Journal of Peptide Science, Vol. 3, 145–154 (1997)

Lipopeptides with Improved Properties: Structure by NMR, Purification by HPLC and Structure–Activity Relationships of New Isoleucyl-rich Surfactins

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Received 9 April 1996
Accepted 18 July 1996

Abstract: The biosynthesis of bacterial isoleucyl-rich surfactins was controlled by supplementation of L-isoleucine to the culture medium. Two new variants, the [Ile4,7]- and [Ile2,4,7]surfactins, were thus produced by *Bacillus subtilis* and their separation was achieved by reverse-phase HPLC. Amino acids of the heptapeptide moiety were analysed by chemical methods, and the lipid moiety was identified to β-hydroxy *anteiso* pentadecanoic acid by combined GC/MS. Sequences were established on the basis of two-dimensional NMR data. Because conformational parameters issuing from NMR spectra suggested that the cyclic backbone fold was globally conserved in the new variants, structure–activity relationships were discussed in details on the basis of the three-dimensional model of surfactin in solution. Indeed, both variants have increased surface properties compared with that of surfactin, and this improvement is assigned to an increase of the hydrophobicity of the apolar domain favouring micellization. Furthermore, the additional Leu-to-Ile substitution at position 2 in the [Ile2,4,7]surfactin leads to a substantial increase of its affinity for calcium, when compared with that of [Ile4,7]surfactin or surfactin. This effect is assigned, from the model, to an increase in the accessibility of the acidic side chains constituting the calcium binding site. Thus, the propensities of such active lipopeptides for both hydrophobic and electrostatic interactions were improved, further substantiating that they can be rationally designed.

Keywords: biosurfactant; lipopeptide; surfactins; *Bacillus subtilis; NMR*

INTRODUCTION

Surfactin, a lipopeptide produced by several strains of *Bacillus subtilis* [1], is well known to be one of the most powerful biosurfactants [2]. In addition to its high surface properties, surfactin forms a stable 1/1 complex with divalent cations [3], penetrates phospholipid monolayers [4] and induces selective cationic pores in membranes [5]. Such properties illustrate the propensity of surfactin for both electrostatic and hydrophobic interactions, especially at

Abbreviations:AcCys, *N*-acetyl-L-cysteine; CMC, critical micellar concentration; DQF-COSY, double quantum filtered correlation spectroscopy; DMSO, dimethylsulphoxide; LSIMS, liquid secondary ion mass spectrometry; NOESY, nuclear Overhauser enhancement spectroscopy; OPTA, *ortho*-phthalaldehyde; TOCSY, total correlation spectroscopy; 1D, 2D, 3D, one, two, three-dimensional.

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CCC 1075-2617/97/020145-10 $17.50

the membrane level. They are doubtless at the origin of its wide spectrum of biological activities. Surfactin inhibits fibrin clotting [6] and lyses erythrocytes [7]. It has moderate antibiotic [8], antitumoral [9] and anti-HIV activities [10]. When it is associated with the antibiotic/antifungal iturin A, another lipopeptide co-produced by *Bacillus subtilis*, surfactin has strong synergistic effects [11].

Surfactin is a cyclic heptapeptide linked to a β-hydroxy fatty acid via an amide bond and an ester bond. The length (C_{13} to C_{15}) and the structure (*iso*, *anteiso* or *n*) of the lipid moieties are variable, and their proportions depend on both culture conditions and strains, as exemplified with *Bacillus subtilis* [12] and *Bacillus subtilis natto* [13]. The peptide sequence of the most usual component is L-Glu1 → L-Leu2 → D-Leu3 → L-Val4 → L-Asp5 → D-Leu6 → L-Leu7, but the coexistence of several variants has been demonstrated in the past few years. These variants constitute the surfactin family and their properties are very sensitive to a single substitution in the peptide moiety [14–17]. On the one hand, several variants substituted at position 4 or 7 have been isolated and we have shown that their critical micellar concentration (CMC) steadily decreases in the order [Val7] > [Leu7] or [Ala4] > [Val4] > [Ile4]. On the other hand, the much more lytic [methylester-Glu1]surfactin does not bind calcium in a 1/1 ratio [18] while the [Gln1] surfactin from marine *Bacillus* species has none of the antibiotic and antitumoral activities [19]. All this suggests that a surfactin variant, with an heptapeptide sequence including the two acidic residues and several isoleucine residues, could lead to more industrial and biological applications with respect to the usual component. Besides, its study should provide valuable data to establish detailed structure–activity relationships, which would be especially useful since production of surfactin variants have become accessible from genetic modifications [20].

The biosynthesis and the type of surfactins have been found to be highly dependent on the nitrogen source of the culture medium [21]. When *Bacillus subtilis* was grown with L-isoleucine, the surfactant mixture contained surfactin, [Val7]surfactin, [Ile4]-surfactin and 30% of a new surfactant material. In the present paper, we demonstrate by chemical analysis, MS and NMR that this material is a mixture of the [Ile4,7]- and [Ile2,4,7]surfactins in equal proportions. Our report concerns the chromatographic procedure that has been developed to acquire an effective separation of such very similar components in amounts large enough (mg) so as to study their properties. We include data on the significantly improved properties (both surface–activity and calcium-binding) of the [Ile2,4,7]variant. The role of acidic and hydrophobic residues is discussed in detail on the basis of structure–property relationships established from the three-dimensional (3D) model of surfactin in solution [22].

MATERIALS AND METHODS

Isolation

Surfactins were produced and isolated from the culture of *Bacillus subtilis* S499 as previously described [17]. Briefly, a preculture, grown in brain heart infusion, was used to inoculate a medium of Landy *et al.* [23] in which L-glutamic acid was replaced by L-isoleucine. Surfactins were extracted, separated by column chromatography and further isolated by two steps of preparative TLC on silica gel. The surfactin material studied hereafter, named B2 in a previous report [17], corresponded to 30% of the total surfactins and was subjected to MS, NMR and chemical analysis to determine its constitution qualitatively and quantitatively. Because of evidence of heterogeneity, two lipopeptides (S1 and S2) were further purified by semi-preparative isocratic reverse-phase HPLC. HPLC was performed on a Lichrosorb 7 C_{18} column (250 × 15 mm) with a Varian 5000 apparatus. The mobile phase was water/methanol (15/85, by vol.) containing 0.01% trifluoroacetic acid, at a flow rate of 4 ml/min, and detection was made at 214 nm. Amino acid analyses were performed on S1 and S2 and their respective surface and calcium-binding properties were studied.

MS, NMR and Fatty Acid Analysis Prior to Separation of S1 and S2

The mass spectrum was recorded on a Kratos MS80 RF instrument. The instrument was operated in the liquid secondary ion mass spectrometry (LSIMS) mode using a cesium-ion gun at 20 kV. A sample (3 μg) was introduced on a copper-probe tip using thioglycerol (2 μl) as matrix. Data were acquired with a DS-90 data system.

NMR data were acquired on a Bruker AMX-500 spectrometer. Lipopeptides (2 mg) were studied as ionized forms from dissolution of the mixture in an NaOH solution pH 10. After lyophilization, the ionized lipopeptides were dissolved in $^2H_6$-dimethyl-

sulphoxide ($^2H_6$-DMSO, 99.9%) for NMR. Data were obtained at temperatures between 19 and 33 ± 0.1°C and the residual ^{1}H-resonance of DMSO was calibrated at 2.49 p.p.m. Double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY, mixing time of 80 ms) and NOE spectroscopy (NOESY, mixing times of 120 and 250 ms) experiments were acquired with a slight presaturation of the residual water arising at 3.30 p.p.m., a spectral width of 5050.5 Hz and a matrix size of 2k × 512 points. Prior to Fourier transform, 2D data were zero-filled to obtain a digital resolution of 1.2 Hz/point in the *f*2 dimension and 4.8 Hz/point in the *f*1 direction.

For fatty acid analyses, the mixture of lipopeptides was hydrolysed with 6 M HCl at 110 °C for 18 h. Lipid moieties were extracted with chloroform. Fatty acid methylesters were obtained by the action of gaseous diazomethane and analysed by combined GC/MS. GC was performed on a DB 5 capillary column (0.32 mm × 30 m) with temperature programming from 60 to 180 °C at 30 °C/min, then followed by an isotherm of 20 min at 180 °C. Electron impact MS was performed at 200 °C and 70 eV. *β*-Hydroxy fatty acid methylesters were identified by their retention times and molecular masses, in comparison with standards.

Amino Acid Analyses of S1 and S2

After HPLC purification, S1 and S2 were hydrolysed with 6 M HCl at 110 °C for 18 h. The resolution of enantiomers and analyses of amino acids were carried out by a precolumn derivatization procedure using *ortho*-phthalaldehyde (OPTA) and *N*-acetyl-L-cysteine (AcCys) as reagents. For this, hydrolysates (40 μg) were dissolved in 100 μl of 0.1 M sodium borate pH 9.2 and mixed for 2 min at room temperature with 50 μl of OPTA-AcCys reagent [24]. Analyses were carried out by HPLC on a Kromasil 5 C_{18} column (150 × 4.6 mm) completed by a 1 cm-long guard column. Elution was performed by the mixture of 50 mM sodium acetate pH 5.9 and methanol, the proportions of which being

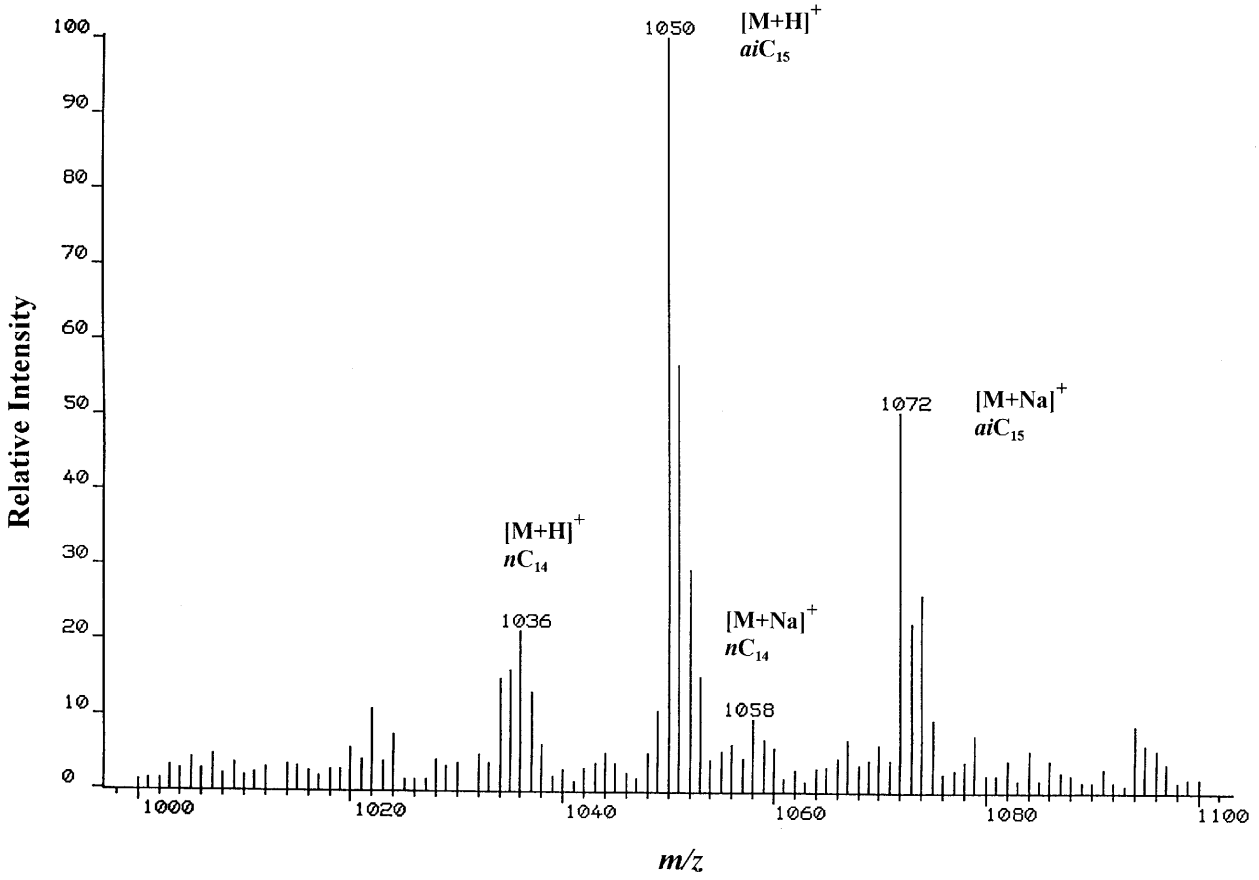

Figure 1 Part of the LSIMS spectrum of the new surfactant material. $[M+H]^+$ and $[M+Na]^+$ peaks are labelled with their corresponding lipid moiety: *anteiso*C_{15} or *n*C_{14}.

controlled by a microprocessor gradient program. Amino acids were identified by comparison with standard amino acid enantiomers.

Surface and Cation-binding Properties

For each lipopeptide, surface tension data were acquired in the conditions previously described [17]. Briefly, surface tension (γ) was measured at 25 °C with a Krüss tensiometer, according to the well-known procedure of Du Nouy. CMC was then determined from the γ versus log concentration curve. Cation binding was studied by a conductimetric method [25]. Schematically, a thermostatically-controlled conductimetric cell was filled with 4 ml of 5 mM Tris solution of pH 9.5 containing 0.3 mM lipopeptide. A constant volume (10 µl) of a Ca^{2+} solution was successively added into the cell while the conductance was recorded. Comparison was made with Ca^{2+} conductance changes in absence of lipopeptide. From this, concentrations of bound and free cations were deduced as described in [3].

RESULTS

Isolation and Characterization

Analysis of the R_F values by analytical TLC on silica gel with chloroform/methanol/propan-1-ol/0.25%KCl/ethyl- acetate (25/13/25/9/25, by vol.) has shown that the new material thoroughly studied in this paper was different from previously characterized surfactins: $R_F = 0.69$ versus 0.66 (surfactin), 0.61 ([Val7]surfactin) and 0.73 ([Ile4]-surfactin). Moreover, a substitution in the basal peptide sequence was easily located at position 7, by identification of Ile instead of Leu as the C-terminal residue, as determined by hydrazinolysis of the acyclic material obtained after alkaline hydrolysis of the lactone bond.

The GC/MS analysis of fatty acid methylesters revealed that the lipid moiety is mainly constituted of β-hydroxy *anteiso* pentadecanoic acid (β-OH *anteiso*C_{15}; peak at m/z 272). Small amounts of β-hydroxy *iso* pentadecanoic acid (β-OH *iso*C_{15}; peak at m/z 272) and β-hydroxy *n* tetradecanoic acid (β-OH nC_{14}; peak at m/z 258) were also detected. Furthermore, the LSIMS mass spectrum of the new surfactins displayed a major $[M+H]^+$ peak at m/z 1050 accompanied with a $[M+Na]^+$ peak at m/z 1072 (Figure 1). With a majority ($>90\%$) of β-OH C_{15}

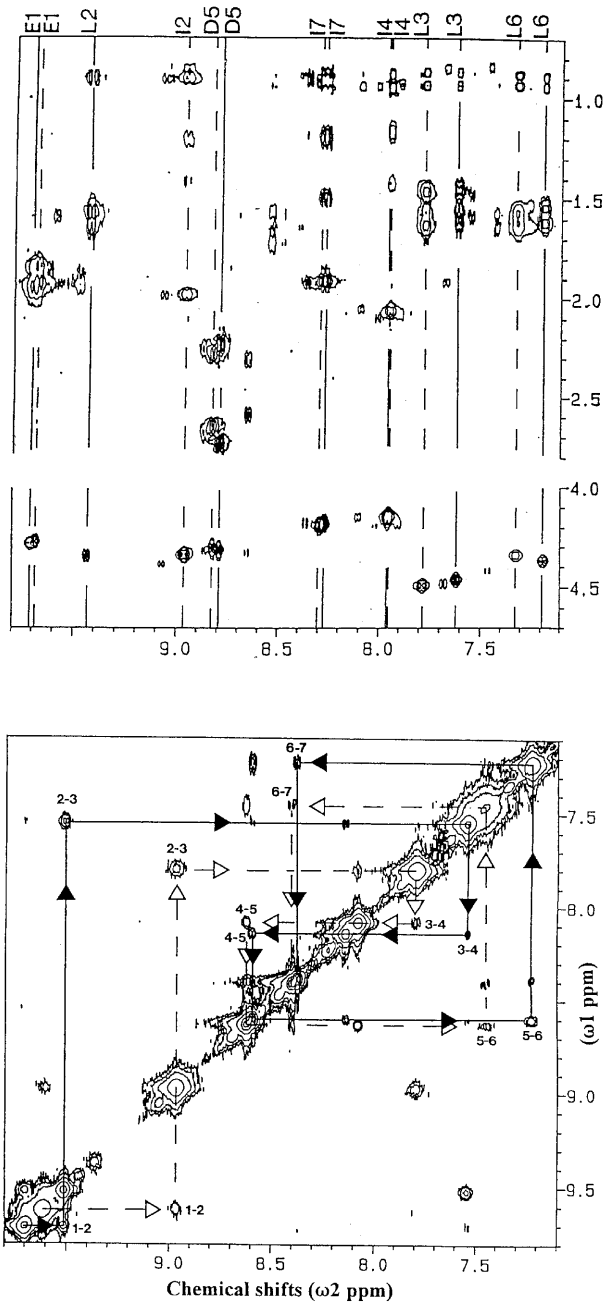

Figure 2 Parts of 2D-NMR spectra of the new surfactant material (2 mg) in 0.5 ml of $^2H_6$-DMSO. Top: TOCSY data (80 ms, 33 °C) of NH-αH and NH side-chain regions. Spin systems for S1 and S2 are shown by solid and broken lines, respectively, and residue assignments are given at the top. Bottom: NOESY data (250 ms, 19 °C) of the NH–NH region where sequential connectivities are labelled. Sequential pathways are illustrated for S1 and S2 by solid and broken lines, respectively. In both spectra, a few minor signals correspond to the presence of minor ($<5\%$) conformers.

Table 1 ^{1}H Chemical Shifts of the Ionized Forms of [Ile4,7]- and [Ile2,4,7]surfactins in $^{2}H_6$-dimethylsulphoxide at 19°C. For Assignment of the C_{15}-lipid Chain, Carbon Atoms are Numbered From 1 to 15, Degenerate CH_2 Resonances are Noted $C^{d}H_2$, and CH_3 Resonances of the *anteiso* Extremity are Noted $C^{ai}H_3$. Chemical Shifts are Accurate to ±0.01 p.p.m.

Residue	Chemical shifts (ppm)					
	NH	αH	βH	γH	γH_3	δH_3
[Ile4,7]surfactin						
L-Glu1	9.65	4.12	1.70,1.68	1.78,1.77		
L-Leu2	9.46	4.17	1.43,1.41	1.42		0.77,0.75
D-Leu3	7.49	4.30	1.40,1.29	1.31		0.80,0.73
L-Ile4	8.09	4.03	1.91	1.28,1.03	0.81	0.73
L-Asp5	8.55	4.20	2.57,2.05			
D-Leu6	7.17	4.26	1.44,1.36	1.40		0.81,0.75
L-Ile7	8.33	4.02	1.78	1.33,1.06	0.77	0.73
Lipid chain	$C^{2}H_2$ 2.70, 2.25; $C^{3}H$ 4.86; $C^{4}H_2$ 1.51; $C^{d}H_2$ 1.15; $C^{ai}H_3$ 0.75					
[Ile2,4,7]surfactin						
L-Glu1	9.56	4.13	1.69,1.67	1.83,1.79		
L-Ile2	8.92	4.16	1.82	1.27,1.04	0.75	0.74
D-Leu3	7.74	4.32	1.44,1.33	1.43		0.81,0.73
L-Ile4	8.03	4.01	1.89	1.26,1.01	0.79	0.73
L-Asp5	8.58	4.20	2.47,2.12			
D-LEU6	7.40	4.24	1.45,1.39	1.41		0.80,0.74
L-Ile7	8.36	4.04	1.78	1.33,1.06	0.77	0.73
Lipid chain	$C^{2}H_2$ 2.70, 2.14; $C^{3}H$ 4.83; $C^{4}H_2$ 1.50; $C^{d}H_2$ 1.15; $C^{ai}H_3$ 0.75					

fatty acid, the molecular mass of the lipopeptide is therefore 14 Da higher than that of surfactin ($[M+H]^+$ peak at m/z 1036, data not shown). Thus the variation in the mass spectrum was not due to a difference in the lipid composition but suggested a second substitution: Leu or Ile instead of the single L-Val4 residue. The minor $[M+H]^+$ peak at m/z 1036 was assigned to the presence of small amounts of lipopeptide with β-OH C_{14} fatty acid as lipid moiety.

For a complete structural elucidation, the material was subjected to NMR spectroscopy. 14 strong NH-signals arising between 7 and 10 p.p.m. were detected in 1D spectra recorded at several temperatures, this instead of the seven NH-signals expected for a single lipopeptide. They corresponded to the 14 spin systems assigned from scalar couplings in DQF-COSY spectra and reported in the TOCSY data of Figure 2. Two glutamate (near $\omega 2 = 9.7$ p.p.m.), two aspartate (near $\omega 2 = 8.8$ p.p.m.), five leucine and five isoleucine residues demonstrated the presence of two heptapeptide moieties. Here, Ile residues were distinguished from Leu residues by the presence of a lower-field shifted βH resonance near $\omega 1 = 1.9$ p.p.m., and by the presence of two well-separated γH and γH′ resonances between $\omega 1 = 1.5$ and 1 p.p.m. The 14 residues were unambiguously located within two heptapeptide sequences, namely S1 and S2, from sequential connectivities in NOESY maps. An illustration is given in Figure 2 where two continuous sequential pathways are depicted from NH(i)-NH($i+1$) connectivities. Thus, S1 is the [Ile4,7]surfactin and S2 is the [Ile2,4,7]surfactin. Interestingly, a substitution at position 2 is observed in S2 for the first time. S1 and S2 were detected in the 1/1 ratio from integration of NH-signals, and their chemical shifts are reported in Table 1.

When compared with the NMR data of surfactin studied in the same experimental conditions, chemical shifts of S1 and S2 were moderately affected. More precisely, NH chemical shifts changed within ±0.1 p.p.m. for the non-substituted residues and within ±0.2 p.p.m. for the substituted residues. Note the exception of residue at position 2 for which the NH resonance was shifted by 0.57 p.p.m. when comparing S2 and surfactin. Comparisons made on $^{3}J_{NH-\alpha H}$ coupling constants did not reveal significant differences at 1.2 Hz resolution. Comparisons were also made on the basis of the network of NOE

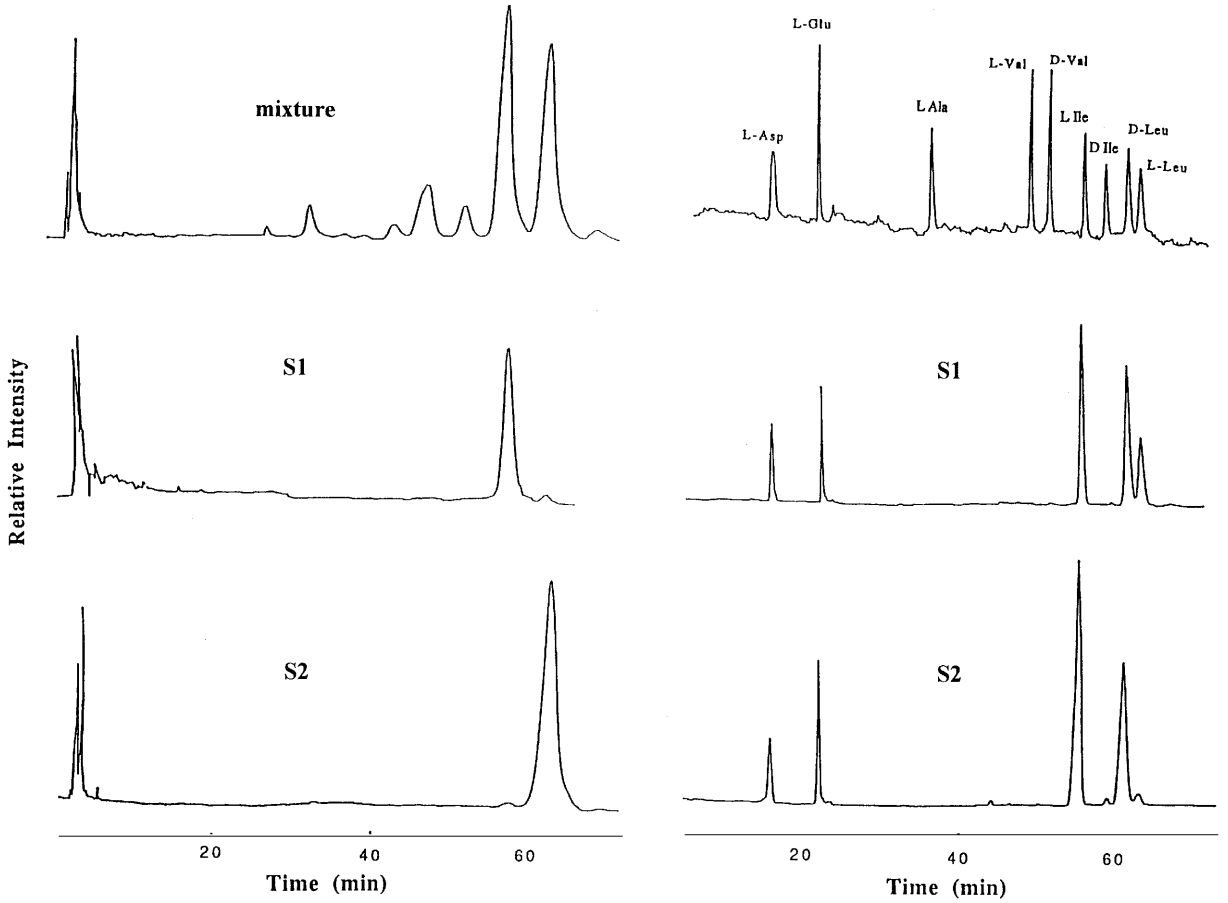

Figure 3 HPLC chromatograms from the purification and analyses of surfactins. Left: the reverse-phase HPLC (on the Lichrosorb column) profile of the surfactant mixture is shown at the top, whereas individual profiles for S1 and S2 are shown below. Right: the HPLC (on the Kromasil column) profile of the mixture of OPTA-AcCys derivatives of standard amino acid enantiomers is shown at the top, whereas profiles for S1 and S2 hydrolysates are shown below.

connectivities. Here, more than 80% of NOEs were found conserved, especially those involving backbone hydrogens. For instance, the sequential NH(i)–NH($i+1$) pathways of Figure 2 are very similar to that observed for surfactin. In addition, connectivities involving NH1 with C^2H, C^2H' or C^3H were found to be similar as well. Concerning medium range connectivities, only the NH3–NH5 and NH5–NH7 connectivities were different from one variant to another.

The HPLC profile of the material revealed two major peaks in a 1/1 ratio, at elution times of 58.3 and 64.5 min (Figure 3). Individual profiles indicate a satisfactory separation of the two lipopeptides S1 and S2. by such a semi-preparative HPLC, S1 and S2 were obtained with a final yield of about 7 mg from 0.5 l of culture medium. In both cases, the lipid moiety is *anteiso*C_{15}. The amino acid composition and chirality, further studied by HPLC, confirmed the NMR evidences: S1 is the [Ile4,7]surfactin since it contains L-Asp, L-Glu, L-Ile, D-Leu and L-Leu in the ratio of 1 : 1 : 2 : 2 : 1, whereas S2 is the [Ile2,4,7]surfactin since the ratio is 1 : 1 : 3 : 2 : 0 (Figure 3).

Properties

Surface tension values for surfactin and for the [Ile4,7] and [Ile2,4,7] variants are printed against the logarithm of each lipopeptide concentration in Figure 4A. Curves show a first break assigned to a premicellization phenomenon occurring in the 10–20 μM range. The second break is characteristic of the CMC. For surfactin, CMC is 0.23 mM and the lowest surface tension, γ_{CMC}, is 37 mN/m. CMC steadily decreases to less than 0.1 mM for the two Ile-rich variants, accompanied with a slight decrease

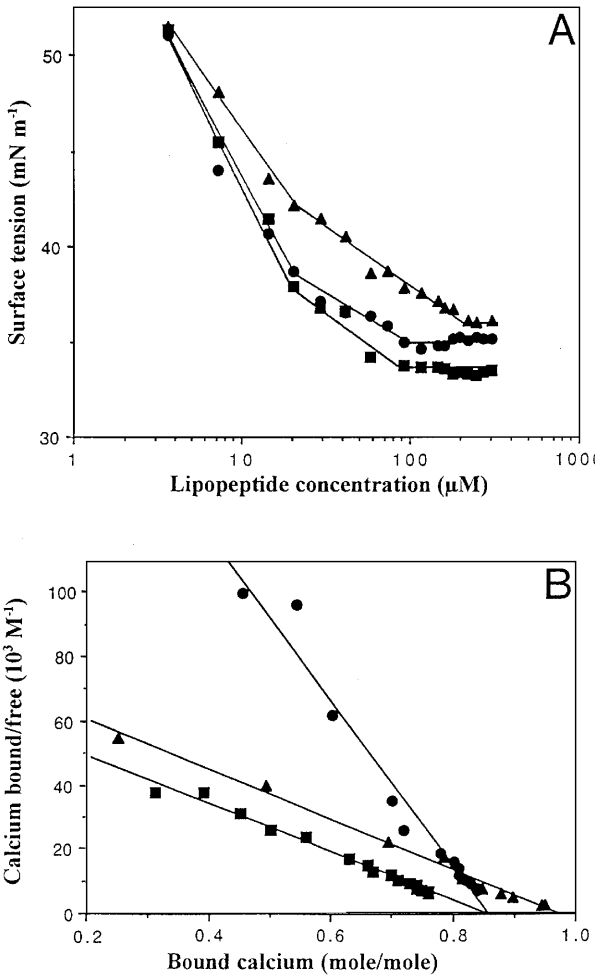

Figure 4 Surface and calcium-binding properties of (▲) surfactin, (■) [Ile4,7]surfactin and (●) [Ile2,4,7]surfactin. (A) Surface tensions of lipopeptide solutions at 25 °C, Tris 5 mM, pH 9.5 (B) Scatchard plots of Ca^{2+} binding by lipopeptides 0.3 mM, Tris 5 mM, pH 9.5. The dissociation constant (K_d) is the inverse ratio of the slope (absolute value) and the molecular bound calcium/lipopeptide ratio (n) is the value at the origin of the vertical axis.

Table 2 Surface and Calcium-binding Properties of Four Surfactins. γ_{CMC} is the Surface Tension Close to CMC and K_d is the Dissociation Constant for the Equimolar Complex with Calcium

Lipopeptide	CMC (mM)	γ_{CMC} (mN m)	K_d (10^{-6} M)
Surfactin	0.23 ± 0.02	37 ± 0.5	11 ± 4
[Ile4]surfactin	0.10 ± 0.01	35 ± 0.5	nd
[Ile4,7]surfactin	0.09 ± 0.01	34 ± 0.5	11 ± 1
[Ile2,4,7]surfactin	0.09 ± 0.01	35 ± 0.5	4 ± 0.2

nd: not measured.

of γ_{CMC}. Calcium-binding properties, monitored by conductimetry measurements at pH 9.5, are reported through Scatchard's representations in Figure 4B. A linear fit was interpreted in terms of a dissociation constant (K_d) and a molecular bound calcium/lipopeptide ratio (n). Close to 1, n indicates that each lipopeptide associates with one calcium. K_d lies in the range of 10^{-5} M for surfactin and [Ile4,7]surfactin, whereas it significantly decreases to 4×10^{-6} M for the [Ile2,4,7] variant (Table 2).

DISCUSSION

Bacillus subtilis produces various typical lipopeptides including the surfactin family [8]. Production and isolation of surfactin variants in suitable amounts to determine their structures and properties have been achieved by using modified culture media. As planned, isoleucyl-containing variants were obtained by using L-isoleucine as the unique nitrogen nutriment. The combination of MS, NMR and fine chromatographic techniques demonstrated that the total surfactant mixture is composed of surfactin (20%), [Val7]surfactin (40%), [Ile4]surfactin (10%), [Ile4,7]surfactin (15%) and [Ile2,4,7]surfactin (15%). It is noteworthy that substitutions were restricted to L-hydrophobic residues. These surfactins, like others studied in our group, have the conserved LLDLLDL heptapeptide chirality and the D chirality at the attachment of the lipid moiety. So far, nine natural heptapeptide moieties have been discovered in this family (Table 3) while the lipid moiety varies in type (*anteiso*, *iso* and *n*) and length (C_{13-15}) because of its biosynthesis mechanism from a starting amino acid [26]. As expected, a majority of β-OH *anteiso*C_{15} have been obtained starting with isoleucine.

Surfactin variants show various activities and both surface and cation-binding activities appeared as relevant parameters. On the one hand, surface activity was monitored by surface tension measurements. A major correlation emerged between CMC values and the hydrophobicity of the fourth residue [17]. It is confirmed in Table 2 where the improved surfactant power is reached for the [Ile4] variant whereas further Leu-to-Ile substitutions at positions 7 and 2 do not cause a more marked decrease of CMC and γ_{CMC} values. On the other hand, the affinity of surfactins for cations was monitored by conductimetric method. Calcium was chosen because it led to the lowest dissociation constant [3] and because biological activities are enhanced by

Table 3 Peptide Sequences of Nine Surfactins from *Bacillus* Species. Only the Heptapeptide Sequences of the Cyclic Moieties are Shown with Linear Representations for Clarity. Substituted Residues are Underlined. Surfactins have the LLDLLDL Chiral Sequences with the Possible Exception of [Gln1]surfacting for which no Data on Chirality have been Reported. A Few Surfactin Variants, Produced from Genetic Modifications and Modified at Position 7, are not Listed [20]

Sequence	Name	Ref.
-Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7-	Surfactin	[6]
-Gln1-Leu2-Leu3-Val4-Asp5-Leu6-Ile7-	[Gln1]surfactin	[19]
-Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Val7-	[Val7]surfactin	[14]
-Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Ile7-	[Ile7]surfactin	[15]
-Glu1-Leu2-Leu3-Ala4-Asp5-Leu6-Leu7-	[Ala4]surfactin	[16]
-Glu1-Leu2-Leu3-Leu4-Asp5-Leu6-Leu7-	[Leu4]surfactin	[17]
-Glu1-Leu2-Leu3-Ile4-Asp5-Leu6-Leu7-	[Ile4]surfactin	[17]
-Glu1-Leu2-Leu3-Ile4-Asp5-Leu6-Ile7-	[Ile4,7]surfactin	This work
-Glu1-Ile2-Leu3-Ile4-Asp5-Leu6-Ile7-	[Ile2,4,7]surfactin	This work

low concentration of divalent cations [27]. The dissociation constant highly depends on the hydrophobic residue at position 2, and even the very conservative Leu-to-Ile substitution significantly reduces its values.

Structure–Activity Relationships

Circular dichroism and NMR data have shown that the 3D structure of surfactin differs when the lipopeptide is dissolved in polar or apolar solvents (chloroform, methanol, pyridine or DMSO). Two quite different models have been proposed, one in DMSO [22, 28] and another one in pyridine [10]. In pyridine, surfactin and [Ile7]surfactin could have different conformations, but only small differences in the properties have been detected. But yet, in DMSO, NMR data show that the backbone fold should be conserved when conservative substitutions occur at positions 2, 4 and 7 (i.e. when one hydrophobic residue is replaced by another one). This is supported by a large data set including chemical shifts, coupling constants and the networks of intra- and inter-residue NOEs involving backbone hydrogens. Obviously, a few NOEs were different in S1 and S2 when compared with surfactin. But these NOEs probably reflect on slight reorientations of side chains, not on drastic changes of the ring topology. Thus, changes in properties resulting from local substitutions provide valuable information to further investigate and validate structure–activity relationships established from the 3D model in DMSO.

The cyclic backbone of surfactin in DMSO has a particular topology related to the sequence of L- and D-conformations (Figure 5). The model exhibits a major apolar side including Leu3, Val4, Leu7 and the lipid chain. At the opposite is a minor polar side where Glu1 and Asp5 are close to Leu2 and Leu6. Especially, Glu1 and Asp5 face each other to constitute a well-suited binding site for divalent cations [22]. Since surface properties increase steadily when increasing the hydrophobicity of residues at positions 4 and 7 (Ala, Val, Leu or Ile), it becomes clear from the 3D model that the hydrophobic character of the apolar domain governs intermolecular hydrophobic interactions. Thus, the very conservative (i.e. same hydrophobicity) Leu-to-Ile substitutions at position 2, 4 or 7 have only small effects on CMC and γ_{CMC}. In contrast, the Leu2-to-Ile2 substitution leads to a significant decrease of the dissociation constant for calcium. This effect is understood from the 3D model as the consequence of a local change in the vicinity of the polar domain. Since Leu and Ile residues differ by the location of a methyl group, the Leu2-to-Ile2 substitution principally affects the steric hindrance of this side chain. More precisely, the substitution should affect either its orientation and/or dynamics, and consequently favour the accessibility of carboxylate groups which constitute the Ca^{2+} binding site.

Surfactins are amphiphilic molecules and their properties are very dependent on the apolar/polar residue distribution. From comprehensive structure–activity relationships, their properties can be rationally improved by selective substitutions. The

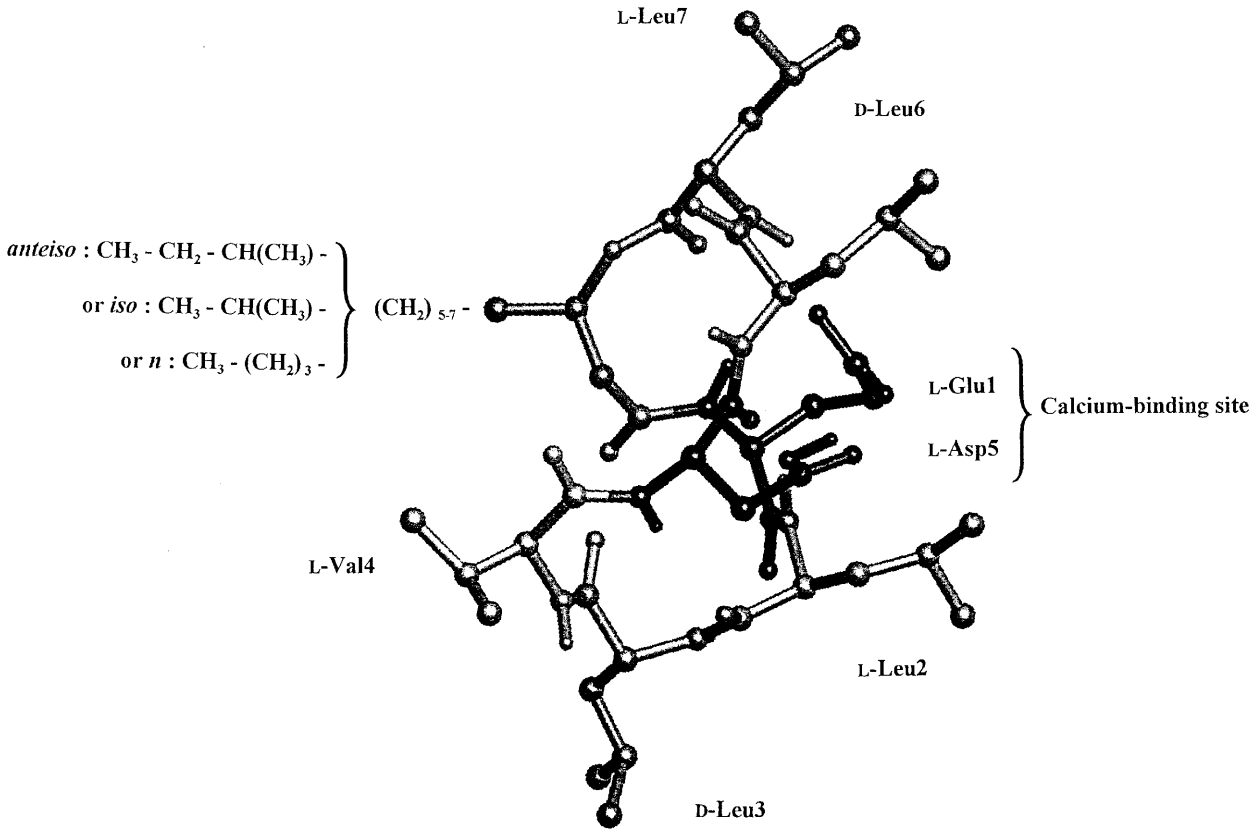

Figure 5 Solution structure of protonated surfactin in DMSO. The model was obtained from NMR data including $^{3}J_{NH-H\alpha}$ coupling constants, NOE-derived distances, NH temperature coefficients and ^{13}C relaxation times. Molecular modelling was achieved by distance geometry and restrained molecular dynamics [22]. On the major apolar side, the extended part of the lipid chain is symbolized depending on its *anteiso*, *iso* or *n* structure. On the minor polar side, Glu1 and Asp5 residues, constituting the calcium-binding site, are shaded.

[Ile2,4,7]variant is the first example for which surface and cation-binding properties are enhanced together, and it should have more applications in industry and biology. Its existence also provides valuable data on the biosynthesis system of surfactins. It is now clear that all the L-leucine binding sites of the surfactin synthetase multi-enzyme complex can also recognize L-isoleucine. In particular, our results confirm those of Galli *et al.* which have shown that each Leu binding site of the three purified subunits can accept, beside Leu, other similar hydrophobic amino acids [29]. Finally, production of other rationally designed variants is already under consideration, although substitution of D-residues remains an open question (e.g. D-leucine inhibits the cell-free biosynthesis [30]) which we will try to resolve in our future projects.

Acknowledgements

Authors dedicate this article to the memory of Prof. Georges Michel for his invaluable contribution in the science of lipopeptides.

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