ACTA MICROBIOLOGICA BULGARICA

Purification of Arabinomannan Synthesized by *Cryptococcus laurentii* AL_{100}

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Abstract

The Antarctic yeast strain *Cryptococcus laurentii* AL_{100} was selected as an active arabinomannan producer (Pavlova *et al*., 2011). The synthesized biopolymer was subjected to purification by two methods. In the application of molecular-sieve purification with Sephadex G75, two separate carbohydrate fractions were detected, while the use of gel-filtration system on Sepharose DEAE CL-6B showed three distinct carbohydrate fractions. The protein content of the fractions was also established.

Key words: exopolysaccharide, yeasts, purification, *Cryptococcus laurentii,* carbohydrate

Резюме

Cryptococcus laurentii AL_{100} беше селекциониран като активен продуцент на екзополизахарида арабиноманан (Pavlova *et al*., 2011). Синтезираният биополимер беше подложен на пречистване чрез използването на два метода. При приложението на молекулно-ситово пречистване със Sephadex G75 бяха установени две отделни въглехидратни фракции. Използването на гел-филтрираща система със Sepharose DEAE CL-6B показа три отделни въглехидратни фракции. Определено беше и протеиновото съдържание във фракциите.

Introduction

Polysaccharides are high molecular compounds composed of a large number of monosaccharide residues joined to each other by glycosidic linkages. The composition of microbial polysaccharides is various, which determines the diversity of their physical and chemical properties (Sutherland, 1990; Nishinari, 2006). They are composed of long chains with molecular weight of $2.0x10^6$ Da. Most often, the composition of the EPS includes glucose, galactose and mannose (Petersen *et al*., 1990; Pavlova and Grigorova, 1999; Pavlova *et al.,* 2004; Radchenkova *et al*., 2013). Taking into account the diversity of sugar components, there is a wide range of molecular structures. The study of the EPS structure is crucial for the understanding of their physicochemical and biological properties, as well as for the use of EPS-producing organisms for industrial or medical purposes (Kumar *et al*., 2007; Freitas *et al*., 2011). Therefore, their application in various industries, such as pharmaceutics, requires that these biomolecules should be purified.

Polysaccharides were purified by means of chromatographic techniques, taking into account the charge, solubility, and molecular weight of the polysaccharide molecule. Chromatographic separation based on particle size was applied for the separation of large molecules from macromolecular complexes in a solution.

Materials and methods

Biosynthesis of exopolysaccharide

The selected producer of arabinomannan *Cryptococcus laurentii* AL_{100} was used for extracellular polysaccharide biosynthesis (Pavlova *et al*., 2011). The biopolymer substance was obtained in bioreactor cultivation conditions. The nutrient medium contained (g/L): sucrose - 40; $(NH_4)_2SO_4$ - 2.5; KH_2PO_4 - 1.0; $MgS0_4.7H_20$- 0.5; NaCl - 0.1; $CaCl_2.2H_20$ - 0.1 and yeast extract - 1.0. A Sartorius bioreactor with a working capacity of 5L was used, equipped with a turbine stirrer, oxygen (Hamilton, Bonaduz AG, Switzerland) and pH (Hamilton, Bonaduz AG, Switzerland) electrodes.

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The temperature, agitation speed, air flow rate, foam and levels in the vessel were monitored by a software program (BioPAT ® MFCS/DA 3.0). Cultivation was performed at 22°C, with mechanical stirring -400 rpm and pneumatic -1.25 L/L/min, for 96h. The inoculum was prepared in 500 mL flasks during periodic deep cultivation on a rotary shaker with 220 rpm mechanical stirring, at 22ºC for 48 h.

Isolation and purification of the EPS from the cultural liquid

After completion of the fermentation, the biomass was separated from the cultural liquid by centrifugation at 6000×g for 30 min. The cultural liquid was concentrated at 60° C in a vacuum evaporator. The cooled concentrate was precipitated with two volumes of cold 95% ethanol and the samples stayed for 24 h at 4°C. The exopolysaccharide was separated from the ethanol solution by centrifugation at 5000×g for 10 min.

Two methods were used for the biopolymer purification. The first method was implemented through a preparative chromatography column with Sephadex G75, with NaCl as an eluent. For the second molecular-sieve purification a DAEC Sepharose CL-6B column was used, where the sample was filtered through a 2 μm pore size membrane before being let in. The system used for fractionation was ÄKTAprime plus. Elution was carried out with distilled water in 4 ml stream, and with a linear NaCl gradient at a concentration of 0 to 1.0 M. The fractions obtained were analyzed for carbohydrate (by the colorimetric method of Dubois *et al*. (1956), using glucose as a standard) and protein (using the colorimetric method of Bradford (1976)) content. The solutions were subjected to dialysis with a membrane for 24 hours.

Results

After the analysis of the exopolysaccharide molecule was carried out, its chemical composition was determined. It showed 79.1% carbohydrate, -11.7% protein (Pavlova *et al*., 2011). The heterogeneous chemical composition determined the need for the removal of the ingredients that were different from the core molecule. According to the figures outlined above, the synthesized biopolymer is a proteoglycan, as it has a protein component.

The method of automatic preparative gel chromatography with Sephadex G75 was used for the purification of arabinomannan. The data in Figure. 1 report two fractions containing carbohydrates with different molecular weight.

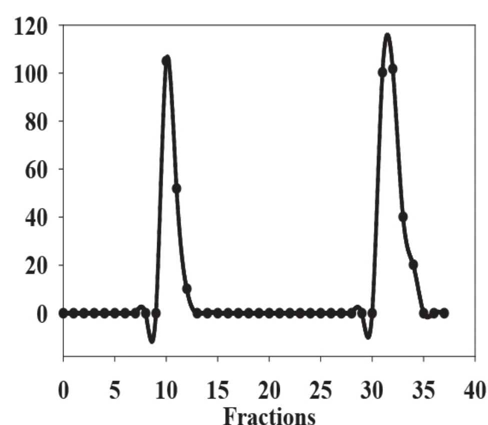

Fig. 1. Carbohydrate amounts in the fraction after purification by Sephadex G75

The first peak was obtained at 10, 11 and 12 fraction and it was a high molecular one, while the second peak appeared between 30 and 35 fractions and was determined as one of low molecular fraction. 25% of all carbohydrates were determined in the first peak, and 54% of them - in the second one. The total yield from both peaks amounted to 79.3% of the initial carbohydrates. There was about 3% of protein present in both fractions, where a carbohydrate component was established.

Purification was done using DEAE- Sepharose CL-6B, which allowed simultaneous performance of gel-chromatography and ion-exchange chromatography.

The initial color of the dissolved polysaccharide was pale pink, while after the filtration through a filter the solution became colorless, which showed that the pigment and part of the polymer had not pass through the pores of the filter.

After purification of the polymer fraction, the elution profile showed the presence of 3 different polysaccharides: the electrically neutral EPS 1 and EPS 2 (Fig 2, A), as well as the negatively charged EPS 3 (Fig.2, B). The first one was from 9 to 19 fractions, as the carbohydrates amount varied from 11.61 μg/mL to 26.32 μg/mL. The second peak was reported from 27 to 34 fractions with carbohydrate content at the highest point of 48.51 μg/mL. Elution with NaCl showed another peak with more carbohydrate content. It extends from 30 to 47 fractions and the highest content of carbohydrate - 88.77 μg/mL was in the sample 39 (Fig 2, B). The data were obtained after a 24-hour dialysis. This peak proves that part of the EPS came out after elution with NaCl through the column. Since sepharose was molecular-sieve and anion- exchangeable, the hanging parts of the polysaccharide molecule were negatively charged.

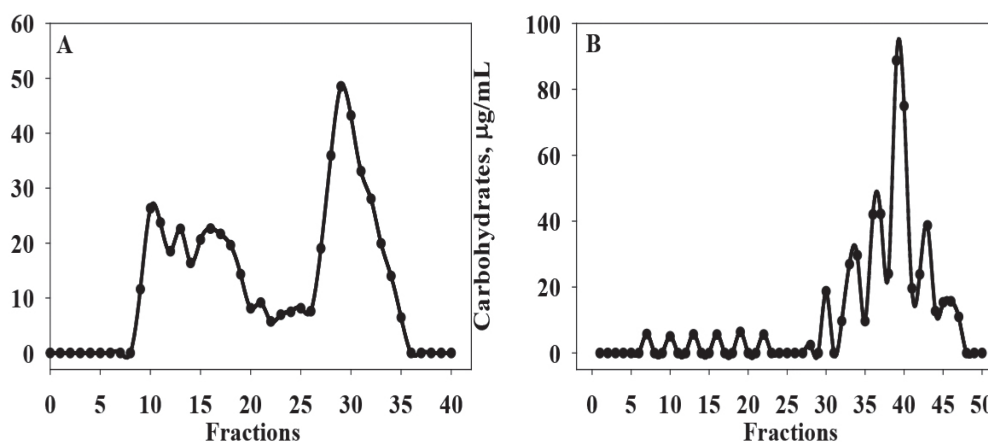

Fig. 2. Carbohydrate amounts in the fractions after purification by Sepharose DEAE CL-6B with eluent A) water and B) NaCl

The first peak contained approximately 19% of the carbohydrate, its content in the second one was 20%, and 40% was recovered in the peak after the elution with NaCl. The protein content analyses showed that during the first peak the protein content was 11.11 µg/mL, at the second peak it was 18.05 µg/mL, and at the NaCl elution peak - 45.83 µg/mL.

Discussion

Since all fractions obtained after purification with Sephadex G75 and Sepharose DEAE CL-6B contained protein (in small amounts), they could probably be determined as proteoglycans.

A more commonly used method for purification of polysaccharides is by Sepharose. Purification of exopolysaccharides by *Bifidobacterium animalis* RH was carried out in a similar manner, by anion-exchange chromatography, wherein the two peaks with a carbohydrate content were detected (Xu *et al*., 2011). The purification of an acidic polysaccharide from *Bifidobacterium longum* BB-79 was accomplished by ion-exchange chromatography and gel-filtration (Roberts *et al*., 1995; Andaloussi *et al*., 1995). Microbial glucan, synthesised from *Geobacillus tepidamans* V264, isolated from a Bulgarian hot spring in Velingrad, was purified by gel filtration on Sepharose DEAE CL-6B (Kambourova *et al*., 2009).

The results of the purification by Sepharose DEAE CL-6B of glucomannan, synthesized from *Sporobolomyces salmonicolor* AL_1, showed the presence of two fractions - the basic one with 90% carbohydrate content and low presence of protein, uronic and nucleic acids after elution with water, and the second one - after NaCl elution with 49% carbohydrate content (Poli *et al*., 2010).

Through the two types of arabinomannan purification, the presence of two, and in the second case - three carbohydrate fractions based on molecular weight and charge was detected. Purification by Sepharose DEAE CL-6B proved that the EPS studied contained groups with negative charge. There is a presence of proteins in both methods used.

Acknowledgements

The study was supported by Grant DTK 02/46 from the National Fund Scientific Investigation.

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