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Preparative separation of echinocandin B from *Aspergillus nidulans* broth using macroporous resin adsorption chromatography

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ABSTRACT

Echinocandin B (ECB), an echinocandin type of lipopeptide antibiotic produced by *Aspergillus nidulans*, is a precursor for the synthesis of novel anti-fungal drug – anidulafungin. In this work, a separation strategy involving one-step macroporous resin adsorption chromatography was established for ECB purification from *Aspergillus nidulans* CCTCC M 2010275 fermentation broth. Among nine macroporous resin adsorbents tested, the non-polar resin HP-20 had the best adsorption and desorption performance. The static equilibrium adsorption data fitted well with the Langmuir equation, and the adsorption kinetic followed the pseudo-second order model. The separation parameters of ECB from broth were optimised by dynamic adsorption/desorption experiments with the column packed with HP-20 resin. Under optimal conditions, the purity increased by 3.8-fold from 23.2% in broth to 88.5% in eluent with 87.1% recovery yield by a one-step treatment. Our study provided a one-step and effective method for large-scale production of ECB, and offered references for separating other echinocandins from broth.

1. Introduction

Echinocandins, the newest class of drugs approved for the treatment of invasive fungal infections, are inhibitors of beta-(1,3)-glucan synthesis, an action that damages fungal cell walls [1]. Currently there are three echinocandins available for clinical use. Caspofungin (Cancidas™) was approved by the FDA in 2001, followed by micafungin (Mycamine™) in 2005 and anidulafungin 39 (Eraxis™) in 2006 [2]. Echinocandin B (ECB), a fermentation product of *Aspergillus nidulans* and the starting point for the development of novel anti-fungal drug anidulafungin [3]. ECB was discovered in 1974 as the first representative of the echinocandins [4]. It is known as one of the natural cyclic hexapeptides that have a linoleoyl side chain. ECB showed potent activity against *Candida tropicalis* and *Candida albicans*, and in vivo activity was demonstrated in an animal model of candidiasis [2].

In the past several decades, both the incidence and the types of fungus infection severely harmful to human health were continuously increased, especially for the immunosuppressed patients [5]. At the same time, the clinical application of certain commonly used clinical antifungal agents, such as imidazoles, triazoles and amphotericin, was restricted because of significant neurotoxicity, drug resistance [2,5]. Due to the low toxicity, strong fungicidal activity, and as well as excellent pharmacokinetic property of the echinocandin type anti-fungal drug, its production via microbial fermentation has been paid great attentions [6]. A great number of reports are available on the echinocandins fermentation [7–12]. However, there are a lack of information on the separation and purification of echinocandins from fermentation broth. Therefore, developing an efficient strategy for echinocandins separation from broth is of significant importance.

The purification of microbiological metabolites from broth by using traditional chromatographic media like silica gel and polyamide gel is not a recommended approach due to low content of the constituents of interest, large volume of broth and metabolite diversities [13]. Therefore, using macroporous resin to process fermentation broth could be a good choice in this field. The primary advantages of these low cost resins are high loading capacities and easy to operation and regeneration make them ideal matrices for separating low concentrations of target compounds from complex solutions [14]. Their capability of removing impurities is also excellent due to their high selectivity resulting from by the suitable structures and polarities of resins, and the multiple chemical interactions involving the resin and

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Nomenclature

q_e	amount of ECB adsorbed at equilibrium (mg/g dry resin)
q_d	desorption capacity (mg/g dry resin)
V_1	volume of ECB solution (ml)
V_d	volume of the desorption solution (ml)
m	dry weight of resin (g)
c_0	initial concentration of ECB (mg/ml)
c_e	concentration of ECB in solution at equilibrium (mg/ml)
c_d	concentration of ECB in the desorption solution (mg/ml)
D	desorption ratio (%)
q_m	theoretical maximum ECB adsorption capacity under experimental condition (mg/g dry resin)
K_L	Langmuir isotherm constant *KF*;
n	Freundlich isotherm constants
t	temperature (K)
q_t	amount of ECB adsorbed at time t
K_1	constant for the pseudo-first order rate equation
K_2	constant for the pseudo-second order rate equation
K_3	constant for the liquid film diffusion model K_4;
C	constants for the intra-particle diffusion model

adsorbents [15,16]. In fact, macroporous resin has been used to separate metabolites from fermentation broth [15,16,14,17–21]. For example, macroporous resins have been successfully used in the separation of α-arbutin from spiropreussione A from endophytic fungal *Preussia* sp. broth [14], α-arbutin from *Xanthomonas* CGMCC 1243 broth [17], brefeldin A from *Eupenicillium brefeldianum* broth [18], fengycin from *Bacillus amyloliquefaciens* ES-2 broth [19], gamma-aminobutyric acid from *Enterococcus raffinosus* TCCC11660 broth [20], surfactin from *Bacillus subtilis* ATCC 21332 broth [15], erythromycin (EM) from fermentation broth [21], etc. However, no document on the ECB separation using macroporous resin is available.

The purpose of this research is to investigate the feasibility of purifying ECB from the fermentation broth of *A. nidulans* CCTCC M 2010275 using one-step macroporous resin adsorption chromatography. The adsorption and desorption properties of ECB on macroporous resins, and a macroporous resin adsorption chromatography method for ECB isolation were investigated.

2. Materials and methods

2.1. Chemicals

Echinocandin B (ECB) standard was purchased from BioAustralis (Smithfield, NSW, AUS). Acetonitrile and methanol were HPLC grade and purchased from Honeywell Burdick & Jackson Chemical Ltd., USA. Ultra-pure water used for analytical and preparative HPLC was produced by Mili-pore Q System (Millipore, USA). All other chemicals and reagents were analytical grade and purchased from Mike Chemical Reagents Co., Ltd. (Hangzhou, China).

2.2. Adsorbents

Nine macroporous resins including HP-20, X-5, AB-8, HZ816, HPD722, HPD400, ADS-7, S-8 and NKA-9 were used in this work. HP-20 was purchased from Mitsubishi Chemical Industries Ltd. (Tokyo, Japan), and X-5, AB-8, HZ816, HPD722, HPD400, ADS-7, S-8 and NKA-9 were purchased from Zhengzhou Qinshi science and technology Co., Ltd. (Zhengzhou, China). All of them were first soaked in 95.0% (v/v) ethanol for 12 h, washed thoroughly with distilled water, and vacuum dried prior to use. Their physical properties and specification are shown in Table 1.

Table 1
Physical properties of macroporous resins.

Name	Polarity	Particle size (mm)	Surface area (m^2/g)	Average pore diameter (nm)
HP-20	Non-polar	0.3–1.25	600–650	24.0–26.0
HZ-816	Non-polar	0.3–1.25	450–550	6.5–7.0
X-5	Non-polar	0.3–1.25	500–600	21.0–23.0
HPD-722	Weak-polar	0.3–1.25	485–530	13.0–14.0
AB-8	Weak-polar	0.3–1.25	450-530	13.0–14.0
ADS-7	Polar	0.3–1.25	100–120	25.0–30.0
ADS-5	Polar	0.3–1.25	520–600	20.0–30.0
S-8	Polar	0.3–1.25	100–120	28.0–30.0
NKA-9	Polar	0.3–1.25	250–290	15.5–16.5

2.3. Preparation of ECB solution

The fungus *A. nidulans* CCTCC M 2010275 was cultured on potato dextrose agar (PDA) plates at 25 °C for 13 days. The agar plugs (0.5 cm in diameter) were inoculated into 250 ml Erlenment conical flasks containing 100 ml of liquid media (10 g glycerol, 10 g peptone, 40 g soybean meal, 20 g peanut oil, 5 g L-Proline, 5 g $CaCl_2$, 8 g K_2HP0_4, 0.05 g $FeSO_4{\cdot}7H_20$, 5 g $MgSO4{\cdot}7H_2O$, 0.1 g $MnSO4{\cdot}H_2O$, 0.6 g $CuSO_4{\cdot}5H_2O$, and 1,000 ml water, initial pH 6.0) and cultured in a rotary shaker at 200 rpm and 25 °C. When the production of ECB in fermentation broth reached the required level (ECB concentration was 1.5 g/L), fermentation broth was centrifuged at 5000 rpm for 20 min to collect the mycelia. Then, the mycelia (100 g) of *A. nidulans* were extracted by 1 L of ethanol solution. The extracts were purified by membrane filtration and then evaporated to dryness. Distilled water was added to get ECB solutions at the concentration of 1.0 mg/mL.

2.4. Analytical methods

The concentrations of ECB were determined by HPLC. The chromatographic conditions were as follows: ODS column (Shimpack, VP-ODS, 4.6mm × 250 mm, Shimadzu Corporation, Kyoto, Japan); UV detector; detection wavelength 223 nm; flow rate 1.0 ml/min; mobile phase, Methanol: Acetonitrile: Water in the ratio of 70:10:20 (v/v); injection volume 10 μl. The retention times for ECB were around 13.5 min. Moreover, all samples were filtered through a 0.45 μm one-off filtration membrane prior to injection into HPLC system to get accurate results. Concentrations of ECB were calculated from the peak areas using a calibration curve.

2.5. Resin selection

Resin selection was conducted based on their adsorption and desorption capabilities for ECB. In adsorption experiment, 50.0 mg of each macroporous resin HP-20, HZ-816, HPD-722, HPD-400, ADS-7, ADS-5, X-5, S-8, AB-8 and NKA-9 was weighted into 250 ml flasks and exposed to 50.0 ml of 1.0 mg/ml ECB solution, agitated for 24 h with a blade stirrer at 150 rpm, 25 °C. In the subsequent desorption test, ten resins saturated with ECH were first washed with deionized water, and then desorbed with 20.0 ml ethanol, by agitating under the same conditions.

The equilibrium adsorption capacity was calculated by Eq. (1).

$$q_e = \frac{(c_0 - c_e)V_1}{m} \quad (1)$$

where q_e is the equilibrium adsorption capacity (mg/g dry resin), V_1 is the volume of ECB solution (ml), c_0 is the initial concentration of ECB (mg/ml), c_e is the concentration of ECB in