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Isolation and characterization of a C_{12}-lipopeptide produced by *Bacillus subtilis* HSO 121

XIANG-YANG LIU, SHI-ZHONG YANG and BO-ZHONG MU*

Laboratory for Advanced Materials and Institute of Applied Chemistry, East China University of Science and Technology, Shanghai 200237, China

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Abstract: A new lipopeptide with C_{12} fatty acid has been isolated from the cell broth of *Bacillus subtilis* HSO121 by chromatographic methods, which is believed to be the homologue of lipopeptides. The fatty acid portion was methylated and analyzed by GC/MS, ESI Q-TOF MS and ^{1}H-NMR. The peptide portion, of which the amino acid composition was obtained by HPLC combined with a phenyl isothiocyanate (PITC) derivatization methods, was analyzed by ESI Q-TOF MS. Comparing the obtained results with surfactin C_{13} showed that the new lipopeptide has a peptide moiety similar to that of surfactin and the difference exists in the fatty acid portion, which is an iso-C_{12} β-hydroxy fatty acid. The critical micelle concentration (CMC) of this new homologue is estimated to be 6.27×10^{-5} mol/l in 10 mmol/l phosphate buffer solution (PBS, pH 8.0) at 30 °C, and the surface tension at CMC (γ_{CMC}) achieved is as little as 27.71 mN/m. The hemolytic activities of the C_{12}-lipopeptide on 2% human erythrocytes showed a HC_{50} of 26.5 μmol/l. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *Bacillus subtilis*; lipopeptide; surfactin; structure; CMC; hemolysis

INTRODUCTION

Microbial lipopeptides have received much attention because of their important role in biotechnological and pharmaceutical fields [1–4]. It is well-known that these amphiphilic compounds with heterogeneous structures varying from the lipid and the peptide chains because of the varieties of strains and culture media [5]. According to the properties of most lipopeptides, many of them are both biosurfactants and antibiotics. In 1968, the first cyclic lipopeptide biosurfactant (surfactin) produced by *Bacillus subtilis* was isolated and proved to possess powerful surface activity and inhibiting the formation of fibrin clot [6]. After that, most of the interest has been focused on the structure and properties of surfactin, which has been proved to be a property of genus *Bacillus* [7]. Surfactin consists of a heptapeptide moiety bonded to the carboxyl and hydroxyl groups of a β-hydroxy fatty acid. It has strong surfactant properties such as largely reducing surface tension, low critical micelle concentration (CMC), emulsification, foam formation and other interfacial properties [8–10]. In addition to the powerful surfactant properties, surfactin showed broad biological activities such as hemolytic activities, modification of the performance of enzymes and interaction with membranes [11–13]. These interesting properties promoted many scientific researchers to explore new surfactin-like lipopeptides. As a result, many hybrid surfactins have been found. As to the peptidic variants, the peptide sequence which has been identified to be N-Glu(1)-Leu(2)-Leu(3)-Val(4)-Asp(5)-Leu(6)-Leu(7)-C, are normally variable on the position 2, 4 and 7 according to the directed and/or combinatorial biosynthesis strategies [7]. The fatty acid moieties are usually composed of *n*, iso and *anteiso* types with the chain lengths of C_{13}–C_{15} [5]. The existence of a homologous surfactin with an alkyl chain of 16 carbon atoms has also been demonstrated [14].

Till 2005, there have been 23 families of lipopeptides originated from microorganisms, of which 21 are cyclic lipopeptides [15]. Therefore, both basic and applied studies on lipopeptides which originated from microorganisms are of particular importance.

However, few papers are devoted to the isolation and characterization of lipopeptides with short chain length fatty acids. In an attempt to discover new lipopeptides produced by microorganisms, *B. subtilis* HSO 121 has been proved to be a rich resource [16–19]. This article describes the isolation, purification and characterization of a new lipopeptide homologue produced by *B. subtilis* HSO 121. The molecular mass was characterized by ESI-MS. The structure was obtained using GC-MS, ^{1}H-NMR, HPLC and ESI Q-Tof mass. The surface properties and hemolytic activities of this new lipopeptide are also evaluated.

MATERIAL AND METHODS

Strain and Medium

B. subtilis HSO121 [16], isolated and identified in our lab, was used to produce lipopeptides on a sucrose culture medium containing: sucrose, 20 g/l; KH_2PO_4, 3 g/l; Na_2HPO_4, 10 g/l; NH_4NO_3, 2 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; yeast extract, 0.2 g/l;

*Correspondence to: Bo-Zhong Mu, Laboratory for Advanced Materials and Institute of Applied Chemistry, East China University of Science and Technology, Shanghai 200237, China; e-mail: bzmu@ecust.edu.cn

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$CaCl_2$, 0.077 mg/l; $MnSO_4 \cdot H_2O$, 0.0017 g/l; and EDTA $\cdot$ Na_2, 0.0015 g/l. The medium was sterilized at 121 °C for 20 min. The culture medium was incubated with an inoculum size of 5% (v/v) at 35 °C for 72 h.

Isolation, Purification and Preparation

Bacteria cells in broth were removed from the culture by centrifugation at 5000 rpm for 30 min. The supernatant was subject to acid precipitation by the addition of 6 mol/l HCl to final pH of 2.0 and allowing the precipitate to settle at 4 °C. The acid precipitate was recovered by centrifugation at 3500 rpm for 20 min and lyophilized overnight. The lipopeptides were extracted from the powder into the methanol under ultrasonic. The methanol extracted lipopeptides were further separated by chromatographic methods. Isolation of the lipopeptide mixtures from the colored impurities was carried out on a C_{18} reversed phase column (50 µm, Φ 3.0 × 10 cm) at a flow rate of 3.5 ml/min with a loading volume of 1.5 ml (300–900 mg). Methanol and 0.05% TFA/water (v/v) were used as the solvent system. The column was first washed by 80% methanol. Then, 100% methanol was used to elute the lipopeptide mixtures. The eluate was detected at 220 nm. Lipopeptide components were characterized on an analytical Hypersil ODS C_{18} column (4.6 mm Φ × 250 mm, particle size 5 µm) according to the retention behavior of the lipopeptides already known.

Further purification and preparation were performed on a HiQ sil C18W column (21.2 mm Φ × 250 mm, particle size 5 µm, pore size 120 Å) at a flow rate of 17 ml/min. The eluate was measured at 214 nm with an UV 2075 detector. Lipopeptides were collected by a CH121SA fraction liquid collector in time mode (30 s/tube). The mobile phase was a mixture of methanol (A) and 0.05% TFA in water (B). The gradient (A/B) was maintained at 90% in the first 24 min, then from 90 to 100% in 10 min, and maintained at 100% from 34 to 40 min. Each fraction was evaporated under vacuum by a rotary evaporator (RE-52A, Shanghai) at 35 °C.

Fatty Acid Analysis

The lipopeptide (2.7 mg) was hydrolyzed with 1 ml 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. Extraction was performed three times and the organic phase was rinsed two times with double-distilled water. For ESI-MS analysis, the fatty acid residue was dissolved in methanol and mixed with 0.1% formic acid, of which 1 µl was used in ESI Q-TOF MS system [17]. The remaining fatty acid residue in methanol was esterified by adding 10% (v/v) sulfuric acid and maintained at 50 °C for 4 h. After adding double-distilled water to the ester solution (3 : 1, v/v), the fatty acid methyl ester was obtained by extracting three times with diethyl ether and was rinsed two times by double-distilled water. The fatty acid methyl ester was dissolved in 200 µl methanol, of which 1 µl was applied on GC-MS system [18]. ^{1}H-NMR spectrum of the fatty acid methyl ester was obtained on a Bruker 400 MHz spectrometer in $CDCl_3$ at 25 °C with tetramethylsilane as the internal standard.

Amino Acid Analysis

Following the extraction of the hydrolysis solution, the aqueous phase was heated to remove the remaining diethyl ether. The analysis of amino acids was carried out by a precolumn derivatization procedure using phenyl isothiocyanate (PITC) [20]. For this, 10 µl hydrolysis solutions were diluted 60 times by double-distilled water, of which 200 µl was mixed with 100 µl 1 mol/l TEA in acetonitrile and 100 µl 0.1 mol/l PITC in acetonitrile. The mixture was vortexed for 1 min and then allowed to settle for 20 min. After 400 µl of *n*-hexane was added into the mixture, they were vortexed for 2 min and stayed for 5 min. The lower phase was filtered through a 0.22-µm filter (Millipore, USA) prior to the injection. Each time 20 µl was injected in the HPLC system. Analysis was performed on a Hypersil C_{18} column (Φ 4.6 × 250 mm) completed by 1-cm guard column at a follow rate of 1 ml/min. HPLC spectra were detected at 254 nm. Solvent system was a mixture of A: acetonitrile–water (4 : 1, v/v) and B: 0.1 M sodium acetate (pH was adjusted to 6.35 by acetic acid)–acetonitrile (97 : 3, v/v), of which the proportions were controlled by the gradient program: 0 min, 0% A; 3 min, 0% A; 16 min 7% A; 26 min, 23% A; 32 min, 35% A; 38 min, 40% A; 43 min, 100% A; 48 min, 100% A; 50 min, 0% A.

Mass Spectrometry

Mass spectrum was obtained with a Q-Tof micro analyzer tandem mass spectrometer equipped with a standard ESI source, following the method described by Yang *et al.* [17]. The spectrum was acquired in the positive ion mode for the lipopeptide and in the negative-ion mode for the fatty acid residue. Full mass spectra were acquired by scanning MS-I and leaving MS-II fixed to pass all ions. ESI spectra were obtained by selecting the desired ion with MS-I, and scanning MS-II to obtain the spectra of the daughter ions formed as the result of ESI of the parent ions.

Surface Tension Measurement

The surface tension of the lipopeptide in 10 mmol/l phosphate buffer solution (PBS, pH 8.0), with concentrations ranging from 2.51×10^{-6} mol/l to 2.64×10^{-4} mol/l, was measured at 30.0 °C with a DCA 315 series system (Thermo-Cahn Instruments, Inc., USA). Each value was the average of three measurements. The surface tension *versus* concentration plot was used to determine the CMC and γ_{CMC} values. CMC was the concentration at which a sharp increase in surface tension is observed.

Hemolysis Assay

Erythrocytes were obtained from the blood of healthy volunteers from Hospital Clinic (Shanghai, China). They were washed five times in 0.15 mol/l NaCl by centrifugation (1000 rpm, 5 min). The cells were then suspended in a PBS containing 137 mmol/l NaCl, 2.68 mmol/l KCl, 9.74 mmol/l $Na_2HPO_4 \cdot 12H_2O$ and 1.47 mmol/l KH_2PO_4 in distilled water (pH 7.4) at a concentration of 2%.

PBS (720 µl) was added to 80 µl DMSO solution containing 1.6 mg N0. The 450 µl aliquots of erythrocyte suspension were added to 50 µl of various concentrations of the lipopeptide dissolved in PBS (from 0.1 µmol/l to 1000 µmol/l) in a total volume of 500 µl. Following incubation at 37 °C for 30 min and centrifugation at 3000 rpm for 10 min, the supernatant was measured directly in the plate reader at 570 nm and

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a reference wavelength of 655 nm (Microplate Reader, 550 Bio-Rad, USA). Each read was performed three times. The percentage of hemolysis was determined by comparing the absorbance of the samples with that of the positive control (100%) totally hemolyzed with 50 µl 10% Triton-X100 and the negative control (0%) with 50 µl PBS containing 5% DMSO instead of the lipopeptide solution. Dose-response curves were determined according to the equation described by Deleu *et al.* [21] and the concentrations inducing 50% hemolysis (HC_{50}) were calculated.

RESULTS

Isolation and Purification of Lipopeptides

The separation and preparation chromatograms of lipopeptides are shown in Figure 1. The elution profile of the normal pressure column is shown in Figure 1(A) During the process of purification on normal pressure column, most of the colored impurities were first washed by 80% methanol. Then, the mixture of lipopeptides, which is more hydrophobic than the colored impurities, was eluted by 100% methanol. It was shown that most of the impurities were separated from lipopeptide mixtures after being treated with normal pressure column. The Prep-HPLC profile of lipopeptide mixture is shown in Figure 1 The main components are peak 1, 2, 3 and 4 with molecular masses of 1007, 1021, 1021, and 1035, respectively, which have been found in our previous work [19] and also reported by other researchers [5]. In addition to peak 1, 2, 3 and 4, a small peak (N0) with retention time of 14 min was collected and applied on ESI-MS.

Structural Characterization

ESI-MS of N0 is shown in Figure 2. The molecular mass of N0 is proved to be 993 by the major $[M + Na]^+$ peak at m/z 1016.63 and the other two minor peaks: $[M + H]^+$ peak at m/z 994.65 and $[M + K]^+$ peak at m/z 1032.61. The three m/z peaks frequently occur in positive ion mode of ESI process of lipopeptides.

The mass spectrum of the fatty acid residue obtained from hydrolyzed N0 is shown in Figure 3. The molecular mass of 216 was first deduced from deprotonated molecular ion ($[M - H]^-$) of 215.8 and this peak was selected for ESI-MS/MS analysis. The ion of $m/z = 59$ dominates the daughter ion spectrum and is probably $C_2H_3O_2^-$, which also occurs in the FAB-MS/MS of hydroxyl fatty acids [22]. The loss of 46 mass units from the precursor ion is rationalized as characteristic of the fatty acid with hydroxyl groupbecause of the loss from carboxyl part of the molecule [23]. The above data indicates that the fatty acid is a saturated monohydroxy fatty acid with a chain length of 12. GC/MS of fatty acid methyl esters of N0 is shown in Figure 4. Compared with the retention behavior of β-hydroxy fatty acid methyl esters in gas chromatography [19], the fatty acid has 12 carbon atoms. The base peak at m/z 103 in EI/MS is due to $[CHOHCH_2COOCH_3]^+$, which is the characteristic fragmentation ion of a β-hydroxy fatty acid. Other characteristic peaks were also assigned in Figure 4. The ^{1}H-NMR of the fatty acid methyl ester derived from N0 is presented in Figure 5. A doublet signal at 0.8–0.9 ppm corresponding to six hydrogen atoms suggests the presence of a terminal isopropyl group. The assignments of other signals are given in Figure 5. This spectrum is very similar to that of the iso-C_{14} β-hydroxy fatty acid [24]. All the data suggests that the fatty acid residue from lipopeptide is an iso-C_{12} β-hydroxy fatty acid.

Figure 6 shows the HPLC profiles of PITC derivatives of amino acids from N0. Comparison with standard L-amino acid residues and negative control profiles, it is concluded that hydrolyzed N0 contains four kinds of

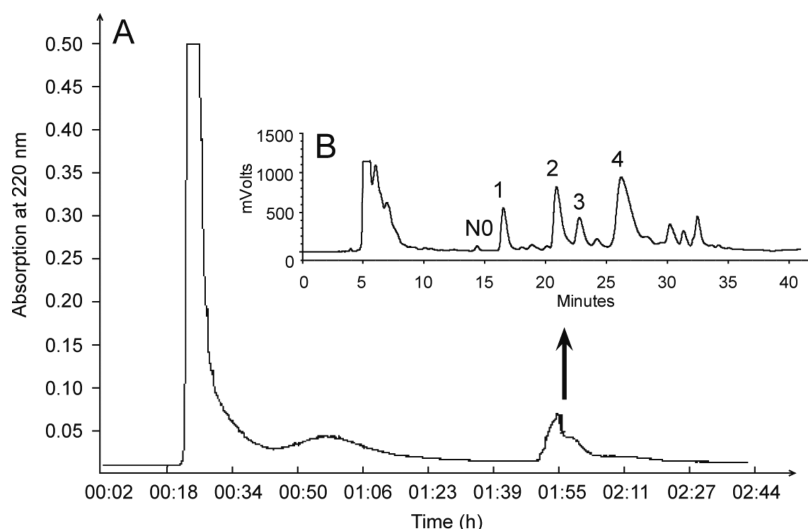

Figure 1 Separation, purification and preparation profile of lipopeptides. (A) Normal pressure column of the methanol extraction of acid precipitation from cell broth of *Bacillus subtils* HSO121. (B) Preparative RP-HPLC of lipopeptide mixtures.

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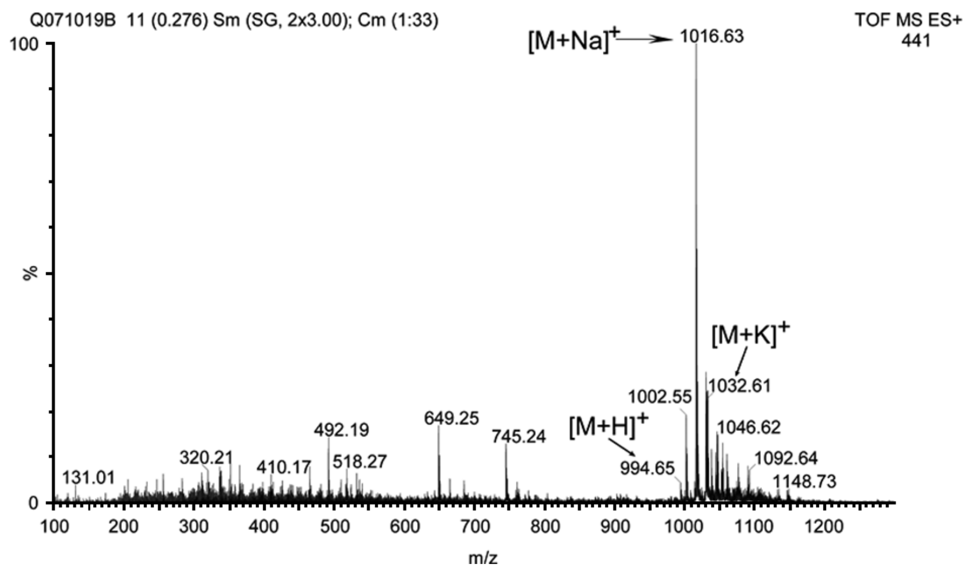

Figure 2 Electro spray mass spectrum of N0.

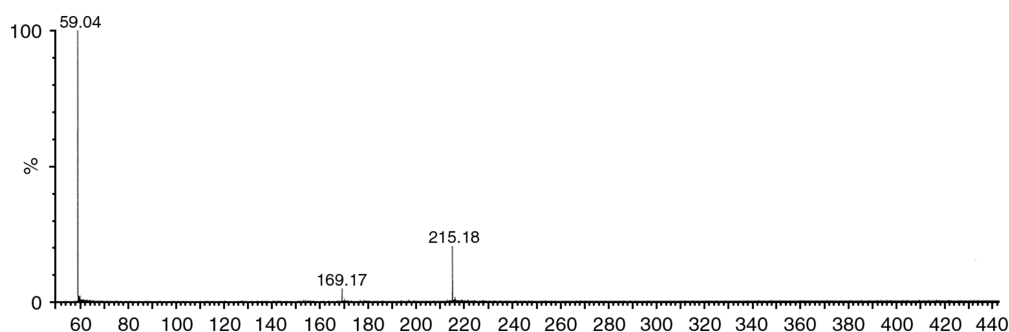

Figure 3 Electro spray mass spectrometry of fatty acid residue from N0 in negative-ion mode.

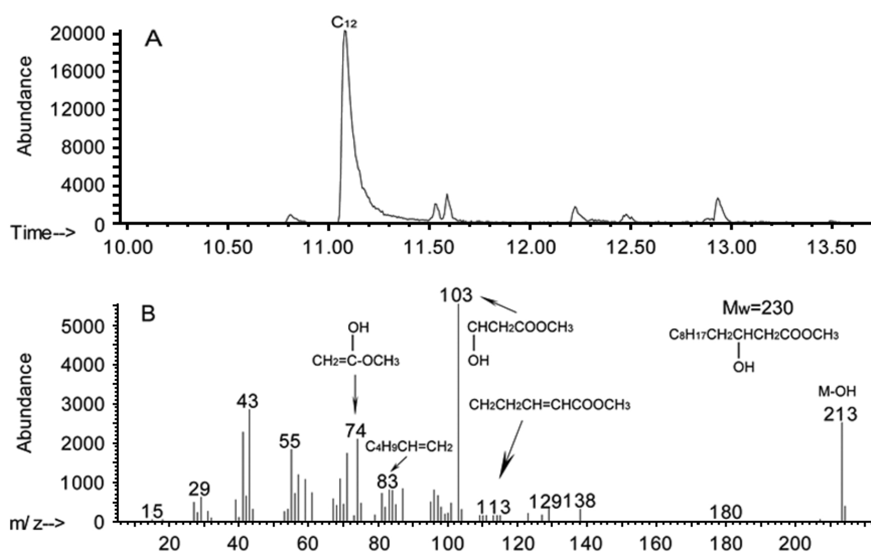

Figure 4 GC/MS of fatty acid methyl ester from N0. (A) Gas chromatography of methylated fatty acid of N0. (B) EI-MS of methylated fatty acid of N0.

amino acids: Asp, Glu, Val and Leu. The proportion of each peak area allowed for the determination of the molar ratio of Asp : Glu : Val : Leu to be 1 : 1 : 1 : 4.

The tandem mass analysis of the sodium-ionized molecular ion, with a mass of 1016.63, is presented in Figure 7. The fragment ions of 1016.51, 903.50 and

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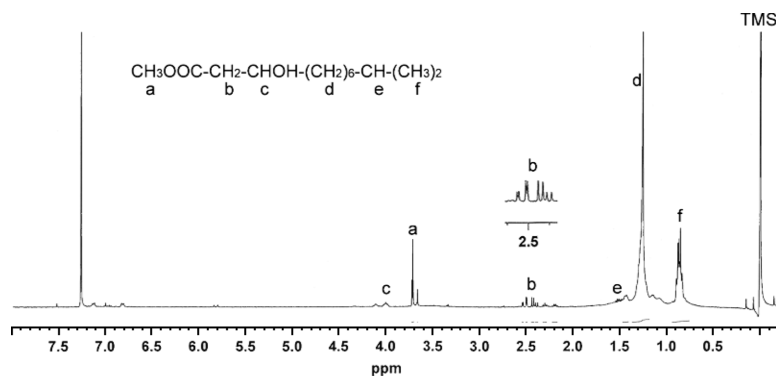

Figure 5 ^{1}H-NMR spectrum of the methyl ester of fatty acid from hydrolyzed N0.

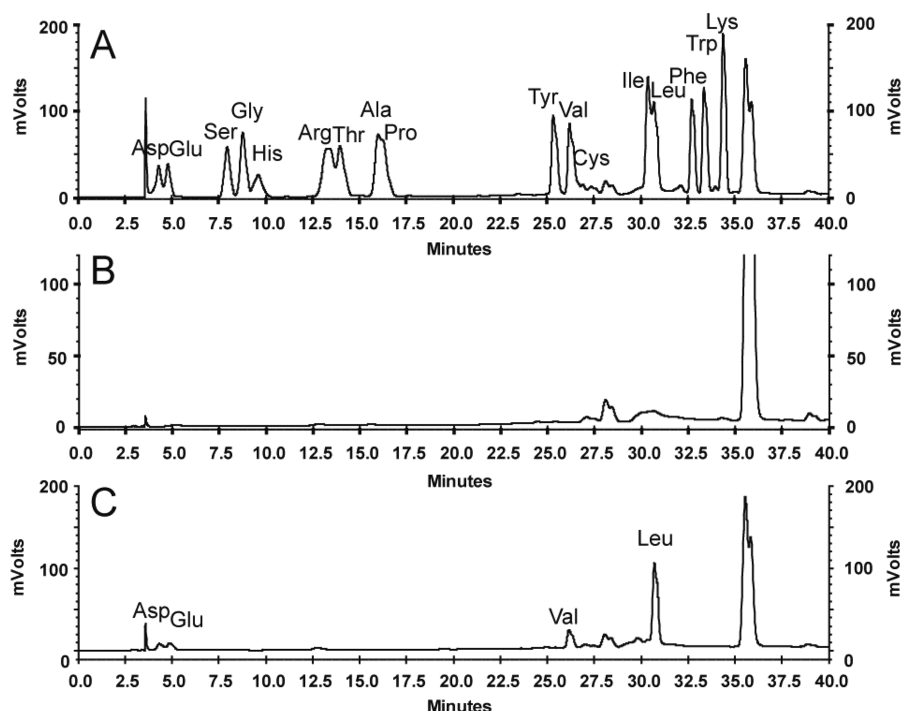

Figure 6 Separation of PITC derivatives of amino acids on a C_{18}-reversed-phase column. N0 was hydrolyzed at 105 °C for 25 h. (A) Standard amino acid analysis; (B) Negative control; (C) Sample from hydrolyzed N0.

790.40 showed a first loss of amino acid residue, which suggested the existence of a cyclic structure. Two sets of sequences are found from the mass spectrum of amino acids: chain A: Val-Asp-Leu-Leu (Figure 8(B)) and chain B: Leu-Leu-Asp-Val (Figure 9(B)). Since there are four Leu residues in N0, the connection of the two sets of amino acids in the peptide moiety is Leu-Leu-Val-Asp-Leu-Leu. According to the double hydrogen transfer mechanism in the ESI-MS/MS of cyclic lipopeptides [17], the difference between fragment ions 1016.51 and 772.38 belongs to Leu-Leu-H_2O (Figure 9(C)). Thus the Leu residue in chain A is a *C*-terminal amino acid residue and participates in the formation of lactone ring of lipopeptide. Furthermore, the 18-unit increase implies that the fatty acid in N0 is a hydroxyl fatty acid [17], which agrees with the analysis result of fatty acid derived from N0. Since there is no hydroxyl residue in Asp, Glu, Val and Leu, the other portion which participates in the formation of lactone ring should be the hydroxyl group originating from fatty acid [17].

A comparison between the tandem mass spectrometry of surfactin C_{13} and N0 is shown in Table 1. In addition to the same fragments resulting from double hydrogen transfer (DHT) and simple cleavage, it showed a 14 units decrease of N0 in the corresponding fragments containing fatty acid residue. This implies that N0 is a homologue of surfactin C_{13-15}. As a result, N0 is an iso-C_{12} β-hydroxy fatty acid connected via the *N*-terminal amino acid residue of the heptapeptide with a lactone ring and closed through the *C*-terminal amino acid residue and the β-hydroxy residue of C_{12} fatty acid (Figure 10), a lipopeptide homologue.

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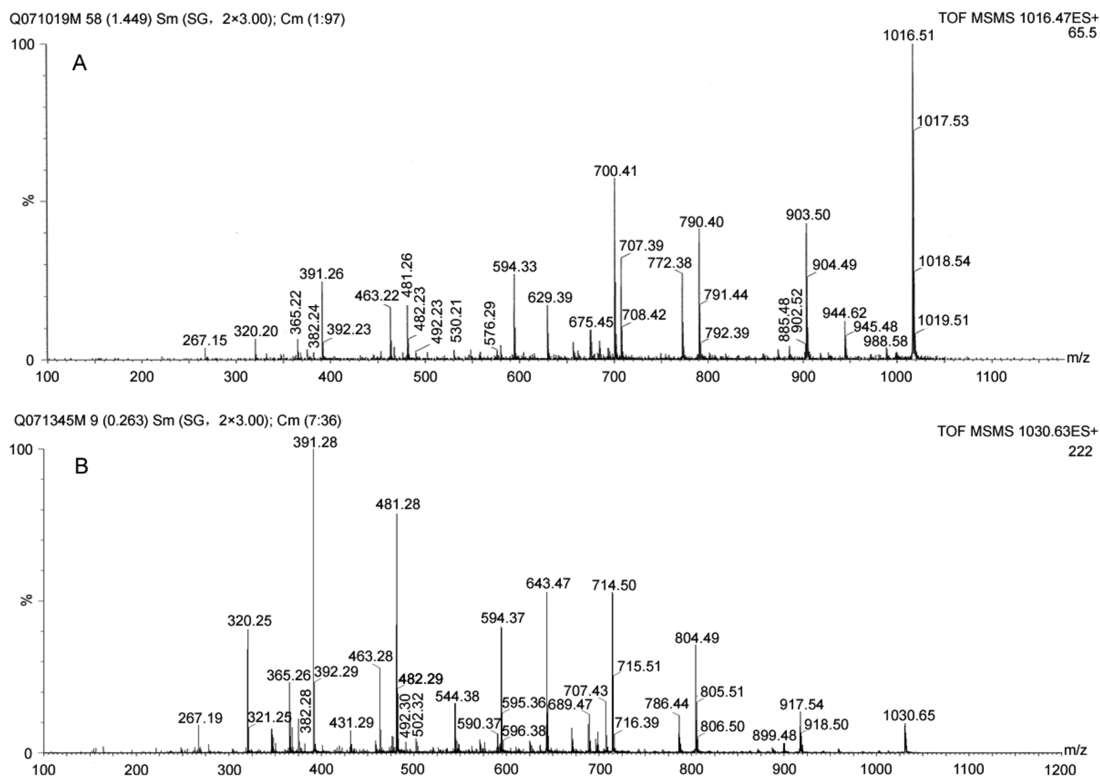

Figure 7 Mass spectrograms of sodium-ionized lipopeptides and their sodium-ionized fragments; (A) N0; (B) Surfactin C_{13}.

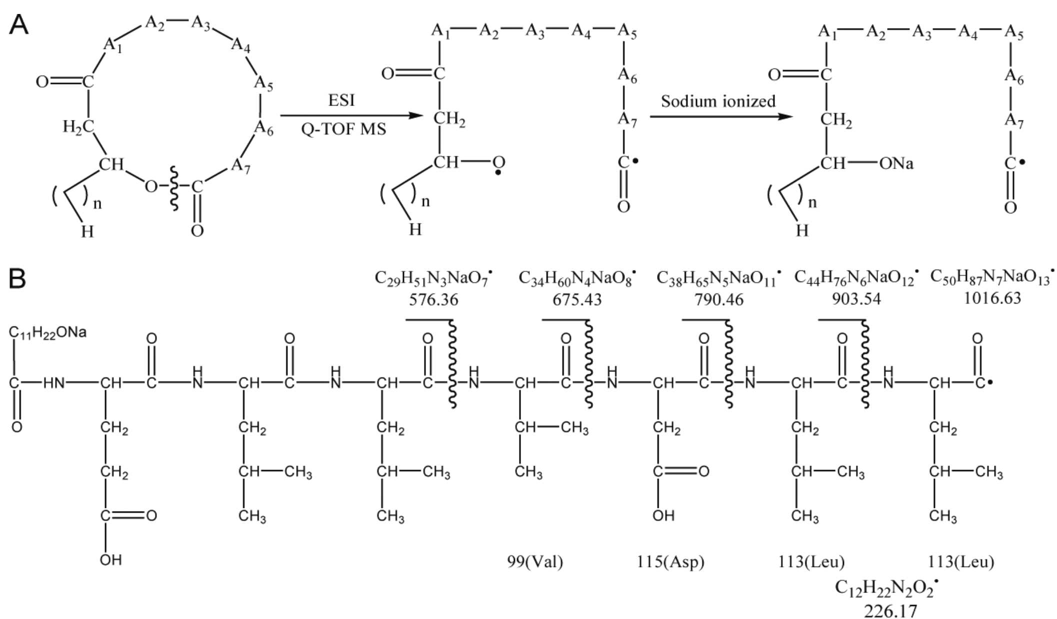

Figure 8 Simple cleavage of lipopeptide in ESI Q-Tof MS. (A) Simple cleavage of usual lipopeptides. (B) a set of fragment ions produced after simple cleavage of N0.

Surface Active Properties

The variation of surface tension values for N0 against the concentration is depicted in Figure 11. The break in the curve is characteristic of CMC, which is 6.27×10^{-5} mol/l and the surface tension at CMC (γ_{CMC}) is 27.71 mN/m.

Hemolysis of Human Erythrocytes

The dose-response curve of N0 is shown in Figure 12(A). Results of each concentration of N0 after centrifugation at 3000 rpm for 10 min is shown in Figure 12(B). At the concentration lower than 20 μmol/l, N0 didn't shown hemolysis activity. However, the hemolysis property of

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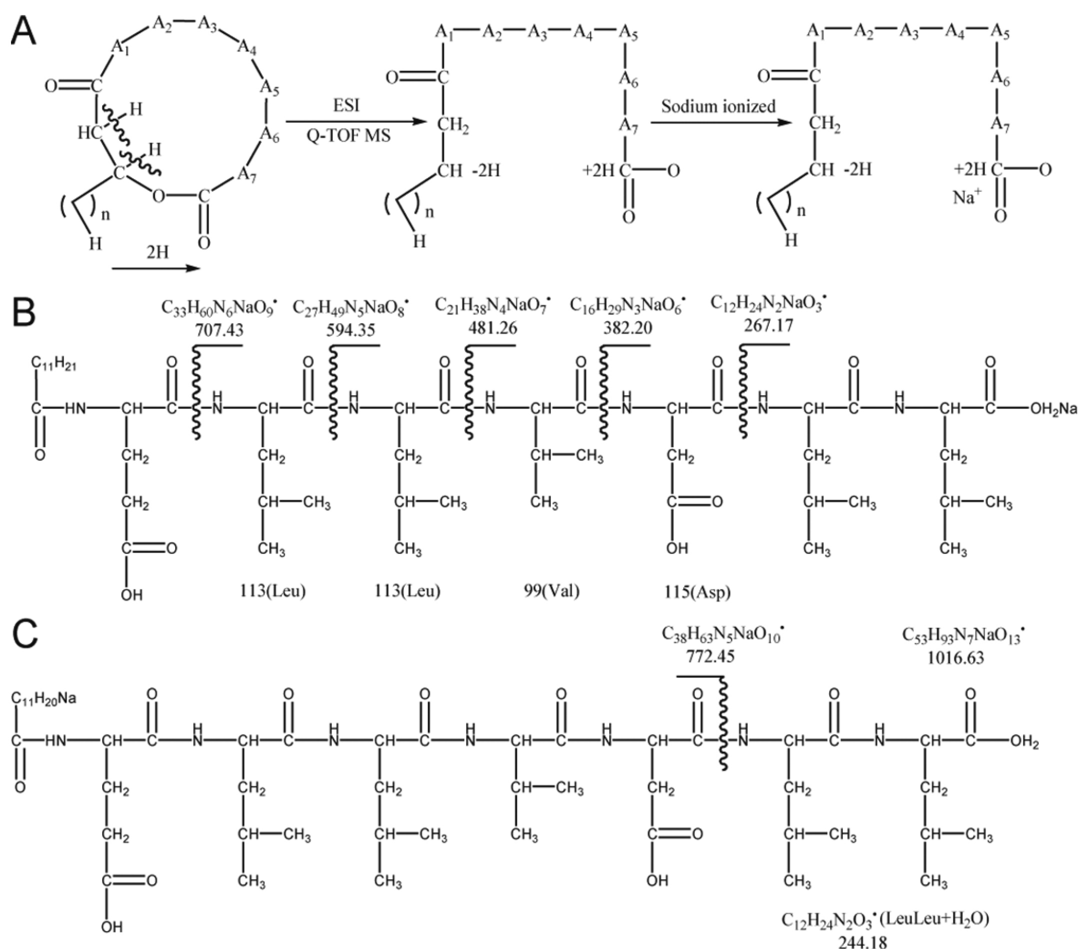

Figure 9 Cleavage of lipopeptides in ESI Q-Tof MS according to the double hydrogen transfer mechanism of aliphatic esters. (A) Cleavage mode of a lipopeptide according to the double hydrogen transfer mechanism. (B) A set of fragment ions produced after cleavage of N0. (C) The difference between the fragments produced after double hydrogen transfer mechanism is 18 units higher than that after simple cleavage (Figure 8(B)).

N0 has a sharp increase from the concentration of 20–30 μmol/l. At the concentration above 30 μmol/l, N0 showed 100% hemolysis.

DISCUSSION

Isolation and Purification

There are several reports on the isolation and preparation of bacterially produced lipopeptides with diversified structures in fatty acid moieties and peptide moieties. The separation of these lipopeptides by chromatographic procedure requires the establishment of appropriate conditions in order to collect all of the lipopeptides. In addition to the methods of ion-exchange chromatography, silica gel chromatography and ultrafiltration [25–27], many scientific researchers have developed rapid HPLC procedure with the pre-treatment methods such as solid-phase extraction, preparative TLC and size-exclusion chromatography [28–30]. Considering the need of the purification of target compounds with small amounts, we developed a reversed phase chromatographic procedure in this article. This procedure uses methanol to replace acetonitrile; making it easier to prepare and characterize N0, and allowing the recycling of methanol. Actually, water/methanol (15/85, by volume) containing 0.01% TFA has been used in the separation and preparation process of isoleucyl-rich surfactins [26]. Although some researcher mentions that the utilization of methanol probably leads to the methylation of lipopeptides [28], there are no literature reports focusing on this assumption as per our knowledge. However, during the course of preparation of lipopeptides the treatment time, temperature and the acid concentration should be reduced in order to avoid the methylation of lipopeptides when methanol is used.

Structures of Lipopeptides

The molecular mass of N0 has a 14-unit difference from 1007, of which the fatty acid part has been proved to have a chain length of 13 which implies that N0 would

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Table 1 Comparison of sodium-ionized fragments of lipopeptides in ESI Q-Tof MS[a]

Lipopeptide		Fragments after DHT		Fragments containing aliphatic acid residues		Fragments after simple cleavage
N0		Na^+LLOH_2	576	Na^+R_1ELL	320	Na^+LLV-CO
		Na^+DLLOH_2	675	Na^+R_1ELLV	365	Na^+LVDNH
	267	$Na^+VDLLOH_2$	790	Na^+R_1ELLVD		or Na^+VDLNH
	382	$Na^+LVDLLOH_2$	903	$Na^+R_1ELLVDL$	391	$Na^+LLVCONH$
	481	$Na^+LVDLLOH_2$	1016	$Na^+R_1ELLVDLL$	463	Na^+LLVD
	594		885	Na^+N0-(LOH_2)		or Na^+VDLL
	707		772	Na^+N0-($LLOH_2$)		or Na^+LVDL
			629	Na^+N0-($DLLOH_2$)	492	$Na^+ELLVNH$
			530	Na^+N0-($VDLLOH_2$)		
			590	Na^+R_2ELL		Same as above
Surfactin (C_{13})		Same as above	685	Na^+R_2ELLV		
			804	Na^+R_2ELLVD		
			917	$Na^+R_2ELLVDL$		
			1030	$Na^+R_2ELLVDLL$		
			899	Na^+Sf-(LOH_2)		
			786	Na^+Sf-($LLOH_2$)		
			643	Na^+Sf-($DLLOH_2$)		
			544	Na^+Sf-($VDLLOH_2$)		

[a] Glu: E; Asp: D; Val: V; Leu: L, Sf: Surfactin C_{13}. R_1: fatty acid of N0, R_2: fatty acid of Surfactin C_{13}.

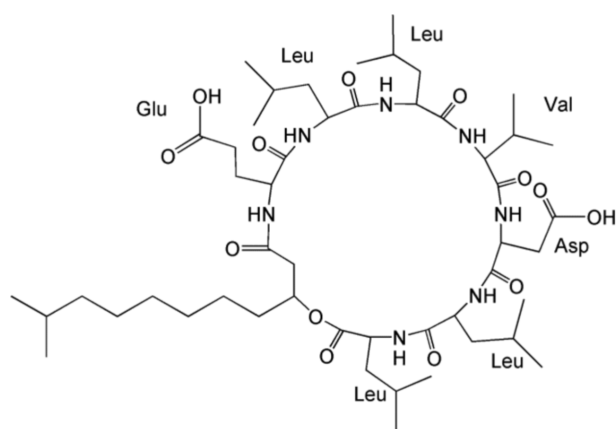

Figure 10 Schematic representation of the primary structure of N0.

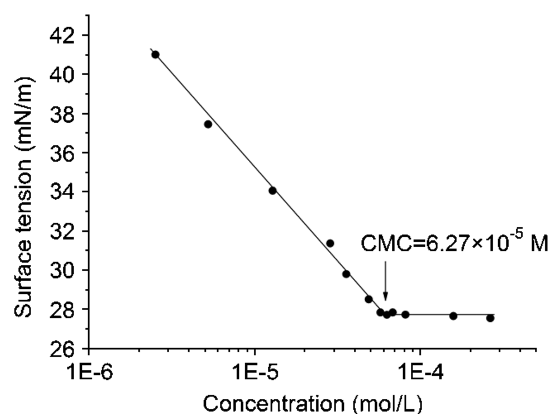

Figure 11 Surface tension of N0 in 10 mmol/l PBS (pH 8.0) at 30.0 °C.

most probably be a lipopeptide homologue. However, amongst the diverse structures of lipopeptides with molecular mass series of 993, 1007, 1021, 1035 and 1049, the 14-unit difference may occur on the following assumptions: (i) the CH_2 difference in the fatty acid homologues; (ii) the difference is located in the peptide portion of which only one amino acid is changed and the difference of molecular mass is exactly 14 (Table 2); (iii) one amino acid is methyl esterified in the peptide part, after the hydroxyl group in the amino acid residue is replaced by methoxyl group, the molecular mass difference will be 14 units. Therefore, the molecular mass with difference of14 units does not mean that it is the homologue of other lipopeptides; it is necessary to analyze the fatty acid part and/or amino acid residue. These analytical ideas may be useful to researchers focusing on the structures of lipopeptides produced by *B. subtilis*.

The identification of fatty acid moiety of N0 was carried out by using three kinds of analytical methods. First, we used ESI-MS/MS to identify the presence of monohydroxy saturated fatty acid according to the characteristic peak of and the *quasi*-molecular ion peak of monohydroxy fatty acid in the tandem mass spectrum. Second, we used GC/MS to distinguish the position of hydroxyl fatty acid. The base peak at m/z 103 in EI MS is characteristic of a β-hydroxy fatty acid. Third, the ^{1}H-NMR spectroscopy was carried out because the position of the methyl group can be reflected on the chemical shift of the proton.

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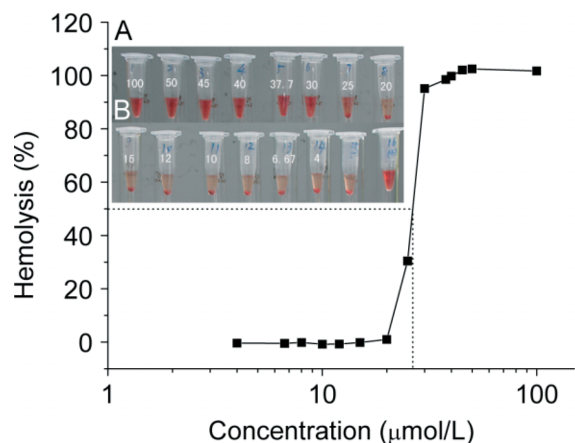

Figure 12 Effects of N0 on 2% human erythrocytes at 37 °C for 30 min (A) the dose-response curve of hemolytic activity of N0. (B) the hemolysis results of each concentration of N0 after centrifugation (3000 rpm, 10 min).

The characterization of the methyl substitutions on the fatty acid by ^{1}H-NMR spectroscopy has been previously performed by several authors [24,33]. The three analytical methods support each other and reasonably make us to reach the conclusion that the fatty acid moiety of N0 is a C_{12} β-hydroxy saturated fatty acid.

Frequently, many strains produce a complex of lipopeptides of which the lipid moieties are β-hydroxy fatty acids [7]. The fatty acids with even-numbered carbon atoms are usually predominated by those with *n* and iso-branched chains; while fatty acids with odd-numbered carbon atoms are mainly composed of those with iso- and *anteiso*-branched chains except for the existence of normal chains in C_{15} fatty acids [34]. However, it appears that the microheterogeneity in β-hydroxy fatty acid chain length is different according to the microbial species. For instance, Bonmatin *et al.* [5] summarized that *B. subtilis* S499 produce surfactins with fatty acid chain length of C_{13}–C_{15}, whereas fatty acid moieties of the lipopeptides produced by *B. subtilis* HSO121 are C_{12}–C_{17} [19]. Yakimov *et al.* [35] demonstrated that *Bacillus licheniformis* BAS50 produce lichenysins with lipid chain length of C_{12}–C_{17}. While Batrakov *et al.* [36] showed that *B. licheniformis* strain 603 produce lipopeptides of which the chain length of fatty acid is C_{13}–C_{17}. In spite of these differences, the common existences of most of fatty acids indicate the similarity of genus *Bacillus*. The iso-branched fatty acids have been proven to be the characteristic lipid composition of bacteria [37]. According to Yakimov's results [35], the effect of the β-amino acid in the culture medium to the fatty acid composition of lipophilic moiety of lipopeptides and the cellular fatty acids of bacilli are similar, which suggests that common enzyme is responsible for the biosynthesis of β-hydroxy fatty acids. These findings imply that hydroxy fatty acids of lipopeptides are probably genus specific. If it does so, the hydroxy fatty acid analysis will contribute to the detection of microbial communities under certain conditions.

Surfactant Properties of Lipopeptides

In fact, the CMC of surfactin has been widely studied. Morikawa's results showed a surfactin CMC of 7.0×10^{-5} mol/l in 10 mM PBS (pH 8.0) at 25 °C [38]. The CMC of surfactin C15 (purchased from Wako Pure, molecular weight is proved to be 1035 by FAB-MS) tested by Ishigami *et al.* [39] is 9.4×10^{-6} mol/l in 0.1 M $NaHCO_3$ (pH 8.7) at 25 °C. The CMC of surfactin from Sigma measured by Heerklotz *et al.* [40] is 7.5×10^{-6} mol/l in buffer (100 mmol/l NaCl, 10 mmol/l Tris, 1 mmol/l EDTA; pH 8.5) at 25 °C. The CMC of surfactin sodium salts (mixtures) estimated by Yoneda *et al.* [8] is 3×10^{-6} mol/l, which is about 10 000 times that of SDS. The CMC of surfactin C_{15} (purity 99%) tested by Razafindralambo *et al.* [41] is 1.95×10^{-6} mol/l in Tris Buffer (pH 8.0, 5 mM) at 20 °C. The isolated C_{12}-lipopeptide showed a purity of 90% on the analytical RP-HPLC (Figure 13). It seems that the CMC of C_{12}-lipopeptide in this article is larger than that of surfactin C_{15}.

According to Heerklotz's results [40], the CMC of surfactin goes down with increase in temperature. Although the CMC was influenced by the solution temperature, and measuring methods, especially the purity of the sample and concentration of the buffer used in the experiment, it is worthy to note that the fatty acid part is important for biosurfactant activities of lipopeptides produced by many strains [42]. Increasing the percentage of branched-chain fatty acids in lichenysin A decreases the activity of the biosurfactant. Deleu *et al.* [21] and Razafindralambo

Table 2 Structures of surfactins with molecular mass of 993 produced by *Bacillus subtilis*

Name	β-hydroxy fatty acids	Peptide sequence	Molecular mass	Ref.
[Val7]surfactin	C_{13}	*N*-Glu-Leu-Leu-Val-Asp-Leu-Val-*C*	993	26,30,31
[Ile2, Val7] surfactin	C_{13}	*N*-Glu-Ile-Leu-Val-Asp-Leu-Val-*C*	993	30
[Ala4]surfactin	C_{14}	*N*-Glu-Leu-Leu-Ala-Asp-Leu-Leu-*C*	993	32

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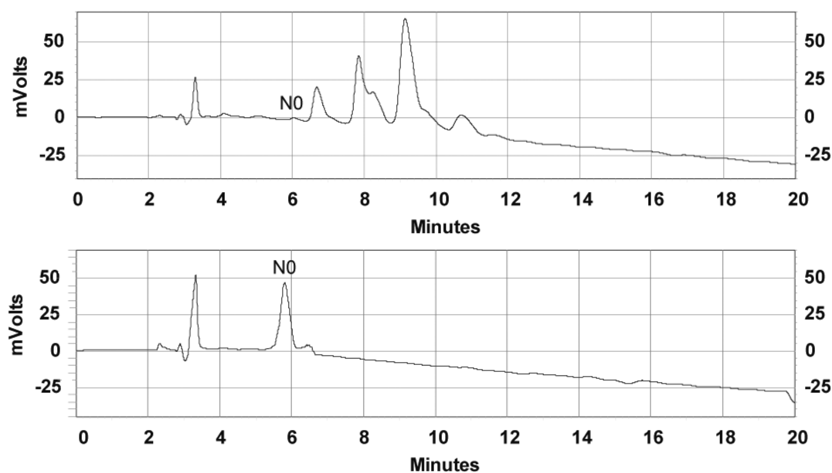

Figure 13 HPLC profile of the ODS C_{18} column (Φ 4.6 × 250 mm) at 30 °C. (A) lipopeptide mixture after being treated by normal pressure column. (B) N0 obtained by preparative HPLC. Each HPLC spectrum was detected at 210 nm. The solvent system was a mixture of (a) acetronitrile and (b) 0.05% TFA in water, of which the proportions were controlled by the gradient program: 0 min, 80% A; 20 min, 100% A; 30 min 100% A.

et al. [41] suggest that the CMC of surfactin increases with the decreasing of the number of carbon atoms of fatty acids. However, the Yakimov *et al.* [35] proposed that the surface activity of lichenysin A is influenced by both the chain length and the branching type. The increasing order was *normal* > iso > *anteiso*, whereas the activity of n-C_{14} was greater than that of iso or *ante*iso C_{15}. Additionally, the small chain length of C_{12}-lipopeptide decreases the hydrophobicity, which will increase the solubility of the whole molecule. Considering these influencing factors, it is easy to explain the higher CMC of iso-C_{12}-lipopeptide in comparison to the surfactin-C_{15}.

Hemolytic Activities of Lipopeptides

Emerging evidence has demonstrated that surfactin-like lipopeptides show hemolytic activities in which the detergent effect involved is related to the concentration of lipopeptides [21]. In this study, we found that the hemolytic activities of N0 examined on human erythrocytes showed a sharp increase near the concentration of CMC, which indicates the micelle play an important role in the hemolytic activities of lipopeptides. Hemolytic potency of N0 (26.5 μM) on human erythrocytes is similar to those of other surfactins (C_{13-15}) ranging from 13–70 μM as examined by Kracht *et al.* [43] and 20–80 μM as examined by Deleu *et al.* [21] However, the hemolytic activities of surfactin C_{14} on sheep erythrocytes reported by Dufour *et al.* showed a larger HC_{50} of 300 μM [11]. The difference can be interpreted to be due to the different source of erythrocytes as indicated by Bernheimer *et al.* [44]; sensitivity of red cells from horse, pig, cat and chicken was less than that of rabbit cells but greater than that of cells from calf, sheep and goat. The different hemolysis results implied different membrane composition of red cells. In addition, the different physicochemical properties of different lipopeptides with different chain lengths will influence their interactions with cell membranes [45]. The lipopeptide homologues with short chain lengths have large HC_{50} [21,43].

CONCLUSIONS

This article describes the isolation, preparation and characterization of a new lipopeptide homologue with a molecular mass of 993 produced by *B. subtilis* HSO121. The preparation is successfully performed with a methanol solvent system in HPLC procedure. The combined use of chemical, chromatographic and mass spectrometric techniques enabled elucidation of the novel lipopeptide without the necessity for opening of the ring to produce a free NH_2-terminus. Combined with the comparison method, the novel C_{12}-lipopeptide is proved to be a homologue of surfactins. The existence of this smaller fatty acid moiety in lipopeptides produced by *B. subtilis* and *B. licheniformis* implies that the β-hydroxy fatty acids are probably characteristic of genus *Bacillus* which can produce lipopeptides. The CMC and γ_{CMC} values of N0 suggest that it has a similar surfactant activity with surfactin C_{15}. The smaller hydrophobic chain will increase the solubility in water solution. Furthermore, the C_{12}-lipopeptide with short hydrophobic lipid chain has low toxicity on red blood cell.

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REFERENCES

1. Van Hamme JD, Singh A, Ward OP. Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnol. Adv.* 2006; **24**: 604–620.
2. Singh A, Van Hamme JD, Ward OP. Surfactants in microbiology and biotechnology: Part 2. application aspects. *Biotechnol. Adv.* 2007; **25**: 99–121.
3. Singh P, Cameotra SS. Potential applications of microbial surfactants in biomedical sciences. *Trends Biotechnol.* 2004; **22**: 142–146.
4. Cameotra SS, Makkar RS. Recent applications of biosurfactants as biological and immunological molecules. *Curr. Opin. Microbiol.* 2004; **7**: 262–266.
5. Bonmatin JM, Laprevote O, Peypoux F. Diversity among microbial cyclic lipopeptides: iturins and surfactants. activity-structure relationship to design new bioactive agents. *Comb. Chem. High Throughput Screen.* 2003; **6**: 541–556.
6. Arima K, Kakinuma A, Tamura G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhititior of fibrin clot formation. *Biochem. Biophys. Res. Commun.* 1968; **31**: 488–494.
7. Peypoux F, Bonmatin JM, Wallach J. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.* 1999; **51**: 553–563.
8. Yoneda T, Tsuzuki T, Ogata E, Fusyo Y. Surfactin sodium salt: an excellent biosurfactant for cosmetics. *J. Cosmet. Sci.* 2001; **52**: 153–154.
9. Razafindralambo H, Popineau Y, Deleu M, Hbid C, Jacques P, Thonart P, Paquot M. Foaming properties of lipopeptides produced by *Bacillus subtilis*: effect of lipid and peptide structural attributes. *J. Agric. Food Chem.* 1998; **46**: 911–916.
10. Deleu M, Razafindralambo H, Popineau Y, Jacques P, Thonart P, Paquot M. Interfacial and emulsifying properties of lipopeptides from *Bacillus subtilis*. *Colloids Surf., A: Physicochem. Eng. Asp.* 1999; **152**: 3–10.
11. Dufour S, Deleu M, Nott K, Wathelet B, Thonart P, Paquot M. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. *Biochim. Biophys. Acta* 2005; **1726**: 87–95.
12. Kikuchi T, Hasumi K. Enhancement of reciprocal activation of prourokinase and plasminogen by the bacterial lipopeptides surfactins and iturin Cs. *J. Antibiot.* 2003; **56**: 34–37.
13. Carrillo C, Teruel JA, Aranda FJ, Ortiz A. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochim. Biophys. Acta* 2003; **1611**: 91–97.
14. Kanatomo S, Nagai S, Ohki K, Yasuda Y. Study on surfactin, a cyclic depsipeptide. I. Isolation and structure of eight surfactin analogs produced by *Bacillus natto* KMD 2311. *J. Pharm. Soc. Jpn.* 1995; **115**: 756–764.
15. Liu XY, Yang SZ, Mu BZ. Molecular structures of microbial lipopeptides. *Biotechnol. Bull.* 2005; **4**: 18–26.
16. Lü YN, Yang SZ, Mu BZ. Isolation and identification of a lipopeptide. *Microbiology* 2005; **32**: 67–73.
17. Yang SZ, Wei DZ, Mu BZ. Determination of the amino acid sequence in a cyclic lipopeptide using MS with DHT mechanism. *J. Biochem. Biophys. Methods* 2006; **68**: 69–74.
18. Yang SZ, Wei DZ, Mu BZ. Determination of the structure of the fatty acid chain in a cyclic lipopeptide using GC-MS. *J. Biochem. Biophys. Methods* 2006; **70**: 519–523.
19. Liu XY, Namir H, Yang SZ, Mu BZ. Structural characterization of eight cyclic lipopeptides produced by *Bacillus subtilis* HSO121. *Protein Pept. Lett.* 2007; **18**: 766–773.
20. Yang Q, Sun LG, Bai XZ, Zhou HT. Simultaneous determination of 18 amino acids by reversed-phase high performance liquid chromatography with precolumn phenyl isothiocyanate derivatization. *Chin. J. Chromatogr.* 2002; **20**: 369–371.
21. Deleu M, Bouffioux O, Razafindralambo H, Paquot M, Hbid C, Thonart P, Jacques P, Brasseur R. Interaction of surfactin with membranes: a computational approach. *Langmuir* 2003; **19**: 3377–3385.
22. Tomer KB, Jensen NJ, Gross ML. Fast atom bombardment and tandem mass spectrometry for determining structural modification of fatty acids. *Anal. Chem.* 1986; **58**: 2429–2433.
23. Kerwin JL, Torvik JJ. Identification of monohydroxy fatty acids by electrospray mass spectrometry and tandem mass spectrometry. *Anal. Biochem.* 1996; **237**: 56–64.
24. Besson F, Tenoux I, Hourdou ML, Michel G. Synthesis of β-hydroxy fatty acids and β-amino fatty acids by the strains of *Bacillus subtilis* producing iturinic antibiotics. *Biochim. Biophys. Acta* 1992; **1123**: 51–58.
25. Vater J. Lipopeptides, an attractive class of microbial surfactants. *Prog. Colloid Polym. Sci.* 1986; **72**: 12–18.
26. Peypoux F, Bonmatin JM, Labbé H, Das BC, Ptak M, Michel G. Isolation and characterization of a new variant of surfactin, the [Val7]surfactin. *Eur. J. Biochem.* 1991; **202**: 101–106.
27. Lin SC, Jiang HJ. Recovery and purification of the lipopeptide biosurfactant of *Bacullus subtilis* by ultrafiltration. *Biotechnol. Tech.* 1997; **11**: 413–416.
28. Razafindralambo H, Paquot M, Destain J, Thonart P. Purification of antifungal lipopeptides by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 1993; **639**: 81–85.
29. Grangemard I, Peypoux F, Wallach J, Das BC, Labbé H, Caille A, Genest M, Maget-Dana R, Ptak M, Bonmatin JM. Lipopeptides with improved properties: structure by NMR, purification by HPLC and structure-activity relationships of new isoleucyl-rich surfactins. *J. Pept. Sci.* 1997; **3**: 145–154.
30. Kowall M, Vater J, Kluge B, Stein T, Franke P, Ziessow D. Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB 105. *J. Colloid Interface Sci.* 1998; **204**: 1–8.
31. Nakayama S, Takahashi S, Hirai M, Shoda M. Isolation of new variants of surfactin by a recombinant *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 1997; **48**: 80–82.
32. Peypoux F, Bonmatin JM, Labbe H, Grangemard I, Das BC, Ptak M, Wallach J, Michel G. [Ala4]surfactin, a novel isoform from *Bacillus subtilis* studied by mass and NMR spectroscopies. *Eur. J. Biochem.* 1994; **224**: 89–96.
33. Oka K, Hirano T, Homma M, Ishii H, Murakami K, Mogami S, Motizuki A, Morita H, Takeya K, Itokawa H. Satisfactory separation and MS-MS spectrometry of six surfactins isolated from *Bacillus subtilis* natto. *Chem. Pharm. Bull. (Tokyo)* 1993; **41**: 1000–1002.
34. Yakimov MM, Timmis KN, Wray V, Fredrickson HL. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Environ. Microbiol.* 1995; **61**: 1706–1713.
35. Yakimov MM, Fredrickson HL, Timmis KN. Effect of heterogeneity of hydrophobic moieties on surface activity of lichenysin A, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. *Biotechnol. Appl. Biochem.* 1996; **23**: 13–18.
36. Batrakov SG, Rodionova TA, Esipov SE, Polyakov NB, Sheichenko VI, Shekhovtsova NV, Lukin SM, Panikov NS, Nikolaev YA. A novel lipopeptide, an inhibitor of bacterial adhesion, from the thermophilic and halotolerant subsurface *Bacillus licheniformis* strain 603. *Biochim. Biophys. Acta* 2003; **1634**: 107–115.
37. Fautz E, Rosenfelder G, Grotjahn L. Iso-branched 2- and 3-hydroxy fatty acids as characteristic lipid constituents of some gliding bacteria. *J. Bacteriol.* 1979; **140**: 852–858.
38. Morikawa M, Daido H, Takao T, Murata S, Shimonishi Y, Imanaka T. A new lipopeptide biosurfactant produced by *Arthrobacter sp.* strain MIS38. *J. Bacteriol.* 1993; **175**: 6459–6466.

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39. Ishigami Y, Osman M, Nakahara H, Sano Y, Ishiguro R, Matsumoto M. Significance of β-sheet formation for micellization and surface adsorption of surfactin. *Colloids Surf., B Biointerfaces* 1995; **4**: 341–348.
40. Heerklotz H, Seelig J. Detergent-like action of the antibiotic peptide surfactin on lipid membranes. *Biophys. J.* 2001; **81**: 1547–1554.
41. Razafindralambo H, Thonart P, Paquot M. Dynamic and equilibrium surface tensions of surfactin aqueous solutions. *J. Surfactants. Deterg.* 2004; **7**: 41–46.
42. Youssef NH, Duncan KE, McInerney MJ. Importance of 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. *Appl. Environ. Microb.* 2005; **71**: 7690–7695.
43. Kracht M, Rokos H, Özel M, Kowall M, Pauli G, Vater J. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. *J. Antibiot.* 1999; **52**: 613–619.
44. Bernheimer AW, Avigad LS. Nature and properties of a cytolytic agent produced by Bacillus subtilis. *J. Gen. Microbiol.* 1970; **61**: 361–369.
45. Bouffioux O, Berquand A, Eeman M, Paquot M, Dufrene YF, Brasseur R, Deleu M. Molecular organization of surfactin-phospholipid monolayers: effect of phospholipid chain length and polar head. *Biochim. Biophys. Acta* 2007; **1768**: 1758–1768.

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