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Structural analysis of *Bacillus licheniformis* 86 surfactant

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SUMMARY

A tentative structure and composition of a surfactant, BL-86, produced by *Bacillus licheniformis* 86 is described. The surfactant is a mixture of lipopeptides with the major components ranging in size from 979 to 1091 Da and varying in increments of 14 Da. The variation in molecular weight represents changes in the number of methylene groups in the lipid and/or peptide portion of the surfactant. There are 7 amino acids per molecule. The peptide portion is composed of the following amino acids: glutamic acid or glutamine (glx), aspartic acid or asparagine (asx), valine, leucine, and isoleucine at a ratio of 1.0 : 1.0 : 1.4 : 3.0 : 0.6, respectively. The leucine is present as both the D and L isomers at a ratio of about 2 : 1, respectively. Forty percent of the molecules contain L-valine instead of L-isoleucine. The glx and asx are present as a combination of L-glutamic acid and L-asparagine and/or L-glutamine and L-aspartic acid. The N-terminus of the peptide is blocked, most likely by a peptide bond to the lipid portion. An ester carbonyl structure is present, which could be a part of a lactone ring connecting the β position of the lipid to one of the carboxyl groups in the peptide. The lipid portion is composed of, on average, 8–9 methylene groups, and contains a mixture of linear and branched tails. Results of DCI-MS and FAB-MS analyses, as well as surface tension measurements, of purified BL-86 HPLC fractions support the proposed composition.

INTRODUCTION

A surfactant produced by *Bacillus licheniformis* 86, designated BL-86, was was found to lower the surface tension (ST) of water to 27 dynes/cm, have a critical micelle concentration (CMC) of 10 μg/ml and lower the interfacial tension (IFT) between water (4% NaCl) and *n*-hexadecane to 0.36 dynes/cm [5]. The surfactant is stable to a wide range of pH, temperatures, and NaCl concentrations [5]. The surfactant is an excellent dispersant, dispersing β-SiC and AlN slurries far better than commercial dispersants (Horowitz and Currie, unpublished data).

Similar surfactants, surfactin and lichenysin, are produced by strains of *B. subtilis* and *B. licheniformis*, respectively [1–4,8,15]. Surfactin is a cyclic lipopeptide containing a carboxylic acid (3-hydroxy-1,3-methyl tetradecanoic acid being the major component) and seven amino acids. The peptide includes glutamic acid (in a peptide bond from the amino terminus to the carboxylate of the fatty acid)-L-leucine-D-leucine-L-valine-L-aspartic acid-D-leucine-L-leucine (esterified from the carboxy terminus to the 3-hydroxyl function of the fatty acid) [10–12]. The major component of surfactin is a lipopeptide of molecular weight (MW) 1036 [6,7,10,12,19]. Another lipopeptide surfactant, lichenysin, produced by *B. licheniformis* JF-2, has been shown to have structural and physiochemical properties similar to surfactin [8,9,15].

In order to establish the structure of BL-86, a series of analytical tests were performed an the data compared to those obtained for surfactin and lichenysin. A tentative structure for BL-86 is presented.

MATERIALS AND METHODS

Microorganisms. Bacillus licheniformis 86 was obtained from J.E. Zajic (Petroleum Bioresources, Inc., El Paso, TX) [5]. *Bacillus subtilis* ATCC #21332 and *B. licheniformis* JF-2 ATCC #39307 were obtained from the ATCC. All bacteria were stored lyophilized.

Growth conditions. Bacillus lichenifromis 86, *B. subtilis* ATCC #21332, and *B. licheniformis* JF-2 ATCC #39307 were grown aerobically in Cooper's medium [4]. Small scale batch fermentation (1.6 l) were performed using a 2-l fermentation vessel (MultiGen, New Brunswick Scientific). The cultures were grown at 30 °C, for 20 h stirred at 275 rpm, with an air supply of 1.0 vvm. Large scale fermentations (16 l) were performed for *B. licheniformis* 86 and *B. subtilis* ATCC #21332 using a 20-l fermentation vessel (L.H. Feremention series 2000) at 30 °C, for 20 h, stirred at 500 rpm, with an air supply of 0.75 vvm. The foam produced by the growing cultures and containing the biosurfactants was continuously collected.

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Surfactant isolation. Bacterial cells were removed from the surfactant containing foam by centrifugation (13000 × *g*, 10 °C, 15 min) for the 1.6-l fermentation and by continuous centrifugation (Sorval KSB-R continuous flow system, 17000 × *g*, 5 °C) for the 16-l fermentation until the supernatant was clear. The supernatants were then subjected to acid precipitation by adding concentrated HCl to final pH of 2.0 and allowing the precipitate to settle at 4 °C. The acid precipitates were recovered by centrifugation (11000 × *g*, 4 °C, 20 min). The pellets were washed several times with acid water (pH 2.0, by HCl) and lyophilized overnight. The surfactants were then extracted from the powder into methanol (for the 1.6-l fermentation products), into tetrahyrofuran (THF) for *B. licheniformis* 86, 16-l preparation [5], or into dichloromethane (for *B. subtilis* ATCC #21332, 16-l preparation). The THF extracted BL-86 was further processed as previously described [5]. Surfactin was further purified as described by Cooper et al. [4].

Infrared analysis. Samples for infrared analysis were prepared by mixing approximately 1 mg of surfactant with 300 mg of KBr and pressing the mixture into a pellet at 20000 psi, for 2–3 min. Infrared spectra of the pellets were obtained on the Nicolet 7199 FT IR spectrometer.

NMR analysis. The ^{1}H-NMR spectra were acquired at 400 MHz on a Varian VXR-400 NMR spectrometer. The samples were prepared as solutions in 100% dimethylsulphoxide-d6-(dmso-d6) using approximately 1–3 mg of surfactant in 0.5 ml of dmso-d6. A two-dimensional homonuclear ^{1}H correlation spectrum (COSY) was acquired using the standard 90° -D-90° pulse sequence. A data matrix of (256 D increments) × (256 data points) was generated. The resulting matrix was zero-filled to a 1024 × 1024 matrix and Fourier processed. The data are presented as a symmetrized contour plot.

Reverse phase HPLC. Analytical reverse phase HPLC was performed using a 30 cm C_{18} μBondapak column (Waters) at 25 °C. Surfactant concentration was 5 mg/ml in methanol. Injection volume was 10–50 μl. The mobile system employed was 60% to 90% gradient of acetonitrile in 0.01 M ammonium acetate, pH 4.8. The eluted peaks were detected by following UV absorbance at 210 nm and by a mass detector at 60 °C (ACS light scattering vapor phase detector). Preparative reverse phase HPLC was performed using a semi preparative C_8 or C_{18} μBondapak column (Waters) at 30 °C, mobile phase: 60–65% to 90% gradient of acetonitrile in 0.01 M KH_2PO_4 or ammonium acetate, pH 4.5–4.8.

BL-86 fraction preparation. Fractions collected from several preparative HPLC runs were pooled, evaporated, desalted by extraction into THF or CH_2Cl_2 and re-evaporated. The powdered fractions were dissolved in alkaline-H_2O (pH 10.5 by NaOH) for ST measurements.

Mass spectroscopy analysis. Surfactant samples were analyzed by direct probe chemical ionization mass spectroscopy (DCI-MS) technique (Finnigan TSQ mass spectrometer) using isobutane as reagent gas. The source pressure was maintained at 1×10^{-5} torr and the source temperature was set at 40 °C. The probe filament was ramped at 50 mA/s and the mass spectrometer was scanned from 800 to 1200 amu at 1-s intervals. The mass range on the MS was calibrated with Tris(perfluorononyl)-*s*-triazine (MW 1485.2) (PCR Research Chemicals, Inc., Gainsville, FL). Fast atomic bombardment mass spectrospcopy (FAB-MS) analysis were performed on the Finnigan TSQ 4600 mass spectrometer (Quad 3), using a glycerol matrix. The mass spectrometer was scanned from 500 to 1200 amu. The mass range was calibrated as for the DCI-MS technique.

LC-MS analysis. LC-MS was performed using reverse phase HPLC and thermospray MS (Oneida Research Services, Whitesboro, NY). The following conditions were used. Thermospray ion source: Vestec Company; source temperatures: inlet temperature 130 °C, probe temperature 255 °C, vapor temperature 235 °C, and block temperature 295 °C; mass spectrometer: Finnigan TSQ 46; scan parameters: liquid chromatograph: Waters gradient system operated in isocratic mode; solvent conditions: gradient elution with solution A varying from 40% to 90% in 20 min: solvent A: acetonitrile, solvent B: 0.1 M ammonium acetate, pH 4.8; LC column: C_{18} μBondapak. The surfactant sample was dissolved in methanol prior to the separation.

Peptide and amino acid analysis. Acid hydrolysis of the surfactant was performed in 6 N HCl, at 110 °C, for 24 h. Ninhydrin assay was performed by spotting a sample of the surfactant dissolved in methanol onto a silica gel 60 plate, spraying with 0.3% ninhydrin in *n*-butanol (Gelman Sciences, Inc., Ann Arbor, MI), and heating for 10 min at 100 °C. When positive, a pink color developed. Amino acids were analysed on a 1-dimensional TLC (silica gel 60), using the following solvents. Solvent system F: ethanol : ammonium hydroxide (34%) : water, 70:30:5, or solvent system C: butanol : acetic acid : water, 80 : 20 : 20 [17]. The amino acids were identified by the R_f, shape, and color of spots, in comparison to standards. The amino acid composition was determined using anion-exchange chromatography (Woodson-Tenent). The amino acids were separated on a Dionex CS4 column and detected by reaction with ninhydrin. A step gradient with four eluents was used. Eluent one was 0.25 mN nitric acid. Eluent two was 6 mN potassium oxalate with 5.6 mN nitric acid. Eluent three was 9 mN potassium oxalate. Eluent 4 was 5 mN nitric acid. Optical isomers of amino acids (D and L) were separated and quantitated by reverse phase HPLC following derivatization with Marfey's rea-

gent [14], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (Pierce Chemical Co.), as instructed by the manufacturer. The surfactant was acid hydrolyzed as above. A sample (3 mg, in 100 μl) was mixed with 200 μl of 1% solution of FDAA in acetone and 40 μl of 1.0 M sodium bicarbonate solution. The mixture was incubated at 40 °C for 1 h, cooled, and then 20 μl of 2 N HCl were added. The derivatized amino acids were separated by reverse phase HPLC (μBondapak C_{18} column, Waters), using a 10% to 50% gradient of acetonitrile in 50 mM TEAP (triethylamine phosphate) buffer, pH 3.0, as the mobile phase, and detected by UV absorbance at 340 nm.

Surface tension measurements. ST measurements were made at 22.5 °C, using a plate tensiometer (Universal Transducer Readout model SC1001, Gould Statham).

RESULTS

Infrared analysis

Infrared analysis of BL-86 revealed a pattern similar to that of surfactin, indicating that BL-86 is a lipopeptide (Fig. 1). The BL-86 infrared spectrum (Fig. 1A) indicates the presence of a peptide component (wavenumber 3430, amide NH streching; wavenumber 1655, amide C=O; and wavenumber 1534, CNH) and an ester carbonyl group (wavenumber 1733, ester C=O), suggesting a lactone ring. This spectrum suggests the presence of an aliphatic chain (wavenumber 3000–2800 C-H stretch modes). Computer deconvoluting of the spectrum suggests the presence of aspartic and/or glutamic acids (carbonyl absorption in the region characteristic of carboxylic acid).

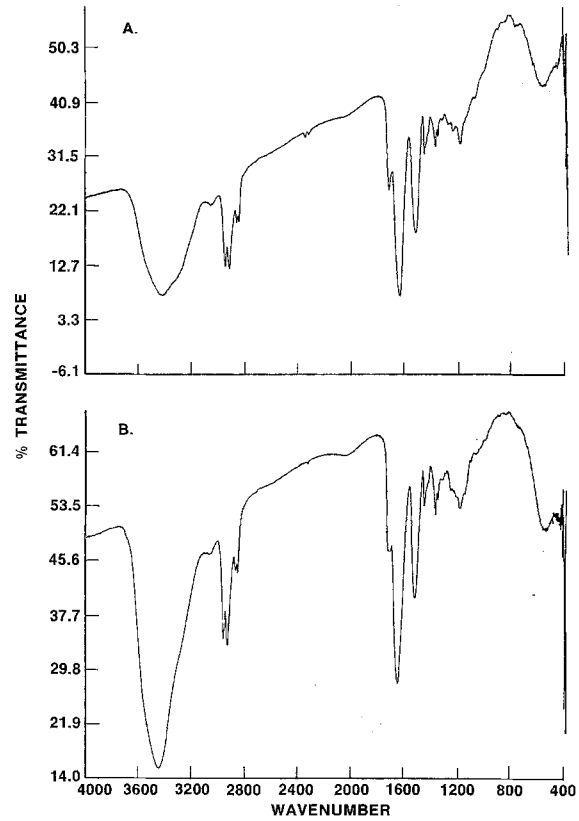

Fig. 1. Infrared analysis of biosurfactants. (A) BL-86 surfactant. (B) Surfactin.

NMR analysis

The ^{1}H-NMR spectrum of BL-86 is presented in Fig. 2A. The spectrum confirms the presence of a long aliphatic chain (CH_2 at 1.5–1.2 ppm) and a peptide backbone (NH at 8.6–8.0 ppm and CH at 4.8–4.2 ppm). The spectrum indicates the presence of the following amino acids: valine with peptide CH at 4.6, CH at 1.7, and 2 CH_3 at 0.9 ppm; leucine with peptide CH at 4.3, CH_2 at 1.7, CH at 1.3, and 2 CH_3 at 0.9 ppm; isoleucine with peptide CH at 4.2, CH at 2.2, CH_2 at 1.9, and 2 CH_3 at 0.9 ppm; aspartic acid or asparagine with peptide CH at 4.7 and CH_2 at 2.8 ppm; and glutamic acid or glutamine with peptide CH at 4.3, internal CH_2 at 1.9, and CH_2 α to the carbonyl at 2.1 ppm. Due to the complexity and overlap of the NMR spectrum, most of these assignments are the result of a detailed analysis of a 2-dimensional COSY spectrum (2-D-homonuclear correlation) (Fig. 2B). A resonance at 5.2 ppm indicates the presence of an ester group which may be a part of a lactone ring. Integration of the spectrum (assuming a value of 1.00 to the H of the resonance at 5.2–5.1 ppm, which is believed to be part of the lactone group) suggests the presence of 7 amino acids, based on the number of peptide CHs. Eleven to 12 terminal CH_3 groups (average of 11.57) were calculated (1.2–0.8 ppm region). The lipid chain seems to consist of mostly 8–9 CH_2 groups (average of 8.6) (1.5–1.2 ppm region).

Reverse phase HPLC

BL-86 was found to be a mixture as demonstrated by reverse phase HPLC (Fig. 3A). The chromatographic profile reveals several, different sized peaks, arranged in groups of singles, doublets, and a triplet (a total of at least 9 peaks). The spectrum derived by mass detection is in agreement with that obtained by UV 210, confirming that all the BL-86 components absorb at UV 210, and to the same extent. When compared to surfactin and lichenysin, the HPLC pattern of BL-86 was found to be significantly different from that of the other two surfactants. Moreover, the HPLC chromatogram of surfactin seemed identical to that of lichenysin (Fig. 3A–C).

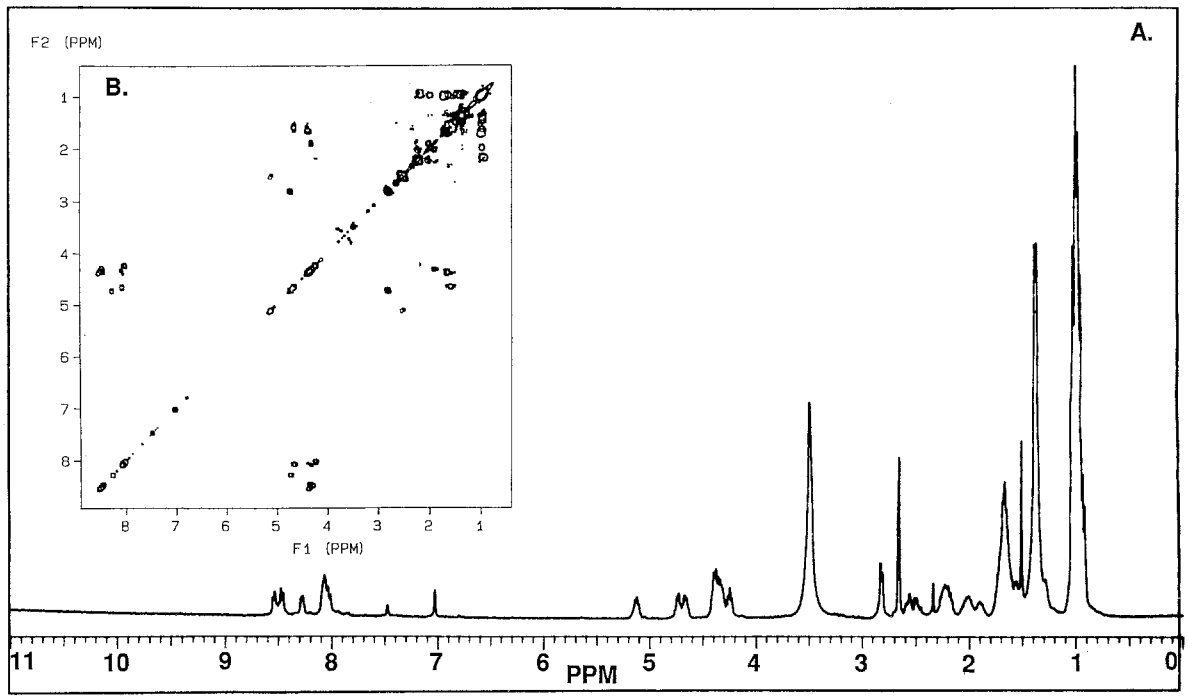

Fig. 2. NMR analysis of BL-86 surfactant. (A) Proton NMR spectrum. (B) Two dimensional COSY analysis.

DCI-MS analysis

DCI-MS analyses of BL-86 revealed that the surfactant is a mixture of molecular ion species with the major components ranging in size from 993 to 1091 Da, varying in increments of 14 Da (Fig. 4A, presented as mass of the protonated molecular ion species). The most abundant components are of 1021, 1035 and 1049 Da (Fig. 4A). The distribution varied slightly among different preparations. Thus, in some preparations there was a shift towards molecular ion species of 1007, 1021 and 1035 Da being the most abundant. The presence of DCI fragmentation products of 32 Da lower than the reported molecular weights, which was detected in some DCI-MS, is a result of breakage, due to high source temperature.

Surfactin, initially characterized as having only one molecular ion species of 1036 Da [2,10] and later described as a mixture of lipopeptides [6,7,12,19], was found to be composed of a series of even MW components, ranging in size between 1022 to 1120 Da, in increments of 14 Da. The major components are 1036, 1050 and 1064 Da (Fig. 4B). None of the odd MW molecular ion species of BL-86 are present in surfactin. Lichenysin has been described as surfactin-like [8]. DCI-MS analysis indicates that lichenysin has the same molecular ion species and composition as surfactin (Fig. 4C).

LC-MS analysis

A sample of the BL-86 surfactant was analysed using thermospray LC-MS under HPLC conditions similar to those used earlier, in an attempt to correlate the molecular weight of the mixture components with the peaks in the HPLC chromatogram. The LC/MS chromatogram of the BL-86 was slightly different from that of the analytical HPLC and had six broad peaks, some of which were partially resolved to contain several narrower peaks. The mass spectra obtained were similar to the DCI-MS spectrum described above. The components of the six peaks had molecular weights corresponding to 979, 993, 1007, 1021, 1035 and 1049 Da.

Peptide and amino acid analysis

Ninhydrin assays performed on BL-86 were negative. Following acid hydrolysis, BL-86 was positive to ninhydrin, indicating the presence of a peptide with a blocked N-terminus.

The amino acids present in acid-hydrolyzed BL-86 were identified by two TLC systems. In each case, the R_fs of the spots matched those of the following standards : aspartic acid, glutamic acid, leucine/isoleucine and valine. Isoleucine and leucine could not be separated under these conditions. In addition, acid hydrolysis would prevent

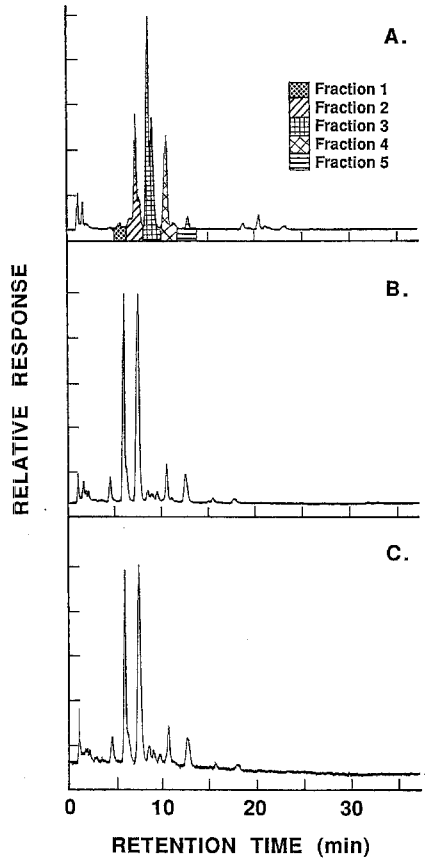

Fig. 3. Reverse phase HPLC analysis of surfactants. (A) BL-86. (B) Surfactin. (C) Lichenysin.

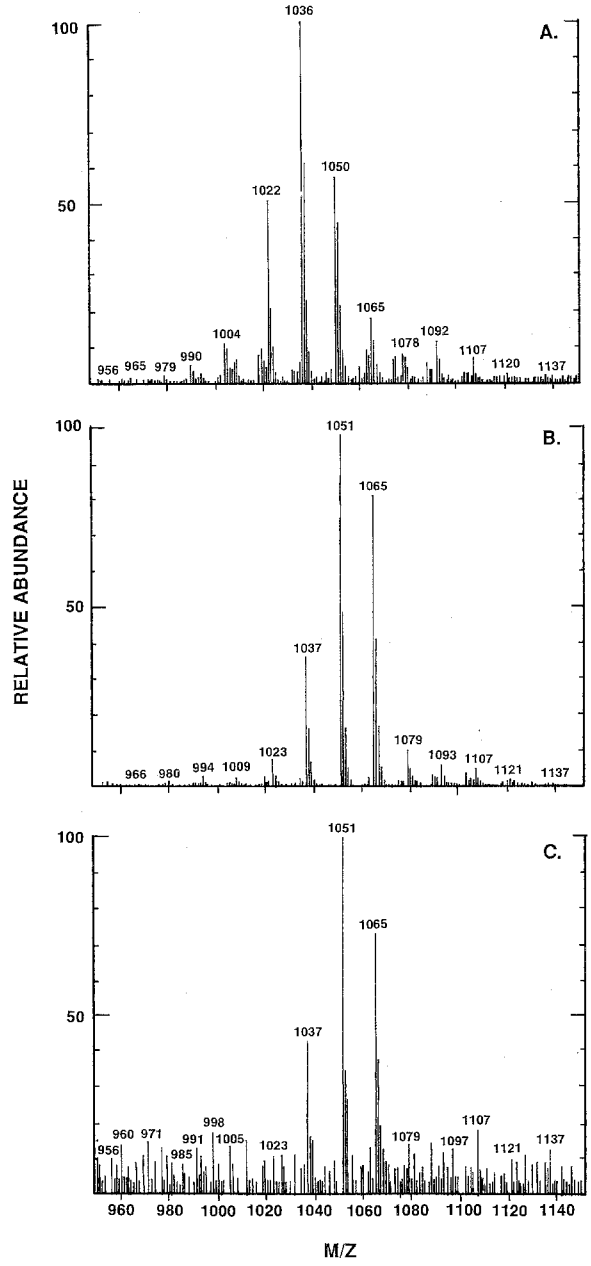

Fig. 4. DCI-MS analysis of surfactants. (A) BL-86. (B) Surfactin. (C) Lichenysin.

detection of glutamine and asparagine due to conversion to glutamic acid and aspartic acid, respectively.

Positive identification and the ratio of the amino acids in the acid-hydrolysed BL-86 were obtained using an ion exchange column coupled to a ninhydrin assay. Both isoleucine and leucine were detected. The following amino acid composition was obtained: glutamic acid, aspartic acid, valine, leucine, isoleucine, 1.0 : 1:1.4 : 3.1 : 0.6, respectively.

The presence and quantity of D amino acids were analysed, using Marfey's method. The results revealed the presence of D and L isomers of some, but not all, of the amino acids present in the peptide portion of BL-86. The most striking is leucine, having a ratio of 60 : 40, D : L isomers, respectively. D-Glutamic acid, D-aspartic acid and D-valine isomers were detected in small, non-molar levels of 10 : 90, 25 : 75 and 1 : 99 D : L ratio, respectively. No D-isoleucine was detected.

Analysis of BL-86 fractions

The BL-86 mixture was fractionated by preparative HPLC for analysis of the mixture components. Five fractions were collected, evaporated, washed and dried. Purity of the fractions was confirmed by analytical HPLC. The separations yielded clean fractions. However, individual fractions may represent more than one component, as they contain single, double or triple peaks.

MS analysis was performed to assign molecular weights to the individual fractions and to identify the number of molecular ion species present in each fraction. Each fraction contained only one major molecular ion

species, even though some of the fractions actually represented more than one major peak. More specifically, FAB-MS analysis revealed the presence of molecular ion species of 1007, 1021, 1035 and 1049 Da, for fractions 2, 3, 4 and 5, respectively (fraction 1 was not analysed because of insufficient material) (Fig. 3A). In another, slightly different fractionation, DCI-MS analysis revealed an additional component of higher molecular weight, 1063 Da.

The ability of the BL-86 fractions to lower the surface tension of water from 72 dynes/cm was tested. Fractions corresponding to molecular ion species of 1007, 1021, 1035, 1049 and 1063 Da had a surface tension lowering activity of 27, 28, 36, 34 and 27 dynes/cm, respectively.

DISCUSSION

The structure and composition of the lipopeptide surfactant BL-86 was studied by a variety of analytical techniques. The IR and NMR data indicated that the peptide portion of BL-86 surfactant contained the amino acids valine, leucine, isoleucine, asx and glx. The presence of these amino acids was confirmed by amino acid analysis. Integration of the NMR results predicted the presence of a total of 7 amino acids per molecule. This prediction is in agreement with the amino acid analysis data in which the sum of the amino acid molar ratios is 7. The ratio of these amino acids was determined to be 1.0 : 1.1 : 1.4 : 3.1 : 0.6 for glx, asx, valine, leucine and isoleucine, respectively. Analysis of the amino acid isomers revealed that 60% of the leucines are in a D form. Thus, within the method's detection range, most of the molecules have a D-leucine : L-leucine ratio of 2 : 1, respectively. The amount of D-glx and D-asx which was also detected was very small, significantly below molar amounts. The presence of a lactone ring in the surfactant was indicated by the IR and NMR spectra, which detected an ester carbonyl group, and was supported by the fact that the peptide has a blocked N terminus (negative ninhydrin results on intact surfactant). The location of the closing of the lactone ring in the peptide was not determined. It could be either to the C-terminal amino acid carboxy group or to side chain carboxy groups of aspartic acid or glutamic acid at a location within the peptide sequence.

The IR and NMR spectra detected the presence of a long aliphatic chain. The NMR integration indicates an average length of 8–9 methylene groups. It is also suggestive of a mixture of linear and branched lipids. Eleven to 12 terminal methyl groups (average 11.57) were calculated. The peptide portion could contribute 10 of the terminal methyl groups (i.e. five branched amino acids). That leaves an average of 1.57 for the number of terminal methyl groups of the lipid, hence the possibility of having a mixture of linear and branched lipids. It is not clear whether the branch is iso-methyl and/or anteiso-methyl in structure.

The information gained by the DCI-MS and LC-MS analyses of the surfactant mixture, as well as the DCI-MS and FAB-MS analyses of the separated surfactant fractions, confirmed that the surfactant is a mixture of structurally similar components. The major components vary in size from 979 to 1091 Da, in increments of 14 Da. The extremes of the molecular weight range were better detected by some techniques than others (e.g. the LC-MS detected better the lower end of the MW distribution, and the DCI-MS of the mixture detected more components at the high range of the MW distribution). The most abundant components of the mixture have molecular weights of 1021 and/or 1035 Da depending on the preparation. Molecular ion species of lower and higher MWs are present in smaller amounts, the least of which are those at the extremes of the MW distribution range. The difference of 14 Da (i.e. a methylene group) between the molecular ion species of BL-86 can be explained by differences in the lipid chain length, a structure of branched vs. linear lipid tail (by addition of a methyl group to a linear lipid chain), isoleucine or leucine vs. valine, and possibly in small amounts also asx vs. glx. A small number of molecules having a MW 1 Da higher than the BL-86 series is indicated by a variance from the standard calculated mass distribution of some the components in the DCI-MS spectra. The source and the structure of the even MW components could arise via substitution of glutamine or asparagine to glutamic acid or aspartic acid, respectively.

HPLC separation revealed at least 9 peaks. Seven molecular ion species were assigned to specific HPLC peaks by the accumulative results of the LC-MS, DCI-MS and FAB-MS analyses of BL-86: 979, 993, 1007, 1021, 1035, 1049 and 1063. The less abundant BL-86 molecular ion species detected by DCI-MS, 1077 and 1091, were not assigned to any specific HPLC peak. In addition, the HPLC fractions which had more than one peak were found to represent only one molecular ion species each. The results indicate the presence of several molecular structures for each given molecular ion species, possibly reflecting the presence of normal and/or various branched structures for the same carbon number. Such changes may result in a slightly different HPLC migration. An additional possibility for this slightly different HPLC migration could be micelle formation during separation.

Reverse phase HPLC and DCI-MS analyses clearly showed that BL-86 is different from surfactin and lichenysin. The DCI-MS analysis indicates that the structural difference between BL-86 and surfactin and lichenysin involves a change in the number of nitrogens in the surfactant molecule. Since BL-86 contains an odd MW

series of components, the structures must contain odd number of nitrogens in them, whereas surfactin and lichenysin must have an even number of nitrogens. This difference can be accounted for by either a different number of amino acids or a substitution of amino acid having one nitrogen vs. one containing two nitrogens.

The closest published lipopeptide structures that resemble the BL-86 as indicated from the cumulative data obtained are surfactin [2] and esperin [18]. Both contain the four amino acids, glutamic acid, aspartic acid, valine and leucine, and in both lipopeptides leucine is present in L and D isomers. The amino acid ratios of L-glutamic acid, L-aspartic acid, L-valine, L-leucine, and D-leucine for surfactin and esperin are 1.0 : 1.0 : 1.0 : 2.0 : 2.0, and 1.0 : 1.0 : 1.3 : 1.7 : 2.0, respectively. However, neither contain isoleucine as part of the molecular structure, nor do they contain asparagine of glutamine. The major differences between surfactin and esperin are: (1) the location of the closing of the lactone ring, which is to the C-terminal, backbone carboxy group of L-leucine in surfactin, and to the side chain carboxy group of the internal aspartic acid in esperin; (2) the presence of L-valine instead of L-leucine as the terminal amino acid in 30% of the molecules in esperin in comparison to surfactin which always has the same terminal amino acid L-leucine; and (3) the heterogeneity of the lipid tail length in esperin vs. a fixed structure and size in the surfactin lipid portion. However, recent data from our laboratory, as well as other laboratories [13,19], indicate variability in the lipid portion of surfactin as well. Recently, an enzyme has been isolated from *B. subtilis* ATCC #21332 which might be involved in surfactin biosynthesis, since its activation pattern is consistent with that of a peptide synthesizing multienzyme [13]. In addition, a genetic locus (*sfp*) has been identified in the *B. subtilis* ATCC #21332 chromosome which is responsible for surfactin production [16]. It is possible that the enzyme(s) that synthesize the peptide portion of this family of lipopeptides in bacteria belonging to the genus *Bacillus* has evolved in such a way that it synthesizes the closely related surfactin, esperin and BL-86 molecules with differences in specificity at the C-terminal amino acid of the chain, as well as differences in selectivity towards aspartic acid or glutamic acid vs. asparagine or glutamine. Thus, the distribution of the C-terminal amino acid in surfactin is 100% L-leucine, in esperin is 30% L-valine and 70% L-leucine, and in BL-86 seems to be 40% L-valine and 60% L-isoleucine. This distribution of asx and glx is 1/1 glutamic acid : aspartic acid in surfactin and esperin, and proposed 1 : 1 glutamine : aspartic acid or glutamic acid:asparagine in BL-86.

Based on the analytical data obtained for BL-86 surfactant and previous literature concerning lipopeptides produced by the genus *Bacillus* we propose the following

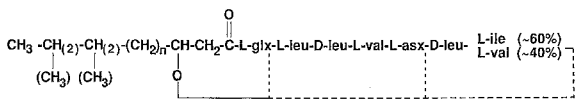

Fig. 5. Tentative structure of BL-86 surfactant.

description of BL-86 surfactant (Fig. 5). The surfactant is a mixture of lipopeptides with the major components ranging in size from 979 to 1091 Da in increments of 14 Da having more than one structure for some molecular ion species. The variations are due to changes in the lipid portion and/or the amino acid composition. The peptide portion contains 7 amino acids: L-glx : L-leucine : D-leucine : L-valine : L-asx : D-leucine : L-valine (40%) or L-isoleucine (60%), where one of the glx and asx is in the amine form (glutamine or asparagine) and the other is in the acid form (aspartic acid or glutamic acid). The sequence of the amino acids is undetermined, but is written tentatively based on the amino acid sequence of surfactin and esperin, with the unique portion put at the C-terminus of the peptide. The peptide is most likely cyclic in nature, having a lactone ring between the β position of the lipid and a carboxy group in the peptide. Either the peptide terminal carboxy group or the secondary carboxy group of aspartic acid or glutamic acid is involved. It is possible also that more than one kind of a lactone ring closure is present in the population of the surfactant molecules. The lipid portion of BL-86 is a long aliphatic chain, averaging 8–9 methylene groups, but possibly representing an even wider size distribution. The lipid is a mixture of linear and branched structures (possibly iso-methyl and/or anteiso-methyl).

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REFERENCES

1 Arima, K., A. Kakinuma, and G. Tamura. 1968. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun. 31: 488–494.

2 Arima, K., G. Tamura, and A. Kakinuma. 1972. Surfactin. United States Patent No. 3,687,926.

3 Bernheimer, A.W., and L.S. Avigad. 1970. Nature and properties of a cytological agent produced by *Bacillus subtilis*. J. Gen. Microbiol. 61: 361–369.

4 Cooper, D.G., C.R. MacDonald, S.J.B. Duff, and N. Kosaric. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42: 408–412.

5 Horowitz, S., J.N. Gilbert, and W.M. Griffin. 1990. Isolation and characterization of a surfactant produced by *Bacillus licheniformis* 86. J. Ind. Microbiol., In Press.

6 Hosono, K. and H. Suzuki. 1983. Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase I. Purification, physicochemical properties and structures of fatty acid residues. J. Antibiot. 36: 667–673.

7 Hosono, K. and H. Suzuki. 1983. Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase II. Amino acid sequence and location of lactone linkage. J. Antibiot. 36, 674–678.

8 Javaheri, M., G.E. Jenneman, M.J. McInerney, and R.M. Knapp. 1985. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. Appl. Environ. Microbiol. 50: 698–700.

9 Jenneman, G.E., M.J. McInerney, R.M. Knapp, J.B. Clark, J.M. Feero, D.E. Revus, and D.E. Menzie. 1983. A halotolerant, biosurfactant-producing *Bacillus* species potentially useful for enhanced oil recovery. Dev. Ind. Microbiol. 24: 485–492.

10 Kakinuma, A., M. Hori, H. Sugino, I. Yoshida, M. Isono, G. Tamura, and K. Arima. 1969. Determination of the location of lactone ring in surfactin. Agr. Biol. Chem. 33: 1523–1524.

11 Kakinuma, A., M. Hori, M. Isono, G. Tamura, and K. Arima. 1969. Determination of amino acid sequence in surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*. Agr. Biol. Chem. 33: 971–972.

12 Kakinuma, A., H. Sugino, M. Isono, G. Tamura, and K. Arima. 1969. Determination of fatty acid in surfactin and elucidation of the total structure of surfactin. Agr. Biol. Chem. 33: 973–976.

13 Kluge, B., J. Vater, J. Salnikow, and K. Echart. 1988. Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. FEBS Lett. 231: 107–110.

14 Marfey, P. 1984. Determination of D-amino acids. II. Use of a reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Res. Commun. 49: 591–596.

15 McInerney, M.J., G.E. Jenneman, R.M. Knapp, and D.E. Menzie. 1985. Biosurfactant and enhanced oil recovery. U.S. Patent No. 4,522,261.

16 Nakano, M.M., M.A. Marahiel, and P. Zuber. 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. J. Bacteriol. 170: 5662–5668.

17 Stahl, E. 1969. Thin Layer Chromatography, p. 745. Springer-Verlag (2nd).

18 Thomas, D.W., and T. Ito. 1969. The revised structure of the peptide antibiotic esperin, established by mass spectrometry. Tetrahedron 25: 1985–1990.

19 Vater, J. 1986. Lipopeptides, an attractive class of microbial surfactants. Prog. Colloid Polymer Sci. 72: 12–18.