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Production and characterization of a C_{15}-surfactin-O-methyl ester by a lipopeptide producing strain *Bacillus subtilis* HSO121

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ABSTRACT

A surfactin mono-methyl ester was isolated from the cell broth of *Bacillus subtilis* HSO121 by acid precipitation, methanol extraction and two-step chromatographic methods. The structure was analyzed by GC/MS, HPLC, ESI Q-TOF MS/MS and NMR. It indicates that this ester is a C_{15}-surfactin-O-methyl ester of which the fatty acid portions are composed of *iso*-C_{15} and *anteiso*-C_{15} β-hydroxy fatty acids. Based on the critical micelle concentration (CMC) in phosphate buffered saline (PBS, pH 8.0) and the IC_{50} value on Hela cell lines, the C_{15}-surfactin-O-methyl ester has a stronger surface activity and lower antitumoral effect than C_{15}-surfactin does. It is found that the Glu residues of surfactin-like lipopeptides play a role in their anti-proliferative effects on Hela cells. The possible antitumoral activity of surfactin is discussed in relation to their structures.

1. Introduction

Lipopeptides are a group of microbial secondary metabolites with amphiphilic properties and remarkable biological properties, isolated from the cell broth of various kinds of microorganisms such as genus *Bacillus*, *Pseudomonas*, and *Streptomyces* [1–4]. The hydrophobic parts contain lipophilic fatty acids and non-polar amino acids, while the hydrophilic parts are composed of the polar amino acids. Surfactin-like lipopeptides produced by genus *Bacillus* are one of the most important classes of these natural compounds. They have low critical micelle concentration (CMC), stable emulsification properties and excellent foamability [5]; surfactins also have hemolytic, anticoagulant, antimicrobial and antitumoral activities [6–8]. These activities are related with their interactions with biomacromolecules such as enzymes [9,10] and lipopolysaccharide [11,12]. The surfactant properties and biological activities of surfactin analogues appear very interesting in the perspective of their utilization both in cosmetic and in pharmaceutical fields.

Surfactins are a large variety of isoforms which differ by variation of the length and branching of their fatty acid components as well as by amino acid replacements in their peptide ring [13]. For *Bacillus subtilis*, different strains can produce a different lipopeptide family and a single strain can also produce lipopeptides with different peptide cores, such as surfactin and iturin. In addition, the diversity of surfactin was enhanced by the combinatorial biosynthesis and precursor-directed biosynthesis. These lead to the exploration of surfactin derivatives with a linear structure or small ring size [14,15]. Therefore, novel surfactin-derivatized lipopeptides with modified properties from *Bacillus* sp. can be continuously explored [16,17]. These studies facilitate the design and building of novel lipopeptide products. Recently, a *B. subtilis* strain was found to produce lipopeptides and [Val7] surfactin methyl ester [18]. This interesting peculiarity of *B. subtilis* makes it feasible to produce lipopeptides with modified amino acids. However, little is known about how many kinds of lipopeptides one strain can produce. We have studied the isolation and structural characterization of the lipopeptides produced by *B. subtilis* HSO121 [19,20]. To further extend our knowledge on the diversity of lipopeptides produced by this strain, we have isolated another two components. One is the C_{12} lipopeptide, which has the shortest chain length among the lipopeptides produced by *B. subtilis* [21]. The other is the C_{15}-surfactin-O-methyl ester, which is a kind of esterified lipopeptide.

In the present paper, the C_{15}-surfactin-O-methyl ester from *B. subtilis* HSO121 was reported. Its production was improved by the optimization of the carbon source and nitrogen source. Its structure was characterized by GC/MS, HPLC, ESI Q-TOF MS/MS and NMR. The surface tension of this compound and its effect on Hela cells were compared with those of surfactin.

2. Materials and methods

2.1. Bacteria strain and culture condition

The strain was isolated from the formation water of Daqing oil fields, China. It was identified as *B. subtilis* at the Institute of Microbiology, Chinese Academy of

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Sciences according to the Bergey's Manual of Determinative Bacteriology [22]. In the investigation of culture medium, a one factor-at-time and orthogonal experiment design methods L_9 (3^3) were applied to optimize the production of surfactin by *B. subtilis* HSO121. The biomass, weight of acid precipitation and reduced surface tension values were selected as evaluating parameters. Growth was carried out in a basal medium containing 0.3% $K_2HPO_4{\cdot}3H_2O$, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% yeast extract and 2 ppm $MnCl_2{\cdot}4H_2O$ (pH 7.2), and supplemented with 2% (w/v) of various carbon sources and 50 mmol/L nitrogen sources to be investigated. The carbon sources investigated included glucose, sucrose, sodium citrate, and olive oil. The nitrogen sources included peptone, sodium nitrate, ammonium nitrate and ammonium sulfate. One hundred milliliters of the resultant medium in a 250-mL Erlenmeyer flask was aerobically cultured at 30 °C for 48 h on a rotary shaker (150 rpm).

2.2. Purification of the lipopeptides

After centrifugation, the cell free supernatants were collected and suffered to the acid precipitation. The dry weight of crude lipopeptides was obtained after the lyophilization of the acid precipitation. For further purification, the lipopeptide components were extracted from the yellow powder to methanol. The lipopeptides components thus obtained was applied on a reversed-phase normal pressure column to remove the color contaminants [21]. The obtained lipopeptide mixtures were further purified by reversed-phase high performance liquid chromatography using a JASCO LC-2000 HPLC system (Tokyo, Japan) equipped with a 5 μm HiQ sil C18W column (Φ 21.2 mm × 25 cm). The column was eluted at a flow rate of 17 mL/min by a first mobile phase of 90% methanol and 10% TFA solution (0.05%) for 24 min, and then increased to 100% methanol from 24 min to 34 min.

2.3. Fatty acid and amino acid analysis

The lipopeptide was hydrolyzed with 6 mol/L HCl at 105 °C for 25 h in a sealed tube. The fatty acid residue was obtained by extracting the hydrolysate with ether and was esterified by sulfuric acid/methanol (1: 10, v/v) [23]. The fatty acid methyl ester was dissolved in methanol and analyzed by GC–MS. The Micromass GCT was equipped with an EI source and a capillary column of HP-5 ms (30 m × 0.32 mm × 0.25 μm). The EI-MS was operated at 70 eV and the source temperature is 250 °C. The column oven temperature started at 80 °C and kept for 3 min, then reached to 240 °C at a rate of 8 °C/min [24]. Following the extraction of the hydrolysate, the aqueous phase was heated to remove the remaining ethyl ether. The analysis of amino acid composition was carried out by a precolumn derivatization procedure on HPLC using phenyl isothiocyanate (PITC) [21].

2.4. Mass spectrometry

Mass spectrum was obtained with a Q-TOF microanalyzer tandem mass spectrometer equipped with a standard ESI source. Its capillary voltage, sample cone voltage, ion energy and collision energy were 3200 V, 80 V, 1.6 V and 60 V, respectively [25].

2.5. Nuclear magnetic resonance

The ^1H and ^{13}C NMR spectra of 5 mg lipopeptides in 0.5 mL deuterochloroform were acquired on a Bruker 400 MHz spectrometer at 25 °C (with tetramethylsilane as internal standard). The assignment of the signal observed in the ^1H NMR and ^{13}C NMR of spectrum was done according to the literature [26,27].

2.6. Surface tension measurement

The surface tension of the lipopeptides in 10 mmol/L phosphate buffered saline (PBS, pH 8.0), with concentrations ranging from 2.51×10^{-6} mol/L to 2.64×10^{-4} mol/L, was measured at 25.0 °C by Du Noüy ring method with a DCA 315 series system (Thermo-Cahn Instruments, Inc., USA). The measurement precision of the surface tension value is ±0.001 mN/m. The surface tension versus concentration plot was used to determine CMC and γ_{CMC}.

2.7. Cytotoxicity assay

The cytotoxicity assay of lipopeptides was performed by the methyl thiazolyl tetrazolium (MTT) colorimetric assay, as described by Mosmann [28]. The lipopeptides was firstly dissolved in DMSO, then, it was diluted by RPMI1640 culture medium, in which the DMSO concentration was 0.1%. The vehicle control was the RPMI1640 culture medium containing 0.1% DMSO, which was believed to present the same pH and ionic strength as the positive control. Also, it was found that vehicle control with 0.1% DMSO does not have the inhibitory effect on the Hela cells. Hela cells were placed within a 96-well culture plate (10^4 cells/well), and allowed to attach for 24 h (37 °C, 5% CO_2) before treatment. The cells were treated with lipopeptide ranging from 6.25 μg/mL to 50 μg/mL or without (vehicle control, 0.1% dimethyl sulfoxide (DMSO)). Lipopeptide cytotoxicity was measured using the MTT assay after the 24 h treatment. Absorbance in control and lipopeptide-treated wells was measured in an Automated Microplate Reader (Bio-Rad 550) at 570 nm.

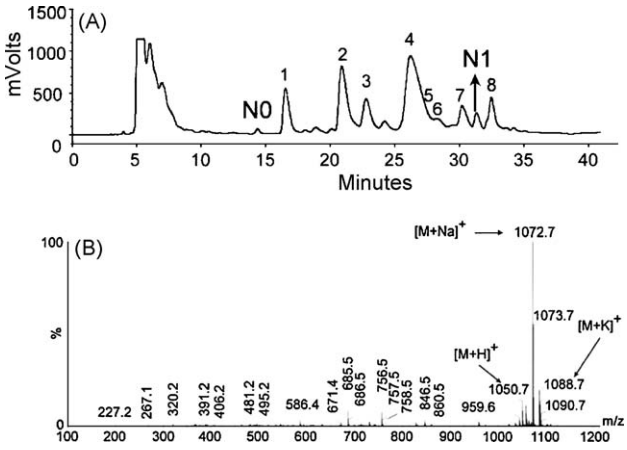

Fig. 1. Isolation, preparation results of lipopeptides. (A) Semi-preparative RP-HPLC and (B) electro spray ionization mass spectrum of N1. Methanol was used in the isolation process.

3. Results

3.1. Culture conditions and surfactin production

In our preliminary experiments, we found that surfactin production was a growth-associated production in which a parallel relationship is obtained between cell growth and surfactin concentration [29]. The culture medium for *B. subtilis* HSO121 was optimized. The concentrations of carbon source and nitrogen source were optimized to be 2% (w/v) sucrose and 0.2% (w/v) NH_4NO_3. Grown in the optimized medium for 2 days, *B. subtilis* HSO121 can produce crude lipopeptides with a yield of 1.8 g/L.

3.2. Isolation and preparation

During the isolation and preparation process of lipopeptides from *B. subtilis* HSO121 (Fig. 1A), 10 fractions were collected, of which nine (N0, 1–8) have been reported in our previous work [20,21]. In the present study, the fraction N1 was collected and applied on ESI-MS.

3.3. Structural characterization

ESI-MS of N1 in Fig. 1B reveals a molecular mass of 1049, corresponding to the $[M+Na]^+$ ion at *m/z* 1072.7, $[M+H]^+$ ion at *m/z* 1050.7 and $[M+K]^+$ ion at *m/z* 1088.7, which frequently occur in the positive mode of ESI-MS.

There are two fatty acids in hydrolyzed N1, as shown in gas chromatogram (Fig. 2A). The two fatty acid methyl esters have same molecular mass according to their EI-MS (Fig. 2B). The base peak at *m/z* 103 corresponds to $[CHOHCH_2COOCH_3]^+$, which is the characteristic fragment ion of a β-hydroxy fatty acid. Other ions were also assigned in Fig. 2B. Comparing with the retention behavior and molecular mass of the C_{12-17} fatty acid methyl esters [20], the fatty acid portions of N1 is determined to be a mixture of *iso*- and *anteiso*-β-hydroxy C_{15} fatty acids.

Comparing with the HPLC profiles of standard PITC-derivatized L-amino acids (Fig. 3A) and negative control (Fig. 3B), the hydrolyzed N1 contains four kinds of amino acids: Asp, Glu, Val and Leu (Fig. 3C). Calculating the proportion of each peak area indicates that the molar ratio of Asp:Glu:Val:Leu is approximately 1:1:1:4, respectively. Based the molecular mass of 1049 on the nitrogen rule, the N1 should contain nitrogen atoms with an odd number. Combined with the analysis results of amino acids and fatty acids, the peptide moiety of N1 is composed of 1 Asp, 1 Glu, 1 Val and 4 Leu. It should be noticed that when the lipopeptides are