Single Kernel Sampling Method for Maize Starch Analysis While Maintaining Kernel Vitality

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ABSTRACT

A nondestructive protocol for maize kernel starch sampling was developed, enabling starch preparation from a single kernel for analysis of starch structure while also maintaining the vitality of the seed. To develop the single kernel sampling (SKS) method, maize genotypes varying in starch structure including *ae, wx, su2, du* and normal in the W64A inbred line were used. Crude endosperm material was removed from the kernel crown, soaked, ground, washed, and dissolved in 90% DMSO. The sample represented ≈10% of the total kernel. Endosperm starch was also isolated from the same genotypes by a standard multikernel isolation (MKI) method. Starches isolated by the two methods were debranched and analyzed by high-performance size-exclusion chromatography (HPSEC) and fluorophore-assisted carbohydrate electrophoresis (FACE). HPSEC and FACE showed similar results for the two sampling methods for degree of polymerization (DP) ≤ 50. We concluded that the material obtained by SKS could be used for identifying amylopectin structural differences among genotypes. Kernel sampling for SKS had no effect on germination, thus plants could be grown for subsequent genetic crosses and analysis. The SKS method may be useful for the screening of populations of maize kernels from genotypes producing novel amylopectin structure, and allow the growth of novel genotypes for further analysis.

In maize research on starch biosynthesis, starch structure is often investigated using samples from homozygous seeds from several ears combined. This requires the use of homozygous lines to obtain large samples and also retention of seed for subsequent planting. Additionally, some mutant genotypes are seedling or seed lethal, making it difficult, if not impossible, to obtain homozygous plants or ears. We reasoned that a method of single seed sampling that retained seed vitality would be an important tool for accelerating the pace of our research. With such a method, we could analyze segregating seeds from a single ear, allowing us to save the time of producing an extra generation by selfing to get homozygous ears. Additionally, we would be able to evaluate starches in seeds that show a lethal phenotype. Requirements of such a system would include 1) a starch structure analytical method comparable to the methods commonly used when starch from multiple kernels is available and 2) seed vitality after sampling. Vitality could be retained if the germ of the kernel remains intact and enough endosperm is retained to support seedling growth. This might be achieved by removing a portion of the endosperm tissue from the crown of a mature kernel, a strategy that we defined as single kernel sampling (SKS). An important question is whether starch structure analysis after SKS would be comparable to that for starches obtained by a standard laboratory multikernel isolation (MKI).

The chain length profiles of debranched starch can be analyzed using high-performance size-exclusion chromatography (HPSEC) (Hizukuri 1986; Klucinec and Thompson 1998), high-performance anion-exchange chromatography (HPAEC) (Wong and Jane 1997), and fluorophore-assisted carbohydrate electrophoresis (FACE) (O'Shea et al 1998). HPAEC, as routinely done without post-column derivatization, and FACE data are based on molar detection. HPAEC gives baseline resolution for oligosaccharides with degrees of polymerization (DP) up to 77 (Wong and Jane 1997). FACE gives baseline resolution up to DP 100 (Morell et al 1998). In contrast, HPSEC is unable to resolve individual chains, but it describes the entire sample of debranched starch including the residual long chains from amylose (Klucinec and Thompson 1998). In the present work, using both HPSEC and FACE analyses, we analyzed chain length distribution after SKS or MKI for normal endosperm starch and several mutant genotypes. We also determined the effect of SKS on germination of these genotypes.

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MATERIALS AND METHODS

Maize Kernels

Backcross conversions in W64A inbred line for the endosperm mutants *waxy* (*wx*), *amylose-extender* (*ae*), *sugary-2* (*su2*), and *dull* (*du*) were produced by standard procedures in the maize genetics program at The Pennsylvania State University. Genotypes were identified by those gene loci homozygous for recessive mutant alleles. The normal genotype in W64A was used as a control. Mature ears were harvested in the summer of 1997 and stored at room temperature.

Preparation of Crude Starch Dispersions by SKS

Tissue (10–20 mg) was cut from the crown of a single kernel using a razor. The tissue was then soaked in 200 µL of steeping solution (0.02*M* NaAc, 0.01*M* $HgCl_2$, pH 6.5) at 22°C for 24 hr and then ground with a pestle in a 1.7-mL centrifuge tube. The pericarp pieces were removed from the crude suspension using a pipette. The crude starch was washed three times by a series of centrifugations (2,000 × *g*, 5 min) and resuspended in deionized water. After centrifugation, the supernatant was decanted, the volume of each pellet was estimated and then dissolved in 9 volumes of DMSO (v/v) in a boiling water bath for 10 min. For each genotype, three kernels were randomly selected from the same ear followed by independent crude starch dispersion preparations.

Preparation of Starch Dispersions by MKI

As a control method for starch isolation, endosperm starch from multiple dried kernels was obtained by the method of Boyer and Liu (1985) with modifications. The kernels were randomly selected from the same ears as for the SKS. Twenty kernels were soaked in 50 mL of steeping solution (0.02*M* NaAc, 0.01*M* $HgCl_2$, pH 6.5) at 22°C for 48 hr. The germ was removed by hand, and the mixture of endosperm and pericarp was homogenized for 10 min using a mortar and pestle. The starch granules were washed through a 105-µm mesh to remove pericarp, followed by 10 extractions with a mixture of 0.05*M* NaCl aqueous solution and toluene (1:1, v/v) to remove the protein. The purified starch was then washed with deionized water (three times), 95% ethanol (one time), and acetone (one time) before being dried at 22°C. Triplicate 5% (w/v) starch dispersions for each genotype were prepared in 90% DMSO by heating the suspensions in a boiling water bath.

Debranching of Starches

Warm (50°C) NaAc buffer (pH 3.75, 0.05*M*, 880 µL) was added to 120 µL of the warm starch dispersion in DMSO. The mixture was vortexed, heated for 10 min in a boiling water bath, and

cooled to 22°C. A solution (100 µL, 0.5 units/mL, 0.05*M* NaAc, pH 3.75) of isoamylase (EC 3.2.1.68 from *Pseudomonas* sp., Megazyme Inc., Ireland) was added to the mixture. The mixture was then digested for 24 hr at 37°C with constant agitation. After digestion, the dispersion was concentrated in a vacuum evaporator (Speed-Vac SC100, Savant) to remove the water, leaving a dispersion of 2–5% (w/v) of carbohydrate in DMSO. For starch prepared by SKS, the starch suspensions from three kernels were debranched independently. For samples prepared by MKI, three subsamples of each isolated starch were debranched.

FACE Analysis

To analyze debranched starch samples by the FACE method, the fluorophore was first covalently attached to the reducing terminus of chains according to O'Shea et al (1998). To a 200-µL well in a 96-well PCR plate (PGC Scientifics Corp., Frederick, MD), 2.5 µL of the concentrated debranched starch dispersion in DMSO, 4.0 µL of 0.10*M* APTS (1-aminopyrene-3,6,8-trisulphonate), and 6.0 µL of 1.0*M* $NaBH_3CN$ (sodium cyanoborohydride) were added. The plate was wrapped in aluminum foil and incubated at 37°C for 24 hr. After the incubation, the samples were diluted by the addition of 112.5 µL of starch sample buffer (6*M* urea, 0.04*M* boric acid, 0.04*M* Tris-base, pH 8.6). The diluted dispersion was stored in a −80°C freezer as a stock dispersion. Before gel loading, the stock dispersion was diluted 100× using 2.0% of blue dextran in starch sample buffer. Labeled maltoheptaose was run separately on the same gel as a standard for DP determination. The instrument used was a ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA). The 7.5% polyacrylamide slab gel (37.5:1 ratio of acrylamide to N,N'-methylene-bisacrylamide as cross-linker, containing 8.9*M* urea) for electrophoresis was 360 mm long and 0.2 mm thick. The running buffer contained 0.089*M* boric acid, 0.089*M* Tris-base,

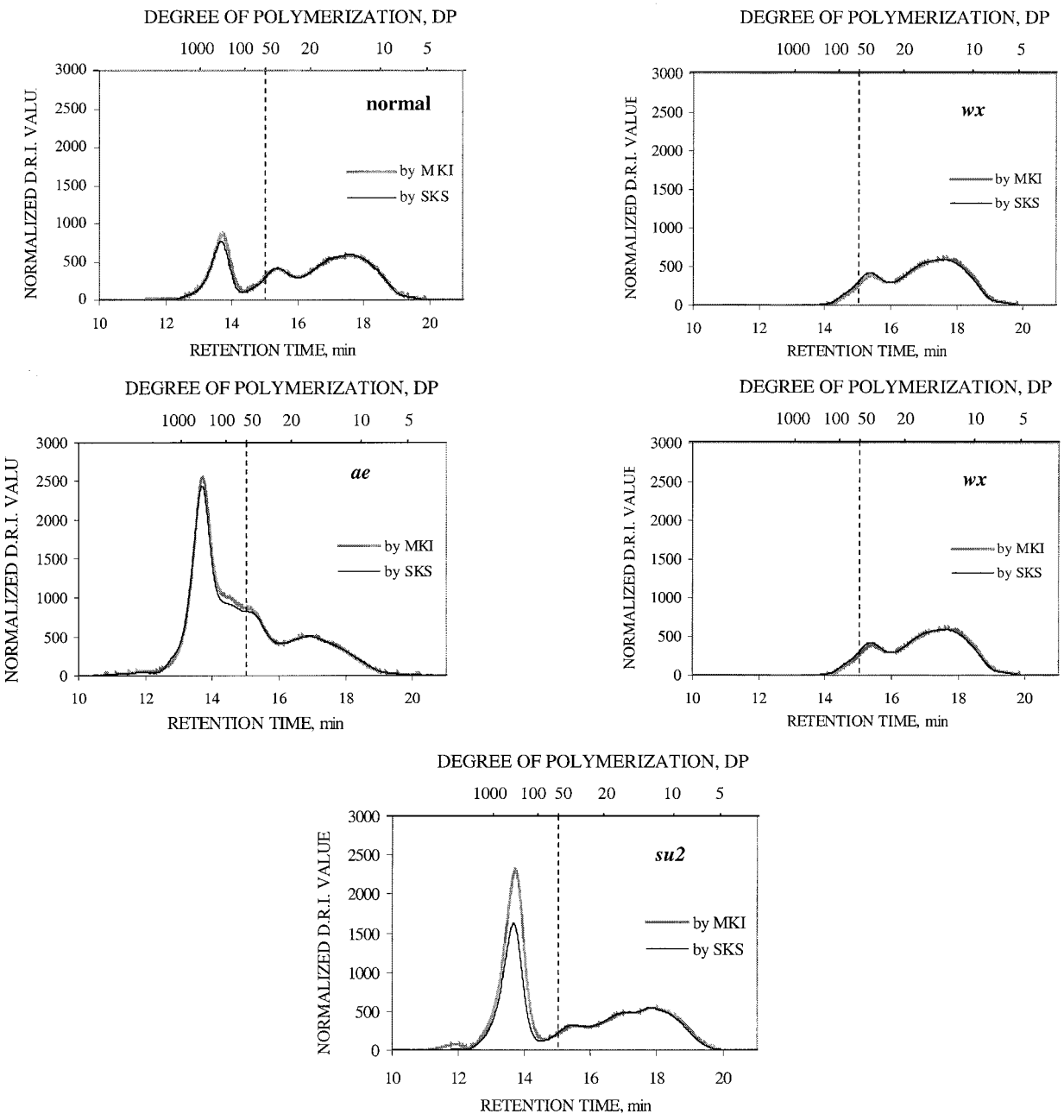

Fig. 1 High-performance size-exclusion chromatography (HPSEC) of debranched starches isolated by standard multikernel isolation (MKI) and single kernel sampling (SKS) from normal, *ae*, *wx*, *su2,* and *du* starches. Chromatograms were normalized to give equal total chromatogram areas for the region DP ≤ 50.

and 0.002*M* EDTA. Operating parameters were 1,500V and a running time of 12 hr. The apparatus allowed a throughput of 25 samples/gel. The data were collected and analyzed by Genescan software version 3.1.2 (Foster City, CA). In the electrophoregrams, the peak areas were baseline resolved to DP ≥ 50. For comparison among electrophoregrams, the total areas of peaks from DP 6 to 50 were normalized. The normalized electrophoregrams were used for further analysis. For starches prepared by MKI, FACE analyses were performed for the three starch suspensions from each genotype. For starches prepared by SKS, the suspensions from the three kernels were analyzed. Figures represent the mean data for each chain length.

HPSEC Analysis

Concentrated debranched starch dispersions were diluted 30 times (v/v) with 90% DMSO, and a 50-µL aliquot was injected in the HPSEC system. DMSO was the mobile phase. The system included two columns (Zorbax PSM 60-S, Mac-Mod Analytical, Palo Alto, CA) and a differential refractometer (model 410, Waters, Milford, MA). The flow rate was 0.5 mL/min, column temperature was 35°C, and detector temperature was 40°C. The molecular weight standards used for column calibration included maltotriose and maltoheptaose (Sigma, St. Louis, MO) and several pullulan standards (P-5, M_w 5800; P-10, M_w 12200; P-20, M_w 23700; P-50, M_w 48000) (Shodex, Japan). The raw data collected using Millennium software (Waters) were exported to and processed in Microsoft Excel. To be comparable with the corresponding FACE electrophoregram, the *y*-axis of the chromatogram was converted from the differential refractive index value (which is a mass response) to a molar response using the calibration curve (Yuan et al 1993). For comparison among starches and for comparison with the FACE data, chromatograms were normalized according to the total area of the region DP ≤ 50. For starches prepared by MKI, analyses were performed for the three starch suspensions from each genotype. For starches prepared by the SKS procedure, the debranched starches from the three kernels were analyzed. Chromatograms represent mean data for each genotype.

Difference Chromatograms and Electrophoregrams

Difference chromatograms and difference electrophoregrams are presented to compare results of analysis from SKS and MKI. The chromatogram or electrophoregram of normal starch from the W64A line is used as the basis for the comparison. The chromatogram or electrophoregram data of normal starch was subtracted from that of a mutant genotype, and the resulting difference profile is expressed as (*mutant* – normal). The subtractions were only performed to compare starches prepared by the same method.

Germination Test

Twenty-five kernels were sampled by SKS for genotypes of normal, *wx*, *ae*, *su2*, and *du* in the W64A inbred line. Twenty-five intact kernels for each genotype were used for germination test controls. All seeds were planted in germination pots in the greenhouse controlled at 25°C and 40% rh. Time of light was ≈10 hr/day.

RESULTS

HPSEC Analyses of Starch Samples After SKS and MKI

Figure 1 shows a comparison of HPSEC results for analyses after MKI and SKS. Generally, little difference is seen between SKS and MKI for chain profiles in the region DP ≤ 50 for the genotypes studied. Because the total area in this region had been normalized, the only differences that might be observed in this region would be in the relative distribution of chains. Some differences are observed in chromatogram region DP > 50 (residual long chains of amylose) for some samples, particularly for *su2*, where the response is greater for starch prepared by MKI.

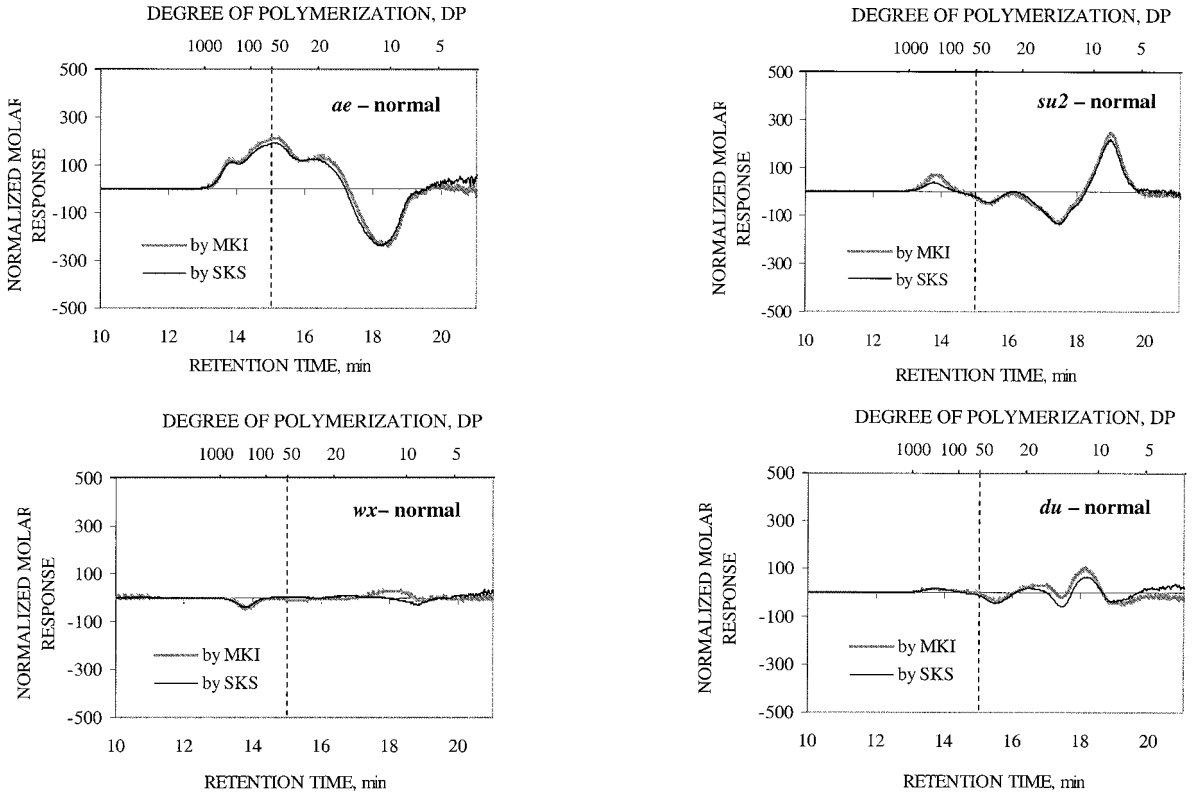

Fig. 2. Difference high-performance size-exclusion chromatography (HPSEC) of debranched starches after first converting the mass response to a molar response and then normalizing the molar response to give equal total chromatogram areas for the region DP ≤ 50. Chromatogram data for normal starch were subtracted from data for *ae*, *wx*, *su2,* and *du* starches, respectively. Results from standard multikernel isolation (MKI) and single kernel sampling (SKS) methods are shown for comparison.

Because the starch granules have been dispersed in DMSO, we think amylose-lipid complex formation is unlikely to be related to the observed difference. It is conceivable that a gradient of amylose in a kernel (Shannon, *in press*) may cause the discrepancy in amylose content after SKS and MKI. Further work will be necessary to investigate the possibility of an amylose gradient in the kernel.

Figure 2 shows the difference chromatograms between starches from mutant and normal genotypes, as evaluated by analysis after either MKI or SKS. The response after 20 min retention time (corresponding to DP 5) is due to amplification of noise in the data transformation from mass to molar response. For the comparison between the *ae* mutant and normal starch, the information about the comparison of starch structure is essentially the same for SKS and MKI in the region DP ≤ 50. Compared to the normal, the *ae* amylopectin has fewer chains of DP 6–15, and more chains of DP > 15. There was no appreciable difference in amylopectin chain length distribution between the *wx* mutant and normal regardless of the starch preparation method used. More chains of DP 6–11 are observed in *su2* than in normal. In contrast, fewer chains of DP 11–25 and DP 30–90 were observed compared with normal. Again, the difference chromatograms after MKI and SKS give similar patterns at DP < 100, which indicates a similar ability in differentiating amylopectin structure. Also, a slightly greater difference in amylose content is observed by MKI than by SKS. The two difference curves are similar throughout for the comparison between *du* and normal.

FACE Analyses of Starch Samples After SKS and MKI

Figure 3 shows the FACE electrophoregrams for debranched starches after isolation by the MKI and SKS procedures. Baseline resolution for individual DP up to at least 50 was observed in all samples. The DP quantification limit depends on both the linear region of the fluorescence detector and relative molar amount of

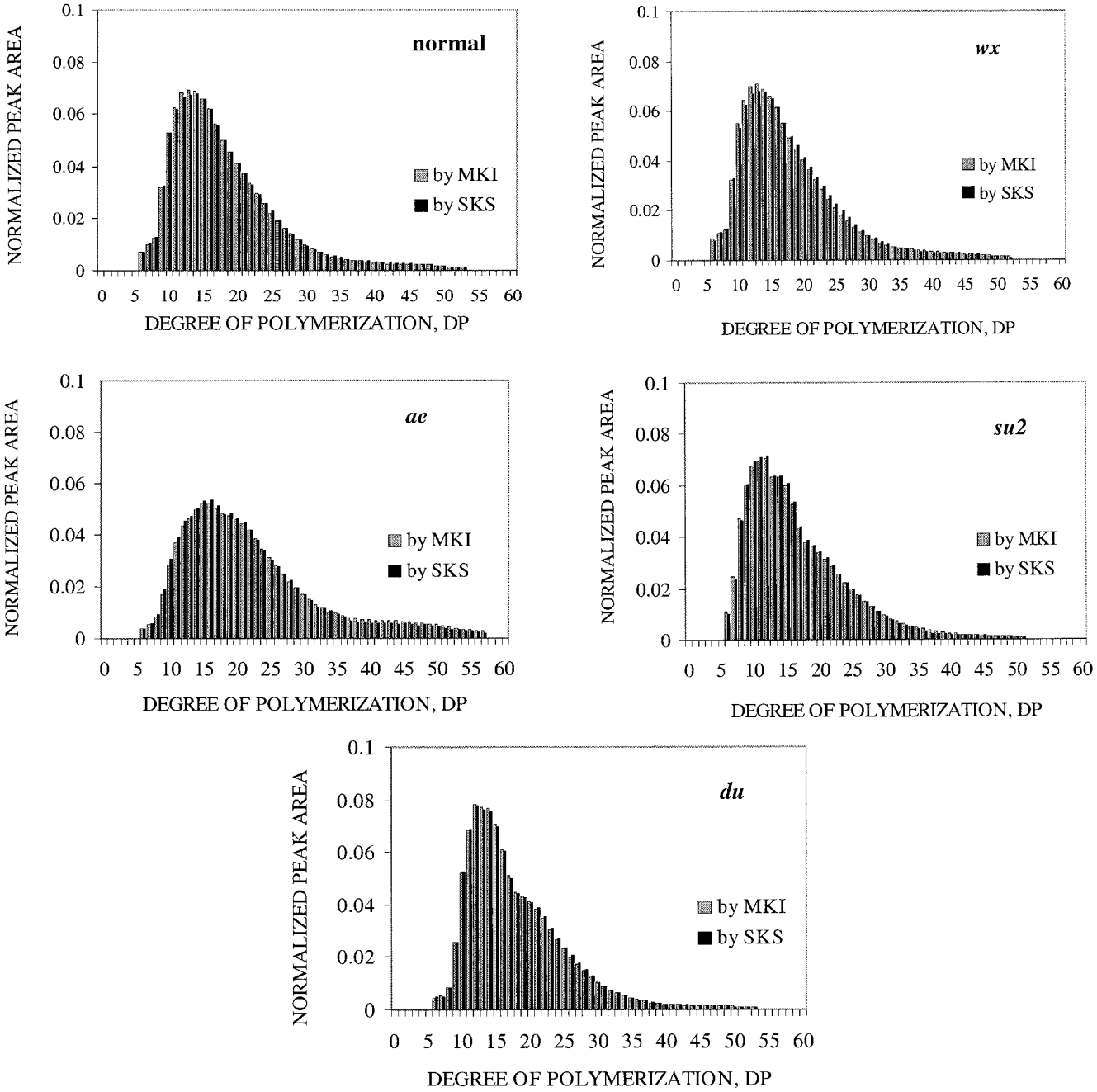

Fig. 3. Fluorophore-assisted carbohydrate electrophoresis (FACE) of debranched starches isolated by standard multikernel isolation (MKI) and single kernel sampling (SKS) from normal, *ae*, *wx*, *du* and *su2* starches. Electrophoregrams were normalized to give equal total areas for the region DP ≤ 50.

chains with a particular DP. For normal, *wx*, *su2*, and *du*, the DP limits given by the data analysis software were ≈52; while for *ae* it was >60. These constraints limit our use of FACE for the analysis of constituent chains of amylopectin. For each genotype studied, preparation by SKS gives a normalized electrophoregram similar to preparation by MKI.

Figure 4 indicates that as for HPSEC analysis, FACE also shows essentially the same amylopectin profile for normal and wx starches. For each mutant genotype, the difference electrophoregram by FACE gives a similar pattern as the difference chromatogram by HPSEC for DP ≤ 50.

Vitality of Seeds After SKS Sampling

For all genotypes tested, germination was 100% (25/25) after SKS. For two genotypes (*ae* and *du*), control germination was < 100% (23/25), but for the others germination was 100%. Whether this difference is meaningful is unclear.

DISCUSSION

HPSEC and FACE in Starch Structure Analysis

HPSEC has been used in determining the chain length distribution of debranched starch (Hizukuri 1986; Klucinec and Thompson 1998). Chains from both amylose and amylopectin can be separated and detected by a proper combination of columns, mobile phase, and operating parameters. The system described here can detect all starch chains and can estimate chain length up to DP ≈ 1,000. However, baseline resolution of individual chains, even those as low as DP6, is not possible.

FACE is a more recently developed method for analyzing debranched starch (Morell et al 1998; O'Shea et al 1998). The baseline resolution capability of FACE enables its quantification of chains with different DP. Due to the molar detection of chains, chains in very low molar amount (e.g., amylose chains) are difficult to detect. FACE using a slab gel appears to be a satisfactory method for quantifying the molar amount of chains with DP < 50, and is thus useful for analyzing all but the longest of the constituent chains of amylopectin. Even though the very long chains of amylopectin may be important, the convenience of the method argues for its utility in screening for novel amylopectin structure.

Due to their complementary abilities, HPSEC and FACE would ideally be used together to obtain comprehensive information about a debranched starch sample. However, it is difficult to use HPSEC for efficient screening of a large number of samples.

Difference Chromatograms and Electrophoregrams

When the HPSEC chromatogram of a reference starch (e.g., debranched normal starch) is subtracted from that of a specific mutant genotype, the difference chromatogram can provide important information concerning structure. FACE electrophoregrams may be similarly compared. This strategy of presenting difference chromatogram has been used with anion-exchange chromatography of debranched starch (Dinges et al 2001). Figures 2 and 4 show differences between each mutant and normal genotype by HPSEC and FACE, respectively. The two preparation methods, MKI and SKS are also compared. Among genotypes studied, only *wx* showed little or no difference compared to normal for DP ≤ 50, either by HPSEC or by FACE. Yun and Matheson (1992) showed that the chain length distribution of normal amylopectin and of *wx* starch is very similar. For starches from *ae*, *su2*, and *du*, characteristic difference chromatograms and electrophoregrams were obtained. Two important conclusions can be made from these results: 1) preparation by SKS

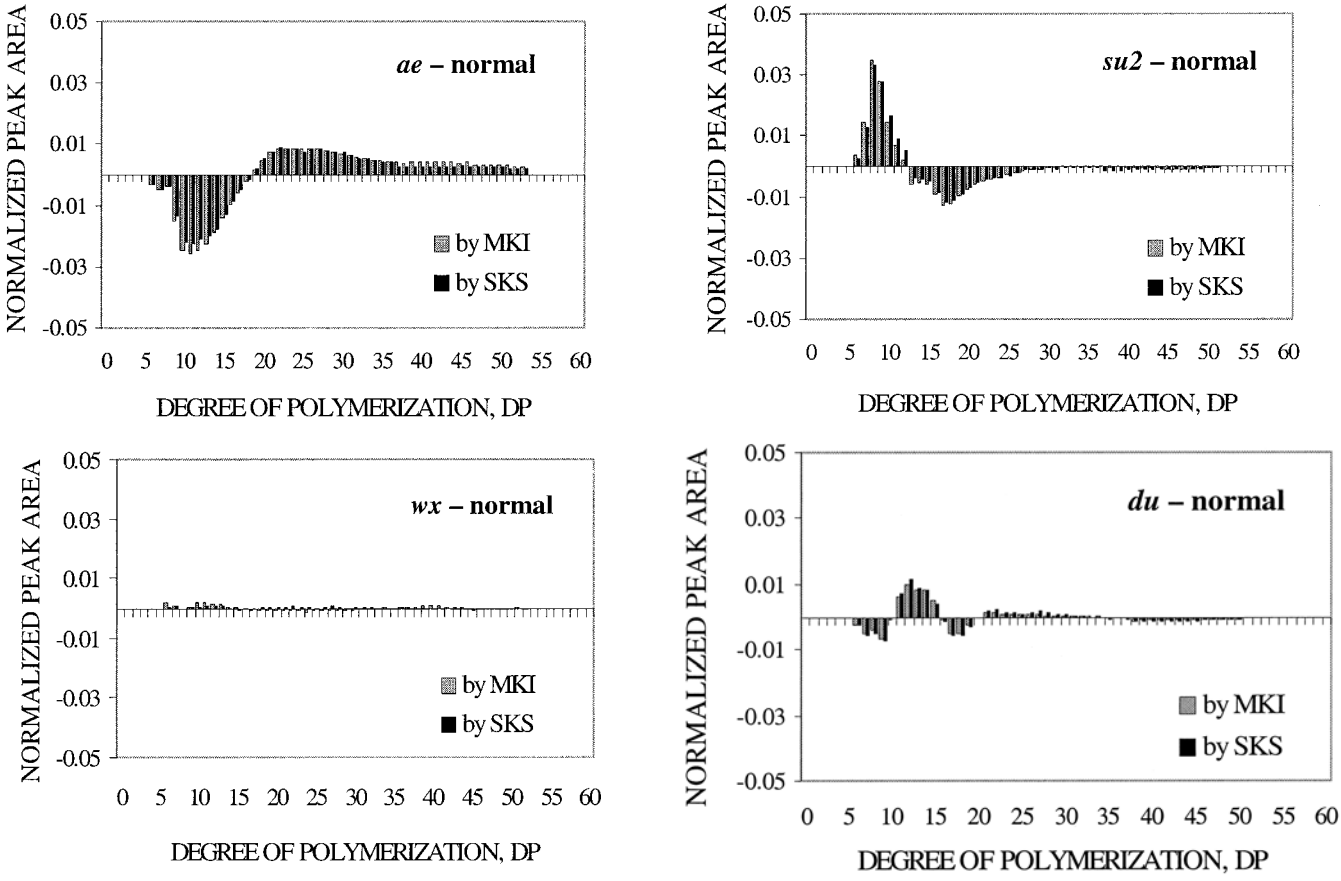

Fig. 4. Difference fluorophore-assisted carbohydrate electrophoresis (FACE) between debranched starches from maize mutants (*ae*, *wx*, *su2* and *du*) and normal. Column data were obtained by subtracting normal electrophoregram from those of mutants. Results from standard multikernel isolation (MKI) and single kernel sampling (SKS) methods are shown for comparison.

and MKI leads to similar difference chromatogram or electrophoregram profiles in the region of DP < 50, which indicates their essentially equivalent ability in differentiating the chain length distribution of amylopectin; and 2) for the same sample, the results given by HPSEC and FACE are similar. The discrepancy between shapes of the difference chromatograms and electrophoregrams is due to the different nature of the *x*-axis for each. At DP > 20, the higher response for the difference chromatogram is due to the log scale, which compresses the data at higher DP. Consequently, the comparison between difference chromatograms and difference electrophoregrams shows a similar fluctuation pattern at DP < 50.

Throughput Using the SKS Method and Subsequent HPSEC and FACE Analysis

For a single individual, processing a series of sample on a daily basis, the throughput possible for SKS was ≈80 samples/day. The throughput possible for MKI averaged ≈10 samples/day. The throughput for HPSEC was 1 sample/hr, or ≈8 samples in a normal workday. The throughput for FACE was 25 samples/12 hr. The sample loading could be completed in 1 hr, and the gel running and data collecting were performed automatically thereafter. The FACE throughput could be increased to 96 samples/12 hr, if a 96-well gel or a 96-channel capillary electrophoresis (CE) DNA sequencer were used. Little modification of instrument and operational parameters was required for the DNA sequencer for FACE.

Potential Application of SKS-FACE Methods in Starch Screening

Compared with MKI, a major advantage of SKS is its nondestructive nature, allowing the vitality of the sampled kernels to be maintained for further research. Also, SKS has essentially no effect on subsequent analysis of amylopectin structure, as compared with MKI. FACE analysis itself has the ability to differentiate the amylopectin structure, and equally good results were possible for starches prepared by SKS and MKI. Based on high analytical throughput and retention of kernel vitality, a combined SKS-FACE strategy can be useful in large-scale screening of kernels for novel endosperm amylopectin structure. Subsequent HPSEC analysis might be limited to those materials identified to be of sufficient interest by the initial screening.

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