IMPACT OF PHYSICAL AND ENZYMATIC MODIFICATIONS OF GRANULAR RICE STARCH ON STRUCTURAL, PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

Academic Year 2019

ผลการดัดแปรทางกายภาพและเอนไซม์ของสตาร์ชข้าวดิบต่อสมบัติทางโครงสร้าง เคมีกายภาพและสมบัติเชิงหน้าที่

นางสาวเทวิกา กีรติบูรณะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เทวิกา กีรติบูรณะ : ผลการดัดแปรทางกายภาพและเอนไซม์ของสตาร์ชข้าวดิบต่อสมบัติด้านโครงสร้าง สมบัติทางเคมีกายภาพและสมบัติเชิงหน้าที่ (IMPACT OF PHYSICAL AND ENZYMATIC MODIFICATION OF GRANULAR RICE STARCH ON STRUCTURAL, PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุนันทา ทองทา และรองศาสตราจารย์ ดร.แอนเดรียส เบลนนาว, 142 หน้า.

งานวิจัยนี้เป็นการดัดแปรสตาร์ชข้าวดิบด้วยวิธีทางกายภาพและเอนไซม์เพื่อผลิตสตาร์ชข้าวดิบที่มีรูพรุนและเพื่อดัดแปรต่อด้วยเอนไซม์ต่อกิ่ง (branching enzyme, BE) ในส่วนแรกสตาร์ชข้าวดิบถูกทำให้มีรูพรุนด้วยเอนไซม์อะไมโลกลูโคซิเดส (amyloglucosidase, AMG) หรือมอลโตเจนิคแอลฟา-อะไมเลส (maltogenic α-amylase, MA) โดยศึกษาลักษณะพื้นผิวของสตาร์ชกรานูล สมบัติด้านโครงสร้างและสมบัติทางเคมีกายภาพ พบว่า เอนไซม์ทั้ง 2 ชนิด สามารถทำให้เกิดรอยบุ๋มและรูพรุนแต่ไม่ทำให้รูปร่างของกรานูลเปลี่ยนแปลง โดย AMG ทำให้เกิดรูขนาดใหญ่และตื้น ซึ่งขนาดของรูพรุนมีความแตกต่างกันเมื่อเปรียบเทียบกับ MA แต่อย่างไรก็ตามการดัดแปรด้วย MA ส่งผลต่อโครงสร้างของสตาร์ช โดยเพิ่มอะมิโลเพคตินสายสั้นและลดขนาดโมเลกุลของสตาร์ชข้าวดิบ และเมื่อเปรียบเทียบกับตัวอย่างควบคุมที่ไม่มีการดัดแปรด้วย MA พบว่า ความสามารถในการละลายน้ำเพิ่มขึ้นแต่กำลังการพองตัวและปริมาณอะมิโลสลดลง อย่างไรก็ตามการดัดแปรด้วย AMG และ MA ทำให้ความเป็นผลึกและเอนทาลปีของสตาร์ชข้าวดิบเพิ่มสูงขึ้น

ส่วนที่สองเป็นการศึกษาการดัดแปรสตาร์ชข้าวดิบด้วยเทคนิคการเกิดผลึกน้ำแข็งใหม่ร่วมกับการละลายน้ำแข็งโดยการใช้อัลตร้าโซนิค (ultrasound-assisted ice recrystallization, US+IR) จำนวน 7 รอบ จากนั้นนำตัวอย่างสตาร์ชข้าวดิบมาดัดแปรต่อด้วยเอนไซม์ AMG หรือ MA พบว่า US+IR ทำให้เกิดร่องและรอยบุ๋มที่ผิวของกรานูล นอกจากนี้ยังพบว่า US+IR ทำให้ค่าความเป็นผลึก ปริมาณอะมิโลส และกำลังการพองตัวลดลง การดัดแปรด้วย US+IR ตามด้วยเอนไซม์ทำให้สตาร์ชข้าวดิบเกิดรูพรุนมากขึ้นและทำให้อุณหภูมิเจลลาติไนเซชัน ค่าความเป็นผลึกและค่าเอนทัลปีเพิ่มขึ้น ในขณะที่กำลังการพองตัวและปริมาณอะมิโลสลดลงเมื่อเปรียบเทียบกับตัวอย่างควบคุมที่ไม่มีการดัดแปรต่อด้วยเอนไซม์ ซึ่งการเปลี่ยนแปลงของตัวแปรดังกล่าวเห็นเด่นชัดกับตัวอย่าง US+IR→MA นอกจากนี้สตาร์ชข้าวดิบที่ผ่านการดัดแปรด้วยวิธี US+IR มีความไวต่อการย่อยด้วยเอนไซม์ย่อยแป้งมากกว่าตัวอย่างที่ไม่ผ่านการดัดแปร และการดัดแปรด้วย US+IR ตามด้วยเอนไซม์แสดงค่าคงที่อัตราการย่อยต่ำกว่าตัวอย่างสตาร์ชข้าวที่ไม่ผ่านการดัดแปรและสตาร์ชข้าวที่ผ่านการดัดแปรด้วย US+IR

ในส่วนสุดท้ายได้ศึกษาผลของการเตรียมตัวอย่างสตาร์ชข้าวดิบที่มีผลต่อสมบัติด้านโครงสร้าง สมบัติทางเคมีกายภาพและอัตราการย่อยด้วยเอนไซม์ย่อยแป้งเพื่อนำไปดัดแปรต่อด้วยเอนไซม์ BE โดยเตรียมตัวอย่างด้วยการดัดแปรด้วยเอทานอลร่วมกับการใช้ความร้อน (ethanol-heating, ETS) ดัดแปรด้วยเอนไซม์ MA และดัดแปรเอทานอลตามด้วยเอนไซม์ MA (ETS→MA) พบว่า ETS ทำให้กรานูลเชื่อมติดกัน มีกำลังการพองตัวสูงและไม่มีรูพรุนเกิดขึ้นที่ผิวของกรานูล ในขณะที่ MA ทำให้ผิวกรานูลเกิดรอยแตก การดัดแปรด้วย ETS→MA ทำให้สตาร์ชข้าวดิบจับกลุ่มเป็นก้อนและเกิดรูพรุน อย่างไรก็ตามการดัดแปรต่อด้วย BE ส่งผลต่อการเปลี่ยนแปลงของผิวกรานูลเล็กน้อย การลดลงของค่าความเป็นผลึกและค่าเอนทัลปีแสดงอย่างชัดเจนกับตัวอย่างที่ผ่านการเตรียมด้วย ETS และ ETS→MA ขณะที่ค่าดังกล่าวเพิ่มขึ้นในตัวอย่างที่ผ่านการเตรียมด้วย MA การดัดแปรต่อด้วยเอนไซม์ BE ทำให้ค่าความเป็นผลึก อัตราส่วนพันธะ α-1,6 ต่อพันธะทั้งหมดและค่าเอนทัลปีเพิ่มขึ้น ในขณะที่ความไวต่อเอนไซม์ย่อยแป้งลดลง นอกจากนี้สตาร์ชข้าวที่ผ่านการเตรียมด้วยวิธีต่างๆ ตามด้วยการดัดแปรด้วยเอนซ์ BE แสดงกำลังการพองตัวลดลงและกรานูลมีความแข็งแรงโดยแยกออกเป็นกรานูลเดี่ยวๆ เมื่อเปรียบเทียบกับตัวอย่างควบคุมที่ไม่ดัดแปรด้วยเอนซ์ไซม์ BE

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2562

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____

THEWIKA KEERATIBURANA : IMPACT OF PHYSICAL AND ENZYMATIC MODIFICATION OF GRANULAR RICE STARCH ON STRUCTURAL, PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES. THESIS ADVISOR : ASST. PROF. SUNANTA TONGTA, Ph.D., AND ASSOC. PROF. ANDREAS BLENNOW. Ph.D., 142 PP.

POROUS STARCH/AMYLOGLUCOSIDASE/MALTOGENIC α-AMYLASE/ BRANCHING ENZYME/ULTRASOUND TREATMENT/ICE RECRYSTALLIZATION

This research was performed to modify granular rice starch using physical and enzymatic methods mainly producing porous starch and for further modification by branching enzyme (BE). Firstly, porous rice starch was carried out separately by amyloglucosidase (AMG) or maltogenic α-amylase (MA). The granular morphology, structural and physicochemical properties were examined. Both enzymes had generated several dents and holes but had no effect on granular shape. AMG induced big and shallow pores with a diversity of pore diameters compared to MA. However, MA obviously modified the fine structure by increasing the short amylopectin chains and decreasing the molecular weight fractions. As compared to the corresponding control, MA increased the solubility but the swelling capacity and amylose content were reduced. Moreover, enzymatic treatments increased the crystallinity and the enthalpy of starch treated samples.

Secondly, the combination of seven repeated cycles of ultrasound-assisted ice recrystallization (US+IR) following treatment with AMG or MA was studied. Generally, US+IR created grooves and indentations on the surface granules. The

US+IR decreased the crystallinity, amylose content and swelling capacity. The sequential US+IR followed by enzymes hydrolysis exhibited more pores and increased gelatinization temperature, crystallinity and the enthalpy while swelling capacity and amylose content were further decreased as compared to their control. The most obvious change of those parameters was found for US+IR→MA. Moreover, the US+IR treated starch showed a higher susceptibility to amylolytic enzymes than native starch. In addition, the combined treatments, especially US+IR→MA exhibited lower digestion rate constant.

Lastly, the effects of pre-treatments of granular rice starch on structural, physiochemical properties and the rate of digestion to amylolytic enzymes for further BE catalysis were studied. The pre-treatments were performed by ethanol-heating (ETS), MA and sequential ETS→MA. The ETS displayed coapted granules, noticeable swelling with no pores whereas MA caused fissures on granular surface. The sequential of ETS→MA created agglomerated and perforated granules. However, further BE catalysis exhibited minor surface changes. The reduction of crystallinity and the enthalpy was pronounced by ETS and ETS→MA, whereas those of values increased for MA. The subsequent BE catalysis increased the crystallinity, α-1,6 glucosidic linkage ratio, the enthalpy and less susceptibility to amylolytic degradation. Moreover, after BE modification pre-treated starch showed less swelling, more intact granules and granular separation compared to the corresponding control.

School of Food Technology

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Asst. Prof. Dr. Sunanta Tongta and Assoc. Prof. Dr. Andreas Blennow, for their excellent teaching, guidance, patience, encouragement, giving many opportunities and the continuous support throughout my study course and research. Without their contribution, my thesis would not have achieved and completed.

My sincere gratitude also goes to Dr. Baljit Ghotra, Dr. Lijia Zhu, Dr.Ali Halalipour, Dr.Maaran Suntharamoorthy and all members in starch lab, ADM, for kindly giving me into their laboratory and good suggestion during my stay in USA. I am grateful to Dr. Eric Bertoft, Bertoft solutions, Finland, for fruitful suggestions.

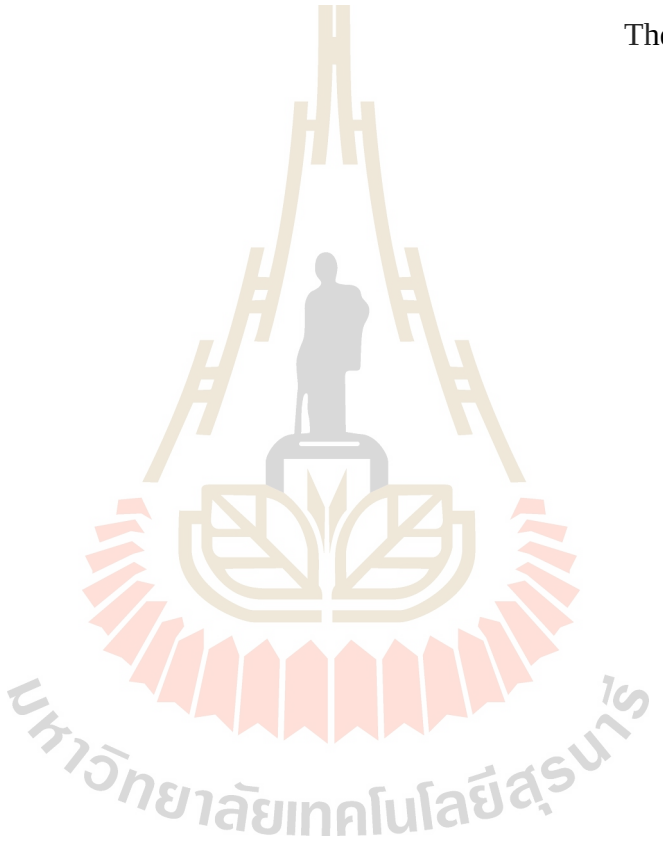
Furthermore, I would like to thank Dr. Aleksander Riise Hansen, Olivia Kofoed-Enevoldsen, Yuyue Zhong and everyone at the section for Plant Glycobiology for sharing knowledge and guidance with the practical laboratory work throughout my study in Denmark. Thanks to all members of "ST group" in Suranaree University of Technology for all their friendship, helps, supports, encouragement and excellent working atmosphere during my Ph.D. study.

Novozymes A/S is gratefully acknowledged for providing MA and BE. General Food Products Co., Ltd., Thailand is also acknowledged for providing rice starch. Especially, I would like to thank the Royal Thai Government Scholarship (Ministry of Science and Technology) for fully funding support. Buriram Rajabhat University, Thailand and ADM, USA are also acknowledged for valuable financial support.

I am also thankful Suranaree University of Technology, Synchrotron Light Research Institute, Thailand, University of Copenhagen, Denmark and Starch lab in ADM, USA for supporting the laboratory facilities.

Finally, I wish to thank my beloved family and my love for their moral support, understanding, inspiration, and encouragement throughout my life.

Thewika Keeratiburana



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CHAPTER I

INTRODUCTION

1.1 Introduction

Rice (*Oryza sativa* L.) is a major cereal crop and a stable food source for half of the world (Amagliani, O'Regan, Kelly, & O'Mahony, 2016; Wani et al., 2012). Starch is the major component of rice, constituting more than 80% of the total dry matter. Rice starch has been used in food and nonfood industries. The functional properties of rice starch are influenced by morphology, crystalline structure, varieties, etc. Rice starch granules are the smallest in cereal grains, the size of which are reported in the range of 2 to 7 μm. Most rice starches had no pores (Fannon, Hauber, & BeMiller, 1992; Sujka & Jamroz, 2007) but some granules possessed small surface pores (Achayuthakan, Suphantharika, & Bemiller, 2012). The smallest granular size of rice starch similar to that of fat globules. Therefore, it is used to replace fat in bakery, salad dressing, soup, sauces and processed meat to keep a full-fat mouth feeling (Wani et al., 2012).

Normally, raw starch has several weak points such as low gel strength, low solubility, high viscosity of the starch gel at room temperature, and irreversible retrogradation. In order to improve the functional properties of native starch, physical, chemical or enzymatic modifications are applied. However, chemical modification can be associated with environmental pollution and increasing concerns from consumers.

Physical and enzymatic modifications are considered to be environmentally friendly as it reduces usage of chemicals, and waste production. Physical modification is a simple method to achieve functional properties suitable for many industrial applications. For instance, freezing-thawing and ultrasonication can be used to make porous starch. Enzymatic modification has the advantages of fewer by-products, increased safety, substrate selectivity, product specificity and environmentally friendly alternative to chemical modification (Le et al., 2009; Oh, Choi, Lee, Kim, & Moon, 2008). In general, enzymatic modification is applied to gelatinized starch rather than granular starch. Due to the tight packing of crystalline zone (amylopectin chains), the granule surface is relatively impenetrable. Direct hydrolysis of starch in native state is desirable because it reduces the costs and saves energy associated with the high temperatures required for gelatinization (Uthumporn, Zaidul, & Karim, 2010).

Nowadays, there is a growing interest in developing porous starch because it has interesting properties for many areas such as food, cosmetics medicines etc. Porous starch is modified starch that consist of pores on the surface and it is possible that these pores could be extended to the center of the granule (Dura, Błaszczak, & Rosell, 2014; Zhang et al., 2012). Porous starch can be produced by physical, chemical, and enzymatic methods. Several researchers have studied porous starch and its properties using freezing-thawing (F/T) method (Tao, Wang, et al., 2015; Tao, Yan, et al., 2015; Yu et al., 2015). Initially, bulk and structured water that crystallizes to ice during the freezing process results in physical stress in the granular matrix (Charoenrein & Preechathammawong, 2012). However, efficient pore and crack formation are facilitated by slow freezing rates and several F/T cycles. Recrystallization of ice is a molecular reorganization process that is characterized by increased mean

size of the ice crystals. Typically, larger ice crystals are created as a result of temperature fluctuations. A typical protocol involves freezing and thawing of starch granules in water for 20-24h F/T cycles which is a slow process. Ice recrystallization can potentially serve as a more efficient procedure to produce porous starch for a shorter time. Ultrasonication is a well-established method for disintegration of biological matter and has also been applied for preparation of porous starch (Qian, Chen, Ying, & Lv, 2011; Wu, Du, Ge, & Lv, 2011). Ultrasound is the mechanical waves with a frequency higher than the threshold of human hearing (>16 kHz). In starch-water system, ultrasound-treatment creates excessive shear force, high temperature, and free radicals, which can break the chains of starch and then change the structure and properties of starch (Sujka & Jamroz, 2013; Zhu, 2015). Therefore, ice recrystallization assisted with mild ultrasonication can produce more pores and can shorten the process time.

Porous starch is commonly obtained by several enzymes such as amyloglucosidase (AMG), α-amylase (AM), cyclodextrin-glycosyltransferase (CGTase), branching enzyme (BE), a mixture of α-amylase and glucoamylase (Benavent-Gil & Rosell, 2017; Chen & Zhang, 2012; Dura et al., 2014; Dura & Rosell, 2016; Uthumporn et al., 2010; Yussof, Utra, & Alias, 2013) and glucoamylase combined with ultrasonic treatment (Wu et al., 2011). In this study, AMG and maltogenic α-amylase (MA) are applied to create the pores in starch granules. AMG is commonly used while there is a lack of information on the possibility of MA to produce porous starch. AMG and MA are exo-acting hydrolase enzymes that catalyze hydrolysis of both α-1,4 and α-1,6 linkages from non-reducing ends of α-glucans. However, their catalytic actions are different. MA produces maltose while glucose is produced by

AMG. MA is also endo-acting and does not require a non-reducing end for activity (Christophersen, Otzen, Norman, Christensen, & Schäfer, 1998). MA displays transglucosylation activity via formation of others glucosidic linkages such as α,1-3 and α,1-6 linkages producing branched oligosaccharides (Cha et al., 1998; Park et al., 1998). Both of AMG and MA can react on raw starch in different ways to "open up" the granular structure permitting further modification using glucanotransferases to obtain highly α-glucans.

The α-glucanotransferases such as branching enzyme (BE) have received considerable attention to modify the structure and its properties of granular starch by cleaving α-1,4- and reforming α-1,6-glucosidic bond (Benavent-Gil & Rosell, 2017; Li et al., 2016; Ren et al., 2017; Van Der Maarel & Leemhuis, 2013). Raw starch modification by BE can increase degree of branching while short chain-length of amylopectin decreased. (Jensen, Larsen, Bandsholm, & Blennow, 2013). Jensen et al. (2013) reported that treatment with BE can stabilize the granular structure. Ren et al. (2017) reported α-1,6 linkages/total linkages ratio of waxy maize starch treated with BE below its gelatinization temperature increased with longer reaction time. Increasing the ratio of α-1,6 linkages/total linkages correlates with a reduction of peak viscosity, setback value, average amylopectin chain length. These results are consistent with data on maize starch treated with BE at 50°C showing an increase in the number of α-1,6 branch point in a time-dependent manner. The average chain length of amylopectin and the amylose content decreased associated with the decrease of pasting peak viscosity and setback with increasing reaction time (Li et al., 2016).

A possible way forward aiming at reducing the digestion time of starch-derived carbohydrates, is to increase the number of short chains at the expense of long chains

in the amylopectin by increasing the amount of α-1,6 linkages. Reducing the molecular size of this highly branched product generates potential prebiotic isomaltooligosacharides, such as isomaltose, panose and isomaltotriose (Ao et al., 2007; Kittisuban, Lee, Suphantharika, & Hamaker, 2014; Martínez, Pico, & Gómez, 2016; Miao et al., 2014). While in its granular state, and followed by opening up their structure by enzymatic or physical modifications, the starch granules are more accessible for enzyme interaction and enzymes, including BE, can use to produce high branched α-glucan. BE has been demonstrated to increase the number of α-1,6 branch points in raw waxy corn starch in a time-dependent manner (Ren et al., 2017). However, the modification of starch containing amylose, such as normal maize and rice starch, is more demanding due to the crosslinking effect of amylose.

As mentioned above, MA possesses both hydrolytic and transglucosylation activities on α-glucan substrates. MA also displays high transglucosylation activity via formation of others glucosidic linkages such as α,1-3 and α,1-6 linkages producing branched α-glucan. This transfer reaction can be stimulated by using low water activity and mild temperature during reaction (Christophersen et al., 1998). Therefore, when starch granules are modified to increase pore formation, BE can permit more efficient production of highly branched α-glucan from granular starch. In this study, granular rice starch was modified by physical or enzymatic modification to increase pore formation and provide accessibility for further enzyme modification.

1.2 Research objective

1.2.1 To study the effect of AMG and MA that are used to prepare porous rice starch on structure and physicochemical properties

1.2.2 To study the effect of ultrasound-assisted ice recrystallization (US+IR) and the combination of US+IR followed by enzymatic hydrolysis to prepare porous structure.

1.2.3. To investigate the morphological, structural and physicochemical properties of granular rice starch affected by US+IR and the combination of US+IR followed by enzymatic hydrolysis.

1.24. To investigate the effect of BE treatment of granular rice starch modified by physical, enzymatic and dual-modifications on structural and physicochemical properties.

1.3 Research hypothesis

Rice starch can be modified by physical and enzymatic methods to produce porous structure. AMG and MA are both hydrolases that possess exo-acting activity that can be used to produce porous starch. Their different catalytic actions on granular starch can provide different granular porous materials for further clean modification using transferases. Moreover, ice recrystallization of granular rice starch can induce local high pressure in the granular matrix to potentially produce porous starch granules. Ultrasound treatment can cause cracks and depressions on the starch granules. Hence, ultrasound-assisted ice recrystallization can serve as a pretreatment for enzymatic hydrolysis to produce porous rice starch. Furthermore, preparation methods, namely ethanol-, MA- and the combination of ethanol and MA can be used to “opening up” the granule before modification with BE, resulting in the increased α, 1-6 linkage.

1.4 Scope of the study

1.4.1 Porous rice starch was modified separately with AMG and MA. The enzyme-action was compared with its control, without enzyme adding of each time of incubation. The morphological, structural and physicochemical properties of porous rice starch were investigated.

1.4.2 The physical modification as ice-recrystallization and ultrasonication (US+IR) 7 repeated cycles was used to produce porous rice starch. Subsequently, US+IR treated starch was further modified with AMG or MA to increase the specific surface area. The morphology, crystalline structure, molecular weight of fraction, thermal properties and the rate of digestion with amylolytic enzymes were determined.

1.4.3 The preparation methods of rice starch for further modification with BE were studied. Maltogenic α-amylase, 50% of ethanol and the combined treatment with ethanol followed by MA treatment were used as a pretreatment. Then, BE was applied to pretreated starches. The morphological, structural and physicochemical properties of modified-starch were investigated.

1.5 Expected results

Results from this research may bring about a fundamental understanding in the preparation of porous rice starch from physical and enzymatic methods. Regarding fundamental knowledge, it will contribute to a better understanding in porous rice starch using ultrasound-assisted ice recrystallization, ethanol techniques and the activities of AMG, MA. In addition, it will lead more understanding the possibility to prepare rice starch for BE modification. Furthermore, they may also show the relationship among the molecular structure, physicochemical properties and amylolytic

digestibility. Regarding this knowledge, it is provided different functionalities depending on the modification methods and extending the range of applications.

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CHAPTER II

LITERATURE REVIEW

2.1 General starch-background

Starch is the main energy reserve in plants and therefore a major source of carbohydrates on earth. Starch is the principal energy source in human diet and affects gut microbiota. In recent years there has been growing interest in potential health effect of starch. An important fraction of starch escapes digestion in small intestine the so-called resistant starch. Resistant starch is fermented by gut microbiota. The major products of this fermentation are short chain fatty acids that are related with several health benefits. Starch is also valuable ingredient being widely used in food and industrial applications as a colloidal stabilizer, thickener, gelling agent and water retention agent (Singh, Kaur, & McCarthy, 2007). There are multiple structure levels of starch structure (Figure 2.1). The first level describes individual linear chain of starch molecules that is anhydroglucose units are linked by α-1,4 glucosidic bonds. Level 2 is that of branched individual starch molecules are joined together by α-1,6 glucosidic bonds to form amylopectin and amylose. Level 3 is described as semi-crystalline lamellae, alternating amorphous and crystalline. This level shows the conformation of starch molecules, the aggregation of starch chains into a helical structure then aggregate to form crystallites. The formation of several alternating crystalline and amorphous lamellae is growth ring structure (level 4). Level 5 is granular structure that consists of growth rings. The last level is whole grain that is

starch granules interact with protein, lipids, non-starch polysaccharides (Tran et al., 2011).

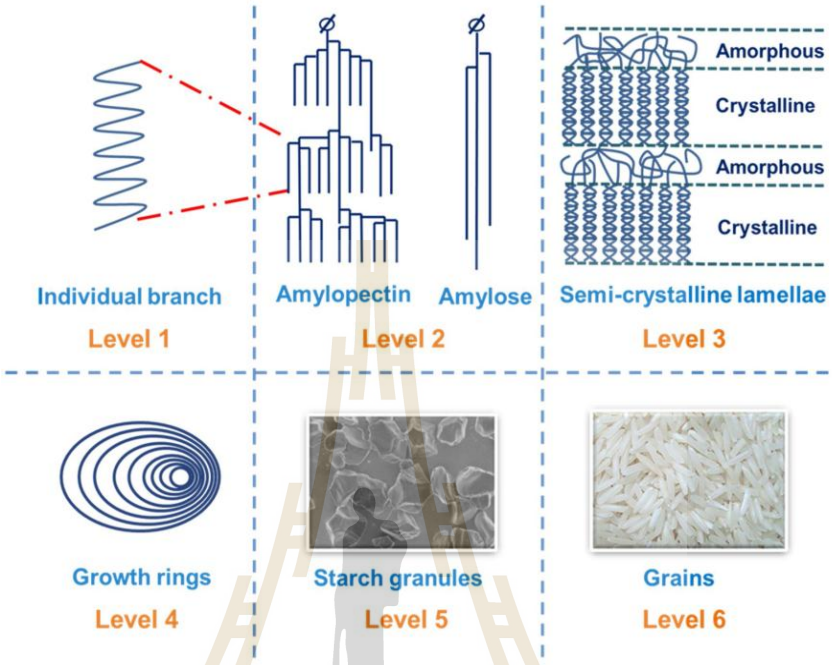

Figure 2.1 The structural level of starch (Li & Gilbert, 2018 adapted from Tran et al., 2011).

2.1.1 The composition of starch

Starch is composed of amylose and highly branched amylopectin. Amylose is mainly linear chain of polymer that connects glucose molecules with α-1, 4-linkages. It also has some branches on the amylose chains (Gunning et al., 2003; Hizukuri, Takeda, Yasuda, & Suzuki, 1981; Juliano, 1998; Takeda, Hizukuri, Takeda, & Suzuki, 1987). The actual location of amylose is still in dispute. The possible locations of amylose are in amorphous lamellae, amorphous growth ring, or interspersed or co-crystallized with amylopectin molecules (Hoover, Hughes, Chung, & Liu, 2010). Amylose is actually helical conformation with six anhydroglucose per

turn. Amylose can form a single helical fold that can complex with free fatty acids, aliphatic alcohols and iodine. The complexation of amylose with iodine change the color of amylose to deep-blue, that can be used to determine the amylose content in starch (Juliano et al., 1981). In rice starch, amylose has the weight-average degree of polymerization (DPw) and the number-average degree of polymerization (DPn) values of 2,750-3,320 and 980-1,110, respectively, and average chain length (CL) of 250-370 (Takeda, Hizukuri, & Juliano, 1986). The amylose contents are classified as waxy (0-2%), very low (5-12%), low (12-20%), intermediate (20-25%) to high (25-33%) (Juliano, 1998). Amylose content is the major factor affecting the physicochemical properties of starch. The high amylose in starch increase starch retrogradation and gelatinization temperature.

Amylopectin is the main component in normal starch (70-80%). It is a highly branched structure that consist of α-1,4 glucosidic backbone and about 5-6% of α-1,6 glucosidic linkages forming branch points (Hizukuri et al., 1981). The organization of amylopectin chain also describes in terminology which characterizes as A, B and C types (Figure 2.2). The outer or A chains are unsubstituted, the shortest CL 6-15 and are α-(1,6)-linked to B chains. The B chains bear one or more A chains and/or B chains. The number of clusters can be classified into B1, B2, B3 and B4 (with one to four clusters) depends on the span and their specific length. For B1 and B2 chains, the CL is essentially 15-25 and 40-50, respectively, with B3 and B4 chains being longer. The C chain, single chain per amylopectin molecule contains the sole reducing terminal residue (Pérez & Bertoft, 2010). The molecular size of amylopectin, extensively branched starch component is much larger than amylose. The Molecular weight (Mw) of amylopectin has been reported to range from 10^7 to 10^9 Da. The Mw

of amylopectin as analyzed by high-performance size-exclusion chromatography suggested that non-waxy and waxy rice have Mw of $2.7x10^9$ and $5.7x10^9$ Da, respectively (Yoo & Jane, 2002) However, the separation and determination of amylose and amylopectin are problematic in determining molecular size. The mainly problems are the inability for solubilization completely and the aggregation of amylopectin and amylose (Blennow, Mette Bay-Smidt, & Bauer, 2001).

2.1.2 The organization of amylopectin

Amylopectin is the main component in starch. Amylopectin consists of numerous chains that is responsible for the semi-crystalline growth ring structure in starch granules. The growth ring consists of stacks of alternating amorphous and crystalline lamellae (Jenkins, Cameron, & Donald, 1993). The originally organized structure of amylopectin has been proposed as “cluster model”, short chains are arranged in clusters and the long chains are interconnect the clusters. Recently, the “building block backbone model” is proposed (Bertoft, 2013), which is the widely accepted to date. In this model, the short chains are form double helices as the building blocks. Building block are outspread along the backbone with consist of internal building blocks that linked by B (short) chains. The B (short) chains probably connect to building outside as external building blocks. The backbone is linked by B (long) chains to form longer backbone (Figure 2.4). Amylopectin has an ability to bond to other substances due to it has many α-glucan chains linked together by generating branch points which more useful for the industries than amylose.

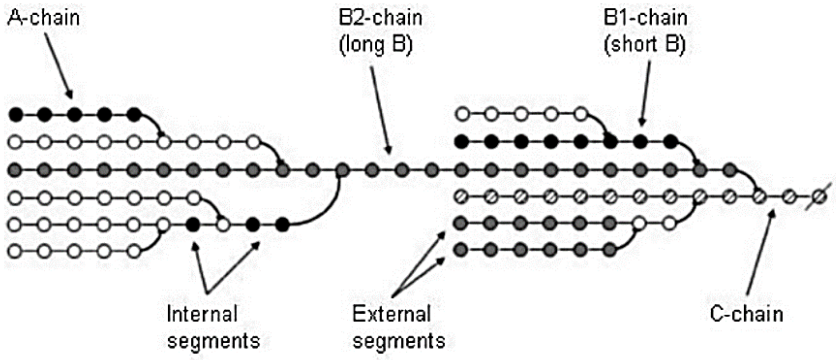

Figure 2.2 The basic organization of the amylopectin chains. Circles denotes glucosyl residues, horizontal lines (1-4) and bent arrows (1-6) linkages. The reducing-end residue is to the right (Pérez & Bertoft, 2010).

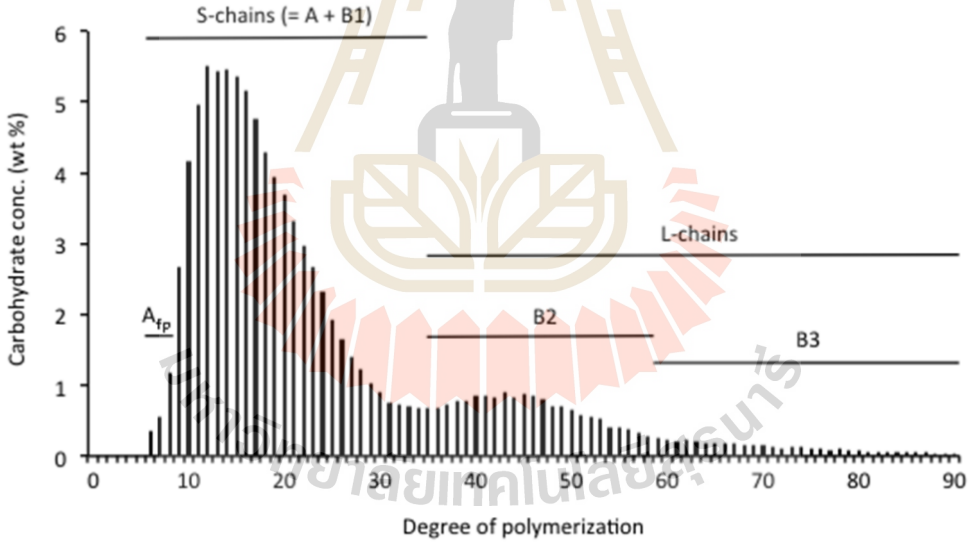

Figure 2.3 Chain length profile of rice starch after debranching (Bertoft, 2017).

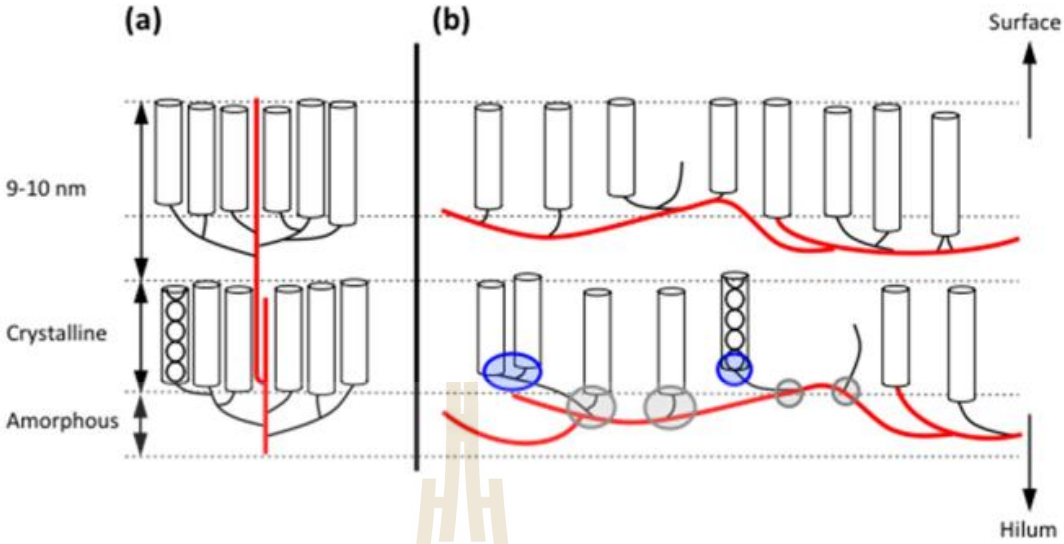

Figure 2.4 Organization od structure unit in amylopectin based on the cluster model (a) or the more recent building block backbone model (b). The thick red lines indicate as long B-chains and black lines indicate as short chains. The external chains are formed double-helices as show in cylinders symbolize. The backbone in (b) carries internal building blocks (enclose in grey) and can carry short BS_{major}-chains that form branches to the backbone and connect to external building blocks (enclose in blue) (Bertoft, 2017).

Table 2.1 Properties of amylose and amylopectin.

Property	**Amylose**	**Amylopectin**
Molecular structure/branches	Mainly linear/	Highly branched/
Molecular weight	$10^5 – 10^6$ Da	$10^7 – 10^8$ Da
Iodine bonds/colour	20%/blue-black	<1%/red-purple
Solubility	Low/barely soluble	High
Gelatinization temperature	high	Low
Amylose-lipid complex	Very high amount	No
Gel formation	Firm, irreversible	Soft, reversible
Viscosity	Low	High
Freezing-thawing stability	Unstable	Stable
Retrogradation rate	High	Low

(Schirmer, Jekle, & Becker, 2015).

2.2 Porous starch produced by physical modification

Porous starch is a modified starch that contains pores on the surface and could be extended to the inner part of the granule (Dura, Błaszczak, & Rosell, 2014). According to International Union of Pure and Applied Chemistry (IUPAC) (Sing et al., 1985), pores are classified as follows: (i) macropores, with diameters larger than 50 nm, (ii) mesopores, with diameters between 2 and 50 nm and (iii) micropores, with diameters smaller than 2 nm. Normally, native starch granules are slowly reacted to enzymatic hydrolysis and chemical reaction (Fannon, Gray, Gunawan, Huber, & BeMiller, 2004; Sujka & Jamroz, 2010). So, it is significant to consider that native

starch is limited for the enzyme accessible inside of the granules (Uthumporn, Shariffa, & Karim, 2012).

2.2.1 Freezing-thawing method

Freezing–thawing (FT) process is considered as one of physical methods to modify starch that is an effective method to produce porous starch (Charoenrein & Preechathammawong, 2012; Yu, Ma, & Sun, 2010; Yu et al., 2015). During the freezing process, water in system turned into ice that applied high pressure to starch granules, resulting in physical stress in the food matrix. It has been reported that freezing–thawing is effective on the structure and functional properties in porous starch production (Tao et al., 2015; Yu et al., 2015). In addition, the effect of freezing–thawing on starch modification depend on many factors such as starch varieties, freezing and thawing temperatures, moisture contents, freeze-thawing cycle and freezing speed (Szymońska, Krok, Komorowska-Czepirska, & Rebilas, 2003; Tao et al., 2015). However, the repeated F/T treatment has been reported. It can enhance the size of cavities and also destroyed the honey-comb structure in starch gel (Wang, Yin, Wu, Sun, & Xie, 2008). Yu et al. (2015) reported that a number of pores on the surface of waxy corn starch granules increased with freeze-thawing cycles. They also concluded that under appropriate condition (-20°C freezing and 25°C thawing, 20 cycles) big pore and clear cracks were obtained. Szymoñska et al. (2003) reported that multiple freezing and thawing process was able to modify potato starch granules, some scratches and roughness could be observed after the modification.

2.2.2 Ultrasonic method

Ultrasound is well known on many processes in food industry. The use of ultrasound is also recognized as the physical modification. Ultrasound is the

mechanical waves with a frequency above the threshold of human hearing (>16 kHz). There are three frequency ranges including power ultrasound (16-100 kHz), high-frequency ultrasound (100 kHz-1 MHz), and diagnostic ultrasound (1-10 MHz) (Patist & Bates, 2008; Zhu, 2015). It is a non-conventional and powerful method to modify starch for environmentally friendly (Carmona-García et al., 2016; Luo et al., 2008; Zhu, Li, Chen, & Li, 2012).

Usually, the ultrasonic application in starch systems is in a liquid-solid bi-phase system with water as the medium. The acoustic ultrasound waves pass through the aqueous medium; it generates a continuous wave-type motion. This motion can be made a pressure that it induces mixing within the liquid. The pressure fluctuations cause the formation, growth and collapse of microbubbles within an aqueous system (Zhu, 2015). The cavitation effect can be occurred by the compression and rarefaction process and the collapse of the bubbles (Patist & Bates, 2008). The occurrence of collapsing bubbles generates a high local turbulence and increased temperature and pressure in the system (Leong, Ashokkumar, & Sandra, 2011). The graphical summary of bubble formation, growth and collapse during sonication is shown in Figure 2.5.

Ultrasonication has been widely used in the starch modification. The effect of ultrasound treatment on structural, physicochemical and functional properties of starch has been studied. Ultrasound treatment affects the morphology of starch granules which can be observed by light microscopy, scanning electron microscopy and transmission electron microscopy. It has been reported that ultrasound is effective on the structure of starch granules by the cavitation effect (Bai, Hébraud, Ashokkumar, & Hemar, 2017; Degrois, Gallant, Baldo, & Guilbot, 1974; Luo et al., 2008; Zhu, 2015).

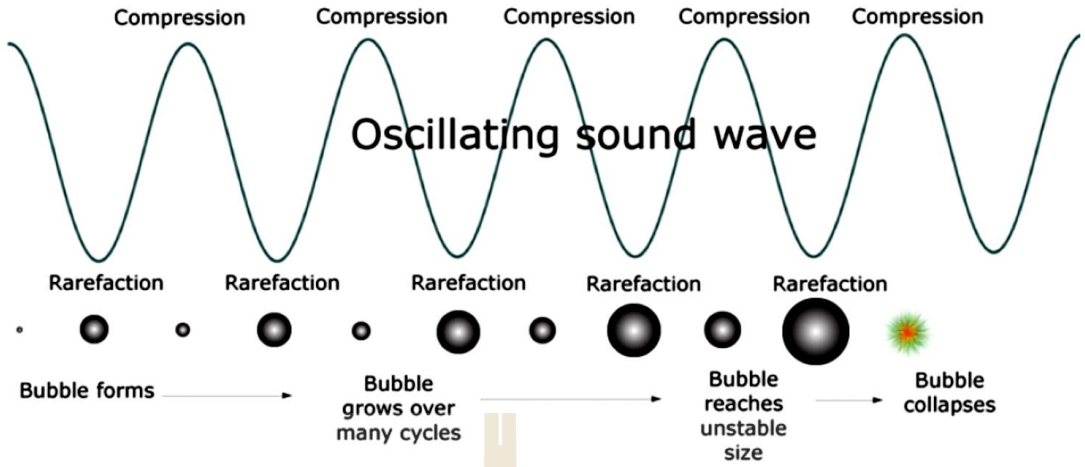

Figure 2.5 The graphical summary of bubble formation, growth and collapse during applied ultrasound waves **(**Leoug et al., 2011).

Sujka & Jamroz (2013) reported that ultrasonication (frequency of 20 kHz and power 170 W for 30 min) causes the formation of crack, pore and harm in starch granules (wheat and rice). Zhu et al. (2012) investigated the effect of ultrasound on supramolecular structure feature of potato starch granules and found that ultrasonication has barely affected on polymorph type of potato (B-type) but the crystalline lamella was reduced. The granules of potato starch showed notch and groove after modified by ultrasound treatment. Huang, Li, & Fu (2007) observed similar changes on the polymorphism and crystallinity of corn starch granules. The results showed that the polymorph type of corn (A-type) and the crystalline structure did not change but it affects the amorphous region. Bai et al. (2017) investigated the pitting of potato starch granules by using high-frequency ultrasound treatment. It showed that the number of pits per starch granules is not affected by amylose content and the maximum number of pits is enhanced with the high frequency of ultrasound. The effect of ultrasonic modification on granules porosity of rice, corn, wheat and

potato was also studied by Sujka (2017). The specific surface area increased for all sonicated starches and the new pores in range of mesopores occurred with ultrasound treatment.

2.3 Starch digesting enzyme

Starch digesting enzymes are enzymes that break down the α-glucans into their smaller chain. Basically, there are four types of starch-digesting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases that it classified on the mechanism on starch chain (Van Der Maarel, Van Der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). Endoamylases such α-amylase (EC 3.2.1.1) are able to cleave α-1,4 glucosidic bonds present in the inner part (endo-) of amylose or amylopectin chain. Hydrolysis occurs in a random fashion at any (1 → 4)-linkage within the starch chain to rapidly reduce the molecular size of starch and the viscosity of the starch solution during pasting (Dura et al., 2014).The second type is exoamylases that act on the external glucose residues of amylose or amylopectin from the non-reducing ends. It cleave an α-1,4 glucosidic bonds such as β-amylase (EC 3.2.1.2) or cleave both α-1,4 and α-1,6 glucosidic bonds like amyloglucosidase or glucoamylase (EC 3.2.1.3) thus producing only glucose. The third type, debranching enzymes hydrolyze α,1-6 glucosidic bonds such as isoamylase (EC 3.2.1.68) and pullanase type I (EC 3.2.1.41). The fourth type of starch digesting enzyme is transferases that cleave an α-1,4 glucosidic bond and then transfer to a glucosidic acceptor with the formation of a new glucosidic bond. Enzymes such as amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) form a new α-1,4 glucosidic bond while branching enzyme (EC 2.4.1.18) forms a new

α-1,6 glucosidic bond. The different enzymes involved in the degradation of starch are shown in Figure 2.6.

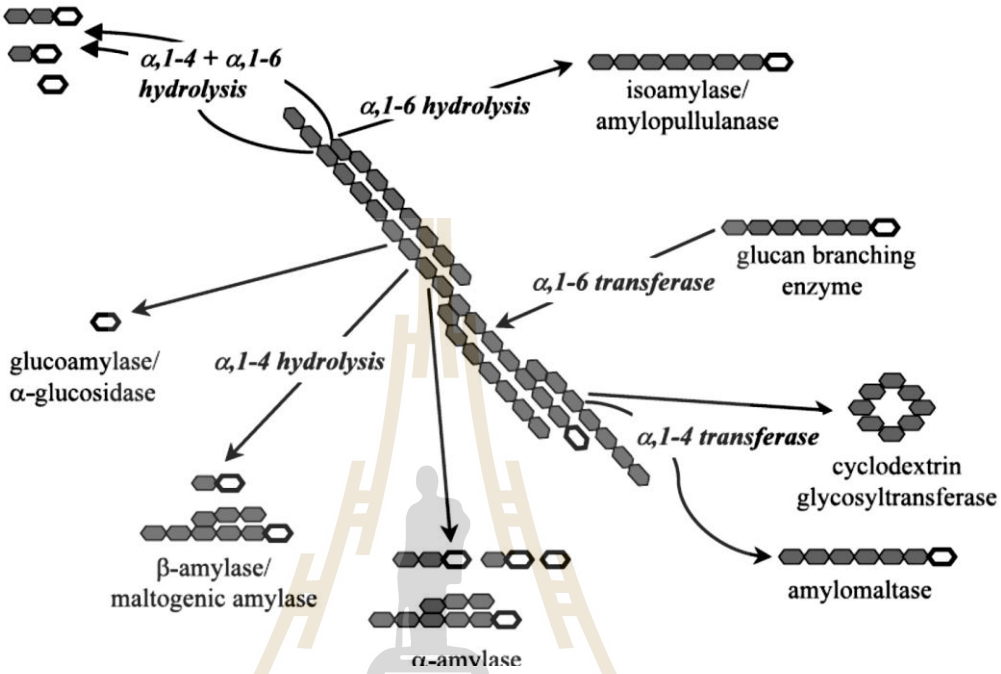

Figure 2.6 Different enzymes involved in the degradation of starch. The open ring structure symbolizes the reducing end of a polyglucose molecule (Van Der Maarel et al., 2002).

2.3.1 Amyloglucosidase (AMG, α-1,4-glucan glucohydrolase, EC 3.2.1.3)

AMG or glucoamylase is produced by a variety of microorganisms. AMG is exo-enzyme, hydrolyzes α-1,4 glucosidic bonds from the non-reducing ends of starch, resulting in the production of glucose. It is able to hydrolyze α-1,6 linkages, also resulting in glucose as the end-product, but the rate more slowly than on α-1,4 linkages (Mertens & Skory, 2007). It has been considered the most importance for worldwide applications (Coutinho & Reilly, 1997). High-glucose and fructose syrups

are produced by AMG. Many researchers have been conducted to characterize properties of raw starch modified by AMG. Synergistic AMG and α-amylase have been studied in raw starch hydrolysis (Fujii, Homma, & Taniguchi, 1988; Uthumporn, Zaidul, & Karim, 2010; Yussof, Utra, & Alias, 2013). AMG is commonly used to produce porous starch. Dura et al. (2014) compared the α-amylase and AMG action on the granules of corn starch. It was concluded that α-amylase produced some holes on the starch granules and its action was not dependent on the granule size while AMG was more active on the starch granules obtaining hollow granules. Huber & Bemiller (2000) observed similar changes on the surface of corn and sorghum starch granules in that AMG produced openings to channels for providing access to the granule interior, and the surface pores enlarged through channels from hilum region toward the outsides.

2.3.2 Maltogenic α-amylase (MA, glucan 1,4-α-maltohydrolase, EC 3.2.1.133)

MA is a glycoside hydrolase cloned from various Gram-positive bacteria (Le et al., 2009). MA hydrolyses α-1, 4glucosidic bonds of starch polymers, releasing maltose residues from the non-reducing ends of starch chains. The mode of its action is unclear. Some reports investigate that MA acts as an exo-activity with removing maltose from the non-reducing end. However, MA has endo-activity, does not require a non-reducing end (Christophersen, Otzen, Norman, Christensen, & Schäfer, 1998; Goesaert, Slade, Levine, & Delcour, 2009; Le et al., 2009; Miao et al., 2014). MA also exhibits high transglycosylation activity via formation of various glucosidic linkages such as α,1-6 linkages producing branched oligosaccharides from liquefied starch (Cha et al., 1998; Park et al., 1998). This enzyme has been studied by

many researchers. It is widely used as an anti-staling agent in bakery products. MA can inhibit the retrogradation of waxy maize starch (Grewal et al., 2015), rice starch and rice meal (Li, Li, Tian, & Park, 2014) in that it increases the proportion of short outer chain of amylopectin. In order to slow down the digestion time of starch-derived carbohydrates, highly branched α-glucan is attempted. The highly branched α-glucan can be obtained by MA (Miao et al., 2014) and the combination of MA with other amylolytic enzymes such as branching enzyme, α-glucanotransferase and transglucosidase have been reported (Ao et al., 2007; Le et al., 2009; Martínez, Pico, & Gómez, 2016).

2.3.3 Branching enzyme (BE, 1,4-α-D-glucan: 1,4-α-D-glucan, 6-α-D-(1,4-α-D-glucano)-transferase, EC 2.4.1.18)

BE is found in plants, animals and microorganisms. It catalyzes both of hydrolytic and transfer reactions to form new α-1,6-glucosidic linkages (Ao et al., 2007; Lee, Yoo, Ryu, Kim, & Yoo, 2008). The branch α-glucan is created by the breaking an α-1,4 glucosidic linkages from the non-reducing end and then transfer it to produce an α-1,6 glucosidic linkage (Sorndech et al., 2015). Moreover, cyclization reaction can be catalyzed by BE, form cycloamylose and cycloamylopectin clusters (Takeda et al., 1986). The smallest cycloglucan (DP 8) was observed from BE treated cassava starch (Sorndech et al., 2015). Jensen, Larsen, Bandsholm, & Blennow (2013) found that the starch structure can be stabilized and degree of branching significantly increase by BE treatment at the extreme starch concentration. BE is able to act on native starch. At below gelatinization temperature, BE can increase the number of α-1,6 branch points of waxy corn starch and this branch points are enhanced with increasing incubation time (Ren et al., 2017). Li et al. (2016) reported that branch chain length distribution of native corn starch change after treated with BE. They

concluded that BE-treated can increase the proportion of A and B1-short chains and decrease in the proportion of B1-long and B2-long chains.

2.4 Native starch modified by enzyme

From an applications point of view, native starch has several disadvantages, such as low solubility, high viscosity of the starch gel at room temperature, and irreversible retrogradation. In order to improve the functional properties, physical, chemical or enzymatic modifications are applied. All types of modifications, enzymatic modification has the advantages of fewer by-products, increased safety, substrate selectivity, product specificity and environmentally friendly alternative to chemical modification (Le et al., 2009; Oh, Choi, Lee, Kim, & Moon, 2008). There is an increasing demand focus on clean technology; thus enzymatic modification has gained much attention (Sun et al., 2010). In general, enzymatic modification is applied to gelatinized starch rather than granular starch. Because of the tight packing of crystalline zone (amylopectin chains), the granule surface is relatively impenetrable, which can act more easily on gelatinized starch than on granular starch. However, during enzymatic hydrolysis some regions of granules are more attacked than others. Those more susceptible areas are the less organized amorphous rings, whereas the crystalline lamella offers higher resistance to enzymatic erosion. Enzymatic reaction consists of three stages: diffusion towards the surface of solid phase, adsorption, and finally catalytic reaction. There are five patterns of enzymes attack have been identified: pin-holes, sponge-like erosion, numerous medium-sized holes, distinct loci leading to single holes in individual granules, and surface erosion. Generally, enzymes can erode the entire granule surface or sections of it (exo-corrosion) or digest channels

from selected points on the surface towards the center of the granule (endo-corrosion) (Sujka & Jamroz, 2007).

Nowadays, there is growing interest in enzyme that can directly digest native starch. Several types of starch digesting enzyme can digest native starch granules below the gelatinization temperature. Hence, in term of starch digesting enzyme that can digest native starch so we call “Raw starch digesting enzyme, RSDE” (Sun et al., 2010). Direct hydrolysis of starch in native state is desirable because it reduces the costs and save energy associated with the high temperatures required for gelatinization. There are many types of starch digesting enzymes that can be used to modify granular starch structure and to achieve desired properties. However, enzymatic hydrolysis of starch in its raw starch is a slow process and poorly hydrolyzed product (Oates, 1997; Yussof et al., 2013). Nevertheless, in view of energy costs, effective utilization of natural resources, and viscosity problems, the direct modification of starch below the gelatinization temperature is desirable (Uthumporn et al., 2010).

An important property of porous starch is increased swelling and solubility which is also related to microstructure and amylose content of starches. This property is very important in the food industry that use as a kind of sorbent (Sun et al., 2010). There are many types of RSDE that can change the size or morphology of the granular starches. A commercial hydrolyzing enzyme (STARGENTM 001, a blend of α-amylase (AM) and glucoamylase (AMG)) was used to hydrolyze native and cross-linked corn, tapioca and sweet potato starches in the granular state. The results showed that granule erosion occurred mainly on the surface and the pores deepened into the interior part of the granules (Yussof et al., 2013). Native corn starch treated

with AM and AMG at sub-gelatinization temperature can produce porous starch. The microstructure of the starch granules was dependent on the enzymatic treatments, but in both cases porous starch granules were obtained (Dura et al., 2014). The effect of AMG hydrolysis on microstructure as analyzed by scanning electron microscopy (SEM) shows that the pore sizes of hydrolyzed starch granules increased gradually with the degree of hydrolysis (Chen & Zhang, 2012).

Various RSDE modifications of starches have been attempted. The major aim to modify the structure of starches is reduced starch retrogradation and increase the α-1,6 linkages. Branching enzyme can be used to moderate the proportion of chain length and increase the number of α-1,6 linkages. Ren et al. (2017) reported α-1,6 linkages/total linkages ratio of maize starch increase with increasing reaction time. Increasing α-1,6 linkages/total linkages ratio was correlated with a reduction in the peak viscosity, setback value, the average amylopectin chain length. These results are consistent with those of other studies which found that corn starch treated with branching enzyme showed increasing α-1,6 branch point with time-dependent, and decrease the average chain length of amylopectin and the amylose content whereas peak viscosity and setback value decrease with increasing reaction time (Li et al., 2016). Recently, Benavent-Gil & Rosell (2017) compared the action of different enzymes (AMG, AM, cyclodextrin-glycosyltransferase (CGTase) and branching enzyme (BE)) with different concentration to produce porous corn starches. It is known that enzymes have ability to attack and act on native starch. Therefore, the characteristics such as microstructure, glucosidic linkage ratio, chain length distribution, pasting and thermal properties of native starch modified by enzyme at below the gelatinization temperature were reported.

2.5 Characteristics of native starch treated with enzymes

2.5.1 Microstructure

The microstructure of porous starch. Native starch granules, following partial treatment with enzyme below their gelatinization temperature, can generate porous starch. Hence, enzyme modification can change the microstructure of the starch granules which can be correlated to diverse functional properties. Amyloglucosidase (AMG) and α-amylase (AM) have the most practical importance enzymes to change the microstructure of starch granules. The microstructure was analyzed using SEM. The effects of two different enzyme, AM or AMG on granular structure to make porous corn starch was studied by using optimal pH conditions, pH 6 for AM and pH 4 for AMG. Corn starches without enzymatic treatment showed that pH did not affect the morphology of starch granules. Enzymatically treated starches showed changes in the surface, but the shape of the granule hardly changes. The microstructure of the starch granules was dependent on the enzymatic treatment, but in both cases porous starch granules were obtained. AM produced some channels on the starch granules and its action was not dependent on the granule size while AMG more active on the starch granules obtaining hollow granules (Dura et al., 2014). Similarly, Huber & BeMiller (2000) reported that in corn and sorghum starch granules, AMG produced openings to channels for providing access to the granule interior, and the surface pores enlarge through channels from hilum region toward the outsides. The effects of incubation time that related to microstructure of granular starch treated with AMG was also studied. The pore sizes of hydrolyzed starch granules increase gradually with longer incubation time (Chen & Zhang, 2012). This was probably due to AMG absorbed to the susceptible surface zones and degraded the external part of the granule by

exo-corrosion. As the pores become larger, channels of endo-erosion sank into the granules. When endo corrosion occurs, the internal part of the granule was corroded through small pores by which enzymes penetrate the granule. At higher degrees of conversion, the granules appear to be shells with the interior hydrolyzed after the inner pores were crossed.

The microstructure of native starch granules treated with enzymes can be related to the swelling power, solubility index and amylose content. Swelling power slightly increased on AM-treated starch. However, this seems not to be the case for AMG-treated starch. This may be because AM is endo-acting enzyme, that randomly cleaved α-1,4 glucosidic linkages inside the structure and degrade the whole starch structure quickly (Sun et al., 2010). Corn starch treated with AM led to small holes and some parts of granules connected with each other while AMG-treated starch resulted in larger holes; thus, AM-treated starch has higher swelling than AMG-treated starch. Solubility index dramatically increase by AMG, followed by AM. They explained that AMG, being an exo-acting enzyme, hydrolyzes both α-1,4- and α-1,6 glucosidic linkages from non-reducing end. Therefore, more soluble compounds were leached. Amylose content was determined by iodine binding that indicated amylose complex formation. In this case, apparent amylose content was not changed during pH and enzyme modification. That was attributed to during corn starch treated with enzyme at 50°C, the granules did not gelatinize hence a small amount of amylose leached into the supernatant.

Other enzymes used for producing porous starches. Not only endo-(AM) and exoamylases (AMG) can change the microstructure of native starch but also transferases. An effect of CGTase on corn starch at sub-gelatinization temperature

(50°C) at different pH conditions, pH 4 and pH 6 was studied. The microstructure of the granules showed an irregular surface with small pinholes. The changes were observed and the extent of the starch modification. CGTase treatment led to make porous corn starch but having smaller and randomly distributed holes (Dura et al., 2014). The effect of BE on granular structure of maize, potato and low-phosphate potato starch, as analyzed by bright field/polarized light and scanning electron microscopy (Jensen et al., 2013) shows that at high BE activity and low substrate concentration the granular structure of maize and low-phosphate potato starch was more intact whereas potato starch loss its granular structure. This might be due to maize starch and low phosphate starch have a tighter structure; therefore, during the treatment the inter-molecular chains can be transferred. The interlinking increases granular stability. Recently, the action of different enzymes, which were AMG, AM, CGTase and BE with different concentration of each enzyme to produce porous corn starches were compared. AMG changed starch granules with bigger pores whereas CGTase led to the lowest pore sizes. When the levels of AM and CGTase were increased, the pore size trended to increase. However, the pore sizes showed no trend with the level of BE. This effect can be explained by that, when starch granules treated with amylolytic enzymes, the enzymes can penetrate through the holes and the hydrolysis occurs in pattern "inside out". Importantly, different porous starches were obtained by different types of amylolytic enzymes or level of enzyme (Benavent-Gil & Rosell, 2017).

2.5.2 The α-1,6 linkage ratio and side-chain length distribution

The α-1,6 *linkage ratio of treated starch.* The changes of the glucosidic linkage and side-chain length distribution of native starch treated with BE display an

increase in α-1,6 linkages and change in the distribution of linear short chain of α-glucan. The α-1,4 to α-1,6 glucosidic linkage ratio can be determined by ^{1}H NMR. The anomeric protons of the free reducing ends show a chemical shift with different from of the anomeric protons at the α-1,4 and α-1,6 glucosidic linkages (Hernández et al., 2008). The effects of BE incubation time on the ratio of glucosidic linkages of corn starch, as analyzed by ^{1}H-NMR (Li et al., 2016) showed that the chemical shift of the anomeric protons at 5.30 ppm refered to α-1,4 glucosidic linkages, while the chemical shift of the anomeric protons at 4.90 ppm refered to α-1,6 glucosidic linkages (Figure 2.7). The result demonstrated that the α-1,6 glucosidic linkage ratio increased with increasing time incubation. For incubation with BE for 10 h, the glucosidic linkage ratio of corn starch was increased by 64.6% compared to the control. Pre-heating the starch at 65°C for 30 min before incubation with BE can promote enzymatic modification of starch (Ren et al., 2017). This might be because the heating process make starch granules swell and adsorb more water; therefore, enzyme more easily accesses to the starch. At the high concentration of substrate, BE also has ability to increase the degree of branching in maize and potato starch (Jensen et al., 2013). Regarding to the mechanism of BE catalysis, an α-1,4 glucosidic bond is cleaved and then transferred to a glucosidic acceptor with the formation of a new α-1,6 glucosidic bond. Hence, BE has been demonstrated to increase the number of α-1,6 branch.

The side-chain length of treated starch. BE also affects the side chain distribution of starches. Due to the α-1,4–1,6 linkages transfer reaction, the side chains of BE treated starch become shorter, resulting in reduced long chain of starch. The chain length distribution of the oligosaccharide chains was divided based on their

DP as follows: A (DP 3-12), B1-short (DP 13-24), B1-long (DP 25-36) and B2 (DP > 36) chains (Kittisuban, Lee, Suphantharika, & Hamaker, 2014). BE treatment can increase proportion of A and B1-short chains and decrease in proportion of B1-long and B2-long chains of normal corn starch (Li et al., 2016). Waxy corn starch also shows an increase of the proportion of B1-short chains while B1-long and B2-long chains decrease with increasing incubation time after BE modification (Ren et al., 2017).

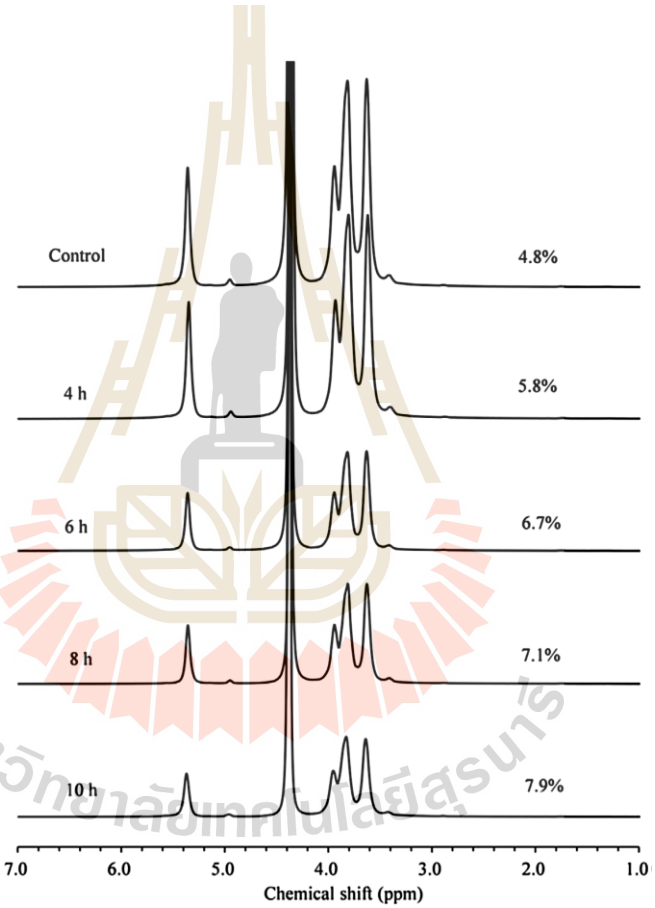

Figure 2.7 ^{1}H NMR analysis of the glucosidic linkage ratio of corn starch treated with BE. Numbers at the left are treatment times; numbers at the right are the percentages of α-1,6 linkages calculated from the peak areas in the NMR spectra (Li et al., 2016).

This result might be due to the fact that the mechanism of BE can act on native starch by cleaving α-1,4 glucosidic bond and then transferring the released chains with forming new α-1,6 glucosidic bonds. The presence of short chains of treated starch also shows this effect on other properties such as pasting and thermal properties.

2.5.3 Pasting properties

The pasting properties of starch is an important benchmark for starch quality. Determination of pasting properties of starches implies the information about gel viscosity, shear resistance and retrogradation behavior. These can be efficiently analyzed using a Brabender amylograph or Rapid Visco Analyzer (RVA) (Dura & Rosell, 2016; Ren et al., 2017). RVA is the mostly used methods which consist of heating/ shearing/ cooling steps. During the measurement, viscosity of starch slurry is measured according by time. The pasting parameters such as pasting parameters, peak viscosity, trough, breakdown, final viscosity, setback, peak temperature and pasting temperature are recorded. At the initial step, the starch granule is heated up to gelatinized temperature in excess water with constant shear rate. The viscosity increases rapidly because of the granules swelling with the increase of temperature. The peak viscosity is defined when the granule is maximum swells before rupturing. With continued stirring and holding temperature, the viscosity decreases as a result of the granules rupture. On the cooling step, amylose and amylopectin chains in gelatinized starch paste partially re-associate to from a gel, that can refer to starch retrogradation.

Native starch treated by enzymes below gelatinization temperature show several changes in pasting properties. Pasting properties of corn starch-treated with AM and AMG were studied. The pasting parameters, the peak viscosity, final viscosity

and setback of AMG treated starch were increased while those values of AM treated starch were decreased compared with the control (Dura et al., 2014). Regarding the enzymes action, AMG treated starch delayed pasting formation and after gelatinization, showed maximum viscosity, higher cooking stability and viscosity after cooling. In contrast, AM treated starch displayed lower maximum viscosity after gelatinized and cooling, that can be related to the molecular size of starch reduction. These effects can be explained by different action mechanism of enzyme can act on native starch that related to different pasting parameter. Subsequently, the effect of CGTase on corn starch at sub-gelatinization temperature (50°C) and at different pH conditions, pH 4 and pH 6 was investigated (Dura & Rosell, 2016). The reaction time did not affect to all pasting parameters except peak time. CGTase treated starch showed a decrease in the peak viscosity. At pH 6 showed lower setback and final viscosity than starch treated at pH 4. Thus, it can be concluded that, starches treated with CGTase at pH 6, showed lower stability than those treated at pH 4. This can be supposed that CGTase at pH 6.0 induced a favour hydrolysis of the amylose chains than at pH 4. CGTase is classified as transferase enzyme that can cleave α-1,4 glucosidic bonds present in the inner part of a starch chains and form a new α-1,4 glucosidic bond and CGTase also has minor hydrolysis activity (Van der Veen, Uitdehaag, Dijkstra, & Dijkhuizen, 2000).

In addition, peak viscosity, final viscosity, breakdown and setback value of starches were abated with BE treatment. Waxy corn starch treated with BE showed the decrease of peak viscosity, final viscosity, breakdown and setback value of starches (Ren et al., 2017). Normal corn starch also showed similar results after BE treatment (Li et al., 2016) and the reduction became more evident with increasing

reaction time. Peak and final viscosity reduced may be attributed to hydrolysis occurring as a side reaction and rearrangement of chains caused by enzymatic modification. Breakdown value represents granule stability. The lower breakdown suggested that starch treated with BE are more densely packed. According to Li et al. (2016), BE-treated led to the reduction of setback value. The setback value of starch treated with BE (after 10 h) was reduced by 45.7% compared with the control. The reduction became more evident with increasing reaction time. The similar results were supported by Ren et al. (2017) in that for 8 h incubated corn starch-treated with BE showed lower setback than that of 4 h. The reduction in setback value can be related to the aggregation of amylose. Hence, it was used to determine the degree of starch short-term retrogradation (Xu et al., 2014).

There is an interesting relationship between pasting properties and the molecular structure of starch. The peak viscosity has a negative correlation with α-1,6 linkages/total linkages ratio whereas it shows a positive correlation with the average chain length. Moreover, the peak viscosity is reduced with increasing α-1,6 linkages/total linkages (Li et al., 2016). This effect is probably due to that the BE-treatment, increasing the number of branch points, weakens the attractive forces within linear segments in the starch granule, making it swell more and easily to rupture. The relationship between setback value and molecular structure indicates that retrogradation occurs more rapidly with fewer α-1,6 glucosidic linkages and long chain length of starch. Hence, corn starch modified with BE increased the α-1,6 linkages/total linkages ratio so it led to a reduction in the degree of chain aggregation. Moreover, BE-treatment also decreased the average chain length of starch, which also had an impact on the decrease of setback values. These results demonstrate that the

retrogradation of corn starch was reduced. On the other hand, corn starch-treated with AMG showed higher setback. This can be related to amylose chain recrystallization. Since AMG is an exo-amylase that can cleave only one glucose residues on the external of amylose or amylopectin from the non-reducing ends, it suggests that a higher amount of amylose chains leached out of the treated granule and they were rapidly able to form helical structures responsible of the gel formation.

2.5.4 Thermal properties

The changes in the thermal properties of starch can be measured using a differential scanning calorimeter (DSC), which measures the energy absorbed as a function of temperature. The thermal parameters involve terms such as the onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), difference between Tc and To, and enthalpy. During heating, To is the temperature which the paste viscosity starts to increase; Tp is the maximum viscosity temperature; and Tc is the final temperature of the viscosity increasing. Gelatinization enthalpy (ΔH) can be related to degree of crystallinity (Thirathumthavorn & Charoenrein, 2006). Some researchers relate the retrogradation among the thermal properties of starch (Li et al., 2016; Ren et al., 2017). The higher gelatinization temperature is required to initiate gelatinization of the starch. That might be due to a delay in the start of gelatinization. Normal corn starch modified by AMG and AM showed delaying the gelatinization process, that started at higher To (Dura et al., 2014). On the other hand, gelatinization temperature of waxy corn starch treated with BE was lower than that non-treated starch (control) (Ren et al., 2017). That might be related to the increased short chains after BE treatment. It can be suggested short chains from weak crystalline network. Hence, gelatinization temperature of BE treated starch is reduced. ΔH of starch treated

with AMG and BE were lower than those of the control. The lower ΔH indicated that hydrolyzed starch required less thermal energy for gelatinization. ΔH is mainly due to the disruption of the double helices rather than the long range disruption of crystalline structure (Cooke & Gidley, 1992). This decreasing trend is probably due to the crystalline structure of treated native starch is decreased. Crystalline structure of starch is mainly caused by amylopectin interactions. So, AMG and BE treatments have affected the double helical conformation of the granule, and likely acting on the amylopectin side chains. On the other hand, ΔH of starch treated with AM was higher than control. This result is probably due to AM acting mainly on the amorphous regions leading to a more ratio of crystalline structure, so it requires higher gelatinization enthalpy. Ren et al. (2017) also found the relationship between structure and ΔH, in which negatively correlated with the α-1,6 linkages/total linkages ratio and it positively correlated with the average chain length. Hence, starches with small amount of branch points and longer chain length have higher gelatinization enthalpy. Moreover, ΔH can be related to the retrogradation. The retrogradation percentage of gelatinized starch after treating with BE then stored for 21 days were decreased. This is probably due to the higher degree of branching and the higher proportion of shorter chains causing that starches are hardly re-associated to form double helices.

2.6 Ethanol treated starch and its properties

Native starch is limited industrial usage due to its exhibit insolubility at room temperature, low gel strength, irreversible retrogradation, etc. Therefore, starch modification is required. Starch modification included physical, chemical and biological methods. Compared with chemical method, physical modification is

promising and attractive method due to the absence of chemical and derivative products, providing no effect on the environment (BeMiller & Huber, 2015).

In order to increase the swelling and solubility of granular starch at low temperature, the simple modified method is heated starch slurries in aqueous ethanol, heated ethanol-treated starch (ETS), commonly called cold-water-swelling starch, CWSS (Jane, Craig, Seib, & Hoseney, 1986; Sarifudin, Soontaranon, Rugmai, & Tongta, 2019; Zhang, Dhital, Haque, & Gidley, 2012). The ETS treatment is considered to be the physical modification since the ethanol can be evaporated from the product after modification (BeMiller & Huber, 2015). The starch products are well called as instant starch due to their promptly adsorb cold water and swell, making the texture like cooked starch.

Many studies have been proposed a mechanism of ethanol to elucidate the structure transformation of ETS during conversion process (Chen & Jane, 1994; Dries, Gomand, Goderis, & Delcour, 2014; Jane et al., 1986; Sarifudin et al., 2019; Zhang et al., 2012). In common, during the process to prepare ETS, starch slurry is heated in the presence of aqueous ethanol to inhibit the swelling of granules, the crystalline structure of native starch melts resulting in the formation of less crystalline structure with granular integrity. The formation of ethanol and amylose and/or amylopectin complex located within single helix cavities and interstices possibly (Zhang et al., 2012).

The study of structural changes of ETS during conversion was first carried out by Jane et al. (1986). A model of ETS conversion was proposed based on the change of A-type starch to V-type structure (Figure 2.8). The model showed that the double helix of amylopectin chains was transformed to single helix structure. They suggested

that ETS had a metastable structure, which was allowed water to absorb easily. A recent study by Sarifudin et al. (2019) reported that during ethanol-treated starch heating, the crystalline structure was realigned into more perfect crystalline before disruption. The loss of birefringence was observed even though granular shape was preserved.

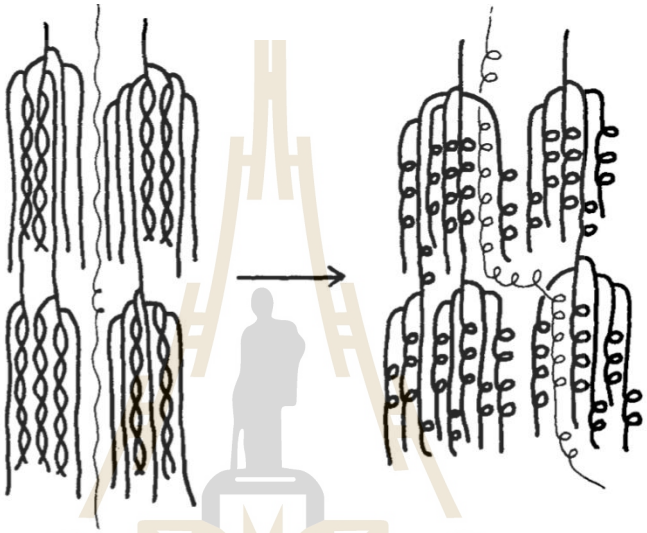

Figure 2.8 The model of an A-type structure converted to V-type structure after conversion by ethanol; the thick and thin lines represent amylopectin and amylose, respectively (Jane et al., 1986).

The morphology of ETS displays totally different compared to native starch granules. As visualized by scanning electron microscopy (SEM), the granules of ETS exhibited broaden, deform and loss of integrity with no changes in shape (Hedayati, Shahidi, Koocheki, Farahnaky, & Majzoobi, 2016). The granular surface changes were observed with wrinkling, indentation and agglomeration features (Zhang et al., 2012). As heating (95°C) maize starch with an aqueous ethanol concentration of 48%, the birefringence still appeared under polarized light microscope (Dries et al., 2014). On

the other hand, Choi, Baik, & Kim, (2017) reported that potato starch did not show the birefringence after modification by 60% ethanol combined with NaOH. Recently, Sarifudin et al. (2019) demonstrated that potato and maize starches were converted to ETS, and their morphologies were transformed. The feature of Maltese crosses of native starches were slightly distorted at 80°C and mostly Maltese crosses did not appeared at 90°C.

The complexation of ethanol and amylose is changed the crystalline polymorph which converted A- and B-type to V-type (Dries et al., 2014; Jane et al., 1986; Sarifudin et al., 2019). It has been proposed that amylose is hold amylopectin during ethanol-heating process, preventing granular integrity (Jane et al., 1986). Thus, the less amount of amylose would make starch granules weaken and tend to disperse during a high temperature processing (Debet & Gidley, 2007). The observation of the crystal structure changes is consistent with enthalpic transitions as measured by DSC. Zhang et al. (2012) reported that the gelatinization enthalpy of ETS from normal maize, waxy maize, potato and tapioca starches was decreased at higher water concentration. The reduction of gelatinization enthalpy implies to the disruption of crystalline part in starch granules, which mainly relates to amylopectin clusters (Miao, Zhang, & Jiang, 2009).

Swelling capacity and the solubility of ETS are prominent properties as compare with native starch. The swelling capacity implies to the ability of starch granules to absorb and hold water with its granule whereas the solubility indicates the amount of solid compounds released from the starch (Dura et al., 2014). The swelling capacity and solubility dramatically increased with ETS. Choi, Baik, & Kim, (2017)

reported that potato starch treated by ethanol exhibited higher swelling and solubility than those of native potato starch.

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CHAPTER III

POROUS HIGH AMYLOSE RICE STARCH MODIFIED BY AMYLOGLUCOSIDASE AND MALTOGENIC α-AMYLASE

3.1 Abstract

Porous starch is attractive by providing high surface area for many applications. In this study amyloglucosidase (AMG) and maltogenic α-amylase (MA) were investigated in direct comparison to elucidate potential effects in producing porous starch using high amylose rice starch as a substrate. Both enzymes generated pores at the surface as illustrated by Scanning Electron Microscopy (SEM). The enzyme-treated granules had higher relative crystallinity as deduced from Wide Angle X-ray Scattering (WAXS). MA treatment increased the number of short amylopectin chains and decreased the molecular weight with extended incubation time. The MA-treated starch had higher solubility whereas swelling capacity, amylose content, peak viscosity, final viscosity, breakdown and setback of both treatments were decreased compared to the control. Enzymatic treatments produced starch with delayed gelatinization temperature and increased the enthalpy. The results demonstrate that porous rice starch can provide different functionalities depending on the enzyme mechanisms, extending the range of applications.

Keywords: rice starch, porous starch, maltogenic α-amylase, amyloglucosidase

3.2 Introduction

Rice starch is a major cereal starch with very diverse functional properties depending on the rice genotypes. Rice starch is considered non-allergen, has small granules which white color and can be found in a wide range of amylose/amylopectin ratios. Typical starch is made up of two distinct polymers of glucose: amylose and amylopectin. Amylose that builds up of 10-40% in typical storage starch is a mainly linear chains of glucose linked by α-1,4 glucosidic bond. Amylopectin consists of an α-1,4 glucosidic backbone and α-1,6 glucosidic linkages forming branch points (Buléon, Colonna, Planchot, & Ball, 1998; Pérez & Bertoft, 2010). The relatively high amylose in starch increases the gelatinization temperature and starch retrogradation in many cases negatively affects the quality of the starch product. In order to improve the functional properties of native starch, physical, chemical or enzymatic modifications are therefore applied. However, chemical modification is associated with environmental pollution and consumer concerns. Enzymatic modification has the advantages of reducing by-products, increasing safety, improving substrate selectivity and product specificity and therefore provides a more environmentally friendly alternative to chemical modification (Le et al., 2009; Oh, Choi, Lee, Kim, & Moon, 2008).

There is a current growing interest to produce porous starches due to their high specific surface area facilitating the access of enzymes and chemical reagents into the starch granules for further modification. Porous starches have applications in various foods as absorbents or carrier agents for volatile compounds, flavorings and protecting the sensitive elements such as vitamin, oil and probiotic (Belingheri, Giussani, Rodriguez-Estrada, Ferrillo, & Vittadini, 2015; Benavent-Gil, Rodrigo, & Rosell, 2018; Li, Ho, Turner, & Dhital, 2016; Xu et al., 2017). Enzyme-assisted modification

has been used as an efficient method for obtaining porous starches. For example, a commercial hydrolyzing enzyme (STARGEN^{TM}001, a blend of α-amylase (AM) and amyloglucosidase (AMG)) has been used to hydrolyze native and cross-linked corn, tapioca and sweet potato starches in their granular state (Yussof, Utra, & Alias, 2013). The results showed that granule erosion occurred mainly on the surface but pores were found in the interior part of the granules. Native corn starch treated with AM and AMG at sub-gelatinization temperature produced porous starch with microstructure that was dependent on the enzymatic treatment (Dura, Błaszczak, & Rosell, 2014). Moreover, the effect of AMG hydrolysis on granular morphology as evaluated by SEM showed that the pore size radius of hydrolysed starch granules gradually increased with higher degree of hydrolysis (Chen & Zhang, 2012).

In this study, AMG and maltogenic α-amylase (MA, glucan 1,4-α- maltohydrolase, EC 3.2.1.133) were investigated to produce porous rice starch. It has been identified both AMG and MA having exo-activities (Van Der Maarel, Van Der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). AMG obligatory and specifically hydrolyzes α-1,4 glucosidic bonds from the non-reducing ends of starch, resulting in the production of glucose. AMG is also capable of hydrolyzing α-1,6 linkages, however at a 80-fold lower rate (Dura et al., 2014; Mertens & Skory, 2007). Catalytically MA is versatile enzyme that in addition to having mainly exo-α-1,4-glucanase activity releasing maltose, also exhibit endo-glucanase activity on accessible intramolecular bonds (Christophersen, Otzen, Norman, Christensen, & Schäfer, 1998; Goesaert, Slade, Levine, & Delcour, 2009; Le et al., 2009). Moreover, transglycosylation activity of MA to form various glucosidic linkages such as α,1-3 and α,1-6 linkages from acarbose and gelatinized starch has been reported (Cha et al., 1998; Lee et al., 2008;

Lee et al., 2002; Park et al., 2000). MA is used mainly as an anti-staling agent by shortening the outermost chains of amylopectin inhibiting retrogradation of amylopectin as shown for waxy maize starch (Grewal et al., 2015), rice starch and rice meal (Li, Li, Tian, & Park, 2014). The use of MA has not been reported for obtaining porous starch. However, MA has been tested in gelatinized systems aiming at modifying starch to reduce amylolytic digestion by increasing branched side chains (Ao et al., 2007; Miao et al., 2014). Moreover, the combination of MA and α-glucanotransferases such as branching enzyme (BE) have received considerable attention to produce highly branched starch (Le et al., 2009; Lee et al., 2008) and attenuated digestion of maize extruded flours (Martínez, Pico, & Gómez, 2016). It is hypothesized that the different catalytic actions of AMG and MA can provide different structural and physicochemical properties of granular rice starch. The aim of this study was to investigate the the different hydrolytic mechanisms of AMG and MA to produce porous starch using high amylose rice starch as a substrate. The morphological, structural and physicochemical properties were characterized.

3.3 Materials and methods

3.3.1 Materials

Rice starch was a gift from General Food Products Co., Ltd. (Nakhon Ratchasima, Thailand). Amyloglucosidase (EC 3.2.1.3, specific activity 300 U/mL) from *Aspergillus niger* was purchased from Sigma-Aldrich (Steinheim, Germany). Maltogenic α-amylase (EC 3.2.1.133, Maltogenase® L) was kindly provided by Novozymes (Bagsvaerd, Denmark).

3.3.2 Preparation of porous starch

Rice starch (10 g dry basis, db) was suspended in 20 mL of 20 mM Na maleate and 5 mM $CaCl_2$ buffer at pH 5.5 (MA treatment) or 20 mM Na acetate buffer at pH 4.5 (AMG treatment). The starch suspension was heated in water bath at 60°C for 0.5 h. Then, AMG or MA (100 U/g starch) was added to the starch suspension. The samples were incubated at 60°C for 3 6 12 and 24 h in a shaking water bath (180 rpm). DI water (40 mL) was added to the suspensions, the starch granules collected on a filter followed by 3 times washing with DI water. The samples were pre-dried at 60°C for 20 min before drying at 130°C for 2 h. The dried starch samples were ground and passed through a 100-mesh sieve then stored in a desiccator for further analysis. The starch control samples were prepared with the same procedure without enzyme addition for all treatments.

3.3.3 Scanning electron microscopy (SEM)

Granular morphology and topography were observed using Field Emission Scanning Electron Microscope (FE-SEM, Carl Zeiss, Oberkochen, Germany). Samples (1%) was suspended in absolute ethanol. One drop of each sample was placed on a coverslip and dried at 50°C for 3h. A thin layer of each sample was placed on aluminum stubs with conductive carbon tape and sputter-coated with gold-palladium. The accelerating voltage was 3.0 kV.

3.3.4 Specific surface area and total pore volume using nitrogen sorption isotherms through Brumauer-Emmett–Teller (BET) protocol

Firstly, starch samples were dried at 100°C for 6 h. Then, dried starch samples (200 mg) were degassed at 125°C for 24 h and immersed in liquid nitrogen (-196°C). The measurement was carried out by dispensing a specific portion of the

nitrogen gas (2 L) and measuring the relative pressure p/po. The pressure change was recorded by BELSORP-mini II. Specific surface area and total pore volume were determined. The monolayer value was calculated using the BET equation (Brunauer, Emmett, & Teller, 1938).

3.3.5 Relative crystallinity

The relative crystallinity of starch samples was quantified by wide-angle X-ray scattering (WAXS) at beamline 1.3, Synchrotron light research institute, Nakhon Ratchasima, Thailand, described by Boonna & Tongta (2018).

3.3.6 Determination of amylose content

The amount of apparent amylose was determined by triiodide colorimetry as described by Wickramasinghe, Blennow, & Noda (2009).

3.3.7 High performance anion exchange chromatography (HPAEC)

The samples (5 mg/mL) were gelatinized by boiling and debranched by isoamylase (2 μL, 260 U/mg, Megazyme) and pullulanase (2 μL, 40 U/mg, Megazyme) at 40°C. Samples of 10 μL were injected into a CarboPac PA-200 column on the HPAEC-PAD system, (Dionex, Sunnyvale, CA, USA) at a flow rate of 0.5 mL/min. Chain fragments were eluted from the column using the following profiles: 0-2 min: 150 mM NaOH isocratic, 5 min: 150 mM NaOH isocratic, 110 mM NaOAc linear gradient, 130 min: 150 mM NaOH isocratic, 400 mM NaOAc convex gradient. Single peaks were integrated and corrected for the detector response (Viksø-Nielsen, Blennow, Nielsen, & Møller, 1998).

3.3.8 Molecular weight of fraction analysis by size-exclusion chromatography with triple detection array (SEC-TDA)

Molecular composition was determined by size exclusion chromatography (SEC) using a Viscotek System (Malvern, UK) equipped with a GS-520HQ column (Shodex) attached to a TDA302 module (Triple detector array) consisting of a refractive index detector (RI), a four-bridge viscometer detector (VIS) and a light scattering detector (LS). The LS consisted of a right-angle light scattering (RALS) and a low angle light scattering (LALS).

Samples were first prepared in the form of non-granular starch as described by Klucinec & Thompson (1998). Non-granular starch (5 mg) was dissolved in 25 μL of 2M KOH at 4°C for 24 h, and re-suspended in 975 μL Milli-Q water mechanically shaken (1200 rpm) at 80°C for 5 h to a final concentration of 1 mg/mL. The calibration was made using pullulan (50 kDa, polydispersion 1.07, Showa Denko) as standard and samples components were eluted in ammonium formate (10 mM) with a flow rate of 0.5 mL/min. The injection volume was 50 μL and the column temperature was thermostated at 60°C. Data analysis was performed using the OmniSec Software 4.7 (Malvern Instrument, Ltd.).

3.3.9 Swelling capacity and solubility of starch

The swelling capacity (SC) and solubility (S) were determined by the method described by Rosell, Yokoyama, & Shoemaker (2011) with a slight modification. Briefly, 100 mg db of the starch was weighed in centrifuge tubes followed by addition of 10 mL of MilliQ water. The samples were heated at 60°C for 30 min under continuous mixing. Then the dispersion was centrifuged at 4000g for 15 min. The supernatant was transferred to Petri dishes then dried at 110°C overnight and

weighed. The residue obtained after drying of the supernatant represented the amount of starch solubilized in water. The swelling capacity and solubility were calculated as follows:

$$SC\ (g/g) = \text{sediment weight} / [\text{weight of dried starch} \times (1 - S/100)]$$

$$S\ (\%) = (\text{dried supernatant weight} / \text{weight of dried starch}) \times 100$$

3.3.10 Pasting properties

The pasting properties were monitored using Rapid Visco Analyser (RVA) (Newport Scientific, Warriewood, Australia). MilliQ water (25 mL) was added to 2.5 g of starch sample (14% moisture content) in the aluminum RVA canister. The RVA settings were as follows: heating from 50 to 95°C for 282 s, holding at 95°C for 150 s and cooling to 50°C over 300 s. The initial mixing paddle speed was 960 rpm for 10 s followed by160 rpm speed during analysis. Pasting parameters were recorded using Thermocline software (Perten Instruments, Hägersten, Sweden) for Windows.

3.3.11 Thermal properties

Thermal behavior of the samples was investigated using differential scanning calorimeter DSC 2500 (TA instrument, New Castle, USA), equipped with a thermal analysis data program (Trios, New Castle, USA). Approximately 10 mg of the sample was loaded into the aluminum pan and MilliQ water was added to obtain a water-sample ratio of 3:1. The sample pans were hermetically sealed and equilibrated at room temperature for 24 h before analysis. Thermal analysis performed by heating from 25 to 130°C at a rate of 5°C min^{-1}. Initial temperature (Ti) was defined as the temperature at which the sample started to gelatinize and onset temperature (To), peak

temperature (Tp), conclusion temperature (Tc), gelatinization temperature range (Tp-Ti) and enthalpy (ΔH) were determined.

3.4 Results and Discussion

3.4.1 Granular morphology

The enzymatic modification of rice starch was carried out separately with amyloglucosidase (AMG) or maltogenic α-amylase (MA). To rule out effects of incubation only, the enzyme-action was compared with their controls for each time of incubation, which was performed in the absence of enzyme. SEM micrographs showed that native high amylose rice starch granules displayed polygonal shape and smooth surface with some small holes as evident at high magnification (Figure 3.1A). Enzymatic treatment had a clear effect on the granular surface, as seen by several dents and holes. However, the enzyme had no effect on the general shape of the granules. Specifically, AMG-treated starch displayed big and shallow pores whereas MA-treated starch showed small pores and they seem to be deeper than those of the AMG treatment. The range of pore diameters hydrolysed by AMG was more diverse and wider compared to MA (Figure 3.2). As the reaction time increased, the average pore size became wider by AMG and some granules were fragmented after being 24 h modification (Figure 3.1, B8) that caused the average pore size dropped. Previous studies have demonstrated that corn starch treated with AMG had larger pores and broader pore size distribution compared with α-amylase, showing small pores on the surface (Benavent-Gil & Rosell, 2017).

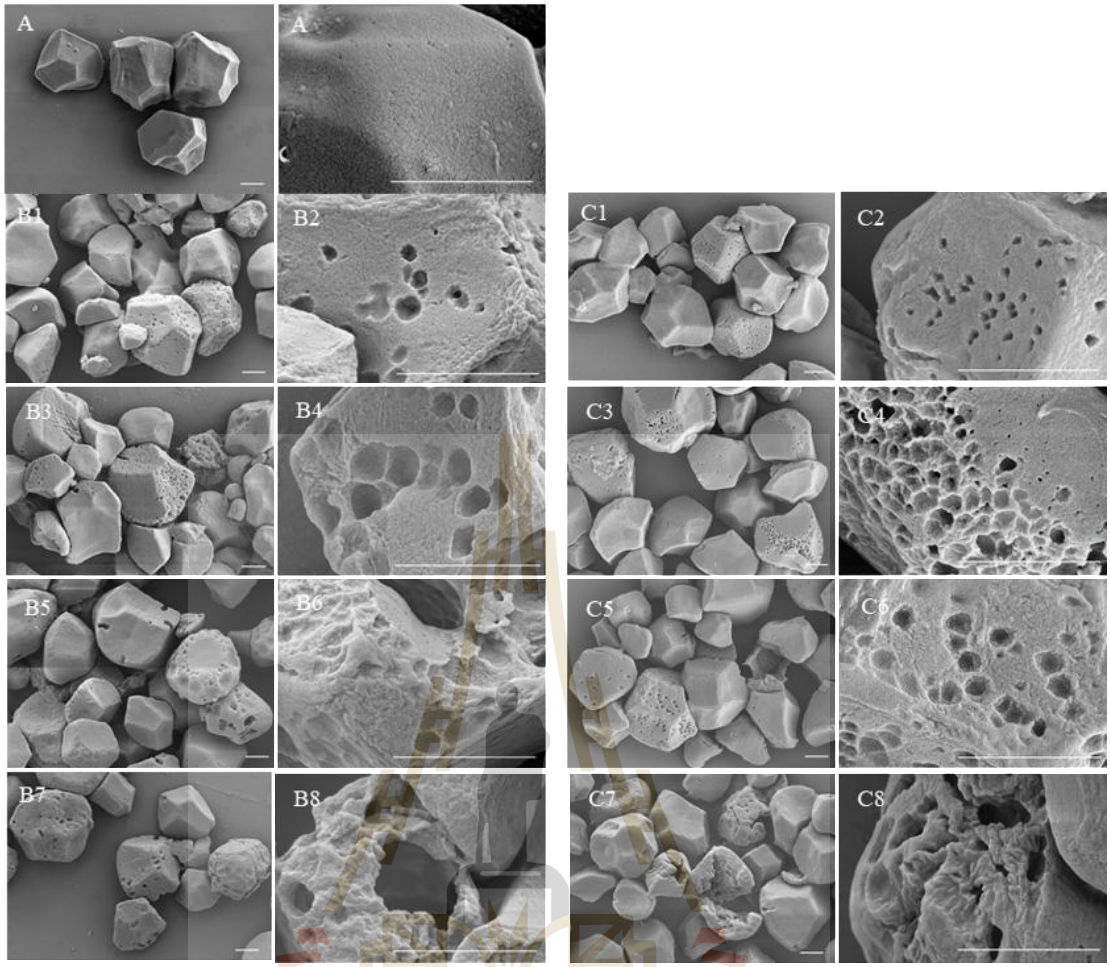

Figure 3.1 SEM images of native rice starch (A) and enzymatically treated starch (B; AMG and C; MA) for 3h (1 and 2), 6h (3 and 4), 12h (5 and 6) and 24h (7 and 8). Scale bar = 2 μm.

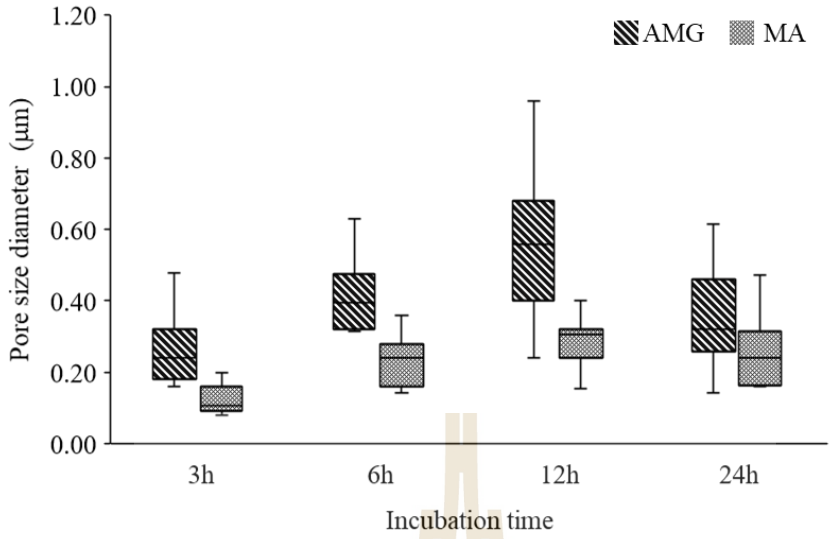

Figure 3.2 Pore size analysis of AMG- and MA-treated starches as analyzed from SEM images.

The specific surface area, $S_{BET,}$ data confirmed that the specific surface areas increased after enzymatic modifications (Figure 3.3), especially for AMG. The highest S_{BET} of AMG and MA treatment was found after 12h and 24h modification, respectively. This result could be attributed to AMG and MA absorption on susceptible surface zones and degraded the external part of the granule by exo-corrosion. As the pore size becomes sufficiently wide by exo-erosion activity, endo-erosion can commence, potentially starting at narrow pore structures at the granule surface, progressing towards to the center of the granule (Sujka & Jamroz, 2007). When endo corrosion occurs, the internal part of the granule was corroded through small pores by which enzymes penetrate the granule. Alternatively, it could be due to that MA is also an endo-acting hydrolase enzyme, and hydrolyses α-glucan chains efficiently in the interior of the starch granule. Therefore, the pores of MA-treated starch seem to be narrower but deeper than those of AMG.

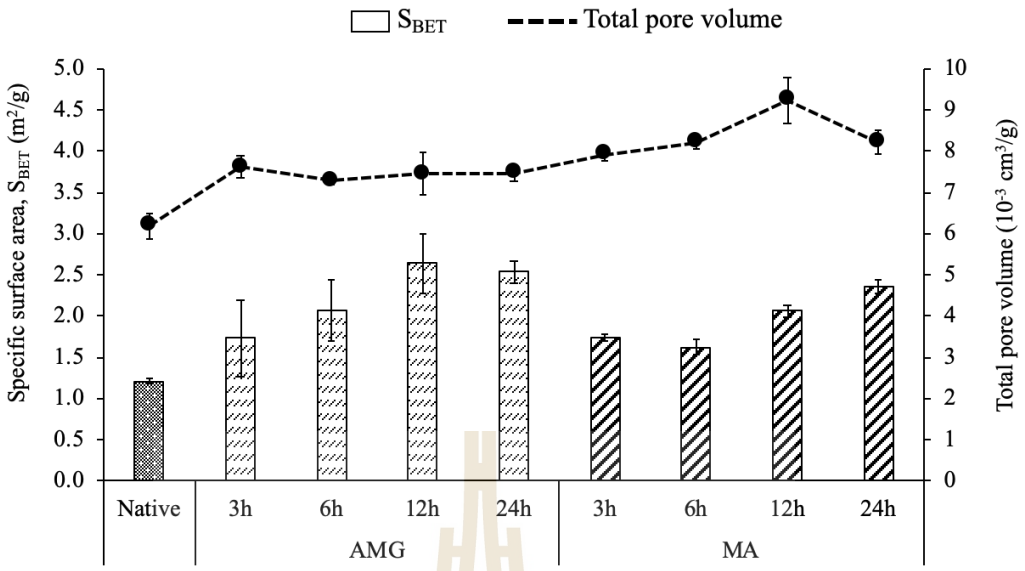

Figure 3.3 Specific surface area (S_{BET}) and total pore volume of native and enzyme-treated starches at 3 6 12 and 24h.

3.4.2 Crystalline structure

To further investigate the effects of enzymatic treatment on the crystalline and amorphous regions, the crystallinity was monitored by wide-angle X-ray scattering. All samples displayed an A-type pattern which strong reflection at 2θ of 15, 17, 18 and 23 (data not shown). The relative crystallinity of the control is not different from that of native starch except some of the MA control (Table 3.1). It should be noted that the control sample for MA, i.e. incubation of the starch at pH 5.5 without MA, resulted in molecular re-arrangement leading to an increase in the relative crystallinity. This increase suggests that annealing took place in a pH dependent manner as suggested (Dura & Rosell, 2016). The relative crystallinity of all enzymatic treatments increased when compared to their buffer controls and native starch, which is consistent with previous report by Zhao et al. (2018). The increase in relative crystallinity of the enzyme treated samples could be an effect of hydrolytic activity mainly in amorphous regions (Dura et al., 2014; Zhao et al.,

2018), thereby increasing the proportion of crystalline structure. The relative crystallinity of AMG treatment steadily increased as treatment time was longer; on the other hand, at the initial time of MA hydrolysis (3 h) the crystallinity was increased to 28.09%. The highest crystallinity was found for the AMG and MA-treated starches, 29.02% and 33.29% after incubation for 24 h and 12 h, respectively. This result implies that AMG gradually hydrolyzes in the amorphous region while MA can hydrolyze faster and MA disrupts the crystalline region when the reaction time longer, thereby at 24h the crystallinity decreased to 27.82%.

3.4.3 Apparent amylose content

The amylose content was determined using iodine complexation, in which its result is summarised in Table 3.1. Native rice starch contained 30.65% apparent amylose that is classified in high amylose rice starch (Juliano, 1992). Although the effect of enzymes on the amylose content was minor both AMG and MA significantly decreased the amylose content compared to their control samples. As treatment time increased, the amylose content steadily decreased by AMG whereas the effects of MA on amylose reduction was obvious at 3h of time incubation as 3.3% reduction. The maximum decrease in amylose content was found for AMG and MA treatments after incubation for 24h and 12h as 5.4% and 5.2% reduction in amylose compared to control, respectively. It has been suggested that the rate limiting step for exo-acting enzymes on starch granules is the accessibility of non-reducing ends (Zhang, Dhital, & Gidley, 2013). Both AMG and MA are exo-acting, while the endo-action of MA would in addition cleave the long chain of rice starch (Grewal et al., 2015), thereby MA treatment might cause the reduction of amylose faster. This result is in agreement with the crystallinity data that increased obviously by MA treatment

Table 3.1 Relative crystallinity, amylose content, swelling capacity and solubility of native, control and enzyme-treated starches.

Samples	Relative crystallinity (%)	Amylose content (%)	Swelling capacity (g/g)	Solubility (%)
Native	23.77 a	30.65 ij	2.89 bcd	0.40 b
AMG-3 control	23.69 a	30.92 k	3.26 ef	1.18 d
AMG-3	25.57 cd	30.65 ij	2.80 bc	0.40 b
AMG-6 control	23.16 a	30.17 h	3.14 de	1.08 d
AMG-6	25.80 cd	29.56 f	2.64 ab	0.39 b
AMG-12 control	24.27 abc	29.46 ef	3.49 fg	1.17 d
AMG-12	28.82 e	28.26 a	3.01 cde	0.12 a
AMG-24 control	22.72 a	30.57 i	3.83 h	1.86 g
AMG-24	29.02 e	28.94 b	2.87 bcd	0.10 a
MA-3 control	26.04 d	30.97 k	3.01 cde	0.46 b
MA-3	28.09 e	29.94 g	2.74 abc	1.45 ef
MA-6 control	25.69 cd	30.43 i	2.93 bcd	0.80 c
MA-6	27.73 e	29.16 cd	2.66 ab	1.67 fg
MA-12 control	25.35 bcd	30.60 i	3.11 de	1.05 d
MA-12	33.29 f	29.00 bc	2.48 a	1.82 g
MA-24 control	23.94 ab	30.83 jk	3.55 g	1.29 de
MA-24	27.82 e	29.30 de	2.76 abc	2.56 h

Values followed by different letters within a column are significantly different ($P < 0.05$)

3.4.4 Chain-length distribution

As expected, the changes in the amylopectin side chain distribution after enzymatic treatments was a minor due to the complexity of starch granular structure that resists to enzyme attack (Božić, Lončar, Slavić, & Vujčić, 2017). However, a clear trend in the chain length distribution was observed (Figure 3.4). At 3h of incubation, the short amylopectin chains with a degree of polymerization (DP) of 6-12 were decreased whereas DP 13-22 were increased for AMG treatment (Figure 3.4, A1). When the incubation time was longer, B-long chains (DP21-30) were hydrolyzed, causing an increase in short so called B-chains (DP13-20) (Figure 3.4, A2). The reduction of short amylopectin chains could be due to that the exo-action of AMG mainly attacks the non-reducing ends of short chains mostly from the external layer of starch granules (Bertoft, 2017; Pérez, Baldwin, & Gallant, 2009). On the contrary, MA treatment increased the proportion of DP2-16 and decreased the proportion of DP17-40 (Figure 3.4, B1). This phenomenon was more obvious with longer incubation time (24h). Miao et al. (2014) proved that maltogenic α-amylase hydrolyzed gelatinized starch with increase the amount of short amylopectin chains. This result is likely attributed to the endo-action of MA that acts mainly on long chains, producing short chains (Grewal et al., 2015).

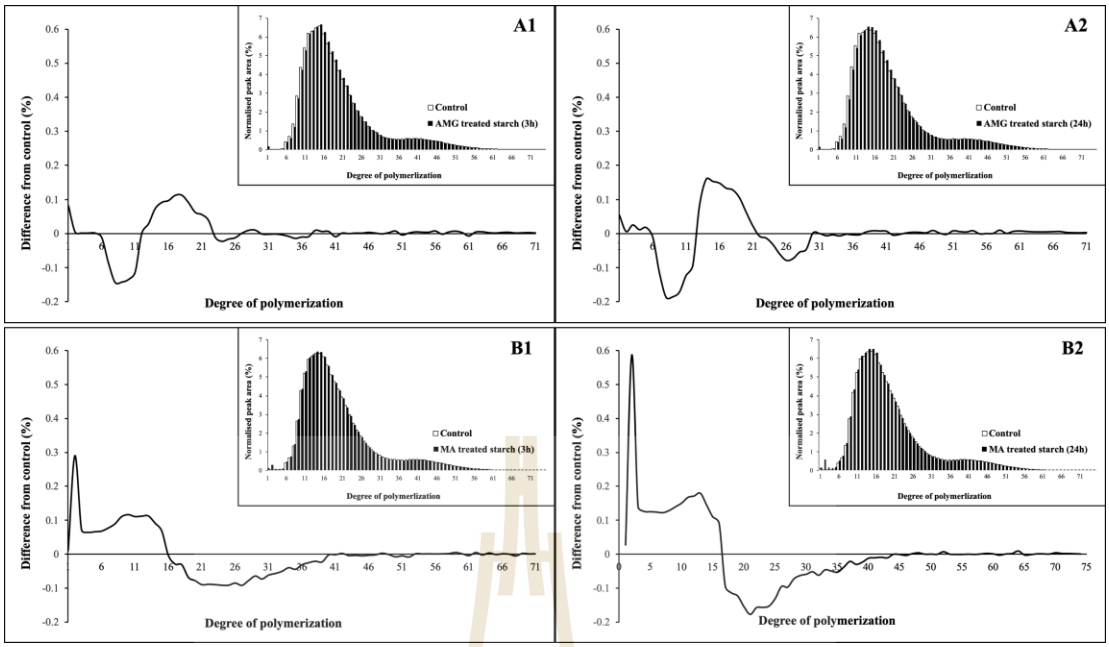

Figure 3.4 Chain length distribution analysis (bar graph) and difference plots (line graph) of enzyme-treated starch in relative with the control. (A1 and A2) AMG-treated starch, (B1 and B2) MA-treated starch after 3h and 24h modification, respectively.

3.4.5 Starch molecular weight fraction

The molecular weight of native, control and the enzymatically treated starch samples was analyzed by SEC. Two starch fractions, I and II, which refer to high and low molecular weight, respectively, were generally detected for all samples (Figure 3.5). For the untreated samples, these corresponded to amylopectin and amylose, respectively. The result showed molecular weight of fraction I and II of native rice starch were 5.8 x 10^7 and 2.4 x 10^7, respectively. After modification with AMG for 6h, fraction I and II decreased moderately. While MA treatment caused a more significantly decreased in fraction I and II as incubation time extended, decreasing from 5.75 x 10^7 to 0.98 x 10^7 and from 2.36 x 10^7 to 0.65 x 10^7 Da,

respectively after incubation for 24 h. The reduction of molecular weight of MA treated starch has reported by several authors (Ao et al., 2007; Christophersen et al., 1998; Grewal et al., 2015; Miao et al., 2014). Interestingly, at 6 h there was a break point in which the molecular weight fractions of MA-treated starch were significantly higher than AMG treated starch but after 24h the MA-treated starch decreased significantly in molecular weight fractions as compared to AMG-treated starch. This could be explained by the AMG has a slower catalytic rate towards α-1,6 glucosidic bonds as compared to the faster hydrolysis of α-1,4 bonds (Mertens & Skory, 2007). On the other hand, the endo acting MA can continue hydrolysis that caused dramatically decreased in the molecular weight of starch sample.

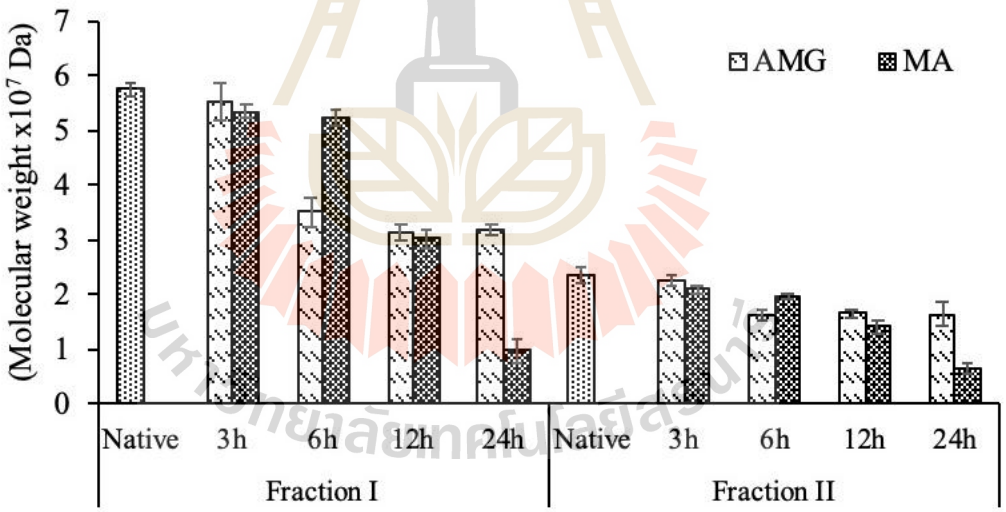

Figure 3.5 Molecular weight of fractions of native, control and enzyme-treated starches.

3.4.6 Swelling capacity and solubility index

The swelling capacity and solubility of the starch samples (Table 3.1) demonstrates that the swelling capacity of the enzyme-treated starches was significantly lower than their corresponding buffer controls, showing that less amount of water was absorbed in the enzyme-treated starch. The decreased swelling capacity of the hydrolyzed starch samples was supposedly directly attributed to the formation of porous structure after enzymatic modification as confirmed by SEM, which weakened the general granular structure of the starch. Alternatively, more hydrophobic surfaces were created at the internal walls of the pinholes thereby restricting binding of water (Dura & Rosell, 2016). Another explanation can be that the decrease of the swelling capacity of the enzyme-treated starch might be an effect of internal molecular rearrangement in the starch granules forming more ordered double helical segments of the amylopectin side-chains (Zavareze & Dias, 2011).

The solubility index was significantly decreased by AMG treatment (Table 3.1). This was in contrast with the increase found for the MA treated starch granules. This phenomenon was more pronounced with longer incubation time. The solubility index indicates the amount of solid compounds released from the starch (Dura et al., 2014). A decrease in solubility of the AMG-treated starch could be explained that AMG attacking amylopectin and releasing glucose occurs at the exterior parts of the molecules. Such trimming can induce the restructuring of starch chains that might be caused more complex structure which decreased the compounds releasing from starch granules (Xie, Li, Chen, & Zhang, 2019). For MA, enhanced solubility can likely be an effect of its endo-activity producing small starch fragments from the starch granules which adheres to the starch granules. These results were

consistent with chain length distribution results that showed that the short chains were increased by MA treatment whereas they were decreased by AMG treatment. Moreover, it was observed that the swelling capacity and solubility index of all the control samples were higher than native starch. This may be explained by the fact that during incubation, water penetrates into the granules, disrupting amorphous region and allowing the granules to swell freely (Zhao et al., 2018) and releasing of soluble compounds from the granule.

3.4.7 Pasting properties

Pasting properties provide information about gel formation dynamics during shear, shear resistance and retrogradation behavior. The pasting parameters of native, control and enzymatically treated starches (Table 3.2) shows that the enzymatic treatments affected the pasting properties of starch by delaying the pasting formation. At all incubation times, pasting temperature was significantly increased by AMG while MA caused the higher pasting temperature during incubation for 12 h and 24 h. These data agree with the swelling capacity since starch swelling is caused by water absorption before gelatinization process (Dura et al., 2014), resulting in that AMG- and MA-treated starches both had lower swelling capacity as compared to their control samples. Enzymatic treatments decreased the peak viscosity, breakdown, final viscosity and setback value compared to their controls. Enzymatic treatments decreased the peak viscosity, breakdown, final viscosity and setback value compared to their controls, which agree with previous report (Dura et al., 2014). Peak viscosity of AMG-treated starch decreased by up to 15% whereas that of MA-treated starch decreased by 31% after 24 h of incubation. The reduction in peak viscosity of the enzyme-treated starches is possibly a direct effect of disintegration of the granular

surface and fragmentation of granules at longer treatment times. The breakdown parameter represents granule stability, indicating the disrupted granules better resisted the shear force during heating (Karim et al., 2008). After being modified by AMG and MA for 24 h, the breakdown value decreased by 27% and 51%, respectively. The reduction could be attributed to the weakened structure after enzymatic treatments. Importantly, the enzyme treatments significantly decreased setback value compared to their control. This effect was pronounced with extended incubation time, showing 50% and 40% decrease after 24 h modification with AMG and MA, respectively, suggesting that low amount of amylose chains disabled the capacity for gel formation (Dura & Rosell, 2016).

3.4.8 Thermal properties

The thermal properties as deduced by DSC (Table 3.3, DSC curve indicated in Supplementary data Figure S1) demonstrated that the initial temperature (Ti), which was the temperature where the starch showed a minor gelatinization, and onset temperature (To) were higher for the enzyme-treated starches than for the non-treated control samples. This suggests that the gelatinization process was delayed, which agrees with the results of the pasting properties (Table 3.2). A significant increase in Ti was observed for the AMG-treated samples but not for the MA-treated starches. Higher gelatinization regimes of enzyme-treated starches have been implied to the structural stability of starch granules due to higher degree of order and crystallinity (Kaur, Singh, Ezekiel, & Sodhi, 2009). Alternatively, the increase of Ti and To found for the enzyme treated samples could be related to the reduction of swelling capacity previously mentioned. Moreover, a significant narrowing in the gelatinization temperature range was observed for the AMG samples, indicating that the

Table 3.2 Pasting properties of native rice starch, control and enzyme-treated starches.

Samples	Pasting temp (°C)	Peak viscosity (cP)	Breakdown (cP)	Final viscosity (cP)	Setback (cP)
Native	79.2 ab	2621 c	550 bcd	2920 de	840 e
AMG-3 control	79.2 ab	3117 f	718 efg	3010 e	611 bcde
AMG-3	80.8 cd	2722 cd	561 cd	2578 c	417 ab
AMG-6 control	78.8 a	2992 ef	717 efg	2802 d	526 abcd
AMG-6	80.8 cd	2599 c	604 cdef	2450 bc	455 ab
AMG-12 control	79.5 abc	2966 ef	631 cdefg	2776 d	441 ab
AMG-12	81.2 d	2759 cd	589 cde	2552 c	382 ab
AMG-24 control	79.6 abc	3126 f	766 g	2968 de	608 bcde
AMG-24	85.6 e	2658 c	559 cd	2403 bc	304 a
MA-3 control	78.8 a	3031 ef	764 g	3028 e	761 de
MA-3	79.2 ab	2204 ab	523 bc	2297 ab	617 bcde
MA-6 control	79.2 ab	2752 cd	697 defg	2788 d	733 de
MA-6	80.0 abcd	2215 b	410 ab	2274 ab	469 abc
MA-12 control	78.8 a	3001 ef	754 fg	3039 e	793 e
MA-12	80.4 bcd	2200 ab	408 ab	2308 ab	517 abcd
MA-24 control	78.8 a	2914 de	691 defg	2941 de	718 cde
MA-24	80.8 cd	2021 a	336 a	2119 a	433 ab

Values followed by different letters within a column are significantly different ($P < 0.05$)

Table 3.3 Thermal properties of native rice starch, control and enzyme-treated starches as determined by DSC.

Samples	Ti (°C)	To (°C)	Tp (°C)	Tc (°C)	Tc-Ti (°C)	ΔH (J/g °C)
Native	60.1 a	69.5 cd	74.9 def	80.7 h	20.6 h	14.0 f
AMG-3 control	63.4 b	69.0 a	74.3 ab	79.8 bcd	16.5 g	10.9 ab
AMG-3	66.4 defgh	69.9 e	74.7 cd	80.2 cdefg	13.9 bcdef	13.7 ef
AMG-6 control	65.0 c	69.0 a	74.0 a	79.2 a	14.2 cdef	10.9 ab
AMG-6	66.3 defg	69.3 bc	74.0 a	79.6 ab	13.3 abcd	14.7 g
AMG-12 control	65.2 cde	69.9 e	74.9 de	79.8 bc	14.6 ef	10.8 ab
AMG-12	67.8 i	70.5 gh	75.0 def	80.2 cdefg	12.5 a	13.7 ef
AMG-24 control	65.2 cde	70.5 gh	75.5 h	80.2 cdefg	15.0 f	10.9 ab
AMG-24	66.7 hi	70.9 ij	75.1 efg	80.4 fgh	12.7 ab	13.0 d
MA-3 control	64.6 bc	69.4 c	74.3 ab	79.5 ab	15.0 f	13.0 d
MA-3	65.5 cdef	69.8 de	74.5 bc	79.9 bcdef	14.5 def	14.0 f
MA-6 control	66.1 defg	70.0 e	74.5 bc	79.9 bcde	14.2 cdef	12.4 c
MA-6	66.4 defghi	70.4 fg	74.9 def	80.3 efgh	13.5 abcde	14.1 f
MA-12 control	66.6 efghi	70.7 hgij	75.1 efg	80.3 defgh	13.8 bcdef	11.4 b
MA-12	67.5 ghi	71.0 i	75.4 gh	80.5 gh	13.1 abc	13.9 f
MA-24 control	66.7 fghi	70.1 ef	75.2 fgh	80.2 cdefg	13.5 abcde	10.5 a
MA-24	67.1 ghi	70.9 ij	75.5 h	80.3 cdefg	13.2 abcd	13.1 de

Ti = initial temperature, To = onset temperature, Tp = peak temperature, Tc = conclusion temperature, ΔH = enthalpy change.
Different small caused letters within a column denote significant differences ($P < 0.05$)

starch granules were more homogeneous and cooperative melting of crystallites took place (Jacobs & Delcour, 1998). In addition, enzyme-treated starches exhibited significantly higher enthalpy (ΔH) than the control, indicating the amounts of molecular order structure was induced (Zhao et al., 2018). Overall, the higher of gelatinization temperature and the enthalpy of enzymatically treated starches made the granules more crystalline which was consistent by relative crystallinity data.

3.5 Conclusions

Porous high amylose rice starch was produced by using AMG and, to our best knowledge, for the first time using MA. The hydrolytic activity affected the granular structure in both the amorphous and crystalline parts. MA specifically increased the short amylopectin chains and increased solubilization. The peak viscosity, final viscosity, breakdown and setback values of pasting properties were reduced by both enzymatic treatments whereas the gelatinization temperature increased. The enzymatic treatments led to an increased relative crystallinity, producing starch granules more homogeneous with respect to thermal properties. Our data provide information on different actions of the two enzymes on granular rice starch with many of the effects generating beneficial physicochemical properties as compared to gelatinized starch.

3.6 Acknowledgements

This study was financially supported by the Royal Thai Government Scholarship (Ministry of Science and Technology), and partly supported by Archer Daniels Midland (ADM) Company, Decatur IL, USA. The SEC was provided by grant

from the Carlsberg Foundation. We gratefully appreciate constructive discussions with Dr. Eric Bertoft on starch molecular structure.

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CHAPTER IV

EFFECT OF CRUDE EXTRACT AND MALTODEXTRIN FROM SWEET CASSAVA ON EXERCISE ENDURANCE CAPACITY IN MALE WISTAR RAT

4.1 Abstract

The effects of multicycle ultrasound-assisted ice recrystallization (US+IR) combined with amyloglucosidase (AMG) or maltogenic α-amylase (MA) catalyzed hydrolysis on structure were investigated. Scanning electron microscopy (SEM) showed that the US+IR produced shallow indentations and grooves on the exterior of granules while the combination US+IR and enzyme hydrolysis created additional pores on starch granules. MA displayed a higher number of pores than AMG. The highest values of specific surface area (S_{BET}) and the total pore volume were obtained for US+IR→MA (1.96 m^2 g^{-1} and 7.26 x 10^{-3} cm^3 g^{-1}, respectively). The US+IR treatment significantly decreased the relative crystallinity, amylose content and swelling capacity. Those parameters were further efficiently decreased following enzymatic hydrolysis. The combined treatments generated products with higher initial gelatinization temperature (Ti) compared to the corresponding controls. The US+IR increased the digestion rate constant (k-value) compared to native starch. However, the combined treatment, US+IR→AMG, significantly decreased the k-value from 2.97 x 10^{-3} to 2.50 x 10^{-3} min^{-1} compared to its control. Our study demonstrates that US+IR treatment in combination with enzyme hydrolysis is a useful method to produce specifically

functionalized porous rice starch that can be used as e.g. absorbents and for further chemical modifications.

Keywords: Porous starch, Ultrasound treatment, Ice-recrystallization, Enzymatic hydrolysis

4.2 Introduction

Rice starch is widely used in food and non-food application. The demand is increasing and important characteristics include hypo-allergenicity, bland taste, white colour and small granules (Amagliani, O'Regan, Kelly, & O'Mahony, 2016). However, the use of native starch is limited due to its compact granules, which is less accessibility of chemicals and enzymes, thereby, impeding its use in some applications. Recently, porous starch has attained increasing interest specifically due to the large specific surface areas of this product that can be used as a carrier or absorbent (Benavent-Gil & Rosell, 2017b; Zhang et al., 2012). A number of methods have been developed to produce porous starch mainly including physical, chemical and enzymatic protocols. However, especially chemical method can be associated with environmental pollution and increasing consumer concerns. Physical and enzymatic methods are considered to be environmentally friendly as it reduces usage of chemicals and waste.

Freezing-thawing (F/T) of granular starch is a clean and feasible way to functionalize starch by generating higher surface area. (Tao, Wang, et al., 2015; Tao, Yan, et al., 2015; Yu et al., 2015). F/T combined with enzyme modification further extends this functionality (Zhao et al., 2018). The F/T principle is based on an initial crystallization of bulk and structured water during the freezing process resulting in

physical stress in the granular matrix inducing pore and crack formation (Charoenrein & Preechathammawong, 2012). The efficiency of this process is facilitated by using slow freezing rates and multiple F/T cycles (Tao, Wang, et al., 2015; Tao, Yan, et al., 2015; Yu et al., 2015). Ice recrystallization (IR) is a molecular reorganization process that it characterized by increased size of the ice crystals. This process is the major cause for physical degradation of e.g. starch using F/T. Typically, larger ice crystals are created as a result of temperature fluctuations. A typical F/T protocol involves freezing and thawing of starch granules in water in 20-24 h F/T cycles which is a slow process and ice recrystallization can potentially speed up the process. Additionally, ultrasonic treatment (US) is a physical method attending attraction due to its capability to reorganize starch chains and create pinholes on starch granular surface. In starch-water systems, ultrasound-treatment creates excessive shear force, high temperature, and free radicals which can break the chains of starch thereby changing, and often improving, the properties of starch (Sujka & Jamroz, 2013; Zhu, 2015).

Enzyme-assisted protocols is gaining in popularity due to the high substrate selectivity, product specificity, mild reaction conditions, reduced by-products and high safety (Le et al., 2009; Lee et al., 2008; Oh, Choi, Lee, Kim, & Moon, 2008). For porous starch, the most common enzymes are α-amylase and amylogucosidase used separate and in combination (Benavent-Gil & Rosell, 2017a; Dura, Błaszczak, & Rosell, 2014; Dura & Rosell, 2016; Yussof, Utra, & Alias, 2013). However, granular rigidity prevents efficient catalysis using enzymes. Thus, high enzyme dosage is required to sustain reasonable reaction rates, resulting in high-costs (Wang et al., 2017). For the purpose to increase reaction rates by increasing the specific surface area of starch, ultrasound treatment have been employed (Li, Li, & Zhu, 2018; Wang et al.,

2017; Wu, Du, Ge, & Lv, 2011). However, the synergy of ice recrystallization and ultrasonic effects might not be sufficient to modify the highly dense and complex starch structure. In this present study, amyloglucosidase (AMG) and maltogenic α-amylase (MA) was used to modify porous rice starch after using physical modifications as a pretreatment. AMG is an exo-acting enzyme that hydrolyzes both α,1-4 and α,1-6 linkages from the non-reducing ends of starch chain releasing glucose. On the other hand, MA is versatile enzyme that mainly has exo-action, cleaving α,1-4 glucosidic linkage, generating maltose but also exhibits endo-action within the starch chain (Christophersen, Otzen, Norman, Christensen, & Schäfer, 1998; Le et al., 2009). In addition, MA also has a minor transglycosylation activity forming linkages such as α,1-3 and α,1-6 linkages producing branched oligosaccharides from gelatinized starch (Cha et al., 1998; Lee et al., 2002).

This study particularly introduces IR and US to prepare granular rice starch for further enzyme treatment producing highly porous rice starch. To the demonstrate the potential of this approach, the morphological, structural, physicochemical and digestibility to amylolytic enzymes were investigated. The results reveal important fundamental mechanisms of combined physical and enzymatic granular starch modification.

4.3 Materials and methods

4.3.1 Materials

Rice starch was a gift from General Food Products Co., Ltd. (Nakhon Ratchasima, Thailand). Amyloglucosidase (EC 3.2.1.3, specific activity 260 U/mL) from *Aspergillus niger* was purchased from Sigma-Aldrich (Steinheim, Germany).

Maltogenic α-amylase (EC 3.2.1.133, Maltogenase® L) was kindly provided by Novozymes (Bagsvaerd, Denmark). Porcine pancreatic α-amylase (PPA, EC 3.2.1.1, specific activity 12 U mg^{-1}) and PGO (peroxidase and glucose oxidase) enzyme kit were purchased from Sigma-Aldrich (Missouri, USA).

4.3.2 Preparation of porous starch

4.3.2.1 Ultrasound-assisted ice recrystallization (US+IR treatment)

Rice starch (10 g, dry basis) samples were dispersed with 20 mL of distilled water for 3 h in an aluminium box at room temperature. Boxes with starch were stored in a temperature range of 0 to -5°C to induce ice recrystallization for 6 h followed by thawing in an ultrasonic bath (Bandelin Sonorex Digitec, Berlin, Germany) at frequency of 20 kHz, power 170 W at temperature range of 25-35°C for 1 h to perform 1 cycle of US+IR. The procedure was repeated in 7 cycles. The starch samples were filtered and dried at 50°C for 12 h. The dried starch samples were gently ground as powder, passed through a 100-mesh sieve and stored in a desiccator until analysis.

4.3.2.2 US+IR followed by enzymatic hydrolysis

Starch samples prepared by US-IR (10 g, dry basis) were suspended in 20 mL of 20 mM Na acetate buffer at pH 4.5 (AMG treatment) or 20 mM Na maleate and 5 mM $CaCl_2$ buffer at pH 5.5 (MA treatment). The starch suspensions were pre-heated in water bath at 60°C for 0.5 h and 100 U/g starch of AMG or MA solutions were added. The samples were incubated at 60°C for 12 h in a shaking water bath (180 rpm). The suspensions were centrifuged (4,000g, 5 min) followed by 3 times washing with 40 mL of MilliQ water. The sediments were pre-dried at 60°C for 20 min before complete drying at 130°C for 2 h. The dried starch

samples were ground and sieves as described above. Control samples were prepared using the same procedures without enzyme.

4.3.3 Scanning electron microscopy (SEM)

Granular morphology was investigated using Field Emission Scanning Electron Microscope (FE-SEM, Carl Zeiss, Oberkochen, Germany). Starch samples (1%) were dispersed in absolute ethanol. One drop of each sample was placed on a coverslip and dried at 50°C for 3 h. A thin layer of each sample was stuck on aluminum stubs with conductive carbon tape and sputter-coated with gold-palladium. The samples were examined at an accelerating voltage of 3.0 kV.

4.3.4 Specific surface area and total pore volume using nitrogen sorption isotherms through Brumauer-Emmett-Teller (BET) protocol.

Prior to measurement, starch samples were dried at 100°C for 6 h and kept in a desiccator. Dried starch (200 mg) was packed in the sample tube and then degassed at 125°C for 24 h. The sample tube was placed in BET instrument with purging nitrogen gas and immersed in liquid nitrogen (-196°C). The measurement was carried out by dispensing a specific portion of the nitrogen gas (2 L) and measuring the relative pressure p/po. The pressure change was recorded by BELSORP-mini II (MicrotracBel Corp., Osaka, Japan). Specific surface area and total pore volume were calculated by BET method (Brunauer, Emmett, & Teller, 1938).

4.3.5 Relative crystallinity

The crystallinity of starch samples was quantified by wide-angle X-ray scattering (WAXS) as described by Boonna & Tongta (2018). The WAXS data were collected in the 2θ range of 8 to 30°. The relative crystallinity was calculated by the ratio of the relative area of crystalline peaks to the total area using a program called SAXSIT

(Small Angle X-ray Scattering Image Tool) developed in-house (BL1.3W: SAXS, Synchrotron Light Research Institute, Nakhon Ratchasima, Thailand) as follows:

$$Relative\ crystallinity\ (\%) = \frac{Area\ of\ crystalline\ peaks}{Total\ area\ of\ crystalline\ and\ amorphous\ peaks} \times 100$$

4.3.6 Determination of amylose content

The apparent amylose content was determined as described by Wickramasinghe, Blennow, & Noda (2009).

4.3.7 Molecular weight fraction analysis by size-exclusion chromatography with triple detection array (SEC-TDA)

Firstly, starch samples were prepared in form of non-granular starch as described by Klucinec & Thompson (1998). Non-granular starch (5 mg) was dissolved in 25 μL of 2M KOH at 4°C for 24 h. The non-granular starch solution was re-dissolved in 975 μL Milli-Q water and shaken in a Thermomixer (Eppendorf, Germany) at 80°C for 5 h at 1200 rpm. Then the sample was diluted to 1 mg/mL. The molecular weight fractions were determined by size exclusion chromatography (SEC) using a Viscotek System (Malvern, UK) equipped with a GS-520HQ column (Shodex) attached to a TDA302 module (Triple detector array) as described by Sorndech et al (2015). Multidetector homopolymer calibration of the RI, scattering and viscosimetere detectors was made using pullulan (Mw 48,800 Da, dn/dc 0.131, polydispersion 1.07, Showa Denko) as a standard. The flow rate was set at 0.5 mL min^{-1} 10 mM ammonium formate containing 0.02% sodium azide as eluent. The data processing was performed using the OmniSec Software 4.7 (Malvern Instrument, Ltd.).

4.3.8 Swelling capacity and solubility of starch

The swelling capacity (SC) and solubility (S) were determined on the basis of the method described by Rosell, Yokoyama, & Shoemaker, (2011) with a slight modification. Briefly, 100 mg db of the starch was weighed in centrifuge tubes followed by addition of 10 mL of MilliQ water and heating at 60°C for 30 min under continuous stirring. After cooling to room temperature, the tubes were centrifuged at 4,000g for 15 min. The supernatant was collected and the precipitate weighed. The supernatants were transferred to Petri dishes then dried at 110°C overnight to constant weight. The swelling capacity and solubility were calculated as follows:

$$SC\ (g/g) = \text{sediment weight}/[\text{weight of dried starch} \times (1 - S/100)]$$

$$S\ (\%) = (\text{dried supernatant weight}/\text{weight of dried starch}) \times 100$$

4.3.9 Pasting properties

The pasting properties were determined using a Rapid Visco Analyser (RVA) (Newport Scientific, Warriewood, Australia). Starch (2.5 g, 14% moisture content) was added to 25 mL of MilliQ water in the aluminum RVA canister. RVA settings were performed by heating from 50 to 95°C in 282 s, holding at 95°C for 150 s and cooling to 50°C. The initial mixing speed was 960 rpm for 10 s and then 160 rpm paddle speed was followed. Pasting parameters were recorded using Thermocline software (Perten Instruments, Hägersten, Sweden) for Windows.

3.4.10 Thermal properties

The thermal properties of starch sample were analyzed using differential scanning calorimeter DSC1 instrument (Mettler Toledo, Switzerland). The starch sample (10 mg, dry basis) was mixed with MilliQ water (1:3, w/v) in the

stainless-steel pan. The sample pan was hermetically sealed and equilibrated at room temperature for 24 h before analysis. An empty sealed pan was used as a reference. The heating profile was from 25 to 130°C at a rate of 5°C min^{-1}. Initial temperature (Ti) defined as the temperature of the sample start to gelatinize, onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), enthalpy (ΔH) were calculated by STAR^e software version 10.0 (Mettler Toledo, Switzerland).

4.3.11 The rate of digestion

The rate of digestion was measured by a modified Englyst method (Englyst, Kingman, & Cummings, 1992). Free sugars were removed by suspending the starch sample (5 mg) in 200 μL of ethanol (80%, w/w), incubation at 85°C for 5 min in a shaking water bath (180 rpm) and centrifugation at 15,000g for 5 min. The starch was collected and incubated in 250 μL of 0.2 M phosphate buffer (pH 6) with 30 U of PPA and 1 U of AMG at 37 °C for 0, 10, 20, 30, 45, 60, 120, 180, 240, 360 and 1440 min in a shaking incubator (120 rpm). Hydrolysis was terminated by adding 250 μL of ethanol (96%, w/w) and sample was centrifuged at 15,000 x for 5 min at 4 °C. The starch digestion rate was measured as the percentage of glucose released over the time course using the PGO enzyme assay (Sigma-Aldrich (Missouri, USA). The experimental data were fitted to a first order kinetic model, $C = 1\text{-}e^{kt}$, where t is the digestion time (min), C is the concentration of product at time t, and k is the digestion rate constant (min^{-1}). The experiments were carried out in triplicate.

4.4 Results and discussion

A 7-cycle ultrasound-assisted ice recrystallization (US+IR) protocol of rice starch was performed followed by additional modification with amyloglucosidase

(AMG) or maltogenic α-amylase (MA). To discriminate effects of the incubation conditions on the starch, control samples were prepared under the same conditions in the absence of enzyme.

4.4.1 Granular morphology, specific surface area and total pore volume

SEM micrographs demonstrate that the native rice starch exhibited a polyhedral morphology and a relatively smooth surface with few pits (Figure 4.1, NR). The US+IR treatment did not have significant destructive effects on the general granular shape (Figure 4.1, A1 and B1). However, this treatment induced granule roughness, additional grooves and shallow indentations at the surface. The specific indentation surface effect is possibly caused by a series of complex events including in granule, ice recrystallization exerting local high pressure in ordered crystalline structures at the surface, resulting in the granular surface indentations (Tao, Yan, et al., 2015; Zhao et al., 2018). Additionally, surface roughness is possibly induced by cavitation force of ultrasonic during thawing process, an effect caused by collage of microbubbles, resulting in a jet of water directed onto the granule surface (Sujka, 2017; Zhu, Li, Chen, & Li, 2012; Zuo, Hébraud, Hemar, & Ashokkumar, 2012).

The combined effects of US+IR and enzymatic hydrolysis were visible in the granular morphology as holes and fissures (Figure 4.1, A2 and B2). MA was demonstrated more active on the starch granules than AMG, resulting in perforated granules seemingly deeper than for the AMG treatment. This discrepancy could be due to the hydrolytic mechanism of MA exhibiting both endo- and exo-activity (Christophersen et al., 1998; Goesaert, Slade, Levine, & Delcour, 2009) as compared to AMG having only exo-activity.

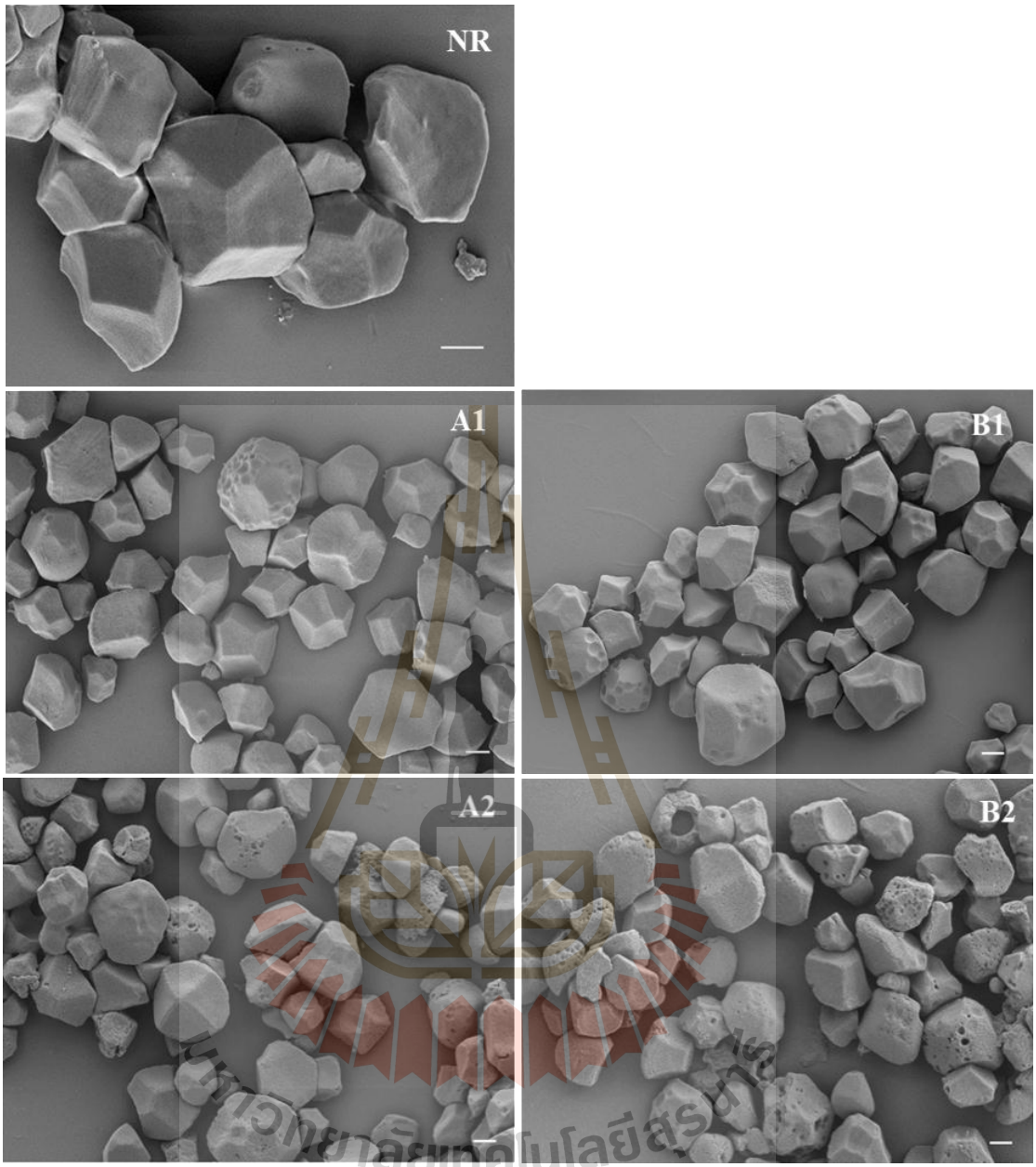

Figure 4.1 SEM micrographs for native rice starch (NR), US+IR treated starch (1) and the combination with AMG and MA (2, A; AMG and B; MA). Scale bar = 2 μm.

The specific surface area and total pore volume of starch samples were calculated based on nitrogen adsorption isotherms using the BET method. Surprisingly, the US+IR treatment had no effect on the specific surface area (S_{BET}) and total pore volume relative to native rice starch (Figure 4.2). However, the combination US+IR and enzymatic hydrolysis resulted in an increase in S_{BET} and total pore volume. The highest S_{BET} and total pore volume were found for the US+IR→MA sample (1.96 m^2/g and 7.26 x 10^{-3} cm^3/g, respectively). These data were in accordance with SEM (Figure 4.2, B2) clearly visualizing more pores at the granule surface of the US+IR → MA sample.

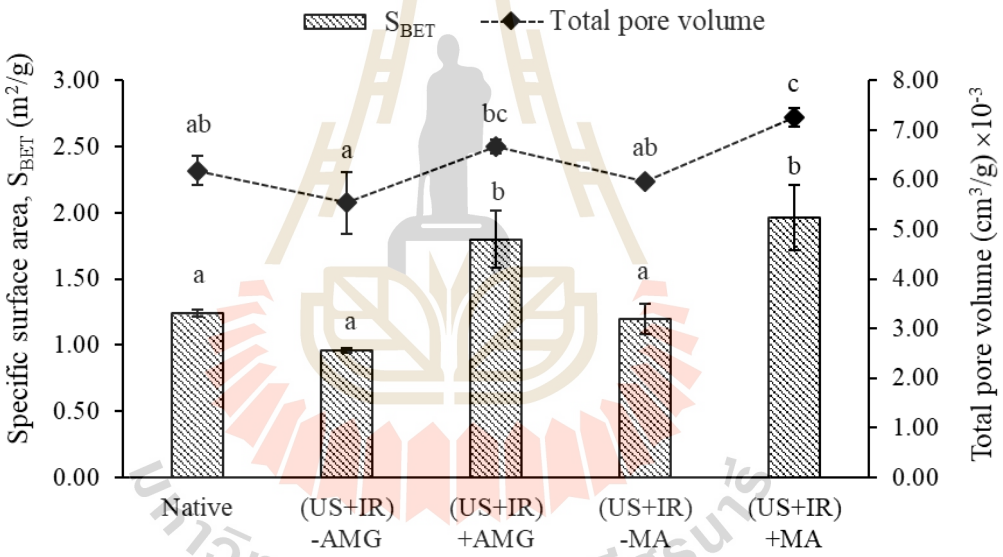

Figure 4.2 Specific surface area and total pore volume of starch samples. Different letters represent that samples are statistically different ($p < 0.05$).

4.4.2 Crystalline structure

The crystalline structure of rice starch processed with US+IR and combined with enzyme hydrolysis was measured by wide-angle X-ray scattering. All starch samples exhibited similar WAXS patterns which strong reflections at 15°, 17°, 18°, and 23° (2θ),

typical for the A-type crystalline polymorph (data not shown). This data documents that the combined treatment did not significantly affect the remaining crystalline arrangement in the starch granules. However, the relative crystallinity, as calculated from the WAXS diffractograms, was significantly decreased after US+IR compared to native rice starch and slightly increased after the combination with enzyme compared to the corresponding control samples (Table 4.1). The crystallinity of native rice starch was 23.8% that fell to 20.6-20.7% after US+IR and increased to 21.8-22.3% following enzyme treatment. This result indicates a synergistic effect of ice crystal growth in the starch granule (Zhao et al., 2018) and the shear force from the ultrasonic energy causing weakening of double helices and partly disruption crystalline starch granule sections (Li et al., 2018; Zhu et al., 2012). The 5.8% and 7.6% increase in crystallinity for AMG or MA, respectively, was likely due to the combined effects of exposure of starch chains to the enzymes, rearranged by the ultrasonic treatment (Li et al., 2018) and ice recrystallization events taking place mainly in amorphous region, as deduced for the increased crystallinity.

Table 4.1 Relative crystallinity, amylose content, swelling capacity, solubility index and rate coefficient (k) for digestion of modified starch.

Samples	**Relative crystallinity (%)**	**Amylose content (%)**	**Swelling capacity (g/g)**	**Solubility index (%)**	**k (min^{-1}) x10^{-3}**
Native	23.8 ± 1.0 c	29.5 ± 0.5 c	2.8 ± 0.1 d	0.47 ± 0.19 a	2.07 ± 0.31 a
US+IR → no AMG	20.6 ± 0.2 a	28.1 ± 0.2 b	2.7 ± 0.1 c	0.94 ± 0.21 b	2.97 ± 0.06 c
US+IR → AMG	21.8 ± 0.2 ab	26.5 ± 0.1 a	2.6 ± 0.0 b	0.98 ± 0.28 b	2.50 ± 0.26 b
US+IR → no MA	20.7 ± 0.0 a	27.9 ± 0.3 b	2.7 ± 0.1 c	0.98 ± 0.26 b	2.60 ± 0.10 bc
US+IR → MA	22.3 ± 0.1 b	26.6 ± 0.1 a	2.1 ± 0.1 a	1.37 ± 0.25 c	2.30 ± 0.17 ab

Values followed by different letters within a column are significantly different ($P<0.05$).

4.4.3 Apparent amylose

For native rice starch, the apparent amylose content was 29.5% (Table 4.1). The apparent amylose of US+IR treated starch samples decreased by approximately 1.5% units. It was noteworthy that the decrease in amylose content can be induced not only by ice crystal growth, which possibly induces exudation of amylose from the amorphous part of the granules (Szymońska & Wodnicka, 2005; Zhang, Han, & Lim, 2018) but also by molecular degradation during US+IR repeated cycles since amylose, mostly located in amorphous part, would be susceptible for ultrasonic degradation. Following the combined hydrolase treatment, the apparent amylose was further decreased to 21.8 and 22.3% for AMG and MA, (an enzyme-related decrease by 1.2 and 1.6% units, respectively. These data suggest that the US+IR treatment has exposed new amorphous granule surfaces allowing increased accessibility of enzymes into starch granules causing more enzymatic efficient hydrolysis of amylose. This result was consistent with the crystallinity data that the combined treatment with enzyme had higher crystallinity than their specific control.

4.4.4 Starch molecular weight fractions

Molecular weight distributions of the starch fractions were obtained using size-exclusion chromatography with triple detection array (SEC-TDA). The chromatogram showed a typical bimodal distribution mainly originating from high molecular mass amylopectin and lower molecular mass amylose (data not show) with fraction I and fraction II that refer to high (mainly amylopectin) and low (mainly amylose) molecular weight, respectively. No significant changes were found for the US+IR treatments (Figure 4.3 and HPLC chromatograms indicated in Supplementary data Figure S2) demonstrating that no significant breakdown of the starch was induced

by this treatment. As expected, hydrolase treatment caused some significant decreases in the molecular weights, but mainly for fraction I (Figure 4.3). Only the MA treatment further decreased the molecular weight of fraction II. This may be explained by different mechanism of the AMG and MA, MA having both endo- and exo-activity (Miao et al., 2014). These data are in compliance with the morphological granule (Figure 4.1) data showing more extensive effect for the MA.

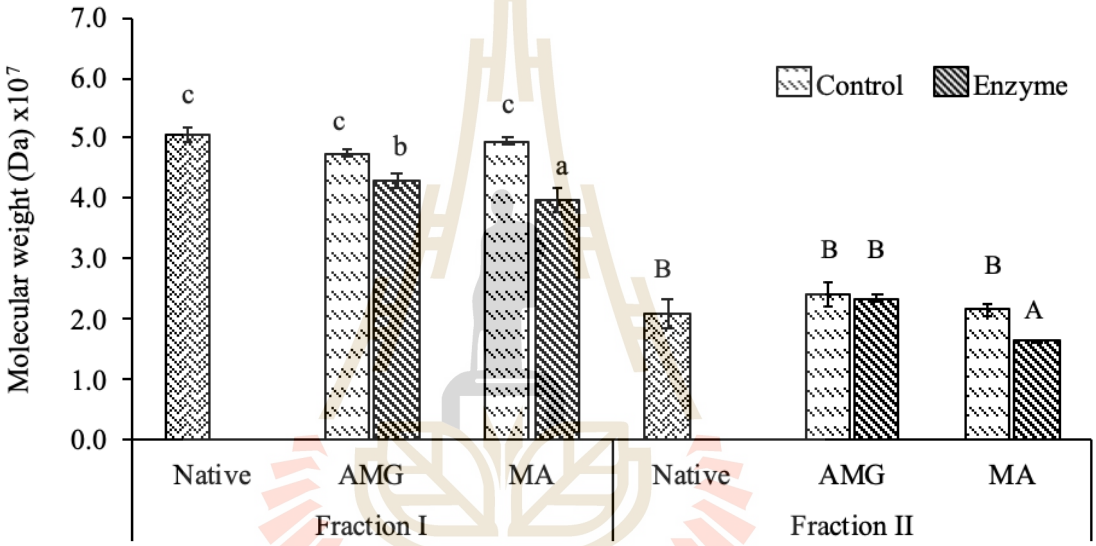

Figure 4.3 Molecular weight of the amylopectin (Fraction I) and amylose (Fraction II) of native and US+IR treated starch with (enzyme, AMG and MA) and without (control) enzymatic hydrolysis.

4.4.5 Swelling capacity and solubility

The swelling capacity of was substantially reduced by the US+IR treatment and enzyme treatment further reduced this parameter (Table 4.1). The most dramatic decrease in swelling capacity was observed for the US+IR → MA treated sample, which was the most affected sample with respect to morphology and reduction

in molecular size. The data suggest that the nature of the modifications are attributed to the internal rearrangement of starch chains in a manner to restrict water absorption and granular swelling (Ding, Luo, & Lin, 2019). The more extensive porous and/or fissure structure of this sample also suggest collapse of inner structures following enzymatic modification (as seen in Figure 4.1, A2 and B2) resulting in reduced capacity for water absorption.

The solubility showed an opposite trend as compared to the swelling capacity, i.e. enhanced solubility following modifications (Table 4.1). For US+IR treatment, increased solubility could be due to a synergistic effect of physical stress induced by ice formation and shear force created by ultrasonic cavitation to induce exudation of small starch fragments. The most prominent increase in solubility was found by US+IR→MA, again explained by endo- and exo-activities of MA producing small molecular and soluble starch fragments.

4.4.6 Pasting properties

Pasting properties are related to the gel viscosity, the stability of starch paste and retrogradation tendency. Both the US+IR and the following enzyme treatments markedly increased the peak temperature from 79.4 up to 93.7°C (Table 4.2). These data agree with the swelling capacity data (Table 4.1) showing suppressed swelling following modification. The US+IR treated sample exhibited an increased peak viscosity, breakdown and final viscosity while setback values were not significantly different. However, following enzyme hydrolysis, those pasting parameters were prominently decrease. The US+IR → MA sample fell down to, or below, that of the native starch sample.

The increase of peak viscosity is supposedly attributed to the weakening of starch interior interactions by US+IR reducing granule integrity, facilitating penetrate of water into the starch granules. However, since the US+IR treatment also caused the amylose leaching (as discussed in section 4.4.3) decreased amylose content can provide the same effects (Jane et al., 1999; Juhász & Salgó, 2008). The enzyme treatments significantly decreased the peak viscosity compared to their corresponding US+IR samples. This reduction is supposedly attributed to the hydrolytic reactions resulting in partly disintegrated the starch granules (Figure 4.1) and decreased molecular weights of the starch fractions (Figure 4.3).

The breakdown parameter is related to the thermal stability of the swollen starch granules in the starch paste during heating and shearing, i.e. higher breakdown indicates less resistance to shear force (Karim et al., 2008; Zhu, Mojel, & Li, 2018). The increased breakdown value of US+IR treated starch, might be due to the weakening or complete rupture of intra- and inter-molecular hydrogen bonds of starch molecules. This view is supported by our WAXS data (Table 4.1) showing reduction of crystallinity after US+IR treatment. However, the breakdown values were decreased following hydrolytic treatment, an effect possibly shortening and solubilization of starch chains causing loss compact granules. Alternatively, the reduction of breakdown value might be because of the peak viscosity decreasing after enzymatic treatment. Final viscosity and setback value are represented to the re-association during cooling of the main amylose that released following. Despite the decreased amylose the US+IR treatments resulted in increased final viscosities which we suggest is an effect release of linear starch fragments by the US+IR treatments prone to re-associate following cooling of the starch paste. Enzyme treatment

expectedly decreased both the final viscosity and setback parameters as expected from the hydrolytic activity. The MA treatment displayed the most substantial effect due to its endo-activity, severely reducing the molecular size.

Table 4.2 Pasting properties of starch samples.

Samples	Pasting Temp (°C)	Peak viscosity (cP)	Breakdown (cP)	Final Viscosity (cP)	Setback (cP)
Native	79.4 a	930 b	100 a	961 b	134 ab
US+IR→no AMG	82.8 b	1150 d	185 c	1100 e	136 ab
US+IR → AMG	88.7 c	1050 c	175 c	1000 c	126 ab
US+IR → no MA	83.6 b	1030 c	149 b	1080 d	146 b
US+IR → MA	93.7 d	770 a	111 a	769 a	110 a

Values followed by different letters within a column are significantly different ($P < 0.05$).

4.4.7 Thermal properties

The thermal properties data, as determined by DSC, were evaluated from the parameters including Ti, identified as a minor increase in gelatinization preceding the major gelatinization point, To: onset temperature, Tp: peak temperature, Tc: conclusion temperature, $\Delta T = Tc - Ti$ and ΔH: enthalpy change (Table 4.3). The Ti of the US+IR treated starch was significantly higher than the native starch and this parameter further increased after enzyme treatment. The increase of Ti could be an effect of molecular annealing taking place during US+IR treatment. The annealing

effect is described as a more ordered structure of the arrangement of double helices that can result in increased granular stability (Jayakody & Hoover, 2008). This result is in agreement with the previous observations showing increased pasting temperature and reduced swelling capacity for the US+IR samples.

Table 4.3 Thermal properties of starch samples.

Samples	**Ti (°C)**	**To (°C)**	**Tp (°C)**	**Tc (°C)**	**Tc-Ti (°C)**	**ΔH (J/g °C)**
Native	60.8 a	70.3 ab	75.5 d	80.2 c	19.4 e	10.1 b
US+IR → no AMG	65.4 b	70.1 a	74.5 a	78.4 a	13.0 c	8.9 a
US+IR → AMG	66.4 c	70.3 ab	74.5 a	78.3 a	11.9 a	9.1 a
US+IR → no MA	65.4 b	70.6 b	74.9 b	78.9 b	13.5 d	9.3 a
US+IR → MA	66.5 c	71.2 c	75.1 c	79.2 b	12.6 b	10.2 b

Ti = initial temperature, To = onset temperature, Tp = peak temperature, Tc = conclusion temperature, ΔH = enthalpy change. Different small caused letters within a column denote significant differences ($P < 0.05$).

Additionally, the of US+IR treated starches showed slightly decreased Tp, Tc and ΔH values while To were insignificantly different from native starch. The decrease could be attributed to disruption of crystalline part of starch granules (Zhao et al., 2018) as shown by our crystallinity data (Table 4.1). Interestingly, the US+IR→MA significantly increased To, Tp, Tc and ΔH compared to buffer control. The higher dissolution transition temperature and ΔH are likely due to the increased perfection of double helices of starch granules caused by the hydrolysis of MA

occurred in amorphous part. This result is also consistent with the crystallinity data which distinctly increased after the combination with MA. Higher degree of crystallinity has been implied to stabilize starch granules (Kaur, Singh, Ezekiel, & Sodhi, 2009). Therefore, the US+IR combined with enzymes, especially MA, are more resistant to thermally processing.

4.4.8 Rate of digestion

To investigate the susceptibility of the modified starch samples, the rate of digestion by PPA (porcine pancreatic amylase) and AMG was carried out. The digestion rate constants (k-values) of US+IR treated starch was markedly higher than of native starch (Table 4.1) demonstrating higher susceptibility to digestive PPA and AMG catalyzed degradation. This increased hydrolytic susceptibility of the US+IR treated starches could be attributed to the combined ice crystallization and ultrasonic-mediated destruction of the crystalline structure, increasing more vulnerable amorphous regions more readily accessed by the dietary hydrolytic enzymes. These data are in accordance with the WAXS data showing decreased crystallinity by US+IR treatment. However, as expected from the action of the hydrolases during preparation of the modified starches, the combined treatments with hydrolases exhibited lower k-value than those of control, i.e. increase enzyme resistance. These results are therefore an effect of the removal of susceptible starch segments and related to the increased crystalline rigidity of the products, having increased crystallinity and melting enthalpy following enzyme treatment.

4.5 Conclusions

Significant differences in the morphological, structural, physicochemical and digestible characteristics of rice starch modified by 7 repeated cycles of US and IR as well as subsequent enzyme-assisted hydrolysis were investigated. The US+IR treatment produced granules with more grooves and shallow indentations. Combined with AMG or MA treatments, created pores and fissures on the granular surface resulting in increased specific surface area. The US+IR treatment decreased the amylose content, relative crystallinity, swelling capacity and the enthalpy of dissolution. Subsequent AMG or MA treatments further decreased amylose content, swelling capacity, molecular weight, peak viscosity, breakdown, final viscosity, setback value and the rate coefficient of amylolytic degradation. Specifically, the US+IR→MA treatment resulted in high crystallinity and enthalpy as compared to AMG making theses treated-starch more thermostable for processing. Overall, the combination of US+IR and enzyme catalysis provides an efficient and new method for producing porous rice starch that can be used in various food applications as carrier agents for volatile compounds, protecting the sensitive substances and for further chemical modifications.

4.6 Acknowledgements

This research was supported by the Royal Thai Government Scholarship (Ministry of Science and Technology), Thailand and partly supported by Archer Daniels Midland (ADM) Company, Decatur IL, USA. The authors would like to thank Dr. Eric Bertoft for fruitful discussions. The SEC was provided by grant from the Carlsberg Foundation. Novozymes A/S is appreciatively acknowledged for providing MA.

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CHAPTER V

PRE-TREATMENT OF GRANULAR RICE STARCH TO ENHANCE BRANCHING ENZYME CATALYSIS

5.1 Abstract

In this study, effects of different pre-treatments of granular rice starch using ethanol (ETS) and maltogenic α-amylase (MA), separately or as combined sequentially ETS→MA were studied for further modification with branching enzyme (BE). The pre-treated samples were characterized with respect to morphology, structure, physicochemical properties and the rate of digestion to amylolytic enzymes. As revealed by scanning electron microscopy (SEM), MA produced pores and also eroded the granular surface whereas ETS caused coapted granules, noticeable swelling but no pores. Most pronounced effects were observed for the ETS→MA sequential treatment, where substantial perforation of the granules was found. This treatment also exhibited the highest specific surface area and decreased peak viscosity, breakdown, final viscosity and setback values. Crystallinity and enthalpy of gelatinization dramatically decreased with ETS and ETS→MA while for MA, those values increased. Subsequent BE catalysis increased the specific surface area, crystallinity and enthalpy. The amount of α-1, 6-glucosidic linkages increased for all the treated starch samples. Interestingly, BE catalyzed branching resulted in more intact granules, less swelling capacity, solubility and granular separation as compared to their control. The most substantia effects were found for the ETS→BE samples. These effects were related to

reduced amylolytic susceptibility. Pre-treatment prior to BE catalysis offers an efficient alternative way to modify granular starch with different structure and properties depending on the pre-treatment protocol.

Keywords: Ethanol-treated starch; Enzymatic modification; Granular rice starch; Branching enzyme

5.2 Introduction

Rice is an important global crop with starch functionality that widely used in food and non-food application such as a gelling agent, fat replacer and colloidal stabilizer (Wani et al., 2012). In general, native starch has several weak points such as low gel strength, low solubility, undesirable gel performance at room temperature and irreversible retrogradation (Abbas, K. Khalil, & Meor Hussin, 2010). To overcome these limitations, physical, chemical or enzymatic modifications are applied. However, chemical modification can be associated with environmental pollution and increasing concerns from consumers (Jensen, Larsen, Bandsholm, & Blennow, 2013; Xie, Li, Chen, & Zhang, 2019). Physical and enzymatic modifications are considered to be environmentally friendly as it reduces usage of chemicals and waste production. Due to the compact structure of native starch, enzymatic modification is applied to gelatinized starch rather than granular starch. Direct modification of starch in its native state is desirable because it reduces the costs and saves energy associated with the high temperatures required for gelatinization (Uthumporn, Zaidul, & Karim, 2010).

In previous study, we reported that branching enzyme (BE) was used to stabilized the starch granules which made a tightly packed granular structure (Jensen,

Larsen, et al., 2013; Jensen, Zhu, et al., 2013). In addition, BE has received attention to produce porous starch and modify structure and its properties by cleaving α-1,4- and reforming α-1,6-glucosidic bond (Benavent-Gil & Rosell, 2017a; Li et al., 2016; Ren et al., 2017; Van Der Maarel & Leemhuis, 2013). However, due to the tight packing of crystalline zone (amylopectin chains), the granule surface is relatively impenetrable, thereby a high enzyme dosage is required to achieve the reaction rate that associate with the high-costs (Wang et al., 2017). Thus, pre-treatment methods aiming at increasing the exposed granule surface are important to opening-up the granular structure before BE catalysis.

Heating starch in aqueous ethanol (ethanol-treated starch, ETS) is well-known procedure to obtain granular cold-water-swelling starch (Chen & Jane, 1994; Jane, Craig, Seib, & Hoseney, 1986; Jivan, Yarmand, & Madadlou, 2014; Sarifudin, Soontaranon, Rugmai, & Tongta, 2019). The method is simplest prepared by heating granule slurries in aqueous ethanol followed by evaporation of the ethanol from the product (Jane et al., 1986; Zhang, Dhital, Haque, & Gidley, 2012). Ethanol usually induces wrinkling of the granular surface, compresses the granules, reduces the swelling temperature and opens up the granular structure by weakening the single and double helical structure in response to complexation by ethanol (Zhang et al., 2012).

Granular starch can also be used as a raw material enzymatic approach. Currently, selected hydrolases and transferases, such as α-amylase, amyloglucosidase, pullulanase, branching enzyme, isoamylase and cyclodextrin-glycosyltransferase have been reported for obtaining starch granules with a porous structure (Benavent-Gil & Rosell, 2017b, 2017a; Chen & Zhang, 2012; Dura, Błaszczak, & Rosell, 2014; Dura & Rosell, 2016; Martínez, Pico, & Gómez, 2016). Among these enzymes, maltogenic

α-amylase (MA, glucan 1,4-α-maltohydrolase, EC 3.2.1.133) has mainly exo-α-1,4-glucanase activity, releasing maltose, but also exhibits endo-glucanase activity (Christophersen, Otzen, Norman, Christensen, & Schäfer, 1998; Goesaert, Slade, Levine, & Delcour, 2009; Le et al., 2009) and transglycosylation activity, which results in the formation of various glucosidic linkages such as α,1-3 and α,1-6 linkages, producing branched maltooligosaccharides from liquefied starch (Miao et al., 2014; Park et al., 1998).

In this study, combinations of pre-treatment protocols were investigated using ETS and MA to opening-up structure before BE catalysis. It is hypothesized that ETS, MA and sequential treatment with ETS and MA can promote different structural and physicochemical properties to allow for enhanced BE catalysis on granular rice starch. The main goal is to, as efficiently as possible, increasing the amount of α,1-6 linkages in the granular state. Major changes in granule morphology, molecular structure, physicochemical properties and the rate of digestion to amylolytic enzymes were found.

5.3 Materials and Methods

5.3.1 Materials

The rice starch was a gift from General Food Products Co., Ltd. (Nakhon Ratchasima, Thailand). Maltogenic α-amylase (MA) and branching enzyme (BE) were kindly provided by Novozymes (Bagsvaerd, Denmark). Amyloglucosidase (AMG, EC 3.2.1.3, specific activity 260 U mL^{-1}) from *Aspergillus niger* was purchased from Sigma-Aldrich (Steinheim, Germany). Porcine pancreatic α-amylase (PPA, EC 3.2.1.1, specific activity 12 U mg^{-1}) and PGO (peroxidase and glucose

oxidase) enzyme kit were purchased from Sigma-Aldrich (Missouri, USA). Analytical grade ethanol was purchased from Carlo Erba, France.

5.3.2 Sample preparation

5.3.2.1 MA treated starch treatment, MA

The rice starch suspension (50% w/v, in 20 mL of 20 mM Na maleate, 5 mM $CaCl_2$ buffer, pH 5.5) was equilibrated in a water bath at 60°C for 0.5 h. MA (100 U/g starch) was added and the mixture incubated at 60°C for 12 h in a shaking (180 rpm) water bath. The starch particles were collected by centrifugation 9,000g, 5 min. The residue was washed three times with 40 mL of DI water. The sample was pre-dried at 60°C for 20 min before drying at 130°C for 2 h. The dried starch samples were ground and passed through a 100-mesh sieve then stored in a desiccator for further analysis.

5.3.2.2 Ethanol treated starch treatment, ETS

The rice starch (10 g dry basis, db) was dispersed in 20 mL of ethanol (50%, v/v). The starch suspension was heated in a water bath at 80°C for 0.5 h and then cooled for 3 h at room temperature. The starch granules collected on a filter followed by 3 times washing with 50% of ethanol and finally dried at 50°C for 12 h. The dried starch was ground and stored as above.

5.3.2.3 The combination of ETS→MA treatment

The rice starch (10 g dry basis, db) was prepared as for the ETS treatment. After removing the ethanol by filtration, the starch was modified as described in section 5.3.2.1.

5.3.2.4 Branching enzyme treatment, BE

Native, MA-treated, ETS-treated and ETS→MA-treated starches (10 g db) were individually suspended in 20 mL of 50 mM phosphate buffer pH 6.5. The starch suspension was incubated at 60°C for 0.5 h and 5000 U/g starch of BE was added and incubated at 60°C for 24 h while mixing (180 rpm). DI water (40 mL) was added to the suspensions, the starch granules collected on a filter followed by 3 times washing with DI water. The samples were pre-dried at 60°C for 20 min before drying at 130°C for 2 h. The samples were gently powdered and sieved as described above.

5.3.3 Scanning electron microscopy (SEM)

Granular morphology was observed using Field Emission Scanning Electron Microscope (FE-SEM, Carl Zeiss, Oberkochen, Germany). Samples (1%) was suspended in absolute ethanol. One drop of each sample was placed on a coverslip and dried at 50°C for 3h. A thin layer of each sample was placed on aluminum stubs with conductive carbon tape and sputter-coated with gold-palladium. The accelerating voltage was 3.0 kV.

5.3.4 Specific surface area, total pore volume and mean pore diameter using nitrogen sorption isotherms through Brumauer-Emmett–Teller (BET) protocol.

The specific surface area, total pore volume and mean pore diameter of starch samples were estimated by nitrogen adsorption method using BELSORP-mini-II sorption isotherm instrument (MicrotracBel Corp., Osaka, Japan). Prior to measurement, samples were dried at 100°C for 6 h. The dried samples (200 mg) were degassed at 125°C for 24 h and immersed in liquid nitrogen (77.35 K). The

measurement was carried out by dispensing a specific portion of the nitrogen gas (2 L) and measuring the relative pressure p/po. Specific surface area, total pore volume and mean pore diameter were determined. The monolayer value was calculated using the BET equation (Brunauer, Emmett, & Teller, 1938).

5.3.5 Relative crystallinity

The relative crystallinity of the starch samples was obtained by wide-angle X-ray scattering (WAXS) as described by Boonna & Tongta, (2018). Samples were equilibrated with saturated LiCl for 7 days. WAXS data were collected in the 2θ range of 8-30°. The relative crystallinity was calculated by the ratio of the relative area of crystalline peaks to the total area using a program SAXSIT (Small Angle X-ray Scattering Image Tool) developed in-house (BL1.3W: SAXS, Synchrotron Light Research Institute, Nakhon Ratchasima, Thailand).

5.3.6 The ratio of α-1, 6-glucosidic linkage

The α-1,4 : α-1,6-glucosidic linkage ratios was analyzed using a Bruker (Fällanden, Switzerland) DRX spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK). Starch sample (10 mg) was dissolved in 500 μL D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) and heated at 99°C for 2 h. ^1H NMR spectra were measured at 80°C. The spectra were processed using Bruker Topspin 2.1 software with zero filling in all dimensions. The ratio of α-1,6-glycosidic linkage was calculated by dividing the peak area corresponding α-1,6-glycosidic linkage by the total peaks area corresponding α-1,4- and α-1,6-glycosidic linkage.

5.3.7 Swelling capacity and solubility of starch

Swelling capacity (SC) and solubility (S) were estimated by Rosell, Yokoyama, & Shoemaker, (2011) with a minor modification. Briefly, starch (100 mg db) was weighed in centrifuge tubes followed by addition of 10 mL of MilliQ water. The slurry was incubated at 60°C for 30 min while mixed (180 RPM) and centrifuged at 4,000g for 15 min. The sediment was weighed and the supernatant transferred to Petri dishes, dried at 110°C overnight and weighed. The residue obtained after drying of the supernatant represented the amount of starch solubilized in water. The swelling capacity and solubility were calculated by the following formulae:

$$S\ (\%) = (\text{dried supernatant weight}/\text{weight of dried starch}) \times 100$$

$$SC\ (g/g) = \text{sediment weight}/[\text{weight of dried starch} \times (1 - S/100)]$$

5.3.8 Pasting properties

The pasting properties were determined using Rapid Visco Analyser (RVA, Newport Scientific, Warriewood, Australia). The starch sample of 2.5 g was mixed with 25 mL of MilliQ water in the RVA canister. The RVA settings were as follows: heating from 50 to 95°C for 282 s, holding at 95°C for 150 s and cooling to 50°C over 300 s. The initial mixing paddle speed was 960 rpm for 10 s followed by 160 rpm speed during analysis. Pasting parameters were recorded using Thermocline software for Windows (Perten Instruments, Hägersten, Sweden).

5.3.9 Thermal properties

Thermal properties of starch samples were investigated using a differential scanning calorimeter DSC 1 (Mettler toredo, Switzerland). Approximately 7 mg of the sample was loaded into the aluminum pan and MilliQ water was added to

achieve a water-sample ratio of 3:1. The sample pans were hermetically sealed and equilibrated at room temperature for 24 h before analysis. Thermal analysis performed by heating from 25 to 130°C at a rate of 5°C min^{-1}. Initial temperature (Ti) was defined as the temperature at which the sample started to gelatinize and onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), gelatinization temperature range (Tp-Ti) and enthalpy (ΔH) were determined by $STAR^e$ software (version 10.0, Mettler Toledo, Switzerland).

5.3.10 ***In vitro*** **digestion**

The rate of digestion was measured following the method by Englyst, Kingman, & Cummings, (1992) with minor modifications. Free sugars were removed by suspending the starch sample (5 mg) in 200 μL of ethanol (80%, w/w), incubation at 85°C for 5 min with shaking (180 rpm) and centrifuged at 15,000g for 5 min. The starch pellet was collected and incubated in 250 μL of 0.2 M phosphate buffer (pH 6.0) with 30 U of PPA and 1 U of AMG at 37 °C for 0, 10, 20, 30, 45, 60, 120, 180, 240, 360 and 1440 min in a shaking incubator (120 rpm). The reaction was terminated by adding 250 μL of ethanol (96%, w/w) and sample was centrifuged at 15,000g for 5 min at 4°C. The starch digestion rate was measured as the percentage of glucose released over the time course using the PGO enzyme assay (Sigma-Aldrich (Missouri, USA). The experimental data were fitted to a first order kinetic model, $C = 1\text{-}e^{kt}$, where t is the digestion time (min), C is the concentration of product at time t, and k is the digestion rate constant (min^{-1}). The experiments were carried out in triplicate.

5.4 Results and discussion

5.4.1 Granular morphology

In this study, rice starch was pre-treated by maltogenic α-amylse (MA), ethanol (ETS) and in sequential step of ethanol and MA (ETS→MA) for further modification with branching enzyme (BE). To confirm the effect of preparation methods for BE catalysis on rice starch granules, the granular morphology was inspected (Figure 5.1).

Rice starch without modification displayed polyhedral morphology with a smooth surface (Figure 5.1, NR). Effect of the different pre-treatments were evident (Figure 5.1, MA, ETS and ETS→MA). MA-treated starch granules displayed channels but no effect was observed on the granular shape. Interestingly, ETS-treated starch showed a more coapted structure but no pores on the granules. Moreover, MA was more active on the ETS treated granules, as seen by several pores and fissures following MA treatment. This effect suggests that the ethanol pre-treatment has created larger granular surface for the MA to act on without disintegrating the granules (Figure 5.1, ETS→MA).

Generally, the BE catalysis of native and modified starches created only minor surface changes (Figure 5.1). The NR→BE and MA→BE treatments resulted in some additional cracks and fissures on the granules. No pores were created by the combined ETS→BE but the starch granules were somewhat more eroded after BE modification. However, BE treatment of the ETS granules, ETS→MA, did not result in further changes in the microstructure. The ETS→BE and ETS→MA→BE starch granules were seemingly more intact as compared to their control without BE (Figure 5.1). These results are in accordance with our previous notion that extensive BE

modification of maize and low-phosphate potato starch can have a stabilising effect on the granules (Jensen, Larsen, et al., 2013).

5.4.2 Specific surface areas, total pore volume and mean pore diameter

To test whether the pre-treatments and subsequent BE catalysis has changed the surface area of the granule preparations, the low-temperature nitrogen adsorption was employed. The specific surface area (S_{BET}), total pore volume and mean pore diameter were calculated using the BET (Table 5.1). Among the pre-treatment protocols, the highest values were observed in the ETS→MA treated starch (2.41 m^2/g, 17.82x10^{-3} cm^3/g and 28.53 nm, respectively) which were almost two-fold higher than for native rice starch. Moreover, the S_{BET} was also increased by MA (1.62 m^2/g) while there was no obvious change by ETS (1.17 m^2/g). These results were consistent with SEM (Figure 5.1, MA and ETS) visualizing more pores of MA treated starch and no pores after ETS modification.

In general, all prepared-starch treated with BE exhibited an increase of S_{BET} as compared to their control without BE. The S_{BET}, total pore volume and mean pore diameter of ETS→MA→BE were the largest (2.54 m^2/g, 21.48x10^{-3} cm^3/g and 37.56 nm, respectively). Interestingly, the pre-treatment by ETS before MA and BE catalysis had larger effect on S_{BET}, showing a 106% and 68% increase, respectively. These data were in accordance with SEM results (Figure 5.1, ETS→MA and ETS→BE) clearly presenting more perforated granules.

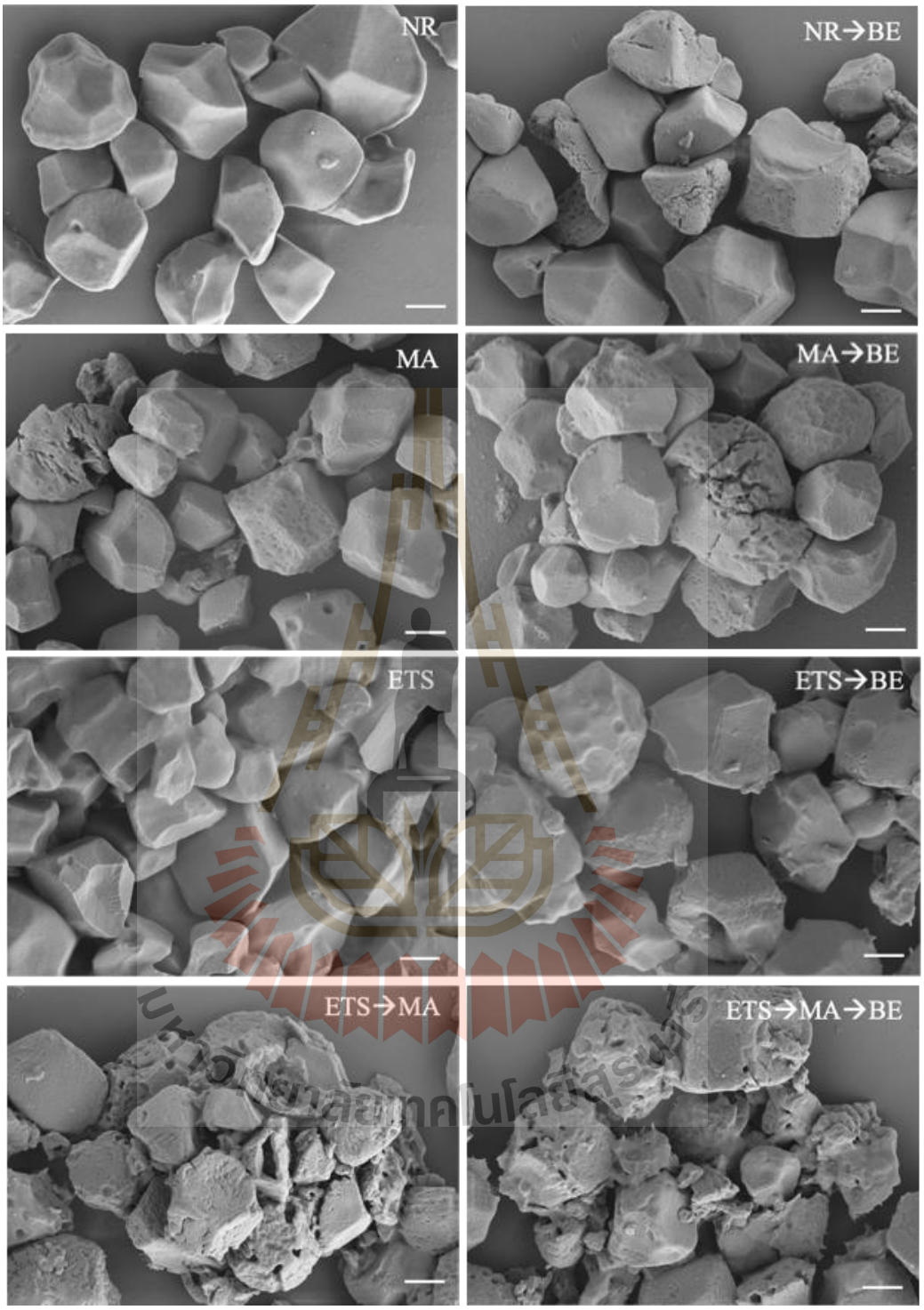

Figure 5.1 SEM micrographs for native rice (NR), maltogenic α-amylase treated (MA), ethanol treated (ETS), sequential step of ethanol and MA (ETS→MA) starches and the combination of those followed by BE treatments. Scale bar = 2 μm.

Table 5.1 Parameters derived from BET protocol of native and starch treated samples.

Samples	S_{BET} (m^2/g)	Total pore volume (cm^3/g) x10^{-3}	Mean pore diameter (nm)
N	1.13 a	8.18 b	19.46 a
MA	1.62 b	9.11 b	23.41 abc
ETS	1.17 a	5.26 a	18.03 a
ETS → MA	2.41 d	17.82 d	28.53 bcd
N → BE	1.24 a	10.30 b	35.56 de
MA→ BE	1.64 b	8.37 b	22.27 ab
ETS → BE	1.96 c	15.33 c	31.35 de
ETS → MA → BE	2.54 e	21.48 e	37.56 e

Letters denote significant difference ($P<0.05$).

5.4.3 Crystallinity

Wide-angle X-ray scattering was conducted to investigate the extent of crystalline and amorphous parts of starch samples. All samples displayed a typical of A-type pattern which exhibited strong peaks at 2θ of 15°, 17°, 18° and 23° (data not shown). However, the relative crystallinity was calculated to quantify the crystallinity of starch samples. The relative crystallinities of NR, MA, ETS and ETS→MA were 21.7%, 23.6%, 14.7% and 17.9%, respectively (Table 5.2). The MA and the combination of ETS→MA treatments exclusively produced porous structure but the crystallinity was slightly increased from 21.7% to 23.6% and 14.7% to 17.9%, respectively. The increase in crystallinity found in the MA treated samples was likely due to that the hydrolytic activity of MA was mainly directed to the amorphous region (Grewal et al., 2015). On the other hand, the relative crystallinity was dramatically

decreased by the ETS treatment. This reduction could be an effect of partial reorganization of the crystalline chain structure due to ethanol complexation (Zhang et al., 2012). However, BE treatment of the ETS samples increased the crystallinity by 41% demonstrating that BE had a significant effect on the granular structure, in agreement with the SEM data (Figure 5.1).

Table 5.2 Relative crystallinity, α-1,6 linkages percentage, swelling capacity and solubility index of starch samples.

Samples	Relative crystallinity (%)	α-1,6 linkages/ total linkages	Swelling capacity (g/g)	Solubility index (%)
N	21.7 bc	4.8	2.09 d	0.87 a
MA	23.6 e	4.7	1.37 a	0.83 a
ETS	14.7 a	4.9	3.32 e	2.82 d
ETS → MA	17.9 b	5.2	2.16 d	2.94 d
N → BE	22.8 de	5.3	1.69 b	0.90 a
MA→ BE	24.1 e	6.1	1.37 a	0.88 a
ETS → BE	20.8 c	5.5	1.94 c	1.83 b
ETS → MA → BE	20.1 c	6.6	2.14 d	2.54 c

Letters within each column denote significant difference ($P < 0.05$).

5.4.4 The α-1,6 glucosidic linkage ratio

The changes in the α-1,4 : α-1,6 glucosidic linkage ratio were determined by ^{1}H NMR (Table 5.2). The α-1,6 glucosidic linkage ratio of NR, MA, ETS and ETS→MA were 4.8%, 4.7%, 4.9% and 5.2%, respectively. It seems that MA and ETS

methods did not alter the α-1,6 glucosidic linkage ratio but the ETS→MA pre-treatment had a more profound effect on this ratio, showing 8.5%, 10.6% and 6.8% increases as compared to native, MA and ETS treated starches.

BE treatment of the native and pre-treated starches significantly increased the α-1,6 glucosidic linkage content. The most evident increase was found for the MA→BE and ETS→MA→BE samples which increased from 4.7 to 6.1% and from 5.2 to 6.6%, respectively (Table 5.2). These data are related to the S_{BET} data showing that MA and ETS→MA treated starch had a notably higher specific surface area (Table 5.1), allowing for better access for the BE in the starch granules. An increase in the α-1,6 glucosidic linkage ratio of the BE treated starches confirms that that BE catalyses an α-1,4 - α-1,6 transglucosidation on non-soluble starch particles as expected (Ao et al., 2007; Guo, Deng, Lu, Zou, & Cui, 2019).

5.4.5 Swelling capacity and solubility

The swelling capacity and solubility of the pre-treated starch samples demonstrated that MA restrained swelling of the granules (1.37 g/g) while ETS treatment resulted in higher swelling capacity (3.32 g/g) (Table 5.2). A decrease in swelling capacity by MA could be attributed to the hydrolytic activity of MA creating a porous structure on the granular surface, decreasing their water-holding capacity. On the other hand, ETS-treated starch exhibited the highest swelling capacity, which could be a direct effect of the disruption of crystalline region, as observed in the reduction of relative crystallinity (Table 5.2), increasing water-starch interaction (Choi, Baik, & Kim, 2017). Interestingly, BE catalysis significantly decreased the swelling of native and ETS treated starch, showing 19.1% and 41.6% decrease compared to their control without BE (Table 5.2), suggesting that BE is capable of catalyzing inter-chain

transfer between amylopectin and amylose generating cross-linking between these entities as suggested before (Jensen, Larsen, et al., 2013). WAXS data confirmed an increase in the relative crystallinity in all BE treated samples suggesting a general re-organization of the starch segments, which itself could explain the suppressed swelling capacity.

For the solubility, no significant difference was found for the native and MA treated starch suggesting that the exo-activity of MA, releasing maltose units, did not have a profound effect on the packing of the remaining starch chains in the starch granule (Xie et al., 2019). However, the solubility of the ETS and ETS→MA treated starches was notably higher than the native and MA treated starches. It could be due to ETS and ETS→MA treatments open-up the starch granule structure, resulting in swollen granules and releasing small and soluble starch fragments from the structure. Following BE catalysis, the solubility of the ETS and ETS→MA treated starches decreased, but no difference was found for N and MA pre-treated samples after BE treatment. Just as for the decreased swelling capacity described above, the reduction of solubility might be due to inter-chains transfer linking amylose and amylopectin segments together (Jensen, Larsen, et al., 2013; Zavareze & Dias, 2011).

5.4.6 Pasting properties

Pasting is an important property of starch that includes thermal processes of starch in water including gel formation, shear resistance and retrogradation. The pasting parameters of native and treated samples (Table 5.3) shows that, among the different pre-treatments, ETS decreased pasting temperature (74.3°C) whereas MA and ETS→MA delayed the pasting formation (82.5 and 82.9°C, respectively). However, following BE treatment, only ETS→BE treated starch showed

an increased pasting temperature while the pasting temperature of native, MA→BE and ETS→MA→BE treated starches were constant. This result is in accordance with the SEM analysis (Fig 1, ETS→BE) and swelling capacity data (Table 5.2) demonstrating that the increased pasting temperature of the ETS→BE sample is connected to the appearance of single starch granules and suppressed swelling. The less swell of starch granules could be related to an increase in pasting temperature since the first step of starch gelatinization is the water absorption that caused starch granules swelling, which mean the less swelling could require more thermal energy to gelatinization (Dura et al., 2014).

The peak viscosity, breakdown, final viscosity and setback values derived from the pasting curves of the pre-treated starches were significantly lower as compared to the native starch. Among the preparation methods, ETS→MA showed the lowest peak viscosity, final viscosity and setback values (105 cP, 100 cP and 19 cP, respectively). The reduction is supposedly attributed to the synergistic effects of ethanol and MA causing dramatic perforation of the starch granules (as seen in Fig 1, ETS→MA), inducing destructed granule surface and leaching of small starch fractions. A substantial decrease in those parameters substantiates that modification with ethanol, resulting in the swollen granules (Table 5.2), permits MA to more efficiently hydrolyze the granular matrix. However, compared to MA and ETS→MA, ETS showed a lower breakdown (17 cP) and higher final viscosity (202 cP) and setback (45cP) indicating that ETS improved the stability of starch paste. This suggests that ethanol-heat treatment, inducing granule swelling, facilitates migration of smaller starch segments and subsequent re-association of these forming a stable gel. The fact that BE catalysis further decreased the peak and final viscosities of both

native and pre-treated starch samples is related to the decreased the swelling capacity described above substantiating that the catalytic action of BE has resulted in even more perforated starch granules.

Table 5.3 Pasting properties of starch samples.

Samples	Pasting Temp (°C)	Peak viscosity (cP)	Breakdown (cP)	Final Viscosity (cP)	Setback (cP)
N	78.4 b	224 g	54 c	225 g	74 e
MA	82.5 c	179 e	26 b	182 e	29 bc
ETS	74.3 a	174 de	17 a	202 f	45 d
ETS → MA	82.9 c	105 b	24 b	100 b	19 ab
N → BE	78.4 b	194 f	55 c	207 f	68 e
MA→ BE	82.5 c	166 d	27 b	175 d	35 cd
ETS → BE	80.9 bc	132 c	16 a	130 c	14 a
ETS → MA → BE	82.2 c	95 a	19 a	90 a	15 ab

Letters within each column denote significant difference ($P<0.05$).

5.4.7 Thermal properties

The thermal properties were evaluated by DSC, presenting the initial temperature (Ti) that is identified as a minor gelatinization temperature, the major gelatinization or onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), gelatinization temperature range ($\Delta T=Tc-Ti$) and the enthalpy change (ΔH) (Table 5.4). Typically, the pre-treatments increased the gelatinization temperatures where ETS→MA exhibited the highest gelatinization temperatures (Ti=73.4°C,

To=76.5°C, Tp=79.4°C and Tc=81.9°C) and the narrowest of ΔT. The narrow and high melting temperatures indicates that ETS→MA had a more ordered and homogeneous crystalline structure, which is also reflected in its granular stability (Zhang, Han, & Lim, 2018). Synergistic effects of ETS, inducing swelling allowing MA to more efficiently hydrolyze mainly the amorphous parts. The increase in Ti and To induced by MA in both of native and ETS treated samples can be attributed to the hydrolytic activity of MA, active mainly in the amorphous parts, can increase the perfection of double helices association in starch granules. This is in accordance with the WAXS data (Table 5.2) showing increased the relative crystallinity and reduced swelling capacity.

After BE catalysis, a significant increase in Ti and To was found for MA→BE and EST→BE treated samples as compared to the two pre-treatments, respectively. Higher gelatinization temperature has been demonstrated to be an effect of higher degree of order and crystallinity (Kaur, Singh, Ezekiel, & Sodhi, 2009). The reduction in swelling capacity of the pre-treated samples (Table 5.2) could be related to the increase in Ti and To. Interestingly, the ΔH values were substantially higher after BE catalysis. Hence, BE helped referring the granular structure of the partly swollen. Especially, ETS→BE treated starch provided higher ΔH and crystallinity. Likewise, the hydrolytic activity of MA on ETS treated starch, producing porous surfaces, reverted the compactness of the granules (Figure 5.1, ETS→MA) and increased the crystallinity. Therefore, pre-treatment with ethanol provides an efficient protocol for further enzyme treatment. It agreed well with the granular morphology (Figure 5.1, ETS→MA and ETS→BE) that observed more individual granules and the increase of crystallinity (Table 5.2). The final product after BE treatment has more

compact structure with increasing the crystallinity, the α-1,6 glucosidic linkage ratio, enthalpy which is different from the native starch.

Table 5.4 Thermal properties of starch samples.

Samples	Ti (°C)	To (°C)	Tp (°C)	Tc (°C)	Tc-Ti (°C)	ΔH (J/g °C
N	66.9 a	72.0 ab	76.0 b	79.6 b	12.8 d	9.325 d
MA	68.7 b	72.9 c	75.7 a	79.2 a	10.5 c	10.225 e
ETS	70.6 d	71.6 a	78.0 d	81.0 d	10.4 c	6.315 a
ETS → MA	73.4 f	76.5 e	79.4 f	81.9 e	8.5 ab	8.485 b
N → BE	67.6 a	72.6 bc	76.0 b	79.9 c	12.3 d	10.695 f
MA→ BE	69.6 c	74.1 d	76.2 c	79.7 bc	10.0 c	10.885 f
ETS → BE	71.8 e	74.7 d	78.2 e	81.0 d	9.2 b	8.610 bc
ETS → MA → BE	73.4 f	76.2 e	79.4 f	81.8 e	8.3 a	8.935 c

Letters within each column denote significant difference (P<0.05).

5.4.8 Rate of digestion

In order to evaluate the susceptibility to amylolytic digestibility of all modified starches in their granular state, the rate of digestion (k-value) by PPA and AMG was applied. MA, ETS and ETS→MA starches showed higher amylolytic susceptibility than the native rice starch (Table 5.5). Among the pre-treatments, the highest k-value was found for ETS treated starch (5.4×10^{-3} min^{-1}) demonstrating very high amylolytic susceptibility for this starch. Based on the swollen nature of these granules, this effect was expected.

As expected from the action of BE following the different pre-treatments, all modified starches exhibited lower k-value than their corresponding

pre-treated controls. However, substantially decreased amylolytic susceptibility were found for the ETS→BE and the ETS→MA→BE samples, showing k-values of 4.6 x 10^{-3} and 3.7 x 10^{-3} min^{-1}, respectively, a reduction by 14.8% and 9.8% respectively due to the activity of the BE (Table 5.5). Again, these results demonstrate that the ETS pre-treatment generates a granular structure suitable for further BE catalysis. This effect is possibly attributed to synergistic effect of ethanol-heat induced swelling, creating disordered structures, readily attached and trimmed by MA leading to a more compact structure. These results are in agreement with the α-1, 4 : α-1, 6 ratio for ETS→MA→BE sample, being very high (6.5%, Table 5.2).

Table 5.5 Digestion rate constant (k) of native and starch treated samples.

Samples	**k (min^{-1}) x 10^{-3}**
N	2.4 ± 0.1 a
MA	2.6 ± 0.3 a
ETS	5.4 ± 0.1 e
ETS → MA	4.1 ± 0.2 c
N → BE	2.3 ± 0.3 a
MA→ BE	2.5 ± 0.1 a
ETS → BE	4.6 ± 0.1 d
ETS → MA → BE	3.7 ± 0.1 b

Letters within each column denote significant difference (P<0.05).

5.5 Conclusions

Pre-treatment of granular rice starch effectively provided a better substrate for BE catalysis. Especially, ETS had a substantial influence on the morphology, generating coapted granules with high swelling capacity, decreased the crystallinity and enthalpy and susceptible to amylolytic digestion. On the other hand, MA increased the specific surface area, relative crystallinity, gelatinization temperature and enthalpy. Combination of ETS→MA exhibited synergistic effects which resulted in highly porous granules, increase in the specific surface area, α-1,6 glucosidic linkage ratio, gelatinization temperature and the enthalpy. All of the pre-treatments led to decrease pasting peak viscosity, breakdown, final viscosity and setback value. BE catalysis increased the specific surface area, crystallinity, α-1,6 glucosidic linkage ratio and the enthalpy of all prepared starches. However, the peak viscosity, final viscosity and the swelling capacity were lower and the pre-treated starches showed less susceptibility to amylolytic degradation following BE modification. Our study provides protocols for pre-treatment granular starch to enhance subsequent BE catalysis, generating beneficial physicochemical properties that can be used in food applications.

5.6 Acknowledgements

This research was financially supported by the Royal Thai Government Scholarship (Ministry of Science and Technology), Thailand and Archer Daniels Midland (ADM) Company, Decatur IL, USA. Novozyme A/S is acknowledged for providing MA and BE.

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CHAPTER VI

SUMMARY

The production of porous rice starch was obtained by using AMG and MA separately. The hydrolytic effects of AMG and MA were compared. Both enzymes created pores at granular surface. AMG-treated starch presented big and shallow pores whereas MA-treated starch displayed small and deeper pores. The range of pore diameters of MA treatment was narrower than that of AMG. The structural changes were clearly observed in both the amorphous and crystalline parts. Specifically, MA lead to increase the short amylopectin chains and solubilization whereas AMG showed an opposite trend. However, both treatments improved the pasting properties by decreased peak viscosity, final viscosity, breakdown and setback values of treated starch. These finding demonstrate that different actions of AMG and MA can produce porous rice starch with various functionalities. Moreover, MA provides a novel alternative enzyme for producing porous starch.

The multicycle (7 repeated cycles) ultrasound-assisted ice recrystallization (US+IR) following treatment with AMG or MA to produce porous rice starch was investigated. This study has found that generally US+IR treated starch exhibited more grooves and shallow indentations on granular surface and decreased the amylose content, relative crystallinity, swelling capacity and the enthalpy of gelatinization. This suggest that a synergistic effect of ice crystal growth in starch granules and the shear force by ultrasonic treatment caused the roughness, surface indentation, weaken double

helices, decreased crystalline region of starch. In addition, the combined effects of US+IR and AMG or MA displayed holes and fissure on granular surface and increased the specific surface area but reduced susceptibility to amylolytic enzymes. Sequential US+IR→MA treatment increased the crystallinity and the enthalpy more than US+IR→AMG. However, US+IR combined with AMG or MA obviously changed the properties of starch, resulting in a reduction of amylose content, swelling capacity, molecular weight, peak viscosity, breakdown, final viscosity and setback value. These results could apply for providing an alternative method to produce porous starch.

The pre-treatments for further BE catalysis were carried out to open-up the starch granules. The ethanol-heating (ETS), MA and sequential ETS→MA were prepared. The results of this investigation show that ETS generated coapted granules with no pores. MA created fissure and some pores on granular surface whereas the ETS→MA exhibited more porous granules and increased the specific surface area. All pre-treatments decreased pasting peak viscosity, breakdown, final viscosity and setback value. However, all of pre-treatments had increased the specific surface area, crystallinity, α-1,6 glucosidic linkage ratio, the enthalpy and less susceptibility to amylolytic degradation after BE catalysis. The most obvious changes on structural and properties of pre-treatments is that ETS increased the swelling capacity, decreased the crystallinity, enthalpy and susceptible to amylolytic digestion. Our data demonstrate that ETS→MA treatment provides an efficient protocol for further BE catalysis.

APPENDIX

SUPPLEMENTARY DATA

CHAPTER III

3.4.8 Thermal properties

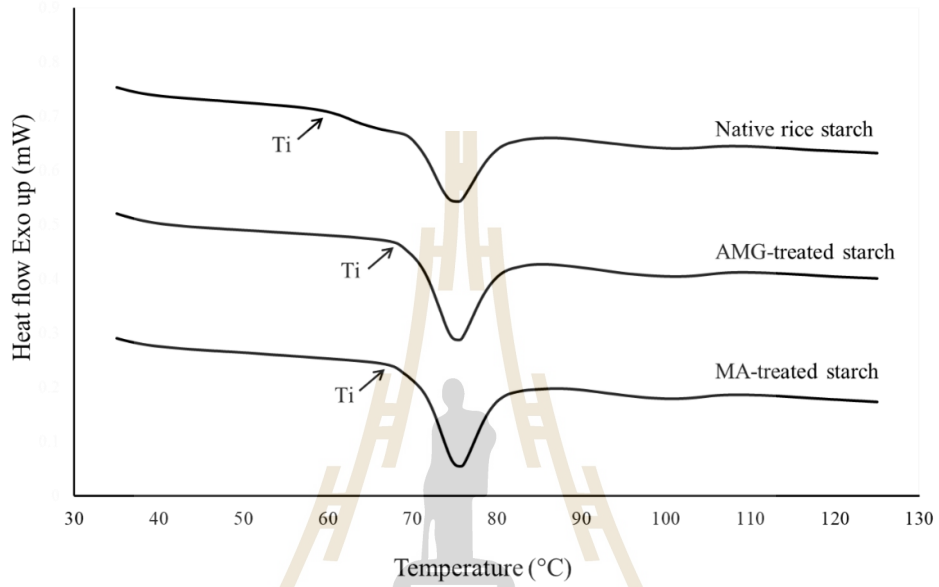

Figure S1 DSC curve of native and enzymatically treated starch after 24h modification. Ti = initial temperature.

CHAPTER IV

4.4.4 Starch molecular weight fractions

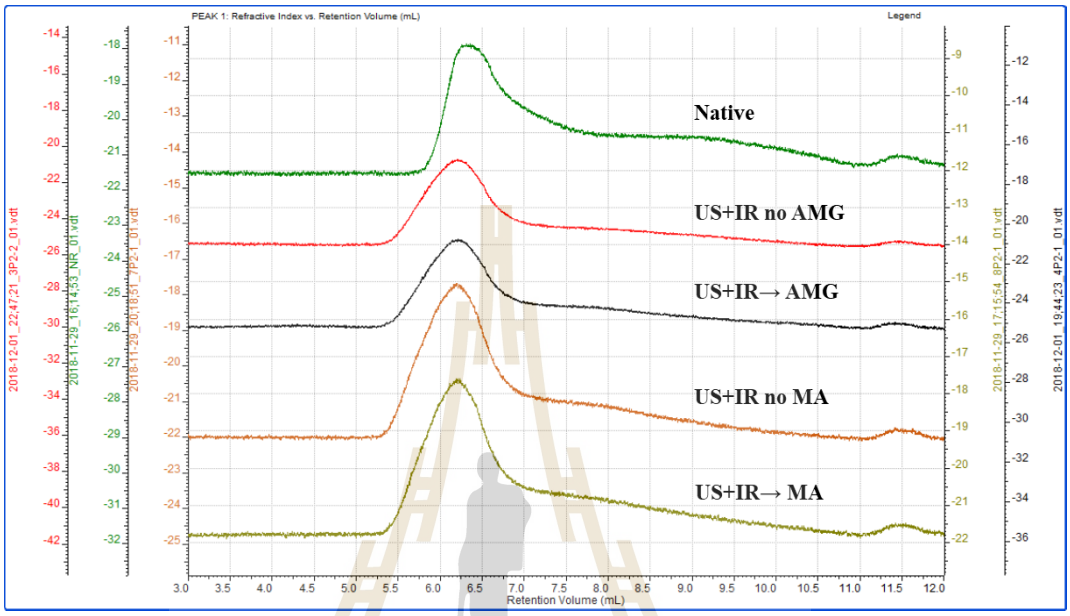

Figure S2 HPLC chromatograms of molecular weight fractions of native and starch treated samples.

BIOGRAPHY

Thewika Keeratiburana was born in October 12, 1986, at Ubon Ratchathani, Thailand. She studied for high school diploma at Lukhamhanwarinchamrab School (1999-2001) and Benchama Maharat School (2002-2004). In 2009, received the degree of Bachelor of Science (Food Technology) with Second Class Honours from Khon Kaen University. In 20011, she received the degree of Master of Science (Food Technology) from Khon Kaen University.

After she finished her Master study, she has worked as a Lecturer at Buriram Rajabhat University, Thailand. In 2015, She received a scholarship from the Royal Thai Government Scholarship (Ministry of Science and Technology) and studied the doctoral degree at Suranaree University of Technology. In 2017, she has enrolled as a double degree PhD student at Department of Plant and Environmental Sciences, University of Copenhagen, Denmark. The agreement on the double degree PhD programme is made together with Suranaree University of Technology, School of Food Technology, Thailand. During her Ph.D. study, she presented "Functionality of porous high amylose rice starch obtained by amyloglucosidase and maltogenic alpha-amylase", Starch Round Table, October 18-20, 2018, Norwich, UK (Oral presentation and poster). She also presented "Porous high amylose rice starch prepared using amyloglucosidase and maltogenic alpha-amylase", Cereals 18: 2018 AACC International Annual Meeting, October 21-23, 2018, London, UK. (Poster).