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# Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization

**Katharina Jenny**[1], **Othmar Käppeli**[2], **and Armin Fiechter**[1]

[1] Institut für Biotechnologie, ETH-Hönggerberg, CH-8093 Zürich, Switzerland
[2] Bideco AG, Im Schörli, CH-8600 Dübendorf, Switzerland

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**Summary.** The surface-active compounds of the strain *Bacillus licheniformis* were isolated and their structure was elucidated. The high surface-active capacity of the crude extract was basically due to traces of long-chain saturated fatty acids, especially of palmitic and stearic acids, to a mixture of small amounts of hydrocarbons with chain lengths of 20 and 22 carbons, and mainly to appreciable amounts of four slightly different lipopeptides. The lipopeptides were found to be a mixture of four closely related compounds. The lipophilic part consisting of *i*-, *n*-$C_{14}$ or *i*-, *ai*-$C_{15}$ $\beta$-OH fatty acids was linked to the hydrophilic peptide moiety, which contained seven amino acids (Glu, Asp, Val, three Leu and Ile) by a lactone linkage. Fifteen milligrams per litre of the purified lipopeptide product decreased the surface tension of water from 72 mN $m^{-1}$ to 27 mN $m^{-1}$, characterizing the product as a powerful surface-active agent that compares favourably to other (bio)surfactants. Antibiotic activity was demonstrated against bacteria and yeasts.

## Introduction

The interest in surface-active agents has increased considerably in recent years (Layman 1985). Besides chemical surfactants, biosurfactants represent a promising group for attempts to enlarge the present range of the product palette for different uses. Most of the biologically produced tensides show outstanding advantages, such as biodegradability and low toxicity, as compared to common synthetic surfactants. Successful applications of biosurfactants were reported for in-situ microbial enhanced oil recovery (Donaldson and Clark 1982; Shennan and Levi 1987), for the handling of oil spills (Gutnick and Rosenberg 1977; Harvey et al. 1990), for the use as food additives (Kudo 1988), for the applications as emulsifiers in agricultural systems (Kachholz and Schlingmann 1987) and in cosmetics (Inoue 1988). Accordingly, the screening for new microbial tensides has intensified. They will become important industrial chemicals as yields of biosurfactants in production processes are improved and the potential for the substitution of existing synthetic surfactants is further evaluated.

Many types of surface-active agents are synthesized by a wide variety of microorganisms. Mostly they exhibit the typical amphiphilic character of lipids but they are generally extracellular. Basically there are six major classes of biosurfactants: glycolipids, lipopeptides/lipoproteins, phospholipids, neutral lipids, substituted fatty acids and lipopolysaccharides. Structure, function and the physiological role of these biological surface-active agents have already been described in several reviews (Cooper and Zajic 1980; Zajic and Seffens 1983; Zajic and Mahomedy 1984; Haferburg et al. 1986; Lang and Wagner 1987).

The most often isolated and most thoroughly studied biosurfactants are the structural homogenous glycolipids, e.g. sophorose lipids, rhamno lipids and trehalose lipids. However, the group of lipopeptides/lipoproteins presents a heterogenous class of biologically active peptides. Most of them are known also to possess antibiotic activity (Umezawa et al. 1978). Surfactin, a cyclic lipopeptide produced by *Bacillus subtilis* is the most effective biosurfactant discovered so far (Cooper et al. 1981a). Only 20 mg $l^{-1}$ of the purified product reduced the surface tension of water from 72 to 27 mN $m^{-1}$. The production of biosurfactants of *B. licheniformis* has been reported under aerobic as well as under anaerobic conditions in batch cultivations (Javaheri et al. 1985; Jenneman et al. 1983; McInerney et al. 1990). However, the structure and properties of the surface-active agents as well as their physiological role remain unclear.

In the present work isolation and structural analysis of different surface-active compounds produced by *B. licheniformis* are reported. The physiology of production in batch and continuous culture will be addressed in a subsequent report.

*Offprint requests to:* O. Käppeli

## Materials and methods

*Microorganism and cultivation conditions.* A strain of *B. licheniformis* was used throughout this work. It was isolated at this institute from soil samples and maintained in frozen glycerol culture at −70° C. The strain was cultivated in a 2-l compact loop bioreactor (MBR, Wetzikon, Switzerland). The reactor was operated under the following conditions: working volume, 1.7 l; temperature, 37° C; stirrer speed, 1200 rpm and pH control at 7.0 with 1 M NaOH. The biosurfactant production was achieved under continuous cultivation in the following minimal medium (all components per litre of medium): 10 g glucose; 2.8 g $NaNO_3$; 0.5 g KCl; 0.2 g $MgSO_4 \cdot 7H_2O$; 2 ml of 84% $H_3PO_4$; 0,03 g $CaCl_2 \cdot 2H_2O$; 200 mg EDTA; 0.8 mg $ZnSO_4 \cdot 7H_2O$; 0.2 mg $MnSO_4 \cdot H_2O$; 60 µg $H_3BO_3$; 20 µg $Na_2MoO_4 \cdot 2H_2O$; 0.1 mg $CuSO_4 \cdot 5H_2O$; 0.1 mg $CoCl_2 \cdot 6H_2O$; 0.3 mg $FeSO_4 \cdot 7H_2O$.

*Isolation.* From the spent culture liquid the cells were separated and the surface-active compounds were subsequently precipitated by adding concentrated HCl. The precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) before loading in the same buffer on an Amberlite XAD-7 column (acrylester resin; Rohm and Haas, Philadelphia, Pa., USA). The adsorption of the active compounds was assayed by measuring the surface tension at the column outlet. The column was washed with distilled water (2–3 bed volumes) and the surface active compounds were eluted with methanol. Surface activity was monitored in each fraction by determination of the surface tension. The active fractions were pooled, lyophilised and washed by the liquid-liquid partition method described by Folch et al. (1957) in a $CHCl_3$-$CH_3OH$-$H_2O$ (8:4:3; v/v/v) mixture for removing non-lipid contaminants. The solvent in the lipid-containing lower phase was removed under reduced pressure and the yellowish crude material was collected for further purification by different chromatographic procedures.

*Adsorption chromatography on silica gel.* Separation of the surface-active compounds was carried out on a silica gel column (2.6 × 46 cm) on a semi-preparative scale with solvents of gradually increasing polarity
($CHCl_3 > CH_2COCH_3 > CHCl_3$—$CH_3OH$ [2:1] > $CH_3OH$)
(Rouser et al. 1976).

*Ion exchange chromatography.* Further purification of the surface-active compounds was achieved by ion exchange chromatography on DEAE-Sepharose CL 6B (Pharmacia, Uppsala, Sweden). The column (0.8 × 15 cm) was equilibrated with 0.01 M TRIS-HCl buffer (pH 7.0) containing 20% (v/v) ethanol and the sample was loaded in the same buffer. The lipopeptides were eluted by a step gradient from 0–1 M NaCl and monitored by spectral analysis in the range 400–190 nm.

*High pressure liquid chromatography (HPLC).* The purified compounds were separated by reversed phase HPLC with a Bruker LC 41 D/CD system (Bruker-Franzen Analytik, Karlsruhe, FRG) on a ODS-Hypersil (0.46 × 25 cm) column. The system was operated at a flow rate of 1.1 ml $min^{-1}$ with a two-step gradient of solvents: solvent 1, 0.1% trifluoroacetic acid (TFA)-0.1% TFA in 80% $CH_3CN$ (2:3); solvent 2, 0.1% TFA in 80% $CH_3CN$.

*Thin-layer chromatography (TLC).* The efficiency of each purification step was analysed by TLC (Si 60 $F_{254}$, 0.25 mm, Merck, Darmstadt, FRG; solvent system: $CHCl_3$—$CH_3OH$—$CH_3COOH$, 80:15:5). Detection was effected with a lipid specific reagent 1,6-diphenyl-2,3,5-hexatriene (DPH; Sigma, München, FRG). The activity of each spot was measured by determining the surface tension of a methanol extract of the scraped-off silica material.

*Fatty acid analysis.* Fatty acids were released from the lipopeptide with 0.5 M HCl in $CH_3CN$—$H_2O$ (9:1) at 105° C for 45 min (Aveldano and Horrocks 1983) and esterified using 5% methanolic HCl (Christie 1982) or diazomethane. The fatty acids were separated on polar and non-polar capillary columns (BP-1, BP-20, 25 m × 0.25 µm, Scientific Glass Engineering, Milton Keynes, UK, and SE 30, 25 m × 0.20–0.25 µm, Mega Glass Capillary Laboratory, Milan, Italy) using two different gas chromatographs (GC; Pye Unicam and HP 5790 A) both equipped with a flame ionization detector and $H_2$ as carrier gas (30 ml $min^{-1}$). As reference standards a bacterial fatty acids methyl ester mixture (Supelco, Bellefonte, Pa., USA) was used. Combined GC/mass spectrometry (MS) was carried out on a Finnigan (San Jose, CA, USA) 1020 B instrument. The subsequent spectra were interpreted according to McCloscey (1970) and Asselineau and Asselineau (1984). Picolinyl esters of the dehydrated-hydrogenated fatty acids (Harvey 1982) were used to confirm the *iso* and *anteiso* branching. Dehydration was achieved by hydrolysis in 6 M HCl at 105° C for 20 h. Hydrogenation was performed in methanol with palladium on charcoal under a hydrogen atmosphere.

*Amino acid analysis.* The amino acid composition of the hydrophilic part was determined on an amino acid analyser (Biotronic LC 600, Maintal, FRG, Mitsubishi CK 10S Quarz, Tokyo, Japan) after total hydrolysis of the lipopeptide in 6 M HCl at 105° C for 24 h.

*Amino acid sequence analysis.* The lipopeptide was partially hydrolysed in 12 N HCl—$CH_3COOH$ (2:1) at 60° C for 7 h. The resulting peptide fragments were separated and isolated on HPLC for further sequence analyses on an amino acid sequence analyser (Applied Biosystems, Foster City, CA, USA, 470 A or 477 A with on-line phenylthiohydantoin amino acid detection model 120 A, Applied Biosystems). Identification of the amino acids was carried out using the original chemicals according to the manufacturer's manual.

*Infrared spectroscopy (IR).* IR spectroscopy was carried out on a Perkin-Elmer (Überlingen, FRG) 580 B instrument in KBr.

*Combined MS.* The structure of the underivatived molecule was verified by Laser Desorption/Fourier Transform Mass Spectrometry LD/FTMS (Nicolet FTMS-2000, Madison, USA). Further confirmation of the structure was achieved by *O,N*-permethylation of the lipopeptides (Thomas and Ito 1969) after the lactone ring was cleaved with 10% NaOH in ethanol at room temperature for 18 h. The subsequent derivatives were analysed by fast atom bombardment (FAB)/MS (ZAB-VSEQ, $Cs^+$-atom beam 2 µA emission, 50 kV accelerating voltage) in a 3-NOBA matrix. Positive ions were detected. The molecular weight was determined by both methods.

*Enzymatic hydrolysis.* Digestion with carboxypeptidase Y (Boehringer, Mannheim, FRG) was modified after Segré et al. (1989) in 0.1 M ammonium bicarbonate-20% ethanol buffer (pH 8.0) at 37° C for 1–20 h at an enzyme/substrate ratio of 1:30.

*Surface activity assay.* The surface tension (ST) and the interfacial tension (IT) were determined with a ring-tensiometer (Krüss Digital-Tensiometer 10, Hamburg, FRG). For IT measurements, a mixture of aliphatic hydrocarbons ranging from tetradecane to octadecane was used as the second liquid phase. The biosurfactant concentration was estimated by multiplying the known critical micelle concentration (CMC) (15 mg $l^{-1}$) with the dilution factor necessary to apply to a particular solution to yield IT values above minimal. The CMC value is defined at that point where surface-active compounds no longer aggregate to form micelles and consequently the IT values start to increase.

*Antibiotic assay.* Antibiotic activity of the purified lipopeptide was tested against 18 microorganisms with the agar diffusion method (Lancini and Parenti 1982). A filter-paper disc soaked with the antibiotic solution in methanol (about 20 µl) was assayed on the surface of an agar medium containing a soft agar culture of the tested microorganisms. Growth inhibition was measured by different diameters of the halos around the paper discs.

*pH and temperature stability.* The ST and IT values of biosurfactant solutions were measured in 0.1 M potassium phosphate buffer at the pH range 3–12 and at 25–60° C.

## Results

The surface-active compounds were isolated from the cell-free culture supernatant of *B. licheniformis* growing continuously at different dilution rates. The ST and IT values of the spent culture liquid, $<27$ m $Nm^{-1}$ and $<0.1$ m $Nm^{-1}$, respectively, indicated conditions of good biosurfactant production, where the product could be collected.

### *Surface-active compounds from the spent medium of B. licheniformis cultures*

The surface-active compounds were isolated from the culture supernatant of continuously growing cells as described in Materials and methods. The step-by-step purification of the raw material (HCl precipitate) to the so-called crude product by using XAD-7 Amberlite column chromatography and liquid-liquid partitioning resulted in a enrichment of the active compounds. A CMC value based on dry matter of up to 400 mg $l^{-1}$ was determined after this purification procedure. Adsorption chromatography of the crude product on a silica gel column by stepwise elution using solvents of increasing polarities resulted in a separation of at least three active products which exhibited distinctly different $R_f$ values (Table 1).

Fraction 1 was directly analysed by GC/MS without any prior treatment. The resulting MS spectra showed the typical signals of long-chain hydrocarbons with the base peak at m/e 57 ($C_4H_9^+$) and a number of fragment ions at m/e 71, 85, 99, 113, etc., due to the loss of a methylene group ($-14$, $—CH_2$). The molecular weight represented by the molecular ions ($M^+$) present ranged from 282 to 310 corresponding to $C_{20}$ and $C_{22}$ aliphatic hydrocarbons.

Fraction 2 was composed of two different compounds. The spot corresponding to an $R_f$ value of 0.96 was due to traces of an unknown glycolipid (detection with orcinol-$H_2SO_4$; Christie 1982). The second compound ($R_f$ 0.83) of fraction 2 was analysed by GC and GC/MS. Fatty acid methyl esters of the untreated as well as of the previously hydrolysed sample showed the same pattern in GC analysis. The chromatogram exhibited two peaks with different retention times. Compared to the standard solutions of fatty acid methyl esters they were identified as saturated, long-chain carboxy acids namely palmitic ($C_{16:0}$) and stearic acid ($C_{18:0}$). The result was verified by GC/MS with the characteristic base peak at m/z 74 typical for saturated fatty acid methyl esters due to the ions formed up on $\alpha$-$\beta$ cleavage with simultanous migration of one hydrogen atom from the lost fragment, known as the McLafferty rearrangement. Signals at M-29, M-31 and M-43 due to the loss of an ethyl, a methoxy and a propyl group and the presence of the molecular ions ($M^+$) at m/z 270 and 298 defined the chain length to be $C_{16}$ and $C_{18}$.

Fraction 3, the main component of the crude product and the most surface-active one was analysed by different procedures after further purification. Ion exchange column chromatography of the main surface-active fraction of *B. licheniformis* (fraction 3 from silica gel column chromatography; Fig. 1) on DEAE sepharose CL 6B resulted in a single peak in the region where surface tension was low (Fig. 1). The active fractions were collected, dried and dissolved in a chloroform-methanol (2:1) mixture before submitting to HPLC. HPLC analysis of the pooled fractions resulted in at least two major peaks. They appeared throughout with a ratio of 2.1:1. The isolated peaks were identified as lipid-positive spots by TLC, both exhibiting the same $R_f$ value as the primary product of the silica gel procedure. This indicated that fraction 3 was made up of two closely related compounds.

### *Identification of the main surface-active compounds*

The IR spectrum of fraction 3 in KBr (Fig. 2) showed strong bands characteristic of peptides at 3300 $cm^{-1}$ (band A), at 1655 $cm^{-1}$ (band F) and at 1535 $cm^{-1}$ (band G) resulting from the N-H stretching mode, the stretching mode of the $>C{=}O$ bond and the deformation mode (combined with the C—N stretching mode)

**Table 1.** Detection of the separated active compounds by thin-layer chromatography (TLC)

| Fraction | $R_f$ value | Minimum surface tension (mN $m^{-1}$) | Detection with DPH | Amount in the crude extract (%) | cmc (mg $l^{-1}$) |
|---|---|---|---|---|---|
| 1 $CHCl_3$ eluate | 0.96 | 29.5 | + | 20 | n.d. |
| 2 Acetone eluate | 0.96 | — | + | Both together | n.d. |
| | 0.83 | 32.0 | + | 3.5 | |
| 3 $CHCl_3$—$CH_3OH$, $CH_3OH$ eluate | 0.54 | 28.0 | + | 67 | 30 |

The solvent system was $CHCl_3$—$CH_3OH$—$CH_3$ COOH (80:15:5). All of the four compounds detected on TLC by their different $R_f$ values showed surface activity and a lipid-positive reaction with 1,6-diphenyl-2,3,5-hexatriene (DPH). The highest amount of active fraction was found however in fraction 3, the $CHCl_3$—$CH_3OH$ eluate. CMC, critical micelle concentration; n.d., not detected

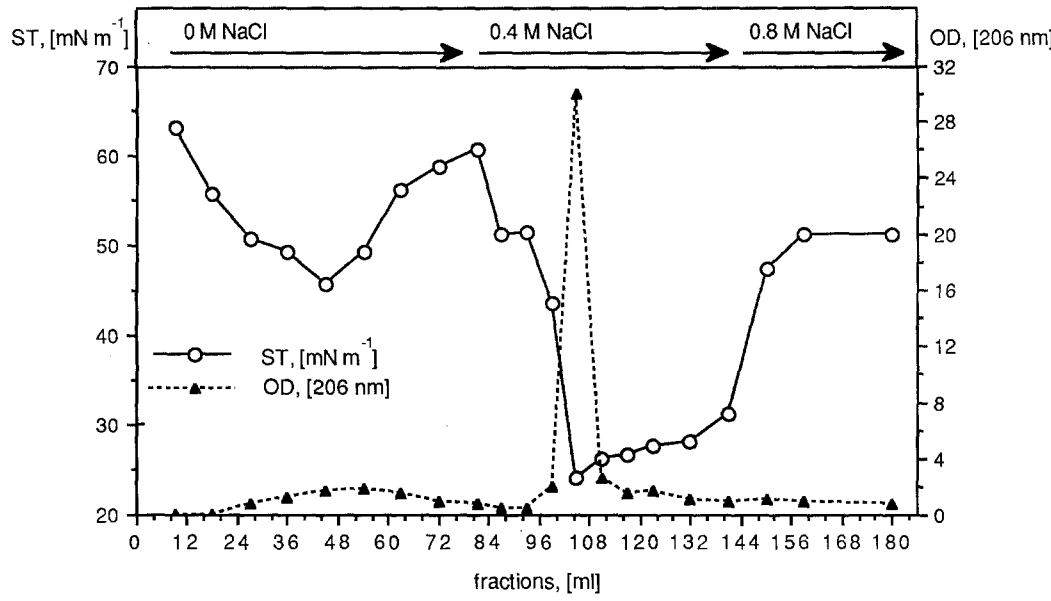


**Fig. 1.** Ion exchange chromatography of the surface active fraction 3 on DEAE-Sepharose. Elution was effected by a gradient from 0 M to 0.8 M NaCl in 10 mM TRIS-HCl containing 20% EtOH (pH 8.0). The absorption (optical density, OD) of the fractions at 206 nm was monitored. The product fraction appeared as a single peak in the region where surface activity was high. ST, surface tension

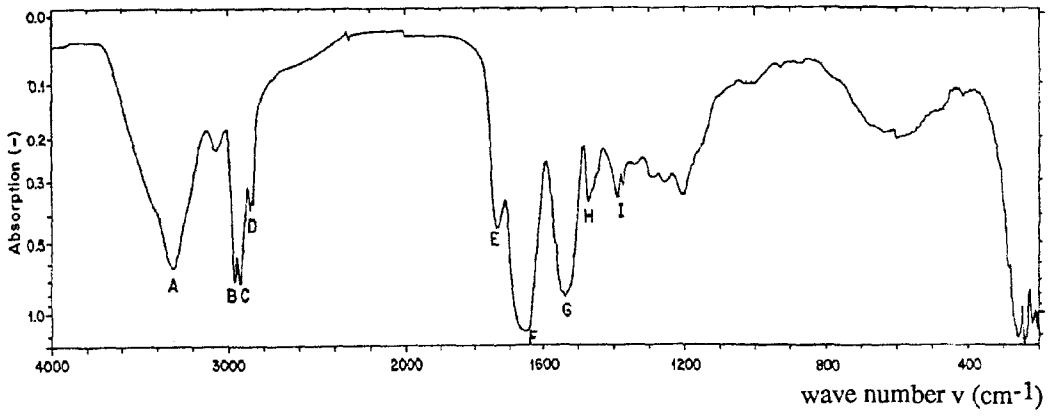


**Fig. 2.** Infrared spectrum of the surface-active fraction 3: for explanation of bands A–I, see text

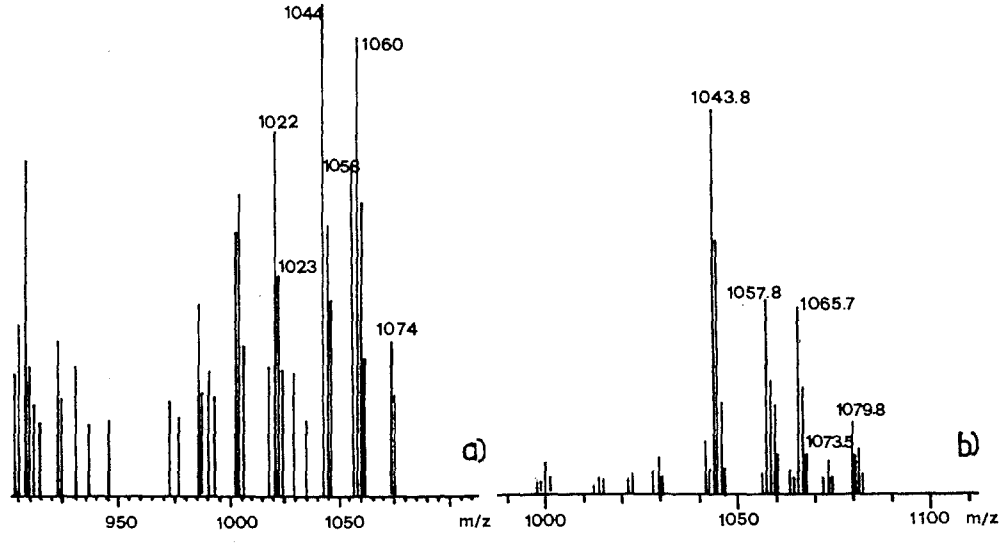


**Fig. 3a, b.** Partial mass spectra of fraction 3 showing the region of the molecular masses by the molecular ions ($M^+$; 1022, 1036) and by abundant ions like ($[M+H]^+$; 1023, 1037), ($[M+Na]^+$; 1044, 1058), ($[M+2Na]^+$; 1066, 1078) and ($[M+K]^+$; 1060, 1074). **a** Fourier transformations mass spectrum. **b** Fast atom bombardment (FAB) mass spectrum

of the N-H bond respectively. The bands at 2960–2930 $cm^{-1}$, 2880–2860 $cm^{-1}$ (bands B, C, D) and at 1470–1430 $cm^{-1}$, 1390–1370 $cm^{-1}$ (bands H, I) reflect aliphatic chains ($-CH_3$, $-CH_2-$) of the fraction. Band E was due to lactone carbonyl absorption. These results indicate that the product contains aliphatic hydrocarbons as well as a peptide-like moiety.

The weight of the complete molecules was determined by both combined LD/FTMS and FAB/MS (Fig. 3). Two molecular weights, 1022 and 1036, were determined by both methods as derived from different attachment of $H^+$, $Na^+$ and $K^+$. The mass difference of 14 units characterizes the lipopeptide as a mixture of closely related molecules varying in their fatty acid residues.

The lipophilic part of the biosurfactant was analysed by combined GC/MS of the fatty acid methyl esters and derivatives it contained. The release of the fatty acids and the subsequent transformation to different derivatives was carried out according to the description in Materials and methods. The resulting GC spectrum was dominated by four main peaks, namely *β*-OH-*iso* $C_{14}$, *β*-OH $C_{14}$, *β*-OH-*iso* $C_{15}$ and *β*-OH-*anteiso* $C_{15}$ with a ratio of 26:45:15:14%. The peaks were identified by MS of the fatty acid methyl esters, giving a characteristic base peak at m/z 103 ($CH(OH)CH_2COOCH_3$) due to the fragment ion caused by *β,γ*-fragmentation commonly known for *β*-OH fatty acid methyl esters and the fragment ion peaks such as M-18, M-50, M-73 for *β*-OH acids. Further elucidation was carried out by MS of the dehydrated hydrogenated forms for determining the chain length (yielding m/z 74, M-31, M-43 for saturated fatty acids) and by MS of the picolinylesters (yielding m/z 92 or 108, +/− M-43 or M-57 characteristic for *iso* and *anteiso* branching).

For the hydrophilic part an amino acid composition of Asp, Leu, Val, Glu and Ile with a ratio of 1:3:1:1:1 was determined. On the basis of the molecular mass and together with the ninhydrin-negative reaction of the native compound a cyclic closed structure of the peptide part was conceivable. For sequence analysis the lipopeptide was hydrolysed in 12 M HCl–$CH_3COOH$ (2:1) and the subsequent fragments were separated by HPLC. The selected ninhydrin positive fragments were identified yielding the sequences shown in Table 2.

Glutamic acid was not detected in any of the fragments but the direct linkage of glutamic acid to the fatty acid part and the position of the dipeptide Leu-Ile at the end of the sequence could be demonstrated by the mass spectrum of the permethylated product (Fig. 4; Table 3). The spectrum of Fig. 4 may be divided into groups representing four compounds: group A shows the permethylated acid itself, which is a mixture of two homologues ($C_{14}$ and $C_{15}$) and group B shows the same mixture which has lost one molecule of methanol from the acyl constituent. Therefore in the lower mass region, peaks form a characteristic fragment peak group of four signals. The mass spectral fragmentation occurred predominantly at the peptide CO–NMe bonds; the resulting peaks, as outlined in Table 3 and in Fig. 4 (for $C_{15}$ constituent only) delineated the sequence.

**Table 2.** Amino acid sequences of peptide fragments

| Peptide fragment | Amino acid sequences |
|---|---|
| 1 | Leu-Val-Asp- |
| 2 | Leu-Val-Asp- |
| 3 | Val- |
| 6 | Leu-Ile |
| 9 | Leu-Leu-Val- |
| 11 | Leu-Val- |
| 17 | Leu- |
| Total sequence | Leu-Leu-Val-Asp-Leu-Ile |

Six of the seven amino acids of the lipopeptide fraction were found in fragments resulting from HPLC separation of an alkaline hydrolysate

In the spectrum the molecular ion peak $M^+$ is only recognized for the $C_{15}$ constituent at m/z 1207.8. The region of high weight shows additionally a complex information due to the attachment with $Na^+$ which leads to the formation of quasi-molecular ions at m/z 1229.9 $(M+Na)^+$ and at m/z 1197.9 and 1183 $(M-OCH_3+Na)^+$ and due to the further loss of OMe from the C-terminal amino acid $(M-OCH_3)^+$. The two isomeric amino acids Leu and Iso and the different branching of the $C_{14}$ and $C_{15}$ fatty acids are difficult to differentiate because they give rise to the same mass increments. An elimination of M-42/M-28 characteristic of Leu/Ile at the C-terminal end was not obvious due to the complex mass region (Biemann and Martin 1987). However, the above results led to the formulation of the structure for the acidic form of the main surface-active fraction:

$R_{1-4}$-$(CH_2)_8$-$CHOHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Ile-OH

The molecular weight and the IR spectrum of the purified compound indicated a lactone-ring bond. The determination of the location of the lactone ring in the compound was carried out by digestion with carboxypeptidase Y, which is known specifically to cleave peptides possessing a free C-terminal residue. The enzyme reaction was controlled on HPLC. The untreated surface active main fraction did not show any reaction after 20 h treatment with the enzyme solution but the free acid form of the lipopeptide derived from alkaline treatment with 10% NaOH in EtOH reacted with the enzyme within 45 min. Here the retention time of the educt was about 1 min shorter than the product. These results excluded a lactone ring bond with the free carboxyl group of the aspartic or glutamic acid and the final structure of the main surface-active fraction was elucidated as is shown in Fig. 5.

### *Characterization of the surface-active lipopeptides*

*Physical product characteristics.* The CMC of the purified lipopeptides was found to be approximately 15–20 mg $l^{-1}$ in 0.1 M potassium phosphate buffer (pH

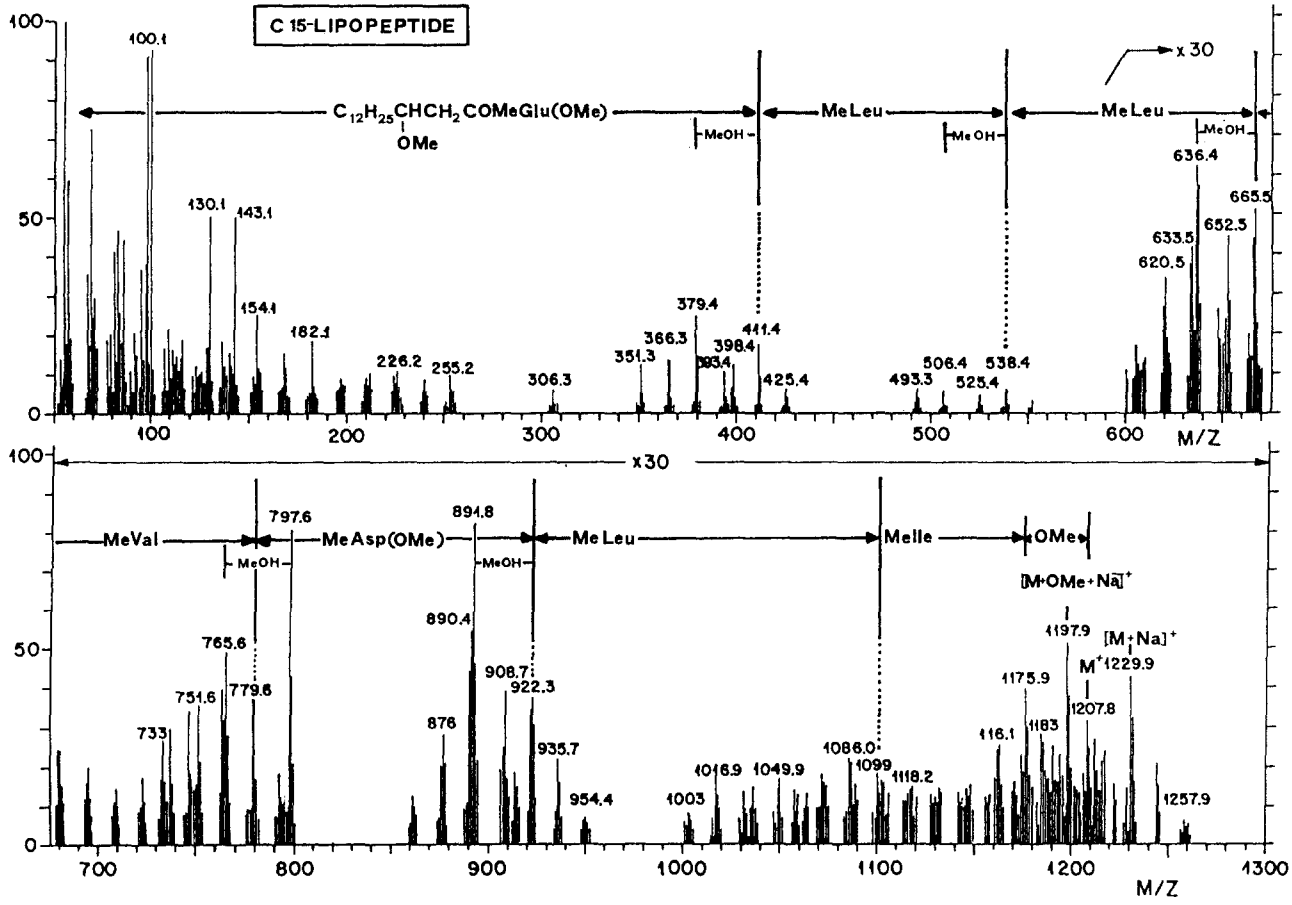


**Fig. 4.** FAB spectrum of the *O,N*-permethylated fraction 3 after alkaline hydrolysis in 10% NaOH in EtOH (3-Nitrobenzylalcohol matrix). The fragmentation ions are outlined by means of the $C_{15}$ constituent

**Table 3.** Identification of the characteristic fragmentation ions of permethylated fraction 3

| Group A | RCHOMeCH$_2$COMeGluOMe | MeLeu | MeLeu | MeVal | MeAsp OMe | MeLeu | Melle | $OCH_3$ | $Na^+$ |
|---|---|---|---|---|---|---|---|---|---|
| $C_{15}$ | 411.4 | 538.4 | 666.4 | 779.6 | 922.3 | 1099 | 1175.9 | 1207.8 | 1229.9 |
| $C_{14}$ | 398.4 | 525.4 | 652.5 | 765.6 | 908.7 | 1086 | 1161 | — | — |
| Group B | RCH=CHCOMeGluOMe | MeLeu | MeLeu | MeVal | MeAsp OMe | MeLeu | Melle | $OCH_3$ | $Na^+$ |
| $C_{15}$ | 379.8 | 506.4 | 634.4 | (746.6) | 890.4 | 1016.9 | (1144) | 1175.9 | 1197.9 |
| $C_{14}$ | 366.3 | 493.3 | 620.5 | 733 | 876 | 1003 | — | 1161 | 1183 |

Group A shows the fragmentation pattern of the permethylated product of each $C_{14}$ and $C_{15}$ constituent. The two homologous constituents in group B are due to a primary loss of MeOH at the acyl end

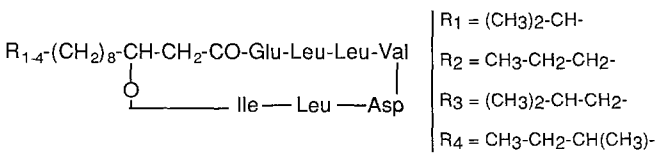


**Fig. 5.** Proposed structure of the surface-active lipopeptides from *Bacillus licheniformis*

7.0). The dependence of the surface activity on temperature and pH was investigated in the same buffer solution at concentrations of 1 CMC (Fig. 6). Temperatures in the range 20–60° C did not show any influence on the surface tension or interfacial tension. An optimal activity was detected within pH 5.0–9.0; below and above these values a slight decrease in activity was observed.

*Biological product characterisation.* Antibiotic activities of the lipopeptides were tested in concentrations of 0.1 to 40 mg $l^{-1}$. A survey of the results is given in Table 4.

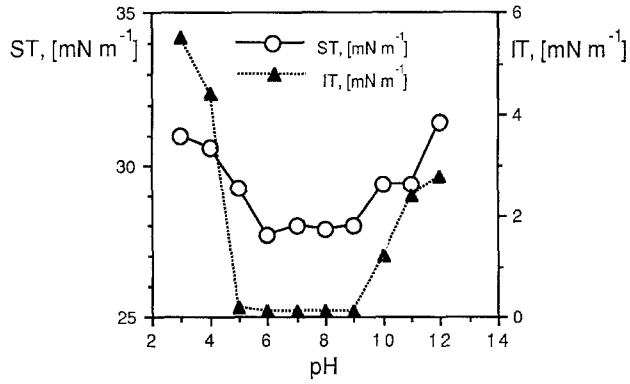


**Fig. 6.** Dependence of ST and interfacial tension (IT) on pH for the purified lipopeptide fraction 3

The lipopeptides showed activity against a variety of yeast strains with older cells being affected by a tenfold lower concentration as compared to exponentially growing cells. Growth of the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* was inhibited by a minimal concentration of 1 mg ml$^{-1}$. *B. licheniformis* itself showed high susceptibility to the product. Concentrations as low as 0.5 mg ml$^{-1}$ inhibited growth. Among the various fungi tested (11 strains) only the growth of *Trichoderma reesei* and *Penicillium oxalicum* was negatively influenced by the lipopeptides. Since these tests were not standardized, the results are only qualitative. Factors such as medium composition, density and age of the inoculum, which could all affect the determination of antibiotic activity, were not investigated.

## Discussion

The spectrum of lipopeptides produced by *Bacillus* spp. is broad but only a few have been reported in relation to their surface activity. Most of them are known for their antibiotic characteristics. Within the peptide antibiotic group the lipopeptides of *B. licheniformis* show analogous structures with representatives of the iturin group (iturin, mycosubtilin, bacillomycin) or of the lactone group (esperin, surfactin, polypeptin) (Umezawa et al. 1978). Most of them show similarities in the hydrophilic part, consisting of a seven-amino-acid cyclic peptide that is linked by a hydroxy or an ester peptide linkage to the fatty acid part.

Surfactin produced by *B. subtilis* shows structurally the highest analogy with the lipopeptides of *B. licheniformis* characterized in this work (Arima 1969; Hosono and Suzuki 1983). Surfactin is already known as a potent surface-active agent and as an antibiotic (Cooper et al. 1981a; Mulligan et al. 1989). This bifunctional nature of surface-active agents is well known for many other biosurfactants, not only for those originating from representatives of the *Bacillus* genus. For example, rhamnolipids and viscosin from *Pseudomonas* spp. as well as the glycolipids of *Torulopsis apicola* are good biosurfactants also exhibiting an antibiotic activity (Hiramoto et al. 1969; Itoh et al. 1971; Hommel et al. 1987; Neu and Poralla 1990). The antibiotic spectrum of the lipopeptides from *B. licheniformis* seems to be identical to the one of surfactin. High antibiotic activity of surfactin against Gram-negative bacteria was reported by Bernheimer and Avigad (1970) and Takahara et al. (1976). The antibiotic activity was not tested against yeast strains, in which in our case growth inhibition was demonstrated.

The structural difference between surfactin and the *B. licheniformis* lipopeptides was established in the C-terminal amino acids (Leu is substituted by Iso) and in

**Table 4.** Antibiotic activity of the surface-active lipopeptides from *Bacillus licheniformis* against yeasts, bacteria and fungi in agar-diffusion tests

| Microorganisms | Test media[a] | Temperature (° C) | MIC[b] (mg ml$^{-1}$) | MIC[c] (mg ml$^{-1}$) | Inhibition zones[d] |
|---|---|---|---|---|---|
| *B. licheniformis* | LB | 37 | 0.5 | — | ++ |
| *Pseudomonas aeruginosa* | LB | 30 | 1.0 | — | +++ |
| *Escherichia coli* | LB | 37 | 1.0 | 0.5 | ++++ |
| *Candida utilis* | YEPD | 30 | 1.0 | 0.1 | +++ |
| *C. tropicalis* | YEPD | 30 | 1.0 | 0.1 | +++ |
| *Trichosporon cutaneum* | YEPD | 30 | 10.0 | — | + |
| *Saccharomyces cerevisiae* | YEPD | 30 | 5.0 | 0.1 | ++ |
| *Trichoderma reesei* | MEA | 30 | 0.5 | 0.1 | ++ |
| *Penicillium oxalicum* | MEA/PDA | 30 | 0.1 | — | ++++ |

No inhibition of growth was obtained with: *Aspergillus nidulans, Phanerochaetae chrysosporium, Bjerkandera adusta, Ustilago maydis, Sordaria fimicola, Botrytis cinerea, Fusarium lycopersicii, Phytophtora infestans, Pythium debarayanum*

[a] Media composition in g l$^{-1}$: LB, yeast extract, 5; glucose, 10; tryptone, 10; NaCl, 5; agar, 15. YEPD, yeast extract, 10; glucose, 20; peptone, 20. MEA, malt extract, 20; agar, 20. PDA, potato dextrose, 20; agar, 20

[b] MIC, minimal inhibitory concentration, against microorganisms during the exponential growth phase

[c] MIC against microorganisms during decreasing and death phase

[d] Maximum diameter of halos: + = 7-10 mm, ++ = 11-14 mm, +++ = 15-18 mm, ++++ = > 18 mm

the composition of the lipophilic part where surfactin shows additionally β-OH-*i,ai* $C_{13}$ and a higher amount of β-OH-*i,ai* $C_{15}$ fatty acids. The biosynthesis of surfactin was studied by Kluge et al. (1988) in a cell-free system. The formation of surfactin occurred non-ribosomally by the thiotemplate mechanism similar to numerous other peptide antibiotics of *Bacillus* spp. and the capsule poly-γ-D-glutamic acid polypeptide of *B. licheniformis* (Troy 1982). The high similarity between surfactin and the lipopeptides described here leads to the conclusion that they are derived from the same enzyme system. Minor variations such as exchange of amino acids in the group Val/Leu/Ile/Thr or Phe/Tyr/Trp are common for peptide antibiotics, e.g. Gramicidin S and Tyrocidin, especially because the substrate amino acid activation of the multienzyme thiotemplate mechanism was not excluded. They can be attributed to overlapping specificities of activation reactions within the thiotemplate mechanism. Due to the non-ribosomal mechanism, specificities of the amino acid activation reactions are considerably lower than in the ribosomal protein synthesizing system (Kleinkauf and Döhren 1986, 1987).

The structure of the fatty acids in the lipopeptides shows a particular characteristic of *Bacillus* spp. (branched and hydroxy fatty acids) whereas the production of long-chain saturated fatty acids such as $C_{16:0}$ and $C_{18:0}$ is rather exceptional for this genus (Kaneda 1977). However, high surface activity of free long-chain fatty acids has already been demonstrated for other species such as *Nocardia erythropolis* and *Corynebacterium lepus* (Cooper et al. 1981b; MacDonald et al. 1981). Since *n*-alkanes were the carbon and energy source in these cases, it was postulated that they derive rather from the substrate than are products of de-novo synthesis.

Hydrocarbons as extracellular products are rather unusual since they function more as cellular components than as extracellular products. The ability of microorganisms to synthesize appreciable amounts of hydrocarbons is limited to a few bacterial, algal and fungal species (Finnerty 1989). Until now *Bacillus* spp. have not been described as characteristic hydrocarbon producers. Since *B. licheniformis* has been cultivated on a water-soluble substrate, the hydrocarbons do not represent an artefact derived from the extraction procedure, as is known for many alkane-utilizing organisms. However, similar substances, described as wax esters, were identified by TLC for a *Bacillus* spp. that showed significant emulsifiying properties (Cooper and Goldenberg 1987).

Surface-active products of *B. licheniformis* were recently described by McInerney et al. (1990). However a structural analysis was not presented. The acid-precipitated fraction was analysed by TLC and shown to be composed of a mixture of four different components. Two of them were lipids, one exhibited an amino acid positive reaction and for the fourth no indication of chemical composition was given. There may exist some similarities between these four components with those identified here. The suggestion made by the authors however, that all four components are needed for full surface activity is refuted by this work. Each fraction we identified showed high surface activity even in small amounts.

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