Surface-Active Properties of Surfactin/Iturin A Mixtures Produced by *Bacillus subtilis*

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Surface-active properties including dynamic adsorption, monolayer stability, micelle forming capacity, and foaming aptitudes of surfactin-C_{15}/iturin A-C_{15} mixtures were studied. Surfactin-C_{15} and iturin A-C_{15} molecules interact in synergism on the most surface-active properties evaluated at 20 °C at the air−water interface and in aqueous solution (pH 8.0). The synergism is positive on the adsorption effect, monolayer stability, foam density, and liquid stability in foam, whereas it is negative on the adsorption rate. No synergism occurs on micelle forming capacity, but surfactin-C_{15} and iturin A-C_{15} form mixed micelles when the solution contains a low proportion of surfactin-C_{15}. In all cases the synergistic effect is maximum when surfactin-C_{15} and iturin A-C_{15} molecules are mixed in a 2:3 ratio. This is attributed to the surfactin/iturin A complex formation resulting from specific interactions among two surfactin-C_{15} molecules and three iturin A-C_{15} molecules. A model of such a complex formation is proposed.

Introduction

Most *Bacillus subtilis* strains coproduce two or three classes of cyclic lipopeptides named iturins, surfactins, and fengycins.[1−4] These compounds can be distinguished by their chemical structure and properties. Surfactins consist of a heptapeptide containing a β-hydroxy fatty acid.[5−7] Iturins are also heptapeptides but they contain a β-amino fatty acid,[8] whereas fengycins are decapeptides containing a β-hydroxy fatty acid.[9] Each lipopeptide class is composed of closely related variants which differ in both amino acid composition and fatty acid alkyl chain varying from C13 to C17. Surfactins are especially potent surface-active compounds[10−12] exhibiting some biological properties such as a cytolytic activity[13] and antiviral properties.[14] Iturins and fengycins are both strong antifungal agents but fengycins are only active against filamentous fungi and less hemolytic than iturins.[9]

In recent studies, it has been demonstrated that lipopeptides belonging to surfactin and iturin classes interact in synergism on biological activities with enhancing hemolytic and antifungal activities up to 40%.[15,16] However, no study has especially been undertaken for evaluating synergism between lipopeptide classes produced by *B. subtilis* on their surface-active properties. This investigation is important with regard to biosurfactant properties, since in most practical applications mixtures rather than individual components are used. Therefore, the influence of the interaction between surface-active substances on the physicochemical properties of such mixtures, including the adsorption behavior and micelle formation, is of a great importance. Indeed, basic surface-active properties control most of the macroscopic or technological relevant aptitudes like foaming, emulsifying, solubilizing, and wetting powers for which biosurfactants are receiving increasing interest.[17,18]

In this paper, we report on the surface-active properties of surfactin-C_{15}/iturin A-C_{15} mixtures in various proportions at the air−water interface and in aqueous solution. Dynamic adsorption, monolayer stability, micelle forming capacity, and foaming aptitudes have been investigated in order to find synergism between such lipopeptides. Interactions between these compounds have also been studied by means of different thermodynamic parameters.

Experimental Section

Lipopeptide Production and Purification. Lipopeptides were produced by fermentation of the *B. subtilis* strain S499 in optimized culture media[19] and extracted from the culture

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(1) Sandrin, C.; Peypoux, F.; Michel, G. *Biotechnol. Appl. Biochem.* **1990**, *12*, 370.
(2) Razafindralambo, H.; Paquot, M.; Hbid, C.; Jacques, P.; Destain, J.; Thonart, P. *J. Chromatogr.* **1993**, *639*, 81.
(3) Ohno, A.; Ano, T.; Shoda, M. *J. Ferment. Bioeng.* **1995**, *80*, 517.
(4) Tsuge, K.; Ano, T.; Shoda, M. *J. Gen. Appl. Microbiol.* **1995**, *41*, 541.
(5) Kakinuma, A.; Ouchida, A.; Shima, T.; Sugino, H.; Isono, M.; Tamura, G.; Arima, K. *Agric. Biol. Chem.* **1969**, *33*, 1669.
(6) Peypoux, F.; Bonmatin J. M.; Labbe, H.; Das B. C.; Ptak, M.; Michel, G. E*ur. J. Biochem.* **1991**, *202*, 101.
(7) Baugmart, F.; Kluge, B.; Ulrich, C.; Vater, J.; Ziessow, D. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 998.
(8) Peypoux, F.; Bonmatin, J.; Labbé, H.; Grangemard, I.; Das B. C.; Ptak, M.; Wallach, J.; Georges, M. *Eur. J. Biochem.* **1994**, *224*, 89.
(9) Vanittanakom, N.; Loeffler, W.; Koch, U. Jung, G. *J. Antibiot.* **1986**, *39*, 888.
(10) Arima, K.; Kakinuma, A.; Tamura, G. *Biochem. Biophys. Res. Commun.* **1968**, *31*, 488.
(11) Razafindralambo, H.; Paquot, M.; Baniel, A.; Popineau, Y.; Hbid, C.; Jacques, P.; Thonart, P. *J. Am. Oil Chem. Soc.* **1996**, *73*, 149.
(12) Razafindralambo, H.; Paquot, M.; Baniel, A.; Popineau, Y.; Hbid, C.; Jacques, P.; Thonart, P. *Food Hydrocoll.* **1997**, *11*, 59.
(13) Bernheimer, A. W.; Avigad, L. S. *J. Gen. Microbiol.* **1970**, *61*, 361.
(14) Itokawa, H.; Miyashita, T.; Morita, H.; Takeya, K.; Hirano, T.; Homma, M.; Oka, K. *Chem. Pharm. Bull.* **1994**, *42*, 604.
(15) Thimon, L.; Peypoux, F.; Maget-Dana, R.; Roux B.; Michel, G. *Biotechnol. Appl. Biochem.* **1992**, *16*, 144.
(16) Maget-Dana, R.; Thimon, L.; Peypoux, F.; Ptak, M. *Biochimie* **1992**, *74*, 1047.
(17) Lin, S. *J. Chem. Technol. Biotechnol.* **1996**, *66*, 109.
(18) Ishigami, Y. *Inform* **1993**, *4*, 1156.
(19) Jacques, P.; Hbid, C.; Vanhentenryck, F.; Destain, J.; Baré, G.; Razafindralambo, H.; Paquot, M.; Thonart, P. *Proc. Eur. Cong. Biotechnol., 6th,* **1994**, *9*, 1067.

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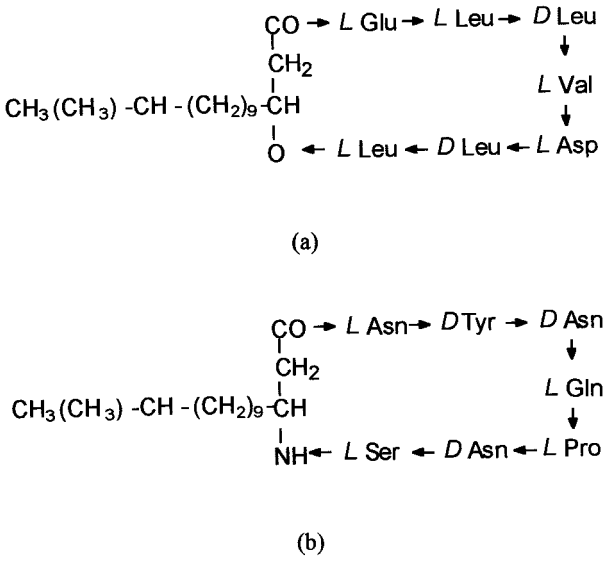

Figure 1. Primary structures of (a) surfactin-C_{15} and (b) iturin A-C_{15}.

supernatant by solid-phase extraction on bond elut C18 (50 g, Varian, CA) as previously described.[2] 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 volume). Surfactin and iturin A containing a fatty acid with 15 carbon atoms (Figure 1) were isolated from crude lipopeptide by reversed phase chromatography using a Chromspher 5 μm C18 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 volume in 25 min) and isocratic (80% by volume) conditions, respectively, for eluting iturin A and surfactin which were detected at 214 and 280 nm simultaneously. Primary structure and purity of the surfactin-C_{15} and iturin A-C_{15} were ascertained by analytical reversed phase high-performance liquid chromatography (chromspher 5 μm C18 column, 1 × 25 cm, Chrompack, Middelburg, The Netherlands), amino acid analysis,[2] and electrospray mass spectrometry measurements using a VG Platform, Fisons Instruments (MA, USA).

Dynamic Surface Tension Measurements. An automated drop volume tensiometer TVT 1 (Lauda-Königshofen, Germany) was employed for measuring the surface tension time dependence of lipopeptide solutions by the dynamic procedure described in detail by Miller.[20] It consists of the continuous formation of drops at the capillary tip with a definite diameter. Reaching a critical volume, the drop falls down and new ones will be created. Thus, the surface tension as a function of the drop time curves ($\gamma = f(t)$) can be established. In this method the surface is continuously renewed while the drop surface ages.

Drops were formed at a capillary tip of 1.055 mm internal radius connected to a Lauda syringe (2.5 mL). The drop forming time was from 0.07 to 4.06 s/μL. The Lauda system includes an option which allows the drop formation rate to be progressively reduced 2, 5, and 20 times as the drop volume increases. The hydrodynamic effects are minimized due to a liquid flow into a detaching drop. All measurements were carried out at 20 ± 0.1 °C. A mean of two drop volumes was used to determine the surface tension as a function of the drop formation time. Variation coefficients were smaller than 1% for both surface tension and drop formation time.

All samples were dissolved in 5 mM Tris buffer at pH 8.0 (Sigma, St Louis, MO) prepared with MilliQ water (Millipore Co., Milford, MA). Surfactin-C_{15} and iturin A-C_{15} samples were mixed in different proportions for total concentrations comprised between 1 and 50 μM. All other reagents were of analytical grade.

Curve $\gamma = f(t)$ Analysis. The $\gamma = f(t)$ curves of surfactin/iturin A mixtures were quantitatively described by the relaxation equation:[21]

$$\gamma_t = \gamma_m + (\gamma_o - \gamma_m)/[1 + (t/t^*)^n] \quad (1)$$

where γ_o is the surface tension of pure solvent, γ_t the surface tension at the time t, γ_m the surface tension at mesoequilibrium, t^* the half-time for reaching γ_m, and n a dimensionless constant. Parameters n, t^*, and γ_m were estimated by computer-fitting of the measured dynamic surface tension data using Sigma-plot software (Jandel, Germany). This model has been used to characterize the dynamic adsorption of surfactant mixtures.[22,23] By differentiating the eq 1 with respect to t and substituting t by t^*, the maximum reducing rate v_{max} of γ is obtained as follows

$$v_{max} = \frac{n(\gamma_o - \gamma_m)}{4t^*} = -(d\gamma_t/dt)_{max} \quad (2)$$

Monolayer Characterization. Monolayer properties were characterized by surface pressure−area isotherms established with an automated Langmuir film balance LFW2 3″5 (Lauda, Königshofen, Germany). Samples were dissolved in a 9:1 (v/v) mixture of hexafluoro-2-isopropanol/dioxan (Sigma, St. Louis, MO) and then spread onto the clean surface of the subphase (5 mM Tris buffer at pH 8.0) by depositing dropwise 30 μL of solution from a Hamilton syringe. Solvents were allowed to evaporate for 15 min after which the film was compressed by the moving barrier at the rate 61.8 $cm^2 \cdot min^{-1}$. All measurements were carried out at 20 ± 0.5 °C. Compression isotherm reproducibility was carefully checked by making at least three measurement sets for each surfactin homologous. The variation coefficients for both molecular area and surface pressure were less than 2.5%.

Equilibrium Surface Tension Measurements. Surface tension (γ) measurements were performed with a drop volume tensiometer TVT1 (Lauda, Königshofen, Germany) using the quasi-static mode as previously described.[24] Equilibrium surface tensions (γ_∞) were estimated by extrapolating the time to infinity for γ vs $t^{-1/2}$ curves. Drops of lipopeptide solution in Tris buffer (pH 8.0, 5 mM) were formed at the tip of the capillary with 1.055 mm internal diameter connected to a Lauda syringe (2.5 mL). The initial and further reductions of drop volume were comprised between 1 and 10% of the initial volume. All measurements were performed at 20 ± 0.1 °C. A mean of two drops was used to determine γ and t. The accuracy of γ and t measurements was less than ±0.05 mN/m and 1 s, respectively.

Determination of Critical Micelle Concentration and Related Parameters. Critical micelle concentration (cmc) was determined at the break of γ_∞ vs concentration curves.

Analysis of Foaming Properties. Foaming properties were analyzed by the bubbling method using an automated apparatus as previously described.[11,12,25] 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. All measurements were carried out at 22 °C. Each analysis was performed at least twice.

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)

(20) Miller, R.; Hofmann, A.; Hartmann, R.; Schano, K. H.; Halbig, A. *Adv. Mater.* **1992**, *4*, 370.

(21) Hua, X.; Rosen, M. J. *J. Colloid Interface Sci.* **1988**, *124*, 652.

(22) Rosen, M. J.; Zhu, Z.; Gao, T. *J. Colloid Interface Sci.* **1993**, *157*, 254.

(23) Rosen, M. J.; Gao, T.; Nakatsuji, Y.; Masuyama, A. *Colloid Surf., A* **1994**, *88*, 1.

(24) Razafindralambo, H.; Blecker, C.; Delahaye, S.; Paquot, M. *J. Colloid Interface Sci.* **1995**, *174*, 373.

(25) Guillerme, C.; Loisel, W.; Bertrand, D.; Popineau, Y. *J. Texture Stud.* **1993**, *24*, 287.

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

(3) half-life time ($T_{1/2}$) of liquid in foam corresponding to the time (s) for the reduction of the liquid in foam to half of the maximum volume.

Results

Dynamic Adsorption Properties. Figure 2 shows some examples of dynamic surface tension (γ_d) vs time plots for surfactin-C_{15}/iturin A-C_{15} mixtures at different molar ratios. Curves of mixtures are situated between those of surfactin-C_{15} and iturin A-C_{15} whatever the total concentration of lipopeptides. At 6×10^{-8} mol/cm^3, mixtures decrease and tend to stabilize more rapidly γ_d within 60 s when the surfactin molar ratio (Xs) increases. It appears that for Xs > 0.5, γ_d vs time plots are superimposed after 15 s.

In order to quantify the adsorption kinetics of surfactin-C_{15}/iturin A-C_{15} mixtures, we have fitted γ_d vs time data by eq 1. Figure 3 illustrates the results of fitting at a total lipopeptide concentration of 6×10^{-8} mol/cm^3. In Figure 4, adsorption parameters resulting from this fitting are plotted as a function of Xs. When Xs increases, γ_m decreases and reaches a minimum value at Xs = 0.4. At the same surfactin molar ratio, t^* reaches a maximum value. These results indicate specific interactions between surfactin-C_{15} and iturin A-C_{15} molecules mixed in a 2:3 ratio which lead to synergistic effects. The synergism is positive for γ_m and negative for t^*. Accordingly, such a mixture is more effective in reducing the dynamic surface tension at the mi-equilibrium but less efficient on the adsorption rate compared to individual components. The n and v_{max} values also reach a minimum for Xs close to 0.4 confirming that at this condition the adsorption rate for mixtures is slower than those for individual components. It is to be noted that constant n has been related to the difference in the adsorption and desorption rates.[26] The higher the n value, the faster the relative adsorption rate than the desorption rate. For Xs > 0.4, γ_m reaches a constant value. However, with increasing Xs from 0.4, t^* decreases while n and v_{max} increase indicating that the adsorption rate increases when the mixture contains further surfactin molecules.

Monolayer Properties. Compression isotherms ($\Pi-A$) of surfactin-C_{15}/iturin A-C_{15} mixed monolayers for different Xs at 20 °C are plotted in Figure 5. The curve shape for mixed monolayers is similar to that of surfactin-C_{15}, which is characterized by a sharp inflection at the surface pressure around 30 mN/m. The mean area A_o for mixed monolayers is always between those of individual components. However, the transition surface pressure (Π_t) of mixed monolayers appears higher than those of pure monolayers indicating the greater stability of mixed monolayers, particularly for Xs close to 0.4.

In order to study the nature and strength of the interactions between surfactin-C_{15} and iturin A-C_{15}, we have calculated a thermodynamic parameter, the excess free energy of mixing (ΔG_m^{ex}) by the Goodrich relation[27]

$$\Delta G_m^{ex} = \int_0^{\pi} A_{12}\, d\pi - x_1 \int_0^{\pi} A_1\, d\pi - x_2 \int_0^{\pi} A_2\, d\pi \quad (3)$$

where A is the mean molecular area, x the molar fraction, subscripts 1, 2, and 12 refer respectively to pure 1 and 2 components and to their mixtures. The ΔG_m^{ex} values were determined for different surface pressures (upper limit of integration) as a function of Xs (Figure 6). As can be seen, the ΔG_m^{ex} value is negative for all mixtures studied and becomes more important with increasing film compression state. A minimum value is observed at Xs = 0.4. Negative values of ΔG_m^{ex} indicate that the interactions between surfactin-C_{15} and iturin A-C_{15} are more important than those between surfactin-C_{15} and surfactin-C_{15} or iturin A-C_{15} and iturin A-C_{15}. Such mutual interaction is maximum for the surfactin-C_{15}/iturin A-C_{15} mixture in a 2:3 proportion.

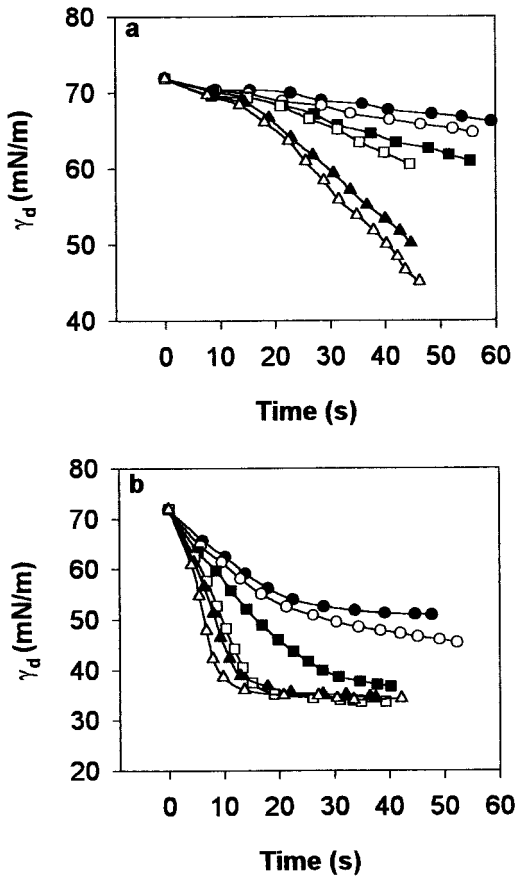

Figure 2. Dynamic surface tension (γ_d) vs time (t) curves of surfactin-C_{15}/iturin A-C_{15} mixtures at lipopeptide total concentration (a) 2.0×10^{-8} mol/cm^3 and (b) 6.0×10^{-8} mol/cm^3: (●) Xs = 0; (○) Xs = 0.2; (■) Xs = 0.4; (□) Xs = 0.6; (▲) Xs = 0.8 (△) Xs =1 (Xs, surfactin molar ratio).

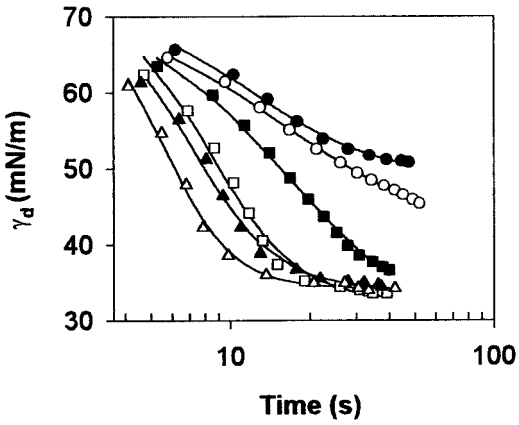

Figure 3. Curve fits calculated from the dynamic surface tension (γ_d) as a function of time for surfactin-C_{15}/iturin A-C_{15} mixtures in different surfactin molar ratios (Xs) at lipopeptide total concentration of 6.0×10^{-8} mol/cm^3: (●) Xs = 0; (○) Xs = 0.2; (■) Xs = 0.4; (□) Xs = 0.6; (▲) Xs = 0.8; (△) Xs =1.

Micelle Forming Capacity. The critical micelle concentrations (cmc) of surfactin-C_{15}/iturin A-C_{15} mixtures

(26) Gao, T.; Rosen, M. J. *J. Colloid Interface Sci.* **1995**, *172*, 242.
(27) Gaines, G. L. *Insoluble Monolayers at liquid-gas Interfaces*; Prigogine, Interscience: New York, 1966; Chapter 6.

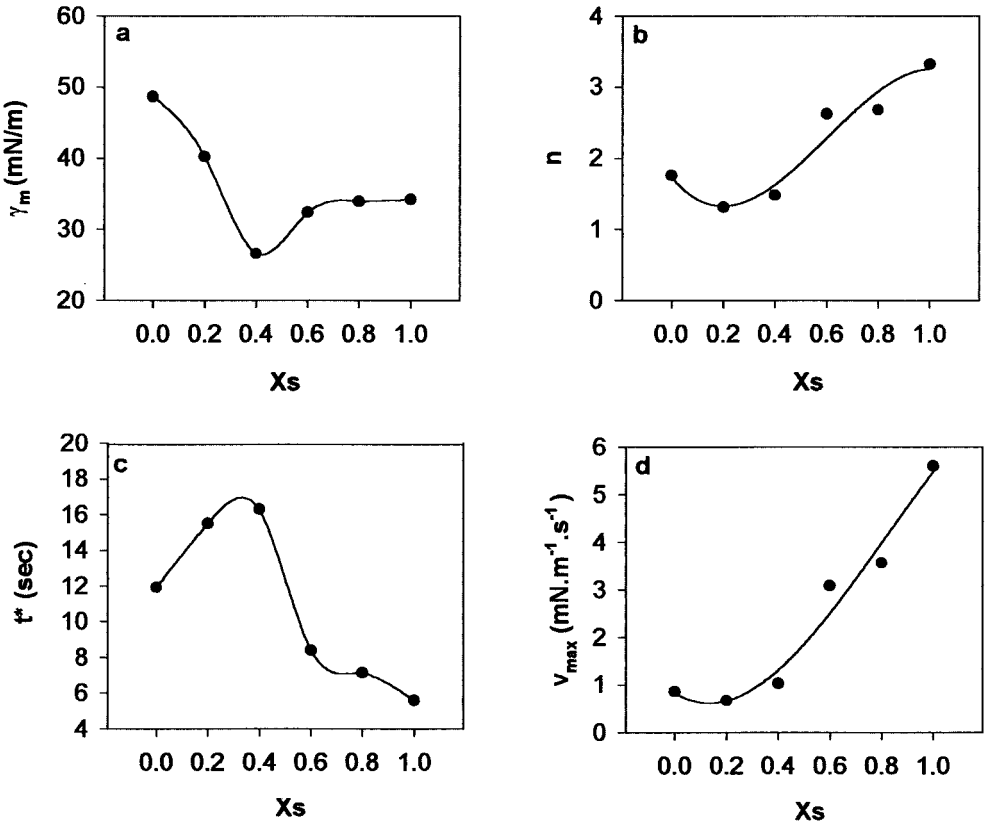

Figure 4. Adsorption kinetic parameters vs surfactin molar ratio (Xs) curves for surfactin-C_{15} /iturin A-C_{15} mixtures: (a) γ_m; (b) n; (c) t^*, (d) v_{max}.

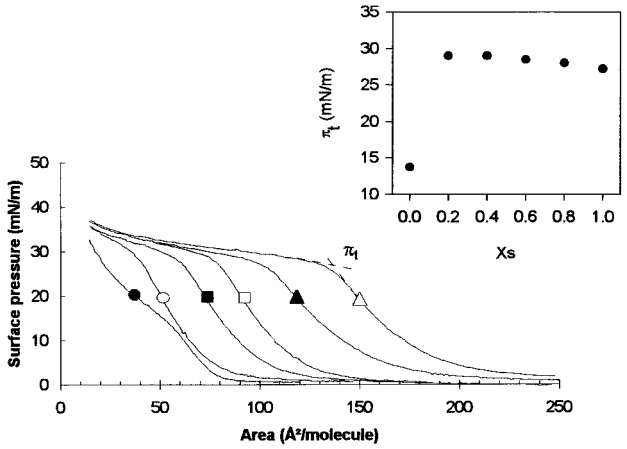

Figure 5. Compression isotherms for surfactin-C_{15}/iturin A-C_{15} mixtures as a function of the surfactin molar ratio (Xs) recorded at 20 °C with subphase constituted by Tris 5 mM pH 8 buffer: (●) Xs = 0; (○) Xs = 0.2; (■) Xs = 0.4; (□) Xs = 0.6; (▲) Xs = 0.8; (△) Xs =1. Insert curve: Change of transition surface pressure (π_t) as a function of Xs.

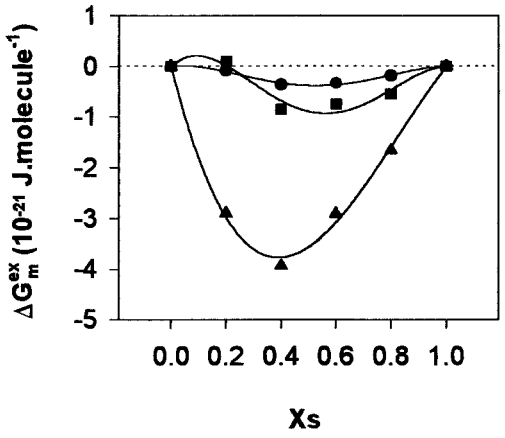

Figure 6. Excess free energy (ΔG_m^{ex}) vs surfactin molar ratio (Xs) for various surface pressure: (●) 10 mN/m; (■) 20 mN/m; (▲) 30 mN/m.

have been determined using the equilibrium surface tension vs log concentration plots. Figure 7 represents the dependence of the surfactin-C_{15}/iturin A-C_{15} cmc on the surfactin molar ratio. As the mixture cmc is never smaller than that of either individual components, at least in the range of composition and conditions studied, it is clear that there is no synergism between surfactin-C_{15} and iturin A-C_{15} on the micelle forming capacity. However, cmc values for Xs 0.2, 0.4, and 0.5 are lower than those predicted for perfectly ideal mixtures. For Xs > 0.5, cmc values coincide with those for perfectly ideal mixtures.

In order to obtain quantitative information, the molar fraction of surfactin-C_{15} (X_1^M) and the molecular interaction parameter (β^M) in surfactin-C_{15}/iturin A-C_{15} mixed micelles have been calculated using the following relations:[28]

$$\frac{(X_1^M)^2 \ln(\alpha C^M{}_{12}/X_1^M C_1^M)}{(1 - X_1^M) \ln[(1 - \alpha)C_{12}^M/(1 - X_1^M)C_2^M]} = 1 \qquad (4)$$

and

$$\beta^M = \frac{\ln(\alpha C^M{}_{12}/X^M{}_1 C^M{}_1)}{(1 - X^M{}_1)^2} \qquad (5)$$

where C_1^M, C_2^M, and C_{12}^M are the critical micelle concentrations of surfactin-C_{15}, iturin A-C_{15}, and their mixture,

(28) Rosen, M. J. *Surfactants and Interfacial Phenomena*; Wiley-Interscience: New York, 1989; Chapter 11.

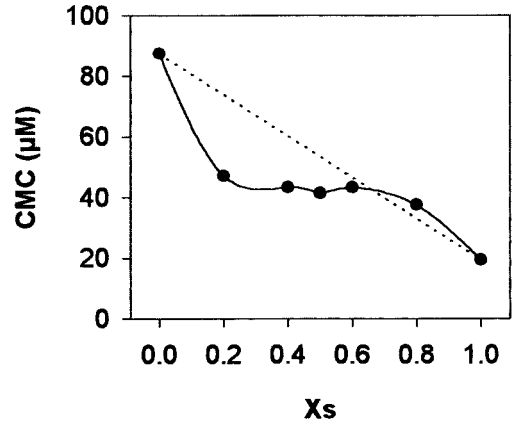

Figure 7. Variation of cmc as a function of the surfactin molar ratio (Xs).

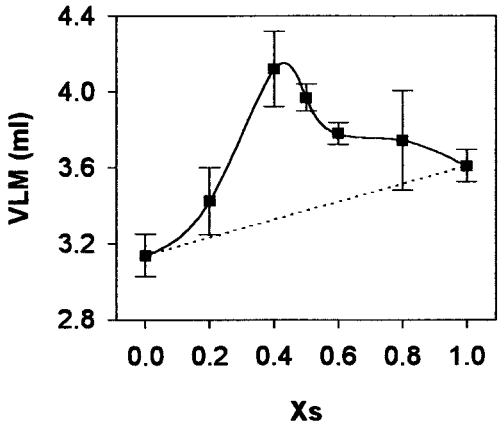

Figure 9. Maximum amount of liquid (VLM) in foam as a function of the surfactin molar ratio (Xs).

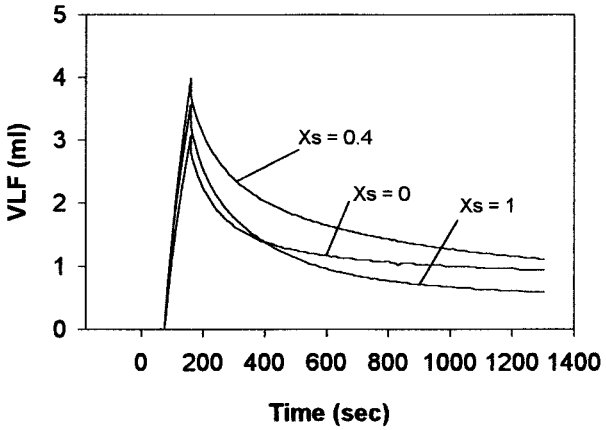

Figure 8. Amount of liquid in foam (VLF) vs time curves of surfactin-C_{15}/iturin A-C_{15} mixture for surfactin molar ratio 0.4 (Xs) compared to each component (Xs = 0, Xs = 1) at 0.2 mg/mL.

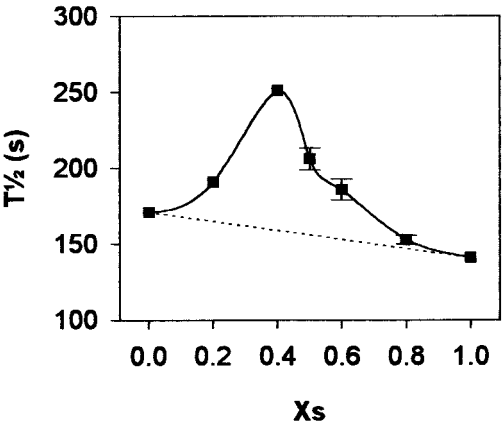

Figure 10. Half-life ($T_{1/2}$) of liquid in foam as a function of the surfactin molar ratio (Xs).

respectively, and α (Xs) the molar fraction of surfactin-C_{15} in solution. Equation 4 is solved iteratively for X_1^M, which is then substituted in eq 5 to obtain β^M. According to the parameter definitions, X_1^M values are equal to 0 and 1 for iturin A-C_{15} and surfactin-C_{15} pure components, respectively. For mixed lipopeptides, eq 4 provided X_1^M values of 0.5 and 0.9 for α = 0.2 and 0.4, respectively. These X_1^M values give respectively β^M values of −0.35 and 1.12 by means of eq 5, indicating the nature and degree of interaction between surfactin-C_{15} and iturin A-C_{15} in mixed micelles. However, only the β^M value for $X_1^M = 0.5$ (α = 0.2) is considered to be significant, taking into account the large errors that eq 4 may induce when X_1^M is close to 0 or 1. For α > 0.4, eq 4 was not solved by iteration for X_1^M, which means from the mathematical form of such an equation that X_1^M is equal to 1, considering the increase of the surfactin molar ratio in mixed micelles (X_1^M) with increasing surfactin molar ratio in solution (α).

Foaming Properties. Figure 8 shows an example illustrating the evolution of the maximum liquid amount in foam prepared with a mixture compared to that of either component. The foaming capacity of surfactin-C_{15}/iturin A-C_{15} mixtures is similar to that of individual components according to the bubbling time required for forming the same foam volume (101 ± 1 s). In contrast, the mixture solutions form foams with a higher maximum amount of liquid in foam (VLM), *i.e.*, with higher maximum density (MD), for the surfactin molar ratios ranging from 0.4 to 0.8 (Figure 9). These results show a synergism between surfactin-C_{15} and iturin A-C_{15} on the foam density. This synergistic effect is maximum at Xs = 0.4 for which the MD is enhanced up to 14% compared to that of pure surfactin (Xs = 1).

Figure 10 shows the dependence of the liquid half-life ($T_{1/2}$) in foam as a function of the mixture composition. As can be seen, the mixtures with Xs = 0.2 to 0.6 exhibit higher liquid stability in foam than individual components. This demonstrates a synergistic effect between surfactin-C_{15} and iturin A-C_{15} on the liquid stability in foam. It clearly appears that the maximum effect is obtained for the mixture with Xs = 0.4. The liquid stability in the foam is enhanced up to 66% compared to that of surfactin-C_{15}.

Discussion

All results presented here clearly indicate that surfactin-C_{15} and iturin A-C_{15} molecules interact specifically in a ratio 2:3 leading in most cases to the synergistic effects on surface-active properties at the air−water interface and in aqueous solution. An evident explanation is the formation of surfactin/iturin A (2:3) complex. In dynamic adsorption properties, all kinetic parameters (t^*, v_{max}, n) indicated the lowest adsorption rate for the mixture at Xs = 0.4. This agrees with the formation of a complex surfactin/iturin A which should adsorb slowly at the air−water interface than individual components because of its higher size and molecular weight. The positive synergism observed on the dynamic surface tension at the mi-equilibrium may be attributed to a greater

intermolecular strength in the adsorbed complex at the air−water interface, which is consistent with the monolayer properties of the mixtures spread at the air−water interface. First, the dependence of the surface pressure transition with the film composition indicates at least the partial miscibility of surfactin/iturin A mixed monolayers. Second, the mixed monolayer exhibits maximum stability for Xs close to 0.4 according to the surface pressure transition value which corresponds to the monolayer stability limit.[27] The negative and minimum value of the mixing excess free energy for Xs = 0.4 confirms that the surfactin-C_{15} and iturin A-C_{15} molecular interaction is maximum in mixed monolayer for Xs = 0.4, and this interaction is very strong when the mixed monolayer is highly condensed. This result is consistent with previous findings,[16] which indicated a maximum negative deviation from the additivity rule in the mean molecular area of surfactin/iturin A mixed monolayers for Xs = 0.4.

Similarly, the negative deviations of cmc from the additivity rule and X_1^M values clearly indicate the formation of surfactin-C_{15}/iturin A-C_{15} mixed micelles for Xs = 0.2 and 0.4. However, the ideal cmc observed for Xs > 0.5 and the trend of X_1^M values to increase with increasing Xs suggest that micelles would only be formed by surfactin molecules when the surfactin molar ratio of the mixture is superior to 0.5.

The synergism observed with the surfactin-C_{15} and iturin A-C_{15} mixtures on foaming properties also indicates that there exist specific interactions which are maximum for Xs = 0.4 among these two lipopeptides. This result suggests the formation of surfactin-C_{15}/iturin A-C_{15} complexes which would have a positive effect on the liquid stability in foam by enhancing the surface film viscosity, because of its voluminous size. Indeed, surface film viscosity is an important factor in foam stabilized by macromolecules like proteins: the greater the liquid film viscosity, the higher the capacity of proteins to stabilize foams.[29−31] Such a mechanism may explain the higher stability with regard to the drainage observed for the mixture with Xs = 0.4. This high viscosity could also explain the synergism in the foam maximum density obtained with the mixture solution. Indeed, it reduces the liquid drainage rate compared to the liquid lamella expansion rate during the bubbling, which enhances the amount of liquid in foam.

On the basis of the information published by other authors, we can propose a hypothesis on the surfactin-C_{15}/iturin A-C_{15} complex formation. Molecular modelizations investigated by Ishigami et al.[32] revealed on the one hand that surfactins form dimers at the air−water interface by hydrophobic interactions between alkyl chains. On the other hand, Bonmatin et al.[33] showed that the surfactin peptide cycle presents a horse "saddle" topology with a "claw" configuration resulting from the two acidic residues of Asp and Glu side chains. By combination of the dimerization and "claw" configuration of surfactin, three anchoring sites for iturin A-C_{15} molecules can be explained. Indeed, two iturin A-C_{15} molecules, or more exactly some of their residues, may be attached to the two main "claws" constituted by the acidic residues of each surfactin molecule of the dimer. A third iturin A-C_{15} molecule may be inserted between the two "claws" which constitute a third "claw". Such a hypothesis is supported by the fact that when the carboxylic groups were neutralized by high salt concentration or protonated by acidification of the media, no interaction occurred in mixed monolayers of surfactin and iturin A.[16] This enables us to account for the inaccessibility of the anchoring sites due to the presence of cations, or the "closing of the claws" by the absence of the electrostatic repulsion between the carboxylic groups. In addition, it has also been shown that iturin A-C_{15} molecules are able to penetrate the surfactin monolayer,[16] which is in agreement with the insertion of iturin A-C_{15} among surfactin-C_{15} molecules. In summary, we propose a schematic model of surfactin/iturin A complex presented in Figure 11.

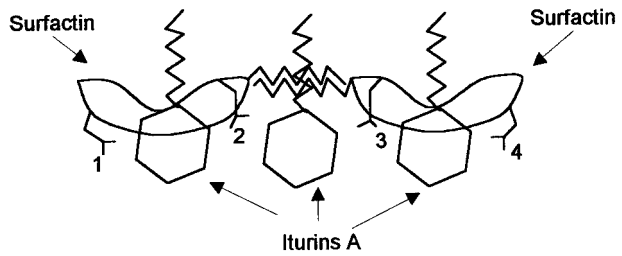

Figure 11. Surfactin/iturin A complex model; 1, 2, 3, 4 indicate carboxylic groups; (1,2), (3,4), and (2,3) constitute three claws.

Conclusion

Surfactin-C_{15} and iturin A-C_{15} interact at the air−water interface and in aqueous solution (pH 8.0) leading to synergistic effects on the most surface-active properties studied at 20 °C. Such synergism is positive on the dynamic adsorption effect, monolayer stability, and foaming properties including foam density and stability but negative on the adsorption rate. The interaction is maximum when two surfactin molecules are mixed with three iturin A molecules. This specific interaction apparently leads to the complex formation which may be explained by the dimerization of surfactin molecules and the "claw" model provided by the carboxylic groups of the surfactin peptide cycle. When the carboxylic groups are ionized, the "claws" are opened allowing the insertion of iturin A molecules. When they are protonated or neutralized by cations, the "claws" are closed or unavailable, and no interaction occurs between surfactin and iturin A molecules. The formation of such a complex may also explain the synergism in biological activity since this improves the adsorption and stability of iturin A at the interface or at the cytoplasmic membrane known as target.

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(29) Halling, P. J. *Critical Rev. Food Sci. Nutrit.* **1981**, *15*, 155.
(30) German, J. B.; O'Neill, T. E.; Kinsella, J. E. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1358.
(31) Walstra, P.; De Roos, A. L. *Food Rev. Int.* **1993**, *9*, 503.
(32) Ishigami, Y.; Osman, M.; Nakahara, H.; Sano, Y.; Ishiguro, R.; Matsumoto, M. *Colloid Surf. B* **1995**, *4*, 341.
(33) Bonmatin, J. M.; Genest, M.; Labbé, H.; Ptak, M. *Biopolymers* **1994**, *37*, 975.