Preparation of a naturally occurring D-*erythro*-(*2S,3R*)-sphingosylphosphocholine using *Shewanella alga* NS-589

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Abstract Sphingosylphosphocholine, an *N*-deacylated derivative of sphingomyelin, has been found to be involved in many cellular events. This paper describes a new method for preparation of a D-*erythro*-sphingosylphosphocholine, which is naturally occurring but difficult to prepare by chemical methods, using marine bacteria as a biocatalyst. When cultured with *Shewanella alga* NS-589 in synthetic medium, sphingomyelin was found to be efficiently converted by sphingomyelin deacylase to sphingosylphosphocholine. Sphingosylphosphocholine was purified with a high yield from the culture supernatant and identified to be a D-*erythro*-(*2S,3R*)-isomer containing d18:1 sphingenine as a long-chain base by fast atom bombardment mass spectrometry and NMR analyses.—**Sueyoshi, N., H. Izu, and M. Ito.** Preparation of a naturally occurring D-*erythro*-(*2S,3R*)-sphingosylphosphocholine using *Shewanella alga* NS-589. *J. Lipid Res.* 1997. *38:* 1923–1927.

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Sphingosylphosphocholine (SPC), an *N*-deacylated derivative of sphingomyelin (SM), is emerging as a new class of signaling molecule for a wide variety of cell activities. In early studies, Desai and Spiegel (1) and Desai et al. (2) reported that SPC is a potent mitogen in a variety of cell types. SPC is accumulated in Niemann-Pick disease type A (3) and stimulates DNA-binding activity of the transcription activator protein AP-1 (4). SPC has been found to stimulate a kinase, probably related to casein kinase II, in Jurkat T cells (5). It was suggested that SPC could be used for therapeutic application in wound healing (6). Recently, SPC has been recognized as a mediator of intracellular Ca^{2+} release from internal stores through a novel intracellular Ca^{2+} channel (7). Mao et al. (8) reported the molecular cloning of a putative receptor for SPC responsible for Ca^{2+} release from endoplasmic reticulum.

SPC is usually prepared by acid methanolysis of SM (9). However, as a consequence of epimerization at C-3 of the sphingosine base during acid hydrolysis, the resulting SPC consists of a mixture of D-*erythro*-(*2S,3R*)- and L-*threo*-(*2S,3S*)-stereoisomers (9). Eventually, almost all studies on SPC used a mixture of these stereoisomers, and thus the physiological functions of naturally occurring D-*erythro* SPC have not been fully clarified.

Here we describe an innovative method for preparing naturally occurring D-*erythro*-SPC using SM-deacylase-producing bacteria as a microbial catalyst. The method consists of the conversion of SM to D-*erythro*-SPC by a newly isolated marine bacterium, *Shewanella alga* NS-589, followed by purification of SPC with conventional column chromatography procedures.

MATERIALS AND METHODS

Materials

SPC (a mixture of D-*erythro*- and L-*threo*-isomers) was obtained from Sigma and Matreya. SM and sodium taurodeoxycholate were purchased from Sigma. Silica gel 60 TLC plates were from Merck (Germany). Recombinant sphingomyelinase (SMase) from *Bacillus cereus* was obtained from Higeta Shoyu Co., Ltd. (Japan) and trypto-soya agar medium was from Nissui Seiyaku Co.,

Abbreviations: SPC, sphingosylphosphocholine; SM, sphingomyelin; SMase, sphingomyelinase; TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; DMe βCD, dimethyl-β-cyclodextrin.

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SM-deacylase-producing bacteria

An SM-deacylase-producing bacterium, *Shewanella* alga NS-589, was isolated from sea mud and maintained on a trypto-soya agar slant medium containing 0.01% SM in order to retain its ability to produce SM-deacylase.

Preparation of D-*erythro*-SPC by using *Shewanella alga* NS-589

NS-589 cells from the agar slant culture were transferred into 100 ml of synthetic medium A (0.05% K_2HPO_4, 0.05% NH_4Cl, 0.1% sodium taurodeoxycholate, 2% NaCl, and 0.1% DMeβCD, pH 7.4) containing 0.1% SM in a 500-ml flask and incubated at 30°C with shaking. After 2 days, the culture was centrifuged at 7,200 *g* for 10 min to remove the bacterial cells. The resulting supernatant from 5 cultures (500 ml) was applied to a reverse-phased preparative C18 column (2.4 × 15 cm), which had been previously washed with chloroform–methanol 1:1 (v/v), and methanol, and equilibrated with distilled water. After washing the column with 500 ml of distilled water, SPC and SM were eluted from the column with 500 ml of methanol. The fractions containing SPC were concentrated to about 10 ml using a rotary evaporator and applied to a Silica Gel 60 column (2.4 × 70 cm), which had been equilibrated with chloroform–methanol–water 5:4:1 (v/v/v). SPC was eluted from the column with the same solvent. Fractions containing SPC were collected, dried with a rotary evaporator, dissolved again in a small amount of distilled water, and finally freeze-dried.

TLC of SPC

Ten μl each of the culture supernatant or eluates from the column was applied to a silica gel 60 TLC, which was developed with solvent I (chloroform–methanol–0.02% aqueous $CaCl_2$, 5:4:1 (v/v/v). For visualizing both SPC and SM, Coomassie brilliant blue R-250 staining was used (10). To examine the purity of the SPC obtained, 10 or 20 μg of sample was applied to TLC which was developed with solvent I, chloroform–methanol–10% acetic acid 5:4:1 (v/v/v) or chloroform–methanol–12 mM $MgCl_2$ 65:25:4 (v/v/v). SPC and other contaminants were made visible by Coomassie brilliant blue R-250, orcinol-H_2SO_4, ninhydrin, Dittmer-Lester reagent, Dragendorff reagent, and primuline.

SMase treatment of SPC

Ten μg of the purified SPC, standard SPC (Sigma) or SM was incubated at 37°C for 16 h with 100 mU of SMase from *Bacillus cereus* in 20 mM Tris-HCl, pH 7.5, containing 0.2% Triton X-100. The reaction products were analyzed by a TLC using solvent I as the developing solvent. The remaining SPC and sphingosine produced were made visible by Coomassie brilliant blue R-250 (10).

FAB-MS analysis of SPC

SPC was analyzed by FAB-MS (JEOL JMS HX-100, JEOL Ltd., Japan) using the positive ion-mode with a 2-nitrobenzylalcohol as the matrix.

NMR analysis of SPC

The ^1H NMR and ^{13}C NMR spectra of the SPC were obtained on a JEOL JNM-A500 spectrometer (JEOL Ltd., Japan). ^1H chemical shifts were measured against $CDCI_3$ at 7.24 p.p.m and ^{13}C chemical shifts were calibrated against $CDCI_3$ at 77.0 p.p.m at 25°C.

RESULTS AND DISCUSSION

We found SM-deacylase activity in the culture fluid of *Shewanella alga* NS-589 which hydrolyzes the *N*-acyl linkage of ceramide in SM to produce SPC. However, the hydrolysis of SM by the enzyme was very slow. Thus large-scale production of SPC by this enzyme was found to be difficult. Instead of using the purified enzyme, we used living NS-589 cells as a microbial catalyst for the conversion of SM to SPC. Surprisingly, 60–70% SM was found to be converted to SPC when 500 mg of egg yolk SM was cultivated with NS-589 at 30°C for 2 days. Neither sphingosine nor ceramide has been detected in the culture, indicating that NS-589 produced SM-deacylase but not SMase. Culture supernatant of NS-589 was applied to a preparative C18 column, from which SPC and SM were eluted by methanol (**Fig. 1A**). Fractions 4 to 9 were pooled, concentrated with a rotary evaporator, and applied to a silica gel 60 column. By chromatography, SPC was found to clearly separate from residual SM and DMeβCD (Fig. 1B), which was used as an enhancer for the conversion of SPC from SM by NS-589, possibly due to the absorption of released fatty acids. Fractions 15 to 30 were collected, concentrated, and finally freeze-dried. In a typical experiment, 154 mg of SPC was obtained from 500 mg of SM.

The final preparation of SPC showed a single band corresponding to the R_f of standard SPC, when the TLCs were developed with solvent I, chloroform–methanol–10% acetic acid 5:4:1 (v/v) or chloroform–methanol–12 mM $MgCl_2$ 65:25:4 (v/v) after being visualized with either Coomassie brilliant blue R-250 (for lipid-containing products, **Fig. 2A**), ninhydrin (for amino group-containing products, Fig. 2B), orcinol-H_2SO_4

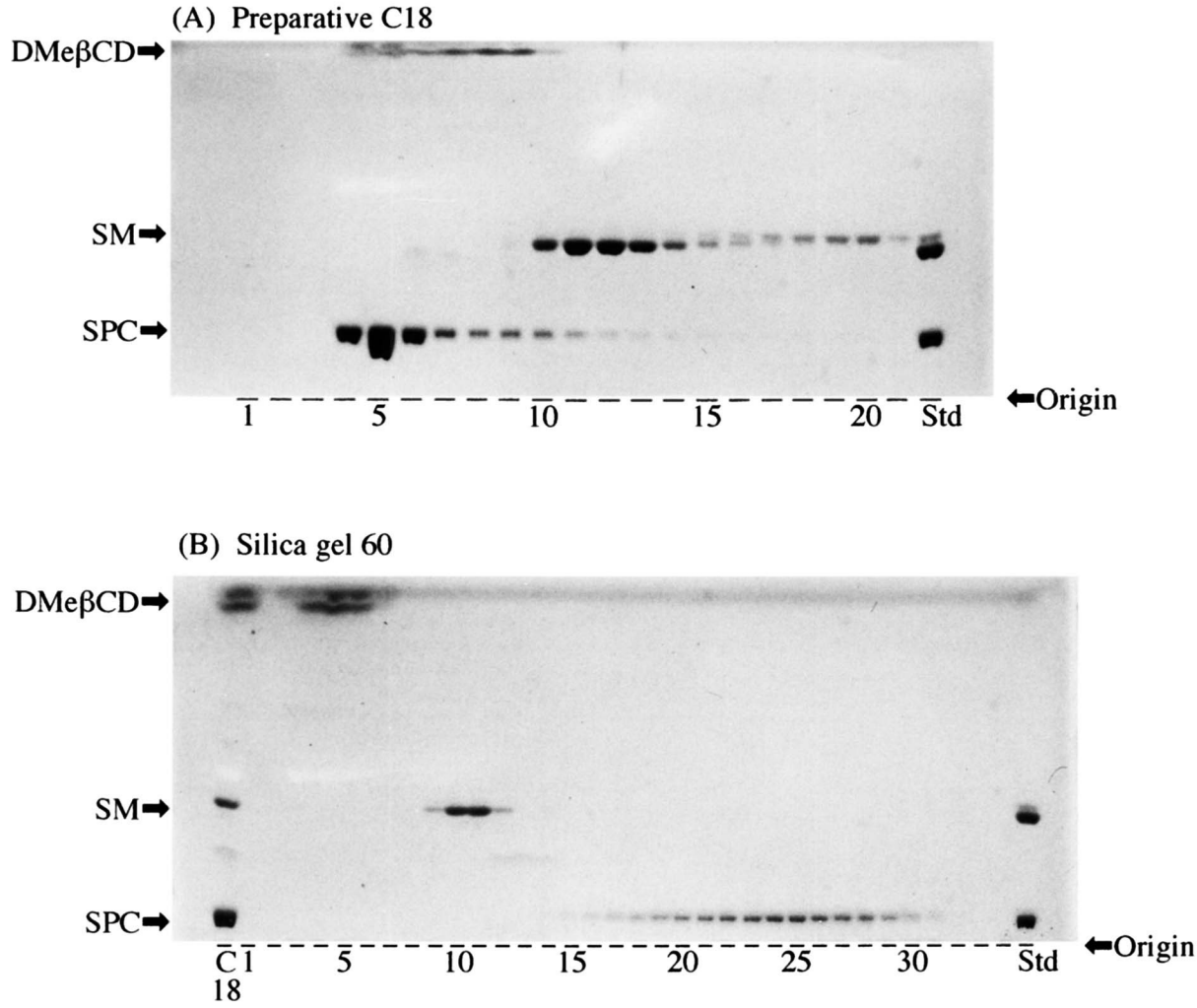

Fig. 1. Purification of D-*erythro*-SPC from the culture supernatant of *Shewanella alga* NS-589. Egg yolk SM (500 mg) was incubated at 30°C for 2 days with NS-589 in synthetic medium A. D-*erythro*-SPC was isolated from the culture supernatant of NS-589 by using (A) a preparative C18 column, followed by (B) a silica gel 60 column. The eluates from the column were checked by TLC as described in Materials and Methods, Std, standard SPC (lower) SM (upper); C18, eluate from preparative C18 column (fractions 4 to 9).

(for carbohydrate-containing products, data not shown), Dragendorff reagent (for choline-containing products, data not shown), Dittmer-Lester reagent (for phosphate-containing products, data not shown), or primuline (for lipid-containing products, data not shown). On the other hand, several impurities were observed in the commercially purchased SPC (Fig. 2A, B). These results indicated that the SPC prepared by the present method was highly purified. Digestion of the SPC with SMase resulted in the production of sphingosine, as observed for standard SPC (Fig. 2C).

The SPC was subjected to FAB-MS using the positive ion-mode with a 2-nitrobenzylalcohol as the matrix. As shown in **Fig. 3,** the characteristic pseudomolecular ions $[M + H]^+$, $[(M-H_2O) + H]^+$ and $[M + Na]^+$ were found at *m/z* 465, *m/z* 447, and *m/z* 487, respectively, indicating that the SPC contains d18:1 sphingenine as a long-chain base.

In ^{1}H NMR spectra of the SPC, the proton on C-4 and C-5 appears as a double doublet at 5.47 p.p.m and a double triplet at 5.77 p.p.m, respectively. These chemical shifts were consistent with those observed on D-*erythro*-(*2S,3R*)-SM (11) and D-*erythro*-(*2S,3R*)-SPC (12). The coupling constant $J_{4,5}$ was 15.3 Hz, suggesting that the two protons on the double bond between carbon atoms 4 and 5 are *trans* to each other. The ^{13}C NMR spectra of two isomers of SPC were reported to be very similar except for a clear difference in chemical shifts on C-3 and C-5 (12). The SPC prepared in the present study gave peaks at 72.56 p.p.m on C-3 and 134.42 p.p.m on C-5, both of which were consistent with those observed on D-*erythro*-(*2S,3R*)-SPC (12). The chemical

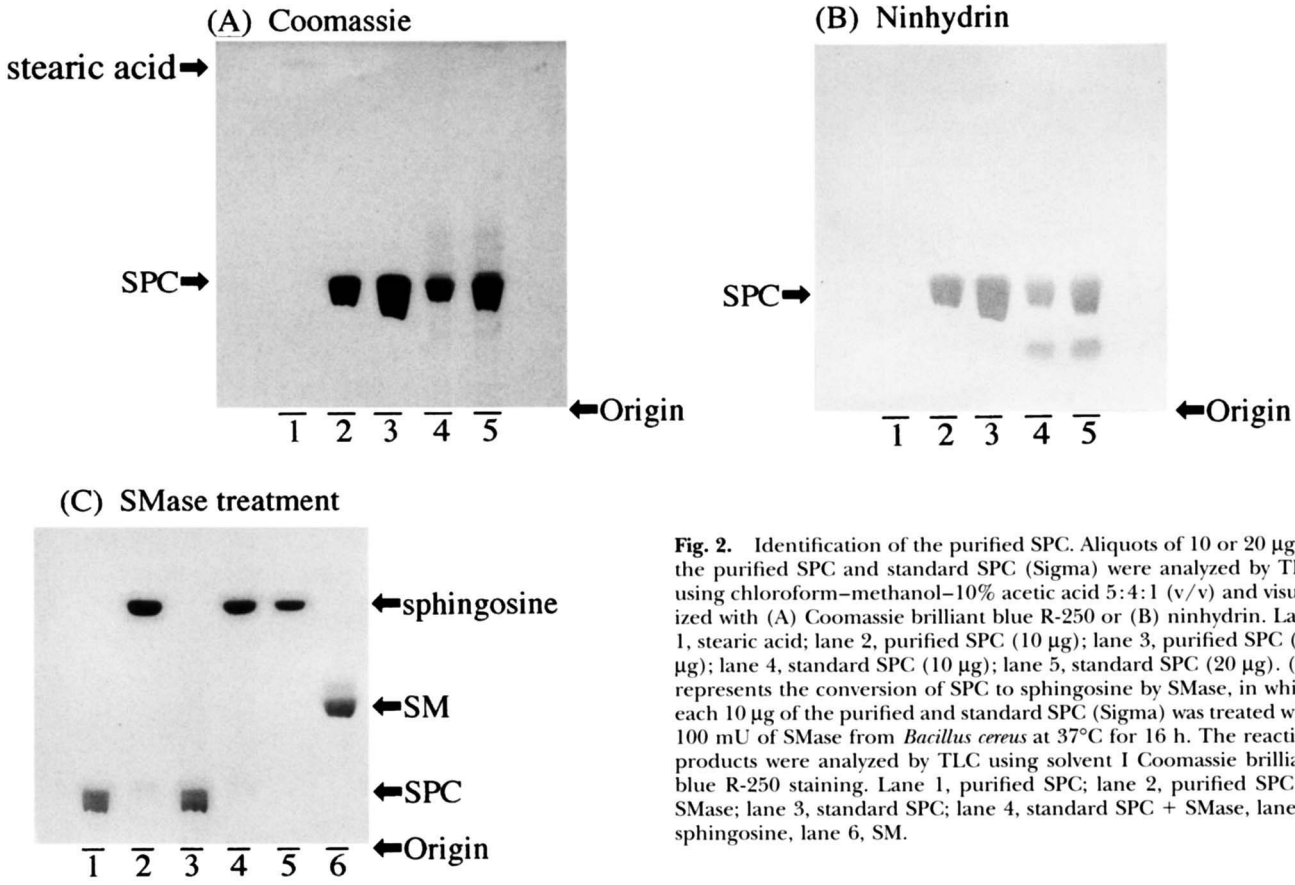

Fig. 2. Identification of the purified SPC. Aliquots of 10 or 20 μg of the purified SPC and standard SPC (Sigma) were analyzed by TLC using chloroform–methanol–10% acetic acid 5:4:1 (v/v) and visualized with (A) Coomassie brilliant blue R-250 or (B) ninhydrin. Lane 1, stearic acid; lane 2, purified SPC (10 μg); lane 3, purified SPC (20 μg); lane 4, standard SPC (10 μg); lane 5, standard SPC (20 μg). (C) represents the conversion of SPC to sphingosine by SMase, in which each 10 μg of the purified and standard SPC (Sigma) was treated with 100 mU of SMase from *Bacillus cereus* at 37°C for 16 h. The reaction products were analyzed by TLC using solvent I Coomassie brilliant blue R-250 staining. Lane 1, purified SPC; lane 2, purified SPC + SMase; lane 3, standard SPC; lane 4, standard SPC + SMase, lane 5, sphingosine, lane 6, SM.

shifts on C-3 and C-5 of L-*threo*-(*2S,3S*)-SPC were found to shift to a lower frequency, as L-*threo*-SPC may form a stable intramolecular hydrogen bond between the amino group on the C-2 carbon atom and the hydroxyl group on the C-3 carbon atom (12). Actually, the standard SPC from Matreya (mixture of D-*erythro*- and L-*threo*-isomers) was found to give two peaks each on C-3 and C-5, respectively, i.e. 72.19 p.p.m and 70.91 p.p.m for C-3 and 134.51 p.p.m and 133.63 p.p.m for C-5. In summary, the prepared SPC was demonstrated to be of the D-*erythro*-(*2S,3R*)-isomer.

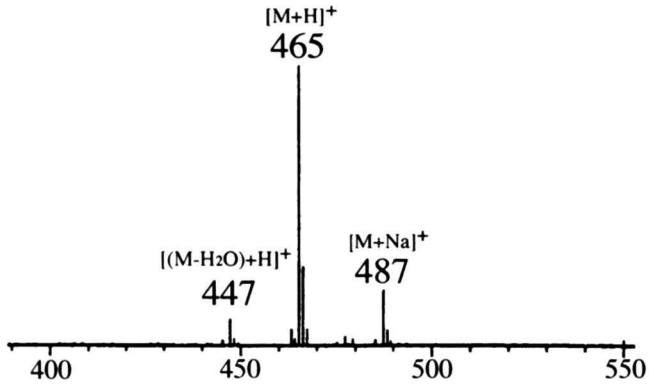

Fig. 3. FAB-MS analysis of the purified SPC. Analysis was conducted with a JMS HX-100 mass spectrometer (JEOL Ltd., Japan) using the positive ion-mode with a 2-nitrobenzylalcohol as the matrix.

The stereochemical configuration of the sphingosyl residue in a naturally occurring SM is always of the D-*erythro*-(*2S,3R*)-type (13). However, acid methanolysis of SM resulted in two stereoisomers, D-*erythro*-(*2S,3R*)- and L-*threo*-(*2S,3S*)-SPC. Very recently Bunemann et al. (12) reported that the two stereoisomers of SPC could be separated by HPLC using a silica-packed Microsorb column. They found that in guinea pig atrial myocytes, D-*erythro*-SPC activated acetylcholine-dependent K^+ conductance, $I_{k(Ach)}$, to an extent 430-times greater than L-*threo*-isomer. However, they pointed out that a trace amount of D-*erythro*-isomer could contaminate the L-*threo*-preparation and would be virtually impossible to detect or eliminate by the methods they have used (12). In contrast, only D-*erythro*-SPC is produced by SM-decrylase from naturally occurring D-*erythro*-SM as shown in this study.

Desai and Spiegel (1) reported that SPC (mixture of D-*erythro*/L-*threo*-isomers) at 10 μM greatly stimulated DNA synthesis and cell division in quiescent Swiss 3T3

fibroblasts (1). We confirmed that D-*erythro*-SPC at 5 μM stimulated the DNA synthesis in Swiss 3T3 cells under the same condition, indicating that D-*erythro* isomer is likely to have a mitogenic activity (data now shown). Other biological activities of D-*erythro*-SPC for other cell lines will be reported elsewhere.

Recently, we reported that *Pseudomonas* sp. TK-4 produces a novel enzyme capable of hydrolyzing the *N*-acyl linkage of ceramides in various glycosphingolipids as well as SM (14). However, TK-4 also produces a large amount of SMase that converts SM to ceramide as well as SPC to sphingosine (Fig. 2C) and thus was not suitable for the method described in this paper. It should be emphasized that the conversion of SM to D-*erythro*-SPC, using a microorganism as a biocatalyst, is now possible with NS-589.

The method described here has been applied to the preparation of a naturally occurring D-*erythro*-SPC at a laboratory scale (mg level) and could be used on an industrial scale (kg level) and thus should greatly facilitate the further study of the physiological functions of D-*erythro*-SPC.

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