ENZYMATIC AND PHYSICAL MODIFICATION OF THAI RICE TO LOW GI RS3 – RICE STARCH

PRAEWPRAN CHAVANON
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A thesis summited to Theophane Venard School of Biotechnology,
Assumption University in part of the Requirements for the degree of
Master of Science in Food Biotechnology
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ABSTRACT

Rice is one of the global staples and a good option for developing an elderly food. However, it is troublesome since rice is digested too quickly which increases body blood sugar level and raises risk of diabetes. Debranching using pullulanase is believed to be an effective solution to solve this problem. This project aimed to modify Thai rice including red rice, Riceberry and Black glutinous rice to become low GI RS3 rice starch by using enzymatic and physical methods. The 10% (w/w) rice starch was gelatinized at 95°C for 15 min. Then, rice starch solution was debranched by pullulanase at 55°C. The concentration of pullulanase was varied into 10, 20, 30, 40, 50, 60 U/g. And debranching time was varied into 1, 2, 3, 4, 5, 6 hours. These debranched samples were determined for reducing ends by Nelson-Somogyi method in order to select the suitable condition for modification of rice starch. Both native and modified rice starches were determined for RAG and SAG after enzymatic and physical modifications. According to results, these enzymatic and physical modifications were effective for increasing resistant starch content in rice starch and making rice starch more resistant to digestive enzymes. Both RAG and SAG in modified rice starch were lower when compared with RAG and SAG in native rice starch. For enzyme modification, the best condition were 60 U/g and 5 hours for enzyme concentration and debranching time respectively. This condition led to the highest concentration of reducing ends which indicated the highest amount of linear chains. These fragments could rapidly produce retrograded starch and increase RS content upon freeze-thaw process. Riceberry was selected as the best type of rice for production of low GI RS3 rice starch since it had the lowest RAG in both native and modified rice starch when compared with other types of rice.

OBJECTIVES

1. To determine rapidly available glucose (RAG) and slowly available glucose (SAG) of 3 different types of rice starch
2. To determine the best condition to produce low GI RS3 rice starch by using pullulanase followed by physical methods
3. To determine the best type of rice used for production of low GI RS3 rice starch

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INTRODUCTION

Aging society in Thailand leads to an increase of health problems and health needs among Thai elderly people. Thai elderly population will meet almost one fourth of total Thai population in 2028 (SCB Economic Intelligence center, 2013). Health problems among the elderly are chronic diseases such as hypertension, diabetes, and cardiovascular diseases. Therefore, the business of developing and selling foods required by senior citizens is promising. The types of food needed are those that are easy to chew and digest, or that increase vitality or appetite. Elderly people tend to consume less per meal, so companies are looking to develop products with smaller serving sizes or in easy-to-open packages (SCB Economic Intelligence center, 2013).

Eating well balanced meals is important to maintaining a healthy lifestyle. Carbohydrates are required for elderly nutrition as they provide the body the fuel to keep your heart, lungs and organs functioning. Rice is one of the global staples and a good option for developing elderly food. It is easy to cook and digest and is comparatively less expensive than other food items offering similar nutritional value. The carbohydrate content of rice is a good source of fuel for the body and brain functioning. Also rice is cholesterol free and low in sodium and helps in managing blood pressure (Cervoni, 2019). However, rice can be a problem since rice is digested too quickly which increases body blood sugar level and raises risk of diabetes in elderly.

Modification of rice to become low GI RS3 rice starch is believed to be an effective solution to solve this problem. This can be done by debranching using pullulanase which is gaining popularity in the processes of starch conversion. The increased degree of debranching would give chains a more opportunity to align and aggregate to form perfect crystalline structure, thereby leading to the formation of more resistant starch content in rice (Babu, 2016). Resistant starch is a carbohydrate that resist digestion in the small intestine and ferments in the large intestine. Because resistant starch is not digested in the small intestine, it does not raise glucose level. Gut health is improved as fermentation in the large intestine makes more good bacteria and less bad bacteria in the

gut. Healthy gut bacteria can improve glycemic control. This low GI RS3 rice starch. It can be used in a large amount of applications including ready-to eat meals, cereal, soup etc.

Therefore, this project aimed to modify different variety of Thai rice to become low GI RS3 rice starch which contains glycemic index less than 55 by using enzymatic and physical methods. The products obtained from this project can be applied to many types of food. Importantly, these products follows elderly food trend since they provide low glycemic index and will not raise risk of several non-communicable diseases.

LITERATURE REVIEWS

1. Red rice, Riceberry and Black glutinous rice

Red rice is a kind of unpolished rice which has higher nutritional value compared to white rice or even polished rice. Cooking time is comparatively more than white rice and it has a nutty taste and more gratifying flavor. It is rich in fiber content, vitamin B1 & B2, iron and calcium. Because of higher nutritive content and health advantages of red rice, it is strongly advised for heart patients as well as diabetics. Additionally it is loved by health gurus as well as fitness fanatics since its high fiber content helps gain less weight (Healthbenefitstimes, n.d.).

Figure 1: Red rice

Riceberry Rice is an across-bred of Hom Nil (Black Fragrant) rice and jasmine (Khao Dawk Mali 105) rice. As a result, it has a long shape (like that of Indica rice), and is purple and soft. Riceberry rice can be grown throughout the year. Riceberry's nutritional characteristics include high antioxidant values, e.g. Beta Carotene, Gamma Oryzanol, Vitamin E, Tannin, Zinc and Folate, with medium to low Glycemic Index (GI). A number of fibers can be found in Riceberry rice. It helps reduce fat and cholesterol levels. In addition to preventing heart diseases, it helps in weight management and maintenance of the excretory system (Sandeerice, 2015).

Figure 2: Riceberry

Black glutinous rice is short grained and sticky when fully cooked. Despite its name, it actually has a dark purple color, and according to the American Chemical Society, it is possibly healthier with more nutritional benefits than brown rice. Black glutinous rice has a dark purple color almost black. Black glutinous rice is rich in antioxidants due to its anthocyanin content. The antioxidant content in rice is claimed to be higher compared to other types of rice. Many researches said that anthocyanins as antioxidants contained in black glutinous rice are believed to have abilities such as: maintaining heart health, preventing cancer caused by free radicals, improving brain function, helping to reduce inflammation, and much more. Not only that, but black glutinous rice also contains other important antioxidants such as vitamin E, which can also help maintain eye health, skin, and body immunity (Pondan, 2019).

Figure 3: Black glutinous rice

2. Starch in rice

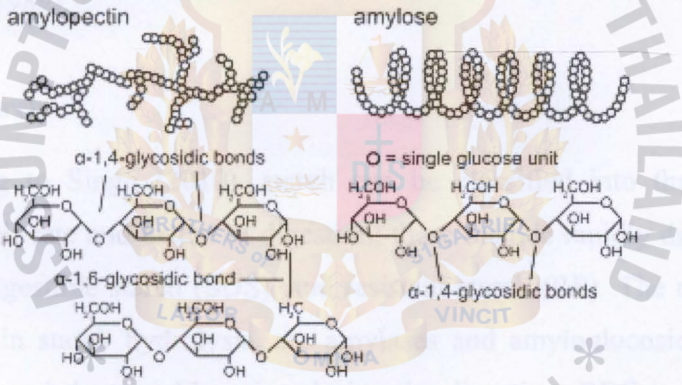

Figure 4: Structure of starch *(Willfahrt, 2019)*

Rice contains approximately 87% carbohydrates, 7 to 8% proteins and very low in fat (Prepare Foods, 1993). Rice starch is made up with amylose and amylopectin. When cooked in water, the starch molecules swell and absorbed the moisture between 61 to 93°C. Amylose is composed largely of unbranched chains of 100 or more glucose members, while amylopectin has a laminated or branched structure, composed of unit chains of about 20 glucose residues. Amylose produces a blue color, while amylopectin yields reddish coloration, with iodine (Rao, 1951).

Starch is a polymer of D-glucose linked α (1-4) and usually consists of an essentially linear fraction, amylose, and a branched fraction, amylopectin. Branch points are α (1-6) linkages. Innovative techniques have now shown rice amylose to have two

to four chains with a number-average degree of polymerization (DPn) of 900 to glucose units and a ß-amylolysis limit of 73 to 87 percent (Hizukuri et al., 1989). It is a mixture of branched and linear molecules with DPn of 1100 to 1700 and 700 to 900, respectively. The branched fraction constitutes 25 to 50 percent by number and 30 to 60 percent by weight of amylose. The iodine affinity of rice amyloses is 20 to 21 percent by weight.

Rice amylopectins have ß-amylolysis limits of 56 to 59 percent, chain lengths of 19 to 22 glucose units, DPn of 5000 to 15,000 glucose units and 220 to 700 chains per molecule (Hizukuri et al., 1989). The iodine affinity of rice amylopectin is 0.4 to 0.9 percent in low- and intermediate-amylose rices but 2 to 3 percent in high-amylose rices. Isoamylase-debranched amylopectins showed more longest chain fractions (DPn >100) (9 to 14 percent) in high-amylose samples with higher iodine affinity than in low- and intermediate-amylose samples (2 to 5 percent) and waxy rice amylopectin (0 percent) (Hizukuri et al., 1989).

3. Resistant starch

According to Singh (2010), starch can be classified into three categories, depending on their rate and extent of digestion; these include rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). The main enzymes, which take part in starch hydrolysis, are amylases and amyloglucosidases resulting glucose, maltose and dextrins liberation during the digestion. RDS is the fraction of starch granules that cause a rapid increase in blood glucose concentration after ingestion of carbohydrates. This fraction of starch in vitro is defined as the amount of starch digested in the first 20 min of a standard digestion reaction mixture. SDS is defined as the starch that is digested after the RDS but in no longer than 120 min under standard conditions of substrate and enzyme concentration. The fraction of starch that escapes digestion in the small intestine, and cannot be digested within 120 min, is defined as resistant starch.

Resistant starch (RS) plays a major role in the health food industry, because it behaves similar to soluble and insoluble dietary fiber in the gastrointestinal tract. As it is resistant to human digestive enzymes, the slow release of glucose results in a reduced energy intake by the intestinal cells. This is evident by the low glycemic index of the non-digested starch. This can help to improve glucose regulation in diabetes and

facilitate better weight control for the obese. The non-digested starch in the large intestine is fermented by colonic microflora producing short chain fatty acids that encourage the growth of beneficial bacteria, indicating a prebiotic functionality. This may lead to healthier colon cells and reduce the development of colon cancer. The metabolic mechanisms of resistant starch was shown in figure 5. In addition, a diet high in RS can reduce blood cholesterol and triglyceride levels, because of higher excretion rates of cholesterol and bile acids. Overall, increasing the RS content in the diet has the potential to provide several significant health benefits and add value to food products (Vatanasuchart, 2009).

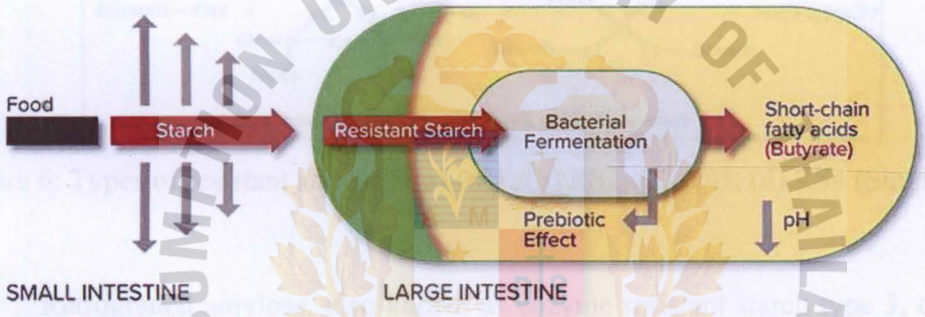

Figure 5: Metabolic Mechanisms of resistant starch (Bird, 2020)

Resistant starch has been classified into four general subtypes (Figure 6) called RS1, RS2, RS3 and RS4. RS1 is heat stable in most normal cooking operations, which enables its use as an ingredient in a wide variety of conventional foods. RS2 is native, uncooked granules of starch. A particular type of RS2 is unique as it retains its structure and resistance even during the processing and preparation of many foods; this RS2 is called high amylose maize starch. RS3 refers to non-granular starch-derived materials that resist digestion. RS3 is of particular interest, because of its thermal stability. This allows it to be stable in most normal cooking operations, and enables its use as an ingredient in a wide variety of conventional foods. RS4 describes a group of starches that have been chemically modified (conversion, substitution, or cross-linking) and include starches which have been etherised, esterified or cross-bonded with chemicals in such a manner as to decrease their digestibility (Singh, 2017).

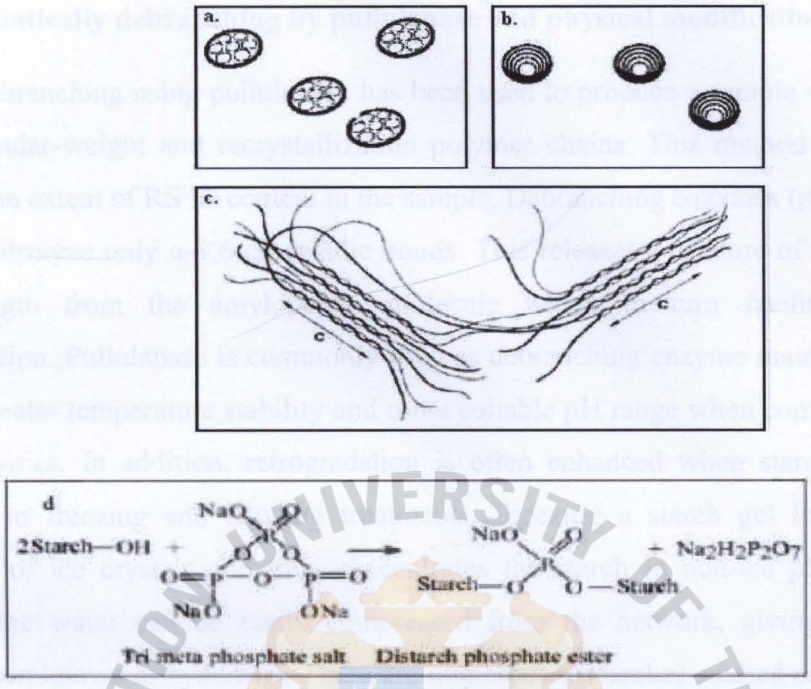

Figure 6: Types of resistant starches: (a) RS1; (b) RS2; (c) RS3; (d) RS4 (Singh, 2017)

Retrograded amylose, also known as enzyme resistant starch type 3, could be formed from native starch granules that have been gelatinized and retrograded afterwards. Resistant starch can be formed through retrogradation. Retrogradation is the precipitation of starch molecules in cooled pastes and gels that contain mainly amylose. The hydrogen bonds within hydrated starch interact and result in physical-chemical changes; however, no permanent chemical bond is created. Amylopectin retrogrades very slowly. The higher the amylose content is, the greater the retrogradation. It is also found that high amylose starch is more resistant to digestion than amylopectin due to its compact linear structure. The rate of retrogradation is dependent on molecular ratio of amylose to amylopectin, structures of the amylose and amylopectin molecules (source of starch), temperature, starch concentration, and presence and concentration of other ingredients such as surfactants and salts (Tan, 2003).

4. Enzymatically debranching by pullulanase and physical modification

Debranching using pullulanase has been used to produce a sample with linear, low-molecular-weight and recrystallization polymer chains. This method is used to improve the extent of RS III content in the sample. 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 facilitate starch retrogradation. Pullulanase is commonly used as debranching enzyme mainly because of their greater temperature stability and more suitable pH range when compared with other enzymes. In addition, retrogradation is often enhanced when starch gels are subjected to freezing and thawing treatments. 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. There are numerous researches studied on the effect of pullulanase on resistant starch content in food samples.

Miao et al. (2009) studied on the effect of pullulanase debranching and recrystallization on structure and digestibility of waxy maize starch. The results showed that increasing pullulanase concentration and decreasing debranching time led to increased maximum SDS, while longer debranching time increase the proportion of RS. A higher SDS content was obtained with debranching 3–6 h at high pullulanase (20 or 40 ASPU/g) then storage at 4 °C for 2 days.

Pongjanta (2009), studied on the effect of debranching enzyme concentration on physicochemical properties and α-amylase hydrolysis rate of resistant starch type III from high amylose rice starch. The results showed that pullulanase hydrolysis improved the degree of syneresis. Resistant starch content increased sharply as the amount of the enzyme increased, reaching the highest (19.81%) for a 12 U/g starch and decreased to 13.16% by 16 U/g starch. The ∝-Amylase hydrolysis rate showed that incompletely-debranched had a lower estimated glycemic index than completely debranched rice starches.

5. Rapidly available Glucose (RAG) and Slowly Available Glucose (SAG)

In principle, the Glycemic Index (GI) is calculated as the measured glycemic response to a portion of test food that contains 50 g "available" carbohydrate (which includes fructose) expressed as a percentage of the glycemic response to the same amount of "available" carbohydrate from a standard food eaten by the same subject. GI values have been published for a wide range of foods and have been used in several studies to design low-glycemic-load diets for diabetic subjects. However, no simple in vitro term is available that defines the carbohydrate in a food in such a way as to characterize its digestion in the gut (Englyst, 1999).

The *in vitro* technique determines RAG, SAG, and starch fractions by measuring the amount of glucose released from a test food during timed incubation with digestive enzymes under standardized conditions. GI is a direct measure of the glycemic response to a food, and thus reflects all the mechanisms that can influence the glycemic response. For most carbohydrate foods, however, the RAG content is almost certainly a major determinant of the magnitude of the GI, and the results have shown a strong correlation between published GI values and RAG values for a wide range of starchy foods.

Rapidly available Glucose (RAG) represents the amount of glucose released after digestion by enzyme in digestive system within first 20 minutes after meal. This part of glucose is then absorbed in duodenum which affects strongly on blood glucose level and insulin level. For Slowly Available Glucose (SAG), it represents the amount of glucose released after digestion by enzyme in digestive system during 20-120 minutes after meal. This part of glucose is then absorbed in jejunum and lleum which affects on blood glucose level and insulin level less than first part that is absorbed in duodenum (Wasusun, 2017).

Both GI and RAG measurements show how food type and food processing can influence the physiologic properties of dietary carbohydrate. According to Englyst (1999), they reported that the *in vitro* measurement of RAG and SAG is useful for characterizing the test foods with respect to glycemic response. To determine the relation between dietary carbohydrate intake and health associated measurements are being applied in ongoing epidemiologic studies.

The values for thirty-nine foods (figure 7) are expressed on the basis of the available carbohydrate content of these foods, highly significant ($P < 0.001$) positive correlations are observed between GI and RAG. The measurement of RAG *in vitro* provides values for direct calculation of the amount of glucose likely to be rapidly absorbed in the human small intestine and, thus, to influence blood glucose and insulin levels. These values can be used to compare foods, as eaten, on an equal-weight basis. Food-table RAG values would allow simple calculation of the total amount of RAG provided by single foods, by whole meals and by whole diets. According to figure 7, the ranking for many foods are not very different between the two systems. However, there are some differences; for example, the rankings for the instant potato and new potato are quite different under the two systems between GI and RAG. The starch in these products is readily digestible, which resulted in a high GI score, but the solids content of these foods as eaten is low, which led to a low RAG value. Also for the starch in brown rice and white rice, brown rice is digested *in vitro* much more slowly than that in white rice. But the GI values for white (GI = 83) and brown rice (GI = 96) are in contrast to these observations; it is possible that the brown rice was chewed more thoroughly.

Food	RAG (g/100 g)	GI
Haricot bean	4	45
Kidney bean	5	42
Chickpea (garbanzo)	5	52
Frozen pea	5	74
Beans in tomato sauce	6	60
Red lentil	8	42
Marrowfat pea	8	68
Potato (new)	8	101
Pearled barley	8	31
Kidney bean (canned)	8	74
Chickpea (canned)	10	60
Butter bean	10	52
Pinto bean	10	60
Sweet potato	10	70
Buckwheat	12	74
Instant potato	12	116
Macaroni	14	64
White spaghetti	14	67
Brown rice	15	96
Yam	16	74
Sweetcorn	16	87
Parboiled rice	17	67
White rice	17	83
Rye wholemeal bread	25	58
Oat bran	32	84
Wholemeal bread	33	99
All Bran	33	73
White bread	38	100
Digestive biscuit	40	82
Porridge oats	42	71
Potato crisps	43	74
Rich Tea biscuit	48	80
Oatmeal biscuit	49	78
Shredded Wheat	50	97
Ryvita crispbread	50	95
Weetabix	59	109
Puffed Wheat	64	110
Water biscuit	66	91
Rice Krispies	73	117

Figure 7: The thirty-nine starchy foods ranked by rapidly available glucose (RAG) and shown with glycaemic index (GI) (Englysti, 1996)

MATERIALS AND METHODS

1. Materials

(1) Rice

Red rice (Khao Dang doi), Riceberry, Black glutinous rice (Khao kam lan na dam)

(2) Enzyme

Pepsin, Pancreatin, Amyloglucosidase, Invertase, Pullulanase

(3) Rapidly available Glucose (RAG) and Slowly Available Glucose (SAG) testing

0.1 M Sodium acetate was prepared by weighing 5.38 g sodium acetate and dissolved in 400 ml distilled water. 0.9 ml acetic acid was added. Then pH of solution was adjusted to 5.2. Lastly, total volume was adjusted to 500 ml by distilled water.

(4) Nelson- Somogyi method

Alkaline copper tartrate was prepared by mixture of reagent A and B. For reagent A, it was prepared by dissolving 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g anhydrous sodium sulphate in 80 ml water and making up to 100 ml. And for reagent B, it was prepared by dissolving 15 g copper sulphate in a small volume of distilled water and then adding one drop of sulphuric acid and making up to 100 ml. Lastly, 4 ml of reagent B and 96 ml of reagent A were mixed together before use.

Arsenomolybdate reagent was prepared by dissolving 2.5 g ammonium molybdate in 45 ml water. Then 2.5 ml sulphuric acid was added and mix well. Then, 0.3 g disodium hydrogen arsenate was dissolved in 25 ml water. Both of them were mixed well and incubated at 37°C for 24–48 hours.

2. Methods

(1) Rapidly available Glucose (RAG) and Slowly Available Glucose (SAG) testing for determination of Glycemic index

Five ml pepsin was added into 0.1 grams sample. Sample was heated at 37°C in water bath for 30 minutes. After that, 10 ml of 0.1 M sodium acetate was added into sample. Five glass beads with 1.5 cm diameter was added into sample and followed by addition of 2.5 ml enzyme mixtures which include 2450 µl of pancreatin (7000 U/ml), 25 µl of invertase (3000 EU/ml) and 25 µl of amyloglucosidase (140 AGU/ml) enzyme. The sample was shaken by shaker with the control temperature at 37°C for 20 minutes. Next, the sample was collected in order to determine amount of glucose (G20) then the sample was continuously shaken at 37°C for another 100 minutes. After that, the sample was collected again for determination of amount of glucose (G120). Amount of glucose was determined by using blood glucose monitoring system (ACCU-chek instant S blood glucose meter) (Englyst, 1992; Wasusun, 2017).

(2) Production of rice starch

Three varieties of Thai rice which were red rice, Riceberry and black glutinous rice were converted to become rice starch by using following methods.

Rice was washed by stirring rice with hand and washing rice with water for 2 times. Then, rice was soaked in water at room temperature for 4-5 hours. After that, rice was washed for 2 times same as first step. Rice was blended with addition of water at room temperature for 30 seconds by using blender until it became fine texture and homogenous. Then, pour blended rice into clear bowl following by adding water into the bowl. It was leaved for 10-15 minutes to let the starch precipitated. After 15 minutes, water and starch showed the separated layer. Water was removed out and only starch part left in the bowl. The wet starch was poured into tray and spread to become thin layer. It was dried at 60°C for 3 hours until the starch became completely dried. After drying, the dried starch was blended by using blender to make it become powder form as figure 8. After that, rice starches are sieved by using 120 mesh sieve in order to

control the particle sizes. The final rice starch was kept in airtight packaging for further experiments.

(a) (b) (c)

Figure 8: (a) Red rice, (b) Riceberry and (c) Black glutinous rice starch

(3) Determination of RAG and SAG in native rice starch in Red rice, Riceberry and Black Glutinous rice starch

Three different types of rice starch from previous experiment were determined for RAG and SAG by using method as described in (1). But in this experiment, the rice starches must be gelatinized first since they were still uncooked. The results in this experiment were compared with the results in modified rice starches in order to determine the efficiency of modification methods.

(4) Determination of reducing ends in rice starch solution by using Nelson-Somogyi method after pullulanase enzyme debranching

The 10% w/w rice starch solution was prepared and was cooked at 95°C for 15 minutes in water bath with continuous stirring. And then, it was debranched by using Pullulanase enzyme at 10, 20, 30, 40, 50 and 60 unit/gram starch. The debranching time was also varied into 1, 2, 3, 4, 5 and 6 hours. The debranching of samples were performed by temperature control shaker at 55°C and 170 rpm. After debranching, the solution was heated at 100°C for 15 minutes in order to stop the enzyme reaction.

The samples from different debranching condition were then determined for the reducing ends by Nelson-Somogyi method. Firstly, 1 gram of sample was weighed and

extracted sugar with the hot 80% ethanol for twice (5mL each time). After that, supernatant was collected and evaporated by keeping it on water bath at 80°C. Next, 10 ml of water was added to dissolve sugar and then 0.1 ml of sample was pipetted out to separate test tubes. In this method, standard glucose must be prepared in order to make a standard curve. 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard solution (100 mg/ml glucose solution) were pipetted out into a series of test tubes. Then, the volume in both standard and sample tubes were made up to 2 ml with distilled water. And 2 ml distilled water was pipetted out in a separate tube to set a blank. After that, 1 ml of alkaline copper tartrate reagent was added into each tube. The tubes were placed in boiling water for 10 minutes. After 10 minutes, the tubes were cooled and 1 ml of arsenomolybolic acid reagent was added to all the tubes. The tubes was left for 10 minutes before measurement of absorbance (wavelength = 620 nm) by spectrophotometer (Somogyi, 1952).

Lastly, the debranching condition which provided highest % increasing of reducing ends was selected for further experiments.

(5) Enzymatic and physical modification of Red rice, Riceberry and Black Glutinous rice starch

The 10% w/w of 3 types of rice starch solution were prepared and were cooked at 95°C for 15 minutes in water bath with continuous stirring. And then, they were debranched by using Pullulanase enzyme at 60 unit/grams starch for 5 hours. The debranching of samples were performed by temperature control shaker at 55°C and 170 rpm. After debranching, the solutions were heated at 100°C for 15 minutes in order to stop the enzyme reaction. The rice starch solutions were then passed through physical modification by chilling, freezing and thawing at 15°C, -10°C and 35°C for 16, 8 and 3 hours respectively. After that, the samples were dried at 60°C for 3 hours and then were ground thoroughly (Pongjanta, 2009). The 3 different types of modified rice starch were packed in airtight packaging for further experiments. The process flowchart of enzymatic and physical modification of red rice, Riceberry and black glutinous rice starch was shown in figure 9.

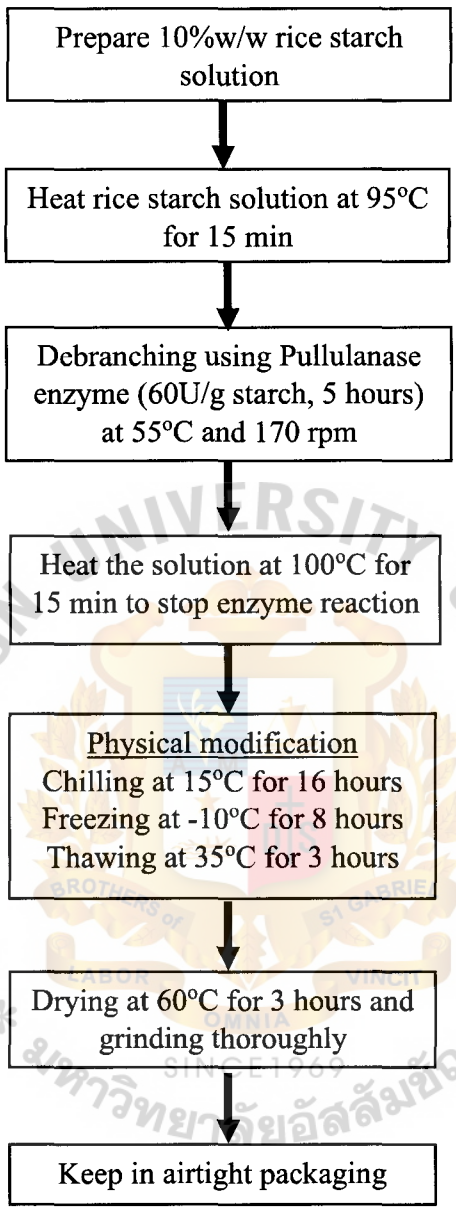

Figure 9: Process flowchart of enzymatic and physical modification of red rice, Riceberry and black glutinous rice starch

(6) Determination of the best type of rice used for production of low GI RS3 rice starch

Three different types of modified rice starch from previous experiment were determined for RAG and SAG by using method as described in (1). The rice starch with lowest RAG was selected as the best type of rice used for production of low GI-RS3 rice starch.

(7) Statistical analysis and experimental design

The statistical analysis will be accomplished by using ANOVA with Duncan's multiple range tests by SAS software version 9.4. Difference at $p \leq 0.05$ was considered to be a significant level.

RESULTS AND DISCUSSION

(1) Preliminary tests of Rapidly available Glucose (RAG) and Slowly Available Glucose (SAG) testing methods.

Before the methods of RAG and SAG described in (1) in Materials and Methods section was used in this project, several preliminary tests were performed to find the best methods.

(1.1) RAG and SAG testing was done by following the methods of Englyst, 1996 which called 'In vitro Rapidly available glucose (RAG) and slowly available glucose (SAG)'. 0.5 g sample was used. Pancreatin, Invertase and Amyloglucosidase were used in same concentration (1 mg/ml) since the research did not mention about the enzyme concentration. After digestion, glucose was determined by using both dinitrosalicylic acid (DNS) method and blood glucose monitoring system (ACCU-chek instant S blood glucose meter). The results showed that RAG and SAG from both methods were too low. These might be caused by too low enzyme concentration that lead to very low amount of glucose released after digestion. When comparing between DNS method and blood glucose monitoring system (ACCU-chek instant S blood glucose meter), the results showed that values of each replication from ACCU-chek were not much different. But for DNS method, values of each replication were different. These showed that using ACCU-chek led to more accurate results. Furthermore, the values from DNS method were much higher than the values from ACCU-chek as shown in Table 1. This might be because ACCU-chek was specific to detection of glucose only. But for DNS method, it was the method testing for the presence of free carbonyl group (C=O), also called reducing ends. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Also, DNS method was time-consuming and more complicated than using ACCU-chek. Therefore, DNS method was not suitable for using in RAG and SAG tests.

Table 1. RAG and SAG measured by using DNS method and ACCU-chek

Types of rice	RAG (g/100 g starch)		SAG (g/100 g starch)	
	DNS	ACCU-chek	DNS	ACCU-chek
Jasmine rice 100%	3.07	0.560	2.61	2.100
Brown rice	3.37	1.015	0.98	2.345
Sao hai rice	2.87	0.805	1.75	2.345
Sticky rice	5.50	1.330	1.78	2.905
RD43	2.78	1.190	2.64	2.870

(1.2) For 2nd Preliminary test, the enzyme concentration was changed from 1 mg/ml for all enzymes to new concentration followed (Englyst H. , 1992) [pancreatin (7000 U/ml), of invertase (3000 EU/ml) and amyloglucosidase (140 AGU/ml)]. The 0.5 g sample was used same as the 1st Preliminary test. The results showed that, RAG and SAG were a bit higher than previous experiment but the values were still low when compared with other researches. For this case, the amount of sample used might be the problem since enzyme concentration was not enough for too much amount of sample. So, the amount of sample used for 3rd preliminary test was decreased to 0.1 g. And the results from this preliminary test showed that using 0.1 g sample led to the applicable range of results. RAG and SAG were much higher than 1st and 2nd preliminary tests.

(1.3) Therefore, RAG and SAG test in this project was done by using 0.1 g sample, enzyme concentration followed Englyst (1992) and the glucose concentration was determined by using blood glucose monitoring system (ACCU-chek instant S blood glucose meter).

(2) Determination of reducing ends in rice starch solution by using Nelson-Somogyi method after pullulanase enzyme debranching

Rice starches were debranched by pullulanase enzyme with different conditions and then amount of reducing ends in sample after debraching was determined by using Nelson-Somogyi method. This method is one of the classical and widely used methods for the quantitative determination of reducing ends. For the principle, the reducing ends are heated with alkaline copper tartrate reduce the copper from the cupric to cuprous state and cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic

acid, the reduction of molybdic acid to molybdenum blue takes place. The blue color developed is compared with a set of standards in a colorimeter at 620nm.

All of these were done for determination of the best condition for modification of rice starch in order to increase RS3 content in rice starch. The enzyme concentration was varied into 10, 20, 30, 40, 50 and 60 unit/gram starch and the debranching time was varied into 1, 2, 3, 4, 5 and 6 hours.

According to the results from Table 2, all conditions showed higher concentration of reducing ends than control (rice starch solution without modification) with significant difference ($p<0.05$). When compare the values among different debranching time in each enzyme concentration, the concentration of reducing ends in different debranching time were significantly different and the values increased with longer debranching time. And when compare the values among different enzyme concentration in each debranching time, the concentration of reducing ends in different enzyme concentration were significantly different ($p<0.05$) and the values increased with increasing enzyme concentration. But when they reached one point that shows the highest concentration of reducing ends, the values then stopped increasing since the reaction of enzyme was rose to a maximum which was also known as substrate inhibition. When comparing among all conditions, the highest concentration of reducing ends was showed in the condition with 60U/g of enzyme concentration and 5 hours of debranching time. Also, the values were significantly different ($p<0.05$) when compared with other conditions. According to figure 10, the results were absolutely showed that the highest % increasing of reducing ends was also shown in the enzyme condition with 60 U/g of enzyme concentration and 5 hours of debranching time. And from Table 3, it was shown that both enzyme concentration and debranching time had effect on concentration of reducing ends in samples. But for replication, there was no significant difference.

This highest concentration of reducing ends shows that pullulanase enzyme hydrolyzed α-1, 6-glucosidic bonds in the structure of rice starch and released the highest amount of linear polymers linked by α-1,4-glucosidic bonds. These fragments could rapidly produce retrograded starch and increase resistant starch content upon aging and freeze-thaw process in further steps (Pongjanta, 2009). Therefore, this enzyme condition with 60 U/g of enzyme concentration and 5 hours of debranching time was selected as the best condition used for modification of rice starch in further experiments.

Table 2. Concentration of Reducing ends (µg/ml) (glucose equivalent) in rice starch solution after different conditions of pullulanase enzyme debranching.

Debranching time (hour)	Enzyme concentration (U/g)					
	10U/g	20U/g	30U/g	40U/g	50U/g	60U/g
0 (Control)	7.55 ± 0.37^{Af}	7.55 ± 0.37^{Ag}	7.55 ± 0.37^{Af}	6.67 ± 0.54^{Ag}	6.67 ± 0.54^{Ag}	6.67 ± 0.54^{Ag}
1	13.38 ± 0.57^{Fe}	16.33 ± 0.18^{Ef}	23.1 ± 0.1^{De}	54.43 ± 0.2^{Cd}	65.8 ± 0.36^{Ae}	62.85 ± 0.67^{Be}
2	15.38 ± 0.72^{Fc}	56.96 ± 0.53^{Ca}	48.13 ± 0.36^{Ea}	53.25 ± 1.06^{De}	70.33 ± 0.1^{Bd}	76.57 ± 0.18^{Ac}
3	25.74 ± 1.18^{Fa}	27.4 ± 0.1^{Ee}	31.23 ± 0.1^{Dc}	72.27 ± 0.27^{Aa}	71.27 ± 0.18^{Bc}	65.15 ± 0.21^{Cd}
4	10.26 ± 0.27^{Fd}	50.84 ± 0.62^{Db}	29.87 ± 0.21^{Ed}	66.56 ± 0.37^{Cb}	119.21 ± 0.27^{Aa}	114.44 ± 0.27^{Bb}
5	23.27 ± 0.37^{Fb}	44.06 ± 0.18^{Cc}	31.46 ± 0.37^{Ec}	35.58 ± 0.35^{Df}	112.49 ± 0.57^{Bb}	168.92 ± 0.44^{Aa}
6	24.28 ± 0.36^{Fb}	39.17 ± 0.37^{Dd}	33.35 ± 0.27^{Eb}	59.14 ± 0.27^{Bc}	61.73 ± 0.18^{Af}	44.24 ± 0.18^{Cf}

Note: Different capital letters in the same row mean significantly different ($p\leq0.05$)

Different small letters in the same column mean significantly different ($p\leq0.05$)

Table 3. Interaction effect between enzyme concentration and debranching time on concentration of reducing ends

Factor	Reducing ends content
Enzyme concentration	*
Debranching time	*
Replication	ns
a:b	*

Note: * = significant difference, ns= no significant difference

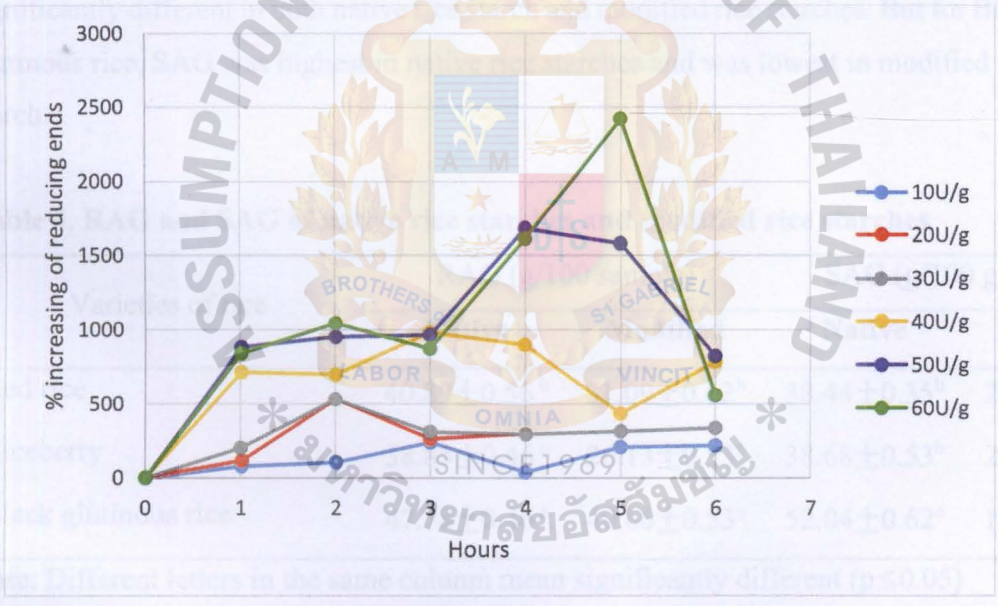

Figure 10: % increasing of reducing ends in rice starch solution after different conditions of pullulanase enzyme debranching

(3) Determination of the best type of rice used for production of low GI RS3 rice starch

Three types of rice starch were modified by using enzymatic and physical modifications. For enzymatic modification, rice starches were debranched by pullulanase enzyme at 60 U/g for 5 hours which was the best condition selected from previous experiment. And for physical modification, rice starched were passed through

chilling, freezing and thawing at 15°C, -10°C and 35°C for 16, 8 and 3 hours respectively. After modification was finished, rice starches were dried at 60°C for 2 hours and then were ground thoroughly. These modified rice starches were then determined for Rapidly available Glucose (RAG) and Slowly Available Glucose (SAG). The native rice starches were also determined for RAG and SAG in order to compare with modified rice starches for the differences between rice starches with and without modification.

According to the results of RAG and SAG of native rice starch and modified rice starches from Table 4, both native and modified rice starches had same tendency for RAG that the highest RAG was shown in black glutinous rice and followed by red rice and Riceberry respectively. For SAG, SAG in red rice and Riceberry were not significantly different in both native rice starch and modified rice starches. But for Black glutinous rice, SAG was highest in native rice starches and was lowest in modified rice starch.

Table 4. RAG and SAG of native rice starches and modified rice starches

Varieties of rice	RAG (g/100 sample)		SAG (g/100 g sample)	
	Native	**Modified**	**Native**	**Modified**
Red rice	40.89 ± 0.56^{b}	31.09 ± 0.62^{b}	38.44 ± 0.35^{b}	26.95 ± 0.53^{a}
Riceberry	38.85 ± 0.46^{c}	26.13 ± 0.53^{c}	38.68 ± 0.53^{b}	27.71 ± 0.61^{a}
Black glutinous rice	47.78 ± 0.46^{a}	40.66 ± 0.53^{a}	52.04 ± 0.62^{a}	19.83 ± 0.53^{b}

Note: Different letters in the same column mean significantly different ($p \leq 0.05$)

When comparing between native and modified rice starches, the results from figure 11 and 12 showed that RAG and SAG in native starches were higher than modified rice starches with significant difference ($p \leq 0.05$). These indicated that rice starches which were modified by enzymatic and physical modification could be more resistant to digestive enzymes because the amount of glucose released after digestion was lower.

Both modifications led to an increasing of resistant starch content in rice starches. The increased resistant starch is primarily owed to an increased level of

amylose through debranching amylopectin molecules by pullulanase enzyme and followed by retrogradation upon freeze-thaw process. Retrogradation permits the amylose molecules to form a more tightly packed crystalline structure, which is in turn difficult for enzymatic hydrolysis. Therefore, aggregation and arrangement of double helices of the short chain amylose molecules increase the resistant starch content (Babu, 2016).

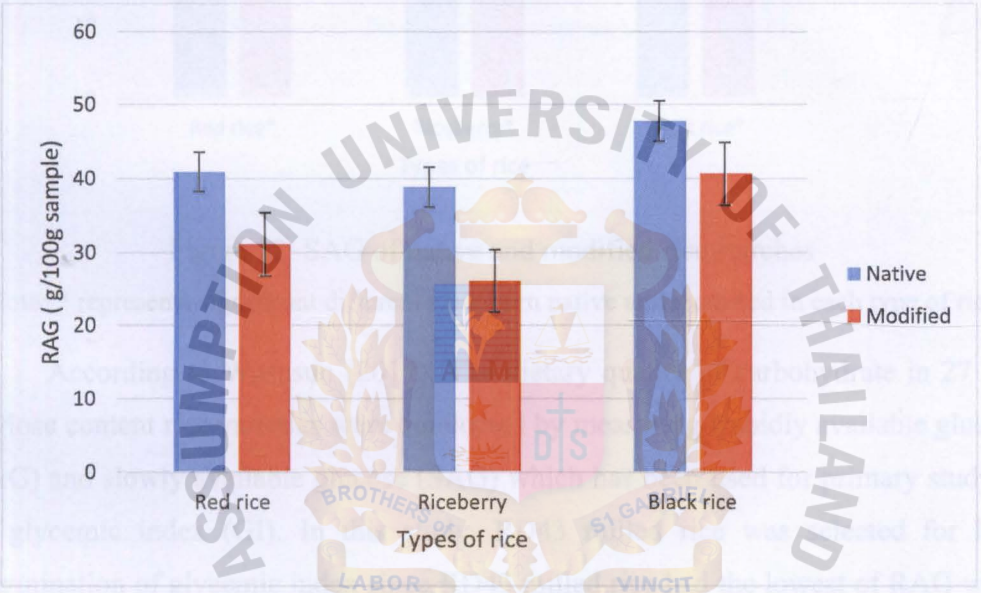

Figure 11: RAG of native and modified rice starches

Note: * represents significant difference between native and modified in each type of rice

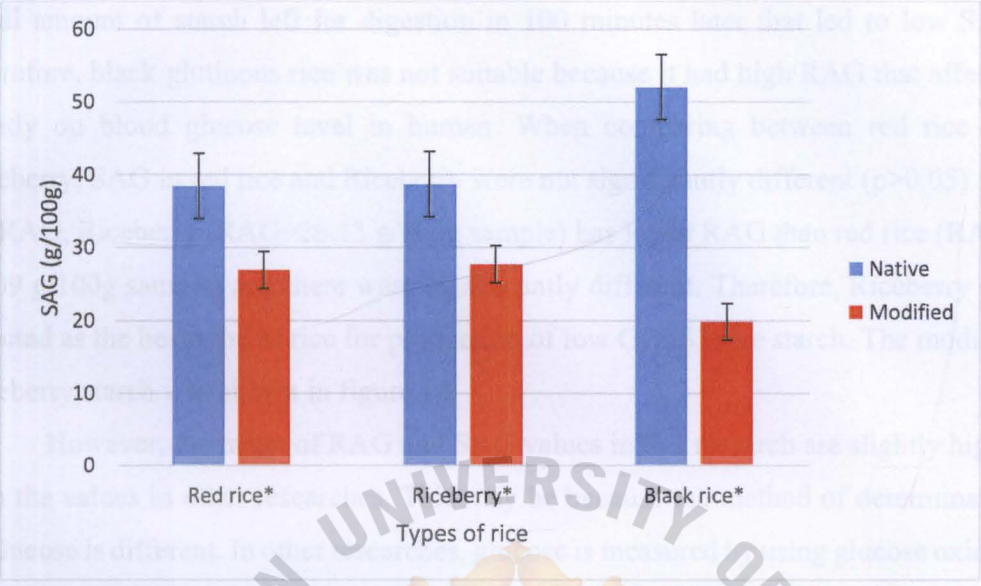

Figure 12: SAG of native and modified rice starches

Note: * represents significant difference between native and modified in each type of rice

According to Wasusun (2017), The dietary quality of carbohydrate in 27 low amylose content rice varieties were conducted by measuring Rapidly available glucose (RAG) and slowly available glucose (SAG) which has been used for primary study of the glycemic index (GI). In this study, RD43 milled rice was selected for later determination of glycemic index since RD43 milled rice had the lowest of RAG when compare with other rice varieties. And for SAG, RD43 milled rice had 3rd highest SAG when compare with other rice varieties. These indicated that in first 20 minutes, starch was slowly digested, and glucose was released in lowest amount and there was large amount of starch left for digestion in 100 minutes later that lead to high SAG. These results were also affirmed by comparing with glycemic index (GI). The results indicated that RD43 has medium GI with higher digestion resistance than other low amylose content rice varieties.

In order to determine the best type of rice for production of low GI RS3 rice starch, RAG and SAG of 3 types of modified rice starches were determined and compared as shown in figure 13. The results showed that Black glutinous rice had highest RAG (40.66 g/100g sample) and had lowest SAG (19.83 g/100g sample). These indicated that in first 20 minutes, starches were quickly digested and glucose was released in highest amount. Since most of starch was digested in first 20 minutes, only

small amount of starch left for digestion in 100 minutes later that led to low SAG. Therefore, black glutinous rice was not suitable because it had high RAG that affected greatly on blood glucose level in human. When comparing between red rice and Riceberry, SAG in red rice and Riceberry were not significantly different ($p>0.05$). But for RAG, Riceberry (RAG=26.13 g/100g sample) has lower RAG than red rice (RAG= 31.09 g/100g sample) and there were significantly different. Therefore, Riceberry was selected as the best type of rice for production of low GI RS3 rice starch. The modified Riceberry starch was shown in figure 14.

However, the range of RAG and SAG values in this research are slightly higher than the values in other researches. This may be because the method of determination of glucose is different. In other researches, glucose is measured by using glucose oxidase colorimetric kit. But for this research, glucose is measured by using ACCU-chek or blood glucose monitoring. Both methods are specific for glucose but glucose oxidase colorimetric kit is expensive, more complicated and also time consuming. The results of RAG and SAG in this research just indicated that the rice with lower RAG will also has lower GI since RAG value and GI value are strongly correlated but the results cannot specify the actual GI value.

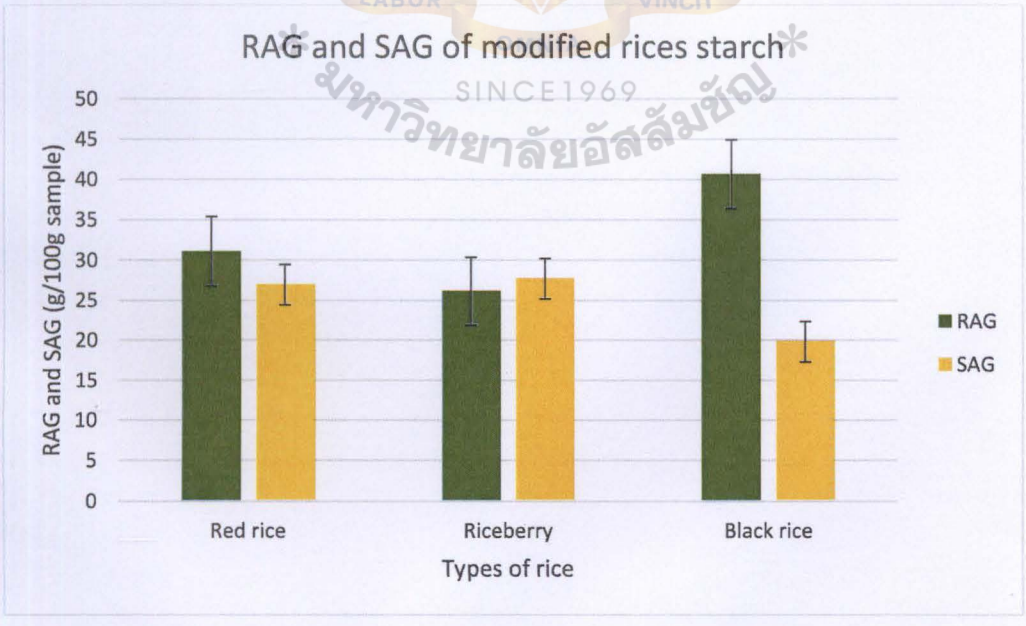

Figure 13: RAG and SAG of modified rice starches

Figure 14: Modified Riceberry starch

CONCLUSION

The results obtained in this study showed that enzymatic modification by pullulanase enzyme and physical modification by freeze-thaw process were effective for increasing resistant starch content in RS3-rice starch and making rice starch more resistant to digestive enzymes because both RAG and SAG in modified rice starch were lower when compared with RAG and SAG in rice starch without modification. The best condition for enzymatic modification by pullulanase enzyme was the condition with 60 U/g for enzyme concentration and 5 hours for debranching time. And Riceberry was selected as the best type of rice for production of low GI RS3 rice starch since it had lowest RAG in both native and modified rice starch when compared with other types of rice.

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APPENDICES

Determination of reducing ends in rice starch solution by using Nelson-Somogyi method after pullulanase enzyme debranching

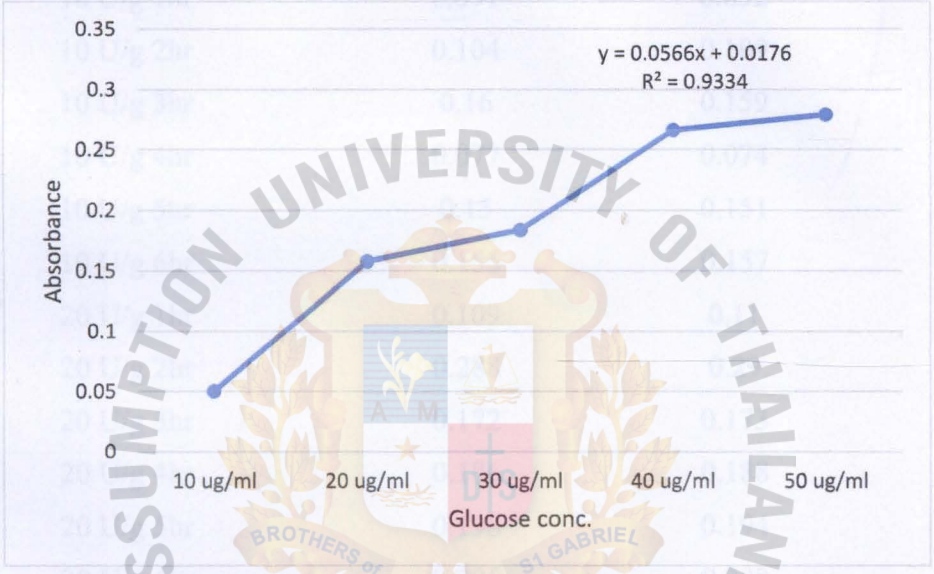

Figure 12: Standard curve of glucose concentration

Table 5. Absorbance of standard curve of glucose concentration

Glucose concentration	Absorbance
10 µg/ml	0.050
20 µg/ml	0.158
30 µg/ml	0.184
40 µg/ml	0.266
50 µg/ml	0.279

Table 6. Absorbance of reducing ends in rice starch solution after different conditions of pullulanase enzyme debranching

Enzyme concentration & debraching time	Absorbance		
	Rep 1	Rep 2	Rep 3
Control	0.058	0.061	0.062
10 U/g 1hr	0.091	0.092	0.097
10 U/g 2hr	0.104	0.109	0.101
10 U/g 3hr	0.16	0.159	0.171
10 U/g 4hr	0.077	0.074	0.076
10 U/g 5hr	0.15	0.151	0.147
10 U/g 6hr	0.155	0.157	0.153
20 U/g 1hr	0.109	0.11	0.111
20 U/g 2hr	0.288	0.29	0.292
20 U/g 3hr	0.172	0.173	0.173
20 U/g 4hr	0.186	0.188	0.186
20 U/g 5hr	0.198	0.194	0.195
20 U/g 6hr	0.205	0.208	0.206
30 U/g 1hr	0.148	0.148	0.149
30 U/g 2hr	0.337	0.34	0.343
30 U/g 3hr	0.194	0.194	0.195
30 U/g 4hr	0.305	0.309	0.302
30 U/g 5hr	0.266	0.267	0.268
30 U/g 6hr	0.241	0.24	0.237

Enzyme concentration & debraching time	Absorbance		
	Rep 1	Rep 2	Rep 3
Control	0.058	0.056	0.052
40 U/g 1hr	0.325	0.327	0.325
40 U/g 2hr	0.313	0.319	0.325
40 U/g 3hr	0.428	0.427	0.425
40 U/g 4hr	0.396	0.392	0.395
40 U/g 5hr	0.217	0.219	0.221
40 U/g 6hr	0.351	0.352	0.354
50 U/g 1hr	0.388	0.39	0.392
50 U/g 2hr	0.416	0.416	0.415
50 U/g 3hr	0.421	0.422	0.42
50 U/g 4hr	0.691	0.694	0.692
50 U/g 5hr	0.652	0.653	0.658
50 U/g 6hr	0.368	0.366	0.367
60 U/g 1hr	0.376	0.375	0.369
60 U/g 2hr	0.45	0.451	0.452
60 U/g 3hr	0.387	0.385	0.387
60 U/g 4hr	0.667	0.664	0.665
60 U/g 5hr	0.971	0.974	0.976
60 U/g 6hr	0.268	0.267	0.269

Table 7. Concentration of reducing ends (µg/ml) in rice starch solution after different conditions of pullulanase enzyme debranching

Enzyme concentration & debraching time	Concentration of reducing ends (µg/ml)		
	Rep 1	Rep 2	Rep 3
Control	7.14	7.67	7.84
10 U/g 1hr	12.97	13.14	14.03
10 U/g 2hr	15.27	16.15	14.73
10 U/g 3hr	25.16	24.98	27.10
10 U/g 4hr	10.49	9.96	10.32
10 U/g 5hr	23.39	23.57	22.86
10 U/g 6hr	24.28	24.63	23.92
20 U/g 1hr	16.15	16.33	16.50
20 U/g 2hr	56.43	56.96	57.49
20 U/g 3hr	27.28	27.46	27.46
20 U/g 4hr	50.78	51.48	50.25
20 U/g 5hr	43.89	44.06	44.24
20 U/g 6hr	39.47	39.29	38.76
30 U/g 1hr	23.04	23.04	23.22
30 U/g 2hr	47.77	48.13	48.48
30 U/g 3hr	31.17	31.17	31.34
30 U/g 4hr	29.75	30.11	29.75
30 U/g 5hr	31.87	31.17	31.34
30 U/g 6hr	33.11	33.64	33.29

Enzyme concentration & debraching time	Reducing ends content (µg/ml)		
	Rep 1	Rep 2	Rep 3
Control	7.14	6.78	6.08
40 U/g 1hr	54.31	54.66	54.31
40 U/g 2hr	52.19	53.25	54.31
40 U/g 3hr	72.51	72.33	71.98
40 U/g 4hr	66.86	66.15	66.68
40 U/g 5hr	35.23	35.58	35.94
40 U/g 6hr	58.90	59.08	59.43
50 U/g 1hr	65.44	65.80	66.15
50 U/g 2hr	70.39	70.39	70.21
50 U/g 3hr	71.27	71.45	71.10
50 U/g 4hr	118.98	119.51	119.15
50 U/g 5hr	112.08	112.26	113.14
50 U/g 6hr	61.91	61.55	61.73
60 U/g 1hr	63.32	63.14	62.08
60 U/g 2hr	76.40	76.57	76.75
60 U/g 3hr	65.27	64.91	65.27
60 U/g 4hr	114.73	114.20	114.38
60 U/g 5hr	168.45	168.98	169.33
60 U/g 6hr	44.24	44.06	44.42

Table 8. % increasing of reducing ends in rice starch solution after different conditions of pullulanase enzyme debranching.

Debranching time (hour)	Enzyme concentration (U/g)					
	10U/g	20U/g	30U/g	40U/g	50U/g	60U/g
1	76.32	114.47	203.95	711.94	882.09	838.806
2	102.63	532.89	650	695.522	949.254	1043.28
3	238.16	260.53	310.53	979.104	964.179	871.642
4	35.53	293.42	568.42	894.03	1679.1	1607.46
5	206.24	314.47	480.26	431.343	1579.1	2420.9
6	219.74	338.16	415.79	782.09	820.896	559.701

Table 9. The ANOVA table (Factorial in RCBD) of concentration of reducing ends

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Enzyme conc.	5	71831.15324	14366.23065	77470.1	<.0001
Debranching time	5	12709.82403	2541.96481	13707.6	<.0001
a:b	25	35801.77263	1432.07091	7722.46	<.0001
Block	2	0.64184	0.32092	1.73	0.1847

Calculation of concentration of reducing ends (µg/ml)

From standard curve ; $x = \frac{y-0.0176}{0.0566}$

(when x is concentration of reducing ends , y is absorbance)

Ex; At 10 U/g and 2 hours Absorbance = 0.105

$x = \frac{y-0.0176}{0.0566} \times 10$ (because the interval of each concentration = 10)

$x = \frac{0.105-0.0176}{0.0566} \times 10 = 15.4$ µg/ml

Calculation of % increasing of reducing ends

$$\%\text{increasing} = \frac{glucose\ concentration\ of\ sample - glucose\ concentration\ of\ control}{glucose\ concentration\ of\ control} \times 100$$

RAG and SAG of native rice starch and modified rice starch

Native rice starch

Table 10. Glucose concentration (mg/dl) of native rice starch

Varieties of rice	G20 #1	G20 #2	G20 #3	G120 (G120-G20) #1	G120 (G120-G20) #2	G120 (G120-G20) #3
Red rice	235	230	236	456(221)	449(219)	459(223)
Riceberry	222	225	219	445(223)	444(219)	436(217)
Black glutinous rice	270	275	274	571(301)	572(297)	568(294)

Table 11. RAG and SAG of native rice starch (g/100g sample)

Modified rice starch

Varieties of rice	RAG #1	RAG #2	RAG #3	SAG #1	SAG #2	SAG #3
Red rice	41.13	40.25	41.30	38.68	38.33	39.03
Riceberry	38.85	39.38	38.33	39.03	38.33	37.98
Black glutinous rice	47.25	48.13	47.95	52.68	51.98	51.45

Table 12. Glucose concentration (mg/dl) of modified rice starch

Varieties of rice	G20 #1	G20 #2	G20#3	G120 (G120-G20)	G120 (G120-G20)	G120 (G120-G20)

				#1	#2	#3
Red rice	178	181	174	335(157)	335(154)	325(151)
Riceberry	146	150	152	308(162)	308(158)	307(155)
Black glutinous rice	235	233	229	349(114)	343(110)	345(116)

Table 13. RAG and SAG of modified rice starch (g/100g sample)

Varieties of rice	RAG #1	RAG #2	RAG #3	SAG #1	SAG #2	SAG #3
Red rice	31.15	31.68	30.45	27.48	26.95	26.43
Riceberry	25.55	26.25	26.60	28.35	27.65	27.13
Black glutinous rice	41.13	40.78	40.08	19.95	19.25	20.30