Separation and quantification of neoagaro-oligosaccharides

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Revised: 31 May 2011 / Accepted: 28 June 2011 / Published online: 2 August 2011

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Abstract Oligosaccharides were obtained from agar by enzymatic hydrolysis. Activated carbon adsorption separation was used to extract oligosaccharides, and gel chromatography separation was applied to further purify oligosaccharides. The result showed that activated carbon adsorption could remove the most salt impurities, and gel column chromatography could give the separation of the two kinds of oligosaccharides. ESI-MS, ^{13}C -NMR revealed that the molecular weight (Mw) of two oligosaccharides were 630 and 936, which were identified as neoagarotetraose and neoagarohexaose respectively.

Keywords Neoagaro-oligosaccharides · Separation · Gel chromatography · Structure Identification

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 (Kazlowski et al. 2008). In recent years, bioactivity studies have demonstrated that the above oligomer forms derived from agar or agarose exhibit a variety of physiological activities. Neoagarobiose is a rare agent with moisturizing and whitening effects on melanoma

cells (Kobayashi et al. 1997). Wang et al. (2004). found that agar oligosaccharides (AOS) exhibited antioxidative activities in scavenging hydroxyl free radical, scavenging superoxide anion radical and inhibiting lipid peroxidation. Recently, the anti-tumor, anti-oxidation and immunological effectiveness of AOS has been confirmed both in vivo and in vitro (Wang et al. 2005). Chen et al. (2007). found that AOS possesses the anti-angiogenic effects, which are associated with apoptosis induction of endothelial cells. Neoagaro-oligosaccharides (NAOS) were reported with higher degrees of polymerization (DP) showed better prebiotic activity (Hu et al. 2006). Wu et al. (2008). confirmed NAOS with whitening effects on B16 murine melanoma cells, these results suggested that the NAOS can be considered as a non-cell-toxic whitening agent for the skin. 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 purpose of this study was to develop methods for the preparation and purification of oligosaccharides. Many methods have been reported for the preparation and purification of oligosaccharides, including: ethanol fractionation (Wang et al. 2004), activated carbon adsorption (Chen et al. 2005), gel chromatography (Rochas et al. 1986; Mao et al. 2001; Chen et al. 2004; Hu et al. 2006; Li et al. 2007). and high-performance liquid chromatography (HPLC) (Kazlowski et al. 2008). However the productions prepared through the methods of ethanol fractionation and activated carbon adsorption are not pure enough and the method of HPLC can only used for analyzing. The most commonly used method is gel chromatography, such as gel chromatography Bio-gel P2 (Rochas et al. 1986; Hu et al. 2006; Li et al. 2007), Superdex 30 (Mao et al. 2001), Sephadex G-10 (Chen et al. 2004), Sephadex G-25 (Chen

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Fig. 1 Pictures of three dry samples, (a) sample of hydrolyzed solution; (b) sample of hydrolyzed solution after activated carbon adsorbed (c) sample of mixture of oligosaccharides separated by activated carbon adsorption from hydrolyzed solution

et al. 2004). But to our knowledge gel chromatography Sephadex LH-20 has not reported for purification of NAOS before. The first step in this study was to prepare low-molecular-weight polysaccharide from agar, which was achieved by enzymatic hydrolysis, followed by activated carbon adsorption and gel Sephadex LH-20 chromatography purification. Then the obtained oligosaccharides were analyzed by TLC, ESI-MS, ^{13}C -NMR.

Materials and methods

Preparation of oligosaccharides by enzymolysis

A 2 g/L solution of agar in distilled water was hydrolyzed by an excess agarase (produced by *Paenibacillus* sp. WL, the CCTCC NO. was M2010049) for 2 h at 45°C, and the enzyme activity was stopped by heating the solution in boiling water for 10 min, and then the solution was centrifuged at 8,000×g for 10 min to remove the precipitation. The solution of oligosaccharides was obtained.

Extraction of oligosaccharides by activated carbon adsorbing

Each 20 ml solution of oligosaccharides containing 1 g activated carbon was whisked for 30 min, then centri-

fuged at 8,000×g for 15 min to separate activated carbon from the solution. Activated carbon was mixed with 10% alcohol (20 ml 10% alcohol per 1 g activated carbon) and whisked for 30 min, then centrifuged at 8,000×g for 15 min to separate activated carbon from the aqueous alcohol. The alcohol solution was concentrated and freeze-dried. Then the mixture of oligosaccharides was obtained.

Purification of oligosaccharides

The mixture of oligosaccharides was separated on a column (100×1 cm) of Sephadex LH-20 with distilled water as the eluant at a flow rate of 40 mL/h, the oligosaccharides in elution was detected by the classical phenol–H₂SO₄ method (Dubois et al. 1956), and the oligosaccharide eluates recovered were concentrated in *vacua* and freeze-dried.

The mixture of oligosaccharides was separated on a column (100×1 cm) of Sephadex G-25 with distilled water as the eluant at a flow rate of 40 mL/h, the oligosaccharides in elution was detected by the classical phenol–H₂SO₄ method.

Thin layer chromatography (TLC) analysis of oligosaccharides

The purified oligosaccharides were analyzed by TLC with a solvent of n-butanol–acetic acid–water (2:1:1) on silica gel

Fig. 2 Chromatogram of oligosaccharides separated by Sephadex LH-20, two kinds of oligosaccharides were obtained, marked oligosaccharide-A and oligosaccharide-B

Fig. 3 Chromatogram of oligosaccharides separated by Sephadex G-25

Fig. 4 Thin layer chromatography (TLC) of oligosaccharide-A (a) and oligosaccharide-B (b), 1 to 5 were different concentration of the same sample

plate, and visualized by spraying with thymol reagent (Duckworth and Yaphe 1970).

Fig. 5 ESI-MS spectrum of oligosaccharide-A

Fig. 6 ESI-MS spectrum of oligosaccharide-B

ESI-MS analysis of oligosaccharides

Mass spectrometer: Agilent 6210 TOF LC/MS (Agilent, America), ionization source: electrospray-ionization (ESI).

¹³C-NMR analysis of oligosaccharides

Samples (20 mg) were dissolved in D₂O (0.5 mL) in 5 mm NMR tubes. Spectra were recorded on a AVANCE III

Fig. 7 ^{13}C -NMR spectrum of oligosaccharide-A, Resonances at about 97 and 93 ppm correspond to neoagaro-oligosaccharides. G represents the 3-O-linked β -D-galactopyranose; A represents the 4-O-linked 3,6-anhydro- α -L-galactopyranose; r and nr denote residues at the reducing and non-reducing end, respectively; a/b for anomer

500 MHz (Bruker, Switzerland) apparatus set at 500 MHz with 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as the internal standard.

Results and discussion

Separation and quantification of neoagaro-oligosaccharides

The hydrolyzed solution contains salts and oligosaccharides (Fig. 1a). Activated carbon adsorption separation can remove the most salt impurities (Fig. 1b). Oligosaccharides (Fig. 1c) can be extracted after adsorbing activated carbon from the hydrolyzed solution, while the salts still remained in the hydrolyzed solution

The mixture of oligosaccharides was purified with a Sephadex LH-20 column. As shown in Fig. 2, Two kinds of oligosaccharides were obtained, marked oligosaccharide-A and oligosaccharide-B. As shown in Fig. 3, the mixture of oligosaccharides could not be separated by Sephadex G-25. In the paper, Sephadex LH-20 was used to purify the oligomers for the first time. Sephadex LH-20 is obtained

by Sephadex G-25 cross-linked with hydroxypropyl, and is extensively used to separate low Mw natural product (Si et al. 2011). In this case, Sephadex LH-20 was much better than Sephadex G-25 in separation of neoagaro-oligosaccharides at the same condition (Figs. 2 and 3), which probably due to the mechanism of Sephadex LH-20 separated neoagaro-oligosaccharides was not only size exclusion chromatography, but also adsorption chromatography (Chen 2003). Then oligosaccharide-A and oligosaccharide-B shown in Fig. 4 were analyzed by TLC. The R_f of oligosaccharide-A was 0.5, the R_f of oligosaccharide-B was 0.35.

As shown in Fig. 5, ion at m/z 629.2 was the quasi-molecular ion $[\text{M}-\text{H}]^-$ of oligosaccharide-A. And the molecular weight (Mw) of oligosaccharide-A was 630. With the result of ^{13}C -NMR we know ion at m/z 485.2 was the ion $[\text{M}-\text{A}-\text{H}]^-$ of neoagarotetraose. As shown in Fig. 6, ion at m/z 935.3 was the quasi-molecular ion $[\text{M}-\text{H}]^-$ of oligosaccharide-B. And the Mw of oligosaccharide-B was 936. With the result of ^{13}C -NMR we know ion at m/z 791 was the ion $[\text{M}-\text{A}-\text{H}]^-$, ion at m/z 629 was the ion $[\text{M}-\text{A}-\text{G}-\text{H}]^-$, ion at m/z 485 was the ion $[\text{M}-\text{A}-\text{G}-\text{A}-\text{H}]^-$ of neoagarohexaose.

Fig. 8 ^{13}C -NMR spectrum of oligosaccharide-B, Resonances at about 97 and 93 ppm correspond to neoagaro-oligosaccharides. G represents the 3-O-linked β -D-galactopyranose; A represents the 4-O-linked 3,6-anhydro- α -L-galactopyranose; r and nr denote residues at the reducing and non-reducing end, respectively; a/b for anomer

¹³C nuclear magnetic resonance spectroscopy (¹³C-NMR)

The ¹³C-NMR spectrum of oligosaccharide-A and oligosaccharide-B showed the typical pattern for neoagaro-oligosaccharides (Figs. 7 and 8). Resonances at about 97 and 93 ppm were characteristic of β and α anomeric forms of galactose residues at the reducing end of the neoagaro-oligosaccharides, respectively (Rochas et al. 1986). The signal at 90.72 ppm, characteristic of agaro-oligosaccharides, the main products of α-agarase degradation of agarose was not found (Rochas et al. 1994; Li et al. 2007). The spectra for the oligosaccharide-A and oligosaccharide-B showed similar patterns, but with different intensities. This ¹³C-NMR and ESI-MS indicates that oligosaccharide-A was neoagarotetraose, oligosaccharide-B was neoagarohexaose.

Conclusion

The study has established feasible approaches for the preparation of neoagaro-oligosaccharides by agarase digestion. Activated carbon adsorption separation can remove most salt impurities, and there is no need to purify agarase first. This method can also be used to extract oligosaccharides from agarose solution hydrolyzed by acid. Gel chromatography of Sephadex LH-20 could purify neoagaro-oligosaccharides. The data of ¹³C-NMR and ESI-MS proved that oligosaccharide separated from agar enzymolysis were neoagarotetraose and neoagarohexaose. The results suggest that agarase used in this study was β-agarase.

Acknowledgements This work was supported by the financial support from the Science and Technology Department of Zhejiang Province (2009C14027)

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