Determination of Molecular Size Distribution of Starch and Debranched Starch by a Single Procedure Using High-Performance Size-Exclusion Chromatography[1]

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ABSTRACT

A single procedure, using high-performance size-exclusion chromatography (HPSEC), was developed for the analysis of starch and debranched starch. Starch was solubilized by heating in dimethyl sulfoxide (90%) and debranched with isoamylase in buffer at pH 3.8. Separation of the reaction products of starches using a two-column HPSEC system gave a high molecular weight peak corresponding to that of linear amylose and two or three lower molecular weight peaks corresponding to those of linear maltooligosaccharides from the branched amylopectin fraction. For the range of genotype corn starches monitored in this study, chain lengths of the lower molecular weight linear fractions ranged from number-average degree of polymerization (DP_n) 16 for waxy sugary 2 to DP_n 88 for amylose extender waxy. The same chromatographic conditions for separating debranched starch fractions also separated starches from dent corn and amylomaize V into two distinct peak fractions corresponding to amylopectin and amylose. This separation was unexpected in view of the molecular weight exclusion limits for the two columns, which were significantly lower than the molecular weight of these fractions. The separation thus allowed for the direct comparison of the elution behaviors of starch and debranched starch using the same chromatographic conditions. In this way, it was shown that the amylose fractions in the dent and amylomaize V corn starches were mainly linear because their elution times were unchanged after debranching. The linear amylose contents of these starches, as determined by this HPSEC procedure, were lower than those determined by the iodine colorimetric procedure. This indicates some contribution of long linear chains of amylopectin to the amylose values determined colorimetrically. As expected, the linear amylose fraction was absent in four waxy genotype corn starches that contained only branched amylopectin fractions with a broad range of chain length distribution.

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Size-exclusion chromatography (SEC) has become the technique most often used for determining molecular weight profiles of starch (Ebermann and Schwarz 1975). A series of procedures that use this technique have been developed (Ebermann and Praznik 1975, Suzuki et al 1981, MacGregor and Morgan 1984). Although excellent separations have been obtained, elution times are lengthy, which restricts the number of samples that can be analyzed. In recent years, developments in SEC supports compatible with the high pressure requirements of high-performance (HP) liquid chromatography columns have led to the application of high-performance size-exclusion chromatography (HPSEC) to the analysis of starch (Hizukuri and Takagi 1984, Takeda et al 1984, Kobayashi et al 1985, Chuang and Sydor 1987, Jackson et al 1988). The HPSEC analyses can be performed rapidly, allowing convenient comparison between large numbers of samples.

Although the molecular composition of starch can be approximately described as a mixture of branched molecules of amylopectin (average molecular weight of at least 10^8) and amylose (average molecular weight between 10^5 and 10^6), the molecular structures of these components are quite complex. For example, it has been shown that about one-half of the amylose molecules in corn starch are branched (Takeda et al 1988). In addition, some amylopectins, such as in amylose extender waxy (*aewx*) corn starch (Boyer et al 1976), contain long chains that bind iodine in a manner similar to amylose and contribute to apparent amylose contents.

The use of debranching enzyme, followed by liquid column chromatography of the linear maltooligosaccharides, has become the standard procedure for determining the chain length distribution in starch. Various gel-permeation chromatography (GPC) procedures (Lee et al 1968, Hood and Mercier 1978) and subsequent HPSEC procedures (Hizukuri 1985, Suzuki et al 1992) have been developed to analyze chain length distribution. However, because of differences in magnitude of molecular size, different chromatographic conditions have been applied for separation of starch and debranched starch. More recently, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC) has also been used to characterize chain lengths of debranched starch (Koizumi et al 1991, Shi and Seib 1992, Suzuki et al 1992).

The HPSEC technique described in this study uses the same conditions to separate starch and debranched starch. This technique can directly monitor the effect of debranching on the molecular size distribution of starch and determine the high molecular weight linear amylose content of starch. In this way, the contribution of long chain amylopectin fractions to apparent amylose content can also be estimated.

MATERIALS AND METHODS

Starch Samples

Starches from dent (normal), amylomaize V, waxy, dull waxy (*duwx*), *aewx*, and waxy sugary 2 (*wxsu*-2) corn genotypes were used. The dent and waxy starches were obtained from A. E. Staley Co. (Decatur, IL); the *duwx* and amylomaize V starches were obtained from American Maize-Products Co. (Hammond, IN); the *wxsu*-2 starch (VO starch) was obtained from National Starch and Chemical Co. (Bridgewater, NJ); and the *aewx* starch was provided by Charles Boyer of The Pennsylvania State University (University Park, PA).

Starch Solubilization

Corn starches (20 or 100 mg) were solubilized in 90% (v/v) dimethyl sulfoxide (DMSO, 4.0 ml) by heating the dispersion in a 4-ml screw-top vial at 90°C with continuous stirring for 16 hr using a heating block with magnetic stirrer (Pierce Chemical Co., Rockford, IL). The solubilized starch was centrifuged (3,000 × *g*) and filtered through a 0.45-μm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI) before chromatographic analysis.

Enzymatic Debranching

Starch was debranched with isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* (Sigma Chemical Co., St. Louis, MO) by digesting a mixture of a 1-ml aliquot of solubilized starch (20 mg/ml 90% DMSO), 2 ml of 0.1*M* acetate buffer (pH 3.8), and 25 μl of isoamylase (~540 units) at 37°C for a minimum of 30 hr. The reaction mixture was heated at 100°C for 5 min to inactivate the enzyme upon completion of debranching. The completion of enzymatic debranching was verified by the absence of glycosidic protons at the α 1-6 glycosidic position using nuclear magnetic resonance analysis (Gidley 1985). The debranched starch

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solutions were maintained at 40–50° C before centrifugation, filtration, and chromatographic analysis to prevent formation of insoluble precipitate.

HPSEC

An aliquot (100 μl) of starch (5 mg/ml) or debranched starch (6.7 mg/ml) solution was injected into a two-column (Bio-Sil TSK or SEC 125, 300 × 7.5 mm, Bio-Rad, Richmond, CA) HPSEC system (Waters Div., Millipore, Milford, MA) using 30% DMSO as the eluent at a flow rate of 0.5 ml/min. The Waters liquid chromatographic system includes an automatic sample injection module (WISP model 710B), a programmable solvent delivery module (model 590), a data and chromatography control station (model 840), and a differential refractometer (model 410). The refractive index detector sensitivity (32), scale factor (75), and internal temperature (40°C) were maintained at constant settings for all determinations. The software used for the acquisition, storage, and processing of data was the Waters Expert LC/GPC data reduction software.

The two-column series was maintained at 30°C and preceded by a guard column (Bio-Sil TSK or SEC 125 Guard, 75 × 7.5 mm, Bio-Rad). It was calibrated using mol. wt. 853,000 to 5,800 pullulan standards with dispersivity (mol. wt./number-average mol. wt. [M*n*]) ranging from 1.06 to 1.14 (Polymer Laboratories, Ltd., Stow, OH). No loss of resolution of the standards (Fig. 1) was observed when a single set of columns (including the guard column) was used regularly over a period of two years. The log molecular weight vs. elution-time plot for the pullulan standards was linear (r = 0.998) at the lower molecular weight range (5,800–48,000).

The molecular size distribution of debranched starch was calculated from the elution profiles of the pullulan standards, assuming that linear starch chains exhibit elution behavior similar to that of the pullulans (Hizukuri 1985). The number-average degree of polymerization (DP_n) of the linear fractions in debranched starch was calculated as M*n* divided by 162.

The amylose content of starch was determined by HPSEC as the ratio between total peak area and the peak area corresponding to amylose (Kobayashi et al 1985). The linear amylose content of debranched starch was calculated as the normalized area of the high molecular weight peak fraction. For the purpose of quantitation, it was also assumed that peak area (refractive index detector response) was proportional to weight fraction, irrespective of molecular weight (Hizukuri and Takagi 1984).

Apparent Amylose Content of Starches

Apparent amylose content was determined colorimetrically by a procedure based on those of McCready and Hassid (1943) and Gilbert and Spragg (1964). Starch (5,000 mg per approx. apparent amylose content) was treated with ethyl alcohol (95%, 1 ml); 1*N* NaOH (1 ml) was added, and the starch was dissolved by heating to 100° C. The solution was made up to 100 ml and a 3-ml aliquot was treated with 0.1*N* HCl (0.3 ml), potassium hydrogen tartrate (150 mg), and iodine reagent (0.5 ml) made up to 100 ml, allowed to stand for 20 min, and read at 680 nm. Potato amylose (type III, Sigma) was used as standard.

RESULTS AND DISCUSSION

Starch Solubilization

Most of the starches solubilized to give clear solutions (Craig et al 1989). Some opacity was observed in a few starches, particularly high-amylose types, but storage under refrigeration or centrifugation did not produce any residue. Jackson (1991) obtained maximum dispersibility of corn starch in DMSO at the concentration (90%) used for solubilization in this study.

Debranching Starch

Under the reaction conditions used, 540 units of isoamylase was considered sufficient to debranch 20 mg of starch, based on the absence of α-(1-6) glycosidic protons after debranching (Fig. 2). This quantity was determined from the quantity of enzyme required to debranch a range of corn starches (waxy, dent, and high amylose). It is possible that a few α 1–6 linkages that may have survived the debranching reaction, such as those reported in corn amylose on debranching with isoamylase (Takeda et al 1988), are below the limit of nuclear magnetic resonance detection.

Precipitation was a factor in solutions of debranched starches, particularly, with the high-amylose starches. It was minimized by maintaining the debranched solutions at 40–50° C before centrifugation, filtration, and injection onto the HPSEC column.

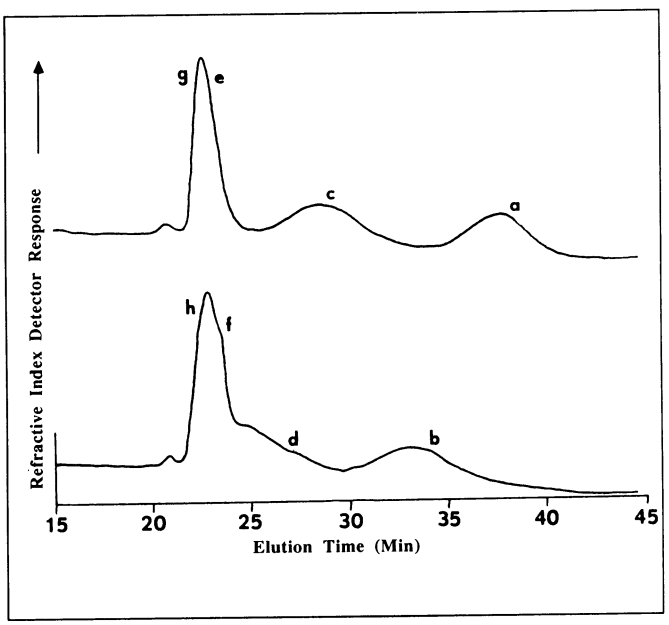

Fig. 1. Elution profiles of eight pullulan standards (**a, c, e, g** and **b, d, f, h**) using two high-performance size-exclusion chromatography columns and 30% (v/v) dimethyl sulfoxide as eluent at a flow rate of 0.5 ml/min. The weight-average molecular weights of the pullulan standards were: **a** = 5,800, **b** = 12,200, **c** = 23,700, **d** = 48,000, **e** = 100,000, **f** = 186,000, **g** = 380,000, and **h** = 853,000.

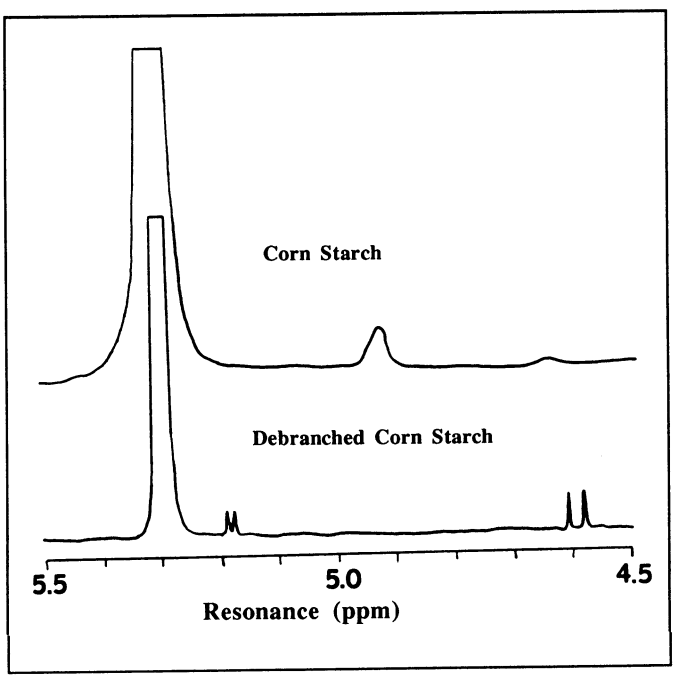

Fig. 2. 1H-nuclear magnetic resonance spectra (75°C) of starch and isoamylase debranched starch in Me_2SO-d_6 recorded at 300 MHz using a Varian XL-300 spectrometer. Peaks at 5.30, 4.95, 5.20, and 4.60 ppm are assigned to H-1 of (1,4)- and (1,6)-α-linked units and the a and b forms of reducing units, respectively.

Formation of insoluble precipitate can be attributed to aggregation and retrogradation of the linear amylose and long DP linear oligosaccharide chains in debranched starch solution (Mercier 1973). The enzyme also produced a small yield of precipitate as shown in a starch-free blank sample.

Structural Properties of Solubilized and Debranched Starches

After injecting a solubilized dent corn starch on to the two-column HPSEC system, separation of two major peaks, attributed to amylopectin (15–20 min of elution time) and amylose, was obtained (Fig. 3). A high-amylose corn starch, amylomaize V, separated into two major peak fractions (amylopectin and amylose) and a minor peak of intermediate molecular size (Fig. 3). Upon isoamylase debranching, these amylose-containing starches were separated by the two-column HPSEC system into distinct peak fractions, consisting of a high molecular weight linear amylose (20–25 min of elution time) and a range of lower molecular weight linear oligosaccharides (Fig. 3). The resolution of the linear oligosaccharide peak fractions revealed two (dent) or three (amylomaize V) populations of chain length distributions from the amylopectin molecules; chain lengths ranged from DP_n 20 for the dent to DP_n 80 for the amylomaize V starches (Table I).

The HPSEC-determined chain length values of the linear oligosaccharide peaks in debranched dent corn starch (DP_n 20 and 45) were generally in agreement with those determined by gel-filtration HPLC of isoamylase debranched amylopectins (peak DP_n of 17–18 and 45–48) from three corn starches (Takeda et al 1988). The values for the amylomaize V starch (DP_n 21, 47, and 80), except for the DP_n 80 chain, were in the upper range of the values (DP_n 16–21 and 41–46) determined by GPC of isoamylase debranched amylopectin from high-amylose V corn starch (Jane and Chen 1992).

Comparing the elution profiles of the dent and high-amylose starches and their debranched products shows that the peaks corresponding to the amylose fractions for each corn starch eluted at about the same time (Fig. 3). The absence of a shift in elution time on debranching was unexpected in view of the work of Takeda et al (1988), who found that amylose in corn starch consists of approximately equal numbers of linear and branched molecules; the linear molecules tended to have the lower molecular weight.

The major elution peak of the waxy starches corresponded to higher molecular weight amylopectins, whereas the tailing shallow peak corresponded to lower molecular weight amylopectins (Figs. 4 and 5). The lower molecular weight fractions and the amylose fractions in dent and amylomaize V corn starches appear to have similar molecular size and shape, based upon their elution times. However, the high molecular weight linear amylose of debranched dent and amylomaize V starches was conspicuously absent in the elution profiles of all the debranched waxy starches (Figs. 4 and 5). Thus, the waxy starches contained only branched molecules, even though peaks were present with elution times similar to that of amylose.

The amylopectin molecules in the *aewx* starch contained the longest branch chain length (DP_n 88), followed by that of amylomaize V starch (Table I). The average branch size or chain length of amylopectin in these starches were similar (Table I), probably because of the amylose-extender (*ae*) gene in both starches (Boyer et al 1976, Yamada et al 1978).

The chain length distribution of amylopectin for all the waxy

TABLE I
Branch Chain Length Distribution of Amylopectins in Some Genotype Corn Starches

Corn Starch	Branch Mode[a]	Branch Molecular Size (DP_n)[b]	Molar Ratio	Average Branch Size (DP_n)
Dent	2	45, 20	1:10	22.3
Waxy	2	47, 19	1:9	21:8
duwx[c]	3	49, 27, 17	1:7:20	20.6
aewx	3	88, 49, 21	1:6:30	27.4
wxsu-2	3	49, 27, 16	1:4:1	20.8
Amylomaize V	3	80, 47, 21	1:8:36	26.9

[a]Branch distribution mode = number of peaks for low molecular weight oligosaccharides in debranched starch as distinguished by high-performance size-exclusion chromatography.
[b]Degree of polymerization.
[c]*duwx* = dull waxy, *aewx* = amylose extender waxy, *wxsu*-2 = waxy sugary.

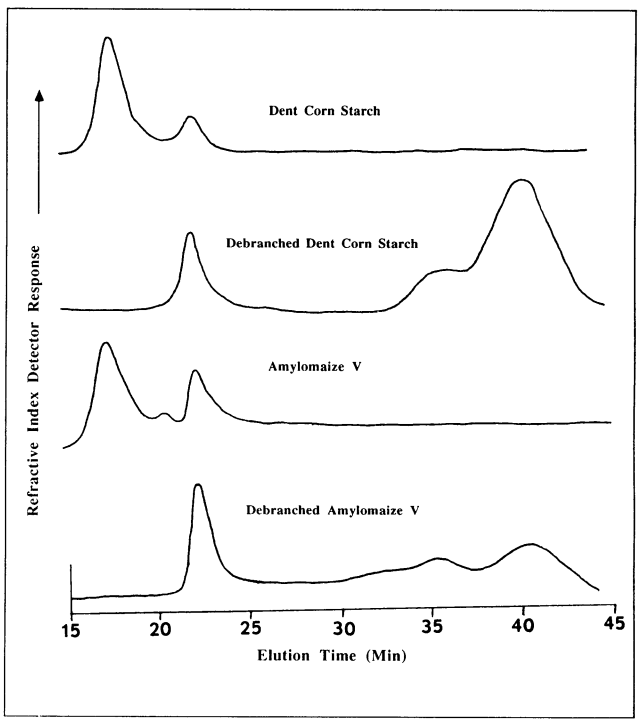

Fig. 3. Elution profiles of dent and amylomaize V corn starches and their debranched products using two high-performance size-exclusion chromatography columns and 30% (v/v) dimethyl sulfoxide as eluent at a flow rate of 0.5 ml/min.

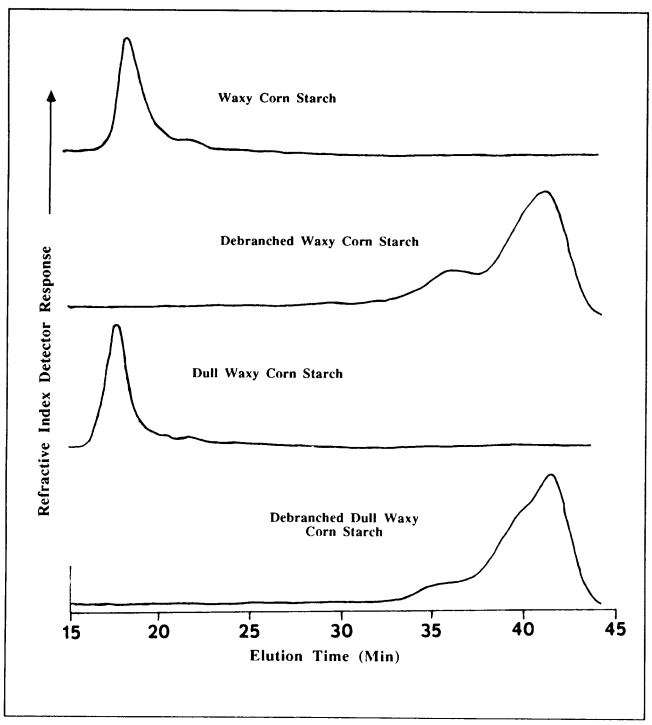

Fig. 4. Elution profiles of waxy and dull waxy genotype corn starches and their debranched products using two high-performance size-exclusion chromatography columns and 30% (v/v) dimethyl sulfoxide as eluent at a flow rate of 0.5 ml/min.

starches (except *aewx*) ranged from DP_n 16–19 for the short unit chains to DP_n 47–49 for the long chains (Table I). The average chain length of amylopectins were similar for all the waxy starches (DP_n 21–22) except *aewx* starch (DP_n ~27). These values, including that of dent corn starch, are in agreement with those determined for corn amylopectins (DP_n 21.4–22.0) by the Smith rapid degradation method and by gel-filtration HPLC on debranching with isoamylase (Takeda et al 1988). Also, the values for the waxy, dent, and amylomaize V starches are in the same order as that determined for the same starches and reported as weight-average chain length of whole amylopectin (Hizukuri 1985). The HPSEC profiles (refractive index detector response) obtained for isoamylase-debranched waxy and *duwx* starches by Yuan et al (1993) were very similar to those shown for the same starches in Fig. 4, although lower values were reported for their chain length distribution and average chain length.

Amylose Content of Starches

The amounts of HPSEC-determined amylose in all the starches were higher than those of the debranched starches (Table II). This was attributed to the slightly branched nature of some amylose molecules and to the coelution of lower molecular weight amylopectins with the amylose fractions. The amylose fractions in the dent and amylomaize V corn starches were mainly linear (Table II). The contribution of lower molecular weight amylopectin fractions to the HPSEC-amylose values was evident by the lack of the high molecular weight linear amylose fractions in all of the debranched waxy starches (Table II).

The apparent amylose contents of all the starches, as determined by the blue value method, were also higher than the HPSEC-based linear amylose contents of the starches (Table II). This indicated that branched amylopectin molecules contributed to the values obtained by the iodine-binding method. The contribution of long chains in the amylopectin fraction was obvious in the analysis of the *aewx* corn genotype (Table II), where a starch that contains only amylopectin (Boyer et al 1976) has linear regions long enough to form the blue iodine complex (Yamada et al 1978), i.e., at least 40 glucose units (John et al 1983).

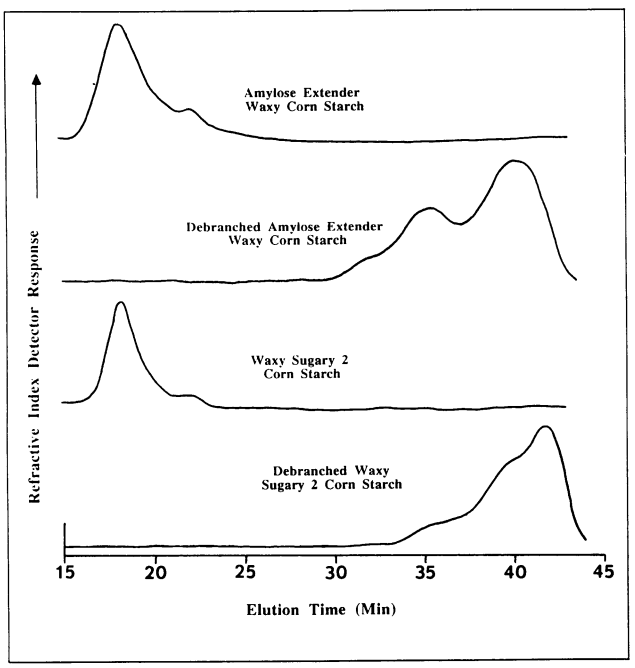

Fig. 5. Elution profiles of amylose extender waxy and waxy sugary 2 genotype corn starches and their debranched products using two high-performance size-exclusion chromatography columns and 30% (v/v) dimethyl sulfoxide as eluent at a flow rate of 0.5 ml/min.

TABLE II
Amylose Contents of Genotype Corn Starches as Determined by High-Performance Size-Exclusion Chromatography (HPSEC) and Colorimetric Procedures

Corn Genotypes	HPSEC Amylose %		Apparent Amylose (%)[c]	Iodine-Binding Amylopectin (%)[d]
	Starch[a]	Debranched Starch[b]		
Dent	28.0	26.0	28.0	2.0
Waxy	7.0	0	1.0	1.0
duwx[e]	6.0	0	3.0	3.0
aewx	18.0	0	17.0	17.0
wxsu-2	5.0	0	2.0	2.0
Amylomaize V	41.0	33.0	47.0	14.0

[a] Includes both high molecular weight branched and linear amyloses and low molecular weight amylopectin fractions.
[b] High molecular weight linear amylose fraction only.
[c] Determined by a colorimetric iodine-binding procedure.
[d] Contribution of long chains in amylopectin fractions to apparent amylose determined by difference.
[e] *duwx* = dull waxy, *aewx* = amylose extender waxy, *wxsu*-2 = waxy sugary.

Separation of Amylose and Amylopectin

The larger molecular size amylopectin molecules apparently eluted at or near the void volume of the column, whereas the amylose molecules, which are smaller, eluted about 5–10 min later. The reported molecular weight of corn starch amylopectin ranged from 10–30 $\times$ 10^6 apparent molecular weight (Jackson et al 1988, 1989) to 40–80 $\times$ 10^6 weight-average molecular weight (Erlander and French 1956). The molecular weight of the amylose fraction ranges from 1.2–4.1 $\times$ 10^5 molecular weight (Ebermann and Praznik 1975, Takeda et al 1988, Takeda et al 1989) up to one million (Banks and Greenwood 1968). In view of the size-exclusion limits of the columns used (mol. wt. 20,000 based on dextran standards), the degree of separation of the dent and amylomaize V starches into their component fractions (amylose and amylopectin) was surprising. In this regard, the columns differed from other columns with similar size-exclusion limits that were also evaluated but did not give the separation (data not shown).

Although not determined, the high extent of starch solubilization by heating in 90% DMSO may have contributed to the exactly reproducible separation of the dent and amylomaize V corn starches and those of other starches. The elution profiles and the relative peak areas of both starches, and for all other starches, were also exactly reproducible, with or without centrifugation and filtration through a 0.45-μm filter, regardless of the clarity of the solutions. This is true even when 100 μl of more concentrated starch solution (6.7 mg/ml vs. 5.0 mg/ml of 90% DMSO) was injected after solubilization without filtration. This is also true for refrigerated starch solutions stored for up to one year.

Rollings and coworkers (Yu and Rollings 1987, Corona and Rollings 1988) noted that, due to various interactions such as polymer-polymer, polymer-solvent, and polymer-support, the accepted theories on the mechanism of separation of biopolymers such as amylose, amylopectin, and glycogen by SEC are not generally applicable to systems using polar solvents (e.g., DMSO in water). Kobayashi et al (1985) suggested that the retention of amylopectin molecules in the two-column SEC system, which separated amylose and amylopectin using DMSO as an eluent, probably resulted from polymer-support interactions. They showed that despite the high molecular weight of amylopectin molecules, this fraction was eluted after the void volume of the column.

Banks and Greenwood (1968, 1972) applied the Mark-Houwink relationship to amylose solutions and obtained a higher exponent in DMSO than in water, suggesting that the former was a better solvent for amylose. On this basis, they suggested an expanded coil structure for amylose in DMSO, whereas a random coil was present in water. This indicated more polymer-solvent interaction in DMSO and more polymer-polymer interaction in water. However, these interactions do not seem to be dominant in the present study because the elution times of both amylose and amylopectin were significantly increased as the DMSO content

of the eluent was increased (data not shown).

The role of polymer shape in solution was also shown by Yu and Rollings (1987), who defined a branching factor related to hydrodynamic volume. They showed that, at a common elution volume (i.e., hydrodynamic volume), the molecular weight of an amylopectin-type molecule would be four to five times that of an amylose molecule. This effect would tend to lessen the separation between amylose and amylopectin and does not seem to be a major factor in the DMSO-water system used in this study.

The shape of the molecules in solution could, however, affect the degree of polymer-support interaction. The more linear amylose and pullulan fractions result in stronger interactions and, thus, a larger elution volume than the branched amylopectin fraction does. Hizukuri and Takagi (1984) showed that the hydrodynamic volume of pullulan is about 35% larger than that of amylose in aqueous solution at the same molecular weight, which is attributable to more expansion of the pullulan molecules. However, in this study, the pullulan standards, evidenced by the similar elution behavior of the mol. wt. 380,000 and 853,000 samples (Fig. 1) and the amylose fractions (Fig. 3), tend to reach the same limiting elution volume, indicating structural similarity of the amylose and pullulan molecules in the DMSO-water solvent.

CONCLUSION

The two-column HPSEC procedure described in this study is suitable for determining the molecular size distribution of starch and debranched starch. Thus, the same chromatographic conditions can conveniently monitor the effect of debranching on the molecular weight profiles of starch and estimate molecular weight-based amylose and amylopectin contents of starches. The high molecular weight linear-amylose contents of certain genotype corn starches, as determined by the HPSEC procedure, were lower than the apparent amylose values determined by the colorimetric iodine-binding method.

The HPSEC procedure was also cost effective, in view of the reproducibility and reliability of the two columns (for up to two years) in separating starch and debranched starch products into their component fractions.

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