Surface Activity and Ca\(^{2+}\)-Dependent Aggregation Property of Lichenysin Produced by Bacillus licheniformis NBRC 104464

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Abstract: Bacillus licheniformis NBRC 104464 produces a cyclic lipopeptide different from surfactin. After we performed liquid chromatography fractionation and purification, we used structural analyses to identify the cyclic lipopeptide as lichenysin. Surface tension measurements of lichenysin sodium salt in water yielded a critical micelle concentration (CMC) of 1.0×10\(^{-5}\) M. The surface tension at the CMC was 28.9 mN/m. Comparative analysis of Ca\(^{2+}\)-influenced micellar aggregation of lichenysin and surfactin revealed that the formation rate of the lichenysin-Ca\(^{2+}\) complex aggregate remained low up to a [Ca\(^{2+}\)]/[lichenysin] molar ratio of 80, whereas the surfactin-Ca\(^{2+}\) complex formed micellar aggregates at the same molar ratio. Further excessive addition of Ca\(^{2+}\) to the micellar solution of lichenysin induced higher turbidity than surfactin.

Key words: cyclic lipopeptide, surfactin, lichenysin, Bacillus licheniformis, biosurfactant

1 INTRODUCTION

Cyclic lipopeptides (CLPs) are microbial surface-active compounds and one of the major classes of biosurfactants\(^3\). With their low critical micelle concentrations (CMCs), CLPs have excellent interfacial properties and engage in biological activities by virtue of their antiviral, antifungal, and antitumor properties\(^4\). Among CLPs, surfactin, iturin, and fengycin produced by Bacillus subtilis are the most studied\(^5,6\).

One interesting and attractive functional property of surfactin is its action as an ionophore, wherein divalent as well as monovalent cations can be trapped in the cyclic peptide\(^7\). Previously, surfactin was demonstrated to have a divalent cation-complexing property owing to the two negative charges derived from the aspartyl and glutamyl residues in its peptide ring; these face each other, forming the binding site for calcium ion (Ca\(^{2+}\))\(^8\). Indeed, surfactin with Ca\(^{2+}\) forms a stable surfactin-ca\(^{2+}\) complex\(^9\). Mulligan \textit{et al.} reported that surfactin micelles can bind divalent heavy metal ions, such as Cu\(^{2+}\) and Zn\(^{2+}\), and can remove the elements from contaminated soils using the membrane filtration technique\(^10\). The effect of monovalent cations on surfactin-Ca\(^{2+}\) micelle solution was investigated by fluorescence, dynamic light scattering (DLS), and freeze-fracture transmission electron microscopy measurements\(^11\). The counterions enhanced the surface activity and reduced the CMC value of surfactin-C\(_{16}\).

In contrast, lichenysin produced by Bacillus licheniformis is structurally similar to surfactin\(^12,13\), but it differs from other well-known families of CLPs, such as the iturins\(^14,15\) and the fengycins\(^16,17\). Based on species-specific variations, lichenysin A, B, C, D, and G and surfactant BL86 were designated\(^18\). The main differences between lichenysin and surfactin are the presence of glutamine residue ([Gln]/at position 1 of the lichenysin peptide sequence in place of glutamic acid (Glu) of surfactin and the resulting changes in the physicochemical properties, such as the CMC value and cation chelating ability. Grangemard \textit{et al.} reported that lichenysin has a more reduced CMC value and is a better chelating agent toward Ca\(^{2+}\) than is surfactin\(^19\). These changes are thought to be induced by an increase in the accessibility of the carbonyl group to Ca\(^{2+}\) owing to the transition of the side chain structure caused by the Glu/Gln exchange. Lichenysin applications in various industries are promising; however, the effect of Ca\(^{2+}\) -binding by lichenysin on water solubility is still not completely understood.

The goal of this study was to investigate the interfacial
properties of lichenysin and the effect of Ca\(^{2+}\) addition on the formation of lichenysin micelle aggregation. To these ends, we first screened a lichenysin-producing \textit{B. licheniformis} strain and detected gene fragments for lichenysin synthesis by polymerase chain reaction (PCR). Then, lichenysin was purified by liquid chromatography, and its structure was determined. The effects of Ca\(^{2+}\) addition on the micelle solutions of both lichenysin and surfactin were compared.

## 2 EXPERIMENTAL

### 2.1 Materials

All reagents and solvents were of the highest purity commercially available. Authentic surfactin sodium salt was kindly supplied by Kaneka Corporation, Japan.

### 2.2 Apparatus

Liquid chromatography-mass spectrometry (LC-MS) analysis of CLPs was performed using a Shimadzu LC-MS 2020 system (Shimadzu, Otsu, Japan) equipped with a TSK-GEL ODS-100V column (4.6 × 150 mm, TOSOH, Tokyo, Japan). The mobile phase composed of acetonitrile: 2% (v/v) acetic acid (4:1) was used at a flow rate of 1.0 mL/min, and the column was kept at 30°C during analysis. The samples were detected and quantified at 210 nm using a UV detector. Effluents were ionized by electrospray-ionization (ESI) and detected in negative ion mode with a m/z range of 50–2000. In contrast, LC-MS-MS analysis was carried out by Genomine, Inc. (Gyeongsangbuk-do, Korea) using an ESI-Mass Spectrometer, Q-TRAP 2000 (Applied Biosystems).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was carried out with the Autoflex Speed TOF/TOF (Bruker Daltonics, Inc.), and synaptic acid was used as the matrix. The instrument was operated in reflector positive-ion mode employing a mass range of 400–3,000 m/z. One μL of fractionated and concentrated CLP solution was spotted on top of 1 μL of saturated matrix solution composed of acetonitrile: water (1:1) over the MALDI plate using a droplet spotting method to ensure that mixing occurred within the drop.

Nuclear magnetic resonance (NMR) analysis of CLPs produced by the \textit{B. licheniformis} strain was carried out using an Avance II 400 MHz NMR spectrometer (Bruker BioSpin KK). The surface tension of the aqueous solution was determined by the Wilhelmy plate method at 25°C using a DI-500 surface tension meter (Kyowa Kaimen Kagaku Co., Niiza, Japan); the accuracy was intermittently verified using ultrapure water.

The assembly sizes of lichenysin were evaluated by DLS measurement with a DLS-7000 instrument (Otsuka Electronics Corporation, Osaka, Japan) using a 488 nm wave length 75 mW Ar laser as a light source at 25°C. The DLS intensity data were processed using the instrumental software to obtain the hydrodynamic diameter, polydispersity index, and mass diffusion coefficient of each sample.

The effect of Ca\(^{2+}\) on micellar solutions of lichenysin was evaluated based on optical density (OD) measurement at 600 nm using a V530 UV/VIS spectrophotometer (V560, JASCO Corporation, Tokyo, Japan).

### 2.3 Bacterial strains, media, and cultivation

Eighteen \textit{B. licheniformis} strains were obtained from the National Institute of Technology and Evaluation (NITE) of Japan. The NBRC numbers are as follows: 12107, 12195, 12197, 12198, 12199, 12200, 12201, 12202, 14206, 15647, 104437, 104447, 104462, 104464, 104469, 104474, 109103, and 109105. Stock cultures were grown using media and temperatures recommended by the NITE. All strains were successfully cultured on NITE medium number 702 (designated as 702 medium) containing 10 g/L of hipolypepton (Wako Pure Chemical Industry, Osaka, Japan), 2 g/L of yeast extract (Difco), and 1 g/L of MgSO\(_4\).7H\(_2\)O (pH 7.0). For the analysis of lichenysin-producing strains, a single colony of each strain was added to 5 mL of 702 medium or sucrose medium [20 g/L of sucrose, 2 g/L of (NH\(_4\))\(_2\)SO\(_4\), 6 g/L of KH\(_2\)PO\(_4\), 2.4 g/L of Na\(_2\)HPO\(_4\), 0.3 g/L of MgSO\(_4\), 0.001 g/L of CaCl\(_2\), 0.018 g/L of CoCl\(_2\)6H\(_2\)O, 0.007 g/L of NiCl\(_2\).6H\(_2\)O, 0.002 g/L of CuCl\(_2\)-2H\(_2\)O 0.083 g/L of FeSO\(_4\)-7H\(_2\)O, and 0.5 g/L of yeast extract (Difco) (pH 7.0)] in a test tube and incubated aerobically at 37°C on a reciprocal shaker (200 rpm) for 24 h. When bacterial growth was observed, the seed cultures (0.5 mL) were transferred to 300-mL Erlenmeyer flasks containing 30 mL of 702 medium or sucrose medium. The flasks were incubated for another 24 h at 37°C on a rotary shaker (200 rpm). After removing the cells by centrifugation, 1 mL of the respective supernatants were acidified with concentrated HCl and extracted with 1 mL of ethyl acetate. The ethyl acetate extracts were dried and then dissolved in an appropriate amount of methanol for LC-MS.

### 2.4 PCR amplification of the lichenysin-synthesis genes

Total DNA was isolated from \textit{B. licheniformis} NBRC 104464 grown on 702 medium according to standard protocols. Using total DNA from the strain as a template, the non-ribosomal peptide synthase-encoding genes were amplified by PCR with the forward and reverse primer sets LchhA5′-ACTGAGGATTCCGAATGTT-3′ and LchhAA5′-TCGCTTCATATTGTGCGTTC-3′ for \textit{ichhA} (lichenysin, 472 bp PCR product) [21]. PCR reactions were performed using Premix Taq™ (Takara Shuzo, Kyoto, Japan) as recommended by the manufacturer, and the PCR conditions were as described previously [20].
2.5 Production, separation, and characterization of lichenysin

For large-scale production of lichenysin for separation and subsequent characterization, *B. licheniformis* NBRC 104464 was grown in a 5-L jar fermenter (Model MDL; B.E. Marubishi). Jar fermenter experiments were performed as follows: the strain was pre-cultivated for 24 h in test tubes containing 5 mL 702 medium (30°C, 200 rpm). The cultures (six test tubes, 30 mL in total) were transferred to a 5-L jar fermenter containing 3 L of the same medium and cultivated for another 24 h. The aeration rate was set to 1.0 volume of air per volume of medium per minute (vvm), and the agitation speed was set to 300 rpm. The temperature was maintained at 37 ± 1°C.

After removing the *B. licheniformis* cells by centrifugation, the supernatant was adjusted to pH 2.0 with concentrated HCl until the supernatant produced a precipitate. The supernatant was then centrifuged at 8,000 rpm for 5 min, and the precipitate was collected and extracted with ethyl acetate. The ethyl acetate extracts were treated with granular activated charcoal (Wako Pure Chemicals), concentrated in vacuo, and then dried. The resultant material was dissolved in an appropriate amount of 1 M NaOH, and the pH of the solution was 8. The crude material was purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a C18 column [acetonitrile: water (4:1)] followed by evaporation and drying under reduced pressure. After filtration, the resultant lichenysin sodium salt was recovered by lyophilization. The mass spectrum and structure of the sodium salt was confirmed by MALDI-TOF-MS, LC-MS-MS, and 1H NMR.

For amino acid composition analysis, the lichenysin was hydrolyzed with 6 M HCl at 100°C for 18 h. The amino acids were treated with phenyl isothiocyanate (PTC) to provide phenylthiohydantoin derivatives. The resultant amino acid derivatives were analyzed by liquid chromatography with a Shimadzu LC-MS 2020 system (Shimadzu, Otsu, Japan) equipped with a WakoPack® WakoSil-PTC (4 × 200 mm, Wako Pure Chemical Industry). The mobile phase, composed of solvent A (PTC-Amino acids Mobile Phase A, Wako Pure Chemical Industry) and solvent B (PTC-Amino acids Mobile Phase B, Wako Pure Chemical Industry), was used at a flow rate of 1.0 mL/min, and the column was kept at 40°C during analysis. The samples were detected and quantified at 254 nm using a UV detector. The retention times of the respective peaks in the sample were compared with those of PTC-treated Amino Acids Standard Solution Type H (Wako Pure Chemical Industry).

2.6 Ca$^{2+}$ titration

The effects of Ca$^{2+}$ on lichenysin micellar solutions were measured by the spectrophotometric turbidity method. In this method, the aqueous solutions containing surfactant (1.0 mM lichenysin sodium salt or surfactin sodium salt) and various amounts of CaCl$_2$ were prepared in a 10-mm optical cell, and the transmittance was measured at 600 nm (JASCO V-560) where the surfactant does not absorb. The absorbance at 600 nm was monitored against the molar ratio of [Ca$^{2+}$]/[surfactant].

3 RESULTS AND DISCUSSION

3.1 Screening of a *Bacillus licheniformis* strain producing lichenysin

In the first screening, 18 *B. licheniformis* strains were examined for their ability to produce CLPs using both 702 medium and sucrose medium. The amounts of CLPs analyzed by LC-MS differed among strains as well as media. In 14 strains, CLPs were only traceable or were undetected, probably because the two media types used were not optimally suitable for all strains.

Among the other four strains, *B. licheniformis* NBRC 104437 and 104464 gave a major peak for CLP at retention time 11.9 min when using the sucrose medium. In contrast, *B. licheniformis* NBRC 104464, 104469, and 104474 each gave a major peak at retention time 12.3 min with the 702 medium. As the peaks for CLPs could be detected in both media when using strain NBRC 104464, this strain was used for further analysis owing to its stable production ability.

Figure 1 shows the LC profile of the CLP products extracted from *B. licheniformis* NBRC 104464 culture supernatant (702 medium) with ethyl acetate after acidification. In addition to the major peak at 12.3 min, there were two other CLP peaks, at 7.3 min and 9.4 min. Considering that LC analysis of authentic surfactin sodium salt under the same conditions gave three peaks at 5.9 min, 7.5 min, and 9.4 min (a series of homologues having C$_{13}$, C$_{14}$, and C$_{15}$ β-hydroxy fatty acids, respectively), the CLPs from strain NBRC 104464 differed from those of surfactin. MS analysis suggested that [M] - [m/z = 1006.5, 1020.5, and 1034.5 for peaks at 7.3 min, 9.4 min, and 12.3 min, respectively] were...
the main ion forms of lichenysin isoforms, because the mass values of surfactin isoforms with C\textsubscript{13}, C\textsubscript{14}, and C\textsubscript{15} β-hydroxy fatty acids are 1007.6, 1021.7, and 1035.7, respectively\textsuperscript{26}. The differences in mass of 1 Da suggest the CLP sodium salts are lichenysin isoforms with C\textsubscript{13}, C\textsubscript{14}, and C\textsubscript{15} β-hydroxy fatty acids. From the peak area of the LC profile (Fig. 1), more than 80% of the isoforms have C\textsubscript{15} β-hydroxy fatty acids.

To investigate the types of CLPs produced by strain NBRC 104464 at the molecular level, the presence of the first structural genes of the lichenysin synthetase operon (\textit{lchAA}) was examined by PCR. The designated DNA regions within \textit{lchAA} were amplified, which is consistent with a previous report in which \textit{lchAA} gene fragments were detected in all 53 \textit{B. licheniformis} strains tested\textsuperscript{24}. Hence, the results suggest that \textit{B. licheniformis} NBRC 104464 produces lichenysin.

### 3.2 Purification and structure analysis of lichenysin produced by \textit{B. licheniformis} NBRC 104464

For subsequent structural characterization, the CLP was purified from culture. After 5-L jar fermenter experiments, the supernatant of a 3-L culture was acidified to form a CLP-containing precipitate was extracted with ethyl acetate, and the supernatant of a 3-L culture was acidified to form a precipitate, which was treated with activated charcoal. After concentration in vacuo, the dried extract was dissolved in NaOH solution to be approximately pH 8. Approximately 60 mg of white powder was obtained from the 3-L culture.

MALDI-TOF-MS spectral analysis showed that the CLP sodium salts contained several molecules having \textit{m/z} 1029.5, 1043.5, and 1057.5. As the spectra of major molecules\textsuperscript{26} observed in authentic surfactin sodium salt were 1030.6, 1044.7, and 1058.7\textsuperscript{26}, the differences in the mass of 1 Da suggest that the CLPs were isoforms of lichenysin sodium salts with C\textsubscript{13}, C\textsubscript{14}, and C\textsubscript{15} β-hydroxy fatty acids.

LC-MS-MS analysis revealed a main signal at \textit{m/z} 1057.5 corresponding to \([\text{M} + \text{Na}]^+\) molecules. Additionally, negative full-scan mode spectra showed a main peak at \textit{m/z} 1056.5, which corresponded to deprotonated \([\text{M} - \text{H}]^-\) molecules. The most abundant molecule at \textit{m/z} 1057.5 resulted in the following product ions: \textit{m/z} 1038.4, 944.5, and 813.4. Ions at \textit{m/z} 1038.4 corresponded to the loss of H\textsubscript{2}O (−18 Da) from \textit{m/z} 1056.5. The remaining product ions corresponded to the losses of Ile (−113 Da; for \textit{m/z} 944.5) and Ile-Leu-H\textsubscript{2}O (−244 Da; for \textit{m/z} 813.4) from \textit{m/z} 1057.5. This appearance of product ions in LC-MS-MS is consistent with experiments using authentic surfactin (data not shown) and previous studies\textsuperscript{27,28}.

The \textsuperscript{1}H NMR measurement of the CLP sodium salts in DMSO-\textit{d}_\textit{6} showed that they consisted of a cyclic peptide, an aliphatic CH proton, and a long aliphatic chain, respectively (Fig. 2). In the \textsuperscript{1}H NMR spectrum, the seven doublets corresponding to NHs in the peptide backbone were observed at δ = 9.84, 9.69, 8.51, 8.28, 8.26, 7.39, and 7.05 ppm, in a 1:1:1:1:1:1:1 area ratio. Two singlets at 7.21 and 6.69 ppm in a 1:1 area ratio can be assigned to CONH\textsubscript{2} of α-Gln1. The NMR spectroscopy enabled distinction of the isomeric forms of the amino acid sequence\textsuperscript{35}. The quintet signal at 4.90 ppm can be assigned to the CH proton of a lactone ring where the hydroxyl group at the C-3 position of the fatty acid residue was attached to the carboxyl group by an ester bond. The signals around δ = 4.02–4.38 with total integration corresponding to seven protons can be assigned to aliphatic αCH protons of the peptide backbone. The signals around 1.00–1.70 ppm indicate the presence of methylene protons of an aliphatic chain and amino acid residues. The large signals around 0.7–0.9 ppm can be assigned to terminal CH\textsubscript{3} protons.

The amino acid compositions of the CLPs were analyzed by LC after PTC-derivatization of the CLP hydrolysate. The CLP contained Lue, Val, Ile, Asx, and Glx in a molar ratio of 2:1:1:1:1 (Fig. 3), which are the same amino acids of lichenysin in previous reports\textsuperscript{28}. Compared to the amino acid composition of authentic surfactin (data not shown), one difference is the existence of an isoleucine residue, which is often found in lichenysin isoforms\textsuperscript{30}. Together with the obtained results, the structure of CLP from \textit{B. licheniformis} NBRC 104464 was determined to be lichenysin, as shown in Fig. 4.

### 3.3 Surface activity of lichenysin from \textit{B. licheniformis} NBRC 104464

The surface activity of lichenysin in water was confirmed by the Wilhelmy plate method. The surface tension of water decreased with increasing concentrations of lichenysin from 72 to 28.9 mN/m (Fig. 5A). The CMC obtained from the intersection of the two fitted lines was 1.0 × 10\textsuperscript{−5}.
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M, which is same order of surfactin\((2.7 \times 10^{-5} \text{ M})\)^9. The light scattering intensity measurements were also performed at varying concentrations of lichenysin. As shown in Fig. 5B, the relative scattering intensity suddenly increased above the concentration of the intersection point observed in Fig. 5A, indicating that lichenysin starts to form micelles above that concentration.

### 3.4 Effect of Ca\(^{2+}\) on micellar solutions of lichenysin

Considering the structural characteristics, lichenysin, which possesses L-Gln instead of the L-Glu in surfactin, could exhibit a different self-assembly from surfactin in the presence of metal cation. To compare the effect of Ca\(^{2+}\) on micelle solution, titration experiments were conducted (Fig. 6). Aqueous micellar solutions\((1.0 \times 10^{-4} \text{ M})\) of lichenysin and surfactin provided clear transparent solutions, and their absorbances at 600 nm\((\text{OD}_{600})\) were almost zero.

When adding Ca\(^{2+}\) to the surfactin solution, the \text{OD}_{600} increased with increasing amounts of Ca\(^{2+}\) to reach 0.21 at a \([\text{Ca}^{2+}] / [\text{surfactin}]\) molar ratio of 80, and the value did not change with further addition of Ca\(^{2+}\). In contrast, in the case of lichenysin, the \text{OD}_{600} values remained low up to the same molar ratio\(80\) of\([\text{Ca}^{2+}] / [\text{lichenysin}]\). However, further addition of Ca\(^{2+}\) to the solution led to an increase in the \text{OD}_{600}, and the value reached to 0.41 at\([\text{Ca}^{2+}] / [\text{lichenysin}]\) of 540. These results indicate that Ca\(^{2+}\) significantly influences the self-assembly via formation of surfactin-Ca\(^{2+}\) complex, whereas lichenysin micelle is hardly affected when the concentrations of the respective surfactants are the same\(([\text{Ca}^{2+}] / [\text{surfactant}] < 80)\). Owing to the two carboxylic acids as well as the distinctive cyclic peptide inner space, surfactin can bind Ca\(^{2+}\) at a molar

![HPLC profile of phenylisothiocyanate derivatives of amino acids derived from lichenysin from Bacillus licheniformis NBRC 104464.](image1)

**Fig. 3** HPLC profile of phenylisothiocyanate derivatives of amino acids derived from lichenysin from *Bacillus licheniformis* NBRC 104464.

![Structure of the cyclic form of lichenysin from Bacillus licheniformis NBRC 104464.](image2)

**Fig. 4** Structure of the cyclic form of lichenysin from *Bacillus licheniformis* NBRC 104464.

![Relationships between surface tension and concentrations of lichenysin at 25°C.](image3)

**Fig. 5** (A) Relationships between surface tension and concentrations of lichenysin at 25°C. (B) Light scattering intensity-concentration plot of lichenysin at 25°C. The light scattering intensity of lichenysin was detected using an Ar laser (488 nm).

![Ca\(^{2+}\)-dependent turbidity changes in micellar solutions of lichenysin (circle) and surfactin (triangle) monitored by UV-vis spectroscopy at 600 nm.](image4)

**Fig. 6** Ca\(^{2+}\)-dependent turbidity changes in micellar solutions of lichenysin (circle) and surfactin (triangle) monitored by UV-vis spectroscopy at 600 nm.

![Abs at 600 nm vs Ca\(^{2+}\)/surfactant (molar ratio).](image5)

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ratio of 1:1\(^{-7-9}\). It has been suggested that Ca\(^{2+}\) has a special interaction with surfactin-C\(_{16}\), even at low concentrations, probably because the size of Ca\(^{2+}\) is fitted to its structure\(^{10}\).

As for lichenysin, Grangemard et al. reported that the association constant of lichenysin with Ca\(^{2+}\) is four-fold higher than that of surfactin. Also, the formation of lichenysin-Ca\(^{2+}\) complex in a molar ratio of 2:1 suggests that the intermolecular salt bridge of lichenysin is stronger than the intramolecular complexation in a 1:1 ratio with surfactin\(^{21}\). Since the lichenysin-Ca\(^{2+}\) complex in a 2:1 ratio is a neutral dimer complex, salt-bridge formation at the air-water interface can influence the micellar aggregation in the presence of excessive amounts of Ca\(^{2+}\). Further comparative analysis of the microenvironment properties in each complex micelle will be necessary to explain the differences between their Ca\(^{2+}\)-dependent aggregation properties.

4 CONCLUSION

Among 18 tested strains, we used Bacillus licheniformis NBRC 104464, which stably produced a CLP different from that of surfactin on two screening media. After extraction and purification of the CLP from bacterial culture, structural analyses by LC-MS, LC-MS-MS, MALDI-TOF-MS, \(^{1}H\)-NMR, and amino acid composition revealed the CLP to be lichenysin. Lichenysin sodium salt can reduce the surface tension of water to 28.9 mN/m at a CMC of 1.0

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