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FINAL YEAR PROJECT THESIS: EFFECTS OF SINGLE AND DUAL MODIFICATION ON THE PHYSICO-CHEMICAL PROPERTIES OF RICE FLOUR

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	**Caption**
α	alpha
C	carbon
DMF	Dual-modified rice flour
DMT	Dual-modified treatment
DSC	Differential Scanning Calorimetry
FTIR	Fourier Transform Infrared
HMF	Heat-moisture treated rice flour
HMT	Heat-moisture treatment
hr	hour
KOH	Potassium hydroxide
kV	kilovolt
L	Litre
mL	Milliliter
mg	miligram
min	minute
NaOH	Sodium hydroxide
NRF	Native rice flour
Rpm	Revolutions per minute
SEM	Scanning Electron Microscope
T_o	Onset temperature
T_p	Peak temperature
T_c	Conclusion temperature
µm	micrometer
UV	Ultraviolet
XRD	X-Ray Diffraction

ABSTRAK

Dalam kajian ini, tepung beras dimodifikasi secara dua kali menggunakan kaedah yang menggabungkan pengubahsuaian fizikal dan pengubahsuaian enzim, iaitu *enzyme-debranching* diikuti dengan *heat-moisture treatment* (HMT). Untuk pengubahsuaian tunggal, tepung beras telah menjalani HMT. Sifat fizikokimia dan kandungan kanji tahan sampel yang asli dan diubah suai disiasat dan dibandingkan. Kajian menunjukkan pengurangan kandungan amilosa yang ketara sepanjang rawatan yang digunakan, dengan tepung beras asli (NRF) mempunyai kandungan amilosa tertinggi (34.52%), diikuti dengan tepung beras yang dirawat dengan HMT, dikenali sebagai HMF (28.91%), dan tepung beras yang diubahsuai dengan *dual-modification* (DMF) (20.82%). HMT dan *enzyme-debranching*-HMT mengurangkan daya pembengkakan tepung beras secara signifikan manakala menunjukkan korelasi terbalik dalam hasil untuk kelarutan. Mikroskopi elektron pengimbasan menunjukkan bahawa DMF memperlihatkan butiran poligonal yang lebih halus dan lebih besar dengan bucu struktur tiga dimensi yang lebih pasti, sedangkan tepung asli dan HMF mempunyai struktur kasar yang tidak sama, menunjukkan tidak ada penggantian morfologi butiran setelah HMT. Suhu gelatinasi dan entalpi lebur kristal (ΔH) jauh lebih tinggi pada sampel HMF dan DMF berbanding dengan tepung asli. Kedua-dua NRF dan HMF menunjukkan corak sinar-X jenis A dengan puncak pada sekitar 15°, 17°, 18°, dan 23°. Sementara itu, DMF memperlihatkan campuran kristal jenis B- dan V dengan puncak intensiti jenis-B pada kira-kira $2\theta = 17.13$ °, 22.56 ° dan 24.01 °, dan juga puncak pada 14.93 ° dan 19.89 ° dari kristal jenis-V. Kristaliniti relatif NRF, HMF dan DMF berdasarkan XRD masing-masing adalah 43.4%, 38.6% dan 49.5%. Analisis lain, seperti daya pembengkakan, pemeriksaan XRD, dan SEM, meramalkan bahawa kandungan RS akan meningkat berikutan oleh pengubahsuaian kanji, yang diimplikasikan oleh peningkatan daya tahan terhadap pencernaan hasil dari kristalinitas yang lebih tinggi.

ABSTRACT

In this study, rice flour was dually-modified using a method that combined physical modification and enzyme modification, namely enzyme-debranching followed by heat-moisture treatment (HMT). For single modification, the rice flour had undergone HMT. The physicochemical properties and resistant starch content of the native and modified samples were investigated and compared. The results showed a significant reduction of amylose content along the treatment(s) used, with the native rice flour (NRF) having the highest amylose content (34.52%), followed by heat-moisture treated rice flour (HMF) (28.91%), and dual-modified rice flour (DMF) (20.82%). HMT and enzyme debranching-HMT significantly reduced the swelling power of rice flour while showing an inverse correlation in the result for solubility. Scanning electron microscopy revealed that DMF displayed smoother and larger polygonal granules with a more definite edges of three-dimensional structure, whereas native flour and HMF had similar rough irregular structure, indicating no alternation of the morphology of the granules after HMT. The gelatinization temperature and crystallite melting enthalpy (ΔH) was significantly higher in HMF and DMF samples compared to the native flour. Both NRF and HMF exhibit A-type X-ray patterns with peaks at around 15°, 17°, 18°, and 23°. Meanwhile, DMF displayed a mixture of B- and V-types crystallites with peaks of B-types crystal patterns at approximately $2\theta = 17.13°$, 22.56° and 24.01°, and also peaks at 14.93° and 19.89° of the V-type crystalline. The relative crystallinities of the NRF, HMF and DMF samples based on XRD were 43.4%, 38.6% and 49.5%, respectively. Other analyses, such as swelling power, XRD, and SEM examination, suggest that the RS content will be increased following modification, implied by higher crystallinity that increased digestion resistance.

CHAPTER 1 INTRODUCTION

1.1 Background Research

Rice which is also known as Oryza sativa L. has been a predominant staple food in Asia and Pacific region, including Malaysia. Malaysians consumed an average of two and half plates of rice per day, as according to food consumption pattern of the adult population in Malaysia (Norimah et al., 2008). Rice has starch as the main component which consists of 25–30% amylose and 70–75% amylopectin (Zhao et al., 2018). Rice can be divided into several types such as waxy rice, brown rice and others, which contains different components and properties. Waxy rice starch contains approximately 100% of amylopectin which mainly contributes to its unique stickiness of starch (Guo et al., 2021). Meanwhile, brown rice which composed of a bran layer, embryo, and endosperm, and has a higher content of certain nutritional components compared to white rice which primarily consists of starchy endosperm (Lee et al., 2015).

Starch is the principal carbohydrate and important source of nourishment for humans. Starch can be classified into rapidly digestible starch (RDS), slowly digestible (SDS), and resistant starch (RS) according to the rate and extent of its digestibility (Englyst et al., 1992). RDS increases the blood glucose level rapidly after consumption and SDS is slowly but completely digested in the small intestine. Meanwhile, RS is the fraction of starch that cannot be digested in the small intestine of healthy individuals but is actually fermented in the large intestine by resident colonic microflora (Haralampu, 2000).

Resistant starch is defined as starch and starch degradation products (Jeong & Shin, 2018). It can be classified into five types in accordance to its physical and chemical characteristics, in which are called RS1, RS2, RS3, RS4 and RS5. As reported by Ding et al. (2019), resistant starch in rice can fall into R2 and R3 category for raw and cooked rice respectively. RS2 is type of starch that is naturally resistant due to the nature of the starch granule, where the starch does not well-gelatinized and hydrolysed during cooking. Meanwhile, RS3 represents retrograded starch which formed after the starch has been cooked and cooled.

At present, there are several modification of starch that have been developed to increase resistant starch content. Hydrothermal treatments (HTT), mainly annealing and heat-moisture treatment (HMT) are some of the physical modifications to modify resistant starch (Juansang et al., 2012; Zavareze et al., 2010). These treatments can modify functional properties of starch without destroying their starch granular structure (BeMiller, 2018). In HMT, starch is subjected to a temperature above the gelatinization temperatures with insufficient moisture to gelatinize (below 35% water w/w) (Jiranuntakul et al., 2011). HMT also have been regarded as green technique and eco-friendly process due to the absence use of chemical reagents (BeMiller, 2018).

Meanwhile, for the chemical modification, a lots of study has also been focused on using phosphorylation method (Dupuis et al., 2014) and acetylation method (Sha et al., 2012) to produce RS. However, there is some concerns about the use of chemical in food industrial, as it may involve the use of corrosive chemicals which may trigger safety problem issues (Lee et al., 2015). U.S. Food and Drug Administration (FDA) has prescribed a regulation, 21 CFR 172.892, to ensure the safe quantities and conditions for the use of chemically modified starch in food products (Chen et al., 2018). This chemically modified starch is classified as a food additive and must be labelled on the package as “food starch–modified”. In Malaysia, The Ministry of Health will only approves the use of food additives that are safe and do not pose any hazard to human health. The modified starch used as food additives are only permitted to be added to certain foods at a maximum level specified in the Food Regulations 1985. For instance, in food products, only 3% octenyl succinic anhydride (OSA) is permitted for modifying starches (Punia, 2020). Nevertheless, consumer preferences to avoid modified starches in favour of more 'clean-label' ingredients indicate that health worries have not totally subsided.

Recently, dual modification methods with combination of two modifications have gain lots of interest as it has shown positive results in increasing the resistant starch in food and. Many studies have been conducted on these methods of modification including but not limited to, autoclaving/retrogradation (Ashwar et al., 2016); double HMT (Gong et al., 2017); and sonification/annealing (Babu et al., 2019). Zhou et al. (2014) used dual treatment of enzyme and heat moisture treatment to produce resistant starch from rice starch, which showed significant increase in resistant starch content (47.0%). Babu et al. (2019) modified starch by annealing and ultra-sonication treatments and found that RS content was significantly enhanced up to 45%.

1.2 Rationale of Study

Malaysia recorded highest levels of obesity rates across Southeast Asia. The increase prevalence of overweight and obesity has accompanied the rise in type 2 diabetes (T2D), affecting 2.8 million individuals (Hussein et al., 2015). High consumption of rice starch/flour can be harmful to obese and diabetes patients due to easily digestible starch. Hence, there is an interest in modifying rice starch/flour as to increase RS content that could resist digestibility. Resistant starch has been shown to have several benefits to gastrointestinal health, in reducing the risk of diabetes, obesity, high cholesterol and other chronic diseases related with insulin resistance risk (Arcila & Rose, 2015; Sha et al., 2012). RS contain almost zero calories (Zhou et al., 2014) and has been listed by Food and Agriculture Organization (FAO) as dietary fibre. RS can decrease insulin secretion and control postprandial blood glucose to prevent diabetes

(Weickert et al., 2005). Hence, the preparation and production of RS has seemingly become increasingly important as it can be of real public health value.

As rice starch/flour is produced locally in Malaysia, the production of RS from it can help increase the economic value. Rice could be a cheap alternative starch source for high RS production in Malaysia. Although the utilization of rice starch/flour is much less than the other cereal starches from corn and wheat, increment of RS content could spark the interest of various food producers to further use rice starch/flour. Rice starch has several advantages, including hypoallergenicity, bland flavour, small granules, white colour, greater acid resistance, spreadability, and relatively good freeze-thaw stability (Wani et al., 2013). These unique characteristics of rice starch make it ideal for various food applications. Lastly, the dual modification of starch which consist of physical and enzymatic modification is a green method, which can be used safely without safety concern for food application.

1.3 Objectives

The objectives of the study are:

1. To modify the rice flour by using single modification of HMT and dual modification of enzymatic debranching-HMT treatment.

2. To analyse the physiochemical properties and resistant starch content of the modified rice flour.

CHAPTER 2 LITERATURE REVIEW

2.1 Starch

Starch plays an important role as one of the main sources of carbohydrates in human diet. Second only to cellulose as the most natural abundant of polysaccharides, starch serves as a major energy source for plants, animals as well as for human consumption. It is a semi-crystalline biopolymer which is made up of a lot of glucose units, is granular in form and serves as energy reserve for plants (Schafranski et al., 2021). Starch presents in most of green plants and can be obtained from edible parts of cereals, legumes, tubers, roots, and rhizomes. Due to its characteristic properties such as low in price, readily available, has nontoxic nature, is renewable and biodegradable, starch has been extensively utilized in food and non-food industry. It has become an important industrial ingredient for many different applications including for food, feed, paper, textile, chemical, cosmetics, pharmaceutical and petrochemical industries (Maniglia et al., 2020). In the food industry, starch can act as a thickener, stabilizer, bulking and gelling agent, texturizer, encapsulation, moisture retention and processing aid. According to Sui, & Kong, (2018), starches with industrial significance are mainly extracted from maize, potato, cassava, wheat, and rice. Depending on the origin of the starch, different starch sources may also vary in terms of their functional and physical properties which includes the shape, size, granular structure, chemical composition as well as amylose-amylopectin proportion. Understanding the starch composition, structure, and physicochemical properties is essential to fully-optimize the processing and utilization of starch for various applications.

2.1.1 Compositions Of Starch

Starch is a branched homopolymer of glucose which is mainly composed of two different glucose polymers: linear amylose and branched amylopectin, linked together through glycosidic bonds. Starch is found in granules form in reserve plants where the granules are synthesized in amyloplasts and deposited in the form of tiny granules in various depots. Since starch is made up by various kinds of components, it can be classified into two main groups, namely major components and minor components. Amylose and amylopectin are the two major components of starch whereas minor components include lipid, phosphorus, proteins and minerals. These two major glucose polymers are responsible for the characteristics of biodegradable and thermoplastic polymer (Yazid et al., 2018). Amylose is an essentially linear molecules composed of α-1,4 glycosidic bonds with a small amount of side chain structure, whereas amylopectin is highly branched by α-1,6 glycosidic bonds connecting the main chain consisting of α-1,4 glycosidic bonds units with the branch chain. Amylose has a smaller molecular weight approximately at $10^{5–6}$ in comparison with amylopectin which has relatively larger molecular weights of around ~$10^{7–8}$, making up the majority of the starch. The way these two polymers distributed and organized throughout the starch granules is what form its semi-crystalline structure, such that the amylose found in the amorphous region are randomly distributed among the crystalline region where amylopectin presents.

2.1.1a Amylose

Amylose is essentially a linear chain polymer composed of D-glucose residues linked through α-1,4-glucosidic bonds, behaving as a non-branched molecule. Recent studies revealed that amylose is not defined as just a straight chain molecule as some α-1,6 branches do present on it. Amylose is considered as an unbranched linear polymer, due to the lengthy chain which contains several hundreds or even thousand glucosyl units and also due to the high molecular weight characteristics possessed by the side branches in amylose. Amylose molecules has natural helical structure in which the interior of the helix contains hydrogen atoms making the interior part hydrophobic while the exterior hydrophilic due to the hydroxyl group presented. This hydrophobicity of interior helical structure is what provides the ability of amylose to form inclusion complexes with polar lipids such as iodine, some alcohol and fatty acid components of glycerides. During complex formation, amylose is thought to transform from coil to helix and guest molecules enter the center cavities of amylose helices, promoting aggregation of helices which in turn forming a partially crystalline amylose structure (Zhang et al., 2012).

Another well-known attribute of amylose includes the ability to form gel after cooking process which is renowned as set back or retrogradation process. The gelling characteristics possessed by amylose showed when it is crystallized during cooling while forming gels once it is solubilised in hot water. Amylose content is also liable in determination of the structural, functional properties and physical properties of starch in pastes and gels. Amylose can form stronger and stiff gel which can negatively influence the quality of the starch product as it requires higher gelatinization temperature and longer retrogradation of starch. In addition to that, starch digestibility may also impacted by amylose content. There is a positive correlation between amylose and resistant starch content, and higher amylose concentration correlates with increased enzyme resistance, slowing hydrolysis.

2.1.1b Amylopectin

In contrast with amylose, amylopectin is a highly branched polymer where the glucose molecules are being linked together by both α-(1,4) glycosidic bond and α-(1,6) glycosidic bond (for about 5%-6%). Amylopectin is a much larger molecule than amylose with an average molecular weights of 1×10^6 to 5×10^8 Da but has shorter linear chain lengths consists of 25-30 glucose units on the major chains (Robyt, 2008). The amylopectin chains have a tree-like arrangement of double helices which forms crystal in the starch granule. Infinite number of potential models for the branched macromolecules of amylopectin has been proposed: laminated, comb-like, tree-like, dichotomous and folded (Frey-Wyssling, 1969). Due to this vast model proposal, it has been very difficult to determine the exact structure that could represents amylopectin. The tree-like type of K. H. Meyer (1952) emphasises the unique position of the molecule's single reducing aldehydic end. However, till now, the way amylose and amylopectin are distributed within a starch granule has yet to be determined (Luallen, 2018).

Certain starch properties are highly influenced by the amylopectin chain length distribution which may varying depending on the botanical source. Amylopectin contributes to the stringiness of food products as it is known to be the non-gelling portion of the starch. As such, pastes made from starches that are almost entirely made up of amylopectin (also known as waxy starches) are considered to be non-gelling but have a cohesive and gummy texture. In general, normal starches contain around 20–30% amylose and 70–80% amylopectin, but amylose content can range from < 1% in waxy starches and > 70% in certain high amylose starches (Cornejo-Ramírez et al., 2018).

2.1.2 Resistant Starch (RS)

Starches can be classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) according to the rate of glucose release and their digestive fate in the gastrointestinal tract (Hung et al., 2016). The term ‘resistant starch’ was created by Englyst *et. al*. (1989), which describe a small fraction of starch that possessed the ability to be resistance towards enzymatic digestion by α-amylase and pullulanase treatment in vitro. RS can be defined as starch that cannot be hydrolysed by the human enzymes, is not absorbed in the small intestine of healthy individuals and, thus, might be fermented by microorganisms in the colon, producing short-chain fatty acids and other products (reference). RS is able to resist to be hydrolysed by the pancreatic and brush border enzyme, resisting upper gastrointestinal digestion within the 120 minutes after consumption. There is also possibility that some of the digestible starch able to pass through the small intestine without being digested, thus, contributing to the amount of RS (Birkett & Brown, 2007). Recently, RS has been classified as a dietary fiber as it has some physiologic effects that are similar to those of dietary fiber (Chen et al., 2020). Contrary to RS, the other fractions of starch, RDS and SDS represent the those that are completely digested.

Resistant starch can be categorized into five different sub-types; types 1, 2, 3, 4, and 5 according to the mechanism by which the starch resists digestion or based on its physical and chemical characteristics. RS type 1 (RS1) is the starch that is physically trapped in whole or partially ground grains, making it physically inaccessible to digestion by entrapment in a non-digestible matrix (Shi & Gao, 2011). This type of RS can be found in unfractionated and unrefined starchy foods, mostly in pulses and some cereals which have thick cell walls (Tacer-Caba & Nilufer-Erdil, 2019). RS type 2 (RS2) is the type of starch that is naturally resistant because of the composition and conformation of the granule. It is found in native resistant starch granules where the starch is eaten raw, for instances raw potato and green banana, or ungelatinized starch such as high-amylose corn starch where the granules did not gelatinize during normal cooking temperature.

As for RS type 3 (RS3), it is the retrograded starch in which the gelatinized starch is cooked and cooled or kept at room temperature 4 °C (fridge) or – 20 °C (freezer) from a few hours to several days or months. RS3 generally formed as a result from normal food processing or during the manufacturing of RS-rich ingredients (Tacer-Caba & Nilufer-Erdil, 2019). After the gelatinised starch is cooled, this retrogradation process leads to recrystallization of some single chains to form double helices via hydrogen bonds, therefore producing a regions that are very resistant to digestive enzymes. Because of its thermal stability during cooking and preserved nutritional functionality, RS3 appears to be of particular interest as it may be used as a heat-stable prebiotic ingredient in a wide range of traditional and developing food products (Ma et al., 2020). A common example of RS3 includes cooked and cooled potato, bread and cornflakes. The fourth class, RS type 4 (RS4) includes chemically modified starch such as that produced by approved chemical modification methods for use in foods. It is prepared by introducing or modifying different or existing chemical structures to the starch polymer, aiming to interfere with the action of digestive enzymes. Examples of chemical modification methods are dextrinization, etherification, esterification, oxidation, and cross-linking with difunctional reagents in which they could decrease starch digestibility.

In addition to that four main types, a new sub-type of RS, namely RS5, has also been introduced recently, arising from the formation of amylose-lipid complexes. RS5 is a starch complex formed between amylose and lipids that is resistant to enzymatic digestion (Shi & Gao, 2011). It is presented as high-amylose starch that are preheated, debranched with isoamylase and complexed with palmitic acid to form inclusion complexes with polar lipids (Shi & Gao, 2011).

Besides increasing the RS content and total dietary fiber content, incorporating RS-rich ingredient in food products may also enhance physiological benefits as well as improving the food quality. The amount of RS present in starchy foods is very small and limited, hence, formulating foods with ingredients rich in RS has been the focus for researchers, scientists and companies that are interested in the development of functional or fortified food. RS can be made into functional food material having low calorific value and low glycemic index (Zhou et al., 2014). Production of RS-rich ingredients is mostly in powdery form so that it can be widely utilized in various kind of foods, mostly moisture-free food products such as breads, buns, breakfast cereals, extruded foods, cereal bars, pasta, noodles, biscuits, confectionery, beverages and yogurt (Shi & Gao, 2011). RS can be considered as functional ingredient as it provides superior handling and improves texture in the finished product due to its physical qualities, specifically its low water-holding capacity

2.2 Rice Flour

2.2.1 Composition Of Rice Flour

Starch is a main constituent of rice and a crucial structural constituent in many rice products (Zavareze et al., 2010). Flours, in general, are considered to be fine and powdery, consists of almost the same components as the raw materials, except for the moisture content and certain minor components (Puncha-arnon & Uttapap, 2013). In rice flour, starch is the main ingredient that makes up 60% - 80% of the total content, thus, is responsible in determining the acceptability of a rice cultivar in terms of its physicochemical properties and cooking characteristics (Lee et al., 2015). Other components present in rice flour include non-starch polysaccharide, sugar, protein, lipid, and inorganic materials. Rice flour is made by dry or wet milling broken rice, whereas rice starch is made by alkaline steeping method with multi-stage purification, in which the purification reduces significant amount of protein content as well as other components in rice starch (Puncha-arnon & Uttapap, 2013).

2.2.2 Processing Of Rice Flour

In general, there are three methods that can be used to prepare rice flour: dry-milling, semi-dry-milling and wet-milling (Qian & Zhang, 2013). Rice milling is a process of dehulling the paddy step-by-step to make brown rice and then remove the bran layer and embryo to finally obtain white rice. In order to produce rice flour, grinding and sifting the starch-containing plant organelles, such as those from grains, seeds, roots, tubers, and fruits will follow after the milling (Puncha-arnon & Uttapap, 2013). As wet-milling method is thought to yield flour with excellent quality, rice flour is usually manufactured by the method even though it generates a large amount of waste water. On the other hand, studies have reported that dry-milled flour retains components such as protein, lipid and ash at higher levels than wet-milled flour (Leewatchararongjaroen & Anuntagool, 2016). In the dry-milling process, polished rice kernels are directly ground with turbo, cyclone or hammer mill whereas in the wet-milling process, rice kernels are soaked in water for one hour before being processed with water in a double-disk stone mill. The slurry is then placed into a thick cloth bag and centrifuged to remove any remaining water before being dried in a hot-air oven at 40° C for 12 hours to reduce the moisture content to 13%. Different milling methods will influence the properties of flour thus producing a range of distinct properties, including varied particle size, colour state of the starch granule and gelatinization properties.

2.2.3 Usage Of Rice Flour

Rice flour which is made from finely milled rice has been traditionally used as a vital ingredient in producing different rice products for generations. In these recent years, there has been considerable amount of attempt to increase rice flour utilization in producing novel foods such as gluten-free bread, noodle, cake, beverages, processed meat, surimi, vinegar, puddings, salad dressing, and tortilla (Kadan et al., 2008; Kim, 2013). This is due to unique functional properties possessed by rice flour in which it is hypoallergenic, colourless and bland (Wani et al., 2013). Another important feature of rice is its easy digestibility (Kylie 2000). Rice starch has a digestibility rate of between 98 and 100%, making it one of the most easily digestible starches available (Rosniyana et al., 2016). This digestibility, along with being entirely hypoallergenic, is one of the main reasons why rice is used so widely in baby food and other special dietary foods (Rosniyana et al., 2016).

Rice is the world's second most important crop after wheat and is a staple food for more than half of the world’s population, with Asia being the major producer and consumer (Akinbile et al., 2011). Asia accounts for over 95% of global rice production with Malaysia ranked 25th, having a total rice production of 2.4 million tonnes and a relatively stable cultivable land area of approximately 0.7 million hectares since the 1980s (Akinbile et al., 2011). In Malaysia, rice is being grown on 673,745 hectares of land and producing paddy grain valued at RM 2 billion yearly, contributing to an average annual growth rate of 3.7% over the last five years (Siwar et al., 2014). This, with the fact that rice was consumed by 97% of Malaysia population twice daily, proved the significant of rice as a critical staple food crop in Malaysia.

2.3 Starch Modification

At present, starch can be modified by physical, chemical and biological methods and/or the combinations thereof to increase its RS content. This RS-rich powdered ingredient produced from the modification can be used to develop food products that could contribute to a healthy digestive system, providing benefits to the consumer. In the physical method, starch is usually performed by the use of moisture, heat, shear force or radiation. The main procedure includes repeated heating–cooling cycles which produces starch with improved resistance to enzymatic hydrolysis, more resistant to heat treatment and can be used as a functional ingredient for fortification of food products. Chemical modification is often implemented in food industry which includes acid hydrolysis, enzyme hydrolysis, linearization, acetylation, phosphorylation, stabilizing (etherification and esterification) and cross-linking. The chemical treatments can reduce the susceptibility of rice to amylolysis by preventing enzymes from binding properly to the starch. Even though widely implemented, there is a growing health concern over the chemical reagents used and their residues presents in the finished product, therefore physical and enzymatic methods which are safe, non-toxic and considered as green method, have become the preferred technology for starch modification.

2.3.1 Heat-Moisture Treatment

Heat–moisture treatment (HMT) and annealing (ANN) are hydrothermal treatments under physical modifications of starch. HMT is carried out at temperatures above the gelatinization temperatures (90–120 °C) with insufficient moisture to gelatinize (10–30%), whereas ANN occurs under a large excess of water (50–60%) and at relatively low temperatures that fall below the gelatinisation temperature. In these modification, starch to moisture ratio, temperature and heating time are the critical parameters that need to be controlled (Chung et. al., 2009). HMT can alter the physicochemical properties of starch without disrupting its granular structure, by improved the thermal stability and solubility, enhanced RS content, or decreased the extent of retrogradation and viscosity. According to Molavi et. al. (2018), the extent of HMT modification depends on the composition, morphology, amylose content and source of the starch.

HMT promotes retrogradation and formation of RS3. By limiting the amount of water, it allows control of molecular mobility at high temperatures. A sealed environment prevents moisture from escaping through evaporation during heating. The created pressure contributes to the rise of thermal energy, which is continuously converted to kinetic energy by water molecules (Schafranski et al., 2021). This results in large-scale segmental motions (entanglement slippage) and enables starch to transition from a glassy to a more flexible state (Wang et al., 2021). HMT increases starch chain interactions, causing the crystalline structure to break and separate the double helical structure. The broken crystals then subsequently rearrange themselves, forming a compact structure that is less susceptible to digestive enzyme attack, thus facilitate resistant starch formation (Pratiwi et al., 2018). This crystalline disruption near the granule's surface can facilitate the attack of α-amylase in the interior.

HMT causes interactions between amylose-amylopectin, amylose-amylose, and amylose-lipids, which inhibits starch chain mobility in the amorphous region. In other words, HMT promotes starch molecule dispersion by facilitating the interweaving of amylose and amylopectin (through intra/intermolecular connections). HMT also results in the formation of starch crystallites (Schafranski et al., 2021).

There have been numerous researches conducted to study the effect of HMT in starch samples, including rice (Zhao et. al., 2014), sago starch (Adawiyah et al., 2017), wheat (Chen et al., 2015), breadfruit (Capron et al., 2007), potato (Varatharajan et al., 2010), as well as unconventional sources such as bananas (Hoyos et.al., 2015). Zavareze et al. (2010) reported that HMT reduced the swelling power and solubility of the rice starches of varying amylose content. Besides that, for the breadfruit starch, HMT have been reported to enhance the formation of SDS and RS and show greater thermostability while reducing the digestibility of starch (Tan et. al., 2014). Chung et. al. (2009) also revealed that the resistant starch contents of heat-moisture treated corn starch increased to 10.5% compared to native with only 4.6% during HMT treatment.

2.3.2 Enzymatic Modification

Enzymatic modification followed by retrogradation process is proven to be effective modification method, as high concentration of RS3 is formed after the modification. This modification involves the use of debranching enzyme such as pullulanase or isoamylase to debranch the a-1-6 amylopectin bonds, resulting in rearrangement of the structure later in the retrogradation process. As a result of debranching, short-chain and long-chain amyloses are produced from amylopectin molecules, creating a new and strong crystalline structure upon cooling. This in turn promotes the formation of RS as the chain reassociation provides greater molecular alignment and aggregation opportunities thus enhancing the resistance towards digestion. This enzymatic modification is recognized as a clean green label together with physical modification as they produce no chemical residues.

A study made by Guraya et al. (2001) revealed that the RS content in waxy and non-waxy rice starch gel increased through pullulanase hydrolysis and the effects were more pronounced in the cold storage debranched gel. Another study has also presented an increase in the RS content of maize starch up to 44.7% (Zhang & Jin, 2011). There are many factors to influence the efficiency of the enzymatic debranching, such as degree of gelatinization, reaction temperature, pH, enzyme activity, enzyme/substrate (E/S) ratio, and more.

2.3.3 Dual-Modification Treatment

Dual-modification treatment is adopted as there is a need to lessen any shortcomings of native or single-modified starch and/or to further improves the properties and functionality of single-modified starch. In certain cases, single modification has shown to be insufficient in utilizing the starch optimally. Chen et al. (2020) reported that, dual modification of enzymatic debranching and heat moisture treatment able to produce thermally stable and digestive enzyme resistant flour of japonica compared to single modification. Debranching alone only improved the RS content to 42%, however, with further HMT method, the RS content has increased to as high as 55%.

There are two types of dual modification, namely homo-dual modification and hetero-dual modification. The combination of two similar modification will be referred to as the former classification while combination of two different modification referred as the later.

Hetero-dual modification can be of dual chemical/physical, chemical/enzymatic or the reversed dual modification processes. The examples of dual modified starches includes double HMT (Gong et al., 2017); autoclaving/retrogradation (Ashwar et al., 2016); debranching-HMT (Mutungi et al., 2009; Satmalee & Matsuki, 2011); cross-linking/HMT (Park et al., 2018; Sudheesh et al., 2020); and sonication/acetylation (Abedi et al., 2019).

The findings from Mutungi et al. (2009), revealed that the resistant starch yield was increased in starch treated with debranching followed by temperature cycling (heating at 121 or 135°C followed by incubation at 60°C). Satmalee and Matsuki (2011) observed that a combination of debranching and HMT enhanced the starch resistance of low amylose Thai rice flour. Zhou et al. (2014) used dual treatment of enzyme debranching followed by HMT to produce resistant starch from rice starch, which showed significant increase in resistant starch content (35.2%) compared to native (2.52%). HMT alone only increased the RS content to 15.3%. This debranching-HMT treatment produces RS by mechanism that amylopectin molecules are cut into short chain amylose and amylose is re-associated which leads to a new and strong crystalline structure upon cooling, so RS3 is formed. The HMT following the enzymatic debranching would further facilitate retrogradation of short linear molecules.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

Rice flour was bought locally from Lotus's Mall Sungai Dua, Pulau Pinang and kept in airtight container in the research laboratory for the project. Pullulanse, microbial (1000 NPUN/g) was bought from Sigma–Aldrich (M) Sdn Bhd. (Selangor, Malaysia). All other chemicals and reagents were of analytical grade.

3.2 Sample Preparation

3.2.1 Heat-Moisture Treatment

Heat-Moisture Treatment (HMT) was performed by adopting method from Chen et al. (2020) with slight modification. The flour (dry basis) was conditioned into a moisture content of 25%, and heated at 100 °C in air-force oven for 16 hour, and then dried at 40 °C for 24 hour to constant weight. The dried sample was then pulverized using mortar and pestle and sieved through a 60-mesh sieve.

3.2.2 Dual-Modification Treatment

Dual-modified flour was prepared by going through enzyme debranching method followed by HMT. In enzyme treatment, 10g of rice flour was put into 100ml phosphate citric buffer solution and then stirred in hot plate using double boil method (Sun, Li, et al., 2014). The mixture is boiled at 100°C for 30 minutes before cooling it down to 46°C. At 46°C, 3% Pullulanase (3mg in 100g starch) was put into the mixture to debranch the sample. The sample was then placed in orbital shaker at 200 rpm for 16 hour. Then, the sample was centrifuged at 3000rpm for 15 min prior to storing at 4°C in the refrigerator overnight. The retrograded starch was then dried at 40°C in the oven for 24 hour to constant weight. Following that, the dried sample performed in the same manner as HMT in Section 3.2.1.

3.3 Amylose Content Determination

Amylose content of starch was determined by following the method of Duan *et. al.*, (2011), with slight modification. About 10 ml of 0.5 mol/L KOH was added to 20 mg starch sample (dry basis), and the suspension was mixed thoroughly by vortex. The dispersed sample was diluted to 100 mL by using distilled water. 10 mL of the diluted sample was then transferred to a 50 ml volumetric flask and 5 ml of 1 mol/L HCl was added, followed by 0.5 ml of iodine reagent. Distilled water was added to the solution to made up to 50 ml. The absorbance was measured at 625 nm on a UV/visible spectrophotometer (UV-160A, SHIMADZU, Kyoto, Japan) after 20 min. The determination of AC was calculated according to a standard curve developed using different ratios of amylose and amylopectin blends.

Regression equation obtained: $y = 0.0047x+0.1214$

$$\text{Amylose content (\%)} = \frac{Absorbance+0.1214}{0.0047}$$

3.4 Swelling And Solubility Index

The swelling and solubility index were determined by adopting the method of Ashwar *et. al.* (2016) with slight modification. Starch samples (1 g dry basis) were taken in pre-weighed centrifuge tubes with 50 mL of distilled water. The starch suspensions were then incubated in a shaking water bath for 15 min at 90 °C. The tubes were then centrifuged at 3000rpm for 15 min after cooling the samples to room temperature. Supernatant was transferred to pre weighed moisture dishes. The gain in weight of centrifuge tubes was expressed as swelling index.

Moisture dishes were dried at 100 °C for 12 h and then cooled in a desiccator to room temperature. The gain in weight of moisture dishes was expressed as solubility index.

$$\text{Swelling power} = \frac{Weight\ of\ wet\ sedimeny}{Initial\ weight\ of dry\ starch}$$

$$\text{Solubility} = \frac{Weight\ of\ dried\ supernatant}{Initial\ weight\ of\ dry\ starch}$$

3.5 Scanning Electron Microscopy (SEM)

The morphological characteristics of starch granules were viewed using a scanning electron microscope (FESEM, Leo Supra 50 VP, Carl-Ziess SMT, Orbekochem, Germany) according to Li *et. al.*, 2018. The sample starch granules were mounted onto aluminium specimen stubs using double-backed cellophane tape and then sprayed with a thin layer of gold. The microstructure of starch particles were observed and photographed at accelerating voltage of 3K. The micrographs were observed at 1000x magnification.

3.6 X-Ray Diffraction (XRD)

The crystalline pattern of the samples were observed using an X-ray Diffractometer (D8 Advancem Bruker, Massachusetts, United States) using the method reported by Chen *et. al.*, (2011). Sample was placed into the sample holder and diffractograms were recorded in the reflection mode in the angular range of 4-40° (2θ) with a rate of 0.02°/s.

3.7 Differential Scanning Calorimetry

Gelatinization characteristics were analysed and recorded on Differential Scanning Calorimetry, DSC-Q100 (TA Instruments, Lukens Drive, New Castle) as described by Jiang et al. (2012) with slight modification. Dry sample starch (approximately 2 mg) was weighed in a small aluminium pan, and distilled water was added at a ratio of 1:3. The pan was then hermetically sealed and allowed to equilibrate at room temperature for 24 h. The samples were scanned from 20 °C to 150°C at 10°C/min. An empty pan was used as a reference and the calorimeter was calibrated with indium. The initial temperature (To), peak temperature (Tp), finishing temperature (Tc), and enthalpy of gelatinization (ΔHgel) were calculated.

CHAPTER 4 RESULT AND DISCUSSION

In this study, three samples were used in analysis, namely native rice flour (NRF), heat-moisture treated rice flour (HMF), and dual-modified rice flour (DMF). HMF was subjected to heat-moisture treatment (HMT) while DMF was modified using enzyme-debranching and HMT. The abbreviations used in this chapter are listed as in Table 4.1:

Table 4.1: Abbreviations

Abbreviation	Caption
NRF	Native rice flour
HMF	Heat-moisture treated rice flour
DMF	Dual-modified rice flour

4.1 Amylose Content

The changes in amylose contents of all samples are displayed in Table 4.2. From the results, all the samples contained amylose content in the range of 20% to 35%. This is in agreement with previous study by Shi and Gao (2011), who had reported amylose content of rice to be in the range of 20% to 30%.

The result from Table 4.2 showed a significant decrease in trend along the treatment(s) used, with the native rice flour having the highest amylose content (34.52%) followed by HMF (28.91%) and DMF sample (20.82%) having the lowest amylose content.

This result is in contrast with most of the studies that found the correlation between amylose and resistant starch contents, in which increasing RS enhanced the amylose content of starch (Khamthong & Lumdubwong, 2012; Li et al., 2020; Li et al., 2019; Shu et al., 2006). According to starch Annison and Topping (1994), the amount of resistant starch should be proportional to the amylose content in the starch. During HMT, the hydrogen bonds between co-crystallized amylose and amylopectin may be disrupted, enhancing the iodine binding ability of amylose thus resulted in increased amount of amylose content (Varatharajan et al., 2011).

Table 4.2: Amylose content of native rice flour (NRF), heat-moisture treated rice flour (HMF), and dual-modified rice flour (DMF).

Sample	Amylose Content (%)
NRF	$34.52^{a} \pm 0.14$
HRF	$28.91^{b} \pm 0.52$
DMF	$20.82^{c} \pm 0.03$

Data represented means ± SD (N=3). Means followed by different superscripts in a column are significantly different ($p < 0.05$).

However, some previous studies also showed decrement in amylose content after HMT. According to Brian & Chung (2016), HMT may decreased the amylose content in rice starch. The reduction in amylose content could due to some amylose interacts with newly-formed chains via strengthened molecular bonds during HMT, or HMT favours amylose entanglement with lipids, lowering its affinity for iodine, thus, interfere with amylose level determination (Wang et al., 2021). The fact that rice flour contained noticeably higher amounts of protein, lipid and phosphorus compared to rice starch would also influence their properties.

Moreover, other previous studies have demonstrated some differences in the physicochemical properties of the HMT flour and its corresponding HMT starch, as the modification can also affect proteins, lipids, and non-starch polysaccharides of the flour (Khamthong & Lumdubwong, 2012; Sun, Gong, et al., 2014a). This is probably the reason for the declination of amylose content in DMF flour, as compared to its native flour. Enzyme modified starches by pullulanase would produce more short-chain and long-chain by hydrolysing the α-1,6-glycosidic linkages on amylopectin, resulted in increased amylose content. However, as rice flour contained other components such as lipid and protein, iodine may compete with lipids in producing helical inclusion complexes in the α -helices of amylose, thus, making amylose measurement to be reduced. Moreover, protein in DMF samples were reported to be significantly higher than native starch, as debranching enzyme destroyed the structure of starch granules to promote protein release (Chen et al., 2020). Proteins tend to agglomerate with starch in cereal flour, which further increases the structural intricacy (Wang et al., 2020). Therefore, other components in flours should be investigated in future studies as they might influence the properties of rice flour.

4.2 Swelling and Solubility Index

The swelling power and solubility of the native rice (NRF), heat-moisture treated rice (HMF) and dual-modified rice (DMF) flour samples are presented in Table 4.3. Swelling power showed the trend of NRF > HMF > DMF, with NRF sample showed the highest swelling power compared to the other modified flour. It can be observed that the swelling power of the rice starch decreases after HMT, and further reduced after DMT. This reduction might be due to the additional interactions that occurred during the treatment such as amylose-amylose and amylose-amylopectin chains (Gunaratne & Hoover, 2002), increased crystallinity, reduced hydration (Waduge et al., 2006), and alterations in the arrangements of the crystalline regions of starch (Zavareze & Dias, 2011). The ordered rearrangement of the starch molecule caused by HMT has been reported to limit the starch hydration and swelling capacity. To be specific, swelling power has been shown to be influenced by amylopectin structure (Sasaki & Matsuki, 1998), amylose content, and the extent of amylose–amylose- and/or amylopectin–amylopectin-chain interactions (Tester et al., 2000).