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Name of University	University of Queensland
Country of Study	Australia
Major	Agriculture
Degree	PhD
Course Title	
Type of Document	Journal Paper
Year	2014
Keywords	rice; physicochemical property; starch fine structure; size-exclusion chromatography; growth location

Rice Science, 2014, 21(1): 47−58
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DOI: 10.1016/S1672-6308(13)60163-8

Effects of Rice Variety and Growth Location in Cambodia on Grain Composition and Starch Structure

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Abstract: The effects of variety and growth location on grain composition and starch structures were investigated using three rice (*Oryza sativa* L.) cultivars (Phka Romduol, Sen Pidao and IR66) with different amylose contents. All the three cultivars were planted in three different agro-climatic zones (Phnom Penh, Coastal and Plateau) of Cambodia. The protein content of polished grains increased when rice was planted at a location with higher average temperature, but their lipid content decreased. The amylose content and degree of branching were not greatly affected by the minor temperature differences among the growing locations. Starch fine structures characterized by the chain-length distribution were significantly different among the cultivars, but not significantly among different locations. The results suggested that protein and lipid biosyntheses were more sensitive to the environmental temperature than that of starch in rice grains.

Key words: rice; physicochemical property; starch fine structure; size-exclusion chromatography; growth location

Starch composition and structures are factors controlling rice grain qualities, especially eating and cooking quality. The physicochemical properties of starch in rice grains and the chemical composition of rice grains are influenced by variety (genetic background) and growth environment (McCall et al, 1953; Aboubacar et al, 2006; Inukai and Hirayama, 2010). Growth temperature has been shown to influence the activities of starch biosynthetic enzymes during grain developmental stage in many crops, and then result in changing of the physicochemical properties of their starches (Hawker and Jenner, 1993; Denyer et al, 1994; Lu et al, 1996; Wallwork et al, 1998; Jiang et al, 2003). High growth temperatures (above 22 °C) during grain filling can result in the reduction of starch accumulation in rice endosperm (Inukai and Hirayama, 2010). Low temperatures (< 22 °C) during grain filling seem to reduce the total starch content in rice endosperm slightly (Umemoto et al, 1995; Ahmed et al, 2008). The protein content in rice endosperm is also influenced by both variety and growth environment (McCall et al, 1953). The protein content of japonica rice varieties increases at high temperatures (at an average of 24.5 °C, ranging from 18.9 °C to 30.1 °C) during grain filling, compared with lower temperatures (at an average of 21.3 °C, ranging from 16.9 °C to 25.7 °C) (Jin et al, 2005). Furthermore, the protein content of brown rice increases with increasing temperature during the ripening stage (Resurreccion et al, 1977). The lipid content of rice endosperm is also affected by both variety and growth environment (McCall et al, 1953).

Starch comprises mainly two types of glucose homopolymers: amylopectin (Ap) and amylose (Am). Ap, a much larger molecule than Am, has a far higher proportion of branch points than that of Am, as the former has a molecular weight between 10^7 to 10^8, while Am reaches a molecular weight of 5×10^5 to 10^6. Similar to the grain composition, the Am content of rice starch is greatly affected by variety and growth environment. A few genes related to Am content in rice grains have been identified, such as *waxy* (*wx*) and *amylose extender* (*ae*) genes (Sano, 1984; Yano et al, 1985; Hirano and Sano, 1998). Furthermore, the decreasing temperatures during the grain filling (Jin et al, 2005; Aboubacar et al, 2006) and ripening stages (Resurreccion et al, 1977; Inukai and Hirayama, 2010) result in higher Am content in rice endosperm, which could be attributed to the higher activation of *wx* gene expression at lower temperatures (18 °C vs. 28 °C) (Hirano and Sano, 1998).

Growth temperature is found to affect the fine structural features of starch, *viz*., the chain (branch)

Received: 14 February 2013; **Accepted:** 15 May 2013

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length distribution (CLD) of Ap in rice grains (Asaoka et al, 1985; Umemoto et al, 1999; Jiang et al, 2003; Suzuki et al, 2003; Aboubacar et al, 2006). In general, the Ap of starch developed at higher temperatures (> 25 °C) consists of more long branches (degree of polymerization (DP) > 25) and less short branches (DP < 25) compared with that at lower temperatures (≤ 25 °C). However, the Ap of starch in the rice endosperms grown at 29 °C night / 35 °C day contains less long branches of DP ≥ 64 than those grown at 22 °C / 28 °C during the grain filling and ripening stages (Jiang et al, 2003). The changes in the chain length of Ap branches developed at different growth temperatures can be attributed to the distinct optimum temperatures of starch branching enzyme isoforms. Each of the isoforms is responsible for synthesizing specific lengths of Ap branches (Takeda et al, 1993; Ohdan et al, 2010).

The differences in starch structure and grain composition caused by genetic background and growth environmental factors can affect the properties of starch in flour and grains, such as gelatinization and pasting properties as well as digestibility (Asaoka et al, 1984; Morrison and Azudin, 1987; Jin et al, 2005; Aboubacar et al, 2006; Syahariza et al, 2013). As rapidly digestible starch has been associated with increasing incidence of metabolic syndrome, including obesity and diabetes, rice grain containing larger amounts of slowly digestible and enzyme-resistant starches are desirable to prevent and alleviate these complications (Lehmann and Robin, 2007). This result indicates the importance of starch structures in determining the nutritional values of a starchy food. Furthermore, the digestibility of cooked rice may be associated with fine structures of both Ap and Am components (Syahariza et al, 2013). The effects of Am content on the relationships between Am and the properties of starch have been reported in many studies (Syahariza et al, 2013). However, no study on the character of CLDs in Am branches has yet been reported.

There are more than 2 000 rice varieties in Cambodia and people from different agro-climatic zones tend to grow and consume different rice varieties (Sar et al, 2012). Little is known about the starch structures of Cambodian rice varieties, which may have effects on health for rice consumers. Therefore, to clarify the relationship between fine structure of starch with growth conditions and genetic background is very important. The objective of this study is to investigate the effects of rice variety and growth location on grain composition and starch structures of Cambodian rice, including the CLD of Am branches. Although many studies have been reported on the effects of environmental temperatures on starch fine structures of rice grains characterized by the fine structures (or branch structure) of Ap, no study has been published about the effect of environmental temperatures on fine structure of Am.

MATERIALS AND METHODS

Materials

Three normal (or non-waxy) rice varieties, namely Phka Rumduol (PR), Sen Pidao (SP) and IR66, were planted at three different agro-climatic zones of Cambodia: Phnom Penh, coastal (Kampot Province), and plateau (Kratie Province), the project sites of Cambodian Agricultural Research and Development Institute (CARDI). The grain samples were all provided by CARDI Plant Breeding Division with collaboration. All the three varieties are consumed widely in Cambodia (Sar et al, 2012) and of different Am content and starch digestibility (Syahariza et al, 2013), which may have different health effects. During the experiment, the annual average minimum/maximum temperatures were 24.8 °C / 33.9 °C, 23.6 °C / 31.5 °C, and 22.8 °C / 30.5 °C for Phnom Penh, coastal and plateau zones, respectively. The annual total rainfall levels were similar between coastal and plateau zones (about 1 320 mm in total), but it was higher in Phnom Penh (a total of 1 551 mm). In contrast, the number of rainy days was comparable between Phnom Penh and coastal zones (126 and 136 d, respectively), but was less in the plateau zone (90 d). The details of the temperature, rainfall level, and number of rainy days in the three agro-climatic zones at the year of sampling are shown in Supplementary Table S1.

Brown rice was obtained by dehulling rough rice with a Satake THU-35 dehuller (Satake, Hiroshima, Japan), and polished using a Satake TM-05 pearler (Satake Engineering Co. Ltd., Tokyo, Japan) to produce polished rice. The polished rice was ground into flour using a cryogenic mill (Freezer/Mill 6850 SPEX, Metuchen, NJ, USA) in a liquid nitrogen bath (Syahariza et al, 2010), and the flour was used to analyze the chemical composition and to characterize the molecular structures of native starch in the rice grains. The mill was set to run at 10 revolutions per second and the milling process was carried out by two cycles of 5 min grinding, with a 2-min re-freezing break. The harvested rough rice contained about 14% moisture before the de-hulling and polishing processes.

Dimethyl sulfoxide (DMSO, GR for analysis) was 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 from Sigma–Aldrich Pty Ltd. (Castle Hill, NSW, Australia). The Total Starch (AA/AMG) assay kit and isoamylase of *Pseudomonas sp*. were from Megazyme International Ltd. (Bray, Co. Wicklow, Ireland). Other chemicals were reagent grade and used as received.

Chemical composition of rice grains

The Megazyme Total Starch assay kit was used to analyze the starch content. An LECO CNS2000 auto analyzer (LECO Corporation, St. Joseph, MI, USA) was used to measure the content of crude protein in rice grains, which was calculated based on the nitrogen content of rice flour (Jung et al, 2003) multiplying by a conversion factor of 5.95 (Jones, 1941). The crude lipid content was measured by Soxhlet extraction, following AOAC method 920.39C for cereal (AOAC International, 2002). All measurements were performed in duplicate.

Extraction and enzymatic disbranching of starch molecules from rice flour

The extraction and dissolution of starch molecules from ground rice flour were carried out following the method described by Syahariza et al (2013) by removing non-starch components, i.e. proteins, lipids, and non-starch polysaccharides, using protease, sodium bisulfite, ethanol, and DMSO with 0.5% LiBr (DMSO/LiBr) solutions. All the chemical ingredients do not degrade starch molecular structure. The extracted starch molecules were enzymatically disbranched using isoamylase, following the method described by Syahariza et al (2013). The samples were frozen in liquid nitrogen for freeze-drying, and then dissolved in DMSO/LiBr solution. The disbranched starch in DMSO/LiBr solution was stored at room temperature for size-exclusion chromatography (SEC, also termed gel permeation chromatography, GPC) analysis.

Chain length distribution of disbranched starch and Am content

The CLD of enzymatically disbranched starch was analyzed using an Agilent 1100 Series SEC system (Agilent Technologies, Waldbronn, Germany) equipped with GRAM 100 and 1000 analytical columns (Polymer Standards Service GmbH, Mainz, Germany) following the methods described by Syahariza et al (2010) and Tran et al (2011).

Since the separation of SEC was based on hydrodynamic volume (V_h), or the corresponding hydrodynamic radius (R_h), a series of pullulan standards with known peak molecular weights from 342 to 2.35 × 10^6 were used for calibration to obtain the correlation between V_h and SEC elution volume. The Mark–Houwink equation was used to convert the molecular weights of pullulan to V_h and then to convert the V_h of disbranched (linear) starch to the corresponding DP (X), as follows:

$$V_h = \frac{2K[(X-1)\cdot M_0 + 180]^{(1+\alpha)}}{5\mathrm{N_A}} \qquad (1)$$

where M_0 = 162.2 is the molecular weight of the anhydroglucose repeating monomer unit and 180 is that of the glucose at the reducing ends, $\mathrm{N_A}$ is Avogadro's constant, and K and α are the Mark-Houwink parameters. In DMSO/LiBr at 80 ºC, K is equal to 0.02427 and 0.01500 mL/g, α is equal to 0.6804 and 0.7430 for pullulan and disbranched starches, respectively (Liu et al, 2010). The DP obtained by the Mark-Houwink equation is prone to error due to small glucan chains, and the inferred value is also with uncertainties due to SEC band broadening. Thus, the DP value is only semi-quantitative. As for any linear polymers, the SEC weight [$w_{de}(\log X)$] and number [$N_{de}(X)$] distributions of disbranched starch are related using the following equation (Castro et al, 2005b):

$$w_{de}(\log X) = X^2 \cdot N_{de}(X) \qquad (2)$$

The size distribution of disbranched starch, or CLD, is thus presented as both SEC weight distribution $w_{de}(\log X)$, 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.

The Am content of rice starch was determined by the area under the curve (AUC) of the Am branches to the AUC of overall Ap and Am branches in the SEC weight distribution of disbranched starch, as there was clear separation between Ap and Am components (International Standardization Organization, 2011; Vilaplana et al, 2012).

Parameterization of starch chain length distribution

Following our previous results (Witt et al, 2012; Syahariza et al, 2013), the molecular fine structures of Ap and Am, obtained from the weight distribution of disbranched starch, were parameterized in two ways in this study.

The first was an empirical analysis. It comprised the DP at each local peak maximum (two peak maxima for Ap, denoted by X_{Ap1} and X_{Ap2}, and two peak maxima for Am, denoted by X_{Am1} and X_{Am2}) and the ratio of peak height of each local peak maximum to the height of the first Ap (Ap1) peak maximum, denoted by $h_{\mathrm{Ap2/Ap1}}$, $h_{\mathrm{Am1/Ap1}}$ and $h_{\mathrm{Am2/Ap1}}$, respectively. The peak height ratio was used to determine the relative amount to Ap1 branches by the amount of each branch group. This was obtained by normalizing the SEC weight distributions of disbranched starch from different samples to yield the same height of Ap1 peak, which was also the highest peak in the weight distribution of disbranched normal rice starch. The AUC was not used to determine the amount of each branch group because there were some overlappings between the two populations of Ap branches as well as between the two populations of Am branches.

The second way for parameterization was a biosynthesis-based analysis. The CLD of Ap branches was fitted using a model designed for those aspects of Ap biosynthesis controlling the CLD (Wu and Gilbert, 2010). In this model, the number distribution (i.e., the relative number of chains with a given degree of polymerization) is assumed to be controlled solely by three types of starch biosynthetic enzymes: starch synthase (SS), starch branching enzymes (SBE) and starch disbranching enzymes (DBE). Kinetic equations were formulated to analyze the disbranched number distribution, i.e. the CLD, $N_{\mathrm{de}}(X)$ of Ap branches in terms of the rates of the enzymatic processes catalyzed by each type of the starch biosynthetic enzymes. The overall $N_{\mathrm{de}}(X)$ of Ap branches is assumed to be resulted from the actions of a number of enzyme sets, where each set contains all the three types of the biosynthetic enzymes, but with different combinations of enzyme isoforms. For example, there are three isoforms of SBE in rice grains: SBEI, SBEIIa and SBEIIb. In this study, the $N_{\mathrm{de}}(X)$ was obtained by SEC. However, the fine structure in the CLD would be influenced due to band broadening during SEC. This precludes the use of more than two enzyme sets to fit each $N_{\mathrm{de}}(X)$. Using the mathematical modelling, three fitting parameters were obtained from $N_{\mathrm{de}}(X)$ for each of the two enzyme sets: the ratio of SBE activity to SS activity (this parameter being termed as β), and two minimum-DP parameters (X_0 and X_{min}) for the action of SBE. X_0 (about 10) was the minimum DP left on the parent chain after the action of SBE, and X_{min} (about 4) was the minimum DP transferred from the parent chain to another chain to form a new branch. The two enzyme sets were denoted by enzyme sets 1 and 2 for (short) single-lamellar and (long) trans-lamellar branches, respectively. Hence, there were five parameters for each $N_{\mathrm{de}}(X)$ to be used for comparison among different samples: β_1, β_2 (a higher β by giving a steeper slope in the approximately linear section of $\log N_{\mathrm{de}}(X)$), X_{min1}, X_{min2} (increasing the DP at the peak and shoulder regions increases X_{min}), and the relative contributions of enzyme set 2 to that of set 1 (the relative heights of the peak and shoulder, denoted by *RC*). The X_{01} and X_{02} were assigned the values of 4 and 1 throughout, respectively (Wu and Gilbert, 2010). The non-linear least-squares fitting procedure used to obtain an experimental $N_{\mathrm{de}}(X)$ is reported in detail by Wu and Gilbert (2010).

Degree of branching

Fully branched starch molecules were extracted from ground rice flour following the method of Syahariza et al (2013). The degree of branching (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 nuclear magnetic resonance (NMR) spectroscopy following the method of Tizzotti et al (2011). The extracted starch molecules were dissolved in DMSO-d_6, and a small amount of TFA-d_1 was added to the sample medium to improve the signals of ^{1}H NMR. While DB can also be determined from the reciprocal of the average of $N_{\mathrm{de}}(X)$, obtaining DB directly by NMR is more accurate than averaging an experimental $N_{\mathrm{de}}(X)$ because of the experimental uncertainties in the latter from band broadening and the Mark-Houwink relation.

Statistical analysis

All experiments were conducted in duplicate for each sample. All data were reported as the mean ± standard deviation (*SD*). Two-way analysis of variance (ANOVA) was used to examine the effects of variety, growth location and the variety-by-growth location interactions, followed by Tukey's pairwise comparisons of the means using SPSS V16.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of variety and growth location on chemical composition of rice grains

The grain compositions of the three normal rice varieties grown at three different agro-climatic zones in Cambodia are summarized in Table 1. Total starch contents ranged from 80.0% to 83.0% for all the three tested varieties. There were no significant effects of variety, growth location, and the interaction between variety and growth location on starch content of rice grains (Table 2).

There were some significant variations for the crude protein content among the three rice varieties and among the three growth locations (Table 1). The protein contents of rice grains ranging from 6.25% to 9.29% were within the ranges reported by the International Rice Research Institute (IRRI) (Gomez, 1979) and other studies (Zhang et al, 2007; Chung et al, 2010). The crude protein content was significantly influenced by both variety and growth location, but not by the interaction between variety and growth location (Table 2). In general, IR66 contains less crude protein than PR and SP (the average data from the three agro-climatic zones of each variety are listed in Supplementary Table S2). In addition, for each variety, the grains from Phnom Penh had the highest crude protein content and those from plateau zone had the lowest one. The crude protein content of PR had no significant differences among the three agro-climatic zones (Table 1). Since the Phnom Penh zone has the highest average growth temperature and the plateau zone has the lowest average growth temperature (Supplementary Table S1), the results suggested that the crude protein content of rice grains tended to increase at higher temperatures.

The contents of crude lipid were significantly different among the varieties and the three agro-climatic zones. However, the effect was not significant by the interaction between variety and growth location (Table 2). The crude lipid contents of all the three rice grain samples (ranged from 0.51% to 1.02%) were within the range in the literatures (Zhang et al, 2007; Chung et al, 2010). The crude lipid contents of PR and SP were lower than that of IR66 (Supplementary Table S2). Furthermore, for each variety, the grains from the Phnom Penh were of the lowest crude lipid content, and those from the plateau zone were of the highest (Table 1).

Table 1. Chemical composition of grains from three rice varieties grown at three agro-climatic zones.

Variety	Zone	Starch (%)	Crude protein (%)	Crude lipid (%)
IR66	Phnom Penh	83.0 ± 1.5 a	8.05 ± 0.09 ab	0.83 ± 0.06 abc
	Coastal	81.3 ± 2.1 a	7.09 ± 0.90 bc	0.90 ± 0.03 ab
	Plateau	81.2 ± 1.2 a	6.25 ± 0.81 c	1.02 ± 0.03 a
PR	Phnom Penh	80.0 ± 0.4 a	8.54 ± 0.26 ab	0.51 ± 0.02 d
	Coastal	81.5 ± 1.4 a	7.71 ± 0.73 bc	0.63 ± 0.03 cd
	Plateau	82.2 ± 0.6 a	7.12 ± 0.45 bc	0.75 ± 0.08 bcd
SP	Phnom Penh	81.6 ± 0.9 a	9.29 ± 0.39 a	0.69 ± 0.09 bcd
	Coastal	82.1 ± 2.6 a	8.01 ± 0.27 ab	0.70 ± 0.02 bcd
	Plateau	80.8 ± 1.2 a	7.52 ± 0.70 bc	0.77 ± 0.02 bcd

PR, Phka Rumduol; SP, Sen Pidoa.

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

Table 2. Level of significance of *F*-values for the effects of variety, growth location, and the interaction between variety and growth location on chemical composition of grain.

Composition	Variety	Growth location	Variety × Growth location
Starch	0.09	0.43	0.61
Crude protein	12.81**	27.29**	0.28
Crude lipid	35.64**	14.48**	0.08

** indicates significant effect at the 0.01 level.

Effects of variety and growth location on starch molecular structure

For each variety, the representative SEC weight distributions of disbranched starch, $w_{\mathrm{de}}(\log R_{\mathrm{h}})$, are presented in Fig. 1-A. The results for all varieties grown in all the three agro-climatic zones are given in Supplementary Fig. S1. The weight distributions were normalized to yield the same peak height of the highest

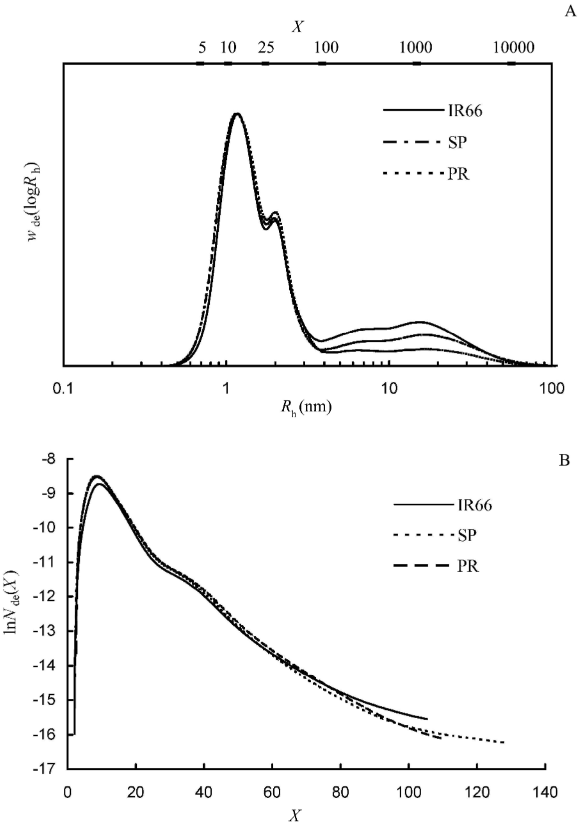

Fig. 1. Representative weight distributions, $w_{\mathrm{de}}(\log R_{\mathrm{h}})$, of disbranched starch (A) and number distributions, $N_{\mathrm{de}}(X)$, of Ap branches (DP ≤ 100) for each rice variety (B), normalized to the height of the highest peak.

PR, Phka Rumduol; SP, Sen Pidoa; X, Degree of polymerization at each local peak maximum; R_{h}, Corresponding hydrodynamic volume.

peak. It was shown as typical weight distributions of disbranched rice starch, with two large Ap-branch peaks and two small Am-branch peaks. The two peaks of Ap-branch represented the branches confined to a single lamella (Ap1, 0.4 nm < R_h < 1.8 nm, DP 4–30) and those spanning more than one lamella (Ap2, 1.8 nm < R_h < 4.0 nm, DP 30–110), respectively. A feature is clearly visible and reproducible in the weight distribution of Am branches (4 nm < R_h < 100 nm, DP 110–30 000). With the presence of at least two obvious peaks, the Am branches clearly visible and reproducible in weight distribution were denoted as Am1 and Am2.

Table 3 lists the empirical parameterization of the CLDs, including the DP at each local peak maximum (X_{Ap1}, X_{Ap2}, X_{Am1} and X_{Am2}), the ratio of the peak height of each local peak maximum to the height of the Ap1 peak maximum ($h_{Ap2/Ap1}$, $h_{Am1/Ap1}$ and $h_{Am2/Ap1}$), and the Am content calculated from the ratio of the AUC of Am branches to the AUC of the whole distribution. There were significant differences among the varieties in X_{Ap1}, X_{min1}, X_{min2}, $h_{Ap2/Ap1}$, $h_{Am1/Ap1}$ and $h_{Am2/Ap1}$, and in the corresponding biosynthesis parameters for the Ap component, β_1, β_2, X_{min2} and *RC*. Significant differences were also detected in Am content. Table 4 shows the fine molecular structures of Ap and Am. The Am content was significantly affected by variety, but with no significant effects of growth location and the interaction between variety and growth location. However, there were no consistent trend between the DP at each local peak maximum and the rice varieties. For example, the X_{Ap1} of IR66 was higher than those of PR and SP, whereas the values of X_{Am1} of IR66 and SP were higher than that of PR (Supplementary Table S2). Furthermore, PR had the highest value of X_{Am2} and IR66 had the lowest among the three rice varieties. Similarly, the $h_{Ap2/Ap1}$ of SP was higher than those of IR66 and PR, but both values of $h_{Am1/Ap1}$ and $h_{Am2/Ap1}$ of IR66 were the highest and those of PR were the lowest. The results suggested that there were no co-dependency among the DPs at different local peak maxima and the peak heights of local peak maxima. It might be the reason that the different branches of Ap and Am were synthesized by distinct enzymes under different genetic background. Both $h_{Am1/Ap1}$ and $h_{Am2/Ap1}$ trended consistently as Am content, suggesting that the two kinds of Am branches contribute identically to the Am content of rice starch from different varieties.

The average values of Am content for PR, SP and IR66 were 15.3%, 21.9% and 27.1%, respectively. Small

Table 3. Molecular structures of starch in rice grains from the three rice varieties grown at the three agro-climatic zones.

Variety	Zone	X_{Ap1}	X_{Ap2}	X_{Am1}	X_{Am2}	$h_{Ap2/Ap1}$	$h_{Am1/Ap1}$	$h_{Am2/Ap1}$	Am (%)	DB (%)	β_1	β_2	X_{min1}	X_{min2}	*RC*
IR66	Phnom Penh	13.50 ± 0.01 a	36.92 ± 3.57 a	340.87 ± 3.02 a	1 150.47 ± 26.84 c	0.52 ± 0.00 b	0.15 ± 0.00 a	0.17 ± 0.00 a	27.5 ± 0.5 a	2.73 ± 0.04 b	0.135 b	0.076 c	7.00 ± 0.00 a	6.50 ± 2.12 b	0.038 a
	Coastal	13.49 ± 0.01 a	34.50 ± 1.90 a	349.50 ± 1.34 a	1 163.62 ± 13.86 c	0.55 ± 0.02 b	0.14 ± 0.00 a	0.17 ± 0.01 a	26.8 ± 0.9 a	2.73 ± 0.00 b	0.136 b	0.077 c	7.00 ± 0.00 a	6.50 ± 2.12 b	0.037 a
	Plateau	13.47 ± 0.03 a	33.90 ± 0.89 a	344.93 ± 8.13 a	1 171.93 ± 24.50 c	0.54 ± 0.01 b	0.15 ± 0.01 a	0.17 ± 0.01 a	26.9 ± 1.5 a	2.86 ± 0.01 b	0.136 b	0.075 c	7.00 ± 0.00 a	7.00 ± 0.00 b	0.037 a
PR	Phnom Penh	13.25 ± 0.04 b	34.26 ± 0.10 a	271.03 ± 1.07 b	1 364.12 ± 38.71 a	0.54 ± 0.01 b	0.06 ± 0.01 c	0.07 ± 0.00 c	15.9 ± 1.1 b	3.61 ± 0.04 a	0.141 a	0.080 a	7.00 ± 0.00 a	8.00 ± 0.00 a	0.036 b
	Coastal	13.18 ± 0.01 b	34.12 ± 0.18 a	281.07 ± 8.10 b	1 418.11 ± 74.66 a	0.55 ± 0.00 b	0.07 ± 0.01 c	0.08 ± 0.00 c	14.4 ± 0.1 b	3.69 ± 0.05 a	0.141 a	0.083 a	7.00 ± 0.00 a	8.00 ± 0.00 a	0.034 b
	Plateau	13.26 ± 0.04 b	35.38 ± 1.61 a	279.45 ± 3.64 b	1 389.06 ± 133.30 a	0.56 ± 0.01 b	0.06 ± 0.00 c	0.08 ± 0.00 c	15.4 ± 0.2 b	3.67 ± 0.01 a	0.143 a	0.082 a	7.00 ± 0.00 a	8.00 ± 0.00 a	0.035 b
SP	Phnom Penh	13.25 ± 0.02 b	33.27 ± 0.10 a	332.09 ± 6.88 a	1 240.74 ± 19.76 b	0.58 ± 0.01 a	0.10 ± 0.00 b	0.13 ± 0.00 b	21.2 ± 0.5 c	2.93 ± 0.02 b	0.141 a	0.080 b	7.00 ± 0.00 a	7.00 ± 0.00 b	0.036 b
	Coastal	13.22 ± 0.01 b	33.84 ± 0.13 a	337.12 ± 9.35 a	1 266.81 ± 36.83 b	0.57 ± 0.01 a	0.10 ± 0.00 b	0.13 ± 0.01 b	22.7 ± 0.1 c	2.95 ± 0.23 b	0.140 a	0.079 b	7.00 ± 0.00 a	7.00 ± 0.00 b	0.036 b
	Plateau	13.28 ± 0.06 b	33.64 ± 0.22 a	334.45 ± 0.59 a	1 260.48 ± 10.65 b	0.58 ± 0.02 a	0.11 ± 0.01 b	0.13 ± 0.01 b	21.9 ± 1.3 c	3.02 ± 0.00 b	0.141 a	0.077 b	7.00 ± 0.00 a	6.50 ± 2.12 b	0.036 b

Mean ± *SD* is calculated from duplicate measurements. Mean values with different letters in the same column are significantly different at the 0.05 level.

PR, Phka Rumduol; SP, Sen Pidoa; Am, Amylose; Ap, Amylopectin; *X*, Degree of polymerization at each local peak maximum; *h*, Ratio of peak height of each local peak maximum to that of the first Ap peak maximum; DB, Degree of branching; *β*, Ratio of starch branching enzymes activity to starch synthase activity; *RC*, Relative contributions of enzyme set 2 to that of set 1.

differences (ranged from 0.7% to 1.5%) were detected among the samples grown in different agro-climatic zones within the same variety (Table 3 and Supplementary Table S2). The Am contents of a number of rice grain samples obtained from the AUC of the Am branches in the SEC weight distribution of disbranched starch were found to have the same tendency as those obtained using iodine colorimetric and Concanavalin A methods, which had lower actual values due it's limitations (Vilaplana et al, 2012; Syahariza et al, 2013). In this study, the data indicated that the Am content was significantly affected by the variety (Table 4), consistent with the results of the Am fine structures.

The average DB values of IR66, SP and PR were 2.77%, 2.97% and 3.66%, respectively. Within the same variety, small differences (ranged from 0.08% to 0.13%) were shown in DB values among the samples grown in the three agro-climatic zones (Table 3 and Supplementary Table S2). Similar to the Am content, the DB value was significantly affected by variety, but not by growth location and the interaction between variety and growth location (Table 4). This could be attributed to the increase in Am content with reducing amount of Ap, which contains most of the branches.

Representative CLDs, $N_{\mathrm{de}}(X)$ of Ap branches ($X \leq 100$) for each variety are presented as $\ln[N_{\mathrm{de}}(X)]$ in Fig. 1-B (Castro et al, 2005a; Wu and Gilbert, 2010). The different representations of the same CLD by weight and number distributions brought out different features of the distribution. The number distributions of both Ap and Am branches ($X \leq 30\ 000$) for all the three varieties grown in the three agro-climatic zones are demonstrated in Supplementary Fig. S2. Since the significant differences in Am branches were observed from the weight distributions $w(\log R_{\mathrm{h}})$ of disbranched starch, the number distributions were used for analyzing the fine structure of Ap only. For $X < 60$, the number distributions of disbranched starch for all samples were superposable, and the fine differences would be masked by the band broadening inherent in SEC measurements. For longer Ap branches, IR66 had a larger amount of branches than PR and SP when X were at 60 to 100.

Table 4. Level of significance of *F*-values for the effects of variety, growth location, and the interaction between variety and growth location on starch structures.

Parameter	Variety	Growth location	Variety × Growth location
X_{Ap1}	137.49**	3.26	1.81
X_{Ap2}	1.62	0.32	1.24
X_{Am1}	257.25**	4.04	0.64
X_{Am2}	25.32**	0.49	0.09
$h_{\mathrm{Ap2/Ap1}}$	16.40**	1.82	2.09
$h_{\mathrm{Am1/Ap1}}$	168.91**	0.47	1.13
$h_{\mathrm{Am2/Ap1}}$	402.71**	1.46	0.90
Am	216.65**	0.10	1.32
DB	201.45**	1.88	0.50
β_1	7.06*	1.43	2.09
β_2	31.34**	2.70	1.64
X_{min1}	-	-	-
X_{min2}	19.00**	0.00	1.00
RC	12.04**	2.83	1.15

* and ** indicate significant effects at the 0.05 and 0.01 levels, respectively.

Am, Amylose; Ap, Amylopectin; X, Degree of polymerization at each local peak maximum; h, Ratio of peak height of each local peak maximum to that of the first Ap peak maximum; DB, Degree of branching; β, Ratio of starch branching enzymes activity to starch synthase activity; RC, Relative contributions of enzyme set 2 to that of set 1.

In order to compare the values among the samples from different varieties and growth locations, the number distribution of individual sample was fitted with the model developed by Wu and Gilbert (2010). Five specific parameters: β_1, β_2, X_{min1}, X_{min2} and RC were adopted in the model (Table 3). The data were fitted up to meet $X \leq 70$, where Ap2 was shown in the non-linear concave-up behavior due to an artifact of SEC band broadening (van Berkel et al, 2005). A typical fit of the number distribution of Ap branches in rice starch (SP as an example), demonstrating the contributions of two enzyme sets, is shown in Fig. 2, and those of all the three rice varieties grown in the three agro-climatic zones are presented in Supplementary Fig. S3. Most of the parameters (β_1, β_2, X_{min2} and RC) from the mathematical model, except X_{min1}, were significantly different among some samples (Table 3) and were significantly affected by variety, but not by the growth

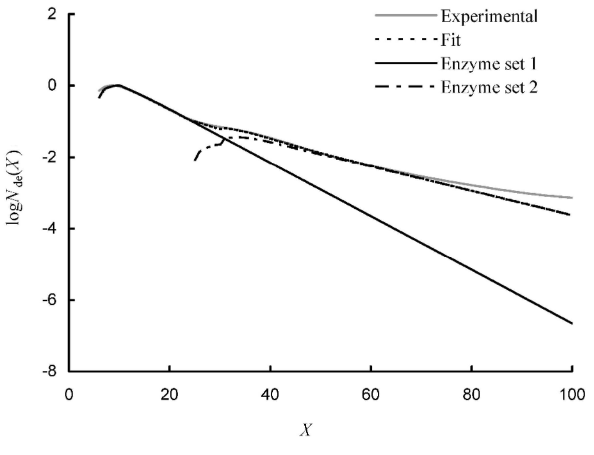

Fig. 2. Typical number distribution, $N_{\mathrm{de}}(X)$, of amylopetin (Ap) branches, fitted to the Wu-Gilbert model with the contributions of two enzyme sets.

location and the interaction between variety and growth location (Table 4). In addition, the β_1 of IR66 was lower than those of PR and SP, with the value at *RC* (Supplementary Table S2). PR had the highest value of β_2 and IR66 had the lowest. Also, PR had higher X_{min2} value than those of IR66 and SP. The results suggested that the enzymatic processes required for starch biosynthesis, for Ap in particular, were not significantly affected by environmental conditions.

Relationship among chemical compositions and starch structures of rice grains

Table 5 lists the correlation coefficients among chemical compositions and various structural parameters. The values of the Ap CLD (X_{Ap1}, X_{Ap2} and $h_{\text{Ap2/Ap1}}$) by the empirical parameterization had high correlations with the biosynthesis-based ones (β_1, β_2, X_{min1}, X_{min2} and *RC*), which could be used to reconstruct the CLD accurately. Furthermore, the DB was the reciprocal of the mean value of $N_{\text{de}}(X)$, which was dominated by the vast number of Ap branches, and was thus related to the biosynthesis parameters (β_1, β_2, X_{min1}, X_{min2} and *RC*). The Am CLD parameters (X_{Am1}, X_{Am2}, $h_{\text{Am1/Ap1}}$ and $h_{\text{Am2/Ap1}}$) were also related to the Am content.

Protein and lipid contents were negatively correlated, which indicated that the content of crude protein increased while that of crude lipid decreased in the synthesis process of rice grains. The same trend was reported also (Juliano et al, 1964). This could be due to the temperature-sensitive biosynthetic enzymes of protein and lipid, probably to ensure the complete grain development and the survival of the grain in harsh environment.

Lipid content was significantly correlated with the amount of Am chains (β_1, β_2 and *RC*), the parameters for the Am CLD (X_{Am1}, X_{Am2}, $h_{\text{Am1/Ap1}}$ and $h_{\text{Am2/Ap1}}$) and the Am content. Although the same observation has been reported for isolated starch granules due to Am-lipid complexes (Zhou et al, 1998), it is not the case for whole grain. Most lipids in grain are triglycerides that cannot form complex with Am and they are located mainly in embryo, whereas starch granules are located in the endosperm. Further study is needed to understand the relationship between the biosyntheses of lipid and Am in rice grain.

Significant correlations could be detected among several biological parameters (β_1, β_2, X_{min1}, X_{min2} and *RC*). To explain this phenomenon, further study would be required and a larger data set along with the parameterization using this model on more accurate CLDs based on fluorophore-assisted carbohydrate

Table 5. Pearson correlation coefficients (*R*) among chemical compositions and starch structures of rice grains.

Parameter	Starch	Crude protein	Crude lipid	X_{Ap1}	X_{Ap2}	X_{Am1}	X_{Am2}	$h_{\text{Ap2/Ap1}}$	$h_{\text{Am1/Ap1}}$	$h_{\text{Am2/Ap1}}$	Am	DB	β_1	β_2	X_{min1}	X_{min2}
Crude protein	NS															
Crude lipid	NS	-0.793**														
X_{Ap1}	NS	NS	0.771**													
X_{Ap2}	NS	NS	NS	NS												
X_{Am1}	NS	NS	0.612**	0.580*	NS											
X_{Am2}	NS	NS	-0.647**	-0.772**	NS	-0.808**										
$h_{\text{Ap2/Ap1}}$	NS	NS	NS	-0.543*	-0.575*	NS	NS									
$h_{\text{Am1/Ap1}}$	NS	NS	0.764**	0.833**	NS	0.890**	-0.903**	NS								
$h_{\text{Am2/Ap1}}$	NS	NS	0.756**	0.812**	NS	0.923**	-0.903**	NS	0.988**							
Am	NS	NS	0.697**	0.828**	NS	0.906**	-0.896**	NS	0.966**	0.979**						
DB	NS	NS	-0.577*	-0.673**	NS	-0.961**	0.869**	NS	-0.907**	-0.934**	-0.940**					
β_1	NS	NS	-0.524*	-0.610**	NS	NS	0.552*	0.540*	-0.661**	-0.622**	-0.614**	0.507*				
β_2	NS	NS	-0.762**	-0.786**	NS	-0.807**	0.826**	NS	-0.885**	-0.875**	-0.875**	0.813**	0.751**			
X_{min1}	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		
X_{min2}	NS	NS	NS	-0.589*	NS	-0.869**	0.776**	NS	-0.815**	-0.814**	-0.836**	0.847**	NS	0.737**	NS	
RC	NS	NS	0.540*	0.809**	NS	0.577*	-0.820**	NS	0.766**	0.731**	0.792**	-0.720**	-0.683**	-0.801**	NS	-0.617**

NS, None significant. * and ** indicate significant correlations at the 0.05 and 0.01 levels, respectively.

Am, Amylose; Ap, Amylopectin; *X*, Degree of polymerization at each local peak maximum; *h*, Ratio of peak height of each local peak maximum to that of the first Ap peak maximum; DB, Degree of branching; β, Ratio of starch branching enzymes activity to starch synthase activity; *RC*, Relative contributions of enzyme set 2 to that of set 1.

electrophoresis (FACE) would be necessary (O'Shea et al, 1998; Wu and Gilbert, 2010).

DISCUSSION

Our results indicated that there were no significant effects of variety and growth location on starch content in rice grains, which are consistent with the result reported by McCall et al (1953), indicating that variety or genetic background of non-glutinous rice have no effects on starch content. However, the deficiency in one or more enzymes and/or low resistance against diseases and pests could reduce the starch content of rice grains due to the ineffective starch biosynthesis, such as in waxy and high-Am varieties (Juliano et al, 1987; Inukai and Hirayama, 2010). However, starch content has been shown to decrease at high growth temperatures (Inukai and Hirayama, 2010), probably due to early termination of grain filling (Jiang et al, 2003). The results of the present study were entirely different. It might be attributed to the small variations in the uncontrolled outdoor temperatures (average differences of < 4 °C) and similar rainfall patterns across the three agro-climatic zones in Cambodia (Supplementary Table S1), compared with the controlled growth temperatures in the greenhouse studies (average differences of 7–10 °C). This implies that the starch content of rice grains is not affected significantly by a minor variation of environmental temperatures.

Significant effects of both variety and growth location on protein content were detected in this study. The effect of variety on protein content in the endosperm among normal rice varieties was also reported by McCall et al (1953). Our results suggest that the crude protein content of rice grains tends to increase when rice is grown at higher temperatures, consistent with the results reported by others (Resurreccion et al, 1977; Jin et al, 2005). However, other studies found that lower temperatures increased protein content in rice grains (Hirano and Sano, 1998; Ueda et al, 1998).

A significant effect of variety on crude lipid content of rice grains was found in the present study. It is consistent with a previous study reported by McCall et al (1953). In addition, there was a trend in this study that the crude lipid content of rice grains decreased when rice was grown at a higher temperature condition. Despite it, there was no significant difference among growth locations within the same variety. Probably, this phenomenon is due to the small amount of lipids in rice grains, which leads to no significant differences among growth locations comparing the whole chemical composition of the grains. However, a significant effect of growth location on the crude lipid content of rice grains was reported by McCall et al (1953) using a larger number of rice varieties. In this study, there was no significant effect of growth location on fine structures of Ap molecules, which was apparently inconsistent with the former report, suggesting that the amount of long branches (DP > 25) of Ap increases at higher growth temperatures (> 25°C) and the short branches (DP < 25) decreases compared with lower growth temperatures (≤ 25 °C) (Asaoka et al, 1985; Umemoto et al, 1999; Jiang et al, 2003; Suzuki et al, 2003; Aboubacar et al, 2006). This discrepancy was attributed to the small variation in the environmental temperatures among the three agro-climatic zones (average differences of < 4 °C) in Cambodia, differed from the larger differences in the controlled growth temperatures carried out in glasshouse, reported by others (average differences of 7–10 °C).

The Am content was mostly determined by the variety or genetic background (Table 4) since there were a few genes that controlled Am content in rice grains, including *wx* and *ae* genes (Sano, 1984; Yano et al, 1985; Hirano and Sano, 1998). Furthermore, the Am content of endosperm starch increases at lower growth temperatures during the filling and ripening stages of rice grains (Resurreccion et al, 1977; Aboubacar et al, 2006; Inukai and Hirayama, 2010), probably due to increased expression of *wx* gene encoding granule-bound starch synthase (GBSS) I in response to lower temperature (18 °C) compared with that at a normal temperature (28 °C) (Hirano and Sano, 1998). The Am content from the present study, however, was significantly affected neither by growth location nor by the interaction between variety and growth location (Table 4), probably due to the small variation in the environmental temperatures and rainfall patterns among the three agro-climatic zones in Cambodia. In addition, the minimum temperature in Cambodia is 22 °C, while the expression of *wx* gene encoding GBSSI would be increased at a lower temperature (18 °C).

The effects of variety and growth location on Am fine structures have not been reported before. Our results suggested that the effect of variety was significant on Am fine structure, i.e. The different Am contents among the varieties were due to the differences in the activities of the underlying biosynthetic processes (Tables 3 and 4). The effects of growth location and the interaction between variety and growth location on

Am fine structure were not significant, this could be due to the small variation in the environmental temperatures among the three agro-climatic zones in Cambodia, similar with the results of Am content and Ap fine structures.

The CLD of Ap branches is the result of the concerted actions of various biosynthetic enzymes, mainly soluble SS (SSS), SBE and DBE (Myers et al, 2000). In this study, the enzyme sets and other parameters derived from CLD of Ap branches were affected by variety only. However, previous reports indicated that the activities of sucrose synthase, ADP-glucose pyrophosphorylase, SBE and SS in rice endosperm are affected by growth temperature (Umemoto et al, 1995; Jiang et al, 2003). The expression of *wx* gene encoding GBSSI varies with temperature (Hirano and Sano, 1998), changing the Am content at different growth temperatures. In addition, the activities of SBE isoforms (SBEI, SBEIIa and SBEIIb), each synthesizing branches of specific lengths, have different optimum temperatures (Takeda et al, 1993; Ohdan et al, 2010), which could affect the chain length of Ap branches when the crop is grown at different temperatures. The results from the current study suggest that the enzymatic processes required for starch biosynthesis, Ap in particular, are not affected by minor changes in the environmental conditions.

CONCLUSIONS

The present study showed that there were no significant effects of variety, growth location and the interaction between variety and growth location on starch content in polished grains of normal rice varieties from Cambodia. However, other components of rice grains, such as protein and lipid contents, were affected both by variety and growth location, consistant with the previous studies. Crude protein content in rice grains increased when the rice was grown in a location with higher temperatures, whereas crude lipid content increased when the rice was grown in a location with lower temperatures. The fine structural features of both Ap and Am were different among the three tested rice varieties, but had no significant difference among locations because of slight variation for temperatures and rainfalls. For Ap, the result from this study was confirmed with previous study (Wu and Gilbert, 2010), but not for Am. These features included DPs at each peak maximum and peak heights of each local peak maximum of both Ap and Am branches (obtained from the SEC weight distribution of the disbranched starch), the biosynthesis-based parameterization parameters (enzyme activity ratios) controlling chain length distribution, Am content and degree of branching. The non-significant differences for most structural parameters among the three growth locations suggested that the growth locations had no obvious impact on the structures of starch when the temperatures and rainfall patterns had no significant differences. The results suggest that the structures of the cooked rice grains in Cambodia are mainly affected by variety. Therefore, the rice consumers could have different health implications if they consume different rice varieties, but would have similar health implications from the same rice varieties grown in different growth locations in Cambodia.

ACKNOWLEDGEMENTS

The authors thank the Cambodian Agricultural Research and Development Institute (CARDI) for their assistance in the sample collection process and the University of Queensland, Australia, for the international postgraduate scholarship. The authors greatly appreciate the technical assistance of Ms. Sarah CHUNG. This work was partially supported by Australian Research Council Linkage (Grant No. LP0990769).

SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Monthly minimum and maximum temperature (°C), rainfall level (mm) and number of raining day in different growth locations of Cambodia in 2011 (Provided by Department of Meteorology, Ministry of Water Resources and Meteorology, Cambodia).

Supplemental Table 2. Chemical composition of grains and molecular structures of starch of different rice varieties averaged from the three agro-climatic zones of Cambodia.

Supplemental Fig. 1. Comparisons of SEC weight distributions, $w_{\mathrm{de}}(\log R_{\mathrm{h}})$, of debranched starch from all rice grain samples of the three varieties, each grown in the three agro-climatic zones.

Supplemental Fig. 2. Comparisons of SEC number distributions, $N_{\mathrm{de}}(X)$, of debranched starch from all rice grain samples of three varieties, each grown in the three agro-climatic zones.

Supplemental Fig. 3. Experimental number distributions of Ap branches and the biosynthesis based model fit.

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