Interdigitated Lamella and Bicontinuous Cubic Phases Formation from Natural Cyclic Surfactin and Its Linear Derivative

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1 INTRODUCTION

Surfactin, which is secreted by various stains of Bacillus subtilis, is one of the most powerful biosurfactants so far known. It is relatively abundant in nature and food products such as Natto (fermented soybeans) and consists of a heptapeptide linked to a β-hydroxy fatty acid and closed via a lactone bond as shown in Fig. 1 (a). Earlier studies showed that surfactin was able to reduce the surface tension of aqueous solution from 72 to 27 mN/m at the concentration of the order 10^{-5} M. Surfactin is also known to exhibit antiviral, antifungal, antitumor, and hemolytic properties. These remarkable physicochemical and biological properties hold promise for the potential application of surfactin in microbial enhanced oil recovery or bioremediation of the environmental pollutant, and in pharmacological research.

The unique cyclic structure as a headgroup is considered

Abstract: The lyotropic phase behavior of the cyclic form surfactin (CS) produced by Bacillus subtilis and its linear derivative in aqueous solution was evaluated for the first time by using polarized light microscopy and small-angle X-ray scattering (SAXS). By polarized light microscopy, the aqueous solutions of CS at the concentrations above 50 wt% were optically anisotropic and gave mosaic textures, suggesting the formation of lamella structures, while those of the linear surfactin (LS) were optically isotropic and no distinctive textures were observed. SAXS diffractograms of the CS solution above 50 wt% clearly gave the three peaks whose spacing ratio of 1: 2: 3, indicating the presence of the lamellar (L_α) phase, while those of the LS solution gave multiple peaks whose spacing ratios of √2: √3: √4: √6: √8, confirming the bicontinuous cubic (V_β) phase of the symmetry Pn3m. It was also found that the lamellar phase with CS was composed of not ordinary bilayer but interdigitated bilayer with the unusual packing of the acyl chain region. These results clearly demonstrated that the cyclic peptide structure plays a key role in regulating their self-assembly, and naturally occurring CS is likely to form lamellar structure by balancing bulky peptide headgroups with interdigitated packing of their acyl chains.

Key words: surfactin, cyclic peptide, lamella phase, cubic phase, biosurfactant, small-angle X-ray scattering (SAXS)
to induce above physicochemical and biological properties of surfactin. In aqueous solution, it was also found that the peptide ring of surfactin constructs a "β-sheet structure", often called "horse-saddle" topology\(^{12, 13}\). Where the two negatively charged amino acid residues such as Glu and Asp constitute a minor polar domain, Val residue faces the fatty acid chain, making up a major hydrophobic domain. Consequently, surfactin has a strong self-assembling ability to form micelles and the micelles tend to from larger aggregates. Knoblich et al. also reported the micellar shape and size of surfactin changed with the pH values by cryo-transmission electron microscopy\(^{14}\). However, most of the physicochemical studies performed on surfactin were limited only to the properties of dilute aqueous solutions.

Another unique feature of amphiphilic molecules such as surfactant is to form various lyotropic liquid crystals including lamellar, cubic, hexagonal structures\(^{15, 16}\). Such molecular behavior in aqueous solution with relatively high concentrations is also crucial not only for the application of surfactin in various fields, but also to understand its role in a biological system.

In this study, we reported for the first time the lyotropic phase behavior of surfactin\(^{\text{CS, Fig. 1(a)}}\) and its linear derivative\(^{\text{LS, Fig. 1(b)}}\) in aqueous solution by using polarized light microscopy and small-angle X-ray scattering (SAXS).

### 2 EXPERIMENTAL

#### 2.1 Materials and sample preparation

Surfactin was kindly supplied by KANEKA Co. Ltd, and was used without further purification. The structure and purity of the surfactin were ascertained by analytical RP-HPLC\(^{17}\) (Inertsil SIL-100A 5 μm C18 column, 0.46×25 cm, GL Sciences Inc.) using a mobile phase consisting of acetonitrile/water\(^{\text{(80/20)}}\) containing 0.1% trifluoroacetic acid\(^{\text{(TFA)}}\)\(^{17}\). MALDI TOF mass spectrometry\(^{17}\) (autoflex speed TOF/TOF, Bruker Daltonics Inc.) measurements were also performed using sinapic acid as a matrix.

A linear form of surfactin\(^{\text{LS}}\) was obtained by chemical cleavage of the heptapeptide cycle from the natural surfactin. The cleavage of the lactone ring was performed by dissolving the cyclic surfactin\(^{\text{(4.8 mmol)}}\) in 100 mL of methanol including sodium methoxide\(^{\text{(14.4 mmol)}}\) with stirring at 65°C for approximately 30 min. The solvent was then evaporated, and 40 mL of 1N HCl aq were added to the reaction mixture. Obtained precipitate was washed with water, and separated by centrifugation\(^{\text{(13000 rpm, 1 h)}}\). Sodium salt of LS was prepared by the addition of 2 equivalents of 1N NaOH aq.

#### 2.2 Visual inspection and polarized optical microscopy

Optically isotropic or anisotropic phase was identified by observation of the sample through cross polarizers. A polarized optical microscope\(^{\text{(ECLIPSE E600, Nikon, Japan)}}\) with crossed polarizing filters equipped with a charge-coupled device camera\(^{\text{(DS-5M, Nikon, Japan)}}\) was used to observe the anisotropic liquid crystalline phases. A halogen lamp\(^{\text{(100 W)}}\) was used as a light source. The type of the lyotropic liquid crystals was determined by their birefringent textures.

#### 2.3 Small-angle X-ray scattering (SAXS)

Measurements were performed using a SAXSess camera\(^{\text{(Anton Paar Co., Ltd., Graz, Austria)}}\). The measuring system consisted of a PW3830 laboratory X-ray generator with a long fine focus sealed glass X-ray tube\(^{\text{(PANalytical Co., Ltd., Almeo, Netherland)}}\)\(^{\text{(Cu-Kα (wavelength of 0.154 nm)}}\)\), a multilayer film Goebel mirror, a block collimator, a semi transmissible beam stop, a TCS120 temperature controller, a vacuum housing, and an imaging plate detector. Samples were sealed into a paste cell and the apparatus was operated at 40 kV and 50 mA. The measurable range of scattering vector\(^{\text{(q)}}\) was 0.06<q/\(\text{nm}^{-1}\)<27 and all measurements were corrected against transmittance by normalizing the primary beam intensity after the ray transmitted through a sample.

### 3 RESULTS AND DISCUSSION

The structure and the fatty acid composition of natural CS was firstly assigned by RP-HPLC and MALDI TOF mass spectrometry, and the CS produced by Bacillus subtilis\(^{\text{J. Oleo Sci. 62, (7) 499-503 (2013)}}\) was found to have the fatty acid composition of C13\(^{\text{(17%)}}\), C14\(^{\text{(52%)}}\), and C15\(^{\text{(31%)}}\). After chemical cleavage of CS with alkaline hydrolysis, the retention times of three RP-HPLC peaks observed were shifted lower, suggesting that LS are more hydrophilic than that of CS. Moreover, the molecular ion peaks corresponding to three HPLC peaks were detected at 1048.2, 1062.7, and 1076.3 m/z, which were well consistent with the sodium adducts of LS with different alkyl chain of C13, C14, and C15, respectively.

The lyotropic phase behavior of CS or LS in aqueous solution was then investigated by polarized microscopy and SAXS. The CS or LS aqueous solution with relatively high concentrations were prepared by vortexing for 1 min at 25°C. The solution was temperature-cycled several times between 25 and 70°C and then equilibrated at 25°C for at least 1 week.

Since both samples became viscous at the concentrations above 50 wt%, the samples above this concentration were observed by polarized microscopy. Interestingly, the aqueous solutions of CS were optically anisotropic and...
gave mosaic textures, suggesting the formation of lamella structures, while those of LS were optically isotropic and no distinctive textures were observed. This implies that the cyclic structure of the peptides on the headgroup plays an important role in the formation of lyotropic liquid crystals in aqueous solution.

To directly determine the structure of the observed lyotropic liquid crystals, SAXS measurements were performed at 25°C. Figure 2(a) shows the diffractograms of the liquid crystals at the CS concentrations above 50 wt%. There is no doubt that the cyclic form of natural surfactin forms a lamella structure whose spacing ratio of the first and second Bragg peaks are 1: 2: 3.

SAXS patterns obtained from LS solutions at the concentrations above 50 wt% were also shown in Fig. 2(b). From the diffractogram, the linear form of the surfactin was found to exhibit a bicontinuous cubic (V2) phase of the symmetry Pn3m (diamond type, QD) at 70 wt% as reported in other systems such as glyceryl monooleate (GMO)/water mixtures. These results clearly demonstrated that the cyclization of the peptide moiety regulates the spontaneous curvature of the assemblies: while cleaved surfactin forms inverse bicontinuous (V2) or hexagonal (H2) phases composed of negatively curved molecules, CS having a more bulky headgroup forms lamellar phase (Lα) generally obtains with almost zero curved molecules whose packing parameter is nearly 1.

To provide further insight into the lamellar structure composed of naturally occurring CS, the lamellar d-spacings of the [100] plane were estimated from the primary peak position (q") of the reflections of various concentrations using the equation of \( d_{100} = \frac{2\pi}{q}\). The obtained lamellar d-spacings were plotted as a function of CS concentrations (Fig. 3(a)). The figure shows that the d-spacing of the CS lamellar structure decreased with an increase in CS concentration, resulting from the shrinkage of water pool in the bilayer.

To estimate the bilayer thickness composed of CS, the d-

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**Fig. 2** SAXS patterns for the lyotropic liquid crystals of (a) CS and (b) LS.

**Fig. 3** The relationship between d-spacing from the primary Bragg peak of Lα and (a) CS concentration or (b) 1/mS.
spacings were further plotted as a function of $1/m_\text{s}$ according to the following equation (Fig. 3(b))\(^{20}\):

$$d = \left(1 - \frac{\rho_s}{\rho_w}\right)d_s + \frac{\rho_s}{\rho_w}m_\text{s}$$

(1)

Where $m_\text{s}$ is the mass fraction of surfactant, $\rho_s$ and $\rho_w$ are the densities of surfactant and water, and $d_s$ is the thickness of the surfactant bilayer. The thickness of CS bilayer, $d_s$, was estimated from a linear fit of the data to be 3.01 nm. Here the molecular length of CS with the energy minimized structure was estimated to be 2.16 nm by the calculation of the semi-empirical molecular orbital method (CAChe v. 6.1, PM5). Because the bilayer thickness generally becomes almost twice molecular length of CS (2.16 nm), the obtained experimental value (3.01 nm) was shorter than expected (4.32). This demonstrated that CS forms not an ordinary bilayer but an interdigitated bilayer as illustrated in Fig. 3(a). Unusual packing arrangement of the acyl chain probably leads the spontaneous curvature of CS with almost zero whose packing parameter is nearly 1 in spite of its bulky cyclic peptide headgroup. In general, interdigitated bilayers give a peak at wide angle region ($q = 15 \text{ nm}^{-1}$) derived from lateral dense packing of molecules in plane by SAXS\(^{21}\). However, no SAXS peak at wide angle region was detected for the present interdigitated bilayers (data not shown). This is probably due to the lose packing of the acyl chains of surfactin caused by bulky cyclic peptide moiety.

The formation of an interdigitated bilayer from natural CS is very interesting in the viewpoint of biology, because and anesthetics by polarized light microscopy, suggesting the formation of lamella structures, while those of the linear surfactin (LS) were optically isotropic and no distinctive textures were observed.

SAXS diffractograms of the CS solution above 50 wt% clearly gave the three peaks whose spacing ratio of 1 : 2 : 3, indicating the presence of the lamellar ($L_\alpha$) phase, while those of the LS solution gave multiple peaks whose spacing ratios of $\sqrt{2}$ : $\sqrt{3}$ : $\sqrt{6}$ : $\sqrt{8}$, confirming the bicontinuous cubic ($V_\gamma$) phase of the symmetry $Pn\overline{3}m$. It was also found that the lamellar phase with CS was composed of not ordinary bilayer but interdigitated bilayer with the unusual packing of the acyl chain region. This is the first observation of interdigitated lamella and bicontinuous cubic phase from natural cyclic surfactin and its linear derivative.

These findings could contribute not only to clarifying the physiological significance of surfactin but also to its practical applications toward various industries.

4 CONCLUSION

The formation of the lyotropic phase behavior of the cyclic form surfactin produced by Bacillus subtilis and its linear derivative in aqueous solution was evaluated. The aqueous solutions of CS at the concentrations above 50 wt% were optically anisotropic and gave mosaic textures by polarized light microscopy, suggesting the formation of lamella structures, while those of the linear surfactin (LS) were optically isotropic and no distinctive textures were observed.

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