

## Enzymes-Resistant Starch (RS III) from Pullulanase- Debranched High Amylose Rice Starch

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### ABSTRACT

In this study, a debranching enzyme (Pullulanase, EC. 232-983-9P, 8U/g starch at 55°C for 0 to 48 hr) was introduced to modify the amylopectin molecules of 15% (w/w) high amylose (32.10%) rice starches suspension which were gelatinized at 75°C for 30 min. The result showed that the retrogradation of debranched starches with different degree of hydrolysis were yielded 0.14 to 1.55% which each samples was then induced at 4°C for 16 hr. Afterward, the one freeze-thaw cycle process (-10/30°C) was applied to promote syneresis of retrograded starches. It was shown that pullulanase hydrolysis improved the degree of retrogradation from 28.10 to 54.53%. The resistant starch content of the RS III sample increased from 4.80 to 12.33% by 0 to 48 hr, respectively. Results showed that after debranching, starch molecule had rearranged and changed their crystal pattern from A to V-type by X-ray diffraction analysis. The RS III sample formed a coarse honeycomb-like and filamentous network structure was observed with Scanning Electron Microscope. The estimated hydrolysis index and glycemic index of the RS III samples were between 35.61% to 62.70% and 59.29% to 73.47% respectively.

**Key words:** resistant starch type III, pullulanase-debranched, high amylose rice starch

### INTRODUCTION

Rice (*Oryza sativa* L.) is a major cereal of Thailand. Commercial rice starch composed of up to 90 % carbohydrates and 0.5-1.5 % protein with negligible fat or dietary fiber (Masaki *et al.*, 2005). Rice flour and starch become popular food ingredients since they are hypoallergenic, low fat and neutral in flavor. However, rice starch has high glycemic index (GI) food compared to starch based

foods. Freshly cooked rice contains a lower percentage of resistant starch (RS), below 3%, but the RS tends to increase with amylose content and processing treatments (Walter *et al.*, 2005).

Enzyme-resistant starch has been recently defined as the sum of starch and its degradation products that are not absorbed in the small intestine of healthy individuals (Englyst *et al.*, 1999). The reduced bioavailability of resistant starch in the human gastrointestinal tract has

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particular significance for diabetics because lowers the insulin response (Englyst *et al.*, 1999). Besides physiological benefits in human, RS has been reported to have potential use as a unique ingredient with improved oral tactile perception, taste palatability, color and texture. RS can be found in nature and its contents of RS are varied by botanical source and starch granular structure (i.e. resistant starch type I and II). In addition, resistant starch (RS III) can be produced from retrogradation of gelatinized starch known as resistant starch type III. The extents of RS III of most starches are, however, are quite low, depending on the molecular structure of starch glucans, namely amylose and amylopectin. High amylose content produced higher resistant starch than low amylose content. Debranching using pullulanase have been used to produce a sample with linear, low-molecular-weight and recrystallization polymer chains (Guraya *et al.*, 2001 and Yin *et al.*, 2007). Debranching enzymes (pullulanase) rapidly hydrolyze only  $\alpha$ -1,6-glucosidic bonds. This releases a mixture of varied unit chain length from the amylopectin molecule which in turn facilitated starch retrogradation. In addition, retrogradation is often enhanced when starch gels are subjected to freezing and thawing treatments (Tovar *et al.*, 2002). 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 *et al.*, 2002).

The objectives of this study was to improve the extent of RS III content by an enzymatically debranching of high amylose rice starch solution and accelerate retrogradation of starch pastes by using a freeze-thaw cycle. The effects of these treatments on physicochemical properties, resistant starch content, and glycemic index were investigated.

## MATERIALS AND METHODS

High amylose rice starch was kindly supplied by Cho Heng Rice Vermicelli Factory Co., Ltd., Nakornprathom, Thailand. Pullulanase enzyme from *Bacillus acidopullulyicus* (E.C. 232-983-9P;  $\geq 400$  international units/ml), pepsin (EC 3.4.23.1; 2,980 unit/mg),  $\alpha$ -amylase (E.C. 3.2.1.1; 20.4 unit/mg) and amyloglucosidase (A-3042; 69.65 unit/mg), Glucose (GO) assay kit (GAGO-20) and potato amylose were purchased from Sigma Chemical Company, USA. Resistant starch assay kit (Megzyme) was obtained from Megazyme International Ireland Ltd. Ireland.

### Resistant starch type III formation

An aqueous high amylose rice starch (15% w/w) was prepared by mixing starch sample in distilled water. The slurry samples were kept at 30°C for 1 hr with occasional vigorous shaking. The annealed samples were partially gelatinized at 75°C for 30 min and cooled to 55°C. The starch samples were debranched (using 8 unit pullulanase enzymes per gram starch) at 55°C for 0, 2, 4, 8, 16, 24, and 48 hr in water bath shaker (170 rpm). The debranched gelatinized samples are then autoclave at 121°C for 15 min and tested for the degree of hydrolysis. The debranched starches with different degrees of hydrolysis were stored at 4°C for 16 hr. Afterward, the one cycle of freezing and thawing process (-10/30°C) of the samples were applied to promote syneresis of the retrograded starches. The retrograded starch was dried at 45°C to approximately 10% moisture content. The RS III samples were ground and passed through a 100-mesh sieve and packed in plastic bags for further determinations.

### Chemical composition of raw material

Moisture, protein, carbohydrate, fat, ash and fiber content (%) of high amylose rice starch (HARS) were determined followed AOAC method (AOAC, 2000). Amylose content (%) was

determined colorimetrically after iodine binding (Juliano, 1997).

### Degree of pullulanase hydrolysis and degree of debranching

Reducing sugar (*Rds*) and total sugar (*Ts*) in the samples, debranching for specific times, were analyzed according to the Park-Johnson method (Hizukuri, 1995) and the phenol-sulfuric acid reagent method (Dubois *et al.*, 1956), respectively. The extent of debranching of rice starch, using pullulanase enzyme, was evaluated in terms of degree of hydrolysis (DH) as the ratio of reducing sugar (*Rds*) / total sugar (*Ts*) (in percentage) as follows: (*Rds after hydrolysis* / *Ts after hydrolysis*)  $\times$  100.

### Degree of Syneresis (DS)

The freeze-thawed samples were then subjected to a vacuum filtration. Exudates water from triplicate retrograded gel samples was collected and weighed. The weight of retrograded gel and water was used to calculate degree of retrogradation by the equation (Tovar *et al.*, 2002):

$$DS (\%) = (\text{Weight of exudates water} / \text{Weight of retrogradation gel}) \times 100.$$

### Scanning electron microscopy (SEM)

The native high amylose rice starch, annealed and 75°C heated starch, 16-hr debranching and RS III samples were dried to less than 4% and milled to fine powder and then deposited on a copper disc and coated with gold. The specimens were examined by scanning electron microscope (JM-560LV model). All samples were observed at 6,000x magnification.

### X-ray diffraction

X-ray diffraction patterns of native rice starch and the RS III samples were measured with copper K<sub>2</sub> radiation ( $\lambda = 0.154$  nm) using a diffractometer (JEOL, JDX-3530, Japan). Diffractometer was operated at 300 mA and 30 kV, 2 $\theta$  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 to 30° Theta.

### Resistant starch and estimated glycemic index analysis

Resistant starch (RS) was determined using a Megazyme Resistant Starch kit (AOAC Method 2002.02). The samples were incubated in a shaking water bath with pancreatic  $\alpha$ -amylase and amyloglucosidase for 16-hr at 37°C to hydrolyse 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 for two times to remove the digested starch (DS). The sediment was solubilized in 2ml of 2 M KOH in an ice bath, neutralized with 8 ml sodium acetate (1.2 M) and the RS hydrolyzed to glucose with enzyme amyloglucosidase (0.1 ml, 3300 Uml<sup>-1</sup>, 50°C). The glucose oxidase/peroxidase reaction was used to measure glucose released from the digested starch and resistant starch. Absorbance was read at 510 nm after a 20 minute incubation period at 50°C. RS and DS were calculated as glucose  $\times$  0.9. The total starch was calculated as the sum of RS and DS.

*In vitro* starch hydrolysis and glycemic index (GI) were determined according to Gōni, *et al.*, (1997). An equation:  $C = C_{\infty}(1 - e^{-kt})$  was used to describe the kinetics of starch hydrolysis, where *C*, *C<sub>∞</sub>* 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 released from white bread. The glycemic index of the samples was estimated according to the equation:  $GI = (39.71 + 0.549) \times HI$ .

### Experimental Design and Statistical Analysis

The data obtained for the pullulanase hydrolysis, physicochemical, resistant starch, hydrolysis index and glycemic index were subjected to analysis of variance (ANOVA). Duncan's multiple range tests (DMRT) procedure was used to make specific comparison between treatments. Pearson correlations between dependent variable were investigated.

## RESULTS AND DISCUSSION

### Chemical composition of native rice starch

Carbohydrate, protein, fat, ash and moisture content on wet weight basis of high amylose rice starch were  $80.38 \pm 0.52\%$ ,  $1.18 \pm 0.06\%$ ,  $1.0 \pm 0.34\%$ ,  $1.0 \pm 0.28\%$ , and  $13.0 \pm 1.05\%$ , respectively. Total starch and amylose content on dry weight basis were  $95.21 \pm 1.2\%$  and  $32.10 \pm 0.67\%$ , respectively.

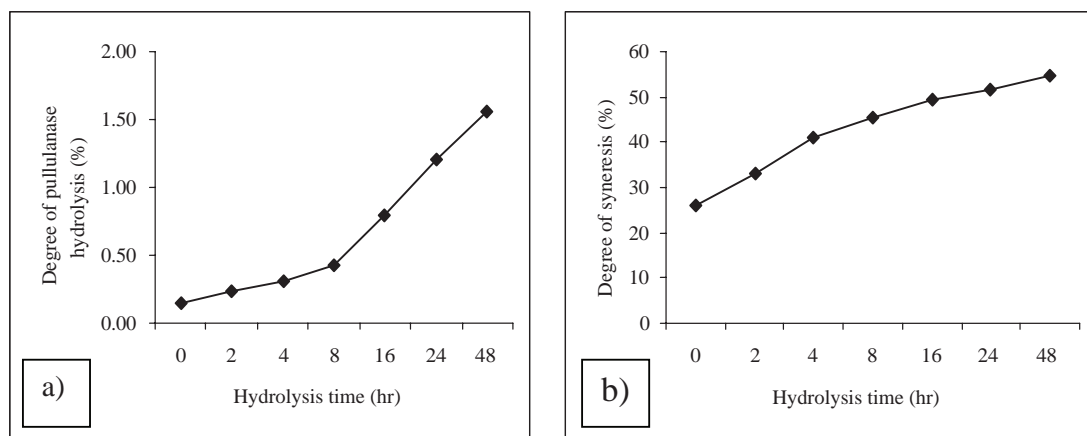
### Degree of pullulanase hydrolysis and degree of syneresis

Degree of hydrolysis and degree of syneresis from pullulanase hydrolysis of high amylose rice starch are shown in Figure 1. The degree of hydrolysis obtained at various debranching time which related to the number of

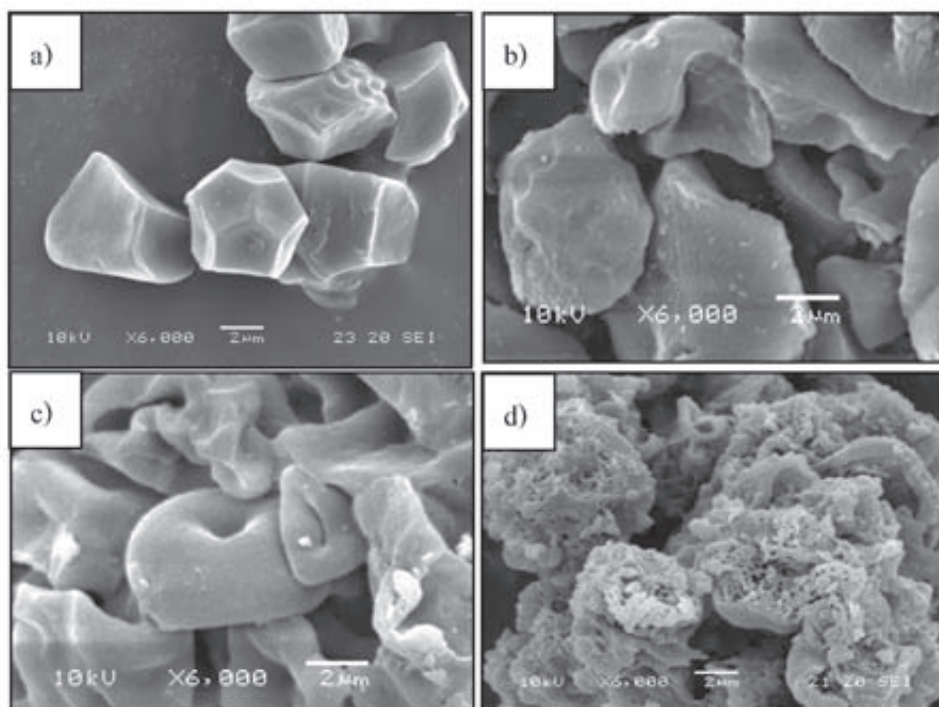
reducing groups produced by action of pullulanase hydrolysis on rice starch from 0 to 48 hr were increased from 0.44 to 1.55%, respectively (Figure 1a). The high degree of pullulanase hydrolysis can be attributed to the high degree of syneresis (28.10 to 54.53%). It could be due to the pullulanase enzyme hydrolysis of  $\alpha$ -1,6-glucosidic bonds and released linear polymers linked by  $\alpha$ -1,4-glucosidic bonds. These fragments could rapidly produce retrograded starch. In addition, the higher degree of retrogradation upon aging and freeze-thaw process was due to a progressive re-association of the starch molecules. This recrystallization could reduce digestibility of the starch (Tovar *et al.*, 2002).

### Morphological properties

SEM micrographs of native high amylose rice starch, annealed and 75°C preheated, 16-hr debranched of 75°C preheated and RS III samples are shown in Figure 2. Native high amylose rice starch granules have polygonal shapes with diameter between 3-5  $\mu\text{m}$  (Figure 2 a). The surface of the native granules was smooth without observable pores. The annealed and 75°C preheated starches (Figure 2 b) exhibited swelling and partial gelatinization at the surface of the starch granules. This might permit the enzyme to access



**Figure 1** Degree of pullulanase hydrolysis (a) and degree of syneresis (b) from pullulanase hydrolysis of 75°C preheated high amylose rice starches from 0 to 48 hr at 55°C.



**Figure 2** Scanning electron micrographs (magnification at 6000x) of native (a), annealed and 75°C preheated (b), 16-hr debranched of starch preheated at 75°C (c) and resistant starch type III (d).

to the molecules more easily and uniformly debranched the amylopectin molecules. After pullulanase hydrolysis for 16 hr, the starch granules showed surface erosion and slightly damaged (Figure 2 c). When the retrogradation starch drying it's shown a coarse honeycomb-like and filamentous network structure as observe with SEM micrographs (Figure 2 d).

#### Starch digestibility and glycemic index

Resistant starch (RS) is used as a predictor of the release rate of glucose and glycemic index (GI), which can be predicted through *in vitro* starch hydrolysis model (Göni *et al.*, 1997; Englyst *et al.*, 1999). The RS content of RS III from 48 hr pullulanase hydrolysis was significant ( $P \leq 0.05$ ) higher than the 0 to 24 hr hydrolysis time (Table 1). The RS contents at the 0 to 48 hr hydrolysis were 4.80, 5.88, 6.61,

9.05, 11.24, 11.43 and 12.33 % dwb, respectively. The digestible starch content was decreased with the increasing resistant starch content. Corresponding to the degree of pullulanase hydrolysis results, the rate and extent of starch hydrolysis and glycemic index were different among RS III formation (Table 1). The high RS content (12.33%) in the RS III samples was highly resistant to hydrolysis, and hydrolysis was complete in a low degree (35.61%) after 180 min, whereas 62.70% of the non debranched samples had been hydrolyzed. In addition, the GI based on HI was between 59.29 to 74.28%. The high resistant starch content in 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). For most starchy food products, a reduction in GI appears to be accompanied by a

**Table 1** Resistant starch, digestible starch, total starch content, hydrolysis index and glycemic index of resistant starch type III from pullulanase debranched high amylose rice starch.

Hydrolysis time (hr)	Resistant starch (% dwb)	Digested starch (% dwb)	Total starch (% dwb)	Hydrolysis index (%)	Glycemic index (%)
0	4.80 <sup>e</sup>	90.39 <sup>a</sup>	95.54 <sup>ns</sup>	62.70	73.47 <sup>a</sup>
2	5.88 <sup>de</sup>	88.55 <sup>b</sup>	95.24	53.75 <sup>b</sup>	64.43 <sup>b</sup>
4	6.61 <sup>d</sup>	89.05 <sup>b</sup>	95.66	46.49 <sup>c</sup>	63.10 <sup>c</sup>
8	9.05 <sup>c</sup>	85.99 <sup>c</sup>	95.04	41.54 <sup>d</sup>	62.55 <sup>d</sup>
16	11.24 <sup>b</sup>	84.04 <sup>d</sup>	95.28	39.09 <sup>e</sup>	61.20 <sup>e</sup>
24	11.43 <sup>b</sup>	84.15 <sup>d</sup>	95.26	36.12 <sup>f</sup>	59.87 <sup>f</sup>
48	12.33 <sup>a</sup>	83.52 <sup>e</sup>	95.86	35.61 <sup>g</sup>	59.29 <sup>f</sup>
Native HARS	6.52 <sup>d</sup>	88.67 <sup>d</sup>	95.20	46.26 <sup>c</sup>	65.10 <sup>b</sup>

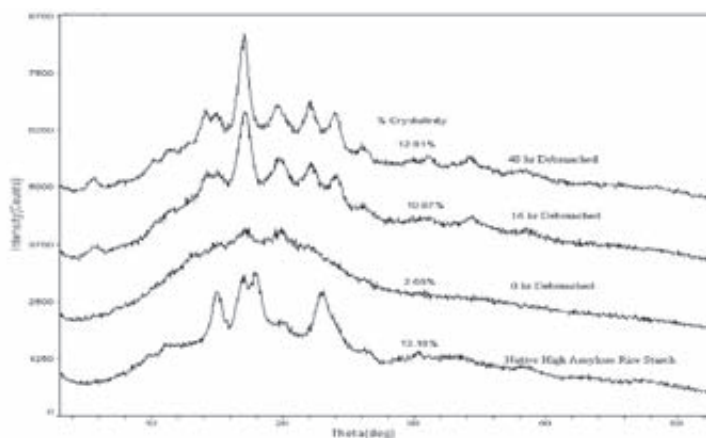
\* Mean values in the same column with different letters are significantly different ( $p \leq 0.05$ )

higher content of resistant starch (Bjorck *et al.* 2000).

#### Crystallinity of RS III by X-ray diffraction

X-ray diffraction patterns of native rice starch and three RS III samples (0, 16 and 48-hr pullulanase hydrolysis and freeze thaw process) are shown in Figure 3. The diffraction pattern obtained from native rice starch was classified as an A-type pattern as indicated by typical peaks at 15.0, 17.5, 20.0 and 23.2° of diffraction angle  $2\theta$ . The calculated crystallinity of native rice starch was 13.18%. The value was in agreement with

those reported for native rice starch (Ornanong *et al.*, 2006). When the HARS was subjected to pullulanase debranching and syneresis treatments, the 0, 16 and 48 hr hydrolysis treatments, the results showed totally different diffraction pattern, from the native rice starch. The RS III sample from non debranching showed very low degree of crystallinity (2.58%) due to the loss in crystallinity during thermal treatment. The RS III formation from 16 and 48 hr hydrolysis displayed V-type diffraction pattern, with 10.67 and 12.91% crystallinity, respectively. This was attributed to debranching and retrogradation which reorganized

**Figure 3** X-ray diffraction patterns of native and RS III samples from debranched and retrograded of high amylose rice starch.



the structure of starch into a helical complex to that of V-amylose (Cui and Oates, 1999). Occurrence of V-diffraction patterns is caused by the presence of amylose and lipids in the starting material.

### CONCLUSION

The results obtained in this study showed that the high degree of pullulanase debranched were closely related to high degree of retrogradation and resistant starch content of the RS III formation. The RS contents of the 0 to 48 hr hydrolysis were 4.80 to 12.33%. X-ray diffraction patterns of the RS III from 16 and 48 hr hydrolysis showed more crystalline structure with V-type pattern. The RS III sample formed a coarse honeycomb-like and filamentous network structure was observed by Scanning Electron Micrograph. The estimated hydrolysis index and glycemic index value of the RS III samples were between 35.61 to 62.70% and 59.29 to 73.47% of GI values, respectively.

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