methods

# A facile method for controlling the reaction equilibrium of sphingolipid ceramide N-deacylase for lyso-glycosphingolipid production[S]

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**Abstract Lyso-glycosphingolipids (lyso-GSLs), the N-deacylated forms of glycosphingolipids (GSLs), are important synthetic intermediates for the preparation of GSL analogs. Although lyso-GSLs can be produced by hydrolyzing natural GSLs using sphingolipid ceramide N-deacylase (SCDase), the yield for this reaction is usually low because SCDase also catalyzes the reverse reaction, ultimately establishing an equilibrium between hydrolysis and synthesis. In the present study, we developed an efficient method for controlling the reaction equilibrium by introducing divalent metal cation and detergent in the enzymatic reaction system. In the presence of both $Ca^{2+}$ and taurodeoxycholate hydrate, the generated fatty acids were precipitated by the formation of insoluble stearate salts and pushing the reaction equilibrium toward hydrolysis. The yield of GM1 hydrolysis can be achieved as high as 96%, with an improvement up to 45% compared with the nonoptimized condition. In preparative scale, 75 mg of lyso-GM1 was obtained from 100 mg of GM1 with a 90% yield, which is the highest reported yield to date. The method can also be used for the efficient hydrolysis of a variety of GSLs and sphingomyelin.[JLR] Thus, this method should serve as a facile, easily scalable, and general tool for lyso-GSL production to facilitate further GSL research.—** Huang, F.-T., Y.-B. Han, Y. Feng, and G.-Y. Yang. **A facile method for controlling the reaction equilibrium of sphingolipid ceramide N-deacylase for lyso-glycosphingolipid production. *J. Lipid Res.* 2015.** 56: **1836–1842.**

**Supplementary key words** enzymology • gangliosides • sphingolipids • endocytosis • mass spectrometry • lyso-GM1 • sphingolipid engineering

Glycosphingolipids (GSLs) are amphipathic compounds that function as essential structural components of mammalian cell membranes. They play very important roles in numerous cellular functions (1–3), including signal transduction, cell adhesion/recognition, endocytosis, apoptosis, autophagy, brain aging, neurodegeneration, and neuroprotection. Lyso-glycosphingolipids (lyso-GSLs), which are N-deacylated derivatives of GSLs, are present in trace amounts in normal tissues but accumulate in several lysosomal diseases (4). Lyso-GSLs such as glucosylsphingosine, globotriaosylsphingosine, and lyso-GM2 are biomarkers for type 1 Gaucher disease (5), Fabry disease (6), Tay-Sachs disease (7), and Sandhoff disease (7). Lyso-GSLs have also been observed to inhibit the activity of protein kinase C (8) and to induce apoptosis (9). Importantly, lyso-GSLs can be used as starting material for the synthesis of various GSL analogs, which are valuable tools for GSL biology research (10–18), pharmaceutical development (19, 20), drug delivery (21), bacterial toxin detection (22), and enzyme assay (23–25). Chemical methods for preparing lyso-GSLs from their parent GSLs have been reported (19, 26–30). However, these methods result in poor product yields due to the formation of by-products resulting from the removal of acetyl groups from sialic acid and N-acetylhexosamine (19, 26–30). For example, the product yields are 54–60% for lyso-GM1 (19, 27) and 36–40% for lyso-GM3 (30). Enzymatic hydrolysis of GSLs, on the other hand, may provide an attractive alternative because of its inherent substrate specificity and high catalytic efficiency.

Sphingolipid ceramide N-deacylase (SCDase) catalyzes reversible reactions in which the amide linkages in GSLs are either hydrolyzed or synthesized (**Fig. 1**). Four SCDases from different prokaryotic microbes have been reported

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Abbreviations: GalCer, galactosylceramide; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; GSL, glycosphingolipid; lyso-GSL, lyso-glycosphingolipid; lyso-SM, sphingosylphosphorylcholine; PS_SCD, SCDase from *Pseudomonas sp.* TK4; SCDase, sphingolipid ceramide N-deacylase; SA_SCD, SCDase from *Shewanella alga* G8; SM, sphingomyelin; TDC, taurodeoxycholate hydrate.

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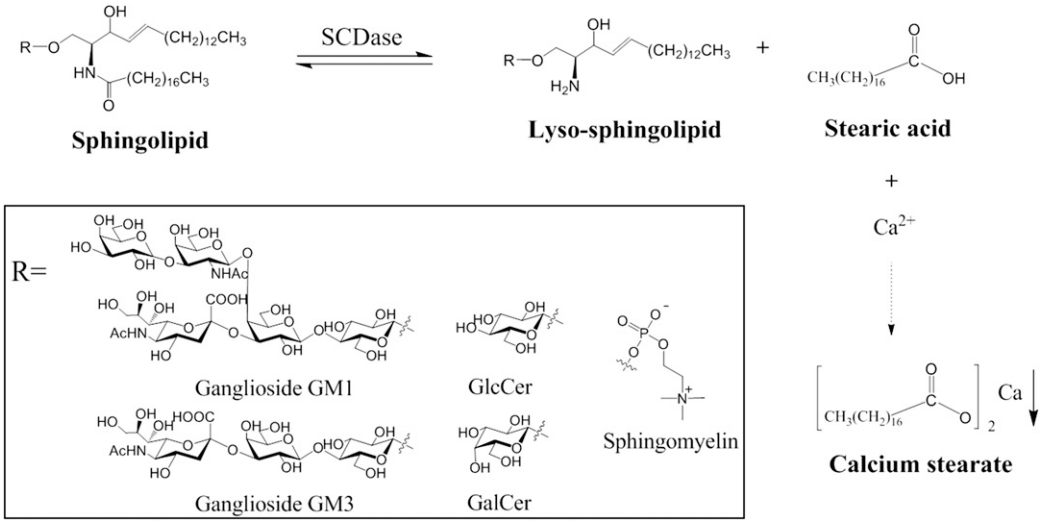


**Fig. 1.** SCDase catalyzes reversible reactions in which the amide linkage in the ceramide moiety of various GSLs is hydrolyzed or synthesized. In the presence of $Ca^{2+}$ and TDC, the reaction equilibrium can be pushed toward hydrolysis by the formation of insoluble stearate salt.

(31–34), but only those from *Pseudomonas sp.* TK4 (PS_SCD) and *Shewanella alga* G8 (SA_SCD) have been well characterized (33–35). Both enzymes have been used in the preparation of lyso-GSLs (22, 36, 37). Previously, we found that SA_SCD showed higher catalytic efficiency and broader fatty acid specificity, making it a better biocatalyst than the commercial PS_SCD (35). However, enzymatic hydrolysis yields were generally low as a result of the equilibrium between hydrolysis and synthesis. Kurita et al. (38) reported that GSL hydrolysis has been improved by an aqueous-organic biphasic system in which the fatty acids released from the hydrolysis reaction were diffused into the water-immiscible organic phase, enhancing GSL hydrolysis in the aqueous phase. However, the yield of the biphasic system is decreased in preparative-scale reactions (22, 36), in which case the extraction of fatty acid is not so efficient because of higher substrate concentration and larger reaction volume.

In the present study, we developed an easier, alternative method that can also improve SCDase hydrolysis efficiency but is more compatible with preparative-scale reactions. We found that the combination of $Ca^{2+}$ and taurodeoxycholate hydrate (TDC) enhanced GSL hydrolysis significantly. The utility of this method for the hydrolysis of various GSLs and SM, as well as in the large-scale preparation of lyso-GM1, was demonstrated. The mechanism of the method was discussed.

## MATERIALS AND METHODS

### Materials

Ganglioside GM1 was obtained from Qilu Pharmaceutical (Jinan, China). Ganglioside GM3 was prepared as described by Rich et al. (39). Glucosylceramide (GlcCer), glucosylsphingosine (GlcSph), galactosylceramide (GalCer), galactosylsphingosine (GalSph), SM, and sphingosylphosphorylcholine (lyso-SM) were purchased from Avanti Polar Lipids Inc.. Triton X-100, TDC, and sodium cholate were purchased from Sigma-Aldrich. Tween 80 was purchased from Beijing Dingguo Changsheng Biotech, China. All of the other chemicals were of analytical or higher grade. Sep-Pak tC18 cartridges (500 mg sorbent) were purchased from Waters.

### Protein expression and purification

Recombinant SA_SCD was heterologously expressed in *Escherichia coli* BL21 (DE3) (35). *E. coli* cells containing pET23b-SA_SCD were grown overnight at 37°C in Luria-Bertani medium containing 100 μg/ml ampicillin. Auto-induction medium (ZYM-5052) (40) containing 100 μg/ml ampicillin was then inoculated with the cultures, which were grown at 37°C. When the cultures reached 2.2–2.4 $OD_{600}$, they were transferred to 16°C and grown for another 20 h. After induction, the cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 20 mM imidazole (20 ml buffer per 1 g cell pellet). The cells were lysed by sonication and centrifuged at 12,000 rpm for 30 min. The resulting supernatant was collected, and SA_SCD was purified using a $Ni^{2+}$-chelating affinity column. The purified protein was dialyzed against storage buffer (25 mM Tris-HCl [pH 7.4] and 10% glycerol) and stored at −80°C. The protein concentration was determined with the Bradford method using BSA as a standard.

### Enzyme activity assay

SCDase activity was measured using GM1 as the substrate. The reactions contained 100 nmol of GM1 and appropriate amounts of enzyme solution in 100 μl of 40 mM sodium acetate buffer (pH 5.8) and 0.08% Triton X-100. After 5 min of incubation at 37°C, the enzymatic reactions were terminated by boiling for 5 min. The reactions were analyzed by HPLC as described below. One unit of SCDase activity was defined as the amount of enzyme that released 1 μmol of lyso-GM1 per minute under the above conditions.

### Optimization of enzymatic hydrolysis

To improve GSL enzymatic hydrolysis, 100 μl reactions were assembled in 35 mM sodium acetate buffer (pH 5.8) as described below. The divalent metal cations were $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, and

$Mg^{2+}$. $Ca^{2+}$ concentrations were 2.5–100 mM. The detergents were Triton X-100, TDC, sodium cholate, and Tween 80; TDC concentrations were 0.07–0.7% (w/v), and GM1 concentrations were 0.5–8 mM. Reactions containing approximately 74 mU of SA_SCD were incubated at 37°C for 12 h before HPLC analysis of the reaction products as described below.

### Quantitative HPLC analysis of GM1 and GM3

GM1 and GM3 hydrolysis was analyzed by HPLC using an Eclipse Plus C18 column (5 μm, 4.6 mm × 100 mm; Agilent Technologies) (supplementary Fig. 1; supplementary Table 1). For analysis of GM1 hydrolysis, the mobile phase consisted of solvent A (0.03% trimethylamine dissolved in water, adjusted to pH 7.5 with phosphoric acid) and solvent B (acetonitrile). A gradient was used at a 1 ml/min flow rate: 0–2 min with 20% A and 80% B, 2–7 min with 20–15% A and 80–85% B, 7–9 min with 15% A and 85% B, and 9–13 min with 20% A and 80% B. For analysis of GM3 hydrolysis, the mobile phase consisted of solvent A/solvent B (18:82) at a 1 ml/min flow rate. The UV detection wavelength was set to 205 nm for both GM1 and GM3, and hydrolysis was calculated as follows:

$$\text{hydrolysis}(\%) = \frac{([B]-[A])\times 100}{[B]}$$

in which $[B]$ and $[A]$ represent the concentrations of either GM1 or GM3 before and after hydrolysis, respectively.

### Quantitative HPLC analysis of GlcCer, GalCer, and SM

The hydrolytic products (GlcSph, GalSph, and lyso-SM) of GlcCer, GalCer, and SM, respectively, were derivatized using o-phthalaldehyde and analyzed by HPLC using a Zorbax SB-C18 column (5 μm, 4.6 mm × 150 mm; Agilent Technologies) (supplementary Fig. 1; supplementary Table 1). The mobile phase consisted of methanol/$H_2O$ (93:7) at a flow rate of 1 ml/min, and the o-phthalaldehyde derivatives were detected with a fluorescence detector ($E_x$ = 340 nm; $E_m$ = 450 nm). Hydrolysis of GlcCer, GalCer, or SM was calculated as follows:

$$\text{hydrolysis}(\%) = \frac{[P]\times 100}{[S]}$$

in which $[P]$ is the concentration of the product (GlcSph, GalSph, or lyso-SM) and $[S]$ is the initial concentration of substrate (GlcCer, GalCer, or SM).

### Analysis of fatty acids in the precipitate

The reactions were centrifuged at 12,000 rpm for 5 min, the precipitates were collected and combined with 10 μl of 20 mM heptadecanoic acid in dimethoxyethane as an internal standard, and the mixtures were evaporated to dryness. The samples were sonicated and resuspended in 100 μl of 2 M HCl in 2-propanol, and the fatty acids were extracted using 200 μl of hexane. Quantitative analysis of fatty acids was performed using HPLC after derivatization with 2-bromoacetophenone. Derivatization was accomplished by drying 50 μl of the extracted supernatant, mixing it with 50 μl of 25 g/l 2-bromoacetophenone in acetone and 50 μl of 25 g/l triethylamine in acetone for 30 min incubation at 70°C, and adding 50 μl of 10 g/l acetic acid in acetone, followed by a final 30 min incubation at 70°C. The derivatized samples were then dried and dissolved in 100 μl of methanol. The Zorbax SB-C18 column was used for fatty acid quantification (supplementary Fig. 1; supplementary Table 1). The mobile phase consisted of methanol/$H_2O$ (97:3) at a flow rate of 1 ml/min, and the UV detection wavelength was set to 244 nm.

### Large-scale lyso-GM1 preparation

GM1 (100 mg) was dissolved in 20 ml of 35 mM sodium acetate buffer (pH 5.8) containing 0.4% TDC, 100 mM $CaCl_2$, and 3.9 U of SA_SCD. The reaction mixture was incubated at 37°C for 12 h. After centrifugation at 12,000 rpm, the supernatant was applied to four Sep-Pak tC18 cartridges (500 mg sorbent). The cartridges were washed with water, with 65% methanol to remove TDC, and finally with 85% methanol to elute lyso-GM1. The lyso-GM1 fractions were pooled, evaporated, and lyophilized.

## RESULTS

### High concentrations of divalent metal cations significantly enhance GM1 hydrolysis

SCDase catalyzes reversible reactions in which the N-acyl linkage of the ceramide moiety to various GSLs is either cleaved or synthesized. The equilibrium between the hydrolytic and synthetic reactions generates a low hydrolytic yield for lyso-GSLs, typically 40–60%. In our previous studies, we found that the presence of 5 mM divalent metal cations (e.g., $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Mg^{2+}$) could enhance SA_SCD hydrolytic activity and inhibited the reverse activity (35). It is suggested that these divalent metal cations might play a critical role in controlling the reaction equilibrium. Here, we did a systematic study for the effects of various divalent metal cations ($Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Mg^{2+}$) on the reaction. As shown in **Fig. 2A**, addition of all the tested cations led to a significant improvement of GM1 hydrolysis, and $Ca^{2+}$ shows the strongest effect. GM1 hydrolysis increased with increasing $Ca^{2+}$ concentration. Compared with the 40.7% yield in the absence of $Ca^{2+}$, the

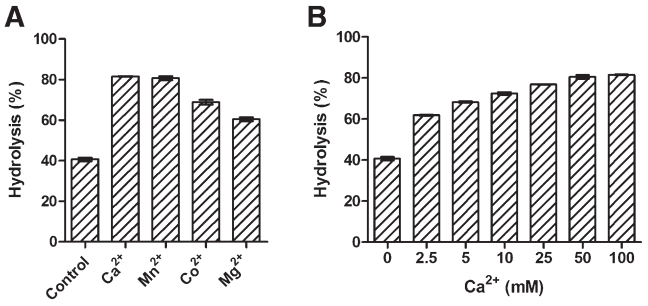


**Fig. 2.** Effects of divalent metal cations on GM1 hydrolysis. A: The effects of high concentrations of various divalent metal cations ($Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Mg^{2+}$) on GM1 hydrolysis. Reactions contained 1 mM GM1 and 100 mM $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, or $Mg^{2+}$ in 35 mM sodium acetate buffer (pH 5.8) with 0.07% Triton X-100. B: The effects of various concentrations of $Ca^{2+}$ on GM1 hydrolysis. Reactions contained 1 mM GM1 and various concentrations of $Ca^{2+}$ in 35 mM sodium acetate buffer (pH 5.8) with 0.07% Triton X-100. Values represent the mean ± SD (n = 3).

addition of 100 mM $Ca^{2+}$ enhanced the yield to 81.6% (Fig. 2B).

### Detergents are essential for efficient enzymatic hydrolysis

Detergents have also been shown to play important roles in enzymatic hydrolysis of GSLs (38). Although the GM1 hydrolysis yield was 81.6% in the presence of 0.07% Triton X-100 and 100 mM $Ca^{2+}$, it dropped sharply to 17.5% in the absence of Triton X-100 (**Fig. 3A**). Therefore, we further investigated the effects of various detergents on the lyso-GM1 production. As shown in Fig. 3A, TDC, sodium cholate, and Tween 80 also improved GM1 hydrolysis in the presence of $Ca^{2+}$, further demonstrating the crucial role of detergents. TDC had the strongest effects of the tested detergents, improving the hydrolysis to 88.2% (Fig. 3A). Interestingly, TDC-based enhancement of hydrolysis required the presence of $Ca^{2+}$: in the absence of $Ca^{2+}$, GM1 hydrolysis was only 2.9%, 14-fold lower than the same case of Triton X-100 (Fig. 3A), suggesting that the enhancement of hydrolysis results from the joint effects of TDC and $Ca^{2+}$.

To further enhance GM1 hydrolysis, the concentration of TDC in the reaction mixture was optimized. As shown in Fig. 3B, hydrolysis of 1 mM GM1 increased with increasing TDC concentrations. Maximal GM1 hydrolysis (~95%) was achieved in the presence of 0.28% TDC and was not further increased with the addition of up to 0.7% TDC.

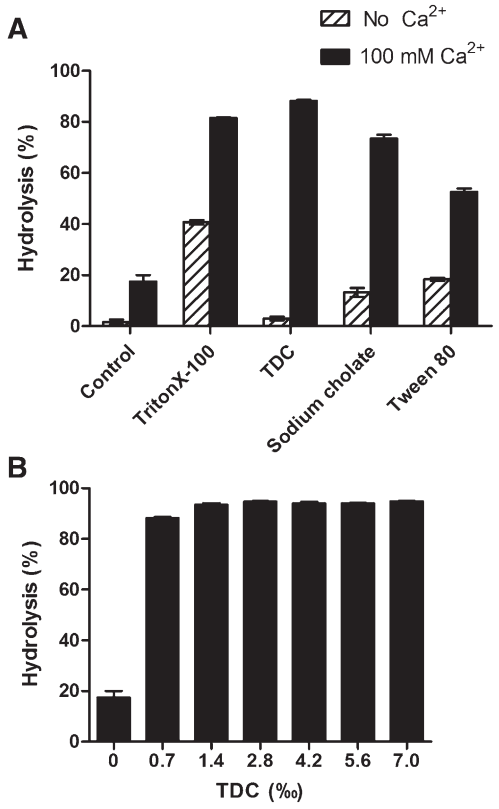


**Fig. 3.** Effects of detergents on GM1 hydrolysis. A: The effects of various detergents (Triton X-100, TDC, sodium cholate, and Tween 80) on GM1 hydrolysis. Reactions contained 1 mM GM1, 100 mM $Ca^{2+}$, or no $Ca^{2+}$ in 35 mM sodium acetate buffer (pH 5.8) with 0.07% Triton X-100, TDC, sodium cholate, or Tween 80. B: The effects of various concentrations of TDC on GM1 hydrolysis. Reactions contained 1 mM GM1 and 100 mM $Ca^{2+}$ in 35 mM sodium acetate buffer (pH 5.8) with various concentrations of TDC. Values represent the mean ± SD (n = 3).

### Adequate TDC is crucial for efficient hydrolysis at high substrate concentrations

To facilitate the production of lyso-GSLs on a preparative scale, higher substrate concentrations are preferred for the reactions. The hydrolysis of various concentrations of GM1 was investigated, and the optimal TDC concentration was revealed to be dependent on substrate concentration (**Fig. 4**). Although 0.07% TDC was sufficient to promote the hydrolysis of 0.5 mM GM1, the efficiency began to decrease for concentrations of substrate greater than 1 mM and declined to 29.5% at 4 mM GM1. The addition of 0.28% TDC enhanced the hydrolysis of 4 mM GM1 to 90.8%, and hydrolysis decreased again when the substrate concentration was increased to 6 mM. Further increasing the TDC concentration to 0.56% significantly improved hydrolysis at high substrate concentrations, reaching levels of 91.3% hydrolysis even at 8 mM GM1 (~12.7 mg/ml).

### Hydrolysis of various sphingolipids is also enhanced by this method

The hydrolysis of various GSLs (GM1, GM3, GluCer, and GalCer) and SM was significantly improved using the optimized conditions (**Table 1**). The hydrolysis of the gangliosides GM1 and GM3 was enhanced to as high as 96%, and the hydrolysis of GluCer, GalCer, and SM improved from 3.07, 8.22, and 23.32% to 66.34, 85.44, and 66.08%, respectively. These results demonstrate that this new method can be used as a general strategy for improving the hydrolysis of various sphingolipids.

### Application to large-scale preparation of lyso-GM1

To test the feasibility of this method on a preparative scale, 100 mg of GM1 was hydrolyzed using the optimized hydrolysis conditions. After 12 h of reaction time, 75 mg of pure lyso-GM1 was isolated using Sep-Pak tC18 cartridges (90% yield). The yield was much larger than has previously reported for the aqueous-organic biphasic system (62–72% yield with 2 weeks of reaction time) (22, 36). In

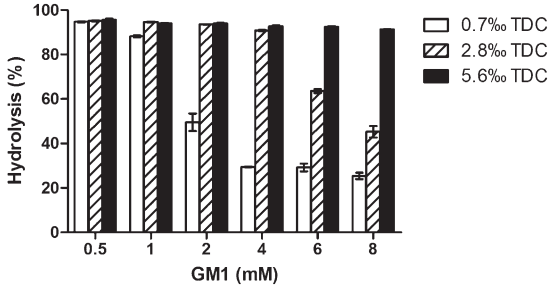


**Fig. 4.** Hydrolysis of various concentrations of GM1. Reactions contained various concentrations of GM1 and 100 mM $Ca^{2+}$ in 35 mM sodium acetate buffer (pH 5.8) with 0.07, 0.28, or 56% TDC. Values represent the mean ± SD (n = 3).

TABLE 1. Comparison of the hydrolysis of various sphingolipids

| Substrate | Hydrolysis (%) | |
|---|---|---|
| | Before optimization | After optimization |
| GM1 | 50.92 ± 0.63 | 96.02 ± 0.17 |
| GM3 | 52.89 ± 0.33 | 96.12 ± 0.05 |
| GluCer | 3.07 ± 0.11 | 66.34 ± 2.68 |
| GalCer | 8.22 ± 0.67 | 85.44 ± 1.46 |
| SM | 23.32 ± 2.02 | 66.08 ± 2.57 |

Reactions contained 0.5 mM GM1, 0.5 mM GM3, 0.2 mM GluCer, 0.2 mM GalCer, or 0.2 mM SM in 35 mM sodium acetate buffer (pH 5.8) with 0.07% Triton X-100 before optimization or in 35 mM sodium acetate buffer (pH 5.8) with 0.28% TDC and 100 mM $CaCl_2$ after optimization. Values represent the mean ± SD (n = 3).

fact, to the best of our knowledge, it is the highest reported yield of lyso-GM1.

### Enhancement of hydrolysis might result from the precipitation of stearic acid

After the hydrolysis of GM1, an obvious precipitate was observed at the bottom of the reaction tube (**Fig. 5A**). We speculated that this precipitate could be stearic acid calcium salt (Fig. 1). To test this hypothesis, we used an HPLC-based method to quantitatively analyze the stearic acid in the precipitate. Indeed, stearic acid was detected in the precipitate, and the amount observed was directly proportional to the amount of GM1 hydrolyzed in the reaction (Fig. 5B), explaining why the reaction equilibrium was shifted toward hydrolysis.

## DISCUSSION

SCDase can hydrolyze GSLs to generate lyso-GSLs, but its application has been limited as a result of the low yield caused by the reaction equilibrium. In this study, we present an easy method for controlling the reaction equilibrium through the addition of $Ca^{2+}$ and TDC to the reaction.

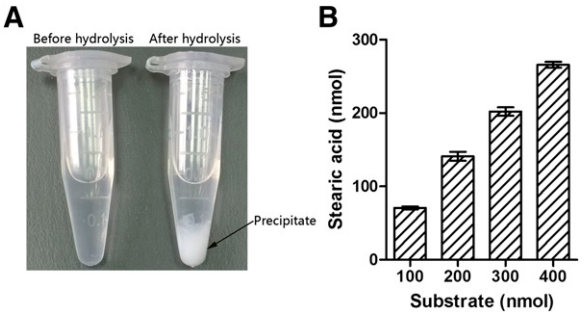


**Fig. 5.** Analysis of fatty acids in the precipitate. A: An obvious precipitate at the bottom of the tube after hydrolysis. Reactions (1 ml) contained 2 mM GM1 and 100 mM $CaCl_2$ in 35 mM sodium acetate buffer (pH 5.8) with 0.28% TDC. B: Quantification of fatty acids in the precipitate. Reactions (100 µl) contained 100, 200, 300, or 400 nmol substrate (GM1) in 35 mM sodium acetate buffer (pH 5.8) with 0.28% TDC and 100 mM $CaCl_2$. After hydrolysis, the precipitate at the bottom of the tube was isolated and analyzed by HPLC as described in Materials and Methods. Values represent the mean ± SD (n = 4).

This method is applicable to many GSLs and is easily scalable for large-scale preparation of lyso-GSLs.

Kurita et al. (38) developed an aqueous-organic biphasic system to increase GSL hydrolysis. The fatty acids released from GSLs were extracted from the aqueous phase with an organic solvent to push the reaction equilibrium toward hydrolysis; however, it was necessary to keep a low substrate concentration, or else the effect of fatty acid extraction would be reduced. Additionally, at least a 5–10 times greater volume of organic solvent relative to the aqueous buffer was required for efficient enhancement of hydrolysis. Consequently, on a preparative scale, the reaction system would necessarily be very large, which would be difficult to handle for downstream purification. These limitations make the aqueous-organic biphasic system inefficient for the preparation of lyso-GSLs, especially on a larger scale. As two examples, lyso-GM1 was prepared at the 10 to 20 mg scale using the biphasic system (22, 36). After 2 weeks of reaction, the yield of lyso-GM1 was 62–72% (22, 36), which is similar that produced by chemical catalysis (19). In contrast, 90% lyso-GM1 yield was achieved after 12 h of reaction time in this report, highlighting the increased efficiency of the new method.

Moreover, the principle of the strategy we used is entirely different from that used by Kurita et al. (38). It has been known for a long time that fatty acids can form water-insoluble complexes with divalent cations (41), so we speculated that the addition of divalent metal cations might reduce the level of free fatty acids in the reaction and promote hydrolysis. Indeed, all of the tested divalent metal cations ($Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Mg^{2+}$) enhanced GSL hydrolysis, and high concentrations of $Ca^{2+}$ were optimal. A considerable quantity of released fatty acids was observed in the precipitate at the bottom of the reaction tube, which is consistent with our explanation.

Interestingly, we also found that the presence of detergent was crucial for the enhancement of GSL hydrolysis. Without detergent in the reaction, GSL hydrolysis was very low even with high $Ca^{2+}$ concentrations. The importance of detergent has also been found in other GSL-hydrolyzing enzymes (42, 43), although the mechanism is not known (42). However, the conformation of GSL substrate is clearly affected by detergent, which may assist the enzyme to recognize the substrate (42, 43). Furthermore, the efficient hydrolysis of high concentrations of GM1 required an adequate amount of TDC. This might be because the GSL has a better accessibility for SA_SCD when detergent and GSL were mixed at an optimal ratio in the micelle. Similar effects have been observed for other lipid-processing enzymes, such as sphingomyelinase (44), snake venom phospholipase A2 (45), and phospholipase C (46, 47).

Lyso-GSLs are important molecules in sphingolipid research because they can be used as intermediates for synthesizing GSL analogs, which are useful tools for GSL biology study and drug development, among other uses. The new method described here provides an easy, efficient, and general means of producing lyso-GSLs on a preparative scale and might facilitate GSL research and the development of new drugs.

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