

Molecular Characterization of Corn Starch Using an Aqueous HPSEC-MALLS-RI System Under Various Dissolution and Analytical Conditions

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ABSTRACT

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Molecular characteristics based on absolute weight-average molecular weight (M_w) and z -average radius of gyration (R_g) of normal corn starch were analyzed by high-performance size-exclusion chromatography (HPSEC) attached to multiangle laser-light scattering (MALLS) and refractive index (RI) detectors under different starch dissolution and analytical conditions. Autoclaving (121°C, 20 min) or microwave heating (35 sec) provided better HPSEC recovery and higher M_w for starch molecules than simple dissolution in hot water. The M_w for the autoclaved corn amylopectin and amylose fractions separated with a TSK G5,000 column at 60°C were 201×10^6 and 3.3×10^6 , respectively. The specific volume for gyration (SV_g) calculated from M_w and R_g could be used for the comparison of molecular compactness which was inversely related to the degree

of branching. The SV_g values of amylopectin and amylose fractions in the chromatogram (TSK G5,000, autoclaved for 20 min) were 0.092 and 0.529, respectively. But a portion (20–30%) of large amylopectin molecules did not pass the injection membrane filter (3.0 μ m) and the SEC column, resulting in incomplete recovery. The unfiltered portion varied according to the dissolution treatment. Homogenization (7,000 rpm, 5 or 10 min) of the starch solution improved the recovery of the amylopectin fraction, but significantly increased the M_w of the amylose fraction (17×10^6). Sonication for 5 min degraded starch molecules. For accurate analysis of a native starch using an aqueous SEC, the starch should be fully dissolved with proper treatment such as autoclaving or microwaving, and the column should be improved for full recovery of large amylopectin molecules.

Starch, which is biosynthesized in granular forms by plants, is often used as a functional ingredient in a variety of foods and industrial materials. Macromolecules comprising the starch granules are normally categorized as amylopectin and amylose, based on their linearity (French 1984; Zobel 1988a,b). Molecular structure of both fractions plays a determining role in the physical properties of starch in a dry state or in an aqueous medium.

For the analysis of the molecular structure, disruption of the granular arrangements followed by complete dissolution of the starch chains in a proper medium is a prerequisite. As starch dissolution solvents, dimethyl sulfoxide (DMSO) and aqueous solutions of inorganic alkali are commonly used (Bradbury and Bello 1993, Jackson 1991). Although starch granules in these solvents are ruptured with mechanical and thermal treatments, the starch molecules often are not completely dispersed (Banks et al 1972). Radosta and Vorwerg (1997) emphasized the importance of additional physical treatments for complete starch dissolution during structural analysis with high-performance size-exclusion chromatography (HPSEC). They suggested sufficient heating with mechanical stirring or pressure to effectively break the intramolecular bonds between starch chains. For the structural analysis of starch with HPSEC, various dissolution processes including autoclaving (Aberle et al 1994, Hanselmann et al 1995), microwave heating (Fishman et al 1996; Bello-Perez et al 1998a,b), steam jet-cooking (Klavons et al 1997, Millard et al 1997), and sonication (Jackson et al 1988, Jackson 1991) have been proposed.

Recently, light scattering (LS) properties of natural and synthetic macromolecules have been used in the molecular characterization and solution behavior analysis of the macromolecules. Information obtained from the LS analysis includes the absolute molar mass (M_w), radius of gyration (R_g), and solvent interaction (A_2) of the macromolecules. Before LS analysis, polymers in sample solution can be fractionated on the basis of specific properties such as hydrodynamic volumes, polarity, sedimentation velocity, and specific affinity to the column. Size-exclusion chromatography (SEC) and field flow fractionation (FFF) are commonly used for the fractionation of starch. Waxy corn starch in aqueous DMSO (90%) was treated under different conditions (gentle stirring at 100 rpm, heat-

ing in an autoclave at pH 3.8 and 7.0, or steam jet-cooking), and the molecular weight was measured in a batch mode LS detector (Klavons et al 1997, Millard et al 1997). The average values of M_w and R_g for the waxy starch differed widely at $(30\text{--}750) \times 10^6$ and 150–350 nm, respectively, depending on the dissolution conditions.

Aberle et al (1994), using a SEC column (Ultrasphere) and LS detector, tested the starches from various sources autoclaved at different temperatures (135–155°C) and reported wide ranges of M_w and R_g values: for amylopectin and amylose: $(53.8\text{--}112) \times 10^6$ and 170–237 nm, and $(2.09\text{--}19.6) \times 10^6$ and 19.0–60.1 nm, respectively. Hanselmann et al (1995, 1996) measured the M_w of autoclaved (175°C) waxy corn amylopectin using a sedimentation FFF with LS and reported values at $(37.5\text{--}360) \times 10^6$, depending on the heating period. Klavons et al (1997) steam jet-cooked waxy corn starch and then fractionated the starch molecules with a hydrodynamic column. They measured the M_w and R_g of amylopectin under LS at $(20\text{--}224) \times 10^6$ and 121–320 nm, respectively.

Microwave-heated (80 sec) waxy corn starch was also analyzed by using Shodex OH-Pak columns connected to an LS detector (Fishman et al 1996), and the M_w and R_g values of amylopectin were reported at 26×10^6 and 76 nm, respectively. These values were lower than those determined by autoclaving or steam jet-cooking.

Recently, Yokoyama et al (1998) analyzed waxy corn starch in an organic system with DMSO solution containing LiBr (50 mM). They heated the starch at 95°C and fractionated it in a hydrophobic SEC column packed with polystyrene-divinyl-benzene particles of relatively large size (20 μ m diameter). Under their experimental conditions, the values of M_w and R_g for waxy corn starch were reported at $(215\text{--}239) \times 10^6$ and 252–300 nm, respectively.

You et al (1999) found that there were significant differences in the M_w data between microbatch and HPSEC modes for LS measurement in the analysis of wheat amylopectins. They also pointed out the low recovery from the HPSEC as well as the possible degradation of starch molecules as they pass through the pressurized columns.

The M_w and other structural features of waxy corn starch reported from HPSEC and LS analyses are inconsistent among researchers. This could be due to the sensitivity of the LS analysis, but differences in sample preparation and analytical conditions also may be major contributors. Several possible causes for the discrepancies have been suggested. Many researchers signified the importance of complete dissolution of starch in a medium. Insufficient heating or mechanical stirring may leave some starch molecules

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entangled or aggregated, giving erroneous values. Another possible cause is that the HPSEC column or a frit in the system could retain some of the large molecules (Barth and Carlin 1984, Yokoyama et al 1998). Recovery of a lower amount of starch than that injected into HPSEC proved this. Still another possible cause is from molecular degradation when starch is exposed to high pressure and shear friction during elution through the columns.

In the present study, molecular characterization of amylopectin and amylose of normal corn starch was performed using an aqueous HPSEC system with attached multiangle laser light scattering (MALLS) and refractive index (RI) detectors. Various treatments for starch dissolution (autoclaving, homogenization, sonication, microwave heating), and analysis conditions (columns, flow rate, temperature, injection filters) were compared for the structural analysis.

MATERIALS AND METHODS

Materials

Normal corn starch was obtained from Samyang Genex Co. (Seoul, Korea). Bovine serum albumin (BSA, 68 K), pullulan (98 K), and dextran (2,000 K) were purchased from Sigma Chemical Company (St. Louis, MO). Reagent-grade sodium nitrate (NaNO_3) and sodium azide (NaN_3) were purchased from Showa Chemical Inc. (Tokyo, Japan).

Sample Preparation

Corn starch purified in DMSO and ethanol was dissolved in an aqueous NaOH solution and neutralized with HCl following the procedure of You et al (1999).

The alkaline-dissolved starch solution was further treated by various physical processes: autoclaving (121°C) for 20 or 40 min (HanShin Medical Co., Seoul, Korea), homogenization for 5 or 10 min at 7,000 rpm (Ace Homogenizer, Nissei AM-3, Tokyo, Japan), sonication for 5 min (SaeHan Ultrasonic Co., Seoul, Korea), or microwave heating for 35 sec (900W, NN6585A, Panasonic,

Franklin Park, IL) in a polycarbonate microwave bomb (model 4782, Parr Instrument Co., Moline, IL).

HPSEC-MALLS-RI System

The mobile phase used for HPSEC was aqueous NaNO_3 solution (0.15M) that had been filtered through 0.1- μm cellulose acetate filters (Whatman, UK) and degassed by a vacuum pump for 2 hr before use. The HPSEC-MALLS-RI system consisted of a pump (P2,000, Spectra System, San Jose, CA), an injector valve with a 500- μL loop (model 7,072, Rheodyne, Cotati, CA), a guard column (TSK PWH, Tosoh Corp., Tokyo, Japan), two SEC columns (TSK G4,000 and 5,000 PWXL, 7.8×600 mm), a multiangle laser light scattering detector (MALLS, 632.8 nm, DAWN DSP-F, Wyatt Technology, Santa Barbara, CA), and a refractive index detector (RI, Shodex SE71, Tokyo, Japan). Column temperature and flow rate were 60 or 70°C , and 0.4 or 0.5 mL/min, respectively. The starch solution was filtered through 0.8- or 3.0- μm cellulose acetate filters while it was hot ($\approx 70^\circ\text{C}$) before injection into the HPSEC system.

Detector Calibration

The calibration constant of the MALLS detector was obtained by measuring the light scattering intensity of toluene at a scattering angle of 90° and normalizing with BSA. The calibration constant for the RI detector was determined by injecting five concentrations (0.05–0.5 mg/mL) of aqueous NaCl solution.

To test the system, commercial pullulan (P-100) and dextran (D-2,000) were analyzed. Recovery for both polysaccharides was $>95\%$; M_w was 1.05×10^5 for P-100 and 2.0×10^6 for D-2,000, in agreement with reported values.

Experimental Conditions

Three sets of experimental conditions (Experiments I–III) with different sample treatments and analytical conditions were examined in this study (Table I). The HPSEC system for Experiment I consisted of TSK G5,000 and G4,000 columns serially connected

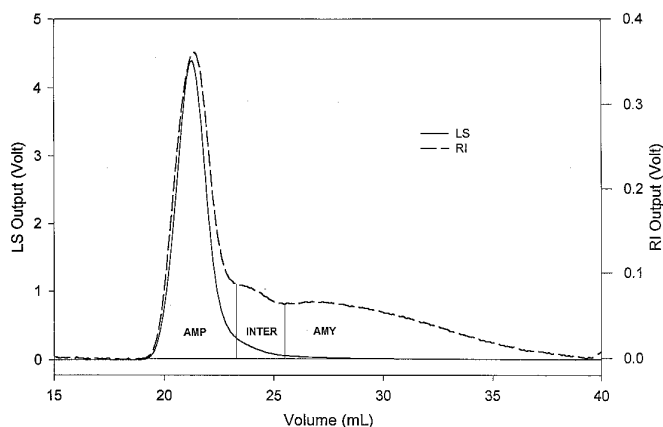


Fig. 1. Light scattering (LS) and refractive index (RI) chromatograms of untreated normal corn starch under conditions for Experiment I.

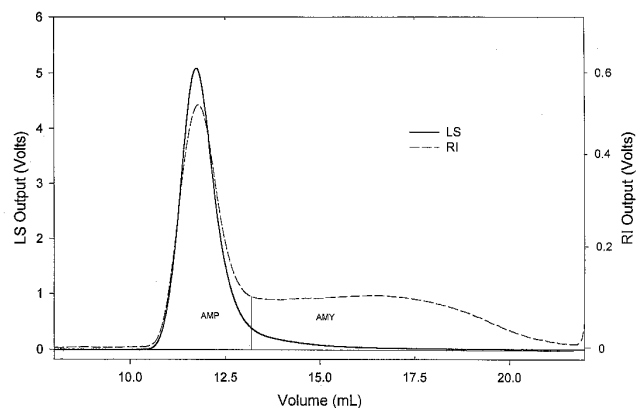


Fig. 2. Light scattering (LS) and refractive index (RI) chromatograms of untreated normal corn starch under conditions for Experiment III.

TABLE I
Starch Dissolution Treatments and High-Performance Size-Exclusion Chromatography (HPSEC) Conditions for Experiments I, II, and III

Experiment	Treatment	Columns	Column Temp. ($^\circ\text{C}$)	Flow Rate (mL/min)	Filter Pore Size (μm)
I	Autoclaving	TSK G4,000 and G5,000 PWXL	60 or 70	0.5	0.8
II	Autoclaving Homogenization Sonication	TSK G4,000 and G5,000 PWXL	70	0.4	3.0
III	Autoclaving Homogenization Sonication Microwave	TSK G5,000 PWXL	60	0.4	3.0 or centrifuge (8,000 $\times g$)

to the injector and maintained at 60 or 70°C. The flow rate of the eluent (0.15M NaNO₃) was 0.5 mL/min. Before sample injection, the starch solution was autoclaved for 20 or 40 min at 121°C and filtered through a 0.8-µm cellulose acetate filter.

In Experiment II, flow rate was reduced from 0.5 to 0.4 mL/min and a membrane filter with a larger pore size (3.0 µm) was used. Also, homogenization (7,000 rpm, 5 or 10 min) and sonication (5 min) treatments were added to the autoclaving treatment. All other experimental conditions were the same as in Experiment I.

In Experiment III, only one column (TSK G5,000) was used and, except for the addition of microwave heating treatment, all experimental conditions were the same as in Experiment II.

RESULTS AND DISCUSSION

Experiment I

Superimposed chromatograms from RI and 90° LS detectors for the corn starch sample that had been alkaline-dissolved without additional physical treatments (untreated) are shown in Fig. 1. On the RI chromatograms, three fractions (amylopectin, intermediate, and amylose) were observed. The M_w values of the fractions were 133×10^6 , 7.72×10^6 , and 2.54×10^6 , respectively. The R_g values of the fractions were 179, 65, and 110 nm, respectively (Table II). However, the HPSEC recovery for the untreated corn starch based on the RI response was <50%, indicating that more than half of the starch in the sample did not elute. However, with autoclave treatment (20 or 40 min at 121°C), the HPSEC recovery increased to 77.8%, but the M_w and R_g values were reduced (A20 or A40 in Table II). When autoclaved for 20 min (A20), the amylopectin fraction in the chromatogram exhibited slightly reduced M_w and R_g ; extended autoclaving (A40) resulted in further reductions of M_w and R_g to 107×10^6 and 167 nm, respectively. The M_w of the autoclaved amylopectin and amylose fractions were similar to those reported by Aberle et al (1995), but the R_g of amylose was significantly higher than the reported value.

Although HPSEC recovery was improved by autoclaving, 20–30% of the starch was still not measured by the RI detector. An attempt to increase recovery was made by raising the column temperature from 60 to 70°C. Recovery was almost complete with this temperature change, but there were significant reductions in M_w and R_g values of the accompanying amylopectin fraction (A20 at 70°C in Table II). The reduction of M_w and R_g values of amylopectin increased the M_w and the percentage of amylose on the chromatogram (2.55×10^6 and 41.8%, respectively, Table II). This result indicates that molecular degradation occurred mainly within the amylopectin fraction. Starch degradation can be caused by pressurized shearing when bulky starch molecules pass through the columns (Klavons et al 1997).

Even at 60°C, where no significant degradation occurred, the amylose content (35.3%) determined by RI was higher than that (≈25%) normally reported by analyses with gel-permeation chromatography or iodine binding (Takeda et al 1987, Hizukuri 1996). The

amylose content obtained from this experiment might not be correct because the HPSEC recovery was not complete. The starch molecules not recovered might be large amylopectins that would make the amylose ratio higher than the actual value.

From the values of M_w and R_g , specific volume for gyration (SV_g) could be calculated as:

$$SV_g = 4/3\pi(R_g \times 10^8)^3/(M_w/N) = 2.522 R_g^3/M_w \quad (1)$$

when the units for SV_g , M_w , and R_g were cm³/g, g/mol, and nm, respectively. In the equation, N was the Avogadro's number (6.02×10^{23} /mol). The SV_g was inversely related to the degree of molecular compactness. The SV_g that a macromolecule exhibits in a solution is significantly reduced by branching (Whistler and BeMiller 1997). The R_g (theoretical probability of finding the molecule at a given distance from the center) has no basis in mass. But the SV_g provides the theoretical gyration volume per unit of molar mass that can give the mass-based information on the density and degree of branching.

Table III presents the SV_g values of untreated and corn starches autoclaved at A20 and A40 under the conditions described for Experiment I. The SV_g of the amylose fraction was 0.82–1.53, which was 7–14× higher than that of amylopectin (0.11), exhibiting the substantial effects of branching on molecular compactness.

The intermediate fraction gave an SV_g value of 0.05–0.09, which was lower than that of amylopectin (0.11), indicating that the molecules in the intermediate fraction were more compact than those in amylopectin. The A20 or A40 intermediate fraction had a lower SV_g value than the untreated intermediate fraction, and this SV_g value was almost half that of the amylopectin fraction in the same treatment. But the molecular weight of the intermediate fraction was <10% of that amylopectin. The starch molecules making up the intermediate fraction could have a greater degree of branching and smaller chain lengths than amylopectin.

Starch molecules with a high degree of branching and small chains might inherently exist in the native granules as a distinct fraction. Phytoglycogens, which have configurations similar to these starch molecules, have been found in mutant rices as biosynthesis intermediates for amylopectin (Nakamura 1996). The low pressure gel-filtration columns, which are normally used for the separation

TABLE III
Specific Volume for Gyration ($SV_g \pm SD$ [cm³/g])^a of Normal Corn Starch Under Conditions for Experiment I

Treatment ^b	Amylopectin	Intermediate	Amylose
60°C			
Untreated	0.109 ± 0.022	0.091 ± 0.004	1.530 ± 1.300
A20	0.106 ± 0.003	0.056 ± 0.013	0.821 ± 0.488
A40	0.110 ± 0.003	0.053 ± 0.003	1.000 ± 0.164
70°C			
A20	0.077 ± 0.052	0.037 ± 0.022	0.053 ± 0.195

^a $2.522 \times R_g^3/M_w$; SD = standard deviation.

^b Treated with autoclaving (121°C) for 20 min (A20) and 40 min (A40).

TABLE II
Weight-Average Molecular Weight and z-Average Radius of Gyration ($M_w \pm SD$ [$R_g \pm SD$]), Amylose Content, and High-Performance Size-Exclusion Chromatography (HPSEC) Recovery of Normal Corn Starch Treated with Autoclaving (121°C) for 20 min (A20) or 40 min (A40) Under Conditions for Experiment I^a

Treatment	Starch Fractions			Amylose Content (%)	HPSEC Rec. (%)
	Amylopectin	Intermediate	Amylose		
60°C					
Untreated	133 ± 8.3 (179 ± 8.5)	7.72 ± 0.12 (65.0 ± 0.72)	2.54 ± 0.02 (110 ± 25)	38.6 ± 3.3	45.3 ± 6.6
A20	121 ± 8.3 (172 ± 1.1)	7.76 ± 0.42 (55.6 ± 3.1)	1.73 ± 0.32 (80.8 ± 12)	35.3 ± 0.7	77.8 ± 0.6
A40	107 ± 4.5 (167 ± 1.1)	7.85 ± 0.84 (54.7 ± 0.74)	1.56 ± 0.34 (85.1 ± 3.3)	37.1 ± 3.0	74.0 ± 1.0
70°C					
A20	60.0 ± 19 (119 ± 20)	6.83 ± 1.5 (45.3 ± 6.9)	2.55 ± 0.72 (79.7 ± 7.3)	41.8 ± 0.22	97.1 ± 3.0

^a TSK G5,000 and 4,000 columns serially connected from injector to detector; flow rate 0.5 mL/min. All starch solutions filtered by cellulose acetate filter (0.8 µm pore size) before injection. SD = standard deviation.

of amylopectin and amylose, elute whole amylopectin molecules as one void volume fraction. In this experiment, two SEC columns serially connected (TSK G5,000 and 4,000) would be capable of fractionating the intermediate molecules.

Another explanation regarding the intermediate fraction is that this short-chained fraction might be generated from amylopectins by shear-induced fragmentation inside the columns. But the chromatogram did not show a smooth shift toward reduced molecular weight as shown in an ordinary starch degradation pattern. The intermediate fraction could be located as a distinct elution peak between amylopectin and amylose fractions, which may imply that the degradation was not random if the intermediate fraction was produced by amylopectin degradation. More research should be done to clarify the origin of the intermediate fraction.

Experiment II

To eliminate or reduce the molecular degradation, the flow rate was reduced from 0.5 to 0.4 mL/min in Experiment II. Also, the starch sample solution was filtered through a membrane filter with a larger pore size (3.0 μm) to reduce any possible filtering loss of the large starch molecules. Under the conditions for Experiment II, the untreated and A20 samples showed higher M_w and R_g values than those measured in Experiment I (Table IV). But HPSEC recovery was not improved even with the higher pore size of the membrane filter. The increased M_w and R_g of the three fractions on the chromatogram may be caused by reduced or eliminated shear degradation. But >20% of the injected starch molecules could not elute from the HPSEC system.

Radosta and Vorwerg (1997) suggested that stirring intensity affected the molecular dispersion of starch in an aqueous medium. They demonstrated that high stirring intensity increased HPSEC recovery, especially for the amylopectin fraction. In this experiment also, the M_w and R_g and HPSEC recovery of starch were significantly raised by homogenization (7,000 rpm for 5 or 10 min) (Table IV). The M_w values of the intermediate and amylose fractions were significantly higher with homogenization as compared with autoclaving (A20). By homogenizing for 5 min at room temperature (H5), the M_w of the intermediate fraction was 27.3×10^6 , which was nearly 4 \times greater than that of the A20 sample. The M_w of the amylose fraction was 16.9×10^6 , nearly 8 \times greater than that of the A20 sample. In addition, homogenization (H5, H10) increased the HPSEC recovery by 5–10% more than that of the A20 sample. The amylose content measured on the chromatogram was 27–28%, which is in accordance with reported values for normal corn starch. This amylose content, which was less than that measured in the autoclaved samples, might have resulted from increased recovery of amylopectin. Intensified stirring might be needed to separate entangled amylopectin molecules in the solution, although the concentration was very low.

The SV_g value clearly demonstrated the conformational differences among the treatments (Table V). The SV_g difference between the autoclaved and homogenized samples was more substantial with the intermediate and amylose fractions than with amylopectin fraction. The SV_g of the intermediate fraction was approximately doubled by homogenization (from 0.068 to 0.13). However, the SV_g of amy-

lose dropped from 2.33 to 0.51 or 0.58. Therefore, it was concluded that the amylose fraction had a totally different conformation of increased compactness, whereas the intermediate molecules were made less compact by homogenization. It is unclear what caused these conformational differences. One possible explanation would be that the mechanical shearing made the short-branched starch chains of the intermediate fractions stretch outward, resulting in raised hydrodynamic volume. Or the high shearing might have made the amylose fraction entangle more readily with the intermediate branches, raising both the M_w and R_g . Due to their linearity, amylose molecules lined up in the direction of mechanical shearing, and these intermolecular linkages increased M_w and decreased SV_g . But it is doubtful that the entangled or lined-up conformations developed during the sample preparation and remained unchanged while eluting from the column. More studies should be undertaken to understand the molecular behaviors induced by homogenization.

Sonication treatment (5 min) showed M_w and R_g values similar to those obtained by autoclave treatment. But, as suggested by the higher standard deviations of the data, the results lacked reproducibility, and molecular degradation was frequent. Depolymerization of amylopectin molecules by ultrasonic shear was also reported by Jackson (1991). When the sonication period was >5 min, the molecular degradation appeared substantial, showing significantly reduced values of M_w and R_g (data not shown).

Experiment III

Considering the starch loss (low HPSEC recovery) and the shear-degradation by the columns in Experiments I and II, only one column (TSK G5,000) was used in Experiment III. The column temperature and flow rate were adjusted to 60°C and 0.4 mL/min, respectively. The starch solutions were filtered through a 3.0- μm cellulose acetate filter as in Experiment II.

The RI chromatogram of the untreated starch showed only two fractions (Fig. 2), indicating that the resolution by one column was not as good as with two (Experiment II). By using one column, the amylose content value was increased from 35.4% (Experiment II) to 51.5% for the untreated sample (Table VI). In addition, the M_w of the amylose fraction also increased markedly. These changes might be caused by the migration of the intermediate fraction found in Experiment II to the amylose fraction. However, the amylose content for the A20 sample (38.4%) was similar to the value obtained in Experiment II, indicating that the intermediate fraction was included in the amylopectin fraction. In the homogenized (H5 and H10) and sonicated samples (S5), the intermediate fraction appeared as a separate fraction on the chromatogram, and this caused the lower amylose content of 24–26% (Fig. 2 and Table VI).

The most interesting result observed in Experiment III was that the M_w and R_g values for the amylopectin fraction (201×10^6 and 194 nm, respectively) in the A20 sample were substantially higher than the values found in Experiment II (144×10^6 and 187 nm). These values were similar to those of waxy maize amylopectin reported by Bello-Perez et al (1998b), Yokoyama et al (1998), and Klavons et al (1997). The waxy maize amylopectin, however, became significantly smaller (26×10^6) when microwaved for 80 sec and

TABLE IV
Weight-Average Molecular Weight and z-Average Radius of Gyration ($M_w \pm \text{SD}$ [$R_g \pm \text{SD}$]), Amylose Content, and High-Performance Size-Exclusion Chromatography (HPSEC) Recovery of Normal Corn Starch Treated with Autoclaving (121°C) for 20 min (A20), Homogenization (7,000 rpm) for 5 and 10 min (H5 and H10), and Sonication for 5 min (S5) Under Conditions for Experiment II^a

Treatment	Starch Fractions			Amylose Content (%)	HPSEC Rec. (%)
	Amylopectin	Intermediate	Amylose		
Untreated	170 \pm 16 (185 \pm 0.63)	8.83 \pm 0.52 (64.5 \pm 4.8)	2.85 \pm 0.24 (107 \pm 1.4)	35.4 \pm 2.3	54.9 \pm 3.2
A20	144 \pm 0.94 (187 \pm 1.4)	7.25 \pm 0.62 (57.8 \pm 2.5)	2.23 \pm 0.02 (125 \pm 17)	37.6 \pm 5.6	70.3 \pm 6.0
H5	232 \pm 3.4 (196 \pm 0.94)	27.3 \pm 14 (109 \pm 20)	16.9 \pm 11 (149 \pm 15)	26.6 \pm 2.1	75.7 \pm 4.8
H10	205 \pm 12 (184 \pm 6.8)	25.9 \pm 10 (108 \pm 19)	12.7 \pm 6.3 (140 \pm 19)	28.2 \pm 3.1	80.2 \pm 3.6
S5	124 \pm 68 (171 \pm 40)	7.68 \pm 1.1 (55.0 \pm 13)	2.65 \pm 1.4 (98.9 \pm 27)	33.1 \pm 1.1	77.9 \pm 20

^a TSK G5,000 and 4,000 columns serially connected and maintained at 70°C; flow rate 0.4 mL/min. All starch solutions filtered by cellulose acetate filter (3.0 μm pore size) before injection. SD = standard deviation.

filtered with a 0.4- μm membrane filter (Fishman et al 1996). Aberle et al (1994) also reported the lower M_w (112×10^6) of amylopectin with autoclaved and filtered normal corn starch (155°C for 20 min, 0.8- μm membrane filter). These low values for the corn amylopectin fraction might be caused by molecular degradation during the starch dissolution treatments or by a low recovery due to the loss of large amylopectin molecules.

The relatively high M_w and R_g values for the amylopectin fraction of the A20 sample in Experiment III, when compared to the values in Experiment II could be attributed to the reduced amylopectin degradation from using only one column (Barth and Carlin 1984, Yokoyama et al 1998) or to the increased recovery of large amylopectins. But the overall recovery of the A20 sample was slightly less than that in Experiment II, proving that the degradation was reduced. Homogenization (H5, H10) increased the M_w and R_g values of the intermediate and amylose fractions, but sonication treatment (S5) induced considerable molecular degradation of amylopectins (Table VI), as found in the previous experiment (Experiment II, Table IV).

Bello-Perez et al (1998b) found that microwave heating (35 sec) was one of the most appropriate dissolution methods for the analysis of starch. Under identical conditions, however, the M_w and R_g values for normal corn amylopectin obtained from this experiment were lower than the values given by Bello-Perez et al (1998b). Furthermore, the amylose content in this experiment was higher (48.5%). This increase could be an indication of possible degradation of the amylopectin fraction from the microwave treatment.

The effects of various physical treatments used for starch dissolution were more evident on the M_w distribution curves (Fig. 3). When the differential weight fraction of untreated, A20, H5, S5, and M35 were compared based on M_w , the H5 starch had a narrow M_w range ($\approx 10^7$ to 10^8) which was a higher M_w region than other starches. But the A20 starch had a wider molecular weight region, ($\approx 10^4$ to 10^8). M35 starch showed a distribution similar to that of A20, but the amylopectin peak was positioned in the lower M_w range and the amylose region became greater, proving amylopectin degradation. Sonicated starch (S5) showed a movement of the amylopectin fraction to a low M_w region, and the separation with amylose was less clear, also indicating the degradation of amylopectins. The homogenized starch sample displayed a unique distribution pattern in which the amylopectin molecules were located in a very narrow range and the amylose fractions had moved to the higher M_w region. This distribution pattern agrees with the previous results of M_w and SV_g . The increased portion of the amylopectins indicates the improved recovery of the molecules. The migration of the amylose fraction to the high M_w region could indicate that high shear during homogenization induced chain associations among the amylose molecules.

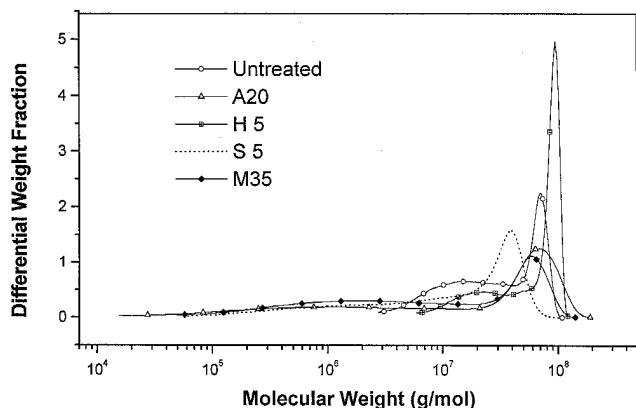


Fig. 3. Weight-average molecular weight (M_w) vs. differential weight fraction of untreated and normal corn starches autoclaved (121°C) for 20 min (A20), homogenized (7,000 rpm) for 5 and 10 min (H5 and H10), sonicated for 5 min (S5), and microwaved for 35 sec (M35) under conditions for Experiment III.

In a separate experiment, starch loss by membrane filtration before SEC injection was tested. Total sugar content in the filtrate, which was passed through a 3.0- μm membrane filter while the solution was hot ($\approx 70^\circ\text{C}$), was measured by the phenol-sulfuric acid method (Dubois et al 1956), and the unfiltered residual starch was calculated. Because of the inhibitory effect of NaNO_3 on the phenol colorization, distilled water was used instead. The filtered starch was significantly different among starch solutions of different dissolution treatments. In a simple dissolution in hot water without any treatment (untreated), 48.8% of the starch in the solution could not pass the membrane (3.0 μm). After autoclaving for 20 min (A20) or homogenization for 5 min (H5), however, only 21 and 31% of the starch resided on the filter, respectively.

Therefore, this experiment revealed that filtration before injection was a major cause of incomplete HPSEC recovery. Autoclaving and homogenization facilitated membrane filtration, allowing starch molecules in the solution to pass more readily through the filter. But there was a significant portion of starch in the sample solution ($>20\%$) that was not filtered. The pore size (3.0 μm) of the filter was significantly larger than the average R_g of the amylopectin fraction. The unfiltered starch might be exceptionally large amylopectin molecules or undissolved starch aggregates. Sonication treatment showed the least filtering loss (3.5%), but was caused by molecular degradation.

To eliminate the filtering loss, autoclaved or microwaved starch solutions (A20 or M35) were centrifuged at $8,000 \times g$ for 15 min at room temperature. There was no precipitate observable by the naked eye in the centrifuge tube. An aliquot was carefully taken from the upper part of the solution and injected into the SEC column without filtration. HPSEC recovery, however, was increased only by 10–15% when compared with the filtered sample, and 20% of the starch still remained undetected. The M_w and R_g values were not significantly changed by centrifuging (Table VI). We proposed that some the large amylopectins that had not been eliminated by membrane filtration could not elute from the SEC column. The SEC column, which was packed with porous beads (1,000 Å pore size, 17 μm bead size), might behave like a filtering device, where large molecules were clogged in the pores or between the column beads. This would explain why waxy starches gave exceptionally low HPSEC recovery when compared with normal starches (Roger and Colonna 1993, Bello-Perez et al 1998). If this is true, for the full recovery of starch without loss of the large amylopectin molecules, the SEC column should be composed of large pore-sized beads.

CONCLUSIONS

High-pressure size-exclusion chromatography (HPSEC) is one of the most popular tools for the fractionation of starch, which is usually done before molecular characterization. But it is clear that the physical treatments for starch dissolution and the analytical conditions in the HPSEC and LS systems significantly affect the structural results. Autoclaving (≈ 20 min) or microwave heating (35 sec) of an aqueous starch sample improved the starch dissolution and gave more accurate structural data than homogenization or sonication (5 min). Homogenization tends to cause the shear-induced

TABLE V
Specific Volume for Gyration ($SV_g \pm SD$ [cm^3/g])^a of Normal Corn Starch Treated in Experiment II

Treatment ^b	Amylopectin	Intermediate	Amylose
Untreated	0.094 ± 0.001	0.078 ± 0.024	1.085 ± 0.060
A20	0.115 ± 0.004	0.068 ± 0.012	2.331 ± 1.282
H5	0.082 ± 0.002	0.132 ± 0.094	0.509 ± 0.212
H10	0.077 ± 0.012	0.134 ± 0.093	0.575 ± 0.316
S5	0.118 ± 0.103	0.064 ± 0.056	1.126 ± 1.093

^a $2.522 \times R_g^3/M_w$; SD = standard deviation.

^b Treated with autoclaving (121°C) for 20 min (A20), homogenization (7,000 rpm) for 5 and 10 min (H5 and H10), and sonication for 5 min (S5).

TABLE VI
Weight-Average Molecular Weight and z -Average Radius of Gyration ($M_w \pm SD [R_g \pm SD]$), Amylose Content, and High-Performance Size-Exclusion Chromatography (HPSEC) Recovery of Normal Corn Starch Treated with Autoclaving (121°C) for 20 min (A20), Homogenization (7,000 rpm) for 5 and 10 min (H5 and H10), Sonication for 5 min (S5), and Microwaving for 35 sec (M35) Under Conditions for Experiment III^a

Treatment	Starch Fractions			Amylose Content (%)	HPSEC Recovery (%)	Filtering Loss
	Amylopectin	Intermediate	Amylose			
Untreated	158 ± 12 (181 ± 5.9)	nd ^b	6.05 ± 2.8 (100 ± 24)	51.5 ± 3.4	48.9 ± 1.6	48.8 ± 2.8
Normal corn (filtering 3.0 μm)						
A20	201 ± 4.0 (194 ± 6.0)	nd	3.03 ± 0.33 (87.1 ± 14)	38.4 ± 2.7	67.0 ± 2.0	20.9 ± 6.4
H5	216 ± 5.0 (185 ± 2.1)	27.4 ± 0.43 (112 ± 1.2)	12.2 ± 0.75 (164 ± 6.4)	24.4 ± 0.75	74.1 ± 0.3	30.7 ± 6.5
H10	195 ± 4.0 (176 ± 0.6)	25.9 ± 2.9 (109 ± 6.9)	11.6 ± 2.3 (154 ± 7.5)	23.6 ± 1.9	79.3 ± 7.3	22.3 ± 4.7
S5	73.8 ± 2.3 (136 ± 1.6)	7.01 ± 0.42 (49.3 ± 0.4)	2.19 ± 0.56 (86.2 ± 1.4)	25.7 ± 2.4	81.3 ± 0.7	3.5 ± 2.2
M35	141 ± 31.1 (180 ± 9.5)	nd	2.57 ± 0.02 (73.5 ± 5.5)	48.5 ± 4.7	67.8 ± 0.3	20.1 ± 0.4
Centrifuge (10,000 rpm)						
A20	173 ± 11.5 (188 ± 5.5)	nd	3.73 ± 0.41 (98.5 ± 3.8)	36.8 ± 4.9	77.2 ± 2.7	
M35	153 ± 28 (179 ± 9.5)	nd	3.65 ± 0.53 (82.5 ± 6.4)	41.9 ± 4.1	82.9	

^a TSK G5.000 column connected and maintained at 60°C; flow rate 0.4 mL/min. All starch solutions filtered by cellulose acetate filter (3.0 μm pore size) before injection.

^b Not detected.

TABLE VII
Specific Volume for Gyration ($SV_g \pm SD [cm^3/g]$)^a of Normal Starch Treated in Experiment III

Treatment ^b	Amylopectin	Intermediate	Amylose
Untreated	0.095 ± 0.013	nd	0.489 ± 0.433
Normal corn (filtering 3.0 μm)			
A20	0.092 ± 0.012	nd	0.529 ± 0.378
H5	0.074 ± 0.004	0.129 ± 0.006	0.916 ± 0.151
H10	0.071 ± 0.001	0.128 ± 0.034	0.800 ± 0.164
S5	0.086 ± 0.004	0.043 ± 0.001	0.738 ± 0.051
M35	0.105 ± 0.023	nd	0.396 ± 0.124
Centrifuge (10,000 rpm)			
A20	0.097 ± 0.009	nd	0.654 ± 0.075
M35	0.096 ± 0.018	nd	0.400 ± 0.092

^a $2.522 \times R_g^3/M_w$; SD = standard deviation.

^b Treated with autoclaving (121°C) for 20 min (A20), homogenization (7,000 rpm) for 5 and 10 min (H5 and H10), sonication for 5 min (S5), and microwaving for 35 sec (M35).

aggregations of amylose, and sonication causes starch degradation. But even with autoclaving or microwave heating, the starch recovery from the SEC column was not complete (≈70–80%). Membrane filter used for HPSEC injection and the column retaining large amylopectin molecules caused the incomplete recovery. Therefore, improvement of the SEC column to provide better elution of the large molecules, possibly by raising the pore and bead sizes, is needed for the accurate structural analysis of starch.

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