Structural Properties of Starch Fractions Isolated from Normal and Mutant Corn Genotypes Using Different Methods

Amalia Tziotis,[1] Koushik Seetharaman,[2] Kit-sum Wong,[1] Jeffrey D. Klucinec,[3] Jay-lin Jane,[1] and Pamela J. White[1,4]

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ABSTRACT

The objectives of this research study were to isolate, evaluate, and compare the fine structures of starch fractions obtained from a wild-type (normal) corn starch and *amylose-extender25*, *dull39*, *sugary2, and sugary1* corn mutants in the same genetic background using three different fractionation procedures based on gel-permeation chromatography or alcohol-precipitation methods. Starch fractions obtained from each of the three methods were enzymatically debranched and analyzed using high-performance anion-exchange chromatography with a postcolumn amyloglucosidase reactor and a pulsed amperometric detector. The separations were performed by fractionation on a GPC column, by precipitation with 1-butanol, and by preferential precipitation with 1-butanol and isoamyl alcohol. Using any of these methods, no apparent differences in the molecular weight distributions of amylopectin or of amylose among the different starches were observed. The proportions of branch chain lengths of the starch components obtained by the various fractionation methods were very similar among methods for each of the starch types analyzed, such as the predominance of long branch chains in *ae25* corn and that of the short branch chains in *su2* corn. Overall, the effect of the corn mutations was more important to the differences observed among the starch types than was the method of fractionation used.

The endosperm mutants of corn (*Zea mays* L.), including amylose extender (*ae*), dull (*du*), sugary-1 (*su1*), and sugary-2 (*su2*), create unique, but what has been thought to be predictable, starch structures. For example, the *ae* mutation causes a loss of starch-branching enzyme IIb activity (Boyer and Preiss 1978), resulting in starch with an apparent amylose (AM) content of up to 80% (Mercier 1973; Banks and Greenwood 1975; Ikawa et al 1978; Boyer et al 1980; Ikawa et al 1981). The *ae* starches also contain branched molecules with a higher proportion of longer chains (DP > 30) than found in the amylopectin (AP) of common corn starch (Takeda et al 1993; Klucinec and Thompson 1998). The *du* mutation results in loss of starch synthase II activity and affects the activity of at least one other starch biosynthetic enzyme (Gao et al 1998), causing *du* corn starches to have a relatively high AM content of starch (depending on the genetic background) and low total carbohydrate content (Creech 1965; Creech and McArdie 1966). The recessive *su2* allele in corn, identified by Eyster (1934), resides on chromosome six. The *su2* starch has a higher percentage of AM than does normal corn starch (Pfahler et al 1957; Kramer et al 1958; White et al 1994; Li and Corke 1999; Perera et al 2001). Pan and Nelson (1984) first reported that the *su1* phenotype is caused by the loss of activity of a pullulanase-type starch-debranching enzyme, suggesting that the debranching enzyme also is involved in starch biosynthesis. In addition to starch, the *su1* mutants of corn accumulate a novel form of water-soluble polysaccharide known as phytoglycogen (Summer and Somers 1944).

Recently, variations in the functions of mutant starches related to structural differences were noted when the mutant was placed in corn with different genetic backgrounds. For example, Li and Corke (1999) developed five different corn inbred lines and evaluated the thermal, pasting, and gel textural properties of each isogenic line consisting of normal corn along with its *du* and *su2* mutants. Differences were reported that related to the amylose percentage (29.5–43.2% for *su2*, and 29.0–37.6% for *du*), swelling power, solubility, digestibility, onset, peak, and conclusion temperatures of gelatinization, change of enthalpy of gelatinization, pasting properties, and gel firmness and adhesiveness. The ability to study differences in the structures of these different starches has been limited by the methodology, which is still evolving. Schoch (1942) developed a method for separating starch into two fractions based on the differential ability of the fractions to precipitate with aqueous 1-butanol. Later, a combination of isoamyl alcohol and 1-butanol for the initial precipitation was introduced (Wilson et al 1943). Lansky et al (1949) proposed that a third component in normal corn starch exists, called intermediate material (IM), with properties different from those of AM and AP. Since then, various structures of IM fractions have been reported after treatment of starch with different reagents (Perlin 1958; Whistler and Doane 1961; Erlander et al 1965; Banks and Greenwood 1967; Adkins and Greenwood 1969; Inouchi et al 1987; Wang et al 1993). Takeda et al (1986) fractionated starch and separated a component they called IM by precipitating with isoamyl alcohol and 1-butanol in an initial precipitation. AM was separated by redispersing the mixture with 1-butanol alone. Most recently, Klucinec and Thompson (1998) employed differential alcohol precipitation using isoamyl alcohol and 1-butanol to obtain fractions designated as AP, IM, and AM from *ae* mutant-containing corn starches. With this method, the IM had structural properties intermediate to those of AP and AM measured by more traditional procedures.

At issue is that various starches likely are composed of a continuum of starch structures, ranging from highly branched, large MW molecules to small MW straight chain molecules. For example, among the many corn mutants affecting starch structure, one probably can find a variety of structures, including highly branched short chains, highly branched long chains, linear chains with many short branches, and linear chains with practically no short branches, all of high, medium, or low MW.

How these starch components then separate depends on both their structure and the fractionation method used. Thus, the current research study was designed to evaluate this new fractionation method along with previous ones when applied to various corn endosperm mutant starches all in the same genetic background. More specifically, the objectives of this study were to isolate, evaluate, and compare the fine structures of starch fractions obtained from a wild-type (normal) corn starch as well as *amylose extender25*, *dull39*, *sugary2, and sugary1* corn mutants in the same genetic background using three different fractionation

[1] Department of Food Science and Human Nutrition and Center for Crops Utilization and Research, 2312 Food Sciences Building, Iowa State University, Ames, Iowa 50011.
[2] Department of Food Science, The Pennsylvania State University, 107A Borland Laboratory, University Park, PA 16802.
[3] BASF Plant Science, L.L.C., Ames Research, 2901 S. Loop Drive, Building 3, Suite 3800, Ames, IA 50010.
[4] Corresponding author. Phone: 515-294-9688. Fax: 515-294-8181. E-mail: pjwhite@iastate.edu

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procedures based on gel-permeation chromatography or alcohol-precipitation methods.

MATERIALS AND METHODS

Corn (*Zea mays* L.) kernels from the ExSeed68 line (wild type [normal] and *dull*39 [*du39*], *amylose extender* [*ae25*], *sugary2* [*su2*], and *sugary1* [*su1*] genotypes) were provided by BASF Plant Science, LLC, Ames Research, IA. All corn endosperm mutants were developed from the normal corn and grown in the summer of 1999 under the same environmental conditions near Ames, IA. The reagents used in the starch analyses were ACS reagent-grade (Fisher Scientific; Fair Lawn, NJ).

Starch was extracted from the corn kernels using the modified 100-g procedure previously described by Singh et al (1997). The extraction procedure was performed twice for all corn types, except for the *su1* mutant, which was available only in limited quantity; thus only one extraction of 50 g was performed.

Amylopectin and Amylose Content Determination

The ConA assay for the measurement of the AM and AP contents was performed as indicated in an amylose/amylopectin assay kit (Gibson et al 1997) purchased from Megazyme International Ireland Ltd; Co. Wicklow, Ireland. Duplicate analyses of starch types pooled after each extraction were performed.

Gel-Permeation Chromatography of Native Starches

The molecular weight distribution of each whole, native starch of each starch type was performed as previously described (Jane and Chen 1992). The starch solution was loaded onto a gel-permeation chromatography column (1.5 cm i.d. × 50 cm) packed with Sepharose CL-2B using a sample applicator. The mobile phase in the system was 0.01*M* sodium hydroxide containing 0.02% (w/v) sodium azide. The column was run in the ascending mode with a flow rate of 0.30 mL/min and 1-mL fractions were collected. The procedure by Dubois et al (1956) was used to determine total carbohydrate content in starch, with the absorbance of each fraction read at 470 nm on a spectrophotometer (model U-2000, Hitachi Instruments). The tubes also were subjected to the blue value analysis conducted following the general procedure by Schoch (1964), and wavelength scanning at 700–400 nm according to Klucinec and Thompson (1998). Total CHO is the absorbance measured for the total carbohydrate content, BV is the absorbance obtained for the blue value test, and λ_{max} is the wavelength at which the highest absorbance was recorded. At least two complete analyses were performed for each starch type pooled after duplicate starch isolations.

Starch Fractionation

Method I: Gel-permeation column chromatography of starches for the isolation of large (L) MW, medium (M) MW, and small (S) MW starch fractions. Starch samples (150 mg, dwb) were dispersed in 3 mL of 90% DMSO and heated with stirring for 1 hr, after which the power was turned off and the samples were stirred for 24 hr. The starch was precipitated with the addition of 3× its volume of ethanol (200 proof) and centrifuged at 6,000 × *g* for 15 min at room temperature. The washing step was repeated three times. The last step involved washing and drying with acetone, after which the samples were left to dry overnight. The samples were dispersed in 15 mL of 0.1*M* sodium hydroxide for 24 hr. The dispersed starches were diluted with 20 mL of deionized water (total volume 35 mL), and the entire solution loaded onto a column (2.6 cm i.d. × 80 cm) containing Sepharose CL-2B (Pharmacia, Piscataway, NJ) using a sample applicator (model SA-5, Pharmacia). The column was run in the ascending mode with a mobile phase of 0.01*M* sodium hydroxide containing 0.02% (w/v) sodium azide, and 5-mL fractions were collected with a fraction collector. The flow rates during loading of the starch solutions onto the column were 0.25, 0.5, 0.5, 0.4, and 0.25 mL/min for wild type and *du39*, *ae25*, *su2*, and *su1* mutants, respectively. Different flow rates during loading were used to accommodate the differences in viscosity of the starch solutions related to the different amylose contents of the starch types (wild type and *su1* were the most viscous, whereas the *du39* and *ae25* were the least viscous). After the samples were loaded onto the column, the flow rate was set to 0.3 mL/min for all samples. A small portion from each fraction (200 μL) was diluted with deionized water to 1 mL, and 200 μL from that solution was used to determine total carbohydrate concentration. Each 5-mL fraction was adjusted to pH 6 by the addition of 0.1*M* HCl solution. Based on the retention volume, the contents of the tubes were pooled into three round-bottom flasks according to MW range: large (L), medium (M), or small (S). A quantity of 20 mL of 90% DMSO was added to each of the flasks to improve starch dispersion, and the samples were concentrated to one-fifth of the initial volume using a rotary evaporator. Finally, the starch in each flask was precipitated with the addition of three times its volume of ethanol and centrifuged at 6,000 × *g* for 25 min. The washing step with ethanol and the centrifugation were repeated three times on the precipitated starch to remove impurities, and the samples were left to dry overnight. These starch fractions later were analyzed by enzymatic debranching (duplicate analyses of one replicate per starch type) for the determination of branch chain length using HPAEC-ENZ-PAD.

Method II: Isolation of soluble and insoluble starch fractions. Starch fractions were separated according to soluble and insoluble fractions following the procedure of Schoch (1942) with modifications by Jane and Chen (1992). A 20% 1-butanol solution was added to the starch solutions. The AM was assumed to form a complex with 1-butanol and settle out as a precipitate (insoluble fraction [Insol]). AP was present in the supernatant (soluble fraction [Sol]). The separation of the two fractions was accomplished by centrifugation at 6,000 × *g* for 40 min. The starch fractions were further purified according to Jane and Chen (1992), with the procedure being performed in duplicate for each starch type.

Method III: Separation of starch into soluble I, soluble II, and insoluble starch fractions. Starch was fractionated using the method of Klucinec and Thompson (1998). Briefly, the fraction remaining as a supernatant after the treatment of starch with an aqueous solution of 6% 1-butanol and 6% isoamyl alcohol was labeled soluble I fraction (SI). The starch precipitated from this first step was treated with a 6% 1-butanol aqueous solution with the supernatant termed the soluble II fraction (SII) and the precipitate was termed insoluble fraction (IS). All three starch fractions were further purified according to Klucinec and Thompson (1998).

Enzymatic Debranching and Branch Chain Length Distribution of Starch Fractions

Starch fractions obtained from each of the three methods were enzymatically debranched according to Jane and Chen (1992). Branch chain lengths were determined using high-performance anion-exchange chromatography with a postcolumn amyloglucosidase reactor and a pulsed amperometric detector (HPAEC-ENZ-PAD) (Wong and Jane 1997). The weight-average chain lengths (CL_w = Σ(DPn × relative percentage)/the number of peaks detected, *n* = 6,…, greatest DP (degree of polymerization) detected) and the greatest DP found also were reported. The debranched components of starch fractions obtained from all starch types by each of the three methods were compared.

RESULTS AND DISCUSSION

Starch Isolation and Amylose Content

Starch isolated from the different corn types gave average total percentage starch yields (dwb) of 56.6 for the wild type, 44.6 for *du39*, 41.7 for *ae25*, and 47.4 for *su2*, with yield deviations very similar (±5%) from isolation to isolation. The starch yield for the

su1 corn was 8% and was lower than that of the rest of the corn types. This result is in agreement with the results reported by Inouchi et al (1983), where starch yields were 8–10% for *su1* corn in the Oh43 genetic background, but lower than 35.4% for *su1* corn in the W23/L17 genetic background (Inouchi et al 1983) or 33.9% for Golden Cross Bantam sweet corn (*su1*) (Creech 1965). The average apparent AM contents of the starch from the wild type, *du39*, *ae25*, *su2*, and *su1* corn were 21.0, 28.6, 51.9, 29.7, and 25.4%, respectively. The wild type and *su2* corn starches were previously reported (Perera et al 2001) to contain 21.6 and 33.5% apparent AM, respectively, based on the iodine affinity of starch as measured using a potentiometric autotitrator (Schoch 1964).

Gel-Permeation Chromatography of Native Starches

Molecular-size distributions of native corn starches obtained from gel-permeation chromatography (GPC) are displayed in Fig. 1. All starches displayed two peaks typically classified as AP (first peak) and AM (second peak). A carbohydrate-rich fraction was also observed between the AM and AP peaks (fractions 25–33). No apparent differences in the molecular weight distributions of AM among the different starches were observed except for the obviously greater total amount of AM in the *ae25* starch. The high ratio of BV to total CHO content for the first peak of the *ae25* mutant starch profile indicated a high proportion of long branch chains in AP, in agreement with previous results (Takeda et al 1993; Klucinec and Thompson 1998) for high-amylose containing starches.

Starch Fractionation by Different Methods

Method I. The fractionation of starch by GPC was based on molecular size distribution of the starch molecules. Fractions were collected according to the total CHO profile (Fig. 2) and these collected materials were isolated and compared among starch types. The total number of fractions (100) collected for all starch types was based on the total CHO profile obtained from *ae25* mutant starch, where the amount of AM present was greater than in the other starches; thus the M area was apparent and easier to assign. The first set of fractions (33–49) was L, the second set of fractions (50–69) was M, and the third set of fractions (70–100) was S. The same fraction numbers then were used for the separation into L, M, and S fractions of all starch types, so that consistency within the range for each fraction could be obtained. The percentage recovery of starch (including L, M, and S fractions) was based on the amount loaded on the GPC column and varied from 69.4 to 96.0% (73.4% for wild type, 69.4% for *du39*, 79.3% for *ae25*, 96.0% for *su2*, and 87.3% for *su1*). The wild type starch had the greatest L fraction concentration and *ae25* the least, as measured by calculating the area under the curve of the chromatograph (Fig. 2). The lowest percentage of M fraction was found in the *ae25* starch and the greatest in the wild type starch. The *ae25* starch had the greatest concentration of S fraction.

Method II. Fractionation by 1-butanol precipitation was based on the branching pattern of the starch components, because the ability of the starch materials to complex and to precipitate in the presence of alcohols is related to the degree of branching (Schoch 1942; Jane and Chen 1992; Klucinec and Thompson 1998). The recovery of the starch fractions (including Sol and Insol fractions), calculated from the amount of starting starch material used, was variable (68.9% for the wild type starch, 61.7% for *du39*, 61.8% for *ae25*, and 60.5% for *su2* starch). The Sol fraction (generally thought to be AP) from the wild type starch consisted predominantly of a single HMW fraction, unresolvable by GPC (Fig. 3). On the other hand, Sol fractions from *du39* and *ae25* mutant starches contained both a HMW fraction and a LMW fraction of branched materials which was resolved by GPC. The presence of the smaller branched M materials was indicated by the CHO content in that region (fractions 28 to 53 for *du39* and fractions 28 to 60 for *ae25*), whereas the higher degree of branching of these materials was evident from the low ratio of BV to total CHO content, and by the λ_{max} obtained, which were of the same range as that of the Sol fraction peak. A similar LMW fraction was noted for the *su2* mutant Sol fraction, with the exception that the λ_{max} obtained for the contaminating materials was lower than that of AP, indicating

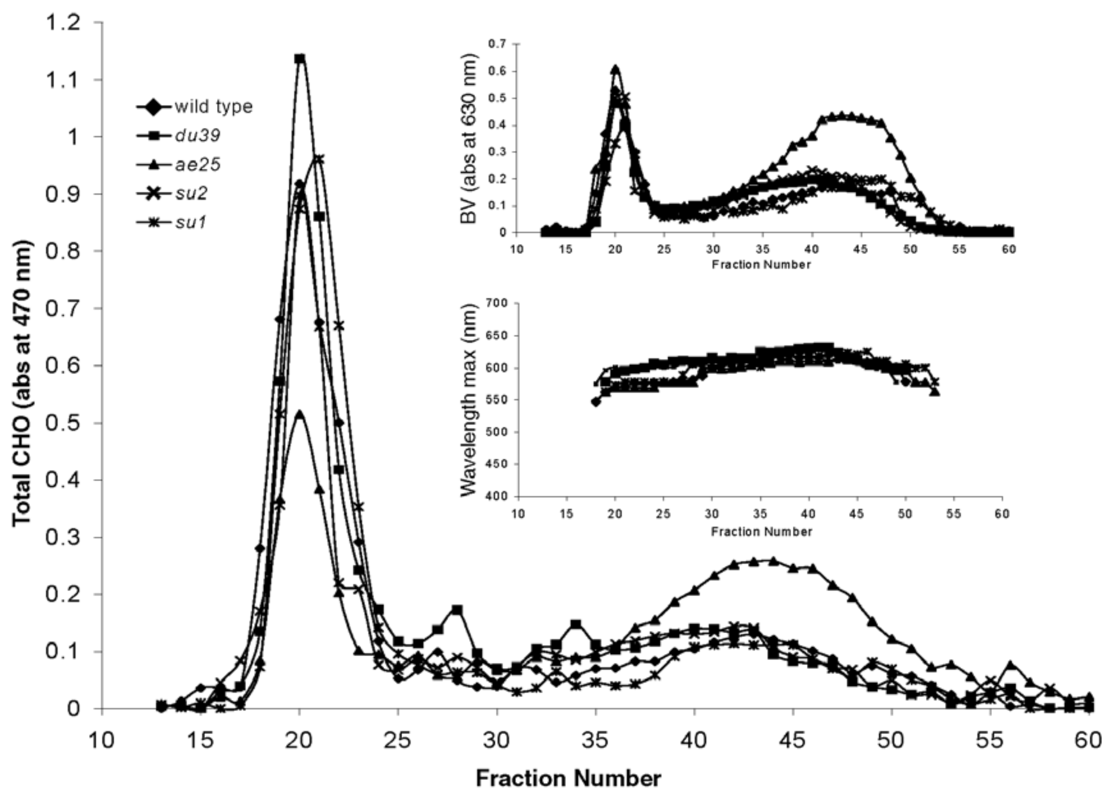

Fig. 1. Gel-permeation chromatography profiles of native starches.

the presence of very short branch chains in the *su2* starch. The Insol fraction, which is typically thought to be AM from all starch types, contained noticeable amounts of starch unresolvable by GPC, characteristic of AP (Fig. 4), even though there was no insoluble material in starch solution before addition of 1-butanol. The Insol fractions had a greater λ_{max} than did the Sol fractions, indicating that the population of branched molecules with long branch chains could have formed a complex with 1-butanol, resulting in coprecipitation with the Insol fraction.

The Insol fraction from all starch types had a greater ratio of BV to total CHO content than did the Sol fraction, indicating a higher linear character for the Insol fraction than for the Sol fraction.

Method III. Fractionation based on treatment with both 1-butanol and isoamyl alcohol resulted in relatively pure SI fractions (generally considered to be AP) from the wild type and *su2* starches, with no contamination from S molecules, thus providing a better separation of soluble and insoluble fractions than Method II (Fig. 5). In addition, because of the isoamyl alcohol addition in Method III, a second soluble fraction for each starch was obtained (SII). The percentage starch recovery (including SI, SII, and IS fractions) based on weight of the starting starch material was greater than for Method II (81.1–88.2%). These data agree with fraction recoveries that were reported by Klucinec and Thompson (1998) for Method III. The *du39* SI fraction contained a small amount of

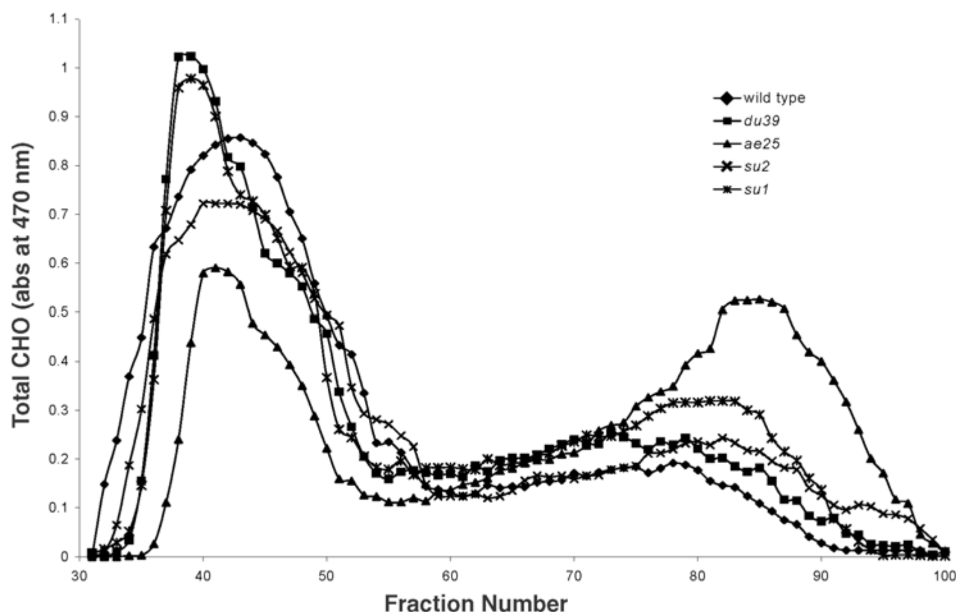

Fig. 2. Gel-permeation chromatography profile of native starches for fractionation into large, medium, and small MW components (Method I).

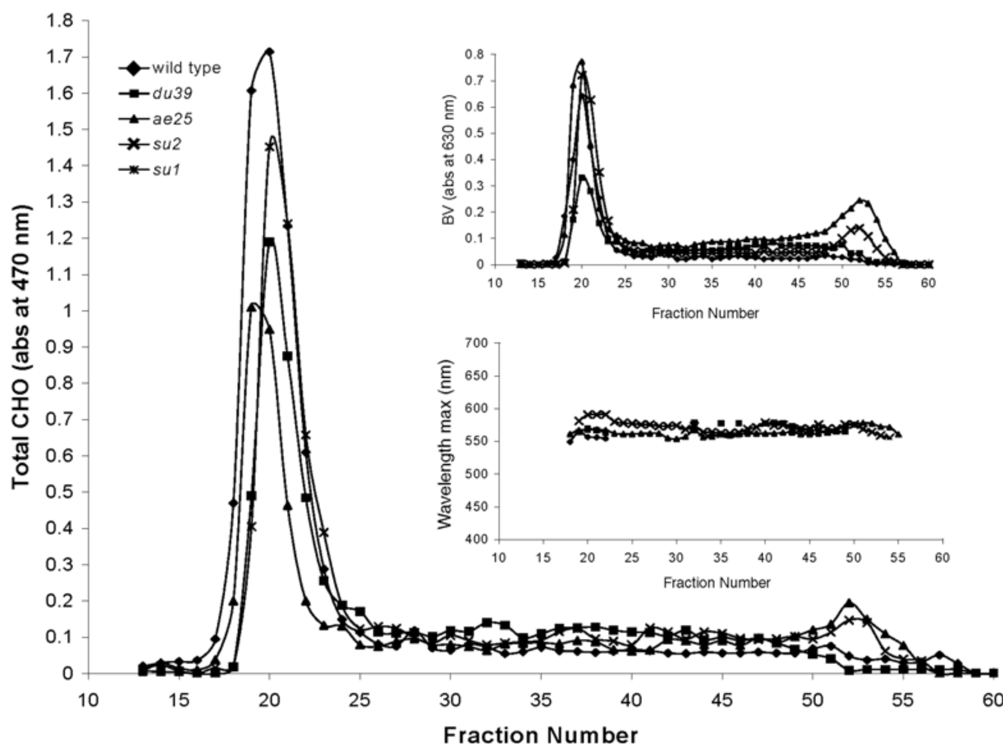

Fig. 3. Gel-permeation chromatography profiles of the Sol starch fractions (Method II) obtained by using 1-butanol precipitation.

LMW components resolvable by GPC and more clearly observable by the elevated BV of the later fractions collected from the GPC column. Additionally, the λ_{max} values of these fractions from *du39* indicates the intermediate complexable chain length of these molecules compared with AP and AM from the wild type starch. On the other hand, the SI fraction from *ae25* mutant starch had a considerable LMW component that was clearly visible from total CHO analysis. Further, the intermediate structural nature of this LMW component is indicated by the low BV to total CHO content ratio as well as by the λ_{max} (565–600 nm).

The common feature among all SII fractions of all starch types was that the λ_{max} observed was 565–600 nm (Fig. 6), which was similar to that for the SI fraction, as also noted by Klucinec and Thompson (1998). The fact that both λ_{max} and the MW range of both SI and SII fractions were the same suggests that reasons other than MW or branch chain length, such as spatial organization or size (gyration radii) contributed to the complex formed between these molecules and the isoamyl alcohol.

The IS fractions (generally considered to be AM) from all starch types all contained a component with a MW unresolvable by

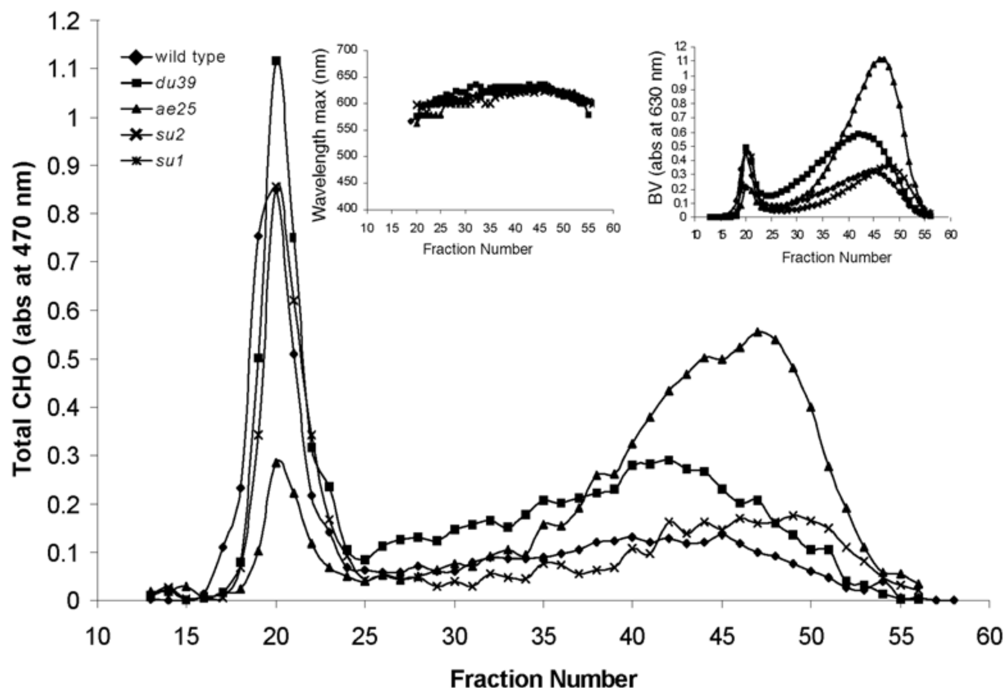

Fig. 4. Gel-permeation chromatography profiles of the Insol starch fractions (Method II) obtained by using 1-butanol precipitation.

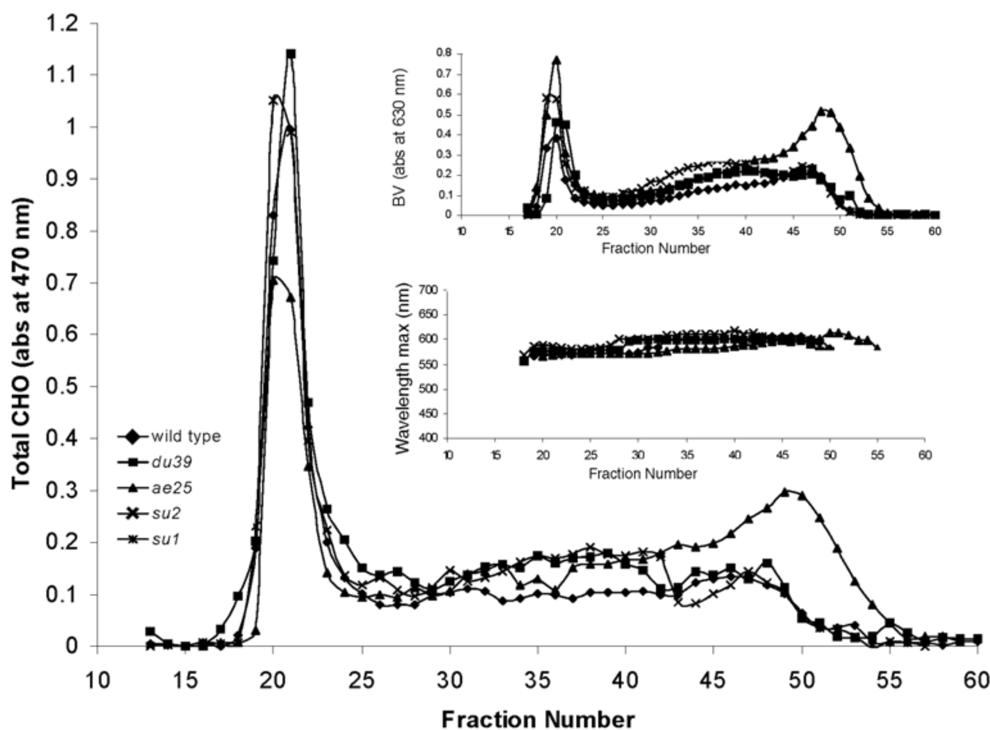

Fig. 5. Gel-permeation chromatography of SI starch fractions (Method III) obtained by using preferential 1-butanol and isoamyl alcohol precipitation.

GPC, characteristic of AP, but the quantity of this HMW component was much lower in the IS from Method III than with the IS from Method II (1-butanol precipitation) as indicated by the total CHO content noted on the GPC chromatograms (Fig. 7). The quantity of alcohol in the aqueous solution (total 12% v/v) added to the starch to be fractionated for Method III was lower than that used for Method II (20% v/v). Perhaps the low amount of alcohol present allowed fewer longer chained molecules of the AP to complex with the alcohols, thus reducing the amount of precipitate with Method III.

Analysis of Enzymatically Debranched Starch Fractions

Method I. All starch fractions (L, M, and S) obtained in Method I contained branched molecules. The branch chain length distributions obtained from all starch types after enzymatic debranching were bimodal with two distinct regions, each represented by a

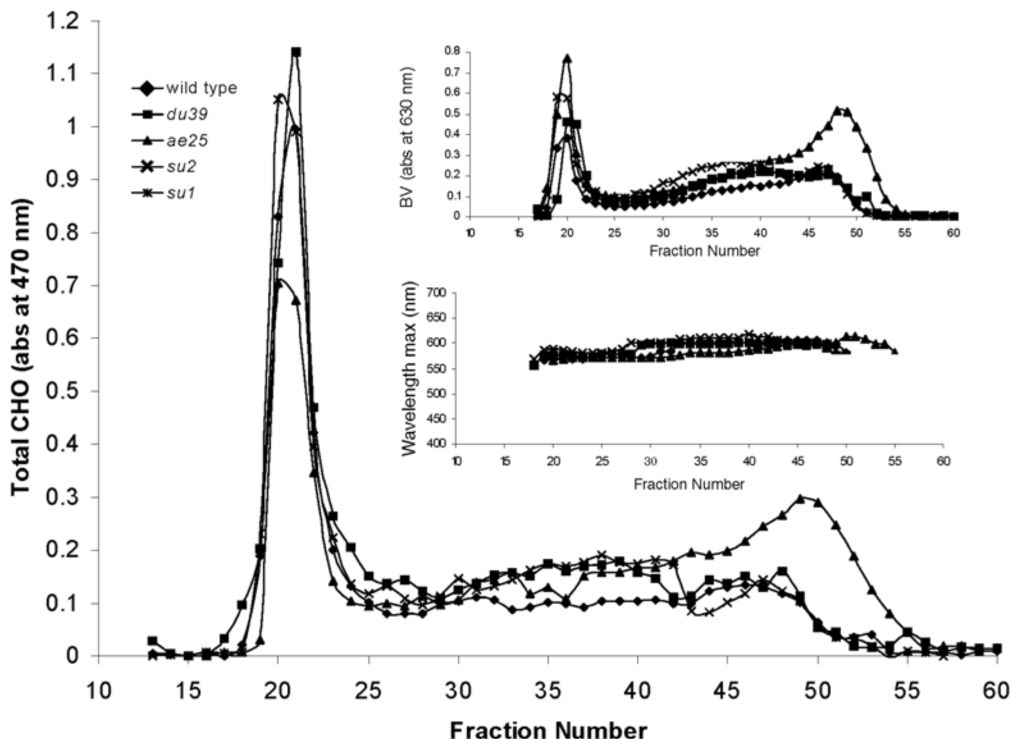

Fig. 6. Gel-permeation chromatography profiles of the SII starch fractions (Method III) obtained by using preferential 1-butanol and isoamyl alcohol precipitation.

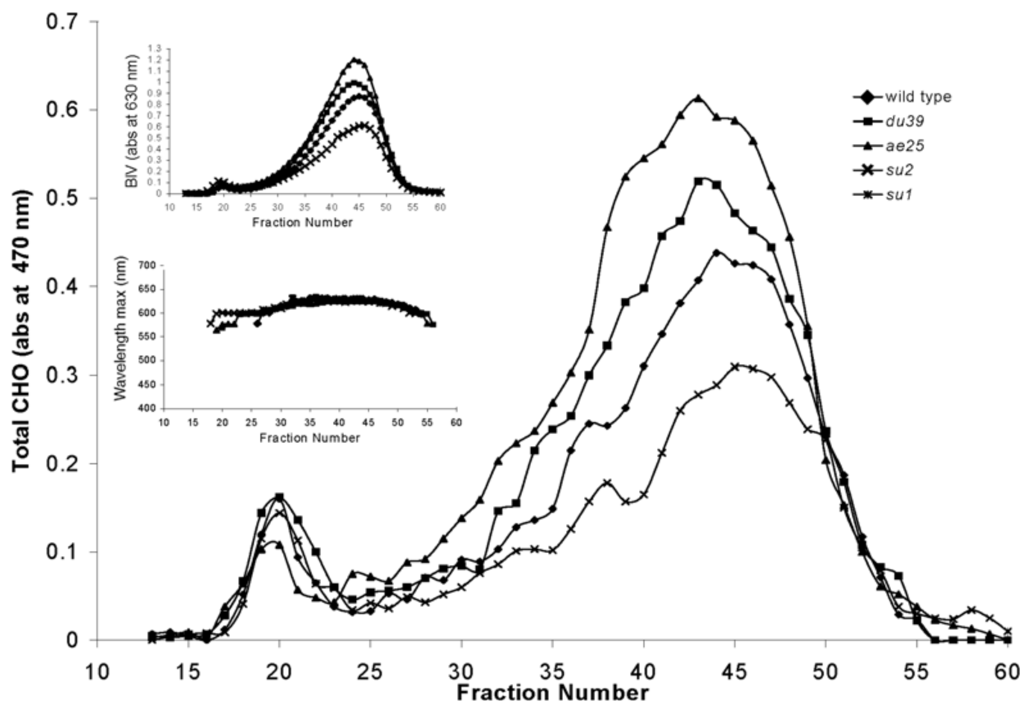

Fig. 7. Gel-permeation chromatography profiles of the IS starch fractions (Method III) obtained by using preferential 1-butanol and isoamyl alcohol precipitation.

peak. The degrees of polymerization (DP) at the maximum height of each peak (DP_{peak}) reported in Table I were identical in the L and M fractions within each starch type for Peak I (DP_{peakI}). For Peak II, the (DP_{peakII}) of L and M were identical only in the wild type and *su1* starches. The DP_{peakII} for M fractions of *ae25* and *su2* was greater than the DP_{peakII} in the L fractions, whereas the DP_{peakII} of *du39* M fraction was lower than that of the *du39* L fraction. All the S fractions from each starch type, except for the wild type starch, had different DP_{peakI} and DP_{peakII} than did the corresponding L and M fractions. It is important to note that the data obtained for the S fraction, containing mainly amylose, reflects a combination of chains from any residual branched molecules in the IS fraction in addition to short branch chains from the precipitated amylose molecules but not the long component chains from amylose, which would be too large for detection with the HPAEC-ENZ-PAD system.

The distributions of DP obtained within the L, M, and S starch fractions for each starch type were grouped into four categories: DP 6–12, 13–24, 25–36, and >36 (Table I). The M fractions tended to contain a greater proportion of chains with DP of 6–12 than did the L fractions from the same starch type. The S fractions from each starch type exhibited an even greater percentage of branch chains with DP 6–12 than did the M and L fractions, because the short branch chains associated with the amylose molecule were present in this fraction. The percentage of chains with DP 13–24 of the M fraction from wild type, *du39*, *su2*, and *su1* starches was the same or slightly lower than that of the corresponding L fraction. For the *ae25* starch, the percentage of DP 13–24 chains was slightly greater in the M fraction than in the L fraction, but still represented the lowest relative percentage of chains with DP 13–24 among all starch types. The percentage of branch chains with DP 13–24 for the S fraction was greater than that of the L and M fractions within a starch type, except for *su1* starch, where the percentage of these chains was lower.

The percentage of chains with DP 25–36 within a starch type was greater in the M fraction than in the L fraction, except for *su2* starch, in which the percentage was lower in the M fraction and *su1* in which the distribution was the same. The S fractions generally contained fewer percentage of branch chains with DP 25–36, except for the *ae25* starch, where the percentage was higher, again reflecting the character of the components in the S fraction. The chain population of DP > 36 was lower in the M than in the L fraction for most starch types, except for *du39* and *su1* starches. The *ae25* L and M fractions had the greatest proportion of DP > 36 branch chains among all L and M fractions, whereas the *du*39 L and M fractions had the lowest proportion of these chains. The S fraction within a starch type had the least percentage of chains with DP > 36. The greatest CL_w among all starches were obtained from the *ae25* L and M starch fractions. The greatest detectable DP for the M fraction from each starch was lower than that for the corresponding L fractions, except for *ae25* and *su2*. The greatest detectable DP for the S fractions was lower than the DP reported for the M fractions within a starch type. Results regarding branch chain length distribution of the *su1* mutant are similar to those reported for mature endosperms of Kinmaze and Taichung-65 *su1* mutants of rice (Nakamura et al 1997); the branch chains with DP ≤ 12 were more numerous and intermediate (DP 13–36) and long chains (DP > 37) were fewer than in the normal counterpart.

TABLE I
Branch Chain Length Distributions of Large, Medium, and Small MW Fractions of Starches Obtained with Gel-Permeation Chromatography (Method I)[a]

Sample	$DP_{Peak\ I}$	$DP_{Peak\ II}$	% Distribution				CL_w	Greatest DP
			DP 6–12	DP 13–24	DP 25–36	DP > 36		
wild L	13	46	18.0 ± 0.9	45.7 ± 1.0	13.2 ± 0.5	23.1 ± 1.4	36.2	75
wild M	13	46	20.9 ± 0.6	45.8 ± 0.9	14.5 ± 0.2	18.8 ± 1.7	38.8	66
wild S	13	46	26.6 ± 0.6	53.3 ± 0.2	11.3 ± 0.3	8.7 ± 0.6	36.2	58
du39 L	13	47	19.2 ± 0.1	50.7 ± 0.4	14.6 ± 0.1	15.5 ± 0.6	34.1	72
du39 M	13	45	20.3 ± 0.1	48.5 ± 1.5	15.3 ± 0.2	15.7 ± 1.2	37.3	66
du39 S	12	44	26.0 ± 0.1	55.9 ±1.1	12.2 ± 0.5	5.9 ± 0.8	34.9	58
ae25 L	15	49	9.2 ± 0.4	36.6 ± 0.1	14.6 ± 0.3	39.6 ± 0.0	44.7	80
ae25 M	15	50	9.3 ± 0.2	37.2 ± 0.0	16.2 ± 0.1	37.4 ± 0.4	43.0	83
ae25 S	16	46	16.7 ± 0.8	46.9 ± 3.4	17.6 ± 0.9	18.7 ± 3.5	36.8	72
su2 L	12	37	24.9 ± 0.0	41.5 ± 0.1	15.1 ± 0.0	18.5 ± 0.2	36.3	69
su2 M	12	43	27.9 ± 1.6	41.4 ± 0.9	14.0 ± 0.5	16.7 ± 0.0	33.8	71
su2 S	11	47	34.8 ± 0.4	43.4 ± 0.1	12.7 ± 1.2	9.2 ± 0.7	38.0	54
su1 L	12	39	21.4 ± 0.3	45.9 ± 0.0	14.5 ± 0.4	18.2 ± 0.1	36.0	70
su1 M	12	39	21.5 ± 0.4	45.5 ± 0.9	14.5 ± 0.2	18.5 ± 0.4	39.5	64
su1 S	11	40	29.8 ± 0.2	39.4 ± 0.3	13.9 ± 0.5	16.9 ± 0.9	36.0	56

[a] Numbers reported are averages of two replicates ± standard deviation. L, large molecular weight; M, medium molecular weight; S, small molecular weight. DP_{Peak}, degree of polymerization at the maximum height of the peak. CL_w, weight-average chain-length.

TABLE II
Branch Chain Length Distributions of Starch Fractions Obtained from Preferential Precipitation with 1-Butanol (Method II)[a]

Sample	$DP_{Peak\ I}$	$DP_{Peak\ II}$	% Distribution				CL_w	Greatest DP
			DP 6–12	DP 13–24	DP 25–36	DP > 36		
$wild_{Sol}$	13	48	17.1 ± 0.3	47.7 ± 0.5	15.8 ± 0.8	19.4 ± 0.0	37.7	70
$wild_{Insol}$	13	44	21.7 ± 0.1	41.5 ± 0.5	17.5 ± 0.1	19.3 ± 0.8	43.1	60
$du39_{Sol}$	13	46	20.7 ± 3.7	48.0 ± 2.8	15.9 ± 1.3	15.1 ± 0.3	34.0	72
$du39_{Insol}$	13	44	21.0 ± 0.9	43.9 ± 1.1	20.5 ± 1.3	14.5 ± 0.8	43.4	57
$ae25_{Sol}$	15	45	10.3 ± 0.0	47.4 ± 1.0	21.7 ± 0.1	20.6 ± 1.0	34.4	84
$ae25_{Insol}$	13	46	18.9 ± 0.2	40.1 ± 0.1	17.9 ± 1.2	23.2 ± 1.5	46.8	59
$su2_{Sol}$	12	44	23.4 ± 0.9	39.9 ± 1.3	16.7 ± 0.3	19.9 ± 0.2	36.1	70
$su2_{Insol}$	12	44	25.7 ± 0.2	40.6 ± 1.1	19.0 ± 0.5	14.7 ± 0.9	38.7	62

[a] Numbers reported are averages of two replicates ± standard deviation. Sol, soluble fraction; Insol, insoluble fraction. DP_{Peak}, degree of polymerization at the maximum height of the peak. CL_w, weight-average chain-length.

Method II. The DP_{peakI} was the same for both the Sol and Insol fractions within a starch type except for *ae25* starch, where the DP_{peakI} for the *ae25* Insol fraction was lower than that for the Sol fraction (Table II). Similarly as for the S fraction of Method I, it is important to note that the data obtained for the Insol fraction, containing mainly amylose, reflects any branch chains from these amylose molecules, and not the long chain amylose portion, which would be too large for detection with the HPAEC-ENZ-PAD system. Any residual branched molecules in the Insol would also influence this chromatogram.

Within a starch type, the branch chain length distributions of the Sol fractions were similar to that of the L fractions from Method I. The relative branch chain length distributions of the Sol fraction revealed that *su2* starch tended to contain the greatest proportion of chains with DP 6–12 (23.4%) and the least proportion of chains with DP 13–24 among the Sol fractions of all starches. The *ae25* starch had the greatest proportion of longer branch chains (DP 25–36 and DP > 36) and *du*39 starch had the least proportion of chains with DP > 36 (15.1%) among all the Sol fractions. The greatest DP_n was found in the *ae25* Sol fraction. The Insol fractions from all starch types, except for *du39* starch, had a greater percentage of branch chains with DP 6–12 than did their corresponding Sol fractions. This finding suggested some similarity in composition to the branch chains in the S fraction of Method I. Generally, Sol fractions had a greater percentage of DP 13–24 than did the Insol fractions from the same starch types, except for *su2* starch. There was no difference between the percentage of chains with DP > 36 between the Sol and Insol fractions from the same starch types, except for the *su2* starch fractions. Unlike the data for the S fraction of Method I, the CL_w of all Insol fractions was greater than that of the Sol fractions for all starch types, indicating that molecules of more linear character were present in the Insol fraction as also observed by the λ_{max} of these fractions. Perhaps Method II allowed a greater percentage of linear components to separate into the Insol fraction than did Method I into the S fraction. However, the highest DP values within all the Insol fractions were still lower than that found for their Sol fractions.

Method III. The greatest DP_{peakI} was found in the *ae25* starch fractions (Table III). Generally, the DP_{peakI} was lower for the SII and IS fractions than for the corresponding SI fractions within a starch type. Similarly as for the S fraction of Method I and the Insol fraction of Method II, it is important to note that the data obtained for the IS fraction, containing mainly amylose, reflects branch chains from these amylose molecules, and not the long chain amylose portion, which would be too large for detection with the HPAEC-ENZ-PAD system. Starch types had greater DP_{peakII} for SII than for SI and IS fractions, except for the wild type starch, which had identical DP_{peakII} in the SI and SII fractions. The percentage distributions showed that IS fractions contained a greater proportion of chains with DP 6–12 than did the SI and SII fraction from the same starch type, reflecting the short nature of the branches on the amylose present in the IS fraction. Considerably more chains with DP 13–24 were present in the IS fraction than in the SI and SII fractions for the wild type and *du39* starches, whereas the SII fractions of *ae25* and *su2* had slightly greater or almost equal percentages of DP 13–24 chains compared with their other fractions.

The percentage of chains with DP 25–36 within a starch type was lower for all SII fractions than for the SI fractions, except for *du39* starch where it was greater (16.9% vs. 14.9%). In addition, except for *ae25* starch, IS fractions had a smaller percentage of branch chains with DP 25–36 than did the SI and SII fractions from the same starch type. The DP > 36 chain population clearly was lower in the IS fractions than in the SII and SI fractions for all starch types, similar to what was found in the S fraction of Method I. The *ae25* SI fraction had the greatest proportion of branch chains with DP > 36 among all SI and SII fractions. Wild type SII fraction had the greatest proportion of these chains among all SII fractions, whereas the *du*39 SI had the least percentage of DP > 36 chains among the SI fractions (16.7%), *su2* SII had the least percentage among the SII fractions (8.6%), and *du39* IS fraction had the least percentage of branch chains with DP > 36 among all starch fractions. In general, the proportion of chains with DP 6–12 for a particular starch type was greater in the SII than in the SI fraction, whereas the percentage of chains with DP > 36 was greater in the SI fractions than in the SII fractions. It is likely that the branched starch molecules of the IS fraction did not stay in the supernatant after 1-butanol addition, as did molecules of the SII fraction, but precipitated, because of the contribution of their long chains, the detection of which was beyond the range measured with the HPAEC system. Among all starch types, the *ae25* SI fraction had the greatest CL_w, whereas the *su2* SII fraction had the lowest CL_w. The greatest DP value within the SI fraction of a starch was always greater than for SII and IS fractions.

CONCLUSIONS

Three methods were used to characterize the starch molecules residing in corn starch granules obtained from different corn mutant types. The separations were performed by fractionation on a GPC column (Method I), by precipitation with 1-butanol (Method II), and by preferential precipitation with 1-butanol and isoamyl alcohol (Method III). All methods revealed that a clear separation among the starch molecules is not possible because there are no strict limits for the fractionation. As noted with all methods, each starch type had a wide range of structures, from

TABLE III
Branch Chain Length Distributions of Soluble I and Soluble II Starch Fractions Obtained from Preferential Precipitation with 1-Butanol and Isoamyl Alcohol (Method III)[a]

Sample	% Distribution						CL_w	Greatest DP
	$DP_{Peak\ I}$	$DP_{Peak\ II}$	DP 6–12	DP 13–24	DP 25–36	DP > 36		
wild SI	13	45	18.1 ± 0.2	43.9 ± 0.1	13.9 ± 0.2	24.1 ± 0.2	36.9	75
wild SII	12	45	24.7 ± 0.3	45.8 ± 0.2	12.7 ± 0.1	16.8 ± 0.3	34.8	69
wild IS	12	40	31.3 ± 2.3	49.4 ± 2.1	10.2 ± 1.9	9.1 ± 2.5	34.0	60
du39 SI	13	42	20.4 ± 0.6	48.0 ± 0.1	14.9 ± 0.3	16.7 ± 1.0	35.4	70
du39 SII	12	45	25.8 ± 0.1	46.9 ± 0.3	16.9 ± 0.2	10.4 ± 0.5	36.5	60
du39 IS	12	42	29.0 ± 1.6	54.5 ± 1.4	11.9 ± 0.3	4.6 ± 0.1	32.5	60
ae25 SI	15	48	13.7 ± 0.4	35.7 ± 0.1	14.9 ± 0.3	35.7 ± 0.6	44.9	74
ae25 SII	15	50	15.7 ± 0.1	53.6 ± 0.7	14.4 ± 0.2	16.2 ± 0.3	34.8	72
ae25 IS	14	46	20.2 ± 2.5	50.9 ± 1.2	16.7 ± 0.8	12.1 ± 0.4	36.2	65
su2 SI	12	40	23.0 ± 0.1	34.3 ± 0.8	15.1 ± 0.1	27.6 ± 0.0	41.1	70
su2 SII	12	43	31.3 ± 0.2	46.6 ± 0.0	13.5 ± 0.3	8.6 ± 0.1	31.3	66
su2 IS	12	41	33.0 ± 1.4	46.7 ± 1.1	12.5 ± 0.1	7.8 ± 0.2	32.5	62

[a] Numbers reported are averages of two replicates ± standard deviation. SI, soluble I fraction; SII, soluble II fraction. DP_{Peak}, degree of polymerization at the maximum height of the peak. CL_w, weight-average chain-length.

highly branched to more linear, with wide MW distributions. The proportions of branch chain lengths of the starch components obtained by the various fractionation methods were very similar among methods for each of the starch types analyzed, such as the predominance of long branch chains in *ae25* corn and that of the short branch chains in *su2* corn. Method I gave information on how the relative branch chain length populations differed from the larger to the smaller molecular weight molecules. Method II separation, based on the behavior of starch molecules when interacting with 1-butanol, provided information about the structure of molecules that complex and precipitate compared with the ones that do not. Method III served the same objective as Method II, but further provided structural characterization of the branched molecules that differentially precipitated with the addition of two separate alcohols, 1-butanol and isoamyl alcohol. Therefore, different pieces of information regarding the branch chain length of the starch molecules were collected depending upon the characterization approach of analysis. Differences in percentages of branch chain lengths among the fractions from the various methods were related to the different demarcations of separation.

In general, Methods I and III separated the branch chains from the various mutant starches into fractions with relatively similar starch components (Tables I and III), where L and SI, M and SII, and S and IS were somewhat matched. In particular, the CL_w and DP were similar, except for M of *ae25* and the CL_w of *su2*. These differences further illustrate that the separation of starches into various fractions is relative to the structure of the starch being measured and varies with the method used. It was difficult to compare the two fractions from Method II with the three fractions from Methods I and III, considering the overlap of components in the Method II fractions with material likely separating into M of Method I and SII of Method III.

In general, the structural characteristics of starches from the corn endosperm mutants revealed in this study agreed with data provided by others in the literature (Wang et al 1993; Wang and White 1994; Kasemsuwan et al 1995; Klucinec and Thompson 1998). In addition, the current data shows that the population of short chains observable by HPAEC-ENZ-PAD in "intermediate materials" in various starch sources, independent of how the intermediate material was defined, varies little from the amylopectin fraction obtained. Furthermore, the amylose, which is defined as either small molecules using chromatographic fractionation or as an insoluble component of precipitation-based fractionation, is an important contributor to the chain length distribution in these amylose-rich fractions. Overall, the effect of mutations was greater than the variations created using different methods of analysis.

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