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Centrifugal partition chromatography: A preparative tool for isolation and purification of xylindein from *Chlorociboria aeruginosa*

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ABSTRACT

A centrifugal partition chromatography (CPC) method was developed for the preparative-scale isolation and purification of xylindein from the wood-staining fungi, *Chlorociboria aeruginosa*. Xylindein, a blue–green pigment naturally secreted from the hyphae and fruiting bodies of the fungus, has great value in the decorative wood industry and textile coloration. Xylindein has great potential for use as a fluorescent labeling agent as well as in organic semiconductor applications. However, a primary limitation of xylindein is its poor solubility in most common HPLC solvents. Consequently, it is arduous to purify using preparative liquid chromatography or solid-phase extraction (SPE). Support-free, liquid–liquid chromatographic methods, including CPC, where solutes are separated based on their different distribution coefficients (K_D) between two immiscible solvent systems, are promising alternatives for the purification of the compound on a preparative scale. In this work, a new biphasic solvent system suitable for CPC separation of xylindein was developed. Various groups of solvents were assessed for their suitability as xylindein extractants. A new solvent system suitable for CPC separation of xylindein, composed of heptane/THF/MEK/acetonitrile/acetic acid/water, was developed. This solvent system yielded a K_D value for xylindein of 1.54 ± 0.04, as determined by HPLC ($n = 3$). The compositions of the upper phase and lower phase of the solvent system were determined by Heteronuclear Single Quantum Correlation (HSQC) NMR and proton NMR. A CPC system, equipped with a fraction collector, was used for the isolation of xylindein from crude extracts. The xylindein fractions isolated by the CPC were then analyzed using HPLC and presented as a fractogram. Based on the CPC fractogram, the purified xylindein fractions were achieved after 30 min CPC separation time, yielding 71% extraction efficiency. The developed CPC method allowed for isolation of this naturally sourced xylindein in amounts suitable for further study.

1. Introduction

Xylindein [1] (8,16-Dihydroxy-3,11-dipropyl-3,4,11,12-tetrahydro-pyrano[4,3-*h*]pyrano[4′,3′;5,6] xantheno[2,1,9,8-klmna]xanthene-1,7,9,15-tetraone) (Fig. 1), a planar, heavily conjugated blue-green pigment with an extended π-system, shows significant promise for use as a fluorescent labeling agent as well as in organic semiconductor (OSCs) applications. The chemical structure of xylindein was elucidated in the mid-1960s [2,3] and more recently, the (3S, 3′S) absolute configuration of xylindein was established [4]. The core structure of the xylindein molecule is *peri*-xanthenoxanthene (PXX), a derivative of which was incorporated into a highly flexible thin film organic light emitting diode (OLED) device by Sony in 2010 [5–7]. Based on its known structure, it is likely that xylindein would be amenable to derivatization for further optimization and applications.

Blue-green wood stained with xylindein has been utilized commercially for decorative wood products, such as intarsia inlays, since the 1600s [8]. Several applications using xylindein as coloration dye in spalting and textile have been studied [9,10]. In natural environments and in culture, xylindein is produced and secreted by the non-pathogenic wood-staining fungi *Chlorociboria aeruginosa* and *C. aeruginascens* as a secondary metabolite [11–13]. Attempts at chemical synthesis of xylindein have not yet advanced to completion [14–16]. Isolation from an abundant natural source may therefore be the most cost-effective, efficient, and nearest-term alternative. An effective separation technique is necessary to optimize and isolate naturally sourced xylindein in amounts suitable for further study.

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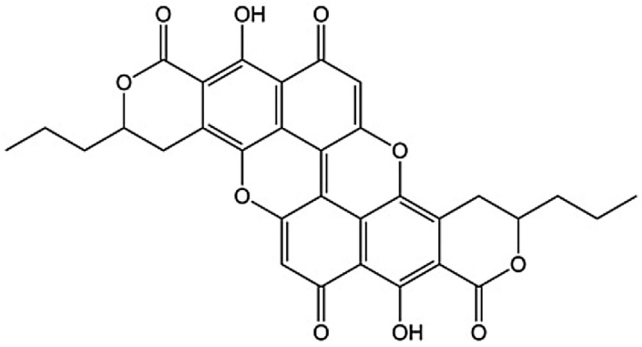

Fig. 1. Structure of xylindein.

Isolation of xylindein from fungal cultures can be done by liquid–liquid extraction using halogenated solvents such as chloroform [4] or dichloromethane [17]. Solid phase extraction (SPE) with a phenyl-bonded column also serves as a useful approach to extract the pigment. However, extraction time, high cost, and low sample loading volume are drawbacks of this technique, suggesting that alternative means of preparative isolation of the pigment for further applications merit consideration. Preparative HPLC could be one of these alternatives for isolation of xylindein from culture, as it has proven to be useful for the isolation of other compounds from natural products [18–20]; however, the low solubility of xylindein in aqueous solutions and most polar organic HPLC solvents and the cost of preparative scale columns makes this approach less attractive.

Centrifugal partition chromatography (CPC) is one of the embodiments of countercurrent chromatography (CCC) [21–23]. CPC is a preparative, support-free, liquid–liquid chromatographic technique. The separation mechanism in CPC is based on differences in distribution coefficients (K_D) of analytes between two immiscible solvent systems. While one liquid remains in the column as the stationary phase by conforming to the application of a centrifugal field, the other is pumped through as the mobile phase. CPC possesses many advantages such as high selectivity attained by skilled design of a solvent system, high loading capacity, low solvent consumption, high sample recovery, no adsorption onto a solid support, and relatively easy scale-up [24,25]. Therefore, CPC serves as an efficient alternative liquid chromatographic technique for preparative separations and purifications at a laboratory scale.

In addition to the advantages of the CPC technique, with the wide variety of solvent combinations available to form a biphasic solvent system and skilled design of such systems, high selectivity for the targeted compounds can be obtained. Once the solvent combination for a freely soluble analyte has been determined in both organic and aqueous phases, it is important to adjust the solvent ratio to obtain a K_D of the target analyte between 0.5 and 2 [26]. The solvent system plays the critical role in selectivity of compounds separated in CPC.

In this work, a CPC method for preparative isolation and purification of xylindein was developed. Due to the low solubility of xylindein in common HPLC solvents, development of a biphasic solvent system as is commonly used in CPC can be a major challenge. Solubility information for xylindein in various solvents would therefore provide valuable insight for the solvent system study; thus the solubility of xylindein in various pure solvents was determined. Solvent system compositions for xylindein isolation using CPC were then explored and optimized, resulting in a new solvent system appropriate for CPC purification of xylindein. The xylindein-containing fractions isolated from the fraction-collector equipped CPC were analyzed using HPLC and presented as a fractogram. The CPC separation technique was optimized and used to isolate naturally sourced xylindein in amounts suitable for further study.

2. Experimental

2.1. Chemicals

All organic solvents used for CPC separation were chromatography grade except for acetic acid, which was analytical grade. Acetonitrile, tetrahydrofuran (THF), and dichloromethane (DCM) were purchased from EMD Millipore (Billerica, MA). Methyl ethyl ketone (MEK) was obtained from Sigma-Aldrich (St. Louis, MO). Heptane and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Chloroform used for HPLC characterization was obtained from Avantor (Center Valley, PA). Phosphoric acid (H_3PO_4) and monosodium phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) were purchased from Mallinckrodt (St. Louis, MO). Water was ultrapure grade (Milli-Q EMD, Billerica, MA). NMR experiments were performed in chloroform-d, acetone-d6, and DMSO-*d*6 (Cambridge Isotope Laboratories Inc., Andover, MA).

2.2. Fungal culture

The fungi *Chlorociboria aeruginosa* (Oeder) Seaver strain UAMH 11657 (isolated from a hardwood log from Ontario, Canada) were kindly provided by Dr. Sara C. Robinson, Department of Wood Science and Engineering, Oregon State University [9–13,17,27]. The fungi were initially cultured on a 10 cm plate of active-growth fungal media (2% malt, 1.5% in deionized water) with 5-point inoculation. After a 24-week incubation at room temperature ($23 \pm 1\,^{\circ}C$), the pre-cultured fungal materials were chopped into approximately 5 mm cubes and transferred into a 500 mL flask containing 250 mL liquid culture media (2% malt in deionized water). The liquid culture flask was allowed to incubate at room temperature on a shaker (MaxQ model 2000, Thermo Scientific, Waltham, MA) at 110 rpm for a period of 10 weeks.

2.3. CPC apparatus

CPC separations were performed on an FCPC® 50 Kromaton Technologies apparatus (Robatel Inc., Pittsfield, MA) with a 50 mL rotor. The FCPC rotational speed was variable between 200 and 2000 rpm and the system supported pressures up to 900 psi (62 bar). Solvents were pumped using a gradient HPLC pump (Model 500 G, Analytical Scientific Instruments Inc., Richmond, CA), which can deliver programmable flow rates from 0.1 mL/min to 80 mL/min with a maximum pressure of 1000 psi (69 bar). Samples were introduced manually into the FCPC column via a 6-port high-pressure injection valve (Rheodyne 7725i, IDEX Health & Science, Oak Harbor, WA) equipped with a 5 mL sample loop. CPC fractions were collected using a Bio-Rad model 2110 fraction collector (Hercules, CA). The CPC experiments were conducted at room temperature ($23 \pm 1\,^{\circ}C$).

2.4. Preparation of the crude xylindein extract

The crude xylindein was prepared from the 10-week incubation liquid fungal culture by liquid–liquid extraction using DCM at room temperature ($23 \pm 1\,^{\circ}C$), an adaptation of a method previously developed in our group [27]. In brief form: The liquid culture (250 mL) was first ground using a house blender (Oster Precise Blend model 200, Boca Raton, FL) for approximately 30 s or until the agar cultural media was transferred into a fine colloid to increase surface area and homogeneity to promote extraction. The ground liquid culture was transferred back into the culture flask and DCM (100 mL) was then directly added. The flask was stirred (300 rpm)