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Molecular weights and gyration radii of amylopectins determined by high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors ☆

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Abstract

High-performance size-exclusion chromatography (HPSEC) equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors was used to determine weight-average molecular weight (M_w) and z-average radius of gyration (R_z) of amylopectin of selected starches. Ranges of M_w and R_z values of amylopectin were 7.0×10^7–5.7×10^9 g/mol and 191–782 nm, respectively. Amylopectins of waxy starches had substantially larger M_w than did those of normal starch counterparts. Based on the dispersed-molecular density (M_w/R_z^3), waxy amylopectins displayed, in general, larger dispersed-molecular density than did normal amylopectin counterparts, and amylopectins of the A-type starches had larger dispersed-molecular density than did those of the B-type starches. These results suggested that amylopectins of waxy starches had more branch-chains and no extra long chains, which resulted in more densely packed molecules than did those of normal starch counterparts. The amylopectin of B-type starch had longer but fewer branch-chains, which resulted in smaller dispersed density than did that of the A-type starch. M_w and R_z values of amylose isolated from amylomaize VII starch were also determined to be 2.8×10^5 and 43 nm, respectively.

Keywords: Amylopectin; Weight-average molecular weight (M_w); z-Average radius of gyration (R_z); Multi-angle laser-light scattering (MALLS)

1. Introduction

Starch is one of the most important biopolymers. There are two major components of starch: amylopectin that is a highly branched gigantic molecule and amylose, a primarily linear molecule. Functional properties of starch are affected by molecular weight of amylose and amylopectin. Larger molecular weight (DP_n) of amylose and amylopectin resulted in higher pasting peak viscosity in wheat (Shibanuma, Takeda, & Hizukuri, 1996) and sago starches (Takeda, Takeda, Suzuki, & Hizukuri, 1989). Jane and Chen (1992) reported that the long branch chain-length of amylopectin and the intermediate size of amylose produced the greatest synergistic effect on pasting viscosity of reconstituted starch.

Branch structures of amylopectin molecules have been studied using various chromatographic techniques such as gel permeation chromatography (Craig & Stark, 1984; Jane & Chen, 1992; Wang, White, Pollak, & Jane, 1993), high-performance size-exclusion chromatography (Hizukuri, 1985; Ong, Jumel, Tokarczuk, Blanshard, & Harding, 1994; Yuan, Thompson, & Boyer, 1993), and high-performance anion-exchange chromatography (Jane et al., 1999; Koizumi, Fukuda, & Hizukuri, 1991; Wong & Jane, 1997). Determination of amylopectin molecular weight is challenging because of its gigantic molecules, which are larger than any other synthetic and natural polymers. Lack of calibration standards causes difficulties in determination of the molecular weight of amylopectin using size-exclusion chromatography.

HPSEC equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors has been applied to determine absolute molecular weight of starches (Aberle, Burchard, Vorwerg, & Radosta, 1994; Fishman, Rodriguez, & Chau, 1996). The MALLS detection technique combining with HPSEC is a powerful tool for determining the absolute molecular weights of such macromolecules. To obtain an accurate molecular weight of amylopectin

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using this technique, complete separation of amylopectin from amylose is required.

Methods used to disperse starch molecules for chromatography are critical because entangled amylose/amylopectin molecules cannot be separated by SEC and will affect the molecular weight and gyration radius determined by the MALLS technique. It has been reported that total starch solubility increased with increasing proportion of amylose (Jackson, 1991). Waxy-type starches, on the other hand, are more susceptible to shear-induced fragmentation than are normal starches (Bello-Pérez, Roger, Baud, & Colonna, 1998; Hanselmann, Ehrat, & Widmer, 1995; Millard, Dintzis, Willett, & Klavons, 1997). M_w of waxy maize was reported to be 6.5×10^7 or 5.9×10^8 depending on dispersing conditions (Millard et al., 1997). Jackson (1991) studied the extent of solubility of maize starches in dimethyl sulfoxide (DMSO) using various conditions. He confirmed that maximum dispersibility was achieved using a solution of 90% DMSO/10% water solution. In this study, we used a gentle procedure to prepare starch dispersion in an aqueous solution containing 90% DMSO and separated amylopectin from amylose using an HPSEC system. Molecular weights and gyration radii of amylopectins were determined by using on-line MALLS and RI detection. Branch structures of amylopectin molecules were proposed to explain the relationship between the molecular weights and gyration radii obtained for starches of A- and B-type polymorphisms.

2. Materials and methods

2.1. Materials

Chinese taro, mung bean, waxy rice, sweet rice, green leaf canna, lotus root, water chestnut, cattail millet, normal and waxy barley (from Dr C. W. Newman), and waxy wheat (from Dr R. A. Graybosch) starches were isolated in our laboratory. Other starches were gifts from Dr A. R. Bonilla (green banana), Cerestar, USA (waxy, *ae*, and *du* waxy maize), National Starch and Chemical (tapioca, normal maize, and amylomaize V and VII), and Lykkeby Starkelsen Food and Fiber AB (waxy potato). Normal rice (from Matheson Coleman and Bel), normal potato and wheat (from Sigma Chemical) starches were purchased. Pullulan standards (Shodex STANDARD P-82) were purchased from Showa Denko K.K. (Tokyo, Japan). Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* was purchased from Hayashibara Biochem. Lab. (Okayama, Japan). Deionized water (18.2 MΩ cm) used as an eluent and for sample preparation was obtained from a Milli-Q Reagent Water System (Millipore, Bedford, MA). Other chemicals were reagent grade and used without further treatment.

2.2. Preparation of starch aqueous dispersions for HPSEC

Starch (120 mg) was evenly wetted with 1.2 ml of water and then dispersed in 10.8 ml of dimethyl sulfoxide (DMSO). The suspension was mechanically stirred while heating in a boiling water bath for 1 h and then stirred for 24 h at 25°C. An aliquot (0.4 ml) of starch dispersion (1.0%, w/v) was mixed with five volume of ethanol (2 ml) to precipitate starch. Ethanol-precipitated starch was separated by centrifugation at $6750 \times g$ for 20 min. The starch pellet was then redissolved in boiling water (10 ml) and stirred for 30 min in a boiling water bath. The hot sample solution was filtered through a nylon membrane filter (5.0 μm) and then injected into an HPSEC system. The final concentration of the starch solution filtrate injected (100 μl) was 0.4 mg/ml.

2.3. Molecular weight distribution of amylopectin determined by an HPSEC-MALLS-RI system

An HPSEC system consisted of an HP 1050 isocratic pump (Hewlett Packard, Valley Forge, PA) equipped with an injection valve (100 μl sample loop, Model 7125, Rheodyne), a multi-angle laser-light-scattering detector (Dawn DSP-F, Wyatt Tech. Corp., Santa Barbara, CA) with a He–Ne laser source ($\lambda = 632.8$ nm) and a K-5 flow cell, and an HP 1047A RI detector (Hewlett Packard, Valley Forge, PA). To separate amylopectin from amylose, Shodex OH pak KB-G guard column and KB-806 and KB-804 analytical columns (Showa Denko K.K., Tokyo, Japan) were used. The temperature of the injector and columns was maintained at 55.0°C using a CH-460 column heater and a TC-50 controller (Eppendorf, Madison, WI). Temperature of RI detector was set at 30.0°C. The mobile phase was distilled–deionized water (18.2 MΩ cm) passed through in-line membrane filters (0.2 and 0.1 μm, Millipore, Bedford, MA) at a flow rate of 0.7 mL/min.

2.4. Data analysis

A pullulan standard, P-20 ($M_w = 2.28 \times 10^4$, 5 mg/ml), was used for normalization of multiangle photodiode detectors and for determination of delay volume (0.222 ml) between MALLS and RI detections. Data obtained from MALLS and RI detectors were analyzed using Astra software (Version 4.7.07, Wyatt Technology, Santa Barbara, CA). M_w was calculated using the following equation:

$$K^* c/R_\theta = 1/[M_w P(\theta)] - 2A_2 c \quad (1)$$

where R_θ is the excess intensity of scattered light at angle θ, c the sample concentration, M_w the weight-average molecular weight, A_2 a second viral coefficient; K^* an optical parameter equal to $4\pi^2 n_0^2 (dn/dc)^2/(\lambda_0^4 N_A)$ where n_0 is the solvent RI and dn/dc is the RI increment, N_A Avogadro's number and λ_0 is the wavelength of the scattered light in vacuum. Function $P(\theta)$ describes the angular dependence of scattered light. Expansion of $1/P(\theta)$ to first order gives:

$$1/P(\theta) = 1 + (16\pi^2/3\lambda^2)\langle r_g^2\rangle \sin^2(\theta/2) + f_4 \sin^4(\theta/2) + \cdots \quad (2)$$

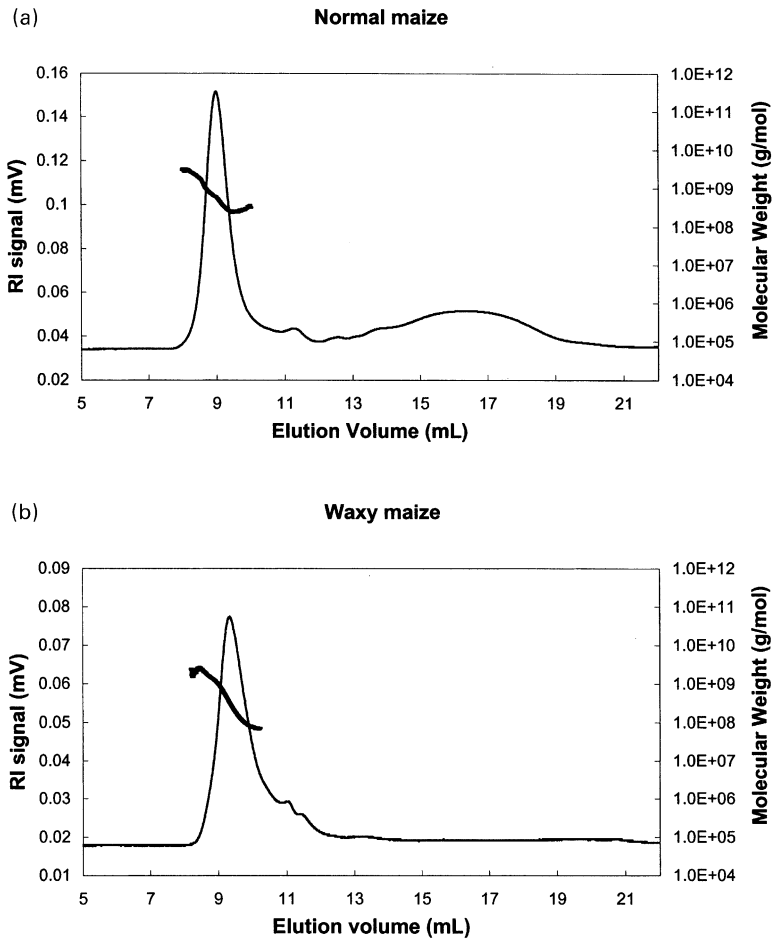

Fig. 1. Amylopectin molecular weight distributions (—) of maize (A) and waxy maize (B) determined by an HPSEC–MALLS–RI system. The RI signal profile (—) is shown throughout the elution volume.

Curve fitting method in this study was based on a second order Berry method ($\sqrt{(K^*c/R_\theta)}$ vs. $\sin^2(\theta/2)$), and a second virial coefficient, A_2 was set at zero (Wen, Arakawa, & Philo, 1996; Yokoyama, Renner-Nantz, & Shoemaker, 1998). M_w and R_z (equal to r_g in Eq. (2)) values were calculated from intercept and slope of the Eq. (1) by extrapolating multiangle signals to zero angle, respectively. The dn/dc value of 0.146 ml/g was used in this calculation (Bello-Pérez et al., 1998; Fishman et al., 1996; Roger & Colonna, 1993).

3. Results and discussion

Molecular weight distributions of amylopectins of normal and waxy maize starches, eluted by distilled–deionized water as the mobile phase and traced by an RI detector, are shown as examples in Fig. 1A and B, respectively. A good separation of amylopectin from amylose was achieved by using the HPSEC system with the operating conditions reported in materials and methods, which enabled us to conduct accurate measurements of amylopectin molecular weights (Fig. 1A). Weight-average molecular weights (M_w) of amylopectins of different starches, calculated using a second order Berry method, are shown in Table 1. Molecular weights and gyration radii of amylopectins obtained in this study were larger than those reported in other studies (Aberle et al., 1994; Fishman et al., 1996). Differences between these results and others can be attributed to the methods of preparing starch dispersions. Millard et al. (1997) reported that methods used to prepare starch dispersion significantly affected molecular weight determination. Using a mild condition, such as direct dispersion in 90% DMSO, they reported M_w of waxy maize starch to be 7.5×10^8 g/mol, which was in agreement with our result (8.3×10^8 g/mol). Millard, Wolf, Dinzis, and Willett (1999) dispersed starch in 90% DMSO and compared the M_w of waxy maize starch obtained from static light scattering with that obtained from analytical ultracentrifugation. In their study, the M_w analyzed by latter method (5.93×10^8 g/mol), calculated using the Svedberg equation, was in good agreement with the light scattering result (5.60×10^8 g/mol). The same authors also reported R_z value (342 nm) for waxy maize starch, which was comparable to R_z (372 nm) calculated from our result.

Another important factor affecting molecular weight

Table 1
Amylopectin molecular weights and gyration radii of selected starches (data were averages of at least two injections)

	M_w ($\times 10^8$)[a]	R_z (nm)[b]	ρ (g/mol/nm^3)[c]
A-type starches			
Normal maize	4.9 (0.8)[d]	312 (23)	16.1
Waxy maize	8.3 (0.2)	372 (11)	16.1
du wx maize	4.9 (0.5)	312 (13)	16.1
Normal rice	26.8 (2.9)	581 (41)	13.7
Waxy rice	56.8 (9.3)	782 (36)	11.9
Sweet rice	13.9 (1.0)	486 (5)	12.1
Normal wheat	3.1 (0.3)	302 (3)	11.3
Waxy wheat	5.2 (0.4)	328 (6)	14.7
Barley	1.3 (0.1)	201 (8)	16.0
Waxy barley	6.8 (0.1)	341 (3)	17.1
Cattail millet	2.7 (0.2)	278 (6)	12.6
Mung bean	3.8 (0.2)	312 (3)	12.5
Chinese taro	12.6 (3.6)	560 (15)	7.2
Tapioca	0.7 (0.1)	191 (25)	10.0
B-type starches			
ae wx maize	3.2 (0.2)	306 (8)	11.2
Amylomaize V	2.4 (0.0)	357 (24)	5.3
Amylomaize VII	1.7 (0.0)	389 (57)	2.9
Potato	1.7 (0.2)	356 (36)	3.8
Waxy potato	2.0 (0.2)	344 (37)	4.9
Green leaf canna	3.4 (2.2)	436 (85)	4.1
C-type starches			
Lotus root	1.5 (0.4)	280 (57)	6.8
Water chestnut	7.1 (1.5)	230 (25)	58.4
Green banana	1.9 (0.8)	286 (29)	8.1
Glycogen			
Cyanobacterial glycogen[e]	0.2 (0.0)	55 (4)	99.2

[a] Weight-average molecular weight.
[b] z-average radius of gyration.
[c] Density (ρ) = M_w/R_z^3.
[d] Standard deviation.
[e] Glycogen was isolated from *Synechocystis* sp. PCC6803 in our laboratory.

calculation is the data fitting method. Yokoyama et al. (1998) applied three different methods (Berry, 1966; Debye, 1947; Zimm, 1948) to analyze M_w and R_z. The authors found that the M_w calculated by the Zimm method was significantly larger than that calculated by Berry and Debye methods. The Zimm method can yield unreasonable results (Aberle et al., 1994), but the Berry method is demonstrated to determine M_w of larger molecules with greater accuracy (Hanselmann et al., 1995). Thus, we chose the second order Berry method (Millard et al., 1997) for our study. The second-order Berry method gave a good curve fitting with laser signals obtained at different angles as shown in amylopectin of sweet rice starch at the peak of the RI signal (Fig. 2).

Among the M_w of the amylopectins reported in Table 1, amylopectins of waxy starches consistently displayed larger M_w than did those of normal starch counterparts. Carbon flux at a form of ADP-Glc (adenosine-5′-diphosphate glucose) is partitioned between amylose and amylopectin in normal starch biosynthesis. Granule-bound starch synthase I (GBSSI) is primarily involved in amylose biosynthesis of starch (Buléon, Colonna, Planchot, & Ball, 1998; Smith, Denyer, & Martin, 1997). In waxy mutants, GBSSI is missing and no amylose is synthesized. It is plausible that ADP-Glc is exclusively incorporated into amylopectin molecules resulting in amylopectin molecules with larger M_w in waxy mutants. Carbon partitioning could also explain substantially smaller M_w of amylopectins of amylomaize V and VII than those of normal and *ae wx* maize. Amylomaize VII has been reported to have less amylopectin of large molecular weight than does normal maize amylopectin revealed by HPSEC (Takeda, Takeda, & Hizukuri, 1993). Fishman et al. (1996) also found, in the analysis of waxy, normal, amylomaize V, and amylomaize VII starches, that the molecular weight of amylopectin decreased with the increase in the amylose content in maize starch. It can also be postulated that space limitation in the normal starch granule because of the presence of ca. 25% by mass of amylose molecules results in smaller M_w of normal amylopectin.

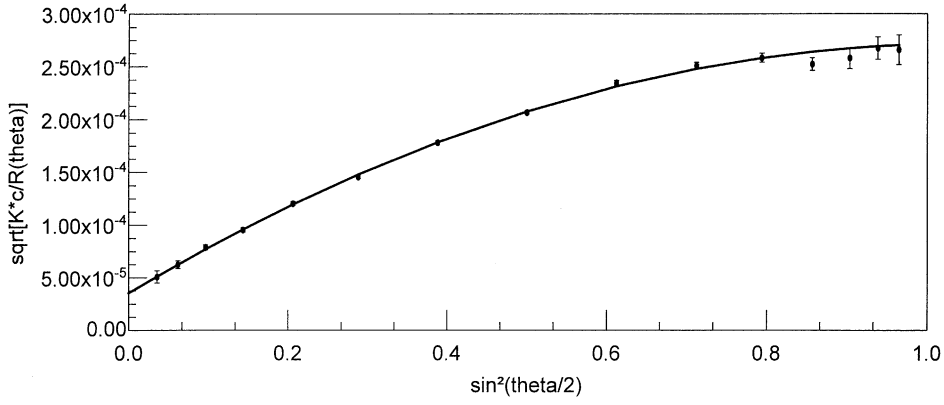

Fig. 2. Light scattering data plot ($\sqrt{(K^*c/R_\theta)}$ vs. $\sin^2(\theta/2)$) of sweet rice amylopectin at the peak concentration based on the second order Berry method. Three detectors at the lowest angle were not used for the curve fitting.

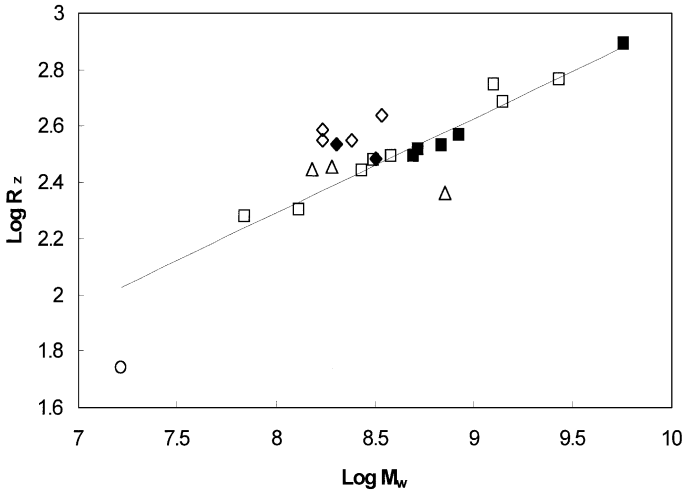

Fig. 3. Relationships of amylopectins between the weight-average molecular weight (M_w) and z-average radius of gyration (R_z). Data are plotted on Log–Log scale: A-type (□); B-type (◇); C-type (△); waxy A-type (■); waxy B-type (◆) amylopectins; glycogen (○). The linear regression line on the graph comprises data of A-type amylopectin.

The M_w of amylopectins isolated from A-type starches varied to a larger range ($0.7–56.8 \times 10^8$) than did those isolated from B-type starches ($1.7–3.4 \times 10^8$). Many amylopectins of A-type starches had larger M_w than did those of B-type starches. Amylopectins of B-type starches also had larger R_z than did those of A-type starches having comparable molecular weights (Fig. 3). Because R_z is related to the volume occupied by the molecule in a solution (Millard et al., 1997), the branch chain-length and branching pattern of the amylopectin molecule are expected to affect the R_z of amylopectin in the solution. Glycogen, being highly branched and compact molecules, displayed a substantially larger dispersed-molecular density ($\rho = M_w/R_z^3$) than did amylopectin (Table 1).

When the log R_z was plotted against log M_w of amylopectin (Fig. 3), A-type starches showed a linear relationship with a slope of 0.334 and a correlation coefficient of $r = 0.98$ ($P < 0.05$). The strong linear relationship between log R_z and log M_w was likely due to similar branching structures of amylopectins among A-type starches. The slope (0.334) indicated that the A-type amylopectins have highly compact spherical forms (Wyatt, 1993). Data of the B-type amylopectins, however, did not give a linear relationship, which could be attributed to differences in branch structures

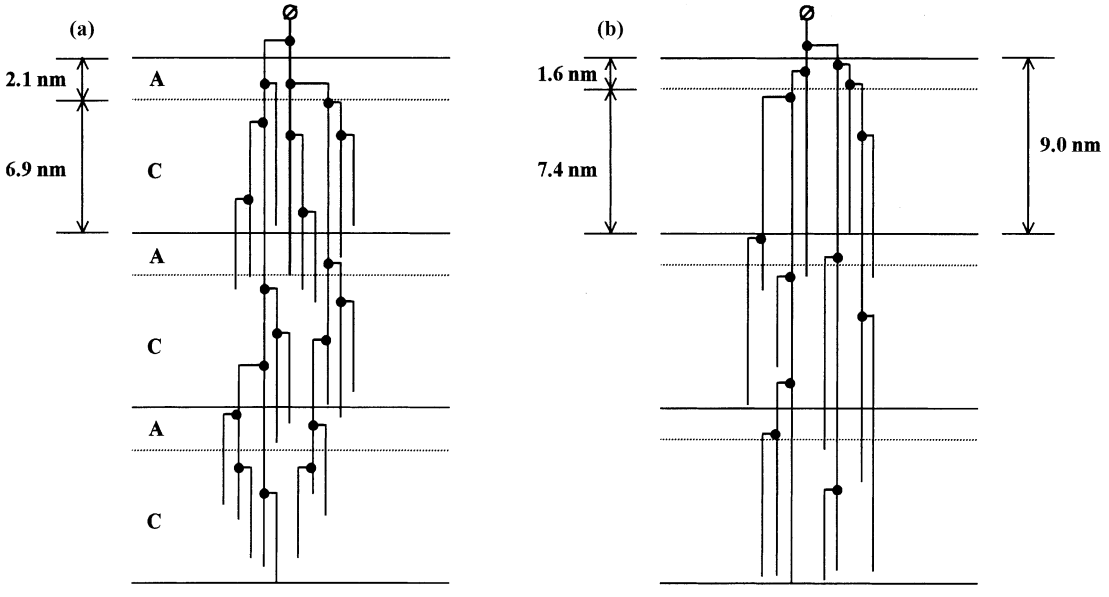

Fig. 4. Structure models of amylopectins of a, normal maize (A-type) and b, amylomaize VII (B-type) starches. A and C stand for the amorphous and crystalline regions, respectively. A repeating distance of 9.0 nm for the cluster (Jenkins et al., 1993; Jenkins & Donald, 1995) and A:B chain ratio of 1.2:1 (Yun & Matheson, 1993) for both starches are used for the models. Average branch chain-lengths of normal maize and amylomaize VII amylopectins are 24 and 31, respectively (Jane et al., 1999).

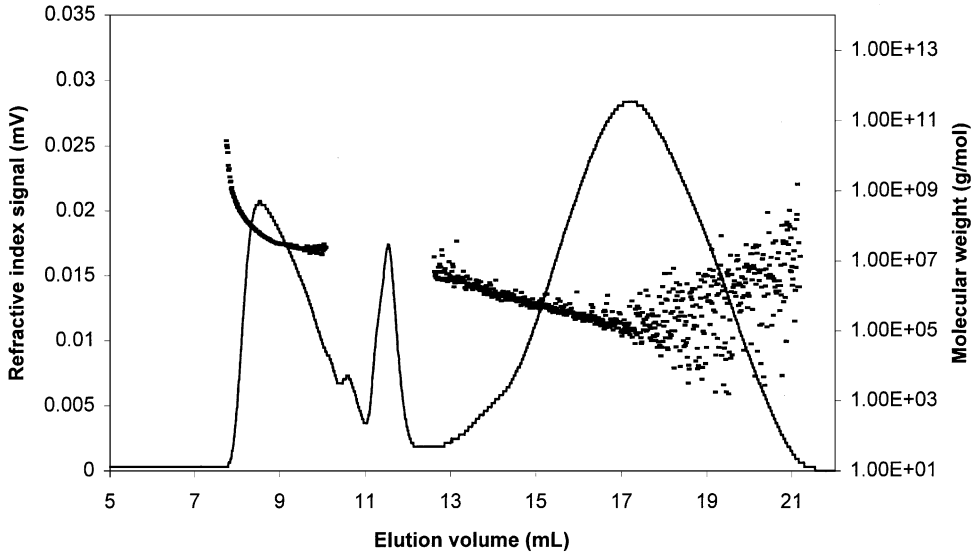

Fig. 5. Molecular weight distribution (—) of amylomaize VII starch determined by an HPSEC–MALLS–RI system. The RI signal profile (—) is shown throughout the elution volume.

of B-type amylopectins. Low density of dispersed B-type amylopectin molecules could result in undefined conformation in aqueous solutions.

Most amylopectins of waxy starches displayed larger dispersed-molecular densities than those of normal starch counterparts (Table 1). The difference might be attributed to extra-long chains of amylopectins. Extra-long chains (ELC), branch-chains that have structures resembling amylose (DP770 or larger) and carry few branches, were found in normal amylopectin but not in waxy amylopectin (Takeda & Hizukuri, 1987; Takeda, Shitaozono, & Hizukuri, 1988; Yoo & Jane, submitted). The presence of ELC that carry few branches in normal amylopectin is likely to decrease the dispersed-molecular density of normal amylopectin. The ELCs that carry few branches can also contribute to the smaller M_w of normal amylopectin. Two A-type amylopectins that displayed smaller dispersed-molecular densities were Chinese taro and tapioca; both are root starches.

Molecular densities of dispersed B-type amylopectins were all smaller than those of the A-type amylopectins except *ae wx* amylopectin (Table 1). Amylopectins of B-type starches comprise longer branch-chains and larger proportions of long B chains than do those of the A-type starches (Hizukuri, 1996; Jane et al., 1999; Takeda et al., 1993). Hizukuri (1985) reported a ratio of short chains to long chains of normal maize amylopectin to be 10, which was at least three times larger than the ratio (3) for amylomaize amylopectin. Differences in branch structures between the A-type and the B-type starches were proposed by Jane, Wong, and McPherson, (1997). They suggest that there are more branches in A-type amylopectins and branch linkages of the A-type amylopectin are scattered and located both in the amorphous and the crystalline regions, whereas there are fewer branches in the B-type amylopectins and most branch linkages are clustered in the amorphous region.

The differences in the molecular weight, gyration radius, and dispersed-molecular density of the A- and B-type amylopectin can be explained by molecular structures of the respective molecules. Structural models constructed using parameters of repeating distance of 9.0 nm (Jenkins & Donald, 1995; Jenkins, Cameron, & Donald, 1993), A/B chain ratio of 1.2:1 (Yun & Matheson, 1993), and the chain-length distribution and molar chain ratio (Jane et al., 1999) for both normal maize (A-type) and amylomaize VII (B-type) amylopectins are shown in Fig. 4. Amylomaize VII has much longer exterior chains (Cheetham & Tao, 1997; Takeda et al., 1993) and larger long-chain/short-chain ratios (Hizukuri, 1985; Jane et al., 1999) than does normal maize. The structure models clearly demonstrate the larger dispersed-molecular density of the A-type amylopectin than that of the B-type.

It was difficult to calculate M_w and R_z of amylose by using the same on-line HPSEC–MALLS–RI system. With the sample concentration of 0.4 mg/ml used in this study and amylose molecules being highly polydispersed, we could not obtain sufficient laser-light scattering signals to calculate molecular weight distributions of amylose of most starch samples. We could, however, determine M_w and R_z of amylose of amylomaize VII that contained about 70% of apparent amylose. The weight-average molecular weight (M_w) and z-average radius of gyration (R_z) of amylose of amylomaize VII were 2.8×10^5 and 43 nm, respectively. The molecular weight was similar to the M_w (1.8×10^5) of amylose calculated by Fishman et al. (1996) using HPSEC-viscometry (Fishman & Hoagland, 1994). Suortti, Gorenstein, and Roger (1998) reported the M_w of amylose to be $4.0–5.0 \times 10^5$ using laser-light scattering technique. The molecular weight distribution of amylomaize VII is shown

in Fig. 5. The weight-average molecular weight of amylose was calculated by using first order Zimm method. The amyloses of amylomaize V and VII have been reported to have the smallest molecular weights among all starches studied (Hizukuri, 1996; Jane & Chen, 1992).

4. Conclusion

Molecular weights (M_w) of amylopectins varied from 7.0×10^7 to 5.7×10^9, depending on the botanical source. Amylopectins of waxy starches had larger molecular weights, and most had larger dispersed-molecular densities than did those of normal amylopectin counterparts. The M_w of A-type amylopectins varied to a larger range (7.0×10^7 to 5.7×10^9) than did that of B-type amylopectins ($1.7–3.4 \times 10^8$). At the same molecular weight, amylopectins of B-type starches had larger R_z than did those of A-type starches. Different branch structures of amylopectins of A- and B-type starches resulted in different dispersed-molecular densities in dilute solutions. The M_w and R_z of amylose from amylomaize VII were 2.8×10^5 and 43 nm, respectively.

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