Carbohydrate Research 343 (2008) 2443–2450

Contents lists available at ScienceDirect

Carbohydrate Research

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

Separation and quantification of neoagaro- and agaro-oligosaccharide products generated from agarose digestion by β-agarase and HCl in liquid chromatography systems

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ARTICLE INFO

Article history:
Received 19 March 2008
Received in revised form 22 June 2008
Accepted 25 June 2008
Available online 2 July 2008

Keywords:
Neoagaro-oligosaccharides
Agaro-oligosaccharides
Isolation
HPLC
ELSD

ABSTRACT

A series of neoagaro-oligosaccharides (NAOS) were separated and isolated by β-agarase digestion and agaro-oligosaccharides (AOS) by HCl hydrolysis from agarose with defined quantity and degree of polymerization (DP). Profiles of the oligomer length in the crude product mixtures were monitored by two high-performance liquid chromatography (HPLC) systems: size-exclusion chromatography (SEC) and NH2-column chromatography (NH2-HPLC), coupled with an evaporative light-scattering detector (ELSD). Calibration curves were established separately to identify the DP and quantify the amount of the oligomer products analyzed in the two systems. Each system was optimized to generate a spectrum of saccharide oligomers with various DP, where the reaction yield for NAOS was 52.7% by 4 U/mg β-agarase and for AOS was 45.6% by 0.4 M HCl. SEC resolved the product in size ranges consisting of DP 1–22 for NAOS and DP 1–14 for AOS. NH2-HPLC clearly resolved both distinct saccharide product sizes within DP 12. The optimized system was connected with a fraction collector to isolate and quantify these individually separated products. The total product yields of the recovered NAOS of DP 1–22 and AOS of DP 1–14 by the SEC system were 84.7% and 82.9%, respectively. NH2-HPLC recovered NAOS and AOS, both with a DP of 1–10 with total product yields of 48.9% and 90.0%, respectively. Isolated NAOS and AOS product fractions were inspected by ^{1}H NMR spectroscopy and ESIMS spectrometry to confirm structure, molecular mass, and purity. This study established feasible systems for the preparation and qualitative and quantitative measurements, as well as for the isolation of various sizes of oligomers generated from agarose.

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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).[1] Two types of oligosaccharides can be derived from agarose by agarases and acid (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),[2–4] 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).[5,6]

In recent years, bioactivity studies have demonstrated that the above oligomer forms derived from agar or agarose exhibit a 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 (abbreviated as N2) was reported to possess moisturizing and whitening effects on melanoma cells.[7] Neoagarotetraose (abbreviated as N4), derived from porphyran, was reported to be utilized in vitro by intestinal bacteria, which stimulated the growth of *Bacteroides*, as well as *Eubacterium* and *Lactobacillus*.[8] Recently, the prebiotic effectiveness of NAOS with DP 4–12 has been confirmed both in vivo and in vitro. They showed augmented growth of *Bifidobacterium* and *Lactobacillus*.[9] The AOS with DP 2–4 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.[10,11] The AOS, especially agarohexaose, (abbreviated as A6), could scavenge reactive oxygen species (ROS) generated by electron leakage and protect cells against apoptosis induced by ROS in a human liver cell L-02 system.[12] The potential of further applications of each different DP of the NAOS and AOS could be discovered if there are qualitative and quantitative methods established to prepare and determine the identity of individual oligomers.

The aim of this study was to develop methods for the preparation, separation, isolation, and production of oligosaccharides, both

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doi:10.1016/j.carres.2008.06.019

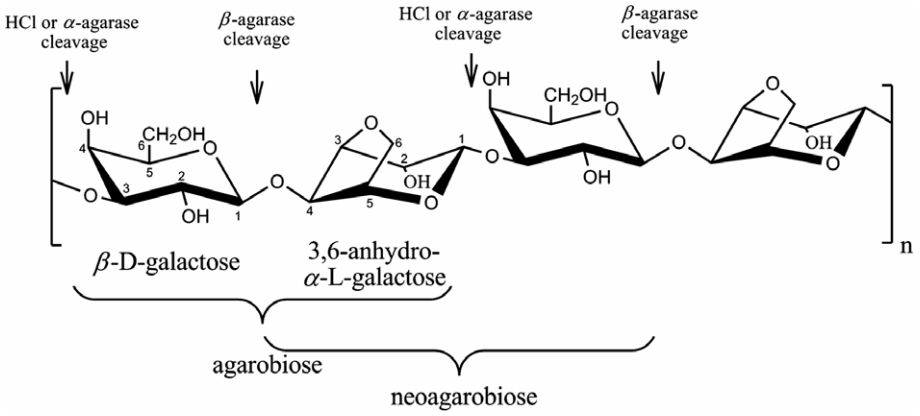

Figure 1. Structures of the agarose molecule and the two product types derived from HCl hydrolysis and digestion with agarases. The cleavage sites are indicated by arrows. The marked agarobiose represents the basic unit of the AOS released by HCl or α-agarase action. The marked neoagarobiose represents the basic unit of the NAOS products digested by β-agarase. The agarose structure was adapted from Lahaye et al. (1989).[1]

NAOS and AOS with different chain lengths, from agarose degraded by β-agarase and HCl, respectively. The length of the oligo-products in the crude mixtures was determined by two high-performance liquid chromatography (HPLC) systems: multi-mode size-exclusion chromatography (SEC) and NH2-column chromatography (NH2-HPLC), coupled with an evaporative light-scattering detector (ELSD). Individual NAOS or AOS was then isolated by SEC or NH2-HPLC equipped with a fraction collector, whereby the separation performance could be inspected. Structures, molecular mass and purity of the isolated products were confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR) and electrospray-ionization mass spectrometry (ESIMS). 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 large quantity, making it possible to conduct further studies on their bioactivities.

2. Results

2.1. Establishing the calibrations of NAOS and AOS in two HPLC systems

Calibration curves (Fig. 2) constructed from retention time versus molecular mass of the Gal, malto-oligomers (DP 2–7), neoagarobiose (N2), neoagarohexaose (N6), and pullulan (5900 Da) standards were used to extrapolate into the DP of NAOS and AOS in the SEC and NH2-HPLC systems. Calibration curves (Inlet figures in Fig. 2) constructed from standard concentrations versus peak area were used for quantification of NAOS and AOS. In the SEC system (Fig. 2A), both the molecular mass and quantity signals of the malto- and neoagaro-oligomer standards with similar molecular size (maltose, 342 Da vs N2, 324 Da, and maltohexaose, 990 Da vs N6, 936 Da) responded in linear correlations with the retention time and sample concentration. Such linearity was extended to larger polymers when a pullulan size of 5900 Da was used. Therefore, determination of the approximate DP of oligomers in the SEC system throughout the study was extrapolated from the A line (Fig. 2A). Quantification of the amount of the identified oligomer peak was calculated based on the A′ regression line ($Y = 8087.7x + 741.6$; $R^2 = 0.9994$, Y, peak area and x, concentration) (Fig. 2A, inlet).

In the NH2-HPLC system (Fig. 2B), however, these malto- and neoagaro-oligomer standards with similar molecular size showed different correlation patterns. Since this column separation is

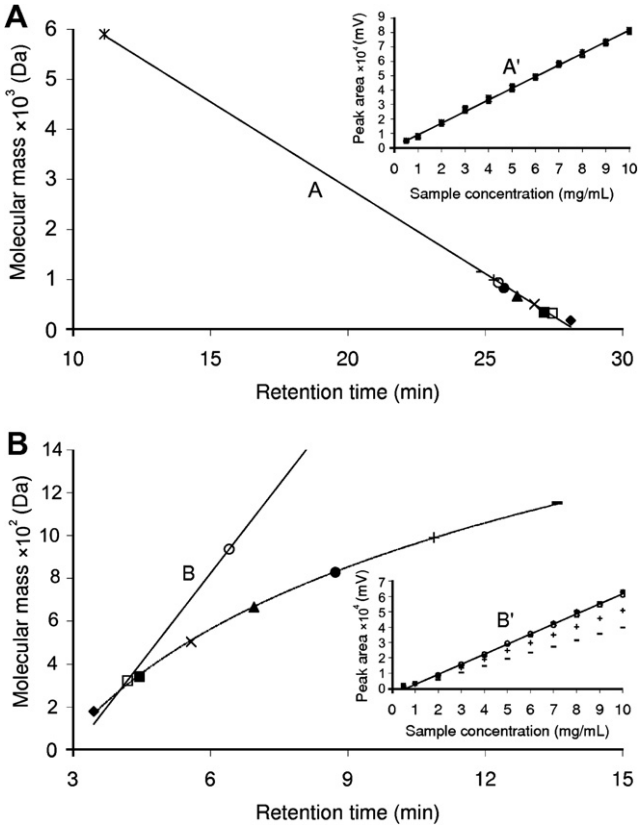

Figure 2. Calibration curves for calculating the DP and quantity of oligomer products separated in the SEC–HPLC (A) and NH2-HPLC (B) systems. Graphs were plotted by retention time versus molecular mass of the standards, and standard concentrations versus peak areas (inlets). Oligomer standards: (◆) galactose, (□) N2, (■) maltose, (×) maltotriose, (▲) maltotetrose, (●) maltopentose, (○) N6, (+) maltohexose, (−) maltoheptose, (∗) Pullulan 5900.

based on the interaction between the basic amino groups of the resin and the surface structure of the oligomer molecules, when compositions among similar sized malto-oligo and NAOS or AOS differ, these would interact differently. In Figure 2B, the less hydrophilic N2 and N6 are shown to elute earlier than their malto-oligo counterparts. The linear line drawn between the N2 and N6 (the B line) standards was deviated from the curved line drawn from the malto-oligostandards. In addition, the peak area also responded

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