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Determination of Siderophore from *Bacillus Mojavensis* Using Liquid Chromatography quadrupole Time-of-flight Tandem Mass Spectrometry

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Recently, it has been reported that *Bacillus mojavensis* possesses antifungal properties and plant growth-promoting activities, which are similar to the characteristics of siderophore. In this study, the siderophore produced by *B. mojavensis* was assessed using a solid phase extraction (SPE) cartridge and liquid chromatography quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS). After *B. mojavensis* was incubated in phenol medium for 16 hr and lyophilized, the sample was dissolved in water and loaded to an SPE cartridge to remove interferences. The cartridge was washed with 5% methanol in water and eluted with 2% formic acid in methanol sequentially. The eluted solution was evaporated under a stream of nitrogen gas and reconstituted in methanol. The reconstituted sample was filtered, and 1 μl of the sample was assessed using Q-TOF MS/MS. The mass spectrometer was operated using the positive electrospray ionization mode. Based on the mass spectrum and tandem mass spectrum, the siderophore produced by *B. mojavensis* was bacillibactin, one of the catechol types of siderophore with a molecular weight of 882.2556. This siderophore analysis could provide a justification for the study of *B. mojavensis* as a functional food and for pharmaceutical applications.

Key words : Bacillibactin, *Bacillus mojavensis*, liquid chromatography-quadrupole time-of-flight mass spectrometry, siderophore

Introduction

The genus *Bacillus* produces a wide range of biologically active molecules [9]. This genus has a variety of species, including *B. subtilis*, *B. thuringiensis, B. atrophaeus, B. velezensis, B. licheniformis, B. clausii, B. amyloliquefaciens*, and *B. mojavensis*. Among them, *B. mojavensis* has been recently discovered in the sand of Mojave Desert [13]. It is a non-pathogenic bacterium with endospore that can endure environmental stress for a long time. Besides of recent discovery, this strain has been researched in various fields. Its remarkable antibiosis activities including antibacterial and antifungal effects have been reported [1]. Furthermore, *B. mojavensis* has been utilized in plant cultivation as plant growth-promoting Rhizobacterium (PGPR) and plant growth promoting endophyte (PGPE) [8, 11]. These biological activities of *B. mojavensis* are similar to that of siderophore, which suggest that *B. mojavensis* produce specific siderophore [10, 15].

There are more than 500 kinds of siderophore. Sidrophore is a chelate compound which has a high affinity to iron oxide. Some bacteria produce siderophore when exposed to limited amount of iron to increase iron utilization [7]. The most common extraction method is by using highly polymerized solid phase extraction (SPE) cartridge such as C18 cartridge and Isolute ENV+ cartridge [2, 4]. In this study, hydrophilic-lipophilic balance (HLB) cartridge (water-wettable, reversed-phase sorbent) was applied to remove various kinds of interferences from culture medium. This extraction process removed medium interferences for efficient isolation of target siderophore because this HLB cartridge has high selectivity for catecholate compounds [12].

In previous studies, nuclear magnetic resonance (NMR), electrophoresis, thin layer chromatography (TLC), X-ray, and amino acid analysis have been employed for siderophore identification [5, 14]. These analytical techniques require siderophore standards with long preconditioning time. In addition, they are accompanied by low sensitivity and accuracy. However, Q-TOF-MS could analyze siderophore without using standards with femtogram level mass sensitivity

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and high accuracy. Therefore, recent siderophore analyses have used high sensitive liquid tandem mass chromatography (LC-MS/MS) or Q-TOF-MS/MS [2, 6, 16]. For these reasons, the siderophore produced by *B. mojavensis* was determined with Q-TOF mass spectrometry in this study.

Materials and Methods

To prepare siderophore, bacterial strain *B. mojavensis* was grown in 10 ml of tryptic soy broth (TSB, Bacto) on a rotating shaker (150 rpm/min) at 45℃ for 16 hr. Phenol medium (2.1 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 4.35 g K_2HPO_4, 1.7 g KH_2PO_4, and 1.0 g phenol in 1 l distilled water) was used as carbon source for siderophore manufacture. Cells cultured in TSB medium were incubated on a rotating shaker (150 rpm, 45℃). After 24 hr incubation, the phenol medium was centrifuged at 10,000 g for 10 min. The supernatant of medium was loaded to Centricon Plus-70 (Millipore, Billerica, MA, UFC700308) with 3,000 nominal molecular weight limit (NMWL) and centrifuged at 3,500 g for 10 min to remove high molecular interferences. The filtered sample was lyophilized for 24 hr by freeze-drying. The freeze-dried sample was diluted in 3 ml of distilled water and residue was removed after centrifugation at 10,000 g for 5 min. HLB cartridge (3 cc, Oasis®, Waters, USA) was preconditioned with 3 ml of methanol and distilled water sequentially. The sample (pH 2.5, 6 N HCl) was loaded to cartridge. The cartridge was then washed with 3 ml of 5% methanol and dried under vacuum. After 5 min, 3 ml of 2% formic acid in methanol was employed for target compound elution. The eluted solution was evaporated to dryness under nitrogen gas. It was reconstituted in 100 µl of methanol and filtered using 0.2 µm PVDF syringe filter. The filtered sample was directly injected to Q-TOF-MS/MS. Q-TOF analysis conditions are shown Table 1.

Results and Discussion

For sample ionization, ESI positive mode was employed. Extracted mass chromatograms of *B. mojavensis* are shown in Fig. 1A. The chromatogram of the extracted-ion chromatography (EIC) showed m/z of 883.2634 ion with retention time of siderophore at 5.112 min. The scan mass spectrum at 5.112 min is shown in Fig.1B with $[M+H]^+$ ion at m/z 883.2637. This mass ion was determined to be bacillibactin ($C_{39}H_{42}N_6O_{18}$, $[M+H]^+$ m/z 883.2634), one of siderophore in *Bacillus* species [3]. In addition, the error ppm (parts per million) value of the measured TOF was 0.23 ppm, assuring the accuracy of this result.

Tandem mass spectrum was performed to obtain fragment information and the pattern of product ion (Fig. 1C. Product ions indicated threonine group, glycine (Gly), and dihydroxybenzoic acid (DHB). The fragmentation pattern of the 883.2637 ion as mass spectrum was dominated by product ions of M-2(DHB+Gly)-NH, M-2(DHB)-Gly, M-(DHB-Gly), and M-(DHB). Synthetically, the pattern of ion product was confirmed to be secondary metabolite of bacillibactin. Therefore, the siderophore of *B. mojavensis* in medium was determined to bacillibactin of catecholate type.

In this study, SPE and centrifugal filter were employed for siderophore isolation and purification. The purified siderophore was analyzed by Q-TOF and identified as bacillibactin. The siderophore identified in this study could be

Table 1. LC-Q TOF-MS/MS condition

Apparatus	Agilent Technologies 6530 Accurate-Mass (Agilent Technologies, Santa Clara, CA, USA) with an Agilent Technologies 1,200 Series (Agilent, Waldbronn, Germany)
Column	XDB-C18 (4.6×50 mm, 1.8 µm)
Mobile phase A	0.1% formic acid in DW
Mobile phase B	0.1% formic acid in ACN
Gradient	B (15, 0.1 min) → B (100, 7.0 min) → B (100, 10.0 min) → B (15, 11.0 min) → B (15, 20 min)
Flow rate	0.5 ml/min
Injection volume	1 µl
Column temperature	30℃
Polarity	ESI positive mode
Fragment energy	150 V
Collision energy	20 V
Mass range	m/z 100 to 1,000
Data acquisition	Mass Hunter software Workstation data Acquisition Software (Agilent Technologies)

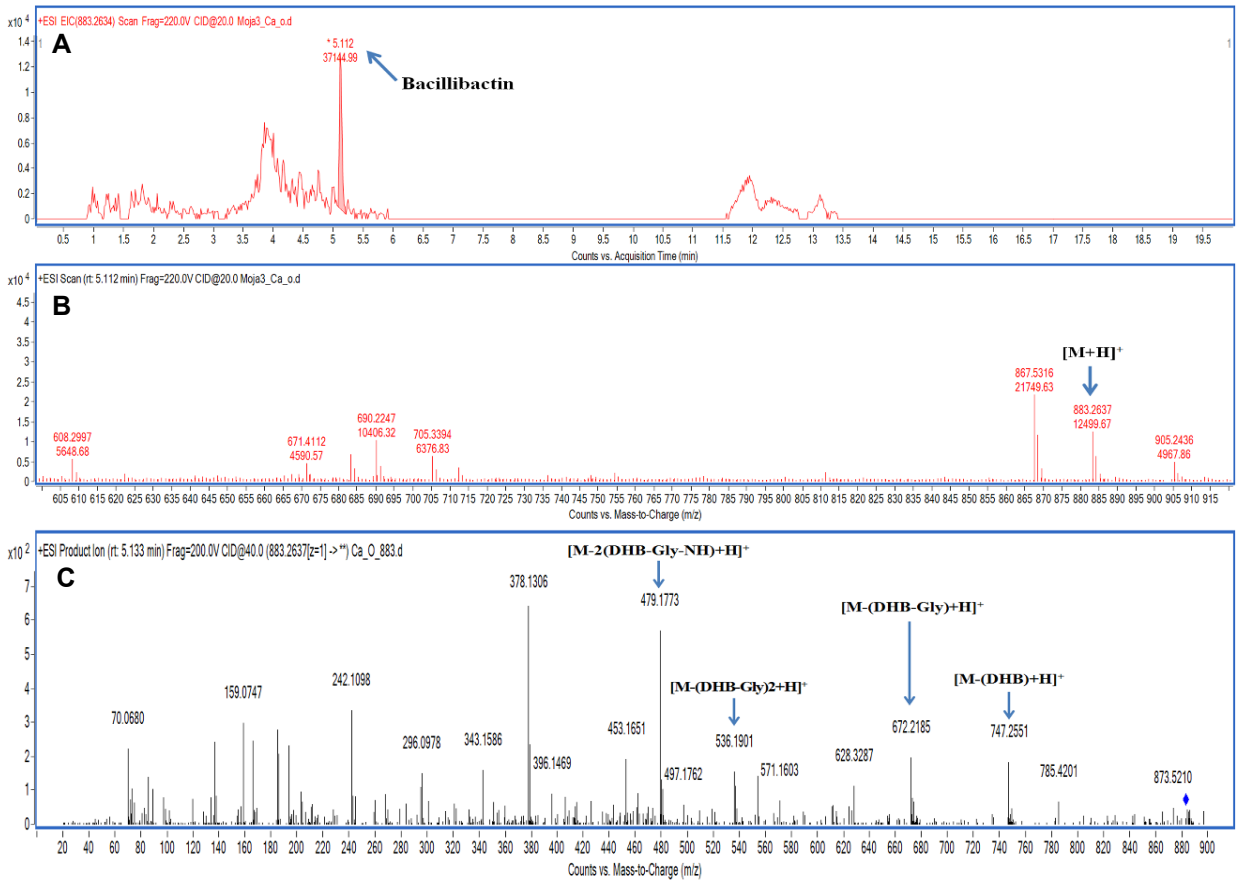

Fig. 1. (A) The extracted ion chromatogram (EIC) as m/z 883.27 ion in positive ESI mode. (B) The mass spectral peaks of detection and extraction in 5.112 min. (C) Product ion mass spectra of sample obtained by Q-TOF-MS/MS in m/z 20-900.

used to explain results of previous *B. mojavensis* research studies. Furthermore, bacillibactin produced by this strain suggest that *B. mojavensis* has potential in advanced biological applications.

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초록 : 액체크로마토그래피-사중극 비행시간형 탠덤질량분석기를 이용한 *Bacillus mojavensis* 균주 속 사이드로포어 규명

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*Bacillus mojavensis*를 이용한 항진균성 및 식물 생장 촉진 활성이 최근 보고되었다. 이런 활성은 사이드로포어의 일반적 특성과 일치하여, 본 연구에서는 *Bacillus mojavensis*가 생산하는 사이드로포어를 고체상추출 카트리지와 액체크로마토그래피-사중극 비행시간형 탠덤 질량분석기를 이용하여 규명하였다. *Bacillus mojavensis*를 페놀 배지에서 16시간 동안 배양하고 동결 건조 시킨 후, 물에 용해시켜 고상추출 카트리지에 로딩하였다. 카트리지는 5% 메탄올로 세척하고 2% 포름산을 이용해 용출 시켰다. 용출액은 메탄올에 재용해 후 분석을 하였다. *Bacillus mojavensis*의 사이드로포어는 질량 스펙트럼의 결과를 바탕으로 882.2556의 분자량을 갖는 카테콜타입의 사이드로포어 중 하나인 bacillibactin으로 확인되었다. 이 사이드로포어 분석은 *Bacillus* 연구 및 기능성 식품 그리고 *Bacillus mojavensis*의 약학 응용 분야에 큰 기여를 할 것으로 예상된다.