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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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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.

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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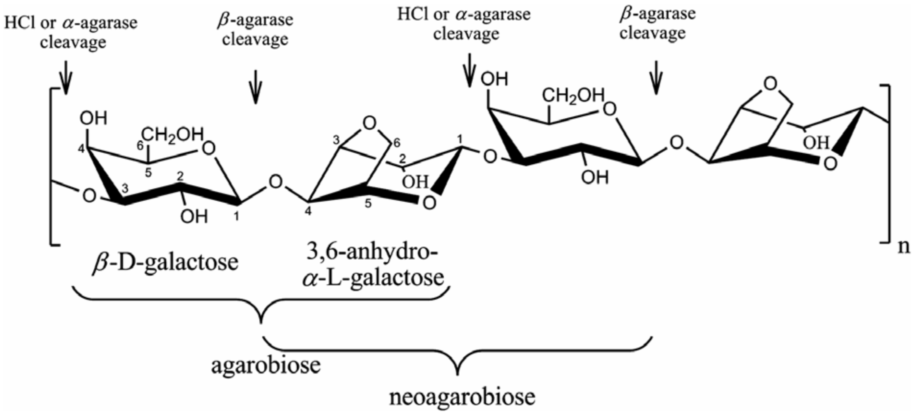

Figure 1. Structures of the agarose molecule and the two product types derived from HCl hydrolysis and digestion with agarases. The cleavage sites are indicated by arrows. The marked agarobiose represents the basic unit of the AOS released by HCl or α-agarase action. The marked neoagarobiose represents 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.

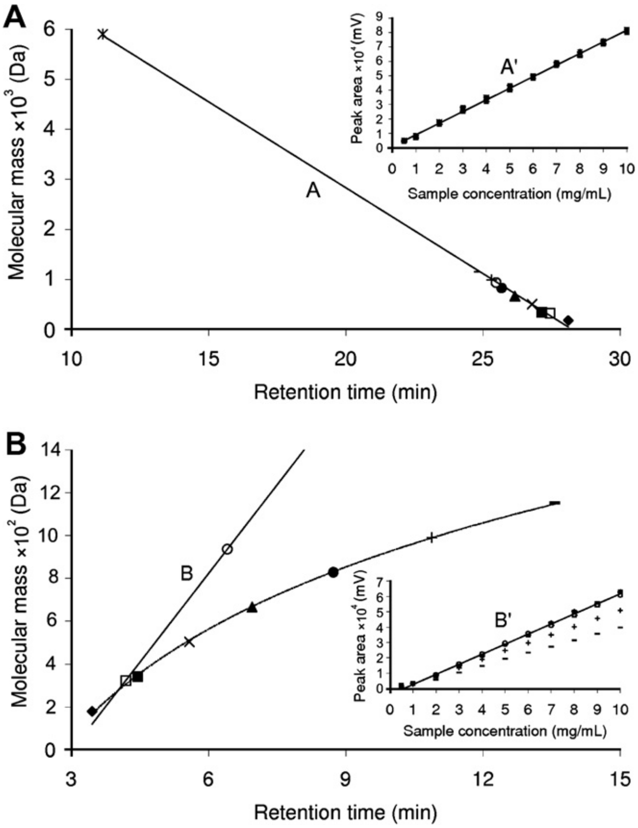

Figure 2. Calibration curves for calculating the DP and quantity of oligomer products separated in the SEC–HPLC (A) and NH2-HPLC (B) systems. Graphs were plotted by retention time versus molecular mass of the standards, and standard concentrations versus peak areas (inlets). Oligomer standards: (◆) galactose,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

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 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 differently in the NH2-system when a different standard was used, even though the correlation lines appear linear. It showed similar peak areas for the size of the Gal, DP 2–5 malto-oligomer, and N2, N6 standards when using the same concentrations (the B′ line). Under the same concentration of maltohexaose and maltoheptose, higher peak areas were detected for maltohexaose than heptoses. In this regard, the identification for the approximate DP of the oligomer peak analyzed in the NH2-HPLC system was extrapolated from the B line, and their quantification were calculated according to the B′ regression line ($Y = 6569.5x - 3989$; $R^2 = 0.9997$; Y, peak area and x, concentration) (Fig. 2B inset). Furthermore, for accurate identification of the Gal, the DP 2 and DP 6 peaks of the expected NAOS and AOS products, these were compared with the corresponding exact retention time from the Gal, N2, and N6 commercial standards in both HPLC systems (data not shown).

2.2. Optimization and determining the DP of the NAOS products from β-agarase digestion

β-Agarase reactions were optimized to generate a spectrum of saccharide oligomers with various DPs. A progressive downsizing of agarose into groups of oligomer population of different size ranges in the crude product was clearly shown when a larger amount of β-agarase was used (Fig. 3A, a–d). The reaction yields for 3, 4, 5, and 6 U/mg were DP 1–24, 45.3%; DP 1–22, 52.7%; DP 1–10, 57.3%; and DP 1–6, 66.6%, respectively. The best β-agarase reaction conditions, 4 U/mg (Fig. 3A, b), was chosen for further analysis in which the SEC–ELSD peak profile resolved the presence of a wide product range of DP 1–22, where DP 4–16 were clearly identified, and larger DP 18–22 products were generated. When the 4 U/mg crude products were analyzed by NH2-HPLC–ELSD, apparent individual peak resolution for monomer Gal and 3,6-AG, and individual N2, N4, N6, neoagarooctaose (N8), neoagarodecaose (N10), neoagarododecaose (N12) products were achieved. However, oligo-product sizes larger than DP 12 were excluded by the system (Fig. 3B).

2.3. Preparation of NAOS with defined DP

For further detailed determination, separation and isolation of individual monomer and oligomers, two independent approaches, SEC and NH2-HPLC, each equipped with a fraction collector, were performed. The size and homogeneity in those individually collected and pooled fractions were inspected by re-injecting into the same systems while connected with the ELSD (Fig. 4A and B). The aligned profiling of each collected NAOS in SEC exhibited moderate peak separation between the 3,6-AG and Gal monomers, and

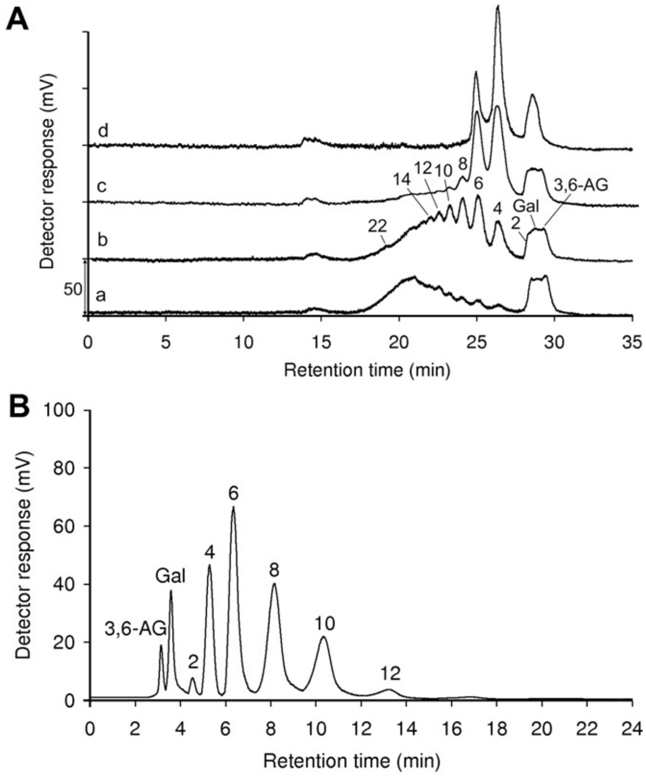

Figure 3. HPLC chromatograms of NAOS crude product mixtures generated by different units of agarase cleavage on agarose. (A) SEC–HPLC system: (a) 3 U/mg, (b) 4 U/mg, (c) 5 U/mg, (d) 6 U/mg. The 0.05% (w/v) melted agarose substrate solution was added with β-agarase and incubated at 40 °C for 24 h. Column, Asahipak GS-320 HQ multi-mode column (300 × 7.6 mm); column temperature, 30 °C; mobile phase, water; flow-rate: 0.4 mL/min; ELSD detector nebulizer temperature, 80 °C. (B) NH2-HPLC system: 4 U/mg agarose. Column, Asahipak NH2P-50 4E multi-mode column (250 × 4.6 mm); column temperature, 40 °C; mobile phase, 65:35 MeCN–water; flow-rate, 1 mL/min; detector nebulizer temperature, 80 °C. Peak identification: 3,6-AG, 3,6-anhydrogalactose; Gal, galactose; 2–22, NAOS with different DP. The labeled DP numbers are the approximate sizes extrapolated from the retention time versus molecular mass standard curves (Fig. 2).

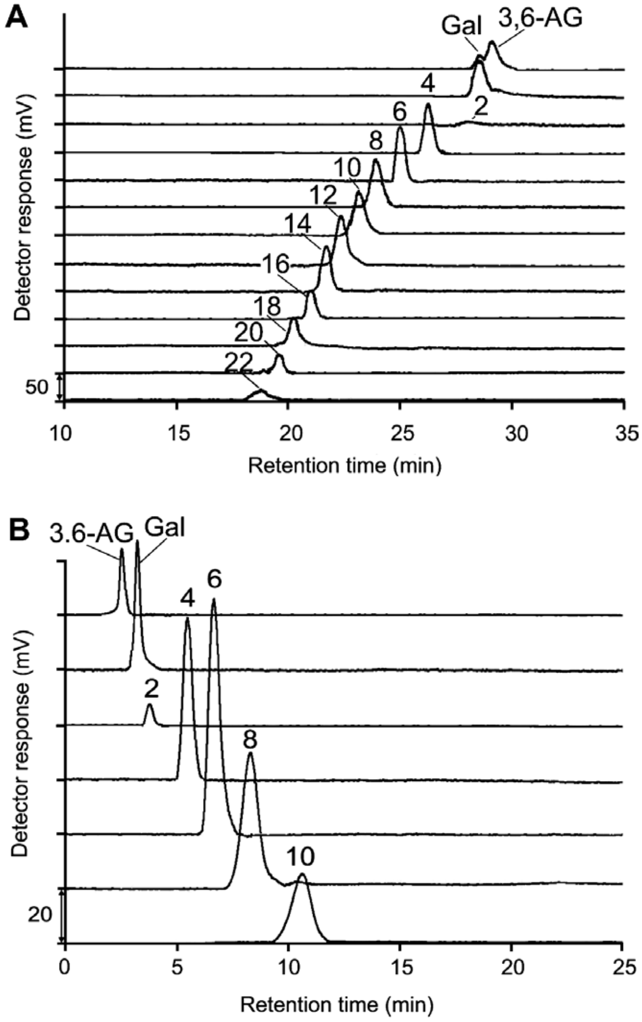

Figure 4. HPLC inspection of the isolated NAOS fractions. Each isolated fraction was re-injected into the same HPLC system and ELSD detected, from which their chromatograms were aligned. (A) SEC–HPLC system; (B) NH2-HPLC system. The chromatographic conditions were the same as in Figure 3.

Table 1
The isolated oligomer product yields from the SEC- and NH2-HPLC systems

Products	NAOS[a]	NAOS[b]	AOS[a]	AOS[b]
3,6-AG		3.4		4.9
Gal	12.8[c]	6.8	10.7[c]	10.2
DP2	0.7	1.2	27.6	30.3
DP4	8.9	9.0	16.9	20.7
DP6	9.8	13.0	12.4	11.0
DP8	12.8	8.5	6.2	8.3
DP10	8.3	7.0	4.5	4.6
DP12	8.5	—	2.8	—
DP14	8.0	—	1.8	—
DP16	5.1	—	—	—
DP18	5.2	—	—	—
DP20	3.0	—	—	—
DP22	1.6	—	—	—
Production yield (%)	84.7	48.9	82.9	90.0

[a] Products prepared by SEC–HPLC system.
[b] Products prepared by NH2-HPLC system.
[c] 3,6-AG and Gal.

finely resolved oligomers from DP 2 to DP 22 (Fig. 4A). The product yield of a particular isolated NAOS was calculated from calibration line A′ (Fig. 2A), and total product yield of these isolated DP 1–22 NAOS by SEC thus was 84.7% (Table 1). On the other hand, NAOS crude products isolated by NH2-HPLC showed distinct resolution among 3,6-AG, Gal, N2, N4, N6, N8, and N10 (Fig. 4B). The product yield of a particular isolated NAOS was calculated from calibration line B′ (Fig. 2B), and total product yield of these isolated DP 1–10 NAOS by NH2 chromatography thus was 48.9% (Table 1).

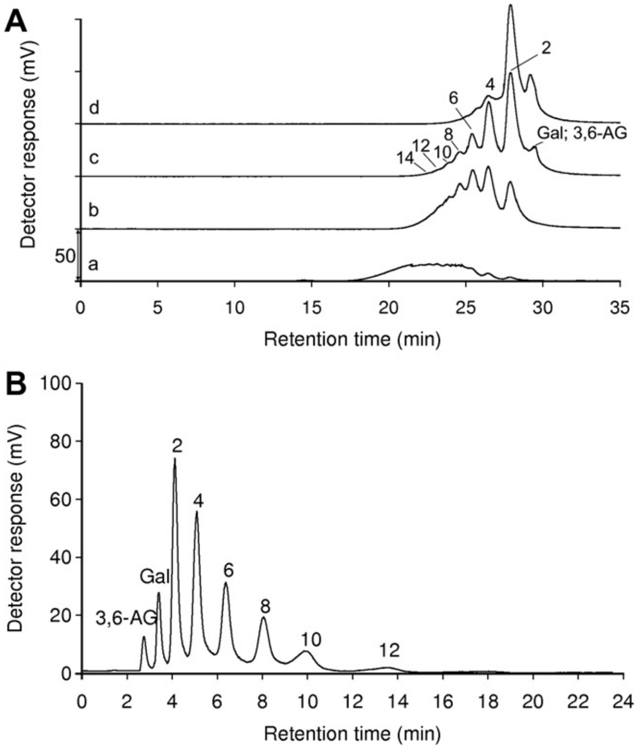

Figure 5. HPLC chromatograms of AOS crude product mixtures derived from HCl hydrolysis under different concentrations. (A) SEC–HPLC system: (a) 0.1 M HCl, (b) 0.2 M HCl (c) 0.4 M HCl, (d) 0.8 M HCl; (B) NH2-HPLC system separation of the 0.4 M HCl lysate. Each set of conditions used 1.5% agarose to react at 50 °C for 6 h. The chromatographic conditions were the same as those in Figure 3. Peak identification 1, 3,6-anhydrogalactose and galactose; 2–22, AOS with different DP. DP numbers of oligomers were extrapolated from the retention time versus molecular mass standard curves (Fig. 2).

2.4. Optimization and determining the DP of the AOS products from HCl hydrolysis

A comparison of the crude AOS product mixtures from the 0.1, 0.2, 0.4, and 0.8 M HCl lysate is shown in the aligned SEC chromatograms (Fig. 5A, a–d). These also show a progressive downsizing of agarose, that is, treatment with higher acid concentration generated smaller products. Product DP ranges and reaction yields for different treatments were: DP 2–22, 14.5% (0.1 M); 2–16, 28.3% (0.2 M); 1–14, 45, 6% (0.4 M), and 1–6, 47.0% (0.8 M). The optimized conditions chosen for generating a spectrum of AOS populations from DP 1–14 was 0.4 M and was used for a parallel analysis in the NH2-HPLC–ELSD system (Fig. 5B). The NH2-HPLC–ELSD clearly resolved individual peaks from monomers to DP 12 of the AOS products (Fig. 5B) as found in NAOS analysis (Fig. 3B).

2.5. Preparation of AOS with defined DP

AOS products were separated, purified, and isolated by SEC and NH2-HPLC as performed for NAOS. The separation of an AOS crude mixture with DP 1–14 by SEC demonstrated distinct partitioning of AOS products of DP 2–14 into individual fractions (Fig. 6A). The product yield of a particular isolated AOS was calculated from

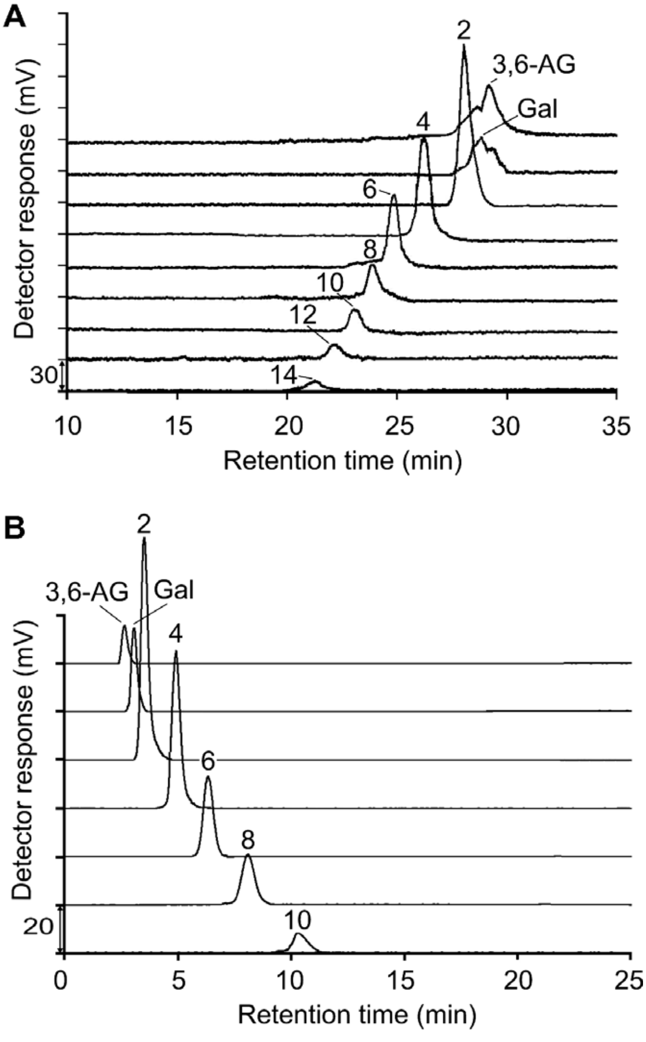

Figure 6. HPLC inspection of the isolated AOS fractions. Each isolated fraction was re-injected into the same HPLC system and ELSD detected, from which their chromatograms were aligned. (A) SEC–HPLC system; (B) NH2-HPLC system. The chromatographic conditions are the same as in Figure 3.

calibration line A′, and the total product yield of these isolated DP 1–14 AOS by SEC was 82.9% (Table 1). The AOS fractions of DP 1–10 obtained by NH2-HPLC had a similar aligned partition profile and peak sharpness as those fractions collected previously for NAOS (Figs. 4B vs 6B). Yields of individual AOS products from NH2-HPLC were calculated according to calibration line B′, and total product yield of these isolated DP 1–10 AOS by NH2-HPLC was 90.0% (Table 1).

2.6. Structure, molecular mass, and purity confirmation of AOS and NAOS

The structures of the isolated NAOS fractions from N2 to N22 and AOS fractions from A2 to A14 were scrutinized by ^{1}H NMR spectroscopy. It is apparent that the difference between the NAOS and AOS series is located in the G1α resonance environment on the α-D-galactosyl reducing end. The ^{1}H NMR spectrum showed that individual NAOS (DP 2–22) have weak signals for G1α at δ 5.28 ppm, as opposed to individual AOS (DP 2–14) where no response for that hydrogen on G1α is observed (Fig. 7). The spectrum also indicated the presence of both the α and β anomers of Gal in NAOS, whereas in AOS only the β anomer was found. These positions of ^{1}H NMR chemical shifts (ppm) of protons in NAOS from N2 to N22 and AOS from A2 to A14 were repeatable (data not shown).

The molecular mass and purity of 12 NAOS and AOS fractions (each DP 2–12) were evaluated by ESI–MS analysis in negative-ion modes to observe distinct target mass signals. The mass spectra showed the expected base mass peak for each oligomer fraction, which was normalized as 100% intensity. For the disaccharides (N2, A2), the base peaks were found at m/z 322.87, which correspond to the singly charged anions $[\mathrm{N2}]^-$ or $[\mathrm{A2}]^-$. The ions found at 629.13, 935.13, 1241.20 appeared to be clusters of DP 2 formed by side ionization reactions (Fig. 8, the N2, A2 samples). Similar clusters were also found in commercial N2, N6 standards (Fig. 8), thus, the clustered oligomer mass formed by ionization process in the ESIMS spectrometry was considered as the artifacts from the monomer mass.

In the mass spectra of the tetrasaccharides (N4 and A4), the base peaks were found at m/z 629.33 and 629.07, which correspond to the charged anions $[\mathrm{N4}]^-$ and $[\mathrm{A4}]^-$. The N4 mass spectrum was contaminated by 6% relative intensity of the N6 found at m/z 935.40. The ions found at 1241.20 of relative intensity 71% and 68% in N4 and A4 were seemingly the dimeric clusters formed from these DP 4 products (Fig. 8). Similar situations were observed for N6 and A6, where the base peaks occurred at m/z 935.40 and 935.13, which corresponded to the charged anions $[\mathrm{N6}]^-$ and $[\mathrm{A6}]^-$. The ions found at m/z 1853.40 and 1853.33 of relative intensity of 7% and 8% were the dimeric clusters formed from DP 6 (Fig. 8). For oligomers with DP 8 the base peaks were found at m/z 1241.47 $[\mathrm{N8}]^-$ and 1241.18 $[\mathrm{A8}]^-$ (Fig. 8), where N8 also contained 6% of relative intensity of N10 (m/z 1547.40). The oligomers with DP 10 (1547.07 $[\mathrm{N10}]^-$; 1547.20 $[\mathrm{A10}]^-$) and DP 12 (1853.27 $[\mathrm{N12}]^-$; 1853.33 $[\mathrm{A12}]^-$) also contained a minor amount (5–8%) of DP 8, DP 12 and 5–10% of DP 10, respectively (Fig. 8, the N10, A10, N12, and A12 samples). From the ESIMS analysis, when the artifact cluster signals were not counted, the purity of these isolated neoagaro- and agaro-oligomers was 92–100%.

3. Discussion

Features of the approaches used in the current study and the comparison with those used in the literature are discussed. Previously, NAOS or AOS oligomers have been predominantly separated and purified by size-sieving based gel-permeation chromatography (GPC)[13–17] and SEC.[14] One method prepared NAOS by SEC (Toyopearl HW-40S column); however, it was limited only to preparing pure N4.[14] For acquisition of separated fractions from mixtures of oligomers, columns and conditions have been reported as follows: DP 2–6 using Bio-Gel P-2,[13,17] DP 2–4 using Sephadex G-10,[14–16] DP 6–8 using Sephadex G-25,[15] DP 4–12 using Bio-Gel P-10.[17] However, obtaining oligomer fractions was somewhat strenuous as

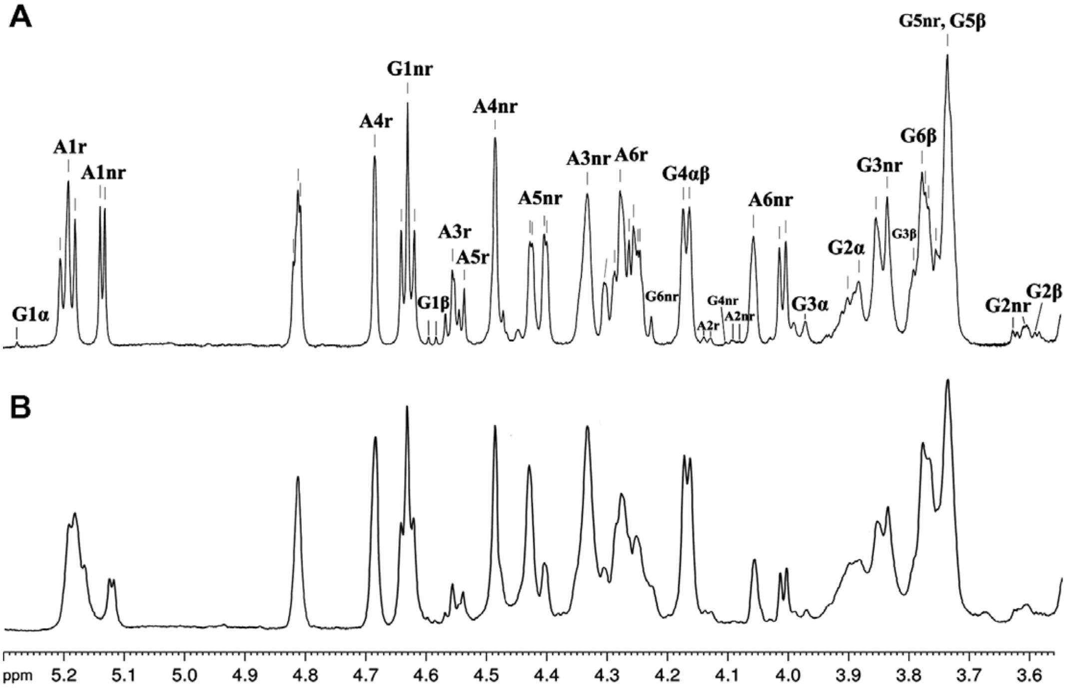

Figure 7. ^{1}H NMR spectra of the isolated N4 (A) and A4 (B) fractions. Peak labels: A, 3,6 anhydrogalactose; G, galactose; nr and r refer to the non-reducing and reducing ends; α, β refer to positions of protons on reducing ends; numbers from 1 to 6 refer to place of protons.

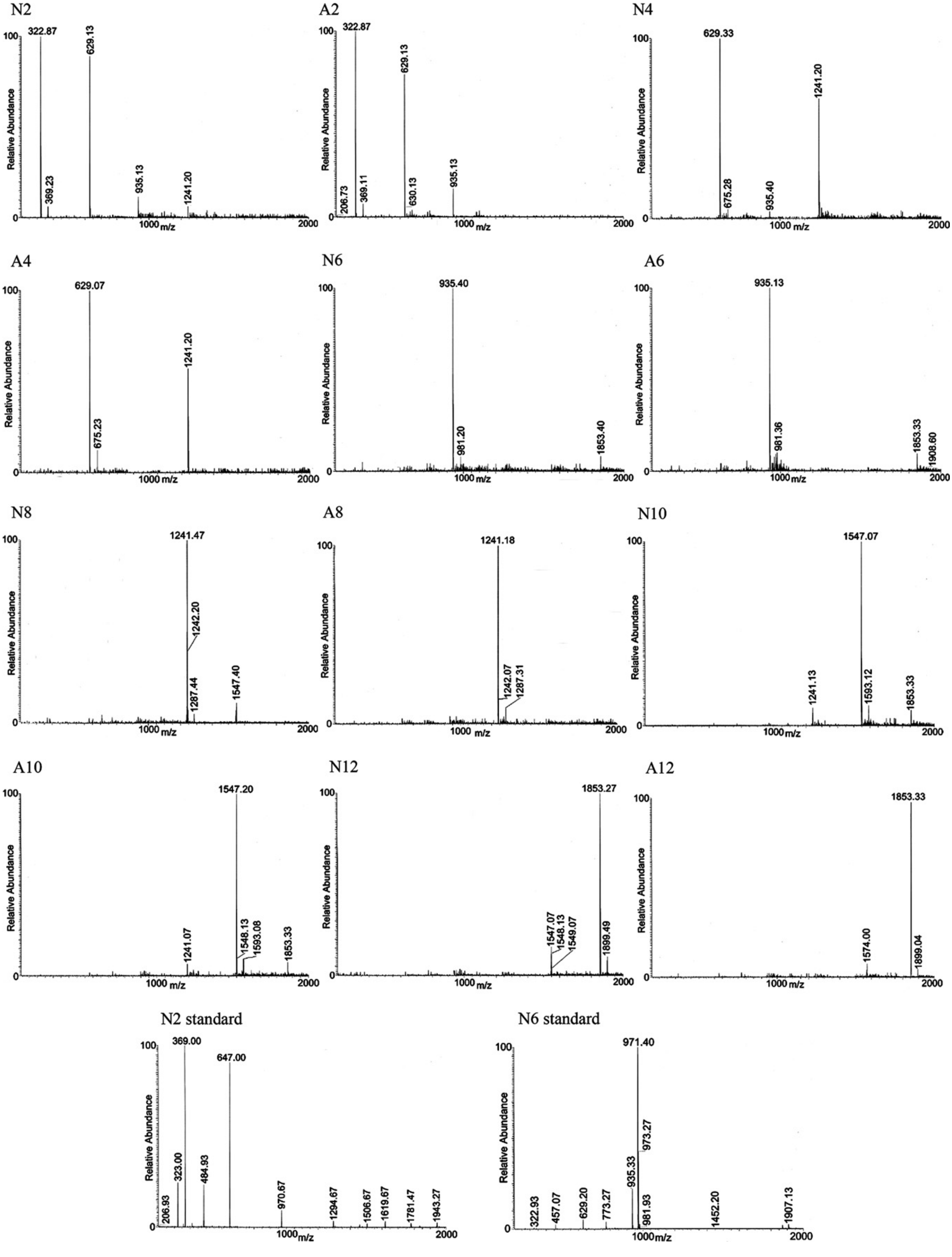

Figure 8. The ESIMS spectra of 12 isolated NAOS and AOS fractions from DP 2–12 and two commercial standards, N2 and N6.

each separated fraction needed to be pooled and re-chromatographed using the same column or subjected to a second column.[15,17] Further removal of salt (such as NH_4HCO_3) in the aqueous buffer was necessary before lyophilizing the oligomers.[17] These methods found in literature were used in preparing oligosaccharides mainly with $DP \leqslant 12$.[13–17]

Therefore, to our knowledge, our work is the first methodology that attempts to acquire well-separated oligomers with $DP \leqslant 22$. The solvent system we used in the SEC system was water, which is easily evaporated and is environmentally friendly. The solvent used in the NH2-HPLC system consists of the general 65:35 MeCN–water, which is easily evaporated to obtain the dried oligomer material for further use. In addition, agaro-product analysis via the NH2-HPLC system successfully separated compounds with a DP of 1–12 with high resolution and purity, although each injection with a small volume of 0.02 mL was allowed to monitor the oligosaccharide composition and to recover a small amount of separated product on an analytical scale. The system should be applicable to scaleup using a similar preparative column.

Different detection approaches commonly used in carbohydrate analysis were adapted for determining NAOS and AOS content in previous reports.[13–21] The phenol–sulfuric acid method was used to measure oligosaccharide amount in terms of total sugar content,[16,18] or it was combined with ferricyanide reagent to measure in terms of reducing sugar content.[13,19,20] Optical methods using RI[14] or UV[15] detectors were also used for these oligosaccharide analyses, where samples were derivatized with α-naphthylamine fluorescent reagent for UV detection. Recently, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD)[21] and fluorophore associated carbohydrate electrophoresis (FACE)[17] have also been used for NAOS determinations. According to our knowledge, this work is the first where ELSD is used for the detection of NAOS and AOS. In comparison to previous detection systems, especially the phenol–sulfuric acid and total reducing sugar methods, ELSD allows a fast, efficient, precise, and repeatable measurement of these oligomers. When ELSD is compared to RI and UV detection, it provides superior sensitivity up to 0.2 μg/mL to detect any signals that possess molecular masses from contaminating materials other than sugars that may be present in the sample.[21]

The ^1H NMR spectra of the isolated NAOS and AOS in this study are consistent with those previously published for the neoagaro-[22,23] and agaro- series,[12] where 28 and 27 major peak signals were assigned. It has been reported that recombinant β-agarase AgaAc from *Zobellia galactanivorans* cleaves the β-(1→4) linkages of agarose in a random manner, resulting in the retention of the β-anomeric-bond configuration only for a short time, and then equilibrates and forms progressively into an α-anomeric configuration because mutarotation takes place in solution.[22] Therefore, both α and β anomeric forms were present in the NAOS fractions as we observed in N2–N22. The presence of G1α and G1β anomeric forms of NAOS was also observed after digesting agar extracted from *Gracilaria textorii* by the β-agarase from *Pseudomonas atlantica*.[23] On the contrary, only the G1β anomeric form was reported in previous publication where agar was digested by acid hydrolysis into AOS.[12] ESIMS used in this study confirms the DP and purity of neoagro- and agaro-oligosaccharides DP 2–12 of molecular mass ⩽2000 Da. (The mass scan range was 200–2000 *m*/*z*). The ions found at 629.13, 935.13, 1241.20 for N2 and A2; 1241.20 for N4 and A4; as well as 1853.40 for N6 and 1853.33 for A6 were not the mass of individual oligosaccharides, indicating they were apparently clusters formed by ion reactions. The cluster formation phenomena were also observed in previous publications.[24,25] When the artifact cluster signals were not counted, the purity of our isolated neoagaro- and agaro-oligomers was 92–100%.

In conclusion, this study has established feasible approaches for the preparation of a series of neoagaro-oligosaccharides (NAOS) by β-agarase digestion and agaro-oligosaccharides (AOS) by HCl hydrolysis from agarose, as well as their qualitative and quantitative measurements and isolation by two HPLC systems. NAOS and AOS of defined quantity, DP and high purity were successfully prepared for further testing their biological activity potentials. This system would also be applicable in the production of oligosaccharides from natural sources such as algae.

4. Experimental

4.1. Chemical and reagents

Agarase[3.2.1.81] from *Pseudomonas atlantica* (Cat. no. A6306, CAS no. 37288-57-6), monosodium phosphate monohydrate and disodium phosphate heptahydrate for buffer preparation, and HPLC-grade solvents (acetonitrile, acetone, methanol, water) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Galactose (180 Da), malto-oligomers (maltose, 342 Da; maltotriose 504 Da; maltotetraose, 666 Da; maltopentaose, 828 Da; maltohexaose, 990 Da; maltoheptaose, 1152 Da), neoagarobiose (N2, 324 Da), and neoagarohexaose (N6, 936 Da), pullulan (5900 Da) used in HPLC calibration were also from Sigma Chemical Co. Hydrogen chloride (HCl) was bought from J.T. Baker (Deventer, Holland). Agarose (162-0133) was from Bio-Rad Laboratories (Hercules, CA, USA). DMSO-d_6 was from E. Merck.

4.2. 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 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 per mg of agarose and incubated at 40 °C for 24 h. The reaction mixture was purified by passing through Sephadex G-25 (Sigma) resin to remove salts, followed by filtering through 0.22-μm membranes (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 HPLC analysis.

4.3. 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 purified, and the crude product was lyophilized as described above.

4.4. Product detection by SEC–ELSD and NH2-HPLC–ELSD systems

The SEC–ELSD system consisted of a pump PU-1580 from Jasco (Tokyo, Japan), an Asahipak GS-320 HQ multi-mode column (300 × 7.6 mm) preceded by an Asahipak GS-2G 7B guard column (50 × 7.6 mm) (Shodex, Showa Denko, Tokyo, Japan), and an ELSD ZAM 3000 (Schambeck SFD GmbH, Bad Honnef, Germany). The temperature settings for the column oven and detector nebulizer were 30 °C and 80 °C, respectively. Samples (1 mg/mL) were injected via a CO-150 sampler with a 20-μL sample loop (Rheodyne, Cotati, CA, USA). The mobile phase for the SEC was deionized water with a flow-rate of 0.4 mL/min. For the NH2-HPLC–ELSD system, the same pump, ELSD injector sampler and sample loop were used as above. However, an Asahipak NH2 P-50 4E multi-mode column (250 × 4.6 mm) and an Asahipak NH2P-50G 4A (10 × 4.6 mm) (Shodex, Showa Denko, Tokyo, Japan) were used, where the temperature settings for the column oven and detector nebulizer were 40 °C and 80 °C, respectively. The mobile phase was isocratic (65:35 MeCN–water) with a flow-rate of 1 mL/min. The commercial Gal, N2, N6, and malto-oligomers with DP 2–7 were used as calibration standards for both systems. Chrom Manager 5.8 software from Analytical Based Development Center (Taichung, Taiwan) was used for online data monitoring and analysis.

4.5. Purification and isolation of the NAOS and AOS product series by SEC and NH2-HPLC

The SEC and NH2-HPLC systems used for product purification and isolation consisted of the same pump and columns as mentioned above, except for connecting with a fraction collector (Amersham Pharmacia Biotech, Uppsala, Sweden). NAOS and AOS product mixtures were prepared as described in Sections 4.2 and 4.3, and the sample injection procedure was the same as described in Section 4.4 except that the sample loop was changed into 1 mL (Rheodyne, Cotati CA, USA) for SEC. Lyophilized crude products of 10 mg were taken and dissolved in 1 mL of deionized water (1% w/v) in which 1 mL for SEC and 0.02 mL for NH2-HPLC was injected for each batch of collection. SEC–ELSD fractions of different DP were collected at time intervals of 18.5–19.5 min, 19.5–20 min, 20–20.5 min, 20.5–21.5 min, 21.5–22.5 min, 22.5–23 min, 23–23.5 min, 23.5–24.5 min, 24.5–25.5 min, 25.5–26.5 min, 26.5–27.5 min, 27.5–28 min, and 28–29 min for the NAOS series, and at 20–21 min, 21–22 min, 22–23 min, 23–24 min, 24–25 min, 25–26.5 min, 26.5–27.5 min, 27.5–28 min, and 28–29 min for the AOS series. These were then freeze dried. For NH2-HPLC, fractions of different DP were collected from retention time intervals of 2.5–3.5 min, 3.5–4.5 min, 4–5 min, 5–6 min, 6–7 min, 7–9 min, and 9–11 min into individual tubes, which were then evaporated and freeze dried. Homogeneity of each collected fraction was checked either by SEC–ELSD or NH2-HPLC–ELSD in the same way as described in Section 4.4. Individually collected peaks of size within DP 12, which were seen in the SEC–ELSD, would be injected into NH2-HPLC–ELSD for dual checks.

4.6. ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR)

^{1}H NMR spectroscopy was conducted in the Instrumentation Center of National Taiwan University (Taipei, Taiwan). Samples were prepared according to the published method in which 2 mg of expected NAOS from N2–N22 and AOS from A2–14 isolated fractions (obtained by SEC–HPLC, as in Section 4.5) were dissolved in 0.6 mL of DMSO-d_6.[26] The dissolved sample was filtered by 0.22-μm membrane and loaded into an NMR tube (178 × 5 mm) (Schott-Duran, Wertheim, Germany). The NMR spectra were recorded on a Bruker AC-300 instrument at 300 MHz (^{1}H), or an Avance 500 FTNMR spectrometers.[27]

4.7. Electrospray-ionization mass spectrometry (ESIMS)

ESIMS analysis of the oligosaccharide fraction was performed with the VG Platform Electrospray ESIMS in the Instrumentation Center of National Taiwan University (Taipei, Taiwan). The VG platform single-quadruple mass spectrometer (Micromass, Altrincham, UK) was equipped with a capillary electrophoresis (CE) interface. The interface utilized a triaxial flow arrangement whereby the CE eluent was mixed with a suitable sheath liquid at the probe tip and then nebulized using N_2 gas. The high voltage applied on the buffer reservoir was 120 kV and 220 kV in normal and reversed electro-osmotic flow CE–MS, with the 3.5 kV ESI voltage applied on the outlet of the separation column. The peaks (Fig. 8) with relative intensities of 5–14% occurred at 46 m/z after base mass peaks resulted from the junction of sample and nebulization N_2 gas.

4.8. Data analysis

Reaction yield was expressed as the percent of the total amount (mg) of NAOS (derived from agarase digestion) or AOS (derived from HCl digestion) crude product (mg) to the total weight of agarose input in the reaction mixture. The total amount of NAOS or AOS crude product was weighted after vacuum lyophilization as described in Sections 4.2 and 4.3. Product yield of particular isolated NAOS or AOS by HPLC systems was expressed as the percent of the amount of individual isolated oligomer (mg) to the total amount of NAOS or AOS crude product injected. The amount of individual oligomer isolated by the SEC system was calculated from the calibration line A′ (Fig. 2A). The amount of individual oligomer isolated by the NH2-system was calculated from the calibration line B′ (Fig. 2B). Production yield of the overall isolated NAOS or AOS was the sum of their individual product yields, expressed in percent.

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