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

Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2016-0057.R2
Manuscript Type:	Article
Date Submitted by the Author:	12-Oct-2016
Complete List of Authors:	Matsumiya, Kentaro; Kyoto University, Laboratory of Quality Analysis and Assessment Suzuki, Yasushi; Saraya Co. Ltd., Biochemical laboratory Hirata, Yoshihiko; Saraya Co., Ltd, Biochemical Laboratory Nambu, Yuko; Kyoto University Matsumura, Yasuki; Kyoto University, Laboratory of Quality Analysis and Assessment
Keyword:	Lactoferrin, Sophorolipids, Protein-surfactant interaction, Complex, Electrostatic interaction

SCHOLARONE™
Manuscripts

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

3

4 Kentaro Matsumiya¹, Yasushi A. Suzuki², Yoshihiko Hirata², Yuko Nambu¹, Yasuki
5 Matsumura¹

6 ¹Graduate School of Agriculture, Kyoto University, Japan, ²Lactoferrin Laboratory,
7 Saraya Co.,Ltd., Japan

8

9 *To whom correspondence should be addressed. Tel: +81-774-38-3748. Fax: +81-774-
10 38-3746. Email: matsumiya@kais.kyoto-u.ac.jp

11 Mailing address: Laboratory of Quality Analysis and Assessment, Division of
12 Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University,
13 Gokasho, Uji, Kyoto 611-0011, Japan

1 Abstract

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

10

11 Keywords

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

14

15 1. Introduction

16 Lactoferrin, first discovered in bovine milk, is a red-colored glycoprotein that
17 can bind iron ions with high affinity (Sørensen and Sørensen 1939; Groves 1960).
18 Bovine lactoferrin (bLf) is a single-chain protein consisting of 689 amino acid residues,
19 with a molecular weight of 78,000 Da and an isoelectric point of pH 8.8 (Moore et al.
20 1997; Shimazaki 1993). Since lactoferrin is associated with a diverse range of
21 biochemical functions, it is widely accepted that it is a multi-functional protein that can
22 be used in the manufacture of different products including foods, drugs, and cosmetics.
23 Some of its important biological functions include anti-inflammatory activity,
24 bacteriostatic activity, growth factor action, and bone formation (Lönnerdal 2013).

1 Amongst the various functions of lactoferrin, researchers have particularly
2 focused on its role in stimulating skin keratinocyte function and wound
3 re-epithelialization, originally reported by Tang et al. (2010). In our previous study, we
4 demonstrated that bLf significantly increased tropoelastin expression in cultured
5 fibroblasts, and this expression is efficiently promoted by addition of a novel skin
6 penetration enhancer, acid-type SLs (Ishii et al. 2012). In the same study, we also
7 observed that on addition of SLs, transdermal absorption of bLf through a model skin
8 was enhanced up to 1.7-fold, possibly due to complex formation between bLf and SLs.
9 However, the exact protein-surfactant interactions remain to be understood.

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

21 In the current study, to investigate the protein-surfactant interactions between
22 bLf and SLs, structural changes in bLf induced by SLs were analyzed using
23 spectroscopic, microscopic, and biochemical methods. bLf-SL complexes formed at pH
24 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_2PO_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):

$$[\theta]_{\lambda} = \frac{\theta_{obs} \times MRW}{10 \times d \times c}$$

where λ = wavelength; θ_{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

1 SLs was roughly 10 nm (Fig. 2d and e). This indicates that bLf and SLs dissolved well
 2 in the solutions. On mixing, they had a tendency to form aggregates of about 17 nm (Fig.
 3 2f). The isoelectric point of bLf is pH 8.8 (Moore et al. 1997; Shimazaki 1993), making
 4 the protein positively charged at pH 5.0 and pH 7.0 and giving it a tendency to bind to
 5 negatively charged molecules in solutions with these pH values. At pH 5.0 or higher, the
 6 solubility of SLs increases with increasing pH, which is closely related to the
 7 electrolytic dissociation of the linked hydroxyl fatty acids (Fig. 1) (Hu and Ju 2001; Van
 8 Bogaert et al. 2011). A similar tendency was also observed for the SLs used in this study.
 9 These data suggest that SLs are negatively charged both at pH 5.0 and at pH 7.0.
 10 Therefore, it is reasonable that bLf would interact with SLs to form complexes under
 11 the tested conditions. bLf contains 101 basic amino acid residues such as His, Lys, and
 12 Arg that can interact with the hydroxyl fatty acid residue of SLs. Since the molar ratio
 13 of bLf and SLs was approximately 1:125, all the basic residues, though not exposed to
 14 the aqueous phase, can be potentially capped by adequate amounts of SLs by
 15 electrostatic interactions.

16 3.2. Microstructure

17 To gain insight into the structural features of the possible complexes,
 18 Cryo-SEM was conducted to observe bLf or/and SLs present in the sample solutions.
 19 Fig. 3a describes Cryo-SEM images obtained from the sample solutions at pH 5.0
 20 containing 0.1 wt% of bLf, SLs, or a combination of bLf and SLs. bLf had a weed-like
 21 fibrous structure (A) that did not seem to aggregate. SLs self-organized into a
 22 ribbon-like architecture (B), as reported by Zhou et al. (2004), and fibrous networks (C).
 23 In solutions where bLf and SLs existed together, well-structured sheet-like materials (D)
 24 were prominently observed. The structures formed by bLf, SLs, and bLf and SLs were

1 observed in most fields of view. Our results suggest that bLf and SLs form continuous
2 giant complexes, similar to the original self-assembly of SL molecules reported
3 previously (Zhou et al. 2004), and do not appear to form a significant amount of
4 vesicle-like complexes.

5 Fig. 3b shows the Cryo-SEM micrographs of the sample solutions at pH 7.0.
6 Unlike the results shown in Fig. 3a, similar structures were found in bLf, SLs and
7 solutions containing bLf & SLs. However, on magnification, some small sheet-like
8 structures were seen in the SLs solution (E). Additionally, at the low magnification
9 (1,000x), the fibrous network in SLs appeared to be finer than the bLf network, while in
10 solutions containing a mixture of bLf and SLs, the width of the fibrous network ranged
11 between the width observed in the bLf and SLs solutions. Therefore, we can assume that
12 in neutral solutions, SLs interact with bLf to form relatively small complexes that
13 depend on electrostatic attractions (Fig. 2f), but does not induce significant changes in
14 the bLf structures. Since the interactions were not strong, it was not possible to identify
15 them under the microscope. Therefore, in order to further investigate interactions
16 between bLf and SLs, a biochemical enzymatic digestion was carried out.

18 3.3. Proteolysis

19 Based on research by Brines and Brock (1983), enzymatic digestions were
20 performed to confirm the structural properties of bLf-SLs complexes. If bLf is included
21 in the vesicle-like self-organization of SLs then it would be quite resistant to proteolytic
22 degradation and would remain structurally intact. Fig. 4 shows SDS-PAGE profiles of
23 enzymatically-digested bLf using Protease K and a pancreatic trypsin under weakly
24 acidic and neutral conditions with or without SLs. The concentration of bLf and SLs

1 were 0.1 wt%. In acidic pH and in the absence of SLs, bLf was extensively digested by
 2 Protease K and trypsin (Fig. 4a). However, in the presence of SLs, specific undigested
 3 fractions of bLf were generated on treatment with Protease K and trypsin (Fig. 4b). At
 4 neutral pH, both enzymes also efficiently digested bLf in the absence of SLs (Fig. 4c),
 5 whereas there were clear undigested protein bands in Protease K-treated or
 6 Trypsin-treated samples (Fig. 4d). These results indicate that bLf interacted with SLs
 7 but was not completely protected by the surrounding SLs. These results also support the
 8 Cryo-SEM observations that bLf was not incorporated into vesicles organized by SLs
 9 but was associated with the ribbon-like self-organization that occurred at pH 5 (Fig. 3a)
 10 and the network-like self-organization that occurred at pH 7, and was mostly exposed to
 11 the aqueous phase in both structures. In addition, the digestion patterns at pH 7.0 and
 12 pH 5.0 were quite different, supporting the observation that bLf was incorporated into
 13 different structures at these two pHs (Fig. 3a, 3b).

15 3.4. Tertiary structural changes

16 To investigate tertiary structural changes in bLf caused by SLs, the surface
 17 hydrophobicity of bLf in the presence of SL molecules was evaluated using ANS-Na as
 18 a fluorescent probe. This type of approach is often used for estimating the
 19 conformational change in globular proteins caused by heat treatment, interaction with
 20 lipid membranes, etc. (Haynes and Staerk 1974; Alizadeh-Pasdar and Li-Chan 2000;
 21 Haweet et al. 2008). Since lactoferrin is a globular protein, inner hydrophobic sites tend
 22 to not be exposed to the hydrophilic phase in the aqueous solution. If conformational
 23 changes induced by environmental factors, such as heat, or chemical factors, such as
 24 protein-surfactant attractive interactions, result in exposure of hydrophobic residues,

1 ANS-Na binding to the exposed hydrophobic sites can significantly increase ANS
2 fluorescence (De et al. 2005). The hydrophobicity indexes of bLf, SLs, and bLf + SLs
3 were defined as the slopes obtained from the ANS fluorescence vs bLf, SLs, and bLf +
4 SLs concentration curves (Kato and Nakai 1980; Hayakawa and Nakai 1985). The
5 hydrophobicity index obtained from the bLf solution at pH 5.0 was significantly
6 different from that of the solution containing both bLf and SLs (*t*-test, $p < 0.05$). The
7 values for the bLf solution and the solution containing both bLf and SLs were $370.7 \pm$
8 1.6 and 1092.3 ± 71.7 , respectively (Fig. 5a). Similarly, at pH 7.0, the hydrophobicity
9 index of the solutions containing both bLf and SLs (478.1 ± 25.8) was much higher than
10 the solution containing only bLf (222.4 ± 3.4) (Fig. 5b)

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

18

19 3.5. Secondary structural changes

20 Fig. 6 describes the CD spectra of the bLf solutions with and without SLs in
21 acidic and neutral conditions. At pH 5.0, wavelength-dependent molar ellipticity of
22 solutions containing only bLf ranged between approximately $-10,000$ to $0 \text{ deg-cm}^2/\text{dmol}$
23 (Fig. 6a and b), similar to the result described by Hu et al. (2008). The CD spectrum of
24 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.

1 References

- 2 Alizadeh-Pasdar, N., and Li-Chan, E. C. Y. 2000. Comparison of protein surface
3 hydrophobicity measured at various pH values using three different fluorescent
4 probes. *J. Agric. Food Chem.* **48**: 328–334.
- 5 Brines, R. D., and Brock, J. H. 1983. The effect of trypsin and chymotrypsin on the in
6 vitro antimicrobial and iron-binding properties of lactoferrin in human milk
7 and bovine colostrum. Unusual resistance of human apolactoferrin to
8 proteolytic digestion. *BBA - Gen. Subj.* **759**: 229–235.
- 9 De, S., Girigoswami, A., and Das, S. 2005. Fluorescence probing of albumin-surfactant
10 interaction. *J. Colloid Interface Sci.* **285**: 562–573.
- 11 Fasman, G. D. 1978. Circular dichroism analysis of chromatin and DNA–nuclear
12 protein complexes. In *Methods in cell biology*, vol 18: Chromatin and
13 chromosomal protein research III, eds. Gary Stein, Janet Stein, and Lewis J.
14 Kleinsmith. New York: Academic press, Inc., p. 327–350.
- 15 Groves, M. L. 1960. The isolation of a red protein from milk. *J. Am. Chem. Soc.*
16 **82**:3345–3350.
- 17 Hawe, A., Sutter, M., and Jiskoot, W. 2008. Extrinsic fluorescent dyes as tools for
18 protein characterization. *Pharm. Res.* **25**: 1487–1499.
- 19 Hayakawa, S. S., and Nakai, S. 1985. Relationships of hydrophobicity and net charge to
20 the solubility of milk and soy proteins. *J. Food Eng.* **50**: 486–491.
- 21 Haynes, D. H., and Staerk, H. 1974. 1-Anilino-8-naphthalenesulfonate: a fluorescent
22 probe of membrane surface structure, composition and mobility. *J. Membr.*
23 *Biol.* **17**: 313–340.
- 24 Hu, F., Pan, F., Sawano, Y., Makino, T., Kakehi, Y., Komiyama, M., et al. 2008. Studies

- 1 of the structure of multiferric ion-bound lactoferrin: A new antianemic edible
- 2 material. *Int. Dairy J.* **18**: 1051–1056.
- 3 Hu, Y., and Ju, L. K. 2001. Purification of lactonic sophorolipids by crystallization. *J.*
- 4 *Biotechnol.* **87** :263–272.
- 5 Imura, T., Morita, T., Fukuoka, T., Ryu, M., Igarashi, K., Hirata, Y., et al. 2014.
- 6 Spontaneous vesicle formation from sodium salt of acidic sophorolipid and its
- 7 application as a skin penetration enhancer. *J. Oleo Sci.* **63**: 141–147.
- 8 Ishii, N., Kobayashi, T., Matsumiya, K., Ryu, M., Hirata, Y., Matsumura, Y., et al. 2012.
- 9 Transdermal administration of lactoferrin with sophorolipid. *Biochem. Cell*
- 10 *Biol.* **90**: 504–512.
- 11 Kato, A., and Nakai, S. 1980. Hydrophobicity determined by a fluorescence probe
- 12 method and its correlation with surface properties of proteins. *Biochim.*
- 13 *Biophys. Acta - Protein Struct.* **624**: 13–20.
- 14 Konishi, M., Fukuoka, T., Morita, T., Imura, T., and Kitamoto, D. 2008. Production of
- 15 new types of sophorolipids by *Candida batistae*. *J. Oleo Sci.* **57**: 359–369.
- 16 Lang, S. 2003. Surfactants produced by microorganisms. In *Novel surfactants:*
- 17 *Preparation applications and biodegradability*, second edition, revised and
- 18 expanded, Surfactant Science, ed. Krister Holmberg. New York, USA: CRC
- 19 Press, p. 279–316.
- 20 Lönnerdal, B., and Suzuki, Y. A. 2013. Lactoferrin. In *Advanced Dairy Chemistry*
- 21 *Volume 1A: Proteins: Basic Aspects*, eds. Paul L. H. McSweeney and Patrick F.
- 22 Fox. New York, USA: Springer, p. 295–315.
- 23 Moore, S. A., Anderson, B. F., Groom, C. R., Haridas, M., and Baker, E. N. 1997.
- 24 Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *J.*

- 1 Mol. Biol. **274**: 222–236.
- 2 Nuñez, A., Ashby, R., Foglia, T. A., and Solaiman, D. K. Y. 2001. Analysis and
3 characterization of sophorolipids by liquid chromatography with atmospheric
4 pressure chemical ionization. *Chromatographia*. **53**: 673–677.
- 5 Shimazaki, K. 1993. Analysis of Human and Bovine Milk Lactoferrins By Rotofor and.
6 **25**: 1653–1658.
- 7 Sørensen, M. and Sørensen, S.P.L. (1939). The protein in whey. C. R. Trav. Lab.
8 Carlsberg **23**: 55–99.
- 9 Tang, L., Wu, J.J., Ma, Q., Cui, T., Andreopoulos, F.M., Gil, J., et al. 2010. Human
10 lactoferrin stimulates skin keratinocyte function and wound re-epithelialization.
11 *Br. J. Dermatol.* **163**(1): 38–47.
- 12 Van Bogaert, I. N. A., Zhang, J., and Soetaert, W. 2011. Microbial synthesis of
13 sophorolipids. *Process Biochem.* **46**: 821–833.
- 14 Zhou, S., Xu, C., Wang, J., Gao, W., Akhverdiyeva, R., Shah, V., and Gross, R. 2004.
15 Supramolecular assemblies of a naturally derived sophorolipid. *Langmuir*,
16 **20**(19): 7926–7932.
- 17

1 Figure captions

2

3 Fig. 1. Chemical structure of an SL.

4 Molecular weight of the displayed chemical compound is 622 Da.

5

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

8

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

11

12 Fig. 4. SDS-PAGE patterns of bLf digested by proteolytic enzymes with or without SLs.

13 Enzymatic treatments were carried out at 37°C for 30, 60, and 90 min. M, C, and the
14 numbers shown above the gels refer to molecular weight markers (M), untreated bLf
15 under the specified pH conditions serving as a control (C), and the incubation time in
16 minutes with Protease K or trypsin. The concentrations of bLf and SLs were 0.1 wt%.

17

18 Fig. 5. Surface hydrophobicity of bLf, SLs, and bLf + SLs under acidic and neutral
19 conditions.

20 Hydrophobicity indexes were calculated based on the slopes of fluorescence intensity vs
21 sample concentration curves. Data are expressed as mean $\pm$ standard deviation (n = 3).

22

23 Fig. 6. CD spectra of the pH 5.0 and pH 7.0 bLf and bLf-SLs solutions. Measurements

- 1 were performed at 25°C on a spectropolarimeter using a quartz cell with 0.1 mm optical
- 2 path length. Values are expressed as the mean of the data obtained from three samples,
- 3 with measurements repeated three times for each sample.

Draft

1

Draft

1 Figure 1

2

Draft

1 Figure 2

1 Figure 3

(a) pH 5.0

x 1,000

x 3,000

bLf

SLs

bLf & SLs

2

1 Figure 3 (continued)

(b) pH 7.0

x 1,000

x 3,000

bLf

SLs

bLf & SLs

2

1 Figure 4

pH 5.0

(a) bLf

(b) bLf & SLs

pH 7.0

(c) bLf

(d) bLf & SLs

1 Figure 5

2 (a) pH 5.0

3

4 (b) pH 7.0

5

Draft

Figure 6

(a) pH 5.0

(b) pH 7.0