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Comprehensive characterization of sphingolipid ceramide *N*-deacylase for the synthesis and fatty acid remodeling of glycosphingolipids

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Abstract Sphingolipid ceramide *N*-deacylase (SCDase) catalyzes reversible reactions in which the amide linkage in glycosphingolipids is hydrolyzed or synthesized. While SCDases show great value for the enzymatic synthesis of glycosphingolipids, they are relatively poorly characterized enzymes. In this work, the enzymatic properties of SCDase from *Shewanella alga* G8 (SA_SCD) were systematically characterized and compared with the commercially available SCDase from *Pseudomonas* sp. TK4 (PS_SCD). The optimal pH values for the hydrolytic and synthetic activity of SA_SCD were pH 6.0 and pH 7.5, respectively. Both activities were strongly inhibited by Zn^{2+} and Cu^{2+}, while Fe^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} promoted the hydrolytic activity but inhibited the synthetic activity. SA_SCD showed very broad substrate specificity both in hydrolysis and synthesis. Importantly, SA_SCD has a broader specificity for acyl donor acceptance than does PS_SCD, especially for unsaturated fatty acids and fatty acids with very short or long acyl chains. Further kinetic analysis revealed that the k_{cat}/K_M value for the hydrolytic activity of SA_SCD was 8.9-fold higher than that of PS_SCD for GM1a, while the values for the synthetic activity were 38-fold higher for stearic acid and 23-fold higher for lyso-GM1a (d18:1) than those of PS_SCD, respectively. The broad fatty acid specificity and high catalytic efficiency, together with the ease of expression of SA_SCD in *Escherichia coli*, make it a better biocatalyst than is PS_SCD for the synthesis and structural remodeling of glycosphingolipids.

Keywords Glycosphingolipids · High-performance liquid chromatography · Sphingolipid ceramide *N*-deacylase · Substrate specificity

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Introduction

Glycosphingolipids (GSLs) are a class of amphipathic compounds on the cell surface that play extremely important roles in cellular signaling (Wennekes et al. 2009). They are involved in a range of pathological processes (Butters et al. 2000; Hakomori 1998; Willison and Yuki 2002) and some GSLs have even shown potential therapeutic effects on cancer and neuronal degenerative diseases (Geisler et al. 1991; Kaneko et al. 2007; Schorsch et al. 2013). However, comprehensive study of their biological functions and their development as new drugs have been severely hampered by challenges associated with their large-scale synthesis. While the isolation of GSLs from natural sources is commonly done,

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products generated in this way are subject to potential transmission of disease, heterogeneity, and scarcity (Rupčić and Marić 2004). The chemical synthesis of complex GSLs structure requires many activation, coupling, protection, and deprotection steps, making it extremely challenging and target specific (Vankar and Schmidt 2000). Enzymatic assembly of GSLs, on the other hand, offers significant advantages due to their much simpler syntheses, milder reaction conditions, and more facile product purification (Rich et al. 2011; Rich and Withers 2012).

Sphingolipid ceramide *N*-deacylase (SCDase) is an enzyme that can serve as a biocatalyst in the enzymatic synthesis of GSLs since it catalyzes the reversible hydrolysis/synthesis of the amide linkage between the fatty acid and the sphingosine base in the ceramide moiety of GSLs (Fig. 1a). The synthetic activity of SCDase has been used previously for the assembly of various GSLs (Mitsutake et al. 1997, 1998; Nakagawa et al. 1999, 2005; Kita et al. 2001; Xu et al. 2009; Kuchař et al. 2010), while its hydrolytic activity has been used to produce *N*-deacylated form of GSLs (Kurita et al. 2000). These so-called lyso-GSLs can serve as intermediates for the production of pharmaceuticals. For example, lyso-GM1a has been used as an intermediate in the synthesis of LIGA-20, a semi-synthetic ganglioside that showed potent multimodal neurotrophic effects (Manev et al. 1990; Mocchetti 2005). Therefore, SCDases have attracted attention recently for their use in the enzymatic processing of GSLs.

Four SCDase have been reported from *Nocardia* sp. (Hirabayashi et al. 1988), *Pseudomonas* sp. (Ito et al. 1995), *Streptomyces* sp. (Ashida et al. 1995), and *Shewanella alga* G8 (Furusato et al. 2002), respectively. Among them, only the genes for the enzymes from *Pseudomonas* sp. TK4 (PS_SCD) and *S. alga* G8 (SA_SCD) have been cloned (Furusato et al. 2002; Ito et al. 2004), but these two enzymes share no

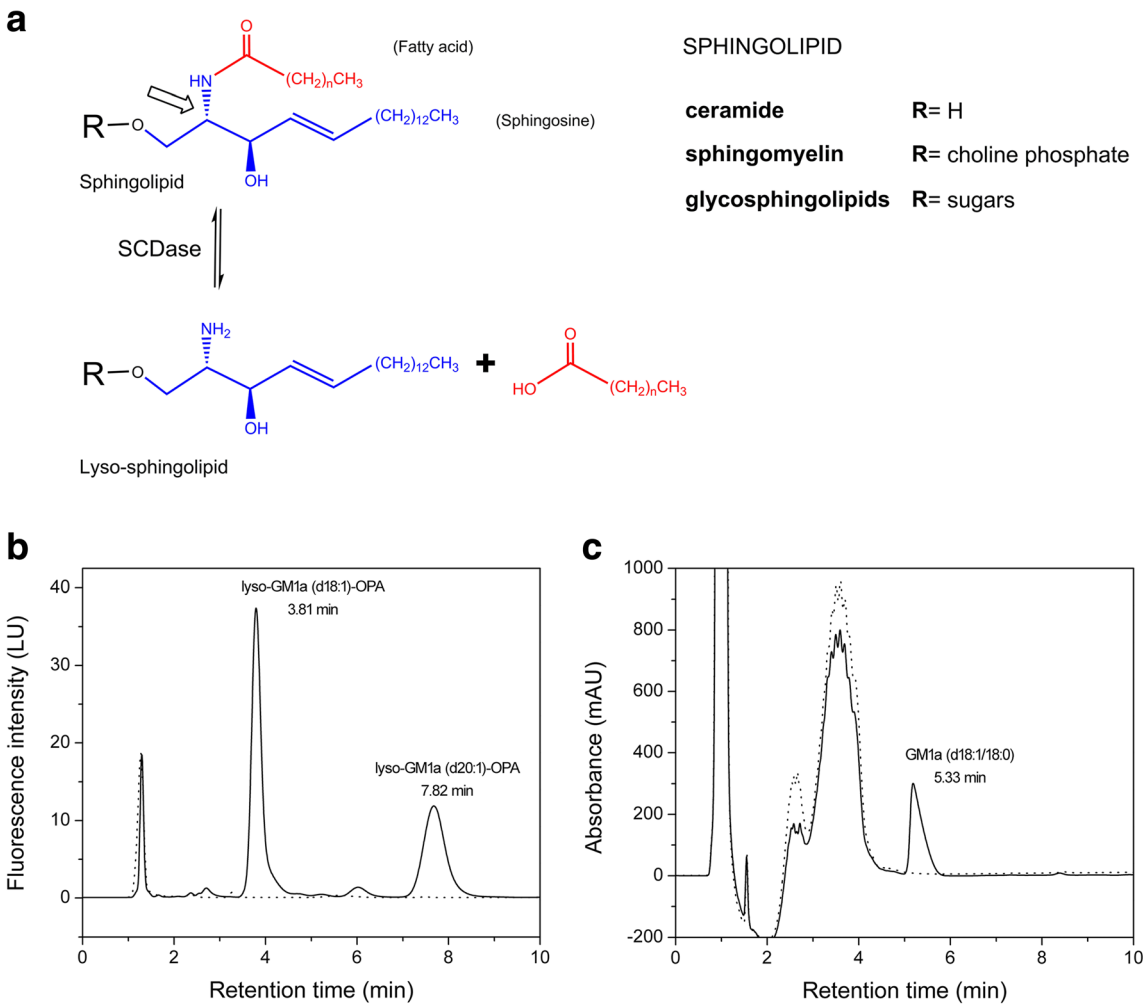


Fig. 1 **a** SCDase catalyzes reversible reactions in which the amide linkage in GSLs is hydrolyzed or synthesized. **b** The representative HPLC chromatograms of the assay for the hydrolytic SCDase activity in the absence (*dotted line*) and in the presence (*solid line*) of SA_SCD. The reaction mixture was derivatized with OPA and detected using a fluorescence detector (E_x=340 nm, E_m=455 nm). **c** The representative HPLC chromatograms of the assay for the synthetic SCDase activity in the absence (*dotted line*) and in the presence (*solid line*) of SA_SCD. The reaction mixture was detected using a UV detector at 195 nm

sequence homology. PS_SCD is now commercially available; however, production of this enzyme is based on the fermentation of *Pseudomonas* sp. TK4 since it has not yet been heterologously overexpressed. By contrast, SA_SCD can be readily overexpressed in *Escherichia coli*, which opens the possibility of mechanistic analysis and enzyme engineering through site-directed mutagenesis (Furusato et al. 2002). However, little is known about the enzymatic properties of SA_SCD since only its hydrolytic activity has been characterized, and this only partially, while its synthetic activity has not been studied. A more extensive characterization of SA_SCD is therefore needed in order for it to be optimally used in the enzymatic synthesis of GSLs.

In this study, we first describe the development of two fast, practical, and precise HPLC-based methods for assay of SCDase. We then use these assays to determine the specificities and kinetic properties of SA_SCD and compare them with those of the commercially available PS_SCD. The results suggest that SA_SCD has superior properties to those of PS_SCD in several respects, making it a valuable biocatalyst for manipulation of GSLs.

Materials and methods

Chemicals and enzymes

The sphingolipids were purchased from Avanti Polar Lipids (Alabaster, USA), except for globotetraosylceramide (Gb4Cer), which was purchased from Wako Pure Chemical Industries (Osaka, Japan) and GM1a, which was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, China). Orthophthalaldehyde (OPA) was purchased from Sigma-Aldrich (St. Louis, USA). Lyso-GM1a standard and PS_SCD were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The lyso-GM1a, lyso-GM3, lyso-sulfatide, lyso-Gb4Cer, and ω-position-modified fatty acids were synthesized in our lab, and methods for this will be published elsewhere. Sep-Pak tC18 reversed phase silica gel cartridges were obtained from Waters (Milford, USA). HPLC solvents were purchased from Anpel Co., Ltd. (Shanghai, China). All other reagents were of the highest purity available.

Protein expression and purification of SA_SCD deletion mutant

The gene encoding the mature SA_SCD protein, which lacks its 38-residue *N*-terminal secretion signal sequence and from which the 277-residue C-terminal sequence has been deleted, was codon-optimized for *E. coli* and synthesized (Genscript Corporation, Nanjing, China). The gene sequence was subcloned into pET23b vector (Novagen, Madison, USA) using the *Nde*I/*Xho*I restriction sites and was transformed into *E. coli* BL21 (DE3) pLysS. Transformants were grown at 37 °C in Luria-Bertani medium containing 100 μg/mL ampicillin until the optical density at 600 nm reached about 0.8. Then, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM at 16 °C for 12 h. The cells were harvested and disrupted by sonication, and the enzyme was purified by Ni^{2+}-chelating affinity chromatography. Protein concentration was determined using the bicinchoninic acid protein assay with BSA as a standard.

HPLC-based assay for SCDase-catalyzed hydrolysis

In a standard assay, the hydrolytic activity of SCDase was measured using GM1a as substrate. The reaction mixture contained 15 nmol of GM1a and an appropriate amount of the enzyme in 30 μL of 25 mM sodium acetate buffer (pH 6.0) with 0.1 % Triton X-100. Following incubation at 37 °C for 5 min, the reaction was stopped by heating in a boiling water bath for 5 min. An aliquot (10 μL) of reaction solution was then taken out, mixed with 20 μL of OPA reagent (7.5 mM), and incubated at 30 °C for 5 min for the derivatization. After centrifugation at 13,000 rpm, the supernatant was transferred to a glass vial and an aliquot (10 μL) was injected onto a reverse-phase HPLC column (Zorbax SB-C18, 4.6 mm ID×150 mm, 5-μm particle size, Agilent Technologies, Santa Clara, USA) using an auto-sampler (Agilent 1260 ALS) with methanol/H_2O (70:30, *v*/*v*) as the mobile phase at a flow rate of 1.0 mL/min. The OPA-derivatized product was detected using a fluorescence detector (Agilent 1260 FLD, E_x= 340 nm, E_m=455 nm).

HPLC-based assay for SCDase-catalyzed synthesis

In a standard assay, the synthetic activity of SCDase was measured using lyso-GM1a (d18:1) and stearic acid as the substrates. The reaction mixture, containing an appropriate amount of SCDase, 15 nmol of lyso-GM1a (d18:1), and 15 nmol of stearic acid, was incubated in 30 μL of 25 mM Tris-HCl buffer (pH 7.5) containing 0.1 % Triton X-100 and 10 % dimethyl sulfoxide (DMSO) at 37 °C for 10 min. Reaction was terminated by heating in a boiling water bath for 5 min, and the generation of GM1a (d18:1/18:0) was measured by HPLC using a reverse-phase column (Zorbax Eclipse Plus C18, 4.6 mm ID×100 mm, 3.5-μm particle size, Agilent Technologies, Santa Clara, USA). The mobile phase contained acetonitrile and water (80:20, *v*/*v*), plus 0.03 % triethylamine, with its pH adjusted to 7.5 using phosphoric acid. GM1a (d18:1/18:0) was eluted from the column at a flow rate of 1.0 mL/min and detected

at a wavelength of 195 nm using a variable wavelength detector (Agilent 1260 VWD).

General characterization of SCDase

The effects of pH on the activities of SA_SCD were measured across the pH range from 4.5 to 10.0 using a wide-range pH buffer containing HEPES, TAPS, CAPS, MES, and acetic acid, each at 40 mM.

The effects of metal ions on the activities of SA_SCD were assayed in the presence of 5 mM metal cations ($FeCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$, and $MgCl_2$) or EDTA.

The effects of organic solvents on the synthetic activity of SA_SCD were measured in the presence of organic solvents (DMSO and dimethoxyethane (DME)) at different concentrations (*v*/*v*).

The effects of detergents on SA_SCD synthetic activity were measured by the standard method except that Triton X-100, sodium deoxycholate (DOC) or taurodeoxycholate (TDC) were used at different concentrations (*v*/*v*). The effects of Triton X-100 on SA_SCD hydrolytic activity were measured by the standard method except that Triton X-100 was used at different concentrations (*v*/*v*) with the GM1a concentration fixed separately at 0.1, 0.5 or 1.0 mM.

In all the reaction mixtures above, 15 ng of enzyme was used in hydrolytic activity assays, and 30 ng was used in synthetic activity assays. The activity is normalized relative to the maximal value in each experiment, except the effect of metal ions in which the activity was normalized relative to the control with no metal ions added.

Substrate specificities of SCDases

The hydrolytic activities of SCDases against sphingolipids with different hydrophilic head groups (Fig. 2) were measured with 15 ng of SA_SCD or 35 ng of PS_SCD by the standard assay. The substrate specificity towards hydrophilic head groups was represented by the specific hydrolytic activity.

The synthetic activities of SCDase towards lyso-GSLs containing different hydrophilic head groups were determined using 15 nmol of lyso-GSL and 30 nmol of stearic acid in 30 μL of 25 mM HEPES buffer (pH 7.0) containing 0.1 % Triton X-100 and 10 % DMSO. Reactions were performed at 37 °C for 12 h with 30 ng of SA_SCD. The synthetic specificity towards hydrophilic head groups was represented by reaction yield (%), calculated as follows: (peak area for total lyso-GSL−peak area for remaining lyso-GSL)×100/peak area for total lyso-GSL. The HPLC detection for lyso-GSLs after OPA derivatization was similar to that for lyso-GM1a except that the mobile phase ratio of methanol/H_2O was adjusted according to the polarity of lyso-GSLs.

The fatty acid specificities of SCDases in the synthetic reaction were determined using a variety of fatty acids. The reaction mixtures containing 15 nmol lyso-GM1a (d18:1) and 30 nmol of fatty acid were incubated at 37 °C for 12 h with 30 ng of SA_SCD or 70 ng of PS_SCD in 30 μL of 25 mM buffer (HEPES buffer for SA_SCD or phosphate buffer for PS_SCD), pH 7.0, containing 0.1 % Triton X-100 and 10 % DMSO.

Kinetic analysis of SCDase

For the kinetic analysis of hydrolytic activity, GM1a (0.02–2.0 mM) was incubated with 15 ng SA_SCD or 35 ng PS_SCD for 2 min in 30 μL of 25 mM sodium acetate buffer (pH 6.0) with 0.1 % Triton X-100 under standard conditions. Kinetic analysis of synthetic activity was performed using lyso-GM1a (d18:1) and stearic acid as substrates in the presence of 30 ng SA_SCD for 3 min or 70 ng PS_SCD for 20 min in 30 μL of 25 mM Tris-HCl buffer (pH 7.5) containing 0.1 % Triton X-100 and 10 % DMSO under standard conditions. Kinetic parameters for stearic acid were determined using concentrations between 0.02 and 2.0 mM at a fixed lyso-GM1a (d18:1) concentration (1 mM). Kinetic parameters for lyso-GM1a (d18:1) were determined using concentrations between 0.06 and 1.0 mM at a fixed stearic acid concentration (1 mM). The parameters K_M and k_{cat} were obtained by fitting the experimental data to the Michaelis-Menten kinetics model using Origin 8.0.

Nucleotide sequence accession number

Nucleotide sequence data of SA_SCD deletion mutant has been deposited in the GenBank database under accession number KM986461.

Results

Development of two HPLC assays of the hydrolytic and synthetic activities of SCDase

The activities of SCDase are typically determined using TLC-based methods by using radioisotopically labeled substrates (Kita et al. 2001; Mitsutake et al. 1998). Although sensitive, such assays are difficult to implement since the labeled reagents are not readily available and require special facilities to handle. Therefore, two HPLC-based methods were developed for measurement of the hydrolytic and synthetic activities of SCDase, respectively (Fig. 1b, c). These methods allowed fast and precise assays of SCDase without the use of radioactive substrates.

The SCDase-catalyzed hydrolysis of GSLs generates a free amine, which can be derivatized with OPA and quantified by a fluorescence detector on HPLC (Fig. 1b). Conditions for the derivatization were optimized as described in Fig. S1. The

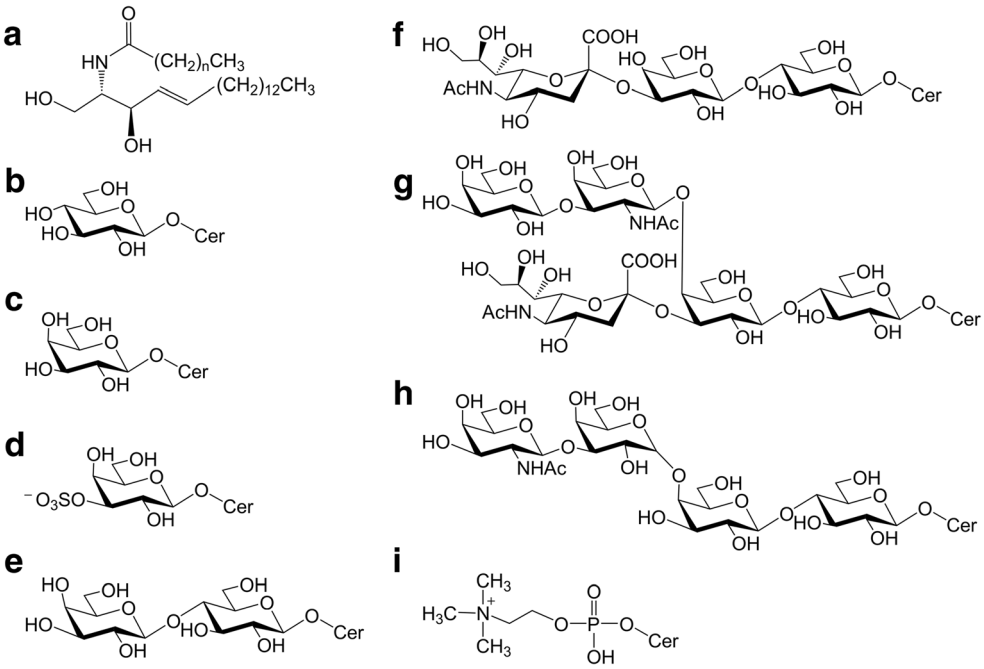


Fig. 2 Sphingolipids with different head groups. Ceramide (**a**). GlcCer (**b**). GalCer (**c**). Sulfatide (**d**). LacCer (**e**). GM3 (**f**). GM1a (**g**). Gb4Cer (**h**). SM (**i**)

method is compatible with the analysis of various substrates, including ceramide (Cer), glucosylceramide (GlcCer), galactosylceramide (GalCer), sulfatide, lactosylceramide (LacCer), GM3, GM1a, Gb4Cer, and sphingomyelin (SM) (Fig. S2). This new method has good precision with intra- and inter-assay relative standard deviations (RSDs) all less than 5 % (Table S1). The HPLC-based method therefore provides a reliable assay for the hydrolytic activity of SCDase.

The synthetic activity of SCDase can be directly determined by monitoring the amide bond formation at 195 nm using a UV detector on the HPLC. To achieve good separation and detection of the product, we established a modified version of the reverse-phase HPLC method reported by Gazzotti et al. (1984): instead of Gazzotti's LiChrosorb RP-8 column, we employed a reverse-phase Zorbax Eclipse Plus C18 column for HPLC analysis. When using lyso-GM1a (d18:1) and stearic acid as substrates, the product GM1a (d18:1/18:0) could be clearly separated from the substrate and buffer components using a mobile phase containing acetonitrile and water (80:20, *v/v*) with 0.03 % triethylamine, pH 7.5 (Fig. 1c). The RSDs of intra-assay and inter-assay for synthetic activity assay were all less than 5 % (Table S2), indicating that this new method also has good precision.

Basic enzymatic properties of SA_SCD

SA_SCD was found in a marine bacterium, *S. alga* G8, and its gene was cloned by Furusato et al. (2002). It is the only known SCDase that can be functionally expressed in *E. coli*. However, it is also a relatively poorly characterized enzyme. The hydrolytic activity of recombinant SA_SCD has been partially characterized (Furusato et al. 2002), but the synthetic activity has not been studied in detail. Herein, we therefore systematically characterized both the hydrolytic and synthetic activities of SA_SCD, and then compared these results with those of the commercially available SCDase from *Pseudomonas* sp. TK4 (PS_SCD) to gain improved insights into the catalytic behavior of SCDases.

SA_SCD was expressed in *E. coli* and purified to homogeneity before characterization (Fig. S3). The effect of pH on the activities was investigated over pH range 4.5–10 using a broad range of buffers (Fig. 3a). The enzyme maintained >80 % of its hydrolytic activity over a broad pH range from 5.5 to 8.0, with its pH optimum at pH 6.0, which is consistent with the results reported by Furusato et al. (2002). The synthetic reaction performed most efficiently between pH 7.0 and 7.5 and decreased sharply when the pH dropped below 6.5 or increased above pH 7.5.

The effects of chelating agent EDTA and various divalent metal-ions on SA_SCD were also examined. EDTA slightly inhibited both the hydrolytic and the synthetic activity. Cu^{2+} and Zn^{2+} strongly inhibited both the hydrolytic and the synthetic reactions (Fig. 3b). However, Fe^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Ca^{2+} activated hydrolysis while significantly inhibiting the synthesis. Mg^{2+} activated hydrolysis and had no obvious effect on the synthesis.

Since most of the fatty acid substrates for the synthetic activity of SCDase are hydrophobic and do not dissolve well in aqueous solution, organic co-solvents provide a means to facilitate the reaction. To identify the optimal conditions for synthesis, the effects of organic solvents on the synthetic activity were examined. While no detectable activity was

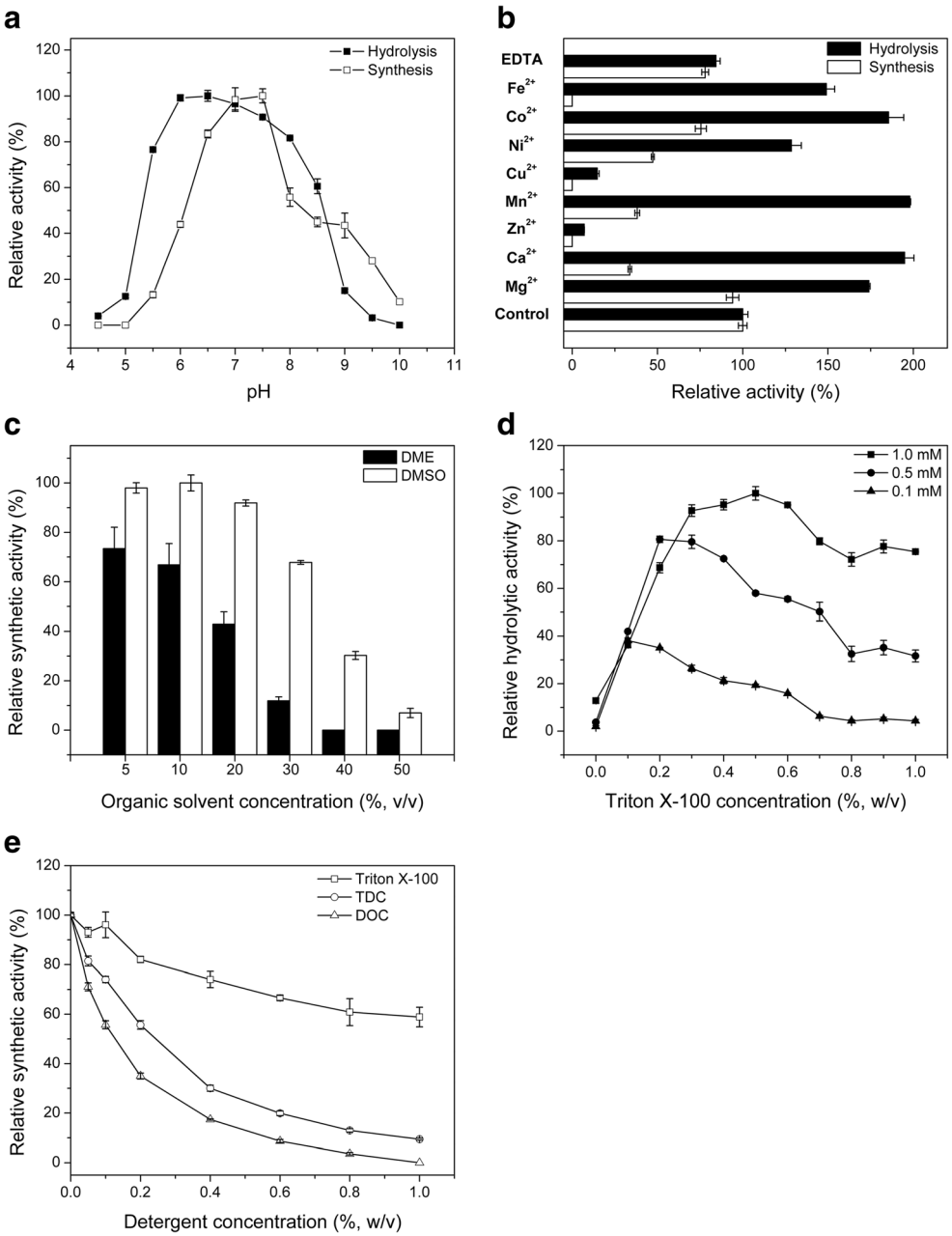


Fig. 3 Characterization of the recombinant SA_SCD. **a** Effects of pH. **b** Effects of metal cation and EDTA. **c** Effects of organic solvents on the synthetic activity of SA_SCD. **d** Effects of Triton X-100 on the hydrolytic activity of SA_SCD at different GM1a concentrations. **e** Effects of detergents on the synthetic activity of SA_SCD. *Values* represent the mean±SD (n=3)

observable without organic co-solvent, addition of 5 % of DME or DMSO strongly promoted the activity. Further increasing the DME concentrations progressively inhibited synthetic activity (Fig. 3c). However, low concentrations of DMSO, up to a maximum at 10 %, enhanced synthetic activity, while higher concentrations inhibited.

The effects of various detergents on the hydrolytic and synthetic reactions of SA_SCD were also examined. Ionic detergents, DOC, and TDC completely inhibited hydrolytic activity (data not shown). Interestingly, the non-ionic detergent Triton X-100 enhanced hydrolysis, but the effect depended on GM1a concentration (Fig. 3d). For low GM1a concentration (0.1 mM), the optimum concentration of Triton X-100 is low (0.1 %, *w/v*), but higher GM1a concentrations (1.0 mM) require a correspondingly higher concentration of Triton X-100 (0.5 %, *w/v*) for the best enhancement. Synthetic activity proceeded most efficiently in the absence of detergents. Addition of Triton X-100, DOC, or TDC inhibited the synthetic reaction (Fig. 3e): no synthesis occurred when DOC or TDC concentration reached 1 %, while 60 % of the activity was still observed with 1 % Triton X-100.

Head group specificities of SA_SCD and PS_SCD

The substrate specificity of SCDase is crucial for its practical applications, but little information is available

for both SA_SCD and PS_SCD. Therefore, the substrate head group specificities of SA_SCD and PS_SCD were studied in detail to make a systematic comparison. For hydrolysis, SA_SCD preferred GSLs with larger sugar moieties (GM3, GM1a, and Gb4Cer) or charged head groups (SM and sulfatide) over those with smaller neutral sugar moieties (GlcCer, GalCer, and LacCer) (Table 1). PS_SCD also preferred GSLs possessing larger sugar moieties, but it did not show preference towards GSLs with charged head groups (e.g., SM). Moreover, PS_SCD hydrolyzed sulfatide faster than GM1a and GM3, whereas SA_SCD hydrolyzed GM1a and GM3 faster. Interestingly, it was apparent that SA_SCD hydrolyzed GSLs much faster than did PS_SCD, indicating greater efficiency. For example, specific activities of SA_SCD for GM3, GM1a, and SM were 25-, 39-, and 69-fold higher, respectively, than those for PS_SCD.

The substrate head group specificities of the SCDases in the synthetic reaction were also examined (Table 1). While PS_SCD showed similar activity towards various lyso-GSLs (Kita et al. 2001), SA_SCD preferred substrates with more polar head groups, such as lyso-SM (54 %), lyso-GM3 (79 %), and lyso-GM1a (81 %), while yields were low for Gal-Sph (20 %), Glc-Sph (39 %), and Lac-Sph (30 %). SA_SCD barely catalyzed the formation of ceramide by condensation of stearic acid to Sph, whereas reaction yields of up to 64.4 % were reported previously for PS_SCD (Kita et al. 2001). The distinct substrate specificities of these two enzymes imply different enzyme-substrate binding modes.

Fatty acid specificities of SA_SCD and PS_SCD

The fatty acid moieties of GSLs can significantly affect their physiological properties (Xu et al. 2009; Chinnapen et al. 2012; Manev et al. 1990). Therefore, remodeling of the fatty acid moieties of GSLs may help in understanding their structure/function relationships and in facilitating the development of GSLs with new functions. Despite the obvious potential of SCDases in the enzymatic synthesis of GSLs, their ability to utilize different fatty acids hasn't been studied in detail. We therefore systematically determined the substrate specificities of SA_SCD and PS_SCD towards fatty acids and their analogs, and thereby generated a library of GM1a derivatives. In these studies, lyso-GM1a (d18:1) was used as the acceptor substrate, and generation of the corresponding GSLs was confirmed by mass spectrometry (Table S3). SA_SCD accepted all ten saturated fatty acids tested as substrates, with reaction yields ranging from 30 to 86 % (Table 2). By contrast, only the fatty acids with medium acyl chain lengths (C14–C20) gave comparable yields with PS_SCD: when the acyl chain is shorter than 14 or longer than 20, the conversion for PS_SCD is much lower than that for SA_SCD. Indeed, PS_SCD barely reacted with caproic acid (1) and lignoceric acid (10), while SA_SCD achieved ~30 and 55 % conversion, respectively. SA_SCD also showed much higher activity towards unsaturated fatty acids than did PS_SCD (Table 3). Oleic acid (11), eicosapentaenoic acid (EPA, 12), and docosahexaenoic acid (DHA, 13) can all be efficiently

Table 1 Specificities of the hydrolytic and synthetic reactions of SA_SCD and PS_SCD for hydrophilic head groups

Name	Structure	Hydrolysis (nmol/min/μg)[a]		Synthesis (%)	
		SA_SCD	PS_SCD	SA_SCD[b]	PS_SCD[c]
Cer	Ceramide (d18:1/18:0)	0.20±0.01	0.04±0.01	0.0	64.4
GlcCer	Glcβ1-1′Cer	1.46±0.1	0.31±0.01	39.0±9.4	75.5
GalCer	Galβ1-1′Cer	2.01±0.03	0.26±0.02	20.1±7.3	76.8
Sulfatide	HSO_3-3Galβ1-1′Cer	7.36±0.4	0.88±0.01	–[d]	61.8
LacCer	Galβ1-4Glcβ1-1′Cer	2.23±0.2	0.23±0.01	29.9±3.3	62.7
GM3	NeuAcα2-3Galβ1-4Glcβ1-1′Cer	20.9±0.8	0.84±0.02	78.8±0.9	–
GM1a	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer	28.7±0.8	0.73±0.02	81.3±0.3	65.5
Gb4Cer	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer	38.9±2.0	1.93±0.03	–[d]	49.1
SM	Choline phosphate-Cer	8.98±0.3	0.13±0.01	54.2±2.0	60.5

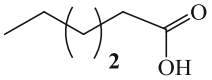
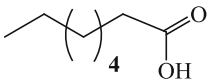
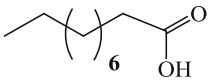
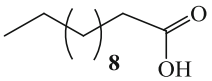
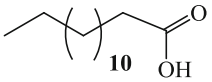
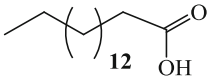
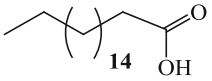
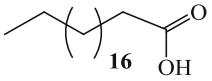
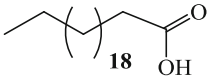
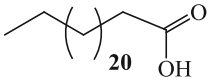
[a] The hydrolytic specificity towards hydrophilic head groups is represented by the specific hydrolytic activities towards various GSLs. Values represent the mean±SD (n=3)

[b] The synthetic specificity towards hydrophilic head groups is represented by the reaction yield (%), calculated as follows: (peak area for total lyso-GSL − peak area for remaining lyso-GSL)×100/peak area for total lyso-GSL. Values represent the mean±SD (n=3)

[c] The data were taken from Kita et al. (2001)

[d] The substrates were not tested

Table 2 Specificity of the synthetic reaction of SA_SCD and PS_SCD for saturated fatty acids

Entry	Structure	Lyso-GM1a (d18:1) Reaction yield (%)[a]	
		SA_SCD	PS_SCD
1		29.7 ± 6.1	0
2		64.3 ± 2.5	17.8 ± 4.1
3		77.9 ± 1.4	50.7 ± 1.4
4		84.8 ± 0.7	67.2 ± 1.3
5		86.1 ± 0.3	81.7 ± 1.5
6		85.9 ± 0.4	85.2 ± 2.2
7		83.5 ± 1.5	82.8 ± 2.4
8		82.5 ± 1.1	76.9 ± 4.7
9		80.7 ± 0.9	36.4 ± 5.9
10		54.5 ± 0.7	9.2 ± 6.2

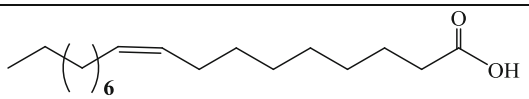
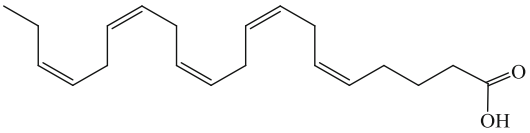
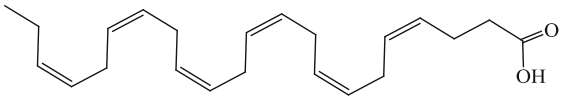
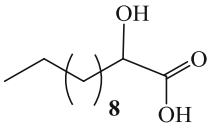
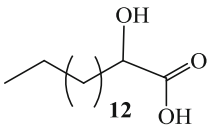
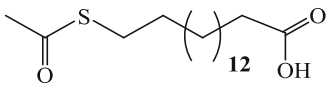
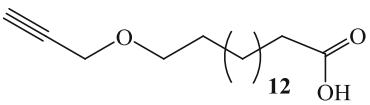
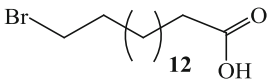
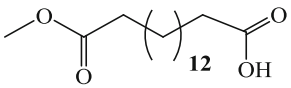
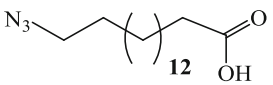
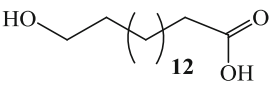
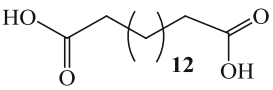
[a] The extent of conversion of lyso-GM1a (d18:1) was calculated as follows: reaction yield (%)=(peak area for total lyso-GM1a (d18:1)−peak area for remaining lyso-GM1a (d18:1))×100/peak area for total lyso-GM1a (d18:1). Values represent the mean±SD (n=3)

utilized by SA_SCD with greater than 70 % conversion, whereas conversion by PS_SCD was much lower (16–35 %). Both enzymes efficiently used various 2-hydroxylated and ω-position-modified fatty acids, but the reaction efficiency decreased for fatty acids possessing a more polar terminal group (Table 3). For example, the reaction yield for 16-hydroxyhexadecanoic acid (21) is lower than that for palmitic acid (6), while hexadecanedioic acid (22) was processed with an even lower yield. However, efficiency increased significantly (up to 85.5 %) when the terminal carboxyl group was protected as its methyl ester (19).

Kinetic parameters of SA_SCD and PS_SCD

Apparent kinetic parameters for hydrolytic and synthetic activities of SA_SCD and PS_SCD were determined (Table 4). The kinetic parameters for the hydrolytic reaction are termed apparent because the GM1a used in this assay is a mixture of GM1a (d18:1/18:0) and GM1a (d20:1/18:0). The K_M for the hydrolytic reaction was similar for the two enzymes, while the k_{cat} and the k_{cat}/K_M of SA_SCD for GM1a are 8.2- and 8.9-fold higher than those of PS_SCD (Table 4). Substrate inhibition was observed for SA_SCD when the GM1a concentration was

Table 3 Specificity of the synthetic reaction of SA_SCD and PS_SCD for a variety of fatty acids

Entry	Structure	lyso-GM1a (d18:1) Reaction Yield (%)[a]	
		SA_SCD	PS_SCD
11		84.4 ± 0.4	35.4 ± 4.6
12		73.4 ± 0.5	21.7 ± 6.3
13		82.3 ± 0.2	15.5 ± 4.2
14		63.0 ± 3.2	57.5 ± 0.9
15		70.0 ± 1.8	74.7 ± 4.7
16		85.5 ± 0.6	65.2 ± 4.2
17		83.0 ± 0.9	71.3 ± 0.3
18		85.0 ± 0.8	75.7 ± 2.3
19		83.8 ± 0.9	75.8 ± 1.7
20		85.8 ± 0.5	81.3 ± 1.5
21		64.3 ± 5.8	56.2 ± 2.9
22		4.0 ± 0.8	23.3 ± 6.1

[a] The extent of conversion of lyso-GM1a (d18:1) was calculated as follows: reaction yield (%)=(peak area for total lyso-GM1a (d18:1)−peak area for remaining lyso-GM1a (d18:1))×100/peak area for total lyso-GM1a (d18:1). Values represent the mean±SD (n=3)

higher than 0.3 mM, whereas this phenomenon was not seen for PS_SCD up to 2.0 mM.

Lyso-GM1a (d18:1) and stearic acid were used as substrates for kinetic analysis of the synthetic reaction. When measuring kinetic parameters for each substrate, the concentration of the co-substrate was fixed at an arbitrary 1.0 mM concentration. The values reported in Table 4 are therefore only estimates of the actual kinetic parameters, but provide a good relative evaluation. The K_M values of SA_SCD for stearic acid and lyso-GM1a (d18:1) were 2.7-

Table 4 Apparent kinetic parameters for SA_SCD and PS_SCD

SCDase	Hydrolytic substrate			Synthetic substrates					
	GM1a[a]			Stearic acid			Lyso-GM1a (d18:1)		
	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1}/mM)	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1}/mM)	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1}/mM)
SA_SCD	3130±260	0.22±0.03	14,160	6800±370	0.49±0.07	13,780	7870±1040	0.63±0.17	12,470
PS_SCD	380±14	0.24±0.03	1590	64±3	0.18±0.03	360	60±2	0.11±0.01	540

The kinetic assays were performed in triplicate. The fitting curves for kinetic parameters are presented in Fig. S4

[a] GM1a is a mixture of GM1a (d18:1/18:0) and GM1a (d20:1/18:0)

and 5.7-fold higher than those of PS_SCD, while the k_{cat} values were 106- and 131-fold higher than PS_SCD. Thus, the k_{cat}/K_M of SA_SCD for stearic acid and lyso-GM1a (d18:1) were 38- and 23-fold higher, respectively, than those of PS_SCD.

Discussion

SCDase has considerable potential in the synthesis of GSLs, especially those bearing unnatural fatty acids, and thus is an important biocatalyst in the enabling of GSL research. Several lines of evidence have recently suggested that the fatty acid moieties of GSLs could significantly affect their biological function. Fucosyl-GM1a bearing polyunsaturated fatty acids inhibits the proliferation of human leukemia-derived HL-60 cells, whereas parental fucosyl-GM1a bearing a stearic acid does not (Xu et al. 2009). GM1a, as the cholera toxin receptor, only effectively relocates from the plasma membrane to the *trans*-Golgi and the endoplasmic reticulum when coupled with an unsaturated acyl chain moiety (Chinnapen et al. 2012). LIGA-20, a semisynthetic GM1a derivative with modified fatty acid moiety, showed greater efficacy and has fewer side effects than GM1a in preventing or slowing down neurodegenerative processes in humans (Lipartiti et al. 1992; Kharlamov et al. 1994; Mocchetti 2005; Bachis et al. 2002). New methods for enzymatic synthesis of GSLs are therefore important for the development of drugs of this class for the treatment of cancer and neuron degenerative diseases.

SA_SCD is the only known SCDase that can be functionally expressed in *E. coli*, at present, thereby opening prospects for protein engineering. However, it is a relatively poorly characterized enzyme. Using the newly developed HPLC-based assay methods, the enzymatic properties of SA_SCD were systematically investigated for the first time. The results for the hydrolysis reaction were similar to those published by Furusato et al. (2002) using the TLC-based method, validating the HPLC methods. More detailed investigation revealed that Fe^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Ca^{2+} enhanced the hydrolytic activity of SA_SCD while inhibiting its synthetic activity, most likely due to the divalent cations forming insoluble stearate salts with the stearic acid released from GM1a by hydrolysis, thus pushing the reaction equilibrium towards the hydrolytic direction. This behavior may prove important for the preparation of lyso-GSLs. Interestingly, Zn^{2+} and Cu^{2+} inhibited both the hydrolytic and synthetic activities significantly, which is different from other divalent cations. The inhibition of excess zinc has been observed in several zinc enzymes, e.g., carboxypeptidase A (Larsen and Auld 1989), thermolysin (Holland et al. 1995), and amidohydrolase (Wakayama et al. 1995). The inhibition effect was specific for zinc and a few other heavy metals such as Pb^{2+}, Cd^{2+}, and Cu^{2+} and was attributed to the possibility that these cations might form an inhibitory metal monohydroxide ($MeOH^+$) complex with the catalytic Zn^{2+} through a stabilizing hydroxide bridge while regular divalent cations did not (Larsen and Auld 1991). The extra metal cation probably perturbs the substrate positioning and stereochemical rearrangements required for substrate cleavage during catalysis (Gomez-Ortiz et al. 1997). It is possible that SA_SCD is also a zinc enzyme, given the fact that both the hydrolytic and synthetic activity of SA_SCD can be inhibited by the chelating agent EDTA. This might explain why Zn^{2+} or Cu^{2+} especially inhibited the enzyme while similar divalent cations did not. However, more studies are needed to explain this phenomenon, such as solving a crystal structure of SA_SCD.

Interestingly, the effects of Triton X-100 on hydrolytic activity depended on the concentration of GM1a, much as seen previously for other lipid processing enzymes, such as sphingomyelinase (Yedgar and Gatt 1976), snake venom phospholipase A_2 (Dennis 1973), and phospholipase C (Eaton and Dennis 1976; Sundler et al. 1978), which all have an optimal Triton X-100/lipid ratio for their hydrolytic activity. This phenomenon can be explained on the basis of surface dilution kinetics, as proposed by Dennis and co-workers (Deems et al. 1975; Roberts et al. 1977; Carman et al. 1995). In this theory, the true substrate for the enzyme is not the free GSL in solution but a mixed micelle of Triton X-100 and the GSLs. The molar ratio of Triton X-100 to GSLs is crucial for the hydrolytic activity of these enzymes. When the

ratio is lower than the optimal value, the activity decreases because fewer molecules are present in the form of micelles. When the ratio is higher than the optimal value, increasing the concentration of Triton X-100 decreases the substrate concentration at the micelle surface, which also decreases the enzymatic activity. This finding is important for the enzymatic preparation of lyso-GSLs: by identifying the optimal molar ratio of Triton X-100/GSLs, the available concentration of GSLs can be optimized, thereby significantly improving the hydrolytic efficiency.

While Furusato et al. (2002) have carried out a limited investigation of substrate selectivities for SA_SCD in the hydrolysis of GSLs, even 10 years after the identification of this enzyme, a clear picture of its overall substrate specificities was still not available. Our results show that both SA_SCD and PS_SCD have quite broad substrate specificities for the hydrolysis of GSLs with different head groups. Both enzymes prefer substrates possessing larger, more polar head groups, suggesting the presence of hydrophilic substrate binding subsites. However, SA_SCD seems to have a narrower sugar moiety specificity for its synthetic activity than does PS_SCD, as it synthesizes GlcCer, GalCer, and LacCer much more slowly and showed no observable synthesis of Cer (Table 1). This suggests that SA_SCD needs a larger, more hydrophilic head group binding in the active site for efficient catalysis of the synthetic reaction, while this is not necessary for PS_SCD.

SA_SCD and PS_SCD also showed broad specificity towards fatty acid substrates, with that for SA_SCD being noticeably broader as it is more tolerant to fatty acids with very short and very long acyl chains, and is more efficient at converting unsaturated fatty acids. Both SA_SCD and PS_SCD accept 2-hydroxylated and ω-modified fatty acids as substrates, but the reaction efficiency is lower for fatty acids with a more hydrophilic tail. This is illustrated by the fact that esterification of the terminal carboxylic acid of 22 dramatically enhances conversion, suggesting that the enzymes possess a long, hydrophobic binding site for the acyl chain with a terminus that accommodates a range of ω-substituents. These broad specificities render the two SCDases, especially SA_SCD, useful catalysts for the fatty acid remodeling of GSLs for many applications. For example, GSLs possessing a terminal thiol (derivative of 16) or carboxyl (22 and derivative of 19) functionality can be anchored to gold- or amino-functionalized surfaces, thus providing the opportunity to create cell membrane mimics for the study of protein-GSLs interactions (Ohlsson and Magnusson 2000). Moreover, GSLs carrying terminal azido- (20) or alkyne-groups (17) can be further modified by click chemistry (Kolb et al. 2001), thereby opening up tremendous possibilities for the chemical modification and fluorescent labeling of GSLs.

Comparison of kinetic parameters for the two SCDases reveals that K_M values are similar, but SA_SCD has a much higher k_{cat} for GM1a hydrolysis than does PS_SCD. As a result, the reaction efficiency (k_{cat}/K_M) of SA_SCD is about 10-fold higher than that of PS_SCD. However, the activity of SA_SCD suffers from substantial substrate inhibition when the concentration of GM1a employed is above 0.3 mM, thus use of SA_SCD to prepare lyso-GSLs on a large scale will require further optimization to minimize this inhibition, possibly through aliquoted additions. SA_SCD is also a more efficient catalyst for synthesis, with k_{cat} values being 106- and 131-fold higher for stearic acid and lyso-GM1a (d18:1), respectively, than those of PS_SCD. Although SA_SCD has lower affinity for the substrates (higher K_M), the catalytic efficiencies (k_{cat}/K_M) are still ~20–40 times higher than those of PS_SCD.

In conclusion, the broad specificity, high catalytic efficiency, and ease of production of SA_SCD expressed in *E. coli* render it a valuable addition to the repertory of enzymes for manipulation of glycosphingolipids. Future attention will focus on the engineering of this enzyme to optimize it for these tasks.

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Conflict of interest The authors have no conflict of interest.

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