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Antioxidant Activity of Germicidin A and B Isolated from the Marine-Derived *Streptomyces* sp. SCS525

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Abstract

Oxidation of lipid compounds in foods reduces their shelf-life, and oxidation in living cells causes damage through the release of free radicals. Synthetic antioxidants for food preservation are phenolic in nature, and thus their use is tightly regulated. On the other hand, naturally occurring antioxidants that have been successfully isolated are limited by low efficacy and high cost. Therefore, the development of safe and low-cost natural antioxidants for use in food production and preservation is needed. Here, we investigated antioxidant activity in extracts from isolated marine bacteria derived from marine sediments. Among them, *Streptomyces* sp. SCS525 showed strong antioxidant activity as measured by free radical scavenging assays. Bioassay-guided fractionation and spectroscopic data analyses led to the identification of gramicidin A (1) and B (2) as strong antioxidant compounds produced by *Streptomyces* sp. SCS525. These two compounds are known to inhibit spore germination, and the polyketide type III pathway-related protein Gcs. These results suggest that safe and low-cost antioxidants can be produced from marine bacteria on a large scale.

Keywords

Germicidin A; Germicidin B; Marine bacteria; Antioxidant; Marine sediment

Introduction

Peroxides in foods are produced by oxidation of constituent lipid compounds. Free peroxides bind to proteins, carbohydrates, vitamins, pigments, and flavor components, which influences their function. In particular, certain peroxides target enzymes, cell membranes, and organelles and induce the generation of free radicals, which can damage living cells [1]. Antioxidants either remove or inactivate the free radicals generated during lipid oxidation by inhibiting the process [2]. They are frequently used for preservation of foods and medicines, offering particular benefit to the preservation of foods containing oils [3].

Currently, the most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), tertiary-butyl hydroquinone (TBHQ), and propyl gallate (PG). These synthetic antioxidants are phenolic and consequently subject to increasing regulation by the FDA [4]. Natural antioxidants, such as tocopherol, are less frequently used as additives owing to their insufficient antioxidative effects and high cost. Therefore, researchers have investigated the development of new antioxidants that are non-toxic and effective. Many new plant-derived antioxidative substances have been reported, but these studies were limited to the investigation of the properties of such substances in relation to plant function [5,6].

Studies on antioxidative substances produced by microorganisms were conducted for the first time in 1957 by Forbes et al., who isolated such substances from yeast. Smith et al. subsequently reported the presence of antioxidative substances in benzene extracts of *Pseudomonas fragi*, *Micrococcus* sp., and *Bacillus cereus* [7,8]. However, these studies are in the stage of distribution and screening of microorganisms. Therefore, the development of safe and low-cost natural antioxidants for use in food production and preservation is still needed.

Recently, we isolated antioxidative substances from *Actinomycetes* derived from marine sediments. By providing a natural antioxidative substance that is safe and low in price from natural sources and cultivable sources. We concluded that such substances could contribute to the expansion of food, cosmetics, and other related industries as well as to the improvement of public health.

Materials and Methods

Collection of marine sediments

Marine sediments were collected from Songnim shore (36°00′42.7″N 126°39′41.8″E) in Janghang, Seocheon, Chungcheong, South Korea (May 2016). The samples were transported to the laboratory, dried and diluted 20-fold with sterile seawater.

Isolation and culture of SCS525 marine bacterium

The diluted sediment suspension (100 µl) was spread on a Marine agar 2216 (Difco Laboratories, Detroit, MI, USA). A single bacterial colony was isolated on a Marine agar plate, cultured at 27°C for 2 weeks, and further cultured in 25 ml Marine broth 2216 (Difco) at 25°C for 7 days. The isolated bacterial strain was designated SCS525. Stocks of all cultures were maintained at -80°C in culture medium containing 15% glycerol.

Extraction and chemotyping of strain SCS525 using HPLC

Strain SCS525 was cultured in 2.5-L Ultra Yield Flasks each containing 1 L of the medium (10 g/l soluble starch, 2 g/l yeast, 4 g/l peptone, 10 g/l $CaCO_3$, 20 g/l KBr, and 8 g/l $Fe_2(SO_4)_3.4H_2O$ dissolved in 750 ml of natural seawater and 250 ml of distilled water) at 25°C with shaking at 120 rpm. After 7 days, the broth was extracted with ethyl acetate. One liter of solution yielded 63 mg of ethyl acetate extract. HPLC analysis was carried out on an Agilent Technologies 6120 quadrupole instrument (Santa Clara, CA, USA) using a reversed-phased column (Phenomenex Luna C-18 (2) 100 Å, 100 × 4.6 mm, 5 µm; Torrance, CA, USA). The column was maintained at 25°C, and the flow rate was set at 1 ml/min. The crude extract was dissolved in methanol (2 mg/ml), and 30 µl of this sample was injected

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onto the column. Mobile phases consisted of water (solvent A) and CH_3CN (solvent B) solutions containing 0.1% (v/v) TFA. Initially, an A:B ratio of 95:5 was maintained for 2 min, which was then decreased linearly to 0:100 from 2 to 12.5 min, maintained for 2.5 min, reversed to 95:5 (A:B), and then maintained from 15 to 18 min before the next injection.

16s rRNA gene sequencing of SCS525

The chromosomal DNA of the cultivated SCS525 bacterial strain was isolated using a LaboPassTM tissue genomic DNA isolation kit (Cosmogenetech, Daejeon, Korea). PCR was employed to amplify the 16S rRNA gene using the primers 27F and 1492R [9], the products of which were purified using a LaboPassTM PCR purification kit (Cosmogenetech), according to the manufacturer's protocol. Sequencing was performed on a capillary electrophoresis instrument (Applied Biosystems 3730XL, Foster City, CA, USA). The 16s rRNA gene sequence derived from the isolated bacteria was aligned with previously described sequences by performing BLAST searches against the GenBank/EMBL/DDBJ database [10].

Mass culture and extraction

SCS525 was cultured in a total of 20 L of the same growth medium used for smaller cultures and under the same conditions. After 7 days, the broth was extracted with ethyl acetate (20 L total), yielding 1.3 g of ethyl acetate extract. The ethyl acetate extract was fractionated using C18 silica vacuum column chromatography and elution with a step gradient from 20% to 100% methanol in H_2O. The third fraction, at 50% methanol (101 mg yield), was subjected to reversed-phase HPLC with 30% aqueous acetonitrile (Watchers 120 ODS-BP, 250 × 10 mm, 5 µm, 2.0 ml/min, UV=280 nm; Osaka, Japan) to identify germicidin A (1, 6.4 mg) and germicidin B (2, 6.5 mg), with retention times of 42 and 26 min, respectively.

Free radical scavenging assays for antioxidant activity

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay was performed according to the method described by Blois [11]. Briefly, 100 µl of SCS525 strain extract, fractions and pure compounds at various concentrations (25, 50, 100, 200 and 400 µg/ml) and 100 µl of 0.15 mM DPPH were dispensed into 96-well plates. The mixture was shaken carefully and left in the dark at 25°C for 30 min, after which absorbance was measured at 516 nm with ascorbic acid (1.25, 2.5, 5 and 10 µg/ml) as a positive control. DPPH scavenging activity was expressed as the half-maximal inhibitory concentration (IC_{50}). The 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was performed according to the method described Re et al. [12]. Briefly, 7 mM ABTS and 2.45 mM potassium peroxodisulfate were mixed and incubated at 25°C for 16 h. Next, 100 µl of this mixture and 100 µl of SCS525 strain extract, fractions, and pure compounds at varying concentrations (25, 50, 100, 200 and 400 µg/ml) were dispensed into a 96-well plate, and the absorbance of the reaction mixture was measured at 734 nm. Ascorbic acid (1.25, 2.5, 5 and 10 µg/ml) was used as a positive control.

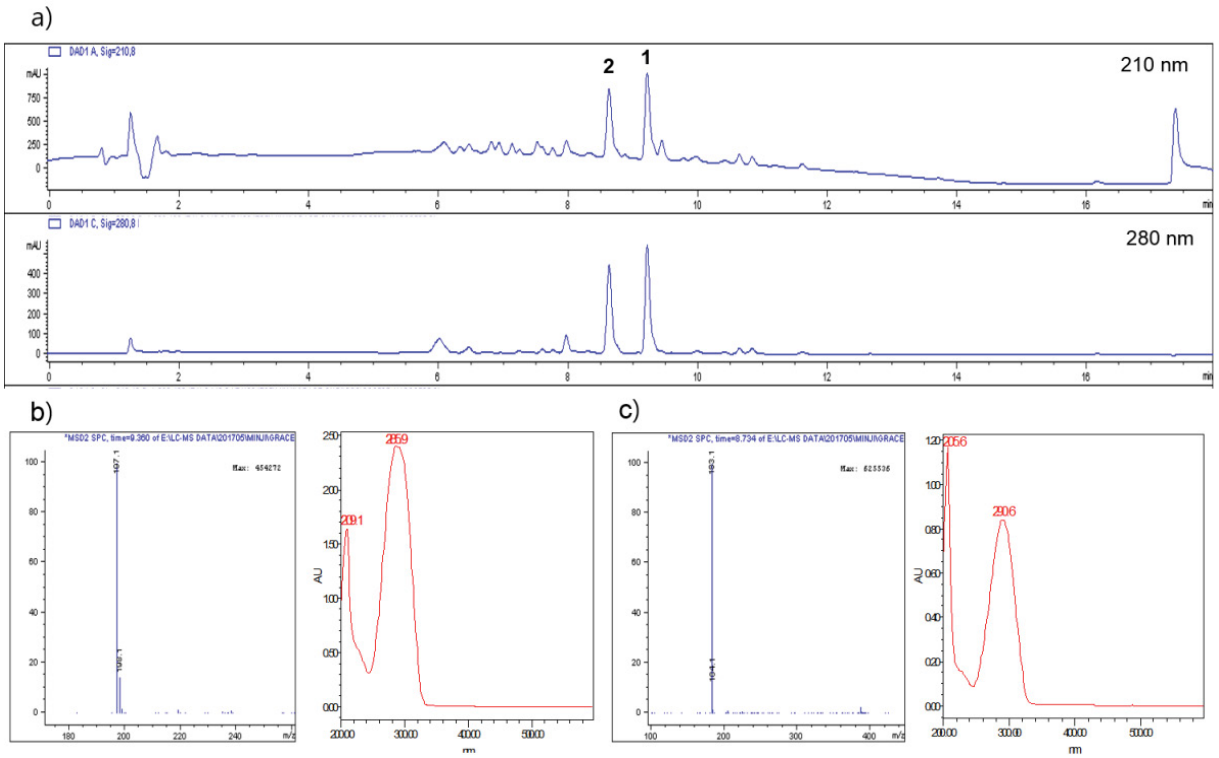


Figure 1: Chemotyping of the crude extract of strain SCS525 using LC-MS. **a):** Germicidin A (1): and Germicidin B; (2): had retention times of 9.3 and 8.7 min, respectively (AU; absorbance unit, UV=210 and 280 nm, respectively). **b):** LR-ESI-MS and UV spectroscopy of compound 1. The *m/z* peak was observed at 197 $[M+H]^+$ and UV absorptions (209, 286 nm) of compound 1. **c):** The *m/z* peak at 183 $[M+H]^+$ and UV absorptions (205, 290 nm) of compound 2 were also observed in the LR-ESI-MS and UV spectroscopic data.

Results

Secondary metabolites produced by SCS525

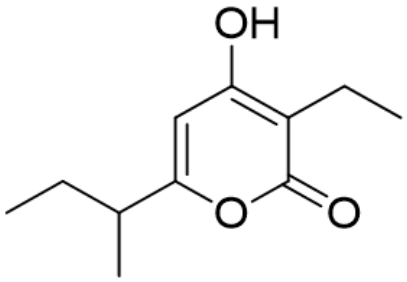
LC traces of the crude extract indicated that this strain produced metabolites with two distinct peaks and retention times of 8.7 (compound 2) and 9.3 min (compound 1), respectively (Figure 1). The UV absorption of these two compounds at 280 nm suggested that they contained conjugated double bonds. By comparison to an in-house spectral library, the UV absorption spectra of two peaks were found to be a good match with those of previously reported natural products, germicidins (Figure 2).

16s rRNA gene sequence of SCS525 and bacterial species identification

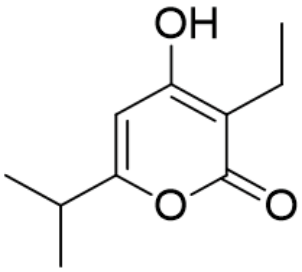
The PCR product of the 16S rRNA gene of SCS525 was approximately 1000 bp on a 1% TBE agarose gel. We successfully obtained a 16S rRNA gene sequence of 1095 bp by cloning and sequencing the PCR product. The SCS525 strain showed 99.8% similarity with *Streptomyces fradiae* SMS_SU23 according to a BLAST search of the GenBank/EMBL/DDBJ database [10]. The cultured SCS525 strain was deposited in the Korean Culture Center of Microorganisms (KFCC11793P). The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SCS525 is MK402126.

Structure determination and chemical properties of germicidin A and B

The chemical structures of the two separated compounds were determined by comparing their UV, MS, and ^{1}H NMR spectroscopic data. ^{1}H NMR of compound 1 showed H-4 (δ_H 5.98, s), H-6 (δ_H 2.47,



Germicidin A (**1**)



Germicidin B (**2**)

Figure 2: Chemical structures of germicidin A (1) and germicidin B (2).

Table 1: Free radical scavenging activity of crude extract, fractions, and pure compounds from strain SCS525, measured by DPPH and ABTS assays.

Sample	IC_{50} (mg/ml) of DPPH	IC_{50} (mg/ml) of ABTS
SCS525-total crude extract	0.516 ± 0.002	0.002 ± 0.000
SCS525-F1	0.621 ± 0.010	0.045 ± 0.004
SCS525-F2	0.373 ± 0.003	0.038 ± 0.000
SCS525-F3	0.305 ± 0.004	0.012 ± 0.000
SCS525-F4	0.318 ± 0.002	0.015 ± 0.000
SCS525-F5	0.327 ± 0.013	0.017 ± 0.000
SCS525-F6	0.546 ± 0.017	0.032 ± 0.001
SCS525-F7	>2.5	0.068 ± 0.001
SCS525-W1	>2.5	0.051 ± 0.000
SCS525-W2	>2.5	0.055 ± 0.001
SCS525-W3	>2.5	0.101 ± 0.001
SCS525-F3-1 (Germicidin B)	1.659 ± 0.163	0.020 ± 0.001
SCS525-F3-2	>2.5	0.043 ± 0.000
SCS525-F3-3	1.454 ± 0.021	0.085 ± 0.000
SCS525-F3-4	1.181 ± 0.035	0.193 ± 0.001
SCS525-F3-5 (Germicidin A)	0.707 ± 0.017	0.006 ± 0.000
SCS525-F3-6	0.730 ± 0.013	0.074 ± 0.001
SCS525-F3-7	0.476 ± 0.001	0.056 ± 0.001
SCS525-F3-8	0.607 ± 0.015	0.063 ± 0.001
SCS525-F3-9	0.351 ± 0.001	0.071 ± 0.002
SCS525-F3-10	0.208 ± 0.001	0.021 ± 0.002
SCS525-F3-11	>2.5	0.433 ± 0.002
SCS525-F3-12	0.934 ± 0.019	0.059 ± 0.001
Ascorbic acid (positive control)	0.007 ± 0.000	0.016 ± 0.000
Values represent the mean ± SD (standard deviation) of three measurements		

q, *J*=7.1 Hz), H-7 (δ_H 1.03, t, *J*=7.5 Hz), H-8 (δ_H 2.39, m), H-9 (δ_H 1.21, d, *J*=7.0 Hz), H-10a (δ_H 1.67, m), H-10b (δ_H 1.55, m), and H-11 (δ_H 0.89, t, J=7.5 Hz) (Figure S1). The ^{1}H NMR data, as well as the m/z peak at 197 $[M+H]^+$ were in agreement with the structure of 6-(2-butyl)-3-ethyl- 4-hydroxy-2-pyrone (germicidin A) [13].

In the LR-ESI-MS spectrum of compound 2, a m/z peak at 183 $[M+H]^+$ was observed. ^{1}H NMR spectroscopic data of compound 2 displayed one olefinic proton (δ_H 5.88, s), three methyl protons [δ_H 1.14 (t, *J*=7.6 Hz, 3H), 1.24 (d, *J*=6.9 Hz, 6H)], a methine proton (δ_H 2.72, m, ^{1}H), and methylene protons [δ_H 2.47 (q, *J*=7.5 Hz)] (Figure S2). The chemical structure of compound 2 was identified as 3-ethyl-4-hydroxy-6-(2- propyl)-2-pyrone (germicidin B) based on the comparison of its ^{1}H NMR and MS spectroscopic data with previously reported data [13].

Antioxidant activity of germicidin A and B

The crude extract, 10 eluted fractions, and 12 pure compounds (including germicidin A and B) from SCS525 were tested for their antioxidant activity by DPPH and ABTS assays. The crude extract showed strong antioxidant activity as 2 μg/ml (IC_{50} of radical scavenging) according to the ABTS assay (Table 1). Among the 10 eluted fractions, the third fraction showed high activity in both assays (Table 1). Compounds 1 and 2 (germicidin A and B) showed higher radical activity in the ABTS assay [germicidin A (1) at 6 μg/ml and B (2) at 20 μg/ml] than in the DPPH assay (Table 1).

Discussion and Conclusion

In this study, we isolated the bacterium *Streptomyces* sp. SCS525 from marine sediments and cultured it in large quantities in a liquid medium. We then identified strong antioxidants in fractions of the strain extract. Germicidin A (1) and B (2) were separated and identified using organic solvents, and their antioxidant activity was confirmed using DPPH and ABTS assays. Germicidin A (1) and B (2) were first isolated from *Streptomyces coelicolor* A3 (2) and *Streptomyces viridochromogenes* NRRL B-1551 [13,14]. The two compounds are known to inhibit spore germination, and the polyketide type III pathway-related protein Gcs [15]. They are also known to have antibacterial effects against several Gram-positive bacteria and inactive in breast and lung cancer cell lines [16]. Our results presented here demonstrate that germicidins derived from bacteria are useful natural antioxidants and can be obtained through simple large scale cultivation.

Conflict of Interest

The authors have declared that no competing interests exist.

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