การดัดแปรแป้งโดยรีคอมบิแนนท์ 4-แอลฟา-กลูคาโนทรานสเฟอเรสจากมัน
สำปะหลัง *Manihot esculenta* Crantz

นางสาวปรียาภรณ์ กิจพิบูลย์

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STARCH MODIFICATION BY RECOMBINANT 4-α-GLUCANOTRANSFERASE FROM CASSAVA *Manihot esculenta* Crantz

Miss Preyapon Kitpibun

A Thesis Submitted in Partial Fulfillment of the Requirements
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By	Miss Preyapon Kitpibun
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Associate Professor Tipaporn Limpaseni, Ph.D.

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ปรียาภรณ์ กิจพิบูลย์ : การดัดแปรแป้งโดยรีคอมบิแนนท์ 4-แอลฟา-กลูคาโนทรานสเฟอเรสจากมันสำปะหลัง *Manihot esculenta* Crantz (STARCH MODIFICATION BY RECOMBINANT 4-α-GLUCANOTRANSFERASE FROM CASSAVA *Manihot esculenta* Crantz) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ทิพาพร ลิมปเสนีย์, 96 หน้า.

Disproportionating enzyme (D-enzyme, DPE) (E.C.2.4.1.25) เป็นเอนไซม์ในกลุ่ม 4-แอลฟา-กลูคาโนทรานสเฟอเรส ทำการโยกย้ายหมู่ไกลโคซิลจากสาย 1,4 กลูแคนไปยังตำแหน่ง C4 ของกลูโคสใน 1,4 กลูแคนอีกสายหนึ่ง DPEI เร่งปฏิกิริยาการย้ายหมู่มอลโทซิล จากสายออลิโกแซคคาไรด์สายให้ไปยังออลิโกแซคคาไรด์สายรับ มีบทบาทเกี่ยวกับการสลายแป้งในพืช งานวิจัยนี้ทำการดัดแปรแป้งมันสำปะหลังด้วยรีคอมบิแนนต์ MeDPEI จากมันสำปะหลังที่ทำให้บริสุทธิ์ พบว่าการบ่มด้วยเอนไซม์ 30 ยูนิตต่อแป้งหนึ่งกรัม ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง ให้ผลิตภัณฑ์ที่ดีที่สุด โดยได้ผลิตภัณฑ์เป็นไซโคลแอมิโลสขนาดใหญ่ตั้งแต่ 18-57 หน่วยกลูโคส ผลจากการวิเคราะห์การกระจายตัวของสายกิ่งแอมิโลเพกติน ด้วย HPAEC-PAD โดยใช้คอลัมน์ Carbopac (PA-1) พบว่าสายกิ่งของแอมิโลเพกตินมีปริมาณลดลง วิเคราะห์ปริมาณแอมิโลสปรากฏ โดยวัดสีจากการจับของแอมิโลสกับไอโอดีน เมื่อเทียบกับแป้งที่ไม่ผ่านการดัดแปร พบว่ามีปริมาณแอมิโลสปรากฏลดลง ผลจากการวัดการรีโทรเกรดของแอมิโลเพกติน พบว่าพลังงานที่ใช้ในการสลายผลึกแอมิโลเพกตินลดลง ความคงทนต่อการแช่เยือกแข็งและการละลายน้ำแข็งเพิ่มขึ้น และมีสมบัติ thermo-reversibility ที่สามารถเปลี่ยนสถานะกลับไปมาระหว่างของแข็งและของเหลวได้ นอกจากนี้ยังได้ทำการดัดแปรแป้งข้าวโพดด้วยสภาวะที่ใช้ในการดัดแปรแป้งมันสำปะหลัง พบว่าได้ผลการทดลองคล้ายกัน ซึ่งเป็นผลมาจากแป้งทีผ่านการดัดแปรด้วย MeDPEI มีปริมาณแอมิโลสและปริมาณสายกิ่งแอมิโลเพกตินลดลง จึงส่งผลต่อโครงสร้างและสมบัติต่างๆ ซึ่งสามารถนำแป้งที่ดัดแปรไปศึกษาการใช้งานในอุตสาหกรรมต่างๆ ได้

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PREYAPON KITPIBUN: STARCH MODIFICATION BY RECOMBINANT 4-α-GLUCANOTRANSFERASE FROM CASSAVA *Manihot esculenta* Crantz. ADVISOR: ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D., pp.

4-α-Glucanotransferase or disproportionating enzyme (D-enzyme, DPE) (E.C.2.4.1.25) catalyzes the α-1,4 glycosyl transfer between oligosaccharides. Type I D-enzyme (DPEI) can transfer maltosyl unit from one 1,4-α-D-glucan to an acceptor mono- or oligo-saccharide, which reflects its physiological role in plant starch degradation. In this study, the purified recombinant DPEI from *Manihot esculenta* Crantz cultivar KU50 (MeDPEI) was used to modify cassava starch. Optimum conditions for cassava starch modification was achieved at 30 units/g starch of enzyme at 37 °C for 12 hours and 5% w/v cassava starch. Cycloamyloses were produced with size distribution in the ranges 18-57 glucose units. When side chain distribution were analyzed by HPAEC-PAD on Carbopac PA-1 column, the treated starch showed lower amount of side chains distribution compared to untreated starch but the size distribution were similar. Apparent amylose content of the treated starch, determined by iodine binding, was also lower. Retrogradation of the treated starch determined by Differential Scanning Calorimeter (DSC) showed lower retrogradation of amylopectin. Percentage of syneresis from freeze-thaw process was lower compared to native starch. Modified corn starch at the same modification condition showed the same results as modified cassava starch. Starches modified by MeDPEI showed suitable properties for further studies in industrial applications.

Department:	Biochemistry	Student's Signature .....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature .....
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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CAs	cycloamyloses
CDs	cyclodextrins
CGTase	cyclodextrin glycosyltransferase
D-enzyme	disproportionation enzyme
DP	degee of polymerization
DPEI	disproportionating enzyme isoform 1
DPEII	disproportionating enzyme isoform 2
DSC	differential scanning calorimeter
EDTA	ethylenediaminetetraacetic acid
g	gram
G'	storage modulus value
G"	loss modulus value
G"/G'	tan δ value
GH	glycoside hydrolases family
HPAEC-PAD	high performance anion exchange chromatography
Hz	Hertz
J/g	Joules per gram
kDa	kilo Dalton
l	litre
LR-CDs	large-ring cyclodextrins

M	molar
mA	milliampere
mg	milligram
ml	millilitre
mM	millimolar
MW	molecular weight
N	normal acid or base
nm	nanometer
OD	optimum density
Pa	Pascals unit
Rf	relative mobility
rpm	revolution per minute
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
U	unit
v/v	volume by volume
w/v	weight by volume
T_c	time at the end
T_o	time at the onset
T_p	time at the midpoint
µl	microliter
µg	microgram
µm	micrometer
ΔH_{retro}	enthalpy of retrogradation

CHAPTER I

INTRODUCTION

1.1 Starch

Starch is found in plant storage organs such as tubers, seeds and roots as the primary source of stored energy. It is found in many plants source such as cassava, rice, maize, peas, potatoes, rice, barley, peas, sago (palm starch) and the leaves of all green plants (Young 1984, Wurzburg 1986). Starch, a major component of starch granules, consists of two types of polysaccharide polymers, amylose and amylopectin (Figure 1.1) (Wurzburg 1986). Amylose is a linear polymer of glucose residue linked with α-1,4 glycosidic linkage and is found in the amorphous regions of starch granules. Amylopectin is a branched polymer of glucose residue linked with α-1,4 glycosidic linkage with branching occus with α-1,6 glycosidic linkage at intervals of 20-30 glucose units (Table 1.1) and arranged in the crystalline lamellae of starch granules (Figure 1.2) (Buléon *et al.* 1998). The content of amylose and amylopectin varies depending on the source of starch. Amylopectin is usually the major component, with ~25% amylose and ~75% amylopectin (ratio of 1 : 3). Starch granules also contain minor components of 0.10 to 0.40% (w/w) of proteins, 0.01 to 0.80% (w/w) of lipids, 0.09 to 0.63% (w/w) of phosphate, minerals and moisture (Table 1.2). The lipids are found in the cereal starch such as rice, wheat, maize, waxy maize and the phosphate are the part of phospholipids in starch. The storage starch in tuber such as potato, shoti, cassava, has the phosphate attaching to the primary alcohol in glucose residues of amylopectin (Robyt 2008). All starches in plant tissues are in water-insoluble form. Scanning electron micrographs of starch granules with various sizes and shapes are shown in (Figure 1.3), with different size of granules and

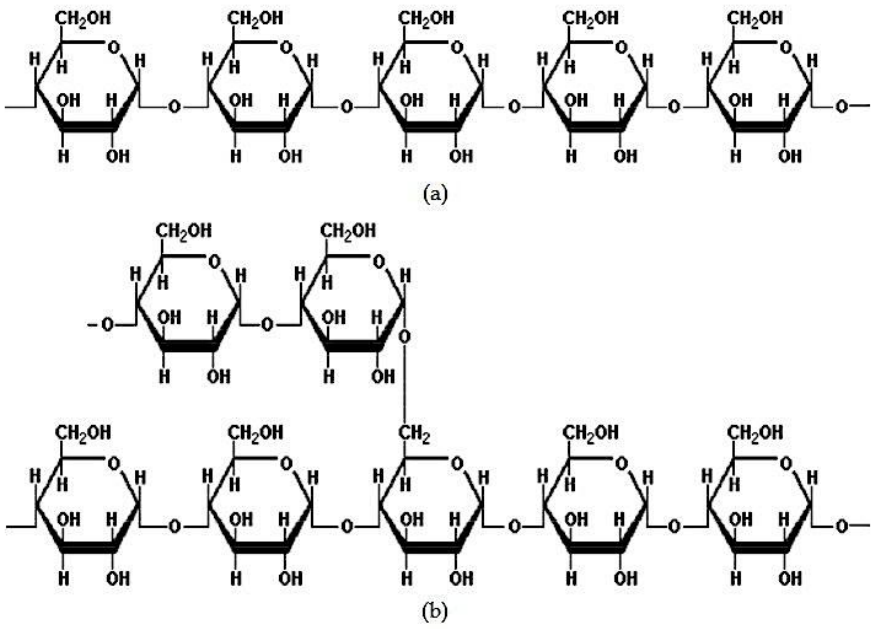

Figure 1.1 Starch components: amylose (a) and amylopectin (b)

(Saunders *et al.* 2011)

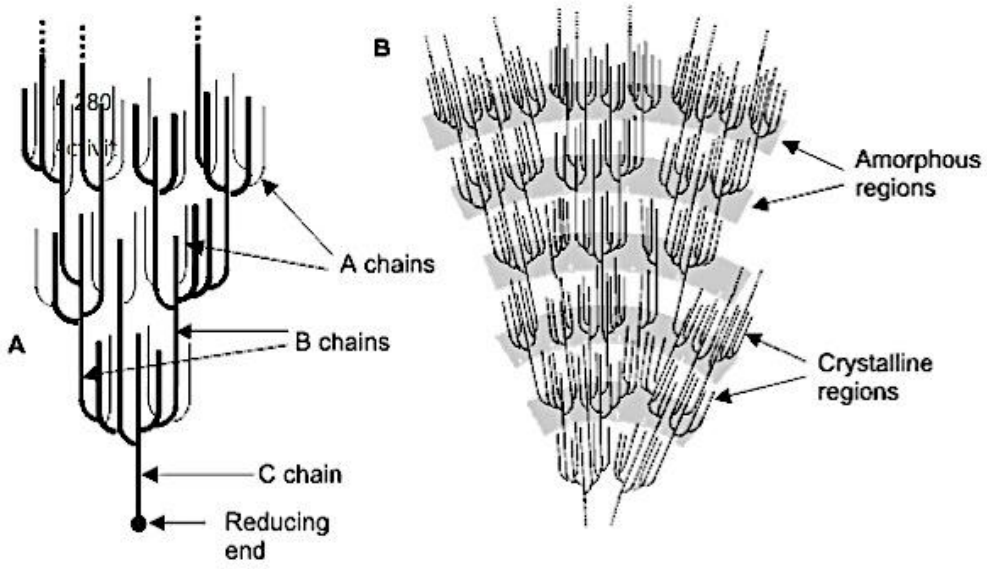

Figure 1.2 A-, B- and C-branch chain types in amylopectin (A) and the amorphous and crystalline regions of the structure (B) (Bertoft 2004)

Table 1.1 Chain length of amylopectin (Hizukuri 1996)

Amylopectin	Average Chain Length
Potato	23
Maize	22
Wheat	20-21
Cassava	21

Table 1.2 Components of starch: the percentage of major components amylose and amylopectin (A) and minor components (B) (Gracza 1965)

Starch	Amylose[A] (%)	Amylopectin[A] (%)	Lipid[B] (%)	Protein[B] (%)	Phosphate[B] (%)
Potato	22	78	0.01	0.10	0.210
Maize	25	75	0.80	0.35	0.090
Waxy maize	0	100	0.20	0.25	0.024
Wheat	23	77	0.90	0.40	0.180
Rice	19	81	0.59	0.30	0.090
Cassava	17	83	0.02	0.10	0.009

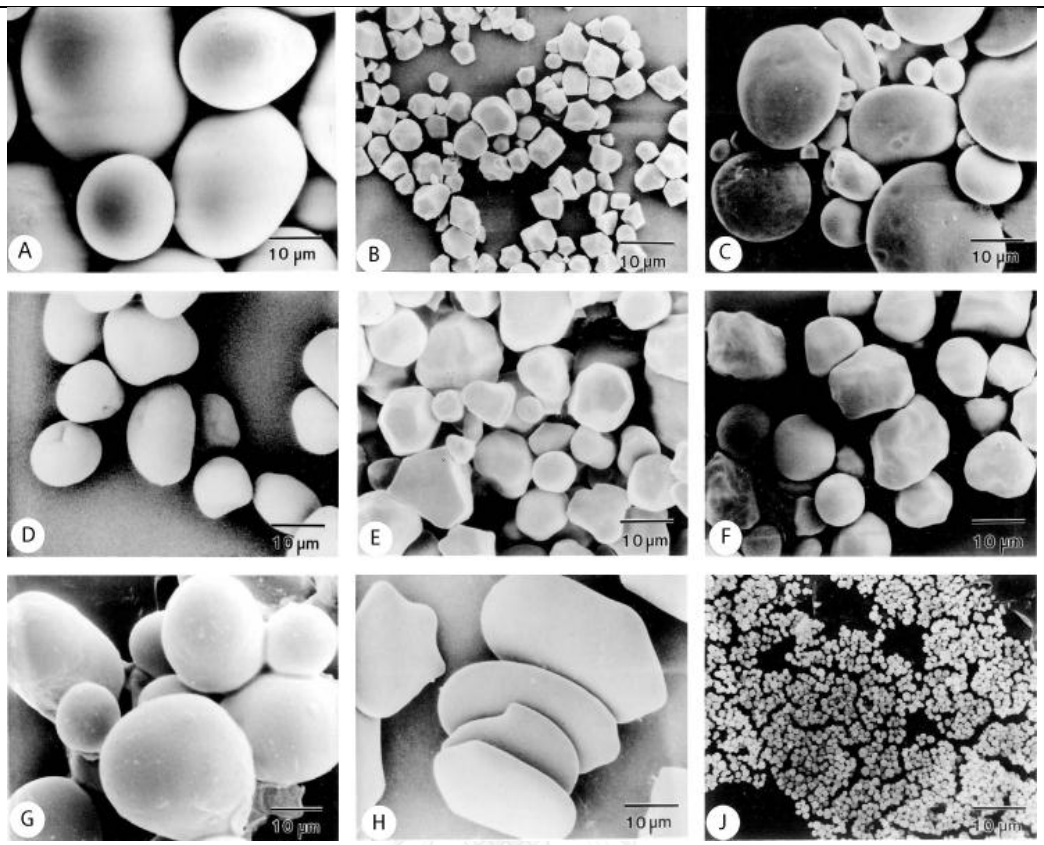

Figure 1.3 Scanning electron micrographs (SEM) of starch granules: potato (A), rice (B), wheat (C), mung bean (D), maize (E), waxy maize (F), tapioca (G), shoti (H) and leaf starch (J) (Jane *et al.* 1994)

different morphologies (Table 1.3) such as oval, round and spherical shapes. Starch granules in leaves are very small about 1 μm in diameter. Starch granules in storage organ are generally bigger than the ones in leaves (Preiss 1996). The cereal starch granules such as maize, waxy maize and oats are around 15-25 μm of diameter except rice starch granules that are smaller with 3-5 μm of diameter. Potato starch granules are very large about 20x75 μm of diameter (Jane *et al.* 1994). The amylose and amylopectin complex formed hydrogen bonds and hydrophobic bonds by, intramolecularly and intermolecularly make a water-insoluble granules. The amylose/amylopectin ratio, size and shape of starch granules and the other components such as protein, lipid, ash and minerals determine characteristic properties such as gelatinization temperature, degrees of crystallinity and swelling of each kind of starches (Table 1.4).

Thailand produces large amount of cassava starch. Both raw and modified starch are used as surface sizing and paper coating in paper industry, warp sizing in textiles industry, dusting agent and dispersing agent in cosmetic and pharmaceutical industries, production of organic compounds by fermentation, and many applications in food industry. For food and pharmaceutical industries, enzymatic modified starch is usually safer than chemical modifications.

Table 1.3 Granule size and average size of starches (Buléon *et al.* 1998, Kim and Huber 2008)

Source	Average size (μm)	Granule Size Range (μm)	Granule Shape
Potato	36	10-100	Large oval
Maize	14	5-25	Polyhedral and rounded
Wheat	7 and 20	3-35	Discs
Cassava	14	3-30	Round

Table 1.4 Starch gelatinization temperature range (Swinkels 1985)

Starch	Gelatinization Temperature Range (°C)
Potato	57-87
Maize	70-89
Waxy maize	68-90
Wheat	50-86
Cassava	64-92

1.2 Physico-chemical characteristic of starches

Structure and composition of each kind of starch or modified starch lead to their suitability in industrial applications.

1.2.1 Thermal properties

Geletinization process is a heating process which starch granule is broken free and allow water to enter into crystalline structures to swell amylopectin and migrate amylose out of the granule to surrounding water. This process changes the viscosity of starch mixture to become a gel (Lai and Kokini 1991). When temperature is cooled down, linear part of starch molecules such as amyloses and linear parts of amylopectins can retrograde and rearrange themselves to more crystalline structure. Retrogradation ratio depends on amylose/amylopectin ratio in starch granules, temperature, concentration of starch and the kind of starch. Amylose chain length with glucose residue 80-100 shows the best retrogradation, at least 8-10 glucose residues of amylose chain length can retrograde and a short chain length of amylopectin with glucose residues longer than 15 residues can retrograde (Eliasson and Gudmundsson 1996). High amylose ratio can retrograde more than low amylose ratio. Therefore, maize and wheat starches which contain high amylose content than cassava and waxy maize starches show degree of retrogradation in the order maize, wheat>cassava starch>waxy maize starch, respectively.

1.2.2 Freeze-thaw stability

After gelatinization, water is released from gel starch (Syneresis) which is a disadvantage for frozen food products. Freeze-thaw stability shows percentages of water released out of gel. The ice crystals form during the freeze process, the

amount of freezable water which increase significantly in every cycle of freeze-thaw process can damage the protein structure (Varriano-Marston 1980) while amylose is migrate out to surrounding water during thaw process of freeze-thaw cycles. Different starches show different percentages of syneresis such as cassava starch shows low percentage of syneresis in first cycle and gradually increased after the first cycle (Table 1.5).

1.2.3 Thermo-reversibility

Thermo-reversibility of gel was determined as the viscosity behavior with change of shear rate, concentration and temperature (Nurul I *et al.* 1999) determined by measuring the stored energy in the material and its recovery per cycle. The energy charges are expressed as storage modulus value (G'), the energy dissipated or lost per cycle as the loss modulus value (G") and the ratio of the energy lost/the energy stored for each cycle defined as tan δ value (G"/G') (Table 1.6). High tan δ value indicates liquid-like state and low tan δ value indicates solid-like state. It was reported that the large granules of potato starch show higher storage and loss modulus and lower tan δ values than the small granule such as maize, waxy maize and cassava (Singh and Soni 2001). Corn starch showed a lower G′ and G″ than potato starch, because of the effect of phospholipids and more rigid granules present in corn starch (Singh and Soni 2001). Rheology defines a relationship between the stress acting on a testing material and the deformation and/or flow that takes place (Figure 1.4). Stress (force per area) and strain (deformation per length) are the key of rheology. Stress (r) is a measurement of force per unit of surface area (shows as Pascals unit: Pa). Rheological properties of food is about $0° < \delta < 90°$.

Table 1.5 Percentage of syneresis of various starches (Srichuwong *et al.* 2012)

Starch	% Syneresis		
	Cycle 1	Cycle 3	Cycle 5
Potato	60.0	71.5	75.6
Maize	35.4	69.5	73.3
Waxy maize	9.6	33.7	53.9
cassava	-	38.9	51.5

Table 1.6 Storage modulus value (G'), Loss modulas value (G") and tan δ of various states (Mezger 2006)

Ideal Viscous Flow Behavior	Behavior of a Viscoelastic Liquid	Viscoelastic Behavior Showing 50/50 ratio of the Viscous and Elastic Portions	Behavior of a Viscoelastic Gel or Solid	Ideal Elastic Deformation Behavior
$\delta = 90°$ $\tan \delta \longrightarrow \infty$ $G' \longrightarrow 0$	$90° > \delta > 45°$ $\tan \delta > 1$ $G'' > G'$	$\delta = 45°$ $\tan \delta = 1$ $G" = G'$	$45° > \delta > 0°$ $\tan \delta < 1$ $G' > G"$	$\delta = 0°$ $\tan \delta \longrightarrow 1$ $G" \longrightarrow 0$

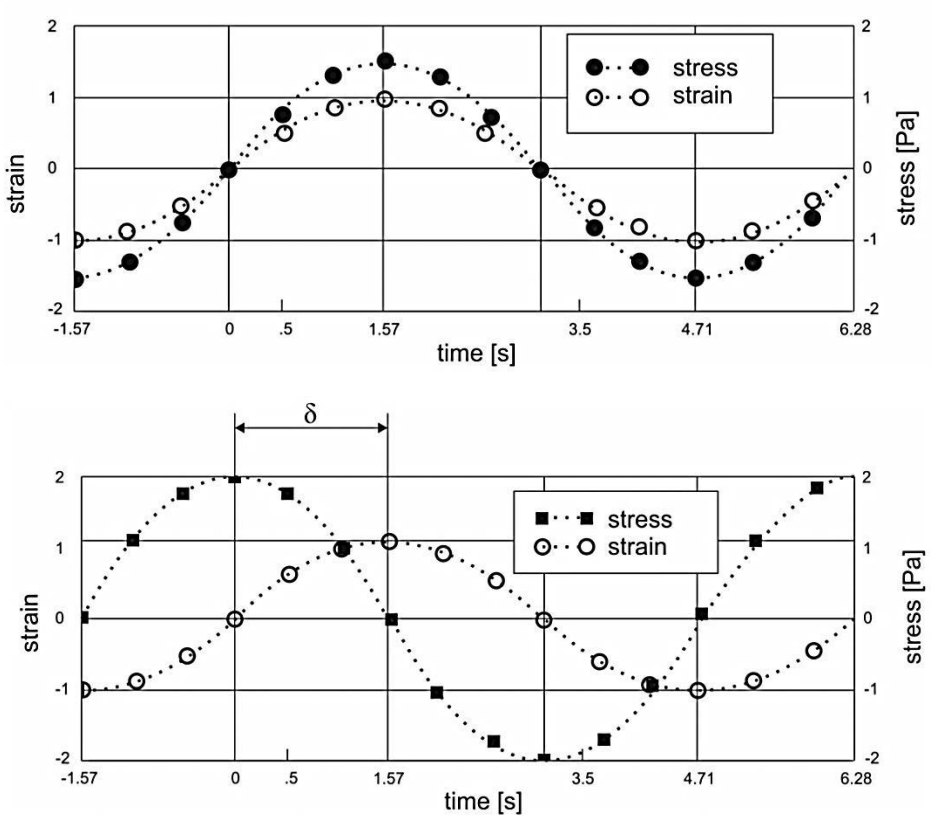

Figure 1.4 Dynamic measurement: strain and stress in an elastic solid (A), strain and stress in the viscoelastic body (B) (Boczkowska and Awietjan 2012)

1.2.4 Apparent amylose content

The quantitation of amylose is based on the starch-iodine-blue value method of (Williams *et al.* 1958). Colorimetric measurement is performed on blue color of the amylose-iodine complex (Figure 1.5). It was reported that color of iodine-stained amylose is blue and iodine-stained amylopectin is purple to red. Long amylose chain had higher iodine absorption values than high branched and short branched amylopectin. A decreased absorption indicated a decrease in the amylose content and the branched chain length of modified starch (Thomas 1999). The complexity with iodine has a blue color when the amylose length over degree of polymerization (DP) 40, has a blue-purple color when the amylose length with DP 30-40, has a purple-red color when the amylose length with DP less than 30, has a red color when the amylose length with DP less than 20, and has a yellow color when the amylose length with DP less than 12. Amylose content with length shorter than DP 47 cannot detect when measuring at 620 nm which is the absorbance for detecting blue color (Kearsley 1995).

1.3 Enzyme involved in starch degradation

1.3.1 The enzyme in α-amylase family

The enzyme in α-amylase family or glycoside hydrolases family 13 (Table 1.7), act on α-glycosidic bonds by hydrolysing the bonds to produce mono- or oligosaccharides (hydrolysis), or form α,1-4 or α,1-6 glycosidic linkages (transglucosylation). There are four groups of enzymes, endoamylase, exoamylase, debranching enzymes and transferases (Figure 1.6) in this family. The first group is, endoamylases hydrolyze α,1-4 glycosidic bonds in the inner part of amylose or

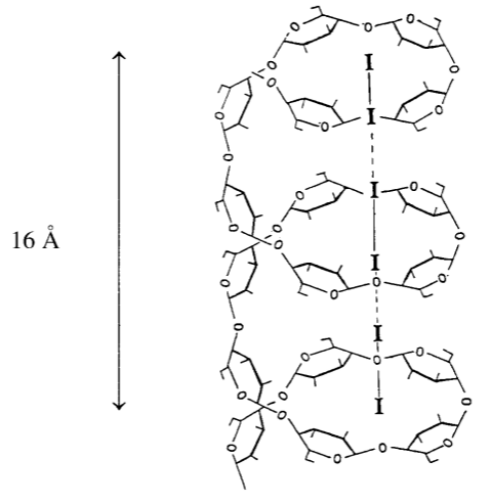

Figure 1.5 The amylose-iodine complex: Schematic structure of the amylose-iodine complex (Minick *et al.* 1991)

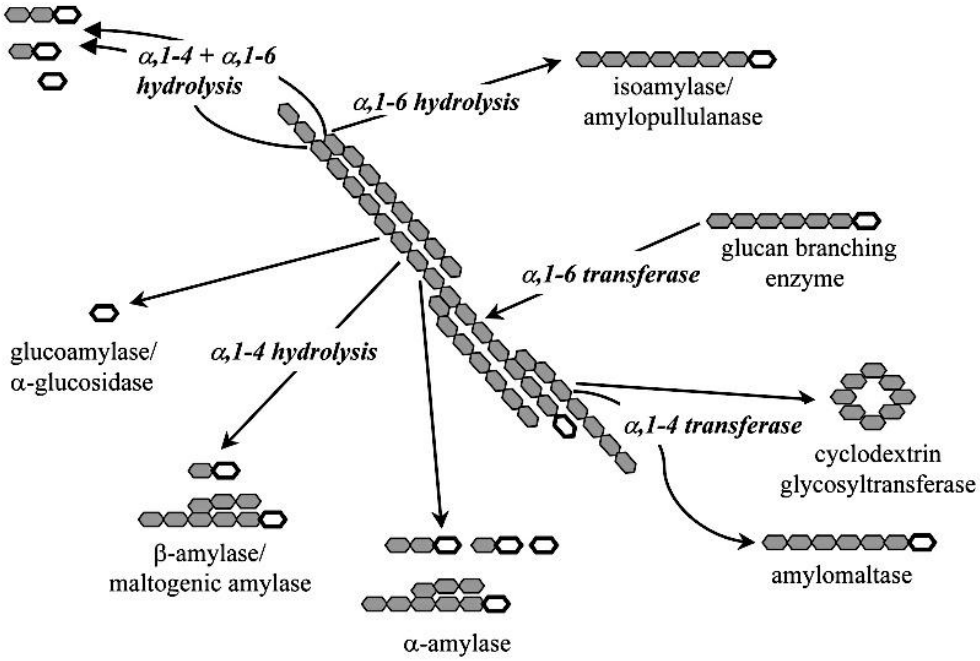

Figure 1.6 Enzyme reactions in the starch degradation (Van Der Maarel *et al.* 2002)

Table 1.7 Glycoside hydrolase family 13 enzymes (Kuriki and Imanaka 1999)

(Van Der Maarel *et al.* 2002)

Enzyme	EC number	Main substrate
Amylosucrase	EC 2.4.1.4	Sucrose
Sucrose phosphorylase	EC 2.4.1.7	Sucrose
Glucan branching enzyme	EC 2.4.1.18	Starch, glycogen
Cyclomaltodextrin glycosyltransferase	EC 2.4.1.19	Starch
Amylomaltase	EC 2.4.1.25	Starch, glycogen
Maltopentaose-forming α-amylase	EC 3.2.1.-	Starch
α-amylase	EC 3.2.1.1	Starch
Oligo-1,6-glucosidase	EC 3.2.1.10	1,6-α-D-glucosidic linkage in some oligosaccharides
α-glucosidase	EC 3.2.1.20	Starch
Amylopullulanase	EC 3.2.1.41	Pullulan
Cyclomaltodextrinase	EC 3.2.1.54	Linear and cyclomaltodextrin
Isopullulanase	EC 3.2.1.57	Pullulan
Isoamylase	EC 3.2.1.68	Amylopectin
Maltotetraose-forming α–amylase	EC 3.2.1.60	Starch
Glucodextranase	EC 3.2.1.70	Starch
Trehalose-6-phosphate hydrolase	EC 3.2.1.93	Trehalose
Maltohexaose-forming α–amylase	EC 3.2.1.98	Starch
Maltogenic amylase	EC 3.2.1.133	Starch
Neopullulanase	EC 3.2.1.135	Pullulan
Malto-oligosyl trehalose hydrolase	EC 3.2.1.141	Trehalose
Malto-oligosyl trehalose synthase	EC 5.4.99.15	Maltose

amylopectin chains producing linear oligosaccharides, branched oligosaccharides and limit dextrins. The well-known endoamylase is α-amylase (EC 3.2.1.1), which was found in microorganisms such as Archaea and Bacteria (Pandey *et al.* 2000). The second group group is the exoamylases which hydrolyze α,1-4 glycosidic bond from the non-reducing end of glucans such as β-amylase (EC 3.2.1.2) producing maltose and limit dextrin, or hydrolyze both α,1-4 and α,1-6 glycosidic bonds on the external of amylose or amylopectin chains such as glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20) which produce only glucose. Glucoamylase and β-amylase are found in a variety of microorganisms (Pandey *et al.* 2000). The third group of α-amylase is debranching enzymes hydrolyze α,1-6 glycosidic bonds, such as pullulanase type I (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). Pullulanases hydrolyze α,1-6 glycosidic bonds in pullulan and amylopectin, but isoamylase can only hydrolyze only α,1-6 in amylopectin. Both enzymes produce long chain oligosaccharides as products (Bender *et al.* 1959). Transferases, the fourth group, hydrolyze α,1-4 glycosidic bond of a donor molecule and transfer the cleaved chain to another chain acting as forming acceptor a new α,1-4 glycosidic bond. The enzymes in this group are amylomaltase (EC 2.4.1.25) which forms a new α,1-4 glycosidic bond and produces a linear oligosaccharides from transglycosylation reaction, cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) forms a new cyclic molecular form α,1-4 glycosidic oligosaccharides (Takaha and Smith 1999).

1.3.2 4-α-Glucanotransferase

4-α-glucanotransferase (EC 2.4.1.25) is the group of enzymes, cyclodextrin glycosyltransferase (CGTase), amylomaltase (AM) or disproportionating enzyme (D-enzyme) and Glycogen debranching enzyme (GDE), can catalyze four reactions (Figure 1.7) cyclization reaction, coupling reaction, hydrolysis and disproportionating reaction. Cyclization reaction the first, an intramolecular transglucosylation with a single linear glucan to produce a cyclic product, cyclodextrins (CDs). Coupling reaction the second, transfers glucosyl units from cyclodextrin to short chain oligosaccharide producing longer chain oligosaccharide. Hydrolysis reaction the third, transfers glucosyl units to an acceptor water. Disproportionating reaction the fourth, an intermolecular transglucosylation reaction, transfers glucosyl units from one oligosaccharide to another oligosaccharide that produces a new longer oligosaccharide (Takaha *et al.* 1998). Amylomaltase and disproportionating enzyme are in glycoside hydrolase (GH) family 77 that show 4-α-glucanotransferase activity (Cantarel *et al.* 2009).

1.3.3 Disproportionating Enzyme

Disproportionating enzyme is found in many plants such as potato (Takaha *et al.* 1998), sweet potato, pea, rice and *Arabidopsis* (Critchley *et al.* 2001, Stettler *et al.* 2009). Disproportionating enzyme is found in two isoforms DPEI and DPEII (Figure 1.8). DPEI catalyzes an intermolecular transglycosylation reaction by transfering a maltosyl unit from non-reducing end of a donor oligosaccharide to an acceptor oligosaccharide while DPEII transfers glycosyl unit from donor oligosaccharide to an acceptor oligosaccharide. There were reported that DPEI from potato and amylomaltase from bacteria can produce cycloamyloses

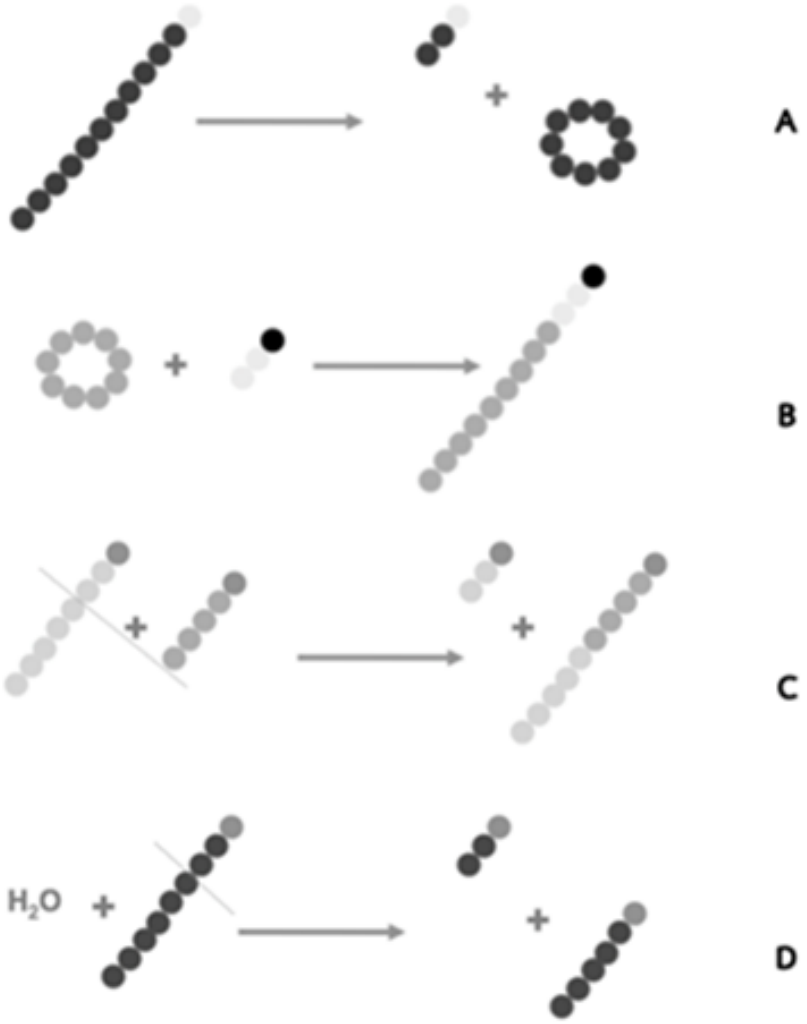

Figure 1.7 Four reactions catalyzed by 4-α-glucanotransferase: cyclization reaction (A), coupling reaction (B), disproportionating reaction (C) and hydrolysis reaction (D) (Van der Veen 2000)

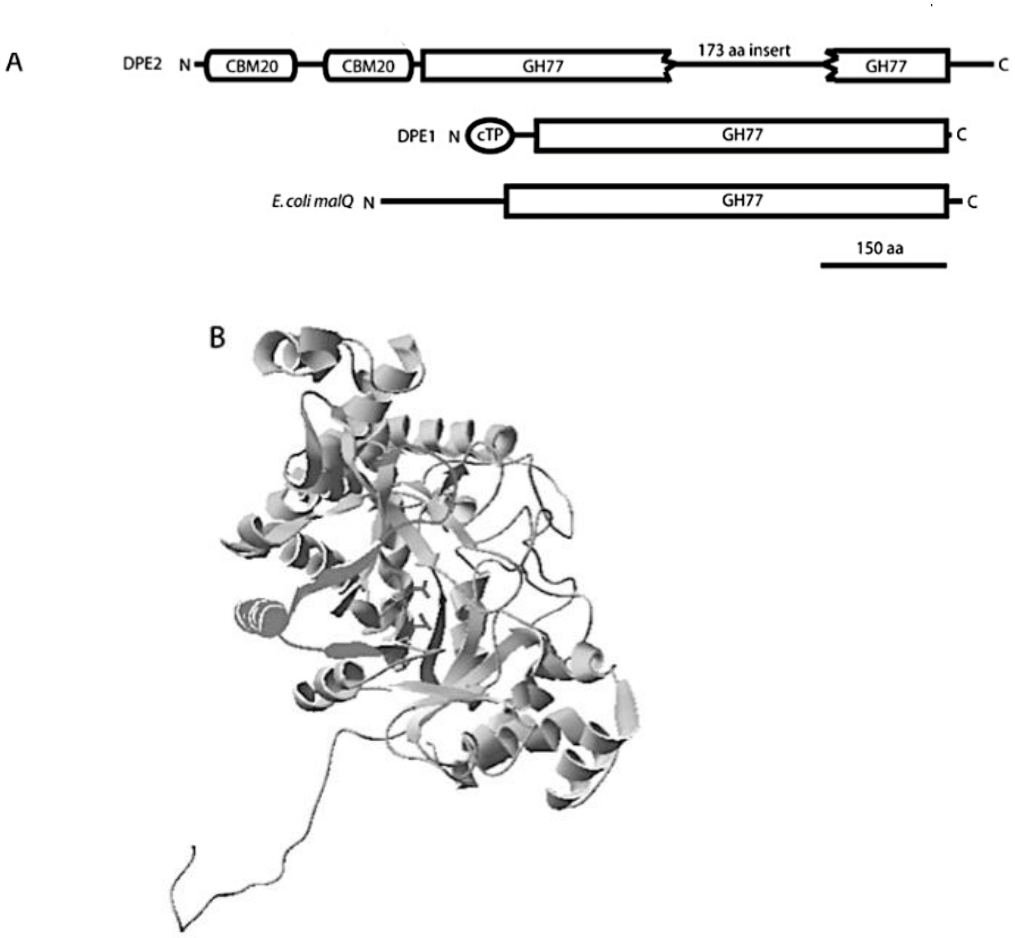

Figure 1.8 Structure of the DPEII and DPEI type 4-α-glucanotransferases: DPEII is organized into 3 modules with an insert of 173 amino acids splitting the GH77 domain (A), the structure of DPEI (PDB: 1X1N) shows the location of the α-helix that has been replaced by 173 amino acids in the DPEII-type enzymes (B) (Steichen *et al.* 2008)

(Takaha *et al.* 1996) (Takaha and Smith 1999). Tantanarat, O'Neill at al. (2014) was also reported that the recombinant MeDPEI from cassava and AtDPEI from *Arabidopsis thaliana* showed a disproportionating activity with maltotriose as substrate by transfering a maltosyl unit. Cycloamyloses with 18-57 glucose units were produced from incubating recombinant MeDPEI with amylose and cycloamyloses with 17-59 glucose units were produced from incubating recombinant AtDPEI with amylose.

1.4 Application of disproportionating enzymes

There are a few reports on the use of disproportionating enzymes in starch modification. Takaha, Yanase et al. (1996) reported that potato D-enzyme catalyzed the cyclization of amylose to produce cycloamyloses with DP ranging from 17 to several hundred and suggested a role of D-enzyme in breakdown of linear or cyclic glucans to produce substrates for hydrolytic or phosphorytic enzymes. Modification of rice grain starch to produce lump-free cooked rice using thermostable disproportionating enzymes: cyclodextrin glucanotransferase from *Pyrococcus furiosus* and α–glucanotraansferase from *Thermus aquaticus* was also reported. The products showed a decrease in retrogradation, viscosity and stickiness of cooked rice and produced lump-free cooked rice with less glucose (Nguyen *et al.* 2014). Van der Maarel, Capron et al. (2005) produced a novel themoreversible gelling product by treating potato starch with hyperthermophilic *Thermus thermophilus* HB8. The amylomaltase-treated potato starch showed the disappearance of long chain amyloses and the shorter side chains of DP 15 in amylopectin and also showed thermoreversible gelation. Van der Kaaij, Yuan et al. (2007) showed that α-glucanotransferase enzymes from *Aspergillus niger*: AgtA and AgtB prefer longer

donor molecules with minimum length of 5 glucose residues while amylomaltases can use smaller donor and acceptor molecules as substrates. AgtA and AgtB can use α-(1,3)-glucooligosaccharides nigerose and nigerotriose as acceptor, and AgtA also produced a amall amount of panose. Modification of corn starch with 4-α-glucanotransferase from *Thermotoga maritime,* treated-corn starch showed lower amount of amyloses and amylopectins, and increase in short branched-chains amylopectin, high solubility and thermo-reversibility of the modified starch (Oh *et al.* 2008). Rice starch modified by 4-α-glucanotransferase from *Thermus scotoductus* showed increased starch concentration, decreased gelation time, increased the strength gel and thermo-reversibility properties (Lee *et al.* 2008). Apart from disproportionating enzymes, other starch hydrolytic enzymes were also used in industrial applications. Van der Maarel, Van der Veen et al. 2002 showed α-amylase can be used as a replacement of acid hydrolysis for removal of starch in fruit juices, beer and clothes in starch-processing industries. Cyclodextrin glycosyltransferase is used to produce cyclodextrins for non-food applications and for hydrolysis of starch in the saccharification process. Thermostable pullulanase, the debranching enzyme, is used as anti-staling agent to prevent the retrogradation of starch in bakery products.

1.5 Aim of the thesis

Recombinant disproportionating I enzymes from cassava tubers (MeDPEI) was produced in our laboratory (Tantanarat *et al.* 2014). The enzyme was well characterized since there have been many reports using disproportionating enzyme from microorganism to treat starches which showed favorable characteristic for use in many industries. Thus we are interested in using our recombinant MeDPEI which is of

plant origin in starch modification and investigating the characteristics of the resulting modified starch. The aims of this thesis are :

1.5.1 To modify starches with MeDPEI

1.5.2 To find the optimal condition for starch modification

1.5.3 To characterize the modified starches

Research steps:

1. Preparation and purification of recombinant MeDPEI from *Manihot esculenta* Crantz cultivar KU50

2. Determination of the optimized conditions for starch modification

3. Characterization of product properties

CHAPTER II
MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HV-110, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Balance: Model PB303-S, Mettler Toledo, Switzerland

Bandelin Sonoplus Sonicator: Model HD220, Bandelin Electronic, Germany

CentriVap Concentrator: Model 79700-01, Labconco Corporation, USA

Differential Scanning Calorimeter (DSC): Perkin Elmer, Diamond DSC, USA

Electrophoresis power supply: Model EC135-90, Thermo Scientific, USA

Electrophoresis Unit: Mini protein, Biorad, USA

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

Hot plate stirrer: Becthai Bangkok Equipment & chemical, Thailand

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD): model DX600, Dionex Corporation, Sunnydale, USA

- Column Dionex CarboPac® PA1 column 4×250 mm, 4 µm, with a 4×50 mm guard, Pulsed Amperometric Detector: Dionex Corporation, Sunnydale, USA
- Column Dionex CarboPac® PA100 column 4×250 mm, 4 µm, with a 4×50 mm guard, Pulsed Amperometric Detector: Dionex Corporation, Sunnydale, USA

Magnetic stirrer: Model AGE, VELP® Scientifica, Italy

Membrane filter: Whatman, England

Microcentrifuge: Model 5430R, Eppendorf, Germany

Oven: Contherm Thermotec 2000, Contherm Scientific, New Zealand

pH meter: MP220, Mettler Toledo, Switzerland

Refrigeration Centrifuge Beckman Coulter Avanti J-30I, Beckman Instrument, USA

Rheometer: Thermoscientific, HAAKE MARS Rotational rheometer, USA

Incubator Shaker: Model Innova® 40, New Brunswick Scientific, USA

Incubator Shaker: Model Innova® 44, New Brunswick Scientific, USA

Streamline Vertical Larminar Flow Cabinets: Model SCV-4A1, Streamline Laboratory, Singapore

UV-VIS spectrophotometer: Model GENESYS 10S, Thermo Scientific, USA

Vortex: Model K-550-GE, Scientific industry Incorporation, USA

Water bath: Memmert, Germany

Water bath shaking: Model SBS30, UK

2.2 Chemicals

Acetic acid: Prolabo, France

Acrylamide: Merck, Germany

Agar: Scharlau, Spain

Ampicillin: Sigma-Aldrich, USA

Amylose from Potato starch: Sigma-Aldrich, USA

Amylopectin from Potato starch: Sigma-Aldrich, USA

Bovine serum albumin (BSA): Sigma-Aldrich, USA

Bromophenol blue: Merck, Germany

n-Butanol: Fisher Chemicals, UK

Cassava Rayong 9 caltivar: Rayong Field Crops Research Center, Thailand

Coomassie Brilliant Blue G-250: Sigma-Aldrich, USA

Coomassie Brilliant Blue R-250: Sigma-Aldrich, USA

Corn starch: Mc Garrette, Thailand

Cycloamylose: Ezaki Glico, Japan

Dialysis tube: Sigma-Aldrich, USA

Dipotassium hydrogen orthophosphate: Ajax Finechem, Australia

Ethanol: Carlo Erba, Italy

Ethylacetate: Carlo Erba, Italy

Ethylenediaminetetraacetic acid (EDTA): Ajax Finechem, Australia

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

Dithiothreitol (DDT): USB corporation, USA

Glucoamylase: SORACHIM, Switzerland

Glycerol: Ajax Finechem, Australia

Hydrochloric acid: Carlo Erba, Italy

Imidazole: Fluka, Switzerland

Iodine: Baker Chemical, USA

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

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

Lactose: Ajax Finechem, Australia

Lysozyme from chicken egg white: Sigma-Aldrich, USA

Maltoheptaose: Hayashibara Biochemical Laboratories Incorporation, Japan

Maltohexaose: Sigma-Aldrich, USA

Maltopentaose: Tokyo Chemical Industry Company Limited, Japan

Maltose: Conda, Spain

Maltotetraose: Hayashibara Biochemical Laboratories Incorporation, Japan

Maltotriose: Sigma-Aldrich, USA

D-(+)-Mannose: Sigma-Aldrich, USA

Methanol: Mallinckrodt, USA

Potassium dihydrogen phosphate: Ajax Finechem, Australia

Potassium iodide: Mallinckrodt, USA

Propanol: Feuergefährlichl, Germany

Pyridine: Carlo Erba, Italy

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

Standard protein marker: Sigma-Aldrich, USA

Thermo-reversible starch: Germany

Sulfuric acid: RCI Labscan limited, Thailand

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

Tris (hydroxymethyl)-aminomethane: Carlo Erba, Italy

Tryptone: HiMedia, India

Yeast extract: Scharlau, Spain

2.3 Medium preparation

LB (Luria Bertani) medium was prepared consisted of 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. Sterilization was prepared by autoclave at 121 °C for 15 minutes.

2.4 Preparation of recombinant MeDPEI

The DPEI gene from *Manihot esculenta* Crantz cultivar KU50 was cloned in vector TrcHis2C and transformed into *E.coli* DH5α. The transformants were cultured on LB medium plate containing 100 µg/ml ampicillin at 37 °C for 18 hours, a single colony of transformants was inoculated in 2 liters of LB broth starter containing 100 µg/ml ampicillin at 37 °C for 4 hours. When the absorbance at 600 nanometer (OD_{600}) reached 0.4-0.6 0.1 mM IPTG was added, and the culture was further incubated at 16 °C for 18 hours. Cells were then collected by centrifugation at 5,000 rpm, 4 °C for 15 minutes (Tantanarat *et al.* 2014)

2.5 Purification of recombinant MeDPEI

Cell pellet was resuspended with lysis buffer containing 10 mM Tris-HCl pH 7.0, 2 mM DTT and 0.3 mg/ml lysozyme. The intracellular enzyme was released by sonication and collected by centrifugation at 12,000 rpm for 45 minutes. The DPEI enzyme was purified by HisTrap affinity column chromatography. Crude enzyme was filtered through 0.45 µm membrane and loaded into HisTrap column. The column was washed with 40 mM imidazole in 20 mM Tris pH 7.4 containing 0.5 M NaCl until the absorbance at 280 nm nearby zero. The enzyme was then eluted with 0.5 M NaCl, 500 mM imidazole in 20 mM Tris pH 7.4 at flow rate of 1 ml/min. The eluted enzyme was collected and dialyzed at 4 °C with 20 mM phosphate buffer pH 7.0.

2.6 Disproportionating activity

Disproportionating activity was determined by the glucose oxidase method with maltotriose as substrate, measuring the glucose released in the reaction. The enzyme (10 µl) was incubated with 25 µl of 5% (w/v) maltotriose in 0.1 M Tris-HCl pH 7.0 100 µl at 30 °C for 10 minutes, 50 µl of reaction mixture was taken, and

added with 30 µl of 1 N HCl to stop the reaction. Glucoseoxidase reaction kit (920 µl) was added and incubated at 30 °C for 10 minutes. The reaction was followed by measuring absorbance at 505 nm (Tantanarat *et al.* 2014).

One disprportionating activity unit was defined as the amount of enzyme that can produce 1 µmol glucose per minute.

2.7 Starch transglucosylation activity

Starch transglucosylation activity was determined by the iodine method by incubation of 100 µl of enzyme at 30 °C in reaction mixture containing of 250 µl of 0.2% (w/v) soluble starch in 0.5 M phosphate buffer pH 7.0, 50 µl of 1% (w/v) maltose and 600 µl of 0.5 M phosphate buffer pH 7.0 for 10 min. The reaction was stopped by boiling, 100 µl of the reaction mixture was added with 1 ml of iodine solution (0.02% I_2 in 0.2% KI) and absorbance at 600 nm was measured (Park *et al.* 2007).

One transglucosylation activity unit was the amount of enzyme that caused a decrease in the blue color of starch-iodine complex at 1% per minute.

2.8 Determination of protein concentration

Protein concentration was determined by Bradford protein assay (Bradford 1976) with Bovine serum albumin (BSA) as standard. One milliliter of Bradford solution containing 0.1% (w/v) Coomassie Brilliant Blue G-250 in absolute EtOH with 85% (v/v) H_3PO_4 was added into 100 µl of sample. The reaction was incubated in darkness for 5 minutes, then absorbance at 595 nm was measured.

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

Molecular weight of protein was determined by SDS-PAGE (Bollag 1996). Solution consists of 7.5% acrylamide separating gel and 5% stacking gel. (Appendix A,B) Protein samples and low molecular weight standard protein marker contained SDS-dye were boiled for 5 minutes and loaded into the sample wells and electrophoresed using Tris-glycine buffer pH 8.3 as electrode buffer with 15 mA of electric current per slab gel. After electrophoresis, the gel was stained with staining solution (1 g of Commassie Brilliant Blue R-250 in absolute methanol with glacial acetic acid, adjusted to the volume of 1 L) for 2 hours and destained by destaining solution.

2.10 Determination of the optimum conditions for starch modification and characterization of product properties

2.10.1 Optimum temperature for cassava starch modification

5% (w/v) cassava starch was gelatinized at 90 °C in DI (deionized) water for 30 minutes then incubated with 20 units/g starch of tranglucosylation activity of purified MeDPEI enzyme at different temperatures: 30, 37 and 45 °C for 24 hours. The reactions were stopped by boiling for 30 minutes and the products were dried in a tray-dryer at 45 °C for 2 days. Then the product properties were characterized (Cho *et al.* 2009).

2.10.2 Optimum enzyme concentration for cassava starch modification

Gelatinized 5% (w/v) cassava starch was incubated with purified MeDPEI enzyme at optimum temperature determined from previous step with different enzyme concentrations: 10, 20 and 30 units/g starch of tranglucosylation activity for 24 hours. The reactions were stopped by boiling for 30 minutes, the products were

dried in a tray-dryer at 45 °C for 2 days. Then the product properties were characterized.

2.10.3 Optimum time for cassava starch modification

Gelatinized 5% (w/v) cassava starch was incubated at optimum temperature and optimum enzyme concentration determined from previous steps at different times: 6, 12 and 24 hours. The reactions were stopped by boiling for 30 minutes, the product was dried in a tray-dryer at 45 °C for 2 days. Then the product properties were characterized.

2.10.4 Corn starch modification

Gelatinized 5% (w/v) corn starch was incubated with previous condition determined from cassava starch modification steps. The reactions were stopped by boiling for 30 minutes, the products were dried in a tray-dryer at 45 °C for 2 days. Then the product properties were characterized.

2.11 Thermal properties

The enthalpy of amylopectin crystal was measured by Differential Scanning Calorimetry (DSC) and the thermal properties calculated with Pyris program. Two milligrams of modified starch was weighed in aluminum volatile pan, then 6 µl of DI water was added and the pan was sealed. Sample was heated from 30-85 °C at 10 degrees per minute by differential scanning calorimeter (DSC) for gelatinization then stored at 4 °C for 14 days for observation of retrogradation and reheated by DSC from 30-85 °C at 10 degrees per minute (Cho *et al.* 2009).

2.12 Freeze-thaw stability

Freeze-thaw stability was determined by measuring the % water released (% syneresis) during the freeze-thaw process (Lee *et al.* 2006). Modified starch (5% w/v)

was gelatinized in DI water for 30 minutes and weighed in pre-weighed eppendorf. Sample was then frozen at -20 °C for 24 hours and thawed at 30 °C in water bath, then centrifuged at 10,000 rpm for 30 minutes and the water separated from the gel was removed. The remaining sample was refrozen at -20 °C and rethawed at 30 °C for 5 cycles. Then % syneresis was determined.

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

2.13 Apparent amylose content

The apparent amylose content was determined by the absorption of blue color in amylose-iodine complex at 620 nm (Juliano 1971). Modified starch (100 mg) was weighed and mixed with 1 ml of 95% (v/v) EtOH and 9 ml of 2 M NaOH in 100 ml volumetric flask and gelatinized in boiling water for 10 minutes. The mixture was adjusted to 100 ml with DI water and 5 ml of the solution was transferred to another 100 ml of volumetric flask containing 2 ml of 1 M acetic acid and 2 ml of iodine solution (0.02% iodine in 0.2% potassium iodine). The mixture was adjusted to 100 ml with DI water and absorbance at 620 nm was measured. The % apparent amylose was determined by comparing with the standard curve of amylose-iodine complex. (Appendix C)

2.14 Thermo-reversibility

Thermo-reversibility of gel was determined as the viscoelasticity between stress value (force) and strain value (deformation) by Rheometer (Lee *et al.* 2006). Modified starch (5% w/v) was gelatinized in DI water for 30 minutes. Sample was measured by rheometer at 70 °C with a 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 measuring at 70°C, sample was stored at 4 °C for 24 hours and measured storage modulus value (G'), loss modulus value (G") and tan δ value (G"/G'). The sample was reheated at 70 °C and restored at 4 °C and measured storage modulus value (G'), loss modulus value (G") and tan δ value (G"/G') for 3 cycles.

2.15 Detection of side chain distribution and size of cycloamyloses by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Detection of side chain distribution by HPAEC-PAD column was performed with CarboPac PA-1. Modified starch (9 mg) was weighed in a test tube, 3.2 ml of UP water was added and sample was gelatinized. Isoamylase (1770 units) and 200 µl of 0.1 M acetate buffer pH 3.5 were added, then the reaction mixture was incubated with shaking at 40 °C for 48 hours. The reaction was stopped by boiling and centrifuged at 10,000 rpm for 30 minutes. The supernatant was filtered for HPAEC-PAD analysis (Kuakpetoon and Wang 2006).

The PA-1 column was equilibrated with 150 mM NaOH. Sample was eluted by linear gradient of 600 mM sodium acetate in 150 mM NaOH with rate 1 ml/min. (Appendix E)

Detection of sizes of cycloamyloses by HPAEC-PAD column CarboPac PA-100. Modified starch (2% w/v) was gelatinized in DI water, 40 units of glucoamylase was added and the reaction mixture was incubated with shaking at 40 °C for 24 hours. The reaction was stopped by boiling and centrifuged at 10,000 rpm for 30 minutes. Supernatant was taken and precipitated by 4 volumes of absolute EtOH for 2 days,

then centrifuged at 10,000 rpm for 30 minutes to collect pellet. The pellet was dissolved in ultrapure (UP) water at 2 mg/ml and filtered for HPAEC-PAD analysis (Tantanarat *et al.* 2014).

The PA-100 column was equilibrated with 150 mM NaOH. Sample was eluted by linear gradient of 200 mM sodium nitrate in 150 mM NaOH with rate 1 ml/min. (Appendix F)

CHAPTER III
RESULTS

3.1 Preparation and purification of recombinant MeDPEI

The MeDPEI transformant from *Manihot eslucenta* Crantz was cultured and was expressed and purified by HisTrap affinity column chromatography according to section 2.4 and 2.5 recombinant MeDPEI. The chromatographic profile is shown in Figure 3.1, Unbound protein fraction was washed by binding buffer until the absorbance at 280 nm was close to zero, the column was then eluted with elution buffer. The fractions with disproportionating activity were pooled and dialyzed with 20 mM phosphate pH 7.0 and the purification result is shown in table 3.1. The purified MeDPEI showed specific activity of 12.6 U/mg protein, 4.3 folds purification and 25.5 % yield. Molecular weight of purified enzyme determined by SDS-PAGE was 61 kDa (Figure 3.2).

3.2 Modification of cassava starch

The purified recombinant MeDPEI was used to modify cassava and the products from the modification were characterized. The optimum conditions for treatment of cassava starch by MeDPEI were determined.

3.2.1 Optimum temperature for cassava starch modification

5% (w/v) cassava starch was gelatinized at 90 °C then incubated with 20 units/g starch of purified MeDPEI enzyme at various temperatures: 30, 37 and 45 °C for 24 hours. Then the product properties could be characterized.

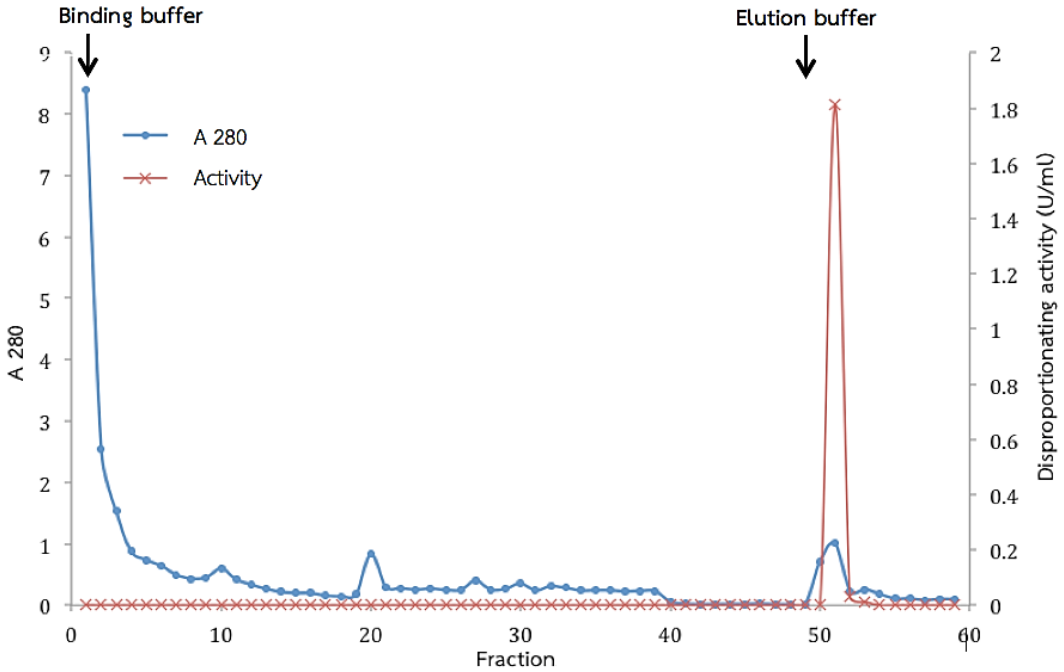

Figure 3.1 Purification of recombinant D-enzyme from *Manihot esculenta* Crantz by HisTrap column chromatography

Table 3.1 Purification Table of recombinant D-enzyme from *Manihot esculenta* Crantz

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	fold	Yield (%)
Crude	2431.7	7077.6	2.90	1.0	100
Purified	143.50	1801.5	12.6	4.3	25.5

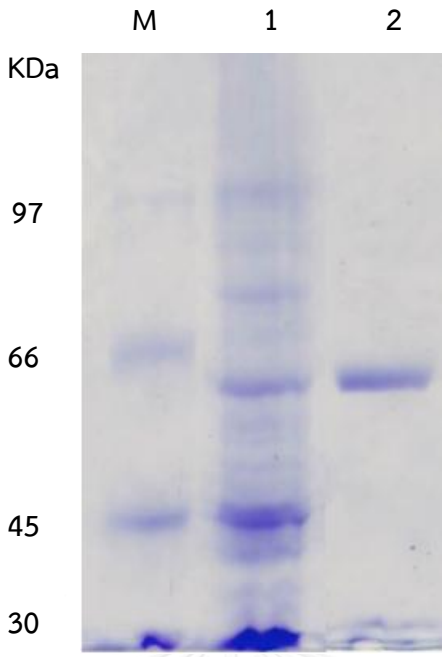

Figure 3.2 Determination of molecular weight of MeDPEI by SDS-PAGE

Lane M = Low molecular weight protein markers:

Phosphaorylase b (97 kDa)

Albumin (66 kDa)

Ovalbumin (45 kDa)

Carbonic anhydrase (30 kDa)

Lane 1 = Crude enzyme 15 µg

Lane 2 = Purified MeDPEI 3 µg

3.2.1.1 Thermal properties

The enthalpy for retrogradation of amylopectin crystal was measured after gelatinization and storage at 4 $^{\circ}$C process for 14 days by Different Scanning Calorimetry (DSC). From the results (Table 3.2), initial temperature for hydrolysis after retrogradation process of modified cassava starch at 30, 37 and 45 ℃ were higher than the non-modified native starch. The modified cassava starch at 37 ℃ showed the highest initial temperature for hydrolysis. Temperature of maximum rate of hydrolysis after retrogradation process of modified cassava starch at 30, 37 and 45 ℃ were lower than the native one, with modified cassava starch at 45 ℃ showing the lowest temperature of maximum hydrolysis rate. Final temperature for hydrolysis after retrogradation process of modified cassava starch at 30, 37 and 45 ℃ were higher than the native one, with modified cassava starch at 37 ℃ had the highest final hydrolysis temperature. Temperature range indicating complexity of modified cassava starch at 30, 37 and 45 ℃ were lower than the native one, with modified cassava starch at 37 ℃ had the lowest temperature range. Enthalpy of retrogradation of modified cassava starch at 30, 37 and 45 ℃ were lower than the native one, indicating modified cassava starch at 37 ℃ had the lowest enthalpy of retrogradation.

3.2.1.2 Freeze-thaw stability

Freeze-thaw stability was determined by measuring the percentage of water released (percentage of syneresis) during the freeze-thaw process. From the results (Figure 3.3), percentages of syneresis of modified cassava starch at 30, 37 and 45 ℃ were significantly increased at cycles 1-4 and the tendency of percentages of syneresis were stable after cycle 4. Percentage of syneresis of native cassava starch was gradually increased at cycles 1-5 and the tendency of continuing increase

Table 3.2 Thermal properties of native and modified cassava starch at various temperatures after gelatinization and storage at 4° C for 14 days

condition	T_o[a] (°C)	T_p[b] (°C)	T_c[c] (°C)	$T_c - T_o$[d]	ΔH_{retro}[e] (J/g)	Retrogradation value (mJ)
Native cassava starch	45.4±0.56	54.9±0.24	62.9±0.25	17.5±0.31	4.97±0.69	2.48
Modified starch at 30 °C	49.5±1.25	54.7±0.00	63.5±1.15	14.0±0.41	0.91±0.31	0.46
Modified starch at 37 °C	51.7±0.03	54.6±0.12	64.1±0.22	12.4±0.41	0.91±0.40	0.43
Modified starch at 45 °C	46.3±0.62	54.3±0.74	63.6±0.60	17.3±0.58	1.36±0.17	0.68

[a]Initial temperature for hydrolysis of amylopectin, [b]Temperature of maximum rate of amylopectin hydrolysis, [c]Final temperature for amylopectin hydrolysis, [d]Temperature range indicating complexity of amylopectin, [e]Enthalpy of retrogradation of amylopectin.

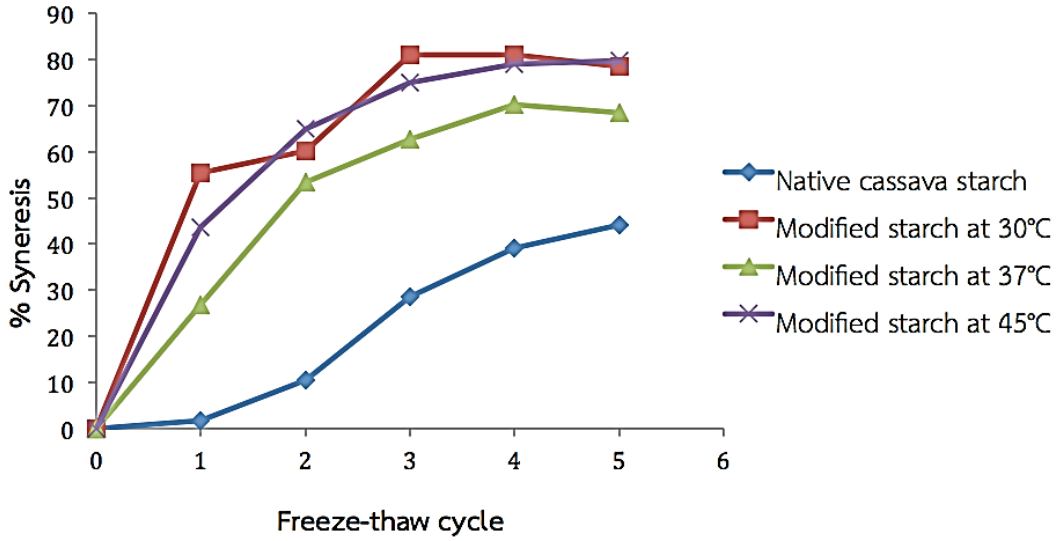

Figure 3.3 Comparison of % syneresis between native and modified cassava starch at various temperatures in the freeze-thaw process

percentages of syneresis. Modified starch at 37 ℃ showed the lowest percentage of syneresis in every cycles of the process compared to the others modified starch.

3.2.1.3 Apparent amylose content

The apparent amylose content was measured by the presence of blue color in the complex with iodine at 620 nm. The results were shown (Table 3.3). Modified cassava starch at 30, 37 and 45 ℃ contained lower percentages of apparent amylose content than the native cassava starch, modified starch at 37 ℃ showed the lowest percentage of apparent amylose content.

3.2.1.4 Thermo-reversibility

Thermo-reversibility of gel was determined as viscoelasticity measured by Rheometer. The results showed (Figure 3.4) that modified cassava starch at 30, 37 and 45 ℃ had high tan δ value as liquid-like state at temperature 70 ℃ and low tan δ value as solid-like state at temperature 4 ℃ and was able to reverse the state when the temperature was changed, while tan δ value of native cassava starch was quite stable.

3.2.1.5 Detection of side chain distribution and size of cycloamyloses by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The results from PA-1 column (Figure 3.5) showed that side chain distribution with DP 6-25 in modified cassava starch at 30, 37 and 45 ℃ were decreased and side chain distribution with DP 12-18 were increased in comparison to native cassava starch, with modified starch at 45 ℃ showing the lowest amount of side chain distribution. Cycloamyloses with DP 18-53 were produced in modified cassava starch at 30, 37 and 45 ℃, as determined by PA-100 column (Figure 3.6) compared to native

Table 3.3 Apparent amylose content of native and modified cassava starch at various temperatures

condition	Apparent amylose content (%)	Decreased in apparent amylose (%)
Native cassava starch	35.4±0.32	-
Modified starch at 30 ℃	32.7±0.74	7.74
Modified starch at 37 ℃	30.7±0.14	13.4
Modified starch at 45 ℃	32.6±0.29	8.05

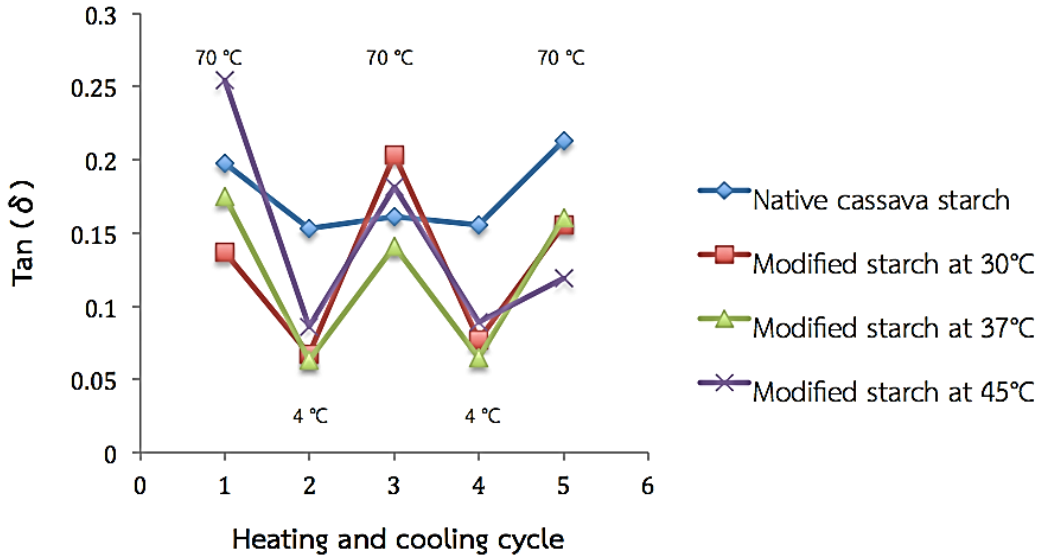

Figure 3.4 Tan (**δ**) values of native and modified cassava starch at various temperatures in heating-cooling cycles

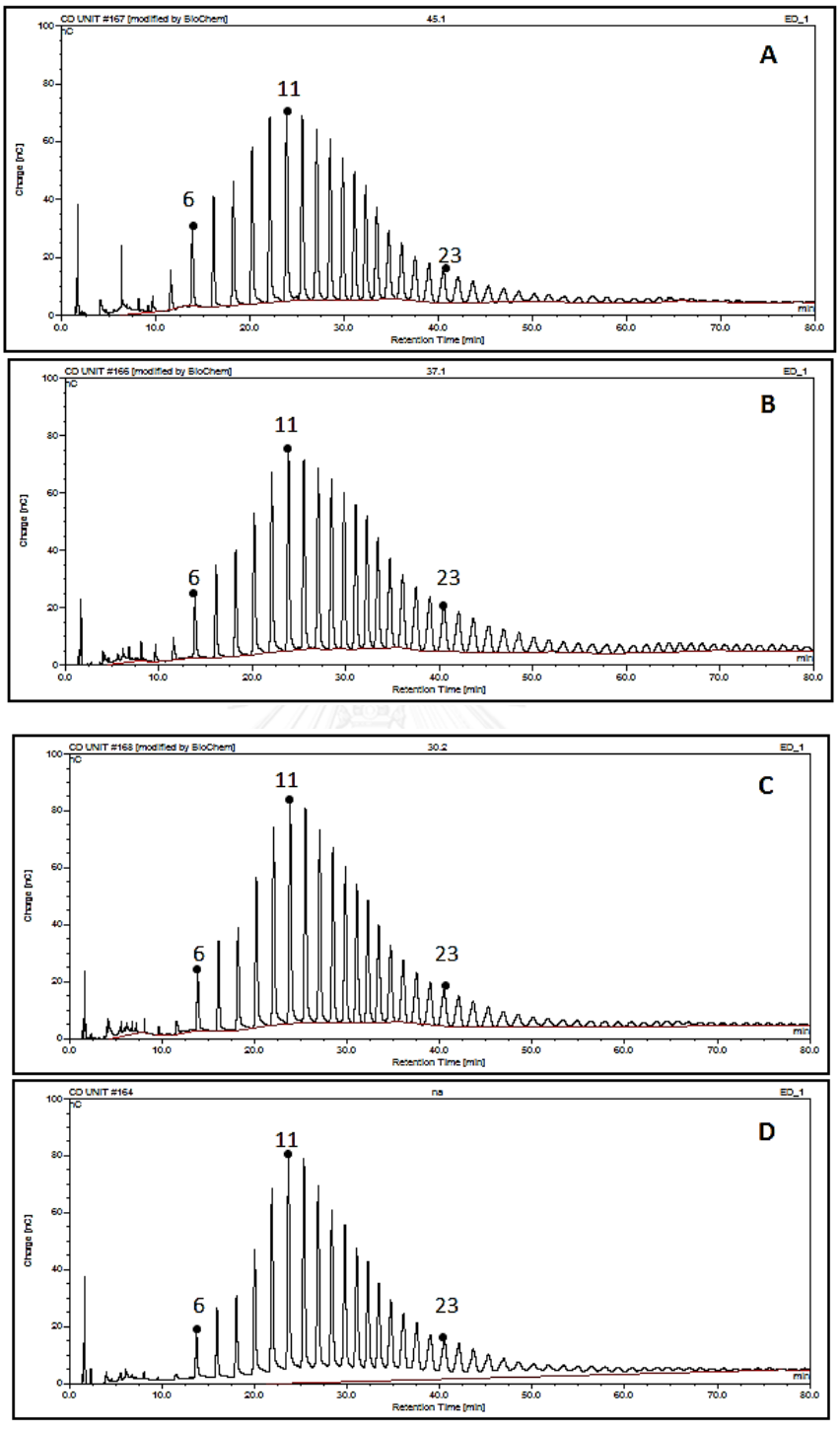

Figure 3.5 Side chain distribution of native and modified cassava starch at various temperatures determined by PA-1 column on HPEAC-PAD: cassava starch modified at 45 °C (A), cassava starch modified at 37 °C (B), cassava starch modified at 30 °C (C) and native cassava starch (D)

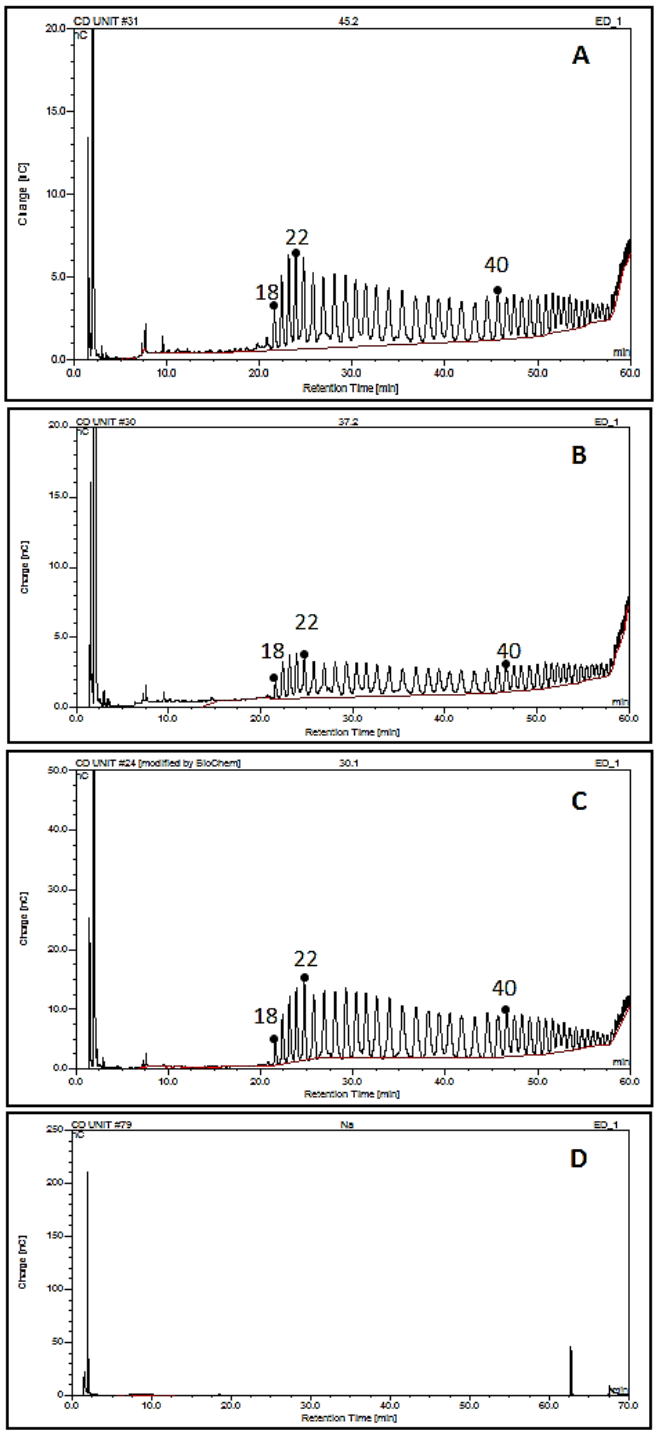

Figure 3.6 Cycloamyloses produced in native cassava starch and modified cassava starch at various temperatures determined by PA-100 column on HPAEC-PAD: cassava starch modified at 45 °C (A), cassava starch modified at 37 °C (B), cassava starch modified at 30 °C (C) and native cassava starch (D)

cassava starch which produced no cycloamyloses. Starch modification at 45 °C produced the highest amount of cycloamyloses.

3.2.2 Optimum enzyme concentration for cassava starch modification

Cassava starch (5% w/v) was gelatinized at 90 °C and incubated at 37 °C for 24 hours with purified MeDPEI at concentrations of 10, 20 and 30 units/g starch. Properties of the product were characterized.

3.2.2.1 Thermal properties

The enthalpy of amylopectin crystal was measured after gelatinization and storage at 4° C for retrogradation process for 14 days by Different Scanning Calorimetry (DSC). From the results (Table 3.4), initial temperature for hydrolysis after retrogradation process of cassava starch modified by 10, 20 and 30/g starch of enzyme were higher than the native one, with cassava starch modified by 30 units/g starch of enzyme showing the highest initial temperature for hydrolysis. Temperatures of maximum rate for hydrolysis after retrogradation process of cassava starch modified by 10, 20 and 30 units/g starch of enzyme were higher than the native one, with cassava starch modified by 30 units/g starch of enzyme showing the highest temperature of maximum rate for hydrolysis. Final temperature for hydrolysis after retrogradation process of cassava starch modified by 10, 20 and 30 units/g starch of enzyme were higher than the native one, cassava starch modified by 10 and 20 units/g starch of enzyme showed the highest final temperature for hydrolysis. Temperature range indicating complexity of cassava starch modified by 10, 20 and 30 units/g starch of enzyme were lower than the native one, with cassava starch modified by 30 units/g starch of enzyme showing the lowest temperature range. Enthalpy of retrogradation of cassava starch modified by 10, 20 and 30 units/g starch

Table 3.4 Thermal properties of native and modified cassava starch at various enzyme concentrations after gelatinization and storage at 4°C for 14 days

condition	T_o [a] (°C)	T_p [b] (°C)	T_c [c] (°C)	$T_c - T_o$ [d]	ΔH_{retro} [e] (J/g)	Retrogradation value (mJ)
Native cassava starch	32.8±0.78	49.0±0.21	61.5±0.07	28.7±0.70	9.05±0.00	4.35
Modified starch by 10 units/g starch of enzyme	39.2±1.32	50.7±0.72	62.3±0.49	23.1±1.59	4.79±0.37	2.32
Modified starch by 20 units/g starch of enzyme	42.1±1.02	51.9±1.03	62.3±0.20	20.2±0.85	3.82±0.15	1.91
Modified starch by 30 units/g starch of enzyme	42.7±1.15	53.2±0.67	62.1±0.92	19.4±0.80	3.75±0.24	1.86

[a]Initial temperature for hydrolysis of amylopectin, [b]Temperature of maximum rate for hydrolysis of amylopectin, [c]Final temperature for hydrolysis of amylopectin, [d]Temperature range indicating complexity of amylopectin, [e]Enthalpy of retrogradation of amylopectin.

of enzyme were lower than the native one with cassava starch modified by 30 units/g starch of enzyme had the lowest enthalpy of retrogradation.

3.2.2.2 Freeze-thaw stability

Freeze-thaw stability determined as percentage of water released (percentage of syneresis) during the freeze-thaw process showed that percentage of syneresis of cassava starch modified by 10 and 20 units/g starch of enzyme were gradually increased at cycles 1-5 (Figure 3.7) while it was quite stable from cycles 1-5 in cassava starch modified by 30 units/g starch of enzyme. Percentage of syneresis of native cassava starch was lower than all modified starch and gradually increased at cycles 1-5. Starch modified by 30 units/g starch of enzyme showed lowest percentages of syneresis in every cycles of the process compared to the other modified starch.

3.2.2.3 Apparent amylose content

The apparent amylose content was measured by the presence of blue color in the complex with iodine at 620 nm. The results showed (Table 3.5) that there was no significant difference in amylose content of native and modified cassava starch at any enzyme concentration.

3.2.2.4 Thermo-reversibility

Thermo-reversibility of gel determined as viscoelasticity by Rheometer. Figure 3.8 showed that cassava starch modified by 10, 20 and 30 units/g starch of MeDPEI had high tan δ value as liquid-like state at temperature 70 °C and low tan δ value as solid-like state at temperature 4 °C. The tan δ value changed when the temperature was changed, while tan δ value of native cassava starch was quite stable.

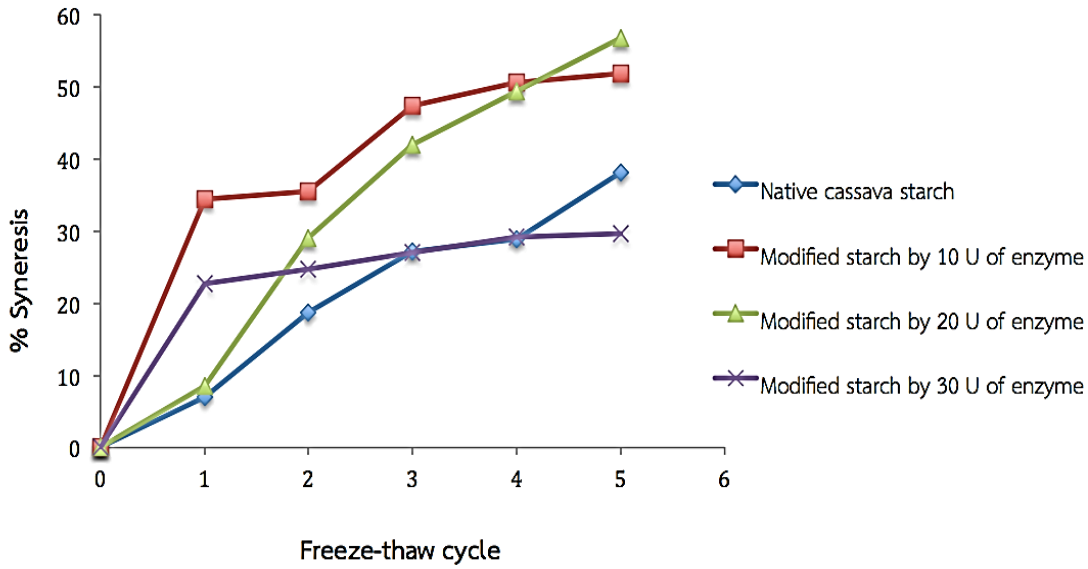

Figure 3.7 Comparison of % syneresis between native and modified cassava starch at various enzyme concentrations in the freeze-thaw process

Table 3.5 Apparent amylose content of native and modified cassava starch at various enzyme concentrations

condition	Apparent amylose content (%)	Decreased in apparent amylose (%)
Native cassava starch	33.2±0.09	-
Modified starch by 10 units/g starch of enzyme	33.0±0.28	0.60
Modified starch by 20 units/g starch of enzyme	33.0±0.57	0.60
Modified starch by 30 units/g starch of enzyme	32.3±0.09	2.71

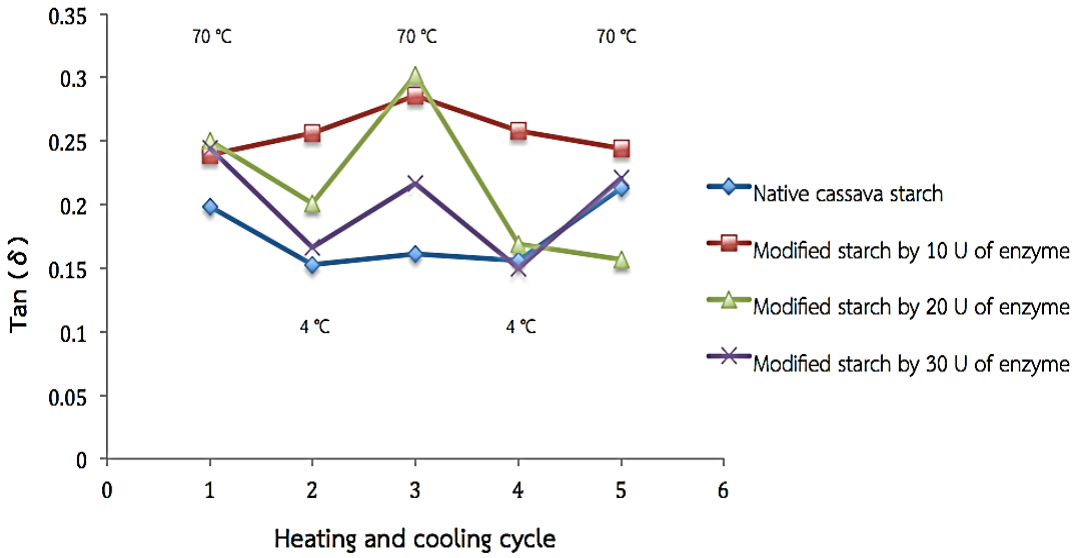

Figure 3.8 Tan (**δ**) values of native and modified cassava starch at various enzyme concentrations in heating-cooling cycles

3.2.2.5 Detection of side chain distribution and sizes of cycloamyloses by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Chromatographic profiles from PA-1 column (Figure 3.9) showed that side chains with DP 6-25 in cassava starch modified by 10, 20 and 30 units/g starch of enzyme decreased and side chain distribution with DP 12-18 were increased in comparison to native cassava starch with starch modified by 30 units/g starch of enzyme had the lowest amount of side chains. Cycloamyloses with DP 18-53 detected by PA-100 column were produced by cassava starch modified with 10, 20 and 30 units/g starch of MeDPEI (Figure 3.10) whereas native cassava starch produced no cycloamyloses. Starch modified by 30 units/g starch of enzyme produced the highest amount of cycloamyloses.

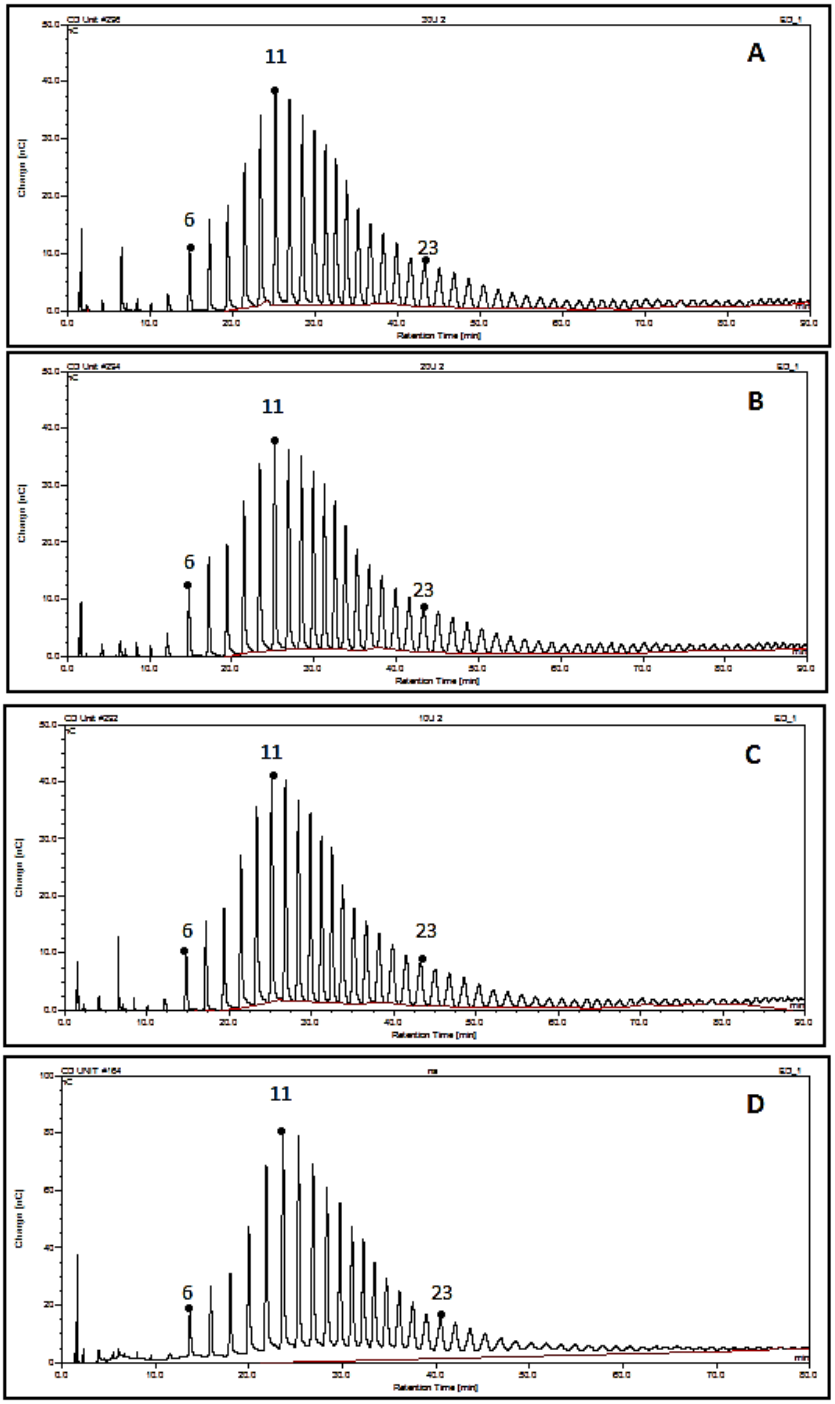

Figure 3.9 Side chain distribution of native and modified starch at various enzyme concentrations determined by PA-1 column on HPEAC-PAD: cassava starch modified for by 30 units/g starch of enzyme (A), cassava starch modified by 20 units/g starch of enzyme (B), cassava starch modified by 10 units/g starch of enzyme (C) and native cassava starch (D)

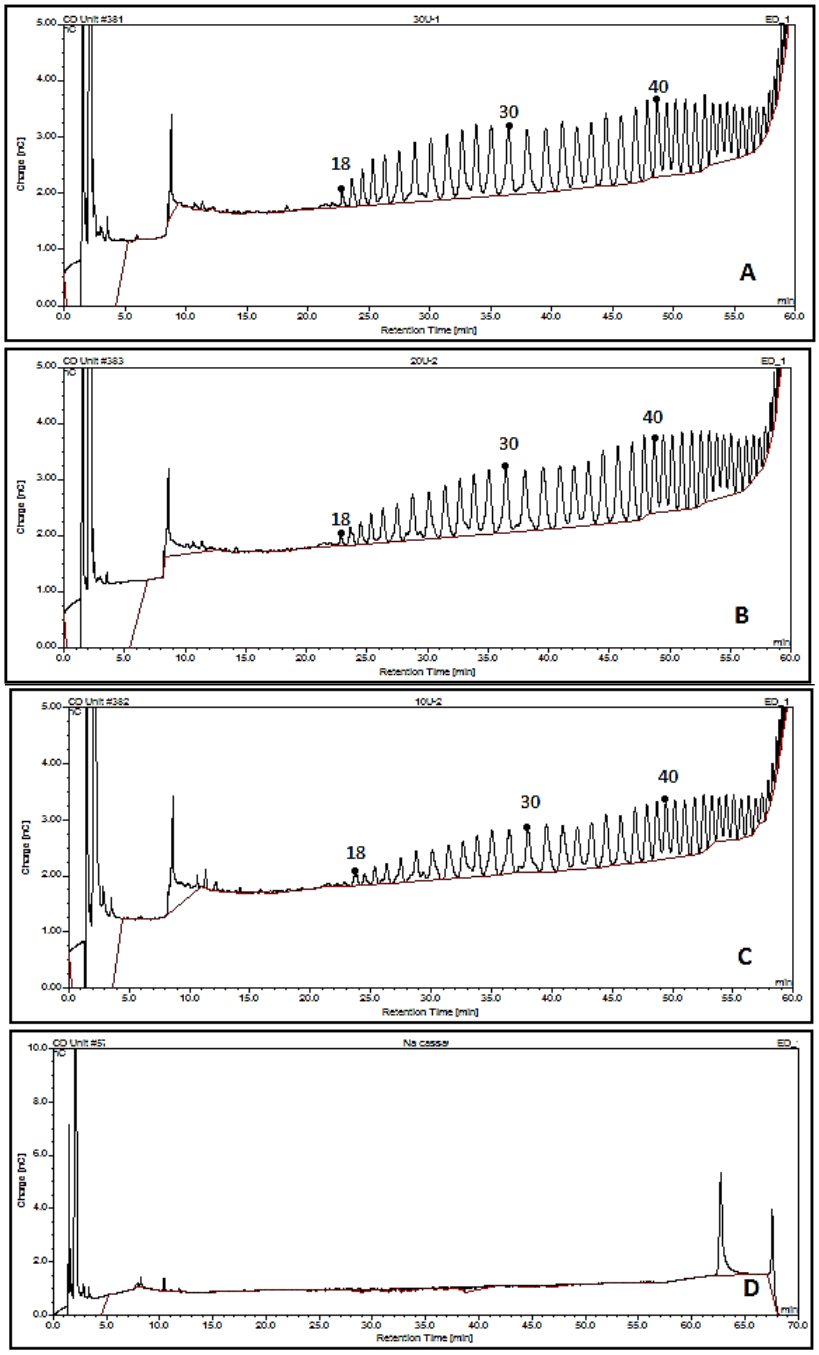

Figure 3.10 Cycloamyloses produced in native and modified cassava starch at various enzyme concentrations determined by PA-100 column on HPAEC-PAD: cassava starch modified for by 30 units/g starch of enzyme (A), cassava starch modified by 20 units/g starch of enzyme (B), cassava starch modified by 10 units/g starch of enzyme (C) and native cassava starch (D)

3.2.3 Optimum incubation time for cassava starch modification

Cassava starch (5% w/v) was gelatinized at 90 °C then incubated at 37 °C with 30 units/g starch of purified enzyme at various incubation time at 6, 12 and 24 hours. The products were characterized.

3.2.3.1 Thermal properties

The enthalpy of amylopectin crystal was measured by Different Scanning Calorimetry (DSC) after gelatinization and storage at 4° C for retrogradation process for 14 days. From table 3.6, initial temperature for hydrolysis after retrogradation process of cassava starch modified at 6, 12, 24 hours were higher than the native one with cassava starch modified at 12 hours showing the highest initial temperature for hydrolysis. Temperatures of maximum rate for hydrolysis after retrogradation process of modified cassava starch for 6, 12, 24 hours were higher than the native cassava starch with cassava starch modified at 6 hours showing the highest temperature of maximum hydrolysis rate. Final temperature for hydrolysis after retrogradation process of cassava starch modified for 6, 12, 24 hours were higher than the native one with modified cassava starch at 12 hours showing the highest final temperature for hydrolysis. Temperature range indicating complexity of cassava starch modified for 6, 12, 24 hours were lower than the native one with modified cassava starch at 24 hours had the lowest temperature range. Enthalpy of retrogradation of cassava starch modified for 6, 12, 24 hours were lower than the native one with cassava starch modified for 24 hours showing the lowest enthalpy of retrogradation.

Table 3.6 Thermal properties of native and modified cassava starch at various incubation times after gelatinization and storage at 4°C for 14 days

condition	T_o[a] (°C)	T_p[b] (°C)	T_c[c] (°C)	$T_c - T_o$[d]	ΔH_{retro}[e] (J/g)	Retrogradation value (mJ)
Native cassava starch	33.3±1.56	48.9±1.55	61.4±0.00	28.1±1.55	9.01±0.08	4.53
Modified starch at 6 hours	45.3±0.56	55.3±1.48	61.9±0.28	16.6±0.84	4.21±0.15	2.11
Modified starch at 12 hours	46.5±0.85	54.8±1.20	62.5±0.14	16.0±0.98	3.94±0.05	1.99
Modified starch at 24 hours	46.2±0.14	54.5±0.70	62.0±0.21	15.8±0.07	3.93±0.31	1.97

[a]Initial temperature for hydrolysis of amylopectin, [b]Temperature of maximum rate for hydrolysis of amylopectin, [c]Final temperature for hydrolysis of amylopectin, [d]Temperature range indicating complexity of amylopectin, [e]Enthalpy of retrogradation of amylopectin.

3.2.3.2 Freeze-thaw stability

Freeze-thaw stability as determined by measuring the percentage of water released (percentage of syneresis) during the freeze-thaw process. Figure 3.11 showed that percentage of syneresis of all modified cassava starch significantly increased at cycles 1-2 and increase more slowly from cycles 2-5. Percentage syneresis of cassava starch modified for 6 hours was significantly higher than the other samples and stable from cycle 2-5. Percentage of syneresis of native cassava starch gradually increased at cycles 1-5. Pattern of percentages of syneresis in every cycles of starch modified for 12 and 24 hours were almost the same.

3.2.3.3 Apparent amylose content

The apparent amylose content measured by the presence of blue color of amylose-iodine complex at 620 nm. The results as shown Table 3.7 showed that cassava starch modified for 6, 12 and 24 hours had lower percentages of apparent amylose content than the native cassava starch with starch modified for 24 hours showing the lowest percentage of apparent amylose content.

3.2.3.4 Thermo-reversibility

Thermo-reversibility of gel determined as the viscoelasticity by Rheometer. The results (Figure 3.12) showed that cassava starch modified at 6, 12 and 24 hours had high tan δ value as liquid-like state at temperature 70 °C and low tan δ value as solid-like state at temperature 4 °C and each state was reversible when the temperature was changed. While tan δ value of native cassava starch was quite stable.

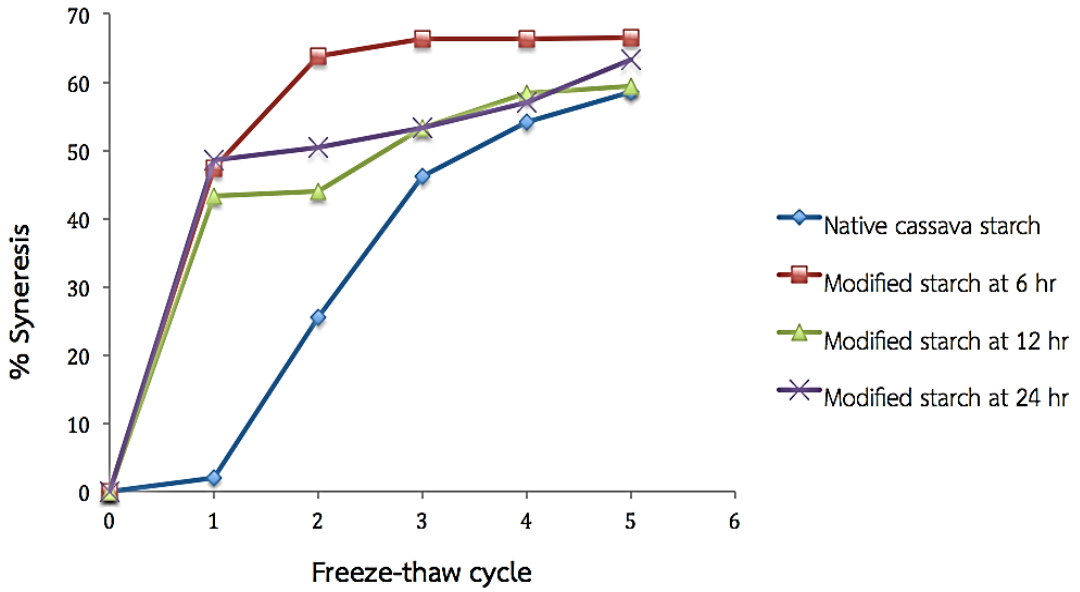

Figure 3.11 Comparison of % syneresis between native and modified cassava starch at various incubation times in the freeze-thaw process

Table 3.7 Apparent amylose content of native and modified cassava starch at various incubation times

condition	Apparent amylose content (%)	Decreased in apparent amylose (%)
Native cassava starch	35.0±0.46	-
Modified starch at 6 hours	33.6±0.06	4.00
Modified starch at 12 hours	33.0±0.12	5.89
Modified starch at 24 hours	31.9±0.33	8.80

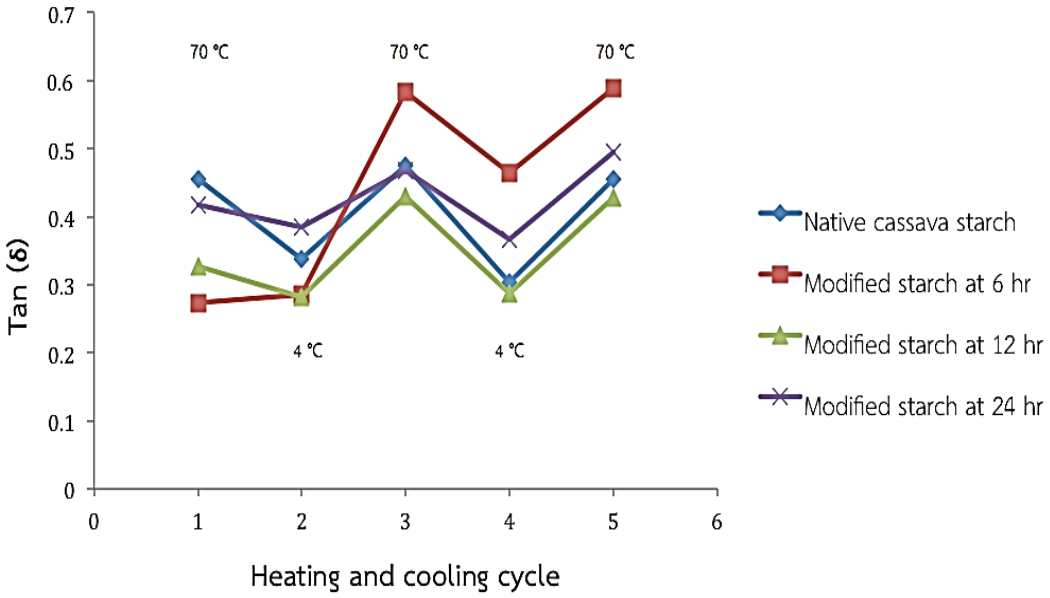

Figure 3.12 Tan (**δ**) values of modified cassava starch at various incubation time and native cassava starch in heating-cooling cycles

3.2.3.5 Detection of side chain distribution and sizes of cycloamyloses by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The results from PA-1 column (Figure 3.13) showed that side chains with DP 6-25 in cassava starch modified for 6, 12 and 24 hours decreased non-significant different and side chain distribution with DP 12-18 were increased in comparison to native cassava starch, with starch modified for 24 hours showing the lowest amount of side chains. Cycloamyloses with DP 18-53 were produced in cassava starch modified for 6, 12 and 24 hours, as determined by PA-100 column (Figure 3.14) whereas native cassava starch produced no cycloamyloses. Starch modified for 12 hours was produced the highest amount of cycloamyloses.

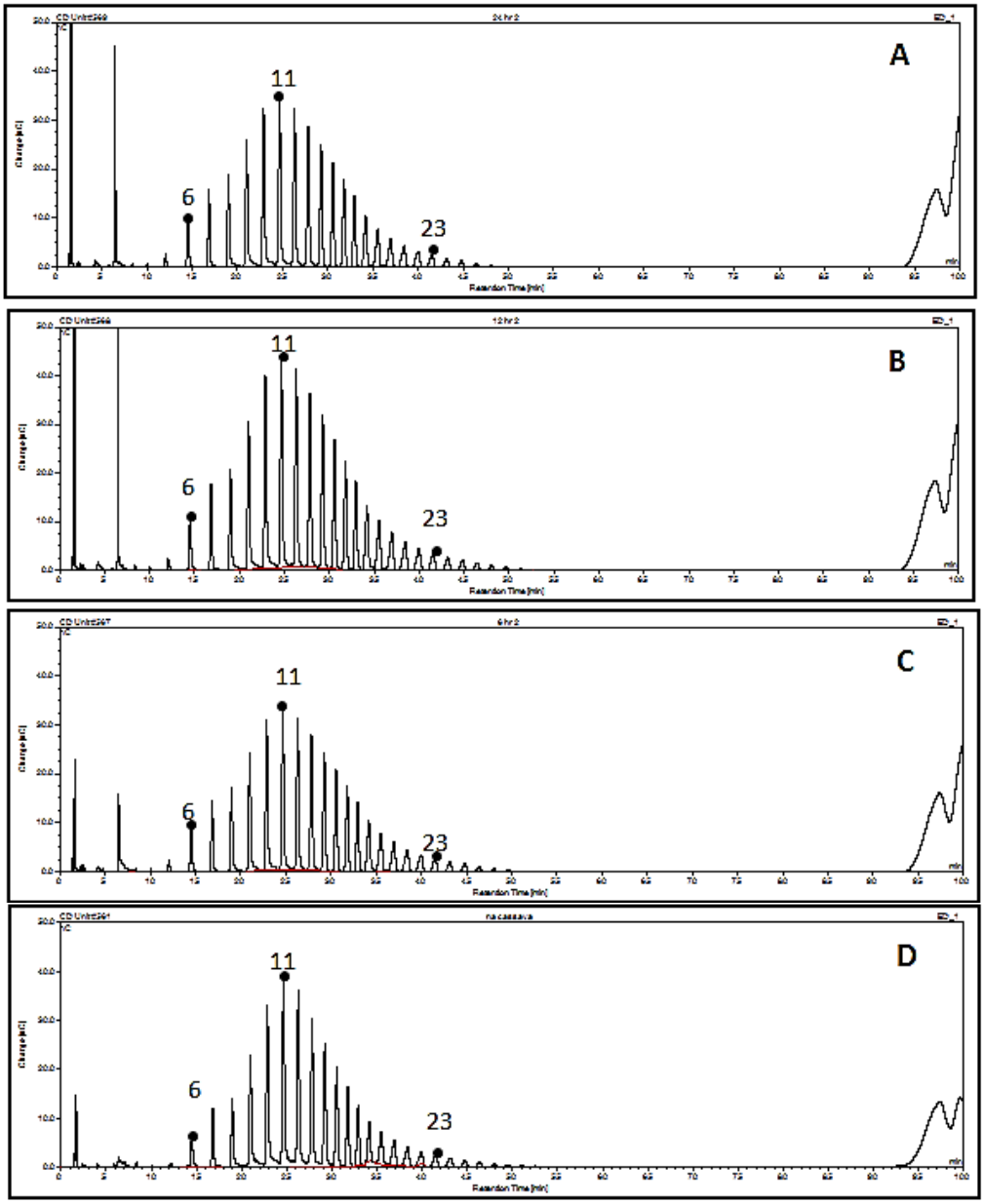

Figure 3.13 Side chain distribution of native and modified starch at various incubation times determined by PA-1 column on HPEAC-PAD: cassava starch modified for 24 hours (A), cassava starch modified for 12 hours (B), cassava starch modified for 6 hours (C) and native cassava starch (D)

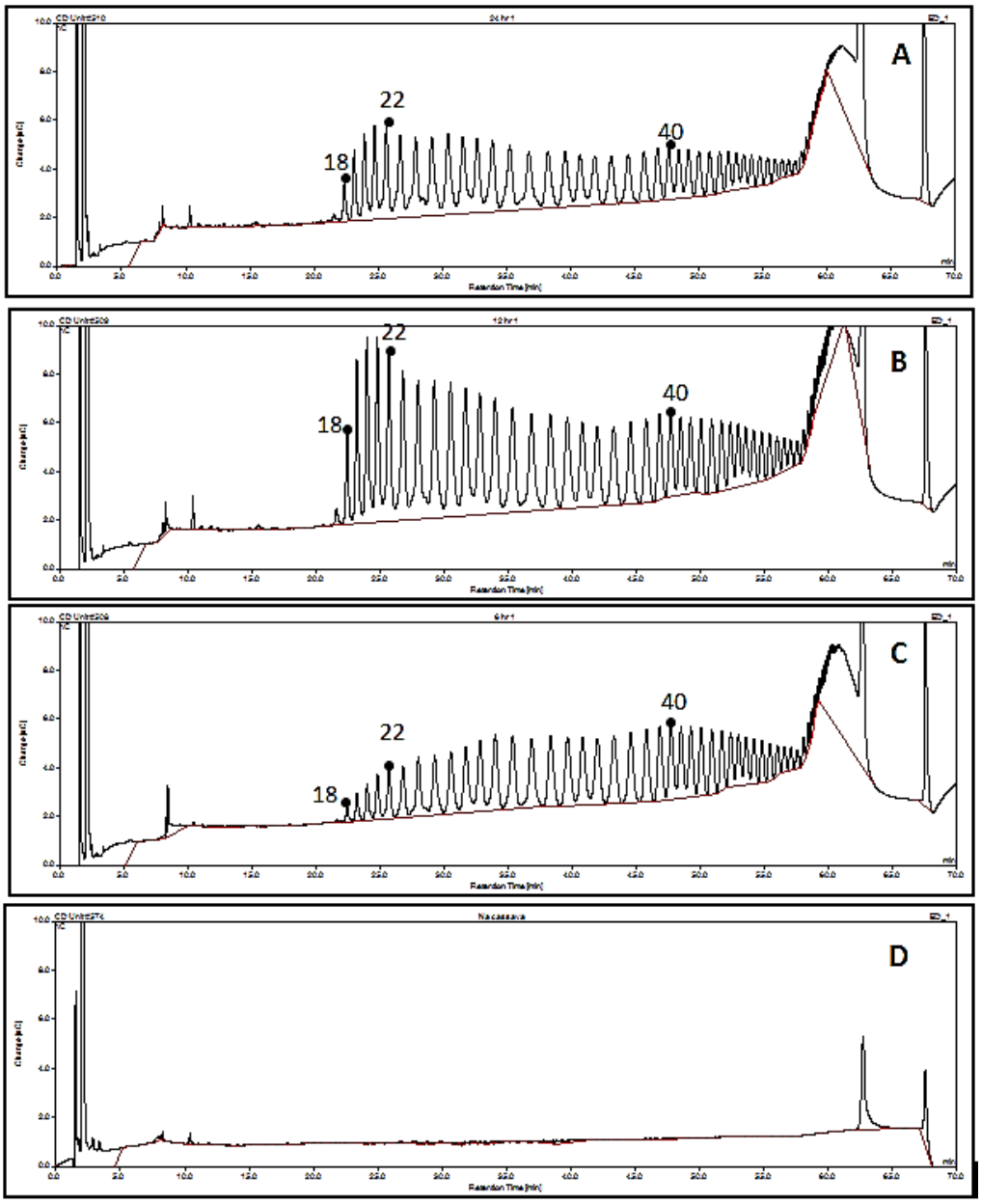

Figure 3.14 Cycloamyloses produced in native and modified cassava starch at various incubation times determined by PA-100 column on HPAEC-PAD : cassava starch modified for 24 hours (A), cassava starch modified for 12 hours (B), cassava starch modified for 6 hours (C) and native cassava starch (D)

3.3 Modification of corn starch

To compare the action of recombinant MeDPEI to other kind of starch, corn starch was modified by MeDPEI at the optimum conditions for cassava starch determined in section 3.2

5% (w/v) corn starch was gelatinized at 70 °C then incubated at 37 °C with 30 units/g starch of purified enzyme for 12 hours. Then the product properties were characterized.

3.3.1 Thermal properties

The enthalpy of amylopectin crystal measured by Different Scanning Calorimetry (DSC) after gelatinization and storage at 4° C for retrogradation process for 14 days were shown in Table 3.8. In comparison with native corn starch, modified corn starch showed higher values in all thermal parameters: the initial temperature of hydrolysis, the temperature for maximum rate for hydrolysis and the final temperature of hydrolysis. Temperature range indicating complexity of modified corn starch was lower than the native one. Enthalpy of retrogradation of modified corn starch was lower than the native one. Native cassava showed highest ΔH_{retro} and cassava treated with MeDPEI showed drastic decrease in ΔH_{retro} compared to modified corn starch.

3.3.2 Freeze-thaw stability

Freeze-thaw stability of modified corn starch was determined by measuring the percentage of water released (percentage of syneresis) during the freeze-thaw process. From the result in Figure 3.15, percentages of syneresis of both modified corn and cassava starches were significantly increased in cycles 1 and remained rather constant though cycles 2-5. Percentage of syneresis of both native cassava and

Table 3.8 Thermal properties of native cassava starch, modified cassava starch, native corn starch and modified corn starch after gelatinization and storage at 4°C for 14 days

condition	T_o[a] (°C)	T_p[b] (°C)	T_c[c] (°C)	$T_c - T_o$[d]	ΔH_{retro}[e] (J/g)	Retrogradation value (mJ)
Native cassava starch	33.3±1.56	48.9±1.55	61.4±0.00	28.1±1.55	9.01±0.08	4.53
Modified cassava starch	46.5±0.85	54.8±1.20	62.5±0.14	16.0±0.98	3.94±0.05	1.99
Native corn starch	46.3±0.77	55.2±1.34	61.7±0.92	15.4±0.14	4.86±0.95	2.47
Modified corn starch	48.3±0.42	57.3±0.14	63.3±0.49	15.0±0.91	2.99±0.07	1.44

[a]Initial temperature for hydrolysis of amylopectin, [b]Temperature of maximum rate for hydrolysis of amylopectin, [c]Final temperature for hydrolysis of amylopectin, [d]Temperature range indicating complexity of amylopectin, [e]Enthalpy of retrogradation of amylopectin.

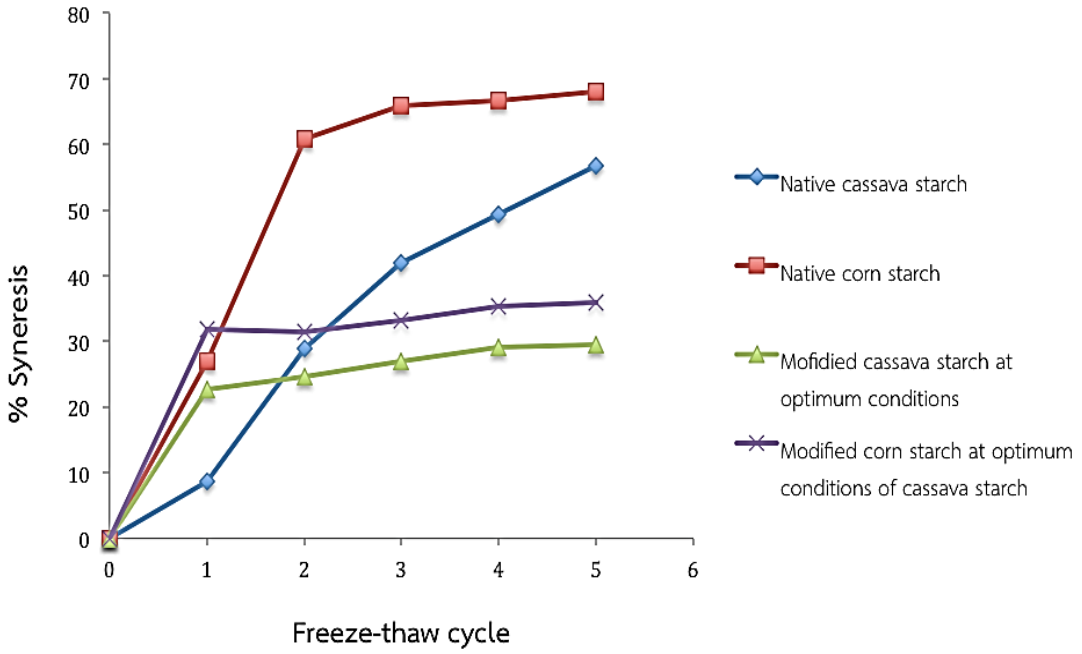

Figure 3.15 Comparison of % syneresis between native cassava starch, modified cassava starch, native corn starch and modified corn starch in the freeze-thaw process

corn starches increased through the freeze-thaw cycles, with highest % syneresis in native corn starch. Modified starch showed significant decrease in % syneresis.

3.3.3 Thermo-reversibility

The viscoelasticity of all starch measured by Rheometer showed that both modified starches had high tan δ value as liquid-like state at temperature 70 °C and low tan δ value as solid-like state at temperature 4 °C (Figure 3.16) and was able to reverse the state when the temperature was changed. The tan δ value of native corn starch was lower and smaller change between states. Cassava starch, both native and modified, showed quite similar patterns of thermo-reversibility while the patterns of native and modified corn starch were quite different. Both native and modified cassava starch had high tan δ than native and modified corn starch.

3.3.4 Apparent amylose content

The apparent amylose content of all starches were measured by the presence of blue color amylose-iodine complex at 620 nm, the results were shown in Table 3.9. Corn starch, both native and modified, showed higher amount of amyloses than cassava starch. Incubation with MeDPE1 caused a decrease in % apparent amylose in both types of starch.

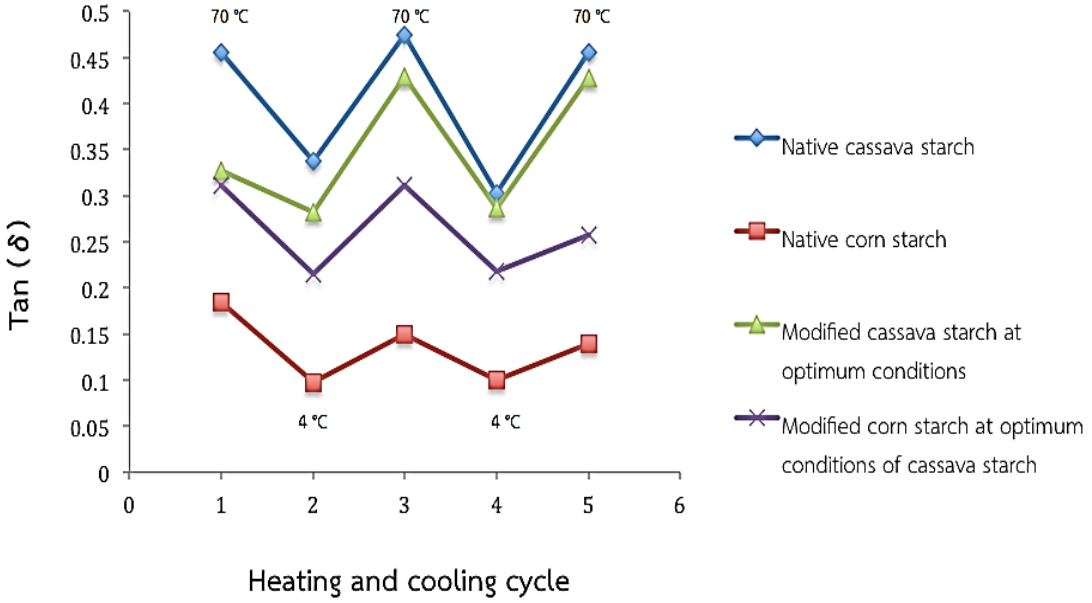

Figure 3.16 tan (**δ**) values of native cassava starch, modified cassava starch, native corn starch and modified corn starch in heating-cooling cycles

Table 3.9 Apparent amylose content of native cassava starch, modified cassava starch, native corn starch and modified corn starch

condition	Apparent amylose content (%)	Decreased apparent amylose (%)
Native cassava starch	35.0±0.46	-
Modified cassava starch	33.0±0.06	5.89
Native corn starch	39.0±0.46	-
Modified corn starch	37.0±0.28	5.13

3.3.5 Side chain distribution and sizes of cycloamyloses measured by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

PA-1 column profile (Figure 3.17) showed that side chain distribution with DP 6-27 were observed in modified corn starch with very small differences. Cycloamyloses with DP 18-55 were produced in modified corn starch, as determined by PA-100 column (Figure 3.18) in comparison to native corn starch which produced no cycloamyloses. Modified cassava starch showed significant decrease in side chain distribution compared to native starch. Cycloamyloses in the range of DP 18-30 appeared to be more in modified corn starch while DP 35-50 appeared to be more in modified cassava starch.

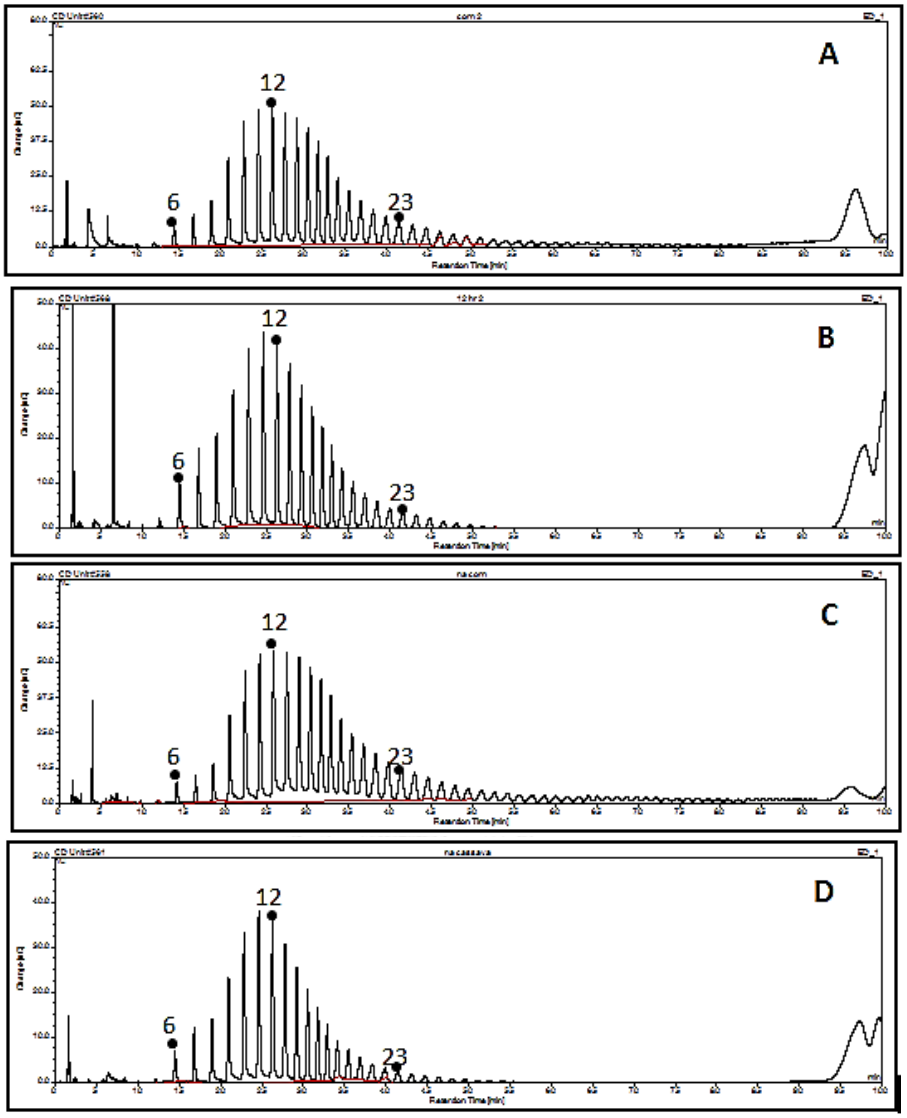

Figure 3.17 Side chain distribution of native cassava starch, modified cassava starch, native corn starch and modified corn starch determined by PA-1 column on HPAEC-PAD: modified corn starch (A), modified cassava starch (B), native corn starch (C) and native cassava starch (D)

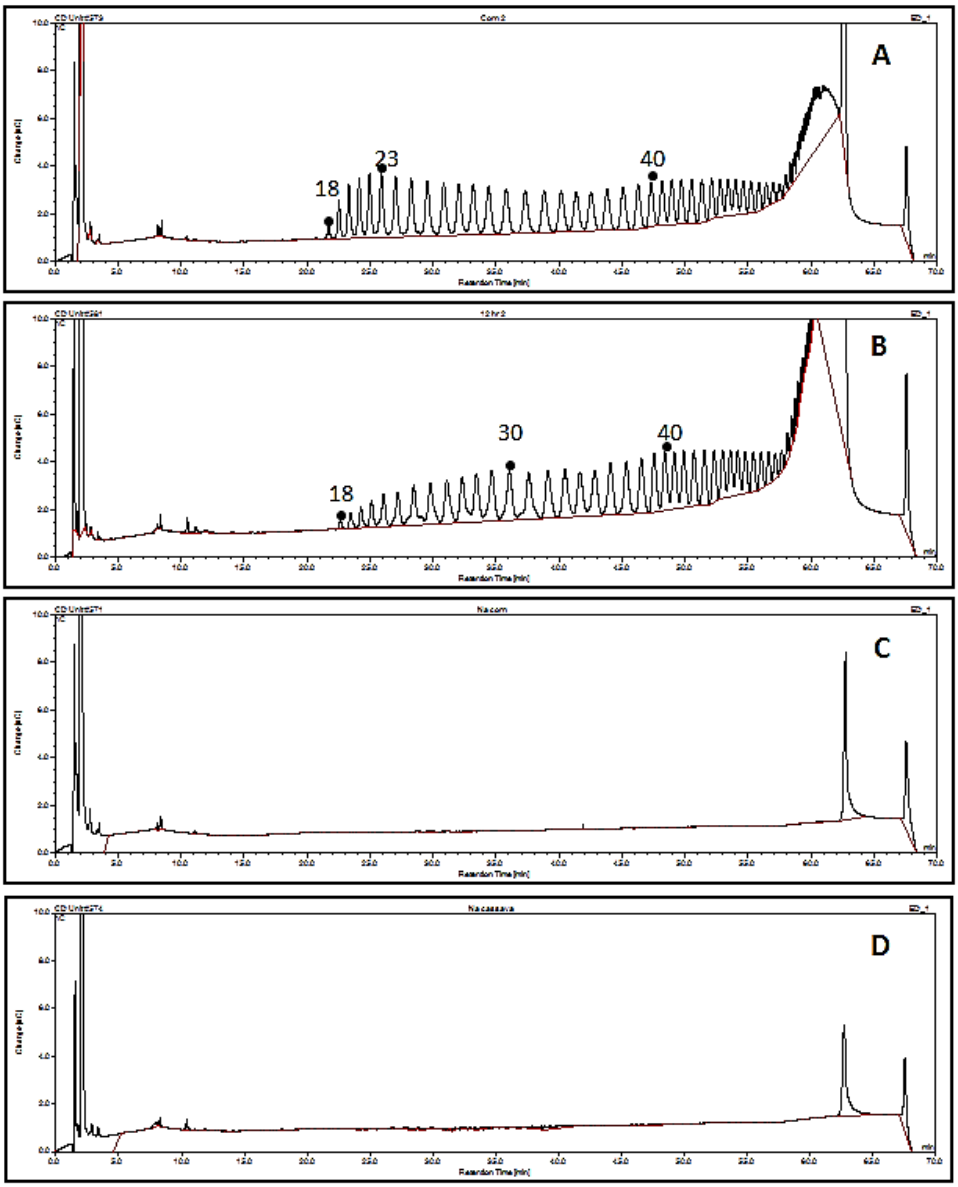

Figure 3.18 Cycloamyloses produced in native cassava starch, modified cassava starch, native corn starch and modified corn starch determined by PA-100 column on HPAEC-PAD: modified corn starch (A), modified cassava starch (B), native corn starch (C) and native cassava starch (D)

CHAPTER IV
DISCUSSION

4.1 Preparation and purification of recombinant MeDPEI

Tantanarat, O'Neill et al. (2014) successfully cloned DPEI gene from *Manihot esculenta* Crantz cultivar KU50 in vector TrcHis2C and transformed into *E.coli* DH5α. The intracellular enzyme was expressed as an intracellular enzyme. The transformant was used to express and collect MeDPEI by inoculated in LB broth starter at 37 °C, induced with IPTG, and the culture was further incubated at 16 °C to prevent formation of inclusion body. Following the method described by Tantanarat, O'Neill et al. (2014), the recombinant MeDPEI enzyme from *Manihot eslucenta* Crantz was expressed as an intracellular enzyme. The crude enzyme was assayed by glucose oxidase reagent and purified by HisTrap affinity column chromatography. Nickel-charged attached to stationary phase of HisTrap affinity column can bind to the imidazole ring of histidine of MeDPEI enzyme, the chromatographic profile of recombinant MeDPEI was shown in Figure 3.1. The purification result was shown in Table 3.1, the enzyme was purified with specific activity of 12.6 U/mg protein, 4.3 folds purification and 25.5 % yield. The molecular weight and enzyme purification were confirmed by SDS-PAGE as shown in Figure 3.2. The purified MeDPEI was used for starch modification.

4.2 Optimization of condition for cassava starch modification

In optimizing the condition for modifying cassava starch with MeDPEI, three parameters were varied: reaction temperatures, incubation time, and enzyme concentrations. Gelatinized 5% cassava starch, the cassava starch concentration which was found to be a suitable preparation used in starch modification (personal

communication), was used. Gelatinization temperature of 90 °C (Swinkels 1985) was in the normal range for cassava starch gelatinization between (64-92 °C) that allowed water to absorb into the starch granule and the starch granule swelling. During heating, starch granule was broken free and water allowed to entering into crystalline structures to swell amylopectin and amylose migrates out of the granule to surrounding water. This process changed the viscosity of starch mixture to become a gel (Suriyakul Na Ayudhaya 2012). After gelatinization process, starch gel was used to determine the optimum conditions. In determining the optimum value of each parameters, the physico-chemical properties of the enzyme-treated starch were monitored.

4.2.1 Determination of optimum temperature

When optimum enzyme concentration had not been determined, 5% cassava starch and 20 units/g starch of MeDPEI were used. Gelatinized starch was incubated with 20 units/g starch of purified MeDPEI at various temperatures: 30, 37 and 45 degree Celsius for 24 hours. The resulting starch was dried and characterized.

From previous studies on cassava starch, the concentration at 5% w/v was normally used. It was the concentration that the starch suspension had the favorable properties for a reaction mixture. Higher concentration of starch may increase the substrate molecules but movement of the molecules in the high starch concentration was more limited due to viscosity.

4.2.1.1 Thermal properties

The enthalpy of amylopectin crystal was measured after gelatinization and stored at 4 °C to follow retrogradation process for 14 days. From the results (Table 3.2). Temperature range (T_c - T_o) indicating complexity of amylopectin of all modified

starch and enthalpy of retrogradation of amylopectin (ΔH_{retro}) were lower than the native cassava starch. The result indicated that the amount of retrograded amylopectin crystal in starch decreased after modification. Oh, Choi et al. (2008) reported that in modification of corn starch with 4-α-glucanotransferase from *Thermotoga maritime,* treated-corn starch showed lower amount of amylopectin which indicated lower retrogradation of amylopectin. OH, Choi et al. (2008) and Suriyakul Na Ayudhaya et al. (2012) also reported that cassava treated with amylomaltase showed lower retrogradation of amylopectin. Starch modified at all temperatures showed higher initial temperature for amylopectin hydrolysis (T_o) and final temperature for amylopectin hydrolysis (T_c) than native starch while the temperature of maximum rate for amylopectin hydrolysis (T_p) were lower. DPEI catalyzes the transfer of maltosyl units from one 1,4-α-D-glucan to an acceptor monosaccharide or oligosaccharide via α-1,4 glycosidic linkages. When MeDPEI was incubated with cassava starch which contains high amount of amylopectins, the α-1,4 glucans in amylopectins were changed causing decrease in the crystalline strength. Thus, the enthalpy of retrogradation of amylopectins decreased and also the variety of crystal structure of starch (Oh *et al.* 2008, Suriyakul Na Ayudhaya 2012). Modified starch at 37 °C showed the best thermal properties due to the lowest variety of amylopectin crystal structure after modification and the lowest enthalpy of retrogradation of amylopectin indicate low amount of amylopectin.

4.2.1.2 Freeze-thaw stability

Freeze-thaw stability was determined by measuring the percentage of water released during the freeze-thaw process or percentages of syneresis. Of all modified cassava starch at 30, 37 and 45 °C, % syneresis significantly increased and more water

was released out of gel than the native cassava starch. Modified starch at 37 °C showed the lowest percentages of syneresis compared to the other modified starch at other temperatures. The disproportionating activity of DPEI transferred maltosyl units to acceptor α-1,4 glucans, amyloses and branches in amylopectins resulting in shorter chains and more suitable sizes of α-1,4 glucans for cycloamylose formation. The remaining starch consisted of shorter amylose chains and shorter branches in amylopectin which consequently led to lower retrogradation (Lee *et al.* 2006). The long amylose chains had higher tendency to retrograde than the short and highly branched amylopectins (Park *et al.* 2007). After heating, amyloses migrate out to surrounding water in thaw process changing the gel structure for water absorption, indicating that the higher the amylose content, led to lower freeze-thaw stability. Thus, in each freeze-thaw cycle, the amylose was gradually removed in the water released resulting in gradual decrease in retrogradation of the starch. Lee, Kim et al. (2006) reported that rice starch modified by 4-α-glucanotransferase showed high freeze-thaw stability which was different from our result. The difference may be due to the conditions in this experiment were not optimum in many parameters such as the optimum enzyme concentration, amylose content could not be reduced enough to have a good freeze-thaw stability.

4.2.1.3 Thermo-reversibility

Thermo-reversibility of gel was determined as the viscoelasticity by Rheometer, and expressed as tan δ values. High tan δ values indicates liquid-like state and low tan δ values indicates solid-like state. The results showed (Figure 3.4) that all modified cassava starch at 30, 37 and 45 °C had high tan δ value in liquid-

like state at temperature 70 °C and low tan δ value in solid-like state at temperature 4 °C and was able to reverse the state when the temperature changed. The change in tan δ value of native cassava starch was less than modified starch. Disproportionating reaction of MeDPEI enzyme, transferring maltosyl units from one 1,4-α-D-glucan to an acceptor monosaccharide or oligosaccharide via α-1,4 glycosidic linkages, can transfer α-1,4 of amylopectin that changes branch length of amylopectin. The short branch length amylopectin had a weaker structure so it is easier to turn liquid-like at high temperature and solid-like at low temperature (Oh *et al.* 2008). The shorter branch length of amylopectin resulted in higher thermo-reversibility as observed in modified starch at 37 °C.

4.2.1.4 Apparent amylose content

The apparent amylose content was measured by the absorption of the amylose-iodine complex at 620 nm. The percentages of apparent amylose content of modified cassava starch at 30, 37 and 45 °C were lower than the native cassava starch. OH, Choi et al. (2008) and Suriyakul Na Ayudhaya et al. (2012) reported similarly. The modified starch at 37 °C had the lowest percentage of apparent amylose content. It was reported that long amylose chain had higher iodine complex absorption values than high branched and short branched amylopectin. A decreased in absorption at A_{620} indicated a decrease in the long chain glucans in content of amylose and the branch of amylopectins in modified starch (Thomas 1999). The amylose-iodine complex appears as blue color when the amylose length is over DP 40, blue-purple color when the amylose length is DP 30-40, purple-red color when the amylose length with DP less than 30, red color when the amylose length is less than DP 20, and yellow color when the amylose length with DP less than 12.

Amylose content with length shorter than DP 47 cannot be detected at 620 nm which is the absorbance for detecting blue color (Kearsley 1995). Thus apparent amylose content of all modified starch value was decreased. Disproportionating reaction of MeDPEI, transfers maltosyl units from one 1,4-α-D-glucan to an acceptor monosaccharide or oligosaccharide via α-1,4 glycosidic linkages, can transfer α-1,4 glucan from amylose, resulting in the increase in shorter amylose chains.

4.2.1.5 Detection of side chain distribution and size of cycloamyloses by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Figure 3.5 showed that side chain distribution with DP 6-25 in all modified cassava starch at 30, 37 and 45 °C decreased in comparison to native cassava starch. OH, Choi et al. (2008) and Suriyakul Na Ayudhaya et al. (2012) reported the presence of oligosaccharide chains with DP 1-5 in similar modification. The action of MeDPEI enzyme which transferred α-1,4 glucan between chains resulting in changes in branch length of amylopectins. MeDPEI can catalyze cyclization reaction of amylose to produce cycloamyloses with DP 18-53 (Tantanarat *et al.* 2014). Longer chains of α-1,4 glucans produced by disproportionating reaction of MeDPEI which reached suitable size to act as substrate for intramolecular transfer reaction or cyclization reaction of MeDPEI would be used up to produce cycloamyloses. Consequently, the chains on amylopectins or soluble oligosaccharides detected in the modified starch were less than the native starch. Detection of cycloamyloses by PA-100 on HPAEC showed the presence of cycloamyloses DP 18-53 in modified starch performed at 30, 37 and 45 °C with similar profiles at 30 and 37 °C.

From all results, the optimum temperature for starch modification was 37 °C.

4.3 Optimum enzyme concentration for cassava starch modification

Gelatinized 5% cassava starch was incubated at 37 °C, the optimum temperature determined in section 3.2.1, and added with varying amount of MeDPEI (10, 20 and 30 units/g starch) for 24 hours. Characterization of all parameters performed on starch modified at various temperatures were also performed on starch modification with different MeDPEI concentration, using the optimum temperature of 37 °C as determined in section 3.2.1. It was concluded that at 37 °C, the most effective MeDPEI concentration which produced modified starch with suitable thermal properties, thermo-reversibility, freeze-thaw stability was 30 units/g starch of enzyme. At this amount of enzyme, the apparent amylose content was lowest (Figures 3.7-3.8 and Tables 3.4-3.5), the side chain distribution decrease in quantity and cycloamyloses production increase (Figure 3.9, 3.10). Thus, next experiments on optimization were performed at 37 °C and 30 units/g starch MeDPEI.

4.4 Optimum incubation time for cassava starch modification

In optimization experiments to determine the optimum temperature and optimum enzyme concentration, incubation time was set at 24 hours and 5% starch concentration. To determine the suitable incubation time, similar experiments as 3.2.1 and 3.2.2 were performed with gelatinized 5% cassava starch, 37 °C and 30 units/g starch MeDPEI at various time intervals, 6, 12 and 24 hours. From characterization results of the resulting modified starch (Figures 3.11-3.14, Tables 3.6-3.7), optimum incubation time was set at 12 hours.

4.5 Comparison of modified starch from cassava and corn starch

After optimized condition for modification of starch were determined to be 5% gelatinized starch, 30 units/g starch MeDPEI, 37 °C and 12 hours incubation, the

conditions were applied to modify 5% corn starch. The comparative data (Table 4.1) indicated that both modified starch showed the characteristics suitable for applications in industries such as food and cosmetics. Although the cassava starch obtained in experiment 3.2.1 showed inferior characteristics of modified starch in term of % syneresis, the starch modified at all determined optimum conditions showed favorable % syneresis. Corn starch had lower values of all parameters compared to cassava starch. Corn starch is composed of more amyloses than amylopectins (Table 1.2), therefore, amylose content decreased upon treatment with MeDPEI. In consequence, all parameters representing the lower amylose content were observed. In conclusion, cassava starch treated with recombinant cassava DPEI yielded starch with properties suitable for applications in frozen food industries such as low retrogradation, high freeze-thaw stability, high thermo-reversibility or low % syneresis, in comparison with native cassava starch. In addition, the production of large ring cycloamyloses in the modification process introduced alternative source for cycloamyloses which may be useful in cosmetic industries.

Table 4.1 Comparative data of optimum conditions for modified cassava and corn starch

Source	Native cassava	Modified cassava	Native corn	Modified corn
Thermal properties	4.53 mJ of retrogradation value	1.99 mJ of retrogradation value	2.47 mJ of retrogradation value	1.44 mJ of retrogradation value
Freeze-thaw stability	55% Syneresis at the last cycle	30% Syneresis at the last cycle	65% Syneresis at the last cycle	35% Syneresis at the last cycle
Apparent amylose content	35.0±0.46% Apparent amylose content	33.0±0.06% Apparent amylose content 5.89% decreased in apparent amylose	39.0±0.46% Apparent amylose content	37.0±0.28% Apparent amylose content 5.13% decreased in apparent amylose
Thermo-reversibility	Non-thermo-reversibility	Thermo-reversibility	Non-thermo-reversibility	Thermo-reversibility
Side chains distribution by HPAEC-PA1	Low short chain-length of amylopectin	Decreased branched-chains distribution and increased short chain-length of amylopectin	Low short chain-length of amylopectin	Decreased branched-chains distribution and increased short chain-length of amylopectin
Cycloamyloses by HPAEC-PA100	No products	Cycloamyloses with DP 18-57 were produced	No products	Cycloamyloses with DP 18-55 were produced

CHAPTER V
CONCLUSIONS

1. Recombinant MeDPEI enzyme from cassava *Manihot esculenta* Crantz was purified, with specific activity of 12.6 U/mg protein , 4.3 folds and 25.5 % yield. The recombinant enzyme was used to modify cassava and corn starch.
2. Optimum conditions were established to yield modified cassava starch with best characteristics of retrogradation, thermo-reversibility, freeze-thaw stability and low apparent amylose content.
3. The optimum condition for cassava starch modification were: 5% cassava starch (w/v), 30 units/g starch of DPEI, at 37 °C and 12 hours incubation time.
4. The modified cassava starch at the determined modification condition showed low retrogradation, high freeze-thaw stability, low apparent amylose content, high thermo-reversibility and high cycloamyloses product.
5. Modified corn starch at the same optimum modification condition as cassava starch also showed low retrogradation, high freeze-thaw stability, low apparent amylose content, high thermo-reversibility and cycloamyloses products.

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APPENDIX

Appendix A

Stock solution for SDS-PAGE

1. 2.0 M Tris-HCl, pH 8.0

Tris (hydroxymethyl)-aminomethane	9.1 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 50 ml by distilled water

2. 1.0 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane	6.0 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 50 ml by distilled water

3. 10% (w/v) SDS

Sodium dodecyl sulfate	5.0 g

Adjusted volume to 50 ml by distilled water

4. 10% (w/v) ammonium persulfate

Ammonium persulfate	1.0 g

Adjusted volume to 10 ml by distilled water

5. 50% (v/v) glycerol

Glycerol	5.0 ml

Added 5.0 ml distilled water

6. Solution A

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

7. Solution B

1.0 M Tris-HCl, pH 6.8	25 ml
10% (w/v) SDS	2 ml
Distilled water	23 ml

Appendix B

Working solution for SDS-PAGE

7.5% separating gel

40% acrylamide	1.41.ml
Solution A	2.50 ml
Distilled water	3.48 ml
10% (w/v) ammonium persulfate	60 µl
TEMED	6 µl

5.0% stacking gel

40% acrylamide	0.32 ml
Solution B	0.50 ml
Distilled water	1.652 ml
10% (w/v) ammonium persulfate	25 µl
TEMED	3 µl

Sample buffer

1.0 M Tris-HCl, pH 6.8	0.6 ml
50% (v/v) glycerol	5.0 ml
10% (w/v) SDS	2.0 ml
2-mercaptoethanol	0.5 ml
1% (w/v) bromophenol blue	1.0 ml
Distilled water	0.9 ml

Sample was mixed with sample buffer (4:1) and heated for 5 minutes before loading to the gel

Electrophoresis buffer

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
Sodium dodecyl sulfate	1 g

Adjusted volume to 1 liter by distilled water

Coomassie Gel Stain

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

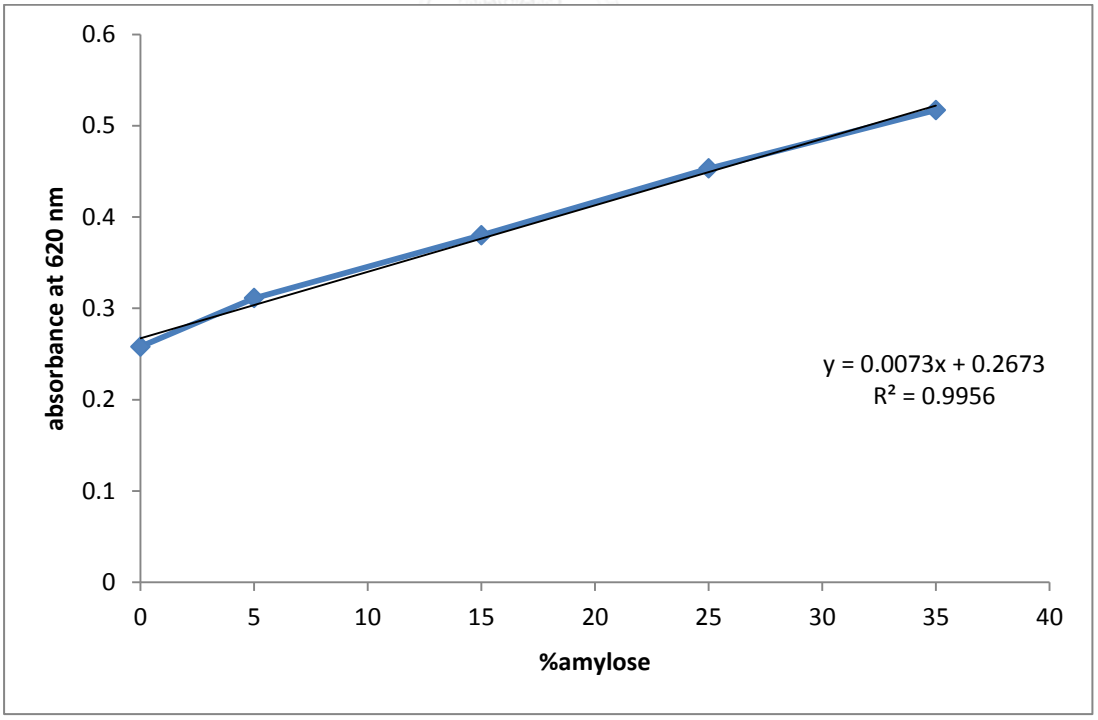
Coomassie Gel Destain

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

Appendix C

Standard amylose for apparent amylose content method

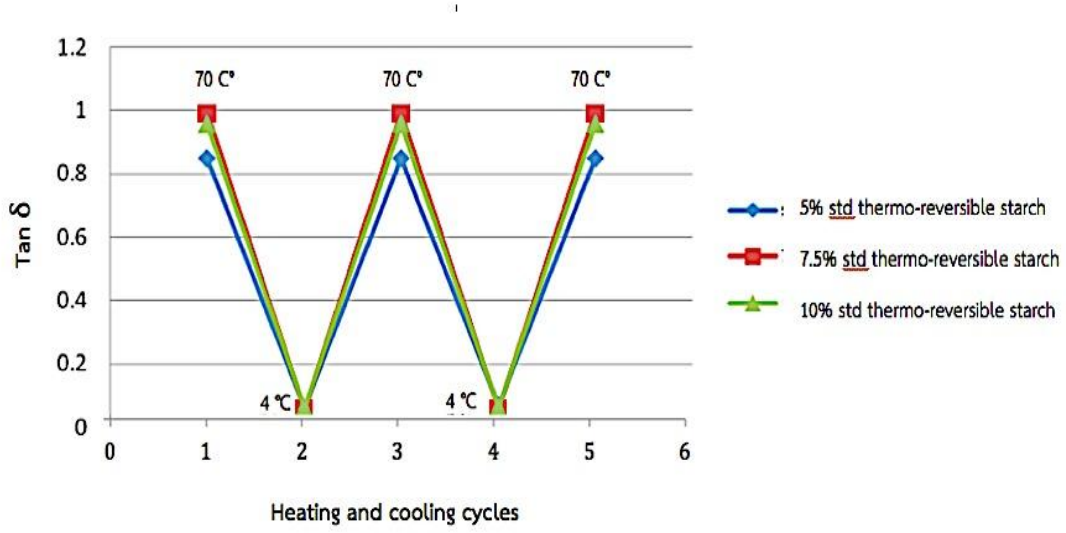
standard	amylose		amylopectin	
	%	mg	%	mg
1	0	0	100	100
2	5	5	95	95
3	15	15	85	85
4	25	25	75	75
5	35	35	65	65



Appendix D

Standard thermo-reversible starch for thermo-reversibility method

Standard thermo-reversible starch from Department of Microbiology and Bioprocess Technology, Institute of Biochemistry, University of Leipzig, Leipzig, Germany.



Appendix E

Working solution for HPAEC column PA1

Working solution

150 mM Sodium hydroxide

Sodium hydroxide	11.88 ml

Adjusted volume to 1.5 liter by ultrapure water

600 mM sodium acetate in 150 mM Sodium hydroxide

Sodium acetate	73.8 g
Sodium hydroxide	11.88 ml

Adjusted volume to 1.5 liter by ultrapure water

500 mM Sodium hydroxide

Sodium hydroxide	26.40 ml

Adjusted volume to 1 liter by ultrapure water

Linear gradient of sodium acetate for HPAEC-PAD column PA1

Sodium acetate (%)	Time (min)
0 - 20	0 - 5
20 - 45	6 - 30
45 - 55	31 - 60
56 - 60	61 - 80
61 - 65	81 - 90
66 - 80	91 - 95
81 - 100	96 - 100

Appendix F

Working solution for HPAEC column PA100

Working solution

150 mM Sodium hydroxide

Sodium hydroxide	11.88 ml

Adjusted volume to 1.5 liter by ultrapure water

200 mM sodium nitrate in 150 mM Sodium hydroxide

Sodium nitrate	25.80 g
Sodium hydroxide	11.88 ml

Adjusted volume to 1.5 liter by ultrapure water

1 M sodium nitrate

Sodium nitrate	42.50 g

Adjusted volume to 1 liter by ultrapure water

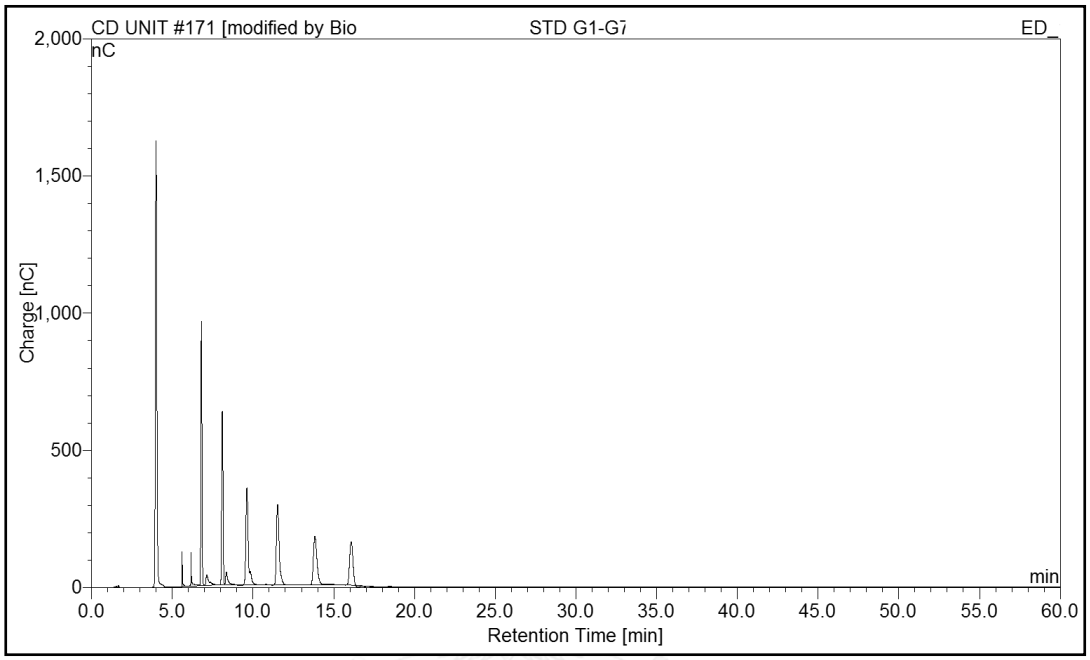
Linear gradient of sodium nitrate for HPAEC-PAD column PA100

Sodium nitrate (%)	Time (min)
4 - 8	0 - 2
8 - 18	3 - 11
18 - 28	12 - 22
28 - 35	23 - 43
35 - 45	44 - 59
45 - 63	60 - 65

Appendix G

Standard for HPAEC column PA1

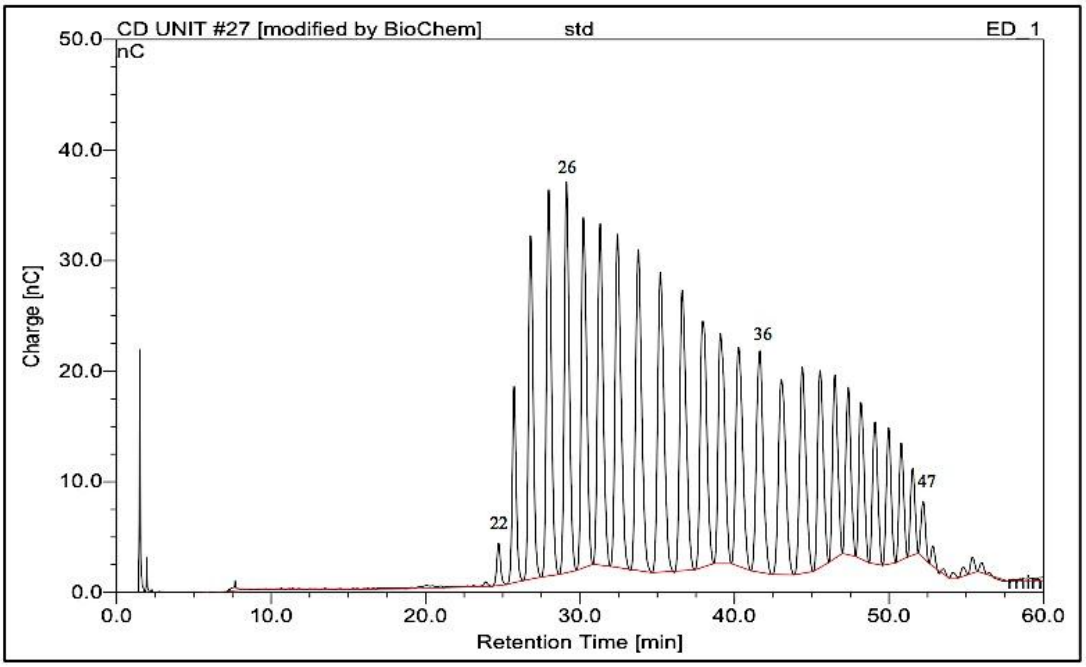
Standard glucose G1-G7



Appendix H

Standard for HPAEC column PA100

Standard large ring cyclodextrins (LR-CDs, CD22-CD48) from Amylomaltase from *Thermus aquaticus* ATCC 33923.



VITA

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