การดัดแปรแป้งด้วยสตาร์ชบรานชิงเอนไซม์จากมันสำปะหลัง *Manihot esculenta* Crantz

นางสาวสุชาดา รักผึ้ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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STARCH MODIFICATION USING STARCH BRANCHING ENZYMES FROM CASSAVA *Manihot esculenta* Crantz

Miss Suchada Rakphung

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Advisor	Associate Professor Tipaporn Limpaseni, Ph.D.

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สุชาดา รักผึ้ง : การดัดแปรแป้งด้วยสตาร์ชบรานชิงเอนไซม์จากมันสำปะหลัง *Manihot esculenta* Crantz (STARCH MODIFICATION USING STARCH BRANCHING ENZYMES FROM CASSAVA *Manihot esculenta* Crantz) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ทิพาพร ลิมปเสนีย์, 120 หน้า.

โซ่กิ่งในแอมิโลเพกติน ถูกสร้างจากเอนไซม์สร้างโซ่กิ่ง (starch branching enzyme : α-1,4-glucan-6-glucosyltransferase, EC 2.4.1.18) เอนไซม์สร้างโซ่กิ่งอยู่ในกลุ่มของ α-amylase มีหน้าที่เร่งปฏิกิริยาการสังเคราะห์ แอมิโลเพกติน โดยการสลาย แอลฟา 1,4-กลูแคน แล้วนำไปเชื่อมกับกลูโคสด้วยพันธะ แอลฟา 1,6 ไกลโคซิดิกที่ด้านปลายรีดิวซ์ของกลูโคสในสายออลิโกแซ็กคาไรด์เดียวกัน ในงานวิจัยของหน่วย มีการโคลนยีน *sbe* จากมันสำปะหลังได้เป็นรีคอมบิแนนท์เอนไซม์ของเอนไซม์สร้างโซ่กิ่งคือ SBERI และ SBERII ในการศึกษานี้ได้ทำการแสดงออก SBERI และ SBERII และทำให้บริสุทธิ์ จากนั้นนำมาดัดแปรแป้งมันสำปะหลัง, แอมิโลส และแอมิโลเพกติน และตรวจสอบผลิตภัณฑ์ที่ได้ด้วย HPAEC-PAD โดยใช้คอลัมน์ carbopac PA-1 และ PA-100 พบว่าภาวะที่เหมาะสมที่จะให้ผลิตภัณฑ์ที่ดีที่สุดในการดัดแปรแป้งมันสำปะหลัง คือ อุณหภูมิ 37 องศาเซลเซียส, เอนไซม์ 15 ยูนิต, ความเข้มข้นแป้งมันสำปะหลัง 10% (w/v) โดย SBERI ใช้เวลาบ่ม 12 ชั่วโมง ในขณะที่ SBERII ใช้เวลาบ่ม 3 ชั่วโมง เมื่อใช้เอนไซม์ทั้งสองดัดแปรแอมิโลส และแอมิโลเพกติน พบว่า SBERI สามารถดัดแปรแอมิโลสได้ผลิตภัณฑ์ที่ 37 องศาเซลเซียส โดยใช้แอมิโลส 2% (w/v) SBERI 10 ยูนิต เวลาบ่ม 12 ชั่วโมง ในขณะที่ SBERII สามารถดัดแปรแอมิโลเพกติน โดยใช้ความเข้มข้น 2% (w/v) ที่ 37 องศาเซลเซียส ใช้เอนไซม์ 10 ยูนิต และเวลาบ่ม 3 ชั่วโมง และผลการวิเคราะห์การกระจายตัวของสายกิ่งแอมิโลเพกติน ในแป้งด้วยคอลัมน์ carbopac PA-1 พบว่ามีปริมาณกิ่งที่มากขึ้นเมื่อเทียบกับแป้งที่ไม่ได้ผ่านการดัดแปร และพบไซโคลแอมิโลสขนาด 9-25 DP ในผลิตภัณฑ์ที่ได้จาก SBERII เมื่อวิเคราะห์ด้วยคอลัมน์ carbopac PA-100 เมื่อนำผลิตภัณฑ์ที่ดัดแปรมาวิเคราะห์สมบัติต่างๆ พบว่าค่าพลังงานที่ใช้ในการสลายผลึกแอมิโลเพกตินลดลง และมีสมบัติที่สามารถเปลี่ยนสถานะกลับไปกลับมาระหว่างของแข็งและของเหลวเมื่อเปลี่ยนอุณหภูมิจากต่ำไปสูงได้ แต่มีค่าความคงทนต่อการแช่เยือกแข็งและการละลายต่ำ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SUCHADA RAKPHUNG: STARCH MODIFICATION USING STARCH BRANCHING ENZYMES FROM CASSAVA *Manihot esculenta* Crantz. ADVISOR: ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D., 120 pp.

Starch branching enzymes (SBE) is a member of the α-amylase family. SBE, an α-1,4-glucan-6-glucosyltransferase (EC 2.4.1.18), introduces branch points in the amylose and amylopectin molecules by hydrolysis of α-1,4-glucan chain from the non-reducing end and catalyzes the formation of an α-1,6 cross linkage between the reducing end of the cleaved chain and a glucose residue on the same oligosaccharide chain. In our laboratory, a few cassava starch metabolizing enzymes have been isolated and their genes were cloned. One of the enzymes was SBE cloned from cassava *sbe* genes. In this research work, two recombinant SBEs, SBERI and SBERII, were expressed and purified. The purified recombinant SBEs were used to modify cassava starch, amylose and amylopectin. SBERI and SBERII were incubated with cassava starch, amylose and amylopectin. The products were identified by HPAEC-PAD technique on a carbopac PA-1 and PA-100 columns. Optimum condition for SBERI activity on cassava starch was 37^{o}C, 15 units of activity, 10% (w/v) of cassava starch and 12 hours incubation time while SBERII showed optimum activity at the same condition but at 3 hours incubation time. When substrate was changed to amylose or amylopectin, SBERI showed activity towards amylose but not amylopectin and the optimum conditions was 37^{o}C, 10 units activity, 2% (w/v) of amylose and 12 hours incubation time. SBERII was active to only amylopectin and the optimum conditions for SBERII activity was 37^{o}C, 10 units activity, 2% (w/v) of amylopectin and 3 hours incubation time. At the determined optimum conditions, analysis of side chain distribution on PA-1 showed that the starch modified by SBERI and SBERII contained higher branches than unmodified substrates and SBERII also yielded medium rings cycloamyloses when analyzed with PA-100 column on HPAEC-PAD. Characterization of the physical properties of the modified product of cassava starch, amylose and amylopectin showed low retrogradation, high thermo reversibility and low freeze-thaw stability.

Field of Study: Biotechnology Student's Signature .....

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LIST OF ABBREVIATIONS

A	absorbance
CH_3COONa	sodium acetate
cm	centimeter
°C	degree Celsius
DI water	deionized water
DP	degee of polymerization
g	gram
HCl	hydrochloric acid
hr	hour
KI	potassium iodide
kDa	kilo Dalton
M	molar
mA	milliamp
min	minute
mg	milligram
ml	milliliter
mm	millimolar
N	normal
NaCl	sodium chloride
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide

nm	nanometer
µl	microliter
µm	micrometer
rpm	revolutions per minute
U	unit
UP water	ultrapure water

CHAPTER I

INTRODUCTION

1.1 Starch

Starch is synthesized in plastids, which are storage organs committed primarily to starch production called amyloplasts, which develop directly from proplastids and have little internal lamellar structure. Starch may also be synthesized in plastids that have other specialized functions such as chloroplasts (photosynthetic carbon fixation), plastids of oilseeds (fatty acid biosynthesis) and chromoplasts of roots such as carrot (carotenoid biosynthesis). It is also synthesized transiently in other organs such as meristems and root cap cells, but its major site of accumulation is in storage organs including seeds, fruits, tubers and storage roots.

Starch can be chemically fractionated into two types of glucan polymer: amylose and amylopectin. Amylose consists of predominantly linear chains of α-l,4-link glucose residues each around 1000 residues long with small number of α-l,6 branches (approximately one branch per 1000 residues). Amylose can makes up to 30% of starch, but proportion may vary considerably with the plant species (a range of 11 to 35% was found in a survey of 51 species (Detherage et al., 1995) and varieties. A range of 20 to 36% was found in a survey of 399 maize

varieties (Detherage et al., 1995) in some plant organs, the developmental age of that organ and to some extent the growth conditions of the plant (Shannon and Garwood, 1984). The amylose chain forms helical structure (Figure 1.1). Once extracted from plants and in solution amylose forms hydrogen bonds between molecules resulting in rigid gels. However, depending on the concentration, degree of polymerization and temperature, it may crystallize and shrink (retrogradation) after heating (Shewmaker and Stalker, 1992). Amylopectin, which consists of highly branched glucan chains, makes up 70% of starch. The ratios of amylose to amylopectin many in different kind of plants (Table 1.2). Chains of roughly 20 α-l,4-linked glucose residues are joined by α-l,6-linkages to other branches. The branches themselves form an organized structure (Figure 1.2). Some are not substituted on the six positions and are called A chains. These chains are α-l,4-linked to inner branches (B chains), which may be branched at one or severa1 points. A single chain per amylopectin molecule has a free reducing end (the C chain). The branches are not randomly arranged but are clustered at 7 to 10 nm intervals. An average amylopectin molecule is 200 to 400 nm long (20 to 40 clusters) and 15 nm wide (Martin and Smith, 1995; Preiss, 1988). After extraction amylopectin has more limited hydrogen bonding than amylose in solution and is more stable, remaining fluid and giving high viscosity and elasticity to

pastes and thickeners. Some starch most notably that from potato tuber, is also phosphorylated. Potato tuber amylopectin carries up to one phosphate per 300 glucan residues (Takeda and Hizukuri, 1982).

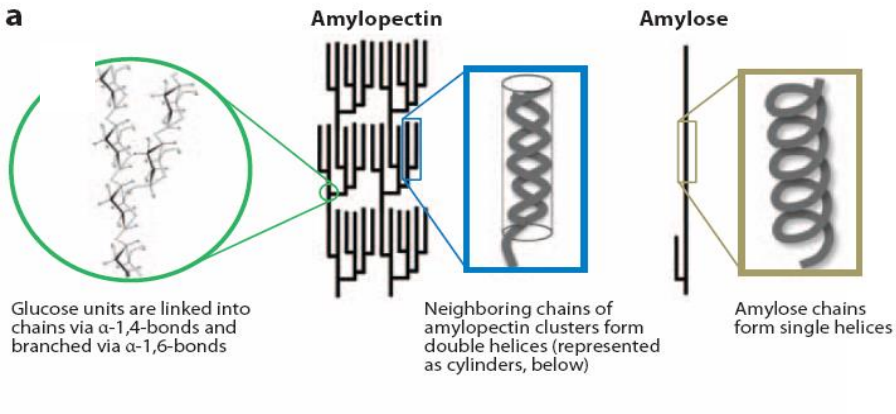


Figure 1.1 A schematic representation of amylose and amylopectin, and the structures adopted by the constituent chains (Zeeman et al., 2010).

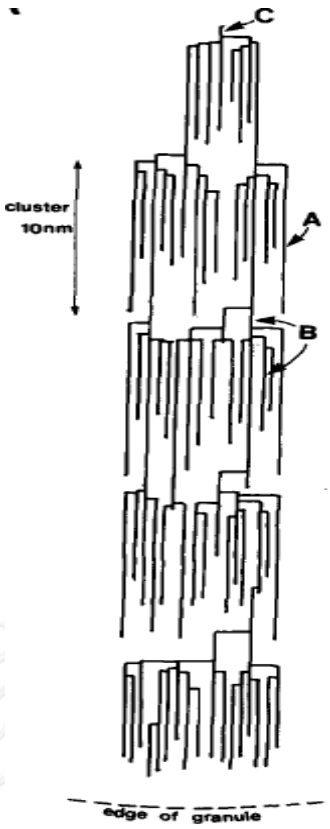


Figure 1.2 Diagrammatic representation of an amylopectin molecule (Martin and Smith, 1995).
α-l,4-link glucans are attached by α-l,6-linkages to form a highly branched structure. Short glucan chains (A chains) are unbranched but linked to multiple branched B chains. There is a single reducing end to the C chain glucan.

Table 1.1 Percent of amylose and amylopectin in reserve plant starch (Zeeman et al., 1984).

Starch	Amylose (%)	Amylopectin (%)
Rice	18.5	81.5
Barley	22	78
Wheat	28	72
Oat	27	73
Corn	28	72
Cassava	17	83
Potato	20	80
Sweet potato	17.8	82.2

1.2 Starch biosynthesis

Starch biosynthesis is well established in the chloroplast of leaves and transported to amyloplasts in storage organs (Myer et al., 2000). Starch biosynthesis in plants occurs in amyloplasts. Many enzymes are involved in the process (Figure 1.3). ADP-glucose, produced by ADP-glucosepyrophosphorylase (AGPase, EC 2.7.7.27) transfers glucose unit to a growing oligosaccharide chain via α-1,4-glycosidic bond. The reaction is catalyzed by starch synthase (SS, EC 2.4.1.21). Starch branching enzyme cut an α-l,4-linked glucan chain and form an α-1,6-linkage between the reducing end of the cut chain and the C6 of another glucose residue in an α-l,4-linked chain, thus creating a branch. Debranching enzyme (EC 2.4.1.25) was reported to be involved in trimming excess branches in amylopectin, to allow packaging in starch granules (Martin and Smith, 1995). Some evidence indicate dispropotionation enzyme (EC 2.4. 1.25) is also involved in starch biosynthesis.

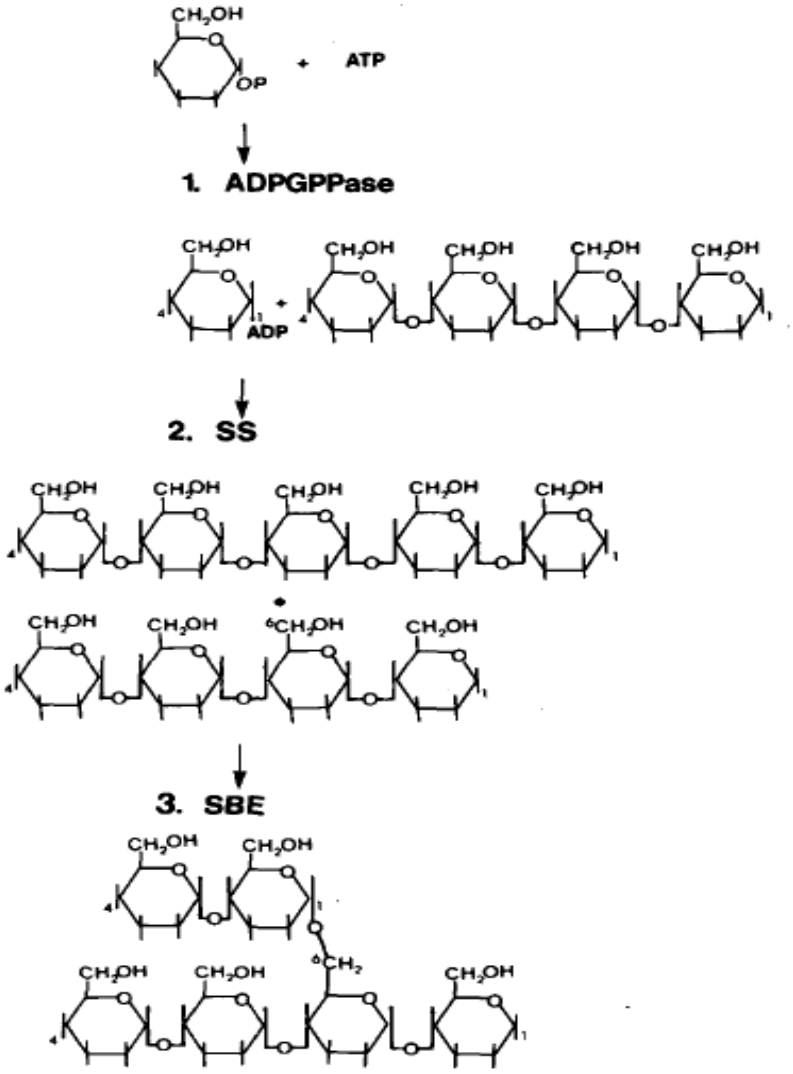


Figure 1.3 Steps of starch biosynthesis (Martin and Smith, 1995).

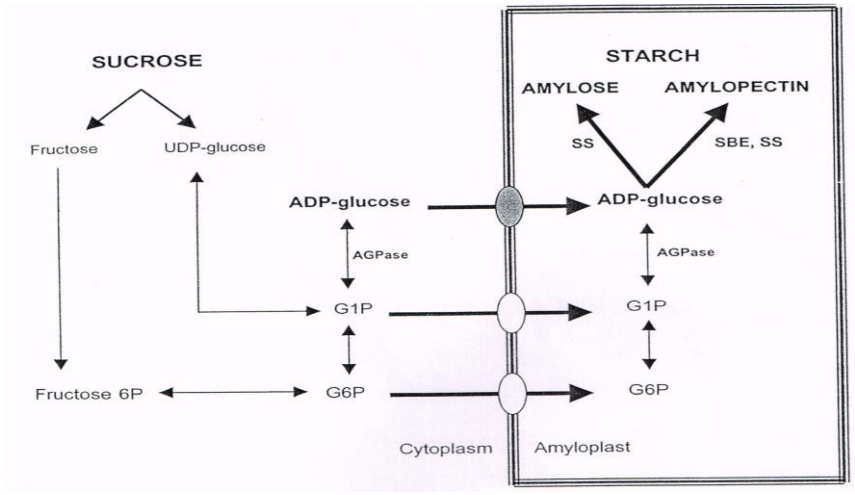


Figure 1.4 General pathway of starch biosynthesis beginning with sucrose the product of photosynthesis (Myer et al., 2000).

From Figure 1.4, sucrose is changed into glucose-6-phosphate for ADP-glucose in the cytoplasm and transported via transporter on amyloplast membrane into amyloplast and synthesize amylose and amylopectin. Glucose-6-phosphate can be converted in amyloplast to ADP-glucose catalyzed by ADP-glucose pyrophosphorylase. ADP-glucose transfer glucose unit to increase the length of polysaccharide. Starch synthase, a reaction catalyzed by forming α-l,4 linkage. Branching is catalyzed by starch branching enzyme.

1.2.1 Amylose synthesis

Amylose consists of glucose subunits linked by α-l,4 bonds froming linear chains, with occasional α-1,6 branch points connecting additional α-l,4-linked chains onto a backbond. The general features of amylose and the other main component of starch are well established.The α-1,4 links in both amylose and amylopectin are made by the starch synthase. The enzyme occurs in multiple forms, but all forms use ADPglucose as the glucose donor to the growing chain. In the storage organ, namely endo-sperm, cotyledons and tubers, amylose is synthesized by the granule-bound starch synthase I (GBSS or GBSSI). The α-1,6 branch points in amylose are not synthesized by GBSSI or GBSSII but may derive from the action of a starch SBE or from branched oligosaccharide as the starch-synthetic substrate, with the poorly branched product subsequently elongated by GBSS. The

substrate for amylose biosynthesis remains controversial and in vivo may be either amylopectin chains or soluble maltro-oligosaccharide (Tiirola et al., 2002) (Figure 1.5**)**.

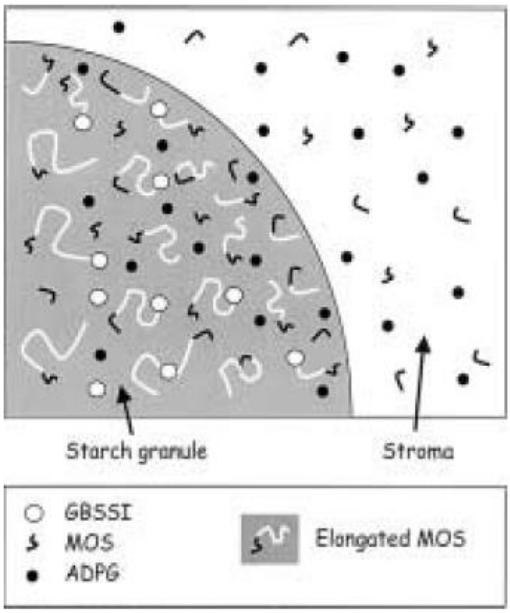


Figure 1.5 A model to explain the synthesis of amylose from malto-oligosaccharide primers (Denyer et al., 2001).
GBSSI binds tightly to the amylopectin matrix of starch granules. In this location, it has a high affinity for MOS. It elongates MOS processively to make long glucan chains. These long glucans are trapped within the amylopectin matrix and are further elongated to form amylose.

1.2.2 Amylopectin synthesis

Amylopectin is considerably more complex as a molecule and its biosynthesis is commensurately more intriguing (Tiirola et al., 2002). The substrate for starch biosynthesis in higher plants is ADPglucose. The glucosyl moiety is transferred onto existing glucan chains by starch synthases. The branching of amylopectin proceeds concurrently with chain elongation (Pan, 1995). Branching is catalyzed by branching enzymes, which cut existing α-1,4-glucan chains and transfer the cut segment of six or more glucose units to the C6 position of a glucosyl residue of another (or the same) glucan chain (Zeeman et al., 2010). The other two enzymes, which are potentially involved in amylopectin biosynthesis are debranching enzyme (DBE) and disproportionating enzyme (D-enzyme) (Myer et al., 2000). DBE catalyzes the hydrolysis of α-1,6- linkages and D-enzyme catalyzes the transfer segment of one linear chain to another.

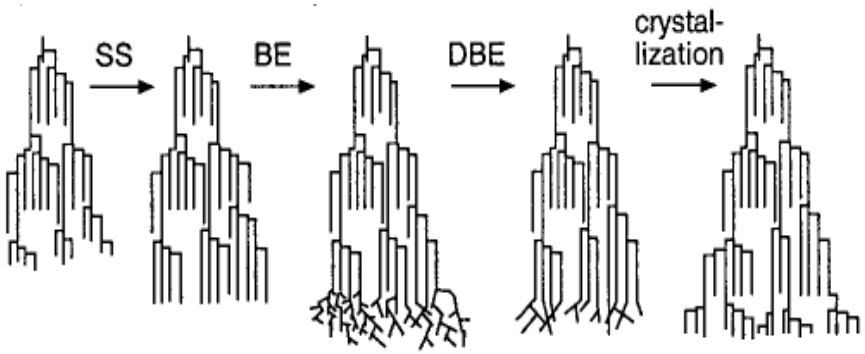


Figure 1.6 Models proposed to explain function in amylopectin biosynthesis (Myer et al., 2000).

1.3 Starch branching enzyme (SBE)

Branching in amylopectin is catalyzed by SBE. SBE is a member of the α-amylase family. SBE an α-1,4-glucan-6-glucosyltransferase, (EC 2.4.1.18) introduces branch points in the amylose and amylopectin molecules by hydrolysis of α-1,4-glucan chain from the non-reducing end and catalyzes the formation of an α-1,6 cross linkage between the reducing end of the cleaved chain and a glucose residue along the remaining chain and glucose residue on the same oligosaccharide chain and glucose residue on the same oligosaccharide chain (Munyikwa et al., 1997).

SBE is found in many plants such as cassava (Yaiyen et al., 2003), potato (Borovsky et al., 1975), Spinach leaf (Hawker et al., 1974), maize endosperm and leaf (Boyer and Preiss, 1978; Dang and Boyer, 1988), rice endosperm (Smyth, 1988) and pea embryo (Matters and Boyer, 1982).

SBE is found in two genes families (families A and B) and It is found in two isoforms (A and B) (Hamada et al., 2002). The two SBEs isoforms create chains with different length or branch points at different frequencies. Multiple forms of SBEs could thus give rise to the branching pattern and polymodal distribution of chain length that underline the cluster structure of the amylopectin. The A and B isoforms of SBEs differ both in their substrate affinities and in the length of

branches they preferentially create. In vitro, A isoform preferentially branches amylopectin and B isoform preferentially branches amylose. With amylose as a substrate, B isoform preferentially transfers longer chain than A isoform (Guan and Preiss, 1993).

SBE was identified in cassava cultivar KU 50 (Yaiyen et al., 2003) and *sbeI* and *sbeII* genes from cassava tubers were cloned in vector pET28 and transformed into *Escherichia coli* rosetta gami (Yaiyen et al., 2010) and identified. The in vitro activities of purified recombinant starch branching enzyme I (SBERI) and recombinant starch branching enzyme II (SBERII) were compared using several assay methods such as substrate specificity, number of branching of linear dextrin. It was found that SBERI and SBERII had the same molecular weight of 90 kDa, but SBERI was more active on amylose substrate whereas SBERII was more active on amylopectin.

1.4 Physical properties of starch

Starches from different sources and different treatment show different physio-chemical characteristics which determine the suitability of each kind of starches in industrial applications.

1.4.1 Thermal properties

Starch undergoes gelatinization under excess moisture above 63% (ratio of 1 glucose : 14 water for complete gelatinization (Lai and Kokini, 1991). It is generally accepted that during gelatinization water

first penetrates the amorphous region, initiating swelling and resulting in a decrease in birefringence. Additionally, water strips starch chains from the surface of crystallites as the temperature is increased. With increasing temperature, the thermal motion of the molecules and the solvation by the swelling forces lead to decreasing order and disruption of the crystalline regions with uncoiling of the double helices until the granular structure is disrupted nearly completely and a solvation is obtained (Figure 1.7) (Donovan and John, 1979; Lineback, 1986; Smith, 1979).

Gelatinization of starch can be followed by differential scanning calorimetry (DSC) (Donovan and John, 1979; Donovan and Mapes, 1980; Eliasson and Gudmundsson, 1996) in which retrogradation of gels from nongranular mixtures with different amylose/amylopectin ratios were studied. Synergistic interactions were seen between amylopectin and amylose at a high amylose content (melting endotherm) (Gudmundsson and Eliasson, 1990). DSC has been widely used to characterize the thermally conversion of starches as a function of moisture content (Biliaderis et al., 1980; Donovan and John, 1979; Donovan and Mapes, 1980; Eliasson, 1980; Wang et al., 1989). In this method, a sample and an inert material are both heated in a uniform way. The temperature difference between the sample and the reference is then converted to the enthalpy change. The results are then interpreted

for starch systems as being related to the breaking of starch-starch hydrogen bonds in favor of starch-water bonds with increase in entropy (Table 1.2).

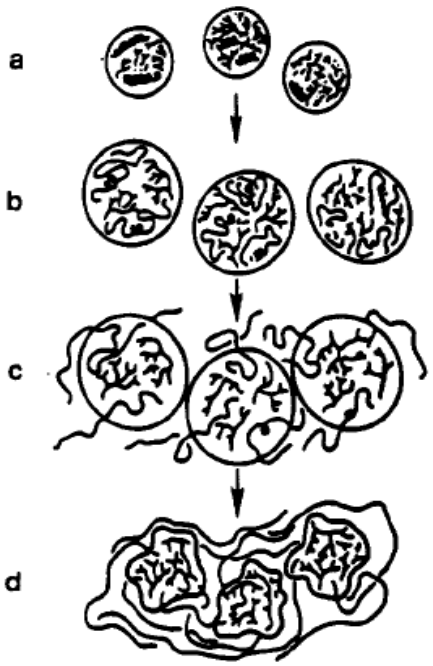


Figure 1.7 Mechanism of starch gelatinization (Remsen and Clark, 1978).
a : Raw starch granules made up of amylose (helix) and amylopectin (branched).
b : Addition of water breaks up amylose crystallinity and disrupts helices.
c : Addition of heat and more water causes more swelling. Amylose begins to diffuse out of granule.
d : Granules, now containing mostly amylopectin, have collapsed and are held in a matrix of amylose forming a gel.

Table 1.2 DSC Characteristics of Several Starches (Lai and Kokini, 1991).

Starch	Endotherm temp, °C		
	T_0	T_p	T_c
Cassava	68	78	92
Potato	57	72	87
Corn	60	67-78	89
Waxy corn	50	68	86

T_0 = The initial gelatinization temperature
T_p = The endotherm peak temperature
T_c = The temperature at which gelatinization ceased

1.4.2 Freeze-thaw stability

Starch has been incorporated in many foods including sauce, soup, frozen-batter, ice cream, cream-based product and dessert. These products usually expose to series of temperature fluctuation during long-term storage or freeze–thaw process before consumption (Srichuwong et al., 2012).

Retrogradation in frozen starch gels was affected by hydrocolloid addition and freezing rate. A freeze–thaw cycle treatment was used in this study to determine the effects of hydrocolloids and freezing rates on the stability in tapioca gels. Analysis of retrogradation of starch gels was performed by freeze–thaw cycle and based on syneresis measurements (Muadklay and Charoenrein, 2008). When starch pastes or gels are frozen in the freezing process, phase separation occurs upon formation of ice crystals. Upon thawing, the pastes or gels are composed of a starch-rich or a starch-deficient aqueous phase. The extent of phase separation increases with additional freeze–thaw cycles due to an increase in amylopectin retrogradation in the starch-rich phase (Yuan and Thompson, 1998). Upon thawing, a phenomenon known as syneresis occurs with starch pastes and gels because the water can be easily expressed from the dense network (Karim et al., 2000). Thus, the amount of syneresis can be used as an indicator of the tendency of starch to retrograde (Karim et al., 2000). Freeze–thaw stability is an important

property that is used to evaluate the ability of starch to withstand undesirable physical changes occurring during freezing and thawing. This property may be simply evaluated by gravimetric measurement of the water of syneresis that separates from starch pastes or gels (Schoch, 1968; Seib, 1990). Repeated freeze–thaw cycles that involve subjecting samples to repeated freezing and intermittent thawing are known to accelerate retrogradation and syneresis drastically (Radley, 1976) (Table 1.3). The rate of freezing is also known to affect the retrogradation rate (Slade, 1986). Slower freezing rates increase both the starch molecular associations and precipitation (BeMiller, 1998). Consequently, different freezing rates used in a freeze–thaw study would also be expected to give rise to different degrees of syneresis (Karim et al., 2000). Hydrocolloids are commonly used to improve the texture and rheological properties of starch-based products (Shi and BeMiller, 2002), because they can be modified to have higher viscosity and less syneresis by using small quantities of hydrocolloids (Mali et al., 2003). According to previous investigations, addition of hydrocolloids reduces syneresis and physical property changes that were induced by freeze–thawing cycles (Lee et al., 2002).

Table 1.3 Syneresis (%) of starch gels after freeze–thaw cycle (Srichuwong et al., 2012).

Starch	% Synneresis		
	Cycle 1	Cycle 3	Cycle 5
Cassava	-	38.9	51.5
Potato	60	71.5	75.6
Corn	35.4	69.5	73.3
Waxy corn	9.6	33.7	53.9

1.4.3 Thermo reversibility

Thermo reversibility was determined as the viscosity behavior. The starch gel is characterized with certain moduli, such as shear modulus (G), storage modulus (G') (elasticity), loss modulus (G") (viscosity), or complex modulus (G*). The phase angle (δ) is used to indicate the degree of elasticity (Eliasson and Gudmundsson, 1996). The ratio of loss modulus G" to the storage modulus G' is called the loss angle (damping) and is expressed by the tangent of the phase angle (δ) (Wilczyński et al., 2012).

The loss angle tan (δ) is the ratio between the energy lost and stored during the deformation and determines the viscoelastic material's ability to dissipate (damp) energy.

$$\tan(\delta) = \frac{G''}{G'}$$

In the case of completely viscous material value of tan (δ) is greater than 1, and the loss modulus values are higher than the storage modulus G" > G'. When the value of tan (δ) is less than 1 it means supremacy of the elastic properties over the viscous, which means higher values of storage modulus in relation to the loss modulus G' > G". The lower the value of the tan (δ), the more elastic the material. A zero value of tan (δ) indicates that the material is perfectly elastic.

The course of deformation and the induced stress in the perfectly elastic solid is in-phase (Figure 1.8). However, in the viscoelastic material phase shift angle (δ) occurs in the range 0 - 90°, between the stress and strain that induces it (Figure 1.9). It is the result of delayed reaction of the material to an applied strain. The response to an applied strain consistent with the phase is called the elastic (material entirely elastic – behaves according to Hooke's law), shifted in phase by 90° - viscous (material entirely viscous - behaves like a Newtonian fluid), and between 0 and 90° - viscoelastic (viscoelastic material) (Boczkowska, 2012).

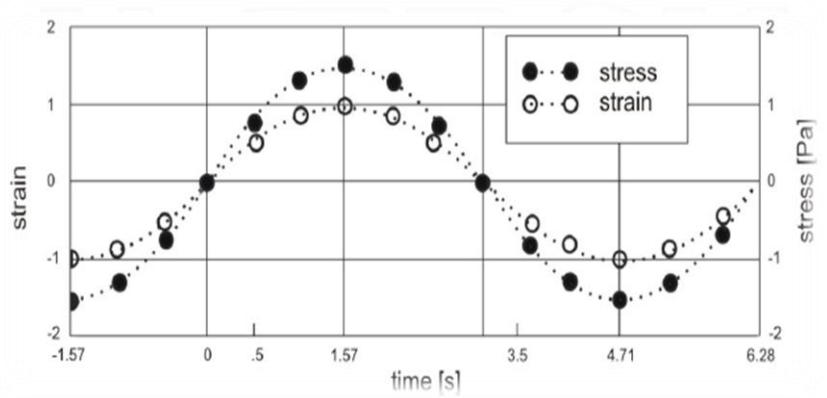


Figure 1.8 Dynamic measurement: strain and stress in an elastic solid (Boczkowska, 2012).

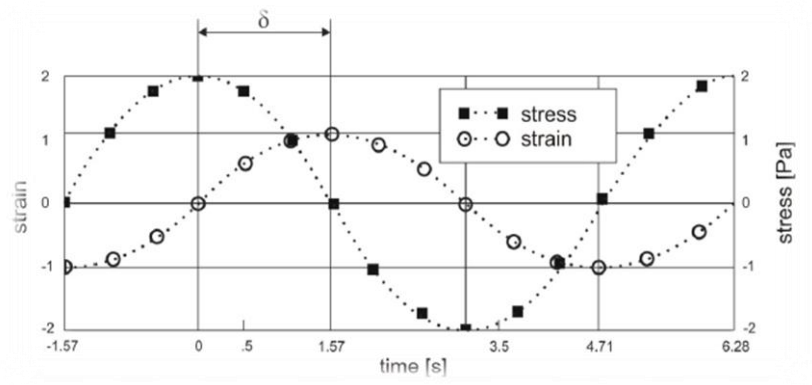


Figure 1.9 Dynamic measurement: strain and stress in the viscoelastic body (Boczkowska, 2012).

1.5 Enzymatic starch modification

Modification of starch is generally carried out to enhance the positive attributes and eliminate the shortcomings of the native starches. Modification of starch is an ever evolving industry with numerous possibilities to generate novel starches which includes new functional and value added properties as a result of modification and as demanded by the industry. The modified starches can be used in foodstuffs, cosmetics, pharmaceutics, detergents, adhesives and drilling fluids. (Kaur et al., 2012). Starch modification can be performed either chemically or enzymatically. Due to safety measure, enzymatic modification is performed especially in food, pharmaceutical and cosmetic industries. Many enzymes have been employed in starch modification. Amylomaltase, an enzyme catalyzes the transfer of α-1,4-glucan via inter- and intramolecular transglucosylation reaction, is one of the enzyme used to modify starch.

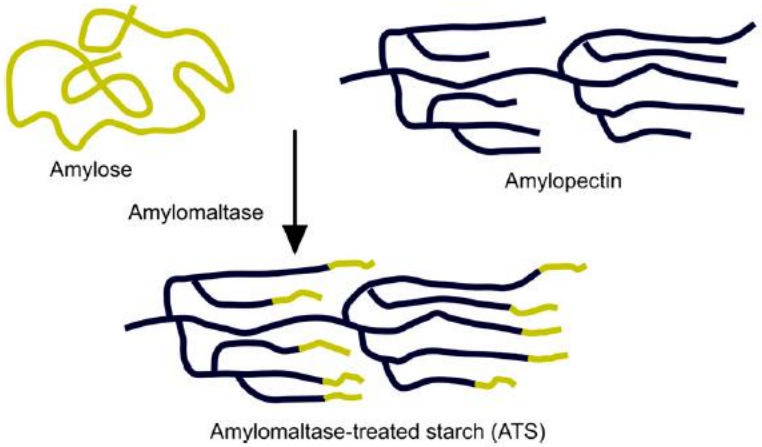


Figure 1.10 Schematic representation of the enzymatic conversion of potato-starch-derived amylose and amylopectin into amylomaltase-treated starch (ATS) by amylomaltase (Alting et al., 2009).

The use of amylomaltase (AM) (EC 2.4.1.25) to modify starches is expected to find applications in the food industry as a plant and chemical free alternative to gelatine (Euverink and Binnema, 1998). Starch treated with α-1,4-α-1,4 glucosyl transferases, also known as AM is used in forming a thermoreversible gel. An AM-modified potato starch has been used as a fat replacer and enhancer of creaminess in yoghurt (Alting et al., 2009). Modification of potato starch with pullulanase resulted in decrease in the average molecular weight, as well as increases in polydispersion and in the amylose content in biomodified starch and find application in manufacturing films and fibres (Kazimierczak et al., 2007). Lee et al. (2008) used *Thermus scotoductus* α-glucanotransferase (TSαGT) and *B. stearothermophilus* maltogenic amylase (BSMA) in modification of rice starch to produce highly branched amylose and amylopectin. The products were highly stable and water soluble. Modification of barley starch by α-amylase and pullulanase showed accumulation of smaller-size hydrolysis products (Lauro et al., 1993). Hansen et al. (2008) reported an improvement in gel texture compared to the native starch in the modification of potato, high-amylose potato, maize and pea starch with AM isolated from the hyperthermophilic bacterium *Thermus thermophilus*.

Branching enzyme from *Bacillus stearothermophilus* TRBE14 was previously reported to be able to catalyze the cyclization of amylose and amylopectin (Takata et al., 1997). The product was highly soluble and stable. The result suggested that branching enzyme might be useful as a new starch-processsing enzyme. Wheat, potato and waxy maize starches were treated by branching enzyme in order to increase the degree of branching of the amylopectin fraction and thereby change the starch degradation profile towards a higher proportion of slowly digestible starch (Kasprzak et al., 2012). Maize starch treated with branching enzyme produce a novel branched structure with slowly digestible character (Li et al., 2014).

In our laboratory, starch metabolizing enzyme from cassava tuber such as starch branching enzymes, starch debranching enzyme and pullulanase have been cloned and characterized (Yaiyen et al., 2010), (Tantarat et al., 2014) and (Kerdpitak et al., 2014). Transformant cassava debranching enzyme (MeDPEI) was used to study starch modification. Kitpibun, (2014) reported large ring cycloamylose production in cassava and corn starch treated with MeDPEI. The product was low in amylose content, lower tan value, low % syneresis, high freeze-thaw stability and high thermo-reversibility.

1.6 Aims of the Thesis

Recombinant starch branching enzymes (SBERs) were produced in our laboratory. This work aimed at using SBERs to treat cassava starch, amylose and amylopectin and characterized the products. The product of interest can then be further used in suitable industry.

Research steps

1. Preparation and purification of starch branching enzymes (native starch branching enzymes and recombinant starch branching enzymes)
2. Determination of the optimum conditions for starch modification
3. Physical properties of modified products

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Hirayama Manufacturing Cooperration, Japan

Autopipette: Pipetman, Gilson, France

Balance: Mettler Toledo, Switzerland

Bandelin Sonoplus Sonicator: Bandelin Electronic, Germany

Differential Scanning Calorimeter: Perkin Elmer, Diamond DSC, USA

Electrophoresis power supply: Thermo Scientific, USA

Electrophoresis Unit: Biorad, USA

Fraction collector: LKB

High Performace Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD): Dionex Corporation, Sunnydale, USA

- Column Dionex CarboPac® PA1 column 4x250 mm, 4 µm, with a 4x50 mm guard, Pulsed Amperometric Detector: Dionex Corporation, Sunnydale, USA
- Column Dionex CarboPac® PA100 column 4x250 mm, 4 µm, with a 4x50 mm guard, Pulsed Amperometric Detector: Dionex Corporation, Sunnydale, USA

HisTrap^{FF} TM column: GE Healthcare, UK

Hot plate stirrer: Becthai Bangkok Equipment & Chemical, Thailand

Incubator: Memmert, Germany

Incubator Shaker: New Brunswick Scientific, USA

Magnetic stirrer: VELP® Scientifica, Italy

Membrane filter: Whatman, England

Microcentrifuge: Eppendorf, Germany

Oven: Contherm Scientific, Newzealand

pH meter: Mettler Toledo, Switzerland

Refrigeration Centrifuge: Avati J-30I: Backman Instrument, USA

Rheometer: Thermoscientific, HAAKE MARS Rotational rheometer, USA

Strealine Vertical Laminar Flow Cabinets: Strealine Laboratory, Singapore

UV-VIS spectrophotometry: Thermo Scientific, USA

Vortex: Scientific industry Incorporation, USA

Water bath: Memmert, Germany

Water bath shaking: UK

2.2 Chemicals

Absolute ethanol: Sigma-Aldrich, USA

Acetic acid: Prolabo, France

Acrylamide: Merk, Germany

Agar, Scharlau, Spain

Ampicillin: Sigma-Aldrich, USA

Amylopectin from Potato starch: Sigma-Aldrich, USA

Amylose from Potato starch: Sigma-Aldrich, USA

Bovine serum albumin: Sigma-Aldrich, USA

Bromophenol blue: Merck, Germany

Cassava tuber (KU50 caltivar: Rayong Field Crops Research Center, Thailand)

Coomassie Brilliant Blue G250: Sigma-Aldrich, USA

Coomassie Brilliant Blue R250: Sigma-Aldrich, USA

Cycloamylose: Ezaki Glico, Japan

D-(+)-Glucose: Fisher chemicals, UK

DEAE-Toyopearl, Tosho

Dialysis tube: Sigma-Aldrich, USA

Dithiothreitol (DTT): USB Corporation, USA

Ethanol: Carlo Erba, Italy

Ethylenediaminetetraacetic acid (EDTA): Ajax Finechem, Australia

Glucoamylase: SORACHIM, Switzerland

Glycerol: Ajax Finechem, Australia

Glycine: Amersham Bioscience

Hydrochloric acid: Carlo Erba, Italy

Imidazole: Fluka, Switzerland

Iodine: Baker chemical, USA

Isoamylase from *Pseudomonas* sp.: Sigma-Aldrich, USA

Isopropyl-β-D-1-thiogalactopyranoside (IPTG): Sigma-Aldrich, USA

Matoheptaose: Sigma-Aldrich, USA

Matohexaose: Sigma-Aldrich, USA

Matopentaose: Tokyo Chemical Industry Company Limited, Japan

Matose: Conda, Spain

Maltotetraose: Hayashibava Biochemical Laboratotries Incorporation, Japan

Maltotriose: Sigma-Aldrich, USA

Methanol: Mallinckrodt, USA

N, N, N', N'-Tetramethylethylenediamine (TEMED): Carlo Erba, Italy

Phenylmethanesulfonylfluoride (PMSF): Applichem, USA

Phosphoric acid: Sigma-Aldrich, USA

Potassium dihydrogen phosphate: Ajax Finechem, Australia

Potassium iodide: Mallinckrodt, USA

Sodium acetate: BDH Chemical Limited, England

Sodium chloride: Ajax Finechem, Australia

Sodium dodecyl sulfate (SDS): Sigma-Aldrich, USA

Sodium hydroxide, Merck, Germany

Sodium nitrate: Ajax Finechem, Australia

Soluble potato starch: Scharlau, Spain

Soluble cassava starch (Rayong 9 caltivar: Rayong Field Crops Research Center, Thailand)

Standard protein marker: Sigma-Aldrich, USA

Tris (hydroxymethyl)-aminomethane: Carlo Erba, Italy

Tryptone: HiMedia, India

Yeast extract: Scharlau, Spain

2.3 Preparation of starch branching enzymes

2.3.1 Preparation and purification of native starch branching enzymes

Native starch branching enzymes was extracted from cassava tubers (*Manihot esculenta* CRATNZ cv. KU50) (Yaiyen, 2003). Crude enzyme prepared by homogenization of parenchyma in cassava tubers and separated by centrifugation at 8000 rpm, 4°C for 30 minutes. The supernatant was purified by DEAE-Toyopearl column chromatography. DEAE-Toyopearl was washed with 0.5 N NaOH followed with distilled water and packed into a glass column (2x15 cm) and equilibrated with starting buffer (0.05 M Tris-HCl pH 7.5 containing 2 mM EDTA and 1 mM DTT). The crude enzyme was loaded into the column and eluted with 0.15 M NaCl at flow rate 60 ml/hr. Fractions were collected using fraction collector. The eluted fractions were monitored for protein by measuring the absorbance at 280 nm and the enzyme activity was

detected by spectrophotometric method. The fractions with enzyme activity were pooled and dialyzed at 4°C with distilled water for second DEAE-Toyopearl step. The enzyme was loaded into the column and eluted stepwise with 0.04M, 0.08 M and 0.1 M NaCl at flow rate 60 ml/hr.

2.3.2 Preparation of recombinant starch branching enzymes

sbeI and *sbeII* genes from cassava tubers (*Manihot esculenta* CRATNZ) were cloned in vector pET28 and transformed into *E. coli* rosetta gami (Yaiyen et al., 2010). *E. coli* transformants were cultured on LB medium plate (Appendix A) containing 100 µg/ml ampicillin at 37°C for overnight, a single colony was inoculated in 5ml LB broth medium (Appendix A) containing 100 µg/ml amplicilin at 37°C overnight. 1.0% cell culture from starter was inoculated in 600 ml of same medium at 37°C with shaking at 250 rpm. When the tubidity at A_{600} reached 0.4-0.6, IPTG was added to a concentration of 0.4 mM and cultivation was continued for 4 hours. The cells were harvested by centrifugation at 8000 rpm, 4°C for 30 minutes. The cell pellet was washed in extraction buffer (0.05 M Tris-HCl pH 7.4 containing 0.1 mM PMSF, 1 mM DTT and 2 mM EDTA) and centrifuged again. Cell pellet was resuspended in extraction buffer (1 g/ 5 ml). Cells were disrupted by sonication and supernatant was collected by centrifugation at 10000 rpm, 4°C for 30 minutes and collected for purification.

2.4 Purification of recombinant starch branching enzymes

The recombinant starch branching enzymes was purified by HisTrap column chromatography. The column was washed with filtrated distilled water followed with filtrated binding buffer (0.05 M Tris-HCl pH 7.5 containing 0.2 mM imidazole and 1 mM DTT) until the absorbance at 280 nm appeared zero. The enzyme was added with 0.2 mM imidazole and filtered. It was loaded into the column at flow rate 0.75 ml/min and eluted with filtrated elution buffer (containing 0.3 M NaCl, 0.5 mM imidazole and 1 mM DTT) until the absorbance at 280 nm appeared zero. The eluted enzyme was collected and dialyzed.

2.5 Protein determination

Protein was determined by Bradford protein assay (Bradford, 1976), using Bovine serum albumin (BSA) as standard. 1 ml of Bradford solution (containing 0.1% (w/v) Coomassie Brilliant Blue G-250 in absolute ethanol with 85% phosphoric acid) was added into 100 µl of enzyme. The reaction was incubated for 5 minutes and absorbance was measured at 595 nm.

2.6 Polyacrylamide gel electrophoresis (PAGE)

2.6.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight of protein was determined by SDS-PAGE.

Solution consists of 10% (w/v) SDS in 7.5% (w/v) separating gel and 5% (w/v) stacking gel (Appendix B) and Tris-glycine buffer pH 8.3 was used as electrode buffer. Protein sample and standard proteins contained Coomassie Brilliant Blue R-250 were boiled for 5 minutes and loaded into gel. Electrophoresis was performed at constant current of 20 mA per slap at room temperature. The gel was stained with staining solution for 1 hour and destained overnight with destaining solution.

2.6.2 Non-denaturing starch-polyacrylamide gel electrophoresis (non- denaturing-PAGE)

Activity of enzyme was qualitatively checked by non-denaturing-PAGE. Solution consists of 7% (w/v) separating gel with 0.6% (w/v) soluble starch and 4% (w/v) stacking gel (Appendix B) and Tris-glycine buffer pH 8.3 was used as electrode buffer. Protein samples containing dye were boiled for 5 minutes and loaded into gel. Electrophoresis was performed at constant current of 16 mA per slap at 4°C for 1 hour. The gel was rinsed with distilled water and soaked in 50 mM NaOH pH 7.0 for 2 hours at room temperature. After that, incubated for 15 minutes in iodine solution (0.1% I_2 in 1% KI). The activity bands appeared as sharp red-brown band on blue background.

2.7 Starch branching enzymes activity assay

SBE activity was determined by a modified spectrophometric method using amylose as substrate for SBERI and amylopectin as

substrate for SBERII (Yaiyen et al., 2003), based on the spectral change of the glucan-iodine complex formed in the reaction (Boyer and Preiss, 1978). The enzyme (100 µl) was incubated with 400 µl of 1 mg/ml amylose or amylopectin at 37°C for 24 hours. The reaction was stopped by boiling for 2 minutes. It was added with 300 µl of distilled water and 2.6 ml of iodine solution (0.005% I_2 in 0.05% KI). The absorbance was measured at 660 nm for amylose and 520 nm amylopectin.

One SBE activity unit was defined as the amount of enzyme that decrease in the blue color of starch-iodine complex per minute at 37°C.

2.8 Determination of the optimum conditions for starch modification

Three kinds of substrates were used in starch modifications: soluble cassava starch (cv. Rayong 9), amylose and amylopectin with the purified SBERI and SBERII. Each kind of substrate was prepared as slurry by boiling the starch in boiling water with stirring for 30 minutes and cooled to 30ºC.

2.8.1 Optimum incubation time

Slurry of 2% (w/v) cassava starch, amylose or amylopectin was incubated with SBERI or SBERII (1 g of starch/15 units of SBE) and the reaction mixture was incubated in shaking water bath at 37ºC at 3, 6, 12 and 24 hours. The reaction was stopped by heating in boiling water for 30 minutes. The modified starch slurry was dried at 45ºC for 48

hours (Lee et al., 2008). The products were prepared for analysis HPAEC-PAD (PA-1 column) according to section 2.9.1.

2.8.2 Optimum substrate concentration

Slurry of cassava starch, amylose or amylopectin was prepared at 2, 5 and 10% (w/v). The substrate slurry was added with 15 units of SBERs. The reaction mixture was incubated in shaking water bath at 37ºC at the determined optimum time of each SBERs (section 2.8.1). Reaction was stopped by heating in boiling water for 30 minutes. The modified starch slurry was dried at 45ºC for 48 hours (Lee et al., 2008). The products were prepared for analysis HPAEC-PAD (PA-1 column) according to section 2.9.1.

2.8.3 Optimum enzyme concentration

To determine the optimum concentrations of SBERI and SBERII for starch modification reaction, the reaction mixture was performed at the determined optimum time and optimum substrate concentration of each enzyme from section 2.8.1 and 2.8.2. The enzyme concentration was varied at 5, 10, 15 and 20 units. Reaction mixture was incubated in shaking water bath at 37ºC. The reaction was stopped by heating in boiling water for 30 minutes. The modified starch slurry was dried at 45ºC for 48 hours (Lee et al., 2008). The products were prepared for analysis HPAEC-PAD (PA-1 column) according to section 2.9.1.

2.9 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection technique (HPAEC-PAD)

Each reaction mixture was prepared the determined optimum time, optimum substrate concentration and optimum concentration of each enzyme. Reaction mixture was incubated in shaking water bath at 37ºC. The reaction was stopped by heating in boiling water for 30 minutes. The modified starch slurry was dried at 45ºC for 48 hours (Lee et al., 2008). The products were prepared for analysis HPAEC-PAD (PA-100 column) according to section 2.9.1 and 2.9.2.

2.9.1 Detection of side chain distribution by CaboPac PA-1 column

The reaction product solution was prepared (4.5 mg in 1.6 ml UP water) and boiled in water with stirring, cooled to room temperature and added with 200 µl of 0.1 M acetate buffer pH 3.5. 2 µl of isoamylase from *Pseudomonas* (1770 units) was added and incubated in shaking water bath at 40ºC for 48 hours. The reaction was stopped by heating in boiling water for 20 minutes and centrifuged at 10000 rpm for 30 minutes. The supernatant was applied to PA1 column (Kuakpetoon and Wang, 2006) equilibrated with 150 mM NaOH and sample was eluted by 600 mM CH_3COONa in 150 mM NaOH and 500 mM NaOH with flow rate 1 ml/min (Appendix D).

2.9.2 Detection of sizes of cycloamyloses by CaboPac PA-100 column

The reaction product solution was prepared (4.5 mg in 1.6 ml UP water) and boiled in water with stirring for 1 hour, cooled to room temperature, added with glucoamylase (40 units) and incubated in shaking water bath at 40ºC for 24 hours. The reaction was stopped by heating in boiling water for 20 minutes and centrifuged at 10000 rpm for 30 minutes. The supernatant was applied to PA100 column (Kuakpetoon and Wang, 2006) equilibrated with 150 mM NaOH and sample was eluted by 200 mM $NaNO_3$ in 150 mM NaOH and 1M $NaNO_3$ with flow rate 1 ml/min (Appendix E).

2.10 Physical properties of modified products

2.10.1 Thermal properties

Thermal properties (The entralpy of amylopectin crytal) was measured by DSC (Cho et al., 2009). It was calculated with Pyris program. The **s**ample was prepared with 2 mg of modified starch in aluminum volatile pan and 10 µl of DI water was added. After that, the pan was sealed. Sample was heated from 30-85ºC at 10ºC/min by DSC for gelatinization, then stored at 4ºC for 14 days for observation of retrogradation and reheated again at the same conditions.

2.10.2 Freeze-thaw stability

Freeze-thaw stability was determined by measuring the % water

released (% synersis) during the freeze-thaw process (Lee et al., 2006).

$$\% \text{ synersis} = \left(\frac{\text{gel weight - gel weight after thaw}}{\text{gel weight}} \right) \times 100$$

The sample was prepared with 5% (w/v) modified starch,] gelatinized in DI water and heated in boiling water for 30 minutes. One ml of **s**ample was weighed in microcentrifuge tube. It was frozen at -20ºC for 24 hours, then thawed at 30ºC in water bath for 2 hours and centrifuged at 10000 rpm, 25ºC for 20 minutes. Water in sample was removed from the gel and weighed. Sample was frozen and thawed again for 4 cycles.

2.10.3 Thermo reversibility

Thermo reversibility was determined by measuring viscoelasticity of gel by rheometer between stress value and strain value (Lee et al., 2006). The sample was prepared with 5% (w/v) of modified starch and gelatinized in DI water, heated in boiling water for 30 minutes. Viscoelasticity was measured by rheometer at 70ºC with 35 mm diameter parallel plate, 1 mm gap size, 1% stress and frequency of 1 Hz. Time sweep program was used to calculate storage modulus value (G'), loss modulus value (G'') and tan (δ) value (G'/ G''). After that, sample was stored at 4ºC for 24 hours and measured by rheometer at 4ºC. Sample was reheated at 70ºC and measured by rheometer at 70ºC. The process between 4ºC and 70ºC was repeated and sample collected for measurement with rheometer.

CHAPTER III

RESULTS

3.1 Preparation and purification of starch branching enzymes

3.1.1 Native starch branching enzymes (SBE)

Crude SBE was prepared by homogenization of parenchyma in cassava tubers *Manihot esculenta* CRATNZ (section 2.3.1). The crude enzyme was loaded on DEAE-Toyopearl column chromatography according to section 2.3.1. Fractions of 18 ml were collected. Unbound proteins were washed with starting buffer and SBE was eluted with 0.15 M NaCl. The fractions with enzyme activity were pooled and enzyme activity was detected by spectrophotometric method (section 2.7) for second DEAE-Toyopearl step. The enzyme was loaded into the column and eluted stepwise with 0.04M, 0.08 M and 0.1 M NaCl. Purification result was shown in Table 3.1.

3.1.2 Recombinant starch branching enzymes (SBER)

sbeI and *sbeII* genes from cassava tubers *Manihot esculenta* CRATNZ were cloned in vector pET28 and transformed into *E. coli* rosetta gami (Yaiyen et al., 2010). The transformants were cultured and crude SBERs were collected by SBE activity (section 2.7) and purified by HisTrap column chromatography according to section 2.3.2 and 2.4.

Unbound proteins were washed with binding buffer and eluted with elution buffer (containing 0.3 M NaCl, 0.5 mM imidazole and 1 mM DTT). The fractions with enzyme activity were pooled and enzyme activity was detected by spectrophotometric method (section 2.7). Purification result was shown in Table 3.1. Two recombinant SBEs were obtained, named SBERI and SBERII respectively.

Molecular weight of native and recombinant SBEs were determined by SDS-PAGE. SBERI and SBERII were 95 kDa as determined from standard protein markers (Figure 3.1A).

Activities of SBEs were checked by non-denaturing-PAGE. Activity bands of SBERI and SBERII were observed (Figure 3.1B).

Table 3.1 Purification of starch branching enzymes from cassava *Manihot esculenta* Crantz

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification fold	Yield (%)
SBE					
Crude	985	150	0.15	1	100
1st DEAE	238	186	0.78	5	124
2nd DEAE	122	155	1.27	4	103
SBERI					
Crude	480	685	1.4	1	100
Histrap column	153	960	6.2	4	140
SBERII					
Crude	395	525	1.3	1	100
Histrap column	110	755	6.8	5	143

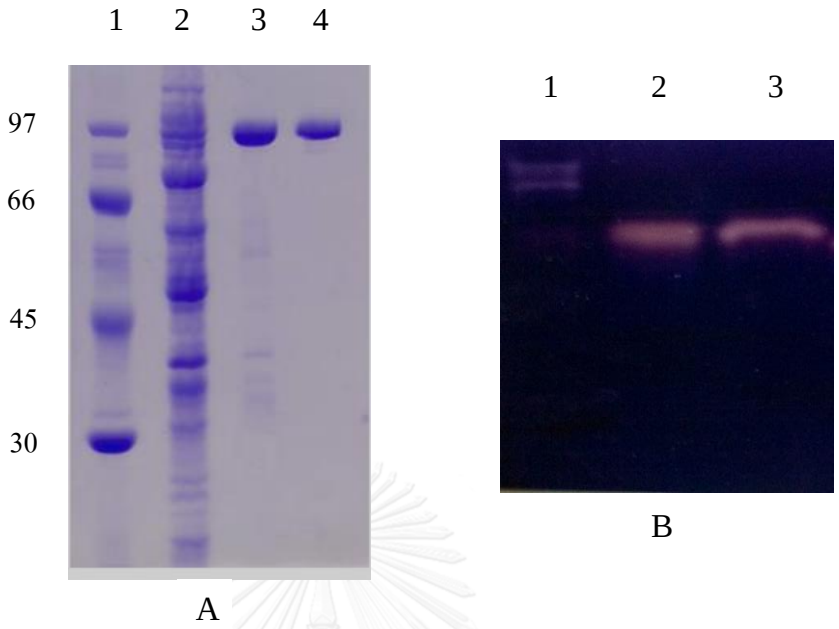


Figure 3.1 SDS-PAGE and non-denaturing-PAGE patterns of recombinant starch branching enzymes.

A : Determination of molecular weight of SBER by SDS-PAGE
Lane 1 = Low molecular weight protein markers, MW in KDa
Lane 2 = Crude enzyme
Lane 3 = SBERI
Lane 4 = SBERII

B : Activity staining of SBER on non-denaturing-PAGE
Lane 1 = Crude enzyme
Lane 2 = SBERI
Lane 3 = SBERII

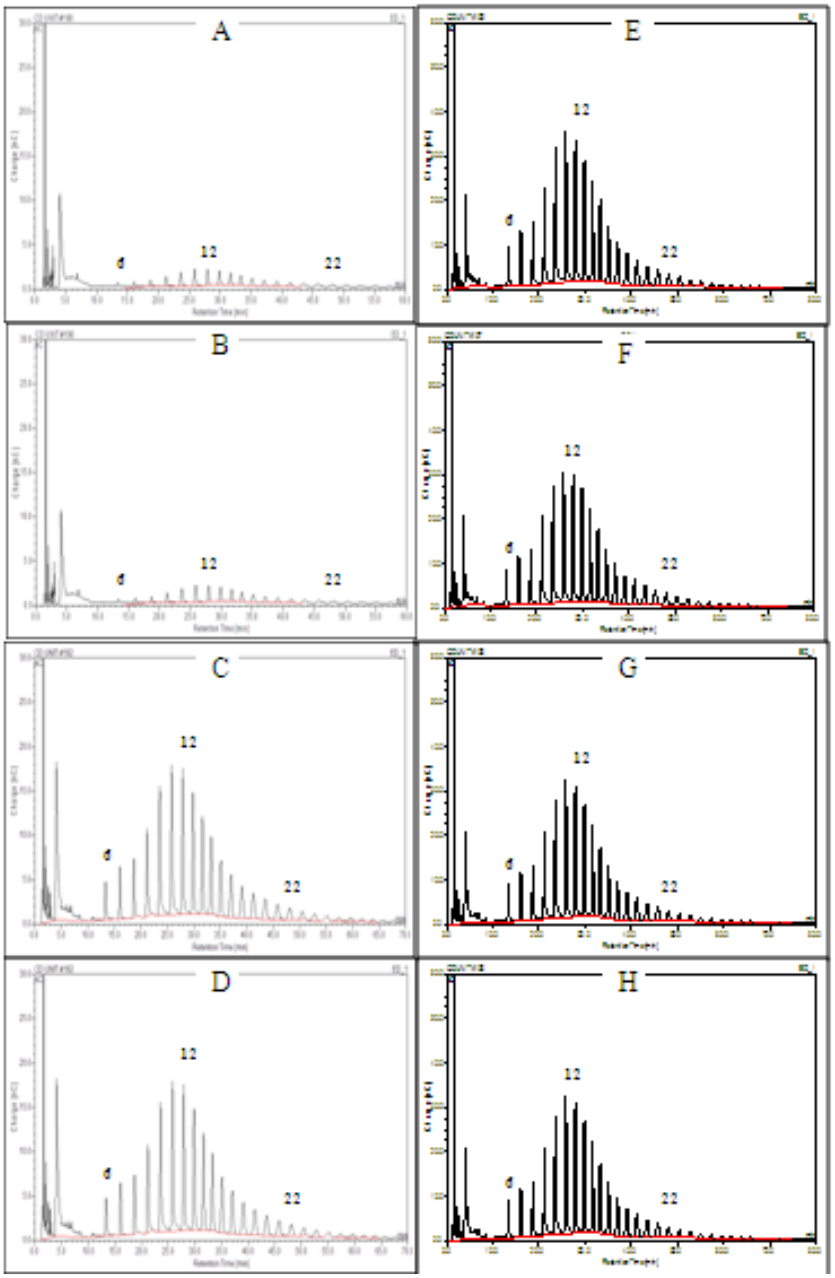
Since recombinant SBEs was obtained with higher activity and the preparation process were more convenient and required less time, they were used for starch modification studies.

3.2 Modification of cassava starch

Previous characterization of SBERI and SBERII by Yaiyen et al. (2010) reported the optimum temperature of SBERI and SBERII at 37^{o}C for both enzymes. Thus, we used the optimum temperature at 37^{o}C as the incubation temperature in our study. The purified SBERs were used to modify cassava starch and determined the optimum conditions using 37^{o}C as reaction temperature. The products were analyzed by HPAEC-PAD.

3.2.1 Optimum incubation time

Reactions were performed with 15 units of SBERI and SBERII and 2% (w/v) of cassava starch at various incubation times (3, 6, 12 and 24 hours). The highest product detected by PA-1 column was observed for SBERI at 12 hours (Figure 3.2C) and 3 hours for SBERII (Figure 3.2E), indicating highest degree of branching was obtained with SBERII. Incubation time up to 24 hours were also performed with both enzymes in which no significant increase was observed. Side chain distribution of all incubations were in the same range of DP 6 to 22.

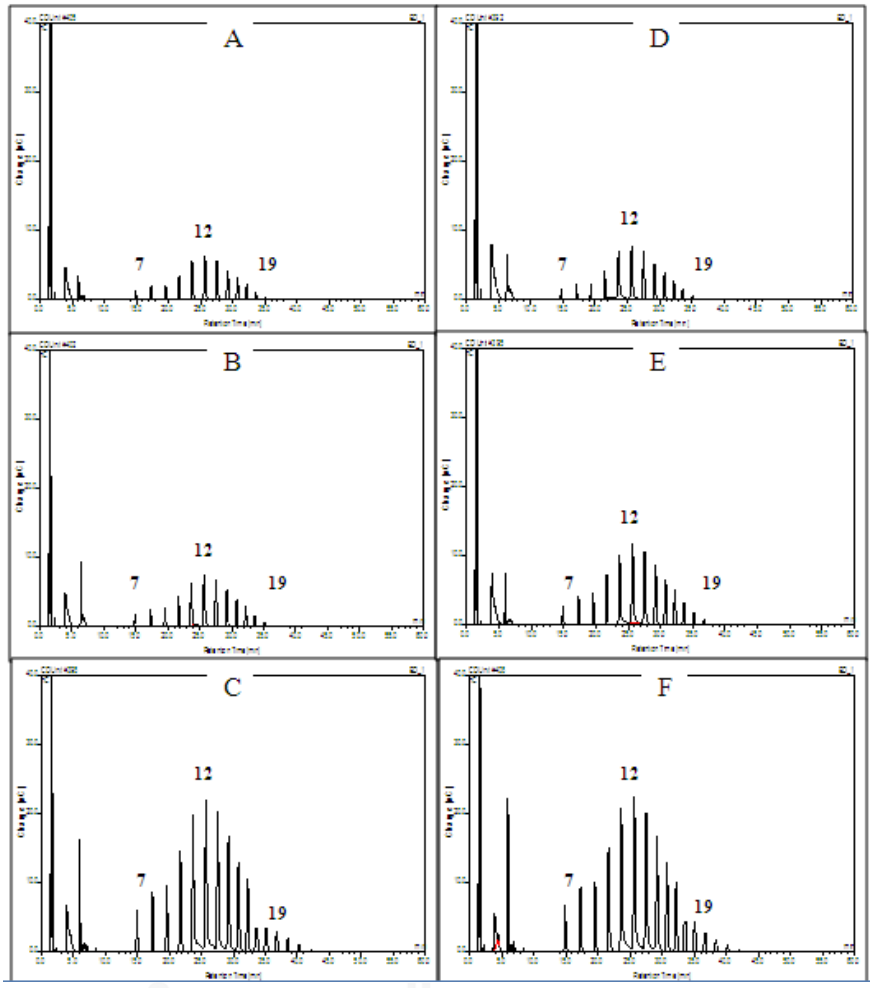


SBERI SBERII

Figure 3.2 Determination of side chain distribution of SBERs-treated cassava starch on PA-1 column at different times: A, E incubation time at 3 hours, B, F incubation time at 6 hours, C, G incubation time at 12 hours and D, H incubation time at 24 hours.

3.2.2 Optimum cassava starch concentration

In the experiment determining optimum starch concentration, reaction times were fixed at 12 hours for SBERI and 3 hours for SBERII. From Figure 3.3, the side chain distribution of modified products at all cassava starch concentrations analyzed by PA-1 column at 10% (w/v) cassava starch for both SBERI and SBERII (Figure 3.3C and 3.3F) were about the same. Thus, 10% (w/v) cassava starch was sufficient for the reaction. Side chain distribution of all incubation was in the same range of DP 7 to 19. Experiments on starch concentration higher than 10% was not performed due to very high viscosity of the starch solution.

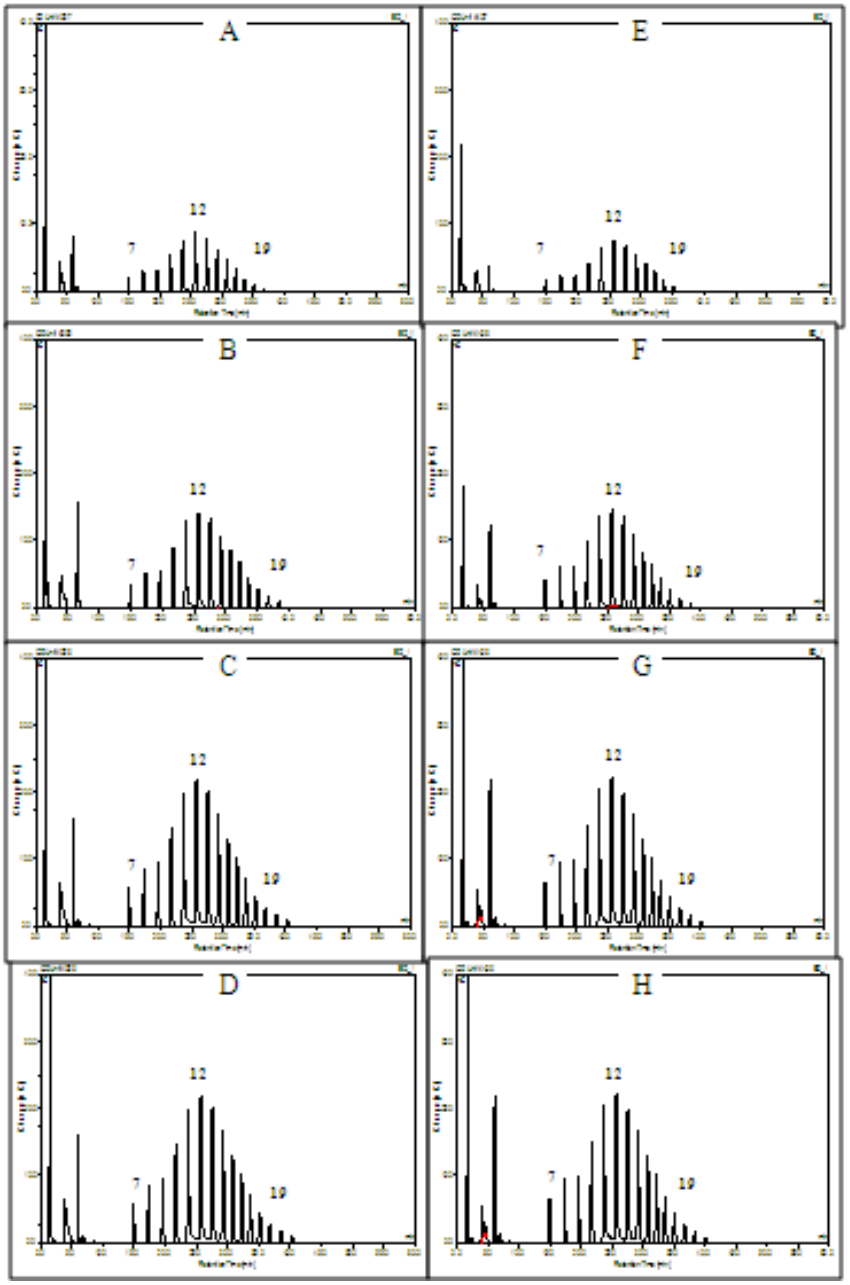


SBERI SBERII

Figure 3.3 Determination of side chain distribution of SBERs-treated cassava starch on PA-1 column at different starch concentration: A, D 2% (w/v) cassava starch, B, E 5% (w/v) cassava starch and C, F 10% (w/v) cassava starch

3.2.3 Optimum enzyme concentration

The determination of optimum enzyme concentrations were performed by using 10% (w/v) cassava starch as determined from the result shown in Figures 3.2 and 3.3. The concentrations of SBERI and SBERII were varied at 5, 10, 15 and 20 units. The reaction of SBERI was incubated for 12 hours and SBERII for 3 hours. At enzyme higher than 15 units, no significant increase in products was observed. The results showed that SBERI and SBERII yielded the highest branches at enzyme concentrations of 15 units (Figure 3.4C and 3.4G). Side chain distribution of all incubation was in the same range of DP 7 to 19.



SBERI SBERII

Figure 3.4 Determination of side chain distribution of SBER-treated cassava starch on PA-1 column at different enzyme concentration: A, E 5 units, B, F 10 units, C, G 15 units and D, H 20 units.

3.3 Modification of amylose

Previous characterization of SBERI by Yaiyen et al. (2010) reported the specificity of SBERI to amylose with optimum temperature at 37^{o}C. Thus, we used the optimum temperature at 37^{o}C as the incubation temperature in our study. The purified SBERI was used to modify amylose and determine the optimum conditions using 37^{o}C as reaction temperature. The products were analyzed by HPAEC-PAD.

3.3.1 Optimum incubation time

Reactions were performed with 15 units of SBERI and 2% (w/v) of amylose at various incubation times (3, 6, 12 and 24 hours). The highest product detected by PA-1 column was observed for SBERI at 12 hours (Figure 3.5C), Incubation time up to 24 hours was also performed but no significant increase was observed. Side chain distribution of all incubation was in the same range of DP 6 to 23.

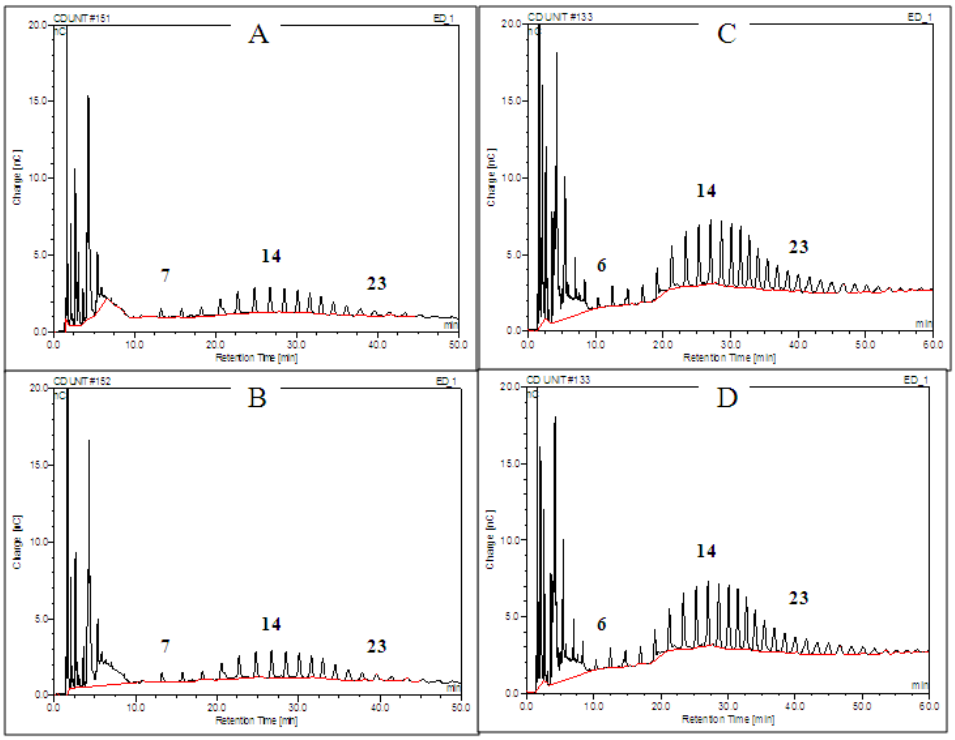


Figure 3.5 Determination of side chain distribution of SBERI-treated amylose on PA-1 column at different times: A incubation time at 3 hours, B incubation time at 6 hours, C incubation time at 12 hours and D incubation time at 24 hours.

3.3.2 Optimum amylose concentration

In the experiment determining optimum amylose concentration, reaction time was fixed at 12 hours for SBERI. From Figure 3.6, the side chain distribution of modified products at all amylose concentrations analyzed by PA-1 column for SBERI (Figure 3.6A) was about the same. Thus, 2% (w/v) amylose was sufficient for the reaction. Side chain distribution of all incubation was in the same range of DP 6 to 23.

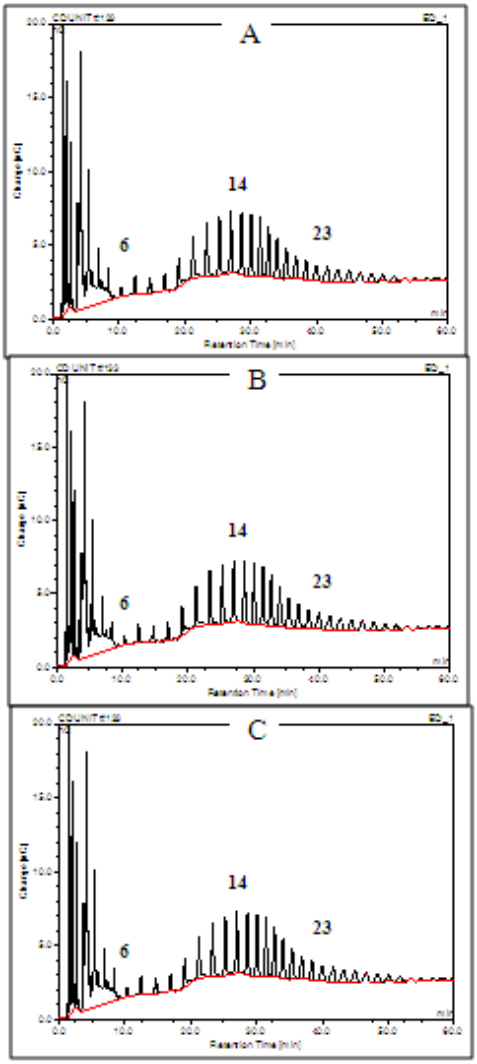


Figure 3.6 Determination of side chain distribution of SBERI-treated amylose on PA-1 column at different starch concentration: A 2% (w/v) amylose, B 5% (w/v) amylose and C 10% (w/v) amylose.

3.3.3 Optimum enzyme concentration

The determination of optimum enzyme concentrations was performed by using 2% (w/v) amylose as determined from the results, shown in Figure 3.6. The concentration of SBERI was varied at 5, 10 and 15 units. The reaction of SBERI was incubated for 12 hours. At enzyme higher than 10 units no significant increase in products was observed. The results showed that SBERI yielded the highest branches at enzyme concentration of 10 units (Figure 3.7B). Side chain distribution of all incubation was in the same range of DP 10 to 20.

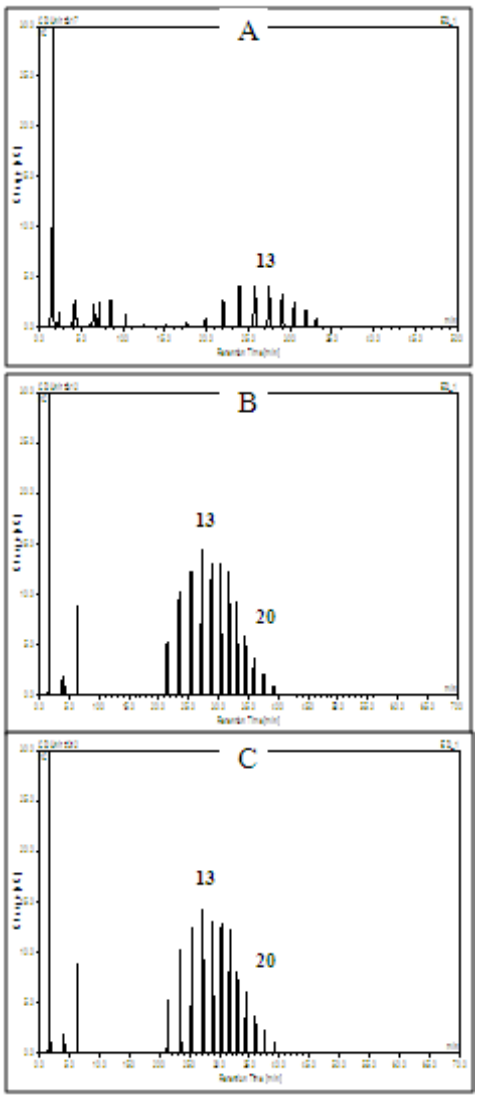


Figure 3.7 Determination of side chain distribution of SBERI-treated amylose on PA-1 column at different enzyme concentration: A 5 units, B 10 units and C 15 units.

3.4 Modification of amylopectin

Yaiyen et al. (2010) reported the specificity of SBERII to amylopectin with optimum temperature at 37^{o}C. Thus, we used the optimum temperature at 37^{o}C as the incubation temperature in our study. The purified SBERII was used to modify amylopectin and determine the optimum conditions using 37^{o}C as reaction temperature. The products were analyzed by HPAEC-PAD.

3.4.1 Optimum incubation time

Reactions were performed with 15 units of SBERII and 2% (w/v) of amylopectin at various incubation times (3, 6, 12 and 24 hours). The highest product detected by PA-1 column was observed for SBERII at 3 hours (Figure 3.8A), Incubation time up to 24 hours was also performed but no significant increase was observed. Side chain distributions of all incubations were in the same range of DP 6 to 22.

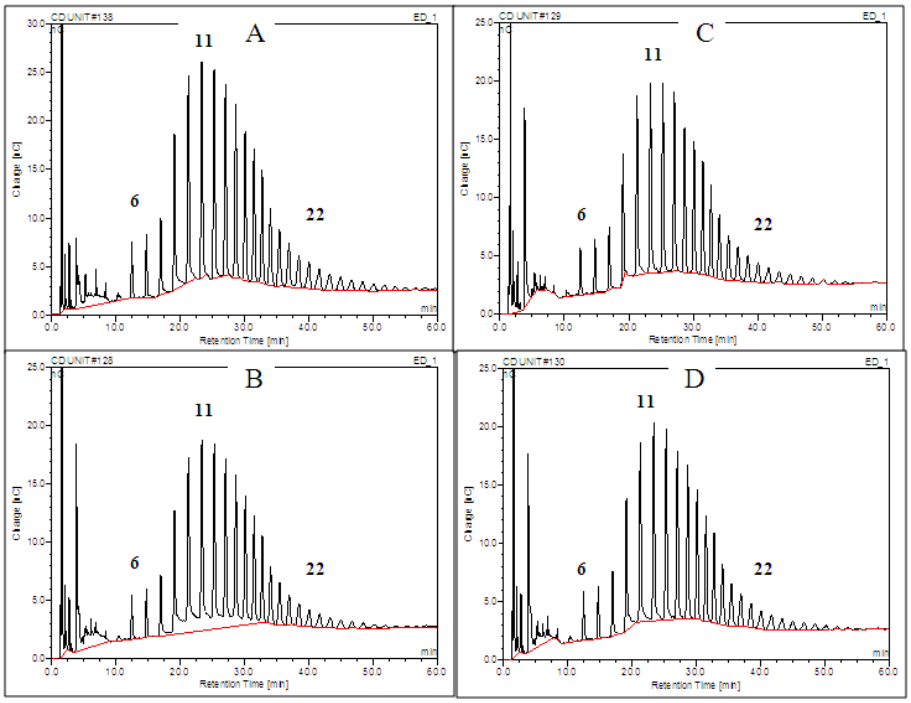


Figure 3.8 Determination of side chain distribution of SBERII-treated amylopectin on PA-1 column at different times: A incubation time at 3 hours, B incubation time at 6 hours, C incubation time at 12 hours and D incubation time at 24 hours.

3.4.2 Optimum amylopectin concentration

In the experiment determining optimum amylopectin concentration, reaction time was fixed at 3 hours for SBERII. From Figure 3.9, the side chain distribution of modified products at all amylopectin concentrations analyzed by PA-1 column for SBERII (Figure 3.9A) was about the same. Thus, 2% (w/v) amylopectin was sufficient for the reaction. Side chain distribution of all incubation were in the same range of DP 6 to 22.

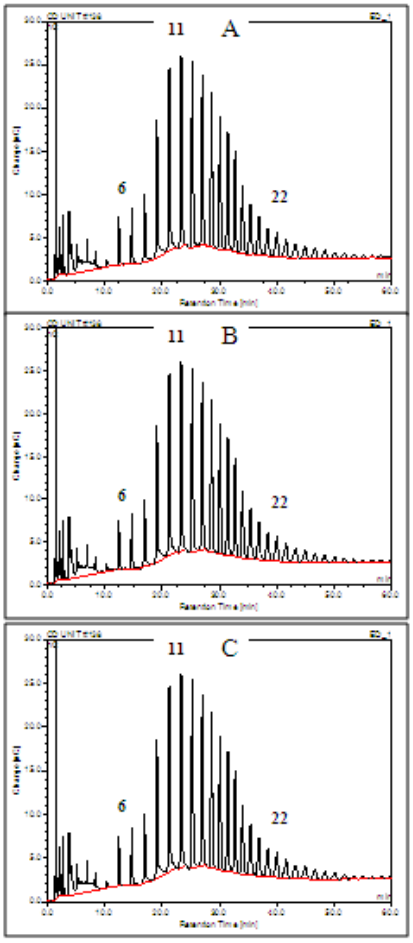


Figure 3.9 Determination of side chain distribution of SBERII-treated amylopectin on PA-1 column at different starch concentration: A 2% (w/v) amylopectin, B 5% (w/v) amylopectin and C 10% (w/v) amylopectin.

3.4.3 Optimum enzyme concentration

The determination of optimum enzyme concentrations was performed by using 2% (w/v) amylopectin as determined from the results, shown in Figure 3.9. The concentration of SBERII was varied at 5, 10 and 15 units. The reaction of SBERII was incubated for 3 hours. At enzyme higher than 10 units no significant increase in products was observed. The results showed that SBERII yielded the highest branches at concentration of 10 units (Figure 3.8B). Side chain distribution of all incubation was in the same range of DP 7 to 24.

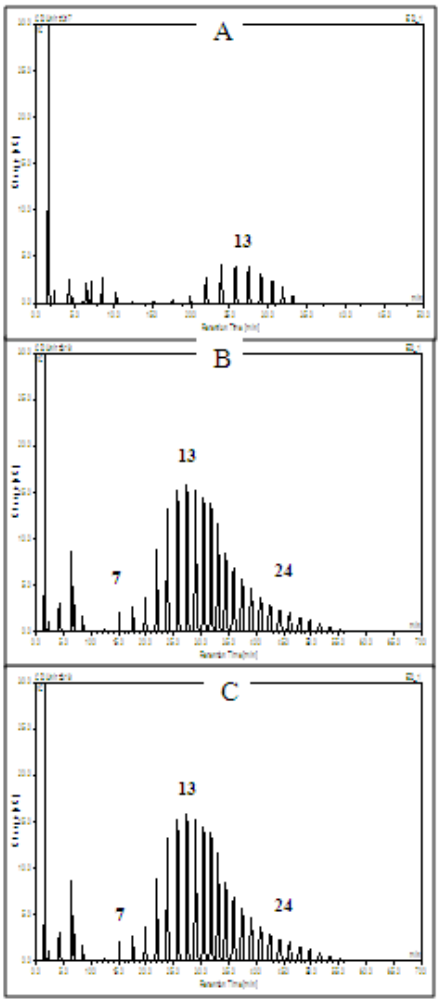


Figure 3.10 Determination of side chain distribution of SBERII-treated amylopectin on PA-1 column at different enzyme concentration: A 5 units, B 10 units and C 15 units.

Thus, the optimum conditions for following the modification of cassava starch by SBERs were 10% (w/v) of cassava starch at 37^{o}C with 15 units of SBERI for 12 hours or 15 units of SBERII for 3 hours. When the reaction products under these conditions were further detected by PA-1 on HPAEC, it was found that side chain distribution with DP 6-22 in modified cassava starch (Figure 3.11) was increased in comparison to control (unmodified cassava starch), with modified cassava starch showing the higher amount of side chain distribution. Notably, SBERII yielded the higher amount of side chain distribution than SBERI.

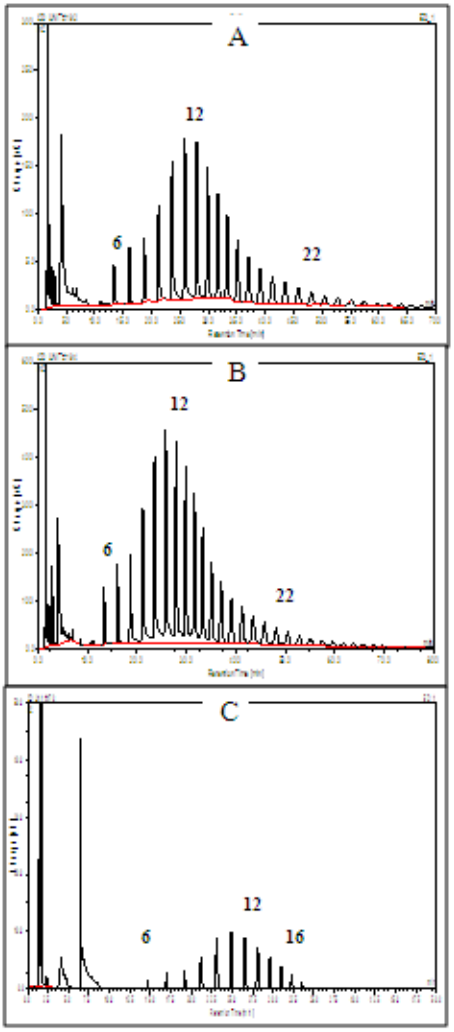


Figure 3.11 Determination of side chain distribution of SBERs-treated cassava starch on PA-1 column at optimum conditions: A 15 units of SBERI with 10% (w/v) cassava starch incubated at 37^{o}C for 12 hour, B 15 units of SBERII with 10% (w/v) cassava starch incubated at 37^{o}C for 3 hours and C unmodified cassava starch.

The optimum conditions for following the modification of amylose by SBERI were 2% (w/v) of amylose at 37^{o}C with 10 units of SBERI for 12 hours. When the reaction products under these conditions were further detected by PA-1 on HPAEC, it was found that side chain distribution with DP 6-26 in modified amylose (Figure 3.12) were increased in comparison to control (unmodified amylose), with modified amylose showing the higher amount of side chain distribution. Figure 3.12C showed that no products was observed when amylase was treated with SBERII.

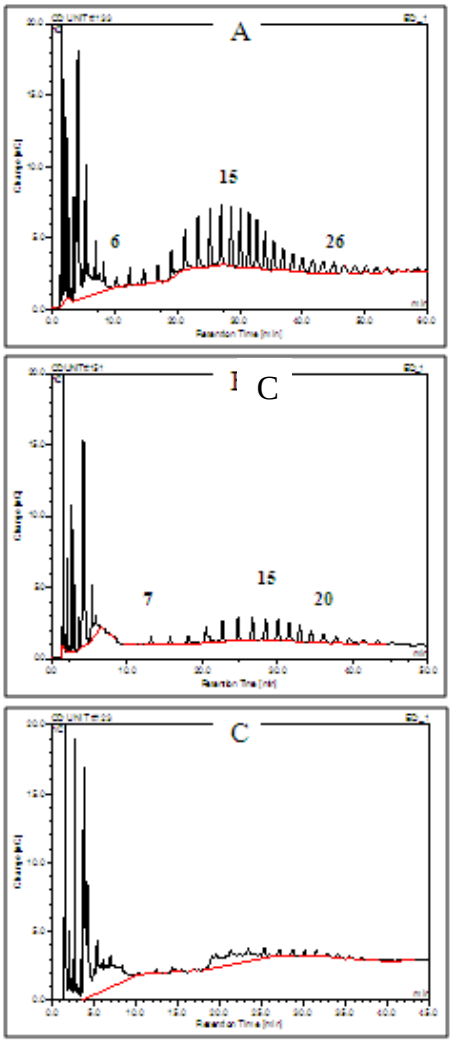


Figure 3.12 Determination of side chain distribution of SBERI-treated amylose on PA-1 column at optimum conditions: A 10 units of SBERI with 2% (w/v) amylose incubated at 37^{o}C for 12 hours, B unmodified amylose and C 10 units of SBERII with 2% (w/v) amylose.

The optimum conditions for following the modification of amylopectin by SBERII were 2% (w/v) of amylopectin at 37^{o}C with 10 units of SBERII for 3 hours. When the reaction products under these conditions were further detected by PA-1 on HPAEC, it was found that side chain distribution with DP 6-25 in modified amylopectin (Figure 3.13) were increased in comparison to control (unmodified amylopectin), with modified amylopectin showing the higher amount of side chain distribution.

SBERI showed minimal activity on amylopectin as shown in Figure 3.13C.

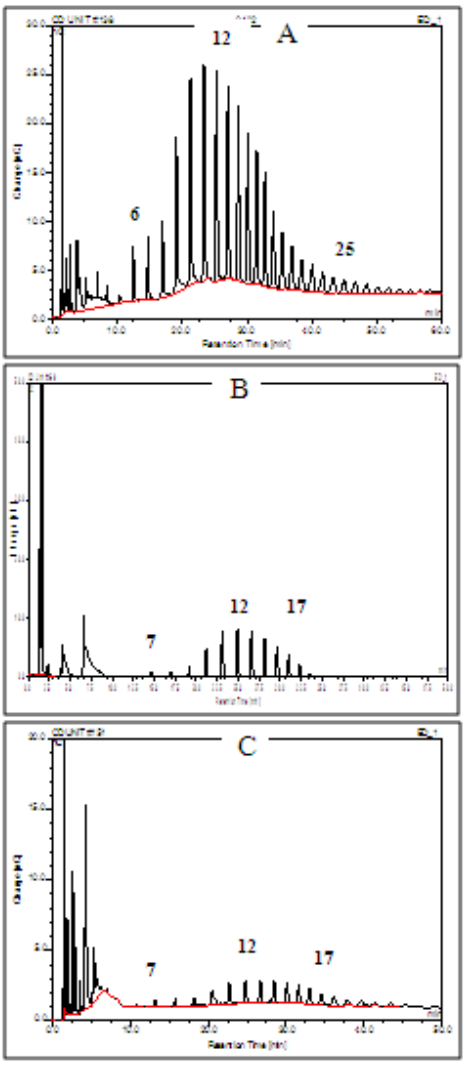


Figure 3.13 Determination of side chain distribution of SBERII-treated amylopectin on PA-1 column at optimum conditions: A 10 units of SBERII with 2% (w/v) amylopectin incubated at 37^{o}C for 3 hours, B unmodified amylopectin and C 10 units of SBERI with 2% (w/v) amylopectin.

Thus, the optimum conditions for following the modification of cassava starch by SBERs were 10% (w/v) of cassava starch at 37^{o}C with 15 units of SBERI for 12 hours or 15 units of SBERII for 3 hours. When the reaction products under these conditions were further detected by PA-100 on HPAEC, it was found that cycloamyloses were detected with size range of 20-31 glucose units for SBERI (Figure 3.14 A) and size range of 9-25 glucose units for SBERII (Figure 3.14 B) compared to control (unmodified cassava starch) which produced no cycloamyloses. Notably, SBERII yielded the higher amount of cycloamyloses than SBERI.

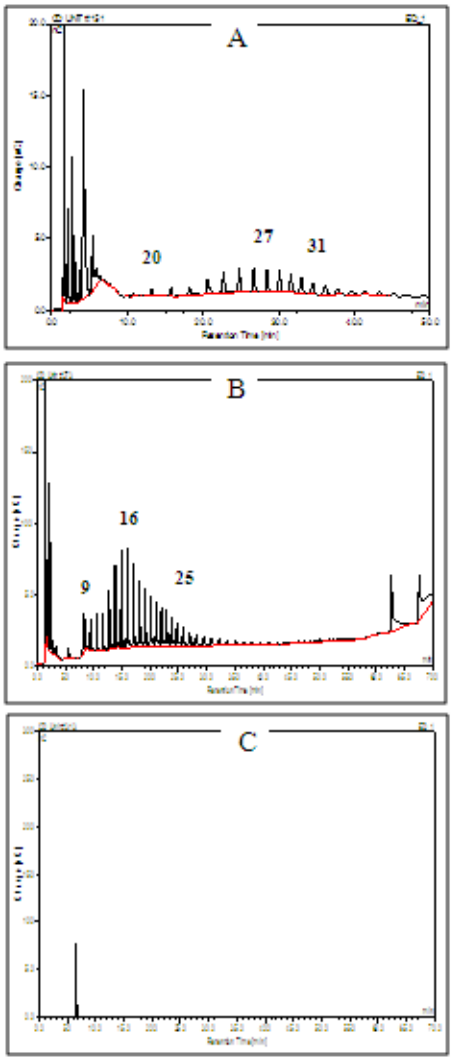


Figure 3.14 Cycloamylose products from SBERs incubated with cassava starch analyzed by PA-100 column at optimum conditions: A 15 units of SBERI with 10% (w/v) cassava starch incubated at 37^{o}C for 12 hours, B 15 units of SBERII with 10% (w/v) cassava starch incubated at 37^{o}C for 3 hours and C unmodified cassava starch.

The optimum conditions for following the modification of amylose by SBERI were 2% (w/v) of amylose at 37^{o}C with 10 units of SBERI for 12 hours. When the reaction products under these conditions were further detected by PA-100 on HPAEC, it was found that trace amounts of cycloamyloses were detected with size range of 23-30 glucose units for SBERI (Figure 3.15) compared to control (unmodified amylose) which produced no cycloamyloses.

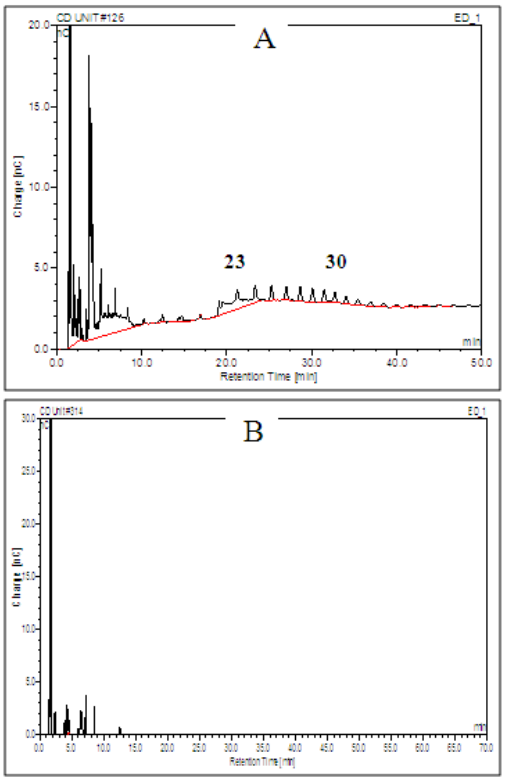


Figure 3.15 Cycloamylose products from SBERI incubated with amylose analyzed by PA-100 column at optimum conditions: A 10 units of SBERI with 2% (w/v) amylose incubated at 37^{o}C for 12 hours and B unmodified amylose.

The optimum conditions for following the modification of amylopectin by SBERII were 2% (w/v) of amylopectin at 37^{o}C with 10 units of SBERII for 3 hours. When the reaction products under these conditions were further detected by PA-100 on HPAEC, it was found that small amounts of cycloamyloses were detected with size range of 20-31 glucose units for SBERII (Figure 3.16) compared to control (unmodified amylopectin) which produced no cycloamyloses.

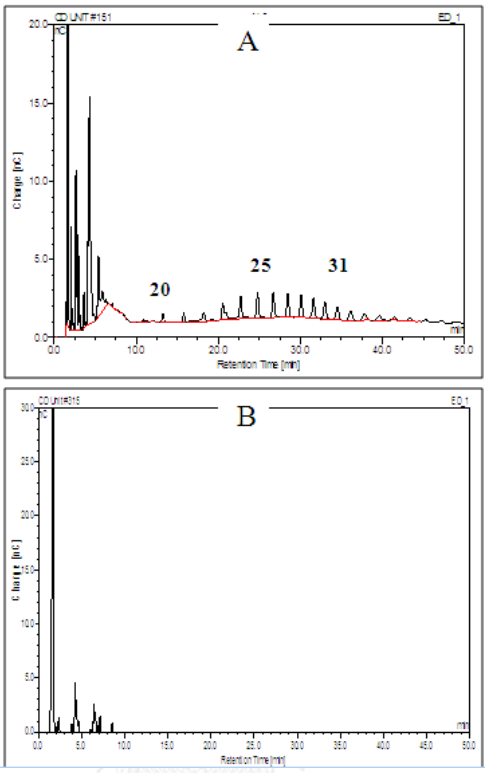


Figure 3.16 Cycloamylose products from SBERII incubated with amylopectin analyzed by PA-100 column at optimum conditions: A 10 units of SBERII with 2% (w/v) amylopectin incubated at 37°C for 3 hours and B unmodified amylopectin.

3.5 Physical properties of modified products

Modification products of each recombinant enzymes with each substrate were obtained from reactions at optimum conditions.

3.5.1 Thermal properties

Thermal properties (The enthalpy of amylopectin crytal) was measured by DSC after gelatinization and retrogradation process according to the method described in section 2.10.1.

From the results in Table 3.2, initial temperatures for hydrolysis of modified products were higher than unmodified cassava starch, unmodified amylose and unmodified amylopectin. The endotherm peak temperature (T_p) was not significantly changed in modified and unmodified cassava starch and amylose, while T_p of modified amylopectin was slightly lower. The temperature at which gelatinization ceased of all treated products were higher than unmodified substrates. Temperature range indicating complexity of modified products from cassava starch and amylose treated with SBERI and amylopectin treated with SBERII were higher than all unmodified substrates. Enthalpy of retrogradation of modified cassava starch was lower than unmodified cassava starch.

Table 3.2 Thermal properties of unmodified and modified substrates

Condition	T_0 (°C)	T_p (°C)	T_c (°C)	Tc - T_0 (°C)	$\triangle H_{retro}$ (J/g)
Unmodified cassava starch	45.9	55.9	60.0	14.1	2.635
Modified cassava starch treated with SBERI	46.3	55.1	63.4	17.1	1.949
Modified cassava starch treated with SBERII	46.9	55.7	61.3	14.4	1.645
Unmodified amylose	47.0	55.7	61.0	14	1.436
Modified amylose	47.6	54.3	63.8	16.2	1.394
Unmodified amylopectin	44.9	55.0	61.2	16.3	3.656
Modified amylopectin	46.6	53.7	63.1	16.5	1.761

T_0 = The initial gelatinization temperature
T_p = The endotherm peak temperature
T_c = The temperature at which gelatinization ceased
Tc - T_0 = Temperature range indicating complexity
$\triangle H_{retro}$ = Enthalpy of retrogradation (determined by peak area)

3.5.2 Freeze-thaw stability

Freeze-thaw stability was determined by measuring the % water released (% syneresis) during the freeze-thaw process according to the method described in section 2.10.2.

From the results in Figure 3.17A, percentage of syneresis of modified cassava starch by SBERI slightly increased at cycles 1-2 and the tend to be stable after cycle 2. The percentage of syneresis of modified cassava starch treated with SBERII increased at cycles 1-2 and was stable after cycle 3. Percentage of syneresis of unmodified cassava starch increased at cycles 1-3 and was stable after cycle 3. Modified cassava starch showed higher percentage of syneresis in every cycles of the process compared to the unmodified cassava starch.

Percentages of syneresis of modified amylose and unmodified amylose were stable after cycle 1. Modified amylose showed slightly higher percentage of syneresis in every cycles of the process compared to the unmodified amylose (Figure 3.17B) but followed the same patterns.

Figure 3.17C showed that percentage of syneresis of modified amylopectin was stable after cycle 1 while the percentage of syneresis of unmodified amylopectin decreased at cycles 1-2 and was stable after cycle 3. Modified amylopectin showed significantly higher percentage

of syneresis in every cycles of the process compared to the unmodified amylopectin.

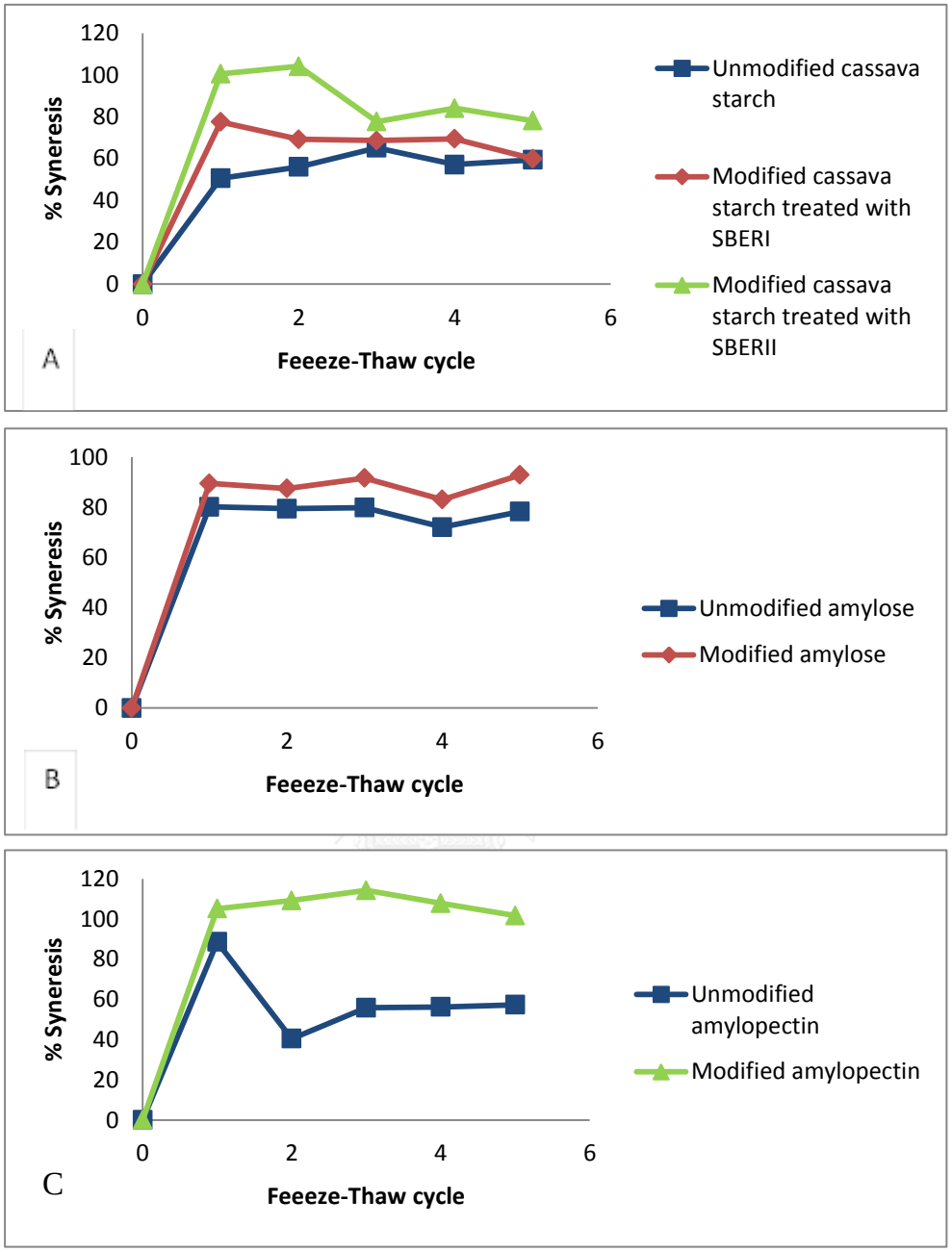


Figure 3.17 Comparison of % Syneresis between unmodified and modified substrates in the feeeze-thaw process: A cassava starch, B amylose and C amylopectin.

3.5.3 Thermo reversibility

Thermo reversibility was determined by measuring of viscoelasticity of gel by rheometer according to the method described in section 2.10.3.

From the results in Figure 3.18A, cassava starch modified by both enzymes showed higher tan (δ) values as liquid-like state at temperature 70°C and lower tan (δ) values as solid-like state at temperature 4°C, with more significant effect in cassava starch treated with SBREI. The process was able to reverse when the temperature was changed. Unmodified cassava starch showed stable tan (δ) values of both states.

Modified amylose showed higher tan (δ) values as liquid-like state at temperature 70°C and lower tan (δ) values as solid-like state at temperature 4°C. The process was able to reverse when the temperature was changed. Unmodified amylose had stable tan (δ) values. (Figure 3.18B)

From the results in Figure 3.18C, modified amylopectin had high tan (δ) values as liquid-like state at temperature 70°C and lower tan (δ) values as solid-like state at temperature 4°C. The process was able to reverse when the temperature was changed. Unmodified amylopectin had stable tan (δ) values.

Table 3.3, summarized and compared the physical properties of unmodified and modified substrates.

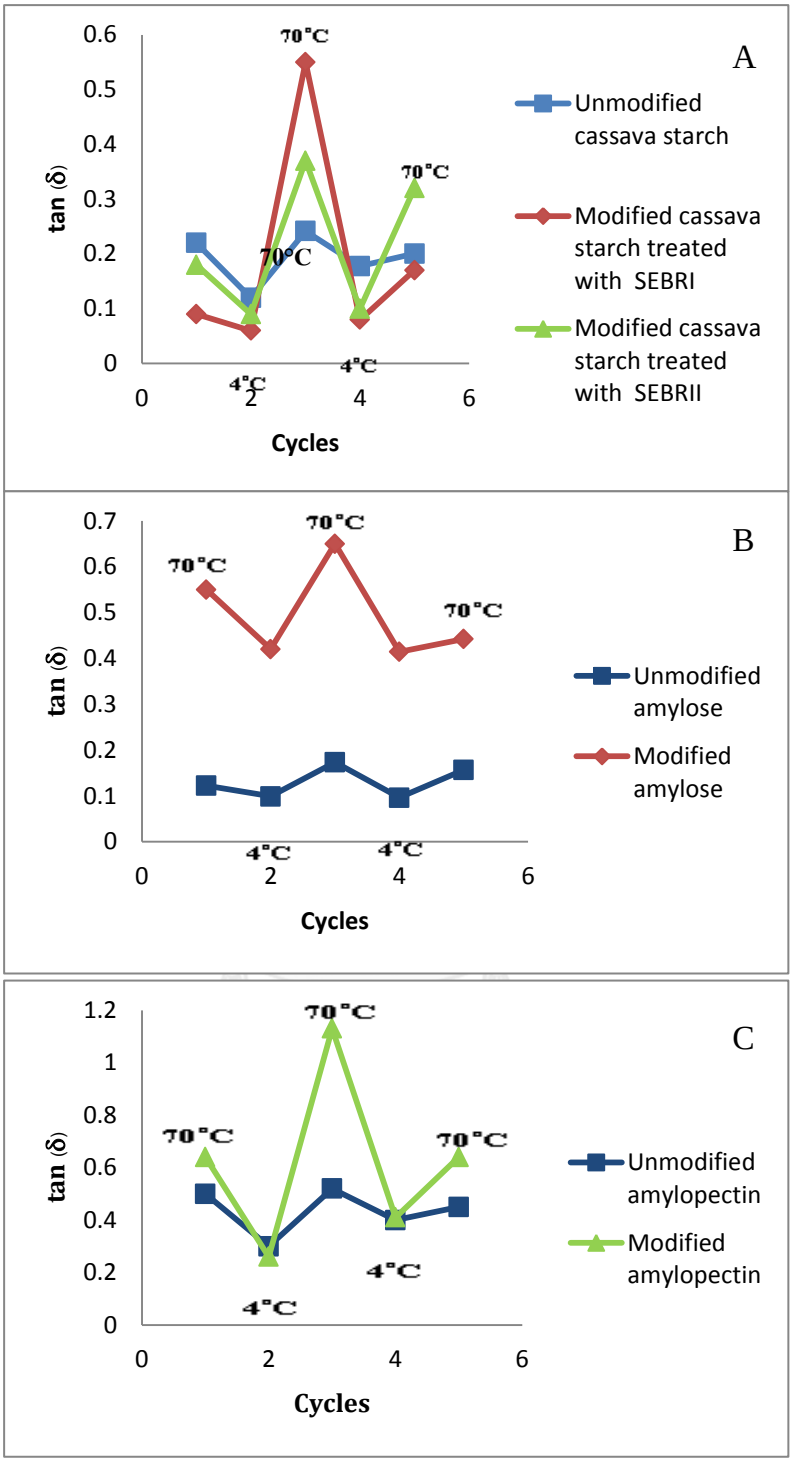


Figure 3.18 tan (δ) values of unmodified and modified substrates in heating-cooling cycles: A cassava starch, B amylose and C amylopectin.

Table 3.3 Comparative data of unmodified and modified substrates

Parameter	Unmodified cassava starch	Modified cassava starch with SBERI	Modified cassava starch with SBERII	Unmodified amylose	Modified amylose	Unmodified amylopectin	Modified amylopectin
Side chain distribution	Low short chain length	DP 6-25	DP 6-25	Low short chain length	DP 6-26	Low short chain length	DP 8-26
Cycloamyloses	No product	20-31 glucose units	9-25 glucose units	No product	23-30 glucose units	No product	20-31 glucose units
Thermal properties	$\triangle H_{retro}$ 2.635 J/g	$\triangle H_{retro}$ 1.949 J/g	$\triangle H_{retro}$ 1.645 J/g	$\triangle H_{retro}$ 1.436 J/g	$\triangle H_{retro}$ 1.394 J/g	$\triangle H_{retro}$ 3.656 J/g	$\triangle H_{retro}$ 1.761 J/g
Freeze-thaw stability	60% syneresis (5 cycles)	60% syneresis (5 cycles)	78% syneresis (5 cycles)	79% syneresis (5 cycles)	91% syneresis (5 cycles)	59% syneresis (5 cycles)	100% syneresis (5 cycles)
Thermo reversibility	No	Yes	Yes	No	Yes	No	Yes

CHAPTER IV

DISCUSSION

Cassava starch has been used as carbohydrate source in tropical food. Application of cassava starch is still restricted due to its composition and structure. Modification of starch has been introduced to enhance functionality of starch. Various methods of starch modification have been employed such as physical, chemical or enzymatic methods. Enzymatic modification of starch has been considered environmental and consumer friendly and a mild process while resulted in fewer by-products (Butler et al., 2004)

Branching enzymes catalyze the cleaving of α-1,4-glucosidic bond and forming α-1,6-glucosidic linkages in amylopectin by transglycosylation reaction. Le et al. (2009) reported modification of amylolytically-resistant tapioca starch by treatment with bacterial branching enzyme and maltogenic amylase. Takii et al. (1999) reported the production of highly branched cyclic dextrins by treating waxy corn starch with branching enzyme. Two forms of recombinant cassava branching enzymes from cassava tubers were cloned and characterized in our laboratory (Yaiyen et al., 2010). In this work, we expressed the two forms of recombinant cassava branching enzymes, SBERI and

SBERII, and used them to modify cassava starch and characterized the products.

4.1 Preparation of recombinant starch branching enzymes

From the purification results in Table 3.1, the yield of recombinant enzymes were higher than native cassava branching enzymes. Moreover, the preparation process of native SBE was tedious, time consuming with inconsistant yield. Thus, SBERI and SBERII were used in the starch modification studies. Both isoforms were used because they had diffferent specificities towards the main compositions of starch, amylose and amylopectin.

4.2 Optimization of starch modification reactions

In optimizing the condition for modifying starch with both SBERI and SBERII, three parameters were varied: incubation time, substrate concentrations and enzyme concentrations. Optimization reactions were performed using three type of substrates: cassava starch, amylose and amylopectin. All substrates were gelatinized to allow the release of amylose and amylopectin from the packing structure such as starch granule into surrounding water (Swinkels, 1985). Primary characterization of the products was determined of the change in degree of branching by PA-1 column on HPAEC-PAD. Cyclic products were determined after optimum conditions were established.

From results in section 3.2-3.4, SBERI was found to show optimum activity when 10% (w/v) cassava starch and 2% (w/v) amylose were used as substrates while little product was observed with 2% (w/v) amylopectin. The reaction was maximum at 12 hours incubation time 37^{o}C with 15 units of SBERI. Similar optimum conditions of cassava starch and enzyme concentration were observed when SBERII was used. However, SBERII was active towards amylopectin but not amylose, with optimum activity at 2% (w/v) amylopectin with 10 units of SBERII. Optimum incubation time of SBERII for 10% (w/v) cassava starch and 2% (w/v) amylopectin were 3 hours which was less than that of SBERI. SBERII was more active towards cassava starch than SBERI at same enzyme concentrations, yielding 3 times higher branching products than SBERI with 10% (w/v) cassava starch at shorter optimum incubation time (Figure 3.11). SBERII was reported to be specific to amylopectin (Yaiyen et al., 2010) while SBERI was more specific to amylose. Cassava starch contains more than 83% amylopectin (Zeeman et al., 1984). Thus, SBERII should be more active on cassava starch which had high amylopectin content. SBERII treated amylopectin yielded branches at DP 7-24 (Figure 3.10B) with higher amount of the products while lower amount of enzyme was used (10 units) compared to SBERII treated cassava starch. SBERII was specific towards branching of amylopectin. The higher activity and higher branches

obtained from amylopectin as substrate may be due to the structure of cassava starch containing both amylopectin and amylose and the branching reaction may be hindered by the starch granules presence in the structure. Gelatinization of the cassava starch helped releasing amylose and amylopectin from the granules and enhance the activity for both SBERI and SBERII. Experiments using amylose and amylopectin as substrates were performed for the purpose of confirming the specificity of SBERI and SBERII. Results from Figure 3.12 and 3.13 confirmed the specificity of SBERI towards amylose and SBERII towards amylopectin.

4.3 Characterization of properties of products from starch modification by SBER

4.3.1 Type characterization on HPAEC-PAD

In the optimization experiments, products from PA-1 column were primarily monitored on PA-1 column to show the branching activity as shown in Figure 3.2-3.13.

Figure 3.11 showed comparison of branching products of SBERI, SBERII at their optimum conditions for cassava starch. Unmodified cassava starch or cassava starch not treated with any SBERs showed small degrees of branching in the chain length 6-16 units. SBERI and SBERII yielded high amount of branches with sizes in the range of DP 6-22 with cassava starch, with the amount obtained from SBERII

showed twice higher than SBERI. With amylose, SBERI showed higher degree of branching compared to minimal branching in untreated amylose and amylopectin (Figure 3.12). On the other hand, SBERII at optimum condition produced higher degree of branching using amylopectin as substrate with much lower degree of branching in untreated amylopectin. Little branching products were observed in amylose treated with SBERII. These results conformed with the specificities of SBERI to amylose and SBERII to amylopectin and the high content of amylopectin in cassava starch.

4.3.2 Cycloamyloses production

Apart from branching products, all the modification reaction mixtures were also analyzed for cycloamyloses on PA-100 column at the optimum conditions. SBERI yielded small amount of cycloamyloses in the range of 20-31 glucose units from cassava starch (Figure 3.14A) and amylose (Figure 3.15A). Significant amount of cycloamyloses with the sizes of 9-25 glucose units were detected in the reaction of SBERII on cassava starch (Figure 3.14B) but only small amount of cycloamyloses (22-31 glucose units) from reaction with amylopectin. However, the conditions used in determining cycloamylose production were the optimum conditions for branching reaction not cycloamylose production. To get higher amount of cycloamylose, optimum conditions for cyclization reaction must be determined.

Therefore, in the modification of cassava starch, SBERI yielded only moderately branches product, presumably amylopectins but only small amount of cycloamyloses with larger ring sizes (20-31 glucose units). The modification products of SBERII on cassava starch yielded more highly branches products compared to SBERI at one fourth incubation time and large amount of medium rings cycloamyloses (9-25 glucose units), as determined on PA-100 column. SBERI appeared to produce large ring cycloamyloses (20-31 glucose units) probably due to its specificity on amylose which contains long chain glycans, resulting in large ring formation. SBERII produced smaller ring cycloamyloses (9-25 glucose units) probably due to ring formation from branches of amylopectin which were shorter glycan chains, resulting in smaller size range of cycloamyloses. Thus, each isoform is suitable for producing different size range of cycloamyloses.

Takata et al. (1996) reported the cyclization reaction of microbial branching enzymes, TRBE14 and proposed the schematic reaction as shown in Figure 4.1. They suggested that branching enzyme changed the structure of amylopectin by transfering multi-branched glucans from one site to another in the same molecule. In addition, BE may produce small cyclic glucans from amylopectin through its intramolecular transglycosylation reaction. From the study on tapioca starch treated with *Bacillus* branching (BE) enzyme, Le et al. (2009) suggested that

BE catalyzed both intermolecular (disproportionation action) and intramolecular (cyclization action) transglycosylation, producing amylopectin with changed in branch distribution and cyclic oligosaccharides or medium rings cycloamyloses (Figure 4.2). Both SBERI and SBERII produced higher degree of branching since cassava starch contained both amylose and amylopectin which both enzymes can act on. However, SBERII was more active towards cassava starch because of its high specificity on amylopectin, the major composition of cassava starch.

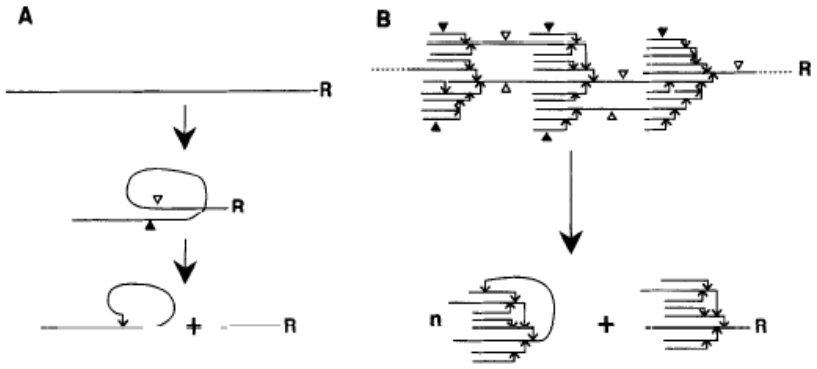


Figure 4.1 Cyclization reactions catalyzed by BE (Takata et al., 1997). Cyclization reactions of amylose (A) and amylopectin (B) are shown. Open and closed triangles indicate the α-l ,4 linkages cleaved by BE and glucosyl acceptors, respectively. Solid lines indicate a 1,4-α-D-glucan chain. Arrows are α-1,6 glucosidic linkages. The reducing end of a glucan chain is shown by "R". Both cyclic molecules and reducing (non-cyclic) molecules are produced by the action on glucan with a reducing end.

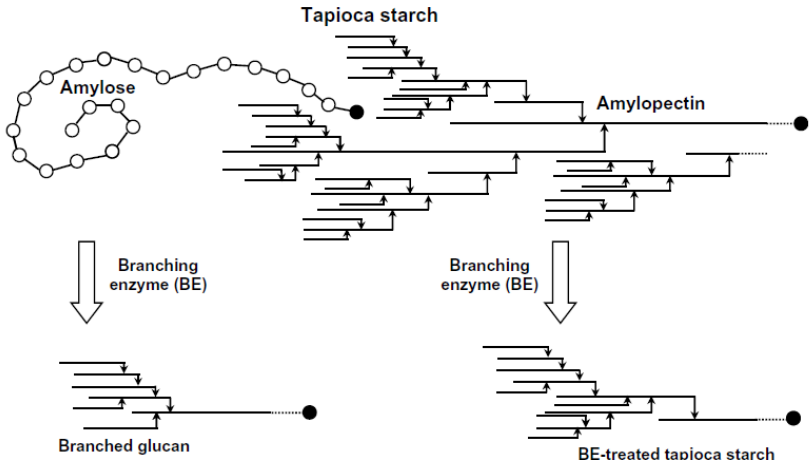


Figure 4.2 Schematic diagram of enzymatic modification of tapioca starch with branching enzyme (Le et al., 2009).
Arrows are a-1,6 glucosidic linkages. The reducing end of a glucan chain is shown by closed circle.

4.4 Physical properties of modified products

4.4.1 Thermal properties

In gelatinization process, starch dissolved in water with heating resulted in disintegration of starch granules. Water is allowed into the crystal structure and amylose released into the surrounding water, allowing gel formation (Lai and Kokini, 1991). When the mixture is cooled down, the remaining linear part of starch molecule such as amylose and linear parts of amylopectins rearrange themselves into crystalline structure, a retrogradation process. The degree of retrogradation depends on factors such as amylose to amylopectin ratio, linear chain length, starch composition and concentration. Apart from long chain amyloses, long chain amylopectin branches (>15 residues) can also retrograde (Eliasson and Gudmundsson, 1996). Modified products of cassava starch and amylopectin may have created more medium branches in amylopectin (DP 7-24), allowing rearrangement into crystals upon cooling resulting in higher retrogradation. Molecular associations of amylose-amylose, amylose- amylopectin, amylopectin-amylopectin occur, with water still embedded in the molecules. High amylopectin starches will form stable and soft gel.

4.4.2 Freeze-thaw stability

During freeze-thaw process, water is released from starch gel, a process called syneresis. All of the modified products obtained in

modification with SEBRI and SEBRII showed higher %syneresis than unmodified starch. After 2-3 cycles of freeze-thawing, %syneresis was stable in all cases (Figure 3.17). This could be due to the lower amylose and higher medium branches (DP 7-24) content in modified cassava starch. Waxy starch (amylose free) remains very stable for long period in refrigerated condition but lost the stability upon application of freeze-thaw process. Syneresis of waxy starch increases with increasing freeze-thaw cycles, an attribute to amylopectin retrogradation (Yaun and Thompson1998; Zheng et al., 1998). This phenomenon may explain the high syneresis observed in modified cassava starch and amylopectin (Figure 3.17).

4.4.3 Thermo reversibility

Thermo reversibility determinines the viscoelasticity of gel by rheometer between stress value and strain value, expressed as tan (δ). High tan (δ) values as liquid-like state and low tan (δ) values as solid-like state. From the results in Figure 3.18, tan (δ) values of all modified starch was high in liquid-like state at temperature 70°C and low in solid-like state at temperature 4°C and was able to reverse the state when the temperature was changed. While the change in tan (δ) value of unmodified starch was less than modified starch. The short branch length amylopectin had weak structure therefore easy to turn liquid-like

state at temperature 70°C and in solid-like state at temperature 4°C (Oh et al., 2008).

In conclusion, SBERII was more active towards cassava starch. Incubation at optimum conditions yielded both highly branched amylopectins and medium rings cycloamyloses, probably due to the high amylopectin content in cassava starch and the specificity of SBERII towards amylopectins. The modified products showed low retrogradation and high thermo reversibility but low freeze-thaw stability.

CHAPTER V

CONCLUSIONS

1. Recombinant starch branching enzymes, SBERI and SBERII and SBE from tubers of cassava *Manihot esculenta* Crantz were expressed and purified. The recombinant enzymes were used to modify cassava starch, amylose and amylopectin.
2. Optimum conditions for modification of cassava starch by SBERI were 10% (w/v) of cassava starch at 37^{o}C with 15 units for 12 hours and 10% (w/v) of cassava starch at 37^{o}C with 15 units for 3 hours for SBERII.
3. The optimum conditions for modification of amylose by SBERI were 2% (w/v) of amylose at 37^{o}C with 10 units for 12 hours while SBERI showed no activity towards amylopectin.
4. The optimum conditions for modification of amylopectin by SBERII were 2% (w/v) of amylopectin at 37^{o}C with 10 units for 3 hours while SBERII showed no activity towards amylose.
5. Determination of side chain distribution showed that modified products of both SBERI and SBERII consisted of higher branches than unmodified substrates. Cassava starch yielded significant amount of medium rings cycloamyloses in the modified products while only minimal amount was observed in amylose and amylopectin.

6. Modification products of cassava starch, amylose and amylopectin showed low retrogradation, high thermo reversibility and low freeze-thaw stability.

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APPENDIX

APPENDIX A

Luria-Bertani (LB) broth media

Tryptone	1% (w/v)
Yeast extract	0.5% (w/v)
NaCl	0.5% (w/v)

The LB plate was added the 1.0-2.0% Agar

APPENDIX B

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

10 % (w/v) ammonium persulfate

ammonium persulfate	1	g

Adjust volume to 10 ml with distilled water.

10 % (w/v) SDS

Sodium dodecyl sulfate	5	g

Adjust volume to 50 ml with distilled water.

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	9.1	g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 50 ml with distilled water.

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6	g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 50 ml with distilled water.

50 % (v/v) SDS

Glycerol	5	g

Added 5 ml distilled water.

Solution B (SDS PAGE)

2 M Tris-HCl pH 8.8	37.5	ml
10% (w/v) SDS	2	ml
Distilled water	10	ml

Solution C (SDS PAGE)

1 M Tris-HCl pH 8.8	25	ml
10% (w/v) SDS	2	ml
Distilled water	23	ml

2. SDS-PAGE

7.5 % Separating gel

40 % Acrylamideml solution	1.41	ml
Solution B	2.5	ml

Distilled water	3.48	ml
10% (w/v) ammonium persulfate	60	µl
TEMED	6	µl

5.0 % Stacking gel

40 % Acrylamide solution	0.32	ml
Solution C	0.5	ml
Distilled water	1.652	ml
10 % (w/v) ammonium persulfate	25	µl
TEMED	3	µl

Sample buffer

1 M Tris-HCl pH 6.8	0.6	ml
50% Glycerol	5	ml
10% (w/v) SDS	2	ml
2-Mercaptoethanol	0.5	ml
1 % (w/v) Bromophenol blue	1	ml
Distilled water	0.9	ml

One part of sample buffer was added to four parts of sample. The mixture was heated 5 min. in boiling water before loading to the gel.

Electrophoresis buffer

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.4	g
SDS	1	g

Dissolve in distilled water to 1 litre. Do not adjust pH (final pH should be 7.8-8.3).

Coomassie Gel Stain

Coomassie Blue R-250	1	g
Methanol	450	ml
Distilled water	450	ml
Glacial acetic acid	100	ml

Coomassie Gel Destain

Methanol	100	ml
Glacial acetic acid	100	ml
Distilled water	800	ml

3. Non- denaturing PAGE

7 % Seperating gel

30 % Acrylamideml solution	1.75	ml
1.5 M Tris-HCl pH 8.8	2.5	ml

Soluble starch	1	ml
Distilled water	2.14	ml
10% (w/v) ammonium persulfate	100	µl
TEMED	10	µl

4 % Stacking gel

30 % Acrylamideml solution	0.67	ml
0.5 M Tris-HCl pH 6.8	1	ml
Distilled water	3.27	ml
10% (w/v) ammonium persulfate	50	µl
TEMED	10	µl

Sample buffer

1 M Tris-HCl pH 6.8	3.1	ml
Glycerol	5	ml
1 % Bromophenol blue	0.5	ml
Distilled water	1.4	ml

One part of sample buffer was added to four parts of sample.

Electrophoresis buffer

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

Dissolve in distilled water to 1 liter. Do not adjust pH (final pH should be 8.3).

APPENDIX C

Iodine's Solution

Iodine solution I
0.05% Potassium iodide; 0.005% Iodine

Potassium iodide	0.05 g
Iodine	0.005 g
Adjust to 100 ml distilled water	

Iodine solution II
1% Potassium iodide; 0.1% Iodine

Potassium iodide	1 g
Iodine	0.1 g
Adjust to 100 ml distilled water	

APPENDIX D

Working solution for HPAEC column PA1

Working solution	
150 mM Sodium hydroxide	
Sodium hydroxide	11.88 ml
Adjust to 1.5 liter ultrapure water	
600 mM Sodium acetate in 150 mM Sodium hydroxide	
Sodium acetate	73.8 g
Sodium hydroxide	11.88 ml
Adjust to 1.5 liter ultrapure water	
500 mM Sodium hydroxide	
Sodium hydroxide	26.4 ml
Adjust to 1 liter ultrapure water	

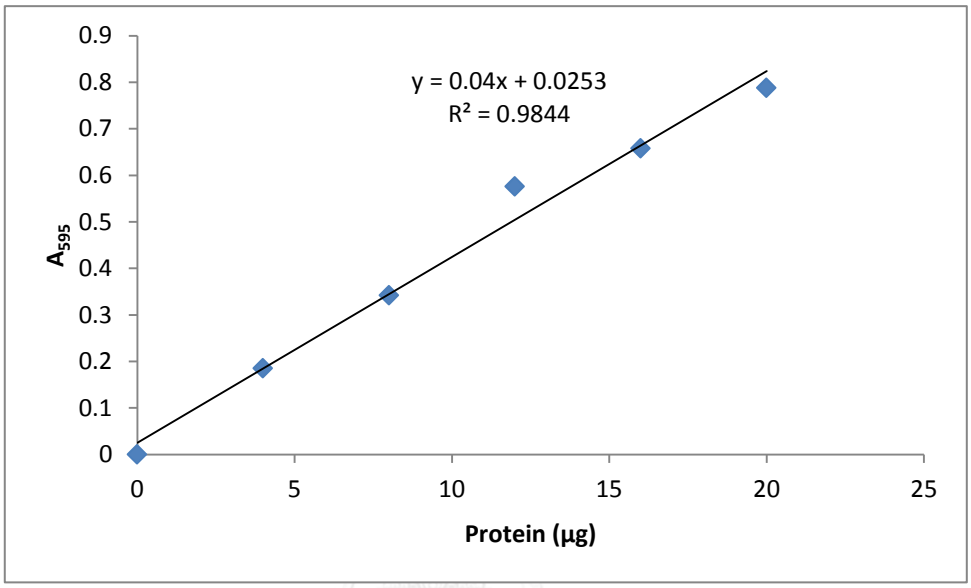
APPENDIX E

Working solution for HPAEC column PA100

Working solution	
150 mM Sodium hydroxide	
Sodium hydroxide	11.88 ml
Adjust to 1.5 liter ultrapure water	
200 mM Sodium nitrate in 150 mM Sodium hydroxide	
Sodium nitrate	25.8 g
Sodium hydroxide	11.88 ml
Adjust to 1.5 liter ultrapure water	
1 M Sodium nitrate	
Sodium nitrate	42.5 g
Adjust to 1 liter ultrapure water	

APPENDIX F

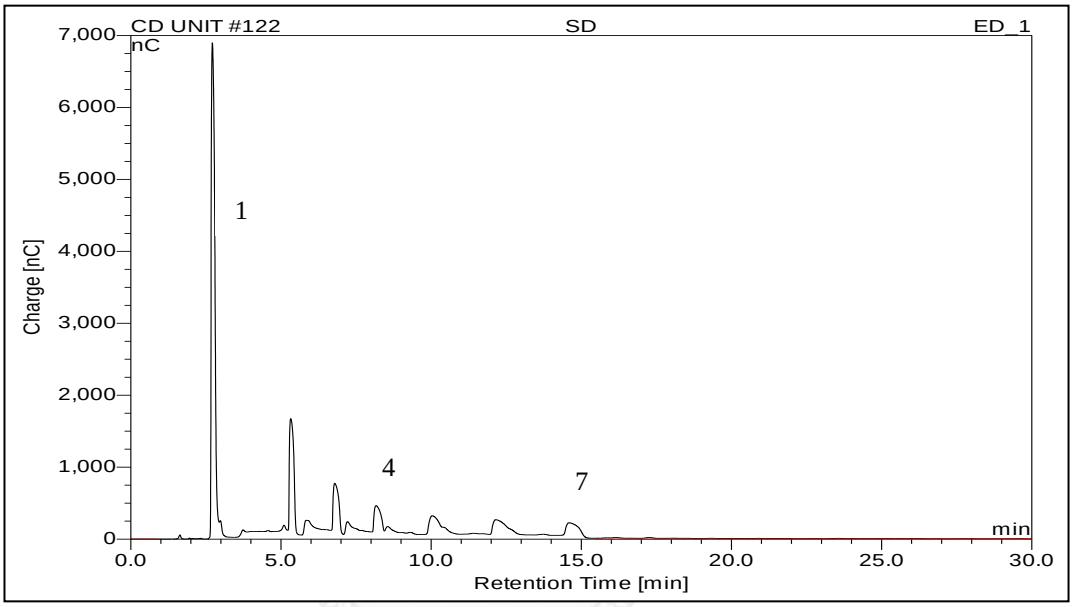
BSA standard curve for protein determination



APPENDIX G

Standard for HPAEC column PA1

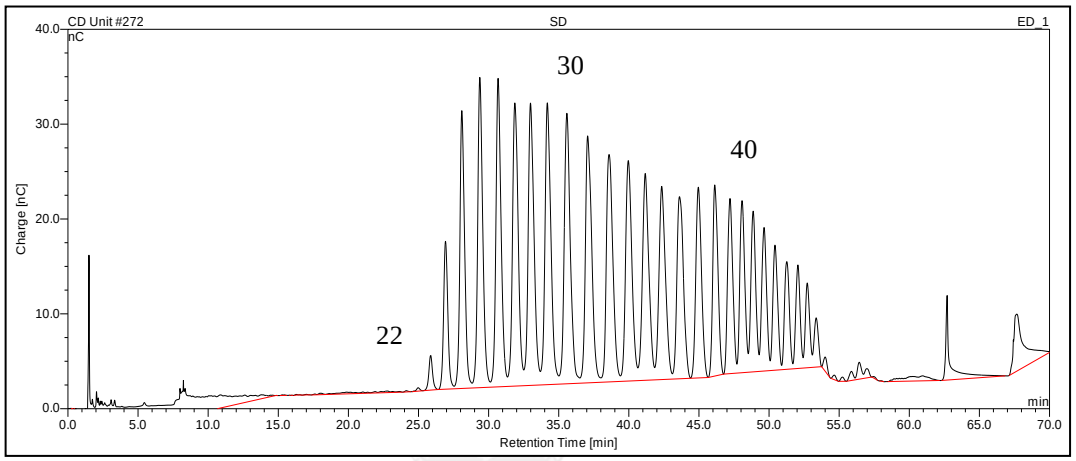
Standard glucose G1-G7



APPENDIX H

Standard for HPAEC column PA100

Standard large ring cyclodextrins (LR-CD) from Amylomaltase from *Thermus aquaticus* ATCC 33923.



VITA

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