A Two-Column Flash Chromatography Approach to Pyoverdinin Production from *Pseudomonas putida* GB1

by

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Graphical Abstract:

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Abstract. Our knowledge of the biological and environmental reactivity of siderophores is limited by the difficulty and cost of obtaining reasonable quantities by purification or synthesis. In this note, we describe a modified procedure for the low-cost, mg-scale purification of pyoverdinsideophores using a dual-flash chromatography (reverse-phase absorption and size exclusion) approach.

Text. Siderophores are a diverse class of chelating agents produced by bacteria, fungi, and grasses to promote uptake of iron and other bioinaccessible nutrient metals (Kraemer et al., 2014; Kraepiel et al., 2009; Neilands, 1995). The pyoverdins (PVDs), a group of fluorescent molecules that are produced by certain bacteria in the *Pseudomonas* genus and characterized by a fluorescent quinoline chromophore (Budzikiewicz, 2010), have been associated with biological and environmental phenomena beyond metal uptake, including pathogenesis, plant disease suppression, contaminant transport, and biomineral formation (Edberg et al., 2010; Lemanceau et al., 2009; Meyer, 2000; Parker et al., 2007).

Currently, PVDs are only available in small quantities at high costs, and thus larger quantities are required for many biological and environmental studies must be produced by purification, e.g., via size exclusion chromatography (Meyer and Abdallah, 1978). In this communication, we describe the application of flash chromatography (compressed gas driven) to allow for PVD production with low-cost chromatographic separation (Still et al., 1978). In addition, we improve upon this methodology by incorporating a desalting/concentrating reverse phase column (Parker et al., 2004).

Pseudomonas putida GB1 cultures (Parker et al., 2004) were grown from frozen stock on half-strength Luria broth (LB) agar plates. A 50 ml iron-free succinate defined medium (SDM) (Meyer et al., 1997) “starter” was inoculated with colonies from the plate and allowed to grow for 24 hr at 28°C. A 10 ml starter aliquot was then used to inoculate 1 L of SDM in sterile Erlenmeyer flasks. The flasks were shaken for 96 hrs at 120 rpm (in stationary phase), and then visually assessed (both by a yellow-green color in the visible and by fluorescence when exposed to a UV-A light) to qualitatively verify PVD production. The culture was distributed into HDPE bottles and centrifuged at 3825 g RCF

for 20 min. The supernatant was decanted and vacuum filtered (Whatman 42) to remove solids to prevent column clogging.

The filtered medium was passed through a flash solid phase extraction (SPE) column (Amberlite XAD-4) to desalt and concentrate the sample. To facilitate sorption to the stationary phase, resin and the filtrate were evenly distributed into foil-coated HDPE bottles, which were shaken at 220 rpm for 24 hr. The solids and media were transferred to a 5×30 cm column, drained by gravity, washed by flash chromatography with 1.5 L of deionized water under 3.4 atm N₂, and then the eluent was changed to a 50/50% vol mixture of methanol and water. In all cases where glassware is pressurized, additional shielding (a hood or column guard) was employed. The first 150 ml of eluent was discarded, and the following 600-750 ml of eluent was collected (until eluent ran clear). The resin was regenerated by rinsing with 1.5 L methanol. The collected eluent was concentrated by rotary evaporation at 60° C (IKA V R10 basic Rotovap), resulting in a final extract volume of 10–15 ml.

The concentrate was further purified by size exclusion flash chromatography. A 2.5×40 cm column (CM-sephadex G-25, General Electric) was preconditioned by flushing with 400 ml of 50 mM pyridine acetate buffer (PyAc) at pH = 5.0. A 5 ml aliquot of PyAc was added to the concentrate and the mixture was applied as a layer to the top of the column. PyAc (50 mM) was then eluted through the column (3.4 atm N₂), splitting the colored PVD extract into three discrete bands. When the first band reached the outlet, the eluent was collected in 10 ml fractions, and the inlet solution was switched to a 750 mM PyAC buffer (pH = 5.0). After the second band had eluted, the inlet solution

was changed to a 2 mM PyAC buffer (pH = 5.0). After elution of the final band, the solution was flushed with 50 mM PyAC to regenerate the column.

The product purity was evaluated by mapping individual fractions (after appropriate dilution) with fluorescence spectroscopy (excitation 200–450 nm, emission 250–600 nm; Perkin Elmer LS 55 fluorescence photometer). The desired chromophore fluoresces at an emission:excitation peak at 460:405 nm (Parker et al., 2014). Only fractions containing this peak at >10:1 intensity over the background were selected for inclusion in the final product. This fraction proved to be 50±10 ml, collected after discarding an initial 100±10 ml of eluent. All of the fractions were eluted with 750 mM PyAc buffer, and amounts to collection of the 50 ml surrounding the middle band, consistent with a previous observations that the front running band contains degradation productions (Meyer and Abdallah, 1978). The selected fractions were pooled and rotary evaporated as described above, resulting in an 80% volume reduction. The product was then frozen and lyophilized until dry. Based on four replicate trials, solid yield averaged $50 \pm 10 \text{ mg l}^{-1}$ growth medium.

Freeze-dried samples from four replicate trials were dissolved in deionized water to an approximate concentration of 0.25 mg ml^{-1} and analyzed using Thermo Scientific Dionex UltiMate 3000 LC system equipped with a thermo C18 column (50 mm × 2.1 mm, 1.9 µm particle size) coupled via heated electrospray ionization (HESI) to a mass spectrometer (Thermo Fisher Scientific Exactive Plus MS) operated in positive ion mode at a mass range of 150-2000 amu. Elution of analytes was performed with deionized water (0.1% FAO) and acetonitrile (0.1% FAO) at a flow rate of 0.5 mL/min. Chromatograms produced a dominant peak with a ~24 s retention time.

Tentative assignments of PVD structure, based on matching masses (Figure 1) to known structures and common motif modifications, are shown in Table 1. Major products included PVD-GB1, an PVD-GB1 -OH β -elimination product, and sulfonated PVD-GB1, as previously shown for this strain (Parker et al., 2014). In trial A, we also note the possible presence of PVD-D47, which has a different protein backbone. However, masses consistent with an added methyl group and sulfonated versions of both the methyl PVD-GB1 (the predominant form in B–D) and the PVD-GB1 -OH β -elimination product are also present. It should also be noted that each run yielded a different combination of products, and that definite identification of these products requires additional analysis, such as by siderotyping (Fuchs et al., 2001) or tandem mass spectrometry (Budzikiewicz et al., 2007). Although their presence has been recognized in many ringed siderophores (Fuchs et al., 2001; Hickford et al., 2004), the causes for formation and biological significance of sulfonated siderophores are unknown.

The procedure described herein provides a method to produce ~100 mg of purified PVD. It should be noted that the purified PVD obtained here is a complicated mixture of closely related structures. This observation suggests that although the approach was optimized for *P. putida* GB1, it may be adapted to other Pseudomonads to produce specific PVDs of interest. It may also provide a generalized method for scaling up the procedure for industrial and technological applications (Ahmed and Holmstrom, 2014; Rajkumar et al., 2010).

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Major Peak (amu)	Relative abundance (%)	Tentative Assignment
1218	A = 100; B = 0; C = 0; D = 16	PVD-D47 ^a
1234	A = 53; B = 18; C = 35; D = 45	OH β -elimination of 1251 ^b
1251	A = 15; B = 59; C = 40; D = 30	PVD-GB1 ^b
1265	A = 32; B = 13; C = 9; D = 24	methylation of 1251
1316	A = 59; B = 72; C = 32; D = 79	sulfonated 1234
1332	A = 22; B = 100; C = 100; D = 100	sulfonated 1251 ^b
1346	A = 26; B = 22; C = 8; D = 16	sulfonated 1265

Table 1. Tentative assignment of major peak masses of purified siderophores from Figure 1. Relative abundance refers to height of major in Figure 1A-D. Masses with abundance <20% in all mass spectra (A-D) are not assigned. ^aBudzikiewicz 2010; ^bParker et al. 2014.

Figures

Figure 1. Mass spectra of samples from four replicate trials of PVD purification (A-D). Peaks highlighted in gray are tentatively assigned in Table 1.

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Figure 1.