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# Sphingosine Kinase Assay System with Fluorescent Detection in High Performance Liquid Chromatography

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Activation of Sphingosine kinase (Sphk) increases a bioactive lipid, sphingosine 1-phosphate (S1P) and has been observed in a variety of cancer cells. Therefore, inhibition of Sphk activity was an important target for the development of anticancer drugs. As a searching tool for Sphk inhibitor, we developed fluorescent Sphk activity assay combined with high performance liquid chromatography (HPLC). Previously we established murine teraticarcinoma mutant F9-12 cells which lack S1P lyase and stably express Sphk1. By using F9-12 cells, optimal assay conditions were established as follows; 100 μM of $C_{17}$-Sph and 30 μg protein of F9-12 cells lysate in 20 min. Sphingosine analog $C_{17}$-Sph was efficiently phosphorylated by Sphk activity ($K_m$ :67.08 μM, $V_{max}$ :1507.5 pmol/min/mg). New product $C_{17}$-S1P was separated from S1P in reversed-phase HPLC. In optimized conditions, 300 nM of phorbol 12-myristate 13-acetate (PMA) increased Sphk activity approximately twice while 20 μM of N,N-dimethylsphingosine (DMS) reduced 70% of Sphk activity in F9-12 cells lysate. In conclusion, we established non-radioactive but convenient Sphk assay system by using HPLC and F9-12 cells.

**Key words:** Sphingosine 1-Phosphate, HPLC, Activity, Sphingosine kinase

## INTRODUCTION

Activation of sphingosine kinases type 1 (Sphk1) and type 2 (Sphk2) rapidly phosphorylated sphingosine (Sph) to sphingosine 1-phosphate (S1P). S1P is a bioactive lipid as it binds to the G-protein-coupled receptors recently renamed $S1P_1$~$S1P_5$ to elicit several biological responses, cell proliferation, angiogenesis and calcium immobilization (Lee *et al.*, 1996). Sphk1 controls the endogenous lipids balance between proapoptotic lipid ceramide and S1P. The ceramide-S1P rheostat has been suggested to determine whether cells undergo apoptosis or proliferation. Sphks and ceramidase are activated by a number of growth factors or intracellular oncoproteins, leading to rapid increases in the intracellular S1P levels and reduction of ceramide. This situation promotes cell proliferation and inhibits apoptosis in tumor cells. Therefore, Sphks in this pathway provide potential targets for the development of new anticancer drugs (Claus *et al.*, 2000).

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Inhibition of Sphks gave profound antiproliferative effects on various tumor cells because S1P is a strong mitogen in this pathway. This issue is substantiated by a recent report that Sphks can directly transform cells (Xia *et al.*, 2000). Indeed, the levels of mRNA encoding Sphks are approximately 2-fold higher in tumors of the breast, colon, lung, ovary, stomach, uterus, kidney, and rectum compared with normal tissue from the same patient (French *et al.*, 2003).

To inhibit Sphk activity, synthetic sphingosine analogs, dimethylsphingosine (DMS), D,L-*threo*-dihydrosphingosine (DHS) and *N,N,N*-trimethylsphingosine (TMS) have mainly used for pharmacological studies. However, these compounds are not specific inhibitors of Sphks as they are known to affect protein kinase C (Igarash *et al.*, 1989), sphingosine-dependent protein kinase (Megidish *et al.*, 1995), 3-phosphoinositide-dependent kinase (King *et al.*, 2000), and casein kinase (McDonald *et al.*, 1991).

Therefore, for developing selective and potent inhibitors of Sphks, sensitive and convenient assay system on Sphk activity has been required. Sphk activity is commonly determined by using radiometric assays (Kono *et al.*, 2000, 2001). The most widely used protocol involves

incubation of Sphk enzyme with Sph and [$^{32}$P] ATP; after extraction of lipids into an organic phase under acidic conditions followed by thin-layer chromatography (TLC), [$^{32}$P] S1P is identified by autoradiography or phosphorimaging (Ogretmen *et al.,* 2001). Alternatively, [$^{3}$H]-labeled Sph (Kono *et al.,* 2000, 2001) has also used, and the product [$^{3}$H] S1P is recovered in the aqueous phase and identified by TLC or directly quantified using liquid-scintillation counting. This procedure has a number of limitations since it lacks the ability to resolve structurally similar radio-labeled species, and can have considerable variability which almost arises due to the sub-quantitative (70-80%) recovery of S1P in the solvent extraction.

In this study, a non-radioactive, sensitive, and highly reproducible fluorescent assay for Sphk activity was developed using a synthetic $C_{17}$-Sph as a substrate plus ATP, and the reaction products $C_{17}$-S1P was dephosphorylated with alkaline phosphatase. The liberated $C_{17}$-Sph were labeled with o-phthalaldehyde (OPA) and then analyzed in HPLC system.

## MATERIALS AND METHODS

### Materials

The Fumonisin $B_1$ ($FB_1$), *d-erythro*-sphingosine (Sph), S1P were purchased from Biomol Research, Inc. (Plymouth Meeting, PA, U.S.A.). $C_{17}$-sphingosine ($C_{17}$-Sph) and $C_{17}$-S1P were obtained from Avanti Polar Lipids, Inc. (Alabastar, AL, U.S.A.). Alkaline phosphatase (APase), DMS and phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO, U.S.A.). Serum and culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.). HPLC-grade acetonitrile was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, U.S.A.).OPA was obtained from Nacalai Tesque (Kyoto, Japan). All organic solvents and chemicals were of analytical grade.

### Cell culture

Mouse embryonal carcinoma F9-12 cells (Sphk overexpressed and S1P lyase knocked-out) were kindly supplied by Dr. A. Kihara in Hokkaido University, Japan. Cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) FBS and 1% penicillin-streptomycin in 0.1% gelatin-coated dishes. Cells were cultured at 37°C in a humidified 5% $CO_2$ atmosphere and routinely subcultured every other day using a solution of trypsin-EDTA from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.).

### Preparation of cells lysate and protein assay

Cells were immediately washed twice with ice-cold phosphate-buffered saline (PBS) and then scraped in 3 mL of lysis buffer (1 mM PMSF, 1x protease inhibitor cocktail, and 1 mM dithiothreitol) after centrifugation at 10000 rpm for 3 min. The pellet lysated in 1 mL assay buffer (12 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail, 5 mM sodium orthovanadate, 2 mM dithiothretol, 0.5 mM 4-deoxypyridoxine) was then sonicated and centrifuged at 1500 rpm for 3 min at 4 .and the resulting supernatant was determined the protein concentration by performing Bradford assay. The cell lysate was diluted to approximately 1 mg/mL total cell protein.

### Sphk activity measurement

In brief, 30 μg protein from cell lysate was incubated in total volumes of 160 μL containing 10 μL of 40 mM ATP in 200 mM $MgCl_2$ and 10 μL of 100 μM $C_{17}$-Sph in 5% Triton X-100. Incubations with cell lysates contained 5 mM NaF and $Na_3VO_4$, included as inhibitors of S1P phosphatase and lyase, respectively, to prevent potential degradation of $C_{17}$-S1P. Reactions were incubation for 20 min at 37°C and reactions terminated by addition of 20 μL 1 N HCl and 0.8 mL $CHCl_3$/Methanol/HCl (100:200:1, v/v) in cold ice. Samples were vigorously vortexed and centrifuged after adding 200 pmol S1P as internal standard. Next, 250 μL of $CHCl_3$ and 250 μL of 2 M NaCl were added, and the mixture was vortexed vigorously for 10 min. Samples were centrifuged for 4 min at 12,000 rpm at 4 and the upper phase was removed. The organic phase was transferred to a fresh tube and left on ice. The $C_{17}$-S1P was extracted by addition of 400 μL $H_2O$ and 40 μL of 3 N NaOH. The tube was then vortexed for 10 min and centrifuged at 12,000 rpm for 4 min. The alkaline aqueous phase containing $C_{17}$-S1P, was mixed thoroughly with 130 μL of dephosphorylation reaction buffer (200 mM Tris-HCl (pH 7.4), 75 mM $MgCl_2$ in 2 M glycine buffer, pH 9.0) and 50 units of APase. The tube was incubated at 37°C for 1 h. The dephosphorylated $C_{17}$-Sph was extracted twice with 500 μL of $CHCl_3$ and 300 μL of $CHCl_3$ then washed 3-times with alkaline water (pH 10.0). The washed $CHCl_3$ phase was transferred to a fresh tube and dried completely under a nitrogen stream.

### HPLC analysis for Sphk activity

The residues were dissolved in 120 μL ethanol, and incubated it at 50°C for 10 min and then derivatized with 15 μL OPA reagent (50 mg OPA, 1 mL ethanol, 100 μL 2-mercaptoethanol, and 50 mL 3% (w/v) boric acid solution). After incubation of the tubes for 40 min in dark room at room temperature, 100 μL aliquots was injected into the HPLC, which consisted of a Jasco (Tokyo, Japan) Model PU 9850 pump, Cosmosil 5C 18-AR packed with Nova-Pak C18 (4.6 mm i.d.x 150 mm), Jasco FP-920 fluorescence spectrophotometer (λex. 340 nm, ëem. 455 nm)

with Jasco autosampler. The isocratic mobile phase of 90% acetonitrile was pumped at a flow rate of 1 mL/min. The resulting data was evaluated using the Borwin chromatographic system manager software. The Sphk specific activity was calculated from the newly synthesized amount of $C_{17}$-S1P and expressed as pmol/mg/min.

### Statistics

All values were expressed as a mean ± standard deviation (SD). Differences between treatments were analyzed statistically by unpaired Student's *t*-test. Differences with $p<0.01$ were defined as statistically significant.

## RESULTS

### Optimized Sphk activity assay

The Sphk activity was measured from F9-12 cells lysate with $C_{17}$-Sph in 5% Triton X-100 and ATP for 20 min. The Sphk specific activity in F9-12 cells was 1.5 nmol/mg/min ($n$=4). The good linear relationship between Sphk activities and the protein amounts of cell lysate was observed in the range of 0-50 μg protein (Fig. 1).

Next, to know the optimal reaction time for $C_{17}$-S1P production, the Sphk activity in 30 μg protein of F9-12 cell lysate was measured for indicated incubation time (Fig. 2). As expected, the Sphk activity exhibited linearity during the incubation from 0-30 min. However, the synthetic rate of $C_{17}$-S1P was gradually lower with prolonged incubation over 30 min. Therefore, the incubation for 20 min was optimal in quantitative range for measuring $C_{17}$-S1P production.

To know the affinity of $C_{17}$-Sph as a substrate and rate of $C_{17}$-S1P production, a wide concentration range of $C_{17}$-Sph (0~1,000 μM) was tested. The synthetic rate of $C_{17}$-S1P was nearly maximized at 500 μM of substrate concentration when 30 μg protein of F9-12 cell lysate was used. From the curve showing a fit of the data to the Michaelis-Menten equation, $K_m$ value and $V_{max}$ were calculated 67.08 μM and 1507.5 pmol/min/mg, respectively (Fig. 3). The inset shows an Eadie-Hofstee plot of the data with linear fit. We chose 100 μM of $C_{17}$-Sph concentration because the $C_{17}$-S1P production rates were quickly lowered in higher $C_{17}$-Sph concentration over 100 μM.

Accordingly, for conventional Sphk assay in F9-12 cells,

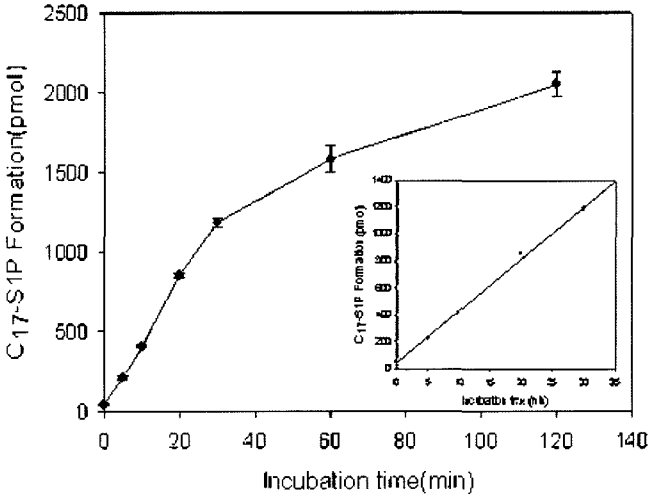


**Fig. 2.** Time courses of the $C_{17}$-S1P production in F9-12 cells lysates. SPHK activity was measured with 30 μg protein of F9-12 cells lysates plus 1.0 nmol $C_{17}$-Sph for the indicated time. The inset shows a linear fit for 0 to 30 min. Values represent the mean ± S.D. from triplicate samples in a typical experiment.

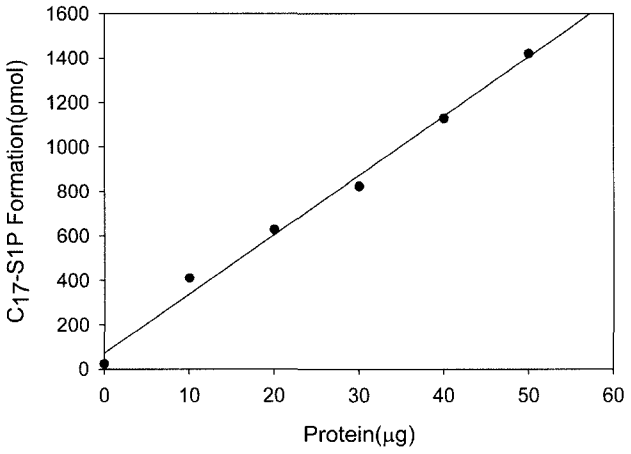


**Fig. 1.** The $C_{17}$-S1P was newly synthesized by the Sphk activity in F9-12 cells lysate. The linear relationship of Sphk activity to protein amount was obtained (average value from duplicated data). The Sphk activity was measured as described in Materials and methods by adding substrate, 1.0 nmol of $C_{17}$-Sph for 20 min.

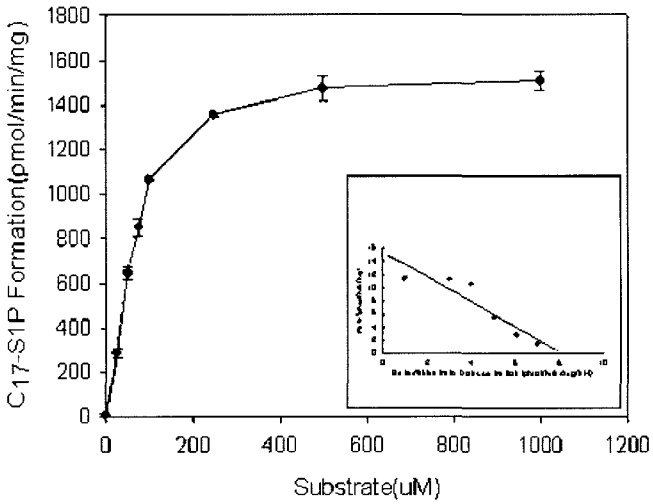


**Fig. 3.** The $C_{17}$-S1P production rate depends on the concentration of substrate, $C_{17}$-Sph. $K_m$ (67.08 μM) and $V_{max}$ (1507.5 pmol/min/mg) values were calculated from the Michaelis Menten equation. The inset shows an Eadie-Hofstee plot of the data with linear fit. Values represent the mean ± S.D. from triplicate samples in a typical experiment.

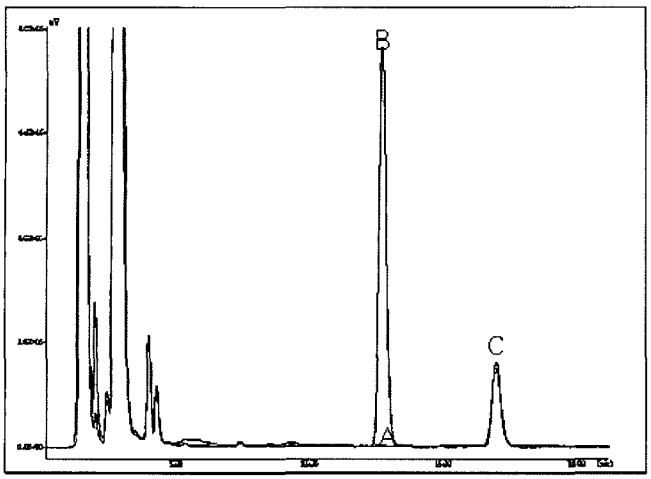


**Fig. 4.** Typical chromatographic profile of $C_{17}$-S1P product synthesized at 37°C for 20 min in 30 μg protein from F9-12 cell lysate. A, blank (without $C_{17}$-Sph); B, $C_{17}$-S1P product from 1.0 nmol $C_{17}$-Sph as a substrate and C, 200 pmol of S1P as an internal standard.

the reaction conditions were optimized with 100 μM of $C_{17}$-Sph and 30 μg protein of F9-12 cells lysate in 20 min. Under these conditions, about 99% of substrate $C_{17}$-Sph was phosphorylated. Typical chromatographic profiles of phosphorylated products ($C_{17}$-S1P) from $C_{17}$-Sph by Sphk activity in F9-12 cells were shown in Fig. 4. In this condition, $C_{17}$-S1P was eluted 12.8 min and well separated from internal standard, S1P (17.1 min). The small peak corresponding to the peak of $C_{17}$-S1P was also observed in blank chromatogram. This experimental error from extraction process plus sample injection was calculated approximately 1.2% and was not so significant when 100 μM of $C_{17}$-Sph was supplied.

### The regulation of Sphk activity by PMA or DMS

Phorbol 12-myristate 13-acetate (PMA) typically increases Sphk activity in early step after the treatment (Mazurek *et al.*, 1994). To evaluate this assay as a screening tool for the Sphk activating materials, F9-12 cells treated with 300 nM PMA for 1h were collected and measured the $C_{17}$-S1P products. The measured $C_{17}$-S1P amount from control and PMA-treated cells were 766.4±69.4 and 1308.8±35.4 pmol/min/mg, respectively. PMA increased Sphk activity 2-fold compared to control cells. (Fig. 5).

Potent Sphk inhibitor, *N,N*-dimethylsphingosine (DMS) has been used to reduce Sphk activity in intact cells (Yatomi *et al.*, 1996). F9-12 cells were treated with 0 μM, 10 μM, 15 μM and 20 μM of DMS and incubated for 24 h at 37°C. SPHK activity was greatly reduced in 20 μM DMS-treated cells (231.6 ± 4.1 pmol/min/mg) compared to control cells (775.9 ± 45.3 pmol/min/mg) (Fig. 6). DMS inhibited Sphk activity in a dose-dependent manner ($IC_{50}$: 16.36 μM) in F9-12 cells. This system will be a useful

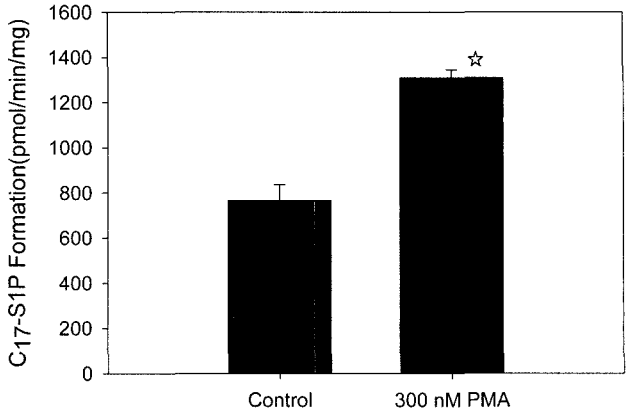


**Fig. 5.** PMA increases Sphk activity. Whole cells lysate from F9-12 cells stimulated by 300 nM of PMA for 1h were measured. The data are expressed by mean ± SD of three independent experiments. *, $p<0.01$ for difference from control.

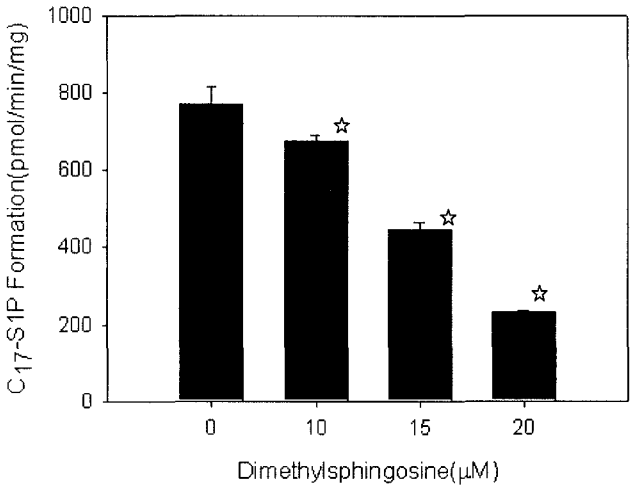


**Fig. 6.** DMS treatment decreases Sphk activity. F9-12 cells were treated with 10, 15 and 20 μM of DMS for 24 h. *$p<0.01$ for difference from control.

method as a screening tool for the development of specific anticancer drugs by inhibiting Sphk activity.

## DISCUSSION

Since S1P was known as a second messenger (Olivera *et al.*, 1993) and inhibits ceramide-induced apoptosis (Cuvillier *et al.*,1996), Sphk enzyme is a potential target in cancer therapeutics. Recently, specific Sphk inhibitors bearing benzofuran-3-one structures efficiently reduced Sphk activity and tumor volume in BALB/c mice (French *et al.*, 2006).

Previous Sphk activity measurements using radio-labeled [$^{32}$P]-ATP or [$^{3}$H]-Sph were semi-quantitative and has limited to measure the activity in Sphk-overexpressed cells (Edsall *et al.*,1999). Therefore, rapid and low-cost

method for the measurement of Sphk activity has been required. Our assay conditions for Sphk activity were optimized with 100 μM of $C_{17}$-Sph and 30 μg protein of F9-12 cells lysate in 20 min.

In general, Triton X-100 has been used to stabilize a number of lipid enzymes, such as phospholipase $A_2$, ceramidase, acid sphingomyelinase, and phosphoinositide 4-kinase, since it prevents aggregation and nonspecific adsorption to surfaces. In our study, addition of Triton X-100 but not Nonidet P-40 or β-octyl glucopyranoside, improved both the Sphk assay recovery and the dissolution of proteins (data not shown).

We used mouse embryonal carcinoma cells (F9-12 cells) which lack the S1P-degrading enzyme S1P lyase (SPL) and stably overproduce Sphk1a as a hemagglutinin (HA)-tagged protein (Kariya *et al.,* 2005). F9-12 cells gave the best circumstance to observe Sphk activity, because they easily accumulate S1P with the lack of SPL, S1P degrading enzyme. Although Km value of cells lysate for $C_{17}$-Sph was 20-fold below to Km value (3.7 μM) of purified mouse Sphk1 (Olivera *et al.,* 1998), fluorescent derivative of $C_{17}$-S1P was detected in high sensitivity. Importantly, the addition of 200 pmol of S1P as an internal standard before lipid extraction profoundly increased the reproducibility in chromatographic profiling (Fig. 4). The typical Sphk activator PMA or Sphk inhibitor DMS were used for the evaluation of this method. PMA increased Sphk activity almost 2-fold in 1 h. DMS reduced 70% of total Sphk activity for 1 day. These data suggested that Sphk assay with HPLC was not required specific reagents but was shown enhanced selectivity for phosphorylated sphingosine analogs. Furthermore, this Sphk assay can also distinguish the type of Sphk (type I and II) depending on the different assay buffer (data not shown). However, this method was required more time to analyze lipids than previous radio-labeled method, because analytical time in HPLC was about 30 min per sample.

This fluorescent HPLC method will provide the improved validation tool for the measurement of Sphk activity as a cancer therapeutic target, as well as screening small molecule inhibitors for this enzyme.

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