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IDENTIFICATION OF IMPORTANT AMINO ACID RESIDUES OF
AMYLOMALTASES THROUGH CHEMICAL MODIFICATION TECHNIQUE

Miss Wanitcha Rachadech

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry
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Thesis Title   IDENTIFICATION OF IMPORTANT AMINO ACID RESIDUES OF AMYLOMALTASES THROUGH CHEMICAL MODIFICATION TECHNIQUE

By   Miss Wanitcha Rachadech

Field of Study   Biochemistry

Thesis Advisor   Professor Piamsook Pongsawasdi, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…………………………………………….Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

……………………………………………….Chairman

(Professor Anchalee Tassanakajon, Ph.D.)

……………………………………………Thesis Advisor

(Professor Piamsook Pongsawasdi, Ph.D.)

………………………………………….Examiner

(Associate Professor Tipaporn Limpaseni, Ph.D.)

………………………………………….Examiner

(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

………………………………………….External Examiner

(Associate Professor Jarunee Kaulpiboon, Ph.D.)

วณิชชา ราชาเดช : การระบุกรดอะมิโนสำคัญของแอมิโลมอลเทสด้วยเทคนิคการดัดแปรทางเคมี.
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ศ.ดร. เปี่ยมสุข พงษ์สวัสดิ์, 133 หน้า.

งานวิจัยนี้มีวัตถุประสงค์ในการเปรียบเทียบและบ่งชี้กรดอะมิโนที่สำคัญของเอนไซม์แอมิโลมอลเทส
จากแบคทีเรีย 2 ชนิด (CGAM จากเชื้อ *Corynebacterium glutamicum* และ THAM จากยีน AM ที่คัดเลือกจาก
ดีเอ็นเอในดิน) และจากพืชหนึ่งชนิดคือ MeDPE1 จากหัวมันสำปะหลัง *Manihot esculenta* Crantz ด้วยวิธีการ
ดัดแปรทางเคมี โดยบ่มเอนไซม์บริสุทธิ์กับสารเคมีที่จำเพาะกับหมู่ฟังก์ชันต่างๆ พบว่าแอมิโลมอลเทสทุกชนิด
ถูกยับยั้งแอกทิวิตีเกือบทั้งหมดเมื่อดัดแปรด้วยสาร *N*-bromosuccinamide (NBS) แถบแอกทิวิตีจากพอลิอะคริลา
ไมด์เจลอิเล็กโทรโฟรีซิสในระบบไม่ทำให้โปรตีนเสียสภาพหายไปหลังจากดัดแปรด้วย NBS หรือ Succinic
anhydride (SAH) ในขณะที่การเคลื่อนที่ของแถบแอกทิวิตีช้าลงเล็กน้อยหลังจากดัดแปรด้วย 1-ethyl-3-(3-
dimethylaminopropyl)-carbodiimide (EDC) จากผลการทดลองแสดงว่า กรดอะมิโนทริปโทเฟน แอสพาร์ทิก/
กลูทามิก และไลซีน มีความสำคัญต่อแอกทิวิตีของเอนไซม์ทั้งสาม เมื่อทำการป้องกันบริเวณเร่งด้วยซับสเตรท
มอลโทไทรโอส (G3) ได้ผลที่ทำให้สันนิษฐานว่ากรดอะมิโนเหล่านี้มีตำแหน่งอยู่หรือใกล้เคียงบริเวณเร่งของ
เอนไซม์ ยกเว้นไลซีนจาก MeDPE1 จากนั้นศึกษาอิทธิพลของการดัดแปรกรดอะมิโนเหล่านั้นด้วยสารเคมีต่อ
สมบัติของเอนไซม์ โดยทำการยับยั้งเอนไซม์ด้วยสารเคมีดังกล่าวที่ความเข้มข้น IC$_{50,}$ pH 6.0 จากค่าคงที่
จลนพลศาสตร์ของปฏิกิริยาการยับยั้งเสมือนอันดับหนึ่ง พบว่า MeDPE1 มีความว่องไวมากที่สุดต่อสารเคมีทุก
ชนิดยกเว้น NBS ในขณะที่ THAM มีความว่องไวน้อยที่สุด ค่า pI ของเอนไซม์ดั้งเดิมและเอนไซม์ดัดแปรด้วย
NBS ของ CGAM และ THAM มีค่าเหมือนกันที่ 6.9 และ 5.8 ตามลำดับ เอนไซม์ดั้งเดิมและเอนไซม์ดัดแปรทุก
ชนิดชอบซับสเตรท G3 ที่สุดเมื่อทำปฏิกิริยา disproportionation แต่ชอบกลูโคสเมื่อทำปฏิกิริยา starch
transglucosylation จากการศึกษาจลนพลศาสตร์พบว่า k_{cat}/K_m ของ CGAM ดัดแปรมีค่าลดลงในทั้งสองปฏิกิริยา
ส่วนค่า K_i ต่อตัวยับยั้งอะคาร์โบสของเอนไซม์ดัดแปรมีค่าเพิ่มขึ้น เมื่อหาจำนวนของทริปโทเฟนที่ถูกดัดแปรใน
บริเวณเร่ง พบว่ามี 1 และ 3 ตัวใน CGAM และ THAM ตามลำดับ จากการศึกษาโครงสร้างทุติยภูมิของ CGAM
และสภาวะแวดล้อมของทริปโทเฟนพบว่าเปลี่ยนแปลงเล็กน้อยหลังถูกดัดแปรด้วย NBS เมื่อตรวจสอบผลิตภัณฑ์
ออลิโกแซ็กคาไรด์สายตรงและไซโคลเดกซ์ทรินวงใหญ่ พบว่าไม่มีผลกระทบจากการดัดแปรทริปโทเฟน
ผลิตภัณฑ์หลักไซโคลเดกซ์ทรินวงใหญ่ของเอนไซม์ดั้งเดิมและเอนไซม์ CGAM ดัดแปร อยู่ในช่วง CD24-CD32
โดยมี CD27-CD28 สูงที่สุด ในขณะที่ THAM ให้ผลิตภัณฑ์ในช่วง CD22-CD28 และมีปริมาณของ CD23-CD24
สูงที่สุด

ภาควิชา.............ชีวเคมี.......................ลายมือชื่อนิสิต...
สาขาวิชา.........ชีวเคมี.......................ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.......................
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    PROF. PIAMSOOK PONGSAWASDI, Ph.D., 133 pp.

The aim of this study is to compare and identify important amino acid residues in two bacterial amylomaltases (AMs): CGAM from *Corynebacterium glutamicum* and THAM from AM gene screened from soil DNA, and a plant MeDPE1 from cassava *Manihot esculenta* Crantz tuber using chemical modification method. The purified AMs were treated with various modifying reagents specific for different functional groups. For all AMs, almost total activity loss was observed with *N*-bromosuccinamide (NBS) modification. The activity band on native-PAGE of CGAM was disappeared after treatment with NBS or Succinic anhydride (SAH), while a small decrease in migration was observed after modification by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). These results suggest the importance of Trp, Asp/Glu and Lys for AMs activity. Substrate protection using maltotriose (G3) suggests that these residues are at or around active site region of these AMs, except for Lys in MeDPE1. Then, the effect of modification of these residues on AMs' properties was analyzed. The inactivation of AMs by these reagents was performed at IC_{50} concentrations at pH 6.0. From the pseudo-first order kinetic constant of the inactivation reaction, MeDPE1 was most sensitive to all reagents except for NBS while THAM was the least sensitive. pI of native and NBS-modified AMs were similar with 6.9 and 5.8 for CGAM and THAM, respectively. Native and NBS-modified of all AMs prefer G3 in disproportionation but G1 in starch transglucosylation reaction. The k_{cat}/K_m values of the modified CGAM were significantly decreased in both reactions. The K_i values of acarbose inhibitor for the modified enzyme were increased. The number of modified Trp in the active site was about one and three residues for CGAM and THAM, respectively. The secondary structure of CGAM and the Trp environment showed a slight change after NBS treatment. The product pattern of linear oligosaccharides and LR-CDs was not much affected by Trp modification. The major LR-CDs from native and modified CGAM was CD24-CD32 with CD27-CD28 as maximum while THAM yielded CD22-CD28 with CD23-CD24 maximum product.

Department: ……Biochemistry…….….…Student's Signature ……….….…………..

Field of Study: ....Biochemistry…………....Advisor's Signature ……………..……...

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LIST OF ABBREVIATIONS

AM	amylomaltase
BSA	bovine serum albumin
°C	degree Celsius
CDs	cyclodextrins
Da	Dalton
D-enzyme	Disproportionation enzyme
DP	degree of polymerization
g	gram
4αGTase	4-α-glucanotransferase
h	hour
l	litre
μl	microliter
M	molar
mA	milliampere
min	minute
mg	milligram
ml	millilitre
mM	millimolar
MW	molecular weight
n.d.	not detectable
rpm	revolution per minute
μg	microgram
U	unit

CHAPTER I

INTRODUCTION

1.1 Starch

Starch is a polymer of α-1, 4-D-glucans which occurs widely in nature. This polymer serves the function as carbon store of plants when glucose is plenty. Plant can utilize starch which consists of two types of molecules: the linear- helical amylose and the branched amylopectin. The dominant industrial sources of starch are potato, wheat, maize, rice and tapioca, which are economically important crops. In the last century, the emergence of a large-scale starch industrial processing to produce various saccharides of beneficial use was occurred. The acid hydrolysis of starch has been shifted to the use of starch-degrading enzymes to produce maltodextrin, modified starch and glucose in the past decades. Many organisms could produce extracellular or intracellular enzymes which are able to convert glycogen or starch into carbon sources as energy for the cells (Figure 1.1). Besides the use in starch industry, starch-converting enzymes are also used in several other industrial applications, such as laundry and dish detergents or as anti-staling in baking industry (Maarel *et al*., 2002).

1.2 Starch-degrading enzymes

Starch-degrading enzymes can be divided into 4 groups: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases (Maarel *et al*., 2002). The first three groups are hydrolases. Endoamylase such as α-amylase (EC 3.2.1.1), is able to randomly hydrolyze the α-1,4-glycosidic bonds in the inner part (endo-action) of starch chain (amylose or amylopectin) to produce various length of oligosaccharide
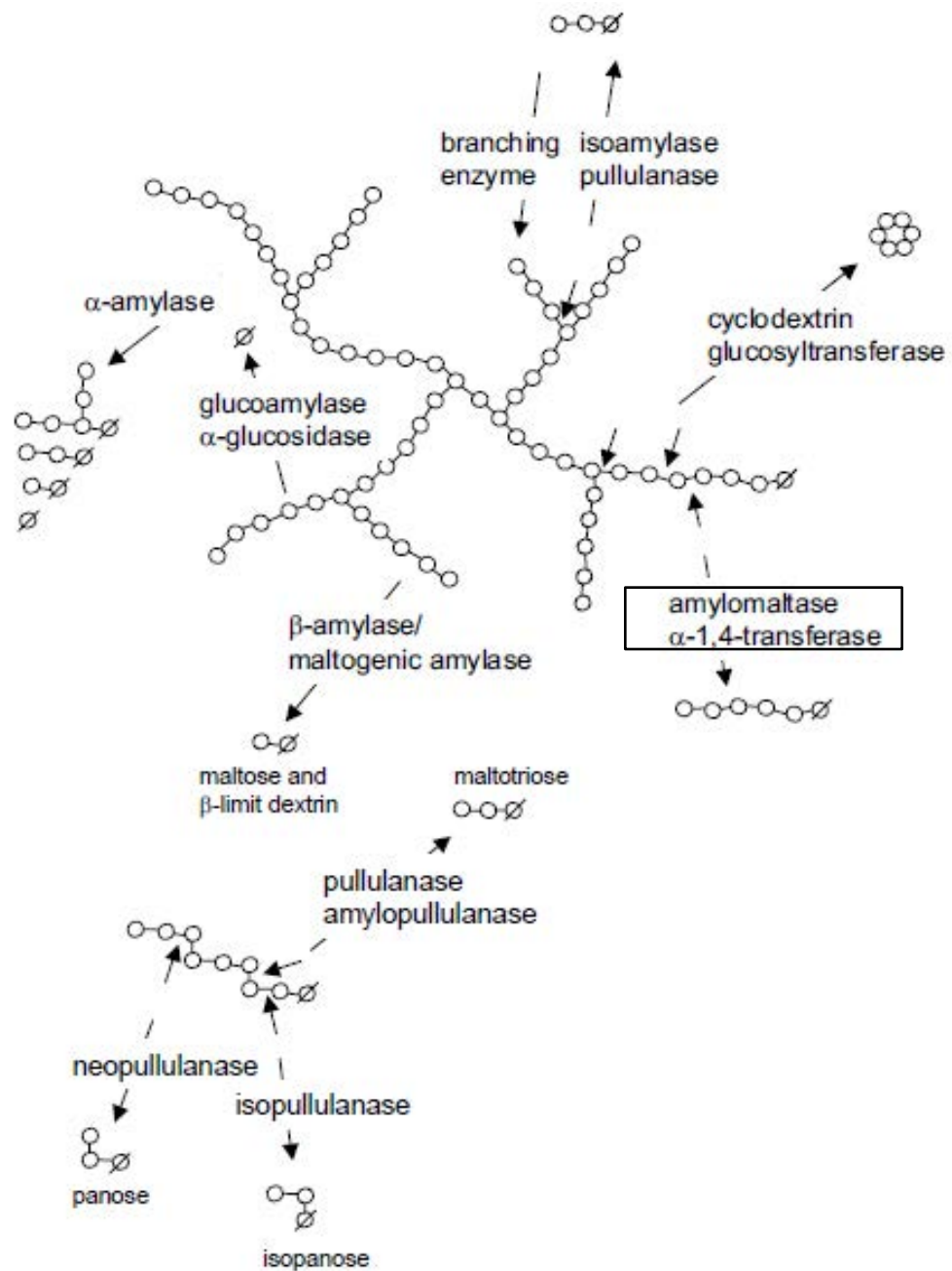
Figure 1.1 Different actions of starch-degrading enzymes. Glucose molecules are indicated as circle while reducing ends are marked by a line through the circle (Turner *et al*., 2007).

as products. The second group is exoamylase which hydrolyzes glycosidic bond from the non-reducing end residues (exo-action) of starch chain to produce glucose, maltose or β-limit dextrin. The example of this group is β-amylase (EC 3.2.1.2), which is able to clave α-1,4-glycosidic bonds to produce maltose while glucoamylase or amyloglucosidase (EC 3.2.1.3) hydrolyzes both of α-1,4 and α-1,6 glycosidic bonds to produce glucose. Debranching enzyme is the third group that can exclusively hydrolyze α-1,6-glycosidic bonds by exo-action, the examples are isoamylase (EC 3.2.1.68) (Abe *et al*., 1999) and pullulanase type I (EC 3.2.1.41) (Ben Messaoud *et al*., 2002), which produce linear oligo/polysaccharides. The transferase group acts by breaking an α-1, 4-link and transfers the resulting glucan moiety to an acceptor molecule through formation of a new α-1,4- link (Takaha and Smith, 1999), the main examples are amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19). These enzymes mainly show transglycosylation reaction to produce cyclic oligosaccharide with 6, 7, 8 unit of glucose or higher, while their hydrolytic activity are very low (Maarel *et al*., 2002). The α-amylase family has the following characteristics: (i) act on α-glycosidic bonds to produce α-anomeric mono- or oligosaccharides; (ii) hold a TIM or $(\beta/\alpha)_8$ barrel structure; (iii) show four conserved regions in the sequence that encode the important amino acids that form the catalytic site; (iv) and display 1 Glu and 2 Asp at the catalytic site (Table 1.1) (Maarel *et al*., 2002; Takashi *et al*., 2006; Takata *et al*., 1992).

1.3 The 4-α-glucanotransferase (4αGTase) group

4-α-glucanotransferase (EC 2.4.1.25) is a group of enzymes catalyzing the reaction that transfers a segment of a 1,4-α-D-glucan to an acceptor molecule, which

Table 1.1 The four conserved regions and the corresponding β-sheets found in the

amino acid sequence of α-amylase family enzymes. (Maarel *et al.*, 2002)

	I β2	II β4	III β5	IV β7
Amylomaltase	EALGIRIIGDMPIFVAED	LFHLVRIDHFRG	VPVLAEDLGVI	VVYTGTHDNDT
Amylosucrase	HEAGISAVVDFIFNHTSN	GVDILRMDAVAF	VFFKSEAIVHP	VNYVRSHDDIG
CGTase	HAKNIKVIIDFAPNHTSP	GIDGIRMDAVKH	VFTFGEWFLGV	VTFIDNHDMER
CMDase	HDNGIKVIFDAVFNHCGY	DIDGWRLDVANE	AIIVGEVWHDA	FNLIGSHDTER
BE	HQAGIGVILDWVPGHFCK	HVDGFRVDAVAN	ILMIAEDSTDW	FILPFSHDEVV
Isoamylase	HNAGIKVYMDVVYNHTAE	GVDGFRFDLASV	LDLFAEPWAIG	INFIDVHDGMT
M.amylase	HQKAIRVMLDAVFNHSGY	DIDGWRLDVANE	AYILGEIWHDA	FNLLGSHDTPR
Pullulanase	HAHGVRVILDGVFNHTGR	GVDGWRLDVPNE	AYIVGEIWEEA	MNLLTSHDTPR
Sucrose Pase	LGECSHLMFDFVCNHMSA	GAEYVRLDAVGF	TVIITETNVPH	FNFLASHDGIG
BLamylase	HERGMYLMVDVVANHMGY	SIDGLRIDTVKH	VYCIGEVLDGD	GTFVENHDNPR

Highlighted alphabets represent the conserved catalytic amino acid residues. β2, β4,

β5, and β7 indicate the β-sheet region. The following enzymes were used for the

alignment: amylomaltase of *Thermus aquaticus*; amylosucrase of *Neisseria*

polysaccharea; CGTase: cyclodextrin glycosyltransferase of *Bacillus circulans* 251;

CMDase: cyclomaltodextrinase of *Clostridium thermohydrosulfuricim* 39E; BE:

branching enzyme of *Bacillus stearothermophilus*; isoamylase of *Pseudomonas*

amyloderamosa ; M. amylase: maltogenic α-amylase of *Bacillus stearothermophilus*;

pullulanase of *Bacillus flavocaldarius* KP 1228; Sucrose Pase: sucrose phosphorylase

of *Escherichia coli* K12; BLamylase: α-amylase of *Bacillus licheniformis*

may be glucose or a 1,4-α-D-glucan, resulting in formation of a new α-1,4-link. There are three types of 4αGTase; CGTase (Type I), Disproportionating enzyme (D-enzyme) or amylomaltase (Type II) and Glycogen debranching enzyme (GDE) (amylo-1,6-glucosidase/ 4-α-glucanotransferase) (EC 3.2.1.33 + EC 2.4.1.25) (Type III), respectively (Takaha and Smith, 1999). The summarized action of 4αGTase is as shown in Figure 1.2. A unique cyclization reaction, an intramolecular transglucosylation, converts linear oligosaccharide to form cyclic glucan, small-ring and large-ring cyclodextrins for CGTase and amylomaltase, respectively. The three other reactions are intermolecular transglucosylation reaction. Coupling is the reversible reaction of cyclization whereby a linear short-chain oligosaccharide acts as an acceptor. Disproportionation reaction transfers glucosyl units from donor substrate to an acceptor, glucose or glucan molecule to form linear oligosaccharides of different size. Hydrolysis involves the transfer of glucosyl group to water molecule, the activity which is usually low for this group of enzyme especially the amylomaltase (Takaha and Smith, 1999).

1.4 Application of Amylomaltase and LR-CDs

Amylomaltases have been used in many applications. The first application of this enzyme is for production of LR-CDs with a degree of polymerization (DP) at least 17 (DP ≥ 17) (Takaha and Smith, 1999; Terada *et al.*, 1999). The prominent point of LR-CDs is their high water solubility, they can form complexes with organic (Takaha and Smith, 1999) or inorganic molecules (Endo and Ueda, 2004; Kitamura *et al.*, 1999), which have potential to be applied in several applications such as food science, pharmaceuticals and biotechnology (Singh *et al.*, 2002). LR-CD (CD9) was
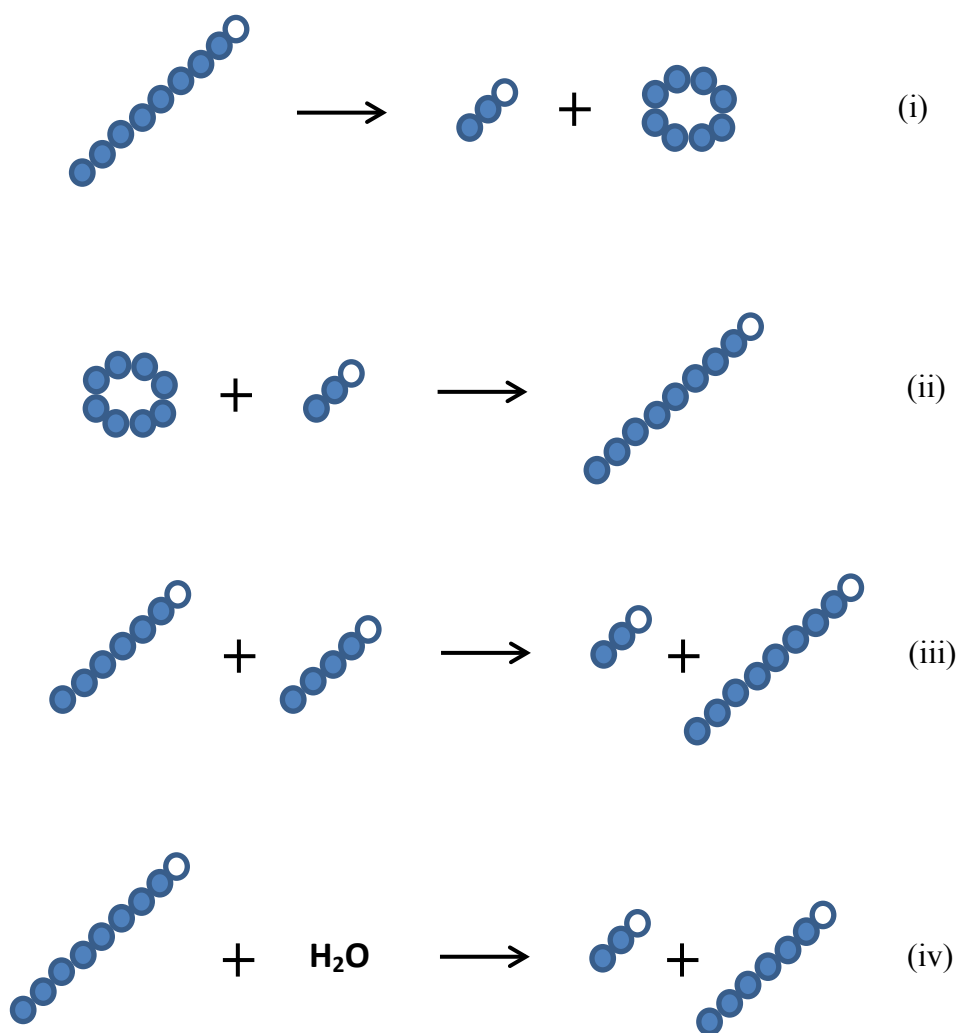
Figure 1.2 The summarized action of 4αGTase groups; (i) Cyclization reaction, (ii) Coupling reaction, (iii) Disproportionation reaction and (iv) Hydrolysis reaction. Glucose molecules are indicated as dark circle while reducing ends are marked by white circle (modified from Takaha and Smith, 1999).

used to form complexes with guest molecules such as digitoxin and spironolactone, the obtained complexes were shown to be more soluble than the corresponding α-cyclodextrin complexes (Ueda *et al.*, 2002). Furuishi and co-workers studied the complexation of C70 Buckminsterfullerene with CD9 (Furuishi *et al.*, 2008). The CD21-CD31 was used in the complex formation with triiodide, and the properties of the complex was investigated by isothermal titration calorimetry (Kitamura *et al.*, 1999). Furthermore, Takaha and Smith (1999) reported that the LR-CDs with a degree of polymerization larger than 50, showed the ability to form complexes with butanol, octanol and oleic acid. In addition, LR-CD was used as an artificial chaperone for protein refolding (Kaper *et al.*, 2004) by acting as the aggregation inhibitor to improve the protein folding problem stems from the proteins overproduced that are commonly obtained as inclusion bodies (Haase-Pettingell and King, 1988). LR-CDs (DP 22 to DP 45 or larger than DP50) as the ingredient of the commercial 'Protein refolding kit' have the ability to strip a detergent molecule from protein-detergent complex and then assist the protein refolding to native conformation or active state (Machida *et al.*, 2000).

The second application of AM is its use in the synthesis of a prebiotic isomalto-oligosaccharides (IMOs) that can be applied as a substitute sugar for diabetics, and to be consumed as neutraceuticals for health benefits such as to improve the internal microflora, or to reduce risk of cancer (Kaper *et al.*, 2004). The synthesis of an anticariogenic maltooligosylsucrose by AM has been recently reported (Saehu *et al.*, 2013). This oligosaccharide can be applied as sucrose substitute, cannot be utilized by *Streptococcus mutans*, a flora known to cause dental caries in humans and experimental animals.

The third application of AM is for the production of a thermoreversible starch gel that can be applied as a substitute for gelatin or agar in foods. The product could be dissolved in water, become 'sol' after heating and form a firm gel after cooling. The action of AMs on starch was as shown in Figure 1.3, starch transglucosylation activity will transfer short chain of glucosyl group to yield shortened or elongated starch side chain (Kaper *et al*., 2004).

Large-ring CDs (LR-CDs) are cyclic oligosaccharides which compose of more than 9 D-glucose units. Interestingly, LR-CDs are produced with different degrees of polymerization (DP) depending on the source of enzyme (Figure 1.4). AM from *E. coli* or *T. aquaticus*, D-enzyme from potato, GDE from *Saccharomyces cerevisiae* and CGTase can produce LR-CDs which have the minimum DP of 17, 22, 17, 11 and 9, respectively (Taira *et al*., 2006; Takaha and Smith, 1999). Ueda *et al*., reported that LR-CDs can be produced in the initial stage of CGTase cyclization reaction, after that it is converted into smaller CDs: CD6 or α-CD, CD7 or β-CD, and CD8 or γ-CD (Terada *et al*., 1997). CD10 to CD21 have been separated and purified from LR-CD mixture produced by the initial action of CGTase (Ueda *et al*., 2002). And the larger ones from CD22 to CD39 have also been purified from LR-CD mixture of *Thermus aquaticus* ATCC 33923 (Terada *et al*., 1999). The physicochemical properties of LR-CDs in comparison to α-, β-, γ- CDs have been reported (Table 1.2) (Taira *et al*., 2006). LR-CDs, especially CD11 onwards, showed significantly higher aqueous solubility than small-ring CDs, with the exception of CD14 and CD26 which do not display any surface activity. There are no noted difference in the rotation or the acid-catalyzed hydrolysis rate between CD36~CD39 but which is a slightly faster than other LR-CDs (CD9~CD35).
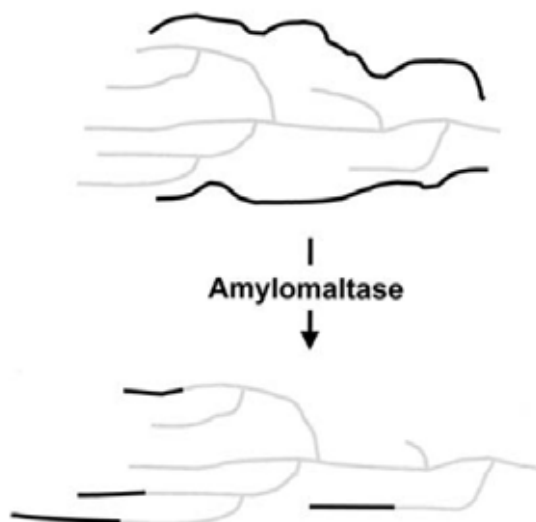
Figure 1.3 Action of AM on starch (starch transglucosylation activity) in the explanation of forming a thermoreversible starch gel. The black and gray lines represent amylose and amylopectin, respectively. Some side chains of the final products have been shortened while others have been elongated (Kaper *et al*., 2004).
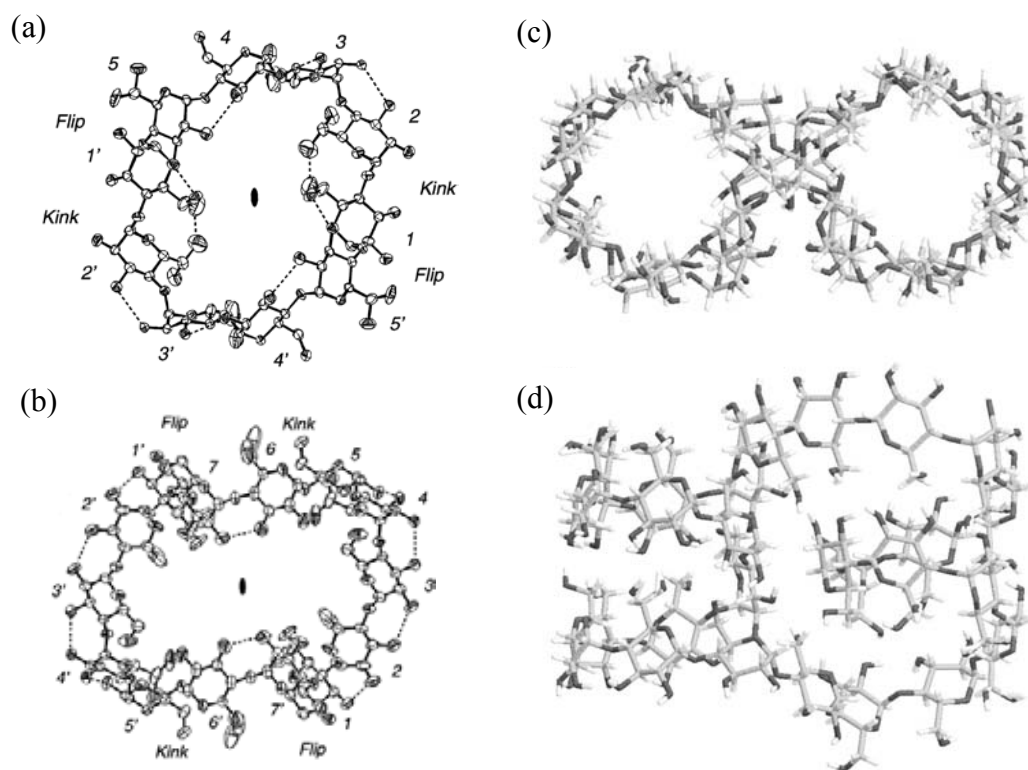
Figure 1.4 The molecular structures of large-ring cyclodextrins (LR-CDs) with degree polymerization of 10 (CD10) (a); 14 (CD14) (b) (Jacob *et al.*, 1999) or of 26 (CD26) with top (c) and side (d) views (Jung *et al.*, 2011). CD10 and CD14 showed two band flips and a perverted form, while CD26 showed two band flips and a helical form. A band flip was defined as a 180 ° reversed glycoside linkage (Taira *et al.*, 2006).

Table 1.2 Physicochemical properties of CDs (Taira *et al.*, 2006)

Number of D-glucose unit	Molecular weight [a]		Aqueous solubility (g/100 mL) [b]	Surface tension (mN/m) [b]	Specific rotation $[\alpha]_D^{25}$	Half-life of ring opening (h)[c]
	Theoretical	Experimental				
α-CD 6	973	973	14.5	72	+147.8	33
β-CD 7	1135	1135	1.85	73	+161.1	29
γ-CD 8	1297	1297	23.2	73	+175.9	15
CD9 9	1459	1459	8.19	72	+187.5	4.2
CD10 10	1621	1621	2.82	72	+204.9	3.2
CD11 11	1784	1783	>150	72	+200.8	3.4
CD12 12	1946	1946	>150	72	+197.3	3.7
CD13 13	2108	2107	>150	72	+198.1	3.7
CD14 14	2270	2270	2.30	73	+199.7	3.6
CD15 15	2432	2432	>120	73	+203.9	2.9
CD16 16	2594	2594	>120	73	+204.2	2.5
CD17 17	2756	2756	>120	72	+201.0	2.5
CD18 18	2919	2919	>100	73	+204.0	3.0
CD19 19	3081	3081	>100	73	+201.0	3.4
CD20 20	3243	3243	>100	73	+199.7	3.4
CD21 21	3405	3405	>100	73	+205.3	3.2
CD22 22	3567	3567	>100	73	+197.7	2.6
CD23 23	3729	3729	>100	73	+196.6	2.7
CD24 24	3891	3891	>100	73	+196.0	2.6
CD25 25	4054	4053	>100	73	+190.8	2.8
CD26 26	4216	4215	22.4	73	+201.4	2.9

Table 1.2 (continued) Physicochemical properties of CDs (Taira *et al.*, 2006)

Number of D-glucose unit	Molecular weight [a]		Aqueous solubility (g/100 mL) [b]	Surface tension (mN/m) [b]	Specific rotation $[\alpha]_D^{25}$	Half-life of ring opening (h) [c]
	Theoretical	Experimental				
CD27 27	4378	4375	>125	72	+189.4	2.8
CD28 28	4540	4537	>125	72	+191.2	2.6
CD29 29	4702	4699	>125	72	+190.2	2.5
CD30 30	4864	4860	>125	72	+189.1	2.3
CD31 31	5026	5023	>125	71	+189.0	2.4
CD32 32	5188	5185	>125	71	+192.7	2.4
CD33 33	5351	5349	>125	71	+192.1	2.2
CD34 34	5513	5510	>125	72	+189.6	2.2
CD35 35	5675	5671	>125	71	+193.7	2.1
CD36 36	5837	5835	>100	71	+190.6	1.9
CD37 37	5999	5995	>100	71	+189.9	1.8
CD38 38	6161	6158	>100	71	+190.1	1.9
CD39 39	6323	6321	>100	70	+188.1	1.8

[a] Theoretical masses were calculated as 162.1406 x n, where n is the number of glucose unit. Experimental masses of α-CD ~ CD26 and CD27 ~ CD39 were determined as the average mass and the monoisotopic mass, respectively.

[b] Observed at 25 ºC.

[c] In 1 mol/L HCl at 50 ºC.

1.5 The structure and physiological roles of Amylomaltase and D-enzyme

The α-amylase family was classified into three groups of glycoside hydrolase families, GH 13, 57 and 77 (Henrissat, 1991). Amylomaltase (AM) was first found in *Escherichia coli*, as the essential enzyme for the metabolism of maltose (Kaper *et al*., 2007; Kaper *et al*., 2004). Similar enzymes in plants are called disproportionation enzyme (D-enzyme). AM and D-enzyme are classified as GH77 while CGTase and α-amylase are part of GH13 (Kaper *et al*., 2004). GH77 enzymes are efficient 4α-GTases with remarkably low hydrolytic activities than those of GH13 (Takaha and Smith, 1999). AM shows similar catalytic reaction to CGTase but it is an intracellular enzyme which forms cycloamyloses (CAs) or large-ring cyclodextrin (LR-CDs) as the major cyclization product, while the final products of CGTase consist mainly of small cycloamyloses or small-ring cyclodextrins with 6-8 glucose units. In *E. coli*, the smallest substrate that AM recognizes is maltotriose (Palmer *et al*., 1976) while maltose is reported as smallest substrate for plant D-enzyme (Takaha and Smith, 1999). AM from *E. coli* is encoded by *malQ* gene, is a dextrinyl transferase that could transfer maltosyl group and longer dextrinyl residues onto glucose, maltose or longer maltodextrins. AM is a part of maltooligosaccharide transport and utilization system that consists of maltodextrin phosphorylase and maltose transport proteins (Park *et al*., 2000) (Figure 1.5). AMs from *Haemophilus influenza* and *Aquifex aeolicus* were reported to be involved in the part of the glycogen metabolism (Takaha and Smith,1999). In photosynthetic cells; *Arabidopsis thaliana* the pathway of starch breakdown and conversion to sucrose by knocking out AM to block the conversion from maltose to sucrose was studied (Figure 1.6).
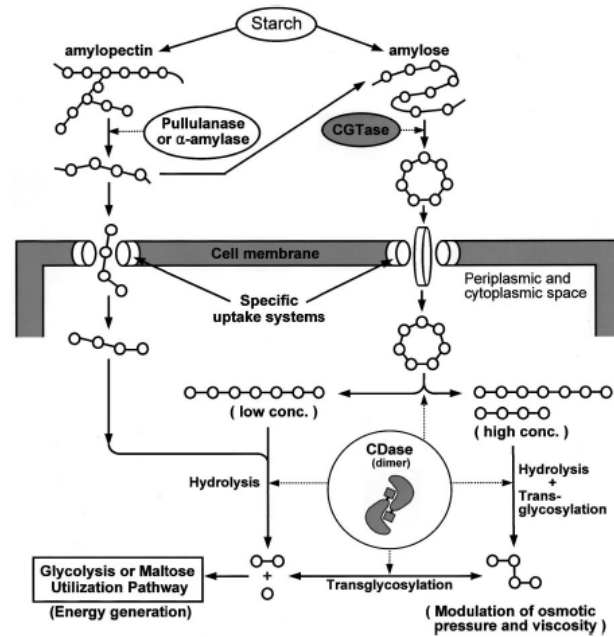
Figure 1.5 The proposed starch utilization pathway in bacteria. An alternate route consisted of the activities of CGTase and CDase is supported by the finding of CD transport protein at the cell membrane (Park *et al*., 2000).
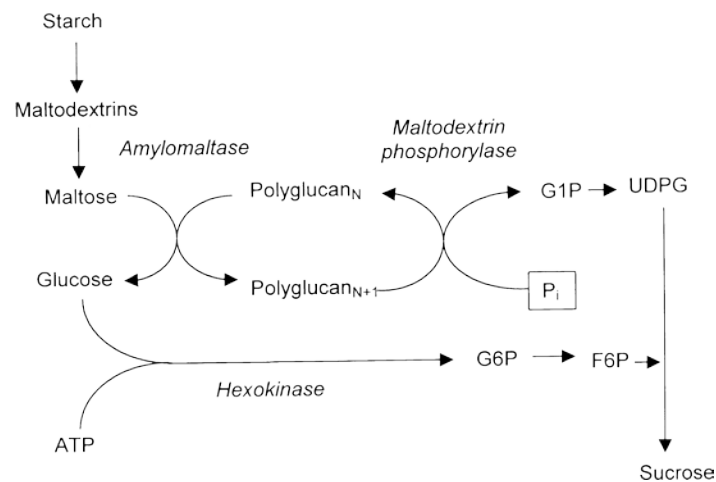
Figure 1.6 The hypothetical pathway for starch breakdown and conversion to sucrose in *Arabidopsis thaliana*. It was proposed that the glucan part of the cytosolic polysaccharide is the direct intermediate between maltose and sucrose (Lu and Sharkey, 2004).

It was proposed that maltose metabolism in the cytosol of *Arabidopsis* leaves is similar to that in the cytoplasm of *E. coli*. (Lu and Sharkey, 2004).

The general GH77 family consists of the $(\beta/\alpha)_8$-barrel (domain A) and three subdomains (B1, B2 and B3) attached around the barrel (Figure 1.7) (Kaper *et al.*, 2007). Three invariable catalytic residues are Asp (nucleophile), Glu (general acid/base catalyst) and Asp, which have been reported in AMs of the 3 *Thermus* species studied, namely *T. aquaticus, T. thermophilus* and *T. brockianus* (numbering in *T. aquaticus* are Asp293, Glu340 and Asp395). These are conserved residues for enzymes in α-amylase family (Takashi *et al.*, 2006). From the structural comparison between AM, α-amylase from pancreatic pig and CGTase from *Bacillus circulans*, it revealed that AM contains a core domain of $(\beta/\alpha)_8$ chain fold barrel and several inserts (subdomain B1, B2 and B3) between core domain (Figure 1.7). Subdomain B2 is present in AM and plant D-enzyme but absent in CGTase and α-amylase. Therefore, it is suggested that this domain has a unique role in AM (Fujii *et al.*, 2005).

The 3-dimensional structures of only six AMs have been reported. Three are from *Thermus: T. aquaticus* (Sträter *et al.*, 2002)*, T. thermophilus* (Barends *et al.*, 2007) and *T. brockianus* (Jung *et al.*, 2011). The others are from *Thermococcus litoralis* (Imamura *et al.*, 2003b), *Thermotoga maritima* (Zhang *et al.*, 2002), and *Aquifex aeolicus* (Fujii *et al.*, 2005).

From the crystal structure of AM from *T. brockianus*, the enzyme showed a structural fold and catalytic mechanism similar to the GH13 of α-amylase family (Jung *et al.*, 2011) though it is classified as GH77. Surprisingly, the active site of α-amylase was exposed as an open catalytic cleft, but AM displayed a flexible lid which
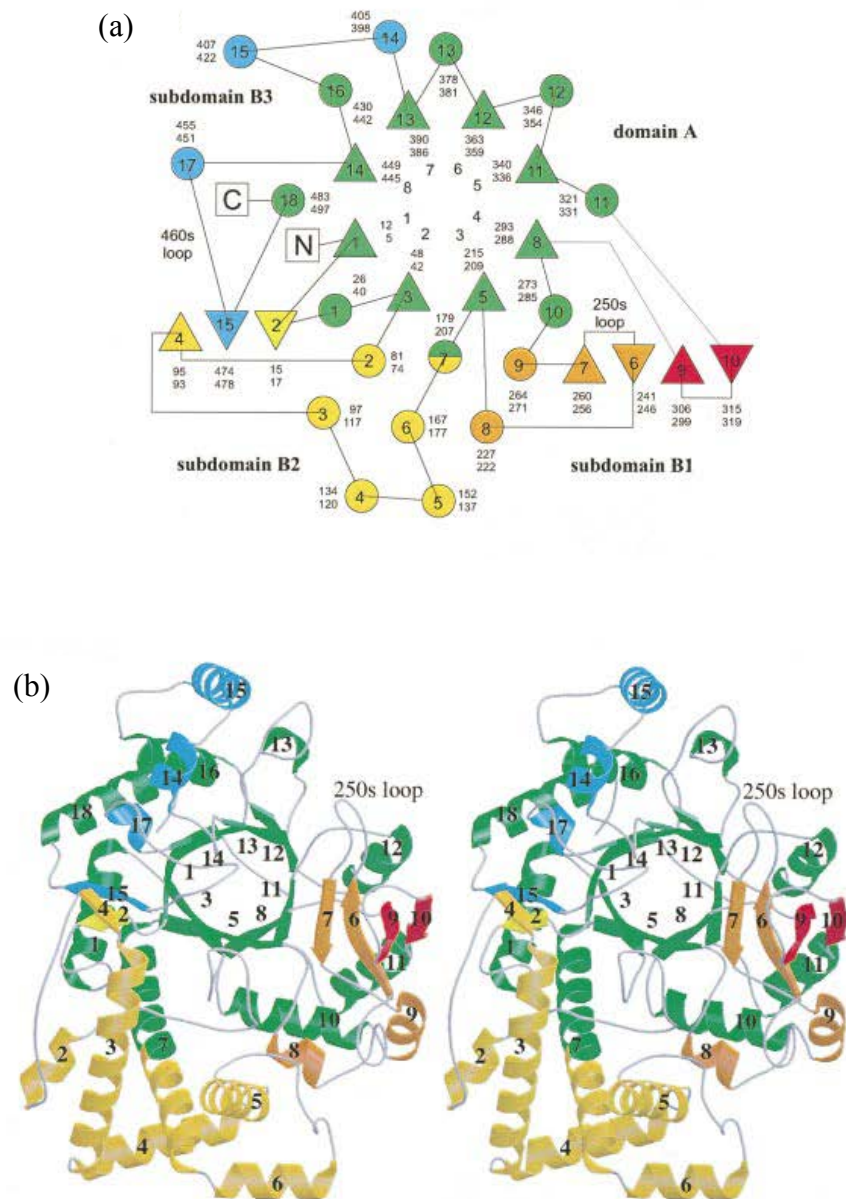
Figure 1.7 Fold pattern of AM from *T. aquaticus*. (a) Topography diagram. β-sheet and helicles are represented by tri-angles and circles. Number 1-8 in the center stand for the position of the first to eight barrel strand. Domain A is colored in green, while subdomain B1-B3 is in yellow, red and blue, respectively. (b) Ribbon presentation of the fold AM as in (a) (Przylas *et al.*, 2000b).

contains a long extended loop (250s loop) (Jung *et al.*, 2011). The conformational flexibility of the 250s loop from a thermostable amylomaltase of *T. brockianus* (TBGT) is shown in Figure 1.8. In *T. aquaticus*, in addition to the core of the catalytic cleft that contains seven residues, two loops (250s and 460s) containing many important hydrophobic residues lied close to the active center were proposed (Figure 1.9). A uniquely long extended 250s loop in *Thermus* partially covers the active site and is proposed to be important for binding of substrates and dissociation of products. Jung *el al.,* reported about the three conserved catalytic residues and the flexible 250s loop of TBGT (Figure 1.10). Trp258 residue in the 250s loop, which exists only in the AM family and not in other glycoside hydrolases or α-amylase family, has been suggested to play a role in the formation of cyclic products. The 250s loop is conserved in GH77 family but is absent in GH13 and GH57 family of α-amylase (Przylas *et al.*, 2000b).

1.6 Investigation of active site residues of 4αGTase enzymes

Active site directed chemical modification is an important tool for studying structure-function relationship of enzymes and other biologically active proteins (Gote *et al.*, 2007). This modification technique can be used to identify active residues without knowing the protein structure. On the contrary, site-directed mutagenesis, though a more direct way to identify functional residues and a more confidence information is given, but the three-dimensional structure of the protein is needed. Several works on chemical modification of proteins have been reported. In the study of CGTase from *Bacillus circulans* A11, chemical modification and substrate protection of carboxyl group (aspartic and glutamic acid), histidine, tryptophan and
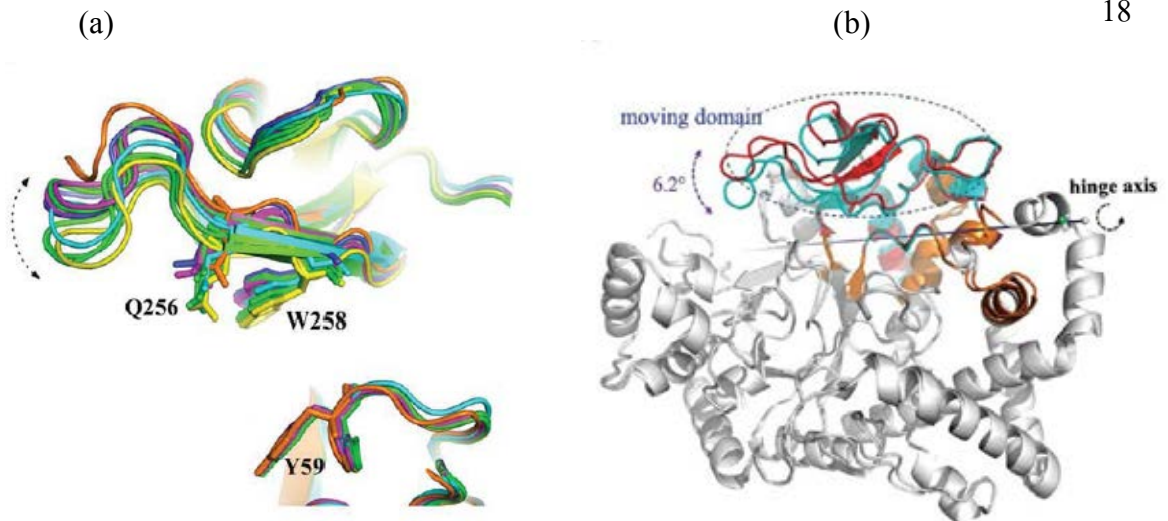
Figure 1.8 The conformational flexibility of the 250s loop in AM. (a) Conformational flexibility of the 250s loop observed in superposition of a thermostable amylomaltase from *T. brockianus* TBGT (magenta) with various AM structures. The dotted arrow represents the regular pattern of the segmental flexibility. (b) Flexible moving domain between two AM structures analyzed by a program DynDom (Jung *et al*., 2011).
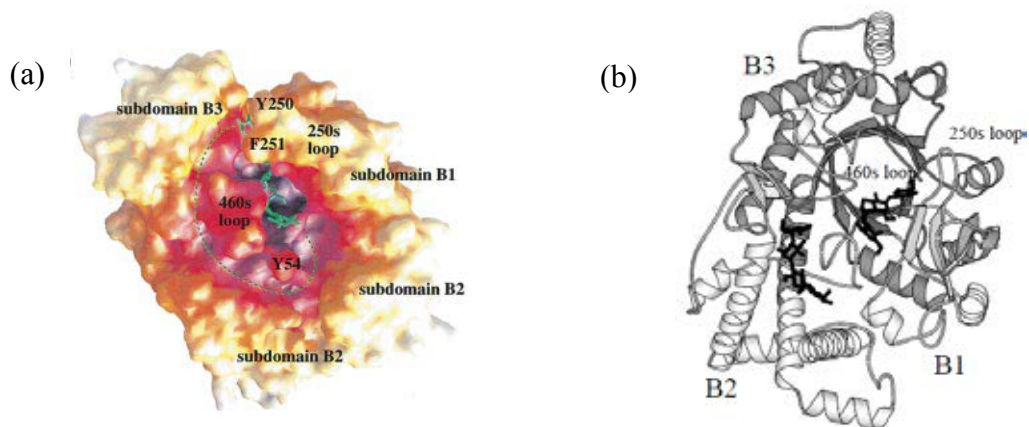
Figure 1.9 Molecular surface of AM from *T. aquaticus* with a modeled binding mode of a maltohexaose (a) (Przylas *et al*., 2000b). Stereo diagram of AM from *T.aquaticus* with acarbose at the active site (b) (Strater *et al*., 2002). Black color stands for two molecules of acarbose. Subdomain A represents in dark grey while subdomains B1-B3 are in light grey.
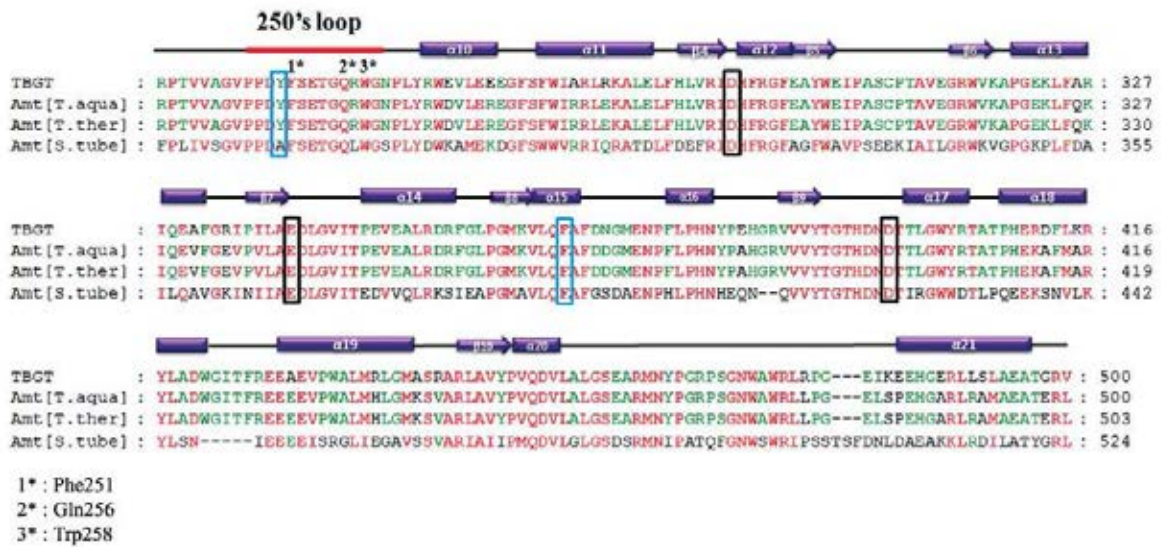
Figure 1.10 Comparison of sequence alignment from TBGT (AAY89060, a thermostable AM from *T. brockianus*) with other AMs (BAA33728; AM from *T. thermophilus*), ZP03497171 (AM from *T. aquaticus*) and Q06801 (D-enzyme from *Solanum tuberosum*). The residues conserved in 100, 75, and 50% of the proteins are shown in red, green, and black, respectively. While three conserved catalytic residues and the flexible 250s loop are boxed in black and blue, respectively (Jung *et al*., 2011).

tyrosine residues suggested that all of these amino acid residues are at the enzyme active site and important for the binding/catalytic function (Tongsima, 1998). When lysine and N-terminal amino acid of CGTase from *Thermoanaerobacter* sp. 501 were modified by succinic anhydride, the transferase (disproportionation) activity of the modified CGTase was significantly increased. In the contrary, the synthesis of the coupling of CDs with short-chain oligosaccharides, and the hydrolysis of starch decreased after succinylation (Alcalde *et al*., 2001). In 2003, essential histidines in isoform 1 of CGTase from *Paenibacillus* sp. A11 were modified by treatment with Diethylpyrocarbonate (DEP). The study showed that the histidine modification could be protected by methyl-β-cyclodextrin substrate prior to DEP treatment. When combined with analysis of tryptic peptide, His-40 and His-327 were identified as essential residues in the active site of isoform 1 (Kaulpiboon and Pongsawasdi, 2003). Up to now, only one report on the study of active site of AM is found. The thermostable AM from *Pyrobacculum aerophilum* IM2 was modified with dithiothreitol (DTT), the result suggested that at least one cysteine disulfide bridge is important for thermostability of AM (Kaper *et al*., 2005).

From the known three-dimensional structure of AM of *T. aquaticus*, besides the three catalytic residues (Asp293 and 395 and Glu340), the 2nd substrate binding site was also proposed. The important amino acid residues were also suggested by site-directed mutagenesis technique. Tyrosine 54 and 101 (Y54 and Y101) in this region were proposed as the residues controlling the specificity of the reaction. The removing of tyrosine side chain by site-directed mutagenesis increased cyclization activity (intramolecular transglucosylation reaction) but decreased disproportionation,

coupling and hydrolytic activities (intermolecular reactions). These amino acid residues are located in the second glucan binding site that is 14 °A away from the catalytic site (Fujii *et al.*, 2007). Srisimarat *et al.*, (2012) reported that site-directed mutagenesis of AM from *Corynebacterium glutamicum* (*Cg*AM) at Tyr-172 to Ala-172 (Y172A) resulted in the decrease in disproportionation, cyclization, and hydrolysis activity of the mutated enzyme compared to wild type. When the LR-CD product profile of both enzymes were investigated, the major LR-CD product of Y172A mutated enzyme were cycloamylose mixture with a degree of polymerization of 28 or 29 (CD28 or CD29), while the wild type enzyme gave CD25 as maximum at 0.05 U of AM. They suggested that Tyr-172 plays an important role in the LR-CD production of this novel *Cg*AM (Srisimarat *et al.*, 2012). The site-directed mutagenesis at Trp of AM form *T. brockianus* has been reported (Jung *et al.*, 2011). The properties of W258G mutant AM were analyzed and showed that no hydrolytic activity with amylose substrate or very low activity with G3 substrate compared with wild type AM. This result suggested that Trp258 plays an important part in the hydrolysis and starch stransglucosylation activities of AM.

1.7 Background of the research group on AMs and D-enzyme

Our research group has characterized a few bacterial AMs, they are a novel enzyme from a mesophilic *C. glutamicum* (Srisimarat *et al.*, 2011) with low homology (21%) to those from *Thermus,* a thermostable enzyme transcribed from AM gene screened from soil DNA which has high homology to *T. thermophilus* (Sawasdee *et al.*, 2012), and a thermostable enzyme from *T. filiformis* with 71% homology to *T.aquaticus* (unpublished data). A novel plant D-enzyme from *Manihot*

esculenta Crantz, with the amino acid sequence identity of 36 %, in comparison to the AM from *T. aquaticus* was also characterized (Srisimarat *et al*, 2011; Sawasdee *et al*., 2012; Tatanarat, 2012). The sizes of the 4 enzymes are 84, 58, 55 and 61 kDa, respectively. We are currently in the process of determining the three- dimensional structure of AM from *C. glutamicum* by X-ray crystallography. In view of so much difference in enzyme properties of *Corynebacterium* AM and cassava D-enzyme in comparison to the AMs from *Thermus,* structural understanding of these two enzymes for the structure-function relationship is our interest.

1.8 The objective of this study

The Amylomaltase (AM) or D-enzyme is classified as GH77 which are able to form cycloamyloses (CAs) or large-ring cyclodextrins (LR-CDs) as the major unique cyclization product. Both the AM and LR-CDs have many potential applications. Only six AMs have been investigated for their 3-dimentional structures. Our research group has found a few novel bacterial and plant AMs. In this study, we aim to compare and identify the important amino acids at or near the active site of three AMs, two from bacteria (one mesophilic and one thermophilic) and one from cassava plant (D-enzyme) using chemical modification technique. The effect of modification will also be investigated by characterization of the native AMs compared with modified enzyme. The basic knowledge of AMs will be useful for further basic and application study.

The objectives:

1 To purify the recombinant bacterial AMs and plant D-enzyme

2 To identify important amino acid residues for activity by chemical modification technique

3 To identify essential amino acid residues at the active site by substrate protection technique

4 To investigate the effect of chemical modification on enzyme characteristics

CHAPTER II

MATERIAL AND METHODS

2.1 Equipment

Amicon:

- Ultra- 0.5 ml Centrifugal Filters Unit (10 kDa cut off)

- Ultra - 15 ml Centrifugal Filter Units (30 kDa cut off)

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan

Auto pipette: Gilson, Pipetman, France

Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., USA

Electrophoresis Unit:

- Mini protein, Bio rad, USA

- Model 111 mini IEF cell, Bio-Rad, USA

FPLC AKTA Amersham Pharmacia Biotech unit:

- Column: Histrap FF^{TM}, HiPrep 16/60 Sephacryl S-100 HR (1 x 120 ml)

- Detector: UPC-900

- Pump: P-920

- Fraction collector: Frac-900

Gel support film for polyacrylamide, Bio-Rad, USA

HPAEC DX-600: Dionex Corp., Sunnydale, USA

- Column: Carbopac PA-100^{TM} (4 x 250 mm)

- Pulsed amperometric detector: DIONEX ED40

- Autosampler: DIONEX AS40

- Column oven: DIONEX ICS-3000 SP

Incubator shaker: Innova^{TM} 4080, USA

Magnetic stirrer and heater: Model Fisherbrand, Fisher Scientific, USA

Microcentrifuge: Eppendorf, Germany

pH meter: Model PHM95, Denmark

Power supply: Model POWER PAC 300, Bio-Rad, USA

Sonicator: Bendelin, Germany

Spectrophotometer: Biomate 3, Thermo scientific, USA

Vortex: Model K-550-GE, Scientific Industries, Ins., USA

2.2 Chemical reagents

N-acetylimidazole (NAI): Sigma, USA

Acrylamide: Merck, USA

Ammonium persulphate: Sigma, USA

Ampicillin: Sigma, USA

Bovine serum albumin: Sigma, USA

N-bromosuccinamide (NBS): Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

Copper sulphate: Carlo Erba Reagenti, Italy

Diethylpyrocarbonate (DEPC): Sigma, USA

Dimethyl sulfoxide (DMSO): Merck, Germany

Dithiothreitol (DTT): usb, Canada

di-Potassium hydrogen phosphate: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC): Sigma, USA

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glucose: BDH, English

Glucose oxidase kit: HUMAN, Germany

Hydrochloric acid: Carlo Erba Reagenti, Italy

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

D (+)-maltose monohydrate: Fluka, Switzerland

Maltotriose: Fluka, Switzerland

β- Mercaptoethanol: Fluka, Switzerland

N, N'-Methylene-bis-acrylamide: Sigma, USA

N, N, N', N'-Tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagenti, Italy

Pea starch: Emsland-Stärke GmbH, Germany

Potato starch (soluble): Sigma, USA

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Potassium iodide: Mallinckrodt, USA

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Riboflavin: BDH, England

Sodium acetate: Merck, Germany

Sodium chloride: Carlo Erba Reagenti, Italy

Standard protein marker: Amersham Pharmacia Biotech Inc., USA

Trinitrobenzenesulfonic acid (TNBS): Sigma, USA

Succinic anhydride (SAH): Fluka, Switzerland

2.3 Bacterial cultivation and Crude enzyme preparation

2.3.1 Starter inoculum

A single colony of three recombinant *E. coli* BL21 (DE3) clones, each harbouring the AM gene from *C. glutamicum* (CGAM) (Srisimarat *et al*., 2011) , the AM gene isolated from soil DNA which showed 99% amino acid sequence identity with AM from *T. thermophiles* (THAM) (Sawasdee *et al*., 2012), or the gene of *Manihot esculenta* D-enzyme (MeDPE1) (Tantanarat, 2012), was cultured in 5 ml LB broth medium (0.5% NaCl, 0.5% Yeast extract and 1% Tryptone, w/v) containing 100 $\mu g.ml^{-1}$ ampicillin at 37 ºC with 250 rpm rotary shaking for overnight (16-18 h). One percent (v/v) starter was transferred into 50 ml fresh LB containing 100 $\mu g.ml^{-1}$ and further cultivated at the same condition for overnight.

2.3.2 Enzyme production and crude enzyme preparation

One percent (v/v) each of starter culture was inoculated into 300 ml fresh LB containing 100 $\mu g.ml^{-1}$ ampicillin in a 1 L flask. Incubation at 37 ºC with 250 rpm rotary shaking was performed until A_{600} reached 0.4-0.6 or about 2.5-3 h. Expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.4, 0.8 and 0.1 mM for CGAM, THAM and MeDPE1, respectively (Srisimarat *et al*., 2011; Sawasdee *et al*., 2012; Tantanarat, 2012). After induction, MeDPE1 was incubated at 16 ºC overnight, while CGAM and THAM were incubated at 37 ºC for 2 and 4 h, respectively, before cell harvesting. The cells were collected by centrifugation at 12,000 x g, 4 ºC for 30 min and consecutively washed by 0.85% (w/v) NaCl and extraction buffer (Appendix 1), respectively, for two times. Pellet cells were

resuspended in extraction buffer. The cells were broken by sonication (Bandelin sonopuls) of 30% pulse for 1 min and stopped for 3 min for CGAM and MeDPE1 or 20% pulse for 5 min and stopped for 5 min for THAM. The process was repeated for 10 cycles for CGAM and MeDPE1 or 5 cycles for THAM. The removal of cell debris was performed by centrifugation at 12,000 x g for 1 h, 4 ºC. The supernatant was dialyzed against 20 mM phosphate buffer, pH 7.4.

2.4 Purification of AMs

All AMs were purified by Histrap column as described (Srisimarat *et al.*, 2011; Sawasdee *et al.*, 2012; Tantanarat, 2012). Crude AMs prepared from section 2.3 were added 20 mM imidazole, filtrated through a 0.45 µm polyethersulfone (PES) membrane filter and then loaded onto a nickel-charged Histrap column equilibrated with binding buffer containing 20 mM imidazole (Appendix 1). Elution was carried out by the same buffer containing 500 mM imidazole at a flow rate of 1 ml/min. Column operation was performed at 4 ºC.

For THAM, one additional purification step was performed. The pool from Histrap column was desalted and concentrated by Centricon (30 kDa), then loaded onto a gel-filtration (1x120 ml, HiPrep 16/60 Sephacryl S-100 HR, GE Healthcare) column. The column was pre-equilibrated with 50 mM phosphate buffer, pH 7.4, containing 50 mM NaCl. Two ml fractions were collected at a flow rate of 0.2 ml/min. Fractions with enzyme activity were pooled, concentrated and dialyzed against 20 mM phosphate buffer pH 7.4.

The purity of all AMs was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5 Enzyme assay

2.5.1 Starch degrading activity

The enzyme activity was determined by measuring the degraded starch in the reaction using iodine method (Srisimarat, 2010).

Fifty microliter of enzyme solution was added into 100 µl of 0.75% (w/v) soluble potato starch and 100 µl of 50 mM phosphate buffer, pH 6.0. Incubation was at 30 ºC for CGAM or at 70 ºC for THAM for 10 min and stopped by adding 500 µl of 1 N HCl. One hundred µl of reaction was withdrawn and mixed with 900 µl of iodine solution (0.005% I_2 in 0.05% KI, (w/v)), Appendix 2. The absorbance was measured at 660 nm.

One unit of enzyme was defined as the amount of enzyme which degraded starch (1 mg/ml) in 10 min incubation time under the assay conditions used.

2.5.2 Starch transglucosylation activity

The enzyme activity was determined by the ability to transfer glucosyl group from starch donor to maltose acceptor. The remained starch was detected by adding iodine solution (modified from Park *et al*., 2007).

The reaction mixture contained enzyme solution (100 µl), 0.2 % (w/v) soluble starch (250 µl), 1.0 % (w/v) maltose (50 µl) and 50 mM phosphate buffer pH 6.0 (600 µl). The reaction mixture was incubated at 30 °C (CGAM) or 70 ºC (THAM) for 10 min, and terminated by heating the solution at 100 °C for 10 min. Aliquot of

100 µl was withdrawn and mixed with 1.0 ml of iodine solution (0.02 % I_2 in 0.2 % KI (w/v), Appendix 2. The absorbance was measured at 600 nm.

One unit of enzyme was defined as the amount of enzyme that produced 1% decrease in the color of starch-iodine complex per min under the assay conditions used.

2.5.3 Disproportionation activity

The enzyme activity was measured by glucose oxidase reagent which detected glucose released in the reaction (Srisimarat *et al.*, 2011).

Twenty µl of enzyme solution was added into 30 µl of 5% (w/v) maltotriose substrate. The reaction mixture was incubated at 30 ºC for CGAM and MeDPE1 or 70 ºC for THAM for 10 min and stopped by adding 30 µl of 1 N HCl. Then, 920 µl of glucose oxidase reagent was added, rapidly mixed and incubated at room temperature for 10 min. The absorbance was measured at 505 nm.

One unit of enzyme activity was defined as the amount of enzyme which produced 1 µmol of glucose per min under the assay conditions used.

2.5.4 Cyclization activity

The cycloamylose (CA) product formed was measured by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection technique (HPAEC-PAD) (Srisimarat, 2010).

The enzyme solution (0.05 U of starch degrading activity) was incubated with 0.2% (w/v) pea starch at 30 ºC for 4 h, stopped by boiling for 10 min. Then,

4 U of glucoamylase was added into reaction mixture and incubated at 40 ºC for 24 h, and inactivated the reaction by boiling for 10 min.

One unit of enzyme activity was defined as the amount of enzyme which produced 1 nC of CD34 per min under the assay conditions used.

2.6 Protein determination

Protein concentration was determined by Bradford (1976) method, using bovine serum albumin (BSA) as standard.

One hundred µl of sample was mixed with 1 ml of Bradford working solution that contained Coomassie blue G-250 (Appendix 3), left for 10 min, and the absorbance was measured at 595 nm.

2.7 Polyacrylamide gel electrophoresis (PAGE)

2.7.1 SDS-PAGE

The denaturing gel was performed on a 5 % (w/v) stacking gel and 12.5% (w/v) separating gel that consisted of 0.1% SDS (w/v), electrophoresis was carried out on a Bio-Rad Mini-Protein III gel apparatus (Bio-Rad Laboratories, Hercules, MA, USA) using the Laemmli buffer system (Appendix 4). The sample buffer was added into samples and boiled for 5 min prior to loading into the gel. The electrophoresis was run at constant current of 20 mA per slab from anode to cathode at room temperature.

2.7.2 Native PAGE

The native-PAGE was performed at 4 ºC. The gel was consisted of 12.5 % (w/v) acrylamide separating gel (containing 0.3 % (w/v) glycogen for MeDPE1) and 5.0 % (w/v) acrylamide of stacking gel. The electrophoresis buffer is Tris-glycine buffer pH 8.3, preparation method was shown in Appendix 5. The sample buffer was added into AM solutions before loading into the gel. The electrophoresis was run at constant current of 16 mA per slab from anode to cathode at 4 ºC on a Mini-Gel electrophoresis unit (Bio-Rad).

2.7.3 Detection of protein bands

2.7.3.1 Coomassie blue staining

After electrophoresis, the gel was stained for proteins with coomassie blue R-250 staining solution (Appendix 4) at room temperature for 3 h. And then, it was destained with destaining solution (Appendix 3) until the background was cleared.

2.7.3.2 Activity staining

The activity of AMs was determined by agitating the gel with substrate solution (0.2 % (w/v) soluble starch in 50 mM phosphate buffer, pH 6.0) at 30 ºC for CGAM and 70 ºC for THAM for 10 min. For MeDPE1, the gel was agitated with 0.3% (w/v) maltodextrin in 50 mM phosphate buffer pH 6.0 at room temperature (25 ºC) for overnight (16-18 h) (modified from method of Tantanarat, 2012). Then, the gel was rinsed 2-3 times with distilled water and stained with iodine solution (0.2% I_2 in 2.0 % KI, (w/v)). The activity band was compared with the protein band(s) to investigate the position of AMs.

2.8 Effect of group-specific reagents on AMs activity

The purified enzyme solutions (1 U of starch transglucosylation activity for CGAM and THAM or disproportionation activity for MeDPE1) in 20 mM phosphate buffer, pH 7.4 were incubated with 5 mM of various modifying reagents that are specific for different functional groups at 30 ℃ for 30 min. The reagents used were *N*-Bromosuccinamide (NBS), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), Phenylmethylsulfonyl fluoride (PMSF), *N*-Acetylimidazole (NAI), Diethylpyrocarbonate (DEPC), Dithiothreitol (DTT), *N*-Ethylmaleimide (NEM), Succinic anhydride (SAH) and Trinitrobenzenesulfonic acid (TNBS). The final volume of reaction mixture was adjusted to 20-50 µl with 50 mM phosphate buffer, pH 6.0. The remaining activity was determined by methods as described in section 2.5. Only for CGAM, remaining activity was assayed by all of the three methods (starch transglucosylation, starch degrading and disproportionation activity).

2.9 Identification of essential amino acid residues at the catalytic site of AMs by substrate protection technique

2.9.1 Modification of AMs

The purified enzyme solutions (1 U of starch transglucosylation activity for CGAM and THAM or disproportionation activity for MeDPE1) in 20 mM phosphate buffer, pH 7.4 were treated with various concentrations of NBS, SAH or EDC and adjusted the final volume to 20-100 µl with 50 mM phosphate buffer, pH 6.0. The reaction was incubated at 30 ℃ for 30 min. The remaining activity of each modified AM was analyzed by method as described in section 2.5.

2.9.2 Substrate protection

The concentration of NBS, SAH or EDC which gave rise to 50% remaining activities (obtained from 2.9.1) was selected for this study. The enzyme solution was pre-incubated with maltotriose (G3) substrate at 30 ºC for CGAM and MeDPE1 or at 70 ºC for THAM, for 5 min. In the case of MeDPE1 before determining enzyme activity, the excess G3 in the reaction was removed by filtration through a 0.5 ml ultrafiltration membrane unit (10 kDa cut off) and centrifuged at 12,000 rpm at 4 °C for 10 min. The retentate solution was collected and measured for disproportionation activity as described in section 2.5.3. For CGAM and THAM, the activity was determined by starch transglucosylation reaction as described in section 2.5.2.

2.10 Inactivation kinetics of AMs with NBS, SAH or EDC

All of modified AMs were prepared as described in section 2.9.1 by treated with NBS, SAH or EDC at concentrations which 50 % of activities remained. The reaction was incubated at 30 ºC in the range of 0-10 min. The remaining activity of each modified AM was analyzed by method as described in section 2.5.2 (for CGAM and THAM) or 2.5.3 (for MeDPE1). The pseudo-first order rate constant was obtained from the slope of the plot between relative activity (%) versus time of reaction (Deshpande *et al*., 1990).

2.11 Characterization of native and modified AMs

    2.11.1 Determination of pI of AMs

        2.11.1.1 Preparation of gel support film

Drops of water were dripped onto the glass plate. The hydrophobic side of the gel support film was placed against the glass plate and the excess air bubbles or water was removed by rolling by a test tube. After that, it was transferred and placed on the casting tray with facing down and resting the spacer bars.

        2.11.1.2 Matrix preparation

The solution of monomer-ampholyte was prepared as described in Appendix 6, then it was degassed for 5 min by vacuum. The catalyst solution was prepared and it was added to the degassed monomer solution by gentle swirling. The solution was carefully pipetted into the spacer between the casing tray and the glass plate. The solution was released slowly for avoid trapping air bubbles. The gel was polymerized by leaving for 45-60 min. Then, it was carefully lifted by spatula. The sample template was laid on the center of the polymerized gel.

        2.11.1.3 Sample preparation and running the gel

The sample solution in distilled water (3 µg protein/ well) was dropped on the sample template, and left until it was completely diffused. The standard protein markers with known pI were loaded on the gel. Then, the sample template was removed from the gel. The gel with gel support film was turned upside-down which was directly placed on the top of electrodes. Focusing was performed under constant voltage at 100 V for 30 min, 200 V for 15 min and 450 V for 60 min, respectively.

After electrofocusing, the gel was removed from electrodes and statically stained with fixative solution and staining solution for 30 and 120 min. The gel was rinsed several times with distilled water, then it was destained with destaining solution until the background was cleared.

2.11.2 Determination of substrate specificity and kinetics of AMs

2.11.2.1 Substrate specificity in disproportionation reaction

The efficiency of native and modified AMs to transfer maltooligosaccharides in disproportionation reaction was analyzed by using maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentose (G5), maltohexaose (G6) and maltoheptaose (G7) as substrate. The 0.5 U of enzyme solution (starch transglucosylation activity) in 50 mM phosphate buffer, pH 6.0 was treated with 50 mM substrate and incubated at 30 ºC for CGAM and MeDPE1 or 70 ºC for THAM, for 10 min. The reaction was inactivated by boiling for 10 min. The amount of glucose released in the reaction was detected by glucose oxidase reagent as described in section 2.5.3.

2.11.2.2 Substrate specificity in starch transglucosylation reaction

The ability of native and modified AMs to transfer glucosyl group from starch donor to linear maltooligosaccharide acceptors (using G1 to G7) was compared. The 0.5 U of enzyme solution (starch transglucosylation activity) in 50 mM phosphate buffer, pH 6.0 was incubated with 1.5 mM of linear maltooligosaccharides, 250 µl of 0.2 % (w/v) soluble starch and adjusted to final volume of 1.0 ml with 50 mM phosphate buffer, pH 6.0. The reaction mixture was incubated at 30 ºC for CGAM or

70 ºC for THAM for 10 min, stopped by boiling for 10 min. Then, the remaining activity of AMs was measured by iodine method as described in section 2.5.2

2.11.2.3 Kinetics of AMs in disproportionation and starch transglucosylation reaction

The rates at which AMs catalyze disproportion and starch transglucosylation reactions were determined, the activities were measured as described in section 2.5.3 and 2.5.2. The purified enzyme solution of CGAM (0.7 or 45 μg protein for disproportionation and starch transglucosylation activity) was incubated with various concentrations of substrate maltotriose (G3), ranging of 5-40 mM (for disproportionation activity) or glucose (G1), ranging of 0.1-5 mM in combination with 0.2% (w/v) soluble starch (for starch transglucosylation activity) at 30 ºC for 10 min in 50 mM phosphate buffer, pH 6.0. The inhibition by acarbose was also investigated. The activity of native or NBS-modified CGAM was measured in the presence of various acarbose concentrations, ranging from 3-80 or 0.25-1.0 mM for disproportionation or starch transglucosylation reaction, respectively. Kinetic parameters were determined from the Lineweaver-Burk plot of the Michaelis-Menten equation.

2.11.3 Determination of the number of essential tryptophan residues
2.11.3.1 Using spectrophotometry

Oxidation of AMs with NBS in the presence and the absence of a protective substrate were performed. Pre-incubation with G3 (50 mM) or 50 mM phosphate buffer, pH 6.0 (as blank) was carried out at 30 ºC for CGAM and MeDPE1 and at

70 ºC for THAM for 5 min. Then the activity was determined as described in section 2.5.2. The appropriate concentration of AM was put into a quartz cell, 50 mM phosphate buffer, pH 6.0 was then added to a final of 1.0 ml to obtain A_{280} of about 0.3. Then 10 µl of 5 mM NBS was added with rapid mixing. After 5 min, the absorbance was recorded. Addition of 10 µl increment of NBS was continued until no further decrease in absorbance was observed. The number (n) of modified-Trp residues per mole was calculated by the following equation:

$$n = \frac{\Delta OD \ \times \ 1.31 \ \times \ M.W. \ \times \ V}{W \ \times \ 5500}$$

Where   n = the number of tryptophan residues per mole of protein

ΔOD = the decrease in A_{280}

M.W. = molecular weight of the protein

V = the initial volume of titrated solution (ml)

W = weight of the protein (mg)

and 1.31 is the empirical factor (Patchornik *et al*., 1958), while 5500 is the molar extinction coefficient of Trp at A_{280} (Spande and Witkop, 1967). The graph was constructed by plotting the relative activity (%) versus the number of modified Trp.

2.11.3.2 Using mass spectrometry (MALDI-TOF)

About 100 µg each of dried AMs was dissolved in 20 µl of ACN-TFA mixture solution (30% (v/v) acetonitrile (ACN) in 0.1% (v/v) trifluoroacetic acid (TFA)). Then 0.5 µl of these sample solutions were withdrawn and mixed with 10 µl of saturated sinapinic acid in ACN-TFA mixture solution. One µl of mixed sample was

dotted on plate and analyzed with MALDI-TOF mass spectrometer (Autoflex ll, at the Department of Biotechnology, Faculty of Science, Mahidol University). Mass spectra were recorded in linear position mode at 20 kV voltage and delayed extraction time of 600 ns.

2.11.4 Circular dichroism spectra

Circular dichroism spectra were obtained using a spectropolarimeter (Jasco J-175, Italy) at the Institute of Molecular Bioscience, Mahidol University. Then 20 µl of native or modified enzyme solution in 20 mM phosphate buffer, pH 7.4 that contained 1.4 and 1.5 mg/ml of protein, respectively, was deposited into the cell (0.2 mm) and measured in the range of 190-250 nm. Each CD spectrum was monitored in three scans at 20 nm/min with constant time at 2 sec and 2 nm bandwidth.

The CD spectra of AMs were calculated from the method of Kelly *et al*., (2005). The Mean Residue Weight (MRW) for estimating the quantity of the peptide bond in protein is as shown in equation (1).

$$MRW \quad = \quad \frac{M}{(N-1)} \qquad (1)$$

Where M stands for the molecular weight of the polypeptide chain (Da), N stands for the number of amino acid residues in the chain and N - 1 stands for the number of peptide bonds. The mean residue ellipticity (MRE) at each of wavelength λ ($[\theta]_{mrw,\lambda}$) is given by equation (2).

$$[\theta]_{mrw,\lambda} \quad = \quad \frac{MRW \times \theta\lambda}{10 \times dc} \qquad (2)$$

Where θλ = ellipticity (degrees) at each of wavelength λ

d = the path length of the cuvette (cm)

and c is the protein concentration (g/ml). The unit of MRE and molar ellipticity is deg.cm^2.dmol^{-1} (Kelly *et al*., 2005). The quantity of β-structure and α-helix in the protein was predicted by using the K2D3 (http://www.ogic.ca/projects/k2d3/) that was an online server program for analysis of the protein secondary structure from CD data.

2.11.5 Fluorescence spectroscopy

The fluorescence spectra of the native and modified AM in 50 mM phosphate buffer, pH 6.0 were measured (Fluorescence spectrometer, LS 55, Perkin Elmer, USA). The protein concentration of AMs used was previously measured to have an A_{280} of about 0.3. Excitation wavelength was set at 295 nm to excite tryptophan and the emission spectrum was recorded between 320 and 400 nm (Konozy *et al*., 2002). The graph was constructed by plotting the fluorescence intensity versus wavelength.

2.11.6 Analysis of linear oligosaccharides and large-ring cyclodextrins
2.11.6.1 Thin layer chromatography (TLC)

To analyze the linear oligosaccharide products, TLC was performed (modified from method of Postaire *et al*., 1991). The sample solution was prepared as described section 2.11.2.1. Then, 2 μl of sample was spotted onto a silica gel TLC plate (F$_{254}$ 20 x 20 cm, Merck, Germany). The mobile phase consisted of n-butanol: pyridine: water (5: 4: 1) by volume. The oligosaccharide in the reaction mixture was

detected by heating at 110 ºC for 10 min after spraying with the mixture solution of sulphuric acid: methanol (1: 2).

2.11.6.2 High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Linear oligosaccharides were analyzed by using HPAEC-PAD as described (Koizumi *et al*., 1999). The sample solution was prepared by the enzyme solution 1 or 2 U (starch transglucosylation activity assay) was incubated with 50 mM maltotriose (G3), maltotetraose (G4) or maltopentaose (G5), and incubated at 30 ºC for 4 h and stopped by boiling for 10 min. Then, 25 µl of sample was injected into the column (Carbopac PA1, 4 x 250 mm, Dionex, USA) and eluted with a linear gradient of sodium acetate (60-65 min, increasing from 30 to 100%) in 150 mM NaOH with a flow rate of 1 ml/min (Srisimarat *et al*., 2011). The size and concentration of linear oligosaccharide products were estimated by comparison with standard G1 to G7.

For large-ring cyclodextrins (LR-CDs), were analyzed by using HPAEC-PAD as described (Koizumi *et al*., 1999). The sample solution was prepared as described in section 2.5.4. Then, 25 µl of sample was injected into the column (Carbopac PA-100, 4 x 250 mm, Dionex, USA) and eluted with a linear gradient of sodium nitrate (0-2 min, increasing from 4 to 8%; 2-10 min, increasing from 8 to 10%; 10-20 min, increasing from 18 to 28%; 20-40 min, increasing from 28 to 35%; 40-55 min, increasing from 35-45%; 55-60 min, increasing from 45-63%) in 150 mM NaOH with a flow rate of 1 ml/min (Srisimarat *et al*., 2011). The size and concentration of LR-CDs products were estimated by comparison with standard LR-CDs.

CHAPTER Ⅲ

RESULTS

3.1 Purification of AMs

3.1.1 Preparation of crude AMs

In the preparation of crude CGAM, THAM and MeDPE1, 4.2, 10.9 and 6.4 g cell pellets of recombinant *E. coli* harboring these respective AM genes were obtained from 1.2, 2.4 and 1.8 liters, of LB broth medium. Cell pellets were dissolved in extraction buffer (~1 g per 2.5 ml), then sonicated and centrifuged to get crude supernatant. Total protein in crude CGAM, THAM and MeDPE1 were 269, 639 and 327 mg protein. Specific starch transglucosylation activity for CGAM and THAM were 2.5 and 2.8 U/mg protein while specific disproportionation activity for MeDPE1 was 10.2 U/mg protein (Table 3.1). MeDPE1 cannot catalyze starch transglucosylation reaction, thus its activity in all experiments was measured by disproportionation reaction. All crude enzymes were then purified by Histrap FF^{TM}.

3.1.2 Purification by Histrap FF^{TM} column

The chromatographic profile of CGAM is shown in Figure 3.1. The unbound proteins were washed off the column by the binding buffer as a bulky broad protein peak. Then, the His-tag protein was eluted by elution buffer in a relatively small and narrow protein peak. The fractions that displayed enzyme activity were pooled and dialyzed against 20 mM phosphate buffer, pH 7.4. The column profiles of THAM and MeDPE1 were similar to that of CGAM. The purified AMs obtained showed specific

activities of 55.2, 40.6 and 429 Unit/mg protein with 65, 21 and 1.8 % recovery for CGAM, THAM and MeDPE1, respectively (Table 3.1).

3.1.3 Purification by gel filtration column

The purified THAM after Histrap FF^{TM} column contained more than one bands on SDS-PAGE (Figure 3.2) so it was loaded into a gel-filtration (S-100 HR) column as described in section 2.4. The specific activity of THAM was increased to 53.6 Unit/mg protein with 4.8 % recovery (Table 3.1).

3.1.4 Determination of purity of AMs

All of enzymes from each purification step were checked for protein pattern and purity by SDS-PAGE. CGAM and MeDPE1 showed a major band at 84 and 61 kDa on SDS-PAGE (Figure 3.2) which indicated the achievement of one step purification by affinity chromatography. In contrast, THAM was not highly purified after Histrap affinity column, a few protein bands were observed on SDS-gel. However, one major band on the gel at 60 kDa was obtained after further purified by gel filtration chromatography.

3.1.5 Non-denaturing gel electrophoresis (Native-PAGE)

The activity band of all purified AMs was examined on native-PAGE as described in section 2.7.3.2 (Figure 3.3). CGAM (I, a) and THAM (II, a) catalyze the conversion of starch to short chain oligosaccharides that cannot form complex with iodine and occur as clear zone band on the dark brown background after staining by iodine. While, MeDPE1 (III, a) transfers the glucan units from maltodextrin to

glycogen. The activity band (dark band) on dark brown background was detected when the gel was stained with iodine. For all AMs, one major band was observed in both protein and activity stain and the activity band were corresponded to the protein band (Figure 3.3, b).

3.2 Effect of group-specific reagents on activities of AMs

The preliminary study to determine essential amino acid residues in AMs was by modification of the enzymes with various modifying reagents that are specific for different functional groups. From the result (Table 3.2), modifying agents that inhibited all 3 enzymes with relatively similar degree of inhibition were NBS and EDC. Modification with 5 mM NBS resulted in total or significant loss of all AM activities, indicating the essential of Trp on enzyme catalysis. EDC, which modified Asp/Glu, showed more inhibition for CGAM (60% remaining activity) than for THAM and MeDPE1 (80% remaining activity), suggesting the more important role of Asp/Glu in CGAM activity. The reagents that affected only one type of AM were NAI, PMSF, and DTT. Modification by NAI on Tyr residues resulted in 40% loss of CGAM activity, suggesting the role of Tyr in enzyme catalysis of CGAM. PMSF and DTT which is specific for Ser and Cys, showed only inhibition on MeDPE1. DEPC which mainly modified His significantly affected MeDPE1 while showed less inhibition on CGAM. The last group of reagents affected all AMs but to a different extent were SAH, TNBS and NEM. SAH and TNBS which modifies Lys mainly inhibited CGAM and MeDPE1, thus His and Lys were more important to these enzymes than to THAM. While NEM affected more on CGAM and MeDPE1.

Table 3.1 Purification of recombinant AMs

Purification step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg protein)	%Yield	Purification fold
Crude CGAM	269	669[a]	2.5	100	1
HisTrap FF^{TM}	8.0	433[a]	55.2	65	22
Crude THAM	639	1771[a]	2.8	100	1
HisTrap FF^{TM}	9.0	365[a]	40.6	21	15
Gel filtration	1.6	86[a]	53.6	4.8	19
Crude MeDPE1	327	3333[b]	10.2	100	1
HisTrap FF^{TM}	0.5	60[b]	120	1.8	42

Crude CGAM, THAM and MeDPE1 were prepared from 1.2, 2.4 and 1.8 liter of cell culture, respectively, which produced 4.2, 10.9 and 6.4 g of cell wet weight.

a = Assayed by starch transglucosylation activity

b = Assayed by disproportionation activity
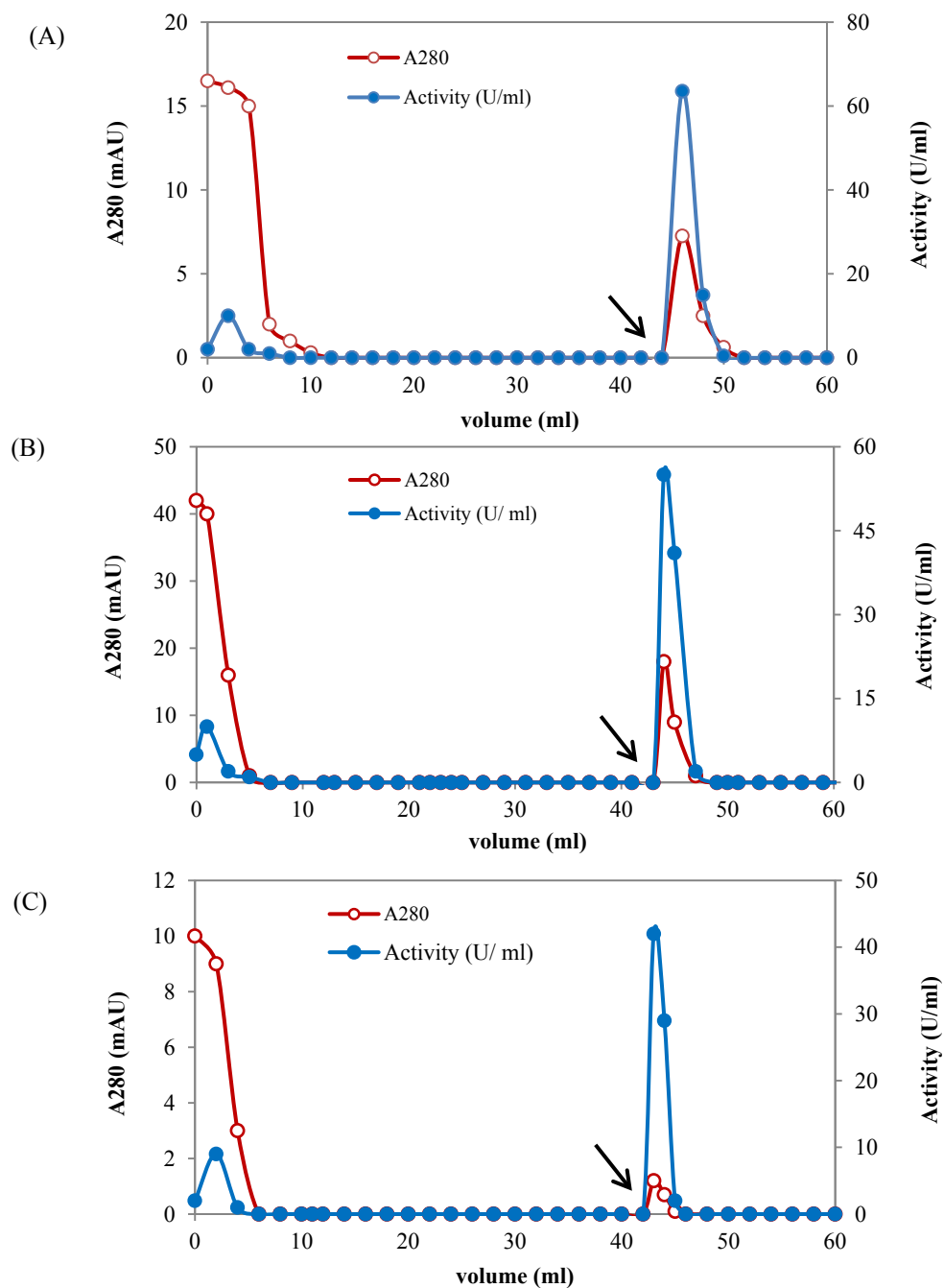
Figure 3.1 Purification profile of CGAM (A), THAM (B) or MeDPE1 (C) by Histrap FF^{TM} column chromatography (1 ml). Unbound proteins were washed off by binding buffer with 20 mM imidazole, pH 7.4. Elution was by the same buffer containing 500 mM imidazole at a flow rate of 1 ml/min. Fraction size was 2 ml. The arrow represents the starting point of the elution of bound proteins.
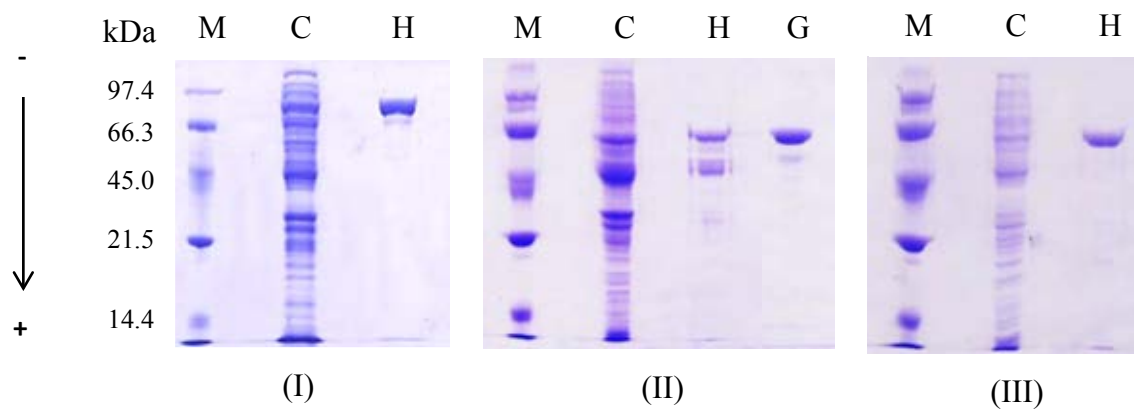
Figure 3.2 SDS-PAGE of recombinant AMs stained by coomassie blue. CGAM (I), THAM (II) and MeDPE1 (III).

> M = protein marker
>
> C = 30 μg of crude CGAM or THAM
>
>   15 μg of crude MeDPE1
>
> H = 6 μg of purified AM (after Histrap FF^{TM} column)
>
> G = 6 μg of purified AM (after gel filtration column)
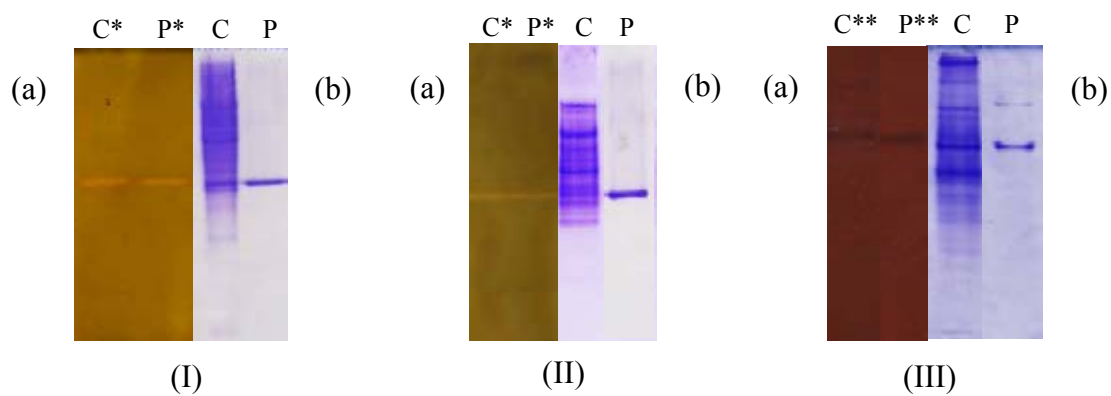
Figure 3.3 Native-PAGE of crude recombinant AMs (C) and purified AMs (P) from, CGAM (I), THAM (II) and MeDPE1 (III). Activity stain by iodine (a) and protein stain by coomassie blue (b).

(a)

    C = 1 U of crude AMs

    P = 1 U of purified AMs

(b)

    C = 15 µg of crude AMs

    P = 3 µg of purified AMs

    *   Starch transglucosylation unit

    **   Disproportionation unit

These results suggested that Trp, Asp/Glu, Cys and Lys were important residues for all the three enzymes but with subtle difference in catalytic role.

To investigate more on the effect of modifying reagents on different activity of CGAM, the enzyme was treated with 5 mM of various modifying reagents as described in section 2.8 and the remained activity was measured by three methods as described in section 2.5.1-2.5.3. The results showed that NBS which modified Trp residue(s) could significantly inhibit all three CGAM activities (5-25% remained activity) (Table 3.3), while EDC, NAI and DEPC which mainly modified Asp/Glu, Tyr or His residue(s), respectively, led to moderate loss of all activities (50-75% remaining activity). For EDC modification, the reagent showed more sensitive inhibition on starch-degrading activity with 34% remaining activity, while the two remaining activities were about two folds higher (60-70% remaining activity). SAH and TNBS which modified Lys, gave similar effect on starch transglucosylation and starch-degrading activities with 20-30% remaining activity but notably differed in the inhibition of disproportionation reaction. Starch-degrading and disproportionation activities were affected by PMSF while no effect on starch transglucosylation activity. DTT and NEM which modify Cys residue(s) showed different result. Lacking of inhibition on all activities by DTT was observed, while NEM could completely inhibit starch-degrading activity and also significantly affected starch transglucosylation activity. It should be noted that starch-degrading activity was more sensitive to most reagents tested than the other two activities.

3.3 Effect of chemical modification on Native-PAGE pattern

Chemical modification may result in changing of the net charge, protein folding and conformation of the modified proteins. The effect of chemical modification on CGAM was investigated by native-PAGE with protein and activity stains (Figure 3.4). The pattern of protein was significantly changed by treatment with NBS, PMSF, DEPC and SAH (Panel A: lanes 2, 4, 6 and 8), as compared to the untreated control (lane 1). More than one protein bands in lanes 2 and 4 were observed with modification by NBS and PMSF. The disappearance of the activity band (Panel B, lane 2 and 8) of CGAM after treatment with NBS or SAH was clearly seen whereas trace of activity was still obtained as a result of PMSF and DEPC modification. Inactivation by EDC was also investigated though moderate inhibition was observed since Asp and Glu are known to be catalytic residues for enzymes in α-amylase family (Takashi *et al.*, 2006). For modification by EDC, a small decrease in relative mobility on native-PAGE of both the protein and activity band was noticed. These modified enzymes by NBS, SAH, and EDC were then selected for further investigation.

3.4 Identification of essential amino acid residues at the catalytic site of AMs by substrate protection technique

The chemical modification by NBS, EDC or SAH affected mainly at Trp, Asp/Glu or Lys residues, respectively. In order to investigate whether these modified residues are at or around the active site, substrate protection experiment was performed. The suitable concentration of each reagent to be used in substrate protection experiment for each enzyme was firstly determined. All of AMs were

Table 3.2 Effect of modifying reagents on amylomaltases. The recombinant CGAM, THAM or MeDPE1 (1 U of starch transglucosylation activity for CGAM and THAM or disproportionation activity for MeDPE1) was incubated with 5 mM modifying reagents at 30 °C for 30 min. Determination of the remaining activity was by starch transglucosylation for CGAM and THAM or disproportionation reaction for MeDPE1.

Modifying reagent (5 mM)	Amino acid involved	Remaining amylomaltase activity (%)		
		CGAM	**THAM**	**MeDPE1**
None	-	100 ± 4.0	100 ± 2.0	100 ± 5.6
N-bromosuccinamide (NBS)	Tryptophan	23.4 ± 5.7	0 ± 4.0	0 ± 0
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)	Carboxylic amino acids	60.4 ± 3.2	80.6 ± 2.8	82.7 ± 1.6
Phenylmethylsulfonyl fluoride (PMSF)	Serine	92.6 ± 14.5	94.9 ± 2.8	22.1 ± 0.3
N-Acetylimidazole (NAI)	Tyrosine	63.4 ± 2.4	103.4 ± 5.2	100.4 ± 3.3
Diethylpyrocarbonate (DEPC)	Histidine	74.3 ± 1.6	101.1 ± 2.8	25.5 ± 1.0
Dithiothreitol (DTT)	Cysteine	93.6 ± 0.7	104.8 ± 1.2	24.9 ± 7.2
N-Ethylmaleimide (NEM)	Cysteine	17.1 ± 4.9	84.3 ± 2.0	40.0 ± 0.7
Succinic anhydride (SAH)	Lysine	22.3 ± 5.7	102.9 ± 3.6	33.3 ± 1.6
Trinitrobenzenesulfonic acid (TNBS)	Lysine	25.4 ± 23.0	74.4 ± 0.4	32.6 ± 4.9

Table 3.3 Effect of modifying reagents on CGAM activities. The recombinant CGAM (1 U of starch transglucosylation activity) was incubated with 5 mM modifying reagents at 30 °C for 30 min. Determination of the remaining activity was by starch transglucosylation, starch-degrading and disproportionation activities.

Modifying reagent (5 mM)	Remaining activity (%)		
	Starch transglucosylation	Starch-degrading	Disproportionation
Control	100 ± 4.0	100 ± 8.3	100 ± 12.7
N-bromosuccinamide (NBS)	23.4 ± 5.7	5.5 ± 7.8	7.6 ± 1.6
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)	60.4 ± 3.2	33.9 ± 8.1	69.1 ± 3.6
Phenylmethylsulfonyl fluoride (PMSF)	92.6 ± 14.5	24.3 ± 8.7	43.9 ± 11.8
N-Acetylimidazole (NAI)	63.4 ± 2.4	48.5 ± 8.6	52.5 ± 0.4
Diethylpyrocarbonate (DEPC)	74.3 ± 1.6	69.6 ± 7.0	52.9 ± 13.9
Dithiothreitol (DTT)	93.6 ± 0.7	100.8 ± 27.7	135.3 ± 5.5
N-Ethylmaleimide (NEM)	17.1 ± 4.9	0 ± 1.2	65.9 ± 10.1
Succinic anhydride (SAH)	22.3 ± 5.7	31.7 ± 19.1	9.6 ± 0
Trinitrobenzenesulfonic acid (TNB**S**)	25.4 ± 23.0	31.5 ± 6.7	82.4 ± 0

(A)   (B)
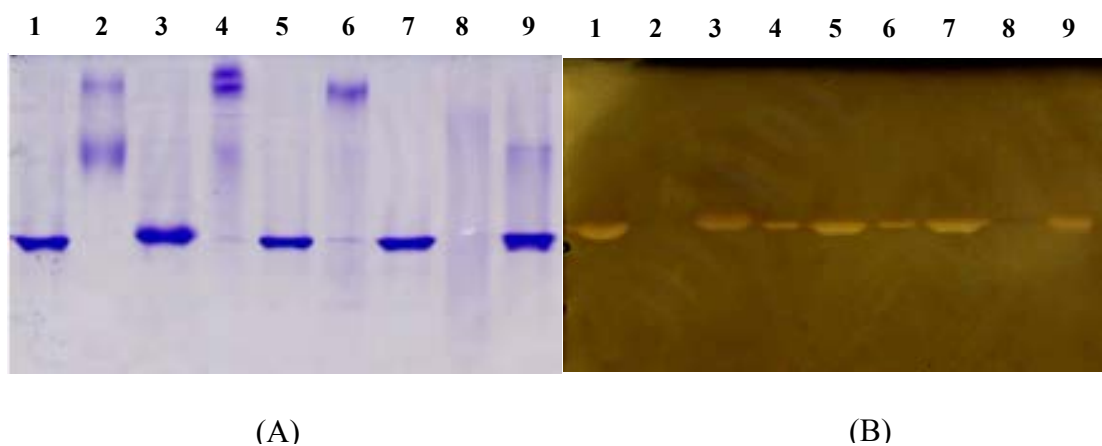
Figure 3.4 Non-denaturing PAGE of native and modified recombinant CGAM. (A)

protein stain by coomassie blue (B) activity stain by iodine reagent.

Lane 1   =   Control (CGAM without modifying reagent)

Lane 2   =   CGAM treated with NBS

Lane 3   =   CGAM treated with EDC

Lane 4   =   CGAM treated with PMSF

Lane 5   =   CGAM treated with NAI

Lane 6   =   CGAM treated with DEPC

Lane 7   =   CGAM treated with DTT

Lane 8   =   CGAM treated with SAH

Lane 9   =   CGAM treated with TNBS

*Modifying reagents used were 5 mM

inhibited at different concentration of each modifying reagent. The total starch transglucosylation activity of CGAM and THAM was completely inhibited after treatment with 1 mM NBS; 30 and 450 mM EDC; 8 and 250 mM SAH, respectively. While nearly all MeDPE1 disproportionation activity was inhibited by 20 mM NBS, 200 mM EDC and 10 mM SAH. Half of CGAM, THAM and MeDPE1 activities were inhibited by; 0.3, 0.5 and 8.0 mM NBS; 10, 75 and 30 mM EDC; and 4.5, 150 and 3.0 mM SAH, respectively (Table 3.7). These concentrations of reagents at half activities of all enzymes (IC_{50} or inhibitory concentration at which 50% of activity was remained) were then used to determine the involvement of modified residues at the active site of AMs. NBS, EDC or SAH were modified in the presence or the absence of maltotriose (G3) substrate at various concentrations as described in section 2.9.2, then activities were compared.

In the presence of G3, decreasing activity of CGAM was found in relation to the increase in G3 from 5 to 75 mM. Up to 40% starch transglucosylation activity of CGAM was decreased in the presence of 50 mM G3 (Table 3.4-3.6). In contrast, the relative activity of THAM was slightly increased, up to 15% in the presence 75 mM G3. For MeDPE1, additional of G3 gave complicate results because G3 is the substrate in disproportionation reaction which was used in the assay for activity. About 5% and 130% of activity were increased when 20 and 50 mM G3 were added, respectively.

Despite of the interfering behavior of G3 added to the enzyme in the 5 min pre-incubation period, it is the best choice used for substrate protection experiment. We then proceeded to compare inhibition by modifying agents in the presence or

absence of G3. From Table 3.4, the result showed that G3 could protect inactivation by NBS. About 30% 70% and 55% of CGAM, THAM and MeDPE1, respectively, were protected by 50 mM G3.

For EDC modification (Table 3.5), inactivation was also protected by G3 for all enzymes. 50 mM G3 could protect 12%, 45% and 40% of CGAM, THAM and MeDPE1, respectively. When SAH modification was investigated, G3 protection was best seen in THAM, about 30% at 50 mM concentration. No protection was observed in MeDPE1 while the result from CGAM was complicated. At 20 mM G3, about 20% protection was observed, surprisingly no protection was seen at 50 mM, (Table 3.6).

These overall results suggested that G3 showed the ability to protect essential Trp, Asp/Glu or Lys residue(s) in all AMs from inactivation by NBS, EDC or SAH modification, with the exception of SAH-inactivated MeDPE1.

3.5 Inactivation kinetics of AMs with NBS, SAH or EDC

To determine inactivation kinetics, the enzymes were incubated with the IC_{50} concentrations of NBS, SAH or EDC at various times (Table 3.7). The plots between the remaining activity and various incubation times were made. The pseudo-first order kinetic constant of each modified AMs was estimated from the slope (Figure 3.8). The inactivation of MeDPE1 was most sensitive to all reagents tested (lowest IC_{50} and highest k_{inact}) except for NBS. In contrast, THAM exhibited the least sensitivity, especially to SAH.

Figure 3.5 Effect of NBS on activity of AMs, CGAM (a), THAM (b) and MeDPE1 (c). AMs were incubated with various concentrations of NBS at 30 ºC for 30 min, as described in section 2.9.1, measurement of the remaining activity after incubation was by starch tansglucosylation for CGAM and THAM or disproportionation reaction for MeDPE1.

Table 3.4 Effect of maltotriose substrate on the inactivation of AMs by NBS

Compound added	% Relative activity		
	CGAM	THAM	MeDPE1
1) None	100	100	100
2) Maltotriose (mM)			
5	97.4 ± 1.1	106.5 ±0.4	N.D.
10	88.3 ± 3.0	111.7 ± 5.2	N.D.
20	77.7 ± 2.2	113.7 ± 0.8	101.5 ± 24.9
50	60.4 ± 0.0	114.2 ± 1.0	230.4 ± 11.2
75	N.D.	115.7 ± 3.2	N.D.
3) Maltotriose (mM), then NBS *			
5	49.1 ± 5.4	61.2 ± 4.9	N.D.
10	55.8 ± 2.9	77.1 ± 1.5	N.D.
20	71.2 ± 3.6	85.4 ± 1.3	46.5 ± 4.1
50	71.7 ± 1.1	92.5 ± 0.3	76.7 ± 3.9
75	85.0 ± 1.2	92.2 ± 0.6	N.D.
4) NBS *	44.0 ± 4.3	23.14 ± 3.0	21.3 ± 2.2

The enzyme was pre-incubated for 5 min with maltotriose substrate at 30 °C for CGAM and MeDPE1 or at 70 °C for THAM. Then NBS at the concentration which gave rise to 50% remaining activity (*0.3, 0.5, and 8.0 mM for CGAM, THAM, and MeDPE1, respectively) was added. After 30 min, remaining activity was measured by starch transglucosylation for CGAM and THAM or disproportionation for MeDPE1.

N.D. = not determined
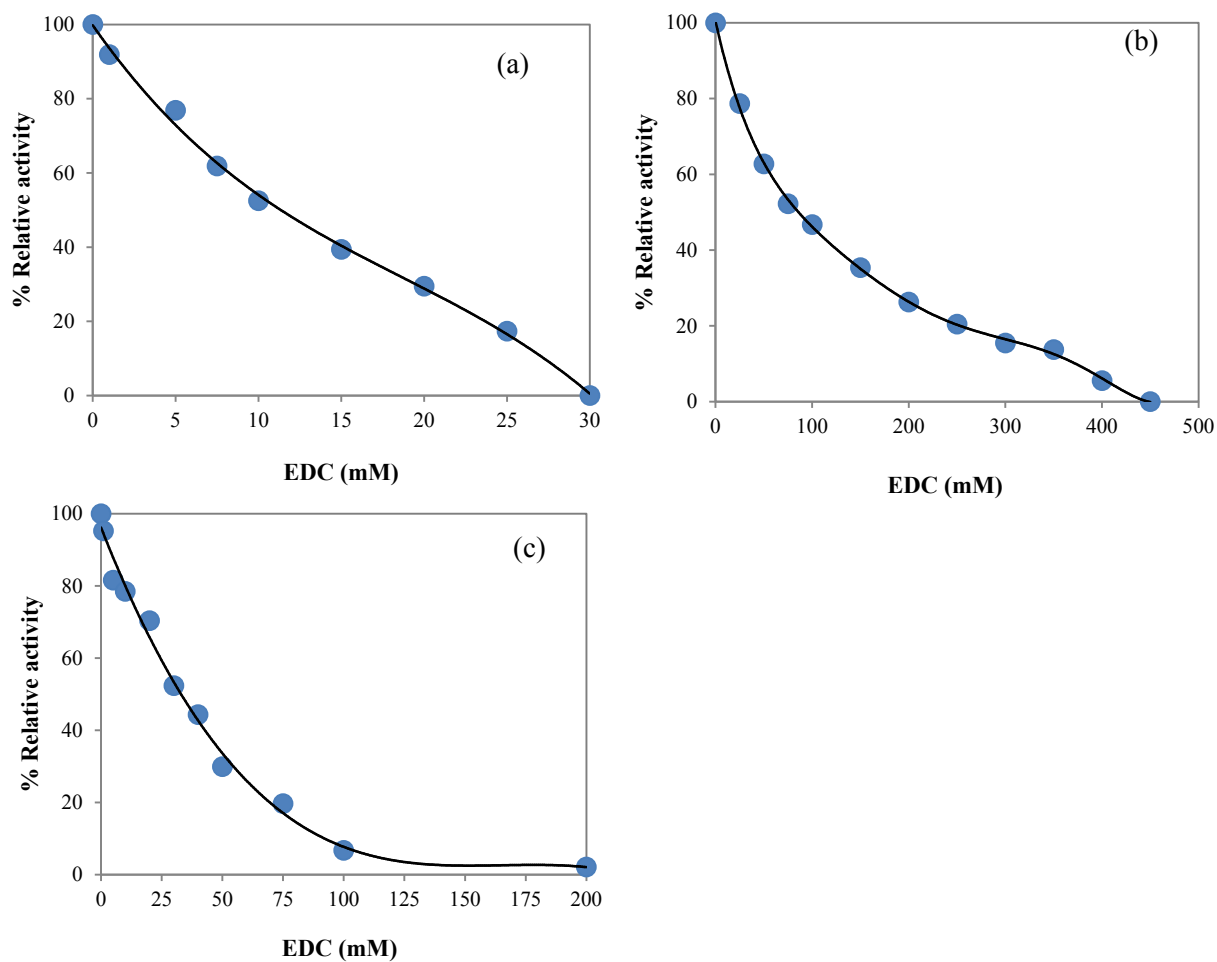
Figure 3.6 Effect of EDC on activity of AMs, CGAM (a), THAM (b) and MeDPE1 (c). AMs were incubated with various concentrations of EDC at 30 ºC for 30 min, as described in section 2.9.1, measurement of the remaining activity after incubation was by starch tansglucosylation for CGAM and THAM or disproportionation reaction for MeDPE1.

Table 3.5 Effect of maltotriose substrate on the inactivation of AMs by EDC

Compound added	% Relative activity		
	CGAM	**THAM**	**MeDPE1**
1) None	100	100	100
2) Maltotriose (mM)			
5	97.4 ± 1.1	106.5 ±0.4	N.D.
10	88.3 ± 3.0	111.7 ± 5.2	N.D.
20	77.7 ± 2.2	113.7 ± 0.8	101.5 ± 24.9
50	60.4 ± 0.0	114.2 ± 1.0	230.4 ± 11.2
75	N.D.	115.7 ± 3.2	N.D.
3) Maltotriose (mM), then EDC *			
5	47.1 ± 2.5	72.9 ± 0.3	N.D.
10	52.2 ± 2.5	79.0 ± 0.9	N.D.
20	44.5 ± 8.5	86.0 ± 0.6	17.7 ± 1.2
50	46.1 ± 6.3	95.1 ± 1.1	50.8 ± 10.0
75	N.D.	99.3 ± 0.6	N.D.
4) EDC *	32.5 ± 6.1	48.3 ± 1.7	12.0 ± 1.5

The enzyme was pre-incubated for 5 min with maltotriose substrate at 30 °C for CGAM and MeDPE1 or at 70 °C for THAM. Then EDC at the concentration which gave rise to 50% remaining activity (*10, 75, and 30 mM for CGAM, THAM, and MeDPE1, respectively) was added. After 30 min, remaining activity was measured by starch transglucosylation for CGAM and THAM or disproportionation for MeDPE1.
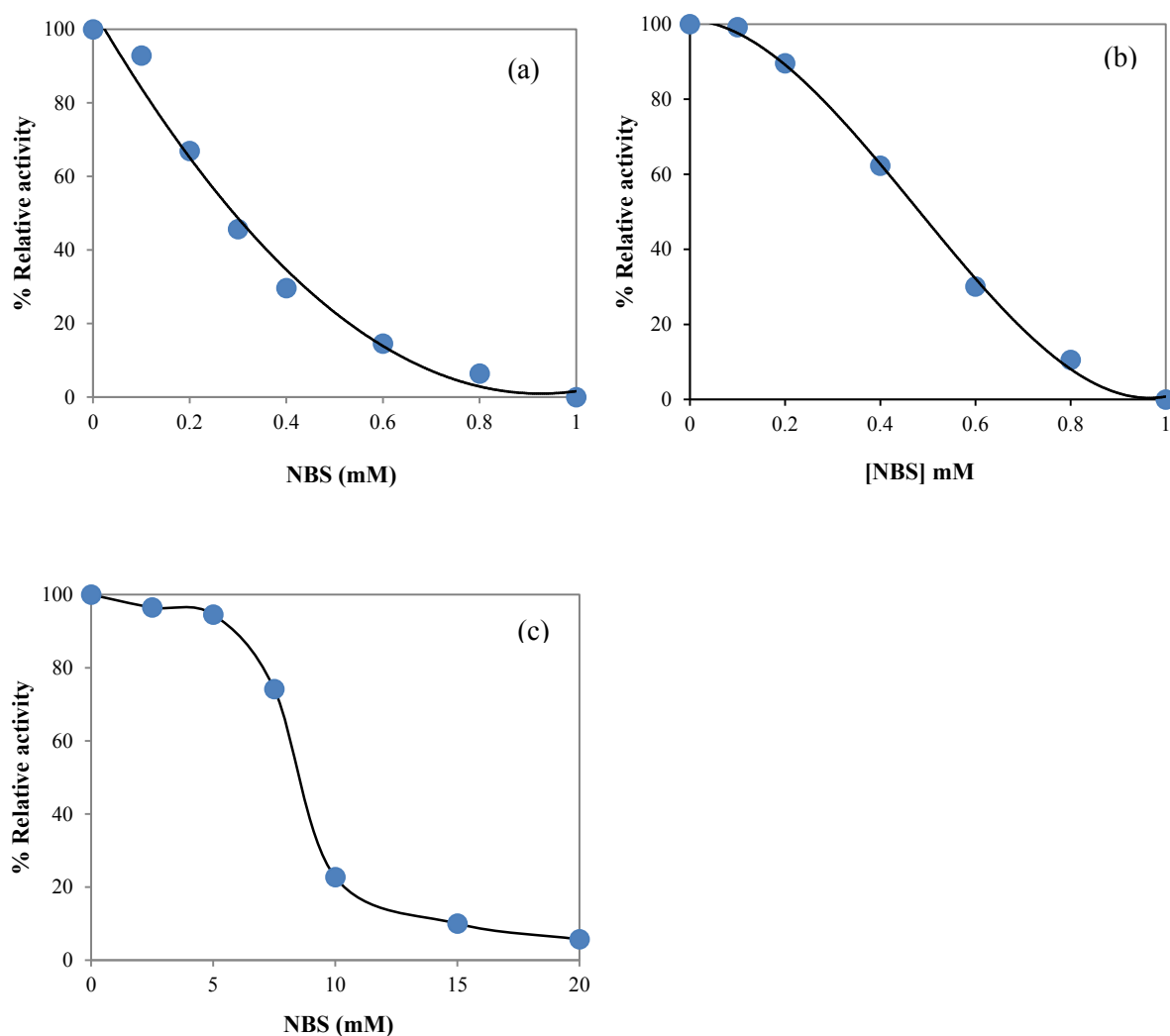
N.D. = not determined
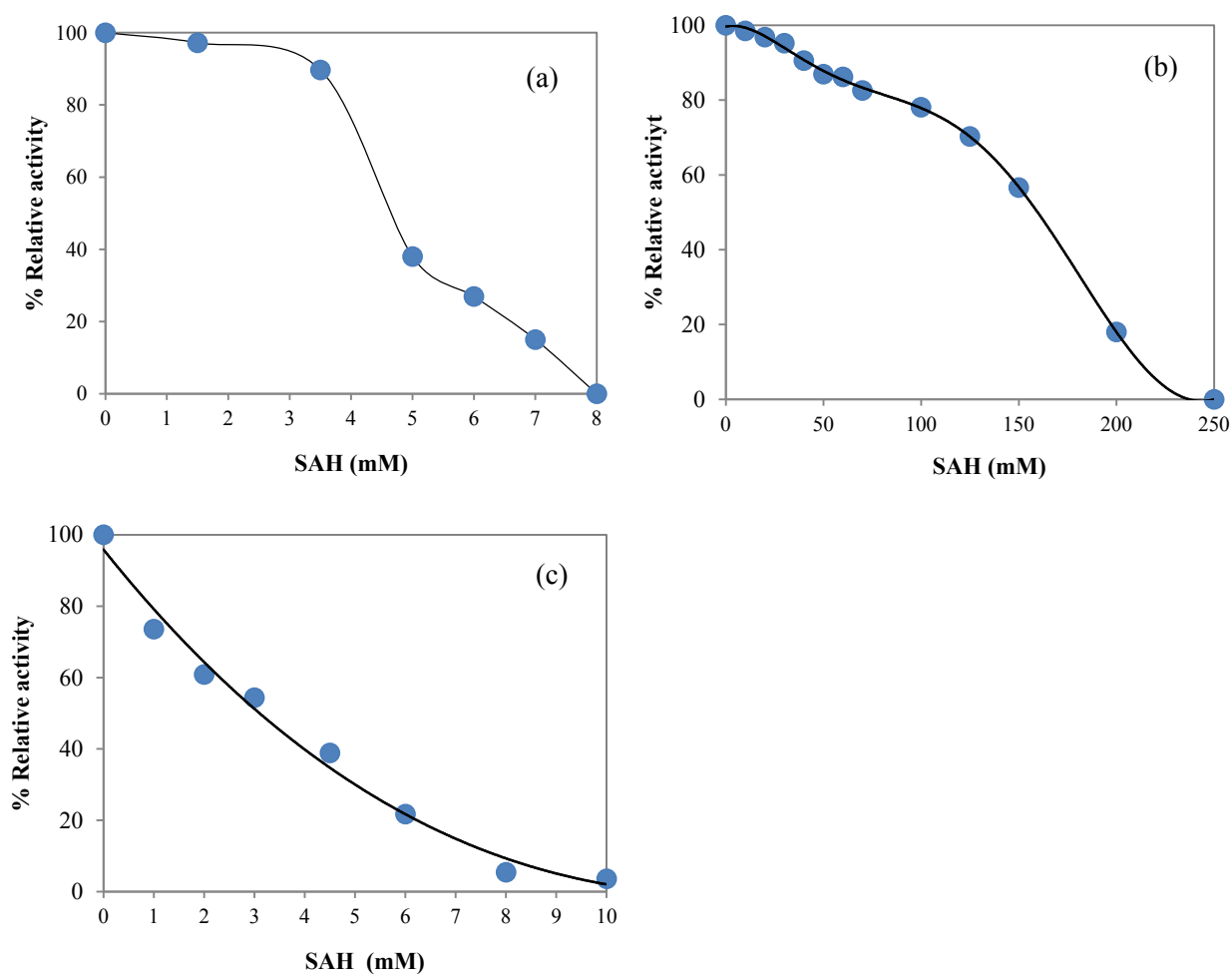
Figure 3.7 Effect of SAH on activity of AMs, CGAM (a), THAM (b) and MeDPE1 (c). AMs were incubated with various concentrations of SAH at 30 ºC for 30 min, as described in section 2.9.1, measurement of the remaining activity after incubation was by starch tansglucosylation for CGAM and THAM or disproportionation reaction for MeDPE1.

Table 3.6 Effect of maltotriose substrate on the inactivation of AMs by SAH

Compound added	% Relative activity		
	CGAM	**THAM**	**MeDPE1**
1) None	100	100	100
2) Maltotriose (mM)			
5	97.4 ± 1.1	106.5 ±0.4	N.D.
10	88.3 ± 3.0	111.7 ± 5.2	N.D.
20	77.7 ± 2.2	113.7 ± 0.8	101.5 ± 24.9
50	60.4 ± 0.0	114.2 ± 1.0	230.4 ± 11.2
75	N.D.	115.7 ± 3.2	N.D.
3) Maltotriose (mM), then SAH *			
5	69.4 ± 4.9	66.5 ± 1.2	N.D.
10	67.7 ± 10.8	72.5 ± 1.5	N.D.
20	60.9 ± 3.5	77.3 ± 3.2	36.8 ± 1.1
50	31.1 ± 3.2	82.4 ± 2.8	27.0 ± 8.0
75	N.D.	84.4 ± 0.4	N.D.
4) SAH *	41.0 ± 2.5	54.9 ± 2.4	51.8 ± 4.3

The enzyme was pre-incubated for 5 min with maltotriose substrate at 30 °C for CGAM and MeDPE1 or at 70 °C for THAM. Then SAH at the concentration which gave rise to 50% remaining activity (*4.5, 150, and 3.0 mM for CGAM, THAM, and MeDPE1, respectively) was added. After 30 min, remaining activity was measured by starch transglucosylation for CGAM and THAM or disproportionation for MeDPE1.

N.D. = not determined

3.6 Characterization of native and modified AMs

To determine the effect of modifying reagents on enzyme characteristics, the effect of NBS on bacterial AMs was chosen for this study due to two reasons. First, CGAM is a novel AM recently reported with low amino acid sequence identity to those previously reported AMs (Srisimarat *et al.*, 2011) and active site characterization has not been resolved while THAM, though showed 99% amino acid sequence identity to *T.thermophilus*, has a unique alkaline pH optimum (Sawasdee *et al.*, 2012). MeDPE1 was left out in most experiment except for substrate specificity due to very low yield of purified enzyme obtained. Secondly, NBS significantly inhibited all AMs tested (Table 3.2). In the modification, all AMs were treated with IC_{50} concentrations of NBS. Only in the pI determination, CGAM was modified with NBS both at IC_{50} (0.3 mM) and at 5 mM which completely inhibited the activity.

3.6.1 Determination of pI

Native and NBS-modified AMs were analyzed for their pIs by electrofocusing gel (Figure 3.12), the pI standard curve was as shown in Figure 3.13. When treated with 0.3 or 0.5 mM NBS (IC_{50} concentrations for CGAM or THAM, respectively), both native and NBS-modified AMs displayed the same pI of 6.9 and 5.8 for CGAM and THAM, respectively, (Figure 3.9, m-II, (a) and (b)). For CGAM, when 5 mM NBS which resulted in a complete loss of enzyme activity (Table 3.2) was used, the protein band was disappeared (Figure 3.9, m-I, (a)). This result was corresponded to the result from native-PAGE analysis of the NBS-modified CGAM stained for protein and activity (Figure 3.4).

Table 3.7 Inactivation kinetics of AMs with NBS, EDC and SAH

Modifying reagent	CGAM	THAM	MeDPE1
1. NBS 1.1 IC_{50} (mM) 1.2 k_{inact} (min^{-1})	0.3 4.58	0.5 2.49	8.0 6.83
2. EDC 2.1 IC_{50} (mM) 2.2 k_{inact} (min^{-1})	10 3.31	75 2.48	30 6.27
3. SAH 3.1 IC_{50} (mM) 3.2 k_{inact} (min^{-1})	4.5 1.52	150 2.39	3.0 4.06

IC_{50} = 50 % inhibitory concentration of modifying reagent

k_{inact} = pseudo-first order rate of inactivation = 0.693/ $t_{1/2}$
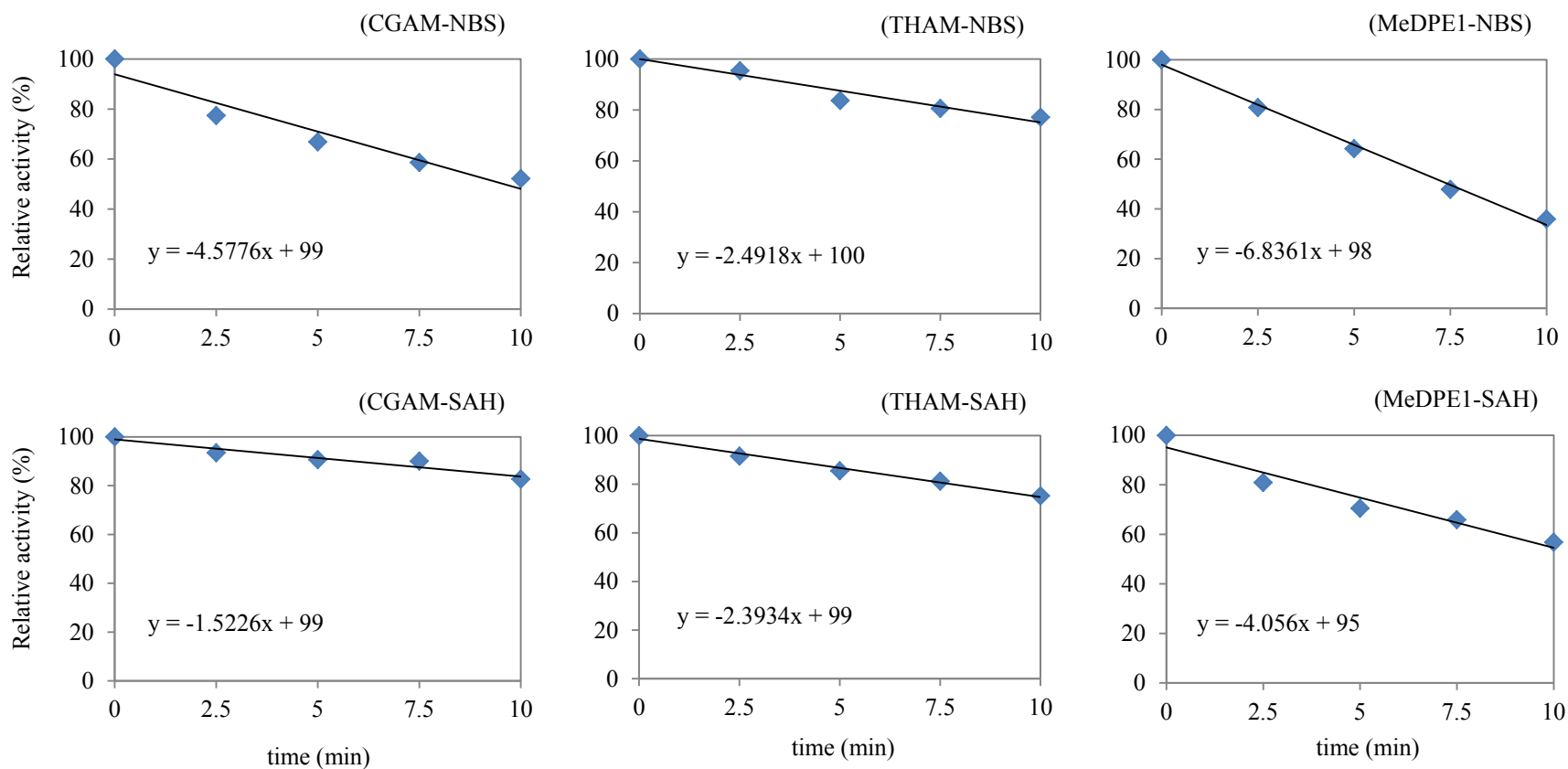
Figure 3.8 Inactivation of AMs with selected modifying reagents at various incubation times. All of native and modified AMs were prepared as described in section 2.10 by treatment with IC_{50} concentrations of NBS, SAH or EDC. The slope of each line indicates the pseudo first-order rate constant of inactivation (k_{inact}).
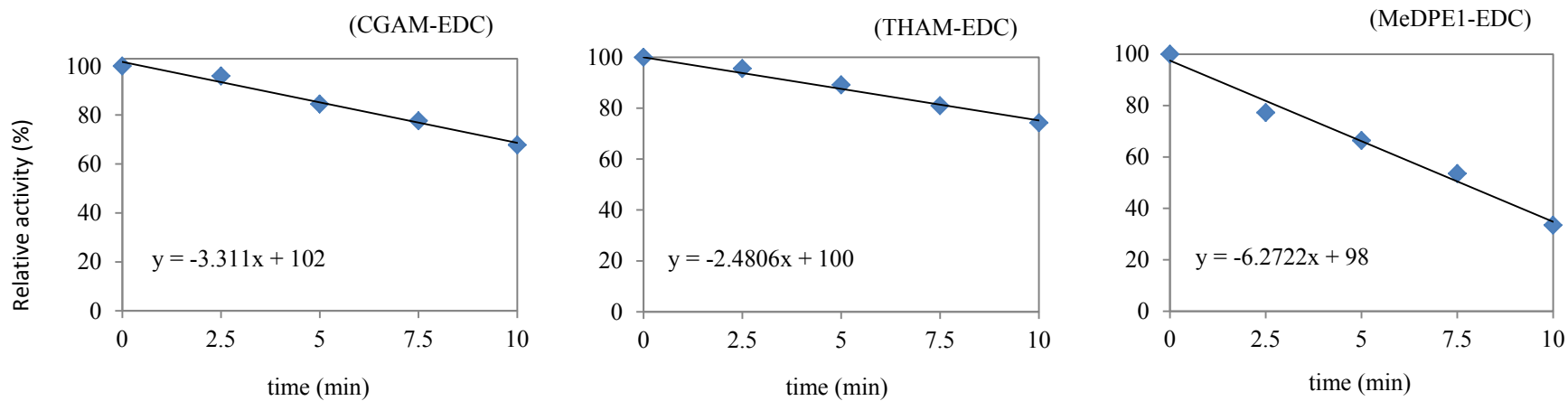
Figure 3.8 (continued) Inactivation of AMs with selected modifying reagents at various incubation times. All of native and modified AMs were prepared as described in section 2.10 by treatment with IC_{50} concentrations of NBS, SAH or EDC. The slope of each line indicates the pseudo first-order rate constant of inactivation (k_{inact}).
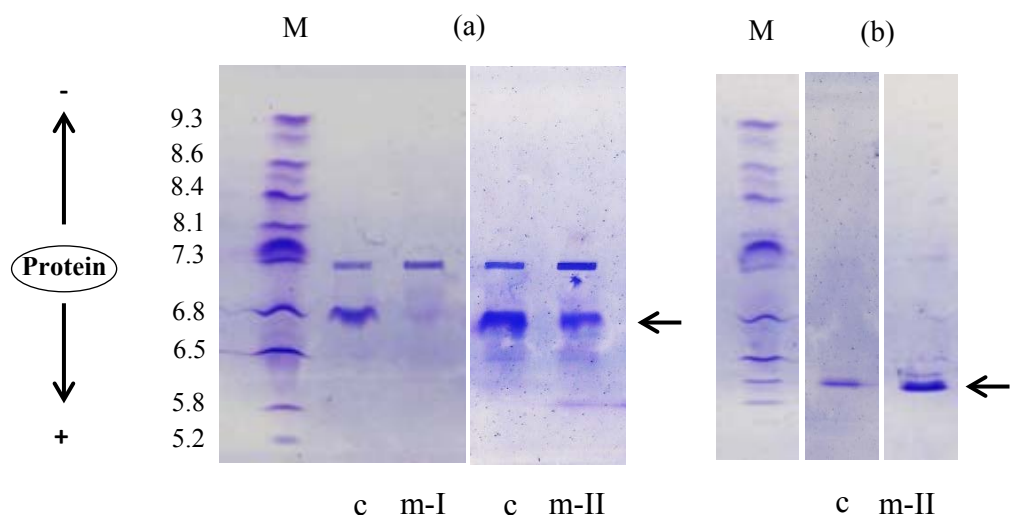
Figure 3.9 The isoelectric point (pI) of native (c) and NBS-modified AM (m), (a) CGAM and (b) THAM. For CGAM, the activity was completely inhibited by 5 mM NBS (m-I) or 50% inhibited (m-II) by 0.3 mM NBS. For THAM, 50% of activity was inhibited by 0.5 mM NBS (m-II).

The arrow represents a determined pI of native and NBS-modified AM.

Lane M   =   Standard pI protein markers;

Trypsinogen (pI 9.30)   Horse myoglobin-acidic band (pI 6.85)

Lentil lectin-basic band (pI 8.65)   Human carbonic anhydrase B (pI 6.55)

Lentil lectin-middle band (pI 8.45)   Carbonic anhydrase B (pI 5.85)

Lentil lectin-acidic band (pI 8.15)   β-lactoglobulin A (pI 5.20)

Horse myoglobin-basic band (pI 7.35)

Panel a, lane c   = 3 μg protein of native CGAM

    lane m-I   = 3 μg protein of NBS-modified CGAM

        (total activity loss)

    lane m-II   = 3 μg protein of NBS-modified CGAM

        (50% relative activity remained)

Panel b, lane c   = 3 μg protein of native THAM

    lane m-I   = 3 μg protein of NBS-modified THAM
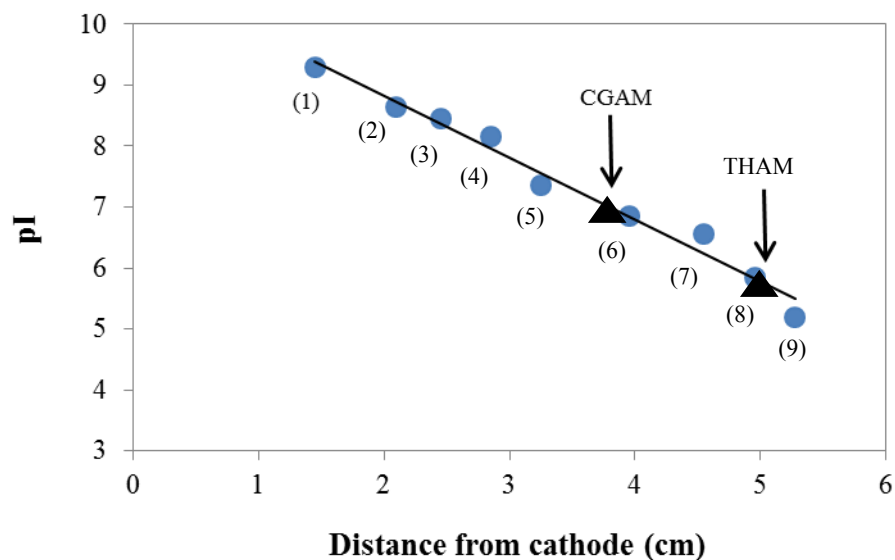
Figure 3.10 The pI calibration curve of standard protein markers

The marginal distance of pI protein markers was determined from cathode and plotted against pI value

(1)	=	Trypsinogen	pI 9.30
(2)	=	Lentil lectin-basic band	pI 8.65
(3)	=	Lentil lectin-middle band	pI 8.45
(4)	=	Lentil lectin-acidic band	pI 8.15
(5)	=	Horse myoglobin-basic band	pI 7.35
(6)	=	Horse myoglobin-acidic band	pI 6.85
(7)	=	Human carbonic anhydrase B	pI 6.55
(8)	=	Carbonic anhydrase B	pI 5.85
(9)	=	β-lactoglobulin A	pI 5.20

Arrow represents estimated pIs of CGAM and THAM.

3.6.2 Determination of substrate specificity and kinetics of AMs

To compare substrate specificity of the native and NBS-modified AMs, the enzyme solution was incubated with short chain maltooligosaccharide substrate (G2-G7) and the activity was measured by disproportionation reaction as described in section 2.11.2.1. The glucose concentration released in the reaction was detected by glucose oxidase method. The results showed that native and NBS-modified of all AMs preferred maltotriose (G3) as the best substrate for this reaction (Figure 3.11.1). However, there was some difference in the order of substrates with low activity between the native and modified CGAM. The decreased order of substrate was G3 > G4 > G5 > G6 ≈ G7 ≈ G2 for native CGAM but G3 > G4 > G5 > G7 > G6 ≈ G2 for modified enzyme (Figure 11.1, a). While both forms of THAM and MeDPE1 showed similar result. When compared to CGAM, the difference was in the order of substrates with low activity; THAM prefers G6 > G7 > G2, while MeDPE1 shows the preferred order of G6 > G2 > G7 (Figure 3.11.1, b and c).

The substrate specificity for starch transglucosylation reaction was also determined. The reaction was performed as described in section 2.11.5.2. From the results, glucose (G1) was the most preferred substrate for both AMs (CGAM and THAM) (Figure 3.11.2). For native and NBS-modified CGAM, the decreasing order of substrate of CGAM was about the same (G1 > G2 ≈ G3 > G4 > G5 > G6 > G7). For THAM, substrate order of the native enzyme was G1 > G3 > G2 ≈ G4 > G5 > G6 > G7 while the NBS modified THAM showed the order of G1 > G3 > G4 > G5 ≈ G2 > G6 > G7.

To determine the rate of the catalyzed reaction by native and NBS-modified (IC_{50}*) CGAM and the inhibitory effect of acarbose, disproportionation and starch transglucosylation reactions were investigated. For kinetics of disproportionation reaction, various concentrations of maltotriose substrate was incubated with CGAM for 10 min in the absence or presence of acarbose inhibitor (Figure 3.12.1). The concentration of acarbose which gave a 50% remaining activity compared to the reaction without acarbose was selected. For starch transglucosylation reaction, various concentrations of glucose (G1) substrate which was used as glucosyl acceptor (soluble starch as glucosyl donor) were incubated with the enzyme for 10 min in the absence or presence of acarbose inhibitor (Figure 3.12.2). Kinetic parameters were calculated from Lineweaver-Burk plots and shown in Table 3.9.

The apparent K_m and k_{cat} values of native CGAM for G3 in disproportionation reaction (19.88 mM and 228 x 10^3 min^{-1}) was significantly higher than that for G1 in starch transglucosylation reaction (0.04 mM and 274 min^{-1}), and the k_{cat}/K_m value was also higher (11.5 x 10^3 compared to 6.85 x 10^3 mM^{-1} min^{-1}). The kinetic parameters for the NBS-modified CGAM were significantly different from the native enzyme. The catalytic efficiency of the modified enzyme for disproportionation and starch transglucosylation reactions decreased nearly 9- and 2- fold, respectively. In the presence of acarbose, the catalytic efficiency (k_{cat}/K_m) of both forms of enzyme in both reactions profoundly decreased. However, inhibitory action seemed to be differ, mixed inhibition in disproportionation but competitive inhibition in starch transglucosylation reaction, respectively. When [acarbose] was varied, inhibition constant K_i was determined (Figure 3.12.3). It was found that the K_i values of native

and modified CGAM were 70.9 and 156.9 mM in the disproportionation reaction and 0.55 and 1.15 mM in the starch transglucosylation reaction, respectively (Table 3.8).

3.6.3 Determination of the number of essential Trp residues of AMs and analysis of native and modified AMs

3.6.3.1 Using spectrophotometry

Titration of AMs with NBS in the absence or presence of G3 substrate was performed in order to determine the number of essential Trp at or around the active site (Kaper *et al.*, 2007). From the result (Figure 3.13 a), in the absence of G3, modification of about 0.8 Trp residue in CGAM led to almost total activity lost. THAM gave a different result (Figure 3.13, b), about four Trp residues were modified in the absence of G3 while only one residue was modified when G3 was present (see Appendix 11 for the calculated number of modified-Trp residue at each incubation time).

3.6.3.2 Using MALDI-TOF mass spectrometry

The mass of native and NBS-modified AMs was analyzed by MALDI-TOF (Figure 3.9.1 and 3.9.2). The mass of 80,896 Da (Figure 3.14.1, a) obtained for native CGAM was slightly lower than the mass of NBS modified CGAM which was 81,244 Da (Figure 3.14.2, b). While the mass of native and modified THAM were 58,708 Da and 58,842 Da, respectively (Figure 3.14.2, a and b). After NBS modification, the mass of CGAM and THAM was increased about 348 and 134 Da, respectively.
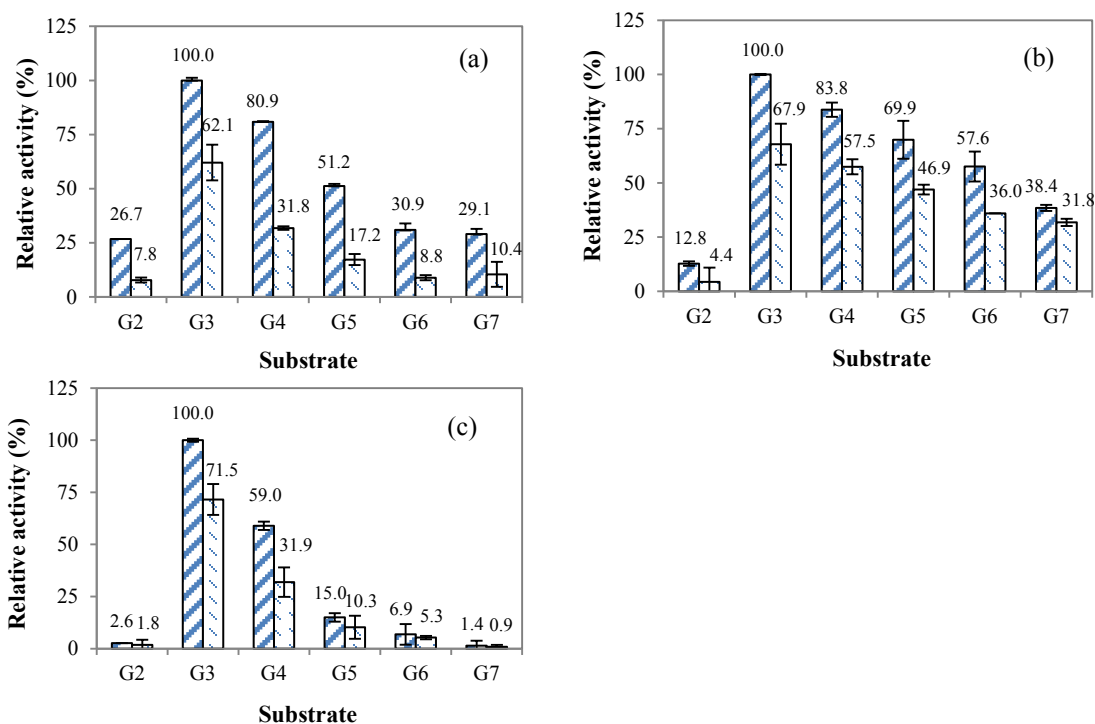
Figure 3.11.1 Substrate specificity of native (▨) and NBS-partially modified (▨) AMs in disproportionation reaction, CGAM (a), THAM (b) and MeDPE1 (c). NBS concentrations used to treat AMs were at IC_{50} for each enzyme. The activity of native AMs for G3 was set as 100%.
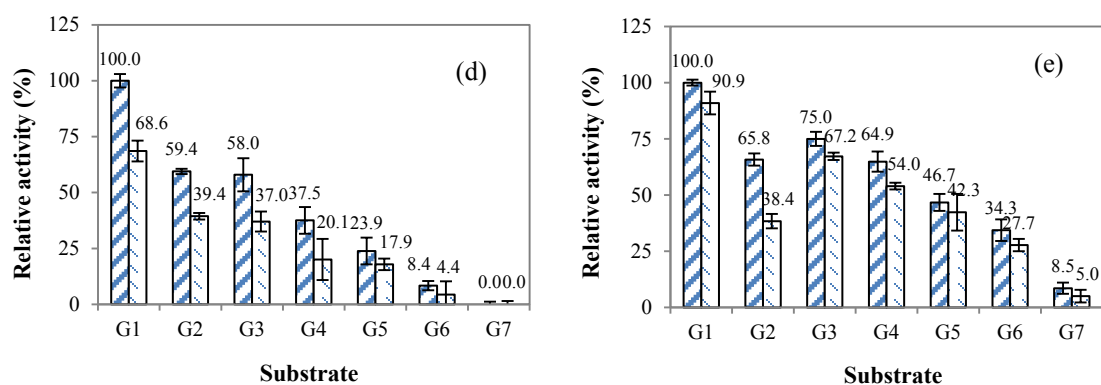
Figure 3.11.2 Substrate specificity of native (▨) and NBS-partially modified (▨) AMs in starch transglucosylation reaction, CGAM (d) and THAM (e). NBS concentrations used to treat AMs were at IC_{50} for each enzyme. The activity of native AMs for G3 was set as 100%.

IC_{50} = inhibitory concentration of modifying reagent at which 50% of activity is remained.

(a)

$y = 18.217x + 0.6416$
$R^2 = 0.9964$

$y = 11.091x + 0.5579$
$R^2 = 0.9967$

1/ v
(ug glucose/min/ug protin)

1/ [G3] mM^{-1}

(b)

$y = 170.36x + 36.989$
$R^2 = 0.9602$

$y = 92.514x + 18.105$
$R^2 = 0.9693$

1/ v
(ug glucose/min/ug protein)

1/ [G3] mM^{-1}
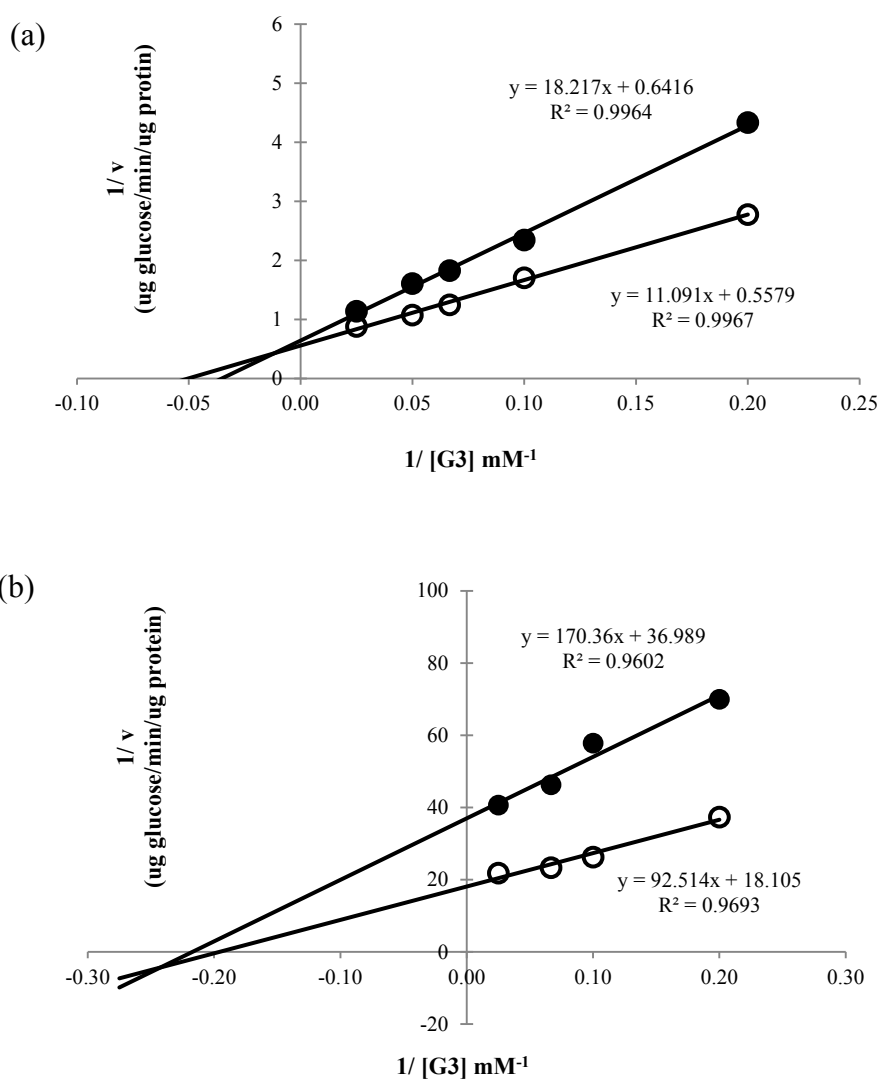
Figure 3.12.1 Lineweaver-Burk plot of native (a) and NBS-modified (*IC_{50}) (b) CGAM with maltotriose substrate in disproportionation reaction in the absence (opened circle) or presence (closed circle) of 10 mM (a) or 5 mM (b) of acarbose. Glucose released was detected by glucose oxidase method.

IC_{50} = inhibitory concentration of modifying reagent at which 50% of activity is remained.
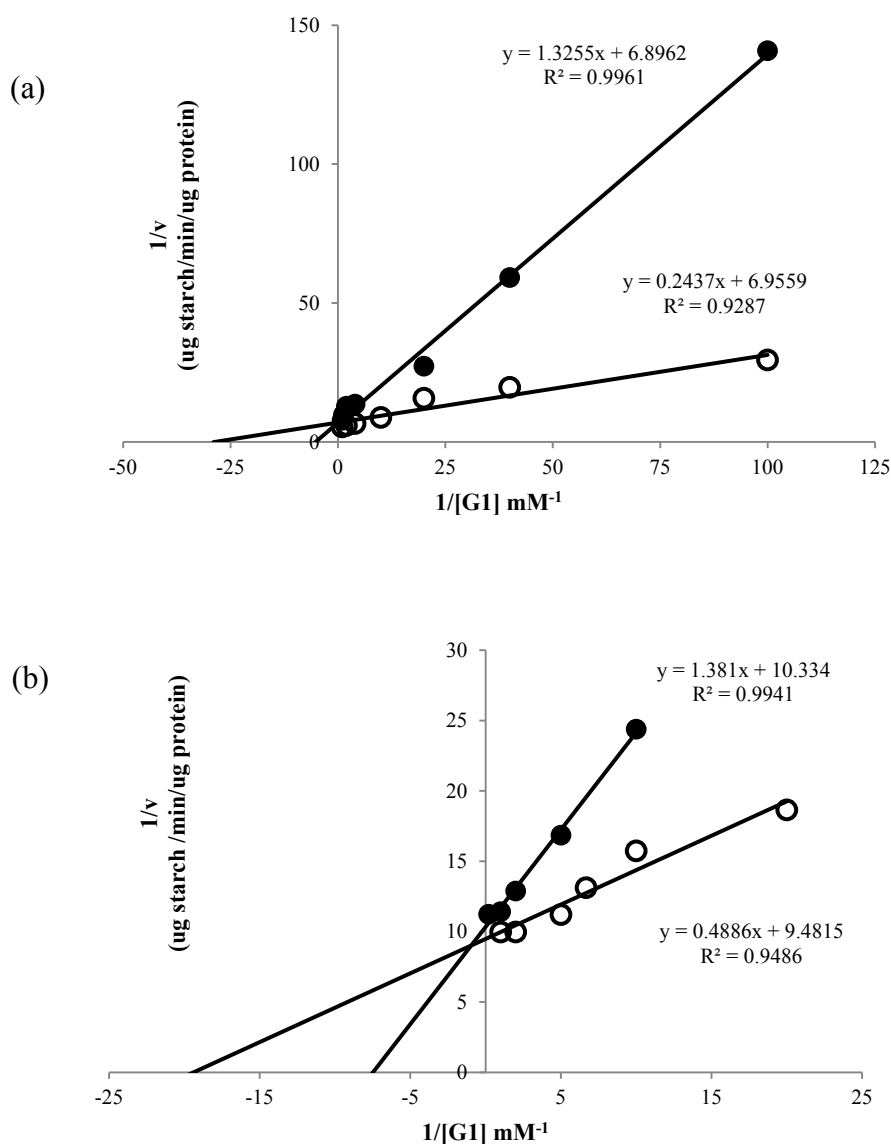
Figure 3.12.2 Lineweaver-Burk plot of native (a) and NBS-modified (*IC$_{50}$) (b) CGAM with glucose as acceptor substrate and soluble starch as glycosyl donor in starch transglucosylation reaction in the absence (opened circle) or presence (closed circle) of 0.75 mM (a) or 0.5 mM (B) of acarbose. Starch remained was detected by iodine method.

IC$_{50}$ = inhibitory concentration of modifying reagent at which 50% of activity is remained.

(a)

$y = 0.0195x + 3.061$
$R^2 = 0.9846$

$y = 0.0262x + 1.8573$
$R^2 = 0.9206$

1/v
(ug glucose/min/ug protein)

acarbose (mM)

(b)

$y = 12.908x + 14.874$
$R^2 = 0.9347$

$y = 13.534x + 7.4939$
$R^2 = 0.9879$

1/v
(ug starch/min/ug protein)
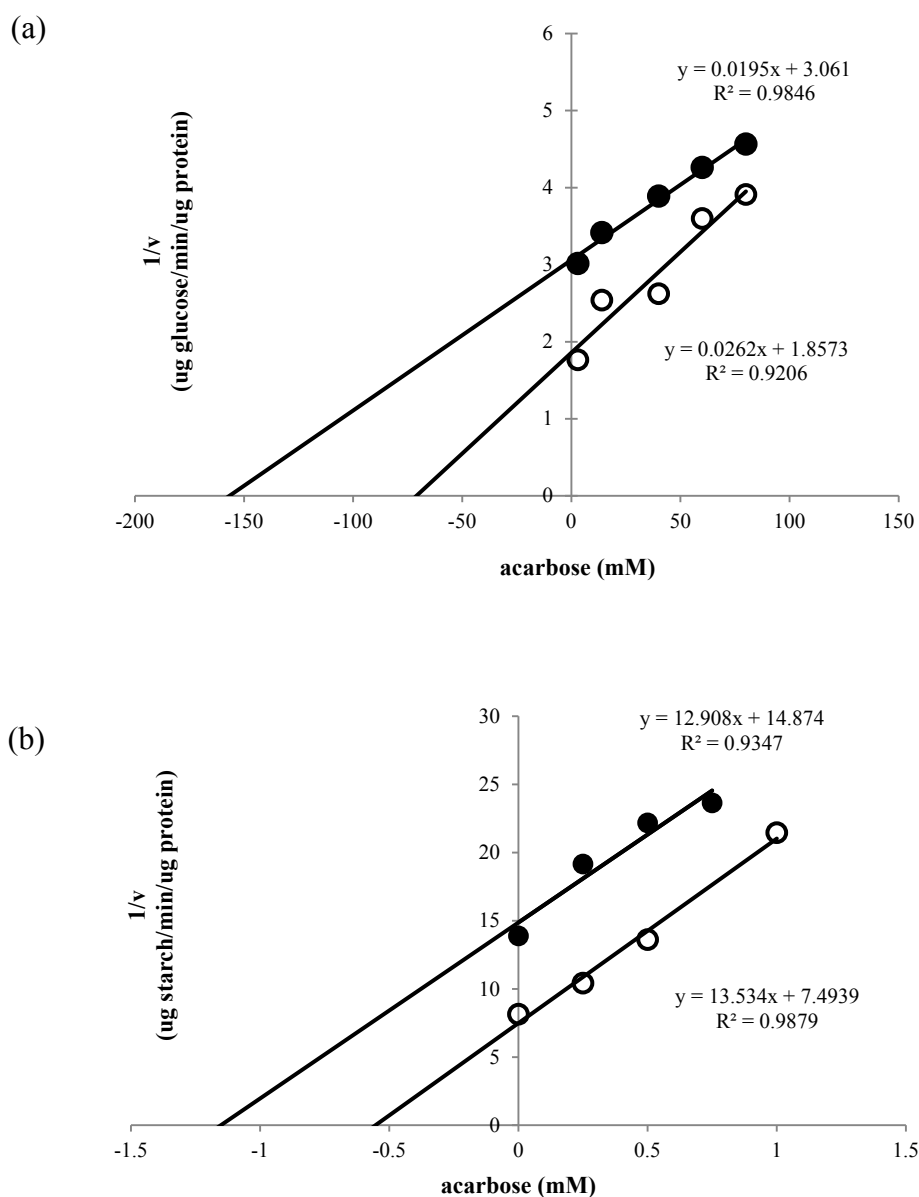
acarbose (mM)

Figure 3.12.3 The plot between acarbose concentration and 1/v of native (opened circle) and NBS-modified (closed circle) CGAM at fixed concentration of G3 substrate (5 mM) in the disproportionation reaction (a) and fixed concentration of G1 substrate (0.1 mM) in the starch transglucosylation reaction (b). NBS used to modify CGAM was at IC_{50} concentration.

Table 3.8 Kinetic parameters of native and NBS-modified (*IC$_{50}$) CGAM on maltotriose substrate (disproportionation activity) (a) or on glucose as acceptor substrate (starch transglucosylation activity) (b).

CGAM (a)	K_m (mM)	Vmax (μg glucose.min^{-1} .μg protein^{-1})	k_{cat} (10^3) (min^{-1})	k_{cat}/K_m (10^3) (mM^{-1}min^{-1})	K_i (mM)
Native CGAM (without acarbose)	19.88	1.79	228	11.5	-
Native CGAM (with 10 mM acarbose)	28.38	1.56	198	7.0	70.9
NBS-modified CGAM (without acarbose)	5.08	0.06	7.03	1.4	-
NBS-modified CGAM (with 5 mM acarbose)	5.57	0.03	3.44	0.6	156.9

CGAM (b)	K_m (mM)	Vmax (ug starch.min^{-1} .ug protein^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (10^3) (mM^{-1}min^{-1})	K_i (mM)
Native CGAM (without acarbose)	0.04	0.14	274	6.85	-
Native CGAM (with 0.75 mM acarbose)	0.19	0.14	277	1.46	0.55
NBS-modified CGAM (without acarbose)	0.05	0.10	201	4.02	-
NBS-modified CGAM (with 0.5 mM acarbose)	0.13	0.10	185	1.42	1.15

IC$_{50}$ = inhibitory concentration of modifying reagent at which 50% of activity is remained.
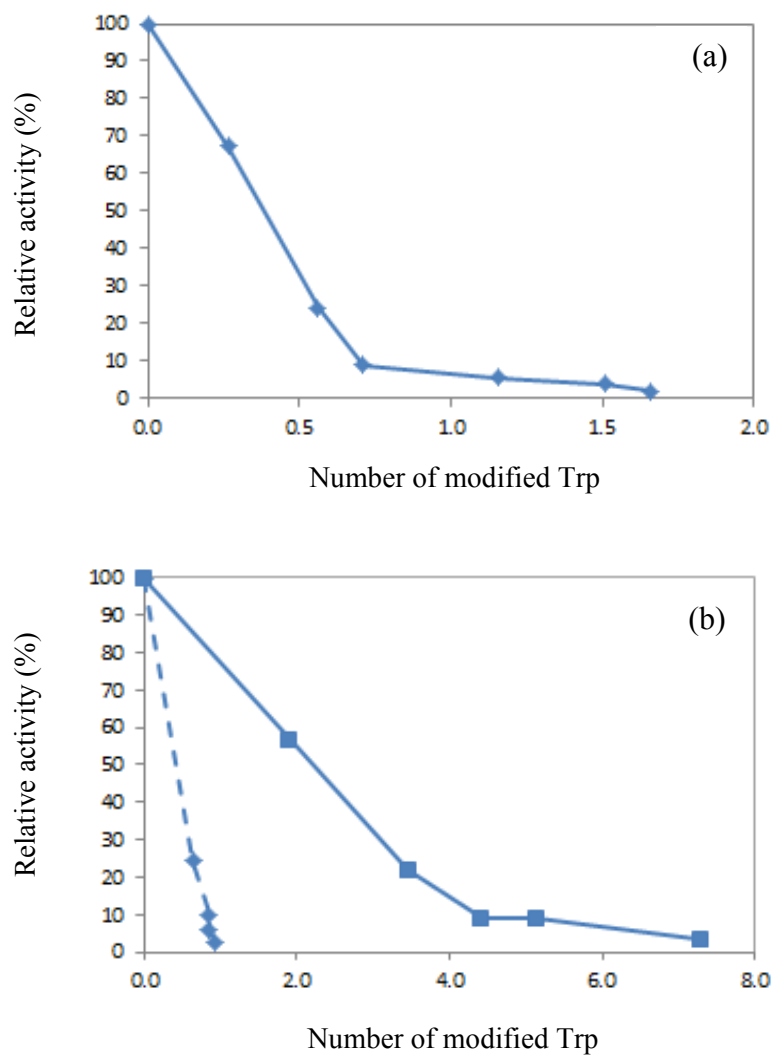
Figure 3.13 Titration of AMs with NBS in the absence (solid line) or presence (dash line) of G3. (a) CGAM and (b) THAM.
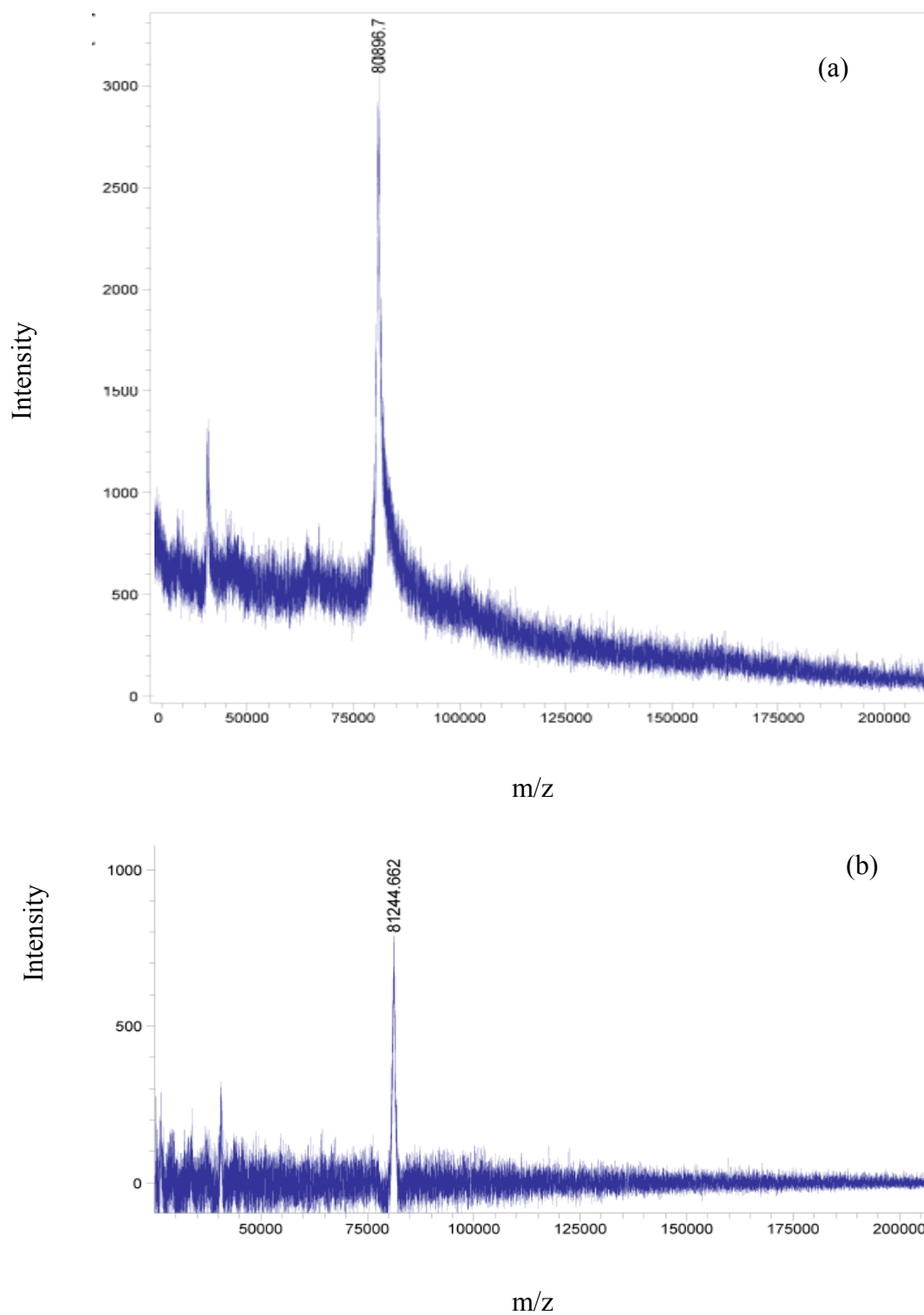
Figure 3.14.1 MALDI-TOF mass spectra of native CGAM (a) and NBS modified (*IC$_{50}$) CGAM (b).
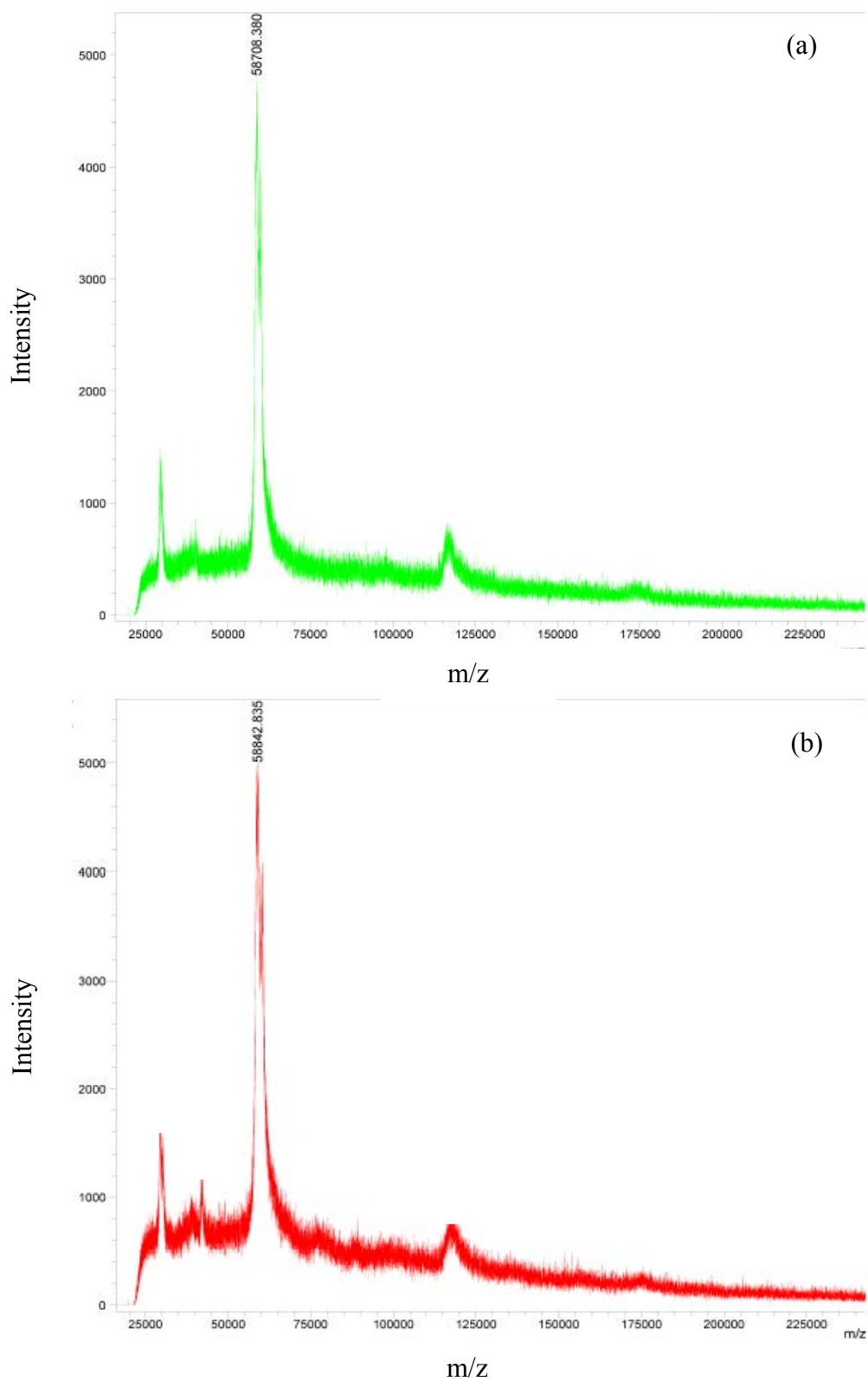
Figure 3.14.2 MALDI-TOF mass spectra of native THAM (a) and NBS modified

(*IC$_{50}$) THAM (b)

3.6.4 Circular dichroism spectra

The secondary structure of the native and NBS-modified AMs was compared by analysis of the CD spectra. From the result, NBS-modified CGAM showed a slightly different spectrum from the native enzyme (Figure 3.15, a), while no significant change was recognized in CD spectrum after modification in the case of THAM (Figure 3.15, b). For MeDPE1, it is difficult to do this experiment because too low protein concentration was obtained in the preparation of purified enzyme. The content of secondary protein structure was predicted by K2D3 online server program. The β-sheet content of the native and NBS-modified AMs was not different, CGAM showed 22.68 and 23.59% while THAM showed 11.60 and 11.17%, respectively. In contrast, α-helix of NBS-modified CGAM and THAM was slightly increased about 2-3% (Table 3.9).

3.6.5 Fluorescence spectroscopy

To analyze the difference in the Trp environment among the AMs, fluorescence spectra were compared. The fluorescence emission maxima of native CGAM (a) and THAM (b) was at 345 nm. A decrease in the fluorescence emission was observed when Trp residue (s) of AMs were modified by NBS (Figure 3.16, a). Modification of Trp residue in CGAM shifted the emission maximum to 352 nm and decreased the fluorescence intensity. For THAM, the emission maximum was not changed, only a decrease in fluorescence intensity was observed.
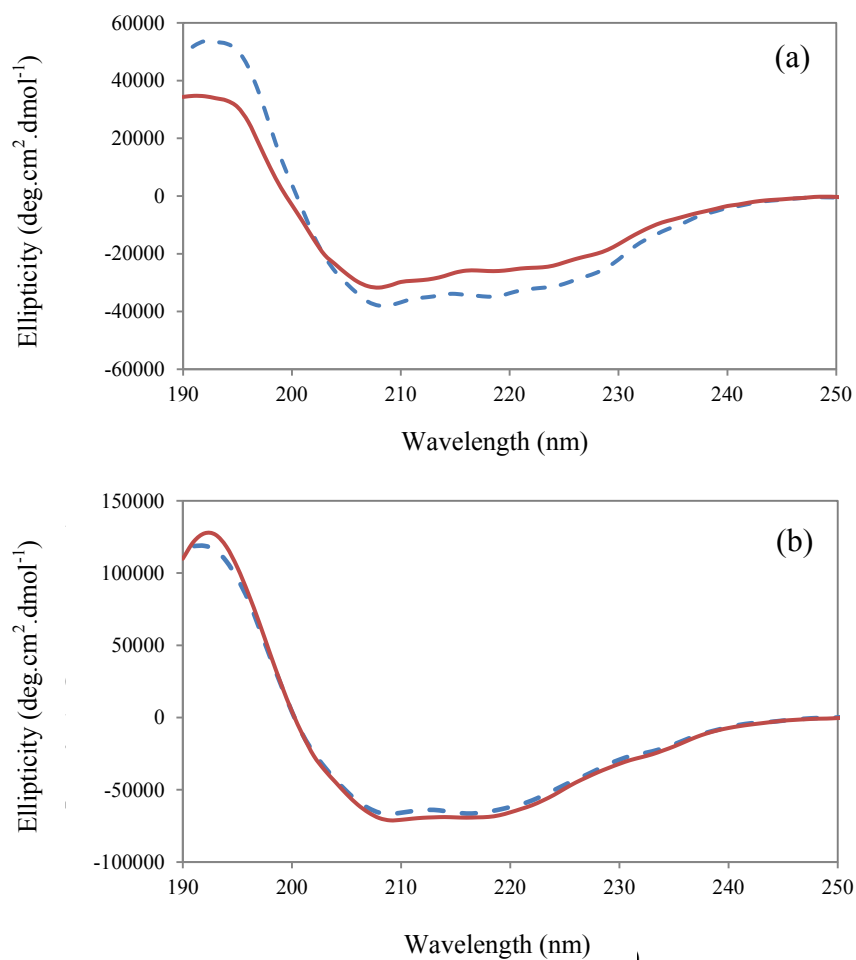
Figure 3.15 CD spectra of native (dash line) and NBS-modified (*IC_{50}) (solid line) AM. (a) CGAM and (b) THAM.

Table 3.9 The content of predicted secondary structure of AMs

AM	Percent	
	β-sheet	α-helix
Native CGAM	22.68	24.56
NBS-modified CGAM (*IC_{50})	23.59	22.77
Native THAM	11.60	44.05
NBS-modified THAM (*IC_{50})	11.17	47.36

Using $\Delta\varepsilon$ mode (molar circular dichroism), predicted by K2D3 program
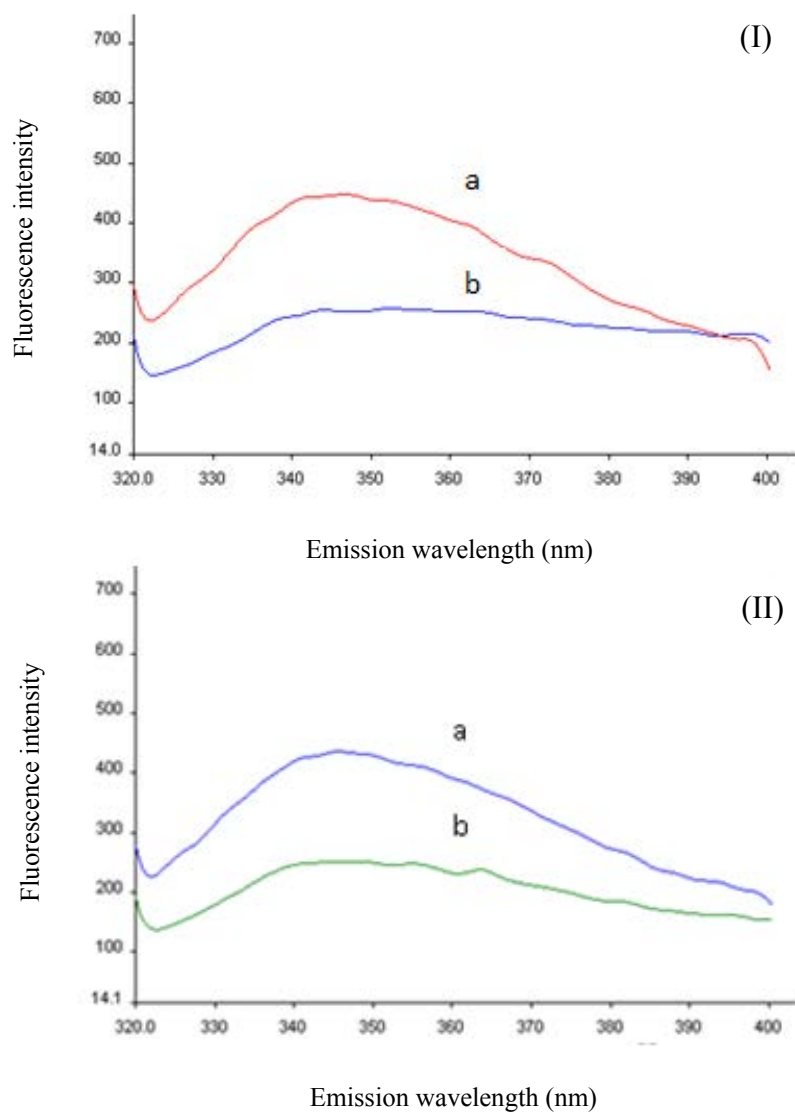
Figure 3.16 Fluorescence emission spectra of the native (a) and modified (b) AM, (I) CGAM, (II) THAM. Excitation was at 295 nm, and emission spectra were recorded between 320 and 400 nm.

3.6.6 Analysis of oligosaccharide products

3.6.6.1 Analysis of linear maltooligosaccharides

The ability to synthesize linear oligosaccharide products of the native and NBS-modified CGAM was compared by TLC and HPAEC. The result from TLC (Figure 3.17) was rather difficult to analyze for differences. From HPAEC, it was found that the product pattern of native and NBS-modified CGAM was similar (Figure 3.18.1-3.18.2). The major product of both forms of CGAM at 4 hours incubations was found in the range of G1 to around G18 with glucose as maximum. The decreased order of products for both native and NBS-modified CGAM was G1 > G3 > G2 > G4 > G5 > G6 > G7 > G8 > G9 > G10 with G3 substrate (Figure 3.18.1, I-a and I-d). When G4 substrate was used, both forms of CGAM showed G1 > G3 > G2 ≈ G4, while the amount of G5 to G10 was about the same (Figure 3.18.1, I-b and I-e). When G5 substrate was used, both forms of CGAM showed G1 > G3 > G2 ≈ G4 ≈ G5, while G6 to G10 was similarly produced (Figure 3.18.1, I-c and I-f). When increased enzyme solution form 1 to 2 U, both forms of native and NBS-modified CGAM still gave the same product order, however, the order was changed to G1 > G2 ≈ G3 > G4, G1 > G2 > G3 > G4 or G1 > G3 > G2 > G4 with G3, G4 or G5 substrate, respectively (Figure 3.18.2). For the products higher than G10, they could not be separated in this column condition.

3.6.6.2 Analysis of large-ring cyclodextrins (LR-CDs)

The ability to form LR-CD product of the native and NBS-modified CGAM or THAM was compared. It was found that the product pattern of native and NBS-modified CGAM was similar (Figure 3.19.1-3.19.2). At 4 hours incubation, the major

large-ring cyclodextrin product of both forms of CGAM was CD24-CD32 with CD27-28 as maximum while THAM gave CD22-CD28 with CD23-24 as maximum. The smallest LR-CDs which were produced from both forms of CGAM and THAM were CD19 and CD20, respectively. The quantity of LR-CDs formed from native and NBS-modified CGAM was about the same, with values of 215.6 and 213.2 mg LR-CDs.g starch^{-1}. In contrast, native and NBS-modified THAM yielded different LR-CDs formed, with the amount of 269.7 and 198.7 mg LR-CDs.g starch^{-1}, respectively.
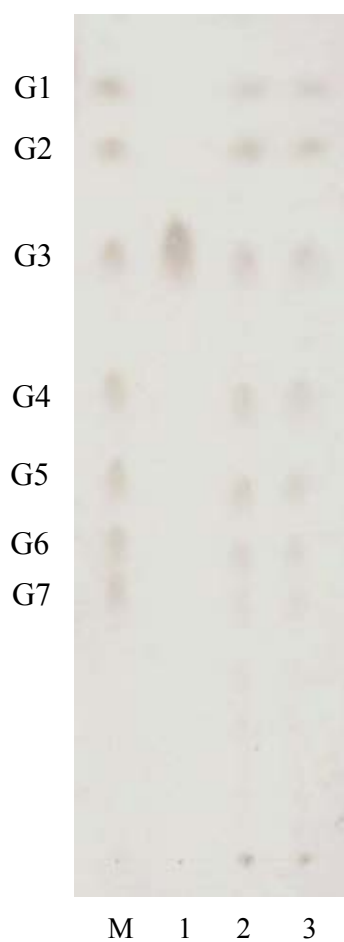
Figure 3.17 TLC chromatogram of products from native or NBS-modified CGAM. The reaction mixture contained 0.25 U of enzyme solution (starch transglucosylation activity) and 0.2% (w/v) maltotriose at 30 ºC for 2 h.

Lane M	=	standard G1-G7
Lane 1	=	without CGAM
Lane 2	=	native CGAM
Lane 3	=	NBS-modified CGAM
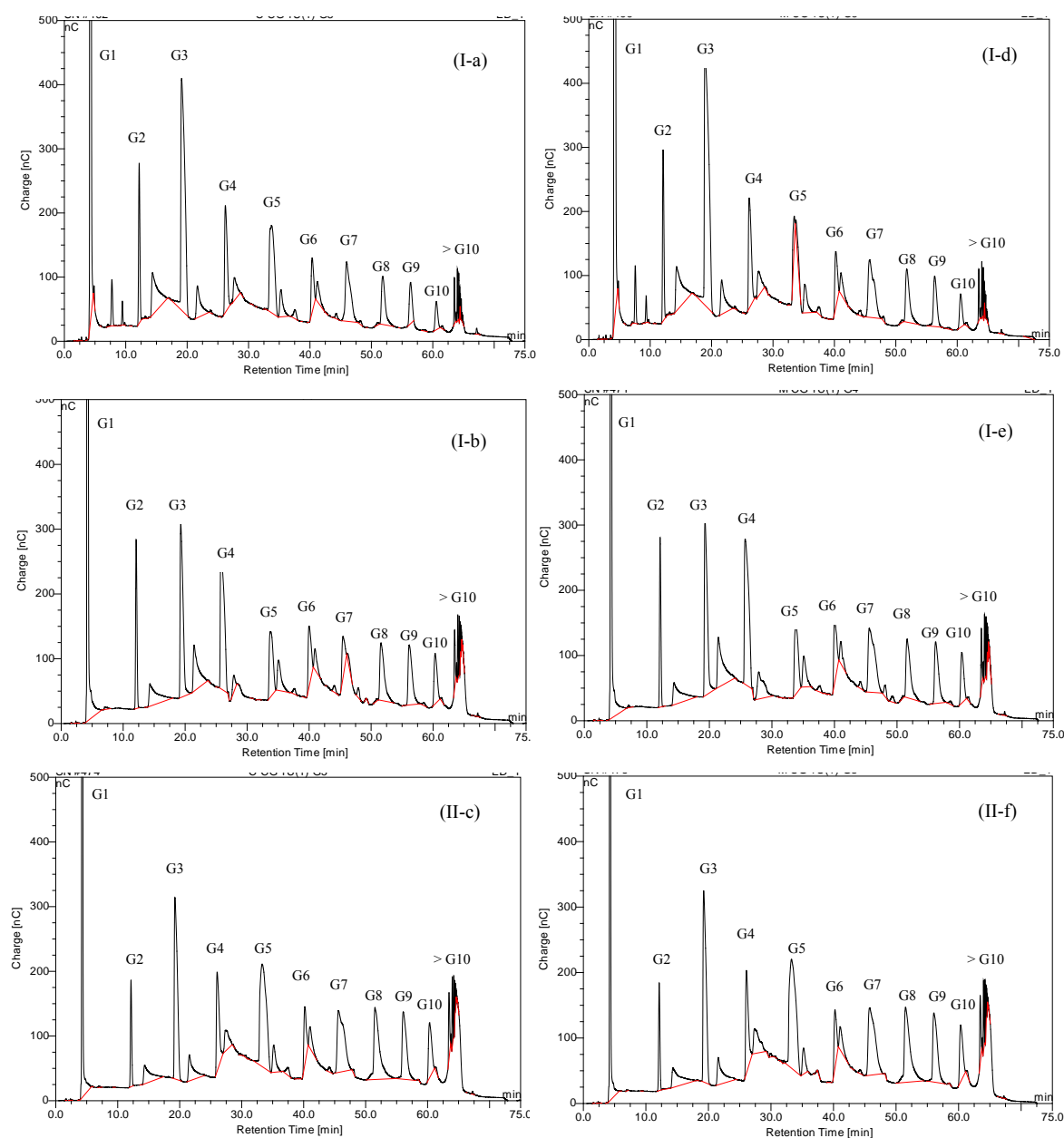
Figure 3.18.1 HPAEC-PAD analyses of synthesized linear oligosaccharides by native (I, a-c for G3, G4 and G5 substrate) and NBS-modified (IC_{50}*) CGAM (I, d-f for G3, G4 and G5). The G3/G4/G5 at 50 mM concentration was incubated with 1 U (starch transglucosylation activity assay) of native and NBS-modified CGAM at 30 ºC for 4 hours. Peak number indicates the analyzed linear oligosaccharide by comparison of R_t with standard G1 to G7.
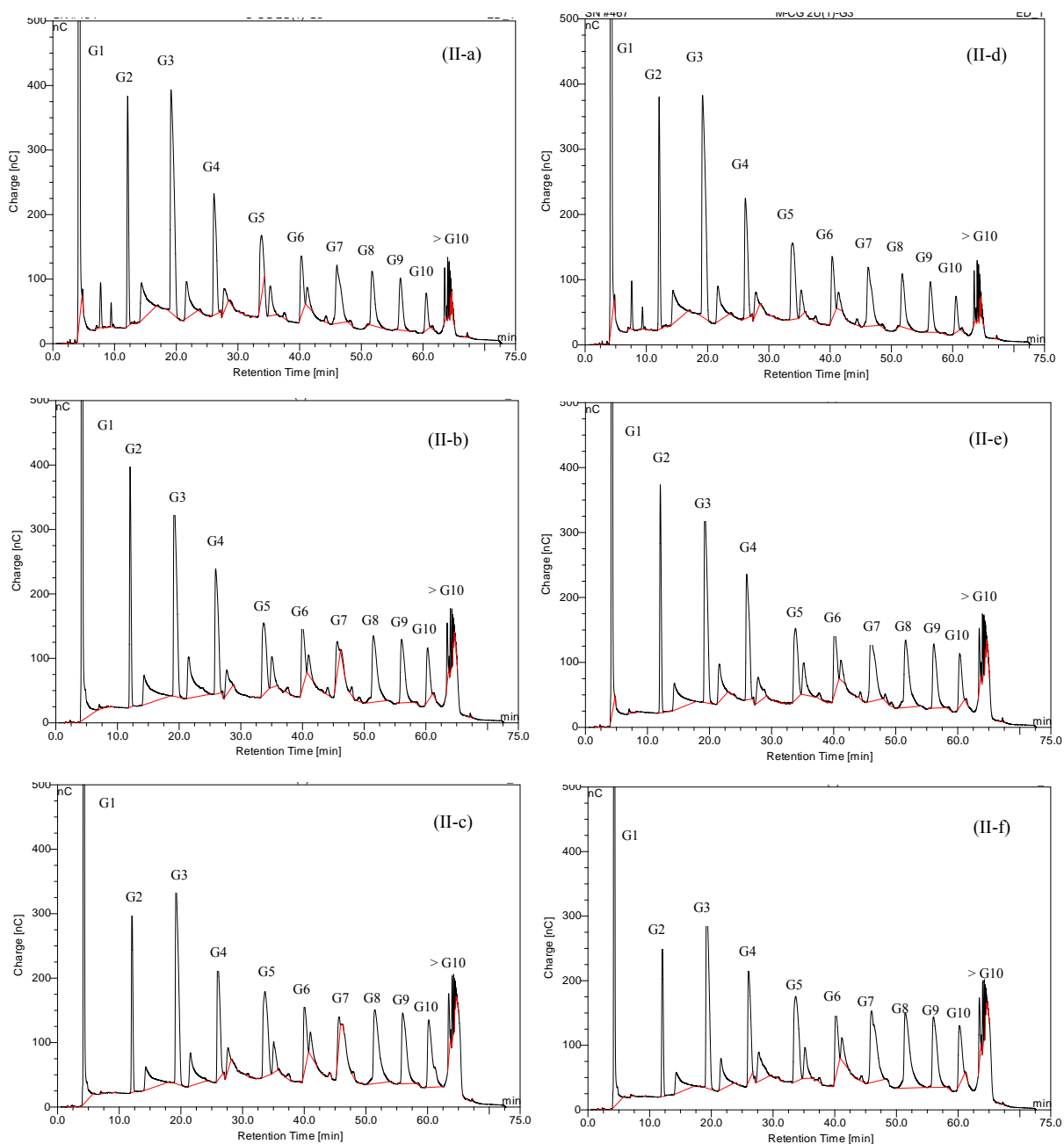
Figure 3.18.2 HPAEC-PAD analyses of synthesized linear oligosaccharide by native (II, a-c for G3, G4 and G5 substrate) and NBS-modified (IC$_{50}$*) CGAM (II, e-f for G3, G4 and G5). The G3/G4/G5 at 50 mM concentration was incubated with 2 U (starch transglucosylation activity assay) of native and NBS-modified CGAM at 30 ºC for 4 hours. Peak number indicates the analyzed linear oligosaccharide by comparison of R$_t$ with standard G1 to G7.
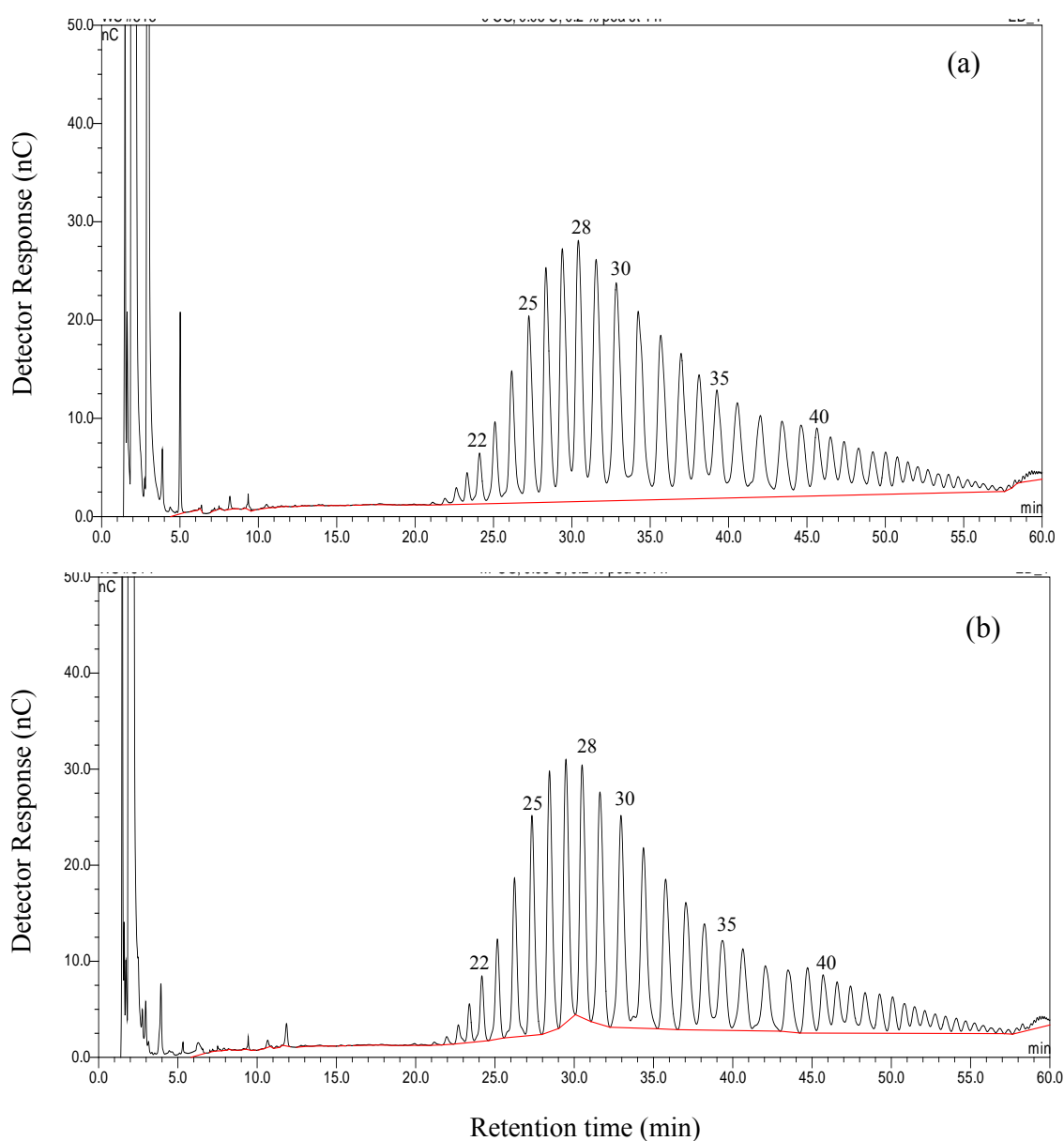
Figure 3.19.1 HPAEC-PAD analyses of synthesized large-ring cyclodextrins by native (a) and NBS-modified (IC_{50}*) CGAM (b). The pea starch solution (0.2%, w/v) was incubated with 0.05 U (starch-degrading activity assay) of native and NBS-modified CGAM at 30 ºC for 4 hours. Peak number indicates the degree of polymerization of LR-CDs by comparison of R_t with standard LR-CDs.

*IC_{50} = inhibitory concentration of modifying reagent at which 50% of activity is remained.
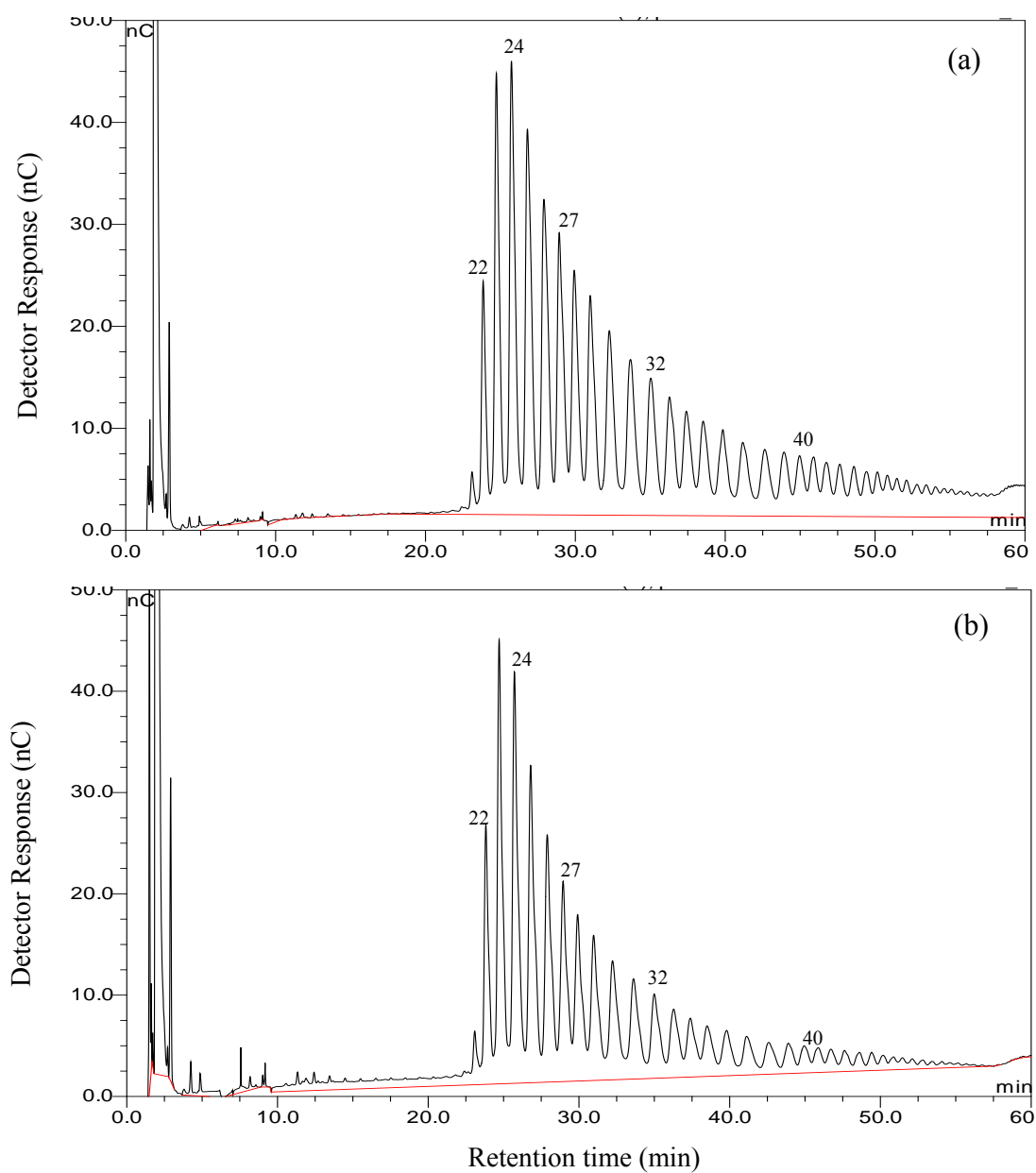
Figure 3.19.2 HPAEC-PAD analyses of synthesized large-ring cyclodextrins by native (a) and NBS-modified (IC$_{50}$*) THAM (b). The pea starch solution (0.2%, w/v) was incubated with 0.05 U (starch-degrading activity assay) of native and NBS-modified THAM at 30 ºC for 4 hours. Peak number indicates the degree of polymerization of LR-CDs by comparison of R$_t$ with standard LR-CDs.

* IC$_{50}$ = inhibitory concentration of modifying reagent at which 50% of activity is remained,

CHAVTER IV

DISCUSSIONS

4.1 Expression and purification of AMs from 3 sources

Two bacterial AMs and a cassava AM were used to compare active site residues in this study. The recombinant AM gene from *C. glutamicum* (CgAM) (Srisimarat *et al.*, 2011), from soil DNA which was proved to be highly homologous to that of *T. thermophilus* (AMY) (Sawasdee *et al.*, 2012) and the gene of *Manihot esculenta* D-enzyme (DPE1) (Tantanarat, 2012) was cloned and expressed as described. *E. coli* has been commonly used as host for AM expression. Gene expression was induced by appropriate concentration of IPTG for each AM source. All expressed AMs are intracellular enzyme as previously reported (Kang *et al.*, 2010). We named the three AMs as CGAM, THAM, and MeDPE1. All recombinant AMs in this study contain histidine residues tagged at the N-terminal. After expression, crude AMs was purified by Histrap column which contains agarose bead with an immobilized chelating group. The bead is charged with nickel ion which can bind histidine-tag protein (Bang *et al.*, 2006; Srisimarat, 2010). We obtained CGAM with two times higher in specific activity than the previouly reported CgAM (Srisimarat *et al.*, 2011). But THAM and MeDPE1 expression and purification were with less efficiency, shows lower than AMY and DPE1 about 3.3 and 2 fold, respectively. This result was corresponded to the determining purity of AMs by SDS-PAGE. CGAM showed a major protein band on the gel, while the purified THAM still had other proteins contaminated after Histrap affinity column (AMY which had higher purification fold than THAM showed a single protein band). Interestingly,

DEP1 was purified with Histrap affinity column and then gel filtration column, respectively, were still contained other proteins on gel SDS-PAGE. While, MeDPE1 which have half purification fold and showed a major protein band on the gel after purification by Histrap affinity column. The native-PAGE of all purified AMs showed a single band of activity corresponded to the protein band which was similar to previous reports (Srisimarat *et al*., 2011; Sawasdee *et al*., 2012; Tantanarat, 2012).

In terms of the assay used, starch transglucosylation activity (using starch as glucosyl donor and maltose as acceptor) and disproportionation activity (using G3 as substrate) are used throughout the study for the two bacterial AMs. For the cassava D-enzyme, MeDPE1, only disproportionation activity is measured since we found that starch transglucosylation activity could not be measured for this enzyme. This suggests the difference in the active site geometry of the bacterial and the plant enzyme.

The three AMs have different properties as summarized in Table 4.1. (Srisimarat *et al*., 2011; Sawasdee *et al*., 2012). No three-dimensional structures have been solved for these enzymes at present, so the identification of active site residues by chemical modification was the aim of this study in order to understand the difference in enzyme catalysis and structure-function relationship of these enzymes.

Table 4.1 Summarized the characterization of AMs

Source of AM	Size		Optimum		Main LR-CD at 24 h	amino acid sequence identity
	amino acid	kDa	pH	Temperature (ºC)		
C. glutamicum	706	84	6	30-40 (St.Tr.) 40-45 (Disprop)	CD27-CD28	24% to *T. aquaticus*
T. filiformis	485	55	7	70 (St.Tr.)	CD24-CD26	71% to *T. aquaticus*
Soil bacterial DNA	523	70	9	70 (St.Tr.)	CD29	99% to *T. thermophilus*
Cassava D-enzyme	585	61	6-7	40 (Disprop)	CD20	37% to *T. aquaticus,* 71% to Potato

St.Tr.   = Starch transglucosylation activity

Disprop   = Disproportionation activity

4.2 Chemical modification

To preliminary identify important amino acid residues for enzyme catalysis, chemical modification with group-specific reagents was performed. The enzyme solutions were treated with various modifying reagents that are specific for different functional groups under mild condition. From the result, (Table 3.2-3.3) it was found that Trp, Asp/Glu, Cys and Lys were important residues for all the three enzymes and which were selected for further investigation. The plant D-enzyme, MeDPE1, differed from the two bacterial enzymes, CGAM and THAM, in its inhibition by PMSF, DEPC, and DTT which suggests that Ser, His, and Cys were important for its activity. The inhibition by DTT suggests that MeDPE1 contains important disulfide bridge while CGAM and THAM do not. Only one AM, the thermostable enzyme from *Pyrobaculum aerophilum* IM2 (PyAMase) was reported to contain two disulfide bridges, proved by modification by DTT which resulted in the decrease in its stability (Kaper *et al*., 2005). AMs from *Thermus* and *P. aeolicus* also were reported to have no disulfide bridge (Kaper *et al*., 2005; Mallick *et al*., 2002). When compared the two bacterial AMs, CGAM were far more sensitive to NAI, NEM, and SAH than THAM suggesting that Tyr, Cys, and Lys were more important for CGAM activity.

The change in the protein pattern and activity after group-specific modification was investigated by native-PAGE technique. From the result we found that, the pattern of protein was significantly changed after treatment with NBS, PMSF, DEPC (Figure 3.4) suggesting the change in net charge and total conformation. EDC which modifies Asp/Glu reduced negative charge (Means and Feeney, 1971) so the modified CGAM run a little slower on the gel. For the activity stain, the band of NBS- and SAH-modified CGAM was disappeared indicating the

complete loss of activity which suggests the essence of Trp and Lys for CGAM activity.

NBS modification is usually conducted in an acidic condition (Spande and Witkop, 1967), which is restricted to modification of Trp residues. The indole group of Trp is oxidized by NBS and produces oxindole derivative (Spande and Witkop, 1967) while SAH reacts with ε-amino groups of Lys and converts to modified derivative that showed negative charge at pH > 5 (Alcalde *et al*., 2001; Gote *et al*., 2007). The activity of AMs was significantly decreased after modifying with SAH, suggesting that the net positive charge on amino acid group of Lys act as important function in the active site (Gote *et al*., 2007). Asp/Glu residues are widely known as catalytic residues of enzymes in α-amylase family (Kaper *et al*., 2007; Przylas *et al*., 2000b), they are modified by EDC at carboxyl group and convert to less negative charge (Alcalde *et al*., 2003). For AM, Asp249 and Glu256 were reported to be localized in the 250s loop (Kaper *et al*., 2007).

From this step, NBS, EDC and SAH were selected for further investigation to demonstrate whether these important amino acid residues are at the active site.

4.3 Identification of essential amino acid residues at the active site of AMs by substrate protection technique

Substrate protection technique is the method to investigate that the amino acid residues proved to be important for activity by chemical modification are or are not located at the enzyme active site. The prevention of enzyme activity loss from modifying reagent by substrate or competitive inhibitor is used to confirm the amino acid residue(s) at the active site. If in the presence of substrate, the activity is

recovered, suggesting that a group at the active site was protected (Means and Feeney, 1971). However, if the activity is not recovered, that residue should not be in the active site, there is possibility that upon modification of that residue by group-specific reagent, a conformational change occurs that results in activity loss.

In this study, maltotriose (G3) was used as preventive substrate from NBS, EDC or SAH modification. From the results (Table 3.4-3.6), G3 which was added in the pre-incubation step to block the active site before chemical modification interfered with the assay by competing with maltose acceptor to react with glucosyl donor and enzyme in starch transglucosylation reaction (Figure 3.11.2). Despite of the interfering action, G3 displayed protection from inactivation by all reagents used for the three enzymes, with the exception for SAH-inactivated MeDPE1. The result suggests that Trp, Asp/Glu and Lys residues were essential amino acid residues at the active site of all enzymes, except for Lys in MeDPE1 (Jung *et al*., 2011).

The importance of Trp in AM activity was reported. Trp258 in the 250s loop of AM from *T. aquaticus* is one out of seven Trp residues and is highly conserved in known sequences of amylomaltase and D-enzyme, but not found in other GH13 enzymes (Przylas *et al*., 2000b). Asp and Glu are confirmed as catalytic residues in all AMs with three-dimensional structures solved by x-ray crystallography. However, Lys has never been reported as important residues in AMs while at least two Tyr (Tyr 54 and Tyr 101 in *T. aquaticus*) has been found to be important for activity.

4.4 Inactivation kinetics of AMs

Incubation of AM solutions with the NBS, SAH or EDC concentrations which gave 50% remained activity (Table 3.7) at various times resulted in a time-

dependent loss of AM activity observed from the plot of the remaining activity versus incubation times (Figure 3.8). The slope from the graph indicates the type of order reaction. If the reaction is pseudo first-order kinetics of inactivation, the activity decreased with increased incubation time (Kaulpiboon and Pongsawasdi, 2003). From the result, all of AMs displayed pseudo first-order kinetics of inactivation. The IC_{50} and the k_{inact} values suggest that the three AMs showed different inactivation sensitivity and different rate of inactivation by these group-specific reagents.

No inactivation kinetics of amylomaltase or D-enzyme has been previously reported. For other related enzymes, CGTase from *Bacillus circulans* was incubated with various concentrations of diethylpyrocarbonate (DEPC) and showed a time dependent loss of enzyme activity, suggesting that inactivation of His residue(s) at or near the enzyme active site follows a pseudo first-order reaction. The second-order rate constant for CGTase was 3.2 $M^{-1}s^{-1}$ (Mattsson *et al*., 1992), while that for CGTase isoform 1 from *Paenibacillus* sp. A11 was at 29.5 $M^{-1}s^{-1}$ (Kaulpiboon and Pongsawasdi, 2003).

4.5 Characterization of native and NBS-modified AMs

The effect of NBS modification on characteristics of CGAM and THAM was investigated. Only in substrate specificity experiment that MeDPE1 was also investigated, this is due to limited amount of MeDPE1 obtained for the study.

pI

Modification of CGAM and THAM by IC_{50} concentrations of NBS did not change the pI values suggesting no significant change in net charge of the enzymes.

Both native and NBS-modified forms of CGAM and THAM showed the pI values of 6.9 and 5.8, respectively. The pI of the recombinant CGAM with 6 His-tag residues at the N-terminal in this study was shifted from 4.7 (Srisimarat, 2010) to 6.9. Surprisingly, the His-taq containing CGAM was more significantly basic than the enzyme without His-taq. However, from the predicted pI values calculated using ExPASy program, pI of 4.94 and 5.93 was obtained for CGAM and THAM, respectively.

Substrate specificity and kinetics of AMs

Substrate specificity of native and NBS-modified AMs in disproportionation and starch transglucosylation was compared. G3 was the best substrate for all enzyme forms in disproportionation reaction while G1 was best for native and modified forms of CGAM and THAM in the starch transglucosylation reaction. The slight difference in order of substrates with low activity was found in nearly all cases when compared the native with NBS-modified enzyme.

The substrate specificity of amylomaltase has been reported. AM form *C. glutamicum* showed similar result as obtained in this study with G3 as the most preferred substrate for disproportionation reaction. The decreased order of substrate was G3 > G4 > G5 > G6 > G7 ≈ G2 (Srisimarat *et al*., 2011) which was slightly different from this study with G6 ≈ G7. In another study of AM from *Thermus thermophiles* HB8 (TtAMase), the descending order of preferred substrate was G3 > G4 > G5 > G6 > G7 > G2 (Kaper *et al*., 2007).

Kinetic parameters of native and NBS-modified CGAM in the presence or absence of acarbose inhibitor for disproportionation and starch transglucosylation

reactions were investigated. In the absence of acarbose, the K_m, k_{cat}, and k_{cat}/K_m values of native CGAM in both reactions were higher than those of modified enzyme (Table 3.8). This suggests the binding of substrate and the reaction rate were changed upon NBS treatment. The K_m value of native CGAM in disproportionation reaction was corresponded to the previously reported value of 19.6 mM (Srisimarat *et al.*, 2011). In the presence of acarbose, a pseudotetrasaccharide which mimics the transition state of the reaction mechanism of GH13 (CGTase and α-amylases) (Kaper *et al.*, 2005; Mosi *et al.*, 1998; Przylas *et al.*, 2000a), kinetic parameters for native and modified CGAM in both reactions were also determined. The k_{cat}/K_m(app) values of the modified enzyme in both reactions were significantly decreased. The k_{cat}/K_m value is the catalytic efficiency of the enzyme which indicates the ability of an enzyme to convert a substrate to product (Stropoplo *et al.*, 2001). The results suggest that Trp residue(s) modified caused the change in the kinetic parameters.

The inhibition pattern by acarbose in disproportionation and starch transglucosylation reaction was different as observed from the Lineweaver-Burk plot (Nelson and Cox, 2005). Mixed-type inhibition in disproportionation but competitive inhibition in starch transglucosylation reaction was obtained. This is the first report on different action of acarbose inhibitor in different activity of AM. The K_i values of acarbose for the modified CGAM were about two times higher than those for the native enzyme in both reactions. The K_i value of acarbose for disproportionation reaction (70.9 mM) was over 100 fold higher than that for starch transglucosylation reaction (0.55 mM), indicating the higher efficiency of acarbose in inhibiting starch transglucosylation reaction. α-amylase and CGTase of the GH13 family were reported to be inhibited by acarbose in the μM range (Kaper *et al.*, 2005; Kim *et al.*, 1999;

Yoon and Robyt, 2003). When the sensitivity to be inhibited by acarbose of PyAMase (AM from *Pyrobaculum aerophilum*) was compared with TtAMase (AM from *Thermus thermophiles* HB8) (Kaper *et al.*, 2007), the structural differences in the active sites of the two enzymes was suggested (Kaper *et al.*, 2005). Interestingly, the comparison between structures of unrelated GH57 4-α-glucanotransferase from *T. litoralis* and PyAMase with acarbose inhibition indicated similar mechanism of the two enzymes (Imamura *et al.*, 2003a).

Number of essential Trp residues of AMs

Trp residue contains indole functional group as chromophore which absorbs strongly at 280 nm (Spande and Witkop, 1967). In contrast, oxidation with NBS produced oxindole which is a weaker chromophore at this wavelength. The side reaction sometimes encountered; oxidation of Met, Cys, His, Lys, or Arg residues (Spande and Witkop, 1967) which cannot interfere the Trp determination, their oxidation products could slightly interrupted the absorbance at 280 nm. After NBS modification, the absorbance at 280 nm was significantly decreased. The number of modified Trp was preliminary estimated form this property according to the equation derived (Spande and Witkop, 1967; Patchornik *et al.*, 1958). To examine if the Trp residues modified by NBS are involved at or around the active site of enzyme, substrate protection was performed. The result of the inactivation of CGAM and THAM by NBS in the absence or presence of G3 substrate suggests that one Trp is in the active site of both enzymes.

CGAM and THAM contain 14 and 21 total Trp residues in the molecules (Srisimarat *et al.*, 2011; Sawasdee *et al.*, 2012), respectively. The number of Trp

modified should be accurately determined by MALDI-TOF mass spectrometry. Each of NBS oxidation adds 16 Da of an oxygen atom (Tsai *et al*., 2006). The mass difference between native and NBS-modified AMs was determined. From the result, the mass of modified-CGAM and -THAM increased by 348 and 134 Da (Figure 2.14.1-2.14.2), respectively. The mass increased was respected to the native AMs with the integration of 22 and 8 oxidized Trp residues in CGAM and THAM with 50% degree of inhibition by NBS (NBS at concentration of 38 or 32 μM was used to modify CGAM or THAM per nmol protein was used; NBS concentration to inhibit total activity per nmol protein were 99.5 or 267 μM, respectively). The calculated number of Trp modified (22) in CGAM was impossible since only 14 Trp exist in a molecule. For THAM, the calculated number of Trp modified (8) is possible since THAM contains total of 21 Trp, however, the number modified determined by spectrophotometry was 4 residues. This unexpected result may be caused by inappropriate ionization in MALDI-TOF analysis.

Secondary structure of proteins

To estimate the structure of unknown proteins and monitor conformation change, circular dichroism (CD) is commonly used (Greenfield, 2006). Alpha-helix and beta-sheet are the main structure type in the protein (Louis-Jeune *et al*., 2011). Alpha-helical protein was determined at 222 and 208 nm with negative bands, at 193 nm with positive band (Holzwarth and Doty, 1965). Beta-sheet was determined at 218 nm with negative band and at 195 nm with positive band (Greenfield and Fasman, 1969). From the results, NBS-modified CGAM showed slightly different spectrum from native (Figure 3.15, a). For THAM, no significant change was detected in CD

spectrum in both forms of enzyme (Figure 3.15, b). This suggests that, after modification with NBS, the conformational of modified AMs was not significant changed. This result corresponds with the values of calculated alpha-helix and beta-sheet content of native and modified CGAM and THAM which was not significantly different (Table 3.9). This implied that, change in enzyme activity should not be involved with an alteration of the protein secondary structure (Alcalde *et al*., 2001).

Tryptophan environment

The decreasing of the fluorescence emission was observed when Trp residues of AMs were modified by NBS (Figure 3.16). NBS-modified CGAM shifted the emission maximum while NBS-modified THAM was not changed. The change in emission maximum suggests that indole group in Trp was oxidized by NBS to oxindole which exerts higher polarity. Therefore, a conformational of protein changed by bringing the indole residue(s) to more polar environment or to charged groups. If after NBS modification, no change in the emission maximum was observed, suggesting that modified Trp residues cannot alter the environmental of these residues (Gote *et al*., 2007; Turk *et al*., 1992). For that reason, NBS modification affected Trp environment in CGAM but not in THAM.

Formation of linear oligosaccharides and large-ring cyclodextrins

The product formation of recombinant CGAM in native and NBS-modified forms was compared (Figure 3.18.1-3.18.2). For linear oligosaccharide product, both forms gave G1 as the principle product from all of substrates used (G3 to G5). AM from *T. thermophilus* HB8 can convert 10 mM of G3, G4 or G5 substrate to produce

G1, G1 and G3 (G4 or G5 substrate), respectively, as the main product (Kaper *et al.*, 2007), while a thermostable AM from *T. brockianus* (TBGT) gave G1 as main product with G5 substrate (Jung *et al.*, 2011). Interestingly, G5 substrate used in AM from *P. aerophilum* IM2 yielded G7 and G3 as the major products (Kaper *et al.*, 2005). The product pattern from G3 to G5 substrate used for both forms of CGAM was similar, suggesting that Trp residue(s) was not involved in production of linear malto-oligosaccharides, but it may be important for the formation of cyclic oligosaccharide (Przylas *et al.*, 2000b).

Comparison of the LR-CDs production from recombinant CGAM and THAM were studied (Figure 3.19.1-3.19.2). The result showed that, the amount of LR-CDs from native and NBS-modified CGAM was similar. For THAM, native AM could produce higher amount (1.3 fold) of LR-CDs than NBS-modified enzyme. The product pattern of native and NBS-modified AMs was similar. CGAM and THAM gave different product pattern. The size of principle LR-CDs formed from CGAM was larger than that from THAM. And the smallest LR-CDs formed were CD19 from CGAM which agrees with the previous report Srisimarat *et al.*, 2011) and CD20 from THAM. While AM from *T. aquiticus* could produce CD22 as smallest LR-CDs (Przylas *et al.*, 2000a; Takaha and Smith, 1999; Terada *et al.*, 1999).

In conclusion, Trp, Asp/Glu localized in the active site region of all three enzymes are important for their catalytic function. For the two bacterial enzymes, Lys is also identified as active site residue. One Trp was identified in the active site of CGAM and THAM. After Trp modification, the significant change observed was in the kinetic parameters of the enzyme-catalyzed reactions.

CHAPTER V

CONCLUSIONS

The purified AMs from two bacterial amylomaltases; CGAM and THAM, and a plant MeDPE1 were treated with various modifying reagents, the result showed that almost total activity loss for all enzymes was observed after NBS modification. Since NBS is specific for indole group of Trp, the inactivated effect suggested that Trp was important for enzyme catalysis. From native-PAGE, the activity band of CGAM disappeared after modifying with NBS or SAH, while a small decrease in the migration of protein band was observed after EDC modification, these findings suggested the importance of Trp, Lys and Asp/Glu for AMs activity. By protection with maltotriose substrate, Trp, Asp/Glu and Lys residues were found to be at or around active site region of AMs, except for Lys in MeDPE1. Then, the NBS-modified AMs were characterized to compare with native AMs. By spectrophotometric method, the number of modified-Trp in the active site of CGAM and THAM was estimated to be about one and three residues, respectively. When the pI values, substrate specificity, Trp environment, secondary structure and product pattern of linear oligosaccharides or LR-CDs of NBS-modified AMs were compared with those of native AMs, no significant difference was observed which suggested that these properties were not affected by NBS modification. It was found that only kinetic parameters were affected. The k_{cat}/K_m values of disproportionation and starch transglucosylation reactions of the modified CGAM were significantly decreased while the K_i values of acarbose inhibitor for the modified enzyme were increased. Interestingly, acarbose displayed competitive inhibition pattern for starch

transglucosylation reaction but mixed type inhibition for disproportionation reaction. The change in kinetic parameters suggested that the binding of substrate and the reaction rate were altered upon NBS modification of CGAM.

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APPENDICES

APPENDIX A: Preparation of solution for cell preparation and enzyme purification

Stock solution

- 1 M Monopotassium phosphate (100 ml)

The pellet of monopotassium phosphate 13.6 g was dissolved in distilled water to final 100 ml

- 1 M Dipotassium hydrogen phosphate (500 ml)

The pellet of dipotassium phosphate 87.1 g was dissolved in distilled water to final 500 ml

- 1 M potassium phosphate buffer, pH 7.4

1 M potassium phosphate buffer, pH 7.4 was prepared by mixing 401 ml of 1 M Dipotassium hydrogen phosphate with 99 ml of Monopotassium phosphate.

1. **Extraction buffer**

   (0.1 mM PMSF, 0.01% (w/v) β-mercaptoethanol and 1 mM EDTA and in 50 mM potassium phosphate buffer, pH 7.4)

1 M potassium phosphate buffer, pH 7.4	5	ml
0.1 mM PMSF in 95% absolute ethanol	0.1	ml
100% (v/v) β-mercaptoethanol	10	μl
0.5 M EDTA	0.2	ml

   Adjusted volume to 100 ml with distilled water

2. 0.85% (w/v) NaCl

  Sodium chloride   0.85 g

  Adjusted volume to 100 ml with distilled water

3. Binding buffer

(20 mM imidazole and 0.5 M NaCl in 20 mM phosphate buffer, pH 7.4)

    Imidazole   0.3 g

    Sodium chloride   5.8 g

    1 M potassium phosphate buffer, pH 7.4 4.0 ml

    Adjusted pH to 7.4 with 1 N HCl and adjusted volume to 200

ml with distilled water

4. Elution buffer

(500 mM imidazole and 0.5 M NaCl in 20 mM phosphate buffer, pH 7.4)

    Imidazole   6.8 g

    Sodium chloride   5.8 g

    1 M potassium phosphate buffer, pH 7.4 4.0 ml

    Adjusted pH to 7.4 with 1 N HCl and adjusted volume to 200

ml with distilled water

APPENDIX B: Preparation for Iodine solution

Stock solution (10x, 100 ml)

0.2 (w/v) I_2 in 2.0 % (w/v) KI

Potassium iodide	2	g
Iodine	0.2	g

Adjusted to 100 ml with distilled water

Iodine solution (1x, 100 ml)

0.2 (w/v) I_2 in 2.0 % (w/v) KI	10	ml
Distilled water	90	ml

APPENDIX C: Preparation of Bradford solution

(Bollag and Edelstein, 1999)

1) **Stock solution**

Ethanol	100	ml
Phosphoric acid	200	ml
Coomassie blue G-250	350	mg

Stable indefinite at room temperature

2) **Working solution**

Ethanol	15	ml
Phosphoric acid	30	ml
Stock solution	30	ml
Distilled water	425	ml

Filter through Whatman No. 1 paper, store at room temperature in brown glass bottle. Unstable for several weeks but may need to be refiltrated.

APPENDIX D: Preparation for polyacrylamide gel electrophoresis

1) **Stock reagent**

   2 M Tris-HCl, pH 8.8

   Tris (hydroxymethyl)-aminomethane   24.2   g

   Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100

   ml with distilled water

   1 M Tris-HCl, pH 6.8

   Tris (hydroxymethyl)-aminomethane   12.1   g

   Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100

   ml with distilled water

   10% (w/v) SDS

   Sodium dodecyl sulfate   10   g

   Adjusted volume to 100 ml with distilled water

   50% (v/v) Glycerol

   100% Glycerol   50   ml

   Added 50 ml distilled water

   1% (w/v) Bromophenol blue

   Bromophenol blue   100   mg

   Added volume to 10 ml with distilled water and stir until

   dissolved. Filtration was performed to remove aggregated dye.

2) **Working solution**

   Solution A

   30% Acrylamide, 0.8% bis-acrylamide, 100 ml

   Acrylamide   29.2   g

N,N'-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water

Solution B

4x Seperating Gel Buffer

2 M Tris-HCl pH 8.8	75	ml
10% (w/v) SDS	4	ml
Distilled water	21	ml

Solution C

4x Stacking Gel Buffer

1 M Tris-HCl pH 6.8	50	ml
10% (w/v) SDS	4	ml
Distilled water	46	ml

10% Ammonium persulfate

Ammonium persulfate	1.0	g
Distilled water	10	ml

Electrophoresis Buffer

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

Adjusted volume to 1 liter with distilled water

5x Sample Buffer

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

Coomassie Gel Stain (1 liter)

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

Coomassie Gel Destain (1 liter)

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

APPENDIX E: Preparation for native-polyacrylamide gel electrophoresis

1) Stock reagent

2 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminomethane   24.2   g

Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane   12.1   g

Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100 ml with distilled water

10% (w/v) SDS

Sodium dodecyl sulfate   10   g

Adjusted volume to 100 ml with distilled water

50% (v/v) Glycerol

100% Glycerol   50   ml

Added 50 ml distilled water

1% (w/v) Bromophenol blue

Bromophenol blue   100   mg

Added volume to 10 ml with distilled water and stir until dissolved. Filtration was performed to remove aggregated dye.

1) **Working solution**

Solution A

30% Acrylamide, 0.8% bis-acrylamide, 100 ml

Acrylamide	29.2	g
N,N'-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water

Solution B

4x Seperating Gel Buffer

2 M Tris-HCl pH 8.8	75	ml
Distilled water	25	ml

Solution C

4x Stacking Gel Buffer

1 M Tris-HCl pH 6.8	50	ml
Distilled water	50	ml

10% Ammonium persulfate

Ammonium persulfate	1.0	g
Distilled water	10	ml

1% (w/v) Glycogen

Glycogen from oyster	0.05	g
Distilled water	5	ml

Electrophoresis Buffer

Tris(hydroxymethyl)-aminomethane	3	g
Glycine	14.4	g

Adjusted volume to 1 liter with distilled water

5x Sample Buffer

1 M Tris-HCl pH 6.8	3.1	ml
50% (v/v) Glycerol	5	ml
1% (w/v) Bromophenol blue	0.5	ml
Distilled water	1.4	ml

APPENDIX F: Preparation solution for isoelectric focusing (IEF)

1) Stock solution

24.25 % Acrylamide, 0.75% bis-acrylamide

Acrylamide	24.25	g
N,N'-metheylene-bis-acrylamide	0.75	g

Adjusted volume to 100 ml with distilled water

0.1% (w/v) Riboflavin, 50 ml

Riboflavin	50	mg
Distilled water	45	ml

Heating until dissolved and adjusted the volume to 50 ml with distilled water

Monomer-ampholyte solution, 5 ml

25% Acrylamide	1.0	ml
25% (v/v) Glycerol	1.0	ml
Distilled water	2.75	ml
Ampholyte	0.25	ml
0.1% (w/v) Riboflavin	50	μl
10% (w/v) Ammonium persulfate	15	μl
TEMED	5	μl

Fixative solution, 100 ml

Ethanol	30	ml
Trichloroacetic acid	12.5	g

Sulfosalicylic acid	4	g

Soaked gel in the solution for 30 min

Staining solution, 100 ml

Ethanol	27	ml
Acetic acid	10	ml
Coomassie blue R-250	40	mg
$CuSO_4$	50	mg
Distilled water	63	ml

$CuSO_4$ was dissolved in distilled water before adding ethanol. The gel was immersed in this solution approximately 1-2 h.

Destaining solution

First destaining solution, 100 ml

Ethanol	12	ml
Acetic acid	7	ml
$CuSO_4$	50	mg
Distilled water	81	ml

$CuSO_4$ was dissolved in distilled water before adding ethanol. The gel was immersed in this solution with 2-3 changes or until the background was nearly clear, approximately 1-2 h.

Second destaining solution, 100 ml

|		
Ethanol	25	ml
Acetic acid	7	ml
Distilled water	68	ml

The gel was immersed into the solution until the background was clear.

APPENDIX G: Abbreviation for amino acid residues (Voet, 1990)

Amino acid	3 Letter-Abbreviation	1 Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspatic acid	Asp	D
Cystein	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown	-	X

APPENDIX H: Standard curve for protein determination by

Bradford's method
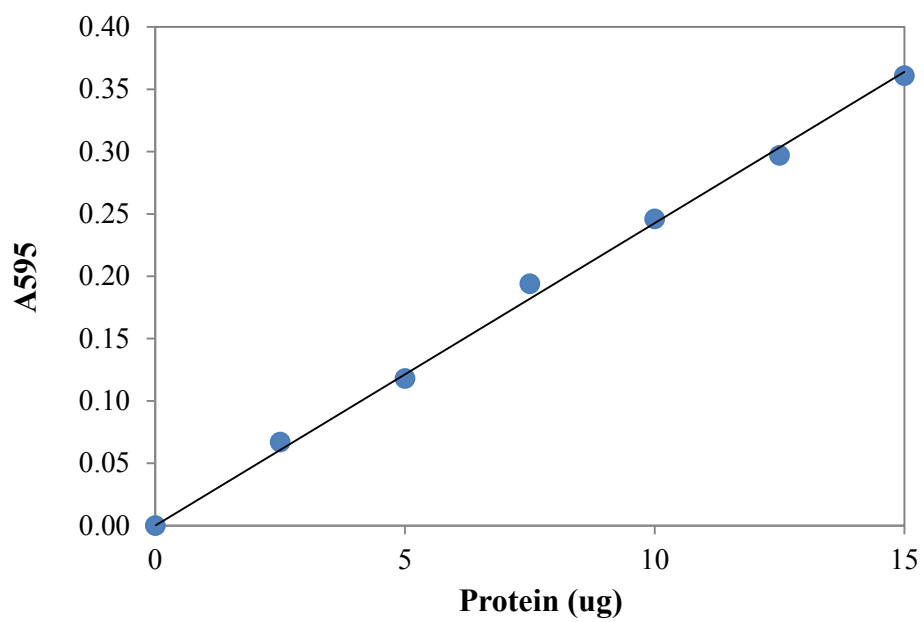
APPENDIX I: Standard curve of glucose determination by glucose oxidase assay
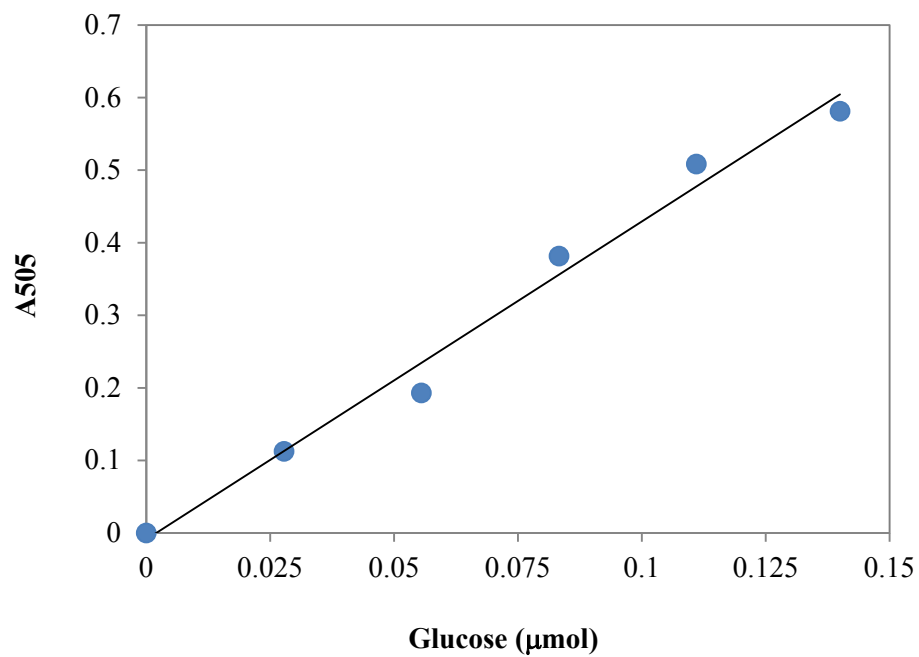
APPENDIX J: Standard curve of starch degrading activity assay
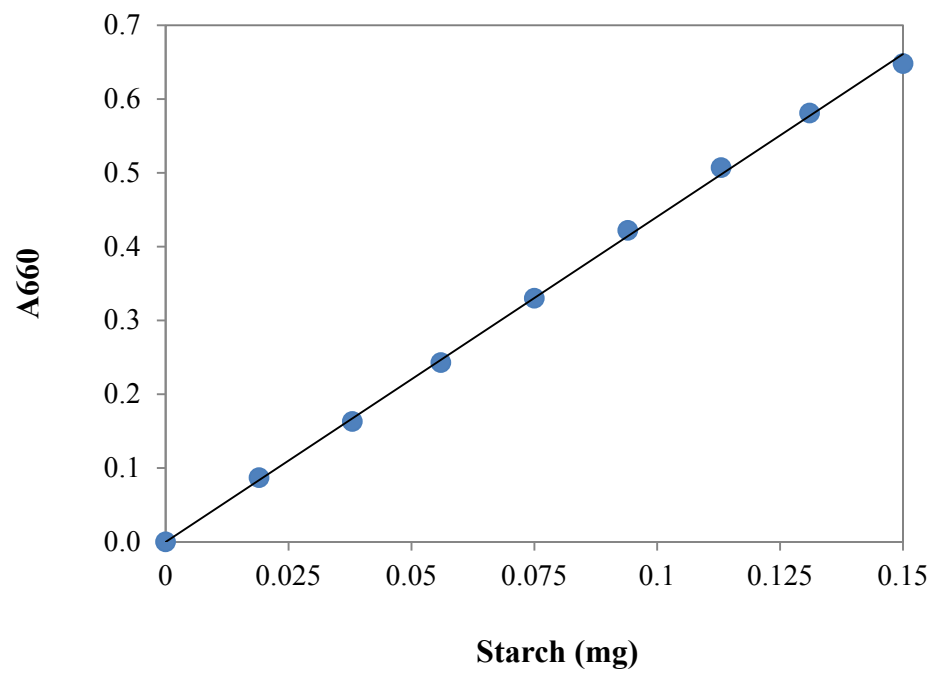
APPENDIX K: The calculated number of modified-Trp residues

CGAM (0.54 mg protein, without maltotriose)

Incubation time (min)	ΔOD	n	U/mg protein	% Relative activity
0	0.054	0.0	34.7	100
5	0.064	0.3	23.4	67.4
10	0.069	0.6	8.4	24.3
15	0.084	0.7	3.1	8.8
20	0.096	1.2	1.9	5.6
25	0.108	1.5	1.4	4.0
30	0.120	1.7	0.7	2.1

THAM

(0.15 mg protein, without maltotriose)

Incubation time (min)	ΔOD	n	U/mg protein	% Relative activity
0	0.044	0.0	218.2	100
5	0.073	1.9	123.9	56.8
10	0.085	3.5	47.6	21.8
15	0.091	4.4	19.9	9.1
20	0.097	5.1	19.9	9.1
25	0.102	7.7	7.6	3.5

(0.15 mg protein, pre-incubated with 50 mM maltotriose)

Incubation time (min)	ΔOD	n	U/mg protein	% Relative activity
0	0.034	0.0	54.8	100
5	0.045	0.6	13.3	24.3
15	0.045	0.8	5.6	10.2
20	0.049	0.8	3.2	5.9
25	0.049	0.9	1.4	2.6
30	0.049	0.9	0	0.0
35	0.049	0.9	0	0.0

*U = starch transglucosylation activity unit

BIOGRAPHY

Miss Wanitcha Rachadech was born on September 30, 1986. She graduated with the Bachelor's degree of Science from the Biochemistry at Chulalongkorn University in 2008, and continued studied for the master degree in Biochemistry at the Department of Biochemistry, Faculty of Science, Chulalongkorn University until 2012.

Publications:

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