Evaluation of Yield and Surface Tension-lowering Activity of Iturin A Produced by *Bacillus subtilis* RB14

Hiroshi Habe1*, Toshiaki Taira2, Yuya Sato1, Tomohiro Imura2, and Takashi Ano3*

1 Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, JAPAN
2 Research Institute for Chemical Process Technology, AIST, Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN
3 Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, Kindai University, 930 Nishimitani, Kinokawa-city, Wakayama 649-6493, JAPAN

Abstract: *Bacillus subtilis* RB14 produces the lipopeptide antibiotic iturin A by submerged and biofilm fermentation. In this study, we optimized the conditions for iturin A production in a jar fermentor. The maximum yield of iturin A was 932 mg L⁻¹ after 120 h. The surface tension of water decreased from 72.0 to 39.0 mN m⁻¹ as the concentrations of C₁₄ iturin A increased, indicating that C₁₄ iturin A behaves as a surfactant in water. The critical micellar concentration obtained from the intersection of two fitted lines was 1.2 × 10⁻⁴ M. Moreover, the surface tension of water decreased as the length of the alkyl chain of iturin A increased.

Key words: cyclic lipopeptide, surfactin, iturin A, *Bacillus subtilis*, biosurfactant

1 Introduction

Cyclic lipopeptides (CLPs) are a major class of biosurfactant, with low critical micellar concentrations (CMCs), which gives them excellent interfacial properties. The surface activity of the CLP surfactin has been investigated, and it was found to act as an ionophore and to have antiviral activity. Other CLPs produced by *Bacillus subtilis* (e.g., iturin and fengycin) have considerable antifungal activity, indeed, an iturin-like compound produced by *B. subtilis* YM 10-20 permeabilizes fungal spores, inhibiting their germination.

Few studies have evaluated the surface-active properties of iturin, likely because iturin A is a weaker surfactant than surfactin. Razafindralambo et al. investigated the surface-active properties of surfactin-C₁₄/iturin A-C₁₅ mixtures in terms of their dynamic adsorption, monolayer stability, and micelle-forming ability, and found that a 2:3 mixture of surfactin-C₁₅ and iturin A-C₁₅ exerted the greatest synergistic effect. The dynamic surface tension of iturin A-C₁₅ was ~50 mN m⁻¹, whereas that of surfactin-C₁₅ was ~30 mN m⁻¹, at a total lipopeptide concentration of 6 × 10⁻⁴ mol cm⁻³. These values were obtained using a Lauda drop volume tensiometer, which differs from our method of using the CMC value of surfactins.

Iturin and surfactin differ in terms of the presence in iturin of a β-amino fatty acid in place of a β-hydroxy fatty acid in surfactin. This may affect the physicochemical properties of the compound, including the stability of the heptapeptide ring structure. In general, amides and cyclic amides (lactams) are exceptionally stable to hydrolysis. Straight et al. reported that iturin A is resistant to enzymatic hydrolysis while surfactin was not. These properties suggest iturin to be useful for some industrial applications.

*Bacillus subtilis* RB14 produces a large quantity of iturin A (yield 4,450 mg L⁻¹) by submerged fermentation and 5,050 mg L⁻¹ by biofilm fermentation using maltose and fish protein as sources of carbon and nitrogen, respectively. These data were obtained using 40 mL of medium in a 200 mL flask. However, mass production of iturin A, which is a prerequisite for evaluation of its surface active properties, requires the use of jar fermentor.

We investigated the surface tension-lowering activity of three iturin A homologues (C₁₄, C₁₅, and C₁₆ fatty acid chains) of *B. subtilis* RB14 using the pendant drop method. To this end, we produced iturin A in a 5 L jar fermentor.

*Correspondence to: Hiroshi Habe. Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, JAPAN: Takashi Ano. Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, Kindai University, 930 Nishimitani, Kinokawa-city, Wakayama 649-6493, JAPAN

E-mail: hiroshi.habe@aist.go.jp (HH), tano@waka.kindai.ac.jp (TA)

Accepted August 2, 2019 (received for review July 4, 2019)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
Three iturin A homologues with C_{14} to C_{16} fatty acid chains were separated by high-performance liquid chromatography (HPLC) and their surface tension-lowering activity was assayed.

2 Experimental Procedures

2.1 Materials

Authentic iturin A (from B. subtilis, >95% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were of the highest purity commercially available.

2.2 Apparatus

HPLC was carried out using a Shimadzu HPLC system equipped with an LC-20AD HPLC pump and an SPD-20AV detector (Shimadzu, Kyoto, Japan). The iturin A homologues were detected and fractionated, respectively, by a SOURCE™ 15RPC ST 4.6/100 and a SOURCE™ 15RPC HR 16/10 (GE Healthcare, Buckinghamshire, UK). The mobile phase, acetonitrile/0.1% (v/v) acetic acid (35:65), was used at a flow rate of 1.0 mL min^{-1} for the SOURCE™ 15RPC ST 4.6/100 and of 3.0 mL min^{-1} for the SOURCE™ 15RPC HR 16/10. The columns were maintained at 30°C. The absorbance at 210 nm was measured using an ultraviolet detector.

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using an Autoflex Speed TOF/TOF instrument (Bruker Corp., Billerica, MA, USA) with 2,5-dihydroxybenzoic acid as the matrix. The instrument was operated in reflector positive ion mode at a mass range of 400–3,000 m/z. One microliter of fractionated and concentrated iturin A solution was spotted on top of 1 µL of saturated matrix solution (1:1 acetonitrile:water) over the MALDI plate using the droplet spotting method to ensure that mixing occurred within the drop.

The surface tension of C_{14} iturin A at 25°C was determined by the pendant drop method using an apparatus consisting of an automatic interfacial tensiometer (DM500; Kyowa Interface Science Co., Ltd., Niiza, Japan) and Drop Shape Analysis software (FAMAS ver. 2.01; Kyowa Interface Science Co. Ltd.). We prepared the samples by diluting the stock solutions with ultrapure water and aging them over night. A setscrew was used to force the solution from a syringe to form a drop at the tip. Drop shape analysis was performed as follows: a drop profile was extracted from the image of a drop and curve fitting software was used to compare the experimental and theoretical drop profiles (Young-Laplace method), yielding the corresponding surface tension value. The changes in drop surface tension caused by C_{14} iturin A at a variety of concentrations were monitored for 10 min.

2.3 Iturin A production by B. subtilis RB14 in a jar fermentor

For large-scale iturin A production, B. subtilis RB14 was cultured in a 5 L jar fermentor (Model MDL; B.E. Marubishi, Tokyo, Japan). Strain RB14 was precultured for 24 h (30°C, 200 rpm) in test tubes containing 5 mL of L-medium comprised of 10 g L^{-1} polypeptone (Nihon Pharmaceutical Co., Tokyo, Japan), 5 g L^{-1} yeast extract (Difco Laboratories Inc., Franklin Lakes, NJ, USA), and 5 g L^{-1} NaCl, adjusted to pH 7.0. The cultures (six test tubes, total volume 30 mL) were transferred to a 5 L jar fermenter with 2 L of base medium containing 67 g L^{-1} maltose monohydrate, 80 g L^{-1} dipropylene glycol (Nihon Pharmaceutical Co.), 5 g L^{-1} K_{2}HPO_{4}, 0.5 g L^{-1} MgSO_{4}·7H_{2}O, 0.025 g L^{-1} FeSO_{4}·7H_{2}O, 0.022 g L^{-1} MnSO_{4}·7H_{2}O, and 0.184 g L^{-1} CaCl_{2}(pH 7.0), and cultured for 120 h. The base medium was prepared on the basis of the medium composition previously reported^{28} with a modification of nitrogen source. The aeration rate was set to 0, 0.5, or 1.0 volumes of air per volume of medium per minute (vvm), and the agitation speed was set to 100, 150, 300, or 500 rpm. The temperature was maintained at 30 ± 1°C, but pH was not controlled. Growth was evaluated by measuring the optical density at 600 nm.

Bacillus subtilis RB14 was sedimented by centrifugation and the supernatant was adjusted to pH 2.0 using concentrated HCl until a precipitate was produced. The supernatant was centrifuged at 8,000 rpm for 5 min, and the precipitate was collected and dissolved in a volume of methanol equal to that of the supernatant. After centrifugation to remove insoluble substances, 1 mL of the supernatant was subjected to HPLC to detect and quantify iturin A using authentic iturin A as a standard.

2.4 Separation and characterization of iturin A homologues

Methanol solutions containing iturin A were concentrated in vacuo and dried. The resultant material was dissolved in an appropriate volume of methanol, and three iturin A homologues were fractionated by HPLC. After acetonitrile and acetic acid had been allowed to evaporate, three iturin A homologues were recovered by lyophilization. The purities of three iturin A homologues in respective fractions were approximately 99.0% for C_{14}, 89.2% for C_{15}, and 83.0% for C_{16} as calculated by HPLC peak area. The mass spectra of the three homologues were analyzed by MALDI-TOF MS and their surface activities were investigated.

3 Results and Discussion

Prior studies of iturin A production by B. subtilis RB14 involved the use of Erlenmeyer flasks^{18, 29}; however, large-scale production using jar fermentor is needed for investi-
Production and Surface Activity of Three Iturin A Homologues

J. Oleo Sci.

Fish protein (Suzuhiro Co., Ltd., Odawara, Japan) reportedly increased the production of iturin A by *B. subtilis* RB14 (maximum yield 4,450 mg L$^{-1}$ by submerged fermentation and 5,050 mg L$^{-1}$ by biofilm fermentation)\(^{18}\). Indeed, fish protein contains a larger amount of nitrogen than commercially available peptones\(^{18}\); however, the quality of fish protein could differ among manufacturing lots, and it may not be available in some countries. Hence, to evaluate the production of iturin A by *B. subtilis* RB14 we used the widely available hipolypepton S peptones (Nihon Pharmaceutical Co.). The supernatants of 2 L cultures were acidified to form a precipitate, which was harvested by centrifugation. The precipitate was dissolved in methanol, and iturin A was quantified by HPLC.

The iturin A yield and the growth of *B. subtilis* RB14 in a 5 L jar fermentor are shown in Table 1. When the strain grows and degrades nitrogen source of protein hydrolysate (hipolypepton), ammonium ions are gradually released into the culture broth, resulting in an increase in pH value. First, we examined iturin A production with agitation at 100 and 300 rpm and an aeration rate of 0 vvm (without aeration) (runs 1 and 2), because the slower growth of *B. subtilis* RB14, compared to *B. subtilis*, would increase the iturin A yield.

Table 1 Production of iturin A in jar fermentor experiments.

<table>
<thead>
<tr>
<th>Run number</th>
<th>Aeration rate (vvm)</th>
<th>Agitation speed (rpm)</th>
<th>Final pH of culture</th>
<th>Optical density at 600 nm</th>
<th>Concentration of iturin A (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>100</td>
<td>6.36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>300</td>
<td>6.42</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>100</td>
<td>7.23</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>300</td>
<td>9.45</td>
<td>155</td>
<td>932</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>300</td>
<td>9.2</td>
<td>145</td>
<td>353</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>300</td>
<td>9.2</td>
<td>149</td>
<td>753</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>300</td>
<td>6.32</td>
<td>44</td>
<td>378</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>500</td>
<td>8.49</td>
<td>91</td>
<td>853</td>
</tr>
</tbody>
</table>

The surface activity of *C14* iturin A in water was evaluated by the pendant drop method (Fig. 2). The surface tension of water decreased from 72.0 to 39.0 mN m$^{-1}$ as the concentrations of the *C14* iturin A homologues increased. The CMC obtained from the intersection of the two fitted lines was 1.2×10$^{-4}$ M. To assess the effect of
alkyl chain length on surface activity, we assayed the surface tension of aqueous solutions of C_{14–C16} iturin A at 5.0 \times 10^{-4}, 3.0 \times 10^{-4}, and 1.5 \times 10^{-4} M (Fig. 3). At each concentration, the surface tension of water decreased as the length of the alkyl chain increased. Li et al.\textsuperscript{21} reported that surfactin possesses considerable surface tension-lowering activity; i.e., the surface tension of surfactin decreased as the length of the alkyl chain increased: \( \gamma_{\text{CMC}} = \begin{align*} 35.6 \text{ mN m}^{-1} (C_{12} \text{ surfactin}), & \quad 30.0 \text{ mN m}^{-1} (C_{13} \text{ surfactin}), \\ 28.8 \text{ mN m}^{-1} (C_{14} \text{ surfactin}), & \quad 28.1 \text{ mN m}^{-1} (C_{15} \text{ surfactin}), \\ \text{and } 27.3 \text{ mN m}^{-1} (C_{16} \text{ surfactin}). \end{align*} \) To our knowledge, this is the first study of the CMC and \( \gamma_{\text{CMC}} \) values of C_{14} iturin A and of the effect of alkyl chain length on its surface tension-lowering activity.

4 Conclusion

Aeration rate and agitation speed are important factors in the production of iturin A by \textit{B. subtilis} RB14 in a 5 L jar fermentor. After the extraction and purification of iturin A by HPLC, three iturin A homologues were identified as C_{14–C16} iturin A by MALDI-TOF MS. Purified C_{14} iturin A reduced the surface tension of water to 39.0 mN m^{-1}, for a CMC of 1.2 \times 10^{-4} M. Also, the surface tension of water decreased as the length of the alkyl chain of iturin A increased. Our data on the production and surface activity of three iturin A homologues will enable further characterization of the surface activity of iturin A.
Production and Surface Activity of Three Iturin A Homologues

Acknowledgments
This work was financially supported by Kaneka Corporation, Japan. The authors thank Mariko Kamata and Shohei Kawamoto for providing technical assistance.

References