Journal of Industrial Microbiology, 6 (1990) 243–248
Elsevier

SIM 00286

Isolation and characterization of a surfactant produced by *Bacillus licheniformis* 86

Sarah Horowitz, J.N. Gilbert and W. Michael Griffin

BP Research, Research Center Warrensville, Cleveland, OH, U.S.A.

(Received 12 October 1989; revised 11 December 1989; accepted 19 December 1989)

Key words: Biosurfactant; Lipopeptide; Surfactin; Lichenysin

SUMMARY

Surfactant (BL86) was isolated from foam produced during growth of *Bacillus licheniformis* 86 by acid-precipitation followed by extraction into tetrahydrofuran or methanol. The surfactant is anionic and dissolves in tetrahydrofuran, methanol, chloroform, dichloromethane, xylene, toluene, and alkaline water. The surfactant lowers the surface tension of water to 27 dynes/cm, and achieves the critical micelle concentration with as little as 10 μg surfactant/ml. Its interfacial tension can reach 0.36 dynes/cm when measured in 4% sodium chloride against *n*-hexadecane. The surfactant is stable from pH 4.0 to 13.0, at temperatures ranging from 25 to 120 °C, and in salt solutions ranging from 0 to 30% NaCl. Preliminary analytical results indicate that the surfactant is a mixture of lipopeptides different from previously reported *Bacillus* produced surfactants.

INTRODUCTION

Surfactants are molecules that tend to concentrate at the phase-boundary and alter the interfacial properties. A surfactant is an amphipathic molecule having two functional parts: a polar, hydrophilic head group, and a non-polar, lipophilic tail. The character of the surfactant is determined by the balance between its hydrophilic and lipophilic components. In solution, surfactant molecules tend to aggregate either with each other (micelle formation) or between phases of different polarity, such as oil/water. Surfactants are characterized by their surface tension (ST) reducing ability, critical micelle concentration (CMC), Gibbs surface excess, interfacial tension (IFT), and hydrophilic-lipophilic balance (HLB) [5,9,10,17,20,23,24].

There are two broad classes of surfactants: chemically synthesized surfactants and biologically produced surfactants, biosurfactants. Chemically synthesized surfactants are usually classified according to the nature of the polar group: cationic, anionic, and nonionic types. Although there are ionic and nonionic biosurfactants, usually they are categorized by their chemical composition and/or the producing organism. Five categories of biosurfactants have been reported: (1) glycolipids, (2) lipopolysaccharides and polysaccharide-lipid complexes, (3) lipopeptides, (4) phospholipids, and (5) fatty acids and neutral lipids [5,10,17,20,23,24]. Theory suggests that the natural role of microbial surfactants may involve adhesion to substrate and nutrients emulsification, desorption from surfaces, antibacterial and antifungal activities, and receptors for bacteriophage [20].

Several surfactants produced by different species and strains of *Bacillus* have been reported [5,10,20,22,23,24]. Many of these are lipopeptides [22]. The best characterized lipopeptide surfactant is surfactin (also named subtilysin and serolysin) and is produced by some strains of *Bacillus subtilis* [1,3,6]. Surfactin was patented in 1972 [2]. Suggested uses for surfactin include inhibition of stationary growth in bouillon of microorganisms belonging to the genus *Mycobacterium*, inhibition of fibrinogen-thrombin reaction (i.e., inhibition of fibrinogen clot formation), increasing the antifungal activity of antifungal agents, treating or preventing hypercholesterolemia, and inhibiting loss of activity of various active substances. Surfactin has also been used in promotion of plasmin-catalyzed and trypsin-catalyzed fibrinolysis [15], as a cytolytic agent in haemolysis (i.e., lysis of erythrocytes) [3], and as an antibiotic in lysis of protoplasts and spheroplasts derived from several bacterial species [3]. A surfactin like lipopeptide surfactant, lichenysin, is produced by *B. licheniformis* JF-2 [13,14] and was patented in 1985 [16] as an enhanced oil recovery agent.

Other peptidelipids are produced by strains of *B. subtilis*. For instance, NLF-I, which has a promoting effect on the lysis of Gram-negative bacteria (e.g.,

Correspondence: S. Horowitz, BP Research, Research Center Warrensville, 4440 Warrensville Center Rd., Cleveland, OH 44128, U.S.A.

Pseudomonas aeruginosa) [21] and Mycosubtilin [18,19] and Bacillomycin L [4], which are used as antifungal agents (antibiotics). *Bacillus circulans* produced the peptidelipid, NLF-II and the peptidelipid has been shown to promote lysis of Gram-negative bacteria [21].

We are currently investigating a novel lipopeptide surfactant produced by *B. licheniformis* 86. The surfactant has been designated surfactant BL86. This paper describes the initial isolation and characterization of the surfactant.

MATERIALS AND METHODS

Microorganisms. Bacillus licheniformis 86 was isolated by and obtained from J.E. Zajic (Petroleum Bioresources, Inc., El Paso, TX as strain PBR 1177). *Bacillus subtilis* 21332 and *B. licheniformis* 39307 were obtained from the American Type Culture Collection. The microorganisms were stored lyophilized and working stock cultures were maintained on Nutrient Agar (Difco) slants at 4 °C.

Culture conditions. The organisms were grown aerobically on Cooper's medium [6]. Small scale fermentations were performed using a 2-l fermentation vessel (MultiGen, New Brunswick Scientific) with a 1.6-l working volume. The cultures were grown at 30 °C, for 20 h, agitated at 275 rpm and air sparged (1.0 vvm). Large scale fermentations (16 l) were performed for production of *B. licheniformis* 86 surfactant and *B. subtilis* 21332 surfactin using a 20-l fermentor (L.H. Fermentation Series 2000) at 30 °C, for 20 h, agitated at 500 rpm and air sparged (0.75 vvm). The foam produced by the growing cultures, which contained the surfactants, was continuously collected.

Surfactant isolation. Bacterial cells were removed from the surfactant-containing foam by centrifugation (13000 × *g*, 10 °C, 15 min) for the 1.6-l fermentations and by continuous centrifugation (Sorvall KSB-R continuous flow system, 17210 × *g*, 5 °C, until the supernatant was clear) for the 16-l fermentations. The supernatant was then subjected to acid precipitation by adding concentrated HCl to a final pH of 2.0 and allowing the precipitate to settle at 4 °C. The acid precipitate was recovered by centrifugation (11000 × *g*, 4 °C, 20 min). The pellet was washed 4 times with acid-water (pH 2.0, by HCl) and lyophilized overnight. The surfactant was extracted from the powder into methanol for the 1.6 l fermentations and tetrahydrofuran (THF, spectrograde) or dichloromethane for the 16-l fermentations of *B. licheniformis* 86 or *B. subtilis* 21332, respectively. Surfactin was further purified as described by Cooper et al. [6].

The THF extracted surfactant BL86 was further processed by drying, using vacuum distillation at 40 °C, and washing 3 times in 5 ml of *n*-hexane each. The dried powder was an off-white color. The material was stored desiccated below 0 °C. In some cases the acid precipitate was neutralized to pH 7.0 prior to lyophilization. Tetrahydrofuran was replaced by methanol in extracting the surfactant BL86 in several preparations.

Surface and interfacial tension measurements. The ST measurements were done using a plate tensiometer (Universal Transducer Readout model SC1001, Gould Statham). Critical micelle dilution (CMD^{-1}) and CMC were determined by measuring the ST of 15 ml samples of a serial (1 : 1) dilution in water of the collected, cleared foam or the dissolved surfactant BL86, respectively. To evaluate the salt stability of surfactant BL86, the ST of 1 mg of surfactant (in 100 µl methanol)/15 ml alkaline-water was measured for 0, 5, 10,15, 17.5, 20, 22.5, 25 and 30% NaCl solutions.

The IFT measurements were done using a Spinning Drop Interfacial Tensiometer model 5000 (Gaertner Scientific Corporation).

Thin layer chromatography. One dimensional thin layer chromatography (TLC) was performed using silica gel G (Fischer Brand Redi/Plate). Samples of surfactant BL86 and surfactin were dissolved in methanol at a concentration of 25 mg/ml and 13 µl of each were spotted on the TLC plates. The solvent system was chloroform: methanol: 28% ammonium hydroxide, 65 : 25 : 4 [6]. Spots were visualized by sulfuric acid charring at 125 °C.

Reverse phase high performance liquid chromatographic analysis. Reverse phase high performance liquid chromatography (HPLC) was performed using a 30 cm C_{18} µ-Bondapak column (Waters) at 25 °C. The mobile phase was a 60 to 90% linear 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).

RESULTS

Isolation and characterization of the surfactant from B. licheniformis 86 fermentations

The 16-l fermentation results are presented in Table 1. On the average 284 mg of methanol purified surfactant BL86 was produced during the 20 h fermentations. The cell mass production was relatively consistent for the four fermentations. However, the amount of surfactant produced varied considerably ranging from 0.6 to 1.2 mg surfactant/g glc consumed. There appeared to be no obvious reason for the variability. The calculated volumetric productivity using these data was 22 mg/l/day.

Surfactant BL86 was recovered from the foam produced during the fermentation. Surface tension measurements on the collected foam prior to any manipulations showed a surface tension of 27 dynes/cm and had a

TABLE 1

Production of surfactant BL86 by *Bacillus licheniformis* 86[a]

Run no.	Surfactant (mg)	Yield cell mass (g CDW/g glc[b])	Yield of surfactant (mg surf./g glc)
1	221	0.26	0.6
2	349	0.29	1.2
3	213	0.28	0.8
4	354	0.25	1.2
Mean	284	0.27	0.95

[a] Fermentation conditions: volume, 16 l; agitation, 500 rpm; aeration, 12 l/min; initial pH, 6.8; time, 20 h; initial glc, 640 g; temperature, 35 °C; surfactant was acid precipitated and extracted into methanol.

[b] CDW/g glc, cell dry weight per gram of glucose.

CMD^{-1} value of approximately 32. No significant level of surface tension lowering activity remained in the spent medium ($CMD^{-1} < 1$).

Surfactant BL86 was found to be soluble in alkaline-water, THF, methanol, chloroform, dichloromethane, xylene, and toluene. It was not soluble in *n*-hexane. Extraction of the surfactant from the lyophilized acid-precipitate into methanol, chloroform, dichloromethane, or THF, does not change significantly the biosurfactant recovery, nor does it change the composition of the surfactant as demonstrated by HPLC analysis (data not shown).

Purified surfactant BL86 has excellent surface tension lowering activity. It reduces surface tension of water from 72 to 27 dynes/cm. Critical micelle concentration measurements performed on serial dilutions of the THF purified surfactant in alkaline water (pH 10.0 by NaOH) and measured in water (pH 7.0 by NaOH) reveal activity at very low concentrations, reaching a CMC value of 10 μg/ml (Fig. 1). Dissolving the THF purified surfactant in methanol, instead of water, does not changes these values. Extraction of the surfactant from lyophilized acid precipitate into methanol, chloroform, or dichloromethane, instead of THF, does not have any significant effect on the surfactant's surface tension reducing activity, nor on the measured CMC values.

The interfacial tension of the THF extracted surfactant BL86 was measured in alkaline-water in the presence or absence of 4% NaCl, against *n*-hexadecane. The lowest measured IFT in water was 2 dynes/cm (Table 2). Addition of 4% NaCl improved the interfacial tension reducing activity of the surfactant, reaching a value of 0.36 dynes/cm (Table 2).

The stability of surfactant BL86 was tested over a wide range of pH, temperature, and salt concentrations. The surfactant retained its surface tension lowering activity (27 dynes/cm) at 0 to 30% NaCl. Higher NaCl concentrations were not tested. The surfactant is stable for 20 min incubation at a temperature range of 25 to 120 °C (Table 3). The surface tension began to rise slightly above 75 °C. The effect of temperatures higher than 120 °C is not known because of equipment limitations. The surfactant is active in water up to a pH of 13.0. At the lower end of the pH scale (<4.0) activity is reduced due to precipitation of the surfactant (Table 4).

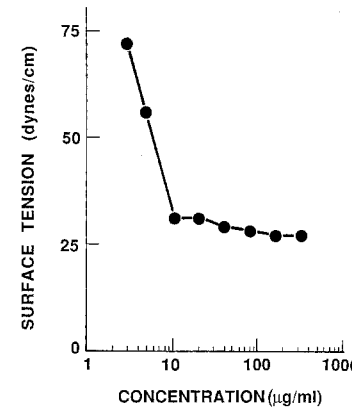

Fig. 1. Surface tension characteristics of surfactant BL86 as a function of concentration.

Comparison of surfactant BL86 with surfactin and lichenysin

One-dimensional TLC performed on the THF purified surfactant BL86 and dichloromethane purified surfactin revealed a significantly different chromatographic pattern. Surfactin migrated as a major spot on the plate, having a R_f of 0.34, which is very close to the reported R_f of 0.37 [6]. Surfactant BL86, on the other hand, separated

TABLE 2

Interfacial tension (IFT) of surfactant BL86 from *Bacillus licheniformis* 86[a]

Surfactant concentration (μg/ml)	IFT in alkaline H_2O (dynes/cm)	IFT in alkaline H_2O with 4% NaCl (dynes/cm)
166.0	5.45	0.455
41.6	2.07	0.363
21.0	2.08	2.365
10.4	7.16	ND[b]
1.3	13.3	ND

[a] IFT measurements were determined against *n*-hexadecane using a spinning drop interfacial tensiometer (Model 500, Gaertner Scientific Corporation).

[b] ND, not determined.

TABLE 3

Stability of the surfactant BL86 at various temperatures[a]

Temperature (°C)	Surface tension (dynes/cm)
25	27
50	27
75	29
100	29
120	33

[a] Samples of 1 mg surfactant/100 μl methanol in 15 ml alkaline-H_2O were subjected to 20 min incubation at different temperatures and then cooled to room temperature prior to surface tension measurements.

TABLE 4

Stability of the surfactant BL86 at various pHs[a]

pH	Surface tension (dynes/cm)
12.91	27
11.97	27
11.08	27
10.18	27
8.96	27
7.03	28
6.06	28
5.11	28
4.02	30
3.03	34
2.01	34

[a] Samples of 1 mg surfactant/100 μl methanol, dissolved in 15 ml alkaline-H_2O, were adjusted with HCl or NaOH to different pHs prior to surface tension measurements.

into two spots, having R_f values of 0.96 and 0.73 for the major and minor components, respectively.

The methanol extracted surfactants of *B. licheniformis* 86, *B. subtilis* 21332 and *B. licheniformis* 39307 were compared by analytical reverse-phase HPLC. The results, obtained by using a mass detector, are presented in Fig. 2. The pattern, which was consistent over several determinations, indicates that surfactant BL-86 is a complex mixture containing at least 8 major components. The other two surfactants, surfactin and lichenysin, appear to be complex mixtures having retention patterns different from that of surfactant BL86. However, some of the components of surfactant BL-86, surfactin, and lichenysin have similar retention values. Thus, the possibility exists, that along with the unique components in surfactant BL-86, similar compounds to those found in surfactin and lichenysin may be present in the BL-86 mixture, but in different proportions. Further analysis will be required to clarify this point.

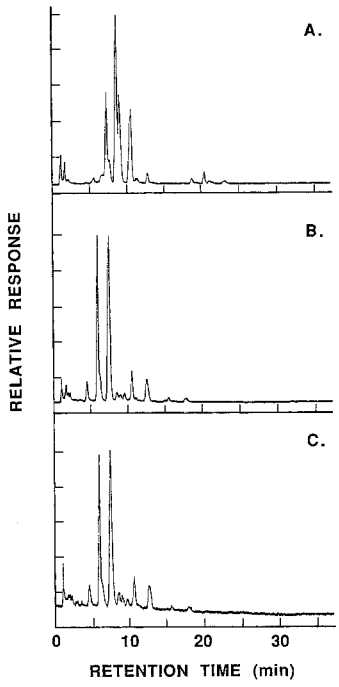

Fig. 2. Reverse phase HPLC analysis of methanol extracted surfactants. (A) Surfactant BL86. (B) Surfactin. (C) Lichenysin.

DISCUSSION

Surfactant BL86 can be easily isolated from foam of glucose based fermentations using acid precipitation. The highest final surfactant amount produced from any single fermentation run was 0.02 g of purified surfactant/l of culture volume. Other investigators have shown final concentrations of 0.1–0.7 g acid precipitated surfactant/l of culture volume for lichenysin [13,16] and up to 0.8 g of purified surfactant/l of culture volume for surfactin [6]. Using data from Cooper et al. [6] an estimated productivity of 220 mg/l/day can be calculated for surfactin production. This value is about 10 times greater than the average productivity for *B. licheniformis* surfactant production. The low productivity and final surfactant amount would need to be addressed before an economic production of such a material could be considered. Improvements, however, may be possible through growth and fermentor manipulations.

The surface tension lowering activity of surfactant BL86, 27 dynes/cm, is extremely good, and as low as that which has been obtained with any other biosurfactant [17]. Surfactant BL86 can, thus, be added to a small group

of biosurfactants which demonstrate such good activity, including surfactin (27 dynes/cm) [1,2,6], and lichenysin (28 dynes/cm in water and 27 dynes/cm in 5% NaCl) [13,14,16]. Surfactant BL86 is an extremely efficient surfactant, having a CMC of 10 μg/ml. For comparison the CMC of surfactin [6] and lichenysin [13] is 25 and ~20 μg/ml, respectively.

The interfacial tension reducing ability of surfactant BL86 against *n*-hexadecane is also very good, reaching a value of 2.1 dynes/cm in alkaline water and 0.36 dynes/cm in alkaline water containing 4% NaCl. As a reference, the IFT of surfactin against *n*-hexadecane at <1% NaCl is 1.0 dyne/cm [6], and that of lichenysin at 5% NaCl is 0.75 dynes/cm [14]. It may be possible to achieve better IFT values by optimizing the conditions under which the IFT is measured by the addition of salt and/or cosurfactants.

Surfactant BL86 is stable under extremes of temperature, salt concentration, and pH. The surface tension remains at 27 dynes/cm with 0 to 30% NaCl. Lichenysin, in comparison, has ST of 27 dynes/cm at 4% and 5% NaCl and increases slightly from 27 to 31 dynes/cm as the salt concentration decreases from 4 to 0%. However, more significant loss of activity occurs beginning at 7% NaCl and reaching 39 dynes/cm at 20% NaCl [14,16]. Although no data has been reported for surfactin one may assume similar properties as lichenysin, since they appear to have a similar composition as shown in Fig. 2. Surfactant BL86 was shown to be resistant to 20 min of incubation at temperatures ranging from 25 to 120 °C. Similar results have been reported for lichenysin and surfactin [2,14,16]. The *B. licheniformis* 86 surfactant retains its activity at pHs from 4.0 to 13.0. A slight loss of activity is observed at pHs below 4.0 due to surfactant precipitation. Surfactin and lichenysin show a similar pattern of pH stability. However, the pH range of activity is narrower: pH 6.0 to 12.0 for surfactin [6], and pH 6.2 to 10.0 for lichenysin [14,16].

Surfactant BL86 is a complex mixture as shown by HPLC. In addition, the HPLC indicates that surfactin and lichenysin are also mixtures. Data for surfactin supporting this result has been previously reported [8,11,22]. The HPLC pattern of surfactant BL86 is significantly different from that of surfactin and lichenysin, suggesting a different composition for surfactant BL86 than the other two. The pattern indicates that the *B. licheniformis* surfactant has 8 major components.

Preliminary results using preparative HPLC to collect individual fractions of surfactant BL86 and then measuring the ST of each indicated that the individual components had ST lowering activity. The chemical structure of the biosurfactant is presently under study. Results to date suggest that it is a mixture of lipopeptides containing asx, glx, val, leu, and ile. Previously reported amino acid analysis of surfactin indicates the presence of aspartic acid, glutamic acid, valine and leucine [1,7,12]. Isoleucine appears to be unique to surfactant BL-86.

The excellent ST, CMC, and IFT characteristics of surfactant BL86 and its stability over a wide range of pH, temperature, and salt concentrations suggest possible commercial applications. However, the low productivity must be addressed before this possibility can be exploited.

ACKNOWLEDGEMENTS

The authors would like to thank T.C. Li and J.B. Marsek for performing various portions of this work.

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