Fe(III)-based immobilized metal–affinity chromatography (IMAC) method for the separation of the catechol siderophore from *Bacillus tequilensis* CD36

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Abstract

Catechol siderophore plays an important role in microbial ecology, agriculture, and medicine, but its research is often limited by the difficulty in acquisition of it in large quantities. Based on evidence from the coordination chemistry and chemical biology, catechol siderophore could chelate Fe³⁺ with high affinity. Therefore, Fe(III)-based immobilized metal–affinity chromatography (IMAC) was applied to capture siderophore from the culture filtrate of *Bacillus tequilensis* CD36. The ethanol-precipitated sample and the separated sample from Fe(III)-based IMAC were analyzed by liquid chromatography–mass spectrometry. According to the result, the pure siderophore DHB-Gly-Thr could be extracted from the ethanol-precipitated sample. Compared with other purifications, Fe(III)-based IMAC was convenient and had fewer steps. In addition, it also reduced the use of toxic chemical solvents in some traditional extraction process, such as extraction and ion exchange chromatography. Fe(III)-based IMAC was successfully used in separation of the catechol siderophore from *B. tequilensis* CD36. The results revealed that Fe(III)-based IMAC was an efficient and environmentally friendly method for the separation and purification of catechol siderophore.

Keywords Fe(III)-based IMAC · Catechol siderophore · LC–MS · *Bacillus tequilensis*

Introduction

Siderophores (Greek for “iron carriers”) are small organic molecules with high Fe³⁺ affinities ($K_{\text{aff}} \sim 10^{23}–10^{52}/\text{M}$), produced by marine and terrestrial bacteria in response to the poor availability of soluble Fe³⁺ (Stintzi et al. 2000; Butler 2005). Based on their Fe³⁺-coordinating groups, siderophores have been divided into three classes: catecholate (e.g., bacillibactin from *Bacillus subtilis*), hydroxamate (e.g., desferrioxamine B from *Streptomyces pilosus*), and hydroxycarboxylate (e.g., achromobactin from *Erwinia chrysanthemi*) (Saha et al. 2016). It was reported that there are approximately 500 different types of siderophores, but many of them still need be structurally characterized. Catechol siderophore plays an important role in microbial

ecology, agriculture, and medicine (Saha et al. 2016). We developed a high-affinity purification technique, Fe(III)-based IMAC, for separation of catechol siderophore. The technique benefits from the high affinity between Fe³⁺ and catechol siderophore.

IMAC is a sample preparation or separation technique based on the affinities of some proteins to the immobilized metals (Gaberc-Porekar and Menart 2001). IMAC is mainly used to purify proteins, but recently, it has also been used for immobilized metal ion-affinity capillary electrophoresis and other applications (Gaberc-Porekar and Menart 2001; Haupt et al. 1996). After first introduced by Porath (Porath et al. 1975), IMAC has gained wide acceptance and further development. Briefly, a metal ion is immobilized to a support matrix. When sample was loaded, the incoming active substances were bound to the immobilized metal and separated from the rest of sample. Thus, Fe³⁺-immobilized IMAC columns may be successfully used for the extraction of siderophore from complex samples (Simionato et al. 2010). Najwa Braich and Rachel Codd had already successfully captured hydroxamate-containing siderophores and other Fe(III)-binding

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metabolites directly from bacterial culture supernatants with nickel(II)-based IMAC (Braich and Codd 2008), but there are no records of Fe(III)-based IMAC for the separation of catechol siderophore.

The previous studies have shown that *Bacillus tequilensis* CD36 produces catechol siderophore which matches the cyclic trimeric ester [DHB-Gly-Thr]₃. Based on evidence from the coordination chemistry and chemical biology, that Fe³⁺ and catechol siderophore could combined with high affinity. A successful application of Fe(III)-based IMAC was provided in the capture of the pure catechol siderophore. The principle and process are shown in Fig. 1 (bacillibactin as an example).

Materials and methods

Bacteria and culture conditions

Bacillus tequilensis CD36 was isolated from the rhizosphere of *Cycas debaoensis*. The previous studies have shown that *B. tequilensis* CD36 produced catechol siderophore that matches the cyclic trimeric ester [DHB-Gly-Thr]₃. Sugar-aspartic acid (MSA) medium was adjusted, as described by Liu et al. (2015). In brief, the following analytical grade chemicals were added to 1 L of deionized water: 12 g sucrose, 1.8 g glutamine, 1 g K₂HPO₄, and 0.5 g MgSO₄ at the natural pH. After culturing for 3 days at 30 °C and 200 rpm, culture was centrifuged at 7000×g for 2 min and the culture filtrate was harvested. The culture filtrate was then mixed with 4 volumes of ethanol, precipitated overnight, concentrated by a rotary vacuum evaporator, resuspended in deionized water, and subjected to further study.

Fig. 1 Fe(III)-based IMAC for the purification of catechol siderophores

Fe(III)–IMAC procedure

For each IMAC run, the column (HiTrap™ Chelating HP; 1 mL) was washed with 10 CV (column volume) of water and 10 CV of 0.1 M Fe₂(SO₄)₃, and then, 10 CV of water, to load Fe³⁺ to the resin; after loading the sample, the column was washed with 10 CV of binding buffer (20 mM Na₂HPO₄ and 500 mM NaCl, pH 5.3) and then with 15 CV of elution buffer (50 mM EDTA, 20 mM Na₂HPO₄, and 500 mM NaCl, pH 7.2); the Fe³⁺–siderophore complex was eluted by the elution buffer. Fractions containing siderophores were identified by chromogenic reaction, because the Fe³⁺–siderophore complex is yellow–green at pH 5.3–7.2. Fractions with a yellow–green color were collected after 10 runs. Fe³⁺ should be removed at a low pH after each IMAC procedure to avoid the precipitation of insoluble compounds. To prevent the Fe(III)–siderophore complex from precipitating and blocking the column in the next step, the collected fractions were deironed with 8-hydroxyquinoline solution, as described by Ding et al. (2010) and then desalted with a HiTrap™ desalting column according to the manufacturer's instructions. The collected sample was further analyzed by LC–MS.

LC–MS analysis

The samples were subjected to HPLC–MS analysis using a Waters Platform ZMD 4000 system consisting of a Micromass ZMD mass spectrometer (Micromass, Beverly, MA, USA) and a Waters 2690 HPLC (Waters, Milford, MA, USA) equipped with a photodiode detector. The samples were injected by an autosampler and subsequently separated by a C18 column (BEH C18, 2.1 × 150 mm 1.7 μm). The HPLC elution was linear from 5 to 100% mobile phase B in 15 min using mobile phase A (H₂O, 0.1% formic acid) and mobile phase B (acetonitrile). Flow rate 0.3 mL/min. Sample size 5 μL. The Waters Micromass ZMD mass spectrometer was operated using the electrospray ionization (ESI) source. All measurements were carried out using the positive ESI. A source temperature of 100 °C and a desolvation temperature of 250 °C were optimal. The capillary voltage was 3.88 kV, and the cone voltage was 60 V. Data were collected and processed with MassLynx software version 4.1 (Micromass, a division of Waters Corp., Beverly, MA, USA).

Results and discussion

LC–MS analysis of the ethanol-precipitated sample

Catechol siderophore with the dihydroxybenzene group has characteristic absorption peaks around the wavelengths of 270 nm, and MassLynx 4.1 (Waters) was used to find the substances with the characteristic absorption peaks. As shown in Fig. 2, there are four characteristic peaks in the ethanol-precipitated sample. Figure 3 shows the MS analytics of peak D, and the mass peak at m/z 881.7 ($[M-H]^-$) matches three DHB-Gly-Thr units minus $3H_2O$ ($[C_{39}H_{41}N_6O_{18}]^-$), which corresponds to the cyclic trimeric ester, (DHB-Gly-Thr)₃. The calculated exact mass of $[C_{39}H_{41}N_6O_{18}]^-$ is 881. In addition, it has the same fragment ion peak with bacillibactin ($[M-H]^- = 881$ Da) (Hertlein et al. 2014). Thus, peak D ($m/z = 882$) matches $[DHB-Gly-Thr]_3$, bacillibactin. Combined with the MS and

UV absorption spectrum data (Fig. 4), the four substances have almost the same chromophore and the color boosting group as these same characteristic absorption peaks at the wavelengths of 249 and 314 nm. For the other three peaks, according to the m/z ratio of the ion fragment in the MS spectrum and the UV absorption spectrum, and compared with the other reports (Li et al. 2017; Miethke et al. 2006; Yu et al. 2011), peak A ($m/z = 312$) matches DHB-Gly-Thr, peak B ($m/z = 606$) matches $[DHB-Gly-Thr]_2$, and peak C ($m/z = 900$) matches $[DHB-Gly-Thr]_3$ (linear).

Purification results from Fe(III)-based IMAC

The catechol siderophore of *B. tequilensis* CD36 was bound to the IMAC column and eluted by EDTA. The Fe(III)–siderophore complex (yellow–green) was stripped from the column by EDTA. As shown in Fig. 5, the purified catechol siderophore DHB-Gly-Thr ($M = 312$), which is the single unit of bacillibactin, could be obtained by

Fig. 2 LC elution curves of the ethanol-precipitated sample

Fig. 3 MS analytics of peak D

Fig. 4 MS and UV spectra of characteristic peaks. **a1, b1, c1, d1:** MS spectrum; **a2, b2, c2, d2:** UV spectrum

Fe(III)-based IMAC. The IMAC procedure can purify siderophores effectively due to the high affinity of siderophores to Fe^{3+} , which reduces the complexity of the purification procedure and amount of organic solvent needed. IMAC has been successfully developed and improved to separate many metal-affinity complexes, such as His-tagged proteins [Ni(II)-IMAC] (He et al. 2017), copper-complexing ligands [Cu(II)-IMAC] (Gordon et al. 2000; Nixon and Ross 2016), phosphopeptides [Ni(II)-IMAC, Fe(III)-IMAC] (Hart et al. 2002; Barnouin et al. 2005), and hydroxamate-containing siderophores [Ni(II)-IMAC, Fe(III)-IMAC] (Braich and Codd 2008; Simionato et al. 2010; Ejje et al. 2013). As bacillibactin is a secondary metabolite with low yield, and only the single unit of bacillibactin DHB-Gly-Thr was separated in this study. DHB-Gly-Thr, also known as Bengtin, can be used as a siderophore by certain *Bacillus* (Li et al. 2017; Ahire et al. 2011).

Conclusion

In the present study, we aimed to investigate the utility of Fe(III)-based IMAC for separating catechol siderophore.

We developed a new method Fe(III)-based IMAC for the purification of catechol siderophore. This method is based on the high affinity of siderophores to Fe^{3+} . Pure siderophore was obtained after IMAC followed by deironing and desalting (Fig. 5). This Fe(III)-based IMAC method can be used to separate catechol siderophore DHB-Gly-Thr from the broth of *B. tequilensis* CD36 in an efficient and environmentally friendly manner. To the best of our knowledge, it was the first time that Fe(III)-based IMAC method was proposed for the separation of catechol siderophore.

Fig. 5 LC–MS analysis of the sample prepared by Fe(III)-based IMAC. **a** LC elution curves; **b** MS spectrum

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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