สมบัติทางโครงสร้าง และการย่อยของมอลโตเดกซ์ทรินย่อยช้า และต้านทานจากแป้งมันสำปะหลังโดยวิธีการดัดแปรทางเอนไซม์

นางสาววราภรณ์ ศรเดช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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STRUCTURAL AND DIGESTION PROPERTIES OF SOLUBLE-, SLOWLY DIGESTIBLE AND RESISTANT MALTODEXTRIN FROM CASSAVA STARCH BY ENZYMATIC MODIFICATION

Waraporn Sorndech

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

Suranaree University of Technology

Academic Year 2015

STRUCTURAL AND DIGESTION PROPERTIES OF SOLUBLE-, SLOWLY DIGESTIBLE AND RESISTANT MALTODEXTRIN FROM CASSAVA STARCH BY ENZYMATIC MODIFICATION

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

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วราภรณ์ ศรเดช : สมบัติทางโครงสร้าง และการย่อยของมอลโตเดกซ์ทรินย่อยช้า และต้านทานจากแป้งมันสำปะหลังโดยวิธีการดัดแปรทางเอนไซม์ (STRUCTURAL AND DIGESTION PROPERTIES OF SOLUBLE-, SLOWLY DIGESTIBLE AND RESISTANT MALTODEXTRIN FROM CASSAVA STARCH BY ENZYMATIC MODIFICATION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุนันทา ทองทา และ รองศาสตราจารย์ ดร.แอนเดรียส เบลนนาว, 202 หน้า.

เอนไซม์ตัดต่อกิ่ง (Branching enzyme, BE) และเอนไซม์ตัดต่อสายกลูแคน (Amylomaltase, AM) ได้ใช้ร่วมกันเพื่อดัดแปรแป้งมันสำปะหลัง โดยใช้แป้งมันสำปะหลังที่ผ่านการเจลาติไนเซชันดัดแปรด้วย BE หรือ AM→BE หรือ BE→AM→BE หรือ การใช้ AM และ BE พร้อมกัน จากนั้นศึกษาลักษณะโครงสร้างโมเลกุลของผลิตภัณฑ์ คือ ความยาวของสายกลูแคน ปริมาณพันธะกลูโคซิดิกตำแหน่ง 1, 6 น้ำหนักโมเลกุล และความสามารถในการย่อย การดัดแปรโดยใช้ BE พบว่าปริมาณกิ่งมีค่า 7.8% ตัวอย่าง AM→BE มีปริมาณกิ่งน้อยกว่าเมื่อเปรียบเทียบกับตัวอย่าง BE→AM→BE นอกจากนี้การดัดแปรโดยใช้ AM→BE และ BE→AM→BE ทำให้อัตราการย่อยของเอนไซม์แอลฟา-อะมิเลส และเอนไซม์กลูโคอะมิเลสมีค่าลดลง การใช้ BE→AM→BE ทำให้เกิดโครงสร้างของกลูแคนที่มีประสิทธิภาพที่ดีที่สุดต่อการเพิ่มปริมาณกิ่ง และลดอัตราการย่อยได้ดีที่สุด โดยมีค่าคงที่ของอัตราการย่อยต่ำที่สุด

การศึกษาผลของปริมาณอะมิโลสต่อการดัดแปรแป้งโดยใช้ BE และ BE→AM→BE เพื่อผลิตมอลโตเดกซ์ทรินย่อยช้า และต้านทาน โดยใช้แป้งข้าวโพดข้าวเหนียว (WX) และแป้งข้าวบาร์เลย์ที่มีเพียงอะมิโลส (AO) ผสมกันในอัตราส่วนของปริมาณอะมิโลส 0-100% พบว่าตัวอย่าง 0% AO ที่ผ่านการดัดแปรโดยใช้ BE และ BE→AM→BE มีอัตราการสร้างกิ่งน้อยกว่าตัวอย่างที่ใช้ 100% AO รวมทั้งมีขนาดโมเลกุลเล็กลงเปรียบเทียบกับแป้งที่ไม่ได้ดัดแปร ปริมาณกลูโคสที่เกิดขึ้นจากตัวอย่างหลังจากย่อยด้วยเอนไซม์แอลฟา-อะมิเลสจากมนุษย์ และเอนไซม์แอลฟา-กลูโคซิเดส (α-glucosidase) จากหนูมีค่าลดลงเมื่อใช้ซับสเตรทที่มีอัตราส่วนของอะมิโลสเพิ่มขึ้น ดังนั้นการใช้ซับสเตรทที่มีปริมาณอะมิโลเพคตินสูง คาดว่าทำให้เกิดผลิตภัณฑ์ที่มีลักษณะคลัสเตอร์ของอะมิโลเพคตินที่มีกิ่งมากขึ้น รวมทั้งอะมิโลเพคตินที่เป็นวง ในขณะที่เมื่อใช้ ซับสเตรทที่มีปริมาณอะมิโลสสูง ให้ผลิตภัณฑ์เป็นอะมิโลสที่มีกิ่งเพิ่มขึ้น และอะมิโลสที่เป็นวง ซึ่งลักษณะโมเลกุลทั้งหมดมีคุณสมบัติชะลอ และต้านทานการย่อย

การผลิตมอลโตเดกซ์ทรินย่อยช้า และต้านทานที่เป็นไอโซมอลโตโอลิโกแซคคาไรด์ (IMOs) ได้ศึกษาโดยดัดแปรแป้งมันสำปะหลังความเข้มข้น 30% และ 50% ด้วยเอนไซม์แอลฟา-อะมิเลส ร่วมกับ BE แล้วต่อด้วยแอลฟา-ทรานซ์กลูโคซิเดส (α-transglucosidase) (ABT) รวมทั้ง

การใช้ α-amylase ร่วมกับ BE แล้วต่อด้วยเบต้า อะมิเลส (β-amylase) และแอลฟา-ทรานซ์กลูโคซิเดส (ABbT) พบว่าปริมาณกิ่งในกลุ่มของ ABT และปริมาณใยอาหารทั้งหมดต่ำกว่าในกลุ่มของ ABbT รวมทั้งพบว่าไม่มีความแตกต่างขององค์ประกอบของ IMOs เมื่อใช้ความเข้มข้นของแป้งเริ่มต้นต่างกัน ตัวอย่าง ABbT และ ABT สามารถกระตุ้นการเจริญของโพรไบโอติกส์ (probiotics) ได้ดีเมื่อเปรียบเทียบกับแป้งที่ไม่ดัดแปร โดยตัวอย่าง ABbT มีค่าพรีไบโอติกส์ อินเดกซ์ (prebiotics index, PI) และการสร้าง IMOs มากกว่าตัวอย่าง ABT และตัวอย่าง ABbT สามารถกระตุ้นการสร้างกรดอะซีติกในปริมาณสูงกว่ากรดไขมันสายสั้นอื่น ๆ ด้วย

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2558

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____

WARAPORN SORNDECH : STRUCTURAL AND DIGESTION PROPERTIES OF SOLUBLE-, SLOWLY DIGESTIBLE AND RESISTANT MALTODEXTRIN FROM CASSAVA STARCH BY ENZYMATIC MODIFICATION. THESIS ADVISOR : ASST. PROF. SUNANTA TONGTA, Ph.D., AND ASSOC. PROF. ANDREAS BLENNOW, Ph.D., 202 PP.

STARCH/BRANCHING ENZYME/AMYLOMALTASE/ α-TRANSGLUCOSIDASE/SLOWLY DIGESTIBLE/RESISTANT MALTODEXTRIN/PREBIOTIC/PROBIOTIC

The combination of branching enzyme (BE) and amylomaltase (AM) were selected to modify cassava starch. Cassava starch was gelatinized and incubated with BE or AM→BE or BE→AM→BE or simultaneous AM and BE. The molecular analysis of the products including chain length distribution, content of α-1,6 glucosidic linkages, absolute molecular weight distribution and digestibility were examined. Only BE catalysis showed 7.8% branching linkages. The sequential AM→BE-treated starch showed lower branching linkages as compared to sequential BE→AM→BE-treated starch. Moreover, the sequential AM→BE and BE→AM→BE-treated starch retarded the digestion rate of α-amylase and glucoamylase. The sequential BE→AM→BE catalysis resulted in more extensive branching and the products also exhibited the lowest digestion rate constant.

The effect of amylose content on BE and combinatorial BE→AM→BE chain transfer were studied. Well-defined ratios of amylose only-barley starch (AO) and waxy maize starch (WX) with non-granular AO content varied from 0 to 100% were

used as a substrate. For only BE catalysis, an increased rate of branch linkage formation for the 0% AO sample treated with BE and BE→AM→BE were lower than the 100% AO sample and also showed a decrease in $\overline{M}w$ compared to native starch. Glucose released from all modified starches after hydrolysis by human pancreatic α-amylase and further hydrolysis by rat intestinal α-glucosidase was decreased with increasing AO ratios. Amylopectin rich substrates were expected to obtain highly branched-amylopectin and cyclo-amylopectin while amylose rich substrates were expected to obtain branched-amylose and cyclo-amylose which retard and suppress the digestion.

Slowly and resistant maltodextrin conferring isomaltooligosaccharides (IMOs) production was prepared by using 30% and 50% cassava starch substrate concentration with simultaneous α-amylase and BE followed by α-transglucosidase (ABT) or simultaneous α-amylase and BE followed by simultaneous β-amylase and α-transglucosidase (ABbT). The ABT catalysis showed branching linkages content and TDF content lower than that of the ABbT catalysis. There was no difference between substrate concentrations. The modified samples stimulated probiotics growth as compared to native starch. Prebiotics index (PI) and IMOs produced from the ABbT samples was higher than the ABT samples. The acetate content was the highest short chain fatty acids (SCFAs) produced from the ABbT samples.

School of Food Technology Student's Signature_____

Academic Year 2015 Advisor's Signature_____

Advisor's Signature_____

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CHAPTER I

INTRODUCTION

1.1 Introduction

Starch, a polymer of glucose linked by α-linkages is widely distributed in many plants which consist of major and minor components. For major components, amylose and amylopectin are classified in this group. Amylose is found with molecular weights ranging from 10^5 to 10^6 Da and with the number of glucose residues per molecule, (DP) ranging from 500 to 5,000. Although it is considered to be an essentially linear, α-1,4 glucosidic linkages, Hizukuri et al. (1981) demonstrated the present of α-1,6 glucosidic linkages in amylose molecules. For amylopectin, it has molecular weights in the average range from 10^7 to 10^9 Da. The structure contains approximately 5% of α-1,6 glucosidic linkages, leading to the short, α-1,4 glucosidic linkages that occur in a bimodal distribution of A-chains (DP approximately 15) and B-chains (DP approximately 45) (Hizukuri, Takeda, Yasuda, and Suzuki, 1981). Starch exists as the major reserve carbohydrate of higher plants, especially in the tuber crops. Root crops constitute one of the most important groups of tropical crops and cassava is one of the most widely cultivated in several countries. Cassava is used as a food for more than 300 million people in the world (Balagopalan, Padmaja, Nanda, and Moorthy, 1998). Nearly 65% of total cassava starch production is utilised for food and the rest is in feed and non-food industry (Breuninger, Piyachomkwan, and Sriroth, 2009). Cassava starch is easily extractable and the roots which contain

very low quantity of proteins, fat, phosphate, etc. Starch obtained is light-brown to white in color, depending on the extraction process.

Maltodextrin is a generic term applied to a variety of products obtained from carbohydrate-active enzyme modified starch. According to Englyst and Englyst, 2004, slowly digested dextrin or starch (SDS) is the modified starch which released glucose in between 20 and 120 min of hydrolysis (Englyst and Englyst, 2004). Starch, linear and branched maltodextrin were used as a substrate to produce highly-branched maltodextrin that act as a slowly digestible maltodextrin (Kittisuban, Lee, Suphantharika, and Hamaker, 2014; Lee et al., 2013; Lee et al., 2007). Many sources of starch were used as a substrate to generate a slowly digestible maltodextrin product, for example, waxy rice starch (Hiroki Takata et al., 1996), and cassava starch (Le et al., 2009). According to these data, cassava starch showed the appropriate source for highly-branched maltodextrin production. Enzymatic and chemical methods are commonly used to produce highly-branched maltodextrin. For enzymatic method, major enzyme that is used to produce this product is branching enzyme (BE, E.C. 2.4.1.18). Andersson et al. (2002) reported the production of slowly digestible maltodextrin using BE cloned from potato gene (Andersson, Rydberg, Larsson, Andersson, and Åman, 2002). This work is similar to that of Le et al. (2009) who reported about the usage of BE to produce slowly digestible maltodextrin from cassava starch (Le et al., 2009). In addition, not only BE but other enzymes are used to study. These are, for example, amylomaltase (AM, E.C. 2.4.1.25) (Jiang, Miao, Ye, Jiang, and Zhang, 2014), α-transglucosidase (TGase, E.C. 2.4.1.24) (Ao et al., 2007; Miao et al., 2014; Shi et al., 2014), and maltogenic α-amylase (Le et al., 2009), etc. The combinations of enzymes were proposed, such as the combination of BE,

maltogenic amylase, and TGase to produce slowly digestible maltodextrin from rice starch. The properties of this product were lower in retrogradation but higher solubility when compared to native rice starch. The patent of Fuertes et al. (2009) and Deremaux et al. (2010) claimed the process to produce slowly digestible maltodextrin for the use in the food industry and hospital as a peritoneal and dialysis solution (Deremaux, Petitjean, and Wills, 2010; Fuertes, Roturier, and Petitjean, 2009). BE, α-amylase, and TGase were applied in this experiment. The products contained 11.2%-15.2% of α-1,6glucosidic linkages. For chemical method, Ohkuma et al. (1994, 1995, 1997) reported the optimum conditions to produce highly-branched maltodextrin or resistant maltodextrin (Ohkuma, Hanno, Inaba, Matsuda, and Katsuda, 1997; Ohkuma, Hanno, Inada, Matsuda, and Katta, 1994; Ohkuma, Matsuda, and Hanno, 1995). The resistant maltodextrin from this process consists of maltose, isomaltose, and another compound that has α-1,2, α-1,6 glucosidic linkage more than native starch similar to the work of Yamada et al. (1993), many sources of starch are used as a substrate in various hydrochloric acid concentration and temperature. The resistant maltodextrins consisted of α-1,2, α-1,3 and α-1,6 glucosidic linkages (Yamada and Matsuda, 1993). The alternative way to produce resistant maltodextrin is using acid and the end product was called ‘pyrodextrin’. However, enzymatic methods are now emerging as alternative clean technologies to provide more environment and consumer safe solutions for starch modification compared to chemical modification.

Resistant maltodextrin was classified as a sub-group of dietary fiber which is a collective term for a variety of plant substances that are resistant to digestion by human gastrointestinal enzymes (Shi et al., 2014; Shu, Jia, Ye, Li, and Wu, 2009).

Dietary fiber can be classified as either water soluble or water-insoluble fiber. Foods rich in water-soluble fiber are dried beans, oats, barley, and some fruits and vegetables. The average total dietary fiber intake for adults in most industrialized countries is well below 25 g/day; the minimal amount is recommended by various health organizations. Based on total dietary fiber intake, approximately 20% water-soluble and 80% water-insoluble were recommended (Bazzano, He, Ogden, Loria, and Whelton, 2003). Isomalto-oligosaccharides (IMOs) is one of the famous prebiotics substance proved by many scientific researchers (Bharti et al., 2015; Goulas, Fisher, Grimble, Grandison, and Rastall, 2004; Kaulpiboon, Rudeekulthamrong, Watanasatitarpa, Ito, and Pongsawasdi, 2015; Yen, Tseng, Kuo, Lee, and Chen, 2011). It structure is consist of α-D-glucopyranose units linked by α-1,6 glucosidic linkages. Traditionally, IMO are produced by sequential reactions of α-amylase, β-amylase and α-glucosidase. IMO are used as food ingredients which have many health benefits and can be stimulate the good gut microbiota (Nguyen and Haltrich, 2013).

The health benefits of slowly and resistant maltodextrin including IMO on human intestinal motility and function were discussed by several researchers (Jiang et al., 2014; Lehmann and Robin, 2007; Rösch, Venema, Gruppen, and Schols, 2015). Regard to definition, resistant maltodextrin is not metabolized in the upper gastrointestinal tract; however, it can have a substantial influence on digestive processes in the stomach and small intestine. Slowly and resistant maltodextrin delays gastric emptying as a result of their water-holding capacity. These phenomena have two major effects: prolong satiety and retard the rate of nutrient absorption in the small intestine. The two major effects may have implications for weight control and

obesity (Dikeman and Fahey, 2006; Howarth, Saltzman, and Roberts, 2001; Slavin, 2005) and possibly influencing susceptibility to type II diabetes (Chaikomin, Rayner, Jones, and Horowitz, 2006), respectively. Moreover, they exhibit many benefits for the large intestine. Several literatures have reported the interactions between host and the microflora and it is clear that the maintenance of healthy gut microflora is important to whole body and gut health (Kumar, Nagpal, Hemalatha, Yadav, and Marotta, 2016; Quigley, 2016). Like the other types of soluble dietary fiber, health benefits of resistant maltodextrin are protective against weight gain, cardiovascular disease, colon cancer, diabetes type I, II and have beneficial effects on the human gastrointestinal tract (Amara and Shibl, 2015; Daliri and Lee, 2015). The presence of their specific linkages were retard or does not permit the hydrolysis in the small intestine of mammals, and their energy content can be available for colonic microflora and produce short chain fatty acid for human host.

Nevertheless, most of previous studies of slowly and resistant maltodextrin productions were focused mainly on the increasing of branch points in maltodextrin molecules. However, high portions of oligosaccharides which easily digested were still remained. In addition, the consumer safety has become an important issue on the food supplied system in many industries. The synergistic carbohydrate-active enzymes catalysis to retard or suppress modified starch digestibility and reduce portions of oligosaccharides were the new and innovative strategies. These ideas can be solved the problem for the industry and can be used as a strategy to attract the customers to choose the slowly and resistant maltodextrin that produces from enzymatic method. Novel enzymatic processing steps to produce slowly and resistant maltodextrin including IMO were proposed in this research.

1.2 Research objectives

The objectives of this research were:

1.2.1 To generate more compact branching of the modified cassava starch product by employing combinations of AM and BE in sequence or simultaneously to produce a range of differently, highly-branched glucan structures with increased resistance towards important dietary amylases.

1.2.2 To determine the effects of amylose content using well define amylose: amylopectin substrate on BE and the combination of AM and BE modes of action and the production of α-1,6 glucosidic linkages related to the slowly and resistant maltodextrin formation.

1.2.3 To produce slowly and resistant maltodextrin including IMOs and higher complex structure by using the combination of α-amylase, BE, β-amylase and TGase to stimulate probiotics.

1.3 Research hypothesis

1.3.1 Synergistic BE and AM might be able to increase degree of α-glucans branching because of disproportionation activity of AM. The digestion rate could be suppressed.

1.3.2 In the system that consists the high level of amylose, BE may produce α-1,6 glucosidic linkages and yield more branch products than the system which consist of high amylopectin content.

1.3.3 The combination of α-amylase and BE might be able to use with extremely high substrate concentration during saccharification process and can be generate high content of α-1,6 glucosidic linkages. The combination of TGase only

and/or simultaneous β-amylase and TGase might be able to generate high content of slowly and resistant maltodextrin which prefer isomalto-oligosaccharides (IMOs) and higher complex structure.

1.4 Scope of the study

These research were study the effects of amylose content using amylose only barley starch (AO) and waxy maize starch (WX) mixture on BE and the combinatorial BE and AM catalysis for academic knowledge about these enzymes mechanisms. Furthermore, the compact branching and small size distribution of the modified cassava starch products were produced by employing BE and combinations of BE and AM to provide slowly digested maltodextrins. In the last part, the production of slowly and resistant maltodextrin including IMOs and higher complex structure were achieved by using the combination of α-amylase, BE, β-amylase and TGase to stimulate probiotics. Structural, physicochemical properties and digestibility were demonstrated for all modified starches. Prebiotic properties of slowly and resistant maltodextrin including IMOs and higher complex structure were determined using *in situ* hybridization.

1.5 Expected results

Results from this research will lead to develop a fundamental understanding in the production of slowly and resistant maltodextrin from enzymatic methods by using carbohydrate-active enzymes. Slowly and resistant maltodextrin can be used as a biologically-inspired material with applications in the food and pharmaceutical industries. Regarding fundamental knowledge, it will contribute a better

understanding of BE, AM and TGase activity, also molecular structure of this modified starch. In addition, they also showed the relationship among the molecular structure, *in vitro* digestibility and prebiotics property.

1.6 Publications

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CHAPTER II

LITERATURE REVIEW

2.1 Starch - background

2.1.1 Starch structure and composition

Starch is the major carbohydrate source for human and animals served as a food for energy that contains high of nutritional quality depending on their structure and processing. Starch is the predominant composition in green plants which serve more than 80% of the world calories (Keeling and Myers, 2010). It also applied for uses in feed, fuel and non-food industry such as paper, textile, etc. Starch molecules consist of α-D-glucose monomer linked together covalently by *O*-glucosidic linkages (Figure 2.1). The ratio of the two main components (Figure 2); amylose and amylopectin varies depending on the starch sources. Normal starch contains about 20%-30% amylose and 70%-80% amylopectin.

Amylose is a linear glucose polymer which is found with molecular weights ranging from 8.1×10^4 to 9.7×10^5 g/mol (Jane, 2004) and with the number of glucose residues per molecule or degree of polymerisation (DP) ranging from 500 to 5,000. Amylose unit chains were classified into three groups, similar to those of amylopectin which consist of A-, B- and main chains or C-chains (Hanashiro, Abe, and Hizukuri, 1996). Although it is considered to be an essentially linear, α-1,4 glucosidic linkages (Berg, Tymoczko, and Stryer, 2002), Hizukuri et al. (1981) demonstrated the present of α-1,6 glucosidic linkages in amylose molecules, on an

average of 2-8 branch points per molecule and the side-chains ranging in a chain-length from 4 to more than 100 glucose units. In addition, the α-1,4 glucosidic linkages of amylose enhance the random coils configuration which can be formed inclusion complex with other amylose-chains or substances. The amylose chain length has been reported to affect the color of the amylose-iodine complex. The color of the complexes changed from brown (DP 21-24), to red (DP 25-29), red violet (DP 30-38), blue violet (DP 39-40), and finally blue (DP > 47) and when DP was lower than 20, no color was formed (John, Schmidt, and Kneifel, 1983). Double-helix amylose can be easily formed during the retrogradation process which becomes desirable for some starchy food such as resistant starch formation (Wang, Li, Copeland, Niu, and Wang, 2015). Amylose can be hydrolysed by β-amylase yielding maltose which provide 70-90% β-amylolysis limit due to its branch points. For amylopectin, it has molecular weights in the average range from 7.0×10^7 to 5.7×10^9 g/mol (Ma et al., 2007; Zhong, Yokoyama, Wang, and Shoemaker, 2006) with a high branching degree (Nilsson, Gorton, Bergquist, and Nilsson, 1996). The structure contains approximately 5% of α-1,6 glucosidic linkages depending on botanical sources, leading to the short, α-1,4 glucosidic linkages that occur in a bimodal distribution of A-chains (DP approximately 15) and B-chains (DP approximately 45). It has been reported that the branch chains of amylopectin are organised in a semi-crystalline structure which consisting of multiple clusters. The action pattern of α-amylase involves a virtually random hydrolytic multiple attack to cleave starch into linear and branched dextrins (Bijttebier, Goesaert, and Delcour, 2008) and requires at least a four glucose-unit linear segment between two adjacent branch points for catalysis resulting in α- limit dextrins. β-amylase hydrolyses α-glucans removing

maltose unit from the non-reducing end (Bailey and Whelan, 1957) until it reaches one to two glucose units from a branch point resulting in β-limit dextrins. The α-limit and β-limit dextrin structure were used to classify a branch chains as the A, B, and C chains (Figure 2.2) (Hizukuri et al., 1981; Vamadevan and Bertoft, 2015). A-chains are the chains which unsubstituted by the others while B-chains are the chains which substituted by the others and C-chains are the main chain containing reducing end (Pérez and Bertoft, 2010). Since amylopectin has many α-glucan chains linked together by generating branch points. This make amylopectin has an ability to bond to other substances which more useful for the industries than amylose. The differentiation properties between amylose and amylopectin were showed in Table 2.1 which plays importance roles in their application in the industries.

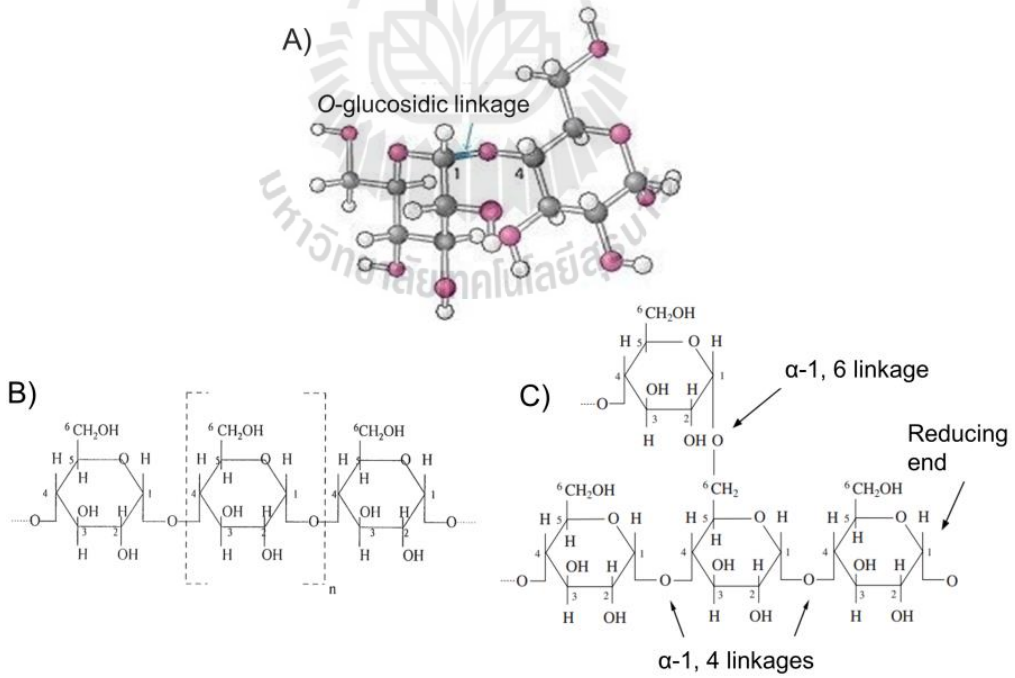

Figure 2.1 A) *O*-glucosidic linkages of maltose which is two glucose molecules linked together. Chemical structure of amylose and amylopectin. B) α-1,4

glucosidic linkages of amylose and C) α-1,4 and α-1,6 glucosidic linkages of amylopectin.

Source: Berg, Tymoczko, and Stryer, 2002.

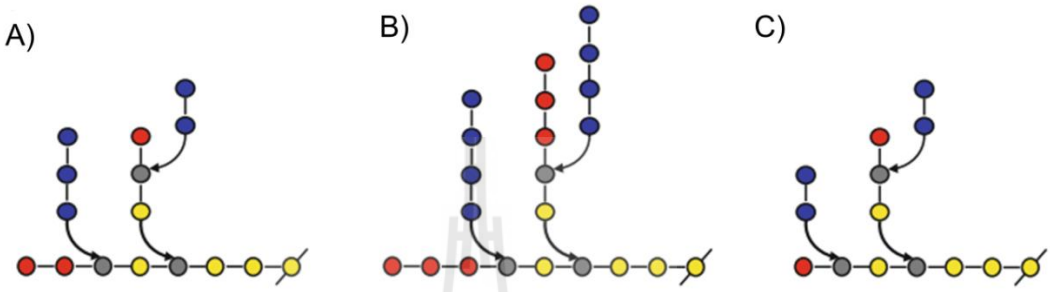

Figure 2.2 Structure of limit dextrins after β-amylase and α-amylase hydrolysis: A) β- limit dextrins, A-chains (blue circles) remain as two or three glucose residues and the external B-chains (red circles) have one or two residues; B) α- limit dextrins, in which all A-chains have four residues and all external B-chain have three residues; C) α, β - limit dextrins, in which all A-chains have two residues and all external B-chain have one residue. Gray circles are glucose residues involved in a branch linkage (arrows), and yellow circles are residues in internal segments of B-chains. Note that the chain segment that carries the reducing end (/) is regarded as an internal segment.

Source: Vamadevan and Bertoft, 2015.

Table 2.1 Properties of amylose and amylopectin.

Property	**Amylose**	**Amylopectin**
Molecular structure/branches	Mainly linear/ primarily α-1,4	Highly branched/ α-1,4; α-1,6
Molecular weight	10^5-10^6 Da	10^7-10^9 Da
Solubility	Low/barely soluble	High
Gelatinization temperature	High	Low
Gel formation	Firm, irreversible	Soft, reversible
Viscosity	Low	High
Retrogradation rate	High	Low

Source: Schirmer, Jekle, and Becker, 2015.

In addition, another starch composition is the intermediate component. It consists of both amylose and amylopectin and has large portion of long: short branch-chains (Takeda, Takeda, and Hizukuri, 1993). The minor components of starches may be considered in three categories. According to their location, there is particulate material which is the fragments of non-starch materials such as proteins, lipids and phosphorus. The second category is surface component which is the material associated with the surface of granules. The third one is internal component which is the material buried within the granule matrix and inaccessible to extraction without granule disruption (Pérez and Bertoft, 2010).

Starch granules were studied by several methods such as x-rays diffraction, electron microscopy, atomic force microscopy, etc. in order to classify the different levels of structural organization (Pérez and Bertoft, 2010). The organization of amylose and amylopectin were arranged in semi-crystalline structure from the origin of the granule called hilum. The growth ring structure consists of crystalline

and amorphous lamellae which rearranged to form A, B and C-crystalline type of starch (Figure 2.3) (Zeeman, Kossmann, and Smith, 2010). Various conformation of starch granules makes them difference in their properties, for example in wheat starch, B-types starch have a negative effect on bread quality while positive effect on pasta-making process (Li et al., 2013). Gelatinization or melting of starch is the process which destroys starch granules and release amylose and amylopectin to the solution (Figure 2.4). It occurs when starch is heated in specific starch to water ratio. Gelatinisation temperature is dependent on sources of starch and their amylose: amylopectin ratio. In the industry, hydrothermal treatment was used to gelatinise starch to make it appropriate for processing.

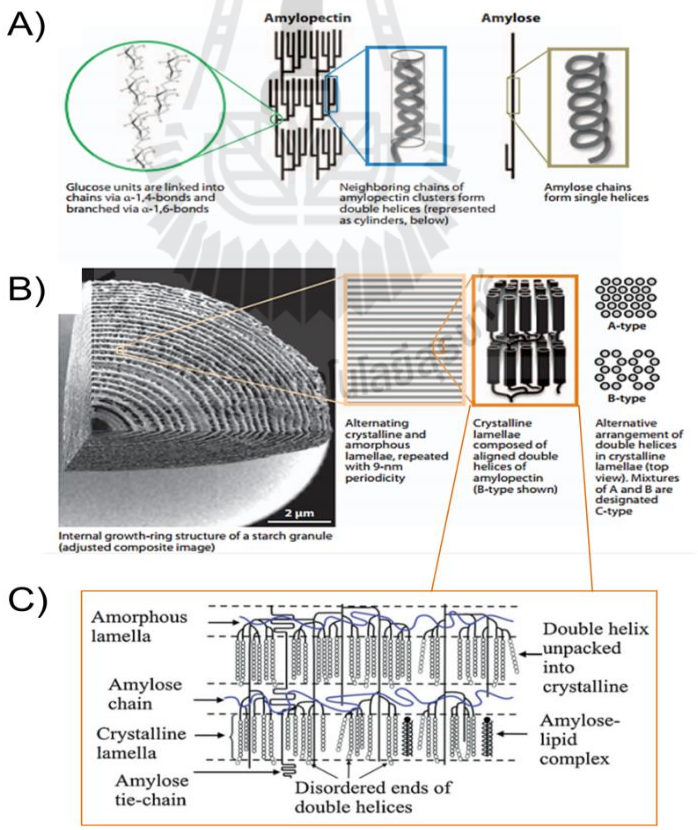

Figure 2.3 The granular compositions of starch. A) Glucose molecules are linked together by α-1,4 and α-1,6 linkages to form amylose and amylopectin.

B) Growth ring structure of a starch granule consisting of crystalline and amorphous lamellae which rearranged to form A-type, B-type and C-type of starch. C) Focusing on the semi-crystalline structure and chains positioned in starch granule. Adapted from Blazek and Gilbert, 2011; Kozlov, Blennow, Krivandin, and Yuryev, 2007; Zeeman et al., 2010.

Source: Blazek and Gilbert, 2011; Kozlov et al., 2007; Zeeman et al., 2010.

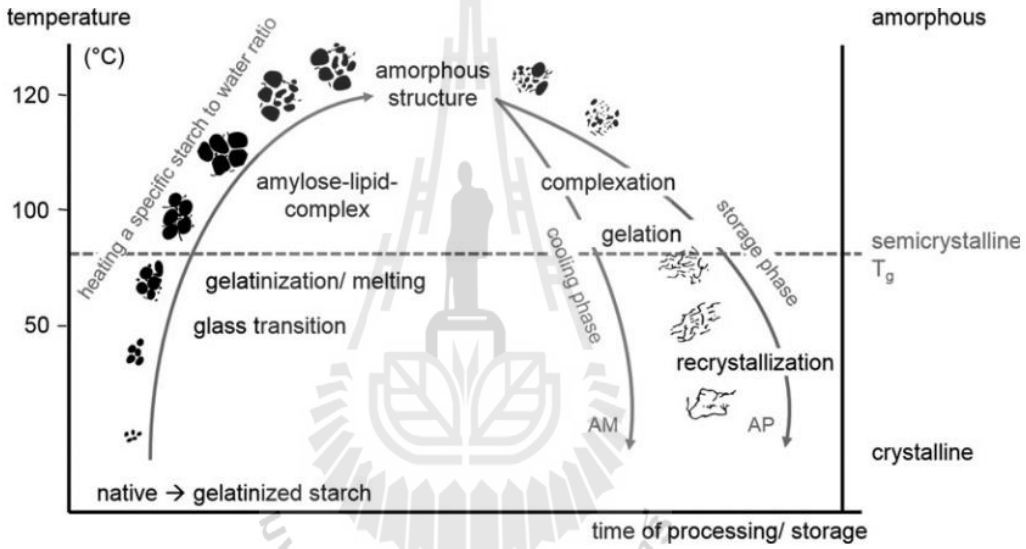

Figure 2.4 Gelatinisation of starch granules.

Source: Schirmer, Jekle, and Becker, 2015.

2.1.2 Cassava starch - cash carbohydrate source in Thailand

Cassava (*Manihotesculenta Crantz*) has become an important industrial crop in Thailand, mainly to produce for consumption, and dried chips and later pellets for the rapidly growing European animal feed market. The size of granules determined by microscopy showed to be 5-40 μm (Moorthy, 2004). Under scanning electron microscopy, granules are round in shape with a flat surface on one side.

Scanning electron microscope of raw cassava starch granules were showed in Figure 2.5 (Prompiputtanaporn, 2015). Gelatinization is the important physical behaviors of starch that are influenced by amylose and amylopectin fractions. For various cassava starch cultivars, diverse amylopectin structural elements resulted in substaintial swelling power, viscoelastic properties, and gel firmness (Charles, Chang, Ko, Sriroth, and Huang, 2005). That makes gelatinization temperature range from 58.5-70^{o}C (Balagopalan et al., 1998). The higher proportion of DP ≥ 67 entanglements with amylose chain lengths to form longer helical structures is confirmed in the high gelatinization starch, better gel shear resistance and higher gel firmness. Nowadays, cassava is one of Thailand's most important economic crops which generate dramatically income for the country because of its several applications. Cassava starch is mostly used for food as basic cooking ingredient. It is a thickener and stabilizer in many kinds of food, for example, sauces, pies, pudding, etc.

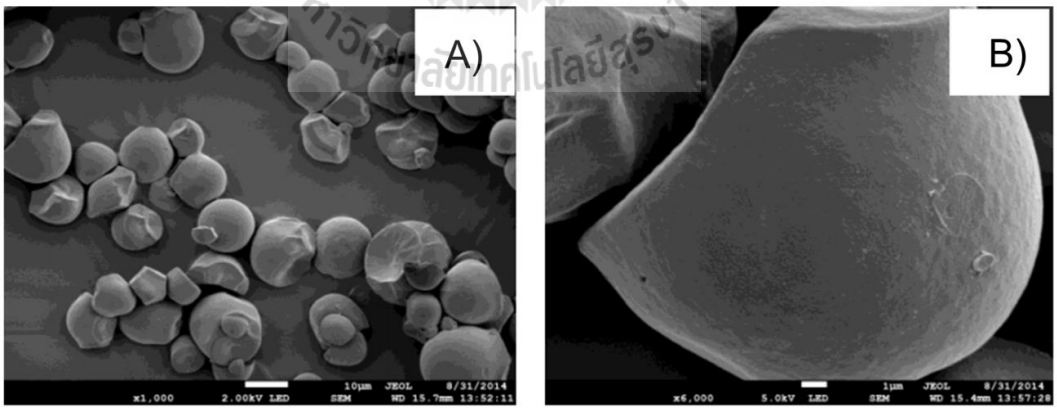

Figure 2.5 Scanning electron microscope of raw cassava starch granules. A) 1,000x, B) 6,000x.

Source: Prompiputtanaporn, 2015.

2.1.3 Amylose only barley starch (AO) - genetically modified starch with ≥ 99% amylose content

Barley starch is a cereal grain, classified in glass family which plays importance roles in agriculture economy as a oldest domesticated crop and mostly used as a plant model for studies of starch biosynthesis (Purugganan and Fuller, 2009; Shaik, 2013). The AO is the transgenic plant which silence genes for starch branching enzymes (SBE I, SBE IIa, SBE IIb) with a single RNAi hairpin (Carciofi et al., 2012). The high yield and pure amylose production from cereal plant system were established. The granular AO compared to native barley starch was no regular size with multilobed, often elongated, rough and globose-shaped granule (Figure 2.6) (Carciofi et al., 2012). There was lower capacity for thermal hydration, swelling and solubilisation which can be solublised in water at 70-80ºC only 20% (Carciofi et al., 2012). This transgenic barley had high possibility to use as a source of valuable nutrition starch or resistant starch and used as a model structure of pure amylose starch in the future study.

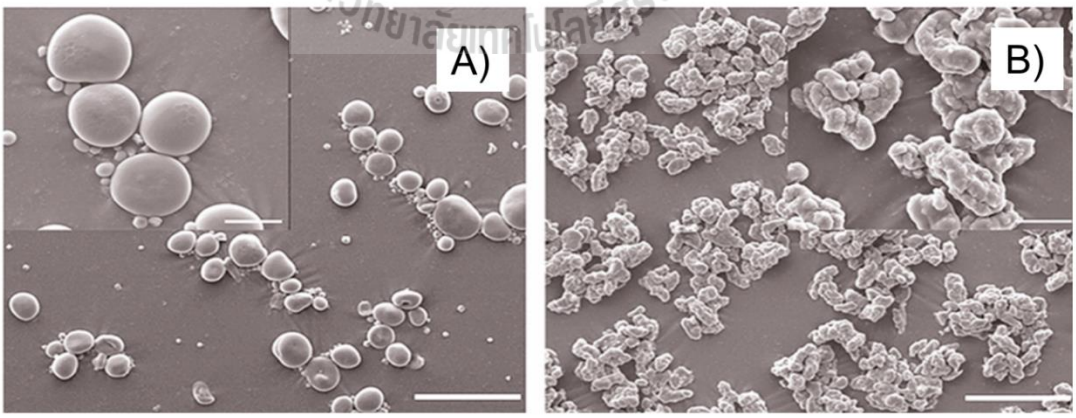

Figure 2.6 Scanning electron microscope of A) native barley starch granular compared to B) AO, with difference magnifications.

Source: Carciofi et al., 2012.

2.1.4 Waxy maize starch (WX) - natural high amylopectin source

WX is wildly utilised in many food for both human and animals. It has amylose content ranging from 1.4-2.7% depending on botanical sources, climatic condition and soil type during growth (Sandhu and Singh, 2007). Branch amylopectin structure of WX gives steric hindrance resulted in it slowly digested rate by amylolytic enzymes.

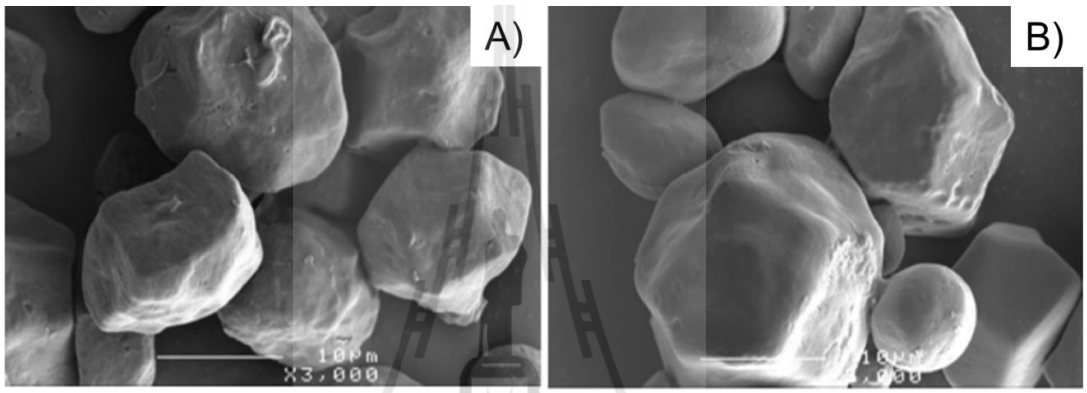

Figure 2.7 Scanning electron microscope of A) waxy maize starch granular compared to B) normal maize starch.

Source: Dhital et al., 2013.

2.2 Clean technology - enzyme modified starch

Enzymatic methods are now emerging as alternative clean technologies to provide more environment and consumer safe solutions for starch modification. In contrast to physical and chemical methods, which often produce unpredicted by-products, enzyme-assisted catalysis can be more specifically controlled and it operates under very mild conditions, thus reducing the risk for producing harmful or unwanted by-products (Butler, van der Maarel and Steeneken, 2004).

2.2.1 Glucanotransferase

Glucanotransferase catalyse the α-1,4 glucan chain transfer to a new position in an acceptor carbohydrate. From CaZy database, glucanotransferase is classified in the group EC 2.4. The starch modification is normally converted to soluble starch by saccharification process by hydrolysis or hydrolysis and further transglycosylation (Sako, Matsumoto, and Tanaka, 1999). These types of non-digestible oligosaccharides after enzymatic modification have been used as functional ingredients in many kinds food products (Do et al., 2012) such as beverages, desserts, breads and filling, cake and biscuits, chocolate, confectionary, sauces, baby food, etc. (Wang, 2009). In this chapter, three different types of glucanotransferase that are commercially available are reviewed in order to understand their mechanisms and the resulting products.

2.2.1.1 **Branching enzyme** (EC 2.4.1.18, BE, GH13, Q-enzyme, 1,4-α-D-glucan: 1,4-α-D-glucan, 6-α-D-(1,4-α-D-glucano)-transferase)

BE is found in microorganisms, plants and animals. There are 2 types of BE: starch BE (SBE) and glycogen BE (GBE). The SBE is found in plants while GBE found in animals and microorganisms. In plants and algae, BE is involved in the biosynthesis of starch where it introduces α-1,6 branch points to produce compact starch granules (Blennow et al., 2013; Funane et al., 1999). In animals and most microorganisms, BE catalyse the formation of α-1,6 branch points of glycogen molecules (Kim, Ryu, Bae, Huong, and Lee, 2008). This enzyme has been studied by many researchers and it belongs to the α-amylase family (Svensson, 1994). It is first identified in potatoes that convert amylose to amylopectin by the production of α-1,6

branch points (Figure 1A) (Robyt, 1995). The SBE synthesis 3.5% α-1,6 glucosidic linkages in amylopectin while 8-9% in glycogen and the average chain-length of the branches is usually 20-23 glucose residues for amylopectin and 10-12 glucose residues for glycogen (Marshall, 1974). For SBE, There are 2 different types of SBE reported by various research groups (Akasaka et al., 2009; Andersson et al., 2002). One of them preferentially transfers longer chains such as maize BEI and the other transfers shorter chains such as maize BEII (Funane et al., 1999). The branch is created by the breaking an α-1,4 glucosidic linkages from the non-reducing end then transfer it to produce an α-1,6 glucosidic linkage. A block of seven residues is transferred to a more interior site for rice SBE (Nakamura et al., 2010). The new α-1,6 glucosidic linkage must be at least four residues away from a another one (Stryer, 1995). Roussel et al. (2013) reported the ability of BE from *Rhodothermus obamensis* to to transfer α-1,4 linkages of a glucan to create new α-1,4 linkages yielding the elongation of chains (Roussel et al., 2013). In addition, BE can catalyse a cyclization reaction of amylose and amylopectin to form cycloamylose and cycloamylopectin clusters (Takata, Takaha, Okada, Takagi, and Imanaka, 1996). For a dilute amylopectin solution, BE from *Bacillus stearothermophilus TRBE14* transformed the amylopectin into a large cyclic glucan with a limited molecular size. When the substrate is concentrated, cyclisation mechanism may be competitively inhibited by α-1,6 glucosidic linkages formation (Takaha, Yanase, Takata, Okada, and Smith, 1998). The smallest cycloglucan (DP 8) was observed from BE treated cassava starch (Sorndech et al., 2015). At the extreme starch concentration with high temperature treatment, BE can be let to stabilization of starch structure and significantly increase degree of branching and these observation can be applied for

the food industry in order to produce food supplement (Jensen, Larsen, Bandsholm, and Blennow, 2013).

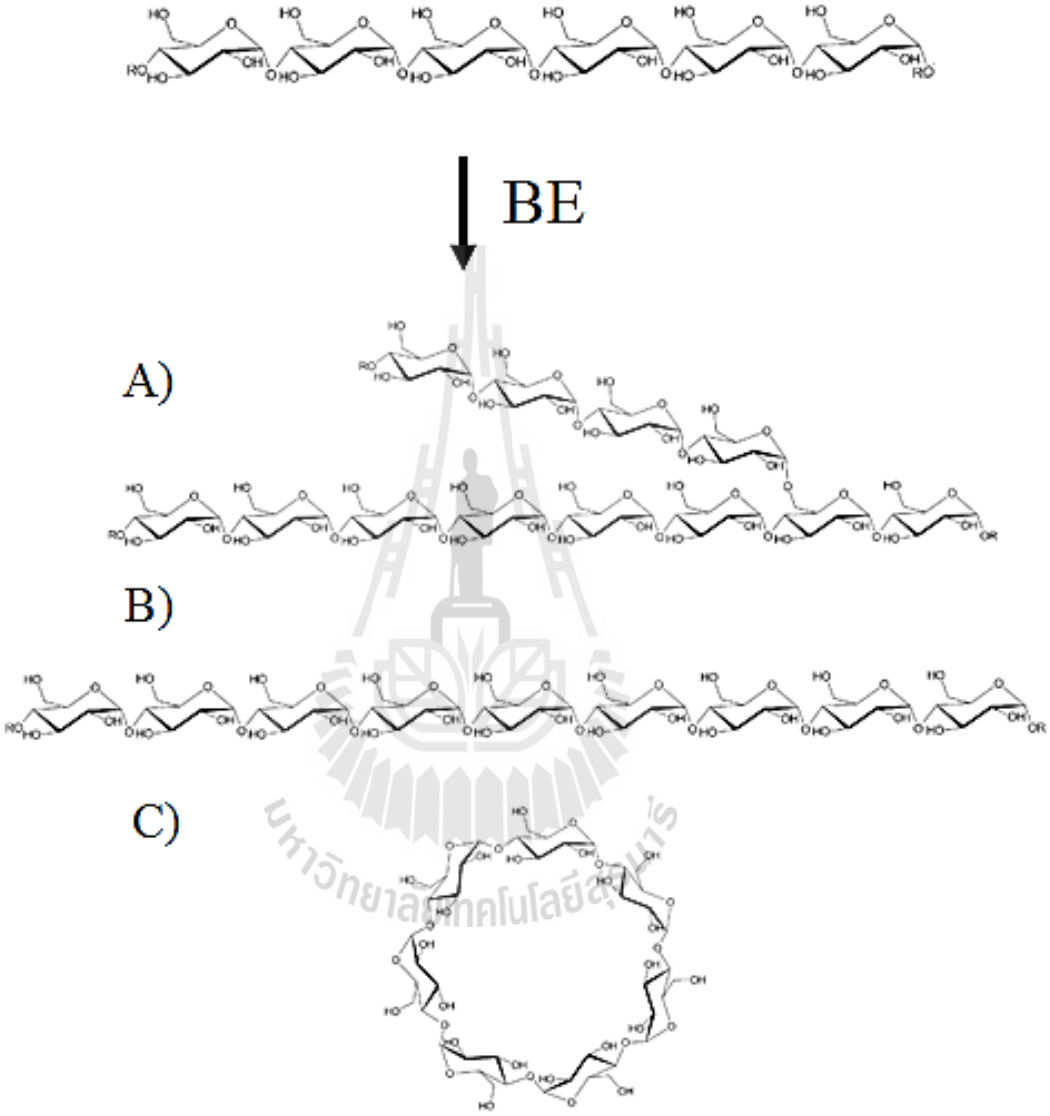

Figure 2.8 BE modes of action on starch and resulting products. A) New α-1,6 glucosidic linkage formation, B) New α-1,4 linkage formation and C) Cycloglucan formation.

Source: Roussel et al., 2013; Sorndech et al., 2015; van der Maarel and Leemhuis, 2013.

2.2.1.2 Amylomaltase (E.C. 2.4.1.25, AM, GH77, D-enzymes, 1,4-α-D-glucan: 1,4-α-D-glucan 4-α-D-glycosyltransferase,)

AM is an intracellular enzymes that involve in starch metabolisms in the bacterial cell (Saehu, Srisimarat, Prousoontorn and Pongsawasdi, 2013). It acts on α-glucan substrates to catalyse transfer of a segment of a α-1,4 glucan to the new position of α-1,4glucosidic linkages (Figure 1B). This molecular glucan transfer reaction is reversible, and is often called disproportionation. They can use high molecular weight amylose and amylopectin in starch as both donor and acceptor (Palmer, Ryman, and Whelan, 1968; Takaha, Yanase, Takata, Okada, and Smith, 1996). The reaction begins with the cleavage of an α-1,4 glucosidic linkage in the substrate glucan. Following the removal of glucan fragment from the donor, AM further attach the glucan segment to the acceptor chains resulting in the formation of the new α-1,4 glucosidic linkage (Ota, Okamoto, and Wakabayashi, 2009). The modification of amylose and amylopectin cause a decrease in amylose chains length, (Hansen, Blennow, Pedersen, Norgaard, and Engelsen, 2008; Park et al., 2007) and change in the chain length configuration of amylose and amylopectin structure (Seo et al., 2007). Also, this enzyme can catalyse intramolecular transfer reactions which creates cycloamylose (Bhuiyan, Kitaoka, and Hayashi, 2003a) with a degree of polymerization (DP) ranging from seventeen to several hundred. In addition, AM can catalyse the formation of cyclo-amylose and amylopectin (Hansen, Blennow, Pedersen, Nørgaard, and Engelsen, 2008; Palmer et al., 1968) also coupling reaction which is the reversible reaction of cyclisation (Jung et al., 2011). Moreover, AM also catalyse the hydrolysis reaction.

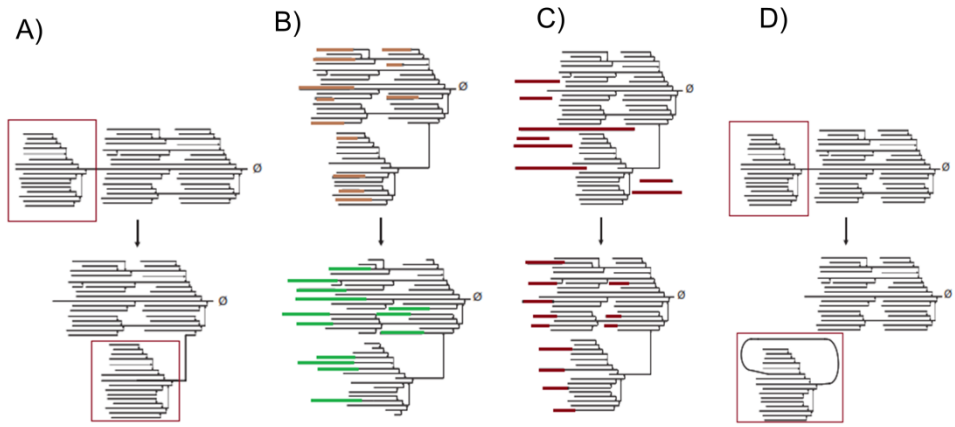

Figure 2.9 AM modes of action on starch and resulting products. A) disproportionation of amylopectin cluster, B) disproportionation of *α*-glucan chains within amylopectin, C) disproportionation of *α*-glucan segments from amylose to amylopectin and D) cyclization of amylopectin.

Source: Hansen et al., 2008.

2.2.1.3 α-transglucosidase (EC 2.4.1.24, TGase, GH13, GH70, 1,4-α-D-glucan: 1,4-α-D-glucan (D-glucose) 6-α-D-glucosyl-transferase)

TGase is widely distributed in microorganisms, plants and animals (Ota et al., 2009). It can catalyse hydrolytic and transfer reactions of non-reducing glucosyl residues of maltose to generate α-1,6 glucosidic linkages of glucose and maltose to form isomaltose and panose, respectively (Figure 2.10A). However, TGase can also transfer of non-reducing glucosyl residues to the α-1,2 glucosidic linkages and α-1,3 glucosidic linkages of glucose to form kojibiose and nigerose or back to α-1,4glucosidic linkages to form maltose. The final products after TGase reaction are the isomalto-oligasaccharides (IMOs) and hydrolysis products such as

glucose (Figure 2.10B) (Mangas-Sánchez and Adlercreutz, 2015) and those have been developed in many food industries. In the traditional methods, TGase is combined with the enzymes from the amylase family to produce IMOs (Lee et al., 2002). Ota et al. (2009) postulated a simple reaction pathway of TGase from *Aspergillus niger* by using [U-^{13}C] maltose and maltoheptaose as a substrate. The result suggested that TGase rapidly transferred glucosyl residues to maltooligosaccharides, and gradually hydrolyzed both α-1,4 and α-1,6 glucosidic linkages at the non-reducing end and transformed them into smaller molecules of mainly α-1,6 glucosidic linkages (Ota et al., 2009) .

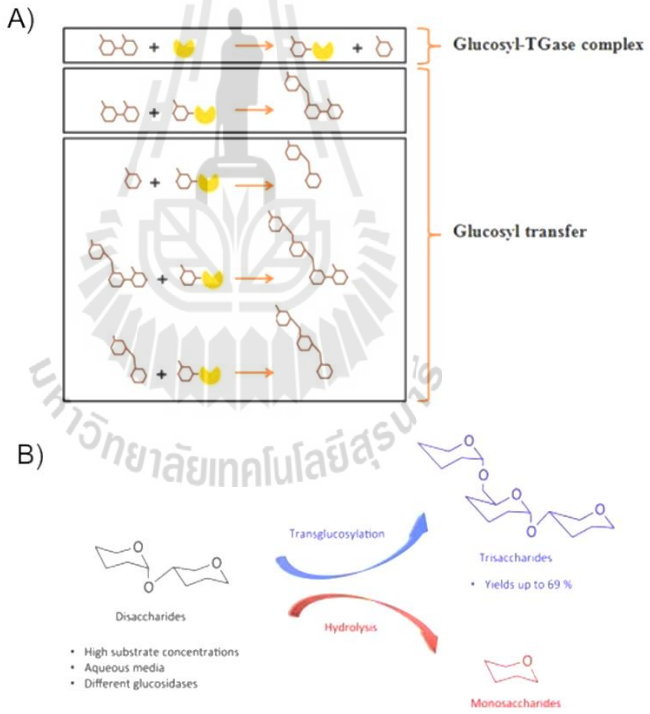

Figure 2.10 TGase modes of action on oligosaccharide substrate and resulting products. A) Yellow symbol is TGase and hexa-cyclic ring is glucose unit. B) TGase mode of actions.

Source: Ota et al., 2009; Mangas-Sánchez and Adlercreutz, 2015.

2.3 Long history: Modified starch products obtained from BE, AM and/or TGase reaction

Enzymatic modification of starch usually involves hydrolysis and transglycosylation by carbohydrate active enzymes. Numerous observations have shown that several carbohydrate active enzymes can modifiy starch structure and change the physicochemical properties of starch. BE, maltogenic α-amylase, TGase, AM, α-amylase, and β-amylase can be combined to obtain novel glucan structures. The principle of using a combination of different carbohydrate active enzymes is discussed in this section. Physicochemical properties after modification and mechanism of carbohydrate enzymes are explained (Ao et al., 2007; Le et al., 2009; Lee et al., 2008; Song, Min, Hwang, and Lee, 2008; Zhang, Ao, and Hamaker, 2006).

The molecular weight distribution of products after modification by carbohydrate enzyme was discussed by Ao et al. (2007). The molecular weight distribution of sample after modified with β-amylase, or maltogenic α-amylase, or both combined with TGase were reported. Normal maize starch and samples after treatment with enzymes were determined by size exclusion column chromatography connected to multi-angle laser light scattering and refractive index detectors analysis (HPSEC-MALLS-RI). These products had lower proportion of larger molecules compared to the control. Starches treated with a combination of β-amylase and TGase or maltogenic α-amylase and TGase showed lower molecular weight of the products when compared to β-amylase treated or maltogenic α-amylase treated only. This implies that TGase mainly catalyse the hydrolysis of starch with β-amylase or maltogenic α-amylase and create new branch linkages with small molecular size. In addition, the combinations of BE and maltogenic α-amylase or AM with maltogenic

α-amylase were studied by Lee et al. (2008). The average molecular weight of the sample determined after treating with enzyme was decreased, indicating that amylose and amylopectin are hydrolyzed into a smaller unit when compared to the control. The reaction occurs by the cleavage of the inter-chain segments of amylose by BE and amylopectin by AM to produce smaller molecules. After being treated with maltogenic α-amylase, the molecular weight became smaller than treatment with only BE or AM. These results indicated that maltogenic α-amylase hydrolyze product in the first step to generate smaller molecules compared to the control. As compared to data from Ao et al. (2007), it can be concluded that the main mechanism of maltogenic α-amylase is the hydrolysis of α-1,4 glucosidic linkages and this enzyme also may exhibit transglycosylation activity via formation of new branch linkages (Le et al., 2009).

Branch chain length distributions of products after enzyme modification were reported by Le et al. (2009) who modified cassava starch using BE isolated from *Bacillus subtilis* strain 168 and maltogenic α-amylase from *Bacillus stearothermophilus*. The final products were analyzed by high pressure anion exchange chromatography (HPAEC) for determination of branch chain length distribution. The number of short side chains with DP 3-5 greatly increased whereas that of DP more than 6 significantly decreased. These results are similar to that obtained by Kim et al. (2008) who used BE to modified rice starch. Branch chains longer than around DP 30 completely disappeared. It was also observed that the new side chains still remained after β-amylase treatment indicating that the new peaks correspond to extra-branched maltooligosaccharides. Furthermore, Ao et al. (2007) also studied the branch chain length distribution of starch sequentially treated with

β-amylase, TGase or maltogenic α-amylase. Mostly short linear chains were produced when using this combination of enzymes and the relative proportion of amylopectin branch chains was reduced. There is a major difference in the substrate efficiency between amylose and amylopectin for BE reported by Le et al. (2009). Rice amylose incubated with BE, produced side-chains from DP6 to DP30, demonstrating that amylose is efficiently branched by BE. Following incubation with maltogenic α-amylase, the number of short chains from DP2 to DP5 increased, whereas chains longer than DP9 decreased. These results demonstrate that the formation of branched side-chain catalysed by BE and maltogenic α-amylase occurs as an effect of transfer of small oligosaccharide by maltogenic α-amylase from long side chains to the other chain via the formation of α-1,6 glucosidic linkages thereby producing highly branched amylose.

Park et al. (2007) reported the modification of amylopectin by AM. The AM treated amylopectin from rice starch show smaller molecular size as compared to the control. This enzyme catalyses the transfer reaction, mainly amylopectin into smaller size. Moreover, the products also comprised an increase in the proportions of apparent amylose and smaller molecules. The transferred products from amylopectin and amylose increased during enzyme treatment has been known to contain modified amylose consisting of linear long-chain material, small cycloamylopectin clusters, including the association of the modified amylopectin clusters reorganized by hydrolysis and disproportionation. (Lee, Kim, Park, and Lee, 2006; Park et al., 2007; Takaha et al., 1998). In addition, it was reported that the AM from *Thermus thermophilus* has the capability to convert starch into a thermoreversible gel by disproportionation and was applied to produce a thermoreversible gel under the trade

name Etenia^{TM} (Euverink and Binnema, 2005). The product was long-chain amylopectin consisting of side chains with approximately DP 35- and longer side chains. This product was also free from amylose. Moreover, the branch-chain length distribution of rice starch treated with AM showed that the proportion of short side-chains (DP 1-8) as well as longer chains (> DP 19) were increased resulting in a flattened chain profile clearly demonstrating a disproportionation reaction catalysed by AM. Interestingly, amylose seems to be a better donor substrate than amylopectin since the amylose content was reduced and a long-chain amylopectin with a broader chain length distribution was produced (Choi et al., 2009).

An additional interesting product resulting from intramolecular AM-catalysed glucan chain transfer was cycloamylose (Przylas et al., 2000). Cycloamyloses are large cyclic compounds consisting of 16 or more α-1,4 linked anhydroglucose units. If the donor molecule in the reverse direction is a cyclic glucan, the reaction termed a coupling reaction (Fujii et al., 2007). To elucidate the product structure from cyclization and coupling reaction, the structure of the glucoamylase-resistant glucan was analyzed by treatment with various sources of amylase. The amount of glucoamylase-resistant glucans increases with catalysis time of AM (Bhuiyan, Kitaoka, and hayashi, 2003b). The cyclic glucoamylase-resistant glucans formed were confirmed by time of flight-mass spectrometry (TOF-MS) demonstrating molecular masses of cyclic products ranging from DP 16 to 50. Hence, the *Aquifex aeolicus* AM produced similar products as AM from *Thermus aquaticus* and *E. coli* but they differs from that of the potato AM which was unable to transfer glucose units for generating cyclic structures. The smallest cycloamylose produced by the *Aquifex aeolicus* AM using amylose as substrate was a DP of 16 which was similar to that of the smallest

cycloamylose produces by the potato D-enzyme with a DP of 17. In contrast, the AM from *Thermus aquaticus* produced cycloamylose with average DP22 (Bhuiyan et al., 2003b). To produce soluble glycogen-like dietary fibre from starch, a combination of BE and AM was used in order to produce glycogen-like molecules (Kajiura, Kakutani, Akiyama, Takata, and Kuriki, 2008; Kajiura et al., 2011; Kajiura, Takata, Kuriki, and Kitamura, 2010; Tafazoli et al., 2010; Hiroki Takata, Kajiura, Furuyashiki, Kakutani, and Kuriki, 2009). By using isoamylase-BE-AM, starch is used as a starting material. First, the branched chains of starch were hydrolyzed using isoamylase to produce a mixture of short chain amyloses. Then, the linear chains were further modified into a highly branched glycogen-like product by the action of BE in the presence of AM. The addition of AM improved the yield of synthetic glycogen several fold. Since a small oligosaccharides were poor substrates for BE, The elongation reaction of the maltooligosaccharide by AM increased the efficiency of BE, resulting in higher yield of the synthetic glycogen product (H. Kajiura et al., 2008). The structural parameters of the enzymatic synthesis glycogen were similar to the natural glycogen. The degradation of this glucan was decreased demonstrating a slow carbohydrate asset. Isomaltooligosaccharides (IMOs) of many different structures can applied in various types of food. This starch modified product has been developed to use in food to prevent dental caries, as substitute sugars for diabetics or to improve the gastrointestinal microflora. These types of oligosaccharides are discussed in term of effect of type of enzyme on IMOs production. The combinatorial use of β-amylase, TGase or maltogenic α-amylase on IMOs production yields glucan products with high isomaltose, isomaltotriose, and panose (IMOs) contents (Ao et al., 2007). TGase catalyses hydrolytic cleavage as well as transfer reactions to convert

maltooligosaccharides to IMOs. The IMO contents can be adjusted depending on the enzyme dosage and incubation time used (Ota et al., 2009). The enzymatic method was demonstrated to be improved for IMOs production (Lee et al., 2002) and HPAEC analysis of generated IMOs demonstrated that cooperative reaction of maltogenic α-amylase and AM certainly had a synergistic effect on the IMOs production. The combined maltogenic α-amylase and AM catalysis using starch as substrate stimulated the formation of IMOs in a cooperative mode.

2.4 Starch digestion

Starch provides the major energy source and glycemic response for human. The starch nutritional quality was measured by using the concept of glycemic index (GI). GI value was obtained from the area under curve of the two hours postprandial blood glucose level after ingestion compared with control carbohydrate food such as glucose or white bread (Lee, Bello-Pérez, Lin, Kim, and Hamaker, 2013). Normally, starch digestion was consists of two phase, there are the intraluminal phase and brush border phase (Lee et al., 2013). The human digestive tract was shown in Figure 2.11 (Widmaier, Raff, and Strang, 2001).

Starch normally enters the body through the mouth. The digestive function is to break down the starch mechanically by chewing and lubricate the starch to make swallowing easier. The salivary α-amylase hydrolyse starch to starch fragments which occurred only 5% here. The bolus of food was passed through the esophagus entering the stomach which has no enzyme for starch digestion. Stomach emptying rate is regulated by some of the hormonal (ghrelin and glucagon-like peptide-I (GLP-I)) and neural mechanisms in order to prevent the damage of the stomach wall or dumped of

acid from the stomach to the small intestine (Hellström, Grybäck, and Jacobsson, 2006; Hellström and Näslund, 2001).

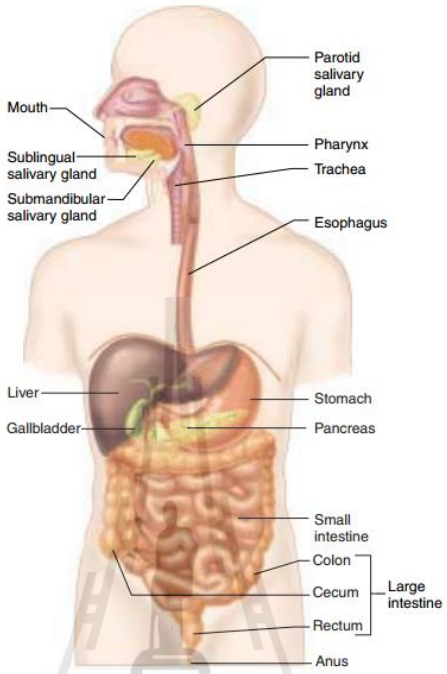

Figure 2.11 Anatomy of the gastrointestinal system.

Source: Widmaier et al., 2001.

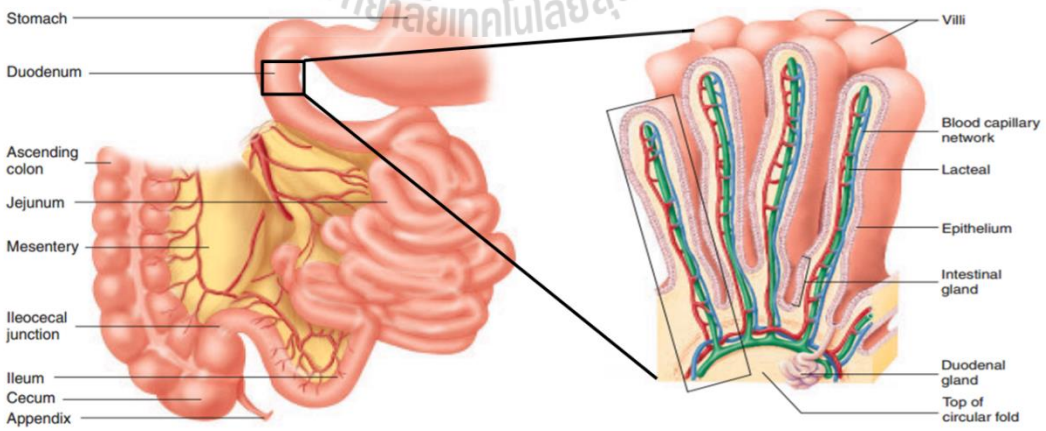

Figure 2.12 Structure of small intestine and the anatomy of villi.

Source: Seeley, Trent, and Stephens, 2004.

The small intestine consists of three parts which are duodenum, jejunum, and ileum. The greatest amount of digestion and absorption occur in the small intestine. Villi were constructed to increase surface area of digestion and absorption. The villi are small and finger-like projections about a millimeter in length that protrude from the circular folds (Figure 2.12) (Seeley, Trent, and Stephens, 2004). The liver and the pancreas are the major accessory glands of the duodenum. The pancreatic duct secretes large quantities of α-amylase in the duodenum which converse starch fragments in to maltose, maltotriose and α-limit dextrins. The brush-border area of the small intestine normally has two exohydrolytic enzyme complexes which are maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) (Lee, Yan, et al., 2013). All of these enzyme complexes have high ability for glucose generation. Maltase usually hydrolyses maltose to glucose and glucoamylase hydrolyse especially, oligosaccharides containing DP 2-9 to generate glucose. Sucrose were hydrolysed by sucrase to release glucose and fructose while isomaltase has high specificity on the α-1,6 glucosidic linkages which can be completely digested to glucose unit (Lin et al., 2012). The excess glucose is converted to glycogen and usually stored in the liver. In the situation that the energy is needed, the glycogen is converted into glucose and used by the cells in cellular respiration. After the indigested starch fragments enter the colon, the colonic microbial ferment them yielding the short chain fatty acid (SCFA), minerals, vitamins and gases which can be absorbed back and transported to the blood stream. The length of large intestine is approximately 1.5-1.8 m. It consists of the cecum, colon, and ends at the rectum (Figure 2.12). The presence of food in the stomach and duodenum stimulate mass movement in the colon and eliminated by the anus.

2.5 Slowly digestible and resistant starch/ maltodextrin

Personal health and well-being life has long been attention. Many modified starches were commercialised in the market in response to consumer demand. Slowly digestible starch/ maltodextrin is a starch portion which completely digested to glucose during 20-120 min by simultaneous α-amylase and glucoamylase *in vitro* measured while resistant starch/ maltodextrin cannot completely digested to glucose at 120 min according to Englyst method (Englyst, Kingman, and Cummings, 1992; Lehmann and Robin, 2007). The starch nutritional fractions (Table 2.2) were classified as rapidly digestible starch (RDS) which completely digested within 20 min in mouth and small intestine because its structure mainly composes of amorphous region. Resistant starch is starch fractions which not completely digested within 120 min (Raigond, Ezekiel and Raigond, 2015). RS provides many health benefits and it actions in the colon to stimulate the colon microbial ecosystem.

Table 2.2 Classification of starch based on nutrition fractions.

Item	**Starch nutrition fractions**		
	RDS	**SDS**	**RS (types I-V)**
Digestion timeline (*in vitro*)/place	Within 20 min/mouth and small intestine	20–120 min/small intestine	>120 min/not in small intestine, main action in colon
Main physiological property	Rapid source of energy	Slow and sustained source of energy and sustained blood glucose	Effects on gut health (e.g. prebiotic, fermentation to butyrate with hypothesised anti-carcinogenic effects)
Structure	Mainly amorphous	Amorphous/crystalline	Dependent on type, mainly crystalline

Source: Raigond, Ezekiel, and Raigond, 2015.

For slowly digestible starch/ maltodextrin, several procedures were used to modified native starches to be slowly digestible starch/ maltodextrin such as annealing and heat-moisture treatment (HMT) which maintained its granular structure and birefringence (Shin, Kim, Ha, Lee, and Moon, 2005). An increase in the relative contents of α-1,6 glucosidic linkages by applying BE, TGase or other carbohydrate hydrolase and transferase enzymes to starch was another approach to generate slowly digestible starch/ maltodextrin (Ao et al., 2007; Kim et al., 2008; Kittisuban et al., 2014; Le et al., 2009; Sorndech et al., 2015). For the *in vivo* definition, there is no existing *in vivo* method that reflects the content of slowly digestible starch/ maltodextrin (Genyi Zhang and Hamaker, 2009). The concept of an extended glycemic index (EGI) was appropriate to explain the slowly digestible starch/ maltodextrin behavior for *in vivo* analysis.

The major importance of slowly digestible starch/ maltodextrin is that it can be extent of digestion and absorption in the small intestine which slow down the postprandial blood glucose level and the glycemic response (Lehmann and Robin, 2007; Genyi Zhang and Hamaker, 2009). The glycemic response is affected by human behavior and food factors. A high content of rapidly available glucose in a meal is correlated to a high rate of blood glucose level. However, a slow glucose release leads to a moderate improve of blood glucose level over an extended time period which showed in Figure 2.13B, C, and D). When slowly digestible or resistant starch / maltodextrin reach the ileum, it can stimulate the secretion of glucagon-like peptide 1 (GLP1) and peptide YY (PYY) hormone. In addition, the SCFA products from the good colon microbiota also stimulating the secretion of those two hormones

resulting in reducing gastric emptying rate and decreasing food intake which showed in Figure 2.14 (Lee et al., 2013).

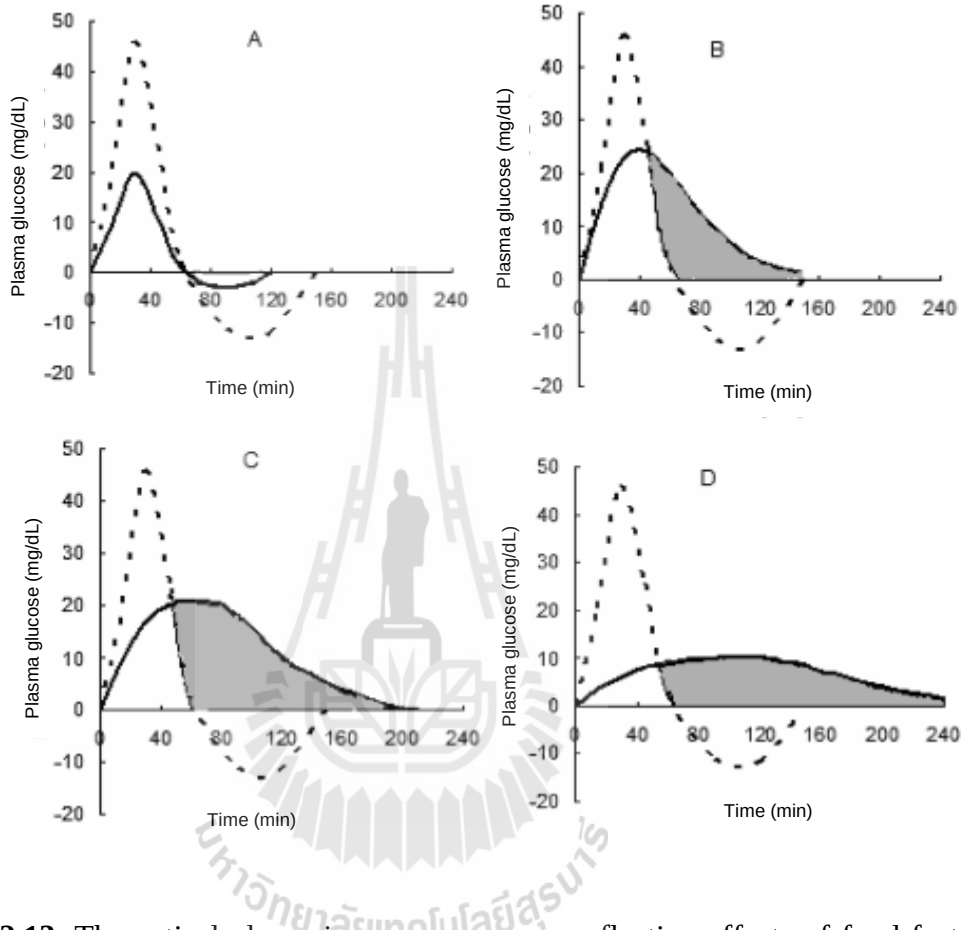

Figure 2.13 Theoretical glycemic response curves reflecting effects of food factors on the glycemic response. Dotted line: glucose control (high GI food), solid lines represent different food samples: low GI food (A); another low GI food (B); high GI food with extended glycemic response (C); long time extension of glycemic response of ideal food (D). The area of shaded region is termed extended glycemic index (EGI) (B, C, D).

Source: Genyi, Zhang and Hamaker, 2009.

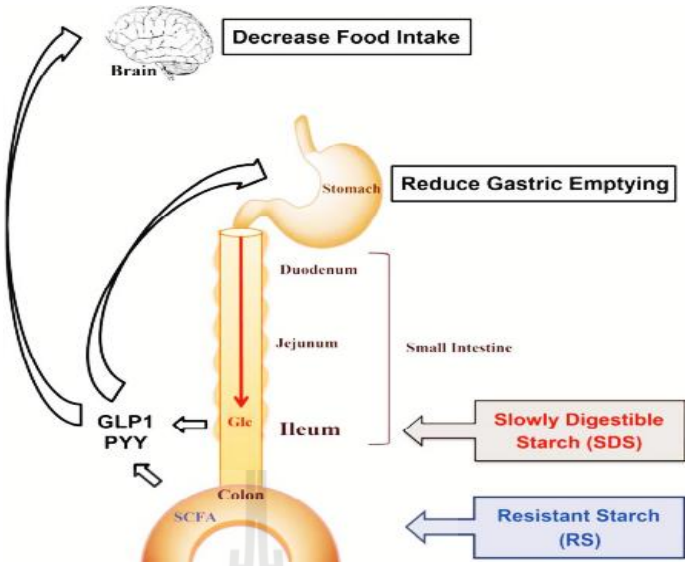

Figure 2.14 Concept of ileal and colonic brake mechanism related to slowly digestible or resistant starches caused by ileal glucose release and SCFA from fermentation. GLP1 = glucagon-like peptide 1; and PYY = peptide YY.

Source: Lee et al., 2013.

Rivellese Giacco and Costabile, (2012) reported the mechanisms of low-GI and fiber foods which refer to slowly digestible or resistant starch/ maltodextrin action in gastrointestinal tract which is composed of multiple processes (Figure 2.15) (Rivellese, Giacco, and Costabile, 2012). They can act on digestion and absorption of nutrients with a decrease in gastric emptying in stomach, slower CHO digestion in the small intestine resulting in lowering fat storage, stimulate the fermentation of non-digested fraction yielding SCFA and improve blood regulation. Additionally, they also increase hypothalamic signals of satiety and then decreasing food intake. Health benefits of slowly digestible or resistant starch/ maltodextrin were reported both *in vitro* and *in vivo* studies. Slowly digestible starch/ maltodextrin can stimulate

secretion of gut incretin hormones (Wachters-Hagedoorn et al., 2006) and recommended for the prevention and management of diabetes (Wolever, 2003), have an impact on satiety-influencing factors (Benton and Nabb, 2003), decrease a risk of diabetes and cardiovascular diseases (Stenvers et al., 2014), decrease a risk of colon and breast cancer (Jenkins et al., 2002), improve metabolic profile, particularly in lower postprandial insulinemia (Axelsen, Arvidsson, Lonnroth, and Smith, 1999), lower levels of circulating triacylglycerols (Harbis et al., 2004) and be also used as source of energy for the athlete (Fuertes et al., 2009). In a food industrial applications aspect, the challenge is to deliver health promoting food products containing slowly digestible or resistant starch/ maltodextrin to overcome organoleptic problems (Lehmann and Robin, 2007).

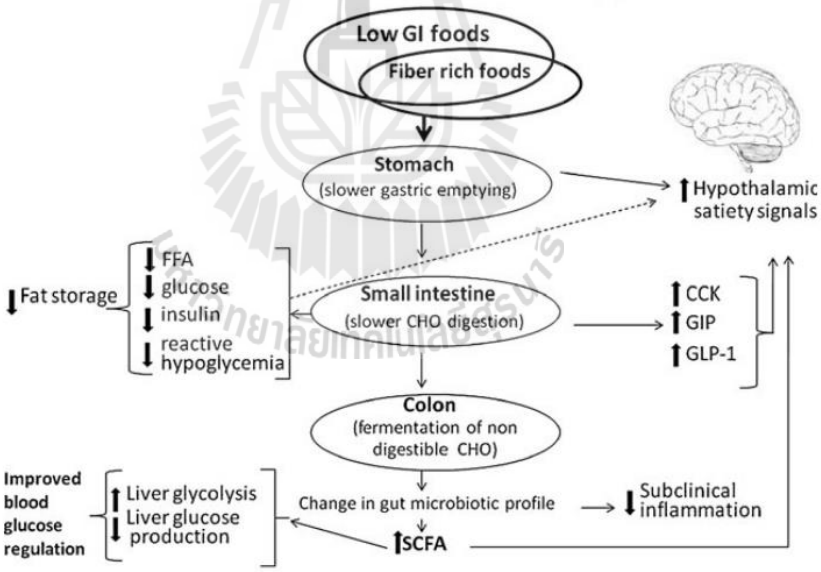

Figure 2.15 Propose mechanism of action of slowly digestible or resistant starches containing foods. CCK: cholecystokinin, CHO: carbohydrate, FFA: free fatty acids, GIP: gastric inhibitory polypeptide, GLP-1: glucagon like peptide 1, SCFA: short chain fatty acids.

Source: Rivellese, Giacco, and Costabile, 2012.

2.6 Along the gastrointestinal tract - diversity of bacteria species is living

The current knowledge on detailed composition of the gut microbiota has been discussed in order to understanding their mechanism which affected the human host. Benefits of the normal flora were the development of innate and adaptive immunity, increased intestinal epithelial integrity, used as an energy source, stimulate vitamin biosynthesis, bile salt transformation, catabolise of dietary glycans, and act as barrier to colonization by microbial pathogens (Clemente, Ursell, Parfrey, and Knight, 2012; Dave, Higgins, and Rioux, 2012). The gut microbiota is dominated by four phyla: Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes (Morgan, Segata, and Huttenhower, 2013). Factors involved in microbiota establishment from newborn to adult were showed in Figure 2.16. At birth, the microbiota diversity and richness were affected by the major factors such as mother gestational age, sanitary conditions or exposure to the antibiotics (Villanueva-Millán, Pérez-Matute, and Oteo, 2015). At the late stages of life, the microbiota was less diversities. Firmicutes and Bacteroidetes were dominated depending on age, diet, stress, culture and living place among other factors.

There were the dominant microbial phyla in oral cavity, distal esophagus, stomach, small intestine and large intestine which showed in Figure 2.17. The members of the phyla Proteobacteria, Bacteroidetes, Actinobacteria, and mostly of Firmicutes were usually found in the oral cavity was similar to that of the distal esophagus. There were some acidified bacteria found in the stomach such as Actinobacteria which was significantly different compared with oral cavity and distal esophagus. *Helicobactor pylori* was reported as a major microbe resident in the stomach (Bik et al., 2006; Dave et al., 2012).

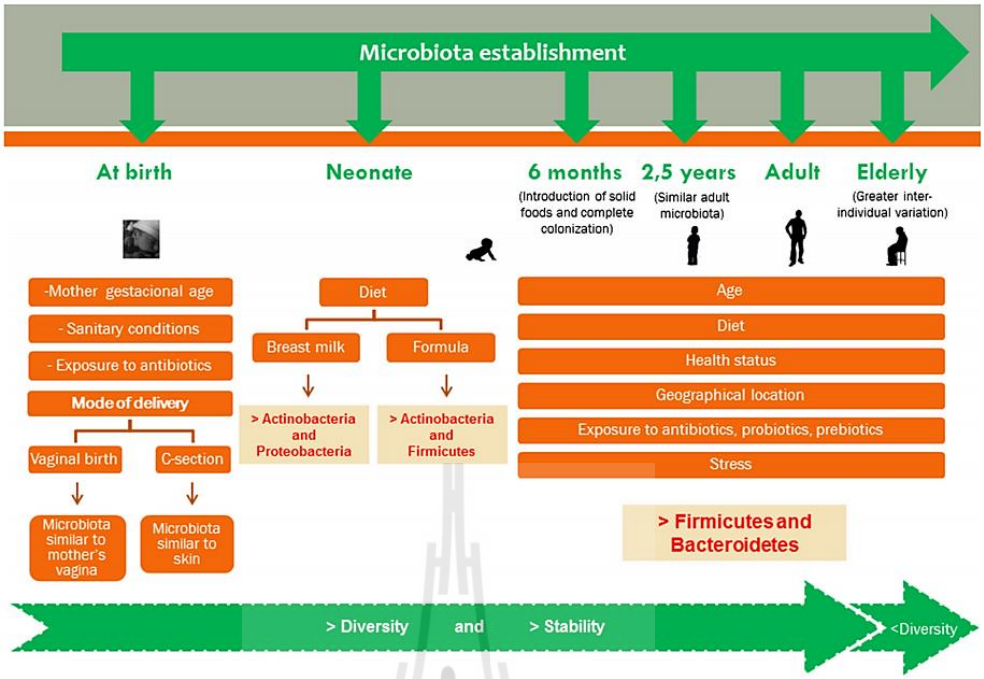

Figure 2.16 Factors involved in microbiota establishment from newborn to adult.

Source: Villanueva-Millán et al., 2015.

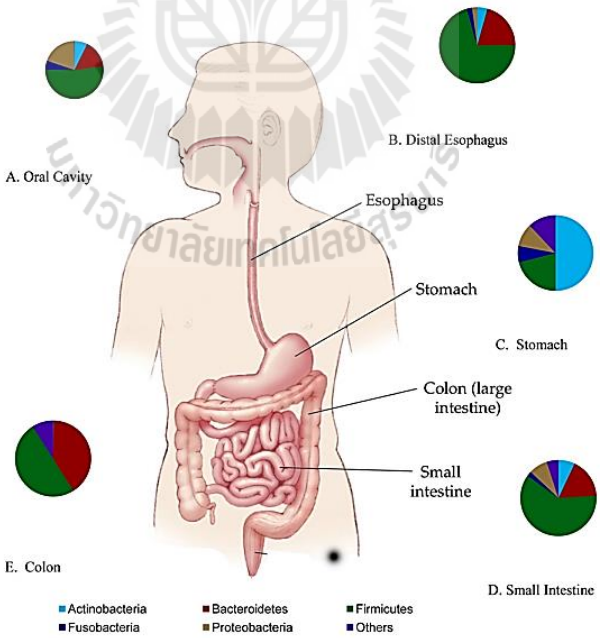

Figure 2.17 The dominant microbial phyla in oral cavity, distal esophagus, stomach, small intestine, and large intestine in the human GI tract.

Source: Dave et al., 2012.

The relatively low-density (10^2-10^7 cells/g) of microbiota in the small intestine were reported elsewhere (El Aidy, van den Bogert, and Kleerebezem, 2015). The microbial diversity in jejunum and ileum were less complex than that of the colon (Hayashi, Takahashi, Nishi, Sakamoto, and Benno, 2005). The phyla Firmicutes and Bacteroides are the dominant phyla in the colon and the phyla were revealed which were the members of Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria, Spirochaetes, and VadinBE97 (Eckburg et al., 2005). All of the phyla play important roles in human health and disease such as strongly affect immune, metabolic and endocrine functions (El Aidy et al., 2015).

2.7 Prebiotics and probiotics: Keys of the healthy life

Resistant starch/dextrin was classified as a prebiotic. Prebiotic term was first lunched as a ‘‘*non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health*’’ (Gibson and Roberfroid, 1995). The criteria was defined for classification of prebiotic substances which consist of *a) resists host digestion and absorption processes*; *b) is fermented by the microflora colonising the gastrointestinal system*; *c) selectively stimulates the growth and/or the activity of one or a limited number of bacteria within the gastrointestinal system* (Gibson, 2004). They are partially or non-digestible which were nonabsorbable in the small intestine. Many food components or substances were classified as prebiotic such as inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomalto-oligosaccharides (IMO), xylo-oligosaccharides (XOS), soybean oligosaccharides, resistant starch/dextrin, etc (Fuentes-Zaragoza et al., 2011; Kumar

et al., 2016). The advantages of prebiotics which using as food ingredients are stable in long shelf life for food and beverage, heat and pH stable which can be applied for various food type. However, intake overdose can cause intestinal bloating, pain, flatulence, or diarrhea and not potent as an antibiotics. The dietary reference intake values for total fiber by life stage were showed in Table 2.3 (Slavin, 2003). Prebiotic has a long history of extraction, production and safe consumption by humans. It was not absorbed in the intestine and then utilized to stimulate the probiotics activity to produce SCFA. Health benefits of prebiotics such as reduce the infectious and antibiotic-associated diarrhea, inflammatory bowel disease, prevent colon cancer, enhance the bioavailability and uptake of minerals, lower some risk factors for cardiovascular disease and related to satiety and weight loss which prevent obesity (Slavin, 2005).

Table 2.3 Dietary reference intake values for total fiber by life stage.

Life stage group	**Adequate intake (g/day)**	
	Male	**Female**
1-3 years	19	19
4-8 years	25	25
9-13 years	31	26
14-18 years	38	26
19-30 years	38	25
31-50 years	38	25
51-70 years	30	21
Over 70 years	30	21
Pregnancy		28
Lactation		29

Source: Salvin, 2003.

Probiotic from the definition of UNFAO/WHO (2001) are "*live microorganisms that when administered in adequate amounts confer a health benefit on the host*". Probiotics must be nonpathogenic, nontoxic, survive in the stomach and small intestine, bile resistance and free of pathogenic, toxic and significantly adverse side effects. For probiotics product, it must be retained stability during the intended shelf life of the product and contain a number of vegetative cells to confer the health benefits. The microorganisms applied in probiotic products for human use were showed in Table 2. Focusing on Lactobacillus and Bifidobacterium, Lactobacillus is lactic acid bacteria which most species were found to apply in many food and agricultural products such as dairy products, pickle vegetable and silage, etc. Lactobacilli are rod-shaped bacteria with non-spore forming. They are strictly fermentative, anaerobic and acidophilic and also have complex nutritional requirements. Lactobacilli are a member of the intestinal tract of human and animal mucosal membranes, and also found on plants, sewage and fermented milk products fermenting or spoiling food. Likewise, Bifidobacterium is also usually applied in many products especially the dairy-based products. Safety of Bifidobacterium was reported. However, they are not classified as a lactic acid bacterium due to its remote phylogenetic position (Huys et al., 2013). Bifidobacterium is a dominant microbiota which represent up to 25% (10^{10}-10^{11} cfu/gram) of the cultivable fecal bacteria in adults (Picard et al., 2005) and this number usually decreases with age.

Table 2.4 Microbial species used as human probiotics.

Genus	Species	Genus	Species
Lactobacillus	*acidophilus*	*Bifidobacterium*	*adolescentis*
	brevis		*animalis*
	delbrueckii		*bifidum*
	fermentum		*breve*
	gasseri		*infantis*
	johnsonii		*longum*
	paracasei	*Streptococcus*	*thermophilus*
	plantarum		*salivarius*
	reuteri	*Bacillus*	*coagulans*
	rhamnosus		*clausii*
	salivarius	*Saccharomyces*	*cerevisiae*
Enterococcus	*faecium*	*Escherichia*	*coli*

Source: Huys et al., 2013.

The probiotic activity to the host was classified in to three levels. It can interfere with growth or survival of pathogenic microorganisms in the gut lumen in the first level then improve the mucosal barrier function and mucosal immune system and the gut and have an effect on the immune system as well as other cell and organ systems at the third level which showed in Figure 2.18 (Rijkers et al., 2010).

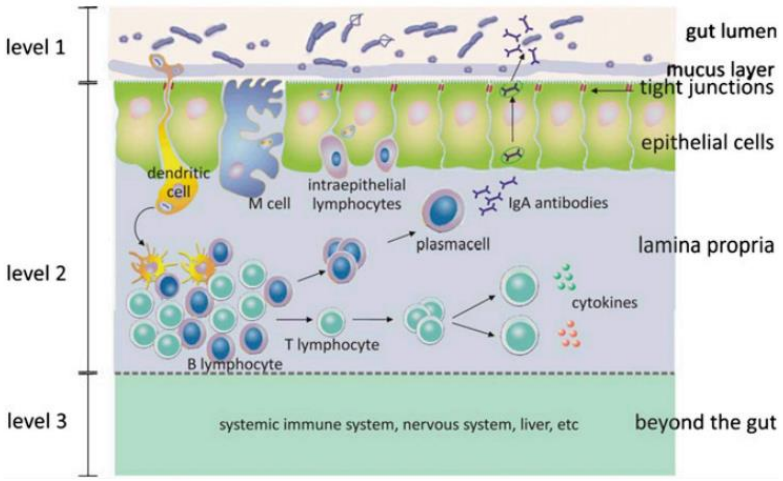

Figure 2.18 The probiotic activity to the host (Rijkers et al., 2010).

The structure of resistance starch/maltodextrin was considered in determining the fermentation ability, transit time and types of SCFA production (Lee et al., 2013). In the colon, the major end products of probiotics fermentation of resistance starch/maltodextrin or prebiotics were SCFA, gases, heat and bacterial cell mass (Topping and Clifton, 2001). The SCFA were identified as acetate, propionate and butyrate that generated from the difference partway which start from pyruvate (Figure 2.19). After pyruvate is reduced to lactate or ethanol, acetate was produced via the Wood-Ljungdahl pathway by hydrolysis of acetyl-CoA or from CO_2 (den Besten et al., 2013). Propionate is formed via the electron transfer chain using phosphoenolpyruvate which converted to oxaloacetate, fumalate, succinate and end up with propionate or by the reduction of lactate which called acrylate pathway. Formation of butyrate begins from condensation of acetyl-CoA molecules followed by reduction to butyryl-CoA. It has been demonstrated that SCFA play an important role in the prevention and treatment of metabolic diseases, intestine disorders, and cancer (Kasubuchi, Hasegawa, Hiramatsu, Ichimura, and Kimura, 2015).

Basic of biological effects of SCFA were reported elsewhere (Hijova and Chemelarova, 2007; Rösch et al., 2015; Vandenplas, Huys, and Daube, 2015). The production of SCFA and other organic acids such as lactic acid can be lowered the pH and increasing the bacteriostatic effect to prevention the pathogens. In addition, the production of bacteriocins and reactive oxygen species, such as H_2O_2 can inhibit the bacteriostatic or bactericidal activity and increase oxidative stress for pathogens (Vandenplas et al., 2015). Additionally, butyrate is the major energy source for colonocytes. Butyrate is a key which used to determine the metabolic activity and growth of colonocytes and function as a protective factor against colonic disorders

(Slavin, 2005). Propionate is taken up by the liver and acetate enters the peripheral circulation which further metabolized by peripheral tissues (Hijova and Chemelarova, 2007). Probiotics can produce vitamins especially, pyridoxine, vitamin K, vitamin B12, biotin, folate, nicotinic acid and thiamine (Fabian, Majchrzak, Dieminger, Meyer, and Elmadfa, 2008) which can be absorbed into the colon. In addition, probiotic can enhance ion absorption by intestinal epithelial cells (Borthakur et al., 2008) and decrease serum cholesterol levels (Begley, Hill, and Gahan, 2006). Probiotics increase IgA secretion resulting in the prevention of gastrointestinal and lower respiratory tract infections (Kabeerdoss et al., 2011) which was benefit to the immune system.

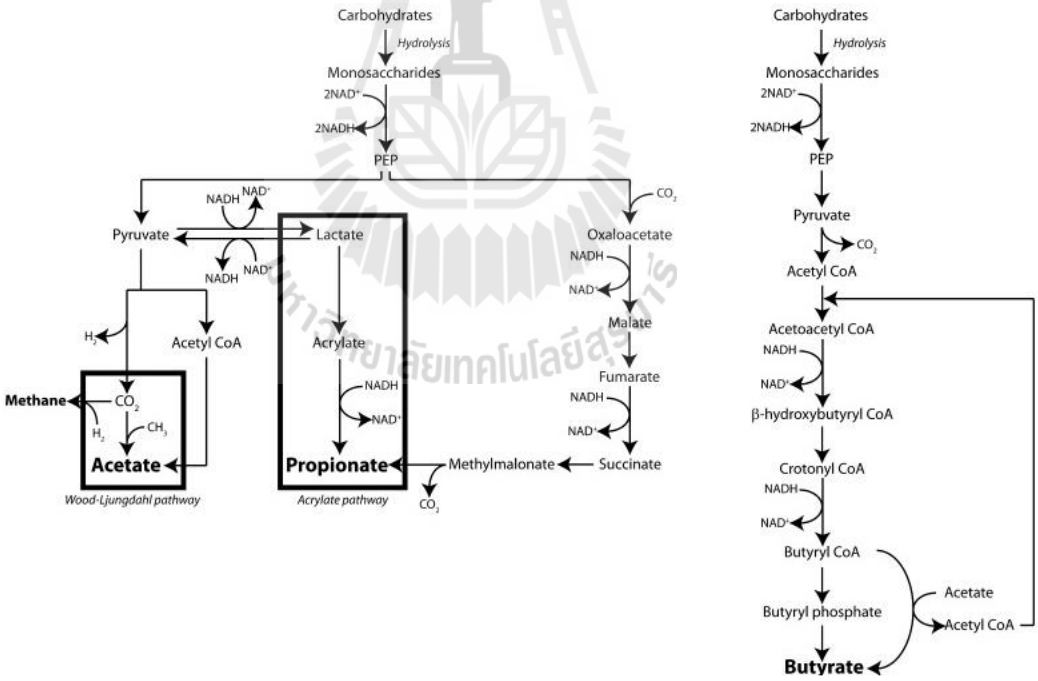

Figure 2.19 Schematic diagram of the pathways that gut microbes use to produce SCFA and other metabolites.

Source: den Besten et al., 2013.

2.8 References

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CHAPTER III

SYNERGISTIC AMYLOMALTASE AND BRANCHING ENZYME CATALYSIS TO SUPPRESS CASSAVA STARCH DIGESTIBILITY

3.1 Abstract

Starch provides our main dietary caloric intake and over-consumption of starch-containing foods results in escalating life-style disease including diabetes. By increasing the content of α-1,6 branch points in starch, digestibility by human amylolytic enzymes is expected to be retarded. Aiming at generating a soluble and slowly digestible starch by increasing the content and changing the relative positioning of the branch points in the starch molecules, we treated cassava starch with amylomaltase (AM) and branching enzyme (BE). We performed a detailed molecular analysis of the products including amylopectin chain length distribution, content of α-1,6 glucosidic linkages, absolute molecular weight distribution and digestibility. Step-by-step enzyme catalysis was the most efficient treatment, and it generated branch structures even more extreme than those of glycogen. All AM- and BE-treated samples showed increased resistance to degradation by porcine pancreatic α-amylase and glucoamylase as compared to cassava starch. The amylolytic products showed chain lengths and branching patterns similar to the products obtained from glycogen. Our data demonstrate that combinatorial enzyme catalysis provides a

strategy to generate potential novel soluble α-glucan ingredients with low dietary digestibility assets.

Keywords: amylomaltase, branching enzyme, clean modification, branched glucan, slow carbohydrate

3.2 Introduction

Starch is the major reserve carbohydrate of higher plants, especially in tubers, roots and grains. Cassava is one of the most widely distributed crops on earth and it is cultivated crops in numerous tropical countries. Cassava starch is easily extractable and very pure products are obtained due to the very low protein and lipid content of cassava. The nutritional quality of starch strongly depends on its structure and to improve functionality chemical or physical processing. Starch normally consists of two discrete molecular fractions. Amylose makes up 25-30% of the starch. This polysaccharide is typically 100-10,000 glucosyl units large, it has an α-1,4 backbone structure, and it is only slightly branched by α-1,6 linkages. Amylopectin makes up 70-75% of the starch, being more than 100-fold larger than amylose, and contains approximately 5% clustered α-1,6 linkages (Damager, Engelsen, Blennow, Møller, and Motawia, 2010).

Nutritionally, starch can be classified into three nutritional types; rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst and Hudson, 1996). The joint FAO/WHO (1998) committee recommended consumption of a diet containing at least 55% of total energy from carbohydrates to maintain health and prevent disease. RDS is the most prominent fraction in the human

diet, however, increased postprandial blood glucose and high risk of diet-related disease ensue. For starch digestion in humans, α-amylase first hydrolyzes starch to produce highly branched glucans, so called α-limit dextrins, followed by complete hydrolysis to glucose by the mucosal α-glucosidases in the small intestine. It is known that α-1,6 linkages in starch are hydrolyzed at a lower rate than are α-1,4 linkages (Lee et al., 2013). Generally, the initial reaction rate of this hydrolysis decreases with increasing degree of polysaccharide branching. This effect is mainly due to steric hindrance in the active site of the hydrolase exerted by α-1,6 bonds (Park and Rollings, 1994). Therefore, SDS has drawn interest because SDS is considered to offer an advantage by inducing only a slow increase of the postprandial blood glucose levels thereby sustaining the blood glucose levels over time (Lehmann and Robin, 2007).

Enzymatic methods are now emerging as alternative clean technologies to provide more environment and consumer safe solutions for starch modification. In contrast to physical and chemical methods, which often produce unpredicted by-products, enzyme-assisted catalysis can be more specifically controlled and it operates under very mild conditions, thus reducing the risk for producing harmful or unwanted by-products (Butler, Van der Maarel, and Steeneken, 2004).

Glucanotransferases belong to specific enzyme families and they catalyse transfer segments of α-glucans in distinct ways. As compared to hydrolases, the use of glucanotransferases has the advantage of retaining higher product yield of high-molecular products. Amylomaltase (AM; 4-α-D-α-glucanotransferase; E.C. 2.4.1.25. belonging to either glucosyl hydrolase family 77, GH77, www.CaZy.org) is an intracellular enzyme that cleaves α-1,4 glucosidic linkages followed by a

condensation of a new α-1,4 linkages within the same α-glucan molecule (intra-molecular) or between the different molecules (intermolecular) (Boos and Shuman, 1998). Due to its transfer of identical linkages, this catalytic reaction is also termed disproportionation. Furthermore, AM can catalyze intra-molecular α-glucan transfer reactions to create cyclic molecules (cyclization) and also has minor hydrolytic activity. As an effect, coupling reactions can occur leading to "reverse cyclization", in which cycloamylose is opened by the enzyme and transferred to an acceptor as a linearized fragment (Fujii et al., 2007). However, the unique action modes of different AMs depend on the species of microorganisms.

Branching enzyme (BE, 1,4-α-D-α-glucan:1,4-α-D-α-glucan6-α-D-(1,4-α-D-α-glucano)-transferase, EC 2.4.1.18, glucosyl hydrolase family 13 or 70, GH13, GH70, www.CaZy.org) acts on α-1,4 glucosidic linkages to produce a branched α-glucan by intra- or inter-molecular α-1,6 glucosidic transfer (Okada, Kitahana, Yoshikawa, Sugimoto and Sugimoto, 1984). Moreover, it has been demonstrated that BE also catalyzes the cyclization of amylose and amylopectin (Takata et al., 1996). Due to the high branching and relatively low molecular size, the products are highly soluble in water, as compared to normal starch, giving a highly stable clear solution and reduced retrogradation (Takata et al., 1996). Very recently, BE was demonstrated to possess a minor α-4-α-D-α-glucanotransferase activity in analogy to AM, yielding elongated linear chains (Roussel et al., 2013).

In this study, we seek to generate more compact branching and homogeneous size distribution of the product by employing combinations of AM and BE in sequence (AM→BE, BE→AM→BE) or simultaneously (AM&BE) expecting to produce a range of differently, highly branched glucan structures with increased

resistance towards important dietary amylases. Our data demonstrate that combinatorial glucanotransferase catalysis provides a clean strategy to generate potential novel soluble α-glucan ingredients with low dietary digestibility assets. The strategy is expected to be applicable also for other combinatorial transferase systems.

3.3 Materials and methods

3.3.1 Materials

Cassava starch was obtained from SanguanWongse Industries Co., Ltd. (NakornRatchasima, Thailand). Preparations of BE (Viksø-Nielsen, Blennow, Nielsen, and Møller, 1998) from *Rhodothermus obamensis* and AM from *Thermus aquaticus* were kindly provided from Novozymes (Bagsvaerd, Denmark). Isoamylase (EC 3.2.1.3, specific activity 210 $U \cdot mL^{-1}$) from *Pseudomonas* sp. was obtained from Megazyme (Wicklow, Ireland). Porcine pancreatic α-amylase (EC 3.2.1.1, specific activity 22 U/mg), glucoamylase from *Aspergillus niger* (EC 3.2.1.3, specific activity 129 U/mg), PGO (peroxidase and glucose oxidase) enzyme kit for glucose determination, potato soluble starch and glycogen type VII from mussel (*Mytilus edulis*) were purchased from Sigma-Aldrich (Missouri, USA). Enzyme activity units of isoamylase, α-amylase and glucoamylase are given according to the suppliers.

3.3.2 Determination of AM and BE activity and optimum substrate concentration

The iodine staining for amylose-iodine assay was performed for BE activity with slight modifications (Takata et al., 1994). The assay was carried out in 50 mM phosphate buffer (pH 6.5) with amylose (1 $mg \cdot mL^{-1}$). BE (100 µL) was added and the reaction mixture was incubated at 60°C for 20 min. Fifty µL of the reaction

mixture were added by 1 mL of iodine reagent containing 0.01% I_2, 0.1% KI, and 0.38% 1 N HCl in water. After 15 min at room temperature for color stabilization, the absorbance at 620 nm was measured.

The activity of branch linkages formation of BE was performed according to Krisman et al. (1985) with slight modification. The branch linkages formation activity of BE was assayed by determination of reducing end content after debranching by isoamylase. Cassava starch (1% $w \cdot v^{-1}$) was used as substrate in 50 mM sodium phosphate buffer (pH 6.5). BE (100 µL) was added and the reaction mixture incubated at 60^{o}C for 20 min. The reaction was terminated by heating in boiling water bath for 30 min and adjusted pH to 4.0 using 50 mM sodium acetate buffer. Isoamylase (0.24 U·g starch^{-1}) was added and incubated at 40^{o}C for 48 h. The reducing sugar content were analysed (Nelson, 1944). One unit of BE was defined as the amount of the enzyme that produces 1 µmole of reducing sugar per min (Krisman, Tolmasky, and Raffo, 1985).

Disproportionation of AM activity was performed according to Jung et al. (2011) and Kaper et al. (2007) with modification. The disproportionation activity of AM was assayed by determination of glucose content. Ten mM maltotriose was used as substrate in 50 mM sodium acetate buffer (pH 6.0) and carried out at 70^{o}C for 10 min. The reaction was stopped by adding 50 µL of 1 M HCl and neutralized with 50 µL of 1M NaOH. The reaction mixture was reacted with 2,500 µL of PGO enzyme solution at 37^{o}C for 30 min to determine the concentration of glucose. The developed color was measured at 440 nm by spectrophotometer (Biochrom Libra S22, Cambridge, UK). One unit of AM was defined as the amount

of the enzyme that produces 1 µmole of glucose per min (Jung et al., 2011; Kaper et al., 2007).

3.3.3 Determination of optimum cassava starch concentration on the content of branch linkage formation

Cassava starch concentration was varied from 5 to 30% ($w \cdot v^{-1}$) and suspended in MilliQ water then adjusted to pH 6.5 with 50 mM sodium phosphate buffer. The suspension was heated to 75°C in a water bath for 15 min and then autoclaved at 121°C for 15 min. BE (4,000 $U \cdot g\ starch^{-1}$) was added and the reaction mixture was incubated at 60°C for 24 h. The reaction was terminated by heating in boiling water bath for 30 min, trace insoluble material removed by centrifugation (1,500 x g for 20 min) and the α-glucan product was recovered and dried at 50°C overnight. The reducing sugar content (Nelson, 1944) and total sugar content (Dubois, Gilles, Hamilton, Rebers, and Smith, 1956) were analysed after debranched by 0.24 U of isoamylase per 5 mg of sample at 40°C. The percentage of branching linkages was calculated using a following equation (Wood and Mercier, 1978):

$$\% \text{ Branch points} = \left(\frac{(\text{Reducing sugar after debranched} - \text{Reducing sugar before debranched})}{\text{Total sugar after debranched}}\right) \text{x } 100$$

3.3.4 Enzymatic modification

3.3.4.1 Cassava starch treated with AM followed by BE (AM→BE)

The AM-treated starch was produced mainly according to van der Maarel (van der Maarel et al., 2005) procedure with modification. Cassava starch was suspended in MilliQ water (10% ($w \cdot v^{-1}$)) adjusted to pH 6.0 with 50 mM phosphate buffer. The suspension was heated to 75°C in a water bath for 15 min and

then autoclaved at 121°C for 15 min. AM (10 U·g starch^{-1}) was added to the gelatinized cassava starch paste and incubated at 70°C for 3 h or 24 h and then terminated by boiling at 100°C for 30 min. The pH was adjusted to 6.5 using 50 mM phosphate buffer and BE (4,000 U·g starch^{-1}) was added. The reaction mixture was incubated at 60°C for 24 h. The reaction was terminated by heating in boiling water bath for 30 min, trace insoluble was removed by centrifugation (1,500 x g for 20 min) and the α-glucan product was recovered and dried at 50°C overnight.

3.3.4.2 Cassava starch treated with BE, AM, and BE in sequence (BE→AM→BE)

A gelatinized cassava starch paste was prepared as mentioned above, pH adjusted to 6.5 with 50 mM phosphate buffer, BE (4,000 U·g starch^{-1}) was added and the mixture incubated at 60°C for 24h. After termination of the reaction at 100°C for 30 min, AM (10 U·g starch^{-1}) was added and incubated at pH 6.0, 70°C for 3 h or 24 h. The reaction was terminated by boiling at 100°C for 30 min. In the last step, BE was added and incubation was performed under optimal condition for each enzyme as described above. The obtained product was then handled for storage as described above.

3.3.4.3 Cassava starch simultaneously treated with AM and BE (AM&BE)

The pH of gelatinized cassava starch paste was adjusted to 6.5 with 50 mM phosphate buffer, AM (10 U·g starch^{-1}) and BE (4,000 U·g starch^{-1}) were added and the mixture incubated at 60°C for 3 h or 24 h. Another set of gelatinized cassava starch was prepared, pH adjusted to 6.0 with 50 mM phosphate

buffer, AM (10 U·g starch^{-1}) and BE (4,000 U·g starch^{-1}) were added and the mixture incubated at 70^{o}C for 3 h or 24 h. The product was collected and dried as above.

3.3.5 Iodine complexation

Iodine colorimetric analysis was carried out mainly as described by Wickramasinghe et al. (2009). Cassava starch, glycogen, potato soluble starch or enzyme-modified starches (20 µg each) were suspended in 1 mL of 1 M NaOH with shaking (1,200 RPM in a Thermomixer, Eppendorf, Germany) overnight until completely dissolved. 50 µL sample was added to 20 µL of the diluted iodine solution (0.26 g I_2 and 2.6 g KI in 10 mL water, diluted 60 times in 100 mM HCl) in a microtiter plate well, and absorbance was recorded (Spectramax M5, Molecular Devices, Sunnyvale, CA, USA) from 350 to 750 nm every 5 nm and the wavelength at maximum absorbance (λ-max) was identified for each scan (Wickramasinghe, Blennow and Noda, 2009).

3.3.6 β-amylolysis limit

The analysis procedure was slightly modified from that of Hood and Mercier (1978). The polyglucan solution (1.5 mL, 0.5% w·v^{-1} in 90% DMSO) was mixed with an acetate buffer solution pH 4.8 (0.3 mL, 0.2 M). The β-amylase solution (0.2 mL, 20 units·mL^{-1}) and deionized water (1.0 mL) were added and mixed, and the solution was incubated at 37^{o}C for 48 h. The reducing sugar content and total sugar content were measured. The percentage of β-amylolysis limit was calculated using a following equation:

$$\text{β-amylolysis limit} = \left(\frac{\text{Reducing sugar after hydrolysed-Reducing sugar of blank}}{\text{Total sugar after hydrolysed-Total sugar of blank}}\right) \text{x100}$$

3.3.7 Chain-length distribution of debranched α-glucan by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Enzyme-modified starches were gelatinized by boiling and enzymatically debranched by using 0.24 U of isoamylase per 5 mg of sample at 40ºC. The obtained linear α-glucan fragments were analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA). Samples of 20 μL (100 μg of linear α-glucan) were injected on a CarboPac PA-200 column using 0.4 $mL \cdot min^{-1}$ flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0-5 min: 0-110 mM linear gradient, 5-130 min: 110-350 mM convex gradient. Single peaks were integrated and, corrected for the detector response (Viksø-Nielsen et al., 1998).

3.3.8 Molecular weight distribution, polydispersity index and intrinsic viscosity analysis by size-exclusion chromatography with triple detection array (SEC-TDA)

The molecular weight distribution (Mw), the polydispersion index (Mw/Mn) and the hydrodynamic volume (Rh) were determined by size exclusion chromatography (SEC) using a Viscotek System (Malvern, UK) equipped with a GS-520 HQ column (Shodex) attached to a TDA302 module (Triple detector array) consisting in a refractive index detector (RI), a four-bridge visco-meter detector (VIS) and a light scattering detector (LS). The LS consisted of a right angle light scattering (RALS) and a low angle light scattering (LALS) that measure the scattered light 7° and 90° with respect to the incident beam. The calibration of the instrument was made using pullulan (50 kDa, polydispersion 1.07, Showa Denko) as a standard, solubilized

in MilliQ water (1 mg/mL) and mechanically shaken at 99ºC for 120 min at 1,000 rpm. Isocratic elution was made using 50 mM ammonium formate (HCO_2NH_4) buffer, with a flow of 0.5 mL/min. Samples were filtered through a 0.22 µm syringe filter and automatically injected (GPC max module) into the column. The injection volume was 50 µL, the column temperature 60ºC. The analysis was performed using the OmniSec Software 4.7 (Malvern Instrument, ltd.).

3.3.9 α-1,6 glucosidic linkages determination by nuclear magnetic resonance spectroscopy (NMR)

The α-glucan samples were dissolved in 500 µL D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) to concentrations of 0.3% ($w \cdot v^{-1}$) under gentle heating. ^{1}H-NMR spectra were recorded on a Bruker (Fällanden, Switzerland) DRX spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK) at 37ºC. Spectra were recorded by sampling 16,384 complex data points during an acquisition time of 1.7 sec, employing 32 transients and a recycle delay of 10 sec for reliable quantifications. NMR spectra were processed using Bruker Topspin 2.1 software with zero filling in all dimensions and mild resolution enhancement. For the latter we employed a time domain Lorentzian-Gaussian Transformation with a line broadening of -1 Hz and a Gaussian broadening of 0.3. Anomeric signals in branch point α-1,6 linkages were quantified relative to anomeric signals in α-1,4 linkages. Integrals of free ^{1}H4 signals from terminal residues of the branches were used to validate the quality of integrations obtained for the α-1,6 anomeric signal. A diffusion ordered spectrum of cassava starch was used to estimate the apparent molecular weight of polysaccharides in the glucan products to yield a weight on the order of ~70 kDa. For 32 gradient amplitudes

that were incremented in steps of gradient squared between 3.6 to 27 G cm^{-1}, 32 transients of 16,384 complex data points (acquisition time 1.63 s) were acquired using a diffusion delay Δ = 300 ms and a gradient pulse duration 1.8 ms. Resultant decays were fitted to the Stejskal-Tanner equation to yield the translational self-diffusion coefficient, which was transformed to an approximate molecular weight (Miller, Klyosov, Platt and Mayo, 2009).

3.3.10 Cyclostructure confirmation by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)

A solution of 2,5-dihydroxybenzoic acid in 30% acetonitrile in water with 0.1% TFA (20 $mg \cdot mL^{-1}$) was prepared as the matrix. The enzyme-modified starch products were dissolved in a minimal volume of DMSO by vortexing and diluted with water to a final concentration of 1 µg/mL. The matrix solution (2 µL) was combined with the diluted analyte solution (2 µL) and this solution (0.5 µL) was added to the target and air-dried. MALDI-TOF MS was performed using a Bruker Daltonics Microflex instrument operating in reflectron mode. A 340 nm laser was used and mass spectra were typically accumulated from 1,000 laser shots. Spectra were generally acquired over a 4,000 m/z range (from 500 Da to 4,500 Da, with matrix suppression up to 400 Da).

3.3.11 *In vitro* starch digestion analysis

In vitro starch degradation was analyzed by a modification of the Englyst method using gelatinized cassava starch, potato soluble starch and glycogen type IV (all gelatinized at 100ºC for 30 min) and modified glucan products. Starch and glucan samples (2% in 250 µL), were incubated in triplicates with 20 U of each α-amylase from porcine pancreas (Sigma A3176) and glucoamylase (Sigma 10113) in

20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.0) at 37ºC for 0, 10, 30, 60, 120, 240, 360, 480, and 1440 min. Enzyme reaction was terminated by adding 30 μL 100 mM HCl. The rate of starch digestion was expressed as % of glucose released from the total starch or α-glucan over the time period (Englyst, Kingman and Cummings, 1992) by using PGO enzymes (Sigma P7119).

The glucose released (%) was calculated using the following equation:

$$\text{Glucose released (\%)} = \left(\frac{\text{Total weight of glucose x 0.9}}{\text{Weight of enzyme-modified starches}}\right) \text{x } 100$$

where 0.9 is the molar mass conversion from glucose to anyhydroglucose (the starch monomer unit).

3.4 Results and discussions

3.4.1 AM and BE unit activity, optimum dosages, cassava starch concentration, and incubation time

Enzyme unit activity was reported following the definition of "The lnternational Union of Biochemistry" which was defined a standard unit of enzyme activity. One unit of enzyme activity is the amount of enzyme which can catalyse the transformation of 1 μmole of the substrate per minute under standard conditions (Units of Enzyme Activity, 1979). AM unit activity was determined by analysing glucose released after AM reaction while BE unit activity was determined by two methods. The first method was measuring a decrease in amylose content by iodine-amylose complex and another method was measuring an increase in reducing sugar content. Unit activities of AM and BE were shown in Table 3.1.

Table 3.1 AM and BE unit activity.

Enzymes	Methodology of activity assay	$U \cdot ml^{-1}$ of enzymes
AM	The increase of glucose content	4,763.45
BE	The decrease of amylose content	7,240.27
BE	The increase of reducing sugar content after debranching by isoamylase	12,067.11

The activity of AM was 4,763.45 $U \cdot ml^{-1}$ which was determined by the method which was generally used. The unit of BE activity which measuring the decrease of amylose content is 7,240.27 $U \cdot ml^{-1}$ of BE, while it has unit activity equal to 12,067.11 $U \cdot ml^{-1}$ with monitoring the increase of reducing sugar content. This result suggested that the accuracy of the two methods were different. The higher accuracy method was measuring an increase in the reducing sugar content because amylose-iodine complex measurement was dependent on the amylose chain length complex with iodine molecule to form color which resulted in various λ_{max} (Yu, Houtman, and Atalla, 1996). Amylose molecule with DP $\leq$ 40 was not detected by this method. Normally, BE catalyse the formation of α-1,6 linkages and leave small amount of reducing end to the system. The detection of the difference of reducing end content between before and after debranching was an appropriate method to determine BE activity.

Table 3.2 The content of α-1,6 glycosidic linkages (%) determined by reducing end analysis with various cassava starch concentration.

Cassava starch concentration (% w·v^{-1})	% Branch linkages
5.0	5.9±1.1
7.5	7.9±0.4
10.0	9.0±0.3
15.0	8.2±0.9
20.0	5.8±0.9
25.0	5.5±0.9
30.0	5.2±0.7

Cassava starch concentration with BE modification was studied in order to observe the content of branch linkages formation (Table 3.2). Generally, BE acts on α-1,4 glucosidic linkages to produce a branched α-glucan by intra- or inter-molecular α-1,6 glucosidic transfer. Starch concentrations ranging from 5.0% to 30.0% were investigated. The result showed that the highest branched linkage formation was 10.0% starch. In addition, increasing starch concentration from 10.0% to 30.0% resulted in the decrease of α-1,6 glycosidic linkage content. It suggested that high starch concentration generated more viscous system and made BE difficulty on diffusion and then retarding BE activity. In addition, the starch concentration from 5.0% to 10.0% showed an increase in branch linkage content. However, branch linkage content of 10.0% substrate were higher than that of 5.0% and 7.5% substrate due to their higher substrate concentration which was efficiently used by BE to create branch point. This result was in agreement with the previous studied which used starch concentration ranging from 5.0%-10.0% (Kasprzak et al., 2012; Le et al., 2009).

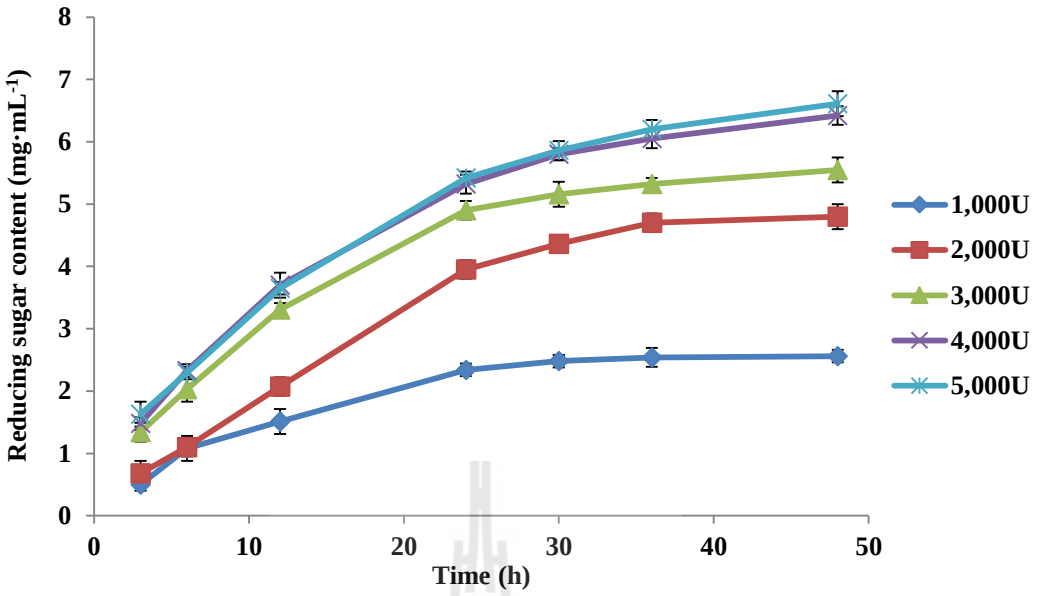

Figure 3.1 Reducing sugar content after debranching by isoamylase of BE treated 10% cassava starch with various BE dosages.

The relationship between starch concentration, incubation time and BE dosages were studied (Figure 3.1). BE dosages ranging from 1,000 U-5,000 U were used and the samples were collected with various time points. The results showed that the reducing end content after debranching was increased when increase in incubation time and BE dosages. Moreover, there was no difference in reducing sugar content between 4,000 U and 5,000 U with various time points. The reducing end content was slightly increased after 24 h of incubation time for 4,000 U and 5,000 U. From this result, the appropriate incubation time and dosage were 48 h and 4,000 U, respectively. However, since BE also has hydrolysis activity, longer incubation time could result in the formation of hydrolysis product. Thus, 24 h was selected for BE incubation time which can be obtained less hydrolysis product and save energy for the industry application compared to 48 h.

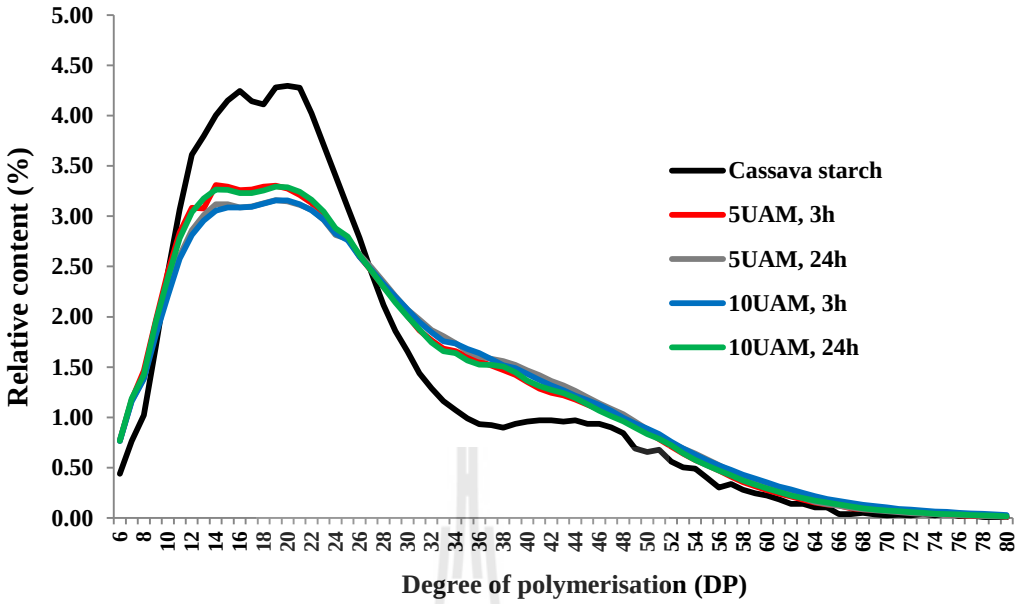

Figure 3.2 Average chain length distribution after debranching by isoamylase of AM treated 10% cassava starch with various AM dosages and incubation time.

The optimum dosages and incubation time of AM were investigated in order to investigate the group of chains which contain high amount of short (DP 6-24) and long chains (DP > 25) α-glucans. Cassava starch was treated with 5 U and 10 U of AM and incubated for 3 h and 24 h for each AM dosages. The average chain length distribution of AM-treated starch showed an increase in chains approximately DP 6-10 and DP > 27 when compared to native cassava starch (Figure 3.2 and Table 3.3). The result indicated that AM has ability to elongate glucan chains and leave some short chains especially DP 6-24 in the system. In addition, AM usually created glucan chain DP > 27 which was long enough for BE to uses as a substrate in the further experiment (Figure 3.2).

Table 3.3 Relative content (%) of degree of polymerization (DP) determined by HPAEC of AM treated 10% cassava starch with various AM dosages and incubation time.

DP	Relative content (%)				
	Cassava starch	**5U, 3h**	**5U, 24h**	**10U, 3h**	**10U, 24h**
DP 6-12	13.04	13.54	12.90	12.72	13.73
DP 13-24	48.43	38.27	36.82	36.68	38.33
DP 25-36	20.83	24.45	25.16	25.03	24.31
DP > 36	16.78	22.04	23.54	23.99	22.29

According to Hizukuri et al. (1981), the relative contents of chains DP 6-12, DP13-24, DP 25-36 and DP > 36 which calculated from Figure 3.2 (Hizukuri, Takeda, Yasuda, and Suzuki, 1981) were shown in Table 3.3. The highest content of group of DP 6-24 were obtained from 10 U-24 h treated samples indicating the disproportionation and hydrolysis of the α-glucan chains. The 10 U-24 h treated samples can be served as short and medium chains substrate for the further experiment. Moreover, the 10 U-3 h treated sample was also chosen because this sample showed the highest content of DP > 25 and can be served as a long chains substrate for further experiment (Table 3.3).

3.4.2 Structural analysis of AM and BE modified products

Cassava starch was treated with AM and/or BE either separately, in sequential steps, or simultaneously for different time periods as indicated in Figure 3.3 and Table 3.4. The λ_{max} of the iodine-α-glucan complexes for all samples were suppressed to values close to the λ_{max} of glycogen (Table 3.4). In many cases, the λ_{max} was lower than 440 nm demonstrating the formation of very densely branched products. The AM→BE and BE→AM→BE modified starches had higher

content of α-1,6 linkages compared to cassava starch and glycogen. Interestingly, the BE→AM→BE product showed the highest content of α-1,6 glucosidic linkages as compared to control BE→X→BE (Table 3.4, X denoting no intermittent treatment) demonstrating an important role of AM to provide efficient substrate for BE to further increase the degree of branching. The five samples with the highest content of α-1,6 linkages were selected for further molecular analysis: AM (3 h, 24 h)→BE, BE→AM (3 h, 24 h)→BE, and AM&BE (60°C, pH 6.5, 24 h).

Table 3.4 Maximum wavelength absorption of iodine-α-glucan complex and content of α-1,6 glycosidic linkages (%) of the control and enzyme-modified starches. X denotes no enzyme treatment in that step. nd: not determined, BE: BE-treated starch for 24 h.

Samples	**λ_{max} (nm)**	**α-(1,6) linkages (%)**
Cassava starch	565	4.9
Glycogen	440	9.7
Potato amylose	630	nd
Potato amylopectin	540	nd
BE	525	7.8
AM 3 h→BE	500	9.9
AM 24 h→BE	470	10.0
BE→X→BE	415	8.3
BE→AM 3 h→BE	420	13.1
BE→AM 24 h→BE	410	10.9
AM&BE 60°C, pH 6.5, 3 h	560	5.1
AM&BE 60°C, pH 6.5, 24 h	425	8.7
AM&BE 70°C, pH 6.0, 3 h	560	4.7
AM&BE 70°C, pH 6.0, 24 h	540	6.4

3.4.3 Chain length distribution

3.4.3.1 Single catalysis

The effects of AM and BE catalysis on the average chain-length distribution or degree of polymerization (DP) of modified cassava starch were investigated by using HPAEC-PAD, permitting a more detailed comparative analysis. Cassava starch only treated with BE for 24h showed a reduced number of chains with DP $\geq$ 20 whilst the proportion of chains shorter than DP 20 were increased as compared to cassava starch (Figure 3.3B). The small branch chains fraction of BE starches modified for 24 h were increased comparable to that of glycogen. Similar structures were reported for BE from *Bacillus subtilis* and *Rhodothermus obamensis* (Kim, Ryu, Bae, Huong, and Lee, 2008; Le et al., 2009) demonstrating that BE generally and specifically catalyzes the formation of extensive α-1,6 linkages. It should be noted that BE, as a minor activity, is also capable of re-distributing α-1,4 linkages in a minor transglycosidation reaction in a similar way as AM (Roussel et al., 2013). When cassava starch was treated with AM, the chain length profile shows a specific increase in DP $\leq$ 7 and DP $\geq$ 25 while DP 7-25 were decreased (Figure 3.3C). The presence of low molecular weight α-glucans (Figure 3.4) in the enzyme-modified starch products indicated a hydrolytic and/or disproportionation activity of AM (Figure 3.3C) that agrees with previous data (Hansen, Blennow, Pedersen, and Engelsen, 2009). The appearance of the low DP chains are supposedly originating from residual segment from the donor chains after transglycosylation and from minor hydrolase activity of BE and AM. The hydrolytic activity of AM is considered minor (Kaper et al., 2007; Kaper, van der Maarel, Euverink, and

Dijkhuizen, 2004; van der Maarel et al., 2005) and in our preparations estimated to 0.73% and 0.13% of the hydrolytic activities for BE and for AM, respectively.

3.4.3.2 AM→BE sequential catalysis

In an attempt to change the relative positions to further increase the number of branch points in the α-glucan product, the cassava starch was subjected to AM treatment for 3 h or 24 h preceding BE treatment (AM→BE). Such modification is expected to re-distribute the chain segments in relation to the α-1,6 linkages providing a different substrate for the following BE catalysis. All AM→BE treated samples showed a substantial amount of chains smaller than DP 25 that were accumulated at the expense of chains longer than DP 25 (Figure 3.3E). These results suggest that BE prefers to use the AM elongated chains of DP ≥ 25 in the amylopectin (Figure 3.3C). The effect of the incubation time with AM was minor as supported by the minor difference found between the AM 3 h→BE and AM 24 h→BE samples (Figure 3.3C). The disproportionation activity of AM demonstrates that AM can provide longer α-glucan chains for further efficient chain transfer catalyzed by BE. The dense branching of the product from the AM→BE treatment (Figure 3.3E) showed that BE can use the outer chains of the AM treated products to create new branch points (Table 3.4).

3.4.3.3 BE→AM→BE sequential catalysis

In order to further investigate the possibility to further increase branching of the α-glucans, samples were sequentially treated with BE for 24 h, AM for 3 or 24 h and last with BE for 24 h (BE→AM→BE, Figure 3.3F). This approach provides new potential α-glucan substrates for BE in the last step, potentially further increasing the branching. In order to investigate the efficiency of isoamylase

debranching, to identify possible closely positioned α-1,6 branch points not accessible for isoamylase catalysis, β-amylolysis was performed (Table 3.5). The β-amylolysis for the BE→AM→BE modified starches spanned 92.8-93.0% as compared to the control glycogen (98.4%) and BE treated starch (99.4%) demonstrating that isoamylase could not completely debranch these highly branched products, supposedly due to closely positioned α-1,6 branch points. For the sample treated with BE →AM 3 h→ BE, a high content of very short α-glucans chains ranging DP 3-12 were formed (Figure 3.3F) and in the raw chromatograms minor peaks were identified as branched α-glucans. Compared to the BE →AM 24 h→ BE sample, BE →AM 3 h→ BE showed lower accumulation of chains ranging from DP 3-15. Furthermore, AM very inefficiently acted on BE treated (highly branched) substrate (Figure 3.3D); hence, very subtle differences in substrate structure in the region DP 8-15 determine the substrate recognition requirements for the BE to act upon to generate substantial differences in BE chain transfer rates seen in Figure 3.1F. Notably, the BE→X→BE sample did not gain high content of α-1,6 branch points despite the extensive BE treatment for two consecutive time periods supporting that AM catalyses the disproportionation and/or cyclization of amylopectin (Hansen, Blennow, Pedersen, Norgaard, and Engelsen, 2008), increasing the catalytic rate of BE. A substantial collected volume of evidence points at health-associated assets of glycogen-like structures and their potential in functional foods (Kajiura, Kakutani, Akiyama, Takata, and Kuriki, 2008).

Table 3.5 β-amylolysis (%) of cassava starch, glycogen and enzyme-modified starches after debranched by isoamylase. BE: BE-treated starch for 24 h.

Samples	**β-amylolysis (%)**
Cassava starch	94.9
Glycogen	98.4
BE	99.4
AM 3 h → BE	96.5
AM 24 h → BE	93.6
BE → AM 3 h → BE	92.8
BE → AM 24 h → BE	92.9
AM&BE 60°C, pH 6.5, 24 h	96.9

Glycogen has a degree of branching that is about twice that of amylopectin (Kajiura et al., 2011) and the combined AM and BE catalysis approach in one case surpassed the chain length distribution of glycogen. This finding highlights the value of combining different enzyme activities to generate products more extreme than found in nature. The BE→AM→BE treated starch generated the highest content of α-1,6 linkages with a chain-length distribution very different to that of cassava starch (Table 3.4). A plausible explanation for this effect could be that the BE→AM-catalysed sequential reaction, provides optimum chain length and differently positioned chains for BE to create further branch points in the last BE catalyzed step. However, previous data suggest that AM preferably catalyses the transfer of longer chains like those found in amylose (Bhuiyan, Kitaoka, and hayashi, 2003). Therefore, for the BE→AM catalysed step, the restriction of such long amylose chains in the BE treated sample would force AM to act on sub-optimal, short chains and amylopectin clusters. This effect was supported by the minor difference found in the

sequential BE→AM (Figure 3D) as compared to the AM treated sample chain profile (Figure 3C). AM is thus potentially mainly involved in transfer of larger fragments e.g. rearrangement of amylopectin clusters and transfer of amylose segments (Hansen et al., 2009). However, rearrangements of amylopectin clusters can enhance BE catalysis in the last step by providing steric access to BE. By using the combined BE→AM→BE protocol, the chain length density of glycogen was surpassed generating a very densely branched α-glucan products.

3.4.3.4 Simultaneous AM&BE catalysis

Finally, we investigated the simultaneous synergistic effects of AM and BE by adding AM and BE simultaneously (AM&BE) in the system using the optimal catalytic conditions for each enzyme respectively, incubating for 3 h or 24 h (Figure 3.3G). The optimum conditions for BE from *Rhodothermus obamensis* activity is 60°C at pH 6.5 while 70°C at pH 6.0 is the optimum condition for AM from *Thermus thermophillus*. Incubation at the optimum condition of BE for 24 h increased the portion of smaller α-glucan chains (DP 3-20) as compared to the AM optimum condition. For this sample, longer chains (DP > 20) were consumed by chain transfer while for the other three incubation conditions (60°C for 3h or 70°C for 3 h or 24 h, respectively) the α-glucan chains of approximately DP 25-40 were increased. As expected, longer incubation time at optimum condition for BE resulted in a highly branched product (Table 3.4). Likewise, after incubation for shorter time at the optimum condition for BE, similar distribution was found as for the optimum condition for AM. Nevertheless, AM and BE still catalyzed chain transfer although the conditions were not optimal, confirming that both amylose and amylopectin are substrates for the AM and BE in the mixed systems, but it should be noted that both

enzymes has relatively higher affinity for amylose than for amylopectin (Shinohara et al., 2001). Most importantly, simultaneous catalysis of AM and BE did not result in the very highly branched product as demonstrated for the sequential action (Table 3.4), suggesting that products generated as intermediates in the AM&BE combined reaction mixture were non-optimal substrates for further branching.

We observed a distinct difference between simultaneous and sequential catalysis of AM&BE. The simultaneous action of AM&BE increased the branch points in the products as compared to control but the effect was less pronounced than for the sequential AM→BE and BE→AM→BE treatments. These data suggest a catalytic competition between AM and BE on long chains during catalysis. At optimal conditions for AM (70^{o}C, pH 6.0), BE activity was sub-optimal and *vice versa*. High BE activity efficiently removes amylose from the system preventing AM to transfer amylose segments to amylopectin or rearrange amylopectin cluster (Bhuiyan et al., 2003; Fujii et al., 2007). This effect potentially leads to lower content of α-1,6 linkages in the simultaneous AM&BE samples (Table 3.4).

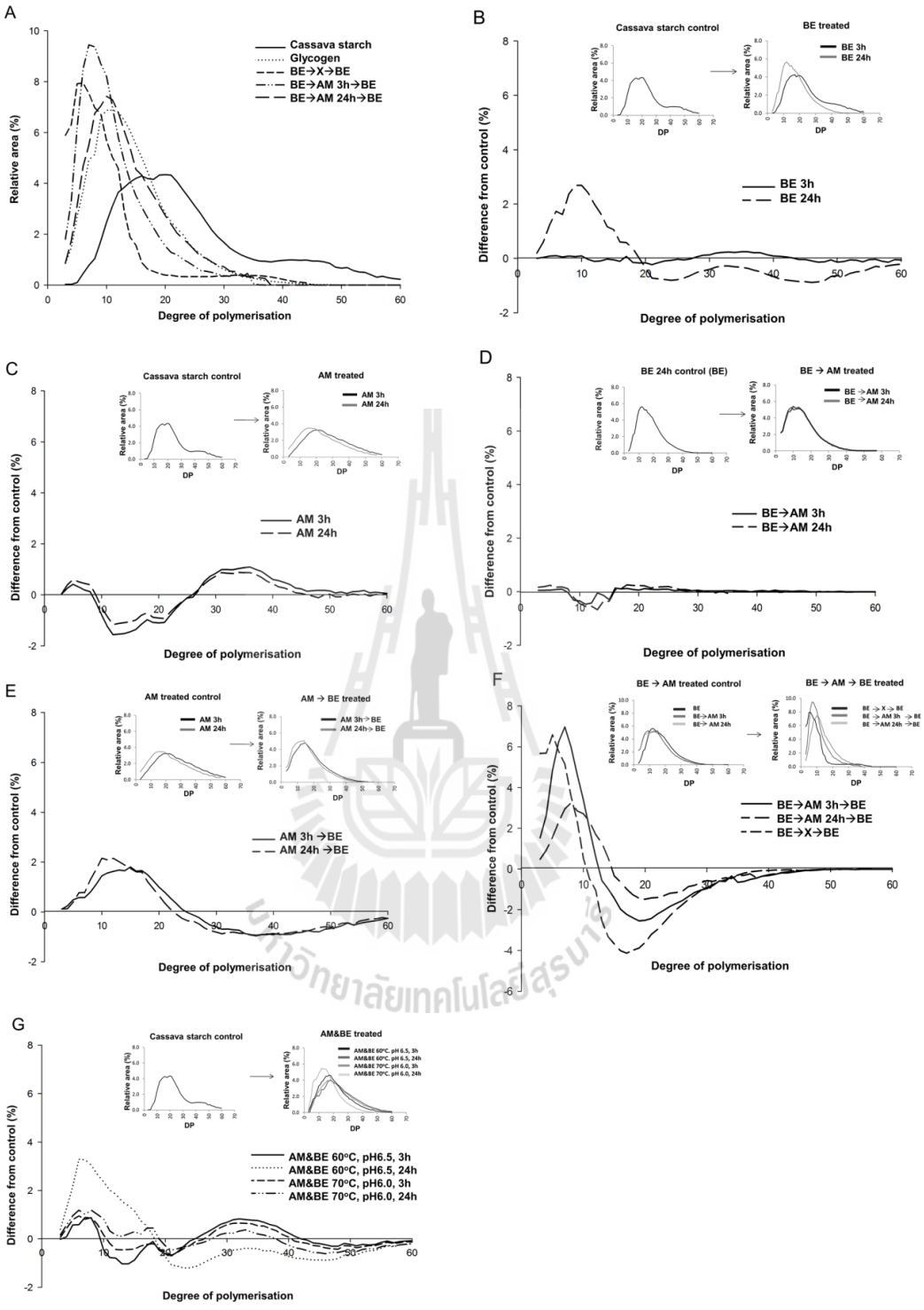

Figure 3.3 Chain length distribution analysis and difference plots of debranched α-glucan samples demonstrating differences due to additional sequential enzyme catalysis with reference to the preceding enzyme step. (A) Chain

length distributions of enzyme treated samples compared to cassava starch and glycogen. (B-G) Difference plots relative to controls as indicated. (B) BE treated samples as compared to cassava starch. (C) AM treated samples. (D) AM treated sample as compared to BE treated cassava starch. (E) BE treated sample as compared to AM treated cassava starches. (F) BE treated sample as compared to BE→AM treated cassava starch using BE treated cassava starch as control. (G) Simultaneous action of AM&BE using cassava starch as control. The Y-axis in A denotes the peak areas standardized to 100%.

3.4.4 Molecular size and cyclo-structures

Intra-chain transfer leads to cyclisation and a reduction in the molecular size. In order to test if any of the enzyme treatments resulted in amylopectin cluster and/or cyclo-amylopectin formation, average molecular weight was analysed by size-exclusion chromatography with triple detection array (SEC-TDA). All AM and BE-treated samples showed decreased average molecular weight except for the AM-treated cassava starch (Figure 3.4). As compared to the glycogen elution profiles, the average molecular weights of the samples were lower, indicating hydrolysis of α-1,4 linkages between amylopectin clusters as mentioned in section 3.2.1 together with the formation of amylopectin cyclo-clusters during the branching reaction. The reduction of molecular size following BE treatment has been reported elsewhere (Kim et al., 2008; Le et al., 2009) and this is the first evidence provided for cyclo-amylopectin cluster formation catalyzed by the *Rhodothermus obamensis* BE. For AM catalysis, cyclization of amylopectin to form amylopectin clusters has been demonstrated

(Hansen et al., 2008). Hydrolytic activities in the AM and BE used in this study were low; 0.73% and 0.13% for BE and for AM, respectively as mentioned above. Hence the reduction of molecular size following AM and/or BE catalysis is likely a combined effect of minor hydrolysis and the formation of amylopectin cluster and cyclo-amylopectin clusters.

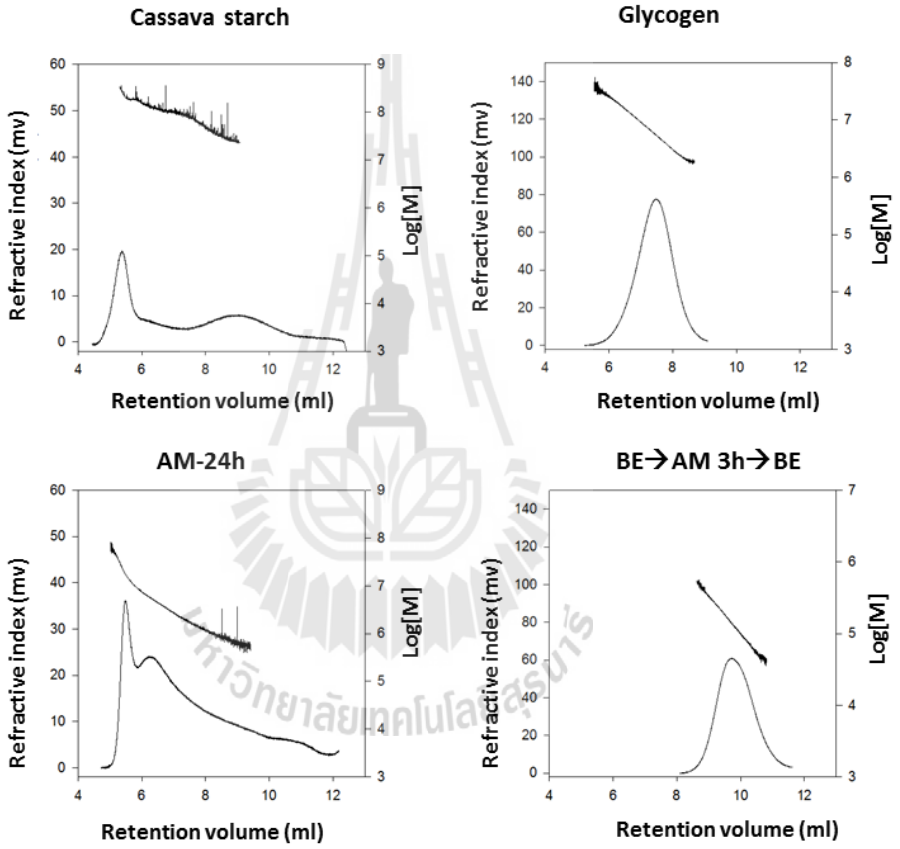

Figure 3.4 Size distributions of cassava starch, glycogen and selected enzyme-modified cassava starch products as analyzed by size-exclusion chromatography with triple detection array (SEC-TDA).

The samples were analysed by MALDI-TOF (Figure 3.5). Interestingly, the smallest cyclic α-glucan found was γ-cyclodextrin (DP 8, m/z 1320) and this

compound was detected in all modified starches and especially in the AM treated samples. Polydispersity index (Mw/Mn) and hydrodynamic volume (Rh) (Table 3.6) showed that the modified starch samples had (Mw/Mn) values as for glycogen and the product obtained after sequential enzyme catalysis shows decreased Rh corresponding to the reduction of the average molecular weight.

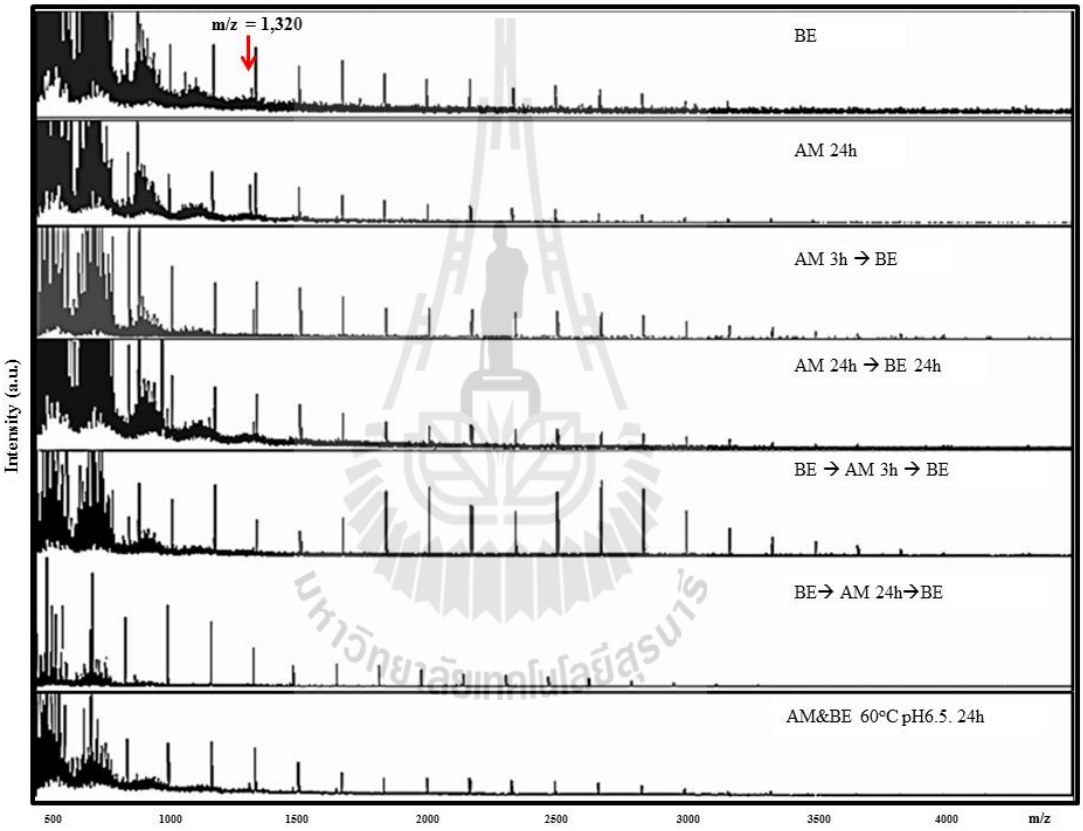

Figure 3.5 MALDI-TOF analysis of cyclostructure formation of enzyme-modified starches.

Table 3.6 Mean molecular weight (Mw), polydispersity index (Mw/Mn) and hydrodynamic volume (Rh) of the enzyme-modified starches. X denotes no enzyme treatment. BE: BE-treated starch for 24 h.

Samples	**Mw (kDa)**	**Mw/Mn**	**Rh (nm)**
Cassava starch	128,800	2.9	137.8
Glycogen	6,870	1.5	45.5
BE	138	1.5	12.4
AM 3 h→BE	137	1.7	12.3
AM 24 h→BE	153	1.8	12.7
BE →X→BE	155	1.6	12.9
BE →AM 3 h→BE	206	1.6	14.1
BE → AM 24 h→BE	179	1.5	13.5
AM&BE 60ºC, pH 6.5, 24 h	179	1.5	13.5

Intra-molecular chain transfer may lead to the generation branched (by BE) or non-branched (by AM) cyclic structures (Bhuiyan et al., 2003; Park et al., 2007; Takata, Takaha, Okada, Takagi, and Imanaka, 1996; Viksø-Nielsen et al., 1998). The smallest cyclo-α-glucan found was the eight glucose-ring γ-cyclodextrin. This is in contrast to earlier findings (Bhuiyan et al., 2003; Park et al., 2007) where no γ-cyclodextrin was detected but the smallest cyclic α-glucans found, following *Aquifex aeolicus* AM treated amylose, was DP16 (Bhuiyan et al., 2003). Amylose treated with AM from *Thermus aquaticus* generated cycloamyloses down to DP 19 (Park et al., 2007). We speculate that this discrepancy can be an effect of the mixed amylose and amylopectin system used in our study providing different types of substrate for the AM potentially with different helical folds.

3.4.5 Amylolytic susceptibility evaluated *in vitro*

To emulate the dietary susceptibility to amylolytic digestion of the AM and BE treated starches, six selected samples were subjected to combined porcine pancreatic α-amylase (PPA) and glucoamylase (GA) treatment. In this type of experiment PPA and GA exert a synergistic digestion of the α-glucans. Generally, the modified products showed less susceptibility to PPA and GA digestion than gelatinised cassava starch. The digestion profiles of the five selected samples compared to cassava starch and glycogen (Figure 3.4) were fitted to a first order kinetic model, $C = 1\text{-}e^{kt}$, where t is the digestion time (min), C is the fraction of digested starch at specified reaction time, and k is the digestion rate constant (min^{-1}). The value of k was obtained from the slope of a linear-least-squares fit of a plot of ln(1 −C) against t (Butterworth, Warren, Grassby, Patel, and Ellis, 2012; Zhang, Dhital, and Gidley, 2013) (Table 3.7 and Figure 3.7). The maximum degradation was found to be lower for all the enzyme-modified samples than for the cassava starch demonstrating the presence of resistant α-glucans. Specifically, the initial hydrolysis rates for the enzyme-modified starches were slower than for the cassava starch control. All enzyme-modified starches showed evidence for first order kinetics ($R^2 > 0.930$, Figure 3.7) indicating rate-limitation in the enzyme-substrate complex formation due to stearic hindrance. This effect can be due to inefficient binding of free maltose and maltotriose, to GA and restricted binding of maltose and maltotriose glucan segments in PPA. The digestion rate coefficients of the modified starches were significantly lower than for cassava starch control supporting a previous study on the modification of starches with BE combination with β-amylase (Le et al., 2009; Lee et al., 2007). A high k-value indicates high susceptibility of PPA and GA catalysis.

The k-value for cassava starch and glycogen were 5.7×10^{-3} min^{-1} and 1.7×10^{-3} min^{-1}, respectively. The corresponding values for the BE→AM 3 h→BE and BE→AM 24 h→BE products were 1.4×10^{-3} min^{-1} and 1.6×10^{-3} min^{-1}, respectively and for the AM 3 h→BE and AM 24 h→BE were 1.9×10^{-3} min^{-1} and 2.1×10^{-3} min^{-1}, respectively. Hence, the BE→AM→BE products had significantly lower k-values compared with the others analysed samples except for glycogen. The difference was more pronounced at maximum digestion reflecting a pronounced effect at the final digestion level by the PPA and GA (Figure 3.6). The levels of glucose released from all samples spanned 64.7-87.7%. The remaining structures can be considered as very resistant towards amylolysis.

Table 3.7 Digestion rate coefficient of cassava starch, glycogen and enzyme-modified starches. BE: Starch treated with BE for 24 h. The data are averages of two measurements±SD. Means in columns with different letters are significantly different ($p<0.05$) by general linear model.

Samples	**k (min^{-1}) $\times10^{-3}$**
Cassava starch	5.7 ± 0.2^{a}
Glycogen	1.7 ± 0.2^{bc}
BE 24 h	2.0 ± 0.3^{b}
AM 3 h→BE	1.9 ± 0.1^{bc}
AM 24 h→BE	2.1 ± 0.2^{b}
BE→AM 3 h→BE	1.4 ± 0.9^{c}
BE→AM 24 h→BE	1.6 ± 0.2^{c}
AM&BE 60°C, pH 6.5, 24 h	2.1 ± 0.1^{b}

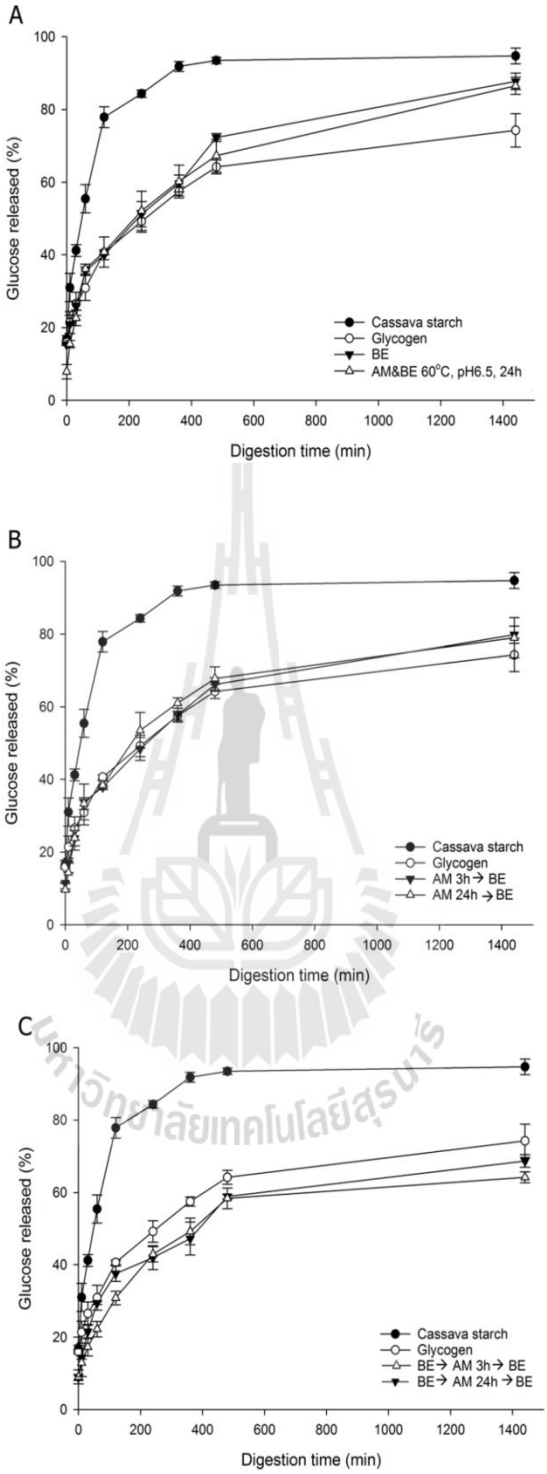

Figure 3.6 Time course digestion profiles of enzyme-modified samples digested with α-amylase (PPA) and glucoamylase (GA) compared with cassava starch.

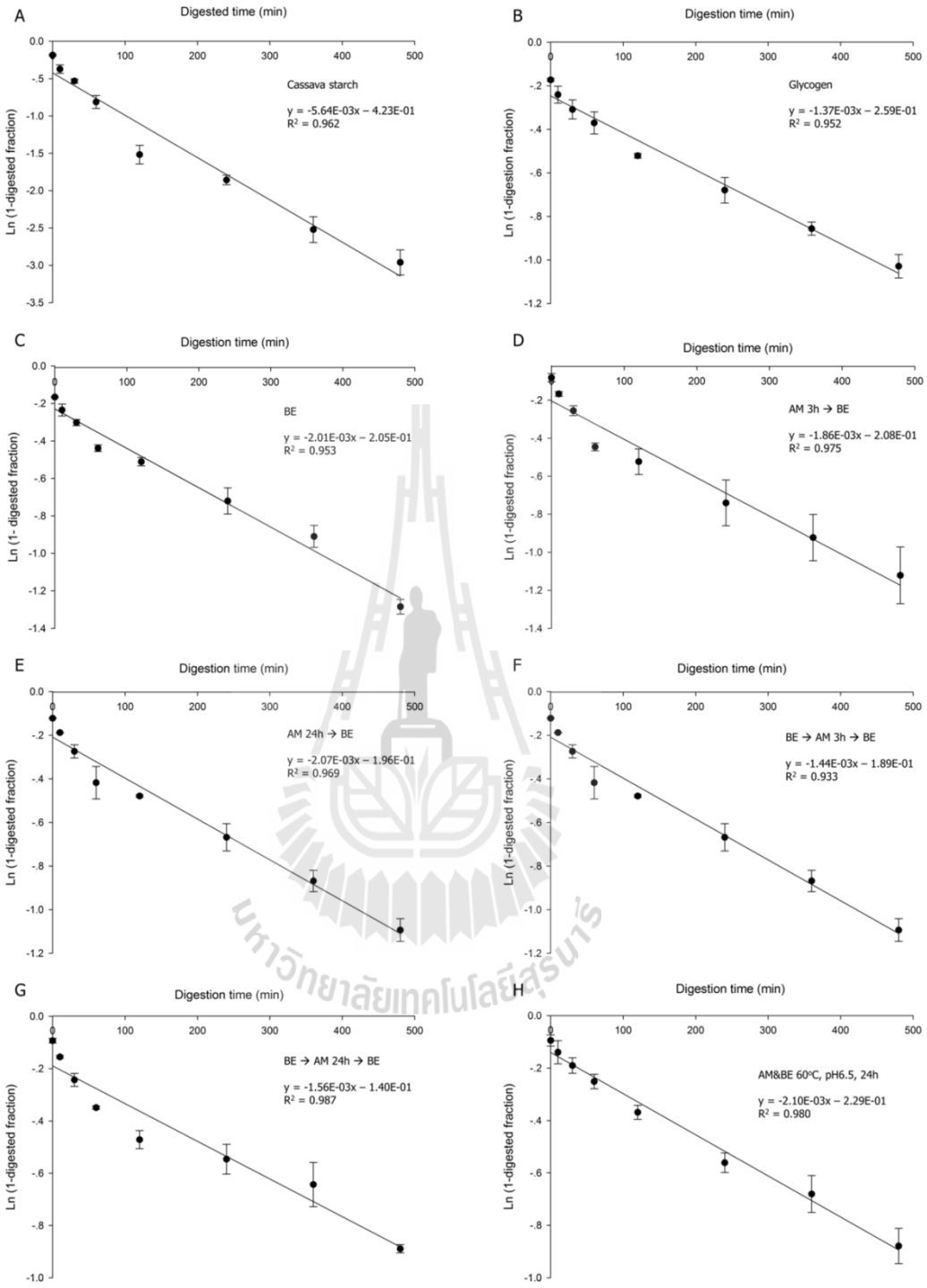

Figure 3.7 First-order kinetics model for combined PPA and GA digestion of cassava starch, glycogen and enzyme-modified starches.

The resistant malto-oligosaccharides obtained after 24 h hydrolysis with PPA and GA were analysed using HPAEC-PAD (Figure 3.8). The chromatographic fingerprint profiles of the products demonstrate high amounts of glucose and maltose. The resistant malto-oligosaccharides included a range of DP ≥ 4. All modified starches and glycogen showed higher content of resistant malto-oligosaccharides as compared to cassava starch. The BE→AM→BE modified starches were initially relatively slowly hydrolyzed by PPA and GA and finally reached lower levels of hydrolysis as compared to the other products. As a conclusion, the difference in the hydrolytic profiles is supposedly directly linked to the presence of densely branched products, especially in the BE→AM 3 h→BE treated starches.

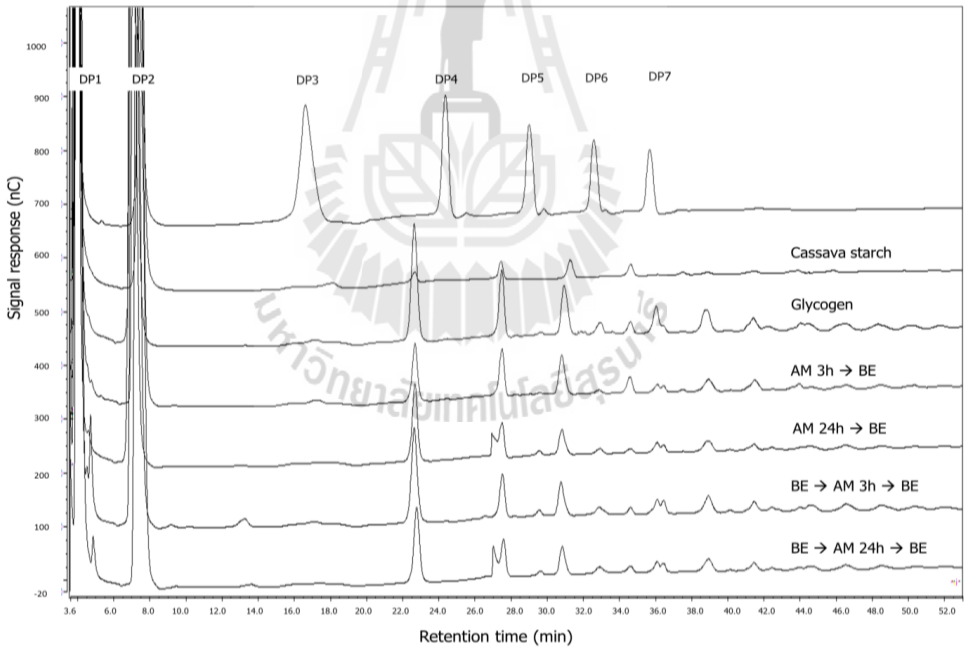

Figure 3.8 Oligosaccharide profiles of enzyme-modified starches by PPA and GA after *in vitro* digestion as analysed by Dionex chromatography. Top chromatogram shows linear standards. Malto-oligosaccharides eluting at intermediate positions indicate the presence of differently branched molecules that are partly resistant towards amylolytic degradation.

Hypothetically, when polysaccharide branching is increased, the subsequent hydrolytic digestion rate should be suppressed as an effect of steric hindrance imparted by the increased and re-positioned α-1,6 linkages as compared to normal starch. As an effect of restrictions in PPA and GA hydrolase recognition of α-1,6 linkages, more densely branched substrates are expected to slow down, or virtually prevent, their enzyme activity. Consistent with this hypothesis, all enzyme modified products showed restricted glucose release following hydrolysis. The BE→AM→BE products were slightly (but each one not significantly) less susceptible than glycogen. All enzyme-modified starches showed slow initial rates of glucose release, indicating that the amylopectin clusters were rearranged. Presumably, more random positions as compared to normal starch and/or highly branched amylopectin clusters and highly branched cyclic-amylopectin clusters were synthesised.

The resistance towards amylolytic degradation is directly related to the affinity of the amylases towards specific linear and branched configurations. PPA needs at least four glucose units between two branch points to cleave this α-glucan molecules (Damager et al., 2005). GA hydrolyses α-1,4 glucosidic linkages at an approximately 30-fold higher rate than α-1,6 glucosidic linkages (Pazur and Ando, 1960). This difference in rates indicates that the α-glucan molecules left after amylolytic digestion may therefore have at least one branch point left from the non-reducing end. The other branch positions must be close to each other and mostly composed of chains branched every two to four glucose units. The presence of such structures provides a mechanistic basis for the lower hydrolytic susceptibility of the branched products as compared to cassava starch. The presence of branched malto-oligosaccharides (Figure 3.8) confirms that α-1,6 linkages were present on the

α-glucan chains every two to four glucose units which PPA could not digest. These are the shortest linear segments that PPA can cleave as mentioned above, resulting in larger branched malto-oligosaccharides with DP ≥ 4 (Figure 3.8). Hydrolytic, branched products likely formed following PPA catalyzed hydrolysis are shown in Table 3.8.

Table 3.8 GA resistant molecules of cassava starch, glycogen and enzyme-modified starches after debranched by isoamylase. BE: BE-treated starch for 24 h, N.A. is not avaiable.

Samples	**GA resistant molecules (%)**
Cassava starch	N.A.
Glycogen	N.A.
BE	1.3
AM 24 h	1.8
AM 3 h→BE	1.7
AM 24 h→BE	1.7
BE→AM 3 h→BE	0.4
BE→AM 24 h→BE	0.1
AM&BE 60°C, pH 6.5, 24 h	0.8

Our results demonstrate the potential of using combinatorial enzyme modification, in this case the glucanotransferases AM and BE, to modify bulk starch polysaccharides and thereby modulate dietary digestibility and functionality. Such products have possible applications as soluble dietary fibers conferring prebiotic properties. The approach also reduces industrial waste as compared to classical chemical modification.

3.5 Conclusions

Diverse structures of highly branched, nearly monodisperse and soluble α-glucan products were produced by different combinations of AM and BE catalysis using cassava starch as substrate. Sequential BE→AM→BE catalysis resulted in more extensive branching as compared to all other enzyme treatment combinations and the products also exhibited higher branching than glycogen. These findings demonstrate the importance of combinatorial catalytic approaches to optimize the synergistic effects of α-glucantransferases in order to optimize molecular structures like α-glucan branching. All α-glucan products had slower amylolytic enzyme digestion rates as compared to native cassava starch making these α-glucans potentially prebiotic. The practical implications are found within new combinatorial and industrially up-scalable ways to produce slowly digestible carbohydrates.

3.6 Acknowledgements

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3.7 Publications

Major content in this Chapter is published in the article:

Sorndech, W., Meier, S., Jansson, A. M., Sagnelli, D., Hindsgaul, O., Tongta, S., and Blennow, A. (2015). Synergistic amylomaltase and branching enzyme catalysis to suppress cassava starch digestibility. **Carbohydrate Polymers.** 132: 409-418.

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CHAPTER IV

EFFECT OF AMYLOSE CONTENT ON BRANCHING ENZYME AND AMYLOMALTASE CHAIN TRANSFER

4.1 Abstract

Thermostable branching enzyme (BE, EC 2.4.1.18) from *Rhodothermus obamensis* in combination with amylomaltase (AM, EC 2.4.1.25) from *Thermus thermophilus* was used to modify starch structure exploring potentials to extensively increase the number of branch points in starch. Amylose is an important constituent in starch and the effect of amylose on enzyme catalysis was investigated using amylose-only barley starch (AO) and waxy maize starch (WX) in well-defined ratios. All products were analysed for amylopectin chain length distribution, α-1,6 glucosidic linkages content, molar mass distribution and digestibility by using rat intestinal α-glucosidases. For each enzyme treatment series, increased AO content resulted in a higher rate of α-1,6 glucosidic linkage formation but as an effect of the very low initial branching of the AO, the final content of α-1,6 glucosidic linkages was slightly lower as compared to the high amylopectin substrates. However, an increase specifically in short chains was produced at high AO levels. The molar mass distribution for the enzyme treated samples was lower as compared with substrate WX and AO, indicating the presence of hydrolytic activity as well as cyclisation of the substrate. For all samples, increased amylose substrate showed decreased α- and β-amylolysis. Surprisingly, hydrolysis with rat intestinal α-glucosidases was higher

with increasing α-1,6 glucosidic linkage content and decreasing $\overline{M}_w$ indicating that steric hindrance towards the α-glucosidases was directed by the molar mass rather that the branching density of the glucan *per se*. Our data demonstrate that a higher amylose content in the substrate starch efficiently produces α-1,6 glucosidic linkages and that the present of amylose generates a higher $\overline{M}_w$ and more resistant product than amylopectin. The combination of BE→AM→BE provided somewhat more resistant α-glucan products as compared to BE alone.

Keywords: amylose content, branching enzyme, amylomaltase, starch degradation

4.2 Introduction

The biosynthesis of starch and glycogen are complex processes consisting of a multitude of enzyme catalyzed transfer and hydrolysis reactions. In general, starch consists of two main components: amylose and amylopectin. Amylose is composed of long and mainly linear backbone α-glucan chains linked together by α-1,4 glucosidic linkages while amylopectin is a branched component linked together by α-1,4 glucosidic backbone with α-1,6 glucosidic branch points (Pérez and Bertoft, 2010). For the industry, such branching pattern is important and starches are selected and further modified to enhance its versatility and satisfy consumer demand (Tharanathan, 2005). Typically, increased branch density can increases solubility of the starch and can suppress amylolytic hydrolysis providing health-associated functionality (Sorndech et al., 2015). However, such glycogen-like functionality is not available in pure bulk quantity from starch crops.

Enzymatic modification of starch is gaining an interest as a clean and safe alternative to chemical modification (Blennow et al., 2013). Such enzymatic starch modification may be carried out either by the use of recombinant enzymes acting on purified starches, or alternatively the enzymes may be expressed directly in the starch producing crops by GM technology (Hebelstrup et al., 2015). However, the GM crop approach may sometimes modify the starch to an extend where its function as a storage of biomass and energy is jeopardized so that biological functions such as grain germination and seedling establishment are deficient (Shaik et al., 2014). Amylomaltase (AM, 4-α-D-α-glucanotransferase; E.C. 2.4.1.25. glucosyl hydrolase family 13 and 70, GH13 and GH70, www.CaZy.org) transfers α-1,4 glucosidic linkages mainly within amylopectin and amylose, amylopectin cluster, or from amylose to amylopectin (Hansen, Blennow, Pedersen, Nørgaard, and Engelsen, 2008). AM has been proven to add value to starch including its modification to impose gelatin-like functionality (Hansen et al., 2008) and increased degradative resistance (Jiang, Miao, Ye, Jiang, and Zhang, 2014). Branching enzyme (BE, 1,4-α-D-α-glucan:1,4-α-D-α-glucan 6-α-D-(1,4-α-D-α-glucano)-transferase, EC 2.4.1.18, glucosyl hydrolase family 13, GH13, www.CaZy.org) catalyses the formation of branch points in starch and glycogen biosynthesis by the cleavage of existing α-1,4 glucosidic linkages followed by transfer to 1,6 hydroxyl groups to form the tree-like amylopectin and glycogen structures (Roussel et al., 2013; Shinohara et al., 2001). *In vitro*, BE also catalyses a cyclisation reaction to form cyclo-amylose and cylo-amylopectin cluster (Kelly, Dijkhuizen, and Leemhuis, 2009; Takata et al., 1996). Starch modification with BE demonstrates an increase in solubility, reduced viscosity and for AM, increase degradative resistance of the product (Blennow et al., 2013).

The thermostable BE from *R. obamensis* has drawn interest in starch modification to efficiently produce highly-branched α-glucans (Roussel et al., 2013; Shinohara et al., 2001). Both amylose and amylopectin have been tested as substrate to identify possible different mechanisms. Some evidence suggests that *R. obamensis* BE activity was higher towards amylose than amylopectin (Shinohara et al., 2001). However, the substrate used was amylose from starch fractionation (Lee et al., 2008) which had amylopectin contamination or was enzymatic synthesized glucans containing DP 2-60 (Roussel et al., 2013), none of which the chain length was long enough compared to natural amylose. Different types of natural starch differ in their amylose content but there is only limited information on the effect of amylose on BE catalysed formation of α-1,6 glucosidic linkages and cyclo-glucans (Andersson, Rydberg, Larsson, Andersson, and Åman, 2002; Roussel et al., 2013; Takata et al., 1997; Takata, Takaha, Okada, Takagi, and Imanaka, 1996). In a previous study, we modified cassava starch by using combinations of AM and BE to increase the content of α-1,6 branch points. The results showed that the highest content of α-1,6 branching points was achieved when using specific serial combinations of AM and BE.

In the present work we investigate the effects of amylose ratios of the substrate starch on AM and BE catalysis. As model substrates, we use the transgenic barley amylose-only (AO) starch which consists of more than 99% amylose (Carciofi et al., 2012) and 100% amylopectin waxy (WX) maize starch in well-defined ratios. The efficiency of α-1,6 branch formation for BE only and sequential BE→AM→BE treatment was investigated and the molecular structures and amylolytic digestibility analysed *in vitro* using rat intestinal α-glucosidases. The study provides additional

information to better understand how BE only and optimized combinations of BE and AM treatments affect the structure and digestibility of the enzyme-modified α-glucan.

4.3 Materials and methods

4.3.1 Materials

Waxy maize starch (WX) was obtained from Cerestar-AKV I/S (Vodskov, Denmark). Amylose-only (AO) barley starch was obtained from Aarhus University (Aarhus, Denmark). BE, AM and β-glucanase were kindly provided from Novozymes (Bagsvaerd, Denmark). Isoamylase (EC 3.2.1.68, specific activity 210 $U \cdot mL^{-1}$) and β-amylase (EC 3.2.1.2, specific activity 620 $U \cdot mL^{-1}$) was obtained from Megazyme (Wicklow, Ireland). Porcine pancreatic α-amylase (EC 3.2.1.1, specific activity 22 $U \cdot mg^{-1}$), and glucoamylase from *Aspergillus niger* (EC 3.2.1.3, specific activity 129 $U \cdot mg^{-1}$) were purchased from Sigma-Aldrich (Missouri, USA). Proteinase K, recombinant, PCR grade was purchased from Roche (Hvidovre, Denmark). Enzyme activity units of isoamylase, α-amylase and glucoamylase are given according to the supplier.

4.3.2 AO barley starch extraction

Amylose-only barley grains (Carciofi et al., 2012) were ground into fine powder and 200 g of flour were mixed in 2 L of 1 mM DTT (dithiotreitol), and 1% SDS (sodium dodecyl sulfate) for 30 min while stirring. The pellet was collected by sediment the starch granules on ice for 1-2 days. The washing procedure was repeated once. The pellet was resuspended in 2 L of deionized water and the slurry sieved through a 70 μm sieve. To remove trace of cell-wall and protein contaminants, the granular starch preparation was subjected to β-glucanase and proteinase K

treatments and the sedimented starch was washed 3 times in distilled water, once in 96% ethanol and finally air dried at room temperature.

4.3.3 Non-granular AO starch preparation

Non-granular starch was prepared according to Kong et al. (2008). Granular AO starch (5 g) was dissolved in 100 mL of 90% DMSO (dimethyl sulfoxide) by heating the mixture in a boiling water bath with constant stirring for 3 h. The AO slurry was placed at room temperature and 200 mL of 95% ethanol was added with continuous stirring. A further 200 mL of 95% ethanol was added, the slurry was left at room temperature and then centrifuged at 2,500 x g for 10 min. The precipitate was suspended with 25 mL of 95% ethanol and pelleted at 2,500 x g for 10 min. The washing procedure was repeated once with 95% ethanol and finally with acetone. The final non-granular AO precipitate was freeze-dried (Kong, Bertoft, Bao, and Corke, 2008).

4.3.4 Enzymatic modification

4.3.4.1 BE action on substrates with well-defined amylose: amylopectin ratios

BE-modified starch was produced mainly as described (van der Maarel et al., 2005) with slight modifications. The WX and AO mixtures (2% $w \cdot v^{-1}$) with non-granular AO content varied from 0, 20, 50, 80, and 100% (0% AO, 20% AO, 50% AO, 80% AO, and 100% AO) were suspended in 50 mM phosphate buffer, pH 6.5. The suspension was heated to 120^{o}C in an oil bath for 3 h while stirring by magnetic stirrer then cooled to 80^{o}C for 2 min. BE (4,000 $U \cdot g\ starch^{-1}$) was added to the gelatinised starch paste and incubated at 80^{o}C for 30 min, then 60^{o}C for 24 h. The reaction was terminated by heating in boiling water bath for 30 min.

The denatured protein and trace insolubles were removed by centrifugation (1,500 x g for 20 min). The soluble α-glucan product was recovered and dried at 50ºC overnight.

4.3.4.2 BE→AM→BE treatments with well-defined amylose: amylopectin substrate ratios

A gelatinised starch paste was prepared as mentioned above, pH adjusted to 6.5 with 50 mM phosphate buffer, BE (4,000 U·g starch^{-1}) was added and the mixture incubated at 60ºC for 24 h. After termination of the reaction at 100ºC for 30 min, AM (10 U·g starch^{-1}) was added and incubation was performed at pH 6.0, 70ºC for 3 h. The reaction was terminated by boiling at 100ºC for 30 min. In the last step, BE was added and incubation was performed under optimal conditions for BE as described above. The BE reaction was terminated and insoluble materials were removed by centrifugation and the product was dried at 50ºC overnight.

4.3.5 β-amylolysis limit

The β-amylolysis limit is the degree of hydrolysis of the α-glucans by β-amylase which express as a percentage conversion of α-glucans to maltose (Patil, 1976). The procedure was slightly modified from that of Wood and Mercier (1978). The α-glucan solution (1.5 mL, 0.5% w·v^{-1} in 90% DMSO) was mixed with an acetate buffer solution pH 4.8 (0.3 mL, 0.2 M). The β-amylase (4 units) and MilliQ water were added to a total volume of 2.7 mL, the reaction mixture was vortexed and incubated at 37ºC for 48 h. The reducing sugar content (Nelson, 1944) and total sugar content (Dubois, Gilles, Hamilton, Rebers, and Smith, 1956) were measured. The percentage of β-amylolysis limit was calculated as follows (Wood and Mercier, 1978):

$$\text{β-amylolysis limit} = \left(\frac{\text{Reducing sugar after hydrolysed-Reducing sugar of blank}}{\text{Total sugar after hydrolysed-Total sugar of blank}}\right) \text{x}100$$

4.3.6 Chain-length distribution of debranched α-glucan

The chain-length distribution of debranched α-glucan samples were analysed by high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The α-glucan samples were gelatinised by boiling and enzymatically debranched by using 0.24 U of isoamylase per 5 mg of sample at 40ºC. The obtained linear α-glucan fragments were analysed by HPAEC-PAD (Dionex, Sunnyvale, CA, USA). Samples of 20 μL (100 μg of linear α- glucan) were injected on a CarboPac PA-200 column using 0.4 $mL \cdot min^{-1}$ flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0-5 min: 0-110 mM linear gradient, 5-130 min: 110-350 mM convex gradient. Single peaks were integrated between DP 3-60 and corrected for the detector response (Viksø-Nielsen, Blennow, Nielsen, and Møller, 1998). The average DP was calculated from the corrected values of the relative content of each chain.

4.3.7 Molar mass distribution, hydrodynamic radius and dispersity analysis by size-exclusion chromatography with triple detection array (SEC-TDA)

The weight-average molar mass distribution ($\overline{M}_w$), the hydrodynamic radius (R_h) and the dispersity ($\overline{M}_w/\overline{M}_n$) were determined by size exclusion chromatography (SEC) using a Viscotek system (Malvern, UK) equipped with a GS-520 HQ column (Shodex, Showa Denko, Japan) attached to a TDA302 module (Triple detector array) consisting of a refractive index detector (RI), a four-bridge visco-meter detector (VIS) and a light scattering detector (LS). The LS consisted of a right angle light scattering (RALS) and a low angle light scattering (LALS). The calibration of the instrument was made using pullulan (50,000 $g \cdot mol^{-1}$, dispersity

1.07, Showa Denko) as a standard which solubilised in MilliQ water (1 mg·mL^{-1}) and mixed at 99ºC for 120 min at 1,000 rpm. Elution was carried out using 50 mM ammonium formate (HCO_2NH_4) buffer, pH 4.5, at 0.5 mL·min^{-1} flow rate. Samples were filtered through a 0.22 µm syringe filter and automatically injected (GPC max module) into the column. The injection volume was 50 µL and the column temperature was 60ºC. The analysis was performed using the OmniSec Software 4.7 (Malvern Instrument, ltd.).

4.3.8 Molar mass distribution, hydrodynamic radius and dispersity analysis by asymmetrical flow field-flow fractionation coupled with multiangle laser light scattering (AF4-MALLS)

AF4-MALLS was used to study high molecular and shearing-sensitive samples like unmodified starch. AO were dissolved in 95% DMSO, precipitated with ethanol and dried (Bello-Pérez, Colonna, Roger, and Octavio, 1998; Rolland-Sabaté, Guilois, Jaillais, and Colonna, 2011). WX was solubilised in water by microwave heating under pressure (Bello-Pérez et al., 1998; Rolland-Sabaté et al., 2011) and filtered (5 µm). AO starch was directly solubilised at a concentration of 10 mg·mL^{-1} in 1 M KOH, for 2 h at 4ºC. The solution was diluted ten times with water and filtered through a 0.45 µm membrane filter. Solubilisation recoveries were calculated by comparing carbohydrate concentrations after and before filtration (Rolland-Sabaté et al., 2011). Aliquots (50 µL and 100 µL) were injected on to the AF4-MALLS instrument using an autosampler WPS-3000SL (Thermo Scientific, Waltham, USA). The equipment included a long AF4 channel, a ThermosPRO oven thermostated at 25ºC, and Eclipse to control the flows in the channel (Wyatt Technology Corporation, Santa Barbara, USA). The channel geometry was trapezoidal with a tip-to-tip length

of 291 mm. A 350 µm polyester spacer and a regenerated cellulose membrane with a cutoff of 10,000 $g \cdot mol^{-1}$ was from Merck Millipore (Darmstadt, Germany). The two on-line detectors comprised a MALLS instrument (Dawn® HELEOS™) fitted with a K5 flow cell and a He-Ne laser (λ = 658 nm) and an Optilab refractometer operating at the same wavelength (Wyatt Technology Corporation, Santa Barbara, CA). The carrier (Millipore water containing 0.2 $g \cdot L^{-1}$ sodium azide) was filtered through 0.1 µm and degassed. Starches were eluted with the flow method described in Rolland-Sabaté et al. (2011). Elution recovery rates were calculated from the ratio of the mass eluted from the channel and the injected mass. $\overline{M}_w$, R_h and dispersity ($\overline{M}_w/\overline{M}_n$) were calculated using ASTRA® software from WTC (version 6.1.2.84 for PC). A value of 0.146 $mL \cdot g^{-1}$ was used as the refractive index increment (dn/dc) for glucans.

4.3.9 Determination of α-1,6 glucosidic linkages by ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR)

α-Glucan samples (0.3% $w \cdot v^{-1}$) were prepared in 500 µL D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) under gentle heating. Bruker (Fällanden, Switzerland) DRX spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK) were used to record the ^{1}H-NMR spectra at 37ºC. Spectra were recorded by sampling 16,384 complex data points during an acquisition time of 1.7 sec, employing 32 transients and a recycle delay of 10 sec for reliable quantifications. Bruker Topspin 2.1 software with zero filling in all dimensions and mild resolution enhancement were used to process NMR spectra.

4.3.10 Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS were used to validate cyclo-structures of the samples. The matrix was 20 $mg \cdot mL^{-1}$ of 2,5-dihydroxybenzoic acid in 30% acetonitrile in water with 0.1% TFA. The α-glucan samples were dissolved in DMSO and diluted with water to a final concentration of 1 $\mu g \cdot mL^{-1}$. The diluted analyte solution (2 µL) was combined with the matrix solution (2 µL) and this solution (0.5 µL) was added to the target and air-dried. MALDI-TOF MS was performed on a Bruker Daltonics Microflex instrument operating in reflectron mode. A 340 nm laser was used and mass spectra were typically accumulated from 1,000 laser shots. Spectra were generally acquired over a 4,000 m/z range.

4.3.11 Hydrolysis properties by human pancreatic α-amylase

Human pancreatic α-amylase hydrolytic degradability of the α-glucan samples was investigated following solubilisation of the α-glucan in 10 mM PBS buffer (pH 6.9) at 1% ($w \cdot v^{-1}$) concentration by boiling for 20 min. After cooling, an aliquot of the sample (1 mL) was incubated with 500 U human pancreatic α-amylase (Meridian Life Science, Inc., Saco, Maine) at 37ºC for 24 h with mixing. The reaction was terminated by boiling for 20 min. The hydrolysed samples containing α-limit dextrins were analysed for their apparent molar mass by HPSEC-RI (Zhang, Ao, and Hamaker, 2006) using pullulans as standard (Polymer Laboratories Inc. Amherst, MA).

4.3.12 Determination of glucose release from modified α-glucan samples by rat intestinal α-glucosidases

An aliquot of the α-limit dextrins (200 µL) was further incubated at 37ºC with the rat intestinal α-glucosidases (500 U, one unit (U) enzyme activity

arbitrarily defined as 1 µg of glucose released from 1% maltose per 10 min at 37ºC). The amount of released glucose at 240 min was determined by the glucose oxidase/peroxidase (GOPOD) method (Vasanthan, 2001).

4.4 Results and discussions

4.4.1 α-1,6 glucosidic linkages

Mixtures of well-defined amylose:amylopectin ratios ranging 0, 20, 40, 60, 10, and 100% amylose generated by mixing AO and WX starches were treated with BE separately and AM and/or BE in sequential steps (BE→AM→BE). The α-1,6 linkage contents of substrates and products as determined by ^{1}H-NMR (Table 4.1) showed that the modified starches had a higher content of α-1,6 linkages ranging 7.0-8.7% for BE-treated starch and 8.0-9.7% for BE→AM→BE-treated starch compared to the original AO (0.2%) and WX (3.5%) substrates. For each enzyme treatment series, a more AO content resulted in a higher rate of α-1,6 linkage formation (Table 4.1). An increase in α-1,6 linkage formation for the 0% AO sample treated with BE was 1.5-fold while the 100% AO sample showed a 34.0-fold increase as compared to the original substrates (Table 4.1). These results are in agreement with those obtained by Shinohara et al. (2001) who suggested that *R. obamensis* BE has a six-fold higher activity for amylose than for amylopectin (Shinohara et al., 2001). However, another study (Roussel et al., 2013), using synthetic amylose DP 2-60 as a substrate for *R. obamensis,* BE showed that BE preferentially used branched substrates as acceptors. Our data provides a possible explanation for this apparent discrepancy in that BE preferably uses the slightly branched AO chain segments as both donors and acceptors substrates. The BE activity on the WX starch was possibly

limited by branching steric hindrance and we suppose that new branch points in the 0%AO sample might be mainly located on the outer chains of glucan structure and a higher AO ratios in AO:WX mixture provides a more optimal donor substrate for BE to create new branch points.

Table 4.1 Content (%) and the increased ratio of α-1,6 linkages of the enzyme-modified starches. WX, waxy maize starch; AO, amylose-only barley starch; BE, branching enzyme-treated starch; and BE→AM→BE, starch treated with branching enzyme followed by amylomaltase and completed with branching enzyme.

Samples/ Enzymes treatment	**AO content (%)**	**α-1,6 (%)**	**Increase in ratio of α-1,6 (fold)****
WX	0	3.5	-
AO	100	0.2*	-
BE	0	8.7	1.5
	20	8.4	2.0
	50	8.2	3.4
	80	7.2	7.4
	100	7.0	34.0
BE→AM→BE	0	9.7	1.8
	20	8.9	2.1
	50	8.7	3.7
	80	8.5	8.9
	100	8.0	39.0

*Degree of branching calculated from ^{1}H-NMR except for the AO substrate, which was determined by reducing end analysis.

**The increment of α-1,6 as compared to the initial values calculated from the substrate mixture ratios (Table 4.2).

Table 4.2 The increment of α-1,6 as compared to the initial values calculated from the substrate mixture ratios.

Samples/ Enzymes treatment	AO content (%)	Initial α-1,6 (%)
WX	0	3.5
AO	100	0.2*
BE or BE→AM→BE	0	3.5
	20	2.8
	50	1.9
	80	0.9
	100	0.2*

*determined by reducing end analysis.

The BE→AM→BE modified starches showed a higher content of α-1,6 linkages as compared to the BE only (Table 4.1). This is in agreement with our previous study using cassava starch as substrate (Sorndech et al., 2015). An increase in α-1,6 linkages for the 0% AO (only WX, amylopectin) treated sequentially with BE→AM→BE was 1.8-fold as compared to the WX substrate while the 100% AO showed a 39.0-fold increase. These increments are slightly higher than the BE only catalyzed branching described above and demonstrates that AM provides a more efficient substrate for BE to slightly further increase the degree of branching by its disproportion activity. It should be noted that as an effect of the very low initial branching of the AO starch, the final content of α-1,6 linkages was slightly lower with increasing AO content (Table 4.1). Both BE and BE→AM→BE treated glucans showed that a higher AO content resulted in the more α-glucose monomers or reducing end groups. Support for such a minor hydrolytic activity was provided by ^{1}H-NMR data of anomeric hydrogens in α-glucose monomers or end group located at 5.30 ppm (Figure 4.1). The presence of these segments may be a consequence during transfer activity leaving the un-attached glucan chains.

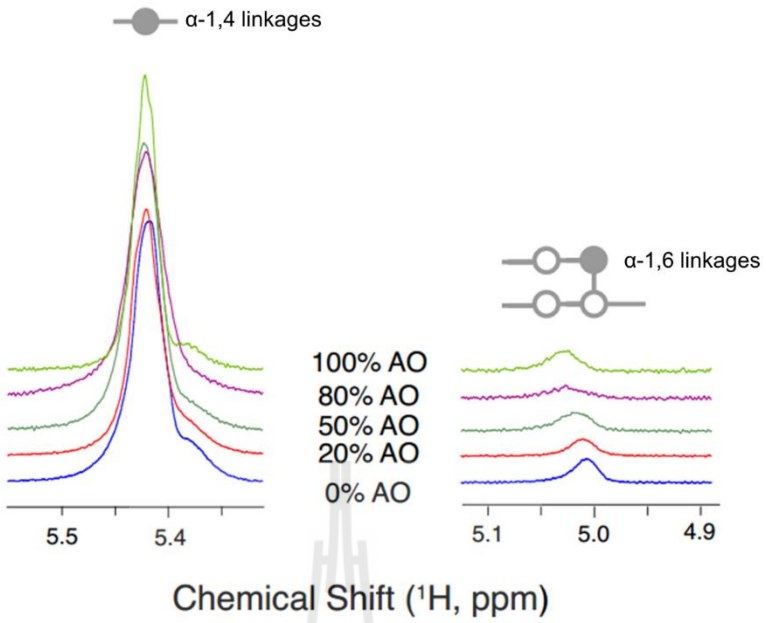

Figure 4.1 ^{1}H NMR spectra of enzyme-treated samples. Different glucose spin systems and the corresponding structural motifs are indicated by circles. Filled circles symbolize the glucose spin system that yield the NMR signal in a structural motif indicated by open circles. Horizontal lines symbolize α-1,4 linkages, while vertical lines symbolize α-1,6 branch points of BE→AM→BE treated samples. AO, amylose only barley starch; BE, branching enzyme-treated starch, BE→AM→BE, sequential BE/AM/BE treatments.

4.4.2 Chain-length distribution

The native and the modified α-glucan products were debranched using excess isoamylase in order to determine the chain length distribution profiles between DP 3-65 by HPAEC-PAD. The unit chain distribution can be classified into A-chains (DP 6-12), B1-chains (DP 13-24), B2-chains (DP 25-36) and long B3-chains (DP > 37) (Hanashiro, Abe, and Hizukuri, 1996). The BE and BE→AM→BE

modified starches had higher number of short chains of approximately DP 3-12, corresponding to A-chains, as compared to AO and WX substrates (Figure 4.2A, Table 4.3). For the BE modified starches, the depletion in the chains of DP > 12 (Table 4.3) suggests the consumption of B1, B2 and long chains, which were used as donor substrates to produce new branch points by BE creating the A-chains. The long chains (mainly B3-chains) were virtually absent after enzyme modification suggesting a complete consumption of B3 chains. Especially, for the 0% AO starch treated only with BE, the DP 3-13 chains were abundant after debranching while chains of DP ≥ 14 were decreased (Figure 4.2D). However, for the 100% AO starch treated the same, this decrease affected the slightly longer chains (DP ≥ 17, Figure 4.2D). This difference, though minor, may explain the relatively good correlation between the increased AO and the maximum length of donor substrate chains, which increased from DP 13 for the 0% AO substrate to DP 16 for the 100% AO substrate. These results suggested that BE prefers long chain glucans present in amylose as donor substrate (Figure 4.5C). However, a minor part of the DP 3-12 chain pool was already present in the modified α-glucans before debranching (data not shown). The amount of these chains was increased with the more AO substrate content supporting that BE prefers to use AO as a donor substrate for the branching reaction cleaving off donor residual segments until the chains were too short to accommodate in the BE catalytic subsites. As deduced from our data, the optimal chain length for BE substrate binding was DP 16 and for chain transfer DP 6-7 from the non-reducing end (Figure 4.2D). These results are in agreement with previous investigations (Kittisuban, Lee, Suphantharika, and Hamaker, 2014; Roussel et al., 2013) using *R. obamensis* BE with selected types of starches as substrate. Interestingly, the chains in the BE→AM→BE modified starches clearly showed a distributional trend

characterized by an increase in short chains of DP 3-12 with higher AO substrate content (Figure 4.2C). For the debranched products produced from the 0% AO substrate treated with BE, the DP 3-12 chain pools were dramatically increased while chains DP ≥ 12 were lower (Figure 4.2E). For the corresponding 100% AO substrate this decrease occurred at somewhat longer chains i.e., DP ≥ 15 (Figure 4.2C and E). The BE→AM→BE treated starch showed a shorter optimum donor chain length (DP 15) compared to the BE-treated starch mixtures (DP 17), indicating an efficient AM catalysis on AO. The dominant binding mode of AM from *Thermus thermophilus* (Kaper et al., 2007) indicates the transfer of chains at least DP 3 long. Thus, as compared to the BE only treated starch, the optimum donor chains for BE was presumably consumed after BE→AM treatment resulting the presence of shorter transferred glucan chains by BE in the last step. These results differ from data for Bacillus sp. BE for which the optimum donor chain length is considerably shorter i.e. DP 12 (Kiel, Boels, Beldman, and Venema, 1991).

The apparent non-coherence with the amount of α-1, 6 linkages analysed by NMR and the high amount of short chains at approximately DP 3-15 (Table 4.1 and Figure 4.2), can be attributed to minor hydrolysis exerted by the transferases. However, already for non-debranched samples we observed an increase in short chains (approximately DP 3-8) with higher AO:WX ratios. For the debranched samples, DP 6 was the smallest branch side chains created by BE (not shown), however, only in minute amounts. Some long chains may have been lost following gelatinization of the AO and subsequent cooling and aggregation. Such a fraction would not be resolved by HPAEC-PAD but can contribute to the α-1, 4 linkages content in the NMR signal.

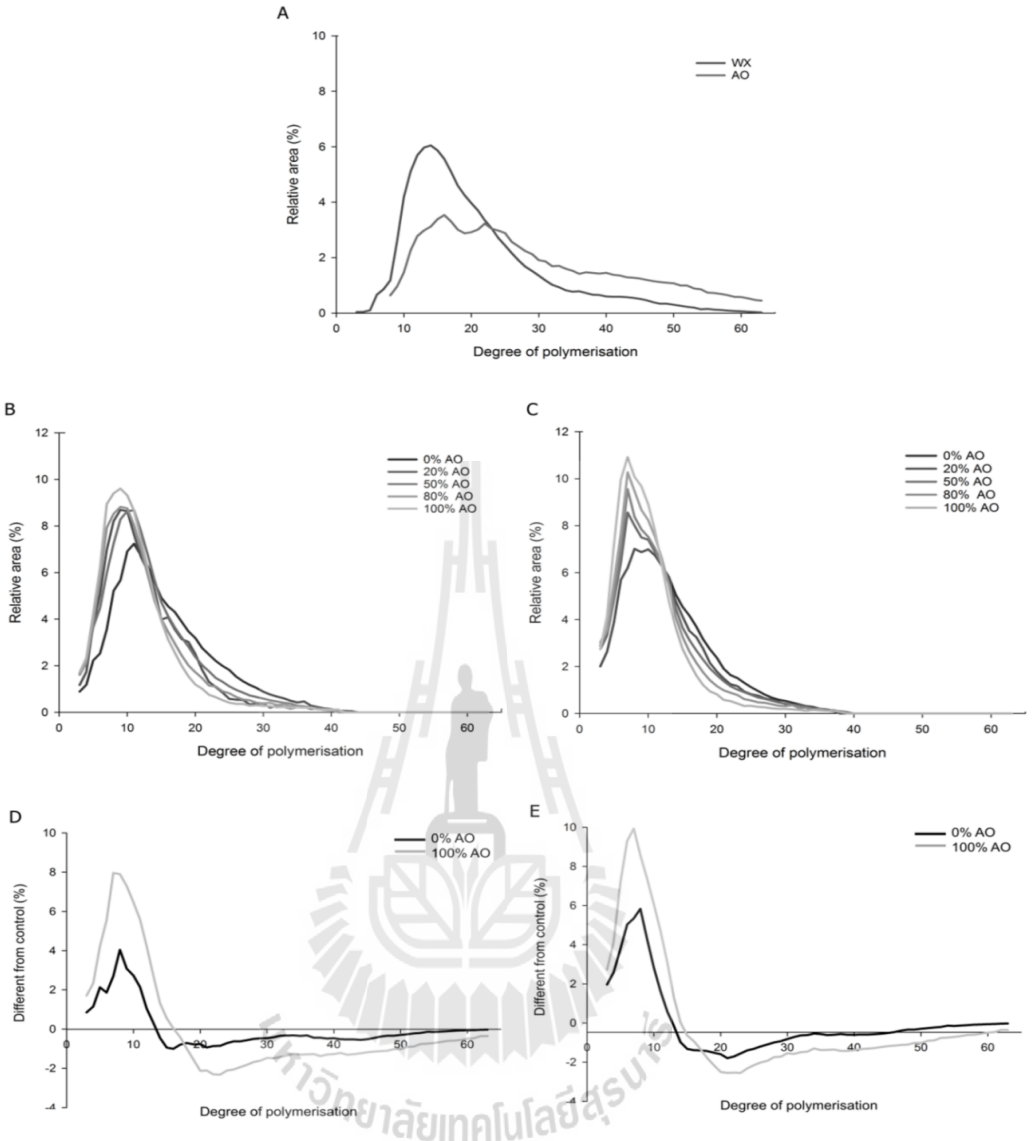

Figure 4.2 Relative area (%) of debranched glucan samples. (A) WX and AO control substrates, (B) BE treated sample, (C) BE→AM→BE treated sample with specific AO content. (D-E) Difference plots relative to controls as indicated, (D) BE treated samples as compared to corresponding control WX and AO substrates, (E) BE→AM→BE treated samples as compared to control WX and AO. WX, waxy maize starch; AO, amylose only barley starch; BE, branching enzyme-treated starch; and BE→AM→BE, sequential enzyme treatments.

Table 4.3 Average chain length distribution (%) of α-glucan products and control AO and WX starches.

% Distribution	WX	AO	BE treated					BE→AM→BE treated				
			0% AO	20% AO	50% AO	80% AO	100% AO	0% AO	20% AO	50% AO	80% AO	100% AO
DP 3-12	20.5	14.6	42.2	58.6	58.5	62.5	67.8	54.3	61.9	65.0	70.6	77.7
DP 13-24	54.2	37.8	45.6	36.7	40.0	31.9	28.4	37.9	32.8	29.8	25.3	19.8
DP 25-36	16.6	22.0	11.2	4.1	7.2	5.1	3.3	6.5	5.2	5.1	3.8	2.3
DP > 37	8.7	25.6	1.0	0.7	0.7	0.5	0.7	0.3	0.1	0.2	0.3	0.2

Note: WX = waxy maize starch; AO = amylose only barley starch; BE = branching enzyme-treated starch; and BE→AM→BE = sequential enzyme treatments.

4.4.3 Molar mass distribution, hydrodynamic radius, dispersity, intrinsic viscosity, and cyclo-structures formation

The molar mass distribution ($\overline{M}_w$), hydrodynamic radius (R_h), dispersity ($\overline{M}_w/\overline{M}_n$) and intrinsic viscosity (IV) of the enzyme-modified starches were determined by SEC-TDA. The $\overline{M}_w$ of AO and WX starch substrates were determined by AF4-MALLS as summarized in Table 4.4. The $\overline{M}_w$ of WX was 3.99×10^8 g·mol^{-1} while for AO was 5.50×10^6 g·mol^{-1}. All BE and BE→AM→BE treated starches had lower $\overline{M}_w$ as compared to the non-modified WX and AO. This indicates that, during the transfer reaction, hydrolysis is also taking place and/or highly branched-amylopectin clusters were produced (Table 4.4 and Figure 4.3). The IV was indifferent for all modified samples and the resulting products derived from the lower AO substrate for both enzyme-treatments showed higher molecular density compared to the use of higher AO substrate. Comparative analysis of synthetic amylose of DP 2-60 and potato amylopectin as a substrates *R. obamensis* BE showed that the potato amylopectin was a better acceptor substrate than the synthetic amylose and those chains larger than DP 11 were optimal donor chain lengths (Roussel et al., 2013). This is in accordance with our data since BE had high activity towards slightly branched and long-chain glucans represented by the AO starch. Minor low molar mass peaks were also observed for the higher AO:WX ratio substrates before the end of the SEC-TDA chromatogram (not shown) indicating various species of molecules generated during enzyme catalysis when using high amylose substrate. The reduction of $\overline{M}_w$ following BE treatment has been reported elsewhere (Kim, Ryu, Bae, Huong, and Lee, 2008; Le et al., 2009). However, an important effect observed for both BE and BE→AM→BE treated starches was an increase in molar mass of the product

with increasing AO:WX substrate ratio (Table 4.4). Supposedly, this effect is due to the high specificity of BE preferably using AO for both donor and acceptor chains as discussed above. The presence of steric hindrance in the WX structure may prevent BE to penetrate the branched structure to access and catalytically act on the acceptor chains during the transfer reaction compared to the more linear AO acceptor chains.

Table 4.4 Molar mass distribution ($\overline{M}_w$), hydrodynamic radius (R_h), dispersity index ($\overline{M}_w/\overline{M}_n$), intrinsic viscosity (IV) and molecular density of the control and enzyme-modified starches. AF4-MALLS was used for AO and WX while SEC (Viscotek-TDA system) was used for enzyme-modified starches.

Enzymes treatment	**AO content (%)**	$\overline{M}_w$ **(×10³ g·mol⁻¹)**	R_h **(nm)**	$\overline{M}_w/\overline{M}_n$	**IV (dL·g⁻¹)**	**Molecular density (g·mol⁻¹·nm⁻³)**
WX*	0	399,000	N.A.	1.4	N.A.	N.A.
AO*	100	5,500	N.A.	6.2	N.A.	N.A.
BE	0	154	12.5	1.7	1.0	18.8
	20	175	13.3	1.6	1.0	17.8
	50	261	15.2	1.6	1.0	17.7
	80	426	17.9	1.6	1.0	17.7
	100	1,000	27.1	1.6	1.0	12.0
BE→AM→BE	0	166	13.0	2.9	1.0	18.0
	20	291	15.9	2.6	1.0	17.3
	50	368	17.3	1.4	1.0	17.0
	80	517	19.1	1.8	1.0	17.7
	100	912	23.9	2.0	1.0	15.9

*The radius of gyration, Rg for WX and AO are 281.0 and 139.0 nm, respectively.

N.A.; Not Available.

The possible production of small cyclodextrins was tested by MALDI-TOF (Figure 4.3). The smallest cyclic α-glucan found was γ-cyclodextrin (DP8, m/z 1,320) and it was detected in all modified starches, especially the AM treated samples. A decrease in $\overline{M}_w$ for all the products indicates the production of cyclostructures with varying $\overline{M}_w$ including cyclo-amylose (Figure 4.5D) and cyclo-amylopectin clusters (Figure 4.5B). Thus, a decrease in $\overline{M}_w$ with decreasing AO ratio is supposedly mainly due to the production of cyclo-amylopectin clusters. However, the high melting point of the AO starch should also be considered. Thermal analysis by differential scanning calorimetry (DSC) of AO revealed that it gelatinised above 100ºC (Carciofi et al., 2012). Eventhough the AO starch was fully gelatinised prior to the following enzyme treatment at 80ºC and then 60ºC, minor aggregation might occur after cooling and during the transfer reactions (Figure 4.5E). The $\overline{M}_w/\overline{M}_n$ ranged 1.4-2.9 for the products demonstrating some degree of dispersity. However, the high $\overline{M}_w/\overline{M}_n$ value (6.2) of the AO substrate was extensively reduced by enzyme treatment. Hence, the reduction in $\overline{M}_w$ following AM and/or BE catalysis is likely a combined effect of hydrolysis and the formation of amylopectin cluster and cyclic α-glucan. However, the monodispersity of the products suggests that the hydrolytic reaction was very minor.

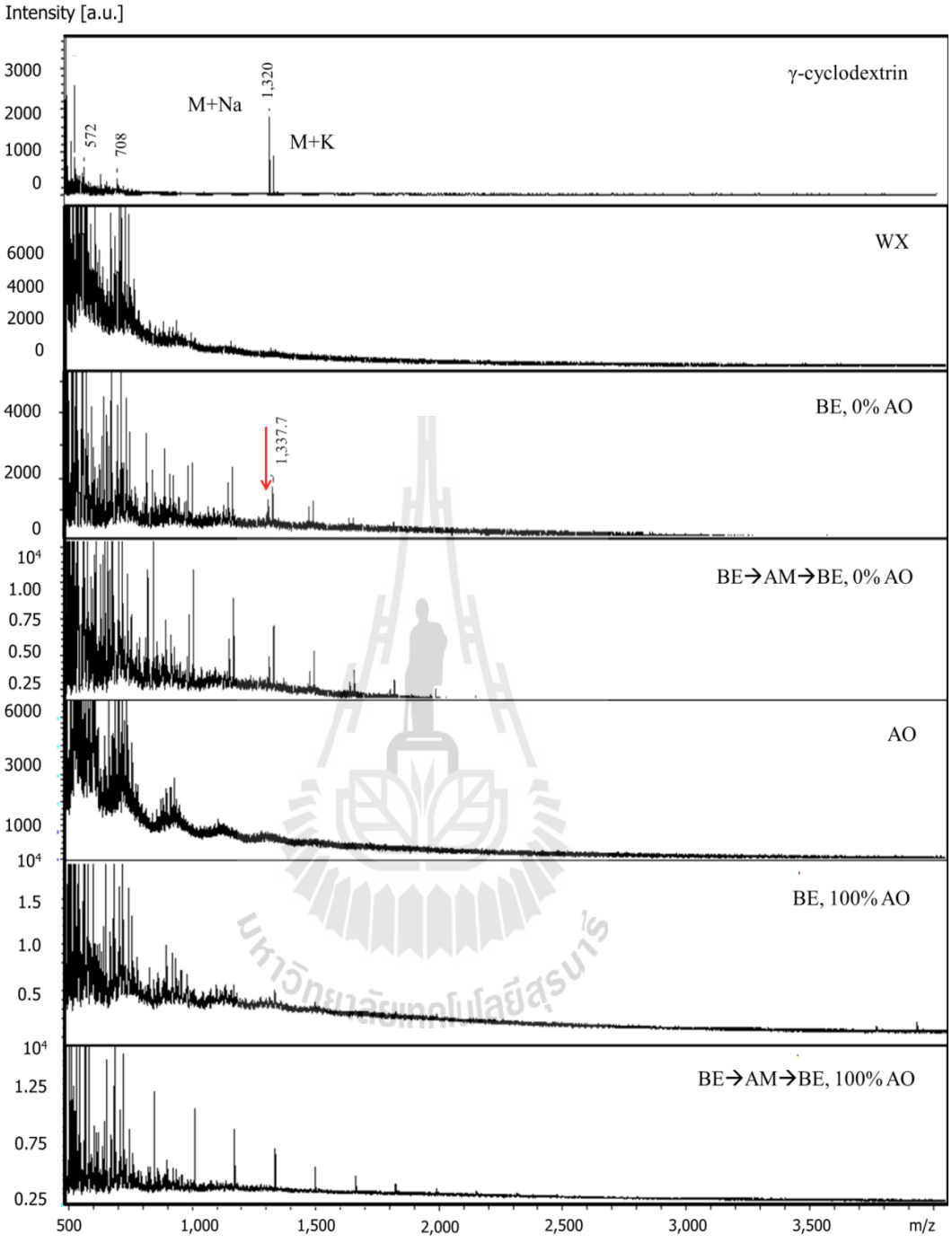

Figure 4.3 MALDI-TOF analysis to identify cyclo-structure (red arrow) formation of enzyme-modified starches.

4.4.4 α-limit dextrin structure and β-amylolysis limit

The content of α-limit dextrin and β-amylolysis limit were determined in order to elucidate their molecular structure. The α-limit dextrin content was determined by HPSEC-RI while the β-amylolysis limit was determined by reducing end analysis (Nelson, 1944). The increased branching of the BE and AM treated products increased the production of α-limit dextrins as compared to the corresponding AO and WX substrates (Table 4.5). However, the β-amylolysis limit decreased with the same treatments demonstrating that the products after BE and AM treatment had lower β-amylase susceptibility than the starch substrate. All BE and BE→AM→BE treated starches produced smaller amounts of α-limit dextrin and lower β-amylolysis limit at higher AO:WX ratios. The decrease in α-limit dextrin was similar for both treatments. The BE-treated samples showed ranging from 32.3% to 6.5% as for the BE→AM→BE-treated samples (from 34.5% to 7.3%, Table 4.5, Figure 4.4). This trend demonstrates that less branched structures were obtained as AO increased which is associated with the branch linkage analysis (Table 4.1). The action pattern of α-amylase involves a virtually random hydrolytic multiple attack to cleave starch into linear and branched dextrins (Bijttebier, Goesaert, and Delcour, 2008) and requires at least a four glucose-unit linear segment between two branch points for catalysis (Damager et al., 2005). The low α-limit dextrin content of the samples having low branch point density is in accordance with the specific action of α-amylase on linear α-1,4 chains. A previous study (Kandra, Gyémánt, Remenyik, Hovánszki, and Lipták, 2002) demonstrated that the maximum frequency of α-amylase attack site is shifted towards the reducing end with longer chain length.

Therefore, our data do not exclude that the distance between each branch points in the products were shorter or equal to four glucose units (Figure 4.5C).

The β-amylase degradation of BE-treated samples was decreased from 20.0% to 33.3% with decreasing AO substrate. This implies that BE preferentially uses AO, initially creating new branch points expected to be located mostly on the non-reducing end of the α-glucan acceptor chains where β-amylase is less active (Figure 4.5C). In addition, the β-amylolysis limit from BE-treated samples was higher than that of the BE→AM→BE-treated samples which decreased from 21.4% to 12.2% with increasing AO (Table 4.5). This result confirms that the disproportionation activity of AM in the BE→AM→BE-treated samples promotes the production of more complex structures as compared to the BE-treated samples. Such branch structures are more β-amylase resistant than randomly branched glucans. The combine results of α-limit dextrin content and β-amylolysis limit provides evidence that the product following BE chain transfer in high amylose:amylopectin systems causes longer distance between the adjacent branch point in the product. The new branch points were located mostly on the non-reducing end of the acceptor α-glucan chains.

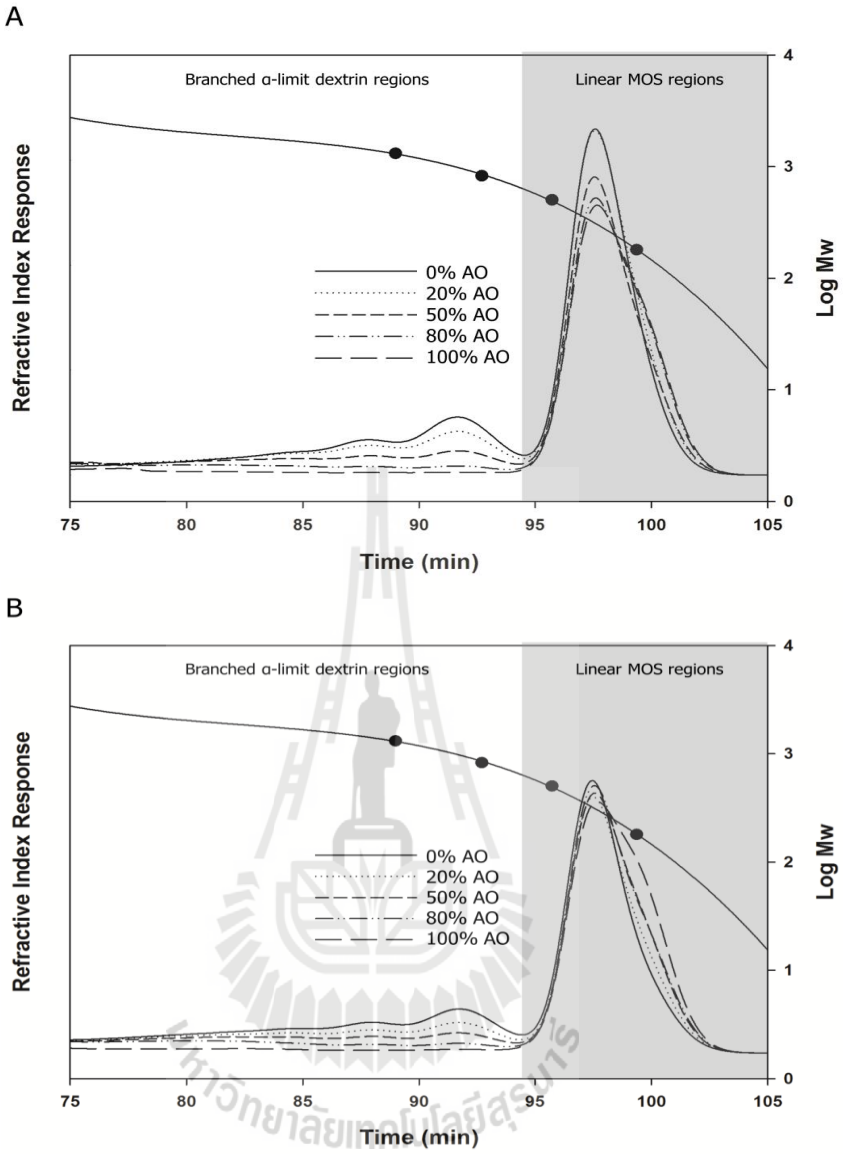

Figure 4.4 Molar mass distribution of enzyme-modified starches by HPSEC-RI after human pancreatic α-amylase treatment. The two different regions indicate branched and linear α-limit dextrins, respectively, as verified elsewhere (Lee et al., 2013). The peak area of branched α-limit dextrins are interpreted in Table 4.5) BE treated samples and B) BE→AM→BE treated samples. Log $\overline{M}_w$ corresponds to the $\overline{M}_w$ calculated with strictly linear pullulan standards.

Table 4.5 The peak area (%) of branched α-limit dextrin regions after human pancreatic α-amylase digestion from Figure 2 compared to the β-amylolysis limit.

Samples/ Enzymes treatment	AO content (%)	Area for branched α-limit dextrin (%)	β-amylolysis limit (%)
WX	0	27.0	47.9
AO	100	3.8	64.8
BE	0	32.3	33.3
	20	27.8	29.3
	50	23.6	25.8
	80	14.3	21.1
	100	6.5	20.0
BE→AM→BE	0	34.5	21.4
	20	29.5	18.7
	50	23.1	14.7
	80	14.9	13.2
	100	7.3	12.2

4.4.5 Glucose released after rat intestinal α-glucosidases hydrolysis

In order to investigate the internal branch structure of the α-limit dextrins (Figure 4.2) obtained from the enzyme modified starches, the samples were subjected to further hydrolysis by rat intestinal α-glucosidases after hydrolysis by human pancreatic α-amylase. These data would also indicate the potential degradative pattern of the α-limit dextrins in the intestinal part of gastrointestinal tract. These amylases consist of maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) and they are mainly active on α-1,4 and α-1,6 linkages, respectively (Lin et al., 2012).

Hydrolysis of α-limit dextrin is expected to specifically slow down the MGAM and SI activities due to the highly-branched structures of these glucans (Lee et al., 2013). Glucose released from all BE and BE→AM→BE treated starches after hydrolysis by human pancreatic α-amylase and rat intestinal α-glucosidases were 4.3-5.8 $mg \cdot mL^{-1}$ and 3.8-5.1 $mg \cdot mL^{-1}$, respectively with increasing AO:WX ratios (Table 4.6). A positive relationship was found between the amount of branched α-limit dextrins in the hydrolysis product and the amount of glucose generated. The α-limit dextrins obtained from the BE→AM→BE treated starches released lower glucose as compared to the BE treated starches. This result demonstrates an effect of AM on arrangement the α-glucan structure due to it disproportionation activity of the α-glucan linear chains or clusters. In addition, a previous study demonstrated that α-glucan hydrolysis rate by MGAM-SI is low on densely branched α-limit dextrin (Kittisuban et al., 2014; Lee et al., 2013). Particular chemical structures including tightly branched clustered in the α-limit dextrin can affect the MGAM-SI catalysis (Lin et al., 2010).

The samples after BE and BE→AM→BE treatment were analysed by HPAEC-PAD in order to investigate the formation of isomaltose, isomaltotriose, and highly branched oligosaccharides. All of them are slowly digested by isomaltase in human body (Gropper, Smith, and Groff, 2009). Only low amounts of isomaltose and isomaltotriose were detected (data not shown). This finding indicates that the fraction denoted as “linear” found in the HPSEC-RI chromatograms possibly consisted of both isomaltose and isomaltotriose and were hence slowly digested. The MGAM has a preference for short α-1,4 linkages while SI has a broader specificity for both α-1,4 and α-1,6 linkages (Sim et al., 2010). The 100% AO product might contain long

chains with highly branch points, and some of them might be in a form of double helical segments which can resist MGAM-SI hydrolysis. Our results are in agreement with those of Ao et al. (2007) who proposed that the slowly digested and resistant properties of the enzyme-modified starch were not affected only from the higher content of α-1,6 linkages and shortened chains. It was also likely to be a minor effect from short amylose fragments that were self-associated which reduced enzyme hydrolysis. The propose products generated from BE and BE→AM→BE on WX and AO substrate were represented in Figure 4.5A-E.

Table 4.6 Glucose released ($mg \cdot mL^{-1}$) from enzyme modified starches and control starch by human pancreatic α-amylase followed by rat intestinal α-glucosidases. The AO:WX ratios indicated are for the original substrates prior to enzyme treatment.

Samples/ Enzyme treatment	**AO content (%)**	**Glucose released ($mg \cdot mL^{-1}$)**
WX	0	5.1 ± 0.3^{bcd}
AO	100	2.6 ± 0.3^{g}
BE	0	5.8 ± 0.0^{a}
	20	5.6 ± 0.2^{ab}
	50	5.3 ± 0.1^{abc}
	80	5.0 ± 0.1^{bcd}
	100	4.3 ± 0.1^{ef}
BE→AM→BE	0	5.1 ± 0.1^{bcd}
	20	4.8 ± 0.2^{cde}
	50	4.5 ± 0.1^{ef}
	80	4.3 ± 0.1^{ef}
	100	3.8 ± 0.1^{f}

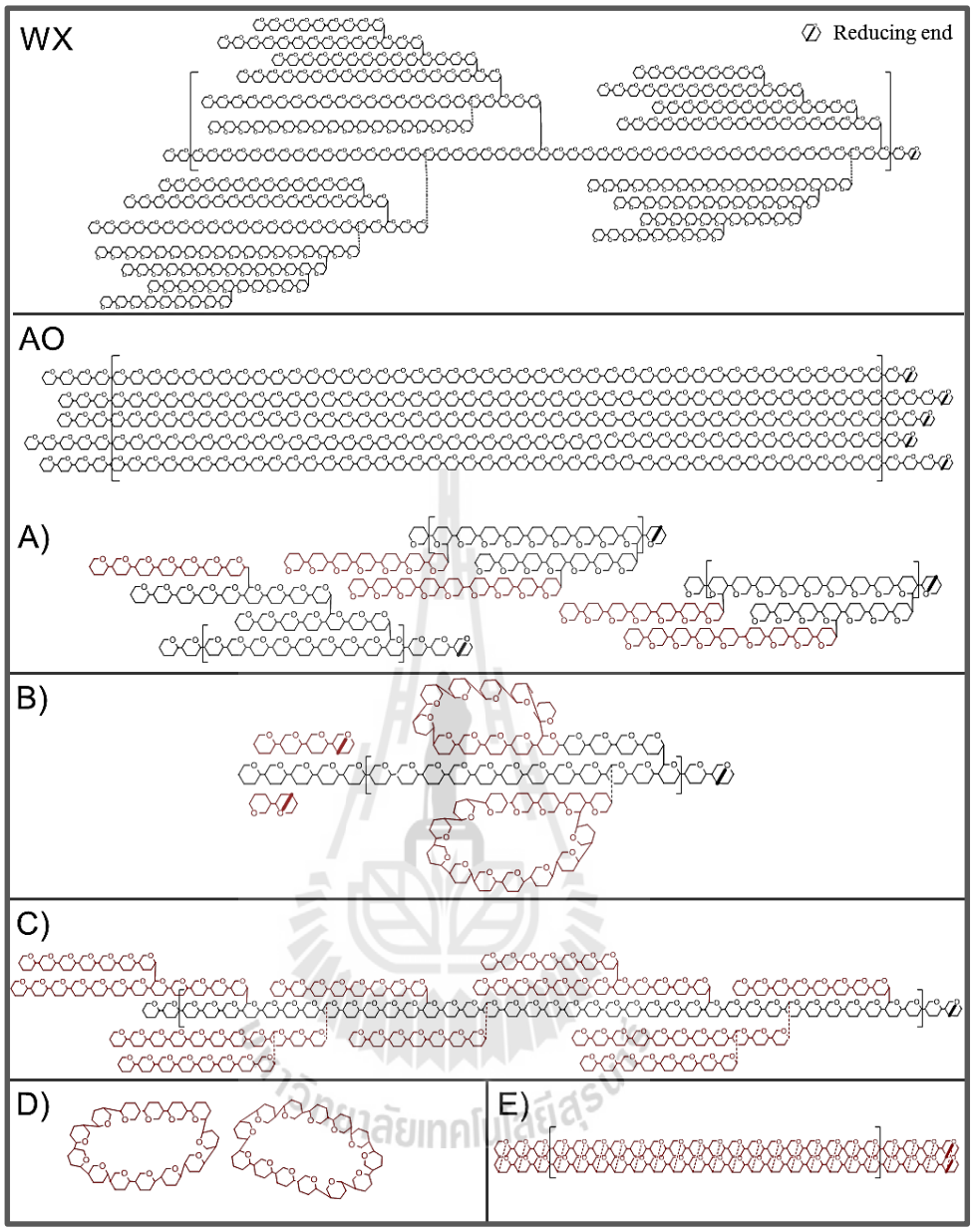

Figure 4.5 Schematic representation the obtained products from BE and BE→AM→BE reaction. The black symbol symbolize α-D-glucose unit and red symbol symbolize newly generated α-glucan. A-B represents enzymes-treated WX products; A: branched-amylopectin cluster; B: cyclo-amylopectin cluster and short α-glucan chains. C-E: represents enzymes-treated AO products; C: branched-amylose; D: cyclo-amylose and E: amylose double helix.

4.5 Conclusions

BE more efficiently catalyzed chain transfer in AO than WX to create new branch points and to produce a low and more monodisperse product. The BE- and BE→AM→BE-treated starches showed higher rate of α-1,6 glucosidic linkages formation and short chains approximately DP 3-16 with higher AO:WX ratios. However, due to a very low initial branch content of the AO starch, the final content of α-1,6 glucosidic linkages was slightly lower at high AO substrate content. Sequential BE→AM→BE catalysis resulted in more extensive branching as compared to using BE alone. This increased branching suppressed amylolytic susceptibility as demonstrated by increased levels of α-limit dextrins for each of the substrates. However, high amylose substrates generated lower level of α-limit dextrin demonstrating that amylose substrate generated less α-amylase resistant products. Further hydrolysis with rat intestinal α-glucosidases showed increased hydrolysis rate with higher α-1,6 glucosidic linkage content and decreased molar mass. This suggests that α-glucosidases is sterically hindered by the molar mass and molecular configuration rather that the branching density of the glucans *per se*. Combined BE→AM→BE produced more resistant α-glucan products as compared to BE alone. Overall, the high amylose starch shows a better potential source to be applied as a raw material for enzymatic modification to produce slowly- and resistant dextrin.

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CHAPTER V

STRUCTURAL AND PREBIOTICS PROPERTIES OF HIGH CASSAVA STARCH CONCENTRATION MODIFIED BY HYDROLASE AND TRANSFERASE ENZYMES

5.1 Abstract

Slowly and indigestible maltodextrin conferring isomaltooligosaccharides (IMOs) production was prepared by simultaneous α-amylase and branching enzyme (BE) followed by α-transglucosidase (ABT) and simultaneous α-amylase and BE followed by simultaneous β-amylase and α-transglucosidase (ABbT) with 30% and 50% ($w \cdot v^{-1}$) cassava starch. ABT catalysis showed branching linkages content of 51.2% and 67.8%, and total dietary fiber (TDF) content was 16.6% and 17.4% when using 30% and 50% substrate respectively. The ABbT catalysis showed branching linkages content of 82.9% and 85.5%, and TDF content was 24.8 and 25.6% when using 30% and 50% substrate respectively. According to Englyst's method, the RDS, SDS and RS from ABT and ABbT samples were approximately 38.1 and 40.7% for RDS, 13.4 and 19.3% for SDS, and 42.1 and 46.0% RS for 30% and 50% substrate respectively. The IMOs composition of the ABT samples were isomaltose, isomaltotriose, panose and higher branched-oligosaccharides which shown lower contents when compared to the ABbT samples. Prebiotics index of 50% substrate

modified by ABbT (3.16) was higher than that of ABT (2.37) and not significant different from FOS but lower than commercial IMOs. All of the samples showed high ability to stimulate the growth of Bifidobacteria and Lactobacillus and inhibit Clostridia (histolyticum subgroup) and Bacteroids. The ABT and ABbT treated smaples can produce higher acetate content when compared to propionate and butyrate. Overall, all α-glucan products had stimulated probiotics activity as compared to native starch suggesting these α-glucans to be potential prebiotics.

Keywords: branching enzyme, α-transglucosidase, isomalto-oligosaccharides, starch nutritional fractions, prebiotics, probiotics

5.2 Introduction

Starch is considered as a major energy source for human and animal that contain varies of nutritional quality depending on their structure and processing based on the glycemic properties (Lee, Bello-Pérez, Lin, Kim, and Hamaker, 2013). Amylose is one of the main components which make up 14-27% of the starch depending on the starch source. This polysaccharide is typically has an α-1,4 glucosidic linkages, and only slightly branched by α-1,6 linkages. Amylopectin makes up 73-86%, being more than 100-fold larger than amylose, and contains approximately 5% clustered α-1,6 linkages . Root starch such as cassava starch is one of the most starches widely used as food because it is easily extractable and very pure products are obtained due to the very low protein and lipid content.

Enzymatic modification of starch is gaining interest as a clean and safe alternative to chemical modification (Blennow et al., 2013). Branching enzyme (BE,

1,4-α-D-α-glucan:1,4-α-D-α-glucan 6-α-D-(1,4-α-D-α-glucano)-transferase, EC 2.4.1.18, glucosyl hydrolase family 13, GH13, www.CaZy.org) catalyses the formation of branch points in starch and glycogen biosynthesis by the cleavage of existing α-1,4 glucosidic linkages followed by transfer to 1,6 hydoxyl groups to form the tree-like amylopectin and glycogen structures (Roussel et al., 2013; Shinohara et al., 2001). Starch modification with BE demonstrates an increase in solubility, reduced viscosity, increase in degradative resistance of the product (Blennow et al., 2013). The thermostable BE has drawn interest in starch modification to efficiently produce highly-branched α-glucans which act as a slowly digested starch (SDS) (Fuertes, Roturier, and Petitjean, 2009; Roussel et al., 2013; Shinohara et al., 2001).

α-Transglucosidase (1,4-α-D-glucan: 1,4-α-D-glucan (D-glucose) 6-α-D-glucosyltransferase, EC 2.4.1.24, GH31, www.CaZy.org) is widely distributed in microorganisms, plants and animals. It catalyses the hydrolytic and transfer reactions of non-reducing glucosyl residues of maltose to generate α-1,6 glucosidic linkages of glucose and maltose to form isomaltose and panose respectively. In addition, α-transglucosidase also transfers non-reducing glucosyl residues to the α-1,2 glucosidic linkages and α-1,3 glucosidic linkages of glucose to form kojibiose and nigerose or back to α-1,4 glucosidic linkages to form maltose. For the traditional methods, α-transglucosidase is combined with the enzymes from the amylase family to produce IMOs (Lee et al., 2002).

SDS is the starch portion which completely digested to glucose during 20-120 min by simultaneous α-amylase and glucoamylase *in vitro* determination according to Englyst method (Englyst, Kingman, and Cummings, 1992; Lehmann and Robin, 2007). The major importance of SDS is that it can extend digestion and

absorption in the small intestine which slows down the postprandial blood glucose level and the glycemic response (Lehmann and Robin, 2007; Zhang and Hamaker, 2009).

IMOs are short α-glucan molecules which linked together by α-1,4 and α-1,6 glucosidic linkages. It consists of predominantly a series of α-1,6 linked IMOs which are isomaltose, panose, isomaltotriose, isomaltotetraose, isomaltopentaose, nigerose, kojibiose, and higher branched oligosaccharides (DP ≤ 10) (Ketabi, Dieleman, and Gänzle, 2011; Koleva, Ketabi, Valcheva, Gänzle, and Dieleman, 2014). IMOs are resistant to amylolytic enzyme digestion and are claimed as a prebiotics substance (Roberfroid, 2007) which is one of the most interesting functions of dietary fiber (Casci and Rastall, 2006). The concept of prebiotics was firstly coiled by Gibson and Roberfroid (2004), "*prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health*" (Al-Sheraji et al., 2013). The prebiotics is related to the term of probiotics which are "*live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host*" (FAO/WHO, 2001). The healthy lifestyle and diet are the most importance factors to protection and prevention of several diseases. The relationships between lifestyle and diet have been reported (Kromhout, Menotti, Kesteloot, and Sans, 2002). Dietary food can influence the metabolic pathways of lowering cholesterol level, decreasing in blood pressure and also saturated fat with high in potassium, calcium, and magnesium absorption (Jenkinsand et al., 2008; Kendall, Esfahani and Jenkins, 2010; Tester, Karkalas, and Qi, 2004). For these reasons, people have become aware of the diet for the health benefits.

In the present work, high cassava starch concentration was used and treated with the combination of α-amylase, β-amylase, BE and α-transglucosidase to produced slowly digested oligosaccharides and IMOs. The structural properties, composition of IMOs and *in vitro* digestion were studied to relate to the prebiotics properties which stimulate probiotics growth. The organic acids and short chains fatty acids (SCFA) were analysed.

5.3 Materials and methods

5.3.1 Materials

Cassava starch was obtained from SanguanWongse Industries Co., Ltd. (NakhonRatchasima, Thailand). A gift of BE (Viksø-Nielsen, Blennow, Nielsen and Møller, 1998) was kindly provided from Novozymes (Bagsvaerd, Denmark). Preparation of α-transglucosidase (TG-L2000) was kindly supplied from Danisco Singapore Pte Ltd- Dupont Industrial Biosciences (Singapore). Porcine pancreatic α-amylase (PPA, EC 3.2.1.1, specific activity 22 $U \cdot mg^{-1}$), glucoamylase (GA, EC 3.2.1.3, specific activity 129 $U \cdot mg^{-1}$) from *Aspergillus niger*, PGO (peroxidase and glucose oxidase) enzyme kit for glucose determination, was purchased from Sigma-Aldrich (Missouri, USA). Enzyme activity units of isoamylase, PPA and GA are given according to the suppliers.

5.3.2 Enzymatic modification

5.3.2.1 Cassava starch simultaneously treated with α-amylase and BE followed by α-transglucosidase

Cassava starch was suspended in deionised water (30% or 50% ($w \cdot v^{-1}$)) adjusted to pH 6.5 with 50 mM phosphate buffer. The suspension was heated

to 80ºC in a water bath and then α-amylase (A, 10 U·g starch^{-1}) and BE (B, 4,000 U·g starch^{-1}) were added to the cassava starch slurry and incubated for 1 h in order to gelatinize starch and then cooled down to 60ºC for 24 h. The reaction was terminated by heating in boiling water bath for 30 min. The pH was adjusted to 4.5 using 50 mM acetate buffer and α- transglucosidase (T, 15 U·g starch^{-1}) was added to the mixture and incubated at 40ºC for 3 h. The reaction was terminated by heating in boiling water bath for 30 min. The α-glucan product was recovered and dried by lyophilisation. The end products from these steps were called as 30ABT and 50ABT.

5.3.2.2 Cassava starch simultaneously treated with α-amylase and BE followed by simultaneously treated with β-amylase and α-transglucosidase

A gelatinised starch paste was prepared as mentioned above. The reaction was terminated by heating in boiling water bath for 30 min. The pH was adjusted to 4.5 using 50 mM acetate buffer. α-transglucosidase (T, 15 U·g starch^{-1}) and β-amylase (b, 30 U·g starch^{-1}) were added to the mixture and incubated at 40ºC for 9 h (for 30% starch) and 15 h (for 50% starch). The reaction was terminated by heating in boiling water bath for 30 min. The α-glucan product was recovered and dried by lyophilisation. The end products from these steps were called as 30ABbT and 50ABbT.

5.3.3 IMOs compositions by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Enzyme-modified starches were gelatinized by boiling and enzymatically debranched at 40ºC for 48 h by using 0.24 U of isoamylase per 5 mg of

sample. The obtained linear α-glucan fragments were analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA). Samples of 20 μL (100 μg of linear α- glucan) were injected on a CarboPac PA-200 column using 0.4 $mL \cdot min^{-1}$ flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0-5 min: 0-110 mM linear gradient and 5-130 min: 110-350 mM convex gradient while for determined the IMOs compositions gradient profile were: 0-5 min: 0-110 mM linear gradient and 5-60 min: 110-350 mM. Single peaks were integrated and, corrected for the detector response (Viksø-Nielsen et al., 1998).

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

Molecular composition was evaluated by size exclusion chromatography (SEC) using a Viscotek System (Malvern, UK) equipped with a GS-220HQ column (Shodex) with a size exclusion limit of 3.0×10^3 $g \cdot mol^{-1}$. The column was attached to a TDA302 module (Triple detector array), a refractive index detector (RI) glucan size was estimated from glucose, maltose and authentic linear glucan standards (maltotriose, maltotetraose maltopentaose, maltoheptaose, and maltooctaose). The analysis was performed using the OmniSec Software 4.7 (Malvern Instrument, ltd.).

5.3.5 α-1,6 glucosidic linkage determination by nuclear magnetic resonance spectroscopy (NMR)

The α-glucan samples were dissolved in 500 μL D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) to concentrations of 0.3% ($w \cdot v^{-1}$) under gentle heating. ^{1}H-NMR spectra were recorded on a Bruker (Fällanden, Switzerland)

DRX spectrometer equipped with a TCI CryoProbe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK) at 37ºC. Spectra were recorded by sampling 16,384 complex data points during an acquisition time of 1.7 sec, employing 32 transients and a recycle delay of 10 sec for reliable quantifications. NMR spectra were processed using Bruker Topspin 2.1 software with zero filling in all dimensions and mild resolution enhancement. For the latter, we employed a time domain Lorentzian-Gaussian Transformation with a line broadening of -1 Hz and a Gaussian broadening of 0.3. Anomeric signals of branch point α-1,6 linkages were quantified relative to anomeric signals of α-1,4 linkages (Miller, Klyosov, Platt and Mayo, 2009).

5.3.6 Total dietary fiber (TDF) analysis

This analysis was investigated according to AOAC Method 2009.01 & 2011.25 & AACC Method 32-45.01 & 32-50.01.

5.3.6.1 Sample preparation

Samples were weighed for 1.000 g into 250 mL round bottles and run two blanks. The samples were wetted using 1 mL of ethanol then 40 mL of pancreatic α-amylase and GA. The bottles were transferred to a shaking water bath and incubated at 37ºC, for 16 h with shaking at 150 rpm. Samples were immediately adding with 3.0 mL of 0.75 M tris-base solution to terminate the reaction. The bottles were placed in a water bath at 95-100ºC, and incubated for 20 min then cooled to 60ºC. Protease solution (0.1 mL) was added and incubated at 60ºC for 30 min. Two M acetic acid was added for 4 mL to adjusted pH to 4.3 and 1 mL of D-sorbitol internal standard solution (100 mg·mL^{-1}) was added.

5.3.6.2 Determination of total high molecular weight dietary fiber (HMWDF)

According to AOAC method 2009.01, the sample was pre-heated to 60ºC and 95% ($v \cdot v^{-1}$) ethanol which pre-heated to 60ºC was further added then precipitated at room temperature for 60 min. One mg of Celite® was weighed and wetted with 15 mL of 78% ($v \cdot v^{-1}$) ethanol in the crucible. The precipitate was filtered through crucible by using vacuum pump then the residue was washed with two 15 mL portions of the following: 78% ($v \cdot v^{-1}$) ethanol, 95% ($v \cdot v^{-1}$) ethanol and acetone. The crucibles were dried overnight in 105ºC hot air oven and cool in desiccator and weighed (Brunt and Sanders, 2013).

5.3.6.3 Protein and ash determination

The residue from crucibles were analysed for protein and ash. Protein analysis was performed on residue using Kjeldahl methods. The factor of 6.25 was used for all cases to calculate mg of protein. For ash analysis, the second residue was incinerated for 5 h at 525ºC then cooled in desiccator.

5.3.6.4 Determination of total low molecular weight dietary fiber (LMWSDF)

The filtrate from 5.2.6.1 were pre-heated to 60ºC and 280 mL of 95% ($v \cdot v^{-1}$) ethanol which was pre-heated to 60ºC were added and mixed thoroughly. After precipitation, 5 mL of 150 mM HCl were added to the samples which were placed into the evaporator flask. Then the samples were evaporated until dryness and 5 mL of deionized water were added. Two mL of this solution was transferred to the column containing 4 g of each Amberlite® FPA 53 (OH-) and Ambersep®200 (H+) (Post, Marshak and DeVries, 2010). The column was eluted at a

rate of 1.0 mL·min^{-1} into a 50 mL test tube. Twenty mL of deionised water were added to the column and eluted at a rate of 1.0 mL·min^{-1}. The eluate was transferred to a 250 mL round bottom rotary evaporator flask and evaporated to dryness at 60ºC. Two mL deionised water was added to the flask and redissolved the sugars by swirling the flask for 2 min then transfer the solution to a storage container. Calculation for HMWDF is following the equation:

$$\text{Blank (B) determination (mg)} = \frac{BR_1 + BR_2}{2} - BP - BA$$

where: BR1and BR2 = residue mass (mg) for duplicate blank determinations respectively.

BP and BA = mass (mg) of protein and ash respectively, determined on first and second blank residues.

$$\text{HMWDF} \left(\frac{\text{mg}}{100\text{g}}\right) = \frac{\frac{R_1 + R_2}{2} - RP - RA - B}{\frac{M_1 + M_2}{2}} \times 100$$

where: R1 = residue mass 1 from M1 in mg; R2 = residue mass 2 from M2 in mg.

M1 = test portion mass 1 in g; M2 = test portion mass 2 in g.

RA = ash mass from R1in mg; RP = protein mass from R2 in mg.

5.3.6.5 Determination of LMWSDF using HPLC

The sample was transferred to a 3 mL disposable syringe and filter through a 0.45 µm filter. Fitty µL of sample was injected to the injection loop on the LC. Standard glucose solution was prepared with concentration of 5, 10, and

20 mg·mL^{-1}. HPLC (Agilent 1260 Infinity LC Systems, Agilent Technologies, USA) were equipped with VertiSep^{TM} Sugar column (6.5×300 mm). Solvent was DI water containing 50 mg/L Na_2Ca-EDTA. The analysis was performed at 80ºC and flow rate of 0.3 mL·min^{-1}.

$$SDFS=Rf\times(Wt\text{-}IS)\times\frac{(PA\text{-}SDFS)}{(PA\text{-}IS)}\times\frac{100}{M}$$

where: Rf is the response factor.

(Response factor (Rf) = (PA-IS) / (PA-Glu) x (Wt-Glu / Wt-IS)

where: PA/IS = peak area internal standard (D-sorbitol).

PA/Glu = peak area D-glucose.

Wt/Glu = mass of D-glucose in standard.

Wt/IS = mass of D-sorbitol in standard.

Wt/IS is weight in mg of internal standard contained in 1 mL of internal standard solution pipetted into sample mixture (100 mg).

SDFS is the dietary fiber which soluble in water and not precipitated in 78% aqueous ethanol.

PA/SDFS is the peak area of the dietary fiber soluble in water and not precipitated in 78% aqueous ethanol.

M is the test portion mass (M1or M2) in grams of the sample whose filtrate was concentrated and analysed by LC.

Integrated TDF (%) = HMWDF (%) + SDFS (%)

5.3.7 *In vitro* α-glucan digestion analysis

In vitro α-glucan degradation was analyzed by a modification of the Englyst method using gelatinized cassava starch, potato soluble starch and glycogen type IV (all gelatinized at 100ºC for 30 min) and modified glucan products. All samples (2% ($w \cdot v^{-1}$) in 250 μL), were incubated in triplicates with 20 U of each PPA and GA in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.0) at 37ºC for 0, 10, 30, 60, 120, 240, 360, 480, and 1440 min. Enzymatic reaction was terminated by adding 30 μL 100 mM HCl. The rate of starch digestion was expressed as % of glucose released from the total starch or α-glucan over the time period (Englyst et al., 1992) by using PGO enzymes. The digestion profiles of the samples compared to cassava starch and commercial IMOs were fitted to a first order kinetic model in order to investigate the digestion rate constant, $C = 1\text{-}e^{kt}$, where t is the digestion time (min), C is the fraction of digested starch at specified reaction time, and k is the digestion rate constant (min^{-1}). The value of k was obtained from the slope of a linear-least-squares fit of a plot of ln(1 −C) against t (Butterworth, Warren, Grassby, Patel, and Ellis, 2012; Zhang, Dhital, and Gidley, 2013). The glucose released (%) after subtraction from the initial glucose content in each samples were calculated using the following equation:

$$\text{Glucose released (\%)} = \frac{(\text{Total weight of glucose released - Blank}) \times 0.9}{(\text{Weight of sample - (Initial glucose in the sample)}} \times 100$$

Where: Blank is the sample treated with PPA and GA and immediately terminated the reaction

0.9 is the molar mass conversion from glucose to anyhydroglucose.

5.3.8 Fecal batch culture fermentation

The pre-sterilised of 90% ($v \cdot v^{-1}$) basal medium (2 $g \cdot L^{-1}$ peptone water, 2 $g \cdot L^{-1}$ yeast extract, 0.1 $g \cdot L^{-1}$ NaCl, 0.04 $g \cdot L^{-1}$ K_2HPO_4, 0.04 $g \cdot L^{-1}$ KH_2PO_4, 0.01 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.01 $g \cdot L^{-1}$ $CaCl_2 \cdot 6H_2O$, 2 $g \cdot L^{-1}$ $NaHCO_3$, 2 mL Tween80, 10 mL vitaminK1, 0.5 $g \cdot L^{-1}$ cysteine HCl, 0.5 $g \cdot L^{-1}$ bile salts, pH 7.0) were inoculated with 10% ($v \cdot v^{-1}$) of fecal slurry, which was prepared by homogenising 10% ($w \cdot v^{-1}$) freshly voided fecal material of three healthy volunteers in 0.1 M phosphate-buffered saline (PBS), pH 7.0 and centrifuged at 2,500 rpm for 10 min to remove the precipitate. Fructo-oligosaccharides (FOS), commercial IMOs (Ingredion Incorporated, Thailand), glucose, and modified samples were *in vitro* digested following to section 5.2.7 in order to eliminate digested oligosaccharides. After digestion, the FOS or commercial IMOs or glucose or modified samples were added to obtain a final concentration of 1% ($w \cdot v^{-1}$). A negative control was performed with no addition of carbon source. Each flask was stirred. The temperature was set at 37ºC and pH was maintained at 6.8. Anaerobic conditions were maintained by using anaerobic chamber with oxygen-free nitrogen at 15 $mL \cdot min^{-1}$. Samples (5 mL) were removed at 0 and 24 h for bacterial count, cells fixation, carbohydrate content analysis and organic acid analysis. Incubations were run on three separated occasions (Ghoddusi, Grandison, Grandison and Tuohy, 2007; Mandalari, Nueno-Palop, Bisignano, Wickham, and Narbad, 2008; Palframan, Gibson, and Rastall, 2002).

5.3.9 Enumeration of bacteria

Bacteria were counted using fluorescent in situ hybridization (FISH) (Palframan et al., 2002). Fermentation samples (375 µL) were diluted four times in 4% ($w \cdot v^{-1}$) filtered paraformaldehyde and fixed at 4ºC overnight. Samples were then washed twice with filtered PBS (0.1 M, pH 7.0) and stored at -20ºC in PBS/ethanol

(1:1, $v \cdot v^{-1}$) until analysis. Hybridization was performed at optimal temperature using genus-specific 16S rRNA-targeted oligonucleotide probes labelled with the fluorescent dye Alexa Flour®-555 dye for the different bacterial groups or with 4,6-diamidino-2-phenylindole (DAPI) for total cell counts. The probes used were Bif164 specific for *Bifidobacterium* (Langendijk et al., 1995), Bac303 specific for *Bacteroides* (Manz, Amann, Ludwig, Vancanneyt, and Schleifer, 1996), Lab158 specific for *Lactobacillus*/*Enterococcus* spp. (Harmsen, Elferrich, Schut, and Welling, 1999), and His150 specific for most species of the Clostridium hystolyticum group (Clostridium clusters I and II) (Franks et al., 1998) which shown in Table 5.1. Sixteen µL of the fixed cell suspension was added to 200 mL of pre-warmed hybridisation buffer (40 mM Tris–HCl, 1.8 M NaCl, pH 7.2) and 64 mL of HPLC grade water. Ninety µL of the hybridisation mixture was then added to 10 mL of each probe (50 $ng \cdot mL^{-1}$) and hybridised for 24 h at either 45ºC (Lab158 and Bac303) or 50ºC (Bif164 and His150). The cells were then washed at their respective hybridisation temperatures for 30 min in 5 mL of wash buffer (20 mM Tris–HCl, 0.9 M NaCl, and pH 7.2). Cells were vacuum filtered onto a 0.2 mm Isopore membrane filter (Millipore Corporation Watford, U.K.). The filter was then examined using an Olympus BX51 fluorescent microscope. The 455 nm-excitation filter was used to illuminate DAPI-stained cells and 555 nm filter was used to count the hybridised cells. At least 15 random fields with 15-150 cells of view were counted on each slide and the average count used for analysis and calculates PI following the equation:

$$PI = \frac{Bif}{Total} - \frac{Bac}{Total} + \frac{Lab}{Total} - \frac{Clos}{Total}$$

Table 5.1 Summarise of DNA probes used for FISH.

Probe	Genus Specification	Sequence (5' → 3')	References
Bif164	*Bifidobacterium* sp.	CATCCGGCATTACCACCC	Langendijk et al., 1995
Lab158	*Lactobacillus* sp.	GGTATTAGCA(C/T)CTGTTTCCA	Harmsen et al., 1999
Bac303	*Bactreoids* sp.	CCAATGTGGGGGACCTT	Manz et al., 1996
Chis150	*Clostridium* sp.	TTATGCGGTATTAATCT(C/T)CCTTT	Harmsen et al., 1999

5.3.10 Organic acids and short-chain fatty acid (SCFA) analysis

One mL of samples were collected from an anaerobic chamber and centrifuged at 15,000 x g for 5 min; 20 µL of the supernatant after diluted was injected into an HPLC system equipped with a refractive index detector. Quantification of the organic acids was carried out using acetic, propionic, butyric, succinic, formic, citric, valeric, isovaleric, oxalic, lactic acids in concentrations between 0.5 and 100 mM as a standard curve, and results were expressed in $mmol \cdot L^{-1}$

5.4 Results and discussions

5.4.1 α-transglucosidase optimum dosages and incubation times

The thirty % and fifty % ($w \cdot v^{-1}$) of cassava starch were selected and simultaneous treated with α-amylase and BE in the first step in order to generate highly-branched glucans. The first set of analyses was designed to investigated the impact of α-transglucosidase on the formation of IMOs after simultaneous treated with α-amylase and BE for 24 h. Table 5.2 demonstrated a relative content of glucan

(%) of the second step, α-transglucosidase treated cassava starch. α-Transglucosidase dosages ranging from 10 to 45 U were studied and the glucose, maltose, maltotriose and IMOs which consist of isomaltose, isomaltotriose and panose were monitored. The α-transglucosidase treated 30% of substrate showed the optimum dosage at 10U which obtain 25.0% of IMOs while that of α-transglucosidase-treated 50% of substrate was 15U where 35.9% of IMOs were obtained.

Table 5.2 Relative content of glucan (%) in the second step, α-transglucosidase-treated cassava starch with various dosages for 1 h after α-amylase and BE treated.

α-Transglucosidase unit	**Relative content of glucans (%)**			
	30% Substrate		**50% Substrate**	
	DP 1-3	**IMOs**	**DP 1-3**	**IMOs**
10	75.0±1.0	25.0±0.7	66.5±0.5	33.5±0.6
15	76.7±1.1	23.3±1.2	64.1±1.0	35.9±0.8
20	80.0±0.7	20.0±0.7	66.3±0.5	33.7±0.7
30	84.2±0.8	15.8±0.9	70.1±0.6	29.9±0.4
45	86.6±1.0	13.5±0.7	76.8±0.7	23.2±0.7

The correlation between α-transglucosidase dosages and incubation time was investigated. The ten U and fifteen U of the second step α-transglucosidase were added to 30% and 50% substrates respectively. As shown in Table 5.3, both substrate concentrations exhibited the highest IMOs concentration at 3 h of incubation time. However, when using higher enzyme dosages and longer incubation times, the IMOs content was lower indicating the non-optimum substrate:enzyme ratios which resulting in more hydrolysis products (Mangas-Sánchez and Adlercreutz,

2015). Moreover, α-transglucosidase hydrolysed maltotriose effectively; however, oligomers longer than DP4 were less favored (Ota, Okamoto and Wakabayashi, 2009).

Table 5.3 Relative content of glucan (%) in the second step, α-transglucosidase-treated cassava starch with various incubation times. The ten U and fifteen U of α-transglucosidase were added to 30% and 50% substrate, respectively.

Incubation time (h)	Relative content of glucans (%)			
	30% Substrate		50% Substrate	
	DP1-3	IMOs	DP1-3	IMOs
3	53.0±1.4	47.0±1.2	56.7±1.1	43.3±1.6
6	55.3±0.9	44.7±0.7	56.8±0.8	43.2±0.9
9	61.7±1.1	38.3±1.2	59.2±1.3	40.9±1.5
12	67.6±1.4	32.4±1.1	62.1±1.2	37.9±0.7
18	70.4±0.9	29.6±0.8	56.7±0.7	43.3±0.9
24	72.3±0.9	27.7±1.0	58.6±1.1	41.5±1.2

5.4.2 α-Transglucosidase:β-amylase optimum dosage and incubation time

As mentioned above (section 5.2.1), 30% and 50% of cassava starch were selected and simultaneous treated with α-amylase and BE in the first step in order to generate highly-branched glucans. Generally, β-amylase hydrolyses α-glucans by removing maltose unit from the non-reducing end (Bailey and Whelan, 1957) until the enzyme reaches one to two glucose units from a branch point. Maltose was reported as the appropriate donor and acceptor substrates for α-transglucosidase (Ota et al., 2009). The combinatorial of α-transglucosidase and β-amylase were used

in the second step of enzyme treatment in order to enhance α-transglucosidase efficiency to produce IMOs. Table 5.4 illustrates the results obtain from the preliminary analysis of α-transglucosidase and β-amylase second step catalysis. Glucose content was measured in order to investigate the α-transglucosidase: β-amylase ratio which provides the lowest of unwanted-glucose content from hydrolysis activity of α-transglucosidase. Both substrate concentrations show the lowest unwanted-glucose content when the α-transglucosidase:β-amylase ratio of 3:1 was used. These results suggested that β-amylase produced the appropriate amount of maltose for α-transglucosidase transferring reaction and released low amount of unwanted-glucose.

Table 5.4 Glucose content ($\mu g \cdot mg^{-1}$) in the second step, α-transglucosidase: β-amylase-treated cassava starch with various enzyme ratios.

α-transglucosidase:β-amylase	**Glucose content ($\mu g \cdot mg^{-1}$)**	
	30% Substrate	**50% Substrate**
1:1	917.3±4.1	716.1±4.9
1:3	792.3±2.4	913.9±5.7
3:1	244.0±3.3	294.0±2.4
3:3	280.3±4.9	298.0±6.5

The correlation between α-transglucosidase:β-amylase ratio (3:1) and incubation time was investigated. The second step α-transglucosidase:β-amylase-treated 30% substrate shows the optimum incubation time of 9 h which obtains 38.2% of IMOs while the 50% of cassava starch shows the optimum incubation time of 12 h which obtains 37.0% of IMOs. However, when using longer incubation times, the

IMOs content was lower indicating more hydrolysis activity was taken place which resulted in the increase of DP 1-3.

Table 5.5 Relative content of glucans (%) in the second step, α-transglucosidase: β-amylase (3:1)-treated cassava starch with various incubation times.

Incubation time (h)	Relative content of glucan (%)			
	30% Substrate		50% Substrate	
	DP 1-3	IMOs	DP 1-3	IMOs
3	77.7±0.7	22.3±0.7	73.7±0.8	26.3±0.7
6	72.1±0.8	27.9±0.9	66.0±0.6	34.0±0.2
9	61.8±0.8	38.2±0.6	69.1±0.7	30.9±0.4
12	69.4±0.5	30.7±0.7	63.0±0.7	37.0±0.6
18	74.2±0.3	25.8±0.3	65.8±0.9	34.3±0.7
24	76.6±0.5	23.4±0.5	73.3±0.7	26.7±0.5

5.4.3 α-1,6 Glucosidic linkages and end groups formation

Simultaneous α-amylase and BE followed by α-transglucosidase (ABT) and simultaneous α-amylase and BE followed by simultaneous β-amylase and α-transglucosidase (ABbT)-treated high cassava starch concentration were studied. Table 5.6 illustrates the relative percent of α-1,4 and α-1,6 linkages in native cassava starch, commercial IMOs and enzymes-modified starch. The 30AB and 50AB samples which were treated with simultaneous α-amylase and BE showed 9.2 and 9.1% of α-1,6 glucosidic linkages respectively indicating the ability of BE to create branch points and produce highly-branched molecules. Generally, the major hydrolysis products of α-amylase are maltose, maltotriose, and α-limit dextrins which contain amount of end-groups (Lee et al., 2013).

Table 5.6 Relative content (%) of α-1,4 and α-1,6 linkages in native cassava starch, commercial IMOs and enzymes-modified starch. 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

Sample	α-1,4 linkages	α-1,6 linkages
Cassava starch	96.4±0.8	3.6±0.4
IMOs	41.6±0.5	58.4±0.6
30AB	90.8±0.5	9.2±1.0
50AB	90.9±1.0	9.1±0.4
30ABT	48.8±1.1	51.2±3.0
50ABT	32.2±0.5	67.8±1.5
30ABbT	17.1±0.3	82.9±0.5
50ABbT	14.5±0.5	85.5±0.8

The 30ABT and 50ABT samples which were treated with simultaneous α-amylase and BE followed by α-transglucosidase showed 51.2 and 67.8 % of α-1,6 glucosidic linkages respectively. These results indicated that the α-transglucosidase utilised the α-amylase hydrolysis products as a donor substrates to generate α-1,6 glucosidic linkages and left some hydrolysis products, such as glucose or oligosaccharides which corresponded to the reducing end formation. In addition, the 50% substrate showed higher α-1,6 linkages compared to the 30% substrate indicated that the higher substrate content and a variety of molecular configurations enhance α-transglucosidase activity to form branch linkages.

The commercial IMOs was used as a positive control in this studied. Normally, starch slurry was liquafaction by α-amylase for 2-2.5 h. The β-amylase and pullulanase were added and incubation for 48-72 h for the first saccharification and

ended up with the second saccharification by α-transglucosidase for 48-72 h (Gibson and Rastal, 2006). The 30ABbT and 50ABbT samples which were treated with simultaneous α-amylase and BE followed by simultaneous β-amylase and α-transglucosidase showed 82.9 and 85.5% of α-1,6 glucosidic linkage respectively which were higher than the commercial IMOs. This results may be explained by the fact that the hydrolysis products of α-amylase which consist of maltose, maltotriose, and α-limit dextrins were further hydrolysed by β-amylase yielding maltose which was appropriate for α-transglucosidase. The α-glucose and β-glucose of ABbT treated samples were increased when compared to other samples (not shown) suggesting the synergistic of β-amylase hydrolysis and α-transglucosidase transferring reaction to generate α-1,6 glucosidic linkages which increased end-groups formation. These results are in agreement with those obtained by Ota et al. (2009) who studied the mechanisms of transglucosidase [EC 2.4.1.24] from *Aspergillus niger* on the oligosaccharides synthesised from a mixture of maltoheptaose and [U–^{13}C]maltose substrates. It showed that after 96 h of incubation, most of α-1,4 linkages molecules were converted into oligosaccharides with DP2 to DP5 consisting of mostly α-1,6 linkages.

5.4.4 Types of IMOs and molecular size distribution

Commercial IMOs and enzymes-modified starch were analysed in order to investigate the types of IMOs formation as shown in Figure 5.1. Commercial IMOs showed high content of isomaltose and moderate content of isomaltotriose, panose and higher branched oligosaccharides. In addition, the high content of mono-, and di-saccharides, and moderate content of higher linear oligosaccharides were observed. As compared to our enzyme-modified samples, the 30AB and 50AB

samples showed a high content of maltose, maltotriose, and higher linear oligosaccharides indicating the hydrolysis activity of α-amylase.

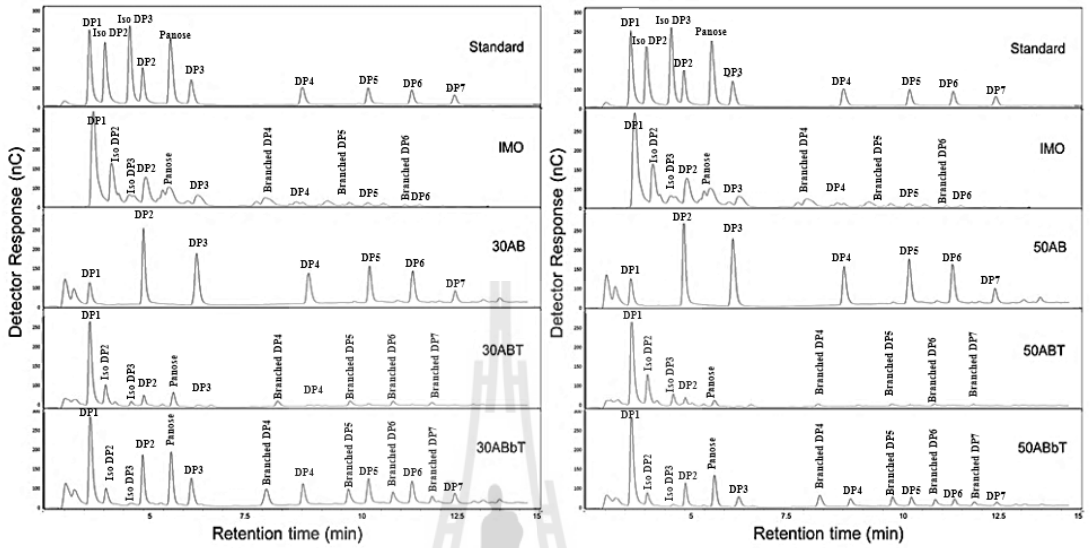

Figure 5.1 HPAEC-PAD analysis of IMOs of commercial IMOs and enzymes-modified starch. A: 30% cassava starch substrate modified starch; B: 50% cassava starch substrate modified starch. Iso DP2: Isomaltose; Iso DP3: Isomaltotriose; 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

Generally, the peaks in-between the linear oligosaccharides in HPAEC-PAD chromatogram showed the formation of branched oligosaccharides. As compared to AB, the 30ABT and 50ABT samples showed an increase in isomaltose, isomaltotriose, panose and higher branched oligosaccharide peak positions as shown in Figure 5.1. The results suggested that α-transglucosidase has activity on short chain α-glucans yielding isomaltose, isomaltotriose, panose, and higher branched oligosaccharides. This observation is in accordance with Ota et al. (2009) who

reported that the *A. niger* transglucosidase can synthesize IMOs DP2-6. Moreover, higher glucose content was noticed when compared to AB sample suggesting that α-transglucosidase had high activity to produce mainly isomaltose and leave glucose as a hydrolysis product (Figure 5.1). However, oligomers longer than DP4 can be utilized by α-transglucosidase but less favored as mentioned in the previous section (5.3.1) resulting in slightly higher content of branch oligosaccharides as showed in Figure 5.1 compared to AB sample. The 30ABbT and 50ABbT samples showed an increase in isomaltotriose, panose and higher branched oligosaccharides DP ≥ 4. This might be due to the fact that during β-amylase hydrolysed the glucan chains, α-transglucosidase also used maltose and short chain α-glucans (DP ≥ 4) to create α-1,6 linkages resulting in more content of isomaltotriose, panose and higher branched oligosaccharides. Mono-, di-, and higher linear oligosaccharides were also observed in this step. It implied that some linear oligosaccharides was not utilised by β-amylase because of the competitive inhibition between β-amylase and α-transglucosidase which may be taken place due to lower substrate concentrations (Thoma and Koshland, 1960). Furthermore, the formation of isomaltose, isomaltotriose, panose, mono-, di- and higher linear- and branched oligosaccharides was confirmed by molecular weight distribution which shown in Figure 5.2. The chromatograms illustrated that all samples consisted of small molecular size products ranging from DP 1-8. The commercial IMOs control, ABT and ABbT sample sets showed higher content of glucose (retention time: 2.5 min) as compared to AB sample sets which was consistent with HPAEC-PAD data (Figure 5.1). The expected branched-oligosaccharides of DP 4-7 were found in between the standard linear peaks (as mentioned as “branched DP 4 to branched DP 7” in Figure 5.1), especially for ABbT sample set. Glucose and linear oligosaccharides were the by-products from

hydrolysis of α-transglucosidase which matched to those observed in earlier studies. Pan and Lee (2005) reported that the production of IMOs using α-transglucosidase obtained high glucose and linear oligosaccharides content.

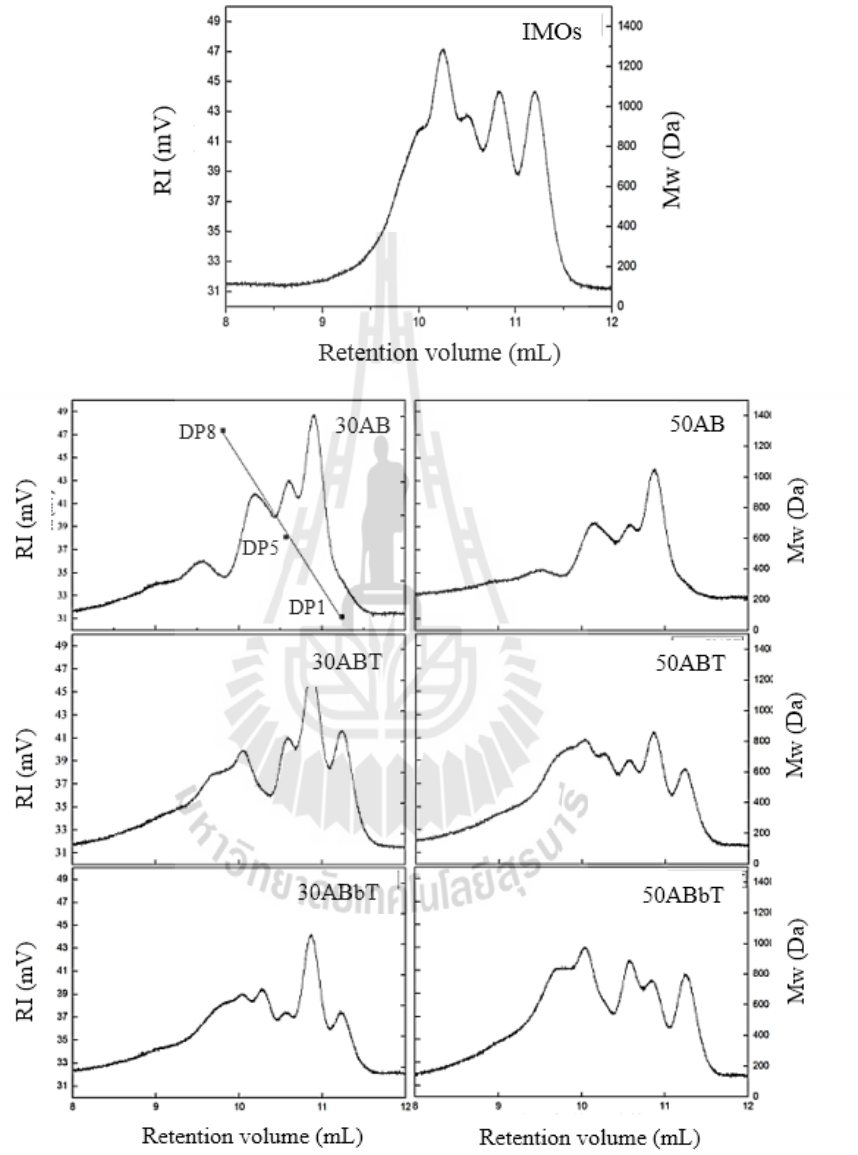

Figure 5.2 Molecular weight distributions of enzyme-modified cassava starch products as analysed by size-exclusion chromatography with triple detection array (SEC-TDA). 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

5.4.5 *In vitro* digestibility

To investigate the dietary susceptibility to amylolytic digestion of the enzymes- treated starches, all samples were subjected to combined porcine pancreatic α-amylase (PPA) and glucoamylase (GA) treatment. In this type of experiment PPA and GA exert a synergistic digestion of the α-glucans. Time course digestion profiles of native cassava starch, commercial IMOs and enzymes-modified starch are presented in Figure 5.3. Generally, the commercial IMOs and modified products showed less susceptibility to PPA and GA digestion than gelatinised cassava starch. Approximately 20% glucose content was observed at 0 h for commercial IMOs, ABT and ABbT sets. However, after the initial glucose content is subtracted, the ABT and ABbT sets showed lower glucose released as compared to AB set. At the end of digestion (300 min), the 30AB and 50AB samples showed 74.8 and 87.4% glucose released. They were higher than that of 30ABT, 50ABT and 30ABbT, 50ABbT sample sets which showed approximately 73.3, 64.0 and 71.3, 60.9% glucose released, respectively. The ABbT sample set showed lower glucose released at the end of digestion as compared to the other enzyme treated samples in the same substrate concentration. These results suggested that the addition of β-amylase improved the slow digesting property of modified starch compared to those of starches treated with α-transglucosidase alone. This result was supported by the previous work of Ao et al. (2007) who reported that the digestion rates of starches treated with one of the amylase alone were higher than those of starches treated with amylase and α-transglucosidase. When compared to commercial IMOs at 300 min of digestion which shown 71.4% glucose released, the 50ABbT sample showed the

lowest glucose released indicating that the complex higher branched structure were formed.

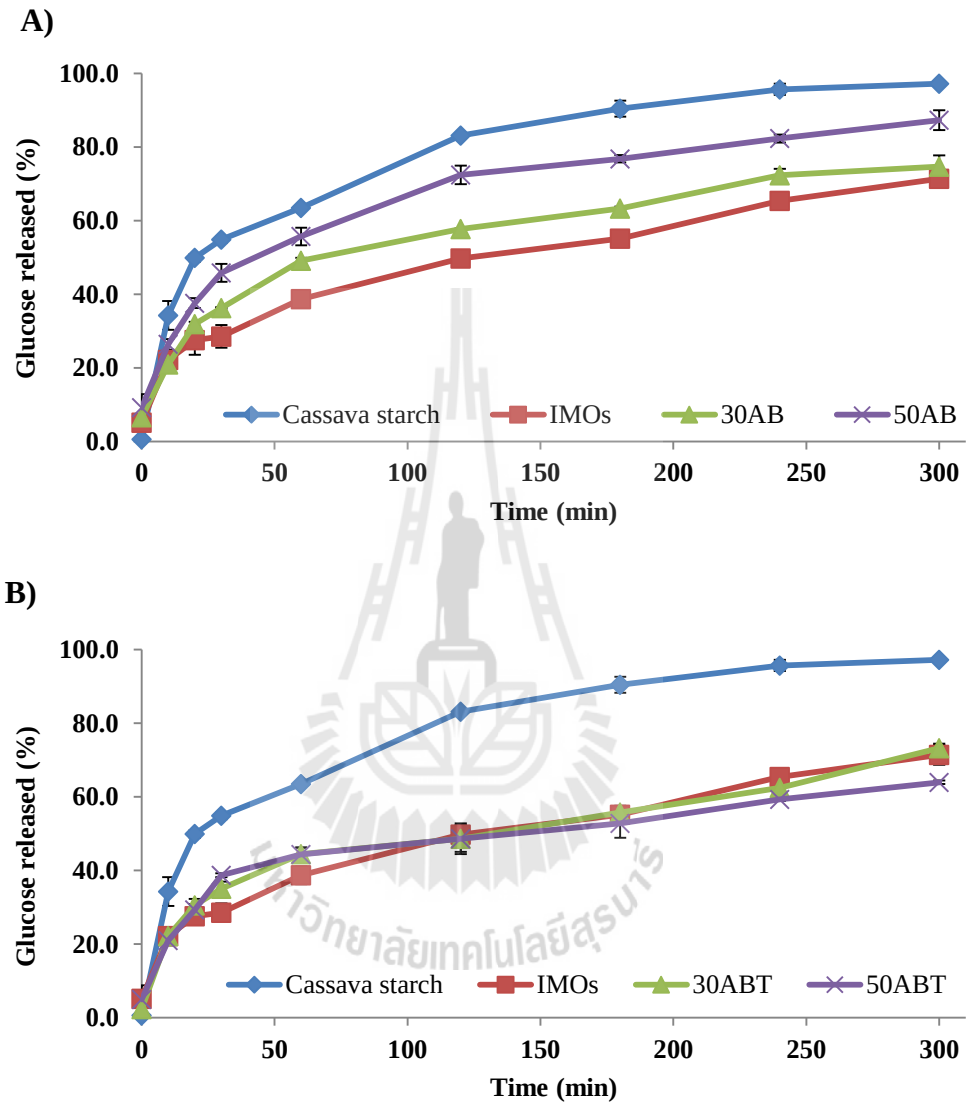

Figure 5.3 Time course digestion profiles of native cassava starch, commercial IMOs and enzymes-modified starch digested with PPA and GA after initial glucose subtraction. A: AB treated starch; B: ABT treated starch; C: ABbT treated starch. 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

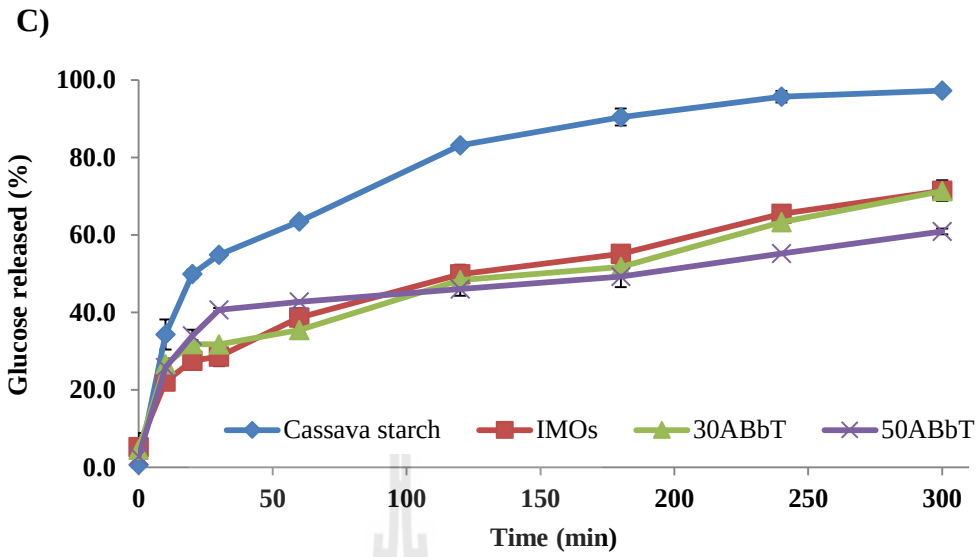

Figure 5.3 Time course digestion profiles of native cassava starch, commercial IMOs and enzymes-modified starch digested with PPA and GA after initial glucose subtraction. A: AB treated starch; B: ABT treated starch; C: ABbT treated starch. 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase (Continued).

The digestion rate constant of the modified starches were significantly lower than that of cassava starch control. A high k-value indicates high susceptibility of PPA and GA catalysis. The k-value for cassava starch and commercial IMOs were 11.3×10^{-3} min^{-1} and 3.5×10^{-3} min^{-1} respectively. The k-values for the 30AB and 50AB products were 4.1×10^{-3} min^{-1} and 6.3×10^{-3} min^{-1}, respectively and for the 30ABT and 50ABT were 4.0×10^{-3} min^{-1} and 3.7×10^{-3} min^{-1}, respectively and for the 30ABbT and 50ABbT were 3.1×10^{-3} min^{-1} and 2.5×10^{-3} min^{-1}, respectively. Therefore, the 50ABbT product had a significantly lower k-values compared with the others analysed samples. The modified starch structures can be considered as very resistance towards

amylolysis which corresponding to the various types of IMOs observed by HPAEC-PAD.

Table 5.7 Digestion rate constant of cassava starch, IMOs and enzyme-modified starches.

Sample	k (min^{-1}) $\times 10^{-3}$
Cassava starch	11.3 ± 0.3^{a}
IMOs	3.5 ± 0.5^{cd}
30AB	4.1 ± 0.2^{cd}
50AB	6.3 ± 0.6^{b}
30ABT	4.0 ± 0.1^{cd}
50ABT	3.7 ± 0.2^{cd}
30ABbT	3.1 ± 0.1^{cd}
50ABbT	2.5 ± 0.2^{e}

Note: Means in columns with different letters are significantly different ($p<0.05$).

Starch nutritional fractions were calculated according to Englyst's method (Englyst et al., 1992) from glucose released after digestions at 20 and 120 min and total dietary fiber content (TDF) are shown in Table 5.8. The commercial IMOs and modified products showed higher RS content than gelatinised cassava starch. Moreover, the 30AB and 50AB showed 25.9 and 26.4% RS respectively which was lower than the other enzyme-treated samples but their higher SDS fraction was observed. The ABT and ABbT sets showed RS content ranging from 42.1 to 45.9% which were not different from the commercial IMOs (46.3% RS). In contrast, commercial IMOs, ABT and ABbT sets represent a lesser TDF content compared to RS content. There were 18.36, 16.62, 17.41, 25.55, and 24.82% for

commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT, respectively. The 30ABbT and 50ABbT showed the highest TDF value which was significantly different from that of commercial IMOs, 30ABT and 50ABT. The differences between RS content and TDF was due to their different digestion time by PPA and GA. TDF analysis was performed according to AOAC 2011.25 and AOAC 2009.01 which digested the sample for 16 h. Therefore, it can imply that all samples contain high RDS and SDS fractions than those calculated according to Englyst's method. These can be related to the SDS structure which is more complex and cannot completely digest within 120 min for this in vitro study.

Table 5.8 The RDS, SDS and RS content of cassava starch, commercial IMOs and enzymes-modified starch calculated from data in Figure 5.4 according to the Englyst's method and total dietary fiber content (TDF).

Samples	RDS (%)	SDS (%)	RS (%)	TDF (%)
Cassava starch	49.9±1.1a	33.2±1.5b	16.9±1.3c	N.A.
IMOs	34.4±0.7c	19.3±1.6c	46.3±1.2a	18.4±1.3b
30AB	30.9±0.6d	43.2±0.5a	25.9±0.4b	N.A.
50AB	38.7±3.9b	34.8±2.5b	26.4±3.2b	N.A.
30ABT	38.1±1.7b	18.1±2.9c	43.9±2.3a	16.6±0.8b
50ABT	38.2±1.0b	16.5±0.5cd	45.3±0.8a	17.4±0.5b
30ABbT	38.6±1.6b	19.3±2.6c	42.1±2.1a	25.5±2.8a
50ABbT	40.7±0.4b	13.4±1.1d	45.9±0.7a	24.8±2.4a

Note: Means in columns with different letters are significantly different ($p<0.05$) by general linear model. N.A. is not avaiable.

5.4.6 Prebiotics properties

5.4.6.1 Optimization of incubation time and substrate content in basal medium for fecal batch culture fermentations

Fructo-oligosaccharides (FOS), commercial IMOs, ABT and ABbT samples were in vitro digested by PPA and GA. The digested samples were recovered and used as substrates for the microbial fermentation from human feces. The pre-sterilised of basal growth medium with 0.5 and 1.0% ($w \cdot v^{-1}$) were inoculated with fecal slurry and incubated for 20 and 24 h in order to optimise the highest total SCFA content and substrate usages by micro-organisms. The results in Table 5.9 demonstrate that the combination of 1% ($w \cdot v^{-1}$) substrate and fermentation for 24 h showed the highest total SCFA content and lower substrate usages by micro-organisms. Total SCFA content were 46.2, 43.2, 41.1, 43.7, 40.4, and 41.3% for FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT, respectively. Substrate usages were 66.5, 61.6, 61.9, 64.5, 61.4, and 63.6% for FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT, respectively. These results are consistent with other reports which found that the optimum concentration of substrate in basal growth medium was 1% ($w \cdot v^{-1}$) (Barczynska, Jochym, Slizewska, Kapusniak, and Libudzisz, 2010; Barczynska, Slizewska, Jochym, Kapusniak, and Libudzisz, 2012; Depeint, Tzortzis, Vulevic, I'Anson, and Gibson, 2008; Ghoddusi et al., 2007; Mandalari et al., 2008; Sanz, Gibson, and Rastall, 2005; Śliżewska, 2013; Wynne, McCartney, Brostoff, Hudspith, and Gibson, 2004).

Table 5.9 Effect of feacal fermentation time and substrate content on organic acids formation and substrate usage. FOS: fructo-oligosaccharides, IMOs: commercial IMOs, 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

Sample Title	Time (h)	Substrate (%)	Organic acids (mM)					Substrate usage (%)
			Lactic	Acetic	Propionic	Butyric	Total SCFA	
FOS	20	0.5	6.1	25.6	5.6	2.2	33.4	76.8
IMOs	20	0.5	1.6	19.5	6.1	7.2	32.8	75.3
30ABT	20	0.5	3.4	21.2	4.9	5.5	31.6	76.3
50ABT	20	0.5	3.7	20.1	4.3	6.8	31.3	75.9
30ABbT	20	0.5	3.0	19.6	5.4	8.0	33.0	76.1
50ABbT	20	0.5	3.6	19.7	5.3	6.7	31.6	76.6
FOS	20	1.0	13.5	42.6	1.9	1.7	46.2	64.1
IMOs	20	1.0	10.2	32.8	1.8	2.2	36.7	59.7
30ABT	20	1.0	12.0	32.5	3.0	1.6	37.1	61.9
50ABT	20	1.0	11.9	33.0	3.7	1.8	38.4	62.4
30ABbT	20	1.0	12.5	35.0	3.8	1.9	40.7	60.5
50ABbT	20	1.0	12.9	34.8	4.7	1.6	41.1	62.5
FOS	24	0.5	6.1	25.6	5.6	2.2	33.4	77.4
IMOs	24	0.5	1.5	19.3	6.8	11.7	37.8	76.4
30ABT	24	0.5	3.5	21.6	5.6	7.6	34.7	80.0
50ABT	24	0.5	2.8	18.7	6.1	8.1	32.9	77.9
30ABbT	24	0.5	3.8	22.2	6.9	7.7	36.8	75.5
50ABbT	24	0.5	3.0	34.9	5.1	2.7	42.6	77.2
FOS	24	1.0	13.5	42.6	1.9	1.7	46.2	66.5
IMOs	24	1.0	11.4	33.6	5.8	3.8	43.2	61.6
30ABT	24	1.0	12.6	33.6	4.9	2.6	41.1	61.9
50ABT	24	1.0	13.4	35.7	5.1	2.9	43.7	64.5
30ABbT	24	1.0	14.3	26.7	6.6	7.1	40.4	61.4
50ABbT	24	1.0	12.4	33.5	5.1	2.7	41.3	63.6

5.4.6.2 Bacterial population and prebiotics index

Bacterial population changes of genus: Lactobacilli, Bifidobacteria, Bacteroides, Clostridia (histolyticum subgroup) and prebiotics index were shown in Figure 5.4 and the bacterial morphology was shown in Figure 5.5. Glucose was used as a negative control while FOS and commercial IMOs were used as positive controls. Total bacterial population at the beginning was 8-9 log cfu·mL^{-1} and end up with 9-10 log cfu·mL^{-1} at 24 h of fermentation. These results are similar to those of Ghoddusi, Grandison, Grandison and Tuohy (2007) who reported the effect of inulin on microbial growth in that the microbial populations was 8.21 log cfu·mL^{-1} at 0 h and 8.83 log cfu·mL^{-1} at 24 h. In addition, Mandalari et al. (2010) reported the effect of almond skin extract on microbial growth and showed that the microbial populations was 9.15 log cfu·mL^{-1} at 0 h and 9.24 log cfu·mL^{-1} at 24 h. Populations of Lactobacilli and Bifidobacteria for all samples were increased after 24 h of fermentation while Clostridia (histolyticum subgroup) and Bacteroides were decreased approximately one log cycle due to the decrease of pH. PI values at 24 h were 1.50, 2.56, 3.58, 2.53, 2.37, 2.64, and 3.16 for glucose, FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT, respectively. Commercial IMOs exhibited the highest PI value. As compared to the other substrates, the PI value of FOS was higher as compared to the others samples except for commercials IMOs which is in agreement with those obtained by Palframan et al. (2002) who got 2.31 of PI value from FOS. The 50ABbT showed the highest PI values compared to 30ABT, 50ABT and 30ABbT. This could be due to the reason that after digested with PPA and GA, the 50ABbT structure contained higher content of small molecules (Figure 5.2) with α-1,6 linkages which was associated with the digestion rate constant (Table 5.6) and

TDF values (Table 5.7). Although α-1,6 linked-glucan was partially hydrolysed by human brush-border enzymes (Lee et al., 2013), some of them can reach and are fermented in the colon. This finding suggested that the 50ABbT structure could be considered to contain higher content of branched oligosaccharides structure from DP > 4 (Figure 5.1) when compared to the other modified samples. These results are supported by Olano-Martin, Gibson and Rastall (2002) and Sanz et al. (2005) who found the positive correlation between α-1,6 linked-glucans and probiotics metabolism.

5.4.6.3 Substrates usage and pH

Total acidity (%), pH, and carbohydrate usage (%) after microbial fermentation from human feces were presented in Table 5.10. Overall, total acidity of all samples was not significantly different. Glucose showed the highest substrate usage and lowest pH which were 85.6% and 3.69, respectively. Substrate usages were 76.5, 77.8, 76.8, 77.4, 72.3, and 78.2 for FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT, respectively. These data are associated with the bacterial population changes (Figure 5.5). The pH was a result of the rate and mechanisms of bacterial enzymes during fermentation on reaching carbohydrates in the colon. The optimum pH can stimulate the probiotics growth resulting in the inhibition of the undesirable bacteria growth (Palframan et al., 2002). In addition, the 50ABbT showed higher carbohydrate usage compared to 30ABT, 50ABT, and 30ABbT which are consistent with PI values. This relationship may be due to the fact that α-1,4 and α-1,6 linked-glucan were desirable for probiotics fermentation. However, these data must be interpreted with caution due to the amount of glucose left after PPA and GA *in vitro* digestion.

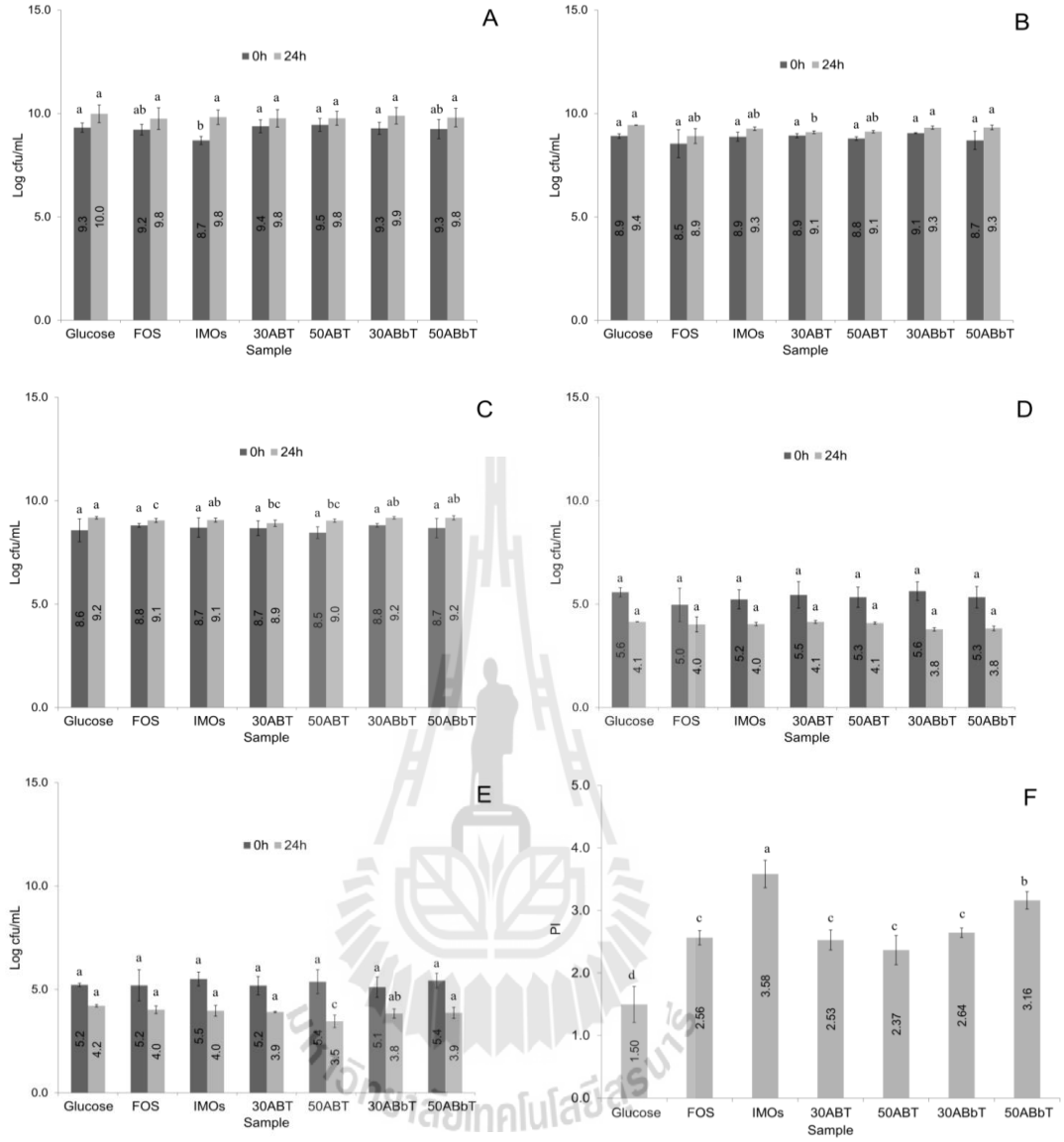

Figure 5.4 Bacterial population (log 10 cfu·mL^{-1}) with each carbohydrate used for 0 h and 24 h of fermentation for left and right respectively. A: total anaerobic bacteria; B: Lactobacillus; C: Bifidobacterium; D: Bacteroides; E: Clostridium (histolyticum subgroup) and F: prebiotics index. Error bars represent standard error. FOS: fructo-oligosaccharides; 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

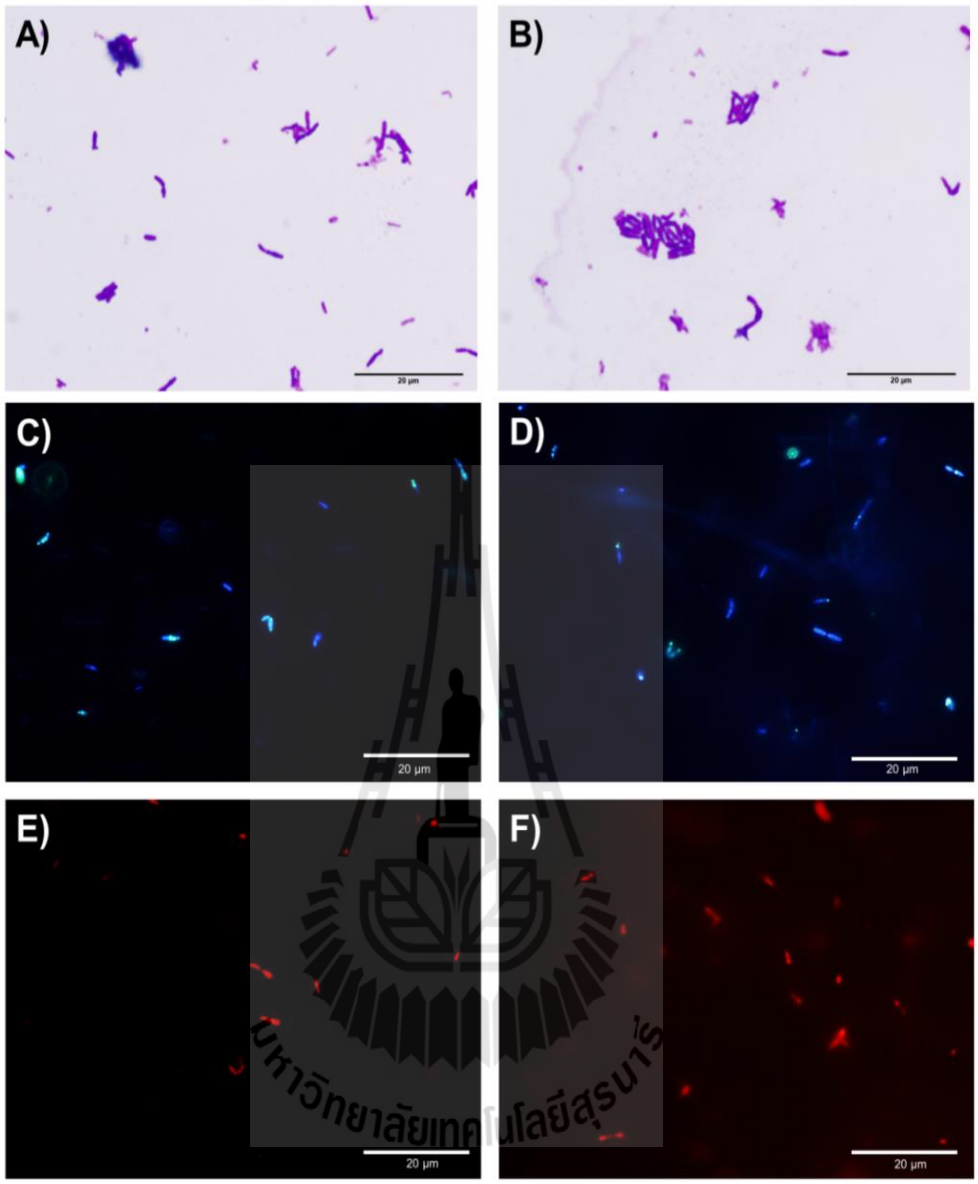

Figure 5.5 Bacterial morphology under bright field and fluorescent microscopy from selected modified samples. All genus strain with crystal violet and safranin A) 0 h, B) 24 h. Total bacterial count with DAPI staining C) 0 h, D) 24 h. Bifidobacterium count using DNA-probe E) 0 h, F) 24 h.

Table 5.10 Total acidity (%), pH, and carbohydrate usage (%) of glucose, FOS, commercial IMOs and enzymes-modified starch after fermentation. 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

Sample Title	Total acidity (%)	pH	Carbohydrate usage (%)
Glucose	0.70±0.04[a]	3.69±0.03[c]	85.6±0.8[a]
FOS	0.73±0.00[a]	3.74±0.02[ab]	76.5±0.9[c]
IMOs	0.72±0.04[a]	3.76±0.01[ab]	77.8±0.7b[c]
30ABT	0.72±0.03[a]	3.76±0.03[ab]	76.8±0.6b[c]
50ABT	0.72±0.04[a]	3.78±0.00[a]	77.4±0.7b[c]
30ABbT	0.74±0.04[a]	3.75±0.01[ab]	72.3±0.6[d]
50ABbT	0.73±0.02[a]	3.74±0.01[b]	78.2±0.6[b]

Note: Means in columns with different letters are significantly different ($p<0.05$).

5.4.7 Organic acids and total SCFA concentration

Organic acids and total SCFA content were investigated by HPLC and showed in Table 5.11. Acetic, propionic, butyric, succinic, formic, citric, valeric, isovaleric, oxalic, lactic acids were found after 24 h of fermentation. Major organic acid obtained from glucose fermentation was lactic acid (Mercier, Yerushalmi, Rouleau, and Dochain, 1992). Glucose, FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT and 50ABbT showed 12.90, 11.45, 11.37, 11.73, 12.26, 11.32, and 12.12 mM of lactic acid content, respectively. High content of lactic acid from commercial IMOs and enzymes-modified starch was partly affects by the amount of glucose left after PPA and GA in vitro digestion. Regarding acetic acid formation, FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT yielded 37.88, 36.52,

38.89, 39.94, 37.25, and 39.99 mM of acetic acid, respectively which were significantly higher than those of propionic and butyric acid formation. These results are likely to be related to the sample structure. Generally, after carbohydrate was fermented in the colon, monosaccharides were released and further used as an energy source for micro-organisms. The monosaccharides were metabolized to be pyruvate, yielding directly acetate or acetyl Co-A which was described by Wood-Ljungdahl partway (den Besten et al., 2013). It is more complex partway for propionic and butyric acid formation compared to acetic acid formation. Hence, acetic acid might be formed from the small molecules and less complex carbohydrate structure, for example, resistant starch, which was reported to obtain high butyric acid yield (Załęski, Banaszkiewicz, and Walkowiak, 2013) due to their large molecules and more complex structure. In addition, FOS and commercial IMOs provide the significantly highest butyric acid contents which were 5.26 and 5.08 mM, respectively. Total SCFA formation of FOS, commercial IMOs, 30ABT, 50ABT, 30ABbT, and 50ABbT were 47.94, 43.25, 43.66, 45.66, 42.28, and 45.37 mM, respectively which were not significantly different (Table 5.11). According to these data, it can infer that the branched DP 2-8 were the optimum carbon source for probiotics metabolism yielding mainly acetic acid but low content of propionic acid compared to FOS.

Table 5.11 Organic acids and total SCFA (acetic acid + propionic acid + butyric acid) content produced after 24 h of fermentation using glucose, FOS, commercial IMOs and enzymes-modified starch. 30: 30% cassava starch substrate; 50: 50% cassava starch substrate; A: α-amylase; B: branching enzyme; b: β-amylase and T: α-transglucosidase.

Sample Title	Organic acid content (mM)							
	Oxalic	**Citric**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Total SCFA**
Glucose	0.79 ± 0.27^{b}	0.30 ± 0.08^{a}	12.90 ± 1.77^{a}	5.57 ± 0.16^{a}	30.31 ± 1.91^{c}	1.54 ± 0.02^{b}	0.00 ± 0.00^{d}	31.85 ± 1.93^{b}
FOS	0.71 ± 0.69^{b}	1.10 ± 1.73^{a}	11.45 ± 2.15^{b}	3.83 ± 1.89^{a}	37.88 ± 1.95^{bc}	4.80 ± 2.11^{a}	5.26 ± 0.60^{a}	47.94 ± 1.44^{a}
IMOs	0.73 ± 0.11^{b}	0.40 ± 0.03^{a}	11.37 ± 1.10^{ab}	5.89 ± 0.59^{a}	36.52 ± 2.85^{ab}	1.64 ± 0.22^{b}	5.08 ± 0.20^{a}	43.25 ± 2.87^{a}
30ABT	1.59 ± 0.53^{a}	0.39 ± 0.05^{a}	11.73 ± 0.23^{a}	7.29 ± 0.60^{a}	38.89 ± 2.94^{a}	1.66 ± 0.08^{b}	3.11 ± 0.39^{c}	43.66 ± 2.48^{a}
50ABT	1.17 ± 0.21^{ab}	0.37 ± 0.04^{a}	12.26 ± 0.34^{a}	7.25 ± 0.71^{a}	39.94 ± 4.49^{a}	1.64 ± 0.06^{b}	4.07 ± 0.55^{b}	45.66 ± 5.08^{a}
30ABbT	0.79 ± 0.21^{b}	0.15 ± 0.11^{a}	11.32 ± 0.50^{ab}	7.10 ± 0.21^{a}	37.25 ± 0.51^{ab}	1.53 ± 0.09^{b}	3.49 ± 0.13^{bc}	42.28 ± 0.39^{a}
50ABbT	0.82 ± 0.19^{b}	0.16 ± 0.11^{a}	12.12 ± 0.09^{a}	7.38 ± 0.16^{a}	39.99 ± 2.46^{a}	1.50 ± 0.07^{b}	3.88 ± 0.38^{b}	45.37 ± 2.89^{a}

Note: Means in columns with different letters are significantly different ($p<0.05$).

5.5 Conclusions

Slowly and resistant maltodextrin conferring IMOs production was prepared by hydrolase and transferase enzymes with high cassava starch substrate concentration. ABT catalysis showed branching linkages content ranging from 51.2% and 67.8%, and TDF content was 16.6 and 17.4% when using 30% and 50% substrate, respectively. ABbT catalysis showed branching linkages content ranging from 82.9% and 85.5%, and TDF content was 24.8 and 25.6% when using 30% and 50% substrate, respectively. The IMOs composition of the ABT sample was isomaltose, isomaltotriose, panose and higher branched-oligosaccharides which shown lower content when compared to the ABbT samples. Prebiotics index of ABbT samples was higher than ABT and not significant different from FOS but lower than commercial IMOs. All of the samples showed high ability to stimulate growth of Bifidobacteria and Lactobacillus and inhibit Clostridia (histolyticum subgroup) and Bacteroids. They can produce higher acetate content when compared to propionate and butyrate. All α-glucan products had stimulated probiotics activity as compared to glucose, especially 50ABT and 50ABbT samples which can be applicable for food industry scale due to the higher substrate content that had slowly digested portion and prebiotics properties compared to the other modified samples.

5.6 Acknowledgements

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CHAPTER VI

SUMMARY

The formations of slowly digestible and resistant maltodextrin were studied by using enzymatic modification. Sequential BE→AM→BE catalysis resulted in more extensive branching as compared to all other enzyme treatment combinations and the products also exhibited higher branching than glycogen. All α-glucan products had slower amylolytic enzyme digestion rates as compared to native cassava starch. These findings demonstrate the importance of combinatorial catalytic approaches to optimize the synergistic effects of BE and AM in order to change α-glucan branching pattern. This strategy of new combinatorial enzyme catalysis can generate potential novel soluble α-glucan ingredients with low dietary digestibility assets.

BE more efficiently catalyzed chain transfer in AO than WX to create new branch points and to produce a more monodisperse product. The BE- and BE→AM→BE-treated starches showed a higher rate of α-1,6 glucosidic linkages formation with higher AO:WX ratios. Sequential BE→AM→BE catalysis resulted in more extensive branching as compared to using BE alone. However, high AO substrates generated more α-amylase resistant products. The higher α-1,6 glucosidic linkage content with lower molar mass showed an increase in rat intestinal α-glucosidases hydrolysis rate. This suggests that α-glucosidases is sterically hindered by the molar mass and molecular configuration rather that the branching density of the glucans *per se*. Overall, the resulting products obtained from high amylose starch

were branched-long chain amylose, cyclo-amylose and amylose double helix while branched-amylopectin cluster and cyclo-amylopectin cluster were obtained from low amylose substrate.

Slow and resistant maltodextrin conferring IMOs production would be prepared by the combination of α-amylase, β-amylase, BE, and α-transglucosidase with high cassava starch substrate concentration. The ABbT catalysis showed higher branching linkages, TDF content for both substrate concentrations compared to ABT sample set. The ABbT samples contained isomaltose, isomaltotriose, panose, and higher branched-oligosaccharides. Prebiotics index of ABbT samples were higher than that of ABT samples which not significant different from FOS but lower than commercial IMOs. All of the samples were capable to stimulate growth of Bifidobacteria and Lactobacillus and inhibit Clostridia (histolyticum subgroup) and Bacteroids. They can produce higher acetic acid content which can be metabolized by peripheral tissues and used as an energy source for muscle cell in the human body when compared to propionate and butyrate. All α-glucan products had stimulated probiotics activity as compared to glucose, especially the ABbT treated samples. This novel approach could be applied for an industry which prefers a high substrate concentration to produce the slow and resistant maltodextrin with prebiotics properties which can be served as an ingredient for various kinds of food and drink.

CURRICULUM VITAE

Waraporn Sorndech was born in November 22nd, 1987 at Samutsongkhram, Thailand. She studied for her high school diploma at Sattha Samut School (2000-2005). In 2009, she received the degree of Bachelor of Science (Food Technology) from Suranaree University of Technology.

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