Surfactin Isoforms from *Bacillus coagulans*

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Bacillus coagulans has been found to produce several surfactins that are powerful lipopeptide surfactants. Four main components with molecular weights 1007, 1021 and 1035 Da were separated. Their structures have been confirmed by spectrometric and spectroscopic studies and by acid hydrolysis. The compounds were found to represent two pairs of surfactin isoforms in which β-hydroxy-*iso*-C_{14} or *anteiso*-C_{15} fatty acids are linked to the [Leu7] or [Val7] heptapeptide moiety by both an amide group and a lactone bond.

Key words: Biosurfactant, Surfactin Isoforms, *Bacillus coagulans*

Introduction

Surfactin, a cyclic lipopeptide, is produced by a large variety of *Bacillus* spp. It is one of the most powerful biosurfactants known and exhibits, together with high surface activity (Cooper *et al.*, 1981), some biological properties, *i.e.*, antibacterial and antifungal activity (Vater, 1986), haemolytic activity (Bernheimer and Avigad, 1970), antitumor activity (Kameda *et al.*, 1974), as well as ionophorous and sequestring properties (Thimon *et al.*, 1993). It was isolated for the first time by Arima *et al.* (1968) and its structure was confirmed by Kakinuma *et al.* (1969) as a cyclic lipopeptide in which a β-hydroxy fatty acid is linked to a sequence of seven α-amino acids: L-Glu–L-Leu–D-Leu–L-Val–L-Asp–D-Leu–L-Leu by an amide group and a lactone bond. In recent years several surfactin isoforms, differing both in heptapeptide moiety and hydrocarbon chain length and structure, have been described and these results have been summarized by Peypoux *et al.* (1999).

Various *Bacillus* species not only produce surfactins but also a number of other lipopeptides such as: iturins (Delcambe *et al.*, 1977), fengycins (Vanittanakom *et al.*, 1986), polymixins (Wilkinson and Lowe, 1964), kurstakins (Hathout *et al.*, 2000) and bacillomycins (Roongsawang *et al.*, 2002). The most powerful biosurfactant is one type of natural surfactin called lichenysin G, produced by *Bacillus licheniformis*, in which the replacement of glutamic acid (in position 1) and leucine (in position 7) of the lipopeptide sequence of surfactin by the glutaminyl and valine residues, respectively, causes at least 10-fold more surface activity when compared to surfactin (Grangemard *et al.*, 1999, 2001).

In this paper we describe the chemical structures of surface-active metabolites from *Bacillus coagulans*, a new surfactins producer, which were reported provisionally as BC1 ÷ BC4 biosurfactants (Huszcza and Burczyk, 2003). Surface properties of individual surfactin isoforms and mixtures of surfactins in comparison with that of commercial surfactin (Sigma) are also described.

Materials and Methods

Microorganism and cultivation conditions

A bacterial strain identified as *Bacillus coagulans* 27 was isolated from oil-contaminated soil (Huszcza, 1995). The biosurfactant production was achieved under cultivation in the following medium (all components per litre of medium): 10 g starch; 9.5 g K_2HPO_4; 1.8 g KH_2PO_4; 2.0 g $NaNO_3$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g KCl; 50 mg $CaCl_2 \cdot 2H_2O$

Abbreviations: LSI-MS, liquid secondary ion mass spectrometry; COSY, correlated spectroscopy; DQF-COSY, double-quantum filtered correlated spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; HMQC, heteronuclear multiple quantum coherence.

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(pH 7.6), in 0.5-l Erlenmeyer flasks with a working volume of 0.1 l at 28 °C on a rotary shaker for 96 h.

Biosurfactants isolation

The cells were removed from the culture by centrifugation. Biosurfactants were precipitated by adjusting the culture medium to pH 2.0 with concentrated HCl, and collected by centrifugation. The precipitate was neutralized with 2 M NaOH and lyophilized to obtain a mixture of biosurfactants designated as "crude product" (Huszcza and Burczyk, 2003; Burczyk and Huszcza, 2001) which was further separated into individual compounds.

Separation procedures

Extraction of the crude product was performed with chloroform/methanol (2:1, v/v) followed by methanol. The extract was subjected to column chromatography on silica gel (kieselgel 60, 230–400 mesh, Merck). Solvents with gradually increased polarity [chloroform < acetone < chloroform/methanol (2:1, v/v) < methanol] were used as eluents. The obtained fractions were analyzed by thin-layer chromatography [TLC, kieselgel 60 F_{254}, Merck, chloroform/methanol/acetic acid 80:15:5 (v/v) as mobile phase] and by reverse-phase high perfomance liquid chromatography (RP-HPLC), using a HPLC chromatograph (Knauer, Germany) with an Ultrasphere ODS, 5 μm (4.6 mm × 25 cm) column (Beckman, USA). The system was operated at a flow rate of 1.0 ml/min with 80% acetonitrile in water (J. T. Baker, Holland). Fractions eluting from the column were detected by their absorbance at 223 nm. Preparative RP-HPLC (HPLC chromatograph Beckman, USA) with a C_{18} Econosil, 10 μm (22 mm × 25 cm) column (Alltech, USA) was used to isolate, after repeated runs, pure homogeneous compounds. The system was operated at a flow rate of 7.0 ml/min with a linear gradient increase from 72–80% acetonitrile in water. Fractions eluting from the column were detected by their absorbance at 223 nm.

Analytical methods

IR spectroscopy was carried out on a Perkin Elmer System 2000 FTIR Spectrometer (Norwalk, CT, USA) in KBr. 2D-NMR measurements: ^{1}H, ^{13}C, DQF-COSY, TOCSY (mixing time 70 ms), ROESY (mixing time 150–240 ms), HMQC and HMQC-TOCSY (mixing time 17 ms) were performed with a Bruker DRX-300 spectrometer at 27 °C. Material from HPLC fractions was dissolved in deuterated acetonitrile, and tetramethylsilane was used as an internal standard. Liquid secondary ion mass spectrometry (LSI-MS) was performed on the Mass Spectrometer AMD-604 with a beam of caesium ions at an accelerating voltage of 8 kV. A mixture of dithioerythritol and dithiothreitol was used as a matrix.

Amino acid hydrolysis

Lipopeptides were hydrolyzed in 6 M HCl at 110 °C for 24 h. The amino acid composition was analyzed with a Mikrotechna type T/339 amino acid analyzer (Praha, Czechoslovakia).

Surface tension measurements

Surface tensions were measured with a Krüss K12e processor tensiometer equipped with a du Nouy Pt–Ir ring. For critical micelle concentration (CMC) determinations, samples were dissolved in 0.1 M $NaHCO_3$. The surface tension concentration data were averages of two independent runs; they were reproducible within ± 0.3 mN/m. The surface tension concentration plots were used to determine CMC. Measurements were taken at (25 ± 0.1) °C at time intervals until no significant change in tension was observed.

Results and Discussion

Isolation and characterization of biosurfactants

Optimization of cell growth of *Bacillus coagulans* led to maximal biosurfactants production with glucose or starch as the organic carbon source at pH 4.0–7.5 and incubation temperature from 20 to 45 °C (Huszcza and Burczyk, 2003; Burczyk and Huszcza, 2001). The metabolites were isolated from the culture broth as described above to obtain a multicomponent mixture designated as "crude product" which yielded a minimal aqueous solution surface tension value of 29 mN/m (Huszcza and Burczyk, 2003). It was extracted with chloroform/methanol (2:1, v/v) followed by methanol, and the obtained, most surface-active fractions were subjected to column chromatography. The fraction obtained with chloroform/methanol (2:1, v/v) as eluent contained surface-active compounds with R_f values in the range from 0.65 to 0.75. This fraction was analyzed by RP-HPLC to show at least 12 components. The main

Table I. ^{1}H chemical shifts (in ppm) of surfactins from *Bacillus coagulans* in acetonitrile at 27 °C (300 MHz, *J* in Hz).

Surfactin residue		BC1	BC2	BC3	BC4
Glu1	Hα	4.11	4.28	4.09	4.30
	Hβ,β'	1.87–2.11	1.54–1.70	1.87–2.11	1.78–2.01
	Hγ,γ'	2.39–2.50	2.35	2.38–2.49	2.38
	HN	7.50 *J* = 7.1	7.50 *J* = 8.8	7.42 *J* = 7.4	7.32 *J* = 8.3
Leu2	H,α	4.49	4.44	4.37	4.46
	Hβ,β',γ	1.50–1.65	1.38–1.68	1.49–1.64	1.50–1.66
	Hδ,δ'	0.86	0.93	0.85	0.93
	HN	7.65 *J* = 9.0	7.56 *J* = 6.3	7.58 *J* = 9.6	7.57 *J* = 8.8
Leu3	Hα	4.23	4.39	4.21	4.41
	Hβ,β',γ	1.57–1.73	1.38–1.68	1.53–1.71	1.60–1.66
	Hδ,δ'	0.94	0.93	0.93	0.95
	HN	7.41 *J* = 7.6	7.58 *J* = 8.9	7.36 *J* = 7.5	7.54 *J* = 8.7
Val4	Hα	3.96	3.96	3.97	3.95
	Hβ	2.24	2.13	2.21	2.15
	Hγ,υ'	0.98	0.93	0.97	0.96
	HN	7.59 *J* = 8.2	7.51 *J* = 7.7	7.48 *J* = 7.5	7.49 *J* = 8.3
Asp4	Hα	4.71	4.64	4.69	4.65
	Hβ	2.77	2.78	2.75	2.83
	Hβ'	2.93	2.87	2.93	2.83
	HN	7.67 *J* = 9.4	7.52 *J* = 9.2	7.64 *J* = 9.6	7.49 *J* = 9.0
Leu6	Hα	4.26	4.44	4.25	4.50
	Hβ,β',γ	1.49–1.86	1.46–1.77	1.42–1.85	1.55–1.75
	Hδ,δ'	0.91	0.92	0.90	0.93
	HN	7.17 *J* = 8.8	7.22 *J* = 9.6	7.11 *J* = 9.2	7.21 *J* = 9.3
Leu7	Hα	4.65	–	4.05	–
	Hβ,β',γ	1.62–1.86	–	1.53–1.85	–
	Hδ,δ'	0.92	–	0.91	–
	HN	7.53 *J* = 9.2	–	7.47 *J* = 8.3	–
Val7	Hα	–	4.19	–	4.18
	Hβ	–	2.16	–	2.18
	Hγ,γ'	–	0.89	–	0.91
	HN	–	7.26 *J* = 9.6	–	7.22 *J* = 9.0
Lipid part	C2H,H′	2.16; 2.41	2.39; 2.64	2.41; 2.61	2.40; 2.64
	C3H	5.31	5.15	5.16	5.16
	C4H,H′	1.35; 1.64	1.64; 1.78	1.29; 1.71	1.66; 1.79
	C*n*H$_2$[a]	1.28–1.44	1.29–1.33	1.28–1.36	1.27–1.35
	C11H$_2$	1.21	1.19	n.d.[b]	n.d.[b]
	C12H	1.54	1.53	1.34	1.35
	C13H,H′	–	–	1.13; 1.34	1.13; 1.34
	C13H$_3$	0.84–0.99	0.85–0.98	–	–
	C14H$_3$	–	–	0.84–0.97	0.84–0.96
	iso C12–CH$_3$	0.84–0.99	0.85–0.98	–	–
	anteiso C12–CH$_3$	–	–	0.84–0.97	0.84–0.96

[a] $n = 5 \div 10$.
[b] n.d., not determined in the present study.

compounds denoted as BC1, BC2, BC3, and BC4 (with retention times 19.8, 22.4, 27.3 and 30.6 min, respectively) constituted about 85% of the analyzed fraction of metabolites or approx. 64 mg/l of the culture broth. They were separated by repeated HPLC runs into pure, homogeneous compounds.

The LSI–MS spectra indicated the MH^+ molecular ions for BC1, BC2, BC3, and BC4, which corresponded to relative molecular masses of the compounds equal to: 1021, 1007, 1035, and 1021, respectively. The IR spectrum of a mixture of this surfactins showed strong bands at 1539, 1649, and 3311 cm^{-1} indicating the presence of a peptide component, whereas the bands at 2872 to 2960 cm^{-1} and 1369 to 1468 cm^{-1} suggested the presence of an aliphatic chain. Amino acid hydrolysis of the peptide fragment indicated the pres-

Table II. ^{13}C chemical shifts (in ppm) of surfactins from *Bacillus coagulans* in acetonitrile at 27 °C (75 MHz).

Surfactin residue		BC1	BC2	BC3	BC4
Glu1	Cα	54.79	53.15	54.83	53.17
	Cβ	26.76	26.65	26.74	26.61
	Cγ	30.10	30.20	30.05	30.06
Leu2	Cα	51.41	52.10	51.37	52.10
	Cβ	39.06	40.01–40.58	39.0–41.46	39.99–40.46
	Cγ	24.70–24.94	24.78–24.95	24.70–24.92	24.76–24.98
Leu3	Cα	53.49	52.65	53.54	52.63
	Cβ	39.82	40.01–40.58	39.0–41.46	39.99–40.46
	Cγ	24.70–24.94	24.78–24.95	24.70–24.92	24.76–24.98
Val4	Cα	61.02	60.98	61.04	61.12
	Cβ	29.45	29.70	29.38	29.61
Asp5	Cα	50.40	50.31	50.43	50.24
	Cβ	35.88	36.03	35.83	36.06
Leu6	Cα	52.51	52.46	52.47	52.47
	Cβ	41.44	40.01–40.58	39.0–41.46	39.99–40.46
	Cγ	24.70–24.94	24.78–24.95	24.70–24.92	24.76–24.98
Leu7	Cα	52.02	–	52.05	–
	Cβ	39.66	–	39.0–41.46	–
	Cγ	24.70–24.94	–	24.70–24.92	–
Val7	Cα	–	57.85	–	57.81
	Cβ	–	31.08	–	31.09
Lipid part	C2	42.47	41.02	42.45	41.04
	C3	71.99	71.99	71.99	72.83
	C4	34.83	34.24	34.80	34.24
	Cn[a]	25.14–29.95	25.20–29.99	25.12–29.78	24.92–30.13
	C11	39.18	39.16	n.d.[b]	n.d.[b]
	C12	28.96	28.13	34.56	34.64
	C13	16.93–23.18	n.d.[b]	36.75	36.49
	C14	–	–	n.d. [b]	11.09
	iso C12–CH_3	16.93–23.18	n.d.[b]	–	–
	anteiso C12–CH_3	–	–	n.d. [b]	18.95

[a] $n = 5 \div 10$.
[b] n.d., not determined in the present study.

ence of four different amino acids: Asp : Glu : Val : Leu in proportion 1:1:1:4 in the biosurfactants BC1 and BC3, and 1:1:2:3 in the biosurfactants BC2 and BC4.

Amino acid sequence in peptide moieties

Both the amino acid composition and sequence were confirmed by two-dimensional NMR spectroscopy. The complete amino acid spin systems were identified from a TOCSY spectrum from the correlation between amide protons and protons bound to Cα and side chain carbon atoms of amino acids (Table I). Five spin systems that exhibited typical methyl resonances near 0.9 ppm were assigned to four leucine and one valine residues in BC1 and BC3, and to three leucine and two valine residues in BC2 and BC4. Sequences of amino acids in biosurfactants were determined from ROESY spectra where correlations between the amide proton of amino acid i and the α-proton of amino acid $i+1$ were observed. Strong interresidue connectivities in the ROESY spectra corresponding to Leu7 and Leu6 for BC1 and BC3, and Val7 and Leu6 for BC2 and BC4 demonstrated that the valine residue instead of a leucine residue occupied the seventh position in BC2 and BC4. Finally, the amino acid sequence in surfactins BC1 and BC3 was determined as Glu-Leu-Leu-Val-Asp-Leu-Leu, and in surfactins BC2 and BC4 as Glu-Leu-Leu-Val-Asp-Leu-Val. The ^{13}C NMR signals which corresponded to all amino acids could be assigned by a HMQC spectrum (Table II).

Table III. Structures of biosurfactants produced by *Bacillus coagulans*.

Biosurfactant	M_W [Da]	M.p.[a] [°C]	Structure	Designation of surfactin
BC1	1021	137–139	$CH_3CH(CH_2)_8CHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Leu (CH3 branch; lactone O)	*iso*-C_{14}
BC2	1007	133–135	$CH_3CH(CH_2)_8CHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Val (CH3 branch; lactone O)	*iso*-C_{14}[Val7]
BC3	1035	134–136	$CH_3CH_2CH(CH_2)_8CHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Leu (CH3 branch; lactone O)	*anteiso*-C_{15}
BC4	1021	131–133	$CH_3CH_2CH(CH_2)_8CHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Val (CH3 branch; lactone O)	*anteiso*-C_{15} [Val7]

[a] Melting points are uncorrected.

Structure of fatty acids residues

On the basis of the composition of the peptide moiety and the molecular weight of the molecules, the lipid chain of surfactins was determined to be a C_{14} fatty acid in BC1 and BC2 and a C_{15} fatty acid in BC3 and BC4. The TOCSY and HMQC spectra allowed to identify the structure of the β-hydroxy fatty acid residues in the biosurfactants. In the HMQC spectra of BC1 ÷ BC4, a cross peak between the carbonyl carbon atom in a fatty acid and the amide protons of Glu1 were observed. A low-field signal at δ 5.15 to 5.31 consistent with C**H**–O of the alcohol moiety of an ester was observed (Table I). In surfactins BC1 and BC2 the fatty acid chain was isobranched and in BC3 and BC4 it was anteisobranched. The ^{13}C signals of the branching CH groups were easily identified at ca. 28 ppm for the *iso* chain type, and ca. 35 ppm for the *anteiso* chain type. The *iso* C12–CH_3 and C13 peaks in BC1 were assigned at 16.93–23.18 ppm. The *anteiso* C12–CH_3 and C14H_3 peaks in BC4 were assigned at 18.95 ppm and at 11.09 ppm, respectively (Table II). Similar signals in BC2 and BC3 could not be unambiguously assigned. The ^{13}C signals of the branching CH group in the fatty acid moiety, however, are easily identified at 28.96 ppm in BC1, 28.13 ppm in BC2, 34.56 ppm in BC3 and 34.64 ppm in BC4. The presence of a lactone ring in the surfactants was identified by the IR and NMR spectra, which indicated ester carbonyl groups.

Natural surfactin is a mixture of structurally similar β-hydroxy C_{13} to C_{16} fatty acid components with *iso*-C_{13}, *anteiso*-C_{13}, *n*-C_{14}, *iso*-C_{14}, *iso*-C_{15}, and *anteiso*-C_{15} branching structures (Besson *et al.*, 1992; Oka *et al.*, 1993; Yakimov *et al.*, 1995).

The presented results show that the bacterium *Bacillus coagulans* is a new producer of known surfactin isoforms: the [Leu7] surfactin described first by Arima *et al.* (1968) and the [Val7] surfactin found by Peypoux *et al.* (1991). Their hydrocarbon chain structures together with molecular masses and melting points are collected in Table III.

Surface properties

The equilibrium surface tensions, γ, of 0.1 M $NaHCO_3$ solutions of four surfactin isoform samples were attained after one hour on an average. Measurements were done at 25 °C and the results were plotted against the logarithm of concentration as shown in Fig. 1. The samples under consideration were: surfactin BC1 (> 99% purity), the mixture of isoforms BC1 and BC2 (2:1 ratio by weight), the mixture that contained 29% BC1, 15% BC2, 25.5% BC3, 13% BC4 and 17.5% of unidentified compounds ("purified product" in Fig. 1), and commercial surfactin. The concentrations near the breaking point of the isoterms were

Table IV. Surface properties of lipopeptides in 0.1 M $NaHCO_3$ at 25 °C.

Biosurfactant	CMC [mg/l]	γ_{CMC} [mN/m]
BC1	17.3 17.6[a]	27.4
BC1 + BC2	18.8 19.1[a,b]	26.7
Purified product	6.79	27.8
Commercial surfactin	10.19	26.6

[a] CMC in $10^6 \times$ mol/l.
[b] Mean molecular weight was calculated on the ground of percentage composition of surfactants mixtures.

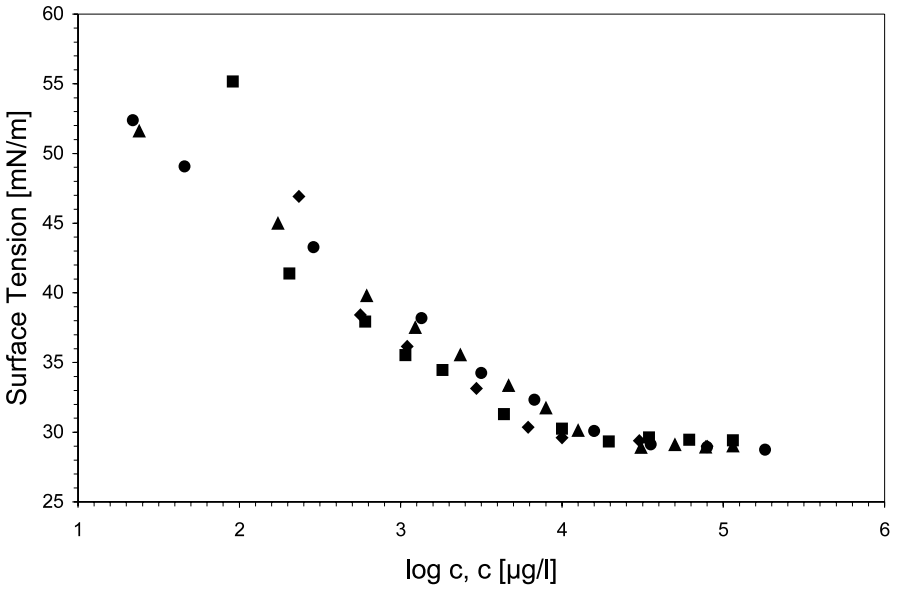

Fig. 1. Surface tension *vs.* concentration (log *c*) isotherms of surfactins: (●), BC1; (▲), BC1 + BC2; (◆), purified product; (■), commercial surfactin at 25 °C.

taken as critical micelle concentration, CMC. The CMC values together with the surface tensions near CMC, γ_{CMC}, are collected in Table IV. The CMC obtained for BC1 (surfactin) was somewhat higher than those reported in the literature for surfactins with normal hydrocarbon chain (Thimon *et al.*, 1992). This is in accord with the finding of Yakimov *et al.* (1996) who found a surface activity order in relation to hydrocarbon chain structure: *normal* > *iso* > *anteiso*. Moreover, no substantial differences occurred between the values obtained for BC1 and for the mixture of surfactins BC1 and BC2, which differ from each other in the amino acid structure at position 7 of the peptide ring, *i.e.*, Leu in BC1 *versus* Val in BC2. This observation is consistent with the finding of other authors (Bonmatin *et al.*, 1995). On the other hand, the CMC value determined for the purified product is markedly lower than that obtained for pure surfactin (BC1). This may be either due to the much higher surface activity of the unidentified compounds present in the sample or due to a synergistic effect observed when dealing with surfactants' mixtures (Rosen, 1988). It is worth mentioning that the CMC value of the purified product mixture of lipopeptides produced by *Bacillus coagulans* is near that of commercial surfactin supplied by Sigma.

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