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Interfacial and emulsifying properties of lipopeptides from *Bacillus subtilis*

M. Deleu [a,*], H. Razafindralambo [a], Y. Popineau [b], P. Jacques [c], P. Thonart [d], M. Paquot [a]

[a] *Unité de Chimie Biologique Industrielle, Faculté universitaire des Sciences agronomiques, 2 Passage des Déportés, 5030 Gembloux, Belgium*

[b] *Laboratoire de Biochimie et de Technologie des Protéines, Institut National de la Recherche Agronomique, 44026 Nantes, Cedex 03, France*

[c] *Unité de Bioindustrie, Faculté universitaire des Sciences agronomiques, 2 Passage des Déportés, 5030 Gembloux, Belgium*

[d] *Centre Wallon de Biologie Industrielle, Université de Liège, B40, 4000 Liège, (Sart-Tilman), Belgium*

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Abstract

The fundamental surface-active properties at the oil/water interface and emulsifying properties of surfactin, iturin A and fengycin, lipopeptides from *Bacillus subtilis*, were investigated. All lipopeptides reduce rapidly the dynamic interfacial tension. Among lipopeptide families, surfactin is the most effective in terms of fundamental dynamic and equilibrium interfacial properties. Lipopeptides present intermediate properties in comparison with sodium dodecyl sulfate and β-lactoglobulin concerning the stabilizing effect towards creaming-flocculation and the resistance to coalescence. Among lipopeptides, iturin A seems to show the best resistance to creaming-flocculation whereas fengycin exhibits the highest resistance to coalescence properties.

Keywords: Lipopeptides; Interface; Emulsifying properties

1. Introduction

Surface-active agents are required for forming and stabilizing disperse systems such foams and emulsions. They find applications in an extremely wide variety of industrial fields involving products formulation in food, cosmetic, road, pesticide, detergent, paper and pharmaceutical industries as well as enhanced oil recovery, transportation of heavy crude oil and bioremediation [1–5].

In molecular terms, surface-active agents are amphiphilic compounds containing both hydrophilic and lipophilic parts [6]. Their efficiency in foaming and emulsifying depends on their amphiphilic structure. In general, two main surface-active agents can be distinguished: small surfactant molecules and amphiphilic macromolecules.

Considering their small size and their simple amphiphilic structure composed by a polar head and a hydrophobic tail, small surfactant molecules diffuse and orient rapidly at fluid–fluid interfaces. They reduce efficiently the interfacial tension and promote the disperse system formation. On the other hand, amphiphilic macromolecules like pro-

* Corresponding author. Tel: +32 81 62 22 29; Fax: +32 81 62 22 31.
E-mail address: deleu.m@fsagx.ac.be (M. Deleu)

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teins have a high molecular weight and a more complex multi-amphiphilic structure. They migrate less quickly to the interface but form a cohesive viscoelastic film via intermolecular interactions for greater long-term stability [7,8]. A perfect surface-active agent should combine the favourable features of proteins with those of the most effective small surfactant molecules [6].

Lipopeptide molecules are typical compounds which could satisfy this condition owing to their hybrid structure and intermediate size in comparison with small surfactant molecules and proteins. Indeed, Razafindralambo *et al.* [9,10] reported in recent papers the excellent foaming properties of surfactin, a lipopeptide from *Bacillus subtilis*, in comparison with sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) and in association with BSA. In addition, as biosurfactant, *B. subtilis* lipopeptides are of increasing interest today because of their environmental compatibility that is their more biodegradability than those of many synthetic surfactants [3,11,12]. Moreover, they show a broad spectrum of molecular structures including isoforms [13–15] and homologous series [16,17] that could offer a wide selection of surface-active agents with properties closely tailored to specific applications. Isoform compounds differ in the amino acid composition of the peptide moiety, whereas homologous series vary in the number of lipidic chain carbon atoms. Three lipopeptide families are excreted by *B. subtilis* strain: surfactins; iturins; and fengycins [18–20].

Several articles reported surface-active properties of iturins and surfactins at the air/water interface [21–25]. But no information is available on lipopeptide properties at liquid/liquid interfaces that concerns emulsion field.

The present paper reports the fundamental surface-active properties at the oil/water interface and emulsifying properties of *B. subtilis* lipopeptides.

2. Experimental

2.1. Materials

Surfactin, iturin A and fengycin were extracted in semi-preparative scale from a culture medium of *B. subtilis* S499 by solid-phase extraction on bond elut C18 (50 g, Varian CA) as previously described [26].

The crude extract was applied to a silica gel 60 column (30×2.5 cm, 45 g, 250–325 mesh, Merck, Darmstadt, Germany) for fractionating the three lipopeptide families. Surfactin and iturin A were eluted with chloroform/methanol/water (65/25/4, by vol.) and fengycin with chloroform/methanol/water/ethanol (7/3/1.5/3.5, by vol.). Identification and purity of lipopeptide families were attested by infrared (IR) spectroscopy, amino-acid analysis and RP-HPLC [26]. Surfactin and iturin A are composed by a mixture of homologous molecules and fengycin is composed by two isoform compounds (A and B) containing homologous molecules. Surfactin, iturin A and fengycin structures are presented in Fig. 1(a–c).

Sodium dodecyl sulfate (SDS) was purchased from Fluka Biochemika (purity >98%, Buchs) and β-lactoglobulin (βlg) from Sigma (St. Louis MO). Dodecane was purchased from Sigma (purity >99%) and hexadecane from Merck (purity for analysis, Darmstadt, Germany). All other reagents were analytical grade. Milli-Q water was prepared by Millipore apparatus (Millipore Co., Milford, MA).

2.2. Preparation of lipopeptides, SDS and βlg solutions

All samples were dissolved in 5 mM Tris buffer prepared with Milli-Q water and adjusted to pH 8.0–8.5. Sample concentrations used were between 1 and 100 mg l^{-1}.

2.3. Dynamic surface tension measurements at the dodecane/water interface

Adsorption kinetics at the dodecane/water interface were monitored continuously by following the decrease in surface tension. The measurements were carried out with a drop volume tensiometer (TVT1, Lauda) used in dynamic mode. Adsorption parameters (n, t^*, v_{max} and γ_m) were determined following a method developed by Hua and Rosen [27] and Filippov [28].

Dynamic interfacial tension versus time plots

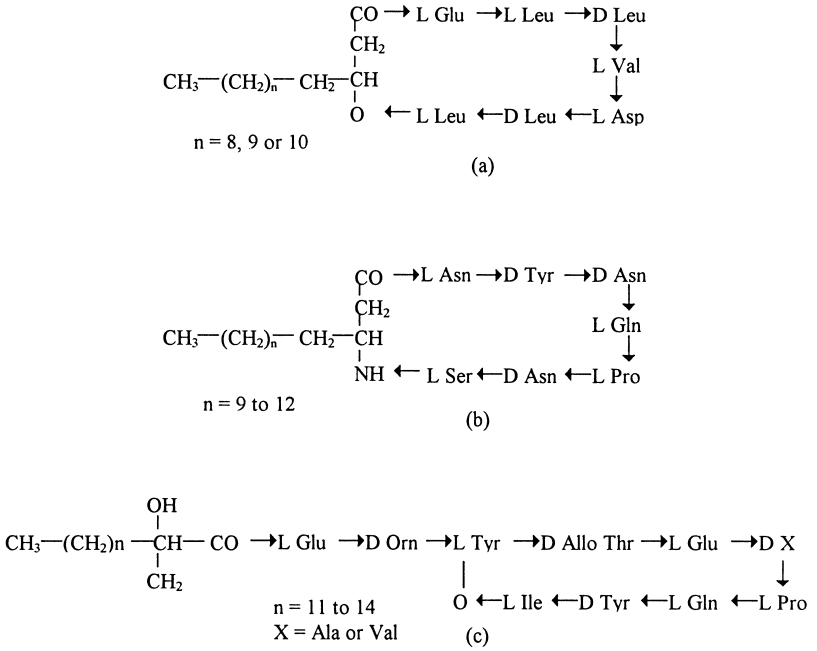

Fig. 1. (a) Structure of surfactin, (b) structure of iturin A, (c) structure of fengycin.

$\gamma = f(t)$ were described by the relaxation equation:

$$\gamma_t = \gamma_m + \frac{\gamma_o - \gamma_m}{1 + (t/t^*)^n} \quad (1)$$

where γ_o is the interfacial tension of pure solvents, γ_t the interfacial tension at the time t, γ_m, the interfacial tension at meso-equilibrium, t^* is half the time taken to reach γ_m, and n is a dimensionless constant. The parameters n, t^*, and γ_m were estimated by computer-fitting of the measured dynamic interfacial tension data using Sigma-plot software (Jandel, Germany). By differentiating Eq. (1) with respect to t and substituting t for t^*, the maximum reducing rate v_{max} of γ is obtained as follows:

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

2.4. Determination of critical micelle concentration

The critical micelle concentration (CMC) and the corresponding interfacial tension (γ_{CMC}) were determined at the intersection point of two linear segments from equilibrium interfacial tension versus concentration logarithm plots. Equilibrium interfacial tension was measured with the drop volume tensiometer used in quasi-static mode [29].

2.5. Emulsifying properties analysis

2.5.1. Determination of "flocculation-creaming" kinetics

An automated conductimetric method was used to determine the kinetics of phase separation [30]. Lipopeptide, SDS and βlg solutions (7.5 ml), and hexadecane (4.5 ml) were poured into a conductimetric cell made of a glass cylinder (diameter 18 mm) with two rod-like stainless steel electrodes precisely positioned at its base. The cells are built in racks of eight identical cells. The height of the electrodes corresponds to the initial level of the aqueous phase in the cell. The conductivity of the solution was measured before the mixing with the hexadecane. Then the emulsion was formed with an ultrasonic cell disruptor (Microson, Heat System), equipped with a probe with a tip 18 mm in diameter. The output power was 35 W and the

frequency 23 kHz. The time of emulsification was 15 s. Droplets formed in these conditions have an average diameter of *ca* 10 μm. Emulsification and measurement of conductivity were automated with the help of a microcomputer, a transfer robot (three axes displacement) and a home-made software. Particularly, the position of the tip of the ultrasonic probe is constant in all the cells, ensuring a good reproducibility of the emulsification. The conductivity was recorded simultaneously in eight cells at pre-programmed time intervals during 5 h. The change of conductivity measures the volume of aqueous continuous phase between the electrodes. It allows the emulsion stability to be determined, because it is directly related to the creaming of hexadecane droplets. Results are reported as the volume fraction of dispersed hexadecane in the emulsion (ϕ), calculated from:

$$\phi = 1 - \left[\frac{7.5}{4.5} \times \left(1 - \frac{C_t}{C_{sol}}\right)\right]$$

where C_{sol} is the conductivity of the aqueous solution before emulsification and C_t is the conductivity of the emulsion at time t.

The rate of destabilization of the emulsion (k_1) was measured between initial ϕ and $\phi + 0.1$ from ϕ versus time plots. Each measurement was only repeated two time but reproducibility tests on BSA show a variation coefficient inferior to 7% and display thus the good reproducibility of the method.

2.5.2. *Evaluation of the resistance to coalescence*

The evaluation of the resistance to coalescence was carried out by measuring the spontaneously separated hexadecane volume after 48 h, without centrifugation. Each measurement was duplicated.

3. Results and discussion

3.1. *Adsorption at the oil/water interface*

Figs. 2–4 show dynamic interfacial tension curves of surfactin, iturin A and fengycin at different concentrations, respectively.

Surfactin, iturin A and fengycin reduce dynamic

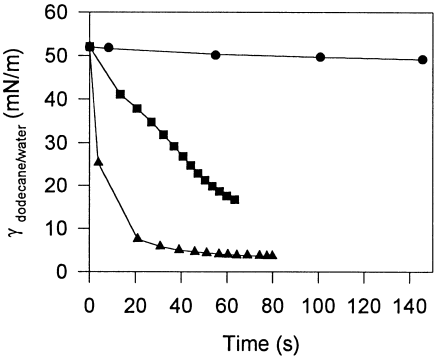

Fig. 2. Curves $\gamma_{int} = f(t)$ of surfactin solutions at different concentrations: ●, 1 mg l^{-1}; ■, 20 mg l^{-1}; ▲, 100 mg l^{-1} in a Tris 5 mM pH 8.0 buffer.

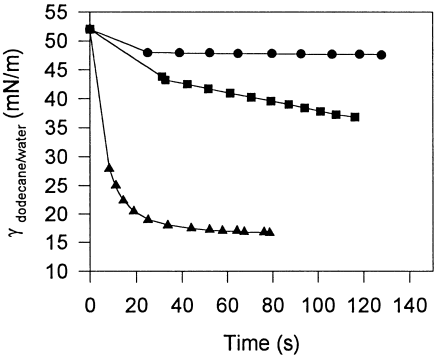

Fig. 3. Curves $\gamma_{int} = f(t)$ of iturin A solutions at different concentrations: ●, 1 mg l^{-1}; ■, 20 mg l^{-1}; ▲, 100 mg l^{-1} in a Tris 5 mM pH 8.0 buffer.

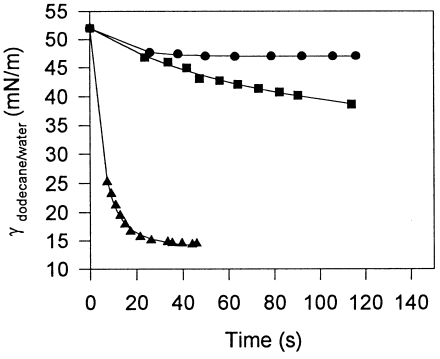

Fig. 4. Curves $\gamma_{int} = f(t)$ of fengycin solutions at different concentrations: ●, 1 mg l^{-1}; ■, 10 mg l^{-1}; ▲, 100 mg l^{-1} in a Tris 5 mM pH 8.0 buffer.

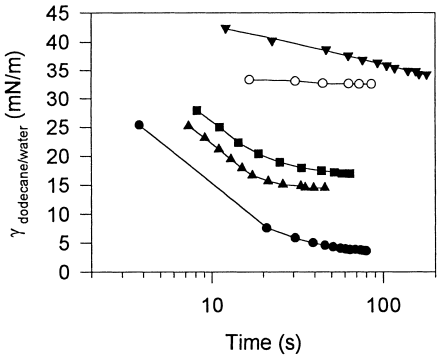

Fig. 5. Curves $\gamma_{int}=f(t)$ of: ●, surfactin; ■, iturin A; ▲, fengycin; ○, SDS; and ▼, βlg solutions at the dodecane/water interface (100 mg l^{-1} in a Tris 5 mM pH 8.0 buffer).

interfacial tension when they are in aqueous solution at concentrations >1 mg l^{-1}. These results reveal that lipopeptides from *B. subtilis* adsorb at the dodecane/water interface and present surface-active properties at this interface. The higher the concentration of lipopeptides, the faster the reduction of γ_{int} and the lower its final value. This observation was already established for lipopeptides at the air/water interface by several authors [22,23,25]. This general property is related to the amphiphilic character of lipopeptides due to the presence of a hydrophobic part consisting of the long chain fatty acid and some lipophilic amino acids, and a hydrophilic part composed by several amino acid residues.

The performance of the three lipopeptides in reducing the dynamic interfacial tension at the dodecane/water interface are compared in Fig. 5. Dynamic interfacial tension data were described by the relaxation equation [27]. Parameters γ_m, n, v_{max} and t^* are listed in Table 1.

Surfactin reduces the dynamic interfacial tension at the dodecane/water interface (γ_m) at a lower value than those of fengycin and iturin A.

According to the t^* value, which is the half time for reaching γ_m, surfactin reduces faster γ_{int} than fengycin and iturin A. Its maximal rate in reducing γ_{int} at t^* (v_{max}) is higher than those of fengycin and iturin A.

Concerning the parameter n, it is lower for surfactin compared to those of iturin A and fengycin. According to Gao and Rosen [31], the parameter n has been related to the difference between the adsorption rate and the desorption rate. The more the n value is near 0, the more the adsorption is near the equilibrium, that is, the adsorption rate is equivalent to the desorption rate. Based on this interpretation, surfactin reaches more quickly the equilibrium adsorption state at the dodecane/water interface in comparison with iturin A and fengycin.

Surface-active properties of fengycin and iturin A are similar in term of rate decay of γ_{int}. Nevertheless, fengycin is more effective than iturin A as regards the effect on the meso-equilibrium γ_m.

From Fig. 5, it appears that lipopeptides are more surface-active than SDS, a classical surfactant, and βlg, a protein, as regards the adsorption effect at the dodecane/water interface.

The difference between the families can be mainly attributed to the variability into the primary structure of the peptide cycle which generates the tridimensional structure at the interface. Previous studies have reported different conformations for iturin A, surfactin and fengycin peptide cycle [32–34]. The tridimensional structure of peptide combined with the presence of the lipidic chain could be a crucial parameter inducing their highest performance compared to small surfactant molecules and proteins such as SDS and βlg, respectively.

Table 1
Characteristic parameters of rate and adsorption effect of surfactin, iturin A and fengycin at 100 mg l^{-1}

Lipopeptides (100 mg l^{-1})	t^* (s)	n	v_{max} (mN m^{-1} s^{-1})	γ_m (mN m^{-1})
Surfactin	3.35	1.17	4.4	2.45
Iturin A	5.84	1.62	2.2	16.27
Fengycin	5.11	1.76	3.0	13.46

3.2. Interfacial properties at equilibrium

Plots of interfacial tension against log (concentration) of surfactin, iturin A and fengycin are shown in Fig. 6. Critical micelle concentrations and interfacial tensions corresponding to the CMC (γ_{CMC}) for the three lipopeptide classes are reported

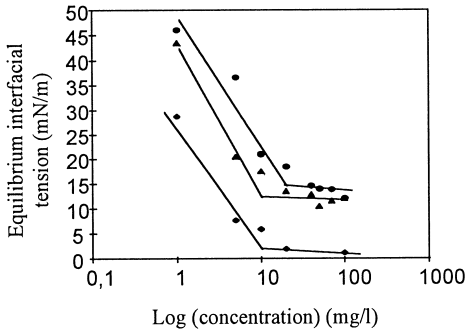

Fig. 6. Plots of equilibrium interfacial tension against log (concentration) of: ♦, surfactin; ●, iturin A; and ▲, fengycin.

in Table 2. CMC values for surfactin and fengycin are similar and lower than that of iturin A. As for the equilibrium interfacial tension, surfactin is more effective than fengycin, which is better than iturin A.

These results could be related to the hydrophobicity of the peptide cycle as already observed in previous works [28,35]. Surfactin peptide cycle ($H_{\phi ave}=1.61$ kcal per residue) is more hydrophobic than that of fengycin ($H_{\phi ave}=1.38$ or 1.49 kcal per residue according to the type of isoform molecule) which is more lipophilic than that of iturin A ($H_{\phi ave}=0.77$ kcal per residue) based on the average hydrophobicity of Bigelow [36]. However, the presence of several different lipidic chains prevents us from further interpretation.

3.3. Emulsifying properties

Three major mechanisms are involved in emulsion breaking: creaming; flocculation; and coalescence. The creaming and flocculation processes are closely related. This is why in our test flocculation cannot be distinguished from creaming and why we use the expression "flocculation-creaming". However, we can distinguish this global phenomenon which occurs quickly from coalescence which happens slowly. The kinetic of emulsion destabilization due to flocculation-creaming (k_1), and evaluation parameters of resistance to coalescence ($\phi_{5\ h}$, $\phi_{48\ h}$ and V_{hexad}) are reported in Table 3. At 0.1 mg ml^{-1}, the rate constant k_1 indicating the initial rate of flocculation-creaming was higher for lipopeptides than for SDS but was lower compared to that of βlg.

Among lipopeptides, iturin A seems to develop the best resistance to flocculation-creaming. For all samples, the emulsion shows no visible coalescence (separated hexadecane at the surface) after 5 h. This is very well reflected in the values of volume fractions ($\phi_{5\ h}-\phi_{24\ h}$). However, some very large droplets and separated hexadecane are observed after 24 h resting. This means that the emulsions are still evolving after 5 h and that coalescence only appears after a longer time of ageing, when water concentration of the cream is sufficiently low for the droplets come into contact. We do not assume a lag phase but a progressive evolution over a long period of time.

After 48 h, SDS releases an hexadecane volume higher than those of lipopeptides. In the other hand, βlg prevents phases separation (release $\cong$0%). Among lipopeptides, fengycin releases an hexadecane volume lower than the others. Thus, it exhibits the highest resistance to coalescence properties.

The flocculation phenomenon mainly depends on repulsion forces between droplets [37].

Table 2
CMC and γ_{CMC} of surfactin, iturin A and fengycin at the dodecane/water interface at 20°C, solutions prepared in a Tris 5 mM pH 8.0 buffer

Samples	CMC (mg l^{-1})	γ_{cmc} (mN m^{-1})
Surfactin	10	2.03
Iturin A	20	14.94
Fengycin	11	11.63

Table 3
Emulsion destabilisation rates, oil volume fractions after 5 h ($\phi_{5\ h}$) and after 48 h ($\phi_{48\ h}$), and percentage of separated hexadecane volume after 48 h of surfactin, iturin A, fengycin, SDS and βlg solutions prepared at a concentration of 0.1 mg ml^{-1} in a Tris 5 mM pH 8.5 buffer (oil phase: hexadecane)

Samples	k_1 (10^{-3} min^{-1})	$\phi_{5\ h}$	$\phi_{48\ h}$	V_{hexad} (%)
Surfactin	0.61	0.52	0.80	25.8
Iturin A	0.53	0.51	0.81	36.1
Fengycin	0.69	0.54	0.81	11.2
SDS	$\cong$0	0.43	0.82	65.7
βlg	1.50	0.66	0.83	0.7

According to our results, repulsions between droplets with lipopeptides are higher than those with βlg but lower than those with SDS.

Among lipopeptides, repulsions are more important in the case of iturin A. Since surfactin and fengycin are ionic lipopeptides whereas iturin A is non-ionic, it seems that repulsion forces involved here are of steric instead of electrostatic nature. Electrical charges of these two lipopeptides should be hidden, due possibly to their conformation at the interface.

For droplets coalescence prevention, the mechanical properties of the interfacial layer are crucial. A stiff and cohesive interfacial film resist better coalescence than a flexible film with a low viscoelasticity [38]. Owing to their ability to form a cohesive and viscoelastic film [39], proteins are more effective to resist coalescence than small molecule surfactants. This is in agreement with our results showing the highest coalescence resistance of βlg.

For molecules having a native structure, layer properties depend on molecule conformation and on residual secondary structures at the interface [40]. According to our results, fengycin presents an interfacial conformation more adapted to resistance to coalescence than surfactin and iturin A.

From the results, lipopeptides present intermediate properties in comparison with SDS and βlg concerning the stabilizing effect towards flocculation-creaming and resistance to coalescence. SDS is a small-molecule surfactant emulsifier often used as reference in emulsifying properties studies [41]. However, it could be interesting to compare lipopeptide emulsifying performance with those of other small-molecule surfactants as Tween for example.

4. Conclusion

It appears from these preliminary results that surfactin, iturin A and fengycin, natural lipopeptides from *B. subtilis*, present attractive fundamental interfacial and emulsifying properties at the oil/water interface. Concerning fundamental dynamic and equilibrium surface properties, surfactin is the most effective. As for emulsifying properties, iturin A seems to develop the best resistance to creaming-flocculation and fengycin exhibits the highest resistance to coalescence properties. These results give initial indications on the performance of lipopeptides as emulsifiers. However, lipopetides used in this study were composed by mixtures of homologous compounds, and also isoforms for fengycin. Further investigation of pure homologous molecules should provide more details on interfacial and emulsifying properties of lipopeptides and on the effect of the lipophilic part on these properties. Such studies are being investigated by our researchers' group.

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