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Monitoring and preparation of neoagaro- and agaro-oligosaccharide products by high performance anion exchange chromatography systems

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ABSTRACT

A series of neoagaro-oligosaccharides (NAOS) were prepared by β-agarase digestion and agaro-oligosaccharides (AOS) by HCl hydrolysis from agarose with defined quantity and degree of polymerization (DP). Chain-length distribution in the crude product mixtures were monitored by two high performance anion exchange chromatography systems coupled with a pulsed amperometric detector. Method 1 utilized two separation columns: a CarboPac™ PA1 and a CarboPac™ PA100 connected in series and method 2 used the PA100 alone. Method 1 resolved the product in size ranges consisting of DP 1–46 for NAOS and DP 1–32 for AOS. Method 2 clearly resolved saccharide product sizes within DP 26. The optimized system utilizing a semi-preparative CarboPac™ PA100 column was connected with a fraction collector to isolate and quantify individually separated products. This study established systems for the preparation and qualitative and quantitative measurements as well as for the isolation of various sizes of oligomers generated from agarose.

1. Introduction

Agarose is a purified linear galactan hydrocolloid isolated from an agar or agar-bearing algae composed of (1 → 4)-linked 3,6-anhydro-α-L-galactose alternating with (1 → 3)-linked β-D-galactopyranose, including neoagaro- and agaro-series (Fig. 1) (Lahaye, Yaphe, Viet, & Rochas, 1989). Two types of oligosaccharides can be derived from agarose by agarase or acid actions (Fig. 1). β-Agarase, the main agarase isolated from marine bacteria, would cleave the β-(1 → 4)-galactosidic bond of the polymer to release neoagaro-oligosaccharides (NAOS, i.e., various units of the neoagarobiose, 3,6-AG-α-(1 → 3)-Gal) (Malmqvist, 1978; Allouch, Helbert, Henrissat, & Czjzek, 2004; Henshaw et al., 2006), while the action of α-agarase or acids (e.g., HCl) would cleave the α-(1 → 3) bond to release agaro-oligosaccharides (AOS, i.e., various units of the agarobiose, Gal-β-(1 → 4)-3,6-AG) (Young, Bhattacharjee, & Yaphe, 1978; Rochas, Potin, & Kloareg, 1994).

In recent years, bioactivity studies have demonstrated that the above oligomer forms derived from agar or agarose exhibit variety of physiological activities. The extent of these activities is also correlated with the degree of polymerization (DP) of the galactosyl groups on the NAOS and AOS. Neoagarobiose (N2) was reported to possess moisturizing and whitening effects on melanoma cells (Kobayashi, Takisada, Suzuki, Kirimura, & Usami, 1997). Sulfated oligosaccharide with degree of polymerization (DP) 4, derived from porphyran, was reported to be utilized in vitro by intestinal bacteria, stimulating the growth of *Bacteroides*, as well as *Eubacterium* and *Lactobacillus* (Osumi, Kawai, Amano, & Noda, 1998). Then, the prebiotic effectiveness of NAOS with DPs 4–12 was confirmed both in vitro and in vivo, showing augmented growth of *Bifidobacterium* and *Lactobacillus* (Hu et al., 2006). Recently, low-DP sulfated saccharides (DP 6) from an agar-bearing alga *Gracilaria* sp. has showed a distinctly higher positive effect on survivability in Japanese encephalitis virus infected C3H/HeN mice in comparison to their polysaccharides (Kazłowski, Chiu, Kazłowska, Pan, & Wu, 2012). The in vivo antiviral activity has been connected with better absorption of the sulfated oligosaccharides with DP 6 than undigested PS (Kazłowski et al., 2012). The AOS with DPs 2–4 (A2–A4) are able to suppress the production of the pro-inflammatory cytokine TNF-α and the expression of iNOS, an enzyme associated with the production of NO in in vitro studies (Kato, Enoki, & Sagawa, 2001). The AOS, especially agarohexaose (A6), could scavenge reactive oxygen species (ROS) generated by electron leakage as well as protect cells

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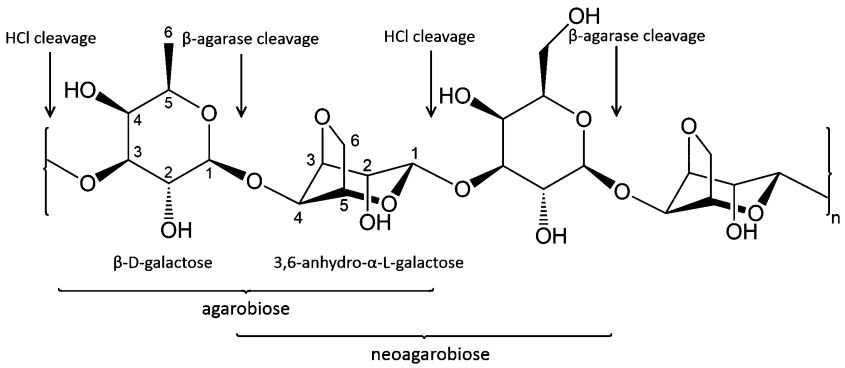

Fig. 1. Structures of the agarose molecule and the two product types derived from digestion with β-agarase and HCl hydrolysis. The cleavage sites are indicated by arrows. The marked neoagarobiose represents the basic unit of the NAOS products digested by β-agarase. The marked agarobiose represents the basic unit of the AOS released by HCl action.

against apoptosis induced by ROS in a human liver cell L-02 system (Chen, Yan, Zhu, & Lin, 2006). The AOS with DPs 2–8 (A2–A8) oral administration inhibited 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice through heme oxygenase-1 induction in macrophages, therefore the A2–A8 might be an important therapeutic agents for inflammatory bowel disease (Higashimura et al., 2013). Algal oligosaccharides are also well known for their industrial applications, e.g. as an energy source alternative to fossil fuels (Haag, 2007; Mascarelli, 2009). The potential of further applications of each different DP of the NAOS and AOS could be discovered if there is a handy qualitative and quantitative method established to prepare and determine the identity of individual oligomers.

The aim of this study was to develop a method for the preparation of oligosaccharides, both NAOS and AOS with different chain lengths, from agarose degraded by β-agarase and HCl, respectively. The length of the oligoproducts in the crude mixtures was determined by two high-performance anion exchange chromatography (HPAEC) systems utilizing a CarboPac™ PA1 and a CarboPac™ PA100 columns connected in series (method 1) or the CarboPac™ PA100 column stand-alone (method 2), coupled with a pulsed amperometric detection (PAD). Individual NAOS or AOS was then isolated by HPAEC equipped with a semi-preparative CarboPac™ PA100 column and a fraction collector, whereby the separation performance could be inspected. The monitoring of the oligosaccharides in the liquid chromatography systems reported herein provides protocols for the preparation and production of each different DP of the NAOS and AOS in high quality and quantity, making it possible to conduct further studies on their bioactivities.

2. Materials and methods

2.1. Chemical and reagents

Agarase [3.2.1.81] from *Pseudomonas atlantica* (Cat. no. A6306, CAS no. 37288-57-6) as well as monosodium phosphate monohydrate and disodium phosphate heptahydrate for buffer preparation were from Sigma-Aldrich Co. (St. Louis, MO, USA). Hydrogen chloride (HCl) was bought from J.T. Baker (Deventer, Holland). Agarose (162–0133) was from Bio-Rad Laboratories (Hercules, CA, USA). Standards: 3,6-anhydro-D-galactose (162 Da), Galactose (180 Da), neoagarobiose (N2, 324 Da), and neoagarohexaose (N6, 936 Da) used in HPAEC calibration were purchased from Sigma-Aldrich Co. For HPAEC eluents preparation, sodium hydroxide (NaOH) was bought from Merck KGaA (Darmstadt, Germany), sodium acetate (NaOAc) and HPLC-grade water were purchased from Sigma-Aldrich Co. Trifluoroacetic acid (TFA) for carbohydrate membrane desalter regeneration was also bought from Sigma-Aldrich Co.

2.2. Preparation of NAOS products

Agarose (100 mg) was boiled in 200 ml of 100 mM phosphate buffer (pH 6.0) to solubilize the solid agarose. It was then cooled to 40 °C for use. β-Agarase was prepared as 10 U × μl^{-1} in phosphate buffer before use. The 0.05% (w/v) melted agarose substrate solution was added with β-agarase to a final concentration of 3, 4, 5 or 6 U enzymes × mg^{-1} of agarose and incubated at 40 °C for 24 h. The reaction mixture was filtering through 0.22-μm membrane (Millipore, Cork, Ireland), and by ultrafiltration with Amicon YM-30 membrane (Millipore, Bedford, MA, USA) to eliminate agarase. The recovered filtrate was the crude product, which was concentrated by vacuum evaporation and freeze dried for HPAEC–PAD analysis.

2.3. Preparation of AOS products

Agarose (1.5 g) was boiled in 100 ml of deionized water to solubilize the solid agarose. It was then cooled to 40 °C for use. HCl was added stepwise into the melted 1.5% (w/v) agarose solution every 1 h at 50 °C for 6 h. The final concentrations of HCl were 0.1 M, 0.2 M, 0.4 M, and 0.8 M. The reacted mixture was filtered, and the crude product was lyophilized as described above in Section 2.2.

2.4. Product detection by HPAEC–PAD system

A Dionex HPAEC–PAD system consisted of an ICS-3000 single pump (SP), an ICS-3000 detector/chromatography module (DC) with an injection valve containing 11.65 μl sample loop, and an electrochemical detector with cell containing Au working and Ag/AgCl reference electrodes (Thermo Fisher Scientific Inc., Waltham, MA, USA). Waveform pulse potentials and durations were as follows: $E_1 = 0.05$ V ($t_0 = 0$ s); $E_2 = 0.05$ V ($t_1 = 0.2$ s); $E_3 = 0.05$ V ($t_2 = 0.4$ s); $E_4 = 0.075$ V ($t_3 = 0.41$ s); $E_5 = 0.075$ V ($t_4 = 0.6$ s); $E_6 = -0.15$ V ($t_5 = 0.61$ s); $E_7 = -0.15$ V ($t_6 = 1$ s). The chain-length distribution of NAOS and AOS was monitored by two methods. Method 1; a CarboPac™ PA1 guard column (2 × 50 mm), a CarboPac™ PA1 analytical column (PA1) (2 × 250 mm) and a CarboPac™ PA100 analytical column (PA100) (2 × 250 mm) were connected in series. Eluents were 165 mM NaOH solution as eluent A and 82.5 mM NaOH solution containing 412 mM NaOAc as eluent B. The conditions for the chain-length distribution monitoring were 0–75 min, linear gradient from 47.5% to 81.3% eluent B; 75–85 min, linear gradient to 83.5% eluent B; 85–90 min, linear gradient to 92.5% eluent B, and 90–120 min, isocratic elution of 92.5% eluent B. Flow rate for these conditions was 0.225 ml/min. Second method; a CarboPac™ PA100 guard column (PA100 guard) (2 × 50 mm) and the PA100 (2 × 250 mm) were connected. Eluents were the same as those in method 1. The conditions for the chain-length