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Aqueous Gel Formation from Sodium Salts of Cellobiose Lipids

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Abstract: Cellobiose lipids (CLs) are asymmetric bolaform biosurfactants, which are produced by *Cryptococcus humicola* JCM 10251 and have fungicidal activity. In this study, the sodium salts of CLs (CLNa) were prepared to improve aqueous solubility of the CLs, and their surface and gelation properties in aqueous solutions were examined by surface tension, rheology, and freeze-fracture transmission electron microscopy (FF-TEM) measurements. The surface tension measurements revealed that the CLNa have high surface activity: CMC_1 and γ_{CMC1} are 0.1 mg/mL and 34.7 mN/m, respectively. It was also found that the CLNa form giant micelles above their CMC, whose average size is 116.6 ± 31.9 nm. Unlike conventional surfactants, the surface tension reduced further with an increase in concentration and the aqueous solution became viscous at the minimum gelation concentration (MGC: 5.0 mg/mL). In rheological studies, the obtained gels proved to be rather soft and their sol-gel temperature was found to be approximately 50℃. FF-TEM observation of the gels showed 3D supramolecular structures with an entangled fibrous network. Since the present CLNa aqueous gels have a degree of fungicidal activity, they could be useful for novel multifunctional soft materials applicable to the food and cosmetic industries.

Key words: glycolipid biosurfactant, cellobiose lipid, surface tension, rheology, freeze-fracture transmission electron microscopy (FF-TEM)

1 INTRODUCTION

Biosurfactants (BSs) are natural amphiphilic molecules, which are produced by microorganisms from a variety of renewable resources[1, 2]. Because of their numerous advantages over synthetic surfactants, they have recently attracted attention as environmentally friendly surfactants that can contribute to a sustainable society. Among a variety of BSs, glycolipid BSs including mannosyl erythritol lipids (MELs)[3–5], sophorose lipids (SLs)[6], and cellobiose lipids (CLs)[7, 8] are promising, not only because they are produced with relatively high yields but also because they exhibit unique biological and surface properties. In fact, MELs and SLs are already commercially available, and they have been used in the cosmetic and detergent industries[9]. However, the number of studies on the properties and the potential application of BSs, especially for CLs, is still limited, which has prevented them from being applied more widely.

CLs are known to have unique "bolaform" structures composed of the cellobiose and carboxylic acid groups as polar headgroups at either end of a hydrocarbon core. Asymmetrical bolaform amphiphiles such as CLs tend to form versatile supramolecular structures under mild conditions and have high biological relevance as mimics of natural transmembrane lipids[10]. Recently, Kulakovskaya *et al*. reported that CLs exhibit fungicidal activity against various yeasts including pathogenic species[11, 12], and hence are potentially useful in animal feed and the protection from fungal spoilage of food. We recently succeeded in a high yield production of a CL, tetra-acetylated cellobiose bearing 2-hydroxy-hexadecanoic acid, by utilizing *Cryptococcus humicola*. The obtained CLs were found to act efficiently as low-molecular-weight (LMW) gelators toward various organic solvents by forming supramolecular struc-

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tures with a continuous three-dimensional (3D) entangled network[8].

In general, many gelators reported so far have a complex structure produced through a synthetic process to control noncovalent interactions including hydrogen bonding, π-π stacking, van der Waals interaction, and coordination[13, 14], and such gelators are not always suitable for large-scale industrial applications. Unlike general synthetic gelators, CLs can be produced simply and efficiently and since they undergo biodegradation and are non-toxic, they are environmentally friendly. Therefore, supramolecular gels including CLs that also have a degree of fungicidal activity have attracted attention for a wide variety of applications in cosmetics, drug delivery, photography, sensors, and food processing.

However, the aqueous solubility of CLs is quite low, and the surface activity and gel formation properties of the CLs in aqueous solutions have not been reported: this has prevented the use of CLs in practical applications. To that end, the sodium salts of the CLs (CLNa) were synthesized in this study to improve aqueous solubility after microbial production of the CLs. The surface and gelation properties of the CLNa in aqueous solutions were then investigated by surface tension, rheology, and freeze-fracture transmission electron microscopy (FF-TEM) measurements.

2 EXPERIMENTAL PROCEDURES

2.1 Production of CLNa

CLNa was produced by Toyobo Co. Ltd., using the following procedure[7]. First, *Cryptococcus humicola* JCM 10251 was obtained from the RIKEN BioResource Center of Japan. Then, seed cultures were prepared by inoculating cells grown on slants into test tubes containing a growth medium [10% (w/w) glucose, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, 0.1% yeast extract] at 27℃ on a reciprocal shaker (180 strokes/min) for 1 d. The seed cultures (60 mL) were finally transferred to a 10-L jar containing 6 L of a basal medium [10% (w/v) glucose, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, 0.1% yeast extract] and incubated (530 rpm) at 27℃ for 6 d.

After cultivation, the CLs that had accumulated in the culture medium were extracted with an equal amount of ethyl acetate/acetone (4/1). The ethyl acetate/acetone fraction was then separated and evaporated off. The obtained product was washed with hexane three times to get rid of impurities. The sodium salts of the CLs were prepared by the addition of 1 N NaOH aq. The structures of the purified CLs were then confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Voyager-DE PRO; Applied Biosystems, Carlsbad, CA) with α-cyano-4-hydroxycinnamic acid as a matrix.

2.2 Gelation test

A weighed amount of CLNa and a measured volume of water were put in a test tube (inner diameter, 1.0 cm); then, the test tube was tightly sealed with a screw cap and heated with shaking at 80℃ until all the solid material had completely dissolved. The solution was set aside and allowed to cool to 4℃ for 4 d. Finally, the test tube was turned upside down to observe if the solution inside could still flow, which is the so-called test tube-inversion method.

2.3 Surface activities

Surface tensions were determined by the Wilhelmy plate method at 25℃ using a DY-500 surface tension meter (Kyowa Kaimen Kagaku Co., Saitama, Japan), whose accuracy was frequently checked with ultra-pure water before use. The Pt plate used was cleaned by flaming and glassware was rinsed sequentially with tap water and ultra-pure water.

2.4 Rheological studies

An Anton Parr MCR302 rheometer with a cone plate was used. The gap distance between the cone and plate was fixed at 1 mm. The gels were scooped onto the plate of the rheometer. A frequency sweep experiment was performed at a constant strain amplitude of 1% and an oscillatory strain sweep was carried out at an angular frequency of 1 Hz. Both the measurements were done at 25℃. The rheometer has a built-in computer which converts the torque measurements into either G' (the storage modulus) or G" (the loss modulus) in oscillatory shear experiments. Heating was performed at the rate of 1℃/min.

2.5 Dynamic light scattering (DLS)

The size of assembled structures was measured with a DLS-7000 (Otsuka Electronics Co., Japan) at 25℃ using a He-Ne laser of 633 nm wavelength as a light source. The time-dependent correlation function of the scattered light intensity was measured at a scattering angle of 90°. The DLS intensity data were processed by using the instrumental software to obtain the hydrodynamic diameter, the polydispersity index, and the mass diffusion coefficient of the samples. The mass diffusion coefficient D was derived from the decay time (τ_C) of the intensity correlation function as $D = (2k_L^2\tau_C)^{-1}$, where k_L is the scattering wave vector. The hydrodynamic mass diffusion coefficient D_0 is obtained as the limit of D as k_L goes to zero. D_0 is found to obey the Stokes-Einstein relation, $D_0 = kT/6\pi\eta R_H$, where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, and R_H is the hydrodynamic radius[15].

2.6 Freeze-fracture transmission electron microscopy (FF-TEM)

Freeze-fracture transmission electron microscopy

(FF-TEM) was used to determine the structure of the assemblies. Some samples were frozen with liquid nitrogen at −189℃. The fracture process was performed with a JFD-9010 (JOEL, Japan) at −130℃ and the surface obtained by following this fracture with an etching process was then shadowed with evaporated platinum at an angle of 60°, followed by carbon at an angle of 90° to strengthen the replica. The replicate was placed on a 400 mesh copper grid after being washed with water, methanol, and chloroform. It was then examined and photographed using a JEM-1010 (JOEL, Japan) transmission electron microscope.

3 RESULTS AND DISCUSSION

3.1 Surface properties

After microbial production of the CLs, the ethyl acetate/acetone (4/1) extracts were neutralized by the addition of 1 N NaOH aq. The obtained CLNa were characterized by MALDI-TOF spectrometry. The molecular weight of the CLNa shown in Fig. 1 is 802.36, and the corresponding molecular ion peaks, 803.38 $[M+H]^+$, and 825.37 $[M+Na]^+$, were detected by MALDI-TOF spectrometry. This demonstrates that *C. humicola* JCM 10251 produces CLs that have the same structure as the CLs produced by *C. humicola* JCM 1461[7]. However, other molecular ion peaks such as 847.42 and 869.41 were also observed by MALDI-TOF spectrometry. These additional peaks probably correspond to $[-CH_2CH_2OH=44]$ adducts somewhere in the hydrophobic region of the CL shown in Fig. 1. Microbial products including CLNa may possibly contain impurities such as pigments, oils, and fatty acids. We confirmed that the HPLC peak of the sample was a single peak, even though the peak was relatively broad.

The produced CLNa were first dissolved in water; all solutions prepared were transparent and well soluble in water at 25℃, unlike the acid form of CLs. The surface tensions of the aqueous CLNa solutions were then measured as a function of CLNa concentration, and the results are shown in Fig. 2. The CLNa lowered the surface tension of water to 34.7 mN/m at a concentration of 0.1 mg/mL (CMC_1), and subsequently the surface tension became constant. This indicates that CLNa exhibit high surface activity. However, unlike that in conventional surfactants, the

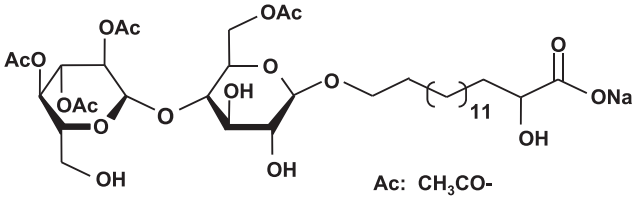

Fig. 1 Structure of sodium salt of cellobiose lipid (CLNa).

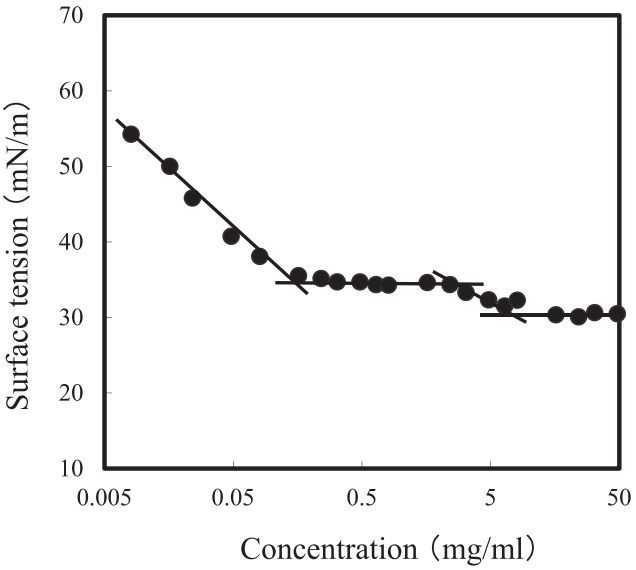

Fig. 2 Relationship between surface tension and concentration of CLNa at 25℃.

surface tension continued to decrease to 30.8 mN/m, becoming constant at a concentration of 12.0 mg/mL (CMC_2). To confirm micelle formation, DLS measurements were also performed at concentrations above the CMCs. The average diameter of the micelles was found to be 116.6 ± 31.9 nm at a concentration of 0.8 mg/mL, which indicates that CLNa forms giant micelles above its CMC_1.

Giant micelle formation has also been reported for other kinds of glycolipid BSs such as MELs[4] and SLs[16]: the MEL, having two medium chains in its structure, produces giant spherical particles with an average diameter of 179.0 nm, and the sodium salts of acidic SLs (SLNa) spontaneously form giant assemblies such as vesicles with an average diameter of 96.2 nm. These lines of evidence suggest that microbial glycolipid BSs tend to form giant assemblies just above their CMC, probably owing to the bulky and complicated structures that are naturally engineered by microorganisms.

In contrast to MELs and SLs, the size of the assemblies of CLNa increased above CMC_2 beyond the upper limit of DLS measurements (>1 μm). Although the detailed structure of the CLNa assemblies is still unclear, the equilibrium of the CLNa in aqueous solutions seems to have changed at the second CMC_2. We also found that the CLNa solutions started to become slightly viscous near the concentrations at which the secondary decay of the surface tension was observed.

3.2 Rheological studies of the gels

The gelation ability of CLNa with water was then examined by the test tube-inversion method. After dissolving CLNa in water by heating to 80℃, the test tube was cooled for 4 d. Subsequently, some samples formed aqueous gels,

Table 1 Gelation properties of CLNa in water at 25℃.

Conc. (mg/mL)	1.0	3.0	5.0	7.0	10.0	20.0	30.0	50.0
Gelation	−	−	○	○	○	○	○	○

and the results are summarized in Table 1. From the table, it can be seen that CLNa gelated with water above 5.0 mg/mL, which is close to their CMC_2. The minimum gelation concentration (MGC) of the gels was then estimated to be 5.0 mg/mL. The value is within a typical concentration range of general LMW gelators (<50.0 mg/mL)[13]. To the best of our knowledge, this is the first example of aqueous gel formation with microbial glycolipid BSs.

An important aspect of viscoelastic samples is their resistance to flow under applied stress. In rheological studies, the storage modulus (G') represents the ability of the deformed material to restore its original geometry, and the loss modulus (G") represents the tendency of a material to flow under stress. For viscoelastic materials such as gels, G' is generally greater than G"[14].

An oscillatory strain sweep was carried out at an angular frequency of 1 Hz at 25℃, and the results are shown in Fig. 3(a). When oscillatory strain was below 3.5%, G' was higher than G", meaning that the CLNa solutions were in the gel state, while above 3.5%, G' was lower than G", indicating that the CLNa solutions were in the sol state. The frequency sweep experiments of a CLNa solution with a concentration of 20 mg/mL were also performed at a constant strain amplitude of 1% at 25℃, and the results are shown in Fig. 3(b). It can be seen from the figure that G' was always higher than G" at the frequencies swept, indicating aqueous gel formation with CLNa as a gelator. Figure 3(c) shows the G' and G" values as a function of temperature at a constant strain amplitude of 1.0 % and an oscillatory strain of 1 Hz. The sample was heated at a rate of 1℃/min. At low temperatures, G' was higher than G", indicating viscoelastic-solid-like behavior of gel formation. The values of both G' and G" decreased sharply at 40℃ and intersected at approximately 50℃, where the sol-gel phase transition occurred.

These results indicate that CLNa provide rather soft aqueous gels at mild preparation conditions. This is an advantage over polymeric conventional gelators. CLNa can be dissolved easily and promptly in water by heating, and a gelator whose sol-gel phase transition temperature is below 100℃ is generally in high demand for practical applications[17].

3.3 FF-TEM observation of the gels

Since LMW gelators are known to self-assemble into fibrous structures with a 3D network, which leads to gel formation with various solvents[18], the self-assembled structures of CLNa in water were then visualized by FF-TEM. Figure 4 shows an FF-TEM image of the CLNa

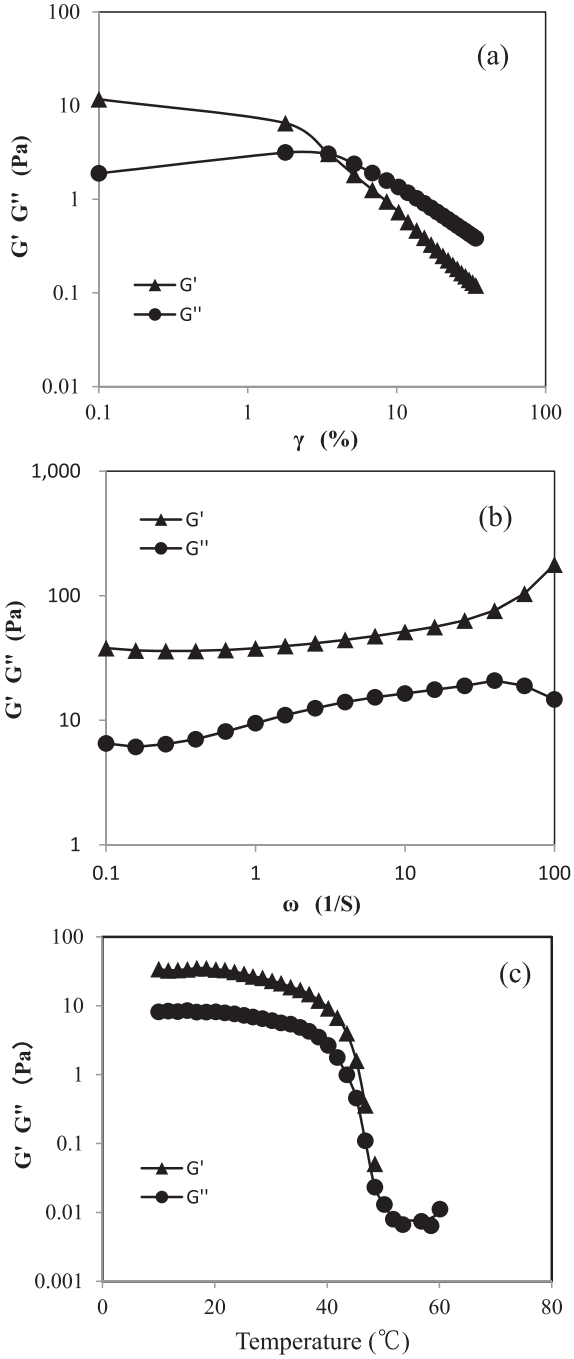

Fig. 3 Change in strage modulus (G') and loss modulus (G'') of aqueous CLNa gel (20 mg/mL) as functions of (a) strain (%), (b) angular frequency (ω), and (c) temperature (℃).

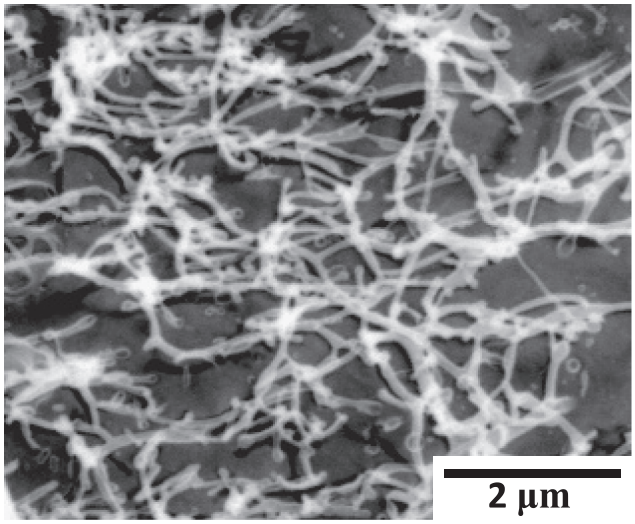

Fig. 4 FF-TEM images of aqueous CLNa gels (20 mg/mL).

gel at a concentration of 20.0 mg/mL. The image clearly exhibits a 3D entangled fibrous network. Although it has already been reported that CLs act as gelators for various organic solvents[8], here we also found that CLNa with asymmetric bolaform structures self-assemble into a fibrous supramolecular structure in water.

It also should be noted that the present CLNa not only provide aqueous gels at mild conditions but also exhibit excellent fungicidal activities toward different yeasts and fungi[11, 12]. Thus, the present aqueous gels derived from glycolipid BSs of CLNa should be useful as novel multifunctional soft materials applicable to the food and cosmetic industries.

4 CONCLUSIONS

In this study, the bolaform glycolipid BSs, cellobiose lipids (CLs), were produced by *Cryptococcus humicola* JCM 10251, and surface and gelation properties of the sodium salts of CLs (CLNa) in aqueous media were examined. The surface tension measurements revealed that the CLNa have high surface activity at quite low concentrations. It was also found that the CLNa salts form giant micelles whose size is 116.6 ± 31.9 nm at concentrations above their CMC_1. Unlike general surfactants, the size of the assemblies increased at lower concentrations, and the aqueous solution became viscous at 5.0 mg/mL.

In rheological studies, the obtained gels were rather soft and their sol-gel temperature was found to be approximately 50℃. FF-TEM observation of the gels showed 3D supramolecular structures with an entangled fibrous network. The CLNa aqueous gels having fungicidal activity would be useful for novel multifunctional soft materials and would be especially applicable to the food and cosmetic industries.

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