The Journal of Microbiology, October 2008, p. 585-589 Vol. 46, No. 5
DOI 10.1007/s12275-008-0114-2

NOTE

Bioluminescent Assay for Sphingolipid Ceramide N-Deacylase Using *Vibrio harveyi* Dark Mutant M-17

Ki Woong Cho

Department of Marine Biotechnology, Anyang University, Incheon 417-833, Republic of Korea

(Received May 2, 2008 / Accepted July 10, 2008)

A new bioluminescent assay method for the activity of sphingolipid ceramide N-deacylase (SCDase: EC 3.5.1.69) as well as ceramidase (CDase: EC 3.5.1.23) was developed using bioluminescent marine bacteria. Enzymatically synthesized ceramide (N-myristoyl sphigosine, C14:0-18:1) and commercial SCDase were used in this demonstration, and myristic (tetradecanoic, C14:0) acid produced by the SCDase hydrolysis was quantified using *Vibrio harveyi* M-17, a dark mutant of *V. harveyi.* The *in vivo* light intensity of M-17 was stimulated up to thousands fold in the presence of myristic acid, was used for this assay. SCDase activity with as little as 10 μU and 5 nM of myristic acid production were detected in less than one min. The assay worked well for the determination of Km and chromatographic fraction assay.

***Keywords*:** bioluminescent assay, *Vibrio harveyi* M-17, SCDase, ceramide, myristic acid

Ceramide is a sphingolipid in which the amino group of sphingosine is acylated with long chain fatty acid, and it is a common biosynthetic precursor of various kinds of sphingolipids such as cerebroside, ganglioside, globoside, and sphigomyelin (Thorpe and Sweeley, 1967). The position of ceramide in sphingolipid family is like that of diacyl glycerol (DG) in glyceride lipid family. Recently, the biochemical and physiological importance of ceramide is increasing as a second messenger in cell differentiation (Taha *et al*., 2006) and apoptosis (Pettus *et al*., 2002). In the epidermis of mammalian skin, ceramide produced from sphingomyelin and glucosylceramide by sphingomyelinase and glucosidase, respectively, is secreted into the extracellular space to form a mantle surrounding individual keratinized cells, and this extracellular ceramide may act as a major lipid molecules of the permeability barrier and a skin water reservoir attracting many attention in cosmedical fields including atopy syndrome treatment (Kita *et al*., 2002).

Sphingolipid ceramide N-deacylase (SCDase) and Ceramidase (CDase) are enzymes which hydrolyze the N-acyl linkage between fatty acids and sphingosine bases in ceramides moiety in various kinds of sphinogolipids producing free fatty acid and lysosphingolipid and sphingosine, respectively. It was strongly suggested that CDase plays a crucial role in the control of cellular and intercellular ceramide content as well as in the regulation of intracellular signal transduction (Coroneos *et al*., 1995). As the importance of CDase increases, many CDases have been reported to be isolated and cloned from various sources (Ito *et al*., 1995; Mitsutake *et al*., 2001; Wu *et al*., 2007).

For these reasons, a sensitive, rapid, and reliable assay method for the CDase activity is needed. The usual assay for CDase is based on the assay of radioisotope labeled fatty acids released from sphingolipids or a change of pH due to released fatty acids. Although sphingolipids containing ^{3}H- or ^{14}C-labeled fatty acids are not commercially available for radioisotope assay procedures, a method to prepare such sphingolipid was reported (Mitsutake *et al*., 1997). Although this method is very sensitive and reliable, it has some drawbacks, such as high-priced substrates, time and labor consuming extraction, and separation procedures, as well as regulations regarding the use of radioisotope chemicals. The assay method to determine a change of pH by generating fatty acid in the SCDase catalyzed reaction has no such problems, but the sensitivity is usually too low to be used in the case where a small amount of SCDase is detected.

Chemiluminescence and bioluminescence assay methods are being used increasingly mainly because of low hazard and high sensitivity. Usually, luminescent bacteria have two enzyme systems for *in vivo* light production. One of these systems is bacterial luciferase which produces light using reduced flavin mononucleotide ($FMNH_2$), long chain aliphatic aldehydes, and molecular oxygen (Hastings *et al*., 1978). The other enzyme system, fatty acid reductase complex, is the supplier of long chain aldehydes from the fatty acid moiety of phospholipids, and is composed of three enzymes, acyl transferase, acyl carrier protein, and reductase. First, acyl transferase takes the fatty acid moiety from phospholipids to the acyl carrier protein using ATP, then the reductase reduces this acyl group to its corresponding aldehyde using

* To whom correspondence should be addressed.
(Tel) 82-32-930-6030; (Fax) 82-32-930-6215
(E-mail) kwcho@anyang.ac.kr

NADPH.

Free fatty acid can bind directly to acyl carrier protein, but the M-17 mutant has defective acyl transferase so it cannot produce a high level of bioluminescence *in vivo*, while in the presence of added myristic acid ($C_{14:0}$), it can produce a normal level of light (Ulitzer and Hastings, 1978). The initial light intensity of *Vibrio harveyi* mutant M-17 induced by addition of exogenous myristic acid is linearly correlated with the amount of myristic acid and can be used in the myristic acid quantitation method. In this report a novel luminescence assay system for SCDase is demonstrated using ceramide with N-myristoyl moiety as a substrate, SCDase from *Pseudomonas* sp. and the luminescent marine bacterium *Vibrio harveyi* mutant M-17.

Materials and Methods

Chemicals and enzymes

Myristic acid and other long chain aldehydes were products of Aldrich Chemical Co. Sphingosine from bovine brain (99%) was purchased from Sigma Chemical Co.. SCDase was purchased from TaKaRa Chemical Co.. One unit hydrolyzes 1.0 μmole of sphingolipids to L-α-lysosphingolipids and a fatty acid per min at pH 6.0 (30 mM acetate buffer with 0.8% Triton X-100) and 37°C.

Substrate ceramide containing N-myristoyl group (C14-ceramide, N-myristoyl-D-sphingosine) was synthesized from D-sphingosine and free myristic acid with *Pseudomonas* SCDase by the methods of Mitsutake *et al*. (1997). The name of the enzyme used in this assay was SCDase, but what really measured is CDase activity so the term CDase instead of SCDase can be used for the name of assay. Instead of radioisotope labeled [^{14}C] stearic acid, myristic acid was used for the condensation reaction (10 mg of myristic acid and 5 mg of D-sphingosine in 0.1 ml of 30 mM sodium phosphate buffer (pH 7.5) in the presence of 0.1% Triton X-100 as recommended by manufacturer and incubated at 40°C for 1 h. After the reaction, the reaction mixture was evaporated, dissolved in 50 μl of Folch solution (chloroform/methanol=2/1, v/v), and unreacted sphingosine and myristic acid was removed by Sep-Pak CM cartridge (500 mg, Alltech) and Sep-Pak Plus silica cartridge (Alltech), respectively. Disposable desalting column Econo 10-DG was a product of BioRad Co. (USA). Ceramide used for TLC standard was obtained from gorgonian *Acabaria undulata* in Korea Ocean Research & Development Institute, marine natural products laboratory.

Culture of luminous bacterium

Vibrio harveyi mutant M-17 was grown in 50 ml of Sea Water Complete medium (5 g Bactotryptone, 3 g yeast extract, 3 ml glycerol, 75% Aged Sea Water per liter, pH 7.0) overnight at 25°C up to OD_{660}=1.5. Cells were harvested by centrifugation (Vision Science Co., Korea) at 25,000×g for 5 min. then washed twice with cold washing buffer (0.1 M Na-phosphate buffer containing 3% NaCl, pH 6.5). The harvesting time is important because myristic acid induced bioluminescence begins to decline after reaching a maximum value in the late logarithmic phase of growth. The washed cell pellet was resuspended in cold washing buffer to final OD_{660}=10, stored on ice, and 0.1 ml of this suspension was diluted to 1 ml with the same buffer at room temperature just before each measurement. This cell suspension gave reproducible results for approximately 3 h.

Luminescent assay of CDase

The free myristic acid (tetradecanoic acid) produced by the reaction of CDase was quantitized with *Vibrio harveyi* M-17 dark mutant. Ten microliter of C14-ceramide stock solution (2 mM in Methanol) was added to 0.5 ml of 50 mM Na-Phosphate buffer (pH 6.0 as recommended by manufacturer) in a 1.5 ml eppendorf tube and the reaction was started by addition of an appropriate amount of CDase (typically 0.2 munit), then incubated at 37°C in a water bath. At every 5 min, 50 μl aliquots were withdrawn using a micropipete, put in a test tube, and heat briefly by immersing boiling water to stop the reaction. Then, a prepared luminous bacterium M-17 cell suspension (1 ml) was added and mixed using a vortex for 5 sec, and the light intensity was measured promptly. The maximum light intensity (Io) was measured using a photomultiplier photometer equipped with a Hamamatsu R-447 phototube with a pre-amplifier (Mitchell and Hastings, 1971) and a high voltage power supply (GW-3000, Bioneer Co.). Records were made with a strip chart recorder (Linear-250). Usually, light peaks were obtained within 1 min. The peak intensity was directly related to the concentration of myristic acid in the mixture. Calibration was done using the continuos light source of Hastings and Weber (1963). One light unit (LU) was defined as 2×10^7 quanta/sec.

Results and Discussion

The growth of the luminous bacterium *V. harveyi* dark mutant M-17 was followed by measuring either the absorbance at 600 nm or the *in vivo* light intensity in the presence of 10 μM of myristic acid, because this bacterium can emit light only in the presence of long chain aldehydes or myristic acid. The level of bioluminescence without addition of myristic acid is approximately 3 LU/ml/A_{600nm}. The luminescence activity induced by addition of myristic acid was a maximum (about 3,500 LU/ml/A_{600nm}) just before cell growth reached the stationary phase. Activity then declined in the stationary phase. For best results, cells should be harvested and washed with buffer just before the beginning of the stationary phase (A_{600nm} is about 1.5). Using a bacterial suspension in phosphate buffer (pH 6.5), the dependence of light intensity on the concentration of added myristic acid was measured, and a standard curve was generated which was linear in the range of 5 nM to 10 μM final concentration of myristic acid. The curve shows a saturation at concentrations of myristic acid higher than 10 μM at the given cell density. Using a suspension with a higher cell density would generate a higher light intensity, but the background signal from the cells also increases so there is no real advantage in sensitivity. The standard curve of light intensity versus myristic acid concentration and the growth curve of *V. harveyi* M-17 were published elsewhere (Cho, 1994).

The synthesis of myristic acid containing ceramide was completed about 2 h, and after purification with Sep-Pak

plus Silica cartridge and Sep-Pak CM cartridge, no free myristic acid was remaining in the sample, which was confirmed with *V. harveyi* M-17 suspension showing no light (data not shown). The hydrolysis of N-myristic acid from ceramide by SCDase was monitored with time using the bioluminescence method (Fig. 1). A plot of light intensity with time was linear up to 25 min. Usually a short delay was observed in myristic acid production during the initial reaction phase, probably due to binding of SCDase to the formed ceramide miscele. This phenomenon was also found in the hydrolysis of phospholipids (Cho, 1994). Ten to fifteen min of fixed assay time is enough to determine SCDase activity, especially for a large number of analyses, such as screening SCDase production or SCDase inhibition assay using bioluminescence. SCDase activity versus light intensity showed good linearity with this luminescent assay, down to 5 mUnit (Fig. 2). SCDase activity versus an increasing concentration of added ceramide substrate exhibited a little distorted hyperbolic pattern of substrate concentration dependence, and from a double reciprocal plot, the Km value of C14-ceramide for SCDase was about 23 μM (Fig. 3).

$$\text{Sphingosine} + \text{Myristic acid} \xrightarrow[\text{at pH 8.0}]{\text{SCDase}} \text{C14-ceramide} \quad (1)$$

$$\text{C14-ceramide} \xrightarrow[\text{at pH 6.0}]{\text{SCDase/CDase}} \text{Sphingosine} + \text{Myristic acid} \xrightarrow{\text{M-17}} \text{light} \quad (2)$$

The usual SCDase or CDase assay system involves quantitation of released free fatty acids or sphingosine after enzymatic hydrolysis. The most popular method uses an artificial ceramide with a radioisotope labeled fatty acid moiety at the amine position (Mitsutake *et al*., 1998). After the reaction, the whole reaction mixture is extracted, concentrated and free fatty acids are separated with thin layer chromatography, scraped off, and the radioisotope activity is measured using a scintillation counter. Another CDase assay method using fluorescent fatty acid derivative, like C12-NBD (7-chloro-4-nitrobenzo-2-oxoa-1,3-diazole)-ceramide as a substrate was also reported (Okino *et al*., 1998). The detection of radioisotope labeled or fluorescent fatty acids released from the reaction is very sensitive method. But increasing concerns about environmental contamination and public health have resulted in a large number of regulations regarding the use of radioisotope labeled chemicals in laboratories. The time and labor cost of the separation process of the free fatty acids from the whole reaction mixture is another drawback of these methods. Assay with HPLC with reversed phase or normal phase column and Evaporative Light-Scattering Detector was also reported (McNabb *et al*., 1999), but each assay needs about 30 min of chromatography.

A bioluminescence assay of SCDase described in this report is a straightforward analysis system using a relatively

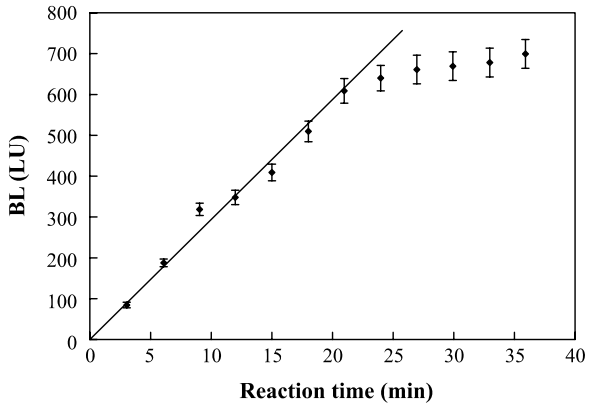

Fig. 1. Time dependent myristic acid production from C14-ceramide by SCDase assayed using M-17. To 1 ml of 50 mM Na-Phosphate buffer (pH 6.0), add 20 μl of 2 mM methanolic C14-ceramide solution, and start the reaction with addition of about 1 munit of SCDase. The reaction mixture was incubated at 37°C, at every 3 min, 50 μl aliquots were taken and added into 1 ml of luminous bacteria suspension in 50 mM phosphate buffer (pH 6.0) and then assayed as described in 'Materials and Methods' section.

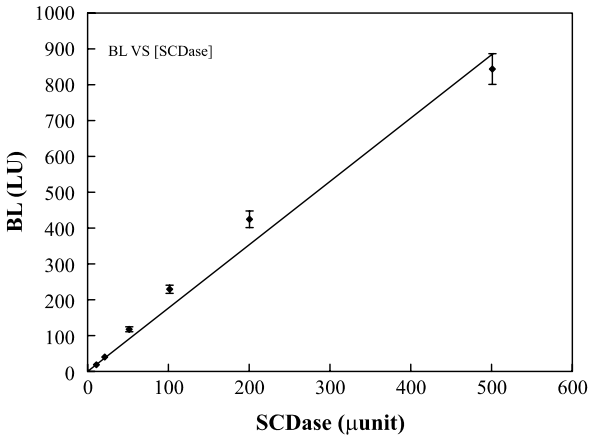

Fig. 2. Various amount of SCDase activity was used in the presence of 50 μM of C14-ceramide in 50 mM Na-Phosphate buffer (pH 6.0 at 37°C), using 15 min fixed time assay procedure.

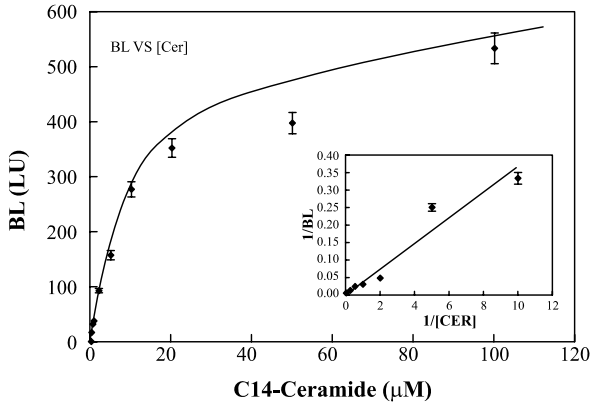

Fig. 3. Luminescent assay of SCDase activity was performed at various concentration of C14-ceramide. 1 munit of SCDase was used in 50 mM Na-Phosphate buffer (pH 6.0 at 37°C), and 15 min fixed time assay were used. (inlet) The double reciprocal plot of light intensity versus C14-ceramide concentration was shown.

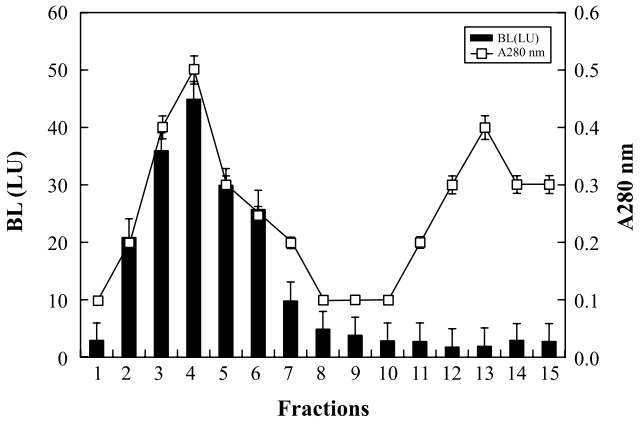

Fig. 4. Bioluminescence assay of CDase chromatographic fractions. Econo 10-DG desalting column was used for demonstration, and 0.2 ml of each fraction (CDase) and 10 μl of C14-ceramide (0.1 mM) were added into 0.5 ml of 100 mM Na-Phosphate buffer (pH 6.0), and after 30 min, *V. harveyi* M-17 suspension (0.1 ml) was and measure the maximum light intensity.

inexpensive substrate and simple equipment. Mitsutake *et al.* (1997) had prepared only [^{14}C]lauroyl-ceramide, [^{14}C]palmitoyl-ceramide, and [^{14}C]stearoyl-ceramide. Although $C_{14:0}$-ceramide was not available commercially and synthesized in this work for the first time, it can be easily synthesized enzymatically using commercial enzyme. Only free myristic acid can induce bioluminescence from luminescence bacterium M-17 unlike radioisotope derivative or fluorescent substrates. The lower limit of myristic acid detection with M-17 is dependent largely on the sensitivity of the photometer used. In this work the limit was 5 pmole of myristic acid using a home-made luminometer. Luminous bacteria, *Vibrio harveyi* M-17, can be easily cultured and used directly as an assaying reagent with relatively little cost and labor. Although the stability of an M-17 suspension is not high and gives reproducible results only for 3 h, bacterial suspensions can be prepared easily. This method can be applied to any myristic acid producing reaction, such as the cholesterol esterase or phospholipase reaction (Cho, 1993, 1994), and alkyl aldehydes like monoamine oxidase and α-oxidase (Cho and Shim, 1993; Cho, 2000). When this assay system is used for crude enzyme source, such as for the screening of SCDase or CDase producing microorganisms or plants, the inclusion of about 1 mM of hydroxylamine in the crude enzyme source is recommended to trap any contaminated natural long chain aldehydes. Although free myristic acid is hardly found in most of fresh cells, some samples such as milk can contain relatively high amount of free myristic acid when decomposed. So a control experiment to minimize the effect of endogenous myristic acid is highly recommended to use this assay method.

The SCDase used in this work was reported to be isolated from *Pseudomonas* sp. with broad substrate specificity. The best substrate reported is sphingolipid GM2 [GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-1'Cer], but simple ceramide without sugar moiety also can be a substrate with about 30% activity of GM2 (TaKaRa Biomedical Co., 2007). By changing the substrate, this method can analyze any sphingolipid deacylase activity including SCDase as well as CDase. One of the most useful application is the enzyme activity assay of fractions collected from any chromatographic separation during the purification process.

References

Cho, K.W. 1993. Bioluminescent assay of bovine liver cholesterol esterase using bacterial luciferase coupled reaction. *Kor. J. Microbiol*. 31, 532-536.

Cho, K.W. 1994. Bioluminescent assay of phospholipase A_2 using luminescent marine bacterium *Vibrio harveyi* mutant M-17. *J. Kor. Biochem*. 27, 522-526.

Cho, K.W. 2000. Bioluminescent assay of α-oxidase from *Cucumis sativas* using bacterial luciferase coupled reaction. *J. Biochem. Mol. Biol*. 33, 353-357.

Cho, K.W. and S.C. Shim. 1993. A new assay method for bovine serum monoamine oxidase: Bacterial luciferase coupled reaction. *J. Kor. Biochem.* 26, 61-65.

Coroneos, E., M. Martinez, S. McKenna, and M. Kester. 1995. Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. Implications for cellular proliferation and differentiation. *J. Biol. Chem*. 270, 23305-23309.

Hastings, J.W., T.O. Baldwin, and M.Z. Nicoli. 1978. Bacterial luciferase: Assay, purification, and properties, p. 135-152. *In* M. DeLuca (ed.), Methods in Enzymology, 57. Academic Press, New York, N.Y., USA.

Hastings, J.W. and G. Weber. 1963. Total quantum flux of isotopic source. *J. Opt. Soc. Am*. 53, 1410-1415.

Ito, M., T. Kurita, and K. Kita. 1995. A novel enzyme that cleaves the N-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms. *J. Biol. Chem*. 270, 24370-24374.

Kita, K., N. Sueyoshi, N. Okino, M. Inagaki, H. Ishida, M. Kiso, S. Imayama, T. Nakamura, and M. Ito. 2002. Activation of bacterial ceramidase by anionic glycerophospholipids: possible involvement in ceramide hydrolysis on atopic skin by *Pseudomonas* ceramidase. *Biochem. J*. 362, 619-626.

McNabb, T.J., A.E. Cremesti, P.R. Brown, and A.S. Fischl. 1999. The separation and direct detection of ceramides and sphingolipid bases by normal-phase high-performance liquid chromatography and evaporative light-scattering detection. *Anal. Biochem.* 276, 242-250.

Mitchell, G. and J.W. Hastings. 1971. A stable, inexpensive solid state photomultiplier photometer. *Anal. Biochem*. 39, 243-250.

Mitsutake, S., K. Kita, T. Nakagawa, and M. Ito. 1998. Enzymatic synthesis of ^{14}C-glycosphingolipids by reverse hydrolysis reaction of sphingolipid ceramide N-deacylase: detection of endoglycoceramidase activity in a seaflower. *J. Biochem*. 123, 859-863.

Mitsutake, S., K. Kita, N. Okino, and M. Ito. 1997. [^{14}C]ceramide synthesis by sphingolipid ceramide N-deacylase: new assay for ceramidase activity detection. *Anal. Biochem*. 247, 52-57.

Mitsutake, S., M. Tani, N. Okino, K. Mori, S. Ichinose, A. Omori, H. Iida, T. Nakamura, and M. Ito. 2001. Purification, characterization, molecular cloning, and subcellular distribution of neutral ceramidase of rat kidney. *J. Biol. Chem*. 276, 26249-26259.

Okino, N., M. Tani, S. Imayama, and M. Ito. 1998. Purification and characterization of a novel ceramidase from *Pseudomonas aeruginosa. J. Biol. Chem*. 273, 14368-14373.

Pettus, B.J., C.E. Chalfant, and Y.A. Hannun. 2002. Ceramide in apoptosis: an overview and current perspectives. *Biochim. Biophys. Acta*. 1585, 114-125.

Taha, T.A., Y.A. Hannun, and L.M. Obeid. 2006. Sphingosine kinase: biochemical and cellular regulation and role in disease. *J. Biochem. Mol. Biol*. 39, 113-131.

Takara Korea Biomedical Co. 2007. Takara Biocatalog. Takara Korea Biomedical Co., Seoul, Korea.

Thorpe, S.R. and C.C. Sweeley. 1967. Chemistry and metabolism of sphingolipids. On the biosynthesis of phytosphingosine by yeast. *Biochemistry* 6, 887-897.

Ulitzur, S. and J.W. Hastings. 1978. Myristic acid stimulation of bacterial bioluminescence in "aldehyde" mutants. *Proc. Nat. Acad. Sci. USA* 75, 266-269.

Wu, B.X., C.F. Snook, M. Tani, E.E. Büllesbach, and Y.A. Hannun. 2007. Large-scale purification and characterization of recombinant *Pseudomonas* ceramidase: regulation by calcium. *J. Lipid Res.* 48, 600-608.