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Lichenysins G, a Novel Family of Lipopeptide Biosurfactants from *Bacillus licheniformis* IM 1307: Production, Isolation and Structural Evaluation by NMR and Mass Spectrometry

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A series of 9 lactonic lipopeptide biosurfactants was isolated from *Bacillus licheniformis* IM 1307 as representatives of the lichenysin group and we propose to name them lichenysins G. They were recovered from the culture medium as complex mixtures of molecules having different peptide sequences and different structures of β-hydroxy fatty acids. Their separation was achieved by a reversed-phase HPLC method leading to eight well-separated compounds. The complete structure of individual isoforms was proposed following the results of amino acid and fatty acid analysis, LSI-MS and 2D NMR spectroscopies. Compared to surfactin, lichenysins G are at least 10 fold more efficient biosurfactants.

The production of lipopeptide biosurfactants by various strains of *Bacillus licheniformis* has been reported for past few years[1]. Interest in such compounds has increased because of their successful applications for *in situ* microbial oil recovery and dispersion of oil spills[2,3]. Elucidation of their structures has shown that the basal structure is similar to that of surfactin, a well-characterized cyclic lipopeptide isolated from *Bacillus subtilis* and one of the most effective biosurfactant so far known. Surfactin, is a macrolide containing β-hydroxy fatty acids (C_{13} to C_{15}) linked to a heptapeptide moiety with the sequence L-Glu1->L-Leu2->D-Leu3->L-Val4->L-Asp5->D-Leu6->L-Leu7[4]. Biosynthetic capacities of *Bacillus subtilis* have been explored by supplementation of selected nitrogen sources to the culture medium. Thus, numerous variants of surfactin, with modified peptide sequences, have been isolated and structurally characterized. It has been demonstrated that the possible variations that finally result, arise only from the replacement of an L-hydrophobic amino acid by another one at positions 2, 4 and/or 7[5~9]. These results are in agreement with the works of Galli *et al.* which have shown that some binding sites of the subunits of surfactin synthetase multi-enzyme complex can accept various hydrophobic amino acids[10]. Furthermore, these variants provided an important basis for structure-activity relationship study in providing an insight into the role of specific side chains and backbone folding on the surface-activity and Ca^{2+} binding capacity[9].

From *Bacillus licheniformis*, only three compounds have been characterized in details. Biosurfactant JF-2 and an unnamed lipopeptide, initially isolated, were further identified as surfactin and as [Ile7]surfactin, respectively[11~13]. Recently, a new surfactant was isolated from *Bacillus licheniformis* BAS 50 and named lichenysin A with reference to the producing strain while

lichenysins B and C are identified as surfactins again. Lichenysin A was reported to have an L-asparagine residue instead of L-aspartic acid at position 5 of the peptide sequence[14]. In the course of our screening for new microbial biosurfactants, we have found that *Bacillus licheniformis* IM 1307 produced a new compound, structurally different from the aforementioned lichenysin A and that we named lichenysin G. Assuming that its biosynthesis occurs by a mechanism involving the action of a multienzyme complex[15] and on the basis of the data obtained with surfactin, it was possible, by a suitable choice of nitrogen sources of culture medium, to produce a series of isoforms defining a family.

In this paper, we report their isolation and their structure determination. As part of the characterisation studies, their surface activities were also measured.

Materials and Methods

Bacterial Strain and Culture Conditions

Bacillus licheniformis IM 1307 was obtained from Institut Mérieux, Lyon, France. A slant culture was inoculated in brain/heart infusion (bioMérieux). After 24 hours at 32°C, the broth (25 ml) was transferred into a grooved 2 liter-Erlenmeyer containing 500 ml production medium. The cultures were grown in the Landy's medium[16] in a rotary shaker for two days at 32°C. In some experiments the L-glutamic acid of Landy medium was replaced by L-isoleucine at the same concentration (5 g/liter).

Extraction and Total Production of Lipopeptides

Extraction of lipopeptide mixtures from culture was carried out according to the procedure used for surfactin[7]. Briefly, after removal of bacterial cells, the medium was adjusted to pH 2.0 with 6 M HCl, then centrifuged. The pellets were neutralised to pH 7.5 and lyophilised overnight. Lipopeptides were extracted from the powder with $CHCl_3$ - MeOH (2 : 1) then with MeOH.

Crude extracts were analysed by silica gel TLC in solvent system I: $CHCl_3$ - MeOH - H_2O (65 : 25 : 4) or system II: $CHCl_3$ - MeOH - PrOH - EtOAc - 0.25% KCl (25 : 13 : 25 : 25 : 9). Lipopeptides were detected as white spots by spraying water and gentle warming. The spots were scrapped off the gel and eluted with methanol. The absorbance of the eluent was monitored at 214 nm and the surfactant yields were estimated by using calibrated authentic standards.

Isolation by Reversed-phase HPLC

Prior to HPLC step, a solid-phase extraction of the crude extract was performed on a Sep-Pak C_{18} cartridge (5 g) as described by RAZAFINDRALAMBO *et al.*[17]. After washing with H_2O, the active fraction was eluted with MeOH. The eluate was concentrated and then submitted to isocratic analytical reversed-phase HPLC with a Varian apparatus equipped with a RP_{18} 5-Nucleosil column (250 × 4.6 mm). The solvent system consisted of CH_3CN - H_2O (70 : 30), containing 0.01% TFA, at a flow rate of 1 ml/minute. The A_{214} of the eluent was monitored. The various compounds were purified to homogeneity by semi-preparative HPLC on a RP_{18} 7-Lichrosorb column (250 × 15 mm) with the analytical solvent, at a flow rate of 4 ml/minute.

Fatty Acid Analysis

Lipid part obtained after acidic hydrolysis (HCl) was esterified by gaseous diazomethane and the fatty acid methyl esters were analysed by GC/MS. GC was performed on a BP 5 capillary column (25 m × 0.22 mm) with helium as carrier gas and temperature programming from 60°C to 180°C at 30°C/minute and then isotherm at 180°C for 20 minutes. EI-MS was performed at 200°C and 70 eV.

Amino Acid Analysis

After HPLC purification, each lipopeptide was hydrolysed with 6 M HCl at 110°C for 24 hours. The lipid moiety was extracted with $CHCl_3$. The composition and the chirality of amino acids of the peptide moiety were determined according to NIMURA and KINOSHITA[18]. Analyses were carried out on a Kromasil 5 C_{18} column (150 × 4.6 mm). Elution was performed by the mixture of 50 mM sodium acetate pH 5.9 and MeOH in proportions controlled by a microprocessor gradient programm. Amino acids were estimated by comparison with calibrated amino acid enantiomers.

NMR Spectroscopy

Lipopeptides were first dissolved in water pH 11, then solubilized as ionized forms in DMSO-d_6 in the 2 ~ 4 mM concentration range. The solvant was used for the lock and to calibrate chemical shifts with respect to the residual ^{1}H-DMSO signal at 2.49 ppm. ^{1}H-NMR data were acquired on a BRUKER AMX500 spectrometer at temperatures ranging from 292 to 303 K. 1D and 2D data were collected with a slight presaturation of the residual ^1H_2O arising near 3.3 ppm. A set of COSY, TOCSY (spin lock time of 80 ms), ROESY (spin lock

time of 120 ms) and NOESY (mixing time of 250 ms) were recorded with 512 t1 increments, each with 2k data points. 2D data were processed with the UXNMR software to give a final matrix size of 2k × 1k data points.

Mass Spectrometry

LSI-MS mass spectra were recorded on a Kratos MS80 RF instrument which was operated in the liquid secondary-ion mass spectrometry mode using a cesium ion gun at 20 kV. Samples (3 μg) were introduced on a copper probe tip using thioglycerol as matrix. Data were acquired with a DS-90 data system.

Surface Tension Measurement

Surface tension (γ) of individual surfactant was measured at 25°C with a Krüss tensiometer using the ring method. The critical micellar concentration was determined from the γ-*vs* log concentration curve. Samples were dissolved in 5 mM Tris solution at pH 9.5[7].

Results

Isolation and Preliminary Structural Characterization

The production of surfactants was screened on the strain *Bacillus licheniformis* IM 1307 grown in the basal medium of Landy and in a modified medium containing L-isoleucine instead of L-glutamic acid, as nitrogen source. The total surface-active compounds were isolated from the culture supernatants and their yields were estimated after TLC analysis with solvent I; it was about 310 mg/liter in the Landy's medium and 125 mg/liter in the Ile medium.

A preliminary characterization was carried out by TLC analysis with solvent II and with surfactin as reference. Analysis of the Rf values has shown, from the extract of the basal Landy's medium, a single spot, L1, with Rf=0.72 and, from the Ile medium extract, two compounds L2 and L3 with Rf=0.72 and 0.77, in the ratio (4:6), respectively. The Rf values of these compounds in the TLC system employed are thus distinctly different from that of surfactin, Rf=0.66 and this result suggested a marked variation in the basal structure of these compounds.

The molecular masses were determined by LSI-MS. In the mass spectrum of L1, the singly protonated molecular ions of four compounds with relative molecular masses of 993, 1007, 1021 and 1035 were observed. The spectra of fractions L2 and L3 displayed $(M+H)^+$ peaks at *m/z* 1035 and 1049, respectively. The observed mass differences of 14 units between all these components suggested that they were a mixture of closely related lipopeptides differing in one or several $-CH_2$ groups.

Identification of Fatty Acids

Prior to a further separation of the extracts in various isoforms by reversed-phase HPLC, the mixtures of lipopeptides were hydrolysed with 6 M HCl at 110°C for 24 hours. The lipid moieties were transformed to methyl esters which were analysed by GC/MS. They were also identified by comparison with β-hydroxy fatty acids from surfactin[6].

The lipid parts issuing from the culture on the Landy's medium and the Ile medium were resolved into six and two components, respectively (Fig. 1). Minor compounds 1 (6%) and 2 (3%) were identified as *iso* and *anteiso* β-OH C_{13}, compounds 3 (26%) and 4 (5%) were *iso* and *n* β-OH C_{14} and compounds 5 (36%) and 6 (24%) correspond to *iso* and *anteiso* β-OH C_{15} (Fig. 1A). Starting with Ile in the culture medium, the chromatogram of the lipid part revealed a major component 6′ (78%) identified as *anteiso* β-OH C_{15} while component 5′ was *iso* β-OH C_{15} (22%) (Fig. 1B).

Reversed-phase HPLC Analysis of Isoforms

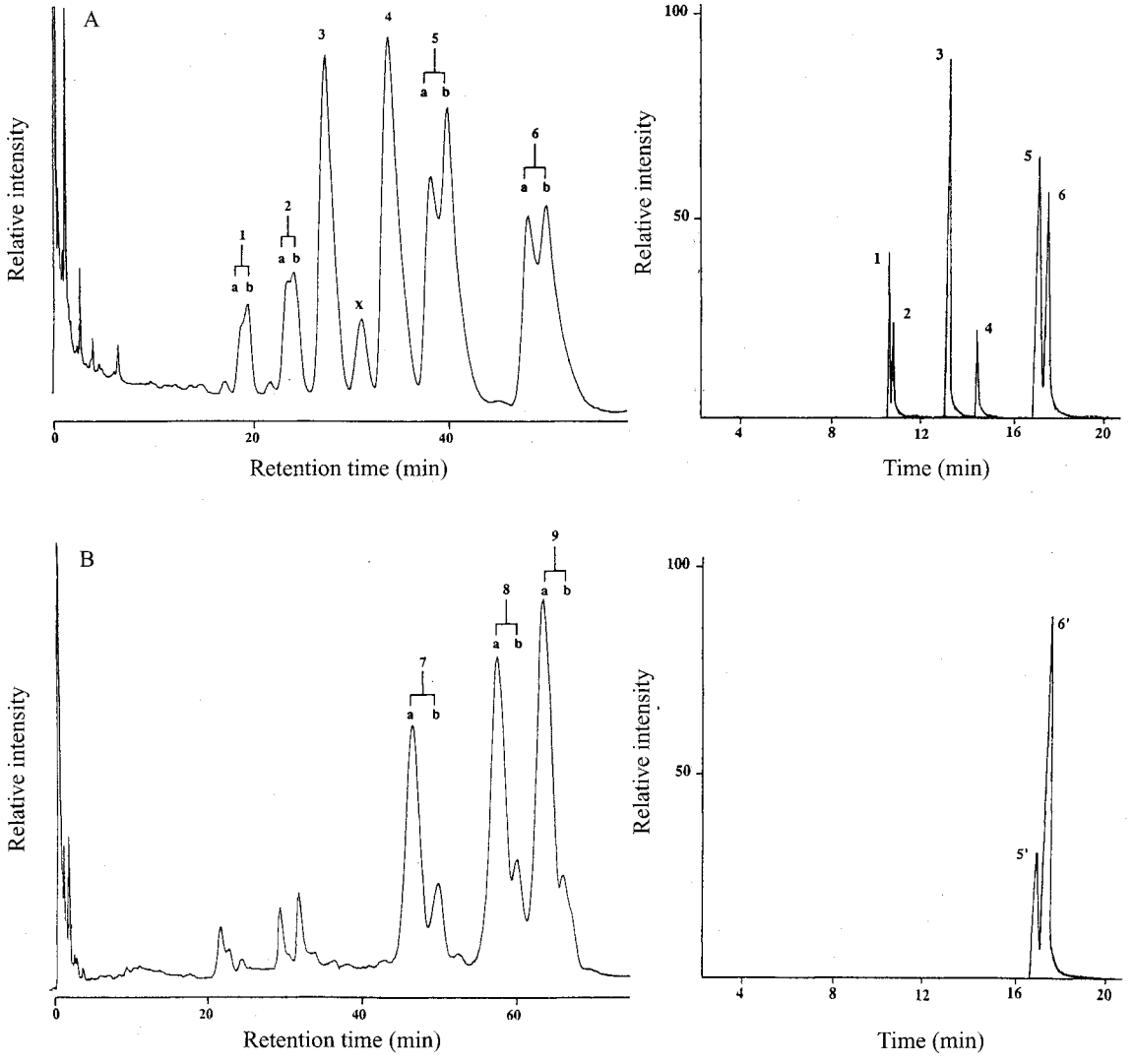
To simply and rapidly separate lichenysin G isoforms we have optimized isolation conditions by semi-preparative isocratic HPLC. This HPLC method allowed us to separate all the constituents of each extract with a purity over 95%.

The HPLC pattern of Landy's medium extract revealed several peaks, designated by 1 to 6 (Fig. 1A) and that of Ile medium extract revealed three major peaks, designated by 7 to 9 (Fig. 1B). Each isoform was purified by semi-preparative HPLC and its amino acid composition was determined (Table 1). The comparison of the amino acid compositions of peptides obtained from Landy's medium revealed the following differences : even peaks, 2, 4, 6, possess a L-isoleucine residue instead of a L-valine residue in odd peaks 1, 3, 5. Identification of the *C*-terminal residues showed that L-isoleucine was the *C*-terminal amino acid of even numbered peaks while L-valine was the *C*-terminal of odd numbered peaks. Thus, the sequences of the two groups of peaks differed by a single L-Ile7/L-Val7 substitution.

In the case of peptides from Ile medium, component 7 has a peptide chain identical with that of 2, 4 and 6. In comparison, component 8 shows a single L-Val4/L-

Fig. 1. Chromatograms from the purification of lichenysins G and analysis of their lipid part.

Left: RP-HPLC profile of homologous isoforms of Landy's medium extract (A) (X: unidentified lipopeptide), and from Ile medium extract (B). Right : Gas chromatograms of the β-hydroxy fatty acid methyl esters after culture in the Landy's medium (A) and after culture in the Ile medium (B): (1) *iso* C_{13}, (2) *anteiso* C_{13}, (3) *iso* C_{14}, (4) *n* C_{14}, (5) and (5'), *iso* C_{15}, (6) and (6') *anteiso* C_{15}.



Ile4 substitution and component 9 shows an additional L-Leu2/L-ILe2 substitution.

Based on the GC data and on amino acid composition of each component, these results suggested, in the Landy's extract, the presence of two compounds with distinct peptide sequences, in addition to homologous lipids (*ai* and iC_{13} in peaks 1 and 2, iC_{14} in peaks 3 and 4, *ai* and iC_{15} in peaks 5 and 6). In the Ile extract, three compounds with distinct peptide sequences and containing the *ai* and *i* β-OH C_{15} as lipid chain were detected. LSI-MS of each isoform supported all these data. The correspondences between HPLC, GC and LSI-MS data are indicated in Table 1.

The HPLC gave rise, therefore, to six lipopeptides from Landy's medium and to three lipopeptides from the Ile medium. As expected, significant differences reside in the amino acid contents and the structure of the acyl groups of these various isoforms, in relation with the structure of the amino acid employed as nitrogen source.

Table 1. Structural characteristics and molecular weights of homologous isoforms.

HPLC peaks	β-OH fatty acids	Amino acid composition: L-Asp	L-Glu	L-Ile	D-Leu	L-Leu	L-Val	C-terminal amino acid	Peptide sequence	Molecular weights (LSIMS) m/z for $[M+H]^+$
1	a aiC_{13} b iC_{13}	1	1	—	2	1	2	L-Val	Gln-Leu2-Leu3-Val4-Asp5-Leu6-**Val7**	993
3	iC_{14}	1	1	—	2	1	2	L-Val		1007
5	a iC_{15} b iC_{15}	1	1	—	2	1	2	L-Val		1021
2	a aiC_{13} b iC_{13}	1	1	1	2	1	1	L-Ile	Gln1-Leu2-Leu3-Val4-Asp5-Leu6-Ile7	1007
4	iC_{14}	1	1	1	2	1	1	L-Ile		1021
6	a aiC_{15} b iC_{15}	1	1	1	2	1	1	L-Ile		1035
7	a aiC_{15} b iC_{15}	1	1	1	2	1	1	L-Ile		1035
8	a aiC_{15} b iC_{15}	1	1	2	2	1	—	L-Ile	Gln1-Leu2-Leu3-**Ile4**-Asp5-Leu6-Ile7	1049
9	a aiC_{15} b iC_{15}	1	1	3	2	—	—	L-Ile	Gln1-**Ile2**-Leu3-**Ile4**-Asp5-Leu6-Ile7	1049

Structure Elucidation

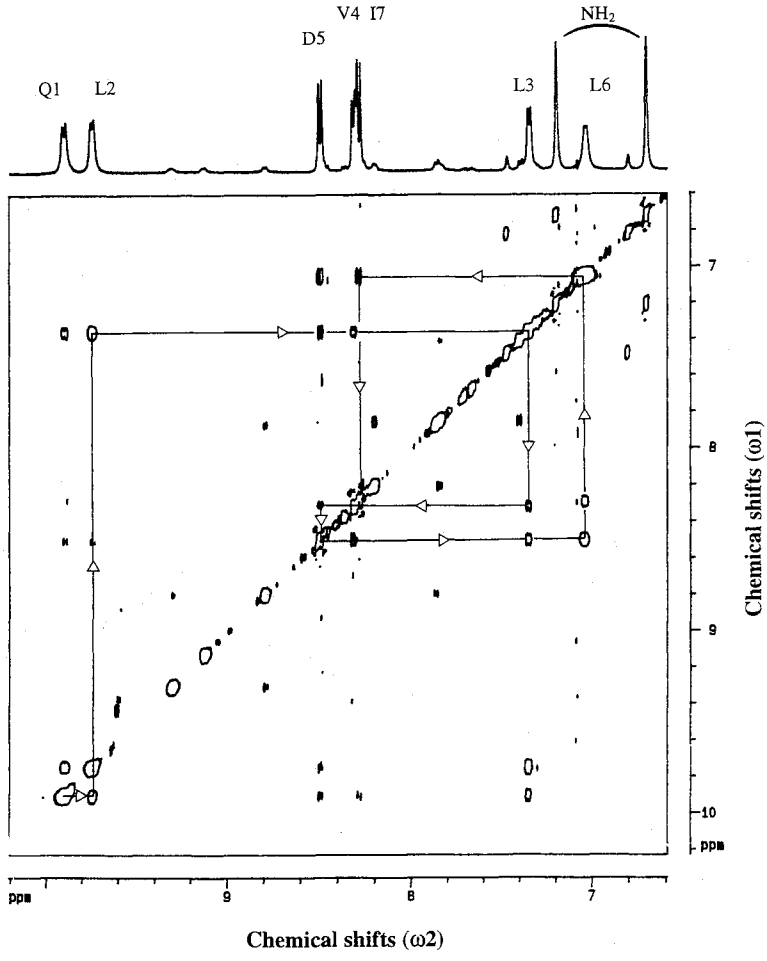
Based on the composition of the peptide moieties and the molecular weights of the molecules, it was suspected that the difference of 1Da, in comparison with surfactin[6], arose from the presence of the amide form of aspartic acid or glutamic acid. The pattern of structural isoforms with mass shifts of 14 Da is due to a mixture of closely related molecules varying both in their lipid moieties and/or in their peptide moieties (see Table 1).

The peptide sequence as well as the lactone position were determined by NMR spectroscopy. Initial structural efforts were focused on the component 7 which was taken as reference. The 6~10 ppm spectral region of the 1D spectrum shown in Fig. 2 first allowed to distinguish nine strong resonances among which seven signals revealed a doublet topology. Then, 2D data demonstrated that the seven doublets corresponded to the NH signals of seven amino acid residues while the two remaining singulets were NεH and NεH′ signals of a $CONH_2$ group. From COSY and TOCSY data, six characteristic spin systems were detected corresponding to one aspartic acid, one isoleucine, one valine and three leucine residues, this in addition to an unique AM(PT)X spin system. The latter was identified to be a glutamine residue and is at the origin of the term lichenysin G. This assignment was confirmed by a set of ROESY crosspeaks involving either NεH or NεH′ protons with αH, βH, βH′, γH and γH′ protons of the AM(PT)X pattern (Fig. 3). Finally, the complete identification and assignment of component 7 was supported by a large set of nOe connectivities including the NH–NH (i, i+1) sequential pathway which is depicted from Gln1 to Ile7 in Fig. 2. Note that such a continuous pathway is somewhat typical of this lipopeptide family[9] and highly suggests that component 7 has a cyclic heptapeptide moiety *via* an ester bond involving the carbonyl group of Ile 7. Actually, this cyclic structure was fully demonstrated by numerous long range nOe connectivities, including connectivities between NH7 and the C^3H, C^2H, C^2H' and NH1 resonances (Fig. 4).

Concerning the other variants, all the sequences were unequivocally determined by the same way (data not shown). As shown in Table 2, NMR spectroscopy allowed particularly to well differentiate the two isomeric amino acids leucine and isoleucine difficult to distinguish by mass spectrometry.

As a result of analysis of all individual chiralities, the

Fig. 2. The NH-NH region of the NOESY spectrum of lichenysin G (component 7) in DMSO-d_6 at 292 K.



A portion of the 1D spectrum is reported at the top with NH assignments (one letter code). In the 2D map, the NH–NH (i, i+1) sequential pathway is depicted by arrows from Gln to Ile7.

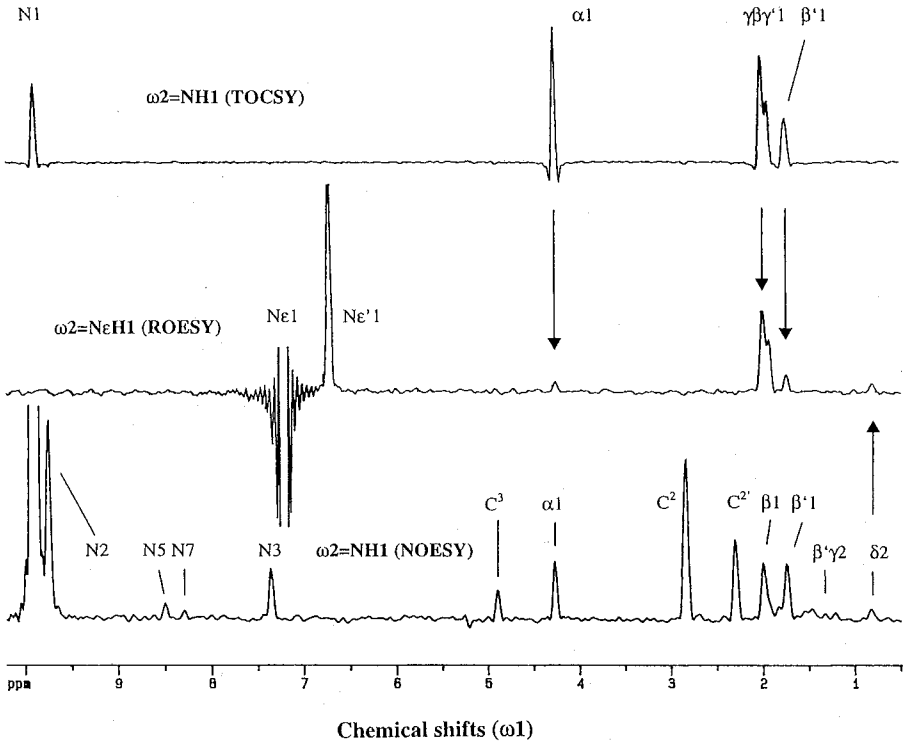
chiral sequence LLDLLDL was deduced (Table 1).

From all these results, the basal structure (peaks 2, 4, 6 and 7) was proposed (Fig. 5). The structure identification of individual isoforms can be interpreted as substitutions at selected positions of the basal peptide sequence. In line with the nomenclature used for surfactin isoforms[6~9] we propose the following names: [Val7]-lichenysin G (peaks 1, 3 and 5), [Ile4]lichenysin G (peak 8) and [Ile2,4]lichenysin G (peak 9) (Table 1).

Surface-activity

Firstly, surface-activity was examined with the basal lichenysin G, in comparison with surfactin. The results are shown in Fig. 6. The amidation of one carboxyl group in this new type of lipopeptide caused substantial modifications in the surface-activity. In fact, a large difference was noted for the critical micellar concentration value which was 220 μM for surfactin and 25 μM for lichenysin G. γ_{CMC} of lichenysin G was 34 mN/m instead of 37 mN/m for surfactin. In addition, the molecular areas, as deduced from the Gibbs equation, significantly differ. The molecular area of lichenysin G decreased to 0.39 nm^2/molecule *vs* 1.01 nm^2/molecule for surfactin. In contrast with surfactin for which Leu to Ile substitutions have led to increased surface-activity, *i.e.* CMC values two fold lower than that of surfactin, no notable difference was observed between the surface-activities of

Fig. 3. 1D slices extracted from TOCSY, ROESY and NOESY data of lichenysin G in DMSO-d_6 at 292 K.



Scalar and dipolar connectivities at the NH1 chemical shift ($\omega 2 = 9.89$ ppm) are labelled at the top and bottom, respectively. ROESY crosspeaks at the NεH1 chemical shift ($\omega 2 = 7.20$ ppm) are indicated by arrows.

lichenysin G variants. Surfactant parameters of all the variants are indicated in Table 3.

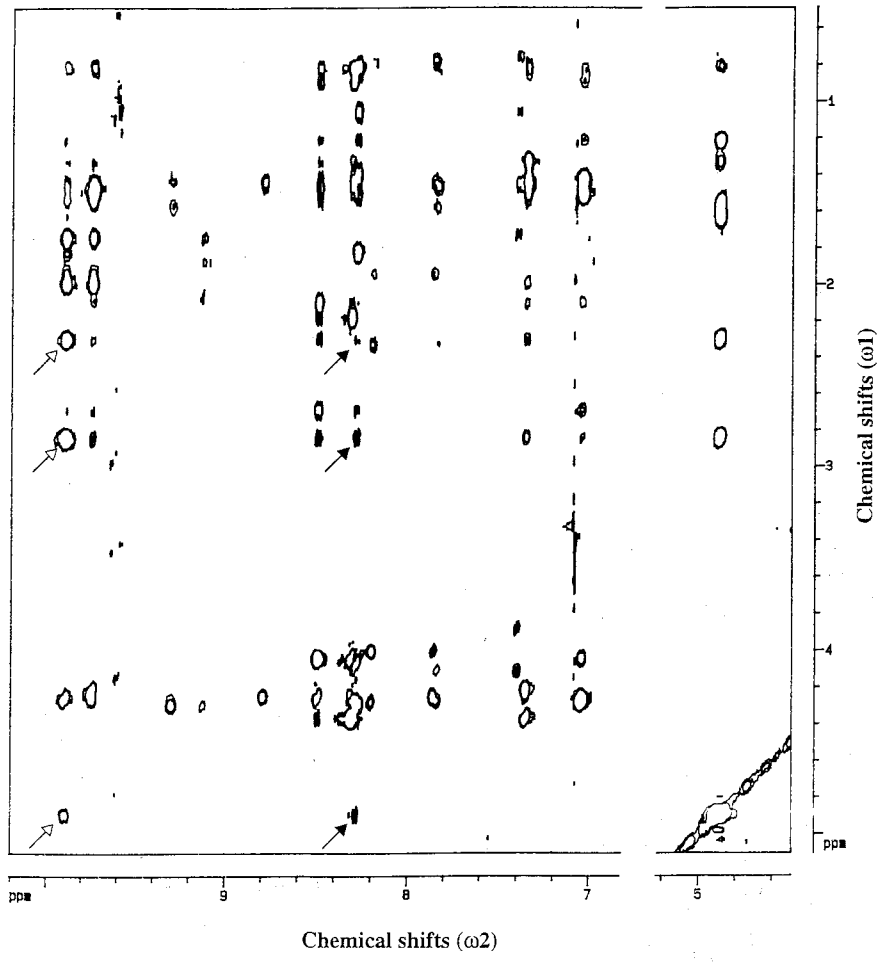
Discussion

Assuming that production of lipopeptides by *Bacillus licheniformis* IM 1307 was under the control of the culture medium, a series of lichenysin G isoforms with a systematic Gln at position 1 was obtained by directed-biosynthesis using L-Glu or L-Ile as nitrogen sources. Numerous variants illustrate the pronounced micro-heterogeneity previously observed for surfactin and more generally for peptides biosynthetized by the poly-enzymic pathway[19,20]. Variants differ by their fatty acid composition, which is a first factor of their property modulation. More interestingly, variants also differ one from each other by amino acids in position 2 (L-Ile/L-Leu substitution), and/or in position 4 (L-Ile/L-Val substitution) or in position 7 (L-Val/L-Ile substitution). These results are similar to those obtained with surfactin for which only the L-hydrophobic amino acids in positions 2 (Leu), 4 (Val) and 7 (Leu) have been replaced by other hydrophobic amino acids, the two D-leucine residues at positions 3 and 6 and the acidic residues at positions 1 and 5 remaining unchange[9,10]. Among the known lipopeptides, lichenysin G reported here structurally resembles halobacillin and isohalobacillin which were isolated from unidentified *Bacillus* sp. strains[21,22]. With reference to the producing strain, we opted for the term lichenysin, previously employed by YAKIMOV *et al.* (1995) to designate a lipopeptide isolated from *Bacillus licheniformis* BAS50 and that possessed a monoanionic structure. Finally, the basic difference between surfactin and lichenysin would turn out to be an amide residue at position 1 (Gln) or 5 (Asn) in lichenysin instead of an acidic one in surfactin. Consequently, lichenysin would become an useful compound for the producing species

Table 2. ^{1}H-NMR chemical shifts of lichenysins G in DMSO-d_6 at 292 K.

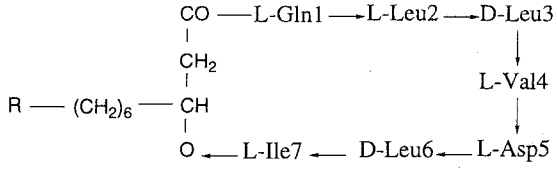
	Residue	NH	αH	βH	γH	γH_3	δH_3	NεH
Lichenysin G	1-L-Gln	9.89	4.24	1.97	1.99			7.20
				1.74	1.92			6.70
	2-L-Leu	9.74	4.19	1.47	1.51		0.82	
				1.45			0.78	
	3-D-Leu	7.36	4.36	1.43	1.39		0.85	
				1.31			0.78	
	4-L-Val	8.31	4.02	2.17		0.89		
						0.81		
	5-L-Asp	8.49	4.24	2.69				
				2.09				
	6-D-Leu	7.04	4.27	1.52	1.50		0.85	
				1.41			0.80	
	7-L-Ile	8.28	4.06	1.82	1.38	0.80	0.76	
					1.05			
	Others	C^2H	C^3H	C^4H	C^5H_2	C^nH_2	CH_3	
		2.83	4.88	1.62	1.32	1.20	0.82	
		2.29		1.54				
[Ile4]lichenysin G	1-L-Gln	9.86	4.25	1.98	2.00			7.19
				1.74	1.93			6.68
	2-L-Leu	9.66	4.21	1.47	1.51		0.82	
				1.43			0.78	
	3-D-Leu	7.39	4.36	1.47	1.44		0.84	
				1.32			0.78	
	4-L-Ile	8.21	4.08	1.98	1.37	0.86	0.78	
					1.09			
	5-L-Asp	8.51	4.24	2.69				
				2.11				
	6-D-Leu	7.02	4.27	1.52	1.51		0.85	
				1.42			0.80	
	7-L-Ile	8.23	4.06	1.81	1.38	0.80	0.76	
					1.05			
	Others	C^2H	C^3H	C^4H	C^5H_2	C^nH_2	CH_3	
		2.87	4.87	1.60	1.31	1.20	0.81	
		2.26		1.54				
[Ile2,Ile4]lichenysin	1-L-Gln	9.95	4.24	1.96	2.03			7.19
				1.76	1.96			6.69
	2-L-Ile	9.04	4.23	1.90	1.27	0.80	0.76	
					1.13			
	3-D-Leu	7.54	4.38	1.47	1.42		0.84	
				1.34			0.78	
	4-L-Ile	8.16	4.05	1.97	1.35	0.85	0.77	
					1.09			
	5-L-Asp	8.54	4.20	2.62				
				2.17				
	6-D-Leu	7.06	4.22	1.54	1.47		0.84	
				1.45			0.79	
	7-L-Ile	8.29	4.07	1.82	1.40	0.79	0.75	
					1.04			
	Others	C^2H	C^3H	C^4H	C^5H_2	C^nH_2	CH_3	
		2.92	4.85	1.60	1.30	1.19	0.81	
		2.16		1.52				

Fig. 4. The NH-side chain portion of the NOESY spectrum of lichenysin G in DMSO-d_6 at 292 K.



Crosspeaks observed between the C^3H proton (4.88 ppm) and the non peptidic part (especially the C^2H at 2.83 ppm and C^2H' at 2.29 ppm) are also reported on the right. Open and filled arrows indicate the crosspeaks involving the C^3H, C^2H and C^2H' protons with the NH1 and NH7 protons, respectively.

Fig. 5. Primary structure of lichenysin G.



R = CH_3–CH(CH_3)–CH_2–, CH_3–CH_2–CH(CH_3)– in the case of *iso* C_{13} or *anteiso* C_{13} β-hydroxy fatty acid, R = CH_3–$(CH_2)_4$–, CH_3–CH(CH_3)–$(CH_2)_2$– in the case of *n* C_{14} or *iso* C_{14} β-hydroxy fatty acid, R = CH_3–CH(CH_3)–$(CH_2)_3$–, CH_3–CH_2–CH–(CH_3)–$(CH_2)_2$– in the case of *iso* C_{15} or *anteiso* C_{15} β-hydroxy fatty acid.

Fig. 6. Tension surface-concentration plots of surfactin$_{15}$ (▲) and lichenysin G_{15} (●). (Surfactin$_{15}$ and lichenysin G_{15} mean that the fatty moiety has 15C atoms.)

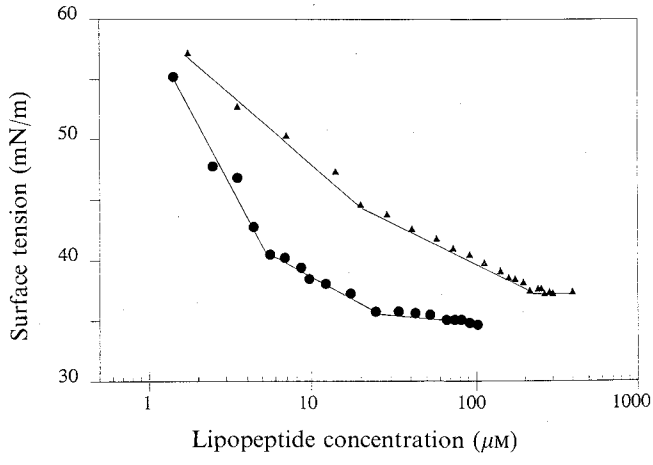


Table 3. Surface-activities of surfactin$_{15}$ and lichenysin G$_{15}$ peptidic variants.

	γ_{CMC} mN/m	CMC μM		γ_{CMC} mN/m	CMC μM
Surfactin	37	220	Lichenysin G	34	25
[Val7]surfactin	38.5	240	[Val7]lichenysin G	34	25
[Ile4]surfactin	35	100	[Ile4]lichenysin G	32	20
[Ile2,4,7]surfactin	35	90	[Ile2,4]lichenysin G	33	20

definition. Presence of an amino acid amide in the lipopeptide biosurfactant BL 86 produced by *B. licheniformis* 86 is consistent with this hypothesis[23]. From the viewpoint of biosynthetic investigation, studies of the relationships between surfactin and lichenysin at the molecular level provide interesting complements to the mechanism of their respective biosynthesis[24].

This is also the first report describing a comparative evaluation between the surface-activities of the two lipopeptides, surfactin and lichenysin G, as well as their peptidic variants (Table 3). The results showed clearly that lichenysins G are more potent surfactants than surfactins, thus emphasizing the predominant role of carboxyl group in the aggregation process. More detailed studies are currently under investigation, especially structure-activity relationships.

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