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Debranching enzyme concentration effected on physicochemical properties and α-amylase hydrolysis rate of resistant starch type III from amylose rice starch

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ABSTRACT

The effect of debranching enzyme concentration on physicochemical properties and α-amylase hydrolysis rate of resistant starch type III from high amylose rice starch were studied. The pullulanase enzyme (8, 10, 12, 14 and 16 U/g starch) was introduced to modify amylopectin molecules of 15% (w/w) gelatinized rice starches at 55 °C for 16 h. The debranched starches with different degrees of hydrolysis (0.14–5.27%), and having 66.60–98.82% β-amylolysis limit were then induced at 4 °C for 16 h, afterward a one cycle of freeze–thaw process (−10/30 °C) was applied. The results showed that a pullulanase hydrolysis improved the degree of syneresis (51.64–54.85% from 8 to 16 U/g starch). Resistant starch content increased sharply as the amount of the enzyme increased, reaching the highest (19.81%) for a 12 U/g starch and decreased to 13.16% by 16 U/g starch. α-Amylase hydrolysis rate showed that incompletely-debranched had a lower estimated glycemic index than completely debranched rice starches. Microstructure of the selected RS III samples using X-ray diffraction and scanning electron microscopy revealed a crystal pattern change from A- to V-type pattern and formed a coarse honeycomb-like and a filamentous network structure.

1. Introduction

The use of starch as a nutrition-valued food product is of great interest among starch industries as Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS) and Resistant Starch (RS). RS has recently been defined as the sum of starch and its degradation products that are not absorbed in the small intestines of healthy individuals (Englyst, Kingman, & Cummings, 1999). RS activities are similar to those of dietary fibers, including pre-biotic effect on colon micro flora, altering metabolism, improving cholesterol metabolism, and reducing the risk of ulcerative colitis and colon cancer. Resistant starch resulting from the highly retrograded amylose fraction has been classified as RS type III.

High amylose rice starch is of particular interest when assessing variability in starch digestibility, it tends to contain higher resistant starch content than waxy rice starch (Walter, Silva, & Denardin, 2005). Miller, Pang, and Broomhead (1995) classified rice as a high glycemic index food with values ranging from 64 to 93 in the freshly cooked form. The freshly cooked rice contains a lower percentage of RS (below 3%) and tends to increase with the amylose content and gelatinized temperatures. Cooling and storing of cooked rice is known to entail starch retrogradation, thus increasing the level of resistant starch type III (RS III) through recrystallization (Englyst et al., 1999). Debranching using pullulanase has been used to produce a glucan with linear, low-molecular-weight and recrystallization polymer chains (Guraya, James, & Champagne, 2001; Yin, Alias, Karim, & Norziah, 2007). This releases a mixture of varied length unit chains from the parent amylopectin molecule that induce retrogradation. In addition, retrogradation is often enhanced when starch gels are subjected to freezing and thawing treatments (Yuan & Thompson, 1998).

Freezing a starch gel leads to the formation of ice crystals and thus concentrates the starch in non-ice phase. Upon thawing, the water can be easily compressed from the network, giving rise to a phenomenon known as syneresis (Tovar, Carmelo, Eggar, Ana, & Elevina, 2002). Pongjanta, Utaipatanacheep, Naivikul, and Piyachomkwan (2007) revealed that the resistant starch content increased quadrupled with debranching and freeze–thaw process (4.07–10.68% and 5.12–19.32% for 0–48 h pullulanase hydrolysis of amylose rice starch preheated at 95 and 121 °C, respectively). Thus, this study was to investigate that neither partially nor completely debranching amylose rice starch has higher predicted resistant starch content. Pullulanase enzyme concentrations effected on physicochemical properties and α-amylase hydrolysis rate of RS III samples from amylose rice starch were analyzed.

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2. Materials and methods

2.1. Materials

High amylose rice starch lot no.1-2006 (containing 83.38% carbohydrate, 1.18% protein, 0.91% fat, 0.82% ash, 13% moisture, 32.10% amylose (iodine method), 95.21% dwb of total starch (enzymatic method) and pH 5.0) was given by Cho Heng Rice Vermicelli Factory Co., Ltd., Nakornpathom, Thailand. Pullulanase enzyme from *Bacillus acidopullulyicus* (E.C. 232-983-9P; ⩾400 U/ml), pepsin (E.C. 3.4.23.1; 2,980 U/mg), α- amylase (E.C. 3.2.1.1; 20.4 U/mg) and amyloglucosidase (A-3042; 69.65 U/mg), Glucose (GO) assay kit (GAGO-20) and potato amylose were purchased from Sigma Chemical Company, USA. A resistant starch assay kit (Megazyme, Cat. no. RSTAR 11/02) was obtained from Megasyme International Ireland Ltd., Ireland.

2.2. Resistant starch type III formation

An aqueous high amylose rice starch (15% w/w, dry basis) was prepared by completely dispersing the sample in distilled water. The slurry samples were annealed at room temperature (30 °C) for 1 h with occasional vigorously shaking. The annealed starch samples were autoclave at 121 °C for 30 min and cooled to 55 °C. The cooked starch samples were debranched using pullulanase enzyme at 0, 8, 10, 12, 14 and 16 U/g of starch at 55 °C for 16 h in a shaker water bath (continuous shaking at 170 rpm). The debranched samples are then heated at 100 °C for 15 min and tested for the degree of hydrolysis and β-amylolysis (%). The debranched starches with different degrees of hydrolysis were stored at 4 °C for 16 h. Afterward, a one cycle of freezing and thawing process (−10/30 °C) of the samples was applied to promote syneresis of the retrograded starches. The retrograded starch was dried at 45 °C to approximately 13% moisture content. The RS III samples were ground and passed through a 100-mesh sieve and packed in plastic bags for further determinations.

2.3. Degree of pullulanase hydrolysis

Reducing sugar (*Rds*) and total sugar (*Ts*) in the samples, debranched for specific times, was analyzed according to the Park–Johnson method (Hizukuri, 1995) and the phenol–sulfuric acid reagent method (Dubois, Hamilton, Rebers, & Smith, 1956), respectively. The hydrolysis treatment was conducted in triplicate. The extent of debranching of high amylose rice starch, using pullulanase enzyme, was evaluated in terms of degree of hydrolysis (D.H.) as the ratio of reducing sugar divide by total sugar (in percentage of glucose) as follow equation:

$$\text{D.H.}(\%) = (\%\ \text{of } \textit{Rds} \text{ after hydrolysis} \div \%\ \text{of } \textit{Ts} \text{ after hydrolysis}) \times 100.$$

2.4. β-amylolysis limit (%) determination

The β-amylolysis limit (%) of native high amylose rice starch and debranched high amylose rice starch at different degrees of hydrolysis was evaluated in order to determine if the high amylose rice starch was completely debranched. The analysis procedure was slightly modified from that of Hood and Mercier (1978). The reducing sugar (*Rds*) and total sugar (*Ts*) of the β-amylolysis product were measured according to the Park–Johnson method (Hizukuri, 1995) and the phenol-sulfuric acid reagent method (Dubois et al., 1956), respectively. The% β-amylolysis limit was calculated using following equation:

$$\frac{\textit{Rds} \text{ of sample after hydrolysis by } \beta\text{-amylase} - \textit{Rds} \text{ of } \beta\text{-amylase blank} \times 100 \times 1.9}{\textit{Ts} \text{ of sample after hydrolysis by } \beta\text{-amylase} - \textit{Ts} \text{ of } \beta\text{-amylase blank}}$$

2.5. Degree of syneresis

Degree of syneresis (D.S.) of the samples, debranched for specific times, was determined according to Karim, Norziah, and Seow (2000) with certain modifications. Portions of each debranched starch paste were transferred into a disposable dish and covered with adhesive tape to prevent moisture loss. The samples were frozen at −10 °C for 16 h and thawed at 30 °C for 2 h. The samples were then subjected to a vacuum filtration after one cycle of the freeze–thaw process. Syneresis water from triplicate retrogradation samples was collected and weighed. The weight of retrograded gel and water syneresis was used to calculate degree of syneresis by the equation:

$$\text{D.S.}(\%) = (\text{Weight of exudates water} \div \text{Weight of retrogradation gel}) \times 100$$

2.6. Resistant starch content determination

Resistant starch content (RS) was determined using a Megazyme Resistant Starch kit (AOAC Method. 2002.02). The samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 h at 37 °C to hydrolyzed digestible starch to glucose. The reaction was terminated with 4 ml ethanol and the indigested RS III was recovered by centrifugation (5000g, 10 min). The supernatant was then decanted and washed with 50% ethanol for twice to remove the digested starch. The sediment was solubillized in 2 ml of 2 M KOH in an ice bath, neutralized with 8 ml sodium acetate (1.2 M) and the RS hydrolyzed to glucose with of amyloglucosidase (0.1 ml, 3300 U/ml). The glucose oxidase/peroxidase reaction was used to measure glucose released from the digested and resistant starches. Absorbance was read at 510 nm after a 20 min incubation period at 50 °C. Resistant starch and digested starch were calculated as glucose × 0.9. The total starch was calculated as the sum of resistant starch and digested starch.

2.7. In vitro starch hydrolysis index and estimated glycemic index determination

In vitro starch hydrolysis rate and hydrolysis index were determined according to Goñi, Garćīa-Alonso, and Saura-Calixto (1997). RS III samples (50 mg) were incubated with 1 mg pepsin in 10 ml HCl–KCl buffer (pH 1.5) at 40 °C for 60 min in a shaking water bath. The digest was diluted to 25 ml by adding Tris maleate buffer (pH 6.9), and then 5 ml of α-amylase solution, containing 2.6 IU of α-amylase in Tris maleate buffer, were added. The samples were incubated at 37 °C in a shaking water bath. 0.1 ml sample was taken from each flask every 30 min from 0 to 3 h and boiled for 15 min to inactivate the enzyme. Sodium acetate buffer (1 ml 0.4 M, pH 4.75) was added and the residual starch digested to glucose by adding 30 μl amyloglucosidase and incubating at 60 °C for 45 min. Glucose concentration was determined by using a glucose assay kit. The rate of starch digestion was expressed as the percentage of starch hydrolyzed at different times. An equation: $C = C_\alpha (1 - e^{-kt})$ was used to described the kinetics of starch hydrolysis, where C, C_α and k were the concentration at time t, the equilibrium concentration and the kinetic constant, respectively. The area under the hydrolysis curve (AUC) was calculated using the equation:

$$\text{AUC} = C_\alpha(t_f - t_0) - \frac{C_\alpha}{k}\left(1 - e^{-k(t_f - t_0)}\right)$$

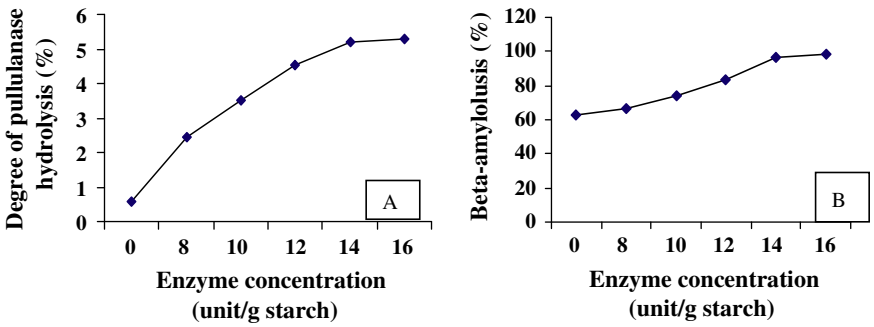

Fig. 1. Effect of pullulanase enzyme concentrations (0–16 U/g starch) on degree of pullulanase hydrolysis (A) and% β-amylolysis limit (B) from 16 h debranching of 15% high amylose rice starch paste.

where, C_α corresponds to the concentration at equilibrium (t_{180}). t_f is the final time (180 min), t_0 is the initial time (0 min) and k is the kinetic constant. A hydrolysis index (HI) was calculated by comparison with the AUC of a reference food (fresh white bread; Farmhouse from local supermarket). Goňī et al. (1997), showed this hydrolysis index to be a good predictor of glycemic response. Expected GI was thus estimated using the model: EGI = 39.71 + (0.549 × HI).

2.8. X-ray diffraction pattern and scanning electron microscopy (SEM)

X-ray diffraction patterns of the selected RS III sample was measured with copper K_2 radiation (λ = 0.154 nm) using a diffractometer (JEOL, JDX-3530, Japan). Diffractometer was operated at 300 mA and 30 kV, 2θ range from 10 to 50.0° with a step size 0.05° and a count time of 2 s. The data was analyzed with program MDI Jade 6.5 (Japan). The crystallinity of the samples was calculated as the proportion of crystalline area to total area at angles between 10 and 30° Theta (Cheetham & Tao, 1998). Morphology of the selected RS III sample was obtained from scanning electron microscope (SEM-JSM-5600 LV, JEOL, Japan). The samples were imaged at 6000× magnifications.

2.9. Experimental design and statistical analysis

The three replications data obtained from the degree of hydrolysis, β-amylolysis limit, degree of syneresis, resistant starch content, α-amylase hydrolysis rate and estimated glycemic index were subjected to analysis of variance (ANOVA). Duncan's new multiple range tests (DMRT) procedure was used to make specific comparison between treatments.

3. Result and discussion

3.1. Effect of enzyme concentration on degree of hydrolysis and β-amylolysis (%)

The degree of hydrolysis and β-amylolysis (%) of 15% (w/w) gelatinized high amylose rice starch reacted with 0–16 U/g starch of pullulanase for 16 h is shown in Fig. 1 The study found that an increase of enzyme concentration from 8 to 14 U/g starch provided the degree of hydrolysis to gradually increase from 2.46 to 5.27% (Fig. 1A). However, there was no significant increase in degree of hydrolysis for the high amylose rice starch samples treated with 16 U/g starch. This indicated that the 15% high amylose rice starch

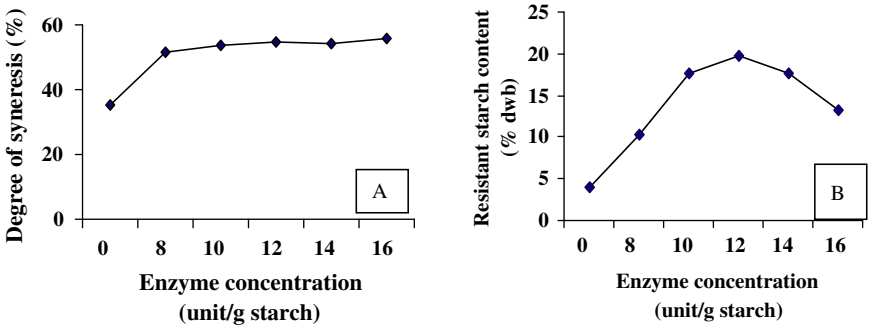

Fig. 2. Effect of pullulanase enzyme concentration (0–16 U/g starch for 16 h) on degree of syneresis (A) and resistant starch content (B) in RS III samples.

Table 1
Effect of pullulanase enzyme concentration (0–16 U/g starch) on physiochemical properties of resistant starch type III samples.

Enzyme concentration (U/g starch)	Physiochemical properties (%)			
	Degree of hydrolysis	β-Amylolysis limit	Degree of syneresis	Resistant starch content
0	0.60 ± 0.03^{e}	62.93 ± 0.75f	35.01 ± 1.43^{d}	3.98 ± 0.46^{e}
8	2.46 ± 0.33^{d}	66.62 ± 1.07^{e}	51.64 ± 1.31^{c}	10.38 ± 0.85^{d}
10	3.49 ± 0.59^{c}	74.48 ± 1.50^{d}	53.54 ± 1.61^{b}	17.68 ± 0.11^{b}
12	4.54 ± 0.41^{b}	83.48 ± 0.78^{c}	54.80 ± 1.50^{a}	19.81 ± 0.28^{a}
14	5.21 ± 0.13^{a}	96.27 ± 3.01^{b}	54.84 ± 1.52^{a}	17.31 ± 0.64^{b}
16	5.27 ± 0.03^{a}	98.82 ± 1.37^{a}	54.85 ± 0. 87^{a}	13.16 ± 0.66^{c}

$^{a,b,c,...}$Means within the same column with different letters indicate significant difference ($P \leqslant 0.05$) by Duncan's new multiple-range test (DMRT).

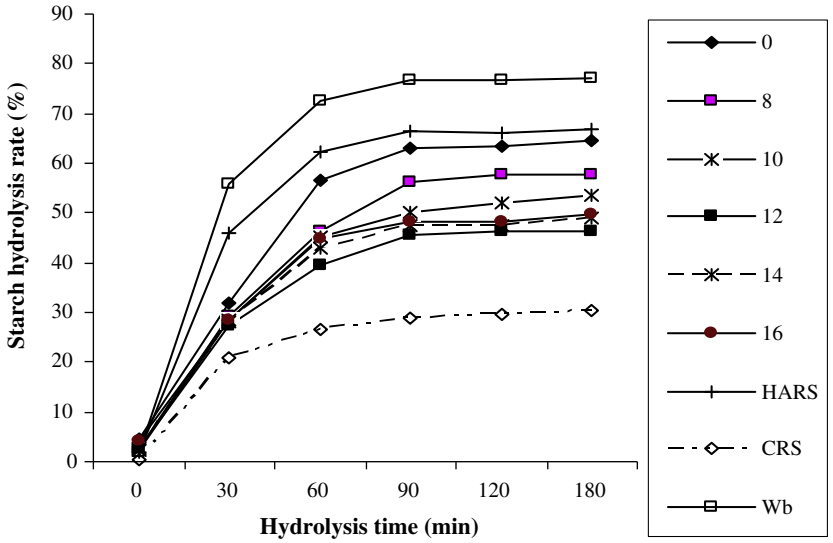

Fig. 3. Effect of pullulanase enzyme concentration (0–16 U/g starch) on α-amylase hydrolysis rate of resistant starch type III samples, native high amylose rice starch (HARS), commercial resistant starch (CRS; Hi-maize) and white bread (WB).

gelatinized was completely debranched by using 14 U/g hydrolysis for 16 h, this was close to completely hydrolyzed to maltose by β-amylase which was 98.82% of β-amylolysis limit (Fig. 1 B). Typically most amylose rice starches would be completely debranched when it had been attacked by β-amylase at least about 95% of β-amylolysis and more particularly at least about 98% of β-amylolysis, debranched by weight (Nagamura, Tolaba, & Suare, 2002).

3.2. Effect of enzyme concentration on degree of syneresis and resistant starch content

The effects of enzyme concentration on the degree of syneresis and resistant starch content of the RS III formation are showed in Fig. 2 and Table 1. The retrogradation from the 8 to 12 U/g starch hydrolysis of the high amylose rice starch was dramatically lost expressible water, which was 35.01–54.80% degrees of syneresis and remained relatively constant thereafter with 12–16 U/g starch (54.80–54.85%).

In addition, resistant starch content increased sharply as the amount of the enzyme increased, reaching highest (19.81%) at 12 U/g starch of pullulanase hydrolysis and dramatically decreased to 17.31 and 13.16% upon 14 and 16 U/g starch of pullulanase enzyme concentration. These results may be explained by analyzing steps in the crystallization process of substances. As Gidley and Bulpin (1987) suggested, a chain length of at least 10 glucose units is required for crystallization and formation of double helices. On the other hand, short chains with DP 6–9 glucose units are known to inhibit retrogradation (Levine & Slade 1986; Shi & Seib, 1992).

Table 2
Effect of pullulanase enzyme concentration (0–16 U/g starch) on hydrolysis index and estimated glycemic index of RS III samples

Enzyme concentration (U/g starch)	Hydrolysis index (%)	Estimated glycemic index (%)
0	55.35[a]	72.58[a]
8	51.51[b]	70.31[b]
10	50.41[b,c]	69.65[b,c]
12	42.63[f]	61.43[f]
14	44.25[e]	65.99[e]
16	45.79[d]	66.90[d]

[a,b,c,...]Means within the same column with different letters are significantly different ($P \leqslant 0.05$) by Duncan's new multiple-range test (DMRT).

Gidley et al. (1995) observed that an approximate relative maximum at DP 20–30 is suitable to form RS III.

3.3. Effect of enzyme concentration on α-amylase hydrolysis rate of RS III

Effect of pullulanase enzyme concentration on α-amylase hydrolysis rate of RS III from high amylose rice starch is presented in Fig. 3. The *in vitro* starch hydrolysis rate (0–180 min) of the treatment with 12 U/g was lowest (2.64–46.24%) among the RS III samples. While, the control (non-debranched rice starch) RS III was highest in α-amylase hydrolysis rate, which was 4.70–64.43% for 0–180 min of hydrolysis time, respectively. These results indicate that the incompletely-debranched RS III sample was resistant to α-amylase digestion. The decrease in the enzymatic digestion of starch after retrogradation had been reported Cui and Oates (1999). They found that the degree of digestion of retrograded sago starch (40% gel) rapidly dropped from 78.3 to 45.4% within 1 h of storage at 5 °C, but extending the storage time to over 6 h had little influence on the degree of enzymatic digestion.

The estimate hydrolysis index and estimated glycemic index of RS III samples was also significantly different among the six treatments as illustrate in Table 2. The treatment with 12 U/g starch of pullulanase hydrolysis was marginally lower in estimated starch hydrolysis index (42.63%) than those of the 14, 16, 10, 8 and 0 U/g starch (44.25, 45.79, 51.51 and 55.35%, respectively). The estimated glycemic index base on hydrolysis index of RS III sample was 72.58, 70.31, 69.65, 61.43, 65.99 and 66.90 for 0, 8, 10, 12, 14 and 16 U/g starch, respectively.

3.4. X-ray diffraction pattern and morphological images

X-ray diffraction patterns of the selected RS III samples (12 U/g starch) are shown in Fig. 4A. The selected RS III sample displayed a V-type diffraction pattern, with 19.74% crystallinity. This was attributed to debranching and retrogradation which reorganized the structure of starch into a helical complex to that of V-amylose. The selected RS III sample formed a coarse honeycomb-like and filamentous network structure was observed with Scanning Electron Micrograph (Fig. 4B). Morphological retrogradation was achieved, bigger, irregularly shaped particles with a white spongy-like porous network. This continuous network structure composed of amy-

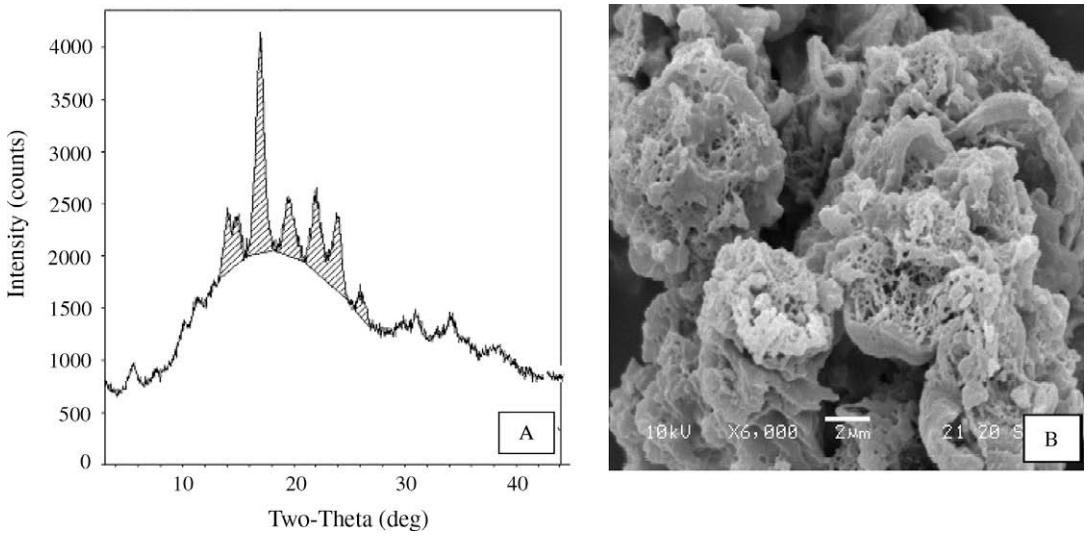

Fig. 4. X-ray diffraction pattern (A) and scanning electron micrographs (6000× magnification) (B) of the selected RS III sample (12 U/g starch).

lose and amylopectin was affected by re-crystallized of the debranched rice starch to form RS III.

4. Conclusions

The present study finding that an incompletely-debranched amylose rice starch has higher predicted resistant starch content than the fully-debranched material. This was attributed to an optimum retrogradation of the rice starch structure into a helical complex that was resistant to human digestive enzymes. The resistant starch content and α-amylase hydrolysis index of the selected RS III samples were 19.81 and 42.63%.

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