

High-performance size-exclusion chromatography (HPSEC) and fluorophore-assisted carbohydrate electrophoresis (FACE) to describe the chain-length distribution of debranched starch

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Abstract—Chain-length (CL) distribution is an important feature of the ‘fine structure’ of starch molecules, which are comprised of amylose and amylopectin. The objective of the present work was to combine data for two methods to achieve a more comprehensive data set that would allow a fuller comparison of the CL distribution for different starches. Both high-performance size-exclusion chromatography (HPSEC) and fluorophore-assisted carbohydrate electrophoresis (FACE) were carried out on endosperm starch isolated from five maize genotypes. For the CL distribution in the range $DP \leq 50$, data in the HPSEC chromatogram were transformed to the form of a FACE electrophoregram, in which the x -axis is DP and the y -axis is the number of chains. The two sets of data in this region were shown to be similar. We conclude that the data sets from HPSEC and FACE may be considered together to describe the CL distribution more completely than for either method alone. We further note that for DP 6–50, data from HPSEC may be transformed to allow a similar presentation as for that obtained by FACE, such that FACE analysis might not be required for comparison of CL distribution of different starches.

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1. Introduction

One of the most commonly reported aspects of starch fine structure is the chain-length (CL) distribution of the starch molecules.^{1,2} When starch is subject to isoamylase hydrolysis, the 1→6 bonds are hydrolyzed to produce a mixture of linear chains. The methods commonly used to characterize the chain-length distribution of the debranched starch are high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC). HPSEC provides a description of all chains in the debranched starch dispersion but cannot resolve peaks for any particular DP.^{2,3} HPAEC provides individual DP information, but only for chains with degree of polymerization (DP) of a maximum (under ideal circumstances) of

about 80.^{4–6} Recently, the technique of fluorophore-assisted carbohydrate electrophoresis (FACE) has been applied in starch structure analysis.^{7,8,11} FACE and HPAEC have a similar upper limit of DP for resolution of individual chains and quantification.^{6,7} We reasoned that it might be possible to combine HPSEC and FACE (or HPSEC and HPAEC) data into a complementary data set to provide a more complete description of the CL distribution. No comparison between these two methods has been published.

In a FACE electrophoregram the area of a baseline-resolved peak is proportional to the number of chains with a particular DP; data are quantified on a molar basis and plotted directly for each DP value. In an HPSEC chromatogram the differential refractive index (DRI) value on the y -axis is a mass response to the carbohydrate in the detection cell at a particular retention time (RT). Within the separation range of the HPSEC media, the RT on the x -axis is approximately proportional to the

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logarithm of the molecular weight (or chain length), and using standards the precise relationship may be determined to generate a standard curve. In order to compare the HPSEC data with data from FACE (or HPAEC), the data from one method must be transformed into the form achieved by the other. We chose to transform the HPSEC chromatogram data into the form of the data obtained by FACE (or HPAEC).

Difference HPAEC chromatograms have been used to compare the CL distributions of starches from different sources, for example, various maize genotypes.⁹ Difference HPSEC chromatograms and FACE electrophoregrams can be constructed as well. By combining data sets from HPSEC and FACE, we hoped to generate more comprehensive difference plots than any currently described.

The objective of the present work was to combine data for the two methods (HPSEC and FACE) to achieve a more comprehensive data set that would allow a fuller comparison of the CL distribution of starches from different sources. A secondary objective was to use the combined analysis to describe differences in CL distributions for endosperm starches from selected maize genotypes.

2. Materials and methods

2.1. Starch structure analysis

2.1.1. Preparation of starch dispersions in 90% Me₂SO. Starch from multiple dried kernels was obtained by the method of Boyer and Liu¹⁰ with modifications. For normal, *ae*, *wx*, *du* and *su2* in the W64A background, 20 kernels were soaked in 50 mL of steeping solution (0.02 M NaAc, 0.01 M HgCl₂, pH 6.5) at 22 °C for 48 h. The germ was removed, and the tissue was homogenized for 10 min using a mortar and pestle. The starch granules were washed through a 105-μm mesh screen, followed by washing 10 times with a mixture of 0.05 M NaCl aqueous solution and toluene (1:1, v/v) to remove the protein. For each genotype, 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 for 24 h. In a 1.7-mL centrifuge tube, 20 mg of dry starch powder was mixed with 380 μL of 90% Me₂SO. The suspension was then heated in a boiling water bath for 10 min to disperse the starch.

2.1.2. Debranching of starches. Warm (50 °C) NaAc buffer (880 μL, pH 3.75, 0.05 M) was added to 120 μL of the starch dispersion. The mixture was vortexed, reheated in a boiling water bath for 10 min, and cooled to 22 °C. A solution of isoamylase (EC 3.2.1.68, from *Pseudomonas* sp., Megazyme Inc., Ireland) (100 μL, 0.5 units/mL, 0.05 M NaAc, pH 3.75) was added to the

dispersion, and the mixture was incubated at 37 °C with constant agitation. After 24 h the mixture was heated in a boiling water bath for 10 min to deactivate the enzyme. The completeness of debranching was confirmed since extended incubation time with fresh isoamylase gave no change on the chromatogram profile. The debranched starch dispersion was concentrated in a vacuum evaporator (SpeedVac SC100, Savant) to remove the water. Concentrated debranched starch dispersions were prepared in triplicate for each genotype.

2.1.3. FACE analysis. In a 200 μL 96-well plate (PGC Scientifics Corp. Frederick, MD), 2.5 μL of concentrated debranched starch dispersion, 4.0 μL 0.10 M APTS (1-aminopyrene-3,6,8-trisulphonate), and 6.0 μL of 1.0 M NaBH₃CN (Caution: toxic!) were added. The wells were sealed, vortexed, and centrifuged (5000g, 10 s), and then wrapped in aluminum foil and incubated at 37 °C. After 24 h, the samples were diluted with 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 preparation. Before gel loading, the stock preparation was diluted 100-fold using 2.0% blue dextrin in starch sample buffer. APTS-labeled maltoheptaose was prepared as a DP standard. The electrophoresis apparatus used was DNA sequencer ABI 377 (Applied Biosystems, Foster City, CA). The 7.5% polyacrylamide slab gel (37.5:1 ratio acrylamide to *N,N'*-methylene-bisacrylamide as cross linker, containing 8.9 M urea) for electrophoresis was 360 mm long and 0.5 mm thick. The running buffer contained 0.089 M boric acid, 0.089 M tris base, and 0.002 M EDTA. Operating parameters were voltage 1500 V and running time 12 h. The capacity of the gel was 25 samples. The data were collected and analyzed using Genescan software version 3.1.2 (Applied Biosystems, Foster City, CA). From the scan of the original electrophoregram, the retention time and area of the peak for each DP were provided. The peak area of each DP is proportional to the number of chains. For each genotype, the FACE analysis was conducted in triplicate.

2.1.4. HPSEC analysis. In general, the procedure was as described previously.³ The concentrated debranched starch dispersion was diluted with 30 volumes (v/v) of 90% Me₂SO, and an aliquot of 50 μL was injected into an HPSEC system with Me₂SO as the mobile phase. The system included two Zorbax PSM 60-S columns (Quantum Analytics Inc., Foster City, CA) and a Waters 410 Differential Refractometer (Waters, Milford, MA). The flow rate was 0.5 mL/min at a column temperature of 35 °C and detector temperature of 40 °C. The molecular weight standards used for column calibration were maltotriose (Sigma), maltoheptaose (Sigma), and pullulan standards (P-5, *M_w* 5800; P-10, *M_w* 12,200; P-20, *M_w* 23,700; P-50, *M_w* 48,000, from

Shodex, Japan). The raw data were collected using Millennium software and then exported to and processed in MS Excel. Best fit for the equation of $\log(M_W)$ versus retention time (RT) was obtained using a polynomial fit of the third order. Based on this standard curve, a M_W value corresponding to each data point for retention time from 10 to 22 min (with a step of 1 s) was calculated and used for the analysis.

2.2. Data treatment

2.2.1. Mean-normalized FACE electrophoregram. DP values and the peak areas corresponding to each DP were exported from the Genescan software to an Excel spreadsheet. For each replicate, the peak area of each DP in the electrophoregram was normalized according to the total peak area from DP 6 to 50. The total peak area from DP 6 to 50 was used as the normalization reference because the length of the shortest chain in starches studied was DP 6, and the baseline-resolved peak area for DP up to 50 could be quantified to result in a standard deviation (SD) of less than 20% of the mean value of the triplicate analyses. In the normalized electrophoregram the x -axis is DP, and the y -axis is the normalized molar fraction of chains at a particular DP. For each genotype, the mean normalized electrophoregram was obtained from the three normalized electrophoregrams of the triplicate analyses.

2.2.2. Mean-normalized HPSEC chromatogram. The HPSEC chromatogram was generated from the raw data exported to the Excel spreadsheet. For each replicate analysis, the differential refractive index (DRI) value at each data point, which corresponds to the mass of material eluted in the interval of two neighboring data points (1 s), was normalized according to the total area (mass amount) in the range of retention time (RT) from 15.1 to 21.0 min (corresponding to DP from 50 to 2; the lower limit of 2 was chosen to include all material in the low-MW region). The mean normalized chromatogram for each genotype was prepared from the normalized chromatograms of the triplicate analyses. The corresponding DP values were calculated from the standard curve and are shown in the secondary x -axis for each chromatogram.

2.2.3. HPSEC data transformation to FACE format. The raw HPSEC data set is the DRI value (normally plotted on the y -axis) as a function of retention time (RT, on the x -axis). These raw data were exported to an Excel spreadsheet. As we have reported previously,² the DRI value (a mass response) for each data point may be transformed into a relative molar response (in this report, this was done only for from DP 2 to 50) using the standard curve to calculate a non-integer DP value corresponding to each RT. For this calculation

each raw DRI value is divided by the non-integer DP value. A plot of relative molar response versus RT is obtained.² The molar response may be then normalized to give equivalent total area, which we did for all samples in the present work.

Using the standard curve the RT values on the x -axis may also be transformed from retention time to non-integer DP. To transform the non-integer DP data to values corresponding to integer DPs to generate a histogram, we did the following further transformation.

For any two sequential RT data points one might calculate the moles eluting *between* those points, meaning the number of moles in the intervening 1-s period. In order to estimate the number of moles associated with the 1-s interval corresponding to a particular RT data point, we chose the molar response at that point, and divided it by the half the 2-s difference in RT for the points before and after the point in question (a trivial calculation in this case). In order to estimate the number of moles associated with a particular non-integer DP value (that corresponded to a particular RT data point), the calculation is no longer trivial. We divided the molar response at the point by half the difference in non-integer DP for the points before and after the data point of interest. (While this response was on a log scale, not a linear scale, within this micro region the error was negligible.)

In order to estimate a molar response that could be associated with a particular integer DP value, we proceeded as follows. By examination of the decreasing non-integer DP values in the spreadsheet, we identified pairs of non-integer DP values that were above and below each unitary DP; for example, the pair of non-integer values of 20.06 and 19.93 spanned DP 20. By the logic in the previous paragraph, we could estimate a molar value corresponding to each of the two non-integer DP values. We assumed a linear relationship between non-integer DP and normalized molar amount in the micro-region between the two non-integer DP values, and we then interpolated to obtain the molar amount corresponding to the unitary DP value.

It was reasonable to compare the molar amounts thus calculated at each unitary DP value because each was determined on a per unit of DP basis. The unity DP value was thereafter treated as if it were an integer for the purpose of plotting the data and making comparisons with FACE data. This interpolated molar amount and the ‘integer’ DP values were thus used to plot the histograms reported in Figure 4 (the data shown in Fig. 4 are the basis for the difference plots of Fig. 7).

This procedure for transforming the continuous non-integer DP data to estimate values corresponding to integer DPs ignores the molar amount that might be calculated for non-integer DP values that do not flank a unitary DP value. Nevertheless, our strategy is useful in that it allows a reasonable approximation of a comparison of molar responses at discrete DPs to be made.

The mathematical treatment for the operations described above is available online as [Supplemental information](#).

After the data transformation of each replicate, the normalization of the transformed HPSEC chromatogram was carried out in the same way as for the normalized FACE electrophoregram. The mean transformed chromatogram for each genotype was obtained from the three normalized transformed chromatograms of the triplicate analyses. The transformed HPSEC chromatogram data and the FACE electrophoregram data for each genotype were compared by subtracting the electrophoregram data from the chromatogram-derived data.

2.2.4. Comparison of starch structures by difference-transformed HPSEC chromatograms and by difference FACE electrophoregrams. The difference-transformed HPSEC chromatogram and the difference electrophoregram are both used to compare the performance of HPSEC and FACE in differentiating starch fine struc-

ture. The transformed chromatogram and electrophoregram of normal starch are used as the reference and subtracted from those of mutant genotypes. The resulted difference profiles are labeled as (mutant—normal).

3. Results

3.1. FACE electrophoregrams

Figure 1 shows the original FACE electrophoregrams of starches from five genotypes. Within the limit of equipment fluorescent response (6000 y-axis unit), a higher sample amount was preferred for a stronger signal-to-noise ratio. The electrophoregram profiles were essentially the same when the height of the highest peak ranged from 2000 to 6000 of the y-axis unit (data not shown). Generally, the upper limit for baseline resolution and reproducible data was between DP 50 and 70, depending on the molar proportion of chains longer than DP 50.

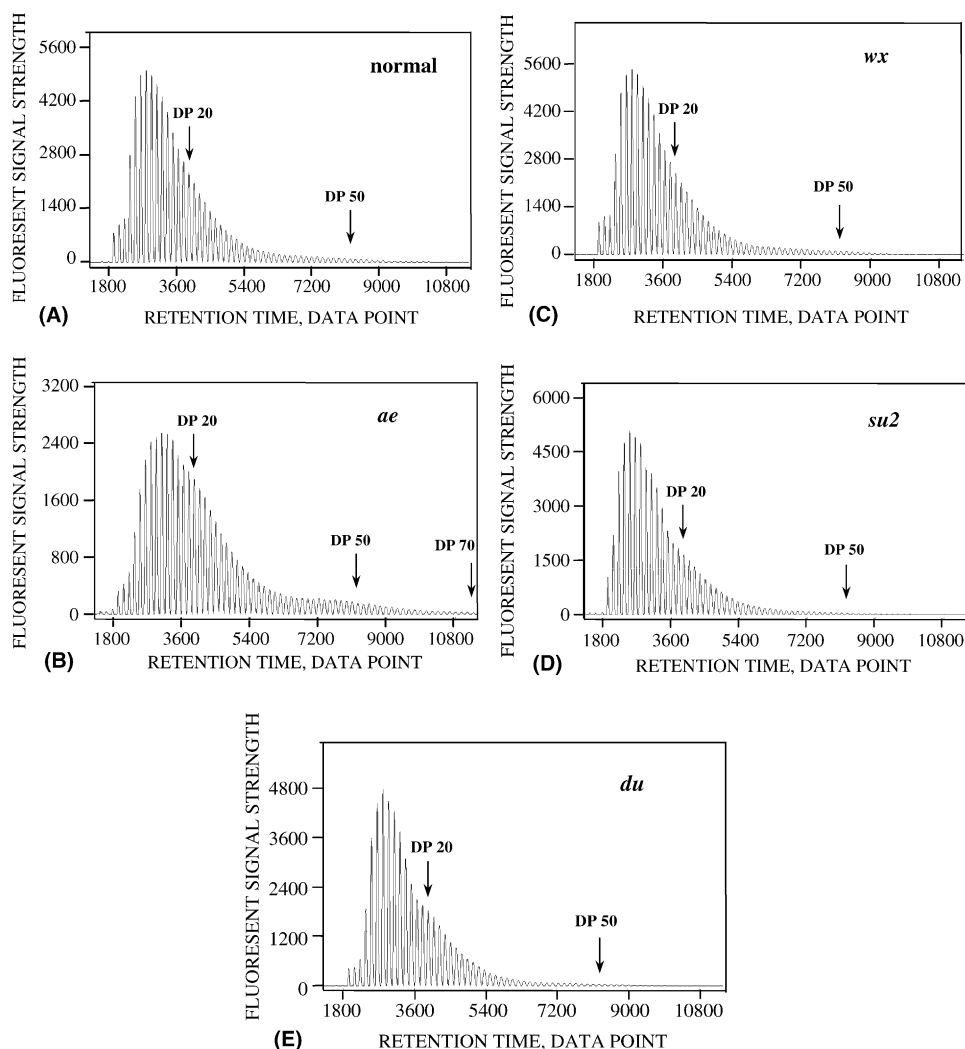


Figure 1. Original FACE electrophoregrams of normal (A), *ae* (B), *wx* (C), *su2* (D), and *du* (E) debranched starches.

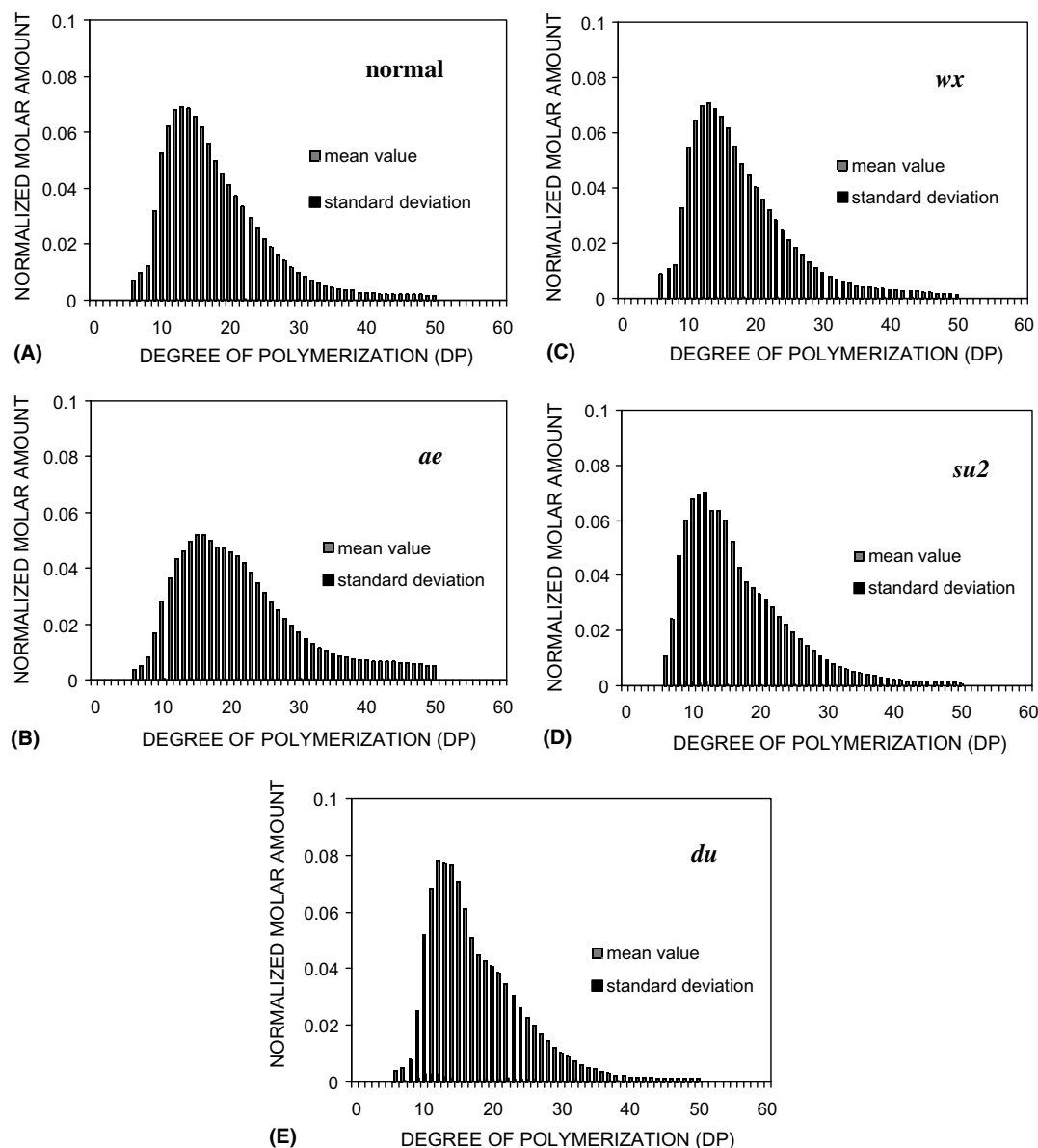


Figure 2. Mean normalized FACE electrophoregrams of normal (A), *ae* (B), *wx* (C), *su2* (D), and *du* (E) debranched starches. Standard deviation of the molar portion at each DP is shown. Data are provided from DP 6 to 50.

Figure 2 shows the mean normalized FACE electrophoregram data of debranched starches. The standard deviation (SD) value is shown beside the corresponding molar proportion for each DP. The molar proportion is provided in the region DP 6–50 since DP 6 is the shortest chain in debranched starch dispersions as indicated in Figure 1, and chains of DP 50 are the longest chains whose molar proportion can be given with a satisfactory SD (<20% of the mean) for all genotypes.

3.2. Mean-normalized HPSEC chromatograms

Figure 3 shows the mean-normalized HPSEC chromatograms of debranched starches. Most chromatogram pro-

files comprise readily distinguishable regions I, II, and III. The long chains (region I) are mainly from amylose, the medium-length chains (region II) are mainly from amylopectin, and the short chains (region III) are also mainly from amylopectin.³ There is no region I depicted for *wx* starch since there is essentially no amylose in this starch. In HPSEC chromatograms resolution of unit DP is not possible.

3.3. Transformed HPSEC chromatograms

Figure 4 shows the mean transformed HPSEC chromatograms of the debranched starches. As for FACE electrophoregrams, each genotype has a distinct profile

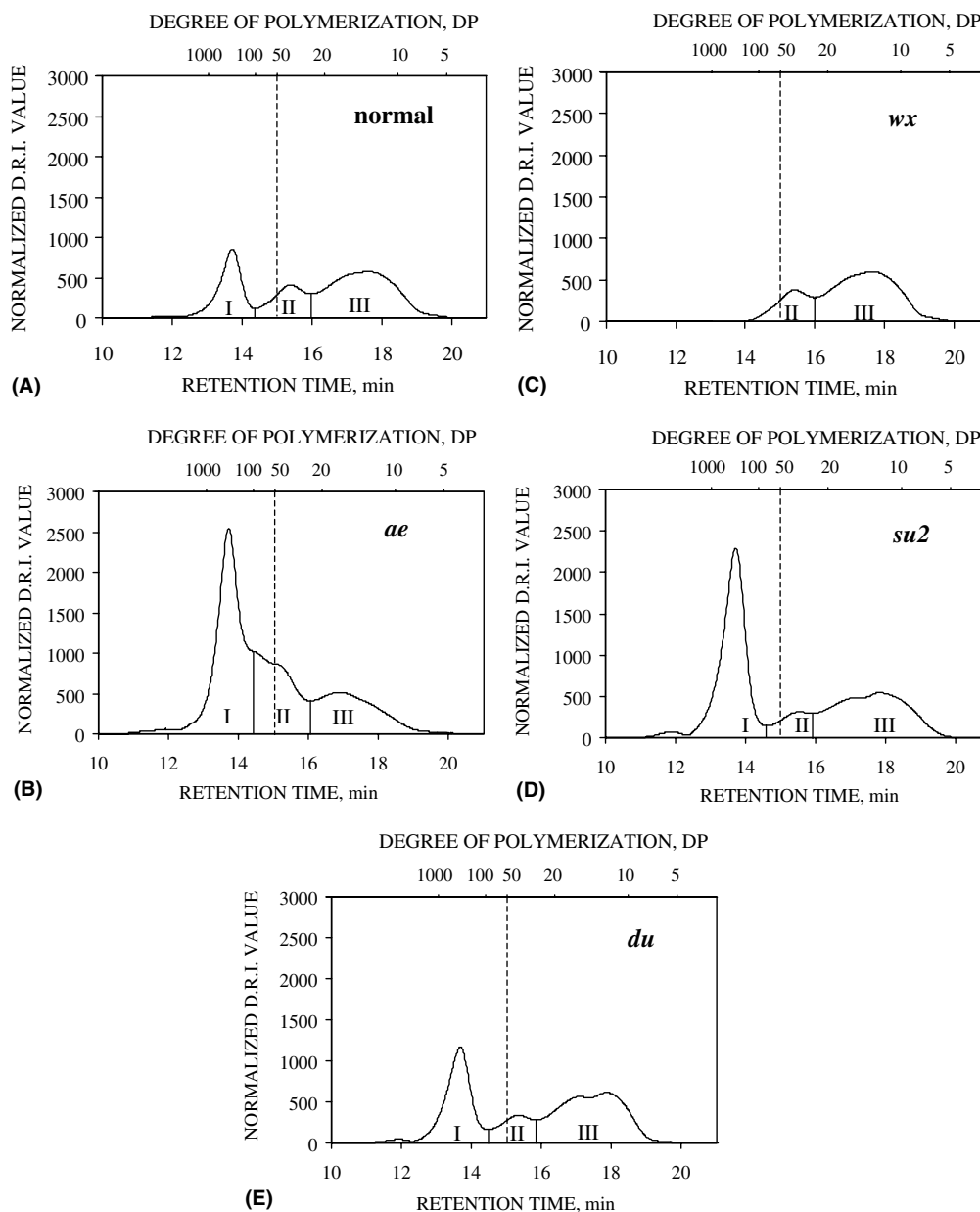


Figure 3. Mean normalized HPSEC chromatograms of normal (A), *ae* (B), *wx* (C), *su2* (D), and *du* (E) debranched starches. The line at DP 50 is used to indicate the portion of the chromatogram (from DP 2 to 50) used for data transformation. Division between regions I and II was based on the minima (normal, *su2*, and *du*) or inflection (*ae*) observed; division between regions II and III was based on the minima observed for all genotypes studied.

pattern, although the differences between any two of them are not evident by visual observation. For each DP, the SD is shown beside the molar portion of chains. We consider that the higher SD values in the region of $DP \leq 11$ (as a % of the mean) originated from the difficulties in using HPSEC to quantify chains with low DP, and the error is made more evident after the data transformation. The length of the shortest chains shown is DP 2, indicating a tailing effect in the HPSEC chromatograms, since by FACE and HPAEC the shortest chains are known to be DP 6.

3.4. Comparison of FACE electrophoregram data and transformed HPSEC chromatogram data

Figure 5 shows the difference between FACE electrophoregrams (Fig. 2) and transformed HPSEC chromatograms (Fig. 4) in the region of DP 6–50. Compared to the FACE electrophoregram, the transformed HPSEC chromatogram gives higher molar proportions for chains from 6 to 17 and very slightly higher proportions for $DP > 38$. In the region of DP from 17 to around 38, the transformed chromatogram

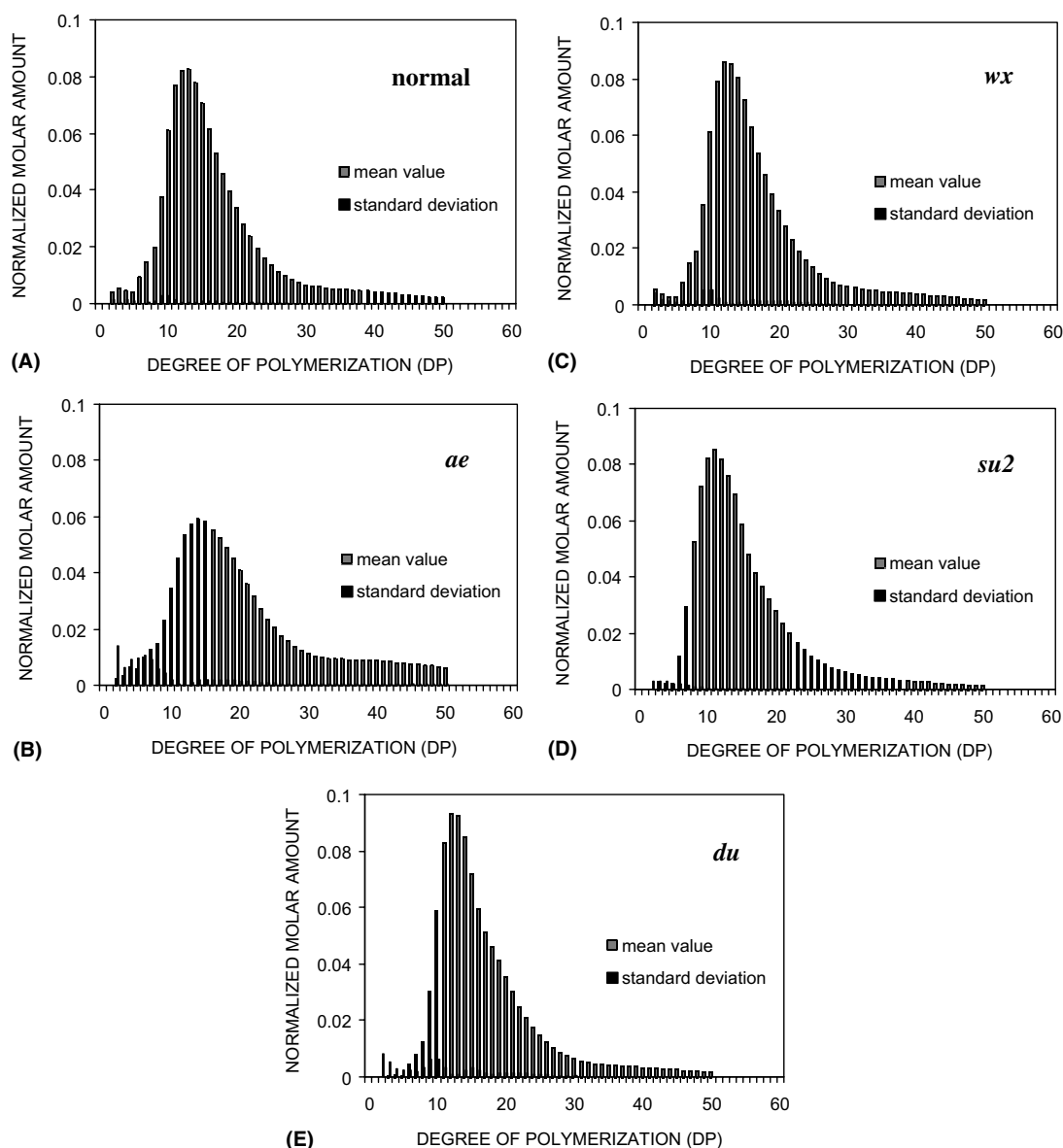


Figure 4. Mean transformed HPSEC chromatograms of normal (A), *ae* (B), *wx* (C), *su2* (D), and *du* (E) debranched starches. Standard deviation of the molar portion at each DP is shown. Data are provided from DP 2 to 50.

gives lower molar proportion than the FACE electrophoregram. These differences are consistent for each genotype, indicating a systematic experimental difference independent of the material being examined.

3.5. Comparison of starches by difference FACE electrophoregrams and by difference-transformed HPSEC chromatograms

While apparently each genotype has its distinct pattern of chain-length distribution, the differences between any two of them are difficult to be described quantitatively from the individual profiles, either for FACE data or for the transformed HPSEC data. For either, differences among starch samples are more evident using dif-

ference electrophoregram plots. Figures 6 and 7 show the difference FACE electrophoregrams and difference-transformed HPSEC chromatograms, respectively. In every case the difference plotted is with respect to the normal starch. Compared to the normal starch, either FACE or the transformed HPSEC shows that the pattern of CL distribution of debranched starches from the four mutant genotypes can be described as follows. For *wx* starch, there is no appreciable difference in the molar portion of chains in the DP region from 6 to 50 (Figs. 6-B and 7-B). For *ae* starch, a comparison of the two types of difference plots shows that FACE and HPSEC provide similar information on CL distribution. The difference FACE electrophoregram (Fig. 6-A) indicated that for *ae* starch, the molar proportion of chains

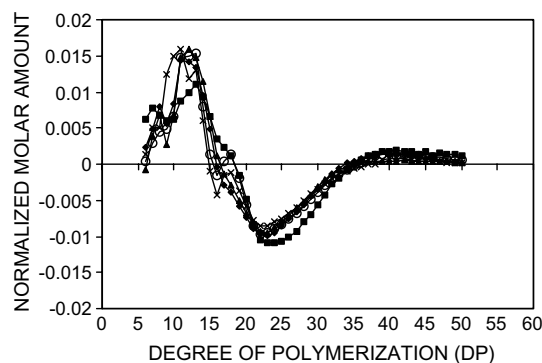


Figure 5. The difference between transformed HPSEC chromatograms and FACE electrophoregrams of normal (\diamond), *ae* (\blacksquare), *wx* (\blacktriangle), *su2* (\times), and *du* (\circ) debranched starches. Data are provided from DP 6 to 50. For each DP, the value on the y-axis was obtained by subtracting the normalized molar amount in the FACE electrophoregram (Fig. 2) from that in the transformed HPSEC chromatogram (Fig. 4).

at each DP (<18) is lower than that of normal starch, and that this situation is reversed for chains DP > 18. In the difference-transformed HPSEC chromatogram (Fig. 7-A), this crossover point is DP17. Thus the two analytical methods provide essentially the same information on the difference between the CL distributions of *ae* and normal starch in the region DP 6–50. Comparisons between *su2* and normal (Figs. 6-C and 7-C) and

between *du* and normal (Figs. 6-D and 7-D) provide further evidence that difference FACE electrophoregrams and difference-transformed HPSEC chromatograms are equally useful in determining differences among the genotypes.

4. Discussion

4.1. Describing the entire chain-length distribution by HPSEC

In the HPSEC chromatograms (Fig. 3), the chains in region I, presumed to be those released from amylose, constitute the longest chains in the debranched starch dispersion. Their DP ranges from around 100 to more than 1000. HPSEC has the advantage of detecting chains in a sufficiently broad range of DP that it allows observation of long linear chains from amylose, as well as medium and short chains from amylopectin. The standard HPSEC chromatogram is not helpful in distinguishing individual DP chains for this sample. Nevertheless, in a previous publication¹¹ the difference HPSEC chromatograms (without transformation) were used to differentiate starches from various genotypes across the entire spectrum of CL.

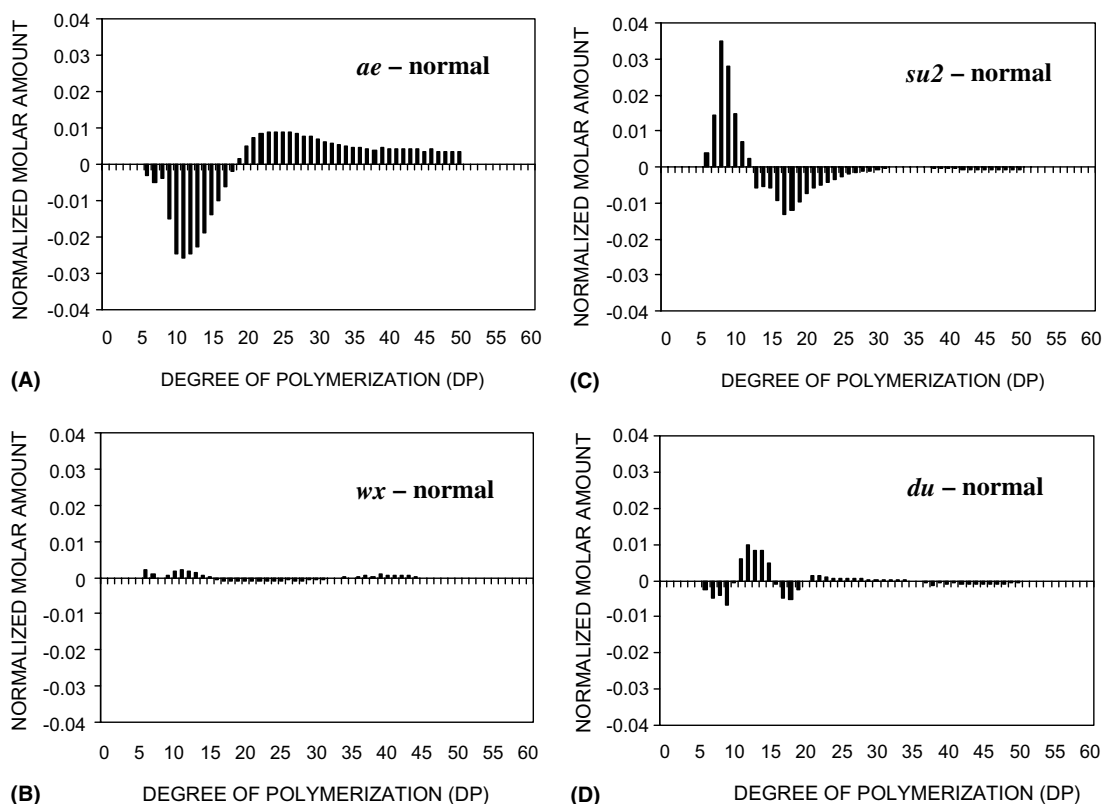


Figure 6. Difference FACE electrophoregrams between normal starch and *ae* (A), *wx* (B), *su2* (C), and *du* (D) starches. Data are provided from DP 6 to 50.

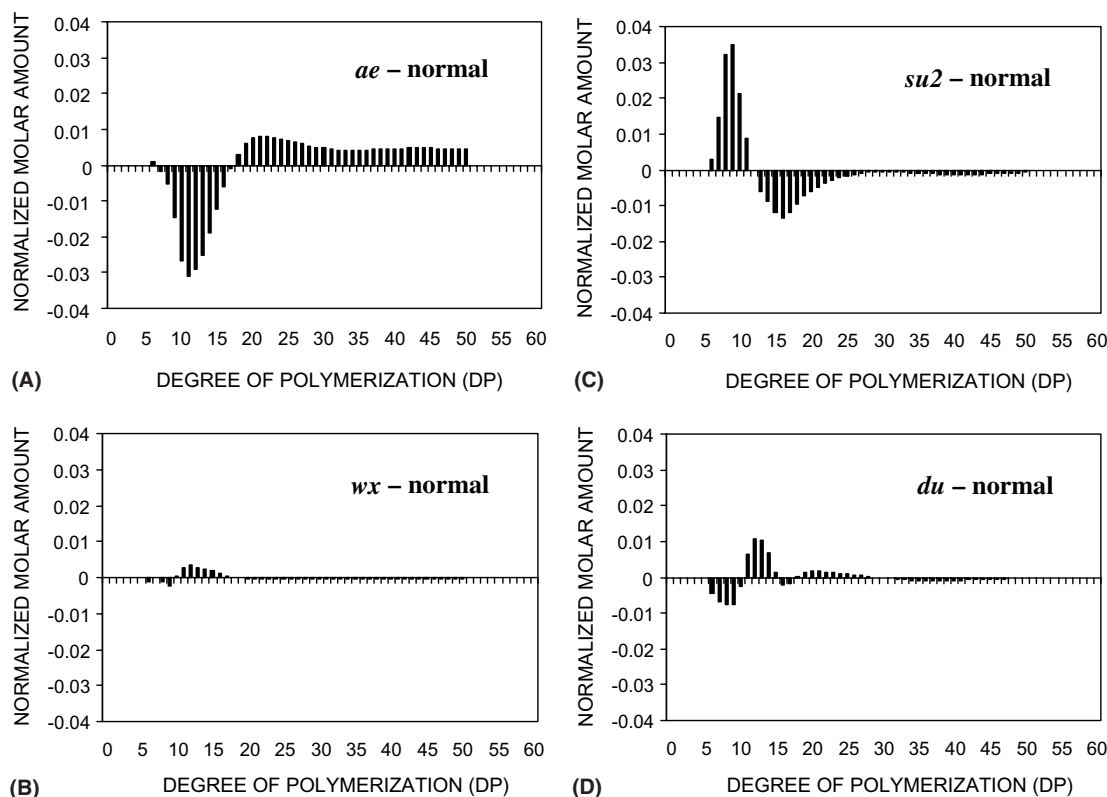


Figure 7. Difference transformed HPSEC chromatograms between normal starch and *ae* (A), *wx* (B), *su2* (C), and *du* (D) starches. Data are provided from DP 6 to 50.

4.2. Baseline resolution of FACE electrophoregram for chains with DP ≤ 50

Figure 1 demonstrates the baseline resolution capability of FACE for chains within a range of DP. The low SD in the range of DP from 6 to 50 resulted from the satisfactory baseline resolution (Fig. 2) and high reproducibility of FACE method. The resolution capability of FACE can provide detailed information about the relative amounts for DP 6–50. This information is useful for detailed investigation of amylopectin branching pattern, especially for the shortest of the two chain populations. While capable of distinguishing individual DP for shorter chains, a major drawback of the use of FACE is the difficulty in detecting longer chains, as the molar amount of chains decreased to under the detection limit for all samples when the chain length exceeded DP 50 (or DP 70 for *ae* starch). Both of these drawbacks limit the use of FACE for quantifying long chains in a debranched starch system.

4.3. Comparison between FACE electrophoregrams and transformed HPSEC chromatograms

The data transformation of the HPSEC chromatogram allows a direct comparison between the HPSEC and the FACE analyses. When the FACE electrophoregrams and transformed HPSEC chromatograms are compared

for each starch (Figs. 2 and 4), the similarity between them is evident.

Although the FACE analysis indicates that chains with DP 6 are the shortest in the debranched starch dispersions, chains from DP 2 to 5 are shown in the transformed HPSEC chromatograms (Fig. 4). Detection in the region of DP 2–5 originated from the tailing effect and was amplified in the process of y -axis transformation from DRI value to molar amount.

Figure 5 shows systematic disagreement between the data in transformed HPSEC chromatograms and the data in FACE electrophoregrams. We consider that the difference between a transformed chromatogram and an electrophoregram originates from the HPSEC standard curve used for both x -axis and y -axis transformations. The standard curve may be somewhat biased due to the conformational difference between the chains in the debranched starch samples and the chains in the pullulan standards (which contain 1→6 linkages in addition to 1→4 linkages) used for calibrating the higher-MW range of the HPSEC columns.

4.4. Difference FACE electrophoregram and difference-transformed HPSEC chromatogram

The advantage of a difference profile for structure analysis is its depiction of nuances of the difference in the CL

profiles of two starches. In the present paper, the chromatogram and electrophoregram of normal starch are used as reference for the starch structure comparison for the other four genotypes.

The systematic difference indicated in Figure 5 has little or no effect on the calculated difference profiles for the four starches (Figs. 6 and 7). By the two analyses the difference profiles for *wx* and normal starch are similar. It is often assumed that the amylopectin from normal and *wx* maize starches is similar. Although the present work suggests small differences, we cannot exclude the idea that some smaller chains from amylose of normal starch might account for the differences.

The difference chain-length distribution is very useful and convenient in screening starches with novel structure. With the database of known starch as the reference, the structure of any unknown starch could be characterized equally well by a difference profile derived from the HPSEC chromatograms or from the FACE electrophoregrams.

4.5. Use of HPSEC and FACE to describe the chain-length distribution of debranched starch

HPSEC has the capability to quantitatively describe the chains longer than DP 50, while FACE has no such capability. Thus only HPSEC can be used to characterize the long chains in the debranched starch. However, the transformed HPSEC chromatogram gives higher SD values (Fig. 4) than the FACE electrophoregram (Fig. 2) in the DP region from 6 to 11 for most genotypes. Thus FACE has the advantage in more precisely quantifying these short chains. Although systematic differences exist between profiles from transformed HPSEC data and FACE data, these systematic differences have little effect on the difference profiles used for structure comparison among the starches analyzed in the present paper.

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Supplementary data

Details on the HPSEC data transformation to FACE format (six pages) is provided in the supplementary data, which is available in the online version, at 10.1016/j.carres.2004.12.033.

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