C-GSL Synthesis by SCDase—Firstly, optimum concentrations of lyso-GSLs for the synthesis of various GSLs were determined using the following conditions: 1 nmol of [^{14}C]stearic acid and various amount of lyso-GSL were incubated with 10 μU SCDase in 20 μl of 25 mM phosphate buffer, pH 7.0, containing 0.1% Triton X-100 at 37°C for 20 h. The concentrations of lyso-GSLs determined (1 nmol for lyso-GalCer, 2 nmol for lyso-Gb4Cer, 4 nmol for lyso-sulfatide, and lyso-GM1a in 20 μl of reaction mixture) were used thereafter. To determine the other optimum conditions, each of the following was varied in turn: incubation time, enzyme amount, concentration of detergent, and pH. For determination of optimum pH, four different buffer solutions were used: 25 mM acetate buffer, pH 3.0-6.0; 25 mM phosphate buffer, pH 6.0-7.5; 25 mM Tris-HCl buffer, pH 7.5-8.5; 25 mM glycine-NaOH buffer, pH 8.5-10.0. After incubation, the mixture was dried, dissolved in 10 μl of solvent A and analyzed by TLC using chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v) as the developing solvent. Each radioactive GSL and fatty acid separated on a TLC was analyzed and quantified with an image analyzer (BAS 1000 model, Fuji Film, Tokyo). The extent of reaction was calculated as follows: reaction (%) = PSL for GSL $\times$ 100/(PSL for GSL + PSL for fatty acid), where PSL = photostimulated luminescence.

Preparation of Various ^{14}C-GSLs Using SCDase—To prepare a large amount of ^{14}C-GSLs, the scale of the reactions was increased as follows. For synthesis of [^{14}C]-GalCer, 100 nmol psychosine and 100 nmol of [^{14}C]stearic acid were incubated in 1 ml of 25 mM phosphate buffer, pH 6.5, containing 0.1% Triton X-100. For [^{14}C]Gb4Cer, 200 nmol of lyso-Gb4Cer and 100 nmol of [^{14}C]stearic acid were incubated in 1 ml of 25 mM phosphate buffer, pH 6.0, containing 0.1% Triton X-100. For [^{14}C]sulfatide, 400 nmol of lyso-sulfatide and 100 nmol of [^{14}C]stearic acid were incubated in 1 ml of 25 mM phosphate buffer, pH 7.0, containing 0.1% Triton X-100. For [^{14}C]GM1a, 400 nmol of lyso-GM1a and 100 nmol of [^{14}C]stearic acid were incubated in 1 ml of 25 mM phosphate buffer, pH 7.0. All incubations were carried out with 1 mU SCDase at 37°C for 20 h.

Removal of Fatty Acids and Lyso-GSLs from Reaction Mixtures—Reaction mixture containing ^{14}C-labeled GalCer, Gb4Cer, or sulfatide was dried with a Speed Vac concentrator and suspended in 1 ml of hexane/diethyl ether/acetic acid (50:50:1, v/v, solvent B), and applied to a Sep-Pak Plus Silica cartridge previously equilibrated with solvent B. Synthesized ^{14}C-GSLs and free lyso-GSLs were adsorbed on the cartridge, while free [^{14}C]stearic acids were eluted with 10 ml of solvent B. Then ^{14}C-GSLs and free lyso-GSLs were eluted from the cartridge with 10 ml of solvent A. The eluate was dried, suspended in 1 ml of distilled water, and applied to a Sep-Pak C18 cartridge to remove salts. After washing with 10 ml of distilled water, ^{14}C-GSLs and lyso-GSLs were eluted from the cartridge with 3 ml of methanol and 10 ml of solvent A. The eluate containing [^{14}C]GalCer was dried, dissolved in 1 ml of chloroform/methanol/distilled water (90:10:1, v/v), and applied to a Sep-Pak CM cartridge equilibrated with the same solvent. [^{14}C]GalCer passed through the cartridge, while psychosine was trapped. Gb4Cer, dissolved in chloroform/methanol/distilled water (65:25:4, v/v, solvent C), was applied to a Sep-Pak CM cartridge and eluted with solvent C. Lyso-Gb4Cer was trapped and eluted with solvent C, but distilled water was replaced by HCl. Sample containing [^{14}C]sulfatide was dissolved in 1 ml of solvent C and applied to a Sep-Pak QMA cartridge. Lyso-sulfatide passed through the cartridge, while [^{14}C]sulfatide was trapped and eluted with 5 ml of chloroform/methanol/0.5 N NaOH (65:25:4, v/v). [^{14}C]Sulfatide was then de-salted using a Sep-Pak C18 cartridge. Sample containing [^{14}C]-GM1a was desalted by a Sep-Pak C18 cartridge as described above, dissolved in solvent C, and applied to HPLC using a normal phase silica column (Inertsil SIL100-5, 4.6 $\times$ 250 mm, GL Science) to remove ^{14}C-fatty acid and lyso-GM1a. The column was equilibrated and eluted with solvent C.

Preparation of GM1a Labeled at the Sugar Moiety—GM1a labeled with ^{14}C at the sugar moiety was prepared as described previously (*3*). Briefly, the sialic acid and GalNAc residues were *N*-deacetylated by hydrazinolysis, and the *N*-deacetylated GM1a was re-*N*-acetylated with 1-[^{14}C]acetic anhydride to obtain the [^{14}C]GM1a.

Extraction of EGCase from a Seaflower—About 300 mg (wet weight) of sample was homogenized with 0.5 ml of 20 mM acetate buffer, pH 6.0, containing 0.2% Triton X-100, 5 mM D-galactonic acid γ-lactone, 5 mM EDTA, 1 mM PMSF, and 1 μg/ml leupeptine. After centrifugation at 10,000 rpm for 10 min, the supernatant was used as the crude EGCase preparation.

Enzyme Digestion of ^{14}C-GSLs—40 pmol ^{14}C-GSLs were treated with 2 mU EGCase II or an extract of a seaflower (100-250 μg protein) in 20 μl of 25 mM acetate buffer, pH 5.5, containing 0.2% Triton X-100. The same amounts of ^{14}C-GSLs were also treated with 0.5 mU SCDase in 20 μl of 25 mM acetate buffer, pH 6.0, containing 0.8% Triton X-100. These reactions were performed at 37°C for 5 h (EGCase II and SCDase) or 16 h (seaflower extracts). After incubation, the reaction mixtures were dried, dissolved in

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10 μl of solvent A and applied to a TLC plate which was developed with chloroform/methanol/25% ammonia solution (90:20:0.5, v/v). The TLC plate was analyzed with an imaging analyzer (BAS 1000, Fuji Film). Protein amounts were determined by BCA assay.

RESULTS AND DISCUSSION

As shown in Fig. 1A, the optimum concentration of lyso-GSLs in the reaction mixture varied depending on the species of the lyso-forms. When 1 nmol of [^{14}C]stearic acid was incubated with 10 μU SCDase at 37°C for 20 h in 20 μl of 25 mM phosphate buffer, pH 7.0, containing 0.1% Triton X-100, the optimum amounts of lyso-GSLs were as follows; 1 nmol for psychosine, 2 nmol for lyso-Gb4Cer, and 4 nmol for lyso-sulfatide and lyso-GM1a. Using each optimum concentration, the following results were obtained. Synthesis of various GSLs by SCDase increased linearly up to 6 h and reached a plateau after 20 h when the reaction efficiency was 50-70% (Fig. 1B). The reaction also reached a plateau when 25 μU SCDase was used and the reaction was carried out at 37°C for 20 h (Fig. 1C). The effects of the detergent on the reverse hydrolysis reaction by SCDase are shown in Fig. 1D. The optimum concentration of Triton X-100 was 0-0.1% and at higher concentrations, the detergent inhibited the synthesis of GSLs (Fig. 1D), while for hydrolysis of asialo-GM1a it was found to be 0.8% (5). This result indicates that the most critical factor determining the direction of the reaction is the concentration of the detergent. The optimum pH for GSL synthesis was found to be around pH 6.0-7.0 (Fig. 1E). SCDase most efficiently catalyzed the condensation of stearic acids (C18:0) to various lyso-GSLs among the fatty acids tested (Fig. 1F).

The optimum conditions determined were applied to obtain a large amount of ^{14}C-GSLs as described in "MATERIALS AND METHODS." It was found that the reaction yield of GalCer, Gb4Cer, sulfatide, and GM1a by SCDase was 67.1, 71.6, 70.3, and 54.4%, respectively (Table I). As shown in Fig. 2, no ^{14}C-GSLs were synthesized when boiled SCDase was used instead of the intact enzyme (lanes 2, 5, 8, and 11), indicating that GSL synthesis is entirely due to the action of SCDase. After incubation with SCDase, reaction mixtures contained ^{14}C-GSLs and free [^{14}C]stearic acid (lanes 1, 4, 7, and 10). Free lyso-GSLs were also found in the reaction mixture when TLC was sprayed with a ninhydrin reagent (data not shown). In this study, the methods for purification of the synthesized ^{14}C-GSLs were extensively examined. The established method consists of three steps. First, [^{14}C]stearic acid is removed from the reaction mixture by a normal phase Sep-Pak Plus Silica. Second, samples are desalted by using a reverse phase Sep-Pak C18 cartridge. Third, free lyso-GSLs are removed by a cation exchange Sep-Pak CM (for GalCer and Gb4Cer) or an anion-exchange cartridge Sep-Pak QMA (for sulfatide). The method using Sep-Pak cartridges is simple, time-saving, and repeatable. However, it is not suitable for GM1a; HPLC using a silica column was required for purification of GM1a as described in "MATERIALS AND METHODS." The purity of each ^{14}C-GSL prepared is shown in Fig. 2. Neither ^{14}C-fatty acids (lanes 3, 6, 9, and 12) nor

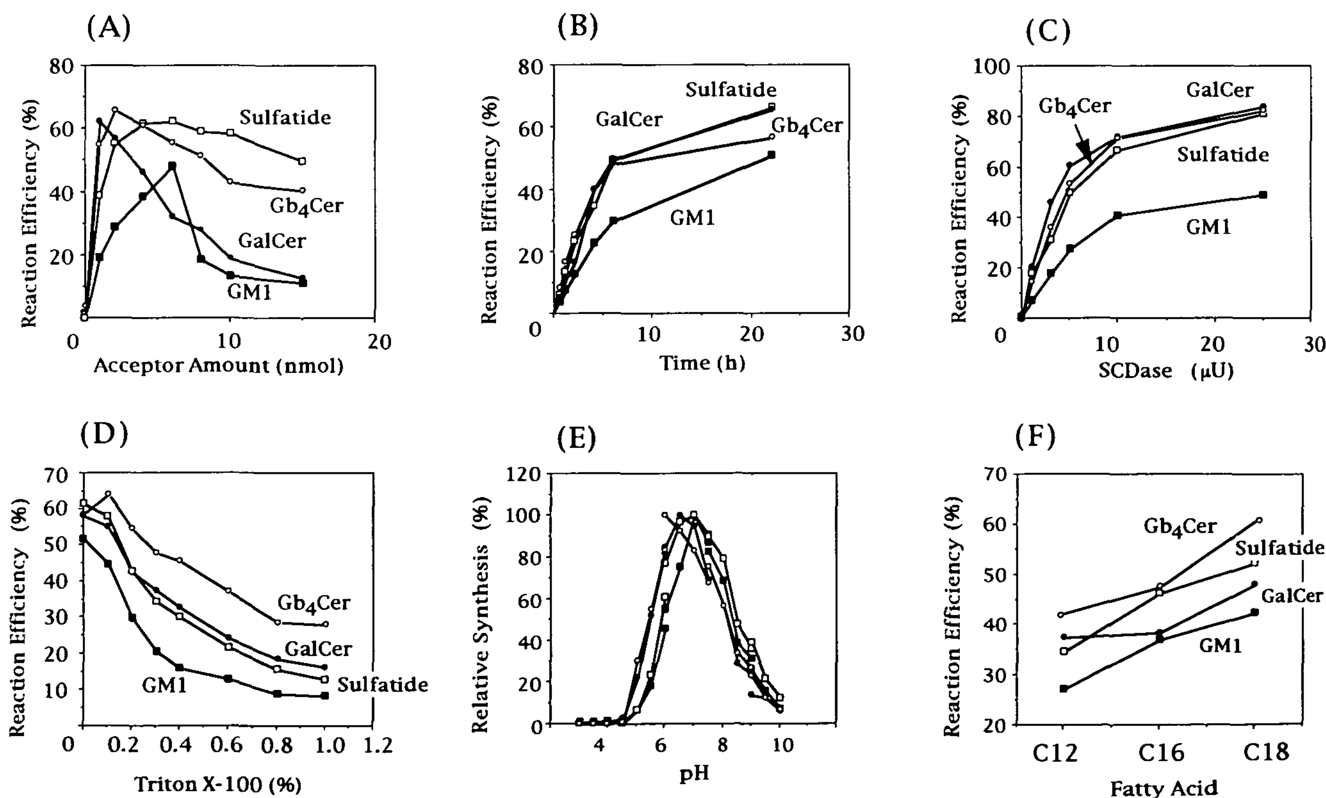

Fig. 1. **Factors in ^{14}C-GSL synthesis by SCDase.** (A), amount of lyso-GSLs; (B), incubation time; (C), enzyme amount; (D), concentration of detergent (Triton X-100); (E), effect of pH; (F), specificity for fatty acids. Values are the means of duplicate determinations. Details are described in "MATERIALS AND METHODS." ●, [^{14}C]GalCer; □, [^{14}C]sulfatide; ○, [^{14}C]Gb4Cer; ■, [^{14}C]GM1a.

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TABLE I. **Synthesis and purification of various GSLs.**

Step	GalCer (nmol)	Sulfatide (nmol)			Purpose
		Gb4Cer (nmol)		GM1 (nmol)	
[14C]Stearic acid	100	100	100	100	14C-GSL synthesis
Lyso-GSL	100	200	400	400	by SCDase[a]
Reaction mixture	67.1[b]	71.6	70.3	54.4	—
Sep-Pak Plus silica[c]	57.0	51.4	59.1	—	Removal of fatty acids
Sep-Pak C18	55.8	50.8	54.8	43.1	Removal of salts
Sep-Pak CM	45.4	43.1	—	—	Removal of lyso-GSLs
Sep-Pak QMA	—	—	48.3	—	Removal of lyso-GSLs
Sep-Pak C18	—	—	41.1	—	Removal of salts
HPLC (silica)	—	—	—	39.4	Removal of fatty acids and lyso-GM1
Final yield	45.4	43.1	41.1	39.4	—

[a]100 nmol of [14C]stearic acid and each amount of lyso-GSLs were incubated with 1 mU SCDase at 37°C for 20 h. [b]Values are the means of duplicate determinations. [c]The synthesized 14C-GSLs were purified as described in "MATERIALS AND METHODS."

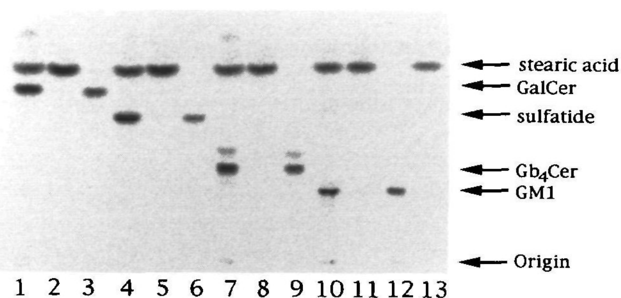

Fig. 2. **Synthesis and purification of 14C-GSLs.** Lane 1, lyso-GalCer+[14C]stearic acid+SCDase; lane 2, lyso-GalCer+[14C]stearic acid+boiled SCDase; lane 3, [14C]GalCer after purification; lane 4, lyso-sulfatide+[14C]stearic acid+SCDase; lane 5, lyso-sulfatide+[14C]stearic acid+boiled SCDase; lane 6, [14C]sulfatide after purification; lane 7, lyso-Gb4Cer+[14C]stearic acid+SCDase; lane 8, lyso-Gb4Cer+[14C]stearic acid+boiled SCDase, lane 9, [14C]-Gb4Cer after purification; lane 10, lyso-GM1a+[14C]stearic acid+SCDase; lane 11, lyso-GM1a+[14C]stearic acid+boiled SCDase; lane 12, [14C]GM1a after purification; lane 13, standard [14C]stearic acid. TLC was analyzed by an image analyzer (BAS 1000). Details are described in "MATERIALS AND METHODS." Arrow indicates the R_f of standard GSLs and fatty acids.

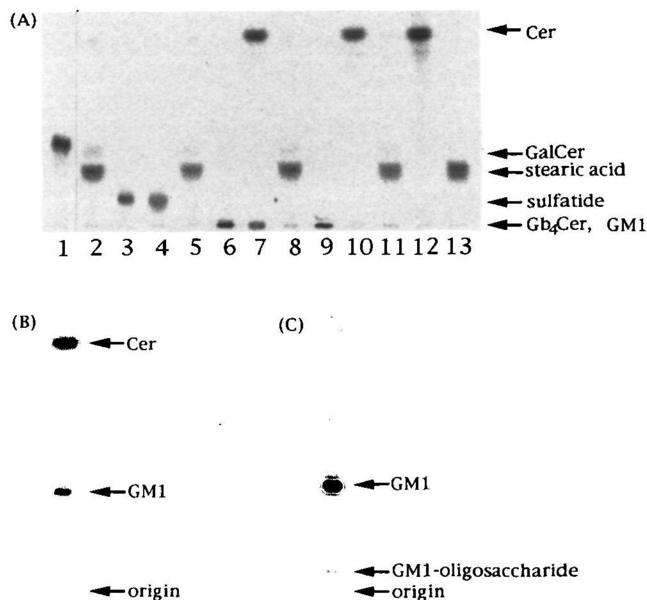

Fig. 3. **Hydrolysis of 14C-GSLs by EGCase or SCDase.** (A) Lane 1, [14C]GalCer; lane 2, [14C]GalCer+SCDase; lane 3, [14C]sulfatide; lane 4, [14C]sulfatide+EGCase; lane 5, [14C]sulfatide+SCDase; lane 6, [14C]Gb4Cer; lane 7, [14C]Gb4Cer+EGCase; lane 8, [14C]-Gb4Cer+SCDase; lane 9, [14C]GM1a; lane 10, [14C]GM1a+EG-Case; lane 11, [14C]GM1a+SCDase; lane 12, standard [14C]Cer; lane 13, standard [14C]stearic acid. (B) [14C]GM1a (labeled at fatty acid)+extract of a seaflower (234 μg protein). (C) [14C]GM1a (labeled at sialic acid and GalNAc)+extract of a seaflower (110 μg protein). TLCs were developed with chloroform/methanol/25% ammonia solution (90:20:0.5, v/v) for (A), chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v) for (B) and (C), and analyzed by use of an image analyzer (BAS 1000). Arrow indicates the R_f of standard GSLs, Cer, fatty acids, and GM1a-derived sugar chains. Details are described in "MATERIALS AND METHODS."

lyso-GSLs (ninhydrin-positive bands, data not shown) were found in purified GSLs. The yield of various GSLs at each step is summarized in Table I. The final yield after purification was 45.4% for GalCer, 43.1% for Gb4Cer, 41.1% for sulfatide, and 39.4% for GM1a (Table I).

We have isolated and characterized the two novel GSL-degrading enzymes: EGCase, which hydrolyzes the glycosidic linkage between oligosaccharide and ceramide of various GSLs (*8, 9*), and SCDase, which hydrolyzes the *N*-acyl linkage between sphingosine and fatty acid in ceramide of various GSLs and sphingomyelin (*5*). As shown in Fig. 3, [14C]Gb4Cer and [14C]GM1a were hydrolyzed by EGCase II, an isoform of EGCase, to release 14C-ceramides (lanes 7 and 10), but [14C]sulfatide was completely resistant to the enzyme (lane 4). All 14C-GSLs tested were hydrolyzed by SCDase to release [14C]stearic acids (lanes 2, 5, 8, and 11). As little as 5 pmol of 14C-ceramide or 14C-fatty acid released by the enzymes can be determined with an imaging analyzer (BAS 1000). These results are consistent with those obtained previously using non-radiolabeled substrates, but the sensitivity by this assay was more than 100 times higher than that reported previously (*9, 5*).

Besides prokaryotes, actinomycetes (*8, 9*), and bacteria (*14*), EGCase (also called ceramide glycanase) has been found in the leech (*10*), earthworm (*11*), and hard-shelled clam (*12*). Using the assay method described above, we found EGCase activity in a coelenterate for the first time. Figure 3B shows the release of 14C-ceramide from [14C]-GM1a by an extract from the seaflower *Condylactis* sp. 14C-labeled GM1a-oligosaccharide was also detected when a GM1a substrate labeled at the sialic acid and GalNAc

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residues was used (Fig. 3C). Hydrolysis of GM1a by the extract therefore appears to be due entirely to the action of EGCase, and not that of exoglycosidases.

Recently we cloned the gene encoding EGCase II of *Rhodococcus* sp. M-777 (*15*). Interestingly, the active site of EGCase II was homologous to that of the endo-β1,4-glucanase family A (*15*). Once we detect the activity of EGCase even in low quantity, it is possible to carry out homology cloning of the enzyme using the known EGCase II gene. In conclusion, the sensitive method for EGCase detection we have developed in this study has considerable potential.

The preparation of ^{14}C-GSLs using the reverse hydrolysis reaction of SCDase can be applied to various species of sphingolipids, not only neutral and acidic GSLs as shown in this study, but also ceramides (*7*), and thus should facilitate sphingolipid research.

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