methods

Enhancement of hydrolytic activity of sphingolipid ceramide *N*-deacylase in the aqueous–organic biphasic system

Toyohisa Kurita,* Hiroyuki Izu,* Mutsumi Sano,[1,*] Makoto Ito,[†] and Ikunoshin Kato*

Biotechnology Research Laboratories,* Takara Shuzo Co., Ltd., Seta 3-4-1, Otsu, Shiga 520-2193, Japan and Department of Bioscience and Biotechnology,[†] Division of Bioresource and Bioenvironmental Sciences, Graduate School Kyushu University, Fukuoka 812-8581, Japan

Abstract Lysoglycosphingolipids were produced from glycosphingolipids by using sphingolipid ceramide *N*-deacylase, which cleaves the *N*-acyl linkage between fatty acids and sphingosine bases in various glycosphingolipids. The enzyme reaction was done in a biphasic media prepared with water–immiscible organic solvent and aqueous buffer solution containing the enzyme. We investigated the effects of organic solvents and detergents on lysoglycosphingolipid production in the biphasic system. Among the organic solvents tested, n-butylbenzene, cumene, cyclodecane, cyclohexane, n-decane, diisopropylether, n-heptadecane, and methylcyclohexane promoted hydrolysis of GM1, whereas benzene, chloroform, ethyl acetate, and toluene inhibited GM1 hydrolysis. Hydrolysis of asialo GM1, GD1a, GalCer, and sulfatide was also enhanced by the addition of n-decane. The hydrolytic activity of the enzyme was enhanced by the addition of 0.8% sodium taurodeoxycholate or sodium cholate to the aqueous phase. The most effective hydrolysis of various glycosphingolipids by the enzyme was thus obtained in the aqueous-n-decane biphasic system containing 0.8% sodium taurodeoxycholate. Under this condition, the fatty acids released from GM1 by the action of the enzyme were trapped and diffused into the organic phase, while lysoGM1 remained in the aqueous phase. Thus the almost complete hydrolysis of GM1 was achieved using the biphasic system, while at most 70% of hydrolysis was obtained using normal aqueous media possibly due to the inhibition of hydrolysis reaction by accumulation of fatty acids in the reaction mixture.—Kurita, T., H. Izu, M. Sano, M. Ito, and I. Kato. **Enhancement of hydrolytic activity of sphingolipid ceramide *N*-deacylase in the aqueous–organic biphasic system. *J. Lipid Res.* 2000.** 41: **846–851.**

Supplementary key words sphingolipid ceramide • *N*-deacylase • lysosphingolipid • biphasic system • organic solvent • detergent

Glycosphingolipids (GSLs) are major constituents of eukaryotic membrane lipids. LysoGSLs, which are *N*-deacylated in the ceramide moiety of GSLs, are detected in normal tissues or cells at quite low levels, although they accumulate in various lysosomal storage diseases, including Gaucher's disease (1), Krabbe's disease (2), metachromatic leukodystrophy (3), and Sandhoff and Tay-Sachs diseases (4). Because lysoGSLs inhibit protein kinase C (5) and they are cytotoxic (6–8), lysoGSLs are suspected to be the substances responsible for the pathology of the lysosomal storage diseases (5, 9).

In addition to their biological activities, lysoGSLs are useful materials for sphingolipid technology. GSLs can be easily labeled by acylation of lysoGSLs using fatty acids labeled with radio isotope or fluorescent reagents. LysoGSLs can be immobilized onto solid phase supports, including glass beads, microtiter plates, and resins, by using primary amine residues of lysoGSLs. LysoGSLs are important starting materials for the preparation of neoglycoconjugates including glycosphingosyl-peptides, proteins, or synthetic polymers.

Although various procedures have been used for the preparation of lysoGSLs (10–12), they include many steps for alkaline hydrolysis and *N*-acetylation, and thus the yield is poor, especially for lysogangliosides.

Sphingolipid ceramide *N*-deacylase (SCDase) hydrolyzes the *N*-acyl linkage between fatty acids and sphingosine bases in the ceramide moiety of various GSLs and sphingomyelin (13). The enzyme also catalyzes the condensation reaction (reverse hydrolysis) between fatty acids and sphingosine bases to form sphingolipids (14, 15). LysoGSLs are easily prepared by using this enzyme, however, the enzyme does not completely hydrolyze GSL substrates even after prolonged incubation, possibly due to the equilibrium between the hydrolysis and condensation reactions.

Abbreviations: Cer, ceramide; GalCer, Galβ1,1′Cer; GD1a, NeuAcα2, 3Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1,1′Cer; GM1, Galβ1, 3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1,1′Cer; GSL, glycosphingolipid; HPLC, high performance liquid chromatography; PDAM, 1-pyrenyldiazomethane; SCDase, sphingolipid ceramide *N*-deacylase; TDC, sodium taurodeoxycholate; TLC, thin-layer chromatography.

[1] To whom correspondence should be addressed.

In the present paper, we describe the enzymatic production of lysoGSLs in the aqueous–organic biphasic system. The conversion yield of lysoGSLs in the biphasic system was much higher than that in the aqueous monophasic media. The present methods should facilitate further studies of lysoGSLs and GSLs.

MATERIALS AND METHODS

Materials

GSLs were obtained from Matreya (Pleasant Gap, PA). n-Decane, n-heptadecane, and sodium taurodeoxycholate (TDC) were purchased from Nacalai tesque, Inc. (Kyoto, Japan). Triton X-100 was from Pierce (Rockford, IL). 1-Pyrenyldiazomethane (PDAM) was purchased from Aldrich (Milwaukee, WI). High performance thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). All reagents were of special grade.

Preparation and assay of SCDase

SCDase was purified from the culture fluid of *Pseudomonas* sp. TK-4 by the method described elsewhere (13) and the same enzyme can be obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan).

SCDase was assayed by incubating with 10 nmol of asialo GM1 for 30 min at 37°C in 10 μl of 50 mm acetate buffer at pH 5.0 containing 0.8% Triton X-100. The reaction was stopped by boiling for 3 min in a water bath. The reaction mixture was then dried using a concentrator, redissolved in 10 μl of chloroform–methanol 2:1 (v/v), and then analyzed by TLC using chloroform–methanol–10% acetic acid 5:4:1 (v/v/v) as a developing solvent. Asialo GM1 and lysoasialo GM1 were visualized using an orcinol-H_2SO_4 spray reagent. The TLC plate was then scanned using an imaging densitometer (Model GS-700, Bio-Rad) to determine asialo GM1 and lysoasialo GM1. The extent of hydrolysis was calculated by: hydrolysis (%) = (spot area for lysoasialo GM1 generated) × 100/(spot area for asialo GM1 remaining + spot area for lysoasialo GM1 generated). One unit of activity was defined as the amount of enzyme needed to release 1 μmol of lysoasialo GM1 from asialo GM1 per min under the above assay conditions.

The SCDase preparation used throughout this study had a specific activity of 31 U/mg protein. The protein was assayed by micro BCA protein assay reagent (Pierce) using bovine serum albumin as a standard.

Hydrolysis of GSLs by SCDase in the biphasic system

A standard reaction mixture contained 100 μl of organic solvent and 10 μl of 50 mm sodium acetate at pH 6.0 containing 10 nmol of GSL, 6 mU of SCDase, and 0.8% TDC or Triton X-100. The mixture was incubated in a 1.5 ml polypropylene tube at 37°C for 16 h. GSL and lysoGSL in the aqueous phase were assayed by the method described below.

Analysis of reaction products

GSLs and lysoGSLs in the reaction mixture were analyzed by TLC using chloroform–methanol–10% acetic acid 5:4:1 (v/v/v) as a developing solvent, and they were visualized using an orcinol–H_2SO_4 spray reagent. The TLC plate was then scanned using the imaging densitometer, and the extent of hydrolysis was calculated by the method described above.

Analysis of fatty acids

The organic phase was separated from the aqueous phase at selected reaction time points, and then each fraction was dried using a concentrator. The fractions were redissolved in 50 μl methanol and sonicated. The fatty acids were labeled by the addition of 50 μl of 1 mg/ml PDAM ethyl acetate solution to the sample solution and the resulting mixture was allowed to stand for 90 min at room temperature. A 50 μl aliquot of the mixture was analyzed by HPLC using a C18-silica column (COSMOSIL 5C18-MS Column, Nacalai tesque). The PDAM-fatty acids were eluted at a flow rate of 1 ml/min with acetonitrile, and they were detected by fluorescence at excitation and emission wavelengths of 340 and 375 nm, respectively. The stearic acid content was determined by comparing the elution position and peak area of the sample with those of authentic standards.

RESULTS

SCDase hydrolyzes the *N*-acyl linkage between fatty acids and sphingosine bases in the ceramide moiety of various GSLs and sphingomyelin. The enzyme also catalyzes the reverse hydrolysis (condensation reaction) between fatty acids and sphingosine bases to form sphingolipids. **Figure 1** shows both reactions. LysoGSLs are easily prepared by using this enzyme, however, the conversion yield of lysoGM1 was at most 50 to 70% in the aqueous monophasic system (**Fig. 2**). This is possibly due to the equilibrium between the hydrolysis and condensation reactions. Thus we tested GM1 hydrolysis by SCDase in the aqueous–organic biphasic system. GM1 hydrolysis was enhanced by the addition of hydrocarbons, such as n-decane, n-heptadecane, cyclodecane, cyclohexane, and methylcyclohexane to the reaction mixture. GM1 hydrolysis was also elevated by the addition of cumene, n-butylbenzene, and diisopropylether. TDC was more effective against GM1 hydrolysis than Triton X-100 in both the aqueous system and biphasic system. GM1 hydrolysis was inhibited by the addition of benzene, chloroform, ethyl acetate, and toluene, and the effects of TDC and Triton X-100 were almost the same in these cases.

Table 1 shows the effects of detergents on GM1 hydroly-

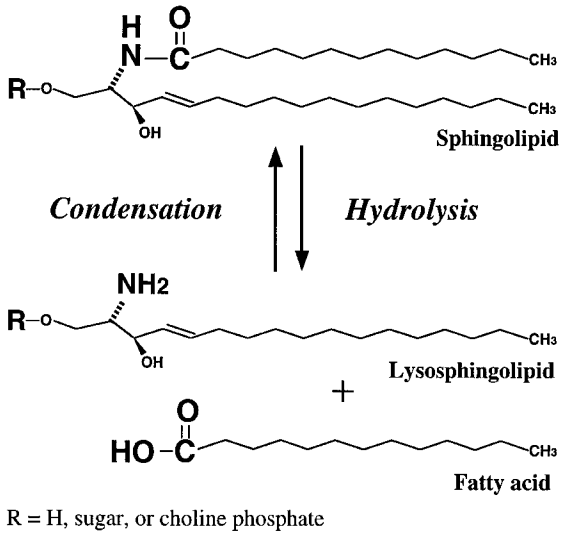

Fig. 1. Hydrolysis and reverse hydrolysis (condensation) reactions catalyzed by SCDase.

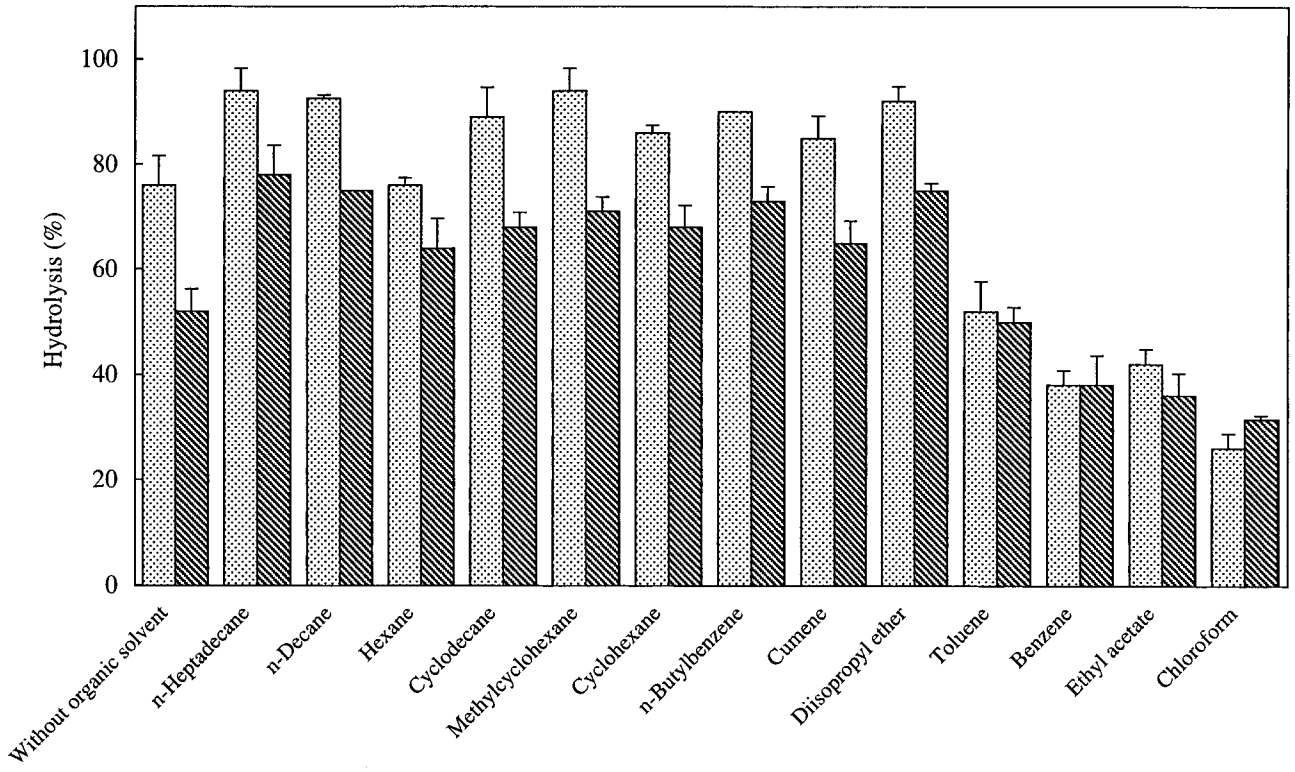

Fig. 2. Hydrolysis of GM1 by SCDase in the biphasic system using various organic solvents. A 100-μl aliquot of organic solvent was added to 10 μl of 50 mm sodium acetate at pH 6.0 containing 10 nmol of GM1, 6 mU of SCDase, and 0.8% TDC (■) or Triton X-100 (▨). The mixture was then incubated at 37°C for 16 h. GM1 and lysoGM1 in the aqueous phase were assayed by the method described in Materials and Methods. Each value is mean ± SD from two independent experiments.

sis by SCDase in the biphasic system. The hydrolysis activity of SCDase was enhanced by the addition of detergents. TDC and sodium cholate were more effective in GM1 hydrolysis than the nonionic detergents such as Triton X-100, Tween 20, Nonidet P-40, Brij 58, and Lubrol PX. Hydrolysis of GM1 was maximum in the presence of 0.8% TDC in the aqueous phase (**Fig. 3**).

GM1 was hydrolyzed in the biphasic system using various ratios of the organic solvent to the aqueous buffer (**Fig. 4**). At least five times as much organic solvent as aqueous buffer was required in the aqueous–organic system for maximal hydrolysis of GM1 by the enzyme.

TABLE 1. Effects of detergents on hydrolysis of GM1 by SCDase in the biphasic system

Detergents	Hydrolysis of GM1
	%
Without detergent	29 ± 4
TDC	97 ± 1
Sodium cholate	97 ± 3
Triton X-100	65 ± 4
Tween 20	48 ± 6
Nonidet P-40	63 ± 1
Brij 58	63 ± 4
Lubrol PX	62 ± 3

A 100-μl aliquot of n-decane was added to 10 μl of 50 mm acetate buffer at pH 6.0 containing 10 nmol of GM1, 6 mU of SCDase, and 0.8% detergent. The mixture was then incubated at 37°C for 16 h. The hydrolysis of GM1 in the aqueous phase was determined by the method described in Materials and Methods. Each value is mean ± SD from two independent experiments.

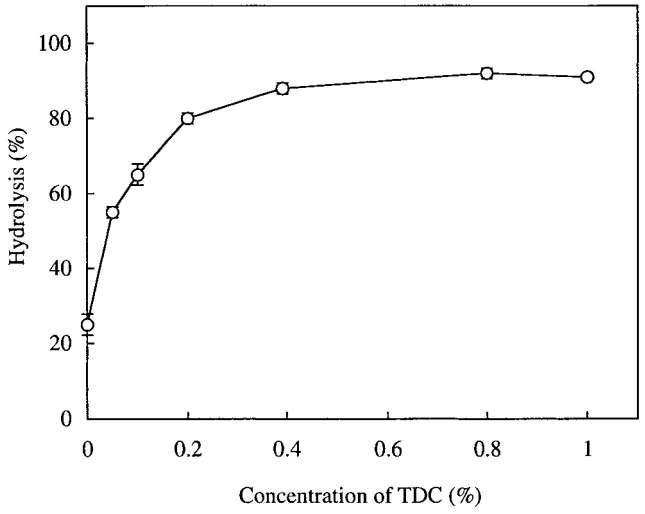

Fig. 3. Effects of TDC on GM1 hydrolysis in the biphasic system. A 100-μl aliquot of n-decane was added to 10 μl of 50 mm sodium acetate at pH 6.0 containing 10 nmol of GM1, 6 mU of SCDase, and 0–1.0% TDC. The mixture was then incubated at 37°C for 16 h. GM1 and lysoGM1 in the aqueous phase were assayed by the method described in Materials and Methods. Each value is mean ± SD from two independent experiments.

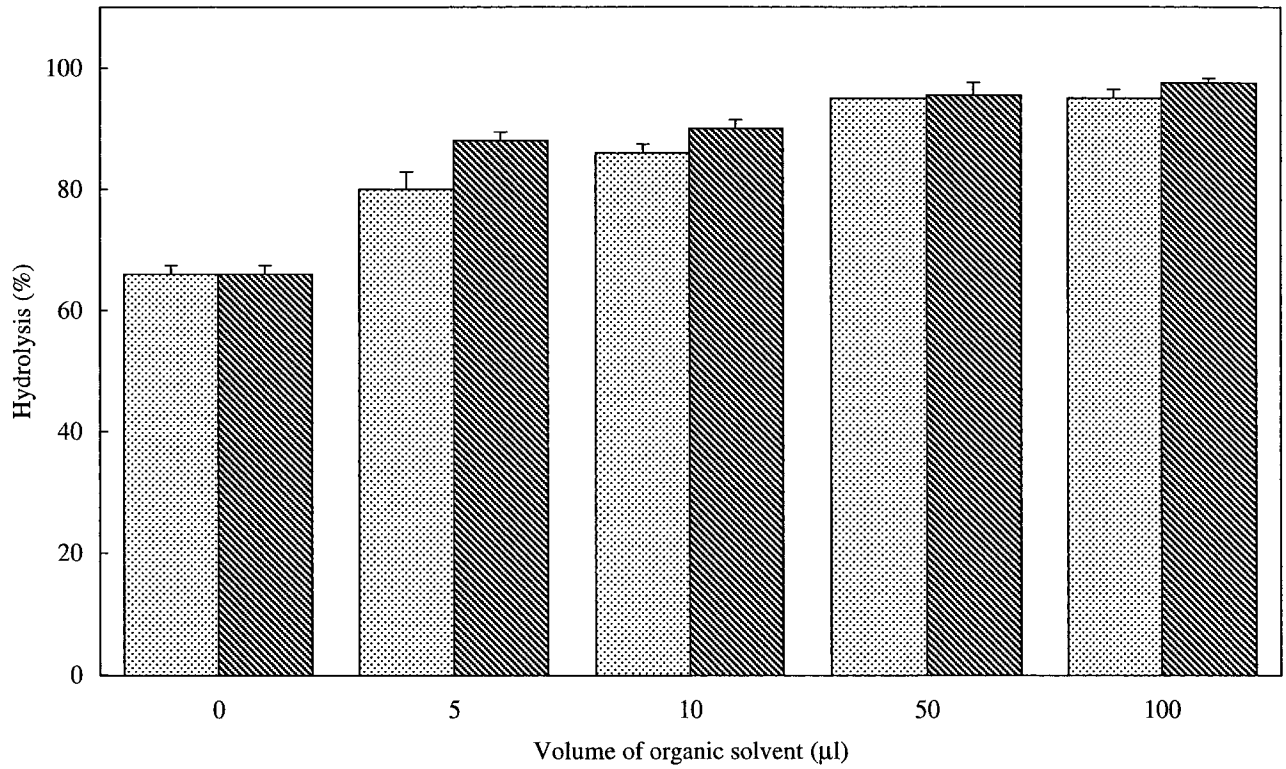

Fig. 4. Effects of organic solvent quantity on hydrolysis of GM1. n-Decane (▩) or n-heptadecane (▨) was added to 10 μl of 50 mm sodium acetate at pH 6.0 containing 10 nmol of GM1, 6 mU of SCDase, and 0.8% TDC. The mixture was then incubated at 37°C for 16 h. GM1 and lysoGM1 in the aqueous phase were assayed by the method described in Materials and Methods. Each value is mean ± SD from two independent experiments.

Hydrolysis of various GSLs in the biphasic system is summarized in **Table 2**. Addition of n-heptadecane was effective against hydrolysis of all GSLs tested, although the extent of enhancement of GalCer and sulfatide was less than that of the other GSLs. Hydrolysis of sphingomyelin also increased more in the biphasic system than in the aqueous monophasic system.

Enzymatic production of lysoGM1 from GM1 was enhanced in the "aqueous-n-decane" biphasic system with a yield of 95%, whereas the yield of lysoGM1 in the aqueous monophasic system was at most 70% (**Fig. 5A**). Fatty acids, which were released from GM1 by SCDase action, in the aqueous and organic phases were analyzed (Fig. 5B). Stearic acid was mainly detected in the n-decane phase and it increased as the reaction progressed. A small amount of stearic acid was detected in the aqueous phase in the early stage of the reaction, but its content then decreased during the course of the reaction. TDC, together with lysoGSL and a small amount of GSL, was mainly recovered in the aqueous phase. Those were separated by a reversed phase HPLC column (Poros R2/H, 10 × 100 mm, PerSeptive Biosystems, Framingham, MA). LysoGSL was eluted at a flow rate of 1.0 ml/min at room temperature with solvents A and B. Solvent A was acetonitrile–water, 1:9 (vol/vol). Solvent B was acetonitrile–methanol 1:9 (vol/vol). The column was equilibrated with solvent A–solvent B 8:2 (vol/vol). After injection of the aqueous phase, the ratio of solvent B to solvent A was held constant for 30 min, and TDC were eluted in the pass-through fraction. The proportion of solvent B was then increased in a linear gradient to 100% in 60 min (fraction size, 1 ml). Each fraction was analyzed by TLC and fractions containing lysoGSL were collected. The purified lysoGSL was free from TDC, buffer salts, and a substrate GSL.

TABLE 2. Hydrolysis of various sphingolipids by SCDase in the biphasic system

Substrates	Hydrolysis	
	Biphasic System	Aqueous System
	%	%
GD1a	92 ± 1	65 ± 0
Asialo GM1	95 ± 3	68 ± 6
GM1	97 ± 1	65 ± 3
Sulfatide	79 ± 1	72 ± 1
GalCer	77 ± 4	70 ± 4
Sphingomyelin	45 ± 4	30 ± 4

The hydrolysis of various GSLs and sphingomyelin in the aqueous phase was determined by the method described in Materials and Methods. Each value is mean ± SD from two independent experiments.

DISCUSSION

SCDase is a unique enzyme that catalyzes both hydrolysis and condensation of the *N*-acyl linkage between fatty acids and sphingosine bases in the ceramide moiety of var-

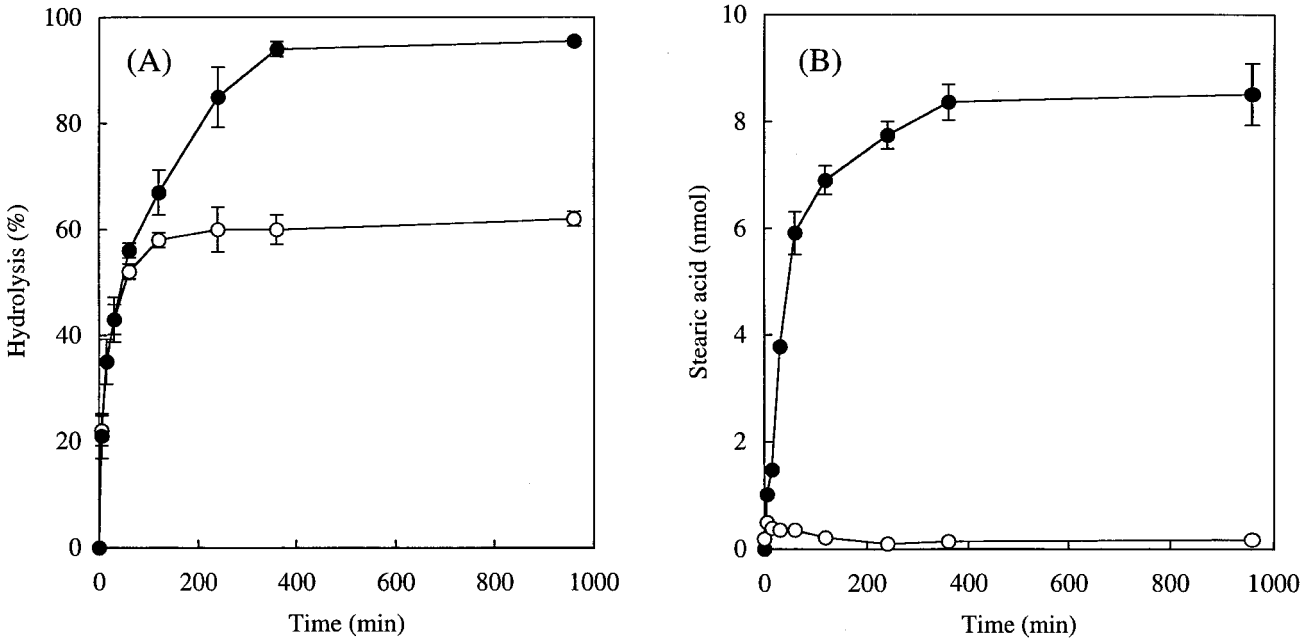

Fig. 5. Fatty acid formation during the course of GM1 hydrolysis by SCDase in the biphasic system. (A), A 10-μl aliquot of 50 mm sodium acetate at pH 6.0 containing 10 nmol of GM1, 6 mU of SCDase, and 0.8% TDC was incubated at 37°C with (●) or without (○) 100 μl of n-decane. GM1 and lysoGM1 in the aqueous phase were assayed by the method described in Materials and Methods. (B), Fatty acids in the organic phase (●) and aqueous phase (○) were labeled with PDAM and analyzed by HPLC by the method described in Materials and Methods. Each value is mean ± SD from two independent experiments.

ious GSLs and sphingomyelin (13–15). The enzyme does not completely hydrolyze GSLs and the yield of lysoGSLs is at most 60–70%. Because SCDase catalyzes the condensation reaction, accumulation of fatty acids and lysoGSLs in the reaction medium may decrease hydrolysis of GSLs.

The aqueous–organic biphasic system has been applied to enzyme reactions (16), in which water-immiscible solvents are used. The system is especially useful when hydrophobic substances are produced because enzymes work in the aqueous phase and do not diffuse into organic phase. We applied the "aqueous–organic" biphasic system to lysoGSL production using SCDase, expecting that the fatty acids, which are produced from GSLs in the aqueous media during hydrolysis, may diffuse into the organic phase, and hydrolysis of GSLs by SCDase in the aqueous phase will increase. Among the organic solvents tested, highly hydrophobic solvents, including n-decane and n-heptadecane, promoted GM1 hydrolysis. Fatty acids, which were released from GM1, diffused into the n-decane phase. In contrast, only a low level of fatty acids was detected in the aqueous phase during the reaction (Fig. 5B). The condensation reaction might be suppressed by diffusion of fatty acids into the organic phase, and as a result the hydrolysis of GSLs is promoted.

Benzene, chloroform, ethyl acetate, and toluene are often used for efficient extraction of such hydrophobic substances as fatty acids, and it is expected that addition of these organic solvents to SCDase reaction media would effectively promote deacylation by decreasing fatty acid content in the aqueous phase. Against expectations, GM1 hydrolysis decreased after addition of these solvents. The reason is unclear at present, but the decrease does not seem to be due to inactivation of SCDase by the organic solvent because the enzyme retained 40–80% of its activity after 3 h incubation in the biphasic system (data not shown).

Lysosphingolipids have been prepared by chemical procedures such as alkaline hydrolysis (10–12). As alkaline treatment releases *N*-acyl residues in both ceramide and sugar moieties, the products lose considerable *N*-acetyl groups in the carbohydrate moiety, and thus the amine residues in the carbohydrate moiety need to be selectively reacetylated. The enzymatic procedure for lysosphingolipids production using SCDase is much easier than the chemical method, and the procedure does not alter the oligosaccharide and sphingoid moieties (13). The primary amine residue generated in lysoGSLs can be used for labeling by ^{14}C- or fluorescent-labeled fatty acids, which are good probes for studies of GSL binding proteins or intracellular transport of GSLs. The primary amine residues in lysoGSLs could be used for conjugation with peptides, proteins, synthetic polymers, or resins, which are useful as artificial GSL antigens or resins for affinity chromatography. LysoGSLs have been used for design and synthesis of bioactive neoglycoconjugates, which have potential as carbohydrate drugs (17). The proposed biphasic system appears to be useful for production of lysoGSLs and lysosphingomyelin, and can facilitate further study of lysosphingolipid function and sphingolipid engineering.

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