Carbohydrate Polymers 150 (2016) 378–384

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Synthesis and characterization of cryogel structures for isolation of EPSs from *Botryococcus braunii*

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ARTICLE INFO

Article history:
Received 18 March 2016
Received in revised form 3 May 2016
Accepted 10 May 2016
Available online 17 May 2016

Keywords:
Cryogel
Exopolysaccharides
B. braunii
Synthesis
PHEMA

ABSTRACT

In this study, the objective was to separate exopolysaccharides (EPSs) released in the broth subsequent to outdoor cultivation of *Botryococcus braunii*. For this, poly(2-hydroxyethyl methacrylate) (PHEMA) cryogels were synthesized. After that, the surface was modified by coupling Concanavalin A. Box-Behnken statistical design was used to evaluate the effect of freezing temperature, Con A concentration and flow rate on Con A binding capacity. Optimum synthesis conditions were elicited as −14.48 °C freezing temperature, 1.00 mg/ml Con A concentration and 0.30 ml/min flow rate yielding 3.18 mg Con A/g cryogel, whereas −16 °C, 1.00 mg/ml and 0.30 ml/min yielded the highest (3.38 mg) binding capacity in experimental cryogel preparation. The EPS adsorption capacity of the optimum cryogel column was found as 3.26 mg EPS/g cryogel corresponding to adsorption yield of 80%. Besides; swelling test, elemental analysis, Micro-CT, SEM and FTIR analysis were carried out for characterization of the synthesized cryogels.

1. Introduction

Cryogels are gel matrices that are formed in frozen solutions of monomeric or polymeric precursors. Cryogels have interconnected macropores or supermacropores with a pore size range of 10–100 μm. These interconnected supermacropores permit the free passage of micro- and nanoparticles without blockage of the gel matrix (Lozinsky et al., 2003; Unluer, Ersoz, Denizli, Demirel, & Say, 2013). High porosity, high mechanical and chemical stability (Tan et al., 2015) make them appropriate carriers for immobilization of biomolecules and cells, thereby creating attractive gel matrices for separation and purification of various molecules (Erturk & Mattiasson, 2014). Cryogels are very good alternatives to remove or purify substances from biological matrices with many advantages such as large pores, short diffusion path, low pressure drop, residence time and cost-effective preparation (Akduman, Uygun, Aktas-Uygun, Akgol, & Denizli, 2013; Baydemir et al., 2009).

Lectins that interact with carbohydrates non-covalently are a group of proteins, which are found in most organisms including viruses, bacteria, plants, and animal (Yavuz, Aksoz, & Denizli, 2012). Immobilized lectins are widely used for affinity purification of glycoproteins and glycopeptides. Concanavalin A (Con A), which is a plant lectin present in the seeds of *Canavalia ensiformis* (Lis & Sharon, 1998), is one of the most studied lectins for this purpose. Con A binds specifically to mannose with high affinity, whereas to glucose with less affinity (Hardman & Ainsworth, 1976).

Extracellular polysaccharides (exopolysaccharides) (EPS) consist of the major part of the organic material released to the environment by microalgae in rivers, lakes and oceans (Weiss et al., 2012). They are soluble in water and form a gel as the viscosity increases, therefore evaluated as alternative food additives (Banerjee, Sharma, Chisti, & Banerjee, 2002). EPSs are found to be involved in pathogenesis, symbiosis, protecting against osmotic shock, toxic stress (De Brito, Caseli, & Nordi, 2011) and also reported to possess immunomodulatory, antibacterial, antiviral, antiulcer, anticancer and antioxidant effects (Parikh & Madamwar, 2006). EPSs are used as stabilizers, emulsifiers, and gelling agents (Mishra, Kavita, & Jha, 2011), help in capturing nutrients, act as anti-corrosive agents and bioflocculants in the food industry (Chowdhury, Basak, Sen, & Adhikari, 2011). In the literature, EPSs have been isolated using various extraction methods (Heperkan, Daskaya-Dikmen, & Bayram, 2014; Razack, Velayutham, & Thangavelu, 2013; Thirugnanasambandham, Sivakumar, & Prakash Maran, 2014). The

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http://dx.doi.org/10.1016/j.carbpol.2016.05.027
0144-8617/

process has several steps; separation of EPS from the culture medium by centrifugation and further purification by additional centrifugation steps, chemical treatment, membrane techniques or combinations of it. *B. braunii* which is green fresh- to brackish-water algae has a unique colonial organization such that individual cells of the colony are embedded in an extracellular matrix (ECM) composed of polymerized and liquid hydrocarbons and exopolysaccharides (Weiss et al., 2012). Cells of *B. braunii* possess an internal fibrillar layer made of mucilaginous polysaccharides which dissolve slowly in the culture medium (Banerjee et al., 2002).

The aim of this study was to prepare a hydrophilic cryogel matrix for efficient separation of exopolysaccharides from *B. braunii* algae. For this, PHEMA based cryogel matrix was prepared as a monolithic form by cryopolymerization of 2-hydroxyethyl methacrylate (HEMA) due to its biocompability, excellent structural and chromatographic properties. Surface modification was carried out by immobilization of Con A in order to provide specificity to synthesized cryogels. We hypothesized that if Con A binding capacity of the cryogel increases, EPS adsorption will be higher due to the increase in the affinity. In this regard, the effect of the binding conditions such as temperature, Con A concentration and flow rate were studied to determine the conditions maximizing exopolysaccharide adsorption which were carried out in a continuous mode. Besides, swelling test, SEM analysis for surface morphology, micro-CT, FTIR and elemental analysis were carried out for characterization. To the best of our knowledge, this is the first study aiming to isolate EPSs using cryogel matrices.

2. Materials and methods

2.1. Materials

N,*N*′-methylene bisacrilamid, amonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylene diamine (TEMED), ethanol and hydrochloric acid were purchased from Sigma Chem. Co. (St. Louis, USA) 2-hydroxyethyl methacrylate (HEMA), triethoxi-3-(2-imidazoline-1-yl)propylsilane (IMEO) were purchased from Aldrich (Munich, Germany) and Concanavalin A was purchased from Sigma-Aldrich. Phenol and sulphuric acid from Merck & Co., Exopolysaccharide native solution (2 g/L) from *Botryococcus braunii* (CCALA778) was supplied from Norsker Investigaciones S.L. (Spain) in the scope of FP7 project, SPLASH.

2.2. Experimental design

Box-Behnken statistical design was used to optimize cryogel preparation. The independent variables were freezing temperature (−12 °C, −16 °C, −20 °C), Con A concentration (0.5, 0.75, 1.0 mg/ml) and flow rate (0.30, 0.45, 0.60 ml/min). These variables were set according to the Box-Behnken experimental design which consists of a group of mathematical approaches for the modelling and analysis of the response influenced by the independent variables. PHEMA cryogels prepared at optimum conditions were used for isolation of EPSs from *B. braunii* culture media.

2.3. Preparation of PHEMA cryogels

The preparation of the PHEMA cryogel has been described elsewhere (Percin, Aksoz, & Denizli, 2013). Briefly, monomers (1.3 ml of HEMA and 0.283 g MBAAm) were dissolved in deionized water (15 ml), and the mixture was degassed under N_2 atmosphere for 5 min to eliminate soluble oxygen. The total concentration of monomers was 10% (w/v). The cryogel was produced by free radical polymerization initiated by TEMED and APS. After adding APS (20 mg), the solution was cooled in an ice bath for 2–3 min. TEMED (25 ml) was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into a plastic syringe (total volume: 5 ml, internal diameter: 0.8 cm) with closed outlet at the bottom. The polymerization solution in the syringe was frozen at the determined temperature for 24 h and then thawed at room temperature. After washing with distilled water, the cryogel was stored in distilled water containing 0.02% sodium azide at 4 °C until use.

2.4. Activation of PHEMA cryogels

Before binding Concanavalin A molecule covalently to PHEMA cryogels, the cryogel was activated with triethoxy-3-(2-imidazoline-1-yl)propylsilane (IMEO), where IMEO/ethanol solution was applied at a rate of 2/1 (v/v) with a peristaltic pump (Watson Marlow SciQ 400) in a continuous system (0.45 ml/min, 3 h, 25 °C).

2.5. Con A binding studies

Con A solutions were prepared with phosphate buffer solution (0.1 M, pH = 7), where the concentration was changed between 0.5 and 1.0 mg/ ml and the flow rate in the range of 0.30–0.60 ml/min to determine the effects of initial concentration and flow rate on the binding capacity. These solutions were subjected to cryogel matrices evenly by a peristaltic pump at room temperature for 2 h. In order to determine the immobilized amount of Con A, initial and final Con A concentrations were determined by monitoring the decrease in UV absorbance at 280 nm (Uygun, Uygun, Ozcaliskan, Akgol, & Denizli, 2012). Con A binding capacities of each cryogel were calculated by using Eq. (1). According to the Box Behnken Design Expert Software, these values were considered as response parameter and optimum conditions were determined.

$$Q = \frac{(C_i - C_f)\,xV}{mx10^{-3}} \quad (1)$$

Q: The amount of covalently bonded Con A on the surface of silanized polymeric cryogels (mg/g)

C_i and C_f: The initial and after concentration of Con A solution, respectively (mg/mL)

V: The total volume of Con A solution (mL)

m: The amount of silanized cryogels (g)

2.6. Characterization of cryogels

2.6.1. Swelling test

The swollen monolithic cryogel matrix (5 ml) was dried in an oven (60 °C) till constant weight (Plieva et al., 2004) to determine the weight of dried polymer sample (m_{drygel}). Subsequently, the dried sample was placed into water and the increase in weight was noted after 1, 5, 10, 15, 30, 60, 120 and 150 min. The ratio of dry polymer and polymer with bound water in the swollen gel was determined as percent of swollen gel weight ($m_{swollengel}$). The swelling degree (S) of cryogel was calculated according to Eq. (2). All analyses were carried out in duplicates and the average values were expressed as the mean ± standard error.

$$S = \frac{\left(m_{wetgel} - m_{drygel}\right)}{m_{drygel}} \quad (2)$$

2.6.2. FTIR

Fourier Transform Infrared (FTIR) Spectroscopy was used to compare the cryogel structures of PHEMA before and after activation by IMEO. Therefore, FTIR spectra of PHEMA and PHEMA-IMEO polymeric cryogels were obtained using FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan).