PRODUCTION OF RESISTANT STARCH TYPE III (RS3) FROM ANTHOCYANIN-RICH FLOUR

METHUS CHUWECH

DOCTOR OF PHILOSOPHY
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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

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THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

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Methus Chuwech

หัวข้อดุษฎีนิพนธ์	การผลิตสตาร์ชทนย่อยชนิดที่ 3 จากแป้งที่มีแอนโทไซยานินสูง
ผู้เขียน	นายเมธัส ชูเวช
ปริญญา	ปรัชญาดุษฎีบัณฑิต (เทคโนโลยีชีวภาพ)
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	ผศ. ดร. จิดาภา ทิน้อย อาจารย์ที่ปรึกษาร่วม

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตสตาร์ชทนย่อยชนิดที่ 3 จากแป้งที่มีแอนโทไซยานินสูง วัตถุดิบที่ใช้ในการศึกษา ได้แก่ ข้าวก่ำ มันม่วง และข้าวโพดม่วง การทดลองเริ่มจากการนำวัตถุดิบทั้ง 3 ชนิดที่มีแป้งเป็นองค์ประกอบหลัก โดยนำส่วนของแป้งมาผ่านกระบวนการสกัดโปรตีนและไขมันออกได้เป็นสตาร์ช เมื่อศึกษาองค์ประกอบทางเคมี พบว่า สตาร์ชทั้ง 3 ชนิดมีปริมาณคาร์โบไฮเดรต 80.28-81.52% ปริมาณอะมัยโลสในสตาร์ชมันม่วงสูงที่สุดคิดเป็น 20.64% รองลงมาคือสตาร์ชข้าวก่ำคิดเป็น 9.36% และต่ำสุดคือสตาร์ชข้าวโพดม่วงคิดเป็น 6.27% สตาร์ชทั้ง 3 ชนิดจะถูกใช้ในการศึกษาขั้นตอนต่อไป

จากการศึกษาการผลิตสตาร์ชทนย่อยชนิดที่ 3 ด้วยวิธี dual-autoclaving retrogradation treatment (DART) พบว่า ปริมาณสตาร์ชทนย่อยของสตาร์ชข้าวก่ำ (ร้อยละ 34.13) มีค่าสูงกว่าสตาร์ชมันม่วง (ร้อยละ 26.15) และสตาร์ชข้าวโพดม่วง (ร้อยละ 20.73) การศึกษาการผลิตสตาร์ชทนย่อยชนิดที่ 3 ด้วยวิธี dual enzyme treatment (DET) ได้ศึกษาภาวะที่เหมาะสมของเอนไซม์ α-amylase พบว่า ระยะเวลาการย่อย 15 นาที ค่า pH 5.5 อุณหภูมิ 90 องศาเซลเซียส ความเข้มข้นของเอนไซม์ 4 ยูนิตต่อกรัม และปริมาณสตาร์ชเริ่มต้นร้อยละ 25 (w/v) สำหรับสตาร์ชข้าวโพดม่วงและสตาร์ชข้าวก่ำร้อยละ 20 (w/v) สำหรับสตาร์ชมันม่วง ขณะที่ภาวะที่เหมาะสมของเอนไซม์พูลลูลาเนส พบว่า ระยะเวลาการย่อย 16 ชั่วโมง ค่า pH 4.5 อุณหภูมิ 50 องศาเซลเซียส ความเข้มข้นของเอนไซม์สูงสุด 12 ยูนิตต่อกรัม สำหรับสตาร์ชข้าวโพดม่วง 10 ยูนิตต่อกรัม สำหรับสตาร์ชข้าวก่ำ และ 8 ยูนิตต่อกรัม สำหรับสตาร์ชมันม่วง เมื่อนำสตาร์ชทั้งหมดไปผ่านกระบวนการเกิดรีโทรเกรเดชันที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง พบว่า ปริมาณสตาร์ชทนย่อยสูงสุดของสตาร์ชข้าวโพดม่วง (ร้อยละ 54.27) มีค่าสูงกว่าสตาร์ชมันม่วง (ร้อยละ 53.36) และสตาร์ชข้าวก่ำ (ร้อยละ 51.34) ซึ่งการดัดแปร

สตาร์ชทั้ง 2 วิธี พบว่า มีปริมาณสตาร์ชทนย่อยที่สูงขึ้นอย่างมีนัยสำคัญ (p<0.05) เมื่อเปรียบเทียบกับสตาร์ชก่อนการดัดแปร

จากการศึกษาลักษณะทางโครงสร้าง สมบัติทางกายภาพ สมบัติทางความร้อน และความสามารถในการต้านอนุมูลอิสระ พบว่า สตาร์ชทนย่อยมีลักษณะเม็ดสตาร์ชที่เปลี่ยนแปลงไป การพองตัว การละลายและความหนืดลดลง พลังงานที่ใช้ในการเกิดเจลาทิไนเซชัน (ΔH) เพิ่มสูงขึ้น และ พบว่า รูปแบบผลึกของสตาร์ชทนย่อยทั้ง 3 ชนิดที่ผ่านการเกิดรีโทรเกรเดชันเปลี่ยนแปลงไปเมื่อเปรียบเทียบกับสตาร์ชก่อนการดัดแปร ความสามารถในการต้านอนุมูลอิสระด้วยวิธี DPPH, ABTS และ FRAP assay จากการศึกษา พบว่า สตาร์ชทนย่อยยังคงมีฤทธิ์ในการต้านอนุมูลอิสระลดลงเมื่อเปรียบเทียบกับสตาร์ชก่อนการดัดแปร นอกจากนี้ เมื่อจำลองสภาวะการย่อยอาหารในมนุษย์ พบว่า การเพิ่มขึ้นของสตาร์ชทนย่อยสามารถลดระดับการย่อยด้วยน้ำลายมนุษย์ สภาวะจำลองกรดในกระเพาะอาหารและสภาวะจำลองในลำไส้เล็ก แสดงถึงความสามารถในการเป็นเส้นใยอาหารได้

Dissertation Title	Production of Resistant Starch Type III (RS3) from Anthocyanin-Rich Flour	
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Degree	Doctor of Philosophy (Biotechnology)	
Advisory Committee	Asst. Prof. Dr. Nopakarn Chandet	Advisor
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ABSTRACT

This research study was aimed to investigate the production of resistant starch type III (RS3) from anthocyanin-rich flour. The raw materials including purple rice, purple sweet potato, and purple corn were studied. The study consisted of all 3 types of raw materials that contain flour as the main component. The purple flours through the process of extracting protein and lipid to be starch. The studied on the chemical composition, it was found that the carbohydrate content of all 3 types of starches was 80.28-81.52%. The highest amylose content in purple sweet potato starch (20.64%), followed by purple rice starch (9.36%) and purple corn starch (6.27%). The 3 types of purple starches were selected for further study.

The studied on the production of resistant starch type III (RS3), after modification by dual-retrogradation treatment (DART), the resistant starch (RS) content of purple rice starch (34.13 %) was higher than that of purple sweet potato (26.15%) and purple corn starch (20.73%). While the dual enzyme treatment (DET) modification was conducted to study the optimum conditions of the α-amylase enzyme, the optimum conditions revealed that the hydrolyzed time was 15 min, pH 5.5, temperature 90°C, enzyme concentration 4 U/g, and initial starch was 25% (w/v) for purple corn, and purple rice starch and 20% (w/v) for purple sweet potato starch. The optimum conditions of pullulanase were 16 h, pH 4.5, temperature 50°C, maximum enzyme concentration of 12 U/g for purple corn starch, 10 U/g for purple rice starch, and 8 U/g for sweet potato starch. After retrogradation at 4°C for 24 h, it was found that the maximum resistant starch content was

purple corn starch (54.27%) was followed by purple sweet potato (53.36%) and purple rice (51.34%). RS obtained from purple starches were significantly higher levels of RS content (p<0.05) compared with native purple starches.

The studied on the characterization of RS including structure by SEM, physical and thermal properties, and enthalpy of gelatinization (ΔH). It was found that the structure of starch granules was changed, swelling and solubility index decreased, the viscosity decreased. In addition, the crystalline pattern from XRD were changed when compared with native purple starch. For antioxidant activity assay, DPPH, ABTS, and FRAP assay were studied. From this study found that RS obtained from purple starches still had lower antioxidant activity compared with native purple starches. The % hydrolysis of RS obtained from purple starches by human saliva, gastric condition, and intestinal condition were decreased when compared to the native purple starches, the result showed that RS obtained from purple starches has similar physiological effects as dietary fiber.

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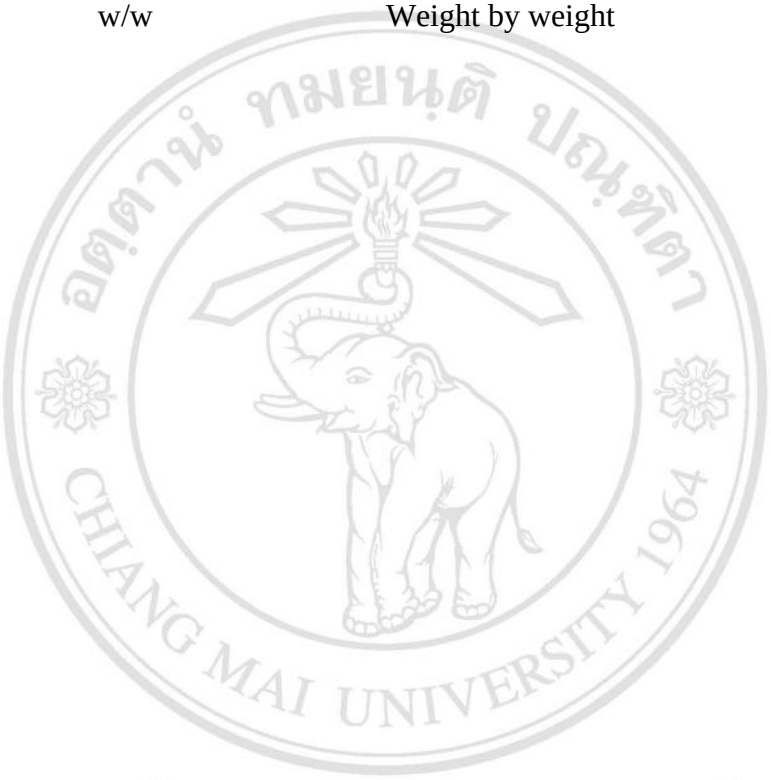
LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
AMG	Amyloglucosidase
cm	Centimeter
cP	Centipoise units
°C	Degree celsius
DART	Dual autoclaving-retrogradation treatment
DET	Dual enzyme treatment
g	G force
GAE	Gallic acid equivalence
GOPOD	Glucose oxidase and peroxidase
h	Hour
KU	Kilo units
µm	Micrometer
µL	Microliter
M	Molarity
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
Mw	Molecular weight
N	Normality
nm	Nanometer
No.	Number
PAA	Pancreatic α-amylase

LIST OF ABBREVIATIONS AND SYMBOLS

U	Unit of enzyme
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight



ข้อความแห่งการริเริ่ม

1) ในปัจจุบันนักวิจัยและโรงงานอุตสาหกรรมอาหารเริ่มให้ความสนใจและศึกษาเกี่ยวกับสตาร์ชทนย่อยชนิดที่ 3 มากขึ้น เนื่องจากสามารถทำหน้าที่เป็นใยอาหารได้ วิทยานิพนธ์นี้เป็นการศึกษาที่แสดงให้เห็นว่าข้าวก่ำ มันม่วง และข้าวโพดม่วง มีศักยภาพในการผลิตสตาร์ชทนย่อยชนิดที่ 3 โดยเปรียบเทียบการผลิตด้วยเทคโนโลยีที่เหมาะสมจำนวน 2 วิธีการ คือวิธีทางกายภาพ และวิธีทางเอนไซม์เทคโนโลยี
2) เพื่อเป็นข้อมูลวิทยาศาสตร์พื้นฐานเพื่อการส่งเสริมและพัฒนาสตาร์ชทนย่อยชนิดที่ 3 ช่วยให้เกิดความเข้าใจและสามารถนำไป ประยุกต์ในการผลิตสตาร์ชทนย่อยชนิดที่ 3 ที่เหมาะสม เพื่อใช้เป็นแหล่งของใยอาหาร และเป็นอาหารฟังก์ชันเพื่อสุขภาพ ซึ่งจะนำไปสู่การพัฒนานวัตกรรมใหม่ของผลิตภัณฑ์ แปรรูปจากข้าวก่ำ มันม่วง และข้าวโพดม่วง จึงเป็นทางเลือกหนึ่งของการเพิ่มมูลค่าของวัตถุดิบให้มีความหลากหลายมากขึ้น

STATEMENTS OF ORIGINALITY

1) Nowadays, many researchers and food industries have tended to pay attention on studying of RS3 because it is dietary fiber for health benefits. Therefore, this research was performed to study the production of RS3 from purple rice, purple sweet potato, and purple corn by comparing two methods between physical and enzymatical technology.
2) Results from this research can be used for more understanding of RS3 production with different methods and for further research and development of products from RS3 as functional foods. This will lead to the development of new product innovations which purple rice, purple sweet potato, and purple corn can be utilized as alternative materials for more value-added products.

CHAPTER 1

Introduction

1.1 Historical Background

The increasing awareness of consumers toward healthy and nutritious foods has encouraged manufacturers to produce functional foods with enhanced nutrition (Koirala and Anal, 2021; Koirala *et al*., 2022). Thai purple rice (*Oryza sativa* L.) is grown in North of Thailand and it has been recognized as a potential cereal grain that contains high amounts of bioactive compounds, which are as usual located in the bran layer (Yamuangmorn and Prom-u-thai, 2021). The two major anthocyanins found in the purple rice pericarp and aleurone layers, are cyanidin-3-glucoside and peonidin-3-glucoside. Moreover, phenolic acids are also found in the outer layers of purple rice grains (Jang and Xu, 2009). Purple potatoes, also known as purple sweet potatoes, are a one year or perennial herb in the family Convolvulaceae. Their flesh is purple to dark purple. Besides the nutrients in ordinary sweet potato, they are also rich in anthocyanins (Bovell-Benjamin, 2007). Purple corn, a special corn variety with high anthocyanin concentration in aleurone layer (Zhang *et al*., 2010), is reported to possess antioxidant ability higher than the non-colored corn (López-Martínez *et al*., 2011). In Thailand, the developed variety of purple corn presents dark purple color due to the considerable the high amount of anthocyanin, phenolic and antioxidant activity, leading to the increased consumption of waxy purple corn as fresh foods (Harakotr *et al*., 2014). It is considered a superfood to produce high value-added commercial products. However, studies on modification of starch from this purple rice, purple sweet potato, and purple corn variant to enhance their functional properties have not been extensively studied.

Starch is a vital carbohydrate for human health in nature. Based on its nutritional properties and release rate of glucose, starch can be classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Ali *et al*., 2014). Among these categories, RS has recently gained much attention in the food industry due

to its ability to enhance satiety, stabilize blood glucose and its important role in combating metabolic diseases and obesity. RS is any starch product that cannot be digested by α-amylase enzymes and absorbed in small intestine (Fuentes-Zaragoza *et al*., 2010). RS can be divided into five categories (RS1-RS5) based on the physicochemical and structural characteristics of starch granules, granularity, cooking operations and interactions with other components. RS1 is inaccessible starch entrapped within whole or partly milled grains or seeds and tubers among all types of RS (Eliasson, 2004). RS2 is a native starch with a B- or C-polymorph granule whose special structure makes them hydrolyze under stringent conditions. RS3 comprises retrograded starch formed during heating and aging. RS4 refers to the starches chemically modified or repolymerized, including conversion, substitution or cross-linking, which resist enzymatic hydrolysis because starch contains specific chemical linkages. RS5 is formed by the interaction of amylose starch with lipids, fats and surface-active agents (Raigond *et al*., 2014). RS3 is highly thermostable and is used as an ingredient in a wide variety of conventional foods (Hernández *et al*., 2008). It can be obtained by hydrothermal treatments and retrogradation of starch derived from cereal grains, roots, tubers and legumes, such as wheat, corn, oat, rice, potato, cassava, and mung bean. Among these starches, high amylose corn starch (HACS) is most often used for preparation of RS3 (Dimantov *et al*., 2004, Herman and Remon, 1989, Sievert and Pomeranz, 1989). The currently accepted mechanism by which RS3 resists amylase digestion is that linear amylose segments align into condensed double helical structures after gelatinization (amylose retrogradation). This arrangement renders α-1,4 glycosidic linkages inaccessible to amylase.

Different methods have been employed for the preparation of RS3. These methods include, but are not limited to, annealing and heat-moisture treatments (Zavareze *et al*., 2010, Juansang *et al*., 2012), chemical modification (Mutungi *et al*., 2010), enzymatic debranching (Miao *et al*, 2009), and temperature-cycled retrogradation (Ratnaningsih *et al*., 2019). Among these methods, physical modification of starch granules by heat and moisture is simple, cheap, and safe with improved water-solubility properties (Ashogbon and Akintayo, 2014), enzyme modification techniques have the advantages that the reaction conditions are mild, and do not require high-pressure and acid-resistant equipment, making it safe for the environment and consumers. Moreover, they have many typical reactions with few types of by-products formed by enzymatic processing (Zhang

and Jin, 2011a). Thermostable α-amylase cleaves α-1,4 glucans into linear chains that can rapidly decrease the viscosity of gelatinous starch solution and promote the production of soluble dextrin and oligosaccharides (Zou *et al*., 2019). Excessive hydrolysis by α-amylase can produce a small amount of glucose and maltose. Pullulanase (pullulan 6-glucanohydrolase) is a debranching enzyme used in starch modification processes. It can specifically cleave α-1,6 glycosidic linkages in pullulan, amylopectin and numerous related polysaccharides (Kim *et al*., 2014). Pullulanase has been used to produce high amylose starches, which have considerable commercial value (Shi *et al*., 2013). The increase in the starch branch density and the crystalline structure of starch by enzymatic modification both contribute to delayed digestion (Ao *et al*., 2007). Dual enzyme treatment of modified starch had higher RS contents than native corn starch (Zhang and Jin, 2011a).

Nowadays, purple rice (*Oryza sativa* L.), purple sweet potato (*Ipomoea batatas*), and purple corn (*Zea mays* L.) and have become popularly utilized as a food material due to the health promoting effects of anthocyanins and phenolic compounds (Hiemori *et al*., 2009). Anthocyanins and phenolic compounds have been recognized as a health enhancing substances due to their antioxidant, anti-inflammatory, anti-atherosclerosis, anti-cancer, hyperlipidemia, and hypoglycemic effects (Abdel-Aal *et al*., 2006; Guo *et al*., 2007; Hiemori *et al*., 2009).

Anthocyanins are water-soluble plant pigments that belong to flavonoids molecule. They are purple, blue, and red color substances found in plant tissues. The appearance of these colors depends on pH values. Anthocyanins are existed in pericarp, seed coat and aleurone layer of rice grains. Cyanidin-3-glucoside, peonidin-3-glucoside and cyanidin chloride were identified as anthocyanins, which are found in purple rice, purple sweet potato, and purple corn. A majority of phenolic compounds were identified as ferulic, protocatechuic, *p*-coumaric and vanillic acid which were found in purple rice, purple sweet potato, and purple corn (Shao *et al*., 2018; Jing and Giusti, 2005; Nems *et al*., 2015). Anthocyanins and phenolic compounds possess antioxidative activities as well as other biological activities such as, inhibition of allergic reaction, reduction of development of cardiovascular diseases, demonstrating antimutagenic activities, and anticancer activities (Sompong *et al*., 2011; Tananuwong *et al*., 2010; Park *et al*., 2008).

However, only a few studies have been conducted to produce RS3 from those anthocyanin-rich flour and a limited number of studies on the preparation of RS3 by dual autoclaving-retrogradation treatment (DART) and dual enzyme treatment (DET) on the formation of RS from purple starches was not reported previously. This problem leads to the search for alternatives to make RS3 into food products by using purple rice, purple sweet potato, and purple corn and as raw material while retaining its color with the antioxidant activity, phenolic compounds and eventually turns into RS3 as the final product.

The objective of this research was to explore the effect of the DART and DET for RS3 production. In this study, purple starches such as purple rice starch, purple sweet potato starch, and purple corn starch subjected to DART and DET were investigated. DART was carried out for the preparation of RS3 by using autoclave and DET modification by using thermostable α-amylase and pullulanase were investigated. Factors affecting enzymatic modifications and retrogradation were optimized by a single factor test on the digestion resistibility. The chemical composition, the RS content, the characterization, bioactive compounds, and *in vitro* starch digestibility will be determined. The results of this study may provide an alternative route for preparation of RS-rich products.

1.2 Starch Granular Structure

Starch granules are composed of two major types of polysaccharides, amylose, and amylopectin but the ratio varies regarding the botanical sources of starch. Amylose comprises linear molecules with α-D-(1,4) glycosidic linkages and some of a few branches, which locates in the amorphous domains. Amylopectin is highly branched molecules with α-D-(1,4) glycosidic-linked short linear chains connected by α -D-(1,6) glycosidic linkages and form the crystalline layers (Boonna, 2016). Amylopectin has a higher molecular weight than amylose (MW=10^7-10^8 for amylopectin and 10^4-10^6 g/mole for amylose) (Mischnick and Momcilovic, 2010). The branch chains of the amylopectin are packed into a semi-crystalline structure of double helices in the starch granules (Jane and Pyun, 1997) (Figure 1.1). The levels of branching points are less dense and ordered (amorphous), whereas crystallinity results from clustered side chains with different lengths (Mischnick and Momcilovic, 2010; Boonna, 2016).

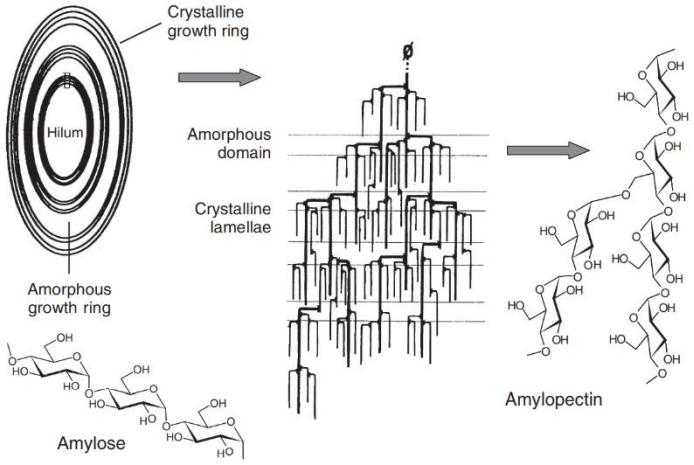


Figure 1.1 Starch granular structure
(Reproduced from Mischnick and Momcilovic, 2010)

The semi-crystalline structures of starch are separated into A-, B-, polymorphs which differ in their packing of double helices and water content (Figure 1.2). The A-type polymorphic crystallizes in a monoclinic unit cell with 4 water molecules per unit cell, which is closely packed and found in cereal starches. In contrast, the B-type polymorphic crystallizes in a hexagonal unit cell, which is relatively loosely packed with an open channel of 36 water molecules in the unit cell, found in many tuber starches and in high amylose starches (Imberty *et al*., 1991). The C-type polymorph is a mixture of A- and B-type polymorphs, which found in legume starches and some tuber starches. In addition, single helices of amylose, which are co-crystallized with other compounds such as iodine, DMSO, alcohol or fatty acid (Sajilata *et al*., 2006), is considered as the V-type polymorph. The different crystalline polymorphs of starch have been identified based on X-ray diffraction patterns (XRD) and illustrated in Figure 1.3. The branching points of the B-type polymorphic amylopectin are mostly located in the amorphous regions, whereas the branching points of the A-type counterpart are scattered in both amorphous and crystalline regions (Jane and Pyun, 1997). As a result of the A-type starch granules show more pinholes on the surface and serpentine-like channels inside the granules than the B-type starch granules (Fannon *et al*., 1992; Huber and BeMiller, 2000), the A-type starch granules are easily hydrolyzed by enzyme than the B- and some C-type starch granules.

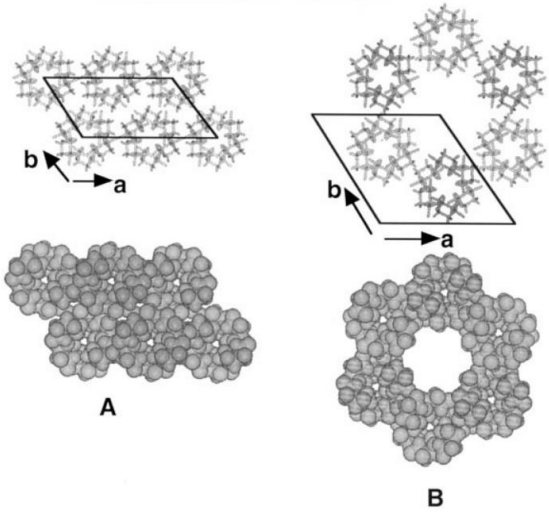


Figure 1.2 A- and B-type polymorphs of amylose (Buléon *et al*., 1998)

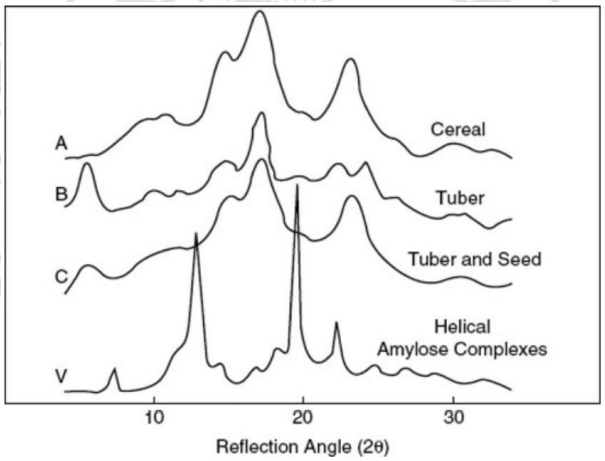


Figure 1.3 X-ray diffractograms of different starches. Labeling refers to (A); A-type from cereal starches, (B); B-type from tuber starch, (C); C-type from seed starches, and (V); V-type from helical amylose complexes (Reproduced from Zobel, 1988)

1.3 The Gelatinization and Retrogradation of Starch

Starch gelatinization is an irreversible process that occurs when starch was heated in a presence of enough water. During heating, the water acts as a plasticizer, is first absorbed in the amorphous regions, leading to granular swelling. This provides sufficient stress connectivity of amorphous regions to tightly bound areas of double helical structures of amylopectin, resulting in the disruption of starch crystallites. The soluble amylose molecules leach into the surrounding water and the granule structure

disintegrates, as evidenced by the loss of birefringence under the microscope with polarized light (Jenkins and Donald, 1998). Normally, differential scanning calorimetry technique is used to observe this endothermic gelatinization phenomenon. The gelatinization temperature of starch is generally dependent on the starch source and amylose content (Boonna, 2016).

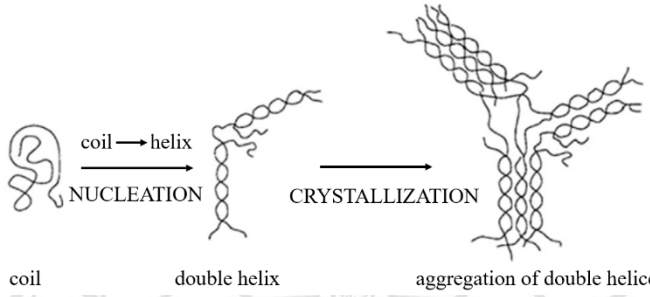


Figure 1.4 Conformational changes during retrogradation (Colonna *et al*., 1992)

Starch retrogradation or recrystallization is a physical process that follows starch gelatinization in which starch molecules (both amylose and amylopectin) can re-associate to form ordered structures of double helices and crystallites during cooling and storage. The typical conformation changes of amylose during retrogradation are illustrated in Figure 1.4. In general, amylose in aqueous solution exists as a random coil. When cooling and storage, amylose can re-crystallize into either A- or B-type double helices which are a spontaneous process. The metastable state with lower free energy occurs. Then, the double helices are infinitely aggregated to form a three-dimensional network with different microstructure features, i.e. crystallinity and porosity (Zhang *et al*., 2015). The storage time and temperature are critical factors influencing the formation of retrograded starch in an excess water. Normally, the crystallization of starch which is a partially crystalline polymer system is governed by glass transition temperature (T_g) and melting temperature of crystal (T_m). The crystallization comprises three steps, including nucleation, propagation, and maturation. The nucleation rate is favored at a temperature near the T_g whereas a higher temperature closed to T_m favored the propagation rate. The maturation rate is dependent on temperature similar to that of propagation rate. The overall recrystallization rate depends mainly on the nucleation and propagation rates (Figure 1.5). For partially crystalline polymer system, crystallization occurred only at a temperature between glass transition temperature (T_g) and melting temperature of crystal (T_m) (Levine and Slade, 1998).

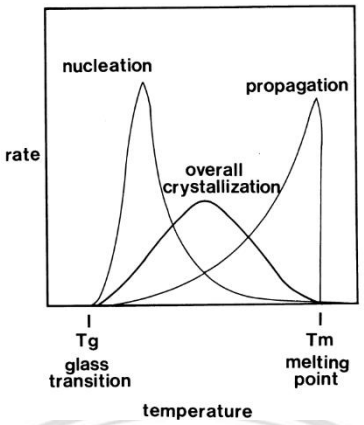


Figure 1.5 Temperature dependence of the nucleation, propagation, and overall crystallization rates according to crystalline polymer system (Eerlingen *et al*., 1993a)

1.4 The Nutritional Classification of Starch

For nutritional characteristics, starch is classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Figure 1.6), according to the rate of glucose release and its absorption in the gastrointestinal tract (Englyst *et al*., 1992). In a recent physiological classification, the fractions RDS and SDS are grouped as glycemic or available carbohydrates, whereas RS is regarded as a non-glycemic carbohydrate (Cummings and Stephen, 2007; Englyst *et al*., 2007; Boonna, 2016).

1.4.1 Rapidly Digestible Starch

Rapidly digestible starch (RDS) is the fraction of starch that causes a sudden increase in blood glucose level after ingestion (Cummings *et al*., 1996). Normally, it consists mainly of amorphous and dispersed starch. *In vitro* experiment of Englyst *et al*. (1992), it is converted to the glucose molecules in 20 min of enzyme digestion. RDS is found in freshly cooked starchy foods such as mashed potatoes and bread. In this case, starch granules are gelatinized and are more accessible to enzymatic digestion. The rapid increases in blood glucose and insulin levels caused by RDS contribute to several health complications, such as diabetes and cardiovascular disease (Brennan, 2005).

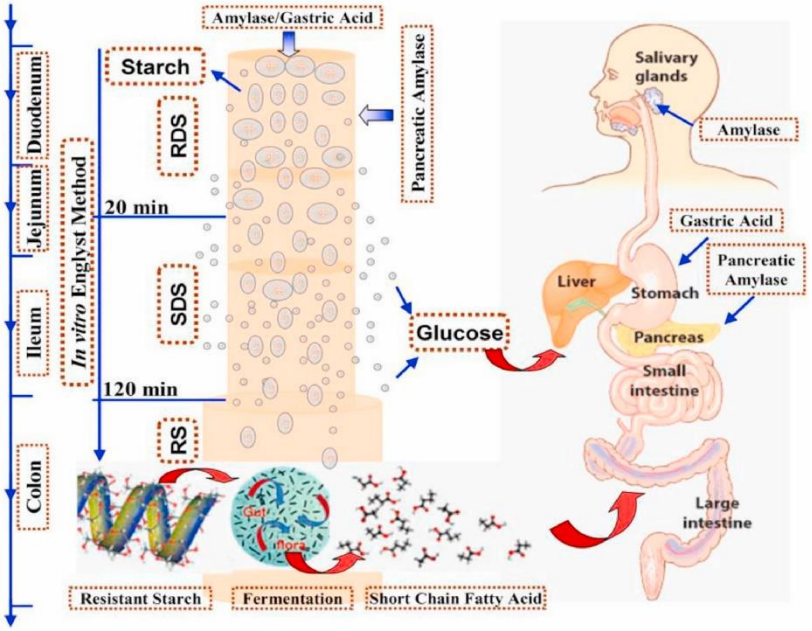


Figure 1.6 Schematic diagram of starch digestion in the human gastrointestinal tract (Liu *et al*., 2017a)

1.4.2 Slowly Digestible Starch

Slowly digestible starch (SDS) is a starch fraction that is completely digested in the small intestine at a lower rate as compared to RDS. Therefore, the potential health benefits of SDS are linked to a stable glucose metabolism, diabetes management, mental performance, and satiety (Lehmann and Robin, 2007). The benefit of products rich in SDS is their moderate impact on the glycemic index (GI). Normally, GI is often used as a measurement of the level postprandial glucose in the food, which can assess the effects of certain food or glucose.

1) The Structure and Formation of SDS

As mentioned, starch granules have a complex and highly ordered semi-crystalline structure. At the molecular level, the crystalline structure and the packing of the amorphous phase influence the enzymatic susceptibility. The A-type starch with shorter double helices are more readily digestible and show a high amount of RDS and SDS compared to B-type starches, which often contain a high amount of RS (Jane and Pyun, 1997). Generally, tuber starches are more resistant to enzymatic hydrolysis than

cereal starches due to a higher granule surface, their surface properties, the channel in cereal starches and the supramolecular arrangement (Lehmann *et al*., 2007). Guraya *et al*. (2001) reported that the SDS fraction of debranched waxy rice starch might result from the formation of imperfect B-type crystallites with lower density, which are more prone to digestion. Moreover, the study of Shin *et al*. (2005) also confirmed that the SDS fraction of debranched waxy sorghum starch might consist of less perfect crystallites and amorphous components. Chung *et al*. (2006) described that the proportion of SDS depends mainly on the rigidity of the amorphous regions in the retrograded starch gel. Zhang *et al*. (2006) studied on the slow digestion property of native cereal starches and proposed that the densely packed of amorphous regions and arranged tightly to the crystalline regions may inhibit a susceptible of an enzyme to hydrolysis.

The formation of SDS involves the step of gelatinization, debranching and retrogradation process similar to the RS formation. More recently, the production of SDS based on physical, chemical, and enzymatic treatments has been reported. According to Guraya *et al*. (2001), SDS formation was most favored with partial debranching of starch. In 2003, Shi *et al*., patented the technology to produce SDS by using enzymatically debranching amylose-containing starches and followed by recrystallization to a highly crystalline form. In addition, Shin *et al*. (2004) observed an increase in SDS up to 22% in cooked waxy sorghum starch after debranched with iso-amylase. Moreover, SDS was also efficiently generated by hydrolysis of starches with α-amylase and followed by partial crystallization of the resulting linear chains (Hamaker and Han, 2004). Hydrothermal treatment has been used to enhance the formation of SDS. According to Niba (2003), the SDS yield of cocoyam, maize, potato and rice starch was increased after heat treatment as compared to the raw starch. The storage at room temperature significantly decreased SDS content, whereas frozen storage increased the SDS yield for maize, potato and yam starches. The heat-moisture treated rice starch showed a slower digestion pattern than that for the native starch (Anderson *et al*., 2002). Furthermore, Shin *et al*. (2005) reported that hydrothermal treatment could be increased the SDS content as compared to the raw starch.

1.4.3 Resistant Starch

Resistant starch (RS) is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Euresta, 1992). RS escapes digestion in the small intestine and is fermented in the large intestine. Several physiological effects have been attributed to RS, which are the prevention of colonic cancer, hypoglycemic effects, hypocholestrolemic effects, prebiotic, inhibition of fat accumulation, and mineral absorption (Ashwar *et al*., 2016a). The product of the fermentation by human colonic bacteria is short-chain fatty acids (SCFA) such as acetate, butyrate, propionate and gasses, CO_2, H_2 and CH_4 (Topping *et al*., 2008). However, butyrate plays an important role in suppressing tumor cells and decreasing the proliferation of colonic mucosal cells.

1) The Classification of RS

According to the mechanism that prevents the enzymatic digestion, RS can be categorized into four types (Englyst *et al*., 1992). RS type I (RS1) represent physically inaccessible to pancreatic α-amylase, which is entrapped within whole or partly milled grains or seeds. RS type II (RS2) is a native granular starch found in food containing uncooked starch (i.e. green banana starch, potato starch, and high amylose maize starch). RS type III (RS3) comprises of retrograded starch formed during processing. RS type IV (RS4) is a group of chemically modified starches such as oxidized starch and cross-linking starch. However, in recent year, amylose-lipid complexes was added to the list of RS as RS type V (RS5), which is the inclusion complexes form between starch and lipids (Topping *et al*., 2010; Boonna, 2016).

2) The Structure and Formation of RS3

RS3 is considered as a retrograded starch, which precipitated from starch pastes or gels after gelatinization and cooling/storage. Some parts of RS3 undergo enzymatic hydrolysis. However, the major part typically behaves resistance to the hydrolysis of amylolytic enzymes. Eerlingen *et al*. (1993b) proposed two possible models of RS3 formation in aqueous amylose solutions, which were micelle form and lamellar structure (Figure 1.7). For the micelle form, it was formed by aggregation of a number of different molecules over a particular region of the chain in an ordered structure

interspersed with an amorphous region. In the case of retrograded amylose, the micelles must be composed of double helices in a hexagonal structure to show a B-type of X-ray diffraction pattern. The folding of the polymer chain led to two-dimensional structures or lamellar shapes. The regions of the folding were amorphous whereas the center of the lamella was crystalline. It demonstrated that hydrolysis with amylolytic enzymes could remove these folding regions, and the molecules with short chain were obtained.

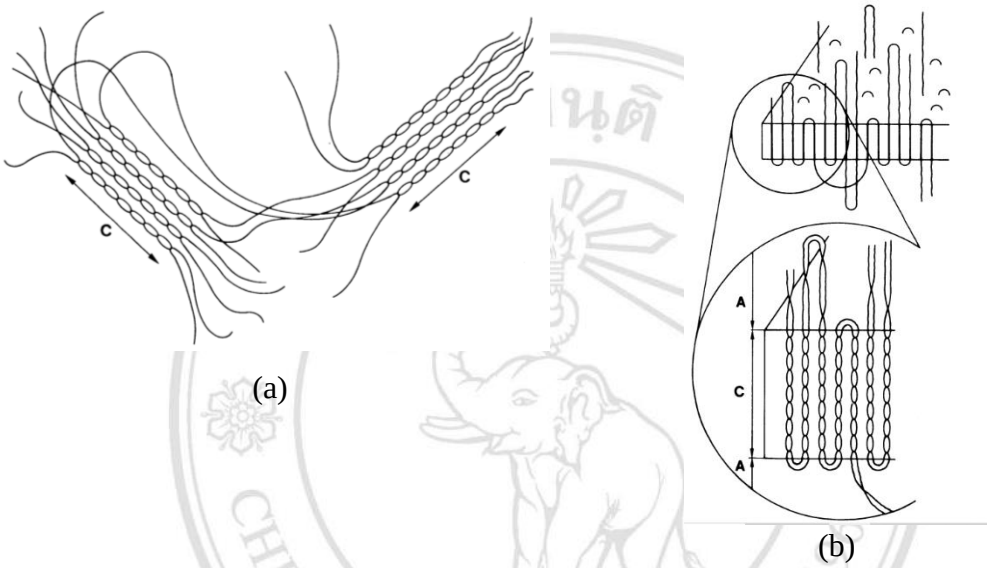


Figure 1.7 The formation of RS in amylose solutions. (a); Micelle model and (b); Lamellar model (Adapted from Eerlingen *et al*., 1993b)

RS3 is formed when the linear glucan of gelatinized starch retrogrades by realigning into double helical strands, and then aggregation there after (Eerlingen and Delcour, 1995). It is known that amylose content was correlated to RS3 yield (Sievert and Pomeranz, 1989). The long-branch chains of amylopectin have properties similar to amylose, which increase the apparent amylose content of the starch. On the contrary, the short-branch chains of amylopectin form double helices that are not long enough to produce stable crystallites.

RS3 contains mainly retrograded amylose (Eerlingen *et al*., 1993a) which consists of short linear segments of α-1,4-glucans arranged in a crystalline structure. Thus, starch with large amounts of amylose and/or long branch chains of amylopectin, such as legume and high-amylose starches (high amylose maize starch), has

been widely used as a starting material to produce RS3 over the last decades (Sievert and Pomeranz 1989; Vasanthan and Bhatty 1998), which is expensive.

Many technologies have been developed to produce RS3 from a group of amylose containing starches, including repeated cycles of autoclaving and heating together with complete debranching. Due to the interference of amylopectin during amylose retrogradation, debranching enzymes, such as iso-amylase and pullulanase was applied to hydrolyze α-D-(1,6) glycosidic branching of amylopectin in order to produce linear molecules that can re-associate, leading to decreasing the susceptible to hydrolysis with amylolytic enzymes. The mild acid hydrolysis of starch is another method to increase the linear chains as well. It hydrolyzes the amorphous regions of the starch granules where the branching points of amylopectin are mostly located, then promoting amylose retrogradation (Vasanthan and Bhatty, 1998; Guraya *et al*., 2001). According to Mutungi *et al*. (2011), the acid treatment of cassava starch before debranching process increased the fraction of linear glucan comprising DP 13-30, resulting in the improvement of RS3 content. Schmiedl *et al*. (2000) reported that DP of chain length about 20-35 was optimal for the formation of RS3 with a high yield and enhanced thermal stability.

The retrogradation or recrystallization conditions also affected the RS3 formation. An increase in starch concentration could increase the RS3 content (Lehmann *et al*., 2002; Schmiedel *et al*., 2003). With respect to storage temperature, Eerlingen *et al*. (1993a) found that isothermal formation of RS3 is favored at 100°C while Eerlingen (1994) reported that incubation at a lower temperature and extended periods of time also produced a higher RS3 yield. A higher temperature generally favored the formation of the more stable A-type rather than B-type starch polymorph (Gidley and Bulpin, 1987). Temperature cycle aging is another method was applied to accelerate the retrogradation and to improve the thermal properties of starch. This method involves a series of temperature and time to induce retrogradation. Haynes *et al*. (2000) demonstrated that the production of particularly thermostable RS3 with peak temperature above 140°C was induced by temperature cycling. The nucleation temperature was 60°C and the propagation was 120°C and was produced RS3 with 35% total dietary fiber (TDF). In 2007, Leong *et al*. demonstrated that the debranched sago starch subjected to temperature cycling and incubation at a series of temperature and time could induce the yield of RS3.

Besides, Park *et al*. (2009) found that storage of gelatinized waxy maize starch at the cycled temperatures of 4°C and 30°C induced a greater amount of RS3 and reduced the *in vitro* glycemic index more effectively than the isothermal storage condition at 4°C. Furthermore, the starch crystal formed under temperature-cycled storage melted at a higher onset temperature (T_o) than those formed at isothermal storage.

The crystalline polymorphic structures of starch are distinguished from each other by several characteristics and properties such as crystallization temperature, molecular structure and amount of bound water where the diversity of polymorph has more influence on thermal and functional properties of RS3. The physical modification by hydrothermal treatments, namely annealing and heat-moisture treatment (HMT) is used to modify the structure of starch because it is consistent with society trends toward natural products and offers the potential to change starch functionality at a low cost and environmental friendly way. Moreover, it also showed an impact on the RS3 formation. According to Jacobasch *et al*. (2006) studied the effect of hydrothermal treatment of novelose 330 (commercial RS3) on the yield and prebiotic properties, they found an increase in the yield of RS3 after subjecting to annealing and HMT up to 75% (as measured by *in vitro* method of Englyst *et al*., 1992) with peak temperature above 120°C. HMT provides a method for the economical production of a high-quality RS3 with dominated prebiotic properties in the distal colon for the health-promoting application. Furthermore, the study of Muntungi *et al*. (2009) showed that time-temperature cycling aging at a temperature of 120/60°C of debranched cassava starch further subjected to HMT increased RS3 content up to 88% and its melting temperature was also improved.

1.5 Production of RS3 by Autoclaving-Cooling Cycles

A starch suspension will form a gel upon heating to constant temperature. When cooled, the gel will turn into a partially crystalline structure. This physical modification is an autoclaving-cooling process that transforms native starch into RS3. The autoclaving process involves heating starch in excess water. During the process, the starch will gelatinize and undergo disruption of its granular structure. It is well-documented that the gelatinization temperature affects the yield of RS3 (Escarpa *et al*., 1997). Upon starch solution cooling, the amylose molecules will recrystallize along with retrogradation (Zabar *et al*., 2008). The starch molecules will re-aggregate during retrogradation to form

a densely packed structure is shown in Figure 1.8 (Haralampu, 2000). Dundar and Gocmen (2013), conducted three cycles of autoclaving-cooling of corn starch and found that the prolonged storage and higher temperature used during autoclaving increased the formation of RS3. This increase in RS after autoclaving-cooling cycles was also observed by Faridah *et al*. (2010), and Sievert and Pomeranz (1989) in arrowroot and high amylose corn starches, respectively. Another study of high amylose corn starch used sequential treatment of acid hydrolysis and autoclaving to increase the yield of RS (Lee *et al*., 1997). Sun *et al*. (2021) revealed the maximum overall thermal stability of purple potato RS prepared via autoclave treatment by examining the formation mechanism. These studies suggest that autoclaving-cooling cycle may effectively increase the RS3 content.

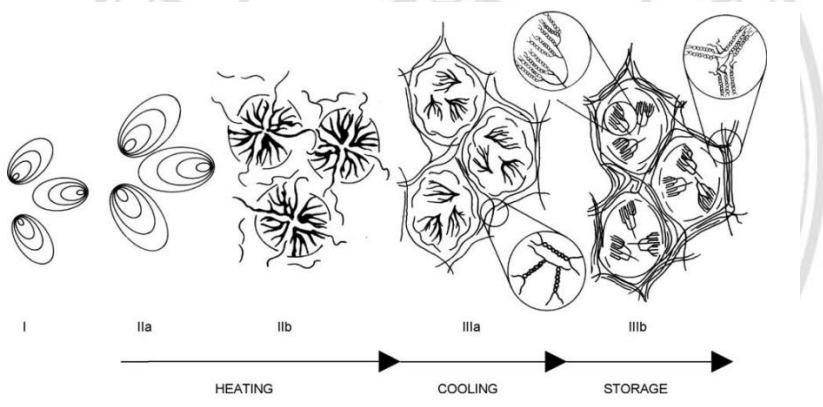


Figure 1.8 Schematic representation of changes that occur in a starch-water mixture during heating, cooling and storage (Reproduced from Goesaert *et al*., 2005)

1.6 Production of RS by Enzyme Treatment

Selective hydrolysis using debranching enzymes produces debranched starch, which contains more linear short amylose (Kiatponglarp *et al*., 2015). The starch chains in debranched starch are able to retrograde more readily into crystalline structures, thereby leading to the formation of RS3 because these compact structures are more difficult for amylases to access (Cai *et al*., 2010). The retrogradation or recrystallization of debranched starch can be induced by temperature-cycling storage or low-temperature incubation, which leads to an increase in the RS3 levels (Figure 1.9). Studies have shown that debranched starch is an important source for the production of RS (Liu *et al*., 2017a). Liu *et al*. (2017b) reported the content of RS increased to 19% after pullulanase

debranching and the crystalline regions of debranched waxy corn starch were enhanced by the short linear glucans produced by pullulanase debranching. In addition to pullulanase, researchers have also utilized amylosucrase to increase the RS content of waxy maize starches (Zhang *et al*., 2017; Kim *et al*., 2016). After amylosucrase treatment, the RS content of waxy maize starch increased to around 74%, which was attributed to the formation of double helices that inhibited the ability of amylase to hydrolyze the starch chains (Zhang *et al*., 2017).

The degree of debranching has a great impact on RS formation. When the degree of debranching is insufficient, it is difficult for the linear chain to align to form enzyme-resistant crystallites (Liu *et al*., 2017a). Thus, RS formation can be promoted by using higher debranching enzyme concentrations and longer debranching times, when the level of pullulanase utilized was increased, the rate of amylopectin debranching in starch is increased, which lead to an increase in crystallinity and RS content (Miao *et al*., 2015; Shi *et al*., 2018). This effect is attributed to the fact that more strongly bound water is held in the crystal structure as the debranching degree increases, resulting in an increase in RS content (Liu *et al*., 2020). However, when DP is too small, the polymers are not long enough to form enzyme-resistant crystallites, the RS yield tends to be low (Onyango *et al*., 2006).

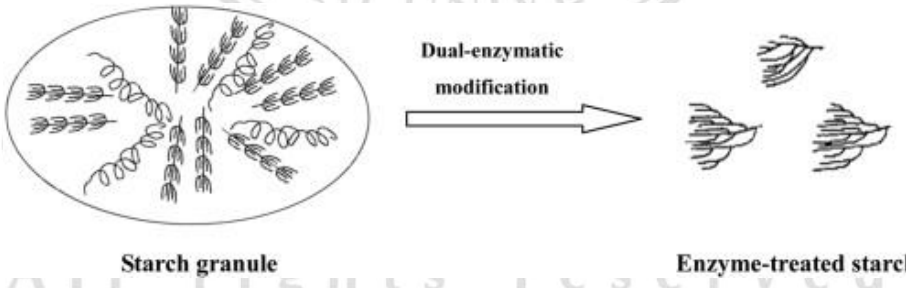


Figure 1.9 Schematic representation of dual-enzymatic treatment increased the content of RS (Reproduced from Miao *et al*., 2014)

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1.7 Factors Influencing RS Formation

1.7.1 Starch Crystallinity

Studies of X-ray diffraction of RS showed that chain fragments were packed in a B type crystalline structure with enlarged crystal lattice which contributes to the formation of RS. Any treatment that eliminates starch crystallinity (e.g., gelatinization) or damages the integrity of the plant cell or tissue structure (e.g., milling) increases access to enzymes and reduces the RS content, whereas recrystallization and chemical modifications increase the RS content (Englyst and Cummings, 1986; Adebowale *et al*., 2009; Kim and White, 2013).

1.7.2 Amylose: Amylopectin Ratio

High amylose content of starch is known to lower starch digestibility (Chung *et al*., 2009). High amylose maize starches with very long chains might be perfectly ordered into double helices to form RS (Ozturk *et al*., 2009). Higher contents of RS were found in Hylon VII than in Hylon V (high-amylose genetically modified corn starches) which might be because of higher amylose content in Hylon VII (Dimantov *et al*., 2004). Leeman *et al*. (2006) claimed that high amylose starch resists enzymatic digestion due to its internal structure and B-type crystallinity.

1.7.3 Retrogradation of Amylose

High amylose starch is rich source of RS2 (Berry, 1986), which after heating and cooling gives RS3 in high yields (Sievert and Pomeranz, 1990) or retrograded starch (Englyst *et al*., 1992). Retrograded amylose in wheat, maize, peas, and potatoes was found to be highly resistant to digestion (Ring *et al*., 1988). Park *et al*. (2009) reported that temperature cycled storage increased the formation of RS and reduced the GI (glycemic index) of waxy corn starch. Borczak *et al*. (2014) claimed that prolonged frozen storage

of wheat-flour rolls significantly increased RS formation. Dual-retrogradation treatment was more efficient as compared to single retrogradation (Tian *et al*., 2013). Repeated autoclaving of wheat starch increased the RS up to10%. Retrogradation of amylose was recognized as the main factor for the formation of RS and higher amounts of RS were obtained with repeated autoclaving (Dundar and Gocmen, 2013). On storage, gelatinized starch pastes undergo retrogradation to semicrystalline structure that resist enzymatic digestion. Wheat bread and cornflakes are rich sources of this type of RS where as cooked and cooled potatoes have only25% of RS3 (retrograded starch) (Englyst and Cummings, 1985).

1.7.4 Enzyme Inhibitor

Enzyme inhibitors like phytic acid, polyphenols, and lectins present in leguminous seeds, have been found to inhibit *in vitro* digestion and hence the glycemic index of starch (Thompson and Yoon, 1984). Both amylase and intestinal maltase activity are inhibited by tannic acid (Björck *et al*., 1987). Since phytic acid inhibits the amylolysis, an increase in phytate content decreases starch digestibility (Thompson and Yoon, 1984).

1.7.5 Ion

Potato starch gels showed decreased yield of RS in the presence of ions like calcium and potassium (Escarpa *et al*., 1997), which may be reflected to the prevention of hydrogen bond formation between amylopectin and amylose chains.

1.7.6 Lipid

Differential scanning calorimetry (DSC) was used to study the effect of sodium stearoyl lactylate (SSL), lysophosphatidylcholine (LPC), and hydroxylated lecithin (OHL) on autoclaved amylomaize starch (Czuchajowska *et al*., 1991). DSC peaks at around 95-110°C indicated the formation of complex compounds between amylose chains and lipid, and the peak at about 155°C indicated the presence of RS. However lower yields of RS were observed from lipid complexed samples as compared to autoclaved and cooled control when subjected to amylolysis by thermostable bacterial α-amylase and amyloglucosidase. Amylose recrystallization which is important in RS formation is adversely affected by complexation of amylose with LPC and SSL. In another study, influence of endogenous lipids on wheat starch showed that defatting of

the starch samples resulted in decrease of the RS content. On addition of SDS to defatted wheat or amylomaize starch, RS yields decreased significantly. X-ray diffraction and DSC techniques confirmed formation of amylose-lipid complexes in the presence of both endogenous lipids as well as added lipids (SDS) (Eerlingen *et al*., 1994).

1.8 Properties of RS

RS has gained importance because of its functional properties. It has many desirable physicochemical properties, e.g. swelling, viscosity, gel formation and water-binding capacity, which make it useful in a variety of foods (Fausto *et al*., 1997). RS can be used to replace starch on a one-to-one basis without significantly affecting dough handling or rheology. RS is present naturally and is bland in flavor, with fine particle size. Because of its fine particle size, RS does not affect the texture of food. RS imparts special characteristics along with dietary fiber fortification in high-fiber foods (Tharanathan and Mahadevamma, 2003). The calorific content of RS is also low (1.6-2.8 kcal g^{-1}), so it can be used to complement reduced-fat and reduced-sugar food formulations. Its physical properties, particularly its low water-holding capacity, provide good handling in processing, as well as crispiness, expansion and improved texture in the end-product. RS has a high gelatinization temperature, good extrusion and film-forming qualities and lower water-holding properties than traditional fiber products. It increases the coating crispness of products and the bowl life of breakfast cereals. Because of the above properties, RS has been used successfully in a range of baked and extruded products. RS is especially suitable for grain-based, low-moisture and moderate-moisture food systems.

RS has attracted the attention of nutritionists and food processors because of its potential physiological benefits and unique functional properties. Owing to increasing awareness about healthy and nutritious foods, consumers are now concerned with supplementary health merits derived from regular ingestion of RS along with traditional nutritional aspects of foods (Aparicio-Saguilán *et al*., 2007). Looking into consumer awareness, food manufacturers, researchers and producers are aiming at the production of improved foods with better digestion and health benefits (Li *et al*., 2015). RS is present naturally in abroad range of starchy products, so it can be added as a functional ingredient. RS-fortified foods are becoming popular, and consumers are accepting food products enriched with RS in order to increase their dietary fiber intake (Buttriss and Stokes, 2008).

RS containing starch ingredients are commonly sold as “resistant starch” at commercial level. A large number of these products are fully digestible and considered as RS suppliers (Xie and Liu, 2004). The first ever commercially available product of RS was reported only during the mid-1990s. Nowadays, RS rich-powders are prepared by a number of companies employing technologies such as that developed at Kansas State University using an amylose-rich starch from maize hybrids (Shin *et al*., 2003). RS products are of high quality and cannot be replaced by traditional insoluble fibers (Baixauli *et al*., 2008). RS is used in the production of moisture-free food products. To improve the textural properties and health benefits, baked products, pasta products and beverages are fortified with RS. Arimi *et al*. (2008) have successfully replaced most or all of the fat in imitation cheese with RS, without adversely affecting meltability or hardness and conferring the well-established benefits of RS as a functional fiber. A large number of products rich in fiber are available on the market, e.g. high-fiber bread and breakfast cereals (Fuentes-Zaragoza *et al*., 2010) but other products such as white bread, biscuits and cakes are not fortified with fiber. The availability of process-tolerant RS has now made it possible to prepare foods rich in dietary fiber. RS has been added in the preparation of pasta and beverages, and dried pasta products containing up to 15% RS can be made with little or no effect on dough rheology during extrusion. Pasta prepared with addition of RS was lighter in color, but a firm texture was obtained in the same cooking time as a control that contained no added fiber (Sajilata *et al*., 2006). RS generally require suspension and add opacity to beverages and may be used in thickened, opaque health drinks where insoluble fiber is desired. Fibers other than RS generally have a strong flavor, coarse texture and poor as well as dry mouth-feel. However, RS imparts a less gritty mouth-feel and masks flavors toa lesser extent.

Table 1.1 summarizes the commercially manufactured RS3 commonly used in food products. The first commercial RS3 was introduced a by Opta Food Ingredients Inc. (USA) of RS such as Crystalean, other commercial sources such as Novelose 330, and Neo-amylose, were introduced to increase the dietary fiber content in foods. Crystalean is an RS3 preparation produced by starch retrogradation of high-amylose maize starch ae-VII hybrid. Hylon-VII, a natural high-amylose maize starch, was introduced by the National Starch and Chemicals Co. (USA). The above mentioned RS3 products have been prepared by heating and cooling high-amylose corn starch under controlled moisture and

temperature conditions; these processes help in the manufacture of granular forms of concentrates containing 47-60% RS. Using maltodextrins as starting material, a natural, highly crystalline RS3 (Actistar Act*-RS3) has also been developed. The taste of Act*-RS3 is very natural owing to the raw material and production process used.

Table 1.1 Commercially manufactured RS3 commonly used in various food products (Raigond *et al*., 2015)

Brand name	RS/TDF content	Physiological and/or health benefits	Manufacturer
Crystalean	41% RS	Prebiotic effect; increases proportion of butyrate; increases cell proliferation in proximal colon, soluble dietary fiber and prebiotic effects; low glycemic index	Opta Food Ingredients Inc., USA
Novelose 300	<30% TDF	Lowers glycemic response when used as a substitute for starch and other rapidly digested carbohydrate	National Starch and Chemicals Co., USA
Act*-RS3	53% RS	Health benefit potential; prebiotic effect; source of butyrate; supports immune system; reduces glycemic response; low calorific value; easily fermentable; very well tolerated	Cerestar (a Cargill Company)
Neo-amylose	87 or 95% RS	Prebiotic; protects against inflammatory intestinal disease; may protect against colorectal cancer; may help control blood glucose levels in diabetics	Protos-Biotech. (Celanese Ventures GmbH)

1.9 Potential Health Benefits of RS

The potential health benefits of consuming RS have been explored. In the past two decades, various promising physiological benefits of RS consumption have been reported, including prebiotic, hypoglycemic, anticancer, anti-obesity, and cholesterol lowering

effects (Figure 1.10) (Bede and Lou, 2021). In this section, a brief overview of some of the most important of these effects is given.

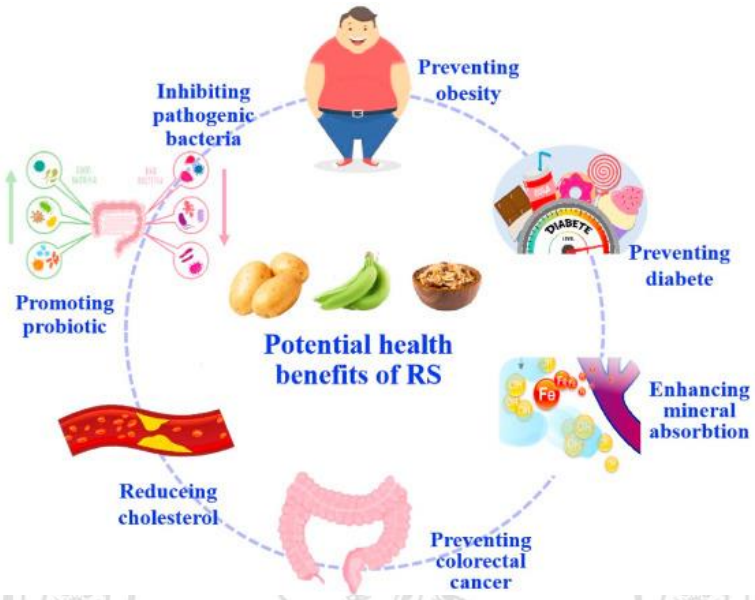


Figure 1.10 The potential health benefits of consuming RS
(Reproduced from Wang *et al*., 2021)

1.9.1 Anti-Diabetic Effects

Studies have reported that more than 5 g/day of RS intake was sufficient to prevent obesity-related diabetes, and most dietary interventions used between 6.5 and 74 g/day of daily RS intake exerted positive anti-diabetic effects (Penn-Marshall *et al*., 2010; Zhou *et al*., 2015). RS could reduce the amplified levels of blood glucose and insulin, because they are not allow for immediate hydrolysis, thus improving postprandial glycemic and insulinemic responses (Kim *et al*., 2003). Moreover, RS acts as an insoluble fiber and is fermented by the colon micro flora into metabolically active short chain fatty acids, which may affect hepatic gluconeogenesis and insulin secretion by the regulation of genes related to glucose and lipid metabolism (Zhou *et al*., 2015). Polakof *et al*. (2013) demonstrated that RS intake through a high-fat feeding could manipulate hyperglycemia, hyperlipidemia, and gene expression related with hepatic glucose and lipid metabolism pathways in diabetic rat. Harazaki *et al*. (2014) fed Otsuka Long-Evans Tokushima fatty rats (an obesity-associated diabetic rat model) with a total diet composed of 350 g/kg of RS for 4 weeks, both plasma insulin concentrations and glycated hemoglobin were

decreased. They thought that the mechanism for the improved insulin resistance by the RS diet involved a reduction of CD11c expression in adipose tissues that were strongly related to the development of insulin resistance. Consequently, RS holds a promise of providing digestion-controlled food materials that can modulate glycemic response or being new dietary fiber sources.

1.9.2 Anti-Obesity Effects

Several studies have investigated the capacity of RS to enhance fat oxidation and inhabit fat accumulation (Meenu and Xu, 2019). Dodevska *et al*. (2016) reported significant decreases in body weight and BMI, as well as waist circumference after the intervention of RS for 12 months. The mechanism of action of RS on obesity were widely studied. It was reported that RS in the diet may affect energy balance through its effect as a stimulator of gut peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) expression, which were intestinally-secreted anti-obesity hormones. They focused on the hypothalamus and inhibited the expression of appetite related genes, thereby playing a role in the prevention of obesity (Lang and Schnabl, 2020). The SCFAs produced by RS fermentation like butyric acid, acetic acid, and propionic acid were reported to inhibit adipose tissue lipolysis (Robertson *et al*., 2003) and in the liver, particularly acetate was proposed to inhibit glycogenolysis, with the result of sparing carbohydrate and increasing fat oxidation (Brown *et al*., 2003). It was also reported that RS intake led to changes in neuronal activity in hypothalamic appetite regulation centers that resulted into the indicative of satiation in mice (So *et al*., 2007), which would reduce the food intake, thus in turn resulting in reduced incidence of obesity and related disorders. Thus, RS exhibits great potential effects on managing metabolic syndrome and reducing obesity.

1.9.3 Benefits for Colon Health

Physiologically, RS behaves like soluble, fermentable dietary fibers that are resistant to digestion in the upper gastrointestinal tract and then selectively stimulate the growth and the activity of beneficial bacterial groups in the colon, such as *Bifidobacteria* and *Lactobacillus* (Ching *et al*., 2021). In addition, the production of SCFAs would reduce the pH in intestinal, which might also inhibit the proliferation of pH-sensitive pathogenic bacteria, such as *E. coli* and *Salmonella* (Topping and Clifton, 2001). Butyrate can reduce cell malignancy risk and RS which ferment in the intestine could produce a

high level of butyric acid or its salts. Besides, butyrate and other short chain fatty acid could also lower the risk of host colorectal diseases by providing energy to the colonocytes and enterocytes (Chen *et al*., 2020). In another study, it has been reported that RS ingestion may have benefits on colonic health by improving crypt cell production and reducing epithelial atrophy in the colon (Raigond *et al*., 2015). Overall, RS consumption exerts beneficial effects on colon health in many ways, which is regarded as a preventive agent for people at high risk for colon cancer.

1.9.4 Aids in Mineral Adsorption

RS was reported to increase absorption of various minerals (Aribas *et al*., 2020). This effect is attributed to the fact that SCFAs reduce the intestinal pH, which may increase the solubility of the minerals and promote the growth of intestinal wall cells. Zeng *et al*. (2017) investigated the impact of lotus seed RS ingestion on the production of short-chain fatty acids (SCFAs) and the absorption of minerals in the colons of mice. The fermentation of the lotus seed RS by the colonic bacteria promoted the production of SCFAs such as formic, acetic, butyric, isobutyric, propionic, and lactic acids. The intestinal absorption of minerals (including calcium, magnesium, and iron) increased as the amount of SCFAs formed was increased.

1.10 Method of RS Determination

The RS assay kit (rapid) method is suitable for the analysis of RS in pure starch, cereal and legume seeds and food samples. This method is an update of the method of McCleary *et al*. (2002) employing incubation conditions similar to those used in AOAC Method 2017.16 for dietary fiber. The enzyme mixture employed pancreatic α-amylase and amyloglucosidase are those used by Englyst *et al*. (1992) except that both enzymes have been purified, standardized and stabilized. Digestion is performed using saturating levels of pancreatic α-amylase and amyloglucosidase with stirring at pH 6 and 37°C for 4 h (Figure 1.11), to simulate *in vivo* conditions in the human small intestine.

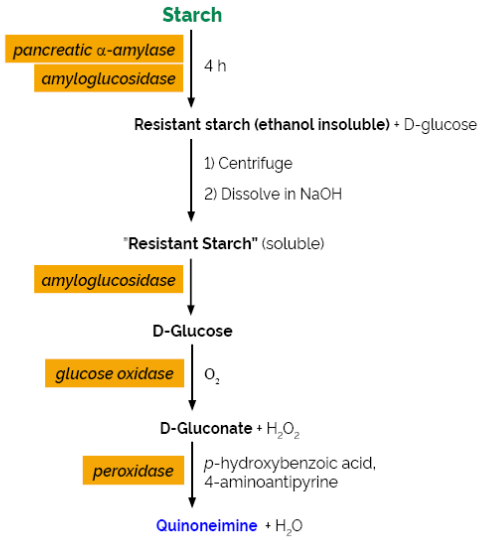


Figure 1.11 Measurement of available RS in food ingredients and products (Megazyme, 2017)

The incubation conditions parallel those used in AOAC Method 2017.16, a new, rapid integrated procedure for the measurement of total dietary fiber (Megazyme method K-RINTDF). This method is physiologically based and designed to service the definition of dietary fiber announced by Codex Alimentarius in 2009.

1.11 Utilization of Anthocyanin-Rich Carbohydrate in Human Foods

1.11.1 Purple Rice

Rice is one of the world's major staple food crops, consumed by more than half of the world's population along with crops such as wheat, maize, and potato; over 90% of the rice production areas and consumption amounts are recorded in Asia (Fairhurst and Dobermann, 2002). At least 175 countries and territories consume rice; the overall consumption is high in the rice-consuming countries, ranging from 100 to 200 kg of paddy rice per person per year according to the FAO (FAO, 1995). This could be one of the reasons why many international programs aimed at boosting human nutrition, e.g., the harvest plus biofortification with high zinc, iron, iodine, and selenium, are focused on rice crops (Prom-u-thai *et al*., 2020). Moreover, it is interesting to observe that among the staple food crops, rice is recognized as potentially containing high amounts of antioxidant compounds such as anthocyanin, especially in pigmented rice varieties with purple

(Figure 1.12). The purple rice is highly valued, particularly among the health-conscious consumers as a functional compound for human health among everyday sources of carbohydrate (Veni, 2019). Additionally, anthocyanin can be directly taken from the concentrate in capsules for convenience, but this can be quite expensive (Norkaew *et al*., 2019). Therefore, purple rice is an advantageous option for anthocyanin intake, especially when dealing with a large number of rice consumers worldwide.

Figure 1.12 Purple rice

The anthocyanin-rich rice grains have been recognized as excellent sources of natural and safe food colorants (Papillo *et al*., 2018). Synthetic colorants can be harmful to humans and the environment via allergic and toxic reactions (Okafor *et al*., 2016), this can raise the demand for naturally pigmented rice such as purple rice that contains high concentrations of anthocyanin. Thus, purple rice not only provides enough anthocyanin to fulfill the growing interest but also can increase the value of rice products. There are numerous rice varieties containing anthocyanins, but only some are accepted as commercial varieties due to yield potential, cooking quality, and other functional properties that may be less acceptable (Yamuangmorn and Prom-u-thai, 2021).

1.11.2 Purple Sweet Potato

Purple sweet potato is a one-year or perennial herb in the family Convolvulaceae. Their flesh is purple to dark purple (Figure 1.13). Besides the nutrients of ordinary sweet potatoes, they are also rich in anthocyanins (Bovell-Benjamin, 2007).

Purple sweet potato is an important source of dietary fiber, minerals, vitamins, anthocyanins, and so on. It can be used by humans and animals (Teow *et al*., 2007). The recommended intake of cereals and potatoes in Dietary Guidelines for Chinese Residents is about 250-400 g/day, of which 50-100 g is potato. The daily dietary structure of American residents showed that the average daily anthocyanin intake per capita was about 12.5 mg/day (Wu *et al*., 2006). Therefore, purple sweet potato can not only be used as a green food to meet people's daily intake of cereal and potato, but also increase the daily intake of anthocyanin to achieve health effects.

Figure 1.13 Purple sweet potato

Purple sweet potato possess attractive purple-red color, high anthocyanin content, high total phenol content, and high antioxidant activity (Steed and Truong, 2008). It is reported that the content of anthocyanin in purple sweet potatoes is significantly higher than that in ordinary orange-fleshed sweet potato (Xu *et al*., 2015), similar to those of anthocyanin crops with the highest yield, such as blueberries, blackberries, cranberries, and grapes (Bridgers *et al*., 2010). Moreover, purple sweet potato is an important source of natural anthocyanin pigments because of its low cost (Jansen and Flamme, 2005).

1.11.3 Purple Corn

Purple corn, a member of the coarse grain family, has attracted extensive attention for its considerable amounts of anthocyanins and other bioactive components (Figure 1.14), which has positive functions in human health as antioxidant, anti-inflammatory, anticancer, antidiabetic, and ocular health enhancing agents (Abdel-Aal *et al*., 2014), purple corn is originally from Peru. It is now widely known throughout the world, especially in Asia, the United States and Europe (Cristianini *et al*., 2020). Except for direct consumption, purple corn is also suitable for processing into flour, which is flexible in the production of food products with pleasant colors, such as muffins, polenta, mush and bakery products. However, bran and germ in flour may have negative impact on end-use products, such as poor palatability and processing characteristics (Niu *et al*., 2014).

Figure 1.14 Purple corn

1.12 Bioactive Compounds

Bioactive compounds are classified as phenolic compounds or non-phenolic compounds such as anthocyanins, carotenoids, and curcumin, Therefore, a systematic classification of bioactive compounds is essential to explore more profound their technological potential. These compounds have nutraceutical properties as antioxidant, antimicrobial, antifungal, anticancer, anti-inflammatory, anti-obesity, antidepressant

activities. However, they are unstable in the presence of oxygen, light, heat, and humidity (Segura Campos, 2018). In addition, the functionality of bioactive compounds is inherently related to their bioavailability, that is, they have to be effectively absorbed in the gut, then carried into the circulatory system, before reach the target cells. Moreover, they can be degraded due the pH change (stomach-small intestine) (Thakur *et al*., 2020).

Bioactive compounds are molecules found at low contents in a wide range of vegetables, cereal grains, and tuber such as purple rice, purple sweet potato, and purple corn. These compounds have been used in several product formulations such as foods, cosmetic and pharmaceutical industries (Segura Campos, 2018).

The bioactive compounds are classified as phenolic compounds or non-phenolic compounds. However, there are contradictions. For instance, some pigments are defined as bioactive compounds; nevertheless, both phenolic compounds and non-phenolic compounds are composed of colored molecules, such as anthocyanins (polyphenol) and carotenoids (non-phenolic compounds). Thus, a systematic bioactive compounds classification, as suggested in Figure 1.15.

Flavonoids are phenolic compounds that interact with other compounds, notably carotenoids and chlorophylls are responsible for the unique color and aroma of fruits and vegetables. These compounds have a phenolic and chemical structure based on a C_6-C_3-C_6 skeleton. Flavonoids can be subdivided into flavones (e.g., rutin, chrysin, apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavan-3-ols (e.g., proanthocyanidins, catechin, epicatechin, and epigallocatechin), flavanones (e.g., flavanone, hesperetin, naringin, and naringenin), isoflavonoids (e.g., genistein and daidzein), anthocyanidins (e.g., apigenidin, malvidin, cyaniding, and delphinidin) (Karak, 2019; Kumar and Pandey, 2013). These compounds have shown biological activities *in vivo*, such as enzymatic activity modulation and cellular proliferation inhibition; *in vitro* and *in vivo* antioxidants (Romani *et al*., 2020).

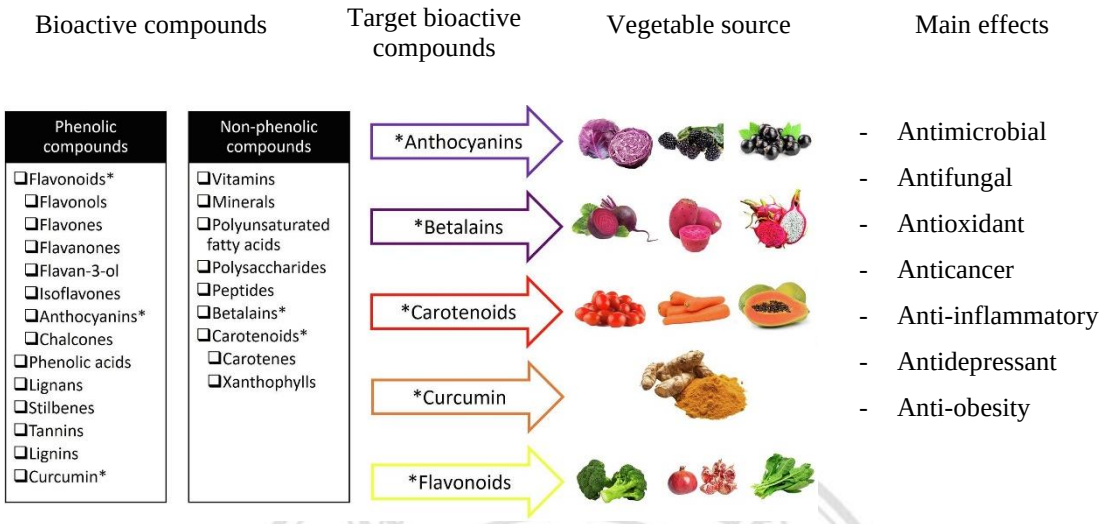
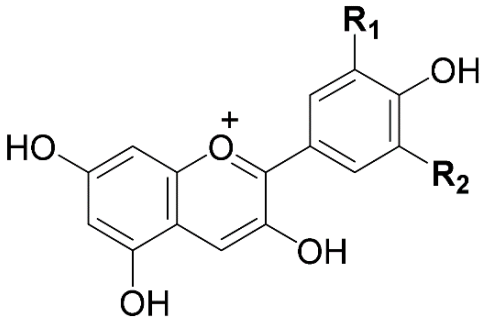


Figure 1.15 Classification and fruit and vegetable sources of bioactive compounds and their properties (Reproduced from Luiza Koop *et al*., 2022).

Anthocyanins receive attention and are studied both because of their impact on food sensory properties, being responsible for the red-blue color of berries, fruits, certain vegetables-based products, and carbohydrate source such as purple rice, purple sweet potato, purple corn, and because of their health-promoting properties (de Pascual-Teresa *et al*., 2010; He and Giusti, 2010; Pojer *et al*., 2013; Chen *et al*., 2016; Li *et al*., 2018; Kang *et al*., 2012).

Anthocyanins and anthocyanin-rich foods have been shown to display several biological activities which may have positive implications on human health, including anti-inflammatory, anti-diabetic, and antioxidant activities (He and Giusti, 2010; Pojer *et al*., 2013). Especially important is their role in preventing cardiovascular health disease through modulation of risk factors such as blood pressure, platelet aggregation, and vascular function (de Pascual-Teresa *et al*., 2010; Fairlie-Jones *et al*., 2017; Garcia-Conesa *et al*., 2018). Furthermore, the intake of anthocyanins seems to have promising effect on cognitive function in humans, with both acute and long-term intakes (Kent *et al*., 2017). Besides, no negative effect of anthocyanin derivatives has been reported, even after ingestion of very high doses (Clifford, 2000).



R1	R2	Anthocyanidin
H	H	Pelargonidin
OH	H	Cyanidin
OH	OH	Delphinidin
OH	OCH3	Petunidin
OCH3	H	Peonidin
OCH3	OCH3	Malvidin

Figure 1.16 Anthocyanidin structure

Anthocyanins are water-soluble pigments belonging to the flavonoid group of polyphenols (Mazza and Miniati, 2017; Andersen, 2005). They have glycosidic structure, that is, one or more sugar molecules (glucose, galactose, rhamnose, xylose, or arabinose) bound to the aglycon, the anthocyanidin. The sugar can be acylated with aliphatic acids (malonic, succinic, malic, and acetic acid) or cinnamic acids (*p*-coumaric, ferulic, and sinapic acid) (Andersen, 2005). Anthocyanins differ in (i) the position and number of hydroxyl groups, (ii) the degree of methylation of hydroxyl groups, (iii) the nature and number of sugar molecules attached to the aglycone, and (iv) and aliphatic or aromatic acids attached to the sugar molecule (Mazza and Miniati, 2017). In nature, more than 600 individual anthocyanins and more than 30 anthocyanidins have been identified (He and Giusti, 2010; Andersen, 2005). but only 6 aglycones are common and widely distributed in foods: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Figure 1.16). Most of the anthocyanins in foods are cyanidin derivatives, followed by delphinidin and pelargonidin and the three methylated anthocyanins, peonidin, malvidin, and petunidin (Andersen, 2005). Cyanidin-3-glucoside is the most widely distributed anthocyanin in edible plants and consequently, is the most studied compound (Wu *et al*., 2006; Koponen *et al*., 2007; Olivas-Aguirre, *et al*., 2016; Eker *et al*., 2019).

1.12.1 Anthocyanins in Purple Rice

Among the anthocyanins accumulated in purple rice varieties, cyanidin-3-glucoside comprised the highest proportion among all anthocyanins accounting for 82.3%, followed by peonidin-3-glucoside (14.6%), cyanidin-3-galactoside (1.2%), cyanidin-3-rutinoside (1.0%), cyanidin (0.7%) and peonidin (0.2%) (Chen et al., 2017). Jiamyangyuen *et al*., 2019 identified cyanidin-diglucoside, cyanidin-3-sambubioside, cyanidin-3-rutinoside, peonidin3-rutinoside and pelargonidin-3-glucoside in Thai purple rice flour, but the levels were quite low. Furthermore, the free and bound extracts showed differences in anthocyanin profiles; most of the cyanidin-3-glucoside and peonidin-3-glucoside was distributed in free form, but other anthocyanins such as cyanidin-3-galactoside, delphinidin, and cyanidin were found only in bound form (Nana *et al*., 2018). A selection of the current data of anthocyanin profiles in various purple rice varieties is provided in Table 1.2.

Table 1.2 Major anthocyanin species (cyanidin-3-glucoside and peonidin-3-glucoside) in various purple rice varieties

Variety	Anthocyanin content (mg/100 g)		References
	cyanidin-3-glucoside	peonidin-3-glucoside	
Kum Doi Saket CMU 125	50	48	Yamuangmorn *et al*., 2019
Hom Nil Riceberry	48	7	Wongsa *et al*., 2019
Hom Nil Leum Pua	2,277	792	Peanparkdee *et al*., 2019
Khao Gam Pah E-Kaw	88	32	Nakagawa *et al*., 2018
Heimi 2420	111	31	Shao *et al*., 2018

1.12.2 Anthocyanins in Purple Sweet Potato

The purple sweet potato is reported to be superior sources of polyphenols, and other functional bioactive components Alam, M.K. (2021). A selection of the current data of anthocyanin profiles in various purple sweet potato varieties is provided in Table 1.3.

Table 1.3 Major anthocyanin species in various purple sweet potato varieties

Variety/Sou	Total anthocyanin content (mg/100 g)	References
Korea	7.4-34.5	Im *et al*., 2021
China	13.73	Sun *et al*., 2018
India	43.4	Vishnu *et al*., 2019
Indonesia	96.98	Herawati *et al*., 2020
China	331.43	Wan *et al*., 2020

1.12.3 Anthocyanins in Purple Corn

The individual anthocyanin of purple corn has been characterized, including cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside (Aoki *et al*., 2002). The anthocyanin profiles in various purple corn are given in Table 1.4.

Table 1.4 Major anthocyanin species in various purple corn

Variety/Source	Total anthocyanin Content (mg/100 g)	References
Amazonas Imports, Inc., Sun Valley, CA	130-380	Cevallos-Casals and Cisneros-Zevallos (2003)
Specialty foods vendor Angelina's Gourmet (Swanson, CT)	113.54	Li *et al*., 2017
Fengyang City, Anhui Province, China	55.8-92.3	Yang and Zhai (2010).

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals	Company
1,1-Diphenyl-2-picrylhydracyl (DPPH)	Sigma-Aldrich, USA
2, 4, 6-Tripyridyl-s-triazine (TPTZ)	Fluka Chemika, Switzerland
2, 2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Sigma-Aldrich, USA
3,5-Dinitrosalicylic acid (DNS)	Sigma-Aldrich, USA
Acetic acid glacial	QReC, New Zealand
Ammonium carbonate	Sigma-Aldrich, USA
α-amylase enzyme (food grade)	Reach Biotechnology, Thailand
Amylose from potato	Sigma-Aldrich, USA
Boric acid	QReC, New Zealand
Calcium chloride dihydrate	RCI Labscan, Thailand
Calcium chloride dihydrate	RCI Labscan, Thailand
Copper sulfate	Ajax Finechem, Australia
Cyanidin chloride	Extrasynthese, France
Cyanidin-3-glucoside	Extrasynthese, France
Ethanol	RCI Labscan, Thailand
Ferric chloride hexahydrate	Loba Chemie, India
Folin-ciocalteu reagent	Merck, Germany
Gallic acid	Merck, Germany
Hexane	QReC, New Zealand

Chemicals	Company
Iodine	Loba Chemie, India
Magnesium chloride hexahydrate	Loba Chemie, India
Maleic acid	Sigma-Aldrich, USA
Methanol	RCI Labscan, Thailand
Pancreatin from porcine pancreas	Sigma-Aldrich, USA
Peonidin chloride	Extrasynthese, France
Peonidin-3-glucoside	Extrasynthese, France
Phenol	Loba Chemie, India
Phosphoric acid	Sigma-Aldrich, USA
Potassium chloride	KemAus, Australia
Potassium dihydrogen phosphate	KemAus, Australia
Potassium iodide	Carlo Erba, Spain
Potassium persulfate	QReC, New Zealand
Pullulanase enzyme (food grade)	Reach Biotechnology, Thailand
Resistant starch assay kit (rapid)	Megazyme, Ireland
Selenium dioxide	KemAus, Australia
Sodium acetate trihydrate	RCI Labscan, Thailand
Sodium bisulfite	Sigma-Aldrich, USA
Sodium carbonate	Loba Chemie, India
Sodium chloride	Loba Chemie, India
Sodium hydrogen carbonate	Loba Chemie, India
Sodium hydroxide	Merck, Germany
Sodium potassium tartrate	Carlo Erba, Spain
Sodium sulfate	QReC, New Zealand
Sulfuric acid	QReC, New Zealand

2.1.2 Instruments

Instruments	Model	Company
Analytical balance	PB 1502-S	Mettler Toledo, Switzerland

Instruments	Model	Company
Autoclave (electric sterilizer)	1925X	All-American, USA
Bench top centrifuge	HERMLE Z 300 K	Hermle Labortechnik GmbH, Germany
Blender	BL-233	Tefal, Thailand
Colorimeter	CR-400	Konica Minolta, USA
Differential scanning calorimeter	DSC823e	Mettler-Toledo, Switzerland
Drying and heating chambers	FP 400	Binder, USA
Grinder	GC-9100	Duchess, Thailand
Hot air oven	500	Memmert, Germany
Hot plate and magnetic stirrer	MR 3001	Heidolph, Germany
High-performance liquid chromatograph	1200	Agilent, USA
Oven and furnace	Eurotherm 91e	Carbolite, UK
pH meter	713	Metrohm, Switzerland
Rapid visco analyzer	RVA-3D	Newport Scientific, Australia
Refrigerator (4°C)	UN-618D	Sanyo, Japan
Scanning electron microscope	JM-5910LV	JOEL, Japan
Spectrophotometer	GENESYS 20	Thermo Scientific, USA
Test sieve	-	Shanghai Jinnuo Metal, China
Vortex mixer	Genie 2 G560E	Bohemia, USA
Water bath	461	Büchi, Switzerland
X-ray diffractometer	MiniFlex II	Rigaku, Japan

2.1.3 Samples

The purple rice was planted at the Mae-Cham district, Chiang Mai, Thailand in June, 2021. The purple sweet potato and purple corn samples were purchased from the Royal Project Foundation, Chiang Mai, Thailand in July, 2022 (Figure 2.1).


(a)


(b)


(b)

Figure 2.1 The typical appearance of samples. (a); purple rice, (b); purple sweet potato, and (c); purple corn

2.2 Methods

2.2.1 Starch Preparation

Purple rice starch was prepared by the alkaline steeping method (Ju *et al*., 2001), with certain changes. Dehulled purple rice grains were steeped in distilled water at 4°C for 24 h. The supernatant was discarded and the soaked purple rice grains were crushed with a mixer and then passed through a 100 μm screen. The slurry was maintained at 4°C for 48 h. The supernatant was removed, and the starch cake was resuspended in a 0.35% sodium hydroxide solution and retained at 4°C for 48 h. The supernatant was decanted, and the starch layer was held in place with water. The slurry was passed through a 100 μm screen, and allowed to stand at 4°C for 24 h. The water washing steps were repeated 3 times. The starch cake suspension with water was returned to suspend in water, neutralized with 0.1 M of hydrochloric acid at pH 7 and stored at 4°C for 24 h. The supernatant was decanted and the neutralized starch was returned to the water and placed at 4°C for 24 h. Finally, the supernatant was removed and the starch cake was dried in a 40°C oven for 24 h. This sample was designated as “native purple rice starch”.

Purple sweet potato starch was isolated by using the methods of Ding *et al*. (2020) and Yong *et al*. (2018) with minor modifications. Briefly, the purple sweet potato was washed, peeled, chopped, then mashed in a blender. The resulting purple sweet potato slurry was treated with a 0.35% sodium hydroxide solution using a reaction time of 4 h. The processed purple sweet potato slurry was filtered through 100 μm screen and centrifuged at 4,000×g for 15 min. Then, the precipitate was washed with distilled water, dried at 40°C for 24 h, crushed, and passed through 100 μm screen. This sample was designated as “native purple sweet potato starch”.

The purple corn starch extraction was performed according to the method described by Sandhu *et al*. (2005) and Paraginski *et al*. (2017) with some modifications. Purple corn grains (200 g) were added to 500 mL of 0.1% sodium metabisulfite in distilled water, and maintained for 20 h at 50°C. After this time, the water was drained and the grains were crushed in a blender until the smallest possible fraction (wet grinding) was reached. The crushed samples were double filtered through 100 μm screen. The protein-starch filtrates were decanted for 4 h. The supernatant was removed and the sedimented protein-starch layer was resuspended in distilled water to be centrifuged at 4000×g for 20 min. The resulting protein-rich supernatant was removed and the remaining starch slurry was returned to suspension in distilled water before further centrifugation to completely remove all remaining protein content. The collected starch was dried at 40°C for 12 h in an oven until 11% of moisture was achieved. This sample was designated as "native purple corn starch".

2.2.2 Determination of Chemical Composition of Purple Starches

1) Determination of Total Protein (AOAC, 1990)

A 1.0 g sample mixed with catalyst (sodium sulfate, copper sulfate, and selenium dioxide: 96:3.5:0.5 w/w) and 20 mL of concentrated sulfuric acid were added into 250 mL round bottom flask containing boiling chips. The mixture was refluxed for 2-3 h or until the solution became clear, and then continued for 20-30 min to ensure complete digestion. The solution was cooled, and 40 mL of distilled water was added slowly into the flask.

In the distillation process, the distillation apparatus was cleaned until free from ammonia. The 50 mL of 2%w/v boric acid solution was transferred into a 500 mL Erlenmeyer flask and 3-5 drops of methylene blue-methyl red indicator solution was added. The flask was placed under the condenser of the distillation apparatus and it was made sure that the condenser tube extended beneath the surface of acid in the flask. For neutralization, 20 mL of 50% of sodium hydroxide was slowly added into the digested solution. The distillation was started with a medium flame after the solution was boiling. The flame was increased until the solution boiled briskly. After distilling 200 mL of liquid, the receiving flask was removed for titration.

The excess hydrochloric acid after reacting with ammonia collected in the receiving flask was titrated with standard 0.05 M of hydrochloric acid with phenolphthalein as indicator. The amount of protein in purple starches are determined by Kjeldahl nitrogen content by a factor of 5.70.

2) Determination of Total Lipid (Williams, 1966)

A weighed dried sample was placed in a Soxhlet thimble and then placed into a Soxhlet extraction tube. The top of the Soxhlet tube was attached to a water-cooled condenser. The bottle of the extraction tube was attached to a weighed flask. The solvent for lipid extraction was hexane. After extraction, the hexane layer was concentrated by vacuum evaporator and weighed. The percentage of total lipid was calculated.

3) Determination of Moisture (Heart and Fisher, 1971)

A metal dish was dried and cooled in a desiccator and then weighed, 5.00 g of the sample was transferred to the metal dish. The dish was placed in the controlled temperature oven (100-105°C) for 3 h. The dish was then removed to the desiccator, cooled, and weighed. The drying was continued for 1 h intervals until a constant weight was obtained. The percentage of moisture was then calculated.

4) Determination of Crude Fiber (Egan *et al*., 1981)

The defatted sample (2-3 g) was transferred into 500 mL Erlenmeyer flask containing boiling chips. 200 mL of 1.25% sulfuric acid was added to the flask and the mixture was boiled on a hotplate for 30 min and stirred. The mixture was filtrated through filter paper No. 54 connected to a Buchner funnel. The residue was washed 3 times with 50 mL of warmed distilled water. The residue was placed into a flask through washed filter paper with 200 mL of 1.25% sodium hydroxide. The mixture was boiled for 30 min and filtrated with filter paper No. 41. The residue was washed 3 times with 25 mL of 1.25% sulfuric acid and with 50 mL warm distilled water and finally washed with 25 mL of 95% v/v ethanol. The residue was placed into a crucible and heated at 600°C for 30 min.

5) Determination of Ash (Heart and Fisher, 1971)

A sample (2-3 g) was placed into a previously ignited and weighed porcelain crucible. The sample was heated with a medium flame until the sample was thoroughly charred. The crucible was transferred into an oven (600°C) and heated for 3 h until the ash has a gray-white appearance. The crucible was cooled in a desiccator, weighed, and was continually reheated for 1 h intervals until a constant weight was obtained, and then, the percentage of ash was calculated.

6) Total Carbohydrate Content

The carbohydrate content was estimated by deducting the total protein, crude fiber, ash and lipid from the total dry matter as: % Total carbohydrate content = 100 - (% moisture content + % ash + % lipid + % crude fiber + % protein).

2.2.3 Determination of Amylose Content (Juliano, 1971)

The amylose content in purple starches was determined by iodine colorimetry. The amylose-iodine complex was measured by spectrometer at 620 nm. The standard curve was made with pure potato amylose. 100 mg of sample was weighed into 50 mL Erlenmeyer flask, followed by adding 1 mL of 95% v/v ethanol and 9 mL of 1.0 N sodium hydroxide. The sample was heated for 10 min in boiling water bath to gelatinize the starch, cooled, and transferred, with several water washings, into a 100 mL volumetric flask; brought up to volume with water; and mixed well.

The purple starch solution at 5 mL was pipetted into a 100 mL volumetric flask, followed by adding 1 mL of 1.0 N acetic acid and 2 mL of iodine solution. The solution was adjusted volume with distilled water, stirred, and kept in room temperature for 20 min. Absorbance of the solution at 620 nm was measured with a spectrophotometer. Amylose content was determined by reference to a standard curve or conversion factor and expressed as a dry weight.

For the standard curve, 40 mg of potato amylose was added with 1 mL ethanol and 9 mL 1.0 N sodium hydroxide, heated for 5 to 10 min in a boiling water bath, cooled, and made up to 100 mL with a pipet, 1, 2, 3, 4, and 5 mL portions are placed in 100 mL volumetric flasks, acidified with 1.0 N acetic acid (0.2, 0.4, 0.6, 0.8, and 1.0 mL,

respectively), and treated as above. The absorbance values at 620 nm were compared to the anhydrous amylose concentration (mg) in order to determine the conversion factor.

2.2.4 Preparation of RS

1) Dual Autoclaving-Retrogradation Treatment (DART) (Ashwar *et al*., 2016b)

DART was carried out for the preparation of RS. Purple starches (20 g) was mixed with 80 mL of distilled water (starch: water, 1: 4), and the mixture was then pressure-cooked in an autoclave at 121°C for 30 min. The autoclaved starch paste was left to cool to ambient temperature and stored at 4°C for 24 h. The autoclaving-retrogradation treatment was repeated again. The samples were finely dried in the 45°C oven and ground using a motor and pestle.

2) Dual Enzyme Treatment (DET) (Zhang and Jin, 2011a)

2.1) Optimized Reaction Conditions for α-Amylase

RS was prepared as shown in Figure 2.2, the purple starches (20%, w/v) were pre-gelatinized with distilled water, stirring for 20 min at 80°C, pH 6.0. The starch paste was treated with thermostable α-amylase in different reaction conditions and then pullulanase was added. The mixture was incubated in a stirred water bath at 46°C for 12 h, cooled in room temperature, and stored at 4°C overnight. The mixture was processed using a thermostable α-amylase (1%, w/w) and incubated in a boiling water bath (95°C) for 45 min then centrifuged (4,000×g, 15 min). The supernatant was discarded. The residue was suspended in 95% v/v ethanol (1:4 residue: ethanol) and shook for 15 min. The ethanol was removed by centrifugation (4000×g, 15 min) and the above procedure was repeated twice more. The residue was dried overnight at 40°C, ground in a centrifugal mill to pass through a 100 μm screen, and kept in airtight containers at room temperature until analysis.

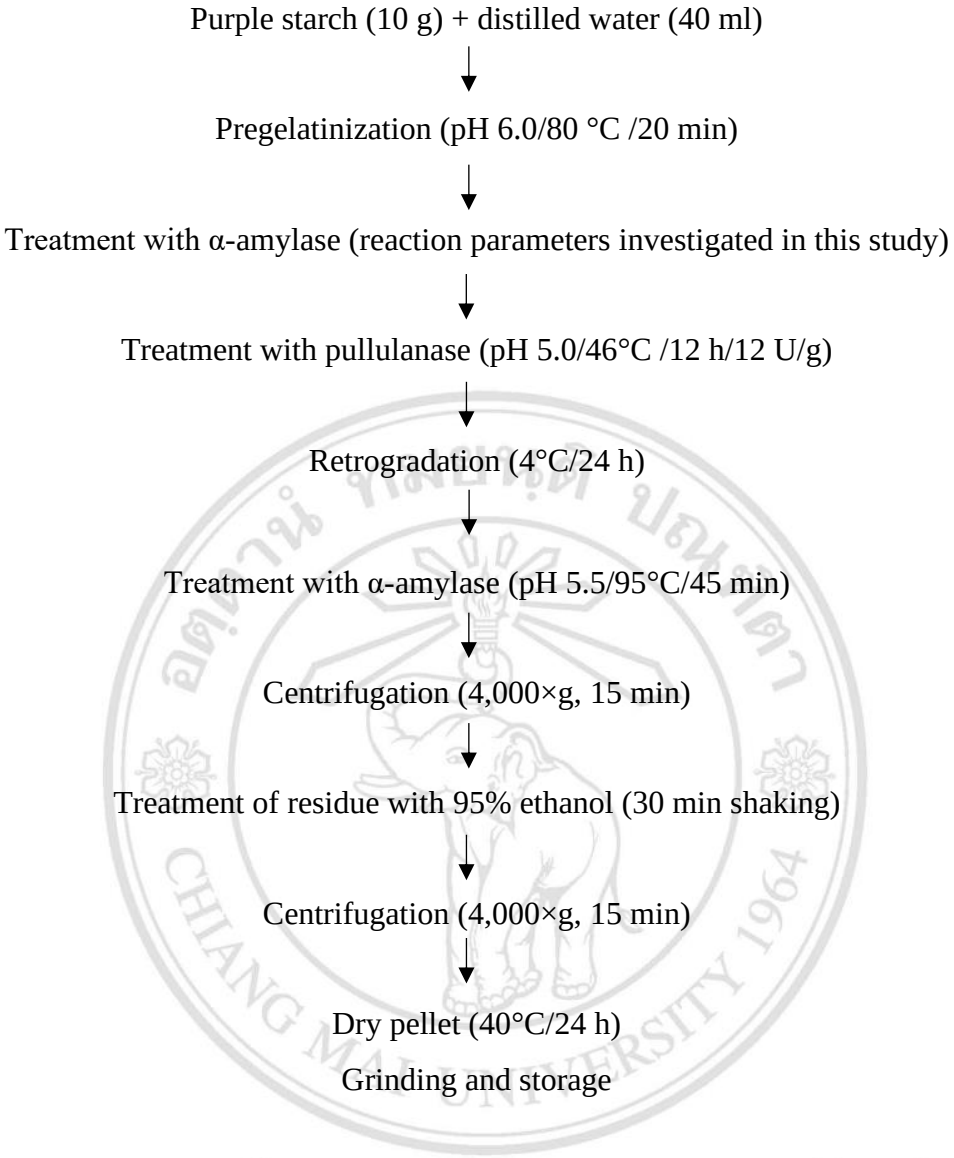


Figure 2.2 Preparation of RS under optimized condition for α-amylase

2.2) Optimized Reaction Conditions for Pullulanase

RS was prepared as shown in Figure 2.3, the purple starches were pregelatinized with distilled water, stirring 20 min at 80°C, pH 6.0. The starch paste was treated with thermostable α-amylase under optimized reaction conditions, the gel was cooled to 44°C, then pullulanase was added in various reaction conditions, cooled at room temperature, and then stored at 4°C overnight. The mixture was processed using a thermostable α-amylase (1%, w/w) and incubated in a boiling water bath (95°C) for 45 min then centrifuged (4,000×g, 15 min). The supernatant was discarded, and the residue was suspended in 95% v/v ethanol (1:4 residue: ethanol) and shaken for 15 min. The

ethanol was removed by centrifugation (4000×g, 15 min) and the above procedure was repeated twice more. The residue was dried overnight at 40°C, ground in a centrifugal mill to pass through a 100 μm screen, and kept in airtight containers at room temperature until analysis.

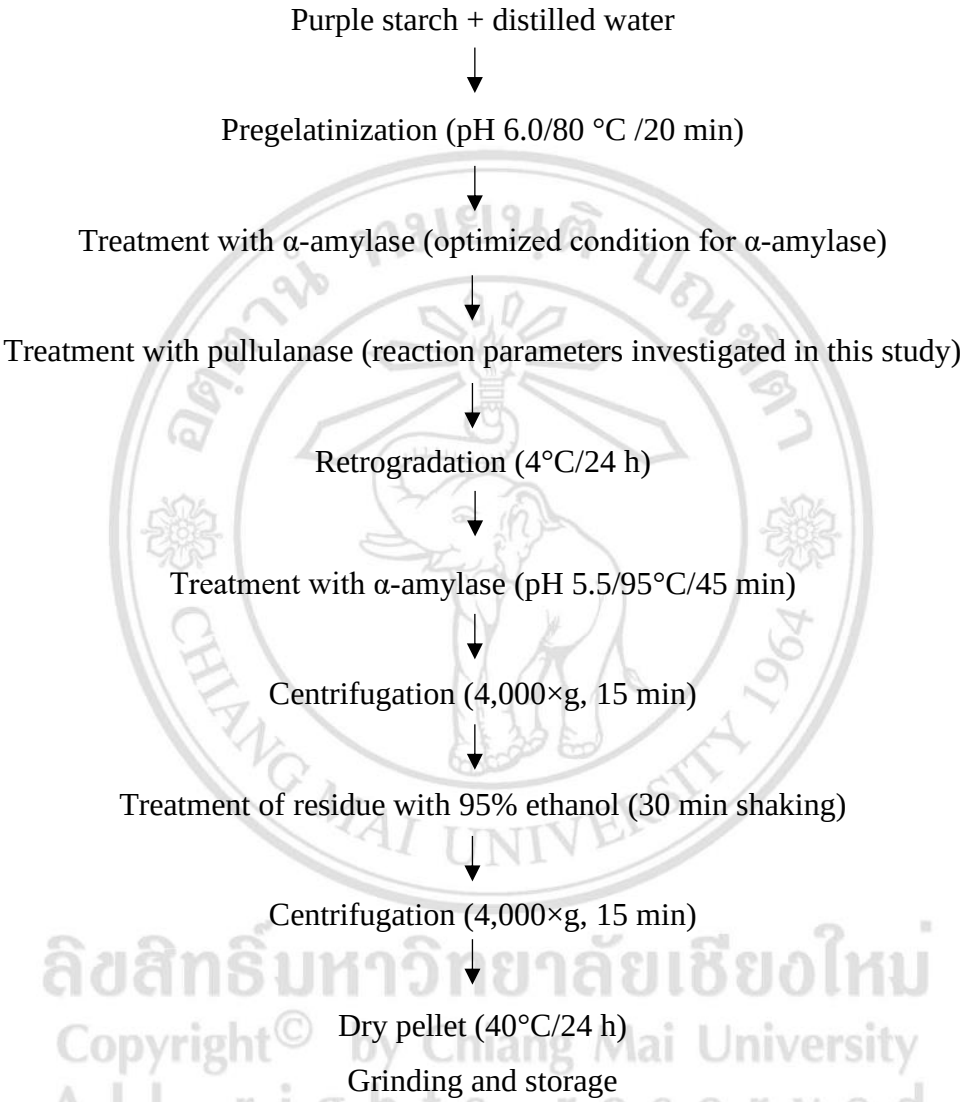


Figure 2.3 Preparation of RS under optimized condition for pullulanase

2.2.5 Measurement of RS Using the Rapid RS Method

RS was measured according to AOAC Method 2002.02/AACC Method 32-40.01 and using the RS method described here. Sample 100 mg was weighed into tube, added 3.5 mL of sodium maleate buffer (pH 6.0) containing 2 mM calcium chloride, mixed on a vortex mixer for 5 sec, and placed in a water bath at 37°C for 5 min to allow the contents to equilibrate to temperature. An aliquot (0.5 mL) of pancreatic α-amylase

(PAA) and amyloglucosidase (AMG) solution (0.4 KU PAA plus 0.17 KU AMG) was added into the tube which was capped tightly, attached horizontally and aligned in the direction of motion in a shaking water bath set at 37°C for 4 h. The tube was removed from the water bath one at a time, added 95% v/v ethanol, capped, and the contents were stirred on a vortex mixer. After removal of caps, centrifuged at 3,250×g for 10 min. The supernatant solution was carefully decanted. The pellets were re-suspended in 2 mL of 50% v/v ethanol and mixed on a vortex mixer. A further 6 mL of 50% v/v ethanol was then added to the tube, the tube was capped, and the contents were mixed by inversion. Tubes were tapped so that all liquid was removed from the caps. Caps were then removed, and the tubes were centrifuged at 3,250×g for 10 min. The supernatant solutions were then carefully decanted. The residue was re-suspended in 8 mL of 50% v/v ethanol, and centrifuged, and the supernatant decanted. The tube containing the residue was inverted on absorbent paper to remove excess liquid, ensuring that the pellets were not dislodged.

Determination of RS, 2 mL of ice-cold 1.7 M sodium hydroxide was added to each tube, and the pellets were re-suspended by stirring for approximate 20 min in an ice/water bath. Sodium acetate buffer (8 mL, 1.0 M, pH 3.8) containing 5 mM of calcium chloride was added to each tube while stirring. AMG (0.1 mL, 3,300 U/mL) was immediately added, mixed well and incubated in a water bath at 50°C for 30 min with intermittent mixing on a vortex mixer. For samples containing <10% RS content, centrifuge aliquots of the undiluted solutions at 6,000×g for 5 min in a microfuge. The final volume in the tube (before removal of an aliquot for centrifugation) was approximately 10.3 mL. For samples containing >10% RS content, the contents of the tubes were quantitatively transferred to 100 mL volumetric flask using a water wash bottle. The volume was adjusted to 100 mL with distilled water, mixed well, and centrifuged at 6,000×g for 5 min. Triplicate aliquots (0.1 mL) were transferred to the bottoms of test tubes, and 3.0 mL of GOPOD reagent was added with mixing, and the tubes were incubated at 50°C for 20 min. A reagent blank solution was prepared by mixing 0.1 mL of 100 mM acetic acid (pH 4.5) with 3.0 mL of GOPOD reagent. Glucose standards were prepared by mixing 0.1 mL of glucose solution (1 mg/mL) with 3.0 mL of GOPOD reagent and incubating at 50°C for 20 min. Absorbance of each solution was measured at 510 nm against the reagent blank. RS content was calculated as follows

$$\text{Resistant Starch (g/100g)} = \Delta A \times F \times \frac{EV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times F \times \frac{EV}{W} \times 0.90$$

Where ΔA = Absorbance of sample solution read against reagent blank

F = Factor to convert absorbance values to mg glucose (100 mg glucose divided by the absorbance value obtained for 100 mg of glucose)

EV = Sample extraction volume (10.3 mL or 100 mL).

0.1 = Volume of sample analyzed

1000 = Conversion from mg to mg

100/W = Conversion to 100 mg sample

W = Sample weight in mg

162/180 = Factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch

2.2.6 Characterization of Native and RS Obtained from Purple Starches

1) Color Measurement

The color of the samples was measured using a colorimeter and the Hunter *L** (lightness), *a** (redness to greenness) and *b** (yellowness to blueness) values using the Hunter system (Hutchings, 1994). Each sample was loaded in a glass Petri dish (6 cm diameter, 1 cm thickness), making sure that the surface presented for analysis at the bottom was as flat as possible. The measuring head was placed in an upright down position and attached directly to the bottom of the sample. The sample was covered with opaque coverage and then measured. All analyses have been triplicated.

2) Scanning Electron Microscopy (SEM)

The surface structure of the samples was observed by SEM using the methodology of Kim *et al*. (2008). A dry, finely ground sample was placed on double-sided adhesive tape, mounted on an aluminum sample holder, and covered with a thin layer of vacuum gold. Samples were examined using a scanning electron microscope.

3) Swelling and Solubility Index (Ashwar *et al*., 2016b)

Samples (0.2 g) were taken in pre-weighed centrifuge tubes with 10 mL of distilled water. The samples suspensions were then incubated in a water bath for 30 min at 60, 70, 80 and 90°C with vortex after every 5 min. After cooling the samples to room temperature, the tubes were centrifuged at 5000×g for 15 min. Supernatant was decanted in pre-weighed moisture dishes. The gain in weight of centrifuge tubes was expressed as swelling index. Moisture dishes were dried at 110°C for 12 h and then cooled in a desiccator to room temperature. The gain in weight of moisture dishes was expressed as solubility index.

4) X-ray diffractometer (Babu and Parimalavalli, 2016)

X-ray diffraction pattern of starch samples were obtained using a X-ray diffractometer. X-ray diffractometer was operated at 40 kV and 30 mA with 0.154 nm CuK radiation (Ni filter). Diffractograms were plotted between the 2θ angles of 5° and 40° at a scan rate of 2°/min. The degree of crystallinity of samples was quantitatively estimated following the method of Nara and Komiya (1983) with the Origin-version 8.5 software (Microcal Software Inc., Northampton, MA, USA).

5) Pasting Properties

The pasting properties of the samples (3.0 g, 14% wet basis) were determined with a rapid visco analyzer. The viscosity was expressed in centipoise units (cP). The samples were held at 50°C for 1 min, heated to 95°C at 3.5 min and held at 95°C for 2.5 min. The samples were then cooled to 50°C in 4 min and held at 50°C for 2 min. The rotating speed was held at 960 rpm for 10 sec and then maintained at 160 rpm during the process. Parameters including pasting temperature, peak viscosity, breakdown, final viscosity and setback were recorded.

6) Differential Scanning Calorimetry (DSC)

The gelatinization characteristics of samples were determined by the method described by Babu *et al*. (2016b) using a differential scanning calorimeter (DSC). A 2 mg sample dwb was weighed in an aluminum pan and 10 μL of deionized water was added. The pan was sealed tightly and then it was allowed to stand for 1 h before carrying out the analysis. An empty aluminum pan was used as a reference. The sample was

subjected to a heating program over a range of temperature from 10 to 125°C and a heating rate of 5°C/min. The onset, peak, and final temperatures (*T*o, *T*p, and *T*c, respectively) and transition enthalpy (ΔH) were determined, where ΔH is the enthalpy change of the reaction, m is the mass of the sample at the beginning of the experiment, K is the calibration coefficient, and A is the area under the peak (Artiaga *et al*., 2005).

2.2.7 Extraction of Bioactive Compounds

Extract preparation before analyzes of total phenolic content (TPC) and antioxidant activity, the samples were extracted by the procedure modified from Burgos *et al*. (2013). The samples (1 g) were extracted with 10 mL of 80% ethanol in 15 mL centrifuge tubes and then shaken for 3 h at room temperature. The mixtures were centrifuged at 4000× g for 10 min. The supernatant was filtered through Whatman No. 1. The extracts were kept under -18°C before analysis.

2.2.8 Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method described by Lemos *et al*. (2015). Briefly, 100 µL of the extract was mixed with 5 mL of 10% (v/v) Folin-Ciocalteu phenol reagent, followed by 0.9 mL of deionized water, the mixture was mixed well and allowed to stand for 5 min. A 3.5 mL of 11.5% sodium carbonate solution was added to the mixture and then allowed to react in the dark for 2 h at room temperature before measuring the absorbance at 765 nm using a spectrophotometer. The TPC value was reported as mg gallic acid equivalents per 100 g sample based on dry basis (mg GAE/100 g, dwb)

2.2.9 Chromatographic Analysis of Anthocyanins

All the samples were analyzed for anthocyanins by reverse-phase HPLC with slight modifications from Pengkumsri *et al*. (2015) using an Agilent 1200 equipped with a multiwavelength detector. Symmetry RP18 Column (4.6 mm of diameter × 250 mm of length, 5 µm particle diameter, Waters Co., Ltd., Milford, MA, USA) was used to separate each form of anthocyanin and the detection wavelength was set at 520 nm. Phosphoric acid (3%) in acetonitrile and phosphoric acid (3%) in deionized water was used as the mobile phase at a flow rate of 1.0 mL/min. The linear gradient elution was operated from

0 to 40 min, with acetonitrile ranging from 10% to 20%. All the samples were tested in triplicate.

2.2.10 Antioxidant Activity

The 1,1-diphenyl-2-picrylhydracyl (DPPH) assay was determined using the method adapted from Burgos *et al*. (2013). A 100 µL of the extract was added to 3 mL of 0.1 M freshly prepared DPPH• free radical in methanol. The absorbance of the mixture was measured at 517 nm after allowing to stand at room temperature for 30 min. The antioxidant activity is expressed in percent inhibition, which is calculated by the formula:

$$\% \text{ Inhibition} = \frac{\text{absorbance blanks} - \text{absorbance of sample}}{\text{absorbance blanks}} \times 100$$

The scavenging ability was determined using 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay modified from Burgos *et al*. (2013). The $ABTS^{\bullet+}$ stock solution was prepared by mixing 7 mM ABTS with 4.75 mM potassium persulfate by the ratio of 1:1 (v/v) for 12 h at room temperature under the dark. Before measurement, the $ABTS^{\bullet+}$ working solution was prepared by mixing the stock solution with DI water until the absorbance reached to 1.0 at 734 nm. A 150 µL of the extract was reacted with 3 mL of working solution and allowed to stand for 10 min. The antioxidant activity is expressed in percent inhibition, which is calculated by the formula:

$$\% \text{ Inhibition} = \frac{\text{absorbance blanks} - \text{absorbance of sample}}{\text{absorbance blanks}} \times 100$$

Ferric reducing ability power (FRAP) assay was measured using the method adapted from Lemos *et al*. (2015). The FRAP reagent was prepared by mixing 300 mM acetate buffer of pH 3.6 with 20 mM ferric chloride solution and 10 mM solution of 2,4,6-tripyridyl-s-triazine reagent in 40 mM hydrochloric acid with the ratio of 10:1:1 (v/v/v). A 100 µL of the extract was added to 3 mL of FRAP reagent, then allowed to react at room temperature for 30 min before measuring the absorbance at 593 nm. $FeSO_4$ was used as standard, and results were expressed as µmol Fe II equivalent per gram sample based on dry basis.

2.2.11 *In vitro* Starch Digestibility (Aisara *et al*., 2021)

1) Simulated Oral Conditions

Samples (50 μL at an initial concentration of 100 mg/mL) was mixed with 375 μL of simulated salivary fluid (SSF) (15.1 mM potassium chloride, 3.7 mM potassium dihydrogen phosphate, 13.6 mM sodium hydrogen carbonate, 0.15 mM magnesium chloride, 0.06 mM ammonium carbonate, 1.5 mM calcium chloride, pH 7.0), 25 μL of 100 U/mL salivary α-amylase dissolved in SSF, 1.25 μL of 0.3 M calcium chloride and 48.75 μL of water. The reaction mixture was incubated at 37°C for 120 min. Samples were periodically taken at 30 min intervals. The samples were then placed in a 100°C water bath for 10 min to terminate the reaction of the enzymes prior to the quantitative analysis of reducing sugars using the DNS method (Miller, 1959), and was determined for total sugar content using the phenol-sulfuric acid method (Dubois *et al*., 1956). Percentage hydrolysis of sample was calculated based on reducing sugar liberated and total sugar content of the sample (Korakli *et al*., 2002):

$$\text{Hydrolysis (\%)} = \frac{\text{reducing sugars released}}{\text{total sugar content} - \text{initial reducing sugar content}} \times 100$$

2) Simulated Gastric Conditions

Samples at an initial concentration of 10 g/L total carbohydrate was adjusted to pH 2.5 with 0.2 M hydrochloric acid and was subsequently incubated at 37°C. After being incubated for 0, 30, 60, 90 and 120 min, samples were taken and neutralized with 0.2 M sodium bicarbonate for further quantitative analysis of reducing sugars by applying the DNS method (Miller, 1959), and was determined for total sugar content using the phenol-sulfuric acid method (Dubois *et al*., 1956). Percentage hydrolysis of sample was calculated based on reducing sugar liberated and total sugar content of the sample (Korakli *et al*., 2002):

$$\text{Hydrolysis (\%)} = \frac{\text{reducing sugars released}}{\text{total sugar content} - \text{initial reducing sugar content}} \times 100$$

3) Simulated Pancreatic Conditions

Samples at an initial concentration of 100 mg/mL (50 μL) was mixed with 375 μL of simulated intestinal fluid (SIF) (6.8 mM potassium chloride, 0.8 mM

potassium dihydrogen phosphate, 85 mM sodium bicarbonate, 38.4 mM sodium chloride, 0.33 mM magnesium chloride, 0.6 mM calcium chloride and 25 μL of 0.3% (w/v) pancreatin solution), 31.25 μL of SSF, 1.25 μL of 0.3 M calcium chloride and 48.75 μL of water. The reaction mixture was incubated at 37°C for 120 min. The samples were then placed in a 100°C water bath for 10 min to terminate the reaction of the enzymes prior to quantitative analysis of reducing sugars using the DNS method (Miller, 1959), and was determined for total sugar content using the phenol-sulfuric acid method (Dubois *et al*., 1956). Percentage hydrolysis of sample was calculated based on reducing sugar liberated and total sugar content of the sample (Korakli *et al*., 2002):

$$\text{Hydrolysis (\%)} = \frac{\text{reducing sugars released}}{\text{total sugar content} - \text{initial reducing sugar content}} \times 100$$

2.2.12 Statistical Analysis

The data were expressed as mean ± SD of three replicate independent experiments were analyzed by one-way and two-way analysis of variance (ANOVA), Duncan's multiple range-test and independent T-test were used to determine significant difference. *p* values of < 0.05 were regarded to be statistically significant. SPSS statistics version 22.0 was used for statistical analysis in this study.

CHAPTER 3

Results and Discussion

3.1 Preparation of Purple Starches

Typically, starch is extracted using physical (wet milled), chemical (acid, alkaline), and enzymatic methods (protease and lipase) (Benmoussa and Hamaker, 2011; Palacios-Fonseca *et al*., 2013; Correia *et al*., 2012). The chemical extraction method, which removes fat and protein and gives high yield and purity of starch (El Halal *et al*., 2017). Purple rice starch and purple sweet potato starch were washed with diluted alkali, followed by washing with water to a neutral pH. The process requires a long time because of sedimentation of the starch and the washing out of the alkali with water. Lum-dubwong and Seib (2000), modified the alkali washing method and isolated rice starch from commercial wet-milled rice starch from Thailand, the isolated starch using this "improved" method was able to have the protein removed successfully. The purple sweet potato, starch was isolated from purple sweet potato using the alkali washing method, which is a common method for starch extraction (Sun *et al*., 2021). In the process of purple corn starch isolation, purple corn was soaked in sodium metabisulphite takes more than 20 h at a temperature of 50°C. According to Taylor, immersion in a solution of sodium metabisulfite could prevent browning reaction on starch, resulting a brighter color. In addition, sodium metabisulphite during extraction would break down the matrix surrounding the starch particles and help release the starch (Taylor *et al*., 1994).

The chemical method has advantages and disadvantages, but it is necessary to have an accurate method with low residual protein content. However, this method generates a large quantity of alkaline waste, resulting in a high cost of wastewater treatment (Wang and Wang, 2001). However, this study was conducted to develop the use of local purple rice, purple sweet potato, and purple corn in Thailand as raw materials to produce the purple starches.

3.1.1 The Appearance Characteristic of Purple Starches

Purple starches had a pink-purple (Figure 3.1), tasteless, odorless powder, can used in food processing. The versatility of starch products is such that they are used as ingredients and functional supplements in a variety of food, as starch is the largest constituent of cereal grains and tubers, there is an increasing interest in the isolation of the product from starch sources (El Halal *et al*., 2017). The utility of purple starches can be increased by developing appropriate processing techniques to prepare RS3 as value-added product.

(a) (b) (c)

(d) (e) (f)

Figure 3.1 The raw materials and purple starches, (a); purple rice, (b); purple sweet potato, (c); purple corn, (d); purple rice starch, and (e); purple sweet potato starch, and (f); purple corn starch

3.1.2 Chemical Composition

The compositional analysis of different purple starches including purple rice starch, purple sweet potato starch and purple corn starch showed the content of carbohydrate (80.28-81.52%), fiber (3.31-4.74%), moisture contents (11.01-12.42%), lipid (0.54-0.86%), protein (0.68-1.09%), and ash (1.37-2.46%) is shown in Table 3.1. These parameters showed significant differences in chemical composition between the three purple starches samples. The low contents of protein, fat and ash indicated that these

starches were extremely pure, and the residual protein was removed during alkali extraction. Previous studies have shown that rice flour contained 84.66% starch, 6.22% protein, 0.24% lipid, and 0.18% ash (Puncha-arnon and Uttapap, 2013), the chemical composition of purple sweet potato flour contained protein, fat, ash, and carbohydrate were 6.53%, 0.21%, 0.85%, and 85.84%, respectively (Asranudin *et al*., 2021). Proximal analysis of purple corn flour showed that protein content was 8.58%, fat content 5.73%, ash content 0.02%, fiber content 2.91%, and carbohydrates content 71.06% (Vilcacundo *et al*., 2020). The difference between starch and flour is that most of the native proteins and lipids in the flour have been removed. The results revealed that the purple starches from the starch extraction process is feasible for using as a substrate in the formation of RS3 by gelatinization and retrogradation process. We therefore expect to use the DART and DET for the direct conversion of starchy substrate to RS3 in the next study in order to utilize purple starches most efficiently.

Table 3.1 Chemical compositions of native purple starches

Parameter	Purple rice starch	Purple sweet potato	Purple corn starch
Moisture (%)	11.01 ± 0.39^{b}	12.42 ± 0.50^{a}	11.77 ± 0.60^{ab}
Ash (%)	1.37 ± 0.11^{c}	2.46 ± 0.14^{a}	2.11 ± 0.14^{b}
Lipid (%)	0.69 ± 0.13^{ab}	0.54 ± 0.08^{b}	0.86 ± 0.15^{a}
Protein (%)	0.89 ± 0.14^{ab}	0.68 ± 0.11^{b}	1.09 ± 0.15^{a}
Fiber (%)	4.74 ± 0.55^{a}	4.00 ± 0.32^{ab}	3.31 ± 0.22^{b}
Carbohydrate (%)	81.52 ± 0.10^{a}	80.28 ± 0.57^{a}	81.21 ± 0.94^{a}
Amylose content (%)	9.36 ± 0.51^{b}	20.64 ± 0.60^{a}	6.27 ± 0.51^{c}

Different superscripts letter within the same column indicates a statistically difference at $p<0.05$

3.1.3 Amylose Content

The amylose content among three different purple starch was significantly different ($p<0.05$) between samples. The amylose content in native starch samples was shown in Table 3.1. The highest amylose content was found in purple sweet potato starch as 20.64%. The amylose content of purple rice starch was 9.36% and the lowest amylose content was found to be purple corn starch (6.27%). The amylose content of white, brown, red, and purple Thai rices ranged from 2.20 to 28.90% (Ponjanta *et al*., 2016, Saikia *et*

al., 2012). Rice with amylose content of 10-20% is classified as low amylose starch. As in the agreement of this study, all purple starched was included in low amylose starch (Juliano *et al*., 1981; Yu *et al*., 2012). Soison *et al*. (2015) found slightly lower results of amylose content in purple sweet potato ranging from 16.5% to 18.5%, compared with the current study. The amylose content of purple corn starch obtained in the present study differed from that determined by the study by Mansilla *et al*. (2019), where they obtained an amylose content ranging from 18.32% to 25.35%. The variation in amylose content of the purple starches to may cause by difference of growing regions and conditions (Wani *et al*., 2013).

One of the most important factors in the formation of RS is the amylose/amylopectin ratio (Garg et al., 2017), a higher content of amylose lowers the digestibility of starch due to positive correlation between amylose content and formation of RS (Sajilata *et al*., 2006). The amylopectin is a much larger molecule than amylose, therefore, it has a much larger surface area per molecule than amylose which makes it a preferable substrate for amylolytic attack. Furthermore, the glucose chains of amylose starch are more bound to each other by hydrogen bonds making them less available for hydrolysis (Singh *et al*., 2010). The greater the content of amylose is, the more difficult the starch is to gelatinize (Biduski et al., 2018) and the more susceptible to retrogradation (Topping *et al*., 2003). Additionally, the *in vitro* and *in vivo* digestibility of high amylose starch containing products was lower than that of the control products without these starches (Gelencsér, 2009). Ao *et al*., (2007) observed that the chain length unit of amylopectin showed correlation with the digestibility. Moreover, the chain length, the degree of polymerization of amylose and amylopectin molecules and the branch density also have an impact on RS content (Perera *et al*., 2010). The rate of starch hydrolysis is controlled by mass transfer rate (influenced by molecular weight distribution, degree of polymerization, content of 1,6 branching bonds of the starches) and the effects of the starch structure are dependent on the substrate concentration (Singh *et al*., 2010).

3.2 Production of RS from Purple Starches by Dual Autoclaving-Retrogradation Treatment (DART)

The order of maximum to minimum amount of RS content in native purple starches was purple sweet potato starch, purple rice starch and purple corn starch (Table 3.2).

DART significantly (p<0.05) increased the RS content from 5.35 to 34.13 g/100 g dwb, 9.11 to 26.15 g/100 g dwb, and 3.18 to 20.73 g/100 g dwb for purple rice starch, purple sweet potato, and purple corn starch, respectively. The result revealed that the RS content of the purple starches was 6.37-, 2.89- and 6.51-fold increase after DART, respectively.

Native purple sweet potato starch showed the highest RS content, this may be due to its high amylose content. After DART, the maximum of RS content was found only 26.15 g/100 g dwb, when the amount of water added was 80 mL (starch 20 g and water 80 mL). In this study, however, we found that the receiving RS content of purple sweet potato starch quite low when compared to the other substrates, this may be involved in an unsuitable of the starch and water added. Ji *et al*. (2010) and Li *et al*. (2010, 2015) also reported that if the amount of water added is low, the starch could not be fully gelatinized, resulting in an incomplete destruction of the starch molecular sequence attributed to insufficient amylose molecules for the formation of a double helix structure and inadequate aging, thus the RS content would be relatively low. In addition, the retrogradation of sweet potato starch is also dependent on the concentration, storage temperature, and chemical compositions of starch. The retrogradation rate accelerates with high starch concentrations and low storage temperatures (Ishiguroa *et al*., 2003). Chung *et al*. (2009) also reported higher levels of resistant starch in high amylose starches like pea and lentil as compared to corn starch. The increase of RS content to the longer cooling time was associated with higher percentage of retrograded starch. Because it was formed by processing, the RS type was RS3. RS3 was a RS that was formed mainly due to food processing (Raigond *et al*., 2015).

Autoclaving-retrogradation treatment is one of the methods that is widely used for production of RS. This modification is performed without addition of chemicals, so it the RS is produced without by-products formation. However, this modification process requires equipment such as an autoclave that can provide high temperature and pressure.

Table 3.2 RS content of purple starches obtained by DART

Sample	Resistant starch content (g/100 g dwb)	
	NS	DART
Purple rice starch	5.35 ± 0.14^{b}	34.13 ± 0.65^{a}
Purple sweet potato starch	9.11 ± 0.12^{a}	26.15 ± 1.02^{b}
Purple corn starch	3.18 ± 0.10^{c}	20.73 ± 0.77^{c}

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$). NS; native starch, DART; dual autoclaving-retrogradation treatment

During starch gelatinization by autoclaving, amylose is leached out from the granules into solution as a random coil polymer. Upon cooling, amylose chains reassociate as double helical coils and can form tightly packed structures that are stabilized by hydrogen bonds that are resistant to enzymatic hydrolysis (Ashwar *et al*., 2015, Simsek and El, 2012). The proposed a concept of starch gelatinization and retrogradation by DART is shown in Figure 3.2.

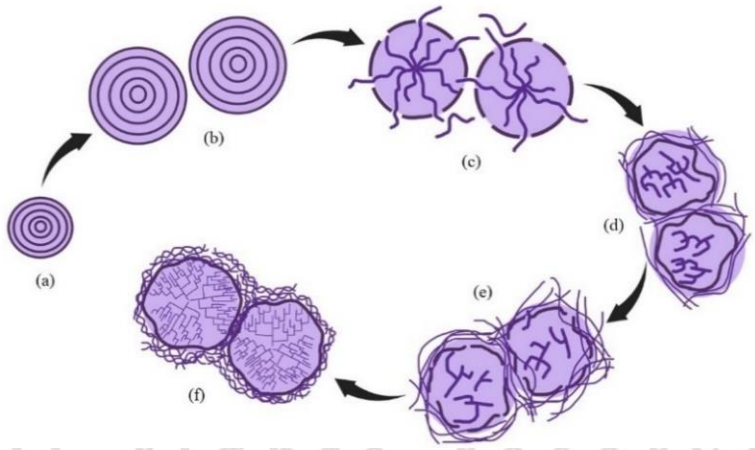


Figure 3.2 The proposed a concept of starch gelatinization and retrogradation during DART, (a); Native starch granules, (b); gelatinization, associated with swelling, (c); amylose leaching and partial granule disruption, resulting in the formation of a starch paste, (d); retrogradation, (e); formation of an amylose network during cooling of the starch paste, and (f); formation of ordered or crystalline amylopectin molecules (amylopectin retrogradation) during storage (Modified from Goesaert *et al*., 2005)

The application of higher temperature along with higher pressure causes faster retrogradation rate, while cooling temperature leads to better retrogradation. The technical conditions used affect the gelatinization and retrogradation process, which subsequently influence the amount of RS produced (Fuentes-Zaragoza *et al*., 2010). In autoclaving-retrogradation treatment, RS can be increased by adjusting processing conditions such as source of sample, heating and cooling temperature and time, also number of autoclaving-retrogradation treatment cycle (Sajilata *et al*., 2006; Faridah *et al*., 2022). Novelose 330 is an example of a commercial RS product produced through autoclaving-cooling cycle process. Several studies stated that the more cycles of autoclaving-retrogradation treatment to increase the higher RS content (Dundar and Gocmen, 2013; Lee *et al*., 2013). However, Ratnaningsih *et al*. (2019) reported that single autoclaving-retrogradation treatment resulted in higher RS content than autoclaving-retrogradation treatment with 3 cycles and 5 cycles. Thus, 3-5 cycles it could be an alternative reprocess for increase RS production. Ashwar *et al*. (2016b) reported that dual autoclaving-retrogradation treatment was optimized for preparing RS. Rahmawati *et al*. (2018) reported that the addition of cycles in the autoclaving-retrogradation treatment did not significantly affect the content of RS. In this work, a dual autoclaving-retrogradation treatment was designed for preparing RS from purple rice starches, can cause reorganization of the amylose and amylopectin chains of starch which significantly improved its physicochemical and digestibility properties. However, re-process more than dual cycle was selected for RS production in order to increase the RS content and for further up-scale utilization.

3.3 Production of RS from Purple Starches by Dual Enzyme Treatment (DET)

3.3.1 Optimization of α-Amylase Hydrolysis Conditions for Dual Enzyme Treatment of Purple Starches

1) Effect of Temperature on RS Content

The α-amylases play many important roles in food industrial applications. The α-amylases, EC 3.2.1.1, are endo-acting enzymes catalyze the hydrolysis of α, 1-4 glucosidic linkages of starch polymers internally, it is impossible to catalyze the hydrolysis of α, 1-6 glucosidic linkages (Paul *et al*., 2021). The released products from hydrolysis are various oligosaccharides and different α-limit dextrins

containing α, 1-6 bonds. A suitable linear chain length is needed for double helix formation and a consequent reduction in starch digestibility. The use of amylases prior to debranching, combined with a method that enhances retrogradation, is an efficient way of forming RS3 (Zeng *et al*., 2015; Villas-Boas *et al*., 2020). The effect of temperature for hydrolysis of α-amylase on RS content is shown in Figure 3.3. RS content in all samples was slightly increase when the reaction temperature of α-amylase was increased from 70°C to 90°C. The maximum RS content at 90°C of purple rice starch, purple sweet potato starch, and purple corn starch were 35.19, 34.58, and 39.91 g/100 g dwb, respectively. The RS content slightly decreased when the hydrolysis was preformed above 90°C. The effects of temperature on enzyme activity seems to be complex procedure, enzyme activity increases with the increasing temperature up to the optimum temperature, due to increasing kinetic energy that increases the chances of collisions between enzyme and substrate resulting higher substrate-product catalysis (Fatoniand and Zusfahair, 2012). After reaching the optimum temperature, increase in temperature decrease the amylase activity. The high temperature will break the secondary, tertiary, and quaternary bonds that maintain the enzyme in its natural structure (Bisswanger, 2014). Retrogradation of starch is impacted by the length of the amylose chains. Longer chains of amylose are hydrolyzed by α-amylase, which attacks the α-1,4 chemical bonds, whereas pullulanase shows greater activity towards short amylose chains. The shorter chains of amylose would facilitate molecular contact to form a crystalline structure. Therefore, the inhibition of α-amylase activity at higher temperatures resulted in decreased yield of RS.

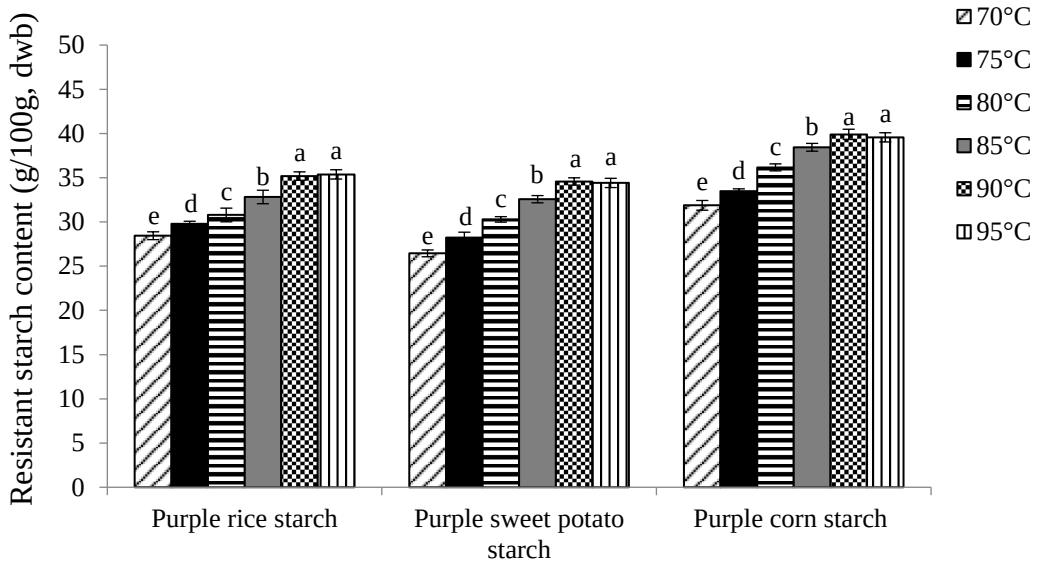


Figure 3.3 Effect of temperature on RS content

2) Effect of pH on RS Content

The pH affects the hydrolytic activity of α-amylase, the activity was increased with pH until it achieves the optimum pH and the activity was decreased after reaching the optimum pH. Changes in pH alter the amino acid side chain ionization on the active side of the enzyme and therefore the enzyme will be in the best conformation at the optimum pH (Yadav and Prakash, 2011). The investigation of pH effect on α-amylase activity was done under the pH ranging from 4.5 to 7.0. As shown in Figure 3.4, The RS content of all purple starches increased when the pH was elevated from 4.5 to 5.5 with the maximum RS content of purple rice starch, purple sweet potato starch, and purple corn starch were 37.30, 36.48, and 41.44 g/100 g dwb, respectively. The amount of RS produced decreased as the pH increased higher than 5.5, reflecting a decrease in α-amylase activity at higher pH. A similar trend was observed when α-amylase was applied to promote RS formation at pH 5.5 (Zhang and Jin, 2011a). iKnowZyme® HTAA is a heat stable α-amylase, it renovates from *Bacillus licheniformis*, the optimum pH of the α-amylase was 5.5-6.5 (Appendix E).

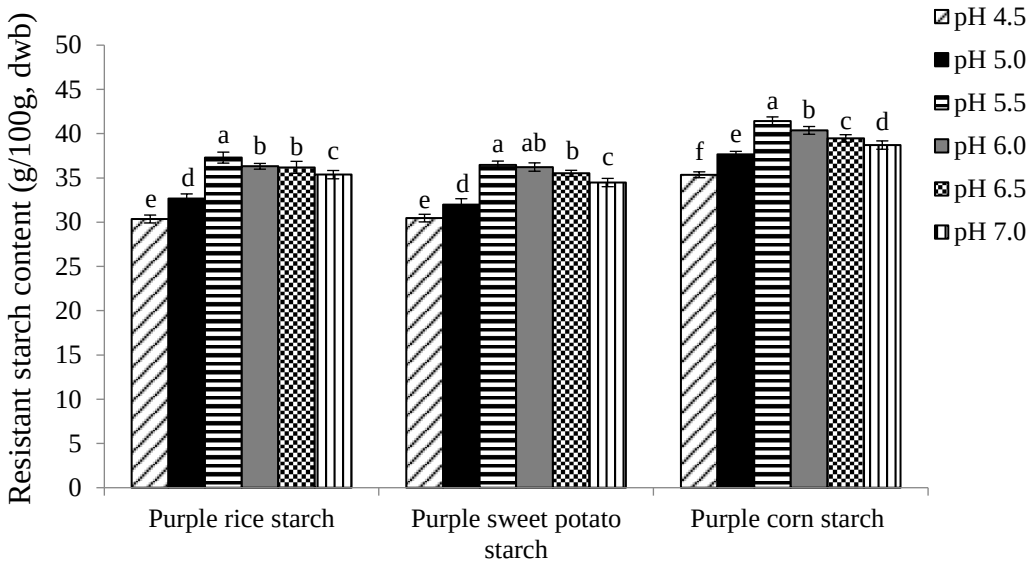


Figure 3.4 Effect of pH on RS content

3) Effect of Reaction Time on RS Content

The reaction time is one of the most critical factors in the RS production by enzymatic method. Given sufficient time, the amylose molecules could be completely degraded into α-limit dextrins, preventing RS formation. The RS content increased gradually as the reaction time increased from 10 to 15 min (Figure 3.5), with the maximum RS content at 15 min of reaction. The highest RS content of 40.35, 42.45, and 43.79 g/100 g dwb was achieved from purple rice starch, purple sweet potato starch, and purple corn starch, respectively. Further prolonged reaction time resulted in a reduction in RS productions, explaining that amylose chains may be hydrolyzed into α-limit dextrins, thereby preventing RS formation. At reaction times longer than 30 min, the RS content decreased more slowly because the hydrolysis reaction tended to balance on substrate consumptions, thereby preventing RS formation. Zhang and Jin (2011a) reported that the initial rate of the dual enzyme reaction increased rapidly. Furthermore, the rate of hydrolysis and RS content decreased, with the duration of the enzyme modification being longer than 25 min. The trend of our results was similar to that of Liu *et al*. (2022). Reddy *et al*. (2013) showed that the RS content increases continuously as the duration of hydrolysis is extended from 10 min to 40 min. The disagreement with our

results might be due to the differences in the type of starch or enzyme hydrolysis conditions and the narrow range of hydrolysis time.

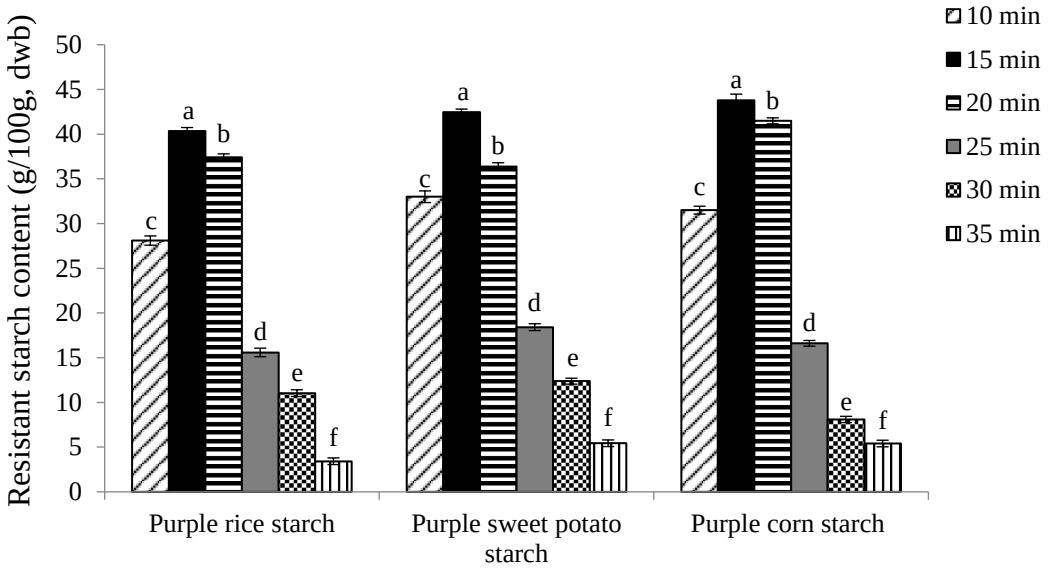


Figure 3.5 Effect of reaction time on RS content

4) Effect of α-Amylase Loading on RS Content

The effects of α-amylase loading on the production of RS were evaluated at 0-10 U/g. As shown in Figure 3.6, the maximum RS content in purple starches were obtained in the reaction using 4 U/g of α-amylase. The highest RS amount in purple rice starch, purple sweet potato starch, and purple corn starch were 42.47, 44.50, and 46.38 g/100 g dwb, respectively. The production of RS gradually increased as the amount of α-amylase increased from 0 to 4 U/g. However, further increases in α-amylase amount resulted in decreased RS production. The trend of the results was similar to that found by Gao *et al.* (2011). The addition of α-amylase increased the RS content compared to purple starches without α-amylase (0 U/g). However, the excessive increase in the amount of added α-amylase resulted in the degree of polymerization of the starch decreasing. Doblado-Maldonado *et al*. (2017). suggested that the difference in the molecular structure of starch can drive the amount of RS, and starch with higher DP and shorter external chains had a higher content of RS. The high amount of added α-amylase led to a significant decrease in the degree of polymerization of starch caused by high branching, thereby leading to the reduction in the RS content. This result suggested that

thermostable α-amylase should be added properly to increase the RS content effectively. These results indicated that the optimum amount of α-amylase was 4 U/g.

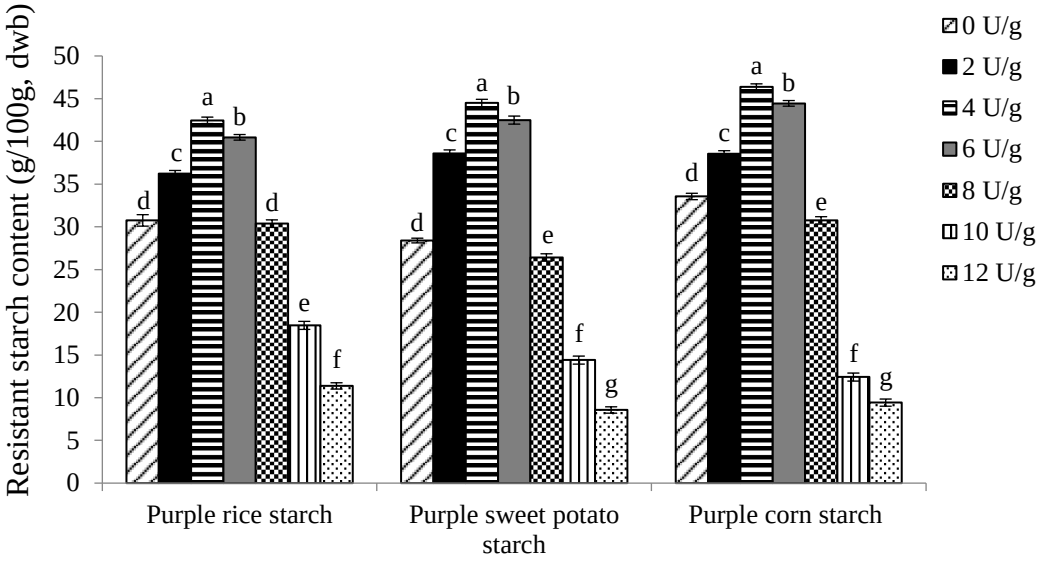


Figure 3.6 Effect of α-amylase loading on RS content

5) Effect of Purple Starches Concentration on RS Content

The effect of the purple starches concentration on the RS content is shown in Figure 3.7, the maximum RS content of purple rice starch, purple sweet potato starch, and purple corn starch were 43.75, 46.37, and 48.27 g/100 g dwb, respectively when the starch concentration was 25, 20, and 25% (w/v). The content of RS initially increased and then decreased as the starch concentrations increased. The RS content obtained from purple rice starch and purple corn starch increased as the starch concentration increased within the range of 15-25% (w/v) and decreased significantly above 30% (w/v). As well as the RS content produced from purple sweet potato increased as the starch concentration increased within the range of 15-20% (w/v) and decreased significantly above 25% (w/v). This finding indicated that the low starch concentration favored enzymatic hydrolysis. At low starch concentrations, the magnitude of the association of thermostable α-amylase and pullulanase with starch molecules. At high concentrations, thermostable α-amylase and pullulanase cannot react evenly with the starch molecules due to difficulty in starch gelatinization, which is not conducive to the mutual proximity and crystallization of amylose, leading to a reduction in the RS content.

This result was similar to that of Liu *et al*. (2022), which showed that the RS content changes significantly with the starch concentration. In conclusion, the purple starch concentration of 25% (w/v) benefited the enzymatic hydrolysis of purple rice starch and purple corn starch and 20% (w/v) of purple sweet potato starch.

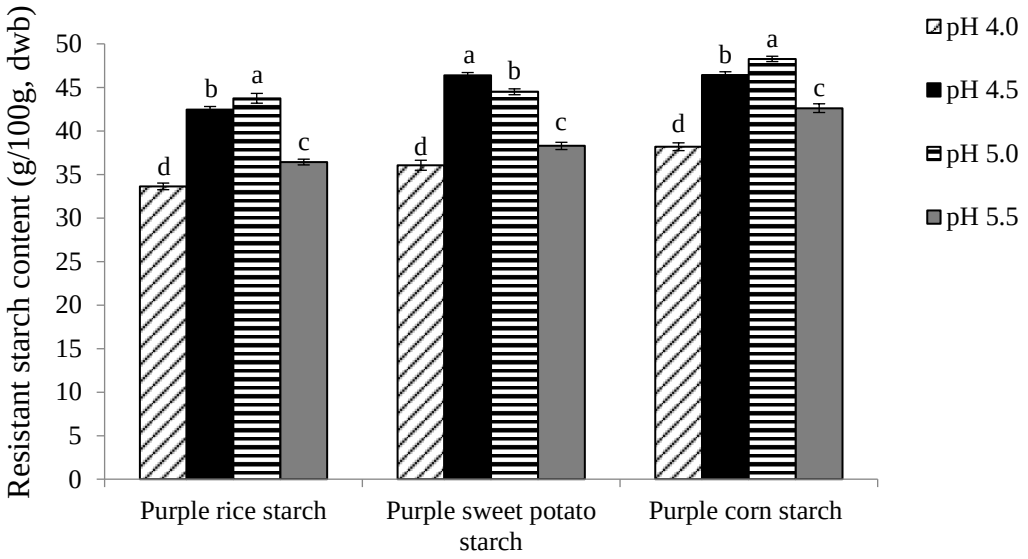


Figure 3.7 Effect of purple starch concentration on RS content

The RS content of foods depends on the botanical source of the starch and the processing conditions (Zhang and Jin, 2011a). In this study, the reaction conditions in the pasting process of purple starches found that RS production could be greatly increased by treatment with thermostable α-amylase before debranching with pullulanase. The conditions for α-amylase hydrolysis were achieved under the following conditions: temperature of 90°C; pH of 5.5; time 15 min; loading of α-amylase, 4 U/g, and starch concentration of 25% (w/v) for purple rice starch and purple corn starch, and 20% (w/v) for purple sweet potato starch, as shown in Table 3.3. The highest RS contents were 43.75, 46.37, and 48.27 g/100 g dwb of purple rice starch, purple sweet potato starch, and purple corn starch, respectively. The result revealed that the RS content was 8.18-, 5.09- and 15.18-fold increase after optimized the condition of α-amylase by DET, respectively.

Table 3.3 The optimal condition of α-amylase hydrolysis on RS preparation from the purple starches

Sample	Condition					RS content (g/100g, dwb)
	Reaction time (min)	pH	Temperature (°C)	Amount of α-amylase (U/g)	Starch concentration % (w/v)	
Purple rice starch	15	5.5	90	4	25	43.75 ± 0.56[a]
Purple sweet potato starch	15	5.5	90	4	20	46.37 ± 0.33[b]
Purple corn starch	15	5.5	90	4	25	48.27 ± 0.31[b]

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$).

3.3.2 Optimization of Pullulanase Debranching Conditions for Dual Enzyme Treatment of Purple Starches

1) Effect of Time on Debranching the Purple Starches for RS Production

The effect of time on the debranching of purple starches with pullulanase was carried out, and the results were shown in Figure 3.8. There was a sharp increase within 12 h and a slow increase in 16-28 h in RS content. The maximum RS content of purple rice starch, purple sweet potato starch, and purple corn starch were 45.48, 47.27, and 49.74 g/100 g dwb, respectively was achieved after 16 h. Therefore, the optimum hydrolyzing time was 16 h. In another study, Wu *et al*. (2009) observed that the optimum debranching time was 6 h when hydrolyzed pullulan with pullulanase. The results of Ozturk et al. (2009) indicated that 48 h of debranching of high amylose maize starches was suitable for RS formation. The results of González-Soto et al. (2004) demonstrated that debranching of banana starch with pullulanase for 5 h was sufficient to obtain a high RS content (18 %). The differences in the results found in this study and those determined by other researchers might be due to the source of the starch and the conditions used for the RS preparation.

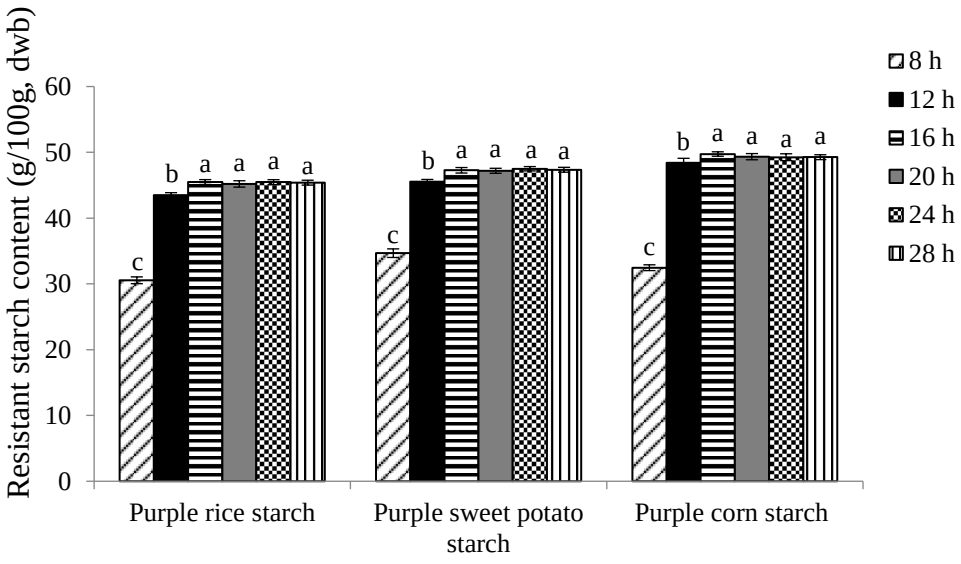


Figure 3.8 Effect of time on pullulanase debranching of purple starches for RS production

2) Effect of pH on Pullulanase Debranching the Purple Starches for RS Production

The pH influences the activity of pullulanase on the debranching of purple starches. Therefore, the different pH values ranging from 4.0 to 5.5 of the reaction mixture on purple starches debranch with pullulanase were investigated. The highest RS content of purple rice starch, purple sweet potato starch, and purple corn starch were 47.37, 49.39, and 51.13 g/100 g dwb respectively were achieved at a pH of 5.5 (Figure 3.9). In other reports, the optimal pH for debranching with pullulanase was 5.0 (Roy *et al*., 2003, Wu *et al*., 2009), 5.9 (Kriegshauser and Liebl, 2000), 6.0 (Kuroiwa *et al*., 2005) and 7.0 (Swamy and Seenayya, 1996). The pullulanase activity was apparent over the acidic ranges, with more than 50% of the maximal activity was detected at pH ranging from 3-6. The enzyme activity was greatly reduced at pH of above 7.0 (Ling *et al*., 2009). iKnowZyme® pullulanase was the pH optimal pH is 4.0-5.0 and effective pH is 3.8-5.5 at 60°C (Appendix E).

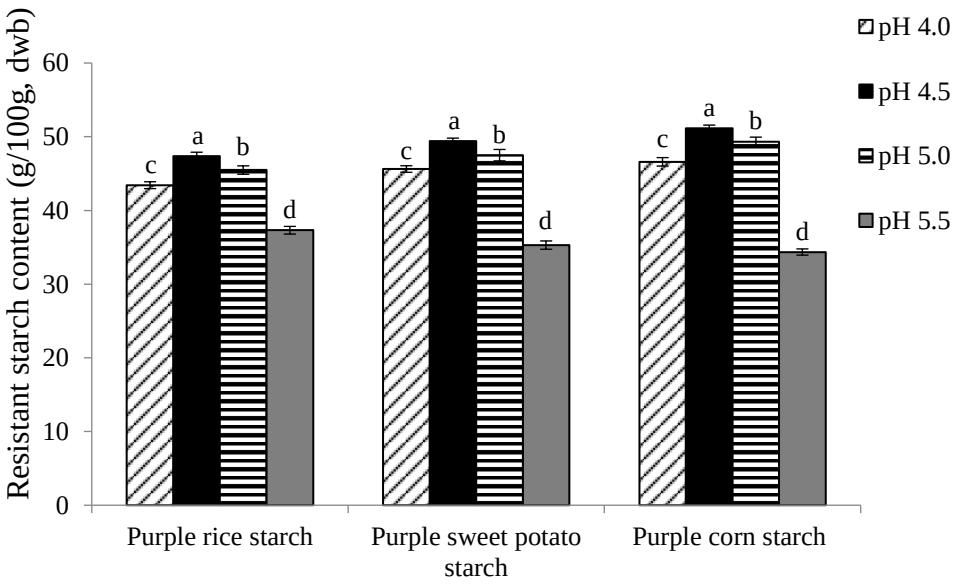


Figure 3.9 Effect of pH on pullulanase debranching of purple starches for RS production

3) Effect of Temperature on Pullulanase Debranching the Purple Starches for RS Production

The temperature plays a role in the activity of pullulanase; thus, it was necessary to study the effects of different temperatures varied from 44 to 52°C of the reaction mixture on pullulanase debranching purple starches with pullulanase. The resulted was shown in Figure 3.10. The highest RS contents were 51.17, 53.34, and 54.38 g/100 g dwb of purple rice starch, purple sweet potato starch, and purple corn starch, respectively. In contrast, the optimal temperature for hydrolyzation with pullulanase were obtained at 45°C (Wu *et al*., 2009), 50°C (Kuroiwa *et al*., 2005), 60°C (Swamy and Seenayya, 1996), 75°C (Kuriki *et al*., 1990, Messaoud *et al*., 2002), and 90°C (Badal *et al*., 1989, Kriegshauser and Liebl, 2000). The different optimal temperature reported may also be due to the differences in substrates and the source of pullulanase. The increasing in temperatures over 50°C caused decreasing in enzyme activity because the tertiary structure of protein was changed, and this performs to denature the active site of the enzyme.

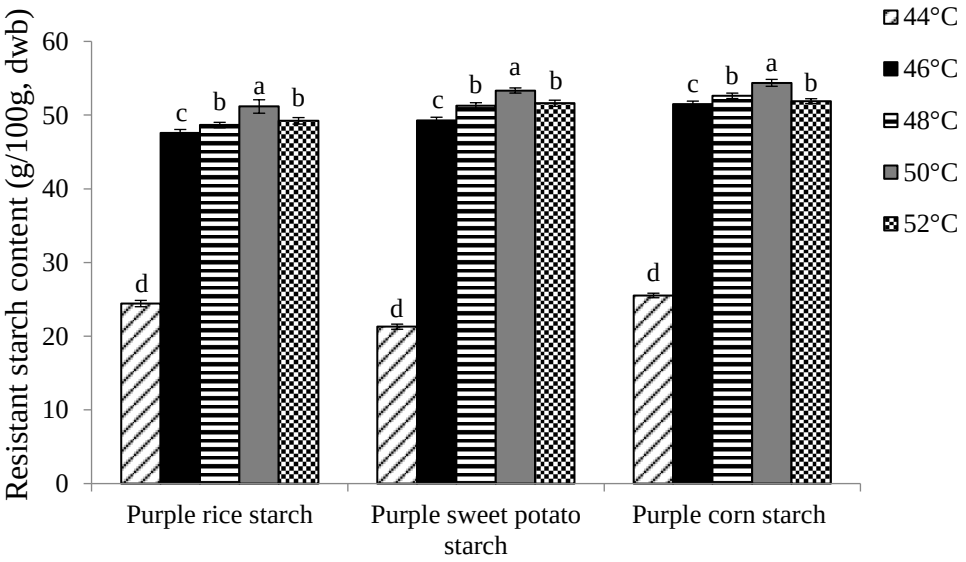


Figure 3.10 Effect of temperature on pullulanase debranching of purple starches for RS production

4) Effect of Amount of Pullulanase Debranching the Purple Starches for RS Production

Figure 3.11 shows the effect of the amount of pullulanase on debranching the purple starches in RS production. At the amount of pullulanase less than 10, 8, and 12 U/g, the RS content of purple rice starch, purple sweet potato starch, and purple corn starch showed a steady increase as the level of pullulanase in the reaction mixture increased. The maximum RS content of purple rice starch, purple sweet potato starch, and purple corn starch were 51.34, 53.36, and 54.27 g/100 g dwb, respectively, observed at the level of 10, 8, and 12 U/g. The results indicated that all purple starches were saturated by pullulanase. The optimum amount of pullulanase under this condition was 10, 8, and 12 U/g for purple rice starch, sweet potato starch, and corn starch, respectively. In a previous report, optimum amount of pullulanase was 8 U/g for high amylose rice starch (Pongjanta *et al*., 2009), 25 U/g for purple sweet potato starch (Zheng *et al*., 2016), the similarly of the optimum amount of pullulanase was 12 U/g for corn starch (Zhang and Jin, 2011a), maybe due to the variation in the substrate. Ren *et al*. (2015) indicated that pullulanase exactly catalyzes the hydrolysis of α-1,6-glycosidic bonds in amylopectin of native starch, resulting in the disruption of double helices and the loss of the characteristic A-type crystal structure and the more the amount of added pullulanase, the greater the interaction of the enzyme with the starch granules in the studied range. The increase in the amount of amylose led to an increase in the content of RS. Shi *et al*. (2013) found that the RS content increased sharply as the amount of the enzyme increased. The highest RS content was obtained with 20 U/g pullulanase, but this content decreased at 30 U/g pullulanase. This disagreement with our results might be due to the differences in enzyme hydrolysis conditions or solvents and the narrow range of enzyme concentrations.

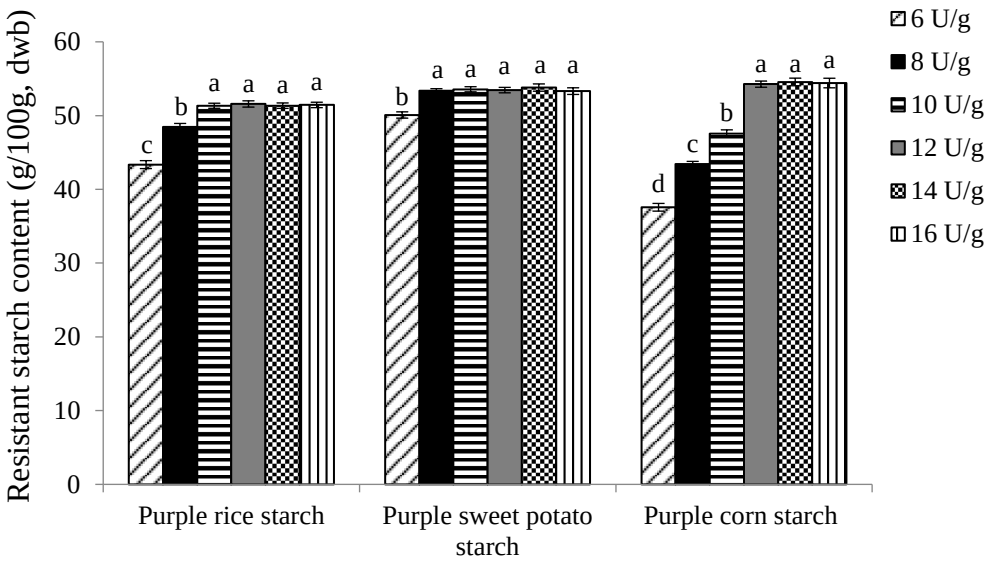


Figure 3.11 Effect of amount of pullulanase debranching the purple starches for RS production

Table 3.4 The optimal condition of pullulanase hydrolysis and debranching on RS preparation from the purple starches

Sample	Condition				RS content (g/100g, dwb)
	Reaction time (h)	pH	Temperature (°C)	Amount of pullulanase (U/g)	
Purple rice starch	16	4.5	50	10	51.34 ± 0.35^{c}
Purple sweet potato starch	16	4.5	50	8	53.36 ± 0.29^{b}
Purple corn starch	16	4.5	50	12	54.27 ± 0.42^{b}

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$)

Purple starches were dually modified using α-amylase and pullulanase to prepare RS. The reaction time, pH, temperature, and the amount of added pullulanase were optimized to increase RS content of the purple starches sample including purple rice starch, purple sweet potato starch, and purple corn starch. The result was shown in Table 3.4, The optimum reaction time were 16 h, pH 4.5, temperature 50°C, and amount of pullulanase was 10, 8, 12 U/g for purple rice starch, purple sweet potato starch, and purple corn starch, respectively. The maximum RS content of purple rice starch, purple sweet potato, and purple corn starch by DET were 51.34, 53.36, and 54.27 g/100g, dwb, respectively. The result revealed that the RS content of the purple starches was 9.60-, 5.86- and 17.07-fold increase after DART, respectively.

Purple starches were subjected to an α-amylase treatment to reduce the length of amylase chains and debranching with pullulanase can generate linear short chains, affording more molecule mobility and an increased opportunity for molecule alignment and aggregation (Zhao and Lin, 2009; Zhao and Jin, 2011). This ordered alignment of starch chains leads to the formation of a perfect crystalline structure (Ozturk *et al*., 2009; Liu *et al*., 2017). During gelatinization, linear short chains and amylose in debranched starch exist as random coil polymers. At low temperature, amylose chains can interact rapidly by forming double helices, while amylopectin need a much longer time to form double helices (Gidley, 1989; Mua and Jackson, 1997). DET can increase the ability of forming double helices. Upon cooling, the flexible linear chains align with other chains to form double helices stabilized by hydrogen bonds and ordered crystalline structure (Haralampu, 2000). Chain length distribution and molecular weight of both amylose and amylopectin and branching patterns of amylopectin can influence the crystalline structure (Gidley, 1989; Mua and Jackson, 1997; Liu *et al*., 2017). The proposed a concept of RS production by DET is shown in Figure 3.2.

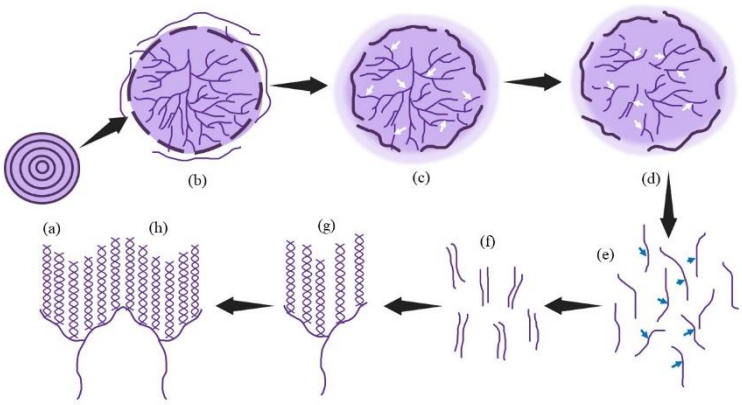


Figure 3.12 The proposed a concept of RS production by DET, (a); native starch granules, (b); gelatinization, associated with swelling, (c); treated with α-amylase, (d); treated with pullulanase, (e); treated with α-amylase, (f); linear short chains, (g); retrogradation forming double helices, and (h); RS3 formation
(Reproduced from Miao *et al*., 2014)

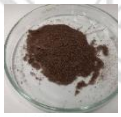
3.4 Characterization of Native and RS Obtained from Purple Starches

3.4.1 Color Measurement

Color measurement of native and RS obtained from purple starches are presented in Table 3.5. The native purple rice, purple sweet potato, and purple corn starches showed that the brightness (*L**) value was in the range of 60.89-68.27, where as *a** value is positive indicates the redness and found to be 4.83, 15.84, and 10.05, respectively. The result indicated that purple sweet potato starch had the reddest color and purple rice starch had the light red color. For the yellowness value *b** values of purple rice, purple sweet potato, and purple corn starches were 4.33, 6.45, and -1.61, respectively. RS obtained from purple starches by DART and DET, it was found that the *L** values of purple rice starches was in the range of 28.25-43.49, reduce from 68.27. Purple sweet potato was 43.32-46.52 reduce form 60.89, and purple corn starch showed that the brightness of RS by DART increased from 67.07 to 74.00, but RS by DET method showed the reduction of brightness from 67.07 to 63.62. The *a** value of RS from purple rice starch was increased in the range of 10.25-13.16, the value of the RS from purple sweet potato starch decreased in the range of 8.96-14.88, and the RS from purple corn starch by DART was lower than native purple corn starch, the value was 6.38, but the RS by DET was 11.35. The change in color values might be caused by the maillard reaction

during autoclaving at high temperature. Similar results were observed for annealed yam starches (Falade and Ayetigbo, 2015).

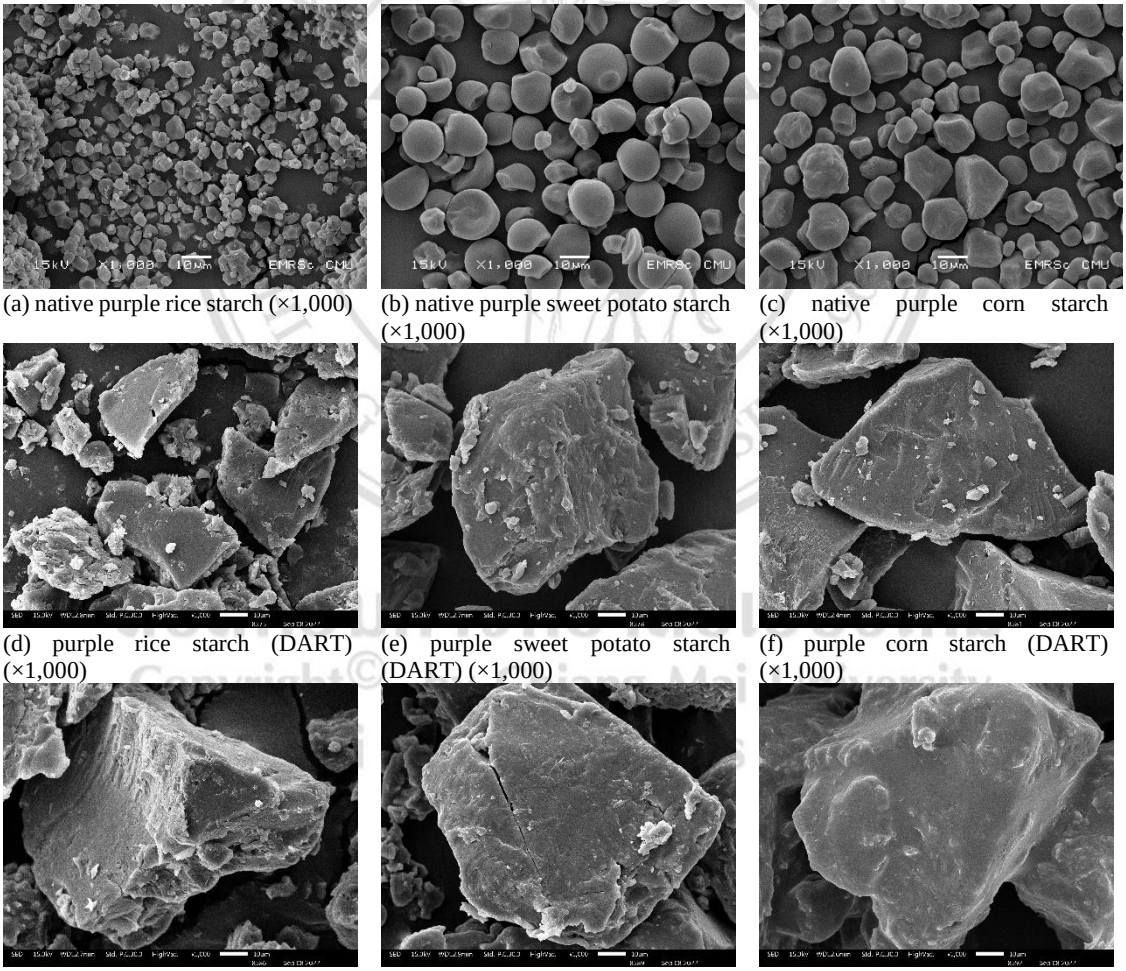
Table 3.5 Appearance and color values of native and RS obtained from purple starches by using different treatment methods

Sample	Appearance	Color		
		*L**	*a**	*b**
Purple rice starch -NS		68.27 ± 0.18^{a}	4.83 ± 0.06^{c}	4.33 ± 0.38^{b}
Purple rice starch - DART		43.49 ± 0.25^{b}	13.16 ± 0.10^{a}	7.14 ± 0.06^{a}
Purple rice starch - DET		28.25 ± 0.50^{c}	10.25 ± 0.32^{b}	3.24 ± 0.19^{c}
Purple sweet potato starch -NS		60.89 ± 0.56^{a}	15.84 ± 0.18^{a}	6.45 ± 0.13^{c}
Purple sweet potato starch -DART		46.52 ± 0.61^{b}	8.96 ± 0.24^{c}	16.95 ± 0.15^{b}
Purple sweet potato starch -DET		43.32 ± 0.88^{c}	14.88 ± 0.20^{ab}	19.55 ± 0.33^{a}
Purple corn starch -NS		67.07 ± 0.16^{b}	10.05 ± 0.10^{b}	-1.61 ± 0.08^{c}
Purple corn starch -DART		74.00 ± 0.65^{a}	6.38 ± 0.13^{c}	15.24 ± 0.24^{b}
Purple corn starch -DET		63.62 ± 0.40^{c}	11.35 ± 0.23^{a}	18.14 ± 0.10^{a}

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different (p<0.05). NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment

3.4.2 The Morphology by SEM

SEM studies revealed that the purple rice starch granules had polyhedral or irregular shapes with size in the range of 5-6 μm (Figure 3.13a). This result was similar to that of Wani *et al*. (2013), which showed that small sized, polyhedral, round or irregular shapes of rice starches (Wani *et al*., 2013). Purple sweet potato starch showed structural differences, the shapes of the various starch granules varied from polygonal, round, and spherical shapes (Figure 3.13b). This similar with previous reports on sweet potato starch granules (Chen *et al*., 2003; Zhu *et al*., 2011). SEM imaged revealed the exposed surface of the purple corn starch was principally smooth with a distinctive polygonal-like shape (Figure 3.13c), similar to the results obtained by Utrilla-Coello *et al*. (2014).



(a) native purple rice starch (×1,000) (b) native purple sweet potato starch (×1,000) (c) native purple corn starch (×1,000)

(d) purple rice starch (DART) (×1,000) (e) purple sweet potato starch (DART) (×1,000) (f) purple corn starch (DART) (×1,000)

(g) purple rice starch (DET) (×1,000) (h) purple sweet potato starch (DET) (×1,000) (i) purple rice starch (DET) (×1,000)

Figure 3.13 Scanning electron micrographs of native and RS obtained from purple starches by using different treatment methods

Starch biosynthesis results in natural variability in amylose and amylopectin molecules, which might be responsible for diversity of starch granules. Other factors responsible for granule diversity may include climate, agronomic conditions, processing etc. (Wani *et al*., 2013). The DART causes the change of native structure (Figure 3.13d-3.13f). The structure of the RS granules becomes shapeless and large complexes forms with uneven surfaces. DART caused the shapeless granules structure and the presence of the starch crystals (Ashwar *et al*., 2016b). This increased density of the crystal structure is largely responsible for its increased resistance to enzyme attack of product RS. Morphological RS by DET was occurred, and the irregular shaped particles formed a white porous network. This continuous network structure was composed of amylose and amylopectin. Similar results have been reported by Jirapa *et al*. (2009). In contrast, RS formed a larger, more compact laminiplantation structure (Figure 3.13g-3.13i). This was attributed to retrogradation of more amylose chains, which resulted in reorganization of the starch structure into a helical complex. This increased density of the crystal structure greatly increased its resistance to enzyme attack.

3.4.3 Swelling Index Properties

When starch is heated in water above its gelatinization temperature, the resulting irreversible changes result in loss of crystallinity, granule swelling and leakage of carbohydrate material (Gerits *et al*., 2015). Variation in the swelling power of the starch under different treatments could be caused by the variations in the associative bonding forces within the starch granules. Swelling power of the native and RS are presented in Table 3.6. Compared to the swelling power of native purple rice starch (10.05-12.50 g/g), the swelling power of RS by DART decreased to 5.20-6.46 g/g, while RS by DET exhibited the lowest swelling power of 2.61-3.93 g/g, native purple sweet potato starch (6.32-8.11 g/g), the swelling power of RS by DART decreased to 5.11-7.01 g/g, while RS by DET showed the lowest swelling power of 3.11-4.16 g/g, for native purple corn starch (11.58-14.10 g/g), the swelling power of RS by DART decreased to 5.82-8.01 g/g, while RS by DET showed the lowest swelling power of 3.86-5.13 g/g, within the temperature range of 60°C to 90°C. DART and DET decreased the swelling index of RS samples significantly ($p<0.05$). During DART, the internal of starch granules was rearranged, and the mobility of the molecules would be increased, and thus cause enhanced associations

between interacting amylose-amylose and amylose-amylopectin chains. The resulting rigid structure could restrict the swelling of starch granules. This result was consistent with previous reports, where the swelling power was reduced during DART of rice starch (Ashwar *et al*., 2016b), sweet potato starch (Zheng *et al*., 2006) and sorghum starch (Sun *et al*., 2014). DET with α-amylase contained shorter amylase chains and decreased steric hindrance among molecules compared with native purple starches. It is advantageous for amylopectin to be debranched by pullulanase, and the short amylose chains released from amylopectin can form double helices, compared with native purple starches, RS by DET showed increased crystalline structures, and thus further reduce the swelling power. The result of DET was similar reduced swelling power for debranched purple sweet potato was reported by Zheng *et al*. (2016). DART and DET *al*so reduced the swelling power index, this is a desirable property in the application of the modified starch in food and allied industries.

Table 3.6 Swelling index of native and RS obtained from purple starches by using different treatment methods

Starch	Process	Temperature			
		60°C	70°C	80°C	90°C
Purple rice	NS	10.05 ± 0.01^{a}	10.54 ± 0.02^{a}	11.79 ± 0.03^{a}	12.50 ± 0.01^{a}
	DART	5.20 ± 0.03^{b}	5.36 ± 0.02^{b}	6.02 ± 0.02^{b}	6.46 ± 0.02^{b}
	DET	2.61 ± 0.01^{c}	2.87 ± 0.01^{c}	3.52 ± 0.02^{c}	3.93 ± 0.02^{c}
Purple sweet potato	NS	6.32 ± 0.01^{a}	6.51 ± 0.01^{a}	7.49 ± 0.02^{a}	8.11 ± 0.02^{a}
	DART	5.11 ± 0.02^{b}	5.37 ± 0.01^{b}	5.91 ± 0.01^{b}	7.01 ± 0.02^{b}
	DET	3.11 ± 0.01^{c}	3.41 ± 0.00^{c}	3.66 ± 0.01^{c}	4.16 ± 0.01^{c}
Purple corn	NS	11.58 ± 0.02^{a}	12.10 ± 0.01^{a}	13.02 ± 0.01^{a}	14.10 ± 0.02^{a}
	DART	5.82 ± 0.07^{b}	6.59 ± 0.01^{b}	7.01 ± 0.01^{b}	8.01 ± 0.03^{b}
	DET	3.86 ± 0.06^{c}	4.02 ± 0.02^{c}	4.51 ± 0.01^{c}	5.13 ± 0.01^{c}

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$). NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment

3.4.4 Solubility Index Properties

The solubility index is an indicator for the amount of soluble molecules leach out from the starch granule (Kumar *et al*., 2019). Solubility index of the native and RS are presented in Table 3.7. Compared to the solubility index of native purple rice starch (0.05-0.11 g/g), the solubility index of RS by DART decreased to 0.03-0.08 g/g, while RS by DET showed the highest solubility index of 0.09-0.14 g/g, native purple sweet potato starch (0.06-0.13 g/g), the solubility index of RS by DART decreased to 0.02-0.08 g/g, while RS by DET showed the highest solubility index of 0.08-0.14 g/g, for native purple corn starch (0.05-0.09 g/g), the solubility index of RS by DART decreased to 0.02-0.06 g/g, while RS by DET showed the increased solubility index of 0.07-0.11 g/g, within the temperature range of 60°C to 90°C. The solubility index of both native and RS was observed to be a function of temperature and the average value of solubility index increased significantly with increase in temperature (60-90ºC) ($p<0.05$). The decrease in solubility index with RS by DART can be attributed to an internal rearrangement of starch granules that provides higher interactions between starch functional groups and formation of more ordered amylopectin clusters (Dias, *et al*., 2010). The increase in solubility index of RS by DET may occur as a result of changes in the molecular structure or as an independent mechanism that leads to the mobility of the starch components, resulting in the leaching of carbohydrates from molecules involved (Govindasamy *et al*., 1996). The DET enhances the starch solubility by reducing the molecular mass of the starch.

Table 3.7 Solubility index of native and RS obtained from purple starches

Parameter	Process	Temperature (°C)			
		60	70	80	90
Purple rice starch	NS	0.05 ± 0.00^{b}	0.08 ± 0.00^{b}	0.10 ± 0.00^{b}	0.11 ± 0.00^{b}
	DART	0.03 ± 0.01^{c}	0.04 ± 0.01^{c}	0.06 ± 0.01^{c}	0.08 ± 0.01^{c}
	DET	0.09 ± 0.01^{a}	0.10 ± 0.01^{a}	0.12 ± 0.01^{a}	0.14 ± 0.01^{a}
Purple sweet potato starch	NS	0.06 ± 0.01^{b}	0.08 ± 0.01^{b}	0.11 ± 0.01^{a}	0.13 ± 0.01^{a}
	DART	0.02 ± 0.01^{c}	0.04 ± 0.02^{c}	0.06 ± 0.01^{b}	0.08 ± 0.01^{b}
	DET	0.08 ± 0.01^{a}	0.10 ± 0.01^{a}	0.12 ± 0.01^{a}	0.14 ± 0.00^{a}
Purple corn starch	NS	0.05 ± 0.00^{b}	0.06 ± 0.01^{b}	0.08 ± 0.01^{b}	0.09 ± 0.02^{ab}
	DART	0.02 ± 0.00^{c}	0.03 ± 0.01^{c}	0.05 ± 0.01^{c}	0.06 ± 0.01^{b}
	DET	0.07 ± 0.01^{a}	0.09 ± 0.02^{a}	0.11 ± 0.01^{a}	0.11 ± 0.01^{a}

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$). NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment

3.4.5 X-ray Diffraction Pattern and Crystallinity

The different types of crystallinities (diffraction pattern) of starch granules are characterized by X-ray diffraction (XRD) analysis. The diffraction patterns are produced by the packages of hexagonal chains of amylopectin (Schafranski *et al*., 2021). Basically, the diffraction patterns found in starches are A-, B-, and C-types. The A- and B-types differ in their compactness and both show a double helical structure. The C-type is considered a mixture of A- and B-types. XRD measures the relative crystallinity of starches, which present a semi-crystalline structure with crystalline and amorphous lamellae in its granule (Khatun *et al*., 2019).

The XRD of native and RS obtained from purple starches is presented in Figure 3.14-3.16, and Table 3.8. Native purple rice starch displayed a A-type crystalline pattern with strong diffraction peaks at 15.01°, 17.02°, 18.05°, and 23.11°. They both have single broad peaks at 15° and 23°, and a dual peak at 17° 18°, which indicate the typical A-type starches (Du *et al*., 2019). Native sweet potato starch displayed a C-type crystalline pattern with strong diffraction peaks at 15.02°, 17.16°, 23.08° and a small peak at 20.15°, which indicate the typical C-type starches (Babu and Parimalavalli,

(2016). Yong *et al*. (2018) investigated the properties of starches of seven purple sweet potato varieties, and the XRD pattern results revealed C-type crystals for all seven native starches, in agreement with the results obtained by Zhang *et al*., 2018, Zheng *et al*., 2016, who also observed C-type crystals in native starch prepared from several varieties of purple sweet potato. Native purple corn starch displayed a A-type crystalline pattern with strong diffraction peaks at 15.02°, 17.02°, 18.04°, and 23.15°. These are also mostly comparable to those review by Li *et al*. (2021) at peak at 15°, 17°, 18°, and 23°in corn starch.

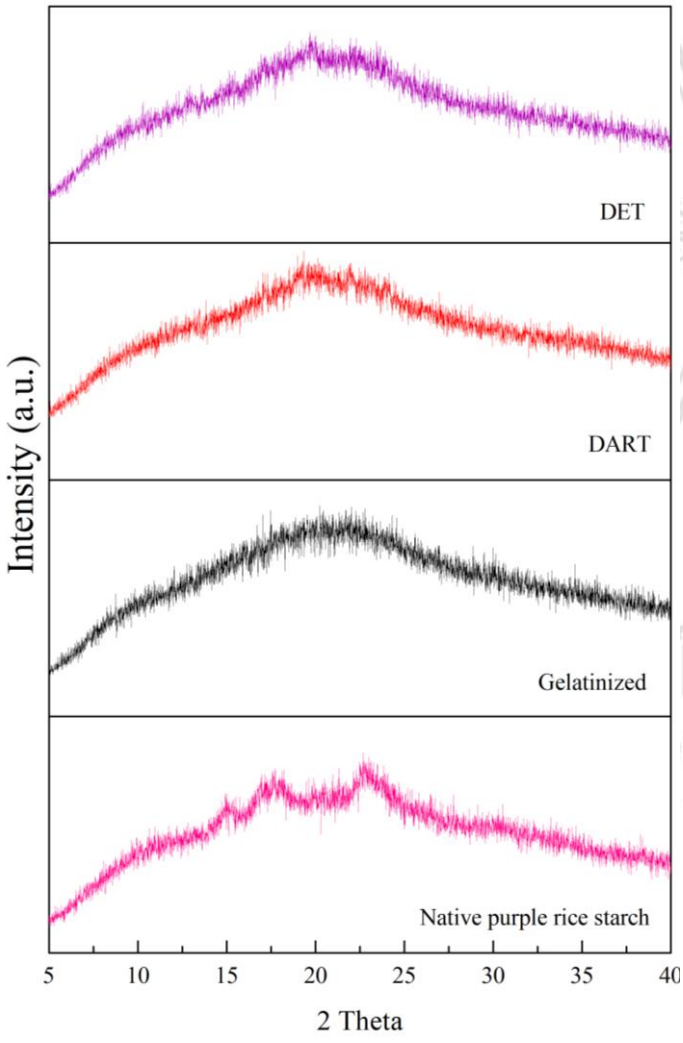


Figure 3.14 X-ray diffraction patterns of native and RS obtained from purple rice starch, DART; dual autoclaving-retrogradation treatment, and DET; dual enzyme treatment

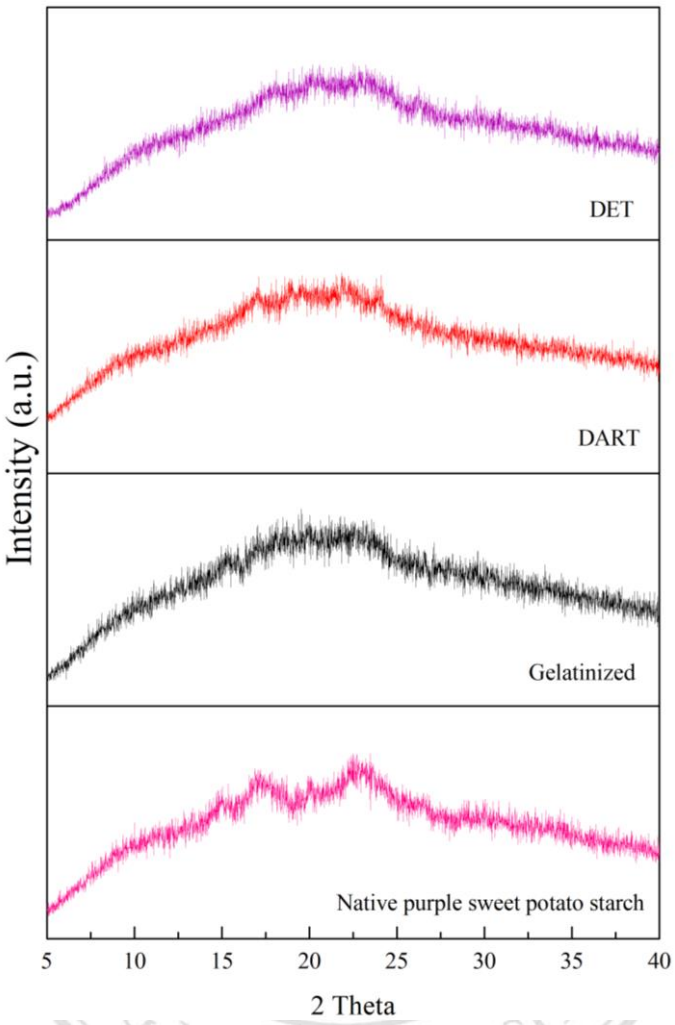


Figure 3.15 X-ray diffraction patterns of native and RS obtained from purple sweet potato starch, DART; dual autoclaving-retrogradation treatment, and DET; dual enzyme treatment

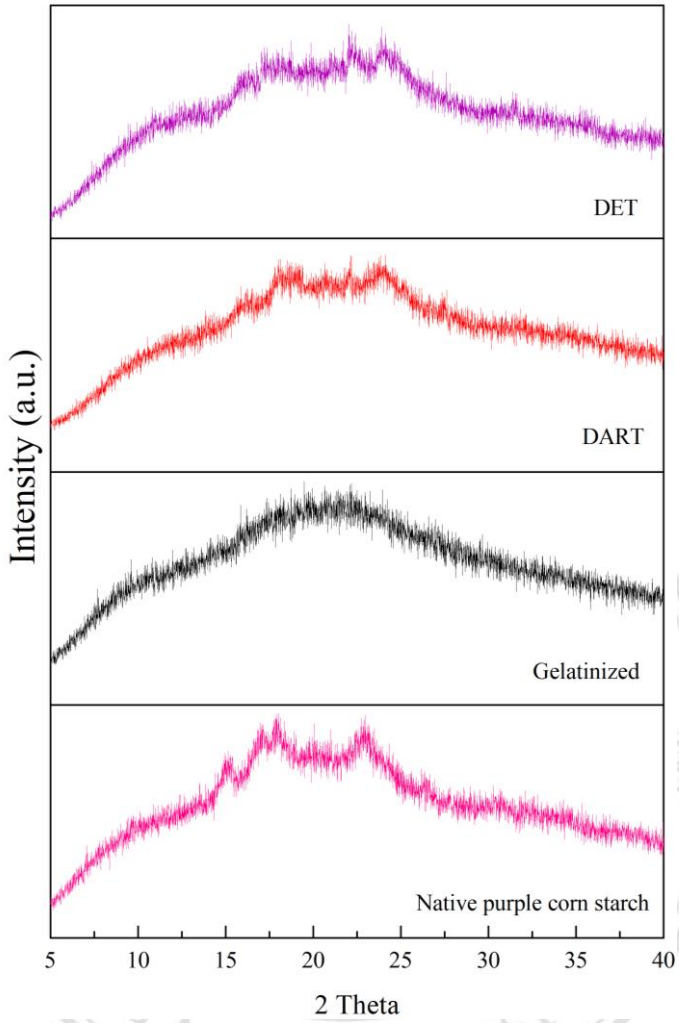


Figure 3.16 X-ray diffraction patterns of native and RS obtained from purple corn starch, DART; dual autoclaving-retrogradation treatment, and DET; dual enzyme treatment

After samples were subjected to DART and DET, all RS showed a B-type diffraction pattern with a sharp peak at 17°, 22°, and 24°, the B-type is typically found in retrograded starch, except RS of purple rice starch by DET, purple sweet potato starch by DART, and purple corn starch showed a combination of B- and V-type diffraction pattern with a sharp peak at 17° and few small peaks at values of 19°, 22°, and 24° which is a characteristic feature of the B+V-type crystallinity (Sievert *et al*., 1991). Interestingly, an additional peak was observed around 19°, the peak in this location is a characteristic of V-type polymorph (Shamaia *et al*., 2003). The peak in this location is a characteristic of B+V-type polymorph. This transformation of diffraction pattern was attributed to debranching and retrogradation which reorganized the structure of starch into a helical

complex to that of V-amylose (Cui and Oates, 1999). Gonzalez-Soto *et al*. (2007) proposed that, retrograded RS developed peaks, because of the process of starch chain re-ordering or retrogradation. Apart from recrystallization of amylose chains, linear debranched amylopectin chains would also associate to form an RS crystalline structure during the period of heating and cooling (Pongjanta *et al*., 2009). Miao *et al*. (2009) observed that when autoclaved debranched corn starches were stored at 4^{o}C for up to 4 days, the X-ray diffraction pattern changed from B-type to B+V-type complex. Occurrence of B+V- type diffraction patterns are caused by the presence of amylose and lipids content in the starting material is shown in Table 3.1.

The degree of crystallinity of native and RS obtained from purple rice starch, purple sweet potato starch, and purple corn starch were shown in Table 3.8. The difference in the crystallinity can be related to difference in crystal size, amount of crystalline regions, orientation of the double helices within crystalline domain, and extent of interaction between double helices (Mutungi *et al*., 2011). The transformation of crystalline pattern of RS by DART could be attributed to the retrogradation at low temperature, which caused increased amylose content and the formation of more ordered double helices within the crystalline domains during DART. The same change was found in the RS by DET. Compared with the relative crystallinity of native purple rice starch sample (30.52%), those of DART and DET starches (7.14% and 11.06%) were both decreased, the relative crystallinity of native purple sweet potato starch sample (26.55%), those of DART and DET starches (11.73% and 13.25%) were both decreased, the relative crystallinity of native purple corn starch sample (32.51%), those of DART and DET starches (24.81% and 28.16%) were both decreased, which was due to the damage of the crystalline region and gelatinization of the starch granules during DART and DET processes. Zhang *et al*. (2014) postulated that the amorphous amylose could have been transformed to a double helix structure during autoclaving-cooling which in turn led to a more orderly packed crystallite However, the crystallinity of all RS samples was not strictly correlated with the RS content. The results suggested that the content of RS was not depends on the crystallinity. Other factors such as morphology, crystal defects, crystal size, swelling, solubility, viscosity, and the orientation of the crystallites should be considered for their ability to resist the enzyme digestibility. During RS3 preparation by debranching followed by different methods of re-crystallization (Mutungi *et al*., 2011), it

was found that the autoclaving, and heat-moisture treatments favored A-type crystalline packing, whereas a higher amount of B-type packing was evident in the annealing samples due to milder re-crystallization conditions. Similarly, the V-type crystalline material was found to be significantly higher in the temperature-cycling and heat-moisture-treated samples, which suggested that the higher temperature conditions enabled stabilization of significantly higher amount of single helical chains into entities capable of diffracting X-rays. However, the exact solid concentration and crystallization temperature required to produce A- or B-type polymorph varied depending on specific material.

The general trends observed for XRD results by different researchers suggested that the RS prepared by molecular mass reduction (enzyme treatment) and hydrothermal treatments of native starch involves the co-crystallization of chains of recrystallized amylose and fragments of linear amylopectin during heating and cooling, consequently leading to a more intense and orderly crystalline structure (Polesi and Sarmento, 2011). However, the crystallinity is not necessarily proportional to the levels of RS from various origins. And the degree of crystallinity is not always linked to resistance to amylolytic enzymes (Mutungi *et al*., 2011; Raigond *et al*., 2015). Based on previous studies, the levels of crystallinity measured in both the enzyme RS (ERS) obtained *in vitro* and physiological RS (PRS) that are obtained *in vivo* fractions have been quite low (Faraj *et al*., 2004). The following two hypotheses are generally accepted theories that can justify the enzymatic resistance of the non-crystalline fraction, one is the attributed by the amorphous regions within the imperfect crystals, the other being the resistance of double-helical structures that are not necessarily being part of crystallites. The low X-ray crystallinity is not necessarily related to poorly ordered starch molecules but may be the result of small-size crystallites in the granules or originate from a combination of molecular and mesoscopic factors, including both recrystallization and an increase in very short branches (Shrestha *et al*., 2015; Ma and Boye, 2018).

Table 3.8 Crystallinity, crystallinity type and diffraction peaks of native and RS obtained from purple starches

Starch	Process	Crystallinity (%)	Crystallinity type	Diffraction peaks (2θ)
Purple rice	NS	30.52 ± 0.46^{a}	A	15.01, 17.02, 18.05, 23.11
	DART	7.14 ± 0.97^{c}	B	17.02, 22.02, 24.03
	DET	11.06 ± 0.74^{b}	B+V	17.05, 19.31, 22.01, 24.15
Purple sweet potato	NS	26.55 ± 0.39^{a}	C	15.02, 17.16, 20.15, 23.08
	DART	11.73 ± 0.55^{c}	B+V	17.07, 19.01, 22.12, 24.15
	DET	13.25 ± 0.85^{c}	B	17.26, 22.01, 24.02
Purple corn	NS	32.51 ± 0.32^{a}	A	15.02, 17.02, 18.04, 23.15
	DART	24.81 ± 0.88^{c}	B+V	16.89, 19.25, 22.15, 24.05
	DET	28.16 ± 0.17^{b}	B	17.07, 22.18, 24.15

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$). NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment

3.4.6 Pasting Properties

Pasting properties of and RS obtained from purple starches by using rapid visco analyzer are given in Figure 3.17 and Table 3.9. All rapid visco amylograph parameters, except pasting temperature. RS produced by DART and DET were significantly ($p<0.05$) lower than those of native purple starches. Peak viscosity of purple starches varied significantly ($p<0.05$) from 2012.1 to 3341.0 cP in which purple corn starch showed the highest and purple sweet potato starch showed the lowest peak viscosity. Gani *et al*. (2012) reported that peak viscosity is influenced by the extent of amylose leaching, amylose-lipid complex formation, friction between swollen granules, granule swelling and competition between leached amylose and remaining ungelatinized granules for free water.

After DART, peak viscosity decreased from 2,367.0 to 554.0 cP for purple rice starch, 2,012.0 to 447.7 cP for purple sweet potato starch, and 3,341.0 to 442.0 cP for purple corn starch. In DET process, peak viscosity decreased from 2,367.0 to 544.0 cP for purple rice starch, 2,012.0 to 447.7 cP for purple sweet potato starch, and 3,341.0

to 442.0 cP for purple corn starch. Significant reduction in swelling index of RS by DART and DET could be responsible for lowering of peak viscosity. The decreases in the viscosity values in the present study might be due to the disrupted starch granules and partial solubilization caused by high autoclaving temperature and enzyme treated (Eerlingen and Delcour, 1995). According to Gelencser *et al*. (2008), the higher the RS content is the lower the viscosity. Final and setback viscosities of native purple starches pastes varied from 3,047.7 to 3,302.7 cP and 1,205.3 to 1679.3 cP respectively. Both final and setback viscosities were significantly reduced after DART and DET. Previous studies claimed that reduced set back values of annealed and heat moisture treated starches limited starch swelling, decrease amount of leached amylose and insufficient reassociation of amylose molecules (Yadav *et al*., 2013). Pasting temperature of native purple starches was observed in the range of 78.3-82.6°C and it increased significantly with treatment. Increase in pasting temperature after treatment supports the fact that DART process tends to increase the region of crystallinity, as a result of reorientation of the starch granules. The strengthening of intragranular bonded forces, allows the starch to absorb more heat before structural disruption and paste formation takes place (Eliasson, 1980). DET show a lower pattern for parameters including peak viscosity, hold viscosity, final viscosity, break down and set back when compared with native purple starches. This attribute could be a result of the enzymatic hydrolysis process which improves the levels of short linear chain molecules and RS. The formation of starch gel ability was reduced in RS by DET when compared with native purple starches.

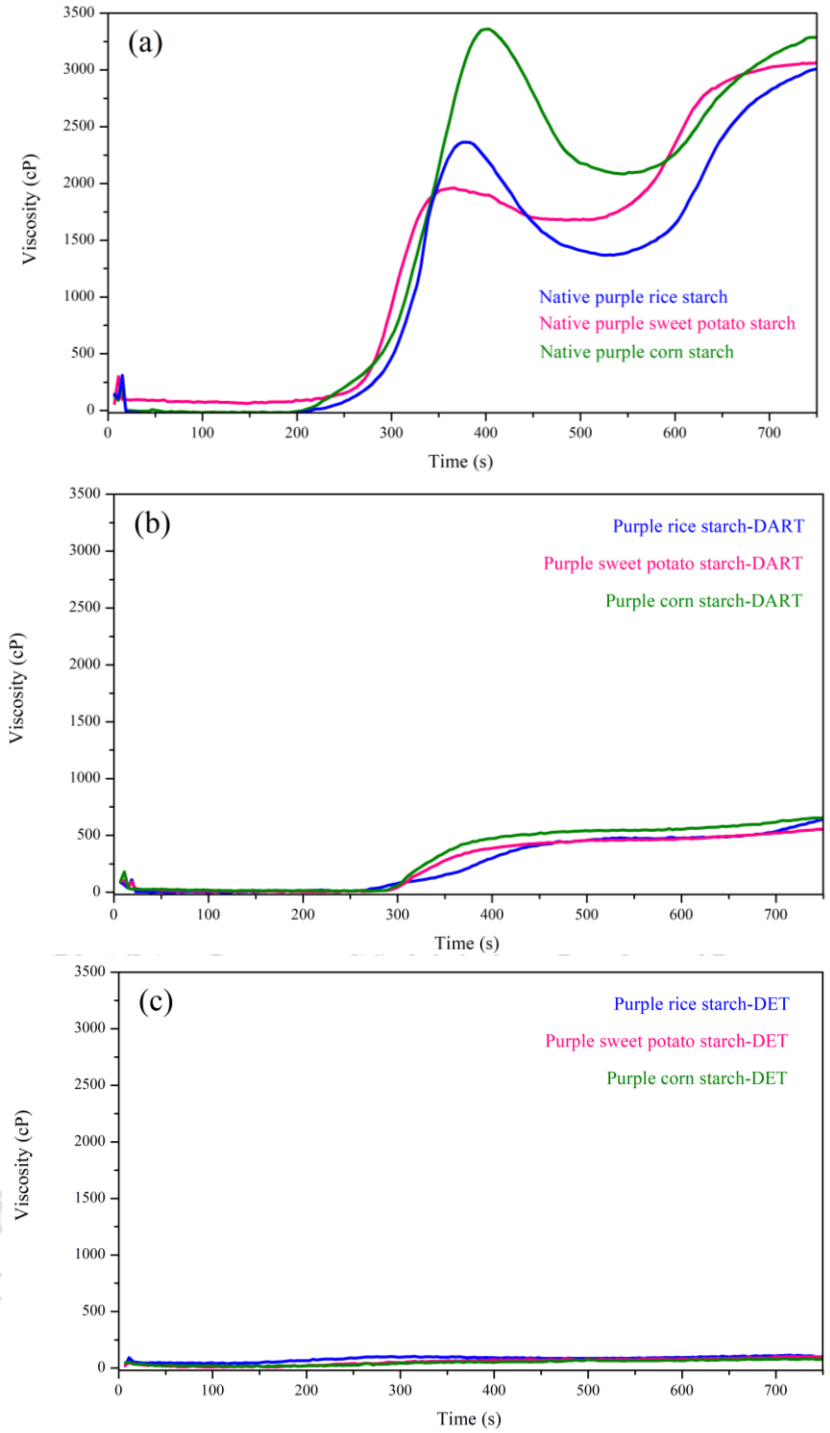


Figure 3.17 Typical RVA starch pasting curves of native and RS obtained from purple starch, (a); native purple starch, (b); DART, dual autoclaving-retrogradation treatment, and (c); DET, dual enzyme treatment

Table 3.9 Pasting properties of native and RS obtained from purple starches

Starch	Process	Pasting temperature (°C)	viscosity (cP)				
			Peak	Hold	Breakdown	Setback	Final viscosity
Purple rice starch	NS	74.25 ± 0.3[b]	2367.0 ± 4.4[a]	1368.3 ± 5.1[a]	998.7 ± 2.1[a]	1679.3 ± 3.2[a]	3047.7 ± 3.1[a]
	DART	82.3 ± 0.1[a]	544.0 ± 2.6[b]	539.0 ± 1.0[b]	5.0 ± 2.0[b]	126.3 ± 2.5[b]	643.3 ± 3.2[b]
	DET	nd	57.0 ± 2.0[c]	54.3 ± 2.1[c]	2.7 ± 0.6[b]	29.3 ± 2.5[c]	83.7 ± 1.5[c]
Purple sweet potato starch	NS	78.3 ± 0.2[b]	2012.0 ± 3.0[a]	1739.7 ± 4.9[a]	272.3 ± 3.1[a]	1370.7 ± 1.2[a]	3110.3 ± 3.8[a]
	DART	82.5 ± 0.2[a]	447.7 ± 1.2[b]	440.3 ± 0.6[b]	7.3 ± 0.6[b]	281.7 ± 2.1[b]	552.0 ± 2.4[b]
	DET	nd	101.7 ± 2.1[c]	86.7 ± 2.5[c]	15.0 ± 3.5[c]	26.7 ± 3.8[c]	113.3 ± 2.1[c]
Purple corn starch	NS	78.5 ± 0.5[b]	3341.0 ± 3.6[a]	2097.3 ± 5.0[a]	1243.7 ± 8.1[a]	1205.3 ± 7.5[a]	3302.7 ± 5.9[a]
	DART	81.4 ± 0.2[a]	442.0 ± 2.6[b]	439.7 ± 1.5[b]	2.3 ± 1.5[b]	143.0 ± 4.6[b]	655.7 ± 3.1[b]
	DET	nd	75.7 ± 2.1[c]	71.7 ± 1.5[c]	4.0 ± 3.6[b]	30.3 ± 1.5[c]	102.0 ± 2.0[c]

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$). NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment

3.4.7 Thermal Properties

The thermodynamic parameters of native and RS obtained from purple starches prepared using the DART and DET methods are shown in Table 3.10. For native purple starches the gelatinization starting temperature (T_o), peak temperature (T_p), ending temperature (T_c), T_c-T_o, and enthalpy change values (ΔH) were 7.19°C, 99.67°C, 108.90°C, 33.71°C, and 1433.71 J/g, respectively for native purple rice starch, 85.57°C, 94.31°C, 104.53°C, 18.96°C, and 1406.89 J/g, respectively for native purple sweet potato starch, 81.53°C, 100.14°C, 108.84°C, 27.31°C, and 1257.87 J/g, respectively for native purple corn starch.

Overall, the T_o, T_p, T_c, T_c-T_o, and ΔH values for RS obtained from purple starches prepared using the DART and DET were greater than the corresponding values for native purple starches. These differences can be explained by the fact that after DART and DET process, the increase in the amylose content in the starch structure increased the crystal melting temperature, which is greater for amylose than for amylopectin. Simultaneously, the increase in short chain amylose content was conducive to the formation of double helical and ordered crystal structures that required more heat energy for their destruction. Thus, increases in amylose content and the subsequent associated effects during the gelatinization process of RS formation can explain the increases in gelatinization temperature and gelatinization enthalpy observed for DART and DET process relative to native purple starches. Similar changes in gelatinization transition temperatures have been reported for heat-moisture treated and annealed starches (Zeng *et al*., 2015).

The decrease in transition temperature range of RS is an indicator of increased homogeneity with perfection of crystallites. This enhanced perfection of crystallites is responsible for the increased RS content against digestion (Park *et al*., 2009). In general, the ΔH values for treated starches showed significant increase over their native counterparts. The increased enthalpy in treated starches confirms that during the retrogradation stage increased interactions between amylose and outer branches of amylopectin resulted in efficient packing by double helix formation. Previous studies have also reported an increase in the ΔH values of the annealed starches from various botanical sources (Alvani *et al*., 2011). Soler, Velazquez *et al*. (2020) found that

autoclaving treatment destroyed the structure of starch granules, enhanced the interaction between amylopectin-amylose or amylose-amylose, and yielded new double helix crystallites with better thermal stability. Li *et al*. (2020) found that the thermal stability of rice RS prepared using a combination of autoclaving and enzyme treatment was better than that native rice starch.

Table 3.10 Thermal properties of native and RS obtained from purple starches

Starch	Process	Gelatinization temperature (°C)				ΔH (J/g)
		T_o	T_p	T_c	T_c- T_o	
Purple rice	NS	75.19	99.67	108.90	33.71	1433.71
	DART	78.61	98.95	107.80	29.19	1526.00
	DET	95.66	106.49	114.08	18.42	1791.28
Purple sweet potato	NS	85.57	94.31	104.53	18.96	1406.89
	DART	91.95	104.18	109.02	17.07	1,442.30
	DET	86.47	102.03	110.36	23.89	1517.17
Purple corn	NS	81.53	100.14	108.84	27.31	1257.87
	DART	97.15	106.53	114.51	17.36	1590.06
	DET	75.93	98.39	108.61	32.68	1516.11

T_o; onset temperature; T_p; peak temperature; T_c; conclusion temperature, ΔH; enthalpy of gelatinization, NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment

3.5 Bioactive Compounds

3.5.1 Total Phenolic and Anthocyanin Contents

As seen in Table 3.10, the total phenolic content in purple rice, purple sweet potato, and purple corn starches were 1,155.01, 275.94, and 330.88 mg GAE/100 g dwb, respectively. Anthocyanin content in purple rice, purple sweet potato, and purple corn starches were 415.53, 8.70, and 9.29 mg/100 g dwb, respectively. Native purple rice starch contained the most total phenolic content and anthocyanin content, followed by purple corn starch and, lastly, purple sweet potato starch ($p<0.05$). The anthocyanins are a subclass of the flavonoids and are recognized as the major phenolic compounds in pigmented rice grains (Kushwaha, 2016), which exist mainly in dark-colored grains rather than among pale-colored grains (Pathak *et al*, 2016). Their color range can be characterized by the degree of hydroxylation in the B-ring of their carbon skeleton; recognized as delphinidin, cyanidin, and pelargonidin-glycoside forms by providing blue-purple, light purplish-red, and orange-red hues, respectively (Glover and Martin, 2012). It has already been confirmed that purple rice contains higher total phenolic content and anthocyanin content than red rice, and cyanidin-3-glucoside was found to be the major anthocyanin detected in pigmented rice grains, with varying concentration among purple grains and red grains (Yao *et al*., 2010).

A dramatic decrease in the levels of the total phenolic content and anthocyanin content of RS obtained from purple starches by using DART and DET when compared with their native purple starches. The reduction was 71.35-76.10% of purple rice starch, 26.38-42.32% of purple sweet potato starch, and 86.52-88.30% of purple corn starch for total phenolic content. The anthocyanin content, after DART and DET was found only RS obtained from purple rice starch, anthocyanin content of RS by DART was 49.93 mg/100 g dwb and RS by DET was 126.18 mg/100 g dwb. The anthocyanin content in all purple starches decreased considerably by both starch preparation process and by RS preparation. Moreover, thermal processes have been shown to result in deterioration of phenolic compounds and anthocyanins, which was associated with breakdown into other products as well as vaporization during heating. (Thuengtung and Ogawa, 2020). As the temperature increases, the hydroxyl group of phenolic compounds would be destroyed. As the temperature increases, the hydroxyl group of phenolic

compounds would be destroyed. Then, phenolic compounds with high antioxidant activity could transform into smaller molecules or other products which may show low antioxidant activity (Saikia *et al*., 2012). A hydrophilic phenolic compound like anthocyanins is mainly found in pigmented rice and labile to the thermal process, light, and oxygen exposure (Surh and Koh, 2014). Herein, the high heating temperature might cause an opening of the pyrylium ring of anthocyanins, leading to cleavage of the glycoside linkage and formation of the chalcone structure (colorless form), indicative of the initial degradation step of anthocyanins (Patras *et al*., 2010). According to the previous study, anthocyanins could be further degraded by the transformation of chalcone structure into coumarin glucoside derivative with loss of B-ring (Patras *et al*., 2010). Moreover, some studied also revealed that anthocyanins could convert into free phenolic acids during heating, which was one of the major anthocyanin degradation products (Yamuangmorn *et al*., 2018).

3.5.2 Antioxidant Activities

Various methods can be used to evaluate the antioxidant activity RS obtained from purple starches (Morales-Soto *et al*., 2014). In the present study, three different methods, the DPPH radical scavenging assay, ABTS radical scavenging activity, and FRAP assay were used to evaluate the antioxidant activity. The antioxidant activities RS obtained from purple starches are presented in Table 3.12. The DPPH scavenging activity of the native purple rice starch, purple sweet potato starch, and purple corn starch showed % inhibition was 70.65%, 54.63% and 38.23% of inhibition, respectively. After DART and DET process, the % inhibition RS from purple rice starch, purple sweet potato starch, and purple corn starch were 48.68-55.23%, 30.36-41.85%, and 17.18-22.88%, respectively. The result revealed that the DPPH radical scavenging activity of RS content of the purple starches decrease was 22.10-31.09%, 23.39-44.42% and 40.15-55.06%, respectively, compared with native starch. The data indicated a significant ($p<0.05$) decrease in DPPH radical scavenging activity compared with native purple starches.

The hydrophobicity of DPPH may influence the activity of hydrophilic compound, the water soluble ABTS radical has also been used to achieve more comprehensive understanding. The % inhibition of ABTS radical scavenging activity of purple rice starch, purple sweet potato starch, and purple corn starch were 66.19%,

57.17%, and 44.62%, respectively. After DART and DET process, the % inhibition RS from purple rice starch, purple sweet potato starch, and purple corn starch were 49.97-54.87%, 36.30-45.64%, and 23.25-29.27%, respectively. The result revealed that the ABTS radical scavenging activity of RS content of the purple starches decrease was 17.10-24.50%, 20.16-36.50% and 34.40-47.89% respectively, compared with native purple starches. The data indicated a significant ($p<0.05$) decrease in ABTS radical scavenging activity compared with native purple starches. Different from DPPH and ABTS which use the anti-oxide compounds to remove specific free radicals, FRAP is based on the integrated reducing ability of the sample as an evaluation of antioxidant ability (Thaipong *et al*., 2006). The FRAP assay of purple rice starch, purple sweet potato starch, and purple corn starch were 4,362.94, 3,838.42, and 3,167.16 µmol Fe II/g dwb. After DART and DET process, the RS production from purple rice starch, purple sweet potato starch, and purple corn starch were 2,822.54-2,921.51, 1,755.37-2,320.58, and 1,928.95-2,111.14 µmol Fe II/g dwb, respectively. The result revealed that the FRAP of the purple starches decrease after modification processes, respectively. The data indicated a significant ($p<0.05$) decrease in FRAP activity compared with native purple starches.

The free radical scavenging activity of purple starches decreased with the decrease of total phenolic content and anthocyanin content in the DPPH, ABTS and FRAP assay. Comparing to the DPPH and ABTS, FRAP was higher in antioxidant activity, but DPPH and ABTS also contained many reduction state compounds, which can assist in stability and quality during processing by DART and DET. The impact of DART and DET on the degradation mechanism of anthocyanins and phenolic acids needs to identify and quantify in further study. The reduction of total phenolic content and anthocyanin content during DART and DET would also decrease the antioxidant activities of RS. The result was consistent with the finding of Gong *et al*. (2017) which indicated that phenolic compounds were responsible for the antioxidant capacity of rice grain. These observations confirm that the DART and DET process is one of the crucial factors influencing the degradation of bioactive compounds and antioxidant activities in RS obtained from purple starches.

Table 3.11 Bioactive compounds of native and RS obtained from purple starches

Starch	Process	Total phenolic content (mg GAE/100g dwb)	Anthocyanin content (mg/100 g dwb)			
			Cyanidin-3-glucoside	Peonidine-3-glucoside	Cyanidin	Peonidin
Purple rice	NS	1155.01 ± 10.79[a]	369.82 ± 1.01	45.71 ± 0.61	ND	ND
	DART	275.94 ± 4.91[c]	ND	29.50 ± 0.82	ND	20.43 ± 0.45
	DET	330.88 ± 6.52[b]	118.56 ± 1.18	ND	7.62 ± 0.34	ND
Purple sweet potato	NS	718.00 ± 7.59[a]	8.70 ± 0.53	ND	ND	ND
	DART	414.13 ± 8.15[c]	ND	ND	ND	ND
	DET	528.53 ± 6.02[b]	ND	ND	ND	ND
Purple corn	NS	962.26 ± 10.49[a]	9.29 ± 1.45	ND	ND	ND
	DART	129.70 ± 2.09[b]	ND	ND	ND	ND
	DET	112.49 ± 2.48[c]	ND	ND	ND	ND

All data were means of triplicates. NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment. Anthocyanin content by using high-performance liquid chromatography

Table 3.12 Antioxidant activities of native and RS obtained from purple starches

Strach	Process	DPPH (% inhibition)	ABTS (% inhibition)	FRAP (μmol Fe II/g dwb)
Purple rice	NS	70.65 ± 0.61[a]	66.19 ± 0.85[a]	4,362.94 ± 118.22[a]
	DART	48.68 ± 0.91[c]	49.97 ± 0.89[c]	2,822.54 ± 111.16[c]
	DET	55.23 ± 0.48[b]	54.87 ± 0.88[b]	2,921.51 ± 227.83[b]
Purple sweet potato	NS	54.63 ± 0.18[a]	57.17 ± 0.95[a]	3,838.42 ± 153.45[a]
	DART	30.36 ± 0.46[c]	36.30 ± 1.26[c]	2,320.58 ± 654.40[c]
	DET	41.85 ± 0.76[b]	45.64 ± 0.97[b]	1,755.37 ± 159.55[b]
Purple corn	NS	38.23 ± 0.77[a]	44.62 ± 0.43[a]	3,167.16 ± 274.39[a]
	DART	22.88 ± 0.73[b]	29.27 ± 0.72[b]	2,111.14 ± 230.20[c]
	DET	17.18 ± 0.86[c]	23.25 ± 1.13[c]	1,928.95 ± 135.05[b]

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different (p<0.05) NS; native starch, DART; dual autoclaving-retrogradation treatment, DET; dual enzyme treatment.

3.6 *In vitro* Starch Digestibility

The hydrolysis of native and RS obtained from purple starches with human salivary α-amylase at pH 7.0 showed that the purple rice starch, the purple sweet potato starch, and purple corn starch gave a significantly higher degree of hydrolysis (p<0.05) when compared with RS. However, there was no significant difference (p<0.05) at 90 min and 120 min. The maximum hydrolysis of purple rice starch, the purple sweet potato starch, and purple corn starch was 11.47%, 10.07%, and 12.11%, respectively at 120 min hydrolysis (Figure 3.18). The maximum hydrolysis of RS production by DART from purple rice starch, the purple sweet potato starch, and purple corn starch was decreased, giving values of 7.50%, 7.87%, and 8.50%, respectively. Moreover, the maximum hydrolysis of RS by DET from purple rice starch, the purple sweet potato starch, and purple corn starch was DART decreased, the values was 5.13%, 5.68%, and 5.96%, respectively. Thus, RS by DART and DET had significantly higher enzymatic resistance compared to native (p<0.05). RS production from purple rice starch by DART and DET methods showed the percentage of resistant hydrolysis was 34.41% and 55.29% for purple rice starch, 21.81% and 43.56% for purple sweet potato, 29.78% and 50.74% for

purple corn starch were resistance to human salivary α-amylase. Starch digestion begins in the mouth and salivary α-amylase acts partially to break down starch (Singh *et al*., 2013).

Purple starches and RS obtained from purple starches were hydrolyzed with artificial human gastric juice (pH 2.5) and showed to be resistant to the juice. Percentage of hydrolysis increased with increasing the incubation time (Figure 3.19). The maximum hydrolysis of purple rice starch, the purple sweet potato starch, and purple corn starch was 7.96%, 7.88%, and 8.60%, respectively, occurred at 120 min of incubation. RS showed high acid resistance compared to the native, the maximum hydrolysis of RS by DART from purple rice starch, the purple sweet potato starch, and purple corn starch was decreased, giving values of 5.96%, 6.22%, and 5.25%, respectively. Moreover, the maximum hydrolysis of RS by DET from purple rice starch, the purple sweet potato starch, and purple corn starch was decreased, the values was 4.32%, 3.93%, and 4.42%, respectively. Under gastric conditions, the purple starches and RS were hydrolyzed were partially digested to both mono and disaccharides.

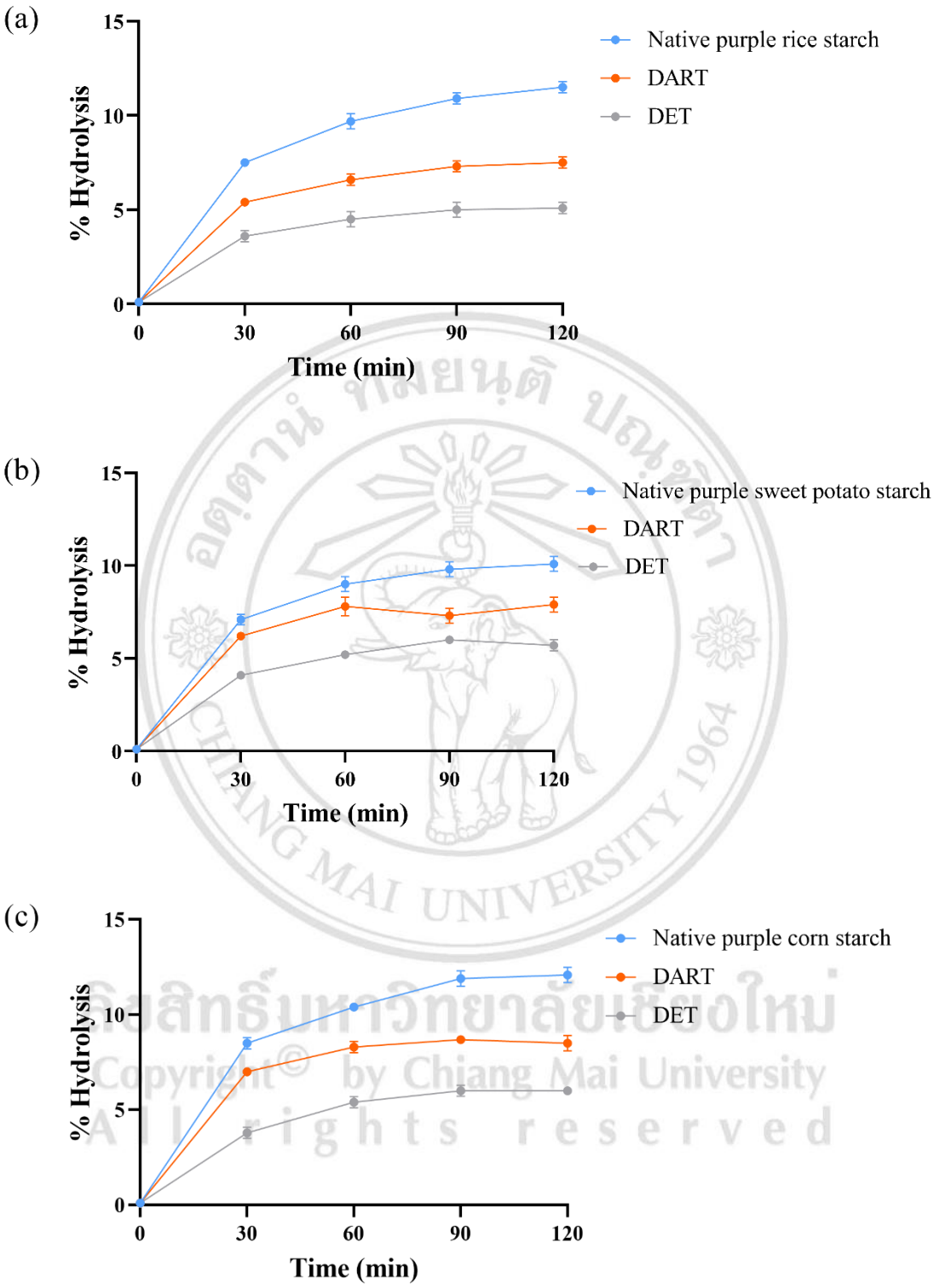


Figure 3.18 % Hydrolysis of native and RS obtained from purple starches by using DART and DET with simulated oral condition. (a); purple rice starch, (b); purple sweet potato starch, and (c); purple corn starch

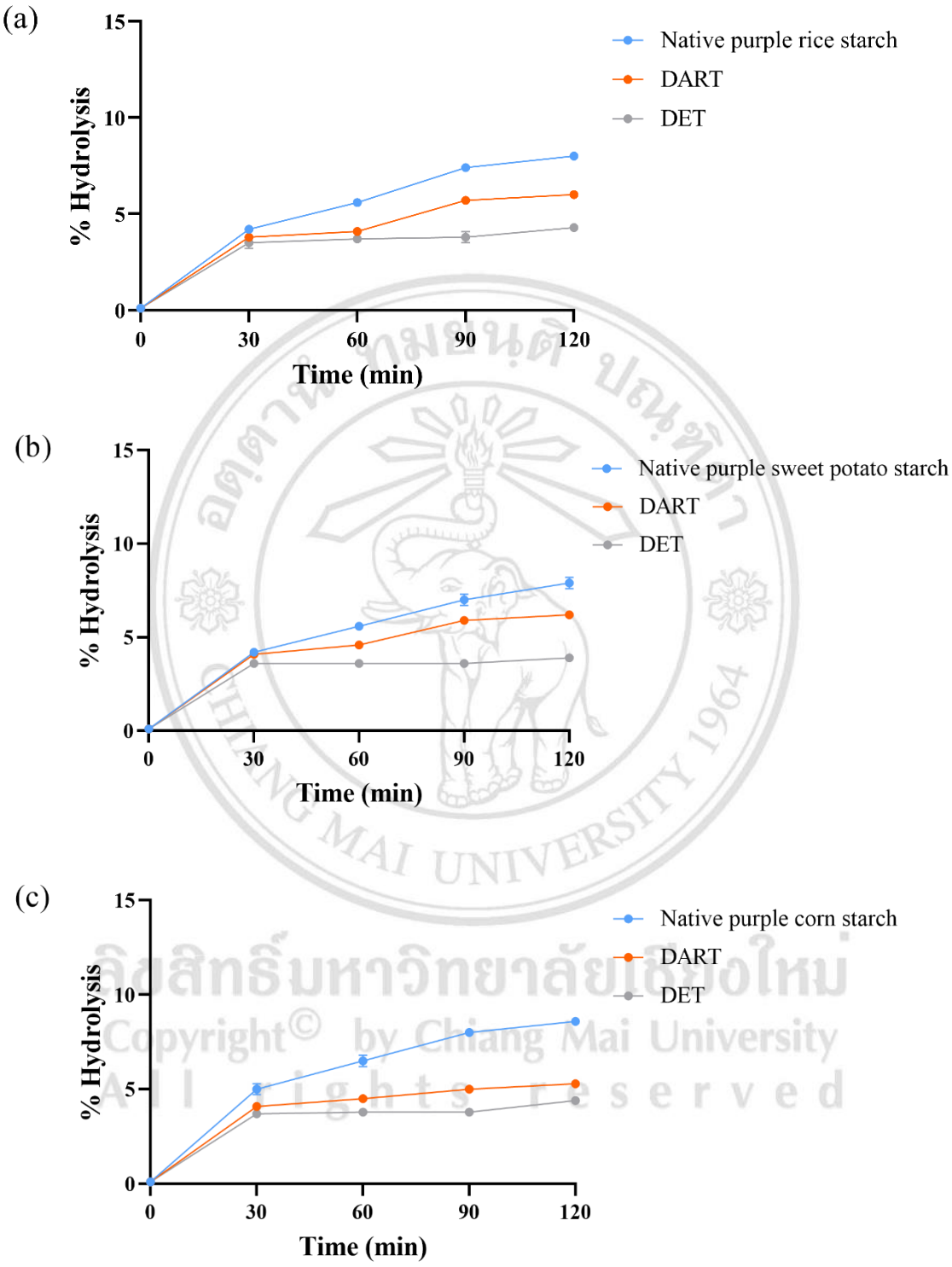


Figure 3.19 % Hydrolysis of native and RS obtained from purple starches by using DART and DET with gastric condition. (a); purple rice starch, (b); purple sweet potato starch, and (c); purple corn starch

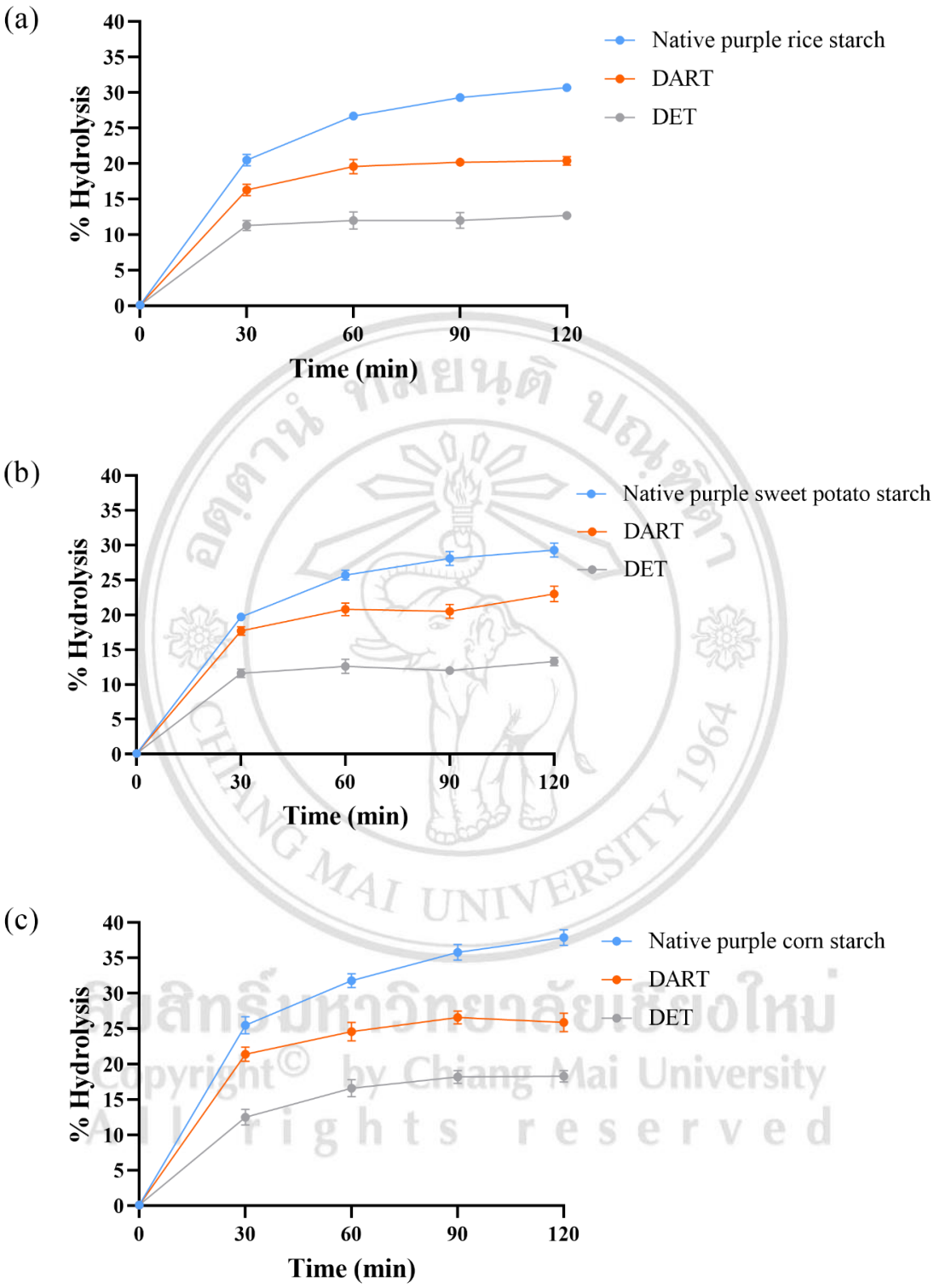


Figure 3.20 % Hydrolysis of native and RS obtained from purple starches by using DART and DET with intestinal condition. (a); purple rice starch, (b); purple sweet potato starch, and (c); purple corn starch

Food is usually retained in the human stomach where gastric juice (pH 2-4) is released within 2 h. Thus, when RS are consumed, 94% of them are estimated to reach the intestine. This result is comparable to that of inulin-FOSs extract, which had 100% resistance to artificial gastric acid (Aisara, 2021).

Under intestinal conditions, native and RS obtained from purple starches was rapidly hydrolyzed by pancreatin after 30 min and hydrolysis increased up to 60 min of incubation as shown in Figure 3.20. The maximum hydrolysis of purple rice starch, the purple sweet potato starch, and purple corn starch was 30.71%, 29.29%, and 37.88%, respectively, implying that native purple starches are more susceptible to enzymatic digestion. For RS that produced by DART, percentage hydrolysis of purple rice starch, the purple sweet potato starch, and purple corn starch at 120 min was 20.42%, 23.04%, and 25.90%, respectively, while percentage hydrolysis of RS produced by DET in purple sweet potato starch, and purple corn starch at 120 min was 12.72%, 13.28%, and 18.26%, respectively, indicating that pancreatin hydrolysis of RS produced by DART and DET method was significantly ($p<0.05$) compared with native purple starches. The results show that only a small part, approximately 12.72% to 25.90% of RS can be hydrolyzed by digestive enzymes in the gastrointestinal track. Therefore, RS obtained from purple starches can reach the colon to promote the function of dietary fiber in the intestine because of their high resistance to α-amylase, gastric juice and pancreatin. It should be noted that DART and DET process had higher levels of RS than native, indicating that the recrystallization of starch, which is one of the key factors for starch digestibility (Adra *et al*., 2022). High RS proportion was induced by the suppressed starch digestive rate, resulting in the decrease of glycemic response (Ovando-Martínez *et al*., 2013). Therefore, the RS obtained from purple starches in this experiment appeared to have partial enzymatic resistance.

3.7 Yields of Purple Starches from Raw Materials

Purple rice starch was produced by using purple rice paddy as raw material. After the processing, the paddy rice 5 kg (25 baht/kg: 125 baht) were removed the protective hull and obtained 3.2 kg of rice kernel. The isolation of purple rice starch was conducted and obtained 2.95 kg starch, representing 92.19% of purple rice kernel (Table 3.13). From the experiment, the yield of purple rice starch was 2.95 kg from 5 kg of paddy rice (0.59

kg starch/ kg paddy rice), and the cost of starch production was 42.37 baht/kg. This price includes only the cost of raw materials without any labor cost.

Table 3.13 Yield of purple rice starch

The portion of purple rice	Weight (kg)	Yield (% of rice kernel)
Purple rice paddy	5.00	-
Purple rice kernel	3.20	-
Purple rice starch	2.95	92.19

Purple sweet potato starch was produced using fresh tuber as raw material. The sweet potato at 5 kg (30 baht/kg: 150 baht) was processed and obtained 2.80 kg of purple sweet potato flour. The purple sweet potato starch was then extracted and obtained at 2.65 kg starch, representing 94.64% of purple sweet potato flour (Table 3.14). From the experiment, the yield of purple sweet potato starch was 2.65 kg from 5 kg fresh tuber (0.53 kg starch/ kg fresh tuber), and the cost of starch production was 56.60 baht/kg. This price includes only the cost of raw materials without any labor cost.

Table 3.14 Yield of purple sweet potato starch

The portion of purple sweet potato	Weight (kg)	Yield (% of flour)
Purple sweet potato (Fresh tuber)	5.00	-
Purple sweet potato flour	2.80	-
Purple sweet potato starch	2.65	94.64

Purple corn starch was produced by using corn ear as raw material. The corn ear at 92.75 kg (50 baht/kg: 4,637.50-baht) was removed husk and corn cob and obtained 34.95 kg of fresh corn kernel. After drying, the corn kernel was 6.35 kg dry weight which represented 18.17% of fresh purple corn kernels. The purple corn starch was then extracted and obtained at 3.12 kg starch, representing 49.13% of dried purple corn kernels, or 8.93% of fresh purple corn kernels (Table 3.15). From the experiment, the yield of purple corn starch was 3.12 kg from 92.75 kg corn ear (0.03 kg starch/ kg corn ear), and the cost of starch production was 1,486.37 baht/kg. This price includes only the cost of raw materials without any labor cost.

Table 3.15 Yield of purple corn starch

The portion of purple corn	Weight (kg)	Yield (%)
Purple corn ear	92.75	-
Husk	31.45	-
Purple corn (fresh kernels and cob)	61.30	-
Purple corn cob	26.35	-
Purple corn kernels (fresh)	34.95	8.93
Purple corn kernels (dried)	6.35	49.13
Purple corn starch	3.12	-

3.8 Yields of RS from Purple Starches by Different Methods

After DART process, the RS yields of purple rice, purple sweet potato, and purple corn were 78.42%, 85.32%, and 81.62%, respectively, by calculating from the initial dry mass of native purple starches. For DET process, the RS yields of purple rice, purple sweet potato, and purple corn were 27.16%, 22.50%, and 23.00%, respectively (Table 3.16).

Table 3.16 Yields of RS from purple starches by different methods

Starch	Yield (%, dwb)	
	DART	DET
Purple rice	78.42 ± 3.21^{a}	27.16 ± 2.02^{a}
Purple sweet potato	85.32 ± 3.69^{a}	22.50 ± 1.67^{b}
Purple corn	81.62 ± 4.46^{a}	23.00 ± 1.54^{b}

All data were means of triplicates. Mean values followed by the different letters within the column are significantly different ($p<0.05$). % yield = 100 × (dry mass of recovered from process/ initial dry mass of starch).

The results indicate that DART process is more appropriate for large-scale production of RS3 than DET because of the higher percentage of dry mass of RS3 recovered from initial dry mass of native starch. The results are in agreement with many researchers who reported that the yield of RS from corn starch increased over four autoclaving-retrogradation treatment, from 20.7 g/100g dwb (after one heating/cooling

cycle) to 31.7 g/100g dwb (after four cycles) (Sievert and Pomeranz, 1989; Yadav *et al*., 2009), similar result was also found in yam (Rosida *et al*., 2016), and black beans (Escobedo *et al*., 2020). Thus, it is possible to increase the amount of RS through the increasing of process cycles. For the further study, the higher number of DART cycles are required for more RS3 content, especially purple sweet potato starch which has high amount of amylose content.

CHAPTER 4

Conclusions

The overarching goal of this thesis project was to isolate and characterize purple rice, purple sweet potato, and purple corn starches from flours and to generate a novel RS3 product from the isolated starches. In Study 1, three most common anthocyanin-rich flours available in the market (e.g., purple rice, purple sweet potato, and purple corn) were selected to isolate starches with 80.28-81.52% total carbohydrate. The highest amylose content was found in purple sweet potato starch as 20.64%. The amylose content of purple rice starch was 9.36% and the lowest amylose content was detected in purple corn starch as 6.27%. In study 2 to develop RS3 through dual-autoclaving retrogradation (DART), and dual enzyme treatment (DET) the derived RS3 product was characterized and compared with native purple starch. The DART method yields 34.13 g/100g dwb of RS3 from the purple rice starch, 26.15 g/100g dwb from purple sweet potato starch, and 20.73 g/100g dwb from purple corn starch. The DET method yields 51.34 g/100g dwb of RS3 from the purple rice starch, 53.36 g/100g dwb from purple sweet potato starch, and 54.27 g/100g dwb from purple corn starch. These results show that the DART and DET developed in this research project was effective in enhancing the enzymatic resistance of RS form purple starch, which could be employed to generate starch-based RS ingredients for the preparation of low-glycemic foods. The new findings as reported in this thesis research will be meaningful for the food processing industry to find new applications of the starch fraction for value-added utilization.

DART can cause reformation of the amylose and amylopectin chains of starch which significantly improved its physicochemical and digestibility properties, thereby increasing their potential use in functional food products. Decrease in breakdown viscosity of RS obtained from purple starches indicates their resistance to shear thinning

during cooking. The XRD pattern and also confirms the efficient packing of double helices within the crystalline lamella. However, the crystallinity is not necessarily proportional to the contents of RS from various source, the degree of crystallinity is not always linked to resistance to amylolytic enzymes. DART also resulted in significant decrease of antioxidant activity by thermal process.

DET mainly generates short linear chains, enabling enhanced molecular mobility for systematic alignment and aggregation of the molecular structure. This conversion into a single uniform system of glucan linear chains leads to the more rapid formation of double helix. The α-amylase splits the α-1,4 glycosidic bond into linear chains that can rapidly reduce the viscosity of the gelatinous starch solution and encourage the production of soluble dextrin and oligosaccharides. Excessive hydrolysis with α-amylase may result in a small amount of glucose and maltose. Pullulanase (pullulan-6-glucanohydrolase) is a debranching enzyme used to modify starch. It can specifically cleave α-1,6 glycosidic linkages in amylopectin. Pullulanase has been used to produce high amylose starches, which have considerable commercial value. The increase in the crystalline structure of starch by enzymatic modification both contributes to delayed digestion. RS3 obtained from DET had a higher RS content than native purple starches. The currently accepted mechanism and the probable logic behind RS is resistance behavior is the failure of the double helix to fit into the active α-amylase site. Even the reduction of smaller linear chains leads to the formation of smaller, denser crystals, which limits the availability of digestive enzymes.

This study also affirmed the DART and DET process as a critical factor affecting the deterioration of total phenolic compound and anthocyanin contents. Consequently, it was responsible for decreasing the antioxidant activities (DPPH, ABTS, and FRAP) of RS obtained from purple starches. Further studies are needed to determine optimal conditions and develop strategies to stabilize anthocyanin, thus improving the quality of RS-rich anthocyanin-containing foods from an industrial perspective.

RS from purple starches have received much attention for both its potential health benefits and functional properties. RS has properties similar to fiber and shows promising physiological benefits in humans, which may result in disease prevention. Because of safety, bright color, and resistant enzyme digestion. In food development, the viscosity is

the main criteria, RS3 from purple starches have the functional properties including low viscosity, and natural based colorants which makes RS3 a high-promising potential ingredient to be incorporated into several food products such as blend with some fiber drink with better acceptability and greater palatability as substitutes of traditional fibers, dairy drinks, bread, cereal products, biscuits, cookies, noodles, snacks, and other food industries, as well as pharmaceutical industry such as use for coloring medical tablets, and use as an encapsulating agent for controlling drug delivery systems. Nonetheless, future work could focus on the effect of RS level on the re-process of autoclaving-cooling cycle treatment, scale-up research, and process improvement for commercial preparation of RS3, studies on the application of physically modified RS3 in food, evaluating the health implications of consuming any RS3 product, and the feasibility of utilizing RS3 from purple starches to increase dietary fiber in novel food products.

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APPENDICES

APPENDIX A

REAGENTS AND SOLUTIONS

A1 Starch Preparations

A1.1 0.35% Sodium Hydroxide Solution

Dissolve 3.5 g of sodium hydroxide with 200 mL of distilled water. Adjust the final volume of solution to 1,000 mL with distilled water and keep in a brown bottle at room temperature.

A1.2 0.01 M Hydrochloric Acid Solution

Mix 10 mL of 1.0 M Hydrochloric acid solution with distilled water. Adjust the final volume to 100 mL. Keep the solution in brown bottle at room temperature.

A1.3 0.1% Sodium Metabisulfite

Dissolve 1.0 g of sodium metabisulfite with 500 mL of distilled water. Adjust the final volume of solution to 1,000 mL with distilled water and keep in a brown bottle at room temperature.

A2 Determination of Chemical Composition

A2.1 50% Sodium Hydroxide Solution

Dissolve 250 g of sodium hydroxide with 200 mL of distilled water. Adjust the final volume of solution to 500 mL with distilled water and keep in a brown bottle at room temperature.

A2.2 2% Boric Acid Solution

Dissolve 20.0 g of boric acid with 200 mL of distilled water. Adjust the final volume of solution to 1,000 mL with distilled water and keep in a brown bottle at room temperature.

A2.3 0.05 M Hydrochloric Acid Solution

Mix 5 mL of 1.0 M Hydrochloric acid solution with distilled water. Adjust the final volume to 100 mL. Keep the solution in brown bottle at room temperature.

A2.4 1.25% Sulfuric Acid Solution

Mix 0.7 mL of 98% sulfuric acid with distilled water. Adjust the final volume to 100 mL. Keep the solution in brown bottle at room temperature.

A2.5 1.25% Sodium Hydroxide Solution

Dissolve 12.5 g of sodium hydroxide with 200 mL of distilled water. Adjust the final volume of solution to 1,000 mL with distilled water and keep in a brown bottle at room temperature.

A2.6 Phenolphthalein Indicator

Dissolve 0.10 g of phenolphthalein was dissolved in 60 mL of 95% ethanol and adjusted volume to 100 mL by distilled water.

A3 Amylose Content

A3.1 1N Sodium Hydroxide Solution

Dissolve 40.0 g of sodium hydroxide with distilled water and adjust to final volume of 1,000 mL. Keep the solution in brown at room temperature.

A3.2 1N Acetic acid

Add 14.354 mL of glacial acetic acid to 50 mL distilled water. Adjust the final volume of solution to 250 mL with distilled water.

A3.3 Iodine Solution

Dissolve 0.2 g iodine and 2.0 g potassium iodide with distilled water and adjust to final volume of 100 mL. Keep the solution in brown at room temperature.

A4 Reagent for Resistant Starch Determination

A4.1 50 mM Sodium Maleate Buffer, pH 6.0 plus 2 mM Calcium Chloride Dihydrate

Dissolve 11.6 g of maleic acid in 1,600 mL of distilled water and adjust the pH to 6.0 with 4 M (160 g/L) sodium hydroxide. Add 0.6 g of calcium chloride dihydrate and dissolve. Adjust the volume to 2 L.

A4.2 1.0 M Sodium Acetate Buffer, pH 3.8 plus Calcium Chloride (5 mM)

Add 57.0 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L with distilled water and store in a bottle.

A4.3 100 mM Sodium Acetate Buffer, pH 4.5

Add 5.7 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 L with distilled water and store in a bottle.

A4.4 1.7 M Sodium Hydroxide Solution

Add 68 g of sodium hydroxide to 800 mL of distilled water in a fume cupboard and dissolve by stirring. Adjust the volume to 1 L with distilled water and store in a bottle.

A4.5 50% (v/v) Ethanol

Add 500 mL of ethanol (95% v/v) to 500 mL of distilled water.

A5 Resistant Starch Assay Kits

Bottle 1

Mixture of purified PAA (40 KU/g) and AMG (17 KU/g)

Bottle 2

Amyloglucosidase (14 mL, 3,300 U/mL on soluble starch)

Bottle 3

GOPOD Reagent buffer

Bottle 4

GOPOD reagent Enzymes

Bottle 5

D-Glucose standard Solution (5 mL, 1.0 mg/mL)

Bottle 6

Resistant starch control

Figure A.1 Resistant starch assay kits

A5.1 Stock PAA/AMG Solution

Add 0.1 g of PAA/ AMG powder mixture (Bottle 1) to 5 mL of sodium maleate buffer and stir on a magnetic stirrer for 5 min. Store on ice during use. Use within 4 h of preparation.

A5.2 Dilute AMG

Add 1 mL of the contents of bottle 2 to 30 mL of 100 mM sodium acetate buffer (pH 4.5). Mix well and divide into 10 mL aliquots and store below -18°C between use, (AMG 100 U/mL).

A5.3 Stock PAA/AMG Solution

Add 0.1 g of PAA/ AMG powder mixture (Bottle 1) to 5 mL of sodium maleate buffer and stir on a magnetic stirrer for 5 min. Store on ice during use. Use within 4 h of preparation.

A5.4 Dilute AMG

Add 1 mL of the contents of bottle 2 to 30 mL of 100 mM sodium acetate buffer (pH 4.5). Mix well and divide into 10 mL aliquots and store below -18°C between use, (AMG 100 U/mL).

A5.5 GOPOD reagent buffer

Dilute the contents of bottle 3 to 1 L with distilled water.

A5.6 GOPOD reagent

Dissolve the contents of bottle 4 in 20 mL of GOPOD reagent buffer and quantitatively transfer this to the bottle containing the remainder of GOPOD reagent buffer. Cover this bottle with aluminum foil to protect the enclosed reagent from light.

A6 Extraction of Bioactive Compounds

A6.1 80% (v/v) Ethanol

Add 421 mL of ethanol (95% v/v) to the bottle. Adjust the final volume of solution to 500 mL with distilled water and keep in a bottle at room temperature.

A7 Total Phenolic Content

A7.1 10% (v/v) Folin-Ciocalteu Phenol Reagent

Add 10 mL of Folin-Ciocalteu reagent with distilled water and adjust to final volume of 100 mL.

A7.2 11.5% Sodium Carbonate Solution

Dissolve 7.50 g of sodium carbonate was dissolved and adjusted volume to 100 mL by deionized water.

A7.3 0.1 mg/mL of Gallic Acid Standard

Dissolve 0.0100 g of gallic acid was dissolved and adjusted volume to 100 mL by deionized water.

A8 Antioxidant Activity

A8.1 0.10 mM of 1,1-diphenyl-2-picrylhydracyl (DPPH)

Dissolve 3.9 mg of DPPH was dissolved and adjusted volume to 100 mL by 95% ethanol.

A8.2 140 mM of Potassium Persulfate

Dissolve 378.4 mg of potassium persulfate was dissolved and adjusted volume to 10 mL by deionized water.

A8.3 7 mM of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

Dissolve 96 mg of ABTS was dissolved and adjusted volume to 25 mL by deionized water.

5.00 mL of 7 mM ABTS and 88 µL of 140 mM potassium persulfate were mixed together and stand in the dark for 16 h. After that there were adjusted volume to 25 mL by deionized water. ABTS cation radical was diluted with deionized water for absorbance at 734 nm in the range of 0.70±0.02 when using.

A8.4 300 mM Acetate Buffer, pH 3.6

Dissolve 3.1 g sodium acetate and 16 mL acetic acid and adjusted volume to 1,000 mL by distilled water.

A8.5 20 mM Ferric Chloride Solution

A 54 mg of ferric chloride is dissolved in 10 mL of distilled water. The solution is transferred to a brown bottle and keeps at 4°C until used.

A8.6 10 mM TPTZ in 40 mM Hydrochloric Acid Solution

Dissolve 31 mg of TPTZ with 10 mL of 40 mM hydrochloric acid solution. Transfer solution into a brown bottle and keep at 4°C until used.

A8.7 1,000 mM Ferrous Sulphate Solution

Dissolve 27.80 g of ferrous sulfate heptahydrate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9 *In vitro* Stach Digestibility

A9.1 Simulated Oral Conditions

A9.1.1 15.1 mM Potassium Chloride Solution

Dissolve 112 mg of potassium chloride is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.1.2 3.7 mM Potassium Dihydrogen Phosphate Solution

Dissolve 50 mg of potassium dihydrogen phosphate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.1.3 13.6 mM Sodium Hydrogen Carbonate Solution

Dissolve 115 mg of sodium hydrogen carbonate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.1.4 0.15 mM Magnesium Chloride Solution

Dissolve 30 mg of magnesium chloride hexahydrate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.1.5 0.06 mM Ammonium Carbonate Solution

Dissolve 90 mg of ammonium carbonate is dissolved in 1,000 mL of distilled water and keep in a bottle at room temperature.

A9.1.6 1.5 mM Calcium chloride solution

Dissolve 22 mg of calcium chloride dihydrate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.2 Simulated Gastric Conditions

A9.2.1 0.2 M Hydrochloric Acid

Mix 20 mL of 1.0 M hydrochloric acid solution with distilled water. Adjust the final volume to 100 mL. Keep the solution in brown bottle at room temperature.

A9.3 Simulated Pancreatic Conditions

A9.3.1 6.8 mM Potassium Chloride Solution

Dissolve 50.6 mg of potassium chloride is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.3.2 0.8 mM Potassium Dihydrogen Phosphate Solution

Dissolve 108.8 mg of potassium dihydrogen phosphate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.3.3 85 mM Sodium Bicarbonate Solution

Dissolve 714 mg of sodium bicarbonate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.3.4 38.4 mM Sodium Chloride Solution

Dissolve 224.2 mg of sodium chloride is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.3.5 0.33 mM Magnesium Chloride

Dissolve 6.7 mg of magnesium chloride is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.3.6 0.6 mM Calcium Chloride Solution

Dissolve 6.6 mg of calcium chloride dihydrate is dissolved in 100 mL of distilled water and keep in a bottle at room temperature.

A9.4 Reducing Sugars Using the DNS Method

A9.4.1 Dinitrosalicylic Acid Reagent (DNS)

Sodium hydroxide 10 g are added into 700 ml of deionized water and mixed in order to add the 300 g potassium sodium tartrate. When the solution dissolved, 3, 5-dinitrosalicylic acid 10 g is then added and continuously stirred. After that, the 0.5 g of sodium sulfite and 2.0 g of phenol are dissolved, respectively. Finally, the volume is adjusted to 1,000 ml by deionized water and kept away from light.

A9.5 Total Sugar Content Using the Phenol-Sulfuric Acid Method

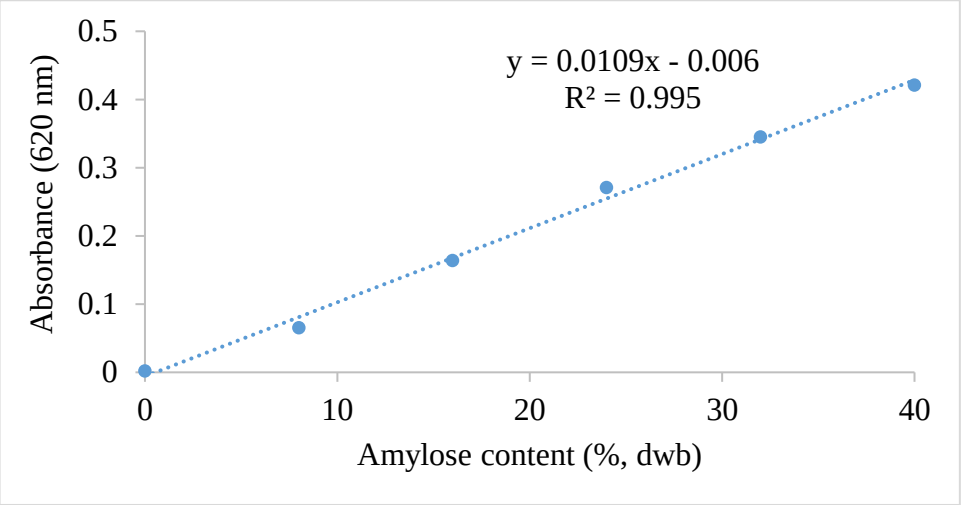
A9.5.1 5% w/v Phenol Solution

5.0 g of reagent grade phenol, crystals is dissolved in 95 mL of distilled water and keep in a brown bottle at room temperature.

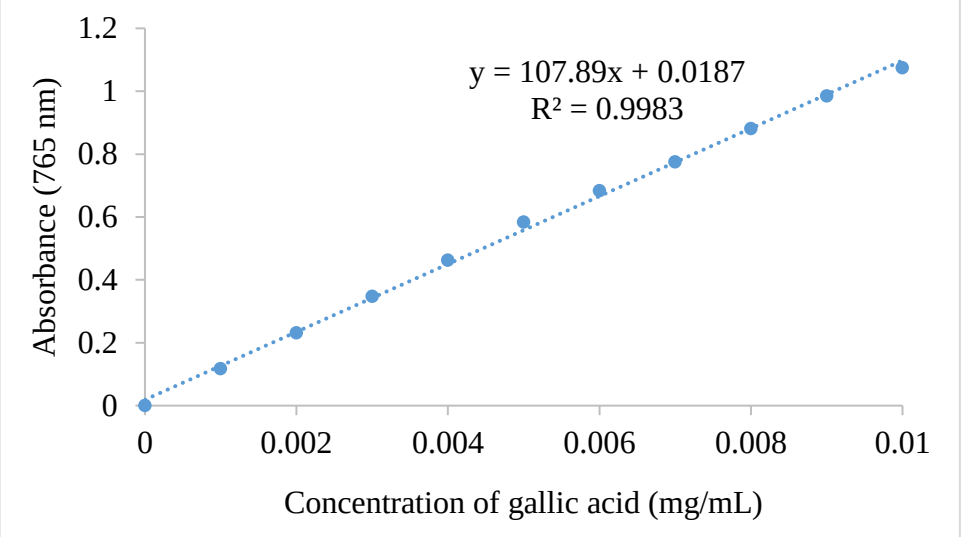
APPENDIX B

STANDARD CALIBRATION CURVES

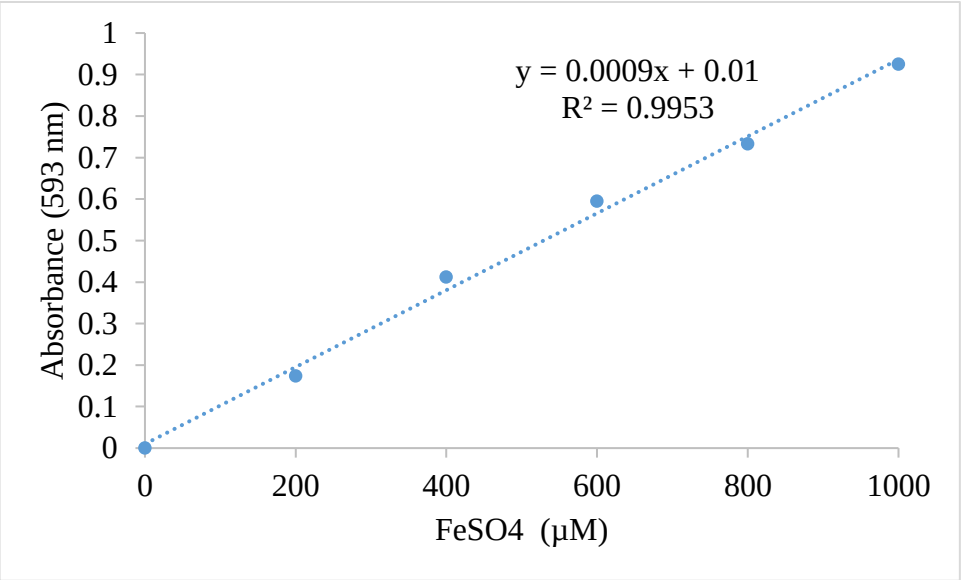
B1 Calibration Curves for the Determination of Amylose Content



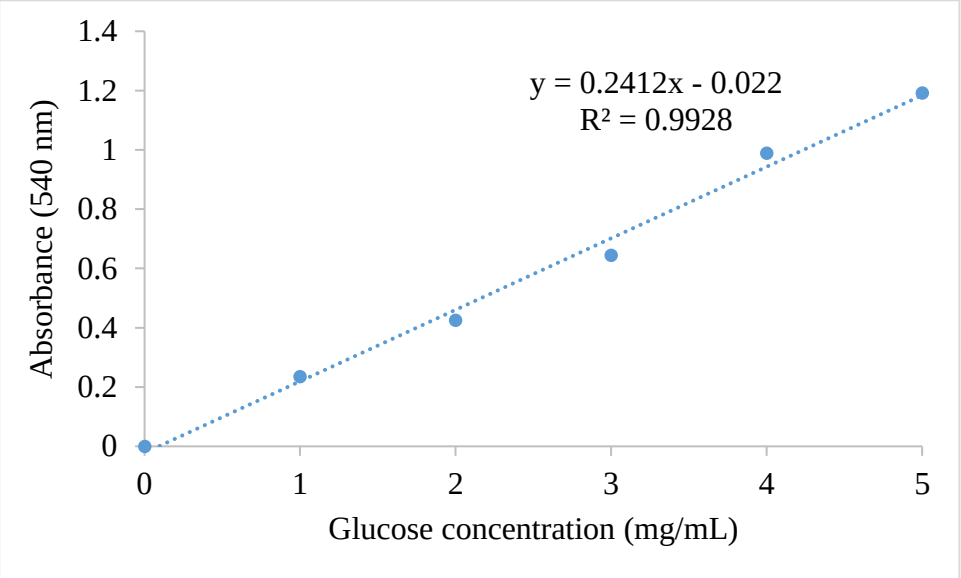
B2 Calibration Curve for the Determination of Total Phenolic Content



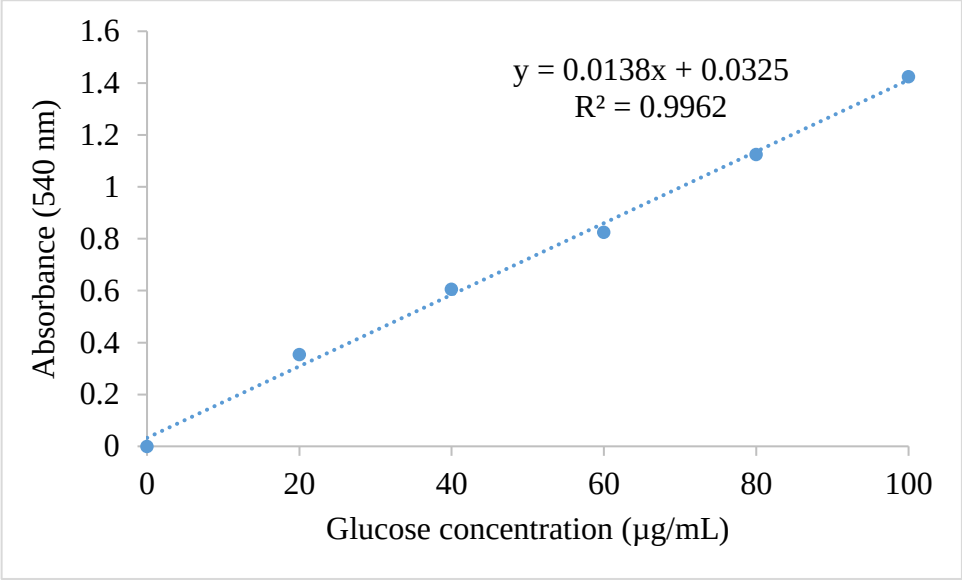
B3 FRAP Assay Standard Calibration Curve



B4 Calculation of Reducing Sugars Using the DNS Method



B5 Calibration Curves for the Determination of Total Sugar by Phenol-Sulfuric Method



APPENDIX C

CHROMATOGRAMS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR ANTHOCYANINS DETERMINATION

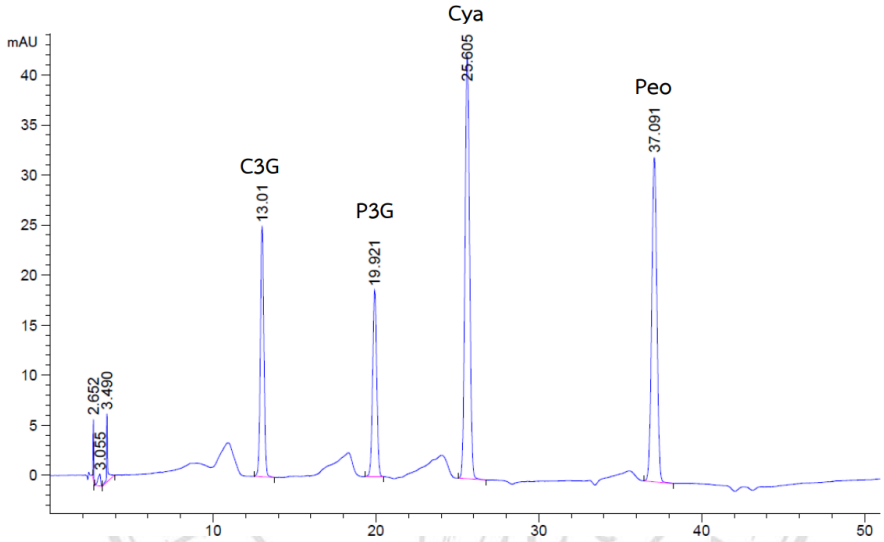


Figure C.1 HPLC chromatogram of standard Cyanidin-3-glucoside (C3G), Peonidin-3-glucoside (P3G), Cyanidin (Cya), & Peonidin (Peo) 25 µg/mL

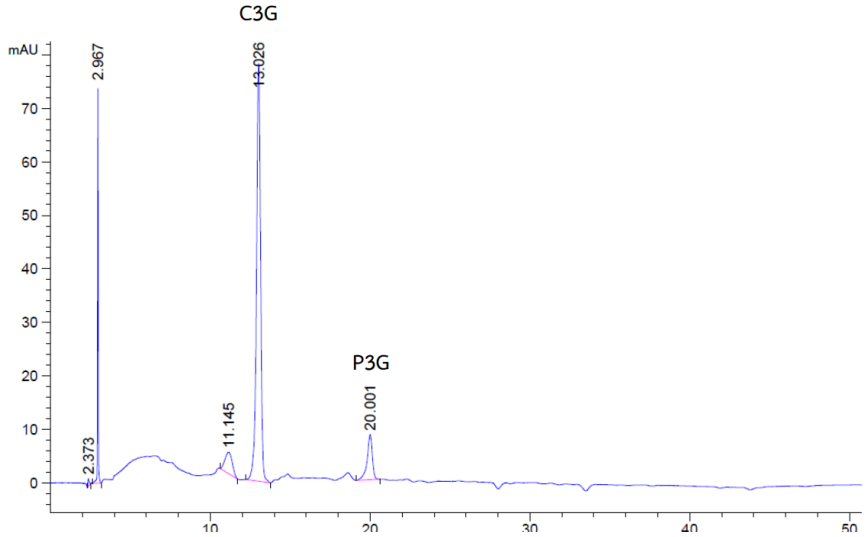


Figure C.2 HPLC chromatogram of native purple rice starch

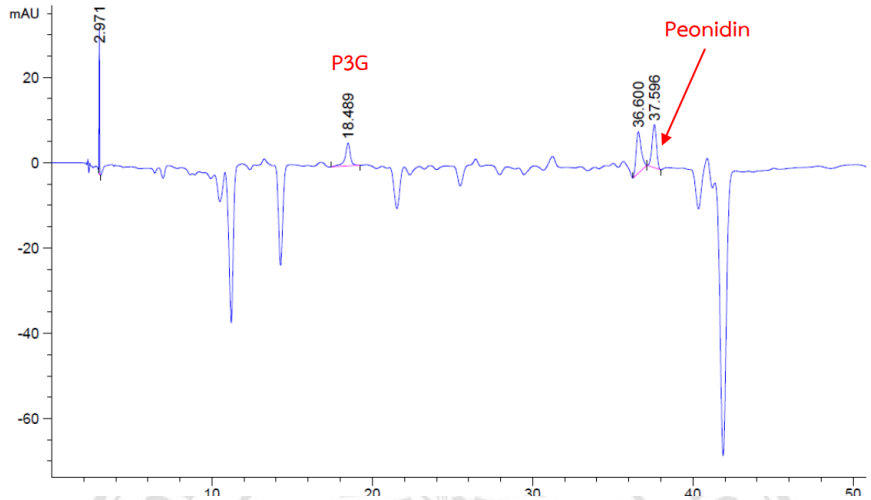


Figure C.3 HPLC chromatogram of RS obtained from purple rice starch by DART

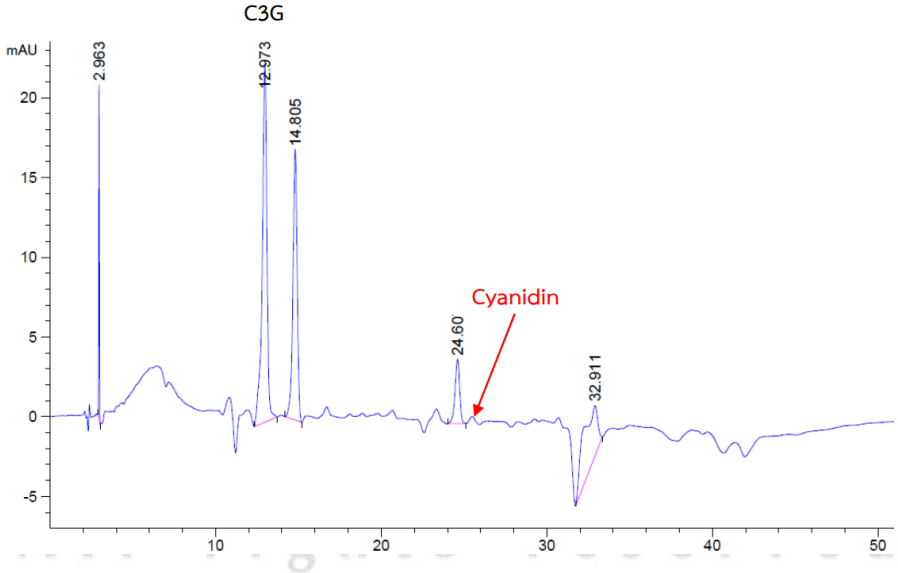


Figure C.4 HPLC chromatogram of RS obtained from purple rice starch by DET

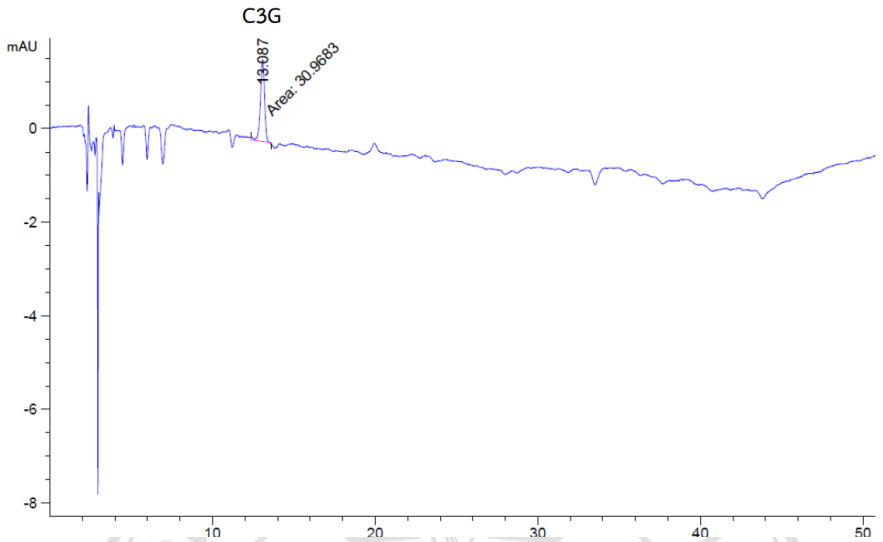


Figure C.5 HPLC chromatogram of native purple sweet potato starch

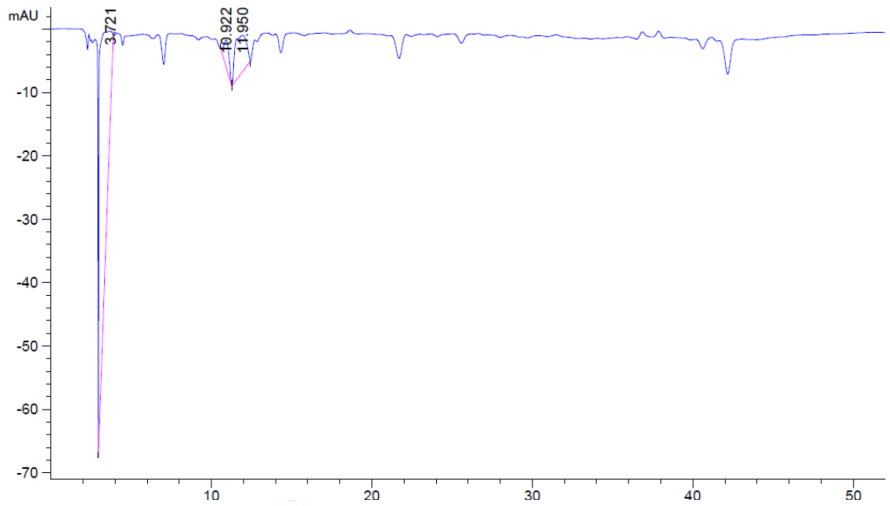


Figure C.6 HPLC chromatogram of RS obtained from purple sweet potato starch by DART

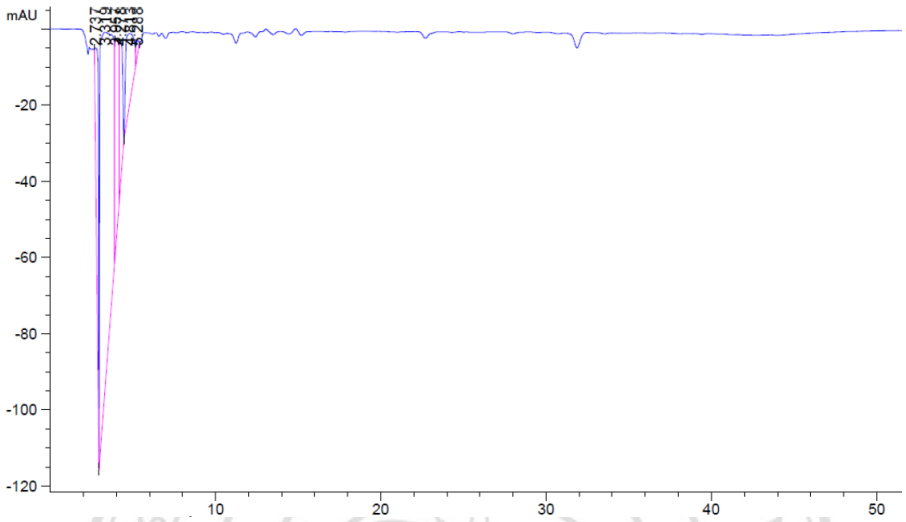


Figure C.7 HPLC chromatogram of RS obtained from purple sweet potato starch by DET

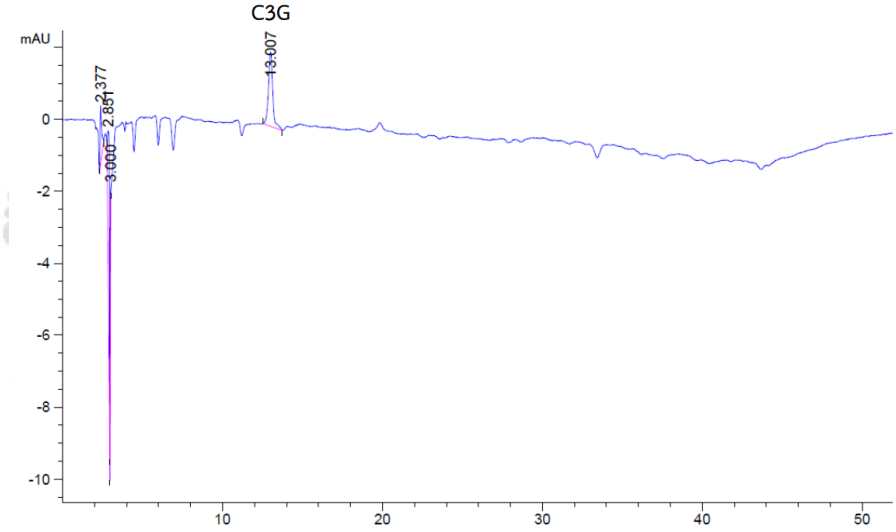


Figure C.8 HPLC chromatogram of native purple corn starch

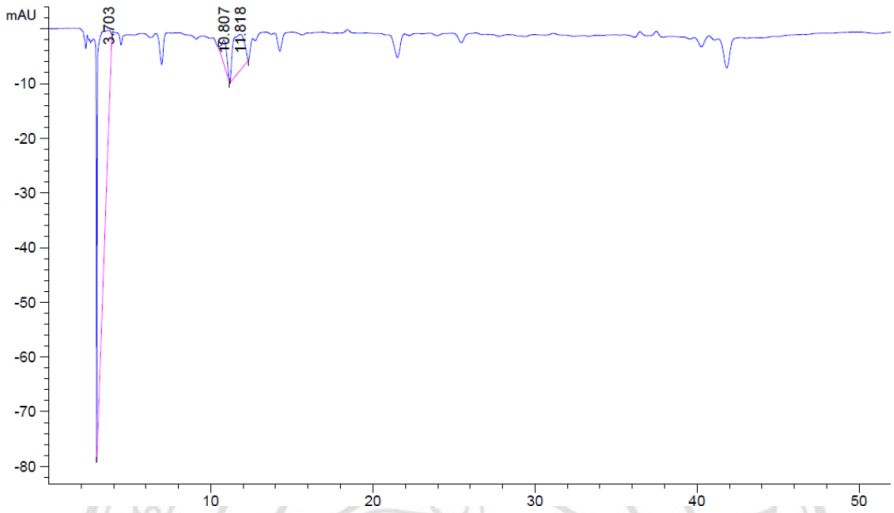


Figure C.9 HPLC chromatogram of RS obtained from purple corn starch by DART

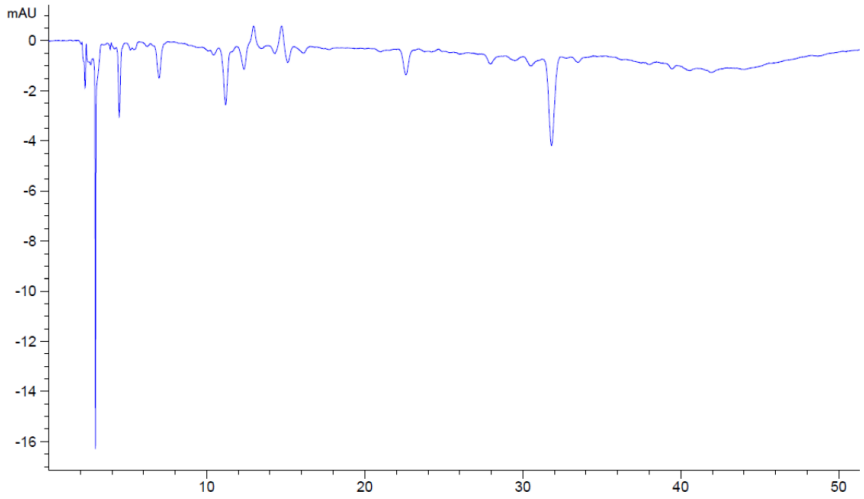


Figure C.10 HPLC chromatogram of RS obtained from purple corn starch by DET

APPENDIX D

DSC THERMOGRAMS

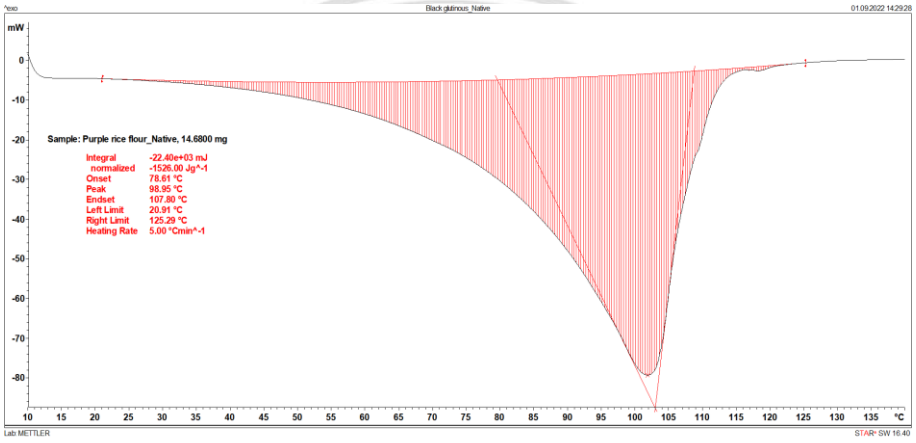


Figure D.1 DSC thermogram of native purple rice starch

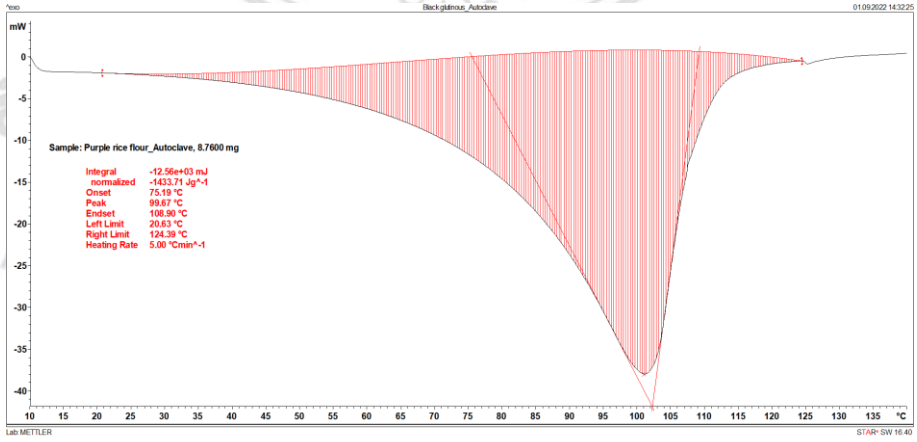


Figure D.2 DSC thermogram of RS obtained from purple rice starch by DART

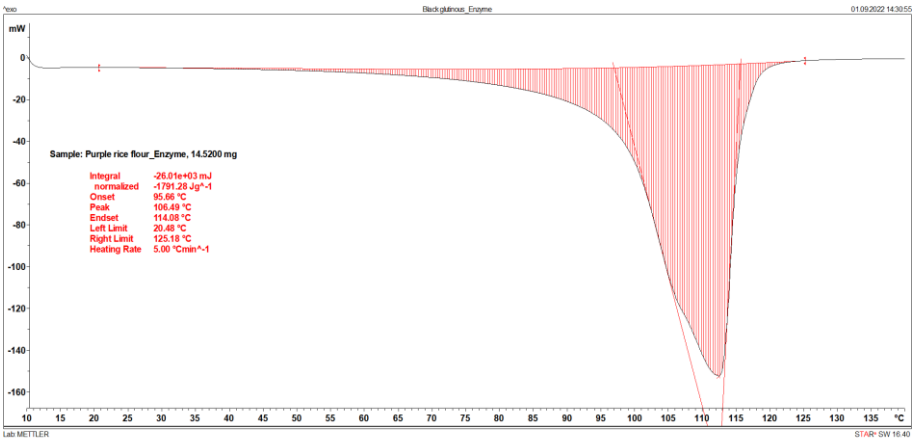


Figure D.3 DSC thermogram of RS obtained from purple rice starch by DART

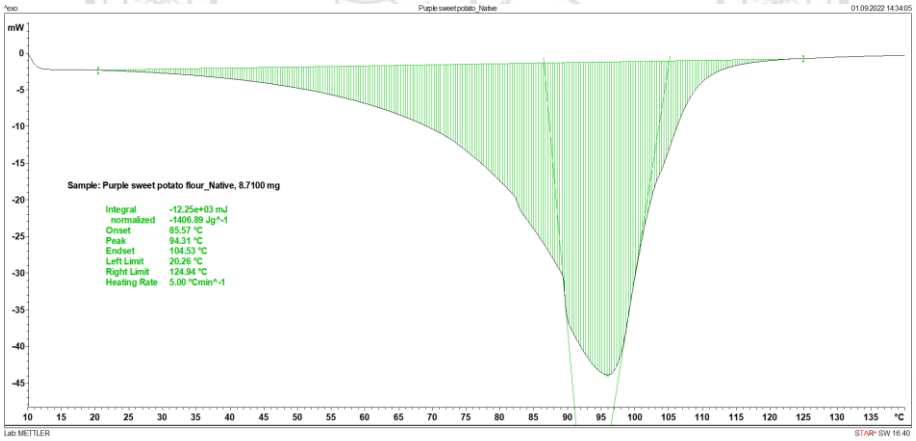


Figure D.4 DSC thermogram of native purple sweet potato starch

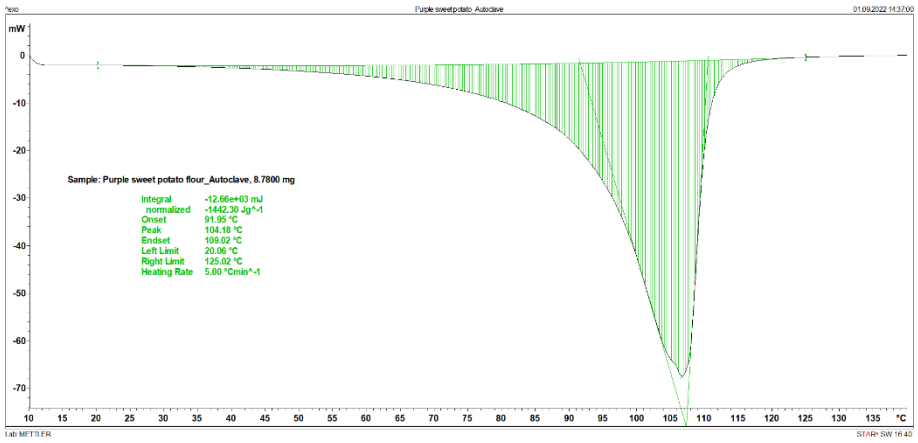


Figure D.5 DSC thermogram of RS obtained from purple sweet potato starch by DART

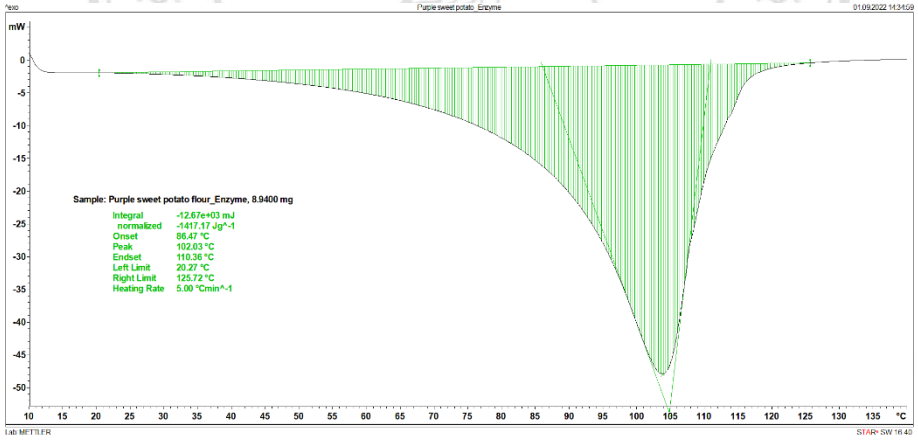


Figure D.6 DSC thermogram of RS obtained from purple sweet potato starch by DET

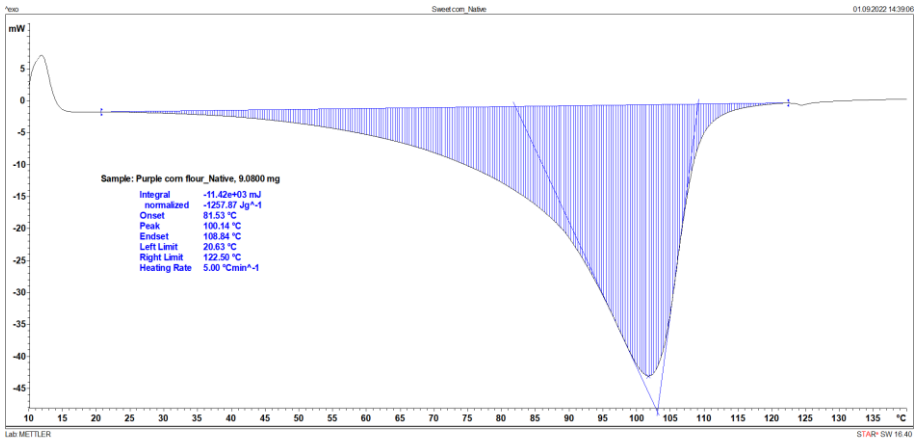


Figure D.7 DSC thermogram of native purple corn starch

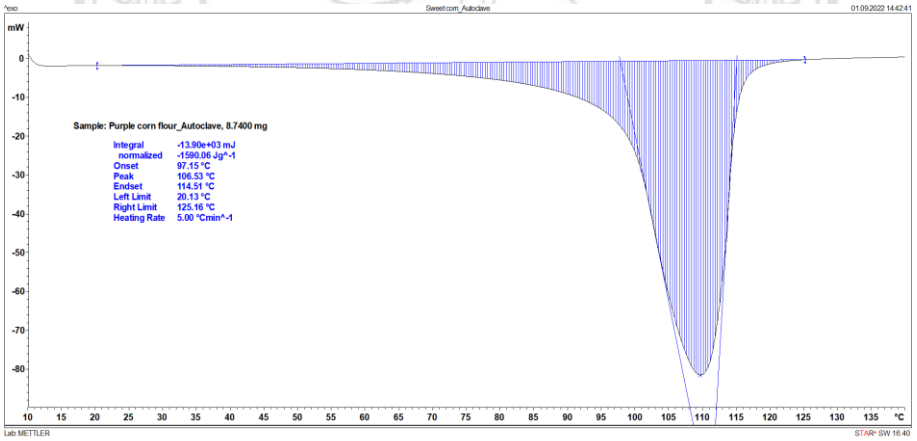


Figure D.8 DSC thermogram of RS obtained from purple corn starch by DART

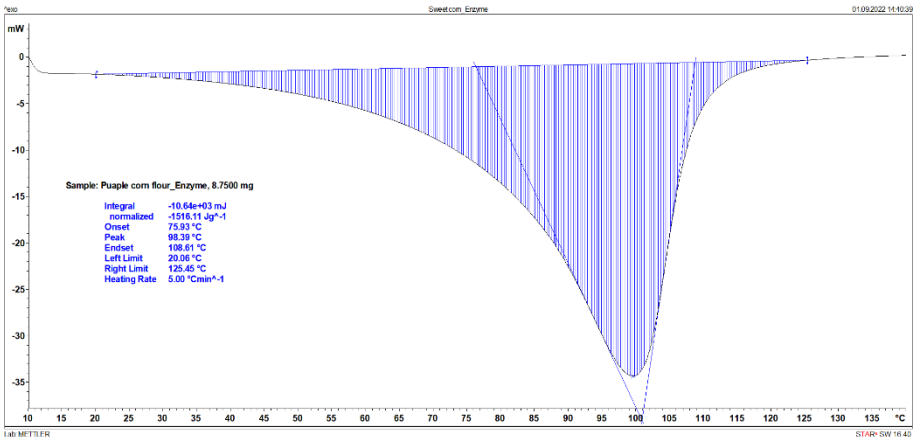


Figure D.9 DSC thermogram of RS obtained from purple corn starch by DET

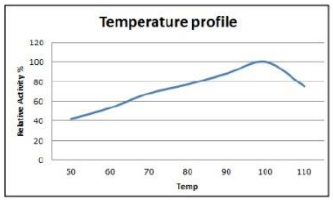
APPENDIX E

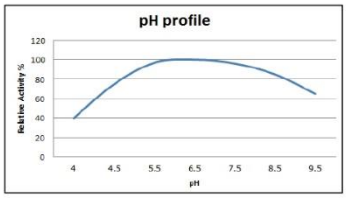
ENZYME SPECIFICATIONS

E1 Specification of α-Amylase

iKnowZyme HTAA(EC Code 3.2.1.1)

CONCENTRATED HIGH TEMPERATURE STABLE ALPHA-AMYLASE





PRODUCT TYPE

Series Type	Activity (IU/ml)	Recommended Dosage (kg /ton of raw starch)
Liquid type		
HTAA 20 L	20,000	0.3 – 0.5 kg
HTAA 40 L	40,000	0.2 – 0.4 kg
HTAA 80 L	80,000	0.1 – 0.3 kg
HTAA 150 L	150,000	0.05 – 0.1 kg
HTAA 180 L	180,000	0.03 – 0.1 kg
Solid type		
	Activity (IU/mg)	**Recommended Dosage (kg /ton of raw starch)**
HTAA 40 P	40,000	0.2 – 0.4 kg
HTAA 200 P	250,000	The high concentration for formulation
	Unit definition	**Conditions**
	The amount of enzyme need to hydrolyze 1 mg of starch in one minute at pH 6,T 70 °C at stared	Adjusting pH to 6.5 – 7.0 Temp: 95 – 105 °C Time : 30 – 100 minutes

REACH BIOTECHNOLOGY CO., LTD.
229/14 ,Radphatthana Road,Saphan Sung, Saphan Sung ,Bangkok. 10240 Thailand.
+66 29173298-9, Fax +66 29173195, www.iknowzyme.com, E-mail: anongk54@yahoo.com

E2 Specification of Pullulanase

iKnowZyme PULLULANASE

CONCENTRATED PULLULANASE

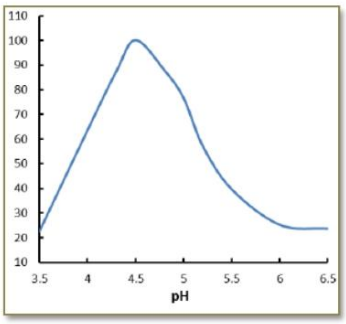


Fig 1 Effect of pH on the relative enzyme activity

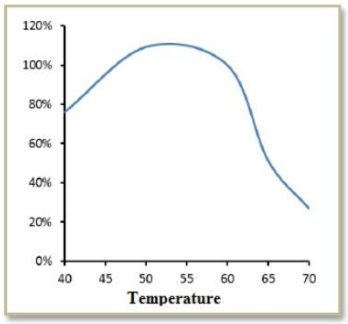


Fig 2 Effect of temperature on the relative enzyme activity

APPLICATIONS

- Effect of pH optimal pH is 4.0-5.0 and effective pH is 3.8 - 5.5 at 60 ℃ (Shown in Figure 1).
- Effect of temperature, as shown in Figure 2, iKnowZyme PULLULANASE performs well at 45-60 °C that also right temperature to produce glucose and maltose.
- Production of ultra high malt syrup

pH 5.0-5.5 is the best for maltose saccharification that also the right pH for iKnowZyme PULLULANASE. Increase malt sugar yield and reduce branched chain dextrin significantly with barley β-amylase (BBA, need to buy another), produce more than 70% of the ultra high malt syrup, achieve more than 80% if used with maltotriose degrading enzyme(need to buy another) , meet the crystalline maltose, maltitol and other special industry demands (Shown in Figure 3).

- Production of high purity glucose syrup

In the production of high purity glucose syrup, DX reach to 96.5% if use iKnowZyme PULLULANASE and glucoamylase together, at the same time reduce the dosage of glucoamylase, as shown in Figure 4.

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CURRICULUM VITAE

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Education

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2014	M.S. (Biotechnology), Division of Biotechnology, the Graduate School, Chiang Mai University, Chiang Mai, Thailand.
2022	Ph.D. (Biotechnology), Division of Biotechnology, the Graduate School, Chiang Mai University, Chiang Mai, Thailand.

Employment History and Research Experience

2015-2020	Lecturer in Public Health Science Program, Nation University, Lampang, Thailand
2015	Joined the Japan-Asia Youth Exchange Program in Science at Fukui University of Technology, Fukui Prefecture, Japan. This Sakura Exchange Program was supported by the Japan Science and Technology Agency (JST).
2018	Research Fund from the National Research Council, Thailand Project title "Development of Modified Purple Rice Starch and Its Application to Produce Functional Food Products for Valued Added Thai Rice"
2021	Certificate of Professional Qualification in Food Biotechnologist Level 5, Thailand Professional Qualification Institute. Thailand.