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Report

The Lower Toxicity and Wider Safety Range of Acidic Sophorolipid Compared to Surfactin and Rhamnolipid as Biosurfactants toward the HaCAT, THP-1, and RAW 264.7

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As the number of consumers suffering from rough hands and dermatitis caused by the frequent daily use of synthetic surfactants increases, biocompatible materials are required. Biosurfactants (BSs), compounds excreted or produced by microbial cells, attract attention as cosmetic substrates suitable for human skin and the environment. This study evaluated the utility of the glycolipid-type sophorolipid (SL) produced by the non-pathogenic yeast *Starmerella bombicola*, as a BS. The cytotoxicity of open-chain acid type SL (SL acid) among SLs, is approximately 100–250 times less than that of commercially available surfactants in all cells. Therefore, SL acid is a promising surfactant with a high safety profile. In contrast, the critical micelle concentration (CMC) of SL acid, surfactin, and rhamnolipid were 1,000 mg/L, 16 mg/L, and 38 mg/L, respectively, indicating that SL acid has lower functionality than the other BSs. Finally, the safety range was analyzed for each BS to indicate practicality. The safety range, the concentration range where each BS can exhibit its function without cytotoxicity, was defined based on the lethal concentration 50 (LC_{50})/CMC value. As a result, the safety range of SL acid is 3.9–4.4 times wilder than that of surfactin and rhamnolipids. Consequently, SL acid could be a promising BS with a wider safety range than other BSs, such as surfuctin and rhamnolipids.

Key words biosurfactants, sophorolipid, safety range

INTRODUCTION

Since the spring of 2020, COVID-19 has increased the opportunities for hand disinfection with alcohol and mask-wearing, such that many people suffer from skin problems such as rough hands, contact dermatitis, and dry skin.[1,2] There is an urgent need to develop skin cosmetics to solve these problems.

Skin cosmetics are frequently used in daily life. Therefore, they must be safe. With carbon neutrality pursued worldwide, environmental friendliness and sustainability are required. Therefore, biosurfactants (BSs), compounds excreted or produced by microbial cells, attract attention as cosmetic substrates suitable for the skin and environment.[3]

BS are amphipathic compounds with hydrophilic and hydrophobic groups, similar to chemically synthesized surfactants. They are classified into lipopeptide, fatty acid, and glycolipid types according to the structure of their hydrophilic group.[4] In general, synthetic surfactants cause environmental pollution owing to their persistent properties. In contrast, BSs are active and biodegradable even at low concentrations. Thus, BSs are considered safe for the skin and environmentally friendly as well.[5] Therefore, BSs are expected to be used as detergents in household products and pharmaceuticals[6] and cosmetics.[7,8]

As one of the BSs, the glycolipid-type sophorolipid (SL) is produced by the non-pathogenic yeast *Starmerella bombicola* and is fundamentally composed of fatty acids with 16- or 18-carbon groups and sophorose, a disaccharide in which glucose is bound at the β-1,2 position.[9] Natural SL produced by *Starmerella bombicola* is a mixture of lactone-type SL (SL lac), in which the carboxyl group in fatty acids is condensed with a hydroxyl group in glucose, and open-chain acid-type SL (SL acid), in which SL lac is hydrolyzed and used. Although the physiological significance of producing sophorolipids is unclear, it has been reported that sophorolipids may have antibacterial activity and may be used to store nutritients by converting carbon sources to SL.[10] Among the various BSs, SL has been reported to be relatively productive and promising. For example, the productivity of SL (70 g/L)[11] is approximately 5–30 times higher than those of other BSs (2.25 g/L for rhamnolipids,[12] 12.4 g/L for mannosylerythritol lipids).[13] In contrast, SL lac is relatively cytotoxic, partly because of its high hydrophobicity.[14]

However, the SL acids' physicochemical properties and biological activities are largely unknown. In this respect, we established a high purification method, which is essential for analyzing SL acids, using our original technology.[15]

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In this study, to understand the safety of SL acid, its cytotoxicity was compared against various cell lines with that of other BSs. In addition, as a function of BSs, the critical micelle concentration (CMC) of various BSs was analyzed from their surface tension. Finally, the safety range was compared to the concentration range in which each BS could exhibit its surface tension without cytotoxicity; thus, the utilization of SL in BS was evaluated in detail.

MATERIALS AND METHODS

Materials Surfactin Na, rhamnolipid, and Tween 20 were obtained from Kaneka Co. Ltd. (Tokyo, Japan), Evonik Co. Ltd. (Essen, NRW, Germany), and Nacalai Tesque (Kyoto, Japan), respectively. Human epidermal keratinization cell line (HaCaT), human acute monocytic leukemia-derived cell line (THP-1), and mouse leukemia macrophage cell line (RAW264.7) were purchased from the American Type Culture Collection (Manassas, VA, USA). HaCaT and RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS) (Biosera Inc., Nuaille, France), supplemented with 1% penicillin-streptomycin-amphotericin B anti biotics suspension (Ab) (FUJIFILM Wako Pure Chemical). THP-1 cells were cultured in RPMI1640 containing 10% FBS, 1% Ab, and 0.1% 2-mercaptoethanol. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The test substances were dissolved and adjusted to pH 7.4 by adding NaOH before use.

Preparation of Natural SL and SL Acid Natural SLs were produced by *Candida bombicola* ATCC22214 using a jar fermenter with 10% glucose and 10% soybean oil as carbon sources and purified as previously reported[15]. Natural SLs with a composition ratio of SL lac to SL acid of approximately 7:3 were dissolved in 0.5 mL of 5N NaOH aqueous solution and placed in a test tube. To purify SL acid, the test tube was tightly sealed with a screw cap and heated (80°C) in a water bath for 2 h for alkaline hydrolysis. The SL acid was purified by ODS column chromatography using a gradient solvent system of water/ethanol (90:10 to 0:100, v/v) and powdered by spray drying. The constituent fatty acids of natural SLs and SL acid are mainly oleic acid (<85%, C18⊿1) and other fatty acid such as palmitic acid, stearic acid and linoleic acid (<15%, C16, C18, C18⊿2).

Cytotoxicity Assay Each cell line was seeded in a 96-well culture plate (Thermo Fisher Scientific K.K., Tokyo, Japan) for the cytotoxicity assay at 5.0 x 10^3 cells/well. After incubating at 37°C for 24 h, the medium was removed and replaced with 100 µL of medium containing test substance at various concentrations ranging from 23.4375 to 48,000 mg/L. The test substance was dissolved in the medium and adjusted to pH 7 by adding NaOH. After incubating for 24 h at 37°C, cell viability was evaluated by WST-8 assay according to the Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) protocol. The LC_{50} was quantified by sigmoid fitting of cell viability at each concentration using KaleidaGraph 3.6 J (Synergy Software, Montgomery, PA, USA).

CMC Determination from Surface Tension The surface tension and critical micelle concentration (CMC) were measured as previously described.[16] Briefly, the surface tension was measured with a CBVP-Z tensiometer (Kyowa Interface Science, Saitama, Japan) according to the Wilhelmy method using a solution with SL concentrations (0.1–10,000 mg/L). Surface tension measurements were conducted at 20°C. The CMC was calculated from the surface tension-logarithmic concentration curve.

RESULTS AND DISCUSSION

Comparative Analysis of the Cytotoxicity of SL Together with Various Surfactants Against Various Types of Cell Lines To understand the safety of SL acid, its cytotoxicity against various cell lines was compared with that of other surfactants and natural SL *in vitro.* For other surfactants, surfactin and rhamnolipid are commercially available BS, and polyoxyethylene sorbitan monolaurate (Tween 20) is a representative synthetic surfactant (Fig. 1). As SL is used on the skin, three human-derived cell lines were selected for the assay: the skin keratinocyte cell line (HaCaT), which is widely used in dermatological research such as epidermal homeostasis analysis;[17] the monocyte cell line (THP-1), commonly used for skin sensitization analysis[18] and the macrophage cell line (RAW 264.7), used for skin inflammation analysis.[19] Twenty-four hours after adding a series of concentrations of various surfactants to the three cell lines, cytotoxicity was assessed. WST8 assay showed that all surfactants were cytotoxic to all cell lines in a concentration-dependent manner (Fig. 2). Table 1 shows the list of LC_{50} of each surfactant for the three cell lines. LC_{50} of surfactin was 80.4 mg/L for HaCaT, 76.7 mg/L for THP-1, and 57.6 mg/L for RAW 264.7, respectively, having an average LC_{50} of 71.6 mg/L. Similarly, the average LC_{50} for rhamnolipid and Tween 20 were 152.3 mg/L and 139.1

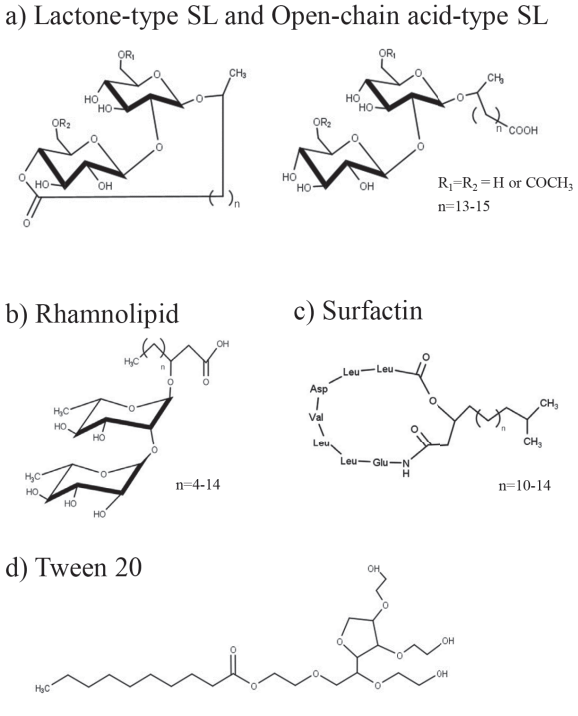

Fig. 1. Structure of Sophorolipid, Rhamnolipid, Surfactin, and Tween 20

a) Sophorolipid is a glycolipid-type biosurfactant composed of fatty acids and sophorose. Natural SL is a mixture of lactone-type SL in which the carboxyl group in fatty acids is condensed with a hydroxyl group in glucose (right panel), and open-chain acid-type SL, in which SL lac is hydrolyzed (left panel). b) Rhamnolipid is a glycolipid-type biosurfactant composed of fatty acids and rhamnose. c) Surfactin is a biosurfactant composed of a peptide with 7 amino acids and fatty acid. d) Tween 20 is a synthetic surfactant composed of nonionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan.

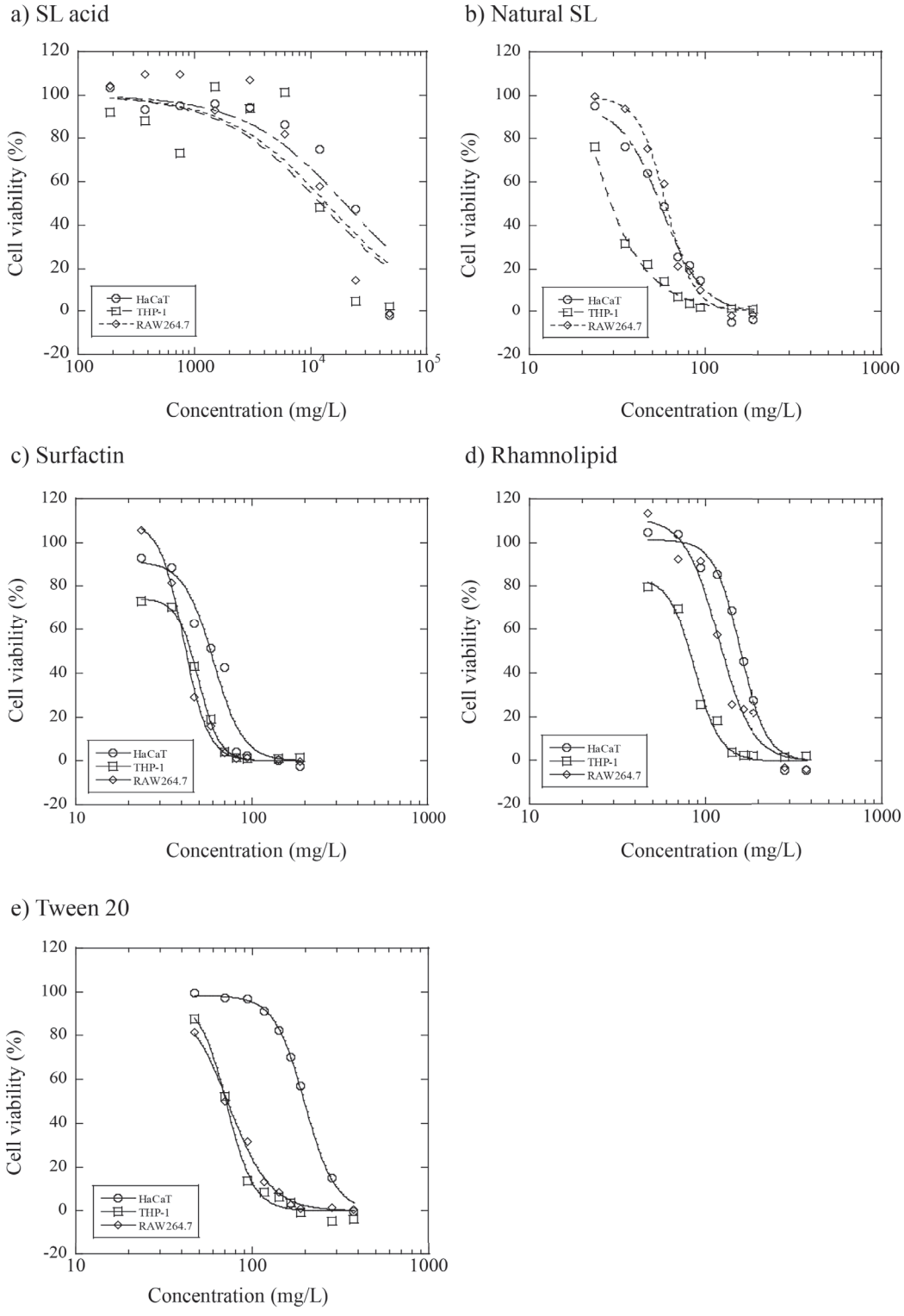

Fig. 2. Cytotoxicity Profile of Various Surfactants

HaCaT, THP-1, and RAW264.7 cell viability at a series of concentrations of a) SL acid, b) Natural SL, c) Surfactin, d) Rhamnolipid, and e) Tween 20 were plotted. KaleidaGraph 3.6 J was used for sigmoidal fitting. The test was performed thrice at least.

mg/L, respectively. In contrast, the LC_{50} of SL acid was 20,930 mg/L for HaCaT, 17,467 mg/L for THP-1, and 14,707 mg/L for RAW 264.7, with an average LC_{50} of 17,701 mg/L. These data suggest that the cytotoxicity of SL acid is approximately 100-250 times less than commercially available surfactants. Therefore, SL acid is a promising surfactant with a high safety profile, although further analysis, including *in vivo* safety tests, is required. In addition, the LC_{50} of natural SL was 65.3 mg/L for HaCaT, 37.2 mg/L for THP-1, and 68.6 mg/L for RAW 264.7, respectively, obtaining an average LC_{50} of 57.0 mg/L. It has been reported that lactone-type SL with a diacetyl group contained in natural SL has high cytotoxicity.[16] However, SL acid has almost no acetyl groups but a carboxyl group, making it more hydrophilic than lactone-type SL with a

Table 1. LC_{50} (mg/L) List of Each Surfactant Calculated from Cytotoxicity Tests

Cell line	SL acid	Natural SL	Surfactin	Rhamnolipid	Tween 20
HaCaT	20,930 ± 747	65.3 ± 5.2	80.4 ± 7.4	165.2 ± 7.8	187.3 ± 38.0
THP-1	17,467 ± 2,402	37.2 ± 8.3	76.7 ± 9.3	126.7 ± 14.8	132.1 ± 20.3
RAW264.7	14,707 ± 2,480	68.6 ± 5.0	57.6 ± 8.8	166.2 ± 24.3	98.0 ± 15.9
Average	17,701 ± 1,852	57.0 ± 6.2	71.6 ± 8.5	152.7 ± 15.6	139.1 ± 24.7

Data are expressed as mean ± S.E. of at least three separate experiments.

Table 2. LC_{50} and CMC Values of SL and Other BSs in Various Cells

	Minimum surface tension (mN/m)	CMC(mg/L)	LC_{50}/CMC			
			HaCaT	THP-1	RAW264.7	Average
SL acid	36.4	1,000	20.9	17.5	14.7	17.7
Surfactin	27.6	16	5.0	4.8	3.6	4.5
Rhamnolipid	28.9	38	4.4	3.3	4.4	4.0

diacetyl group. Therefore, natural SL containing lactone-type SL with a diacetyl group is more hydrophobic and cytotoxic than SL acid. Moreover, Adu SA *et al*. reported that the LD_{50} of diacetyl lactone-type SL is higher than that of non-acetyl acid-type SL, so our data correlating with these reports may be more reliable.[20,21]

These findings indirectly suggest that SL lac is more cytotoxic than SL acid. However, it must be demonstrated that the cytotoxicity of natural SL is not due to trace impurities other than SL acid or SL lac in natural SL.

Determination of CMC from the Surface Tension of Various Surfactants and Comparative Analysis of Safety Ranges To compare the functionality of each surfactant, the CMC was determined from the surface tension. The surface tension of the SL acid was quantified using the Wilhelmy method and compared with those of surfactin and rhamnolipid, which are commercially available BSs. Table 2 showed that the minimum surface tensions of SL acid, surfactin and rhamnolipid were 36.4, 27.7, and 28.9 mN, respectively. Therefore, the CMC of SL acid, surfactin, and rhamnolipid were 1,000, 16, and 38 mg/L, respectively. While the data suggested that SL acid has lower functionality than the other BSs, it had the lowest cytotoxicity and the highest safety *in vitro*.

Finally, the safety range (the concentration range where each BS can exhibit its function without cytotoxicity) was evaluated to compare their practicality. The safety range was defined as the LC_{50}/CMC value,[22,23] indicating that a higher value of LC_{50}/CMC means a wider safety range. Safety range analysis of each BS showed that the average LC_{50}/CMC of SL acid, surfactin, and rhamnolipids were 17.7, 4.5, and 4.0, respectively. The data suggest that the safety range of SL acid is 3.9–4.4 times wilder than surfactin and rhamnolipid. SL acid may be more hydrophilic than surfactin and rhamnolipids because the functional groups forming hydrogen bonds in SL acid are delocalized over a larger volume. Therefore, SL acid may have less membrane permeability and less cytotoxicity.

Furthermore, Tween 20 is approved as a food additive, is used as a cosmetic drug, and is considered a safer ingredient. The safety range of SL acid is 3–10 times wilder than that of Tween 20 (CMC: 20 to 80 mg/L, LC50/CMC: 1.7–7.0), suggesting that the safety of SL acid is certainly considered high. It has been reported that surfactants with lower CMC tend to be more cytotoxic,[24] so our data correlating with this report may be more reliable. Therefore, a balance between the functionality and safety of surfactants is important for their utilization. In particular, it is more practical to have a wider safety range for surfactants used daily, even if their activity is low. Consequently, SL acid could be a promising BS with a wider safety range than other BSs, such as surfactin and rhamnolipids.

Conclusions This study revealed that SL acid has lower toxicity and a wider safety range than surfactin and rhamnolipids as biosurfactants toward HaCAT, THP-1, and RAW 264.7 cell lines. Consequently, SL acid is a promising surfactant with many safety characteristics. By further analyzing *in vivo* safety tests, we hope SL will be used as a substrate for cosmetics and medicines.

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Conflict of interest The results of this study were partially collected using a collaborative research fee from Saraya Co., Ltd.

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