Separation and Purification Technology 143 (2015) 72–79

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Dual gradient macroporous resin column chromatography for concurrent separation and purification of three families of marine bacterial lipopeptides from cell free broth

Gunaseelan Dhanarajan, Vivek Rangarajan, Ramkrishna Sen *

Department of Biotechnology, Indian Institute of Technology Kharagpur, West Bengal 721302, India

ARTICLE INFO

Article history:
Received 4 December 2014
Received in revised form 7 January 2015
Accepted 22 January 2015
Available online 30 January 2015

Keywords:
Lipopeptide families
Column chromatography
Macroporous adsorption resin (MAR)
pH/solvent dual gradient elution
Improved resolution

ABSTRACT

A simple and efficient dual-gradient elution strategy for the separation and purification of marine bacterial lipopeptides was developed in macroporous resin column chromatography. Preliminary batch adsorption studies conducted on four different resins revealed that a non-polar resin, HP-20, exhibited the highest adsorption capacity. The optimal conditions yielding maximum adsorption of lipopeptides on to this resin were as follows: temperature = 40 °C, resin dosage = 10 g L^{-1} and initial lipopeptide concentration = 3 g L^{-1}. While employing a simple stepwise solvent gradient elution under optimal conditions, three lipopeptide families, namely, iturin, fengycin and surfactin could be separated with poor resolution. However, the resolution was significantly improved after implementing a family specific dual-gradient elution strategy, wherein, both the solvent composition and pH of the mobile phase were simultaneously varied. Using this approach, lipopeptides were effectively resolved and purities up to 68.3% (iturin), 77.6% (fengycin) and 91.6% (surfactin) were achieved. To the best of our knowledge, this is the first report on the macroporous adsorption resin (MAR) chromatography employing family specific dual-gradient elution strategy for simultaneous recovery and purification of lipopeptides directly from cell free culture broth.

1. Introduction

Lipopeptides are a group of green microbial surfactants that contain a hydrophilic peptide moiety and a lipophilic fatty acid chain. Based on the amino acid composition of peptide moiety, lipopeptides are classified into various families such as surfactin, lichenycin, iturin, and fengycin [1]. Schematic structures of iturin, fengycin and surfactin are given in Fig. 1. The microbial species determines whether these surface active molecules will be synthesized as a single family [2] or as a mixture of two or more families [3,4]. In addition to their surface and emulsifying activities, these molecules are also endowed with attributes of therapeutic agents. However, it is indeed the level of purity that decides the scope of their applications. For bioremediation and enhanced oil recovery applications, crude lipopeptides after primary recovery from culture broth can be used [5,6]. However, it becomes mandatory to significantly enhance the purity of lipopeptides for food and pharmaceutical applications [7,8]. Furthermore, each family of lipopeptides exhibits specific bioactive properties. For instance, members of iturin family are known for their antifungal activity; fengycins are mostly antifungal with limited antibacterial activity and surfactins are reported showing antiviral, antibacterial and antitumor activities [3,9]. Therefore, it is of great importance to separate and purify the individual lipopeptide families from the crude mixture.

Several techniques such as foam fractionation, ultra-filtration, solvent extraction and gel permeation chromatography have been reported for the separation and purification of lipopeptides [9–11]. However, these methods are not efficient in resolving the lipopeptide mixture into individual families [12]. Recently, macroporous adsorption resin (MAR) chromatography has been successfully used for separation of various bioactive molecules on the basis of hydrophobic/hydrophilic interactions between solute and resin surface [13,14]. Some salient features of MAR include high adsorption capacity, good stability, low fluid resistance and easy regeneration [13,15]. Correctly selected MAR and optimal operating conditions can lead to macroporous resin based adsorption chromatography becoming superior to any of the alternative methods presently available for lipopeptides. Though the use of MAR was known only for the adsorption-based recovery of lipopeptide families as a mixture [12,16], no systematic studies leading to the development of a MAR based chromatographic method for the

* Corresponding author. Tel.: +91 3222 283752.
E-mail address: rksen@yahoo.com (R. Sen).

http://dx.doi.org/10.1016/j.seppur.2015.01.025
1383-5866/

separation and purification of individual lipopeptide families present in the crude mixture have hitherto been reported. Thus, the present work is aimed at developing an efficient chromatographic method for separating and simultaneously purifying three different lipopeptide families, namely, iturin, fengycin and surfactin directly from the cell free broth using the MAR that showed better performance in the studies on the adsorption and desorption characteristics of lipopeptides. To the best of our knowledge, this would be the first attempt to use MAR chromatography to resolve the three lipopeptide families immediately after cell harvesting.

2. Materials and methods

2.1. Adsorbent resins

Four MARs including XAD-4, XAD-7, HP-20 and HP-2MG were purchased from Sigma, USA. The physical properties of the MARs are given in Table 1. The resins were pretreated to remove the monomers, porogenic agents and salts trapped inside the pores during the synthesis process. They were soaked in 95% (*v*/*v*) ethanol for 24 h, washed with deionized water and vacuum dried before use.

2.2. Production and isolation of lipopeptides

Lipopeptide production by marine *Bacillus megaterium* (isolated from Andaman and Nicobar Islands, India) was carried out using noodle processed water supplemented with chemical fertilizers as reported earlier [17]. At the end of fermentation, the culture broth was harvested and the cells were separated by centrifugation. The supernatant was acidified to pH 2 using 6N HCl and was kept at 4 °C overnight. The precipitate was centrifuged and the pellet was lyophilized to get the crude lipopeptide, which was used for further analyses and adsorption studies.

2.3. Analytical methods

Lipopeptide concentration was determined using reverse phase HPLC instrument (Agilent Technologies, CA, USA) equipped with Zorbax C_{18} column and diode array detector. The mobile phase consisted of solvent A (Milli-Q water) and solvent B (HPLC grade acetonitrile with 0.1% trifluoroacetic acid). The lipopeptides were eluted by linearly increasing the percentage of solvent B from 5% to 95% for 60 min at a flow rate of 0.4 mL min^{-1}. The molecular weight of lipopeptides was determined using Voyager DE-Pro MALDI-ToF spectrometer (Applied Biosystems Inc., CA, USA) as reported earlier [7].

2.4. Static adsorption and desorption studies

2.4.1. Selection of adsorbent for lipopeptide recovery

MAR was selected for recovering the lipopeptides based on its adsorption and desorption capacities. Four MARs at 10 g L^{-1} concentration were added individually to flasks containing 20 mL of lipopeptide solution. The flasks were then shaken (150 r/min) at 35 °C until adsorption equilibrium. Samples were withdrawn from the flasks at preset time intervals and analyzed by HPLC. After the resins attained saturation, they were washed with de-ionized water to remove un-adsorbed lipopeptides. In order to desorb the lipopeptides, different solvents such as ethanol, methanol and acetone were tested, and acetone was found to be more efficient (Fig. S1 in supplementary information). Therefore, it was used as the eluent for all further experiments. The concentrations of lipopeptides in eluted samples were measured by HPLC. Adsorption and desorption capacities of the resins were quantified using the following equations:

$$Q_e = \frac{(C_0 - C_e)V_a}{W} \quad (1)$$

$$A_R = \frac{(C_0 - C_e)}{C_e} \times 100\% \quad (2)$$

$$D_R = \frac{C_d V_d}{(C_0 - C_e)V_a} \times 100\% \quad (3)$$

where A_R and D_R are adsorption and desorption ratios, respectively (%); C_0, C_d and C_e are solute concentrations in feed, eluent and effluent solutions, respectively (g/L); V_a and V_d are feed volume and eluent volume, respectively (mL) and W is dry weight of the adsorbent (g).

2.4.2. Factors influencing adsorption

A suitable resin dosage for efficient lipopeptide adsorption was determined by adding various concentrations of pre-selected resin (5–20 g L^{-1}) in 20 mL of lipopeptide solution (1 g L^{-1}). To assess the effect of temperature, flasks with optimal amount of resin and lipopeptide concentration were incubated at different temperatures (25–45 °C) until equilibrium. Although pH plays an important role on the adsorption capacity of MAR, considering the impending use of such a system for *in situ* product recovery, the pH of lipopeptide solution was maintained at 6.7 (which is optimal for lipopeptide production) throughout the studies.

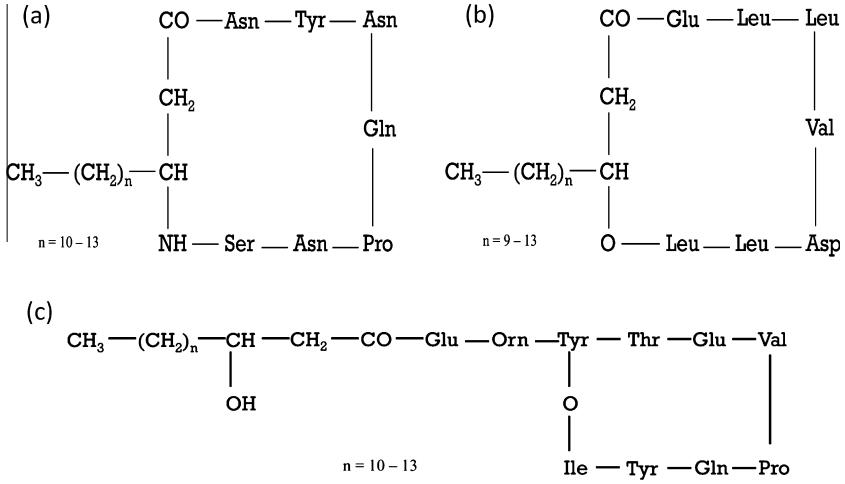

Fig. 1. Schematic structures of (a) iturin, (b) surfactin and (c) fengycin.