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Characterization of maize amylose-extender (*ae*) mutant starches. Part I: Relationship between resistant starch contents and molecular structures

Li Li [a], Hongxin Jiang [a], Mark Campbell [b], Michael Blanco [c], Jay-lin Jane [a,*]

[a] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
[b] Truman State University, Kirksville, MO, USA
[c] USDA-ARS/Plant Introduction Station, Ames, IA 50011, USA

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ABSTRACT

Endosperm starches were isolated from kernels of seven maize amylose-extender (*ae*) lines: three new *ae*-lines, derived from a Guatemalan breeding cross with pedigrees of GUAT209:S13 × (OH43*ae* × H99*ae*) B-B-4-1-2-1-1, GUAT209:S13 × (OH43*ae* × H99*ae*) B-B-4-4-2-1-1, and GUAT209:S13 × (OH43*ae* × H99*ae*) B-B-4-4-2-1-2, designated as GSOH1, GSOH2, and GSOH3, respectively, were developed by the USDA-ARS Germplasm Enhancement of Maize (GEM) Project, and four existing inbred lines, H99*ae*, OH43*ae*, B89*ae*, and B84*ae*. The resistant starch (RS) contents, measured using AOAC method 991.43 for total dietary fiber, showed that the three new-line starches had larger RS contents (39.4–43.2%) than the four inbred lines (11.5–19.1%). This study was conducted to understand relationship between the RS content and molecular structure of the maize ae-mutant starch. Analytical results showed that the three new-line starches had larger apparent (83.1–85.6%) and absolute amylose-contents (57.4–62.6%) than the starches of the inbred *ae*-lines (61.7–67.7% and 35.5–44.7%, respectively). The RS content of the *ae*-mutant starch was positively correlated with both the apparent and absolute amylose-contents of the starch with correlation coefficients of 0.99 and 0.96, respectively. Gel permeation chromatograms revealed that all seven *ae* starches contained large proportions of intermediate components (IC), 22.4%–52.0%. All seven *ae* starches displayed similar onset gelatinization temperatures (64.5–65.8 °C), but the three new-line starches displayed higher conclusion temperatures (122.0–130.0 °C) than the four inbred-line starches (100.5–105.3 °C). These results indicated that the crystalline structure of the three new *ae*-line starches was retained after boiling at ~100 °C. The crystalline structure was resistant to enzyme hydrolysis and resulted in greater RS contents.

1. Introduction

Starch is the major energy reserve in higher plants and is the second largest biomass on earth next to cellulose. Starch is an important ingredient for food and nonfood applications; it serves not only as the major energy source in food and feed, but also as a thickener, a binding agent, a texturizer, a filler, a film forming agent, and feedstock for fermentation of biomaterial and fuel. Starch consists of two major components: amylose and amylopectin. Amylose has mainly linear molecules with α-1,4 linked D-glucosyl units and a few branches of α-1,6 linkages (French, 1973; Hizukuri, Takeda, & Yasuda, 1981). Amylopectin is a highly branched molecule, consisting of about 5% α-1,6 linkages (French, 1973).

Normal maize starch consists of 20–30% amylose. One of the maize mutants, *amylose-extender* (*ae*) mutant, produces starch with a much larger amylose-content and amylopectin with significantly longer branch-chains than the normal maize starch (Baba & Arai, 1984; Baba, Arai, Yamamoto, & Itoh, 1982; Jane & Chen, 1992; Jane et al., 1999; Kasemsuwan, Jane, Schnable, Stinard, & Robertson, 1995; Shi & Seib, 1995; Takeda, Takeda, & Hizukuri, 1993; Yuan, Thompson, & Boyer, 1993). Because the long branch-chains of the amylopectin also bind iodine, form helical complex, and develop dark blue-color, which inflate the value of amylose-content. Thus, the apparent amylose-content of the maize *ae*-mutant starch is substantially larger than the absolute amylose-content. For the determination of absolute amylose-content, the iodine bound by amylopectin is subtracted from the total iodine bound by the starch (Jane et al., 1999). The very long branch-chains of the *ae*-mutant amylopectin are also known for developing the B-type crystallinity of the starch (Hizukuri, Kaneko, & Takeda, 1983; Kasemsuwan et al., 1995). These *ae*-mutant starches contain intermediate components (IC) that consist of branched molecules with molecular weights smaller than amylopectin but similar to amylose (Baba & Arai, 1984; Baba et al., 1982; Kasemsuwan et al.,

* Corresponding author. Fax: +1 515 294 8181.
E-mail address: jjane@iastate.edu (J.-l. Jane).

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1995; Wang, White, Pollak, & Jane, 1993). Studies have shown that starch with a larger amylose-content has less susceptibility to enzyme hydrolysis (Jane et al., 2003; Okuda, Aramaki, Koseki, Satoh, & Hashizume, 2005).

In the human digestive system, a portion of starch cannot be digested and absorbed in the small intestine and is passed to the large intestine for bacteria fermentation. This starch is known as resistant starch (RS) (Englyst & Macfarlane, 1986). RS is classified into four types. Type I RS is starch that is entrapped in plant tissue and not susceptible for enzyme hydrolysis. Type II RS is native raw starch granules having the B-type polymorphism, such as potato, wrinkle pea, and high-amylose maize starches, which are resistant to enzyme hydrolysis. Type III RS is retrograded amylose (Englyst, Kingman, & Cummings, 1992), and Type IV RS is chemically modified starch (Woo & Seib, 2002). Studies have suggested that consumption of RS made from high-amylose maize starch brings a wide range of health benefits, such as lowering the glycemic index and promoting colon health (Sajilata, Singhal, & Kulkarni, 2006). Thus, it is important to understand the molecular structures and properties of the RS in the high-amylose maize starch.

Many methods have been reported in the literature for analyzing RS contents of different foods or raw material. Examples include Englyst's method (Englyst et al., 1992) for prepared or ready-to-eat food products, and AOAC 2002.02/AACC 32–40 method (AOAC, 2005) for analysis of RS contents in raw starch and plant material. For human consumption, starch is used as an ingredient and is subjected to cooking. The resistant starch content of raw starch without cooking, therefore, is misleading and not meaningful. For example, the resistant starch content of raw potato starch is 63.39% (AOAC, 2005), but in practice, people do not eat raw potato. After cooking, the resistant starch content of freshly cooked potato is 3% (Englyst & Cummings, 1987). Because of this misleading result generated from the AOAC 2002.02 method, AOAC 991.43 method (AOAC, 2003) for total dietary fiber analysis is a preferred method for determination of RS content of starch samples (Seib & Woo K., 1999; Shin & Seib, 2004; Shin, Song, & Seib, 2004). When isolated starch samples (purity >99%) were subjected to the AOAC 991.43 analysis, residues remained after thermal stable α-amylase hydrolysis at ~100 °C for 30 min and subsequent glucoamylase hydrolysis are considered true resistant starch according to the AACC Dietary Fiber Definition Committee Report (AOAC, 2001) and other studies (Champ, Langkide, Brouns, Kettlitz, & Collet, 2003; Gray, 2006).

The objective of this study was to understand relationships between RS contents and structures of maize *ae*-mutant starches. Starches were isolated from kernels of four existing maize *ae*-mutant inbred lines and three new *ae*-lines that contained pedigrees of a Guatemalan line (Campbell, Jane, Pollak, Blanco, & O'Brien, 2007) and were developed from the USDA-ARS GEM project at Truman State University. The RS, amylose, amylopectin, and IC-contents of the starches, the molecular weights and the branch structures of the amylopectin and IC, and the thermal properties of the mutant starches were analyzed. The relationship between the RS contents and the starch structures were proposed and discussed.

2. Materials and methods

2.1. Materials

All chemicals were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO). Crystalline *Pseudomonas* isoamylase (EC 3.2.1.68), specific activity about 66,000 U/mg of protein, was purchased from Hayashibara Shoji, Inc. (Okayama, Japan) and was used without further purification. Three new-lines of maize *ae*-mutants, GUAT209:S13 × (OH43*ae* × H99*ae*) B-B-4-1-2-1-1, GUAT209:S13 × (OH43*ae* × H99*ae*) B-B-4-4-2-1-1, and GUAT209:S13 × (OH43*ae* × H99*ae*) B-B-4-4-2-1-2, designated as GSOH1, GSOH2, and GSOH3, respectively, were developed by M. Campbell at Truman State University breeding nursery in Kirksville, MO, and the USDA-ARS Germplasm Enhancement of Maize (GEM) project in Ames, IA. These new *ae*-mutant lines were related F6 inbred lines and derived from the registered germplasm line GEMS-0067 (Campbell, Jane, Pollak, Blanco, & O'Brien, 2007). Other public inbred maize *ae*-mutant starches of lines H99ae, OH43ae, B89ae, and B84ae were used in the study for comparison. Corn was harvested after it was physiologically mature, about 50 days after pollination.

2.2. Starch isolation

Maize kernels of the *ae*-mutant lines were steeped in an aqueous solution of 0.45% sodium metabisulfite at 4 °C overnight. The steeped sample was milled in a microblender for 1 min, and the process was repeated three times. The ground sample was filtered through a nylon screen with a pore size of 53 μm and washed with excess water. The residue was ground again with additional water until no more starch was released. Starch was collected by centrifugation, suspended in an aqueous solution of 0.1 M NaCl containing 10% toluene, and stirred for one hour using magnetic stirrer at a high speed to remove protein. This treatment was repeated until the toluene layer became clear and contained no protein. The purified starch was washed three times with water, twice with 100% ethanol, and dried at 30 °C for 48 h.

2.3. Resistant starch (RS) content

RS contents of the *ae*-mutant starch samples were determined using the AOAC Method 991.43 for total dietary fiber (AOAC, 2003) and Englyst's method (1992) for comparison. For the AOAC 991.43 method, starch (1.0 g, dry-starch basis, dsb) was suspended in a Mes-tris buffer solution (0.05 M, 40 ml) and hydrolyzed with 500 u of α-amylase from *Bacillus licheniformis* (Sigma Chemical, Cat. No. A3403) in a boiling water-bath for 30 min with stir. The sample was then digested with protease from *Bacillus licheniformis* (5 mg, Sigma Chemical, Cat. No. P3910) at 60 °C in a shaker water-bath for 30 min. The sample dispersion was adjusted to pH 4.4–4.6 by adding HCl and then hydrolyzed with amyloglucosidase (300 U, Sigma chemical, Cat. No. A9913) at 60 °C in a shaker water-bath for 30 min. The digested sample was filtered through a celite layer in a crucible and washed twice with 15 ml of 78% ethanol, twice with 15 ml of 100% ethanol and rinsed with 15 ml of acetone. The remaining sample was dried in an oven at 100 °C overnight. The resistant starch content was calculated using the equation: % RS content = Remaining sample weight (g, dsb)/initial sample weight (g, dsb) × 100%.

For RS determined using Englyst's method (1992), Starch (1.000 g, db) in 20 mL of sodium acetate buffer (0.1 M, pH 5.2) was cooked in a boiling water-bath for 30 min. The starch dispersion was cooled down to 37 °C, mixed with an enzyme solution (5 mL) consisting of pancreatin extract and amyloglucosidase, and incubated in a water-bath at 37 °C. The pancreatin extract was prepared as follows; 3.0 g of pancreatin (Sigma, Cat. No. P7545) was suspended in 20 mL deionized water, stirred for 10 min at room temperature, and centrifuged at 1500*g* for 10 min. The enzyme solution was prepared by mixing 13.5 ml supernatant of pancreatin extract, 210 U amyloglucosidase (Sigma, Cat. No. A7095), and 1.0 mL deionized water. The rapid digestible starch (RDS) was defined as the total starch digested within the first 20 min, and the slowly digestible starch (SDS) was the starch digested between 20 and 120 min (Englyst et al., 1992). The resistant starch content was calculated as follows:

$$(\%)\mathrm{RS} = 100\% \times (\text{total starch} - \mathrm{RDS} - \mathrm{SDS})\ (\mathrm{g, dsb}) / \text{totalstarch}\ (\mathrm{g, dsb}).$$

2.4. Fractionation of starch components

Amylopectin and intermediate components (IC) were separated from amylose using 1-butanol. Amylose formed a helical complex with 1-butanol and precipitated (Jane & Chen, 1992; Schoch, 1942). The supernatant, containing amylopectin and IC, was collected, concentrated, and the amylopectin and the IC were precipitated using excess ethanol and further purified by repeating the complex-formation procedure 2–3 times. Removal of amylose from the amylopectin and IC mixture was verified using gel permeation chromatography (GPC).

2.5. Amylose-content of starch

Apparent and absolute amylose-contents of starch samples were determined using potentiometric titration (Song & Jane, 2000). Starch (0.5 g) was defatted and dispersed using 90% DMSO (50.0 ml) following the procedure reported by Song and Jane (2000). The defatted and dispersed starch was precipitated with ethanol, and then dried at 30 °C for 48 h. Iodine affinities of the defatted starch samples and of the mixtures of amylopectin and IC were determined using a potentiometric autotitritor (702 SM Titrino, Brinkmann Instrument, Westbury, NY). The analysis was done in duplicate. The iodine affinity of amylose used in the calculation was 20%. The apparent amylose-content was calculated by dividing the iodine affinity of the starch by 20%. The absolute amylose-content was calculated by using the equation: $(\mathrm{IA_{starch}} - \mathrm{IA_{amylopectin+IC}})/(20 - \mathrm{IA_{amylopectin+IC}}) \times 100\%$ (Takeda et al., 1993).

2.6. Amylopectin and IC-contents of starch

The amylopectin and the IC-contents of the starch were determined using gel permeation chromatography (GPC) followed by total carbohydrate (CHO) analysis (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Starch (15 mg) or a mixture of amylopectin and IC was defatted, dispersed, and precipitated and then re-dispersed in boiling distilled water (5 ml). This dispersion (2.0 ml) was then injected into a Sepharose CL-2B gel permeation column (Pharmacia, Piscataway, NJ) (1 cm ID × 48 cm) and eluted using an eluent containing 25 mM NaCl and 1 mM NaOH at a flow rate of 0.7 ml/min in a descending mode. Fractions of 1.0 ml each were collected and analyzed for total carbohydrate (Phenol-sulfuric acid method) (Dubois et al., 1956) and blue-value (iodine staining) (Juliano, 1971) at 490 and 630 nm, respectively. The analyses were conducted in duplicate. The amylopectin content was calculated using the following equation:

$$\%\ \text{Amylopectin} = \text{Total CHO}_{\text{amylopectin}}/\text{Total CHO}_{(\text{amylopectin+IC+amylose})}$$

The % IC content of the mixture of amylopectin and IC was calculated as:

$$\%\ \mathrm{IC_{of\ (amylopectin+IC)}} = \text{Total CHO}_{\mathrm{IC}}/\text{Total CHO}_{(\text{amylopectin+IC})}$$

The % IC content of the whole starch was calculated using the equation:

$$\%\ \mathrm{IC} = \%\ \mathrm{IC_{of(amylopectin+IC)}} \times \%\ \mathrm{AP}/(100 - \%\ \mathrm{IC_{of(amylopectin+IC)}}).$$

2.7. Molecular weight (M_W) and gyration radius of amylopectin and M_W of IC

The weight-average molecular weight (M_w) and z-average gyration radius (R_z) of amylopectin were determined using a high-performance size-exclusion chromatograph equipped with a multi-angle laser-light scattering detector (Dawn DSP, Wyatt Tech., Santa Barbara, CA) and a refractive index detector (G1362A, Agilent, Santa, Clara, CA) (HPSEC-MALLS-RI). A Shodex OH pak SB-G guard column and SB-806 and SB-804 analytical columns were used to separate molecules of different sizes following the same methods described by Yoo and Jane (2002). The samples were analyzed in duplicate. An aqueous solution containing the mixture of amylopectin and IC (0.4 mg/ml) was freshly prepared and injected to the columns. The molecular weight of IC was determined using a standard curve consisting of a series of pullulan of different molecular weights (P5, P10, P20, P50, P100, P200, P400, and P800).

2.8. Branch chain-length distributions of amylopectin and IC

Amylopectin and IC of different molecular weights (M_W) were separated using GPC. The fractions within the amylopectin peak and within the IC peak of large and small-M_W were combined separately. The combined eluent was concentrated and used for branch chain-length distribution analysis. The collected amylopectin or IC was dispersed using DMSO following the same procedure reported earlier and then dispersed in hot water (3 mg in 2.7 ml), stirred in a boiling water-bath for 20 min, cooled down to room temperature and debranched using isoamylase in an acetate buffer solution (0.01 N, pH 3.5) containing 0.02% sodium azide in a shaker water-bath at 40 °C and 120 strokes per minute for 24 h. The iso-

Table 1
Resistant starch and amylose-contents of maize *ae*-mutant starches

Sample	%RS (AOAC 991.43)[a]	% Starch fraction[b] Englyst et al. 1992)			% Apparent amylose[c]	% Abs. amylose[c]
		RDS	SDS	RS		
GSOH1	42.4 ± 1.7	64.7 ± 4.0	0.9 ± 3.4	34.3 ± 0.7	85.6 ± 1.7	62.6
GSOH2	43.2 ± 0.1	65.2 ± 3.3	3.2 ± 2.7	31.7 ± 0.7	84.2 ± 0.5	57.4
GSOH3	39.4 ± 0.5	66.2 ± 4.4	2.9 ± 1.4	30.9 ± 3.0	83.1 ± 0.1	61.1
H99ae	19.1 ± 0.5	79.5 ± 1.4	1.9 ± 0.7	18.6 ± 2.1	67.7 ± 0.4	44.7
OH43ae	14.0 ± 0.5	77.9 ± 0.8	3.2 ± 1.0	18.9 ± 1.8	61.7 ± 0.1	35.5
B89ae	14.9 ± 0.6	77.9 ± 3.4	2.6 ± 0.1	19.5 ± 3.5	63.3 ± 1.1	39.2
B84ae	11.5 ± 1.4	75.0 ± 0.3	4.0 ± 3.0	20.9 ± 2.7	62.8 ± 0.3	41.4
Correlation coefficient between RS% and amylose-contents					0.99 (AOAC 991.43) 0.97 (Englyst et al. 1992)	0.96 (AOAC 991.43) 0.96 (Englyst et al. 1992)

Apparent amylose-content (%) = $\mathrm{IA_{starch}}/\mathrm{IA_{amylose}} \times 100\%$.
Absolute amylose-content (%) = $(\mathrm{IA_{starch}} - \mathrm{IA_{amylopectin+IC}})/(\mathrm{IA_{amylose}} - \mathrm{IA_{amylopectin+IC}}) \times 100\%$.
[a] Resistant starch (RS) contents were determined using AOAC 991.43 total insoluble dietary fiber.
[b] Resistant starch contents were determined using Englyst et al. 1992. RDS = rapid digestible starch; SDS = slowly digestible starch; % Resistant starch (RS) content = 100 × (total starch mass (db) − RDS–SDS)/total starch mass (db).
[c] Amylose-content was determined using potentiometric titration method.

amylase-hydrolyzed sample was adjusted to pH 7 by adding NaOH and boiled for 15 min to inactivate the enzyme.

The branch chain-length distributions of amylopectin and IC were analyzed using high-performance anion-exchange chromatography (Dionex-300, Sunnyvale, CA) equipped with an on-line amyloglucosidase column and an ED50 pulse-amprometric detector (Dionex, Sunnyvale, CA) (HPAEC-ENZ-PAD). A PA-100 anion-exchange analytical column (4 × 250 mm) and a guard column (Dionex, Sunnyvale, CA) were used for separation. The operational parameters were the same as that described by McPherson and Jane (1999). The data were analyzed by using Chromeleon software (Dionex, Sunnyvale, CA), and the sample was analyzed in duplicate.

2.9. Thermal properties of the ae-mutant starches

Thermal properties of the starch samples were determined using a differential scanning calorimeter (DSC-7, Perkin–Elmer, Norwalk, CT), following the method of Song and Jane (2000). The

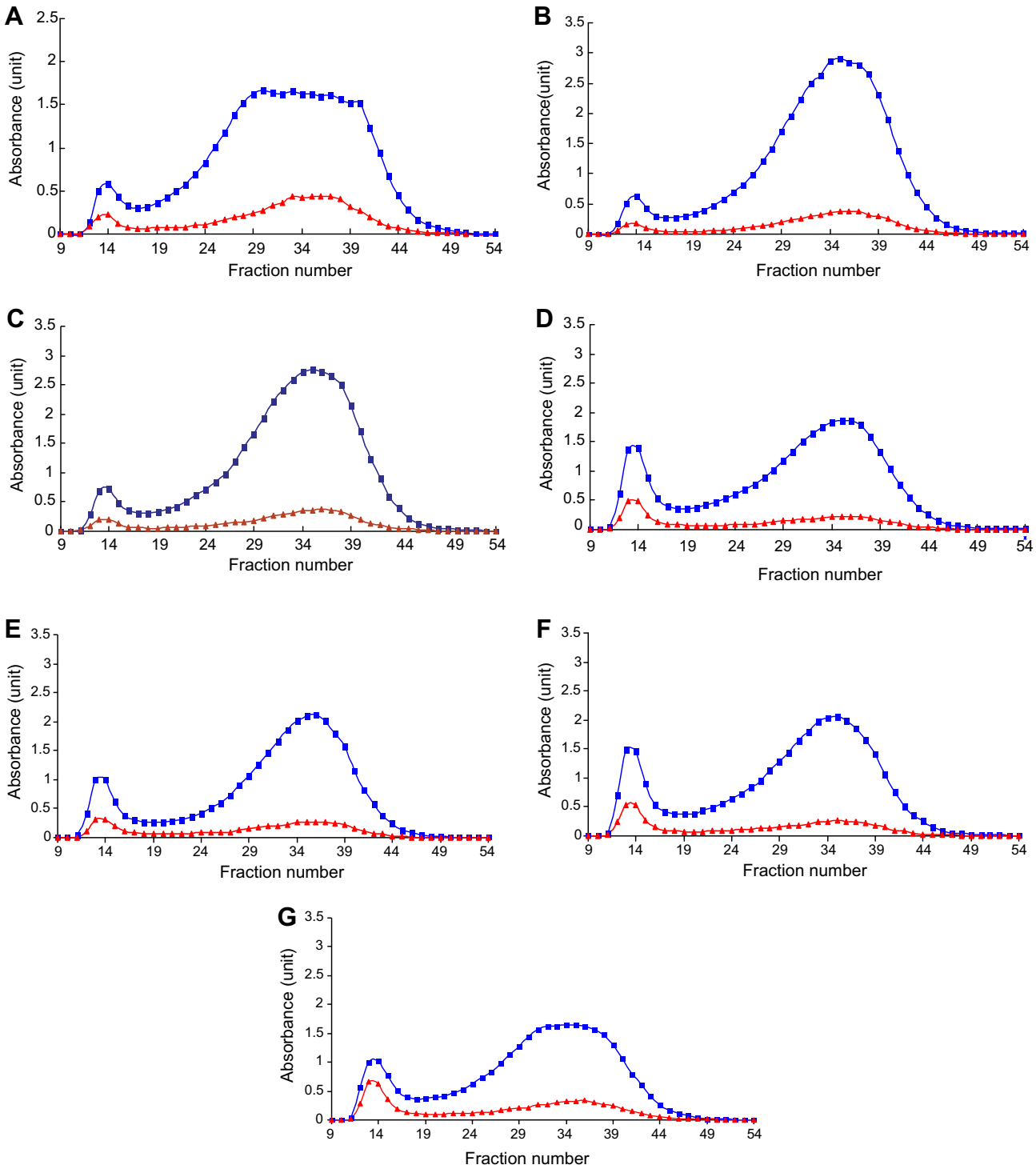

Fig. 1. Sepharose CL-2B gel permeation chromatography profiles of native *ae*-mutant maize starches. (A)GSOH 1, (B)GSOH 2, (C)GSOH 3, (D) H99*ae*, (E) OH43*ae*, (F) B89*ae*, (G) B84*ae*. -■- Blue-value; -▲- Total CHO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

starch sample (~6 mg, dsb) with excess water (3×) was heated at 10 °C/min from 25 to 180 °C in a sealed stainless steal pan. An empty pan was used as the reference. The sample was analyzed in triplicate, and the data were calculated using Pyris software (Perkin–Elmer, Norwarlk, CT). The melting of amylose–lipid complex peak was determined by rescanning the gelatinized sample using the same parameters.

3. Results and discussion

3.1. Resistant starch (RS) content

RS contents of the maize *ae*-mutant starches are shown in Table 1. Results obtained using the AOAC 991.43 method showed that RS contents of the three new *ae*-line starches, GSOH1, GSOH2, and GSOH3, were 39.4–43.2%, which were much greater than that of the four existing inbred *ae*-mutant starches of H99ae, OH43ae, B89ae, and B84ae (11.5–19.1%) (Table 1). RS contents determined using the Englyst's method (Englyst et al., 1992) showed somewhat less RS contents of the new *ae*-line starches (30.9–34.3%) and similar or a little larger RS contents of the four existing inbred *ae*-line starches (18.6–20.9%). In general, both methods gave results in a similar trend.

3.2. Contents of amylose, amylopectin, and IC

Apparent and absolute amylose-contents of the mutant starches were determined using potentiometric titration, and the results are shown in Table 1. The apparent amylose-contents of the mutant starches were substantially larger than the absolute amylose-contents (Table 1). The differences (~21–27%) between the two were results of the very long branch-chains of amylopectin and IC, which also bound iodine (Kasemsuwan et al., 1995). The IC had branched structures, which remained in the supernatant with amylopectin during 1-butanol fractionation of amylose from amylopectin. The IC also had smaller molecular weight, similar to amylose and, thus, was eluted together with amylose in the gel permeation chromatograms. The starches from the three new *ae*-mutant lines had larger apparent amylose-contents (83.1–85.6%) and absolute amylose-contents (57.4–62.6%) than the starches of existing inbred *ae*-lines, (61.7–67.7%) and (35.5–44.7%), respectively. The RS contents were positively correlated with both the apparent and the absolute amylose-contents of the starches, and the correlation coefficients were 0.99 and 0.97 for apparent amylose-contents using the AOAC 991.43 method and the Englyst's method, respectively, and 0.96 for absolute amylose-contents using either method. These results were in agreement with that reported by Jane et al. (2003) and Okuda et al. (2005), showing that starch consisting of a larger amylose-content displayed less enzyme-digestibility.

Gel permeation chromatograms (GPC) of the mutant starches are shown in Fig. 1, and the amylopectin-contents of the mutant starches, calculated from GPC chromatograms, are shown in Table 2. The starches of the three new *ae*-mutant lines had smaller amylopectin-contents than that of the existing inbred *ae*-lines.

After amylose of the mutant starch was removed from the starch using 1-butanol complex formation, the amylopectin and IC in the supernatant were collected and further separated using GPC (Fig. 2). The removal of amylose from the amylopectin and IC mixture was indicated by the ratios of the blue-value to the total carbohydrate content for fractions around 17–23, which were similar to that of the amylopectin peak (fractions 11–16) (Fig. 2). Amylose displayed larger blue-value as shown in fractions 17–23 in Fig. 1. The proportions of the amylopectin and the IC-contents in the mixture, calculated on the basis of the total carbohydrate shown in the peaks of GPC, are summarized in Table 2. The starches of the three new *ae*-lines had larger IC-contents than that of most existing *ae*-lines except OH43*ae*. The OH43*ae* starch contained the largest IC-content (52%) among all the *ae*-mutant starches analyzed.

3.3. Molecular weights of amylopectin and IC

The molecular weight and gyration radius of the amylopectin and the molecular weight of the IC are shown in Table 3. The weight-average molecular weights of the amylopectins varied from 2.5 to 9.0×10^7 g/mol, which were smaller than that of amylopectins of most starches reported (Yoo & Jane, 2002), but were much larger than the average molecular weights of IC (1.5×10^5–1.04×10^6 g/mol).

3.4. Branch chain-length distributions of amylopectin and IC

The branch chain-length distributions of the *ae*-mutant amylopectins are shown in Table 4. Average branch chain-lengths of the amylopectin were DP 32.0–37.6, which were substantially longer than that of the normal maize starch (DP 24.4) (Jane et al., 1999). The longest detectable branch chain-lengths of the amylopectin ranged between DP 80 and 99. The three new *ae*-line starches had shorter average branch chain-lengths (DP 32.0–35.6) than existing inbred *ae*-line starches (DP 36.2–37.6) (Table 4). Starch granules of the B-type polymorph are known to be digested much more slowly by amylase than that of the A-type polymorph (Fuwa, Takaya, & Sugimoro, 1980; Jane, 2006; Jane et al., 2003; Kimura & Robyt, 1995) and are known as the type II resistant starch. Although amylopectin is considered the major component contrib-

Table 2
Amylopectin and the IC-contents of maize *ae*-mutant starches determined using GPC method

Sample	% Amylopectin[a]	% IC in the mixture of IC + AP[b]	% IC[c]	% Large-M_W IC[d]	% Small-M_W IC[e]
GSOH1	11.6 ± 0.4	75.7 ± 1.9	36.1	8.5	27.6
GSOH2	10.7 ± 0.3	79.1 ± 1.3	40.5	8.5	32.0
GSOH3	13.9 ± 1.1	76.4 ± 0.8	45.0	10.6	34.4
H99ae	33.5 ± 0.9	44.6 ± 0.6	27.0	11.5	15.5
OH43ae	25.4 ± 0.6	67.2 ± 0.9	52.0	20.7	31.3
B89ae	33.0 ± 0.6	42.7 ± 2.6	24.6	10.2	14.4
B84ae	32.3 ± 0.7	41.0 ± 2.6	22.4	8.4	14.0

[a] % Amylopectin was calculated by dividing the total carbohydrate content of the amylopectin peak by the sum of that of the amylopectin, IC, and amylose peaks. % amylose + IC mixture was calculated following the same method.
[b] % IC in the mixture of amylopectin and IC was determined using GPC fractionation of the mixture of amylopectin and the IC, calculated by dividing the total carbohydrate content of the IC peak by the sum of that of the amylopectin and IC peaks.
[c] % IC was calculated as % IC in mixture × AP%/(100% − %IC in mixture).
[d] Fractions 17–27.
[e] Fractions 28–41.

uted to the crystallinity of starch granules, the branch chain-lengths of the amylopectins in these starches did not correlate with their large RS contents. This result suggested that amylopectin was not primarily responsible for the enzyme resistance of the starch.

The GPC chromatograms of the mixtures of amylopectin and IC (Fig. 2) showed a distinct peak located between fractions 28 and 41 (M_W between 1.2×10^6 and 2.0×10^3 g/mol), which displayed more intense blue-color than the IC of larger M_W. This peak is designated as the small-M_W IC. The relative content of the small-M_W IC varied with mutant lines and was larger (27.6–34.4%) for the three new *ae*-line starches that consisted of greater RS contents than the existing inbred *ae* starches (14.0–31.3%) (Table 2). This small-M_W IC peak has been observed in the fractionated amylopectin-IC mixtures of dominant *ae*-mutant (Kasemsuwan et al., 1995) and other maize *ae*-mutant starch (Baba & Arai, 1984; Baba et al., 1982; Jane & Chen, 1992; Wang et al., 1993). To understand the structures of IC of different M_W, fractions of IC in the GPC profile were collected separately into two groups according to the M_W. The large-M_W IC

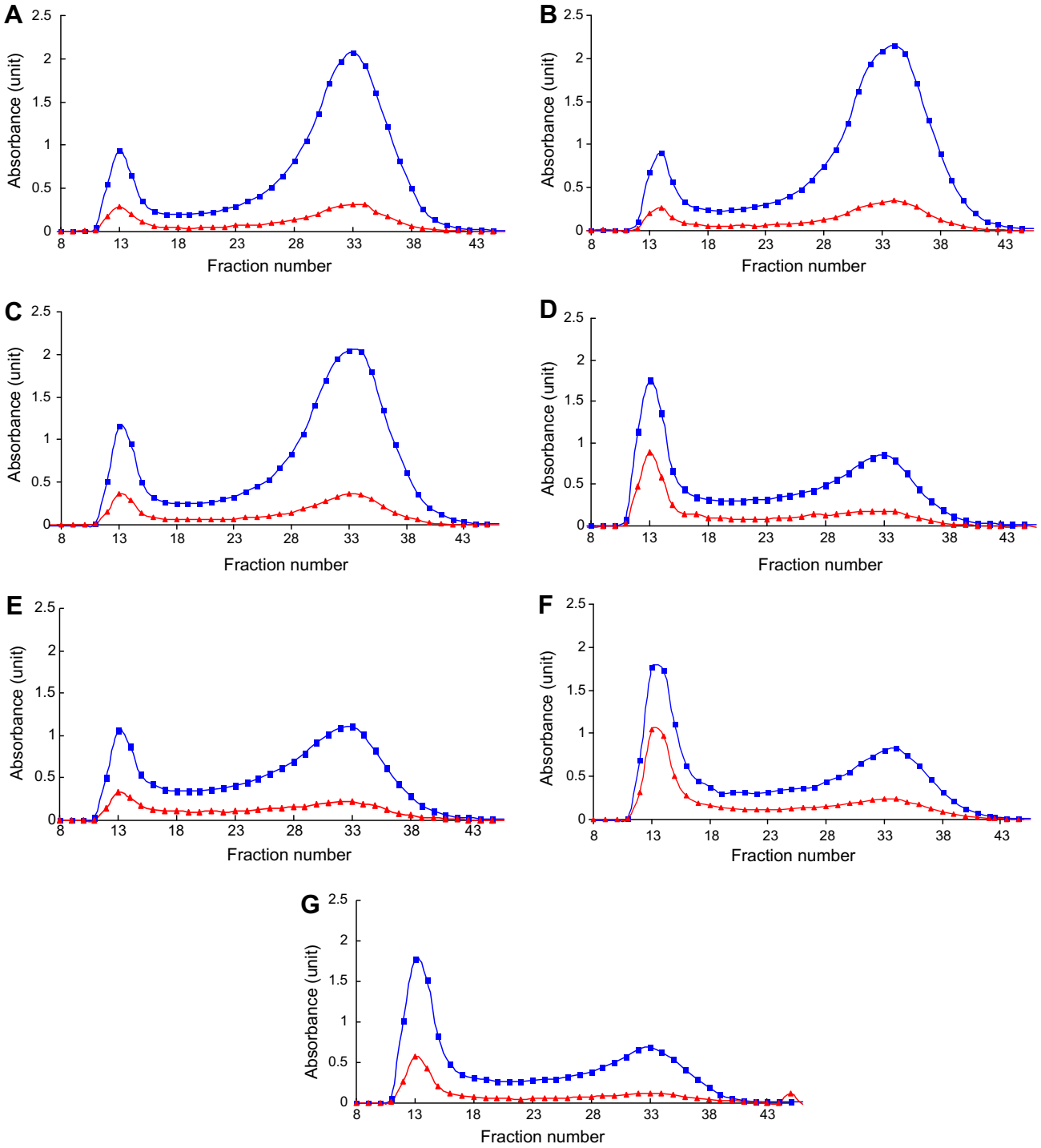

Fig. 2. Sepharose CL-2B gel permeation chromatography profiles of mixtures of amylopectin and intermediate components of *ae*-mutant maize starches. (A) GSOH 1, (B)GSOH 2, (C) GSOH 3, (D) H99*ae*, (E) OH43*ae*, (F) B89*ae*, (G) B84*ae* -■- Blue-value; -▲- Total CHO. The molecular weight of small intermediate components is 2.0×10^3 (fraction 41) to 1.2×10^6 g/mol (fraction 28). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was collected between fractions 17 and 27, and the small-M_W IC was collected between fractions 28 and 41.

The branch chain-length distributions of the large-M_W IC are shown in Table 5. Average branch chain-lengths of the large-M_W IC varied from DP 31.6 to 38.5, which were similar to that of the amylopectin (DP 32.0–37.6). The longest detectable chain-length ranged from DP 74 to 79, which were shorter than that of amylopectin (DP 80–99). These results agreed with the data reported by Kasemsuwan et al. (1995): the IC of the maize

Table 3
Molecular weights and gyration radii of maize *ae*-mutant starch amylopectins and intermediate components (IC)

Sample	Amylopectin		IC
	$M_w \times 10^7$ (g/mol)[a]	R_z (nm)[b]	$M_w \times 10^5$ (g/mol)[c]
GSOH1	4.8 ± 0.1	158 ± 6	1.5 ± 0.1
GSOH2	4.2 ± 0.2	151 ± 7	1.5 ± 0.0
GSOH3	5.2 ± 0.2	173 ± 11	1.9 ± 0.1
H99ae	7.7 ± 0.0	196 ± 3	6.1 ± 0.2
OH43ae	2.5 ± 0.1	105 ± 8	7.5 ± 0.1
B89ae	9.0 ± 0.3	213 ± 24	10.4 ± 0.2
B84ae	6.0 ± 0.3	162 ± 7	7.9 ± 0.2

[a] Weight-average molecular weight.
[b] Z-average radius of gyration.
[c] Average molecular weight of IC calculated using pullulan standard curve.

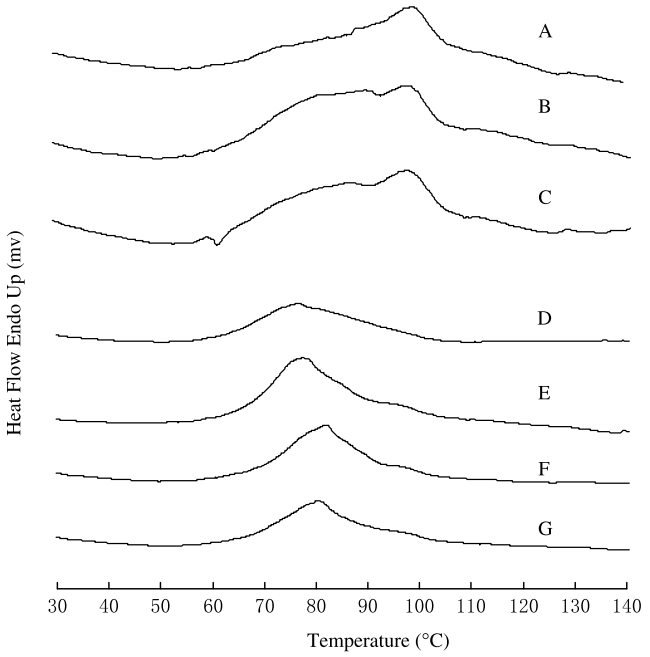

Fig. 3. DSC thermograms of gelatinization of *ae*-mutant maize starches. (A) GSOH 1, (B) GSOH 2, (C) GSOH 3, (D) H99*ae*, (E) OH43*ae*, (F) B89*ae*, and (G) B84*ae*.

Table 4
Branch chain-length distributions of maize *ae*-mutant starch amylopectins[a]

Sample	DP ⩽ 12	DP13–24	DP 25–36	DP ⩾ 37	Average CL (DP)	Longest detectable DP
GSOH1	13.3 ± 1.5	35.2 ± 2.9	14.1 ± 0.2	37.4 ± 4.2	32.0	80
GSOH2	9.2 ± 2.2	37.5 ± 0.2	13.2 ± 1.1	40.2 ± 1.3	35.6	90
GSOH3	10.7 ± 0.1	39.8 ± 0.7	15.1 ± 1.9	35.3 ± 4.1	32.5	83
H99ae	9.7 ± 0.0	36.3 ± 1.3	12.8 ± 0.4	41.2 ± 1.7	36.8	99
OH43ae	10.5 ± 0.6	34.7 ± 1.6	14.1 ± 0.6	40.7 ± 2.9	37.6	89
B89ae	7.7 ± 1.7	36.8 ± 1.4	14.7 ± 1.5	40.8 ± 1.2	36.8	93
B84ae	10.1 ± 0.1	35.4 ± 0.8	13.3 ± 0.0	41.1 ± 0.7	36.2	94

[a] The branch chain-length distribution of amylopectin was analyzed using high-performance anion-exchange chromatography equipped with an on-line amyloglucosidase column and an ED50 pulse-amprometric detector. A PA-100 anion-exchange analytical column (4 × 250 mm) and a guard column were used for separation.

Table 5
Branch chain-length distribution of the large molecular-weight IC[a,b]

Sample	DP ⩽ 12	DP13–24	DP 25–36	DP ⩾ 37	Average CL (DP)	Longest detectable DP
GSOH1	7.0 ± 0.1	30.5 ± 0.8	16.3 ± 0.3	46.1 ± 1.2	36.9	78
GSOH2	6.4 ± 0.0	31.7 ± 0.7	16.6 ± 0.8	45.3 ± 1.5	36.5	78
GSOH3	5.9 ± 0.1	29.1 ± 0.2	15.9 ± 0.2	49.1 ± 0.1	38.5	79
H99ae	7.2 ± 0.2	33.6 ± 2.7	17.1 ± 0.8	42.1 ± 2.2	35.3	75
OH43ae	9.2 ± 0.2	38.0 ± 0.4	19.4 ± 0.6	33.4 ± 1.2	31.6	75
B89ae	7.2 ± 0.2	33.5 ± 2.7	17.1 ± 0.8	42.1 ± 2.2	35.3	74
B84ae	7.6 ± 0.4	34.0 ± 2.2	17.8 ± 1.7	40.1 ± 1.2	34.8	77

[a] The branch chain-length distribution of the large molecular-weight IC was analyzed using the same method described in Table 4.
[b] The sample was collected between fractions 17–27.

Table 6
Branch chain-length distribution of the small molecular-weight IC[a,b]

Sample	DP ⩽ 12	DP13–24	DP25–36	DP ⩾ 37	Average CL (DP)	Longest detectable DP
GSOH1	1.8 ± 0.1	9.9 ± 0.2	14.0 ± 0.8	75.3 ± 1.1	50.6	80
GSOH2	2.2 ± 0.3	12.8 ± 2.1	14.6 ± 2.1	70.5 ± 4.5	47.3	77
GSOH3	2.8 ± 0.3	12.5 ± 0.1	13.3 ± 0.2	71.4 ± 0.1	48.5	77
H99ae	3.4 ± 0.1	18.6 ± 0.4	14.4 ± 0.0	63.5 ± 0.4	45.2	76
OH43ae	4.7 ± 0.0	20.3 ± 0.4	14.9 ± 0.8	60.2 ± 1.3	43.0	74
B89ae	3.8 ± 0.1	19.8 ± 0.4	16.6 ± 0.2	59.8 ± 0.7	43.4	78
B84ae	5.4 ± 0.2	26.8 ± 0.3	18.9 ± 0.1	48.9 ± 0.3	38.7	77

[a] The branch chain-length distribution of the small molecular-weight IC was analyzed using the same method described in Table 4.
[b] The sample was collected between fractions 28–41.

Table 7
Thermal properties of native maize *ae*-mutant starches[a]

Sample	Native starch				Amylose–lipid complex[c]			
	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
GSOH1	65.7 ± 0.7[b]	98.4 ± 0.4	130.0 ± 2.8	13.0 ± 0.3	76.9 ± 6.4[b]	99.4 ± 0.8	104.5 ± 1.4	2.2 ± 0.7
GSOH2	65.2 ± 1.4	96.2 ± 3.3	124.0 ± 5.7	11.7 ± 0.3	81.7 ± 6.4	94.4 ± 4.4	104.6 ± 0.8	1.9 ± 0.4
GSOH3	64.6 ± 0.5	98.2 ± 1.3	122.0 ± 1.4	17.4 ± 0.7	73.4 ± 2.7	95.8 ± 3.9	103.2 ± 0.3	2.5 ± 0.1
H99ae	64.8 ± 0.3	76.9 ± 3.2	105.3 ± 1.1	15.6 ± 0.1	77.5 ± 2.8	97.9 ± 0.6	104.9 ± 0.9	1.2 ± 1.2
OH43ae	65.8 ± 1.3	78.1 ± 2.0	103.3 ± 0.4	14.7 ± 0.5	78.3 ± 2.4	95.9 ± 3.5	107.4 ± 1.1	2.5 ± 0.1
B89ae	65.0 ± 0.1	80.8 ± 0.6	103.6 ± 0.6	13.0 ± 0.1	85.5 ± 5.1	97.7 ± 0.5	104.8 ± 0.3	0.7 ± 0.3
B84ae	64.5 ± 0.6	80.8 ± 1.4	100.5 ± 0.7	17.4 ± 0.6	84.3 ± 6.0	97.8 ± 5.3	105.4 ± 4.2	0.4 ± 0.5

[a] Samples (~6.0 mg, dsb) and deionized water (~18.0 mg) were used for the analysis; T_o, T_p, T_c and ΔH are onset, peak, conclusion temperature, and enthalpy change, respectively.
[b] Values were calculated from three replicates; ±Standard deviation.
[c] Melting of amylose–lipid complex was determined by rescanning the gelatinized starch sample using the same parameters.

dominant *ae*-mutant starch contains no very long branch-chains. The large-M_W IC of the mutant starches had moderately fewer (35.0–47.2%) short branch-chains (DP ⩽ 24) than the amylopectin (44.5–50.5%). These results showed that the large-M_W IC had similar branch structures to amylopectin but with smaller molecular-weights.

The branch chain-length distributions of the small-M_W IC are shown in Table 6. The average branch chain-lengths of the small-M_W IC of the samples varied from DP 38.7 to 50.6, which were longer than that of the amylopectin and the large-M_W IC. These results were in agreement with those reported by Baba and Arai (1984). The three new *ae*-line starches displayed substantially longer average chain-lengths (DP 47.3–50.6) than existing *ae*-line starches (DP 38.7–45.2). These long branch-chain-lengths of the small-M_W IC coincided with their intense blue-color of the peak. The longest detectable chains were between DP 74 and 80, similar to that of the large-M_W IC, but were shorter than that of amylopectin (DP 80–99). The small-M_W IC displayed greater blue-value than the large-M_W IC. The three new *ae*-line starches consisted of the least short-branch-chains (DP ⩽ 24, 11.7–15.3%) and the most long-branch-chains (DP > 37, 70.5–75.3%), which contributed to the large blue-values of the peak and the large apparent amylose-contents of the starch samples. The long branch-chains and the small molecular-weights made these small-M_W IC molecules highly susceptible to retrogradation.

3.5. Thermal properties of the maize ae-mutant starches

Thermograms of the maize *ae*-mutant starches are shown in Fig. 3, and the thermal properties are summarized in Table 7. All samples displayed similar onset gelatinization temperatures (64.5–65.8 °C). The gelatinization ranges of the three new *ae*-line starches, however, were broader than that of the existing inbred *ae* starches and consisted of double or multiple transitions. The conclusion gelatinization temperatures of the three new *ae*-line starches varied from 122.0 to 130.0 °C, which were similar to the results reported by Fergason, Jeffcoat, Fannon, and Capitani (1994) and Shi, Capitani, Trzasko, and Jeffcoat (1998). These results indicated that starch granules of these three new *ae*-lines retained some of the crystalline structures after heating at the boiling-water temperature (100 °C). Those existing inbred *ae*-line starches had conclusion gelatinization temperature slightly above the boiling temperature, ranging between 100.5 and 105.3 °C. Therefore, the RS contents of the three new *ae*-line starches were substantially larger. These high conclusion gelatinization temperatures of the three new *ae*-line starches were results of the large contents of amylose and IC and the long branch-chains of the IC. It is known that retrograded amylose has gelatinization temperature above 150 °C (Sievert & Pomeranz, 1989).

When the *ae*-mutant starches were re-scanned using a DSC, all the thermograms showed a peak between 94.4 and 99.4 °C, which were attributed to the melting of amylose–lipid complex. Thermal transitions of melting the starch–lipid complex are summarized in Table 7. The three new *ae*-line starches and the OH43*ae* starch displayed larger enthalpy-changes of melting the starch–lipid complex than the others. These results coincided with the large IC-contents of these starches. It is plausible that the long branch-chains and the smaller M_W of the IC molecules facilitate the helical complex formation with lipids. The starch–lipid complex is known to be present in a collapsed helical conformation, which is resistant to enzyme hydrolysis (Jane & Robyt, 1984). Further studies on the structure of starch–lipid complex in the *ae*-mutant maize starch and its impact on the RS content will be reported separately.

4. Conclusions

The RS contents of three new maize *ae*-line starches were larger than that of existing inbred *ae*-line starch samples. The RS content was positively correlated with the apparent and the absolute amylose-content. The conclusion gelatinization temperatures of the three new *ae*-line starches were 122.0–130.0 °C, substantially higher than that of the existing inbred *ae* starches (100.5–105.3 °C). The crystalline structures of the new *ae*-line starches were retained after heating at the boiling-water temperature and resulted in the large RS contents.

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