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Isolation of Sphinin, an Inhibitor of Sphingomyelinase, from *Streptomyces* sp. F50970

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Abstract Sphingomyelinase (SMase EC:3.1.4.12) has been suggested to play important roles in the cell cycle, differentiation, apoptosis, inflammation, and the regulation of eukaryotic stress responses. SMase inhibitors may be a powerful tool to elucidate and regulate these cellular responses in which SMase involves. We first isolated an SMase inhibitor, named sphinin, from a strain of soil actinomycetes, F50970. Sphinin inhibited Mg^{2+}-dependent neutral SMase from chicken embryo at 1.2 μg/ml of IC_{50}. Sphinin also inhibited acidic SMase, but it had no inhibitory activity on PI-PLC and PC-PLC, suggesting that sphinin is a specific inhibitor of SMase. The strain F50970 was identified as a *Streptomyces* sp. by its spiral spore chain, LL-diaminopimelic acid, menaquinone patterns of MK-9 (H'6) and MK-9 (H'8), FA-2c type of fatty acid pattern, and other morphological, physiological, and cultural characteristics.

Key words: Sphingomyelinase inhibitor, sphinin, chicken embryo, *Streptomyces* sp. PI-PLC, PC-PLC,

Sphingomyelinase (SMase) has received considerable attention in recent years, mainly because sphingomyelin (SM) hydrolysis products have important signalling effects on multiple cellular functions [4, 6, 7]. SMase hydrolyzes SM to produce phosphorylcholine and ceramide. SMase is known to be activated by several extracellular agonists such as TNF-α, Fas ligand, IL-1, 1,25-dihydroxyvitamine D-3, interferon-γ, retinoic acid, and nerve growth factor [3, 8, 10, 13, 15, 16]. Analogous to the central role of diacylglycerol (DAG), ceramide plays an equally critical role as a second messenger in cell signalling through the action of phospholipase C (PLC) in cellular signal transduction [4, 6, 7, 18]. SMase has been suggested to be involved in cell growth, differentiation, apoptosis, and inflammatory responses. However, little is known about the regulation of SMase. An inhibitor of SMase would be a potential agent to explore the mechanism of the signal transduction in these systems [10]. SMase is expected to be a new target of regulator of cell responses related to cancer, cellular senescence, and inflammatory immune disease. Therefore, regulation of SMase activity would be an available means to control these cellular responses. In the line of the screening program, we first screened a strain of soil actinomycetes F50970 which produces an SMase inhibitor.

In this report, we describe the isolation of SMase-specific inhibitor, sphinin, from *Streptomyces* sp. F50970 and the identification of the strain.

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Materials and Methods

Microorganisms

Strains of soil actinomycetes and their culture broths were a gift from Screening Room, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea.

Chemicals

[*N*-methyl-^{14}C]sphingomyelin (bovine; specific activity 56 mCi/mmol) was purchased from Amersham Pharmacia Biotech. PC-PLC (*Clostridium perfringens*, 45 unit/mg protein), *p*-nitrophenylphosphorylcholine, diaminopimelic acid, phenylmethanesulphonyl fluoride (PMSF), aprotinin, and antibiotics were purchased from Sigma (St. Louis, U.S.A.). TLC plates (silica gel 60 F_{254} and cellulose) silica gel 60 (70-230 mesh), Triton X-100, and solvents were purchased from Merck (Darmstadt, Germany).

Preparation of SMase from Chicken Embryo

Chicken embryos at day 8 after egg laying were removed and washed with lysis buffer containing 20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 10 mM 2-mercaptoethanol, 200 mM sucrose, 0.2 mM PMSF, and 2 μM aprotinin. The embryos were homogenized in a Waring blender for 5 min with 4

volumes of the lysis buffer. The homogenate was centrifuged at 6,000 ×g for 10 min to remove debris, and then the supernatant was further centrifuged at 100,000 ×g for 30 min to pellet particulate. The membrane particulate was resuspended in the lysis buffer and extracted with 1% Triton X-100 containing 0.5 M NaCl for 1 h, and then centrifuged at 100,000 ×g for 30 min. The supernatant was dialyzed against the lysis buffer containing 1% Triton X-100. All procedures were carried out at 4°C and the prepared membrane fraction was stored at -80°C until enzyme assay.

Assay for SMase and Inhibitory Activity

SMase assay *in vitro* was carried out as described previously [12] with a slight modification. The reaction mixture of [*N*-methyl-^{14}C] sphingomyelin (10,000 dpm), membrane fraction (20 μg of protein), 0.1 M Tris-Cl (pH 7.5), 0.5% Triton X-100, 10 mM $MgCl_2$, and 0.2% bovine serum albumin with or without inhibitors in a total volume of 0.1 ml was incubated at 37°C for 30 min for Mg^{2+}-dependent neutral SMase. For acidic SMase, 0.1 M sodium acetate (pH 5.0) and 10 mM $CoCl_2$ were used instead of Tris-Cl and $MgCl_2$ [15]. The reaction was stopped by adding 0.8 ml of chloroform/methanol (2:1, v/v). Then, 50 μl of water was added to the mixture, vortexed, and centrifuged at 6,000 ×g for 1 min to separate the two phases. The 100 μl of the upper phase was counted for radioactivity by a liquid scintillation counter (Packard, U.S.A.). The percent inhibition was calculated by the formula 100(A-B)/A, where A is cpm of liberated radioactive product without an inhibitor and B is that with an inhibitor. IC_{50} value represents the inhibitor concentration at 50% inhibition of enzyme activity.

Assay of Other Phospholipases

PI-PLC was assayed according to Ahn *et al.* [1] using [^{3}H] phosphatidylinositol as a substrate. For the assay of PC-PLC from *C. perfringens*, *p*-nitrophenylphosphorylcholine was used as a substrate [11].

Fermentation

A piece of well sporulated ISP2 medium (1% malt extract, 0.4% yeast extract, and 0.4% glucose) agar slice was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of ISP2 medium. This seed culture was shaken on a rotary shaker at 200 rpm for 4 days at 30°C. Ten ml of seed culture was inoculated into 200 ml of fish meal medium (2% glucose, 1% soluble starch, 1% soybean meal, 1% fish meal, 0.1% beef extract, 0.4% yeast extract, 0.2% NaCl, and 0.005% K_2HPO_4, pH 7.3) in a 1-l flask, and incubated under the same conditions.

Isolation of an SMase Inhibitor

During purification, the SMase inhibitor was detected by its inhibitory activity on SMase of chicken embryos. Since a preliminary test showed that the SMase inhibitory activity was found in both culture filtrate and mycelium cake, we used these fractions to isolate the inhibitor, according to the procedure outlined in Fig. 1.

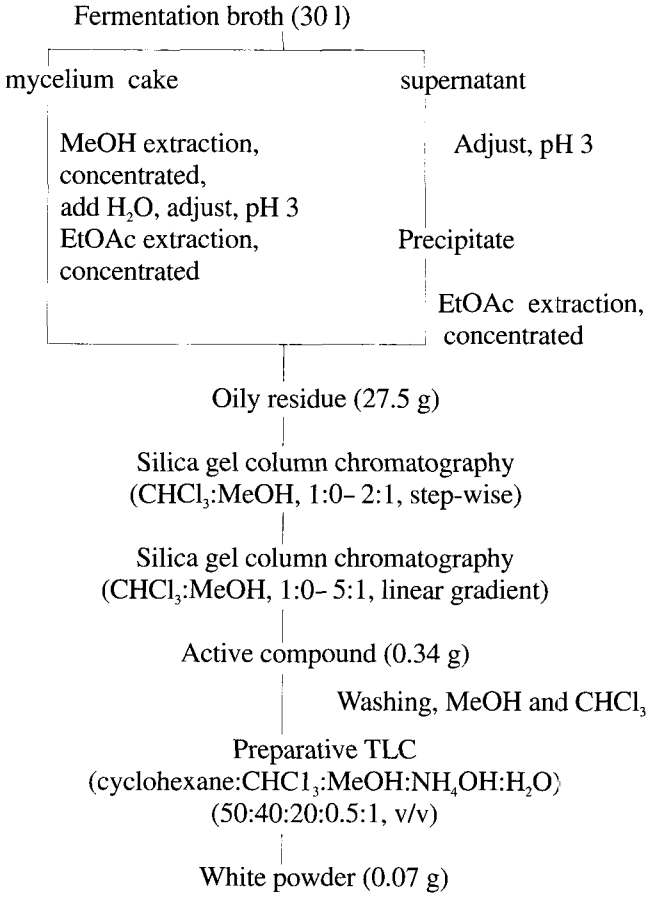

Fig. 1. Isolation procedure for an SMase inhibitor, sphinin, from *Streptomyces* sp. F50970.
EtOAc, ethyl acetate; MeOH, methanol.

Taxonomic Study

Cultural and physiological characteristics of the strain F50970 were examined by using the media and methods of Shirling and Gottlieb [17], and the Bergey's Manual of Systematic Bacteriology, volume 4 [21]. To examine the spore chain morphology, strain F50970 was incubated for 14 days on ISP2 agar medium and ISP 4 agar medium. Spore chain morphology of the strain was examined by light microscopy. Specimen for SEM was prepared by the methods of Williams and Davis [20]. Fatty acids were separated and identified by fatty acid methyl esters (FAMEs) analysis according to the method of Miller and Berger [14]. The FAMEs were analyzed by the gas chromatographic separation on a 25 mm×0.2 mm methyl phenyl silicone fused silica capillary column. Menaquinone homologs were identified by HPLC and LC-Mass spectrometry according to Tamaoka *et al.* [19]. Diaminopimelic

acid (DAP) isomers were analyzed by a cellulose thin layer chromatography according to the method of Yamada and Komagata [22].

RESULTS AND DISCUSSION

Screening of Strains Producing an SMase Inhibitor

We used Mg^{2+}-dependent neutral (pH 7.5) SMase from the membrane particulate of chicken embryos for screening microorganisms which produce SMase inhibitors. From more than 1,000 strains of soil actinomycetes tested, four strains were selected for their inhibitory activity on SMase. Eventually, one strain, named F50970, was selected for its high and stable production of the inhibitor. The production of SMase inhibitor was closely related to the cell growth (data not shown). SMase inhibitory activity was observed both in culture filtrate and mycelium cake.

Isolation and Characteristics of the SMase Inhibitor from F50970

The inhibitor was purified from the culture filtrate and mycelium cake by solvents-extraction, a number of column chromatographies, and preparative TLC (Fig. 1). Figure 2 shows a HPLC profile of purified active compound. An active compound, named sphinin, inhibited SMase in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) of sphinin for Mg^{2+}-dependent neutral (pH 7.5) SMase from chicken embryo was 1.2 μg/ml in our assay system (Fig. 3). Sphinin also inhibited acidic SMase from chicken embryo. However, sphinin had no inhibitory activity on PI-PLC and PC-PLC at a 50 μg/ml concentration (data not shown), suggesting that sphinin might be a specific inhibitor of SMase. From the color reactions [2] of sphinin, it seems not to be related to phospholipids and not to have a choline moiety (Table 1). The physico-chemical properties of sphinin are summarized in Table 2. Sphinin was soluble in chloroform and DMSO but insoluble in hexane, ethylacetate, acetonitrile, and water. The molecular weight of sphinin was shown to be 501.6 based on the FAB-MS data.

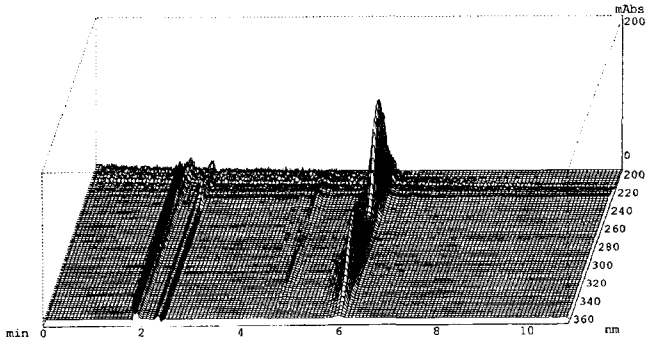

Fig. 2. High-performance liquid chromatogram of sphinin.
Chromatography was performed as follows: column, Hypersil (5 μm, 200×4.6 mm, Hewlett Packard); mobile phase, cyclohexane/$CHCl_3$/methanol/NH_4OH/H_2O (50:40:20:0.5:1, v/v); flow rate, 1 ml/min; and detector, diode array detector (SPD-M10AVP, Shimadzu).

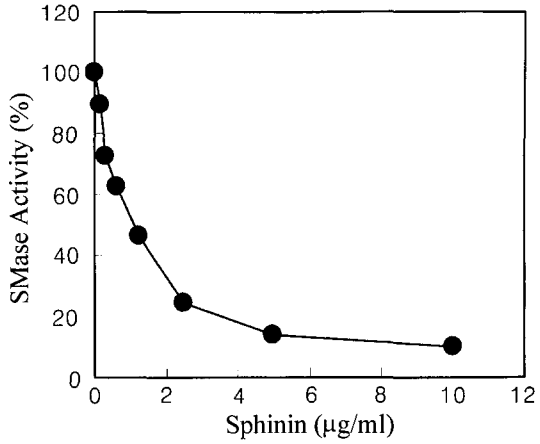

Fig. 3. A dose-dependent effect of sphinin on Mg^{2+}-dependent neutral SMase activity.

Table 1. The comparison of color reaction of sphinin and other lipids[a].

	Sphinin	PC	SM
Iodine	+	+	+
Rhodamine B	+	+	+
Molybdeneum blue reagent (for phospholipids)	-	+	+
Ninhydrins (for free amino group)	-	+	-
Drangendorff's reagent (for choline)	-	+	+

[a]The color reagents were prepared as described in Dawson *et al.* [2].
Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin.

Table 2. Physico-chemical properties of sphinin.

Appearance	White powder
MW	501.6
UV λ_{max} nm (MeOH)	240, 320
IR (KBr) cm^{-1}	3394, 3014, 2957, 2934, 2875, 1659, 1603, 1477, 1237
Solubility[a]	
+	$CHCl_3$, DMSO
±	MeOH, CH_2Cl_2, Dioxane
-	Hexane, EtOAc, CH_3CN, H_2O
R_f (Silica gel 60 F_{254}) $CHCl_3$:MeOH:Acetic acid (6:3.5:0.8, v/v)	0.71

[a]+, soluble; ±, slightly soluble; -, insoluble.
MeOH, methanol; EtOAc, ethyl acetate.

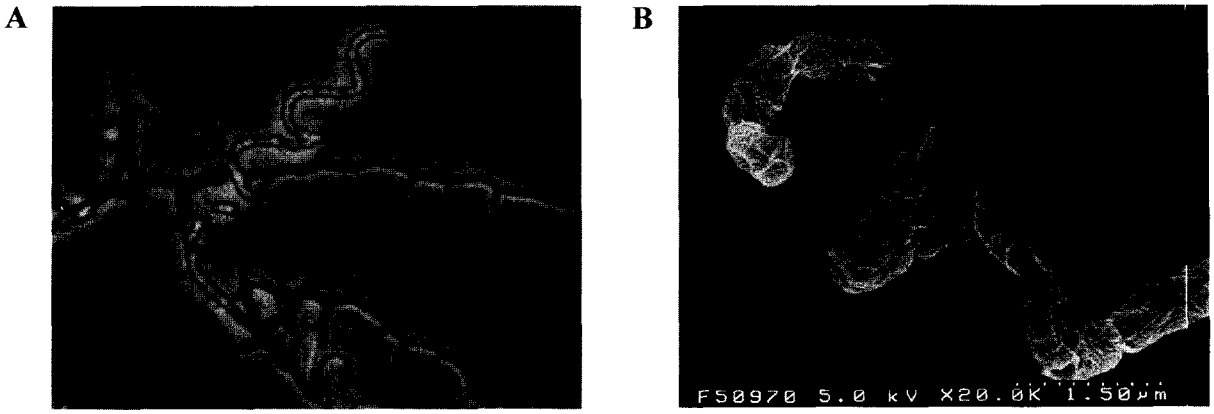

Fig. 4. Phase contrast (× 1 K) (A) and scanning electron micrograph (× 50 K) (B) of spore of the strain F50970.
Strain F50970 was cultivated on inorganic salts-starch agar medium for 14 days at 30°C.

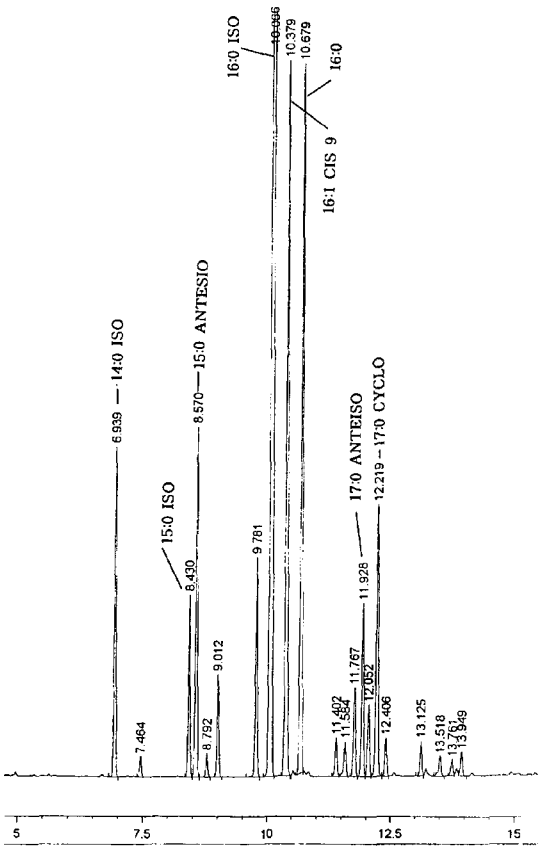

Fig. 5. Gas chromatographic profile of fatty acids of strain F50970.
FAMEs were prepared and analyzed using the standardized procedure described by Miller and Berger [14].

Morphological and Chemotaxonomical Characteristics of Strain F50970

The configuration of the spore chains of strain F50970 grown on inorganic salts starch agar medium (ISP4) had a form of spiral spore chain on aerial mycelium. A spore chain consisted of 50-70 rugose spores (0.5-1.0 µm) (Fig. 4). Aerial spore mass was gray, and the colony was tough and leathery. Strain F50970 showed the characteristics of good growth, pale yellow substrate mycelium, white aerial mycelium, and dark-green soluble pigment. Melanin pigment was observed on peptone yeast iron agar medium (ISP6). The type of diaminopimelic acid (DAP) in the cell wall peptidoglycan of strain F50970 was identified to be LL-DAP, which is identical to those of genus *Streptomyces*. By FAMEs analysis, the fatty acids profile of strain F50970 showed to be FA-2c type (Fig. 5). Menaquinone type of strain F50970 was MK-9 (H_6) and MK-9 (H_8) as a predominant isoprenolog. These results are summarized in Table 3. The utilization of carbon and nitrogen sources and other physiological properties of *Streptomyces* sp. F50970 are summarized in Table 4. From these results, the strain F50970 was identified as *Streptomyces* and named *Streptomyces* sp. F50970 [5].

Table 3. Morphological and chemical characteristics of the strain F50970.

Morphological characters	
Colony surface	leathery
Spore chain morphology	spiral, 30-70 spores
Spore size	0.5×1.0 (µm)
Spore surface	rugose
Color of spore mass	gray
Substrate mycelium	yellow-brown
Melanin pigment	positive
Diffusible pigment	dark green
Chemical characters	
Diaminopimelic acid	LL-DAP
Predominant menaquinones	MK-9(H'_6)/MK-9(H'_8), 4 a' type
Fatty acids	16:0, Iso-14/16, Anteiso-15/17, 2c type

Table 4. Physiological characteristics of strain F50970[a].

Characteristics	score	Characteristics	score
Degradation of			
Allantoin	+	Utilization of C-source (1%)	
Casein	+	Adonitol	+
Elastin	+	L-Arabinose	±
Esculin	+	Cellobiose	+
Hypoxanthine	-	Dextran	+
Starch	+	D-Fructose	+
L-Tyrosine	+	D-Galactose	+
Urea	+	Meso-inositol	+
Xanthine	-	Inulin	+
Xylan	+	D-Lactose	+
		Mannitol	+
Hippurate hydrolysis	+	D-Mannose	+
Pectin hydrolysis	+	D-Melezitose	±
H_2S production	+	D-Melibiose	+
β-Lactamase production	+	Raffinose	+
		L-Rhamnose	+
Antibiosis against		Salicin	-
Bacillus subtilis KCTC 1021	+	Sucrose	+
Micrococcus leuteus KCTC 10240	-	Trehalose	+
Pseudomonas fluorescens KCTC 2344	-	Xylitol	-
Saccharomyces cerevisiae KCTC 1814	+	D-Xylose	+
Candida albicans KCTC 7270	+	Dextrose	+
Streptomyces murinus KCTC 9492	+	Sodium acetate	+
Escherichia coli RK 4936	-	Sodium citrate	+
Aspergilus niger KCTC 2119	+	Sodium malonate	+
		Sodium propionate	+
Resistance to antibiotics (μg/ml)		Sodium pyruvate	+
Dimethylchlorotetracycline (500)	-		
Gentamicin (100)	-	Utilization of N-source (0.1%)	
Neomycin (50)	-	L-Asparagine	+
Olendomycin (100)	+	L-Proline	+
Penicillin G (10iu)	+	DL-α-amino-*n*-butyric acid	-
Rifampicin (50)	+		
Streptomycin (100)	+	L-Arginine	+
Tobramycin (50)	-	L-Cysteine	-
Vancomycin (50)	-	L-Histidine	+
		L-Hydroxyproline	+
Growth with (%)		L-Methionine	-
Crystal violet (0.0001)	+	Potassium nitrate	+
Phenol (0.1)	-	L-Phenylalanine	+
Phenylethanol (0.1)	+	L-Serine	+
(0.3)	-	L-Threonine	+
Potassium tellurite (0.01)	+	L-Valine	-
Sodium azide (0.01)	+		
Sodium chloride (7)	+	Growth at temp. (°C) 4	±
(10)	-	10	+
		37	-
Growth at pH 4.3	-	45	-

[a]The cultural and physiological characteristics of the strain F50970 were examined by using the media and methods according to Shirling and Gottlieb [17] and Williams *et al.* [21].

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