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Interference Prevention in Size-Exclusion Chromatographic Analysis of Debranched Starch Glucans by Aqueous System

Amy Hui-Mei Lin,[†,§] Yung-Ho Chang,[‡] Wen-Bin Chou,[‡] and Ting-Jang Lu[*,†]

[†]Institute of Food Science and Technology, National Taiwan University, 1, Sec. 4 Roosevelt Road, Taipei 10617, Taiwan, Republic of China

[‡]Department of Food and Nutrition, Providence University, 200, Chung Chi Road, Shalu 43301, Taiwan, Republic of China

ABSTRACT: Branch chain-length distribution of amylopectin plays an important role on the characteristics of starch. One of the adapted protocols for determining the chain-length distribution and mass proportion of starch molecules is that starch is debranched with isoamylase and then analyzed by using high-performance size-exclusion chromatography coupled with multiangle laser-light scattering and refractive index detection (HPSEC-MALS-RI). However, ammonium sulfate in commercial isoamylase and acetate in debranching buffer give significant interferences on the chromatograms because of their undesirable ionic interactions with column sorbent materials. This study deals with development for correcting those interferences. A weak anion-exchange resin or selective precipitation with barium acetate was employed to remove sulfate prior to HPSEC determination. The interference of acetate was overcome by means of high ionic strength eluent, 0.3 M sodium nitrate. The specific refractive index increment (dn/dc) of amylodextrin was determined to be 0.147 using the modified conditions and was applied to calculate the molecular weight distribution of debranched starch molecules.

KEYWORDS: starch, chain-length distribution, amylopectin, amylose, size-exclusion chromatography, ionic interaction, refractive index increment dn/dc, ammonium sulfate

INTRODUCTION

Starch, one of the most abundant natural polymers, is composed of two main components: highly branched amylopectin and essential linear amylose. The molecular structures of both amylose and amylopectin play important roles in the physical properties of starch, which is often used as a functional ingredient in a variety of foods and industrial materials. Amylose and amylopectin both contain glucose bound by α-(1→4) linkages to form linear chains. Amylose can be separated into completely linear molecules and branched molecules.[1−3] The degree of polymerization (DP) of amylose ranges from 200 to 20 000,[4] with most of the amylose branches being under DP100.[5] In amylopectin, most chains are short and bound to longer chains by an α-(1→6) linkage, forming a highly branched structure.[6] In general, the branches of amylopectin molecules cover a wide size range from six to several hundreds, and, in some cases, even more than a thousand.[7] Most of the debranched amylopectin molecules show a bimodal distribution of long and short chains,[8] with some having three to four fractions when examined by size-exclusion chromatography (SEC).[7−9] Although the molecular composition of starch can be approximately described as a mixture of amylose and amylopectin, the molecular structures of these components are quite complicated. For example, approximately one-half of the amylose molecules in corn starch are branched.[10] Some amylopectins, such as in the amylose extender waxy corn starch (*aewx*), contain long chains that bind iodine in a manner similar to amylose and contribute to apparent amylose contents.[11] Using debranching enzyme such as isoamylase in conjunction with other measurements is a widely accepted way to examine the chain-length distribution of amylose and amylopectin.

Light scattering properties have been applied to the molecular characterization and solution behavior of both natural and synthetic macromolecules. Information obtained from light scattering analysis includes absolute molar mass (M_w), radius of gyration (R_g), and solvent interaction (A_2) of macromolecules.[12] Low-angle laser-light scattering has been widely used;[7,8,13] however, it has relatively high noise as compared to multiangle laser-scattering (MALS) because of higher interferences from particles and other impurities at low angles. The high noise level has a great effect on small molecules such as short-chains of debranched starch molecules. MALS needs extrapolation to zero-angle to obtain molar masses but provides the choice of the signals from low noise angles; multiangles, on the other hand, additionally provide geometry and dimension information. When light-scattering properties are applied to determine the absolute molecular weight of a polymer, it requires a polymer solution with diluted concentration so that the second viral coefficient is negligibly small, and a low scattering angle near zero so that the error from angular dependence can be ignored. The weight-average molecular weight of the polymer is given[13] in eq 1

$$M_w = k\frac{(\mathrm{LS})}{(\mathrm{RI})}\Big/\frac{\mathrm{d}n}{\mathrm{d}c} \qquad (1)$$

where LS and RI present the laser-light scattering photometer and refractometer response, respectively, k is an instrumental constant, and dn/dc is the refractive index increment of the

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polymer. The value of dn/dc is with respect to polymer composition, molar mass, solvent, and laser wavelength and greatly affects the accuracy of molecular weight calculation.

Before light scattering analysis, polymers can be fractioned on the basis of specific properties such as hydrodynamic volumes, polarity, sedimentation velocity, and specific affinity to the columns. High-performance size-exclusion chromatography (HPSEC)[13−17] is a commonly used technique to analyze starch molecules. It separates molecules on the basis of hydrodynamic volume. The process is a permanently stressed equilibrium (stressed by flow and matrix pores) with the response to be separated molecules under the stress. Hizukuri used TSK size-exclusion chromatographic columns (Tosoh Corp., Tokyo, Japan) eluted with phosphate buffer (0.1 M, pH 6.2) to examine debranched amylopectin from 20 starches.[8] The weight-average chain length distribution of most debranched amylopectin samples (17 specimens) showed that bimodal distributions on size-exclusion chromatogram of fractions contained long and short chains; some starches showed trimodal distributions of which the fractions contained long chains and two groups of short chains. Similar analytical conditions were applied in several reports.[6,18−22] However, the ammonium sulfate in the isoamylase suspension and the acetate buffer used for the debranching reaction became interferences in the system consisting of TOSOH TSK-GEL PW_{XL} columns eluted in series with phosphate buffer. In this paper, we tested several methods to remove the interferences so as to avoid overestimating the molecular weight. We also determined the refractive index increment (dn/dc) of amylodextrin, which has an average DP of 26, and applied it to calculate the molecular weight distribution of debranched amylopectin.

MATERIALS AND METHODS

Materials. Normal corn starch was obtained from National Starch & Chemical Co. (Gauteng, South Africa). Pullulan standards (Shodex Standard P-82) were purchased from Showa Denko (Kawasaki, Japan). Isoamylase (crystal) was from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Reagent-grade sodium nitrate and disodium hydrogen phosphate were purchased from RDH Chemical Co. (Spring Valley, CA), while other sugar standards and chemicals were from Sigma-Aldrich Co. (St. Louis, MO). Deionized water was obtained from a Milli-Q water purification system (Millipore Co., Bedford, MA), and the water was degassed under vacuum followed by filtering through a membrane filter (pore size 0.02 μm) prior to use in the MALS system. The cellulose acetate filter membranes with pore size 0.02 μm were from Whatman Ltd. (UK). The polyvinylidene fluoride (PVDF) syringe filters with pore size 0.45 and 5 μm were from Millipore Corp. (Billerica, MA). The anion-exchange resins including Amberlite IRA-400 and -67 were purchased from Sigma-Aldich Co. (St. Louis, MO). The Vivapure D mini H spin column was from Sartorius Stedim Biotech S.A. (Goettingen, Germany).

Preparation of Debranched Starch Samples. The starch solution was prepared by weighing 50 mg (dry basis) of starch, wetted with 5 mL of water. Next, 45 mL of dimethyl sulfoxide (DMSO) was added and heated in a boiling water bath with stirring for 1 h. The starch solution was continuously stirred for 12 h at room temperature. Starch was precipitated after adding 100 mL of ethanol and recovered by using centrifugation at 3000g for 10 min. The starch was dissolved in 90% DMSO again and heated in a boiling water bath with stirring for 10 min. The starch DMSO (90%) solution was stored at room temperature for further analysis. The starch molecules were reprecipitated with ethanol and dissolved in deionized water and heated in a boiling water bath for 10 min prior to the enzymatic debranching reaction.

Starch solution (25 mg/2.4 mL water) was prepared from the above solution. The acetate buffer (final concentration was 25 mM, pH 3.5), sodium azide (0.02%), and diluted isoamylase solution (5 μL, 5.9 U/μL) were added to the starch solution. The mixture was shaken in a water bath controlled at 45 °C for 1 h. After diluted isoamylase solution (5 μL, 5.9 U/μL) was again added, the mixture was incubated for another 2 h. The solution was neutralized with 25 mM sodium hydroxide and heated in a boiling water bath for 5 min to inactive the enzyme. The debranched starch solution was diluted with deionized water to 5 mL and filtered through a syringe filter (pore size 0.45 μm).

For background observation, a mixture of acetate buffer, sodium azide, heat-deactivated isoamylase suspension (or ammonium sulfate), and maltohexaose was filtered through syringe filters (pore size 0.45 μm) and injected into a HPSEC-MALS-RI system. The concentration was the same as those prepared in the debranching reaction.

The debranched starch solution or the mixture of salts and sugar was loaded into a column (i.d. 1 cm × 5 cm) packed with anion-exchange resin, which includes Amberlite IRA-400 and Amberlite IRA-67, separately. The eluate was filtered through a syringe filter (pore size 0.45 μm) before injection to the HPSEC-MALS-RI system. For using Vivapure D mini H spin columns to desalt, debranched starch solution or the mixture of salts and sugar was loaded onto the spin column and then centrifuged at 3000 rpm for 5 min. Next, the eluate was collected and injected onto the HPSEC-MALS-RI system. The third method we tried to remove the salts was to generate a precipitate with barium acetate. The debranched starch solution or the mixture of salts and sugar was mixed with barium acetate (0.5 M, 40 μL). After being vortexed, the mixture was filtered through the syringe filters (pore size 0.45 μm) before injection into the HPSEC-MALS-RI system.

Preparation of Amylodextrin for dn/dc Determination. Corn starch (15 g) was suspended in a hydrochloric acid solution (7.5%, 1000 mL) and put in an incubator controlled at 35 °C [23] for 25 days. The starch suspension was stirred well twice a day until it reached a constant degree of hydrolysis. The total carbohydrate content of the supernatant was determined by the phenol−sulfuric acid method[24] and was applied to the calculation of the hydrolysis degree. When it reached the constant hydrolysis degree, the acid solution was discarded. The rest of the suspension was neutralized with sodium hydroxide (0.1 M) and stored overnight. The next day, it was washed by deionized water five times to remove salts.

The above amylodextrin was dissolved in water (about 0.5%) and then heated in a boiling water bath for 30 min followed by autoclaving (121 °C, 30 min). The starch solution was filtered through a filter (pore size 5 μm) and then loaded into a column (100 cm × 1.5 cm, Bio-Rad Econo-column, Bio-Rad Lab., Hercules, CA) packed with TSK Gel Toyoperal HW-55F (Tosoh Corp., Tokyo, Japan) and eluted with deionized water with 0.02% sodium azide. The flow rate was 0.8 mL/min. The total carbohydrate and reducing sugar content were determined by the phenol−sulfuric acid method[24] and Somogyi Nelson method,[25,26] respectively. The fraction of average DP 26 was used for the dn/dc determination.

Serial dilutions of the above fraction were prepared with sodium nitrate solution (0.05−0.5 mg/mL). The final concentration of sodium nitrate was 0.3 M. The RI (RI, Optilab DSP, Wyatt Technology Inc., Santa Barbara, CA) detector was set at 690 nm, and temperature was controlled at 35 °C. Samples of 10 different concentrations were injected from low to high concentration. Enough volume was injected to get a flat region for each concentration. The DNDC 5.20 (Wyatt Technology Co., Santa Barbara, CA) was applied to calculate the dn/dc value.

HPSEC-MALS-RI System. The system consisted of a high-performance size-exclusion chromatography apparatus (HPSEC) equipped with a Metrohm 7091C pump (Herisau, Switzerland), a Rheodyne 9725i

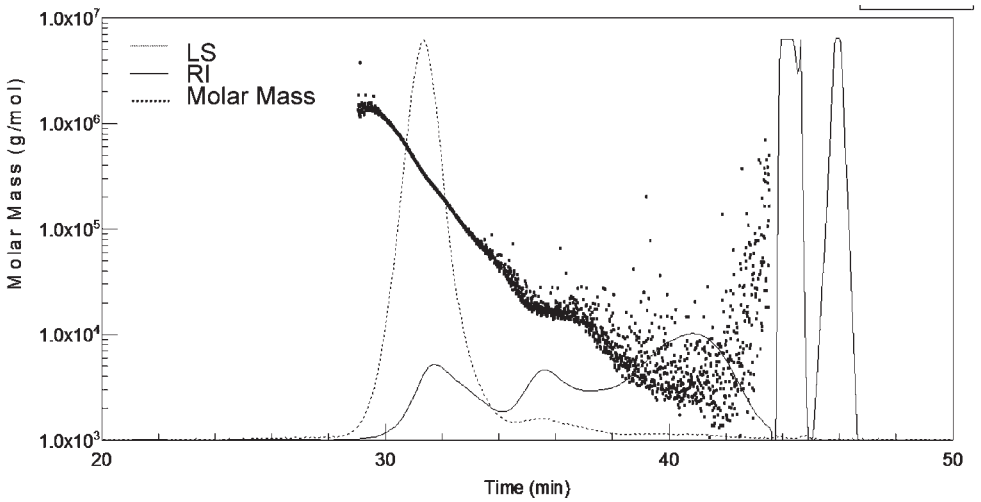

Figure 1. HPSEC-MALS-RI chromatogram of the debranched corn starch molecules eluted with phosphate buffer (0.1 M, pH 6.2) at 0.5 mL/min on a series of TSK-gel G3000PW$_{XL}$−G2500PW$_{XL}$−G2500PW$_{XL}$ columns at 70 °C. RI, —; LS, - - -; MW, ■.

Table 1. Differences in the Determined Values of Chain-Length of Debranched Corn Starch Measured by HPSEC-MALS-RI System Eluted with 0.1 M Phosphate Buffer and 0.3 M Sodium Nitrate

eluent		F1	F2	F3
phosphate buffer	DP$_w$[a]	1549.4 ± 41.5 a	107.9 ± 16.8 b	48.9 ± 13.3 b
	DP$_n$[a]	837.0 ± 39.1 a	98.5 ± 16.1 b	21.9 ± 4.7 b
	mass ratio (%)	22.82 ± 0.13 a	19.59 ± 0.12 a	57.60 ± 0.33
sodium nitrate	DP$_w$	1913.0 ± 49.8 b	54.2 ± 1.0 a	19.6 ± 0.4 a
	DP$_n$	900.0 ± 66.3 b	50.5 ± 1.1 a	18.0 ± 0.2 a
	mass ratio (%)	23.69 ± 0.14 b	20.59 ± 0.12 b	55.71 ± 0.32

[a] DP$_w$, DP$_n$: weight- and number-average degree of polymerization, respectively. F1: amylose. F2 and F3: long- and short-chain branches of amylopectin, respectively. Means of duplicate analyses. Values bearing different alphabetic letters were significantly different by *t*-test ($P < 0.05$).

PEEK injector with 100 μL PEEK sample loop, a precolumn filter with 0.5 μm PEEK frit (A707, Upchurch, Oak Harbor, WA), a multiangle laser-light-scattering photometer (MALS, DAWN EOS, Wyatt Technology Inc., Santa Barbara, CA), and a refractometer (RI, Optilab DSP, Wyatt Technology Inc., Santa Barbara, CA) as detectors. A series of columns of a TSK-gel G3000PW$_{XL}$ and two G2500PW$_{XL}$ (Tosoh Corp., Tokyo, Japan) were connected and maintained at 70 °C. The columns were eluted with deionized water, phosphate buffer (0.1 M, pH 6.2), and 0.1 and 0.3 M sodium nitrate with 0.02% sodium azide at 0.5 mL/min separately for different tests. All of the eluents were filtered (filter pore size 0.02 μm) and degassed. The RI detector was maintained at 35 °C.

The MALS was calibrated with toluene at the angle of 90°, and the laser intensity of multiangles was normalized with pullulan standard (molecular weight 17 300 g/mol). The calibration constant for the RI detector was determined by injecting five concentrations (0.05−0.5 mg/mL) of sodium chloride solution.

RESULTS

Phenomena of Interferences. To examine debranched amylopectin molecules, we adapted our chromatographic conditions to resemble a protocol described in the literature[8] that used TSK columns eluted with phosphate buffer. Phosphate buffer showed a high light-scattering noise level, and the use of phosphate buffer as the eluent led to higher variations and inconsistencies in determining values of the molecular weight distribution in the HPSEC chromatogram of debranched corn starch molecules. The major variation occurred in the fractions of debranched amylopectin molecules. The relationship between the logarithm of molecular-weight and the elution volume deviated from a monotonously decreasing trend with poor reproducibility on the debranched chains of amylopectin fraction (Figure 1, Table 1). To understand the causes of the interference, the debranched starch samples were replaced by maltohexaose, the possible shortest branch chain existing in amylopectin molecules.[27,28] We then observed the effects of buffer, enzyme suspension, and salts used in the sample preparation in the following experiments.

Interference from Acetate Buffer. Acetate buffer was used as the buffer for the debranching reaction and became one of the interferences for the determination of the distribution of starch chain-length in the size-exclusion system. Deionized water, phosphate buffer, and different concentrations of sodium nitrate were used individually as eluents in the HPSEC-MALS-RI determination. The RI signal of acetate buffer overlapped with maltohexaose when deionized water was used as the eluent (Figure 2). While using phosphate buffer as the eluent, the signal of acetate buffer was delayed but still partly overlapped with maltohexaose (Figure 3a). Sodium nitrate (0.3 M) delayed acetate peak further and made the acetate buffer completely separate from maltohexaose molecules (Figure 3b). Therefore, sodium nitrate was chosen for all further experiments for starch chain-length distribution determination. To understand the

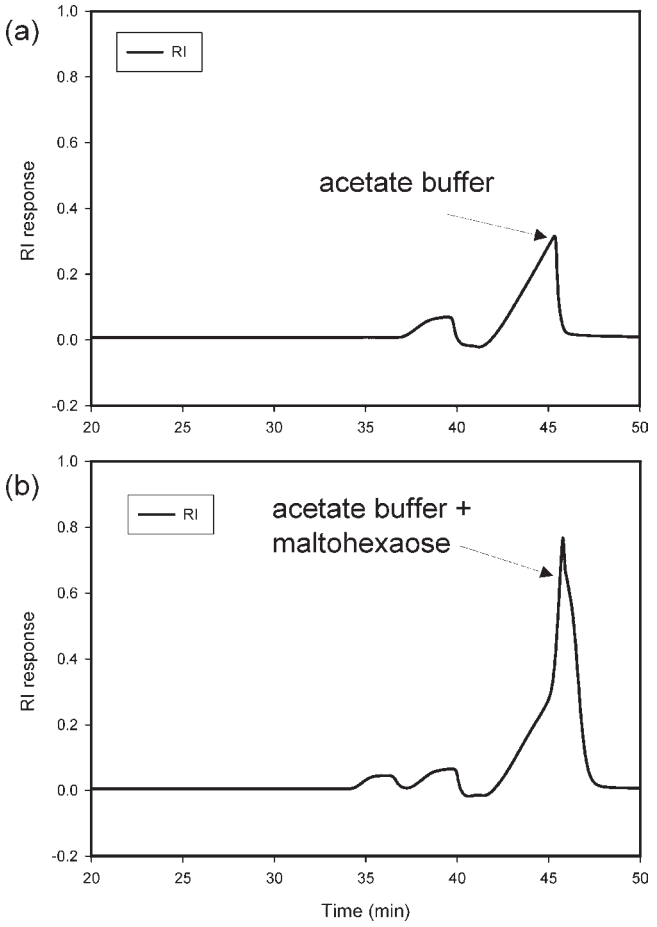

Figure 2. RI signals of (a) acetate buffer and (b) the mixture of maltohexaose, acetate buffer, and isoamylase eluted with deionized water containing 0.02% sodium azide with other conditions the same as those in Figure 1.

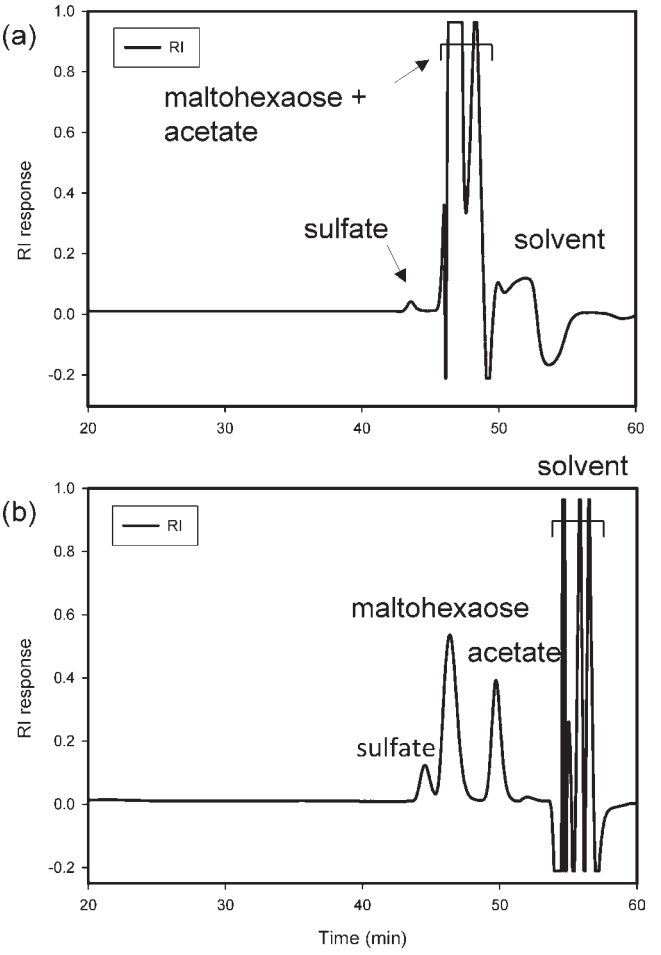

Figure 3. RI signals of a mixture of maltohexaose, acetate buffer, and isoamylase eluted with (a) phosphate buffer (0.1 M, pH 6.2) and (b) sodium nitrate solution of 0.3 M with other conditions the same as those in Figure 1.

influence of sodium nitrate on ion-suppression, concentrations of 0.1 and 0.3 M were used as eluents for debranched corn starch samples. At a concentration of 0.1 M, the signals of acetate buffer and starch molecules were separated but did not reach baseline—baseline separation (Figure 4a). At the higher concentration of 0.3 M, acetate buffer was completely separated from starch molecules (Figure 4b). The use of 0.3 M sodium nitrate as the eluent successfully prevented the interference from acetate buffer in the size-exclusion system. Other benefits of using sodium nitrate included low light-scattering noise and a coherent relationship of the logarithm of molecular-weight and the elution volume (Figure 5).

Interference from Isoamylase Suspension. The commercial isoamylase is suspended in an ammonium sulfate solution. The RI signal of sulfate overlapped with debranched amylopectin molecules and became interference like acetate buffer in a size exclusion system (Figure 6). When a low amount of isoamylase was added for the debranching reaction, the sulfate signal was covered by the amylopectin molecules signal and affected the molecular-weight determination; when a higher amount of isoamylase was added, it was easily misread as an extra fraction (Figure 6, pointed by an arrow) of debranched amylopectin molecules.

An anion exchange resin was employed to remove acetate and sulfate ions in the debranched sample solution. To evaluate the adequacy and efficiency of anion-exchange pretreatment, a mixture of maltohexaose, enzyme suspension, and acetate buffer was loaded onto a column packed with strong anion-exchange resin Amberlite IRA-400. The resin removed the acetate, sulfate, and also maltohexaose (showing its weak anionic property) (Figure 7). A mixed bed resin AG 501-X8 also removed sulfate and part of the maltohexaose after vortexing and resting for 15 min (data not shown). A weak anion-exchange resin Amberlite IRA-67 was tested, and it removed the sulfate, but the sugar molecules remained in the eluate. The major disadvantages of using anion-exchange columns included the time-consuming procedures and the dilution of the sample. The spin column embedded weak anion-exchange material, Vivapure D mini H spin column, also removed the sulfate with less sample dilution of debranched starch molecules (Figure 8). The Vivapure spin column did not completely remove the acetate in the starch sample because of limited column capacity, and breakthrough occurred when a relatively large amount of acetate buffer was applied to the spin column.

Generation of a precipitate of salts is another way to remove sulfate. A barium acetate solution was added to the mixture of debranching enzyme, acetate buffer, and sugar. The barium acetate reacts with ammonium sulfate and produces barium

sulfate, the latter having very low solubility (0.00115 g/L at 18 °C) and can be easily removed by filtration (Figure 9). This method did not interfere with the remaining sugar and did not dilute the sugar concentration.

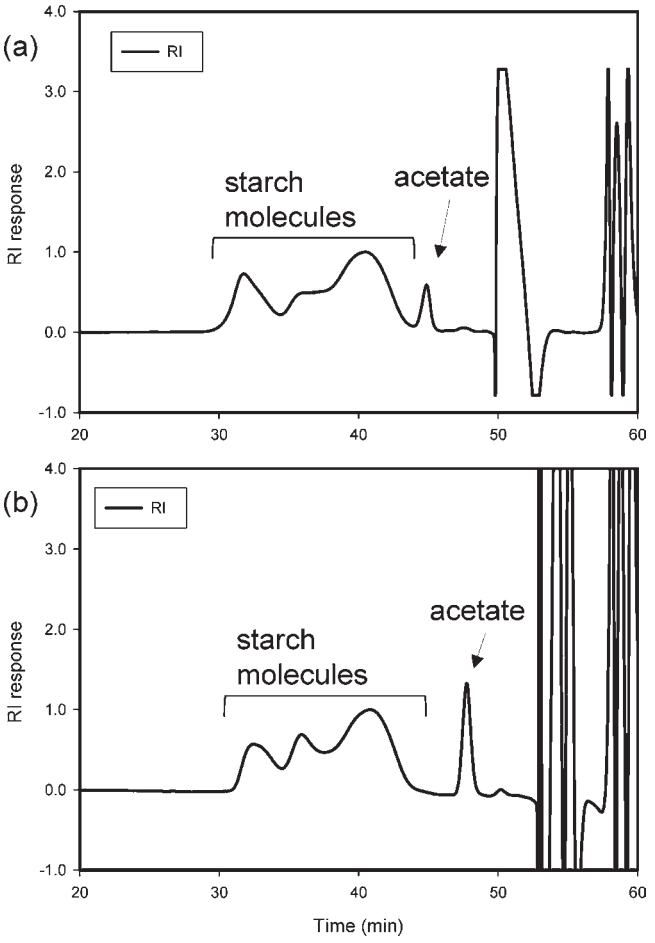

Figure 4. RI signals of debranched corn starch molecules eluted with sodium nitrate solution of (a) 0.1 M and (b) 0.3 M, respectively, with other conditions the same as those in Figure 1.

Values of d*n*/d*c* of Amylodextrin. Amylodextrin molecules with the number average DP 26 were collected by gel filtration column, and then the d*n*/d*c* values were determined by injecting serial concentrations to the RI. The d*n*/d*c* values were 0.147 and 0.133 in solutions of 0.3 M sodium nitrate and deionized water with 0.02% sodium azide, respectively. The d*n*/d*c* value was applied to calculate the molecular weight. The determined molecular weight of maltohexaose was 1022 g/mol, which was less than 10% error.

In our optimized system, which used 0.3 M sodium nitrate as the eluent, sulfate was removed by either ion-exchange or precipitation. We then applied the d*n*/d*c* values determined under the same experiment conditions, and determined values of molecular weight distribution of debranched corn starch molecules, which are listed in Table 1. A comparison of the results obtained with phosphate buffer or sodium nitrate (0.3 M) as the eluents shows that the interferences in the first system affected separation and detection, resulting in overestimated molecular weight of the chain-length of amylopectin molecules.

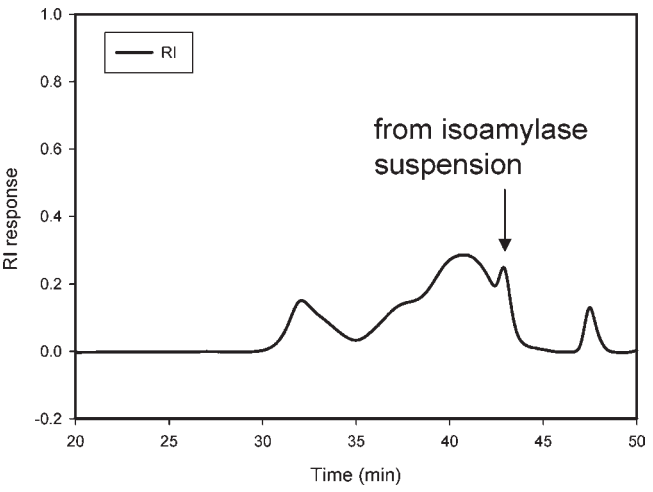

Figure 6. Interference in the HPSEC-MALS-RI chromatogram of debranched corn starch molecules caused by the isoamylase suspension. The chromatographic conditions were the same as those in Figure 5.

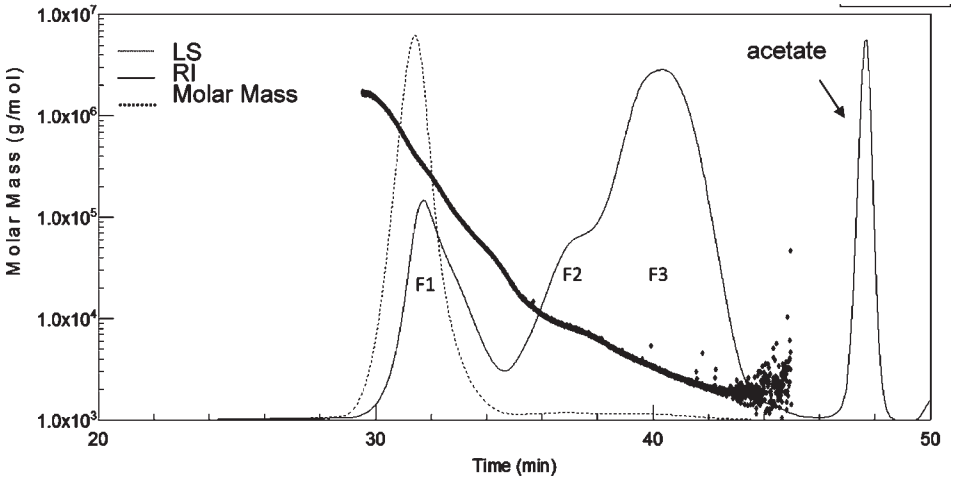

Figure 5. HPSEC-MALS-RI chromatogram of the debranched corn starch molecules eluted with sodium acetate (0.3 M) at 0.5 mL/min on a series of TSK-gel G3000PW$_{XL}$−G2500PW$_{XL}$−G2500PW$_{XL}$ SEC columns at 70 °C. Barium acetate was added to remove sulfate from debranched starch samples. RI, −; LS, - - -; MW, ■.

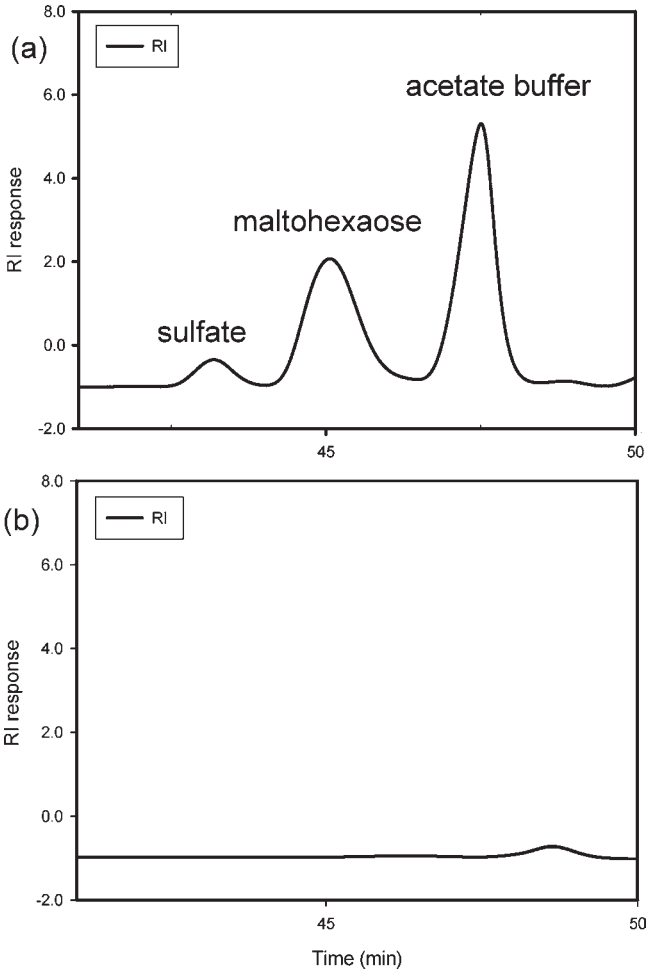

Figure 7. HPSEC-MALS-RI signals of a mixture of maltohexaose and isoamylase (a) before and (b) after elution through Amberlite IRA-400 resin column. The chromatographic conditions were the same as those in Figure 5.

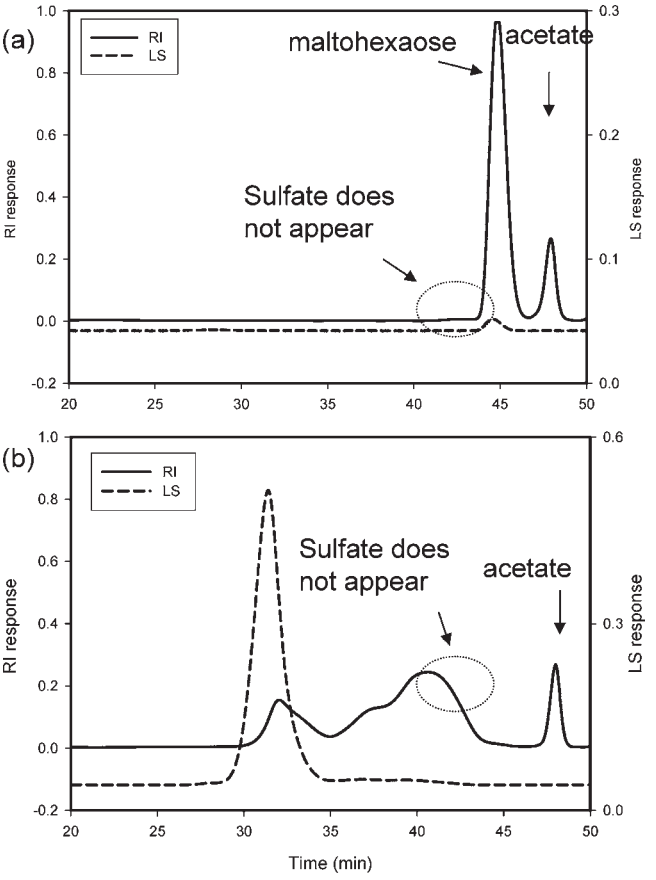

Figure 8. HPSEC-MALS-RI chromatograms of (a) the mixture of maltohexaose, acetate buffer, and isoamylase suspension and (b) debranched corn starch molecules desalted by Vivapure D mini H spin column. The chromatographic conditions were the same as those in Figure 5.

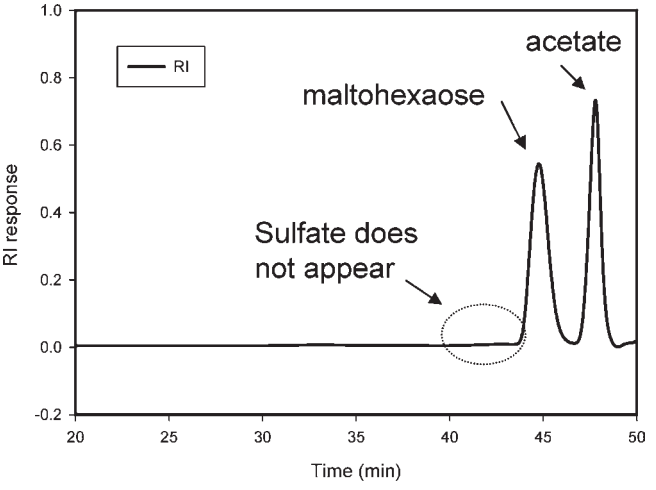

Figure 9. HPSEC-MALLS-RI chromatograms of the mixture of maltohexaose, acetate buffer, and isoamylase reacted with barium acetate. The precipitate had been removed by filtration. The chromatographic conditions were the same as those in Figure 5.

DISCUSSION

Theoretically, a size-exclusion chromatography column separates molecules by hydrodynamic volume affected by molecular size and to some extent by shape. Small molecular-weight salts do not generally interfere with the molecular size determination of polymers. In this study, we noticed that some ionic interference affected the molecular weight determination of debranched starch molecules in a HPSEC-MALS-RI system. Ammonium sulfate from the commercially prepared isoamylase suspension and sodium acetate used as the debranching reaction buffer were the main sources of interferences in the chromatograms because of the ionic interaction between samples and the packing materials of polymeric size-exclusion columns. The common packing materials for SEC columns include carbohydrate matrices such as the cross-linked dextran gels (e.g., Sephadex) or agarose gels (Sepharose), rigid noncarbohydrate matrices such as polyacrylamide gels, the semirigid methacrylate copolymers such as the TSK PW and TSK SW gels, as well as the semirigid styrene and divinylbenzene copolymers, and rigid silica gel. Most sorbents of SEC carry a few residual charged groups on the surface, which can result in undesirable ionic interactions between samples and sorbents.[22,29−31] The ionic interactions include ion exchange, ion exclusion, and ion inclusion.[31−34] It has been reported that TSK PW columns contain approximately 12 μ-equivalents of negative charges per mL resulting from the

presence of carboxyl groups.[35] The distorted peak with a sloping front and a sharp tail obtained with acetate buffer (Figure 2) was the result of the ion-exclusion effect on elution with water. On the leading edge of the peak, the charge sites hindered diffusion of ions into the gel, whereas, on the trailing side, they enhanced diffusion out of the gel interior. With enough ionic strength in the eluent, the acetate buffer exhibited a symmetrical peak (Figure 3b) upon elution with 0.3 M sodium nitrate.

Because of the charge on the surface of the sorbent, substances with the same charge are excluded from the pore by the electrostatic repulsion. Therefore, acetate, a small monovalent anion, eluted faster than expected and overlapped with the debranched starch molecules and maltohexaose if water or phosphate buffer were used as eluent. The effect of electrostatic repulsion was more profound on sulfate, a divalent ion, which eluted prior to maltohexaose even using 0.3 M sodium nitrate to suppress the charges on the surface of the sorbent. Molecules move differently in a size-exclusion column due to the hydrodynamic volume rather than the molecular weight.[36−38] Our study further indicated that ionic interaction significantly altered linear velocity of an ionized molecule in a polymeric size-exclusion column. In some cases, a weak anionic water-soluble polymer, dextran (M_w = 2 kg/mol), could be excluded from the pores of silica gel upon elution with water,[39] but a mixture of pullulans was eluted without a good correlation with molecular size when a silica gel packed column was eluted with water (data not show). In other studies, the addition of ammonium sulfate to the eluent was thought to provide ionic strength and avoid interference peaks from sulfate; however, it has also been mentioned that sulfate ions can enhance the retrogradation of amylose solutions,[40−42] and, therefore, we excluded it from this investigation.

Multiangle laser-light scattering (MALS) equipped with refractive index detection (RI) has been used in the characterization of starch molecules. For chain-length distribution (or molecular weight distribution) studies, MALS provides molecular weight and mass information, and RI provides mass data. The signals from the two detectors may be further processed to generate the absolute molecular weight. The presence of salts significantly affected the RI signal and resulted in overestimation of molecular weight of debranched amylopectin. Molecular weight calculation was another concern. In the past, the limitations of laser strength and wavelength resulted in great noise in the light scattering signals for low molecular weight molecules. A calibration curve of molecular weight and elution volume made by pullulan or dextran standards was applied for the low molecular weight molecules.[43,44] In the present system, the laser detector with a vertically polarized 30 mW gallium-arsenide laser operating at λ = 690 nm gave less noise upon the elution with sodium nitrate (Figure 5). It became possible to read the absolute molecular weight of low molecular weight molecules, despite the systematic deviation in the low molecular-weight region caused by low light scattering signal.[45] The dn/dc value of amylodextrin (0.147) determined at 690 nm at 35 °C on the elution with 0.3 M sodium nitrate is close to 0.146,[17] the value that was determined on the elution with 0.15 M sodium nitrate and has been widely applied in the literature. While this dn/dc value was applied to maltohexaose in the current system, the difference between calculated and theoretical molecular weight was less than 10%. The dn/dc value is affected by the wavelength, temperature, and eluent. The dn/dc of amylopectin is 0.151, 0.155 in water,[46] 0.142 in 1 M potassium hydroxide,[47] and 0.156 in 0.5 M potassium chloride.[48] Thus, our results indicate that the appropriate practice is to determine the dn/dc value with the same system used for the molecular weight analysis.

In polymeric size-exclusion chromatography columns, the few negative charges on the sorbent surface generated undesired ionic interferences between the sample and the packing material. The charges on the chromatography matrix repulsed anions in samples, causing them to elute faster and to overlap with starch sample signals, resulting in deviations of molecular weight determination. Using a weak-anion exchange resin or introducing barium acetate to generate a precipitate can remove the sulfate ion interference. Increasing the ionic strength of the eluent by using 0.3 M sodium nitrate can further diminish acetate ionic interference. Sodium nitrate also provided lower light scattering noise background, which helped to determine the molecular weight of relatively small molecules. The dn/dc of amylodextrin determined in the current system was applied to the calculation and contributed to the improvement of the determination of debranched starch molecules.

Besides aqueous HPSEC, fluorophore-assisted carbohydrate electrophoresis (FACE)[49] and fluorophore-assisted gel-permeation HPLC (F-GPC) using organic solvent (dimethyl sulfoxide)[50,51] as eluent are also useful methods for analysis of starch molecules. These two methods assume that the labeling on reducing ends of molecules is quantitative and independent of the size of glucans[49,52] and is sensitive to small molecules and less sensitive to large molecules.[50−53] Measurement of size distribution of starch molecules is a difficult separation by liquid chromatography, and development of better systems is ongoing in many research groups.[54,55] The interference prevention would be one of the important tasks.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +886-2-3366-4131. Fax: +886-2-2362-0849. E-mail: tjlu@ntu.edu.tw.

Present Addresses

[§]Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, 745 Agriculture Mall Drive, West Lafayette, Indiana 47907, United States.

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