Improved Methods for the Structural Analysis of the Amylose-Rich Fraction from Rice Flour

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Cooking and sensory properties of rice are largely determined by the amylose content and structure. For relationships between functional and structural properties, a more accurate method to determine the structure of amylose is required. Here we calibrate size exclusion chromatography (SEC) columns, using Mark−Houwink parameters for linear starch and pullulan standards, to obtain the true molecular weight distribution of linear starch. When the molecular weight distribution is reported relative to pullulan, rather than the actual molecular weight which is readily obtained from universal calibration, it is seen that the molecular weights of longer amylose chains are greatly underestimated. We validate the SEC method to enable the measurement of the hydrodynamic volume distribution of the starch by examining reproducibility and recovery. Analysis of the starch in the sample pre- and post-SEC shows that 20% of the carbohydrate is not recovered. Comparison of the weight-average degree of polymerization, $\bar{X}_w$, of (undebranched) starch of pre- and post-SEC is made using iodine binding as well as Berry plots of data from multi-angle laser light scattering (MALLS). These both show that current SEC techniques for starch analysis lead to significant loss of high molecular weight material. Indeed, for the systems studied here, the values for $\bar{X}_w$ after SEC are about three times lower than those before SEC. Iodine−starch complexes of pre- and post-SEC samples reveals that the SEC techniques give reliable data for the amylose fraction but not for amylopectin. We address reports in the literature suggesting that the conventional isoamylase method for debranching starch would lead to incomplete debranching and thus incorrect molecular weight distributions. However, it is shown using ^{1}H NMR that isoamylase can completely debranch the amylose (to within the detection limit of 0.5%), and by SEC that successive incubation with isoamylase, α-amylase, and β-amylase can degrade the amylose-rich fraction completely to maltose. We develop a method to obtain a hot water soluble fraction (HWSF), rich in undamaged amylose molecules, directly from rice flour, avoiding the structural degradation of previous techniques. With appropriate sample handling, the formation of associations between starch chains is minimized. With the combination of calibrated and validated SEC methods, and an improved extraction of amylose from rice, the $\bar{X}_w$ for both HWSF and debranched HWSF are found to be much larger than has previously been reported.

Introduction

Starch is the major component of rice grains, and the amylose content of the starch is considered to be the most important factor affecting the cooking quality of rice. However, amylose content is not sufficient to explain variability in cooking properties (e.g., refs 1−3), and the molecular architecture of the amylose molecules is also likely to contribute to these properties. The validity of any structure−function relation is entirely dependent on the quality of the method used to acquire the data. Outstanding in rice chemistry is a method for the most accurate determination of amylose structure. To perform such a study, the following are required: (a) the ability to determine amylose structure with a calibrated SEC system, (b) experimental size exclusion chromatography experimental SEC data that are representative of the true hydrodynamic volume distribution of amylose-rich fractions, (c) the ability to debranch an amylose-rich fraction completely, and (d) a method to obtain an amylose-rich fraction of rice flour with minimal degradation of the starch structure and minimal contribution from amylopectin. Even though in this work only rice is used, it is reasonable to assume that the general methodologies and conclusions will find application in other grains.

A complication of obtaining an accurate amylose structure is that pullulan standards have generally been used to calibrate the SEC. The problem with this is twofold. First, amylose molecules are branched[4] and the elution profile of branched molecules does not directly translate to the molecular weight distribution (MWD) of the amylose.[5] Second, since pullulan standards are not of the same composition as starch, their elution profile will differ from that of starch, and so a calibration curve generated from pullulan standards cannot be used in isolation

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to measure the MWD of either amylose molecules or amylose chains. Here we use and build on earlier techniques[5,6] to quantitatively convert SEC traces to MWDs. The particular system for the methodologies discussed here is SEC with differential refractive index (DRI) detection. Additional detector systems are available, such as in-line MALLS and in-line viscometry, which provide more information than a DRI system alone: respectively the $\bar{X}_w$ for each slice of elution volume (from MALLS) and hydrodynamic volume (from viscometry), the latter being extremely complex to interpret for branched polymers and indeed only able to be interpreted with semi-empirical relations and parameters.[7,8] No combination of these can provide a complete specification of the architecture of a branched polymer.

Aqueous SEC can provide structural information by separating the components based on their hydrodynamic volume and on their molecular weight (after meeting certain conditions). However, it is important to ensure that the SEC data are representative of the true hydrodynamic volume distribution of the sample, as some studies have suggested that recovery of carbohydrate following SEC is incomplete.[9−11] One approach is to measure the recoverable carbohydrates after a sample passes through the SEC system. An alternate approach is to use MALLS to compare the $\bar{X}_w$ value of the initial sample to the sample collected after passage through the SEC column, or again, by iodine assay to identify any differences in the absorbance spectra between the pre- and post-SEC samples. Many studies (e.g., refs 12,13) describe the measurement of amylose by SEC, but few of the methods have been widely adopted, perhaps because none for rice amylose have been rigorously validated.[12,14] This complicates efforts to draw comparisons between different studies.

The analysis of the structure of amylose requires analysis of the components of amylose molecules. Amylose molecules are essentially long linear chains that carry a few long branches.[12,14] Investigation of the chains constituting the molecules is typically done using isoamylase from *Pseudomonas* sp. (EC 3.1.2.9) to hydrolyze the α-(1,6) branch linkages to produce linear α-(1,4) linked chains.[15,16] However, it is generally accepted that isoamylase is not able to access some of the branchpoints of amylose.[17,18] The basis for this inference is that after treating the debranched amylose with β-amylase (EC 3.2.1.2) (to cleave alternate α-(1,4) bonds to produce maltose), high molecular weight molecules remain.[4,14,19] We revisit this problem by exploring the effect of molecular association and pH on the efficiency of both enzymes using ^{1}H NMR analysis[20] and SEC. After optimizing the method to collect a fraction from rice flour that is rich in intact amylose molecules, and determining the conditions required to debranch it completely, we obtain the MWDs, the weight and number average degrees of polymerization ($\bar{X}_w$ and $\bar{X}_n$), and the polydispersity (Q) of the debranched amylose.

Previous methods which have isolated starch from flour using variations of the alkaline precipitation/steeping method[21] have been shown to cause degradation of the large amylose chains.[22] Precipitation of amylose from the starch, using butanol or concavalin A, is not absolute, with incomplete recovery of the amylose[23] and precipitation of some residual amylopectin.[9,12,14,19] The common feature of many methods[6] is to leach out the amylose molecules at a temperature above the gelatinization temperature; this feature will be used to develop a new procedure to extract an amylose-rich fraction directly from rice flour. The compromise with this method is that, while existing lipid−amylose complexes will remain in the insoluble fraction, some free lipids may complex with the leached amylose; the data generated here will eventually be applied to understanding cooking properties, and formation of lipid-amylose complexes is one of the processes of cooking. Here we will investigate the following: how the concentration of the rice flour in the solution influences the proportion of amylose in the leached fraction (supernatant); ways to minimize the contribution of amylopectin to the supernatant; the relationship between gelatinization temperature and amount of amylose leached; and we rigorously investigate a method to measure the hot water soluble fraction (HWSF) and hot water insoluble fraction (HWIF) using SEC. The absorbance of the complex between iodine and the leached compounds, and comparison with the iodine complex between both amylose and amylopectin, can give some indication of the composition of the leached components.[12,24]

Currently, a full description of the complex structure of amylose, on all its levels of organization, is well beyond present technology, but many structure−property relations may hopefully be understood using the descriptors of structure available from current technology, for example, the distribution of the hydrodynamic volume of amylose molecules together with the MWD of the chains comprising the amylose molecules. Not only could such data be used for structure−property relations for purposes of cooking quality and industrial applications of starch (e.g., ref 25), but it could also be used to make qualitative and quantitative inferences about the enzymatic processes in starch biosynthesis.[26] In this paper, we examine methodologies so that these and other quantitative measures of architecture can be obtained reliably for amylose from rice, so that eventually structural information of starch can be related to both physical properties of the starch and to the enzymatic processes of the in vivo starch synthesis.[26]

Materials and Methods

Materials. Samples of rice (*Oryza sativa*), Shimizu mochi, Amaroo and Doongara, were obtained from the 2002/3 harvest at NSW Agriculture, Yanco, Australia. Shimizu mochi is a waxy Japanese variety, and Amaroo and Doongara are both non-waxy Australian varieties. Paddy of each variety was dehulled (THU35A 250V 50 Hz test husker, Satake), milled (McGill no.2 mill), and then ground (Cyclotec 1093 sample mill, Tecator) to pass through a 0.5 mm sieve. Amylose content was measured,[27] and protein content were determined (Dumas method, ASTM E191−64). Gelatinization temperature was determined using a differential scanning calorimeter (Q Series Instrument, Q Series Explorer software, TA Instruments) increasing in temperature from 35 to 120 °C, in increments of 4 °C/min.

The hot water soluble fraction (HWSF) was collected from the flour of each variety, at two concentrations (1.96% and 3.85% w/w). Rice flour (250 mg or 500 mg) from the three varieties was mixed with water (12.5 g), and the HWSF was separated from the flour by rapid viscoanalysis (RVA 3, Newport Scientific) (AACC standard method 61−02). After each run, the mixture was centrifuged immediately (10^4 *g*, 10 min). The supernatant contained the HWSF, and the pellet contained the hot water insoluble fraction (HWIF).

Isoamylase (*Pseudomonas* sp., EC 3.1.2.9, 105 U/mg), β-amylase (Barley, EC 3.2.1.2, 1400 U/mg), α-amylase (*Bacillus licheniformis*, EC 3.2.1.1, 54 U/mg), and a total starch assay kit were obtained from Megazyme and used as supplied. Pullulan standards (from Shodex) were used for SEC calibration, using the universal calibration. High-purity water (MilliQ) was used for all SEC experiments, and deuterium oxide (D_2O) was used for all ^{1}H NMR experiments. All chemicals used were reagent grade.

Calibration and Validation of SEC for HWSF of Flour.

Size Exclusion Chromatography. SEC of the HWSF was performed either on a Waters system consisting of an Alliance (2695) and

differential refractive index (DRI) detector (Waters 2410) with Waters software (Empower) to control the pump and to acquire and process data, or with a Shimadzu system: a Wyatt OPTILAB EOS interferometric refractometer and Astra software. For both systems, the eluent was ammonium acetate (0.05 M, pH 5.2) flowing at 0.5 mL min^{-1}.[19] SEC columns packed with a hydroxylated poly(methyl methacrylate)-based gel, an Ultrahydrogel 500 (UH500) or an Ultrahydrogel 250 (UH250, both from Waters), were used independently and held at 60 °C during each run. Aliquots (40 μL) of the HWSF were injected without further processing, and the run time was 40 min.

Calibration of SEC. Shodex standards (P800, P400, P200, P100, P50, P20, P10, P5) were injected into the SEC into the UH500 and UH250 columns separately. On the basis of retention time, a calibration curve for linear starch was created using the Mark−Houwink−Sakaruda equation and universal calibration.[5] The Mark−Houwink parameters used for this conversion, with ammonium acetate (0.05 M, pH 5.2) as the solvent and the column at 60 °C were recently found to be $K = 0.001\ 26$ mL g^{-1} and $\alpha = 0.733$ for pullulan, and $K = 0.0544$ mL g^{-1} and $\alpha = 0.486$ for linear starch.[5] The value of α is close to the value of $^1/_2$ for a θ solvent; that is, the solvent system chosen here behaves ideally.[28]

Validation of the SEC. As amylose is best separated by the UH500,[16] validation was performed on this system. Reproducibility of the SEC and of the UH500 column were tested by replicate collections, on different days, of the HWSF of Amaroo, prepared at both concentrations. To determine whether the chromatogram represents all the starch molecules in the injection, carbohydrate recovery, $\bar{X}_w$ by MALLS, and λ_{max} of starch-iodine complexes were measured pre- and post-SEC. The pre-SEC sample was prepared by mixing an aliquot of each sample (40 μL) with eluent (5.96 mL) to give the same concentration as the material that eluted from the column. As starch eluted from the DRI detector, the post-SEC sample was collected from the beginning of the void volume until the end of the separating phase (8−20 min) (manually or with a Waters Fraction Collector III).

For measurement of total starch, the pre- and post-SEC samples were evaporated under vacuum (Jouan RCT 60 and RC 10.09) to a weight of 500 mg, and then starch in the samples was measured with the total starch assay kit (Megazyme). Recovery is reported as the difference in the glucose content pre- and post-SEC.

For $\bar{X}_w$ determination, a photon correlation spectrometer (Brookhaven Instruments comprising a BI-200SM version 2 goniometer with BI-APD avalanche photodiode detector, and PC1 B1−9000AT EN correlator) in static (MALLS) mode, with a 35 mW He−Ne laser light, $\lambda = 633$ nm, and 400 μm aperture was used. To obtain reproducible $\bar{X}_w$ data using this technique, filtered eluent (0.1 μm) was used to prepare the samples and as the reference. Defects in sample tubes, coupled with impurities in the Decalin solvent used for MALLS, required that each sample be measured in triplicate. Berry plots[28,29] were generated with BIC Zimm Plot Software (version 3.17). For the pre-SEC Berry plot, the HWSF was diluted with eluent to a concentration range from 0.11 to 0.02 mg mL^{-1}. For the post-SEC Berry plot, different injection volumes were used to create a similar range of concentrations, and the Berry plot was determined from five concentrations measured at six angles (30°, 45°, 55°, 65°, 75°, and 90°). Each value is the average of three replicates.

For starch−iodine complexes, an aliquot of the injection pre- and post-SEC (1 mL) was mixed with citric acid (0.1 M, 2 mL) and iodine solution (1 mL), and then, the volume adjusted to 20 mL with water. Absorbance was measured every 1.25 nm over the visible spectrum using a scanning spectrophotometer (GBC UV/VIS 918).

Is Debranching Complete of the HWSF from Flour?

Amylose was debranched as follows. The HWSF (795 μL, 1.96%) from each variety was mixed with sodium acetate buffer (0.2 M, pH 4, 205 μL) together with glacial acetic acid (6 μL) and isoamylase (10 μL). This mixture was incubated at 50 °C for 2 h, boiled to denature the enzyme, and centrifuged (10^4 *g*, 5 min). The supernatant was desalted with mixed bed resin (BioRad AG 501-X8) for 60 min at 50 °C.

Isoamylase was also incubated with aliquots of the HWSF of flour (795 μL) mixed with sodium acetate buffer at either pH 4 or pH 5 (0.2 M, 205 μL) together with glacial acetic acid (6 μL), or with MilliQ water (205 μL) to maintain a pH of 6. Each reaction mixture (pH 4, 5, and 6) was debranched as above. To one sample with a pH of 4, NaOH (1 M, 200 μL) was added to change the pH to 6. β-amylase (20 μL) was added directly to these mixtures (pH 4, 5, 6, and to the sample with a pH change from 4 to 6), and the mixtures were incubated (60 °C, 20 min). Samples were then boiled to denature the enzyme, centrifuged to remove precipitates (10^4 *g*, 5 min), and desalted (as above). Aliquots of each supernatant (40 μL) were analyzed by SEC.

Analysis of the α-(1,6) linkages was completed on HWSF (1.96%) prepared from the three flours, using D_2O as the solvent instead of MilliQ water, and debranched at pH 4 (as above). ^{1}H NMR (Bruker 300 Ultrashield) was used to detect the proportion of α-(1,4) and α-(1,6) linkages in each sample before and after debranching. Removal of salt by using the mixed bed resin caused poor peak resolution, and was therefore not used in sample preparation. ^{1}H NMR were measured at 300.13 MHz and 360 K.[20] XWIN PLOT software was used for processing data. Chemical shifts were referenced to an internal standard of 3-tris(methylsilyl)sodium propionate-d_4(TSP) with a chemical shift of 0 ppm. The lower limit of resolution of α-(1,6) linkages was determined by subsequent dilution of the sample with D_2O.

Evidence of association, aggregation, or retrogradation of freshly prepared HWSF and debranched HWSF (as above at pH 4) from Doongara (1.96%) was examined. Aliquots of both were injected into the SEC immediately after preparation, and a second aliquot of each was treated to disassociate any associated chains. The second aliquot of each was diluted by half and kept at 50 °C for one week with constant, gentle shaking, during which time aliquots were removed daily and injected into the SEC.

An additional α-amylase incubation between the isoamylase and β-amylase incubations was performed. To one sample debranched at pH 6, α-amylase (20 μL) was added directly. To another sample debranched at pH 4, α-amylase (20 μL) was added after the pH of the debranched sample was changed to pH 6 by the addition of NaOH (1 M, 200 μL). Samples were incubated (60 °C, 60 min), then boiled and centrifuged (10^4 *g*, 5 min); β-amylase was then added to the supernatant, and the samples were incubated and treated as above.

Characterizing Starch in the HWSF from Flour.

Identification of Starch in the HWSF. The HWSF of flour (1.96%) from the three varieties was collected as described earlier, but both the supernatant and the pellet were retained after centrifugation. An aliquot of the HWSF (795 μL) was debranched as described above. To a subsample (40 mg) of each HWIF, NaOH (0.25 M, 1 mL) was added and the samples were solubilized by heating and refluxing (2 min). The weight of each solubilized HWIF was adjusted to 4 g with water. An aliquot (795 μL) of each solubilized HWIF was debranched as described above. The Waters SEC was used, the injection volume was 40 μL, and the run time was 40 min. The yield of the HWSF was determined gravimetrically after drying aliquots of the HWSF (∼50 °C, ∼24 h), and the contribution of amylose and amylopectin to both the HWSF and the HWIF was determined by SEC using the UH250, which suitably separates debranched amylose and amylopectin chains.[16]

Maximizing Amylose in the HWSF. Twelve samples of flour from each variety were mixed with water (1.96%) to determine (i) the temperature at which amylose leaches from the granules, using RVA, (ii) if solubility of amylose is dependent on gelatinization temperature, and (iii) if different heating parameters increase the amylose in the HWSF. The AACC standard method (61−02) was divided into twelve run times, with the first sample run for the first 1 min of the standard method, and the last sample run for the full 12.5 min. Table 1 shows the temperature and time for each of the twelve runs. After each run, the mixture was centrifuged immediately (10^4 *g*, 10 min), and the

Table 1. Time and Temperature Regime Used for the Collection of HWSF throughout the RVA Separation Technique

collection time (min)	collection temperature (°C)
1.00	50
2.50	68
3.00	74
3.50	80
4.00	86
4.50	92
5.00	95
5.33	95
5.66	95
6.00	95
8.33	83
12.00	50

supernatant was retained. Amylose in the supernatants was measured by iodine binding.

For each variety, the HWSF was made at 1.96% and 3.85% to try to maximize the amylose in the HWSF. The heating and stirring profiles used were the full 12.5 min of the AACC standard RVA method (61−02), and viscosity was recorded to determine any resistance to the paddle. The mixtures were centrifuged at either 2000, 10 000, or 14 000 *g* for 10 min. The HWSF was analyzed by SEC and iodine binding.

For starch−iodine complexes, the HWIF was solubilized by the addition of NaOH (10 mL, 1 M). It was then heated with refluxing (2 min), the volume adjusted to 100 mL with water, and an aliquot of 1 mL used. For the HWSF, an aliquot of 0.1 mL was used. Citric acid (0.1 M, 2 mL) and iodine solution (1 mL) were added to each, and then the volume was adjusted to 20 mL with water. Absorbance was measured as described earlier.

Results and Discussion

Calibration and Validation of SEC for HWSF of Flour. Size exclusion chromatography (SEC) separates *branched* and *linear* molecules on the size of the molecule in an aqueous mobile phase. Regardless of composition, the elution time is a function of the hydrodynamic volume.[8,30] The SEC distribution can be expressed simply as hydrodynamic volume, but it is more informative if expressed as degree of polymerization (DP, or *X*). MALLS measures the *weight-average* degree of polymerization ($\bar{X}_w$) for each elution volume; while such information is useful, it gives only an average molecular weight for branched polymers, because SEC separates by hydrodynamic volume, not molecular weight. Moreover, use of MALLS was found to be impractical for the present system, as the concentration high enough for a good DRI response was found to swamp the very sensitive MALLS detectors. Universal calibration[30] assigns a true hydrodynamic volume to the sample eluting at a given elution volume; this relies on the assumption that SEC separation depends only on hydrodynamic volume, not composition. The Mark−Houwink parameters relate the intrinsic viscosity (or hydrodynamic volume) and DP of a *linear* standard such as pullulan, to the viscosity (or hydrodynamic volume) and DP of a second *linear* polymer such as starch.[5] So for a *linear* system, elution time is related to hydrodynamic volume, hydrodynamic volume is related to intrinsic viscosity, and intrinsic viscosity is related to the DP. For a branched system, it has been shown[8] that hydrodynamic volume correlates well with elution volume. Here, the SEC distribution is expressed as "linear equivalent DP", whereby the DP is for a *linear* molecule that has the same hydrodynamic volume of a *branched* molecule. A full description of universal calibration and derivation of K and α values (which relate hydrodynamic volume and X) has recently been published.[5]

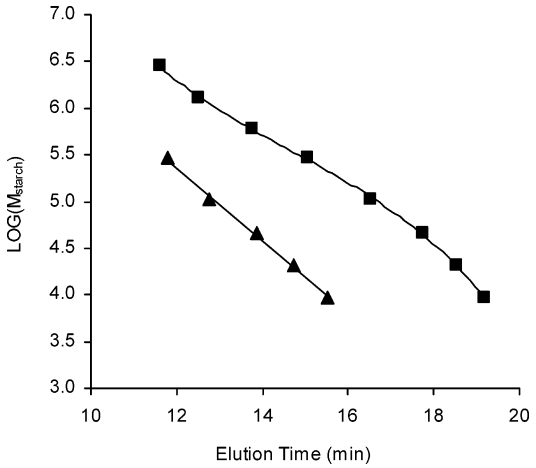

Figure 1. Calibration curves of linear starch for the Waters system using UH250 column (triangles) and UH500 column (squares) created from the Mark−Houwink parameters of pullulan (K = 0.001 26 mL g^{-1} and α = 0.733) and linear starch (K = 0.0544 mL g^{-1} and α = 0.486) in ammonium acetate solution (0.05 M, pH 5.2) at 60 °C.[5]

The calibration curves created for both columns used in this study, the UH250 and the UH500, are shown in Figure 1. When pullulan standards are used in the calibration, the UH250 has a separating range of DP 60 to 1800, but when additional, lower molecular weight standards, like poly(ethylene oxide) are used, the UH250 can separate linear starch to DP 10.[31] The UH500 has a separating range from DP 60 to 17000, and it is suitable for the measurement of amylose molecules. It is also of interest to examine the size of the error that arises if one makes the common practice of referring molecular weight relative to pullulan, rather than of starch, or to use an inappropriate fit to the calibration curve. Figure 2a shows the SEC distributions of a starch sample (debranched Amaroo (1.96%) on the UH500) with the molecular weight relative to pullulan and the true molecular weight found by universal calibration using the above Mark−Houwink parameters. It is seen that determining the molecular weight from pullulan ("relative molecular weight") without universal calibration significantly underestimates the actual molecular weight by a factor of 10. If the UH500 calibration curve is fitted with a linear curve (Figure 2b), as is the case with the UH250 column, rather than a third-order polynomial, then the difference between the pullulan and the starch standard is also distorted at the high molecular weight end of the chromatogram.

Both the separation technique and the SEC technique are very reproducible for both concentrations (Figure 3). However, it is important to ensure that the SEC signal obtained represents the distribution of all the starch in the injection. The first check was to examine the recovery (%) of carbohydrate in HWSF collected post-SEC. The results in Table 2 show a 77−82% recovery of carbohydrate from the SEC. In contrast to previous work,[10] this result was found to be independent of the initial concentrations of flour and of amylose content.

It is important to know if the loss of 18−23% carbohydrate (Table 2) is across the entire distribution, or the loss of a certain component of the HWSF. To quantify any difference in $\bar{X}_w$ pre- and post-SEC, Berry plots were obtained using MALLS for the HWSF of Doongara at both concentrations. Figure 4 shows a typical Berry plot. Table 2 shows that the $\bar{X}_w$ of the pre-SEC HWSF was approximately 3 times larger than that of the post-SEC HWSF for the 1.96% (w/w) preparation and 3.5 times larger for the 3.85% (w/w) preparation. Given that the loss of carbohydrate measured directly after SEC was only about 20%

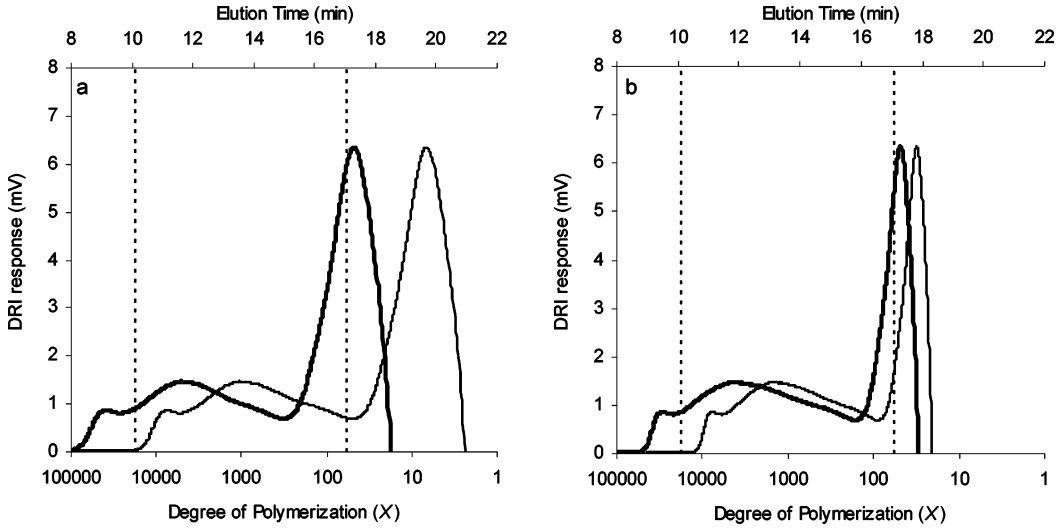

Figure 2. SEC distribution for debranched Amaroo (1.96%) starch, showing two scales: molecular weight relative to pullulan standards (thin line) and absolute molecular weight from universal calibration (thick line). Part a is with a third-order calibration, and part b is with a linear calibration. The vertical dashed lines show the limit of calibration for the UH500 column.

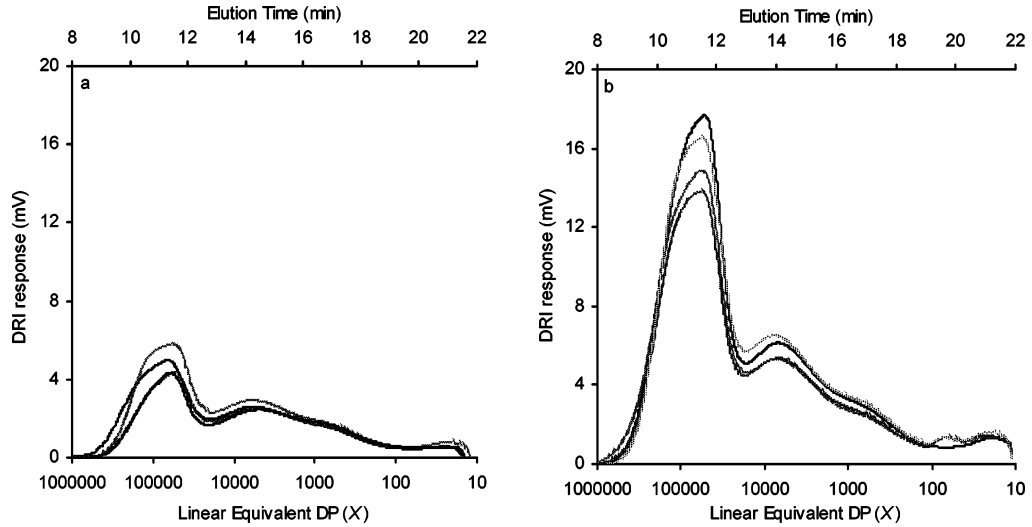

Figure 3. Reproducibility tests: SEC traces of the HWSF of flour of Amaroo prepared and analyzed on four different occasions at both rice slurry concentrations: (a) 1.96% (w/w) and (b) 3.85% (w/w). These SEC traces were obtained from the Waters SEC system.

Table 2. Validation of the UH500/SEC System for Analysis of the HWSF of Flour[a]

carbohydrate recovery (%)	rice flour slurry 1.96%	rice flour slurry 3.85%
Shimizu Mochi	83.5	80.1
Amaroo	83.5	74.8
Doongara	79.3	78.3
average ± SD	82.1 ± 2.4	77.7 ± 2.7
$\bar{X}_w$ − Doongara only[b]	pre-SEC	post-SEC
rice flour slurry 1.96%	$(7.5 \pm 2.0) \times 10^5$	$(2.5 \pm 0.4) \times 10^5$
rice flour slurry 3.85%	$(1.5 \pm 0.5) \times 10^6$	$(4.3 \pm 0.3) \times 10^5$
λ_{max} (nm)[c]	pre-SEC	post-SEC
Amaroo	611	630
Doongara	618	631

[a] Recovery (%) of carbohydrate in the HWSF (from the three varieties at both concentrations) was determined. [b] Values of $\bar{X}_w$ for the HWSF of Doongara prepared as determined from Berry plots of MALLS data (an example of a Berry plot is in Figure 4). [c] Absorption maxima, λ_{max} (nm), of starch−iodine complexes with HWSF from the two non-waxy varieties collected pre- and post-SEC.

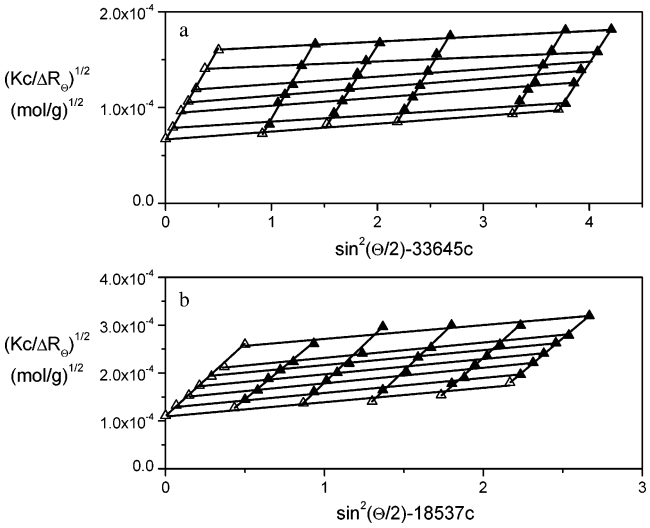

Figure 4. An example of a Berry plot prepared from MALLS data for Doongara HWSF (3.85% (w/w)) prepared (a) pre- and (b) post-SEC.

(Table 2), this loss must have been predominantly from high molecular weight species, like amylopectin, to cause the large decrease in the $\bar{X}_w$ of the molecules in the sample. The λ_{max} of the starch−iodine complex of the HWSF collected pre- and post-SEC, shown in Table 2, shows that λ_{max} of the HWSF post-SEC has shifted to an even higher λ_{max} (toward that of amylose) than the pre-SEC HWSF. While we cannot exclude the possibility that some components may not be recoverable, these

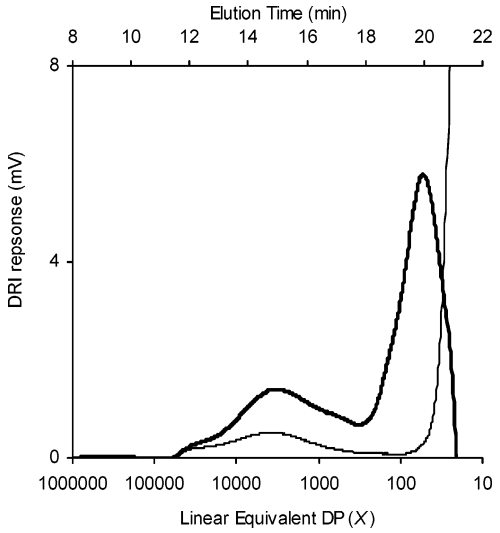

Figure 5. SEC traces of debranched HWSF (thick line) and debranched HWSF treated with β-amylase (thin line). These SEC traces were obtained from the Waters SEC system.

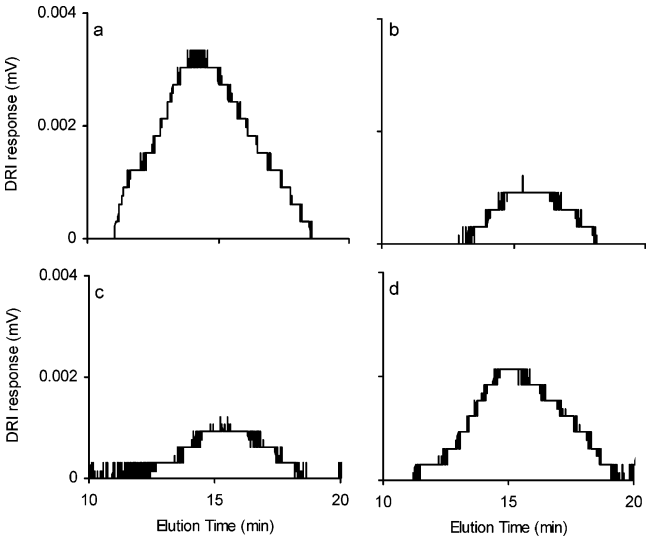

Figure 6. SEC traces of the HWSF of Doongara (1.96% (w/w) slurry) digested by isoamylase, then β-amylase, at pH 4 (a), pH 5 (b), pH 6 (c), and at the correct pH for each enzyme (d). These SEC traces were obtained from the Shimadzu SEC system.

data further indicate that the unrecovered high molecular weight material is likely to be amylopectin. The loss could be due to the poor solubility of the amylopectin,[10,11] or because the 10 μm pore size of the UH500 column (Waters specifications) prevented high molecular weight material from passing through the column (note that this loss of material was observed when the SEC was run without a guard column). Fortuitously, the unrecovered material increases the proportion of amylose in the HWSF.

Is Debranching Complete of the HWSF from Flour?

Here we hope to solve the long-standing problem of apparent incomplete debranching of amylose.[4,14,19] In theory, linear amylose chains should be obtained by hydrolyzing the α-(1,6) linkages with isoamylase. Some studies suggest that not all branch points are accessible to isoamylase, based on the molecular weight of the material remaining after incubations of amylose first with isoamylase, then with β-amylase.[4,14,19] Incomplete debranching would clearly have major implications for qualitative and quantitative interpretations of MWD data of amylose that was assumed to be debranched by isoamylase. The incubation of amylose with isoamylase and β-amylase is replicated in Figure 5, and in our hands, β-amylase completely digests the lower molecular weight chains of amylopectin (DP 10−100) in the debranched HWSF, but incompletely digests the higher molecular weight chains.

In biological systems, pH affects the efficacy of catalytic sites of enzymes. Therefore, the effect of pH on successive incubations of isoamylase followed by β-amylase was examined across the range of pH suitable for these enzymes. Figure 6 shows that independent of pH, high molecular weight material remained. However, as the pH became less acidic, more of the higher molecular weight material was degraded. The remaining high molecular weight material, across the range of pH tested, reacted with iodine to form a blue color, confirming the presence of long chains of starch. There are three possibilities: (i) that isoamylase does not hydrolyze all the α-(1,6) linkages, presenting a barrier to the action of β-amylase; (ii) that debranched chains associate, aggregate, or retrograde, restricting the access and activity of β-amylase; or (iii) that β-amylase is much more efficient at digesting short chains, like the amylopectin chains that are completely digested in Figure 5.

The efficacy of hydrolysis of the α-(1,6) branch linkages of HWSF by isoamylase can be measured by ^{1}H NMR, which can distinguish the α-(1,6) and α-(1,4) linkages of starch, with chemical shifts of 5.40 for the α-(1,4) linkages and 5.00 for α-(1,6) linkages.[20] In Figure 7a,c, and e, the ^{1}H NMR spectra of HWSF show that branching frequency decreases with increasing amylose content (5.6% for Shimizu Mochi, 2.5% for Amaroo, and 1.3% for Doongara). Upon debranching of the HWSF with isoamylase, the α-(1,6) peak disappears altogether, suggesting complete hydrolysis of all branch linkages (Figure 7b,d, and f). However, the lower limit of ^{1}H NMR detection of α-(1,6) linkages was found to be 0.5% (equating to 1 in 200 linkages), which is not sufficient to confirm complete hydrolysis of α-(1,6) linkages.

Retrogradation, entanglement, and association occur immediately after preparation of amylose solutions,[11,13,32] and these have the potential to decrease the efficiency of isoamylase or β-amylase. Debranching of the HWSF (containing both amylose and amylopectin) theoretically produces chains that range from DP 6 (the shortest amylopectin chain[33]) up to several thousand.[14] The rate of retrogradation can be minimized,[32] but rates of aggregation increase for chains around DP 50, and for small molecules (e.g., DP ≈ 110).[32,34] This aggregation can be due either to chain entanglement, or to hydrogen bonding, between chains on different molecules (the latter is termed "association" for this purpose). Association is favored enthalpically, but entropic considerations show that dissociation of both aggregates and enthalpically associated chains must be favored if the solution is sufficiently dilute. This process can be quite slow: for example, it is well-known that dissolution of long branched chains can take many days, an observation which can be rationalized in terms of reptation theory.[35,36] Thus, it is essential that the rate of this dissociation is over a reasonable time and temperature.[32] Here the SEC data were collected daily over 7 days at 50 °C. Normalized SEC traces of fresh and disassociated HWSF and debranched HWSF are shown in Figure 8. Comparison of the SEC traces of fresh and disassociated HWSF (Figure 8a) indicates that the earlier elution of the fresh HWSF suggests some association in this sample. In contrast, the debranched HWSF (Figure 8b) showed no detectable evidence of association in the original sample. However, as isoamylase can hydrolyze the densely located branch linkages of amylopectin, and provided any associations do not involve the branch points, association might not affect isoamylase. The exo-

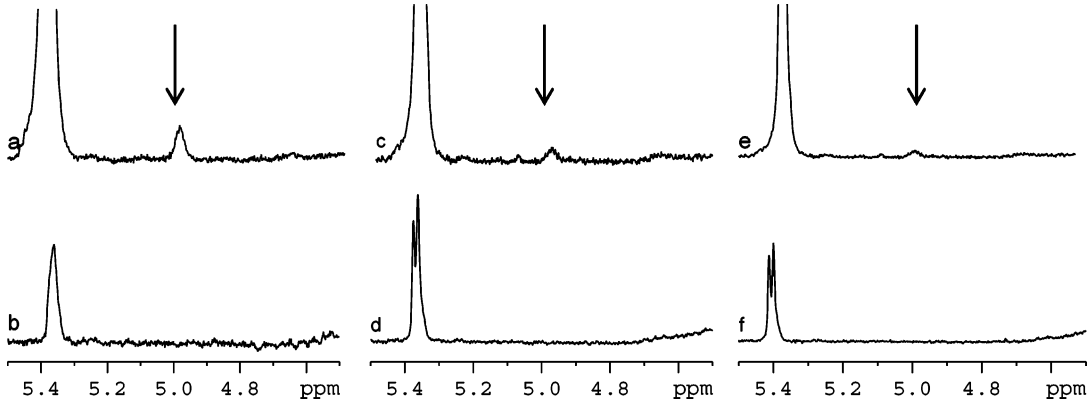

Figure 7. [1]H NMR spectra of HWSF (first row) and debranched HWSF (second row) from three varieties: Shimizu Mochi (panels a and b), Amaroo (panels c and d), and Doongara (panels e and f). The chemical shift for α-(1,4) linkages is 5.40, and 5.00 for α-(1,6) (as denoted by the arrows).[20]

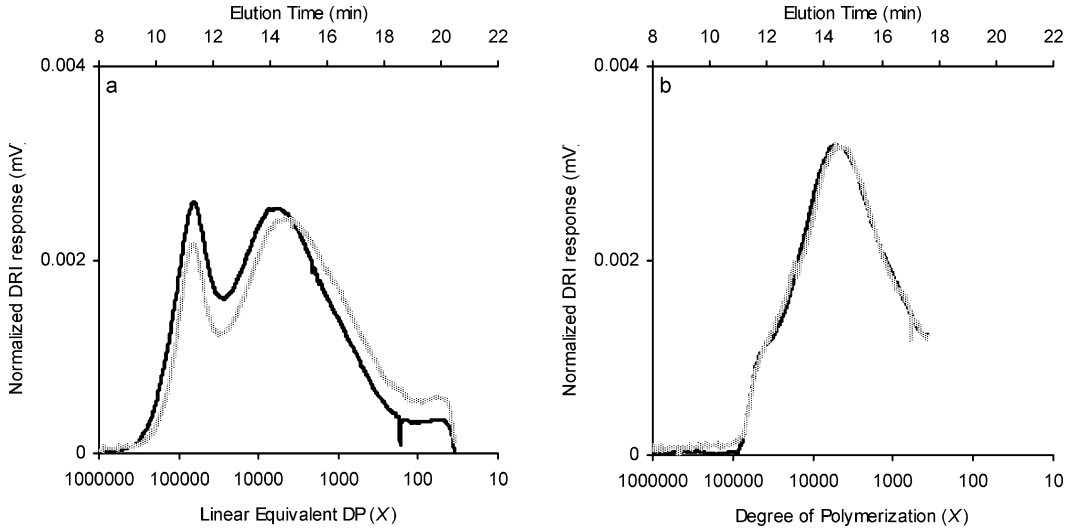

Figure 8. Association tests of the HWSF from Doongara flour 1.96% (w/w) in the native (a) and debranched (b) state. Normalized SEC traces of the fresh HWSF (thick line) and the diluted trace (thin line) are presented.

hydrolyzing β-amylase should be free to hydrolyze the disassociated debranched HWSF.

Figure 5 shows that β-amylase is much more able to digest shorter chains than longer ones, either because the shorter chains associate less,[37] or because the shorter chain length provides a more suitable substrate for β-amylase. The addition of α-amylase to a solution of debranched chains will hydrolyze α-(1,4) linkages randomly and decrease the average chain.[38] SEC traces of these digestions are shown in Figure 9. For each sample, including the negative control (no starch added), a peak eluted between 16 and 20 min (Figure 9). The material reacted with iodine to form a yellow color, confirming absence of starch, and it absorbed strongly at 205 and 280 nm, indicating the presence of protein, probably residual enzyme. Thus, β-amylase was able to digest all of the short chains in the α-amylase-treated debranched solution, either because short chains provide a more suitable substrate or because short chains associate and aggregate more slowly.[37]

The diversity of techniques employed here largely explains why the question of complete debranching of amylose by isoamylase has remained unanswered. To summarize, pH does not affect isoamylase efficiency, [1]H NMR used in isolation is not sufficient to answer this question, and β-amylase cannot digest debranched HWSF without a prior or concurrent incubation with α-amylase to decrease the size of chains. Taken together, these results strongly suggest that isoamylase does indeed completely debranch the starch in the HWSF of flour.

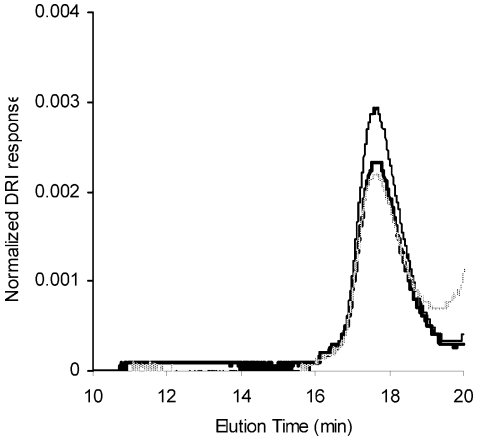

Figure 9. Normalized SEC traces of the HWSF of Doongara (1.96% (w/w) slurry) digested by isoamylase, then α-amylase, and finally β-amylase, at pH 6 (bold line), at the correct pH for each enzyme (dashed line), and a blank of the three enzymes at pH 6 (thin line). These SEC traces were obtained from the Shimadzu SEC system.

Characterizing Starch in the HWSF from Flour. The amorphous nature of amylose allows it to be relatively readily leached from the granules,[39,40] and many studies that separate amylose from starch capitalize on this feature.[23,39,41] Purification of starch from flour carries the risk of damaging and losing amylose molecules;[22] this would make qualitative and quantita-

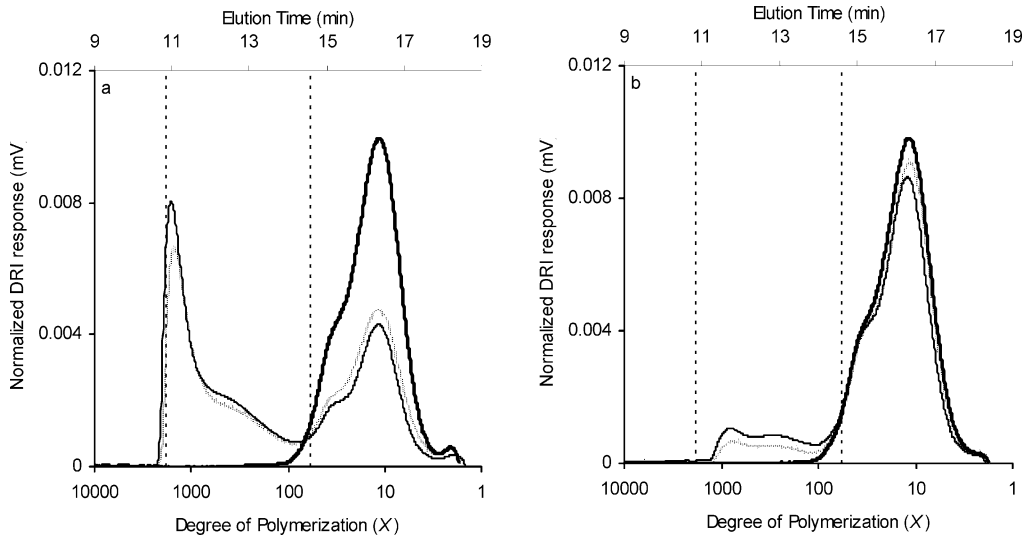

Figure 10. SEC distributions of debranched starch in the (a) HWSF of flour and (b) HWIF of flour of three varieties separated on a UH250 column: Shimizu Mochi (thick line), Amaroo (dotted line), and Doongara (thin line). These SEC traces were obtained from the Waters SEC system, and the vertical dashed lines show the limit of calibration for the UH250 column.

tive interpretation impossible. Here, we attempt to leach an amylose-rich fraction from flour, thus avoiding the long time and other problems encountered when purifying starch from rice flour. The associated lipid−amylose complexes that may be leached will not contribute to the gel/paste of the cooked rice and as such not affect the eating quality, the investigation of which will in the future make use of the methods developed in the present paper. Figure 10 shows the debranched HWSF and HWIF from each variety separated on the UH250. This column elutes amylose chains in the void volume and separates amylopectin chains.[16] Figure 10 also shows that for the waxy variety, all the amylopectin chains elute below DP 100. Figure 10a shows that all the chains greater than DP 1000 are in the HWSF, many of the chains in the range of DP 100−1000 are in the HWSF, and some chains on the order of amylopectin are in the HWSF. Figure 10b shows that the HWIF contains no chains greater than DP 1000, but it does contain chains in the range of DP 100−1000. Thus, the data indicate that some amylopectin is present in the HWSF and that some short amylose chains, or very long amylopectin chains, are within the HWIF. While it is possible for the long chains in the HWIF to be covalently linked to amylopectin molecules, given that amylose chains could be formed by extension of amylopectin chains,[42] we first assume that they are derived from amylose.

To compare the separation of amylose from rice flour, the amount of amylose in the HWSF requires quantification. To do this, the yield of the dried HWSF of flour is measured, and the amylose within this fraction is determined by integrating the total intensity in the SEC traces (see, e.g., ref 43 for an explanation as to why this yields the mass of chains). The chains between DP 100 and 1000 in Figure 10b are assumed to be (short) amylose chains for this calculation. With Amaroo as the example from Table 3, 6.7% of flour comprises amylose in the HWSF, and 7.2% of flour comprises short chains of amylose in the HWIF. In the only comparable study known to the authors, the yield of the HWSF from a starch source ranged from 4-6%, and amylose within this HWSF accounted for only 7−35%;[23] this equates to less than 2% of the starch being amylose in the separated fraction. So while there is slightly more amylose in the HWIF than HWSF using this method, the yield of amylose separated from flour is three to four times higher than that reported elsewhere.[23] Reasons to explain this discrepancy lie in the methodology of the earlier study. Specifically,

Table 3. Proportions in Total Rice Flour[a]

	% total rice flour		
variety	HWSF	amylose in HWSF	amylose in HWIF
Shimizu Mochi	10.8	0.2	0.0
Amaroo	13.9	6.7	7.2
Doongara	17.4	9.7	11.1

[a] Proportion of flour as (a) HWSF, (b) amylose in the HWSF, and (c) amylose in the HWIF, for the three varieties prepared from a 1.96% (w/w) rice slurry.

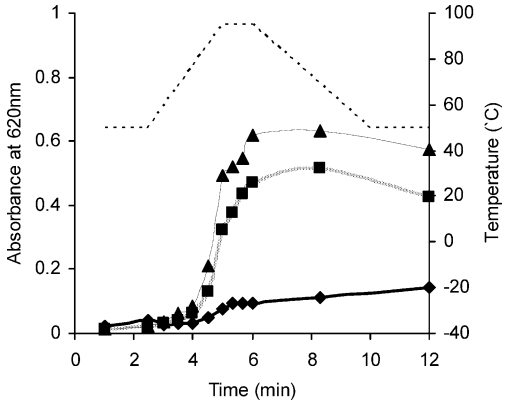

Figure 11. Absorbances of starch−iodine complexes from HWSF obtained across the entire RVA temperature profile (dashed line). The HWSF (1.96% (w/w)) were obtained from three varieties, Shimizu Mochi (diamonds), Amaroo (squares), and Doongara (triangles). The collection regime for this figure is outlined in Table 1.

(a) amylose was extracted by the butanol method from a starch, which was previously prepared by alkaline precipitation,[23] both cause damage to starch, and (b) the leaching temperature of 80 °C was below the critical temperature of 85 °C,[6] which would reduce the amount of amylose extracted.

Assuming that the chains from DP 100−1000 in the HWIF are derived from amylose, we attempted to recover those chains into the HWSF. Figure 11 shows the absorbance at 620 nm of the HWSF from the three varieties (1.96% concentration) taken from twelve different samples, run to different stages of the RVA profile. Figure 11 shows that amylose only begins to solubilize at about 80 °C for both Amaroo and Doongara,[6] despite a difference between the two varieties of 10 °C in gelatinization temperature (Table 4); amylopectin also started to solubilize at that temperature, suggesting that it would be

Table 4. Amylose Content, Protein Content, and Gelatinization Temperature of the three varities used in this study—Shimizu Mochi, Amaroo, and Doongara

	amylose content (%)	protein content (%)	gelatinization temperature (°C)
Shimizu Mochi	0	8.0	66.0
Amaroo	19	6.5	64.7
Doongara	24	6.7	73.1

Table 5. Absorption Maxima, λ_{max} (nm), of Iodine Complexes[a]

λ_{max} (nm)	flour	HWSF 3.85%	HWSF 1.96%	HWIF 1.96%
Shimizu Mochi	plateau at 505	no plateau	no plateau	521
Amaroo	583	613	610	563
Doongara	601	620	621	583

[a] Complexes with starch in the rice flour, and the starch within the HWSF at two concentrations, and HWIF of flour prepared at the lower rice slurry concentration.

difficult to avoid amylopectin contamination in the HWSF. The amount of amylose (and amylopectin) that solubilizes continued to increase until 95 °C, at which point, starch granules lose structure.[44] For Amaroo and Doongara, λ_{max} falls slightly after the plateau, but it continues to increase for the waxy variety. Together, the data suggest either that the decrease in absorbance beyond the plateau for the non-waxy varieties is due to dilution of the amylose, as the amylopectin continues to solubilize, or that, during the cooling phase, some of the amylose precipitates and joins the HWIF. If the latter is true, it is curious that, for both varieties, only short chains precipitate (Figure 10). The data indicate that the optimal time to stop the RVA run is at 7 min (of the AACC standard 61−02) in order to minimize amylopectin and maximize amylose in the HWSF.

Contamination of the HWSF by amylopectin seems inevitable, regardless of the separation technique.[6,12,14,19] There is a range of sizes of amylopectin molecules,[45] and perhaps the smaller ones (DP 700−2100) solubilize more readily. Different speeds of centrifugation did not alter the amount of soluble amylopectin in the HWSF (data not shown). All other samples were centrifuged at 10^4 *g*, and other ways to maximize amylose in the HWSF were investigated.

Two concentrations of flour from each variety were tested to try and increase the proportion of amylose in the HWSF. The most conventional and simplest way to determine the compositions of the HWSF and the HWIF is to measure the maximum absorbance (λ_{max}) of the complex between starch in these fractions and iodine. The λ_{max} of this complex can be used to differentiate between amylose and amylopectin in the starch.[46−48] Table 5 shows the λ_{max} of the starch−iodine complex from gelatinized flour, from the HWSF, and from the solubilized HWIF of flour for all varieties and both concentrations. Amylose content is routinely measured at 620 nm (AACC 61−03).[27] Table 5 shows that λ_{max} of the starch−iodine complex from gelatinized non-waxy flour is ~590 nm and for the amylopectin−iodine complex from Shimizu mochi flour is 505 nm. Table 5 also shows that the maximum absorbance of HWSF is close to that of amylose (~615 nm) and that of HWIF is closer to that of amylopectin (~573 nm). These data indicate that the HWSF is amylose-rich and the HWIF is amylose-poor. Concentration does not affect λ_{max}, suggesting that concentration did not affect the proportion of amylose in the HWSF. SEC was used to analyze the hydrodynamic volume distribution of each fraction.

Having shown that chromatograms of the molecules within the HWSF are reproducible and representative of the amylose (if not the amylopectin), the amylose in the HWSF can be examined with confidence. The chromatograms in Figure 12 show the bimodal SEC traces of the HWSF of flour, as separated by the UH500. The elution profile of Shimizu mochi shows that amylopectin elutes first, and comparison with the other two varieties indicates that the broader second peak must be amylose (with a small amylopectin tail). In fact, upon debranching, the narrow amylopectin peak is removed with all chains eluting below DP 100 (Figure 13). For all three varieties, the HWSF from the high concentration (3.85%) samples contained much more amylopectin than did the HWSF from the lower concentration (1.96%), even though some of the amylopectin molecules are removed during SEC. During preparation of the samples of the higher concentration, some resistance to the stirring of the

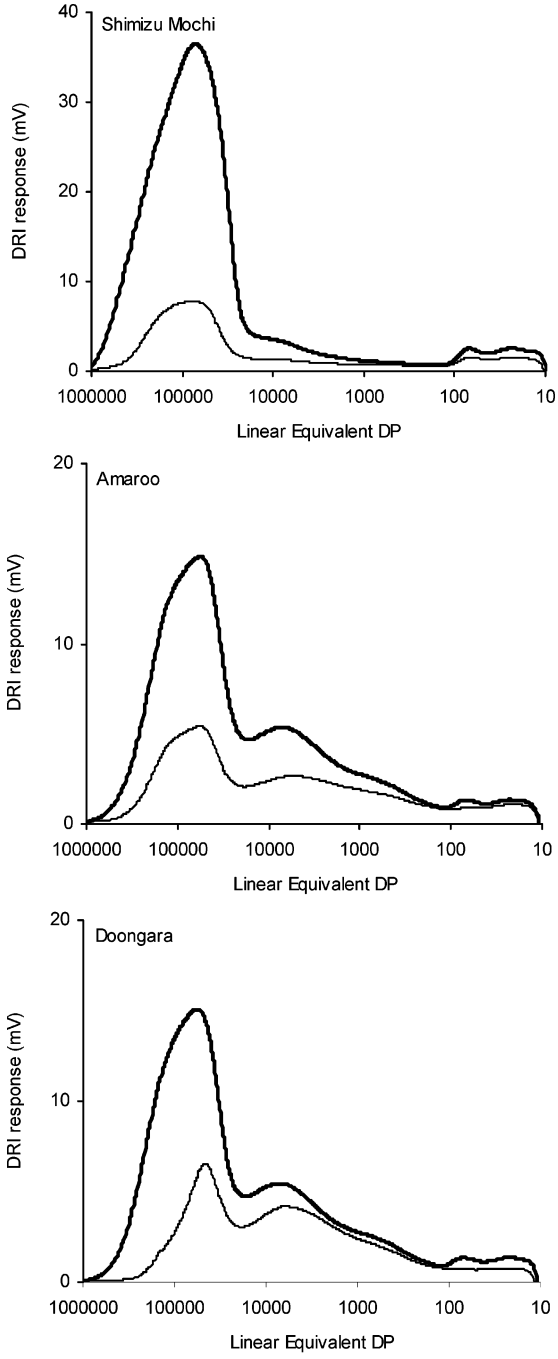

Figure 12. SEC traces of the HWSF of flour of Amaroo, Doongara, and Shimizu Mochi rice flour separated on a UH500 column at two concentrations: 1.96% (thin line) and 3.85% (thick line). These SEC traces were obtained from the Waters SEC system.

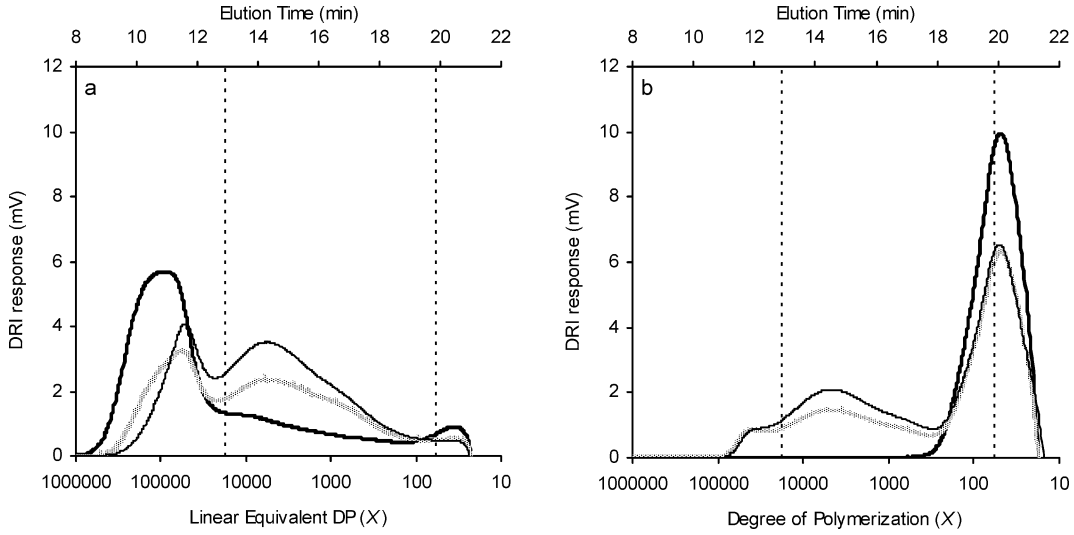

Figure 13. SEC traces of (a) native HWSF from the 1.96% (w/w) preparation and (b) molecular weight distributions of these HWSF, completely debranched by isoamylase, as separated by the UH500 column using the Waters system. Three varieties are presented Shimizu Mochi (thick line), Amaroo (dotted line), and Doongara (thin line), and the calibration limits of the Waters system used here are also presented.

paddle was noted (data not shown), suggesting that the samples experienced shear.[40] Shear assists the removal of outer layers of starch granules as they "cook",[44] and at the higher concentration, greater interaction between molecules probably promoted more solubilization of the outer layers of the granule. Thus, an amylose-rich fraction can be separated from flour, and concentration of flour and degree of heating can be manipulated to minimize amylopectin and maximize amylose in the fraction.

The amylose-rich HWSF, both complete and debranched, for each variety was separated by the UH500 (Figure 13) (in which the abscissa show the equivalent molecular weight of linear starch with the same hydrodynamic volume; Figure 13a). For native amylose, the weight average molecular weight for the HWSF can only be determined by MALLS (Table 2), and the number average cannot be determined by either SEC or MALLS. From the SEC data, the intensity of the DRI response in the amylose region of the native HWSF trace directly reflects the amylose content of the varieties (e.g., Doongara has both the highest intensity and the amylose content). The $\bar{X}_w$ value for Doongara obtained by MALLS reported in Table 3, 7.5×10^5, is much larger than previous reports of 3000 for rice.[12,49,50] Post-SEC values for $\bar{X}_w$ are larger than those of other starches, 7.4×10^3 for cassava to 5.3×10^3 for maize.[9] The smallest sub-fractions of native starch can range from 360 to 840, while the largest ranges between 1.25×10^4 and 5.2×10^3.[51] Reasons for this discrepancy are discussed below.

The HWSF is completely debranched by isoamylase to produce linear starch chains and separated by a UH500. Here, the broad peak between DP 10^5 and 100 is the amylose peak, while the amylopectin elutes in the narrow peak lower than DP 100. The SEC distribution has not been corrected for band broadening, and although this is sufficiently small in modern SEC systems so as not to affect averages significantly, detailed shapes within the distribution may be affected.[5,52] SEC distributions of debranched HWSF are summarized in Figure 13b, and associated weight and number average degrees of polymerization ($\bar{X}_w$ and $\bar{X}_n$) and polydispersity ($\bar{X}_w/\bar{X}_n$) are shown in Table 6. Thus for debranched amylose, MALLS data are not required. An $\bar{X}_w$ value of ~360 reported[12] in an earlier study of debranched amylose was determined by the modified Park–Johnson method and anthrone–sulfuric acid method[4] and can be directly compared to the $\bar{X}_n$ value of ~1800 reported here. The $\bar{X}_w$ value for debranched HWSF was found to be 0.61×10^4 for Amaroo and 1.26×10^4 for Doongara. There are no reports on an $\bar{X}_w$ value for debranched amylose from rice; however it has been reported that the $\bar{X}_w$ value from other starch sources can range from 1.03×10^3 to 4.28×10^3.[24]

Table 6. $\bar{X}_n$, $\bar{X}_w$, and Polydispersity (Q) for Debranched HWSF of Flour (1.96% (w/w) from the Nonwaxy Varieties

HWSF	$\bar{X}_n$	$\bar{X}_w$	$Q = \bar{X}_w/\bar{X}_n$
Amaroo	1.82×10^3	0.61×10^4	3.4
Doongara	1.78×10^3	1.26×10^4	7.1

It is clear that a combination of sample preparation and advances in the understanding of the MWD theory can explain the discrepancies noted above. Amylose from previous studies was sourced from the precipitation of amylose from starch, after the starch itself was prepared from flour. It has been shown that incubation of flour in an alkaline solution,[22] as part of the alkaline precipitation method, causes a significant decrease in the MWD of the amylose. In addition, the $\bar{X}_n$ and $\bar{X}_w$ for debranched amylose quoted here may be slightly biased toward higher molecular weights, as the method to obtain an amylose-rich fraction preferentially leaches the larger amylose molecules, though it cannot be confidently assumed that the molecules of DP 100–1000 in the HWIF are derived from amylose.

Conclusions

The present paper reports improvements in key experimental techniques and analytical instrumentation for obtaining the most accurate hydrodynamic volume distribution for molecules, and the MWD for debranched amylose. It is shown that the common practice of referring to molecular weights relative to pullulan greatly underestimates the actual molecular weight of the starch, and this practice can lead to errors. Analysis of an amylose-rich fraction by SEC, using conditions typical of those used widely in the literature, showed a 20% loss of carbohydrate. By colorimetric analysis of the starch–iodine complexes and by determining $\bar{X}_w$ by MALLS, this loss was found to be predominately from residual amylopectin found in the amylose-rich fraction. Means of avoiding this loss are an important area for future work. With conditions used by many workers, it was shown that isoamylase does in fact hydrolyze all α-(1,6) branch linkages in the amylose-rich fraction to produce linear, α-(1,4)

linked amylose chains. Amylose was separated directly from flour to avoid the starch degradation experienced by previous methods. By using RVA, a separation method was developed to extract an amylose fraction directly from flour, without causing any degradation to the starch. The $\bar{X}_w$ value for native amylose from Doongara is much larger than previous reports for rice. Similarly, the $\bar{X}_w$, $\bar{X}_n$, and polydispersity of debranched HWSF were also much larger. The combination of improved sample preparation and data treatment has yielded what are regarded as the most reliable MWDs of the amylose-rich fraction of rice flour.

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