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Enzymatic synthesis of a novel glycolipid biosurfactant, mannosylerythritol lipid-D and its aqueous phase behavior

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ABSTRACT

Mannosylerythritol lipids (MELs) produced by yeasts are one of the most promising glycolipid biosurfactants. In this study, we succeeded in the preparation of a novel MEL homolog having no acetyl groups, namely MEL-D. MEL-D was synthesized by lipase-catalyzed hydrolysis of acetyl groups from a known MEL, and identified as 4-*O*-[2′,3′-di-*O*-alka(e)noyl-β-D-mannopyranosyl]-(2*R*,3*S*)-erythritol. The obtained MEL-D showed a higher critical aggregation concentration (CAC = 1.2×10^{-5} M) and hydrophilicity compared to known MELs, retaining an excellent surface tension lowering activity (the surface tension at the CAC was 24.5 mN/m). In addition, we estimated the binary phase diagram of the MEL-D–water system based on a combination of visual inspection, polarized optical microscopy, and SAXS measurement. From these results, MEL-D was found to self-assemble into a lamellar (L_α) structure over all ranges of concentration. Meanwhile, the one-phase L_α region of MEL-D was extended wider than those of known MELs. MEL-D might keep more water between the polar layers in accordance with the extension of the interlayer spacing (*d*). These results suggest that the newly obtained MEL-D would facilitate the application of MELs in various fields as a lamellar-forming glycolipid with higher hydrate ability.

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1. Introduction

Mannosylerythritol lipids (MELs, Fig. 1) are one of the most promising glycolipid-type biosurfactants, which are amphiphilic compounds produced by microorganisms from renewable resources.[1–3] MELs are abundantly produced from vegetable oils and sugars by the yeast strains of the genus *Pseudozyma*[1,4] and the fungi of the genus *Ustilago*.[5,6] These glycolipids exhibit not only excellent surface-active properties,[7] but also versatile biochemical actions,[1] including cell-differentiation, induction of different mammalian cells,[8] as well as affinity binding toward different immunoglobulins.[9–11] In addition, MELs markedly increase the efficiency of gene transfection mediated by cationic liposomes via membrane fusion between liposomes and plasma membranes.[12–14] Very recently, we have demonstrated that MELs show a ceramide-like moisturizing activity and cell activating properties toward skin cells,[15,16] as well as hair care properties.[17] MELs have thus great potential not only as environmentally friendly surfactants but also as advanced biomedical and cosmetic materials.

Conventional MELs, namely MEL-A, -B, and -C, have 4-*O*-β-D-mannopyranosyl-erythritol as the hydrophilic part and two fatty acyl groups as the hydrophobic part. In addition, these MEL homologs have one or two acetyl groups at C-4′ and/or C-6′ in the mannose moiety (Fig. 1). Interestingly, these glycolipids show quite different physicochemical and biochemical properties, although the difference in the chemical structure is very small.[18–21] For instance, di-acetylated MEL-A spontaneously forms an L_3 (sponge) phase in a wide range of concentrations, while mono-acetylated MEL-B forms L_α (lamellar) and myelin structures.[18] Moreover,

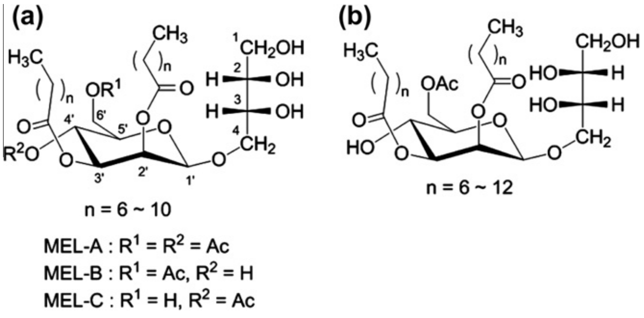

Figure 1. Chemical structures of the conventional mannosylerythritol lipids. (a) MEL-A, B, and C produced by *Pseudozyma antarctica* and (b) MEL-B produced by *Pseudozyma tsukubaensis*.

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self-assembled monolayers of di-acetylated MEL-A gave a high affinity toward immunoglobulins, while that of mono-acetylated MEL-B and MEL-C gave almost no affinity.[11] These results indicate that the acetyl groups on the hydrophilic sugar moiety play a key role in the properties of MELs. Thus, the development of novel types of MELs with different degree of acetylation and investigation of their properties would lead to a broad range of applications for them.

Because of this situation, we have been strongly motivated to investigate the properties of a new type of MEL homolog having no acetyl groups, namely MEL-D. However, MEL-D has not been obtained from microbial products, and we have not succeeded in finding an efficient MEL-D producer. In this study, we have thus focused our attention on the efficient preparation of MEL-D by the selective hydrolysis of acetyl groups from conventional MELs. In addition, we investigated the interfacial properties and phase behavior of the obtained MEL-D in water. This is the first report on the synthesis and physicochemical characterization of MEL-D.

2. Experimental section

2.1. Materials

All reagents and solvents were commercially available and were used as received. Lipase, Novozym® 435 (10,000 PLU/g), Lipozyme® RM IM (150 IUN/g), and Lipozyme® TL IM (250 IUN/g) were kindly donated by Novozymes Japan Ltd (Japan). MEL-A (Fig. 1a), 4-*O*-[4′,6′-di-*O*-acetyl-2′,3′-di-*O*-alka(e)noyl-β-D-mannopyranosyl]-(2*S*, 3*R*)-erythritol, was prepared from soybean oil by the yeast strain of *Pseudozyma antarctica* T-34 in our laboratory according to the literature.[19] MEL-B (Fig. 1b),[22] 4-*O*-[6′-*O*-acetyl-2′,3′-di-*O*-alka(e)noyl-β-D-mannopyranosyl]-(2*R*,3*S*)-erythritol, which is the diastereomer of MEL-B (Fig. 1a) produced by *P. antarctica*, was kindly supplied by TOYOBO Co., Ltd (Japan). It was produced from olive oil by the yeast strain of *Pseudozyma tsukubaensis* NBRC 1940 and was used as received.

2.2. Preparation of MEL-D

The following is a typical procedure for the enzymatic synthesis of MEL-D from MEL-B. A mixture of MEL-B (5 g) and Novozym 435 (2.5 g) was dispersed in 90% ethanol (100 mL), and the reaction mixture was stirred for 7 days at 60 °C. The reaction was traced by thin layer chromatography (TLC) on silica plates with chloroform/MeOH/7 M ammonia solution (65:15:2, by vol). The compounds on the plates were located by charring at 110 °C for 5 min after spraying an anthrone/sulfuric acid reagent as previously reported.[23] The purified MEL fraction including MEL-A, -B, and -C prepared as reported previously[24] was used as a standard. In addition, the reaction conversion was estimated by HPLC on a silica gel column (Inertsil SIL 100A 5 μm, 4.6 × 250 mm; GL Science Inc., Japan) with low-temperature evaporative light-scattering detector (ELSD-LT; Shimadzu, Japan) using a gradient solvent program consisting of various proportions of chloroform and MeOH (from 100:0 to 0:100, v/v) at a flow rate of 1 mL/min.[25]

After the hydrolysis reaction, the starting MEL-B was almost completely consumed and the deacetylated product was quantitatively produced (>99%). The immobilized lipase was removed by filtration and the filtrate was evaporated. The residue was dissolved in a mixture of chloroform and acetone (1:1, vol/vol) and then purified by silica gel (Wako-Gel C-200) column chromatography using a gradient elution of chloroform–acetone (5:5→0:10, vol/vol) mixtures as solvent systems. The purified product, MEL-D (yield: 4 g, 80 wt %), was used in the following experiments.

2.3. Structure determination of MEL-D

Structure determination of the purified product dissolved in $CDCl_3$–CD_3OD (90:10, vol/vol) was performed by ^1H, ^{13}C NMR, and two-dimensional NMR analyses, such as ^1H–^1H correlation spectroscopy (COSY), and heteronuclear multiple quantum correlation (HMQC) using a Varian INOVA 400 (400 MHz).

The molecular weight of the purified product was measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Voyager-DE PRO) with an α-cyano-4-hydroxycinnamic acid matrix.

The fatty acid profile of the purified product was examined by gas chromatography–mass spectrometry (GC–MS) as previously reported. The methyl ester derivatives of fatty acids were prepared by mixing the purified MEL-D (10 mg) with 5% HCl–MeOH reagent (1 mL) (Tokyo Kasei Kogyo, Tokyo, Japan) at 80 °C for 20 min. After the reaction mixture was quenched by the addition of water (1 mL), the methyl ester derivatives were extracted with *n*-hexane (2 mL) and then analyzed by GC–MS (Hewlett Packard 6890 and 5973 N) with a TC-WAX (GL-science, Tokyo) with the temperature programed from 90 °C (held for 3 min) to 240 °C at 5 °C/min. **MEL-D**: ^1H NMR ($CDCl_3$–CD_3OD 90:10, 400 MHz): δ 5.45 (d, 1H, J = 3.2 Hz, H-2′), 5.26–5.43 (br, 1.4H, –CH=CH–), 4.91 (dd, 1H, J = 2.0, 10 Hz, H-3′), 4.74 (s, 1H, H-1′), 3.93 (m, 1H, H-4b), 3.91 (m, 1H, H-4a), 3.82 (m, 2H, H-6′), 3.81 (m, 1H, H-4′), 3.72 (m, 2H, H-1), 3.67 (m, 1H, H-3), 3.60 (m, 1H, H-2), 3.38 (m, H-5′, overlapped with the signal of MeOH), 2.77 (t, 0.1H, J = 6.4 Hz, $-CH{=}CHCH_2CH{=}CH-$), 2.39 (t, 2H, J = 5.6 Hz, $-CH_2C{=}O$ at the C-2′ position), 2.30 (m, 2H, $-CH_2C{=}O$ at the C-3′ position), 1.96–2.12 (br, 2.6H, $-CH_2CH{=}CH-$), 1.55–1.72 (br, 4H, $-CH_2CH_2C{=}O$), 1.20–1.40 (br, 20H, $-CH_2-$), 0.89 (m, 6H, $-CH_3$); ^{13}C NMR ($CDCl_3$/CD_3OD = 90/10, 100 MHz): δ 173.9 and 173.8 ($RC{=}O \times 2$), 131.4 and 128.3 (–CH=CH–), 98.9 (C-1′), 76.9 (C-5′), 73.6 (C-3′), 71.7 (C-4), 71.6 (C-2), 71.1 (C-3), 69.3 (C-2′), 65.2 (C-4′), 63.6 (C-1), 61.6 (C-6′), 34.0 ($-CH_2C{=}O$ at the C-2′ position), 33.9 ($-CH_2C{=}O$ at the C-3′ position), 31.9, 29.5–29.9, and 22.7 ($-CH_2-$), 27.5 and 26.6 ($-CH_2CH{=}CH-$), 24.8–25.2 ($-CH_2CH_2C{=}O$), 14.1 ($-CH_3 \times 2$).

2.4. Determination of surface tension of MEL-D

Surface tension of the obtained MEL-D was determined by a Wilhelmy-type automatic tensionmeter CBVP-A3 (Kyowa-kaimenkagaku, Tokyo, Japan) at 25 °C.

2.5. Investigation of aqueous phase behavior of MEL-D

We investigated the aqueous phase behavior of the obtained MEL-D according to the literature.[20]

2.5.1. Sample preparation

To prepare the samples for phase behavior study, MEL-D was dissolved in an appropriate amount of MeOH–chloroform mixed solution, and the stock solution was distributed into test tubes in different amounts (about 0.1–1.0 g MEL-D each). After the solvent was removed on a rotary evaporator, the sample tubes were kept in a vacuum desiccator at room temperature for over one week to completely remove the solvent. Next, distilled water (Milli-Q) was added to each MEL-D test tube to obtain the MEL-D–water mixtures. Different samples were prepared by varying the concentration of MEL-D. The MEL-D–water mixtures were then vigorously mixed by a vortex mixer and temperature-cycled between 25 and 70 °C several times in a water bath until the samples were mixed thoroughly. Each sample was equilibrated at room temperature for at least one week. At lower MEL-D concentrations ($\leqslant$10 wt %), turbid solutions were obtained. Vesicle formation was confirmed by confocal laser scanning microscopy of these solutions.

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