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Product monitoring and quantitation of oligosaccharides composition in agar hydrolysates by precolumn labeling HPLC

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Abstract

Since the discovery of multiple bioactivities for agarobiose oligomers, a quantitative method has been in great need to monitor the agarobiose oligomers. This report demonstrates that agarobiose oligomers can be separated with high resolution in HPLC after introducing α-naphthylamine into compounds. Agarobiose oligomers ranged from biose to decaose were isolated by Sephadex column. HPLC analysis indicated that each oligomer could be quantified with good linearity and a low detection limit of 0.1–4 μg/ml. The chromatographic profiles of agaro-oligosaccharides with different hydrolysis modes (hydrochloride, citric acid, solid acid, and hydroxyl radical degradation) showed that agarobiose could be obtained more than 57.8% using solid acid mediated hydrolysis, while hydrochloride acid could degrade agar into a series of agaro-oligosaccharides from biose to decaose. The yield of oligosaccharides was low if hydrolyzed by citric acid. The Fenton degradation can increase the speed of hydrolysis, but the product was complex.

Keywords: Agarobiose oligomers; HPLC; α-Naphthylamine; Agaro-oligosaccharides

1. Introduction

Agaro-oligosaccharides are linear molecules built of 1,4-linked 3,6-anhydro-α-L-galactose alternating with 1,3-linked β-D-galactopyranose, including neoagaro series and agaro series (Fig. 1) [1]. Generally, β-agarase (the main agarase isolated from marine bacteria) can cleave β-1,4 bond into neoagaro-oligosaccharides, while acid can cleave the α-1,3 bond into agaro-oligosaccharides [2]. In recent years, bioactivity studies demonstrated that neoagaro-oligosaccharides showed almost no physiological functions. In contrast, the agaro-oligosaccharides exhibited a variety of physiological activities, and the activities are correlated to the degree of polymerization (DP) [3]. The agaro-oligosaccharides 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 [4]. The oligosaccharides with DP 6–8 can elicit physiological response in algae [5]. Therefore, these oligosaccharides could prevent death from endotoxin shock, as well as exhibit anti-cancer and anti-inflammatory effect. Due to their pharmaceutical and agrochemical importance, various hydrolysis protocols have been developed to obtain agaro-oligosaccharides with different DPs. The establishment of a sensitive quantitative analysis for these oligosaccharides is essential not only for monitoring hydrolysis process but also for furthergoing biochemical research of each oligosaccharide.

HPLC has played an important role in carbohydrate analysis. RI detectors are used for underivatized sugar detection, but they are not sensitive enough. Therefore, a highly sensitive and selective method is required for the analysis of agaro-oliogosaccharides. Recently, there have been numerous reports on the use of fluorescent reagents (such as 1-dimethylaminonaphthalene-5-sulfonyl chloride [6], 4-dimethylaminoazobenzene-4′-sulfonyl chloride [7], 2-aminopyridine [8], and *p*-aminobenzoic ethyl ester [9], etc.) to derivatize the saccharides at their reducing ends.

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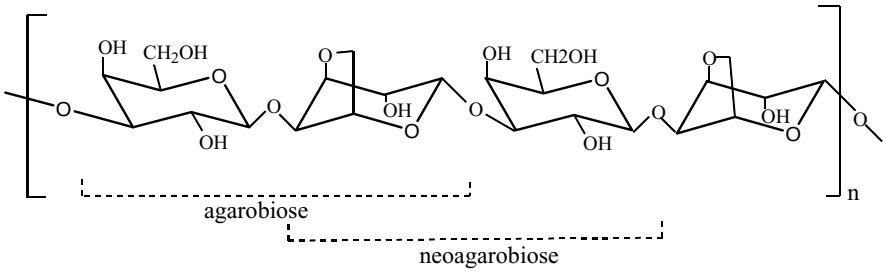

Fig. 1. The structure of agaro-oligosaccharide.

These reagents can improve the detective sensitivity remarkably, however, most of these methods are used for qualitative analysis, only Chaturvedi et al. [10] established a quantitative method for milk oligosaccharides by perbenzoylating those oligosaccharides.

In this work, a pre-column derivatization HPLC method for measuring agarose-derived oligosaccharides was described. We also showed that various agaro-oligosaccharides profiles with different hydrolysis modes could be analyzed using the method described here.

2. Experimental

2.1. Materials

Agar, citric acid, ascorbic acid, α-naphthylamine were all of analytical grade and commercially available. GF254 silica sheets were purchased from Merck Co. (Darmstadt, Germany). Dowex 50 W × 2 cation ion exchange resin and sodium cyanoborohydride were obtained from Sigma Co. (St. Louis, MO, USA).

2.2. The degradation of agar

Four hydrolysis protocols were carried out. (1) Inorganic acid (HCl) degradation: HCl was added into solution containing 1.5% melted agar every 1 h at 50 °C for 6 h. The final concentration of HCl was 0.4 mol/L. (2) Organic acid (Citric acid) degradation: 1.5% agar particles were dissolved in solution containing 0.5 mol/L citric acid at 90 °C for 4 h. (3) Solid acid mediated (cationic exchange resin) degradation: 1.5% agar particles were suspended in water containing 20% cationic exchange resin, holding the solution at 90 °C for 6 h [4]. (4) Fenton degradation or hydroxyl radical degradation: 1.5% melted agar was dissolved in 0.1 mol/L phosphate buffer (pH 7.3), containing 1 mmol/L $FeSO_4{\cdot}7H_2O$, 0.02 mol/L ascorbic acid, and 0.1 mol/L H_2O_2. The reaction was allowed to proceed at 50 °C for 2 h, then terminated by adding catalase [11].

2.3. Purification of agaro-oligosaccharides

Freeze-dried hydrolyzate (0.5 g) was dissolved in 1 ml distilled water. The syrup was loaded onto Sephadex G 25 column (2.6 cm × 100 cm) and eluted with water. The oligosaccharide composition in the eluted fractions (5 ml each) was monitored using thin-layer chromatography, successively, which had been developed by *n*-butanol/ethanol/water at a ratio of 3:2:2 on silica gel plate, and visualized by spraying with thymol reagent [12]. The fractions with DP 2–4 were pooled and re-chromatographed on Sephadex G 10 column (1.0 cm × 50 cm) to obtain the purified biose and tetraose, respectively, while fractions with DP 6–8 were re-chromatographed on Sephadex G 25 column (1.6 cm × 100 cm) to obtain purified hexose and octaose. Each purified agaro-oligosaccharide was lyophilized and will be further used as authentic compound for quantitative analysis.

2.4. Derivatization of agaro-oligosaccharides

Oligosaccharides were derivatized with α-naphthylamine [13]. Briefly, 1 mg oligosaccharide sample was dissolved in 10 μl water, then mixed with 40 μl derivatization reagent and incubated at 80 °C for 30 min. After cooling, the solution was partitioned with chloroform and water. The water phase was used for HPLC analysis. The derivatization reagent was obtained by dissolving 2 mol/L α-naphthylamine, 1.2 mol/L $NaBH_3CN$ in acetic acid/methanol (1:9).

2.5. HPLC analysis and quantification

HPLC analysis were performed on a Waters Millenium HPLC system composed of two waters 515 pumps with a Waters 2996 photodiode array detector. Prior to HPLC, all solvents were degassed. The agarose-derived oligosaccharides were analyzed in isocratic mode on a Waters symmetry C_8 column RP (150 mm × 3.9 mm, 5 μm) at a flow rate of 0.5 ml/min with the mobile phase of 40% MeOH in distilled water. The PDA detection of the effluent was carried out at 243 nm. The peak areas of pure agaro-oligosaccharides and oligosaccharides in crude hydrolysates were calculated automatically when baseline correction was appropriate.

3. Results and discussion

After derivatization, the naphthylamine moiety at the reducing end enhanced the detective sensitivity of HPLC

Table 1
The retention time of agaro-oligosaccharides under different elution conditions

Oligosaccharide	Gradient elution (min)[a]	Isocratic elution (min)			
		MeOH:H_2O			
		0.25:0.25	0.23:0.27	0.15:0.35	0.20:030
Agarbiose	24.19	8.68	11.56	27.56	16.83
Agartetraose	23.37	7.66	10.19	23.87	14.75
Agarhexaose	22.54	6.82	9.04	19.53	12.89
Agarooctaose	21.83	6.16	8.10	16.21	11.35
Agarodecaose	21.26	5.56	7.31	14.06	10.13

[a] A linear gradient of 20–80% methanol over 40 min.

remarkably. Although the derivative showed an absorbance peak around 327 nm in the PDA spectrum, however, maximum absorbance appears at 243 nm, which was chosen as the detection wavelength in following analysis to increase the sensitivity. Several eluting protocols were compared (Table 1): when a linear gradient elution from 20 to 80% MeOH was initially selected as the mobile phase, long retention time was resulted, most peaks were eluted between 20 and 30 min; when the mobile phase was changed to isocratic mode of 50% MeOH, the chromatogram showed that peaks were appeared too early, and most of them were eluted in 10 min. We also examined 27% MeOH, 15% MeOH, and 40% MeOH as mobile phases. Finally, the isocratic 40% aqueous methanol was chosen for all the following analysis based on peak separation efficiency and economic running time. Under this condition, agaro-oligosaccharides with DP 2–10 had an excellent peak resolution affordable for quantitation. Fig. 2 is an example of hydrolysate HPLC chromatogram, which was degraded by 10% solid acid at 70 °C for 6 h. Under this condition, a garo-oligosaccharides with DP from 2 to 10 were separated completely, and the retention time of biose to decaose was 16.83, 14.75, 12.89, 11.35, and 10.13 min, respectively, while the retention time of α-naphthylamine, the fluorescent reagent was 25.15 min, which was eluted more slowly than oligosaccharide derivatives because of its low polarity. The percentage of agarobiose in this hydrolysis mode was rather high. The complete separation allows us for further quantitative analysis. Actually, up to DP = 22 oligosaccharide can be clearly seen in the chromatogram.

However, because α-naphthylamine was only linked to the reducing end of sugar, the mass of oligosacchrides with different DPs is not proportional to their peak areas. We also observed that various oligosaccharides with the same content exhibited different peak areas. Agarbiose showed highest peak area, and the area would decrease with the increasing of DPs. Thus, for the purpose of quantification, the agaro-oligosaccharides from biose to decaose need to be purified, and the relationship between the mass and peak area of each saccharide needs to be investigated.

The saccharides mixture in hydrolysate was separated and purified by multiple gel permeation chromatography on Sephadex G 25 and Sephadex G 10 columns. The purified agaro-biose, tetraose, hexaose, octaose, and decaose were detected by TLC (Fig. 3). Each saccharide was homogeneous. According to our knowledge, agarooctaose and agarodecaose were purified for the first time. Then these oligosaccharides were derivatized by α-naphthylamine for HPLC analysis.

Although fluoresamines have been utilized for UV or fluorescence detection, the fluorescence intensity will decrease

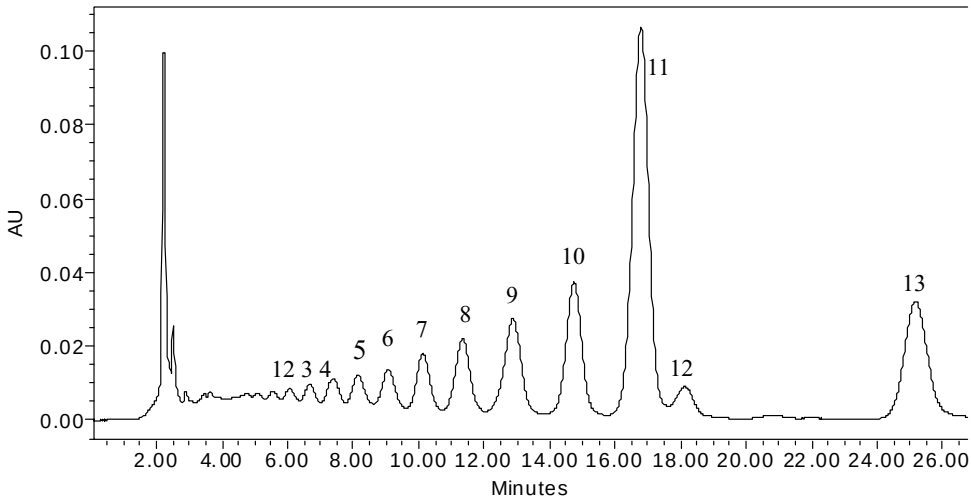

Fig. 2. HPLC separation of α-naphthylamine-labeled agaro-oligosaccharides mixture. Hydrolysis condition: 1.5% agar was hydrolyzed by 10% solid acid at 70 °C for 6 h. Sample was applied to a Waters C_8 RP column with the mobile phase of MeOH:H_2O = 2:3 (v/v) at a flow rate of 0.5 ml/min. Peaks of oligosaccharides: (1) DP = 22; (2) DP = 20; (3) DP = 18; (4) DP = 16; (5) DP = 14; (6) DP = 12; (7) DP = 10; (8) DP = 8; (9) DP = 6; (10) DP = 4; (11) DP = 2; (12) monosaccharide; (13) α-naphthylamine. Note: each oligosacchride is eluted with the degree of polymerization; "11", the biose, is the major sugar component in the hydrolysate, "13", the labeling reagent eluted far late than the labeled oligosaccharide does not interfere in this analysis protocol.

Fig. 3. TLC of purified agaro-oligosaccharides. From left to right: biose, tetraose, hexaose, octaose, decaose, respectively. These purified oligosaccharides are further used as authentic compounds for HPLC quantitation.

after a few hours for some fluoresamines, and the precision and sensitivity of UV detection will decrease too. We examined the stability of the α-naphthylamine derivatives, and found it was stable for at least half day (25 °C).

For the results of quantitative analysis using various amounts of purified agaro-oligosaccharides, the relationship between the peak areas and the contents for each oligosaccharide was linear. The regression equations of the contents and peak areas of these sugars were summarized in Table 2. Each equation for all agarobiose polymers except agarotetraose had high correlation coefficient of greater than 0.999, indicating that they have good linearity. The detection limits at the signal-to-noise ratio of 3 for these saccharides were about 0.1–4 μg/ml from biose to decaose. The limits increased with the increase of the DP of oligosaccharides. It was shown that agarobiose derivative had highest sensitivity. The effect of oligosaccharide chain on the molar absorbance of chromophore will be further considered later.

Table 2
The regression equations of the contents and peak areas of agaro-oligosaccharides

Oligosaccharide	Equation	r^a	Detection limit (μg/ml)
Agarobiose	$y^b = 52.59x^c - 0.18$	0.999	0.184
Agarotetraose	$y = 11.95x - 0.13$	0.997	1.046
Agarohexaose	$y = 4.37x + 0.12$	0.999	1.689
Agarooctaose	$y = 4.90x - 1.75$	0.999	3.303
Agarodecaose	$y = 1.62x - 0.30$	0.999	4.337

[a] Correlation coefficient.
[b] y, peak area $\times 10^{-6}$ (UV $\times$ s $\times 10^{-6}$).
[c] x, the concentration of agaro-oligosaccharide (mg/ml).

These equations were used to calculate the amounts of oligosaccharides in hydrolysates described below.

Four hydrolysates from different protocols were investigated by HPLC using the method described above (Fig. 4).

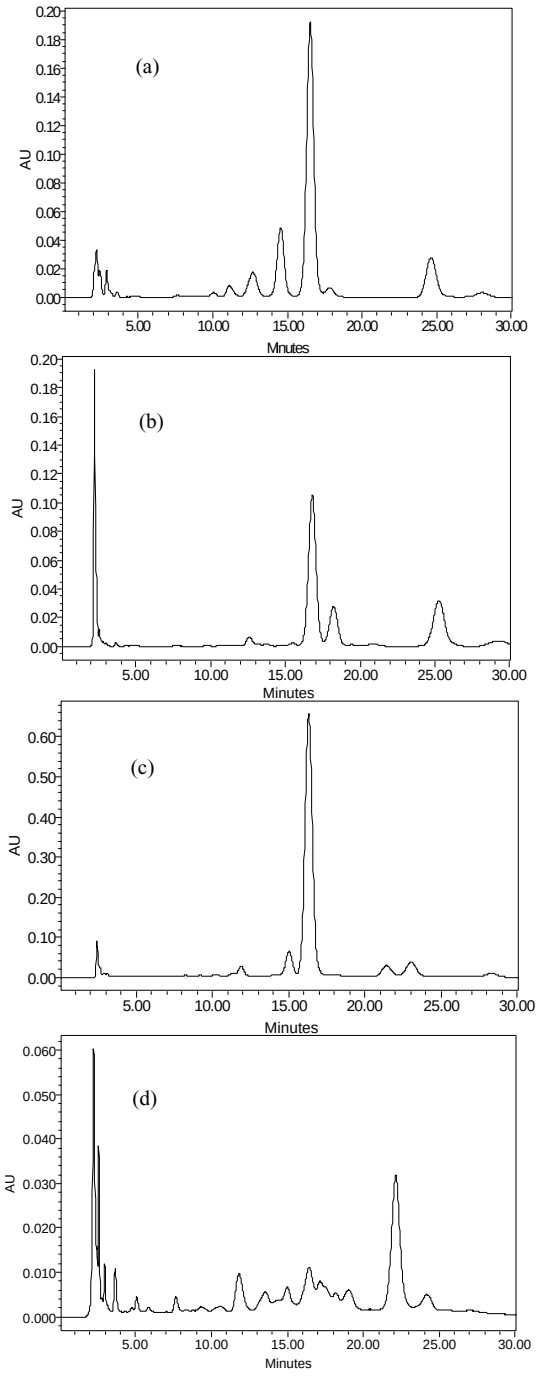

Fig. 4. HPLC separation of four kinds of hydrolysates derivatives. (a) HCl degradation, 1.5% agar, 0.4 M HCl, 50 °C for 6 h. (b) Citric acid degradation, 1.5% agar, 0.5 M citric acid, 90 °C for 4 h. (c) Solid acid degradation, 1.5% agar, 20% solid acid, 90 °C for 6 h. (d) Fenton degradation, 1.5% agar, 1 mM $FeSO_4 \cdot 7H_2O$, 0.02 M Vc, and 0.1 M H_2O_2, 50 °C for 2 h. Note: Only the results under optimized hydrolysis conditions are presented here. Chromatographic conditions are the same as described in Fig. 2.

Table 3
The yield of agaro-oligosaccharides and percentage of agarobiose in four modes of hydrolysates

	HCl	Citric acid	Solid acid	Fenton
Agaro-oligosaccharide (%)[a]	16	5	33.2	4.15
Agarobiose (%)[b]	24.9	89	57.8	5.1

[a] The yield of agaro-oligosaccharides is described as the ratio of oligosaacharide (DP $\leq$ 10) to the total agar input.
[b] The percentage of agarobiose described as the percentage of the content of agarobiose related to the total oligosaccharides (DP from 2 to 10).

All of the hydrolysis conditions utilized here were the optimal conditions to obtain agarobiose (data not shown). Table 3 showed the yields of oliogsaccharides of the four products and the percentage of agarobiose in all oligosaccharides. Data analysis showed that the highest yield of 33.2% was recorded in solid acid mediated hydrolysate. HCl could degrade agar into a wide range of oligosaccharides from biose to decaose with yield of 16%. However, the hydrolysate from citric acid degradation had low yield of oligosaccharides, and a small amount of monosaccharide was also found in the degradation. Under Fenton hydrolysis mode, saccharides were oxidated thoroughly, which resulting in a complex product mixture. Quantitative analysis of the agarobiose fraction in the four hydrolysis modes revealed that citric acid degradation could obtain high percentage of agarobiose with 89%, and solid acid mediated degradation accounted for 57.8%. In general, these results indicated that solid acid mediated hydrolysis mode was a suitable way to obtain agaro-oligosaccharides, especially agarobiose.

In conclusion, a pre-column derivatization HPLC-based assay for measuring agarose-derived oligosaccharides is described and various hydrolysis/cleavage protocols are also compared. Basically, sugar mixtures are derivatized by reductive amination with a naphthylamine group and then subjected to reverse-phase chromatography under isocratic conditions. α-Naphthylamine is a fluorescent reagent. In this study, we used UV to detect it. It can be anticipated that the analytical sensitivity could be greatly improved with fluorescent detector. The methodology seems useful and simple overall for sugars ranging in size from 2 to 12 monomers.

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References

[1] M. Lahaye, W. Yaphe, Carbohydr. Res. 190 (1989) 249–265.
[2] A. Karlsson, S.K. Singh, Carbohydr. Polym. 38 (1995) 7–15.
[3] I. Kato, T. Enoki, H. Sagawa, Food Dev. 36 (2001) 65–71.
[4] T. Enoki, H. Sagawa, T. Tominaga, E. Nishiyama, N. Koyama, T. Sakai, F.G. Yu, K. Ikai, I. Kato, US Patent, 0105029 A1 (2003).
[5] F. Weinberger, G. Dohnert, B. Kloareg, P. Potin, Chembiochemistry 3 (2002) 1260–1263.
[6] J.S. Sawada, Y. Umeda, A. Kondo, I. Kato, Anal. Biochem. 207 (1992) 203–207.
[7] H. Kwon, J. Kim, Anal. Biochem. 215 (1993) 243–252.
[8] T.J. Amiss, K.L. Tyczkowska, D.P. Aucoin, J. Chromatogr. 526 (1990) 375–382.
[9] V. Stocchi, F. Palma, G. Piccoli, B. Biagiarelli, L. Cucchiarini, M. Magnani, J. Liquid Chromatogr. B 17 (1994) 347–357.
[10] P. Chaturvedi, C.D. Warren, G.M. Ruiz-Palacios, L.K. Pickering, D.S. Newburg, Anal. Biochem. 251 (1997) 89–97.
[11] H.M. Chen, X.J. Yan, L. Zheng, W.W. Zhang, W. Lin, J. Zheng, I. Zhou, Tec. 24 (2003) 41–44.
[12] M. Duckworth, W. Yaphe, J. Chromatogr. 49 (1970) 482–487.
[13] W.T. Wang, Q.J. Chen, W.G. Chai, Chin. Biochem. J. 1 (1988) 116–123.