รายงานวิจัยฉบับสมบูรณ์

Production Research of Resistant Starch from Cassava by Enzymatic Reaction and Spray Drying Process

Resistant starch หรือสตาร์ชพลังงานต่ำจากแป้งมันสำปะหลังที่ผลิตโดยวิธีการใช้เอนไซม์ร่วมกับการอบแห้งแบบพ่นฝอย

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และ
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ABSTRACT (บทคัดย่อ)

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ชื่อโครงการ Resistant starch หรือสตาร์ชพลังงานต่ำจากแป้งมันสำปะหลังที่ผลิตโดยวิธีการใช้เอนไซม์ร่วมกับการอบแห้งแบบพ่นฝอย (Production Research of Resistant Starch from Cassava by Enzymatic Reaction and Spray Drying Process

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บทคัดย่อ

ได้ศึกษาสภาวะการย่อยสตาร์ชมันสำปะหลังด้วยปฏิกิริยาเอนไซม์ pullulanase และทำแห้งด้วยวิธีการพ่นฝอย (spray drying) ที่มีผลต่อปริมาณ resistant starch (RS-III) อัตราการย่อยสตาร์ช (*in vitro* starch digestibility) การเปลี่ยนแปลงทางเคมีและกายภาพ รวมทั้งลักษณะโครงสร้างสตาร์ช โดยเปรียบเทียบกับการทำแห้งแบบแช่แข็ง (freeze drying) และ ลมร้อน (hot air drying) ทำการเตรียมส่วนผสมสตาร์ชเข้มข้น 10% ของน้ำหนักแห้ง ปรับความเป็นกรดให้ได้ 5.0 และ 5.5 แล้วทำให้สตาร์ชสุก (gelatinization) ด้วยความร้อนจากความดันไอน้ำ แล้วจึงเติมเอนไซม์ pullulanase เข้มข้น 3%, 5% และ 10% เพื่อย่อยแอมิโลเพกทินเป็นเวลา 8, 16 และ 24 ชม. ทำการคืนตัวสตาร์ช (retrogradation) ด้วยการแช่เย็นที่อุณหภูมิ 4°ซ ใช้เวลา 24 และ 48 ชม. และทำให้แห้งด้วยวิธีการต่างๆ ผลการศึกษาพบว่าเมื่อสตาร์ช มันสำปะหลังผ่านการย่อยด้วย 10 % pullulanase และปรับความเป็นกรดที่ pH 5 ย่อยนาน 8 ถึง 24 ชม. จะมีปริมาณ RS-III เกิดขึ้นสูงกว่า การ ใช้ pullulanase ความเข้มข้นต่ำกว่า และ pH 5.5 และพบว่าปริมาณ RS มีความสัมพันธ์กับน้ำตาลรีดิวซิงส์เพิ่มขึ้น และแอมิโลสลดลงจากค่าเริ่มต้น จากผลของวิธีการทำแห้งต่อปริมาณ RS-III พบว่า ระยะเวลาการย่อย 8 ชม. ร่วมกับวิธีลมร้อน ทำให้เกิด RS-III เท่ากับ 43.4 ± 4.6 g/100g และเมื่อย่อยนาน 24 ชม. จะให้ค่าสูงสุด 50.9 ± 2.9 g/100g สำหรับการทำให้เย็นนาน 24 ชม. และยังพบว่าวิธีการพ่นฝอย จะให้ค่า RS สูงเมื่อทำให้เย็นนาน 48 ชม. จากผลการตรวจสอบอัตราการย่อย RS-III ที่ผลิตได้ พบว่าการทำแห้งแบบลมร้อน มีผลให้อัตราการย่อยสตาร์ชต่ำกว่าตัวอย่างอื่นๆ และเมื่อใช้เวลาย่อยนาน 90 นาที พบว่า อัตราการย่อยลดลงจากสตาร์ชทางการค้าร้อยละ 20 ถึง 30 และพบว่าความร้อนที่ใช้ในการสลายโครงสร้างผลึกมีค่าระหว่าง 167.6 ± 0.7 ถึง 188.4 ± 2.8 J/g ซึ่งต่ำกว่าค่า RS ทางการค้า เนื่องจากเป็นโครงสร้างที่เกิดจากการคืนตัวของแอมิโลเพกทินสายสั้นๆ จะไม่แข็งแกร่งเท่ากับ RS-III ที่เกิดจากแอมิโลส เมื่อตรวจสอบโครงสร้างด้วย X-ray diffractograms พบว่า RS-III มี ลักษณะผลึกแบบ B โดยการเปลี่ยนผ่านด้วยลักษณะผลึกแบบ C ซึ่งบ่งชี้ถึงโครงสร้างผลึกที่แข็งแกร่งมากขึ้นโดยเฉพาะตัวอย่าง RS-III ที่ทำแห้งด้วยลมร้อน และ ภาพจาก SEM ก็ให้ผลที่สอดคล้องกัน ขณะที่การทำแห้งแบบพ่นฝอยทำให้เกิดโครงสร้างสตาร์ชขนาดเล็กกว่าแต่มีลักษณะการยึดเกาะตัวรวมกันของชิ้นส่วนที่มีขนาดเสมอกัน

ABSTRACT

This study was aimed to determine an appropriate condition of pullulanase reactions for preparing resistant starch (RS-III) from the commercial cassava starch. Effects of different dryings of spray, freeze and hot air on physical and chemical properties, the *in vitro* starch digestibility as well as structural properties of the RS-III starches were examined. The 10% starch suspension was gelatinized and hydrolyzed by the 3%, 5% and 10% pullulanase of the total starch (db) adjusted to pH 5 and pH 5.5, then allowed to hydrolyzed for 8 to 24 hrs, at 50 °C and cooling for 24 and 48 hrs, at 4 °C then dried using the tested methods. The result showed that a condition of the 10% pullulanase with pH 5 for the hydrolysis of 24 hrs produced the higher RS-III than the other conditions. This was suitable for partially debranching amylopectin molecules of the cassava and consequently providing small linear fragments and small clusters of the amylopectin molecules for retrogradation / recrystallization and formation of the RS-III. Also it was shown that the formation of the RS-III would be related to the increased reducing sugars and the decreased amylose molecules from the initial values. With the effects of different dryings, the hot air drying enhanced the formation of RS-III greater than the spray and freeze dryings. The hydrolysis time of 8 hrs gave a high yield of RS-III of 43.4 ± 4.6 g/100g and the 24 hrs had the highest content of resistant starch of 50.9 ± 2.9 g/100g. This result was related to the *in vitro* starch digestibility of the hot-air dried samples showing about 20% to 30 % slower than the commercial cassava starch, after amylase digestion of 90 min. Moreover the DSC determination showed an endothermic melting enthalpy over a range of 167.6 ± 0.7 to 188.4 ± 2.8 J/g for the RS-III samples which was lower than those of the commercial cassava starch and resistant starch. This finding implied that degradation of the amylopectin molecules to short linear chains would contribute to the formation of less confined structure for melting. Finally, the structural changes of the type-B crystallites and the scanning electron micrographs of the RS-III obtained with this study could confirm its resistance to enzymatic digestion, particularly with the hot-air dried RS-III samples. Whereas the micrographs of the spray dried RS-III exhibited a fine structure of small starch particles aggregated.

EXCLUSIVE SUMMARY

This present study showed that an appropriate condition for production of the RS-III of cassava starch was the 10% pullulanase of the total starch (db) adjusted to pH 5 for the enzymatic hydrolysis of 8 hrs and cooling for 24 hrs, then dried using hot air. This was suitable for partially debranching amylopectin molecules of the cassava and consequently providing small linear fragments and small clusters of the amylopectin molecules for retrogradation / recrystallization and formation of the RS-III. With the effects of different dryings, the hot air drying enhanced the formation of RS-III greater than the spray and freeze dryings. The hydrolysis time of 8 hrs gave a high yield of RS-III of 43.4 ± 4.6 g/100g and the 24 hrs had the highest content of resistant starch of 50.9 ± 2.9 g/100g. This result was related to the *in vitro* starch digestibility of the hot-air dried samples showing about 20% to 30 % slower than the commercial cassava starch, after amylase digestion of 90 min. Moreover the DSC determination showed an endothermic melting enthalpy over a range of 167.6 ± 0.7 to 188.4 ± 2.8 J/g for the RS-III samples which was lower than those of the commercial cassava starch and resistant starch. This finding implied that degradation of the amylopectin molecules to short linear chains would contribute to the formation of less confined structure for melting. Finally, the structural changes of the type-B crystallites and the scanning electron micrographs of the RS-III obtained with this study would confirm its resistance to enzymatic digestion, particularly with the hot-air dried RS-III samples. Whereas the micrographs of the spray dried RS-III exhibited a fine structure of small starch particles aggregated.

Production Research of Resistant Starch (RS-III) from Cassava by Enzymatic Reaction and Spray Drying Process

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INTRODUCTION

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

Resistant starch defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individual (Englyst, Kingman and Cummings,1992). There are four types of resistant starch; type I represents physically inaccessible starch which is locked in plant cell walls of some foodstuffs such as partially milled grains, seeds and legumes. Type II resistant starch is native granular starches found in food containing uncooked starch such as bananas, raw potatoes and beans. Contents of resistant starch of reference banana flour samples determined by three laboratories averaged at 52.1 % dry matter and lentil flour had 8.2% (Goni et al., 1996). Type III resistant starch (RS-III) is 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 (Eerlingen and Delcour, 1995; Englyst, Kingman and Cummings,1992).

Generally it is known that resistant starch 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 type III resistant starch which joins with short linear segments of alpha-(1-4)-glucans arranged in a crystalline structure. A study on resistant starch formation showed that as the amylose fraction increased, resistant starch yield increased. Also the formation was affected by the water content of starting starch suspension, autoclaving temperature, conditions of enzymatic reaction, cooling and drying process as well as the added ingredients such as lipids, sugars and salts (Eerlingen and Delcour, 1995).

In preparing type IV amylase resistant starch, the starches used are commercially derived from high amylose corn starch containing greater than 40% amylose, for example Hylon V and Hylon VII. 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, Henley and Altieri,1994; Shi and Jeffcoat, 2003; Schmiedel, Konig and Jacobasch, 2003). Resistant starch is a very fine powder, white in color and having bland taste, therefore, it is easy to incorporate into a variety of food products without altering their color and flavor. Use of resistant starch as functional food ingredient for producing snack products showed healthful benefits for people with obeses and diabetes (King, 2004).

In Thailand, cassava starch is a major commercial product used in both foods and non foods, such as thickeners, sweeteners, alcohol and paper. Cassava starch contains a very pure starch relatively free from lipid and protein, so it is a good source for starch modification (Sriroth and Piyachomkwan, 2003). Starch is a mixture of two glucose polymers of amylose and amylopectin. 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 commercial starch sample was 28.8%. Also cassava amyloses have beta-amylolysis limit of 75%, indicating them to be slightly branched molecules (Swinkels, 1985; Champagne, 1996).

Thus, process for resistant starch (RS-III) production should be carried out on cassava starch which has a potential to utilize as a food ingredient for health food manufacturing. The objective of this present study was to investigate the enzymatic process for preparing resistant starch from the commercial cassava starch. Effects of the pH, pullulanase

concentration and reaction times of 2 to 24 hours on the contents of reducing sugars, amyloses and resistant starch were determined. Effects of spray drying on contents of resistant starch as well as the *in vitro* starch digestibility of the tested starches were examined and compared to the freeze and hot air treated samples. Lastly the thermal and structural properties also were examined.

MATERIALS AND METHODS

Materials

Commercial cassava starch donated from the Taiwa Public Co. Ltd. (Thailand) was used in this study. The required specification of sulfur dioxide content was lower than 10 ppm by net weight of the cassava starch. Pullulanase (Promozyme 400PUN/ml, Sigma No.P2986) standard of amylose (Sigma No. A0512) and amylopectin (Sigma No. A8515) and glucose oxidase-peroxidase reagents (Sigma No. G3660) were purchased from Sigma Chemical Co. (Japan). Amyloglucosidase was from Boehringer No.102857 and other chemical agents were of analytical grade from Merck (Germany).

Methods

Process for resistant starch production

Weigh 20 g commercial cassava starch (dry basis) into glass bottle with cap

↓

Bring to total of 200 g with distilled water to obtain 10 % starch suspension (w/w)

↓

Gelatinize by pressure cooking at 120°C for 30 minutes and cool down to 50°C

↓

Add 3%, 5% and 10 % pullulanase of the starch weight to debranch the starch molecules

↓

Place the sample bottles in shaking water bath controlled at 50°C

↓

Allow to hydrolyze for periods of 2, 4, 6, 8, 16, 20 and 24 hours

↓

Deactivate the enzyme by heating the hydrolyzed samples at 90°C for 30 minutes.

↓

Determine the content of reducing sugars in the resulting starch product

↓

Cool in refrigerator for 24 and 48 hours

↓

Centrifuge at 4500 rpm for 15 minutes and collect the starch

↓

Dry the starch by spray, freeze drying and conventional hot air

↓

Determinations

Process conditions for drying the RS-III from the cassava starch

Following the process of centrifuging for the RS-III isolation after the enzymatic reaction and cooling, For spray drying process the sediment was brought to a solid concentration of 5% to 8% with distilled water. The starch suspension was homogenized for 3 min. and then dry in a spray drier (Niro Atomizer, Model P-6.3, Copenhagen, Denmark) with feeding rate of 14.5 rpm, inlet temperature of 180°C and outlet temperature of 80°C (Gonzalez-Soto et al., 2006). The spray dried RS-III samples were stored at room temperature in sealed plastic bags. For process of freeze drying (Heto, Model Lyolab 3000, Japan), the treated starches were frozen at -40 °C for about 48 hours before vacuum freeze drying at -50°C for 12 hours, whereas hot air drying was done at 40°C for 12 hours by using conventional hot air oven.

Determination of reducing sugars and amylose contents

The reducing sugars contents of the cassava starches among the treatments were examined by using the Nelson method (Ghose and Bisaria, 1987). Colorimetric method was used for determining the changes in amylose contents of the treated cassava starch samples (AACC, 2000), as shown in Appendix I.

Determination of resistant starch

Resistant starch was determined by the direct method of Goni et al. (1996). Ground samples (100 mg) were incubated with a solution containing 20 mg pepsin at 40°C for 60 minutes to remove protein. Tris-maleate solution containing 40 mg alpha-amylase was added and incubated at 37°C for 16 hours to hydrolyze digestible starch (DS). The hydrolysates were centrifuged and the residues were solubilized and incubated with amyloglucosidase at 60°C for 45 minutes to hydrolyze resistant starch. The content of glucose was measured using the glucose oxidase-peroxidase kit and the resistant starch comprising fractions of RS-III was calculated as mg of glucose x 0.9. The procedure was shown in Appendix I.

***In vitro* digestibility test**

The resistant starch from the commercial cassava treated with different hydrolysis times of 8 and 24 hours and cooling of 24 and 48 hours were determined for the *in vitro* rate of starch digestibility using the method of Goni et al.(1997). Ground starch samples (50 mg) were incubated with a solution containing 20 mg pepsin at 40°C for 60 minutes to remove protein and volume was completed to 25 ml with Tris-maleate buffer. 5 ml Tris-maleate solution containing 3.3 IU alpha-amylase was added and incubated at 37°C to

hydrolyze digestible starch (DS). One ml aliquot samples were taken from each tube every 30 minutes from 0 to 180 minutes 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 minutes. The content of glucose was measured using the 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 the total starch hydrolyzed at different times of 30, 60, 90, 120 and 180 minutes and the RS-III digestibility curve was plotted. The procedure was shown in Appendix I.

Thermal Properties

The RS-III samples obtained with any drying treatments were examined by using a differential scanning calorimeter (DSC) (Mettler Toledo DSC 822, Switzerland), equipped with a cooling system according to the method adapted from Shamai et al. (2003). Sample in a size of 150 µm (7 - 8 mg, db) was weighed in the aluminum DSC pan. The cover was carefully put on and sealed hermetically using sealing tools. Weights of the sealed pans before and after determination were recorded to check for water leakage due to improper sealing. The sample pan was placed carefully in the DSC and was heated at 10°C/min from a temperature of 0°C to 200°C. An empty pan was used as reference and the instrument was calibrated using indium control. Endothermal curves exhibiting onset, peak and end temperatures (°C) and melting enthalpy (J/g of the sample weight, db) of duplicate samples were recorded.

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 diffraction peak area and 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 the magnification of 100X, 500X, 1000X and 2000X (Vatanasuchart et al., 2005).

Statistical analysis

Data obtained for the reducing sugars, the amylose, and the RS-III obtained from all treatments were evaluated and compared to those of the commercial starch. Means and standard deviations for each treatment were calculated and the ANOVA and DMRT test were used for comparing differences of the mean values at $P < 0.05$.

RESULTS AND DISSCUSION

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

Table 1 shows the effects of pullulanase concentrations (3% and 5% of cassava starch weight, dry matter) and pH of cassava starch suspension (5.0 and 5.5) on the contents of reducing sugars obtained form the process of enzymatic reaction. The results showed that the treatment of 5% pullulanase of both acidic conditions showed more affect on the contents of reducing sugars than the 3% pullulanase when the reaction times reached 8 hours. The reducing sugar contents obtained with any treatments tended to increase with the length of the reaction time. As compared to the initial content (0.62 g/100g), the cassava starches treated with 5% pullulanase for 2, 4, 6 and 8 hours had the reducing sugar contents of 1.72 ± 0.0, 2.75 ± 0.1, 1.71 ± 0.0 and 3.43 ± 1.2 g/100g, respectively, for pH 5.5 and were higher than those obtained with the treatment at pH 5.0. As for a study by Guraya et al. (2001), a slow digestible rice starch (SDS) made with pullulanase debranching process was affected by retrogradation of starch and the reducing value test showed a good indicator. And higher concentrations of pullulanase and short debranching times would be more suitable for producing SDS.

The result in Table 2 illustrates the effects of pullulanase concentrations (3% and 5% of cassava starch on dry weight basis) and pH of starch suspension (5.0 and 5.5) on the amylose contents when determined after treatments of pullulanase, cooling and hot air drying. At pH 5.0 the starches treated with 3% and 5% pullulanase for 4, 6 and 8 hours had no significant difference in the amylose contents but lower than the initial content of 31.8 ± 2.6 g/100g. And the 3% and 5% pullulanase hydrolysis of the 6 and 8 hours showed a higher amylose content than those treated at pH 5.5. The starches treated at pH 5.0 gave the amylose contents of 27.5 ± 0.3 and 27.4 ± 0.1 g/100g for the 3% pullulanase, and 23.2 ± 1.9 and 20.3 ± 0.3 g/100g for the 5% pullulanase when hydrolyzed for 6 and 8 hours, respectively (Table 2). This might involve formation of the resistant starch as showing with a higher content of 12.8 ± 1.3 g/100g for the 3% pullulanase and 17.4 ± 1.5 g/100g for the 5% pullulanase when the hydrolysis of 8 hour with pH 5.0 was employed as well. In contrary, a study on sago starch by Wong et al. (2005) found that raw sago was resistant to the action of pullulanase but caused an increase in the linear long chain dextrin representing a high amylose starch of 33.2%.

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

Hydrolysis times (hours)	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 [b]	1.16 ± 0.0 [b]	1.72 ± 0.0 [b]
4	1.74 ± 0.0 [c]	2.00 ± 0.0 [b]	1.12 ± 0.1 [b]	2.75 ± 0.1 [c]
6	1.55 ± 0.1 [d]	1.48 ± 0.0 [c]	1.26 ± 0.0 [c]	1.71 ± 0.0 [b]
8	1.50 ± 0.0 [d]	1.63 ± 0.1 [c]	1.57± 0.1 [d]	3.43 ± 1.2 [d]

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

Hydrolysis times (hours)	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]

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different at the 0.05 level by ANOVA and DMRT.

Table 3 RS-III formation by the pullulanase reactions for 8 hours*.

Sample treatments	Contents of resistant starch (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]

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different at the 0.05 level by ANOVA and DMRT.

Table 4 Changes in the reducing sugars and amylose contents (g/100g dry weight) of cassava starches by the 10% pullulanase reactions*.

Hydrolysis times (hours)	Reducing sugars		Amylose molecules	
	pH 5.0	pH 5.5	pH 5.0	pH 5.5
0	0.62 ± 0.0 [a]	0.62 ± 0.0 [a]	31.8 ± 2.6 [a]	31.8 ± 2.6 [a]
8	0.80 ± 0.0 [b]	2.92 ± 0.1 [b]	19.7 ± 0.6 [b]	24.2 ± 0.3 [b]
16	0.79 ± 0.0 [b]	3.34 ± 0.0 [c]	15.6 ± 4.5 [bc]	13.9 ± 1.3 [c]
24	0.83 ± 0.1 [b]	3.86 ± 0.2 [d]	8.5 ± 0.7 [c]	18.9 ± 0.7 [d]

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different at the 0.05 level by ANOVA and DMRT.

Table 5 RS-III formation with the 10% pullulanase reactions*.

Hydrolysis times (hours)	Resistant starch formation (g/100g dry weight)	
	pH 5.0	pH 5.5
0	9.2 ± 1.3 [a]	9.2 ± 1.3 [a]
8	41.2 ± 3.5 [b]	32.4 ± 1.4 [b]
16	45.8 ± 2.5 [b]	30.4 ± 4.1 [b]
24	42.5 ± 1.3 [b]	39.0 ± 0.0 [c]

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different at the 0.05 level

Effects of the pullulanase reactions on formation of the RS-III

In this study, the RS-III samples obtained with the pullulanase reaction over the debranching time of 8 hours were compared to that of the commercial cassava starch and the gelatinized starch (50% solid) (Table 3). At pH 5.0 the cassava starches treated with 3% (12.8 ± 1.3 g/100g) and 5% (17.4 ± 1.5 g/100g) pullulanase showed a significant higher contents of RS-III than the gelatinized commercial starch (9.2 ± 0.0 g/100g) and the samples treated at pH 5.5 (7.0 ± 2.6 and 13.0 ± 1.3 g/100g).

Moreover a treatment of 10 % pullulanase reaction was studied on the reducing sugars, amylose and resistant starch contents and the results were shown in Table 4 and 5. The reducing sugar contents of the starches treated at pH 5.0 were slightly changed by the lengthy reaction time of 8, to 24 hours. Whereas the amylose contents obtained with the samples treated at pH 5.0 and 5.5 tended to decrease with the length of the reaction time of 8, 16 and 24 hours. This implied that amylose molecules would be degraded by the

pullulanase rection at some branching sites, in relation to a report of the cassava starch amylolysis of 75%.

Table 5 shows the effect of acidity (pH 5.0 and pH 5.5) on formation of the RS-III treated with 10% pullulanase reaction for 8 to 24 hours. At pH 5.0 the 10% pullulanase treated starches showed a significant increase in the contents of resistant starch observed for 8, 16 and 24 hrs (41.2 ± 3.5, 45.8 ± 2.5 and 42.5 ± 1.3 g/100g, respectively), as compared to that of the gelatinized commercial starch (9.2 ± 0.0 g/100g). At pH 5.5, the resistant starch contents for the samples treated for the same reaction period were lower than those treated at pH 5.0. The values were 32.4 ± 1.4, 30.4 ± 4.1 and 39.0 ± 0.0 g/100g, respectively. This present finding indicated that the RS-III could be produced by the pullulanase reaction at pH 5.0 over the periods of 8 to 24 hrs. Thus, the optimum condition of the 10% pullulanase, pH 5.0 and hydrolysis period of 8 hours was more suitable for debranching the cassava starch molecules and providing more linear chains to form the resistant structure of the RS-III from cassava starch.

Generally the debranching enzyme used is pullulanase that will hydrolyze the alpha-1,6 linkages in amylopectin of starch to eliminate the amorphous regions, thus short linear segments of alpha-(1-4)-glucans were arranged in a crystalline structure (Guraya, James and Champagne, 2001). The resulting retrograded starch or RS-III can be isolated by dehydrating or drying with different methods such as hot air drying, spray drying freeze drying or extrusion process. A study on process for making resistant starch from high amylose starch found that extrusion yielded higher levels of resistant starch than the methods of hot air and spray drying; it was 30.0%, 21.5% and 14.3%, respectively (Chiu, Henley and Altieri, 1994).

Effects of drying treatments on the amylose content of the RS-III samples

This experiment was subjected to determine the amylose contents of the RS-III samples effected by spray drying as compared to freeze and hot air drying. The RS-III samples from the commercial cassava starch were processed by the 10% pullulanase hydrolysis for 8 and 24 hours at 50°C and cooling of 24 and 48 hours at 4 °C. As for the adopted process for RS-III production, the cassava amylopectin molecules were debranched and a short linear molecules were retrograded to form a crystalline structure and dried. The results obtained with different drying methods were compared and shown in Table 6. No effect of the different drying methods on the amylose contents was found with the RS-III hydrolyzed for 8 hours with cooling of 24 and 48 hours. However the

obtained amylose contents were significantly decreased with an increased hydrolysis time from 8 hours to 24 hours. Obviously, the RS-III samples hydrolyzed for 24 hoursr and treated with spray, freeze and hot air drying showed a significantly lower amylose contents than the commercial starch (31.8 ± 2.6 g/100g), for the cooling treatment of 24 and 48 hours. The values were 21.7 ± 1.9, 15.8 ± 0.5, and 14.1 ± 1.4 g/100g for the 24 hour cooling; and 14.4 ± 5.0, 12.5 ± 0.9 and 19.2 ± 0.5 g/100g for 48 hours, respectively.

As the pullulanase enzyme has a specific action on the 1-6 glycosidic branching chains, the amylopectin molecules would be primarily degraded when the hydrolysis took place at the amorphous areas of the cluster zones. The short linear chains and small clusters of amylopectin obtained would be responsible for rercrystallization after cooling and aggregate to form the RS-III structure. Recrystallization of short chain length of amylopectin molecules of 20 to 25 glucose units from the outer chain-A would produce a less resistant structure than the amylose recrystallization (Sajilata et al. 2006). In this study, the 8 hour hydrolysis showed a slight change of the amylose contents, while a distinctive change was found with the 24 hydrolysis for any cooling times. However the lower contents of amylose had not an impact on the RS-III contents and the in vitro starch digestibility tests from the obtained results. This implied that a longer period of pullulanase hydrolysis leaded to degradation of amylose molecules at some branching sites and a report of the cassava starch amylolysis of 75% was related to this result.

Table 6 Effect of drying treatments on the amylose contents (g/100g dry weight) [1,2].

Sample treatments	Cooling time	
	24 hr	48 hr
Commercial cassava starch,	31.8 ± 2.6 [a]	31.8 ± 2.6 [a]
8 hr hydrolysis		
Spray drying	27.0 ± 1.6 [b]	28.9 ± 0.3 [a]
Freeze drying	27.4 ± 1.7 [b]	27.2 ± 4.8 [a]
Hot air drying	29.4 ± 1.6 [ab]	28.3 ± 0.8 [a]
24 hr hydrolysis		
Spray drying	21.7 ± 1.9 [c]	18.8 ± 1.1 [b]
Freeze drying	15.8 ± 0.5 [d]	12.5 ± 0.9 [c]
Hot air drying	14.1 ± 1.4 [d]	19.2 ± 0.5 [b]

[1] Values are means of duplicate analysis.
[2] In a column, means not sharing a common superscript are significantly different at the 0.05 level

Effects of drying treatments on the RS-III content

This experiment was subjected to determine the RS-III contents effected by spray drying as compared to freeze and hot air drying, employing in drying the pullulanase hydrolyzed cassava starch after 24 and 48 hours of cooling. As for the adopted process for resistant starch production in this report, the cassava starch was hydrolyzed by the 10% pullulanase at 50°C for 8 and 24 hours, the debranched molecules were retrograded by cooling at temperature of 4 °C for 24 and 48 hours (Table 7). Then the samples were dried and the content of resistant starch were compared. The results showed that the contents of resistant starch produced with the pullulanase reaction for 8 hours and followed by cooling of 24 hours were 33.0 ± 2.2 g/100g for spray drying, 41.7 ± 2.3 g/100g for freeze drying and 43.4 ± 4.6 g/100g for hot air drying. When the hydrolysis time extent to 24 hours, the contents of resistant starch were 32.9 ± 0.1 g/100g for spray drying, 44.8 ± 0.4 g/100g for freeze drying and 50.8 ± 2.9 g/100g for hot air drying. No significant difference in the resistant starches was shown with the samples hydrolyzed for 8 and 24 hours when dried with spray and freeze drying. However the values from both spray and freeze dried samples were significantly lower than those from the hot air dried samples and the hydrolysis for 24 hours gave a high resistant starch content of 50.9 ± 2.9 g/100g. As for the cooling of 48 hours, the spray drying had more effect on the resistant contents for both of hydrolysis times. The values were increased from 33.0 ± 2.2 g/100g to 37.7 ± 1.0 g/100g for 8 hour hydrolysis and from 32.9 ± 2.2 g/100g to 41.3 ± 0.8 g/100g for 24 hour hydrolysis Whereas freeze and hot air drying treatments showed the less effect on the RS-III with the 48 hours cooling than the 24 hours cooling.

Moreover, the findings indicated that the observed resistant starch contents seemed to increase with the length of the hydrolysis time, although the different drying treatments were used. This implied that the cooling prolonged for 48 hours might lead to more water reabsorbed into the fragmented short chain molecules of the hydrolyzed cassava starch, therefore formation of a resistant structure was disturbed in particular with drying process of direct contact heat exchanger. However this change did not occur with the spray drying treatment.

Table 7 Effect of drying treatments on the RS-III contents (g/100g dry weight) [1,2].

Sample treatments	Cooling time	
	24 hr	48 hr
Gelatinized cassava starch, commercial (50% solid)	9.2 ± 0.2 [a]	9.2 ± 0.2 [a]
8 hr hydrolysis		
Spray drying	33.0 ± 2.2 [b]	37.7 ± 1.0 [c]
Freeze drying	41.7 ± 2.3 [c]	26.8 ± 0.2 [b]
Hot air drying	43.4 ± 4.6 [c]	29.8 ± 3.9 [b]
24 hr hydrolysis		
Spray drying	32.9 ± 0.1 [b]	41.3 ± 0.8 [c]
Freeze drying	44.8 ± 0.4 [c]	40.3 ± 0.1 [c]
Hot air drying	50.9 ± 2.9 [d]	45.2 ± 0.3 [d]

[1] Values are means of duplicate analysis.
[2] In a column, means not sharing a common superscript are significantly different at the 0.05 level by ANOVA and DMRT.

Rate of enzymatic starch digestibility

Table 8 shows profiles of enzymatic starch digestibility of the RS-III samples produced in this study. The effects of spray, freeze drying as compared to the hot sir drying on the RS-III prepared with the 10% pullulanase debranching reaction conditioned at pH 5.0 for hydrolysis of 8 and 24 hours and followed by retrogradation of 24 and 48 hour cooling. As comparison to the commercial cassava starch, most of the RS-III samples dried using the spray and freeze drying were less resist to the enzymatic digestion than those treated with the hot air. While the commercial cassava starch exhibited a very fast starch digestibility since it contains a very pure native starch. Thus, almost fully digestion was observed for the commercial starch showing a rate of 80.81% to 89.40% of the total starch content over the digestion period of 30 to 180 minutes. Whereas the hot air dried RS-III samples hydrolyzed for 8 hours and following with cooling of 24 hours showed the slowest rate of overall starch digestibility as compared to the other samples determined in this study.

According to a study on a starch hydrolysis procedure to estimate glycemic index, the best hydrolysis value to estimate *in vivo* glycemic response would be at 90 minutes (Goni et al., 1997). When starch digestibility of the spray dried samples examined at 90 minutes, a distinctive reduction of the digestion rate was 22.69% from the commercial starch, with

the treatment of the 24 hour hydrolysis following with cooling of 48 hours. Similarly a reduction of the digestion rate of the freeze dried samples was 20.77% from the commercial starch with hydrolysis of 24 hours and following with cooling of the 24 hours.

Table 8 Rates of the *in vitro* starch digestibility (% of total starch content) of the RS-III samples produced with different cooling of 24 and 48 hr.

Sample treatment	Digestion times (min)					
	30	60	**90**	120	150	180
Commercial cassava starch	80.81	83.36	**89.40**	86.22	92.58	89.40
24 hr cooling for retrogradation						
8 hr hydrolysis						
Spray drying	72.89	81.37	**76.69**	70.92	73.65	70.16
Freeze drying	72.42	77.40	**74.33**	76.64	79.51	74.14
Hot air drying	52.88	55.26	**57.32**	55.74	57.01	57.96
24 hr hydrolysis						
Spray drying	76.50	67.54	**74.52**	71.64	65.12	72.55
Freeze drying	53.07	66.71	**66.71**	56.03	55.74	55.74
Hot air drying	53.65	63.54	**68.63**	71.03	69.83	69.83
48 hr cooling for retrogradation						
8 hr hydrolysis						
Spray drying	72.32	75.65	**74.29**	72.93	73.23	77.62
Freeze drying	64.95	74.57	**70.20**	72.82	74.28	73.40
Hot air drying	59.11	71.59	**70.10**	77.53	72.48	64.76
24 hr hydrolysis						
Spray drying	69.08	65.63	**66.83**	66.38	69.38	67.28
Freeze drying	72.30	68.56	**72.02**	72.88	77.78	77.49
Hot air drying	59.64	60.54	**58.14**	64.73	61.44	61.74

[1] Values are means of duplicate analysis.
[2] In a column, means not sharing a common superscript are significantly different at the 0.05 level by ANOVA and DMRT.

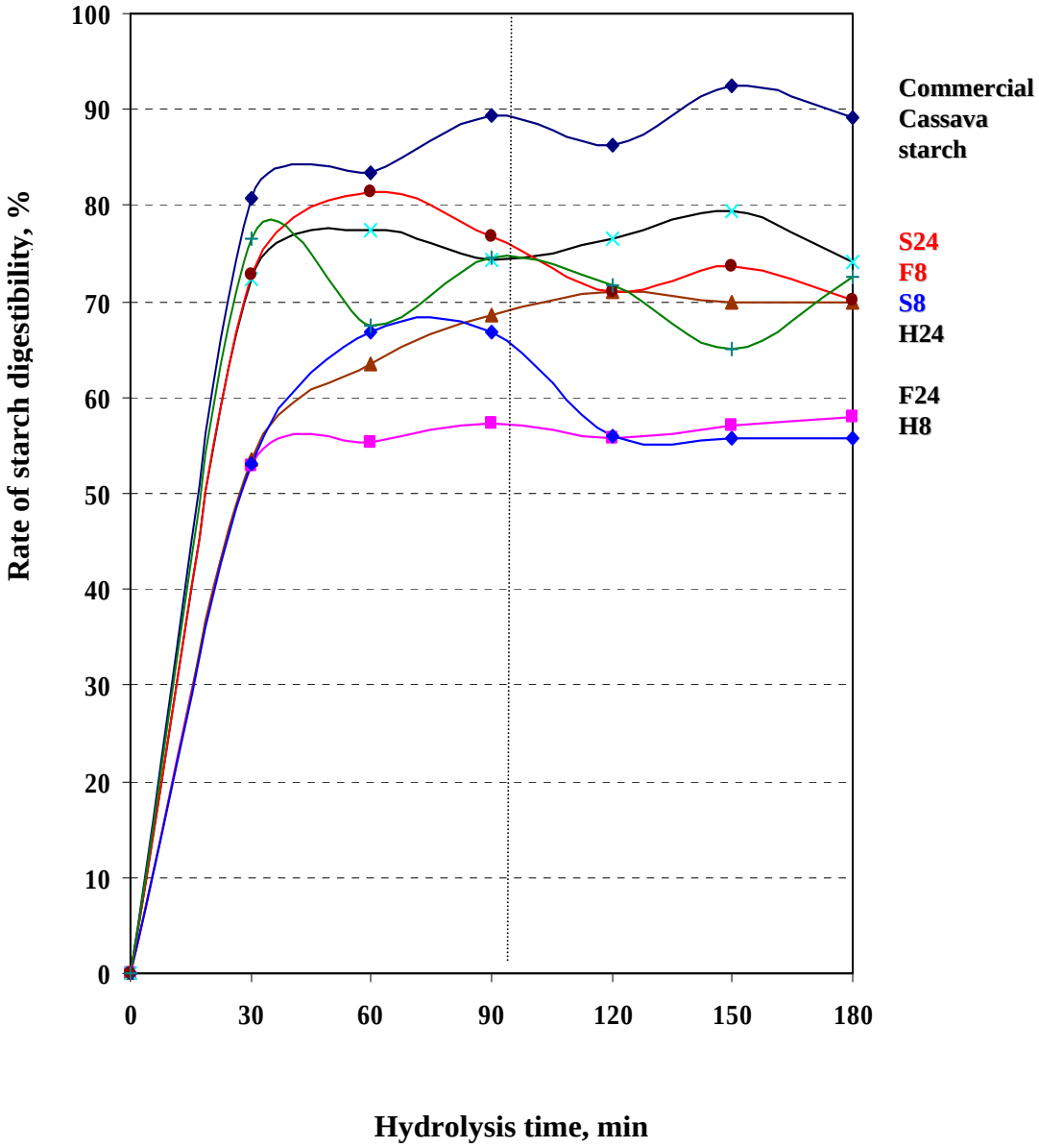

Figure 1 Rate of *in vitro* enzymatic starch digestibility of the spray dried RS-III samples treated with 8 hr (S8) and 24 hr hydrolysis (S24), the freeze dried RS-III samples treated with 8 hr (F8) and 24 hr (F24) hydrolysis and the hot dried RS-III samples treated with 8 hr (H8) and 24 hr (H24) hydrolysis, following by cooling of 24 hr and compared to that of the commercial cassava starch.

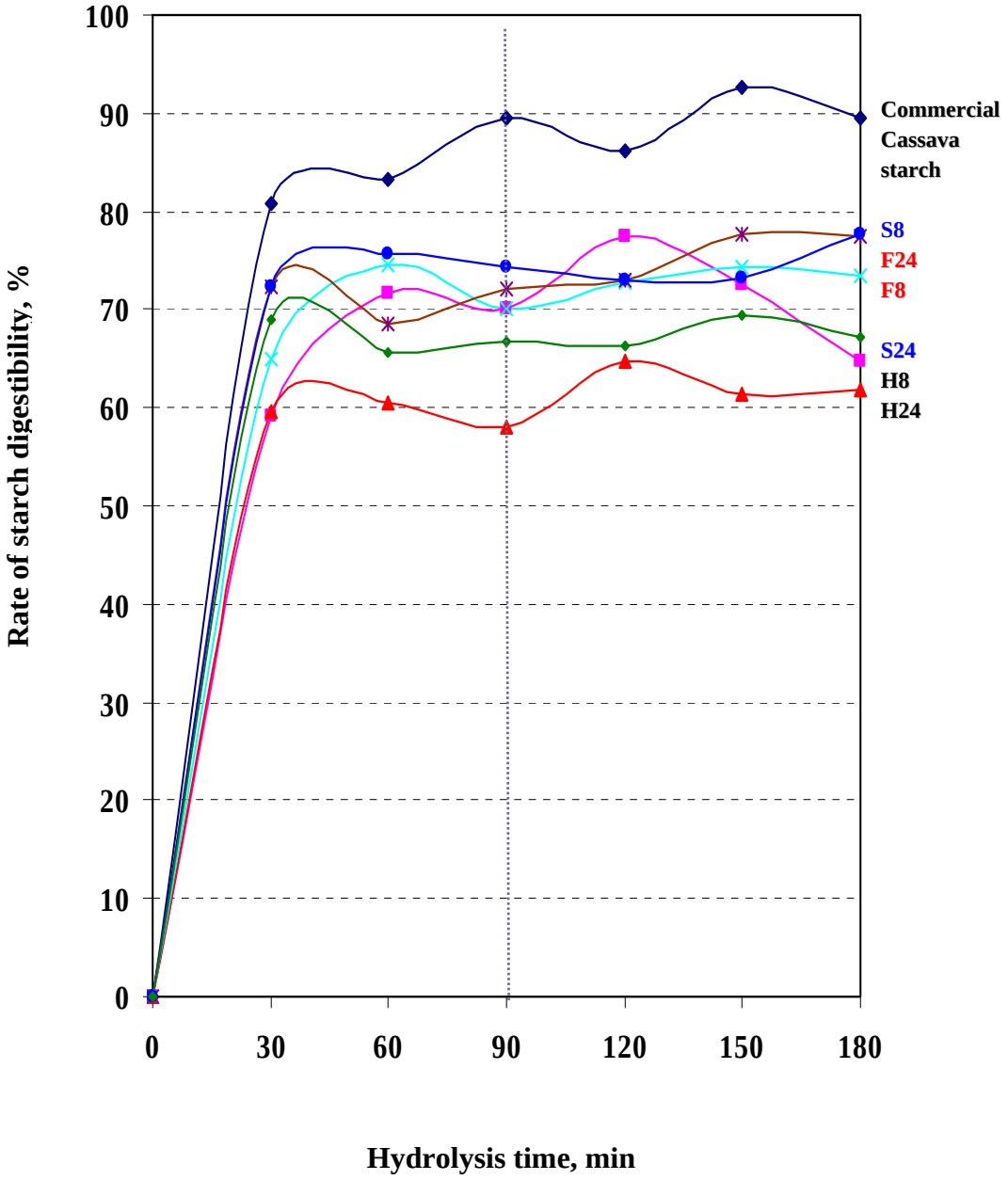

Figure 2 Rate of *in vitro* enzymatic starch digestibility of the spray dried RS-III samples treated with 8 hr (S8) and 24 hr hydrolysis (S24), the freeze dried RS-III samples treated with 8 hr (F8) and 24 hr (F24) hydrolysis and the hot dried RS-III samples treated with 8 hr (H8) and 24 hr (H24) hydrolysis, following by cooling of 48 hr and compared to that of the commercial cassava starch.

Figure 1 exhibits the *in vitro* starch digestibility rate of the spray, freeze and hot air dried RS-III samples produced by using cooling time of 24 hours when compared to the commercial starch. A digestion rate of the RS-III samples obtained with the hot air drying was slower than the spray drying with the hydrolysis of 8 and 24 hours. And treatment of hot air with 8 hour hydrolysis with the cooling of 24 hour produced a digestion rate of 32.08% slower than the commercial starch. Considering a starch digestion for 90 minutes a decreasing rate was found with the spray dried samples treated with a cooling time extent to 48 hours. This result was related to a higher content of RS-III formed when the cooling of 48 hours was used for retrogradation.

Figure 2 exhibits a slow starch digestibility rate of the RS-III samples produced with cooling of 48 hours and treated with any dryings, when compared to the commercial starch. The digestibility curves of the freeze dried samples were similar to those of the spray dried samples and they exhibited faster digestibility than the hot air drying. The digestibility curve of hot air treatment with 24 hour hydrolysis gave the slowest rate among the different drying methods.

Thermal Properties

The thermal behavior of the RS-III produced from the cassava starch treated with 10% pullulanase for 8 and 24 hours with cooling of 24 and 48 hr and followed by spray drying was studied using DSC measurements. The thermograms of the RS-III samples were shown in Figure 3 and the transition temperatures and the corresponding melting enthalpies are demonstrated in Table 9. The commercial cassava starch showed the highest melting enthalpy of 271.7 ± 9.6 J/g and the commercial resistant starch was 255.0 ± 0.1 J/g, when compared to the RS-II samples produced in this study. The RS-III samples exhibited the melting enthalpies over a range of 167.6 ± 0.7 to 188.4 ± 2.8 J/g, lower than those of the commercial cassava starch and resistant starch. As for the cassava starch hydrolyzed with the pullulanase for 8 hour, the RS-III samples obtained showed the melting enthalpies of 188.3 ± 0.8 J/g for cooling of 24 hrs and 188.4 ± 2.8 J/g for cooling of 48 hrs. These enthalpy values were higher than the RS-III hydrolyzed for 24 hrs. Moreover an endothermic transition temperature for the obtained RS-III samples was over a range of 140 to 175 °C. This finding implied that degradation of the amylopectin molecules to short linear chains would contribute to the formation of less confined structure for melting, which was agreed with the result of the digestibility rate. As we known retrograded amylose molecules provided more resistant structure for slow digestion property than the retrograted amylopectin chains.

Table 9 Thermal properties of the spray dried RS-III samples.

Samples	Melting Enthalpy (J/g)	Melting temperature (°C)		
		Onset	Peak	End
Commercial cassava starch	271.7 ± 9.6a	145.1 ± 3.0	148.8 ± 1.6	172.4 ± 2.8
Commercial resistant starch	255.0 ± 0.1b	139.9 ± 0.4	146.2 ± 1.1	175.2 ± 5.4
8 hr hydrolysis				
24 hr cooling	188.3 ± 0.8c	144.1 ± 2.7	146.7 ± 0.8	164.9 ± 12.4
48 hr cooling	188.4 ± 2.8c	147.7 ± 2.9	150.9 ± 4.0	172.7 ± 12.8
24 hr hydrolysis				
24 hr cooling	182.1 ± 1.0c	147.6 ± 9.0	151.2 ± 8.0	174.5 ± 2.3
48 hr cooling	167.6 ± 0.7d	145.9 ± 4.8	149.5 ± 4.3	170.7 ± 3.4

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different at the 0.05 level by ANOVA and DMRT.

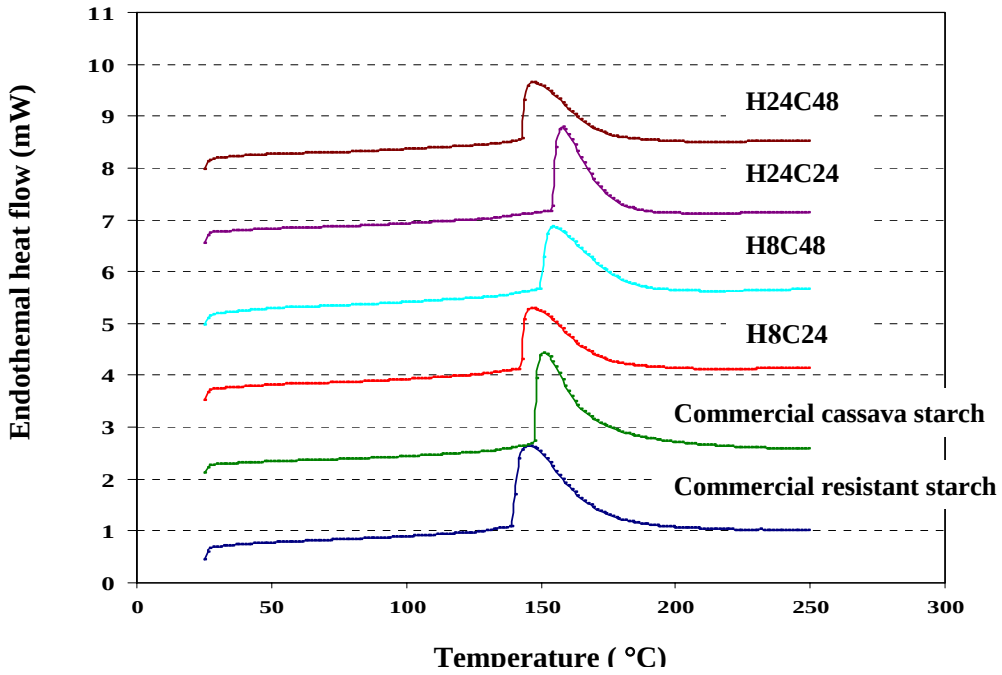

Figure 3. DSC thermograms of the spray dried RS-III samples produced with 8 hr hydrolysis and 24 hr cooling (**H8C24**), 8 hr hydrolysis and 48 hr cooling **(H8C48)**, 24 hr hydrolysis and 24 hr cooling (**H24C24**), and 24 hr hydrolysis and 48 hr cooling (**H24C48**), and compared to the commercial resistant starch and the commercial cassava starch.

Changes in structural properties of the RS-III samples

The diffraction patterns of the RS-III cassava starches treated with the spray and freeze drying when compared with the hot air and commercial cassava starch are shown in Figure 3. In general the commercial cassava starch exhibited typical type A 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 change in the X-ray diffraction profiles was observed. The X-ray diffractograms of the RS-III treated with spray, freeze and hot air drying appeared to have a similar typical B-type pattern indicated the peaks at 2-theta of 24°, 22.5°, 17° and 5.6°. In particular the hot air dried samples clearly showed a transition of crystallinity from A- to B-type pattern and resulted in a C-type structure as exhibited by additional peaks at 2-theta of 19°and 5.5°. This finding showed that the hot air dried RS-III profiles had sharper and larger characteristic peaks at 2-theta of 19.6°and 5.6° than the other diffractographs. Whereas a distinctive peak at 2-theta of 13° was produced with the spray and hot air dried RS-III samples.

This finding was associated with a report on polymorphism of RS-III produced from the native corn flour and wheat starch exhibiting typical A-type diffraction patterns (Shamai et al., 2003). For both starches, low retrogradation temperature (40°C) resulted in a formation of B-type pattern as indicated by the pronounced peaks at 2-theta of 23° and 17°. Whereas retrogradation at high temperature (95°), formation of a mixture of A- and V-type structure was obtained, as indicated from the peak at 2-theta of 19.8° and 13°. As a remark, the peak at 2-theta of 5.5° was considered as a finger print for B-type structure that was also clearly shown with the spray and hot air dried RS-III samples.

With the pullulanase debranching process, retrogradation at 4°C and treatments of the spray, freeze and hot air dryings, a recrystallized structure of the RS-III observed with X-ray diffractometry showed a mixture of B-type and V-type polymorphs. More effect on the polymorph structure was found with the spray and hot air dried RS-III samples.

Considering the cassava starch used for a starting material for the RS-III production, the same A-type pattern as cereal starches was exhibited. While the resulting RS-III starches showed the B-type structure that was more resistant to the enzymatic digestion with α- amylase than the native starch, as we found with the hot air dried RS-III samples. In general the A-type crystallites of native starch were attacked more rapidly by α-amylase than potato starch which exhibits B-type crystallites since the granular size and the differences in the surface to volume ratio (Alonso, 1998). In addition Williamson et al.

(1992) compared the enzymatic resistance of the different polymorph using A and B-type crystallites of amylose with an average DP of 20. They showed that the B-type crystallites were hydrolyzed more slowly than the A-type ones.

When the degree of relative crystallinity was determined, as compared to the commercial starch (36.9%) the spray dried RS-III samples showed a decrease in the relative crystallinity to 29.5% and 28.4% for 8 and 24 hr hydrolysis, respectively. Whereas the freeze dried RS-III samples had a lower in the relative crystallinity of 22.5% and 27.4% for 8 and 24 hrs, respectively. But the hot air treatment with 24 hr hydrolysis showed a slight increase to 38.2%, not much different from the commercial starch of 36.9%. This means that the pullulanase reaction incooperating with the spray and freeze drying produced less crystalline structure, thus it was less responsible for the digestion property than the hot air treated RS-III starches. Likewise, a result of starch digestibility rate of the RS-III samples treated with the spray and freeze-drying showed less resist to digestion than that done with the hot air drying.

Table 10 Degree of relative crystallinities (%) of the RS-III samples of the 24 hr cooling and treated with different dryings [1].

Sample treatments	Relative crystallinities (%)
Commercial cassava starch	36.9
Spray drying	
8 hr hydrolysis	29.5
24 hr hydrolysis	28.4
Freeze drying	
8 hr hydrolysis	22.5
24 hr hydrolysis	27.4
Hot air	
8 hr hydrolysis	33.8
24 hr hydrolysis	38.2

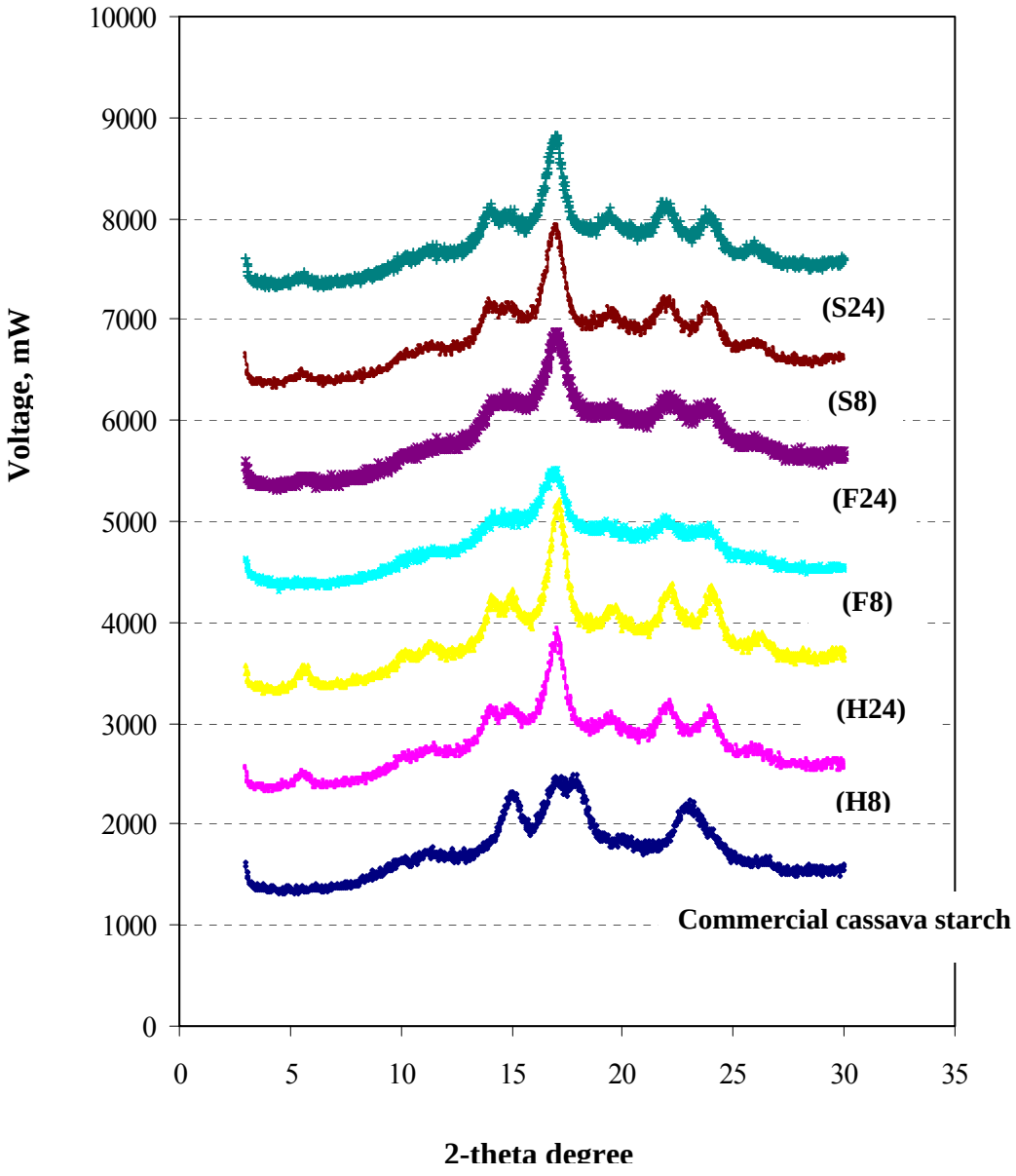

Figure 4 X-ray diffraction patterns of the RS-III cassava starches with 24 hr cooling. (F8): freeze drying with 8 hr hydrolysis, (F24): freeze drying with 24 hr hydrolysis , (H8) : hot air drying with 8 hr hydrolysis ; and; (H24) : hot air drying with 24 hr hydrolysis.

Scanning electron microscopy examination

Microscopic images of the RS-III cassava samples obtained from the tested treatments of hydrolysis for 8 and 24 hours and cooling of 24 and 48 hours were examined using the scanning electron microscope with magnifications of 100X, 500X, 1000X and 2000X. Generally, native cassava starch granules when examined under a microscope are of medium size ranging from 4 to 35 μm and are 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 5).

With the SEM micrographs, the RS-III samples with treatments of spray, freeze and hot drying revealed a loss of the cassava starch granule characteristic which resulted from the debranching process of the pullulanase reaction and aggregation of the starch granular remnants was shown. The electron micrographs of the spray dried RS-III samples clearly exhibited aggregation of small starch particles when viewed with magnifications of 1000X and 2000X, whereas the other drying treatments clearly showed with magnifications of 100X and 500X. In addition formation of the starch aggregate with the spray drying revealed a rather round structure and in a size approximately between 10 to 20 μm. When treated with hydrolysis of 24 hours and cooling of 48 hours, more symmetrical round structure was formed in spit of having a difference in size (Figure 6 and 7).

Figure 8 and 9 shows a sponge-like structure for all of the freeze dried RS-III samples when viewed with magnifications of 100X and 500X. This means that the starch materials produced with freeze drying was formed in a size of between 100 to 200 μm larger than that produced with the spray drying. Moreover evidence of pitting on a rough surface of the freeze dried RS-III samples might be due to a melting of small ice crystallites during the freeze-drying treatment.

When viewed with magnifications of 100X and 500X, a stone-like structure of starch materials with a large size approximately between 100 to 200 μm was exhibited for the hot-air dried RS-III samples (Figure 10 and 11). The image of a layering starch fragments looked quite dense and rigid; this may lead to the slow rate of starch digestibility observed with the hot air drying. Also the *in vitro* starch digestibility tests showed that the hot air dried RS-III samples had a slower rate of starch digestibility than the spray and freeze dried samples.

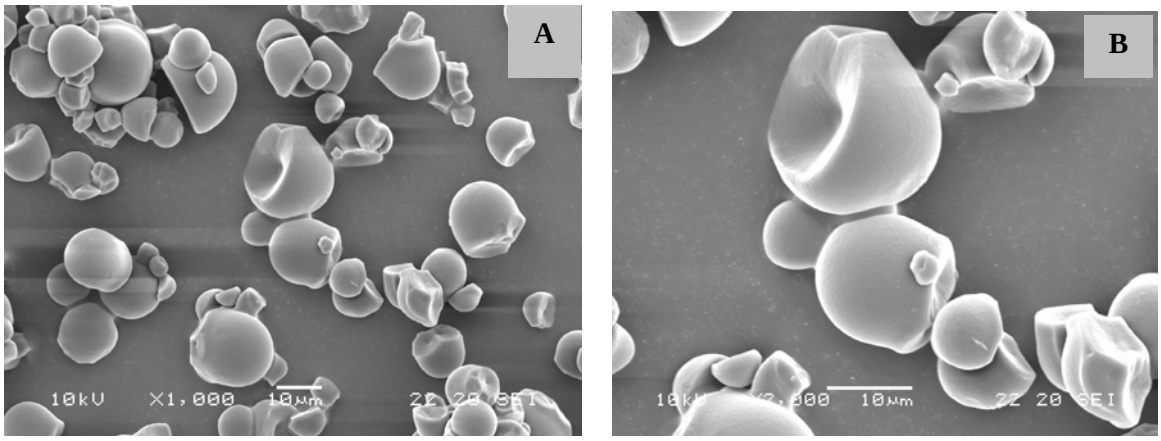

Figure 5 SEMs of the commercial cassava starch granules **(A)** 1000X and **(B)** 2000X.

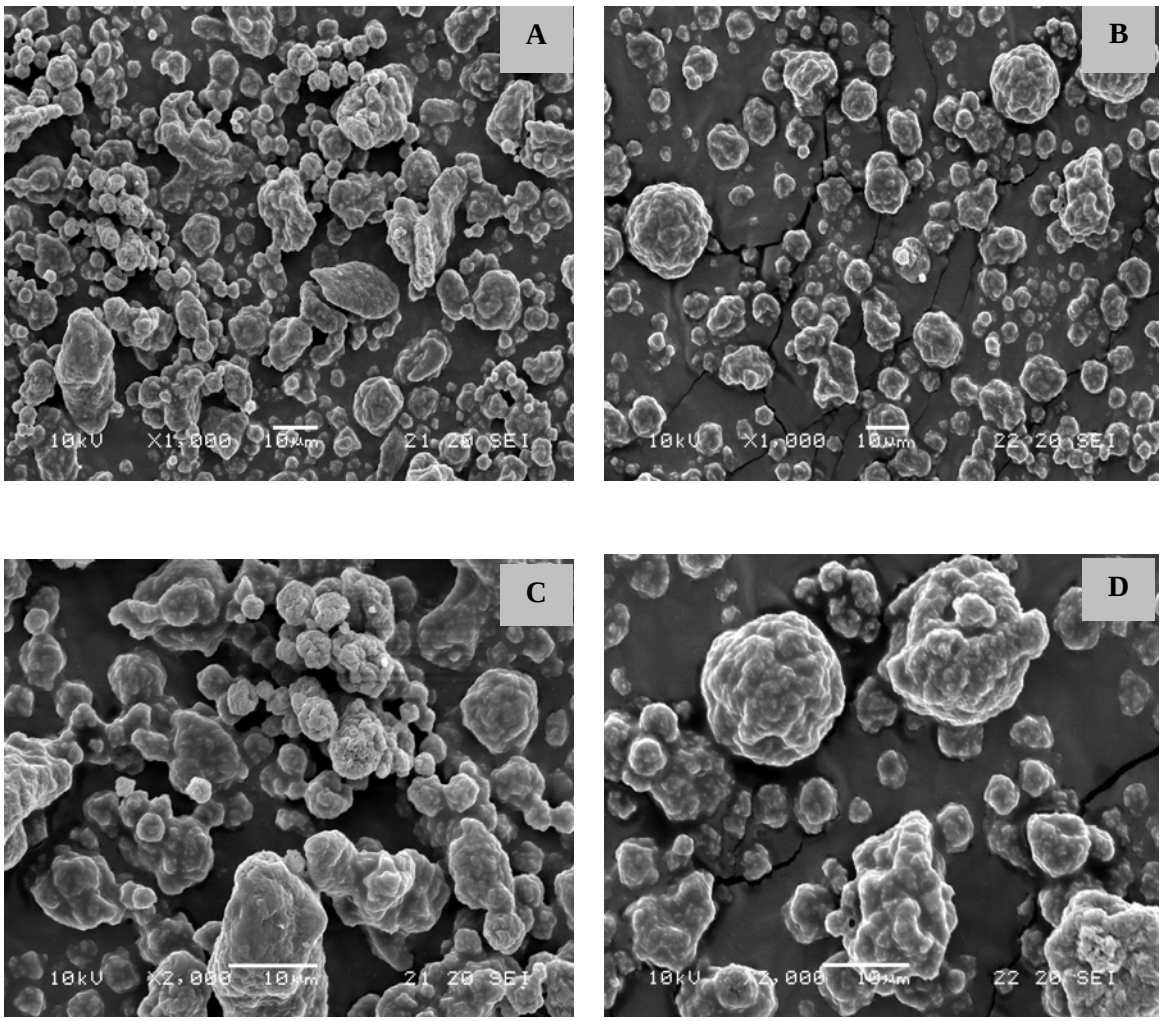

Figure 6 SEMs of the spray dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 hrs with magnification of 1000X; and **(C)** 8 and **(D)** 24 hrs with magnification of 2000X, following with the 24 hr cooling.

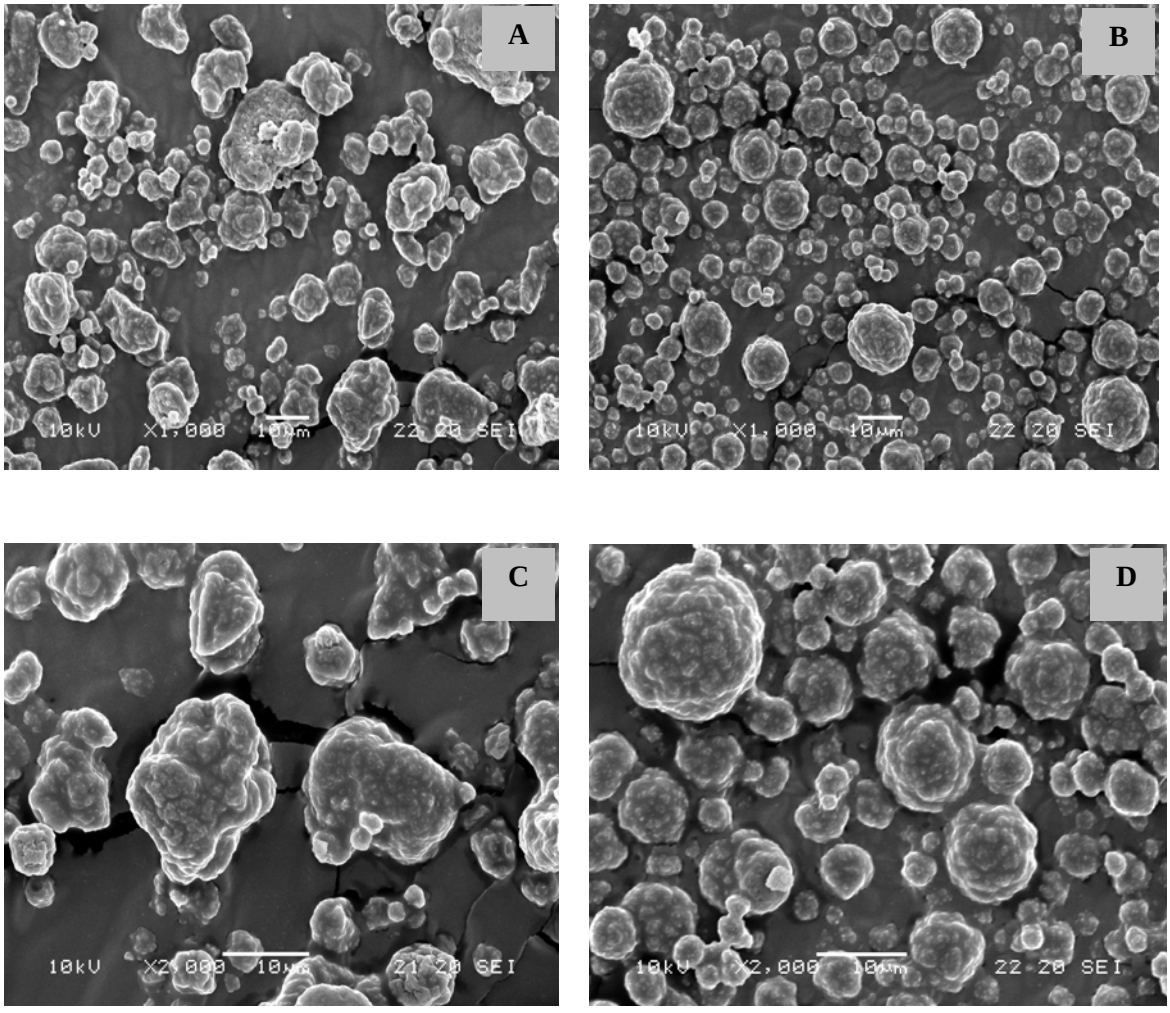

Figure 7 SEMs of the spray dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 hrs with magnification of 1000X; and **(C)** 8 and **(D)** 24 hrs with magnification of 2000X, following with the 48 hr cooling.

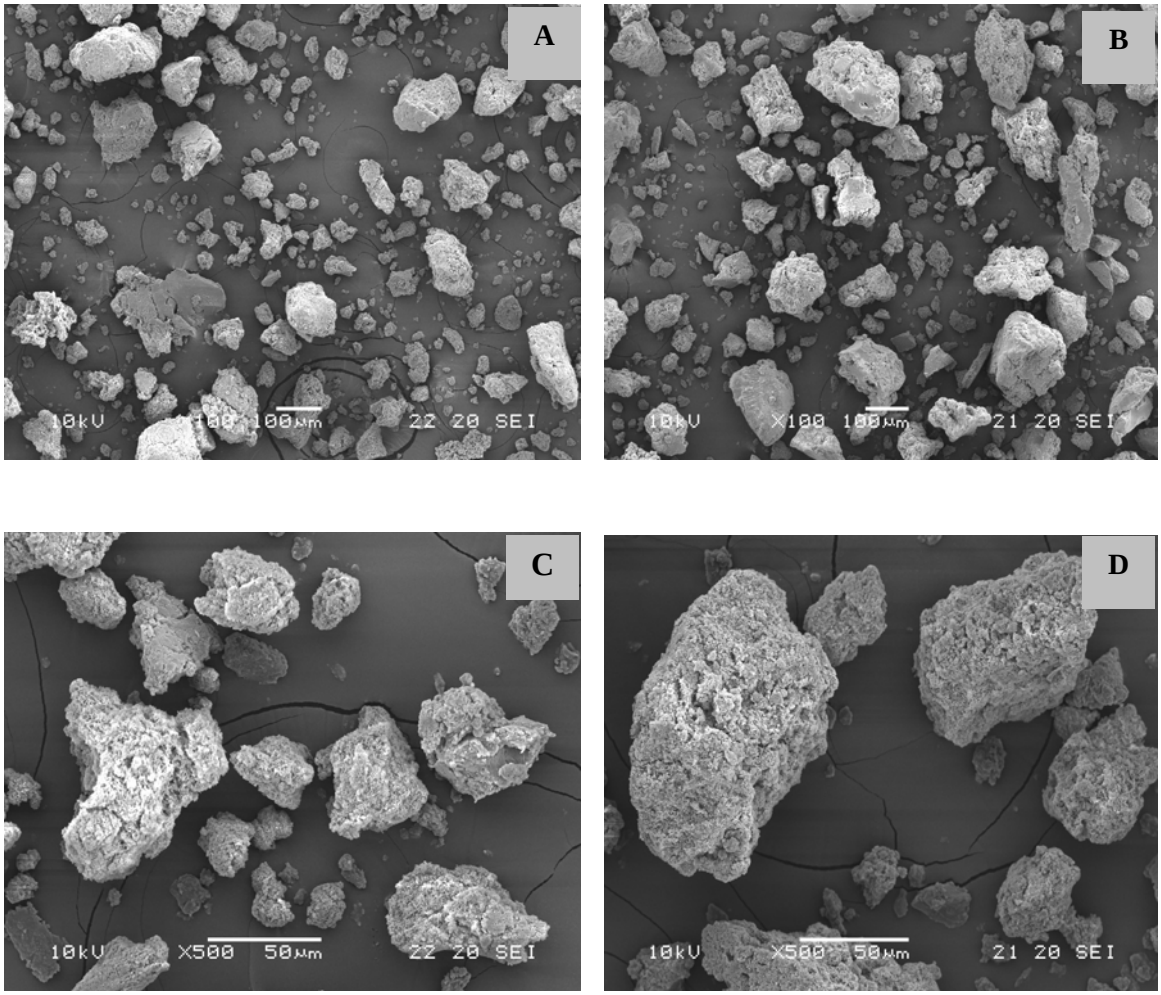

Figure 8 SEMs of the freeze dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 hrs with magnification of 100X; and **(C)** 8 and **(D)** 24 hrs with magnification of 500X, following with the 24 hr cooling.

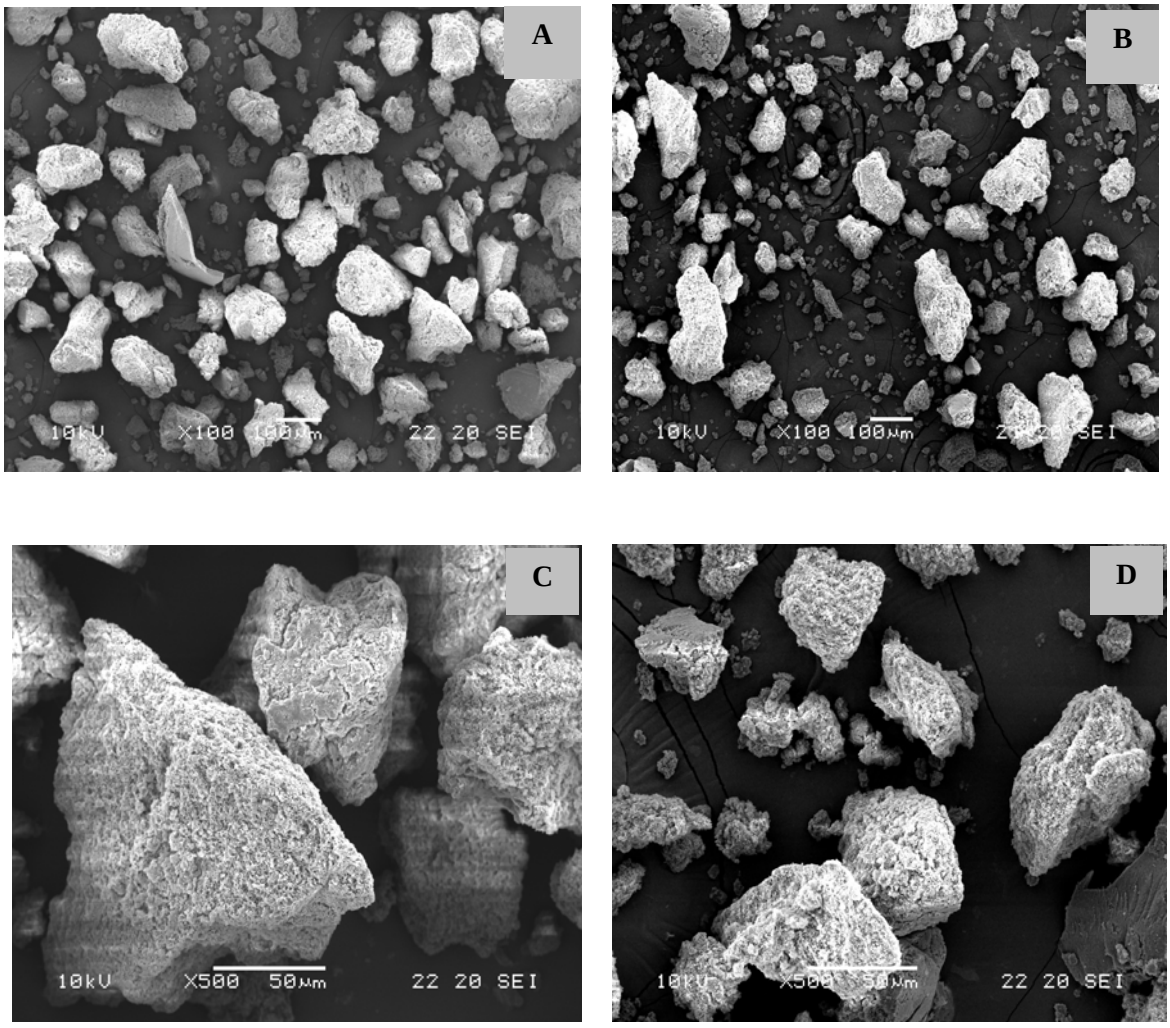

Figure 9 SEMs of the freeze dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 hrs with magnification of 100X; and **(C)** 8 and **(D)** 24 hrs with magnification of 500X, following with the 48 hr cooling.

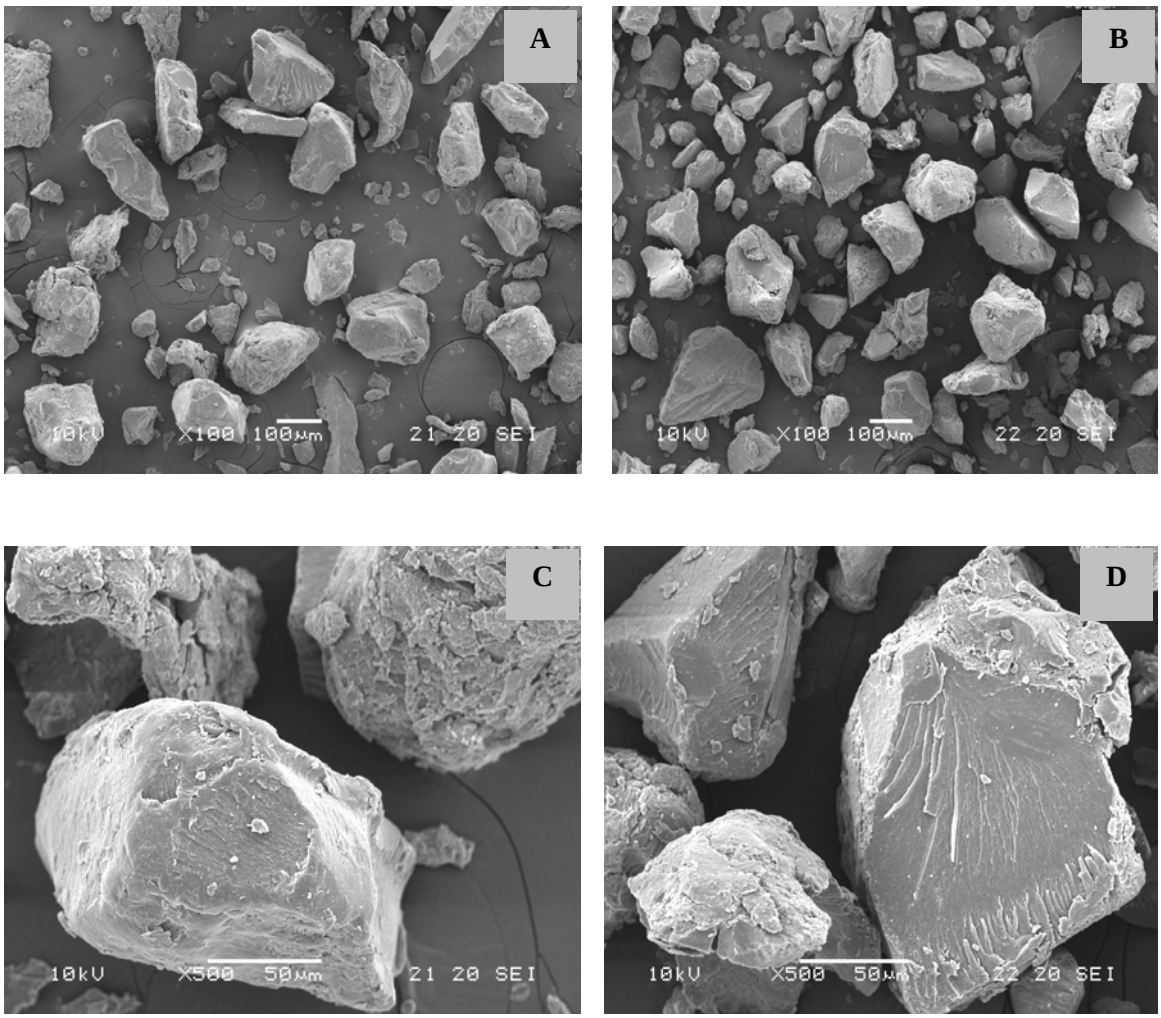

Figure 10 SEMs of the hot air dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 hrs with magnification of 100X; and **(C)** 8 and **(D)** 24 hrs with magnification of 500X, following with the 24 hr cooling.

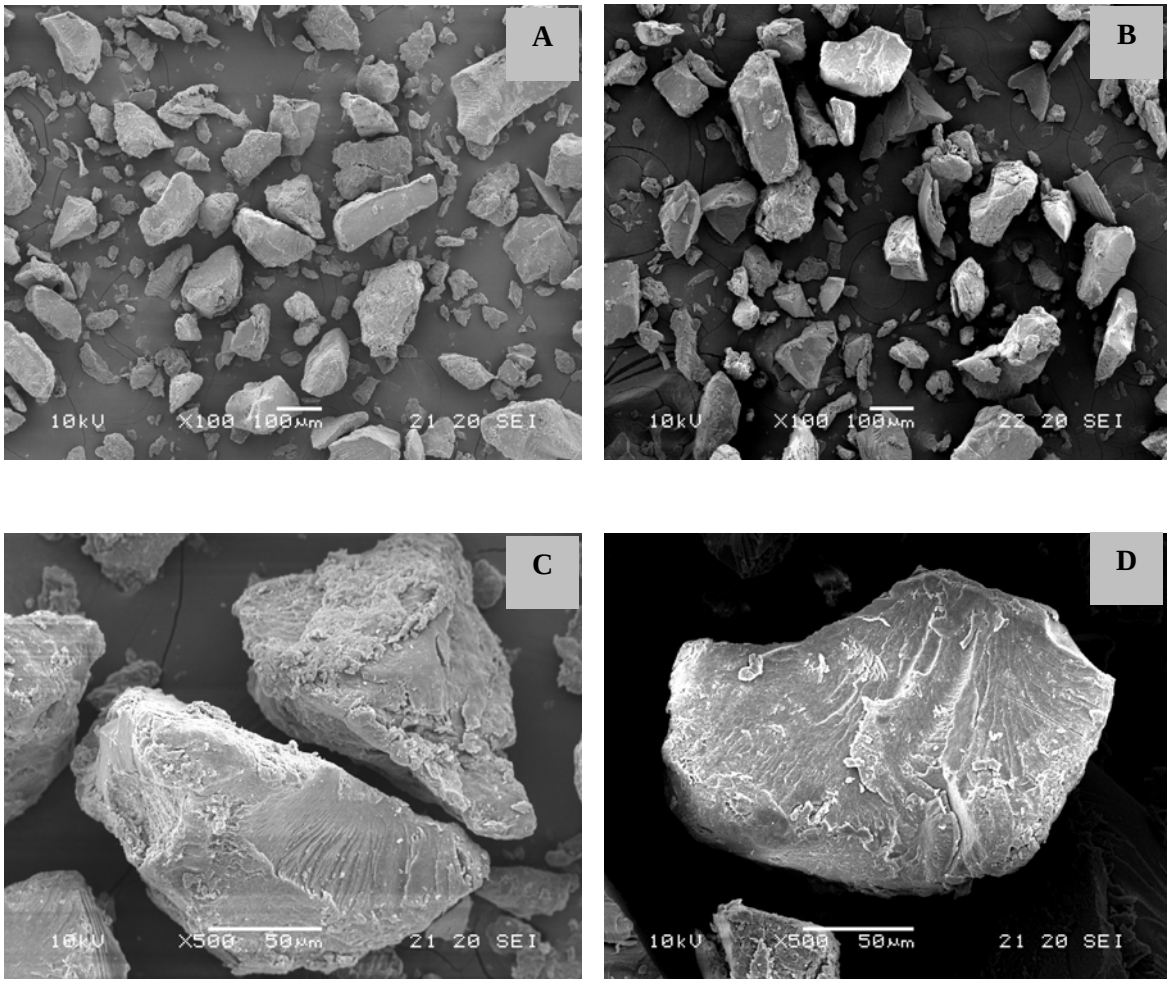

Figure 11 SEMs of the hot air dried RS-III samples after hydrolysis reaction for **(A)** 8 and **(B)** 24 hrs with magnification of 100X; and **(C)** 8 and **(D)** 24 hrs with magnification of 500X, following with the 48 hr cooling.

CONCLUSIONS

This present study showed that an appropriate condition for production of the RS-III of cassava starch was the 10% pullulanase of the total starch (db) adjusted to pH 5 for the enzymatic hydrolysis of 8 hrs and cooling for 24 hrs, then dried using hot air. This was suitable for partially debranching amylopectin molecules of the cassava and consequently providing small linear fragments and small clusters of the amylopectin molecules for retrogradation / recrystallization and formation of the RS-III. With the effects of different dryings, the hot air drying enhanced the formation of RS-III greater than the spray and freeze dryings. The hydrolysis time of 8 hrs gave a high yield of RS-III of 43.4 ± 4.6 g/100g and the 24 hrs had the highest content of resistant starch of 50.9 ± 2.9 g/100g. This result was related to the *in vitro* starch digestibility of the hot-air dried samples showing about 20% to 30 % slower than the commercial cassava starch, after amylase digestion of 90 min. Moreover the DSC determination showed an endothermic melting enthalpy over a range of 167.6 ± 0.7 to 188.4 ± 2.8 J/g for the RS-III samples which was lower than those of the commercial cassava starch and resistant starch. This finding implied that degradation of the amylopectin molecules to short linear chains would contribute to the formation of less confined structure for melting. Finally, the structural changes of the type-B crystallites and the scanning electron micrographs of the RS-III obtained with this study would confirm its resistance to enzymatic digestion, particularly with the hot-air dried RS-III samples. Whereas the micrographs of the spray dried RS-III exhibited a fine structure of small starch particles aggregated.

ACKNOWLEDGEMENTS

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APPENDIX I

METHODS FOR DETERMINATIONS

Determination of Reducing Sugars

Reagents : All chemicals (p.a.) were from E. Merck (Germany)

Nelson A

25 g. Na_2CO_3 (anhydrous)
25 g. K-Na-tartarate (Rochelle salt)
200 g Na_2SO_4 (anhydrous)
20 g. $NaHCO_3$
: dissolve in about 800 ml H_2O and dilute to 1000 ml. Store at room temperature.

Nelson B

15 g. $CuSO_4 . 5H_2O$ in 100 ml water containing two drops of concentrated H_2SO_4

Nelson A & B: Mix 25 ml Nelson A + 1 ml Nelson B

Nelson C

25 g. $(NH_4)_6 Mo_7 O_{24} . 4H_2O$/ 450 ml H_2O; add 21 ml Concentrated H_2SO_4; mix and add 3 g $Na_2 HAsO_4.7H_2O$/ 25 ml.
: keep at 37°C for 2 day and store in a brown bottle at room temperature.

Determination of reducing sugars:

1. Pipet 1 ml of hydrolysate starch sample into a 20 ml test tude
2. Add 1 ml of Nelson A&B mixture and votex.
3. Boil the sample at 100°C for 15 minutes and cool in cold water.
4. Add 1 ml Nelson C, then mix and place in room temperature for 30 minutes.
5. Dilute to 10 ml with H_2O.
6. Measure absorbsnce at 520 nm. For test sample, determine mg glucose from standard curve plotted absorbance against of glucose concentration of 0, 40, 80, 120, 160, and 200 µg/mg.

Adopted from method of Ghose and Bisaria (1987)

Colorimetric Determination of Amylose Contents (AACC, 2000)

Reagents

- Amylose, Type III from potato (Sigma No. A-0512, Japan)
- Amylopectin, from potato (Sigma No. A-8515, Japan)
- Ethanol, 95%
- Sodium hydroxide, 1.0 N
- Sodium hydroxide, 0.09 N
- Acetic acid, 1.0 N
- Iodine solution, 0.2% I_2 and 2% KI in distilled water

Procedure for Sample Determination

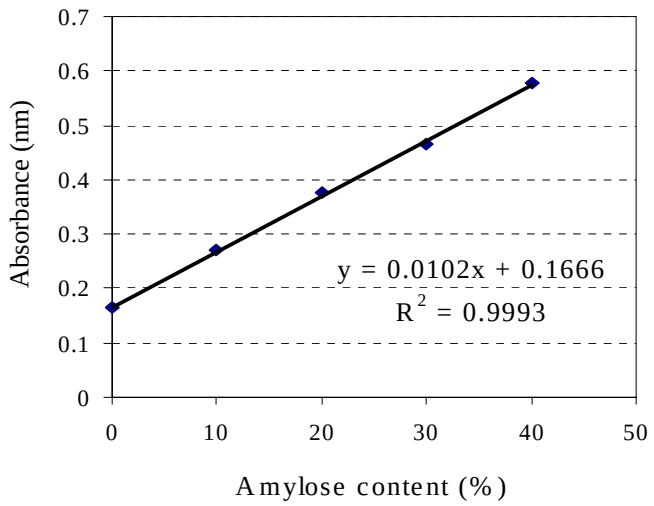
1. Weigh duplicate 100 mg (db) starch samples and quantitatively transfer to 100 ml volumetric flasks.
2. Add 1 ml of 95% ethanol, carefully washing down any sample adhering to side of the flasks.
3. Add 9 ml 1 N sodium hydroxide to each sample and heat in a boiling water for 10 minutes and cool to room temperature.
4. Make the solutions to 100 ml volume with distilled water and votex vigorously and obtain 1 mg/ml solution. Let stand for at least 2 hours before continuing with the next steps.
5. Pipette 5 ml of the sample solutions (or else each of standard mixtures of amylose and amylopectin prepared for working solutions) into 100 ml volumetric flasks, containing about 50 ml distilled water.
6. Add 1.0 ml N acetic acid and mix.
7. Add 2 ml iodine solution.
8. Make up to 100 ml volume with distilled water, mix and let stand for 20 minutes.
9. Read absorbance at 620 nm. For a blank, prepare using 5 ml 0.09 N NaOH, instead of the sample solution in the step 4.3.5.
10. Plot absorbance against amylose concentration of working solutions for a standard curve.

Procedure for Preparation for Standard Curve

1. Weigh 100 mg of standard mixtures of amylose and amylopectin into volumetric flasks following the Table.
2. Add 1 ml of 95% ethanol add 9 ml 1 N sodium hydroxide following the same procedure of step 2 to 4
3. Pipette 5 ml aliquots of each mixture of the working solutions in 100 ml volumetric flasks, each containing about 50 ml of distilled water.
4. Repeat steps 6 to 9.
5. Plot absorbance at 620 nm against the assigned amylose content for the standard curve as shown in Appendix Figure 2.
6. Read % amylose values of the tested samples from the standard curve.

Standard working solutions for amylose determination

Amylose content (% dry weight basis)	Volume ratio of standard working solutions (ml/100ml)	
	Amylose (g)	Amylopection (g)
0	0	0.100
10	0.010	0.090
20	0.020	0.080
30	0.040	0.060
50	0.050	0.050
60	0.060	0.040



Standard curve for amylose determination.

Analysis of Resistant Starch

Adapted from method of Goni, I., L. Gacia-Diz, E. Manas and F. Suara-Calixto (1996)

Chemicals

- KCl-HCl buffer, pH 1.5;
- 0.1 M Tris-maleate buffer. pH 6.9 containing 4 mM $CaCl_2$;
- 0.1 M KOH;
- 0.4 M acetate buffer, pH 4.75, containing 20 mM $CaCl_2$;
- 2 M HCl;
- Pepsin (Merck No. 7190, 2000 FIT-μ/G): solution containing 1 g pepsin in 10 ml KCl-HCl buffer;
- Pancreatic α-amylase (Sigma A-3176): solution containing 40 mg of α -amylase per ml of Tris-maleate buffer:
- Amyloglucosidase (Boeringer Mannheim No. 102857);
- Glucose oxidase-peroxidase kit for determination of glucose (GOD/PAP, Boëhringer Mannheim No. 676543).

Procedure

Samples with low water content are milled to pass through a one mm sieve. If the fat content is ≥5%, samples must be defatted (petroleum-ether extraction).

When the aim of the analysis is the determination of RS in foods as eaten, drying, cooling or storage of samples must be avoided because they could affect the RS content. The samples must therefore be directly homogenized into the centrifuge tube used for analysis.

1. Weigh out 100 mg of dry milled sample into a 50-ml centrifuge tube. Add 10 ml of KCl-HCl buffer, pH 1.5 (pH adjustment with 2 M HCl or 0.5 M NaOH.) In the case of wet samples, weigh a portion equivalent to 100 mg of dry matter, add KCl-HCl buffer, pH 1.5 and homogenize into the centrifuge tube.
2. Add 0.2 ml of the pepsin solution (1 g pepsin/10 ml buffer KCl-HCl). Mix well and leave in a water bath at 40^{o}C for 60 min with constant shaking.
3. Take samples out of the water bath and let them cool to room temperature. Add

   9 ml of 0.1 M Tris-maleate buffer. Adjust to pH 6.9 with 2 M HCl or 0.5M NaOH.
4. Add 1 ml of the α -amylase solution (40 mg α -amylase per ml Tris-maleate buffer). Mix well and incubate for 16 h in a water bath at 37^{o}C with constant shaking.
5. Centrifuge samples (15 min, 4500g) and discard supernatants. Wash at least once with 10 ml of distilled water. Centrifuge again and discard supernatants.
6. Add 3 ml of distilled water to the residue. Carefully moistening the sample. Add 3 ml of 4 M KOH, mix and leave for 30 min at room temperature with constant shaking.
7. Add 3 ml of 0.4 M sodium acetate buffer, pH 4.75. Add approximately 4-5 ml of 2 M HCl and adjust pH to 4.75 with 2 M HCl
8. Add 80 μl of amyloglucosidase. Mix well and leave for 45 min in a water bath at 60^{o}C with constant shaking.

9. Centrifuge (15 min. 4500g), collect supernatant and save it in a volumetric flask. Wash the residues at least once with 10 ml of distilled water, centrifuge again and combine supernatant with that obtained previously. Make up to 50-1000 ml. depending on RS content. (Alternatively, filtration of the samples could be performed instead of centrifugation.)
10. Prepare a standard curve from a glucose water solution (20-80 μg glucose/ml).
11. Pipette 0.5 ml of water, sample and standard solution into test tubes. Add 1 ml of the reagent from the glucose determination kit (GOD-PAP). Mix well and leave for 30 min in a water bath at 37^{o}C.
12. Read the absorbance of the samples and standards at 540 nm against a reagent blank (zero base of the spectrophotometer: reagent blank against reagent blank). Absorbances should be read between 5 and 45 min after incubation.
13. Calculations: use the standard curve to calculate the glucose concentration of the samples.
14. The resistant starch concentration of the test sample is calculated as
    mg of glucose x 0.9 x dilution factor.

Remarks : Digestible starch (DS) was calculated as the difference between total starch and the indigestible starch or resistant starch expressed as percent of the sample weight.

Glucose Determination using Assay kit

Introduction

Enzymes, as analytical tools, have found widespread use in the food, biochemical, and pharmaceutical industry. Enzymatic methods are specific, reproducible, sensitive, rapid, and therefore, ideal for analytical purposes. Due to the high specificity and sensitivity of enzymes, quantitative assays may be done on crude materials with little or no sample preparation. This kit is for the quantitative, enzymatic determination of glucose in food and other materials.

Principle

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{D-Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \underset{\text{(colorless)}}{\text{Reduced o-Dianisidine}} \xrightarrow{\text{Peroxidase}} \underset{\text{(brown)}}{\text{Oxidized o-Dianisidine}}$$

$$\underset{\text{(brown)}}{\text{Oxidized o-Dianisidine}} \xrightarrow{H_2SO_4} \underset{\text{(pink)}}{\text{Oxidized o-Dianisidine}}$$

Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a colored product. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink color measured at 540 nm is proportional to the original glucose concentration.

Reagents

1. Glucose Oxidase/Peroxidase Reagent (Product Code G 3660)

Store the unopened kit reagent at 2-8 ^{o}C. Each capsule contains 500 units of glucose oxidase (*Aspergillus niger*), 100 purpurogallin units of peroxidase (horseradish) and buffer salts. Dissolve the contents of the capsule in an amber bottle with 39.2 ml of deionized water. The solution is stable up to one month at 2-8 ^{o}C and for at least 6 months frozen at-20 ^{o}C. Discard if turbidity develops.

2. o-Dianisidine Reagent (Product Code D 2679)

Store the unopened kit reagent at 2-8 ^{o}C. Minimize exposure to light. The preweighed vial contains 5 mg of o-dianisidine dihydrochloride. Reconstitute the contents of the o-dianisidine vial with 1.0 ml of deionized water lnvert the vial several times to dissolve. Avoid exposing the reagent to light. Solution is stable for 3 months at 2-8 ^{o}C.

3. Assay Reagent

Add 0.8 ml of the o-Dianisidine Reagent to the amber bottle containing the 39.2 ml of Glucose Oxidase/Peroxidase Reagent. Invert bottle several times to mix. Minimize exposure to light. Solution is stable up to 1 month at 2-8 ^{o}C. Discard if turbidity develops or color forms.

4. Glucose Standard Solution (Product Code G 3285)

D-Glucose, 1.0 mg/ml in 0.1% benzoic acid. This standard is traceable to an NIST standard and is supplied ready-to-use. It is stable at 2-8 ^{o}C for at least six months. Discard if turbidity develops.

Procedure for Sample Preparation

Dilute sample with deionized water to approximately 20-80 μg glucose/ml. Filter or deproteinize solution if necessary to clarify. Decolorize solutions that are strongly colored and that have a low glucose concentration. Degas carbonated or fermented products.

Determination

Glucose Concentration from Standard Curve

1. Pipette the following solutions into the appropriately marked test tubes:

Tube	Water (ml)	Sample (ml)	Glucose Standard (ml)
Reagent Blank	1.00	-	-
Standard # 1	0.98	-	0.02
Standard # 2	0.96	-	0.04
Standard # 3	0.94	-	0.06
Standard # 4	0.92	-	0.08
Test	-	1.00	-

2. At zero time, start the reaction by adding 2.0 ml of Assay Reagent to the first tube and mixing. Allow a 40 second interval between additions of Assay Reagent to each subsequent tube.
3. Let each tube react exactly 30 minutes at 37 ^{o}C. Stop the reaction at 40 second intervals by adding 2.0 ml of 12 N H_2SO_4 into each tube. Carefully mix each tube thoroughly.
4. Measure the absorbance of each tube against the reagent blank at 540 nm.

Calculations

For standards, plot Absorbance at 540 nm (y axis) vs mg of glucose (x axis). If the standard curve is not linear, results will be inaccurate. Repeat assay.

For test sample, determine mg glucose from standard curve. Multiply the mg glucose determined above by the dilution factor made in sample preparation.

Method for Determination of *In vitro* Kinetic of Starch Digestion

This is an improved method used to measure the rate of starch hydrolysis in different times, adapted from Goni Isabel, Garcia-Alonso Alejandra and Saura-Calixto, Fulgencio (1997).

Sample preparation

All samples (50 mg) were boiled with water (5 ml) in capped centrifuged tubes and cook for 15 min.

Analysis of total starch (TS)

1. 50 mg samples were dispersed with 6 ml 2 M KOH, shaken at room temp. for 30 min.
2. Add 3 ml of 0.4 M sodium acetate buffer , pH 4.75 and 60 ul amyloglucosidase to the suspension, incubate 45 min. in 60 °C shaking water bath.
3. Measure glucose content and convert to starch content by multiple by 0.9.

Procedure for measurement of *in vitro* starch digestibility

1. 50 mg food portion prepared as explained above.
2. Add 10 ml of HCl-KCl buffer, pH 1.5 , pH adjusted.
3. Add 0.2 ml of solution containing 1 g of pepsin in 10 ml HCl-KCl buffer, incubate at 40 °C for one hour in a shaking water bath.
4. To each sample, bring volume to 25 ml with “Tris-meleate buffer, pH 6.9”
5. Add 5 ml of a solution of alpha-amylase in Tris-meleate containing 3.3 IU.
6. Incubate at 37 °C in a shaking water bath for 30 min to 180 min.
7. **1 ml** aliquote from each tube taken every 30 min. from 0 to 3 hours (7 time points).
8. Each tube placed in 100°C water bath, for 5 min. to inactivate the enzyme action, then refrigerated until the end of incubation time.
9. Centrifuge samples (15 min, 4500g) and collect the supernatants.
10. 3 ml of 0.4M sodium acetate buffer, pH 4.75 was added to each tube.
11. 60 µl amyloglucosidase was added to hydrolyze the digested starch remains into glucose for 45 min. at 60°C in a shaking water bath.
12. Centrifuge (15 min. 4500g), collect supernatant and save it in a volumetric flask. Wash the residues at least once with 10 ml of distilled water, centrifuge again and combine supernatant with that obtained previously. Volume adjusted to 10-100 ml with distilled water.
13. Duplicate aliquots of 0.5 ml used to measure for glucose content by the glucose oxidase-peroxidase kit.

The digestible starch (DS) was calculated as the percentage of sample weight and rate of starch digestion for 30, 60, 90, 120, 150 and 180 minutes was determined

APPENDIX II

OUTPUTS OF THE RESEARCH

Effects of Pullulanase Reaction and Drying Methods on Resistant Starch Type III Formation of Cassava Starch and the Enzymatic Digestion

By

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Presented at

"The 14th World Congress of Food Science & Technology"
October 19-23, 2008
Shanghai, China

What is Resistant Starch ?

Definition : The total amount of starch and the products of starch degradation that resist digestion in the small intestine of healthy people

Classification

RS-I : physically inaccessible starch in the plant cells

RS-II : native granular starch with non-cooking

RS-III : retrograted starch or crystalline of non granular starch

RS-IV : modified by treatments of chemical, physical, thermal.. etc.

Englyst, 1992, 1996; Higgins et al. 2004

Benefits of Resistant Starch

- **Prebiotic effects**
- **Property of low glycemic index (low GI)**
- **Slow release of glucose resulted in**
  - reduced energy uptake by the intestinal cells
  - improved glycemic and insulinemic responses
- **Resemble to properties of dietary fiber,**
  - improved lipid & cholesterol metabolism
- **Good for Diabetes and obeses**

Annison and Topping,1994; Wolever and Mchling, 2002

The Objectives

- To study on pullulanase reactions for preparing the RS-III from the commercial cassava starch
- To examine the effects of spray, freeze and hot air drying on the RS-III formation and change in the amylose molecules
- To compare the *in vitro* digestibility of the obtained RS-III samples
- To examine thermal properties using DSC
- To examine structural properties using X-ray and SEM

Materials and Methods

Process for Resistant Starch Production

Prepare a 10 % suspension of the commercial cassava starch (db)

↓

Gelatinize by pressure cooking at 121°C for 30 min. and cool down to 50°C

↓

Add 3%, 5% and 10 % pullulanase to debranch the amylopectin

↓

Allow to hydrolyze for 8 and 24 h, at 50°C

↓

Deactivate the enzyme by heating at 90°C for 30 min.

↓

Cool for 24 and 48 h, at 4 °C

↓

Centrifuge and collect the starch

↓

Dry by spray, freeze and hot air

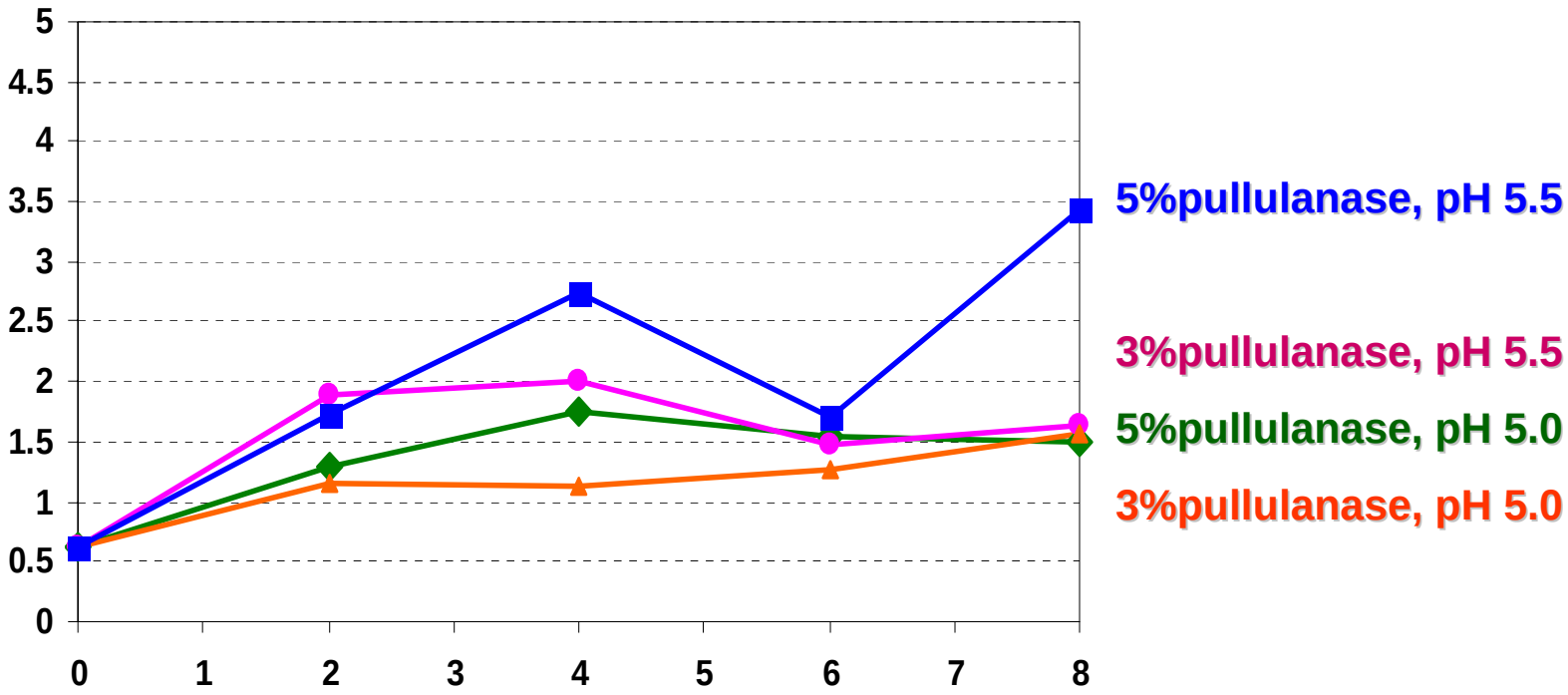
↓

Determinations of the RS-III and properties

Results and Discussion

Change in Reducing Sugars

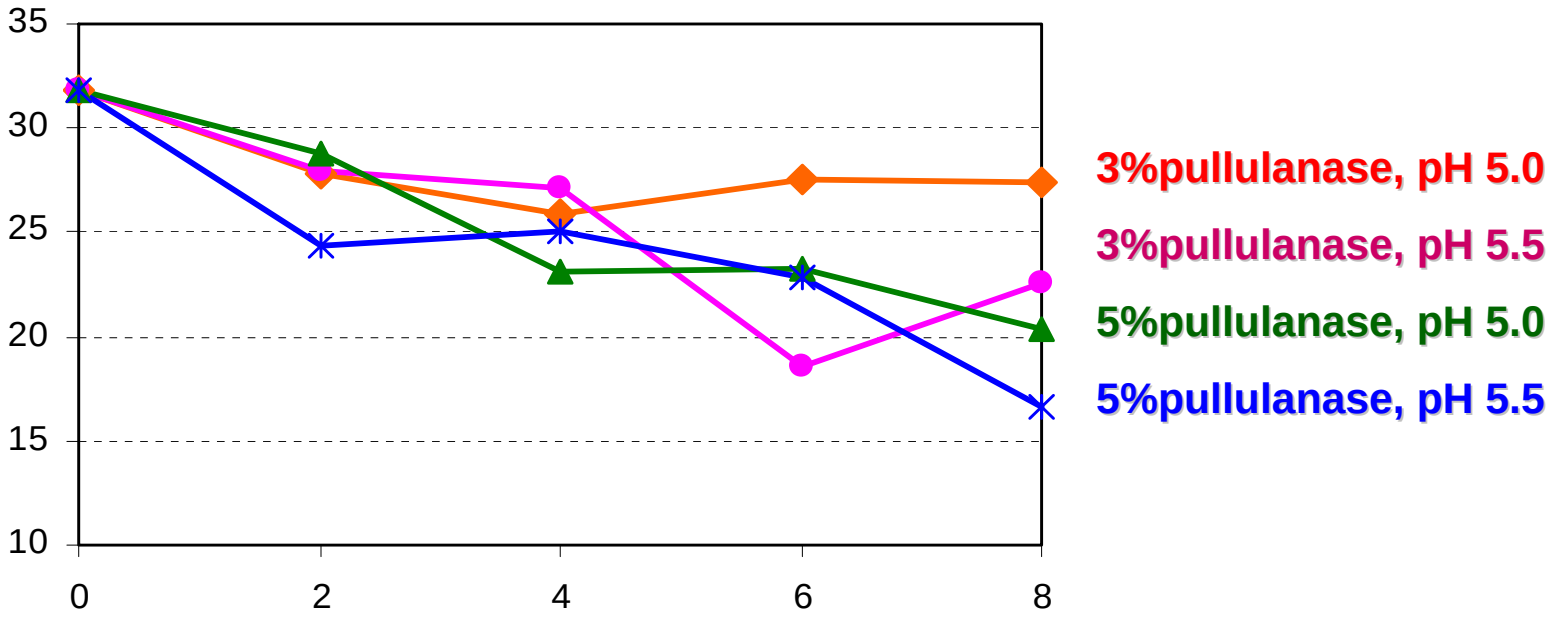
g/100g starch, db



Hydrolysis time, hours

Change in Amylose Contents

g/100g starch, db



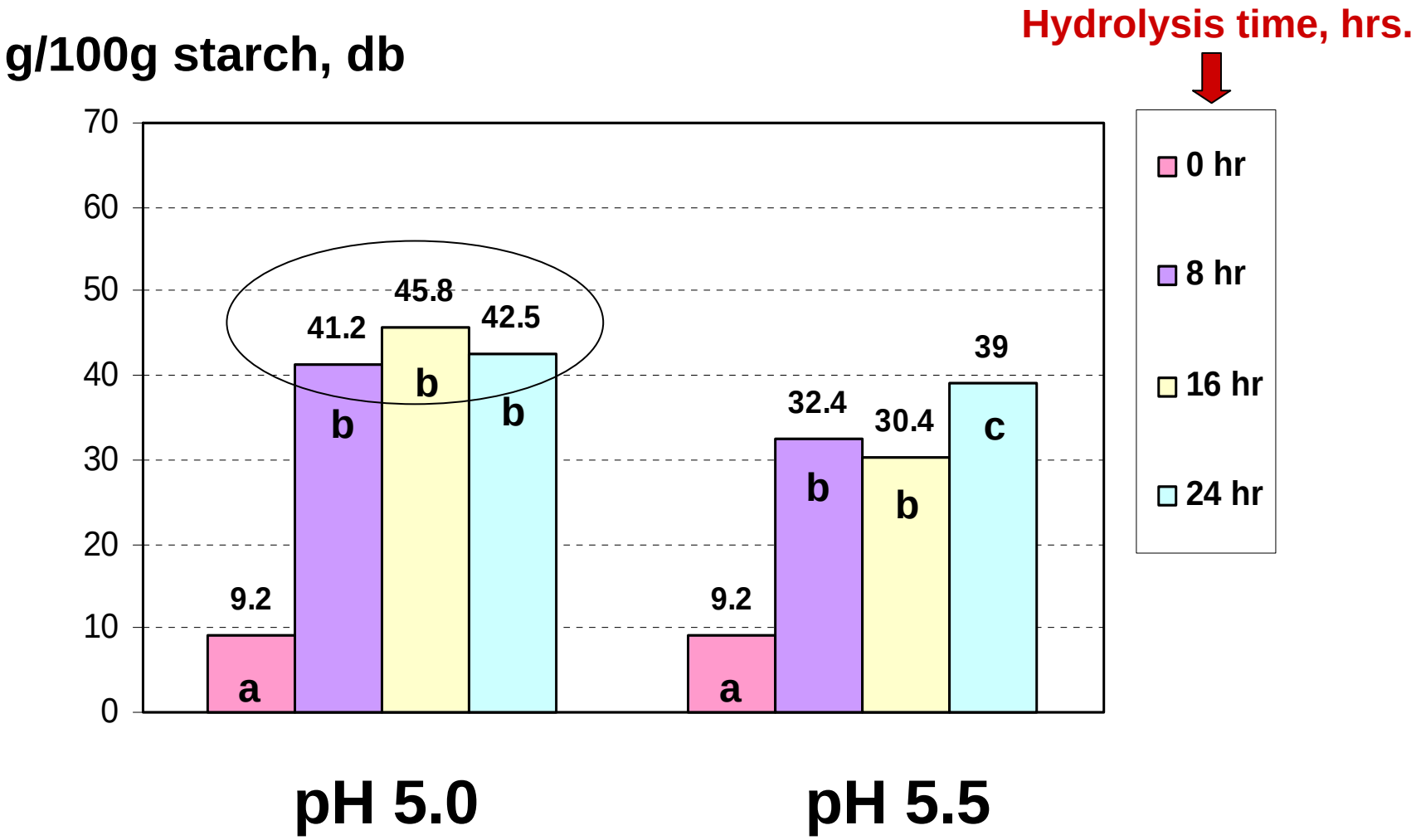
Hydrolysis time, hours

RS-III Formation with the Hydrolysis for 8 hours

Sample treatments	Contents of resistant starch (g/100g, dry weight)
Commercial cassava starch	58.2 ± 1.3 a
Gelatinized cassava starch	
10% starch	0.0
50% starch	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

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different $p < 0.05$

RS-III Formation by the 10% Pullulanase



Drying Effects on Amylose Contents, g/100g

Samples	Cooling time	
	24 hr	48 hr
Com. cassava starch	31.8 ± 2.6 a	31.8 ± 2.6 a
8 hr hydrolysis		
Spray	27.0 ± 1.6 b	28.9 ± 0.3 a
Freeze	27.4 ± 1.7 b	27.2 ± 4.8 a
Hot air	29.4 ± 1.6 ab	28.3 ± 0.8 a
24 hydrolysis		
Spray	21.7 ± 1.9 c	14.4 ± 1.1 b
Freeze	15.8 ± 0.5 d	12.5 ± 0.9 c
Hot air	14.1 ± 1.4 d	19.2 ± 0.5 b

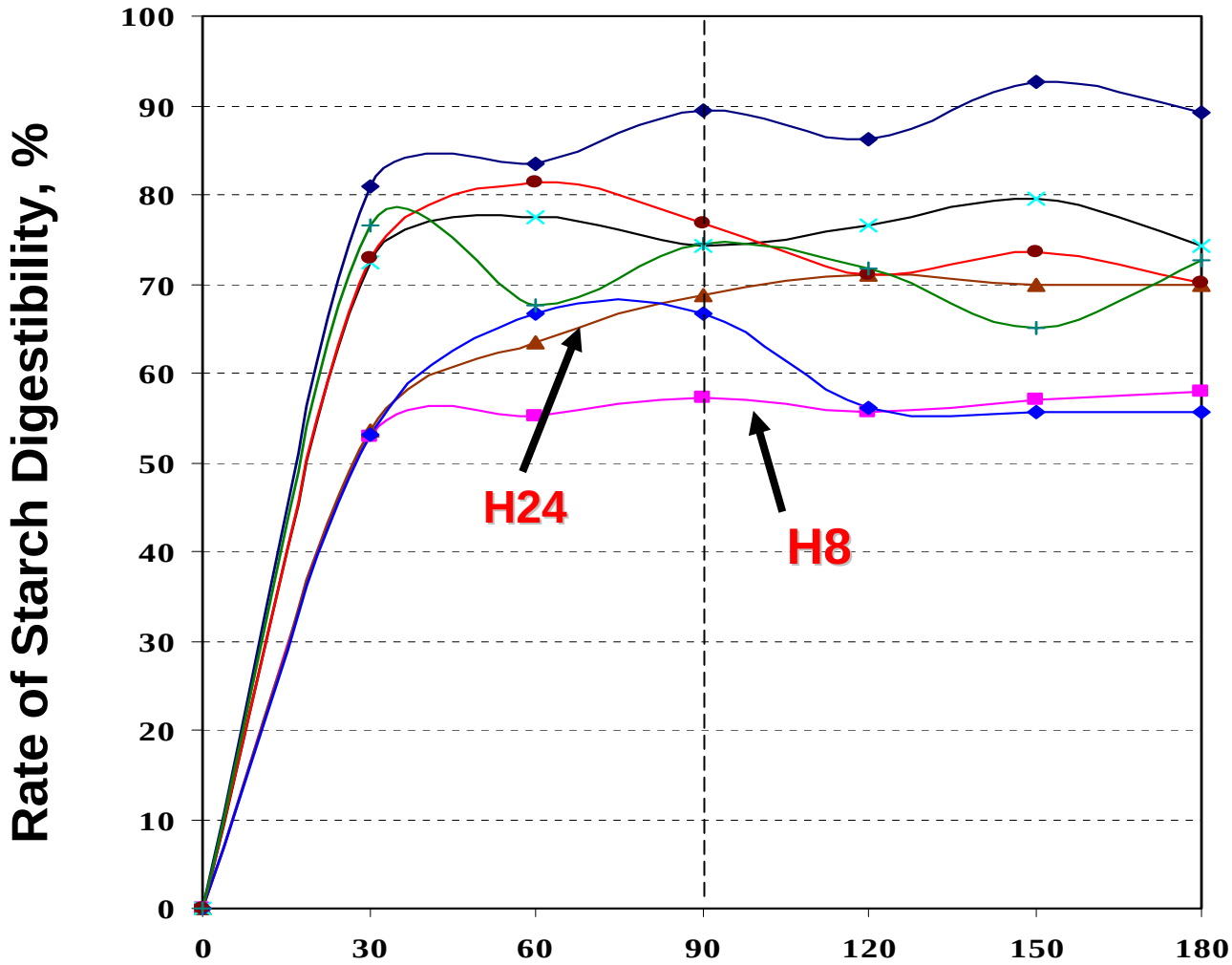
* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different $p < 0.05$

Drying Effects on RS-III Contents, g/100g

Samples	Cooling time	
	24 hr	48 hr
Com. cassava starch	9.2 ± 0.2 a	9.2 ± 0.2 a
8 hr hydrolysis		
Spray	33.0 ± 2.2 b	37.7 ± 1.0 c
Freeze	41.7 ± 2.3 c	26.8 ± 0.2 b
Hot air	43.4 ± 4.6 c	29.8 ± 3.9 b
24 hydrolysis		
Spray	32.9 ± 0.1 b	41.3 ± 0.8 c
Freeze	44.8 ± 0.4 c	40.3 ± 0.1 c
Hot air	50.9 ± 2.9 d	45.2 ± 0.3 d

* Values are means of duplicate analysis.
In a column, means not sharing a common superscript are significantly different, $p < 0.05$

In Vitro Starch Digestibility (24 h cooling)



Com. cassava starch

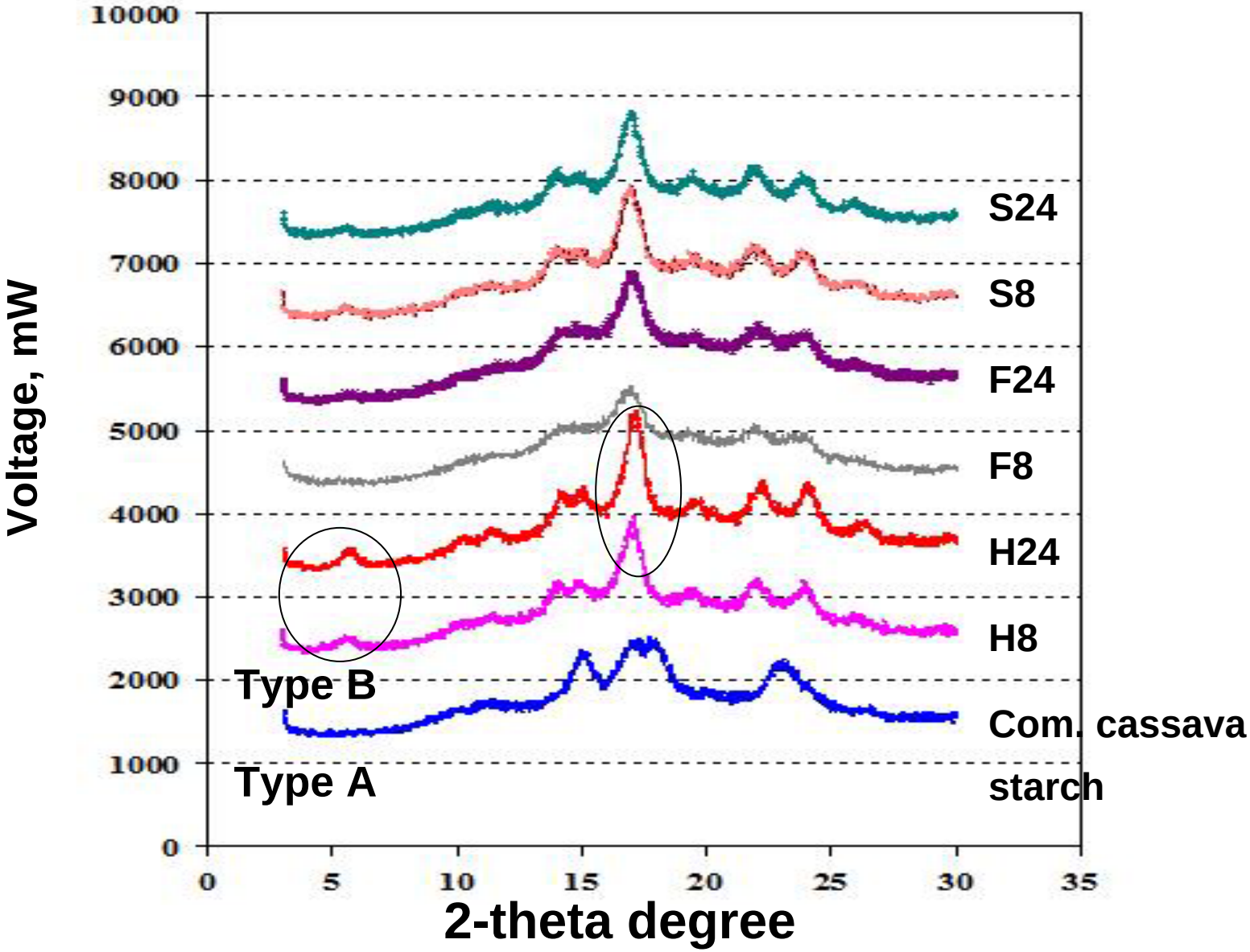
F8, S24, S8,

F24

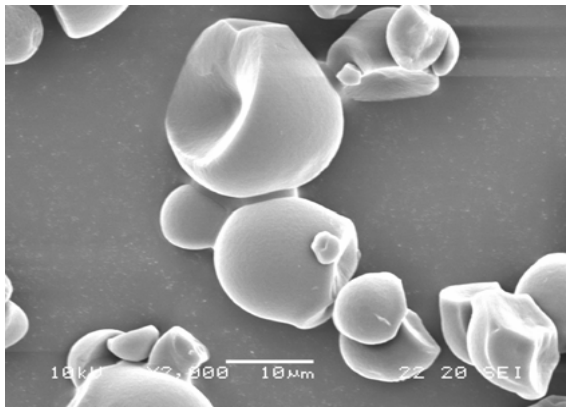
Thermal Properties of Spray-Dried RS-III

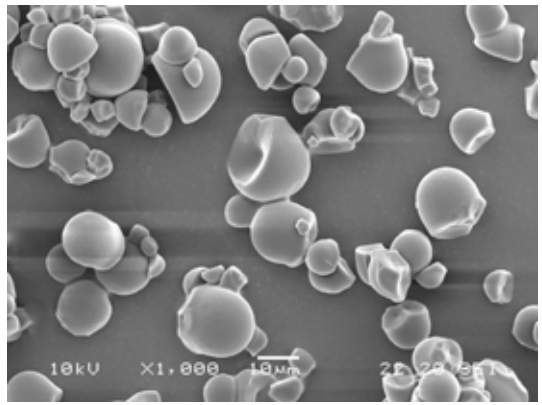
Samples	Melting Enthalpy, J/g	Melting temperature, °C		
		Onset	Peak	End
Com. cassava starch	271.7 a	145.1	148.8	172.4
Com. resistant starch	255.0 b	139.9	146.2	175.2
8 h hydrolysis 24 h cooling	188.3 c	144.1	146.7	164.9
48 h cooling	188.4 c	147.7	150.9	172.7
24 h hydrolysis 24 h cooling	182.1 c	147.6	151.2	174.5
48 h cooling	167.6 d	145.9	149.5	170.7

X-Ray Diffractograms



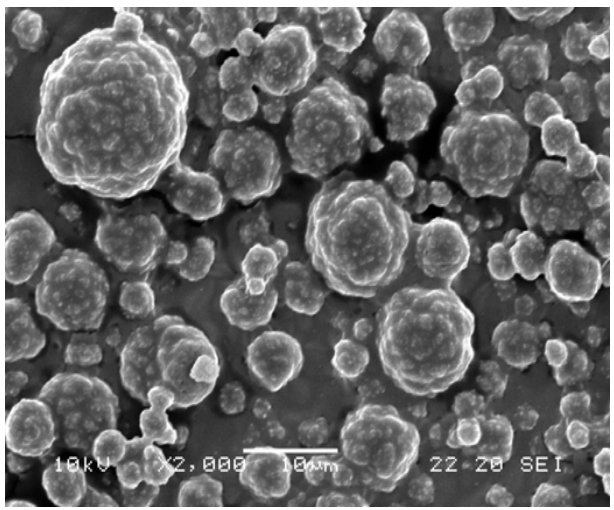
Scanning Electron Micrographs of the RS-III



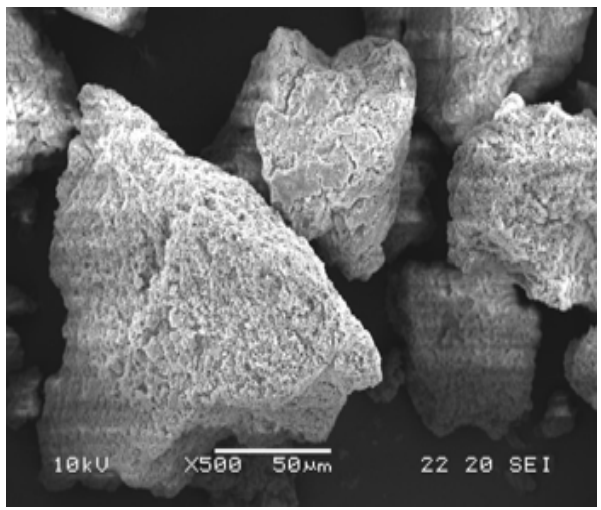


Com. Cassava starch granules,

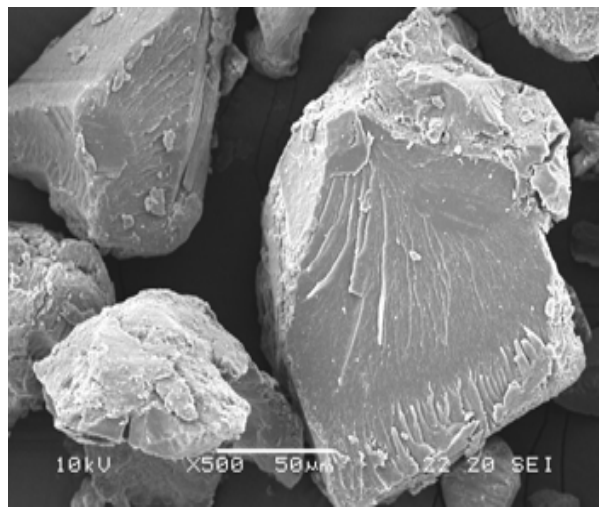
1000X, 2000X



Spray drying, 2000X



Freeze drying, 500X



Hot air drying, 500X

Conclusions

An appropriate condition for production of RS-III from the cassava starch was

- **The 10% pullulanase of the total starch (db) adjusted to pH 5 with hydrolysis for 8 hrs. and cooling for 24 hrs, then dried using hot air.**
- **This was suitable for partially debranching the amylopectin molecules, consequently providing small linear fragments/ small clusters for recrystallization and formation of the RS-III.**
- **A high yield of the obtained resistant starch was 43.4% and the starch digestibility was 32 % lower than the cassava starch.**

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Thank You Very Much