การกลายพันธุ์ยีนไซโคลเดกซ์ทรินกลูคาโนแทรนส์เฟอเรสจาก *Bacillus circulans* A11 เพื่อแปลี่ยนแปลงความจำเพาะของเอนไซม์ต่อการสร้างผลิตภัณฑ์

นายการัณยภาส พรมเคนสา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2550

MUTAGENESIS OF CYCLODEXTRIN GLUCANOTRANSFERASE GENE FROM *Bacillus circulans* A11 TO CHANGE THE PRODUCT SPECIFICTY OF THE ENZYME

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การัณยภาส พรมเดนสา : การกลายพันธุ์ยีนไซโคลเดกซ์ทรินกลูคาโนแทรนส์เฟอเรสจาก *Bacillus circulans* A11 เพื่อเปลี่ยนแปลงความจำเพาะของเอนไซม์ต่อการสร้างผลิตภัณฑ์ (MUTAGENESIS OF CYCLODEXTRIN GLUCANOTRANSFERASE GENE FROM *Bacillus circulans* A11 TO CHANGE THE PRODUCT SPECIFICITY OF THE ENZYME) อาจารย์ที่ปรึกษา : รศ.ดร. วิเชียร ริมพณิชยกิจ, 80 หน้า

ไซโคลเดกซ์ทริน (CD) เป็นออลิโกแซ็กคาไรด์ ที่ประกอบด้วยกลูโคสจำนวน 6, 7 หรือ 8 หน่วย มาเชื่อมต่อกันเป็นวงด้วยพันธะ α-1,4-glycosidic มีชื่อเรียกว่า α-, β- และ γ-ไซโคลเดกซ์ทริน ตามลำดับ CD เป็นผลิตภัณฑ์ที่เกิดจากการย่อยแป้งของเอนไซม์ไซโคลเดกซ์ทรินกลูคาโนแทรนส์เฟอเรส (CGTase) ซึ่งสามารถนำไปใช้ประโยชน์ในอุตสาหกรรมต่างๆ ได้ CGTase ประกอบด้วย 5 โดเมน คือ A, B, C, D และ E โดเมน A/B เป็นโดเมนเร่งปฏิกิริยาในขณะที่โดเมนอื่นทำหน้าที่อื่นๆ ประกอบ CGTase ที่ผลิต CD ได้เพียงชนิดเดียวมีความสำคัญมากสำหรับอุตสาหกรรมการผลิต CD เพราะว่าการแยก CD ต่างชนิดมีต้นทุนสูงและใช้เวลามาก การเปรียบเทียบลำดับกรดอะมิโนของโดเมน A2 ระหว่าง β-CGTase จาก *Bacillus circulans* A11 กับ α- และ γ-CGTase พบว่า 4 บริเวณที่แตกต่าง คือ บริเวณ I, II, III และ IV ที่ตำแหน่งกรดอะมิโน 239-250, 262-271, 284-320 และ 333-342 (ตำแหน่งกรดอะมิโนของ CGTase จาก *B. circulans* A11) ตามลำดับ ยิ่งไปกว่านั้นซีรีนตำแหน่ง 184 กลูตาเมตตำแหน่ง 264 และ 268 ได้ถูกเลือกเพื่อศึกษารูปแบบทางเข้าจับของสับสเตรตด้วย ทั้ง 4 บริเวณที่แตกต่างใน β-CGTase ของ *Bacillus circulans* A11 ได้ถูกกลายพันธุ์โดยวิธี unique site elimination (USE) mutagenesis เช่นด้วยกับซีรีนตำแหน่ง 184 กลูตาเมตตำแหน่ง 264 และ 268 dextrinizing activity และ CD forming activity ของเอนไซม์กลายพันธุ์ถูกวิเคราะห์ พบว่าบริเวณกลายพันธุ์ I ไม่มีอิทธิพลต่อความจำเพาะของการสร้างผลิตภัณฑ์ บริเวณกลายพันธุ์ II และ III สนับสนุนการสร้างผลิตภัณฑ์ β-CD บริเวณกลายพันธุ์ IV แสดงผลกระทบเล็กน้อยต่อการสร้างผลิตภัณฑ์ β-CD และการกลายพันธุ์ซีรีนตำแหน่ง 184 เป็นไลซีนเพิ่มการสร้างผลิตภัณฑ์ α-CD เพียงเล็กน้อยกับลดการสร้างผลิตภัณฑ์ γ-CD

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....การัณยภาส พรมเดนสา.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550.....

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KARANYAPAS PROMCANSA : MUTAGENESIS OF CYCLODEXTRIN GLUCANOTRANSFERASE GENE FROM *Bacillus circulans* A11 TO CHANGE THE PRODUCT SPECIFICITY OF THE ENZYME. THESIS ADVISOR : ASSOC. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D. 80 pp.

Cyclodextrins (CDs) are cyclic oligosaccharides of 6, 7 and 8 glucose units, linked by α-1,4-glycosidic bonds, called α-, β- and γ-cyclodextrins, respectively. CDs are the products of enzymatic conversion of starch and related substrates by cyclodextrin glucanotransferases (CGTases), and are useful carrier molecules for several applications in industries. The CGTase consists of 5 domains, A, B, C, D and E. Domains A/B are the central catalytic domain while others perform accessory functions. CGTases that predominantly produce one type of cyclodextrin are of great importance for industry because the costly and time-consuming separation of different cyclodextrins can be avoided. Amino acid sequence comparison of subdomain A2 between the *Bacillus circulans* A11 β-CGTase and the α- and γ-CGTases, four different regions I, II, III and IV were found at position 239-250, 262-271, 284-320 and 333-342 (*B. circulans* A11 CGTase numbering), respectively. In addition, S184, E264 and E268 were also selected to study the mode of substrate entrance. The four regions as well as S184, E264 and E268 in β-CGTase from *Bacillus circulans* A11 were mutated using the unique site elimination (USE) mutagenesis method. The dextrinizing activity and CD-forming activity of the mutant enzymes were assayed. It was found that region I mutation had no influence on product specificity, regions II and III favored β-CD production, and region IV showed little affect on β-CD production. The S184K mutation slightly increased the proportion of α-CD with the expense of γ-CD.

Department.....Biochemistry..... Student's signature Karanyapas Promcansa
Field of study.....Biochemistry.....Advisor's signature .....
Academic year.....2007.....

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Last but not least, the greatest gratitude is expressed to my family for their love, care, support and understanding that I have spent my time with this thesis rather than with them. Finally, I would like to thanks myself for my patience, intention and attempts.

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ABBREVIATION

BSA	Bovine serum albumin
CDs	Cyclodextrins
CGTase	Cyclodextrin glucanotransferase
ºC	Degree Celsius
µl	Microlitre
ml	Millilitre
mM	Millimolar
µM	Micromolar
M	Molar
µg	Microgram
mg	Milligram
rpm	Revolution per minute
nm	Nanometre
ddH_2O	Double Distilled water

CHAPTER I

INTRODUCTION

Many plants produce starch, a high molecular weight polymer of glucose, for storage as a carbon and energy source. These starch molecules are mostly found in seeds (e.g. wheats) or roots (e.g. potato) in the form of granules consisting of two types of glucan polymers, highly branched amylopectin and linear amylose. Many bacteria are able to use starch as a carbon and energy source for growth. For this purpose, these microorganisms convert starch molecules extracellularly into molecules suitable for uptake and further conversion by the cells. A whole range of starch-degrading enzymes with different reaction specificities has evolved in these organisms yielding a wide variety of products (Fig. 1.1).

1.1. Starch degrading enzymes and CGTase

A number of these enzymes find application in the industrial processing of starch, either for the modification of starch molecules or for the production of specific degradation products. These enzymes are therefore studied extensively, leading to more knowledge of their reaction mechanisms and the factors determining substrate and product specificity.

A particularly interesting enzyme is cyclodextrin glycosyltransferase (CGTase; 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase, EC. 2.4.1.19), which has the unique capability of converting starch into cyclic oligosaccharides, called cyclodextrins (CDs). The enzyme is a member of the α-amylase family (family 13) of glycosyl hydrolases. (van der Veen *et al*., 2000). Since the first report of a *Bacillus*

macerans strain capable of producing CDs from starch, many CGTases have been purified and biochemically characterized. CGTase has received considerable attention, since CDs have many applications in food, cosmetic and pharmaceutical industries (Qi and Zimmermann, 2004). The enzyme is generally found in bacteria and was recently also discovered in archaea. Table 1.1 lists the bacterial species producing CGTase, which can be used for CD production.

When these bacteria excrete CGTases into the starch medium, the CGTases convert starch into CDs, which are subsequently absorbed and hydrolyzed by another enzyme cyclodextrinase [CDase; cyclomaltodextrin dextrin-hydrolase (decyclizing), EC 3.2.1.54].

1.2. Cyclodextrins

CDs are cyclic oligosaccharides composed of α-1,4-glycosidic-linked glucosyl residues produced from starch or starch derivates using cyclodextrin glycosyltransferase (CGTase). There are three major types of CDs according to the number of glucosyl residues in the molecule: α-, β- and γ-CDs consisting of 6, 7 or 8 glucose units, respectively (Fig. 1.2a).

The glucose residues in the CD ring are arranged in a manner that the secondary hydroxyl groups at C2 and C3 are located on one edge of the ring and the primary hydroxyl groups (C6) on the other edge. The apolar C3 and C5 hydrogens and the ether-like oxygens are at the inside and the hydroxyl groups at the outside of these molecules. These results in a torus-shaped molecule with a hydrophilic outside, which can dissolve in water, and a hydrophobic cavity, which can form inclusion complexes with a wide variety of poorly water soluble compounds, called guest

molecules (Fig. 1.2b and 1.2c). The characteristics of the CDs are summarized in Table 1.2, which provide important parameters for complex formation with hydrophobic compounds (Szejtli, 1998; van der Veen *et al.*, 2000a).

The formation of inclusion complexes leads to changes in the chemical and physical properties of the encapsulated compounds. This has led to the various applications of CDs in chemistry, agriculture, pharmaceutical, food, textile, biotechnology and environmental protection as listed in Table 1.3.

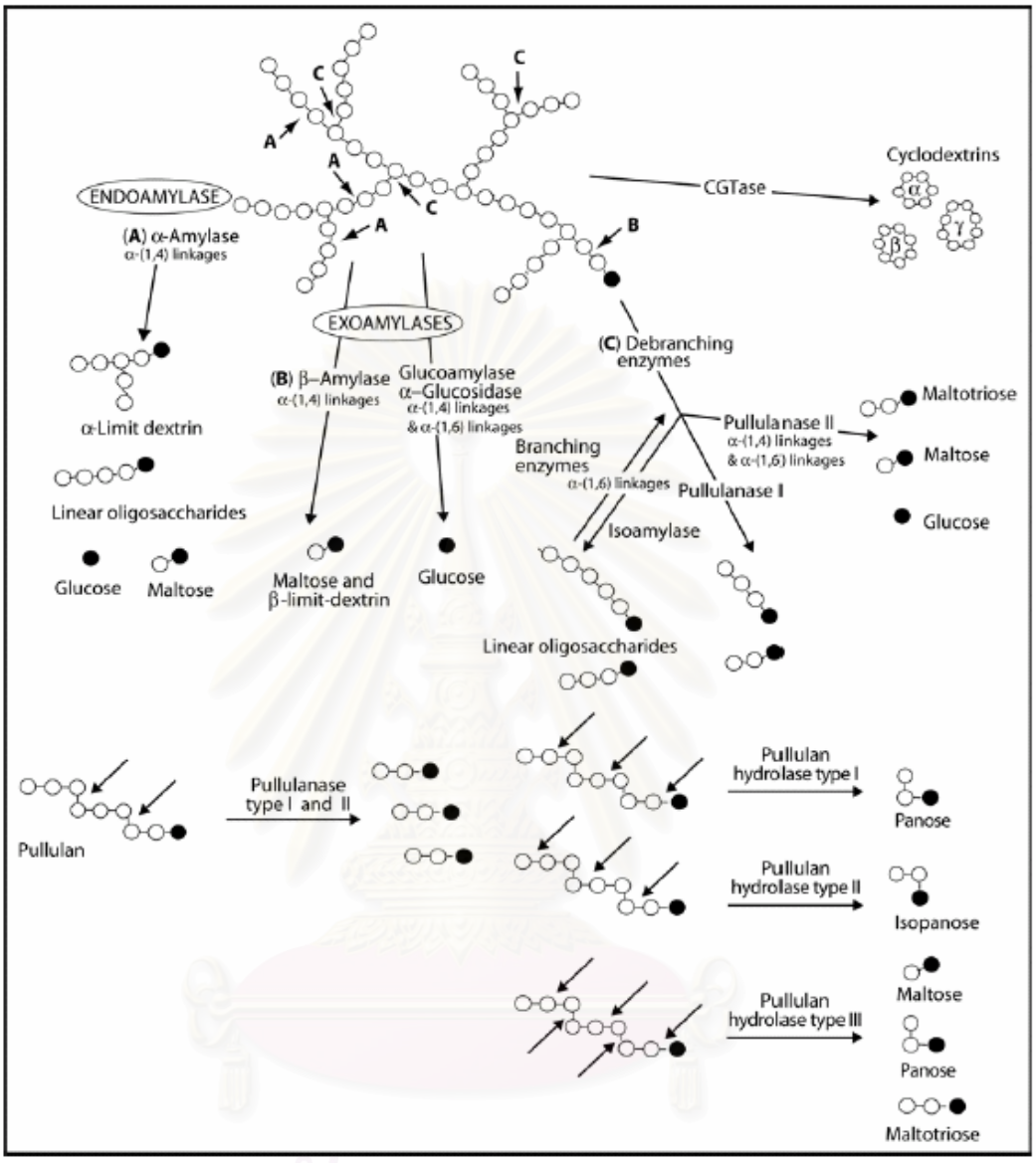

Fig. 1.1. Schematic representation of the action of starch-processing enzymes. (•), The reducing end glucose molecule; (o) the non-reducing end glucose molecule (Hobel, 2004).

Table 1.1. Bacterial species producing cyclodextrin glycosyltransferases (CGTase) for use in CD production (Biwer *et al.*, 2002).

CGTase	Reference
Bacillus macerans	Shiraishi *et al*. 1989; Lee *et al*. 1992; Shieh and Hedges 1994; Starnes 2001
B. megaterium	Bender 1986; Ammeraal 1988; Lee *et al*. 1992; Drauz and Waldmann 1995; Starnes 2001
B. circulans	Bender 1986; Mattsson *et al*. 1991; Lee *et al*. 1992; Van der Veen *et al*. 2000
B. stearothermophilus	Bender 1986; Ammeraal 1988; Lee *et al*. 1992; Drauz and Waldmann 1995; Starnes 2001
B. ohbensis	Bender 1986; Ammeraal 1988; Lee *et al*. 1992; Starnes 2001
B. licheniformes	Kobayashi 1996
B. cereus	Jamuna *et al*. 1993
Klebsiella pneumoniae	Bender 1986; Ammeraal 1988; Lee *et al*. 1992; Drauz and Waldmann 1995; Starnes 2001
K. oxytoca	Lee *et al*. 1992; Choi *et al*. 1996
Micrococcus lutens	Yagi *et al*. 1982; Bender 1986; Lee *et al*. 1992
M. varians	Yagi *et al.* 1982; Bender 1986; Lee *et al.* 1992; Starnes 2001)
Clostridium spp.	Slominska and Sobkowiak 1997
Thermoanaerobacter spp.	Lee *et al*. 1992; Starnes 2001
Thermoanaerobacterium thermosulfurigenes	Wind *et al.* 1995, 1998; Starnes 2001
Anaerobranca gottschalkii	Prowe and Antranikian 2001

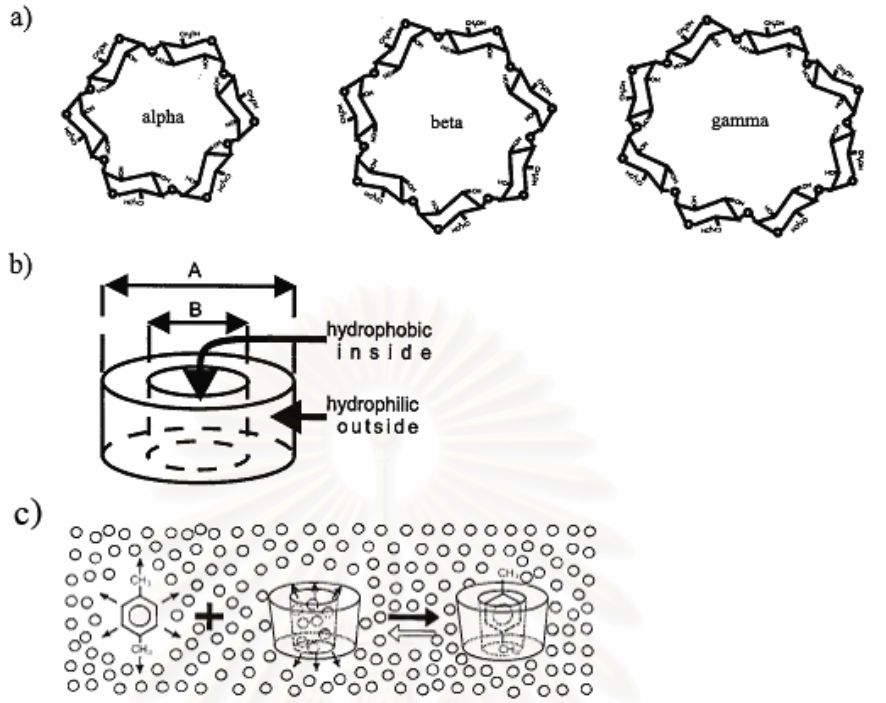

Fig. 1.2. Structures and properties of CDs. (a) α-, β- and γ-CDs; (b) three-dimensional model and property of CD; (c) formation of the inclusion complex of a CD with a guest molecule. p-Xylene is the guest molecule; the small circles represent the water molecules (Szejtli, 1998; van der Veen *et al*., 2000a).

Table 1.2. Characteristics of α-, β-, and γ-CDs. (Szejtli, 1998)

Cyclodextrin	**α**	**β**	**γ**
No. of glucose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water, g 100 mL^{-1} at room temp.	14.5	1.85	23.2
Cavity diameter, Å	4.7-5.3	3.6-6.5	7.5-8.3
Height of torus, Å	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Approx volume of cavity, Å	174	262	427
Crystal forms (from water)	Hexagonal plates	Monoclinic parallelograms	Quadratic prisms

Table 1.3. Applications of CDs in different industries (Biwer *et al.*, 2002).

Application	Industry	Reference
Stabilisation of volatile or unstable compounds	Food, pharmaceutical	Horikoshi *et al*. 1981; Bender 1986; Hedges 1992; Maggi *et al*. 1998; Liese *et al*. 2000
Reduction of unwanted tastes and odour	Food, pharmaceutical	Bender 1986; Hashimoto 1996; Nagai and Ueda 1996; Liese *et al.* 2000; Buschmann *et al.* 2001
Gelling and thickening agent	Food	Hashimoto 1996
Protection from decomposition induced by light, temperature and air	Food	Hedges 1992; Pedersen *et al*. 1995; Hashimoto 1996; Buschmann *et al.* 2001
Removal of cholesterol	Food	Buschmann *et al*. 2001
Dietary fibre and calorie substitute for weight control	Food	Lee *et al*. 1992
Perfuming fabrics or loading with antiseptic substances	Textile	Denter *et al.* 1997; McCoy 1999
Auxiliary material in production processes (e.g. fatty acids, benzyl alcohols, antibiotics)	Biotechnology	Bar 1996
Improvement of bioavailability and reduction of side effects (e.g. Ibuprofen)	Pharmaceutical	Nagai and Ueda 1996; Uekama and Irie 1996; Brunet *et al.* 1998; Maggi *et al.* 1998
Intermediate in drug production	Pharmaceutical	McCoy 1999
Separation of isomers	Chemistry	Hedges 1992; Szejtli 1996b; Brunet *et al.* 1998
Solution of water-insoluble compounds	Chemistry, food	Bender 1986; Hedges 1992; Pedersen *et al*. 1995; Hashimoto 1996; Buschmann *et al.* 2001
Additives in pesticides	Agriculture	Szente and Szejtli 1996
Control of plant growth	Agriculture	Brunet *et al.* 1998
Immobilisation of toxic compounds (e.g. heavy metals, trichloroethene)	Environmental protection	Bar 1996; Szejtli 1996b; Wilson 1999
Improving decomposition of stable compounds (e.g. trichlorfon) and sewage sludge	Environmental protection	Szejtli 1996b

1.3. Limitation of commercial cyclodextrin production

A major disadvantage of CD production by CGTases is that all known wild-type CGTase enzymes produce a mixture of α-, β-, and γ-CDs and are sensitive to product inhibition by these cyclic compounds. The *Thermoanaerobacter* CGTase for instance produces an approximately equal mixture of α- and β-CDs with small amount of γ-CD.

Two different industrial approaches are used to purify the produced CDs: selective crystallization of β-CD (which is relatively poorly water-soluble) and selective complexation with organic solvents. These processes not only serve to purify the CDs, but also result in decreased product inhibition, enhancing the total conversion of starch to CDs from 40% to 60% (Bergsma *et al.*, 1988). Toluene and cyclohexane are commercially used for the complexation and selective precipitation of β-CD. For α-CD 1-decanol can be used, but this compound is difficult to remove from aqueous solution because of its high boiling point (229ºC). Cyclododecanone can be used for complexation and selective precipitation of γ-CD, but this solvent is too expensive for commercial use. Further disadvantages of the use of organic solvents are their toxicity, their flammability, and the need for a solvent recovery process (Pedersen *et al.*, 1995). The use of organic solvents also limits the applications involving human consumption. Also the processes used for β-CD production are not ideal, since they make the production of CDs too costly for many applications and limit the availability of α- and γ-CDs. Consequently, there is a great demand for a process that could produce these CDs economically.

Clearly, the availability of CGTase enzymes capable of producing an increased ratio of one particular type of CD and with reduced product inhibition

would help to avoid the above-described expensive and environmentally harmful procedures involving organic solvents. This situation has strongly stimulated studies of CGTase structure–function relationships, the mechanisms of CGTase catalysed reactions and the product inhibition. Till now, detailed knowledge has become available, allowing rational design of CGTase with improved cyclodextrin product specificity and reduced product inhibition (Pedersen *et al*., 1995; Dijkhuizen *et al*., 1996; Dijkhuizen *et al*., 1999).

1.4. Three-dimensional structure of CGTase

Primary and three-dimensional structural comparisons between CGTases and α-amylases have revealed both common and distinct features among the enzymes. Both CGTases and α-amylases share three structural domains: A, B and C (Rashid *et al*., 2002) (Fig. 1.3 and 1.4). Domain A comprises 300-400 amino acid residues and contains a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices. This $(\beta/\alpha)_8$ or TIM barrel catalytic domain is present in all enzymes of the α-amylase family. Several proline and glycine residues flanking the loops connecting the β-strands and α-helices are highly conserved in these enzymes (Janecek, 1997 and Janecek and Sevcik, 1999). The catalytic residues, Glu257, Asp229 and Asp328 (*B. circulans* A11 CGTase numbering) and substrate binding residues are located in the loops at the C-termini of β-strands in domain A. On the basis of the studies from X-ray crystallography, it has been proposed that the active center of CGTase possesses a tandem subsite architecture in the substrate binding groove, and that it comprises at least nine sugar-binding subsites, designated from the

nonreducing end to the reducing end as -7 to +2 (Fig. 1.5) (van der Veen *et al*. 2000a; Strokopytov *et al*., 1996).

Domain B is an extended loop region inserted between β-strand 3 and α-helix strand 3 of domain A. It is rather large and is regarded as a separate structural domain. This domain B consists of 44-133 amino acid residues and contributes to substrate binding by providing several amino acid side chain alongside a long groove on the surface of the enzyme that interact with the substrate (Uitdehaag *et al*., 2002). Domain C is approximately 100 amino acids long and has an antiparallel β-sandwich fold. This domain C of the CGTase from *B. circulans* strain 251 contains one of the three-maltose binding sites (Lawson *et al.*, 1994). This maltose binding site is found to be involved in raw starch binding (Penninga *et al*., 1996a), suggesting a role of the domain C in substrate binding. Others suggest that this domain is involved in bond specificity, particularly in enzymes hydrolyzing or forming the α-(1,6) bonds, e.g. pullulanase, isoamylase and branching enzymes.

CGTase have two additional domains not found in α-amylases, domains D and E, which have the β-sheet structures (Fig. 1.3). The domain D, consisting of approximately 90 amino acids with an immunoglobulin fold, is almost exclusively found in CGTases and its function is unknown. The domain E consists of approximately 110 amino acids and is found to be responsible for substrate binding. Two maltose-binding sites have been identified in domain E of CGTase from *B. circulans* 251. Evidences that this domain contributes to raw-starch binding have also been obtained from other CGTases (Svensson *et al*., 1989).

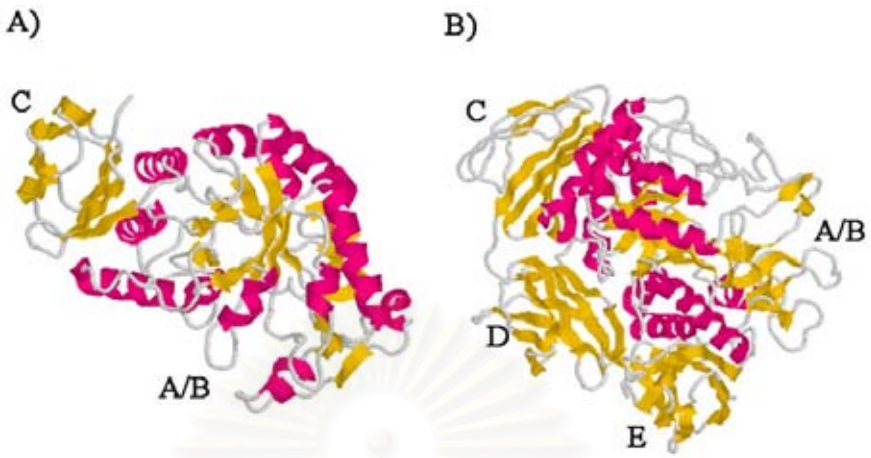

Fig. 1.3. Comparison of the three dimensional structures of α-amylase and CGTase. A) α-amylase from *Bacillus subtilis* (PDB 1BAG); B) CGTase from *Bacillus circulans* strain 251 (PDB 1CDG).

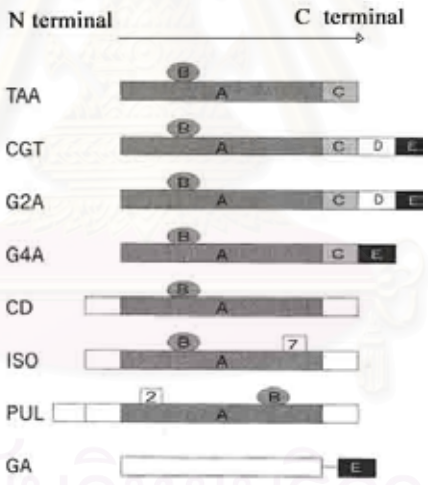

Fig. 1.4. Domain level organization of starch-degrading enzymes. CGT, CGTase from *B. circulans*; G2A, maltogenic α-amylase from *B. stearothermophilus*; G4A, maltotetraose forming α-amylase from *Pseudomonas stutzeri*; TAA, α-amylase from *A. oryzae* (Taka-amylase A); CD, cyclodextrinase from *Klebsiella oxytoca*; ISO, isoamylase from *P. amyloderamosa*; PUL, pullulanase from *K. aerogenes*; GA, glucoamylase (family 15 of glycosyl hydrolases) from *Aspergillus niger* (van der Veen *et al*., 2000a).

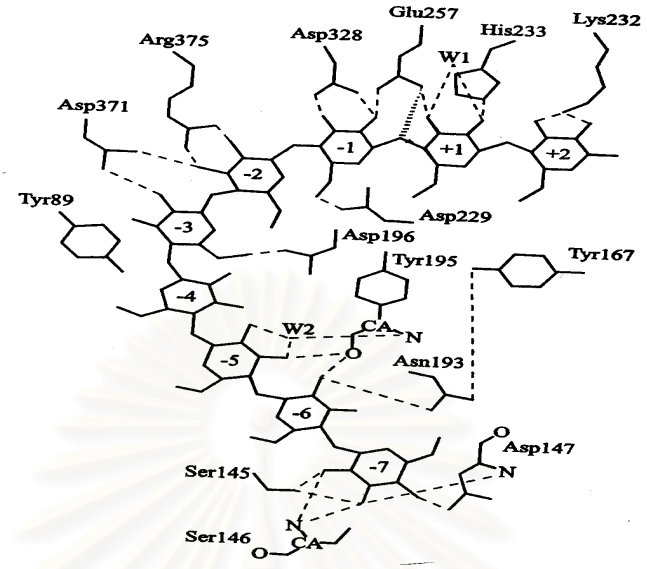

Fig. 1.5. Schematic representation of the hydrogen bonds between the *B. circulans* strain 251 CGTase and a maltononaose inhibitor bound at each subsite of the active site. W1 and W2 indicate water and CA indicates alpha carbon (Source: van der Veen *et al.*, 2000a).

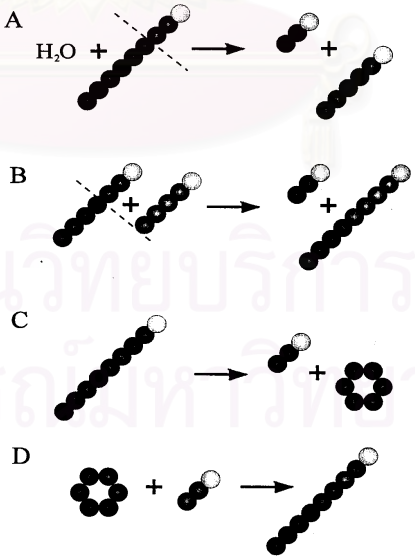

Fig. 1.6. Schematic representation of the CGTase-catalysed reactions. The circles represent glucose residues; the white circles indicate the reducing end sugars. (A) hydrolysis; (B) disproportionation; (C) cyclization; (D) coupling (van der Veen *et al.*, 2000a).

1.5. CGTase reaction and its catalytic mechanism

The CGTase catalyzes four distinctly different reactions, depending on the nature of the acceptor molecule: three transglycosylation reactions (disproportionation, cyclization and coupling) and a hydrolysis reaction (Fig. 1.6).

i) Disproportionation is an intermolecular transglycosylation reaction. In this reaction a linear malto-oligosaccharide is cleaved and one of the products is transferred to another linear acceptor substrate. This reaction yields a mixture of smaller and longer oligosaccharides.

ii) Cyclization is an intramolecular transglycosylation reaction, in which the non-reducing end of the same oligosaccharide is transferred to the reducing end of the same oligosaccharide chain. The products of this reaction are cyclodextrins.

iii) Coupling is the reverse of the cyclization reaction in which the opening of a cyclodextrin ring is followed by the transferring of the oligosaccharide to a linear saccharide acceptor. This reaction produces a longer chain oligosaccharide.

iv) Hydrolysis is a reaction in which part of a linear oligosaccharide is transferred to water. This reaction is much weaker than the transglycosylation reactions.

Like the α-amylase family, CGTase has the catalytic mechanism as the α-retaining double displacement mechanism. This mechanism involves two catalytic residues, an acid/base catalyst (Glu257 in CGTase) and a nucleophile (Asp229) (Fig. 1.7). When substrate binds, Glu257 (as acid) donates a proton to the scissile glycosidic bond oxygen. The scissile bond between subsite +1 and -1 is then cleaved. During the cleavage reaction, the -1 sugar goes through an oxocarbonium ion-like transition state, then collapses into a stable covalent glycosyl enzyme reaction

intermediate which is β-glycosidically linked to the nucleophile Asp229. The leaving group (at subsite +1) is exchanged for a new compound, the acceptor, which gets activated by Glu257 (now a base). This activated acceptor attacks the covalent intermediate, and via another oxocarbonium ion-like transition state, the final α-1,4-glycosidic bond in the product is formed (Uitdehaag *et al*., 2002).

1.6. Sequence comparasion of the CGTase genes and the cyclodextrin products

In general, CGTases from different microorganisms produce all three types of cyclodextrins with different ratios. The CGTases thus can be classified into α-CGTase, α/β-CGTase, β-CGTase, β/γ-CGTase and γ-CGTase corresponding to their major cyclodextrin products. All five types of CGTases show clear similarity in their amino acid sequences, ranging from 47% to 99%. Fig. 1.8 shows the alignment of amino acid sequences of α-, β-, β/γ- and γ-CGTases from different sources (Takada *et al.*, 2003). Table 1.4 lists the amino acid residues that may be involved in catalytic function. The first specific CGTase residues that may be involved in product specificity are found in the region between α-strand 1 and α-helix 1, which consists of a stretch of amino acid residues 27-53 and is involved in calcium binding. Residue 47 is involved in the binding of (semi)cyclic oligosaccharides and is typically an Arg in β-CGTase, Lys in α-CGTase, Thr in β/γ- and γ-CGTases and His in the CGTase producing virtually no α-cyclodextrin (Fig. 1.8) (van der Veen *et al*., 2000c and Takada *et al*., 2003).

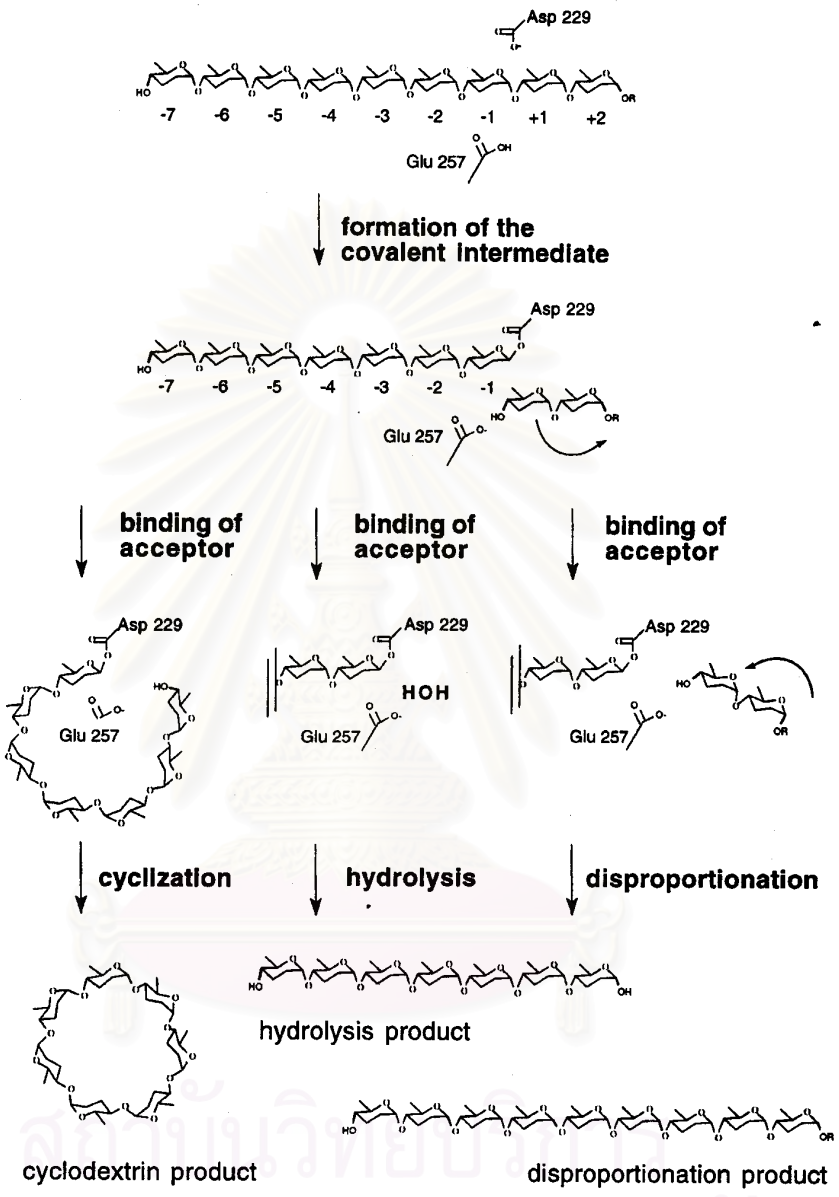

Fig. 1.7. The catalytic reaction of CGTase, which involves cyclization, disproportionation and hydrolysis (Uitdehaag *et al.*, 2000).

The second region is found at subsite -3. Amino acid residues 87-94 at subsites -3 form a hairpin turn. These amino acid residues show hydrophobic interactions with glucose unit bound in this region, and are remarkably different among the four types of CGTases. Both α- and β-CGTases have the sequence INYSGVN but the sequence HP-GGF- is found in β/γ- and γ-CGTases (Fig. 1.8 and Table 1.4) (Takada *et al*., 2003). The fact that the hairpin turn in γ- and β/γ-CGTase are shorter than those in other CGTases indicates that more space for the bound glucosyl chain in this region is needed for a higher level of γ-cyclization activity as shown in Fig.1.9.

Another specific region consists of residues 145-152 of α- and β-CGTases at subsite -7. The amino acids in this region are located in a loop at the start of the B-domain, and are SSTDPSFA and SSDQPSFA for α- and β-CGTases, respectively. The β/γ- and γ-CGTases completely lack the six amino acid residues in this region (D-I) (Fig. 1.8). This indicates that the absence of these amino acid residues may also be necessary for a higher level of γ-cyclization activity as it provides more space for the bound glycosyl chain in this region as shown in Fig.1.9.

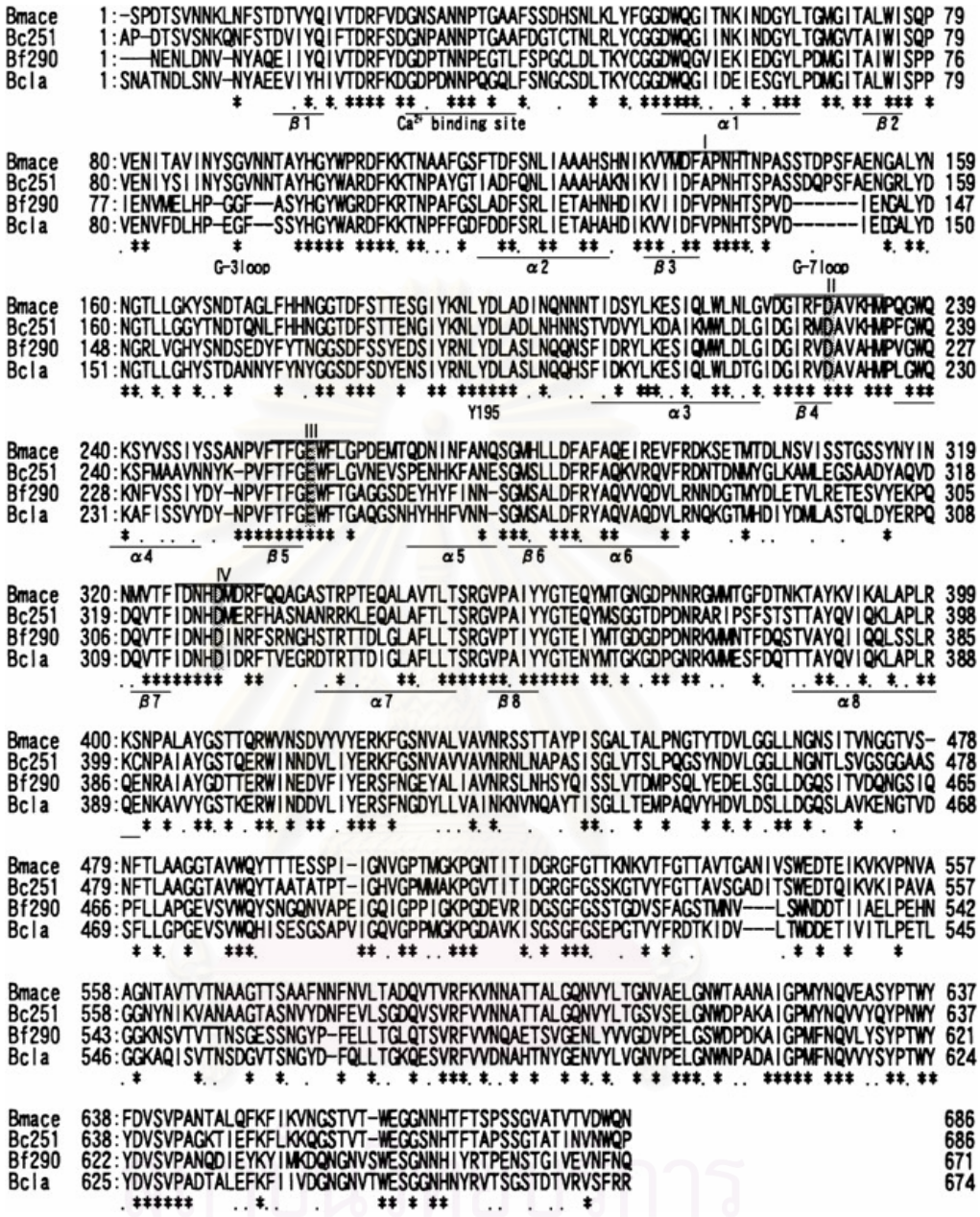

Figure 1.8. Alignment of amino acid sequences of typical α-, β-, β/γ-, and γ-CGTases. Bmac, Bc251, Bf290 and Bcla are the amino acid sequences of α-CGTase from *B (P). macerans*, β-CGTase from *B. circulans* 251, β/γ-CGTase from *Bacillus firmus* 290-3, and γ-CGTase from *B. clarkii* 7364, respectively (Source: Takada *et al.*, 2003).

Table 1.4. Comparison of the amino acid residues around the active center in the four types of CGTases. (Takada *et al.*, 2003 and Leemhuis *et al*., 2003b)

Residue No.	Residue in α-CGTase	Residue in β-CGTase	Residue in β/γ-CGTase	Residue in γ-CGTase	Function
Subsite +2					
183	F	F	F	F	Cyclization
232	K	K	A	A	Disproportionation
259	F/Y	F/Y	F	F	Cyclization and disproportionation
Subsite +1					
194	L	L	L	L	Cyclization
230	A	A	A	A	Transglycosylation*
233	H	H	H	H	General activity
Subsite -1					
The catalytic residues **(D229, E257 and D328)** are completely conserved.					
Subsite –2					
98	H	H	H	H	General activity
101	W	W	W	W	-
375	R	R	R	R	-
Subsite –3					
89	Y	Y	-	-	Cyclization specificity
87-93	INYSGVN	INYSGVN	HP-GGF-	HP-GGF-	Cyclization specificity
371	D	D	D	D	Cyclization
196	D	D	D	D	Cyclization
47	K	R	T	T	Cyclization
Subsite –4 and –5					
No side chain contacts					
Subsite –6					
167	Y	Y	Y	Y	-
179-180	GG	GG	GG	GG	Cyclization
193	N	N	N	N	Cyclization
Subsite –7					
145-152	SSTDPSFA	SSDQPSFA	D------I	D------I	Cyclization, Central specificity
195	Y	Y/F	Y	Y	Cyclization, Ca^{2-} binding site
32-36	NNPTG	NNPTG	NNPEG	NNPQG	Ca^{2+}binding

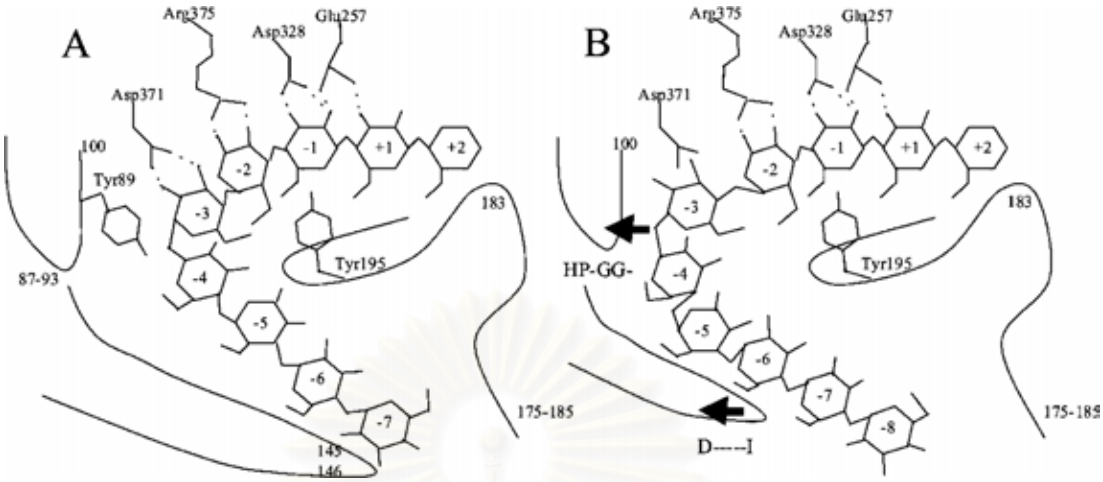

Figure 1.9. Schematic representation of the substrate binding site structures in CGTases. (A) β-CGTase; (B) γ-CGTase (Takada *et al.*, 2003).

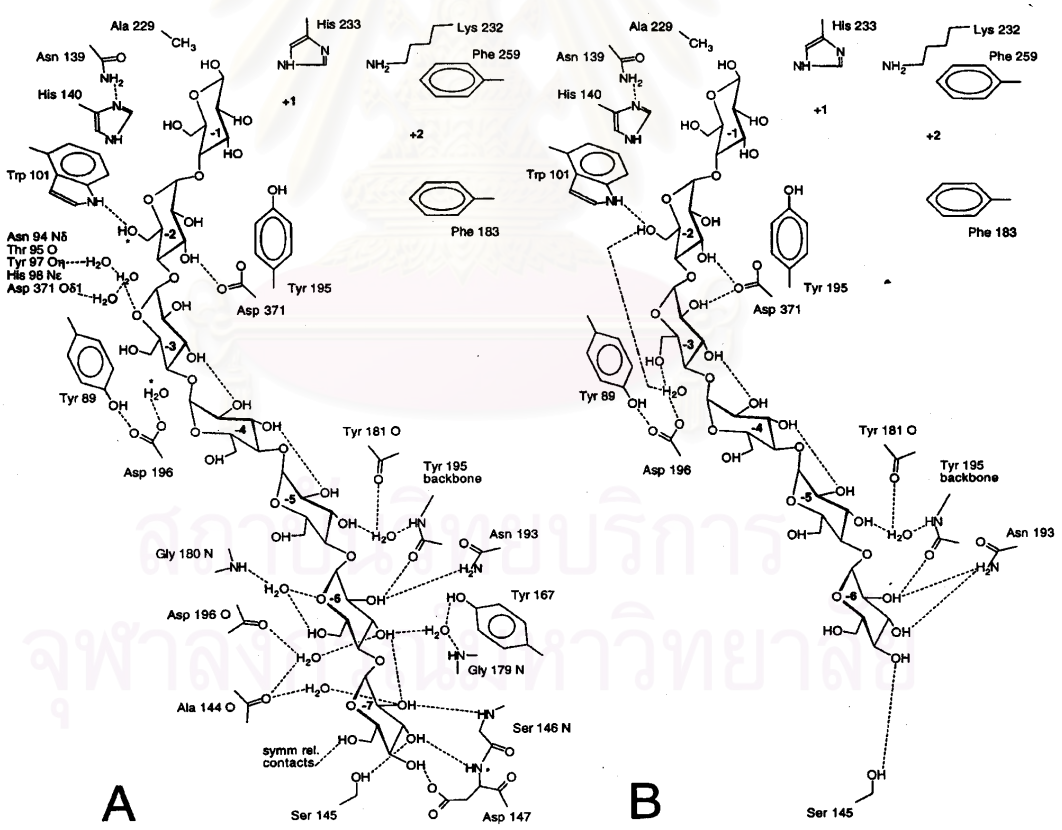

Figure 1.10. Schematic representations of all the interactions of (A) maltoheptaose, and (B) maltohexaose with the CGTase from *B. circulans* strain 251 (Source: Uitdehaag *et al.*, 2000).

1.7. Mutagenesis of CGTase

In the past, it was believed that the aromatic amino acid residue 195 (Phe or Tyr), located in the center of the active site cleft of the enzymes, played an important role in the cyclization reaction of CGTase and a mechanism for the α-(1,4)-glucan chain folding around this residue had been proposed (Strokopytov *et al*.,1996). Attempts had been made to substitute this amino acid residue with other amino acid residues to alter the product specificity. Substitution of this amino acid with Trp (Y188W) (equivalent to Y195 in CGTase of *B. circulans* 251) in *B. ohbensis* (Sin *et al.*, 1994), Y195L in *B. circulans* strain 251 (Penninga *et al.*, 1995) and Y195W in the *B. circulans* strain 8 (Parsiegla *et al.*, 1998) could improve product specificity to a limited extent with higher production of γ-cyclodextrin. However, several other Y188 and Y195 mutations as well as the F191Y (equivalent to Y195 in CGTase of *B. circulans* 251) in the CGTase of *G. stearothermophilus* NO2 did not support the importance of this central amino acid (Fujiwara *et al*., 1992a; van der Veen *et al*., 2000c). Furthermore, the natural α-, α/β-, β-, β/γ- and γ-CGTases all have Tyr or Phe at this position, indicating that this residue is not involved in product specificity.

Kaneko *et al.* (1989; 1990) constructed twelve chimeric CGTases form alkalophilic *Bacillus* sp. strain 38-2 and alkalophilic *Bacillus* sp. strain 17-1. Both types of CGTases produce β-cyclodextrin predominantly. They found that the N-terminal and the C-terminal segment were important for cyclodextrins production, heat stability and the pH activity profile.

Fujiwara *et al.* (1992b) constructed seven chimeric CGTases form *Geobacillus stearothermophilus* NO2 and *Bacillus macerans* IFO3490, which produced α/β- and

α-cyclodextrins as the major products, respectively. They found that the cyclization reaction and cyclodextrin production were conferred by the N-terminal domain of CGTase.

Strokopitov *et al.* (1996) studied the X-ray structure of the CGTase from *B. circulans* strain 251 in complex with a maltononaose inhibitor and suggested that the sugar binding subsites further away from the catalytic site could be important for the enzyme-product specificity and that it might be possible to change the ratio of the produced cyclodextrins by altering the affinities for glucose residues at these sugar binding subsites. Subsites -6, -7 and -8 may be the key sites for the product specificity.

Parsiegla *et al*. (1998) constructed β-CGTase mutant from *B. circulans* strain 8 by replacing residues 145-151 with a single Δ(145-151)D, which removed most interactions at subsite -7. The mutant showed reduced production of β-cyclodextrin and increased production of γ-cyclodextrin. The higher γ-cyclization activity of this mutant were explained as a widening of the active site cleft in the deleted portion of amino acid sequence to produce more space for the bound glucosyl chain (Parsiegla *et al*., 1998). This Δ(145-151)D deletion mutant had a stretch of HTSPADAE similar to a stretch of HTSPVDIE in the β/γ-CGTase from *B. firmus* strain 290-3 (Englbrecht *et al*., 1990) and γ-CGTase from *B. clarkii* 7364 (Takada *et al.*, 2003). This indicates that it is necessary for γ-cyclization activity to have more space for the bound glucosyl chain at subsite -7 (Fig. 1.9).

Uitdehaag *et al.* (2000) determined the X-ray structures of CGTase in complex with maltoheptaose, an equivalent oligosaccharide for β-cyclodextrin, and maltohexaose, an equivalent oligosaccharide for α-cyclodextrin, they found that the conformations of maltoheptaose and maltohexaose were different at subsites -3, -6

and -7 (Fig. 1.10). The conformation differences at specific sugar binding subsites suggested that the determinants for cyclodextrin size specificity are located at subsites -3, -6 and -7. This finding was in agreement with the amino acid sequence comparison of natural CGTases with different size specificities, which showed the highest sequence variation in subsites -3 and -7.

Leemhuis *et al.* (2002a) found that the subsite -6 in the active site of β-CGTase from *B. circulans* strain 251 was of great importance for all three transglycosylation reactions (cyclization, coupling and disproportionation) but not for hydrolysis reaction. They found that G180 and N193 were important for β-and γ-cyclization, while G179 was important for α-cyclization.

Kerdsin (2003) constructed the chimeric cyclodextrin glucanotransferases from *Paenibacillus macerans* (α-CGTase) and *Bacillus circulans* A11 (β-CGTase) and found that the subdomain A2 region most likely contained the determinant for CGTase product specificity.

Rimphanitchayakit *et al.* (2005) constructed the chimeric cyclodextrin glucanotransferases from *Bacillus circulans* A11 and *Paenibacillus macerans* IAM1243 by shuffling the domains A/B, C, D and E. The results showed that the amino acid segment essential for the product specificity was located at the C-terminal half of domains A/B.

Nakagawa *et al.* (2006) mutated A223 at subsite +2 and G255 at subsite +3 in the acceptor site of the CGTase from Alkalophilic Bacillus clarkii 7364. The replacement of A223 by lysine, arginine and histidine strongly enhanced the γ-cyclodextrin forming activity in the neutral pH range. On the other hand, all mutants

obtained on replacing G255 with the above amino acids showed significant decreases in the γ-cyclodextrin forming activity.

1.8. Scope of this study

As mentioned above, the separation of the different cyclodextrins is costly and time-consuming, CGTases that predominantly produce one type of cyclodextrin are of great importance for industry (Englbrecht *et al*., 1990 and Martins *et al*., 2002). Several attempts have been made to select the CGTase with one product specificity. There are two possible ways to obtain such CGTase. The first one is to screen for the microorganisms that produce one type of cyclodextrin. The second is to mutagenize the existing CGTase to create an enzyme with single product specificity. In this study, the β-CGTase gene from *B. circulans* A11 (Rimphanitchayakit *et al.*, 2005) was mutated in four amino acid regions in the subdomain A2, residues 239-250, 262-271, 284-320 and 333-342, that might involve in product specificity (Kerdsin, 2003). In addition, the amino acid at positions 264, 268 and 184 were also mutagenized to study the interaction of substrate to the enzyme. The affects of mutation on product specificity were reported and discussed herein.

CHAPTER II

MATERIALS AND METHODS

2.1. Equipments

Autoclave: Model HA-30, Hirayama Manufacturing Corporation, Japan.

Autopipette: Pipetman, Gilson, France.

Balance: Sartorius LC 620, Germany.

Centrifuge: Refrigerated centrifuge: Model J-21C, Beckman Instrument Inc., USA.

Centrifuge: Microcentrifuge High Speed: Model 1110 Mikro 22R, Hettich Zentrifugen, Germany.

Centrifuge: Minimicro DW 41.

Gel document: Syngene, A division of Synoptics Ltd., UK.

Incubator: Heraeus, Germany.

Incubator shaker: Innova 4000, New Brunwick Scientific Co., Inc., USA.

Laminar flow: Issco, USA.

pH meter: Precisa pH900, Precisa instrument AG company, Switzerland.

Refrigerator: Upright Ultra Freezer; Model MDF-U4086S, Sanyo Electric Trading Co.Ltd., Japan.

Spectrophotometer: Jenway 6400, England.

Vortex: Model 232, Fisher Scientific Inc., USA.

Water bath: MEMMERT GmbH + Co. KG, Germany.

U.V. transilluminator: 2011 MA Crovue, San Gabrial, USA.

Transformation apparatus: Gene Pulser™, Biorad, USA.

High performance liquid chromatography: Shimadzu, Japan.

Power supply: Model EC 135-90, E-C Apparatus Corperation, USA.

2.2. Chemicals

α-, β- and γ-cyclodextrins, Sigma, USA.

Absolute alcohol, Merck, Germany.

Acetonitrile (HPLC grade), Lab Scan, Ireland.

Agar, Merck, Germany.

Agarose, SEAKEM LE Agarose, FMC Bioproducts, USA.

Ampicillin, Biobasic Inc., Thailand.

Boric acid, Merck, Germany.

Bovine serum albumin (BSA), Sigma, USA.

Bromophenol blue, Merck, Germany.

Calcium chloride, Merck, Germany.

Chloroform, Sigma, USA.

Di-Sodium hydrogenphosphate, Fluka, Switzerland.

DNA marker, Lamda (λ) DNA digested with *Hin*dIII: Biobasic Inc., Thailand.

Ethidium bromide, Sigma, USA.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Glacial acetic acid, BDH, England.

Glucose, Sigma, USA.

Glycerol, Univar, Australia.

Hydrochloric acid, Lab Scan, Ireland.

Iodine, Baker chemical, USA.

Kanamycin, Bio101 Inc., USA.

Methanol, Scharlau, Spain.

NucleoTrap Gel Extraction Kit, BD Biosciences, USA.

Orthophosphoric acid 85%, Carlo Erba, Italy.

Phenol, Pierce, USA.

Polyethylene glycol 6000, Fluka, Switzerland.

Potassium iodide, Mallinckrodt, USA.

QIAprep Spin Miniprep Kit, Qiagen, Germany.

Sodium acetate, Carlo Erba, Italy.

Sodium carbonate, BDH, England.

Sodium chloride, Univar, Australia.

Sodium dihydrogen orthophosphate, Carlo Erba, Italy.

Sodium dodecyl sulfate, Sigma, USA.

Sodium hydroxide, Carlo Erba, Italy.

Soluble starch, Scharlau, Spain.

Tetracyclin, Sigma, USA.

Tris-base, USB, USA.

Tryptone, Merck, Germany.

Yeast extract, Scharlau, Spain.

2.3. Bacterial strains

Escherichia coli XL1-Blue [*hsdR17(rk-mk+), recA1, endA1, gyrA96, thi-1, supE44, relA1, lac*[F' *proAB lacl*^{q}*ZΔM15Tn10* (Tet^{r})]] was used for DNA manipulation.

E. coli BMH 71-18 *mutS* [F', *proA*$^{+}$, *proB*$^{+}$, *lacI*^{q}, *lacZΔM15/mutS:Tn10, thi, supE,* Δ(*lac-proAB*)] was used for mutagenesis.

M13K07 bacteriophage was used for preparing single-stranded DNA.

2.4. Plasmid vector

A plasmid pVR328, a derivative of pUC119 containing the β-CGTase gene from *Bacillus circulans* A11 (Pongsawasdi and Yagisawa, 1987), was used for mutagenesis and constructing the recombinant plasmids.

2.5. Enzymes

Restriction endonucleases: *Nde*I, *Eco*RI, *Kpn*I, *Sph*I, *Hin*dIII, *Sac*II and *Bam*HI, were purchased from New England Biolabs Inc.; USA, and *Eco*RV, *Mlu*I and *Stu*I was from Fermentas Inc., USA.

T4 DNA ligase and T7 DNA polymerase was purchased from Fermentas Inc., USA.

T4 polynucleotide kinase was purchased from New England Biolabs Inc., USA.

T7 DNA polymerase was purchased from Fermentas Inc., USA.

Amyloglucosidase [(1→4)-α-D-glucohydrolase, EC 3.2.1.3, from *Aspergillus niger*] was purchased from Fluka, Switzerland.

RNase A was purchased from Sigma, USA.

β-Amylase was purchased from Sigma, USA.

2.6. Media preparation

1. Luria-Bertani broth (LB medium)

LB broth consists of 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl, supplemented with 100 μg/ml ampicillin when needed. LB broth was used to cultivate *E. coli* cells.

2. LB-starch agar plate

LB-starch agar consists of 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.6% (w/v) agar and 1% (w/v) soluble starch, supplemented with 100 μg/ml ampicillin when needed. LB-starch agar plate was used for plate-enzyme assays to detect the starch-hydrolyzing activity of CGTase.

2.7. General techniques in genetic engineering

2.7.1. Preparation of competent cells

A single colony of the *E. coli* XL1-Blue or BMH71-18*mut*S was cultured as a starter in 5 ml of LB medium and incubated at 37ºC with 250 rpm shaking for 24 hours. The starter was 1:100 diluted in 200 ml of LB-broth in 500 ml Erlenmeyer flask, and the culture was incubated at 37ºC with 250 rpm shaking until the optical density at 600 nm of the culture reached 0.5-0.6 (~3-4 hours).

The culture was chilled on ice for 15 minutes and the cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4ºC. The supernatant was removed. The cell pellet was washed twice with 1 volume and 0.5 volume of cold

sterile water, respectively. The cells were resuspended and centrifuged at 6,000 rpm for 15 minutes at 4ºC. The supernatant was discarded. The pellet was washed with 10 ml of ice cold sterile 10% (v/v) glycerol, and finally resuspended in a final volume of 0.6 ml of ice-cold sterile 10% glycerol. The cell suspension was divided into 40 µl aliquots and stored at –80ºC until used.

2.7.2. Electroporation

The competent cells were thawed on ice. The cell suspension of 40 µl was mixed with 1-2 µl of the mutagenesis or ligation mixture, mixed well and placed on ice for 1 minute. The mixture was electroporated in a cold 0.2 cm cuvette with the apparatus setting as 2.5 µF, 200 Ω of the pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were resuspended in 1 ml of LB broth and incubated at 37ºC for 1 hour. The cell suspension was spread on the LB-starch agar plate.

2.7.3. Plasmid preparation (alkaline lysis method)

Cells harboring plasmid were cultured in 1.5 ml LB medium and harvested by centrifugation at 8,000g, 4ºC for 2 minute. The packed cells were resuspended in 100 µl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA and 50 mM glucose) by vigorous vortexing. Then 200 µl of solution II (1% SDS, 0.2 N NaOH) was added, mixed by inversion until the mixture was clear. The mixture was neutralized by adding 150 µl of solution III (3 M sodium acetate, pH 4.7), mixed by inversion and kept on ice for 10 minutes. After centrifugation at 10,000g for 10 minutes, 10 mg/ml of RNaseA was added to the supernatant to give a final concentration of 10 µg/ml and incubated at 37 ºC for 20 minutes. The supernatant was

extracted with one volume of phenol:chloroform (1:1). Two volumes of absolute alcohol were added, mixed by inversion several times and stored at –20ºC for 20 minutes. The plasmid was pelleted by centrifugation at 10,000g, washed with 70% ethanol, and vacuum-dried for 10 minutes. The pellet was dissolved in 50 µl TE buffer and stored at –20ºC.

For DNA sequencing, the QIAprep Spin Miniprep kit was used to prepare the plasmid DNA, and performed according to the kit protocol. Briefly, the plasmid-harboring cells were cultured in 1.5 ml LB medium and harvested by centrifugation at 8,000g, 4ºC for 1 minute. The bacterial cell pellet was resuspended in 250 µl Buffer P1 by vortexing until the cell clumps were not visible. Buffer P2 of 250 µl was added, and gently inverted the tube 4-6 times. The mixture became viscous and slightly clear. Buffer N3 of 350 µl was added. The solution became cloudy after gently inverting the tube 4-6 times. The preparation was centrifuged for 10 minutes at 10,000g. The supernatant was then applied to the QIAprep spin column and centrifuged for 1 minute. The flow-through was discarded. Buffer PB and PE were added and centrifuged for 1 minute, respectively. Finally, the column was placed in a 1.5 ml microcentrifuge tube and added Buffer EB to the center of the QIAprep spin column to elute the DNA, the column was left stand for 1 minute, and then centrifuged for 1 minute.

2.7.4. Agarose gel electrophoresis

DNA was analyzed by using 1% agarose gel in TBE buffer (98 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.2). DNA samples with 1×tracking dye were loaded into the wells. The gels were run at 100 volts for 1 hour or until bromophenol blue reached the bottom of the gel. After electrophoresis, the

gels were stained with 2.5 μg/ml ethidium bromide solution for 2-5 minutes, and the DNA bands were visualized under the UV light. The sizes of DNA fragments were determined by comparing the relative mobilities with those of standard DNA fragments (λ/*Hin*dIII marker).

2.7.5. Extraction of the DNA fragment from the agarose gel

The QIAquick Gel Extraction Kit was used for extracting DNA fragment from the agarose gel, and performed according to the kit protocol. Briefly, the DNA fragment was excised from the agarose gel. The weigh of the gel slice was measured and transferred to a 1.5 ml microcentrifuge tube. Three volumes of Buffer QG to a volume of the gel were added. The mixture was incubated at 50ºC for 10 minutes and vortexed briefly during the incubation time. The mixture was placed in a QIAquick spin column and centrifuged at 10,000 rpm for a minute at room temperature. The column was washed with 750 μl of Buffer PE and centrifuged for a minute, respectively. The column was again centrifuged at 13,000 rpm for a minute. The column was placed into a clean 1.5 ml microcentrifuge tube. Finally, the Buffer EB was added to elute DNA by incubating at room temperature for 2 minutes. The DNA fragment was collected by centrifugation.

2.7.6. Preparation of single-stranded plasmid

Single-stranded plasmid was prepared from pVR328, a pUC119 plasmid carrying the β-CGTase gene from *B. circulans* A11, by using helper phage M13KO7. A single colony of *E. coli* XL1-Blue, containing pVR328, was inoculated into LB broth containing ampicillin 100 μg/ml and incubated with shaking at 37ºC for 2-3 hours or until the cell density was very slightly turbid. Then, the culture was

added the helper phage to 10^7-10^8 pfu/ml and grown at 37ºC for 1-2 hours with vigorous shaking. The culture was added kanamycin to 70 μg/ml to select for the phage-infected cells and further grown at 37˚C for 12-18 hours with vigorous shaking. The 1.5 ml of the cell culture was centrifuged for 5 minutes at 10,000g in a microcentrifuge. The supernatant of 1.2 ml was removed to a new tube. The supernatant was added 200 μl of a PEG solution containing 20% PEG8000 and 2.5 M NaCl, mixed, and incubated to allow the phage particles to precipitate on ice for 30 minutes. The mixture was centrifuged for 15 minutes at 10,000 rpm to collect the phage precipitate. The pellet was resuspended in 90 μl of TE buffer and 10 μl of 3 M sodium acetate, pH 5.5. The suspension was extracted with one volume phenol:chloroform (1:1) and one volume chloroform, respectively. Two volumes of absolute alcohol were added, mixed and stored at –20ºC for 30 minutes. The single-stranded plasmid was pelleted by centrifugation at 10,000 rpm, washed with 70% ethanol and dried. The pellet was dissolved in 30 μl TE buffer.

2.7.7. Phosphorylation of oligonucleotide primers

Three mutagenic oligonucleotides, named KP1, KP6 and KP7 were purchased from Biobasic Inc., Thailand, and the other four mutagenic oligonucleotides, named KP2, KP3, KP4 and KP5 were purchased from Proligo Singapore Pty Ltd, Singapore. They were phosphorylated before use in the mutagenesis procedure. Approximately 100-200 pmol of an oligonucleotide was kinased in 10 μl reaction containing kinase buffer, 1 mM ATP, and 10 U T4 kinase, and incubated at 37ºC for 1 hour. The reaction was stopped by heating at 70ºC for 15 minutes. Then, the reaction was spun for 1 minute and stored at –20ºC.

2.8. Mutagenesis of β-CGTase gene using the USE (Unique Site Elimination) procedure

The eight mutagenic primers, KP1, KP2, KP3, KP4, KP5, KP6, and KP7 were designed according to the amino acid sequence of the A2 regions of β-CGTase, which were different from those of α-CGTase and γ-CGTase. The nucleotide sequences of the eight primers are shown in Fig. 2.1. For the screening of the mutants, primers KP1, KP2, KP3, KP4, KP5, KP6, and KP7 were also designed to contain the *Stu*I, *Eco*RI, *Bam*HI, *Mlu*I, *Sac*II and *Kpn*I, respectively.

PRIMER KP1 (28mer)

***Stu*I**

5′ GGT GGA CAT AAA GGC CTT CTG CCA GCC 3′

PRIMER KP2 (55mer)

***Eco*RI**

5′ GTT GTC CCT GAA CAC TTC CCG GGC TTC CTG GGC GAA TTC GAA ATC GAG CAG GCT C 3′

PRIMER KP3 (64mer)

***Bam*HI**

5′ GGA CTC GTT AGC GAA ATG ATG GAT GTT GGA TCC CTG GGC GCC CGT GAA CCA TTC GCC GAAC G 3′

PRIMER KP4 (59mer)

***Mlu*I**

5′ GCC AGC GCC TGC TCC GTC GTA CGC GTA TCC CGG CCT TCC ACC GTG AAA CGC TCC ATG TC 3′

PRIMER KP5 (33mer)

***Sac*II**

5′ GCG AAT TGA TGG TAC CGC GGA CTA ATC TCA TTG 3′

PRIMER KP6 (41mer)

***Sac*II**

5′ CGA ATT GAT GGT ACC GCG GAC TAA TCT CAT TGA CGC CAA GGA 3′

PRIMER KP7 (39mer)

***Kpn*I**

5′ GTT CTC AAT GGT TT*T GAA* ATC GGT ACC GCC ATA ATG GTG 3′

Fig. 2.1. The mutagenic oligonucleotides used to produce the mutations in the USE mutagenesis procedure. The restriction recognition sites, which allow rapid screening of the potential mutants, are shaded.

The USE mutagenesis procedure is summarized in Fig. 2.2. The procedure works by simultaneously annealing two oligonucleotides primers to a single-stranded plasmid. One primer introduces the desired mutation, and the other primer mutates a restriction site unique to the plasmid for the purpose of selection. The selection primer, called *Sca*I primer, eliminates the *Sca*I site at the ampicillin resistant gene in the plasmid. Each of the seven kinased mutagenic primers, the kinased *Sca*I primer and 40 ng of the single-stranded plasmid were mixed in 10 μl reaction containing annealing buffer (200 mM of Tris-HCl, 100 mM of $MgCl_2$ and 500 mM NaCl). The reaction mixture was heated at 95ºC for 5 minutes, and then placed at room temperature for 15 minutes. Next, it was added 6 μl of an enzyme mixture containing polymerase buffer, 1 U T7 polymerase, 5 U T4 ligase, 2.5 mM dNTPs, and 10 mM ATP. It was incubated at 37ºC for 1-2 hours. The reaction mixture was then transformed into an *E. coli* strain BMH71-18*mut*S. A mixture of plasmids were generated by culturing the transformation mixture in the presence of 100 μg/ml ampicillin overnight, pelleting the cells and preparing the plasmids. The plasmid mixture was digested with *Sca*I to linearize most of the wild type plasmid, and transformed into an *E. coli* XL1-Blue to separate individual clones. Colonies were cultured for plasmid miniprep and screened for the plasmids with the added restriction sites by using restriction enzyme digestion.

The mutation regions were confirmed using DNA sequencing service by Bioservice unit, National Center for Genetic Engineering and Biotechnology (BIOTEC). The correctness of DNA sequences around the mutation regions was also determined. The DNA sequences were subcloned into the same location in the original plasmid (pVR328) to avoid possible other mutated sequenced in the mutated

plasmids. The plasmids were digested with the appropriate restriction enzymes to yield DNA fragments containing the mutant regions. The DNA fragments were gel purified using agarose gel electrophoresis and eluted with QIAquick Gel Extraction Kit. The eluted fragments from mutant plasmids and the restriction enzyme-digested pVR328 were mixed and ligated with T4 DNA ligase. The ligation reactions were incubated for 16-24 hours at 22ºC. The ligation mixture was used to transform the *E. coli* XL1-Blue by electroporation. The transformants were plated on the LB agar containing 100 μg/ml of ampicillin at 37ºC for an overnight. Each transformant was grown in the LB broth in the presence of 100 μg/ml ampicillin. In order to verify the presence of the mutation, the recombinant plasmids were screened for the mutant regions by restriction enzyme digestion. The cloning sites were also checked.

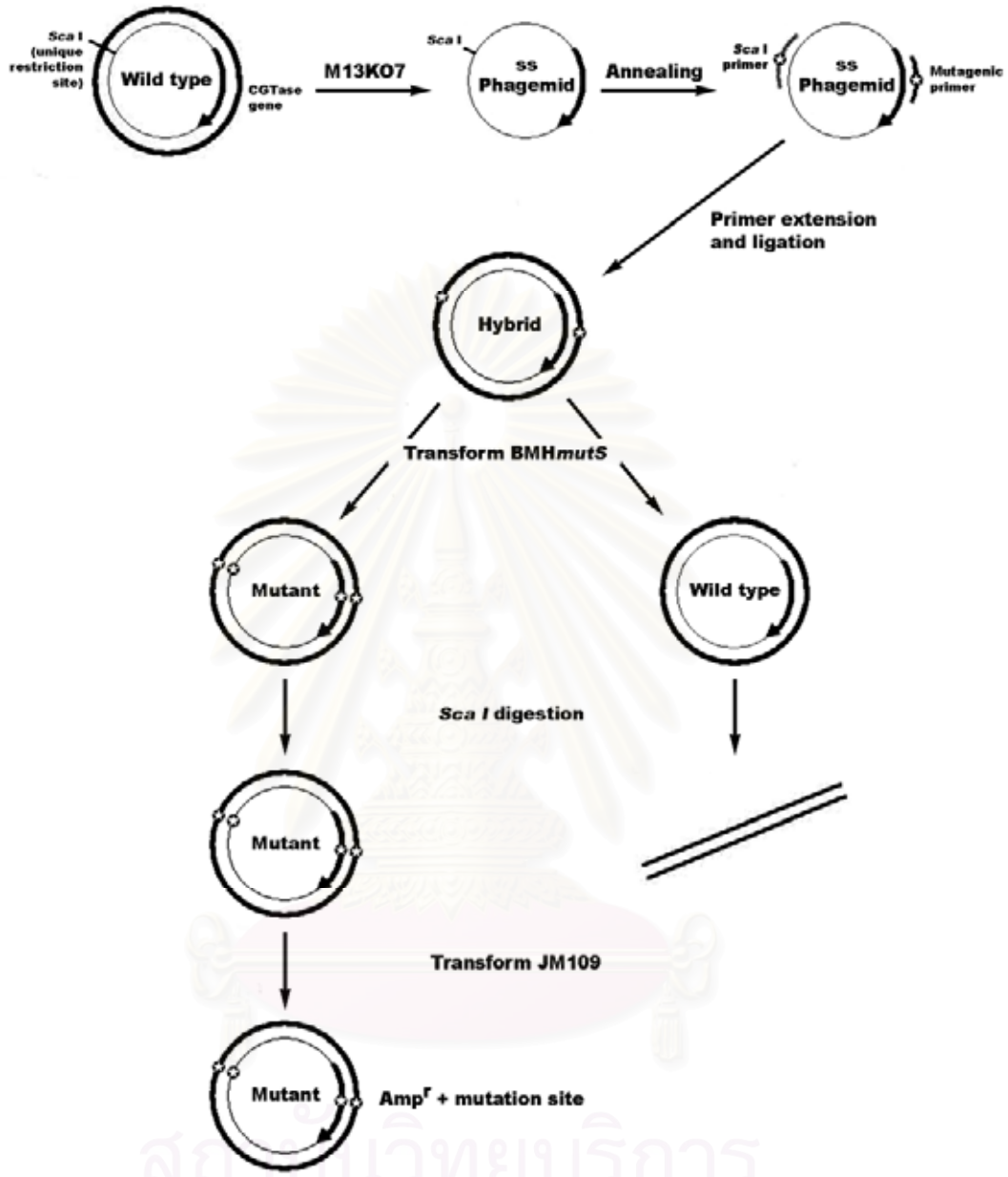

Fig. 2.2. Schematic diagram of USE mutagenesis protocol.

2.9. Detection of the mutant CGTase activity

2.9.1. Dextrinizing activity

1. Halo zone on LB-starch agar

E. coli XL1-Blue cells, containing a CGTase plasmid, were plated on a LB-starch agar plate and incubated at 37ºC for 24 hours. The halo formation was observed after the iodine solution (0.02% (w/v) I_2 in 0.27% (w/v) KI) was poured onto the agar.

2. Dextrinizing activity assay

The *E. coli* XL1-Blue cells, containing the CGTase plasmid, were grown at 37ºC for 16 hours in LB broth containing ampicillin. The culture was centrifuged to remove the cells, and the supernatant liquid was used as crude enzyme preparation.

CGTase activity was measured as the dextrinizing power according to the method of Fuwa (1954) with slight modification. The reaction mixture was performed by incubation 100 µl of the crude enzyme with 500 µl of 0.2% potato soluble starch in 0.2 M phosphate buffer, pH 6.0, at 40ºC for 10 minutes. To stop the enzymatic reaction, 1 ml of 0.2 N HCl was added to the reaction mixture. The iodine-starch complex (blue color) was developed by mixing 400 µl of the reaction mixture with 4 ml of 0.01% (w/v) I_2 in 0.1% (w/v) KI. The solution was placed at room temperature for 20 minutes, and the absorbance at 600 nm was measured. The results were expressed as the mean values of the duplicate experiments.

One unit of enzyme is defined as the amount of enzyme, which produces 10% reduction in the intensity of blue color of starch-iodine complex per

minute under the described condition. The relative activity was calculated using a no enzyme control with the highest absorbance as 0%, and zero absorbance as 100%.

2.9.2. CD forming activity

The *E. coli* XL1-Blue cells, containing the CGTase plasmid, were grown at 37ºC for 18 hours in LB broth containing ampicillin. The culture was centrifuged to remove the cells, and the supernatant liquid was used as crude enzyme preparation. Then, 200 μl of the crude enzyme from each mutant plasmid was incubated with 500 μl of 1% potato soluble starch in 10 mM phosphate buffer, pH 6.0 at 37ºC for 12 hours. The reaction was stopped by boiling for 10 minutes, and clarified by centrifugation. The mixture was treated further for 4 hours with 20 U of β-amylase to digest the remaining starch and oligosaccharides formed from CGTase reaction. The β-amylase reaction was stopped by boiling for 10 minutes, and clarified by centrifugation. The cyclodextrin samples were filtered by syringe through the 0.45 μm Sartolon polyamide membrane (Sartorius, Germany) prior to HPLC analysis. Each sample of 20 μl was analyzed using the Shimazu HPLC with a Hypersil-APS2 (NH_2) column (0.46×250 mm), acetonitrile/water [65:35 (v/v)] at a flow rate of 1.0 ml/min and a refractometric detector. The cyclodextrin standard was a mixture of α-, β- and γ-cyclodextrins (10 mg/ml each). For quantitative analysis, peak area corresponding to each cyclodextrin was used to calculate the cyclodextrin product ratios.

2.10. Protein determination

The protein sample (100 μl) was mixed with 1 ml of Bradford's reagent (Appendix A), and left stand at room temperature for 5-10 minutes. The absorbance at

595 nm was measured. The protein concentration was calculated from a standard curve of bovine serum albumin.

CHAPTER III

RESULTS

3.1. Amino acid sequence comparison among various CGTases and the design of mutagenic primers

Kerdsin (2003) had constructed the chimeras to localize an essential part of enzyme that may be involved in specificity of CD production using *in vivo* homologous recombination between the α-CGTase from *Paenibacillus macerans* and β-CGTase from *Bacillus circulans* A11. He found four variable amino acid sequence regions in the subdomain A2, designated residues 239-250 (I), 262-271 (III), 284-320 (II) and 333-342 (IV) (*B.* circulans A11 β-CGTase numbering), which most likely contained the determinant for CGTase product specificity (Fig. 3.1). In this study, these regions were tested for their involvement in product specificity by mutagenesis. The four regions in the three-dimensional structure of CGTase are depicted in Fig. 3.2.

From the result of the alignment among the CGTases with different product specificities (Fig. 3.1), four primers KP1-4 (Fig. 3.3) were designed to change the four variable amino acid regions towards those of either α- or γ-CGTases. The KP1 introduced S241A substitution in region I, a change towards γ-CGTase. The KP2 mutagenized region II towards α-CGTase. The KP3 introduced both amino acid substitution and deletion of bases in region III towards γ-CGTase. The KP4 introduced amino acid substitutions in region IV also towards γ-CGTase.

Since region III was supposed to lie along one side of the polysaccharide-binding path of the CGTase, it was expected that changes in some charged amino acid residues might affect the activity of the enzyme. The amino acid residues 184, 264 and 268 (Fig. 3.2) were changed for the study. The KP5 and KP6 primers (Fig. 3.3) changed the negatively charged amino acids, glutamate in region III to positively charged amino acid, arginine. The KP7 changed a serine residue at position 184 to lysine. The position 184 was approximately opposite to the region III (Fig. 3.2).

For the screening of the mutants, primers 1-7 also created *Stu*I, *Eco*RI, *Bam*HI, *Mlu*I, *Sac*II and *Kpn*I restriction sites upon mutagenesis, respectively, in the mutant plasmids.

3.2. Mutagenesis of β-CGTase gene from *B. circulans* A11 using USE procedure

The plasmid pVR328, carrying the β-CGTase gene from *B. circulans* A11, was used as a template for the USE mutagenesis procedure (Fig. 2.2). The mutagenic primers KP1-7 along with the reference *Sca*I primer were used to mutate the CGTase genes at 5 positions described above. The mutagenesis procedure generated a mixture of mutated plasmids. After removing the mostly wild type plasmids with *Sca*I digestion, the mutated plasmids were separated into individual clones by transformation. The clones were screened for the presence of restriction sites, *Stu*I, *Eco*RI, *Bam*HI, *Mlu*I, *Sac*II and *Kpn*I, created. The positions of these sites were confirmed by restriction enzyme digestion and agarose gel electrophoresis (Fig. 3.4). Seven mutated plasmids, pKP1-7, were obtained as shown in Fig. 3.5 with the restriction sites, *Stu*I, *Eco*RI, *Bam*HI, *Mlu*I, *Sac*II and *Kpn*I, respectively.

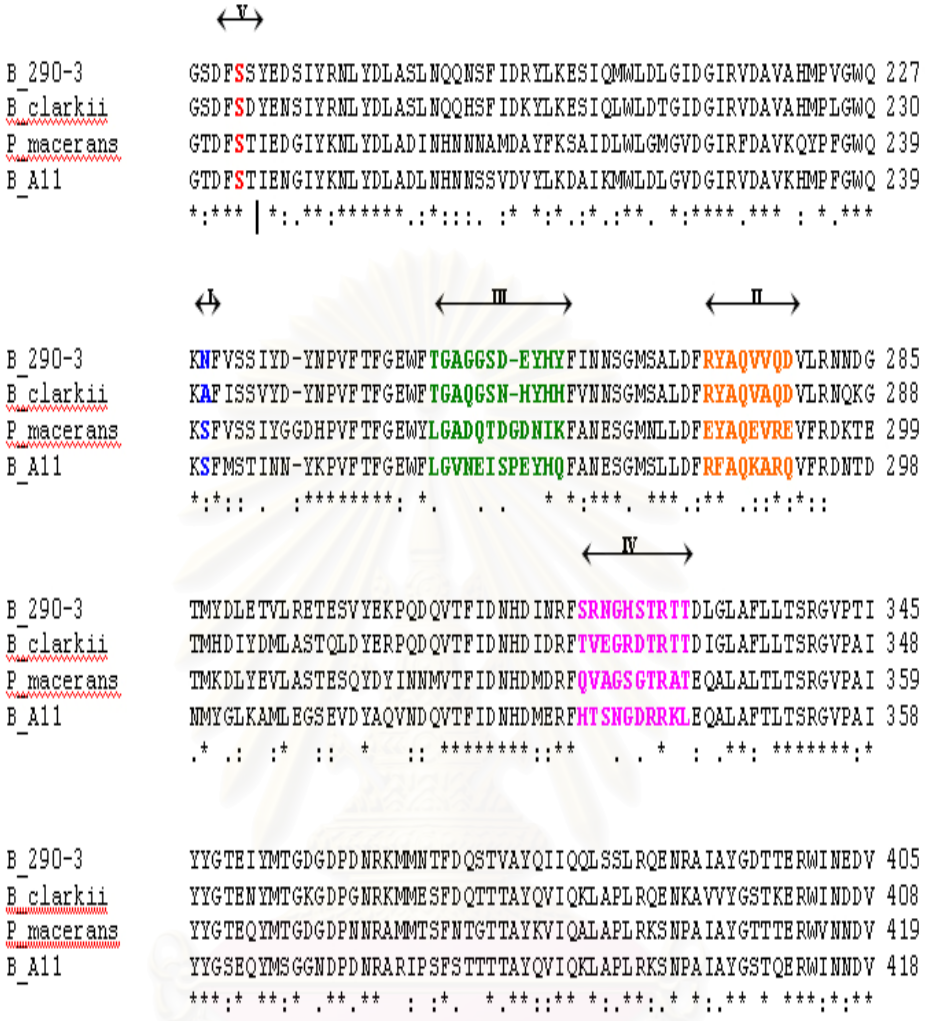

Figure 3.1. Alignment of the amino acid sequences between β-CGTase from *B. circulans* A11 (B_A11) and other typical CGTases, α-CGTase from *P. macerans* IAM1243 (P_macerans), β/γ-CGTase from *Bacillus* sp. 290-3 (B_290-3) and γ-CGTase from *B. clarkii* 7364 (B_clarkii). Double arrows indicate the variable amino acid sequences.

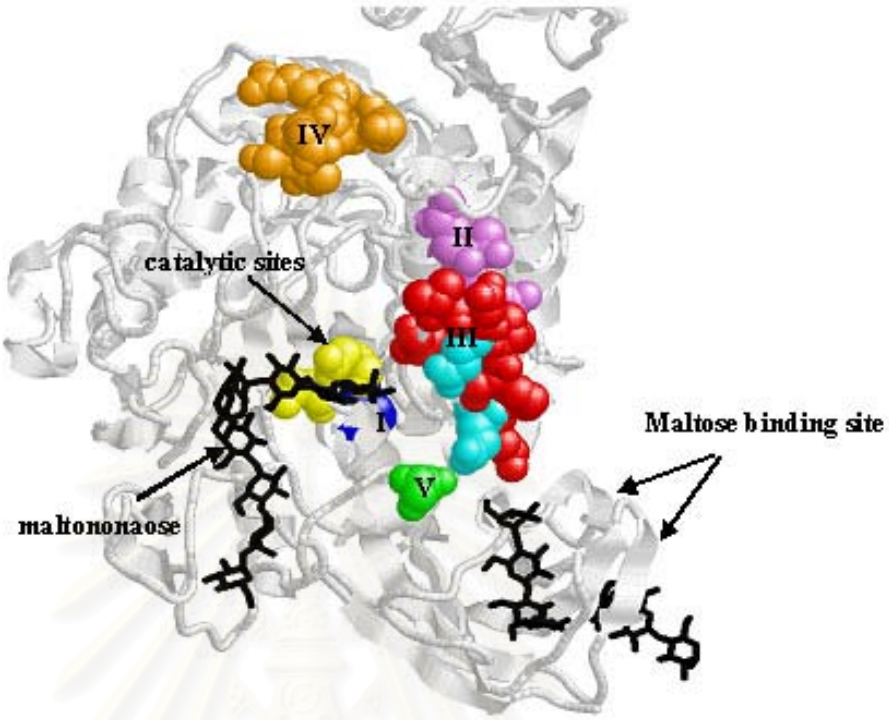

Figure 3.2. Positions of the CGTase regions that were mutagenized in this study. Region I is dark blue. Region II is purple. Region III is red and light blue (E264 and E268). Region IV is orange. The S184 is green. The catalytic residues (D229 and E257) are yellow. The maltononaose and maltotrioses are black.

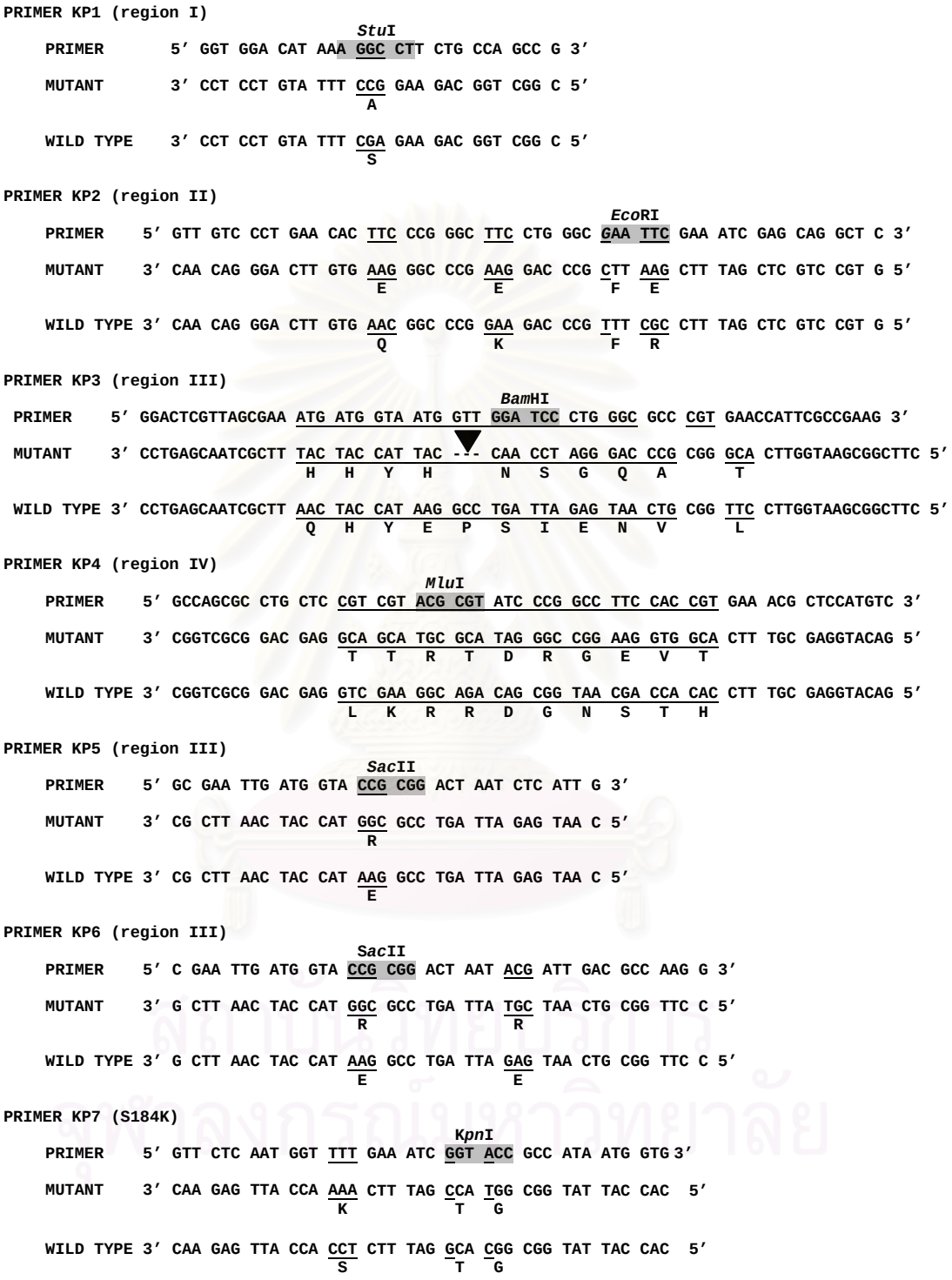

Figure 3.3. The design of oligonucleotides used in USE procedure and the regions of nucleotide sequences of wild type that were used for the designing. Newly created restriction recognition sites are shaded. Triangle indicates the positions of nucleotides deleted.

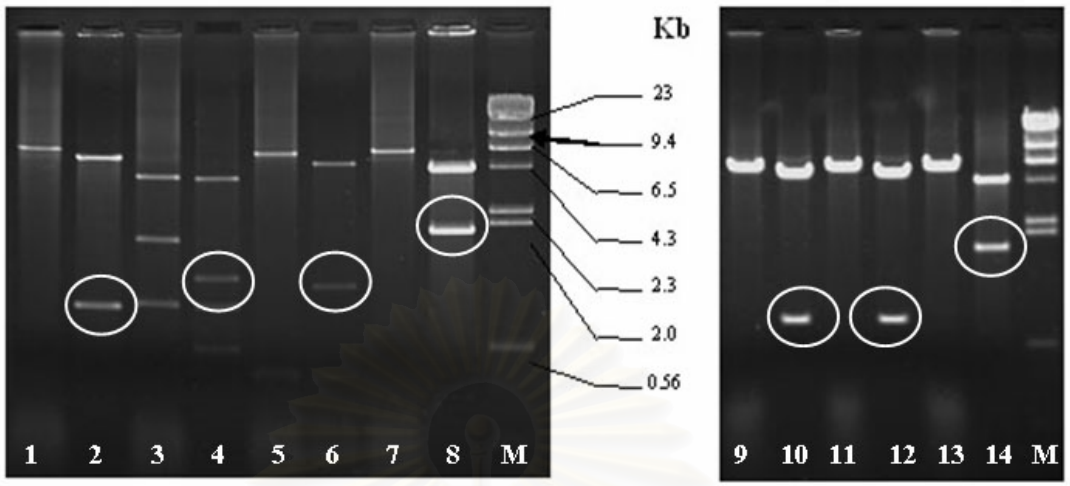

Figure 3.4. Restriction digestion of pKP1-7. Lane M: λ/*Hin*dIII marker; lanes 1, 3, 5, 7, 9, 11 and 13: pVR328 digested with *Stu*I, *Eco*RI, *Bam*HI, *Hin*dIII+*Mlu*I, *Sac*II, *Sac*II and *Kpn*I, respectively; lanes 2, 4, 6, 8, 10, 12 and 14: pKP1-7 digested with *Stu*I, *Eco*RI, *Bam*HI, *Hin*dIII+*Mlu*I, *Sac*II, *Sac*II and *Kpn*I, respectively. The circles indicated the fragment size of the mutants.

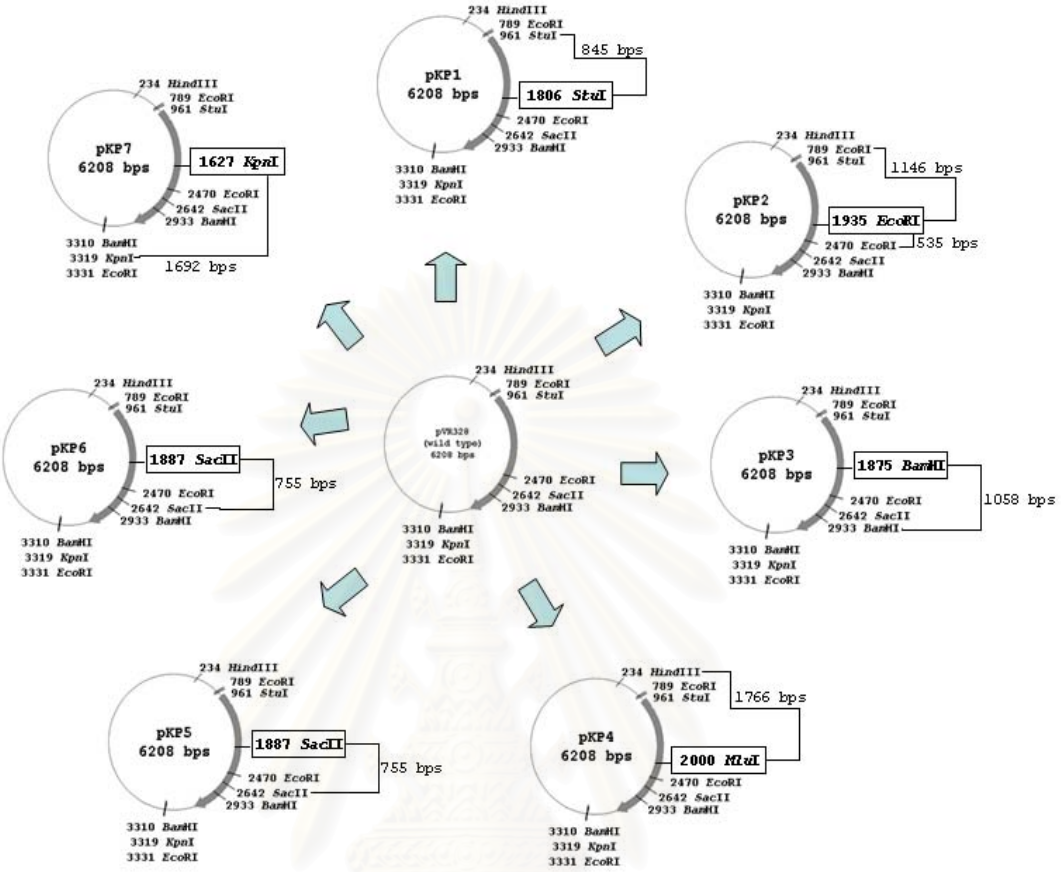

Figure 3.5. The mutated plasmids (pKP1-7). The plasmids were processed to confirm the mutations by restriction digestion and DNA sequencing. The new restriction sites are boxed and the sizes of the DNA fragments analyzed are indicated.

3.3. DNA sequencing determination of the mutation regions and subcloning of the mutant CGTases in pVR328

The precise DNA sequences around the new restriction sites in pKP1-7 were determined using the ABI Prism Big Dye Terminator Cycle Sequencing. The seven mutants had the nucleotide sequences as designed (Fig. 3.6). Nucleotide sequences between the two restriction sites border the mutation sites were also checked to make certain that there were no additional mutations. The restriction sites were used subsequently to subclone the mutation sites.

The DNA fragments containing the new restriction sites were subcloned into the original plasmid (pVR328) to avoid other possible mutation sequences in the mutated plasmids. To subclone the mutation sites into the pVR328, the DNA fragments between two adjacent restriction sites were removed from pKP1-6 as the 987 bps *Nde*I-*Eco*RV fragment and pKP7 as the 322 bps *Sph*I-*Nde*I fragment, replacing the corresponding fragments in pVR328.

3.4. The activities of mutant CGTases

3.4.1. Halo zone on LB-starch agar

E. coli XL1-Blue transformant containing each of the mutant CGTase was tested for dextrinizing activity on the LB-starch agar plate. Cells with dextrinizing activity gave halo zone surrounding the colony upon KI-I_2 treatment. Fig. 3.8 shows such activity. Fig. 3.7 summarizes the halo zone assay of all the transformants. Most of the mutants had similar dextrinizing activity to the wild type, while pKP1 had low dextrinizing activity

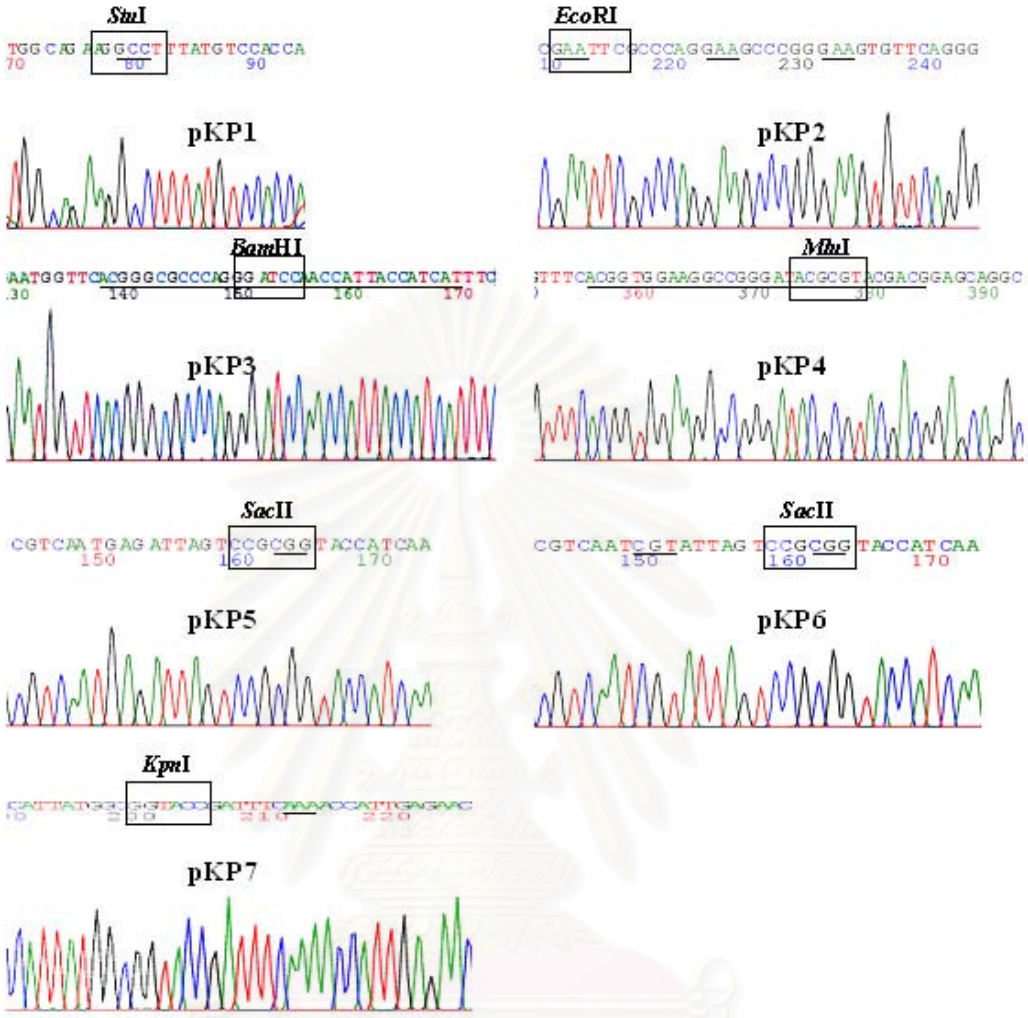

Figure 3.6. The nucleotide sequences of mutant sites in pKP1, pKP2, pKP3, pKP4, pKP5, pKP6 and pKP7. The new restriction sites are boxed. Each mutation region is underlined.

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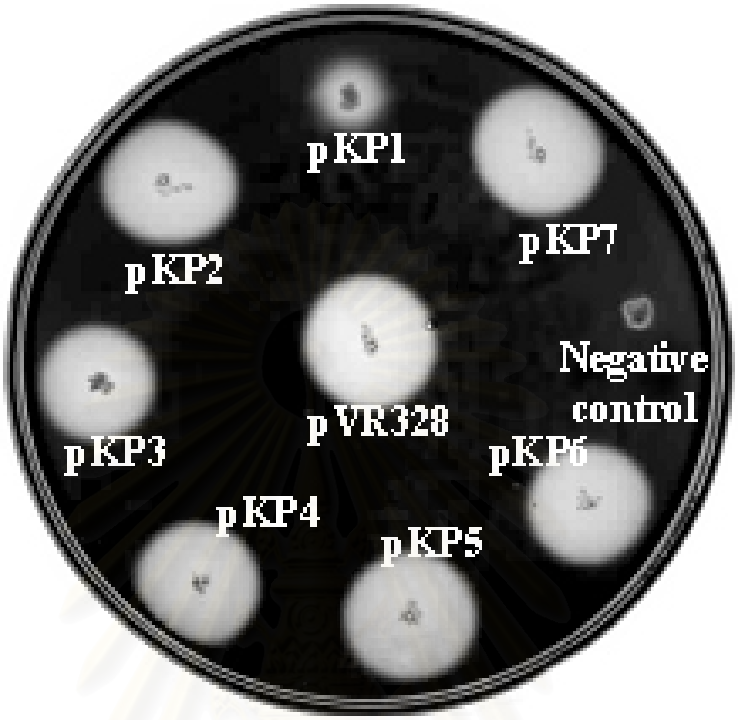

Figure. 3.7. Iodine test for dextrinizing activity of the wild type and mutant CGTases. Clear zones surrounding the colonies indicate starch hydrolysis activity.

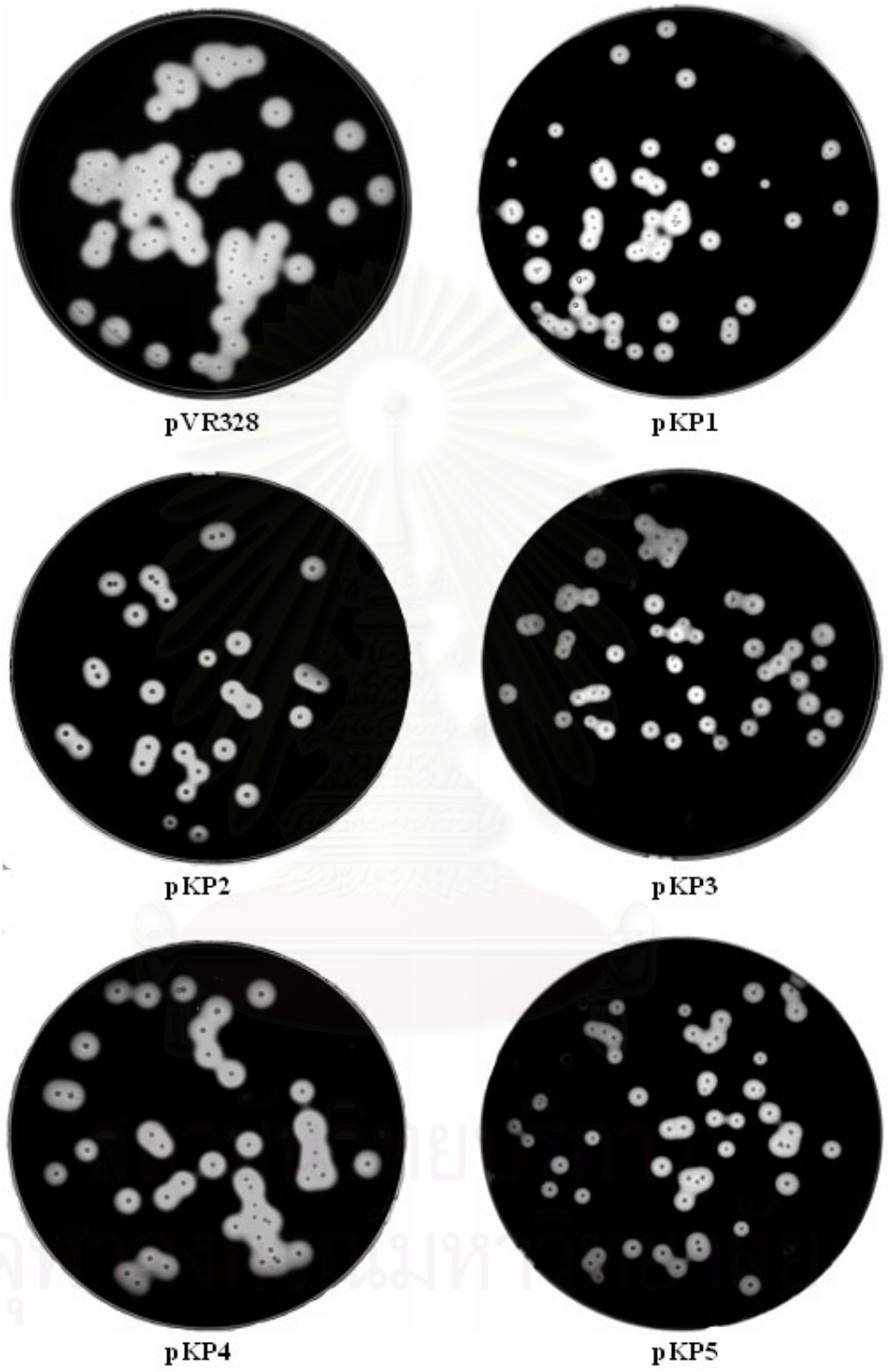

Figure 3.8. Iodine test for dextrinizing activity of wild type and mutant CGTases. Clear zones surrounding the colonies indicate starch hydrolysis activity.

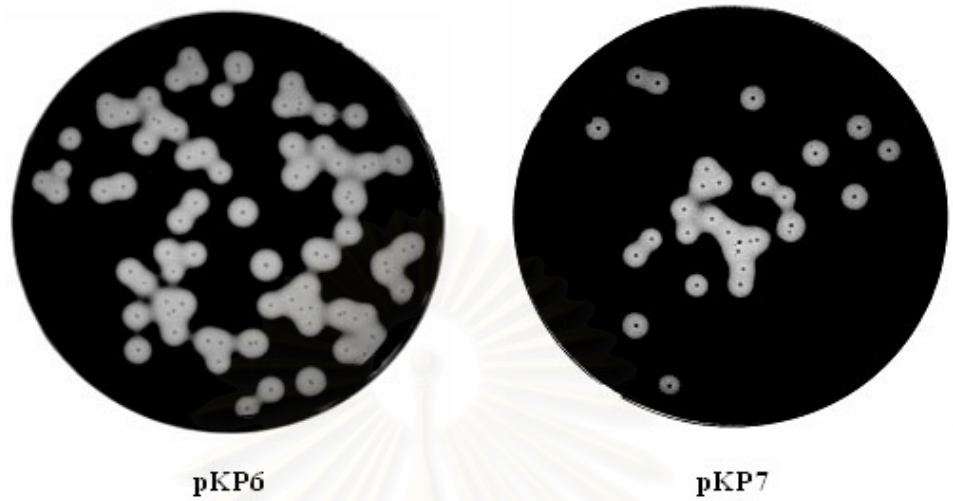

Figure 3.8. (continue) Iodine test for dextrinizing activity of wild type and mutant CGTases. Clear zones surrounding the colonies indicate starch hydrolysis activity.

3.4.2. Dextrinizing activity assay

The crude enzymes in the culture supernatants of the transformants were also assayed for dextrinizing activity using the Fuwa method (Fuwa *et al*., 1954) (Table 3.1 and Fig. 3.9). The pKP1 and pKP7 had slightly lower dextrinizing activity than the wild type, while the pKP4, pKP5 and pKP6 were less active and the pKP2 and pKP3 had the lowest dextrinizing activity.

3.4.3. Cyclodextrin forming activity

The crude enzyme from the culture of each mutant plasmid was incubated with soluble starch at 37 ºC for 12 hours. The reaction was terminated by boiling. Then, β-amylase was added to digest the remaining starch. All mutant enzymes exhibited the cyclodextrin forming activity. To measure the product ratios, the peak areas of each cyclodextrin in the HPLC profiles were determined. The cyclodextrin ratios were then calculated as percentages of mass and mole ratios of α-, β- and γ-CDs.

The results are shown in Fig. 3.10, Table 3.2 and Table 3.3. All the mutant enzymes produced β-CD as a major product. The pKP1 and pKP7 have similar proportion of α-, β-, γ-CD to that of the wild type. The pKP2-6 had decreased proportion of α-CD production, especially, pKP2, pKP3 and pKP6 that α-CD was produced only at 7, 3 and 6 %, respectively. The pKP2-6 had increased proportion of β-cyclodextrin production. Surprisingly, the pKP7 had decreased proportion of γ-cyclodextrin production about 2 folds, while the other mutant CGTases have not changed comparing with the wild type.

Table 3.1. Dextrinizing activity assay of wild type and mutant CGTases.

Specific dextrinizing activity (unit/mg protein)								
No./Name	**Wild type**	**pKP1**	**pKP2**	**pKP3**	**pKP4**	**pKP5**	**pKP6**	**pKP7**
1	128.88	101.22	14.62	25.96	54.34	32.58	32.52	104.41
2	117.87	176.67	19.91	14.86	80.25	77.66	41.33	88.49
3	101.45	82.52	19.70	21.08	64.79	82.25	99.65	130.09
4	181.66	151.86	18.29	38.99	116.72	99.79	36.21	109.25
5	146.65	162.27	8.74	18.89	88.13	63.93	46.07	107.45
6	191.33	176.68	33.91	31.42	81.45	61.27	72.89	119.17
7	155.35	93.96	13.63	19.07	71.20	29.55	74.49	113.14
8	119.38	75.22	35.34	32.60	80.67	26.99	44.26	105.30
9	159.43	77.25	13.03	31.17	44.69	80.30	36.64	103.78
10	142.35	111.75	9.54	18.23	65.44	64.23	42.22	101.99
Average	**144.44**	**120.94**	**18.67**	**25.23**	**74.77**	**61.59**	**53.78**	**110.29**
S.D.	28.60	41.57	9.24	7.96	19.91	26.38	23.07	12.90

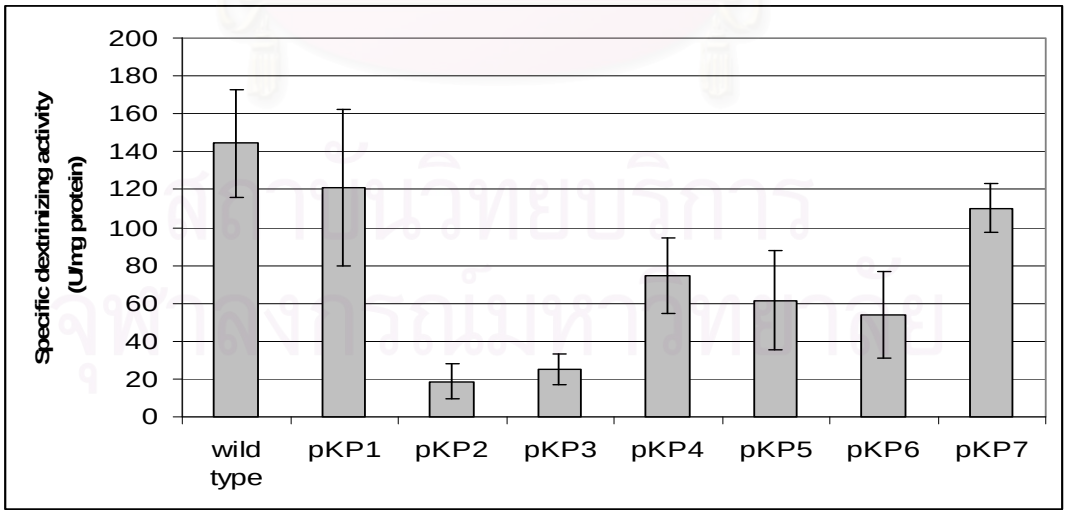

Figure 3.9. Specific dextrinizing activity of wild type and mutant CGTases.

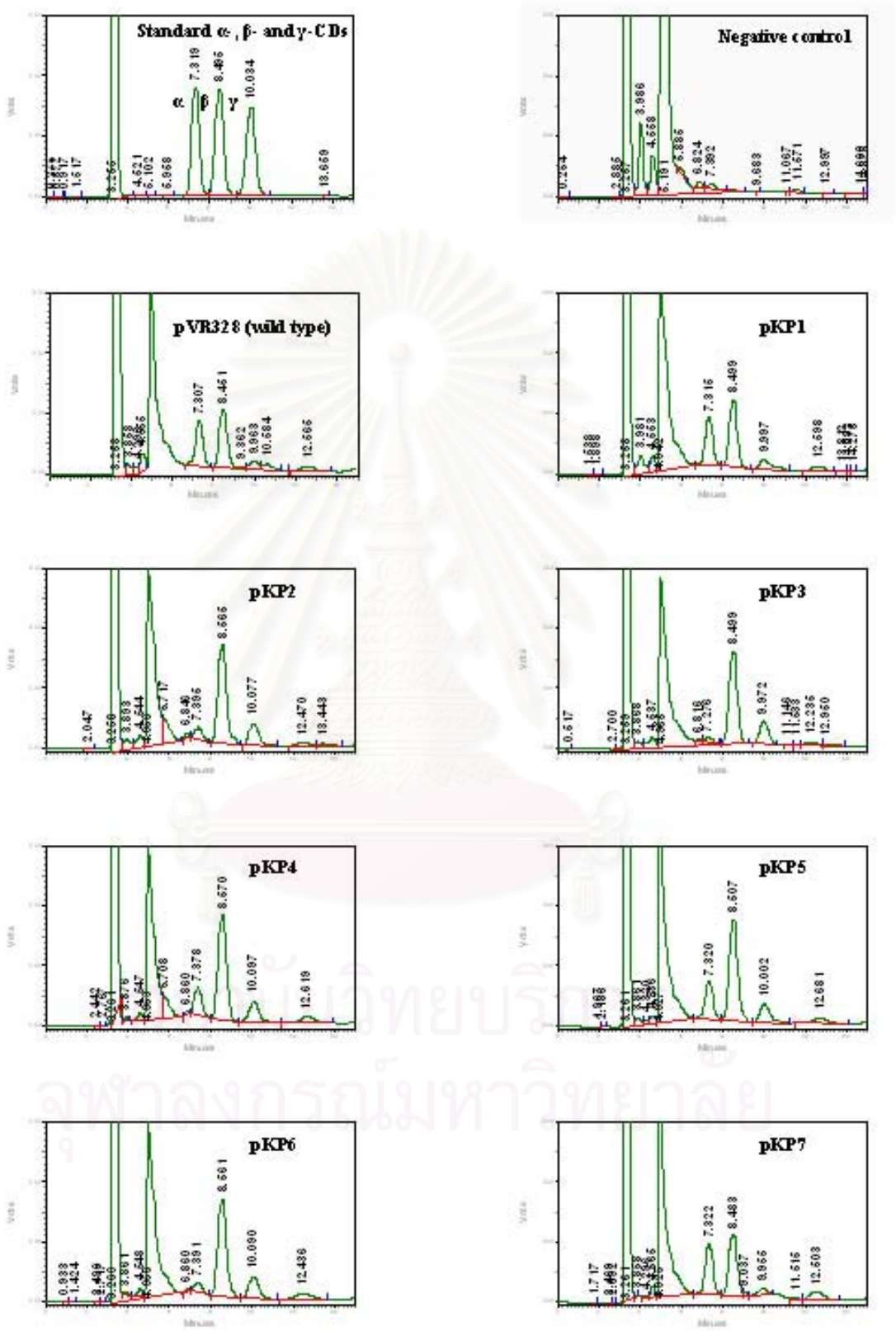

Fig. 3.10. HPLC profiles of cyclodextrins formed by the wild type and mutant CGTases.

Table 3.2. Summary of CD-forming activities of the mutant CGTases.

Plasmid	Mass ratio	mole ratios, α: β:γ
pVR328 (wild type)	32:54:14	36:52:12
pKP1	35:53:12	39:51:10
pKP2	7:75:18	8:76:16
pKP3	3:78:19	4:79:17
pKP4	15:69:16	18:68:14
pKP5	22:64:14	25:63:12
pKP6	6:76:18	8:76:16
pKP7	40:54:6	44:51:5

Table 3.3. Summary of CD production by the mutants

Mutant	Mutation region	Type of replacement	CD production		
			α-CD	b-CD	γ-CD
pKP1	I	γ	No	No	No
pKP2	II	α	Decrease	Increase	No
pKP3	III	γ	Decrease	Increase	No
pKP4	IV	γ	Decrease	Increase	No
pKP5	III	Arginine	Decrease	Increase	No
pKP6	III	Arginine	Decrease	Increase	No
pKP7	V	Lysine	No	No	Decrease

CHAPTER IV

DISCUSSION

This thesis describes the investigations of cyclodextrin glucanotransferase (β-CGTase) from *Bacillus circulans* A11. Recently (Kerdsin, 2003) had suggested that the four stretches of amino acid sequences, 239-250, 262-271, 284-320 and 333-342 residues, may be involved in the cyclodextrin product specificity (Fig. 3.1). The thesis presents the mutation of selected β-CGTase residues in these regions to determine the CD product specificity (Fig. 3.2 and 3.3). Some detailed knowledge of the mechanism of the β-CGTase catalyzed reactions was required for the construction of some mutant enzymes via rational protein engineering.

4.1. Three-dimensional structure of the CGTase

The amino acid sequence of *Bacillus circulans* A11, β-CGTase, (wild type A11 CGTase) is about 98% homology to that of β-CGTase from *Bacillus* sp. 1011 (PDB 1I75), whose the X-ray crystallographic structure is already known. The overall predicted structure of the wild type A11 CGTase was found to be similar to the β-CGTase from *Bacillus* sp. 1011 (Fig. 4.1). The three-dimensional structures of all mutant CGTases (pKP1, pKP2, pKP3, pKP4, pKP5, pKP6 and pKP7) were also predicted and compared with the wild type CGTase. The comparison of the conformation around the mutated residues showed clearly that no large structure arrangement had taken place as a result of amino acid changes. The structures of the wild type CGTase and the mutant CGTases with all the mutated regions were shown in Fig. 4.2. Regions II, III and IV were located on the surface of the enzyme and

residues on the loop regions and region I located on the opposite side. The mutation regions resided outside the active site cleft (catalytic residues Asp229 and Glu257), and perhaps did not have any influence on the active site structure of the enzyme. This may be the reason why all mutant proteins retained their catalytic activity.

4.2. Dextrinizing activity of the CGTase

The *E*. coli XL1Blue transformants harboring each mutant CGTase gene was tested for dextrinizing activity on the LB-starch agar plates (Fig. 3.7). The clear zones surrounding the colonies appeared after exposing to a KI-I_2 indicator solution. It indicated that starch around the colonies had been degraded by the hydrolytic activity of the CGTases. From the result, all mutant clones exhibited the dextrinizing activity. One of the mutant CGTases had decreased dextrinizing activity. The pKP1 mutant had strongly decreased dextrinizing activity while the other mutants had about the same level of dextrinizing activity as the wild type CGTase (Fig. 3.7).

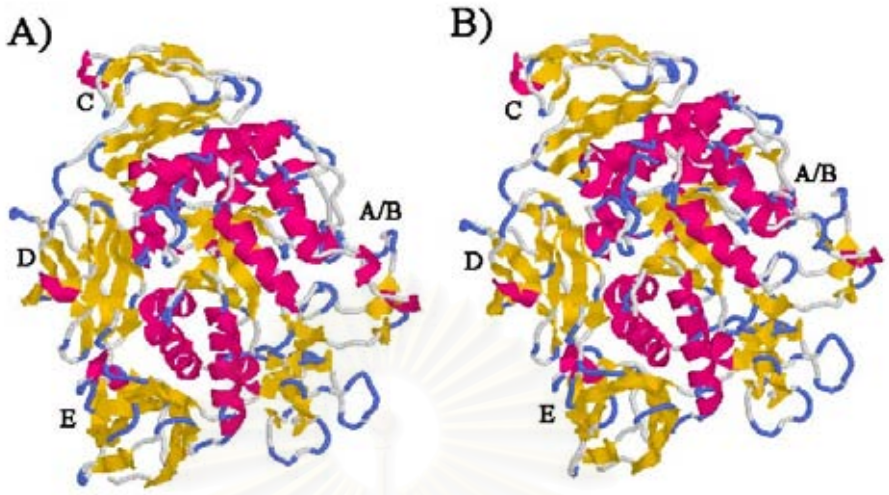

Figure 4.1. Comparison of the three-dimensional structures between the CGTase from *Bacillus* sp.1011 (PDB 1I75) (A) and the CGTase from *B. circulans* A11 (B).

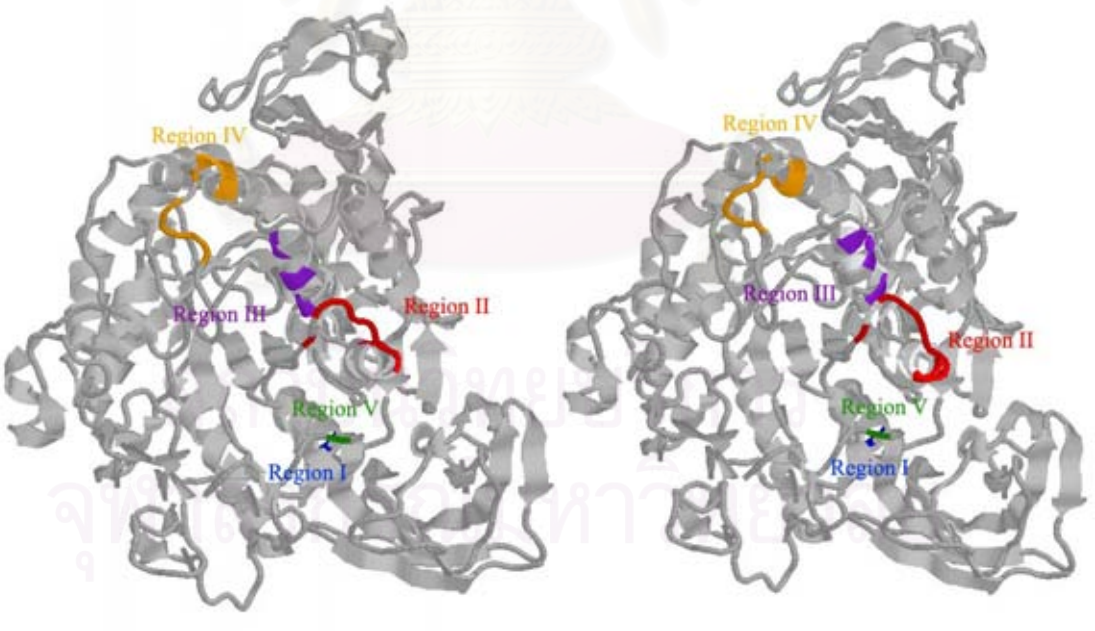

Figure 4.2. Comparison between the three-dimensional structure of the wild type CGTase from *B. circulans* A11 (A) and the predicted structure of mutant CGTases with all mutated residues (B).

Furthermore, the culture media of the transformant were also assayed for dextrinizing activity (Fig. 3.9 and Table 3.1). The mutated regions II (pKP2), III (pKP3, pKP5 and pKP6) and IV (pKP4) had decreased dextrinizing activity, especially the mutated regions II and III. The mutation in region IV farther from the active site cleft had less effect on the dextrinizing activity. This decrease in activity might be due to the fact that the mutations changed the amino acid residues in the surrounding of the active site and the entrance for the oligosaccharide substrate. The single mutations in regions I (pKP1) and S184 (pKP7) seemed to have small or no effect on the dextrinizing activity. The mutagenized amino acid in pKP1 located on the backside of the domain A. However, the result disagreed with that of halo zone assay on a LB-agar for unknown reasons.

4.3. Cyclodextrin forming activity

Upon culturing, the CGTases produced by the *E*. coli XL1Blue transformants were secreted into the medium. The cell culture medium was used as a source of crude enzyme for assaying the CD-forming activity. The crude enzyme from each mutant was incubated with soluble starch at 37 ºC for 12 hours. The remaining oligosaccharides were digested with β-amylase, and the reaction products were subjected to HPLC analysis. HPLC profiles of the cyclodextrin production indicated that the mutant enzymes produced the cyclodextrins at various ratios, and some of them were different from that of the wild type enzyme (Fig. 3.10, Table 3.2 and Table 3.3). The size of the CD product depends directly upon the number of glucose units which are bound in the active site up to subsites -6, -7, or -8 (Strokopitov *et al*., 1996).

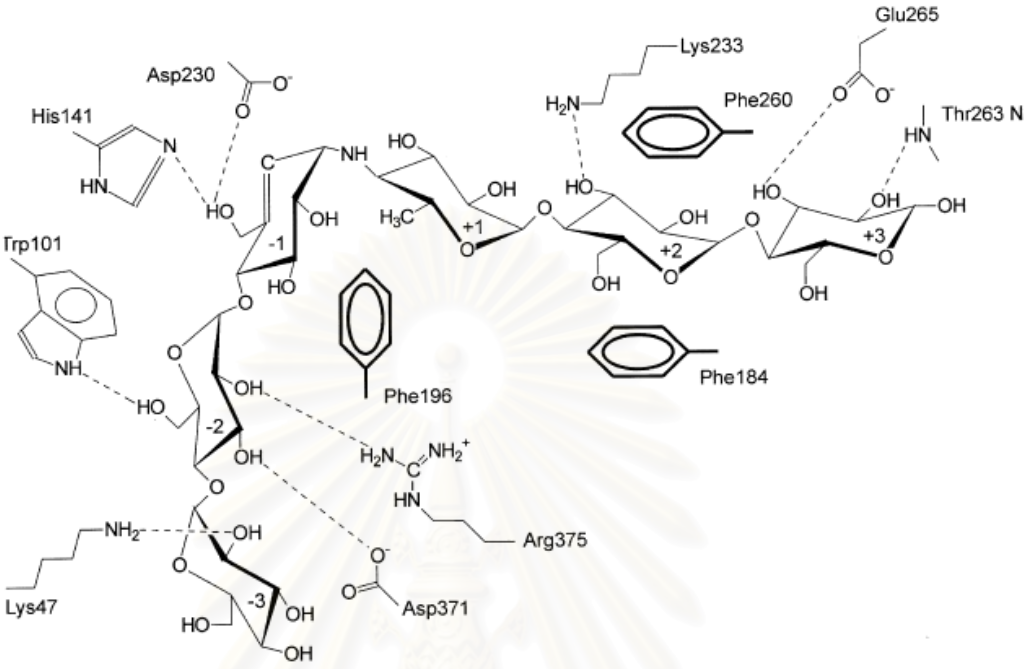

Figure 4.3. Overview of the interactions between Tabium CGTase and a maltohexaose inhibitor bound from subsites -3 to +3. For clarity, not all interactions at subsites -1 and + 1 are shown (Wind *et al*., 1998).

For the mutated region I (pKP1), amino acid residue was changed in favor of residue found in the γ-CGTase group (Fig 3.1) which located on the backside of the active site. The pKP1 seemed to have no influence on the CD product ratio.

The mutated region III, pKP3 and pKP6 (E264R/E268R) increased significantly the production of β-CD, slightly increased the production of γ-CD, while the production of α-CD was drastically diminished. The pKP5 (E264R) had moderate negative effect on the production of α-CD. The region III probably constituted the acceptor binding site equivalent to the proposed acceptor subsite +3 and part of acceptor subsite +2.

Previously, the acceptor subsite + 2 was important for the hydrolysis and transglycosylation reaction specificity of CGTase (van der Veen *et al*., 2001; Leemhuis *et al*., 2002b) while the acceptor subsite +3 of Tabium CGTase bound with the substrate via Thr263 and Glu265 locating on loop 260- 268 (equivalent to region III in this study) (Fig. 4.3). The subsite +3 has a high affinity for sugar acceptors (Leemhuis *et al*., 2002a). On the other hand, both Phe183 and Phe259 (*B.circulans* 251) was involved in substrate binding through stacking interaction with the sugar ring at subsite +2 (van der Veen *et al*., 2001). van der Veen *et al.* (2001) proposed that the Phe183 and Phe259 played important and distinct roles in the transglycosylation reactions catalyzed by the CGTase. The Phe183 affected especially the cyclization and coupling reactions, whereas Phe259 was the most important for the cyclization and disproportionation reactions. The mutated region III (pKP3), amino acid residues changed in favor of γ-CGTase group (Fig. 3.1) were equivalent to mutation in the acceptor subsites +2 and +3 (Wind *et al*., 1998) that might affect the hydrogen bond network, Van der Waals interactions with aromatic residues and hydrophobic effect

from displacement of bound water molecules in these subsites (Johnson *et al*., 1988 and Quiocho, 1986, 1989). The geometry of the both acceptor subsites +2 and +3 might be changed, and affected on the ratios of CD production.

E264 was specifically involved in initial binding of the acceptor subsite +3 in the groove of substrate entrance (van der Veen *et al*., 2001), and E268 also appeared in this groove (Fig. 4.3 and 4.4). It was possible that E268 might be involved in the substrate binding. From the results, the pKP5 and pKP6 had decreased dextrinizing activities. The affect of pKP6 were stronger than that of the pKP5 because of the double mutation in the pKP6. This result suggested that E268 was also part of the substrate binding cleft.

The mutated region II [pKP2 (R284E/K288E/Q291E)] was close to the mutation region III. The effects of mutation in region II might be the same as those of region III. In fact, they reduced strongly the dextrinizing activity and their product specificities were similar.

The mutated region IV (pKP4) had about the same level of negative effect on dextrinizing activity as mutations in pKP5 and 6. The pKP4 significantly decreased the proportion of α-CD production and slightly increased the proportion of β-CD production albeit less pronounces than the pKP2, 3 and 6. This might be because region IV lay farther away from the active site. It could be postulated that the effect of pKP4 was similar to those of pKP2, 3 and 6.

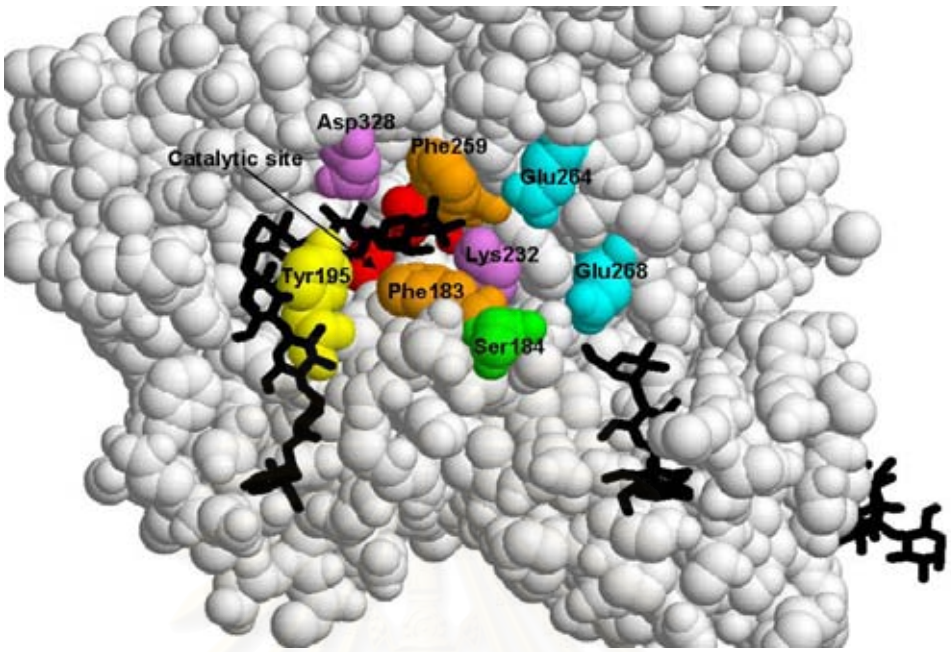

Figure 4.4. Overview of the interactions between a maltononaose substrate and *B. circulans* strain 251 CGTase from subsites -3 to +3. For clarity, not all interactions at subsites -1 and + 1 are shown (PDB 1CXK).

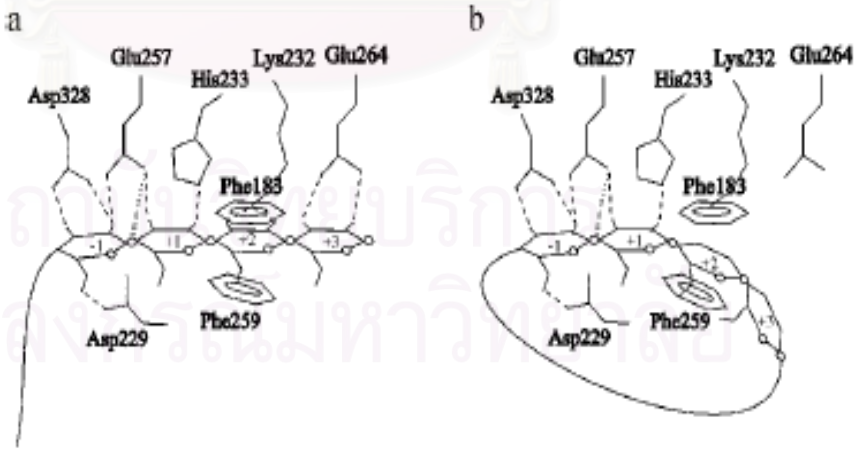

Figure 4.5. Schematic representation of the interactions between the *B.circulans* strain 251 CGTase and sugars bound at the active site. a) binding mode of a linear oligosaccharide (Strokopytov *et al*., 1996, Uitdehaag *et al*., 1999b). b) binding of a cyclodextrin (Uitdehaag *et al*., 1999a).

Phe183 involved the binding of the acceptor subsite +2 in the groove of substrate entrance (Fig. 4.4 and 4.5). Phe183 and Phe259 formed the hydrophobic interaction with substrate at subsite +2 (Strokopytov *et al*., 1996 and Uitdehaag *et al*., 1999a, 1999b). By replacing the S184 with K in pKP7, it was expected to interfere with the substrate binding. Two assumptions of this mutation were made; the mutation at 184 might affect the Phe183 interaction with substrate or it formed salt bridge with one of the two glutamates nearby blocking the possible entrance route for the substrate (Fig. 4.4 and 4.5). The results showed that the dextrinizing activity of pKP7 was similar to those of the wild type, while the pKP7 had decreased proportion of γ-cyclodextrin production about 2 folds. The S184K might interfere with amino acids in the acceptor subsite +2 and +3, but might not block the possible entrance route. Since the pKP7 had no effect on the dextrinizing activity, it was not possible to conclude the effect of the S184 mutation.

CHAPTER V
CONCLUSIONS

1. The mutation in region I had no influence on the product specificity of the CGTase from *Bacillus circulan* A11.
2. The mutations in regions II and III favored β-CD production, and region IV showed little affect on β-CD production.
3. The S184K mutation slightly increased the proportion of α-CD with the expense of γ-CD.
4. The E264R and E268R might interfere in the substrate binding at the acceptor subsite +2 and +3.

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APPENDICES

APPENDIX A

1. Reagents for plasmid preparation

1.1. Lysis solution: 10 mL

50% Glucose	2	mL
0.5 M EDTA	0.2	mL
1 M Tris-HCl (pH 8.0)	0.25	mL
ddH_2O	7.55	mL

1.2. Alkaline SDS solution: 10 mL

5% SDS	2	mL
5 N NaOH	0.4	mL
ddH_2O	7.6	mL

1.3. Hight salt solution: 3 M Sodium acetate (pH 5.2)

$NaOAc.3H_2O$	408.1	g
ddH_2O	700	mL

Adjust pH to 5.2 with glacial acetic and adjust the volume to 1 litre with water.

2. Other reagents for preparation

2.1. RNase A solution

Dissolve RNase A (pancreatic) at a concentration of 10 mg/mL in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, then heat at 100 ºC, 15 min, cool slowly at room temperature, aliquot, and store at -20 ºC.

2.2. 10% Glycerol

Glycerol	10	mL
Water	90	mL

2.3. 5× TBE buffer (for agarose gel electrophoresis)

Tris-base	54	g
Boric acid	27.5	g
0.5 M EDTA (pH 8)	20	mL

2.4. 0.5 M EDTA

EDTA	186.1	g
Water	1000	mL

Dissolve EDTA in 800 mL water and adjust pH to 8.0 with NaOH before adjusting volume to 1 litre, and then autoclave.

2.5. 1M Tris-HCl

Tris-base	121.1	g
Water	1000	ml

Adjust pH to 7-8 before adjusting volume to 1 litre, and then autoclave.

2.6. 5% SDS (store at room temperature)

SDS	5	g
Water	100	mL

2.7. 5N NaOH

NaOH	20	g
Water	100	mL

Dissolve NaOH in 70 ml water before adjusting volume to 100 mL

2.8. Loading buffer (for agarose gel electrophoresis)

Glycerol	20	mL
Bromphenol blue	4	mg
Water	80	mL

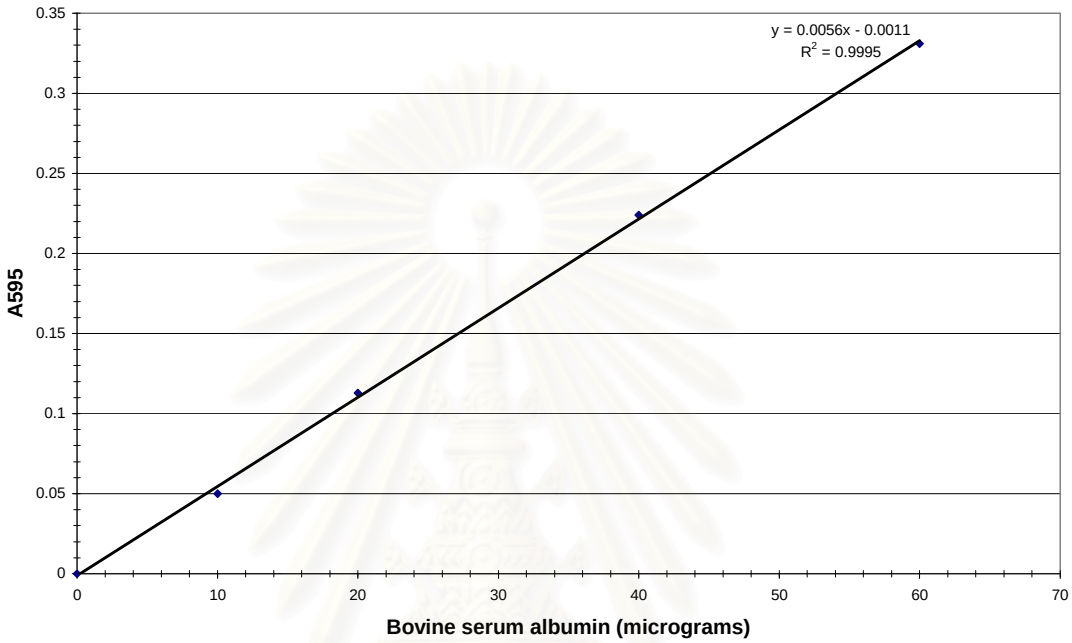
2.9. Bradford's reagent

Coomassie blue G 250	50	mg
95% Ethanol	25	mL
85% H_3PO_4	50	mL

Adjust volume to 500 mL with water.

APPENDIX B

Standard curve for protein determination by Bradford's method.



BIOGRAPHY

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