NOTE

Surface-assisted Laser Desorption/ionization Mass Spectrometry Analysis of the Glycolipid Biosurfactants, Mannosylerythritol Lipids, Using an Ionization-assisting Substrate

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Abstract: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a promising tool for the screening of glycolipid-type biosurfactants (BSs) from a crude extract of microbial products. However, it is unsuitable for the detection of lower molecular weight products because the observed ions are overlapped with matrix-derived ions at lower mass range. In this study, we applied a “matrix-free” surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) analysis using a through-hole alumina membrane as an ionization-assisting substrate. Using this method, we could detect a variety of lower molecular weight products in an extract of a glycolipid BS producer with good sensitivity. In addition, the culture solution could be analyzed directly by this method.

Key words: biosurfactant, ionization-assisting substrate, MALDI-TOF MS, SALDI-MS, screening

1 Introduction

Biosurfactants (BSs), which are amphiphilic compounds produced by a variety of microorganisms, are more multifunctional, more environmentally and biocompatible, than chemically synthesized surfactants. Among these compounds, glycolipid-type BSs are the most promising BSs for many industrial processes because of their availability from renewable resources, such as vegetable oils and sugars, and their versatile interfacial and biochemical properties^{1,2)}. Glycolipid-type BS molecules possess a complex bulky structure and several chiral carbons, leading to excellent surface-active properties, unique self-assembling abilities, and various biological activities. These unique chemical structures of glycolipid-type BSs are formed by biosynthesis and specific to each BS producer strains. The known structural variations of BS are limited, and the identification of novel BS producers has been greatly anticipated.

Screening for BS producers generally includes: (i) en-

richment of microbes from select microbial sources, (ii) evaluation of the products in microbial cultures based on their surface-active properties, and (iii) structural determination of the BSs. Structural determination requires a large amount of product purified through time-consuming methods, such as column chromatography. Recently, we applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) to screen for glycolipid-type BS producers^{3,4)}. MALDI-TOF MS is commonly used to identify the complex structures of organic molecules based on precise mass-to-charge ratio (m/z) measurements. This method is an excellent high-throughput screening tool that requires only a small amount of sample, and it has provided useful information on the structures of glycolipid BSs⁵⁻⁹⁾. In these studies, we subjected small amounts of crude extracts of BS producers to MALDI-TOF MS and verified this analytical tool for the rapid and reliable identification of microbial products.

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However, MALDI-TOF MS analysis is not appropriate for the detection of every BS. It is unsuitable for the detection of lower molecular weight products ($m/z < 500$) due to interference with matrix-derived ions. Additionally, tested BSs are prepared by extraction using an organic solvent, such as ethyl acetate or hexane, to remove impurities from the microbial cultures. Therefore, MALDI-TOF MS might miss lower molecular weight and more hydrophilic microbial products. Thus, in this study, we used an ionization-assisting substrate and performed surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) analysis^{10, 11)} of culture extracts of BS producers as a matrix-free method using a through-hole alumina membrane as an ionization-assisting substrate^{12, 13)}. Mannosylerythritol lipids (MELs; Fig. 1) were selected as a target of the investigation. These are glycolipid-type BSs produced by fungal strains of the genera *Pseudozyma* or *Ustilago*^{14, 15)}. We obtained not only diacylated MELs as a main component, but also monoacylated MELs when the MEL production was carried out using glucose as the sole carbon source¹⁶⁾. These monoacylated MELs are more hydrophilic and of lower molecular weight than diacylated MELs and are assumed to be unsuitable for extraction from microbial cultures with ethyl acetate followed by MALDI-TOF MS analysis. Therefore, if monoacylated MELs could be detected with good sensitivity in SALDI-MS analysis of *Pseudozyma* yeast cultures, this method would be demonstrated to be a powerful analytical tool for the screening of producers of glycolipid BSs with a variety of chemical structures.

2 Experimental Procedures

2.1 Materials and microorganisms

All reagents and solvents were commercially available and were used as received. *Pseudozyma antarctica* JCM10317 was used as a MEL producer. The ionization-assisting substrate (DIUTHAME® chips) is a commercial

product available from Hamamatsu Photonics K. K. (Hamamatsu, Japan). The production procedures, characteristics, and shape of the through-holes are described elsewhere¹⁷⁾.

2.2 Preparation of MELs

Mannosylerythritol lipids were prepared using glucose as the sole carbon source by the yeast strain *P. antarctica* JCM10317 as previously reported¹⁶⁾. Seed cultures were prepared by inoculating cells grown on the slants into growth medium (4% w/w glucose, 0.3% w/w NaNO₃, 0.03% w/w MgSO₄ 7H₂O, 0.03% w/w KH₂PO₄, and 0.1% w/w yeast extract (pH 6.0)) at 25°C on a reciprocal shaker (150 rpm) for 2 days. The seed cultures (1 mL) were transferred to 300-mL Erlenmeyer flasks containing 30 mL of basal medium containing 10% w/w glucose, and then incubated on a rotary shaker (250 rpm) at 25 °C for 7 days. After 7 days of cultivation, the culture broth was extracted with an equal volume of ethyl acetate, followed by mass spectrometry analysis.

2.3 SALDI-MS analysis of extracts from culture broth containing MELs using an ionization-assisting substrate

SALDI-MS analysis was performed according to previous reports^{12, 13)}. The preparation procedures were almost identical for crude ethyl acetate extracts containing MELs from culture broth and supernatant of the culture broth of *P. antarctica* JCM10317. Sample was applied to the back side of the DIUTHAME® chip and dried. For culture broth, ca. 3 µL of methanol was additionally applied onto the chip. Next, the chip was inverted and sodium trifluoroacetate (NaTFA) solution (ca. 1 µL) added onto the front side of the chip and allowed to dry (Fig. 2). Mass spectra were recorded using a JMS-S3000 SpiralTOF instrument (JEOL Ltd., Akishima, Japan).

3 Results and Discussion

3.1 SALDI-MS analysis of MELs using an ionization-assisting substrate

In the MALDI mass spectrum of MELs (Fig. S1 in the Supporting Information), matrix and plasticizer-derived ion peaks were mostly detected in the low mass range ($m/z < 500$). Therefore, MALDI-TOF MS is not usually applied to the mass analysis of low molecular weight samples to avoid the interference of such peaks. In this study, to observe low mass area clearly, we carried out SALDI-MS using an ionization-assisting substrate without matrix. Crude ethyl acetate extracts of culture broths containing MELs were directly applied to the ionization-assisting substrate, and the dried sample substrate was analyzed. The obtained mass spectrum is shown in Fig. 3a. We previously reported that *P. antarctica* produces monoacylated MELs

Conventional (diacylated) MEL: R¹, R² = fatty acyl chain
 monoacylated MEL: R¹, R² = either fatty acyl chain or H
 MEL-A: R³ = R⁴ = Ac
 MEL-B: R³ = H, R⁴ = Ac
 MEL-C: R³ = Ac, R⁴ = H
 MEL-D: R³ = R⁴ = H
 ME: R¹ ~ R⁴ = H

Fig. 1 Chemical structures of mannosylerythritol lipids.

Fig. 2 Workflow for surface-assisted laser desorption/ionization mass spectrometry analysis of culture broth using a through-hole alumina membrane as an ionization substrate.

Fig. 3 Mass spectra of (a) crude ethyl acetate extracts of culture broth and (b) culture broth supernatant from *Pseudozyma antarctica*. Unmarked: the sodium adduct, *: the potassium adduct.

as well as conventional diacylated MELs when using glucose as the sole carbon source¹⁶⁾. In the mass spectrum, peaks corresponding to monoacylated and diacylated MELs were clearly detected over the ranges of m/z 450–600 and m/z 650–750, respectively. In addition, some peaks in the lower mass range (m/z 100–500) were detected. The peak at m/z 203 corresponded to glucose, which was present in the culture broth. The peaks at m/z 307 and m/z 349 were assigned to mannosylerythritol (ME) without esterified acyl groups and acetylated ME, respectively, which were produced as intermediates in MEL biosynthesis. Peaks were observed for both the sodium and potassium adducts. These peaks have been difficult to detect by conventional MALDI-TOF MS analysis because of overlapping matrix cluster ion peaks. Because the present measurement was performed using a matrix-free method, these ion peaks could be detected without interference from matrix cluster ions.

3.2 Direct SALDI-MS analysis of microbial cultures containing MELs using an ionization-assisting substrate

DIUTHAME substrate is made of a through hole porous alumina membrane¹⁷⁾. When a sample solution is applied to the substrate, we expected that organic components in the solution are adsorbed and concentrated on the substrate and a solvent passes through the substrate. Therefore, we tried to perform direct mass analysis of the crude culture broth using the DIUTHAME chip without the extraction process. The culture broth supernatant was freed of yeast cells by gentle centrifugation and then applied to the ionization-assisting substrate and allowed to dry. The obtained mass spectrum is shown in Fig. 3b. The spectral pattern was almost identical to that of ethyl acetate extract of the culture broth, with differences only in the peak intensities. Thus, this method permits direct SALDI-MS analysis of the microbial culture broth without pretreatment. In addition, more hydrophilic products with lower molecular weights (e.g., ME and acetylated ME) were detected with good sensitivity from the culture broth. There was almost no differ-

ence in the peak intensities between monoacylated MELs and diacylated MELs. This method is particularly suitable for the detection of microbial products that are difficult to extract from culture broth with an organic solvent. Our findings indicate that SALDI-MS is a promising high-throughput screening tool for BS producers, with which microbial products in the culture broth can be directly analyzed without pretreatment.

4 Conclusion

In this study, SALDI-MS analysis of glycolipid BSs, i.e., MELs, was performed for the first time using an ionization-assisting substrate without matrix. More hydrophilic and lower molecular weight intermediates of the conventional diacylated MEL biosynthesis, such as monoacylated MELs and mannosylerythritol and its derivatives, were detected without interference by matrix cluster ions. In addition, the culture broth supernatant containing MELs could be directly analyzed without pretreatment, and all extracellular products were detected. The present matrix-free method of SALDI-MS is a significant advance for screening glycolipid BS producers because all microbial products in the culture broth can be rapidly and directly detected without pretreatment, and more hydrophilic microbial products with lower molecular weight can be detected with good sensitivity.

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Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21084

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