

Starch Molecular Mass and Size by Size-Exclusion Chromatography in DMSO-LiBr Coupled with Multiple Angle Laser Light Scattering

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ABSTRACT

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The weight average molar mass (M_w) and root mean square radii of starches from waxy maize (Amioca), waxy rice flour, cassava, Hylon V, Hylon VII, and potato amylose were determined by size-exclusion chromatography (SEC) and multiple-angle laser light scattering (MALLS). Dimethylsulfoxide (DMSO) containing 50 mM LiBr was used to dissolve the starches and also served as the mobile phase. SEC with large particle size polystyrene divinylbenzene packing materials and MALLS instrumentation were evaluated for the ability to separate and determine molar mass (MM)

of starch polymers, respectively. The determination of M_w by MALLS is necessary because the M_w of many cereal starches exceeds the available molecular standards by one or two orders of magnitude. The M_w depends on the method of calculation. The M_w (Berry method) of starch from waxy corn was 2.27×10^8 Da, waxy rice 8.9×10^7 Da, cassava 5.7×10^7 Da, Hylon V 2.7×10^7 Da, Hylon VII 4.8×10^6 Da, and potato amylose 1.9×10^5 Da. Recovery dropped dramatically for molecules with root mean square radii >200 nm.

Mass and size are two fundamental physical characteristics of molecules that are not readily determined in cereal starches. This lack of knowledge of starch structure has retarded the development of structure-function relationships between molecular characteristics of starch, and its food applications and digestive properties. The molecular weight or molar mass (MM) of polymers is commonly determined by size-exclusion chromatography (SEC) (Yau et al 1979). However, several experimental difficulties are encountered in attempting to determine MM and size of cereal starch polymers by SEC (Jackson 1988). Cereal starches are difficult to dissolve in aqueous solutions and they may be easily degraded by shear forces in the chromatographic process. Additional difficulties include the lack of calibration standards with MM exhibiting the same order of magnitude as starches. Aqueous high-performance SEC (HPSEC) with multiangle laser light scattering (MALLS) and differential refractive index (DRI) detectors have been used recently to elucidate the weight average molar mass (M_w), size, and structure of amylopectin and starch from various sources (Bello-Perez et al 1996, Fishman et al 1996). MALLS determines polymer M_w without the need for standards to calibrate column elution volumes as required for DRI detection. This is not only a convenience but a necessity because polymer standards >2 million Da are not available.

Solubilization of many cereal starches in aqueous solvents requires high temperatures and high pH that can result in molecular size reduction due to degradation or depolymerization, oxidation, or high MM due to incomplete disassociation or aggregation. When waxy corn starches were solubilized in water by different techniques, a wide range of M_w values were determined: $10\text{--}30 \times 10^6$ Da (Jackson et al 1988, 1989), $146\text{--}168 \times 10^6$ (Erlander and French 1958) and 400×10^6 Da (Banks et al 1972).

Dimethylsulfoxide (DMSO) or aqueous DMSO solutions have been used to solubilize cereal starches in preparation for analysis of total starch (Libby 1970) (AACC 1995), nuclear magnetic resonance (Gidley 1985, Kasemsuwan and Jane 1996) and SEC (Kobayashi et al 1985, Chuang and Snyder 1987). DMSO has been used previously as the mobile phase in SEC of starch utilizing silica-based column packing material (Stone and Krasonski 1981). Newer pack-

ing materials based on polystyrene-divinylbenzene have been developed. This packing material is available with large pore sizes capable of separating polystyrene polymers with MM as high as 100×10^6 Da. This packing material is also available with large particle sizes (20 μm) and large frit porosity (10 μm) that reduce shear degradation of large starch polymers relative to high-resolution columns. Chromatographic columns for HPSEC of starch molecules >10 million Da must be carefully evaluated to recognize polymer degradation or selectivity as shown in this study.

The purpose of this study was to: 1) determine the MM resolving ability of large pore and particle size polystyrene divinylbenzene columns with large pore frits using DMSO with LiBr mobile phase; and 2) evaluate the suitability of an absolute molecular weight detector (MALLS coupled with DRI) to characterize the MM and size of cereal starches.

MATERIALS AND METHODS

Pullulan (P-10) and dextran molar mass standards (T70 and T2000) were obtained from JM Science (Grand Island, NY) and Pharmacia BioProcess Technology AB (Uppsala, Sweden), respectively. Waxy corn (Amioca), cassava and high-amylose corn (Hylon V and Hylon VII) starches were obtained from National Starch and Chemical Co. (Bridgewater, NJ). Potato amylose Type III (A0512), essentially free of amylopectin, was obtained from Sigma Chemical Co. (St. Louis, MO). Waxy rice was provided by the USDA-ARS, Rice Quality Laboratory, Beaumont, TX.

Sample Preparation for HPLC

All starches were used as supplied. Whole rice grains were reduced to flour by a Wiley mill fitted with a #70 mesh screen. Dimethylsulfoxide, HPLC grade, (Sigma) containing 50 mM purified LiBr (Fisher Scientific, Fair Lawn, NJ) was used as the mobile phase for the HPLC and the solvent for the starch samples. LiBr-DMSO solution was added to test tubes containing 20 mg of starch or flour to obtain a final concentration of $\approx 0.4\%$ (w/w). All samples and standards, except P-10, were heated to 95°C while stirring for 15 min in a Reactitherm stirring heating module (Pierce Chemical Co., Rockford, IL). The starch DMSO dispersions were allowed to cool slowly to room temperature while continuously stirring for ≈ 18 hr. The sample solutions were clear, except for the Amioca and waxy rice, which were slightly cloudy, indicating incomplete solubilization. Samples were centrifuged for 10 min at 10,000 rpm (Eppendorf, model 5415C) before analysis. Recovery of starch in the supernatant was determined by a total starch assay procedure modified for use with DMSO (AACC 1995). Aliquots of supernatant (0.75 mL) were diluted 1:1 using DMSO with 50 mM LiBr to a final concentration of 0.2%. Each sample was made in duplicate.

¹ USDA, ARS, Western Regional Research Center, 800 Buchanan St., Albany, CA 94710. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Chromatography

The mobile phase was pumped (model 1350, Bio-Rad Laboratories, Hercules, CA) through an inline 0.5- μm filter to a Rheodyne injector fitted with a 100- μL injection loop to the chromatography columns to a Dawn DSP-F MALLS photometer with a helium neon laser source ($\lambda = 632.8 \text{ nm}$) and a K-5 flow cell (DAWN DSP-F, Wyatt Technology Corp, Santa Barbara, CA) and through a DRI (Waters 410 differential refractometer). The following sets of columns were evaluated: A) one Plgel Mixed-A (Polymer Labs; Amherst, MA) and two Styragel HMW7 (Millipore Corp. Milford, MA); B) TSK G7000HXL and G5000HXL columns (TosoHaas, Montgomery, PA). The frit porosity and particle packing sizes for the column sets were A) 10 and 20 μm , respectively, and B) 5 and 9 μm , respectively. The columns were placed in a column heater kept at 55°C. The mobile phase was 50 mM purified LiBr in HPLC-grade DMSO (LiBr-DMSO) that was helium sparged. The flow rate was 0.4 mL/min. The refractive index of the mobile phase was determined by refractometer (Milton Roy Co., New York, NY) to be 1.4785 at 550 nm, and 25°C. The dn/dc value of 0.066 for starch in DMSO was used for the M_w calculations.

Data Treatment

Data were analyzed by Astra software (Version 1.4, Wyatt Technology, Santa Barbara, CA). The basis of the determination of M_w and root mean square radius of large polymers in solution (Zimm 1948) by the software is:

$$R_\theta/K^*c = MP(\theta) - 2A_2cM^2P^2(\theta) \quad (1)$$

where R_θ is the Rayleigh ratio or excess scattering of the polymer in solution when compared to that of the solvent in solution at angle θ ; c is the concentration of solute; M is the weight average MM , and A_2 is the second virial coefficient. K^* is the optical constant = $4\pi^2 n_0(dn/dc)^2 \lambda N_A$ where n_0 is the refractive index (RI) of the solvent, dn/dc is the change in RI with polymer concentration at the wavelength (λ) of the scattered light, and N_A is Avogadro's number.

MM (g/mol)

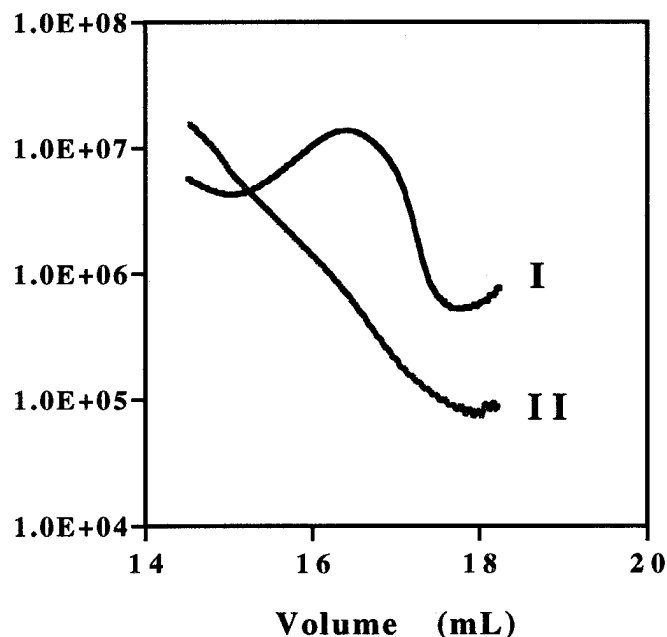


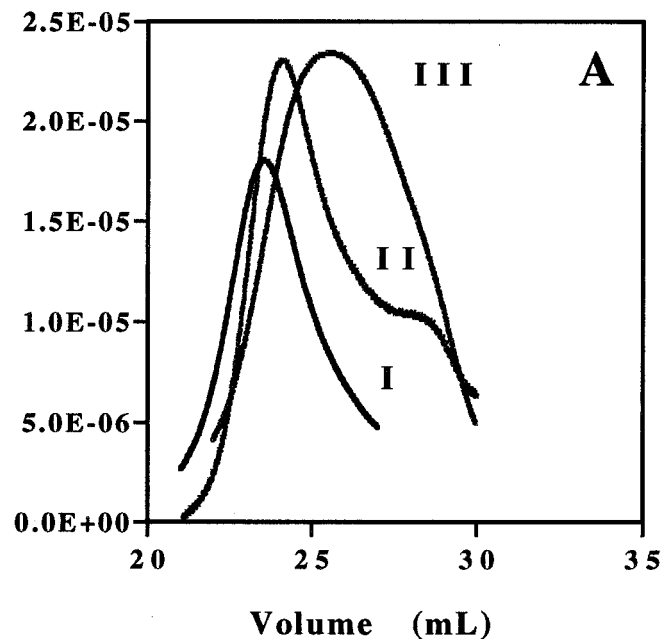
Fig. 1. Molar mass (MM) determined by multiple-angle laser light scattering (MALLS) vs. elution volume plots of a mixture of equal concentrations of dextran standards (70,000 and 2,000,000 Da) in dimethylsulfoxide (DMSO) (I) and in 50 mM LiBr in DMSO (II). Addition of LiBr results in an inverse relationship between MM and elution volume.

$P(\theta)$, the scattering function, is usually applied in the form of the first two terms of the power series shown in Eq. 2, where m is equal to $(4\pi/\lambda) \sin(\theta/2)$ and $\langle r^2 \rangle$ is the mean square radius (MSR).

$$P(\theta) = 1 - 2m^2\langle r^2 \rangle/3! + \dots \quad (2)$$

This is a general result that does not require information about the shape of the polymer (Kratovich 1987).

Conc. (g/mL)



MM (g/mol)

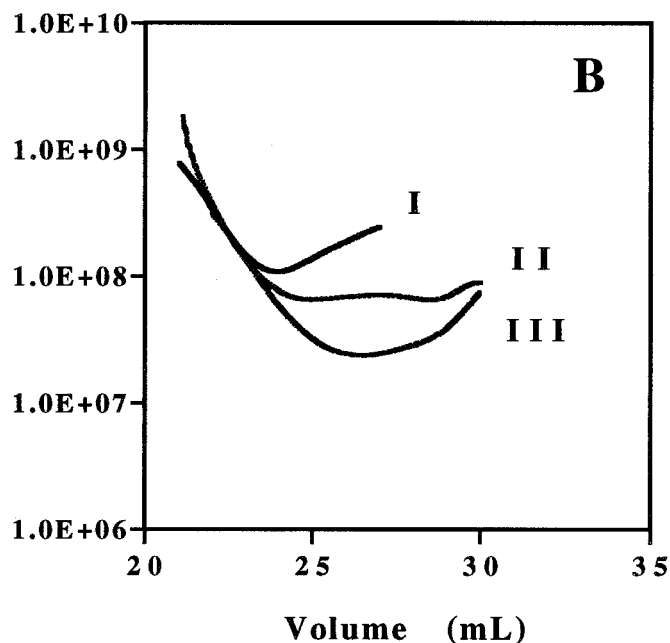


Fig. 2. A, Concentration chromatograms and molar mass (MM) vs. elution volume. **B,** MM vs. elution volume. Waxy corn (I), waxy rice (II), and tapioca (III).

Samples were analyzed for M_w and root mean square radius using three different methods to extrapolate n th degree polynomials fit to Eq. 1 to 0 angle. The extrapolation methods are commonly referred to as : 1) R_θ/K^*c (Zimm 1948); 2) K^*c/R_θ (Debye 1947); and 3) $\sqrt{(K^*c/R_\theta)}$ (Berry 1966) vs. $\sin^2(\theta/2)$. M_w is determined by extrapolation of the functions defined by the three methods to 0 angle. In this study, $A_2 = 0$, and the validity of this assumption was verified by the observation of the same MM at two different concentrations.

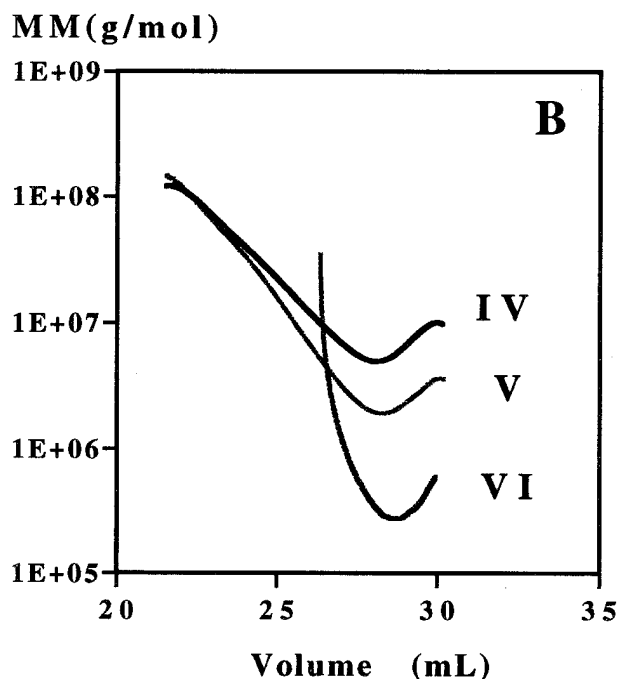
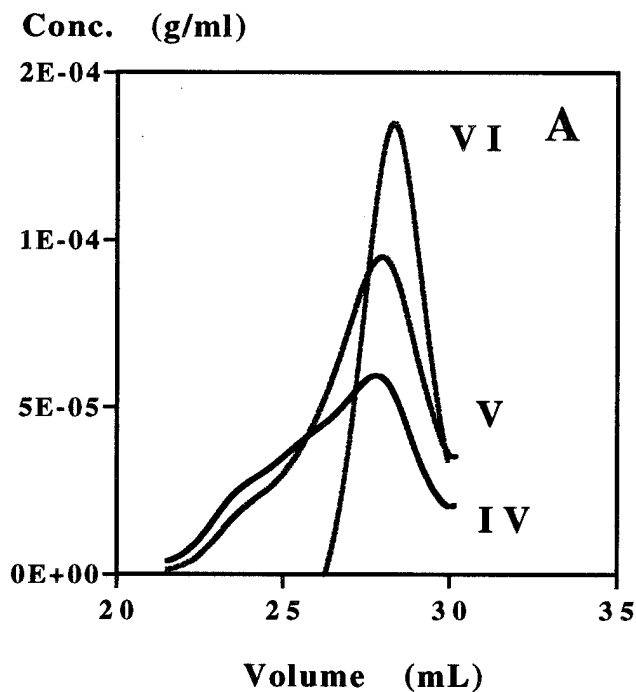


Fig. 3. A, Concentration chromatograms and molar mass (MM) vs. elution volume. B, MM vs. elution volume. Hylon VII (V), Hylon V (IV), and potato amylose (VI).

RESULTS AND DISCUSSION

The linearity of a combination of two 20- μ m particle size columns, the Plgel Mixed-A with a resolving range of 1,000–40,000,000 and Styragel HMW 7 with resolving range of 500,000–100,000,000 was evaluated by MALLS for a mixture of dextran standards: T70 and T2000, with a M_w of 70,000 and 2,000,000, respectively. The mixture of two dextran standards were eluted with DMSO and LiBr-DMSO. The MM vs. elution volume curves from the MALLS detector are shown in Fig. 1. The curve is linear between 40,000 and 8,000,000 when an ionic component (LiBr) is included. This result is consistent with previous studies of SEC of carbohydrate polymers on silica-based column packing material (Chuang and Snyder 1987). A review of semirigid polymer gel packing materials suggested that ionic modifiers are usually necessary to minimize the effects of packing material surface chemistry and

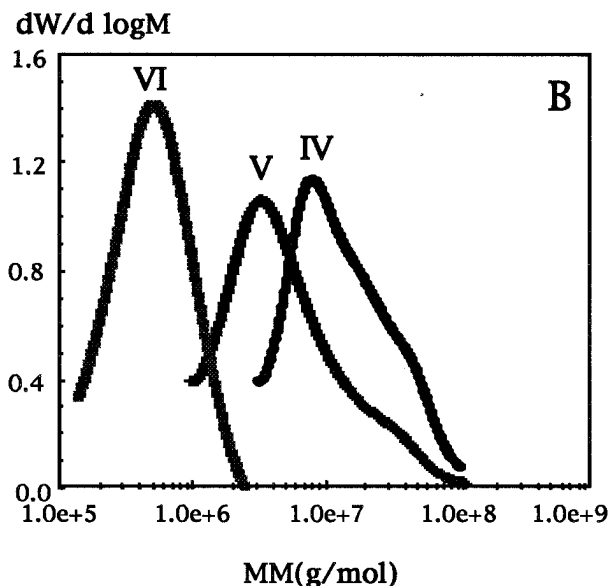
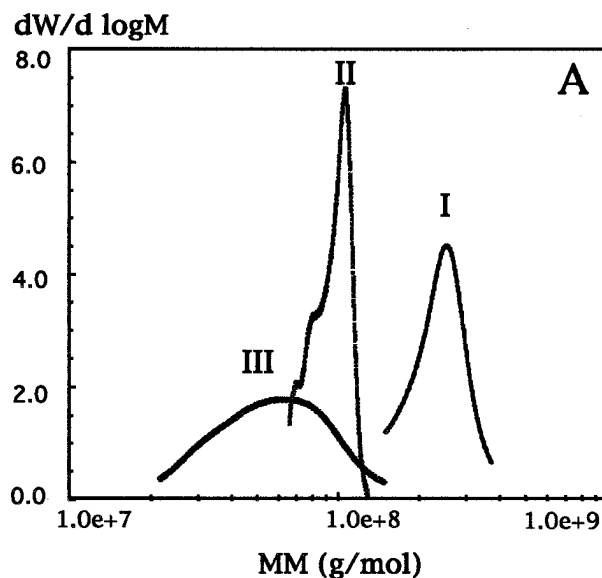


Fig. 4. Differential weight fraction vs. molar mass (MM) plots. A, Waxy corn (I), waxy rice (II), and tapioca (III). B, Hylon VII (V), Hylon V (IV), and potato amylose (VI).

ionic components of the polymer (Meehan 1995). Polymer-polymer, polymer-solvent, and polymer-support interactions could also occur in SEC using polar organic solvent mobile phases (Omorodion et al 1981, Yu and Rollings 1987, Corona and Rollings 1988).

All 18 detectors of the MALLS were normalized to equal scattering signal intensity with a pullulan standard of a known M_w (P-10; 1.22×10^4 Da). The normalization was verified with dextran (T2000; nominal M_w 2×10^6 Da) in 50 mM LiBr in DMSO. For large polymers, proper normalization is achieved if scattering intensities of the laser at the low-angle detectors are much greater than the scattering intensities at larger angles. The light scattering peak heights should decrease uniformly as the scattering angle increases from 0 to 180° (detectors 1–18, respectively). A second-order polynomial fit was used to determine the M_w of the T2000 using the Debye extrapolation method. The M_w for T2000 was determined to be 2.24×10^6 Da. This value agrees with that of Fishman et al (1996), who reported values for T2000 dextran of 2.210×10^6 Da using aqueous HPSEC and second-order polynomial fit using the Debye extrapolation method.

The chromatograms of carbohydrate polymers of starches (cassava, waxy corn, Hylon V, Hylon VII), a waxy rice flour, and potato amylose by polystyrene divinylbenzene columns (set A) eluted with 50 mM LiBr in DMSO are shown (Figs. 2 and 3). The waxy starches eluted before potato amylose as expected. The shoulder of the waxy rice chromatogram (Fig 2A, line II) may be due to distinct populations of amylopectins or method of preparation different from the commercial starches. Water present in native starches chromatographed as a large negative peak, which in all cases was well resolved from the starch peak.

The size of the packing material and the frit porosity can affect recovery of starch. The recoveries of starch chromatographed on the larger packing material and frits (set A) are shown in Table I and the corresponding size of the starch molecules are shown in Table II. Significant retention of starch molecules, which occurs only with radii >200 nm (Berry method), occurred with 20- μ m particle size packing materials. These results suggest that even with the larger packing material and frit porosity, the largest carbohydrate polymers may be retained by the frit or column packing, thus reducing mass recovery. When higher resolution columns (set B: TosoHass G7000HXL, G5000HXL) with smaller particles and frit porosity were used, the M_w for potato amylose, Hylon VII, and waxy corn were lower compared to the larger particle and pore size column system (set A) (Table III). The small frit porosity and particle packing material may induce shear degradation. However, the reason small fragments would not have been accounted for in the recovered mass is that the low MM material may have coeluted with the moisture in the sample which appeared as a large negative peak at the end of the chromatogram. It should be noted that all starches,

except potato amylose, were only partially solubilized (Table I). The degree of self-association that decreased solubility did not affect recovery of the solubilized starch, with the exception of waxy corn (Amioca).

Analysis of the MALLS-DRI signals show a complex relationship between elution volume and MM for extremely high molecular weight starches (Figs. 2B, 3B). The cereal starches show an upward curvature at high-elution volumes, indicating a non-size-exclusion effect. These results show that the solvent system is not optimum for cereal starches. A small amount of water (10–15%) greatly improves DMSO as a solvent (French 1984). However, HPLC column manufacturers do not recommend the use of water in non-aqueous columns due to swelling of column packing material. The phenomena of increasing MM with increasing elution volume has been observed previously with high molecular weight branched dextrans and hyaluronic acid (Wyatt and Short 1991) and highlights the need for MM determination methods that are not dependent on extrapolated calibration standards. Molecular shape, polymer-column interactions, microgel formation, or internal chain density may be responsible.

The weight fraction vs. MM are shown in the differential chromatograms (Fig. 4A and B). The differential chromatograms show that the MM of waxy corn is higher than that of waxy rice or cassava. The differential plot (Fig. 4B) clearly shows that Hylon VII has a distinct peak of lower mass when compared to Hylon V. Using aqueous SEC, researchers reported the MM of the high-amylose corn starches was intermediate between that of potato amylose and the waxy amylopectins (Fishman et al 1996). The polydispersity of Hylon VII is also greater than the other high amylose starches (Table I).

TABLE II
Z-Average Radius (nm) Determined by Three Extrapolation Methods

Sample	Duplication	Berry ^a	Zimm ^a	Debye ^b
Potato amylose	1	82 (6) ^c	96 (5)	128 (5)
	2	69 (9)	86 (6)	118 (6)
Hylon VII	1	124 (3)	154 (2)	138 (3)
	2	124 (3)	151 (2)	137 (3)
Hylon V	1	202 (1)	369 (2)	155 (3)
	2	208 (1)	382 (2)	157 (3)
Cassava	1	217 (1)	283 (1)	138 (3)
	2	226 (1)	286 (2)	165 (3)
Waxy rice	1	199 (1)	345 (2)	157 (3)
	2	211 (1)	345 (2)	158 (3)
Waxy corn	1	252 (1)	511 (2)	170 (4)
	2	300 (1)	500 (2)	171 (4)

^a Second-order fit.

^b Fifth-order fit.

^c Precision of polynomial fit in (%).

TABLE I
Weight Average Molar Mass (M_w) of Starches and Rice Flour Determined Using Three Extrapolation Methods ($\times 10^{-6}$)

Sample	Duplication	Berry ^a	Zimm ^b	Debye ^b	Polydispersity	HPLC Recovery (%) ^c
Potato amylose	1	0.209 (4) ^d	0.221 (5)	0.258(4)	1.7–1.8	100 (100)
	2	0.177 (5)	0.186 (6)	0.223 (6)	1.9–2.0	
Hylon VII	1	4.80 (3)	5.26 (6)	5.32 (2)	3.0–3.5	100 (74)
	2	4.91 (3)	5.49 (3)	5.37 (2)	2.6–3.0	
Hylon V	1	29.1 (3)	38.1 (9)	26.6 (3)	1.9–2.2	100 (64)
	2	25.5 (3)	33.3 (10)	22.9 (3)	1.8–2.0	
Cassava	1	56.9 (3)	82.9 (12)	48.4 (3)	1.3–1.6	97.8 (49)
	2	57.6 (4)	85.9 (11)	47.5 (4)	1.3–1.5	
Waxy rice	1	89.1 (3)	116 (8)	78.7 (3)	1.1–1.2	100 (21)
	2	90.5 (3)	116 (8)	77.8 (3)	1.1–1.2	
Waxy corn	1	215 (3)	383 (16)	156 (4)	1.2–1.5	54 (46)
	2	239 (6)	340 (15)	156 (6)	1.1–1.5	

^a Second-order fit.

^b Fifth-order fit.

^c Solubility in DMSO (%).

^d Precision of polynomial fit (%).

The M_w values are based on a well-established relationship between M_w and light scattering intensity at 0 angle (Eq. 1). However, the method of extrapolation to 0 angle for large molecules is analogous to dividing a nonzero value by a number approaching zero.

M_w calculations by extrapolation of the same experimental data by Berry, Zimm, and Debye methods lead to variable results for large molecules (Table I). Using all three methods, similar results were obtained for the potato amylose, the starch with the lowest M_w and

TABLE III
Comparison of Weight Average Molar Mass (M_w) ($\times 10^{-6}$) Determined by Large and Small Packing Particles

Sample	Duplication	Molar Mass ^a		Recovery (%)	
		Large Particle ^b	Small Particle ^c	Large Particle	Small Particle
Potato amylose	1	0.209 (4) ^d	0.141 (4)	100	86
	2	0.177 (5)	0.147 (5)		
Hylon VII	1	4.80 (3)	3.93 (3)	100	78
	2	4.91 (3)	3.33 (3)		
Waxy corn	1	215 (3)	157 (3)	54	39
	2	239 (6)	161 (3)		

^a Berry method, second-order polynomial fit.

^b 20 μm , 10 μm frit.

^c 9 μm , 5 μm frit.

^d Precision of polynomial fit (%).

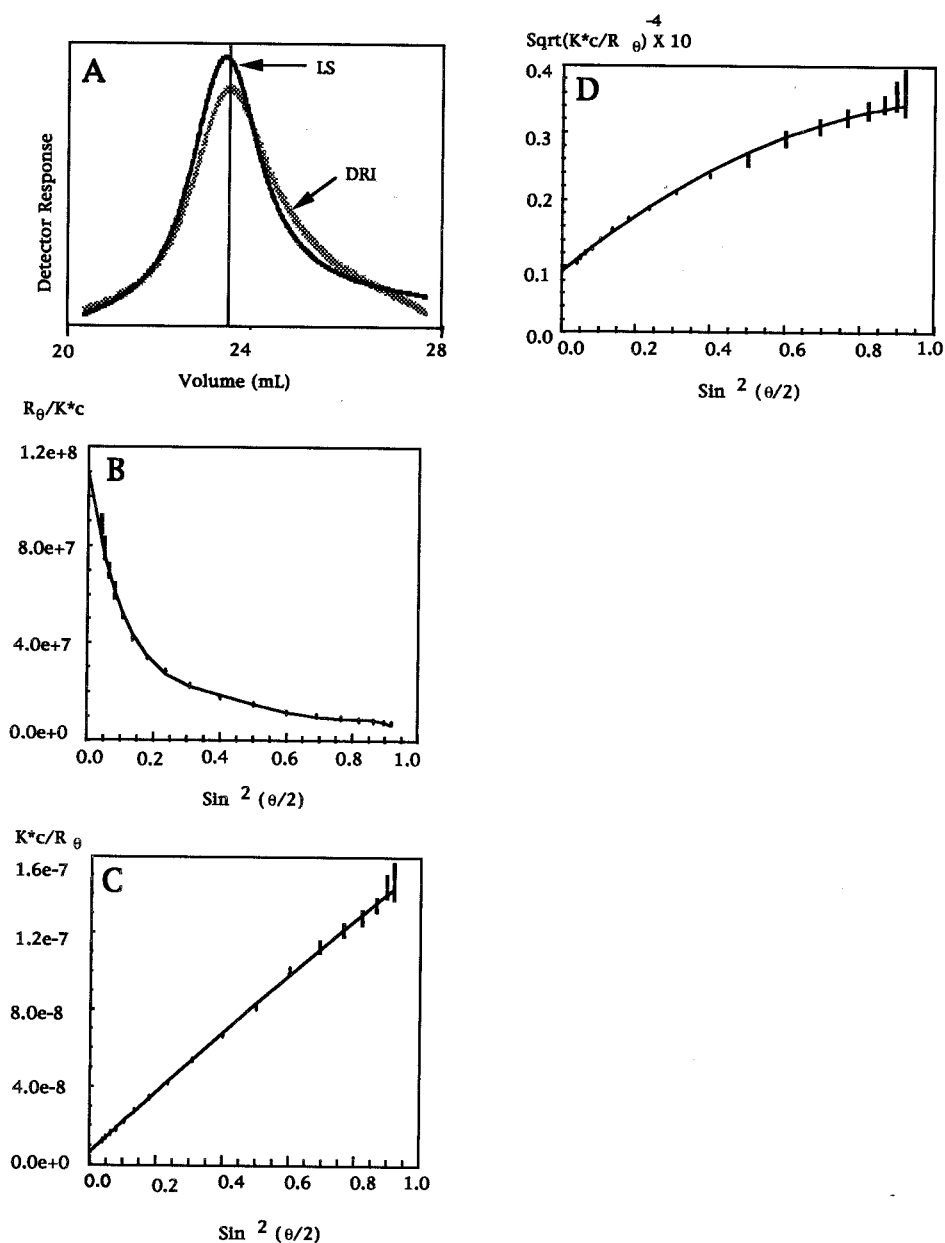


Fig. 5. A, Light scattering (LS) from the 90° angle detector and differential refractive index (DRI) intensity data vs. elution volume for waxy corn starch. B–D, Debye, Zimm, and Berry methods used to determine weight average molar mass (M_w) by extrapolation of light scattering intensity from 18 detector angles to 0 angle. The three methods required polynomials of 5th, 2nd, and 2nd order, respectively, to fit the data.

root mean square radius. The M_w calculated by the Zimm method at >10 million Da increased $\approx 50\%$ more than M_w calculated by the Berry and Debye methods. Shortt (1993) found that for small molecules which only require a first-order fit, the Debye and Zimm methods perform equally well. As molecular size increases, the calculated M_w diverges. A small change in the slope of the line has a dramatic effect on the value of the y-intercept, which is directly related to the M_w (Fig. 5). The collection moment from which the M_w was calculated for all three fit methods for waxy corn, and the graphs used for the determination of waxy corn M_w , which had the largest M_w , by the three methods are shown (Fig. 5)

Samples were analyzed for M_w , polydispersity, and Z-average root mean square radius (Tables I and II) with a fifth-order fit for the Debye method and a second-order fit for the Berry and Zimm methods. The Berry and Debye methods gave similar results for M_w . Shortt (1993) reported that the Zimm method was sensitive to detectors and fit order used in analysis and therefore may lower the precision of the polynomial fit for molecular sizes >100 nm. The fit errors for samples analyzed using the Zimm method were greater than those for the Berry and Debye methods (Table I), indicating increased uncertainty in the M_w values. The M_w values for waxy corn differ from other reported values. In aqueous solvent, Fishman (1996) reported a M_w of 26×10^6 Da for waxy corn starch after heating and then prefiltering through a 0.45- μm filter. Aberle et al (1994) reported radii values of 214–238 nm for waxy corn. Our M_w values were an order of magnitude higher than Fishman's, but our sizes were in agreement with Aberle et al (1994) (Table II).

Large particle- and pore-size polystyrene divinylbenzene SEC columns with large frit porosity eluted with 50 mM LiBr in DMSO can be used to conveniently determine MM distributions of cereal starch molecules. However, the recovery of the samples decreases with root mean square radius >200 nm. This may be related to incomplete solubility of the higher molecular weight starch polymers or to the loss that occurs during the chromatography. The use of MALLS detection is necessary due to the lack of suitable calibration standards and nonlinear MM vs. elution volume curves for starch polymers.

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