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Properties of Pullulanase Debranched Cassava Starch and Type-III Resistant Starch

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ABSTRACT

The objective of this study was to produce type-III resistant starch (RS-III) by the pullulanase reaction. A 10% (dry weight) cassava starch suspension adjusted to either pH 5.0 or 5.5 was gelatinized at 120°C for 30 min. Pullulanase of 3%, 5% and 10% (v/w) of starch weight was added to debranch the starch and placed in a water bath of 50°C for hydrolysis periods of 2 to 24 h. The resulting starch was retrograded by a cooling of 4°C and dried by hot air at 40°C. The starch products were determined for reducing sugars, amylose, RS-III, and for *in vitro* starch digestibility, as well as changes in the structural properties. The results showed that the reducing sugars obtained with any treatments tended to increase with the length of the reaction time. The starches treated at pH 5.0 or pH 5.5 and hydrolyzed with 5% pullulanase for 8 h had significantly higher reducing sugars of 1.57±0.1 and 3.43±1.2 g/100g, respectively, than the initial content (0.62±0.0 g/100g) and showed more effect than the 3% pullulanase. At pH 5.0, the starches hydrolyzed with 3 and 5% pullulanase for 8 h gave a higher amylose content than those treated at pH 5.5. An acidity effect of pH 5.0 related to the content of RS-III formed over the reaction time of 8 h, showing a high value of 12.8±1.3 and 17.4±1.5 g/100g for the 3% and 5% pullulanase, respectively. When the starch was reacted with 10% pullulanase for 8, 16 and 24 h, a significant increase in the RS-III from the gelatinized cassava starch (9.2 ± 0.0 g/100g) resulted with a value of 41.2±3.5, 45.8±2.5 and 42.5±1.3 g/100g, respectively. This result also related to the *in vitro* starch digestibility of the RS-III samples being about 20 to 30 % slower than the starting starch after 90 min of amylase digestion. Finally, the structural changes to the type-B crystallites via type-C and a mixture of the V-type, as well as the scanning electron micrographs of the RS-III could confirm its property of slow enzymatic digestion. Thus, conditions of pH 5.0, hydrolysis of 10% pullulanase for 24 h and hot air drying were suitable for partially debranching amylopectin of the cassava starch, consequently providing small linear fragments and small clusters of the amylopectin for recrystallization and formation of the RS-III.

Key words: cassava starch, type-III resistant starch, pullulanase, *in vitro* starch digestibility, structural properties

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INTRODUCTION

At present, consumers are showing increasing interest in choosing healthy, functional, food products. Resistant starch (RS) plays a major role in the health-food industry because it behaves with properties similar to soluble and insoluble dietary fiber in the gastrointestinal tract. As it is resistant to human digestive enzymes, a slow release of glucose results in reduced energy intake by the intestinal cells, which is evident by the low glycemic index of the non-digested starch. This can help to improve glucose regulation in diabetes and better weight control for the obese (Ohr, 2004). The non-digestible starch in the large intestine is fermented by colonic microflora producing short chain fatty acids that encourage the growth of beneficial bacteria, indicating a prebiotic functionality. This may lead to healthier colon cells and reduce the development of colon cancer. In addition, a diet high in RS can reduce blood cholesterol and triglyceride levels because of higher excretion rates of cholesterol and bile acids. Overall, increasing RS content in the diet has the potential to provide several significant health benefits and value to food products (Wursch, 1999; Croghan, 2004).

RS is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of a healthy individual (Englyst *et al.*, 1992). There are four types of RS. Type I represents physically inaccessible starch, which is locked in the plant cell walls of some foodstuffs, such as partially milled grains, seeds and legumes. Type II is native granular starch found in food containing uncooked starch, such as bananas, raw potatoes and beans. Type III resistant starch (RS-III) is made up of retrograded starch or crystalline non-granular starch, like the starch found in cooked and cooled potatoes, bread crust, cornflakes and retrograded, high-amylose maize starch. Type IV refers to specific chemically and thermally modified or repolymerized starches (Englyst *et al.*,1992; Eerlingen and Delcour, 1995).

Generally, it is known that RS-III is formed when the linear amylose fraction of starch is retrograded or recrystallized after the gelatinization of starch and debranching enzymatic conversion of amylopectin to linear molecules. This refers to the RS-III, which joins with short linear segments of α-(1-4)-glucans arranged in a crystalline structure. A study on RS formation showed that as the amylose fraction increased, RS yield increased. The formation was affected by the water content of the starting starch suspension, autoclaving temperature, conditions of enzymatic reaction and the cooling and drying process, as well as by the added ingredients such as lipids, sugars and salts (Eerlingen and Delcour, 1995).

Commercially, the starches used in preparing RS-III are derived from high amylose corn starch containing greater than 40% amylose. The process consisted essentially of the steps of gelatinizing a slurry of the starch, treating the gelatinized starch with a debranching enzyme, deactivating the enzyme, cooling and isolating the starch product (Chiu *et al.*,1994; Shi and Jeffcoat, 2003; Schmiedel *et al.*, 2003). In Thailand, cassava starch is a major commercial product used for starch modification. Apparently, native cassava amylose ranges from 19.6% to 24.1%, depending on the time of harvest from 6 to 10 months, whereas cassava amylose from a commercial starch sample was 28 to 30% (Vatanasuchart *et al.*, 2005).

Thus, a process for RS-III formation should be carried out on cassava starch, which has potential to be utilized as a food ingredient for manufacturing health food. The objective was to determine the enzymatic process for preparing RS-III from commercial cassava starch. The effects of the acidity of the starch suspension, pullulanase reactions, cooling and hot air drying on the contents of reducing sugar, amylose and RS-III were examined. The *in vitro* starch digestibility and changes in the structural properties of the RS-III obtained were also observed.

MATERIALS AND METHODS

Process for RS-III production

Commercial cassava starch donated by the Taiwa Public Co. Ltd. (Thailand) was used for the RS-III production. The procedure for RS-III production with pullulanase hydrolysis cooling and hot air drying is shown below. The resulting starch product was screened and sieved through 100 mesh size before determinations.

Weigh 20 g commercial cassava starch (dry weight basis) into a glass bottle with a cap
↓
Bring to total of 200 g with distilled water to obtain 10 % starch suspension (w/w)
↓
Adjust the acidity of the starch suspension to pH 5.0 and 5.5
↓
Gelatinize by pressure cooking at 120°C for 30 min
↓
Cool down to 50°C by placing at room temperature
↓
Add 3%, 5% and 10 % pullulanase (Promozyme 400PUN/ml, Sigma No.P2986) of the starch weight, to debranch the amylopectin molecules
↓
Place the sample bottles in a shaking water bath controlled at 50°C
↓
Allow to hydrolyze for periods of 2, 4, 6, 8, 16 and 24 h
↓
Deactivate the enzyme by heating the hydrolyzed samples at 90°C for 30 min
↓
Retrograde the resulting starch by placing in refrigerator at 4°C for 24 and 48 h
↓
Centrifuge at 4500 rpm for 15 min and collect the RS-III
↓
Dry the starch by conventional hot air at 40°C for 8 to 12 h
↓
Determinations

Determination of reducing sugars and amylose content

The contents of the reducing sugars in the cassava starches among the treatments were examined using the Nelson method (Ghose and Bisaria, 1987). The colorimetric method based on an amylose and iodine complex was used for determining the changes in amylose content of the treated cassava starch samples. Standards of amylose (Sigma No. A0512) and amylopectin (Sigma No. A8515) and other chemical reagents from Merck, Germany were used following the AACC method (2000).

Determination of resistant starch (RS)

RS was determined by the direct method of Goni *et al*. (1996). Ground samples (100 mg) were incubated with a solution containing 20 mg pepsin (Merck No. 7190, 2000FIT-μ/g) at 40°C for 60 min to remove any protein. Tris-maleate solution containing 40 mg pancreatic α-amylase (Sigma No.A-3176, 23 IU/mg) was added and incubated at 37°C for 16 h to hydrolyze digestible starch. The hydrolysates were centrifuged and the residues were solubilized by dispersing in 4 M KOH and incubated with 80 μl amyloglucosidase (Boehringer No.102857) at 60°C for 45 min to hydrolyze RS. The glucose content was measured using a glucose oxidase-peroxidase kit (Sigma No. G3660) and the RS was calculated as mg of glucose x 0.9.

The *in vitro* starch digestibility test

The RS from the commercial cassava treated with hydrolysis times of 8 and 24 h and cooling of 24 and 48 h were determined for the *in vitro* rate of starch digestibility using the method of Goni *et a*l. (1997). Ground starch samples (50 mg) were incubated with a solution containing 20 mg pepsin at 40°C for 60 min. To remove the protein and Tris-maleate, a solution containing 3.3 IU pancreatic α-amylase was added and incubated at 37°C to hydrolyze digestible starch.

One ml aliquot samples were taken from each tube every 30 min from 0 to 180 min and were placed in a tube at 100°C to inactivate the enzyme. Then, 60 µl amyloglucosidase was added to hydrolyze the digested starch into glucose at 60°C for 45 min. The glucose content was measured using the glucose oxidase-peroxidase kit and the hydrolyzed starch was calculated as mg of glucose x 0.9. The rate of starch digestion was expressed as the percentage of total starch hydrolyzed at different times of 30, 60, 90, 120, 150 and 180 min.

Structural properties

Wide angle X-ray diffraction patterns of the RS-III were examined using a JEOL, JDX 3530, Japan. The degree of relative crystallinity was calculated from the ratio of the diffraction peak area and the total diffraction area (Shamai *et al*. 2003). The microscopic observation of starch granules was performed using scanning electron microscopy (SEM) with a JEOL, JSM 5600LV, Japan at magnifications of 100X and 500X (Vatanasuchart *et al*., 2005).

Statistical analysis

The SPSS for Windows program, version 10 was employed to analyze the results obtained from at least two replications. Means and standard deviations for the reducing sugars, the amylose and the RS-III of the treated samples were calculated and analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used for comparing differences of the mean values at $p \leq 0.05$.

RESULTS AND DISCUSSION

Changes in reducing sugars and amylose of the pullulanase hydrolyzed cassava starches

Table 1 shows the effects of pH (5.0 and 5.5) of starch suspension and pullulanase concentrations (3% and 5%) on the reducing sugars obtained from the process of the enzymatic reaction. The results showed that the reducing sugars obtained with any treatments tended to increase with the length of the reaction time. For both enzymatic treatments, only pH 5.5 with 5% pullulanase had more effect on the reducing sugars than treating at pH 5.0 when the reaction time reached 8 h. Also the starches treated at pH 5.0 or pH 5.5 and hydrolyzed with 5% pullulanase for 8 h had significantly higher amounts of reducing sugars, 1.57±0.1 and 3.43±1.2 g/100g, respectively, than the initial content (0.62 g/100g). The starch treated at pH 5.5 and hydrolyzed with 5% pullulanase for 2, 4, 6 and 8 h had a reducing sugar content of 1.72±0.0, 2.75±0.1, 1.71±0.0 and 3.43±1.2 g/100g, respectively; these values were relatively higher than those obtained with the treatment at pH 5.0. A study by Guraya *et al*. (2001) pointed out that the reducing value was a good indicator of the pullulanase debranching process and after retrogradation of starch, a slow digestible starch (SDS) and resistant starch (RS) were produced. A higher concentration of 10g of pullulanase per 100 g non-waxy rice starch was more suitable for producing RS than SDS when debranching time increased from 2 h to 24 h.

Table 1 Content of reducing sugars (g/100g dry weight) of the treated cassava starches [1].

Hydrolysis times (h)	3% Pullulanase		5% Pullulanase	
	pH 5.0	pH 5.5	pH 5.0	pH 5.5
0	0.62 ± 0.0 [a]	0.62 ± 0.0 [a]	0.62 ± 0.0 [a]	0.62 ± 0.0 [a]
2	1.29 ± 0.1 [b]	1.90 ± 0.0 [c]	1.16 ± 0.0 [b]	1.72 ± 0.0 [b]
4	1.74 ± 0.0 [d]	2.00 ± 0.0 [c]	1.12 ± 0.1 [b]	2.75 ± 0.1 [c]
6	1.55 ± 0.1 [c]	1.48 ± 0.0 [b]	1.26 ± 0.0 [c]	1.71 ± 0.0 [b]
8	1.50 ± 0.0 [c]	1.63 ± 0.1 [b]	1.57 ± 0.1 [d]	3.43 ± 1.2 [d]

[1] Within a column, means not sharing a common superscript are significantly different at $p \leq 0.05$.

The results in Table 2 illustrate the effects of pH (5.0 and 5.5) of starch suspension and pullulanase concentrations (3% and 5%) on amylose when determined after treatment of pullulanase, cooling and hot air drying. At pH 5.0, the starches treated with 3% and 5% pullulanase for 4, 6 and 8 h showed no significant difference in amylose content, but were lower than the initial content of 31.8±2.6 g/100g. The hydrolysis of 6 and 8 h produced a higher amylose content in comparison with those treated at pH 5.5. For the starches treated at pH 5.0 and hydrolyzed for 6 and 8 h, the amylose contents were 27.5±0.3 and 27.4±0.1 g/100g when hydrolyzed with 3% pullulanase, and 23.2±1.9 and 20.3±0.3 g/100g when hydrolyzed with 5% pullulanase, respectively. This study found that treatments involving a pullulanase reaction caused a reduction in the amylose contents. The explanation is that amylose molecules of cassava starch are composed of some branching sites, which could be degraded by the debranching enzyme, as reported by Sriroth and Piyachomkwan (2003). In addition, this finding showed that amount of the amylose obtained by pullulanase reaction was not related to the formation of the RS-III, which was in contrast to a study by Eerlingen and Declour (1995) reported that the amyloses from different cultivars of rice were positively correlated to RS yield. Whereas a study on sago starch by Wong *et al*. (2005), found that raw sago starch was resistant to the action of pullulanase but caused an increase in the linear long chain dextrin representing a high amylose starch content of 33.2%.

Formation of RS-III by the pullulanase reactions

In this study, the RS of commercial cassava and gelatinized starch (50% solid) were compared to those obtained from the pullulanase treatment over a reaction time of 8 h. At pH 5.0 the RS-III quantities obtained with 3% (12.8±1.3 g/100g) and 5% (17.4±1.5 g/100g) pullulanase were significantly higher than those of the gelatinized cassava starch (9.2±0.0 g/100g) and the starches treated at pH 5.5 (Table 3).

Generally, pullulanase, the debranching enzyme, is frequently used to hydrolyze α-1,6 linkages in amylopectin of starch in order to eliminate amorphous regions, thus short linear segments of α-(1-4)-glucans were arranged in a crystalline structure (Guraya *et al*., 2001). The pullulanase enzyme preferably reacts with a pH from 4.5 to 5.5 at a temperature of 40°C to 60°C. The resulting retrograded starch or RS-III can be isolated by dehydrating or drying with different methods, such as hot air, spray or freeze drying, as well as by the extrusion process. A study on processes for making RS-III from high amylose starch found that extrusion yielded higher levels of RS than the methods of hot air and spray drying, being 30.0, 21.5 and 14.3%, respectively (Chiu *et al*., 1994).

When treatments of 10% pullulanase and an increased reaction time to 24 h were employed for RS-III production (Table 4), the amylose of the starches treated at pH 5.0 and 5.5 tended to decrease with the length of the reaction time. The RS-III obtained was higher than that treated with

Table 2 Changes in the amylose (g/100g dry weight) of the treated cassava starches [1].

Hydrolysis times (h)	3% Pullulanase		5% Pullulanase	
	pH 5.0	pH 5.5	pH 5.0	pH 5.5
0	31.8 ± 2.6 [a]	31.8 ± 2.6 [a]	31.8 ± 2.6 [a]	31.8 ± 2.6 [a]
2	27.8 ± 0.6 [ab]	27.9 ± 0.3 [b]	28.8 ± 0.1 [a]	24.4 ± 0.0 [b]
4	25.9 ± 2.6 [b]	27.1 ± 1.7 [b]	23.1 ± 0.1 [b]	25.1 ± 0.1 [b]
6	27.5 ± 0.3 [ab]	18.6 ± 0.6 [c]	23.2 ± 1.9 [b]	22.9 ± 2.4 [b]
8	27.4 ± 0.1 [ab]	22.6 ± 0.2 [d]	20.3 ± 0.3 [b]	16.6 ± 1.9 [c]

[1] Within a column, means not sharing a common superscript are significantly different at $p \leq 0.05$.

the 3% and 5% pullulanase. The starches treated at pH 5.0 and hydrolyzed with 10% pullulanase for 8, 16 and 24 h showed a significant increase in the RS-III of 41.2±3.5, 45.8±2.5 and 42.5±1.3 g/100g, respectively, as compared to that of the gelatinized cassava starch (9.2±0.0 g/100g). In this regard, pH 5.0 contributed to a more preferable pullulanase reaction for RS-III production than pH 5.5, in particular with high concentration of 10% pullulanase. The amylose molecules, which were degraded by 10% pullulanase enzyme at some branching sites, involved more linear fragments for RS-III formation.

Considering the acidity (pH 5.0 and 5.5) of cassava starch treated together with low (3% and 5%) and high (10%) concentrations of pullulanase, the RS-III formed with the low pullulanase concentration over hydrolysis of 8 h would mainly result from the recrystallized starch products of amylopectin molecules. This occurred because at the beginning stage of 8 h, a fast hydrolysis of the amylopectin by pullulanase occurred at the α-1,6 linkages in amorphous regions, but a slower degradation was shown with more confined structure of amylose, as reported by Oates (1997). However, the pH 5.0 treatment had less effect on reduction of the amylose from the initial content than the pH of 5.5 did.

Furthermore, a distinctive reduction of the amylose was observed with the starch of pH 5.0 when treated with the 10% pullulanase, particularly when the debranching reaction was extended to a longer period of 24 h. Thus, the resulting starch materials could be contributed from both amylopectin and amylose molecules and had a substantial effect on the RS-III formation. Conclusively, the optimum conditions of pH at 5.0 and hydrolysis of 10% pullulanase for 24 h should

Table 3 RS-III formation by the pullulanase reactions for 8 h[1].

Sample treatments	RS-III contents (g/100g, dry weight)
Commercial cassava starch	58.2 ± 1.3 [a]
Gelatinized cassava starch	
10% solid	0.0
50% solid	9.2 ± 0.0 [b]
3% pullulanase reaction	
pH 5.0	12.8 ± 1.3 [c]
pH 5.5	7.0 ± 2.6 [b]
5% pullulanase reaction	
pH 5.0	17.4 ± 1.5 [d]
pH 5.5	13.0 ± 1.3 [c]

[1] Within a column, means not sharing a common superscript are significantly different at $p \leq 0.05$.

Table 4 Changes in the amylose and resistant starch contents (g/100g dry weight) of cassava starches by the 10% pullulanase reactions[1].

Hydrolysis times, (h)	Amylose		RS-III	
	pH 5.0	pH 5.5	pH 5.0	pH 5.5
0	31.8 ± 2.6 [a]	31.8 ± 2.6 [a]	9.2 ± 1.3 [a]	9.2 ± 1.3 [a]
8	19.7 ± 0.6 [b]	24.2 ± 0.3 [b]	41.2 ± 3.5 [b]	32.4 ± 1.4 [b]
16	15.6 ± 4.5 [bc]	13.9 ± 1.3 [c]	45.8 ± 2.5 [b]	30.4 ± 4.1 [b]
24	8.5 ± 0.7 [c]	18.9 ± 0.7 [d]	42.5 ± 1.3 [b]	39.0 ± 0.0 [c]

[1] Within a column, means not sharing a common superscript are significantly different at $p \leq 0.05$.

be most suitable for debranching the cassava starch molecules and providing more linear fragments and small clusters of amylopectin for recrystallization and formation of the RS-III.

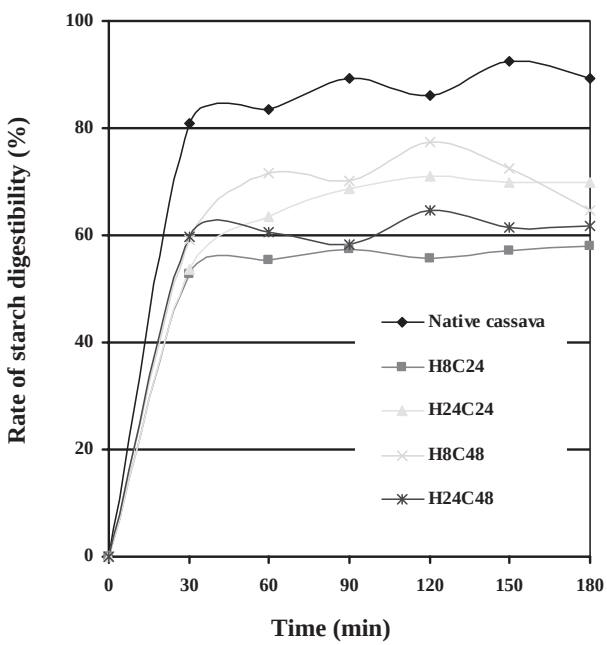
Rate of enzymatic starch digestibility

Table 5 and Figure 1 show the *in vitro* enzymatic starch digestibility of the RS-III produced at pH 5.0 and hydrolyzed with 10% pullulanase for 8 and 24 h, then retrograded by cooling at 24 and 48 h and compared to the commercial cassava starch. With cooling for 24 h, the starches hydrolyzed for 8 and 24 h were similarly resistant to the amylase digestion after

Table 5 Rates of the *in vitro* starch digestibility (% of total starch content) of the RS-III samples produced with different cooling for 24 and 48 h.

Sample treatment	Digestion times (min)					
	30	60	90	120	150	180
Commercial cassava starch	80.8	83.4	89.40	86.2	92.6	89.4
24 h cooling						
8 h hydrolysis	52.9	55.3	57.3	55.7	57.0	58.0
24 h hydrolysis	53.7	63.5	68.6	71.0	69.8	69.8
48 h cooling						
8 h hydrolysis	59.1	71.6	70.1	77.5	72.5	64.8
24 h hydrolysis	59.6	60.5	58.1	64.7	61.4	61.7

[1] Values are means of at least two analyses.



[1] Values are means of at least two analyses.

Figure 1 Rate of *in vitro* enzymatic starch digestibility of the hot-air dried RS-III treated with pullulanase hydrolysis for 8 h (H8) and 24 h (H24), followed by cooling for 24 h (C24) and 48 h (C 48), compared to that of commercial cassava starch.

30 min, showing approximately 52.9% and 53.7% slower digestion rate than the commercial starch, respectively. Obviously, the commercial cassava starch exhibited a very fast starch digestibility as it contains a very pure native starch. Thus, almost full digestion was observed at a rate of 80.8% digestion of the total starch after 30 min of starch digestion. Considering the treatments, the RS-III produced with cooling for 48 h had a faster digestion rate than that treated with cooling for 24 h. This might be caused by a long cooling time of the hydrolyzed starch in excess water resulting in the prevention of the recrystallization of the starch fragments, leading to a disruption of the newly formed resistant structure.

A study on the *in vitro* starch digestibility by Sagum and Arcot (2000) showed that three varieties of raw rice (25.8 to 35.6%), such as *Doongara, Inga and Japonica* were more digestible when boiled (54.3 to 74.6%) or pressure-cooked (65.2 to 75.1%). Also, Anderson *et al.* (2002) hypothesized that when starch is heated to the melting temperature and held at this temperature for a certain time, subsequent cooling and recrystallization would effect a higher degree of starch crystallization, which in turn would result in a decrease in the digestibility of the starch. In addition, the heat moisture treatment induces structural changes and modification of the properties. Therefore, the heat moisture process of rice starches adjusted to 20% moisture and heated to melting temperature (T_m) using DSC would produce heat-stable, slowly digestible rice starch. Results showed that digestibility decreased by 25% and 10%, respectively, for non-waxy and waxy rice starches relative to non-treated starches. In contrast, a conventional oven produced a slight increase in the digestibility of the treated starches.

Likewise, in a study on the effect of the pullulanase debranching enzyme on the formation of slow digestible rice starch, it was found that a high enzyme concentration of 10 g/100 g of starch produced more debranched starch than a lower level did. In addition, the enzyme was fully active after 24 h of incubation, therefore a longer hydrolysis time increased the resistance. Moreover, the incomplete debranching occurred mainly due to the retrogradation of amylose during incubation with the pullulanase; this result was associated with this present study (Guraya *et al.*, 2001).

Structural properties of the RS-III samples

The diffraction patterns of the RS-III samples compared to commercial cassava starch are shown in Figure 2. In general, the cassava starch exhibited a typical A-type pattern with peaks at 2-theta about 23°, 18°, 17° and 15°. When the pullulanase debranched starches were retrograded at 4°C and dried, an enzymatic resistant structure of the RS-III starches was formed and a change in the X-ray diffraction profiles was observed. The RS-III appeared to have a similar typical B-type pattern, indicating peaks at 2-theta of 24°, 22.5°, 17° and 5.5°. In particular, the RS-III clearly showed a transition of crystallinity from an A- to a B-type pattern and resulted in a C-type structure as exhibited by additional peaks at 2-theta of 19.6° and 5.5°. This finding showed that the RS-III profiles had sharper and larger characteristic peaks at 2-theta of 19.6° and 5.5° and a distinctive peak was produced at 2-theta of 13°, which are characteristics of V-type starch.

Therefore, this study found that retrogradation of cassava starch treated with pullulanase led to the formation of a mixture of C- and V-type structures. Whereas, Shamai *et al.* (2003) reported that the slow digestion of corn starch (B-type) with pancreatic α-amylase resulted in larger, ordered, crystalline regions and thus the RS-III residues exhibited the somewhat sharper X-ray patterns of a mixture of A- and V-type polymorphs after retrogradation. Moreover, the RS-III treated with hydrolysis of 8 and 24 h had a degree of relative crystallinity of 33.8% and 38.2%, respectively, when compared to that of the

commercial cassava (36.9%).

Using scanning electron microscopy (SEM) with magnifications of 100X and 500X, microscopic images were examined of the RS-III treated with pullulanase hydrolysis of 8 and 24 h, cooling of 24 and 48 h and dried using hot air. Generally, native cassava starch granules when examined under a microscope were of medium size ranging from 4 to 35 μm and were round or oval with a truncated end. When viewed under SEM, the granules possessed a smooth outer surface and were round with a flat surface on one side, containing a conical pit (Figures 3). In contrast, the RS-III exhibited a stone-like structure of starch materials with a large size approximately between 100 to 200 μm (Figure 4a-d), which was due to aggregation of linear starch fragments after retrogradation and hot air drying. The image of the layered starch fragments looked quite dense and rigid; this may lead to the slow rate of starch digestibility observed with the treatment.

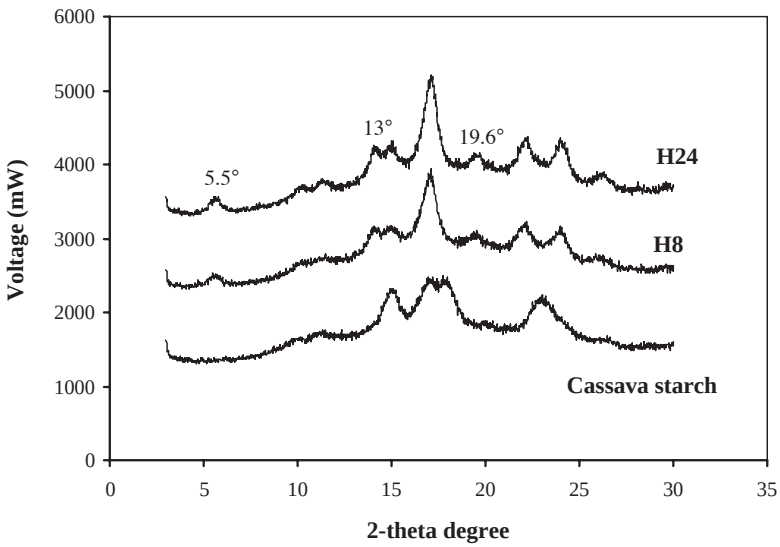


Figure 2 X-ray diffraction patterns of the hot-air dried RS-III of cassava starches treated with 24 h cooling following pullulanase hydrolysis of 8 h (H8) and of 24 h (H24), when compared to cassava starch.

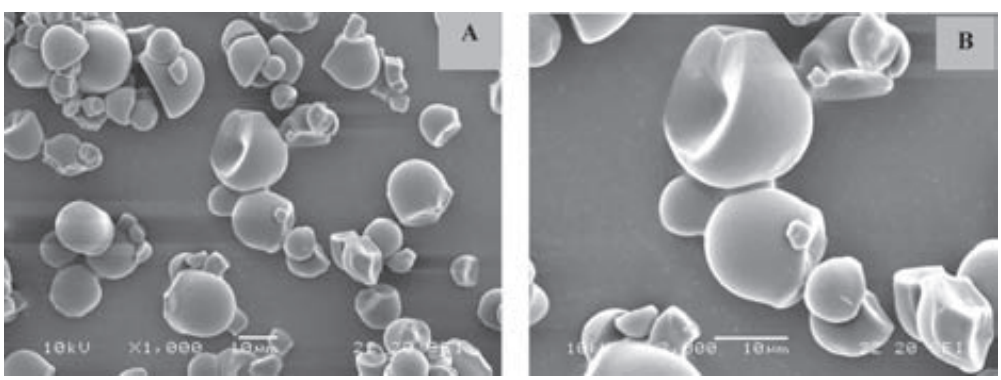


Figure 3 SEMs of commercial cassava starch granules **(A)** 1000X and **(B)** 2000X.

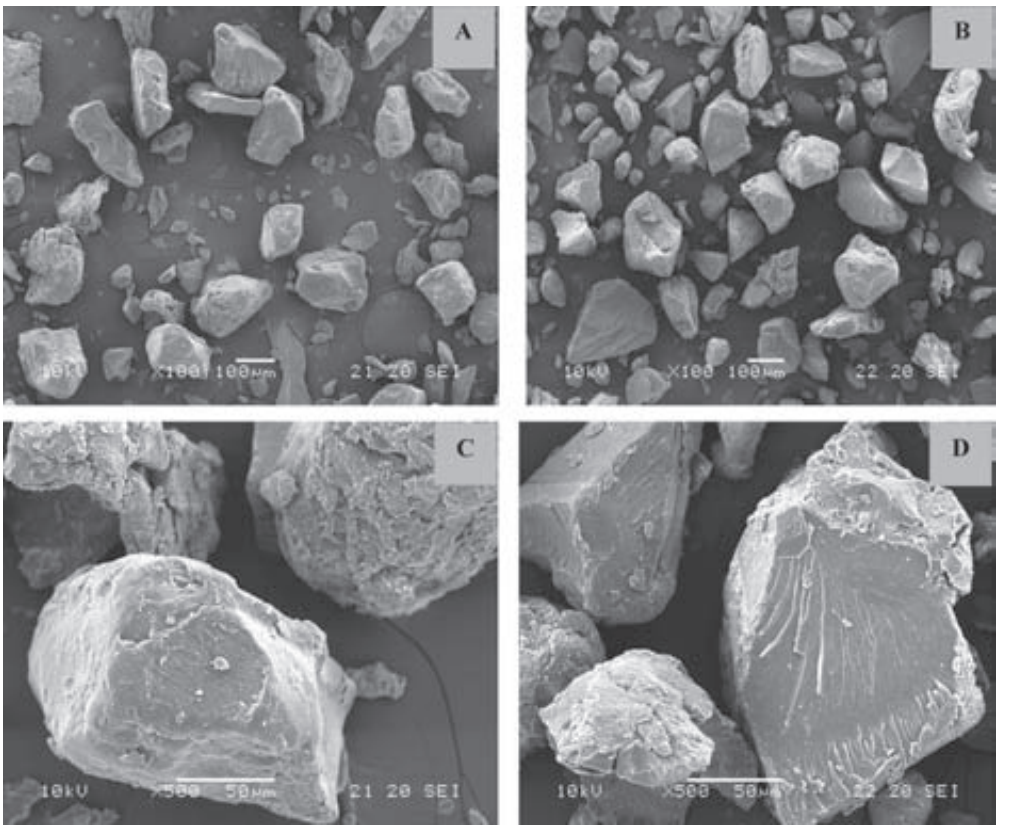


Figure 4 SEMs of the hot air dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 h with magnification of 100X; and **(C)** 8 and **(D)** 24 h with magnification of 500X, following with the 24 h cooling.

CONCLUSIONS

This present study showed that an appropriate condition for the production of the RS-III of cassava starch was pH 5.0 for a starch suspension treated with 10% pullulanase for 8 to 24 h and cooled at 4°C for 24 h, then dried using hot air. This was suitable for partially debranching amylopectin molecules of the cassava starch and consequently providing small linear fragments and small clusters of the amylopectin molecules for retrogradation/recrystallization and formation of the RS-III. The starches treated at pH 5.0 and hydrolyzed with 10% pullulanase for 8, 16 and 24 h showed a significant increase in the RS-III of 41.2±3.5, 45.8±2.5 and 42.5±1.3 g/100g, respectively, as compared to that of the gelatinized cassava starch (9.2±0.0 g/100g). This result related to the *in vitro* starch digestibility of the RS-III obtained, being about 20% to 30% slower than commercial cassava starch, after 90 min of amylase digestion. This finding implied that degradation of the amylopectin molecules to linear chain fragments would contribute to the formation of a less confined structure for melting. Finally, the structural changes to the type-B crystallites via type-C and a mixture of the V- type, as well as the scanning electron micrographs of the RS-III obtained with this study would confirm the property of slow enzymatic starch digestion.

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