Insight into the Selectivity and Mechanism of Surfactin Containing Multiple Dissociated Carboxylic Acids with 1-Bromoacetylpyrene in Fluorescent Derivatization

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Fluorescent derivatization of the carboxylic acids in surfactin peptide rings is an effective way to improve the sensitivity of trace detection of surfactin, but very little is known about the reaction selectivity of surfactin containing multiple carboxylic acids in derivatization. In this paper, the reaction selectivity in fluorescent derivatization of a surfactin containing two carboxylic acids in its peptide ring with 1-bromoacetylpyrene and the catalysis role in the reactions were investigated using electrospray ionization mass spectrometry and tandem mass spectrometry. It showed that only one carboxyl was labeled with 1-bromoacetylpyrene in derivatization reactions, and the connection of the Asp residue with 1-bromoacetylpyrene was confirmed. It also showed that triethylamine as a catalyst was connected with surfactin to liberate more nucleophilic groups beneficial to promote the derivatization rate. This would contribute to better understanding the mechanism of derivatization of surfactin and its analogues with 1-bromoacetylpyrene, and with other fluorescent labeling reagents.

Keywords Selectivity, carboxyl, surfactin, derivatization, catalysis

(Received October 29, 2017; Accepted December 7, 2017; Published May 10, 2018)

Introduction

Surfactin is one of the major lipopeptide families mainly produced by genus Bacillus and Pseudomonas.1–3 It has attracted much attention from both scientific and industrial communities due to their prominent interfacial and biological activities,2,4 and the great potential applications in industrial fields, such as agriculture,5,6 medicine,7,8 food,9 cosmetics,2 environmental protection10 and the petroleum industry to enhance oil recovery.11,12

Surfactin is a cyclic lipopeptide, and typically, it is involved in a peptide ring formed by seven amino acids (Glu-Leu-Leu-Val-Asp-Leu-Leu) bonded to a hydroxyl fatty acid chain by lactone bond, in which only Glu and Asp are the amino acids containing two carboxyl groups,13,14 and as a result, there existed two dissociated carboxyls in a surfactin molecule. In fact, most families of lipopeptides have dissociated carboxyls in their molecules, such as lichenysin (Glu-Leu-Leu-Val-Asp-Leu-Ile),15 iturin (Asp-Tyr-Asp-Glu-Pro-Asp-Ser),16 bacillomycin (Asp-Tyr-Asp-Glu-Pro-Asp-Thr, Asp-Tyr-Asp-Glu-Pro-Asp-Thr or Asp-Tyr-Asp-Glu-Pro-Asp-Glu-Thr),17–19 and fengycin (Glu-Orn-Tyr-Thr-Glu-Ala-Pro-Glu-Tyr-Ile or Glu-Orn-Tyr-Thr-Glu-Val-Pro-Glu-Tyr-Ile).20

For both quantitative and qualitative determination, especially for trace detection of lipopeptides in mixed aqueous solutions, research on lipopeptides is extremely important in view of the low concentration of lipopeptides in environmental samples. Fluorescent derivatization21,22 would be a feasible strategy for trace determination of lipopeptides because of the dissociated carboxyls in the molecule of lipopeptides, and a great effort has been made in both quantitative and qualitative determinations of lipopeptides via fluorescent derivatization. In our previous work, 1-bromoacetylpyrene (BAP) proved to be an effective fluorescent labeling reagent to overcome the limitations of trace detection of surfactin in aqueous solutions.23 However, a few shortcomings have been reported regarding the reaction selectivity of multiple carboxyls in a surfactin molecule in fluorescent derivatization, and knowledge about the active site of surfactin molecule in fluorescent derivatization is still limited.

In this study, the reaction selectivity of multiple carboxyls in a surfactin molecule in fluorescent derivatization and the role of catalyst in this derivatization were analyzed using electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS), and the mechanism of the reaction selectivity in derivatization reactions is discussed in this paper.

Experimental

Chemicals

BAP (97%, Sigma-Aldrich, USA), triethylamine (TEA, >99.0%, LingFeng Chemical Reagent Co., China), and acetonitrile (>99.9%, J&K, China) were used in the fluorescent derivatization reactions.
**Derivatization method**

The surfactin samples extracted from 10.0 mL cell-free broth, 2.00 mg BAP and 0.100 mL TEA were mixed and dissolved in acetonitrile, and the final volume of the reaction mixture was 1.00 mL. The solution was heated at 60°C for 20 min.\(^{23}\)

For the contrasting test, the surfactin samples extracted from 10 mL cell-free broth and 0.100 mL TEA were mixed and dissolved in acetonitrile, and the final volume of the reaction mixture was 1.00 mL. The solution was heated at 60°C for 20 min.

**Determination of molecular mass**

ESI-MS (Thermo Finnigan, USA) was used to determine the molecular weight of the derivative surfactin (D-surfactin). The instrument was operated in the negative mode and its capillary voltage, sample cone voltage and extraction cone voltages were

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![Tandem mass spectrogram](image)

**Fig. 1** Mass spectrogram of the surfactin after derivatization.

![Tandem mass spectrogram](image)

**Fig. 2** Tandem mass spectrogram: (a) D-C\(_{15}\) \((m/z = 1249.1)\); (b) D-C\(_{14}\) \((m/z = 1263.2)\); (c) D-C\(_{13}\) \((m/z = 1277.2)\).
3 kV, 100 V and 6 V, respectively. The scanned area was 200 – 2000 m/z.

Identification of connection of BAP and TEA

A tandem mass spectrometer (Q-TOF Micro YA019, Micromass, UK) was utilized to determine the connection of BAP residues and TEA in the D-surfactins. The electrospray mode was negative, the capillary voltage, sample cone voltage, ion energy and collision energy were 3.2 kV, 80 V, 1.6 V and 60 V, respectively.

Results and Discussion

Number of derivative carboxyl in D-surfactin

The molecular weights (MW) of surfactin samples after derivatization with BAP were determined using ESI-MS are shown in Fig. 1. The surfactins produced by Bacillus subtilis were mainly constituted from C12-surfactin to C17-surfactin. However, C13-surfactin, C14-surfactin and C15-surfactin were the principal components. The molecular weights of C13-surfactin, C14-surfactin and C15-surfactin were 1008, 1022 and 1036, respectively. While after derivatization, BAP (MW = 323) would take the place of carboxyls in the surfactin molecule with a residue of hydrogen bromide (HBr) (MW = 81), resulting in a net increase of m/z by 242. Consequently, the peaks of 1249, 1263 and 1277 corresponded to the relative molecular mass of the three D-surfactin homologs of derivative C13-surfactin (D-C13), derivative C14-surfactin (D-C14) and derivative C15-surfactin (D-C15), respectively, which suggested that only one carboxyl of surfactin was labeled with BAP in the reaction. If a surfactin molecule was connected with two BAP, the relative molecular mass of the three D-surfactin homologs would be 1491, 1505 and 1519, respectively. However, there was not any evidence related to such peaks in Fig. 1, which confirmed that only one of the two dissociated carboxyls of the surfactin molecule was finally reacted with one BAP in this derivatization.

Reaction site of the derivatization

For a further understanding of the reaction site of the derivatives, tandem mass spectrometry was applied to determine the reaction site of the two dissociated carboxyls at which the derivatization reaction was involved. [M–H]– of D-C13.
The amino acid sequence of the surfactin is N-Glu-Leu-Leu-Val-Asp-Leu-Leu-C.\textsuperscript{13,14} As shown in Fig. 2a, the 259 loss between m/z = 1249 and 990, and between m/z = 27 and 668 implied that the BAP residue lost a Br atom and obtained an O atom from the reacted carboxyl. The surfactin molecule has seven amino acids and only Glu and Asp have extra carboxyls for derivatization, the loss of O atom could be found from the Glu residue or the Asp residue. The 323 loss between m/z = 1249 and 926 implied the fraction of C\textsubscript{10}H\textsubscript{21}C=CHCO-Glu. It showed an intact Glu residue connected with the β-hydroxy fatty acid without any changes in the connection and structure. However, the 99 loss between m/z = 763 and 664, and between m/z = 342 and 243 implied the Asp residue (115) lost an O atom. The change of the molecular weight of BAP residue and Asp residue indicated the gain or loss of an O atom, which proved the reaction site of derivatization between surfactin and BAP was the carboxyl in Asp residue, as shown in Fig. 3. In the same way it could be found, in Figs. 2b and 2c, that D-C\textsubscript{14} and D-C\textsubscript{15} showed the same connection site of BAP residue. According to the dimensional structure of surfactin determined by \textsuperscript{1}H-NMR, distance geometry and molecular dynamics,\textsuperscript{24} surfactin exhibited a particular horse saddle folding mode and the side chain of Glu residue was wrapped in the inside of the particular mode. The steric hindrance may prevent the approach of BAP for derivatization. However, the side chain of Asp residue was exposed to the outside, and the carboxyl easily to reacted with BAP as the MS/MS shows.

Catalysis of TEA in the derivatization

TEA was a basic alkaline catalyst and was used in many chemical reactions.\textsuperscript{25–27} In principle, TEA combined with hydrobromic acid in derivatization would promote the reaction rate. However, compared with that in Fig. 1, peaks (m/z) of 1350.1, 1364.1, and 1378.1 showed a 101 increase of molecular weight than that of D-C\textsubscript{13}, D-C\textsubscript{14} and D-C\textsubscript{15}, respectively. Considering that the solution only contained surfactin sample, BAP, TEA and acetonitrile, it is reasonable to assume that TEA was connecting with the D-surfactin molecule. In order to confirm the connection of TEA and D-surfactin, [M–H]\textsuperscript{−} of m/z = 1364.1 and 1378.1 were chosen and subjected to MS/MS analysis, and the results are shown in Fig. 4.

It indicated that there was a 101 difference between m/z = 1364 and 1263, m/z = 1378 and 1277, respectively, which, unfortunately, implied the separation of TEA and the D-surfactin molecule. However, because TEA is known as an organic base and Glu residue has a dissociated carboxyl without derivatization, a connection based on the difference of acidic-basic properties is possible.

Consequently, a verification test was implemented by mixing the surfactin sample and TEA without BAP in acetonitrile solution using ESI-MS, as shown in Fig. 5. [M–H]\textsuperscript{−} of m/z 1006.5, 1020.5 and 1034.6 showed the relative molecular mass of C\textsubscript{13}, C\textsubscript{14} and C\textsubscript{15}, respectively, and compared with those corresponding peaks, m/z 1107.5, 1121.5 and 1135.6 showed a 101 increase versus the corresponding surfactin homologs. This implied that a surfactin molecule connected with a TEA molecule. Similarly, m/z = 1208.5, 1222.5 and 1236.5 implied a surfactin molecule connected with two TEA molecules, which indicated two carboxyls in a surfactin molecule were linked with TEA.

According to the reaction site of surfactin and the role of TEA in derivatization, the process of derivatization could be summarized as shown in Fig. 6 (it is a C\textsubscript{15}-surfactin molecule illustrated in the process). In this reaction, surfactin was activated by TEA and exposed more nucleophilic groups to attack the BAP, which has an α-C and is prone to come up with nucleophilic substitution.\textsuperscript{28–30} Because of the steric-hindrance effect, only the carboxyl in Asp residue was labeled with BAP. After derivatization, the D-surfactin was still connected with TEA in the carboxyl in Glu residue. However, D-surfactin

![Fig. 5 Mass spectrogram of surfactin and TEA mixture: a, C\textsubscript{13}; b, C\textsubscript{14}; c, C\textsubscript{15}; d, C\textsubscript{13} and TEA; e, C\textsubscript{14} and TEA; f, C\textsubscript{15} and 2TEA; g, C\textsubscript{13} and 2TEA; h, C\textsubscript{14} and 2TEA; i, C\textsubscript{15} and 2TEA.](image)

![Fig. 6 Reaction process of derivatization of surfactin with BAP.](image)
connected with TEA could dissociate to D-surfactin and TEA in solution, so the MS result in Fig. 1 shows MS information of D-surfactin and related D-surfactin with 101 m/z increase.

Conclusion

Fluorescent derivatization of surfactin molecules has been adopted for trace measurement of surfactin and its analogues in aqueous solution, but little is known of the mechanism of fluorescent derivatization of surfactin. In this work, the number of derivative carboxyls, the reaction site in surfactin, and the catalysis role of TEA in the derivatization reaction have been investigated. ESI-MS and MS/MS analyses show that, although there were two dissociated carboxyls in the peptide ring of a surfactin molecule, only one carboxyl in the peptide ring of a surfactin molecule was labeled with BAP in the reaction derivatization, and a connection of the Asp residue in the peptide ring with BAP was confirmed by MS/MS. Meanwhile, the results also showed that TEA as a catalyst was connected with surfactin and D-surfactin by acid-base interaction and caused a 101 increase of m/z in the MS after derivatization, and this acid-base interaction occurred before the surfactin reacted with BAP and consequently promoted the derivatization rate. This study contributed to a better understanding of the mechanism of fluorescent derivatization of surfactin and its analogues with BAP, and with other fluorescent labeling reagents.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 51574125, 21203063), 863 program (2013AA064403) and the Fundamental Research Funds for the Central Universities of China (WJ1514313).

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