Foaming Properties of Lipopeptides Produced by *Bacillus subtilis*: Effect of Lipid and Peptide Structural Attributes

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To study the effect of lipid and peptide structural attributes of *Bacillus subtilis* lipopeptides on their foaming properties, the formation, stability, and appearance of foams prepared with surfactins (C_{13}−C_{15}) and iturins A (C_{14}−C_{17}) were characterized. The density and stability of lipopeptide foams depend on both the alkyl chain hydrophobic character and peptide molecular intrinsic properties. A lipidic chain length of 14 carbon atoms provides lipopeptides with the best foaming properties in terms of foam density and liquid stability in foams. Increases in alkyl chain length above 15 carbon atoms result in a drastic decrease of iturin A foam density. With the same alkyl chain, surfactin produces denser foam whereas iturin A exhibits better foaming stability. These results demonstrate the importance of the peptide structural attributes of *B. subtilis* on their foaming properties

Keywords: *Foam; lipopeptide; surfactin; iturin A; structural attributes; foaming properties*

INTRODUCTION

Foams are disperse systems in which gas bubbles are surrounded by a continuous liquid phase (Halling, 1981). These systems are encountered in many applications, such as the product formulation in food, cosmetic, detergent, and pharmaceutical areas, as well as particle transport, fire fighting, and enhanced oil recovery (Aubert et al., 1986; Kraynik, 1988). Their formation and stability require the use of surface-active agents that are amphiphilic compounds. Indeed, foaming properties are among the most important functional properties of surface-active agents (Rosen, 1989).

The efficiency of a surface-active agent for forming and stabilizing a foam depends mainly upon its molecular structure and intrinsic properties (Rosen, 1972; Kinsella, 1981), as well as the environmental conditions of formation and maintenance of the foam (Patino et al., 1995). Among intrinsic factors, the amphiphilic structure and molecular size appear crucial for foaming abilities of surface-active agents. A perfect foaming agent should rapidly adsorb at the air−water interface and reduce efficiently the surface tension for better foamability, but they should also form a cohesive viscoelastic film via intermolecular interactions for greater foam stability (German et al., 1985; Damodaran, 1994). The first two criteria are easily fulfilled by small surfactant molecules, which diffuse and orient rapidly at the air−water interface, considering their small size and their simple amphiphilic structure composed of a polar head and a hydrophobic tail. However, amphiphilic macromolecules such as proteins are more appropriate for the last requirement, because of their high molecular weight, bulky size, and multiamphiphilic structures, i.e., with multiple hydrophilic and hydrophobic sequences.

In recent papers, we reported the excellent foaming properties of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*, in comparison with sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) (Razafindralambo et al., 1996) and in association with BSA (Razafindralambo et al., 1997). We found that surfactin was more efficient than SDS and BSA on both foaming capacity and foam stability and exhibited a synergistic effect on the BSA foam stability with regard to drainage. Owing to their hybrid structure and intermediate size in comparison with small surfactant molecules and proteins, the lipopeptide compounds appear to be an attractive class of foaming agents. Those produced by *B. subtilis* are of particular interest since they exist in a variety of structures including isoforms (Baugmart et al., 1991; Peypoux et al., 1991; Itokawa et al., 1994) and homologous series (Isogai, 1982, Hosono and Suzuki, 1983). Isoform compounds differ in the amino acid composition of the peptide moiety, whereas homologous series vary in the number of lipidic chain carbon atoms.

The aim of the work presented in this paper was to study the relationships between the molecular structure of *B. subtilis* lipopeptides and their abilities to form and stabilize foams. Reliable information concerning the effect of structural elements on foaming properties would enable us to select optimum structures for further development. Also, such information would be helpful to molecular designing of new potent foaming agents for specific purposes.

A special emphasis has been focused on the lipid moiety effect by comparing foaming properties of lipopeptide homologous series. The importance of the

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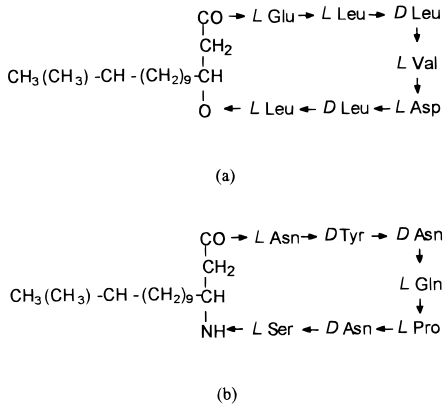

Figure 1. Examples of surfactin (a) and iturin A (b) primary structures.

peptide moiety characteristics has also been pointed out by comparing two lipopeptides having the same lipidic chain. Homologous series of surfactins (C_{13}−C_{15}) and iturins A (C_{14}−C_{17}) were used in this study.

EXPERIMENTAL PROCEDURES

Lipopeptide Production and Purification. Lipopeptides were produced by fermentation of the *B. subtilis* strain S499 in optimized culture media (Jacques et al., 1994) and extracted from the culture supernatant by solid-phase extraction on bond elut C_{18} (50 g; Varian, CA) as previously described (Razafindralambo et al., 1993). The crude extract was applied to a silica gel 60 column (30 × 1.5 cm, 50 g, 250−325 mesh; Merck, Darmstadt, Germany) and then eluted with chloroform/methanol/water (65/25/4, by vol). Three surfactins containing respectively β-hydroxy fatty acids of 13 (SuC_{13}, MW 1007), 14 (SuC_{14}, MW 1021), and 15 (SuC_{15}, MW 1035) carbon atoms and five iturins A containing β-amino fatty acids of 14 ($ItAC_{14}$, MW 1042), 15 ($ItAC_{15}$, MW 1056), 16 ($ItAC_{16}$-*n* and $ItAC_{16}$-*iso*, MW 1070), and 17 ($ItAC_{17}$, MW 1084) carbon atoms, respectively, were isolated from crude lipopeptide fractions by reversed-phase chromatography using a Chromspher 5 μm C_{18} column (1 × 25 cm, Chrompack, Middelburg, The Netherlands). The following conditions were used: flow rate at 4 mL/min, acetonitrile/H_2O/TFA 0.05% as mobile phase, under linear gradient (35−50% by vol in 25 min) and isocratic (85% by vol) conditions, respectively, for eluting iturins A and surfactins, UV detection at 214 and 280 nm simultaneously. The primary structure (Figure 1) and purity of the lipopeptide homologous series (>99%) were ascertained by analytical RP-HPLC (Chromspher 5 μm C_{18} column, 1 × 25 cm, Chrompack, Middelburg, The Netherlands), amino acid analysis (Razafindralambo et al., 1993), and electrospray mass spectrometry measurements using a VG Platform, Fisons Instruments (MA).

Analysis of Foaming Properties. Foaming properties were analyzed by the bubbling method using an automated apparatus as previously described (Guillerme et al., 1993; Razafindralambo et al., 1996; Razafindralambo et al., 1997). It consists of measuring continuously the foam volume and the amount of liquid in foam during and after its formation. Foam was formed by injecting a constant flow of air bubbled (20 mL/min) through a porous disk (pore diameter: 2 μm) situated at the bottom of a column (2 × 20 cm) containing 8 mL of solution. The bubbling was continued until a preset value of foam volume (35 mL) was reached (maximum foam volume). Then, the foam stability was monitored for 20 min.

Lipopeptide solutions (0.05−0.2 mg/mL) were prepared in 5 mM Tris buffer (pH 8.0) using MilliQ water (Millipore Co., Milford, MA). All measurements were carried out at 22 °C. Each analysis was performed at least twice.

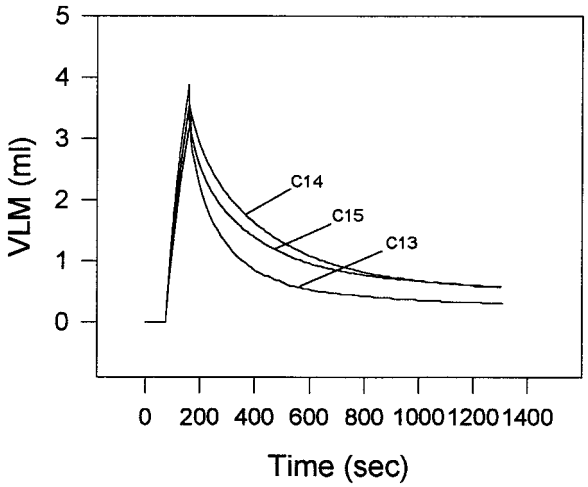

Figure 2. Volume of liquid in foam (VLM) vs time curves of surfactins at 0.2 mg/mL in 5 mM Tris buffer (pH 8.0).

Table 1. Foaming Capacity (FC), Maximum Density (MD), Initial Drainage Rate (LP1), and Half-Life of Liquid ($T_{1/2}$) in Surfactin Foams Prepared with 0.2 mg/mL Solution in 5 mM Tris Buffer at pH 8.0

surfactin	FC	MD	LP1	$T_{1/2}$ (s)
C_{13}	1.03 ± 0.01	0.09 ± 0.00	2.70 ± 0.24	81 ± 0
C_{14}	1.10 ± 0.00	0.11 ± 0.00	2.26 ± 0.03	171 ± 0
C_{15}	1.12 ± 0.00	0.10 ± 0.00	2.50 ± 0.00	141 ± 0

Foaming properties were characterized by various parameters including (1) foaming capacity (FC)

$$FC = \frac{\text{maximum volume of foam (mL)}}{\text{volume of gas injected (mL)}}$$

(2) foam maximum density (MD)

$$MD = \frac{\text{maximum volume of liquid (mL)}}{\text{maximum volume of foam (mL)}}$$

(3) initial drainage rate (LP1) corresponding to the initial slope of the liquid amount vs time curves, and (4) half-life time ($T_{1/2}$) of the liquid in foam corresponding to the time (seconds) for the reduction of the liquid in foam to half of the maximum volume.

RESULTS AND DISCUSSION

Effect of the Lipid Moiety. Surfactins. Figure 2 shows an example of the liquid amount change in surfactin foams during and after bubbling. From these kinetic curves of foam formation and foam stability, various assessment parameters were calculated. Results are listed in Table 1. In terms of foaming capacity (FC), i.e., the amount of gas necessary to form the same foam volume, the surfactin homologous series shows similar performance. Likewise, the maximum densities (MD) of foams are quite equivalent for the surfactin homologous compounds. In contrast, significant differences are observed in their ability to control the liquid drainage according to the drainage initial rate (LP1) and the half-life ($T_{1/2}$) of liquid in the foam. It appears that SuC_{14} exhibits the highest foaming stability against the liquid drainage. Consequently, the hydrophobic character of the alkyl chain does not influence the surfactin foamability but it strongly affects their ability to stabilize the foam against the drainage. In addition, there exists an optimum lipidic chain providing surfactin with the maximum foam stability. This observation

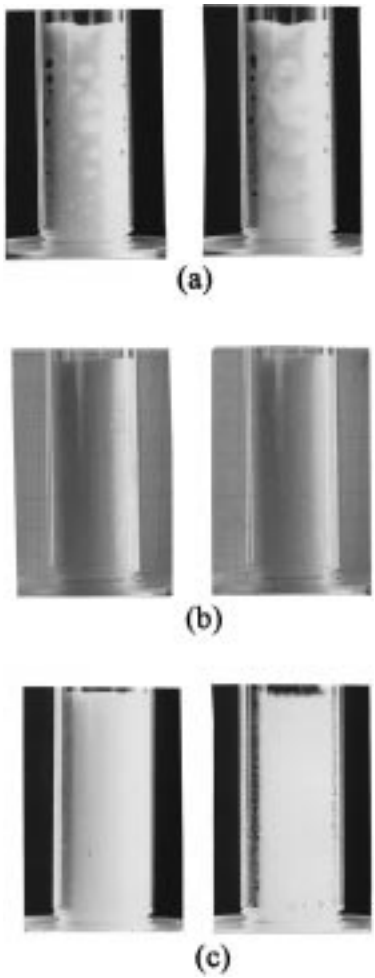

Figure 3. Appearances of surfactin foams at $t = 0$ (on left) and $t = T_{1/2}$ (on right): (a) SuC_{13}; (b) SuC_{14}; (c) SuC_{15}.

is in agreement with the trend reported by Rosen (1989) that good foaming agents have structures of intermediate alkyl chain length. Indeed, a too short chain produces insufficient cohesiveness, whereas a too great length produces too much rigidity for good film elasticity.

Foam appearances for surfactin homologous compounds just after bubbling and at a liquid half-life ($T_{1/2}$) in the foam are shown in Figure 3. As can be seen, all foams maintain their initial volume at $T_{1/2}$, showing the good stabilizing power of surfactins. However, the foam appearance is quite different, especially at $T_{1/2}$, for the three surfactins. Bubbles of different sizes are clearly visible in the SuC_{13}, foam, indicating an important physical destabilization like bubble disproportionation and/or coalescence. SuC_{14} and SuC_{15} foams are macroscopically more homogeneous. However, the SuC_{15} foam appears more dry at $T_{1/2}$, confirming a more important drainage rate observed from Figure 2, compared to SuC_{14}. Despite a notable liquid drainage in SuC_{15}, bubbles seem to be more resistant to physical destabilization, unlike what happened with SuC_{13}. This could be related to a more cohesive film formed by SuC_{15} due to a longer alkyl chain, which ensures greater molecular interactions. A film with sufficient cohesiveness and strength around the air bubbles would be more able to prevent physical destabilization of foam, like the disproportionation process, by retarding air diffusion from small bubbles to large ones (Walstra and de Roos, 1989).

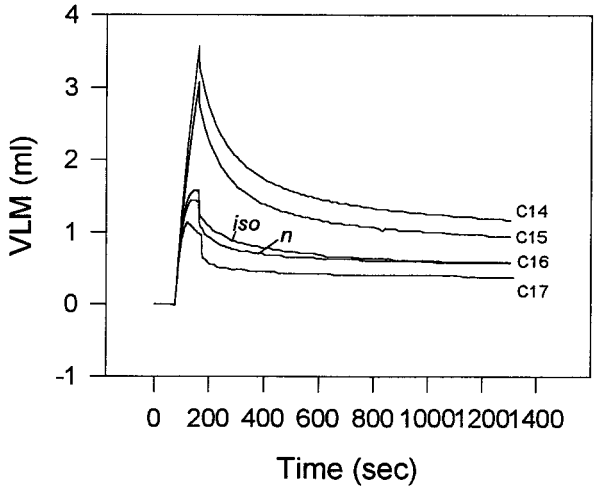

Figure 4. Volume of liquid in foam (VLM) vs time curves of iturins A at 0.2 mg/mL in 5 mM Tris buffer (pH 8.0).

Table 2. Foaming Capacity (FC), Maximum Density (MD), Initial Drainage Rate (LP1), and Half-Life of Liquid ($T_{1/2}$) in Iturin A Foams Prepared with 0.2 mg/mL Solution in 5 mM Tris Buffer at pH 8.0

iturin A	FC	MD	LP1	$T_{1/2}$ (s)
C_{14}	1.10 ± 0.00	0.10 ± 0.00	2.09 ± 0.08	206 ± 13
C_{15}	1.10 ± 0.01	0.09 ± 0.00	2.08 ± 0.08	176 ± 0
C_{16}-*iso*	1.10 ± 0.01	0.04 ± 0.00	1.52 ± 0.09	175 ± 35
C_{16}-*n*	1.08 ± 0.01	0.04 ± 0.00	1.57 ± 0.06	186 ± 34
C_{17}	0.99 ± 0.01	0.03 ± 0.00	2.04 ± 0.27	81 ± 8

Iturins A. Figure 4 compares the evolution of the liquid amount in iturin A foams during and after bubbling. Table 2 lists the foaming capacity (FC), maximum density (MD), drainage initial rate (LP1), and half-life ($T_{1/2}$) of liquid in foams prepared with iturin A solutions. As observed with surfactins, iturin A homologous compounds have the same FC. On the other hand, the amount of liquid in the foam over all the time, and then the MD, depend strongly upon the hydrophobic character of the lipidic chain. MD decreases with the increasing number fatty acid carbon atoms. However, the relationship between the hydrophobic character of the fatty acid and MD is not linear. A sharp break is observed when the alkyl chain passes from C_{15} to C_{16}, the MD value being reduced by half. These results suggest a significant change in the behavior of iturin A molecules at the air−water interface when the fatty acid contains more than 15 carbon atoms. This is in agreement with the dynamic adsorption and monolayer properties, which showed clear discrepancies between iturins A with shorter chains (C_{14}, C_{15}) and those with longer chains (C_{16}, C_{17}) (Razafindralambo, 1996). Indeed, iturins A with shorter chains are more efficient in reducing the dynamic surface tension than those with longer chains, but the latter form a more rigid film at the air−water interface than the former.

Moreover, the results show that $ItAC_{16}$ with linear and *iso* fatty acid chains exhibit equivalent foaming properties, indicating that the branching in the hydrocarbon chain does not influence the ability of iturin A to form and stabilize the foam.

Concerning the foam stability, a general trend in the decrease of the liquid stability with increasing lipidic chain length occurred according to the $T_{1/2}$ values, except for $ItAC_{16}$, for which the liquid stability appeared higher than that of $ItAC_{15}$. On the basis of the initial drainage rate values (LP1), ItC_{16} compounds give the

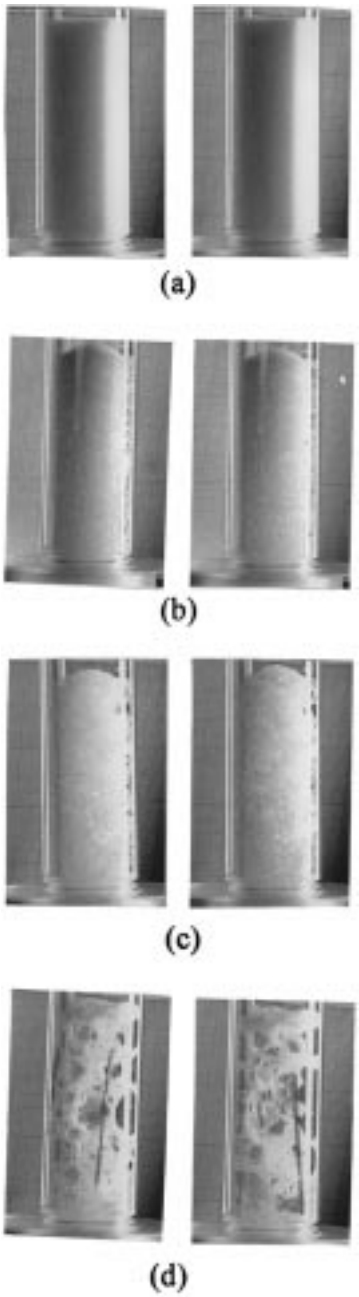

Figure 5. Appearances of iturin A foams at $t = 0$ (on left) and $t = T_{1/2}$ (on right): (a) $ItAC_{14}$; (b) $ItAC_{15}$; (c) $ItAC_{16}$-*i*; (d) $ItAC_{17}$.

highest liquid stability in the foam. This could be related to the lower liquid amount in the foam prepared with $ItAC_{16}$. Indeed, the drainage initial rate is mainly influenced by gravity, especially when the foam, is first formed (Rosen, 1989). The smaller the amount of liquid in the foam, as with ItC_{16}, the weaker is the gravity effect on the initial drainage rate. In the case of $ItAC_{17}$, the $T_{1/2}$ is very short since the liquid amount in foam is extremely low, resulting in fast bubble collapse. The alkyl chain length effect on the stability of iturin A foams is well illustrated by foam appearances just bubbling ($t = 0$) and at the half-life ($t = T_{1/2}$) of liquid in the foam (Figure 5). It clearly appears that the longer the alkyl chain of iturin A, the lower is the bubble stability. Bubble destabilization mainly occurs during the foam formation since the appearance of foams is quite similar at $t = 0$ and $t = T_{1/2}$. This also indicates that the initial drainage rate does not significantly affect

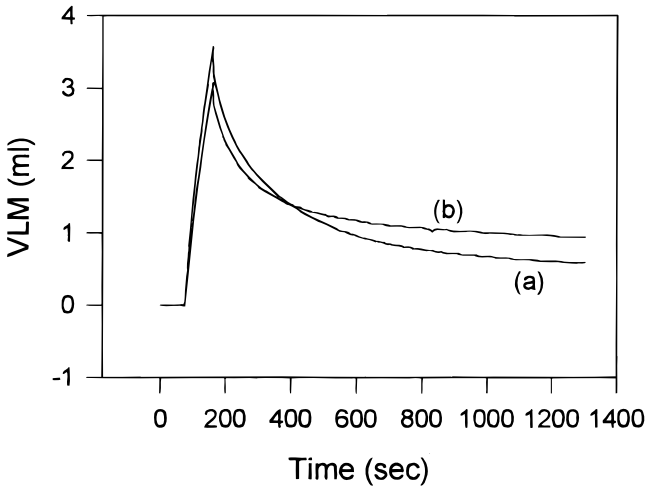

Figure 6. Comparison of the volume of liquid in the foam (VLM) vs time curves of SuC_{15} (a) and $ItAC_{15}$ (b) at 0.2 mg/mL in 5 mM Tris buffer (pH 8.0).

Table 3. Comparison of the Initial Drainage Rate and Half-Life of Liquid in SuC_{15} and $ItAC_{15}$ Foams Prepared at Different Concentrations in 5 mM Tris Buffer at pH 8.0

	initial drainage rate (mL/s)		liquid half-life (s)	
concn (mg/mL)	surfactin-C_{15}	iturin A-C_{15}	surfactin-C_{15}	iturin A-C_{15}
0.05	2.35 ± 0.08	1.07 ± 0.10	57 ± 3	98 ± 2
0.1	2.23 ± 0.04	1.52 ± 0.05	91 ± 0	330 ± 5
0.2	2.50 ± 0.00	2.08 ± 0.12	141 ± 0	171 ± 0

the bubble stability of iturin A foams. The negative effect of too long an alkyl chain length ($>C_{15}$) on bubble stability could be explained by a loss of film elasticity to the detriment of film rigidity, as already mentioned in the case of surfactin homologous compounds.

Effect of the Peptide Moiety. By comparison of the foaming properties of surfactin and iturin A having the same lipidic chain, the effect of the peptide moiety can be deduced. In Figure 6, the liquid amount in the foam vs time curves for SuC_{15} and $ItAC_{15}$ at 0.2 mg/mL are compared. Their ability to control the drainage was evaluated by the initial drainage rate (LP1) and the half-life of liquid in the foam ($T_{1/2}$). Such parameters are listed for SuC_{15} and $ItAC_{15}$ at different concentrations in Table 3. During a period covering the end of bubbling and the beginning of the drainage, the foam of SuC_{15} contains much more liquid than that of $ItAC_{15}$. Therefore, SuC_{15} is able to form a denser foam than $ItAC_{15}$ since the foam volumes with both lipopeptides are equivalent for this period. This result is not surprising considering the higher surface activity of surfactin compared to iturin A. Indeed, surfactin adsorbs faster at the air−water interface and reduces the surface tension more than iturin A (Thimon et al., 1992; Razafindralambo, 1996).

In contrast, $ItAC_{15}$ is more efficient at stabilizing the liquid in the foam than SuC_{15} according to the LP1 and $T_{1/2}$ values, whatever the concentration. The lower stability of liquid in the surfactin foam could be related to its fast adsorption rate at the air−water interface and, then, a too rapid decrease of the surface tension, which does not allow the liquid lamella thickness to be restored by the well-known Marangoni effect (Rosen, 1989). Indeed, the surface tension gradient between thicker portions and a thinned spot of liquid lamella

Table 4. Molecular Intrinsic Properties of the Peptides Constituting Surfactin and Iturin A

peptide characteristic	surfactin	iturin A
ionic nature	anionic	non ionic
hydrophobicity (kcal)[a]	1.614	0.778
secondary structure	β-sheet[b]	3 β-turns[c]

[a] Calculated according to Bigelow's method (1967). [b] Ishiganmi et al. (1995). [c] Marion et al. (1986).

should not disappear before the liquid has moved in to restore the lamella thickness.

Moreover, the initial liquid drainage rate (LP1) in $ItAC_{15}$ foam increases, and its liquid half-life goes through a maximum with increasing concentration from 0.05 to 0.2 mg/mL. In contrast, LP1 remains constant while $T_{1/2}$ lengthens with increasing concentration in the case of SuC_{15}.

The increase of lipopeptide concentration induces two opposite effects. It improves the rheological properties of interfacial films, which oppose the liquid flow, but it also induces the growth of the liquid amount in foams, which promotes liquid drainage by the gravity effect. Therefore, the results of LP1 values as a function of lipopeptide concentration indicate that SuC_{15} is able to control the gravity effect in a large range of concentrations, which could be related to its possibility to lower greatly the surface tension, even at low concentrations. This allows the pressure difference through the liquid lamella (Laplace pressure), which is one of two main factors causing drainage of liquid (Rosen, 1989), to be low. Consequently, the increase of $T_{1/2}$ with increasing surfactin concentration possibly arises from a better liquid lamella viscosity.

In the case of iturin A, the increase of concentration also improves the rheological properties of its interfacial films. This probably explains the increase of $T_{1/2}$ when the concentration passes from 0.05 to 0.1 mg/mL. However, the drop of $T_{1/2}$ at a higher concentration (0.2 mg/mL) indicates that the gravity effect becomes more important than that of the liquid lamella viscosity. This could be related to an insufficient reduction of the surface tension, inducing a high pressure difference through the liquid lamella, which promotes drainage of liquid.

All these results demonstrate the importance of the peptide moiety on foaming properties of lipopeptides produced by *Bacillus subtilis*. Among the peptide molecular attributes that differ in surfactin and iturin A, we can mainly distinguish the ionic nature, molecular hydrophobicity according to Bigelow's parameter, and secondary structure (Table 4). Indeed, surfactin is an anionic lipopeptide by the presence of two carboxylic groups (Asp, Glu), whereas iturin A is a non ionic lipopeptide. On the other hand, the surfactin peptide moiety seems to be organized in β-sheet secondary structure at the air−water interface (Ishigami et al., 1995), while the iturin A peptide backbone appears in a more rigid conformation by the presence of 3 β-turns (Marion et al., 1986). The importance of these structural and physicochemical properties on foaming properties of proteins has been reported in several review papers (Halling, 1981; Kinsella, 1981; Damodaran, 1994).

CONCLUSIONS

Foaming properties of *B. subtilis* lipopeptides depend both on the alkyl chain hydrophobic character and peptide molecular intrinsic properties. It clearly appears that the existence of an optimum lipidic chain length of 14 carbon atoms provides lipopeptides with excellent foamability and high ability in stabilizing foams. Increases in alkyl chain length above 15 carbon atoms result in a drastic decrease of iturin A foam density and then a lower foam stability. Concerning the effect of the peptide molecular attributes, it is difficult to establish precise relationships with foaming properties without further systematic investigation with isoform lipopeptides that only differ by one amino acid. Moreover, studies of lipopeptide conformation at the liquid interfaces should provide further insight into the structure−function relationships for such particular amphiphilic compounds. These works are now under investigation by our research group.

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