ความสัมพันธ์ระหว่างโครงสร้างและหน้าที่ของแอมิโลมอลเทส

จาก *Corynebacterium glutamicum*

นางสาวสุทิพาพรรณ ตุ้มหอม

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STRUCTURE AND FUNCTION RELATIONSHIP OF AMYLOMALTASE FROM

Corynebacterium glutamicum

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สุทิพาพรรณ ตุ้มหอม : ความสัมพันธ์ระหว่างโครงสร้างและหน้าที่ของแอมิโลมอลเทสจาก *Corynebacterium glutamicum* (STRUCTURE AND FUNCTION RELATIONSHIP OF AMYLOMALTASE FROM *Corynebacterium glutamicum*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. เกื้อการุณย์ ครูส่ง, 212 หน้า.

แอมิโลมอลเทส (EC 2.4.1.25) เร่งปฏิกิริยาการโยกย้ายหมู่กลูโคซิลภายในหรือระหว่างสายโมเลกุลของกลูแคนทำให้เกิดไซโคลเดกซ์ทรินวงใหญ่ (LR-CD)และออลิโกแซ็กคาไรด์สายตรงตามลำดับ งานวิจัยนี้ต้องการศึกษาความสัมพันธ์ระหว่างโครงสร้างและหน้าที่ของแอมิโลมอลเทสจาก *Corynebacterium glutamicum* (*Cg*AM) โดยทำการกลายยีนเฉพาะตำแหน่งที่กรดอะมิโน Y418 (อยู่บริเวณปลาย loop 410s ที่ปกคลุมบริเวณเร่ง) ตำแหน่ง H461 (ในบริเวณเร่ง) และตำแหน่ง 287 (ในบริเวณ second glucan binding) จากการเปรียบเทียบสมบัติทางชีวเคมีของเอนไซม์กลายที่ทำให้บริสุทธิ์กับเอนไซม์ดั้งเดิม (WT) พบว่าเอนไซม์ทุกรูปแบบมีขนาด 84 kDa มีภาวะเหมาะสมที่ 40 ˚ซ pH 6.0 และ 30 ˚ซ pH 6.0 สำหรับปฏิกิริยาดิสพรอพพอร์ชันเนชันและไซโคลเซชันตามลำดับ เอนไซม์ WT เอนไซม์กลาย Y418 และ N287 มีความจำเพาะต่อซับสเตรตเหมือนกัน คือ ชอบมอลโทไทรโอส ในขณะที่เอนไซม์กลาย H461 ชอบมอลโทส โดยการกลายเฉพาะตำแหน่ง ทำให้ทรานส์กลูโคซิเลชันแอกทิวิตีของเอนไซม์กลายลดลงอย่างเห็นได้ชัด โดยเฉพาะไซโคลเซชันแอกทิวิตีของเอนไซม์กลาย H461 หายไป โดยประสิทธิภาพในการเร่งปฏิกิริยา, k_{cat}/K_m ของเอนไซม์กลายทุกชนิดต่ำกว่า WT จากการลดลงของ k_{cat} ในปฏิกิริยาดิสพรอพพอร์ชันเนชันและไซโคลเซชัน ในขณะที่ K_m ของเอนไซม์กลาย Y418 ไม่แตกต่างจาก WT แต่ค่า K_m ของเอนไซม์กลาย H461 และ N287 สูงกว่า WT ในปฏิกิริยาดิสพรอพพอร์ชันเนชัน และเมื่อใช้แป้งถั่วเป็นซับสเตรตในปฏิกิริยาไซโคลเซชันพบว่า K_m ของเอนไซม์กลาย Y418 และ N287 สูงกว่า WT และมีการเปลี่ยนแปลงขนาดและผลผลิตผลิตภัณฑ์ LR-CD โดยพบว่าเอนไซม์กลายทุกตัวให้ปริมาณ LR-CD ที่ลดลงด้วยเมื่อเทียบกับ WT และเอนไซม์กลาย Y418A/S/D ให้ขนาดผลิตภัณฑ์ที่ใหญ่ขึ้น (CD36 - CD40) เอนไซม์กลาย Y418R และ W ให้ช่วงขนาดผลิตภัณฑ์ที่กว้าง ขณะที่เอนไซม์กลาย N287A/S/D/R และ W ให้ขนาดผลิตภัณฑ์ CD ที่เล็กลง (CD26 - CD28) เมื่อเทียบกับ WT (CD29-CD33) จากการวิเคราะห์ CD spectra พบว่าการกลายไม่มีผลเปลี่ยนแปลงโครงสร้างทุติยภูมิของเอนไซม์ เมื่อทำการศึกษาสมบัติชีวฟิสิกส์ของเอนไซม์ด้วยวิธี ITC และ DSC พบว่า เอนไซม์ WT ผลิตความร้อนได้มากกว่าเอนไซม์กลาย Y418A/W ในปฏิกิริยาดิสพรอพพอร์ชันเนชันและเกิดภาวะการยับยั้งด้วยผลิตภัณฑ์เมื่อเพิ่มความเข้มข้นของมอลโทไทรโอส จากการศึกษารายละเอียดความจุความร้อนของเอนไซม์ด้วยเทคนิค DSC พบว่าเอนไซม์ WT และ Y418A มีค่า Peak temperature (T*p*) ที่คล้ายกัน ในขณะที่เอนไซม์กลาย Y418W มีค่า T*p* ต่ำกว่า 4 ˚ซ นั่นคือ เอนไซม์ WT และ Y418A มีความเสถียรต่ออุณหภูมิมากกว่าเอนไซม์กลาย Y418W เราได้สร้างโครงสร้างจำลองของเอนไซม์กลายโดยใช้โครงสร้างผลึกเอนไซม์ WT เป็นต้นแบบ เพื่อใช้ช่วยอธิบายสมบัติของเอนไซม์ที่เปลี่ยนไปเมื่อทำการกลาย จากผลการทดลองทั้งหมด สรุปได้ว่า ทั้ง 3 ตำแหน่งสำคัญต่อแอกทิวิตีของเอนไซม์ในระดับที่ต่างกัน โดยตำแหน่ง Y418 ต้องคงปฏิสัมพันธ์ไฮโดรโฟบิคและขนาดทางเข้าที่เหมาะสมของของซับสเตรตในบริเวณ loop ส่วนตำแหน่ง N287 ต้องคงโซ่ข้างประจุบวก โดยทั้ง 2 ตำแหน่งนี้ควบคุมขนาดผลิตภัณฑ์และปริมาณ LR-CDs ด้วย ในขณะที่ H461 มีความจำเป็นยิ่งต่อการเร่งปฏิกิริยาโดยเฉพาะปฏิกิริยาไซโคลเซชัน

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SUTHIPAPUN TUMHOM: STRUCTURE AND FUNCTION RELATIONSHIP OF AMYLOMALTASE FROM *Corynebacterium glutamicum*. ADVISOR: PROF. PIAMSOOK PONGSAWASDI, Ph.D., CO-ADVISOR: ASST. PROF. KUAKARUN KRUSONG, Ph.D., 212 pp.

Amylomaltase (EC 2.4.1.25) catalyzes intramolecular and intermolecular transglucosylation reactions of glucan to yield large-ring cyclodextrin (LR-CD) and linear oligosaccharide products, respectively. The aim of this work is to investigate structure and function relationship of amylomaltase from *Corynebacterium glutamicum* (*Cg*AM). Three target amino acid residues of *Cg*AM, Y418 (at the tip of 410s loop which lies over the active site), H461 (in active site) and N287 (in second glucan binding site) were selected for site-directed mutagenesis. Biochemical characterization of all purified mutated *Cg*AMs were compared to wild-type (WT). All enzymes had an approximate size of 84 kDa on SDS-PAGE, the same optimum temperature and pH of 40 $^{\circ}$C, pH 6.0 and 30 $^{\circ}$C, pH 6.0 for disproportionation and cyclization reactions, respectively. For substrate specificity in disproportionation reaction of WT, Y418 and N287 mutated *Cg*AMs preferred maltotriose while maltose was suitable for H461 mutants. Significant decrease in transglucosylation activities of mutated *Cg*AMs was observed especially cyclization activity which was lost in H461 mutants. The catalytic efficiency, k_{cat}/K_m values of all mutated *Cg*AMs were lower than that of WT, mainly due to the significant decrease in k_{cat} for disproportionation and cyclization reactions. K_m values of Y418 mutants were not changed much while values of H461 and N287 mutants were higher than WT. In addition, K_m values of Y418 and N287 mutants were higher than WT with pea starch substrate in cyclization reaction. The changes in size and yield of LR-CD products were observed as the result of Y418 and N287 mutations. Y418A/S/D mutated *Cg*AMs gave a larger CD size (CD36 - CD40), Y418R/W showed a broad product pattern while N287A/S/D/R/W gave a smaller size (CD26 - CD28), all mutated enzymes showed lower product yield in comparison to WT with CD29-CD33 as main products. The secondary structure of all enzymes as analyzed was not significantly changed upon mutations. Biophysical properties of WT and Y418A/W *Cg*AMs were determined by ITC and DSC techniques. Heat production of WT was higher than those of Y418A/W in disproportionation reaction. The product inhibition was occurred as a result of increase in maltotriose concentration. The heat capacity profile from DSC showed that peak temperatures (Tp) of WT and Y418A were similar while a shift of Tp of Y418W towards 4 °C lower was observed. The results indicated that WT and Y418A showed higher stability than Y418W *Cg*AM. The model structures of mutated *Cg*AMs were constructed using WT X-ray structure as template in order to help explain change in properties upon mutation. The overall results suggested that all three residues of *Cg*AM are important for enzyme activity to a different extent. Y418 has to retain hydrophobic interactions and the suitable distance of loop channel for substrate entering, N287 should remain positively charge side chain. Both positions are also involved in controlling the amount and size of LR-CD products. While H461 is essential for enzyme catalysis especially in cyclization reaction.

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

A	absorbance
AM	amylomaltase
BSA	bovine serum albumin
° C	degree celcius
CDs	cyclodextrins
LR-CDs	large-ring cyclodextrins
Da	Dalton
D-enzyme	Disproportionation enzyme
DP	Degree of Polymerization
g	gram
4αGTase	4-α-glucanotransferase
GH	glycoside hydrolase family
h	hour
l	litre
µg	microgram
µl	microliter
M	molar
mA	milliampere
min	minute
mg	milligram
ml	milliliter

mM   millimolar

MW   molecular weight

ND   not detectable

PAGE   polyacrylamide gel

                            electrophoresis

rpm   revolution per minute

SDS   sodium dodecyl sulfate

U   unit

CHAPTER I
INTRODUCTION

1.1 Starch converting enzymes

A variety of different enzymes involving in the synthesis and degrading of starch have been studied (Van der Maarel *et al*., 2002). Most of them belong to α-amylase family based on the amino acid sequence homology; (Table 1.1). They are basically classified into 4 groups (Figure 1.1); (i) endoamylase such as α-amylase with four highly conserved regions in their primary sequences (Van der Maarel *et al*., 2002) and an (α/β)$_8$ or TIM barrel in their structures (Banner *et al*., 1975), the enzyme hydrolyzes α-1,4-glycosidic bond from inner chain of amylose or amylopectin, producing linear or branched oligosaccharides. α-amylase is a well-known endoamylase found in a wide variety of organisms. (ii) exoamylase which catalyzes the hydrolysis of α-1,4 and α-1,6-glycosidic linkages from non reducing end of amylose and amylopectin results in short chain products (Robyt and Ackerman, 1971); (iii) debranching enzyme such as isoamylase and pullulanase type I which hydrolyze α-1,6-glycosidic bond resulting in long chain oligosaccharides as products (Bender *et al*., 1959); (iv) transferase or 4-α-glucanotranferase which hydrolyzes α-1,4-glycosidic linkages of donor and transfer to glycosidic acceptor so a new glycosidic bond is formed such as cyclodextrin glycosyltransferase (CGTase) and amylomaltase (AM) (MacGregor *et al*., 2001).

1.2 The 4-α glucanotransferase

4 - α glucanotransferase is a specific type of transferase consisting of amylomaltase, CGTase and glycogen debranching enzyme (Takaha and Smith, 1999). 4-α glucanotransferase catalyzes inter- and intra-molecular transglucosylation reactions consisting of 4 different reactions (Figure 1.2). The first reaction is disproportionation reaction which is an intermolecular transglucosylation reaction. The enzyme transfers the glucosyl units from short chain oligosaccharides resulting in oligosaccharides of various lengths as in the following equation:

$(\alpha\text{-1,4-glucan})_m + (\alpha\text{-1,4-glucan})_n \longleftrightarrow (\alpha\text{-1,4-glucan})_{m-x} + (\alpha\text{-1,4-glucan})_{n+x}$

The second is cyclization reaction, an intramolecular transglucosylation. The enzyme converts a single linear glucan to a cyclic glucan which is called cyclodextrins (CDs) as shown:

$(\alpha\text{-1,4-glucan})_n \longleftrightarrow \text{cyclic } (\alpha\text{-1,4-glucan})_x + (\alpha\text{-1,4-glucan})_{n-x}$

The third reaction is coupling, a reverse reaction of cyclization. In this reaction, CD ring is opened and act as a donor, the enzyme transfers glucosyl units from CD to short chain oligosaccharides producing longer oligosaccharides as product. Finally, hydrolysis reaction, the enzyme catalyzes and transfers glucosyl residues to water which acts as acceptor. Among these reactions, amylomaltase has high activity in disproportionation and cyclization reactions, but low activity in coupling and hydrolysis reactions (Van der Maarel *et al*., 2000). All of these reactions can be catalyzed by both amylomaltase and CGTase, but there is a different in the size of products from cyclization reaction. CGTase produces small ring cyclodextrins (SR-CDs) while amylomaltase produces large-ring cyclodextrins (LR-CDs). SR-CDs are

cyclic oligosaccharides consisting of 6, 7 and 8 glucose units linked by α-1,4-glycosidic bond which is called α, β and γ-CDs, respectively (Takaha and Smith, 1999) while LR-CDs comprise of glucose residues linked by α-1,4-glycosidic bond with degree of polymerization (DP) from 9 onwards (Van der Maarel *et al*., 2000). Cyclization specificity is one of the most important properties of amylomaltase to catalyze the formation of cyclic glucans with degree of polymerization (DP) larger than 16 while DP9 - DP15 are produced from CGTase at short reaction period (Takaha and Smith, 1999).

1.3 Amylomaltase (AM)

Amylomaltases or AM (EC 2.4.1.25) is an intracellular 4-αGTase catalyzed transglucosylation reactions to yield linear oligosaccharides and LR-CD products as described in section 1.2. (Takaha and Smith, 1999). The enzymes have been classified into glycoside hydrolase family 77 (GH77) (Coutinho *et al*., 2003), which forms the α-amylase superfamily together with GH13 and GH70, sharing a similar fold and the active site residues (MacGregor et al., 2001). Amylomaltases have been found in microorganisms as well as in plants, where they are known as disproportionating enzyme or D-enzyme (Tantanarat *et al*., 2014). Amylomaltase was firstly found in *Escherichia coli* as maltose inducible enzyme which involved in maltose metabolism (Boos and Shuman, 1998). In plants, D-enzyme is involved in starch metabolism, a role has been implicated in nocturnal maltose metabolism in the cytoplasm (Smith *et al*., 2004).

Amylomaltase gene was reported in many bacterial strains such as *Streptococcus pneumoniae* (STASsI *et al*., 1981), *Clostridium butyricum* NCIMB7423 (Goda *et al*., 1997), *thermophillic bacteria Thermus aquaticus* (Terada *et al*., 1999), *Aquifex aeolicus* (Bhuiyan *et al*., 2003) and some archaea such as hyperthermophilic *Thermococcus litoralis* (Jeon *et al*., 1997) and hyperthermophillic *Pyrobaculum aerophilum* IM2 (Kaper *et al*., 2005). D - enzyme in plant was found in potato tubers (Peat *et al*., 1956), carrot roots (Manners and Rowe, 1969), spinach leaves (Okita *et al*., 1979), arabidopsis (Tantanarat *et al*., 2014), pea leaves (Kakefuda and Duke, 1989) and cassava *Manihot esculenta* Crantz tuber (Tantanarat *et al*., 2014). The three dimensional structures of amylomaltases from *T. aquaticus* (Przylas *et al*., 2000b), *Thermotoga maritime* (Roujeinikova *et al*., 2002), *Thermus thermophilus* (Barends *et al*., 2007), *Thermus brockianus* (Jung *et al*., 2011), *A. aeolicus* (Barends, 2005) , *E. coli* (Weiss *et al*., 2015), *Corynebacterium glutamicum* (Joo *et al*., 2016) Recently, the X-ray structure from *T. aquaticus* in complex with cycloamylose with a degree of polymerization of 34 (CA34) at resolution range 1.73-37 Å have been reported (Roth *et al*., 2017).

1.4 Large-ring cyclodextrins (LR-CDs)

Cyclodextrins (CDs) or cycloamylose (CAs) is composed of glucose units joining to form a cyclic structure with 1,4 - glycosidic linkages (Figure 1.3). These molecules have unique structure with a hydrophophilic rim, formed by hydroxyl groups, at both side of the cone (Van der Maarel *et al*., 2002). The central hydrophobic cavity of CDs can trap hydrophobic guest molecules (Del Valle, 2004). The inclusion

complexes formed can change the solubility, reactivity and stability of the guest substances and have found many applications in the food, chemical and pharmaceutical industries (Takaha and Smith, 1999).LR-CDs apparently experience steric strain which results in a distortion of the previously annular molecule to form various structures due to possibility of different H-bonding. In CD10, the macrocyclic rings are deformed elliptical shape while cavity of CD14 is a narrow groove with a boat and butterfly-shaped. (Figure 1.3). CD9 has an intermediate structure between CD10 and CD14, it displays a distorted elliptical macrocyclic ring without a band flip (Endo, 2011). The largest cycloamylose that could be characterized by X-ray crystallography is CA26 (Gessler *et al*., 1999). CA-26 folds into two short left handed V-amylose helices in an anti-parallel arrangement with a hydrophobic channel of 5.0-5.5 Å in diameter (Figure 1.4). This channel may form complexes with a large variety of guest molecules, in particular with long extended molecules. The physicochemical properties of CD26 to CD39 are shown in Table 1.2. The aqueous solubilities of LR-CDs, except for CD9, CD10, CD14 and CD26 are higher than those of α-, β- and γ-CD. This may be a consequence of high structural flexibility, on the basis of the formation of intramolecular and intermolecular hydrogen bonds. There are no marked differences in specific rotation among various sizes of LR-CDs (CD10 to CD39).

1.5 Applications of LR-CDs and amylomaltase

Due to the hydrophilic surface, LR-CDs can dissolve in water and form inclusion complexes with guest molecules such as fatty acid, alcohol (Zheng *et al*., 2002), surfactant (Mun *et al*., 2009) and benzene (Harata, 1981) by trapping guest

molecules into their hydrophobic cavity to improve solubility, stability or biological properties of guest molecules (Kim *et al*., 2011). LR-CDs have been used in various applications: in food, phamarceutical, cosmetic and paper industries (Takaha and Smith, 1999). In pharmaceutical application, LR-CDs form inclusion complexes with flubiprofen and ibuprofen resulted in 14 - and 2 - folds increase in drug solubility, respectively (Baek *et al*., 2011). LR-CDs are also used as an artificial chaperone for protein refolding (Machida *et al*., 2000). Table 1.3 summarizes studies on the inclusion complex formation between LR-CDs or mixture of LR-CDs and guest compounds (Endo, 2011).

Amylomaltase is used to modify starch to produce themoreversible gel. The enzyme hydrolyzes α-1,4-glycosidic linkage of amylose chain and transfers the glucosyl units to amylopectin chain, resulting in a change in side chain distribution and thermoreversible properties (Kaper *et al*., 2005). The modified starch is used in foods to replace gelatin from animal to make food edible for vegetarian and muslim. Moreover, the modified starch is used to replace fat or cream in dairy products to improve creaminess in low-fat yogurt (Alting *et al*., 2009). Amylomaltase is also used to produce glucoside products through intermolecular transglucosylation such as isomalto-oligosaccharides (IMOs) with DP2-DP6 (Rudeekulthamrong *et al*., 2013). IMOs are non digestible saccharides of glucose-based consisting of α-1,6-glycosidic linkage, they act as prebiotics that activate the growth of normal flora in digestive system which benefit human health. Amylomaltase was also reported to be used in the synthesis of maltosylsucrose with anticariogenic property (Saehu *et al*., 2013).

1.6 *Corynebacterium glutamicum*

Corynebaterium glutamicum (*C. glutamicum*) is a mesophilic gram positive bacteria which has attracted biotechnology industry (Joo *et al*., 2016). It is employed in large-scale fermentation due to its remarkable ability to produce amino acids, nucleotides and vitamins (Vertès *et al*., 2005). *C. glutamicum* cells grow in a medium containing sugar accumulate glycogen and degrade that polymer when sugar becomes a limiting factor (Seibold *et al*., 2007). Unlike other gram positive bacteria, *C. glutamicum* has a glycogen degradation pathway similar to that in *E. coli* (Seibold *et al*., 2009). During the degradation of glycogen which is intertwined with maltose metabolism, it forms maltodextrins. Enzymes such as glycogen phosphorylase, glycogen debranching enzyme, glucokinase, α-phosphoglucomutase, maltodextrin glucosidase, maltodextrin phosphorylase and 4-α-glucanotransferase participate in maltose/maltodextrin metabolism and glycogen degradation in *E. coli* (Boos and Shuman, 1998; Seibold *et al*., 2009) as shown in Figure 1.5. In *C. glutamicum*, maltose/maltodextrins serve as substrates in synthesis of trehalose. This disaccharide is involved in the response to osmotic stress and also in the biosynthesis of mycolic acids, which are an important component of bacteria cell wall (Wolf *et al*., 2003).

Table 1. 1 Enzymes belonging to the α-amylase family with four highly conserved regions and the corresponding β-sheets found in their amino acid sequences. The five invariable catalytic sites are those highlighted (Van der 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

CMDase = Cyclo-maltodextinase, BE = Branching enzyme, M. amylase = maltogenic α-amylase, Sucrose Pase = Sucrose phosphorylase, BLamylase = α-amylase from *Bacillus licheniformis*
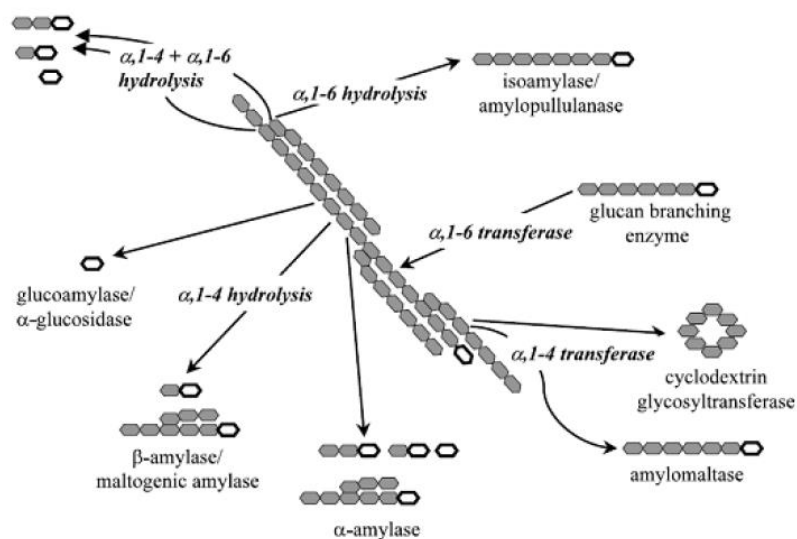
Figure 1. 1 The different enzymes involved in the degradation of starch. The open ring structure symbolizes the reducing end of a glucan molecule (Van der Maarel *et al*., 2002).
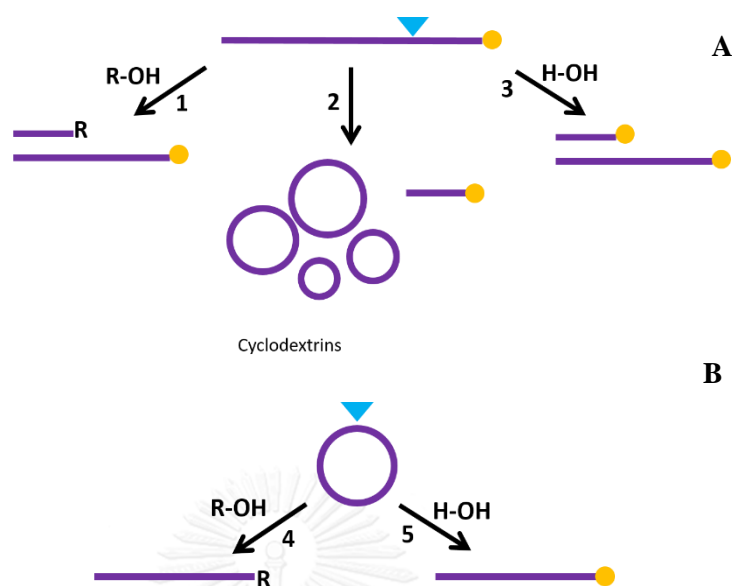
Figure 1. 2 Model of action of amylomaltase on linear and cyclic α-1,4 glucans. Reactions catalyzed by amylomaltase on amylose (A) and cyclodextrins (B). Lines and circles indicate linear and cyclic α-1,4 glucans chains, respectively. Blue closed triangles, attacked points by amylomaltase; yellow closed circles, glucosyl residues at reducing ends; R, α-1,4 glucans; 1, disproportionation reaction; 2, cyclization reaction; 3,5 hydrolysis reaction; 4, coupling reaction.
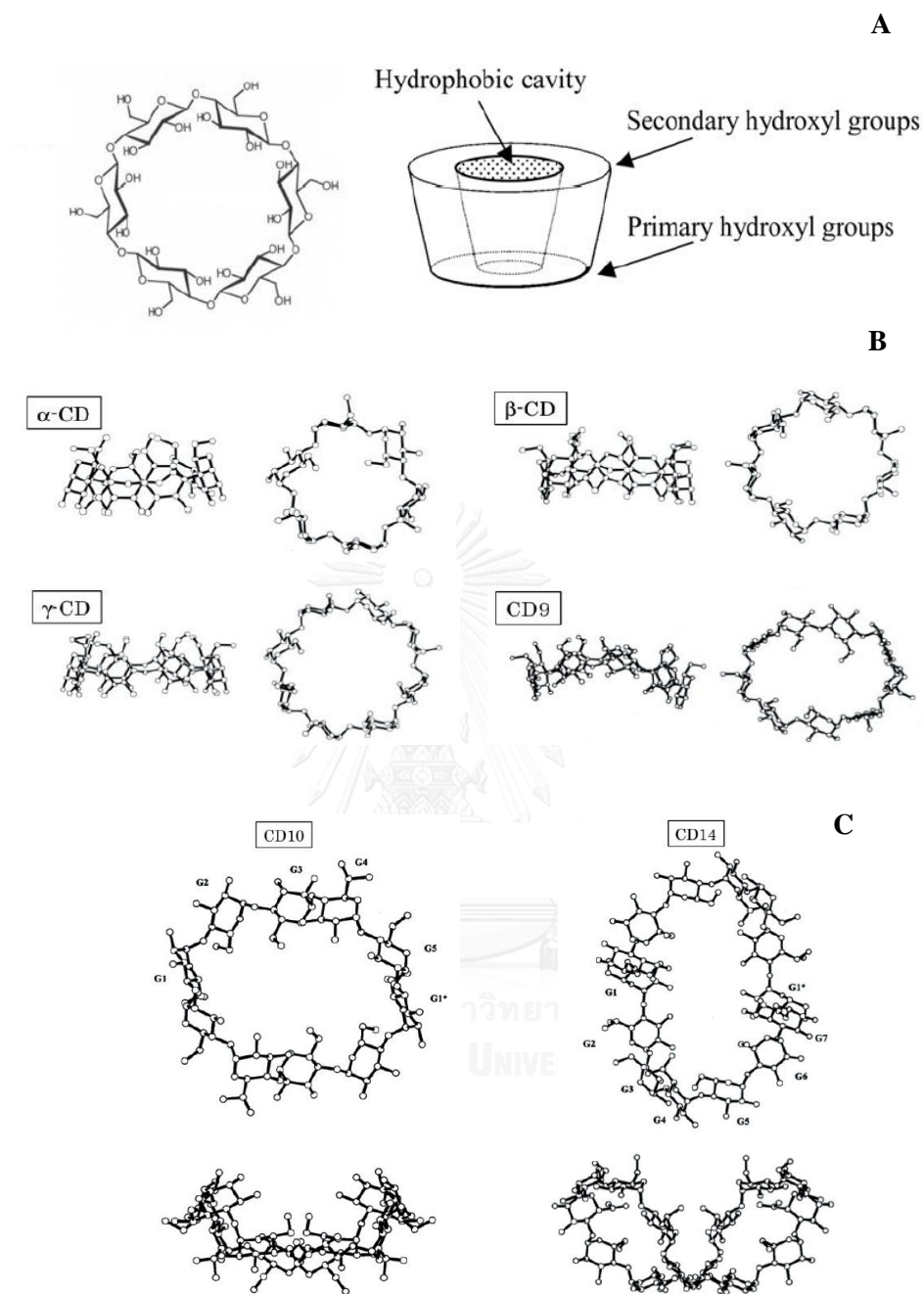
Figure 1. 3 Schematic presentation of α-CD (A) (Larsen, 2002). Molecular structures (side and top views) of α-CD, β-CD, γ-CD, CD9 (B) CD10 and CD14 (C) (Endo, 2011).
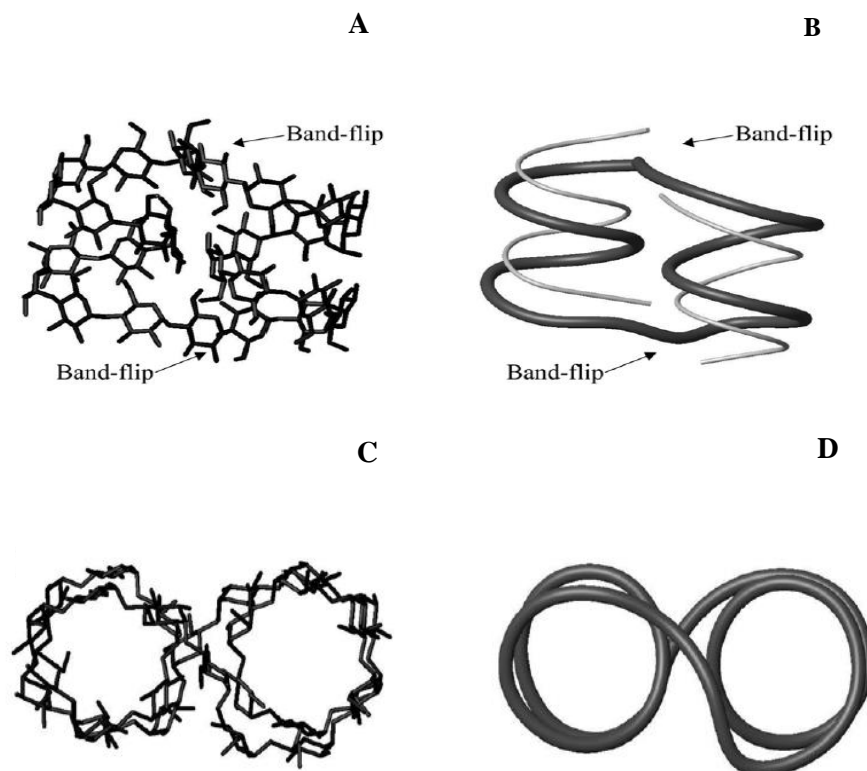
Figure 1. 4 Solid state structure of LR-CDs (CD26). Structure of CD26 indicating the positions of the band-flips and the V-amylose like segments (A). Same structure as A, the thick dark tube traces the position of C1, whereas the thin grey tube traces the position of C6. The band-flipped positions are clearly seen (B). CD26 viewed from the top (C). Same structure as C, the thick dark tube traces the position of C1 (D) (Larsen, 2002). A band flip was defined as a 180° inverted glycoside linkage (Taira *et al*., 2006).

Table 1.2 Physicochemical properties of native small-ring CDs and LR-CDs (Endo, 2011).

	Theroritical[a] molecular weight	Aqueous[b] solubility	Surface[b] tension	Specific rotation	Half-life of[c] ring opening		Theroritical[a] molecular weight	Aqueous[b] solubility	Surface[b] tension	Specific rotation	Half-life of ring opening
		(g/100 mL)	(mN/m)	$[\alpha]_D^s$	(h)			(g/100 mL)	(mN/m)	$[\alpha]_D^s$	(h)
α-CD	972.8	14.5	72	+147.8	33	CD23	3729.2	>100	73	+196.6	2.7
β-CD	1135.0	1.85	73	+161.1	29	CD24	3891.4	>100	73	+196.0	2.6
γ-CD	1297.1	23.2	73	+175.9	15	CD25	4053.5	>100	73	+190.8	2.8
CD9	1459.3	8.19	72	+187.5	4.2	CD26	4215.7	22.4	73	+201.4	2.9
CD10	1621.4	2.82	72	+204.9	3.2	CD27	4377.8	>125	72	+189.4	2.8
CD11	1783.5	>150	72	+200.8	3.4	CD28	4539.9	>125	72	+191.2	2.6
CD12	1945.7	>150	72	+197.3	3.7	CD29	4702.1	>125	72	+190.2	2.5
CD13	2107.8	>150	72	+198.1	3.7	CD30	4864.2	>125	72	+189.1	2.3
CD14	2270.0	2.30	73	+199.7	3.6	CD31	5026.4	>125	71	+189.0	2.4
CD15	2432.1	>120	73	+203.9	2.9	CD32	5188.5	>125	71	+192.7	2.4
CD16	2594.2	>120	73	+204.2	2.5	CD33	5350.6	>125	71	+192.1	2.2
CD17	2756.4	>120	72	+201.0	2.5	CD34	5512.8	>125	72	+189.6	2.2
CD18	2918.5	>100	73	+204.0	3.0	CD35	5674.9	>125	71	+193.7	2.1
CD19	3080.7	>100	73	+201.0	3.4	CD36	5837.1	>100	71	+190.6	1.9
CD20	3242.8	>100	73	+199.7	3.4	CD37	5999.2	>100	71	+189.9	1.8
CD21	3405.0	>100	73	+205.3	3.2	CD38	6161.3	>100	71	+190.1	1.9
CD22	3567.1	>100	73	+197.7	2.6	CD39	6323.5	>100	70	+188.1	1.8

a) Calculated as 162.1406 * n, where n is the number of glucopyranose unit.
b) Observed at 25 °C.
c) In 1 mol/L HCl at 50 °C.

Table 1.3 Studies of inclusion complex formation between purified LR-CD or mixture of LR-CDs and guest molecules (Endo, 2011).

CD	(Ref. No.)	Indicator or Method	Compound
(Pure LR-CD)			
CD9	(10)	Enhancement of solubility (UV/VIS absorption)	Anthracene
			Amphotericin B
			Ajmalicine
			Ajmaline
			Carbamazepine
			Digitoxin
			Spironolactone
			9,10-Dibromoanthracene
			Perylene-3,4,9,10-tetracarboxylic dianhydrate
		Solubility method	Spironolactone
CD9	(51, 53)	Enhancement of solubility	Fullerene C_{60}
	(50, 52)	(Spectrophotometry)	Fullerene C_{70}
CD9	(45)	Enhancement of solubility (Spectrophotometry)	Reserpine
			[2,2]-Paracyclophane
			Perylene
			Triphenilene
			1,8-Naphthalic anhydride
			Naphthalene-1,4,5,8-tetracarboxylic dianhydride
			Digitoxin
			Gitoxin
			Digoxin
			Methyldigoxin
			Lanatoside C
			G-Strophanthin
			Proscillaridin A
		Solubility method and NMR	Digitoxin
CD9	(87)	Simple precipitation	1,5-Cyclooctadiene
			Cyclononanone
			Cyclodecanone
			Cycloundecanone
			Cyclododecanone
			Cyclotridecanone
			Cyclopentadecanone
		Powder X-ray diffraction	Cycloundecanone
		DSC	Cyclododecanone
CD9 - CD13	(46-48)	Capillary electrophoresis	Benzoate
			2-Methy benzoate
			3-Methyl benzoate
			4-Methyl benzoate
			2,4-Dimethyl benzoate
			2,5-Dimethyl benzoate
			3,5-Dimethyl bezoate
			3,5-Dimethoxy benzoate
			Salicylate
			3-Phenyl propionate
			4-*tert*-Butyl benzoate
			Ibuprofen anion
			1-Adamantane carboxylate

Table 1.3 (continue) Studies of inclusion complex formation between purified LR-CD or mixture of LR-CDs and guest molecules (Endo, 2011).

CD	(Ref. No.)	Indicator or Method	Compound
(Pure LR-CD)			
CD14 - CD17	(49)	Capillary electrophoresis	Salicylate 4-tert-Butyl benzoate Ibuprofen anion
CD21 - CD32	(56)	Isothermal titration calorimetry (ITC)	Iodine
CD9	(55)	X-ray crystallography	Cycloundecanone
CD12	(54)	NMR	Single wall carbon nanotube (SWNT)
CD26	(57, 88)	X-ray crystallograpy	NH_4I_3 $Ba(I_3)_2$ Undecanoic acid Dodecanol
(Mixture of LR-CDs)			
CA(S)[*] and CA(L)[**]	(89)	Spectrofluorometry	8-Anilino-1-naphthalene sulfonic acid
CA(S)[*] and CA(L)[**]	(25, 89)	Simple precipitation	1-Octanol 1-Butanol Oleic acid
CA (with oligomerization degree of 22 to around 60)	(64)	Enhancement of solubility (Spectrophotometry)	Fullerene C_{60}
CD21 - CD40	(90)	ITC	Sodium dodecyl sulfate Sodium myristoyl sulfate
CA	(91)	Simple precipitation	SB3-14[***] SB3-16[****]

[*] CA(S) : Mixture of LR-CDs with oligomerization degree around 20 to 55, mainly oligomerization degree of 25 to 50.

[**] CA(L) : Mixture of LR-CDs with average oligomerization degree of ca. 150, except CDs with smaller than oligomerization degree of 50.

[***] SB3-14 : 3-(*N,N*-dimenylmyristylammonio)propansulfonate

[****] SB3-16 : 3-(*N,N*-dimenylpalmitylammonio)propansulfonate
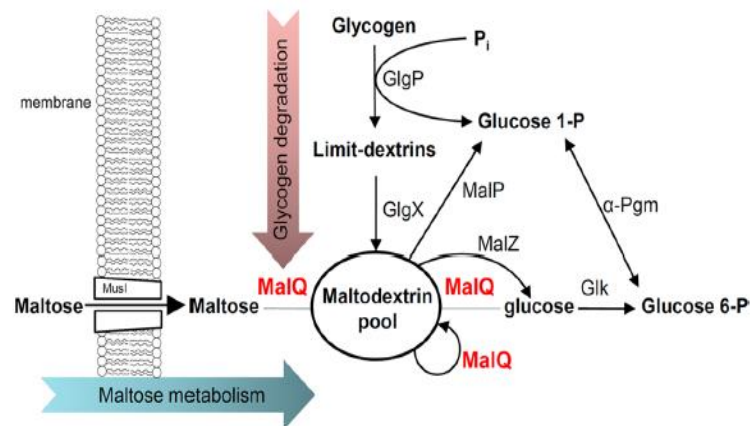
Figure 1. 5 A pathway of maltose/maltodextrin and glycogen degradation in E. coli (Seibold and Eikmanns, 2007). The compounds with bold black font are the key metabolites of the pathway. Proteins and enzymes encoded by genes shown are GlgP, glycogen phosphorylase; GlgX, glycogen debranching enzyme; MusI, a maltose transporter; Glk, glucose kinase; α-Pgm, α-phosphoglucomutase; MalZ, maltodextrin glucosidase; MalP, maltodextrin phosphorylase; MalQ, 4-α-glucanotransferase (Joo *et al*., 2016).

1.7 Structure of Amylomaltase from *C. glutamicum*

A novel amylomaltase from a mesophilic bacteria *C. glutamicum* ATCC 13032 (*Cg*AM) was characterized, analyzed, engineered and crystallized by our group to investigate functional roles of amino acid residues for the understanding of catalytic mechanism and enzyme improvement (Nimpiboon *et al.*, 2016a; Srisimarat *et al.*, 2012; Srisimarat *et al.*, 2013). *Cg*AM is different from amylomaltase *T. aquaticus* (*Ta*AM) in amino acid sequence and protein size with a different LR-CD product profile, having CD19 as the smallest product (Srisimarat *et al.*, 2011). We reported crystallization of *Cg*AM at 1.7 Å resolution but not yet submitted the structure to the Protein Data Bank.

In 2016, the Korean group reported crystallization of *Cg*AM complexed with bis-tris molecule at 1.70 Å resolution, and deposited the structure in Protein Data Bank (PDB code 5b68) (Joo *et al.*, 2016). However, the first three residues of the protein were invisible in the electron density map, 703 amino acids (Agr4-Asp703) out of 706 was obtained from their crystal structure. The amino acid sequence alignment of *Cg*AM, *E. coli* (*Ec*AM), *A. aeolicus* (*Aa*AM), *T. thermophilus* (*Tt*AM) and disproportionating enzyme DPE1 from *A. thaliana* (*At*DPE1) was compared Figure 1.6. Interestingly, *Cg*AM showed an overall folding similar to *Ec*AM Figure 1.6. The *Cg*AM structure consists of two distinctive domains: the N-terminal domain (N-domain; Met1-Arg165) and the C-terminal domain (C-domain; Leu166-Asp706) as shown in Figure 1.7A. The N-terminal domain is divided into two subdomains: Subdomain I (N1-subdomain, Met1-Pro72) which consists of three α-helices and one

double strand antiparallel β-sheet. The three α-helices form a helix bundle like shape and the β-sheet attached to the C-domain. Subdomain II (N2-subdomain, Leu73-Arg165) composed of two four-stranded β-sheets that form a β-sandwich. The N1 subdomain interacts directly with the C-domain whereas the N2-subdomain is loosely attached to the C-domain by forming independent folds.

The C-domain consists of four subdomains; one core subdomain and three auxiliary subdomains (Figure 1.7B). The core subdomain (CC-subdomain) is located at the center of C-domain and forms a $(β/α)_8$ -barrel (TIM barrel) fold with eight helices (α5, α9, α12-14, η9, α8 and α20) covering the eight stranded β-barrels (β12-14, β17, and β20-23). The CC-subdomain forms a catalytic site and also contributes to the substrate binding site. Auxiliary subdomain I (CA1-subdomain; Met221-Try358) comprises of three 3_{10}-helices and three α-helices and is located at the edge of $(β/α)_8{}^1$ and $(β/α)_8{}^2$. Auxiliary subdomain II (CA2-subdomain; Val391-Gly439 and His461-Phe485) comprises of two 3_{10}-helices and a three-stranded twisted β-sheets, and attached to the $(β/α)_8{}^3$ and $(β/α)_8{}^4$ of the CC-subdomain. The CA2-subdomain connects to the N-domain by interacting with N1-subdomain. Auxiliary subdomain III (CA3-subdomain: Thr558-Gly625 and Leu650-Asn693) comprises of 3_{10}-helices and four α-helices, covering the edge of $(β/α)_8{}^7$ and $(β/α)_8{}^8$ and also contacts the CA1-subdomain. The auxiliary subdomains are heavily involved in the substrate-binding pocket by which CA2-subdomain is a main contributor.

1.7.1 Structural comparison of *Cg*AM with other amylomaltases

   Overall structure

The *Cg*AM structure is homologous to amylomaltases from *E.coli* (*Ec*MalQ or *Ec*AM, PDB code 4S3P), *T. aquaticus* (*Ta*AM, PDB code 1CWY), *T. thermophilus* (*Tt*AM, PDB code 2OWX) and disproportionating enzyme (DPEs) from *A. thaliana* (*At*DPE1, PDB code 5CPQ) (Holm and Sander, 1998). Both amylomaltase and DPE1 from plant belong to 4-α-glucanotransferase type. However, the superimposed structures of *Cg*AM, *Ec*AM, *Tt*AM and *At*DPE1 revealed the striking difference of N-terminal domains whereas C-domains are closely matched and the N-domain interacting regions are similar (Figure 1.8A-B). In *Cg*AM and *Ec*AM, the extended domain from the N-terminal is found which is completely absent in the thermostable *Ta*AM or *Tt*AM. However, *At*DPE1 has a long N-terminal arm region that stretches toward the neighboring subunit to form a dimer of enzyme (Joo *et al*., 2016; O'Neill *et al*., 2015). The function of N-terminal extension domain of *Cg*AM remains unclear.

   Active site

In the active site cleft of *Cg*AM structure (PDB Code 5B68), one bis-tris methane molecule and one sulfate ion were bound (Figure 1.9). The catalytic mechanism of *Cg*AM was proposed from structural and biochemical studies on *Ec*AM (Weiss *et al*., 2015) and *Tt*AM (Barends *et al*., 2007) with the three catalytic conserved residues, two aspartates (D) and one glutamate (E). In *Ec*AM, D448 acts as a base (the catalytic nucleophile), E496, a proton donor (acid/base catalysis) and D548, a transition state stabilizer (Weiss *et al*., 2015). In *Cg*AM, the catalytic residues D460, E508 and D561 are located at CC subdomain at the corresponding positions with D448, E496 and

D548 of *Ec*AM, which suggested that *Cg*AM catalyzes enzyme reaction by the mechanism similar to *Ec*MalQ (Figure 1.9). In addition, the model for substrate (maltoheptaose; G7) binding of *Tt*AM showed seven subsites (-4 to +3) in the active site of enzyme (Figure 1.12) (Kaper *et al*., 2007). Three catalytic residues of *Cg*AM are corresponded to catalytic residues of *Tt*AM on the basis of the conserved residues at the active site of amylomaltase (Figure 1.6).

Unique loop

In *Cg*AM structure, the 410s loop (residues 414-422) is the flexible lid connecting the helices α15 and α16 in CA2-subdomain (Figure 1.6). It is located on top of the active site cleft covering subsite +1 to +3 of the substrate-binding groove (Joo *et al*., 2016) (Figure 1.10). The amino acid sequence around this loop is highly conserved compared to the 250s loop in *Ta*AM and 400s loop in *Ec*AM indicating that *Cg*AM also contains the flexible lid which undergoes conformation change during substrate binding as observed in *Ta*AM and *Ec*AM (Figure 1.6). In *Ta*AM, two solvent exposed hydrophobic residues at the tip of the loop: F251 and F366 residues, showed some disorder (flexibility) in the unliganded enzyme structure as well as in the complex with acarbose (Przylas *et al*., 2000a). Since this loop is highly conserved in amylomaltases, these findings suggested that this loop is involved in the substrate binding (Jung *et al*., 2011; Strater *et al*., 2002). Possibly the loop changes conformation or becomes less flexible when larger substrate binds closely to the loop.

Second glucan binding site

In order to study substrate binding to the enzyme, crystals of *Ta*AM were soaked with high concentration (100 mM) of acarbose, the potent inhibitor of many enzymes

in the α-amylase family (Przylas *et al*., 2000a). It was found that beside the acarbose bound at the active site, a second acarbose molecule was located in a groove (near Y54 and Y101) close to the active site center of *Ta*AM (Figure 1.11). The distance between the reducing end of acarbose and nonreducing end of the substrate analog bound to the active site is about 14 Å. Moreover, hydrophobic contacts of Y54 with glucose unit B and Y101 with unit C of the inhibitor are probably the most important interactions that determine the conformation and binding of the inhibitor to this site. Y101 of *Ta*AM is involved in a hydrophobic stacking interaction with glucose unit C (Figure 1.13). The second acarbose has significantly fewer interactions with the protein compared to the acarbose bound to the active site (Przylas *et al*., 2000a). Surprisingly, no second glucan binding site was reported in other amylomaltases with the X-ray crystal structures studied e.g. in thermophilic *Tt*AM and *Tb*AM, or the mesophilic *Ec*AM and *Cg*AM (Barends *et al*., 2007; Joo *et al*., 2016; Jung *et al*., 2011; Srisimarat *et al*., 2013; Weiss *et al*., 2015). From the superimposed structures, Y23 and N287 or Y290 of *Cg*AM are corresponding to Y54 and Y101 in the second glucan binding site of *Ta*AM, respectively (Joo *et al*., 2016; Przylas *et al*., 2000a; Srisimarat *et al*., 2013). It is observed that the residues around the second acarbose binding site are not strictly conserved compared to the acarbose binding to the active site (Strater *et al*., 2002).

1.8 Objectives

For a better understanding of enzyme mechanism and a way to improve enzyme performance, we are interested in investigation of a structure-function relationship of *Cg*AM. Our previous works on site-directed mutagenesis of residues in *Cg*AM are summarized in Discussion section. In the present work, we aim to focus on the three different regions of *Cg*AM; 410s loop, the active site, and the second glucan binding areas. Important residues will be selected to study and explore their functions involving in transglucosylation activity and LR-CD products profile. So, the work aims

1. To select the residues in the three regions and construct the mutated amylomaltase genes from *C. glutamicum* ATCC 13032 by site directed mutagenesis.

2. To express mutated *CgAM* genes in *E. coli*.

3. To purify and characterize recombinant mutated *Cg*AMs.

4. To construct the model structures of mutated *Cg*AMs by homology modeling program.

5. To compare structures and properties of mutated *Cg*AMs with the wild-type enzyme and identify important residues related to functions of the enzyme.
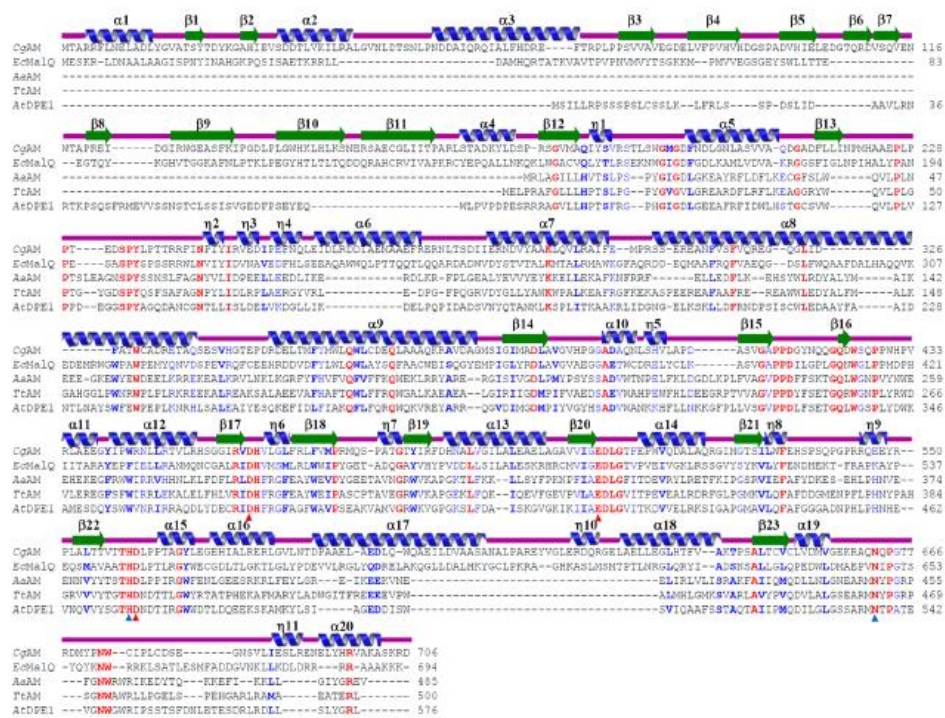
Figure 1. 6 The amino acid sequence alignment of amylomaltases from *C. glutamicum* (*Cg*AM), *E. coli* (*Ec*AM), *A. aeolicus* (*Aa*AM), *T. thermophilus* (*Tt*AM) and disproportionating enzyme DPE1 from *A. thaliana* (*At*DPE1). The secondary structure elements are marked on top of the alignment based on the *Cg*AM structure: α-helices and 3_{10}-helices by a helix and β-strands by an arrow. The η symbol refers to a 3_{10}-helix. Conserved residues are boxed in white on a red background; similar residues are boxed in red with white background. Residues involved in enzyme catalysis and maltose binding are indicated with triangles colored with red and blue, respectively (Joo *et al*., 2016).

A

B
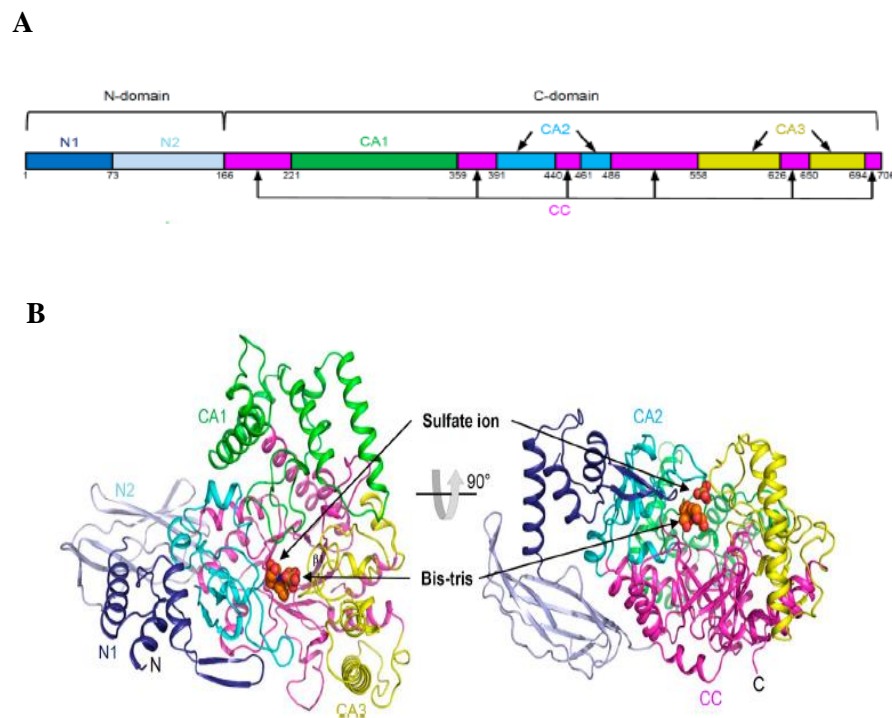
Figure 1.7 The Domain identification of *Cg*AM. N1 and N2 represent N1- and N2-subdomains of N domain, CC is core subdomain of C-domain, and CA1, CA2 and CA3 are auxiliary subdomains I, II and III of C-domain, respectively (A). Overall structure of *Cg*AM complexed with bis-tris and sulfate ion (B), the structure of *Cg*AM is represented as a secondary structure diagram. The right side figure is rotated 90 degree in horizontal direction. A bis-tris molecule and sulfate ion bound in *Cg*AM are shown as sphere models and labeled. The subdomains of *Cg*AMs are distinguished with color scheme as in panel A (Joo *et al*., 2016).
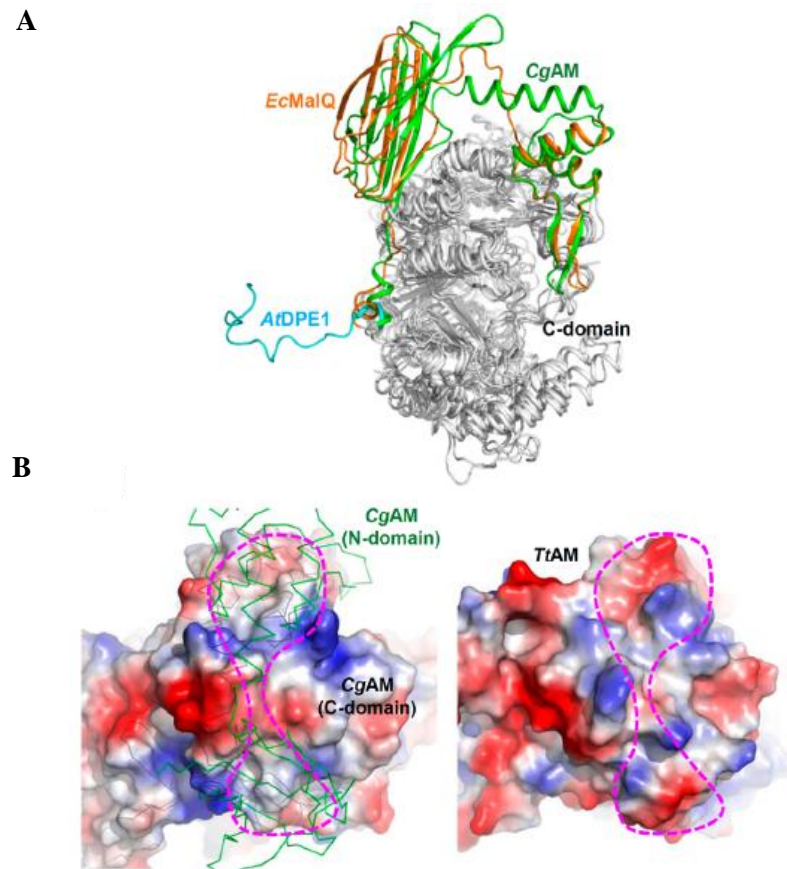
Figure 1. 8 Comparison of N-domains of amylomaltases. Structures of *Cg*AM, *Ec*AM, *Tt*AM and *At*DPE1 are superimposed. C-domains of four enzymes are shown in grey while N-domains of *Cg*AM and *Ec*AM in green and orange, respectively, and N-terminal arm region of AtDPE1 is shown in cyan (A). Charge distribution on the surfaces of C-domains of *Cg*AM and *Tt*AM. The N-domain and C-domain of *Cg*AM are shown as ribbon and electrostatic potential surface mode (left), and *Tt*AM as an electrostatic potential surface mode (right).N-domain interacting region on the C-domain is indicated as a magenta-colored dotted line (left), and the corresponding region of *Tt*AM is indicated as in *Cg*AM (B) (Joo *et al*., 2016).
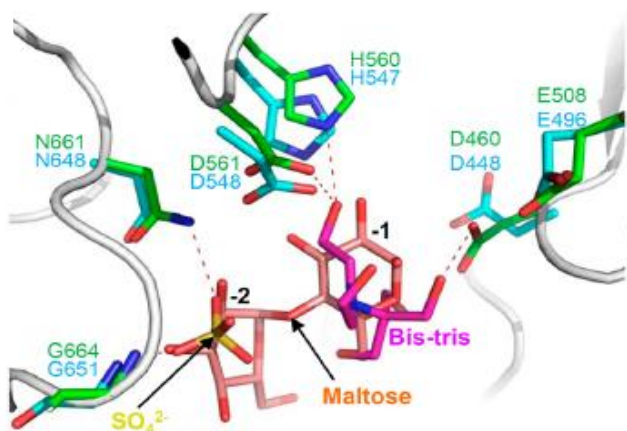
Figure 1. 9 Active site of *Cg*AM. The superimposed amylomaltase structures of *Cg*AM and *Ec*AM with bis-tris methane and maltose complex, respectively. The bis-tris and sulfate molecule bound in *Cg*AM are shown in pink and yellow, respectively. The maltose molecule bound in *Ec*AM is shown as salmon-colored stick. Catalytic residues in *Cg*AM (D460, E508 and D561) and *Ec*AM (D448, E496 and D548) are shown in green and cyan-colored stick, respectively (Joo *et al*., 2016).

A

B
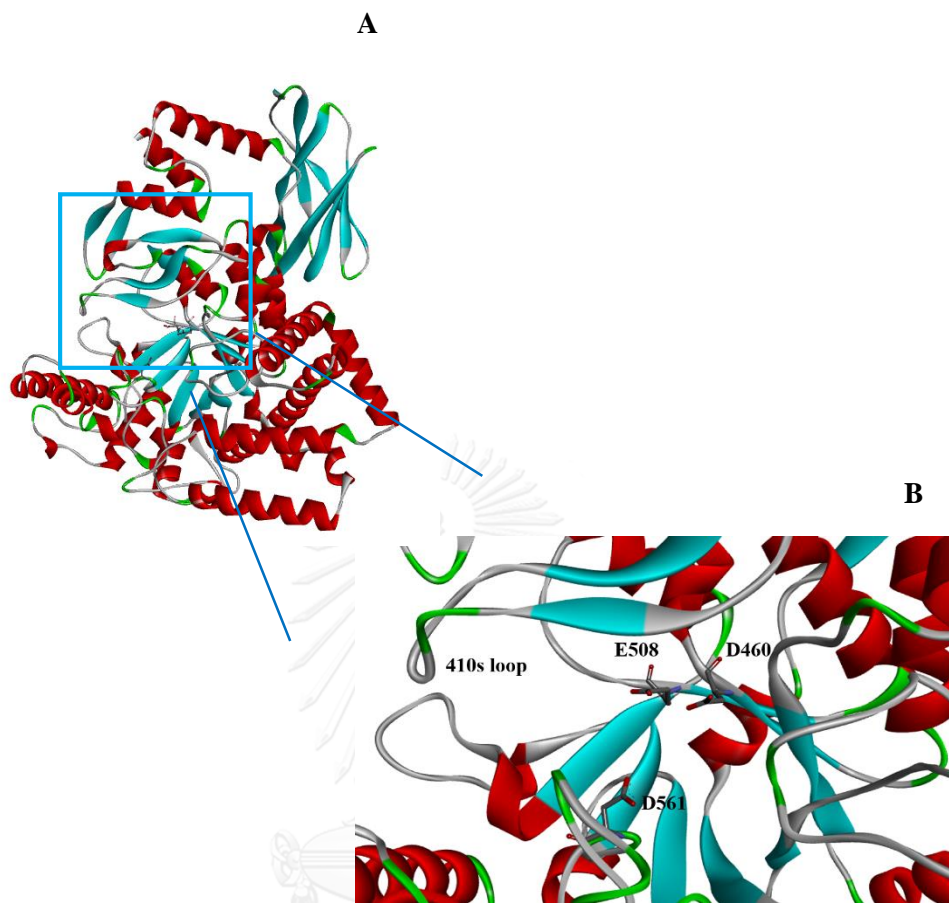
Figure 1. 10 Structure of amylomaltase from *C. glutamicum* (*Cg*AM) (PDB code 5B68) .α -helix, β-sheet and loop are red, cyan and green, respectively. The expanded structures in the box in (A) are the three catalytic residues (D460, E508 and D561) and 410s loop (B) (Joo *et al*., 2016).
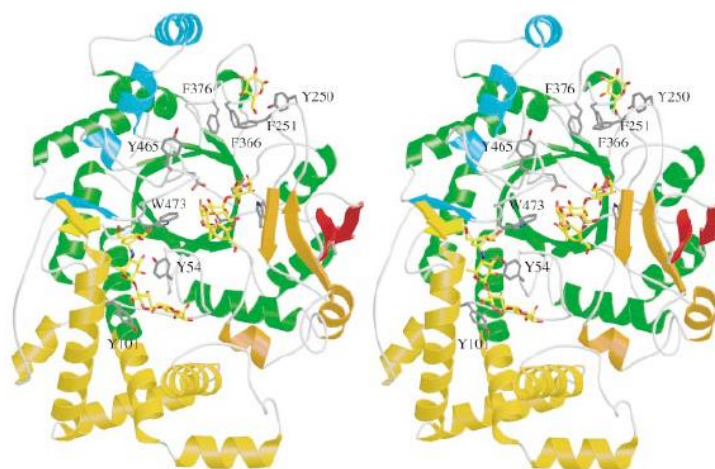
Figure 1. 11 Ribbon representation (stereo view) of the fold of amylomaltase *T. aquaticus* (*Ta*AM) with acarbose. In addition to the two acarbose molecules (yellow carbon atoms), selected protein side chains (dark gray carbon atoms) are shown. The acarbose molecule bound to the active site is on the upper right side, close to W473. The catalytic side chains (D293, E340 and D395) and W258 which are partially hidden by acarbose or protein residues are not labelled. The (β/α)₈ barrel core structure (subdomain A) is colored in green, insertions between the first and fifth strand of the barrel (subdomains B2 and B1) are painted in a gradient going from yellow to red. Additional small insertions are shown in blue (subdomain B3) (Przylas *et al*., 2000a).
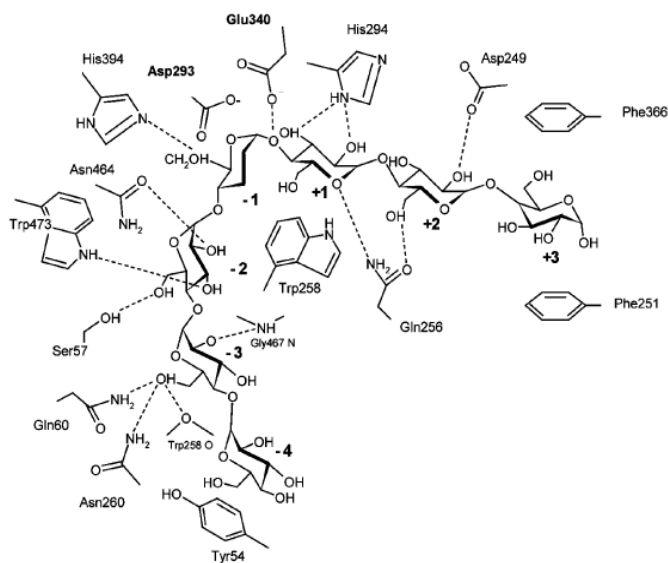
Figure 1. 12 Proposed binding of maltoheptaose (G7) in the active site of amylomaltase from *Thermus thermophilus* (*Tt*AM). Catalytic residues are shown in boldface (D293 and E340) (Kaper *et al.*, 2007).
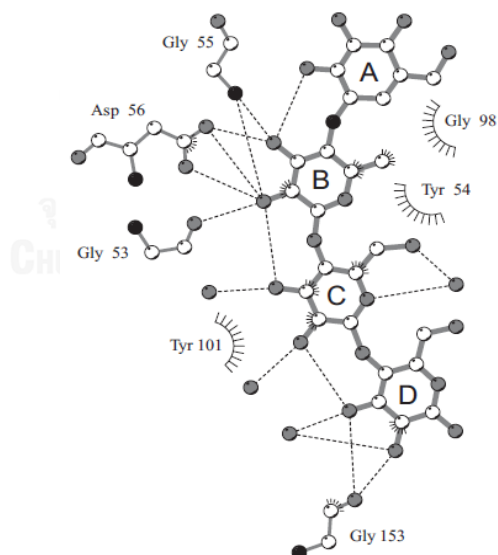
Figure 1. 13 Scheme of the interactions of acarbose bound to the secondary glucan binding site of amylomaltase from *T. aquaticus* (*Ta*AM) (Przylas *et al.*, 2000a)

CHAPTER II
MATERIALS AND METHODS

2.1 Equipments

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

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model AvantiTM J30-1I, Beckman Instrument

Inc., USA

Electrophoresis unit:

- Mini protein, Bio-Rad, USA

- Agarose gel electrophoresis, Bio-Rad, USA

Differential scanning calorimeter, Microcal, LLC, Nothampton, USA

Gel Document: SYNGEND, England

Gel support film for polyacrylamide, Bio-Rad, USA

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

- Column: Carbopac PA-100TM 4 x 250 mm

- Pulsed amperometric detector: DIONEX ED40

- Autosampler: DIONEX AS40

- Column oven DIONEX ICS-3000 SP

Incubator, waterbath:

- Model M20S, Lauda, Germany and BioChiller 2000, FOTODYNE Inc., USA

- ISOTEMP 210, Fisher Scientific, USA

Incubator shaker: Innova^{TM} 4080, New Brunswick Scientific Co., Ltd., England

Isothermal titration calorimeter, Microcal, LLC, Nothampton, USA

Laminar flow: HT123, ISSCO, USA

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA

Membrane filter: polyethersulfone (PES), pore size 0.45 µm, Whatman, England

Microcentrifuge: Eppendorf, Germany

 pH meter: Model PHM95, Radiometer Copenhegen, Denmark

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

 Shaking waterbath: Model G-76, New Brunswick Scientific Co., Inc., USA

Sonicator: Bendelin, Germany

 Spectrophotometer: DU Series 650, Beckman, USA

Thermal cycler: Mastercycler, Eppendorf, Germany

 UV detector: Monitor UV-1, GE Healthcare Bio-Sciences AB, Sweden

Vortex: Model K-550-GE, Scientific Industries, Inc, USA

2.2 Chemicals

Acrylamide: Merck, Germany

Agar: Merck, Germany

Agarose: FMC Bioproducts, USA

Ammonium persulphate: Sigma, USA

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, USA

Bovine serum albumin (BSA): Sigma, USA

Boric acid: Merck, Germany

Coomassie brilliant blue R-250: Sigma, USA

Copper sulfate: Carlo Erba Reagenti, Italy

4,4-Dicarboxy-2,2-biquinoline: Sigma, USA

Dimethyl sulfoxide (DMSO): Merck, Germany

di-Potassium hydrogen phosphate anhydrous: Carlo Earba Reagenti, Italy

di-Sodium ethylene diamine tetra acetic acid: M&B, England

1 kb DNA ladder^{TM}: New England BioLabs Inc., USA and Fermentas, Canada

DNA extraction kit: Geneaid Biotech Ltd., Taiwan

dNTP: Stratagene, USA

Ethidium bromide: Sigma, USA

Ethyl alcohol absolute: Carlo Earba Reagenti, Italy

Ethylene diamine tetra acetic acid (EDTA): Merck, Germany

Gel extraction kit: Geneaids Biotech Ltd., Taiwan

Glacial acetic acid: Carlo Earba Reagenti, Italy

Glucose: BDH, England

Glucose liquicolor (Glucose oxidase kit): HUMAN, Germany

Glycerol: Merck, Germany

Glycine: Sigma, USA

Hydrochloric acid: Carlo Earba Reagenti, Italy

Iodine: Baker chemical, USA

Isopropylthio-β-D-galactoside (IPTG): Sigma, USA

Maltoheptaose (G7), Maltohexaose (G6), Maltopentaose (G5), Maltotetraose (G4),
and Maltotriose (G3): Wako Pure Chemical Industries, LTD., Japan

Maltose: BDH, England

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

Peptone: Scharlau Microbiology, Spain

Phenol: Fisher Scientific, England

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Potassium iodide: Mallinckrodt, USA

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Sodium acetate: Merck, Germany

Sodium carbonate anhydrous: Carlo Earba Reagenti, Italy

Sodium chloride: Carlo Earba Reagenti, Italy

Sodium citrate: Carlo Earba Reagenti, Italy

Sodium dodecyl sulfate: Sigma, USA

Sodium hydroxide: Merck, Germany

Soluble starch (potato): Scharlau Microbiology, Spain

Standard Large-ring cyclodextrin (LR-CD): Ezaki Glico Co., Ltd., Japan

Standard protein marker: Amersham Pharmacia Biotech Inc., USA

Tris (hydroxymethyl)-aminomethane: Carlo Earba Reagenti, Italy

Tryptone: Scharlau Microbiology, Spain

Yeast extract: Scharlau Microbiology, Spain

2.3 Enzymes, Restriction enzymes and Bacterial strains

Corynebacterium glutamicum ATCC 13032, Thailand Institute of Scientific and

Technological Research, Thailand

E. coli BL21 (DE3): Novagen, Germany

Glucoamylase from *Aspergillus niger*: Fluka, Switzerland

Pfu Thurbo® DNA polymerase: Promega, USA

Plasmid pET-19b: Novagen, Germany

Restriction enzymes

- *Dpn* I: New England BioLabs Inc. USA

- *Nde* I: Fermantas, Canada.

- *Xho* I: Fermantas, Canada.

2.4 Construction of mutated amylomaltase gene from *C. glutamicum*

2.4.1 Selection of target amino acid residues

Target amino acid residues of *Cg*AM were selected by using the amino acid

multiple sequence alignment and superimposed of X-ray structures between *Cg*AM

(Joo *et al*., 2016; Srisimarat *et al*., 2013) and well-known characterized amylomaltase

from *Thermus* sp. (*Ta*AM) (Przylas *et al*., 2000b). Three amino acid residues were

chosen as followed;

1. Y418 residue at the tip of 410s loop which lies over the active site

(corresponded to F251 in 250s loop in *Thermus* sp.)

2. H461 residue near the catalytic site of amylomaltase corresponded to H294 in *Thermus sp*.

3. N287 residue near the second binding site of amylomaltase corresponded to Y101 in *Thermus sp.*

2.4.2 Site-directed mutagenesis of *CgAM* gene

Site directed mutatgenesis of *CgAM* gene was performed by amino acid substitution using mutated primers. A plasmid (pET-19b) carrying wild type (WT) *CgAM* gene was used as template to design mutated primers. Mutated *CgAM* genes were constructed by PCR mediated site-directed mutagenesis using Quick-Change kit (Stratagene, USA) and mutated primers which contained a mutation site at three residues (Y418, H461 and N287). Changes to alanine (A), aspartic acid (D), serine (S), arginine (R) and tryptophan (W) were performed. For Y418, substitution by phenylalanine (F) was also performed. The mutated primers were shown in Table 2.1, 2.2 and 2.3.

PCR conditions were pre-denaturation for 2 min at 95 $^{\circ}$C, denaturation for 1 min at 95 $^{\circ}$C, annealing for 30 sec at 68 $^{\circ}$C, extension for 16 min at 72 $^{\circ}$C and post-extension for 10 min at 72 $^{\circ}$C. PCR product was digested with *Dpn* I endonuclease (target sequence: 5'-GA$_{CH3}$^TC-3') which is specific for methylated as well as hemimethylated DNA. The enzyme was used to digest parental DNA template to select the mutation-containing DNA synthesized for 1 h. DNA isolated from almost all *E. coli* strain is dam-methylated which is susceptible to *Dpn* I. The nicked plasmid of *Dpn* I treated was then transformed into *E. coli* BL21 (DE3) electrocompetent cells.

2.4.3 Preparation of competent cells for electroporation (Sambrook and Russell, 2006)

A fresh overnight culture of *E. coli* BL21 (DE3) was inoculated into 100 ml of LB medium with 1% inoculum size. The cell culture was cultivated with shaking at 250 rpm until OD_{600} reached 0.3 to 0.4. The culture was chilled on ice for 15 min at 4 ℃. The cells were washed by cold sterilize water, centrifuged at 5,000 x g for 3 times at 4 ℃. After centrifugation, the cells were resuspended in approximately 15-20 ml of 10% glycerol in distilled water and centrifuged at 5,000 x g for 10 min at 4 ℃. Finally, the cells were resuspended again in 10 ml of 10% glycerol in distilled water and then 20-30 µl of cell suspension was aliquoted into each eppendorf tube and stored at -80 ℃ until use.
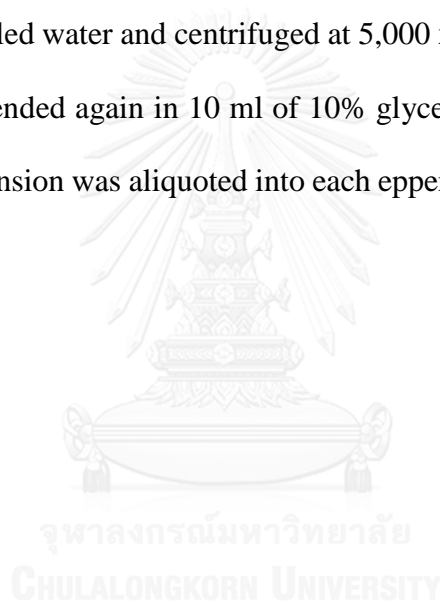
Table 2. 1 Y418 mutated *CgAM* gene primers for PCR mediated site - directed mutagenesis. The mutation positions were underlined.

Y418A_FWD	[5'- CGCCCCACCAGTTGGA<u>GC</u>CAACCAGCAGGGCCAAG -3']
Y418A_REV	[5'- CTTGGCCCTGCTGGTTG<u>GC</u>TCCATCTGGTGGGGCG -3']
Y418S_FWD	[5'- CACCAGATGGAT<u>C</u>CAACCAGCAGGG -3']
Y418S_REV	[5'- CCCTGCTGGTTG<u>G</u>ATCCATCTGGTG -3']
Y418D_FWD	[5'- CCACCAGATGGA<u>G</u>ACAACCAGCAGG -3']
Y418D_REV	[5'- CCTGCTGGTTGTCTCCAT<u>C</u>TGGTGG - 3']
Y418R_FWD	[5'- CCACCAGATGGA<u>CG</u>CAACCAGCAGGGC -3']
Y418R_REV	[5'- GCCCTGCTGGTTG<u>CG</u>TCCATCTGGTGG -3']
Y418W_FWD	[5'- CACCAGATGGAT<u>GG</u>AACCAGCAGGGCCAAGACTGG -3']
Y418W_REV	[5'- CCAGTCTTGGCCCTGCTGGTT<u>CC</u>ATCCATCTGGTG -3']
Y418F_FWD	[5'- CCCACCAGATGGAT<u>TT</u>AACCAGCAGGGCC -3']
Y418F_REV	[5' - GGCCCTGCTGGTTA<u>AA</u>TCCATCTGGTGGG -3']

Table 2. 2 H461 mutated *CgAM* gene primers for PCR mediated site - directed mutagenesis. The mutation positions were underlined.

H461A_FWD	[5'- GCGGAATCCGCGTGGAC<u>GC</u>CGTTCTTGGTTTGTTCAG- 3']
H461A_REV	[5'- CTGAACAAACCAAGAAC<u>GG</u>CGTCCACGCGGATTCCGC- 3']
H461S_FWD	[5'- CGGCGGAATCCGCGTGGAC<u>AG</u>CGTTCTTGGTTTGTTCAGG- 3']
H461S_REV	[5'- CCTGAACAAACCAAGAACGCT<u>GTC</u>CACGCGGATTCCGCCG- 3']
H461D_FWD	[5'- GCGGAATCCGCGTG<u>GA</u>CGACGTTCTTGGTTTGTTC- 3']
H461D_REV	[5'- GAACAAACCAAGAAC<u>G</u>TCGTCCACGCGGATTCCGC- 3']
H461R_FWD	[5'- CGGAATCCGCGTGGACC<u>G</u>CGTTCTTGGTTTGTTC- 3']
H461R_REV	[5'- GAACAAACCAAGAACG<u>C</u>GGTCCACGCGGATTCCG- 3']
H461W_FWD	[5'- GCGGAATCCGCGTGGAC<u>TGG</u>GTTCTTGGTTTGTTCAGG- 3']
H461W_REV	[5'- CCTGAACAAACCAAGA<u>ACC</u>CAGTCCACGCGGATTCCGC- 3']

Table 2. 3 N287 mutated *CgAM* gene primers for PCR mediated site - directed mutagenesis. The mutation positions were underlined.

N287A_FWD	[5'- GACATCATTGAGCGC<u>GC</u>TGACGTCTACGCTGC -3']
N287A_REV	[5'- GCAGCGTAACGTCAGCGC<u>GC</u>TCAATGATGTC -3']
N287S_FWD	[5'- GACATCATTGAGCGC<u>TC</u>TGACGTCTACGCTGC- 3']
N287S_REV	[5'- GCAGCGTAGACGTCA<u>GA</u>GCGCTCAATGATGTC- 3']
N287D_FWD	[5'- CATCATTGAGCGC<u>G</u>ATGACGTCTACGC- 3']
N287D_REV	[5'- GCGTAGACGTCAT<u>C</u>GCGCTCAATGATG- 3']
N287R_FWD	[5'- CCTCAGACATCATTGAG<u>CG</u>CCGCGACGTCTACGCTGCAAAGC- 3']
N287R_REV	[5'- GCTTTGCAGCGTAGACGTC<u>GC</u>GGCGCTCAATGATGTCTGAGG- 3']
N287W_FWD	[5'- GACATCATTGAGCGC<u>TGG</u>GACGTCTACGCTGC- 3']
N287W_REV	[5'- GCAGCGTAGACGT<u>CCC</u>AGCGCTCAATGATG TC- 3']

2.4.4 Plasmid transformation

The recombinant plasmids containing mutated *CgAM* gene from section 2.4.2 were introduced into *E. coli* BL21 (DE3) competent cells by electroporation technique. In the electroporation step, 0.2 cm cuvettes and sliding cuvette holder were chilled on ice before use. The Gene Pulser® apparatus was set to the 25 µF capacitor, 2.5 kV, and the pulser controller unit was set to 200. The competent cells which were prepared as described in 2.4.3 were gently thawed on ice. One to five microliter of recombinant plasmid was mixed with 30 µl of the electrocompetent cells and then placed on ice for 3 min. This mixture was transferred into a chilled cuvette. The cuvette was applied one pulse at the above settings. Subsequently, one milliliter of LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) was added immediately to the cuvette. The cells were quickly resuspended, then the cell suspension was transferred to new sterilize tube and incubated at 37 ℃ for 45 min with 150 rpm shaking. Finally, the cell suspension was spread onto the LB agar plate containing 100 µg/ml ampicillin. After incubation at 37 ℃ for 16 h, the colonies were picked.

2.4.5 Selection of mutated transformant clones

Transformation colonies were cultured in LB medium containing 100 µg/ml ampicillin for 12 h. The recombinant mutated plasmids were extracted, and size check was performed by agarose gel electrophoresis. The point mutation was confirmed by nucleotide sequencing.

2.4.6 Nucleotide sequencing

About 50-100 ng of the recombinant mutated plasmids from section 2.4.5 were subjected to automated DNA sequencing (Wardmedic, Thailand). The sequencing was performed using T7 promoter and T7 terminator for DNA sequence 5' terminus and 3'

terminus of the inserted *CgAM* gene, respectively. The obtained DNA sequence was used to design internal primers of f2CgAM and r2CgAM for determining the residual sequence which was localized in the middle of the gene. The internal primers were used as shown below;

F2CgAM_FWD: [5'- TCTACTCTGTGCGTTCCACGTTG -3']

R2CgAM_REV: [5'- CCTGCGAGTTCTGCTTATAGG - 3']

The mutated gene was compared with WT- *CgAM* gene to determine point mutation by Clastal O program (1.2.4).

2.5 Expression and purification of recombinant amylomaltase

2.5.1 Expression of amylomaltase in *E. coli*

E. coli BL21(DE3) cells harboring WT or mutated *CgAM* genes were grown in LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) containing 100 µg/ml ampicillin at 37 ℃, expression was triggered by 0.4 mM of isopropylthio-β-D-galactoside (IPTG) as described (Srisimarat *et al*., 2011). Cells were cultured at 16 ℃, 150 rpm for 20 h and harvested by centrifugation at 5000 x g for 15 min at 4 ℃ then washed by 0.85% NaCl and extraction buffer, respectively as described (Srisimarat *et al*., 2011). Bacterial cells were resuspended in extraction buffer then disrupted by 15 cycles sonication (30% pulse for 1 min and stopped for 3 min for each cycle). Bacterial cells debris was removed by centrifugation at 12,000 x g, 4 ℃ for 45 min. The supernatant carrying crude enzyme was collected and dialyzed against 20 mM phosphate buffer pH 7.4.

2.5.2 Purification of amylomaltases

The crude amylomaltase (containing His-tag fragment) from section 2.5.1 was purified by Histrap FF^{TM} affinity at 4 ℃. The column was equilibrated with 20 mM phosphate buffer pH 7.4 containing 20 mM imidazole and 0.5 M NaCl at the flow rate of 1ml/min. The crude enzyme solution was applied to Histrap FF^{TM} column (1 ml, two columns consecutively) and washed with the same buffer until A$_{280}$ of eluent decreased to baseline. The purified enzyme was eluted by the same buffer supplemented with 500 mM imidazole (Srisimarat *et al*., 2011). Fractions were collected and the enzyme activity was determined. The active fractions were pooled and dialyzed against 20 mM phosphate buffer pH 7.4 and protein bands, protein concentration and enzyme activity were determined as described in section 2.6, 2.7 and 2.8 respectively.

2.6 Polyacrylamide gel electrophoresis (PAGE)

2.6.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using protein gel apparatus (Bio-rad Laboratory, USA). Before loading the sample on the gel, the samples were mixed with loading buffer which contained 60 mM Tris-HCl pH 6.8; 25% (v/v) glycerol; 2% (w/v) SDS; 14.4 mM 2-mercaptoethanol; 1% (w/v) bromophenol blue and boiled for 5 min. 7.5% Stacking gel and 5% separating gel were performed. Pre-stained Protein Ladder (Thermo Scientific Page Ruler), a mixture of proteins of various sizes (10 to 180 kDa), was used as protein marker. Protein bands were observed by Coomassie blue staining.

2.6.2 Detection of protein bands by Coomassie blue staining

The gel was stained by agitating with Coomassie staining solution (1%, w/v Coomaasie blue R-250, 45%, v/v methanol and 10%, v/v glacial acetic acid) for 1-2 h. Then, destained by destaining solution (10%, v/v methanol and 10%, v/v glacial acetic acid) for several times until gel background was cleared.

2.7 Protein determination

Protein concentration was determined by coomassie blue according to Bradford method, using bovine serum albumin as standard (Bollag *et al*., 1996). One hundred microliter of sample was mixed with 1 ml of Bradford working solution containing Coomassie blue G-250 for 10 min and the absorbance was measured at 595 nm.

2.8 Enzyme assay

All assays were performed for wild- type (WT) and mutated *Cg*AMs. Amylomaltase activity was determined by six assays as described below.

2.8.1 Starch transglucosylation activity

The starch transglucosylation activity was measured by iodine method. 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 (Park *et al*., 2007). The reaction mixture containing 250 µl of 0.2% soluble potato starch, 50 µl of 1% (w/v) maltose, 100 µl of enzyme and 600 µl of 50 mM phosphate buffer, pH 6.0. The reaction was incubated at 30 °C for 10 min and stopped by boiling for 10 min. Then, 100 µl of reaction mixture was aliquoted and mixed with 1 ml of

iodine solution (0.02% (w/v)) I_2 in 0.2% (w/v) KI) and the absorbance at 600 nm was measured.

One unit is defined as the amount of enzyme required to degrade 1 mg starch/ml per min under the described condition.

2.8.2 Disproportionation activity

This activity was measured by following glucose produced from glucosyl transfer between short oligosaccharides using glucose oxidase method (Miwa *et al.*, 1972). The 50 µl of reaction mixture, containing 5% (w/v) maltotriose (G3) and enzyme in 50 mM phosphate buffer, pH 6.0 was incubated at 40 ºC for 10 min, the reaction was stopped by boiling for 10 min. An aliquot of 10 µl was mixed with glucose oxidase reagent kit and incubated at 30 ºC for 10 min. The absorbance at 505 nm was measured.

One unit defined as the amount of enzyme required for the production of 1 µmole of glucose per min under the described condition.

2.8.3 Starch degrading activity

This activity was determined by measuring the degraded starch in the reaction by iodine method (Srisimarat *et al.*, 2011). The 250 µl of reaction mixture, containing 100 µl of 0.75% (w/v) potato soluble starch, 50 µl of the enzyme and 50 mM phosphate buffer pH 6.0. The reaction was incubated at 30 ºC for 10 min, stopped by adding 500 µl of 1 N HCl. Then, 100 µl of reaction mixture was aliquoted and mixed with 900 µl of iodine solution (0.005% (w/v)) I_2 in 0.05% (w/v) KI) and the absorbance at 660 nm was measured.

One unit is defined as the amount of enzyme required to degrade 1 mg starch/ml per min under the described condition.

2.8.4 Cyclization activity

This activity was determined by measuring LR-CD product using high-performance anion exchange chomatography with pulse amperometric detection (HPAEC-PAD) (Srisimarat *et al*., 2011). The reaction mixture contained 150 μl of 2% (w/v) pea starch and 50 μl of enzyme in 50 mM phosphate buffer pH 6.0 in a total volume of 1.5 ml. The reaction was incubated at 37 ºC for 1.5 h, then stopped by boiling for 10 min. After cooling, 8 U of glucoamylase was added and further incubated at 40 ºC for 18 h. The reaction was inactivated by boiling for 10 min, then the reaction was cooled down and analyzed by (HPAEC-PAD) (model ICS-5000 single pump, Dionex, USA), using Carbopac PA-100 (4x250 mm). Elution was by six continuous steps linear gradient of sodium nitrate in sodium hydroxide as previously described (Srisimarat *et al*., 2011). The size of LR-CD products was compared with standard LR-CDs (CD22-CD50).

One unit is defined as the amount of enzyme required for the production of 1 nC of CD31 per min under the described condition.

2.8.5 Coupling activity

This activity is a reverse of cyclization activity, measured by DNS method (Zhu *et al*., 2005). The 500 µl of reaction mixture, containing 100 µl of 3 mg/ml of LR-CDs, 100 µl of 1 mg/ml (w/v) of glucose and 100 µl of the enzyme in 50 mM phosphate buffer pH 6.0. The reaction was incubated at 30 ºC for 10 min, then stopped by boiling. 8 U of glucoamylase was added to reaction, incubated at 40 º C for 30 min and inactivated by boiling. DNS reagent (3,5-dinitrosalicyclic acid, 500 µl was added and boiled for 5 min, cooled down the reaction at room temperature and added 4 ml of distilled water. The absorbance at 540 nm was measured.

One unit is defined as the amount of enzyme required for the production of 1 µmole of reduced glucose per min under described condition.

2.8.6 Hydrolytic activity

This activity was determined by measuring glucose produced from hydrolysis of LR-CDs by bicinchoninic acid (BCA) assay (Srisimarat *et al*., 2011). The 50 µl of reaction mixture, containing 30 µl of 0.5 mg/ml of LR-CDs and 10 µl of the enzyme in the total volume of 50 µl of 50 mM phosphate buffer pH 6.0. The reaction was incubated at 30 ºC for 10 min, then stopped by adding 30 µl of 1 N HCl. Bicinchoninic acid reagent was added to make the final volume of 1 ml, incubated at 80 ºC for 25 min, inactivated the reaction on ice for 5 min. The absorbance at 562 nm was measured.

One unit is defined as the amount of enzyme required for the production of 1 µmole of reduced glucose per min under the described condition.

2.9 Characterization of amylomaltase

2.9.1 Effect of temperature on amylomatase activity

The effect of temperature on starch transglucosylation, disproportionation and cyclization activities of WT and mutated CgAMs were determined at optimum pH of 6.0 using 50 mM phosphate buffer. The temperature was varied in the range of 25-70 °C. The activity was determined as described in section 2.8.1, 2.8.2 and 2.8.4. The maximum activity observed for each enzyme was set as 100%.

2.9.2 Effect of pH on amylomaltase activity

The effect of pH on enzyme activity for starch transglycosylation, disproportionation and cyclization activities were determined as described in section 2.8.1, 2.8.2 and 2.8.4, respectively. WT and mutated CgAMs were incubated in various buffers: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and tris-HCl buffer (pH 8.0-9.0) at each optimum temperature before determination of enzyme activity. The results were shown as percentage of relative activity. The highest activity was defined as 100%.

2.9.3 Effect of temperature on amylomaltase stability

The effect of temperature on enzyme stability for disproportionation reaction was determined. WT and mutated CgAMs were incubated at various temperatures (30-40 °C) for 0-180 min and then enzyme activity was measured under the standard condition as described in section 2.8.2. The results were shown as percentage of relative activity. The highest activity was defined as 100%.

2.9.4 Effect of pH on amylomaltase stability

The effect of pH on enzyme stability for disproportionation reaction was determined as described in section 2.8.2. WT and mutated CgAMs were incubated at 30 ºC in phosphate buffer pH 7.0 and tris-HCl buffer pH 8.0 for 0-120 min before determination of enzyme activity. The results were shown as percentage of relative activity. The highest activity was defined as 100%.

2.9.5 Substrate specificity

Substrate specificity was determined by measuring disproportionation activity using maltose (G2) to maltoheptaose (G7) as substrates. 0.2 U starch transglucosylation activity of CgAMs were incubated with 50 mM substrate in phosphate buffer pH 6.0 and the amount of glucose product was determined by glucose oxidase method as described in 2.8.2. The relative activities were calculated by using WT activity with G3 substrate as the control.

2.9.6 Determination of kinetic parameters

2.9.6.1 Disproportionation reaction

Kinetic study of disproportionation reaction was performed by incubating various concentrations of G3 substrate (0-40 mM) with CgAMs at 30 ºC for 5 min. The kinetic parameters K_m and V_{max} were determined from Lineweaver-Burk plot, k_{cat} and k_{cat}/K_m values were calculated. The activities of mutated enzymes were determined by glucose oxidase method as described in section 2.8.2.

2.9.6.2 Cyclization reaction

0.05 U starch degrading activity of *Cg*AMs was incubated with various concentrations of pea starch substrate (0-0.5%) in 50 mM phosphate buffer, pH 6.0 at 30 °C for 90 min then reaction was stopped by boiling for 10 min. 8 U of glucoamylase was added and incubated at 40 °C for 18 h. LR-CD products were analyzed by HPAEC-PAD as described in 2.8.4. The kinetic parameters of *Cg*AMs on cyclization activity, K_m and V_{max} were determined from Lineweaver-Burk plot, k_{cat} and k_{cat}/K_m values were calculated.

2.9.7 Analysis of pattern of LR-CD products

LR-CD products were determined by HPAEC-PAD as described in 2.8.4.

2.9.7.1 Effect of incubation time on LR-CD profile

The reaction mixtures contained 0.2% (w/v) pea starch and 0.10 U starch degrading activity of *Cg*AMs in 50 mM phosphate buffer, pH 6.0. The reaction was incubated at 30 ºC for 1.5, 6, 12 and 24 h and then stopped by boiling. 8 U of glucoamylase was added and further incubated at 40 ºC for 18 h and inactivated by boiling. The reaction products of *Cg*AMs were analyzed by HPAEC-PAD.

2.9.7.2 Effect of the amount of enzyme on LR-CD profile

The reaction mixtures contained 0.2% (w/v) pea starch with various amounts of 0.5, 0.10 and 0.20 U starch degrading activity of *Cg*AMs in 50 mM phosphate buffer, pH 6.0. The reaction was incubated at 30 ºC for 6 h and then stopped by boiling. 8 U of glucoamylase was added, then incubated at 40 ºC overnight and inactivated by boiling. The reaction products of *Cg*AMs were analyzed by HPAEC-PAD.

2.9.8 Circular dichroism spectra

The spectra were obtained using a J-815 CD spectrometer (Jasco, Japan). A protein concentration of 0.2 mg/ml was used in the wavelength scanning range of 190-250 nm at 25 °C. Each CD spectrum was monitored in thee scans at 20 nm/min with constant time at 2 sec and 2 nm bandwidth.

For the CD spectra, the Mean Residue Weight (MRW) for estimating the quantity of the peptide bond in protein as shown in equation (1) was calculated (Kelly *et al.*, 2005).

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

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

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

Where θ_λ = the observed ellipticity (degrees) at each wavelength

d = pathlength of cuvette (cm)

c = the protein concentration (g/ml)

If the molar concentration (m) of solute was known, the molor ellipticity at wavelength λ ($[\theta]_{molar, \lambda}$) is given by equation (3)

$$[\theta]_{molar, \lambda} = \frac{100 \times \theta_\lambda}{m \times d} \qquad (3)$$

The unit of MRE and molar ellipticity is deg.cm^2.dmol^{-1} (Kelly *et al.*, 2005). The percentages of protein secondary structures were calculated using the Dichroweb server program.

2.10 Biophysical characterization of amylomaltase

2.10.1 Isothermal titration calorimetry (ITC)

The enthalpy associated with *Cg*AM activity interacted with G3 substrate in disproportionation reaction was determined by a Microcal VP-ITC isothermal titration calorimeter (Microcal, LLC, Nothampton, USA). Buffer and sample solutions were degassed immediately by centrifugation at 10,000 x g 4 ºC for 45 min prior to conducting the assay. The reference cell, sample cell and titration syringe were filled with degassed milliQ water, 10 mM G3 in 20 mM phosphate buffer, pH 6.0 and 20 µM of each enzyme in 20 mM phosphate buffer pH 6.0, respectively. The system was equilibrated at 30 ºC which is the optimum condition of *Cg*AMs with stirring at 329 rpm prior to titration of enzyme. Titration was performed by single injecting 20 µl of 20 µM purified WT and Y418A/W mutated *Cg*AMs in 20 mM phosphate buffer containing 100 mM NaCl pH 6.0 into a stirred (329 rpm) cell of 1.3187 ml which contained 10 mM G3 in the same buffer, with reference power of 34.5 µcal/sec, initial delay of 100 sec for 4500 sec spacing time. For each titration experiment, buffer was titrated as a control instead of using enzyme. Titrations of both WT and Y418A/W mutated *Cg*AMs were carried out similarly and continued until no significant signal response was observed on enzyme activity. The mean values of activities were calculated from three independent experiments.

To confirm the amylomaltase activity from ITC, disproportionation activity by glucose oxidase method was performed using the same condition of ITC experiment. The reaction mixtures containing various concentrations of substrate G3 (10 - 30 mM) were incubated with 20 μM of CgAMs for 0, 1, 3, 5, 10, 20, 40 and 75 min. Then the enzyme activity was measured by glucose oxidase method as previously described in 2.8.2.

2.10.2 Kinetic meaurements by ITC

All reactions were carried out at 30 ºC in 20 mM phosphate buffer containing 100 mM NaCl pH 6.0. The cells were filled with similar solutions as described in 2.11.1 except that three sets of sample cells were filled with G3 at 10, 20 and 30 mM. Titration parameters of WT and Y418A/W mutated CgAMs were as described in 2.10.1. Origin software program was used to analyze the data.

2.10.3 Differential scanning calorimetry (DSC)

DSC analysis was performed using VP-DSC calorimeter (MicroCal, Northampton, MA, USA) with the cell volume of 0.52061 ml at every scan rate of 0.75, 1.0 and 1.5 ºC/min in the temperature range of 10 - 90 ºC. WT and mutated CgAMs were dissolved in 50 mM phosphate buffer containing 100 mM NaCl pH 6.0. All samples were determined for protein concentration by measuring absorbance at 280 nm using the Thermo Scientific Nanodrop 2000C spectrophotometer and also degassed by centrifugation at 3,000 x g, 4 ºC for 30 min. Protein concentration of all CgAMs used was around 80 μM. Base-line scan collected with buffer in the sample cells while degassed water was filled in the reference cell. The reversibility of enzyme thermal transition was evaluated by checking the reproducibility of scan rate on immediate cooling and rescanning. Origin program software with DSC add-on module was used

for data analysis, T_m (unfolding temperature, defined as the temperature of maximum apparent heat capacity) and T_p (peak temperature) were obtained. Thermal transition curves were obtained from the plot of heat capacity against temperature.

2.11 Structural Modeling

The X-ray structure of *Cg*AM was previously reported (Joo *et al*., 2016). In this study, the model structures of all mutated *Cg*AMs were constructed by the Discovery Studio 3.5 Client program (Chikan *et al*., 2013) using WT *Cg*AM X-ray structure (PDB code 5B68) (Joo *et al*., 2016) as template. The acarbose molecule bound at the active site of the X-ray structure of *Ta*AM (PDB 1esw) (Przylas *et al*., 2000a) was used to superimpose with WT and *Cg*AM model structures.

The distances between the two residues Y418 and F534 at the loop entrance of WT and Y418 mutated *Cg*AMs were determined. The interactions within enzyme molecule and between enzyme and acarbose at the active site through H-bond were examined and compared to WT enzyme..

CHAPTER III
RESULTS

The present work is to investigate the structure-function relationship of *Cg*AM, particularly focuses on the structure important for controlling activity and LR-CD product formation of the enzyme. Site-directed mutagenesis was performed by substitution of amino acid residues of interest and their effects on enzyme structure and activities were monitored.

3.1 Construction of mutated amylomaltase gene from *C. glutamicum*

3.1.1 Selection of target amino acid residues of *Cg*AM

Three amino acid residues from three regions of thermophilic amylomaltase (F251; H294 and Y101 in 250s unique loop, active site and 2nd binding glucan, respectively) (Jung *et al*., 2011; Kaper *et al*., 2007; Przylas *et al*., 2000a) were selected as template residues in this study (see detail in Discussion section 4.1.1). By comparison of the amino acid sequence of *Ta*AM with *Cg*AM, our target residues for point mutation: F251, H294 and Y101 of *Ta*AM are corresponded to Y418, H461 and Y290 of *Cg*AM Figure 3.1. However, the result from superimposed structures of *Ta*AM PDB code 1CWY (Przylas *et al*., 2000b) with *Cg*AM (Unpublished data), (Srisimarat *et al*., 2013), which is more convincing than sequence comparison showed that F251, H294 and Y101 of *Ta*AM are corresponded to Y418, H461 and N287, not Y290 of *Cg*AM (Figure3.2).

The first residue of interest was Y418 which was at the tip of 410s loop in *Cg*AM (corresponded to F251 in 250s loop of AM from *Thermus* sp. (Jung *et al.*, 2011). The second target was H461 which was near the catalytic site of amylomaltase corresponded to H294 in *Thermus sp*. (Kaper *et al.*, 2007). The third target was N287 which is near the second glucan binding site corresponded to Y101 in AM from *Thermus sp.* (Przylas *et al.*, 2000a). To investigate the effect of these positions (Figure 3.3) on the structure and function relationship of *Cg*AM, site-directed mutagenesis of *CgAM* gene was performed as described in 2.4.2.

3.1.2 Extraction of recombinant plasmid pET - 19b - *CgAM*

The recombinant plasmid pET-19b containing *CgAM* gene (Srisimarat *et al.*, 2011) was extracted from *E. coli* DH5α and DNA fragments were checked by agarose gel electrophoresis (Figure 3.4 Lane 1). After double digestion with *Nde* I and *Xho* I, it was found that the sizes of pET-19b vector and *CgAM* gene were around 5.7 kb and 2.1 k, respectively (Figure 3.4 Lane 2). The measured ratio of A260/A280 was 1.8 indicated the sufficient purity of this extracted recombinant plasmid for use as a template for PCR amplification.

3.1.3 Site-directed mutagenesis of *CgAM* gene

Through site-directed mutagenesis of *CgAM* gene, all mutants were constructed by a single point mutation at three positions: Y418, H461 and N287.All target residues were substituted by alanine, A (non polar aliphatic), tryptophan, W (aromatic), serine, S (polar uncharge), aspartic acid, D (negative charge) and arginine, R (positive charge). In addition, Y418 was also substituted by phenylalanine, F to mimic its corresponding residue of *Thermus* sp. amylomaltase.

The recombinant plasmid pET-19b containing *CgAM* gene was used a template for site-directed mutagenesis. The product from PCR amplification was found as a single band on agarose gel electrophoresis as shown in Figure 3.5, 3.7 and 3.9 for Y418-, H461- and N287-mutated *CgAM* genes, respectively. The size of PCR product was 7.8 kb as expected for *CgAM* gene and pET-19b.

3.1.4 Transformation

The PCR products after digestion with *Dpn* I were purified and transformed into the competent cells of *E. coli* BL21 (DE3) by electroporation. Two hundred microliters of transformants were spread on LB agar plate containing 100 μg/ml ampicillin and incubated overnight at 37 ℃. The *E. coli* BL21 (DE3) containing pET-19b vector harboring each mutated *CgAM* gene was grown on the plate. To confirm the insertion of mutated *CgAM* genes into the pET-19b, the transformant clones were picked for plasmid extraction and digested with *Nde* I and *Xho* I. The agarose gel pattern of recombinant plasmid containing mutated *CgAM* genes was shown in Figure 3.6, 3.8 and 3.10 for Y418-, H461- and N287-mutated *CgAM* genes, respectively.

3.1.5 Nucleotide sequencing

To investigate the result of mutation on *CgAM* gene, the recombinant plasmids were subjected to DNA sequencing. T7 promoter and T7 terminator primers were used to sequence the 5' terminus and 3' terminus, respectively. The residual sequence of *CgAM* gene was extended by two primer pairs of f2*CgAM* and r2*CgAM* (sequence shown in section 2.4.6). The nucleotide sequences of Y418-, H461- and N287-mutated *CgAM* genes were aligned with WT-*CgAM* gene by Clustal O (1.2.4) program (Figure 3.11, 3.13 and 3.15), respectively. It was found that the nucleotide sequences of Y418, H461 and N287 mutated *CgAMs* contained 2,121 bp which could be deduced to 706

amino acids similar to WT gene. The deduced amino acid sequences of mutated *CgAM*

genes were then compared to the WT sequencing using Clustal O program. Y418 was

changed to Ala (A), Glu (D), Ser (S), Trp (W), Arg (R) and Phe (F) as shown in Figure

3.12 while H461 and N287 were changed to Ala (A), Glu (D), Ser (S), Trp (W) and

Arg (R), respectively (Figure 3.14 and 3.16). These results confirmed that single point

mutations at Y418, H461 and N287 on *CgAM* gene were successfully performed.

```
TaAM    ------------------------------------------------------------    0
CgAM    MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60


TaAM    ------------------------------------------------------------    0
CgAM    IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP    120


TaAM    -----------------------------MELPRAFGLLLHPTSLPG------------    18
CgAM    REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV    180
                                        *        **:: *: *

TaAM    -----------PYGVGVLGLEARDFLRFLKGAGGRFWQVLPLGPT----GYGDSPYQAFS    63
CgAM    MAQIYSVRSTLSWGMGDFND-LGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTT    239
                   :*:* :.      ::  .:   *. *   : *:  :       ****   :

TaAM    AFAGNPYLIDLRPLAEKGYLV-------------LKDPGFPQGRVDYGWLYAWKWPALKA    110
CgAM    RRFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRA    299
              **  *  *:. : *  . *        :::  .:  .. ::  . :** *   .*:*

TaAM    AYQGFLEKAPRKEREDFPAFQEKEASWLKDYALFMALKAQHGGLPWNRWPLPLRRREEKA    170
CgAM    IFEM---PRSSEREANFVSFVQR--------------EGQGLIDFATWCADRETAQSES    341
         ::       :.. :* :* ::                :* : :  *    .  :.::

TaAM    MKEAEAALAEEVAFHAWTQWLFFEAWKALKEEAEALG--IQIIGDMPIFVAEDSAEVWAH    228
CgAM    VHGTEPDRDELTMFYMWLQWLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGAD----    397
         :: :*     *  . *: * *** *   *  ::.*    *  * *:.*: : *   ..*:

TaAM    PEWFHLDEEGRPLVVAGVPPDYFSETGQRWGNPLYRWDVLEREGFSFWIARLAKALELFH    288
CgAM    --AQNLSHVLAPDASVGAPPDGYNQQGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSG    455
          :*..   *   . .*.*** :.: ** *.:* ::   * .**:  *   *   ..*.

TaAM    LVRVDHFRGFEAYWEIPASCPTAVEGRWVKASGEKLFDR--IQEVFGEVPILADDLGVIT    346
CgAM    GIRVDHVLGLFRLFVMPRMQSP-ATGTYIRFDHNALVGILALEAELAGAVVIGSDLGTFE    514
          :****. *:    : :*       . * :::  . : *..   ::   :. . ::.****.:

TaAM    PEVEPLRDRYGLPGMKVLQFAFDHGMENPFLPHNYPAHGRVVVYTGTHNDTTLGWYRTA    406
CgAM    PWVQDALAQRGIMGTSILWFEHSPSQPGPRRQEEYRPLAL--TTVTTHDLPPTAGYLEGE    572
         * *:    : *: * .:* *  .. .*   .:*    .  . ***    * *:  .

TaAM    SPHERAFLERYLADWGISFRQEEEVPWALMGLCLKSVARLAIYPVQDVLA----------    456
CgAM    HIALRERLGVLNTD--PAAELAEDLQWQAEILDV--AASANALPAREYVGLERDQRGELA    628
          *   *    :*   :.   *:: *     *  : .*     *.:: :.

TaAM    ------------------------LGSEARMNYPGRPSG---NWAWRLLPGQLTQEHA-    487
CgAM    ELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQNQPGTTRDMYPNWCIPLCDSEGNSVLIE    688
                                  :*.:   * **   .   **.  *   .: ..

TaAM    ----ARL-LAMAEATGRT 500
CgAM    SLRENELYHRVAKASKRD 706
            .*    :*:*: *
```

Figure 3. 1 Amino acid sequence alignment of *Ta*AM and *Cg*AM using Clustal O (1.2.14). Three conserved catalytic residues (D293, E340 and D395 in *Ta*AM; D460, E508 and D561 in *Cg*AM) of amylomaltases were shown in red highlight. Y418, H461 and Y290 residues of *Cg*AM were shown in green, blue and yellow, respectively. (*), (.) and (:) represented amino acid identity, semi-conserved sustituition and conserved substituition, respectively.
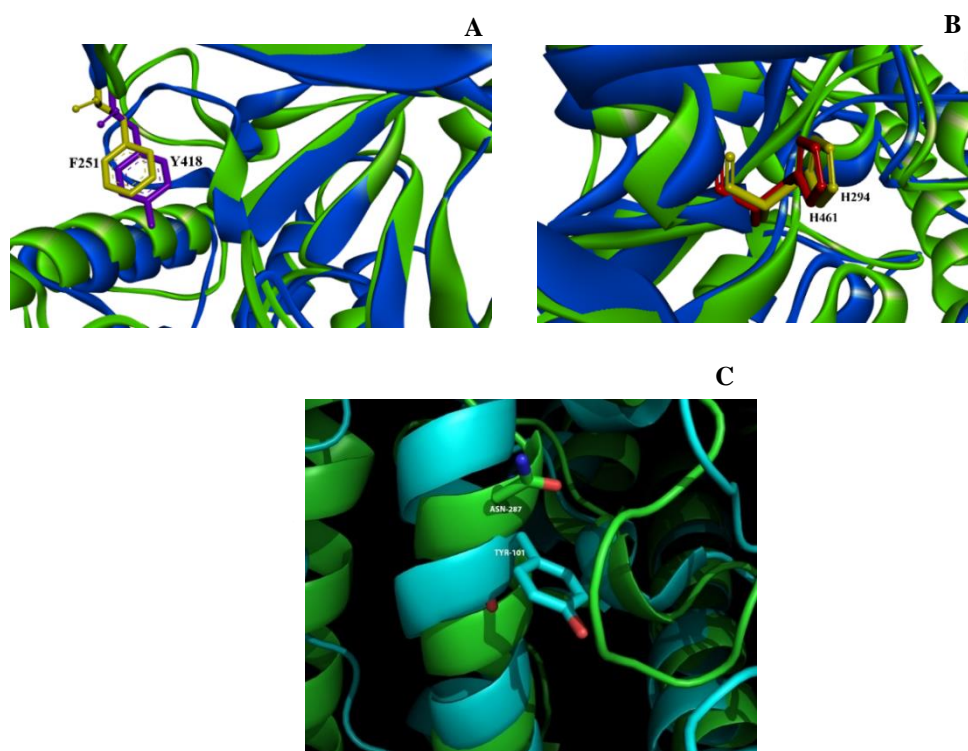
Figure 3. 2 The superimposed structures of *Cg*AM (green) PDB 5B68 (Joo *et al*., 2016; Srisimarat *et al*., 2013) on *Ta*AM (blue) PDB 1CWY (Przylas *et al*., 2000b). The enzyme structures are displayed as secondary structures generated by Discovery Studio Client program 3.5. Corresponding residues are displayed as stick and colored by atom name: Y418-*Cg*AM and F251-*Ta*AM are shown in purple and yellow (A), H461-*Cg*AM and H294-*Ta*AM are shown in red and yellow (B), while N287-*Cg*AM and Y101-*Ta*AM are shown in green and cyan (C).
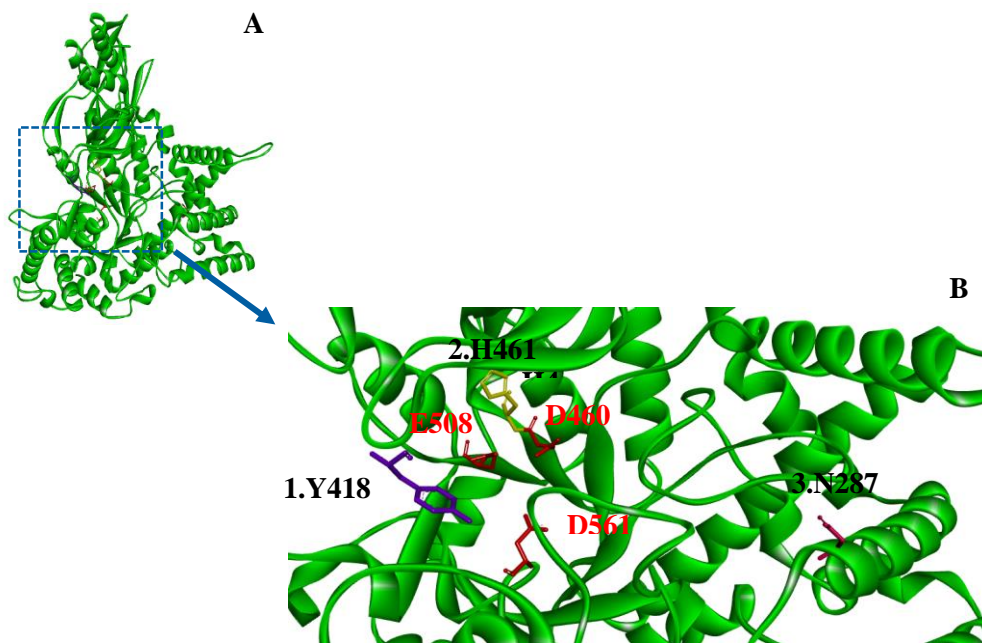
Figure 3. 3 Overall structures of WT *Cg*AM (PDB code 5B68) (Joo *et al*., 2016) (A).

The expanded structure of that in the box in (A) are displayed (B). The three catalytic

residues represented in red (D460, E508, D561) while Y418, H461 and N287 are shown

in purple, yellow and pink, respectively.
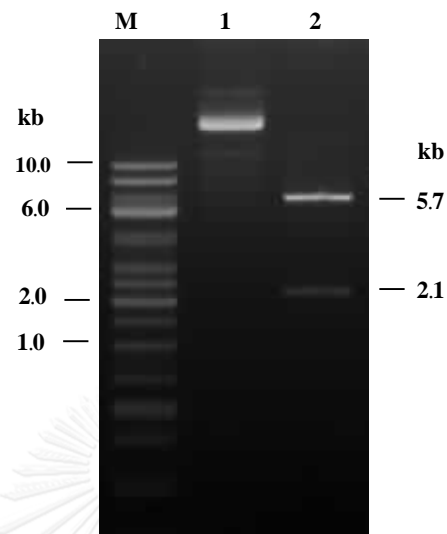
Figure 3. 4 Agarose gel electrophoresis of recombinant plasmid pET-19b-*CgAM*. The

DNA samples were separated on 0.75% agarose gel and visualized by ethidium bromide

staining.

Lane M = GeneRuler[TM] 1 kb DNA ladder (Fermentas, Canada)

Lane 1 = Recombinant plasmid containing *CgAM*

Lane 2 = Recombinant plasmid containing *CgAM* after double digestion with *Nde* I and

Xho I
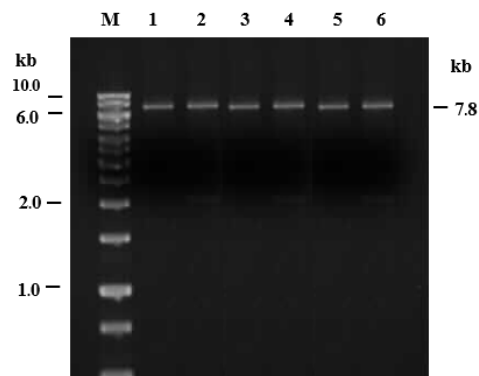
Figure 3. 5 Agarose gel electrophoresis of amplified DNA containing *CgAM* gene with

a single point mutation at Y418. The DNA samples were separated on 0.75% agarose

gel and visualized by ethidium bromide staining.

Lane M = GeneRuler[TM] 1 kb DNA ladder (Fermentas, Canada)

Lane 1, 2, 3, 4, 5 and 6 = PCR product from mutated Y418A-, Y418S-, Y418D-,

Y418R-, Y418W- and Y418F-*CgAM*, respectively.
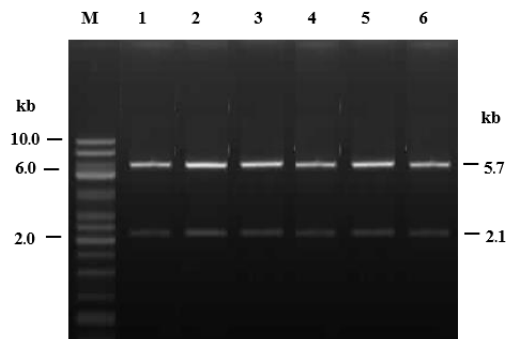
Figure 3. 6 Agarose gel electrophoresis of PCR product from pET-19b vector harboring

CgAM gene with a single point mutation at Y418 after digested with *Nde* I and *Xho* I.

The DNA samples were separated on 0.75% agarose gel and visualized by ethidium

bromide staining.

Lane M = GeneRuler^{TM} 1 kb DNA ladder (Fermentas, Canada)

Lane 1, 2, 3, 4, 5 and 6 = PCR product from pET19-b vector harboring mutated *CgAM*

gene: Y418A, Y418S, Y418D, Y418R, Y418W and Y418F,

respectively.
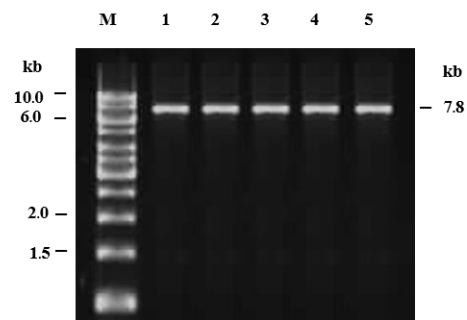
Figure 3. 7 Agarose gel electrophoresis of amplified DNA containing *CgAM* gene

with a single point mutation at H461. The DNA samples were separated on 0.75%

agarose gel and visualized by ethidium bromide staining.

Lane M = GeneRuler^{TM} 1 kb DNA ladder (Fermentas, Canada)

Lane 1, 2, 3, 4 and 5 = PCR product from mutated H461A-, H461S-, H461D-, H461R-

and H461W-*CgAM*, respectively.
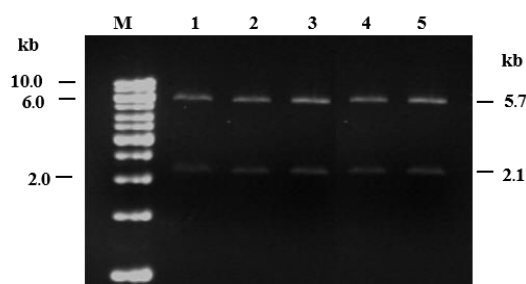
Figure 3. 8 Agarose gel electrophoresis of PCR product from pET-19b vector harboring

CgAM gene with a single point mutation at H461 after digested with *Nde* I and *Xho* I.

The DNA samples were separated on 0.75% agarose gel and visualized by ethidium

bromide staining.

Lane M = GeneRuler^{TM} 1 kb DNA ladder (Fermentas, Canada)

Lane 1, 2, 3, 4, 5 and 6 = PCR product from pET19-b vector harboring mutated *CgAM*

gene: H461A, H461S, H461D, H461R and H461W,

respectively.
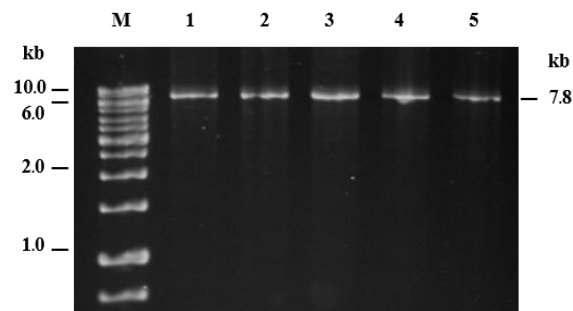
Figure 3. 9 Agarose gel electrophoresis of amplified DNA containing *CgAM* gene with a single point mutation at N287. The DNA samples were separated on 0.75% agarose gel and visualized by ethidium bromide staining.

Lane M = GeneRuler^{TM} 1 kb DNA ladder (Fermentas, Canada)

Lane 1, 2, 3, 4 and 5 = PCR product from mutated N287A-, N287S-, N287D-, N287R- and N287W-*CgAM*, respectively.
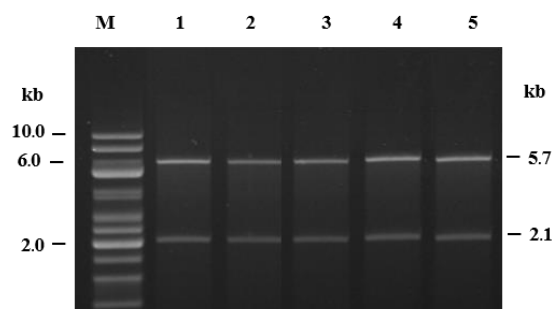
Figure 3. 10 Agarose gel electrophoresis of PCR product from pET-19b vector harboring *CgAM* gene with a single point mutation at N287 after digested with *Nde* I and *Xho* I. The DNA samples were separated on 0.75% agarose gel and visualized by ethidium bromide staining.

Lane M = GeneRuler^{TM} 1 kb DNA ladder (Fermentas, Canada)

Lane 1, 2, 3, 4, 5 and 6 = PCR product from pET19-b vector harboring mutated *CgAM*

gene: N287A, N287S, N287D, N287R and N287W,

respectively.

```
Y418F_CgAM   ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
Y418W_CgAM   ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
Y418R_CgAM   ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
WT_CgAM      ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
Y418D_CgAM   ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
Y418A_CgAM   ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
Y418S_CgAM   ATGACTGCTCGCAGATTTTTGAATGAACTCGCCGATCTCTACGGCGTAGCAACTTCCTAC      60
             ************************************************************

Y418F_CgAM   ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
Y418W_CgAM   ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
Y418R_CgAM   ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
WT_CgAM      ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
Y418D_CgAM   ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
Y418A_CgAM   ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
Y418S_CgAM   ACTGATTACAAAGGTGCCCATATTGAGGTCAGCGATGACACATTAGTGAAAATCCTGCGT     120
             ************************************************************

Y418F_CgAM   GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
Y418W_CgAM   GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
Y418R_CgAM   GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
WT_CgAM      GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
Y418D_CgAM   GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
Y418A_CgAM   GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
Y418S_CgAM   GCTCTGGGTGTGAATTTAGATACAAGCAACCTCCCCAACGATGACGCTATCCAACGCCAA     180
              ***********************************************************

Y418F_CgAM   ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
Y418W_CgAM   ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
Y418R_CgAM   ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
WT_CgAM      ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
Y418D_CgAM   ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
Y418A_CgAM   ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
Y418S_CgAM   ATTGCCCTCTTCCATGATCGAGAGTTCACTCGCCCACTGCCTCCATCGGTGGTTGCAGTT     240
             ************************************************************

Y418F_CgAM   GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
Y418W_CgAM   GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
Y418R_CgAM   GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
WT_CgAM      GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
Y418D_CgAM   GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
Y418A_CgAM   GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
Y418S_CgAM   GAAGGTGATGAACTAGTTTTCCCGGTGCATGTGCACGACGGTTCCCCTGCAGATGTCCAC     300
             ************************************************************

Y418F_CgAM   ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
Y418W_CgAM   ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
Y418R_CgAM   ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
WT_CgAM      ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
Y418D_CgAM   ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
Y418A_CgAM   ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
Y418S_CgAM   ATCGAATTGGAAGACGGCACGCAGCGGGATGTTTCTCAGGTGGAAAACTGGACAGCGCCA     360
             ************************************************************
```

Figure 3. 11 Nucleotide sequences of six Y418 mutated *CgAM*s compared with that of

WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are shaded.

```
Y418F_CgAM    CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
Y418W_CgAM    CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
Y418R_CgAM    CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
WT_CgAM       CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
Y418D_CgAM    CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
Y418A_CgAM    CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
Y418S_CgAM    CGGGAAATTGATGGGATTAGGTGGGGCGAGGCATCGTTTAAGATTCCTGGTGATCTCCCC    420
              ************************************************************


Y418F_CgAM    TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
Y418W_CgAM    TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
Y418R_CgAM    TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
WT_CgAM       TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
Y418D_CgAM    TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
Y418A_CgAM    TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
Y418S_CgAM    TTGGGTTGGCACAAGCTTCACCTTAAATCCAATGAACGCTCAGCTGAGTGCGGTTTGATC    480
              ************************************************************


Y418F_CgAM    ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
Y418W_CgAM    ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
Y418R_CgAM    ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
WT_CgAM       ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
Y418D_CgAM    ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
Y418A_CgAM    ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
Y418S_CgAM    ATCACCCCGGCTCGTCTGTCTACTGCTGATAAGTATCTTGATTCCCCTCGCAGTGGTGTC    540
              ************************************************************


Y418F_CgAM    ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
Y418W_CgAM    ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
Y418R_CgAM    ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
WT_CgAM       ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
Y418D_CgAM    ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
Y418A_CgAM    ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
Y418S_CgAM    ATGGCGCAGATCTACTCTGTGCGTTCCACGTTGTCGTGGGGCATGGGTGATTTCAATGAT    600
              ************************************************************


Y418F_CgAM    TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
Y418W_CgAM    TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
Y418R_CgAM    TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
WT_CgAM       TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
Y418D_CgAM    TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
Y418A_CgAM    TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
Y418S_CgAM    TTAGGAAACTTGGCAAGTGTGGTTGCCCAGGATGGAGCAGACTTCCTGCTCATCAACCCC    660
              ************************************************************


Y418F_CgAM    ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
Y418W_CgAM    ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
Y418R_CgAM    ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
WT_CgAM       ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
Y418D_CgAM    ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
Y418A_CgAM    ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
Y418S_CgAM    ATGCACGCTGCAGAGCCGCTGCCTCCTACTGAGGACTCTCCTTATCTGCCCACAACCAGG    720
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Figure 3.11 (continue) Nucleotide sequences of six Y418 mutated *CgAM*s compared with that of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are shaded.

```
Y418F_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
Y418W_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
Y418R_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
WT_CgAM       CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
Y418D_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
Y418A_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
Y418S_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
              ************************************************************

Y418F_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
Y418W_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
Y418R_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
WT_CgAM       ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
Y418D_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
Y418A_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
Y418S_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
              ************************************************************

Y418F_CgAM    TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
Y418W_CgAM    TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
Y418R_CgAM    TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
WT CgAM       TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
Y418D_CgAM    TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
Y418A_CgAM    TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
Y418S_CgAM    TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
              ************************************************************

Y418F_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
Y418W_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
Y418R_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
WT_CgAM       TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
Y418D_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
Y418A_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
Y418S_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
              ************************************************************

Y418F_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
Y418W_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
Y418R_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
WT_CgAM       GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
Y418D_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
Y418A_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
Y418S_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
              ************************************************************

Y418F_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
Y418W_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
Y418R CgAM     TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
WT CgAM        TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
Y418D_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
Y418A_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
Y418S_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
              ************************************************************
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Figure 3.11 (continue) Nucleotide sequences of six Y418 mutated *CgAM*s compared with that of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are shaded.

```
Y418F_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
Y418W_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
Y418R_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
WT_CgAM       TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
Y418D_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
Y418A_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
Y418S_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
              ************************************************************

Y418F_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
Y418W_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
Y418R_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
WT_CgAM       ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
Y418D_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
Y418A_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
Y418S_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
              ************************************************************

Y418F_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATTTAACCAG    1260
Y418W_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATGGAACCAG    1260
Y418R_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGACGCAACCAG    1260
WT_CgAM       CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
Y418D_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGAGACAACCAG    1260
Y418A_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGAGCCAACCAG    1260
Y418S_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATCCAACCAG    1260
              ******************************************************  ******

Y418F_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
Y418W_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
Y418R_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
WT_CgAM       CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
Y418D_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
Y418A_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
Y418S_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
              ************************************************************

Y418F_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
Y418W_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
Y418R_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
WT_CgAM       ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
Y418D_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
Y418A_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
Y418S_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
              ************************************************************

Y418F_CgAM    CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
Y418W_CgAM    CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
Y418R_CgAM    CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
WT_CgAM       CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
Y418D_CgAM    CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
Y418A_CgAM    CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
Y418S_CgAM    CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
              ************************************************************
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Figure 3.11 (continue) Nucleotide sequences of six Y418 mutated *CgAM*s compared with that of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are shaded.

```
Y418F_CgAM    ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
Y418W_CgAM    ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
Y418R_CgAM    ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
WT_CgAM       ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
Y418D_CgAM    ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
Y418A_CgAM    ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
Y418S_CgAM    ACCTATATCCGCTTCGACCATAATGCGTTGGTAGGCATTCTAGCCCTAGAAGCAGAACTC    1500
              ************************************************************

Y418F_CgAM    GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
Y418W_CgAM    GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
Y418R_CgAM    GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
WT_CgAM       GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
Y418D_CgAM    GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
Y418A_CgAM    GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
Y418S_CgAM    GCAGGCGCCGTTGTCATTGGTGAAGATCTGGGAACGTTTGAGCCTTGGGTACAAGATGCA    1560
              ************************************************************

Y418F_CgAM    TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
Y418W_CgAM    TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
Y418R_CgAM    TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
WT_CgAM       TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
Y418D_CgAM    TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
Y418A_CgAM    TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
Y418S_CgAM    TTGGCTCAGCGTGGCATCATGGGCACCTCGATCCTATGGTTCGAGCATTCCCCAAGCCAG    1620
              ************************************************************

Y418F_CgAM    CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
Y418W_CgAM    CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
Y418R_CgAM    CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
WT_CgAM       CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
Y418D_CgAM    CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
Y418A_CgAM    CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
Y418S_CgAM    CCGGGTCCTCGCCGCCAGGAAGAGTATCGTCCGCTGGCCTTGACCACTGTGACCACTCAT    1680
              ************************************************************

Y418F_CgAM    GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
Y418W_CgAM    GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
Y418R_CgAM    GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
WT_CgAM       GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
Y418D_CgAM    GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
Y418A_CgAM    GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
Y418S_CgAM    GATCTCCCTCCGACTGCTGGTTATTTGGAGGGCGAGCACATTGCTCTTCGTGAGCGATTG    1740
              ************************************************************

Y418F_CgAM    GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
Y418W_CgAM    GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
Y418R_CgAM    GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
WT_CgAM       GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
Y418D_CgAM    GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
Y418A_CgAM    GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
Y418S_CgAM    GGGGTGCTCAACACTGATCCTGCTGCAGAACTCGCTGAGGATCTGCAGTGGCAAGCGGAG    1800
              ************************************************************
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Figure 3.11 (continue) Nucleotide sequences of six Y418 mutated *CgAM*s compared with that of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are shaded.

```
Y418F_CgAM   ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
Y418W_CgAM   ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
Y418R_CgAM   ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
WT_CgAM      ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
Y418D_CgAM   ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
Y418A_CgAM   ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
Y418S_CgAM   ATCCTTGATGTCGCAGCATCTGCCAACGCATTGCCAGCCCGGGAATACGTGGGACTCGAA      1860
             ************************************************************

Y418F_CgAM   CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
Y418W_CgAM   CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
Y418R_CgAM   CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
WT_CgAM      CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
Y418D_CgAM   CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
Y418A_CgAM   CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
Y418S_CgAM   CGCGATCAGCGCGGTGAGTTGGCTGAGCTGTTGGAAGGCCTGCACACTTTCGTTGCGAAA      1920
             ************************************************************

Y418F_CgAM   ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
Y418W_CgAM   ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
Y418R_CgAM   ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
WT_CgAM      ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
Y418D_CgAM   ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
Y418A_CgAM   ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
Y418S_CgAM   ACCCCTTCAGCACTGACCTGTGTCTGCTTGGTAGACATGGTCGGTGAAAAGCGGGCACAG      1980
             ************************************************************

Y418F_CgAM   AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
Y418W_CgAM   AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
Y418R_CgAM   AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
WT_CgAM      AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
Y418D_CgAM   AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
Y418A_CgAM   AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
Y418S_CgAM   AATCAGCCGGGCACAACGAGGGATATGTATCCCAACTGGTGTATCCCACTGTGTGACAGC      2040
             ************************************************************

Y418F_CgAM   GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
Y418W_CgAM   GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
Y418R_CgAM   GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
WT_CgAM      GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
Y418D_CgAM   GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
Y418A_CgAM   GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
Y418S_CgAM   GAAGGCAACTCCGTGCTCATTGAATCGCTGCGTGAAAATGAGCTGTATCACCGTGTGGCA      2100
             ************************************************************

Y418F_CgAM   AAGGCAAGCAAGCGAGATTAG                  2121
Y418W_CgAM   AAGGCAAGCAAGCGAGATTAG                  2121
Y418R_CgAM   AAGGCAAGCAAGCGAGATTAG                  2121
WT_CgAM      AAGGCAAGCAAGCGAGATTAG                  2121
Y418D_CgAM   AAGGCAAGCAAGCGAGATTAG                  2121
Y418A_CgAM   AAGGCAAGCAAGCGAGATTAG                  2121
Y418S_CgAM   AAGGCAAGCAAGCGAGATTAG                  2121
             ********************
```

Figure 3.11 (continue) Nucleotide sequences of six Y418 mutated *CgAM*s compared with that of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are shaded.

```
WT_CgAM      MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
Y418A_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
Y418S_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
Y418D_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
Y418R_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
Y418W_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
Y418F_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ      60
             ************************************************************

WT_CgAM      IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
Y418A_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
Y418S_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
Y418D_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
Y418R_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
Y418W_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
Y418F_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP     120
             ************************************************************

WT_CgAM      REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
Y418A_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
Y418S_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
Y418D_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
Y418R_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
Y418W_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
Y418F_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV     180
             ************************************************************

WT_CgAM      MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
Y418A_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
Y418S_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
Y418D_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
Y418R_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
Y418W_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
Y418F_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR     240
             ************************************************************

WT_CgAM      RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
Y418A_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
Y418S_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
Y418D_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
Y418R_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
Y418W_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
Y418F_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI     300
             ************************************************************

WT_CgAM      FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
Y418A_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
Y418S_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
Y418D_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
Y418R_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
Y418W_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
Y418F_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ     360
             ************************************************************

WT_CgAM      WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ     420
Y418A_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGANQ     420
Y418S_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGSNQ     420
Y418D_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGDNQ     420
Y418R_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGRNQ     420
Y418W_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGWNQ     420
Y418F_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGFNQ     420
             *********************************************************** **
```

Figure 3. 12 The deduced amino acid sequences of six Y418 mutated *Cg*AMs compared with that of WT using Clustal O (1. 2. 4) multiple sequence alignment. Mutated amino acids are shaded.

```
WT_CgAM       QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
Y418A_CgAM    QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
Y418S_CgAM    QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
Y418D_CgAM    QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
Y418R_CgAM    QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
Y418W_CgAM    QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
Y418F_CgAM    QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG      480
              ************************************************************


WT_CgAM       TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
Y418A_CgAM    TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
Y418S_CgAM    TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
Y418D_CgAM    TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
Y418R_CgAM    TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
Y418W_CgAM    TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
Y418F_CgAM    TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ      540
              ************************************************************


WT_CgAM       PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
Y418A_CgAM    PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
Y418S_CgAM    PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
Y418D_CgAM    PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
Y418R_CgAM    PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
Y418W_CgAM    PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
Y418F_CgAM    PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE      600
              ************************************************************


WT_CgAM       ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
Y418A_CgAM    ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
Y418S_CgAM    ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
Y418D_CgAM    ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
Y418R_CgAM    ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
Y418W_CgAM    ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
Y418F_CgAM    ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ      660
              ************************************************************

WT_CgAM       NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
Y418A_CgAM    NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
Y418S_CgAM    NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
Y418D_CgAM    NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
Y418R_CgAM    NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
Y418W_CgAM    NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
Y418F_CgAM    NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD      706
              **********************************************
```

Figure 3.12 (continue) The deduced amino acid sequences of six Y418 mutated *Cg*AM compared with that of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated amino acids are shaded.

```
H461S_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
H461R_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
WT_CgAM       TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
H461A_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
H461D_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
H461W_CgAM    TGGCTATGTGATGAGCAGCTGGCGGCAGCTCAAAAGCGCGCTGTCGATGCCGGAATGTCG    1140
              ************************************************************

H461S_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
H461R_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
WT_CgAM       ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
H461A_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
H461D_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
H461W_CgAM    ATCGGCATCATGGCAGACCTGGCAGTTGGTGTGCATCCAGGTGGTGCTGATGCCCAGAAC    1200
              ************************************************************

H461S_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
H461R_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
WT_CgAM       CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
H461A_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
H461D_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
H461W_CgAM    CTCAGCCACGTACTTGCTCCGGATGCGTCAGTGGGCGCCCCACCAGATGGATACAACCAG    1260
              ************************************************************

H461S_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
H461R_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
WT_CgAM       CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
H461A_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
H461D_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
H461W_CgAM    CAGGGCCAAGACTGGTCCCAGCCACCATGGCATCCAGTGCGTCTTGCAGAGGAAGGCTAC    1320
              ************************************************************

H461S_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
H461R_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
WT_CgAM       ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
H461A_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
H461D_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
H461W_CgAM    ATTCCGTGGCGTAATCTGCTGCGCACTGTGCTGCGTCACTCCGGCGGAATCCGCGTGGAC    1380
              ************************************************************

H461S_CgAM    AGCGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
H461R_CgAM    CGCGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
WT_CgAM       CACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
H461A_CgAM    GCCGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
H461D_CgAM    GACGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
H461W_CgAM    UGGGTTCTTGGTTTGTTCAGGCTCTTTGTCATGCCACGCATGCAATCCCCTGCTACGGGC    1440
              ***********************************************************
```

Figure 3. 13 Nucleotide sequences of five H461 mutated *CgAM*s compared with that of WT using Clustal O (1. 2. 4) multiple sequence alignment. Mutated codons are shaded. (Only the sequences from NT 1081-1440 are shown here, the sequences from NT1-NT1080 and NT1441-NT2121 which are the same in all *CgAM*s are not shown.)

```
WT_CgAM      QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQSPATG     480
H461A_CgAM   QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDAVLGLFRLFVMPRMQSPATG     480
H461S_CgAM   QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDSVLGLFRLFVMPRMQSPATG     480
H461D_CgAM   QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDDVLGLFRLFVMPRMQSPATG     480
H461R_CgAM   QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDRVLGLFRLFVMPRMQSPATG     480
H461W_CgAM   QGQDWSQPPWHPVRLAEEGYIPWRNLLRTVLRHSGGIRVDWVLGLFRLFVMPRMQSPATG     480
             ****************************************  ******************

WT_CgAM      TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ     540
H461A_CgAM   TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ     540
H461S_CgAM   TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ     540
H461D_CgAM   TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ     540
H461R_CgAM   TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ     540
H461W_CgAM   TYIRFDHNALVGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQ     540
             ***********************************************************

WT_CgAM      PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE     600
H461A_CgAM   PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE     600
H461S_CgAM   PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE     600
H461D_CgAM   PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE     600
H461R_CgAM   PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE     600
H461W_CgAM   PGPRRQEEYRPLALTTVTTHDLPPTAGYLEGEHIALRERLGVLNTDPAAELAEDLQWQAE     600
             ***********************************************************

WT_CgAM      ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ     660
H461A_CgAM   ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ     660
H461S_CgAM   ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ     660
H461D_CgAM   ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ     660
H461R_CgAM   ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ     660
H461W_CgAM   ILDVAASANALPAREYVGLERDQRGELAELLEGLHTFVAKTPSALTCVCLVDMVGEKRAQ     660
             ***********************************************************

WT_CgAM      NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD    706
H461A_CgAM   NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD    706
H461S_CgAM   NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD    706
H461D_CgAM   NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD    706
H461R_CgAM   NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD    706
H461W_CgAM   NQPGTTRDMYPNWCIPLCDSEGNSVLIESLRENELYHRVAKASKRD    706
             **********************************************
```

Figure 3. 14 The deduced amino acid sequences of five H461 mutated *Cg*AMs compared with that of WT using Clustal O (1. 2. 4) multiple sequence alignment. Mutated amino acids are shaded. (Only the sequences from AA421-AA706 are shown here, the sequences from AA1-AA420 which are the same in all *Cg*AMs are not shown.)

```
N287R_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
N287W CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
WT CgAM       CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
N287D_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
N287A_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
N287S_CgAM    CGCTTTATCAACCCGATCTACATTCGGGTAGAAGATATTCCGGAGTTTAATCAGCTTGAG    780
              ************************************************************

N287R_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
N287W CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
WT CgAM       ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
N287D_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
N287A_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
N287S_CgAM    ATTGATCTACGCGATGATATCGCAGAGATGGCTGCGGAATTCCGCGAACGCAATCTGACC    840
              ************************************************************

N287R_CgAM    TCAGACATCATTGAGCGCCGCGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
N287W_CgAM    TCAGACATCATTGAGCGCTGGGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
WT_CgAM       TCAGACATCATTGAGCGCAATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
N287D_CgAM    TCAGACATCATTGAGCGCGATGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
N287A_CgAM    TCAGACATCATTGAGCGCGCTGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
N287S_CgAM    TCAGACATCATTGAGCGCTCTGACGTCTACGCTGCAAAGCTTCAAGTGCTGCGCGCCATT    900
              ******************   ***************************************

N287R_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
N287W_CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
WT CgAM       TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
N287D CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
N287A CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
N287S CgAM    TTTGAAATGCCTCGTTCCAGCGAACGTGAAGCCAACTTTGTCTCCTTCGTGCAACGGGAA    960
              ************************************************************

N287R_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
N287W CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
WT CgAM       GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
N287D_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
N287A_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
N287S_CgAM    GGCCAAGGTCTTATTGATTTCGCCACCTGGTGCGCGGACCGCGAAACTGCACAGTCTGAA    1020
              ************************************************************

N287R CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
N287W_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
WT_CgAM       TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
N287D_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
N287A_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
N287S_CgAM    TCTGTCCACGGAACTGAGCCAGACCGCGATGAGCTGACCATGTTCTACATGTGGTTGCAG    1080
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Figure 3. 15 Nucleotide sequences of five N287 mutated *CgAM*s compared with that

of WT using Clustal O (1.2.4) multiple sequence alignment. Mutated codons are

shaded. (Only the sequences from NT 721-1080 are shown here, the sequences from

NT1-NT720 and NT1081-NT2121 which are the same in all *CgAM*s are not shown.)

```
WT_CgAM      MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60
N287A_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60
N287S_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60
N287D_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60
N287R_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60
N287W_CgAM   MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ    60
             ************************************************************

WT_CgAM      IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP   120
N287A_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP   120
N287S_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP   120
N287D_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP   120
N287R_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP   120
N287W_CgAM   IALFHDREFTRPLPPSVVAVEGDELVFPVHVHDGSPADVHIELEDGTQRDVSQVENWTAP   120
             ************************************************************

WT CgAM      REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV   180
N287A_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV   180
N287S_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV   180
N287D_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV   180
N287R_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV   180
N287W_CgAM   REIDGIRWGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITPARLSTADKYLDSPRSGV   180
             ************************************************************

WT_CgAM      MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR   240
N287A_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR   240
N287S_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR   240
N287D_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR   240
N287R_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR   240
N287W_CgAM   MAQIYSVRSTLSWGMGDFNDLGNLASVVAQDGADFLLINPMHAAEPLPPTEDSPYLPTTR   240
             ************************************************************

WT_CgAM      RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERNDVYAAKLQVLRAI   300
N287A_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERADVYAAKLQVLRAI   300
N287S_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERSDVYAAKLQVLRAI   300
N287D CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERDDVYAAKLQVLRAI   300
N287R_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERRDVYAAKLQVLRAI   300
N287W_CgAM   RFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTSDIIERWDVYAAKLQVLRAI   300
             **********************************************  *************

WT_CgAM      FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ   360
N287A_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ   360
N287S_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ   360
N287D_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ   360
N287R_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ   360
N287W_CgAM   FEMPRSSEREANFVSFVQREGQGLIDFATWCADRETAQSESVHGTEPDRDELTMFYMWLQ   360
             ************************************************************

WT CgAM      WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ   420
N287A CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ   420
N287S CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ   420
N287D_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ   420
N287R_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ   420
N287W_CgAM   WLCDEQLAAAQKRAVDAGMSIGIMADLAVGVHPGGADAQNLSHVLAPDASVGAPPDGYNQ   420
             ************************************************************
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Figure 3. 16 The deduced amino acid sequences of five N287 mutated *Cg*AMs compared with that of WT using Clustal O (1. 2. 4) multiple sequence alignment. Mutated amino acids are shaded. (Only the sequences from AA1-AA420 are shown here, the sequences from AA421-AA706 which are the same in all *Cg*AMs are not shown.)

Expression, Purification and Characterization of Y418 mutated amylomaltases

3.2 Expression and purification of Y418 mutated amylomaltases

After *Cg*AMs were successfully expressed by induction with 0.4 mM IPTG, cell pellets were disrupted by sonication, and the supernatant carrying crude enzyme was purified by affinity column. The crude and purified enzymes were subjected to electrophoresis on 7.5% separating SDS-polyacrylamide gel. SDS-PAGE of crude and purified preparations of six Y418 mutated *Cg*AMs were shown in Figure 3.17. All mutated *Cg*AMs showed an overexpression of the enzyme with the major protein band approximately at 84 kDa similar to WT *Cg*AM. This suggested that mutated *Cg*AMs were successfully purified by His- trap column chromatography. The fraction that showed high enzyme activity were pooled and dialyzed against 20 mM phosphate buffer, pH 7.4 at 4 ºC. The specific starch transglucosylation and disproportionation activities of the purified Y418 mutated *Cg*AMs were 1.2-3.0 fold and 1.3-3.0 fold lower than that of the WT, respectively (Table 3.1). For both activities, the specific activities of WT and Y418F mutant were closed (25.0 and 21.0 U/mg protein for starch transglucosylation activity, respectively) while those of Y418A/D/S/W mutants were 1.3-2.3 -fold lower than WT. Interestingly, Y418R mutant was most affected with the lowest specific activity of 8.36 U/mg (Table 3.1). In addition, percent yields of WT and all mutated *Cg*AMs were similar. It is noticeable that the yields of all enzymes assayed by disproportionation activity were 4.0-fold lower than assayed by starch transglucosylation activity (Table 3.1 and 3.2).
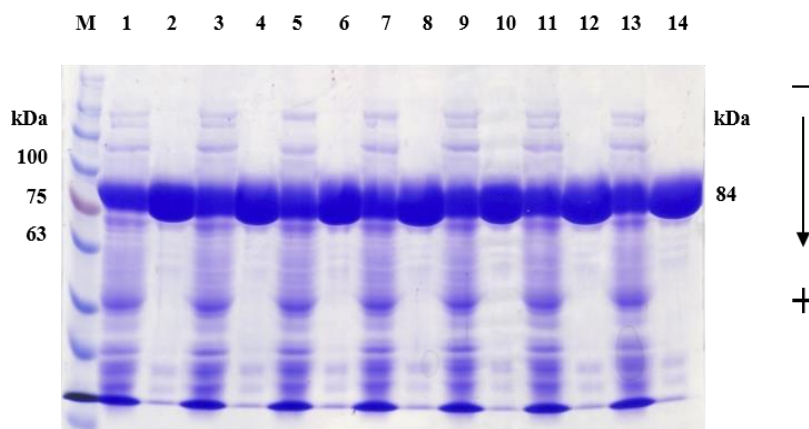
Figure 3. 17 SDS-PAGE of crude and purified enzyme expressed from cells harboring

the series of Y418 mutated *Cg*AMs.

Lane M   = Protein markers

Lane 1, 3, 5, 7, 9, 11 and 13   = 20 µg of crude WT-, Y418A-, Y418S-, Y418D-,

                                                Y418R-,Y418W- and Y418F-*Cg*AMs, respectively

Lane 2, 4, 6, 8, 10, 12 and 14 = 10 µg of purified WT-, Y418A-, Y418S-, Y418D-,

                                                Y418R-,Y418W- and Y418F-*Cg*AMs, respectively

Table 3. 1 Purification of WT and Y418 mutated *Cg*AMs assayed by starch transglucosylation activity.

*Cg*AM	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
WT					
Crude	284	425	1.50	1	100
Purified	7.15	179	25.0	16.7	42.1
Y418A					
Crude	242	254	1.05	1	100
Purified	5.61	94.1	16.8	16.0	37.0
Y418S					
Crude	264	304	1.15	1	100
Purified	8.05	146	11.8	10.2	48.0
Y418D					
Crude	235	305	1.30	1	100
Purified	6.84	130	19.0	14.6	42.6
Y418R					
Crude	252	278	1.10	1	100
Purified	7.42	62	8.36	7.57	22.3
Y418W					
Crude	265	204	0.77	1	100
Purified	6.65	72	10.8	14.1	35.3
Y418F					
Crude	300	415	1.38	1	100
Purified	8.12	170	20.9	15.1	41.0

Table 3. 2 Purification of WT and Y418 mutated *Cg*AMs assayed by disproportionation activity.

*Cg*AM	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
WT					
Crude	284	1785	6.29	1	100
Purified	7.15	196	27.4	4.36	11.0
Y418A					
Crude	242	975	4.03	1	100
Purified	5.61	98.7	17.6	4.37	10.1
Y418S					
Crude	264	964	3.65	1	100
Purified	8.05	112	13.9	3.81	11.6
Y418D					
Crude	235	954	4.06	1	100
Purified	6.84	130	19.0	4.68	13.6
Y418R					
Crude	252	578	2.29	1	100
Purified	7.42	69.4	9.35	4.08	12.0
Y418W					
Crude	265	613	2.31	1	100
Purified	6.65	78.5	11.8	5.10	12.8
Y418F					
Crude	300	1643	5.48	1	100
Purified	8.12	178	21.9	4.00	10.8

3.3 Effect of Y418 mutation on enzyme characteristics

To determine the effect of Y418 mutation on enzyme, various biochemical and biophysical properties together with the product formation of the series of Y418 mutated *Cg*AMs (Y418A/S/D/R/W/F) were measured and compared to the WT.

Biochemical properties of *Cg*AMs

3.3.1 Various amylomaltase activities

The effect of single point mutation at Y418 residue on various activities of *Cg*AM was examined as described in 2.8 and the result was shown in Table3.3. Mutation at Y418 caused an obvious change in enzyme activities; both the inter- and intra-molecular transglucosylation reactions were affected. A significant decrease in the three main activities of AM: starch transglucosylation (1.2-3.0 folds), disproportionation (1.2-2.6 folds) and especially cyclization (1.3-7.3 folds) was observed (Table 3.3). Y418F, Y418A/S/D and Y418W/R mutants showed a slight, moderate and dramatic reduction in those activities, respectively when compared to WT. However, the hydrolysis and coupling activities were mostly not changed except for coupling of R mutant which showed half reduction. From these results, it could be summarized that all Y418 mutated *Cg*AMs showed lower activity of the three mains transglucosylation activities than those of WT *Cg*AM.

3.3.2 Optimum conditions and stability

In this experiment, the optimum conditions for two main activities, disproportionation and cyclization of *Cg*AMs were determined.

*3.3.2.1 Effect of temperature on Cg*AM *activity*

Optimum temperatures of disproportionation and cyclization activities of *Cg*AMs were determined as described in section 2.9.1. For disproportionation activity,

the optimum temperature of Y418 mutated CgAMs was 40 ºC similar to WT enzyme (Figure3.18A).At 55 °C, all CgAMs showed half of the maximum disproportionation activity. The cyclization activity of CgAMs was also investigated, the optimum temperature of WT and Y418 mutated CgAMs was 30 ºC (Figure 3.19A). All enzymes had less than 50% residual cyclization activity at temperature higher than 45 ºC, especially the D, R and W mutants which had less than 10% activity. The cyclization activity of Y418A/S/D/R/W at 50 ºC could not be detected while that of the WT-and Y418F had 10-20% residual activity.

3.3.2.2 Effect of pH on CgAM activity

The effect of pH on enzyme activity was examined by disproportionation and cyclization activities as described in section 2.9.2. The optimum pH for disproportionation activity of WT and all Y418 mutated CgAMs was pH 6.0 Figure3.18B. At pH 9.0, both WT and mutated CgAMs showed similar activity, 60-70% relative activity still remained. While only 10% activity was observed at pH 4.0 and higher than 80% CgAM activity remained in the pH range of 6.0-8.0. In addition, the optimum pH for cyclization activity of WT-and Y418 mutated CgAMs was also investigated (Figure3.19B), the results showed that all CgAMs had optimum pH at pH 6.0. Interestingly, 50-70% of activity of all CgAMs still remained at pH 9.0.

3.3.2.3 Effect of temperature stability of CgAM

Temperature stability of CgAM was determined by following disproportionation activity as a function of time. CgAMs were pre-incubated at different temperature, ranging from 30 ºC, 35 ºC and 40 ºC for 0-180 min as described in 2.9.3. It was observed that WT-CgAM was more stable than all Y418 mutated enzymes at all temperatures tested. All CgAMs well maintained its activity when

incubated at 30 ℃ for 180 min, about 70% activity was still observed (Figure 3.20A).

The loss of 50% activity was found after 180 min at 35 ℃ especially for Y418R and

Y418W mutated *Cg*AMs (Figure 3.20B). At 40 ℃, all enzymes showed nearly 100%

activity at short incubation time of 10 min but their activities were dramatically

decreased to 10% at 90 min incubation time, and lost all activities after 90 min (Figure

3.20C).

3.3.2.4 Effect of pH stability of *Cg*AM

The pH stability of disproportionation activity of *Cg*AM was followed at 30 ℃

as described in 2.9.4. The result was shown in Figure 3.21. At pH 7.0, nearly 90% of

WT and Y418F activities were remained at 120 min incubation time while other

mutated enzymes had 70-80% activity. At pH 8.0 and the same incubation time of 120

min, the residual activities of WT-, Y418F- and Y418A- *Cg*AMs were about 50-60%

while other mutated *Cg*AMs showed 40% remaining activity (Figure 3.21B).

3.3.3 Substrate specificity

The ability of mutated *Cg*AMs to catalyze different substrates (G2-G7) of

disproportionation reaction was determined and compared to that of the WT as

described in 2.9.5 (Fig. 3.22). G3 was the best while G7 was the worst substrate for

both WT and mutated *Cg*AMs. For most of mutated enzymes, specificity for G3 was

close to that of G4 while the WT significantly used G3 better than G4. It is noticeable

here that Y418F showed an increase in preference for G5 to G7 (Figure. 3.22).

3.3.4 Determination of kinetic parameters

3.3.4.1 Kinetic study of disproportionation reaction

The kinetic analysis of *Cg*AMs was performed using G3, the most favorable

substrate of disproportionation reaction as described in section 2.9.6.1. Figure 3.23

represented the Lineweaver-Burk plot of CgAMs. All mutated CgAMs except the F mutant had a lower k_{cat} and k_{cat}/K_m values, the W and R mutants showed the lowest k_{cat}/K_m with 8- and 12-fold lower than the WT's value (Table 3.3). The mutation affected more on the k_{cat}, 1.4 to 10.8-fold lower than that of the WT while the K_m values were not much changed with the values around 16-20 mM.

3.3.4.2 Kinetic study of cyclization reaction

Y418A and Y418W were selected for the study of the kinetics of cyclization reaction compared to WT enzyme as described in section 2.9.6.2. Figure 3.24 represented the Lineweaver-Burk plot of CgAMs. All kinetic parameters were calculated and shown in Table 3.4. The K_m values of Y418A and Y418W CgAMs were about 1.5-fold higher than that of WT, indicating lower affinity towards pea starch substrate. The k_{cat}/K_m of Y418A and Y418W CgAMs were approximately 3.0 -7.8 fold lower than the WT's value.

3.3.5 Analysis of pattern of LR-CDs products

Production of LR-CDs was performed, CgAMs were incubated with 2% (w/v) pea starch at 30 ºC, pH 6.0 which was their optimum condition. LR-CD mixtures in the reaction were analyzed by HPAEC-PAD described in section 2.9.7

3.3.5.1 Effect of incubation time

Effect of incubation time on LR-CDs production was carried out as described in section 2.9.7.1. To compare the LR-CDs production patterns of WT and Y418 mutated CgAMs, the enzymes with 0.10 U starch degradation activity were incubated at various times (1.5, 6, 12 and 24 h). It was clearly observed that the LR-CD yields of all CgAMs were significantly increased at longer incubation time (Figure 3.25). Interestingly, WT and Y418F mutated CgAMs gave CD29-CD33 as main CD product

while Y418A/D/S mutants yielded CD38-CD40 as principle product at 6 and 12 h. In addition, Y418W and Y418R mutants showed broad size of LR-CDs at every incubation time.

3.3.5.2 Effect of unit enzyme

To observe the influence of enzyme concentration on LR-CDs formation, 0.05 U, 0.1 U and 0.2 U starch degradation activities of enzymes were used as described in section 2.9.7.2. The result showed that the increase in enzyme concentration resulted in a shift of the principle CD products to smaller size as shown in Figure 3.26.

Table 3. 3 Activities of WT and Y418 mutated *Cg*AMs

	Specific activity (U/mg)				
	Starch transglucosylation	**Disprop.**	**Cyclization**	**Coupling**	**Hydrolysis**
WT	26.0 ± 1.50	24.4 ± 0.93	0.44 ± 0.08	0.16 ± 0.01	0.03 ± 0.00
Y418A	17.0 ± 0.26	19.4 ± 0.76	0.19 ± 0.04	0.19 ± 0.01	0.04 ± 0.01
Y418S	12.5 ± 1.65	18.8 ± 1.00	0.17 ± 0.06	0.14 ± 0.01	0.03 ± 0.00
Y418D	16.0 ± 1.56	19.0 ± 1.08	0.25 ± 0.07	0.14 ± 0.02	0.02 ± 0.01
Y418R	8.93 ± 1.64	9.56 ± 0.98	0.06 ± 0.02	0.08 ± 0.03	0.03 ±0.02
Y418W	12.1 ± 0.33	11.4 ± 0.51	0.08 ± 0.03	0.13 ± 0.01	0.03 ± 0.01
Y418F	20.9 ± 1.59	20.8 ± 1.28	0.33 ± 0.03	0.13 ± 0.01	0.03 ± 0.01

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
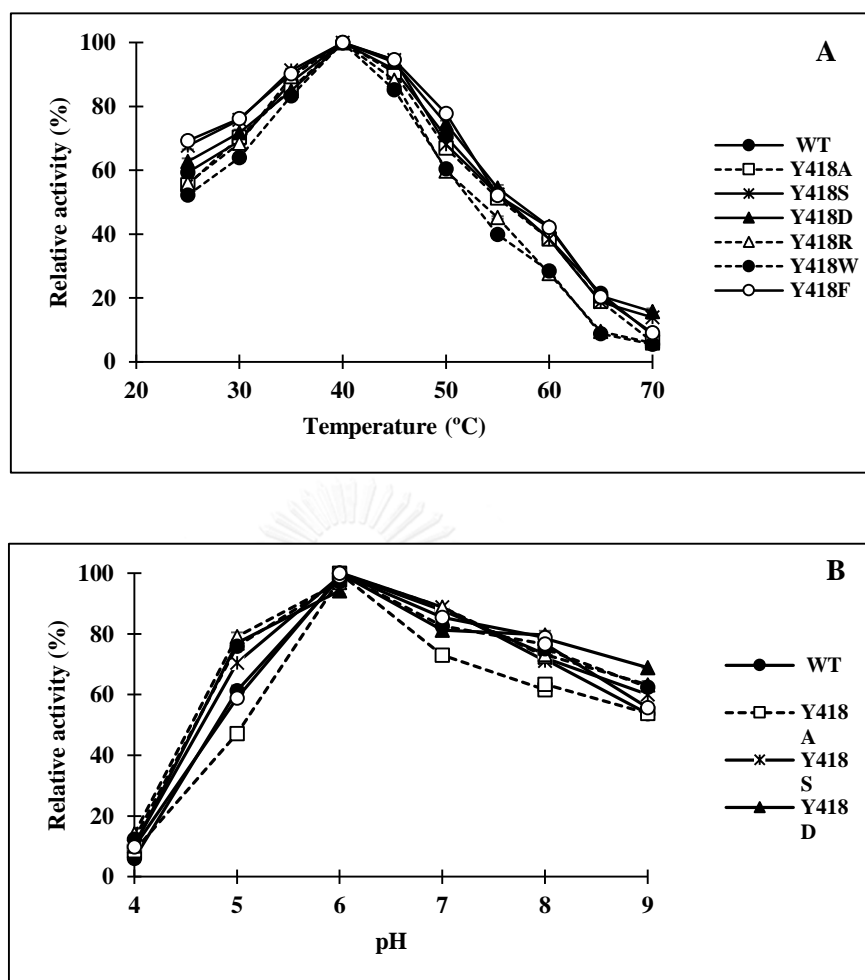
Figure 3. 18 Effect of temperature (A) and pH (B) for WT and Y418 mutated *Cg*AMs

on disproportionation activity. The enzymes were fixed at pH 6.0 in (A) and 30°C (B).

Buffer used were: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and tris-

HCl buffer (pH 8.0 -9.0). The experiments were performed as described in section 2.9.1

and 2.9.2. Data are shown as the mean ± SD and are derived from three independent

repeats.
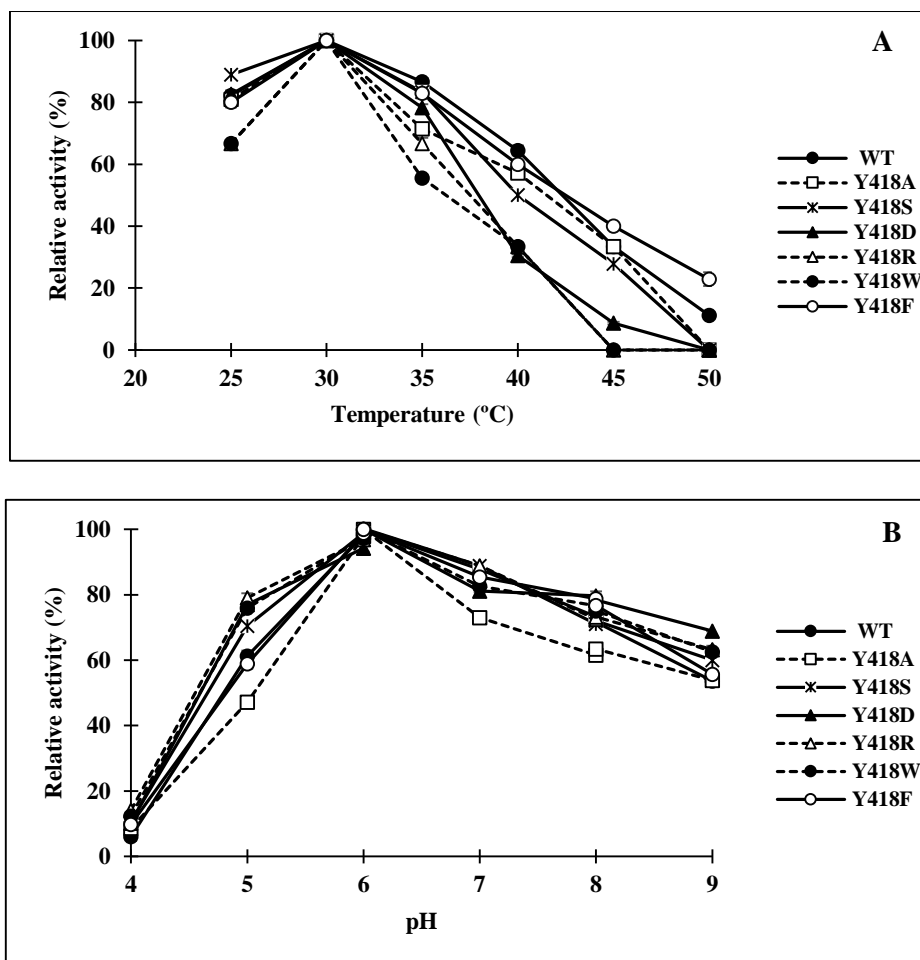
Figure 3. 19 Effect of temperature (A) and pH (B) for WT and Y418 mutated *Cg*AMs

on cyclization activity. The enzymes were fixed at pH 6.0 in (A) and 30°C (B). Buffer

used were: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and tris-HCl

buffer (pH 8.0-9.0). The experiments were performed as described in section 2.9.1 and

2.9.2. WT and Y418 mutated *Cg*AMs were incubated with 2% (w/v) of pea starch for

90 min at various temperature and pH. LR‐CD products were analyzed by HPAEC-

PAD. Data are shown as the mean ± SD and are derived from three independent repeats.
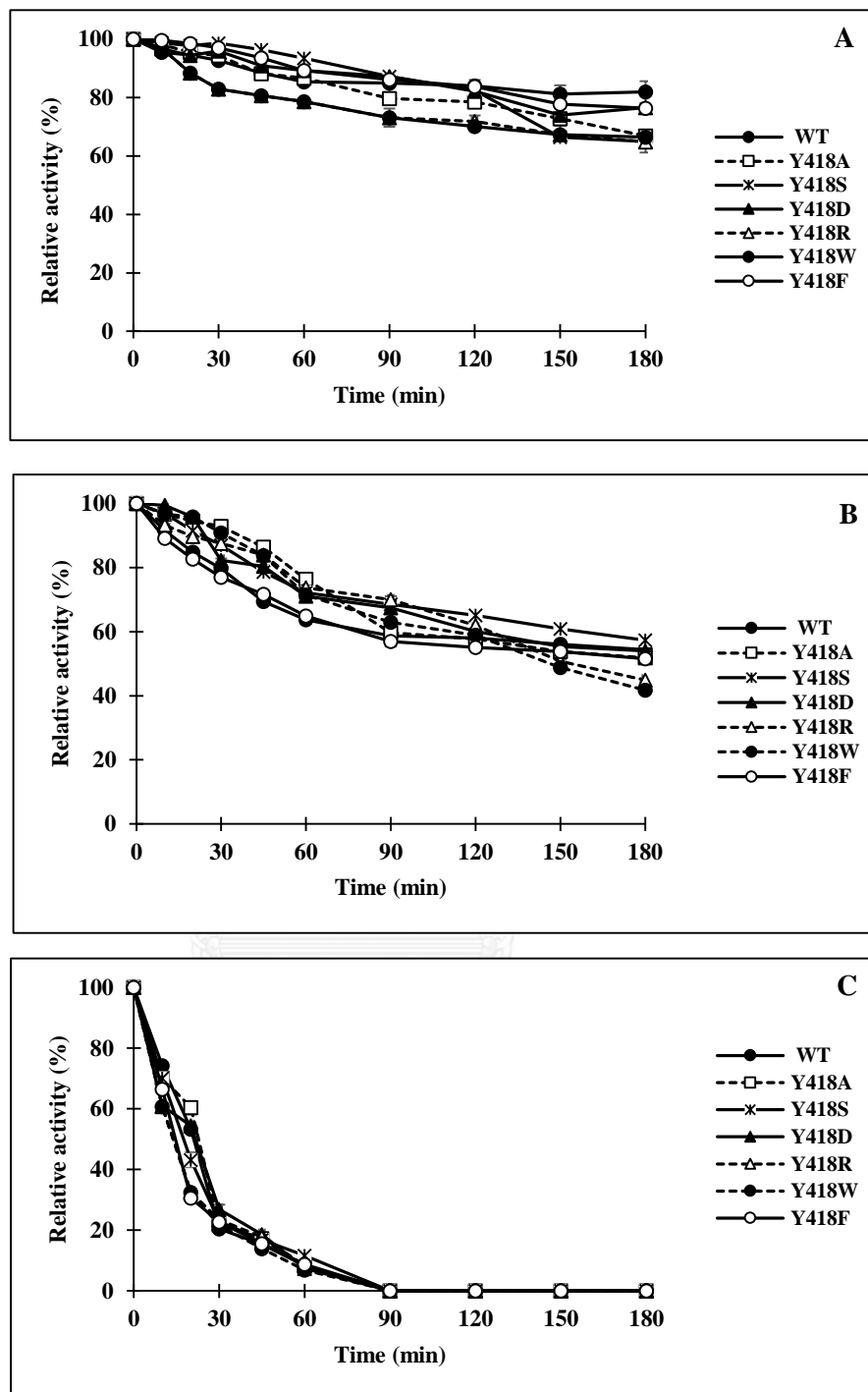
Figure 3. 20 Effect of temperature *Cg*AM stability. WT and Y418 mutated *Cg*AMs

were incubated in phosphate buffer pH 6.0 at 30 ℃ (A), 35 ℃ (B), 40 ℃ (C) for 0 to

180 min. The activity was assayed by disproportionation activity assay as described in

2.9.3
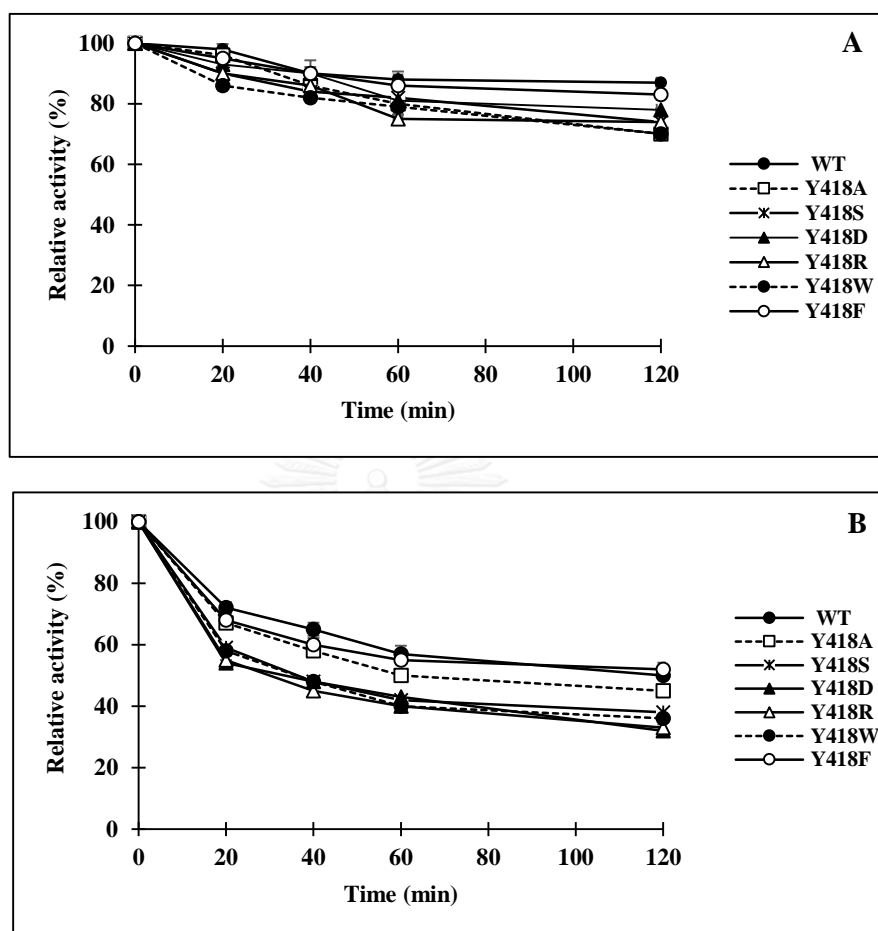
Figure 3. 21 Effect of pH on *Cg*AM stability. WT and Y418 mutated *Cg*AMs were incubated at 30 ℃ in phosphate buffer pH 7.0 (A) and tris-HCl buffer pH 8.0 (B) for 0-120 min. The activity was assayed by disproportionation activity as described in 2.9.4.
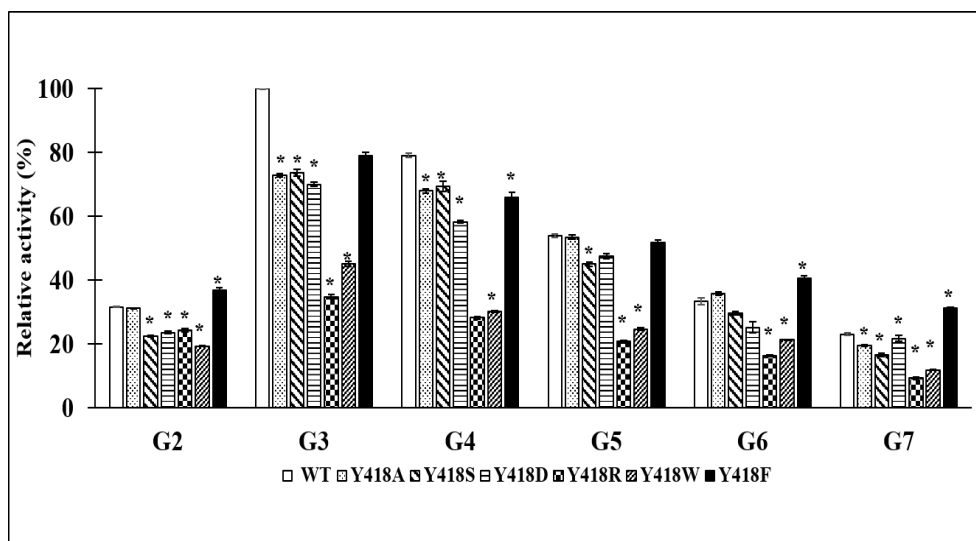
Figure 3.22 Substrate specificity of WT and Y418 mutated *Cg*AMs in dispro

portionation reaction using malto-oligosaccharides (maltose, G2 to maltoheptaose, G7)

as substrate. The activity of WT - *Cg*AM on G3 substrate was set as 100%. *P < 0.05

(Student's t-Test) with respect to the disproportionation reaction of WT - *Cg*AM. Data

are presented as the mean ± SD and are derived from three independent experiments.
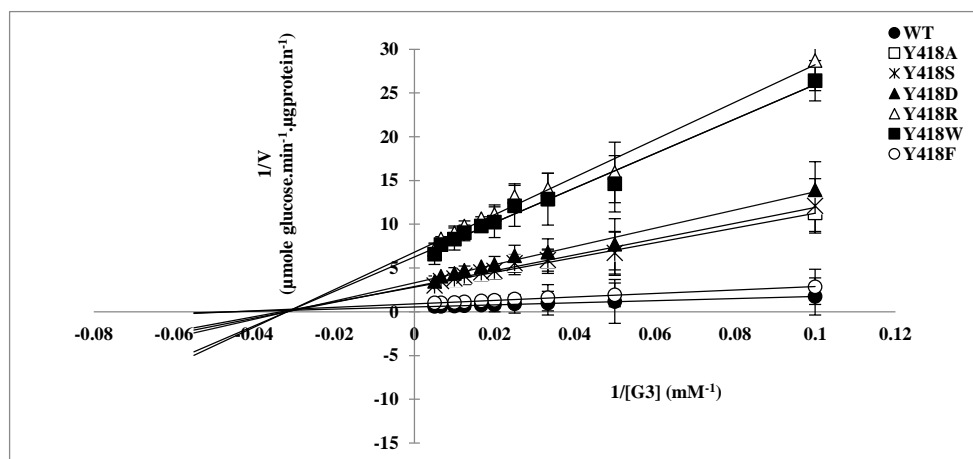
Figure 3. 23 Lineweaver- Burk plot of WT and Y418 mutated *Cg*AMs-catalyzed disproportionation reaction. All *Cg*AMs were incubated with various concentrations of maltotriose (G3) in 50 mM phosphate buffer, pH 6.0 at 40 ºC for 5 min. Glucose oxidase method was used to determine the amount of free glucose produced during the reaction.

Table 3.4 Kinetic parameters of WT and Y418 mutated *Cg*AMs from dispro-portionation reaction using maltotriose substrate.

*Cg*AM	K_m (mM)	V_{max} (µmole glucose.min^{-1}.µgprotein^{-1})	k_{cat} (min^{-1}) [10^3]	k_{cat}/K_m (mM^{-1}min^{-1}) [10^3]
WT	17.2 ± 1.67	2.21 ± 0.31	168 ± 3.30	9.80 ± 1.09
Y418A	15.7 ± 0.58	0.44 ± 0.21	118 ± 2.60	7.52 ± 0.41
Y418S	18.0 ± 0.68	0.43 ± 0.18	110 ± 3.56	6.11 ± 0.42
Y418D	16.3 ± 1.02	0.37 ± 0.11	112 ± 3.34	6.90 ± 0.64
Y418R	19.5 ± 0.82	0.19 ± 0.08	15.5 ± 1.70	0.80 ± 0.09
Y418W	20.4 ± 0.75	0.18 ± 0.05	24.2 ± 3.14	1.18 ± 0.13
Y418F	18.2 ± 0.95	1.88 ± 0.26	160 ± 6.49	8.80 ± 0.21

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
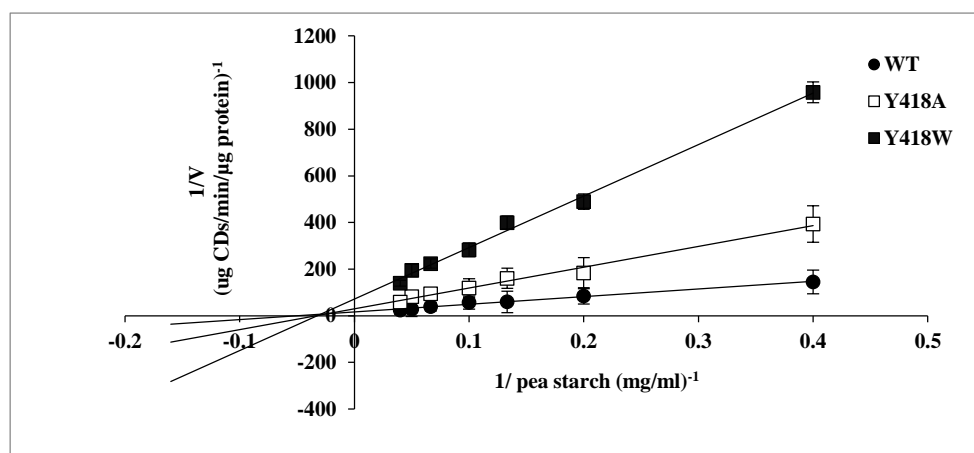
Figure 3. 24 Lineweaver- Burk plot of WT and Y418 mutated *Cg*AMs-catalyzed cyclization reaction. *Cg*AMs (0.05 U starch degrading activity) were incubated with varying concentrations of pea starch at 30 ºC for 60 min. LR-CD products were analyzed by HPAEC -PAD.

Table 3. 5 Kinetic parameters of WT and Y418 mutated *Cg*AMs from cyclization activity using pea starch as the substrate.

*Cg*AM	K_m (mg/ml)	V_{max} (µg CD.min^{-1}. µgprotein^{-1})	k_{cat} (min^{-1}) [10^3]	k_{cat}/K_m (mg.ml^{-1}min^{-1}) [10^3]
WT	19.9 ± 1.78	0.06 ± 0.13	6.12 ± 1.35	0.31 ± 0.06
Y418A	30.0 ± 2.16	0.03 ± 0.06	3.25 ± 1.20	0.11 ± 0.07
Y418W	31.7 ± 3.55	0.01 ± 0.02	1.13 ± 0.26	0.04 ± 0.03

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
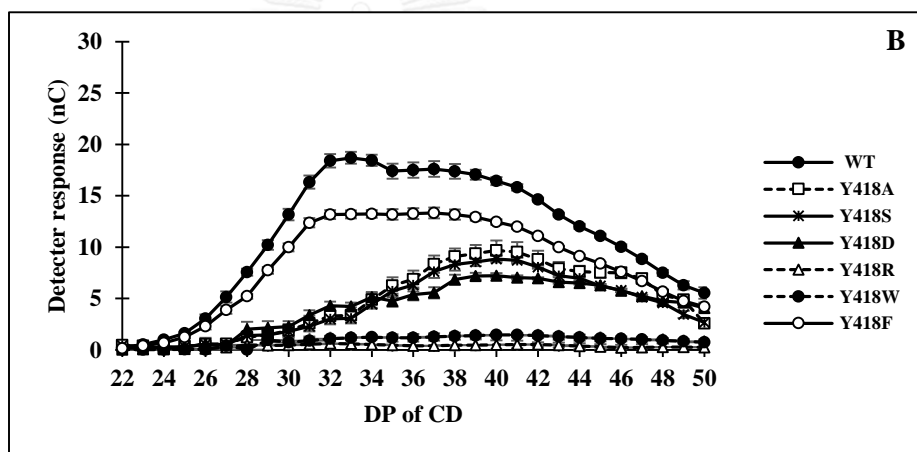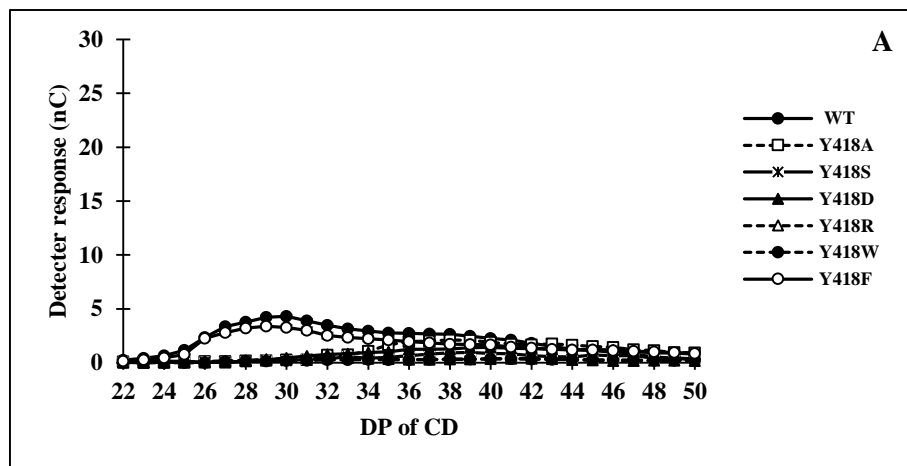
Figure 3. 25 HPAEC‑ PAD analysis of LR‑ CD products synthesized at different incubation time 1.5 h (A), 6 h (B), 12 h (C) and 24 h (D) by WT and Y418 mutated *Cg*AMs (DP = Degree of Polymerization).
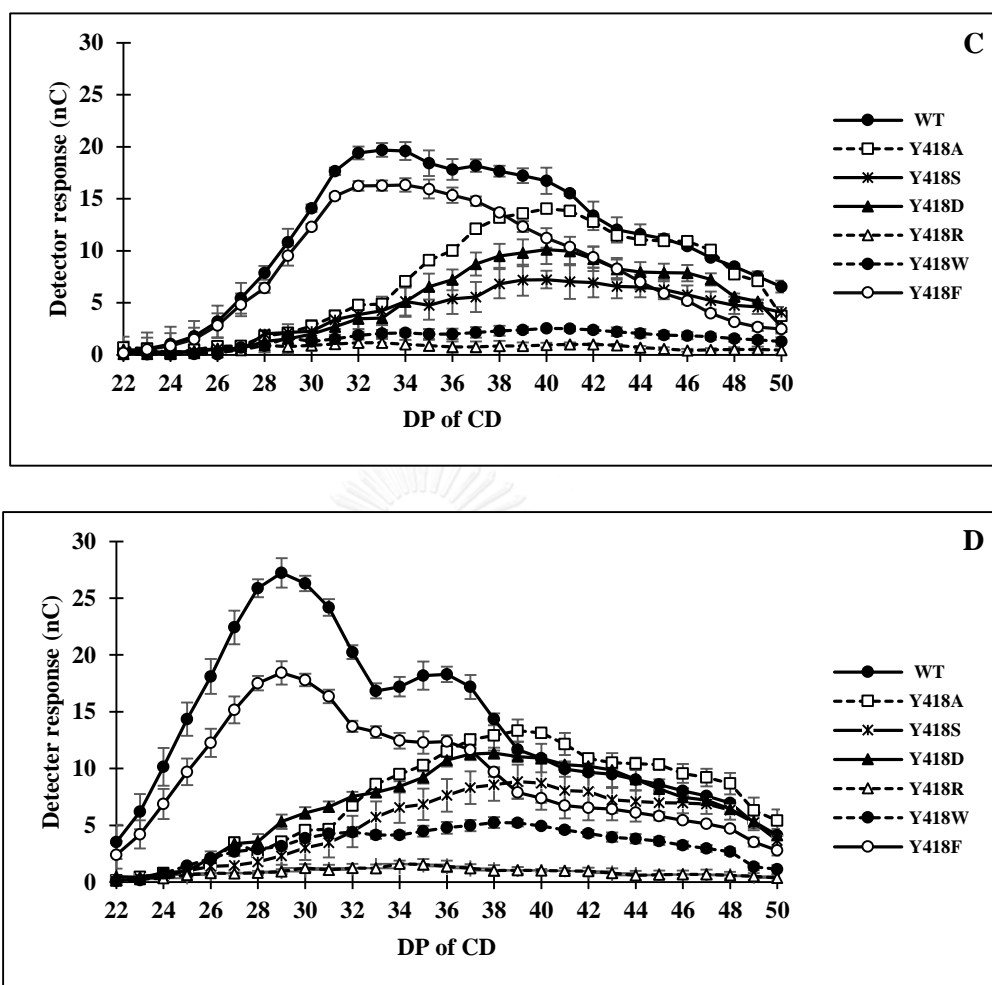
Figure 3.25 (continue) HPAEC-PAD analysis of LR-CD products synthesized at different incubation time 1.5 h (A), 6 h (B), 12 h (C) and 24 h (D) by WT and Y418 mutated *Cg*AMs (DP = Degree of Polymerization).
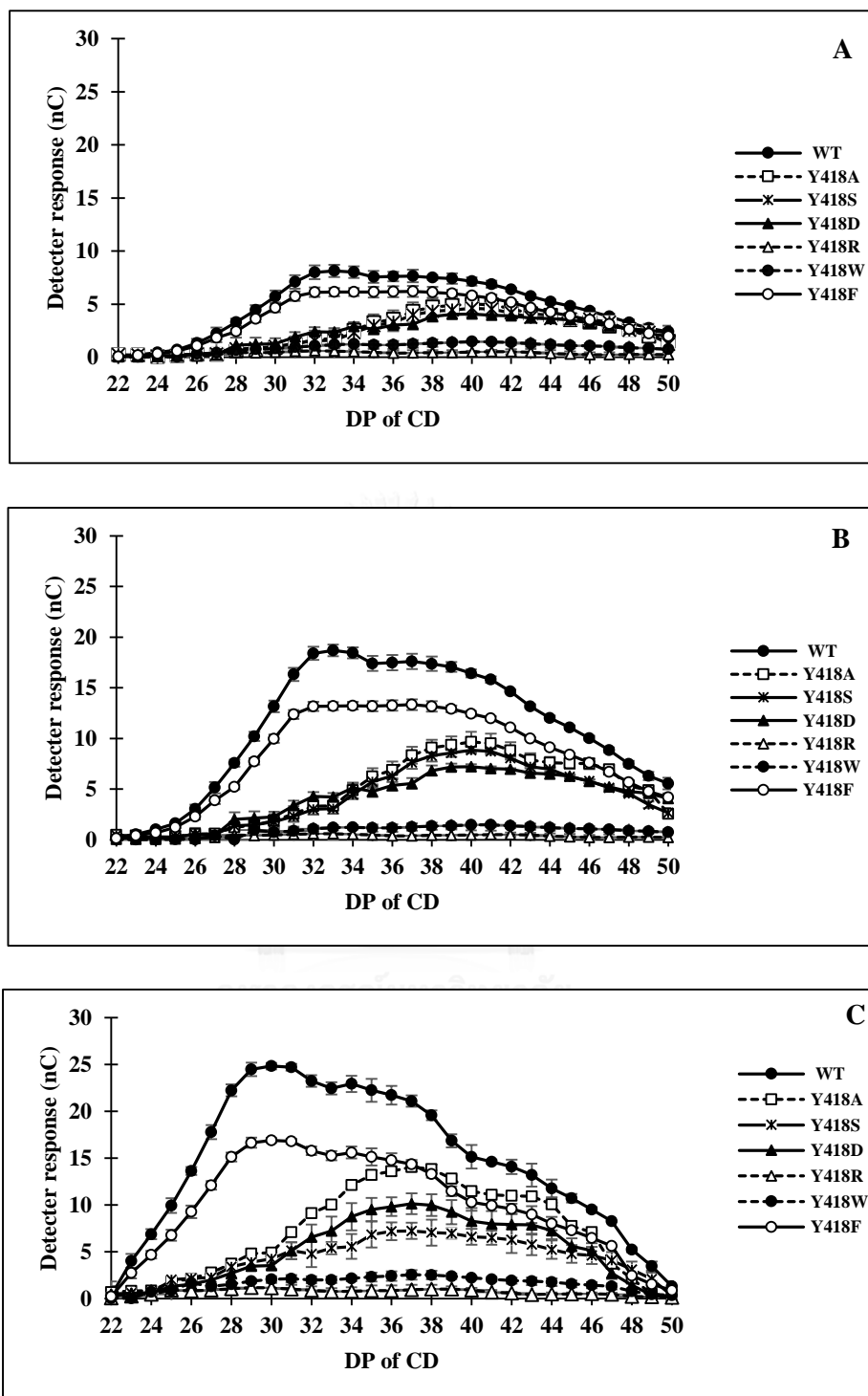
Figure 3. 26 HPAEC-PAD analysis of LR-CD products synthesized for 6 h at different

enzyme unit; 0.05 U (A), 0.10 U (B) and 0.20 U (C) by WT and Y418 mutated *Cg*AMs

(DP = Degree of Polymerization).

3.3.6 Enzyme conformation

In order to investigate the effect of Y418 mutation on secondary structure and folding property of *Cg*AM, circular dichroism (CD) analysis was performed. Purified WT and Y418 mutated *Cg*AMs at 0.2 mg/ml protein concentration were used in the wavelength scanning range from 190 to 250 nm. The CD spectra of Y418 mutated *Cg*AMs were compared with WT as shown in Figure 3.27. The result showed that the CD spectra of the mutated *Cg*AMs and WT were closely overlaid, a slight change was observed in CD spectra of the mutated enzymes. The contents of the secondary structures calculated as described in section 2.9.8 were: α-helix 26-27%, β-sheet 20-22% and random coil 51-52%.

Biophysical properties of *Cg*AMs

3.3.7 Isothermal titration calorimetry (ITC)

In this work, ITC was performed to study the heat change during enzyme catalysis. Maltotriose (G3) was used as a substrate of disproportionation reaction to determine the heat of enzyme reaction between WT and Y418A/W mutated *Cg*AMs as described in section 2.10.1. The thermal power level (μcal/sec) after titration of the enzyme into sample cell showed heat production of enzyme reaction. The average of three replicates gave a thermal power value of WT *Cg*AM which was higher than those of Y418A (1.5 folds) and Y418W (2.5 folds) *Cg*AMs (Figure 3.28). ITC results thus showed a different heat production of WT and Y418A/W enzymes which was corresponded to the activities of *Cg*AMs measured by biochemical method as shown in (Figure 3.29).

To determine the kinetic parameters of CgAMs, the average thermal power prior to the subsequent titration was converted to the rate of G3 catalysis. Every experimental data was fit to the systematic curve fitting analysis (Global fitting program) and estimates of kinetic parameters, the V_{max}/K_m, were obtained. WT CgAM showed the highest value, V_{max}/K_m values of WT, Y418A and Y418W for 10 mM G3 were 1.75, 1.15 and 0.94 mM^{-1}s^{-1}, respectively. (Table 3.6). The graph plotted between V$_{max}/K_m$ against concentration of G3 showed the product inhibition was occurred at high G3 concentration for WT, Y418A and Y418W mutated CgAMs (Figure 3.30).

3.3.8 Differential scanning calorimetry (DSC)

In this work, DSC was performed in the temperature range of 10 ºC to 90 ºC in an attempt to compare the thermal stability between WT and Y418A/W mutated CgAMs. The thermal transition curves from this temperature range of all scanning rate (0.75 ºC/min, 1.0 ºC/min and 1.5 ºC/min) for WT and Y418A/W mutated CgAMs in phosphate buffer pH 6.0 were plotted (Figure 3.31). The results showed that, all CgAMs demonstrated the irreversible transition. The heat capacity profiles of WT CgAM at all scanning rate gave a closer peak temperature (T$_p$) to that of Y418A but a higher T$_p$ than Y418W mutated CgAM. At 1.0 °C/min a shift of peak temperature of Y418W towards 4 °C lower than the value of WT was observed (Table 3.7).

3.4 Structural modeling of Y418 mutated CgAMs

The 3D - structure of CgAM was recently reported (Joo et al., 2016). In this present study, the model structures of all Y418 mutated CgAMs were constructed by the Discovery Studio 3.5 Client program using WT CgAM X-ray structure (PDB code 5B68) (Joo et al., 2016) as template. We found eight residues in the 410s loop including

D416, G417, N419, Q420, R577, V582, Q662 and P663 are within 5 Ǻ away from Y418 position. In the WT and the Y418A/S/D/W/F mutants, no hydrogen bond was observed between residues 418 and these residues, but the R mutant had one H-bond forming with D416 (Fig. 3.32 1A-1G).

The distance between the residues position 418 and F534 which forms the substrate channels was also predicted using the same Discovery Studio Program (Table 3.8). Y418A and Y418W provided a distance of 1.0 Ǻ longer and 5.6 Ǻ shorter than that of the WT, respectively. The molecular surfaces of aromatic edge of CgAMs were determined to show the hydrophobic interaction between Y418 and F534 residues (Fig. 3.32 2A-2G). WT-CgAM showed the strongest hydrophobic interaction (gold color in Figure 3.32-1A) while moderate interaction was found in Y418F/W mutants probably due to the inward direction of F/W side chain. For the Y418A/S/D/R mutants, low or no interaction was observed (grey color in Fig 3.32 - 2B; 2C; 2D; 2E).
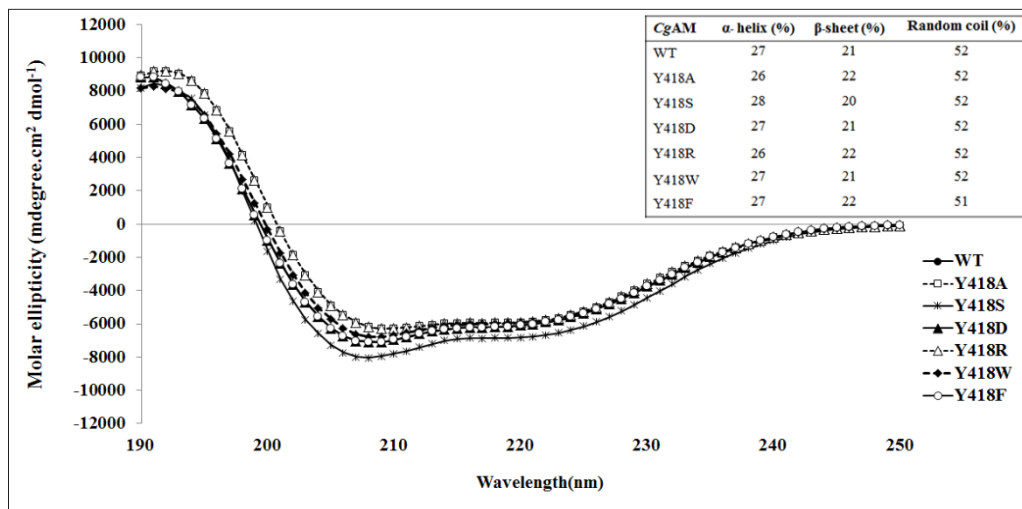
Figure 3. 27 Circular dichroism spectra and the predicted secondary structural compositions of WT and Y418 mutated *Cg*AMs.
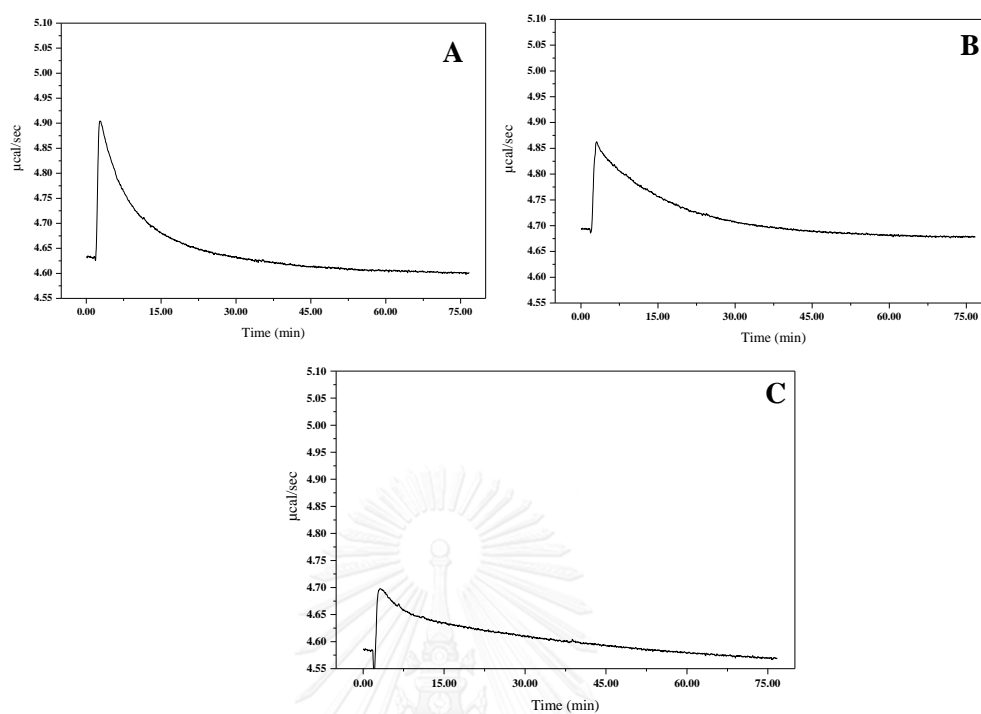
Figure 3. 28 Disproportionation activity of amylomaltase as measured from an enthalpy change when substrate binds to enzyme detected by ITC. Data heat rate as a function of time from a single injection of 20 uM enzymes into 1.4482 ml of 10 mM G3 in 20 mM phosphate buffer pH 6.0 containing 100 mM NaCl, WT (A), Y418A (B) and Y418W(C) *Cg*AMs.

Table 3. 6 Kinetic parameters (V_{max}/K_m) of WT, Y418A and Y418W CgAMs from disproportionation activity determined by ITC.

G3(mM)	V_{max}/K_m app $* 10^{-3}$ (s^{-1})		
	WT	Y418A	Y418W
10	1.748 ±0.024	1.149 ± 0.068	0.940 ± 0.046
20	1.236 ±0.042	0.722 ± 0.030	0.685 ± 0.026
30	0.899 ±0.032	0.687 ± 0.160	0.433 ± 0.120

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
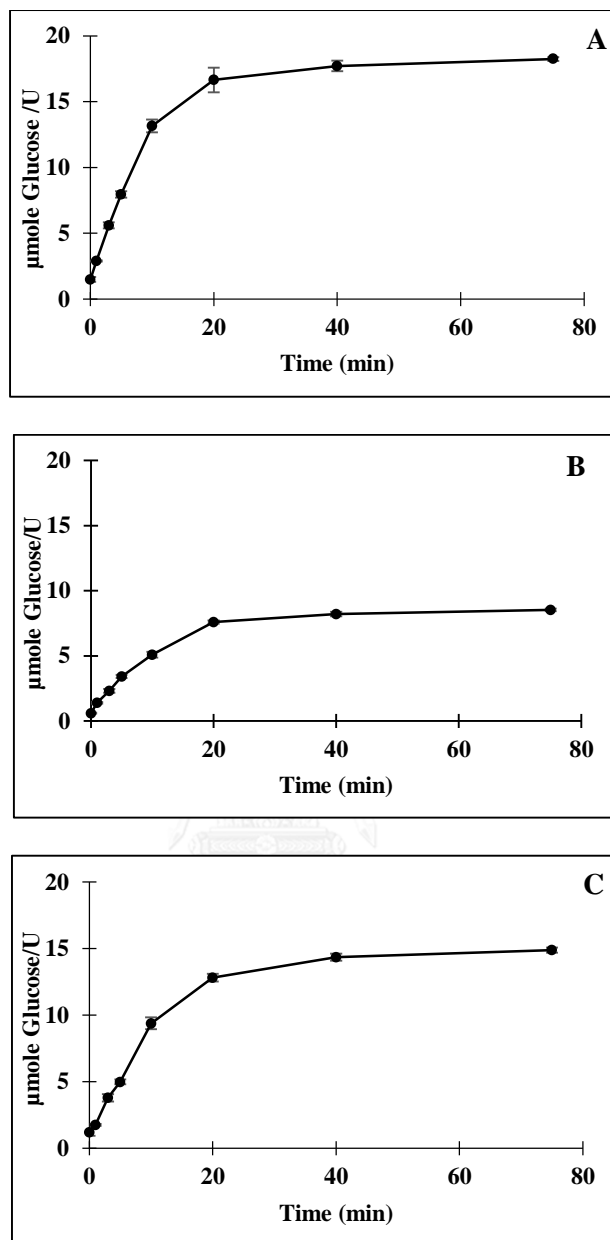
Figure 3. 29 Disproportionation activity of amylomaltase as measured from glucose product released by glucose oxidase assayed spectrophotometrically. G1 liberates as a function of time from incubation of various concentrations of 10 mM G3 with 20 μM WT (A), Y418A (B) and Y418W (C) *Cg*AMs.
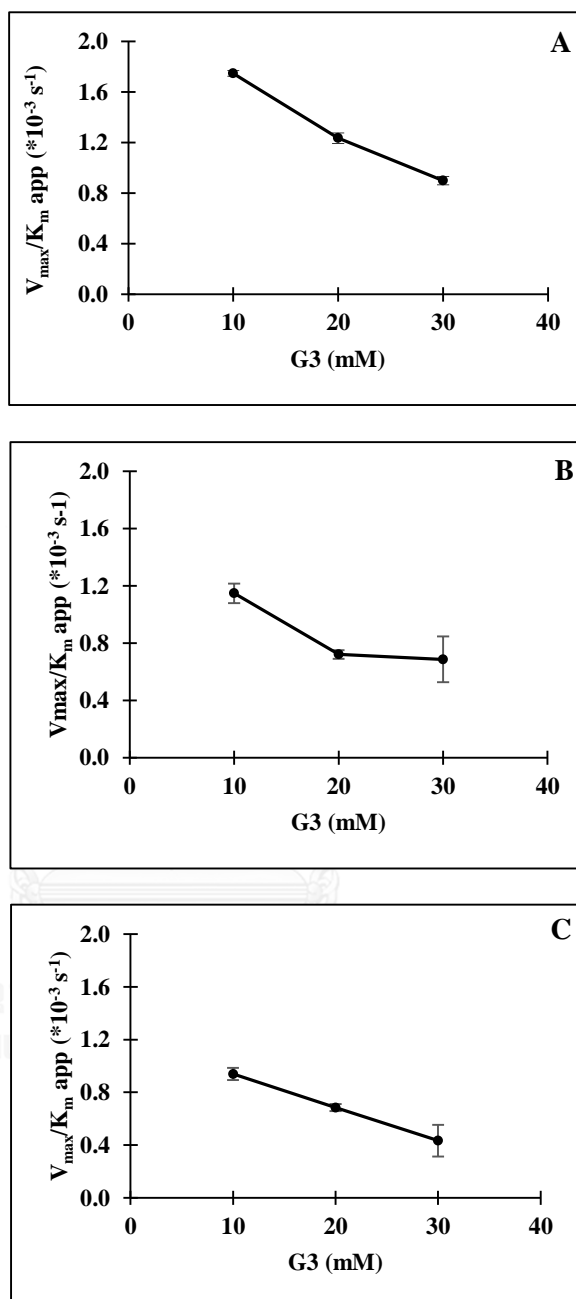
Figure 3. 30 Product inhibition of WT (A), Y418A (B) and Y418W (C) *Cg*AMs in disproportionation reaction as determined by ITC. Error bars are derived from three independent experiments.
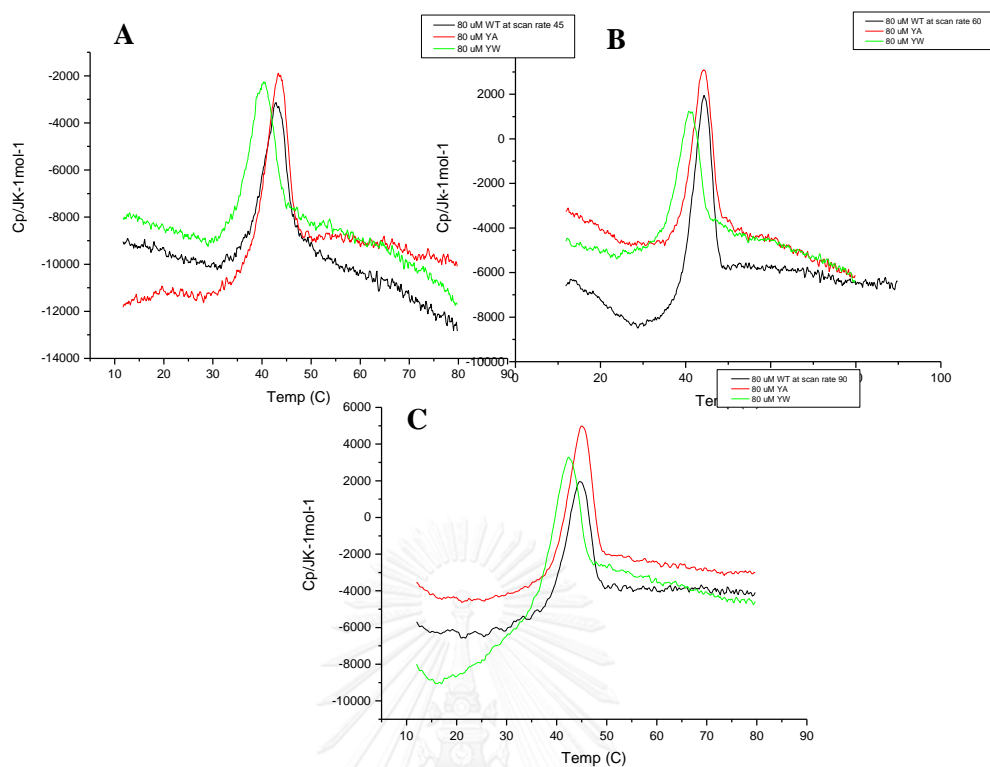
Figure 3. 31 Differential scanning calorimetry (DSC) analysis of *Cg*AMs at a scan rate of 0.75 ºC/min (A), 1.0 ºC/min (B) and 1.5 ºC/min (C). The black, red and green lines represent the thermograms of 80 μM of WT, Y418A and Y418W *Cg*AMs, respectively. Thermal profile of 50 mM phosphate buffer pH 6.0 containing 100 mM NaCl as reference was subtracted from sample profile.

Table 3. 7 Peak temperature of WT, Y418A and Y418W *Cg*AMs at different scan rate of DSC measurements.

	Tp (ºC)		
	Scan rate 0.75 ºC/min	Scan rate 1.0 ºC/min	Scan rate 1.50 ºC/min
WT	42.89	44.26	44.45
Y418A	43.29	44.19	44.96
Y418W	40.45	40.57	42.29
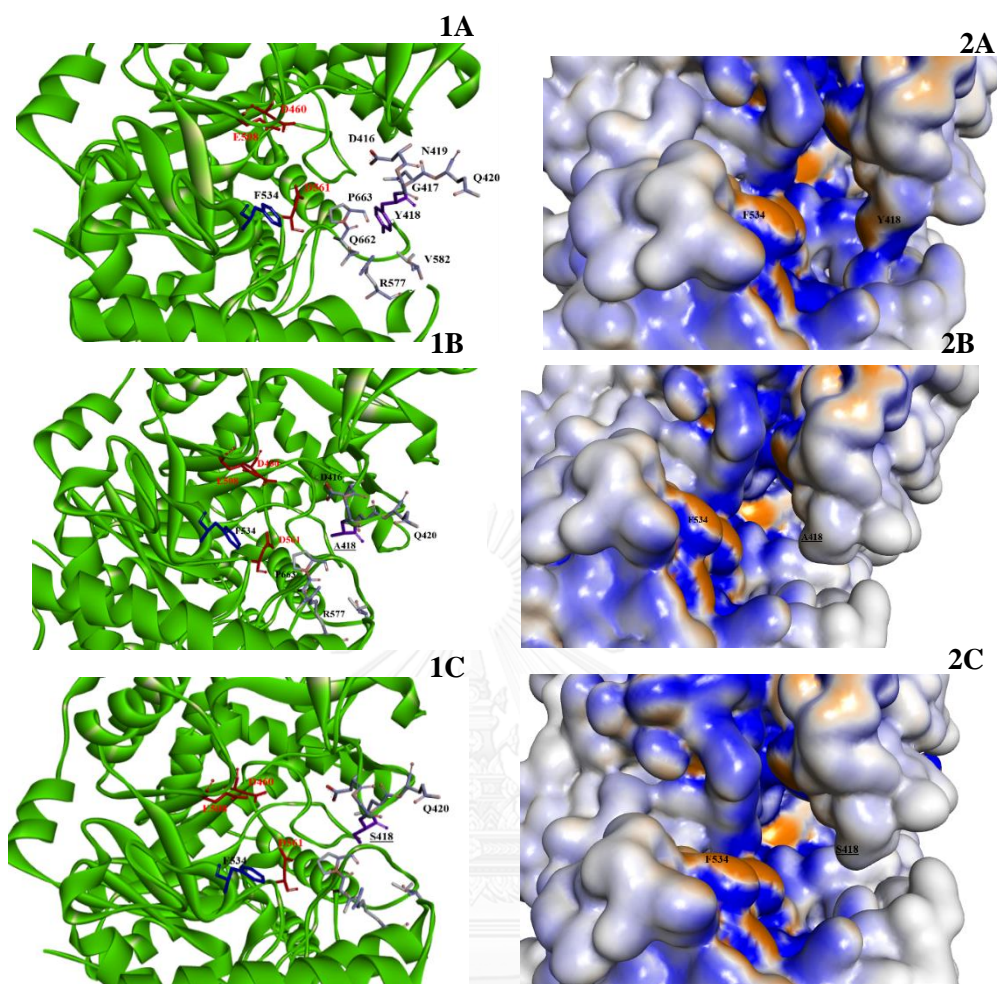
Figure 3. 32 Structures of WT and Y418 mutated *Cg*AMs. The X-ray structure of WT CgAM (PDB code 5B68) (Joo *et al*., 2016) was used as template to prepare the model structures of Y418 mutated *Cg*AMs. Proposed amino acid residues shown are those within 5 Å away from Y418 residues and the three catalytic residues represented in red (D460, E508, D561) of WT (1A), Y418A (1B), Y418S (1C), Y418D (1D), Y418R (1E), Y418W (1F), and Y418F (1G) *Cg*AMs, respectively. Hydrogen bonds are shown as dashed red line. Aromatic surfaces of WT (2A), Y418A (2B), Y418S (2C), Y418D (2D), Y418R (2E), Y418W (2F), and Y418F (2G) *Cg*AMs, respectively, are displayed with low or no hydrophobicity shown in grey, medium in blue and high in gold.

Figure 3.32 (continue) Structures of WT and Y418 mutated *Cg*AMs. The X-ray structure of WT *Cg*AM (PDB code 5B68) (Joo *et al*., 2016) was used as template to prepare the model structures of Y418 mutated *Cg*AMs. Proposed amino acid residues shown are those within 5 Å away from Y418 residues and the three catalytic residues represented in red (D460, E508, D561) of WT (1A), Y418A (1B), Y418S (1C), Y418D (1D), Y418R (1E), Y418W (1F), and Y418F (1G) *Cg*AMs, respectively. Hydrogen bonds are shown as dashed red line. Aromatic surfaces of WT (2A), Y418A (2B), Y418S (2C), Y418D (2D), Y418R (2E), Y418W (2F), and Y418F (2G) *Cg*AMs, respectively, are displayed with low or no hydrophobicity shown in grey, medium in blue and high in gold.

1F

2F

1G

2G

Figure 3.32 (continue) Structures of WT and Y418 mutated *Cg*AMs. The X-ray structure of WT *Cg*AM (PDB code 5B68) (Joo *et al*., 2016) was used as template to prepare the model structures of Y418 mutated *Cg*AMs. Proposed amino acid residues shown are those within 5 Å away from Y418 residues and the three catalytic residues represented in red (D460, E508, D561) of WT (1A), Y418A (1B), Y418S (1C), Y418D (1D), Y418R (1E), Y418W (1F), and Y418F (1G) *Cg*AMs, respectively. Hydrogen bonds are shown as dashed red line. Aromatic surfaces of WT (2A), Y418A (2B), Y418S (2C), Y418D (2D), Y418R (2E), Y418W (2F), and Y418F (2G) *Cg*AMs, respectively, are displayed with low or no hydrophobicity shown in grey, medium in blue and high in gold.

Table 3.8 Distance between amino acids at position 418 and F534 of WT and Y418

mutated *Cg*AMs as measured by Discovery Studio 3.5 Client program.

	Distance (Å)
WT (Y418-F534)	11.2
Y418A - F534	12.2
Y418S - F534	9.7
Y418D - F534	8.4
Y418R - F534	6.7
Y418W - F534	5.6
Y418F - F534	10.8

Expression, Purification and Characterization of H461 mutated *Cg*AMs

3.5 Expression and purification of H461 mutated *Cg*AMs

Five H461 mutated *Cg*AMs were expressed and purified as similar to the WT and Y418 mutated *Cg*AMs. The protein patterns of crude and purified *Cg*AMs on SDS-PAGE were shown in Figure 3.33. All H461 mutated *Cg*AMs showed overexpression of the major protein band approximately at 84 kDa similar to WT enzyme, they were thus successfully purified by only one affinity His-trap column. The fractions that showed high enzyme activity were pooled and dialyzed against 20 mM phosphate buffer, pH 7.4 at 4 ℃. The specific disproportionation activities of the purified H461S/D, H461R/W and H461A mutated *Cg*AMs were significantly lower than that of the WT (for 3.4 - 6.0 and 8.0 folds, respectively) (Table 3.9). It is noticed that when compared to WT, the decrease in starch transglucosylation activity of H461 mutated *Cg*AMs was greater than decrease in disproportionation activity. The specific starch tansglucosylation activities of H461S/D, H461R/W and H461A mutated *Cg*AMs were 5.0, 14 -23 and 44 - fold lower than that of the WT, respectively (Table 3.10).
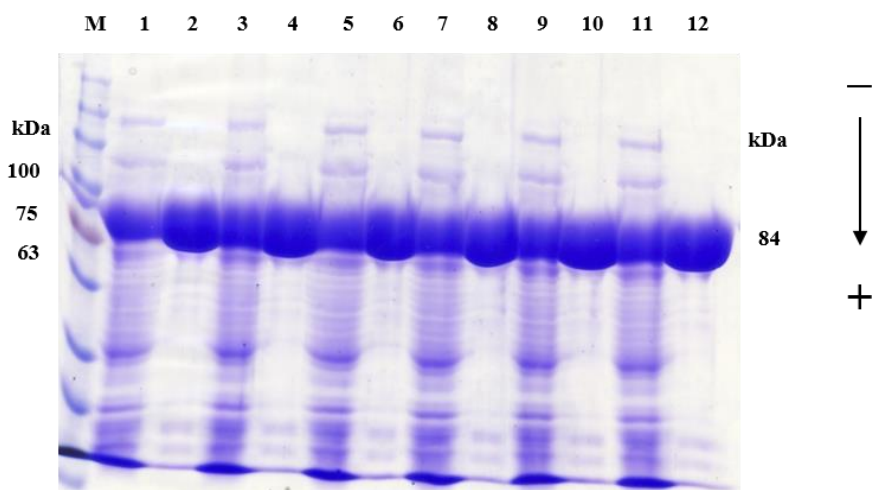
Figure 3. 33 SDS-PAGE of crude and purified enzyme expressed from cells harboring

the series of H461 mutated *Cg*AMs.

Lane M   = Protein markers

Lane 1, 3, 5, 7, 9 and 11 = 20 μg of crude WT-, H461A-, H461S, H461D-, H461R-

                                   and H461W-*Cg*AMs, respectively

Lane 2, 4, 6, 8, 10 and 12 = 10 μg of purified WT-, H461A-, H461S, H461D-, H461R-

                                   and H461W-*Cg*AMs, respectively

Table 3. 9 Purification of WT and H461 mutated *Cg*AMs assayed by starch transglucosylation activity.

*Cg*AM	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
WT					
Crude	294	467	1.59	1	100
Purified	7.68	188	24.5	15.4	40.3
H461A					
Crude	257	114	0.44	1	100
Purified	7.00	3.92	0.56	1.26	3.40
H461S					
Crude	297	184	0.62	1	100
Purified	7.35	34.1	4.64	7.50	18.6
H461D					
Crude	221	227	1.03	1	100
Purified	6.65	31.3	4.66	4.53	13.7
H461R					
Crude	247	88.3	0.36	1	100
Purified	5.28	5.52	1.05	2.92	6.25
H461W					
Crude	283	121	0.43	1	100
Purified	6.30	11.4	1.81	4.22	9.41

Table 3. 10 Purification of WT and H461 mutated *Cg*AMs assayed by dispro-
portionation activity.

*Cg*AM	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
WT					
Crude	294	1670	5.68	1	100
Purified	7.68	169	22.0	3.87	10.1
H461A					
Crude	257	231	0.90	1	100
Purified	7.00	19.5	2.80	3.10	8.44
H461S					
Crude	297	396	1.30	1	100
Purified	7.35	47.6	6.50	4.86	12.0
H461D					
Crude	221	572	2.60	1	100
Purified	6.65	43.4	6.50	2.51	7.59
H461R					
Crude	247	415	1.70	1	100
Purified	5.28	18.9	3.60	2.13	4.55
H461W					
Crude	283	455	1.60	1	100
Purified	6.30	26.2	4.20	2.59	5.76

3.6 Effect of H461 mutation on enzyme characteristics

3.6.1 Various amylomaltase activities

The effect of H461 mutation on various activities of *Cg*AM was investigated as shown in Table 3.11. When compared to WT, transglucosylation activities of all mutated enzymes including starch transglucosylation and disproportionation activities were dramatically decreased especially for H461A (8.7 and 32 -fold lower) and H461R (9.1 and 22 -fold lower). In addition, cyclization activity of H461 mutated *Cg*AMs could not be detected by HPAEC-PAD while hydrolysis and coupling activities were not different from that of WT *Cg*AM.

3.6.2 Optimum conditions and stability

In this experiment, the optimum conditions for starch transglucosylation and disproportionation reactions were determined.

*3.6.2.1 Effect of temperature on Cg*AM *activity*

The optimum temperatures of all H461 mutated *Cg*AMs for starch transglucosylation and disproportionation reactions were 30 ℃ and 40 ℃, respectively (Figure 3.34A and 3.35A). The values were the same as those of WT and Y418 mutated *Cg*AMs. In addition, at temperature higher than 45 ℃ the activity of all H461 mutants was totally lost while WT-*Cg*AM had 50% of relative activity.

*3.6.2.2 Effect of pH on Cg*AM *activity*

The optimum pH of all H461 mutated *Cg*AMs for starch transglucosylation and disproportionation reactions was the same, at pH 6.0 (Figure 3.34B and 3.35B). The value was the same as that for WT and Y418 muated *Cg*AMs. At pH 7.0, H461 mutants completely lost their activities while WT had maintained 90% of relative activity.

Table 3. 11 All activities of H461 mutated *Cg*AMs

	Specific activity (U/mg)				
	Starch transglucosylation	**Disprop.**	**Cyclization**	**Coupling**	**Hydrolysis**
WT	25.9 ± 1.59	24.2 ± 1.02	0.44 ± 0.08	0.15 ± 0.01	0.03 ± 0.00
H461A	0.81 ± 0.51	2.79 ± 035	ND	0.02 ± 0.00	0.02 ± 0.00
H461S	5.58 ± 1.59	5.32 ± 0.49	ND	0.04 ± 0.01	0.03 ± 0.00
H461D	4.87 ± 0.46	6.55 ± 0.54	ND	0.03 ± 0.01	0.02 ± 0.00
H461R	1.18 ± 0.20	2.64 ± 0.53	ND	0.03 ± 0.01	0.02 ± 0.01
H461W	1.75 ± 0.19	3.67 ± 0.58	ND	0.03 ± 0.01	0.02 ± 0.01

Data are shown as the mean ± standard deviation and are derived from three independent experiments. ND = not detectable
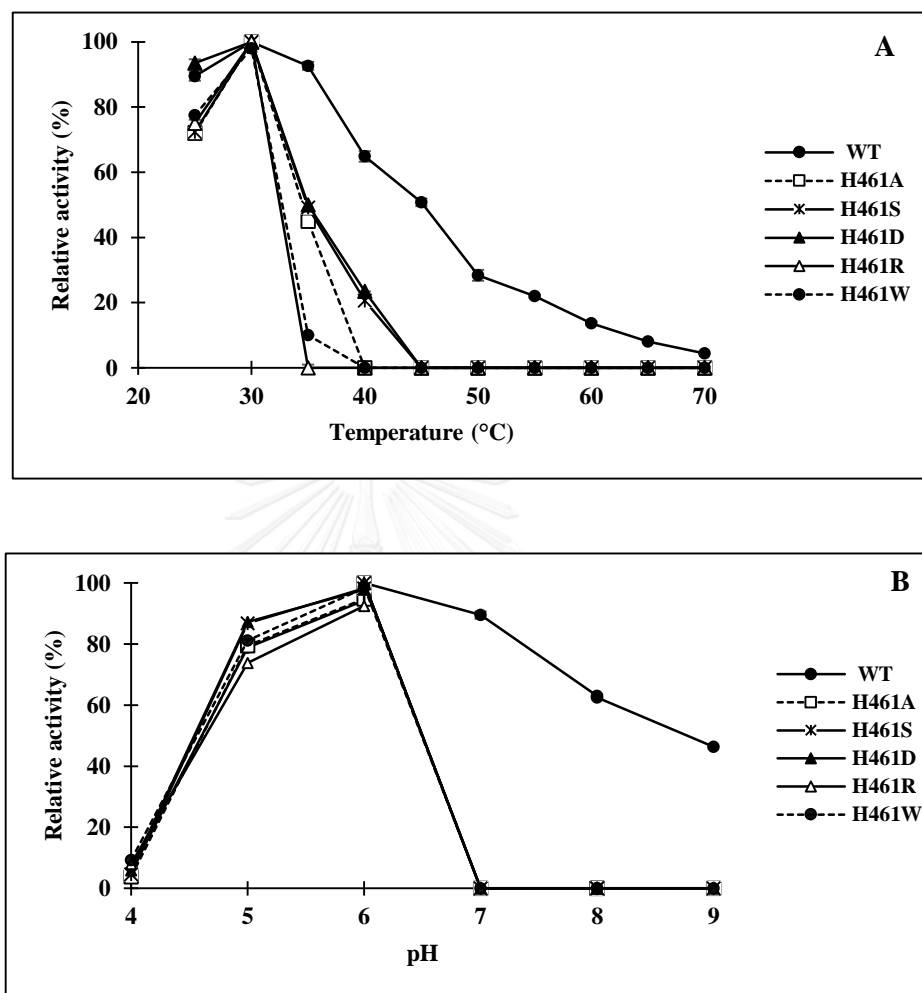
Figure 3. 34 Effect of temperature (A) and pH (B) for WT and H461 mutated *Cg*AMs

on starch transglucosylation activity. The enzymes were fixed at pH 6.0 in (A) and 30°C

(B). Buffer used were: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and

tris-HCl buffer (pH 8.0 -9.0). The experiments were performed as described in section

2. 9. 1 and 2.9.2. Data are shown as the mean ± SD and are derived from three

independent repeats.
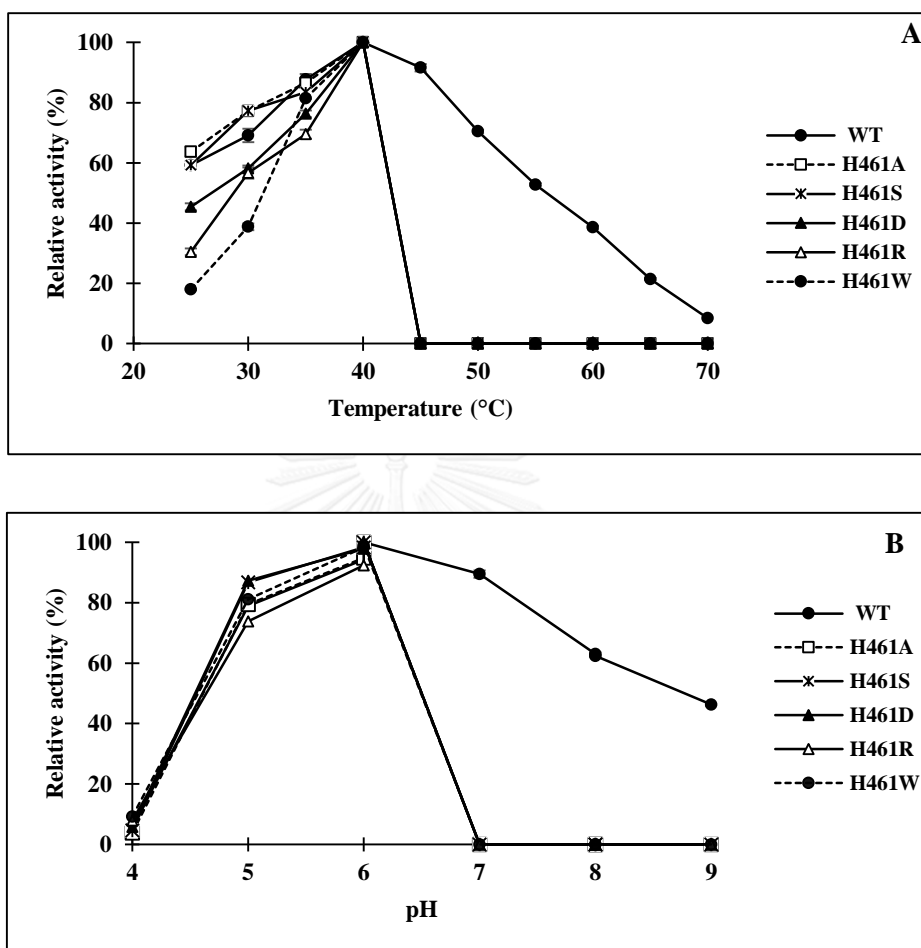
Figure 3. 35 Effect of temperature (A) and pH (B) for WT and H461 mutated *Cg*AMs

on disproportionation activity. The enzymes were fixed at pH 6.0 in (A) and 40°C (B).

Buffer used were: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and tris-

HCl buffer (pH 8.0 -9.0). The experiments were performed as described in section 2.9.1

and 2.9.2. Data are shown as the mean ± SD and are derived from three independent

repeats.

*3.6.2.3 Effect of temperature on Cg*AM *stability*

The effect of H461 mutation on temperature stability was followed by determining disproportionation activity as described in 2.9.3 at different temperature (30 ℃, 35 ℃ and 40 ℃) (Figure 3.36). At 30 ℃, WT enzyme maintained 90% of its activity while H461 mutants lost 50% of their activities after 45 min incubation. When the temperature stability was determined at 35 ℃ and 40 ℃, H461 mutated *Cg*AMs were totally lost their activities at 20 min and 10 min incubation time, respectively. At long incubation time of 180 min at 30 ℃ and 35 ℃, 80% and 60% residual activity of WT, respectively, were observed. The results showed that all H461 mutated *Cg*AMs were significantly less stable than WT enzyme.

*3.6.2.4 Effect of pH on Cg*AM *stability*

The effect of H461 mutation on pH stability was followed by determining disproportionation activity as described in 2.9.4 at pH 7.0 and pH 8.0. The same results were found at both pHs, WT *Cg*AM maintained 90% of its activity while all of H461 mutants totally lost their activities at 20 min incubation time (Figure 3.37). At long incubation time of 120 min at pH 7.0 and 8.0, the WT still showed 90% and 50% residual activity, respectively.

3.6.3 Substrate specificicty

The substrate specificity for disproportionation reaction using malto-oligosaccharide substrate (maltose; G2 to maltoheptaose; G7) was determined. Maltotriose (G3) was the most efficient substrate while maltoheptaose (G7) was poor substrate for WT-*Cg*AM. The substrate specific activity for WT was in order of G3>G4>G5>G6>G2>G7 (Figure 3.38). Interestingly, H461 mutants showed a different

order of G2>G3>G4>G5=G6>G7. It was shown that H461 mutants could use maltose as the best substrate.

3.6.4 Determination of kinetic parameters

Kinetic study of disproportionation reaction

The kinetic analysis of CgAMs was performed using G3, the most favorable substrate in disproportionation reaction of the WT. Figure 3. 39 represented the Lineweaver-Burk plot of CgAMs. All H461mutated CgAMs had a lower k_{cat} and k_{cat}/K_m values, the R mutant showed the lowest k_{cat}/K_m with 36-fold lower than the WT's value (Table 3.12). The mutations were severely affected on the k_{cat}, 14 to 18-fold lower than that of the WT while the K_m values were higher than the WT (18 mM) with the values around 30-37 mM.

3.6.5 Analysis of LR-CD products

To compare the LR-CDs production profile of WT- and H461 mutated CgAMs, the enzymes were incubated with pea starch substrate as described in section 2.9.7 and LR-CD products were determined by HPAEC-PAD. It was found that all H461 mutants could not produce LR-CD products (Figure 3.40).

3.6.6 Enzyme conformation

To investigate the result of H461 mutation on enzyme conformational change, analysis of secondary structure was performed using circular dichroism (CD) spectrometer scanning from 190 to 250 nm. The CD-spectra of all mutated and the WT enzymes were compared at pH 6.0 (Figure 3.41). The result showed that the CD spectra of H461 mutated CgAMs and WT were closely overlaid, only a slight change was observed in the spectra of mutants. The contents of the secondary structure were: α-helix 26-27%, β-sheet 20-22% and random coil 51-52%.

3.7 Structural modeling of H461 mutated *Cg*AM

The model structures of H461 mutated *Cg*AMs were constructed by the Discovery Studio 3.5 Client program using WT *Cg*AM X-ray structure (PDB code 5B68) (Joo *et al*., 2016) as template (Figure 3.42). All *Cg*AM structures were superimposed with acarbose ligand from the *Ta*AM X-ray structure (PDB 1esw) (Przylas *et al*., 2000a) In area of active site, twelve residues including Y23, G412, W425, D460, V462, L463, G464, L465, R467, L468 and E508 are within 5 Å away from H461 position. In the WT and the H461A/D/R/W mutants, no hydrogen bonding was observed between amino acid at position 461 with these residues, but S mutant had one H-bond with D460 (Figure 3.42-C) Interestingly, only WT and H461D mutated *Cg*AMs formed two and one H-bond with acarbose molecule at subsite +1 of the active site (Figure 3.42A and D)
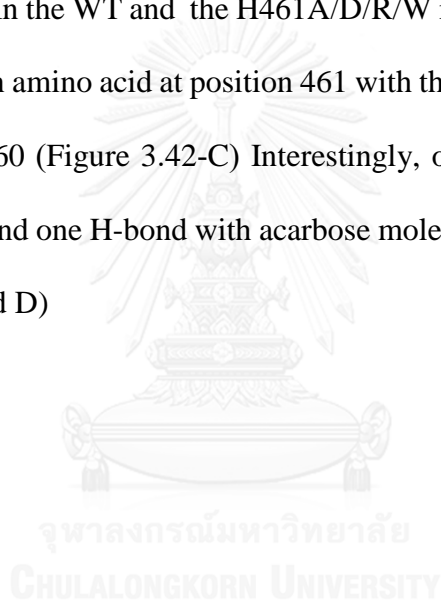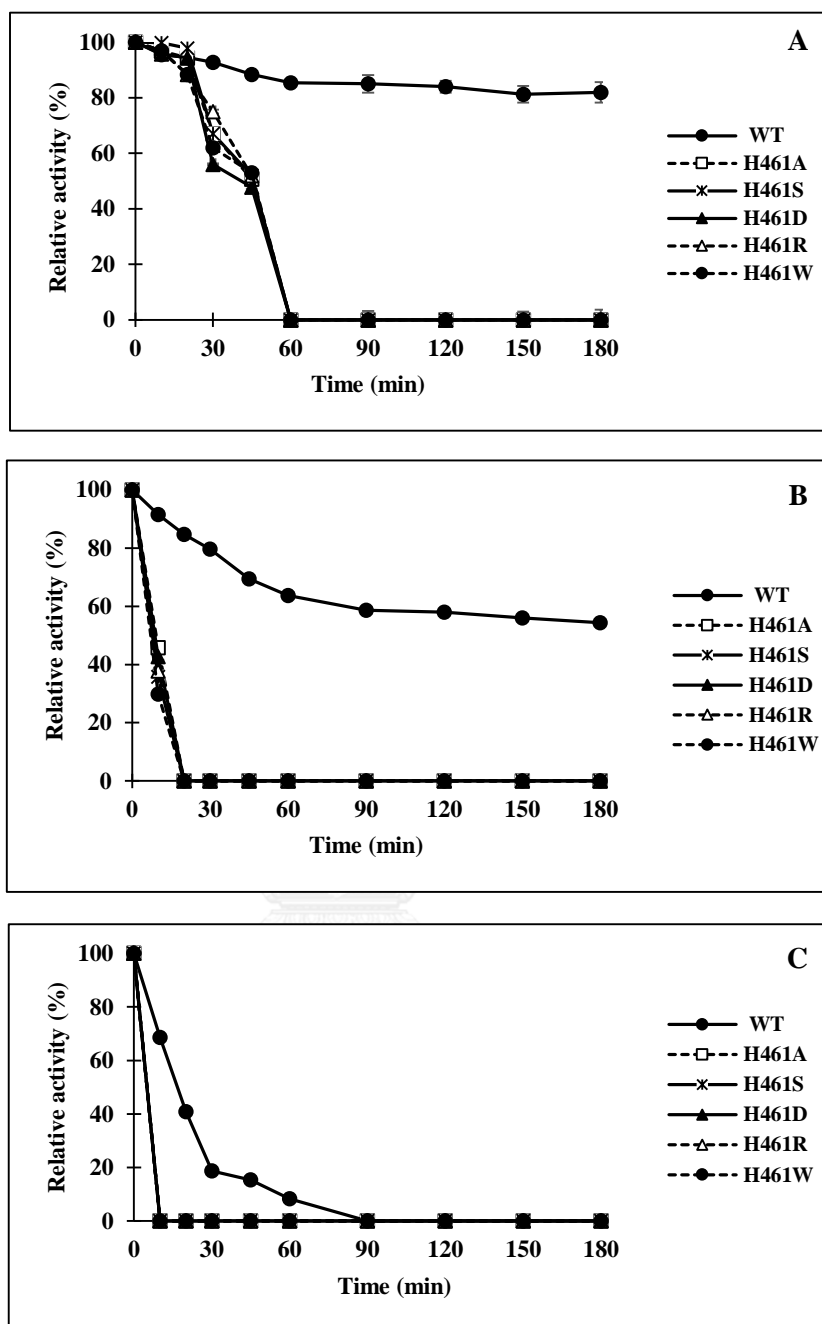
Figure 3. 36 Effect of temperature on *Cg*AM stability. WT and H461 mutated *Cg*AMs were incubated in phosphate buffer pH 6.0 at 30 ℃ (A), 35 ℃ (B), 40 ℃ (C) for 0 to 180 min. Disproportionation activity was assayed as described in 2.9.3. Data are shown as the mean ± SD and are derived from three independent experiments.
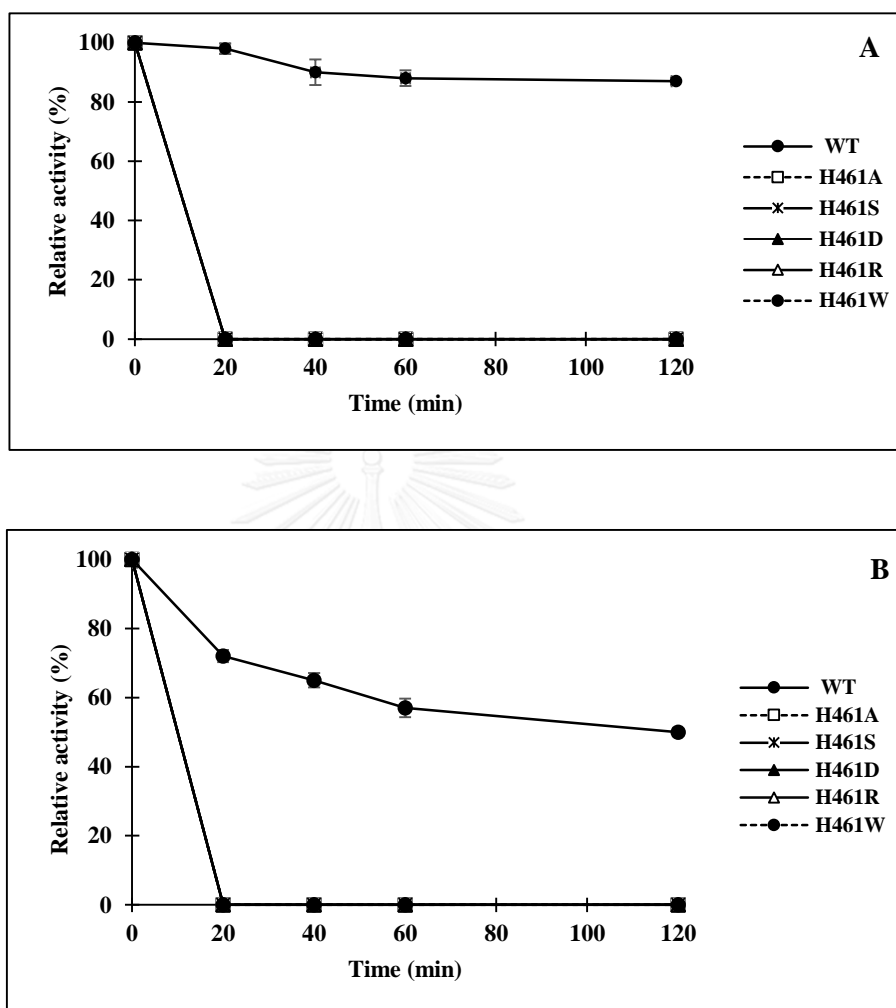
Figure 3. 37 Effect of pH on *Cg*AM stability. WT and H461 mutated *Cg*AMs were incubated at 30ºC in phosphate buffer pH 7.0 (A) and tris-HCl buffer pH 8.0 (B) for 0-120 min. Disproportionation activity was assayed as described in 2.9.4. Data are shown as the mean ± SD and are derived from three independent experiments.
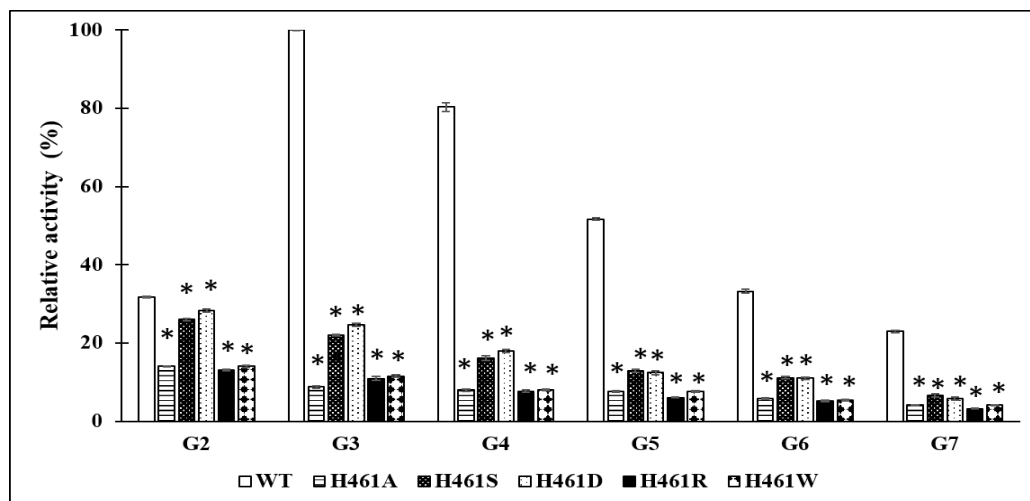
Figure 3. 38 Substrate specificity of WT and H461 mutated CgAMs in dispro-

portionation reaction using malto-oligosaccharides (maltose, G2 to maltoheptaose, G7)

as substrate. The activity of WT-CgAM on G3 substrate was set as 100%. *P < 0.05

(Student's t-Test) with respect to the disproportionation reaction of WT-CgAM. Data

are presented as the mean ± SD and are derived from three independent experiments.
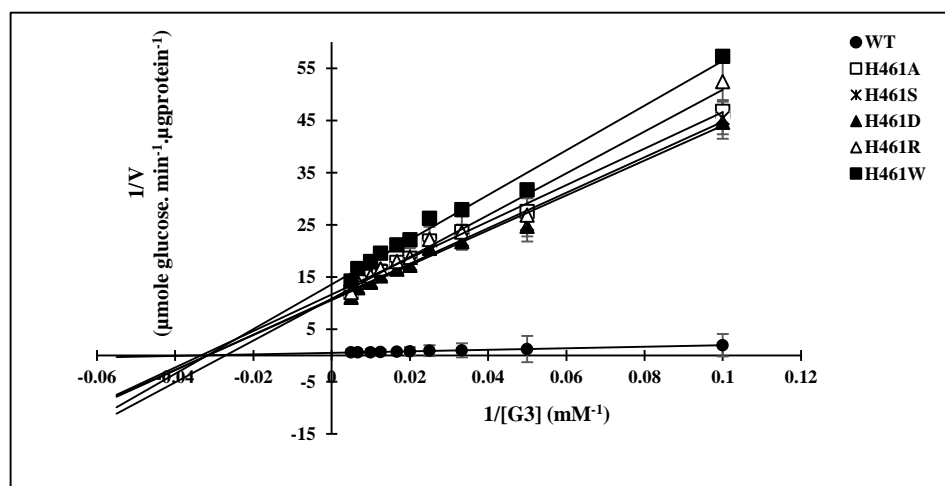
Figure 3. 39 Lineweaver‑Burk plot of WT and H461 mutated *Cg*AMs ‑ catalyzed disproportionation reaction. All *Cg*AMs were incubated with various concentrations of maltotriose (G3) in 50 mM phosphate buffer, pH 6.0 at 40 ℃ for 5 min. Glucose oxidase method was used to determine the amount of free glucose produced during the reaction.

Table 3. 12 Kinetic parameters of WT and H461 mutated *Cg*AMs derived from the disproportionation reaction using maltotriose as the substrate.

*Cg*AM	K_m (mM)	V_{max} (µmole glucose.min^{-1}.µgprotein^{-1})	k_{cat} (min^{-1}) [10^3]	k_{cat}/K_m (mM^{-1}min^{-1}) [10^3]
WT	18.2 ± 1.34	1.98 ± 0.28	157 ± 4.56	8.63 ± 3.45
H461A	29.7 ± 2.45	0.08 ± 0.03	10.2 ± 1.05	0.34 ± 0.25
H461S	31.5 ± 3.26	0.09 ± 0.05	11.0 ± 2.11	0.35 ± 0.31
H461D	32.4 ± 4.55	0.09 ± 0.07	11.2 ± 2.04	0.35 ± 0.18
H461R	36.8 ± 3.27	0.08 ± 0.07	8.94 ± 1.86	0.24 ± 0.17
H461W	33.4 ± 1.28	0.09 ± 0.04	9.32 ± 1.21	0.28 ± 0.19

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
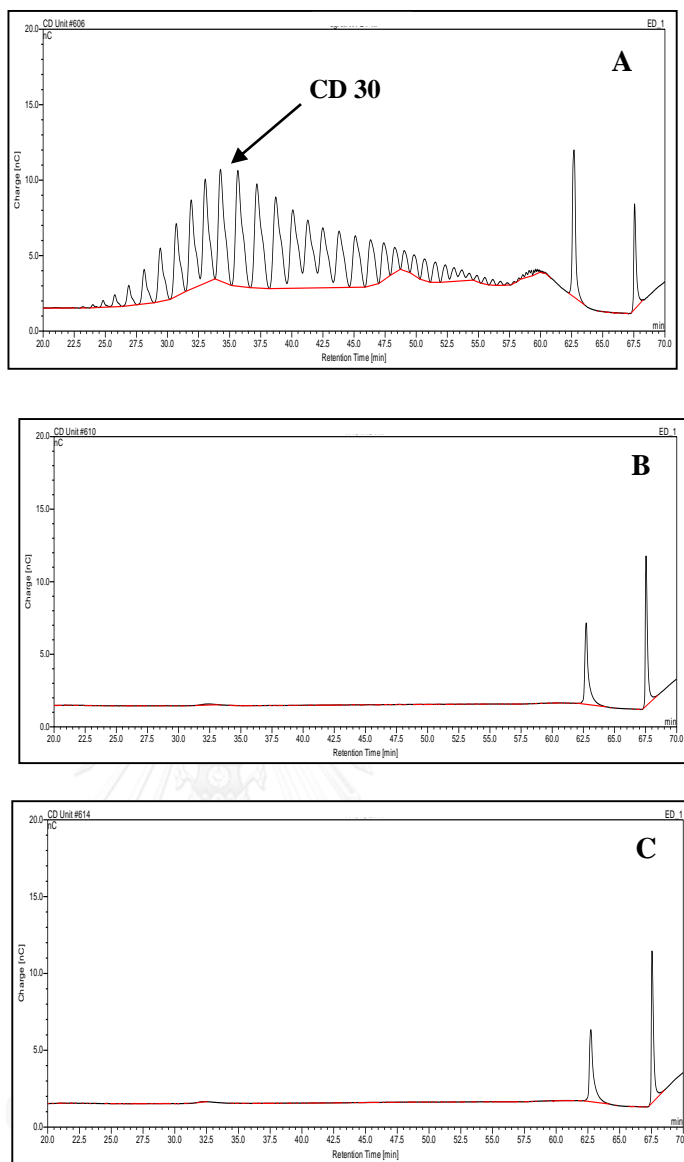
Figure 3. 40 HPAEC-PAD analysis of the LR-CD products synthesized by using 0.2%

pea starch incubated with 0.4 mg protein of enzyme at 30 °C, phosphate buffer pH 6.0

for 90 min. The LR-CD products from WT (A), H461A (B), H461S (C), H461D (D),

H461R (E) and H461W (F) mutated *Cg*AMs, the arrow indicated LR-CD at DP 30.
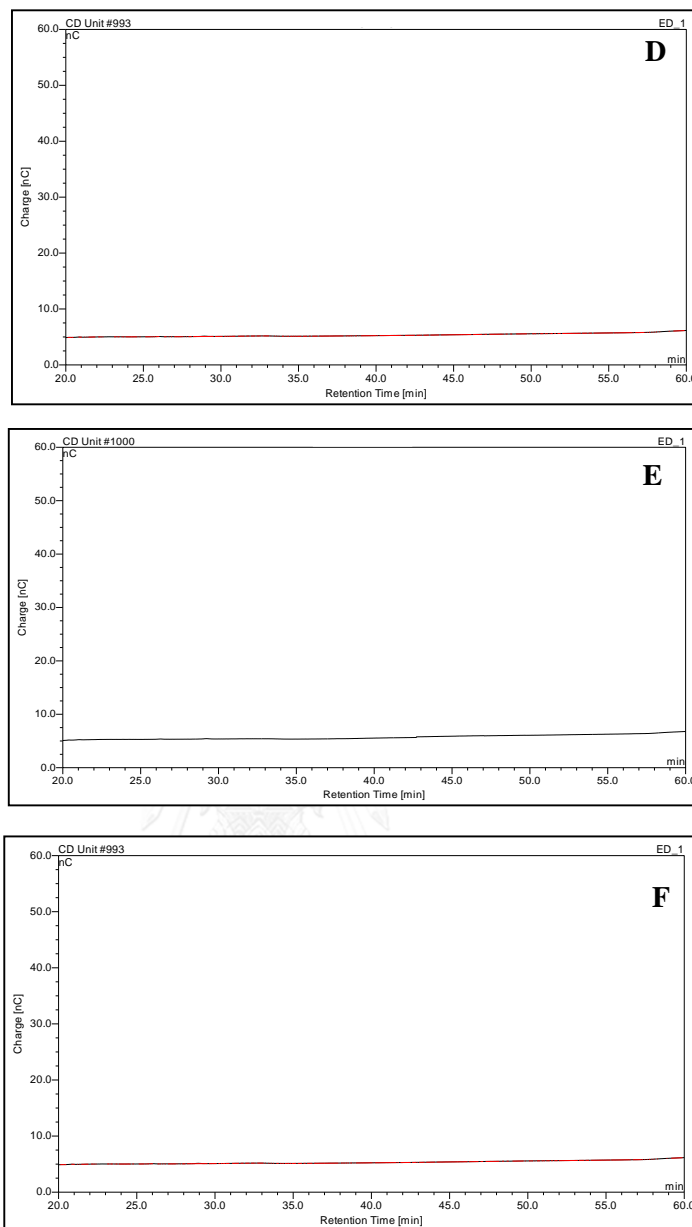
Figure 3.40 (continue) HPAEC-PAD analysis of the LR-CD products synthesized by using 0.2% pea starch incubated with 0.3 mg protein of enzyme at 30 °C, phosphate buffer pH 6.0 for 90 min. The LR-CD products from WT (A), H461A (B), H461S (C), H461D (D), H461R (E) and H461W (F) mutated *Cg*AMs, the arrow indicated LR-CD at DP 30.
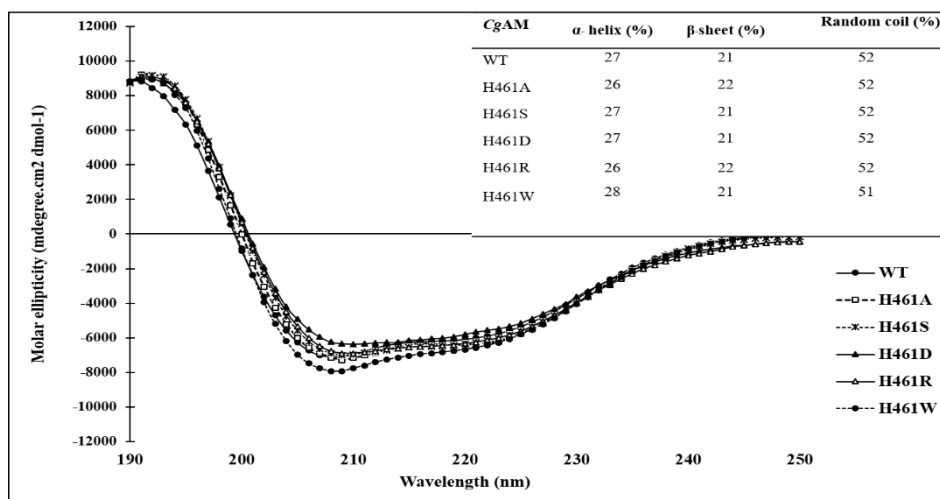
Figure 3. 41 Circular dichroism spectra and the predicted secondary structural compositions of WT and H461 mutated *Cg*AMs.
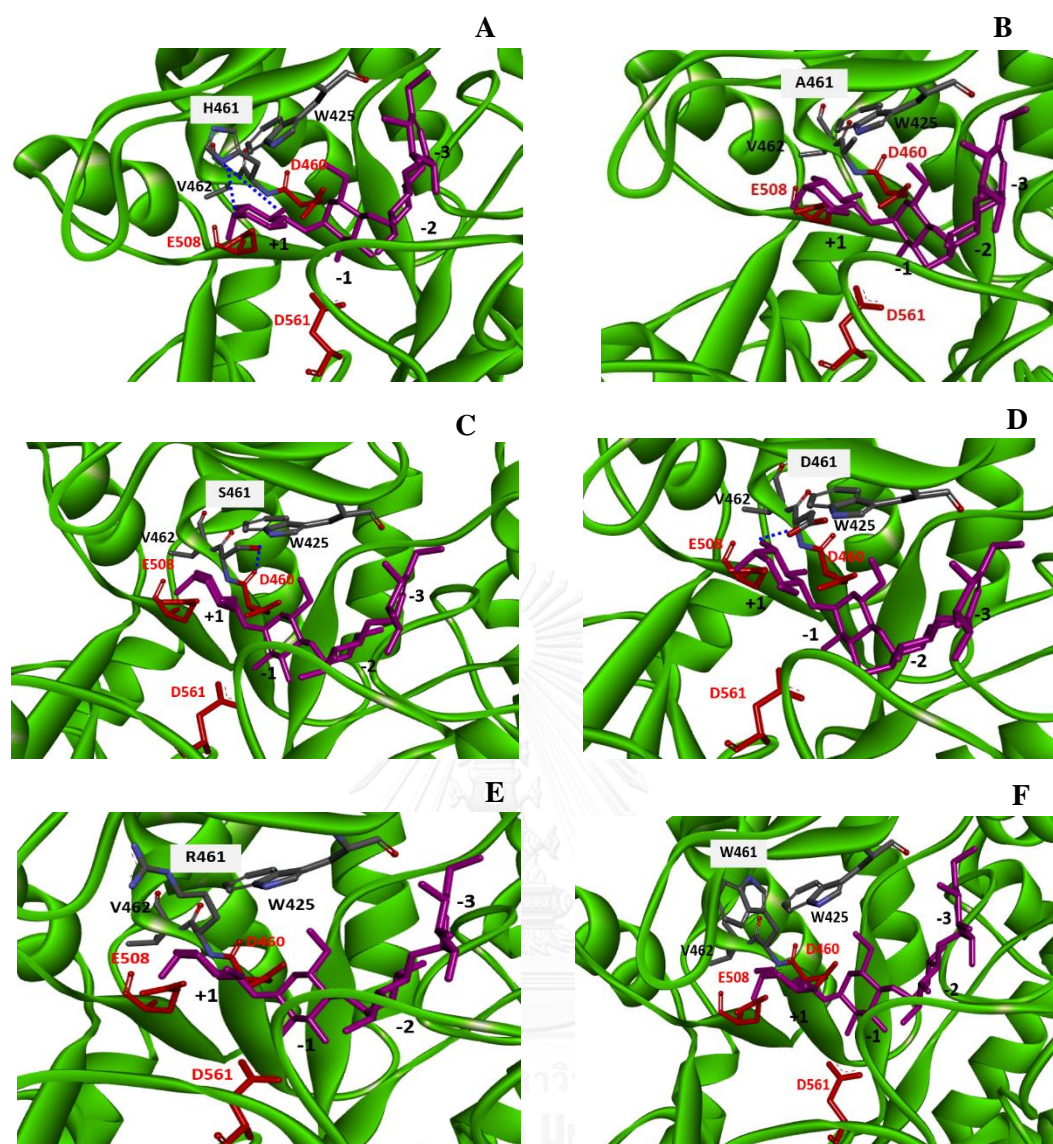
Figure 3. 42 Structures of WT and H461 mutated *Cg*AMs with acarbose (in purple). The X-ray structure of WT *Cg*AM (PDB code 5B68) (Joo *et al.*, 2016) was used as template to prepare the model structures of H461 mutated *Cg*AMs. All *Cg*AM structures were superposed with acarbose ligand bound at active site of the X-ray structure of *T. aquaticus* amylomaltase (PDB 1esw) (Przylas *et al.*, 2000a) Proposed amino acid residues shown are those within 5 Å away from H461 residues of WT (A), H461A (B), H461S (C), H461D (D), H461R (E) and H461W (F) CgAMs, respectively. The three catalytic residues (D460, E508, D561) are represented in red. Hydrogen bonds are shown as dashed blue line.

Expression, Purification and Characterization of N287 mutated *Cg*AMs

3.8 Expression and purification of N287 mutated *Cg*AMs

Overexpression of the major protein band approximately at 84 kDa similar to WT was observed for all five N287 mutated *Cg*AMs (Figure 3.43). The mutated enzymes were thus successfully purified by His-trap column chromatography. The fractions that showed high enzyme activity were pooled and dialyzed as described for Y418 and H461 mutated *Cg*AMs. The specific starch transglucosylation activities of the purified N287S/D/W and N287A/R mutated *Cg*AMs were 4.3-9.0 fold and 14-33 fold lower than that of WT, respectively (Table 3.13). In addition, specific disproportionation activity of all mutated *Cg*AMs was less affected than starch transglucosylation activity, N287S/D/W and N287A/R mutants were 3.0-5.0 fold and 8.0-11 fold lower than WT, respectively (Table 3.14). Interestingly, N287W mutant showed highest specific activity in both reactions than other mutated *Cg*AMs.

3.9 Effect of N287 mutation on enzyme characteristics

3.9.1 Various amylomaltase activities

The effect of N287 mutation on various activities of *Cg*AM was determined, transglucosylation activities of the mutated enzymes including starch transglucosylation and disproportionation were significantly decreased compare to WT enzyme. N287A and R mutants had lowest specific activities of 0.81 and 1.47 U/mg protein for starch transglucosylation activity which was 33 and 19 - fold lower than WT, respectively (Table 3.15). In addition, cyclization activities of all N287 mutated *Cg*AMs were 5.4 to 22 - fold lower than WT, the R mutant showed the lowest specific

activity. Coupling activity was also decreased while hydrolysis activity was not change (Table 3.15).

3.9.2 Optimum conditions and stability

The optimum conditions for disproportionation and cyclization reactions of *Cg*AMs were determined.

3.9.2.1 Effect of temperature on CgAM activity

The optimum temperatures of all N287 mutated *Cg*AMs for disproportionation and cyclization reactions were 40 ℃ and 30 ℃, respectively (Figure 3.44A and 3.45A). The values were the same as those of WT, Y418 and H461 mutated *Cg*AMs. In addition, at temperature higher than 45 ℃, the activities of all N287 mutants were completely lost while WT-*Cg*AM stilled showed 50% of relative activity.

3.9.2.2 Effect of pH on CgAM activity

The optimum pH of all N287 mutated *Cg*AMs for disproportionation and cyclization reactions was the same, at pH 6.0 (Figure 3.44B and 3.45B). The values was the same as those results of WT, Y418 and H461 mutated *Cg*AMs. At pH 7.0, N287R mutant completely lost its activity while N287A/D/S/W lost their activities at pH 8.0. Interestingly, the WT still maintained 40% of relative activity even at pH 9.0.
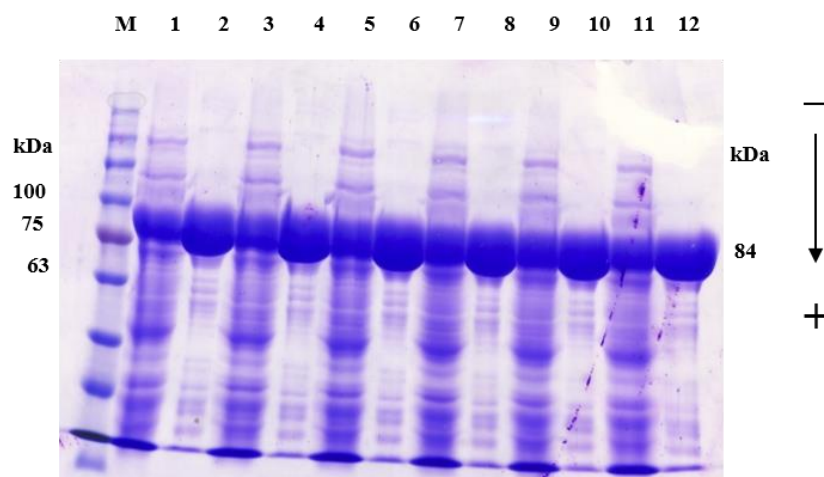
Figure 3. 43 SDS-PAGE of crude and purified enzyme expressed from cells harboring

the series of N287 mutated *Cg*AMs.

Lane M   = Protein markers

Lane 1, 3, 5, 7, 9 and 11   = 20 µg of crude WT-, N287A-, N287S, N287D-, N287R-

                                                and N287W-*Cg*AMs, respectively

Lane 2, 4, 6, 8, 10 and 12 = 10 µg of purified WT-, N287A-, N287S, N287D-,

                                                N287R- and N287W-*Cg*AMs, respectively

Table 3. 13 Purification of WT and N287 mutated *Cg*AMs assayed by starch transglucosylation activity.

*Cg*AM	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
WT					
Crude	295	453	1.54	1	100
Purified	7.86	186	23.6	15.4	41.1
N287A					
Crude	245	123	0.50	1	100
Purified	6.88	4.98	0.72	1.44	4.05
N287S					
Crude	276	176	0.64	1	100
Purified	7.14	29.7	4.16	6.52	16.9
N287D					
Crude	258	217	0.84	1	100
Purified	7.23	18.9	2.61	3.11	8.70
N287R					
Crude	278	114	0.41	1	100
Purified	6.97	12.1	1.74	4.23	10.6
N287W					
Crude	281	185	0.66	1	100
Purified	7.12	36.7	5.15	7.83	19.8

Table 3. 14 Purification of WT and N287 mutated *Cg*AMs assayed by dispro-portionation activity.

*Cg*AM	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
WT					
Crude	295	1748	5.93	1	100
Purified	7.86	223	28.4	4.8	12.8
N287A					
Crude	245	248	1.01	1	100
Purified	6.88	25.8	3.75	3.70	10.4
N287S					
Crude	276	374	1.36	1	100
Purified	7.14	40.3	5.64	4.17	10.8
N287D					
Crude	258	621	2.41	1	100
Purified	7.23	68.4	9.46	3.93	11.0
N287R					
Crude	278	401	1.44	1	100
Purified	6.97	17.5	2.51	1.74	4.4
N287W					
Crude	281	672	2.39	1	100
Purified	7.12	70.3	9.87	4.13	10.5

Table 3. 15 All activities of N287 mutated *Cg*AMs

	Specific activity (U/mg)				
	Starch transglucosylation	**Disprop.**	**Cyclization**	**Coupling**	**Hydrolysis**
WT	26.8 ± 1.52	26.1 ± 2.82	0.43 ± 0.09	0.10 ± 0.04	0.03 ± 0.00
N287A	0.81 ± 0.51	3.51 ± 0.63	0.07 ± 0.03	0.02 ± 0.01	0.02 ± 0.01
N287S	3.04 ± 0.44	5.66 ± 0.54	0.04 ± 0.02	0.04 ± 0.01	0.03 ± 0.00
N287D	2.7 ± 0.64	9.97 ± 1.27	0.10 ± 0.03	0.13 ± 0.01	0.03 ± 0.01
N287R	1.47 ± 0.16	2.92 ± 0.29	0.02 ± 0.03	0.03 ± 0.01	0.02 ± 0.01
N287W	4.87 ± 0.46	8.95 ± 0.2	0.09 ± 0.04	0.03 ± 0.01	0.02 ± 0.00

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
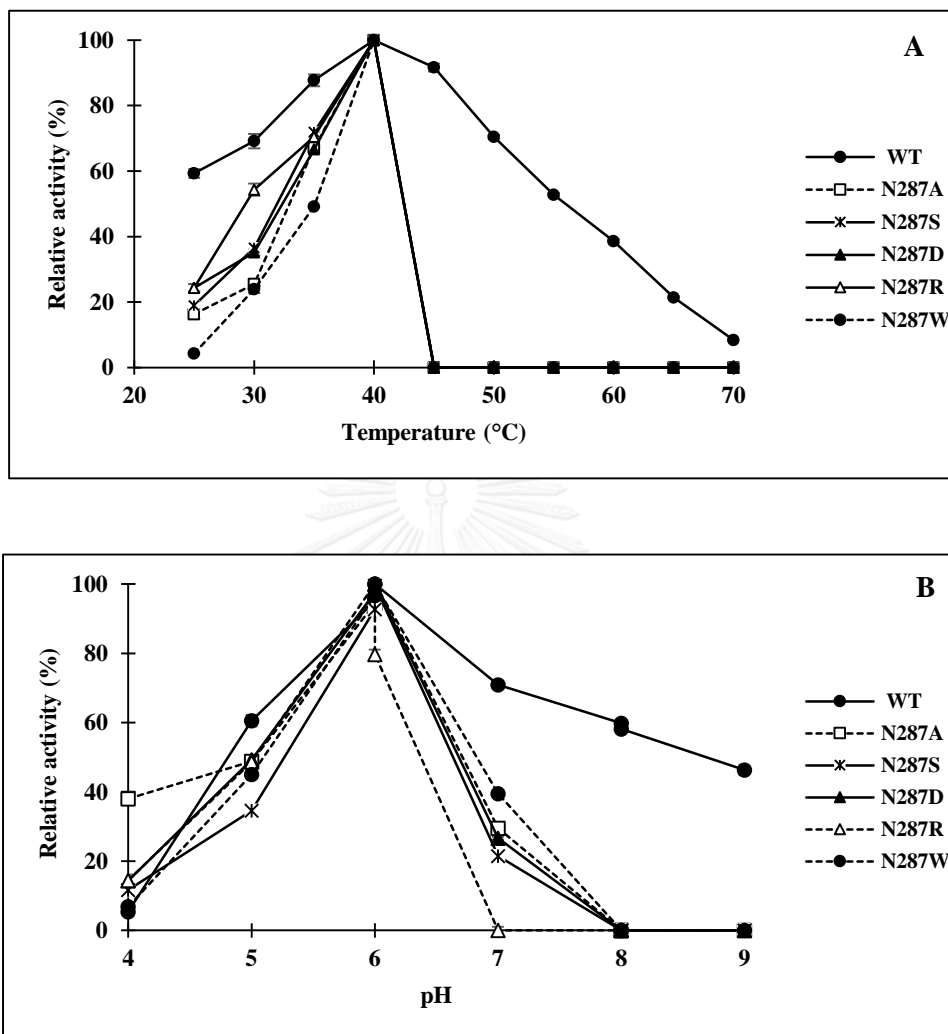
Figure 3. 44 Effect of temperature (A) and pH (B) for WT and N287 mutated *Cg*AMs

on disproportionation activity. The enzymes were fixed at pH 6.0 in (A) and 30°C (B).

Buffer used were: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and tris-

HCl buffer (pH 8.0 -9.0). The experiments were performed as described in section 2.9.1

and 2.9.2. Data are shown as the mean ± SD and are derived from three independent

repeats.
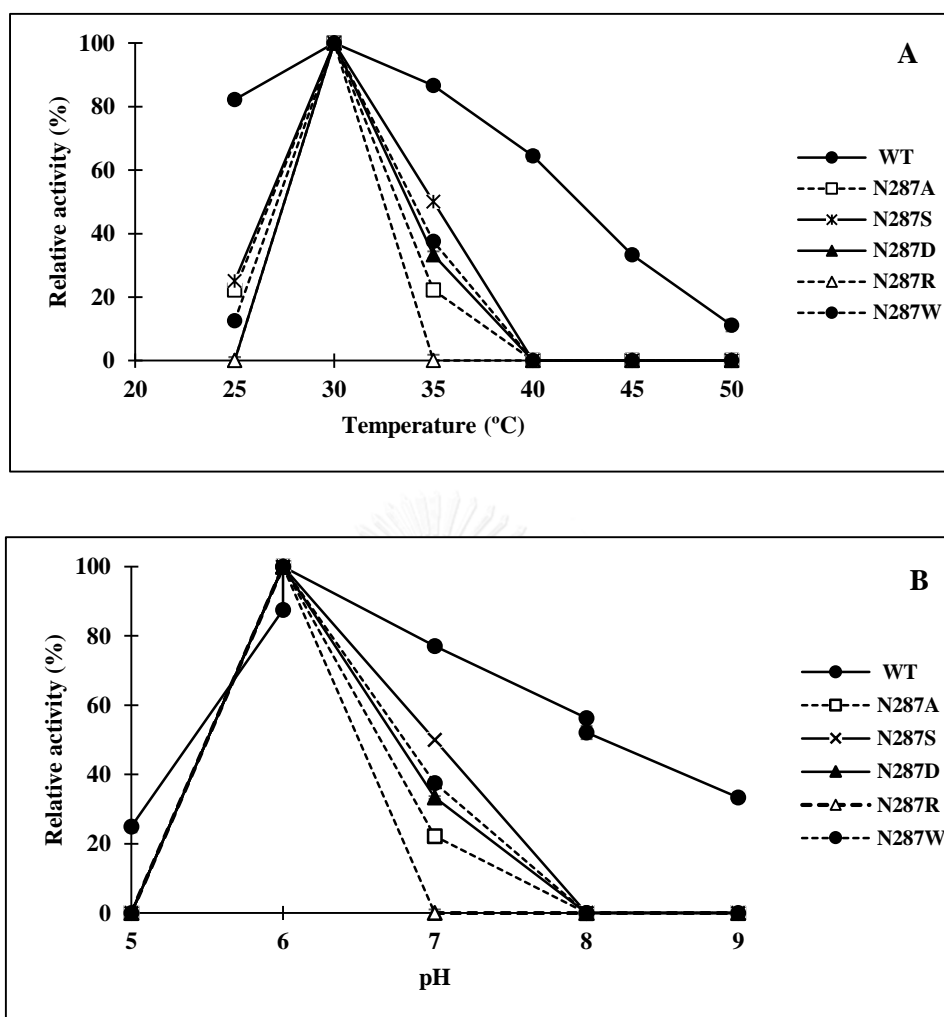
Figure 3. 45 Effect of temperature (A) and pH (B) for WT and N287 mutated *Cg*AMs

on cyclization activity. The enzymes were fixed at pH 6.0 in (A) and 30°C (B). Buffer

used were: acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and tris-HCl

buffer (pH 8.0-9.0). The experiments were performed as described in section 2.9.1 and

2.9.2. WT and N287 mutated *Cg*AMs were incubated with 2% (w/v) of pea starch for

90 min at various temperatures and pHs. LR-CD products were analyzed by HPAEC-

PAD. Data are shown as the mean ± SD and are derived from three independent repeats.

*3.9.2.3 Effect of temperature on Cg*AM *stability*

For the effect of N287 mutation on temperature stability, dispropotionation activity was determined as described in 2.9.3 at different temperature (30 ℃, 35 ℃ and 40 ℃) (Figure 3.46). At 30 ℃, WT enzyme maintained 90% of its activity while all N287mutants showed 50% of their activities at 45 min incubation. All mutated enzymes lost their activities at 90 min while 90% activity of WT still remained up to 180 min (Figure 3.46A). When the temperature stability was determined at 35 ℃, WT enzyme maintained 60% of activity at 180 min while N287 mutants lost 50% of their activities at early incubation time of 20 min. At 40 ℃, activities of WT and N287 mutated *Cg*AMs were totally lost at 90 min and 10 min, respectively. The results showed that N287 mutated *Cg*AMs were significantly less stable than WT enzyme.

*3.9.2.4 Effect of pH on Cg*AM *stability*

The effect of N287 mutation on pH stability was followed by determining dispropotionation activity as described in 2.9.4 at pH 7.0 and pH 8.0. Similar results were found at both pHs, at long incubation time of 120 min, WT *Cg*AM maintained 80% and 50% of its activity at pH 7.0 and 8.0, respectively (Figure 3.47). N287W mutant was more stable than other mutants at 20 min, however, activities of all N287 mutants were totally lost at 40 min incubation time (Figure 3.47).

3.9.3 Substrate specificicty

The substrate specificity for disproportionation reaction using malto-oligosaccharide substrate (maltose; G2 to maltoheptaose; G7) was determined (Figure 3.48). Maltotriose (G3) was the most efficient substrate while maltoheptaose (G7) was poor substrate for both WT and N287 mutated *Cg*AMs. The substrate specific activities

of all enzymes were in the order of G3>G4>G5>G6>G2>G7. The result suggested that N287 residue is not involved in substrate specificity.

3.9.4 Determination of kinetic parameters

3.9.4.1 Kinetic study of disproportionation reaction

The kinetic analysis of CgAMs was performed using G3, the most favorable substrate in disproportionation reaction. Figure 3.49 represented the Lineweaver-Burk plot of CgAMs. All mutated N287 CgAMs had a lower k_{cat} and k_{cat}/K_m values, the R mutants showed the lowest k_{cat}/K_m with 19-fold lower than the WT's value (Table 3.16). The mutation affected more on the k_{cat}, 4.7 to 10 -fold lower than that of the WT while the K_m values were (30-37 mM) higher than WT's value (20 mM).

3.9.4.2 Kinetic study of cyclization reaction

Y418A and Y418W were selected for kinetic study of cyclization reaction compared to WT enzyme as described in section 2.9.6.1. Figure 3.50 represented the Lineweaver-Burk plot of CgAMs. All kinetic parameters were calculated and shown in Table 3.17. The K_m values of N287A and N287W CgAMs were 31.0 and 29.1 mg/ml which were higher than that of WT (19.9 mM), indicating lower affinity towards pea starch which is a substrate. The k_{cat}/K_m of N287A and N287W CgAMs were approximately 2.8-7.8 fold lower than WT's value. Interestingly, N287W showed 2.8 - fold higher catalytic efficiency than N287A.

3.9.5 Analysis of LR - CD products

CgAMs were incubated with 2% (w/v) pea starch at 30 ºC and pH 6.0, the optimum condition of the enzyme. LR-CD mixtures in the reaction were analyzed by HPAEC-PAD as described in section 2.9.7

3.9.5.1 Effect of incubation time

Effect of incubation time on LR-CDs production was carried out as described in section 2.9.7.1. To compare the LR-CDs production patterns of WT and N287 mutated *Cg*AMs, 0.10 U starch degradation activity of *Cg*AM was used and incubation time was varied (1.5, 6, 12 and 24 h). It was clearly found that the LR-CD yields of WT and N287 mutated *Cg*AMs were significantly increased at long incubation time (Figure 3.51). Interestingly, N287 mutated *Cg*AMs showed CD28-CD33 as main CD products while CD32-CD34 were principle products of WT at 24 h. In addition, the amounts of LR-CDs produced by all N287 mutants were significant lower than that of the WT at every incubation time.

3.9.5.2 Effect of unit enzyme

To observe the influence of enzyme concentration on LR-CDs formation, 0.05 U, 0.1 U and 0.2 U starch degradation activities of enzymes were used. The result showed that the increase in enzyme units gave rise to the increase in LR-CD yields. At 6 h, N287 mutated *Cg*AMs showed a shift of the principle CD products to smaller size (CD26-CD29). The yield of CD32 (the maximum product of WT) at 0.2 U enzyme was 2 to 3 - fold increased when compared with using 0.05 U of the same enzyme Figure 3.52.
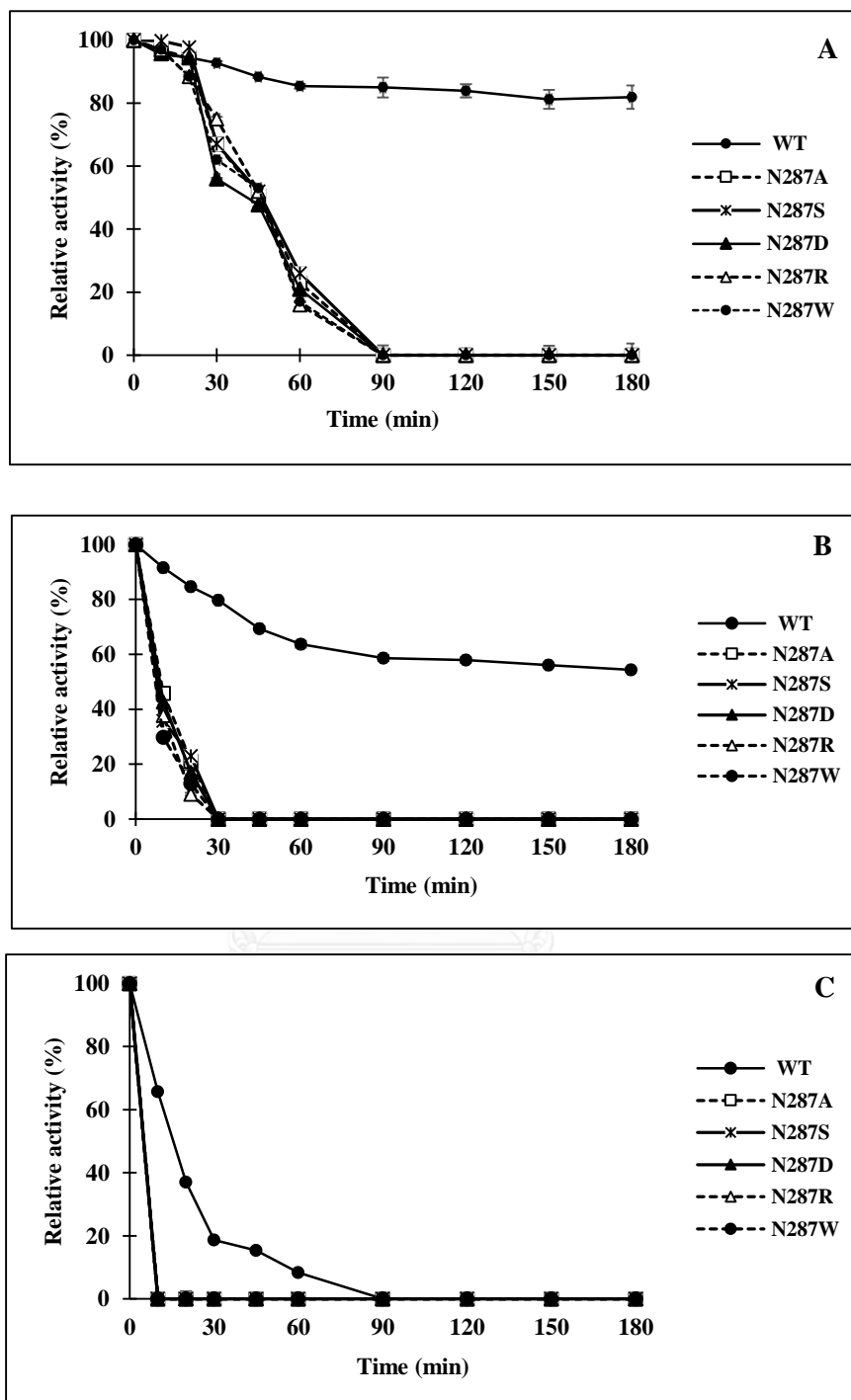
Figure 3. 46 Effect of temperature on *Cg*AM stability. WT and N287 mutated *Cg*AMs were incubated in phosphate buffer pH 6.0 at 30 ºC (A), 35 ºC (B), 40 ºC (C) for 0 to 180 min. Disproportionation activity was assayed as described in 2.9.3. Data are shown as the mean ± SD and are derived from three independent experiments.
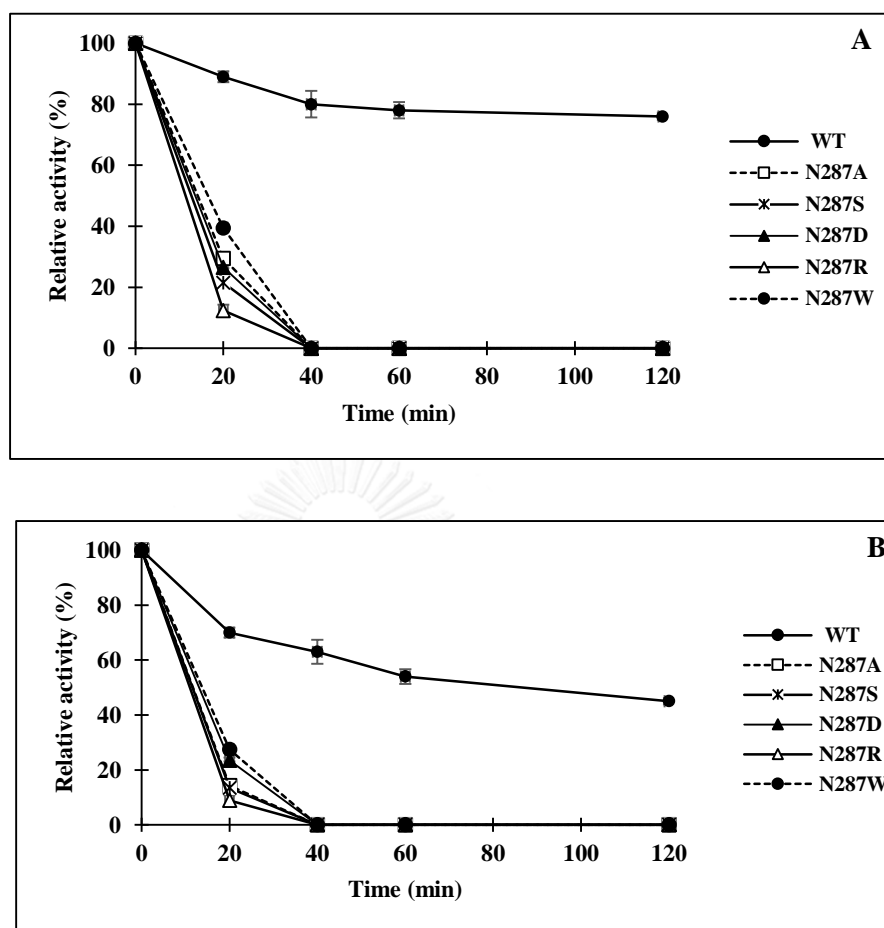
Figure 3. 47 Effect of pH on *Cg*AM stability. WT and N287 mutated *Cg*AMs were incubated at 30 ºC in phosphate buffer pH 7.0 (A) and tris - HCl buffer pH 8.0 (B) for 0- 120 min. Disproportionation activity was assayed as described in 2.9.4. Data are shown as the mean ± SD and are derived from three independent experiments.
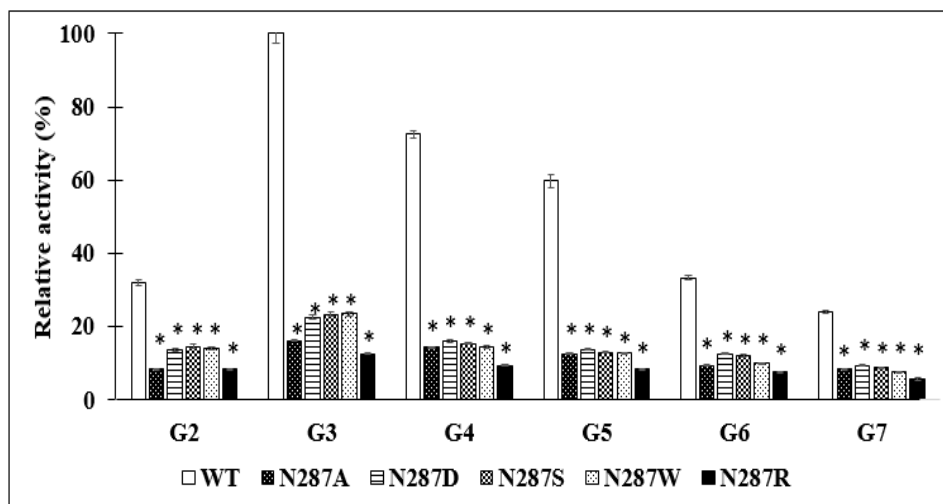
Figure 3. 48 Substrate specificity of WT and N287 mutated *Cg*AMs in dispro-portionation reaction using malto-oligosaccharides (maltose, G2 to maltoheptaose, G7) as substrate. The activity of WT-*Cg*AM on G3 substrate was set as 100%. *P < 0.05 (Student's t-Test) with respect to the disproportionation reaction of WT-*Cg*AM. Data are presented as the mean ± SD and are derived from three independent experiments.
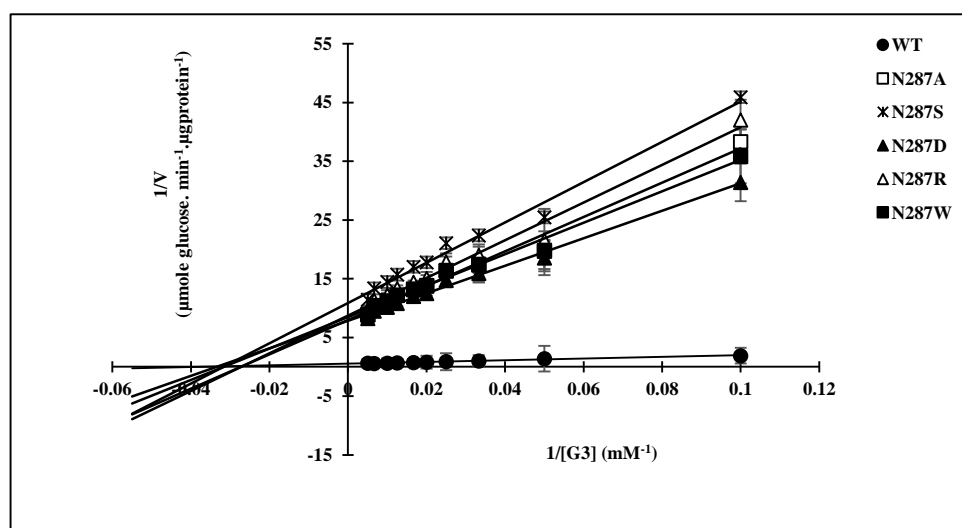
Figure 3. 49 Lineweaver-Burk plot of WT and N287 mutated CgAMs - catalyzed disproportionation reaction. All CgAMs were incubated with various concentrations of maltotriose (G3) in 50 mM phosphate buffer, pH 6.0 at 40 °C for 5 min. Glucose oxidase method was used to determine the amount of free glucose produced during the reaction.

Table 3. 16 Kinetic parameters of WT and N287 mutated CgAMs derived from the disproportionation reaction using maltotriose as the substrate.

CgAM	K_m (mM)	V_{max} (μmole glucose.min^{-1}.μgprotein^{-1})	k_{cat} (min^{-1}) [10^3]	k_{cat}/K_m (mM^{-1}min^{-1}) [10^3]
WT	19.7 ± 1.52	2.05 ± 0.58	182 ± 5.38	9.23 ± 2.76
N287A	35.8 ± 3.21	0.06 ± 0.17	32.7 ± 2.44	0.91 ± 0.31
N287S	35.4 ± 3.52	0.09 ± 0.01	19.3 ± 3.16	0.54 ± 0.23
N287D	29.8 ± 5.21	0.13 ± 0.16	38.5 ± 4.18	1.29 ± 0.21
N287R	37.0 ± 5.77	0.06 ± 0.08	18.2 ± 2.75	0.49 ± 0.27
N287W	32.5 ± 4.27	0.12 ± 0.21	28.5 ± 2.13	0.88 ± 0.12

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
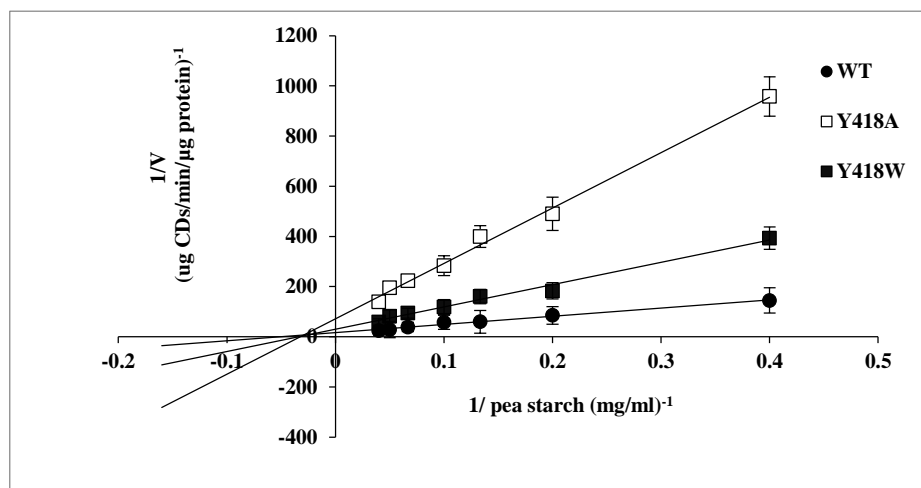
Figure 3. 50 Lineweaver- Burk plot of WT and N287 mutated *Cg*AMs-catalyzed cyclization reaction. *Cg*AMs (0. 05 U starch degrading activity) were incubated with various concentrations of pea starch at 30 ºC for 60 min. LR-CD products were analyzed by HPAEC-PAD.

Table 3. 17 Kinetic parameters of WT and N287 mutated *Cg*AMs derived from cyclization reaction using pea starch as the substrate.

*Cg*AM	K_m (mg/ml)	V_{max} (µg CD.min^{-1}.µgprotein^{-1})	k_{cat} (min^{-1}) [10^3]	k_{cat}/K_m (mg.ml^{-1}min^{-1}) [10^3]
WT	19.9 ± 1.78	0.06 ± 0.13	6.12 ± 1.35	0.31 ± 0.06
N287A	31.0 ± 2.16	0.01 ± 0.06	1.13 ± 1.20	0.04 ± 0.07
N287W	29.1 ± 3.55	0.04 ± 0.02	3.25 ± 0.26	0.11 ± 0.03

Data are shown as the mean ± standard deviation and are derived from three independent experiments.
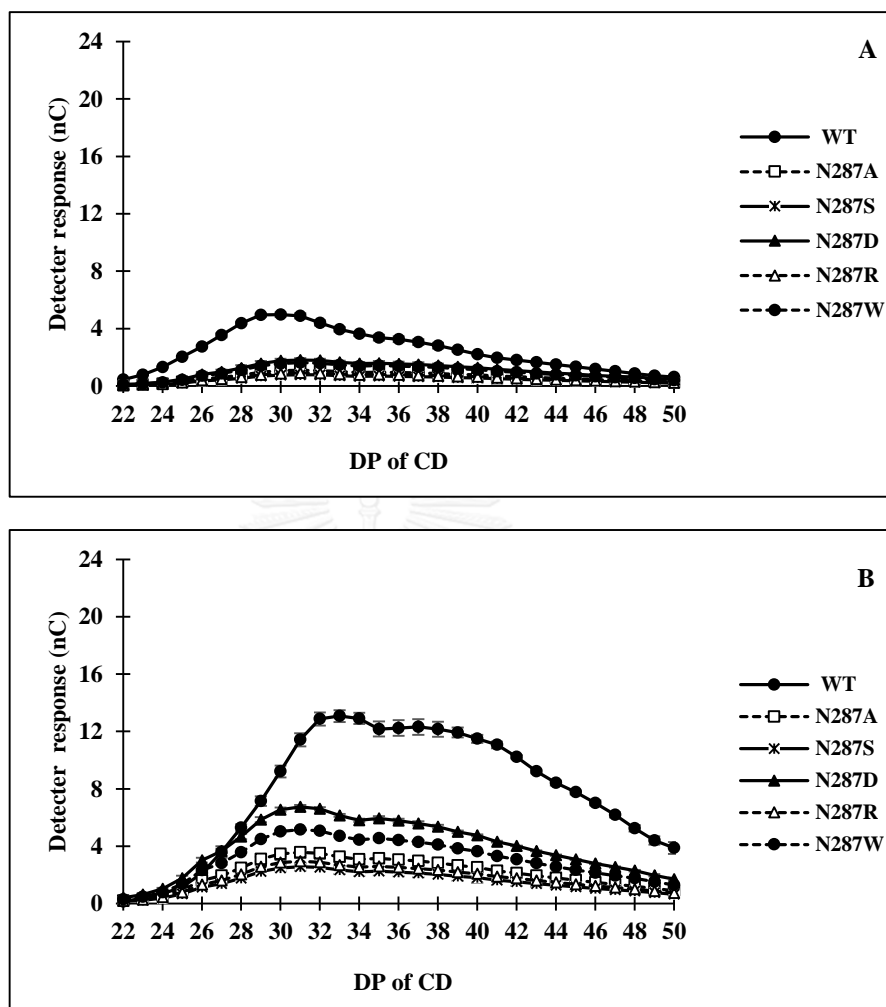
Figure 3. 51 HPAEC- PAD analysis of LR- CD products synthesized at different incubation time 1.5 h (A), 6 h (B), 12 h (C) and 24 h (D) by 0.10 unit of WT and N287 mutated *Cg*AMs.(DP = Degree of polymerization).
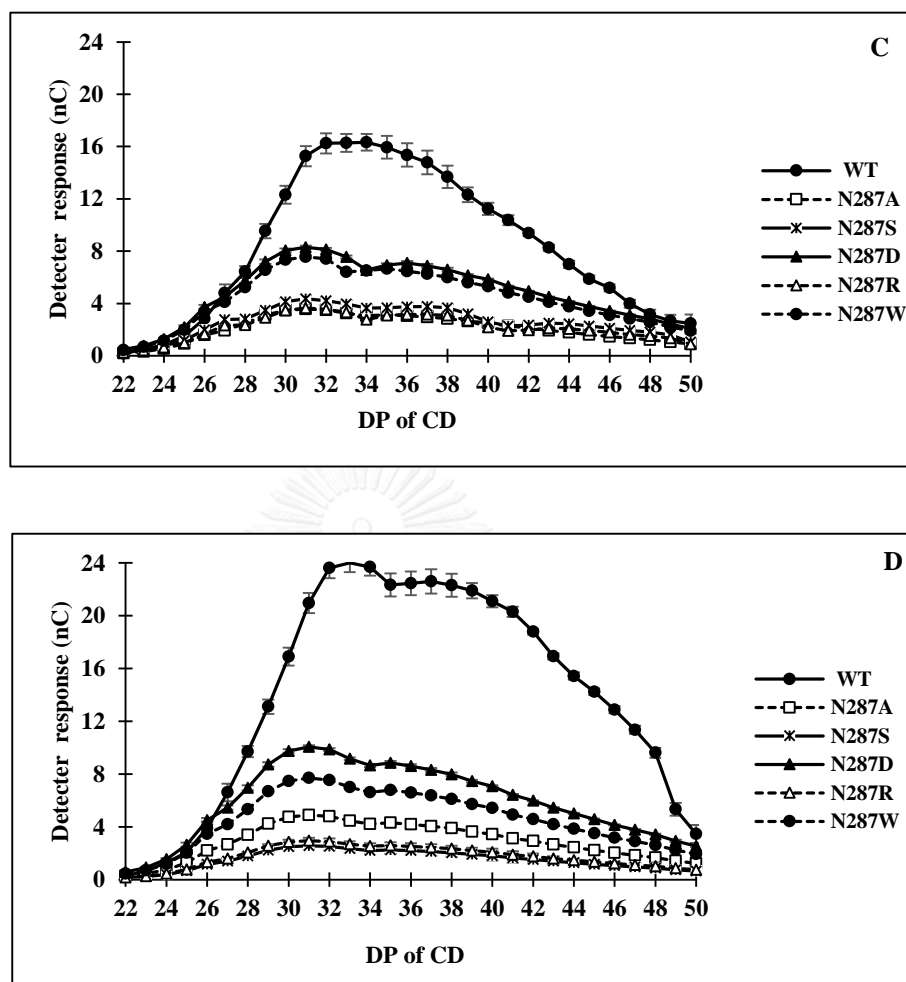
Figure 3.51 (continue) HPAEC-PAD analysis of LR-CD products synthesized at different incubation time 1.5 h (A), 6 h (B), 12 h (C) and 24 h (D) by 0.10 unit WT and N287 mutated *Cg*AMs. (DP = Degree of polymerization).
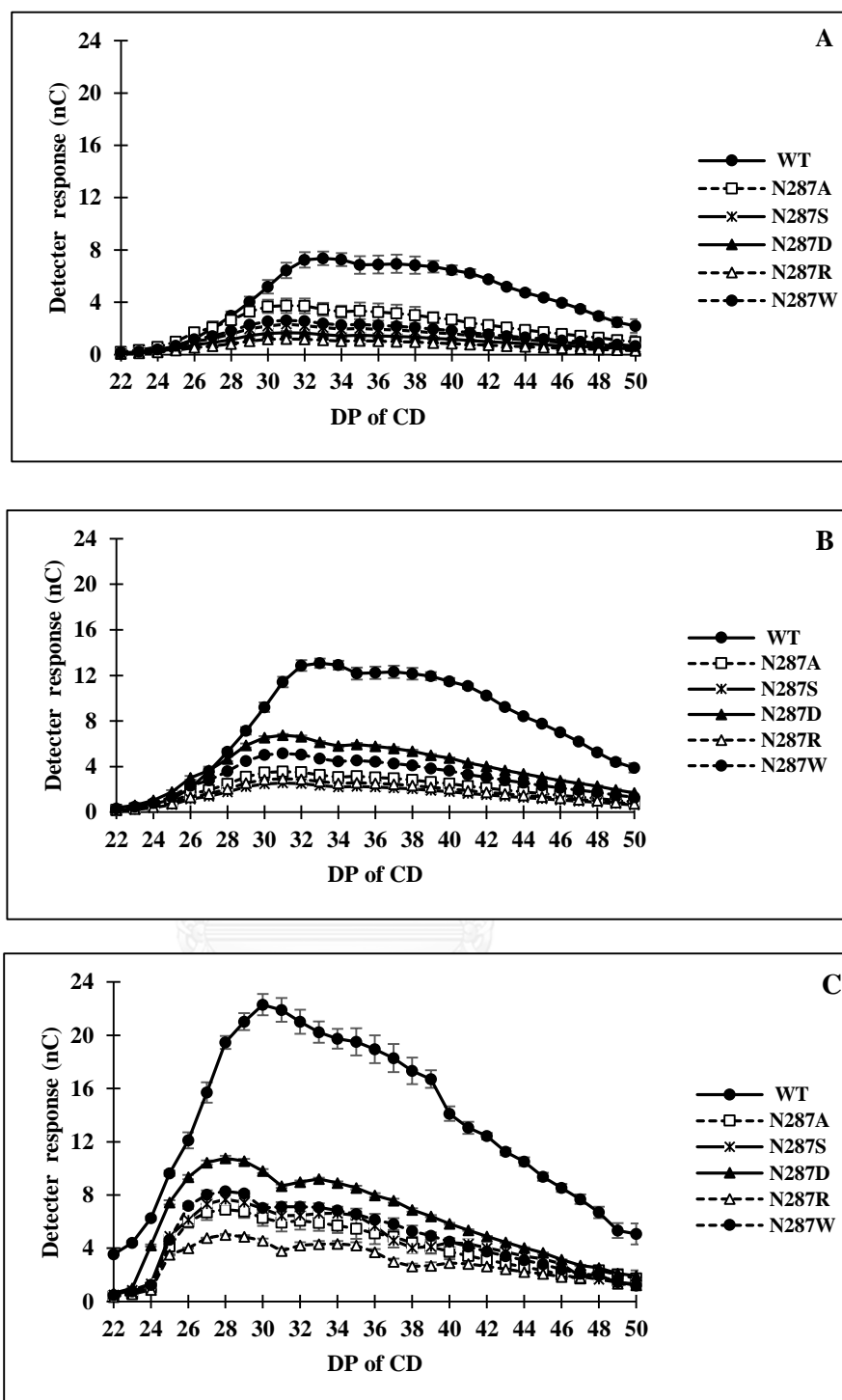
Figure 3. 52 HPAEC-PAD analysis of LR-CD products synthesized for 6 h at different

enzyme unit; 0.05 U (A), 0.10 U (B) and 0.20 U (C) by WT and N287 mutated *Cg*AMs

(DP = 22-50). (DP = Degree of polymerization).

3.9.6 Enzyme conformation

To investigate the effect of N287 mutation on enzyme conformational change, analysis of secondary structure was performed using circular dichroism (CD) as described before. The result showed that the CD spectra of N287 mutated and WT enzymes at pH 6.0 were closely overlaid (Figure 3.53). All mutated CgAMs showed a slight change in CD spectra as compared to the WT. The contents of the secondary structure were: α-helix 26-27%, β-sheet 20-22% and random coil 51-52%.

3.10 Structural modeling of N287 mutated CgAM

The model structures of N287 mutated CgAMs were constructed by the Discovery Studio 3.5 Client program using WT CgAM X-ray structure (PDB code 5B68) (Joo *et al*., 2016) as template. The CgAM model structures were superimposed with acarbose ligand from the TaAM X-ray structure (PDB 1esw) (Przylas *et al*., 2000a) In vicinity of N287 residue, ten residues including E225, P228, P229, T230, D285, R286, D288, V289, Y290 and A291 are within 5 Å away from this position (Figure 3.54). It was observed that amino acid at position 287 could form H-bond with three residues, T230, D285 and Y290 depending on its side chain. We found that all CgAMs had one hydrogen bond between amino acid position 287 with Y290 (Figure 3.54). While only N287D mutant had one hydrogen bond formed with unit C of acarbose (Figure 3.54 D).
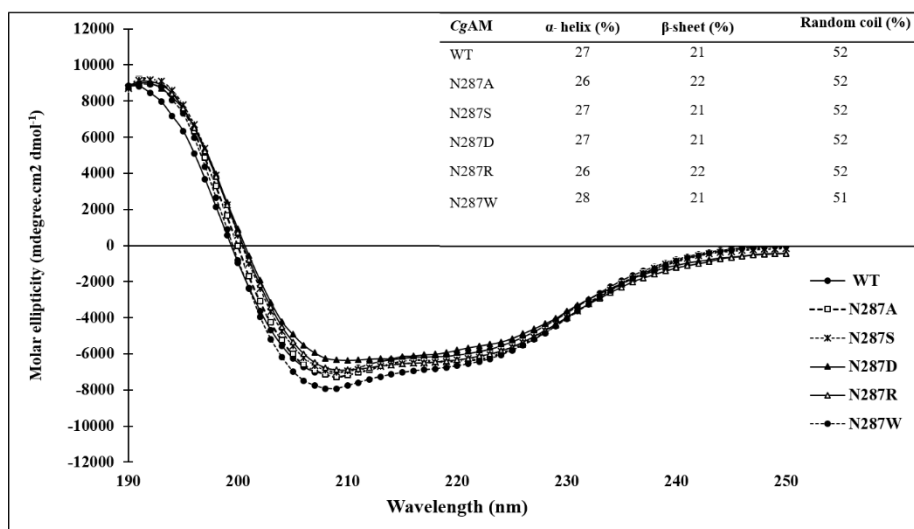
Figure 3. 53 Circular dichroism spectra and the predicted secondary structural compositions of WT and N287 mutated *Cg*AMs.
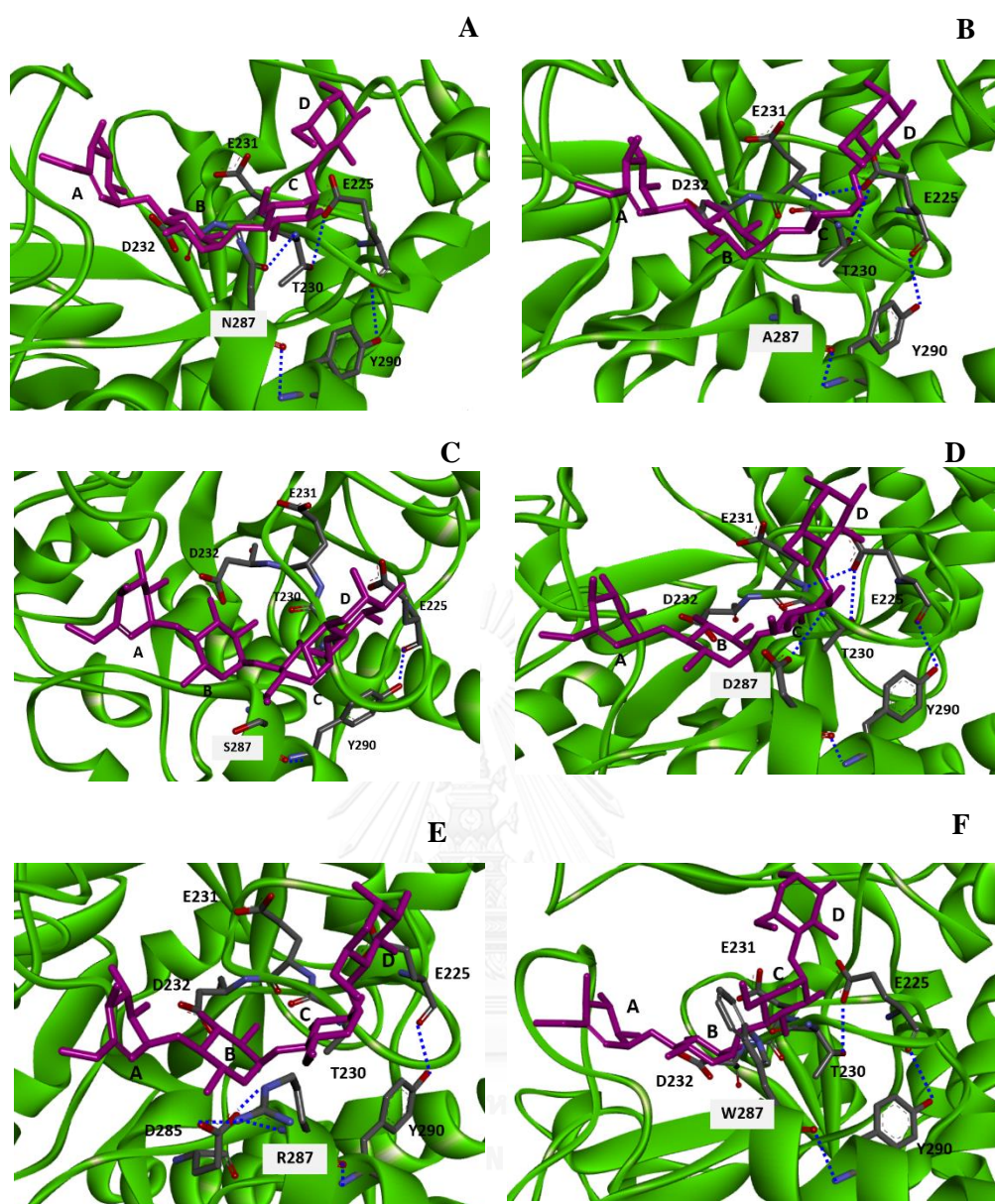
Figure 3. 54 Structures of WT and N287 mutated *Cg*AMs with acarbose (in purple). The X- ray structure of WT *Cg*AM (PDB code 5B68) (Joo *et al*., 2 0 1 6) was used as template to prepare the model structures of N287 mutated *Cg*AMs. All *Cg*AM structures were superposed with acarbose bound at active site of the X-ray structure of *T. aquaticus* amylomaltase (PDB 1esw) (Przylas *et al*., 2000a). Proposed amino acid residues shown are those within 5 Å away from N287 residues of WT (A), N287A (B), N287S (C), N287D (D), N287R (E) and N287W (F) *Cg*AMs, respectively. Hydrogen bonds are shown as dashed blue line.

CHAPTER IV
DISCUSSION

A novel amylomaltase from *C. glutamicum* (*Cg*AM) was previously cloned with the open reading frame (ORF) of 2,121 bps which could be deduced to 706 amino acids, and expressed in *E. coli* BL21(DE3) by our research group (Srisimarat *et al*., 2011). *Cg*AM is larger in size and has a low amino acid sequence identity (about 26% identity) to those well characterized AMs from *Thermus* sp. The X-ray crystal structure of *Cg*AM has been solved by our group but not yet published, then in 2016 the Korean group deposited the structure in Protein database as PDB code 5B68 (Joo *et al*., 2016). *Cg*AM has additional N-terminal domain which was not present in *Thermus* amylomaltases (Joo *et al*., 2016; Przylas *et al*., 2000b).

In recent years, site-directed mutagenesis of *Cg*AM has been previously studied by our group to investigate functional roles of different residues for the understanding of catalytic mechanism and enzyme improvement. We target residues involved with substrate binding. Y172 and N287 in the proposed second glucan binding site were shown to control transglucosylation activities and LR-CD product profile (Nimpiboon *et al*., 2016b; Srisimarat *et al*., 2012). N287Y showed an increase in thermostability as well as A406V/L (Nimpiboon *et al*., 2016a; Nimpiboon *et al*., 2016b). W425 at substrate-binding edge, next to 410s loop is proved to be essential for substrate binding, total loss of cyclization activity was observed in W425A suggesting the importance of hydrophobicity around here (Rachadech *et al*., 2015). In the present study, three residues of *Cg*AMs (Y418, H461 and N287) were our target for mutation to explore

their functions. We here described the construction of mutated *CgAMs* gene by site-directed mutagenesis, sequencing to identify the site of mutation, investigating the functions of target residues by enzyme characterization, comparing the properties of the mutated *Cg*AMs to that of the WT enzyme and construction of the model structures of mutated *Cg*AMs based on the WT structure in order to explain the *Cg*AM function.

4.1 Construction of mutated amylomaltase genes from *C. glutamicum*

4.1.1 Selection of target amino acid residues of *Cg*AM

In this work, amino acid sequence alignment and superimposed structure of *Cg*AM (PDB code 5b68) with that of *Ta*AM (PDB code 1CWY), a well-characterized AM, were compared. Three amino acid residues consisted of F251, H294 and Y101 of *Ta*AM were selected as target residues for study. F251 which is in the 250s loop of *Ta*AM and all *Thermus* AMs were previously reported as important residue for substrate recognition and transglucosylation activity from the result of site-directed mutagenesis study in AM from *T. brockianus* (*Tb*AM) (Jung *et al*., 2011). H294 has no evidence on its function but it was proposed to be important for binding with substrate in *T. thermophilus* amylomaltase (*Tt*AM) (Kaper *et al*., 2007). While Y101 which is in the second glucan binding site was reported to be involved in cyclization activity of amylomaltase as evidenced from site-directed mutagenesis study in *Ta*AM (Fujii *et al*., 2005; Fujii *et al*., 2007).

From our amino acid sequence alignments, the results showed that F251, H294 and Y101 of *Ta*AM are corresponded to Y418, H461 and Y290 of *Cg*AM. The same results were found when the X-ray structures were superimposed except in the third residue (Y101). The multiple sequence alignment showed that Y101 is not conserved

in all species of AM, though in *Cg*AM, this residue seems to be aligned with Y290 (Figure 3.1). By superimposing the X-ray structure of *Cg*AM solved by our group (unpublished structures)(Srisimarat *et al*., 2013) with that of *Ta*AM, N287 rather than Y290 of *Cg*AM has closed interaction with acarbose then was closer to Y101 of *Ta*AM (Figure 3.2). We then decided to investigate the roles of Y418, H461 and N287 of *Cg*AM in structure-function relationship of this enzyme.

4.1.2 Mutation of *CgAM* gene using site-directed mutagenesis

Site-directed mutagenesis is a common technique used for mutagenesis in the attempt to study the importance of amino acid residues on the structure and the function of protein or enzyme. The mutated primers were designed based on *E. coli* codon usage, to anneal with target site of gene then PCR amplification was performed. Substitutions at three residues (Y418, H461 and N287) by six residues representing different amino acid groups as indicated in Result section 3.1.3 were performed.

The series of Y418 (Y418A/S/D/R/W/F), H461 (H461A/S/D/R/W) and N287 (N287A/S/D/R/W) mutated *CgAM* genes were obtained and sequenced to confirm mutation sites (Figure 3.4-3.8). The involvement of Y418, H461 and N287 on *Cg*AM characteristics was explored. From the previous study, we found only one mutation study at F251 (in 250s loop, the unique loop lies over the enzyme active site, see Figure 4.1) of *Thermus* AMs which is corresponding to Y418 (in 410s loop) of *Cg*AM. Site-directed mutagenesis of loop tip residue F251 together with Q256 and W258 in *T. brockianus* AM (*Tb*AM) was reported. The decrease in transglucosylation activity, the changes in substrate affinity and specificity were reported in F251G, Q256G and W258G mutated *Tb*AM (Jung *et al*., 2011). For H461 of *Cg*AM, this residue is corresponded to H294 of *Thermus* AMs of which no previous report on mutagenesis

was found. From amino acid sequence alignment and superimposed structures of *Cg*AM and *Ta*AM, these histidine (H) residues were in the active site area, near the catalytic residues of the two enzymes (Figure 3.1). In the model structure of *Tt*AM, H294 forms two hydrogen bonding with glucose residue (at subsite +1) of G7 substrate (Figure 4.1), thus the role in substrate binding was suggested.

For the third residue of interest which is corresponded to N287 of *Cg*AM, Y101 located close to glucose unit C in the 2nd glucan binding site of *Ta*AM (Figure 4.2) was reported to affect cyclization activity (Y101G caused higher cyclization activity) (Fujii *et al*., 2007). Recent study by our group showed the increase in thermostability, the change in substrate specificity for disproportionation reaction, and a significant decrease in the three main activities of *Cg*AM, (starch transglucosylation, disproportionation and cyclization) when substituted N287 of *Cg*AM with Y (Nimpiboon *et al*., 2016b).

4.2 Expression and purification of the recombinant WT and mutated *Cg*AMs

E. coli has been commonly used as host cell for AMs expression, gene expression was induced by IPTG. All expressed AMs are intracellular enzyme as previously reported (Kaewpathomsri *et al*., 2015; Kaper *et al*., 2007; Srisimarat *et al*., 2011). The recombinant WT, Y418, H461 and N287 mutated *CgAM* genes were expressed in *E. coli* BL21(DE3) using the expression vector pET-19b and 0.4 mM IPTG, cells were cultivated at 16 °C for 20 h. The results revealed that all *Cg*AMs showed higher expression as soluble protein (Figure 3.17, 3.33 and 3.43). The recombinant WT and mutated *CgAM* genes in *E. coli* BL21 (DE3) containing pET-19b harboring *CgAM* genes were expressed as enzymes containing his-tag residues at the

N-terminus. All enzymes were successfully purified by HisTrap FF^{TM} affinity. Specific activities and yields of all mutated CgAMs were decreased to a different extent (Table 3.1, 3.10 and 3.13) depending on mutated position and activity assay.

The molecular weight of the WT, and all mutated CgAMs containing his-tag residues was 84 kDa (Figure 3.17, 3.33 and 3.43) as similar to the previous study (Srisimarat *et al*., 2011), thus the mutations did not affect the size of AM. When compared to other AMs, the size of CgAM was close to those of 4αGTase from *Thermococcus litoralis* (79 kDa) (Xavier *et al*., 1999) and AM from *E. coli*, *Ec*AM (78.5 kDa) (Weiss *et al*., 2015) but different from *Ta*AM (57 kDa) (Terada *et al*., 1999), *Synecocystis sp*. PCC6803 (57 kDa) (Lee *et al*., 2009) and AM from *T. filiformis* JCM 11600 (55 kDa) (Kaewpathomsri *et al*., 2015), as well as plant D-enzyme from Cassava and *Arabidopsis sp*. (59 kDa) (Tantanarat *et al*., 2014). AMs from mesophilic bacteria (*E. coli* and *C. glutamicum*) is larger in size than AMs from thermophilic bacteria and plant D-enzymes.

4.3 Characterization of mutated CgAMs

4.3.1 Various activities of amylomaltase

The effect of Y418, H461 and N287 mutations on various activities of CgAM was investigated, all mutations caused a significant decrease in enzyme activities; both the inter- and intra-molecular transglucosylation reactions were affected. No change in optimum conditions of CgAM was observed upon these mutations. Optimum temperatures and pH of WT and all mutated CgAMs were: 40 ℃, pH 6.0 for disproportionation reaction (Figure 3.18, 3.35, 3.44); and 30 ℃, pH 6.0 for cyclization reaction (values for H461 could not be determined since no cyclization activity could

be detected) (Figure 3.19, 3.45). These values are in accordance with previous reports from the WT (Nimpiboon *et al*., 2016b; Srisimarat et al., 2011).

For Y418 mutated *Cg*AMs, a significant decrease in the three main activities of AM: starch transglucosylation, disproportionation and especially cyclization was observed (Table 3.3). Our result agrees well with the mutation study of the corresponding residue F251 which showed the decrease in the three main activities when substituted by glycine (G) (Jung *et al*., 2011) in *Tb*AM or insertion of the two serines closed by (+SY^{250}F^{251}+S) in *Ta*AM (Roth *et al*., 2017). The reduction in *Cg*AM activities observed can be explained by the inability of the 410s loop to adopt the proper conformation of the enzyme during substrate binding. We propose that hydrophobic interaction is required here and Y418 contributes to suitable conformation flexibility of 410s loop to enable highest catalytic activity of *Cg*AM. The hydrophobicity of 410s loop (with Y418) in *Cg*AM is different from that of 250s loop (with F251) in *Thermus* AM (Joo *et al*., 2016; Jung *et al*., 2011). Interestingly, *Ec*AM had L406 in 400s loop (Weiss *et al*., 2015) as corresponding residue to Y418. Thus hydrophobic environment at this loop tip position of AMs is important, the residues may either be aromatic Y/F or aliphatic L depending on the enzyme. These differences covering the extended edge of substrate-binding site might make each AM differ in recognizing long polysaccharide substrate (Joo *et al*., 2016).

H461 mutations resulted in nearly total loss of all transglucosylation activities. Cyclization activity of H461 mutated *Cg*AMs could not be detected by HPAEC-PAD while hydrolysis activity was not affected (Table 3.11). From multiple sequence alignment, we found that histidine (H) residue in active site area is highly conserved among AMs. And from our proposed modeling structure of *Cg*AM, H461 is very close

to the two catalytic residues (D460 and E508) inside the active pocket of the enzyme (Figure 3.42-A). *Kaper et al*, 2007 reported the model structure of substrate binding sites in the active site area of amylomaltase from *T. thermophilus* HB8, seven subsites forming H-bonds with maltoligosaccharide substrate were proposed. H294 of *Tt*AM found close to catalytic residues (E340 and D249) showed two hydrogen bonding interactions at subsite +1 with maltoheptaose (G7) (Figure 4.1). Due to H461 of *Cg*AM is corresponded to H294 of *Thermus sp.* amylomaltase, which is proposed to be important in substrate binding, we proposed here that H461 in *Cg*AM is an essential residue for enzyme catalysis.

Mutation at the third residue of interest, N287, resulted in a dramatic decrease in all transglucosylation activities but not hydrolysis (Table 3.15). This result was similar to previous work from our group on N287Y mutated *Cg*AM which reported that the transglucosylation activities (starch transglucosylation, disproportionation and cyclization) were diminished (Nimpiboon *et al*., 2016b). N287 of *Cg*AM is corresponded to Y101 of *Ta*AM which locates in the secondary binding site of enzyme (Fujii *et al*., 2007). Y101G mutated *Ta*AM was also reported to affect cyclization and disproportionation activities of the enzyme (Fujii *et al*., 2007; Roth *et al*., 2017) Further explanation that relates how Y418/H461/N287 mutations affect structural change in vicinity of target residues leading to changes in *Cg*AM activity is discussed in 4.4.

4.3.2 Substrate specificity and kinetic values

Specificity for malto-oligosaccharide substrates in disproportionation reaction of all mutated *Cg*AMs was compared to WT (Figure 3.22, 3.38, 3.48). For WT, Y418 and N287 mutated *Cg*AMs, G3 was the best while G7 was the worst substrate. Highest

specificity for G3 was reported in almost all WT AMs, including those from *Thermus* sp. (Srisimarat *et al*., 2012; Terada *et al*., 1999; Van der Maarel *et al*., 2000). It is noticeable here that Y418F showed an increase in preference for G5 to G7 (Figure 3.22). For Y418 residues, our result was different from *Tb*AM study in which substitution of a hydrophobic F251 by a small aliphatic G changed substrate specificity to longer oligosaccharides (G4 to G7) while the WT preferred G3 (Jung *et al*., 2011). Also for N287, our N287A/S/D/R/W mutants preferred G3 as similar to WT, whereas previous study by our group showed that N287Y-*Cg*AM preferred longer oligosaccharide maltopentaose (G5) (Nimpiboon *et al*., 2016b). For H461 mutated *Cg*AMs, these enzymes were different from WT in substrate specificity, they preferred G2 to G3. From these overall results, mutation at H461 caused the change in substrate specificity of disproportionation reaction while mutation at Y418 did not. For mutation at N287, change in substrate specificity was observed when substituted by Y (Nimpiboon *et al*., 2016b) but not by A/S/D/R/W.

Kinetic parameters of disproportionation reaction of the WT with G3 substrate were in accordance with previous report (Nimpiboon *et al*., 2016b). A lower k_{cat} and k_{cat}/K_m values were observed for all Y418 mutated *Cg*AMs while value for the F mutant was similar to the WT. The W and R mutants showed the lowest k_{cat}/K_m (Table 3.4). The mutation affected more on the k_{cat} while the K_m values were not much changed (16-20 mM). Our result is contradicted to the report in F251G *Tb*AM in which the K_m for maltotriose increased 3 folds with 9 folds decrease in k_{cat} (Jung *et al*., 2011).

In this study, kinetics of cyclization reaction was also investigated with the two mutants, Y418A and Y418W, k_{cat} and k_{cat}/K_m were lower than that of WT (Table 3.5) while K_m values with pea starch substrate were higher (30 mM vs 19 mM of WT).

These results suggested that binding of short oligosaccharide (like G3) was not changed but binding of large substrate like pea starch was affected upon Y418 mutation while catalytic rates of both reactions with different substrate were decreased. The aromatic side chain at position 418 is thus required for high CgAM activity especially for long polysaccharide substrate. However, bulky side chain of tryptophan led to activity decrease.

For H461 mutated CgAMs, only kinetics of disproportionation reaction using maltose (G2) as substrate were determined since cyclization activity could not be detected. Mutation at H461 resulted in a significant decrease in k_{cat} and k_{cat}/K_m while the K_m values were larger than that of WT (30-37 mM vs 20 mM of WT) (Table 3.12). The mutation at this position thus affected affinity of substrate binding and enzyme catalytic rate.

For N287, kinetics of disproportionation (with G3 substrate) and cyclization (with pea starch) reactions were performed. For both reactions, mutation at N287 affected binding of small and large saccharide substrates (higher K_m for all mutants), also the reaction rate and catalytic efficiency (lower k_{cat} and k_{cat}/K_m) (Table 3.16 and 3.17). Our results on kinetic analysis of N287 mutated CgAMs were similar to the previous study in N287Y mutant (Nimpiboon *et al*., 2016b).
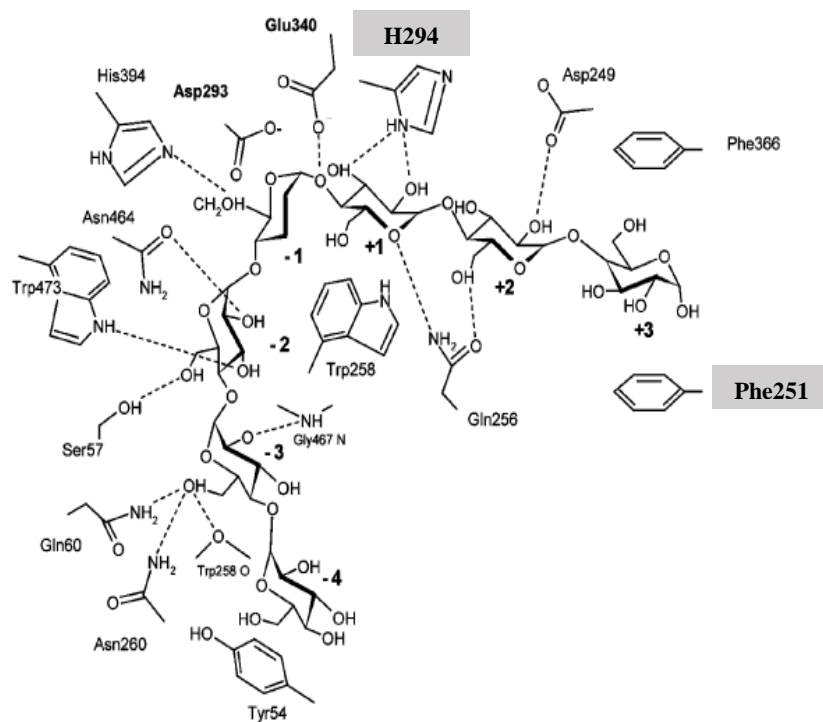
Figure 4. 1 Proposed binding of maltoheptaose (G7) in the active site of amylomaltase from *Thermus thermophilus* (*Tt*AM). Catalytic residues are shown in boldface (D293 and E340). Histidine 294 (His294) and Phenylalanine 251 (Phe251) residues are shaded (Kaper *et al.*, 2007).
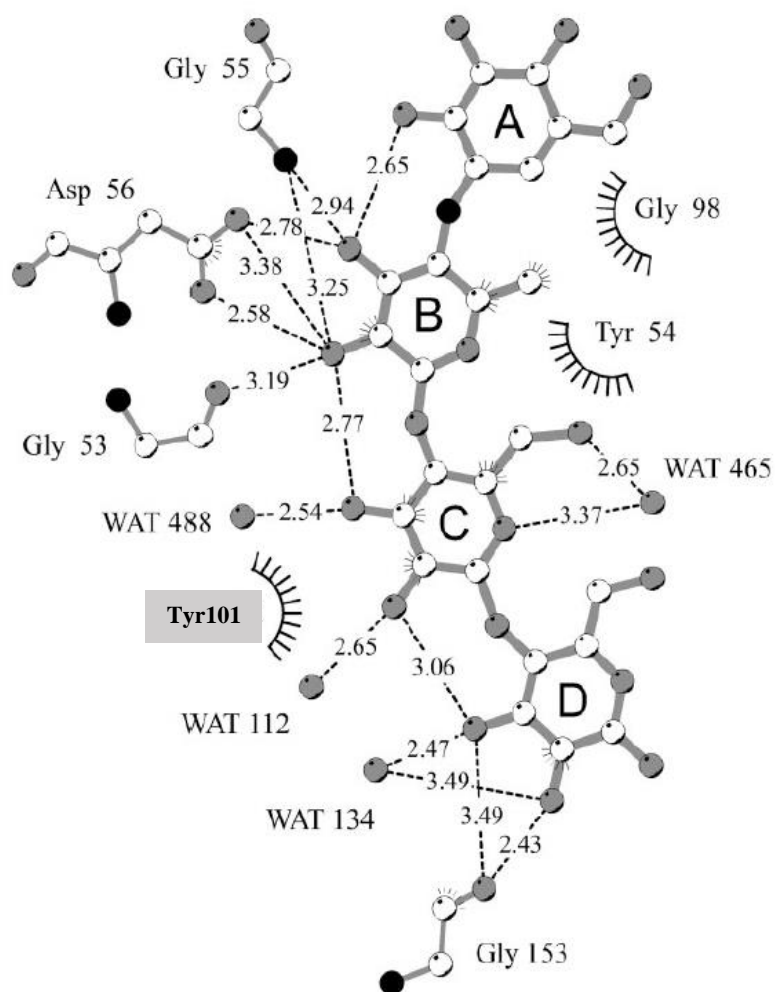
Figure 4. 2 Scheme of the interactions of acarbose bound to the secondary binding site of amylomaltase from *Thermus aquaticus* (*Ta*AM). Tyrosine 101 (Tyr 101) residues are shaded (Przylas *et al*., 2000a).

4.3.3 LR-CD products profile

LR-CD was analyzed by HPAEC-PAD (Srisimarat *et al*., 2012), the total CD yields and product patterns of all *Cg*AMs were depended on incubation time and enzyme concentration. Long incubation time and high enzyme concentration gave high yield and smaller size of LR-CDs. This is in accordance with previous report by our group (Srisimarat *et al*., 2012).

The product patterns of all mutated *Cg*AMs except for Y418F were different from that of the WT. The signal indicated yield from WT reached the highest at 28 nC followed by yields of Y418F, Y418A, Y418D, Y418S, Y418W and Y418R mutated *Cg*AMs (Figure 3.25-D). Interestingly, the main products of the WT and Y418F were CD29-CD33 while Y418A/D/S/R/W - *Cg*AMs showed a shift to the larger product size, CD36-CD40 (Figure 3.25 and 3.26). The amounts of LR-CDs formed from all mutated enzymes, especially from the W and R mutants, were less than that of the WT. Y418R gave the lowest amount of LR-CD. The product yields were corresponded to the cyclization activities observed (Table 3.3).

For N287 mutated *Cg*AMs, the LR-CDs production profiles were different from WT and Y418 mutated *Cg*AMs, the yield was lower and LR-CDs pattern shifted to the smaller CD size (CD26-CD29) (Figure 3.51 and 3.52). Our results are different from N287Y, the main product of N287Y was CD29-CD31 which may be due to increase in hydrophobic interaction (Nimpiboon *et al*., 2016b).

From the results of our study, substitution of Y418 and N287 residues of *Cg*AM thus affected not only the size but also the yield of LR-CD product. We propose that appropriate hydrophobicity with Y/F residues at the position 418 is required for high LR-CD production of *Cg*AM whereby the products are of the relatively large CD size.

However, for the position 287, mutation to A/S/D/R/W caused the decrease in LR-CDs yield and a shift to smaller CD size. On the other hand, an increase in hydrophobicity as in N287Y resulted in larger size of LR-CDs and higher yield at 24 h incubation time due to enzyme thermostability (Nimpiboon *et al*., 2016b).

4.3.4 Secondary structure

The enzyme secondary structure was analyzed by CD spectrophotometry and the content of the structure was determined. All mutated *Cg*AMs showed a slight change in CD spectra as compared to the WT (Figure 3.27, 3.41, 3.53). The proportions of the secondary structure of all *Cg*AMs were: α-helix 26-28%, β-sheet 20-22% and random coil 51-52%. Hence, mutations at Y418, H461 and N287 residues did not result in a massive conformational change of *Cg*AM. The composition of the secondary structure of WT *Cg*AM obtained is the same as that previously reported (Nimpiboon *et al*., 2016a; Rachadech *et al*., 2015). Changes in enzyme activities, kinetic parameters and LR-CDs profile were thus mainly due to a single amino acid substitution at Y418, H461 and N287 suggesting that these residues are required for maintaining such properties of *Cg*AM.

4.3.5 Isothermal titration calorimetry (ITC) and Differential scanning calorimetry (DSC)

To compare biophysical properties of WT and mutated *Cg*AMs, ITC and DSC were performed. This is the first work to use ITC for characterization of amylomaltase. We investigated the molar enthalpy change in disproportionation reaction of WT, Y418A and Y418W *Cg*AMs. The enzyme catalyzed - disproportionation reaction was endothermic (heat was produced) thus producing a large positive deflection in thermal power (Figure 3.28). The return of thermal power to the level matching the initial base

line indicated complete catalysis of G3 in the sample cell. Heat production by WT was higher than those of Y418A and Y418W CgAMs (Figure 3.28), corresponded to the relative activities of the three CgAMs as measured by biochemical method (Figure 3.29). It was observed that Y418W had less molar enthalpy or heat production than the WT and Y418A even though tryptophan **(W)** is in the same aromatic amino acid group as similar to tyrosine **(Y)** of WT. Explanation can be given by comparing the model structures of these two mutants with the X-ray structure of WT CgAM (Joo *et al.*, 2016) as illustrated in Figure 3.32 and further discussed in 4.4. Y418A had a low hydrophobic interaction with F534, the residue that supposed to be involved in the loop entrance area of the enzyme**.** While Y418W though aromatic but the W side chain of mutant was left behind the substrate channel groove (Joo *et al.*, 2016), thus hydrophobic interaction between substrate and enzyme was even lower than that of Y418A mutant leading to extremely low enzyme activity.

Kinetics of disproportionation reaction by G3 substrate were also investigated by ITC. Curve fitting analysis was performed on the thermal power profile (Jeoh *et al.*, 2005) and the endothermic heat rate production of the enzyme-catalyzed reaction was converted to the kinetic parameters of the enzyme **(**Todd and Gomez, 2001). The pseudo steady state assumptions at high substrate to enzyme ratio was used to determine kinetic parameters of the enzyme **(**Bianconi, 2007). The thermogram of WT and Y418A/W mutated CgAMs showed that the thermal power signal was higher when concentration of G3 was increased (Appendix 22). V_{max}/K_m values were in the order of WT > Y418A > Y418W at 10 mM G3 (Table 3.6). This suggested that each enzyme had different efficiency in catalysis. Moreover, all results showed that when concentration of G3 was increased, the product inhibition was occurred, V_{max}/K_m values

were decreased when G3 was changed from 10 to 30 mM (Figure 3.30). This may result from higher production of glucose, a main product from disproportionation reaction, that can also act as substrate for CgAM or glucose itself can be an inhibitor to the enzyme (Jeoh et al., 2005). Similar situation had been reported in ITC analysis of β-D-glucosidase whereby high glucose product produced at high cellobiose substrate could inhibit enzyme activity, V_{max}/K_m values observed at high substrate concentration were decreased (Jeoh et al., 2005).

DSC is one of the most frequently used techniques to determine thermal stability of protein. Its fundamental principle is to measure the heat capacity (C_p) when molecules unfold due to the increase in temperature, generally performed at -10 ºC to 130 ºC. The transition midpoint or melting temperature (T_m) is considered as a temperature, where 50% of the proteins own its native conformation, and the rest remain denatured. Higher T_m values would be representative of a more stable molecule. Besides T_m, T_p value which represents the DSC peak temperature is easily obtained and also can be used to designate thermostability of the molecules (Jelesarov and Bosshard, 1999). In this work, DSC was investigated to determine the thermal stability of WT and Y418A/W mutated CgAMs. At every scanning rate (0.75, 1.0 and 1.5 ºC .min^{-1}), all CgAMs showed the irreversible transition state in accordance with our previous report on the WT enzyme (Nimpiboon et al., 2016a). When compared the thermal transition curves (Figure 3.31), the heat capacity profiles of WT and Y418A were similar while a shift of peak temperature (T_p) of Y418W towards 4 °C lower was observed (Table 3.7). DSC results thus support that CgAM is not a thermostable enzyme in accordance with our previous studies (Nimpiboon et al., 2016a). Moreover, the WT was more stable to temperature than Y418W, which suggested that the aromatic side chain especially

tyrosine at position 418 is involved in controlling thermal stability of CgAM through the aromatic hydrophobic interaction at the substrate entrance.

4.4 The proposed model structures of mutated CgAMs

In the attempt to relate changes in properties of CgAM observed upon mutations to changes in enzyme structure, the X-ray structure of the WT CgAM (PDB code 5B68) (Joo *et al*., 2016)was compared with the model structures of all mutants (Figure 3.32, 3.42 and 3.54). Possible H-bonding and hydrophobic interaction with residues closed by the mutated residues was analyzed and compared with WT. In addition, H-bond interactions between position 461 and 287 with acarbose molecule were also investigated.

Six model structures of Y418 mutated (Y418A/S/D/R/W/F mutants) CgAMs were contructed Figure 3.32. In the 410s loop, eight residues including D416, G417, N419, Q420, R577, V582, Q662 and P663 are within 5 Ǻ away from Y418. It is known that side chain-side chain H-bond can be formed within 3.5 Ǻ (Barlow and Thornton, 1983; McDonald and Thornton, 1994; Sokalingam *et al*., 2012). We found that only the R mutant had one H-bond forming between R418 and D416 while no H-bond was found in WT and other mutants (Figure 3.32-1E). Difference in H-bonding observed here was not corresponded to the difference in properties of Y418 mutated enzymes. When the molecular surfaces of aromatic edge of CgAMs were determined, WT-CgAM showed rather strong hydrophobic interaction (gold color in Figure 3.32-2A) between Y418 and F534 residues that are proposed to form the channel for substrate entering, as similar to situation in TbAM (Jung *et al*., 2011) described below. Moderate interaction was found

in Y418F/W mutants which might be due to the inward direction of F/W side chain. For the Y418A/S/D/R mutants, low or no interaction was observed (grey color in Figure 3.32 2B-2F). Thus, hydrophobic interactions around the loop area were different between the WT and mutated enzymes, and this difference is likely to responsible for change in properties of Y418 mutated enzymes. It was reported that the decrease in hydrophobicity of the environment led to non-optimal binding of the incoming oligosaccharide acceptor substrate (Tang *et al*., 2006). Transglucosylation activities of amylomaltases, as similar to cyclodextrin glycosyltransferases, are facilitated by hydrophobic interaction at the acceptor binding site (van der Veen *et al*., 2001). From sequence alignment of *Cg*AM and *Tb*AM, Y418 and F534 in 410s loop of *Cg*AM are corresponded to F251 and F366 in 250s loop of *Tb*AM that have been proposed to form the hydrophobic stacking interaction between substrate and enzyme (Jung *et al*., 2011). The mutants F251G in *Tb*AM (Jung *et al*., 2011) and F366L in *Tt*AM showed a significant decrease in the activity of both enzymes (Kaper *et al*., 2007). We here propose that upon losing the hydrophobic interaction at the loop entrance, the mutated *Cg*AMs could not stabilize the extended loop due to less suitable conformation that leads to less enzyme activities and product yield.

From the model structure, the substrate entering channel in Y418A was larger (Figure 3.32-2B) while those of Y418R and Y418W were smaller (Figure 3.32-2E, 2F) when compared to the WT ((Figure 3.32-2A)). The distances between the residue position 418 and F534 which form the channels were predicted (Table 3.8); 1.0 Å longer and 4.5 Å shorter than that of the WT were observed in the A and R mutants, respectively. The R and W mutants provided the shorter distances due to having charged and bulky functional groups (Nelson *et al*., 2008; Voet *et al*., 1999), thus

resulting in difficulty of substrate, especially those long polysaccharides, to enter the loop area. In contrast, the distance in the F mutant did not change much (0.4 Å shorter), in accordance with the insignificant change of catalytic activity and product formation compared to the WT. The loop movement is important, as similarly proposed in 250s loop of *Tb*AM (Jung *et al*., 2011). Y418 having proper hydrophobic interaction with F534 might make the 410s loop in closed form, the acceptor substrate was then oriented to the active site and the conformation of *Cg*AM was suitable for catalysis. While in the R and W mutants, the loop might be in open form resulting in activity lost.

The model structures of H461 mutated *Cg*AMs were also constructed. The H461 residue is in the active site area, twelve residues including Y23, G412, W425, D460, V462, L463, G464, L465, R467, L468 and E508 are within 5 Å away from H461 position. In the WT and the H461A/D/R/W mutants, no hydrogen bond was formed with those residues, but the S mutants had one H-bond with D460 (Figure 3.42-C). From our study, we found that H461 is essential for *Cg*AM activity, nearly total loss of all transglucosylation activities were observed upon mutation, especially cyclization whereby LR-CDs formation was nil. Analysis of H-bonding between residue 461 and closed by residues was not totally accounted for changes in activities except for the S mutant which had one H-bonds that might cause local conformational change around active site. From the previous report in *Tt*AM, the model for substrate binding site in active site area showed the interaction of H294 residue with maltoheptaose (G7) substrate at subsite +1 (Kaper *et al*., 2007). Moreover, from multiple amino acid sequence alignment, we observed that this histidine is highly conserved among amylomaltases and disproportionating enzymes (DPE-1) from bacteria and plants, respectively (Appendix 18). When H-bond interactions between position 461 and

acarbose molecule were investigated (Figure 3.42), we found only WT and H461D mutated *Cg*AMs could form two and one H-bond with acarbose molecule at subsite +1 (Figure 3.42A and D) These results suggested that H-bond of H461 with substrate at subsite +1 is essential for *Cg*AM catalysis.

Around N287 residues, ten amino acids residues including E225, P228, P229, T230, D285, R286, D288, V289, Y290 and A291 are within 5 Å away from N287 position. Hydrogen bonds could be formed between residues at position 287 and three residues (T230, Y290 and D285) (Figure 3.54). And only N287D mutant had one hydrogen bond with glucose unit C of acarbose bound at the active site (Figure 3.54-D). The previous study from our group reported that the change in hydrophobicity at this position in N287Y mutants led to decrease in enzyme activity (Nimpiboon *et al*., 2016b). Moreover, mutation at Y101 of *Ta*AM (corresponded to our N287) showed lower disproportionation activity similar to our results. We conclude here that position 287 requires positively charged side group for high *Cg*AM activity.

After we nearly finished mutation experiments at N287, the X-ray structure of *Cg*AM was published by a Korean research group (Joo *et al*., 2016) and deposited as PDB 5B68 around mid 2016 as earlier mentioned. We then used this structure to superimpose with X-ray structure of *Ta*AM (PDB 1CWY) and surprisingly found that N287 of *Cg*AM is corresponding to G98, not Y101 of *Ta*AM. And Y101 of *Ta*AM corresponded to Y290 of *Cg*AM, the same result as we had obtained from our amino acid sequence alignment (Figure 3.1). Our resolved X-ray structure and the PDB 5B68 structure seem to show a three amino acid - shift in the helix turn around the positions 287 to 290. The difference may be due to different based structure used when electron density map was resolved into each X-ray structure of *Cg*AM. At present, we think

Y290 of *Cg*AM is corresponding to Y101 of *Ta*AM. So which residue in *Ta*AM corresponded to N287 of *Cg*AM, sequence alignment suggested G98 (Figure 3.1). The residue G98 is closed to glucose unit A and also one of the important residues in the second glucan binding site of *Ta*AM (Figure 4.2) However, from our results we found that N287 was closer to glucose unit C than unit A (Figure.3.54) Therefore, effects from N287 mutations reflect the importance of second glucan binding site of *Cg*AM.

In early 2017, Roth *et al*. reported the X-ray crystal structure of *Ta*AM in complex with cycloamylose (CA34) at resolution range of 1.73-37 Å. The result showed that 250s loop (F251 residue) and the second glucan biding site (Y54 and Y101 residues) which are close to 460s loop are important for LR-CD formation and enzyme activity via the hydrophobic interaction of amino acid residues around the loops of *Ta*AM   (Roth *et al*., 2017). This work thus supports our results in terms of the importance of the unique loops and the second glucan binding site on their functions involving transglucosylation activity and LR-CD product formation of *Cg*AM

CHAPTER V
CONCLUSIONS

Three target amino acid residues of *Cg*AM consisting of Y418 (at the tip of 410s loop which lies over the active site), H461 (in active site) and N287 (in second glucan binding site) were selected for site-directed mutagenesis. Y418, H461 and N287 mutated *Cg*AMs were constructed, expressed and purified by His-trap column chromatography. The major protein band of 84 kDa similar to WT - *Cg*AM was observed. All mutated *Cg*AMs showed significantly lower transglucosylation activities than WT enzyme, except the Y418F mutant. Interestingly, H461 mutated *Cg*AMs lost an intramolecular transglucosylation (cyclization) activity since LR-CD products could not be detected.

The optimum temperatures and pHs of mutated enzymes were similar to WT, at 40 ℃, pH 6.0 and 30 ℃, pH 6.0 for disproportionation and cyclization reactions, respectively. Thermostability at 30, 35 and 40 ℃ and pH stability at pH 7.0 and 8.0 of all mutated *Cg*AMs were significantly decreased when compared to WT. Substrate specificity in disproportionation reaction showed that G3 was the best substrate for WT, Y418 and N287 mutated *Cg*AMs while H461 mutants preferred G2. The catalytic efficiency, k_{cat}/K_m of all mutated *Cg*AMs was lower than that of WT, mainly due to the significant decrease in k_{cat} for both disproportionation and cyclization reactions. The K_m values for G3 of Y418 mutated *Cg*AMs were not changed much while those of H461 and N287 mutated enzymes were higher than WT. In cyclization, K_m values for pea starch substrate of Y418 and N287 mutated *Cg*AMs were higher than WT. Product analysis demonstrated that all mutated enzymes gave a lower product yield in comparison to WT. Y418A/S/D mutated *Cg*AMs provided a larger LR-CD size (CD36-

CD40) while N287/A/S/D/R/W gave a smaller size (CD26-CD28) compared to WT (CD29-CD33). While Y418R/W showed a broad product pattern.

The secondary structure observed from CD spectra was not significantly changed upon mutations. In biophysical characterization, heat production in disproportionation reaction by WT was higher than those of Y418A and Y418W as observed in ITC. And product inhibition was observed at high maltotriose. From DSC, heat capacity profile of Y418W showed the lowest peak temperature (T_p) than WT and Y418A CgAM. The model structures of all mutated CgAMs were constructed and compared to WT structure to help explain mutation effect on changes in properties of CgAM. From overall results, changes in enzyme activities, biochemical & biophysical properties and LR-CD product pattern resulted from local conformation change caused by alteration in hydrophobicity or hydrogen bonding interactions around mutated residues. Y418 in 410s loop not only contributes to transglucosylation activities but also controls the amount and size of LR-CD products through the proposed hydrophobic stacking interaction and the suitable distance (418 - F534 residue) of loop channel for substrate entering, the shift in product pattern is likely to be the result of the difference of preferable oligosaccharides substrates between WT and mutated CgAMs. H461 is essential for enzyme catalysis, especially in cyclization reaction and N287 could also be involved in enzyme activity and LR-CD products pattern by retaining positively charge side chain.

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APPENDICES

APPENDIX 1: Preparation of polyacrylamide gel electrophoresis

   1) **Stock reagents**

   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

   1.5 M Tris-HCl pH 8.8

   Tris (hydroxymethyl)-aminomethane   18.2 g

   Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with

   distilled water

   2 M Tris-HCl pH 8.8

   Tris (hydroxymethyl)-aminomethane   24.2 g

   Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with

   distilled water

   0.5 M Tris-HCl pH 6.8

   Tris (hydroxymethyl)-aminomethane   6.06 g

   Adjusted pH to 6.8 with 1 M 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 M HCl and adjusted volume to 100 ml with

   distilled water

Solution B (SDS-PAGE)

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (SDS-PAGE)

1 M Tris-HCl pH 6.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

2) **Denaturing PAGE (SDS-PAGE)**

10% separating gel

30% Acrylamide solution	2.50 ml
Solution B (SDS-PAGE)	2.50 ml
Distilled water	2.39 ml
10% $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

5% stacking gel

30% Acrylamide solution	0.84 ml
Solution C (SDS-PAGE)	1.0 ml
Distilled water	3.1 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

5X sample buffer

1 M Tris-HCl pH 6.8	0.6 ml
50% Glycerol	5.0 ml
10% SDS	2.0 ml
2-Mercaptoethanol	0.5 ml
1% Bromophenol blue	1.0 ml
Distilled water	0.9 ml

One part of sample buffers was added to four parts of sample. The mixture was boiled for 5 min prior to loading to the gel.

Electrophoresis (TBE) buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 litre with distilled water (pH should be approximately 8.3)

Coomassie Gel Stain, 1 litre

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

Adjusted volume to 100 ml with distilled water, adjusted to pH 4.0, 5.0 or 6.0 by 0.2 M acetic acid.

APPENDIX 2: Preparation of agarose electrophoresis

Stock Tris-boric acid (TBE) solution (10X)

Tris (hydroxymethyl)-aminomethane	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

Adjusted volume to 1 litre with distilled water

APPENDIX 3: Preparation for buffers solutions

0.2 M Sodium Acetate pH 4.0, 5.0 and 6.0

CH_3COONa	1.21 g

Adjusted volume to 100 ml with distilled water and adjusted pH to pH 4.0, 5.0 and 6.0 by 0.2 M acetic acid

0.2 M Phosphate buffer pH 6.0

KH_2PO_4	3.28 g
K_2HPO_4	0.16 g
Distilled water	100 ml

0.2 M Phosphate buffer pH 7.0

KH_2PO_4	1.35 g
K_2HPO_4	1.67 g
Distilled water	100 ml

0.2 M Phosphate buffer pH 8.0

KH_2PO_4	0.48 g
K_2HPO_4	2.34 g
Distilled water	100 ml

0.2 M Tris-Glycine NaOH pH 8.0, 9.0 and 10.0

Glycine	1.5 g

Adjusted to pH 8.0, 9.0 and 10.0 by 1 M NaOH and adjusted volume to 100 ml
with distilled water

APPENDIX 4: Preparation of solution for cell preparation and enzyme purification

1) **Stock solution**

1 M Monopotassium phosphate 100 ml

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

1 M Dipotassium hydrogen phosphate 100 ml

The pellet of Dipotassium hydrogen phosphate 87.1 g was dissolved in distilled water to final volume 500 ml

1 M Potassium phosphate buffer, pH 7.4

1 M Potassium phosphate buffer, pH 7.4 was prepared by mixing 1 M of monopotassium phosphate and 1 M dipotassium hydrogen phosphate and then pH was adjusted to pH 7.4.

2) **Extraction buffer**

1 M Potassium phosphate buffer, pH 7.4	5.0 ml
0.1 M 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

3) **0.85% NaCl**

Sodium chloride	0.85 g

Adjusted volume to 100 ml with distilled water

4) **Binding buffer**

Imidazole	0.3 g
Sodium chloride	5.8 g
1 M Potassium phosphate buffer, pH 7.4	4.0 ml

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

5) **Elution buffer**

Imidazole	6.8 g
Sodium chloride	5.8 g
1 M Potassium phosphate buffer, pH 7.4	4.0 ml

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

APPENDIX 5: Preparation of Iodine solution

10X Iodine stock solution 100 ml

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

Potassium iodide	2 g
Iodine	0.2 g

1X Iodine solution 100 ml

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

APPENDIX 6: 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 with stirring overnight and filtration by Whatman No.1 paper.

2) **Working solution**

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

Store at room temperature in brown glass bottle.

APPENDIX 7: Preparation for Bicinchoninic acid (BCA) assay

Bicinchoninic acid reagent

Solution A

4, 4'-dicarboxy-2, 2'-biquinoline	0.130 g

Dissolved in 85 ml of distilled water

Na_2CO_3	6.221 g

Adjusted to 100 ml with distilled water

Solution B

Component 1

L-aspartic acid	0.642 g
Na_2CO_3	0.868 g

Dissolved in 15 ml of distilled water

Component 2

$CuSO_4$	0.174 g

Dissolved in 5 ml of distilled water

Mixed component 1 and 2, then adjusted volume to 25 ml with distilled

water

Mixed 24 ml of solution A and 1 ml of solution B and used within 24 h

APPENDIX 8: Preparation for DNS reagent

DNS reagent

2-hydroxy-3, 5'-dinitrobenzoic acid	5 g
2 N NaOH	100 ml
Potassium sodium tartrate	150 g

Adjusted volume to 500 ml with distilled water

APPENDIX 9: Bacterial media culture

LB (Luria-Bertani) medium and plate

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

Adjusted volume to 1,000 ml with distilled water (15 g/l of bacteria agar was added for plate). The medium were sterilized by autoclaving at 15 lb/in2 for 15 min.

APPENDIX 10: Ampicillin antibiotic

100 mg/ml of ampicillin

Ampicillin	1 g
Autoclaved distilled water	10 ml

An aliquot of 100 mg/ml of ampicillin was stored at -20 °C for long time.

APPENDIX 11: Standard curve for protein determination by Bradford's method

$y = 0.0261x$
$R^2 = 0.992$
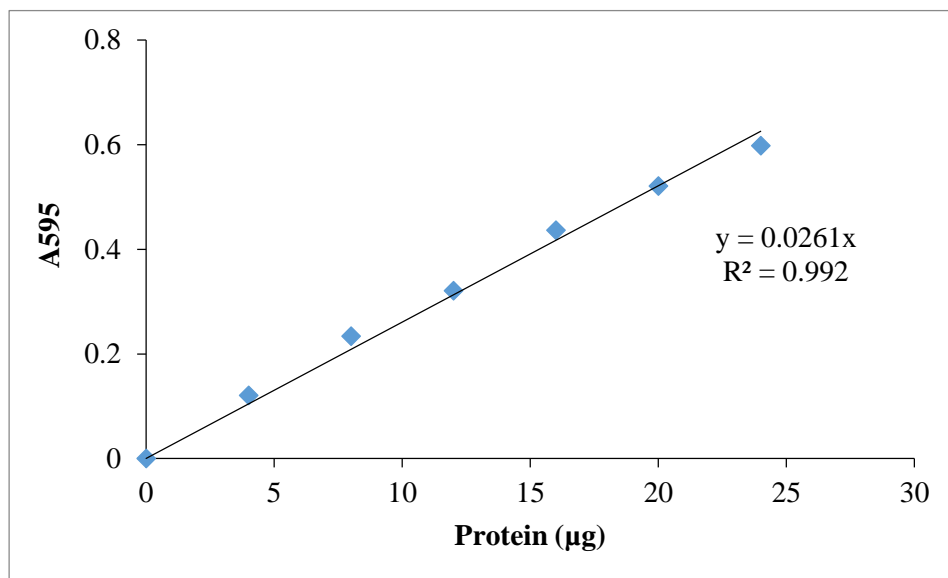
APPENDIX 12: Standard curve for glucose determination by glucose oxidase assay

$y = 6.8078x$
$R^2 = 0.997$
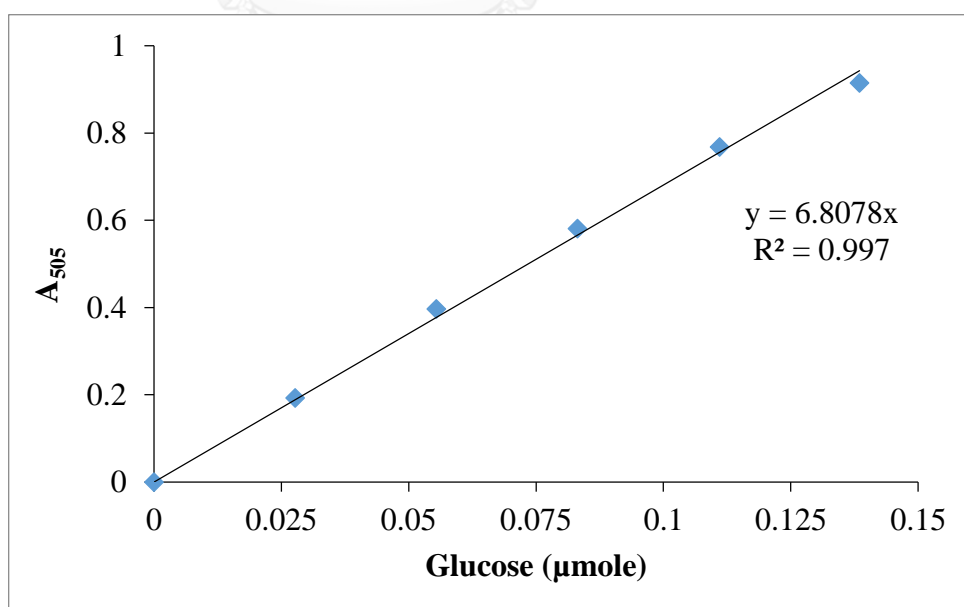
APPENDIX 13: Standard curve for starch degradating activity assay

$$y = 5.115x$$
$$R^2 = 0.994$$
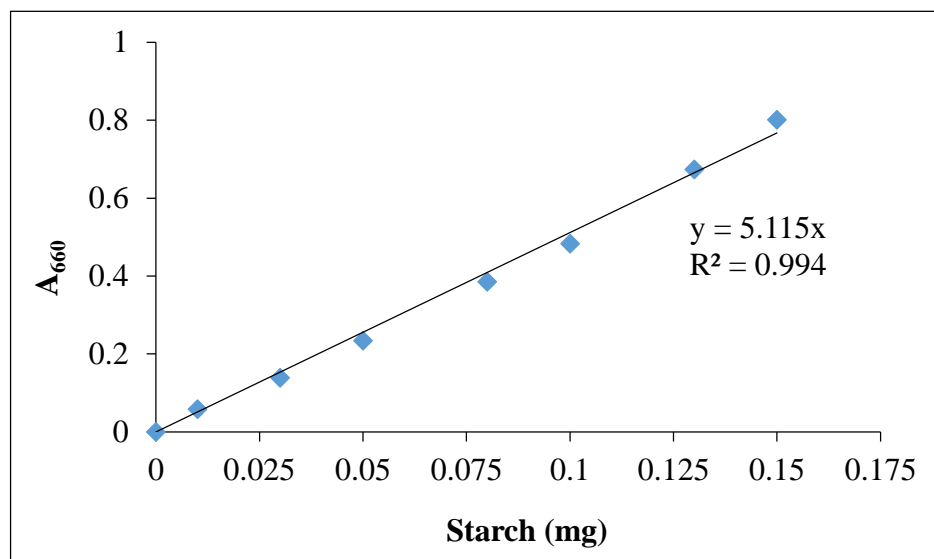
APPENDIX 14: Standard curve for glucose determination by Bicinchoninic acid assay

$$y = 29.361x$$
$$R^2 = 0.993$$

APPENDIX 15: Restriction map of pET-19b vector

pET-19b cloning/expression region
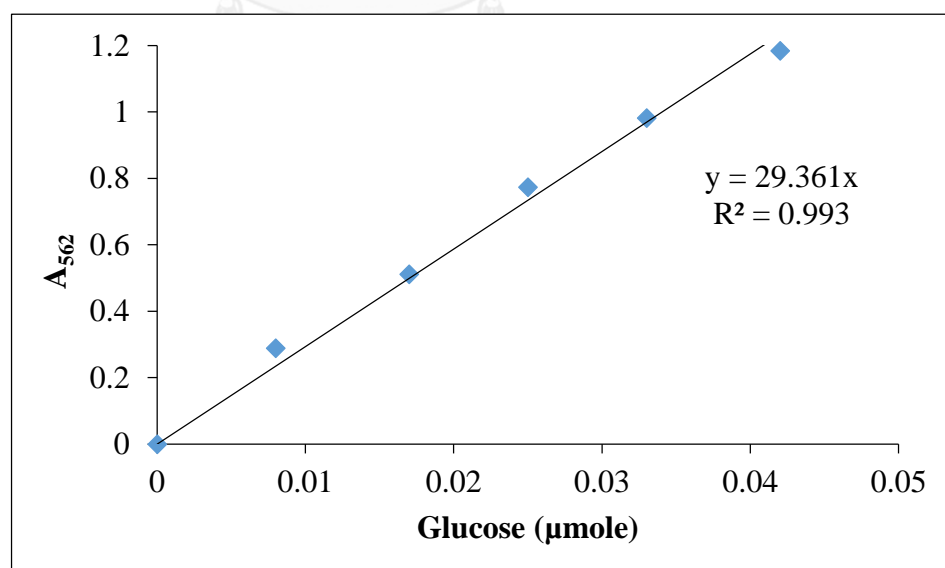
APPENDIX 16: Structure of amino acids commonly found in protein
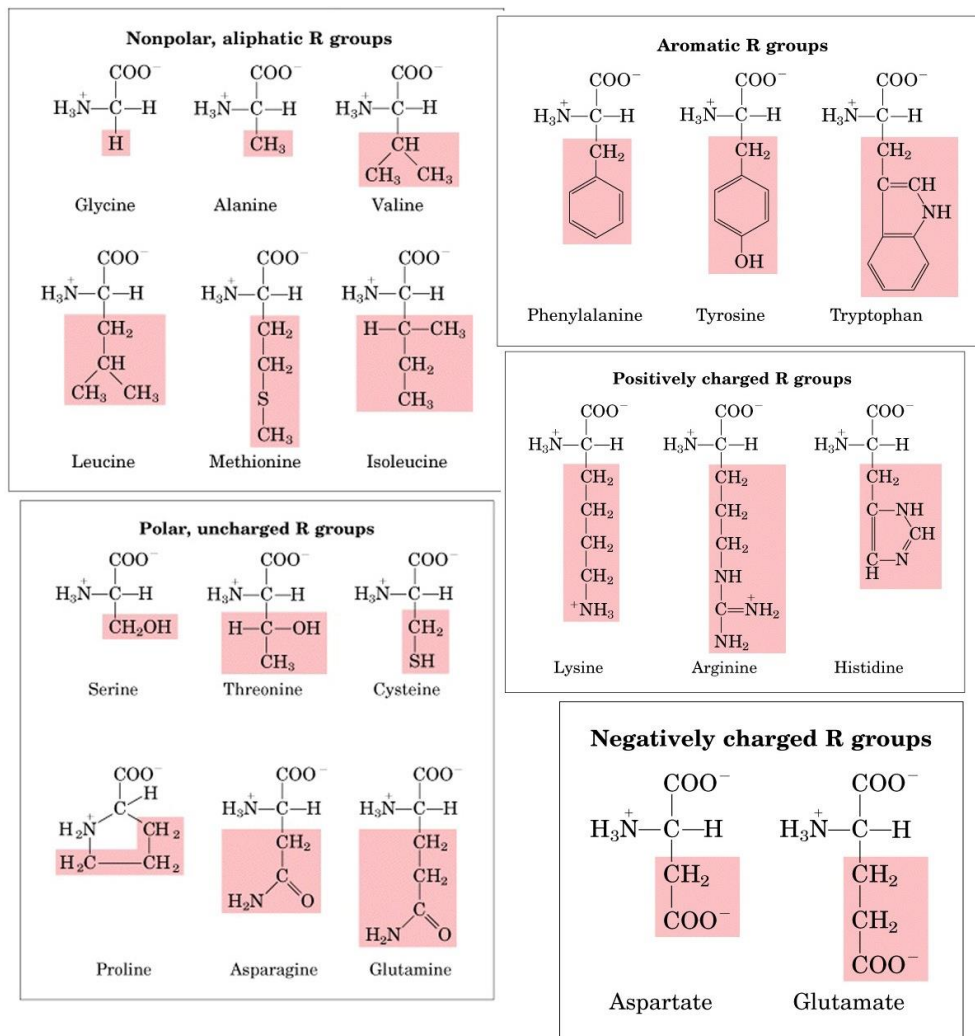
APPENDIX 17: Abbreviation of amino acid residues (Voet, 1990)

Amino acid	3 Letter-Abbreviation	1 Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic aicd	Asp	D
Cysteine	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	Try	W
Tyrosine	Trp	Y
valine	val	V
Unknown	-	X

APPENDIX 18: Amino acid sequences alignment of amylomaltases

```
StDPE1      ---------------------------------------------------------   0
AtDPE1      ---------------------------------------------------------   0
SpAM        ---------------------------------------------------------   0
TaAM        ---------------------------------------------------------   0
TtAM        ---------------------------------------------------------   0
CgAM        MTARRFLNELADLYGVATSYTDYKGAHIEVSDDTLVKILRALGVNLDTSNLPNDDAIQRQ  60
EcAM        MESKR-LDNAALAAGISPNYINAHGKPQSISAETKRRLL-------------------   38


StDPE1      ---------MA--------IHTCFSLIPSSFSSPKLPYPKNTTFQSP-------------   30
AtDPE1      ---------MSILLRPSSSPSLCSSLKLFRLSSPDS-----LIDAAV------------   33
SpAM        ---------------------------------------------------------    0
TaAM        ---------------------------------------------------------    0
TtAM        ---------------------------------------------------------    0
CgAM        IALFHDRE----FTRPLPPSVV-------AVEGDELVFPVHVHDGSPADVHIELEDGTQR  109
EcAM        -DAMHQRTATKVAVTPVPNVMV-------YTSGKKM--PMVVEGSGEYSWLLTTE-----  83


StDPE1      ----IPKLSRPTFMFDRKG-SFQNGTAAVPAVGEDFPIDYAD-----------------  67
AtDPE1      ----LRNRTKPSQSFRMEVVSSNSTCLSSISVGEDFPSEYEQ-----------------  71
SpAM        ---------------------------------------------------------    0
TaAM        ---------------------------------------------------------    0
TtAM        ---------------------------------------------------------    0
CgAM        DVSQVENWTAPREI---DGIR---WGEASFKIPGDLPLGWHKLHLKSNERSAECGLIITP  163
EcAM        ---------EGTQY---KGHV---TGGKAFNLPTKLPEGYHTLTLTQEDQRAHCRVIVAP  128


StDPE1      ---WLPKRDPNDRRRAGILLHPTSFPG--PYGIGDLGPQAFKFLDWLHLAGCSLWQVLPL  122
AtDPE1      ---WLPVPDPESRRRAGVLLHPTSFRG--PHGIGDLGEEAFRFIDWLHSTGCSVWQVLPL  126
SpAM        ----------MKKRQSGVLMHISSLPG--AYGIGSFGQSAYDFVDFLVRTKQRYWQILPL   48
TaAM        ---------MELPRAFGLLLHPTSLPG--PYGVGVLGREARDFLRFLKGAGGRFWQVLPL   49
TtAM        ---------MELPRAFGLLLHPTSLPG--PYGVGVLGREARDFLRFLKEAGGRYWQVLPL   49
CgAM        ARLSTADKYLDSP-RSGVMAQIYSVRSTLSWGMGDFNDLGNLAS-VVAQDGADFLLINPM  221
EcAM        KRCYEPQALLNKQKLWGACVQLYTLRSEKNWGIGDFGDLKAMLV-DVAKRGGSFIGLNPI  187
            *     :   :. .      *:* :.           :           : *:


StDPE1      VPPGKRGNEDGSPYSGQDANCGNTLLISLEELVDDGLLKMEEL-------P---EPLPT  171
AtDPE1      VPPD----EGGSPYAGQDANCGNTLLISLDELVKDGLLIKDEL-------P---QPIDA  171
SpAM        GATS----YGDSPYQSFSAFAGNTHFIDLDILVEQGLLEASDL-------EGVDFGSDA   96
TaAM        GPTG----YGDSPYQAFSAFAGNPYLIDLRPLAEKGYLVLKD-------------PGFPQ   92
TtAM        GPTG----YGDSPYQSFSAFAGNPYLIDLRPLAERGYVRLED-------------PGFPQ   92
CgAM        HAAEPLPPTEDSPYLPTTRRFINPIYIRVEDIPEFNQLEIDLRDDIAEMAAEFRERNLTS  281
EcAM        HALYPANPESASPYSPSSRRWLNVIYIDVNAVEDFHLSEEAQAWWQLPTTQQTLQQARDA  247
            ***        *   * :   : .
```

*St*DPE1 = Disproportionating enzyme from *Solonum tuberlusum*, *At*DPE1 = Disproportionating enzyme from *Arabidopsis thaliana*, *Sp*AM = Amylomaltase from *Streptococcus pneumonia*, *Ta*AM = Amylomaltase from *T. aquaticus*, *Tt*AM = Amylomaltase from *T. thermophilus*, *Cg*AM = Amylomaltase from *C. glutamicum* and *Ec*AM = Amylomaltase from *E.coli*

```
StDPE1    DRVNYSTISEIKDPLITKAAKRLLSSEGE-LKDQLENFRRDPN-ISSWLEDAAYFAA--I    227
AtDPE1    DSVNYQTANKLKSPLITKAAKRLIDGNGE-LKSKLLDFRNDPS-ISCWLEDAAYFAA--I    227
SpAM      SEVDYAKIYYARRPLLEKAVKRFFEVGD---VKDFEKFAQ--D-NQSWLELFAEYMA--I    148
TaAM      GRVDYGWLYAWKWPALKAAYQGFLEKAPRKEREDFPAFQE--K-EASWLKDYALFMA--L    147
TtAM      GRVDYGLLYAWKWPALKEAFRGFKEKASPEEREAFAAFRE--R-EAWWLEDYALFMA--L    147
CgAM      DIIERNDVYAAKLQVLRAIFE--MPRSSER-EANFVSFVQREGQGLI-------------    325
EcAM      DWVDYSTVTALKMTALRMAWKGFAQRDDEQ-MAAFRQFVADHGDSLFWQAAFDALHAQQV    306
                .  ::        :     :      .          :   *
```

```
StDPE1    DNSVNTISWYDWPEPLK-NRHLAALEEVYQSEKDFIDIFIAQQFLFQRQWKKVRDYARSK    286
AtDPE1    DNTLNAYSWFEWPEPLK-NRHLSALEAIYESQKEFIDLFIAKQFLFQRQWQKVREYARRQ    286
SpAM      KEYFDNLAWTEWPDADARARKASALESYREQLADKLVYHRVTQYFFFQQWLKLKAYANDN    208
TaAM      KAQHGGLPWNRWPLPLR-RREEKAMKEAEAALAEEVAFHAWTQWLFFEAWKALKEEAEAL    206
TtAM      KGAHGGLPWNRWPLPLR-KREEKALREAKSALAEEVAFHAFTQWLFFRQWGALKAEAEAL    206
CgAM      -------DFATWCADRE-TAQSESVHGTEPDRDELTMFYMWLQWLCDEQLAAAQKRAVDA    377
EcAM      KEDEMRWGWPAWPEMYQ-NVDSPEVRQFCEEHRDDVDFYLWLQWLAYSQFAACWEISQGY    365
                :  *          .   :.       :    .    *::         :
```

```
StDPE1    --GISIMGDMPIYVGYHSADVWANKKQFLLNRKGFPLIVSGVPPDAFSETGQLWGSPLYD    344
AtDPE1    --GVDIMGDMPIYVGYHSADVWANKKHFLLNKKGFPLLVSGVPPDLFSETGQLWGSPLYD    344
SpAM      --HIEIVGDMPIYVAEDSSDMWANPHLFKTDVNGKATCIAGCPPDEFSVTGQLWGNPIYD    266
TaAM      --GIQIIGDMPIFVAEDSAEVWAHPEWFHLDEEGRPLVVAGVPPDYFSETGQRWGNPLYR    264
TtAM      --GIRIIGDMPIFVAEDSAEVWAHPEWFHLDEEGRPTVVAGVPPDYFSETGQRWGNPLYR    264
CgAM      GMSIGIMADLAVGVHPGGADAQNLSHVLAPD------ASVGAPPDGYNQQGQDWSQPPWH    431
EcAM      EMPIGLYRDLAVGVAEGGAETWCDRELYCLK------ASVGAPPDILGPLGQNWGLPPMD    419
            :  :  *:  : *     .::     .      .        * ***  .  ** *. *
```

```
StDPE1    WKAMEKDGFSWWVRRIQRATDLFDEFRIDHFRGFAGFWAVPSEEKIAILGRWKVGPGKPL    404
AtDPE1    WKAMESDQYSWWVNRIRRAQDLYDECRIDHFRGFAGFWAVPSEAKVAMVGRWKVGPGKSL    404
SpAM      WEAMDKDGYKWWIERLRESFKIYDIVRIDHFRGFESYWEIPAGSDTAAPGEWVKGPGYKL    326
TaAM      WDVLEREGFSFWIARLAKALELFHLVRVDHFRGFEAYWEIPASCPTAVEGRWVKASGEKL    324
TtAM      WDVLEREGFSFWIRRLEKALELFHLVRIDHFRGFEAYWEIPASCPTAVEGRWVKAPGEKL    324
CgAM      PVRLAEEGYIPWRNLLRTVLRHSGGIRVDHVLGLFRLFVMPRMQ-SPATGTYIRFDHNAL    490
EcAM      PHIITARAYEPFIELLRANMQNCGALRIDHVMSMLRLWWIPYGE-TADQGAYVHYPVDDL    478
                :     :    :       :       *:**. .:    : :*      * :      *
```

```
StDPE1    FD--AILQAVGKINIIAEDLGVITEDVVQLRKSIEAPGMAVLQFAFGSDAENPHLPHNHE    462
AtDPE1    FD--AISKGVGKIKIIAEDLGVITKDVVELRKSIGAPGMAVLQFAFGGGADNPHLPHNHE    462
SpAM      FA--AVKEELGELNIIAEDLGVMTDEVIELRERTGFPGMKILQFAFNPEDESIDSPHLAP    384
TaAM      FD--RIQEVFGEVPILAEDLGVITPEVEPLRDRYGLPGMKVLQFAFDHGMENPFLPHNYP    382
TtAM      FQ--KIQEVFGEVPVLAEDLGVITPEVEALRDRFGLPGMKVLQFAFDDGMENPFLPHNYP    382
CgAM      VGILALEAELAGAVVIGEDLGTFEPWVQDALAQRGIMGTSILWFEHSPSQPGPRRQEEYR    550
EcAM      LSILALESKRHRCMVIGEDLGTVPVEIVGKLRSSGVYSYKVLYFENDHEK-TFRSPKAYP    537
              .  :        ::.****  .   :    .   .   :* *  .  .        .
```

(Continued) *St*DPE1 = Disproportionating enzyme from *Solonum tuberlusum*, *At*DPE1 = Disproportionating enzyme from *Arabidopsis thaliana,* *Sp*AM = Amylomaltase from *Streptococcus pneumonia*, *Ta*AM = Amylomaltase from *T. aquaticus*, *Tt*AM = Amylomaltase from *T. thermophilus*, *Cg*AM = Amylomaltase from *C. glutamicum* and *Ec*AM = Amylomaltase from *E.coli*

```
StDPE1      Q--NQVVYTGTHDNDTIRGWWDTLPQE-EKSNVLKYLSN-----IEEEEISR--------    506

AtDPE1      V--NQVVYSGTHDNDTIRGWWDTLDQE-EKSKAMKYLSI-----AGEDDISW--------    506
SpAM        A--NSVMYTGTHDNNTVLGWYRNEIDDATREYMARYTNR-----KEYETVVH--------    429
TaAM        AHGRVVVYTGTHDNDTTLGWYRTASPH-ERAFLERYLADWGISFRQEEEVPW--------    433
TtAM        AHGRVVVYTGTHDNDTTLGWYRTATPH-EKAFMARYLADWGITFREEEEVPW--------    433
CgAM        P--LALTTVTTHDLPPTAGYLEGEHIALR-ERLGVLNTDPAAE-L-AEDLQW-QAEILDV    604
EcAM        E--QSMAVAATHDLPTLRGYWESGDLTLG-KTLGLYPDEVVLR-GLYQDRELAKQGLLDA    593
                :     ***      *:                                     :

StDPE1      --------------------------GLIEGAVSSVARIAIIPMQDVLGLGSDSRMNI    538
AtDPE1      --------------------------SVIQAAFSSTAQTAIIPMQDILGLGSSARMNT    538
SpAM        --------------------------AMLRTVFSSVSFMAIATMQDLLELDEAARMNF    461
TaAM        --------------------------ALMGLCLKSVARLAIYPVQDVLALGSEARMNY    465
TtAM        --------------------------ALMHLGMKSVARLAVYPVQDVLALGSEARMNY    465
CgAM        AASANALPAREYVGLERDQRGELAELLEGLHTFVAK--TPSALTCVCLVDMVGEKRAQNQ    662
EcAM        LHKYGCLLKR--AGHKASLMSMTPTLNRGLQRYIAD--SNSALLGLQPEDWLDMAEPVNI    649
                               .:     .  :  *:  :     :.      *

StDPE1      PATQ---FGNWSWRIPSSTSFDNLDAEAKKLR---DILATYGRL---------    576
AtDPE1      PATE---VGNWGWRIPSSTSFDNLETESDRLR---DLLSLYGRL---------    576
SpAM        PSTL---GGNWSWRMTEDQLTP---AVEEGLL---DLTTIYRRINENLVDLKK    505
TaAM        PGRP---SGNWAWRLLPGQLTQ---EHAARLL---AMAEATGRT---------    500
TtAM        PGRP---SGNWAWRLLPGELSP---EHGARLR---AMAEATERL---------    500
CgAM        PGTTRDMYPNWCIPLCDSE-------GNSVLIESLRENELYHRVAKASKRD--    706
EcAM        PGTS-YQYKNWRRKLSATLETMFADDGVNKLLKDLDRR---RR-AAAKKK---    694
                *.        **   :           *           *
```

(Continued) *St*DPE1 = Disproportionating enzyme from *Solonum tuberlusum*, *At*DPE1 = Disproportionating enzyme from *Arabidopsis thaliana, Sp*AM = Amylomaltase from *Streptococcus pneumonia*, *Ta*AM = Amylomaltase from *T. aquaticus*, *Tt*AM = Amylomaltase from *T. thermophilus*, *Cg*AM = Amylomaltase from *C. glutamicum* and *Ec*AM = Amylomaltase from *E.coli*

APPENDIX 19: HPAEC profile of standard 0.8 mg/ml LR-CD
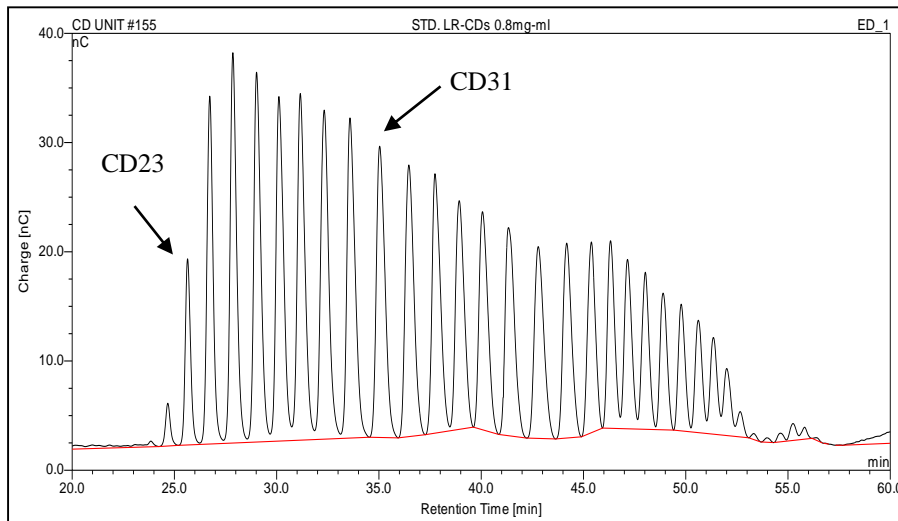
APPENDIX 20: Isothermal titration calorimetry (ITC)

ITC is the direct method to measure the heat change that occurs when two molecules are interacted at a constant temperature (Jelesarov and Bosshard, 1999). Heat is released or absorbed as a result of the redistribution of non-covalent bonds when the interacting molecules go from free to the bound state. The experiment is performed by titrating one binding partner (titrant or L) in syringe into a solution containing the other binding partner (titrand or M) in the sample cell of the calorimeter. After each addition of a small aliquot of L, the heat released or absorbed in the sample cell is measured with respect to a reference cell filled with buffer or control sample. The heat change is expressed as the electrical power (μcal/sec) required to maintain a constant small temperature difference between sample cell and reference cell, which are both placed in adiabatic jacket (Freyer and Lewis, 2008).

ITC is a one particular type of calorimeter of several that can be used for enzyme kinetic analysis (Hansen *et al*., 2016). In general, kinetic data obtained from ITC are in the form of the rate of heat production, $(dQ_{measured}/dt)$ (Hansen *et al*., 2016). Two types of kinetics of enzyme-catalyzed reactions by ITC was divided into 2 method; multiple injection method and single injection method. The multiple injection method is done by successive injections of substrate solution into an enzyme solution which is commonly used to investigate binding property of biomolecules and enzyme kinetics (Jelesarov and Bosshard, 1999). While single injection method is used for determining enzyme activity which usually requires less experiment time than multiple injection method (Transtrum *et al*., 2015).
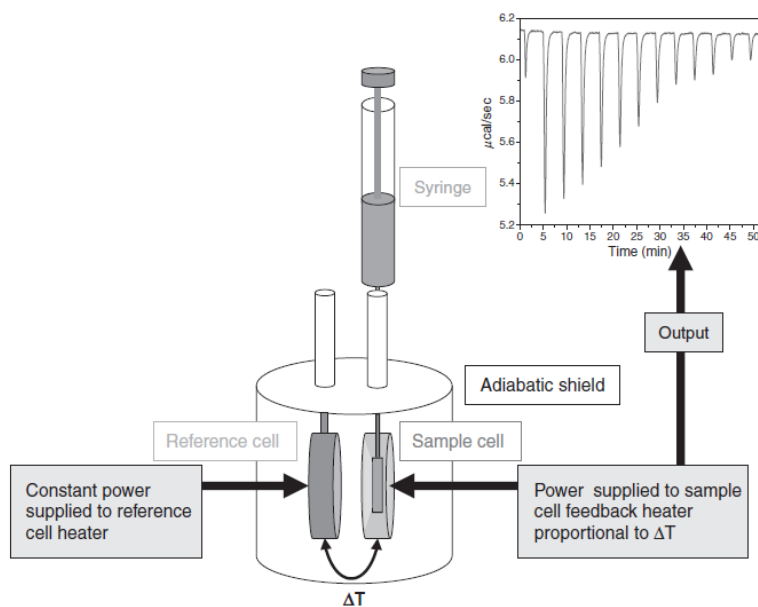
Represent diagram of ITC instrument. Major features of instrument such as the references and sample cells, syringe for adding titrant and the adiabatic shield are noted in figure. This diagram shows an oversimplification how the power applied by the instrument to maintain constant temperature between the references and sample cells is measured resulting instrument signal (Freyer and Lewis, 2008).

**APPENDIX 21: Differential scanning calorimtery (DSC)**

DSC is used to study thermal transitions of biological macromolecules such as protein when temperature increases (Freire, 1995). DSC instrument monitors a small temperature difference between a reference cell that is filled with water or buffer and sample cell that contains the protein of interest in an identical solvent (Johnson, 2013). As the temperature of both cells is increased thermally induced process occurring in the sample cell resulted in heat being absorbed and this produces a change in the temperature difference relative to the reference cell (Jelesarov and Bosshard, 1999). Heaters on the sample cell surface in a feedback circuit input additional electrical power to return the temperature difference to its initial value. This is additional heat is proportional to the excess heat capacity of the thermally induced process. For simple proteins a single heat absorption peak is often observed in the scan.

DSC experiment gives the partial specific heat capacity and transition midpoint or melting temperature (T_m) which is considered as the temperature that half of the protein structure is in native stage and another is denatured (Johnson, 2013). Beside T_m, T_p or peak temperature can be determined. T_p represents the highest value of DSC peak temperature which is easily obtained and also be designated as the thermal stability of molecules (Gill et al., 2010).

A   **B**
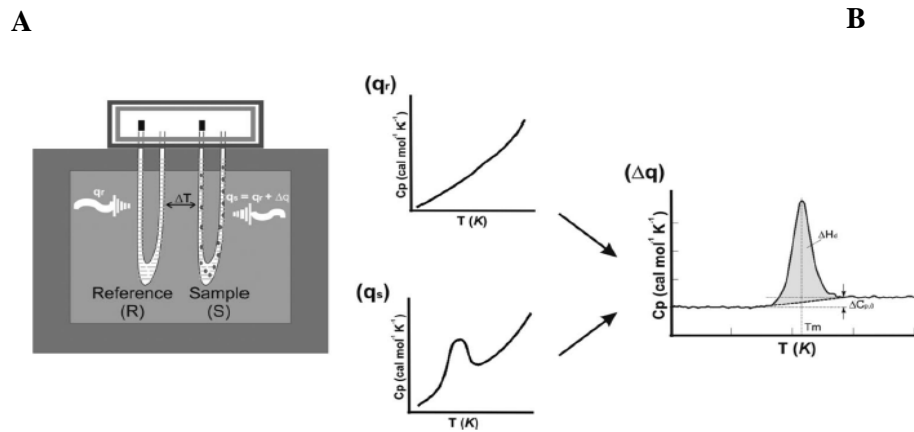
Represent diagram for a DSC experiment. The amount of heat required to increase the temperature by the same increments (ΔT) of a sample cell (qs) is higher than that required for the references cell (qr) by the excess heat absorbed by the molecules in the sample (Δq) (A). The resulting DSC scans with the reference subtracted from the sample show how this excess heat changes as a function of temperature. T (K), temperature Kelvin, ;ΔH_d, change in enthalpy; $\Delta C_{p,d}$, change in C_p; T_m, transition and melting point; $_d$, denatured (B) (Gill *et al*., 2010).

APPENDIX 22: Heat production of amylomaltase from disproportionation reaction detected by ITC technique

WT *Cg*AM   Y418A   Y418W
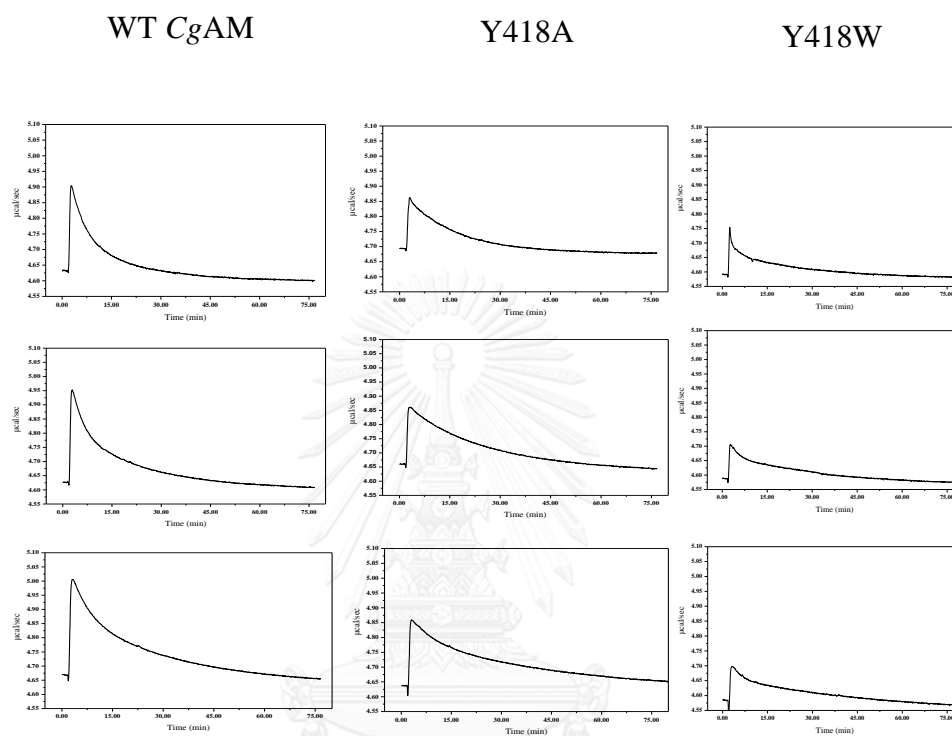
VITA

Miss Suthipapun Tumhom was born on April 16th, 1985. She graduated with the first class honors in Bachelor degree of Science (Biology) from Faculty of Science, Silpakorn University in 2007. Then she is studying in Ph. D program of the Biochemistry and Molecular Biology, Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2011. She has received Science Achievement Scholarship of Thailand since her undergraduate study till Doctoral degree.

Publications:

Suthipapun Tumhom, Kuakarun Krusong, and Piamsook Pongsawasdi, Y418 in 410s loop is required for high transglucosylation activity and large-ring cyclodextrin production of amylomaltase from Corynebacterium glutamicum, Biophysical and Biochemical Research Communication, 488 (2017), 515-521.

Suthipapun Tumhom, Kuakarun Krusong, Shun-ichi Kidokoro, Etsuko Katoh and Piamsook Pongsawasdi, Biophysical characterization of wild type and Y418 mutated amylomaltases from Corynebacterium glutamicum (under submission to PlOSONE).

Awards:

2015   The 1st Runner up for poster presentation in "Cell and Molecular Biology" theme in the 20th Biological Science Graduate Congress, Chulalongkorn University, Bangkok, Thailand

2016   Outstanding Oral Presentation Award in "The 5th International Biochemistry and Molecular Biology Conference, Prince of Songkla University", Songkla, Thailand.

Grants and fellowships:

2003 - 2006   Science Achievement Scholarship of Thailand (SAST) for Bachelor degree of Science in Biology, Silpakorn University, Thailand

2011 - 2016   Science Achievement Scholarship of Thailand (SAST) for Doctorate degree of Science in Biochemistry and Molecular biology, Chulalongkorn University

2016   Research Grant "Japan Student Service Organization (JASSO)" at Nagaoka University of Technology, Nagaoka, Japan

2016   The 90th anniversary of Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University

2017   The oversea research experience scholarship, Graduate school and Faculty of Science, Chulalongkorn University