Determination of Molecular Weight Distribution and Average Molecular Weights of Oligosaccharides by HPLC with a Common C18 Phase and a Mobile Phase with High Water Content

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Size Exclusion Chromatography (SEC) has been extensively used to monitor molecular weight distribution of polymer samples. A typical phase of SEC is composed of uniform-sized porous beads with pores of wide dispersity. A solute whose size is larger than the largest pore of the phase will elute first, and a solute whose size is smaller than the smallest pore of the phase will elute last in SEC. In other words, the size factor governs elution order. Larger solutes will elute faster than smaller solutes.

SEC has been used in analysis of polysaccharides, too. For example, a review of SEC of cellulose and related polysaccharides[1] and a review of SEC of aloe vera polysaccharides[2] appeared recently in the literature. A very unique SEC system was also recently introduced where 3 on-line detectors including right-angle laser light scattering, refractometer (RI), and viscometer were used.[3] However, separation efficiency of SEC alone is not satisfactory in general, thus there are efforts to improve such problem. For example, a HPLC-SEC-RI system was suggested.[4]

Application of SEC for oligosaccharides is widespread, too. However, complete separation of individual oligosaccharides composed of even single monosaccharide by SEC is not possible yet. Thus SEC is rather used for purification of oligosacchrides. Determination of molecular weight by SEC is dependent upon calibration curves of standard materials. The whole task will be hampered if no standard is available.

There are some other separation techniques to enable baseline separation of individual oligosaccharides composed of a single monosaccharide. They are high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) and capillary electrophoresis (CE) coupled with reductive amination of the sample. In HPAEC-PAD, individual oligosaccharides ionize in a mobile phase of strong base and have different ion exchange interactions with the stationary phase to give baseline separation.[5,6] However, this method also requires calibration standards since the signal intensity is not proportional to the molar concentration of each oligosaccharide component. In the CE method, individual oligosaccharides are chemically modified to have a chromaphore with a charge, then they have different mobilities since they all have the same charge but different masses, and they are base-line separated. A typical reagents of reductive amination for this purpose is 8-aminonaphthalene-1,3,6-trisulfonate (ANTS)[7] or 1-aminophenanthrene-3,6,8-trisulfonate (APTS)[8] since they are strongly fluorescent and charged –3. Only the reducing end of each oligosaccharide molecule is subject to reductive amination, and the signal intensity of each derivatized oligosaccharide is assumed to be proportional to its molar concentration regardless of its molecular weight. The problem of CE method is its low sensitivity when UV detection is used. Fluorescence detection may be employed to overcome this problem, but the limited range of signal linearity of fluorescence may mislead in measurement of molecular weight distribution and average molecular weight. However, in the above techniques and the technique introduced in this study, good separation is possible for only oligomers the DP of which is less than 20-30. It should also be noted that SEC is not a technique designed to work well for low MW samples. SEC should be a rather good technique for high MW polymers.

In this study, a simple method has been developed for determination of molecular weight distribution and average molecular weights. The sample was modified by reductive amination with 4-aminobenzoic acid ethyl ester (ABEE),[9,10] and ABEE-oligosaccharides were separated in a HPLC system with a common C18 column and a mobile phase of high water content.

Experimental Section

Chemicals. Acetonitrile and water were of HPLC grade and purchased from Fisher (Pittsburgh, PA, USA) and used without purification. Dextran 1500, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, ABEE (4-aminobenzoic acid ethyl ester), sodium cyanoborohydride, and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Three brands of Korean beer (Hite, Cass, OB Blue) were purchased from a near grocery store.

Chromatographic system. The chromatographic system we used for ABEE-derivatized oligosaccharides, was composed of a Shimadzu (Tokyo, Japan) LC-10AD pump, a Rheodyne (Cotati, USA) sample injection valve with a 0.5 μL loop, a Samsung (Seoul, Korea) SLC-2000 UV detector,

an Alltima C_{18} (0.5 mm I.D. × 300 mm length, 5 μm particle size) column, and a computer equipped with the Youlin-Gisul (Sung Nam, Korea) Multichro 2000 software. The eluent was 10/90 (v/v %) acetonitrile/water. The flow rate was fixed at 0.01 mL/min, and the detector wavelength was set at 307 nm. An Alltech (Deerfield, IL, USA) slurry packer was used to pack the microcolumns. The slurry was made by mixing 150 mg particles with 2 mL methanol, and was sonicated for 20 min before packing. The slurry was transferred to the slurry reservoir (1.2 mL), and the pressure of the slurry packer was raised to 14,000 psi instantly. The pressure was maintained for 2 min., and decreased to 10,000 psi, and the final pressure was maintained for 10 min.

Preparation of a synthetic mixture of oligosaccharides. A synthetic mixture of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose was prepared by measuring 1.0 mg of each component and dissolving the components in 100 μL water. An 30 μL aliquot was taken and diluted to 100 μL for reductive amination.

Preparation of dextran and beer samples. 1.0 mg dextran was measured and dissolved in 100 μL water for reductive amination. Each beer was filtered through a 0.45 μ membrane filter, and used for reductive amination.

Derivatization procedure to obtain ABEE-oligosaccharides. An aliquot of 100 μL sample (or a standard solution) was mixed with 80 μL of acetic acid, 80 μL of 1.4 M $NaBH_3CN$, and 400 μL of 0.6 M ABEE in methanol, and the mixture was heated at 80 °C for 2 hr, and cooled to room temperature.[9,10]

Principles of separation of oligosaccharides based on molecular weight by UVD-HPLC. Saccharides are very polar molecules. A nonpolar moiety (ABEE) is introduced in the molecule upon reductive amination. The relative contribution of the nonpolar moiety to the whole molecule will be more significant as the molecular weight of the saccharide gets lower. In other words, ABEE-saccharide gets more nonpolar as the molecular weight of saccharide gets lower. In reversed phase liquid chromatograpy (C18 column), a more polar ABEE-saccharide (higher molecular weight) elutes faster than a less polar ABEE-saccharide (lower molecular weight). This behavior is similar to that of size exclusion chromatography. In a mobile phase of very high water content (90%), baseline separation was observed for oligosacchrides.

Determination of average molecular weight. It is assumed that the signal intensity of each derivatized oligosaccharide is proportional to its molar concentration regardless of its molecular weight. In addition, baseline separation of individual oligosaccharides is enabled in this study. Thus average molecular weights can be calculated with the measured peak areas of individual oligosacchrides. Theoretical average molecular weights can be computed for the synthetic sample as follows:

$$M_n = (\Sigma n_i M_i)/(\Sigma n_i) \quad (1)$$

$$M_w = (\Sigma w_i M_i)/(\Sigma w_i) \quad (2)$$

M_n and M_w are number average molecular weight and weight average molecular weight, respectively, n_i and w_i, number of mol and weight fraction of each oligosaccharide, and M_i, molecular weight of each oligossachride.

Experimental average molecular weights based on chromatographic data can be computed as follows:

$$M_n = (\Sigma A_i Mi)/(\Sigma A_i) \quad (3)$$

$$M_w = (\Sigma A_i M_i^2)/(\Sigma A_i M_i) \quad (4)$$

A_i is the peak area of each oligosaccharide peak.

Results and Discussion

Comparison of theoretical average molecular weights with experimental average molecular weights for the synthetic sample. ABEE-derivatized oligosaccharides were nicely separated under the optimized separation conditions with the Alltima C_{18} stationary phase. The obtained chromatogram is shown in Figure 1. Theoretical and experimental values are in a good agreement within a few % error. The experimentally determined M_n and M_w (three batches) were 451.2 ± 2.1 (theoretical value; 463.6) and 651.3 ± 2.2 (theoretical value; 662.1), respectively.

However, the assumption of equal sensitivity of derivatized oligosaccharides regardless of molecular weight has been found rough. A systematic trend of decreased peak intensity (for equivalent molar concentration of each oligosaccharide, A_iM_i) with increase of molecular weight was observed. We found that complete derivatization of oligosaccharides of higher MW requires longer reaction time. The problem was partially solved by increasing the reaction time of ABEE derivatization from 30 min (given in the literature) to 2 hr. There still remained a systematic trend of decreased peak sensitivity (A_iM_i) with increase of molecular weight although the discrepancy is minimized to about 10%. This is

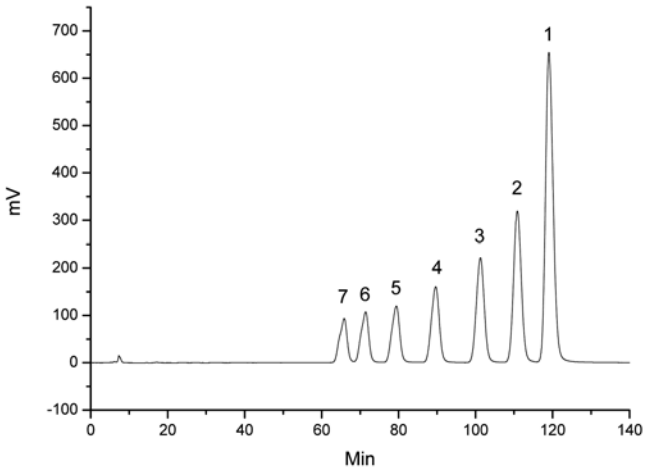

Figure 1. The chromatogram of ABEE-derivatized oligosaccharides of the synthetic sample obtained with an Alltima C_{18} column (0.5 mm I.D × 300 mm, 5 μm) in 10/90 (v/v %) acetonitrile/water at a flow rate of 0.01 mL/min at a wavelength of 307 nm. 1; Glucose, 2; Maltose, 3; Maltotriose, 4; Maltotetraose, 5; Maltopentaose 6; Maltohexaose 7; Maltoheptaose

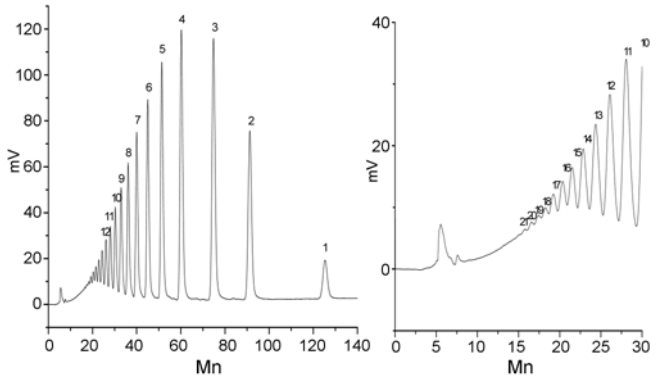

Figure 2. The whole chromatogram (left) and expanded view of front peaks (right) of ABEE-derivatized dextran 1500 obtained with the Altima C_{18} column (0.5 mm I.D × 300 mm, 5 μm) in 10/90 (v/v %) acetonitrile/water at a flow rate of 0.01 mL/min.

why the experimental M_n and M_w were smaller than the theoretical values although the difference is only a few %. Further extension of reaction time caused appearance of many small impurity peaks (unstable baseline).

Measurements of molecular weight distribution and average molecular weights of dextran. The chromatogram of ABEE-dextran 1500 is shown in Figure 2. Dextran is partially branched oligosaccharides composed of only glucose. As shown in Figure 2, separation of oligosaccharides of higher DP(degree of polymerization > 10) is not very good, and peaks are not identifiable for DP > 21. Thus the molecular weight of DP 21 is assigned for the unresolved oligosaccharides (DP ≥ 21) for calculation of average molecular weights. For other overlapped peaks, vertical lines are drawn from all the valleys to the baseline to define boundaries of individual peaks. We assumed that all the individual peaks represent for oligodaccharides of different MW. This is only an assumption although it can be somehow supported by the mass spectrum (see below). The calculated M_n and M_w were 851.2 ± 13.5 and 1305 ± 15.2 (three batches) under the above assumption. The suggested M_w by the vendor for dextran 1500 is 1500 but this is not a certified value. The mass spectrum of dextran 1500 (5 mg/mL in ESI solvent, 5 μL injection) was obtained by flow injection ESI/MS in 70/30 (v/v %) methanol/water at a flow rate of 30 μL/min for another reference of molecular weight distribution (Figure 3). The peak intensity pattern of mass spectrum is very similar to that of HPLC chromatogram. The base peak corresponds to DP of 4 (m/z of $M+Na^+$ = 689).

It should be noted that the elution times of the peaks of Figure 2 (dextran) are far off the elution times of the oligosaccharides in Figure 1 (synthetic mixture). The retention time of peak 2 in Figure 2 is similar to that of peak 4 (maltotetraose) in Figure1, and the retention time of peak 3 in Figure 2 is similar to that of peak 5 (maltopentaose) in Figure 1. If it is assumed that the peak 2 of Figure 2 is maltotetraose and the peak 3 of Figure 2 is maltopentaose, then the M_w will be closer to 1500. It also implies that there are no disaccharide and trisaccharide in dextran 1500 even though there is a monosaccharide (glucose), which is not

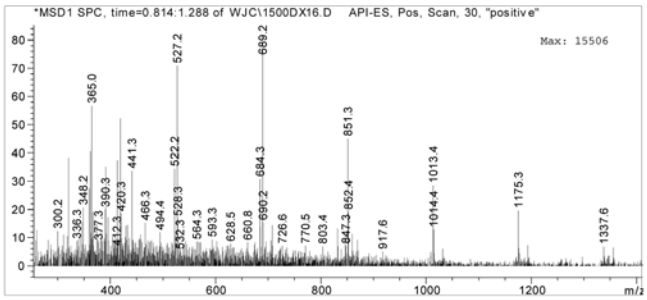

Figure 3. The mass spectrum of dextran 1500 obtained by ESI/MS in 70/30 (v/v %) MeOH/water with 10^{-5} M NaCl.

only unnatural but also inconsistent with the mass spectrum. In addition, the dextran sample is different from the synthetic sample in that it is composed of partially branched oligasaccharides. Nevertheless, this argument does not confirm the assumption that all the individual peaks represent for oligodaccharides of different MW, either. We had better note that there is some uncertainty in determination of M_n

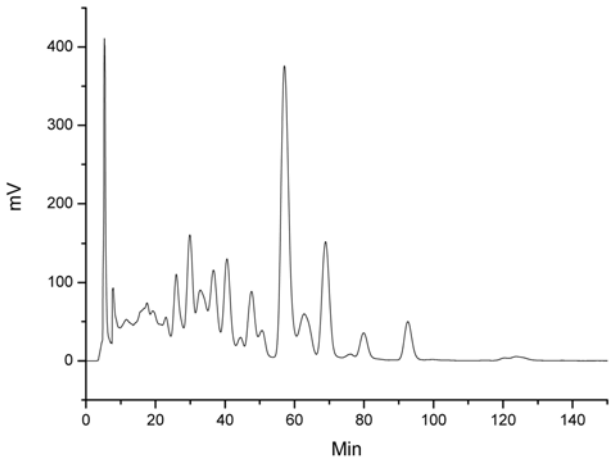

Figure 4. The chromatogram of ABEE-derivatized Cass beer obtained with an Alltima C_{18} column (0.5 mm I.D × 300 mm, 5 μm) in 10/90 (v/v %) acetonitrile/water at a flow rate of 0.01 mL/min.

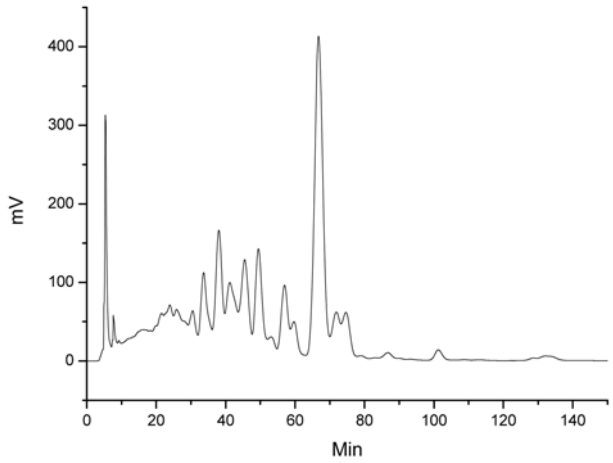

Figure 5. The chromatogram of ABEE-derivatized Hite beer obtained with an Alltima C_{18} column (0.5 mm I.D × 300 mm, 5 μm) in 10/90 (v/v %) acetonitrile/water at a flow rate of 0.01 mL/min.

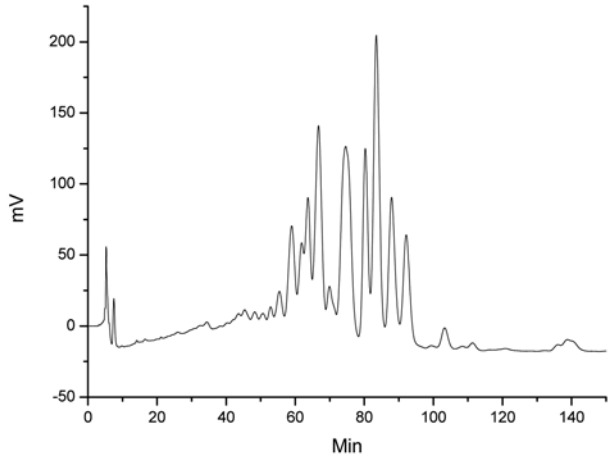

Figure 6. The chromatogram of ABEE-derivatized OB Blue beer obtained with an Alltima C_{18} column (0.5 mm I.D × 300 mm, 5 μm) in 10/90 (v/v %) acetonitrile/water at a flow rate of 0.01 mL/min.

and M_w of dextran 1500.

Measurement of molecular weight distribution of beer samples: fingerprint concept. Beer is known to contain some oligosaccharides. The method of this study was applied to monitor molecular weight distribution of beer samples. Three brands of Korean beer were examined-Cass, Hite, and OB Blue. Their chromatograms obtained after ABEE derivatization were shown in Figures 4, 5, and 6, respectively. Beer saccharides may not be composed of single monosaccharide considering the complicated peak patterns. The average molecular weights of beer oligosaccharides cannot be determined at present because of the complex peak patterns. The masses of individual peaks should be determined by LC/MS for calculation of average molecular weights, which may be a good topic of future study. Nevertheless, the obtained chromatogram bears a character of fingerprint, thus this analytical technique may serve as a quality control protocol in beer production.

Conclusion

A simple method has been developed for determination of molecular weight distribution and average molecular weight by a HPLC system. A common C18 column and a mobile phase of high water content (90%) were used in this system, and the oligosaccharide sample was derivatized with ABEE. In this system, a larger solute elutes faster than a smaller solute. The merit of this method is the fact that standards are not required since the signal intensity of each ABEE-oligosaccharide is generally proportional to its molar concentration regardless of its molecular weight. The applicability of this method has been examined for a synthetic sample, a dextran sample, and beer samples.

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