THESIS

ENZYME-RESISTANT STARCH TYPE III PRODUCTION FROM HIGH AMYLOSE RICE STARCH AND APPLICATION IN LOW GLYCEMIC INDEX BUTTER CAKE

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GRADUATE SCHOOL, KASETSART UNIVERSITY

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THESIS

ENZYME-RESISTANT STARCH TYPE III PRODUCTION FROM HIGH AMYLOSE RICE STARCH AND APPLICATION IN LOW GLYCEMIC INDEX BUTTER CAKE

JIRAPA PONGJANTA

A Thesis Submitted in Partial Fulfillment of
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The purposes of this study were to investigate the production of enzyme-resistant starch type III (RS III) from high amylose rice starch (HARS) and application in low glycemic index butter cake. HARS composed of 95.21% dried weight basis of total starch, 1.18% protein, 0.91% fat, 0.82% ash, 13% moisture content and 32.10% amylose content. The effects of preheated treatments of 15% HARS and enzyme concentration on physicochemical properties of RS III samples were also examined. A debranching enzyme (Pullulanase, EC. 232-983-9P, 8 unit/g starch at 55°C for 0 to 48 hr) was introduced to modify the amylopectin molecules of 15% HARS, which were preheated at 75°C, 95°C and 121°C for 30 min. The retrogradation of debranched starches with different degrees of hydrolysis (0.14 to 3.10%) were heated in boiling bath for 30 min then induced at 4°C for 16 hr. Afterwards, the one cycle of freeze-thaw process (-10/30°C) was applied to promote syneresis of the retrograded starches. The resistant starch content increased from 4.12% to 19.31% of dried weight basis from control to 48 hr debranched of preheated rice starch at 121°C. Effect of pullulanase enzyme concentration (8, 10, 12, 14 and 16 unit/g starch debranched of 121°C preheated 15% HARS and incubated at 55°C for 16 hr) was investigated. Result had shown that the enzyme concentration at 12 unit/g of starch was an optimum for pullulanase hydrolysis (4.54%) allowed more rearrangement and ordered structures of retrograded starches that characterized as V-type pattern, which was 19.81% resistant starch content. The RS III sample formed a coarse honeycomb-like and filamentous network structure was observed by Scanning Electron Micrograph. The estimated hydrolysis index and glycemic index value of the selected RS III sample were 39.50% and 61.43% of GI values, respectively. The effect of using RS III as flour replacement and high fructose corn syrup (HFCS-55) with sucrose substituted on the physiochemical properties and sensory evaluation of the resultant cakes were studied. Results revealed that an optimum formula of butter cakes were 29.79% cake flour, 3.31% RS III, 16.08% HFCS, 10.72% sucrose, 25.13% butter, 0.21% baking powder, 0.10% salt, 1.34% corn flour and 13.40% eggs. The butter cake was accepted by 30 panelists at level of moderately like and the physicochemical properties were not significantly difference from the control cake. The estimated glycemic index of the developed butter cake was 68.16% of GI value and classified as a medium glycemic index food, while the control cake was classified as high glycemix index food (77.10% of GI value).

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Jirapa Pongjanta

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LIST OF ABBREVIATIONS

Avg.	=	average
cm	=	centimeter
cP	=	centripoint
°C	=	degree Celsius
DMRT	=	Duncant's multiple range tests
DM	=	dried matter
DP_n	=	degree of polymerization
DS	=	digested starch
DSC	=	differential scanning calorimetey
dwb	=	dried weight basis
EGI	=	estimated glycemic index
g	=	gram
GI	=	glycemic index
GL	=	glycemic load
GT	=	gelatinization temperature
HARS	=	high amylose rice starch
HFCS	=	high fructose corn syrup
HI	=	hydrolysis index
hr	=	hour
l	=	liter
M	=	molar
µg	=	microgram
µl	=	microliter
µM	=	micromolar
µm	=	micron mesh
mg	=	milligram
mM	=	millimolar
MW	=	molecular weight
rpm	=	rotation per minute

LIST OF ABBREVIATIONS (Continued)

Rds	=	reducing sugar
RS	=	resistant starch
RS I	=	resistant starch type III
RS II	=	resistant starch type II
RS III	=	resistant starch type III
RS IV	=	resistant starch type IV
SEM	=	scanning electron microscopy
TS	=	total starch
Ts	=	total sugar
u/g	=	unit/gram
w/v	=	weight by volume
w/w	=	weight by weigh

ENZYME RESISTANT STARCH TYPE III PRODUCTION FROM HIGH AMYLOSE RICE STARCH AND APPLICATION IN LOW GLYCEMIC INDEX BUTTER CAKE

INTRODUCTION

Rice (*Oryza sativa* L.) is a major cereal of Thailand. Commercial rice starch composed of up to 90 % carbohydrates and 0.5-1.5 % protein with negligible fat or dietary fiber (Juliano, 1992). Rice flour and starch become popular food ingredients since they are hypoallergenic, low fat and neutral in flavor. However, rice starch is high glycemic index (GI) food, which was low in resistant starch (RS) content. Freshly cooked rice contains below 3% of RS content, but the RS tends to increase with amylose content and processing treatments (Walter *et al*. 2005). RS has been recently defined as the sum of starch and its degradation products that are not absorbed in the small intestine of healthy individuals (Englyst *et al*. 1999). The reduced bioavailability of RS in the human gastrointestinal tract has particular significance for diabetics because it lowers the insulin response. Besides physiological benefits in human, RS has been reported to have potential use as a unique ingredient with improved oral tactile perception, taste palatability, color and texture. RS can be found in nature and its contents of RS are varied by botanical source and starch granular structure (resistant starch type I and II). In addition, resistant starch can be produced from retrogradation of gelatinized starch known as resistant starch type III (RS III).

There has been previously published study to produce appropriate technology for RS III formation from high amylose rice starch and its stability in food applications. Thus, the primary purpose of this study was to improve RS III from high amylose rice starch by enzymatically-debranching process. The extents of RS III of most starches are, however, are quite low, depending on the molecular structure of starch glucans, namely amylose and amylopectin. High amylose starch produced higher resistant starch than low amylose starch**.** Debranching using pullulanase have

been used to produce a sample with linear, low-molecular-weight and recrystallization polymer chains (Guraya *et al*. 2001 and Yin *et al*. 2007). Debranching enzymes rapidly hydrolyze only α-1,6-glucosidic bonds. This releases a mixture of varied unit chain length from the amylopectin molecule which in turn facilitated starch retrogradation. In addition, retrogradation is often enhanced when starch gels are subjected to freezing and thawing treatments (Tovar *et al*. 2002). Freezing a starch gel leads to the formation of ice crystals and thus concentrates the starch in non-ice phase. Upon thawing, the water can be easily compressed from the network, giving rise to a phenomenon known as syneresis (Tovar *et al*. 2002). The recrystallinity of rice starch was altered by pullulanase debranching and freeze-thaw process. The effects of these treatments on degree of hydrolysis, degree of syneresis, degree of crystallinity, physicochemical properties, *in vitro* starch digestibility and glycemic index were examined. The optimum condition of RS formation was selected to application in low glycemic index butter cake product.

The food industry is being challenged to redesign traditional foods for optimal nutritional value, in response to some population sectors with particular nutritional necessities and making them as tasty as or better than the original. One way to achieve a healthy food product is to reduce or to omit some of the glucose-laden ingredients, especially flour and sugar since, at present, diabetic and obesity is frequently cited as a serious health problem. At the same time, there is a constant demand for dietetic foods suitable for diabetics, which may have the same calorie-value being also sucrose-free since this sugar cannot be metabolized without insulin. For this reason, it is a challenge to develop food products, which was a cake in this study that low glycemic index for individuals who are intolerant to glucose by using RS III as flour replacement and high fructose corn syrup as sucrose replacement. Many carbohydrate-rich foods have high glycemic indexes (GI.), and certainly are not good in any substantial quantity for people with diabetes. RS III is broken down and degrade more slowly, releasing glucose gradually into our blood streams and are said to have lower glycemic indexes. Fructose is very sweet, is roughly 1.75 times sweeter than sucrose, and is defined as low glycemic ingredient (Jenkin *et al*. 2002a). Because fructose must be changed to glucose in the liver in order to be utilized by the body,

blood glucose levels do not raise as rapidly after fructose consumption compared to other simple sugars and complex carbohydrates. Butter cake is a low dietary fiber, complex fat and water emulsion system containing flour, milk, sugar, fat, eggs and baking powder (Bennion, 1995b). A proper combination of the ingredients can give a high-quality product with desirable flavor and texture. From a physiological standpoint, as low glycemic ingredients, has none of the published report describe the practical use of RS III and high fructose corn syrup in butter cake. The effects of RS III and high fructose corn syrup application in butter cake on physicochemical properties and sensory evaluations of the developed butter cake were investigated. The stability of RS III to tolerate varying processing conditions in the cake on starch digestibility, hydrolysis index and glycemic index were examined using *in vitro* analysis.

OBJECTIVES

Overall objectives:

1. To produce resistant starch type III (RS III) from high-amylose rice starch by enzymatically-debranching process.

2. To produce low glycemic butter cake product by using RS III from high amylose rice starch and high fructose corn syrup.

Specific objectives:

1. To study the physiochemical properties of native high-amylose rice starch

2. To investigate the effect of preheated treatments of rice starch on degree of hydrolysis, degree of syneresis of debranched products and *in vitro* starch digestibility of RS III formation.

3. To investigate the effect of pullulanase enzyme concentrations on degree of hydrolysis, degree of syneresis, β-amylolysis of debranching products and *in vitro* starch digestibility of RS III formation.

4. To examine granule structure and crystallinity of RS III with a scanning electron microscopy (SEM) and X-ray diffraction pattern.

5. To investigate the optimum level of RS III and high-fructose corn syrup in butter cake by sensory evaluation and physicochemical analysis.

6. To examine an *in vitro* starch digestibility, hydrolysis index and estimated glycemic index of butter cake with RS III and high-fructose corn syrup replacement of wheat flour and sucrose.

LITERATURE REVIEW

1. Resistant starch functional and properties

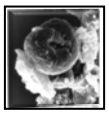
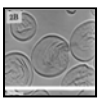
1.1 Definition and classification

Resistant starch (RS) is described as starch that escapes digestion in the small intestine (Englyst and Kingman, 1990; Bravo *et al*. 1998; Topping and Clifton, 2001). Scanning electron microscopy assessment of ileostomy effluents showed that some starch escaped digestion in the small intestine and the granules remained intact (Muir *et a*l. 1995). A EURESTA study defined resistant starch as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Asp, 1992). Experimental data now shows that this definition could be broadened to indicate that resistant starch is not digested by pancreatic enzymes, and hence is not metabolized or absorbed in the small intestine, but rather enters the colon where it is fermented by colonic microflora. On the other hand, dietary fiber consists mainly of plant cell wall material, notably cellulose, hemicellulose, pectins, gums, mucilages and lignin. These are generally not digested by enzyme hydrolyses because of their complex nature.

Classification of four forms of resistant starch and factors affecting their resistance to digestion in the colon are show in Table 1. RS is further partitioned in physically inaccessible starch (RS I), in resistant starch granules (RS II), in retrograded starch (RS III), of which only retrograded amylose is totally resistant (Englyst and Macfarlane, 1986; Englyst *et al*. 1987; Englyst *et al*. 1992; Englyst and Hudson. 1996) and in chemically modified starch (RS IV) (Brown *et al*. 1995). Analyses indicate that RS consists of crystallized, linear, unbranched, short-chain α-glucans (DP about 60-65) (Berry *et al*. 1988; Siljeström *et al*. 1989). Since amylose-lipid complexes significantly reduce the availability to α-amylase, an interrelation of complexation with lipids and resistant starch formation is assumed but it is verified that amylose-lipid complexes are not involved in the formation of RS.

However, it was discovered that a removal of lipids from the starch resulted in higher RS yields. This is probably due to an increase of lipid-depleted "free" amylose crystallized (Berry *et al*. 1988 and Cerletti *et al*. 1993). Derived from heat treated starches, RS displays endothermic transitions between 120 and 177°C in DSC studies that seem to be due to melting of recrystallized amylose (Sievert and Pomeranz, 1989). RS exhibits a B-type X-ray diffraction pattern at low retrogradation temperatures and a mixture of A-type with B-type or V-type pattern if retrograded at high temperatures (95°C) (Siljeström *et al*. 1989, Sievert *et al*.1991, Eerlingen *et al*. 1993 and Shamai *et al*. 2003).

Table 1 Classification of RS and factors affecting their resistance to digestion in the colon

Type	Description	Food sources	Resistant reduced by
RS I	Physically protected 	Whole or partially milled seeds, legumes, pasta	Milling and Chewing
RS II	Non gelatinized granules with B-type crystallinity and are hydrolyzed slowly by α-amylase 	Raw potatoes, green banana, some legumes, high amylose starch	Food processing and Cooking
RS III	Retrograded starch 	Cooked and cooled cereal products with prolong and/ or repeated moist heat treatment	Processing condition
RS IV	Chemically modified starches due to cross-bonding with chemical reagents. 	Some fiber drink, foods in which modified starches has been used (e.g. certain breads and cakes)	Less susceptible to *in vitro* digestibility

Source: Asp, (1992) and Englyst *et al*. (1992)

1.2 Nutritional and health relevance of resistant Starch

Starch is a major dietary component of most diets around the world. Its digestion and metabolism therefore has physiological ramifications. Disparities in the prevalence of diseases related to consumption and digestion of complex carbohydrates such as starch and dietary fiber have been widely described. Epidemiological studies indicate that diets with slowly digestible, less available starch protect against diseases such as type 2 diabetes, colorectal cancer and other diet-related chronic diseases. Differences in levels of starch consumption as well as modes of food processing are certainly of some significance. Muir *et al*. (1996) established the relationship between dietary fiber intake in Japan and occurrence of colon polyps, colon cancer and diverticulosis, further establishing dietary fiber metabolism in the colon as having a primary role in disease prevention. The role of resistant starch was highlighted by observations that fermentation products in the colon surpassed expected amounts from available dietary fiber, indicating that another significant fermentation substrate was probably present (Topping and Clifton, 2001). This was established to be resistant starch.

1.2.1 Resistant starch and colonic function

Resistant starch has been shown to be protective against colorectal cancer. Ahmed *et al*. (2000) attributed low rates of colorectal cancer in a black South African population to high levels of resistant starch in the diet. This population consumes processed maize products as staples, particularly fermented maize porridge. Experimental data in human subjects showed that stale maize porridge (high resistant starch) resulted in higher fermentation in the colon, and subsequently higher levels of short-chain fatty acids and a significant lowering of stool pH. Short-chain fatty acids levels were significantly higher in healthy subjects who consumed stale maize porridge than in subjects who consumed fresh porridge (low resistant starch). Bingham *et al*. (2003) reported that in populations with a low to average intake of dietary fiber (measured as non-starch polysaccharides), doubling of fiber intake could reduce the risk of colorectal cancer by up to 40%.

Cassidy *et al*. (1994), in an international correlative study, found a strong inverse relationship between starch intake and colon ad rectal cancer, even after adjusting for fat and protein intake. Non starch polysaccharides were only significantly correlated when combined with starch. In this study, the authors assumed that 5% of all starch consumed was resistant and that this RS contributed to the protective effect of starch. However, this estimate represents a substantial amount of RS reaching the colon as dietary intakes of starch are approximately 8-10 times higher than intakes of non starch polysaccharides.

1.2.2 Resistant starch and short chain fatty acid production

The fermentation of resistant starch in the colon by colonic bacteria to produce short-chain fatty acids has been associated with various health benefits (Escarpa *et al*. 1996; Topping and Clifton, 2001). The formation of butyrate seems to be especially beneficial, as its function has been linked to lowering cancer risk by various mechanisms. Butyrate inhibits division of cancer cells, induces apoptosis in colon tumor cell lines and inhibits proliferation of colonic mucosal cells (Van *et al*. 1994). Butyrate induces the chemo preventive enzyme, glutathione transferase in rat colon (Escarpa *et al*. 1996).

Furthermore, the toxicity of potential mutagens such as nitrosamide and hydrogen peroxide in human colon cells is inhibited by butyrate. In addition to the production of short-chain fatty acids, the presence of resistant starch in the colon results in lowers concentrations of bile acids such as cholic acid and enhances their excretion, probably protecting against colorectal cancer. In particular, RS II (from raw potatoes starch) is reported to increase the concentration of butyrate in humans and rats (Cummings *et al*. 1996; Martin *et al*. 2000; Henningsson *et al*. 2003). While RS3 (retrograded starch) reported to increase the concentration of acetate in pigs (Martin *et al*. 2000), but not in humans (Cummings *et al*. 1996).

1.2.3 Resistant starch and metabolic responses

Resistant starch is not absorbed and therefore causes minimal increase in post-prandial glucose potential (hence has low glycemic index, GI), and insulin. This is the case with most legume starches which have higher amylose content than cereal starches and are easily retrograded to produce higher levels of resistant starch (Crosby, 2003). Legumes and other complex carbohydrate-based foods constitute a large part of the diet in many non-Western societies, and the protective role of resistant starch may be more obvious. This is significant in the management and control of glucose-metabolism related diseases such as type II diabetes and obesity.

1.2.4 Resistant starch and insulin and glucose metabolism

Insulin is a hormone that enables glucose uptake by muscle and adipose cells, thereby lowering blood glucose levels (Bornet *et al*. 1989). It also inhibits the use of stored body fat and together with an array of other physiological signals can modulate appetite and satiety signals. RS-rich foods release glucose slowly and therefore one would expect this to result in a lowered insulin response, greater access and used of stored fat and potential, a muted generation of hunger signals. Not only would these conditions help in the management of clinical conditions, such as diabetes and impaired glucose tolerance, but also possibly in the treatment of obesity and in weight management (Goddard *et al*. 1984).

1.2.5 Resistant starch and lipid metabolism

Cheng and Lai (2000) investigated the effects of different proportion of rice starch and cornstarch on lipid metabolism in rats fed high dietary cholesterol. They found that the rice starch was aggregation (n= 20 - 60) of smaller granules (3 - 8 micron in diameter), whereas the cornstarch was composed of larger (5 - 15 micron), single granules. The compound rice starch (0.99 kg/L) was larger in size and denser in structure than cornstarch (0.63 kg/L). Serum total cholesterol concentrations in rats

fed both the 45 and 63g/100g rice starch diets were significantly lower than in all other groups. The serum propionate concentration in the rats fed 63 g/100g rice starch diets was significantly higher than that of other groups. Hepatic triglyceride and total cholesterol concentration in rat fed 63 g/100g rice starch diets were significantly lower than the control group. These results suggest that, because the compound rice starch was an aggregation of smaller granules, larger in size and denser in structure than cornstarch, it was digested more slowly and altered lipid metabolism. Resistant rice starch is fermented to produce propionate, which reduces serum and hepatic cholesterol.

1.3 Processing and resistant starch formation

Food processing techniques, particularly those that utilize heat, moisture or chemicals influence the formation and levels of resistant starch. This is attributed to structural changes induced in the amylose in starch. Dehulling legumes by steam treatment and cooking for instance result in higher levels of resistant starch (Tovar *et al.* 2002). The high levels of amylose in legumes are converted to resistant starch with processing. Resistant starch most commonly found in processed foods is largely retrograded starch.

Autoclaving, a moist-heat technique commonly employed in the food industry, involves somewhat extensive processing of the food product, as well as heating and cooling phases. Multi-cycle autoclaving of starch-based products results in retrogradation, and consequently increased levels of resistant starch (RS III), particularly in high amylose foods (Skrabanja *et al*. 2001).

Processing techniques such as canning, extrusion and microwave heating however, are reported to result in lower levels of resistant starch (Mèance *et al*. 1999). These processes utilize moist heat thereby facilitating gelatinization of starch, and with no subsequent retrogradation phase, starch susceptibility to digestion is greatly enhanced. Baking for prolonged periods meanwhile, was shown to increase resistant starch content of wheat bread and rye bread (Rabe and Seivert, 1992).

Storage techniques and conditions of starch-based foods and products may influence resistant starch levels. Retrogradation and restructuring of amylose occurs, particularly with protracted storage. It is also suggested that during storage, starch interacts with other food components, which inhibit starch-degrading enzymes and reduce its digestibility (Kailcheusky *et al*. 1990).

1.4 Structure of resistant starch

The resistance of starch to digestion is influenced by the nature of the association between starch polymers, with higher amylose levels in the starch being associated with slower digestibility rates. Both B and C type starches appear to be more resistant to digestion with high-amylose maize producing RS which has been particularly useful in the preparation of foods (Brown, 2004). Retrograded starches refer to certain structural forms of RS. Retrogradation occurs when starch is cooked in water beyond its gelatinization temperature and then cooled. Amylose is found in the amorphous parts of the starch crystal, while amylopectin gives starch its crystalline structure. Upon heating with excess water and at sufficiently high temperatures, the starch crystalline regions 'melt'. The starch granules gelatinize and the starch is subsequently more easily digested. However, these starch gels are unstable and upon cooling re-form crystals that are resistant to hydrolysis by amylases (i.e. resistant to digestion). Slow cooling of the gelatinized starch favors Type A crystallization while slow cooling in excess water favors Type B crystallization. This process is known as retrogradation (Topping and Clifton 2001). In general, starches rich in amylose are naturally more resistant to digestion and also more susceptible to retrogradation.

1.5 Resistant starch as a functional food ingredient

Starch is used in multiple capacities in the food industry: for bulking, functionality, texture and for nutritional quality, among other uses. It can therefore be easily modified and further exploited to increase its value in disease prevention. However, dietary intake of resistant starch has been very low. Johnson and Gee (1996) for instance, estimated daily intake of resistant starch by an individual in the UK to be

only about 3 g. The physiological benefits of resistant starch indicate great potential for its incorporation in functional food product development. Functional foods are defined as “foods similar in appearance to conventional foods that are consumed as a part of the normal diet and have demonstrated physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions” (Bravo *et al*. 1998). Resistant starch can be exploited for the development of functional foods in various ways:

1. identification of foods high in resistant starch and processing them to be palatable and appealing to consumers;
2. application of processing techniques that increase resistant starch (retrograded) levels in foods;
3. development of resistant starch-based products as prebiotics
4. genetic modification of amylose : amylopectin ratios; and
5. development and recommendation of food science practices that ensure stabilization of resistant starch levels in foods.

Several whole grains possibly contain significant amounts of resistant starch as well as other fermentable carbohydrates, as a consequence of the inaccessibility of the starch to digestion. Utilization of suitable processing techniques that render whole grain products acceptable and palatable will be very useful in contributing to resistant starch incorporation in functional foods. Legumes are good sources of starch (up to 65 per cent on a dry weight basis), and type RS II resistant starch (resistant granules). It has been demonstrated that these levels are modified by processing (Bravo *et al*. 1998 and Englyst *et al*. 1999).

1.6 Measurement of resistant starch

Due to its health benefits, it was aimed to develop a RS production process and thus to enhance the RS content in food. Beforehand a standardized determination method had to be invented and validated. Several *in vitro* methods were utilized (Englyst *et al*. 1982, Berry, 1986, Champ, 1997, Björck *et al*.1987, Englyst *et al*. 1992, Saura-Calixto *et al*. 1993, Englyst *et al*. 1996 and Goni *et al*. 1996) before

McCleary and co-workers developed an official AOAC method for the determination of RS in plant and starch materials (McCleary and Monaghan, 2002).

The main step of any method to measure the content of RS in foods must first remove all of the digestible starch from the product using thermostable α-amylases (McCleary and Rossiter 2004). At present, the method of McCleary and Monaghan (2002 and AOAC method 2002.02) is considered the most reproducible and repeatable measurement of RS in starch and plant materials, but it has not been shown to analyze all RS as defined (Champ *et al*. 2003). It is based on the principle of enzymic digestion and measures the portions of starch resistant to digestion at 37^{o}C that are typically not quantities due to the gelatinization at 100°C followed by digestion at 60^{o}C.

In the US and some other countries such as Japan and Australia, the Association of Official Analytical Chemists (AOAC) method 985.29 for Total Dietary Fiber Determination in Foods (Prosky *et al*. 1985), a gravimetric determination of dietary fiber quantity after enzymic digestion, mimicking human digestion) is commonly used to measure total dietary fiber (De Vries, 2004). This method accounts for some (RS III, the retrograded portion, and RS II as found in high amylose maize) RS present as part of the total dietary fiber value. Therefore, while it does measure some RS as part of the total dietary fiber figure, additional methods are needed for quantification of the other categories of RS (Champ *et al*. 2003). Again, this highlights the need for a universally agreed definition and method of analysis for all of the components of dietary fiber, including RS.

1.7 Commercially manufactured sources of resistant starch

In addition to the natural food sources of RS, some commercially manufactured forms of RS are also available. Hi-maize® was originally obtained from a maize hybrid grown in Australia. It originally contained 80–85% amylose with approximately 30% dietary fiber when commercially released in 1993 but it has since been improved to provide ingredients containing approximately 60% dietary fiber

(Brown *et al*. 1995). This product, high-amylose maize starch and categorized as RS II is now sold throughout the world by National Starch and Chemical Co. and is used in products including cereals, biscuits, other baked goods, dairy products, nutrition bars and breads. In particular, Hi-maize is incorporated into Australian, New Zealand and Swedish breads, such as Wonder-White, Nature's Fresh Fiber White and Pagens 'Bra'. A number of RS III ingredients are available with a dietary fiber content < 30%; in general these are derived from cooked and recrystallized maize or tapioca starch (Crosby 2003). NOVELOSE 330® (National Starch and Chemical Limited) and CrystaLean (Opta Food Ingredients, Inc.) are also examples of commercially developed RS III which is derived from high amylose maize. Research is intensifying regarding RS IV chemically modified starches; RS IV which have been created using dysfunctional phosphate reagents are available for inclusion in foods; however, as yet there is a lack of information regarding their potential clinical and physiological effects (Brown, 2004). There are a number of advantages to using commercially manufactured sources of RS in food products. Unlike natural sources of RS (legumes, potatoes, bananas), commercially manufactured resistant starches are not affected by processing and storage conditions. Such as the amount of RS II in green bananas decreases with increasing ripeness.

1.8 Dietary intakes of resistant starch

Worldwide, dietary intakes of RS are believed to vary considerably. It is estimated that intakes of RS in developing countries with high starch consumption rates range from approximately 30 to 40 g/day (Baghurst *et al*. 2001). Dietary intakes in India and China were recently estimated at 10 and 18 g/day (Platel and Shurpalekar 1994, Muir *et al*. 1998). Intakes in the EU are thought to lie between 3 and 6 g/day (Dyssler and Hoffmann, 1994). Dietary intakes of RS in the UK are estimated at 2.76 g/day (Tomlin and Read 1990) and are believed to range from 5 to 7 g/day in Australia (Baghurst *et al*. 2001).

2 Rice starch

2.1 Rice production

Rice (*Oryza sativa* L.) is the most important cereal crop and the stable food of over half the world's population. The majority of rice varieties grown in Thailand are long grain. In Thailand, rice varieties are divided into 3 types according to custom and usage, such as waxy rice, low amylose rice, and high amylose rice. Rice varieties commonly used in commercial rice flour production in Thailand are waxy and high amylose rice. In 2003 – 2004, Thailand produced 26.124 million metric tons of rice and exported 6.62 million metric tons. Food ingredient usage of rice accounts for 22% of domestic rice sales. The use of rice as a food ingredient has also increased by 3.7% due to the rising popularity and availability of snacks, frozen dish, rice pudding, package mixes and beverage. More research on rice functionality, physical and chemical properties is needed in order to meet the food industry's demand for low-fat, hypoallergenic and nutritious food ingredient. The rice grain (Figure 1) comprises the hull (16-28% dry mass basic) and the caryopsis. The mass distribution of the rice caryopsis is 1-2 % pericarp, 4-6% aleurone plus seed coat and nucellus, 2-3% embryo and 89-94% starchy endosperm (Hoseney, 1990). Further milling to remove the pericarp, seed coat, testa, aleurone layer and embryo to yield milled or white rice results in a disproportionate loss of lipid, protein, fiber, reducing sugars and total sugars, ash, and minor components including vitamins, free amino acids and free fatty acids (Park *et al*. 2001). Diastatic, proteolytic and lipolytic were also reduced by milling. On the other hand available carbohydrates, mainly starch, were higher in milled rice than in brown rice. Starch is the major constituent of milled rice at about 90% of the dry matter. Protein and lipid contents are also significant. The endosperm cells are thin-walled and packed with amyloplasts containing compound starch granules which are evenly distributed, although they are smaller in size near the periphery of the endosperm (Blanshard 1987). The two outer most cell layers (the sub aleurone layer) are rice in protein and lipid and have smaller amyloplasts and compound starch granules than the inner endosperm.

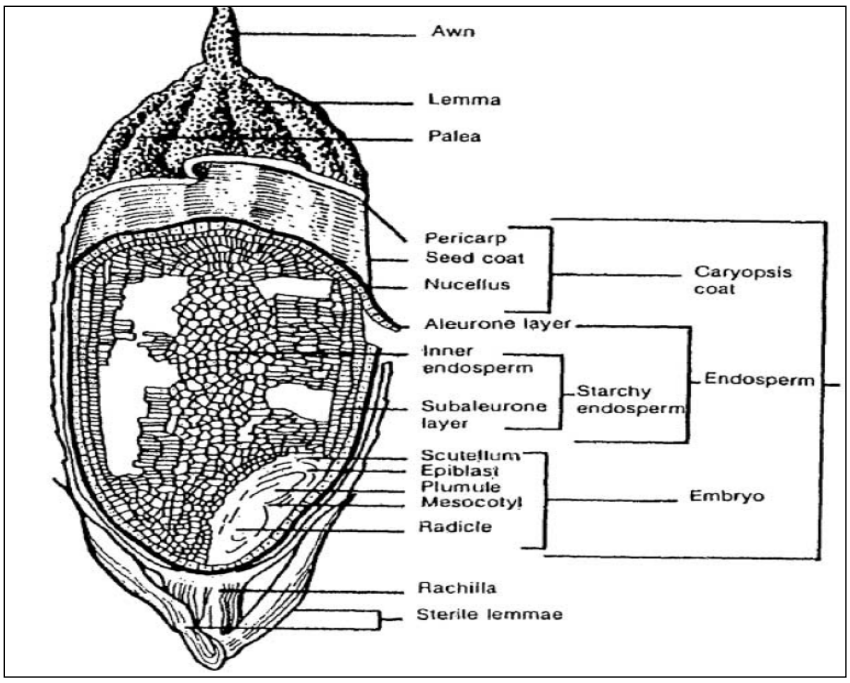


Figure 1 A detailed structure of the rice grain
Source: Blakeney (1984)

Rice starch production involves mainly wet milling of broken with 0.3 - 0.5% sodium hydroxide to remove protein (Juliano, 1984). Broken are steeped in alkali solution for 24 hours and are then wet milled in pin mills, hammer mills or stone-mill disintegrators with the alkali solution. After the batter is stored for 10 to 24 hours, fiber (cell wall) is removed by passing it through screens; the starch is collected by centrifugation, washed thoroughly with water and dried. Protein in the effluent may be recovered by neutralization and the precipitated protein used as a feed supplement. Rice starch is used exclusively as a human food, largely for baby foods and also in extruded noodles.

2.2 Granule structure of rice Starch

The endosperm, the starchy inner portion of rice kernel, is composed of tightly packed compound starch granules with protein interspersed as spherical bodies and crystalline structures. The starch content is particularly high, up to 77.6 % in

milled rice (Juliano and Bechtel, 1985). Starch granules are relatively dense, insoluble, and swell only slightly in cold water. Rice starch has the smallest particle size and the whitest color of all the commercial starches. The average rice starch granule is between 2 - 8 microns in mature grain (Figure 2 A). These granules are polyhedral and constitute approximately 90% of milled rice (dry weight). Starch granules consist of alternating semi-crystalline and amorphous (Myer *et al*. 2001). However, rice contains only compound granules in amorphous rings. Dang and Copeland (2003) suggested that the growth rings in rice are approximately 400 nm apart. Based on scanning electron microscopy observations, one block-let in the semi-crystalline growth ring contains several amorphous and crystalline lamellae (Figure 2 B). Donald *et al*.(1997) reported that the cross striations within the growth rings of rice starch (as observed by AFM) correspond to the blocklet of amorphous and crystalline lamellae. These blocklet have an average size of 100 nm in diameter and are proposed to contain 280 amylopectin side chain clusters. Amorphous lamellae contain branch points of the amylopectin side chains and possibly some amylose, whereas semi-crystalline lamellae are constituted of amylopectin double helices (Figure 2 C).

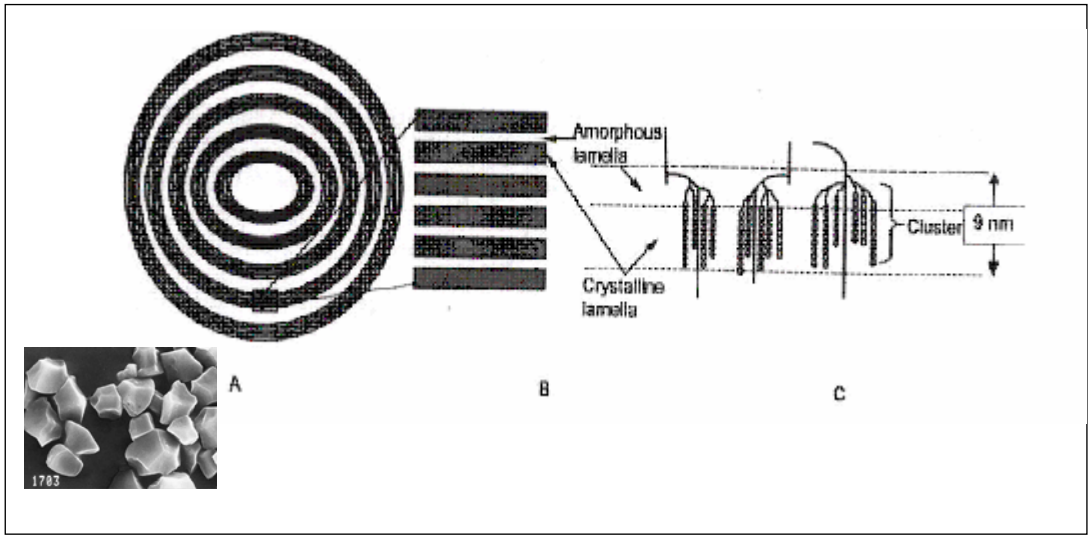


Figure 2 Granule structure of rice starch and lamellar structure of a starch granule
Source: Donald *et al*.(1997) and Dang and Copeland (2003)

2.3 Chemical composition of rice starch

Rice starch is made up of two distinct polymers, amylose and amylopectin; 98.50% of the starch granule is α-glucans. Both amylose and amylopectin are glucose polymers linked by the α-(1,4) linkage (Takeda *et al*. (1993) . Amylopectin also contains 4-5% of α-(1,6) linkages, leading to a branched molecule (Cura and Krisman, 1990). The average chain length for cereal amylopectin is 20-26 glucose units. The rice starch type can vary in certain genotype from regular waxy rice (0-2%), very low amylose rice (5-12 %), low amylose rice (12 – 20%), medium amylose rice (20-25%) and high amylose rice (25-33 %) (Juliano, 1992). Rice starch, as other starch, is composed of two polymeric forms of glucose: amylose and amylopectin (Figure 3). These two molecules are organized into a radically anisotropic, semi-crystalline structure in the starch granule (Lineback, 1984).

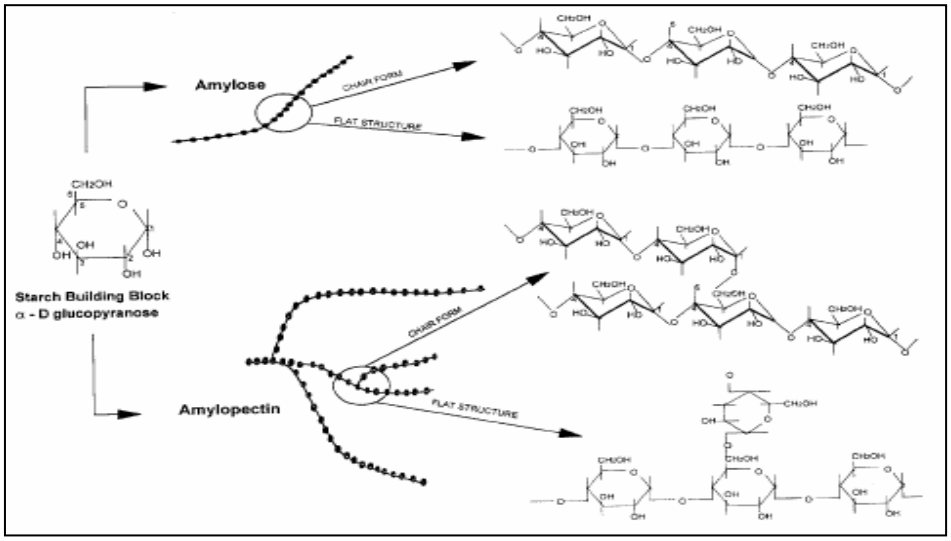


Figure 3 Representative partial structures of amylose and amylopectin
Source: Takeda *et al*. (1993)

2.3.1 Rice amylose

Amylose was long believed to be a linear polymer. However, in recent years it has become known that amylose contains linear and branched chains

(Takeda *et al*. 1986 and Takeda *et al*. 1993). Rice amylose is a mixture of branched and linear molecules with a degree of polymerization (DP_n) of 1,100-1,700 and 700-900, respectively (Hizukuri *et al*. 1989). The branched fraction in rice constituted 25-50 % by number and 30-40% by mass of amylose. Rice amyloses have a β-amylolysis of 73–81 %, indicating them to be slightly branched molecules with three to four chains on average (Hizukuri *et al*. 1988). This β-amylolysis limit is considerably higher than that of amylopectin (55-60%). These structural properties of amylose from rice are similar those of wheat and maize, as indicated in Table 2. The amylose content of the starch granule varies with the botanical source of the starch and is affected by climatic and soil conditions during grain development (Morrison and Azudin, 1987). High temperatures decrease the amylose content of rice, whereas cool temperatures have the opposite effect. Amylose content of rice is specified as waxy (0-2%), very low (5-12%), low (12-20%), intermediate (20-25%) and high (25-33%) (Juliano,1992). Amylose is a roughly linear molecule containing about 99% α-(1,4) and about 1% α- (1,6) bonds with a molecular weight of 500-20,000 (Figure 3).

Table 2 Structural properties of amylose

Source	β-Amylolysis limit (%)	Avg. DP_n	Avg. Number Chain	Avg. Chain Length	Branched Molecules (%)
Wheat	88	1,300	4.8	270	27
Maize	82	930	2.7	340	44
Rice: Indica	73	1,000	4.0	250	49
Japonica	81	1,100	3.4	320	31

Source: Hizukuri *et al*. (1989)

Amylose can form an extended shape (hydrodynamic radius 7-22 nm) but generally tends to wind up into a rather stiff left-handed single helix or form even stiffer parallel left-handed double helical junction zones (Swinkels, 1985). Single helical amylose has hydrogen-bonding O_2 and O_6 atoms on outside surface of the

helix with only the ring oxygen pointing inwards. Hydrogen bonding between aligned chains causes retrogradation and releases some of the bound water (syneresis). The aligned chains may then form double stranded crystallites that are resistant to amylases. These possess extensive inter- and intra-strand hydrogen bonding, resulting in a fairly hydrophobic structure of low solubility. Single helix amylose behaves similarly to the cyclodextrins by processing a relatively hydrophobic inner surface that holds a spiral of water molecules, which are relatively easily lost to be replaced by hydrophobic lipid or aroma molecules. It is also responsible for the characteristic binding of amylose to chains of charged iodine molecules (e.g. the polyiodides; chains of I_3^- and I_5^- forming structures such as I_9^{3-} and I_{15}^{3-}; note that neutral I_2 molecules may give polyiodides in aqueous solution and there is no interaction with I_2 molecules except under strictly anhydrous conditions) where each turn of the helix holds about two iodine atoms and a blue color is produced due to donor-acceptor interaction between water and the electron deficient polyiodides (Davies *et al*. 1980).

2.3.2 Rice amylopectin

Rice amylopectins are highly branched polymer having on the average 96% α- (1,4) bonds and 4-5% α-(1,6) bonds (Figure 4). Enzymatic techniques have been used to obtain structural information and develop models of amylopectin. This branching is determined by branching enzymes that leave each chain with up to 30 glucose residues. Each amylopectin molecule contains a million or so residues, about 5% of which form the branch points. There are usually slightly more outer unbranched chains (called A-chains) than 'inner' branched chains (called B-chains). There is only one chain (called the C-chain) containing the single reducing group. The molecule may have 10,000 – 100,000 individual chains and the ratio of unbranched to branched chains is about 1.0. Approximately 22-25 chains form each cluster, comprising the crystalline regions of starch granules (Figure 4). In waxy rice, 80-90% of the amylopectin chains probably constitute a single cluster, while the remaining 10–20 % from intercluster connections, which are mainly between adjacent clusters (Lineback, 1993).

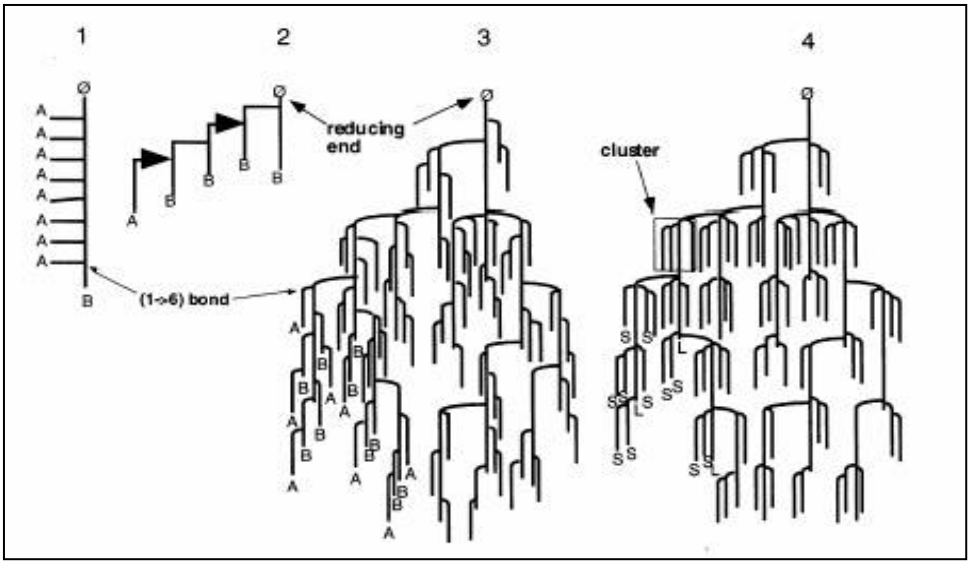


Figure 4 Cluster model of amylopectin. ((= Reducing chain-end). Solid lines indicate (1,4)- α-D- glucan chain; arrows indicate α-(1,6) linkage.

Source: Lineback (1993)

Each amylopectin molecule contains up to two million glucose residues in a compact structure with hydrodynamic radius 21-75 nm (Lineback, 1993). The molecules are oriented radically in the starch granule and as the radius increases so does the number of branches required filling up the space, with the consequent formation of concentric regions of alternating amorphous and crystalline structure (Figure 5). The structural properties of amylopectin, with amylose, vary depending on source (Table 3). Rice amylopectin have ((-amylolysis limits of 56 – 58%, chain lengths of 18-21, and widely different DP and linear chain distribution values (Hizukuri et al. 1989 and Takeda et al. 1987). Low average DP values are observed for indica rice (4,700), as compared to those for japonica rice (12,800) and waxy rice (18,500) amylopectin.

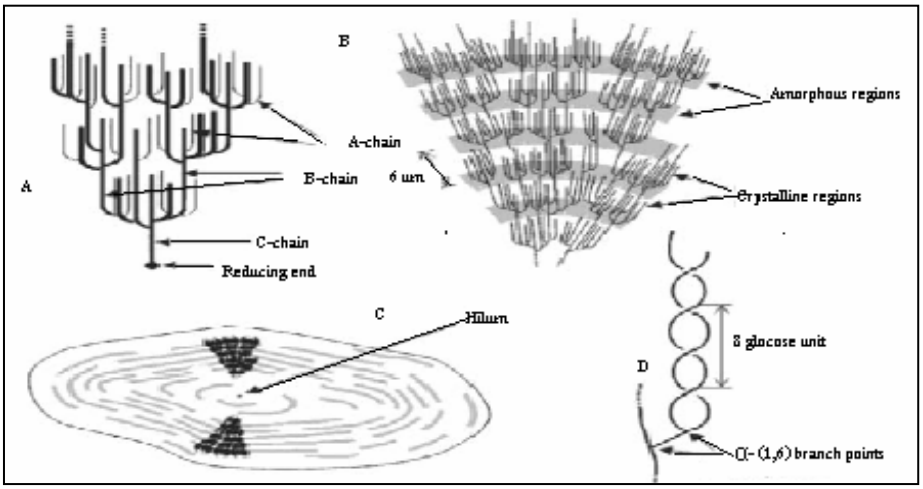


Figure 5 Idealized diagram of the consequent formation of concentric regions of alternating amorphous and crystalline structure of amylopectin.

Source: Martin (2005)

Table 3 Structural properties of amylopectin

Source	Avg. Degree of Polymerization	Avg. Number Chains	Avg. Chain Length	Avg. External Chain Length	Avg. Internal Chain Length
Maize	8,200	370	22	15	6
Rice: Indica	4,700	220	21	14	6
Japonica	12,800	670	19	13	5
Waxy rice	18,500	1,000	18	12	5
Potato	9,800	410	24	15	8

Source: Hizukuri *et al*. (1988)

Amylose and amylopectin are assembled in a cluster structure, in which the granules are composed of starch molecules laid down in concentric rings (Figure 6). The molecules that comprise a layer are deposited in a radial fashion with some sections in highly ordered crystalline regions. These radically ordered crystallites are linked by less structured amorphous regions. Hydrogen bonding is

likely to be a significant force in both regions. Linear portions of amylopectin constitute the crystalline regions, whereas the branch points and amylose are the main components of the amorphous portion (Blanshard, 1987).

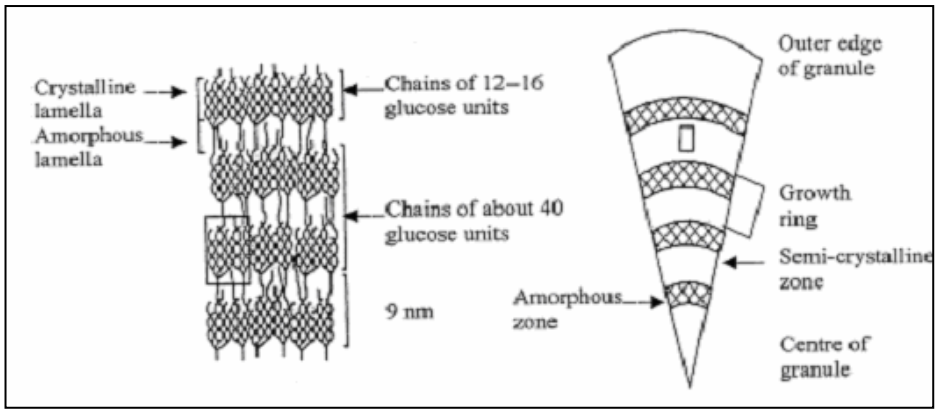


Figure 6 Semi-crystalline and amorphous regions in the starch granule
Source: Smith *et al*. (1997)

2.4 Minor components of rice starch

The main minor components in rice starch granules are lipids and protein. Non-waxy rice starches (12.2-28.6% amylose) contain 0.9–1.3% lipids comprising 29-45% fatty acids and 48% lysophospholipids (Jane *et al*. 1996). Waxy rice starches (1.0-2.3% amylose) contain ineligible amounts of lipids. Starch proteins are mostly either storage protein or biosynthetic or degradative enzymes. Rice storage proteins exist mainly as protein bodies (PB), PB I (prolamin) or PB II (glutelin) (Juliano, 1984). Biosynthetic enzymes are most probably entrapped within the starch granules following starch synthesis (Glaszmann, 1987). Besides lipids and proteins, phosphorus is an important non carbohydrate component of rice starch. In waxy rice starches, it is mainly present as phosphate-monoesters (0.003% on dry basis), whereas non-waxy rice starches predominantly contain phospholipids phosphorus 0.048% on dry basis (Jane *et al*. 1992). Other mineral components of starch are calcium, potassium, magnesium and sodium in their ionic form (Juliano and Viliareal, 1993).

2.5 Rice starch gelatinization

Gelatinization describes the irreversible collapse (disruption) of molecular order within a starch granule when heated in excess water. When an aqueous suspension of starch suspension of starch is heated above the gelatinization onset temperature (T_o), the granules absorb large amounts of water and swell considerably impart a substantial increase to the viscosity of the suspension (Thomas and Atwell, 1999). Further heating of starch granules in excess water results is granule swelling and additional leaching of soluble component (primary amylose). Swelling of granules attains a maximum value at elevated temperatures and is subsequently followed by granular disruption and exudation of the granule contents into the suspension matrix (Whistler *et al.* 1984) as shown in Figure 7. Final gelatinization temperature (GT) of starch granules refers to the water temperature at which at least 90 percent of the starch granules have gelatinized or lost birefringence (Maltese cross) or swollen irreversibly in hot water. GT is classified for rice starch granules as low (55 to 69.5°C), intermediate (70 to 74°C) and high (74.5 to 80°C). GT is indexed in the breeding programmed by the alkali spreading value based on the degree of dispersion of six grains of milled rice in 10 ml of 1.7 percent potassium hydroxide after 23 hours soaking at 30°C (Swinkels, 1985).

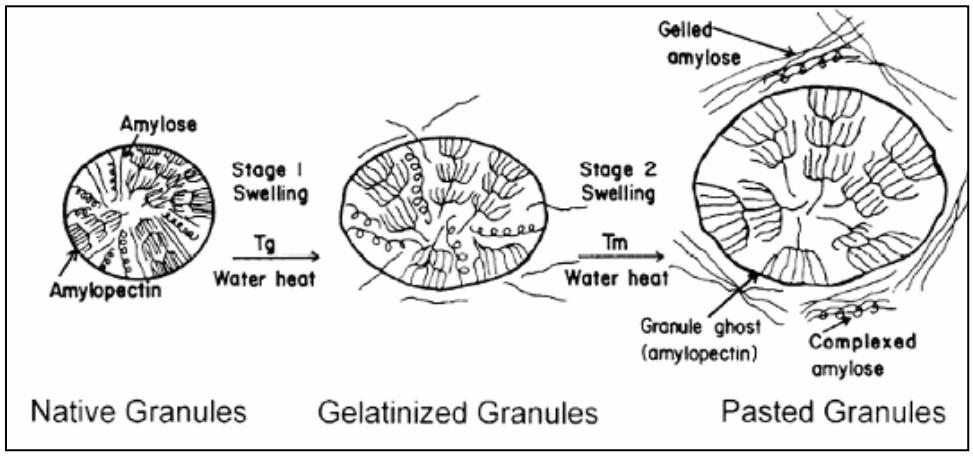


Figure 7 Idealized diagram of the swelling and gelatinization of a starch granule

Source: Swinkels, (1985)

A high GT value is uncommon, particularly in high amylose rice. A low ambient temperature during ripening may increase amylose content and independently reduce GT (Nikuni *et al*. 1969). The GT affects the degree of cooking of rice because of the cooking gradient from the surface to the core of the grain. Because GT correlates directly with cooking time, a low GT favors fuel conservation, provided eating quality is not adversely affected. GT also affects the molecular properties of amylopectin (Donald *et al*. 1997).

2.5.1 Rice starch changes during gelatinization

Rice starch begins to gelatinize between 85^{o}C and 95^{o}C, the exact temperature dependent is the specific varieties (Bhattacharya, 1979). For example, different starches exhibit different granular densities, which affect the granules that can absorb water. Since loss of birefringence occurs at the time of initial rapid gelatinization (swelling of the granule), loss of birefringence is a good indicator of the initial gelatinization temperature of a given starch. The largest granules, which are usually less compact, begin to swell first. Once optimum gelatinization of the grains has occurred, unnecessary agitation may fragment the swollen starch grains and cause thinning of the paste. Further, it occurs in those parts of the grain where the water content is sufficiently high (water to starch ratio ≥ 0.75) (Hoseney, 1990). Starch swell are transformed progressively to an essentially amorphous form with loss of organized structure. Ultimately granule structure is completely lost and a thin paste (< ~ 4%) or gel (> ~ 4%) is formed. Evidence of this loss of order can be seen by irreversible granule swelling, loss of birefringence, and loss of crystallinity.

2.5.2 Loss of birefringence

In polarized light ungelatinized starch granules show birefringence, resulting in the “maltese cross” pattern (Fitt and Snyder, 1984). When T_o is reached the birefringence begins to disappear. One of the most common methods for determining the gelatinization temperature range is to follow the loss of birefringence

in excess water. The loss of birefringence occurs over a broader temperature interval when content is decreased (Lund, 1984).

2.5.3 Loss of crystallinity

The loss of crystalline order during heating is observed by X-ray diffraction. The diffraction pattern disappears, and eventually a pattern indicative of a completely amorphous material is obtained (Zobel *et al.* 1988). The temperature range during which the crystallinity is lost and the rate which it is lost depend on the water content and or the type of starch (Liu *et al*. 1997). The temperature range increases with decreasing water content, and at water content below 50% the temperature for complete loss of crystallinity approaches 100^{o}C. The loss of crystallinity seems to occur in two steps: at first the loss occurs at a very low rate, but then at a temperature typical of the starch the rate increases dramatically (Svensson and Eliasson, 1995).

Tester *et al*. (1994) indicated that low and high gelatinized temperature (GT) starches had very similar amylopectin chain lengths after debranching and on debranching of insoluble residues after lintnerizaton. The low GT starches could be annealed to behave like high GT starches, but the latter also responded a little to annealing. It was concluded that low GT starches have less crystallinity and less perfect crystallites than the high GT starches due to minor amylopectin structural differences. Eliasson *et al*. (1980) reported that amylopectin branch chain lengths and distributions determine starch GT, enthalpy change, and pasting properties. Starch GT increased with increasing branch chain length.

2.5.4 Endothermic transitions

The starch gelatinization is an endothermic process with enthalpy values in the range 10-20 J/g. During the last 10 -15 years DSC has become the most important tool for studying starch gelatinization (Donaovan, 1979; Eliasson, 1980). DSC measures the gelatinization transition temperature and the enthalpy of gelatinization (ΔH). These DSC parameters are influenced by the molecular

architechture of the crystalline region (Noda *et al*. 1996). The effect of water content on the glass transition (T_g) of the native and gelatinized rice starches was studied by DSC (Perdon *et al*. 2000) demonstrated that T_g increased as water content decreases from 27 to 3% (mass basis). Moreover, T_g of native starch was significantly higher than that of gelatinized starch in a low moisture content range (8-18%), and the difference became greater as the moisture content decrease (Noda *et al*. 2003).

With regard to amylopectin chain length distribution, Noda *et al*. (2003) revealed that vary short (DP<12) amylopectin chains related negatively, while somewhat longer (12 < DP < 24) amylopectin chains related positively to gelatinization onset (T_o), peak (T_p) and conclusion (T_c) temperatures of rice starch as measured by DSC. Whether amylopectin chains have a positive or negative influence on gelatinization temperature depends on the way they are packed into the lamellar structure of the starch granules (Chung *et al*. 2002). Short amylopectin chains may destabilize the lamellar structure in several ways. Starches with higher relative amounts of very short amylopectin chains will thus have lower molecular and crystalline order and a non-optimized packing within the crystalline lamellae. Consequently, they will most likely have lower gelatinization T_o, T_p, T_c. For gelatinization to occur the regions of amorphous starch must first melt or undergo glass transition (Slade and Levin, 1988b). The heat energy required to completely gelatinize starch in rice is critical to the rice processor, who must optimize heat input, cooking time, and temperature.

2.5.6 Morphological changes

The swelling of starch granules and the solubilization of macromolecules are the overall effects of the gelatinization process. The process can be characterized by the swelling index and the solubility index. The high swelling power can be related to the sharp consistency increase observed on viscograms in the same temperature range. Simultaneously, a large amount of soluble material is recovered in the supernatant indicating a high solubilization of starch granules. Using light microscopy and scanning electron microscopy several authors observed the

morphological changes occurring at the different temperatures (Lu *et al*. 1996). When the water molecules possessed sufficient kinetic energy to overcome the attractive forces between the hydrogen-bonded starch molecules within the granule, hydration of the grain occurred, with swelling of the amorphous regions between the crystallites. The size of the starch granules increased slightly as the temperature was raised from 35 to 55^{o}C (Yeh and Li, 1996).

2.6 Rice starch retrogradation

Starch retrogradation is a process that occurs when the molecules comprising gelatinized starch begin to re-associate in an ordered structure (Figure 8). In the initial phases of retrogradation, two or more molecules may form a simple juncture point, which then may develop into more extensively ordered regions. Ultimately, under favorable conditions, crystalline order may appear and amylose precipitation from "solution" may occur in dilute solution. Retrogradation of gelatinized starch is a reorganization process that can involve either amylose or amylopectin, with amylose undergoing retrogradation at a more rapid rate than amylopectin (Jacobson *et al*. 1997).

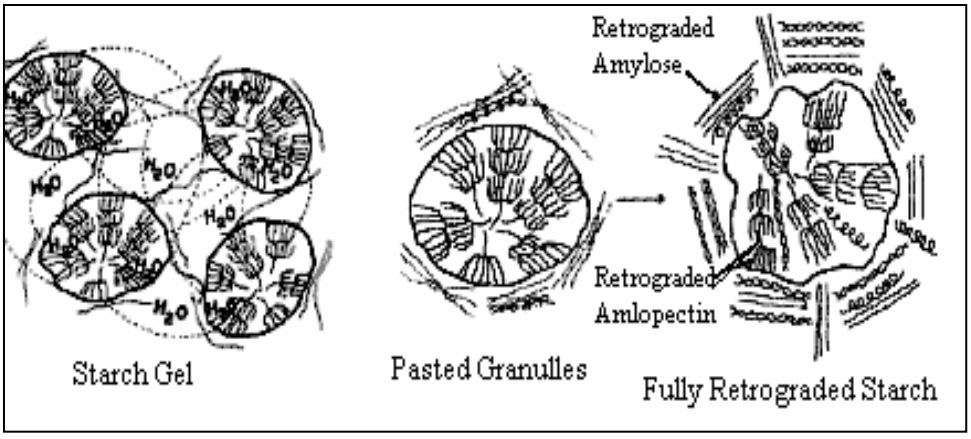


Figure 9 Retrogradation during storage of cooked cereal starch paste leads to recrystallization of the amylose and amylopectin chains

Source: Jacobson *et al*. (1997)

2.6.1 Factors affecting rice starch retrogradation

The rate of rice starch retrogradation depends on a number of variables, including the structures and ratio of amylose and amylopectin, storage temperature, starch concentration, botanical sources of the starch and the presence and concentration of other ingredients, such as surfactants and salts. The retrogradation kinetics of starch has received wide attention though the underlying mechanism of retrogradation has not been concluded. Lai *et al*. (2000) reported the retrogradation kinetics of pure amylopectin from 13 rice cultivars. Generally, the amylopectin systems showed two stages of retrogradation behavior during early (< 7 days) and late (>7 days) storage. Correlation analysis suggested that the kinetics of early stage retrogradation were more correlated than the late stage retrogradation with the number-average molecular weight and chain lengths of the amylopectin molecules. The proportion of short, long and extra long chain fractions appeared to have greater effects on the enthalpy changes and late stage kinetics than the other structural factors.

1) Structures and ratio of amylose and amylopectin

Tako and Hizukuri (2000) proposed some mechanisms for rice starch retrogradations which are based on the formation of hydrogen bonding at various levels. It is assumed that intramolecular hydrogen bonding may take place between OH-6 and the adjacent hemiacetal oxygen atom of the D-glucosyl residues. Intermolecular hydrogen bonding my take place between OH-2 of the amylopectin and an adjacent OH-6 of the amylose. Another intermolecular hydrogen bond may form between OH-2 of a D-glucose residue of the former molecule and OH-6 of a D-glucose residue of short side chain (A and B1) of the latter molecule. After saturation of intermolecular hydrogen bonding between amylose and amylopectin molecules, an intermolecular association may also take between amylopectin molecules through hydrogen bonding. The mechanism of retrogradation is complicated because retrogradation rate may vary from one cultivar to another due to differences in the proportion and interaction of amylopectin and amylose, chain length distribution, and molecular size of branched molecules (Hizukuri, 1986). The 1,4–linked α-D-glucan

amylose is the linear fraction of starch. Commercial samples of amylose usually occur in retrograded, water insoluble form, which can be solubillized by pressure cooking at 150-160^{o}C. On cooling, amylose solutions of 2% or higher concentrations, prepared in this way, rapidly gel, while at concentrations lower than 2% precipitation occurs (Schoch, 1964). Amylose is also soluble in dilute alkali, and rapid neutralization of concentrations greater than 2% results in gel formation. Both the gelation of amylose from concentrated solutions and precipitation from dilute solution can be termed retrogradation. The gels and precipitates formed result from the inherent tendency of amylose molecules to undergo conformational ordering and to tendency of amylose molecules to undergo conformation ordering and to subsequently align or aggregate (Gidley and Bulpin, 1989). The rate of retrogradation increases with increasing amylose concentration and with decreasing temperature and is greatest at pH 5-7. In addition, the retrogradation rate has a sharp maximum at amylose degree of polymerization (DP) of 80, shorter and longer molecules being much more soluble (Ring *et al*. 1987 and Jacobson *et al*. 1997).

Goodfellow and Wilson (1990) studied the retrogradation of amylose using Fourier Transform Infrared (FT-IR). They found that helix formation must occur before the creation of crystallites; therefore double helix must form at the short or early on in the gelation process. Thus in summary, a same time as phase separation, to create a gel network, with subsequent aggregation of these helices producing crystallinity would be consistent with the experimental data (Figure 9). The IR results for amylopectin suggest an initial fast change followed by a much slow change. Previous IR work by Wilson *et al*. (1987) in which the spectra for amylopectin from 6 to 340 hour were obtained suggest that FT-IR and G´ monitor the same process-namely the crystallization of the amylopectin side chains followed by a slow aggregation of these helices to produce crystallinity (Figure 9) .

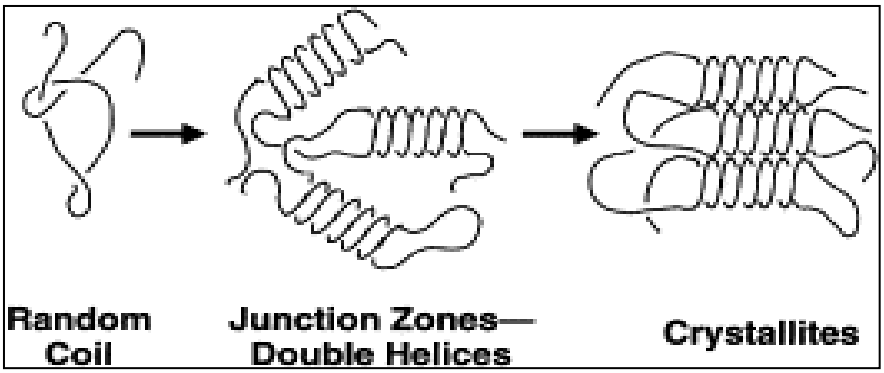


Figure 9 Schematic diagram showing the process occurring the gelation and retrogradation of amylose and amylopectin

Source: Wilson *et al*. (1987)

Cereal amylose may form double-helical associations of 40-70 glucose units, while amylopectin forms shorter double helices (Miles *et al*. 1985a; Goodfellow and Wilson, 1990; and Liu *et al*. 1997). The latter can be attributed to restrictions imposed by the branching structure of the amylopectin molecules and the chain lengths of the branches, Thus, amylose, is responsible for short-term changes while amylopectin is responsible for the longer term rheological and structural changes of starch gel. Double helices may associate and organize into crystallites, most of which are related to association of the amylopectin chain which comprise the bulk of the starch component of rice. The retrogradation of maize amylopectin was proportional to the amount of short chains having a DP of 16-30 and inversely propolytional to the level of short chains with a DP of 6 – 11 (Shi and Seib, 1995).

Lu *et al*. (1997) studied the retrogradation of amylopectin from Taiwan rice varieties. They reported that the short chains of amylopectin with DP 10 - 15 glucose units would result in more retrograded crystalline building blocks. On this basic as well, it would require more energy to disorder the greater number of double helical linkages of retrograded amylopectin. Thus, the plateau enthalpy would be higher (Sander *et al*. 1990). Gidley and Bulpin (1987) suggested that a chain length of at least 10 units is required for crystallinity development and, by inference, for the formation of double helices. On the other hand, short chains with DP 6-9 glucose

units are known to inhibit retrogradation (Shi and Seib, 1992). The retrogradation of waxy rice starch appears to be directly proportional to the mole fraction of unit chains of amylopectin with DP 14-24 and inversely proportional to mole fraction DP 6 - 9. Once crystalline regions are formed, their reversal is dependent on molecular type: amylose, 100^{o}C to 120^{o}C; amylopectin, as low as 50^{o}C for samples having a low degree of order. When formed in gels, crystalline regions act as physical cross links in a gel network. In reviewing crystallinity, the X-ray polymorphic forms have been described in terms of structure, their function in granules, changes that occur with annealing, loss due to melting (gelatinization), and recrystallization upon retrogradation. Through macro-crystalline domains, crystallinity also contributes to granule ultra structure. These structures are often revealed by etching granule examination. Most studies require the use of transmission or scanning electron microscopy but large growth rings can be seen with an optical microscope. Observed structures show periodic density fluctuations that vary in size and shape, depending on the extent to which amorphous regions or imperfect crystallites have been disrupted (Maningat and Juliano, 1980).

2) Starch concentration

Starch concentration influences the extent of retrogradation. The maximum extent of retrogradation is obtained in a starch concentration about 60% (w/w). Retrogradation process is very sensitive to temperature (Zeleznak and Hoseney, 1986). The aging of wheat starch gels at 4, 21, and 30^{o}C was studied with DSC (Lengton and LeGrays, 1981). Crystallinity increased with time and crystallization occurred at the highest rate and to the greatest extent in storage at 4^{o}C. Retrogradation is very sensitive to the water content in starch gels. Lengton and LeGrays (1981) observed that crystallization during aging occurred only in gels with starch content between 10 and 80%, and maximum crystallization, measured with DSC, occurred in gels with 50–55% starch. Other DSC studies have confirmed that maximum crystallinity occurs in gels with 50–60 % starch (w/w) (Zeleznak and Hoseney, 1986).

The recrystallization process depends on the glass temperature (T_g) of amorphous gel because the mobility of the chains determines their amorphous gel. As a plasticizer, water controls the T_g of the amorphous gel. At very low water content, the T_g is above room temperature and the amorphous gel is in a highly viscos glassy state that effectively binders molecular mobility. Recrystallization increases with increasing water content (depress of T_g below room temperature) up to 45 - 50%, because of progressively more effective plasticization (increase molecular mobility); with further increase of water content up to 90%, it decreases, apparently due to excess dilution (Slade and Levine, 1987).

One can visualize the gel as starch chains with layers of water molecules attached by hydrogen bonding. As the starch paste is cooled, the starch chains become less energetic and the hydrogen bones become stronger, giving a firmer gel. As a gel ages or if it is frozen and thawed, the starch chains have a tendency to interact strongly with each other and thereby force water out of the system. The squeezing of water out of the gel is called 'syneresis'. Longer storage gives rise to more interaction between the starch chain and eventually to formation of crystals. This process, called 'retrogradation' is the crystallization of starch chains in the gel. Because the crystalline areas differ from the non crystalline areas in their refractive index, the gel becomes more rigid or rubbery, perhaps partially as a result of crystallization and partially just from the interaction of the starch chains (Hoseney, 1990).

3) Storage temperature

Retrogradation is greatly affected by storage temperature. Compared to storage at room temperature, storage of starch gels containing 45-50 % water at low temperature but still above the glass temperature ($Tg \approx 5.0^oC$) increases the retrogradation, especially during the first day of storage, compared to starch gels stored at room temperature (Gudmundsson, 1994). Storage temperature below Tg virtually inhibits recrystallization. Higher temperatures (above 32 – 40 ^oC) effectively reduce retrogradation. Colwell *et al*. (1969) studied the effect of storage temperature

(-1 to 43^{o}C) on concentrated starch gels by DSC. They found that the mechanism of starch crystallization (instantaneous nucleation followed by rod-like growth of crystals) is the same over the whole range of temperature. Moreover, at higher storage temperatures, a more symmetrically perfect crystalline structure is found. Biliaderis and Zawastowski (1990) studied the effect of temperature on rigidity of 5 % (w/w) amylose and 40% waxy maize starch gels. They observations further support the notion that amylase gelation mainly involves rapid formation of double helical junction zones upon cooling of amylose solutions, whereas a network-based structure for amylopectin is established mainly as a result of separation of partially crystalline structure.

4) Botanical sources of the starch

The botanical source is one of great importance for the retrogradation of starch gels (Gudmundsson and Eliasson, 1993). They dose not concern differences in amylase content, because it has even been observed in starches with very similar amylase content. This indicates that structural differences found in the amylopectin molecule may be the cause of differences in the recrystallization rate. Amylopectin from cereal has also been shown to retrograde to a lesser extent than pea, potato and canna amylopectin which has been attributed to shorter average chain length in the cereal amylopectin (Kailcheusky *et al*. 1990). The structural differences in cereal amylopectin as related to retrogradation can be related either to differences in the amorphous regions or to differences in the ratio of short to long chains and the ratio of A-chains to B-chains. A greater amount of short chains over 15 glucose units and increase ratio of A- chains to B-chains probably promote retrogradation. It has also been reported that very short chain (6-9 glucose units) can inhibit or retard retrogradation of starch gels (Levine and Slade, 1986)

2.6.2 Method used for estimating retrogradation

Retrogradation has been studied using analysis methods of different techniques such as X-ray diffraction (Gidley and Bulpin, 1987), differential scanning

calorimetry (DSC) (Russell, 1983; Mua and Jackson, 1995), Fourier transform infrared spectroscopy (FT-IR) (Van *et al*. 1994) or Raman spectroscopy (Bulkin *et al*. 1987) and rheological techniques (Doublier and Choplin, 1989; Mita, 1992).

1) Retrogradation measured by X-ray diffraction

Early X-ray diffraction studied on aged starch gels showed that the B-type diffraction pattern developed slowly (Zobel, 1973). Crystal growth, as detected by X-ray diffraction, is slower than formation of the gel network and has been proposed as occurring in the polymer-rich regions of the gel (Miles *et al*., 1985a). For both amylose and starch gel, the initial development of crystallinity occurred at similar rate. However, the crystallization of amylose effectively reached a limit rates. However, the crystallization of amylose effectively reached a limit after 2 days whereas the crystallinity of the starch gel continued to increase (Miles *et al*. 1985b). The amylopectin gels show only a slow increase in crystallinity with time and approach a limiting value after 30-40 days (Ring *et al*. 1987). Isolated gelatinized starch granules that have been washed free from all exudates amylose gave no X-ray diffraction pattern immediately after cooling. After two weeks of storage, the B-type pattern is obtained. Amylose gels are firm, white and opaque. They can withstand autoclave at 130 ^{o}C. Retrograded amylose gives the B-type starch X-ray diffraction pattern. Early X-ray diffraction analysis of stretched amylose film in the B-type structure was interpreted in terms of a six fold single helix structure with a repeat distance of 10.4 °A. On this basis it could be postulated that the mechanism of cross linking amylose gels was via junction zones of aggregated single helices. From studies on Naegeli amylodextrins, however, Kainuma and French (1972) proposed a model for B-type starch comprising intertwined double helices. This proposal has recently been confirmed by X-ray diffraction studies (Wu and Sarko, 1978) as showed in Figure 11. This most recent crystal structure of the B-polymorph of amylose is based on double stranded helices. The individual strands are in a six fold helical conformation repeating in 20.8 Å, and are wound parallel around each other. In the case, however, the X-ray data cannot reliably distinguish between right and left-handed models (Cheetham and Tao (1998).

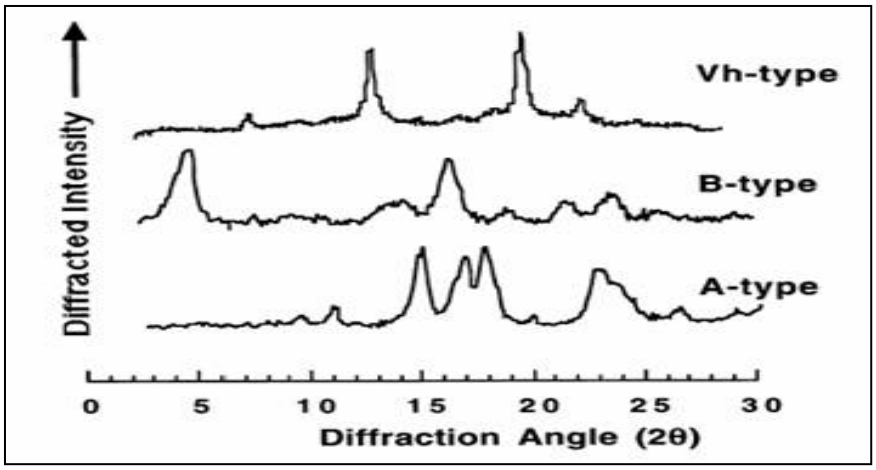


Figure 10 Schematic diagram showing the process occurring the X-ray diffraction pattern of starch

Source: Wu and Sarko (1978)

2) Retrogradation measured by DSC

Thermal methods as DSC are well fitted to follow the rate and extent of retrogradation as the starch molecules progressively re-associate on aging. Nakasawa *et al.* (1984) studied the effect of storage temperature on recrystallization and glass transition temperature (T_g) of non waxy and waxy rice starch gel systems containing 60% moisture content by using DSC. The nucleation and propagation for the recrystallization process were determined by recrystallization degree obtained from crystallite melting enthalpy changes during storage. The recrystallization rate for both rice starch gels within 3 days of storage and its temperature dependence were analyzed by Avrami and Arrhenius equations. They found that the maximum nucleation and propagation for recrystallization of both rice starch gel systems occurred at 4 ^{o}C and 30 ^{o}C, respectively. The T_g slightly increased with increasing recrystallization degree, and the highest T_g was observed in the maximum recrystallization temperature ranges. The T_g and recrystallization rate of non waxy rice starch gel were changed more than those of the waxy one, while the higher activation energy and Q_{10} value were shown in waxy rice starch gel (Krueger *et al.* 1987a).

2.7 Rice starch digestibility

Starch that is resistant to digestion is thought to offer humans some protection from the development of various chronic diseases (Topping and Clifton, 2001). There have been several reports that have linked amylose content to rice starch digestibility (Goddard *et al*. 1984 and Noda *et al*. 2003). However, it has been found that similar hydrolysis of raw starch by amylase occurred with waxy and non waxy rice cultivars. Perez *et al*. (1991) reported that rice cultivars with similar amylose content varied in starch digestibility, the difference being associated with other properties, such as GT, cooking time, amylograph consistency, and volume expansion upon cooking. Therefore, amylose content alone is not a predictor of starch digestion rate (Perez *et al*. 1991). Zhang *et al*. (1996) showed that japonica waxy rice flour with different X-ray diffraction patterns differed in starch digestion, the percent digestion of group II flour was higher than group I flour, either with glucoamylase or with alpha-amylase. The authors suggested that the starch in group II had a looser structure arrangement, at the molecular level, than did those in group I. These allowed the enzymes to penetrate rapidly into the starch granules in group II. Perhaps, then, amylopectin may play a role in conferring the rate of rice starch digestion.

Processing techniques are also reported to impact the rate of rice starch digestion. Parboiling reportedly decreases rice starch rate of digestion (Tetens *et al*. 1997). Rashmi and Urooj (2003) found that the steaming of rice created more resistant starch than boiling or pressure cooking. Storage under refrigeration also has been reported to slow the rate rice starch digestion (Frei *et al*. 2003). Kim *et al*. (2006) studied the effects of amylose content, autoclaving, parboiling, extrusion, and post-cooking treatments on resistant starch (RS) content of different rice cultivars. They found that the extent of RS formation was significantly influenced by the amylose content of rice, volume of water used for cooking and cooling after cooking.

The higher-amylose rice variety had more resistant starch than the lower-amylose variety. The amounts of RS were between 0.1 to 0.26g/ 100g DM for raw rice and 0.13 to 0.14g/100g DM for extruded rice. The resistant starch formation in

rice due to the various cooking and cooling combinations under test ranged from 0.6 g to 5.1 g/100 g DM. Decreasing the rice : water ratio (1:2) and cooling (24 hr at 4^{o}C) after cooking significantly increased the RS content. Extrusion decreased the RS content in the high RS rice only (0.42 – 0.16 g/100 g).

2.8 Glycemic index of rice starch

The glycemic index (GI) is a physiological concept used to classify carbohydrate containing foods. It is closely tied in with the term 'glycaemic response'. Both refer to the ability of a particular food to elevate postprandial blood glucose concentrations. GI is measured as the incremental area under the blood glucose curve after consumption of 50 g of available carbohydrate from a test food, divided by the area under the curve after eating a similar amount of available carbohydrate in a control food (generally white bread or glucose) (Ludwig and Eckel, 2002). Foods with high GI value release glucose rapidly into the blood stream (elicit a rapid glycaemic response), while foods with a low GI value release glucose more slowly into the bloodstream and result in improved glycaemic and insulinaemic responses.

Waxy and low-amylose rice had higher glycaemic indices than intermediate- and high-amylose rice (Goddard and Marcus, 1984; Juliano and Goddard, 1986; Jiraratsatit *et al*. 1987; Tanchoco *et al*. 1990), (Table 4). Processing, such as parboiling and noodle-making, tends to reduce the glycaemic index of rice, particularly which of high- and intermediate-amylose rice (Panlasigui, 1989; Wolever *et al*. 1986). By contrast, Tsai *et al*. (1990) reported that waxy rice, rice gruel, steamed rice and rice noodles had similar glycaemic indices to that of white bread in NIDDM patients. Among high-amylose rice, the low-GT, hard-gel IR42 had a higher glycaemic index than the intermediate-GT, softer-gel IR36 and IR62 (Panlasigui, 1989). By contrast, Srinivasa Rao (1970) reported that the ingestion of hard-gel IR8 resulted in a lower peak plasma glucose level than ingestion of the softer-gel both have high amylose and low GT.

Table 4 Glycemic index (%) of cooked milled rice and rice products of varying amylose content in normal and non-insulin-dependent diabetes mellitus (NIDDM) subjects.

Subjects	Waxy (0-2%)	Gruel, waxy	% amylose content			Products	
			10-20	20-25	>25	Noodles	Parboiled
Normal, USA[a]	96	Not study	93	93	60	Not study	Not study
Normal, Indonesia[b]	87	96	Not study	52	70[e]	78-82	Not study
Normal – NIDDM Canada & Philippines[c]	116	Not study	Not study	Not study	72[f] 84[g]	56-66	66[g]
NIDDM Thailand[b]	75	Not study	71	Not study	Not study	53-55	Not study
Normal Thailand[c]	100	Not study	87	Not study	Not study	Not study	Not study
NIDDM Taiwan[d]	118	124	111	Not study	Not study	110	Not study

[a] = Glycemic index based on insulin response

[b] = Glycemic index based on glucose response, with glucose drink as 100%

[c] = Relative value of glycemic index based on waxy rice response as 100%

[d] = Glycemic index based on white bread as 100%

[e] = Red rice

[f] = Intermediate gelatinized temperature

[g] = Low gelatinized temperature

Source: Goddard and Marcus (1984), Juliano and Goddard (1986), Jiraratsatit *et al*. (1987) and Tanchoco *et al*. (1990)

Recently, there has been a flurry of public and commercial interest in the GI concept and its possible inclusion on food labels, both as an aid for the management of diabetes and to indicate potential foods that may aid weight loss and management (McKevith, 2004). It is known that dietary fiber may contribute to an improved glycaemic response and, in general, high-fiber foods are assigned a lower GI value. Interest is now increasing in assigning GI values to RS rich foods. However, it must be remembered that for foods enriched with truly resistant starches, a reduced glycaemic response may simply result from a lack of available digestible starch, rather than any specific physiological effects (Jenkins *et al*. 2002).

2.9 Measuring glycemic index *in vitro*

Glycemic index evaluation in humans can be difficult and costly, therefore studies measuring in vitro digestion of starch foods have been done in order to predict *in vivo* effects (Grandfelt *et al*. 1992, Englyst and Hudson 1996, Bjŏrck 1996 and Gońi *et al*. 1996). Gońi *et al*. (1997) developed a first order equation from the in vitro kinetics of starch digestion of foods. This model has a high correlation with in vivo glycemic responses ($r=0.909$, $p<=0.05$), in addition to good reproducibility and application in other studies (Table 5).

Table 5 Recent studies using the Goñi *et al*. (1997) model for starch hydrolysis and estimate glycemic index determination.

Food	Authors
Sorghum and maize flours	Ezeogu *et al.* (2005)
Raw and processed mucuna beans	Grandfelt *et al*. (1992)
Rice differing in amylose contents	Hu *et al*. (2004)
Six rice cultivars	Frei *et al*. (2003)
Starchy foods: polished and whole rice, corn, polenta, white spaghetti, potatoes and legumes	Rosin *et al*. (2002)

Source: Ezeogu *et al.*, (2005), Grandfelt *et al*. 1992, and Hu *et al*. (2004)

3. A review of resistant starch type III formation from rice starch

There are no commercial resistant rice starches currently available. However, there have been previously published studies on the developing resistant rice starch. Resistant rice starch results from the highly retrograded amylose fraction, the quantity formed being directly proportional to the amylose content of starch. The degree of formation of RS III in rice starch depends on amylose content and processing conditions as shown in Table 6.

Table 6 The formation degree of RS III in rice starch depends on amylose content and processing conditions, which was analysis follow McCleary and Monaghan (2002) method.

Amylose content (%)	Processing conditions	Resistant starch (%)	Digested starch (%)	Total starch (%)
31.10	Native rice starch	12.90	69.20	82.10
30.80	Rice: water (1:2) boiled	2.20	79.60	81.80
20.20	Native rice starch	9.20	74.4	83.60
34.10	8% rice starch gel and storage at 4^{o}C for 24h-synersis	3.6	93.5	97.1
27.60	Milled white raw rice	5.00	83.30	88.30
	Milled parboiled rice	3.90	75.40	79.30
25.60	Extruded rice	0.16	85.44	85.60
32.80	Retrograded rice flour	3.23	67.60	75.20
26.80	Native indica milled rice	3.20	71.20	74.10

Source: Sagum (2000); Tovar *et al.* (2002), Hu *et al*. (2004), Walter *et al*. (2005), Kim *et al*. (2006), Yang *et al*. (2006) and Hu *et al*. (2004)

3.1 Processing condition on improvement of RS III content

In other process for producing amylase resistant rice starch, Shi and Trzasko (1997) described a method in which a high amylose starch. Having at least 40% (w/w) of amylose and a water content of 10 to 80% (w/w), was heated to temperature between 60 and 160^{o}C to provide a resistant granule starch with a total dietary fiber content of at least 12%. The formation of non-waxy rice starch with lower digestibility and therefore higher resistance to α- amylase on heating to 140^{o}C may be attributed to the formation of several smaller molecular weight fractions from hydrolysis of the starch on heating, with the different fraction re-crystallizing at different temperatures on cool down. Cooling and re-crystallization of these smaller starch fractions my not permit an orderly rearrangement of granules thereby preventing access to α- amylase. When starch is gelatinized under high moisture content and allowed to cool, alignment of ordered amylose molecules with each other leads to the formation of a rigid gel. Resistant starch is produced as the insoluble crystallite formed by the process of controlled retrogradation (Englyst *et al*. 1992). Heat-moisture treated at the melting temperature (T_m) has been used to study the digestibility and pasting properties of rice starch. Anderson *et al*. (2002) found that starches (20% moisture) heated to the temperature of melting (T_m) and held for 60 min in the calorimeter showed a slow digestibility compared to unheated samples. Digestibility decreased by 25% and 10%, respectively, for non-waxy and waxy rice starches relative to non-treated starch (Lui *et al*. 1991)

Kim *et al*. (2006) carried out studies to determine resistant rice starch in different ways, cooked as brown or white, boiled or steamed, whole grain or flour and retrograded while grains and flour from the average amylose content was 22.25%. They summarized that brown rice and retrograded rice appear to containing highest amounts of resistant starch (Table 7). Unfortunately, boiled rice is low, and rice that retrograde a lot are considered low quality. The material lost in boiling is also high in RS, probably because amylose was selectively leached. Leaching of amylose would less starch in the boiled rice and explain the loss in RS in boiled rice. The effect of microwave treatment on the contents of total and enzyme resistant starch in white and

brown rice (three Australian cultivar: Calrose, Doongara and waxy). The starch content was maintained or increased by cooking in a microwave oven except with cultivar waxy. The increase was consistent with starch gelatinization and therefore, with greater susceptibility to enzyme attack. Cooked rice had a higher content of resistant starch than the corresponding raw product, possibly because of the production of retrograded starch. It can be concluded that although the content of the resistant starch in rice increased by the techniques known to increase the resistant starch in other foods (heating, cooking, cooling and freezing), the impact was not as great (Kim *et al*. 2006).

Table 7 Resistant starch content of Doongara variety from cooked and processed

Treatment	Resistant starch Type	% Resistant Starch
Brown rice	Unavailable for digestion	3.7
Steamed Rice	Modified	2.5
Boiled Rice	Modified	1.5
Material leached in boiling	Modified	5.3
Gel from flour	Modified	0.1
Retrograded Rice	Retrograded	4.6
Retrograded Gel	Retrograded/ Modified	0.5

Source: Kim *et al.* (2006)

3.2 Enzymatically treatment on improvement of RS III content

According to a recent review by King and Tan (2005), Enhancing resistant starch (RS) content of rice starch. The studied pointed out that non gelatinization; non refrigerated storage rice starch samples had higher resistant starch contents than gelled samples with and without storage. Most gelled had levels of RS below the original starting material. The starting rice starch material had a RS content of 5%. Pullulanase alone at 4 hr of treatment without gelatinize produced the highest amount

of RS overall, 60%, among the three enzyme combination, followed by α-amylase-pullulanase, then α-amylase alone.

The amount of resistant starch can be increased by enzymatically digesting rapidly digesting starch followed by washing/filtration to remove short chain low molecular weight sugars. Crystallization of all crystallisable substances involves three sequential steps, namely: 1) nucleation – formation of critical nuclei; 2) propagation–growth of crystals from nuclei by intermolecular association; and 3) maturation–crystal perfection and continued slow growth (Wunderlich, 1976). Nucleation is favoured at lower temperatures, while propagation and maturation favour higher temperatures. In retrograded wheat starch gels, low temperature storage (at 5 and 4^{o}C) has been shown to result in recrystallization to lower T_m, less symmetrically perfect polymorphs than those produced by storage at room temperature. Higher crystallization temperature favours formation of the higher T_m, more stable A-type polymorphs. These probably form the resistant starch fraction (Seib and Woo, 1999).

Hizukuri *et al*. (1983) also reported that isoamylase and pullulanase degraded potato amylose rapidly at the beginning and then approached constant values on prolonged incubation with large amounts of enzyme. Debranching non-waxy starch and waxy starch with a higher concentration of enzyme results in higher amount of slowly digestible starch and resistant starch after 2-4 hr of debranching and a subsequent decrease at longer debranching times.

The differences in the amount of debranching are affect the retrogradation process during storage. The degree of retrogradation depends on starch precipitation or aggregation and gelation. Gidley and Bulpin (1989) described these processes in a phase diagram showing the effect of cooling aqueous solution of synthetic amylose. In general, precipitation was favored by shorter chain lengths and gelation is favored by longer chain lengths. Guraya *et al*. (2001a) observed that debranched waxy starch formed white milky precipitates on cooling while non-waxy debranched starch formed a white gel with a fat-like consistency.

Guraya *et al*. (2001b) found that waxy debranched starches in aqueous solution formed particle sizes around 4 μm on cooling while solutions of non-waxy starches formed 45 μm size aggregates. Amylose of degree of polymerization (DP) 40–65 gave fine, dense precipitates while precipitates of DP 90 and 110 are less dense and therefore will have bigger particle sizes (Gidley and Bulpin, 1989). For chains of intermediate length (250<DP<660) both gelation and precipitation are observed, while long chain amylose (DP>1,100) are found to form gel on cooling.

Gidley and Bulpin (1989) formulated a reasonable for the observed effects based on the relative importance of chain alignment and cross-linking in cooled aqueous solution. Thus, for long chains, extensive cross-linking occur which results in the formation of a macromolecular network eventually resulting in gelation. If the chain length over which these interactions occur is substantially shorter than the total chain length, then more than two regions within a single chain could be involved in separate interactions, thereby leading to a cross-linked network structure.

Conversely, if the total chain length is not substantially longer than the interacting chain length, then extensive cross-linking will not occur and chain alignment will predominate, a process which, if followed by lateral aggregation, would eventually lead to precipitation. Lateral aggregations of shorter chains are due to formation of double-helices. These helices further aggregate leading to formation of ordered crystalline arrays which precipitate out of solution. These crystalline arrays are resistant to digestion (Jane and Robyt, 1984). During the cooling of hot aqueous non-waxy debranched starch solution, the longer amylose molecules tend to form a cross-linked network and the smaller molecules form the precipitates.

Guraya *et al.* (2001a) observed that 10% solution of waxy and non waxy starch debranched for 4 hr to 24 hr with 10g of pullulanase per 100 g starch was increased formation of resistant starch from 30% to 44% in waxy starch and from 40% to 50% in non-waxy starch. There is very little change in the rapidly digestible starch for the samples debranched using 10 g of pullulanase per 100 g starch from 4 to 24 hr. This suggests that additional debranching dose not release any more small

chains but liberates longer chains which then are used to form resistant starch. Initially when starch is partially debranched and is allowed to retrograde, imperfect packing of helices in crystallites might be taking place leaving some tight amorphous regions which can be digested slowly.

The cooperatively interacting length which gives rise to an ordered chain structure and the density which results from packing might be playing an important role in the digestibility of these starches. Therefore if the objective is to have high amount of resistant starch, then the starch needs to be highly debranched by using non-waxy starch. Longer and complete debranching times promote formation of resistant starch. Cooling at 15^{o}C had greatest reduction in starch digestibility was observed with the highest amount of resistant starch (Guraya *et al*. (2001a). This temperature favours nucleation as well as propagation and maturation of crystals. Rapidly cooling and storing the debranched starch at 1^{o}C had lower starch digestibility.

4. Starch Digestive Enzymes

Although glucose can be absorbed from the small intestine (especially terminal end of the duodenum and jejunum), but maltose and dextrin generated from starch hydrolysis cannot. Absorptive epithelial cells which line the intestinal villi produce a number of other 'brush border' enzymes to allow for digestion and subsequent absorption of such materials (disaccharides derived from other sources). These enzymes are not released into the lumen of the small intestine but are bound to the membrane of microvilli (Ronald and Matin, 1992).

Consequently, some carbohydrate occurs at the surface of epithelia cells in the small intestine rather than the lumen of the gut. Brush border enzymes include sucrase, converting sucrose to glucose and fructose and lactase converting lactose to glucose and galactose, but these are not relevant to starch metabolism. A trehalase (converts trehalose to two glucose molecule) is also present. However, maltase (converting maltose generated from α-amylase activity to glucose) and isomaltase

(also called α-dextrinase) which hydrolyses α-(1, 6) bonds of isomaltose and α-dextrins) continue the digestive process and thus covert available starch to glucose (Hayashida *et al*. 1990). A representation of amylase and debranching enzyme hydrolysis of starch is shown schematically in Figure 11. The figure described that β-amylase is an exo-acting enzyme cleaving successive, β-maltose molecules from the non-reducing end of amylose or from the outer branches of amylopectin; it cannot by-pass α-(1,6) bonds. While α-amylase is an endo-acting enzyme hydrolysis α-(1,4) bonds at random giving rise to malto-oligosaccharides (linear or branched, typically DP ~2-6); it does not hydrolyze α-(1.6) bonds. Debranching enzymes (e.g. isoamylase or pullulanase) specifically hydrolyze α-(1-6) bonds at the branching point of amylopectin. Amyloglucosidase 9glucoamylase) is an exo- acting hydrolase which releases single glucose molecules from the non-reducing end of α-(1-4) oligosaccharide or polysaccharides. This enzyme is unique because it can hydrolyze α-(1-6) branching points, converting starch completely to glucose (Tester *et al*. 2004).

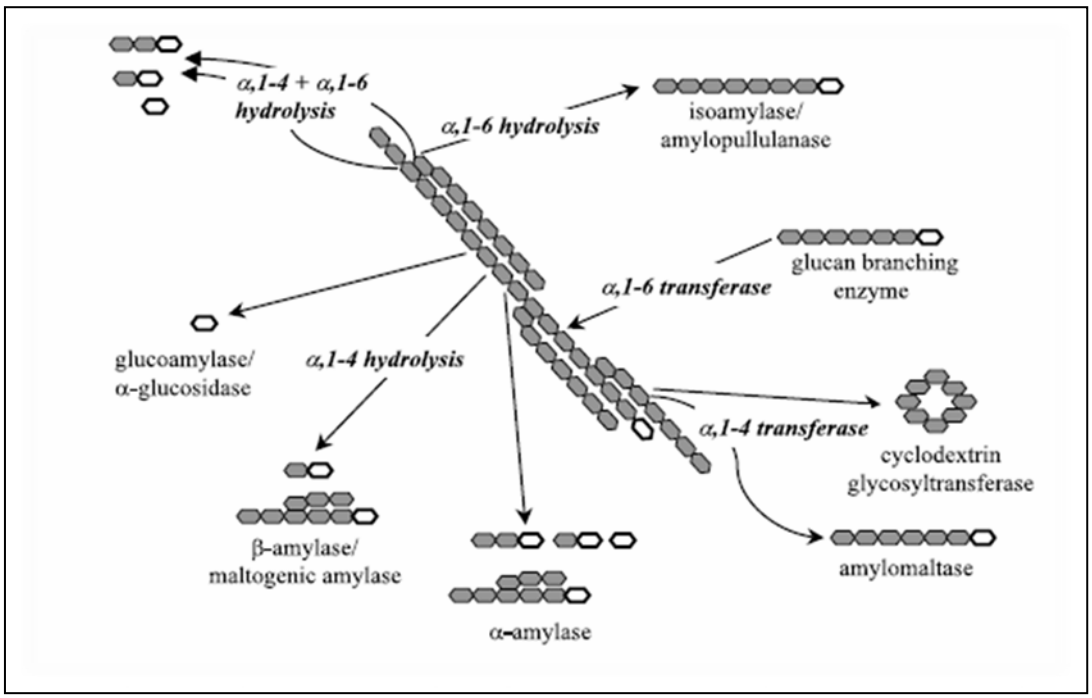


Figure 11 Action patterns of hydrolytic enzymes on amylose and amylopectin
Source: Tester *et al*. (2004)

4.1 Amylolytic enzymes

Three groups of amylolytic enzymes are commonly distinguished on the basis of hydrolytic specificity: enzymes specific for the α-(1,4) linkage, those specific for the α-(1,6) linkage and those that hydrolyze both α-(1,4) and α-(1,6) linkages without discrimination (Tester *et al*. 2004).

4.1.1 Enzymes specific for the α-(1,4) linkage

These enzymes, also known as amylase, are subdivided according to action pattern into: endoenzymes and exoenzymes (Govindasamy *et al*. 1992).

1) Endoenzymes

The α-amylases (α-(1,4-D-glucan-4-glucanohydrolase, E.C. 3.2.1.1) are present in most living organisms (microorganisms, plants, and animals),where they hydrolyze starchy substrates to the oligosaccharide level and capable of random hydrolysis of α-(1,4) linkages in macromolecules. The α-amylases are small proteins, generally in the molecular weight range of 50,000 – 60,000. The amino acid sequences of 18 α-amylases are now known (Raimbaud *et al*. 1989), but only two crystallographic analyses of the three- dimensional structure of α-amylases have been published. These concern the Taka-amylase from *Aspergillus oryzae* (Matsuura *et al*. 1984) and the porcine pancreas α-amylases (Buisson *et al*. 1987).

The pH optimum of the α-amylases is a function of enzyme origin, and is usually situated in the weakly acid pH zone between 4.8 and 6.9. Extremes exist, however, such as the acidophilic α-amylases of *Bacillus acidocaldarius* (pH 3.5), and the basophilic α-amylases of *Bacillus licheniformis* (pH 9.0). The presence of Ca^{2+} sometimes permits improved stability of the enzyme at more extreme pH values.

The temperature optimum for activity is also dependent on the origin of the enzyme. It is usually about 40 – 50 ^{o}C, but may reach values of around

70 -80 ^{o}C for α-amylases of bacteria origin (*Bacillus stearothermophilus*, *Bacillus subtilis*, and *B*. *licheniformis*).

Action pattern of the α-amylases: These endoenzymes hydrolyze α-(1,4) linkages on several chains and in several places along the same chain, releasing glucose and oligosaccharides of two to seven glucosyl units with an α-anomeric reducing end (Figure 11). Their action rapidly reduces the viscosity of starch solutions, hence the occasional use of the term liquefying amylases to describe them. The action pattern of α-amylases is a function of its origin and of the nature of the substrate (Buisson *et al*. 1987).

For amylose, at the end products are essentially maltose and maltotriose. Hydrolysis of maltotriose into maltose and glucose occurs later, since this oligosaccharide is more resistant to hydrolysis. For an amylose chain in solution, it random coil conformation favors the breakage of internal bonds close to one of the ends. As a result, the attack, however repetitive, generates chains with a high degree of polymerization, leaving the analysis of experimental results much more complex. For amylopectin, persistent branched limit dextrins remain, along with the above-mentioned oligosaccharides, glucose, maltose and maltoriose. These limit α- dextrins contain all α-(1,6) linkages from the original macromolecule and adjacent α-(1,4) linkages with a heightened resistance to enzymatic hydrolysis. Depending on the origin of the α-amylase, the limit α-dextrins may contain three (*B. subtilis*), four (pancreas and *A. oryzae*), or five glucosy units (barley malt)(Hayashida *et al*. 1990).

2) Exoenzymes

The exoenzymes are chiefly represented by the β- amylase group (E.C. 3.2.1.2), although new microbial exoenzymes that produce one specific type of oligosaccharide have been recently discovered. These different enzymes are distinguished essentially by the type of hydrolysis product generated that is, maltose, maltotriose, maltoterose, maltopentose, or maltohexose (Abe *et al*.1988).

Structure and properties of β- amylase; Knowledge of β-amylase (α-1,4- glucan maltohydrolase) is still relatively limited. The best known are those of plant origin (barley, wheat, oats, soya, and sweet potato) synthesized in latent form I grains and seeds, and then activated by proteolysis during germination. More recently, β- amylases from bacterial sources (*Bacillu*s, *Pseudomonas*, and *Streptomyces*) have also been isolated (Mercier, 1985). β-amylases are proteins composed of a single polypeptide chain (Molecular weight 60,000), but amino acid sequences are presently known for only two (Bacillus polymyxa and soya seed) (Mikami and Morita, 1988).

The pH and temperature optimum: The β-amylase have a pH optimum generally between 5 and 6, and a temperature optimum of about 50^{o}C. However, bacteria β-amylase show thermo stability properties superior to those of β-amylase of plant origin (Table 8).

Action pattern of β-amylase; Beta-amylase, or saccharifying enzymes, hydrolyze α-(1,4) linkages in amylose and amylopectin chains from their non reducing ends, releasing maltose with a β-anomery. The action of the enzyme is blocked at the α-(1,6) branch-points. Thus, amylose is totally hydrolyzed, whereas under the same conditions 55% of the amylopectin is converted to β-maltose. The residual product of amylopectin hydrolysis is a limit β-dextrin of high molecular weight, containing all of the α-(1,6) linkages present in the initial molecule (Ray, 1996).

Table 8 Characteristics of enzyme β-amylase (E.C. 3.2, 1.2)

Origin of Enzyme	Molecular weight	pH Optimum	Temperature Optimum (^oC)
Plant - Barley	56,000	5.2	-
- Wheat	64,200	5.2-6.2	55
- Soya	57,000	5.4	55
- Sweet potato	50,000	5.6	50-55
Microbial			
- *Bacillus cereus*	58,000	7.0	40
- *B. polymyxa*	42,000	7.5	40
- *B. megaterium*	58,000	6.5	40-50

Source: Ray and Nanda (1996)

4.1.2 Enzymes specific for the α-(1,6) linkage

Debranching enzymes specifically hydrolyze alpha-(1,6) linkages of branched α-glucan (amylopectin, glycogen) and their degradation products. They are present essentially in higher plants and microorganisms, where they play an important role in the total hydrolysis of starch. These enzymes are divided into two groups, based on their action pattern (Fogarty, 1983). Enzymes that can directly hydrolyze α-(1,6) linkages of native amylopectin and glycogen. This group includes the pullulanases (glycogen 6-glucanohydrolase, E.C. 3.2.1.41) and the isoamylases (glycogen 6-glucanohydrolase, E.C. 3.2.1.68), which are distinguished by their respective aptitudes to hydrolyze pullulan (poly α-(1,6)-maltotriose) and glycogen. These enzymes are produced exclusively by microorganisms. More recently, a pullulanase derived from *Bacillus acidopullulyticus* has been found to be sufficiently stable for use at 60^{o}C and the enzyme has been commercialized. The use of this enzyme in saccharification provides an increase in dextrose yield of 0.5 to 1.5% (Jensen and Norman, 1984).

Structure and properties: The pullulanases appear to be composed of a single polypeptide chain (*A. aerogenes*, molecular weight 143,000), but nothing is yet known of the three dimensional structure adopted by the latter. Certain tryptophan groups may be involved in the catalytic action of pullulanase (Jensen and Norman, 1984).

The pH and temperature optimum: Pullulanases and isoamylases have pH optima for activity between 5 to 7, and temperature optimum between 45 to 55^{o}C. The presence of a Ca^{2+} ion seems to improve the activity of these enzymes, whereas heavy metals such as Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+} and Co^{2+} exert a more or less marked inhibitory action (Jensen and Norman, 1984).

Action pattern: The pullulanases hydrolyze the α-(1,6) linkages of starch, glycogen, pullulan, and limit dextrins. However, the precise location of these α-(1,6) linkages is relatively important, since it strongly influences the ability of the enzyme to act on this substrates. In particular, the presence of two α-(1,4) linkages adjacent to the α-(1,6) linkages to be hydrolyzed (6^2-O-α- maltotriose) is necessary Kennedy *et al*. (1987). The isoamylases show some differences when compared to the pullulanases. They are unable to hydrolyze pullulan and cannot cleaveα-(1,6) linkages in molecules containing less than three α-(1,4) linkages (Table 9).

Table 9 Characteristics of pullulanase and isoamylases enzymes

Characteristics	Type of enzymes		
	Pullulanase		Isoamylase
	A. aerogenes	*B. cereus*	*Cytophaga sp.*
Molecular weight	143,000	112,000	120,000
pH optimum	6.5	6.0-6.5	5.0-6.0
Temperature optimum (^{o}C)	50	55	45
Pullulan hydrolysis	+	+	-

Source: Kennedy *et al*. (1987)

4.1.3 Enzymes specific for both of the α-(1,4) and α-(1,6) linkages

The amyloglucosidases (E.C. 3.2.1.3) are those exoenzymes that hydrolyze both types of linkages in α-glucan chains, releasing glucose in β- anomery. These enzymes, also called glucoamylase, glucamylase, or γ-amylase, are mainly produced by microorganisms, especially by molds such as *Aspergillus, Penicillium,* and *Rhizopus* (Hayashida *et al*. 1990).

Structure and properties: Amyloglucoxidases from molds are proteins whose molecular weight varies widely from 27,000 to 112,000, depending on their origin. The amino acid sequences of several glucoamylases (*Aspergillus niger*, *Aspergillus phoenicis*, and *Endomycopsis sp*.) are know, and although the results vary greatly with enzyme source, some characteristics stand out. The amyloglucosidases generally contain methionine and tryptophan residues, and half a cystein residue. Amyloglucosidases are chiefly made up to isoenzymes, I and II, which differ in their capacity to hydrolyze starch in solid form, and in their stability.

Thus amyloglucosidase I adsorb to and hydrolyze solid starch. In contrast, amyloglucosidase II exhibits neither of these two properties (Hayashida *et al*. 1990). *Aspergillus* enzyme is highly recommended for structural analyses because it lacks α-amylase. Two or three isoforms have been found in several enzymes of fungal origin, and the isoform with the highest molecular weight exhibits high raw-starch-digesting activity because it has a starch-binding domain. The enzyme is used for the determination of starch because it hydrolyzes starch specifically and commercial preparations from *Rhizopus* contain weak α-amylase and completely hydrolyze starch (Manelius *et al*. 1997).

The pH and temperature optimum: The properties of the amyloglucosidase are a function of enzyme origin. Their pH optima lie between 4.5 and 5.5, and their temperature optima between 40 and 60^{o}C. The presence of oligosaccharudes in the medium stabilizes the enzymes. In contrast, the presence of Ca^{2+} ions inhibits them, and favors their denaturation (Table 10) (Abe *et al*. 1988).

Action pattern: The amyloglucosidases are exoenzymes that release β-D-glucose by repeated hydrolysis of the α-(1,4) linkages of α-glucan chains from the nonreducing end. They also hydrolyze, albeit at a slower rate (10-30 times more slowly), α-(1,6) and α-(1,3) linkages, which makes them rather unspecific as enzymes. The rate of hydrolysis also depends on the nature of the linkages adjacent to the hydrolyzed glycosidic linkage, and on the size and structure of the substrate. More particularly, long α-glucan chains (amylose and amylopectin) are hydrolyzed more rapidly than maltodextrins and oligosaccharides (Fogarty, 1983).

These enzymes are the only ones capable of assuring total conversion of starch to glucose. However, when the concentration of the medium in β-glucose increases, following hydrolysis of α-glucan chains, the amyloglucosidse are liable to catalyze the condensation of several glucose units (transglycosylase action), producing mostly maltose and isomaltoe. Some amyloglucosidases are able to partly degrade solid starch (starch granules) (Fogarty, 1983 and Abe *et al*. 1988). This ability to degrade starch granules may be a property more peculiar to type I amyloglucosidases, which are capable of adsorption to native starch granules.

Table 10 Characteristics of enzyme amyloglucosidases

Origin of enzyme	Molecular weight	pH optimum	Temperature optimum (^{o}C)
A. niger I	99,000	4.5 – 5.0	-
A. niger II	112,000	4.5-5.0	-
A. oryzae I	76,000	4.5	60
A. oryzae II	38,000	4.5	50

Source: Mercier (1985)

5. Mechanisms of RS III formation during cooling, freezing and drying

5.1 The mechanisms of RS III formation during cooling

The rate of cooling of the gelatinized starch to the nucleating temperature should be as fast as possible and may be at least about 1°C per min on average, preferably at least about 3°C per min on average, most preferably at least about 4°C per min on average. By cooling to the nucleation temperature rapidly, the propagation of undesirable crystal forms, such as amylose-lipid complexes, is substantially reduced or eliminated. Also, rapid cooling generally promotes the generation of large numbers of small seed crystals, rather than fewer, larger crystals. After nucleation, the nucleated crystals of enzyme-resistant starch type III may be propagated or grown by raising the temperature of the gelatinized starch from the nucleation temperature to a crystal-propagating temperature (Eliasson and Kim, 1992).

Propagation of the resistant starch type III crystals may be achieved at a temperature above the melting point of any of amylose-lipid complexes which may have been formed during nucleation. Thus, use of a crystal-propagating temperature above the melting point of the amylose-lipid complexes remelts the complexes, thereby making more amylose available to formation of resistant starch type III. However, the crystal-propagation temperature is maintained below the melting point of the desired crystals of enzyme-resistant starch type III to avoid melting or destroying them. The temperature is preferably raised from the nucleating temperature to the crystal-propagating temperature at a rapid rate to avoid any substantial propagation of undesirable crystals, such as amylose-lipid complexes (Yuan and Thompson, 1998).

5.2 The mechanisms of RS III formation during freeze-thawed process

Freezing is a physical treatment widely applied for preservation, drying and lyophilisation of starchy food (Ahmed and Lelievre, 1978). It is also used for sample preparation in granule structural investigations by means of many physical

methods, for instance in scanning electron microscopy (SEM) or transmission electron microscopy (TEM) (Hayagava *et al*. 1998). It was also considered to cause some changes in the nutritional properties of starch (Guraya *et al*. 2001b).

Freezing of starch solutions resulted in their cooperation and increasing retrogradation, while pregelatinised starch became less sensitive to retrogradation and stayed smooth after the process (Waigh *et al*. 1998) well as Donald *et al*. (2001) reported that some reversible structural disorder of starch granules occurred at sub-zero temperatures. Repeated freeze–thawing procedures slightly influenced the water solubility and the water holding capacity, but did not change the branching characteristics of starch granules (Szymonska *et al*. 2003). Retrogradation is enhanced when starch gels are subjected to freezing and thawing. Freezing of starch gel causes the phase separation upon ice crystal formation. Upon thawing, water can be expressed from the gel known as syneresis.

Resistant starch type III is cooked cooled and retrogradation of starch, some of which is resistant to the enzymatic digestion in the human digestive system. In the previous studied retrogradation of starch gels (starch pastes) is often enhanced when they are subjected to freezing and thawing treatments. During freezing, the gel can separate into fractions by the formation of ice crystals, such that the starch is concentrated in a non-ice phase. When thawed, the ice crystals melt to form a mixture of water and gel. The freeze-thaw stability of starch gels has been evaluated by measuring the quantity of liquid that can be separated from a thawed gel after centrifugation (Hood and Scifried, 1974).

Varavinit *et al*. (2002) investigated the freeze-thaw stabilities of three different rice flour gels (amylose rice flour with 28% amylose, Jasmine rice flour with 18% amylose and waxy rice flour with 5% amylose) by first freezing at -18°C for 22 h and subsequent thawing in a water bath at 30°C, 60°C and 90°C. The freeze-thaw stability was determined for five cycles. Starch gels thawed at higher temperature exhibited a lower syneresis value (percent of water separation) than those thawed at lower temperature. Amylose rice flour gels gave the highest syneresis value (especially in the first cycle). The jasmine rice flour gels gave a highest syneresis

value than the waxy rice flour gel. However, they found that during the first cycle the amylose rice flour gel had changed from a smooth gel to a rough- textured porous gel (rough surface) with a sponge-like structure that allowed it to reabsorb the separated water. Thus, syneresis was not detectable unless this rough-textured gel pressed to squeeze out the absorbed water. This retrogradation phenomenon occurred when the starch gel was frozen and ice crystals spread within the gel. Upon subsequent thawing at a lower temperature (30°C), the ice crystals melted and a rough porous gel was formed and it was not enough to form a spongy structure and a high syneresis value was observed. From the second to the fifth cycle, the quantity of rough-textured gel accumulated curve indicated the formation of a rough and porous gel alter thawing and it formation of a rough and porous gel after thawing and it confirmed the very low freeze-thaw stability of this flour. When thawed at higher temperature (60°C, 90°C and microwave), amylose rice flour gels no longer formed a rough, spongy gel because of re-dissolved of retrogradation starch during heating.

5.3 The mechanisms of RS III formation during drying of the starch

The drying of recrystalized resistant starch may be performed at room temperature or at elevated temperatures. Thus, the recrystalized retrograded starch may be cooled from the crystal-propagating temperature to room temperature or to a drying temperature which is above room temperature. Exemplary drying temperatures may range from about 20°C. to about 130°C., depending on mode of drying, preferably from about 45°C. to about 80°C., for oven-drying. Known drying methods for the drying of starch, which do not substantially destroy or melt the crystals of resistant starch type III, may be employed. Exemplary drying methods which may be used include freeze-drying, oven-drying, vacuum-drying, spray drying, flash-drying, belt-drying and drum-drying. The drying of the high-melting-point resistant starch type III composition is conducted to achieve a shelf-stable water activity or relative humidity of less than about 0.7. The water content of the dried product may approximate that of commercially available flour. Exemplary moisture contents of the dried, bulking agent or flour substitute or replacer may range from about 8% by weight to about 14% by weightb (Relinda, 1994).

6. Low glycemic butter cake products

6.1 Butter cakes production and function of ingredients

Cake is a food well linked by consumers all over the world. According to Thai farmer research center, (2007) the snack cake category of the baking industry represents approximately a 7,100 million Bath and the trend are growing in Thailand. This is due to consumers' increased demand for goods that can be consumed with little or no home preparation. Although the production of bake goods, most notably cakes, is considered by many to be an art, it is also a product of science. Therefore, it is extremely important for those developing new cakes to understand each different ingredient and its purpose in the mixture of the product (Goldstein, 2001).

6.1.1 Optimal cake characteristics

A high-quality cake has a high-specific volume and is symmetrical. The top of a layer cake needs to be flat for stacking (Penfield and Campbell, 1990f). The crumb of a high-quality cake is moist, elastic, has a fine grain, cells of uniform size, and thin cell walls. Crusts should be thin and tender (Bennion, 1995b).

6.1.2 Function of cake ingredients

The main ingredients used in cake formulations are flour, sugar, fat, eggs, liquid, and leavening agents. Flavoring ingredients such as salt, vanilla, spices, coloring agents, etc. are also used in small amounts (Penfield and Campbell, 1990f). Each ingredient has its own function in cakes, and if slightly changed will alter final cake quality. Therefore, a proper balance of ingredients needs to be obtained to produce consistent high-quality cakes.

1) Flour

The main structure of cakes is composed of flour. Flour also binds and absorbs moisture from the mixture (Goldstein, 2001). Different types of flour

have different properties, which are applicable to different types of cake products. However, most flours do contain wheat, which contain varying proportions of glutenin and gliadin proteins. Cake flour, chlorinated soft wheat flour, is used in cakes. Chlorinated cake flour improves the performance in high-ratio cakes (Bennion, 1995g). Chlorine breaks inter- and intramolecular hydrogen bonds and some peptide bonds in flour proteins, resulting in increased dispersibility. The percent of protein in flour determines gluten strength in baked products. Gluten proteins found in wheat flours give structure to baked goods. Soft wheat flours contain less than 10% protein, while hard wheat flours have more than 10% protein. Cake flour usually has about 7.5% protein, whereas all-purpose flour has about 10.5% protein (Patil, 1991). Soft wheat flours are preferred in soft baked goods, such as cakes because soft baked goods require a small amount of gluten formation. All-purpose flour, however, can be substituted in cakes using one cup minus two tablespoons of all-purpose flour to replace one cup of cake flour. Since little gluten is developed in cakes, the gelatinization of starch is more important to a cake's structure (Bennion, 1995b). Flour contributes structure to cakes. If too little flour is used, the cake structure is weak and may fall, and the texture is coarse (Bennion, 1995b). If too much flour is used, a compact, dry cake is produced.

2) Eggs

In cake batters, eggs could account for as much as 70% of the cost of the ingredients in the batter (Goldstein, 2001). In baked goods, eggs impart binding, shortening, leavening capabilities as well as aid in coloring and browning during the baking process. Milk is also an integral part of a cake batter. Because it contains protein, sugar, and butterfat, it adds color, nutritional value, and moistness to the product (Goldstein, 2001).

3) Sweetener

Sugar adds sweetness to cakes. But more importantly, it affects texture, volume, moisture retention and color. Cake tenderness is increased because sugar delays the gelatinization of the starch and interferes with gluten development.

Sugar increases cake volume by decreasing the cohesive forces (resistance to the movement of cake batter during baking) and allowing the batter to move more freely (Bennion, 1995b). Sugar also increases moisture retention and keeping quality of cakes by absorbing water. Finally, sugar adds color through Maillard browning. Alternative sweeteners such as high-fructose corn syrup can replace sugar in cakes. High-fructose corn syrups can reduce caloric content in cakes, and increase moisture retention and color because they are sweeter, more hygroscopic and are reducing sugars. Excessive amounts of sugar produce a coarse, thick-celled, gummy cake with a rough, sugary, and overly brown crust (Bennion, 1995b).

4) Fat or butter

The purpose of the fat or butter in the cake is to provide tenderness, flavor and odor to the product. Also the major point of the differentiation of the type of fat used is whether it is a solid or liquid at room temperature. During the mixing process, air is incorporated into the fat at a stage called creaming. This creates pocket where both air and moisture occur, thus creating texture that is light and airy. This stag, where gas bubbles are incorporated into the mixture, is directly linked to the final texture and volume of the cake which is related to the final quality of the cake product (Sahi and Alava, 2003).

5) Leavening agents

Leavening agents provide both volume and structure to the finished product. These agents help to aerate the mixture through the release of air, steam, and/ or carbon dioxide during the process (Goldstein, 2001). During the whipping process, air is incorporated into the batter. When the acids and base of leavening agents react with the moisture and heat during the baking process, small bubbles are formed in the product, which results in a honeycomb of small air spaces (Goldstein, 2001).

6.1.3 Cake Manipulation

A variety of mixing methods are used to combine shortened cake ingredients. The four most common methods used are: (1) conventional method, (2) conventional sponge method, (3) creaming method and (4) quick-mix method (Bennion, 1995). As cake ingredients improve, differences among mixing methods are not very great. Instead, changes in cake quality occur by the amount of manipulation that is applied. Under-manipulation of a cake batter may yield a cake of good volume, but the texture is coarse and the cell walls are thick (Bennion, 1995b). Optimal amount of manipulation results in a cake of optimum volume, uniform texture, small cells, and thin cell walls (Bennion, 1995b). Over-manipulation of a cake batter may produce a fine texture, but tunnels are likely to be formed, and produce a compact cake of low volume (Bennion, 1995b). Even though sugar and fat in cake formulas retard gluten development, excessive over-manipulation may still toughen cakes

Many different types of cake products are made with batters that have been whipped to incorporate air including sponge, roll and butter cakes (Nielsen, 2002). These items are offer produced on a large scale basis with continuous mixing, baking, and procession lines. In fact, several important steps go into the commercial preparation of baked goods. The first step involves the combination or pre-mixing of ingredients. Next, the batter is stabilized in a feed tank. After this step, the air is incorporated into the batter at a controlled rate. Next, the batter is deposited into containers, baked, cooled and packaged.

6.2 Glycemic index (GI) and glycemic load (GL) in cake products

Many carbohydrate-rich foods have high glycemic indexes (GI.) and certainly are not good in any substantial quantity for people with diabetes. Other carbohydrates break down more slowly, releasing glucose gradually into our blood streams and are said to have lower glycemic indexes. The really shocking results of GI studies are in which foods produce the highest glycemic response. They include many of the starchy foods we eat a lot of, including most bread, most breakfast

cereals, and baked potatoes. Low glycemic foods include beans, peas oats, and some types of rice and acidic fruits (Jenkin *et al*. 2002a).

6.2.1 Glycemic index definition and classification

The glycemic index (GI) is a numerical system of measuring how much of a rise in circulating blood sugar a carbohydrate triggers the higher the number, the greater the blood sugar response. So a low GI food will cause a small rise, while a high GI food will trigger a dramatic spike. Food with GI of 70 or more classified as high, a GI of 56 to 69 inclusive is medium, and a GI of 55 or less is low (Foster *et al*. 2002). Before the development of the glycemic index beginning in 1981, scientists assumed that our bodies' absorbed and digested simple sugars quickly, with producing rapid increases in our blood sugar level (Table 11) (Jenkins *et al*. 2002).

Table 11 Glycemic index and sweet taste value in various sugar

Sugar types	Glycemic index (Glucose = 100)	Glycemic index (Bread = 100)	Sweet taste value (%) (Sucrose = 1%)
Glucose	100	140	0.7
Sucrose	60	95	1
Lactose	46	65	0.4
Fructose	19	15	1.1

Source: Jenkins *et al*. (2002b)

6.2.2 Glycemic load definition

The glycemic load (GL) is a relatively new way to assess the impact of carbohydrate consumption that takes the glycemic index into account, but gives a fuller picture than does glycemic index alone. A GI value identify only how rapidly a particular carbohydrate turns into sugar. It doesn't identify how much of that carbohydrate is in a serving of a particular food. People need to know both things to

understand a food's effect on blood sugar (Jenkins *et al*. 2002). The carbohydrate in watermelon, for example, has a high GI but there isn't a lot of it, so watermelon's glycemic load is relatively low. A GL of 20 or more is high, a GL of 11 to 19 inclusive is medium, and a GL of 10 or less is low Foster *et al*. 2002).

6.2.3 Relationships between glycemic index and glycemic load

Foods that have a low GL almost always have a low GI food with an intermediate or high GL range from very low to very high GI. Both GI and GL in cake products are listed in Table 12. The GI is of foods based on white bread, where it is set to equal 100. The other is the glycemic load, which is calculated from the glycemic index divided by 100 multiplied by its available carbohydrate content (i.e. carbohydrates minus fiber) in grams.

Table 12 Glycemic index and glycemic load representative in cakes.

Cake types	GI (bread = 100)	Glycemic Load	Avail. Carb per serving	Serving Size (grams)
Angel food cake	95 ± 70	27.55	29	50
Banana cake	67 ± 80	25.46	38	80
Chocolate cake	79 ± 10	41.08	52	111
Cupcake, strawberry-iced	104 ± 12	29.12	28	53
Pound butter cake	73 ± 12	18.98	26	38
Sponge cake, plain	66 ± 60	23.76	36	63
Pound cake (Sara Lee)	77 ± 80	21.56	28	53

Source: Jenkins *et al*. (2002a)

6.3 Low glycemic index butter cake

The food industry is being challenged to redesign traditional foods for optimal nutritional value, in response to some population sectors with particular nutritional necessities and making them as tasty as or better than the original. One

way to achieve a healthy food product is to reduce or to omit some of the calorie-laden ingredients, especially sugar and fat since, at present, obesity is frequently cited as a serious health problem. At the same time, there is a constant demand for dietetic foods suitable for diabetes, which may have the same caloric-value being also sucrose-free since this sucrose cannot be metabolized without insulin. For this reason, it is a challenge to develop a cake product that sucrose-free for individuals who are intolerant to glucose (Hicsasmaz *et al*. 2003).

Butter cake is a complex fat and water emulsion system containing flour, sugar, fat, eggs and baking powder. A proper combination of the ingredients can give a high quality product with desirable flavour and texture. Shortening plays an important role in baked products. The functions of shortening in cake were described by several researches (Moncrieff, 1970; Howard, 1972). The major component of flour is starch; starch properties were shown to be important in making cakes. The functionality of starch and sugar in cake contributes structure, increase batter viscosity and cake volume, control moisture, increase shelf life and affected to health problems. Cake is a food well liked by consumers all over the worlds. However, due to its high caloric content, over-consumption may contribute to obesity. Several studies suggest that foods high in added sugar may increase the risk of diabetes. The glycaemic indices of cake products vary continuously from about 66 to over 104 (Table 12). Angel food cake and cub cake show a high GI (95 and 104). Banana cake, sponge cake and cupcake, strawberry-iced give a medium GI (66 to 77). According to the health problem related to the cake product is it important to understand the role of food ingredients to produce low glycemic index (GI) food.

6.3.1 The role of food ingredients on low GI food

Differences in qualitative nutritional properties among starchy foods are particularly intriguing. Elucidating the role of starch qualities in human nutrition requires a greater understanding of how the physicochemical characteristic of food relate to physiological properties. The relationship between the rate of starch digestion and GI has been established by investigations of *in vitro* amylolytic hydrolysis

(O'Dea *et al*. 1981, Jenkins *et al*. 1982, Bornet *et al*. 1989, Granfeldt *et al*. 1992 and Englyst *et al*. 2003). The rate and extent of starch digestion is influenced by many intrinsic food factors. Starch consists of two glucose polymers; amylose amylopectin. The physical arrangement of amylose and amylopectin in food and their interaction and/or interconnection with other food component (protein, lipids and fibre) determine the physicochemical and functional properties of starch and is susceptibility to amylolytic enzymes, and thus its bioavailability. Hydroyhermic food processing has a major impact on starch availability (Bjorck, 2000). The arrangement of starch components changes continuously under the influence of hydrothermic parameters during both food processing and storage.

6.3.2 The role of starch sources and starch granule on low GI food

Common food starch is derived from seed (wheat, maize rice, barley), roots (potatoes, cassava) and legumes (pea, lentils, mungbeen). Native starch in granule form is insoluble in cold water. Its microcrystalline structure gives rise to a characteristic polarization cross under polarized light. Under dry heat conditions, starch granules are not disrupted and the polarization cross remains unchanged. Native starch granules are slowly attacked by salivary and pancreatic amylases. Mechanical processes cause fissures to appear on the surface increase the susceptibility of starch to α- amylase (Bjorck and Asp, 1984).

6.3.3 The role of gelatinized starch on low GI food

Starch is usually eaten after an initial gelatinization step. Dramatic changes occur in the structure of the starch granule when it is heated in the presence of water are descried in above topic (2.4). Amylose content is not a discriminate indicator of α- amylase susceptibility when starch is native (Bornet *et al*. 1989). Tuber starches are highly resistant to amylase despite their low amylose content. However, after gelatinization, amylose content becomes an important factor in determining α- amylase susceptibility. It is explained by the marked tendency of high amylose content starch to produce hard gels, retrograded amylose and amylose lipid

complexes. In the meantime, and inverse relationship appears between amylose content and the degree of glycemic response to processed starchy food (Bornet *et al*. 1989, Granfeldt *et al*. 1995).

6.3.4 The role of starch gel and retrograded starch on low GI food

As temperature decreases, a gel forms progressively under the action of the system and consists of the remaining wrapping of the starch granules (ghost systems) enriched in amylopectin, following immersion in high amylose content; this is called the gelification step. A rearrangement between starches occurs and a three-dimensional network is rapidly constituted. The higher the amylose content of starch had a harder the starch gel. The starch gel structure has lower α- amylase susceptibility than a paste (Eliasson, 1980).

As starch chain rearranges, hydrogen bonds between chains reappear and a novel crystalline structure is created; this is referred to as the retrograded phenomenon. Over time, starch gel retrogradation increases. It is the more marked if the gelatinization of the starch has been conducted well, such as at high temperature, with high moisture and under prolonged and effective stirring. The other factors that promote retrogradation are high amylose content, low starch gel moisture and low storage temperature (4°C). Retrogradation is delayed when mono-or glycerides are added, whereas all the preventing dehydration of starch gel encourages its retrogradation. The crystalline structure of retrograded amylose is acid and heat-resistant. Its melting-point is above 120°C. *In vitro*, the retrograded starch and retrograded amylose fraction are highly resistant to α- amylase. This resistant starch fraction is resistant to amylase digestion in the human digestive tract and exhibits digestive behavior similar to that of the non-starchy polysaccharide fraction (NSP) or indigestible oligosaccharides (Fan and Marks, 1999).

6.3.5 The role of lipid-starch complexes on low GI food

During gelatinization, constitutive monoglycerides of cereal starch and added monoglycerides melt and merge with amylose to create a novel crystalline structure- the amylose lipid complex. This structure is digested by α-amylase more slowly than that of native amylose. The longer the monoglyceride chain, the slower the slower the digestion of the amylose lipid complex (Hibi *et al*, 1990).

6.3.6 The role of simple sugars on low GI food

Food carbohydrates in which the main carbohydrate is fructose or lactose elicit low GIs (Wolever *et al.* 1994). This is the case for dairy products and fruits. Both sugars when eaten alone lead to a low GI; 19 and 46 respectively for fructose and lactose (Spicth *et al.* 2002). An inverse correlation has been observed between the glycaemic index and the amount of sucrose added to cereals in healthy subjects. The quantities of total carbohydrates, fats, proteins, however, remained identical. Insulinaemic response was significantly lower in the case of breakfast cereals containing sugar than for cereals without sucrose (Miller *et al*. 1995).

Fructose or fruit sugar is the primary carbohydrate in many fruits, maple syrup, and honey. In fact, the fructose content of most fruits and many vegetables is roughly 10% of their dry weight. Fructose is very sweet and is roughly 1.75 times sweeter than sucrose (white sugar). Although fructose has the same chemical formula as glucose ($C_6H_{12}O_6$), its structure (shape) is quite different. In order to be utilized by the body, fructose must be converted to glucose within the liver.

At appropriate levels, less than 10g per serving fructose is an acceptable sweetener. Because fructose must be changed to glucose in the liver in order to be utilized by the body, blood glucose levels do not rise as rapidly after fructose consumption compared to other simple sugars or even complex carbohydrates. For example, the glycemic load calculation for 10 grams of fructose it

would be only 2. In comparison, the glycemic load for a slice of bread is 10; an apple is 7, and a cup of white rice 26 (Miller *et al*. 2003).

High fructose corn syrup (HFCS) is manufactured by enzymatically hydrolyzing a high-glucose corn syrup, isomerizing glucose into fructose (Bray *et al*. 2004). A typical HFCS contains 42% fructose. Higher fructose corn syrups (55, 90, and 100%) can be obtained by enrichment of the isomerized syrups (Appl, 1991). High-fructose corn syrup provides many benefits in reduced-calorie and reduced-fat systems. High-fructose corn syrup improves texture, helps retain moisture, lowers water activity, and enhances flavor, color and sweetness. Texturally, fructose has a low tendency to crystallize, reducing the potential for graining (Nonaka, 1997). In addition, fructose does not invert in acidic conditions like sucrose does which eliminates the possibility of changes in texture during storage. Also, fructose's high hygroscopicity increases the perception of tenderness in baked goods. In baking systems, a desirable aroma, color, and flavor develop when the reducing property of HFCS reacts with proteins (Maillard Reaction). The intense sweetness of HFCS, especially of HFCS-90, allows use of 10-20% less sweetener per pound in most soft baked goods. Finally, HFCS is effective at lowering water activity because it is a monosaccharide creating a high osmotic pressure (Hanover and White. 1993).

6.3.7 The role of other factors for low GI food

Fiber type and amount may also affect the blood glucose raising potential of foods. Indeed, the effect of added soluble viscous dietary fibers on the reduction of both glycaemic and insulinaemic responses has been largely reported. Some effects of insoluble fibers and dietary resistant starch on short-term post-prandial glycaemia have been also reported, but the effect is weaker. The lowering effect of resistant starch on blood glucose (by replacing rapidly the digestible starch fraction) has also been underline. However, most of these effects vary greatly depending on the physicochemical properties of fibers and may be affected by the physicochemical parameter of food processes. Co-ingestion of fat with carbohydrates (as part of the food, or added) can lower the post-prandial glycemic response to foods.

Whether the quantity or the quality of lipids influences the glucose response, is not yet described clearly. Recent studies suggest that fat may influence glucose response even at a low dose (Owen and Wolever, 2003). However, while studying a large variety of cereal processed foods, Englyst *et al.* (2003) demonstrated fat is not the major determinant of their glycemic index. Other factors such as structure, texture of foods and content in anti-nutrients or organic acids may also affect their glycaemic index.

6.3.8 The role of resistant starch on low GI food starch

For most starchy food products, a reduction in GI appears to be accompanied by a higher content of resistant starch (RS). A high RS content will add to the total amount of indigestible carbohydrates reaching the colon for fermentation. Lin *et al*. (1994) found that the using resistant starch replacement of fat at 5% of the flour in a yellow cake formulation produced cakes that were softer than the control and that up to 15% of the cake flour could be replaced with no significant effect on cake quality. In addition to replacing cake flour, Lin *et al*. (1994) found that up to 25% of the fat in the formulation could be replaced with resistant starch while maintaining quality equal to the control cakes.

6.4 Low GI diets and their associated health benefits

The degree of glycemic and hormonal response associated with carbohydrate load affects numerous functions within the body. Repeated changes in postprandial glycemic levels may affect the long-term function of organs. A standard sample is that of diabetes. Repeated abnormally pronounced hyperglycaemia in itself constitutes one of the mechanisms responsible for the complications seen during the course of this disease (Willet *et al*. 2002). Figure 14 provides a graphical illustration of the potential impact of reduced post-prandial glycaemic response on various body functions as well as potential dysfunction (Lang *et al.* 2004). Diabetes mellitus is chronic disorder that results from a deficiency in the insulin hormone. This occurs either because of an absolute decrease in the amount hormone. This occurs either

because of an absolute decrease in the amount of insulin produced by the pancreas (type 1 diabetes) or because of a relative deficiency of insulin in patients whose tissues are resistant to the hormone (type 2 diabetes). Insulin resistance is an impairment of insulin action, especially in the insulin-stimulated uptake of glucose by the tissue and the inhibition of glucose output by the liver and of fat breakdown (lipolysis). Insulin resistance is often associated with aberrations in other aspects of metabolism, especially of lipids, as well as obesity. The hallmark of untreated diabetes mellitus is elevated blood glucose concentration. It is estimated that 114 million people re suffering from diabetes. By the year 2010, this figure may reach 333 million worldwide. Among the numerous environmental factors that may interact with genetic potential to develop insulin resistance and type 2-diabetes, the role of dietary carbohydrate and fiber intake has been investigated. There is evidence that rapidly digested starches increase the risk of type 2 diabetes. However, there is support for the contention those eating foods that are rich in slowly digested starch (low GI) and resistant starch or starch high in soluble fiber, as well as the avoidance of excess weight and physical inactivity, reduces the risk of developing type 2-diabetes (Salmeron *et al*. 1997)

6.4.1 Low GI diet and diabetes mellitus

The trivial effects of low GI foods observed in diabetic subjects over the short and long terms comprise reduction of post-orandial blood glucose response (Miller *et al*. 2003). The majority of studies suggest that foods and diets with low GI offer greater benefits for diabetes mellitus patients than foods with a high GI (Willet *et al*. 2002). A recent meta-analysis (Miller *et al*. 2003) based on the results of 14 short- and medium-term clinical studies has shown that low GI diet is liked to a reduction in the concentration of glycosylated haemoglobin and fructisamine, two major markers that reflect glycaemia control and are linked to potential complications of diabetes. Furthermore, many animal and human studies demonstrate greater sensitivity to insulin after a low GI diet than after a high GI diet (Ludwig, 2002).

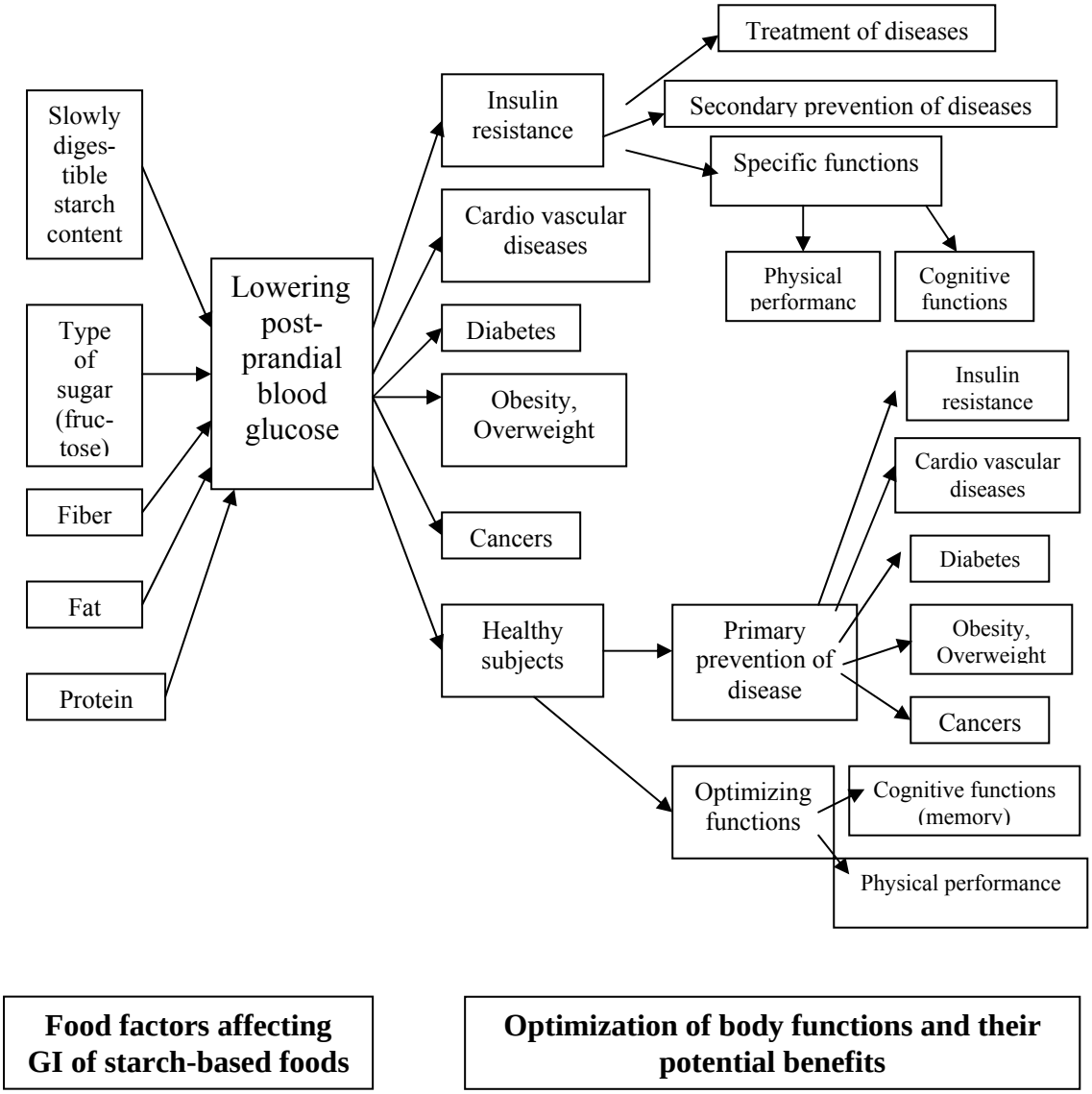


Figure 12 The link between glycaemic response and health outcomes
Source: Lang *et al*. (2004)

6.4.2 Low GI diet, weight and obesity control

Few medium-term studied have compared the weight loss potential of high and low GI high carbohydrate diets. Some studies (Marckmann *et al*. 2000, Slabber *et al*. 1994 and Spieth *et al*. 2000) have shown more marked weight loss with a low GI diet and a high GI diet. This greater satiety and better use of fat reserves by

enhanced fat oxidation at the expense of carbohydrate oxidation. One important issue is the action of low GI foods on modulation of the expression of human appetite and more specifically on satiation (within meals) and satiety (following meals). Several studies have shown that low GI meals or foods producing a low blood glucose response are more satiating than isoenergetic meals or high GI foods inducing high blood glucose (Miller *et al*. 2003 and Ludwig, 2002). Because low GI starchy foods are characterized by a slower rate of digestion and absorption, nutrient receptors in the gastrointestinal tract are stimulated for a longer period of time, resulting in prolonged feedback to the satiety centre in the brain (Lavin *et al*. 1998). A further exciting prospect is the potential of GI foods to modulate oxidation and storage of fats. Post-prandial rises in glucose and insulin concentrations increase carbohydrate oxidation acutely through the rapid activation of key rate limiting enzymes and reduce fat oxidation (Miller *et al*. 2002). High GI meals induce lower fatty acid plasma response throughout the day (Kiens and Richter, 1996) and lower rtes of fat oxidation than do low GI meals (Febbraio *et al*. 2000). To date, emerging evidence exists to claim a beneficial effect of a high fiber low GI starchy diet on weight maintenance or weight loss. However, the limited availability of data requires further research including long-term clinical studies.

6.4.3 Low GI diet on lipid metabolism and cardiovascular disease

Glycaemic index and blood lipids have been studied since the concept of GI was first introduced. A high-carbohydrate diet and high glycaemic indices were linked to the risk of coronary heart disease in women in a large prospective study (Liu *et al*. 2000). Cross-sectional studies showed that low GI diets are associated with high HDL-cholecterol concentration, especially in women (Frost *et al*., 1999). In addition, in a very well controlled study of type 2 diabetes patients, serum cholesterol, LDL cholesterol and apolipoprotein B concentrations fell more significantly after a low GI diet than after a high GI diet (Wolever *et al*., 1992). Proof of the clinical value of low GI diets in primary or secondary prevention of hart diseases awaits prospective intervention trials (Leeds, 2002).

6.4.4 Low GI diet on cognitive performance

The Academic Press Dictionary of Science and technology defined cognition as 'the mental activity by which an individual is aware of and knows about his or her environment, including such processes as perceiving, remembering, reasoning, judging and problem solving'. The brain uses glucose as a main source of energy. Glycogen brain becomes important only in any metabolic state where supply transiently cannot meet demand. Such conditions could occur during prolonged focal activation, sleep deprivation, seizures, and mild hypoxia (Gruetter, 2003).

Under normal circumstances, the brain depends on a continuous supply of glucose from blood. In adult humans, the brain oxidizes about 120 g of glucose per day. In general, cognitive performance tasks can be used to examine a number of skills or abilities concerning the following functions: perception, memory, attention, arousal, information processing accuracy and speed of movement. Each function can be evaluated using specialized tests (Bellisle *et al*. 1998). A number of studies performed in adults, beginning with the Iowa breakfast studies, have demonstrated that missing breakfast can have detrimental effects on performance in terms of reaction-time tasks, spatial memory and immediate word recall (Benton, 1992). While some aspects of memory seem to be susceptible to the effects of missing breakfast, other aspects of performance are not affected (Smith *et al*. 1994). Studies in children and in adolescents have clearly shown deficits in a wide range of performance tasks following omission of breakfast, with greater effects in under and malnourished children. A number of studies have shown that breakfast vs. no breakfast or high- vs. low-energy breakfast causes changes in sustained attention, reaction time and memory. Energy improves performance in all of these and may be related to blood glucose levels, with high-carbohydrate meals producing the best effects (Bellisle *et al*. 1998). A recent literature review concluded that the enhancement of cognitive function by incrementing systemic glucose is limited to relatively complex tasks, and not easy tasks (Benton, 2001).

MATERIALS AND METHODS

Materials

1. Raw Materials

High amylose rice starch (HARS) was kindly supplied by Cho Heng Rice Vermicelli Factory Co., Ltd., Nakornpathom, Thailand. A total of 50 kg in the same batch was used in this study. Commercial resistant starch (HI-MAIZE ® 1043) was donated by the National Starch, Food innovation. Cake flour, shortening, salt, sugar, baking powder, whole eggs, and skim milk were purchased from a local market. High fructose corn syrup (HFCS-55) was kindly supplied by Chao khun Agro products Co., Ltd., Bangkok, Thailand.

2. Chemical Reagents

1. Amylose standard, type III from potato (Sigma No. A-0512)
2. Sodium hydroxide (Merck)
3. Hydrochloric acid (Merck)
4. Acetic acid (Merck)
5. Phosphate buffer (pH 6.89) (Merck)
6. Ethanol, 95% (Merck)
7. Pullulanase enzyme from *Bacillus acidopullulyicus* (Sigma, E.C. 232-983-9P; ≥ 400 units/ml
8. α-amylase (EC 3.2.1.1Type VI-B from porcine pancreas, Sigma Chemicals)
9. Pepsin (EC 3.4.23.1; 2,980 unit/mg)
10. Glucose (GO) assay kit (GAGO-20)
11. Amyloglucosidase, from *aspergillus niger* (A-3042, Sigma)
12. Resistant starch assay kit (Megazyme) was obtained from Megasyme International Ireland Ltd., IRELAND.

3. Equipment and Instruments

1. Balance (BP 211D, Sartorius, Germany)
2. pH Meter (Model C831, Turnhout, Belgium
3. Spectrophotometer (Hitachi 100-6-Japan)
4. Hot air oven, controlled temperature, (Memmert, Germany)
5. Shaker water bath (EYELA, Model SB-651, Tokyo, Japan)
6. Automatic autoclave (ALP Co., LtD, Tokyo, Japan)
7. Refrigerator (Whirlpool)
8. Scanning electron microscopy, SEM, JEOL, JSM 6310F, Japan
9. X-ray diffractometer, JEOL, JDX 3530, Japan
10. Differential scanning calorimetry, Pyris IDSC, Perkin Elmer, U.S.A.
11. Spectro Colorimeter (Model JS 555, Tokyo, Japan)
12. GBX-1 water activity system
13. Texture analyzer , Stable Micro System, TA.XT2i/25, England)
14. Visco Basic Plus (PoLyvisco, Kinmatica)
15. Vacuum filter
16. Glassware for analysis
17. Kitchen Aid Mixers (U.S.A)
18. Kitchen electric oven
19. Kitchenware for processing
20. Tray dryer
21. Cyclotec 1093 sample mill (Tecator, Swenen)
22. Baking equipments

Methods

1. Production of resistant starch type III from high amylose rice starch by enzymatically debranching process

1.1 Physiochemical properties of native high amylose rice starch

1.1.1 Proximate Analysis

Moistures content, protein, carbohydrate, fat, ash and fiber content (%) of high amylose rice starch (HARS) and RS III were determined followed AOAC (2002) methodology. The procedures are shown in Appendix A.

1.1.2 Total Starch Content

Total starch content (glucose × 0.9) was estimated in 2 N KOH pretreated samples by an enzymatic method of AOAC (2002). The procedures are shown in Appendix A.

1.1.3 Amylose content

Amylose content (%) was determined calorimetrically after iodine binding (Juliano, 1971), using potato amylose as standard. The procedures are shown in Appendix A.

1.1.4 Scanning electron microscopy (SEM)

The native high amylose rice starch was dried to less than 4% moisture content and then deposited on a copper disc and coated with gold. The specimens were examined by mean of the scanning electron microscope (JM-560LV model). The sample was imaged at 2,000x and 6,000x magnification (Appendix B).

1.2 Effect of preheated treatments on physiochemical properties of resistant starch type III formation

1.2.1 Resistant starch type III formation

An aqueous high amylose rice starch (HARS; 15% w/w) were prepared by fully dispersing the sample in distilled water. The slurry samples were annealed at room temperature (30°C) for 1 hr with occasionally vigorous shaking. The annealed samples were preheated at different temperatures (75, 95 and 121°C for 30-min) and cooled to 55°C. The starch solution samples were debranched using 8 unit pullulanase enzymes per gram starch at 55°C for 0, 2, 4, 8, 16, 24 and 48-hr in shaker water bath (continuous shaking at 170 rpm). The retrogradation of debranched starches were then induced at 4°C for 16-hr. Afterwards, the one cycle of freeze-thaw process (-10/30°C) was applied to promote syneresis of the retrograded starches. The recrystallization starch was dried at 45°C to approximate 13 % moisture content and ground passed through a 100-mesh sieve.

1.2.2 Degree of pullulanase hydrolysis

Reducing sugar (Rds) and total sugar (Ts) in the samples, debranched for specific times, were analyzed according to the Park- Johnson method (Hizukuri, 1995) and the phenol-sulfuric acid reagent method (Dubois *et al.* 1956), respectively. The procedures are shown in Appendix A. The hydrolysis treatment was conducted in triplicate. The extent of debranching of HARS, using pullulanase enzyme, was evaluated in terms of degree of hydrolysis (D.H.). Degree of pullulanase hydrolysis was calculated by the equation:

$$\text{D.H. (\%)} = \left(\frac{\text{(\% of Rds after hydrolysis- Rds blank)}}{\text{(\% of Ts after hydrolysis - Ts blank)}} \right) \times 100$$

1.2.3 Degree of syneresis

Degree of syneresis in the samples, debranched for specific times, was determined according to Karim *et al.* (2000) with modification. Portions of each debranched starch paste were transferred into each disposable dish and covered with adhesive tape to prevent moisture loss. The samples were frozen at -10°C for 16-hr and thawed at 30°C for 2-hr. The samples were then subjected to a vacuum filtration after freeze-thaw cycles. Water of syneresis from triplicate gel samples was collected and weighed. The weight of retrograded gel and water syneresis was used to calculated degree of syneresis as follows:

$$\text{Degree of syneresis (\%)} = \left(\frac{\text{Weight of water syneresis}}{\text{Weight of retrogradation gel}} \right) \times 100$$

1.2.4 Color value and water activity

The Hunter *L**, *a**, *b** values of RS III samples were measured using a Colorimeter (JS 555, Japan). Water activity (a_w) was determined by Aqualab Water Activity Meter (Series 3) at 25^{o}C (Pullman, USA).

1.2.5 Production yield

The weight of native rice starch and RS III samples were used to calculated production yield of resistant starch type III formation. Production yield (%) of the RS III was determined by using the formula:

$$\text{Production yield (\%)} = \left(\frac{\text{Wt. of resistant starch}}{\text{Wt. of rice starch}} \right) \times 100$$

1.2.6 Microscopic images

Microscopic images of the selected RS III samples were examined using a SEM with magnifications of 2,500X and 6,000X. The procedures are shown in Appendix B (Atichokudomchai *et al*. 2000).

1.2.7 X-ray diffraction

X-ray diffraction patterns of native rice starch and the selected RS III samples were measured with copper K_2 radiation (λ =0.154 nm) using a diffractometer (JEOL,JDX-3530, Japan). The diffractometer was operated at 300 mA and 30 kV, 2θ range from 10 to 70.0^o with a step size 0.05^o and a count time of 2s. The data was analyzed with program MDI Jade 6.5 (Japan). The crystallinity of RS III samples were calculated as the proportion of crystalline area to total area at angles between 10 and 45 ^{o}2θ (Cairns *et al*. 1997). The procedures are shown in Appendix B.

1.2.8 Resistant starch, digestible starch and total starch analysis

Resistant starch (RS), digestible starch (DS) and total starch (TS) content in the RS III samples were determined by using a Megazyme Resistant Starch kit (AOAC Method 2002.02). Briefly, samples (100 mg) were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 hr at 37^{o}C to reduce digestible starch to glucose. The reaction was terminated with 4 ml ethanol and the RS pellet was recovered by centrifugation (5000 g, 10 min). The supernatant was decanted and washed with 50% ethanol for two times (digested starch). The pellet was solubillized in 2ml of 2 M KOH in an ice bath, neutralized with 8 ml sodium acetate (1.2 M) and the resistant starch hydrolyzed to glucose with of AMG (0.1 ml, 3300 Uml^{-1}, 50^{o}C). The glucose oxidase/peroxidase reaction was used to measure glucose released from digested starch and resistant starch. Absorbance was read (510 nm) after a 20 minute incubation period at 50^{o}C. The procedures of resistant starch, digested starch and total starch determination were shown in Appendix A.

1.2.9 Starch hydrolysis rate and estimated glycemic index

In vitro starch hydrolysis and glycemic index were determined according to Goňī, *et al.* (1997). RS III samples (50 mg) were incubated with 1 mg pepsin in 10 ml HCl-KCL buffer (pH 1.5) at 40 ^{o}C for 60 min in a shaking water bath. The digest was diluted to 25 ml by adding Tris maleate buffer (pH 6.9), and then 5 ml of α-amylase solution, containing 2.6 IU of α-amylase in Tris maleate buffer, were added. The samples were incubated at 37 ^{o}C in a shaking water bath. 0.1 ml sample was taken from each flask every 30 min from 0 to 3-hr and boiled for 15 min to inactivate the enzyme. Sodium acetate buffer (1 ml 0.4 M, pH 4.75) was added and the residual starch digested to glucose by adding 30 µl amyloglucosidase and incubating at 60 ^{o}C for 45 min. Glucose concentration was determined by using a glucose oxidase-peroxidase kit. The rate of starch digestion was expressed as the percentage of starch hydrolyzed at different times.

An equation: $C=C_{\infty}(1\text{-}e^{-kt})$ was used to described the kinetics of starch hydrolysis, where C, C_{∞} and k were the concentration at time t, the equilibrium concentration and the kinetic constant, respectively. The area under the hydrolysis curve (AUC) was calculated using the equation:

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

Where, C_{∞} corresponds to the concentration at equilibrium (t_{180}). t_f is the final time (180 min), t_0 is the initial time (0 min) and k is the kinetic constant.

A hydrolysis index (HI) was calculated by comparison with the AUC of a reference food (white bread). By using the *in vitro* starch HI values, the GI was estimated by the following equation established by Goňī, *et al*. (1997):

$$GI = 39.71 + (0.549 \times HI).$$

1.3 Effect of pullulanase enzyme concentration on physicochemical properties of RS III formation

A pullulanase hydrolysis condition for RS III formation was chosen by means of the previous experiment. A concentration of 0, 8, 10, 12, 14 and 16 units / game starch and a hydrolysis period of 16 hr at 55^{o}C were employed for investigation of pullulanase enzyme concentration effects on RS III propertes. The RS III was produced as described in section 1.2.1. Three replicates of all experiments will be performed. Degree of pullulanase hydrolysis and degree of syneresis were examined as described in section 1.2 .2 and 1.2.3.

1.3.1 β-amylolysis (%)

The β-amylolysis (%) of native high amylose rice starch and RS III prepared from the treatments of debranched high amylose rice starch at different degree of hydrolysis was evaluated in order to investigate degree of debranching. The analysis procedure was slightly modified from that of Hood and Mercier (1978). Native high amylose rice starch and debranching treatments with different enzyme concentration (4 mg) dissolved in 1 ml of dimethylsulfoxide by heating in boiling bath for 15 min and then cooling and diluted to 3 ml with distilled water. The solution (0.45 ml) was taken and mixed with an acetate buffer solution pH 6.0 (50 μl; 0.1 M). β-amylase enzyme (1 μl; 20 units /ml) was added and mixed well. The solution was incubated at 30^{o}C for 12-hr and boiled for 5 min for deactivated enzyme. The reducing sugar (Rds) and total sugar (Ts) of the β-amylolysis product were measured according to the Park- Johnson method (Hizukuri, 1995) and the phenol-sulfuric acid reagent method (Dubois *et al.* 1956), respectively. β-amylolysis (%) was calculated:

$$\left(\frac{\text{Rds of sample after hydrolysis by β-amylase} - \text{Rds of β-amylase blank}}{\text{Ts of sample after hydrolysis by β-amylase} - \text{Ts of β-amylase blank}} \right) \times 100$$

1.3.3 Resistant starch content, starch hydrolysis and glycemic index

Resistant starch content, starch hydrolysis and glycemic index were examined as described in section 1.2.8 and 1.2.9.

1.3.4 Production yield, color, water activity and moisture content

Production yield, color value, water activity and moisture content were examined as described in section 1.2.4 and 1.2.5.

1.4 Scaled up production of RS III production from HARS

The scaled up (150 g of rice starch in 850 g distilled water) for RS III formation was chosen by means of the previous experiment (1.3). The RS III was produced as described in the above experiment 1.2.1. Comparative study on degree of hydrolysis, viscosity of the debranched product, degree of syneresis, resistant starch content, water activity and production yield between small and larger-scale production of RS III with three replicates of all experiments were performed. Degree of pullulanase hydrolysis and degree of syneresis were examined as described in section 1.2.2 and 1.2.3.

1.4.1 Viscosity

The viscosity of solutions was measured at room temperature (30°C). Before measurement, samples were mixed for 30s before removal of a 1 ml aliquot for degree of hydrolysis determination and the remained sample was measured a viscosity. The debranched solutions were placed in 250-mL glass beakers with a 10-cm diameter. Viscosity was measured using a Visco Basic Plus (PoLyvisco, Kinmatica) with a No. L 3 spindle at a speed of 100 rpm. Viscosity was measured in cP. Viscosity values were assayed in triplicate.

1.4.2 Production yield, color value, water activity and moisture content

Production yield, color value, water activity and moisture content were examined as described in the previous experiment 1.2.4) and 1.2.5).

1.4.3 Resistant starch content, starch hydrolysis and glycemic index

Resistant starch content, starch hydrolysis and glycemic index were examined as described in the above experiment 1.2.8) and 1.2.9).

2. An application of RS III and HFCS as low glycemic butter cake product

2.1 Preparation of butter cake with HFCS as replacement for sucrose.

High fructose corn syrup (HFCS) (55% fructose) was used to replace 0, 20, 40, 60, 80 or 100% of the sugar in butter cakes in order to reduced glycemic index and improve the moistness of the cake. The sugar replacement was done in a combination of the foam part of the cake formula. Butter cake formulation system (100% component) was set at 33.01% cake flour, 1.34% corn flour, 0.21% baking powder, 13.40% eggs, 26.80% sugar, 0.10% salt, and 25.13% butter (Pongjanta *et al*. 2002). First, the butter was beaten on level 6 (medium speed) with the paddle attachment in a Kitchen Aid® stand mixer for 5 min and than combined with sifted cake flour, corn flour, salt, and baking powder. Second, the whole eggs were whipped until they formed soft peaks. The sucrose and fructose were added in the whole egg bowl and whipped to form firm, moist peaks. The whipped whole eggs were determined the specific gravity of each cake foam. Next, the batter were poured into the foam part and well mixed with an eggbeater until all of the foam parts were incorporated into the batter. The batter was then placed into a greased 9 × 5″ loaf pan and baked for 40-50 minutes in a 350°F Hotpoint electric oven. The cakes were allowed to cool for 3 hr and were packed in low-density polyethylene packages at room temperature 3-8 hr prior to physiochemical quality analysis and sensory evaluation.

2.1.1 Cake sample preparation

Cake quality attributes were evaluated after cooling during 3 hr at 25 ± 2°C. For crust and crumb attribute determinations, whole cakes were halved along the height. The upper half cake was used for crust evaluation and the lower one for crumb cake evaluation. Six cakes from the same batter were analyzed for physical properties, and the other six, for sensory evaluation, by placing them on coded white plastic plates, covered with plastic wrap to prevent drying. Sensory evaluation took place during 2 hr after removing from refrigerator.

2.1.2 Physicochemical quality of the low glycemic butter cake

1) Specific gravity of the cake batter

Specific gravity was determined by dividing the weight of a material by the weight of an equal volume of water (Penfield and Campbell, 1990). First, the weight of an empty container, such as a crystallizing dish or a graduated beaker with the capacity to hold 50 ml, was determined using a Sartorius portable top load balance (Type PT 1200-OUR, Bohemia, NY). Second, 50 ml of distilled water was added to the container and weighed. Third, the weight of the cake batter was measured, after ensuring that no air pockets remained. This was accomplished by pouring approximately 25 ml of cake batter into the container and tapping the container 12 times. Excess batter was then poured until it reached the 50 ml mark, and tapped again 12 times. Any excess batter outside of the container was wiped off. Finally, 50 ml of cake batter, with air pockets removed, was weighed. Specific gravity was calculated as follows:

$$\text{Specific Gravity} = \frac{\text{(Weight of filled container – weight of container)}}{\text{(Weight of water-filled container – weight of container)}}$$

2) Cake volume

Cake volume and color were measured after cooling for 1 hr at room temperature. Volumes of cakes were measured using rapeseed displacement and specific volumes were calculated (Lee *et al*. (1985). The empty cake pan was filled with rapeseed. The empty cake pan volume (V1) was calculated based on the volume of rapeseed as determined by a graduated cylinder. After baking, the rest of the volume of the cake pan (V2) was filled with rapeseed, and the volume of rapeseed was determined by graduated cylinder. The cake volume is V1 – V2.

3) Cake color

Color was measured using a Minolta spectrophotometer CN-508i (Minolta, Co. LTD, Japan). Tristimulus values were automatically calculated from the spectrum by means of a computer programmed. Results were expressed in the CIE $L^*a^*b^*$ color space and were obtained using the D65 standard illuminant, and the 2° standard observer (CIE 1931). Color determinations were made 9 × 5 times in each cake: Crumb or crust cake color was checked at nine different points on each cake and every point was measured five times. The nine points were positioned in the centre of the cake and in the centre of eight imaginary sectors in which it was divided along the diameter.

4) Texture Analyses

Crumb texture was determined by a TA-XT2 texture analyzer (Stable Microsystems, Surrey, UK) provided with the software *"Texture Expert"*. An Aluminium 25 mm diameter cylindrical probe was used in a *"Texture Profile Analysis"* (TPA) double compression test to penetrate to 50% depth, at 1 mm/s speed test, with a 30 s delay between first and second compression hardness (kg), gumminess (g_f), chewiness (g_f), adhesiveness (g_f·s), cohesiveness, springiness and resilience were calculated from the TPA graphic (Appendix Figure 7). Both, springiness and resilience, give information about the after stress recovery capacity.

Nevertheless, the former refers to retarded recovery (after the delay between compressions), the latter concerns instantaneous recovery (immediately after the first compression, while the probe goes up). Texture determinations were carried out, after removing the crust, in (40×40×20) mm-sized samples.

2.1.4 Chemical properties

1) Proximate analysis

Moisture, protein, fat and ash content (%) of the butter cake samples were determined followed AOAC (2002) methodology (Appendix A)

2) *In vitro* starch digestibility

The effect of the replace 0, 20, 40, 60, 80 and 100% of the sugar in butter cakes on *in vitro* starch digestibility was determined as described in above experiment 1.2.(1). Butter cake samples were defatted before enzymatic starch digestion using petroleum ether (25 ml/g x 3, 15 min, ambient temperature) determination. Three replicates of all experiments were performed.

2.1.5 *In vitro* starch hydrolysis index and glycemic index

In vitro starch hydrolysis index, glycemic index of the replace 0, 20, 40, 60, 80 or 100% of the sugar in butter cakes were determined as described in the above experiment 1.2.3. The butter cake samples were defatted before enzymatic starch digestion using petroleum ether (25 ml/g x 3, 15 min, ambient temperature). Three replicates of all experiments were performed.

2.1.6 Sensory evaluation

Hedonic sensory tests were conducted by 30 untrained panelists consisting of Department of Home Economic Faculty of Agriculture,

Kasetsart University staff and students. Cakes were evaluated based on acceptability of their appearance, odor, flavors, texture and overall preference by a hedonic 9-point scale where 9 means most liked and 1 most disliked (Lawless and Heymann, 1998). The control cake was presented simultaneously with the rest of samples and was evaluated in random among panelists. The scoring sheet is shown in Appendix C.

2.2 An application of RS III as a low glycemic butter cake

2.2.1 Butter cake production

Enzyme-resistant starch type III (RS III) was chosen by means of the previous experiment (1.4), the low glycemic butter cake was reformulated. The RS III was added to the cake formulations at levels of 0, 5, 10, 15 and 20% based on cake flour. The butter cake formula was selected from the 2.1 experiment. Sifted flour, baking powder, and RS III were mixed well in butter cream. The butter cake were process following step as described above experiment. At the final mixing stage, the specific gravity of the batter was measured. The batter was then placed into a greased 9 × 5 loaf pan and baked for 40-50 minutes in a 350°F Hotpoint electric oven. The cakes were allowed to cool for 3 hr and were packed in low-density polyethylene packages at room temperature 3-8 hr prior to physiochemical quality analysis and sensory evaluation.

2.2.2 Physiochemical analysis and *in vitro* starch hydrolysis index

Physiochemical analyses were examined as described in the above experiment 2.1.3. The effect of the RS III replace 0, 5, 10, 15 and 20 % of cake flour in butter cake on *in vitro* starch digestibility was determined as described in above experiment 2.1.4 (b) Three replicates of all experiments were performed. *In vitro* starch hydrolysis index and estimate glycemic index were examined as described in the above experiment 2.1.5. The effect of the RS III replace 0, 5, 10, 15 and 20 % of cake flour in butter cake on sensory evaluation were examined as described in the above experiment 2.1.6.

3. Experimental Design and Statistical Analysis

3.1 Resistant starch type III production

Completely Randomized Design (CRD) was used to evaluate the means of physiochemical analysis of debranched products and RS III properties. The data obtained for the degree of pullulanase hydrolysis, degree of syneresis, physicochemical properties, *in vitro* starch digestibility, *in vitro* starch hydrolysis index and glycemic index were subjected to analysis of variance (ANOVA). Duncan's Multiple Range Tests (DMRT) procedure was used to make specific comparison between treatments (Lawless and Heymann, 1998).

Pearson correlations between % crystallinity and resistant starch content of RS III were investigated. The SPSS for Windows program, version 10.0, was employed for analyzing the results obtained from three replications.

3.2 Utilization of RS III in low glycemic butter cake

Completely Randomized Design (CRD) was used to evaluate the means of physiochemical analysis of butter cake samples. The data obtained for the specific gravity, volume, color, texture profile analysis, *in vitro* starch digestibility, *in vitro* starch hydrolysis index and glycemic index were subjected to analysis of variance (ANOVA). Duncan's Multiple Range Tests (DMRT) procedure was used to make specific comparison between treatments (Lawless and Heymann, 1998).

Completely Randomized Block Design (CRBD) for sensory scores of butter cake samples. Mean with standard deviation for each treatments were calculated and the analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) was used for comparing differences of the mean values at the 0.05 confidence level (Lawless and Heymann, 1998).

RESULTS AND DISCUSSION

In this study, the experiments were set in the laboratory and were divided into 2 parts, which consisted of the production of resistant starch type III from high amylose rice starch, by enzymatically debranching process and the second part is an application of the resistant starch type III in low glycemic butter cake.

1. Production of resistant starch type III from high amylose rice starch by enzymatically debranching process

1.3 Physiochemical of the native high amylose rice starch

1.1.1 Chemical composition

The commercial high amylose rice starch donated by the Cho Heng Rice Vermicelli Factory Co., Ltd. was chemical and physically characterized before use as control sample and for resistant starch type III formation by enzymatically debranching process. Chemical compositions of the commercial high amylose rice starch are shown in Table 13.

Moisture, fat, ash, protein and total carbohydrate content of commercial high amylose rice starch were 13.01 ±1.05%, 0.91 ± 0.28%, 0.82 ± 0.34%, 1.18 ± 0.06%, and 83.78 ± 0.52%, respectively. Total starch content of the rice starch as determined by an enzymatic method showed the average at 96.21± 1.2% of the sample weight on dry basis. The rice starch consists mainly of amylose and amylopectin which was 32.10 ± 0.67% and 67.93 ± 0.57%, respectively. Similar values of the amylose content in the commercial high amylose rice starch have been reported earlier (Piyarat, 2003).

The commercial high amylose rice starch had β-amylolysis limits of 62.48 ± 2.49 % released after 12 hr β-amylase hydrolysis (Table 13). Rice starch is composed of two polymer forms of glucose: amylose and amylopectin. Amylose is an

essentially linear polymer of α-(1,4)-linked D-glucopyranosyl units with slightly branched of α- (1,6)-linked (Ghampagne, 1996). Rice starch amylose have DP_n value of 920 – 1110, CL of 250-370 and β-amylolysis limits of 73 -84% (Takeda *et al*. 1986). Rice starch amylopectin consists of α-(1,4)-linked D-glucosyl chains and highly branched with 5 - 6% of α-(1,6)-bonds (Buleon *et al*. 1998). It has a DP_n of 8,200-12,800, CL values of 19 -23 and β-amylolysis limits of 49 - 59% (Takeda *et al*. 1986).

Table 13 Chemical composition and fine structural of the native high amylose rice starch

Chemical composition	**Native high amylose rice starch (% wwb)[a]**
Moisture content	13.01 ± 1.05
Fat	0.91 ± 0.28
Ash	0.82 ± 0.34
Protein	1.18 ± 0.06
Total carbohydrate	83.78 ± 0.52
Total starch (dwb)	95.21 ± 1.20
Amylose content	32.10 ± 0.67
Amylopectin	67.93 ± 0.57
β-amylolysis limits	62.48 ± 2.49
pH	5.50 ± 0.65

[a] Values are means of triplicate measurements ± standard deviations.

1.1.2 Granular structure

Scanning electron micrograph (magnification at 2500x and 6000x) of native commercial high amylose rice starch granules are shown in Figure 13. Native commercial high amylose rice starch granules were observed to be

polyhedral and irregular in shapes. The granular size was small with diameter between 3-6 μm and form parts of compound granules. The surface of the granules was smooth without observable pores or fissure. Piyarat (2003) reported that the granule size mean of Thai rice starches (Supanburee 1, Jasmine rice and commercial rice starch) showed a common size of rice starch of 4 - 6 μm.

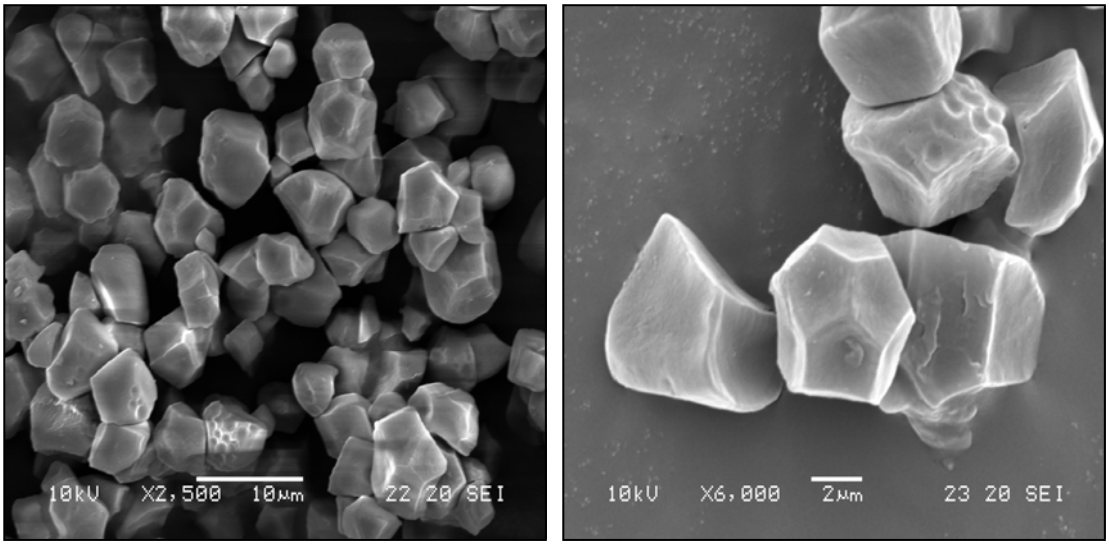


Figure 13 Scanning electron micrographs (magnification at 2500x and 6000x) of native commercial high amylose rice starch

1.2 Effect of preheated treatments on RS III formation

1.2.1 Degree of pullulanase hydrolysis

The reducing sugar content was used to determine the amount of branch chains released by action of pullulanase hydrolysis on the 15% high amylose rice starch preheated at different temperatures (75°C, 95°C and 121°C) shown in Figure 14 and Appendix Table E1. The degree of hydrolysis of the samples increased with increasing incubation time and preheated temperature. The degrees of pullulanase hydrolysis in high amylose rice starch slurry preheated at 121°C were significantly higher than the preheated at 75°C and 95°C. The degree of pullulanase

hydrolysis of the rice starch preheated at 75°C, 95°C and 121°C for 0 to 48-hr were 0.14 to 1.55% and 0.14 to 0.50%, and 0.17 to 3.10%, respectively.

As the reaction time of hydrolysis increased, the degree of hydrolysis in the 121°C and 75°C preheated increased sharply (0.17 to 3.10%, 0.14 to 1.55%) after 2 and 8-hr hydrolysis time, respectively. While, the rice starch preheated at 95°C was remain stable after 8-hr incubation. Because of the 95°C preheated rice starch slurry was sticky and less free water content that lead to incomplete gelatinization of starch granules. These results indicated that pullulanase enzyme was less activity to hydrolyze the limited free water in the starch solution and native rice starch. For rice starch to be gelatinized and provide an accessible substrate for pullulanase hydrolysis the gelatinization process must not be restricted. If water is limiting the amount of gelatinized starch is restricted together with the capacity of enzyme to hydrolyze the starch (Tester *et al*. 2004).

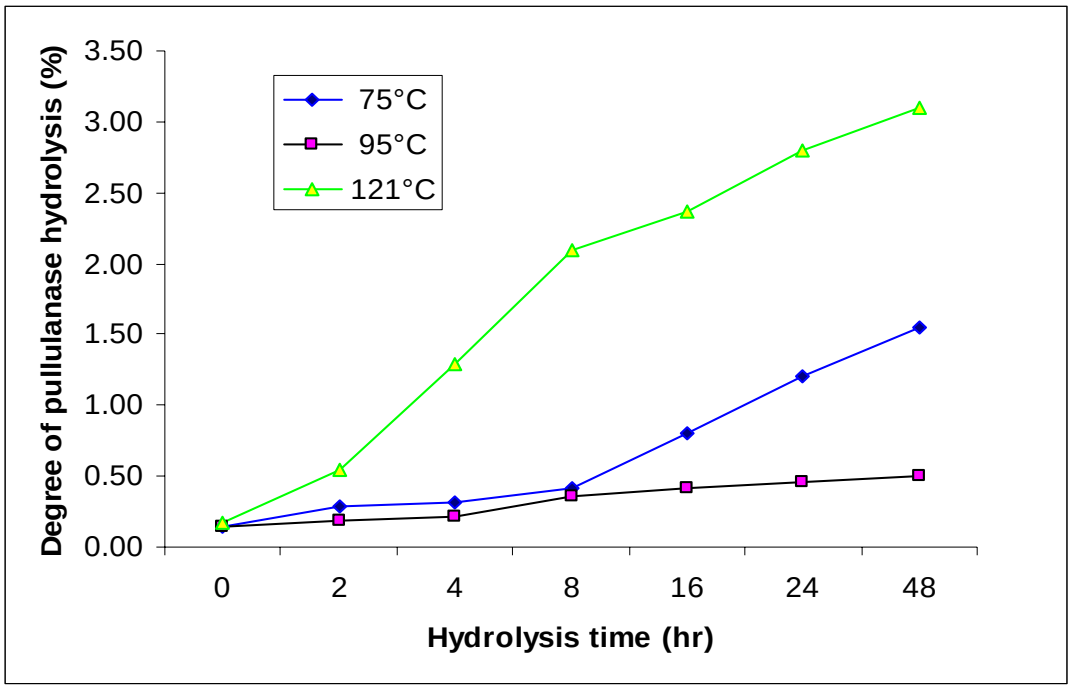


Figure 14 Degree of pullulanase hydrolysis of 0 to 48 hr debranched high amylose rice starch preheated at 75°C , 95°C and 121°C for 30 min

In addition, the 121^{o}C preheated 15% rice starch slurry for 30 min was sufficient to breaking of hydrogen bonds within starch molecules that opens the granules to hydration and enzymatic hydrolysis. In the case of the 75^{o}C preheated 15% rice starch slurry for 30 min was sufficient to swell the native rice starch granules without disrupting the granules. This permits the enzyme to access to the molecule and uniformly debranch the amylopectin molecules as can see clearly in the morphological reveal in the next section. Haralampu *et al*., (1998) reported that the temperatures considered appropriate for swell the starch granule without disrupting starch granule are from about 75^{o}C to 90^{o}C and the time required to sufficiently swelling the starch granules was less than 1 hour. The morphological of 15% high amylose rich starch granules changed after preheated at 75^{o}C and pullulanase hydrolysis for 16-hr could be clearly seen in the images shown in Figure 15.

1.2.3 Morphological image of rice starch preheated-debranched

Scanning electron microscopy (magnification at 2,500x and 6,000x) of the 15% HARS preheated at 75°C, 95^{o}C and 121°C for 30 min are shown in Figure 17. There was a marked difference in shape between the 75^{o}C, 95^{o}C and 121°C preheated HARS samples.

The 75°C preheated of 15% annealed rice starch solution (a) exhibits swollen and partial gelatinization at the surface of the starch granules. This permits the enzyme to access to the molecule more easily and uniformly debranched the amylopectin molecules. While, the 95^{o}C preheated HARS show a dried surface granule without observable pores or fissure. Additionally, at 121°C preheated rice starch shown surface erosion and slightly damaged starch granules. This suggests that a considerable degree of the granular crystalline structure has been destroyed or disrupted during gelatinization at highest temperature.

The difference preheated samples showed that the granular structure swelling and surface collapsed “doughnut-shaped” morphology.

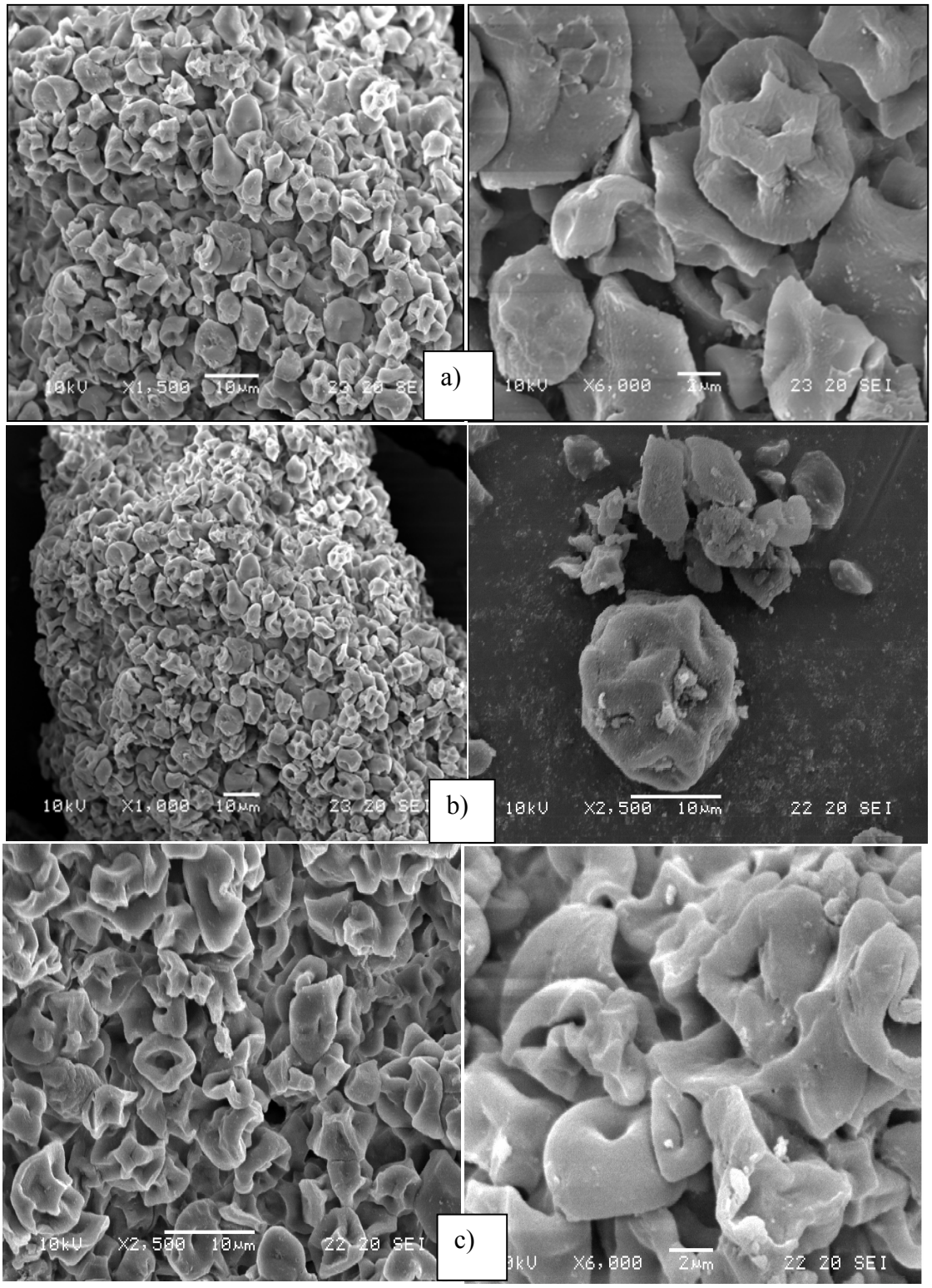


Figure 15 Scanning electron micrographs (magnification at 2,500x and 6,000x) of 15% rice starch solution preheated at 75°C(a), 95°C (b)and 121°C (c) for 30 min.

1.2.4 Retrogradation gel images

Retrogradation gel images of 0 - 48 hr debranched high amylose rice starch preheated at 75, 95, and 121 ^{o}C for 30 min are shown in Figure 16. Briefly, all debranched starch solutions were heated in boiling bath for 30 min to stop any further enzymatic activity and to unwind any double helices, melt the crystallites and retrograde starch which might have formed during the initial heating and incubation at 55^{o}C during 4 to 48 hr debranching. Visible precipitates formed immediately after removing and cooling the debranched solution from the boiling. As the cooling progressed (at 4^{o}C for 16 hr), increases in turbidity and precipitate formation were noticed. The 16 to 48 hr debranched of rice starch preheated at 75^{o}C, 95^{o}C and 121^{o}C had higher turbidity gel than 0 to 4 hr debranched samples that moderate clarity and harder gelation than 8 hr debranched samples.

The turbidity and clarity differences between the studied starches may affected by the degree of retrogradation, granule swelling and the association between amylose and amylopectin (Swinkels, 1985). Very slowly retrogradation gel was visible observed for the control sample (non enzyme) cooling for first hour in an ice bath at 4^{o}C. In contrast, the 16 to 48-hr debranched of rice starch gel retrograded much more rapidly during the first hour of cooling in the same condition.

The major factors influencing rice starch retrogradation would be starch content, ratio of amylose to amylopectin, and the branch chain length of amylopectin. Although both amylose and amylopectin components are involved in the process of starch retrogradation, the linear fraction, amylose, favors recrystallization in a rapid mode (Slade and Levine, 1988a).

When starch is gelatinized under high moisture content and allowed to cool, alignment of order amylose molecules and debranched amylopectin with each other leads to the formation of a rigid gel. A rearrangement between starches occurs and a three-dimensional network is rapidly constituted. The higher the

amylose content of starch had a harder the starch gel. The starch gel structure has lower α- amylase susceptibility than a paste. Resistant starch is produced as the insoluble crystallite formed by the process of controlled retrogradation (Englist *et al.* 1992). The crystalline structure of retrograded amylose is acid and heat- resistant. Its melting-point is above 120°C. *In vitro*, the retrograded starch and retrograded amylose fraction are highly resistant to α- amylase (Tetens *et al*. 1997).

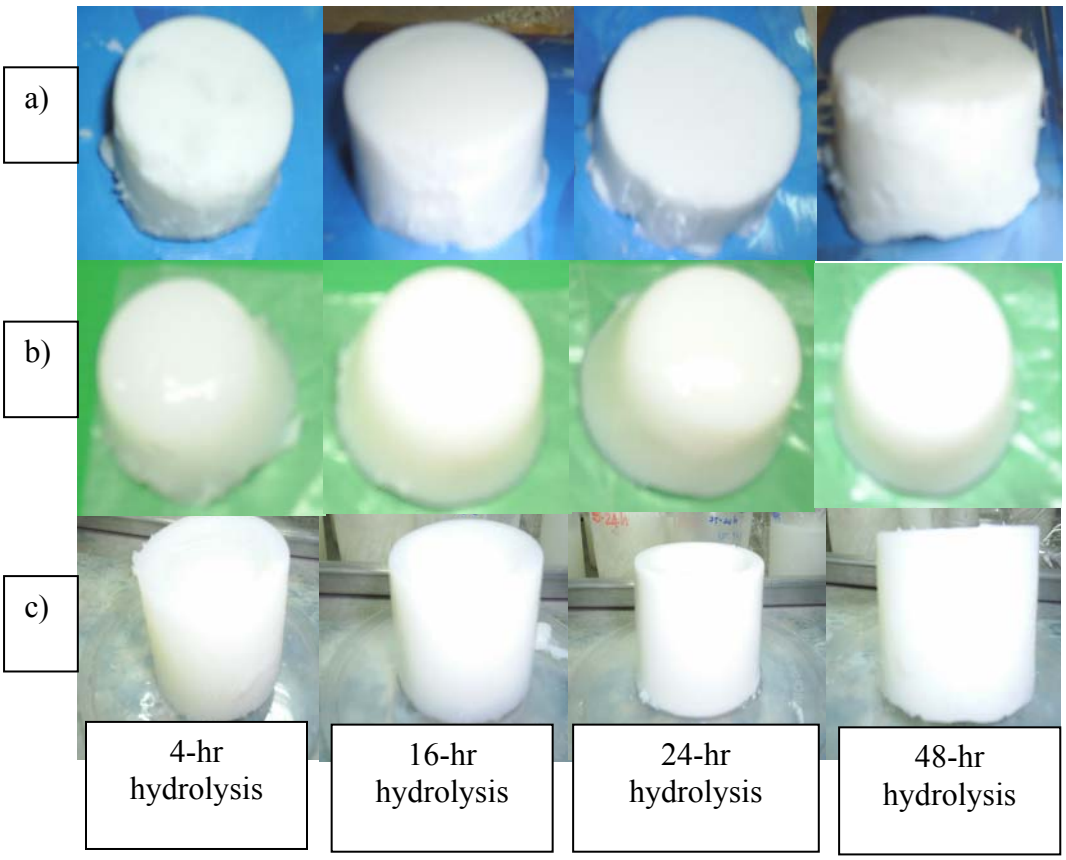


Figure 16 The typical appearance of retrogradation gel from 4, 16, 24 and 48-hr debranched HARS preheated at 75°C (a), 95°C (b) and 121°C (c) for 30 min

1.2.5 Degree of syneresis

The results obtained in this study suggested that the high degree of pullulanase hydrolysis was closely related with high degree of syneresis of the RS III production. It was obvious that by an enzyme hydrolysis with a pullulanase enzyme improved the degree of syneresis as shown in Figure 17 and Appendix Table E2. Degree of syneresis was improved from 30.02 to 51.45, 28.50 to 45.27 and 33.22 to 55.87 % for 0 to 48-hr debranched rice starches preheated at 75, 95 and 121°C for 30 min, respectively. This could be due to the pullulanase enzyme hydrolyze α-1,6-glucosidic bonds, releasing a linear polymers linked by α-1,4-glucosidic bonds. These fragments are linear polymers about 10 to 65 anhydroglucose unit, which were formed by progressive re-association of starch molecules aged during short-term incubation.

Additional, the freeze-thaw cycle was used to promote syneresis of retrograde starch. Yuan and Thompson, (1998) revealed that the rate and extent of retrogradation of starch paste during the freeze-thaw process was increased by temperature reduction (greater nucleation rates). As the starch paste is cooled, the starch chains become less energetic and the hydrogen bones become stronger, giving a firmer gel. As a gel ages or if it is frozen and thawed, the starch chains have a tendency to interact strongly with each other and thereby force water out of the system (Figure 18).

The squeezing of water out of the gel was called “syneresis”. Longer storage gives rise to more interaction between the starch chain and eventually to formation of crystals. This process, called “retrogradation” is the crystallization of starch chains in the gel. Because the crystalline areas differ from the non crystalline areas in their refractive index, the gel becomes more rigid or rubbery, perhaps partially as a result of crystallization and partially just from the interaction of the starch chains (Hoseney,1998).

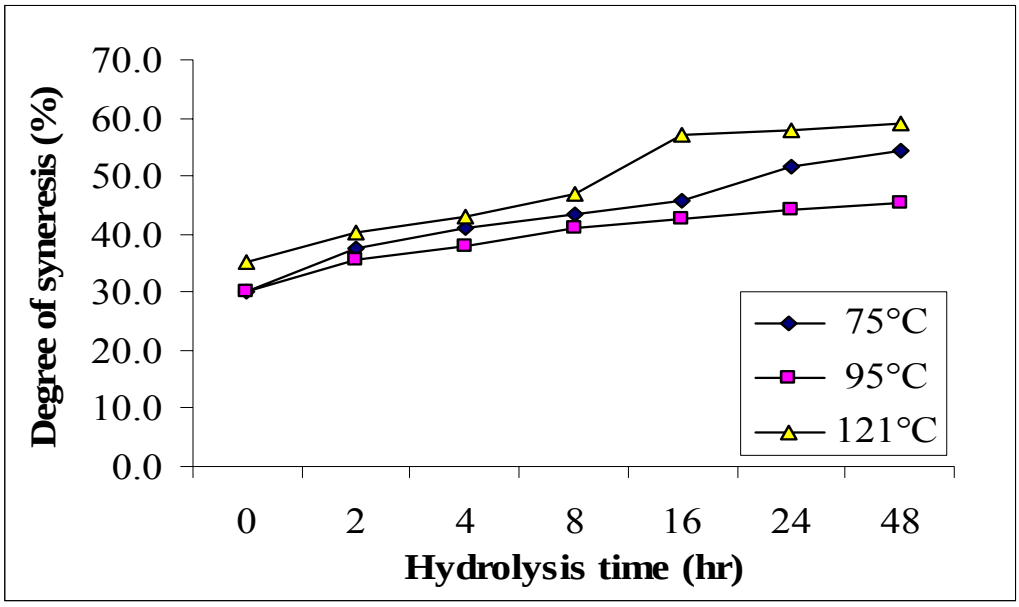


Figure 17 Degree of syneresis of 0 to 48 hr debranched HARS preheated at 75°C, 95°C and 121°C for 30 min

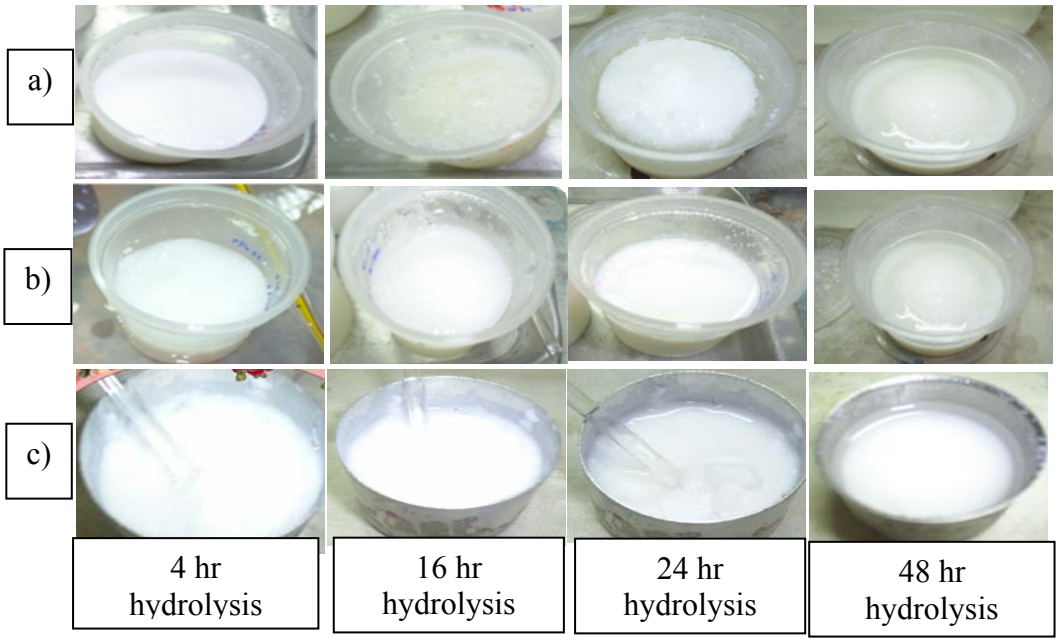


Figure 18 The typical appearance of syneresis gel from 4, 16, 24 and 48 hr debranched HARS preheated at 75°C (a), 95°C (b) and 121°C (c) for 30 min

1.2.6 SEM micrograph and photograph of RS III sample

Scanning electron micrograph (magnification at 6,000 x) and the typical appearance of RS III presented in Figure 19. Morphological changes that occur in rice starch from native to retrograded states were studied by electron microscopy. When retrogradation was achieved, bigger, irregularly shaped particles with a white spongy-like porous network and honeycomb arrangement were observed. This pocked mark or honeycomb arrangement may be due to gelation of the starch dispersion during freezing. This continuous network structure composed of amylose and amylopectin was affected by re-crystallized of the debranched high amylose rice starch to form resistant starch type III. In addition, the fleshy and dried retrogradation of the debranched showed a white color that was small lumps.

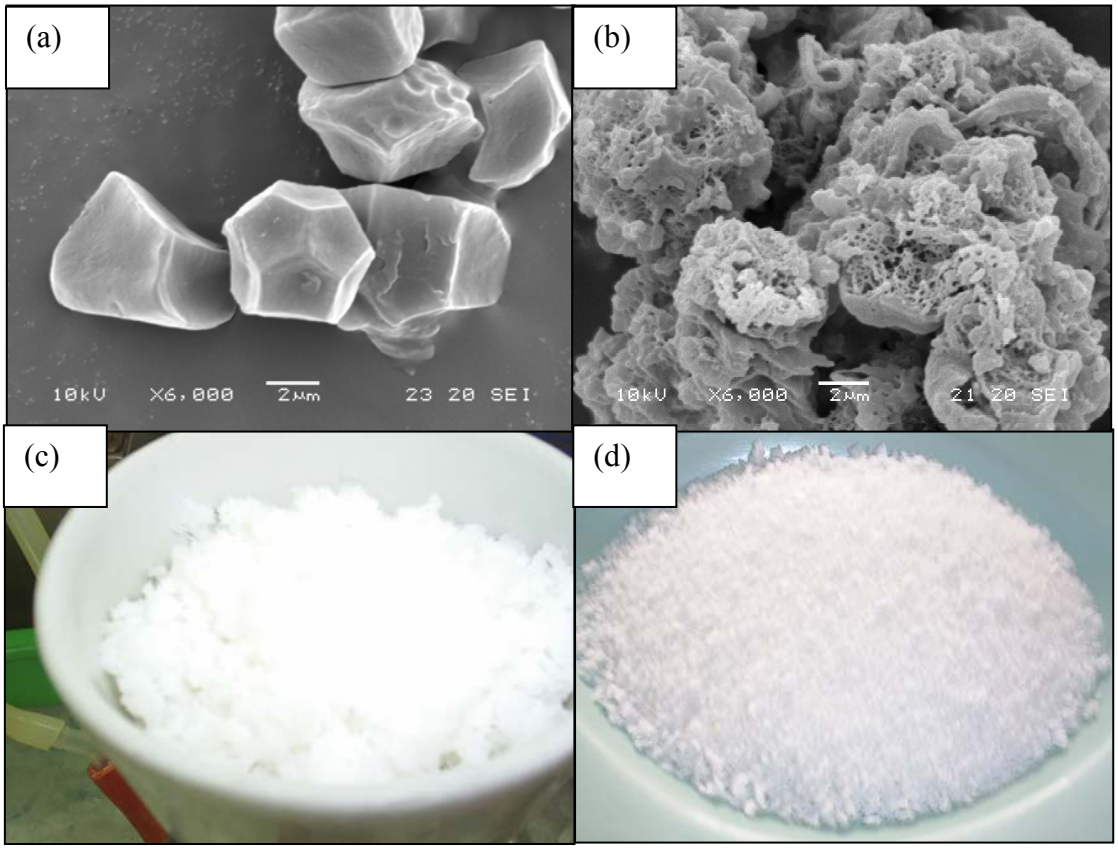


Figure 19 Scanning electron micrograph of native (a), retrogradation rice starch (b) and typical appearance of fresh (c) and dried (d) retrograded from 16-hr debranching and retrogradation of high amylose rice starch

1.2.7 Physicochemical properties of RS III samples

Table 14 shown the physicochemical properties of RS III from pullulanase debranched (0 to 48 hr) of 15% rice starch slurry preheated at 75°C, 95°C and 121°C for 30 min. The production yields and water activity in the RS III samples were not significantly different (P≥0.05) among treatments. The RS III samples had production yield and water activity between 88.13-89.87% and 0.436-0.649% for 0 – 48 hr debranched of rice starch slurry preheated at 75°C, 95°C and 121°C for 30 min, respectively.

The moisture content in the 121°C preheated samples were significantly lower (P≤0.05) than the 75°C and 95°C preheated samples. The moisture content was 5.17 -10.50%, 10.48 -12.22% and 10.67 -12.55% for 121°C, 75°C and 95°C preheated rice starch sample, respectively. It could be clearly seen that the high degree of syneresis trend to lower percentage of moisture content and water activity.

All color data was expressed by the Hunter system. The Hunter *L**, *a** and *b** values correspond to lightness, redness, and yellowness, respectively. Hunter L^*, a^*, b^* values of RS III samples were affected by hydrolysis time (Table 14). The result indicated that the color of the RS III from 0 to 16 hr of hydrolysis in both of preheated rice starch at 75°C, 95°C and 121°C were significantly (p≤0.05) lightener than the 24 to 48 hr hydrolysis rice starch samples. Native high amylose rice starch had the highest lightness (*L**) value at 98.55 and lowest *b** value at 2.03. The 48 hr debranched of preheated rice starch at 121°C was lowest *L** value at 85.79 and highest in *b** value (7.47).

These was likely due to the higher reducing sugar content in the 24 to 48 hr hydrolysis samples which caused by the condensation of an amino group and a reducing compound, resulting complex changes in color of RS III samples (Maillard, 1912).

Table 14 Physicochemical properties of RS III from 0 to 48-hr debranched of HARS preheated at 75°C, 95°C and 121°C for 30 min.

Treatments		Yields (%)	Moisture content (%)	A_w value	Color value		
Preheated treatments	Hydrolysis time (hr)				*L**	*.a**	*.b**
75°C	0	88.13^{ns}	12.22^{ab}	0.617^{ns}	95.51^{a}	-0.88^{ab}	5.03^{b}
	4	89.68	11.11^{b}	0.585	93.67^{ab}	-0.96^{ab}	6.03^{ab}
	8	89.41	10.76^{bc}	0.567	93.61^{ab}	-0.99^{ab}	6.13^{ab}
	16	89.01	10.61^{bc}	0.558	93.58^{ab}	-1.18^{b}	6.13^{ab}
	24	89.59	10.48^{bc}	0.550	91.68^{b}	-1.32^{b}	7.19^{a}
	48	89.62	10.67^{bc}	0.547	89.52^{c}	-2.11^{c}	7.21^{a}
95°C	0	88.34	11.50^{b}	0.649	94.92^{a}	-0.83^{ab}	4.53^{c}
	4	89.87	12.20^{ab}	0.619	93.04^{ab}	-1.01^{b}	6.11^{ab}
	8	89.35	11.35^{b}	0.621	92.78^{ab}	-1.18^{b}	6.63^{ab}
	16	89.49	12.55^{a}	0.608	92.76^{ab}	-1.16^{ab}	6.75^{ab}
	24	88.26	11.39^{b}	0.576	90.72^{b}	-2.06^{c}	6.87^{ab}
	48	88.16	10.67^{c}	0.562	88.87^{b}	-2.14^{c}	7.22^{a}
121°C	0	88.34	10.50^{c}	0.586	89.65^{b}	-0.23^{b}	3.87^{c}
	4	89.87	7.58^{d}	0.529	88.20^{b}	-0.01^{a}	3.84^{c}
	8	89.35	7.35^{d}	0.530	87.93^{b}	-0.11^{a}	3.21^{c}
	16	89.49	6.55^{de}	0.510	88.92^{b}	-0.09^{a}	3.84^{c}
	24	88.26	6.39^{de}	0.536	88.61^{b}	-0.04^{a}	3.66^{c}
	48	88.16	5.17^{e}	0.436	85.79^{c}	-1.18^{b}	7.47^{a}
Native rice starch		100	13.18^{a}	0.651	98.55^{a}	-1.16^{b}	2.03^{d}

$^{a,b,c....}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New multiple-Rang test (DMRT).

^{ns} = Not significantly different ($P \geq 0.05$)

1.2.8 *In vitro* starch digestibility of RS III samples

Effects of preheated treatments and hydrolysis time on resistant starch (RS), digested starch (DS), total starch (TS) content, hydrolysis index (HI) and glycemic index (GI) in native rice starch, RS III sample and commercial control (Hi-maize, National Starch) are presented in Table 15 and 16. RS content and GI have been established as two important indictors of starch digestibility. RS is used as a predictor of the speed of release of glucose and GI can be predicted through *in vitro* starch hydrolysis model (Englyst *et al*. 1999; Goni *et al*. 1997). The RS contents in 48-hr hydrolysis of 121^{o}C preheated rice starch were (19.19 %) highest but not significant difference (P≤0.05) from the 16 and 24-hr hydrolysis time (17.14 and 18.33%). The RS contents in the 0 to 48 hr hydrolysis of 75^{o}C and 95^{o}C preheated high amylose rice starch ranged from 4.04 to 12.33 % and 4.06 to 9.68 % base on dry weight, respectively. They were significantly different from the commercial resistant starch and native high amylose rice starch. The RS content of the commercial resistant starch (Hi-maize starch) was 48.32 % base on dry weight. RS content in the native high amylose rice starch was 4.99 % base on dry weight.

Overall comparison indicated that the higher pullulanase hydrolysis produced the higher RS content as seen in the 48 hr hydrolysis of 121^{o}C preheated treatment. The resistant starch content increased by 4 folds with debranching process (3.37, 4.12 and 19.31 % for native starch, non-debranched retrograded starch and 48-hr pullulanase debranched starch preheated at 121°C). These may be attributed to the formation of several smaller molecular weight fractions from hydrolysis of the starch during debranching, with the different fractions recrystallization at low temperature cool down. Recrystallization of these smaller starch fractions may permit an orderly rearrangement of granules thereby preventing access to α- amylase digestion (Englyst *et al*. (1992). The DS content was highest in the 0 hr of hydrolysis in both of 75^{o}C, 95^{o}C and 121^{o}C heated rice starch which was 90.39, 90.21 and 90.75 %, respectively. All RS III samples contained the same amount (95.04 – 95.87%) of total starch after enzymatically debranched of high amylose rice starch.

Table 15 Effect of preheated treatments and hydrolysis time on resistant starch, digestible starch and total starch content of RS III from 0 to 48 hr debranched HARS preheated at 75°C, 95°C and 121°C

Treatments		Resistant starch (% dwb)	Digested starch (% dwb)	Total starch (% dwb)
Heated temperature	Hydrolysis time (hr)			
75°C	0	4.80^{fg}	90.39^{a}	95.54^{ns}
	4	6.61^{f}	89.05^{b}	95.66
	8	9.05^{e}	85.99^{d}	95.04
	16	11.24^{d}	84.04^{e}	95.28
	24	11.43^{d}	84.15^{e}	95.26
	48	12.33^{d}	83.52^{f}	95.86
95°C	0	4.07^{g}	90.21^{a}	94.28
	4	5.99^{f}	89.68^{b}	95.22
	8	8.89^{e}	87.55^{c}	95.04
	16	10.05^{d}	85.11^{d}	94.71
	24	10.21^{d}	84.15^{e}	95.39
	48	10.68^{d}	83.90^{ef}	95.59
121°C	0	5.12^{f}	90.75^{a}	95.87
	4	7.62^{e}	87.46^{c}	95.18
	8	11.33^{d}	84.60^{e}	95.94
	16	17.13^{c}	78.37^{g}	95.50
	24	18.33^{b}	76.75^{h}	95.09
	48	19.32^{b}	75.89^{i}	95.22
Native rice starch		4.99^{ef}	90.39^{a}	95.38
CRS (Hi-maize)		48.32^{a}	47.78^{j}	96.10

$^{a,b,c....}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New multiple-Rang test (DMRT).

^{ns} = Not significantly different ($P \geq 0.05$)

1.2.9 Hydrolysis index and glycemic index of RS III samples

Figure 20 and Table 16, shows the *in vitro* starch hydrolysis results, including the estimated parameter of the calculated hydrolysis index and glycemic index. The hydrolysis kinetic for the RS III samples showed that the equilibrium concentration ($C\infty$) of the hydrolyzed starch was changed slightly by pre-heated treatment and debranching time. The debranch of 121^{o}C pre-heated rice starch exhibited a lower $C\infty$ than the 75^{o}C preheated and 95^{o}C preheated samples that debranching at 4 to 48 hr.

The kinetic constant (*k)* of the starch samples tested showed that the native high amylose rice starch had highest *k* value, indicating that hydrolysis occurred most rapidly in the solubillized samples. While, the RS III formation by retrogradation of 48 h debranching of 121^{o}C, 75^{o}C and 95^{o}C preheated high amylose rice starch showed lower *k* value (0.018 to 0.020) than other treatments (0.21 to 0.036). In addition, the commercial resistant starch (Hi-maize) was lowest in kinetic constant (0.014), this due to its inherent resistance to enzymatic hydrolysis.

The hydrolysis index (HI) of the RS III samples range between 26.83 to 71.59 % (Figure 20). The RS III samples sowed a lower HI value than the native high amylose rice starch (82.42 %) but higher than commercial resistant starch (12.84 %). This behavior indicates that the developed RS III samples are resistant to enzymatic digestion. The decrease in the enzymatic digestion of starch after retrogradation has been reported by several researchers although the starch type and storage conditions were not identical to those used here. Gui and Oates (1997) found that the degree of digestion of retrograded sago starch (40 % gel) rapidly dropped from 78.3 to 45.4% within 1 hr storage at 5^{o}C, but extending the storage time to over 6 hr had little influence on the degree of enzymatic digestion. They claimed that the digestibility of sago starch was highly sensitive to the retrogradation of amylose that occurred mainly in the early period of storage, whereas amylopectin recrystallization, which occurs more slowly, had little influence on the enzymatic digestibility. It is supposed that the amorphous matrix of starch is readily exposed to the digestive

enzymes whereas most of the crystallites reformed during retrogradation are embedded in the matrix (Granfeldt *et al*. 2000).

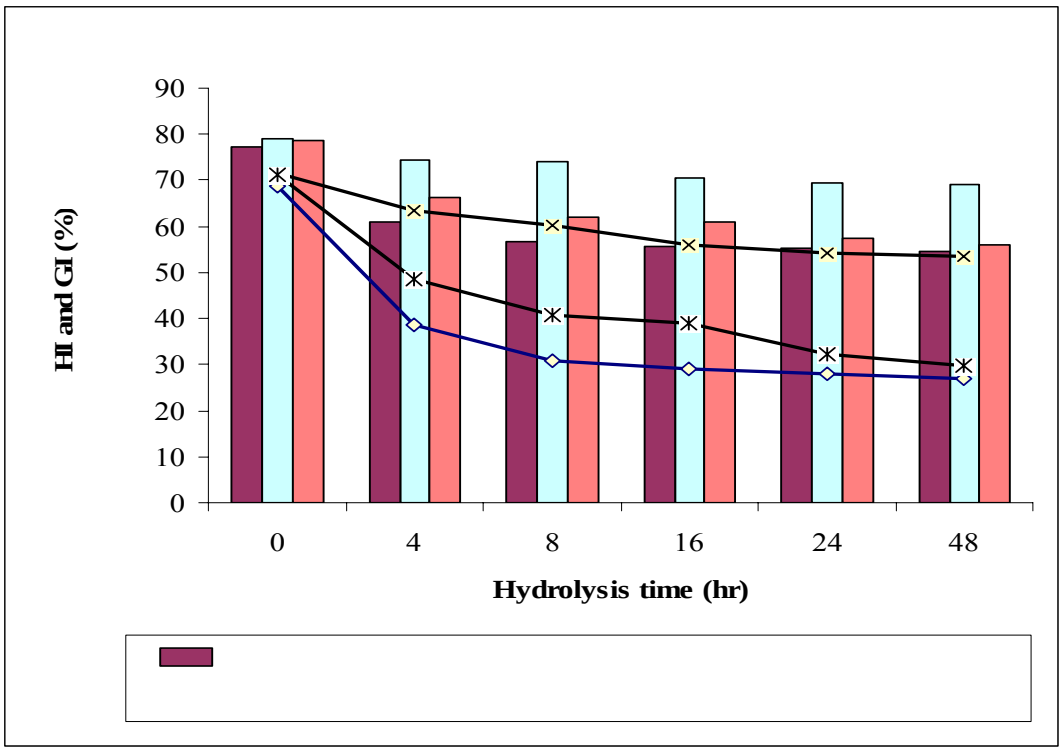


Figure 20 Effect of pre-heated treatments and hydrolysis time on the calculated hydrolysis index (HI) and glycemic index (GI) of RS III from 0 to 48-hr debranched high amylose rice starch preheated at 75°C, 95°C and 121°C

The estimated glycemic index (GI) based on HI of the developed RS III samples ranged from 54.44 to 79.01 GI value (Table 16). Degree of pullulanase hydrolysis had an obvious impact on starch retrogradation and thus on the predicted glycemic response. The high degree of pullulanase hydrolysis and degree of syneresis led to a reduction of HI and estimated GI for all preheated treatments. The lowest GI value was observed in the 4 to 48 hr debranching of 121°C preheated treatment (60.83 to 54.44 GI value) and followed by the 4 to 48 hr debranching of 75°C preheated (61.43 to 56.09 GI value). While, the 4 to 48-h debranching of 95°C preheated treatment was highest this was 69.09 to 62.63 GI values.

Table 16 Effect of preheated treatments and hydrolysis time on the estimated parameter, the calculated hydrolysis index and glycemic index of RS III from 0 to 48 hr debranched HARS preheated at 75°C, 95°C and 121°C.

Treatments		Equilibrium concentration ($C\infty$)	Kinetic constant (k)	Calculated HI (%)	Estimated GI value
Preheated treatment	Hydrolysis time (hr)				
75°C	0	53.80±3.22 ^{b}	0.035 ^{ns}	71.09±2.62^{b}	78.74±1.22^{b}
	4	46.24±1.03^{d}	0.027	46.50± 1.31^{f}	65.43± 3.16^{e}
	8	44.11± 2.13^{e}	0.021	42.76± 1.28^{g}	61.57± 1.77^{f}
	16	42.87± 2.05^{f}	0.021	46.12± 4.42^{f}	59.87± 2.55^{h}
	24	43.39± 3.04^{f}	0.024	35.61± 3.88^{h}	59.29± 2.35^{h}
	48	38.35±1.72^{g}	0.017	29.83± 2.78^{i}	56.09±1.81^{i}
95°C	0	54.35±2.98^{b}	0.036	71.59±2.84^{b}	79.01±2.14^{b}
	4	48.70± 0.12^{c}	0.029	53.52± 1.21^{d}	69.09± 2.21^{c}
	8	46.01± 0.20^{d}	0.026	52.01± 2.65^{d}	68.30± 1.4^{d}
	16	45.73± 0.18^{e}	0.027	51.68± 0.10^{e}	68.13± 0.5^{d}
	24	45.26± 0.30^{e}	0.021	46.45± 1.71^{f}	65.25± 2.4^{e}
	48	43.23±1.26^{f}	0.020	41.69± 2.91^{g}	62.63± 2.7^{g}
121°C	0	52.66±0.34 ^{b}	0.034	68.66±0.23^{c}	78.41±2.76^{a}
	4	26.17±0.46^{h}	0.033	38.47±1.35^{h}	60.83±0.77^{f}
	8	24.82±0.57^{i}	0.028	30.87±0.22^{i}	56.65±2.39^{i}
	16	23.37±0.72^{i}	0.021	28.88±0.89^{i}	55.57±1.07^{j}
	24	23.19±0.76^{i}	0.022	28.14±0.84^{i}	55.15±3.21^{j}
	48	21.17±0.64^{j}	0.018	26.83±4.57^{j}	54.44±2.18^{k}
Native rice starch		78.98±3.80 ^{a}	0.035	82.42±1.67^{a}	84.96±2.34^{a}
CRS (Hi-maize)		19.26±0.27^{k}	0.014	12.84±.3^{k}	46.76±3.71^{l}

$^{a,b,c....}$ Means within the same column with different letters are significantly different (P≤ 0.05) by Ducan's New multiple-Rang test (DMRT).

^{ns} = Not significantly different (P≥0.05)

The low GI value of the 121^{o}C preheated treatment was in recrystallization with its relatively high in resistant starch content (7.62 to 19.32%). Because of this recrystallization was poor in starch digestibility. Thus the high resistant starch content in RS III sample had a low glycemic index because of the slowly release of glucose, which may simply result from a lack of available digestible starch (Jenkins *et al*. 2002, Kim *et al*. 2006 and Shane, 2005). Resistant starch type III promotes slow and moderate postprandial glucose and insulin response (Jenkin *et al*. 1994). For most starchy food products, a reduction in GI appears to be accompanied by a higher content of resistant starch (Bjorck *et al*. 2000). Resistant starch can thus be expected to contribute to the colonic generation of short-chain fatty acids with potential beneficial effects on glucose and lipid metabolism (Thorburn *et al*. 1993).

The commercial resistant starch (Hi-maize from National starch) was lowest in degree of starch hydrolysis and GI value range between 12.84 and 46.76, respectively. While, the native high amylose rice starch was highest in starch hydrolysis rate and GI value, which was 82.42 % and 84.96, respectively. Similar trends, with comparable values of HI and GI, have also been reported in native high amylose rice starch (25 – 33 % amylose content) ranged from 81 to 84, low amylose content (10 - 20 %) ranged from 87 to 93 and waxy rice ranged from 87 to 96 (Juliano and Goddard, 1986; Juliano *et al*. 1989 and Panlasigui, 1989).

GI is a concept comprehensively reflecting the digestibility and utility of RS III samples. Factors affecting the GI of RS III samples such as degree of syneresis and resistant starch content were taken into account in this concept (Yang, 1999). Thesis results showed that different degree of syneresis and resistant starch content had different influence on hydrolysis index. For example, lower degree of syneresis and resistant starch content had high degree of starch hydrolysis rate and consequently high estimated GI value because digestive enzyme could easily reach the structure of starch chain. The opposite was true as to the RS III from high degree of syneresis and resistant starch content. Possible explanation for GI difference of different RS III formation is high recrystallization structure has stronger resistance to digestive enzymes.

1.2.10 Crystallinity of RS III

X-ray diffraction patterns of native high amylose rice starch and RS III samples (0 to 48 hr pullulanase hydrolysis of 75°C and 121°C preheated high amylose rice starch) are shown in Figure 21 and 22. The diffraction pattern obtained from native high amylose rice starch was classified as an A-type pattern as indicated by typical peaks at 15.0, 17.5 and 23.2° of diffraction angle 2θ, with 13.18 % crystallinity (Figure 20). These values are in agreement with those reported for native rice starch and cereal starches. Ornanong *et al*. (2006) reported that the X-ray diffractogram of the native rice starch showed an A-type crystal pattern with strong reflections at 14.9, 17.8 and 22.8 ° of diffraction angle 2θ for all strains of rice.

When the starch was subjected to debranching and retrogradation treatments in this study, the 0, 16 and 48 hr pullulanase hydrolysis treatments showed completely different pattern from the native high amylose rice starch. The RS III sample from 0 hr hydrolysis exhibited very low crystallinity values due to the loss of crystallinity during thermal treatment, and the calculated crystallinity was only 2.58 %. This result suggests that the stored starch contained amorphous region than crystalline region. The RS III formation from 16 and 48-hr hydrolysis of 75°C preheated starch sample displayed a V-type diffraction pattern with 10.67 % and 12.91% crystallinity, respectively. This was attributed to debranching and retrogradation which reorganized the structure of starch into a helical complex to that of V-amylose (Cui and Oates, 1979). Occurrence of V-diffraction patterns is caused by the presence of amylose and lipids in the starting material.

In addition, the X-ray diffraction profile (Figure 21) of the RS III samples from 4, 16, 24 and 48 hr hydrolysis of 121°C preheated starch showed very strong V-type diffraction pattern, which had 14.37, 15.59, 15.80 and 19.50% crystallinity, respectively. RS III samples from 48 hr hydrolysis of 121°C preheated rice starch gave the strongest diffraction peak at around 17° 2θ and a few small peaks at around 2θ values of 20°, 22 ° and 24°. This indicated that highly debranching and retrogradation of high amylose rice starch influenced the crystalline structure.

When such a starch sample is X-rayed, a crystalline V structure is detected. The alternative for V to appear in this case would include migration through the granule of a large amylose molecule and/ or long chain fatty acids. A second limiting factor would be to locate sufficient material in one place for crystallite formation. In general, retrograded amylose gives the V-type starch X-ray diffraction pattern. Early X-ray diffraction analysis of stretched amylose film in the B-type structure was interpreted in terms of a six-fold single helix structure with a repeat distance of 10.4^{o} A (Blackwell *et al.* 1969). On this basis, it could be postulated that the mechanism of cross-linking amylose gels was via junction zones of aggregated single helices.

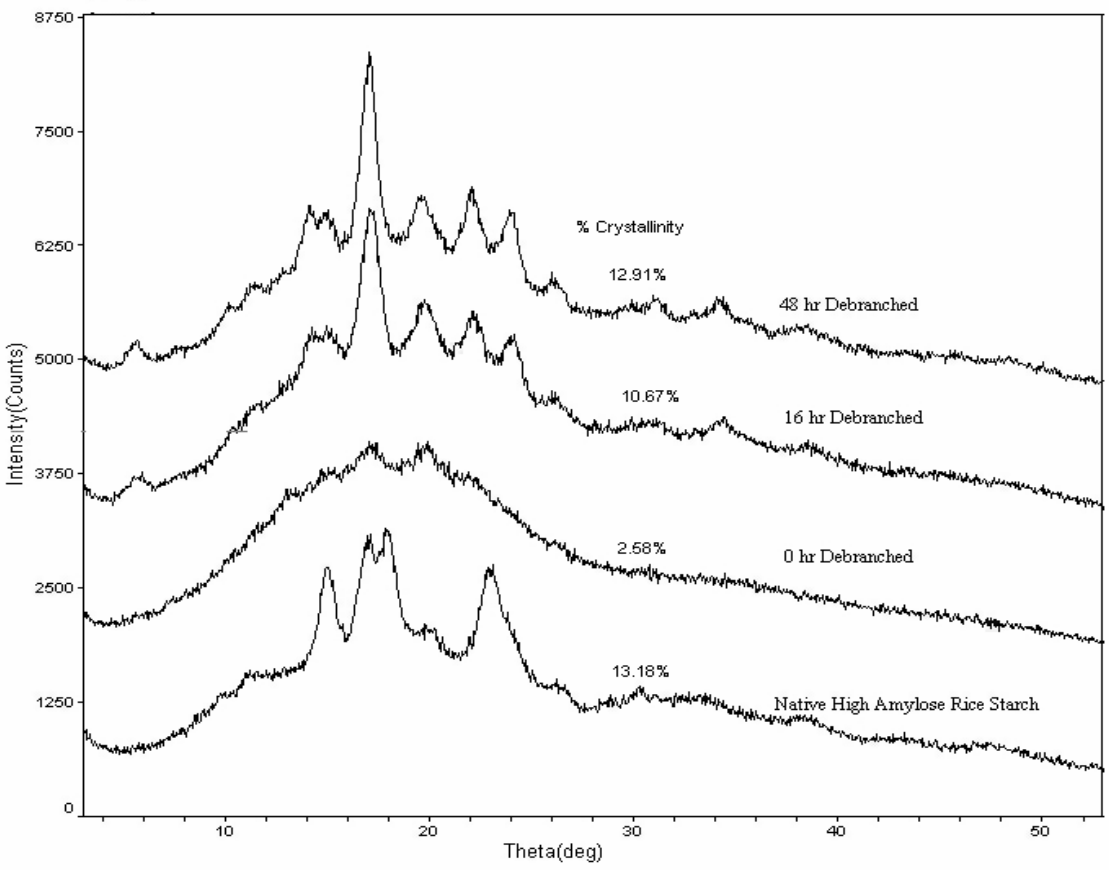


Figure 21 X-ray diffraction pattern of native HARS and RS III from 0, 16 and 48-hr Pullulanase debranching of 75°C preheated HARS for 30 min

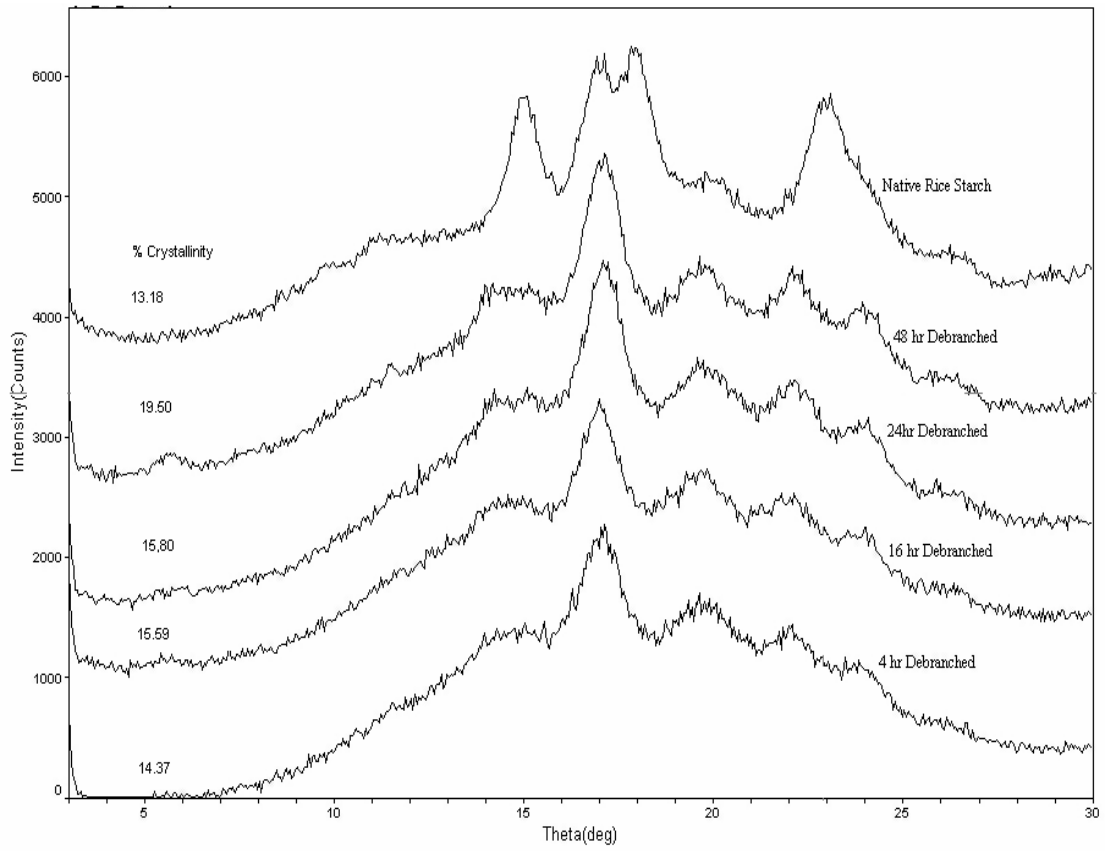


Figure 22 X-ray diffraction pattern of native HARS and RS III from 4, 16, 24 and 48-hr pullulanase-debranching of 121°C preheated HARS for 30 min

1.2.11 Correlation study

1) Degree of syneresis and resistant starch correlation

The correlation between degree of debranching vs resistant starch content and degree of syneresis vs resistant starch content of RS III formation are show in Figure 23. A positive correlation between the degree of debranched and degree of synersis (R^2 0.895) of the RS III from pullulanase debranched of 121^{o}C preheated of 15% HARS slurry are shown in Figure 23 (A). The results obtained in this study suggested that the high degree of pullulanase debranching, is closely related with high degree of syneresis and affected resistant starch content of the RS III

production. This could be due to the pullulanase enzyme hydrolyzed α-1,6-glucosidic bonds, releasing a linear polymers linked by α-1,4-glucosidic bonds. These fragments are rapidly producing retrograded indigestible starch. An application of freeze-thaw process (syneresis) on retrograded gel results in the continuous reassociation and eventual recrystallization of the gel, a process that excludes water from the gel phase (Thomas and Atwell, 1999; Towar *et al*. 2002). Present data show that gels of higher degree of syneresis, developed significantly higher resistant starch content (Figure 24; B). A positive correlation between the degree of syneresis vs dehydration rate (R^2 =0.9634) of the RS III from pullulanase debranching of 121^{o}C were also observed.

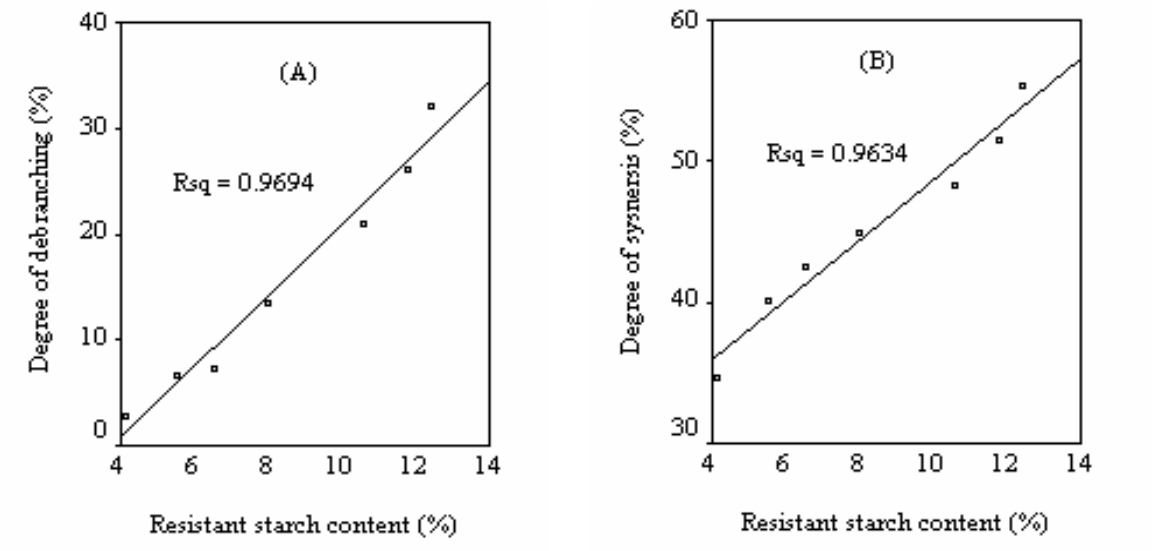


Figure 23 Correlation between degree of debranching vs resistant starch content (A) and degree of syneresis vs resistant starch content (B) relation in RS III from 0- 48 hr debranching of 121°C preheated HARS

2) Percentage of crystallinity and resistant starch content correlation

The calculated crystallinities show strong correlation with resistant starch content (Figure 24).The degree of crystallinity being same proportional to the resistant starch content. RS III from highly resistant starch content (19.32 %) had the highest crystallinity (19.50 %), while RS III with lower resistant starch content (had the lowest value (2.58%). Figure 24 is a plot of crystallinity vs. resistant starch

content. The high correlation factor (r = 0.7218) suggests that resistant starch content plays a role in increasing crystallinity.

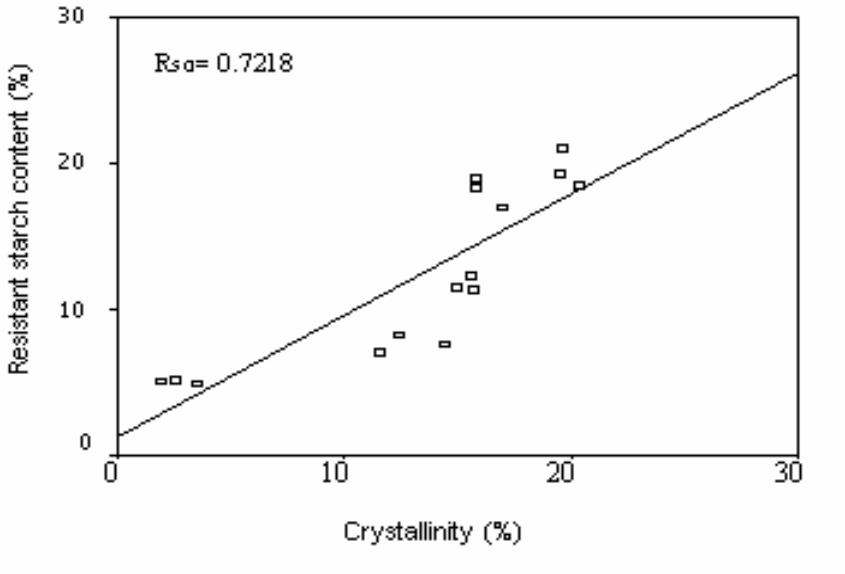


Figure 24 The Crystallinity and resistant starch content relation in RS III formation from 0- 48 hr debranching of 121°C preheated HARS

1.3 Effect of pullulanase enzyme concentration on RS III formation

Overall comparison in the experiment 1.2 indicated that longer incubation of complete gelatinization (121°C preheated) of 15% rice starch solution with pullulanase enzyme produced higher resistant starch content. The extended duration of enzyme treatment increased resistant starch content and yellowish color in 24 and 48-hr over 4 to 16 hr debranched of 121°C preheated the rice starch. Thus, in order to reduce incubation time which was increased resistant starch content, the effect of pullulanase concentration treatments of 121°C preheated the rice starch was investigated. A pullulanase concentration of 0, 8, 10, 12, 14 and 16 units / g starch (dry basic) and a hydrolysis period of 16 hr at 55^{o}C were employed for investigation of enzyme concentration effect on degree of pullulanase hydrolysis, β-amylolysis, degree of syneresis and resistant starch content that was presented in Figure 25, 26, 27, 28 and Appendix Table E 4.

1.3.1 Degree of pullulanase hydrolysis

Degree of hydrolysis of 15% (w/w) gelatinized high amylose rice starch reacted with 0 to 16 unit/g starch of pullulanase for 16 hr are shown in Figure 25. The amount of hydrolysis degree was defined by measuring the concentration of reducing groups which were freed by alpha-1,6-D-glucanohydrolase activity by means of Park and Johnson method. The study found that an increasing of enzyme concentration from 8 to 14 unit/g starches provided the degree of hydrolysis to gradually increase from 2.46 to 5.21 %. However, there was no significantly increased in degree of hydrolysis for the rice starch samples treated with 16 unit/g starch. This indicated that the starch was completely debranched.

When the starch is completely debranched, the monitored measurement will no longer change. The debranched starch will typically have an average chain length of 14 to 25 glucose units and less than about 0.2 %, particularly less than about 0.1 % alpha-1,6-D-glucosidic bonds linkages(Hizukuri *et al.* 1981).

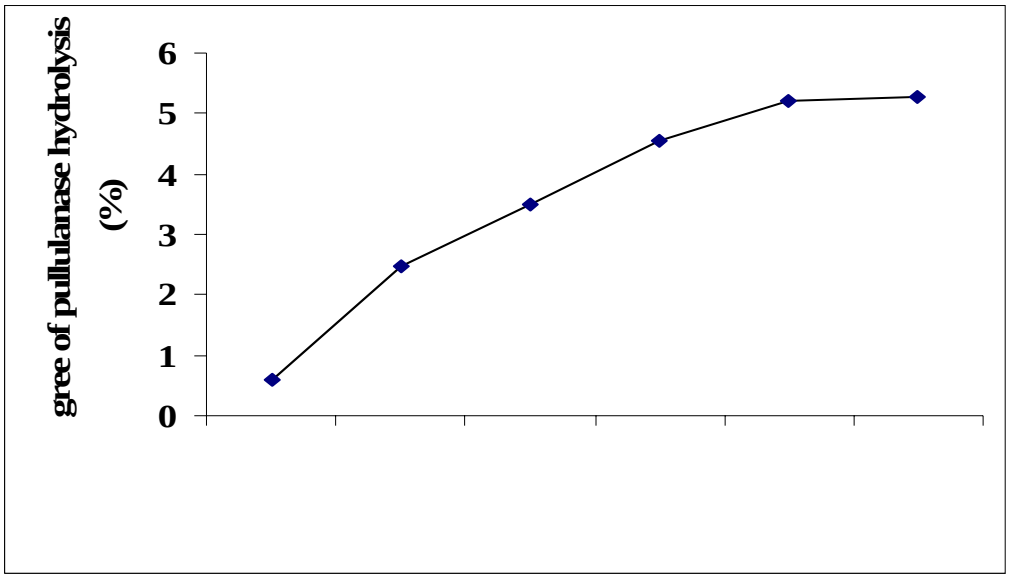


Figure 25 Effect of pullulanase enzyme concentration (0 to 16 unit/g starch) on degree of pullulanase hydrolysis of 16 hr debranching of 15 % HARS preheated at 121°C for 30 min

1.3.2 β-amylolysis (%)

β-amylase attacks the next to last glycosidic linkage from the non- reducing ends on branched chains and specifically releases maltose. However, this enzymatic action is blocked by α-D-(1,6) branched linkages (Zeikus, 1989). As shown in Figure 26, the debranched sample by 14 and 16 unit/g starch of pullulanase hydrolysis for 16 hr showed higher β-amylolysis (96.27 and 98.82 %) than did the 8 to 12 unit/g starch concentration (66.60 to 83.48 %). These results indicated that the 14 and 16 unit/g starch of pullulanase hydrolysis the rice starch was nearly completely debranched with 16 unit/g starch.

Typically, the starch was completely debranched when it has been attacks by β-amylase at least about 95 %, more particularly at least about 98%, most particularly at least about 99 % debranched by weight (Nagamura *el al.* 2002).

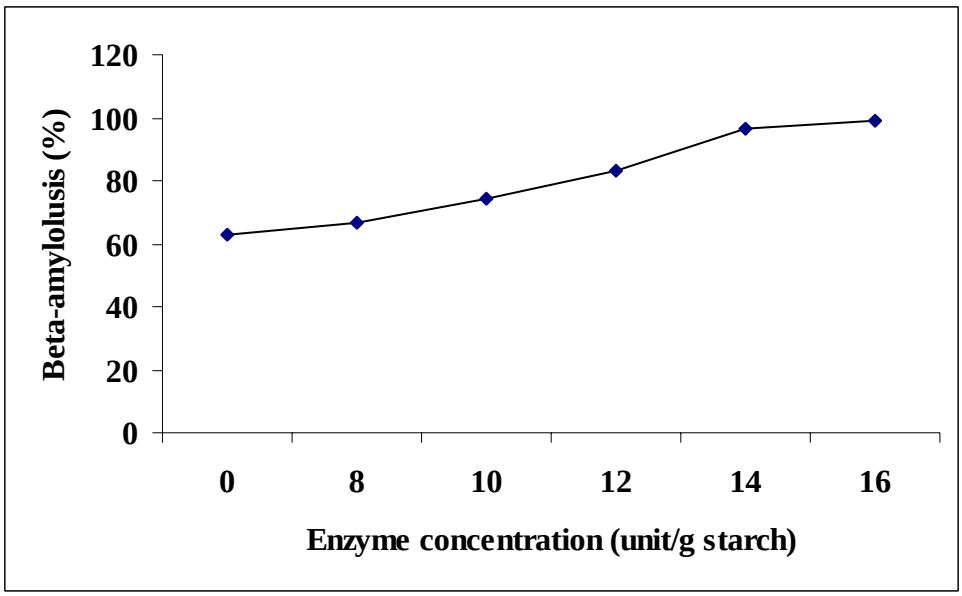


Figure 26 Effect of pullulanase enzyme concentration (0 to 16 unit/g starch) on β-amylolysis (%) of 16 hr debranching of 15% HARS preheated at 121°C for 30 min

1.3.3 Degree of syneresis

The effects of enzyme concentration on the degree of syneresis of debranching and freeze-thaw cycle (-10/30°C) of 15 % rice starch paste preheated at 121°C for 30 min showed in Figure 27. The concentration of pullulanase enzyme was fixed at 0, 8, 10, 12, 14 and 16 unit/g starch were debranched in a water bath at 55°C for 16 hr. The degree of syneresis for the freeze-thawed cycle of debranched rice starch paste in the range of 8 to 16 unit/g starch of pullulanase concentration were higher (53–58 %) than the non debranched freeze-thawed cycle of rice starch paste (34 %). Moreover, the degree of syneresis was not significant difference between rice starches gels treated with 12 to 16 unit/g pullulanase concentrations. The debranched rice starch gel was dramatically lost expressible water in the 8 to 10 unit/g starch hydrolysis and remained relatively constant thereafter indicating a very high concentration of pullulanase treatments. In general, the rate of water syneresis of starch paste after freeze-thaw treatments correlated well with prolonged refrigeration tests and with quality evaluation of frozen starch-thickened foods. In addition, the technique was developed for cold-resistant starches, it has been used on a wide range of starches (Wu and Seib, 1990 and Kim *et al*. 2006).

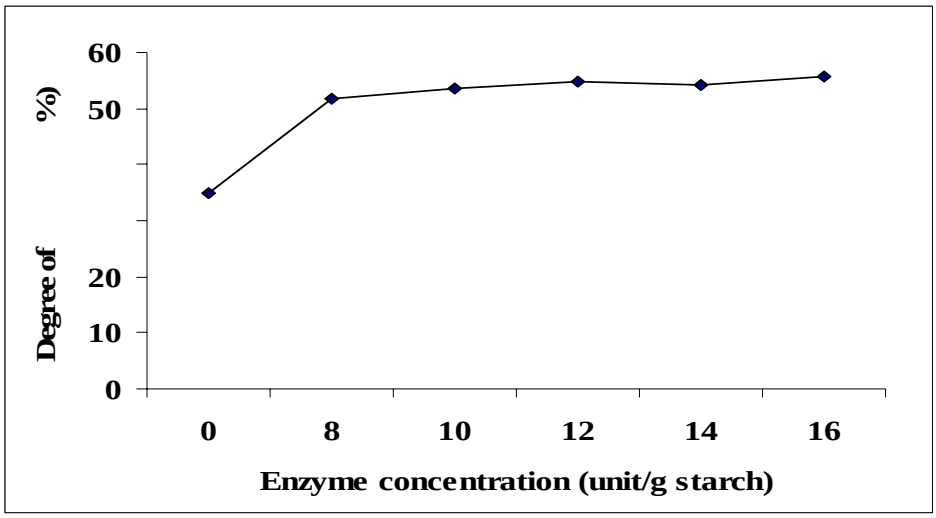


Figure 27 Effect of pullulanase enzyme concentration (0 to 16 unit/g starch for 16h) on degree of syneresis of 15 % HARS preheated at 121°C for 30 min

1.3.4 *In vitro* starch digestibility

Pullulanase enzyme concentration (0, 8, 10, 12, 14 and 16 unit/g starch) dependence of RS III formation was examined, and *in vitro* starch digestibility (%dwb) of RS III formation was presented in Figure 28. Resistant starch content increased sharply as the amount of the enzyme increased to at 12 units/g starch, reaching highest (19.81 %) resistant starch content. Furthermore, resistant starch content in RS III samples dramatically decreased to 17.31 % and 13.16 % upon higher content of the enzyme (14 and 16 units/g starch). Meanwhile, digested starch content decreased as the resistant starch content increase. The digested starch and total starch content in the RS III samples range between 74.29 to 90.09 % and 93.24 to 94.40 %, respectively.

This result indicated that the amount of resistant starch could be related to the degree of pullulanase hydrolysis. The higher degree of pullulanase hydrolysis may be attributed to the formation of short linear polymers linked by α-1, 4-glucosidic bonds fractions that may inhibit rearrangement of granules thereby preventing retrogradation. As Gidley and Bulpin (1987) suggested, a chain length of at least 10 glucose units was required for crystallization and, by inference, for the formation of double helices. On the other hand, short chains with DP 6-9 glucose units are known to inhibit retrogradation (Levine and Slade 1986, Shi and Seib, 1992). Gidley *et al,* (1995) observed that an approximate relative maximum at DP 20 - 30 suitable to form RS III. Eerlingen *et al*. (1993) found that the chain length of the resistant regions (DP 19 - 26) was independent of the amylose average chain lengths originally used to form the RS III.

Many researchers would expect retrogradation to have an effect on the covalent structure of the native starch granules. Faissant *et al.* (1993) and Cairns *et al*. (1995) revealed that resistant starch obtained from retrograded high amylose maize starch consisted of three chain populations. The average chain lengths of these populations depend on the starting materials and enzymatic treatment. However, in both case a high molecular weight fraction ($\overline{DP}_n$ > 100), a medium

molecular weight ($\overline{DP}_n$ ~ 30), and a low molecular weight fraction ($\overline{DP}_n$ ~ 5) was found for the resistant starch type III.

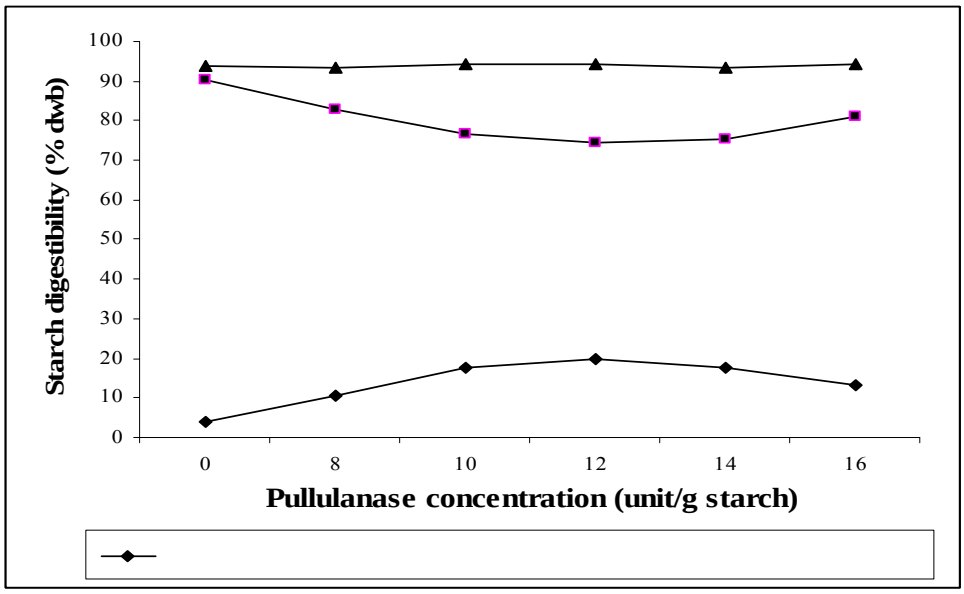


Figure 28 Effect of pullulanase enzyme concentration (0 to 16 unit/g starch for 16h) on starch digestibility (% dwb) of RS III formation from HARS

1.3.5 Physiochemical properties

The effects of enzyme concentration (0 to 16 unit/g starch for 16hr) on the physiochemical properties of debrached rice starch paste preheated at 121°C for 30 min showed in Table 17. Production yield (%) is the total weight of the dry RS III fractions divided by the original weight of the rice starch sample. The production yield, moisture content, and water activity in RS III formation by debraching process with different enzyme concentrations were 84.61 to 86.16%. The slight decreasing in production yield for the lower enzyme concentration (8 to 10 unit/g starch) might be due to some losses of starch fraction during freeze-thawed process and vacuum filtrated. However, no significant differences ($P \leq 0.05$) in production yield was observed among the different debranching treatments.

Non significant differences (P≥0.05) in moisture content and water activity were observed among the debranching treatments. The moisture content and water activity were 8.47 to 9.09 % and 0.326 to 0.510, respectively.

Table 17 showed that Hunter L^*, a^* and b^* values of RS III samples were affected by degree of hydrolysis. This was due to the increased of reducing sugar. The reducing sugar reacts with amino compounds in the starch slurry during hydrolysis and storage. The result indicated that the color of the RS III formation from 16 hr of pullulanase hydrolysis with 10, 12, 14 and 16 unit/g starch were more yellow than those of the 0 and 8 unit/ g starch. The RS III from 0 and 8 unit/g starch of pullulanase enzyme hydrolysis showed a comparable level of L* values (whiteness) a* values (red-green axis) and b* values (yellowness) to that by the higher enzyme concentration (10, 12, 14 and 16 unit/g starch). A comparison with the treatments from higher enzyme concentrations showed a significant increase in red-green axis and yellowness.

Table 17 Effect of pullulanase enzyme concentration (0 to 16 unit/g starch for 16h) on production yield (PY), moisture content (MC), water activity (a_w) and color value of RS III from HARS

Enzyme concentration (unit/g starch)	PY (%)	MC (%)	a_w	Hunter Color value		
				*L**	*a**	*b**
0	84.11^{b}	8.57^{ns}	0.505^{ns}	91.91^{a}	-0.62^{b}	2.96^{b}
8	84.99^{ab}	9.09	0.510	90.11^{b}	-0.68^{b}	3.72^{ab}
10	85.92^{a}	8.82	0.426	90.98^{b}	-0.91^{b}	4.26^{a}
12	85.99^{a}	8.47	0.353	90.44b	-0.41^{b}	4.26^{a}
14	85.94^{a}	8.85	0.328	90.79^{b}	0.73^{a}	4.84^{a}
16	86.16^{a}	8.47	0.326	90.42^{b}	0.78^{a}	4.84^{a}

$^{a, b, c.....}$ Means within the same column with different letters are significantly different (P≤ 0.05) by Ducan's New multiple-Rang test (DMRT).

^{ns} = Not significantly different (P≥0.05)

1.4 Scale up production of RS III by enzymatically debranching process

The investigation of pullulanase enzyme concentration effects on degree of pullulanase hydrolysis, β-amylolysis, degree of syneresis, resistant starch content and physicochemical properties of RS III in the experiment 1.3 indicated that the concentration of pullulanase enzyme with 12 unit/g of starch was an optimum level for RS III formation. It had highest content of resistant starch content and little change in physicochemical properties from the other treatments. Thus, the 12 unit/g starch of pullulanase enzyme debranching at 55°C for 16 hr of 15% high amylose rice starch slurry preheated (121°C for 30 min) were selected for scale up (150 g of rice starch in 850g distilled water) to produced RS III in this experiment.

Comparative study on physiochemical properties of an experiment and the larger- scale production of RS III formation with three replications (Table 18) showed not significantly different ($P \leq 0.05$) among the two production scales. The viscosity of the debranched product, degree of hydrolysis, degree of syneresis, resistant starch content, water activity and production yield of the larger scale was close to the experimental scale as following section.

1.4.1 Viscosity

The viscosity of the larger scale (1-lit starch solution) formation of RS III measured at 100 rpm was lower than the experimental-scale (300-ml starch solution) but not significant difference ($P \geq 0.05$) with an approximately 1,785.15 ± 13.18 and 1,790.50 ± 12.21 cP, respectively. The lower the viscosity, the less was the tendency for any further spontaneous reduction with pullulanase hydrolysis. Viscosity is the flow property of a material, or the resistance of the material to flow under a mechanical stress expressed in units of centipoises (cp) (Elgun *et al*. 1998). Solutions with viscosity of less than 1,000 cp are freely flowing liquids. Between 1,000 and 3,000 cp they have a thick, soup-like consistency, and at higher viscosities, the gruels become progressively thicker (Hsu and Huang, 2000).

1.4.2 Degree of hydrolysis

The degree of hydrolysis of the larger-scale samples was 4.74 ± 0.52 %, which was only 0.20% different from that of the experiment –scale (4.61 ± 0.43%). The addition of pullulanase to the gelatinized rice starch suspensions resulted in a more runny solution after 16-hr of reaction, especially at larger-scaled of starch solution. This was probably due to the higher amount of the rice starch solution more accessible for pullulanase hydrolysis and produce short chain linear glucan. Debranched rice starches, particularly non-waxy rice starch, have been found to be useful for produce high yield of slowly digestible starch and resistant starch (King and Tan, 2005). Guraya *et al*. (2001a) found that, the debranched starch formed a white crystalline product with slowly digestible properties. They observed that both the level of enzyme and time allowed for debranching have a pronounced effect on the amount of starch that is debranched.

1.4.3 Degree of syneresis

The scale up hydrolyzed samples were then induced to 4°C for 16 hr. Afterwards, the freeze-thaw cycle (-10/30°C) was further applied to promote syneresis of retrograded starches. Degree of syneresis in the larger-scale had higher (56.66 ± 1.62) than the experimental-scaled (55.80 ± 2.43) but not significantly ($P \geq 0.05$). This due to the differences for the degree of hydrolysis, that was affected the retrogradation process during storage. The high degree of hydrolysis had high degree of syneresis. Additionally, the degree of syneresis (retrogradation) depends on starch precipitation or aggregation and gelation. Gidley and Bulpin (1989) described these processes in a phase diagram showing the effect of cooling aqueous solution of synthetic amylose. In general, shorter chain lengths favored precipitation and longer chain lengths favor gelation.

1.4.4 *In vitro* starch digestibility

Table 18 shows the resistant starch (RS), digested starch (DS) and total starch (TS) contents of the large and small-scale production of RS III samples. RS content in the larger-scaled was higher but not significant difference (P≤ 0.05) from the small-scale. The RS contents in the small and large-scale ranged from 19.81 ± 1.22 to 20.01 ± 0.79% dwb of the RS III sample, resulting in low DS in the large-scale. The DS content was 74.29 ± 1.07 in the experimental scale and 74.04 ± 0.34% in the larger scale. Relinde (1994) reported that gelatinized starch with high enzyme resistance starch contains, can only be obtained when crystallization can occur over sufficiently long segments of the polymer chains (25 glucose residues). Furthermore, the RS III production from the larger-scale was selected for the next experiment on an application of RS III in low glycemic index butter cake product.

Table 18 Comparative study on physiochemical properties between experimental and larger-scaled production of RS III from HARS

Physiochemical properties	**Batch of RS III production** [ns]	
	Experimental-scaled	**Larger-scaled**
Viscosity (cp; centripoint)	1,790.50 ± 12.21	1,785.15 ± 13.18
Degree of hydrolysis (%)	4.61 ± 0.43	4.74 ± 0.52
Degree of syneresis (%)	55.80 ± 2.43	56.66 ± 1.62
Resistant starch (%)	19.81 ± 1.22	20.01 ± 0.79
Digested Starch (%)	74.29 ± 1.07	74.04 ± 0.34
Total starch (%)	94.10 ± 1.32	94.26 ± 0.91
Water activity	0.26 ± 0.21	0.28 ± 0.02
Production yield (%)	93.69 ± 1.72	94.44 ± 1.67

[ns] = Not significantly different (P≥0.05)

2 An application of resistant starch type III in low glycemic butter cake

Butter cake is a food well liked by consumers all over the world. However, due to it is high caloric content and defined as high glycemic food, over-consumption may contribute to obesity and led to high risk of health problem. A previous study showed that sucrose-rich diets resulted in a steady state of hyperglycemia and incidence of type 2-diabetes (Lombardo *et al*. 1996). In order to produce low glycemic butter cakes, the effect of using RS III as flour replacement and high fructose corn syrup (HFCS-55) with sucrose substituted on the physiochemical properties, *in vitro* starch digestibility, and sensory evaluation of the resultant cakes were study.

2.1 Effect of HFCS-55 with sucrose substituted on butter cake quality

High fructose corn syrup (HFCS-55% fructose) was used to replace 0, 20, 40, 60, 80 or 100% of the sucrose in butter cake to reduced glycemic index and improved the moistness of the butter cake. The sucrose replacement was done in a combination of the foam part of the cake. Replacement of up to 60 to 80% of total sucrose had no significant effect on physiochemical properties and sensory evaluation of the butter cakes. The following sections summarize the results.

2.1.1 Physical properties of HFCS replace of the sucrose in butter cake

The results of physical analysis of HFCS-55 replaced of the sucrose in butter cake are shown in Table 19, 20 and Figure 29. Replacement with HFCS of 40, 60, 80 or 100% for sucrose resulted in foams with lower specific gravities, decreased foam beating time, and cakes with lower volume, browner crusts, yellower crumb and firmer texture when replaced up to 80% as indicated in the following sections.

1) Specific gravity

Specific gravity of the butter cake form and batter were significantly affected by the addition of HFCS-55. The amount of HFCS, which was higher than 40% replacement had significant (P≤0.05) effect on the specific gravity of the butter cake form and batter (Table 19). The higher amount of HFCS replaced (60, 80 and 100%) for sucrose tended to lower specific gravity of the butter cake form and batter (0.27, 0.25, 0.23 and 0.86, 0.78, 0.76 g/ml, respectively). Measurement of a batter's specific gravity estimates the amount of air incorporated into a batter such that a lower specific gravity is indicative for a batter with more air and viscosity (Penfield and Campbell, 1990a). A viscous batter helps keep air bubbles from rising out of the batter while a less viscous batter with a higher specific gravity allows large bubbles to coalesce, rise up to the surface and leave the batter (Bath *et al*. 1992; Kim and Walker, 1992). The specific gravity of a batter can also be related to a cake's volume (Penfield and Campbell, 1990d). That is, the greater the total cake volume, the less its weight per unit volume and the lower its specific gravity. Adequate air incorporation is necessary to produce a cake of high volume.

2) Cake color

Crust color also significantly affected (P≤0.05) by the replacement of sucrose with HFCS. The Hunter *L**, *a** and *b** values correspond to lightness, redness, and yellowness, respectively. Table 19 shows that the crust color parameter a* and b* values of HFCS butter cake increased with increasing HFCS content. The red and yellowness occurred during baking reaction in 100% HFCS more than 100% sucrose. The crust color of control (100% sucrose) cake was lighter (P≤0.05) and less red (P≤0.05) than all other cakes. Crust *L**, *a** and *b** values were not significantly different for 20, 40, 60 and 80% HFCS cakes. Similar, it has been reported that browning reaction affects color during thermal processing of cookie and bread crust (Wang and Peng, 1998). During the heat treatment, sucrose and starch may hydrolyze respectively into glucose and fructose, or maltose and glucose. In addition, the formed of HFCS that less thermo-stable than sucrose and newly formed

sucrose or maltose are reducing sucroses which can further participate in the caramelisation and the maillard reaction when amino-acid are present (Many, 1998). Both reactions produce brown polymers, which contribute to the surface coloration of cakes. Thus, the crusts of 100% HFCS cakes were darker, redder and yellowness than the other cakes (Figure 29).

Crumbs color; *a**-value and *b** values from the cake crumbs indicated that both the red and yellow color was significant ($P \leq 0.05$) increased obviously due to different amounts of HFCS. The HFCS addition (20, 40, 60, 80 and 80%) increase the internal *a** and *b** value as compared to the control (Table 20). In additional, when the level of HFCS increased, the *L**-value decreased gradually, *a** and *b** values increased, indicating a darker, redder and yellowness crumb obtained from 100% HFCS cake (Figure 29). The stable crust color of the cakes supports the work of Mc-Cullogh (1985) and Jonna *et al*. (1990) that the increased browning of cakes prepared with HFCS replacement for sucrose may be control with the addition of cream of tartar.

3) Volume and symmetry shape

Volume and symmetry shape, as can be seen in Table 20 and Figure 29, the effect of HFCS on volume of butter cakes was significantly different ($P \leq 0.05$). The HFCS cakes were decreased in volumes compared to the 100% sucrose ones (control). Sucrose cake had the highest volume (1880.66 cm^3) with significant difference ($P < 0.05$) from HFCS cakes. However, the replacement of 20%, 40%, 60% and 80% HFCS cakes were not significant difference ($P \geq 0.05$). The HFCS cake volumes were 1864.66, 1864.00, 1863.40, and 1862.33 cm^3, respectively. The 100% HFCS cake was significant ($P \leq 0.05$) lowest (1847.23 cm^3) in cake volume among the six treatments. These results are at variance with those found by Murano and Johnson (1998) and Joanna *et al.* (1990) where 50% and 100% HFCS addition led to cakes with lower volumes than the control, which was 1882.80 to 1828.34 cm^3, respectively.

The decrease in volume of HFCS cakes seems to have two main causes. First is decrease in batter stability during the heating stage – related to batter viscosity decrease and foam bubble size increase. Second is changes in the thermosetting mechanism, due to different interactions among the HFCS- sucrose used and starch-proteins of the batter that affect starch gelatinization and protein denaturation temperatures. A decrease in any of these temperatures is expected to cause a premature thermosetting of protein or starch matrix, which will start at the crust due to direct contact with the heating medium. This, then, lowers the heat transfer rate, and produces a vapor pressure build-up, causing inadequate expansion of individual bubbles (Hicsasmaz *et al*. 2003).

In general, butter cakes with higher volume showed higher central loaf height (Figure 29). The high values indicate that the cake has more height in the centre than in the sides. The 60% HFCS cakes showed the higher central loaf than the control. While when 100% HFCS was replaced, a marked decrease in the central height that agree with pervious studied by Murano and Johnson (1998).

4) Texture profile analysis

Several factors are known to affect the texture of cakes, such as the amount of fat, sucrose, manipulation and liquid. In fact, one of the most important functions of fat and sucrose is to tenderize baked products (Penfield and Campbell, 1990e). Texture profile analysis for fresh butter cake samples was presented in Table 20. The amount of HFCS replacement for sucrose was a significant factor ($P \leq 0.05$) in the texture values of the butter cakes. Significant changes occurred on HFCS butter cake in hardness and chewiness. However, the springiness, cohesiveness, gumminess and resilience were not significantly different ($P \geq 0.05$) among the control and HFCS butter cakes. With total sucrose replacement by HFCS, there was a general decrease in hardness of the cake samples, which was related to the low density of the HFCS cakes as suggested above on specific gravity of the cake batter.

The different effect of HFCS on butter cake hardness could be explained by the higher water content in HFCS than sucrose. Thus, the acceptable cake texture could be obtained from batters with high or low viscosity, although water was kept constant for all formulations. Furthermore, the firmest cake (Table 20) resulted in the least aerated (highest specific gravity as revealed in Table 19).

Table 19 Mean values for specific gravity and color value of batter and baked cakes prepared with high fructose corn syrup (HFCS-55) replacement for sucrose.

HFCS-55 replacement (%)	Specific gravity		Baked cake color					
	Foam	Batter	Crust			Crumb		
	(g/ml)	(g/ml)	*L**	*a**	*b**	*L**	*a**	*b**
0	0.34^{a}	0.96^{a}	62.00^{a}	13.75^{c}	32.10^{c}	79.16^{a}	2.28^{c}	30.72^{d}
20	0.31^{a}	0.94^{ab}	46.51^{b}	15.36^{b}	32.45^{b}	77.43^{ab}	6.70^{b}	34.87^{c}
40	0.30^{a}	0.93^{b}	44.76^{b}	15.67^{ab}	34.23^{b}	73.14^{b}	6.75^{b}	35.21^{c}
60	0.27^{b}	0.86^{c}	44.67^{b}	15.83^{ab}	36.11^{b}	72.78^{bc}	6.78^{b}	37.32^{b}
80	0.25^{bc}	0.78^{d}	43.98^{b}	15.91^{ab}	36.65^{b}	71.46^{c}	7.66^{a}	37.65^{b}
100	0.23^{c}	0.67^{e}	41.07^{c}	17.73^{a}	38.28^{a}	71.42^{c}	7.67^{a}	38.82^{a}

$^{a, b, c\cdots}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's Ducan's New Multiple Range Test (DMRT).

Table 20 Mean values for volume and texture analysis of butter cakes prepared with high fructose corn syrup (HFCS-55) replacement for sucrose.

HFCS-55 replacement (%)	Volume (cm^3)	Texture profile analysis					
		Hard-ness (kg)	Sprin-giness (sec)	Cohes-iveness	Gummi-ness (g.force)	Chewi ness (g.force)	Resili-ence
0	1880.66^{a}	0.89^{a}	0.79^{ns}	0.74^{ns}	0.67^{ns}	0.65^{a}	3.40^{ns}
20	1864.66^{b}	0.64^{b}	0.77	0.77	0.68	0.63^{a}	3.36
40	1864.00^{b}	0.63^{b}	0.73	0.74	0.67	0.54^{a}	3.47
60	1863.40^{b}	0.60^{b}	0.66	0.72	0.64	0.48^{b}	3.13
80	1862.33^{b}	0.58^{b}	0.74	0.77	0.66	0.46^{b}	3.54
100	1847.23^{c}	0.56^{b}	0.71	0.69	0.61	0.43^{b}	3.06

$^{a, b, c\ldots}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New Multiple Range Test (DMRT).

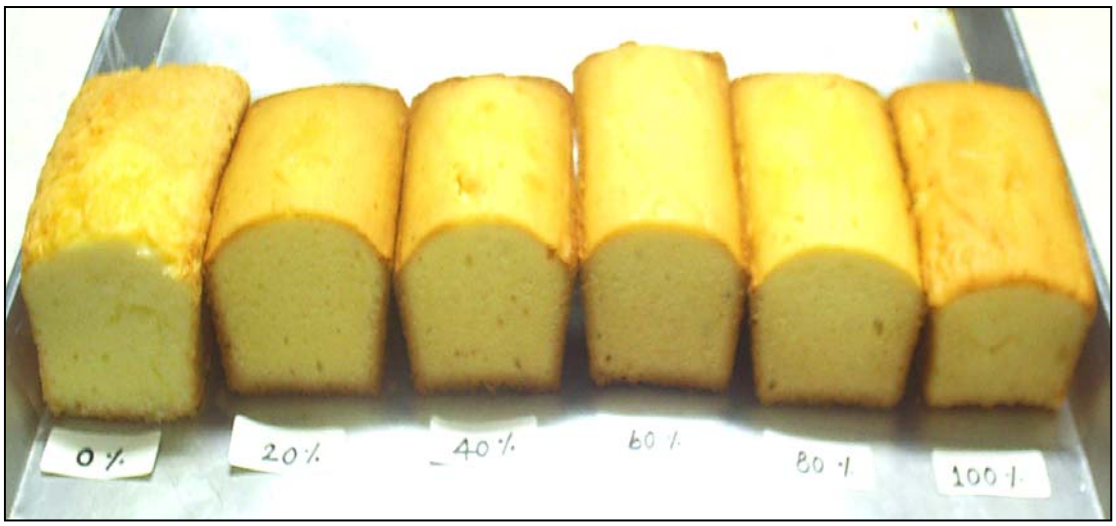


Figure 29 Color and appearances of butter cakes prepared with high fructose corn syrup (HFCS-55) replacement for sucrose at 0, 20, 40, 60, 80 and 100%

2.1.2 Sensory evaluation of HFCS replace of the sucrose in butter cake

According to Bennion (1995a), the food choices that consumers make are influenced by many factors such as income, culture, religion, and health concerns. Yet for most people, most importantly, foods must be palatable, if they are to be eaten. Palatability of foods was determined by different sensory sensations, such as odor, color, taste, and mouth feel. Foods prepared comprise various flavor profiles, partly because individuals vary in their ability to intensify and experience flavor. However, sensitivity to pleasurable encounters with food can be heightened as more about food characteristics is learned. The perceived sensory attributes of foods consist of color appearance, texture (moistness and tenderness), flavor (taste and aroma) and total acceptance (McWilliams, 1993). Panelists in this study generated attributes that fell into the four categories described above. During testing, the sensory panel kept into consideration that high-quality cakes should have a high volume, a moist and tender texture that is not too soggy, sticky or crumbly, and a moderately sweet flavor. The following sections summarize the results.

1) Color

Crust color scores were not significantly different ($P \geq 0.05$) for 0, 20, 40, 60 and 80% HFCS replacement, which was 6.66, 7.26, 6.60, 6.13 and 6.00, respectively. Whereas the crust color scores for 100% HFCS were lowest (4.60) with significant difference ($P \leq 0.05$) from the other treatments. Butter cakes containing only sucrose had yellowish golden crusts, while HFCS cakes had yellow brown crusts. Panelists' scores agreed wit Hunter L^*, a^* and b^* values, which indicated that the crust of butter cakes became more yellow as HFCS content increased and sucrose content decreased (Table 21). Excessive exterior browning in HFCS-containing cakes has been attributed to caramelization (Lin and Lin, 2001). HFCS is high solubility allowed it to decompose and brown rapidly. Sucrose, on the other hand, does not have reducing ability because its reducing groups (glucose's aldehyde carbon and fructose's keto carbon) are tied up (Penfield and Campbell, 1990g).

There was no difference in the sensory scores for crumb color among 20 to 80% HFCS (P≥ 0.05). The crumb color scores of 100% HFCS replace were lower than the other treatments (P≤0.05). The result indicated that the crumb color of 100 % HFCS was yellowish more than those of the other butter cakes. Panelist scores for crumb color agreed with *b** values measured by the color spectrophotometer, which indicated that HFCS yielded a brown yellow crumb color.

2) Texture scores

Texture is an important characteristic in consumer's perception of food and purchasing decisions. Meilgaard *et al*. (1991a) defines texture as the "sensory manifestation for the structure of products in terms of their tactile feel properties measured by the tactile nerves in the surface of the skin of the hand, lips, or tongue (oiliness, tenderness and moistness)". Because the surface of the skin, lips, tongue are more sensitive than other parts of the body, they can detect smaller force, particle size, thermal and chemical differences. Moreover, studies and surveys have shown that certain textures are universally preferred over others (crispy, crunchy and tender.) while others are disliked (tough, soggy and crumbly) (Bennion, 1995a).

a) Moistness score

Panelists' defined moistness (Appendix E) as the amount of wetness perceived within the mouth. The moistness evaluated by panelists among all samples was significant difference (P≤0.05). The results agreed with percentage moisture analysis, which shown the highest moisture content in the 100% HFCS butter cake (Table 22). Panelists assigned the highest moistness score (6.66%) to 100% HFCS butter cake but not significantly different (P≥0.05) from the 60 and 80% HFCS replacement for sucrose (6.13 and 6.40, respectively). Butter cake with 100% sucrose had the lowest moistness score (4.93) on the hedonic scale. Subjective moistness scores correlated with the objective percent moisture values (Table 22): decreasing trend in moistness was generally observed. Moreover, perceived moistness

and objective percent moisture generally increased as the level of HFCS increased. Thus, the HFCS is hydrophillic properties enhanced moisture retention in cakes.

b) Tenderness score

Tenderness was described as the amount of chewing resistance. Significant differences ($P \leq 0.05$) for tenderness were found among the six treatments (Table 22). Mean tenderness scores from panels evaluation was significantly different ($P \leq 0.05$) between HFCS butter cake treatments. Regarding crumb tenderness, 60 and 80% HFCS butter cakes were tendered than 0, 20, 40 and 100% HFCS butter cakes. The tenderness scores were 5.20, 5.46, 5.66, 6.60, 7.13 and 6.73 for 0, 20, 40, 60, 80 and 100% HFCS butter cakes, respectively. The highest score of 40/60% of sucrose-HFCS in butter cake because of HFCS had high humectancy that related to the moisture content of the cakes. Thus, adding an optimum ratio of sucroses and HFCS to a butter cake product would induce tenderness (Philip and Carole, 1982). These results support the work of Joanna (1990) who reported that at the levels of 0 and 50% HFCS replacement for sucrose, fewer differences were observed between cakes made with cake flour and with all-purpose flour. However, when the level of HFCS replacement was increased to 75 and 100%, the use of cake flour produced a cake that was more tender than that prepared with all purpose flour.

Table 21 Mean sensory scores of butter cakes prepared with high fructose corn syrup (HFCS-55) replacement for sucrose

HFCS-55 replacement (%)	Sensory scores					
	Color		Texture		Sweetness	Overall liking
	Crust	Crumb	Moistness	Tenderness		
0	6.66[a *]	5.60[b]	4.93[c]	5.20[b]	5.80[b]	5.86[bc]
20	7.26[a]	6.33[a]	5.73[b]	5.46[b]	6.03[ab]	5.89[bc]
40	6.60[a]	6.26[a]	5.86[b]	5.66[b]	6.23[ab]	6.00[b]
60	6.13[a]	6.66[a]	6.43[ab]	7.60[a]	7.36[a]	7.53[a]
80	6.00[a]	6.53[a]	6.40[ab]	7.13[ab]	6.16[ab]	6.93[ab]
100	4.60[b]	5.13[b]	6.66[a]	6.73[ab]	6.10[ab]	5.26[c]

[a, b, c…] Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New Multiple Range Test (DMRT). Hedonic scale of 1= dislike extremely, 5 = neither like nor dislike, 9 = like extremely.

3) Sweetness scores

Butter cakes with 40 to 60% HFCS replacement for sucrose had the highest mean sweetness scores (6.23 and 7.36, respectively). The sweetness score of 100% sucrose butter cake (5.80) was significantly ($P \leq 0.05$) less than those made with 20, 80 and 100% HFCS (6.03, 6.16 and 6.10 respectively). The effect of HFCS replacement on sweetness could be the reason for the relative sweetness being about 100 – 110 compared to that of sucrose (Alexander, 1998). Bray *et al*. (2004) hyposized that HFCS-55 is much sweeter than sucrose. Consequently, there is a theoretical advantage to using less fructose to provide an equivalent degree of sweetness in baked products which normally contain sucrose. Fructose has an agreeable sweetness with no bitter aftertaste and is claimed to be 15-60% replacement for sucrose (Schallenberger, 1963).

In the dietetic cake mix of Corliss *et al.* (1999), crystalline fructose is combined with baking powder having glucono-delta lactone as the acidic component and emulsifiers such as propylene glycol monostearate and lactated monoglycerides to obtain a product which has an acceptably sweet taste upon baking. In addition, high fructose corn syrup is used to replace the expensive crystalline fructose which is utilized in bakery products (Joanna, 1990).

4) Overall liking scores

The mean overall liking scores of 0, 20, 40, 60, 80 and 100% HFCS replacement for sucrose were 5.86, 5.89, 6.00, 7.53, 6.93 and 5.26, respectively. No difference ($P \geq 0.05$) was found among 60 and 80% HFCS butter cakes in the overall liking scores. The 60% HFCS had highest in total acceptance score, which was moderately like (7.53) by panelist. As can be seen from the results of physical analysis and descriptive liking score, this may be due to the crust and crumb color, where the tenderness and sweetness of 60% HFCS butter cake was higher than the other treatments. However, the overall liking scores of 100% HFCS was lowest but not significantly different ($P \geq 0.05$) from the 0 and 20% HFCS butter cakes. In summarize, the sensory evaluation results showed significant HFCS effect was observed for crust and crumb color, moistness, tenderness, sweetness and overall linking of the butter cakes. Replacement of 60 % sucrose with HFCS did not affect greatly the sensory characteristics studied.

2.1.3 Chemical composition of HFCS replace of sucrose in butter cake

Butter cake is a complex fat and water emulsion system containing flour, sucrose, fat, eggs and baking powder. A proper combination of the ingredients can give a high quality product with desirable flavour, texture and nutritional values.The results of chemical analysis for butter cakes prepared with high fructose corn syrup (HFCS) replacement of sucrose are show in Table 22. The following sections summarize the results.

1) Moisture content

The moisture content (the quantitative determination of total water content) of a food is one indication of a food's stability and quality (Pomeranz and Meloan, 1994). Moistness is a favorable sensory attribute in baked products because it is synonymous with a soft, tender product. However, too much moisture promotes microbial growth (Nonaka, 1997). HFCS, a hygroscopic sweetener, also acts as a humectant by drawing in moisture from the air into the cake. The moisture content was significantly different among the butter cake samples ($P \leq 0.05$). Moisture content of the cake samples were between 18.21 – 27.15%. As the HFCS addition level increased, the moisture content increased gradually. The cakes with HFCS were more hygroscopic because of the humectants nature of the syrup. In this study the cakes with 100% HFCS as a replacement for sucrose had the highest moisture content, which was 27.15%. The major difference between sucrose and HFCS-55 is their moisture content (5% versus 23%, respectively) (Hanover and White, 1993). The high moisture content in the cake samples were contributes to the moistness of the butter cakes (Table 21). This is in agreement with other findings of Joanna *et al*. (1990).

2) Protein, crude fat and ash content

As expected for protein, crude fat and ash content (expressed in wet base) were the same in both butter cakes (Table 21). No difference (($P \geq 0.05$) in fat content (25.06 – 25.43%) was detected between the butter cake samples, which are in accordance with the equivalent butter level used in both formulations. Similarly, the incorporation of HFCS did not produce a noticeable change in the final protein content, which was 12.45 – 12.89%. This type of product was generally characterized by medium protein contents and, evidently, the actual level depends on the formulation employed. Butter cake formulated with HFCS show no difference (($P \geq 0.05$) in ash content (2.70-2.86 %) was detected between cakes, reflecting the low mineral level in this ingredient.

3) *In vitro* starch digestibility

In vitro starch digestibility results (Table 22) showed non-significant HFCS effect was observed for digested starch (DS), resistant starch (RS) and total starch (TS) in the butter cake samples. DS values ranged from 30.30 to 31.86% on a dry matter basis. Small amounts of RS were detected in the butter cake samples ranging from 1.39 to 1.45% on a dry matter basis. Total starch content in the HFCS cakes was lower than in the control cake (31.66 to 32.28% on a dry matter basis), however it were not significantly different ($P \geq 0.05$). Nevertheless, it can be concluded that HFCS did not improve the resistant starch content in butter cake. Lin *et al*., (1994) revealed that the endothermic transition temperature for melting of the amylose-lipid complex was delayed by a high concentration of sucrose. Neither the melting temperature nor the enthalpy of the amylose lipid complex (110 ^{o}C) was significantly changed when the cake samples were stored for 7 days.

The content of resistant starch formed in the butter cakes is probably retrogradation of amylose lipid complex. Because the high values reported for fat content (25%) may contribute to the formation of starch/fatty acid complexes because butter was included in the formulation. In addition, it is generally accepted that amylose retrogrades very quickly after gelatinization. Judiha and Melissa (1996) found that RS content in home-prepared cake was present immediately after baking, and contained about 1.7g/ serving (95 g) of resistant starch content.

Table 22 Mean values for chemical composition of butter cakes prepared with 55-high fructose corn syrup (HFCS-55) replacement for sucrose

HFCS-55 replacement (%)	Moisture content (%)	Fat (%)	Protein (%)	Ash (%)	*In vitro* starch digestibility		
					DS (%)	RS (%)	TS (%)
0	18.21[e]	25.01[ns]	12.59[ns]	2.85[ns]	30.27[a]	1.39[a]	31.66[a]
20	18.67[e]	25.23	12.62	2.76	30.92[a]	1.41[a]	32.43[a]
40	19.43[d]	25.36	12.45	2.78	30.86[a]	1.42[a]	32.28[a]
60	21.94[c]	25.08	12.77	2.86	30.30[a]	1.45[a]	31.75[a]
80	24.28[b]	25.06	12.89	2.78	31.76[a]	1.44[a]	32.10[a]
100	27.15[a]	25.43	12.87	2.70	31.86[a]	1.42[a]	32.28[a]

[a,b,c…] Means within the same column with different letters are significantly different (P≤ 0.05) by Ducan's New Multiple Range Test (DMRT).

[ns] = Not significantly different (P≥ 0.05)

2.1.4 *In vitro* starch hydrolysis rate and its kinetic constant

The *in vitro* starch hydrolysis rate represents the proportion of starch that is theoretically digestible (under the conditions of the study) (Ezeogu *et al*. 2005). All butter cake samples showed a slowly rate of starch digestion compared to the white bread (Figure 30). Meaning that at all sampling times, lower percentages of starch were hydrolyzed in the butter cake products compared to the white bread sample (Table 23 and Figure 30). The hydrolysis rate of HFCS-butter cake samples was not significantly different (P≥0.05) from control (sucrose- butter cake) samples (62.26 to 63.65% at 180 min hydrolysis time). This explains the similarly in digestion rate was due to the same amount of digested starch content in the butter cake samples. Meanwhile, the white bread that analyzed as a reference food showed a higher hydrolysis rate (76.95% at 180 min hydrolysis time) than that of the butter cake samples. These due to the higher content of wheat starch in the bread formula than the butter cake formula.

Table 23 Percentage (dwb) of starch hydrolyzed at different times (min), calculated Equilibrium concentration (C_∞) and Kinetic constant (k) for each type of butter cakes prepared with HFCS-55 replacement for sucrose

HFCS-55 replacement (%)	% Starch hydrolysis rate at various time (min)						C∞	k
	0	30	60	90	120	180		
0	3.70^{ab}	36.95^{b}	54.32^{b}	62.53^{b}	62.80^{b}	63.65^{b}	62.99^{b}	0.041^{ab}
20	2.97^{b}	35.54^{b}	53.63^{b}	62.44^{b}	62.86^{b}	62.99^{b}	62.76^{b}	0.054^{a}
40	2.84^{b}	35.59^{b}	52.35^{b}	62.56^{b}	62.86^{b}	62.96^{b}	62.79^{b}	0.054^{a}
60	2.70^{b}	35.11^{bc}	50.10^{c}	61.04^{b}	62.66^{b}	62.84^{b}	62.51^{b}	0.054^{a}
80	2.78^{b}	35.13^{bc}	49.14^{c}	61.42^{b}	62.48^{b}	62.74^{b}	62.21^{b}	0.055^{a}
100	2.84^{b}	34.59^{c}	48.58^{c}	61.75^{b}	62.26^{b}	62.26^{b}	62.22^{b}	0.056^{a}
White bread	4.81^{a}	55.78^{a}	72.5^{a}	76.54^{a}	76.80^{a}	76.95^{a}	76.76^{a}	0.038^{b}

$^{a,b,c\ldots}$ Means within the same column with different letters are significantly different (P≤ 0.05) by Ducan's New Multiple Range Test (DMRT).

The kinetic parameters that describe the hydrolytic process of starch digestion were obtained (Table 23). C∞ represents the equilibrium concentration, reached after 180 min of hydrolysis and the constant "k" stands for the kinetic (digestibility) constant (i.e. intrinsic susceptibility of the starch in the product to digestion). The equilibrium concentration (C∞) of the hydrolyzed starch in butter cake was not significantly different (P≥0.05) by HFCS replaced for sucrose (62.22 to 62.99 %). However, the HFCS-cakes slightly lower C∞ value than the sucrose-butter cake. The kinetic constant from the butter cake samples was not significantly different (P≥0.05) among the butter cake samples. Sucrose-cake and HFCS-cake had similar starch digestion patterns and gave similar kinetic constants (the intrinsic susceptibility of the starch in the products to digestion was similar) between 0.042 to 0.056. This finding suggests that using the refined fractions of wheat flour and sucrose enhanced

the starch digestibility (fewer amounts of other flour components such as fiber, lipids, ash, minerals).

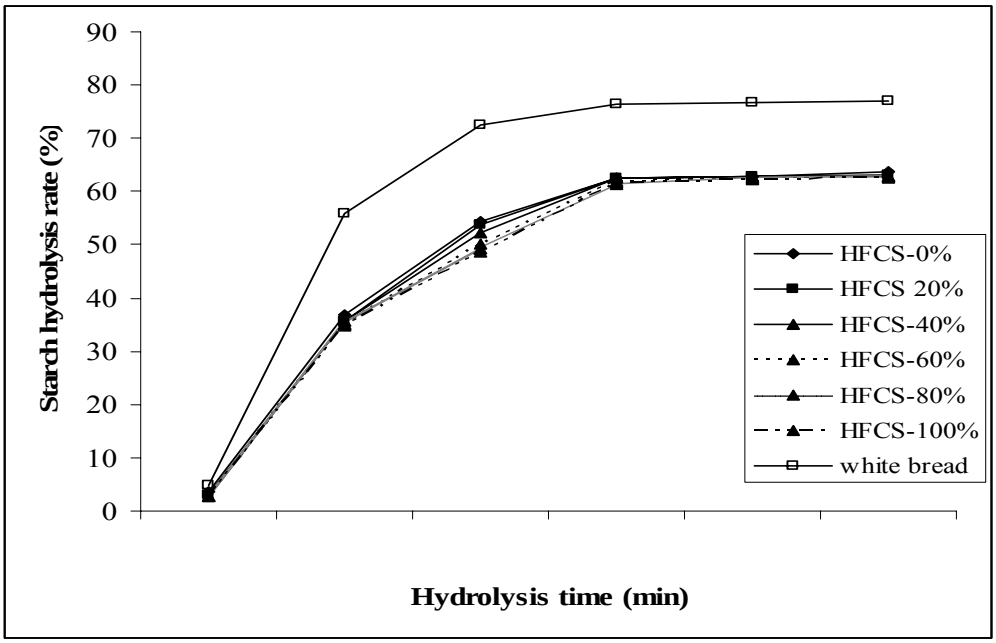


Figure 30 Starch hydrolyzed rate at different times (min),of butter cakes prepared with 55-high fructose corn syrup (HFCS-55) replacement for sucrose and white bread as a referent food.

2.1.5 Hydrolysis index and glycemic index

The starch hydrolysis index (HI) and estimated glycemic index (GI) of the butter cake samples are showed in Table 24. The HI was used in the formula by Goñi *et al*. (1997) to obtain the GI. The GI of all butter cake products was significantly different (P≤ 0.05). The 100% HFCS-butter cake show lowest value of GI (68.77/100 g sample). While, the 100% sucrose butter cake had highest value of GI (77.24/100 g sample).

The factor with slightly effect on butter cake GI values was the HFCS used. Jenkines *et al*. (1981) and Foster *et al*. (2002) revealed that HFCS had lower GI value (89/100g sample) than sucrose (92/ 100g sample). In addition Yang *et*

al. (2006) reported that when glucose was used as the reference (GI of glucose: 100), the GI rank of monosaccharide from high to low was maltose (105.0 ± 5.7) > white sucrose (83.8 ± 12.1)> honey (73.5 ± 13.3) > lactose (46.0 ± 8.0) > fructose (23.0 ± 4.6).

Englyst *et al*. (1999) reported that the rate of digestion of the food was an important determinant of glycemic response. In addition, the nature of the starch, particle size and presence of fiber, fat and proteins were all found to result in difference in the glycemic response (Krezowski *et al*.1986 and Slavin *et al*. 1997).

Jenkins *et al*. (2002) revealed that the glycemic indices of cake products vary continuously from about 66 to over 104. Angel food cake and cub cake show a high GI (95 and 104%). Banana cake, butter cake, sponge cake and cupcake, strawberry-iced give a medium GI (66 to 77%). The GI values of the both tested cake samples were based on white bread, where it is set to equal 100 of hydrolysis index.

In summerised, high fructose corn syrup (HFCS-55% fructose) was used to replace 0, 20, 40, 60, 80 or 100% for the sucrose in butter cake to reduced glycemic index and improved the moistness of the cake. The sucrose replacement was done in a combination of the foam part of the cake. Replacement of HFCS up to 60% of total sucrose had no significant affects on physiochemical properties and sensory evaluation of the butter cakes. Replacement of 60% sucrose with HFCS produced produce cakes that were softer and slightly lower glycemic index value than the control cakes (100% sucrose) which had highest score on overall liking from panelist. Thus, the formulation with 60% HFCS replacement for sucrose was selected for the next experiment.

Table 24 Area under curve (AUC), hydrolysis index and glycemic index (GI) value from ingredients of butter cakes prepared with high fructose corn syrup (HFCS-55) replacement for sucrose.

HFCS-55 replacement (%)	AUC*	Hydrolysis index (%)	GI value** (%)
0	1777.42 ± 91^{b}	63.20^{b}	77.24 ± 0.97^{b}
20	1423.32 ± 15^{c}	50.61^{c}	69.77 ± 0.84^{c}
40	1424.46 ± 42^{c}	50.65^{c}	69.79 ± 4.99^{c}
60	1417.84 ± 36^{c}	50.41^{c}	69.65 ± 5.16^{c}
80	1390.79 ± 24^{d}	49.45^{d}	69.08 ± 3.85^{c}
100	1376.33 ± 94^{d}	48.94^{d}	68.77 ± 5.80^{d}
White bread	2812.47 ± 34^{a}	100.00^{a}	99.10 ± 9.99^{a}

$^{a,b,c\cdots}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New Multiple Range Test (DMRT).

* Parameter of the kinetic equation $AUC = C_{\infty}(t_f - t_0) - (C_{\infty}/K)(1-e^{-kt})$

** GI value $= 39.71 + (0.549 \times HI)$

2.2 The effect of using RS III with flour substituted on butter cake qualities

Enzyme-resistant starch type III (RS III) from high amylose rice starch in the first experiment was replaced at 0, 5, 10, 15 and 20% of cake flour. The effect of RS III replacement on physicochemical properties, sensory evaluation, *in vitro* starch digestibility, starch hydrolysis rate and estimated glycemic index in the developed butter cakes are summarized as following section.

2.2.1 Physical properties of butter cake with RS III replace of cake flour

Effect of using RS III with flour substituted on physical properties of butter cake was studied by investigating specific gravity of cake batter and specific

volume, crust and crumb color of baked cake. The following sections summarize the results.

1) Specific gravity values

Table 25 shows the mean specific gravity values of the five cake batter treatments. Significant differences ($P \leq 0.05$) were found among treatments. The mean specific gravity values were between 0.68 to 0.77 g/ml. RS III added in cake flour is speculated to bind water and control viscosity, which aids in gas retention (Sobczynska and Setser, 1991). In fact, as the percentage of cake flour was replaced with increased levels of RS III, the average specific gravity increased. Since the lower specific gravity ratios, theory predicts a higher volume cake. Specific gravity is a measurement of air incorporated into a cake batter during mixing. This air, along with baking powder gasses released during baking, determines the cake texture and volume (Tenbergen, 2003).

2) Specific volume

RS III was affected on specific volume of butter cake ($P \leq 0.05$) as shown in Table 25 and Figure 31. The result revealed that butter cake with RS III replace of wheat flour had lower specific volume than control (100% cake flour). Due to flour affected the physical structure of baked products by regulating gelatinization of starch. Delay in starch gelatinization during baking allows air bubbles to expand properly due to vapor pressure build up by carbon dioxide and water vapor before the cake sets (Kocer, 2007).

In theory, the decrease in batter cake expansion has two causes: 1) decrease in batter stability during the heating stage that related to batter viscosity decrease and foam bubble size increase 2) changes in the thermosetting mechanism, due to different interactions among the bulking agent used and starch. Proteins of the batter that affect starch gelatinization and protein denaturation temperatures. A decrease in any of these temperatures is expected to cause a premature thermosetting of protein or starch matrix, which will start at the crust due to direct contact with the

heating medium. Then, lower the heat transfer rate causing inadequate expansion of individual bubbles (Ronda *et al.* 2005). The batter was probably not sufficiently viscous to minimize the coalescence of gas bubbles, which causes loss of leavening gases found during baking as large bubbles rise to the surface and escape (Penfield and Campbell, 1990).

In addition, the greater rising volume for the wheat flour cake over the mixed RS III cake probably resulted from the higher ability of gluten to trap CO_2 gas liberated from baking powder. So far, these properties of gluten have not been completely achieved in the RS III from rice starch mixtures tested.

3) Cake color

Crust color values are illustrated in Table 25 and Figure 31. With respect to crust *L** values, control (0% RS III) was significantly whiter ($P \leq 0.05$) than treatment of 10%, 15% and 20% RS III replacement for cake flour, but not significantly different ($P \geq 0.05$) from the 5% RS III replaced treatment. The 5% RS III replacement was significantly whiter ($P \leq 0.05$) than 15% and 20% RS III treatments, but not significantly whiter than 10% RS III replacement. Crust *a* and b** values displayed significant differences ($P \leq 0.05$): treatment with RS III replacement was more yellow compared to control (100% wheat flour) cakes. Overall, when comparing *L* a** and *b** values, control produced the lightest crust and RS III replacement produced the darkest crust. RS III replacement cake flour resulted in the darkest crust because the reducing end in the debranched high amylose rice starch prior RS III formation is promote Maillard browning reaction. This reaction involves the interaction of amino acid and reducing sucrose in HFCS.

Crumb color values are presented in Table 25 and Figure 32. Significant differences ($P \leq 0.05$) were observed in *L** and *b** values among cake treatments. Results of the crumb *L** values indicated that control treatment (100% cake flour) was significantly lighter ($P \leq 0.05$) than the other cake treatments. The crumb *b** values showed that 20% RS III replacement was significantly more yellow

(P≤0.05) when compared to the other cake formulations; and that treatment had a significantly (P≤0.05) yellowish crumb. Overall, both *L** and *b** values indicated that the 20% RS III replaced treatment produced a significantly (P≤0.05) darker crumb when compared to the other treatments. It is interesting to note, however, 20% RS III replaced that, which produced a darker crust, resulted in light crumb values (Table 24).

Table 25 Mean values for specific gravity and color value of batter and baked cakes prepared with RS III replacement for cake flour at 0, 5, 10, 15 and 20% with an incorporated of 40:60 of sucrose: HFCS

RS III replacement (%)	Specific gravity (g/ml)	Baked cakes						
		Volume (cm^3)	Crust color			Crumb color		
			*L**	*a**	*b**	*L**	*a**	*b**
0	0.68^{a}	1833.66^{a}	49.81^{a}	15.34^{c}	34.94^{b}	76.27^{a}	6.62^{ns}	35.42^{b}
5	0.69^{a}	1827.00^{b}	49.45^{a}	16.39^{b}	35.29^{b}	75.99^{a}	6.71	36.75^{b}
10	0.70^{a}	1830.33^{b}	49.02^{ab}	16.70^{b}	35.49^{b}	74.96^{b}	7.44	36.73^{b}
15	0.75^{b}	1826.00^{c}	48.82^{b}	16.69^{b}	36.71^{a}	74.19^{b}	7.45	36.67^{b}
20	0.77^{b}	1814.00^{d}	47.21^{c}	17.89^{a}	37.08^{a}	73.94^{b}	7.81	37.88^{a}

$^{a,b,c\cdots}$ Means within the same column with different letters are significantly different (P≤ 0.05) by Ducan's New Multiple Range Test (DMRT).

^{ns} = Not significantly different (P≥ 0.05)

Figure 30 Crust color appearances of butter cakes prepared with RS III replacement for cake flour at 0, 5, 10, 15 and 20% with an incorporated of 40:60 of sucrose: HFCS

Figure 31 Crumb color appearances of butter cakes prepared with RS III replacement for cake flour at 0, 5, 10, 15 and 20% with an incorporated of 40:60 of sucrose: HFCS

2.2.2 Sensory evaluation of RS III replace of wheat flour in butter cake

The results of sensory evaluation of butter cake prepared with high RS III replacement for cake flour are presented in Table 26. For measuring product liking and preference, the 9-point hedonic scale is a unique scale, providing results that are both reliable and valid (Stone and Sidel, 1993). The perceived sensory attributes of butter cake consist of color appearance, texture (moistness and tenderness), flavor (sweet ness and aroma) and total acceptance. Panelists in this study generated liking score that fell into the four categories described above. During testing, the sensory panel kept into consideration that high-quality cakes should have a golden color, a moist and tender texture that is not too soggy, sticky or crumbly, and a moderately sweet flavor. The following sections summarize the results.

1) Color appearance scores

Color appearance scores of the butter cake samples were significantly different ($P \leq 0.05$) for 0, 5, 10, 15 and 20% RS III replacement for cake flour, which was 7.20, 6.40, 6.73, 6.86 and 6.40, respectively (Table 26). The control butter cake (100% cake flour) had highest score, which was moderated like by panelist. While in the treatment with RS III replacement for wheat flour were little like and not significantly different ($P \geq 0.05$) among for four level RS III replacement. However, the 15% RS III replacement for cake flour had highest score among four treatments. The color appearance of food is very important because the consumers' purchasing decisions were largely based on the expected color appearance of certain foods. Often, the sensory attributed seemed most critical in foods is color. In bakery products, uniform, golden brown crusts are desired (McWilliams, 1993b). Browning of cakes occurs in the crust and crumb, but is most apparent in the crust of a cake. Browning, a result of Maillard reaction and some caramelization, occurs most rapidly when monosaccharides are contained in a cake (McWilliams, 1993d).

2) Texture score

a) Moistness score:

Panelist has defined moistness as the amount of wetness perceived within the mouth. Significant differences (P≤0.05) for moistness (5.30 to 6.93) were found among five treatments (Table 26).The panel rated 20% RS III replacement as the driest (P≤0.05) cake, while control treatment (100% cake flour) was found to be significantly more moist (P≤0.05) when compared to the other treatments. Meanwhile, control did not differ significantly (P≥0.05) from 5% and 10% RS III replaced treatments. Treatment with 15% substitute cake flour was also not significantly different (P≥0.05) from the other treatments. In general, all of the cakes were found to be relatively moist because even the high level of RS III substitute cake flour (20%), had a mean moistness score of 5.30. Subjective moistness scores correlated with the objective percent moisture values (Table 26): decreasing trend in moistness was generally observed. Moreover, perceived moistness and objective percent moisture generally decreased as the level of RS III increased.

b) Tenderness score

Tenderness was described as the amount of chewing resistance. Significant differences (P≤0.05) for tenderness were found among the five treatments (Table 26). Control was rated the tenderest cake (6.85), but was only significantly difference (P≤0.05) from the 20% RS III replaced treatment (5.66). Overall, 5% to 15% RS III replaced treatments were considered tender (sensory scores between 6.66 and 6.86). As expected, tenderness scores decreased as RS III was increased up to 20% replaced. Lin *et al*.(1994) found that replacement of 5 % of the flour in a yellow cake formulation with resistant starch produced cakes that were softer than the control. Replacing 15% of flour by RS (without reducing amount of shortening) had no significant effect on cake quality.

3) Flavor score

Flavor is the most important factor consumers consider when shopping for food (Bruhn *et al*. 1992). Flavor sensations are produced when salty, sweet, sour, or bitter substances dissolved in solution are detected by the taste buds, while aroma or odor is smell detected by the nose receptors. Panelists measured the perception of sweetness and aroma are presented in the following section.

a) Sweetness score

The preference for sweet foods develops at an early age. However, the perception of sweetness varies individually, due to genetics, concentration of the sweetener, temperature, viscosity, and pH (Bennion, 1995). In general, research has shown that individuals have a low sensitivity for sucrose (Meilgaard *et al*. 1991b). Non-significant differences ($P \geq 0.05$) for sweetness were found among butter cake treatments (Table 26). A change in the degree of sweetness was perceived in butter cakes with greatest in 20% cake flour replacement. That is, a control treatment was less like on sweetness (6.40) than the other treatments. However, cake formulations that contained RS III was slightly sweeter than control (100% cake flour). Overall, 5% through 20% RS III replacement for cake flour were perceived as moderately like on sweetness taste. However, sweetness scores for all treatments were rated as being bland (6.40 to 6.96). It appears that RS III is tasteless, has no satiating effect and acts as a mild laxative (Rössler *et al.*, 2002).

b) Aroma score

Non-significant differences ($P \geq 0.05$) for aroma score were found among five butter cake treatments (Table 26). Panelist gave a score for butter cake prepared from 0, 5, 10, 15 and 20% RS III replacement for cake flour were 5.86, 5.66, 6.60, 6.70 and 6.06, respectively. It had a typical RS III aroma without sour, musty, or other objectionable odors. The flavor was bland and was of typical rice flavor with no rancid or off flavors (Anonymous, 2003).

4) Overall liking

The mean overall liking scores are presented in Table 26 for each of five formulations of the butter cake product. The formulation with the highest (6.96) overall liking score was formulation with 15% RS III replacement for cake flour that was not significantly different (P≥0.05) from the 10% RS III replaced treatment (6.83). However, formulation with 5% RS III replacement had the next highest overall liking score (6.63) with not significantly (P≥0.05) from control and 20% RS III replacement that had an overall liking score 6.53 and 6.06, respectively. The lowest score on overall liking in 20% RS III replace cake flour could be due to the sample had sandiness crumb. However, RS III used in this experiment that produce from high amylose rice starch, which was contained of about 20% resistant starch content. It has been experimentally determined that the amount of resistant starch III that can be added to a product varies above 11%, creation of a product acceptable in organoleptic evaluation was unable to be made (Anonymous, 2003).

Table 26 Mean sensory scores of butter cakes prepared with RS III replacement for cake flour at 0, 5, 10, 15 and 20% with an incorporated of 40: 60 of sucrose: HFCS.

% RRS III replacement	Sensory scores					
		Texture		Flavor		
	Color appearance	Moist-ness	Tender-ness	Sweetness	Aroma	Overall liking
Control (0)	7.20[a]	6.93[a]	6.85[a]	6.40[b]	5.86[b]	6.53[b]
5	6.40[b]	6.73[a]	6.66[a]	6.56[a]	5.66[b]	6.63[b]
10	6.73[b]	6.86[a]	6.75[a]	6.66[a]	6.60[a]	6.83[ab]
15	6.86[b]	6.43[ab]	6.83[a]	6.86[a]	6.70[a]	6.96[a]
20	6.40[b]	5.30[b]	5.66[b]	6.96[a]	6.06[a]	6.06[b]

[a,b,c....] Means within the same column with different letters are significantly different (P≤ 0.05) by Ducan's New Multiple Range Test (DMRT). Hedonic scale of 1= dislike extremely, 5 = neither like nor dislike, 9 = like extremely.

2.2.3 Chemical composition of RS III replaced of the flour in butter cake

The moisture content, fat, protein, ash and *in vitro* starch digestibility in butter cake sample using RS III substituted cake flour at 0, 5, 10, 15 and 20% with an incorporated of 40:60 of sucrose: HFCS were analyzed. The following sections summarize the results.

1) Moisture content

The moisture content (the quantitative determination of total water content) of a food is one indication of a food's stability and quality (Pomeranz and Meloan, 1994). An increase in moisture content was observed as the percentage of cake flour was replaced with the RS III (Table 27). However, significant differences ($P \leq 0.05$) were only evident when comparing treatments with 10, 15 and 20% RS III replaced (25.24, 25.37, and 25.60, respectively) to control (23.49%). No significant differences ($P \geq 0.05$) were seen with 5% RS III replaced treatments (24.82) to control (23.49%). Overall, control (100% cake flour) had lower percent moistures content than RS III replaced treatments. The higher moisture content of the cake crumb of the cake flour-replaced samples apparently was caused by the higher water-holding capacity of RS. Lin *et al.* (1994) reported that resistant starch (RS) prepared from amylomaize VII absorbed much more water (water-holding capacity 2.5 ml/g) than did wheat flour (0.5 ml/g).

2) Protein, crude fat and ash content

As expected for protein, crude fat and ash content were slightly different among butter cake samples (Table 27). Crude fat content in RS III replacement for cake flour was slightly less than the 100% cake flour. No significant difference ($P \geq 0.05$) in fat content (25.06 – 24.72%) was detected between 0 to 20% RS III replaced flour in the butter cake samples. Similarly, the incorporation of 5, 10 and 15% RS III did not significant difference ($P \geq 0.05$) from control (100% cake flour) in the final protein content, which was 12.12, 11.43, 11.28 and 12.37%,

respectively. However, the 20% RS III replaced was significant ($P \leq 0.05$) lower in protein content (10.84%) than the other treatments. This may be the result of protein content in RS III was lower (1.18 ± 0.06%) than cake flour (6 - 7%). Butter cake formulated with RS III replacement for cake flour showed no significant difference ($P \geq 0.05$) in ash content (2.62 – 2.27 %) was detected between the butter cake samples.

3) *In vitro* starch digestibility

Digestible starch (DS), resistant starch (RS) and total starch (TS) of the butter cake with 0 to 20% RS III substituted for cake flour are shown in Table 26. The butter cake with RS III substituted had a significantly ($P \leq 0.05$) lower amount of DS due to the substitution of the RS III fraction for the refined wheat flour. The DS content in 0, 5, 10, 15 and 20% RS III replaced were 31.23, 30.84, 29.97, 28.64 and 28.73, respectively. As expected, RS content in RS III-butter cake (5 - 20% replaced) was markedly higher than in the control butter cake. The RS content of the wheat cake was similar to previous experiment (1.79%). RS contents of RS III-supplemented butter cake increased significantly ($P \leq 0.05$) as the RS III addition level increased, which was 2.10 to 4.40%). These values indicated that RS III contributes significantly to the final butter cake RS content and confirm that the baking process dose not alter this indigestible component.

Table 27 Mean values for chemical composition and energy value of butter cakes prepared with RS III replacement for wheat flour at 0, 5, 10, 15 and 20% with an incorporated of 40: 60 of sucrose: HFCS.

RS III replacement (%)	Chemical composition (%)				*In vitro* starch digestibility		
	Moisture content	Fat	Protein	Ash	DS (%dwb)	RS (%dwb)	TS (%dwb)
0	24.06^{b}	25.06^{a}	12.37^{a}	2.62^{a}	31.23^{a}	1.79^{d}	33.03^{a}
5	24.82^{ab}	24.96^{a}	12.12^{a}	2.84^{a}	30.84^{b}	2.10^{c}	32.95^{a}
10	25.24^{a}	24.89^{a}	11.43^{a}	2.92^{a}	29.97^{c}	2.74^{c}	32.72^{a}
15	25.37^{a}	24.76^{ab}	11.28^{ab}	2.96^{a}	28.64^{cd}	3.91^{b}	32.56^{ab}
20	25.53^{a}	24.07^{b}	10.84^{b}	2.27^{b}	27.73^{d}	4.44^{a}	32.17^{b}

$^{a,b,c\ldots\ldots}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New Multiple Range Test (DMRT).

4) Starch hydrolysis rate and estimated glycemic index

a) Starch hydrolysis rate

Average hydrolysis curves of butter cake prepared with RS III replacement for wheat flour were depicted in Table 28 and Figure 33. The corresponding hydrolysis parameters, hydrolysis index and estimated glycemic index value are summarized in Table 28. While bread, used as reference, showed a digestion value of about 76.76% after 180 min, which agrees with the values reported by Goni *et al*., (1997). Hydrolysis rate was markedly slower for 10 to 20 % RS III-butter cake and faster for the 100% wheat flour-butter cake. The lower glucose released in the RS III substituted-butter cakes are noteworthy, since the digestion experiment was performed at RS III level. Thus, the presence of RS III in the butter cake seems to affect the susceptibility to glucose released in starch composition of the butter cakes. The starch hydrolysis rate from 0 to 180 min digestion time for 0, 5, 10 and 15% RS III replaced for wheat flour in butter cake were 3.70 to 67.65%, 2.97 to 64.86%, 2.86

to 59.99%, 2.79 to 55.96 and 2.78 to 53.04%, respectively. While, the raw RS III from high amylose rice starch had a lowest starch hydrolysis rate from 0 to 180 min between 1.53 to 43.61%.

The equilibrium concentration (C∞) of the hydrolyzed starch in butter cake was significantly different (P≤0.05) by RS III replaced for wheat flour. However, the 20% RS III-cakes greatest lower C∞ value than the wheat flour-butter cake. The kinetic parameters that describe the hydrolytic process of starch digestion were obtained (Table 27). The wheat flour-butter cake and the 5 and 10% RS III-substituted butter cake gave slightly lower kinetic constants than white bread sample but not significant difference (P≥0.05). The 15 and 20% RS III-substituted flour in butter cake had significantly (P≤0.05) lower *k* value then the control butter cake. While, the RS III from high amylose rice starch had lowest in *k* value (0.022). This suggests that the addition of RS III had altered the intrinsic susceptibility of the starch component in butter cake to digestion. Therefore, it confirmed that differences in the digestibility of the RS III-butter cake were due to a fraction of enzyme resistant starch type III from high amylose rice starch.

Thus, hydrolysis rate was markedly slow for RS III-butter cake and faster for the control butter cake. The low and slow hydrolysis rate of starch in the 20% RS III-butter cake is noteworthy, since the digestion experiment was performed at equivalent of digested starch content in the butter cakes. Analysis data from starch hydrolysis rate was used to calculated area under curves, hydrolysis index and estimated glycemic index in the developed butter cake samples in the following section.

Table 28 Percentage (dwb) of starch hydrolyzed at different times (min), calculated Equilibrium concentration (C_∞) and Kinetic constant (k) for butter cakes prepared with RS III replacement wheat flour.

RS III replacement (%)	% Starch hydrolysis rate at various time (min)						C∞	k
	0	30	60	90	120	180		
0	3.70^{b}	36.95^{b}	55.32^{b}	66.92^{b}	67.72^{b}	67.65^{b}	67.44^{b}	0.034^{a}
5	2.97^{b}	34.54^{c}	54.63^{b}	63.44^{c}	64.86^{c}	64.90^{c}	64.40^{c}	0.034^{a}
10	2.86^{bc}	32.19^{d}	52.35^{c}	58.76^{d}	59.82^{d}	59.99^{d}	59.52^{c}	0.030^{a}
15	2.79^{c}	30.11^{e}	49.96^{d}	55.46^{e}	55.71^{e}	55.96^{e}	55.71^{d}	0.029^{a}
20	2.78^{c}	31.13^{e}	46.14^{e}	49.45^{f}	52.58^{f}	53.04^{f}	51.69^{e}	0.024^{b}
Raw RS III	1.53^{d}	18.43^{f}	32.04^{f}	42.78^{g}	42.93^{g}	43.61^{g}	43.11^{f}	0.022^{c}
White bread	4.81^{a}	55.78^{a}	72.50^{a}	76.54^{a}	76.8^{a}	76.95^{a}	76.76^{a}	0.038^{a}

$^{a,b,c\ldots\ldots}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New Multiple Range Test (DMRT).

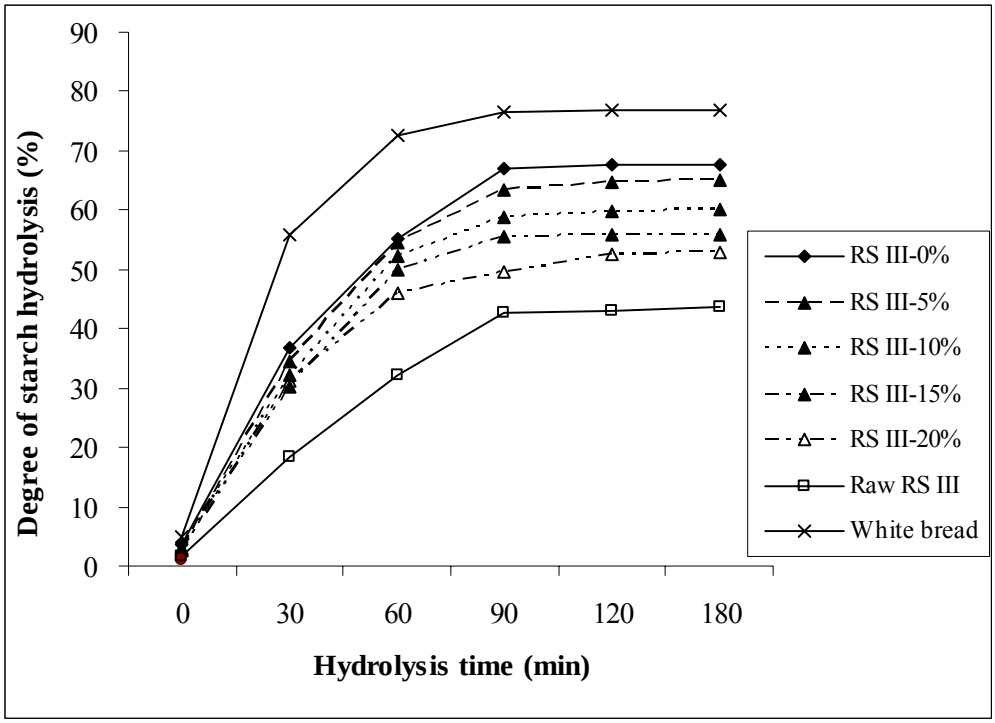


Figure 33 Average hydrolysis curves of butter cake prepared with RS III replacement for wheat flour in butter cake product.

b) Hydrolysis index and glycemic index

Starch hydrolysis index (HI) of each butter cake was obtained by using an Area Under Curve (AUC) in the starch hydrolysis rate from 0 to 180 min of the butter cake samples divided by AUC of white bread. The glycemic index (GI) value of each sample was obtained by using the starch hydrolysis index in the formula by Goni *et al*. (1997). The butter cake with the added RS III had a significantly lower HI compared to the wheat flour cake. A HI of 62.95% was determined in wheat flour cake, which significantly different ($P \leq 0.05$) from that of 5, 10, 15 and 20 % RS III replaced wheat flour-cake (Table 29). The hydrolysis index of 5, 10, 15 and 20 % RS III replaced wheat flour-cakes were 55.72%, 47.90%, 44.74% and 43.57%, respectively. These results showing that glucose liberation from RS III added butter

cake is lower in these samples than in wheat flour cake. This pattern is important in the mechanisms governing post-prandial glycemia for wheat flour based-products.

Estimated glycemic index (GI) value for the 5, 10, 15 and 20% RS III substituted flour in butter cake samples was below that determined in the control butter cake (Table 29). The GI value for 0, 5, 10, 15 and 20% RS III substituted flour in butter cake samples were 71.80%, 69.65%, 68.16%, 66.16% and 65.58%, respectively. Estimated GI value suggests as an important ‘‘slow digestion’’ features for the RS III-cake, which is in line with perceived health-beneficial characteristics of RS III (Asp *et al*. 1996; Champ *et al*. 2003). RS III is an accompanying feature of low-GI foods. When plotting the RS III of 10 food items and their GI value, a very high correlation was seen (Bjorck *et al*. 2000). For starch food products, a reduction in GI appears to be accompanied by a higher content of RS III (Akerberg *et al*. 1998).

The glycemic index (GI) is a numerical system of measuring how much of a rise in circulating blood sucrose a carbohydrate triggers, the higher the number, the greater the blood sucrose response. Therefore, a low GI food will cause a small rise, while a high GI food will trigger a dramatic spike. A list of carbohydrates with their glycemic values was shown below. A GI thay 70 % or more is high, a GI of 56 to 69% inclusive is medium, and a GI of 55% or less is low (Jenkins *et al*. 2002). Thus, the 10, 15 and 20% RS III replacment for wheat flour in the butter cake formala could be produced a medium GI butter cake product, which was 68.16%, 66.16% and 65.58%, respectively. While the control butter cake and 5% RS III replacement were classified as a high GI butter cakes, which was 71.80% and 69.65%, respectively .

Table 29 Area under curve (AUC), hydrolysis index (HI) and glycemic index (GI) value from butter cakes prepared with RS III replacement for wheat flour at 0, 5, 10, 15 and 20% with an incorporated of 40:60 of sucrose: HFCS.

RS III replacement (%)	AUC*	HI (%)	GI value ** (%)
0	1517.05 ± 7.27^{c}	54.72^{c}	71.80±6.45^{b}
5	1417.84 ± 3.69^{c}	50.41^{c}	69.65±5.16^{c}
10	1347.23 ± 3.59^{d}	47.90^{d}	68.16±3.85^{d}
15	1258.34 ± 9.27^{e}	44.74^{e}	66.16±4.59^{c}
20	1225.32 ± 8.54^{f}	43.57^{d}	65.58±3.21^{c}
Raw RS III	711.76 ± 5.66^{g}	25.31^{f}	54.74±2.65^{d}
White bread	2812.47±13.34^{a}	99.89^{a}	99.04±4.99^{a}

$^{a,b,c\ldots\ldots}$ Means within the same column with different letters are significantly different ($P \leq 0.05$) by Ducan's New Multiple Range Test (DMRT).

* AUC Parameter of the kinetic equation $= C_{\infty}\,(t_f - t_0) - (C_{\infty}/K)(1 - e^{-kt})$

** GI value $= 39.71 + (0.549 \times HI)$

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Based on the experimental results and discussion of this study, the major conclusions of this study were as following.

1. Enzyme resistant starch type III production from high amylose rice starch

1.1 Chemical compositions of native high amylose rice starch (HARS), carbohydrate, protein, fat, ash and moisture content of high amylose rice starch were 80.38 ± 0.52%, 1.18 ± 0.06%, 1.0 ± 0.34%, 1.0 ± 0.28% and 13.0 ± 1.05%, respectively. Total starch content of the rice starch as determined by an enzymatic method showed the average at 96.21 ± 1.2% of the sample weight on dry basis. The rice starch consists mainly of amylose, amylopectin and β-amylolysis limits which was 32.10 ± 0.67%, 67.93 ± 0.57 and 62.48 ± 2.49 %, respectively. The high amylose rice starch granules were observed to be polyhedral and irregular in shapes. The granular size was small with diameter between 3 - 6 μm and form parts of compound granules.

1.2 For the effect of preheated treatments on RS III formation, it was obvious that by a pullulanase hydrolysis improved the degree of syneresis (33.22, 51.45, 45.27 and 58.91% for non-debranched and debranched starches preheated at 75, 95 and 121°C for 48 hr). The debranched starches with higher degree of syneresis provided products with higher resistant starch contents which were evaluated by pancreatic α-amylase and glucoamylase at 37°C for 16 hr. The resistant starch content increased by 4 folds with debranching process (5.61, 4.12 and 19.31% for native starch, non-debranched retrograded starch and 48 hr pullulanase debranched starch preheated at 121°C). The high degree of pullulanase hydrolysis and degree of syneresis led to a reduction of HI and estimated GI for all preheated treatments. The lowest GI value was observed in the 4 to 48 hr debranched of 121^{o}C preheated

treatment (60.83 to 54.44%) and followed by the 4 to 48 hr debranched of 75^{o}C preheat (61.43 to 56.09%). While, the 4 to 48 hr debranched of 95 ^{o}C preheated treatment was the highest (69.09 to 62.63%) and classified as medium GI food. Debranching allowed more rearrangement of linear glucans and more ordered structures of retrograded starches which were characterized as V-type pattern, could be obtained as indicated by X-ray diffraction analysis. When the rice starch re-crystallized (RS III) was achieved, bigger, irregularly shaped particles with a white spongy-like porous network and honeycomb arrangement were observed by Scanning Electron Micrograph (SEM).

1.3 The effect of pullulanase enzyme concentration on RS III formation, the study found that an increasing of enzyme concentration from 8 to 14 unit/g starches with 16 hr hydrolysis time provided the degree of hydrolysis to gradually increase from 2.46 to 5.21% and remained stable when treated with 16 unit/g starch. This indicated that the rice starch was completely debranched that has been attacks by β-amylase at 96.27 and 98.82%. Degree of syneresis was dramatically lost expressible water in the 8 to 10 unit/g starch hydrolysis and remained relatively constant in the 12 to 16 unit/g starches. In addition, enzyme concentration was affected to starch digestibility, resistant starch content increased sharply as the amount of the enzyme increased to 12 units/g starch, reaching highest (19.81%) resistant starch content. Furthermore, resistant starch content in RS III samples dramatically decreased to 17.31% and 13.16% upon higher content of the enzyme (14 and 16 units/g starch). This because of the higher degree of pullulanase hydrolysis attributed to the formation of short linear polymers linked by α-1,4- glucosidic bonds fractions that may inhibit rearrangement of granules thereby preventing the starch retrogradation.

1.4 The optimum condition for larger scale production of RS III from high amylose rice starch by enzymatically-debranching process was 15% high amylose rice starch slurry preheated (121°C for 30 min) and debranching at 55°C for 16 hr with 12 unit/g starch of pullulanase enzyme. The optimum debranched rice starch were heated in boiling bath for 30 min for deactivate enzyme then induced at 4°C for 16 hr. Afterwards, the one cycle of freeze-thaw process (-10/30°C) was applied to promote

syneresis of retrograded starches. The viscosities of the debranched starch in the larger-scale (1-lit starch solution) formation of RS III, measured at 100 rpm were 1,785.15±13.18 centripoint. Degree of hydrolysis, degree of syneresis, resistant starch, digested starch, total starch, water activity and production yield were 4.74 %, 56.66%, 20.01%, 74.04%, 94.26%, 0.28 and 94.44%, respectively.

2. An application of RS III from high amylose rice starch to low glycemic index butter cake can be concluded as following.

2.1 Replacement of HFCS up to 60% of total sucrose and substituted of RS III from 5 to 15% wheat flour had no significant affects on physiochemical properties and sensory evaluation of the butter cakes. Butter cakes prepared with RS III replacement for wheat flour at 10% with an incorporated with 40:60 of sucrose: HFCS-55 had highest score on overall liking from panelist and lower glycemic index than the control butter cake with 100% sucrose and wheat flour.

2.2 An optimum formula of the developed butter cakes were 29.79% cake flour, 3.31% RS III, 16.08% HFCS, 10.72% sucrose, 25.13% butter, 0.21% baking powder, 0.10% salt, 1.34% corn flour and 13.40% eggs. The butter cake was accepted by 30 panelists at level of moderately like and the physicochemical properties were not significant difference from the control cake. The estimated glycemic index of the developed cake was 68.16% GI value and classified as medium glycemic index food, while the control cake was classified as high glycemix index food 77.10% GI value.

Recommendations

1. Continuing research in RS III formation by enzymatically-debranching high amylose rice starch is required to:

1.1 Investigate the fine structure and processing properties (molecular weight, water absorption index, water holding capacity, water solubility index, thermo properties and pasting properties) of RS III sample to clearly understand the structure

changed during processing and the end product that can be useful for an application of RS III as a low glycemic ingredient in several food products.

1.2 Investigate the effect of commercial grade of pullulanase enzyme substitute analytical grade that was used in this experiment on physicochemical properties of RS III from high amylose rice starch prior to industrial scale production.

1.3 Transfer this innovation to an appropriate factory that had similarly production equipments such as high fructose corn syrup factory and modified starch factory in order to produce RS III for industrial scale production.

2. In case of an application of RS III as a low glycemic index ingredient in the other food products are needs to:

2.1 Investigate the optimum level in several types of food production such as a batter products (fried coating flour), snack food, healthy cookies.

2.2 Investigate the stability of resistant starch after food processing and its health benefit in both of *in vitro* and *in vivo* methods.

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APPENDICES

Appendix A

Chemical analysis procedures

1. Determination of moisture content

1.1 Apparatus

1.1.1 Metal dish (Diameter approximate 55 mm, height 15 mm, with inverted slip-in cover fitting tightly on inside).

1.1.2 Hot air oven

1.1.3 Air-tight desiccator (Reignited CaO is satisfactory drying agent)

1.2 Method determination

The moisture content of tested samples was determined using the indirect method, according to the AOAC procedures (2002). Dish was heated to 105°C with cover, cooled in desiccator and weighed soon after reaching room temperature. Accurately weight 2.0000 g well-mixed test portion in covered dish. Loosen cover test portion and heat at 105°C to constant weight (about 5 h) in hot air oven. Immediately tighten cover on dish, transfer to desiccator and weigh soon after reaching room temperature. Percent moisture content was calculated as follow:

$$\% \text{ Moisture content} = (\text{Loss weight} / \text{Sample weight}) \times 100$$

2. Determination of protein content

2.1 Regents and apparatus for analysis

2.1.1 Sulfuric acid (H_2SO_4)

2.1.2 Potassium sulfate (K_2SO_4)

2.1.3 Copper sulfate ($CuSO_4$)

2.1.4 Sodium hydroxide (NaOH)

2.1.5 Bromocresol green

2.1.6 Methy red

2.1.7 Digestion and distillation unit (Model B-324, BÜCHI, Switzwerland) with kjeldahl digestion tube

2.2 Method determination

Total nitrogen content of tested samples was determined using the Kjeldahl method, according to the AOAC procedures (2002). Sample (2.0000 g) was weight in the Kjeldahl digestion tube. Fifteen ml of conc. H_2SO_4 and 15 g of catalyst (mixed $CuSO_4$ with K_2SO_4 in a weight ratio of 0.5:1) were added to the sample and digested in a digester at 420°C until a clear solution was obtained. Distilled water (55 ml) was added to the tube and the mixture steam distilled using a distillation unit, while 85 ml of 40% NaOH solution were added automatically via a built in dispensing system. The distillate was collected in a 50 ml saturated boric acid with mixed indicator. The mixed indicator solution was prepared by mixing 0.1% (w/v) of bromocresol green with 0.1% (w/v) of methyl red. Titration was done using 1.0 N HCL until the color changed to pink-red. The total nitrogen content and protein content was calculated as following formula;

$$\text{Total nitrogen} = \frac{\text{Volume of HCL} \times \text{Normality of HCL} \times 14.007 \times 100}{\text{Weight of sample (g)}}$$

$$\text{Protein content} = (\ \% \text{ total nitrogen} \times 5.70)$$

3. Determination of crude fat content

3.1 Regents and apparatus for analysis

3.1.1 Analytical balance (at least 1 mg sensitivity).

3.1.2 Electrical drying oven to be operated at 102ºC± 1ºC.

3.1.3 Soxhlet extraction unit comprising:

- Round bottom flask, 150 mL
- Soxhlet extractor with 60 mL
- siphoning capacity and condenser
- Cellulose extraction thimbles (28 x 80 mm)

3.1.4 Fume cupboard

3.1.5 Heat source, either electric heating mantle, or steam bath 100 mL beaker

3.1.6 Desiccator with silica gel desiccant

3.1.7 Glass rod

3.1.8 Petroleum spirit boiling point 60-80ºC

3.1.9 Cotton wool free of fat

3.1.10 Acid washed sand

3.2 Method determination

Total fat content of tested samples was determined using the Soxhlet method, according to the AOAC procedures (2002). Rinse all glassware with petroleum spirit, drain, dry in an oven at 102ºC for 30 min. and cool in a desiccator. Place a piece of cotton wool in the bottom of a 100 mL beaker. Put a plug of cotton wool in the bottom of an extraction thimble and stand the thimble in the beaker. Accurately weigh 5 g of sample into the thimble. Add 1 - 1.5 g of sand and mix the sand and sample with a glass rod. Wipe the glass rod with a piece of cotton wool and place cotton wool in the top of the thimble. Dry the sample in an oven at 102ºC for 5 hours. Allow the sample to cool in a desiccator. Take the piece of cotton wool from the bottom of the beaker and place it in the top of the thimble. Insert the thimble in a Soxhlet liquid/solid extractor (Figure 1). Accurately weigh a clean, dry 150 mL round bottom flash and put about 90 mL of petroleum spirit into the flask. Assemble the extraction unit over either an electric heating mantle or a water bath. Heat the solvent in the flask until it boils. Adjust the heat source so that solvent drips from the condenser into the sample chamber at the rate of about 6 drops per second. Continue the extraction for 6 hours. For sausage meat and other emulsified products, the extraction should be performed in stages: Extract for about 4 hours, then remove the heat source and drain the solvent from the extractor in the flask. Remove the thimble from the extractor and transfer the sample to a 100 mL beaker. Break up the sample with a glass rod. Return the sample to the thimble and replace the thimble in the extractor. Rinse the beaker with petroleum spirit and pour rinsings into the extract. Continue extraction for a further two hours. Remove the extraction unit from the heat

source and detach the extractor and condenser. Replace the flask on the heat source and evaporate off the solvent. (The solvent may be distilled and recovered). Place the flask in an oven at 102ºC and dry the contents until a constant weight is reached (1-2 hours). Cool the flask in a desiccator and weigh the flask and contents. Total fat content was calculated using following formula:

$$\% \text{ Crude fat} = \frac{(W2 - W1) \times 100}{S}$$

Where, weight of empty flask (g) = W1, weight of flask and extracted fat (g) = W2 and weight of sample = S

4. Determination of ash content

4.1 Apparatus

4.1.1 Ashing dish

4.1.2 Furnace

4.1.3 Air-tight desiccator (Reignited CaO is satisfactory drying agent)

4.2 Method determination

The ash content of tested samples was determined using the direct method, according to the AOAC procedures (2002). Dish was heated to 105°C with cover, cooled in desiccator and weighed soon after reaching room temperature. Accurately weight 3.0000 g well-mixed test portion in ashing dish. Ignite in furnace at approximately 550°C (dull red) until light gray ash results or to constant weigh. Cool in desiccator and weigh soon after reaching room temperature. Percent ash content was calculated as follow:

$$\% \text{ Ash content} = (\text{Ash weight} / \text{Sample weight}) \times 100$$

5. Determination of amylose-amylopectin content

5.1 Apparatus and reagent

5.1.1 Spectrophotometer (Hitachi 100-6 , Japan)

5.1.2 Hot plate

5.1.3 100 ml volumetric flasks

5.1.4 Glassware (Pipettes, beaker, cylinder)

5.1.5 Amylose, Type III from potato

5.1.6 Ethanol, 95 %

5.1.7 Sodium hydroxide, 1.0 N and 0.09 N

5.1.8 Acetic acid, 1.0 N

5.1.9 Iodine solution, .02 % I_2 and 2 % KI in distilled water

5.2 Method determination

Amylose and amylopectin content of tested samples was determined using the colorimetric method, according to the Juliano *et al*. (1971) procedures. Weigh duplicate 100 mg (db) starch samples and quantitatively transfer to 100 ml volumetric flasks. Add L ml of 95 % ethanol, carefully washing down any sample adhering to side of the flasks. Add 9 ml 1 N sodium hydroxide to each sample and heat in a boiling water for 10 minutes and cool to room temperature. 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. 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. Add 1.0 ml N acetic acid and mix. Add 2 ml iodine solution. Make up to 100 ml volume with distilled water, mix and let stand for 20 minutes. Read absorbance at 620 nm. For a blank, prepare using 5 ml 0.09 N NaOH, instead of the sample solution in the previous step. Percent ash content was calculated as follow:

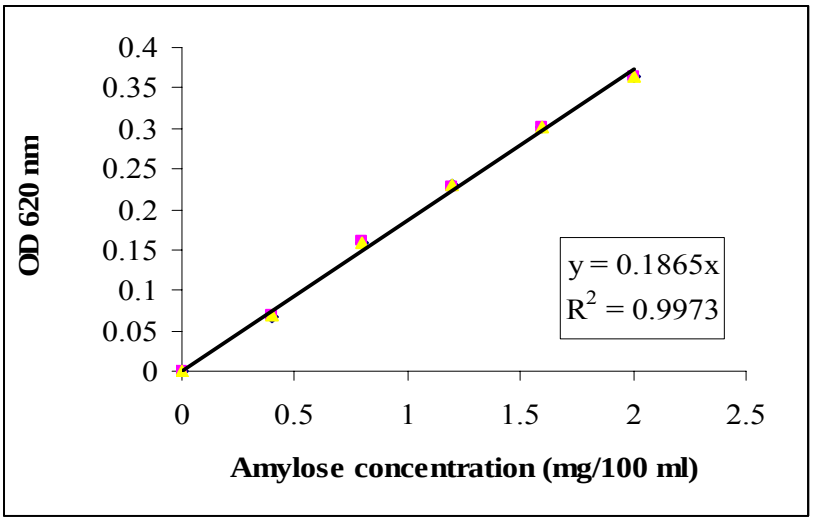
$$\% \text{ Amylose content} = \frac{\text{Amylose concentration from standard curve (mg/100 ml)} \times 10}{5 \times \text{wt of dry rice starch (gram)}}$$

5.3 Procedure for Preparation for Standard Curve

Prepare solutions (1 mg/ml) of standard amylose and amylopectin following the same procedure as in steps 4.3.1 to 4.3.4 for the sample preparation above. Prepare working solutions of mixed amylose and amylopectin for standard curve following Appendix Table A 2. Pipette 5 ml aliquots of each mixture for the working solutions in 100 ml volumetric flasks, each containing about 50 ml of distilled water. Repeat measurement as a sample steps described above. Plot absorbance at 620 nm against the assigned amylose content for the standard curve as shown in Appendix Figure A1. Read % amylose values of the tested samples from the standard curve as in Appendix Table 1. Amylose content was ca

Appendix Table A1 Standard working solutions for amylose determination

Amylose content (% dry weight basic)	Volume ratio of standard working solution (ml/100ml)		
	Amylose (ml)	Amylopectin (ml)	0.09N sodium hydroxide (ml)
0	0	18	2
10	2	16	2
20	4	14	2
30	6	12	2
40	8	10	2



Appendix Figure A 1 Standard curve for amylose determination

6. Determination of total sugar content

6.1 Apparatus and reagent

6.1.1 Spectrophotometer

6.1.2 Water bath

6.1.3 Balance with digital

6.1.4 Vortex mixer

6.1.5 Cuvettes tubes for spectrophotometer

6.1.6 Glassware (Erlenmeryer flask, volumetric flasks, pipette)

6.1.7 Mechanical, adjustable volume pipettors (1000 and 100 μl) with plastic tips

6.1.8 Repipettor (for fast-delivery of 5ml concentrated H_2SO_4

6.1.9 Test tubes, 16-20 mm internal diameter and test tube rack

6.1.10 D-Glucose ($C_6 H_{12} O_6$) for standard glucose solution, 100 μg/ml. Prepare by adding 0.01 gram of dried D-glucose in distilled water and adjusted volume to 100 ml in volumetric flask.

6.1.11 DMSO concentrated

6.1.12 Sulfuric acid (H_2SO_4), concentrated

6.1.13 5% (w/v) Phenol solution ($C_6 H_6 O_6$)
Prepare by dissolve 5 gram of phenol crystal (reagent grade) into distilled water and adjusted volume to 100 ml in volumetric flask.

6.2 Method determination

Total sugar content of tested samples was determined using the Phenol-Sulfuric Acid method, according to the Dobois *et al.,* (1956). Carbohydrates (simple sugars, oligosaccharides, polysaccharides, and their derivatives) react in the presence of strong acid and heat to generate furan derivatives that condense with phenol to form stable yellow-gold compounds that can be measured spectrophotometrically.

1) Standard curve procedure

Using the glucose standard solution (100 μg/ml) and distilled water as indicated in the Appendix Table A2. Pipette aliquots of the glucose standard into clean test tubes (triplicates for each concentration) such that the tubes contain 0, 20, 40, 60, 80 and 100 μg/ml. The 0 μg/ml sample tube was used to prepare the reagent blank. Pipette 1 ml of each glucose concentration into test tube. Add 1 ml of 5% Phenol solution into each tube from Part 2.4.2 and immediately mix on a Vortex test tube mixer. Add 5 ml H_2SO_4, concentrated to each tube from Part 2.4.3. The sulphuric acid reagent should be added rapidly to the test tube. Direct the stream of acid against the liquid surface rather than against the side of the test tube in order to obtain good mixing. Mix on a Vortex test tube mixer. Let the tube 4 to stand for 30 min in hooder. Read absorbance at 485 nm by using Spectrophotometer. Plot absorbance at 485 nm against the assigned glucose content for the standard curve (Appendix Figure A 2).

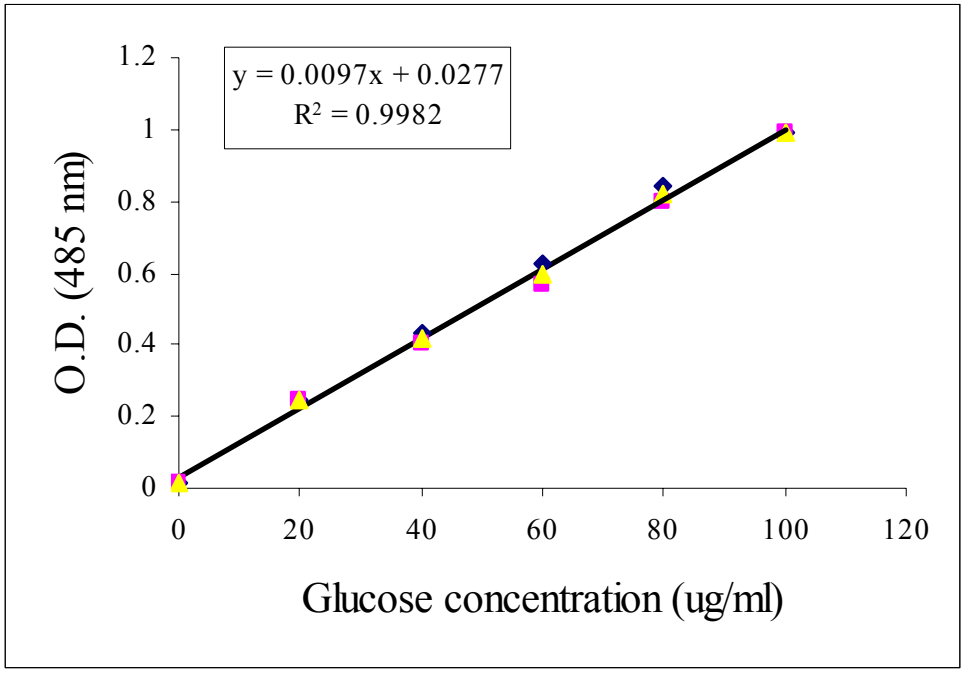
2) Analysis of total sugar in samples

The total sugar for the native starches, debranched products and RS III sample were purified and destructured by dissolving in DMSO prior dilution. In sample tubes, the sample tested was completely solution and contained 20 – 100 μg/ml by dilution. After dilution as indicated, pipette 1.0 ml of sample into a test tube and analysis following step as in standard curve procedure. Analyze each diluted sample in duplicate. Calculate the concentration of glucose in the samples in term of glucose (μg/ml) as following equation:

$$\text{Total sugar } (\mu g/ml) = \frac{\text{Absorbance of sample} \times \text{Dilution}}{\text{Slope}}$$

Appendix Table A 2 Standard working solutions for total sugar determination

Glucose concentration (µg/ml)	Distilled water (ml)	Glucose stock solution (ml)
0	10	-
20	8	2
40	6	4
60	4	6
80	2	8
100	-	10



Appendix Figure A 2 Standard curve for total sugar determination

7. Reducing sugar determination by Park and Johnson Method

7.1 Apparatus and reagent

7.1.1 Spectrophotometer

7.1.2 Boiling bath and ice bath

7.1.3 Balance with digital

7.1.4 Vortex mixer

7.1.5 Cuvettes tubes for spectrophotometer

7.1.6 Glassware (Erlenmeryer flask, volumetric flasks, pipette, test tube)

7.1.7 Mechanical, adjustable volume pipettor (500 and 100 μl) with tips

7.1.8 D-Glucose ($C_6 H_{12} O_6$) for standard glucose solution, 0-5 μg/ml. Prepare by adding 0.02 gram of dried D-glucose in distilled water and adjusted volume to 100 ml in volumetric flask.

7.1.9 $NaCO_3$ –$NaHCO_3$-KCN reagent (for 1 lit preparation)

- Potassium cyanide (KCN) 0.65 gram
- Sodium carbonate ($Na_2 CO_3$) 4.80 gram
- Sodium hydrogen carbonate ($HNaO_3$) 9.20 gram

Prepare by dissolve the Na_2CO_3 KCN chemical reagent into distilled water and adjusted volume to 1000 ml in volumetric flask.

7.1.10 Ferriccyanide reagent (for 1 lit preparation)

- Potassium ferriccyanide $(K_3Fe (CN))_6$ 0.50 garm

Prepare by dissolve the Ferriccyanide reagent into distilled water and adjusted volume to 1000 ml in volumetric flask.

7.1.11 Ferric ammonium sulphate reagent (for 1 lit preparation)

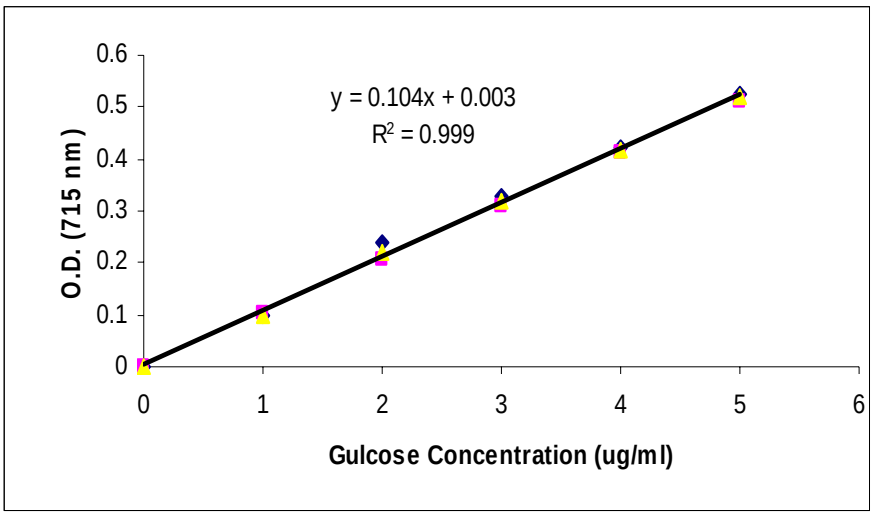
- $FeNH_4 (SO_4)_2.12H_2O$ 3.0 gram
- Sulphuric acid (H_2SO_4) 50 mM 1000 ml

Prepare by dissolve $FeNH_4 (SO_4)_2.12H_2O$ into Sulphuric acid 50 mM and adjusted volume to 1000 ml in volumetric flask with 50 mM Sulphuric acid.

7.2 Method determination

Reducing sugar content of starch hydrolysis samples was determined by the Park and Johnson method, according to Hizukuri (1995). Reducing sugar for the native starches, debranched products and RS III sample were purified and destructured by dissolving in DMSO prior dilution. In sample tubes, the sample tested was completely solution and contained 0 – 5 µg/ml by dilution. Duplicate 1.0 ml of starch solution/ standard glucose/ blank were pipette into test tube. An aliquot of 1.0 ml samples/ blank/ standard with 0.5 ml of ferriccyanide reagent and 0.5 ml of $NaCO_3$ –$NaHCO_3$-KCN reagent were heated in a boiling water for 15 min. This solution was quickly cooled for 10 min, and 2.50 ml of ferric ammonium sulphate reagent were then added and mixed well. After 20 min incubation at room temperature, the absorbance of the colored solution was measured at 715 nm. The absorbance of the samples / standard/ blank were measured using a spectrophotometer (Model UV-160 A, Shimadzu, Japan) with a path length of 10 nm quartz cuvette cell. The reducing sugar content was calculated by comparison to a glucose standard curve (Appendix Figure A3) by using equation:

$$\text{Reducing sugar (µg/ml)} = \frac{\text{Absorbance of sample} \times \text{Dilution}}{\text{Slope}}$$



Appendix Figure A 3 Standard curve for reducing sugar determination

8. Resistant starch determination by AOAC Method

8.1 Apparatus and reagent

8.1.1 Spectrophotometer

8.1.2 Boiling bath and ice bath

8.1.3 Bench centrifuge

8.1.4 Shaking water bath

8.1.5 Balance with digital

8.1.6 Vortex mixer

8.1.7 Magnetic stirrer

8.1.8 pH Meter

8.1.9 Stop-clock

8.1.10 Spectrophotometer

8.1.11 Cuvettes tubes for spectrophotometer

8.1.12 Glassware (Erlenmeryer flask, volumetric flasks, test tube)

8.1.13 Mechanical, adjustable volume pipettor (100 μl) with tips

8.1.14 Sodium maleate buffer (0.1 M, pH 6.0)

8.1.15 Sodium acetate buffer (1.2 M, pH 3.8)

8.1.16 Sodium acetate buffer (100 mM, pH 5.8)

8.1.17 Potassium Hydroxide (2 M)

8.1.18 Stock *Amyloglucosidase* solution

8.1.19 *Pancreatic α- amylase*

8.1.20 *Glucose oxidase-peroxidase-aminoantipyrine* reagent

8.2 Method determination

8.2.1 Principle

Resistant starch was determined by the Megazyme resistant starch assay kit, according to AOAC (2002). Samples are incubated in a shaker water bath with *pancreatic α- amyla*se and *amyloglucosidase* (AMG) for 16 hr at 37°C, during which time non-resistant starch is solubilized and hydrolyzed to glucose by the combined action of the two enzymes. The reaction is terminated by the addition of an equal volume of ethanol, and the RS is recovered as a pellet on centrifugation. This is then wash twice by suspension in aqueous ethanol (50%, v/v), followed by centrifugation. Free liquid is removed by decantation. RS in the pellet is dissolved in 2 M KOH by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution is neutralized with acetate buffer and the starch is quantitatively hydrolyzed to glucose with AMG. Glucose is measured with glucose oxidase/peroxidase reagent, and this is a measure of the RS content of the sample. Non-resistant starch (solubilised starch) can be determined by pooling the original supernatant and the washings, adjusting the volume to 100 mL measuring glucose content with GOPOD.

8.2.2 Preparation of test samples

Grind an approximately 50 g sample of grain or lyophilized plant or food product in grinding mill to pass a 1.0 mm sieve. Transfer all material to wide-mouthed plastic jar and mix well by shaking and inversion, Industrial starch preparation are usually supplied as a fine powder, so grinding is not required. Mince fresh samples(e.g. canned bean, banana, potatoes) in a hand operated or electric meat mincer to pass an ~ 4.5 mm screen. Determine moisture content of dry samples by AOAC Method 925.10 (2000), and of fresh samples by lyophilisation followed by oven drying according AOAC Method 925.10 (2000).

8.2.3 Measurement of resistant starch

Resistant starch content in the RS III samples and control were determined as following section.

1) Hydrolysis and solubilisation of non-resistant starch

1. Accurately weigh a 100 ± 5 mg sample directly into each screw cap tube and gently tap the tube to ensure that the sample falls to the bottom.
2. Add 4.0 ml of pancreatic α-amylase (10 mg/ml) containing AMG (3 unit/ml to each tube.
3. Tightly cap the tubes, mix them on a vortex mixer and attach them horizontally in a shaking water bath, aligned in the direction of motion.
4. Incubate tubes at 37°C with continuous shaking (200 strokes/ min) for exactly 16 hr.
5. Remove the tubes rom the water bath and remove excess surface water with paper towel. Remove the tube caps and treat the contents with 4.0 ml of ethanol (99%) with vigorous stirring on a vortex mixer.
6. Centrifuge the tubes at 1,500 g (approx. 3,000 rpm) for 10 min (non-capped).
7. Carefully decant supernatants and re-suspend the pellets in 2 ml of 50% ethanol with vigorous stirring on a vortex mixer. Add a further 6 ml of 50% ethanol, mix the tubes and centrifuge again at 1,500 g for 10 min.
8. Decant the supernatants and repeat this suspension and centrifugation step once more.
9. Carefully decant the supernatant and invert the tubes on absorbent paper to drain excess liquid.

2) Measurement of resistant starch

1. Add a magnetic stirrer bar and 2 ml of 2 M KOH to each tube and re-suspended the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer.

2. Add 8 ml of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 ml of AMG (3300 unit/ml), mix well and place the tubes in a water bath at 50°C.

3. Incubate the tubes for 30 min with intermittent mixing on a vortex mixer.

4. For samples containing > 10% RS content; quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle). Use an external magnet to retain the stirred bar in the tube while washing the solution from the solution from the tube with the water wash bottle. Adjust to 100 mL with water and mix well. Centrifuge an aliquot of the solution at 1,500 g for 10 min.

5. For samples containing < 10% RS content; directly centrifuge the tubes at 1,500 g for 10 min (no dilution). For such samples, the final volume in the tube is approximately 10.3 ml.

6. Transfer 0.1 mL aliquots (in duplicate) of either the diluted or undiluted supernatants into glass test tubes, treat with 3.0 mL of GOPOD reagent and incubate at 50°C for 20 min.

7. Measure the absorbance of each solution at 510 nm against the reagent blank.

3) Measurement of non resistant (solubilised) starch

1. Combine the supernatant solutions obtained on centrifugation of the initial incubation with the supernatants obtained from the subsequent two 50% ethanol washings and adjust the volume to 100 mL with water in a volumetric flask.

2. Incubate 0.1 mL aliquots of this solution (in duplicate) with 3.0 mL of GOPOD reagent for 20 min at 50°C.

3. Measure the absorbance at 510 nm against a reagent blank.

4. Calculate the content of non-resistant (solubilised) starch.

4) Calculate resistant starch, non-resistant starch

Calculate resistant starch, non-resistant (solubilised) starch total starch content (%, on a dry weight basis) in test samples as follows:

a) Resistant starch (g/ 100g sample)
(Samples containing > 10% RS):

$= \Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180$

$= \Delta E \times F/W \times 90.$

b) Non-Resistant starch (g/ 100g sample)
(Samples containing > 10% RS):

$= \Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180$

$= \Delta E \times F/W \times 90.$

c) Total starch content

= Resistant starch + Non-resistant starch.

Where:

ΔE = absorbance (reaction) read against the sample analyzed reagent blank.

F = conversion from absorbance to micrograms (the absorbance obtained for

100 μg of glucose in the GOPOD reaction is determined and F= 100 (100 μg of glucose) divided by the GOPOD absorbance for this 100 μg of glucose.

100/0.1 = volume correction (0.1 mL taken from 100 mL).

1/1000 = conversion from micrograms to milligrams.

W = dry weight of sample as is" weight × (100 – moisture content)/100.

100/W = factor to present RS as a percentage of sample weight.

162/180 = factor to convert from free glucose, as determined, to anhydro-glucose as occurs in starch.

10.3/0.1 = volume correction (0.1 mL taken from 10.3 mL) for samples containing 0-10% RS where the incubation solution is not diluted and the final volume is ~ 10.3 mL. When wet samples are analysed, this volume will be larger, and this should be Allowed for in the calculations.

Appendix B

Physical analysis procedures

1. Scanning Electron Microscopic Examination

1.1 Apparatus

1.1.1 Scanning Electron Microscope (SEM, JEOL, JSM 6301F, Japan)

1.1.2 Fine coaster (JFC 1200,JEOL, Japan)

1.1.3 Aluminum specimen stub

1.1.4 Double-sided adhesive tape

1.2 Procedure for sample determination

Scanning Electron Microscope (SEM) of native rice starch, pullulanase hydrolysis sample and RS III samples were examined according to Atichokudomchai *et al* .,(2000). All the samples were dried to less than 4 moisture content before examined. Take a dried sample and sprinkle onto double-sided adhesive tape attached to the specimen stub. Remove the excess sample. Place in fine coater for gold coating for 150 seconds. Place the coated sample in sample chamber in the SEM (Appendix Figure B 1). Examine at magnification of 2,500 X and 6,000X with the accelerating voltage of 10 KV.

Appendix Figure B1 Scanning Electron Microscope (SEM) instrument

2. X Ray Diffraction Measurement

2.1 Apparatus

2.1.1 Wide-angle X-ray diffractometer (JEOL,JDX 3530, Japan).

2.1.2 Silicon sample cell

2.1.3 Computer with program MDI Jade 6.5 (Japan)

2.2 Procedure for Sample Preparation

X Ray Diffraction pattern of native rice starch and RS III samples were examined according to Cheetham and Tao (1998).Take a samples for about 5 g. Pack tightly in rectangular silicon cell and spread samples evenly to obtain a smooth surface and place the sample cell in sample holder. Expose to the X-ray beam.

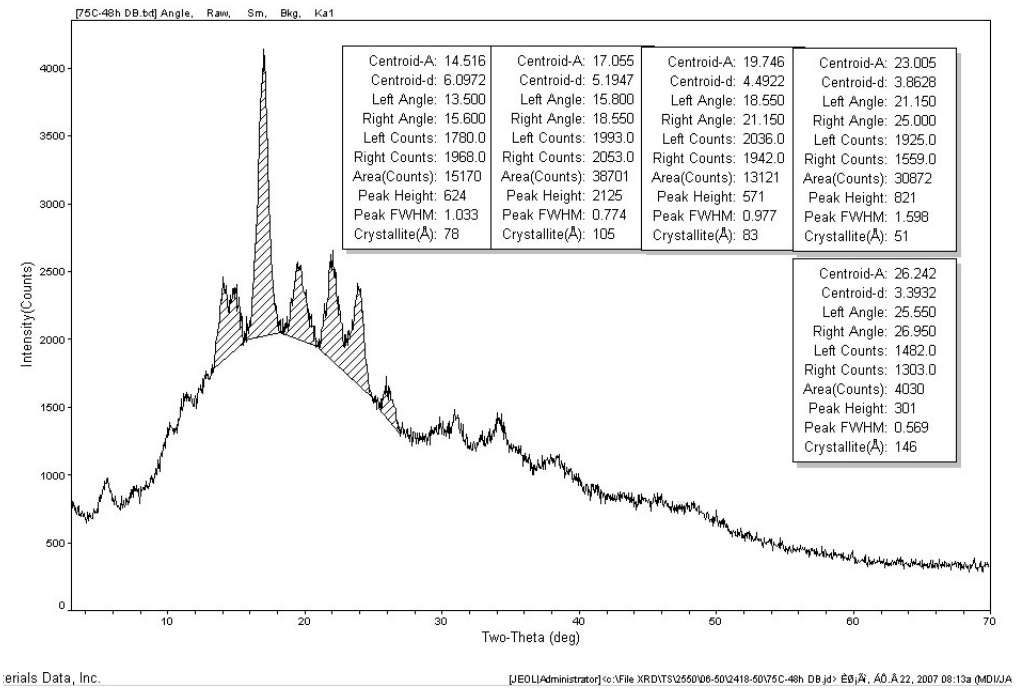
The X-ray diffractometric conditions as following Cairns *et al*., (1997

Monochromatic Cu-K_2 radiation	0.154 nm
X-ray generator power	300 mA, 30 kB
Scanning, 2-theta	10^o to 70.0^o
Step angle	0.05^o
Count time	2 sec
Measurement temperature	ambient temperature

2.3 Crystallinity calculation

Amorphous and crystalline sections were examined from the X-ray diffractograms (Appendix Figure B 2). Peak baseline (white area) and smooth curve (bold area) were computer-plotted on the diffractogram (Appendix Figure B 3). The area above the smooth curve was the crystalline portion and the bold area above the peak baseline was the amorphous portion. The % crystallinity of RS III samples were calculated as the ratio of area of the crystalline sharp peak over the total area at angles between 10 and 45 ^{o} 2θ using a computer program based on the methods of Nara *et al*. (1978).

Appendix Figure B2 X-ray diffractometric instrument



Appendix Figure B3 Peak baseline (bold area) and amorphous area (white area) of RS III formation from enzymatically debranching HARS

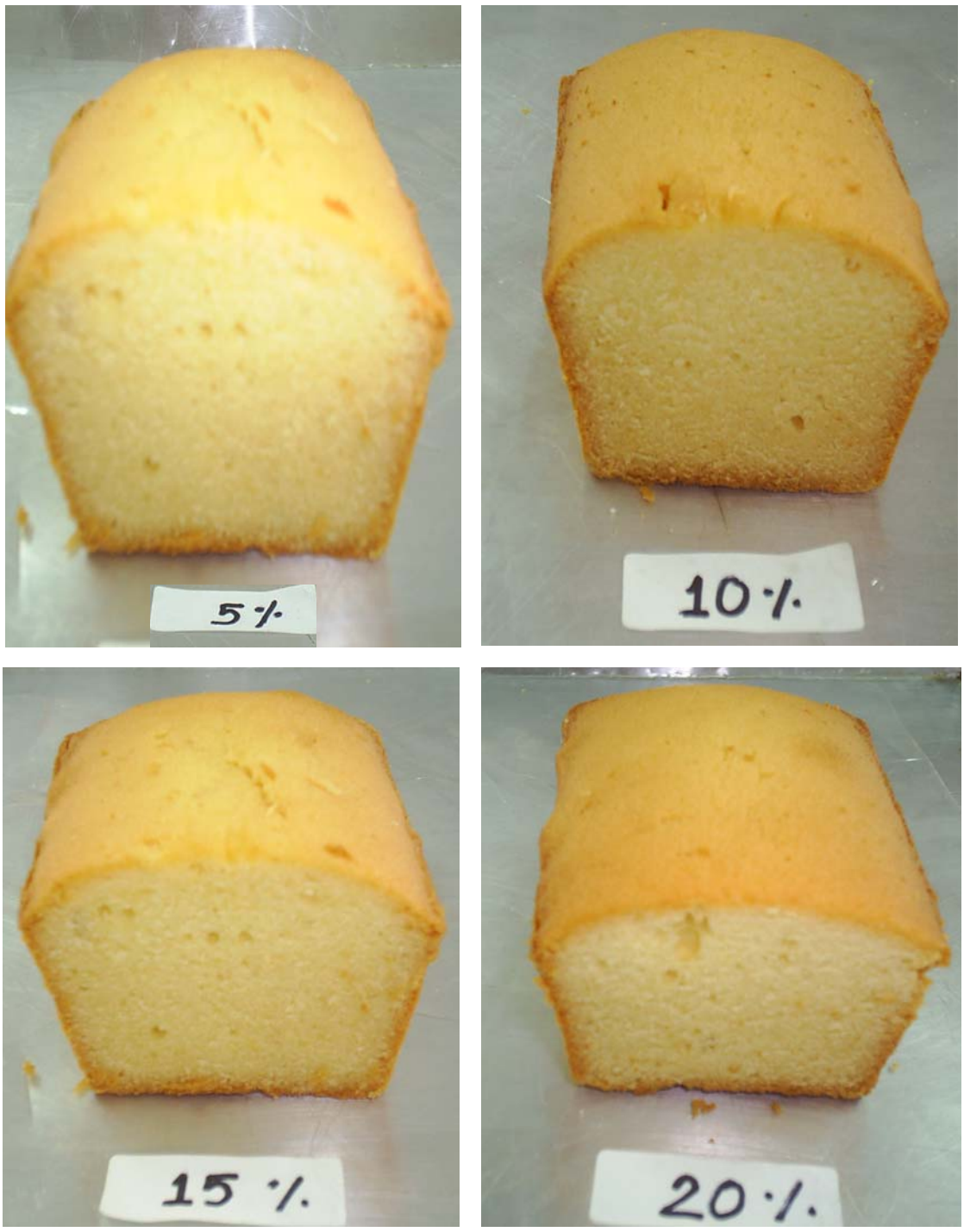
Appendix C

Picture of some experiments

Appendix Figure C1 Pullulanase enzyme, native HARS and some apparatus for RS III production

Appendix Figure C2 Visuals appearance of RS III samples with various processing Steps

Appendix Figure C 3 Visuals appearance of low glycemic butter cake by using HFCS replacement for sugar at 20, 40, 60, 80 and 100%



Appendix Figure C4 Visuals appearance appearances of butter cakes prepared with RS III replacement for cake flour at 5, 10, 15 and 20% with an incorporated of 40:60% sugar and HFCS

Appendix D

Sensory evaluation form

1. Introduction

There are several standard techniques in the sensory evaluation of foods (Harry and Barbara, 1991). It is based on the panellists' ability to verbalise product perception in a reliable manner. After screening, training, developing an language, and scoring the samples, the data are statistical analyses.

One of a sensory method with deliberate relationships to psychological principles is the nine-point hedonic scale. This instrument has proven to be a durable and useful method for the assessment of food likes and dislikes by consumers. It has a number of salient properties; it is balanced, bipolar, contents a neutral point, and has approximately equal psychological spacing between scale points, giving it roughly interval priorities (Stone and Sidel, 1993).

2. Invitation and Selection of Panellist

Panellists were invited and selected based on their responses to questions pertaining to food, interest, availability, and healthy. These subjects were then asked to complete a series of acuity tests including odour matching, basic taste identity, texture ranking, and a series of triangle tests.

3. Sample Presentation

Cake samples were evaluated in a soundroof, humidity-controlled sensory room with individual booths. Panels were held once a day in the mid-morning and afternoon three times a week for a period of 3 weeks. A total of three replications were completed. The sample was placed in cups with plastic lids. All cups were coded with three digit random numbers. Panellists were instructed to evaluate by using the hedonic scale at 9 point.

4. Sensory Evaluation Form

Hedonic Scale

Product Sample _____

Panellist's name _____ Sex _____

Date _____ Time _____

Directions:

1. Please rinse your mouth with water in between samples.
2. Please fill the score for your idea on an appearance, color, aroma, flavour, texture and total acceptance of each product by using the following Hedonic scales:

9 = Like extremely	6 = Like slightly	3 = Dislike moderate
8 = Like very much	5 = Fair	2 = Dislike very much
7 = Like moderately	4 = Dislike slightly	1 = Dislike extremely

Quality	Sample (code number)				
	276	687	277	412	245
Appearance					
Color					
Flavor - sweetness					
- aroma					
Texture - moistness					
-tenderness					
Overall liking					

Comments:……
……..
……..
……..

Appreciated thanks for your kind as panellists

Appendix E

Experimental data

Appendix Table E1 Degree of pullulanase hydrolysis of 0 to 48 hr debranched HARS preheated at 75°C, 95°C and 121°C for 30 min.

Hydrolysis time (hr)	Preheated treatments*		
	75°C	95°C	121°C
0	0.14±0.38	0.15±0.05	0.17±0.07
2	0.23±0.39	0.18±0.06	0.54±0.03
4	0.31±0.43	0.21±0.09	1.29±0.15
8	0.42±0.35	0.36±0.12	2.09±0.09
16	0.80±0.10	0.42±0.15	2.37±0.03
24	1.21±0.67	0.46±0.06	2.79±0.07
48	1.55±0.49	0.50±0.14	3.10±0.15

* Each value in the Table represents the mean ± standard deviation of triplicate determinations.

Appendix Table E2 Degree of syneresis of 0 to 48 hr debranched HARS preheated at 75°C , 95°C and 121°C for 30 min.

Hydrolysis time (hr)	Preheated treatments*		
	75°C	95°C	121°C
0	28.10±1.77	29.96±3.23	35.01±2.42
2	37.67±2.19	35.53±0.86	40.14±1.17
4	40.92±1.23	40.74±3.12	42.99±1.19
8	43.54±1.69	41.15±2.22	47.02±2.02
16	45.92±1.42	42.44±2.13	56.91±2.30
24	51.45±2.01	44.27±3.67	57.78±1.86
48	54.53±2.70	45.55±3.32	58.88±0.26

* Each value in the Table represents the mean ± standard deviation of triplicate determinations.

Appendix Table E3 X-ray diffraction profile and degree of crystallinity of the native HARS and RS III samples from 0, 4, 16, 24 and 48 hr hydrolysis of 75 ^{o}C and 121^{o}C preheated 15% HARS

RS III samples	X-ray diffraction profile		Crystallinity Degree (%)
	Peak area	Total area	
native HARS	96356	731100	13.17±9.59
0 hr DB of 75 °C preheated	19010	736252	2.58±9.96
16 hr DB of 75 C° preheated	77309	724823	10.66±5.91
48 hr DB of 75 °C preheated	93313	722840	12.90±9.22
4 hr DB of 121 °C preheated	51187	356178	14.37±1.19
16 hr DB of 121 °C preheated	56384	361748	15.58±6.54
24 hr DB of 121 °C preheated	62915	372959	16.86±9.15
48 hr DB of 121 °C preheated	71413	366313	19.49±5.08

* Each value in the Table represents the mean ± standard deviation of triplicate determinations.

Appendix Table E4 Effect of pullulanase enzyme concentration (0 to 16 unit/g starch) on physiochemical properties of 16 hr debranching of 15 % rice starch preheated at 121°C for 30 min.

Enzyme concentration (unit/g starch)	Physiochemical properties (%)			
	Degree of hydrolysis	β-amylolysis	Degree of syneresis	Resistant starch content
0	0.60±0.03	62.93±0.75	35.01±1.43	3.98±0.46
8	2.46±0.33	66.62 ±1.07	51.64±1.31	10.38±0.85
10	3.49±0.59	74.48± 1.50	53.54±1.61	17.68±0.11
12	4.54 ±0.41	83.48±0.78	54.80±1.50	19.81±0.28
14	5.21 ±0.13	96.27±3.01	54.14±1.52	17.71±0.64
16	5.27 ±0.03	98.82±1.37	55.85±0. 87	13.16±0.66

* Each value in the Table represents the mean ± standard deviation of triplicate determinations.

Appendix F

List of publications

LIST OF PUBLICATIONS

Pongjanta, J., A. Utaipatanacheep, O. Naivikul and K. Piyachomkwan. 2007. Improvement of resistant starch type III formation from high amylose rice starch by enzymatically debranching process, pp. 245-251. Proceedings Starch Update 2007. **The 4th International Conference on Starch Technology.** Queen Sirikit National Convention Center Bangkok, Thailand.

Pongjanta, J., A. Utaipatanacheep, O. Naivikul and K. Piyachomkwan. 2008. Enzymes-resistant starch type III (RS III) from pullulanase debranched high amylose rice starch, pp 99-106. Proceedings of **the 46th Kasetsart University Annual Conference: Agro-Industry.** Kasetsart University, Bangkok, Thailand.

P-STARCH-12

Improvement of Resistant Starch Type III Formation from High Amylose Rice Starch by Enzymatically Debranching Process

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Abstract

The use of starch as nutrition-valued food products is of great interest among starch industries as Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS) and Resistant Starch (RS). Resistant starch can be found in nature of which the contents are varied by botanical source and starch granular structure (i.e. resistant starch type I and II). In addition, resistant starch can be produced from retrogradation of gelatinized starch which is resistant starch type III (RS III). The extents of RS III of most starches are, however, quite low, depending on the molecular structure of starch glucans, namely amylose and amylopectin. In this work, a debranching enzyme (Pullulanase, EC. 232-983-9P, 8U/g starch at 55°C for 0 to 48-hr) was introduced to modify the amylopectin molecules of high amylose rice starches (32.10%, amylose content) which were partially gelatinized at different temperatures (75, 95 and 121°C for 30-min). The retrogradations of debranched starches with different degrees of hydrolysis (0.14 to 3.10%) were then induced at 4°C for 16 hrs. Afterwards, the freeze-thaw cycle (-10/30°C) was further applied to promote syneresis of retrograded starches. It was obvious that by a pullulanase hydrolysis improved the degree of syneresis (33.22, 51.45, 45.27 and 58.91% for non-debranched and debranched starches preheated at 75, 95 and 121°C for 48-hr). The debranched starches with higher degrees of syneresis provided products with higher resistant starch contents which were evaluated by pancreatic α-amylase and glucoamylase at 37°C for 16-hr. The resistant starch content increased by 4 folds with debranching process (5.61, 4.12 and 19.31% for native starch, non-debranched retrograded starch and 48-hr pullulanase debranched starch preheated at 121°C). It is undoubtedly that debranching allowed more rearrangement of linear glucans and more ordered structures of retrograded starches which were characterized as V-type, could be obtained as indicated by X-ray diffraction analysis.

Keywords: Resistant starch type III, Enzymatically-debranched, High amylose rice starch

Introduction

Rice, being one of the primary dietary sources of carbohydrates worldwide, is of particular interest when assessing variability in starch digestibility. Miller *et al.*, (1992) classified rice as a high glycemic index food with value ranging from 64 to 93 in the freshly cooked form. The freshly cooked rice contains a lower percentage of RS(below 3%) and tends to increase with amylose content and gelatinized temperature (Walter *et al.*, 2005). The cooling and storing of cooked rice is known to entail starch retrogradation, thus increasing the level of enzyme-resistant starch through recrystallization (Englyst *et al.*, 1999). Thus, resistant starch formations from rice starch are excellent functional food ingredients in regulating glucose control for diabetic patients.

Resistant starch (RS) is described as starch that escapes digestion in the small intestine (Cumming *et al.*, 1996; Topping and Clifton, 2001). RS activities are similar to those of dietary fibers, including pre-biotic effect on colon micro flora, altering metabolism, improving cholesterol metabolism, and reducing the risk of ulcerative colitis and colon cancer. Resistant starch resulted from the highly retrograded amylose fraction has been classified as RS type III (RS III). Formation of RS III depends on many factors, such as amylose/amylopectin ratio, pH, temperature, incubation and storage time, number of heating and cooling cycles, and water content (Escarpa, 1996; Kim *et al.*, 2006). Debranching using pullulanase had been used to produce a glucan with linear, low-molecular-weight and recrystallization polymer chains (Guraya *et al.*, 2001; King and Tan, 2005 and Yin *et al.*, 2007). Debranching enzymes such as pullulanase rapidly hydrolyze only α-1,6-glucosidic bonds. This releases a mixture of varied length unit chains from the parent amylopectin molecule that induce retrogradation. In addition, retrogradation is often enhanced when starch gels are subjected to freezing and thawing treatments (Tovar *et al.*, 2002).

The objective of this study was to improve RS III formation from high-amylose rice starch by enzymatically debranching treatments. The effects of these treatments on physicochemical properties and starch digestibility were carried out to determine if resistant starch content could be raised, without contravening food safety regulations.

Materials and Methods

Materials

High amylose rice starch (HARS) (containing 83.38% carbohydrate, 1.18% protein, 0.91% fat, 0.82% ash, 13% moisture, 32.10% amylose (iodine method), 95.21%dwb of total starch (enzymatic method) and pH 5.0) was kindly supplied by Cho Heng Rice Vermicelli Factory Co., Ltd., Nakornpathom, Thailand. Pullulanase enzyme from *Bacillus acidopullulyticus* (Sigma, E.C. 232-983-9P; ≥ 400 units/ml) was used for the preparation of debranched HARS. Resistant starch assay kit (Megazyme) was obtained from Megasyme International Ireland Ltd., IRELAND. Pepsin (EC 3.4.23.1; 2,980 unit/mg), α- amylase (E.C. 3.2.1.1; Type VI-B from hog pancreas; 20.4 unit/mg) and amyloglucosidase (A-3042; 69.65 unit/mg, from *aspergillus niger*), Glucose (GO) assay kit (GAGO-20) and potato amylose were purchased from Sigma Chemical Company, USA.

Resistant starch type III formation

An aqueous high amylose rice starch (HARS; 15% w/w, dry basis) were prepared by fully dispersing the sample in distilled water. The slurry samples were annealed at room temperature (30°C) for 1 hr with occasionally vigorous shaking. The annealed samples were preheated at different temperatures (75, 95 and 121°C for 30-min) and cooled to 55°C. The starch solution samples were debranched using 8 unit pullulanase enzymes per gram starch at 55°C for 0, 2, 4, 8, 16, 24 and 48-hr in shaker water bath (continuous shaking at 170 rpm). The retrogradations of debranched starches were then induced at 4°C for 16-hr. Afterwards, the freeze-thaw cycle (-10/30°C) was further applied to promote syneresis of retrograded starches. The recrystallization starch was dried at 45°C to approximate 10 % moisture content and ground passed through a 100-mesh sieve.

Degree of pullulanase hydrolysis

Reducing sugar (Rds) and total sugar (TS) in the samples, debranched for specific times, were analyzed according to the Park- Johnson method (Hizukuri, 1995) and the phenol-sulfuric acid reagent method (Dubois *et al.*, 1956), respectively. The hydrolysis treatment was conducted in triplicate. The extent of debranching of HARS,

using pullulanase enzyme, was evaluated in terms of degree of hydrolysis (D.H.). D.H. was determined by the equation: *D.H. (%) = (% of Rds after hydrolysis ÷ % of TS after hydrolysis) × 100.*

Degree of syneresis

Degree of syneresis in the samples, debranched for specific times, was determined according to Karim *et al.*, (2000) with modification. Portions of each debranched starch paste were transferred into each disposable dish and covered with adhesive tape to prevent moisture loss. The samples were frozen at -10°C for 16-hr and thawed at 30°C for 2-hr. The samples were then subjected to a vacuum filtration after freeze-thaw cycles. Water of syneresis from triplicate gel samples was collected and weighed. The weight of retrograded gel and water syneresis was used to calculated degree of syneresis as follows:

Degree of syneresis (%) = (Weight of water syneresis / Weight of retrogradation gel) × 100.

Resistant starch and estimated glycemic index analysis

Resistant starch (RS) was determined using a Megazyme Resistant Starch kit (AOAC Method 2002.02). The samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 hr at 37°C to reduce digestible starch to glucose. The reaction was terminated with 4 ml ethanol and the indigested RS III was recovered by centrifugation (5000 g, 10 min). The supernatant was decanted and washed with 50% ethanol for two times (digested starch). The indigested RS III was solubillized in 2ml of 2 M KOH in an ice bath, neutralized with 8 ml sodium acetate (1.2 M) and the RS hydrolyzed to glucose with of AMG (0.1 ml, 3300 Uml^{-1}, 50°C). The glucose oxidase/peroxidase reaction was used to measure glucose released from the digested starch and resistant starch. Absorbance was read (510 nm) after a 20 minute incubation period at 50°C. Resistant starch and digested starch were calculated as glucose × 0.9. The total starch content was calculated as the sum of resistant starch and digested starch.

In vitro starch hydrolysis and glycemic index (GI) were determined according to Goñi, *et al.*, (1997). An equation: $C = C_\infty(1\text{-}e^{-kt})$, was used to described the kinetics of starch hydrolysis, where C, C_∞ and k were the concentrations at time t, the equilibrium concentration and the kinetic constant, respectively. Using the hydrolysis curve (0-180 min), the hydrolysis index (HI) was calculated as the percentage of total glucose released from the samples, to that released from white bread. The glycemic index of the samples was estimated according to the equation: GI = (39.71 + 0.549)× HI (Goñi, *et al.*,1997).

X-ray diffraction

X-ray diffraction patterns of native rice starch and the selected RS III samples were measured with copper K_2 radiation (λ =0.154 nm) using a diffractometer (JEOL,JDX-3530, Japan). The diffractometer was operated at 300 mA and 30 kV, 2θ range from 10 to 70.0° with a step size 0.05° and a count time of 2s. The data was analyzed with program MDI Jade 6.5 (Japan). The crystallinity of RS III samples were calculated as the proportion of crystalline area to total area at angles between 10 and 35 °2θ (Cairns *et al.*, 1997).

Experimental Design and Statistical Analysis

The data obtained for the pullulanase hydrolysis, physicochemical, resistant starch, hydrolysis index and glycemic index were subjected to analysis of variance (ANOVA). Duncan's multiple range tests (DMRT) procedure was used to make specific comparison between treatments (SPSS version 13).

Result and Discussion

Degree of pullulanase hydrolysis and degree of syneresis

The reducing sugar content was used to determine the amount of branch chains released by action of pullulanase hydrolysis on the 15% high amylose rice starch partially gelatinized at different temperatures (75, 95 and 121°C) shown in Fig. 1a. The degrees of pullulanase hydrolysis in high amylose rice starch slurry heated at 121°C were significant higher than the preheated at 75°C and 95°C. The degree of pullulanase hydrolysis of high amylose rice starch preheated at 75°C, 95°C and 121°C for 0 to 48-hr were 0.14 to 1.55% and 0.14 to 0.50%, and 0.17 to 3.10%, respectively.

It was obvious that an enzyme hydrolysis with a pullulanase enzyme improved the degree of syneresis (33.22, 51.45, 45.27 and 58.87% for non-debranched and debranched starches preheated at 75, 95 and 121°C for 48-hr; Fig 1b).This should be due to the pullulanase enzyme hydrolyze α-1,6-glucosidic bonds, releasing a linear polymers linked by α-1,4-glucosidic bonds. The higher degree of syneresis was due to a progressive reassociation of the starch molecules upon aging and freeze-thaw process. This recrystallization is referred to as retrogradation, and may reduce the digestibility of the starch (Gurya *et al*., 2001 and Tovar *et al*., 2002).

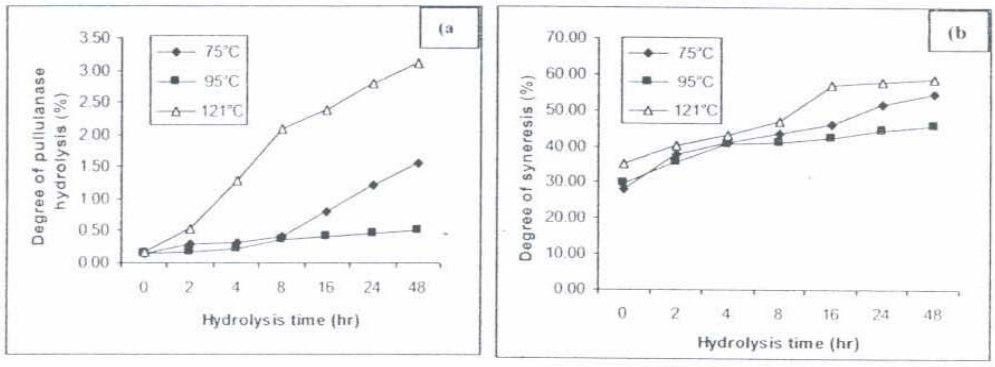

Fig1. Degree of pullulanase hydrolysis (a) and degree of syneresis (b) of 0 to 48-hr debranched high amylose rice starches preheated at 75, 95 and 121°C for 30 min.

Resistant starch and estimated glycemic index

Resistant starch (RS) content and glycemic index (GI) have been established as two important indictors of starch digestibility. RS, HI and GI value of resistant starch type III are presented in Fig 2. The RS contents in 48-hr hydrolysis of 121°C preheated sample were highest (19.19%) but not significant difference ($p > 0.05$) from the 16 and 24-hr hydrolysis of the 121°C preheated (17.14 and 18.33%). The RS content in the 0 hr to 48 hr hydrolysis of 75°C and 95°C preheated ranged from 4.04 to 12.33% and 4.06 to 9.68%, respectively.

The RS III from pullulanase debranched starch preheated at 121°C (0 to 48-hr hydrolysis) was lowest in the HI value (68.66 to 18.17%, respectively). The GI values based on HI values were between 77.40 to 49.68% from 0 to 48-hr hydrolysis. The GI value in the 0 hr to 48 hr hydrolysis of 75°C and 95°C preheated decreased from to 78.73 to 56.08 and 79.01 to 69.09%, respectively. High resistant starch content in RS III sample had a low glycemic index because of the slowly release of glucose, which may simply result from a lack of available digestible starch (Jenkins *et al*., 2002, Kim *et al*., 2003 and Shane, 2005).

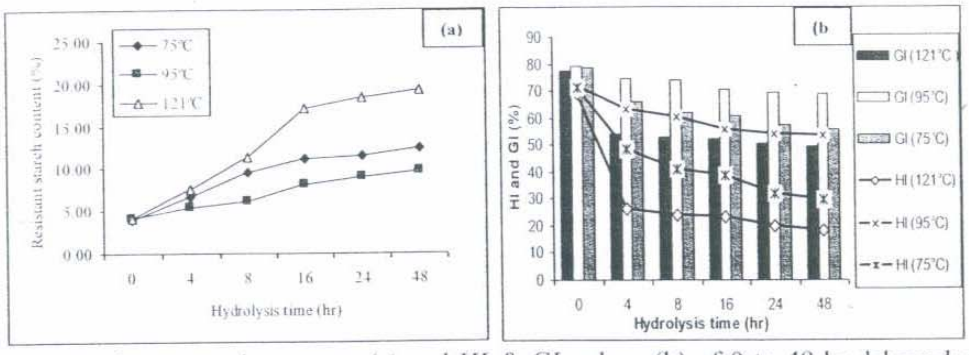

Fig2. Resistant starch contents (a) and HI & GI values (b) of 0 to 48-hr debranched high amylose rice starches preheated at 75, 95 and 121°C for 30 min.

Crystallinity of RS III formation

X-ray diffraction patterns of native high amylose rice and RS III samples (0 to 48-hr pullulanase hydrolysis of 75 and 121°C preheated) are shown in Fig.3. The diffraction pattern obtained from native starch was classified as an A-type pattern as indicated by typical peaks at 15.0, 17.5 and 23.2° of diffraction angle 2θ, with 13.18% crystallinity. These values are in agreement with those reported for native rice starch (Ornanong *et al.*, 2006). When the starch was subjected to debranching and retrogradation treatments in this study, the 0, 4, 16, 24 and 48-hr pullulanase hydrolysis treatments showed completely different pattern from the native starch. The RS III sample from 0 hr hydrolysis exhibited very low values due to the loss of crystallinity during thermal treatment, and the calculated crystallinity was only 2.58%. The RS III formation from 16 and 48-hr hydrolysis of 75°C-preheated starch sample displayed a V-type diffraction pattern with 10.67% and 12.91% crystallinity, respectively. In addition, the RS III samples from 4, 16, 24 and 48-hr hydrolysis of 121°C-preheated starch showed a V-type diffraction pattern, which had 14.37, 15.59, 15.80 and 19.50% crystallinity, **respectively.**

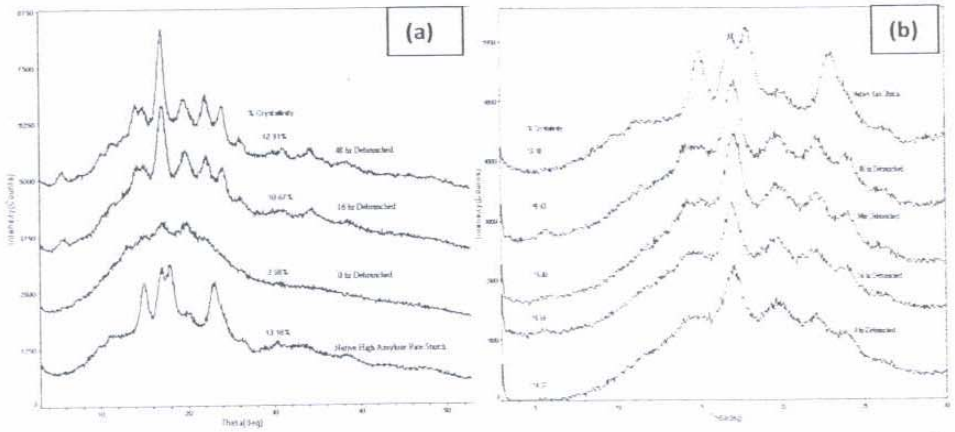

Fig3. X-ray diffraction pattern of native high amylose rice starch and RS III from pullulanase-debranched high amylose rice starch (0 to 48-hr) preheated at 75°C (a) and 121°C (b).

Conclusions

The results obtained in this study show that the higher degree of pullulanase debranching was closely related to a higher degree of syneresis and resistant starch content of the RS III produced from high amylose rice starch. The higher degree of syneresis yield produced with higher resistant starch contents and lower glycemic indices. X-ray diffraction pattern of RS III were displayed a V-type diffraction pattern.

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สตาร์ชที่ทนต่อเอนไซม์ (RS III) จากการดัดแปรสตาร์ชข้าวแอมิโลสสูงด้วยเอนไซม์พูลูลาเนส

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

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บทคัดย่อ

ศึกษาการดัดแปรโครงสร้างสตาร์ชข้าวแอมิโลสสูง (32.10%) ด้วยเอนไซม์พูลูลาเนส (E.C.232-983-9P, 8 ยูนิตต่อกรัมสตาร์ช) ย่อยตัดพันธะกิ่งแอมิโลเพกทินในโมเลกุลสตาร์ชข้าวเข้มข้นร้อยละ 15 โดยน้ำหนัก ซึ่งให้ความร้อน (75°ซ.นาน 30 นาที) ก่อน แล้วย่อยตัดพันธะกิ่งนาน 0 ถึง 48 ชั่วโมง (ที่ 55°ซ.) พบว่าเอนไซม์พูลูลาเนสสามารถตัดพันธะกิ่งแอมิโลเพกทินได้ร้อยละ 0.14 ถึง 1.55 เมื่อนำสตาร์ชที่ตัดพันธะกิ่งมาต้มสุกและทำให้เย็น (4°ซ./16 ชั่วโมง) จนเกิดรีโทรเกรเดชั่น แล้วคืนรูปจากเยือกแข็ง (-10/30°ซ.) เพื่อให้โมเลกุลสตาร์ชจัดเรียงตัวใหม่และเพิ่มการบีบน้ำออก มีผลให้ระดับการเกิดรีโทรเกรเดชันเพิ่มขึ้นจากร้อยละ 28.10 เป็นร้อยละ 54.53 และปริมาณสตาร์ชที่ทนต่อการย่อยด้วยเอนไซม์แอลฟาอะมิเลสเพิ่มขึ้นจากร้อยละ 4.80 เป็น 12.33 เมื่อทำการดัดแปรด้วยพูลูลาเนสนาน 0 ถึง 48 ชั่วโมง ตามลำดับ และการดัดพันธะกิ่งทำให้โมเลกุลสตาร์ชเรียงตัวใหม่มากขึ้นและเปลี่ยนโครงสร้างผลึกสตาร์ชจากแบบ A เป็นแบบ V เมื่อตรวจสอบด้วยเครื่องเอกซ์เรย์ดิฟแฟรกโตมิเตอร์

Abstract

In this studied, a debranching enzyme (Pullulanase, EC. 232-983-9P, 8U/g starch at 55°C for 0 – 48-hr) was introduced to modify the amylopectin molecules of 15% (w/w) high amylose rice starches (32.10%, amylose content) which were partially gelatinized at 75°C for 30 min. The retrogradations of debranched starches with different degrees of hydrolysis (0.14–1.55%) were then induced at 4°C for 16 hrs. Afterwards, the freeze-thaw cycle (-10/30°C) was further applied to promote syneresis of retrograded starches. It was shown that pullulanase hydrolysis improved the degree of retrograded from 28.10 to 54.53%. The resistant starch content of pullulanase debranched starch increased from 4.80% to 12.33% by 0 to 48-hr, respectively. Results had showed that after debranching, starch molecule have rearranged and changed their crystal pattern from A to V-type revealed by by X-ray diffraction analysis.

Key words: Resistant starch type III, Pullulanase-debranched, High amylose rice starch,

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Introduction

Rice (*Oryza sativa* L.) is a major cereal of Thailand. Commercial rice starch composed of up to 90 % carbohydrates and 0.5-1.5 % protein with negligible fat or dietary fiber (Masaki *et al.*, 2005). Rice flour and starch become popular food ingredients since they are hypoallergenic, low fat and neutral in flavor. However, rice starch has high glycemic index (GI) food compared to starch based foods. Freshly cooked rice contains a lower percentage of resistant starch (RS), below 3%, but the RS tends to increase with amylose content and processing treatments (Walter *et al.*, 2005).

Enzyme-resistant starch has been recently defined as the sum of starch and its degradation products that are not absorbed in the small intestine of healthy individuals (Englyst *et al.*, 1999). The reduced bioavailability of resistant starch in the human gastrointestinal tract has particular significance for diabetics because lowers the insulin response (Englyst *et al.*, 1999). Besides physiological benefits in human, RS has been reported to have potential use as a unique ingredient with improved oral tactile perception, taste palatability, color and texture. RS can be found in nature and its contents of RS are varied by botanical source and starch granular structure (i.e. resistant starch type I and II). In addition, resistant starch (RS III) can be produced from retrogradation of gelatinized starch known as resistant starch type III. The extents of RS III of most starches are, however, are quite low, depending on the molecular structure of starch glucans, namely amylose and amylopectin. High amylose content produced higher resistant starch than low amylose content. Debranching using pullulanase have been used to produce a sample with linear, low-molecular-weight and recrystallization polymer chains (Guraya *et al.*, 2001 and Yin *et al.*, 2007). Debranching enzymes (pullulanase) rapidly hydrolyze only α-1,6-glucosidic bonds. This releases a mixture of varied unit chain length from the amylopectin molecule which in turn facilitated staarch retrogradation. In addition, retrogradation is often enhanced when starch gels are subjected to freezing and thawing treatments (Tovar *et al.*, 2002). Freezing a starch gel leads to the formation of ice crystals and thus concentrates the starch in non-ice phase. Upon thawing, the water can be easily compressed from the network, giving rise to a phenomenon known as syneresis (Tovar *et al.*, 2002).

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

Materials and Methods

High amylose rice starch was kindly supplied by Cho Heng Rice Vermicelli Factory Co., Ltd., Nakomprathom, Thailand. Pullulanase enzyme from *Bacillus acidopullulyicus* (E.C. 232-983-9P; ≥ 400 international units/ml), pepsin (EC 3.4.23.1; 2,980 unit/mg), α- amylase (E.C. 3.2.1.1; 20.4 unit/mg)

and amyloglucosidase (A-3042; 69.65 unit/mg), Glucose (GO) assay kit (GAGO-20) and potato amylose were purchased from Sigma Chemical Company, USA. Resistant starch assay kit (Megzyme) was obtained from Megasyme International Ireland Ltd. Ireland.

Resistant starch type III formation

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

Chemical composition of raw material

Moisture, protein, carbohydrate, fat, ash and fiber content (%) of high amylose rice starch (HARS) were determined followed AOAC(AOAC, 2000). Amylose content (%) was determined colorimetrically after iodine binding (Juliano, 1997).

Degree of pullulanase hydrolysis and degree of debranched

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

Degree of Syneresis (D.S.)

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

D.S. (%) = *(Weight ofexudated water / Weight of retrogradation gel) X 100.*

Scanning electron microscopy (SEM)

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

X-ray diffraction

X-ray diffraction patterns of native rice starch and the RS III samples were measured with copper K_2 radiation (λ =0.154 nm) using a diffractometer (JEOL, JDX-3530, Japan). Diffractometer was operated at 300 mA and 30 kV, 2θ range from 10 to 50.0° with a step size 0.05° and a count time of 2 s. The data was analyzed with program MDI Jade 6.5 (Japan). The crystallinity of the samples was calculated as the proportion of crystalline area to total area at angles between 10 to 30° Theta.

Resistant starch and estimated glycemic index analysis

Resistant starch (RS) was determined using a Megazyme Resistant Starch kit (AOAC Method 2002.02). The samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16-hr at 37°C to reduce digestible starch to glucose. The reaction was terminated with 4 ml ethanol and the RS sediment was recovered by centrifugation (5000 g, 10 min). The supernatant was decanted and washed with 50% ethanol for two times (digested starch; DS). The sediment was solubillized in 2ml of 2 M KOH in an ice bath, neutralized with 8 ml sodium acetate (1.2 M) and the RS hydrolyzed to glucose with amyloglucosidase (0.1 ml, 3300 Uml^{-1},50°C). The glucose oxidase/peroxidase reaction was used to measure glucose released from the digested starch and resistant starch. Absorbance was read at 510 nm after a 20 minute incubation period at 50°C. RS and DS were calculated as glucose × 0.9. The total starch was calculated as the sum of RS and DS.

In vitro starch hydrolysis and glycemic index were determined according to Goni *et al.*, (1997). An equation: $C=C_{\infty}(1-e^{-kt})$ was used to described the kinetics of starch hydrolysis, where C, C_{∞} and k were the concentration at time t, the equilibrium concentration and the kinetic constant, respectively. Using the hydrolysis curve (0-180 min), the hydrolysis index (HI) was calculated as the percentage of total glucose released from the samples, to released from white bread. The glycemic index of the samples was estimated according to the equation: $GI = (39.71 + 0.549) \times HI$.

Experimental Design and Statistical Analysis

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

Results and discussion

Chemical composition of native rice starch

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

Degree of debranched and Degree of retrograded

Degree of debranched and degree of syneresis from pullulanase hydrolysis of high amylose rice starch are shown in Fig.1. The absorbance obtained at various debranching time is related to the number of reducing groups produced by action of pullulanase hydrolysis on rice starch from 0 to 48-hr were increased from 0.44 to 1.55% degree of hydrolysis (Fig 1a). The high degree of pullulanase hydrolysis can be attributed to the high degree of syneresis (28.10 to 54.53%). It could be due to the pullulanase enzyme hydrolysis of α-1,6-glucosidic bonds and released a linear polymers linked by α-1,4-glucosidic bonds. These fragments could rapidly producing retrograded starch. In addition, the higher degree of retrograded upon aging and freeze-thaw process was due to a progressive re-association of the starch molecules. This recrystallization may reduce the digestibility of the starch (Tovar *et al.*, 2002).

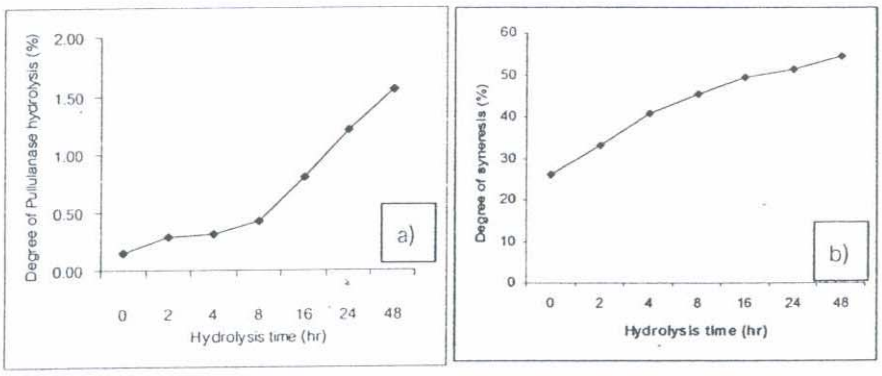

Fig.1. Degree of pullulanase hydrolysis (a) and degree of syneresis from pullulanase hydrolysis of 75°C preheated high amylose rice starch from 0 to 48-hr at 55 °C.

Morphological properties

SEM micrographs of native high amylose rice starch, annealed and 75°C preheated, 16-hr debranched of 75°C preheated and RS III samples are shown in Fig. 2. Native high amylose rice starch granules have polygonal shapes with diameter between 3-5 μm (Fig 2 a). The surface of the native granules was smooth without observable pores. The annealed and 75°C preheated (Fig 2 b) exhibited swelling and partial gelatinization at the surface of the starch granules. This might permit the enzyme to access to the molecules more easily and uniformly debranch the amylopectin molecules. At hydrolysis for 16-hr, the starch showed surface erosion and slightly damaged starch granules. In Fig 2d the RS III sample formed a coarse honeycomb-like and filamentous network structure.

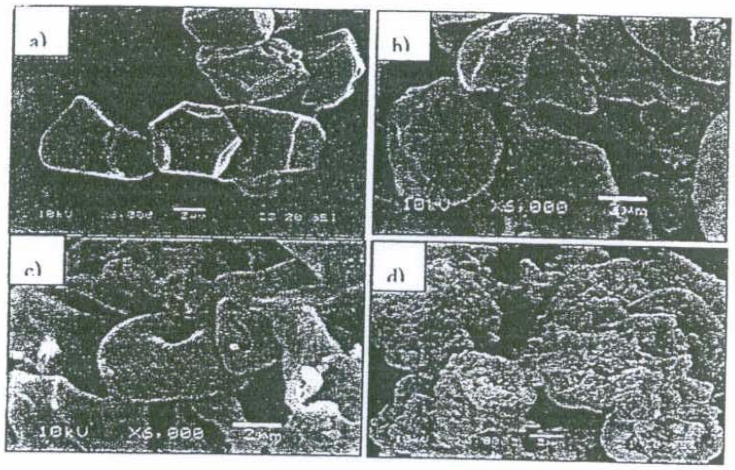

Fig.2 Scanning electron micrographs (magnification at 6000x) of native (a), annealed and 75°C preheated (b), 16-hr debranched of starch preheated at 75C (c) and resistant starch type III (d).

Starch digestibility and glycemic index

Resistant starch (RS) is used as a predictor of the release rate of glucose and glycemic index (GI) which can be predicted through *in vitro* starch hydrolysis model (Englyst *et al.*, 1999; Goni *et al.* 1997). The RS content in hydrolyzed for 48 hrs was not significant difference ($p \geq 0.05$) from the 16 and 24 hr hydrolysis time (Table 1). The RS contents at the 16 to 48-hr hydrolysis were 11.24 and 12.33 % dwb, respectively. The digestible starch content was decrease with the increasing hydrolysis time. Corresponding to the degree of hydrolysis results, the rate and extent of starch hydrolysis and glycemic index were different among RS III formation (Table 1). The high RS content (12.33%) in the RS III samples was highly resistant to hydrolysis, and hydrolysis was complete in a low degree (35.61%) after 180 min, whereas 62.70% of the non debranched samples had been hydrolyzed. In addition, the GI based on HI were between 59.29 to 74.28%.

Crystallinity of RS III by X-ray Diffraction

X-ray diffraction patterns of native rice starch and three RS III samples (0, 16 and 48-hr pullulanase hydrolysis and freeze thaw process) are shown in Fig. 3. The diffraction pattern obtained from native rice starch was classified as an A-type pattern as indicated by typical peaks at 15.0, 17.5, 20.0 and 23.2° of diffraction angle 2θ. The calculated crystallinity of native rice starch was 13.18%. The value was in agreement with those reported for native rice starch (Ornanong *et al.*, 2006). When the HARS was subjected to pullulanase debranching and syneresis treatments, the 0, 16 and 18 hr hydrolysis treatments, the results showed totally different diffraction pattern, from the native. The RS III sample from non debranched showed very low degree of crystallinity (2.58%) due to the loss in crystallinity during thermal treatment. The RS III formation from 16 and 48 hr hydrolysis displayed V-type diffraction pattern, with 10.67% and 12.91% crystallinity, respectively.

Table1. Resistant starch, digestible starch, total starch content, hydrolysis index and glycemic index of resistant starch type III from pullulanase debranched high amylose rice starch.

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

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

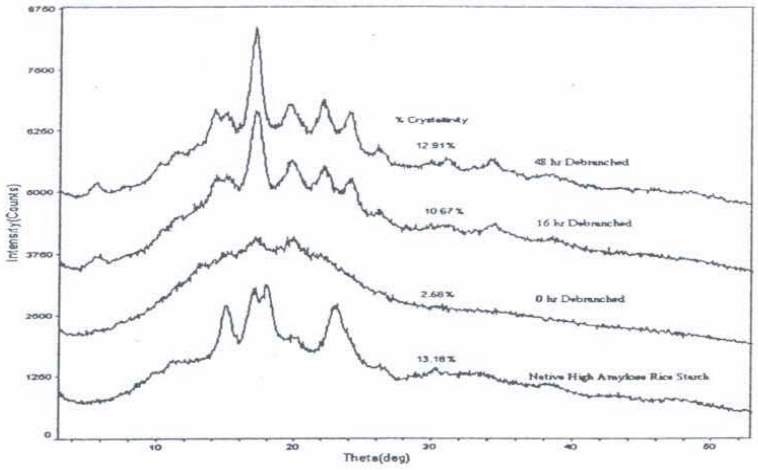

Fig. 3 X-ray diffraction patterns of native and RS III samples from debranched and retrograded of high amylose rice starch.

Conclusion

The results obtained in this study showed that the high degree of pullulanase debranched were closely related to high degree of retrogradation and resistant starch content of the RS III formation. The RS contents of the 0 hr to 48 hr hydrolysis were 4.80 to 12.33%. X-ray diffraction patterns of the RS III from 16 and 48-hr hydrolysis showed more crystalline structure with V-type pattern.

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เรื่อง

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