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Protein-surfactant interactions between bovine lactoferrin and sophorolipids under neutral and acidic conditions

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Abstract

In order to understand the protein-surfactant interactions between naturally derived sophorolipids (SLs) and bovine lactoferrin (bLf), we carried out spectroscopic, microscopic, and biochemical experiments under weakly acidic and neutral pH conditions. Particle size analysis, microscopy, and enzymatic digestion indicated that bLf and SLs interact with each other to form sheet-like and small aggregated structures reflecting the original self-organization of SLs at pH 5.0 and 7.0, respectively. Circular dichroism (CD) showed that SLs did not significantly affect the secondary structure of bLf.

Keywords

Lactoferrin; Sophorolipids; Protein-surfactant interaction; Complex; Electrostatic interaction

1. Introduction

Lactoferrin, first discovered in bovine milk, is a red-colored glycoprotein that can bind iron ions with high affinity (Sørensen and Sørensen 1939; Groves 1960). Bovine lactoferrin (bLf) is a single-chain protein consisting of 689 amino acid residues, with a molecular weight of 78,000 Da and an isoelectric point of pH 8.8 (Moore et al. 1997; Shimazaki 1993). Since lactoferrin is associated with a diverse range of biochemical functions, it is widely accepted that it is a multi-functional protein that can be used in the manufacture of different products including foods, drugs, and cosmetics. Some of its important biological functions include anti-inflammatory activity, bacteriostatic activity, growth factor action, and bone formation (Lönnerdal 2013).
Amongst the various functions of lactoferrin, researchers have particularly focused on its role in stimulating skin keratinocyte function and wound re-epithelialization, originally reported by Tang et al. (2010). In our previous study, we demonstrated that bLf significantly increased tropoelastin expression in cultured fibroblasts, and this expression is efficiently promoted by addition of a novel skin penetration enhancer, acid-type SLs (Ishii et al. 2012). In the same study, we also observed that on addition of SLs, transdermal absorption of bLf through a model skin was enhanced up to 1.7-fold, possibly due to complex formation between bLf and SLs. However, the exact protein-surfactant interactions remain to be understood.

Acid-type SLs are produced from natural resources through fermentation by non-pathogenic yeast, Starmerella bombicola. These are surface-active glycolipids comprising a sophorose unit, which is a glucose disaccharide, glycosically linked to a hydroxyl fatty acid as shown in Fig. 1 (Lang 2003; Ishii et al. 2012). According to a previous study, SL molecules tend to self-organize, forming giant twisted or helical ribbons in acidic conditions (pH < 5.5) (Zhou et al. 2004). The yield of the solid ribbon-like products decreases with an increase in pH, forming short-range ordered micelles under neutral conditions. In contrast, the sodium salt of acidic SLs (SLs-Na) spontaneously forms vesicles in a neutral aqueous solution, and these vesicles can incorporate triterpene glycosides and significantly improve the penetration of triterpene glycosides through an in vitro skin model (Imura et al. 2014).

In the current study, to investigate the protein-surfactant interactions between bLf and SLs, structural changes in bLf induced by SLs were analyzed using spectroscopic, microscopic, and biochemical methods. bLf-SL complexes formed at pH 5.0 (weakly acidic) and pH 7.0 (neutral) were chosen as representative of efficient
delivery agents of bLf in commonly used cosmetics and food products.

2. Materials and methods

2.1. Materials

Medium containing 10% glucose, 10% palm oil, 1% KH$_2$PO$_4$, 0.5% MgSO$_4$, 0.1% NaCl, 0.1% urea, and 0.25% yeast extract was fermented by Starmerella bombicola (NBRC 10243) to produce a mixture of lactone-type and acid-type SLs. The lactone-type SLs were chemically converted to the acid-type SLs, which were purified as previously described (Zhou et al. 2004). The typical chemical structure of SLs used in this study is shown in Fig. 1. The purity and chemical structure of the SLs were confirmed by NMR and liquid chromatography-mass spectrometry as previously described (Konishi et al. 2008 and Nuñez et al. 2000). bLf (purity > 95%) was purchased from Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). The iron saturation level of bLf ranged between 10% and 20% and LPS contamination was undetectable (manufacturer's specifications). Proteolytic enzymes proteinase K obtained from Tritirachium album (EC 3.4.21.64; catalogue No. 160-14001) and trypsin obtained from porcine pancreas (EC 3.4.21.4; catalogue No. 207-19183) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of analytical grade.

2.2. Experiments

2.2.1. Preparation of sample solutions

bLf and SLs solutions were prepared by adding 0.2 wt% of bLf or SLs into 10 mM sodium citrate buffer solution (pH 5.0) or 10 mM sodium phosphate buffer solution (pH 7.0) followed by overnight incubation on an automatic shaker (>12 h) at ambient
temperature to ensure complete dissolution. Sample solutions were prepared by gently mixing the bLf and SLs solutions at a volume ratio of 1:1 and were subjected to assays within 6 h of preparation. The approximate molar ratio of bLf and SLs was 1:125, contingent on the most likely molecular weight of 78,000 Da for bLf and 622 Da for SLs.

2.2.2. Particle size analysis

Particle size distribution of sample solutions was analyzed by dynamic light scattering (DLS) at 25°C (Nicomp 370 submicron particle size analyzer (Particle Sizing System, Inc., Santa Barbara, California, USA)) using the solid particle mode and Nicomp distribution analysis mode assuming multi-modal distributions. A typical particle size distribution is described in the results section. The particle size in the dissolved sample solutions is also reported as volume-weighted mean particle diameter ($d_{4,3}$).

2.2.3. Cryo-scanning electron microscopy (Cryo-SEM)

Sample solutions were rapidly frozen in liquid nitrogen slush and quickly transferred into the specimen chamber of a scanning electron microscope equipped with a cryo-unit (S4100, Hitachi, Ltd., Tokyo, Japan). They were etched at -65°C for 60 min or longer to remove water molecules on the surface of frozen solutions and then sputter coated with gold. All samples were observed at -65°C with an acceleration voltage of 5.0 kV.

2.2.4. Enzymatic digestion

Sample solutions were gently mixed with enzyme solutions containing Proteinase K or trypsin at a volume ratio of 19:1 followed by incubation in a water bath (with constant shaking) at 37°C for 90 min. The enzymatic reactions were terminated by
high-temperature heat treatment at 95°C for 20 min. The enzymatically hydrolyzed samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on a gradient precast slab gel containing 5–20% acrylamide (e-PAGEL E-T520L, ATTO, Tokyo, Japan), followed by staining with Coomassie brilliant blue.

2.2.5. Surface hydrophobicity

Surface hydrophobicity of bLf was evaluated as described previously (Kato and Nakai 1980; Hayakawa and Nakai 1985). In order to exclude the background influence of SLs on fluorescence intensity, the SLs solutions were used as controls for both bLf and SLs solutions. To prepare various concentrations of bLf and SLs solutions, sample and control solutions were diluted with the corresponding buffer solution at a volume ratio of 0:25, 3:22, 6:19, 9:16, and 12:13. Solutions were gently mixed with 1-anilinonaphthalene-8-sodium sulfonate (ANS-Na) in appropriate buffer solutions (8 mg/mL) at a volume ratio of 200:1 and incubated in a water bath at 25°C for 10 min. Using a fluorescence spectrophotometer, fluorescence intensity was recorded at a wavelength of 470 nm with excitation at 390 nm (F-3000, Hitachi, Tokyo, Japan). The hydrophobicity index was calculated from the slopes of the fluorescence intensity vs sample concentration curves.

2.2.6. Circular dichroism (CD)

CD spectroscopy was performed at 25°C on a spectropolarimeter using a quartz cell with 0.1 mm optical path length (J-720, JASCO, Tokyo, Japan). The values were expressed as the mean of the data obtained from three samples: each measurement was repeated three times for each of the three samples. The mean residue ellipticity was calculated according to the following equation (Fasman 1978):
\[ [\theta]_\lambda = \frac{\theta_{\text{obs}} \times \text{MRW}}{10 \times d \times c} \]

where \( \lambda \) = wavelength; \( \theta_{\text{obs}} \) = observed ellipticity in degrees; MRW = mean residue weight of repeating unit; \( c \) = concentration in grams per milliliter; \( d \) = path length in centimeters.

2.2.7. Statistics

All experiments were conducted in triplicate with freshly prepared sample solutions. Mean values and standard deviations of the triplicate measurements are shown unless otherwise stated. Statistical analyses were performed using Microsoft Excel ver. 2013 for Windows.

3. Results and discussion

3.1. Particle size analysis

To analyze the particle size distribution of the sample solutions containing 0.1 wt% of bLf and/or 0.1 wt% of SLs, DLS measurements were carried out using the particle size analyzer at 25 °C. Under weakly acidic condition, the particle size of bLf was approximately 10 nm (Fig. 2a), indicating that bLf completely dissolved in the solution with pH 5.0. In contrast, SLs seemed to form large aggregates of about 1,200 nm in the pH 5.0 solution (Fig. 2b), as described previously (Zhou et al. 2004). When bLf and SLs were mixed at a weight ratio of 1:1, the mono-modal peak originating from the dissolved bLf disappeared, and aggregates slightly larger than the SLs aggregates, probably consisting of SLs and bLf, were observed (Fig. 2c). These results show that bLf and SLs interact with each other and probably form complexes at pH 5.0.

In neutral sample solutions (pH 7.0), the mean particle size of both bLf and
SLs was roughly 10 nm (Fig. 2d and e). This indicates that bLf and SLs dissolved well in the solutions. On mixing, they had a tendency to form aggregates of about 17 nm (Fig. 2f). The isoelectric point of bLf is pH 8.8 (Moore et al. 1997; Shimazaki 1993), making the protein positively charged at pH 5.0 and pH 7.0 and giving it a tendency to bind to negatively charged molecules in solutions with these pH values. At pH 5.0 or higher, the solubility of SLs increases with increasing pH, which is closely related to the electrolytic dissociation of the linked hydroxyl fatty acids (Fig. 1) (Hu and Ju 2001; Van Bogaert et al. 2011). A similar tendency was also observed for the SLs used in this study. These data suggest that SLs are negatively charged both at pH 5.0 and at pH 7.0. Therefore, it is reasonable that bLf would interact with SLs to form complexes under the tested conditions. bLf contains 101 basic amino acid residues such as His, Lys, and Arg that can interact with the hydroxyl fatty acid residue of SLs. Since the molar ratio of bLf and SLs was approximately 1:125, all the basic residues, though not exposed to the aqueous phase, can be potentially capped by adequate amounts of SLs by electrostatic interactions.

3.2. Microstructure

To gain insight into the structural features of the possible complexes, Cryo-SEM was conducted to observe bLf or/and SLs present in the sample solutions. Fig. 3a describes Cryo-SEM images obtained from the sample solutions at pH 5.0 containing 0.1 wt% of bLf, SLs, or a combination of bLf and SLs. bLf had a weed-like fibrous structure (A) that did not seem to aggregate. SLs self-organized into a ribbon-like architecture (B), as reported by Zhou et al. (2004), and fibrous networks (C). In solutions where bLf and SLs existed together, well-structured sheet-like materials (D) were prominently observed. The structures formed by bLf, SLs, and bLf and SLs were
observed in most fields of view. Our results suggest that bLf and SLs form continuous
giant complexes, similar to the original self-assembly of SL molecules reported
previously (Zhou et al. 2004), and do not appear to form a significant amount of
vesicle-like complexes.

Fig. 3b shows the Cryo-SEM micrographs of the sample solutions at pH 7.0.
Unlike the results shown in Fig. 3a, similar structures were found in bLf, SLs and
solutions containing bLf & SLs. However, on magnification, some small sheet-like
structures were seen in the SLs solution (E). Additionally, at the low magnification
(1,000x), the fibrous network in SLs appeared to be finer than the bLf network, while in
solutions containing a mixture of bLf and SLs, the width of the fibrous network ranged
between the width observed in the bLf and SLs solutions. Therefore, we can assume that
in neutral solutions, SLs interact with bLf to form relatively small complexes that
depend on electrostatic attractions (Fig. 2f), but does not induce significant changes in
the bLf structures. Since the interactions were not strong, it was not possible to identify
them under the microscope. Therefore, in order to further investigate interactions
between bLf and SLs, a biochemical enzymatic digestion was carried out.

3.3. Proteolysis

Based on research by Brines and Brock (1983), enzymatic digestions were
performed to confirm the structural properties of bLf-SLs complexes. If bLf is included
in the vesicle-like self-organization of SLs then it would be quite resistant to proteolytic
degradation and would remain structurally intact. Fig. 4 shows SDS-PAGE profiles of
enzymatically-digested bLf using Protease K and a pancreatic trypsin under weakly
acidic and neutral conditions with or without SLs. The concentration of bLf and SLs
were 0.1 wt%. In acidic pH and in the absence of SLs, bLf was extensively digested by Protease K and trypsin (Fig. 4a). However, in the presence of SLs, specific undigested fractions of bLf were generated on treatment with Protease K and trypsin (Fig. 4b). At neutral pH, both enzymes also efficiently digested bLf in the absence of SLs (Fig. 4c), whereas there were clear undigested protein bands in Protease K-treated or Trypsin-treated samples (Fig. 4d). These results indicate that bLf interacted with SLs but was not completely protected by the surrounding SLs. These results also support the Cryo-SEM observations that bLf was not incorporated into vesicles organized by SLs but was associated with the ribbon-like self-organization that occurred at pH 5 (Fig. 3a) and the network-like self-organization that occurred at pH 7, and was mostly exposed to the aqueous phase in both structures. In addition, the digestion patterns at pH 7.0 and pH 5.0 were quite different, supporting the observation that bLf was incorporated into different structures at these two pHs (Fig. 3a, 3b).

3.4. Tertiary structural changes

To investigate tertiary structural changes in bLf caused by SLs, the surface hydrophobicity of bLf in the presence of SL molecules was evaluated using ANS-Na as a fluorescent probe. This type of approach is often used for estimating the conformational change in globular proteins caused by heat treatment, interaction with lipid membranes, etc. (Haynes and Staerk 1974; Alizadeh-Pasdar and Li-Chan 2000; Haweet et al. 2008). Since lactoferrin is a globular protein, inner hydrophobic sites tend to not be exposed to the hydrophilic phase in the aqueous solution. If conformational changes induced by environmental factors, such as heat, or chemical factors, such as protein-surfactant attractive interactions, result in exposure of hydrophobic residues,
ANS-Na binding to the exposed hydrophobic sites can significantly increase ANS fluorescence (De et al. 2005). The hydrophobicity indexes of bLf, SLs, and bLf + SLs were defined as the slopes obtained from the ANS fluorescence vs bLf, SLs, and bLf + SLs concentration curves (Kato and Nakai 1980; Hayakawa and Nakai 1985). The hydrophobicity index obtained from the bLf solution at pH 5.0 was significantly different from that of the solution containing both bLf and SLs ($t$-test, $p < 0.05$). The values for the bLf solution and the solution containing both bLf and SLs were 370.7 ± 1.6 and 1092.3 ± 71.7, respectively (Fig. 5a). Similarly, at pH 7.0, the hydrophobicity index of the solutions containing both bLf and SLs (478.1 ± 25.8) was much higher than the solution containing only bLf (222.4 ± 3.4) (Fig. 5b).

If bLf and SLs did not interact with each other in the bLf + SLs solution, the values for the bLf alone and bLf + SLs solutions would be expected to be similar to each other. The fact that the values of the bLf + SLs solution were significantly higher than the values of the bLf alone solutions indicate that bLf and SLs form a complex, presumably via electrostatic interactions between positively charged sites of bLf and the negatively charged hydroxyl fatty acid residues of SLs. These results are in agreement with the data obtained from the particle size analysis described in section 3.1.

3.5. Secondary structural changes

Fig. 6 describes the CD spectra of the bLf solutions with and without SLs in acidic and neutral conditions. At pH 5.0, wavelength-dependent molar ellipticity of solutions containing only bLf ranged between approximately -10,000 to 0 deg-cm$^2$/dmol (Fig. 6a and b), similar to the result described by Hu et al. (2008). The CD spectrum of bLf at pH 5.0 did not change significantly on addition of SLs (Fig. 6a). This indicates
that at pH 5.0, binding of SLs to bLf is not strong enough to cause marked changes in
the secondary structures of bLf. Similarly, at neutral pH, addition of SLs into the bLf
solutions did not cause marked changes in the CD spectrum, (Fig. 6b), indicating that at
neutral pH, interactions between bLf and SLs are as mild as those observed in the pH
5.0 solution.

4. Conclusions

Under acidic and neutral conditions, bLf is associated with the fibrous
self-organization of SLs, with continuous exposure to the aqueous phase. The size and
shape of the bLf-SLs complexes that form at pH 5.0 and pH 7.0 vary significantly. The
positively charged bLf and the negatively charged SLs seem to form a complex via
electrostatic interactions, but the interactions are not strong enough to alter its secondary
structure.
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Fig. 1. Chemical structure of an SL.
Molecular weight of the displayed chemical compound is 622 Da.

Fig. 2. Particle size distributions of bLf, SLs, and bLf + SLs mixtures under acidic (a-c) and neutral (d-f) conditions with a volume-weighted mean diameter.

Fig. 3. Cryo-SEM images of pH 5.0 and pH 7.0 aqueous solutions of bLf, SLs, and bLf + SLs.

Fig. 4. SDS-PAGE patterns of bLf digested by proteolytic enzymes with or without SLs. Enzymatic treatments were carried out at 37°C for 30, 60, and 90 min. M, C, and the numbers shown above the gels refer to molecular weight markers (M), untreated bLf under the specified pH conditions serving as a control (C), and the incubation time in minutes with Protease K or trypsin. The concentrations of bLf and SLs were 0.1 wt%.

Fig. 5. Surface hydrophobicity of bLf, SLs, and bLf + SLs under acidic and neutral conditions.
Hydrophobicity indexes were calculated based on the slopes of fluorescence intensity vs sample concentration curves. Data are expressed as mean ± standard deviation (n = 3).

Fig. 6. CD spectra of the pH 5.0 and pH 7.0 bLf and bLf-SLs solutions. Measurements
were performed at 25°C on a spectropolarimeter using a quartz cell with 0.1 mm optical path length. Values are expressed as the mean of the data obtained from three samples, with measurements repeated three times for each sample.
Figure 1
Figure 2

(a) bLf  
\(d_{4,3} = 11.0 \pm 0.0\)

(b) SLs  
\(d_{4,3} = 1239.0 \pm 152.3\)

(c) bLf & SLs  
\(d_{4,3} = 2115.5 \pm 432.2\)

(d) bLf  
\(d_{4,3} = 11.3 \pm 0.1\)

(e) SLs  
\(d_{4,3} = 13.7 \pm 2.9\)

(f) bLf & SLs  
\(d_{4,3} = 16.9 \pm 1.2\)
Figure 3

(a) pH 5.0

bLf

SLs

bLf & SLs

A

B

C

D

x 1,000

30.0 µm

x 3,000

10.0 µm
Figure 3 (continued)

(b) pH 7.0

\[ \begin{array}{cc}
\text{bLf} & \text{SLs} \\
\text{bLf & SLs} & \\
\end{array} \]

\[ \begin{array}{cc}
\text{x 1,000} & \text{x 3,000} \\
\end{array} \]

\[ \begin{array}{cc}
\text{30.0 \(\mu\)m} & \text{10.0 \(\mu\)m} \\
\end{array} \]
Figure 4

pH 5.0

(a) bLf

(b) bLf & SLs

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<td>M C 30 60 90</td>
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pH 7.0

(c) bLf

(d) bLf & SLs

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Figure 5

(a) pH 5.0

(b) pH 7.0
Figure 6

(a) pH 5.0

(b) pH 7.0