Effects of Preheated Treatments on Physicochemical Properties of Resistant Starch Type III from Pullulanase Hydrolysis of High Amylose Rice Starch

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Abstract: In this study, the effects of preheated treatments on physicochemical properties of resistant starch type III formation by pullulanase hydrolysis of High Amylose Rice Starch (HARS) were investigated. A debranching enzyme (Pullulanase, 8 U g⁻¹ starch at 55°C for 0-48 h) was introduced to modify the amylpectin molecules of 15% (w/w) HARS suspension (32.10%, amylose content) which had been preheated at 95 and 121°C for 30 min. Retrogradation gels of debranched starches with different degrees of hydrolysis (0.14 to 3.10%) were then induced at 4°C for 16 h. Afterward, one cycle of the freeze-thaw process (-10/30°C) was applied to promote syneresis of the retrograded starches. Results show that pullulanase hydrolysis enhanced the degree of syneresis (33.22, 45.27 and 58.91% for non-debranched and debranched starches which had been preheated at 95 and 121°C for 48 h, respectively). The debranched starches with higher degree of hydrolysis provided products with higher resistant starch contents. The resistant starch content increased quadrupled with debranching and the freeze-thaw process (4.07 to 10.68% and 5.12 to 19.32% for 0 to 48 h pullulanase hydrolysis of HARS preheated at 95 and 121°C, respectively). Results had shown that after debranching and retrogradation, the HARS molecules had rearranged and changed their crystal pattern from A to V-type pattern, as revealed by X-ray diffraction analysis. In vitro starch hydrolysis index of the RS III samples from 0 to 48 h of pullulanase hydrolysis of the HARS which had been preheated at 95 and 121°C were reduced from 71.591 to 41.69% and 68.66 to 26.83%, respectively.

Keywords: Degree of hydrolysis, degree of syneresis, X-ray diffraction pattern, in vitro starch hydrolysis index

INTRODUCTION

The development of new products is a strategic area of the food industry, because consumers are demanding foods that have two properties; firstly the traditional nutritional aspects of any food, whereas, as a second requirement, additional health benefits are expected from its regular ingestion. In a rapidly changing world with altered food habits and stressful life styles, it is becoming more and more recognized that a healthy digestive system is essential for good overall quality of life (Nugent, 2005). Initial clinical studies have demonstrated that Resistant Starches (RS) have activities similar to dietary fiber, including pre-biotic effect on colon micro flora, improving cholesterol metabolism, reducing the risk of colon cancer and low glycemic index (Cheng and Lai, 2000; Nugent, 2005). Besides physiological benefits in humans, RS has been reported to have potential as a unique ingredient with improved oral tactile perception, taste, palatability, color and texture (Brown, 2004). Resistant Starches (RS) was further divided into four types:

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Type I: It is physically inaccessible starch, as in cooked legume seeds
Type II: It is resistant granular starch, such as those in raw potatoes and green banana
Type III: It is retrograded starches, which may be formed in cooked and cooled starches
Type IV: It is chemically modified starches (Englyst et al., 1992)

From these four types, RS III seems to be particularly interesting because it retains its indigestibility when added as an ingredient to processed foods. Formation of RS III depends on many factors, such as amylose/amylopectin ratio, pH, temperature, incubation and storage time, number of heating and cooling cycles and water content (Escarpa et al., 1996; Kim et al., 2006).

Rice (Oryza sativa L.), being one of the primary dietary sources of carbohydrates worldwide, is of particular interest when assessing variability in starch digestibility. The freshly cooked rice contains a lower percentage of resistant starch, below 3%, but this tends to increase with amylose content and gelatinized temperature (Walter et al., 2005). Several researchers stated that debranching using pullulanase had been used to produce rice starch with linear, low-molecular-weight and recrystallization polymer chains, which were enhanced resistant starch content (Guraya et al., 2000; Yin et al., 2007; Pongjanta et al., 2008). Debranching enzymes such as pullulanase only rapidly hydrolyze α-1,6-glucosidic bonds. This releases a mixture of unit chains of varied lengths from the parent amylopectin molecules which induce retrogradation. In theory, a fast and efficient enzyme hydrolysis requires pre-swelling of starch in water and full starch gelatinization. In practice, this can be achieved by incubation of moistened starch at a temperature exceeding 100°C (Fumiko et al., 1983). However, the gelatinization temperature depends on the variety of rice, the growing temperature of the starch grain and starch content in the water (Kazumi et al., 2005). Thus the objective of this study was to investigate the effect of preheated treatment on the rate of pullulanase hydrolysis and RS III formation. Physicochemical properties, in vitro starch digestibility and glycemic index of the RS III formation were examined.

MATERIALS AND METHODS

This research was conducted during June 2006 to November 2007. High amylose rice starch (HARS; lot No. 1/2006) was kindly supplied by Cho Heng Rice Vermicelli Factory Co., Ltd., Nakornprathom, Thailand. Pullulanase enzyme from Bacillus acidopullulolyticus (EC 232-983-9, p; 400 international units ml⁻¹), pepsin (EC 3.4.23.1; 2,980 unit mg⁻¹), α-amylase (EC 3.2.1.1; 20.4 unit mg⁻¹) and amylglucosidase (A-394; 69.65 unit mg⁻¹), Glucose (GO) assay kit (GAGO-20) and potato amylase were purchased from Sigma Chemical Company, USA. Resistant starch assay kit (Megzyme) was obtained from Megazyme International Ireland Ltd. Ireland. Commercial resistant starch (HI-MAIZE ® 1043) was donated by the National Starch, Food innovation, Australia.

Effect of Preheated Treatments on Morphological Image of HARS Granules

An aqueous HARS (15% w/w) was prepared by mixing starch sample in distilled water and annealing them at 30°C for 1 h with occasionally vigorous shaking. The annealed samples were preheated at 95 and 121°C for 30 min and cooled to 55°C. The starch samples were dried to less than 4% moisture content by ethanol-acetone-diethyl ether and grinded into fine powder. The powder sample was deposited on a copper disc and coated with gold. The specimens were examined by means of a scanning electron microscope (JEM-560LV model). The samples were viewed at 2,500x magnification (Aichokudomechai et al., 2000).

Effect of Preheated Treatment on Degree of Hydrolysis

The resistant starch type III (RS III) formation was prepared as described in Pongjanta et al. (2008). The HARS suspension (15% w/w) was annealed at room temperature for 1 h and heated at

80
95 and 121°C for 30 min. The heated samples were cooled to 55°C and debranched with a pullulanase enzyme (8 unit g⁻¹ starchy) for 0, 4, 8, 16 and 24 h in a shaker water bath. At the end of each time interval the debranched samples were boiled for 30 min to deactivate the enzyme. Reducing sugar (Rs) and total sugar (Ts) in the samples, which debranched for specific times, were analyzed according to the Park and Johnson method (Hizukuri, 1995) and the phenol-sulfuric acid reagent method (Dubois et al., 1956), respectively. The extent of the debranching of different preheated HARS, using the pullulanase enzyme, was evaluated in terms of Degree of Hydrolysis (DH) as the ratio of reducing sugar to the total sugar (in percentage) as follows:

\[
DH(\%) = \frac{Rs \text{ after hydrolysis}}{Ts \text{ after hydrolysis}} \times 100
\]

**Effect of Preheated Treatments on Degree of Syneresis**

Degree of Syneresis (DS) of the samples, debranched for specific times, was determined according to Karim et al. (2000) with modification. Portions of each debranched starch paste were transferred into each disposable dish and covered with adhesive tape to prevent moisture loss and were then induced at 4°C for 16 h. Afterward, one cycle of the freeze-thaw process (-10 for 16 h/30°C for 3 h) was further applied to promote syneresis of retrograded starches. Syneresis water from triplicate retrograded gels was collected and weighed. The weight of retrograded gel and water syneresis was used to calculate the degree of syneresis with this equation:

\[
DS = \frac{\text{Weight of water syneresis}}{\text{Weight of retrogradation gel}} \times 100
\]

The drained gel was dried in a tray drier at 45°C. The dried samples were ground and then passed through an 80 mesh screening and kept in a plastic bag for further analysis.

**Effect of Preheated Treatments on Degree of Crystallinity**

The degree crystallinity of the samples was calculated from the X-ray diffraction patterns as the proportion of crystalline area to total area at angles between 10 to 30° 2θ (Linfeng and Ya-Jane, 2001). X-ray diffraction patterns of native HARS and the selected RS III samples were measured with copper Kα radiation (λ = 0.154 nm) using a diffractometer (JEOL, JDX-3530, Japan). Diffractometer was operated at 300 mA and 30 kV, 2θ ranged from 10 to 50.0° with a step size 0.05° and a count time of 2 sec. The data was analyzed with the computer program MDI Jade 6.5 (Japan).

**Effect of Preheated Treatments on Resistant on Resistant Starch Content**

The resistant starch content was determined using a Megazyme Resistant Starch kit (McCleary and Monaghan, 2002). The samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 h at 37°C to hydrolyses digestible starch to glucose. The reaction was terminated with 4 mL ethanol and the RS sediment was recovered by centrifugation (5000 g, 10 min). The supernatant was decanted and washed with 50% ethanol twice to remove the Digested Starch (DS). The sediment was solubilized in 2 mL of 2 M KOH in an ice bath, neutralized with 8 mL sodium acetate (1.2 M) and the resistant starch was hydrolyzed into glucose with amyloglucosidase (0.1 mL, 3300 U mL⁻¹, 50°C). The Glucose assay kit (GAGO-20) was used to measure glucose released from the digested starch and resistant starch. 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.

**Effect of Preheated Treatments on Resistant on in vitro Starch Hydrolysis Rate**

*In vitro* starch hydrolysis rate and estimated glycemic index were determined according to Goni et al. (1997). Following equation was used to describe the kinetics of starch hydrolysis.
where, \( C_0 \), \( C_c \), and \( k \) were the concentration at time \( t \), the equilibrium concentration and the kinetic constant, respectively.

Using the hydrolysis curve (0-180 min), the Hydrolysis Index (HI) was calculated as the percentage of total glucose released from the samples, to be released from white bread (farm house, local market). The Estimated Glycemic Index (EGI) of the samples was estimated according to Goñi et al. (1997) by the equation:

\[
\text{EGI} = 39.71 + (0.549 \times \text{HI})
\]

**Experimental Design and Statistical Analysis**

Completely Randomized Design was used to evaluate the data obtained from the degree of hydrolysis, degree of syneresis, resistant starch content, *in vitro* starch hydrolysis rate and estimated glycemic index. Analysis of Variance (ANOVA) and Duncan’s Multiple Range Tests (DMRT) procedure were used to make specific comparisons between the treatments by using SPSS for Windows Release 12.0.

**RESULTS AND DISCUSSION**

**Effect of Preheated Treatments on Morphological Image of HARS Granules**

There was a marked difference in shape and size between native HARS and the 95 and 121°C preheated HARS samples. The native HARS granules were observed and proved to be polyhedral and irregular in shape. The granular size was small with diameter of between 3 to 6 \( \mu \)m and formed parts of compound granules. The surface of the granules was smooth without observable pores or fissure. The preheated samples at 95 and 121°C showed that the granular structures swelled and their surfaces collapsed in a doughnut-shaped morphology (Fig. 1). In addition, the HARS that had been preheated at 95°C showed a difference in size and shape, some granules were swelled, while some became dried surface granules. However, the 121°C preheated HARS showed surface erosion and slightly damaged starch granules. This suggests that a considerable degree of the granular crystalline structure was destroyed and were sufficient enough to break hydrogen bonds within the starch molecules which then opened the granules to enzymatic hydrolysis (Kazumi et al., 2005).

**Effect of Preheated Treatments on Degree of Pullulanase Hydrolysis**

The degree of hydrolysis increased with an increasing incubation time and preheated temperatures. The Degree of hydrolysis in HARS slurry preheated at 121°C was significantly higher than those preheated at 95°C, which was 0.17 to 3.10% and 0.14 to 0.50% from 0 to 48 h of hydrolysis time, respectively (Fig. 2). As the reaction time of hydrolysis increased, the degree of hydrolysis in those preheated at 121°C increased sharply after 4 to 48 h of hydrolysis time. This was due to the fact that those preheated at 121°C was sufficient to break the hydrogen bonds within the starch molecules that opened the granules to hydration and enzymatic hydrolysis. Meanwhile, the rice starch that was preheated at 95°C remained stable after 8 h of incubation. Because, the sample that was preheated at 95°C was sticky and dried, it led to restricted gelatinization of the starch granules. Tester and Qi (2004) revealed that the rice starch must be gelatinized prior to pullulanase hydrolysis. If water is limited, the amount of gelatinized starch is restricted together with the capacity of the enzyme to hydrolyze the starch.
Fig. 1: Morphological image (X 2,500) of the (a) Native HARS and the 15% HARS solution preheated at (b) 95°C and (c) 121°C
Fig. 2: Degree of pullulanase hydrolysis from 0 to 48 h debranching of 15% HARS preheated at 95 and 121°C

Fig. 3: Degree of syneresis of retrogradation gel from 0 to 48 h debranching of 15% HARS preheated at 95 and 121°C

**Effect of Preheated Treatments on Degree of Syneresis**

The results obtained in this study suggested that the high degree of pullulanase hydrolysis was closely related with the high degree of syneresis of the RS III formation (Fig. 3). Degree of syneresis was improved from 8.50 to 45.27% and 33.22 to 55.87% for 0 to 48 h debranching of HARS preheated at 95 and 121°C for 30 min, respectively. This was due to the pullulanase enzyme hydrolyze α-1, 6-glucosidic bonds, releasing more linear polymers linked by α-1,4-glucosidic bonds. These fragments had approximately contained of 10 to 65 anhydroglucose unit that was formed by progressive re-association of starch molecules during short-term incubation (Guraya *et al.*, 2001). Additionally, the freeze-thaw cycle was used to promote syneresis of retrograded starch. Yuan and Thompson (1998) revealed that the rate and extent of retrogradation of starch paste during the freeze-thaw process was increased by temperature reduction. As the starch paste is cooled, the starch chains become less energetic and the hydrogen bonds become stronger, giving a firmer gel. As a gel ages or if it is frozen
Fig. 4: X-ray diffraction pattern of native HARS and RS III samples from 4, 16, 24 and 48 h pullulanase-debranching of 15% HARS preheated at 121°C

and thawed, the starch chains have a tendency to interact strongly with each other and thereby force water out of the system (Karim et al., 2000; Pongjanta et al., 2008).

**Effect of Preheated Treatment on Degree of Crystallinity**

The diffraction pattern obtained from the native HARS was classified as an A-type pattern that was indicated by typical peaks at 15.0, 17.5 and 23.2° of diffraction angle 2θ with 13.18% crystallinity (Fig. 4). These values are in agreement with those reported for native rice starch and cereal starches. When the starch was subjected to debranching and retrogradation treatments in this study, the RS III samples showed completely different pattern from the native HARS. The RS III samples derived from 4, 16, 24 and 48 h hydrolysis of the HARS preheated at 121°C showed very strong V-type diffraction pattern, which was 14.37, 15.59, 15.80 and 19.50% crystallinity, respectively. RS III samples from 48 h pullulanase hydrolysis of the HARS preheated at 121°C gave the strongest diffraction peak at around 17° 2θ and a few small peaks at around 20 values of 20, 22 and 24°. This indicated that the RS III derived from HARS that was highly degree of hydrolysis and degree of syneresis influenced the crystalline structure. This was attributed to debranching and retrogradation which reorganized the structure of starch into a helical complex to that of V-amylase (Cui and Oates, 1999). On this basis, it could be postulated that the mechanism of cross-linking amylase gels was via junction zones of aggregated single helices (Zobel et al., 1988).

**Effect of Preheated Treatments on Resistant Starch Content**

The RS contents in 48 h hydrolysis of HARS preheated at 121°C was the highest (19.19%) among RS III formation but not significant difference (p<0.05) from the 16 and 24 h hydrolysis time (17.14 and 18.33%). While, the RS contents in the 0 to 48 h hydrolysis of the RS III from HARS preheated at 95°C was the lowest ranged from 4.07 to 10.68% (Table 1). In addition, resistant starch content in the RS III samples was significantly different from the commercial (High-maize) resistant starch (48.32%) and the native HARS (4.99%). Overall comparison indicated that the higher
pulullanase hydrolysis produced the higher RS content than the non debranched HARS. The RS content increased 4 fold with debranched and freeze thawed process (4.99 to 19.31% for the native HARS and the 48 h pulullanase debranched of HARS with has been preheated at 121°C). These may be attributed to the formation of several linear glucan fractions from pulullanase hydrolysis of the HARS that was recrystallized at low temperature cool down. The recrystallization of these linear starch fractions may permit an orderly rearrangement of granules thereby preventing access to α-amylase digestion (Englyst et al., 1992). In addition, the Digested Starch (DS) content had the highest in the control (0 h of hydrolysis) in those of HARS preheated at 95 and 121°C, which was 90.21 and 90.75%, respectively. All the RS III samples contained the same amount of total starch content, which was 95.04-95.87%.

**Effect of Preheated Treatments on Resistant on in vitro Starch Hydrolysis Rate**

The starch hydrolysis rate for the RS III samples showed that the equilibrium concentration (C<sub>eq</sub>) of the hydrolyzed starch was changed slightly by preheated treatment and debranching time. The 4 to 48 h debranched of HARS preheated at 121°C exhibited a lower C<sub>eq</sub> than those of preheated at 95°C (Table 2). Meanwhile, the kinetic constant (k) of the starch samples tested showed that the native HARS had the highest k value, indicating that hydrolysis occurred most rapidly in the solubilized HARS samples (Frei et al., 2003). While, the RS III formation by retrogradation of 48 h debranching of HARS preheated at 121°C and 95°C showed lower k value (0.018 to 0.020) than the other treatments (0.21 to 0.036). In addition, the commercial resistant starch (Hi-maize) had the lowest in kinetic constant (0.014), this due to its inherent resistance to enzymatic hydrolysis (Grandfieldt et al., 2000). The Hydrolysis Index (HI) of each RS III sample was obtained by using an Area Under Curve (AUC) in the starch hydrolysis rate from 0 to 180 min of the each RS III samples divided by AUC of white bread (Goñi et al., 1997). The HI of the RS III samples range between 26.83 to 71.59%. The RS III samples showed a lower HI value than the native HARS (82.42%) but higher than the commercial resistant starch (12.84%). This behavior indicated that the developed RS III samples are resistant to enzymatic digestion. The decrease in the enzymatic digestion of starch after retrogradation has been reported by several researchers. Cui and Gates (1999) found that the digestion rate of retrograded sago starch (40% gel) rapidly dropped from 78.5 to 45.4% within 1 h storage at 5°C, but extending the storage time to over 6 h had little influence on the degree of enzymatic digestion. It is supposed that the amorphous matrix of starch is readily exposed to the digestive enzymes whereas most of the
Table 2: Estimated parameter of starch hydrolysis rate (Ceq and k), calculated Hydrolysis Index (HI) and estimate Glycemic Index (GI) of RS III from 0 to 48 h pullulase-debranching of 15% HARS preheated at 95 and 121°C

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hydrolysis time (h)</th>
<th>Equilibrium concentration (Ceq)</th>
<th>Kinetic constant (k)</th>
<th>Calculated HI (%)</th>
<th>Calculated GI (%)</th>
<th>Estimated GI value</th>
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<tbody>
<tr>
<td>Preheated treatment</td>
<td></td>
<td></td>
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<tr>
<td>95°C</td>
<td>0</td>
<td>54.35±2.98</td>
<td>0.036a</td>
<td>71.59±2.84</td>
<td>79.01±2.14</td>
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<td>4</td>
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<td>69.09±2.21</td>
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<td></td>
<td>8</td>
<td>46.01±0.20</td>
<td>0.026</td>
<td>52.01±2.65</td>
<td>68.30±2.49</td>
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<td>16</td>
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<td>0.027</td>
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<td></td>
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<td>46.45±1.71</td>
<td>65.25±2.40</td>
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<td></td>
<td>48</td>
<td>45.23±1.26</td>
<td>0.020</td>
<td>41.69±2.94</td>
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<td>121°C</td>
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<td>53.66±0.34</td>
<td>0.034</td>
<td>66.66±0.23</td>
<td>78.41±2.70</td>
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<td>55.15±3.21</td>
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<td></td>
<td>48</td>
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<td>Native rice starch</td>
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<td>82.42±1.67</td>
<td>84.96±2.34</td>
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<tr>
<td>CRS (Hi-maize)</td>
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<td>19.66±0.27</td>
<td>0.014</td>
<td>12.84±0.36</td>
<td>46.76±3.71</td>
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</tbody>
</table>

Mean values within the same column with different letter(s) are significantly different (p<0.05) by New Duncan’s Multiple Range Test (DMRT). ns: Not significantly different (p>0.05)

crystallites reformed during retrogradation are embedded in the matrix (Granfeldt et al., 2000). In addition, the Estimated Glycemic Index (EGI) based on HI of the RS III samples ranged from 54.44 to 79.01% (Table 2). The results of this study revealed that the high degree of pullulase hydrolysis and degree of syneresis led to a reduction of HI and EGI for both of preheated treatments. The 4 to 48 h debranching of HARS preheated at 95°C was higher EGI values than those of the HARS preheated at 121°C, which was 69.09 to 62.63% and 60.83 to 54.44% EGI values, respectively. The low EGI value of the HARS preheated at 121°C was due to its relatively high in resistant starch content (19.32%). The high resistant starch content in the RS III sample had a low glycemic index because of the slowly release of glucose, which may simply result from a lack of available digestible starch (Jenkins et al., 2002; Kim et al., 2006). Jenkin et al. (2002) report that resistant starch type III are promotes slow and moderate postprandial glucose and insulin response. For most starchy food products, a reduction in GI appears to be accompanied by a higher content of resistant starch (Bjorck et al., 2000). For the commercial resistant starch (Hi-maize), had the lowest in HI and EGI value range between 12.84 and 46.76, respectively. In contrast, the native HARS had the highest in HI and EGI value, which was 82.42 and 84.96%, respectively. Similar trends, with comparable values of HI and EGI, have also been reported in native HARS (25-33% amylose content) ranged from 81 to 84, low amylose content (10-20%) ranged from 87 to 93 and waxy rice ranged from 87 to 96 (Juliano and Goddard, 1986; Juliano et al., 1989).

CONCLUSION

The RS III formation from enzymatically-debranching of HARS preheated at 121°C had higher resistant content than those of the HARS preheated at 95°C and the native HARS. X-ray diffraction pattern of the RS III were higher degree of crystallinity than the native HARS with a V-type pattern. The extent of resistant starch content was positively corrected with the degree of pullulase hydrolysis and degree of syneresis. The RS III samples exhibit lower starch hydrolysis rate and estimated glycemic index than the native HARS.

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