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The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains

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ABSTRACT

Statistically and causally meaningful relationships are established between starch molecular structures (obtained by size-exclusion chromatography, proton NMR and multiple-angle laser light scattering) and digestibility of cooked rice grains (measured by *in vitro* digestion). Significant correlations are observed between starch digestion rate and molecular structural characteristics, including fine structures of the distributions of branch (chain) lengths in both amylose and amylopectin. The *in vitro* digestion rate tends to increase with longer amylose branches and smaller ratios of long amylopectin and long amylose branches to short amylopectin branches, although the statistical analyses show that further data are needed to establish this unambiguously. These new relationships between fine starch structural features and digestibility of cooked rice grains are mechanistically reasonable, but suggestive rather than statistically definitive.

1. Introduction

Rice (*Oryza sativa* L.) is one of the mostly grown food crops in the world and an important staple food for more than half of the world population. A better understanding on the digestibility of starch in cooked rice grains is important because of the rapid rise of diet-related health complications, particularly obesity, type 2 diabetes, and colorectal cancers. Foods containing starch that is slowly digested to glucose, and where significant quantities reach the lower gut ("resistant starch"), can mitigate, and also prolong the onset of, these diseases.

Starch structure has a bearing on starch digestibility. Starch comprises two types of molecules: amylopectin (Ap) and amylose (Am). Ap is a group of highly branched glucose polymers with a vast number of short branches and molecular weight $\sim 10^{7-8}$, whereas Am has a smaller molecular weight ($\sim 10^{5-6}$) with few long branches. Several studies have demonstrated that the digestibility of rice starch (both isolated starch and that in the grains) is associated with Am content (Chung, Liu, Wang, Yin, & Li, 2010; Frei, Siddhuraju, & Becker, 2003; Zhu, Liu, Wilson, Gu, & Shi, 2011). These studies used rice starches with wide ranges of Am contents (e.g. 1.7% to 55.4% Am), allowing the relationship between starch digestibility and Am content to be easily observed, but not the fine structures; there do not seem to be any literature data on the effects of Am fine structures (branching structure and molecular size) on starch digestibility. The digestibility of starch in grains may also be affected by non-starch components, such as protein and cell-wall matrices which can entrap starch granules, and lipids which can form complex with Am.

The objective of this study is to obtain a mechanistic understanding of the relationship between starch structures and digestibility of cooked rice grains. Since the starch granular and crystalline structures are greatly disrupted by the cooking process, only grain composition and starch molecular structures will be considered here. The molecular structures are the molecular size distributions of individual branches (i.e. debranched starch) – generally termed the chain-length distribution (CLD) – of Am and Ap, the molecular size distributions of whole (fully branched) starch, degree of branching (DB) of starch molecules, and the average molecular size of whole starch. Structural characterisation was performed using size exclusion chromatography (SEC) with refractive index (RI) detection, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, and offline multi-angle laser light scattering (MALLS) (i.e. without size separation). Offline MALLS analysis is necessary because shear scission of whole Ap molecules is unavoidable during SEC separation (Cave, Seabrook, Gidley, & Gilbert, 2009). The rice grain varieties chosen for the present study mainly have a narrow range of Am contents, allowing correlations with fine molecular structures that are normally concealed when other structural differences, such as Am content, are much more pronounced. Digestibility of cooked rice grains is determined from a common

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in vitro method and the digestion profile is fitted to first-order kinetics to obtain a digestion rate coefficient. The results can be used to understand the importance of starch structures in determining various nutritional properties of cooked rice grains.

2. Materials and methods

2.1. Materials

Fourteen polished rice grain samples, which were four Malaysian and ten Cambodian rice varieties, were chosen based on the consumer popularity in both countries. The use of a large number of rice varieties is necessary to obtain the correlations between starch structures and digestibility that are statistically valid and reliable. Since the rice grain samples were collected from various locations in both countries, they allow the observation of the effects of different geographical and environmental factors on the starch structures and digestibility. The complete list of rice samples with their origins and suppliers is summarised in Table 1.

Dimethyl sulfoxide (DMSO, GR grade for analysis) was purchased from Merck Co. Inc. (Kilsyth, VIC, Australia). LiBr (ReagentPlus), DMSO-d_6 (99.5% atom D), and trifluoroacetic acid-d_1 (TFA-d_1, 99% atom D) were purchased from Sigma–Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Total Starch (AA/AMG) assay kit and isoamylase from *Pseudomonas* sp. were purchased from Megazyme International Ltd. (Bray, Ireland). A series of pullulan standards with peak molecular weights from 342 to 2.35×10^6 were from Polymer Standards Service GmbH (PSS, Mainz, Germany). Other chemicals were reagent grade and used as received.

2.2. Cryogenic milling of rice grains

Rice grains were ground into flour using a cryogenic mill (Freezer/Mill 6850; SPEX, Metuchen, NJ) in a liquid nitrogen bath as the cryogenic medium. Ground rice flour was used to analyse the chemical composition of rice grains and to characterise the molecular structures of native starch in the rice grains. The mill was set to run at 10 s^{-1} and the milling process was carried out in 2 cycles of 5-min grinding with a 2-min re-cooling break in between. The resulting flour was sieved through a 250-μm screen and kept in a desiccator for subsequent analyses.

2.3. Composition of rice grains

The starch content of rice grains was analysed from the ground rice flour using the Megazyme Total Starch (AA/AMG) assay kit. The crude protein content of rice grains was calculated from the nitrogen content of ground rice flour, obtained using a LECO CNS2000 auto analyzer (LECO Corporation, St. Joseph, MI) (Jung et al., 2003), with a conversion factor of 5.95 (Breese, 1941). The crude lipid content was measured by Soxhlet extraction, following AOAC method 920.39C (AOAC, 2002). All measurements were performed in duplicate.

2.4. Starch extraction from rice grains

The extraction and dissolution of starch molecules from ground rice flour was performed following a method described elsewhere (Syahariza, Li, & Hasjim, 2010; Tran et al., 2011), which uses a combination of protease, sodium bisulfite, DMSO with 0.5% w/w LiBr (DMSO/LiBr), and ethanol solutions to completely dissolve starch molecules and remove non-starch components, i.e. proteins, lipids, and non-starch polysaccharides, with no or minimal degradation of starch molecules. This method is a better alternative to the starch extraction from rice grains using alkaline solution, as basic pH is a catalyst for starch hydrolysis, especially when heating and mixing are involved (Han & Lim, 2004; Kim, Huber, & Higley, 2006). The extracted starch in DMSO/LiBr solution was stored at room temperature for subsequent analysis by SEC and offline MALLS detector. For NMR analysis, DMSO-d_6 was used instead of DMSO/LiBr solution to dissolve the freeze-dried starch sample, and TFA-d_1 was added to the sample medium right before the NMR analysis to improve ^{1}H signals (Tizzotti, Sweedman, Tang, Schaefer, & Gilbert, 2011).

2.5. Molecular size distributions of whole branched and debranched starches and amylose content

The structure of extracted whole starch molecules was characterised using an Agilent 1100 Series SEC system (Agilent Technologies, Waldbronn, Germany) equipped with GRAM 30 and 3000 analytical columns (PSS) and an RI detector (RID-10A, Shimadzu Corp., Kyoto, Japan) following the method described elsewhere (Cave et al., 2009; Liu, Halley, & Gilbert, 2010). The structure of starch enzymatically debranched using isoamylase, as described elsewhere (Hasjim, Lavau, Gidley, & Gilbert, 2010; Tran et al., 2011), was also characterised using the same SEC system, but using GRAM 100 and 1000 analytical columns (PSS).

The molecular size distribution of whole starch was plotted as weight distribution, $w_{br}(\log R_h)$, against hydrodynamic volume, V_h (the separation parameter for SEC), or the equivalent hydrodynamic radius, R_h. For whole starch molecules, as for any branched

Table 1
Sources and chemical compositions of rice grain samples.*

Variety	Abbreviation code	Supplier/Province	Country of origin	Total starch (%)	Total crude protein (%)	Total crude lipid (%)
MR84	84	MARDI	Malaysia	81.4 ± 0.8^{a}	8.80 ± 0.6^{a,c}	0.34 ± 0.2^{b,c}
MRQ74	74	MARDI	Malaysia	77.6 ± 1.4^{a}	10.26 ± 0.3^{a}	0.16 ± 0.1^{c}
MR220	220	MARDI	Malaysia	79.1 ± 3.3^{a}	6.61 ± 0.1^{c,d}	0.26 ± 0.0^{b,c}
MR219	219	MARDI	Malaysia	76.3 ± 2.3^{a}	7.16 ± 0.1$^{a–d}$	0.43 ± 0.1^{b,c}
Phka Kagney	PK	CARDI	Cambodia	86.4 ± 2.8^{a}	7.28 ± 0.3$^{b–d}$	0.41 ± 0.2^{b,c}
Phka Malis	PM	CEDAC	Cambodia	81.7 ± 2.9^{a}	6.71 ± 0.9^{c,d}	0.54 ± 0.1^{b,c}
Neang Minh	NM	Battambang	Cambodia	79.1 ± 0.2^{a}	7.40 ± 0.0$^{b–d}$	0.39 ± 0.0^{b,c}
Neang Khon	NK	Battambang	Cambodia	81.5 ± 0.7^{a}	6.72 ± 0.1^{c,d}	0.24 ± 0.0^{b,c}
Somali	SM	Battambang	Cambodia	79.0 ± 1.5^{a}	9.13 ± 0.2^{a,c}	0.47 ± 0.1^{b,c}
Sen Pidoa	SP	Battambang	Cambodia	80.8 ± 0.4^{a}	8.95 ± 0.6^{a,c}	0.46 ± 0.1^{b,c}
IR66	IR66	Kampot	Cambodia	81.1 ± 4.0^{a}	6.08 ± 0.1^{d}	0.21 ± 0.0^{b,c}
CAR9	CAR9	Kampot	Cambodia	75.5 ± 1.6^{a}	9.33 ± 1.6^{a,b}	1.01 ± 0.0^{a}
Bei Katam	3K	Kampong Speu	Cambodia	82.5 ± 0. 3^{a}	8.06 ± 0.8$^{a–d}$	0.32 ± 0.2^{b,c}
Phka Rumduol	PR	CARDI	Cambodia	76.7 ± 8.1^{a}	8.03 ± 0.4$^{a–d}$	0.61 ± 0.1^{a,b}

MARDI, Malaysian Agricultural Research and Development Institute; CARDI, Cambodian Agricultural Research and Development Institute; CEDAC, Centre d'Etude et de Développement Agricole Cambodgien.

* Mean ± SD is calculated from duplicate measurements. Values with different letters in the same column are significantly different with $p < 0.05$.

polymer, there is no unique relation between size (V_h or R_h) and molecular weight. On the other hand, for linear polymers such as debranched starch, there is a unique relation between size and molecular weight, or equivalently degree of polymerisation (DP), X. Therefore, the molecular size distribution of debranched starch, or CLD, is presented as both weight distribution, $w_{de}(\log R_h)$ and number distribution, $N_{de}(X)$, the latter as $\ln N_{de}(X)$; the different ways of presenting the same information bring out different features of the distribution (Castro, Dumas, Chiou, Fitzgerald, & Gilbert, 2005), which emphasise the differences in the distributions of a certain branch population among samples that are not obvious in a different way of plotting. For example, differences in the Ap branches are best observed from the weight distribution, whereas those in the Am branches are best observed from the number distribution. Details of data treatment are in Supplementary data. All SEC molecular size distributions were normalised to yield the same height of the highest peak for comparisons among different rice varieties.

Due to unavoidable problems of SEC separation of whole Ap molecules, such as shear scission, lack of high molecular-weight standards for calibration in the Ap size range, the separation limit of SEC columns, and low recovery (Cave et al., 2009; Vilaplana & Gilbert, 2010a), the apparent distribution of the Ap fraction in the SEC weight molecular size distribution of whole starch cannot be used to make structural inferences.

The fine molecular structures of Ap and Am branches are reported as the DP at each peak maximum (two for Ap peaks denoted by X_{Ap1} and X_{Ap2}, and two for Am peaks denoted by X_{Am1} and X_{Am2}) and the peak height of each peak maximum as the ratio to the height of the first Ap peak maximum (denoted by $h_{Ap2/Ap1}$, $h_{Am1/Ap1}$ and $h_{Am2/Ap1}$).

The Am content of rice starch was determined from the SEC weight molecular size distribution of debranched starch as the ratio of the area under the curve (AUC) of Am branches to the AUC of overall Ap and Am branches (International Standardization organization, 2011; Vilaplana, Hasjim, & Gilbert, 2012); comparisons with the results from iodine colorimetric and Concanavalin A methods are given in the Supplementary data.

2.6. Degree of branching

The DB of whole starch molecules, defined as the percentage of α-(1 → 6) glycosidic linkages (branching points) to the total of both α-(1 → 4) and α-(1 → 6) glycosidic linkages, was determined using ^{1}H NMR following the method of Tizzotti, Sweedman, Tang, Schaefer, and Gilbert (2011).

2.7. Weight-average molecular weight and average radius of gyration of whole starch molecules

The weight-average molecular weight ($\overline{M}_w$) and z-average radius of gyration ($\overline{R}_g$) of whole starch molecules were analysed in duplicate using an offline MALLS detector (BI-MwA, Brookhaven Instrument, Corp., Holtsville, NY) in "batch mode" (without size separation) at ambient temperature. Details of sample preparation and data interpretation of the Berry plot to obtain ($\overline{M}_w$) and ($\overline{R}_g$) are included in the Supplementary data.

2.8. Preparation of cooked rice

About 100 g of rice grains were cooked using a household rice cooker (Kambrook Rice Express, VIC, Australia) in distilled water with a rice-to-water ratio of 2:3. The cooking process was conducted using the preset cooking setting of the cooker, followed by a 5-min holding period at the warming setting. The cooked rice grains were cooled at room temperature for 1 min followed by grinding using a commercial food blender (Sunbeam Multiblender Pro., NSW, Australia) for 10 s to simulate human chewing. The cooked samples were divided into two equal parts: one for moisture content measurement and the other for *in vitro* digestion. The moisture content was determined from the weight difference before and after drying the cooked rice sample in a convection oven at 110 °C overnight.

2.9. In vitro starch digestibility

In vitro starch digestion was carried out following the method described elsewhere (Al-Rabadi, Gilbert, & Gidley, 2009; Sopade & Gidley, 2009) with a slight modification (Supplementary data). The starch digestibility of cooked rice grains was determined from the amount of glucose released into the supernatant, which was converted to mass of digested starch by a factor of 0.9, and the degree of *in vitro* starch digestion is presented as g/100 g dry starch in the rice grains. The digestogram was fitted to first-order kinetics as shown in Eq. (1):

$$C = 1 - e^{-kt} \quad (1)$$

where C is the fraction of digested starch at digestion time t and k is the digestion rate coefficient. The value of k was obtained from the slope of a linear-least-squares fit of a $\ln(1 - C)$ plot against t. The digestograms are equally well fitted by numerical least-squares fitting of the exact Michaelis–Menten (MM) equation (Dona, Pages, Gilbert, & Kuchel, 2010) to obtain the MM parameters k_m and v_{max}, but because the fit with a single parameter first-order equation is just as good, this MM two-parameter fit contains redundant information.

2.10. Statistical analysis

For each structural measurement, duplicated analysis was performed for each sample. All data were reported as the means ± standard deviations (SD). Similarly, the digestion rate coefficients were analysed in duplicate for each grain sample. One-way analysis of variance (ANOVA) and Pearson as well as Spearman rank correlation methods were carried out using SPSS V. 16.0 software (SPSS Inc., Chicago, IL). The means of duplicated measurements were used for the correlation analysis.

3. Results and discussion

3.1. Grain composition

The compositions of rice grain samples are presented in Table 1. The starch contents of rice grains range from 76% to 86%, with no significant differences among all rice grain samples. The crude protein contents range from 6% to 10% and the crude lipid contents are between 0.2% and 1.0%. There are some significant differences in the crude protein and lipid contents among different rice grain samples. The starch, crude protein and crude lipid contents of rice grains from the present study are in the same ranges as those in the literature (Chung et al., 2010; Juliano & Hicks, 1996).

3.2. Starch molecular structures

Typical SEC weight molecular size distributions, $w_{br}(\log R_h)$, of whole (fully branched) starch from selected rice grain samples are presented in Fig. 1, normalised to the same height of the highest (Ap) peak; those from all rice grain samples are given in the Supplementary data. All samples show typical bimodal distributions of whole starch, consisting of Ap ($R_h > 100$ nm) and Am ($R_h < 100$ nm) peaks with a small shoulder at $R_h \sim 3$ nm, which may be attributed to residual proteins (Syahariza et al., 2010). As

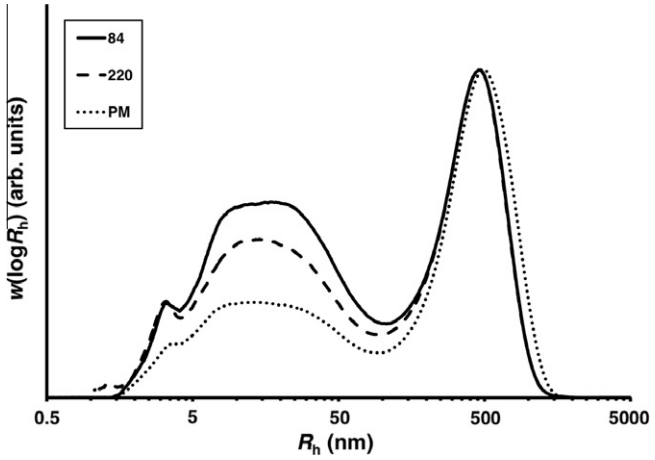

Fig. 1. Weight molecular size distributions of whole starch, $w_{br}(\log R_h)$, extracted from selected rice grain samples as representatives, normalised to yield the same height as the highest peak.

stated, the SEC separation of whole Ap molecules suffers from unavoidable shear scission and calibration problems, so this component of $w_{br}(\log R_h)$ is not considered further. The Am component of the whole molecule distributions was expressed as the value of R_h at the peak maximum and the average R_h, $\overline{R}_h$, of this Am component as defined by Vilaplana and Gilbert (2010b). For the whole Am molecules, there are no significant differences in the R_h at the peak maximum and the $\overline{R}_h$ among different varieties, regardless of the differences in their genetic backgrounds and growing locations (Table 2). This suggests that the whole molecular size of Am in rice grains is unaffected by genetics and environment factors.

There are no significant differences in the $\overline{M}_w$ and $\overline{R}_g$ of whole starch molecules among all samples (Table 2). The $\overline{M}_w$ and $\overline{R}_g$ of whole starch are dominated by the Ap molecules because of their large molecular size. Although the results might suggest that genetic background and growing location are not the determinants for whole starch molecular size, a firm conclusion cannot be drawn from the relatively wide uncertainty in each of these quantities.

A comparison of $\overline{M}_w$ and $\overline{R}_g$ of whole starch molecules in rice grains from the present study with those in the literature is difficult as there is a wide range of $\overline{M}_w$ reported, ranging from 10^6 to 10^9. These differences may be real or artifactual, with different sample preparations, solvent/eluent systems, and extrapolation methods. Degradation of starch molecules may occur during sample preparation when starch is subjected to harsh chemicals, overheating, or excess mechanical energy of milling/mixing, underestimating the actual molecular size (Bello-Perez, Roger, Baud, & Colonna, 1998). Furthermore, aggregates may remain if starch molecules are not completely dissolved, overestimating the molecular size of starch (Jackson, 1991; You & Lim, 2000). Starch can also undergo retrogradation if water or aqueous solution is used as solvent, causing an artefact in the structure characterisation (You & Lim, 2000).

Typical SEC weight molecular size distributions of debranched starch, $w_{de}(\log R_h)$, from selected grain samples are presented in Fig. 2; those from all samples are given in the Supplementary data. The same information is presented in this figure as the CLD, in terms of the number distribution $N_{de}(X)$; these different representations of the same data bring out different features of the distribution. The number distributions of selected grain samples are also presented in Fig. 2 and those for all samples in the Supplementary data. All weight and number distributions are normalised to yield the same height of the highest Ap branch peak.

The weight molecular size distributions of debranched starch from all rice grain samples show two large peaks of Ap branches and two smaller peaks of Am branches. The Ap-branch peaks can be divided into the branches confined to one lamella (Ap1, 0.5 nm < R_h < 2 nm, DP ~ 5–30) and those spanning more than one lamella (Ap2, 2 nm < R_h < 4 nm, DP ~ 30–100) (Vilaplana & Gilbert, 2010b). A feature which is clearly visible and reproducible in the weight molecular size distribution of Am branches (R_h > 4 nm, DP > 100) is the presence of at least two peaks, denoted by Am1 and Am2. While this feature can also be seen in the data reported elsewhere, e.g., Ward, Gao, de Bruyn, Gilbert, and Fitzgerald (2006), it does not seem to have attracted comment. This feature must be due to potentially discrete enzymatic processes of starch biosynthesis in plants. One can speculate on its origin, e.g., different isoforms of granule-bound starch synthase (GBSS) in the amyloplast, responsible for the biosynthesis of Am, but such speculation must await further experiments to elucidate the underlying biosynthetic processes.

Some significant differences are observed in the branch-chain length, X, and the height of each local peak maximum in the fine structures of Ap and Am branches among the different rice varieties (Table 2). The various X at the peak maxima of different groups of Ap and Am branches are denoted by X_{Ap1}, X_{Ap2}, X_{Am1} and X_{Am2}. The X_{Ap1} and X_{Ap2} of SP variety are significantly smaller in size than those of other varieties. Furthermore, there are significant differences in the X_{Am2} among some of the 14 rice varieties. The results suggest that the branching structures of Ap and Am are affected by genetic background and/or growing location.

The heights of local peak maxima of Ap and Am branches in the SEC weight molecular size distribution of debranched starch are presented as the ratios to that of the peak maximum of the short (single-lamellar) Ap (Ap1) branches (denoted by $h_{Ap2/Ap1}$, $h_{Am1/Ap1}$ and $h_{Am2/Ap1}$). The ratios are used to determine the amount of each branch group (Ap1, Ap2, Am1, and Am2) as the relative amount to Ap1 branches. This was obtained by normalising the SEC weight molecular size distribution of debranched starch to yield the same height of the Ap1 peak. The normalisation can be performed using whole AUC or the height of one peak. As long as all distributions are normalised in the same manner, the effect of sample concentration can be eliminated and the amount of each branch population can be meaningfully compared as the relative amount to the whole AUC or to the height of one peak. The height of Ap1 peak was chosen for normalisation because it emphasises the differences of longer (Ap2, Am1, and Am2) branches among different samples and longer branches have been associated with lower starch digestibility (Okuda, Aramaki, Koseki, Satoh, & Hashizume, 2005). The use of peak height, presented as the ratio value, is better than the AUC of the branches because the AUCs of Ap1 and Ap2 peaks are overlapping with each other, as is also the case for those of Am1 and Am2. The peak heights of Am1 and Am2 ($h_{Am1/Ap1}$ and $h_{Am2/Ap1}$) are significantly different among some of the 14 rice varieties. The Am contents determined from the ratio of the AUC of the Am branches to the AUC of the total Ap and Am branches (Table 2) have the same trend as the Am contents obtained by iodine colorimetric and Concanavalin A methods (Table 1 in the Supplementary data), showing significant differences among some of the rice grain samples. However, the Am content calculated from the ratio of the AUC of the Am branches to the AUC of the total Ap and Am branches is higher than those from iodine colorimetric and Concanavalin A methods, possibly due to the incomplete removal of lipids during iodine colorimetric analysis and the co-precipitation of Am by Concanavalin A, underestimating the Am content (Vilaplana et al., 2012). The different heights of the Am-branch peaks among different samples reflect the differences in their Am contents.

The number distribution is presented with two different ranges of X, to show the qualitative differences among the representative samples at shorter DP, $X \leqslant 400$, which are not clearly represented

Table 2
Molecular structures and digestion rates of starch in rice grain samples.*

Variety	Am component of SEC weight molecular size distribution of whole starch distribution		$\overline{M}_w/10^7$	$\overline{R}_g$/nm	DP of peak maximum in SEC weight molecular size distribution of debranched starch				Height of peak maximum in SEC weight molecular size distribution of debranched starch as ratio to Ap1 peak height (SD ~ ± 1.5% for all data)			Am content (%)	DB (%)	$k/10^{-3}$ min^{-1}
	R_h/nm at peak maximum	$\overline{R}_h$/nm			X_{Ap1}	X_{Ap2}	X_{Am1}	X_{Am2}	$h_{Ap2/Ap1}$	$h_{Am1/Ap1}$	$h_{Am2/Ap1}$			
84	17.0 ± 0.1^{a}	8.7 ± 1.3^{a}	3.4 ± 1.5^{a}	166 ± 10.6^{a}	20.1 ± 0.0^{a}	41.2 ± 0.0^{a}	290 ± 50^{a}	934 ± 19^{c}	0.75^{a}	0.23^{a}	0.17^{a}	33.2 ± 0.7^{a}	3.0 ± 0.1$^{c–e}$	6.9 ± 1.2^{b}
74	18.0 ± 0.6^{a}	9.7 ± 0.5^{a}	4.8 ± 3.7^{a}	175 ± 12.7^{a}	18.9 ± 0.2^{a}	40.8 ± 0.3^{a}	317 ± 11^{a}	1064 ± 25^{b,c}	0.66^{a}	0.16^{b}	0.17^{a,c}	30.1 ± 0.9$^{a–d}$	2.9 ± 0.1^{d,e}	5.7 ± 0.4^{b,c}
220	12.4 ± 3.0^{a}	8.0 ± 0.8^{a}	3.7 ± 1.8^{a}	220 ± 43.4^{a}	19.1 ± 0.2^{a}	40.6 ± 0.1^{a}	263 ± 31^{a}	1046 ± 42^{b,c}	0.66^{a}	0.11$^{b–d}$	0.11$^{c–e}$	24.8 ± 0.6$^{c–e}$	3.0 ± 0.0$^{c–e}$	4.8 ± 0.1^{b,c}
219	10.4 ± 0.4^{a}	10.5 ± 0.7^{a}	1.8 ± 0.7^{a}	119 ± 47.4^{a}	19.1 ± 0.3^{a}	40.5 ± 0.0^{a}	244 ± 35^{a}	1061 ± 65^{b,c}	0.70^{a}	0.13^{b,c}	0.13$^{b–e}$	26.3 ± 1.6$^{b–e}$	2.7 ± 0.0$^{c–e}$	5.7 ± 0.9^{b,c}
PK	17.1 ± 2.1^{a}	10.5 ± 0.3^{a}	3.3 ± 1.8^{a}	163 ± 7.0^{a}	19.0 ± 0.0^{a}	40.6 ± 0.1^{a}	304 ± 17^{a}	1124 ± 15^{b}	0.67^{a}	0.13^{b,c}	0.13$^{b–e}$	28.0 ± 0.4$^{a–e}$	3.0 ± 0.1$^{c–e}$	4.8 ± 0.1^{b,c}
PM	16.9 ± 6.3^{a}	8.2 ± 1.9^{a}	4.4 ± 0.3^{a}	167 ± 16.0^{a}	20.2 ± 0.6^{a}	41.2 ± 0.0^{a}	225 ± 14^{a}	1038 ± 6^{b,c}	0.73^{a}	0.09^{c,d}	0.09$^{d–e}$	22.1 ± 1.6^{e}	3.4 ± 0.1^{a,b}	5.5 ± 0.5^{b,c}
NM	18.7 ± 1.8^{a}	10.4 ± 0.8^{a}	2.8 ± 1.2^{a}	129 ± 41.9^{a}	19.4 ± 1.1^{a}	40.7 ± 0.4^{a}	284 ± 16^{a}	1079 ± 7^{b,c}	0.70^{a}	0.15^{b,c}	0.15$^{a–d}$	30.7 ± 0.1^{a,c}	3.0 ± 0.1$^{c–e}$	4.4 ± 0.4^{b,c}
NK	16.6 ± 2.5^{a}	9.3 ± 0.6^{a}	2.6 ± 1.6^{a}	126 ± 43.1^{a}	19.5 ± 0.2^{a}	41.0 ± 0.1^{a}	297 ± 10^{a}	1113 ± 41^{b,c}	0.70^{a}	0.13^{b,c}	0.14$^{b–d}$	28.6 ± 0.4$^{a–e}$	3.2 ± 0.0$^{b–d}$	5.2 ± 0.6^{b,c}
SM	13.4 ± 6.0^{a}	9.7 ± 1.0^{a}	2.5 ± 1.1^{a}	169 ± 8.5^{a}	19.6 ± 0.3^{a}	40.9 ± 0.1^{a}	258 ± 51^{a}	1199 ± 74^{a,b}	0.71^{a}	0.11$^{b–d}$	0.11$^{c–e}$	24.0 ± 1.9^{d,e}	3.2 ± 0.1^{b,c}	4.8 ± 1.1^{b,c}
SP	17.2 ± 0.7^{a}	10.2 ± 1.9^{a}	3.4 ± 0.6^{a}	143 ± 7.8^{a}	15.1 ± 0.6^{b}	33.1 ± 0.2^{b}	335 ± 35^{a}	1087 ± 54^{b,c}	0.73^{a}	0.13^{b,c}	0.09$^{b–d}$	27.6 ± 1.7$^{a–e}$	3.0 ± 0.1^{a,c}	5.9 ± 1.1^{b,c}
IR66	14.7 ± 0.4^{a}	9.8 ± 0.5^{a}	3.8 ± 1.3^{a}	143 ± 8.5^{a}	20.4 ± 1.4^{a}	40.9 ± 0.1^{a}	307 ± 1^{a}	1087 ± 77^{b,c}	0.78^{a}	0.17^{b}	0.18^{a,b}	32.3 ± 4.8^{a,b}	2.7 ± 0.1^{e}	3.7 ± 0.5^{b,c}
CAR9	17.1 ± 0.4^{a}	9.9 ± 2.0^{a}	3.6 ± 1.2^{a}	146 ± 29.7^{a}	19.4 ± 0.4^{a}	40.8 ± 0.0^{a}	255 ± 0.2^{a}	1067 ± 52^{b,c}	0.69^{a}	0.15^{b,c}	0.15$^{a–d}$	30.6 ± 0.4^{a,c}	3.0 ± 0.1$^{c–e}$	5.7 ± 1.3^{b,c}
3K	18.1 ± 0.4^{a}	9.7 ± 0.6^{a}	2.8 ± 0.2^{a}	163 ± 4.9^{a}	18.9 ± 0.0^{a}	40.5 ± 0.0^{a}	327 ± 0.2^{a}	1033 ± 24^{b,c}	0.66^{a}	0.15^{b,c}	0.15$^{b–d}$	28.6 ± 1.3$^{a–e}$	3.0 ± 0.1$^{c–e}$	3.6 ± 0.4^{c}
PR	13.7 ± 5.0^{a}	7.9 ± 1.5^{a}	2.5 ± 0.0^{a}	152 ± 19.1^{a}	20.2 ± 0.6^{a}	40.3 ± 0.1^{a}	265 ± 16^{a}	1363 ± 39^{a}	0.60^{a}	0.06^{d}	0.07^{e}	13.5 ± 0.2^{f}	3.6 ± 0.1^{a}	14.6 ± 1.5^{a}

* Mean ± SD is calculated from duplicate measurements. Values with different letters in the same column are significantly different with $p < 0.05$.

in the plot with wider X range ($0 < X < 10\,000$). The $\ln N_{de}(X)$ representations show the following features. For DP $X < 50$, the number molecular size distributions of debranched starch from all rice grain samples are superimposable (Fig. 2); fine differences would however be masked by the band broadening inherent in SEC measurements. Qualitative differences are observed for DP DP $X \geqslant 50$, e.g. MR84 has a greater amount of branches for $50 < X < 2000$ than MR220 and PM, but the opposite is observed for $X \geqslant 4000$.

The DB values of whole starch extracted from rice grain samples range between 2.7% and 3.6% (Table 2). Similar to the Am content, there are significant differences among some of the 14 rice grain samples. The DB values from ^{1}H NMR analysis are compared with those obtained from inverse relation with number average of $N_{de}(X)$ from SEC molecular size distribution of debranched starch (DB = 1/number average of $N_{de}(X)$). Both methods produce similar DB values (Table 2 in the Supplementary data).

3.3. Starch digestibility of cooked rice grains

Heating of starch granules, flour, or grains in excess water, such as during normal home cooking, causes starch to gelatinise, which is the rupture and disintegration of the compact semi-crystalline granular structure of starch through the disruptions of inter- and intra-molecular hydrogen bonds of starch molecules. Initially, water molecules plasticise the amorphous domains, leading to a small and reversible swelling of the granule. The swelling continues as the heat continues to melt the Ap crystallites. The mainly linear Am molecules then leach out of the swollen granules into the solution phase, surround the granules, and inhibit further swelling (Tester & Morrison, 1990). Gelatinisation increases the susceptibility of starch to digestive enzymes, as the swelling of starch granules increases the accessibility of enzymes to penetrate into the granules.

First-order kinetics is used here to fit the starch digestograms of cooked rice grains. Although cooking gelatinises starch granules and causes them to swell and solubilise, starch granules in cooked rice grains are trapped in the protein and cell-wall matrices, inhibiting the swelling of starch granules and solubility of starch molecules during cooking. Hence the diffusion rate of digestive enzymes into insoluble gelatinised starch granules in the grains largely contributes to the digestibility of starch in cooked rice grains; diffusion-controlled processes are expected to obey first-order kinetics (Al-Rabadi et al., 2009). Fig. 3 shows a typical digestogram of starch in cooked rice grains, together with the first-order kinetics fit. The values of k from all samples are listed in Table 2 and the plots are available in the Supplementary data. The correlation coefficients (R^2) of k range between 0.89 and 0.99.

3.4. Digestibility – structure relationships

Pearson correlation analysis was first used to reveal the linear relationships between starch digestion rate coefficient k and the structural features (both of grain composition and starch molecular structures). In Table 2, the PR sample exhibits significantly different Am content as well as k compared with the other 13 samples. Spearman rank correlation method was also used since it is less sensitive to outliers and makes no assumptions about the distribution of the data. The Spearman method can also detect non-linear relationships which cannot be obtained from the Pearson correlation method. The significant correlations are then rationalised mechanistically.

The correlation results from both correlation methods are presented in Table 3 and the correlation plots are available in the Supplementary data. In addition, the correlations with the exclusion of PR sample are also presented in Table 3 to demonstrate

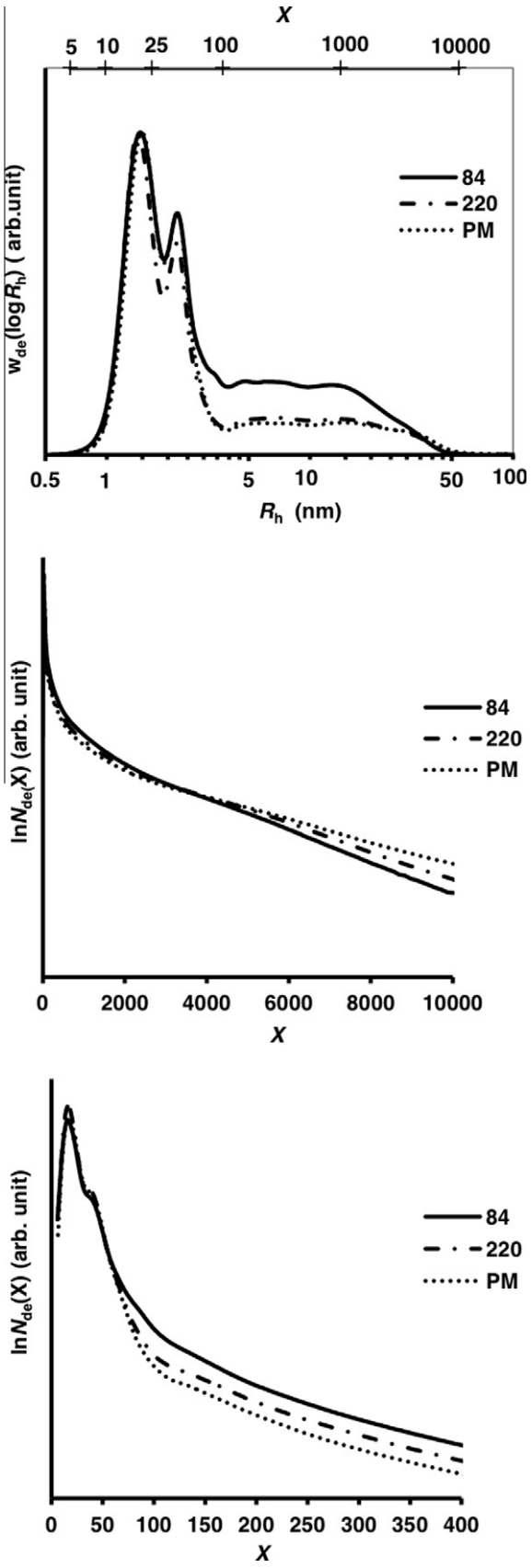

Fig. 2. Representative size distributions of debranched starch, presented in equivalent ways to bring out different features: as weight, $w_{de}(\log R_h)$, and number, $N_{de}(X)$, molecular size distributions (the latter presented with two ranges of X), normalised to yield the same height as the highest peak.

the differences in the correlations when a narrow range of Am contents was used.

3.4.1. Effects of grain composition on the starch digestibility of cooked rice grains

Both Pearson and Spearman correlation test results with the inclusion as well as the exclusion of PR sample show that there are no significant correlations between k and total starch, crude lipid, and protein contents (Table 3 and the correlation plots are available in the Supplementary data). The results are consistent with other studies reported elsewhere (Chung et al., 2010; Okuda et al., 2005).

3.4.2. Effects of starch molecular structures on the digestibility of cooked rice grains

The correlation analysis indicates the relationships between k and what we term gross starch molecular structural features: Am content, DB, $\overline{M}_w$, and $\overline{R}_g$. Some of these correlations have also been reported in the literature. With the inclusion of PR, the Pearson correlation method shows the negatively linear correlation between k and Am content ($R = -0.728$) (Table 3 and the correlation plots are available in the Supplementary data), consistent with the results from other studies, showing that higher Am content decreases starch digestibility (Chung et al., 2010; Frei et al., 2003; Okuda et al., 2005; Zhu et al., 2011). Am, having a more linear and flexible structure than Ap, can form double helices after cooking (retrogradation), which have higher resistance towards amylase hydrolysis than does amorphous starch. Hence, starch containing higher amounts of Am are digested more slowly than that with a lower Am content. This explains the highest k value of PR among all samples because of its lowest Am content. However, no significant correlation is observed when using Spearman test and when PR was excluded.

Similar to Am content, a significant positive correlation is observed between k and DB ($R = 0.656$) when the Pearson correlation test was used with the inclusion of PR. DB is dominated by the branching points in the Ap molecules, together with Am content. Ap dominates the swelling of starch granules, while the presence of Am dilutes the amount of Ap. Furthermore, Am-lipid complexes can restrict granule swelling during cooking (Tester & Morrison, 1990), which limits the accessibility of digestive enzymes into the granules. There are no correlations when the Spearman method is used, as well as when PR is excluded from the analysis. There are

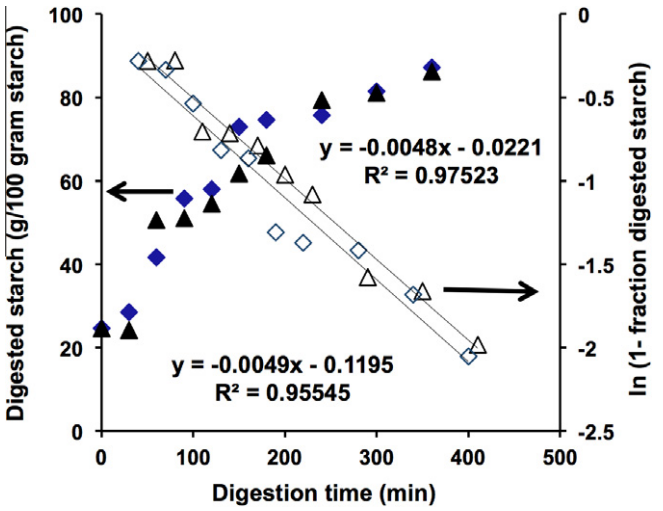

Fig. 3. Typical starch digestograms of cooked rice grains, and first-order kinetics fits of the digestograms.

Table 3
Correlation coefficients (R) between digestion rate coefficient, k, of starch in cooked rice grains and the structural attributes.

Structural attributes	Correlation coefficient			
	All samples		All samples without PR	
	Pearson	Spearman	Pearson	Spearman
Grain composition				
Total starch (%)	−0.379	−0.406	−0.260	−0.300
Total crude protein (%)	0.182	0.401	0.463	0.433
Total crude lipid (%)	0.328	0.419	0.266	0.294
Gross starch molecular structures				
Am content	−0.728**	−0.193	0.050	0.011
DB	0.656*	0.197	0.143	−0.024
$\overline{M}_w$	−0.190	−0.024	0.155	0.15
$\overline{R}_g$	−0.048	−0.020	−0.022	0.006
Fine starch molecular structures				
R_h/nm at whole (fully branched) Am peak maximum	−0.232	−0.155	0.000	−0.064
$\overline{R}_h$/nm of whole (fully branched) Am molecules	−0.521	−0.232	−0.193	−0.036
X_{Ap1}	0.149	0.049	−0.189	−0.109
X_{Ap2}	−0.055	−0.098	−0.200	0.120
X_{Am1}	−0.225	−0.175	−0.219	−0.144
X_{Am2}	0.677**	0.029	−0.412	−0.217
$h_{Ap2/Ap1}$	−0.539*	−0.016	0.149	0.236
$h_{Am1/Ap1}$	−0.445	−0.151	0.266	0.068
$h_{Am2/Ap1}$	−0.536*	−0.311	−0.133	−0.135

* Correlations are significant at $p < 0.05$.
** Correlations are significant at $p < 0.01$.

no significant correlations between k and either $\overline{M}_w$ or $\overline{R}_g$ in the present study.

We now turn to the influence of starch fine structural features on digestibility; this is the first such examination of these effects. Nine independent structural variables are used to cover the fine molecular structures of starch, which comprise R_h at the peak maximum and $\bar{R}_h$ of the Am component in the SEC weight molecular size distribution of whole starch, four branch-chain lengths (X_{Ap1}, X_{Ap2}, X_{Am1}, and X_{Am2}) and three height ratios ($h_{Ap2/Ap1}$, $h_{Am1/Ap1}$, and $h_{Am2/Ap1}$) of peak maxima in the SEC weight molecular size distribution of debranched starch. These fine features essentially encompass the size distributions of both whole and debranched molecules. Thus these nine structural features are truly independent structural variables, and two of the gross features considered above, DB and Am content, are then dependent variables.

The correlations between k and these fine features using the two different correlation methods are listed in Table 3, together with the comparison of the correlations when PR sample was excluded from the statistical analysis. The correlation plots are available in the Supplementary data. The results from the Pearson correlation test show that X_{Am2}, $h_{Ap2/Ap1}$, and $h_{Am2/Ap1}$ are all significantly correlated (absolute values of $R > 0.5$) with k. However, the use of the Spearman rank method and exclusion of PR resulted in no significant correlations.

The significant positive correlation between k and X_{Am2} ($R = 0.677$) suggests a higher digestion rate with longer chain length of longer amylose branches.

The significant negative correlation between k and $h_{Ap2/Ap1}$ ($R = -0.539$) implies that a slower digestion rate with higher ratios of long Ap branches (Ap2) to short Ap branches (Ap1). However, a higher proportion of long Ap branches (DP $\geqslant$ 35) has previously been reported to increase the degree of swelling on wheat starch granules (Sasaki & Matsuki, 1998). The mechanism behind this relationship needs further investigation to be able to draw a robust conclusion.

The significant negative correlation between k and $h_{Am2/Ap1}$ ($R = -0.536$) in the Pearson test suggests higher digestibility with lower ratios of longer Am to short Ap branches, consistent with the results from Am content and DB, and emphasising the effects of Am in slowing digestion rate of starch. The correlations with the molecular size and branching structure of Am molecules do not appear to have been noticed before, and this finding may give a different perspective on the effects of Am fine structure on starch digestibility. However, it is emphasised that the correlations found with Pearson and inclusion of PR is lost with Spearman correlation and exclusion of PR.

4. Conclusions

The Pearson correlation results from this study support the validity of those structural components usually considered important for starch digestibility, which are Am content and DB. The mechanistic reasons for these effects are well known. Our study also shows, for the first time, that fine structural features of both Am and Ap may be significant for the digestion rate of starch in cooked rice grains. Those features which increase the digestion rate coefficient (more rapid digestion) are a longer chain length of long Am branches and a smaller relative amount of long (trans-lamellar) to short (single-lamellar) Ap branches, as well as a smaller relative amount of long Am branches to short Ap branches.

Mechanistic reasons for these correlations may arise from how these structural features affect various rate-controlling steps in starch digestion, such as the degree of granule swelling after cooking (including solubilization of starch molecules into the buffer solution and inhibition of granule swelling by Am-lipid complex), and the rapidity of, and extent to which, retrogradation of Am molecules takes place. These merit separate investigations.

The correlation results are however suggestive rather than definitive, since there are no significant correlations found in Spearman rank test. A larger number of samples with a wider range of amylose contents is recommended to test the correlation results suggested by Pearson correlation analysis.

This finding gives a new perspective on the effects of Am and Ap fine structures on starch digestibility of cooked rice grains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.08.053.

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