การสร้างไซโคลเดกซ์ทรินกลูคาโนทรานสเฟอเรสไคเมอริกโดยอาศัยโฮโมโลกัสรีคอมบิเนชัน
และศึกษาการทำงานของเอนไซม์ไคเมอริก

นายอนุศักดิ์ เกิดสิน

CONSTRUCTION OF CHIMERIC CYCLODEXTRIN GLUCANOTRANSFERASES BY

HOMOLOGOUS RECOMBINATION AND STUDY OF THEIR ACTIVITIES

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อนุศักดิ์ เกิดสิน : การสร้างไซโคลเดกซ์ทรินกลูคาโนทรานสเฟอเรสไคเมอริกโดยอาศัยโฮโมโลกัสรีคอมบิเนชันและศึกษาการทำงานของเอนไซม์ไคเมอริก. (CONSTRUCTION OF CHIMERIC CYCLODEXTRIN GLUCANOTRANSFERASES BY HOMOLOGOUS RECOMBINATION AND STUDY OF THEIR ACTIVITIES) อ. ที่ปรึกษา : ผศ.ดร. วิเชียร ริมพณิชยกิจ, 106 หน้า. ISBN 974-17-3661-4

ไซโคลเดกซ์ทริน (CDs) เป็นโอลิโกแซ็กคาไรด์ที่ต่อกันเป็นวงด้วยกลูโคสจำนวน 6, 7 และ 8 หน่วย มีชื่อเรียกว่า α-, β- และ γ-ไซโคลเดกซ์ทริน ตามลำดับ CDs เป็นผลิตภัณฑ์ที่เกิดจากการย่อยแป้งของเอนไซม์ไซโคลเดกซ์ทรินกลูคาโนทรานสเฟอเรส (CGTase) สามารถนำไปใช้ประโยชน์ในอุตสาหกรรมต่างๆ CGTase ประกอบด้วย 5 โดเมน คือ A, B, C, D และ E โดยโดเมน B จะแทรกอยู่ระหว่างโดเมน A ทำให้แบ่งโดเมน A ออกได้เป็น โดเมนย่อย A1 และ A2 CGTase จากธรรมชาติ โดยทั่วไปจะผลิต CDs ทั้ง 3 ชนิด ในสัดส่วนที่ไม่เท่ากัน ซึ่งต้องเสียเวลาและต้นทุนที่แพงในการแยกไซโคลเดกซ์ทรินแต่ละชนิดให้บริสุทธิ์ ในการศึกษาครั้งนี้ต้องการหาบริเวณสำคัญที่มีผลต่อการสร้างไซโคลเดกซ์ทรินในสัดส่วนที่แตกต่างกันของเอนไซม์นี้ โดยทำการสร้างเอนไซม์ไคเมอริกด้วยวิธีโฮโมโลกัสรีคอมบิเนชัน ระหว่าง α-CGTase จาก *Paenibacillus macerans* และ β-CGTase จาก *Bacillus circulans* A11 ทำการทรานสฟอร์มพลาสมิดที่มียีน β-CGTase อยู่ด้าน 5' ของยีน α-CGTase (pVR321) หรือพลาสมิดที่มียีน α-CGTase อยู่ด้าน 5' ของยีน β-CGTase (pVR388) ซึ่งผ่านการตัดด้วยเอนไซม์ตัดจำเพาะที่เหมาะสม แล้วนำเข้าสู่เซลล์ที่มีศักยภาพในการเกิดรีคอมบิเนชัน จากนั้นทำการศึกษาการทำงานของเอนไซม์ไคเมอริกที่ได้ จากการศึกษาได้พลาสมิดไคเมอริก 2 กลุ่ม คือ พลาสมิดไคเมอริกจาก pVR321 ซึ่งยังคงให้ CD หลักเป็น β-CD พบว่าการเกิดรีคอมบิเนชันที่บริเวณโดเมน C และ D มีผลทำให้ลดการสร้าง α-CD ขณะเดียวกันเพิ่มการสร้าง β- และ γ-CDs นอกจากนี้ยังพบว่าการเกิดรีคอมบิเนชันภายในโดเมนย่อย A2 ที่เหนือจากบริเวณปลายของโดเมนย่อยขึ้นไปมีผลต่อการสร้าง α- และ β-CDs เพียงเล็กน้อย แต่สามารถเพิ่มสัดส่วนการสร้าง γ-CD สำหรับพลาสมิดไคเมอริกจาก pVR388 พบว่าส่วนใหญ่สูญเสียความสามารถในการทำงานยกเว้น pVR402 และ 403 ซึ่งให้ CD หลักเป็น β-CD พบว่าบริเวณเกิดรีคอมบิเนชันอยู่ด้านปลายของโดเมนย่อย A1 โดยมีบริเวณทางปลายอะมิโนของเอนไซม์ประมาณ 100 กรดอะมิโนเป็นของ α-CGTase เมื่อทำการสร้าง pVR404 โดยทำการแทนที่โดเมนย่อย A2 โดเมน C, D และ E ใน pVR402 ด้วย α-CGTase ซึ่งเอนไซม์ไคเมอริกนี้ยังคงบริเวณปลายของโดเมนย่อย A1 และ B ของ β-CGTase พบว่าสามารถสร้าง CD หลักเป็น α-CD เมื่อเปรียบเทียบกับไคเมอริก pVR402, 403 และไคเมอริกจาก pVR321 พบว่าโดเมนย่อย A2 น่าจะมีบทบาทสำคัญต่อความจำเพาะของผลิตภัณฑ์ แต่การมีส่วนของ β-CGTase ในบริเวณนี้ทำให้มีการสร้าง β-CD ได้เพิ่มขึ้นมากและลดสัดส่วนการสร้าง α-CDs ได้พอสมควร ถึงแม้ว่าโดเมน A และ B จะมีความสำคัญต่อความจำเพาะของการสร้าง CDs แต่โดเมนอื่นก็มีส่วนช่วยในการสนับสนุนความจำเพาะของการสร้าง CDs เช่นกัน

ภาควิชา.............ชีวเคมี........................ ลายมือชื่อนิสิต...

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

ปีการศึกษา......... 2546.................... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.................................

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KEY WORD: Cyclodextrin / Cyclodextrin glucanotransferase / Chimeric / Homologous recombination

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Cyclodextrins are cyclic oligosaccharides of 6, 7 and 8 glucose units, called α-, β-, and γ-cyclodextrins (CDs), 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. CGTase is an enzyme consisting of 5 domains, A, B, C, D and E; domain B divides domain A into subdomain A1 and A2. It has been known that natural CGTases produce a mixture of α-, β- and γ-CDs with different ratios and the separation of CDs is time-consuming and employs expensive organic solvents. This study is aimed to determine the essential part of enzyme that may be involved in different ratio of CDs production by using *in vivo* homologous recombination between α-CGTase from *Paenibacillus macerans* and β-CGTase from *Bacillus circulans* A11. Two plasmids, containing a direct repeat of either the β- and α-CGTase genes (pVR321) or α- and β- CGTase genes (pVR388), were used to construct the chimeric CGTase genes. After appropriate restriction enzyme digestion, each linearized plasmid was transformed into a recombination-proficient host. The resulting chimeric clones were characterized. Two series of chimeric plasmids were obtained. For chimeric CGTases from pVR321 series, we found that recombination within the domains C and D decreased the production of α-CD significantly and increased the production of β- and γ-CDs. Recombination within the 3'-half of subdomain A2 had little effect on the production of α- and β-CDs, while the proportion of γ-CD was slightly increased. The chimeric CGTases in this series still produce β-CD as a major product. For the chimeras in pVR388 series, several of them showed neither dextrinizing nor cyclization activities. Interestingly, two chimeras in this series, pVR402 and 403 whose N-terminal regions about 100 amino acid residues (N-terminal half of subdomain A1) derived from α-CGTase, produced β-CD as major product. Replacing the subdomain A2, domains C, D and E in pVR402 with the wild type α-CGTase sequence results in a chimeric plasmid pVR404, whose CGTase gene is equivalent to α-CGTase containing the C-terminal half of subdomain A1 and domain B of the β-CGTase sequence. The pVR404 produced α-CD as major product. By comparing the results from pVR404 with those of pVR402, 403 and pVR321 series, the subdomain A2 region most likely contains the determinant for CGTase product specificity. Nevertheless, the existence of the β-CGTase sequence in this region renders the CGTase produced more β- and less α-CDs. In summary, it can be seen that although the A and B domains are considered to be important for product specificity, the other domains also contributed to this specificity as well.

Department..............Biochemistry ....Student's signature………………………………………………

Field of study...........Biochemistry.............Advisor's signature……………………………………….......

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ABBREVIATION

Å	Aungstrom
BSA	Bovine serum albumin
CDs	Cyclodextrins
CGTase	Cyclodextrin glucanotransferase
$^{\circ}$C	Degree celsius
µl	Microlitre
ml	Millilitre
mM	Millimolar
µM	Micromolar
M	Molar
µg	Microgram
rpm	Revolution per minute
nm	Nanometre

CHAPTER I

INTRODUCTION

Starch is one of the most abundant biopolymers on earth and it is the main source of energy for a wide variety of organisms. This molecule is buit from units of glucose , linked by either α(1,4) and α(1,6) glycosidic bonds. Starch molecules are mostly found in seeds or roots in the form of granules consisting of two types of glucan polymers, linear amylose and branced amylopectin (van der Veen *et al*., 2000d)

Many organisms are able to use starch as carbon and energy sources for growth. The organisms possess different starch processing enzymes, all of which are grouped as the α-amylase family (Fig. 1). This group includes enzymes that display specificity for either of the two types of bonds, α(1,4) and α(1,6), and whose activities classify them as either hydrolases (EC. 3.2.1) or transferases (EC. 2.4.1) (Fig. 2)(del Rio *et al*., 1997).

Cyclodextrin glucanotransferase (CGTase;1,4-α-D-glucan : 1,4-α-D-glucopyranosyl-transferase EC. 2.4.1.19) is a unique extracellular enzyme capable of converting starch and related substrates into cyclic oligosaccharide, known as cyclodextrins (CD). CGTase is produced by a variety of bacteria including (Tonkova, 1998).

- aerobic mesophilic bacteria : *Pseudomonas* spp., *Bacillus cereus, B. megaterium, B. ohbensis, Paenibacillus macerans, Klebsiella oxytoca, K. pneumoniae, Micrococcus luteus, Brevibacillus brevis*
- aerobic thermophilic bacteria : *B. stearothermophilus*
- anaerobic thermophilic bacteria : *Thermoanaerobacterium thermosulfunigens, Thermoanaerobacter* sp.
- aerobic alkalophilic bacteria : *B. circulans , Bacillus* sp. AL-6
- aerobic halophilic bacteria : *B. halophilus*

When these bacteria excrete CGTases into the starch medium. These CGTases convert starch into cyclodextrin, which are subsequently absorbed and degraded by the action of an enzyme cyclodextrinase, a membrane-associated enzyme located at the cytosolic side (Fig. 3). There are two possible explanations for the existance of this complicated system.

(i) The organism may build up an external storage form of glucose, not accessible for most other organisms because they are not able to metabolize cyclodextrins (Penninga, 1996b).

(ii) Cyclodextrins are used to form inclusion complexes with toxic compounds in its environments or with compounds needed for growth (Aeckersberg, 1991).
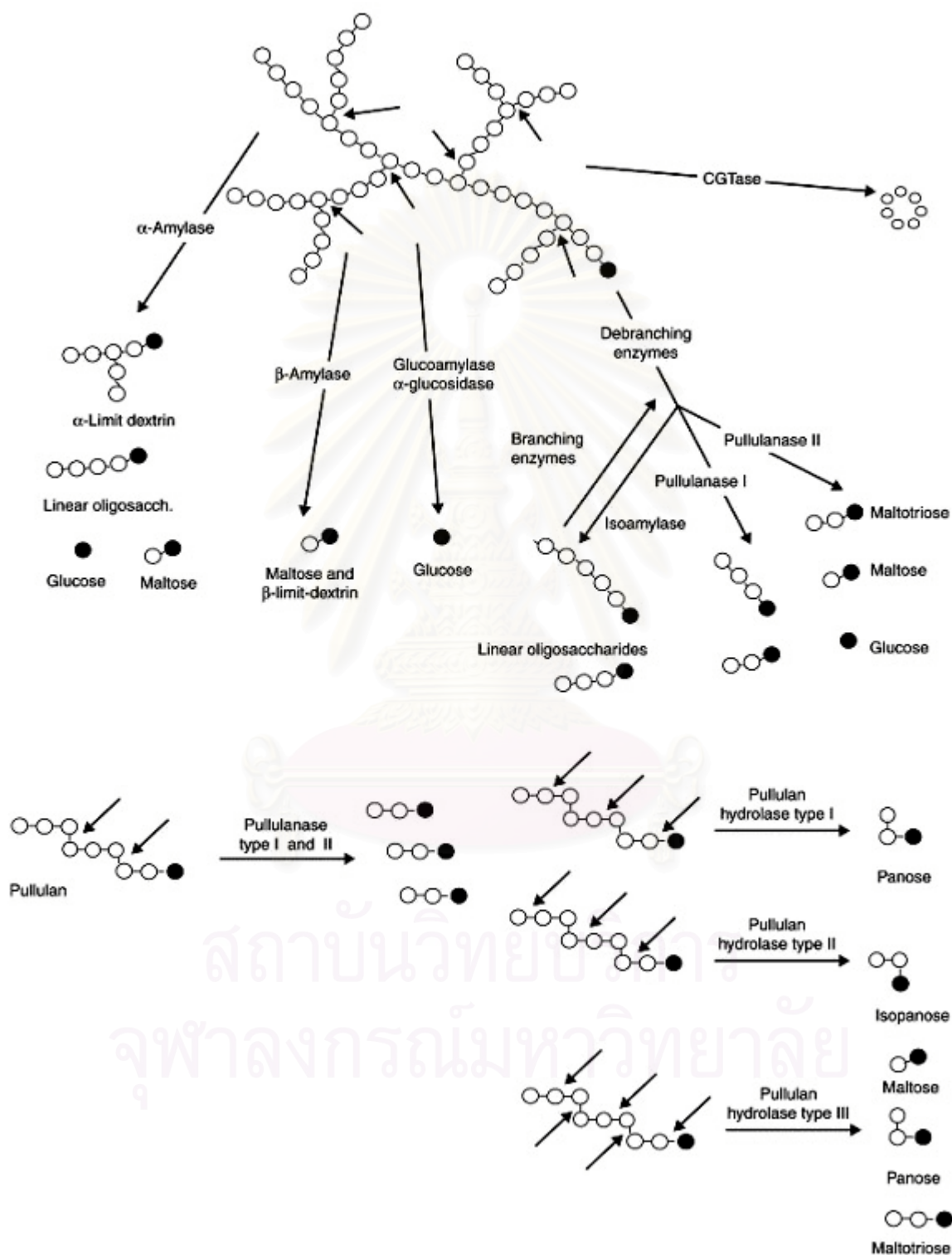
Figure 1 : Schematic representation of the action of starch-processing enzymes. Black circles indicate the reducing sugars (Source : Bertodo and Antrnikian, 2002).
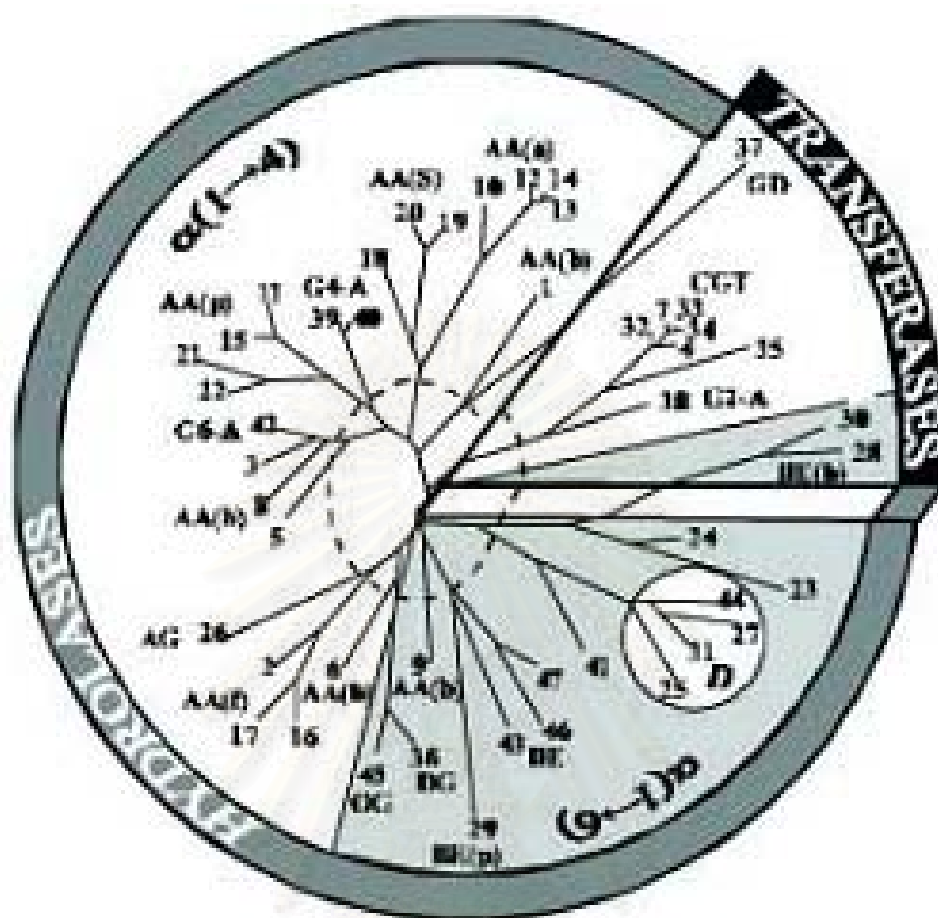
Figure 2 : Distance tree of the starch-processing enzymes. Specificity for α(1,4) bonds is indicated as a white area, α(1,6) bonds as a gray area, and dual bonds as an area with a *D*. The segment sticking out indicates transglycosylation enzymes; other are hydrolyzing enzymes. The letters between brackets indicate the enzyme origin, from animal (a),bacteria (b), fungi (f), plant (p), or *Streptomyces* (s) sources. AA : α-amylase; AG: α-glucosidase; AP: α-amylase-pullulanase; BE: branching enzyme; CGT: cyclodextrin glucanotransferase; DG: dextran glucosidase; GD: glycogen debranching enzyme; G2-A: maltogenic α-amylase; G4-A: malto-tetraohydrolase; G6-A: malto-hexaohydrolase; OG: oligo 1,6-glucosidase. 1-22 are α-amylase; 22-26: α-glucosidase; 27: α-amylase-pullulanase; 28-30: branching enzymes; 31: cyclodextrinase; 32-35: cyclodextrin glucanotransferase; 36: dextran glucosidase; 37 : glycogen debranching enzyme; 38: maltogenic α-amylase; 39-40: malto-tetraohydrolase; 41-42: malto-hexaohydrolase; 43: isoamylase; 44: neopullulanase; 45: oligo 1,6 glucosidase; 46-47: pullulanase (Source : Penninga,1996b ; reproduced from Jespersen *et al.,* 1993).
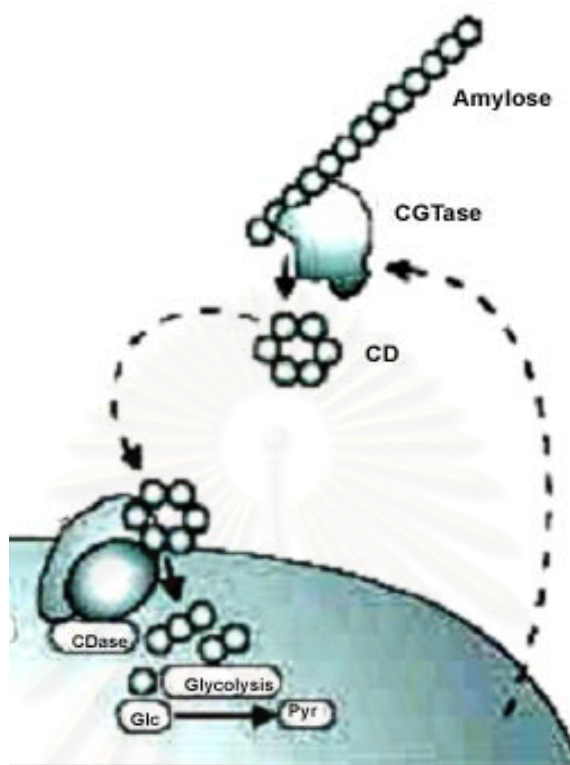
Figure 3 : Schematic representation of the location and action of CGTase and cyclodextrinase (CDase) (Source : Penninga, 1996b).

1. Application of cyclodextrins and CGTases

1.1. Application of cyclodextrins

CGTases are able to convert starch to cyclodextrins via the cyclization reaction. The cyclodextrins are cyclic $\alpha(1,4)$ linked oligosaccharides mainly consisting of 6, 7 or 8 glucose residues, designated as α- , β- and γ- cyclodextrins (α- , β- and γ- CDs), respectively (Fig. 4a). The glucose residues in the cyclodextrin rings are arranged in such 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 (Fig. 4b). The apolar C3 and C5 hydrogens and ether-like oxygens are at the inside and the hydroxyl groups at the outside of these molecules. This results in torus shaped molecule with a hydrophilic outside, which can dissolve in water, and a hydrophobic cavity, which enables cyclodextrins to form inclusion complexes with a wide variety of guest molecules (Fig. 4c). Their sizes are an important parameter for complex formation with various hydrophobic compounds or functional groups (Table 1) (van der Veen *et al.*, 2000d). Thus each type of cyclodextrin is used for complexation with contain group of guest molecules.

Table 1 : Properties of cyclodextrins

	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25 $^{\circ}$C (%W/V)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Inner diameter (Å)	4.7 – 5.3	6.0 – 6.5	7.5 – 8.3
Height of torus (Å)	7.9	7.9	7.9
Approximately cavity volume (Å)	174	262	427

The driving force of inclusion complex formation is the entropic effect of displacement of water molecules from the hydrophobic environment in the cavity. This is probably combined with the fact that the water causes strain on the cyclodextrin ring, which is released after complexation, producing a more stable and lower energy state (van der Veen *et al.,* 2000d). The formation of inclusion complexes leads to changes in the chemical and physical properties of the encapsulated compounds. This has led to various applications of cyclodextrins in analytical chemistry, agriculture, pharmaceutical, food, cosmetics, biotechnology and toiletry.

In analytical chemistry, cyclodextrins are used for the separation of enantiomers by HPLC or GC. In nuclear magnetic resonance (NMR) studies, they can act as chiral shift agents and in circular dichroism as selective agents altering the spectra. In electrochemical chemistry, they can be used to mask contaminating compounds, allowing more accurate determinations. In agriculture, cyclodextrins can be applied to delay seed germination. In food industry, the cyclodextrins have found several applications such as texture-improving of pastry and meat products, reduction of bitterness, ill smell and taste, stabilization of flavors and emulsions like mayonnaise and depletion of cholesterol from milk. In the pharmaceutical industry, cyclodextrins increase the water solubility of several poorly water-soluble substances, improve bioavailability, facilitate the handling of volatile products and reduce the dose of the drug administered. Cyclodextrins also improve the stability of substances *i.e.,* increase their resistance to hydrolysis, oxidation, heat, light and metal salts. The inclusions of irritating medicines in cyclodextrins can also protect the gastric mucosa for the oral route, and reduce skin damage for the dermal uses (Penninga,1996b). Further, cyclodextrins have been successfully applied in the production of vacuolating cytotoxin from *Helicobacter pylori* , a

protein that may have a key role in the pathology of gastric disease associated with a risk for gastric carcinoma (Tonkova, 1998).
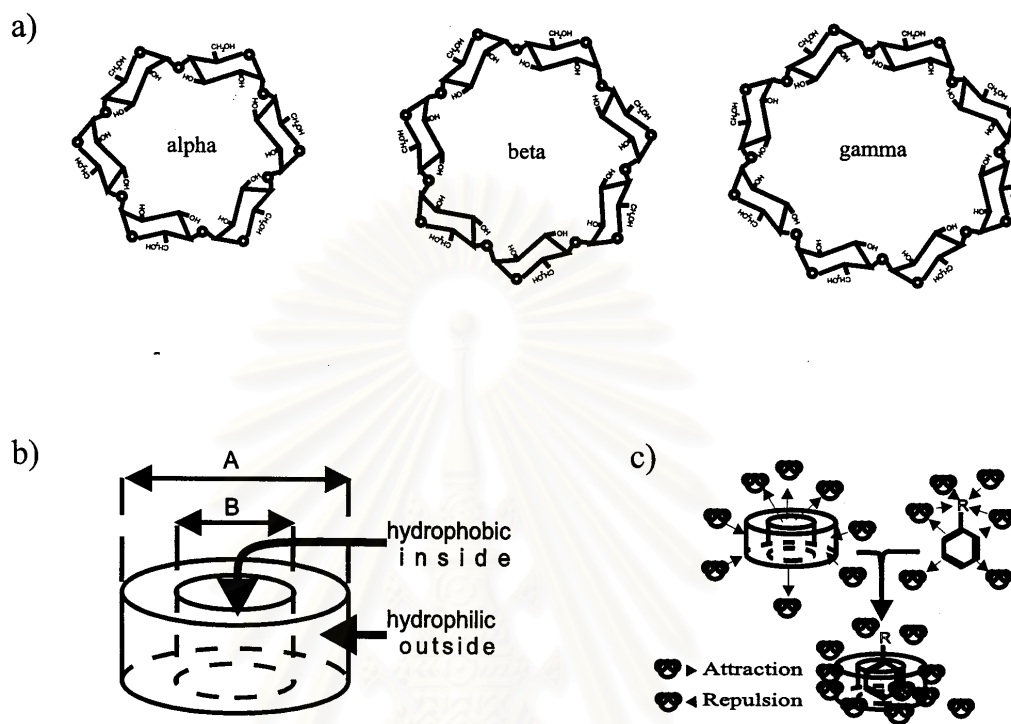
Figure 4 : Structures and properties of cyclodextrins. (a) α-, β-, and γ-cyclodextrins; (b) three-dimensional form and properties of cyclodextrins; (c) formation of inclusion complex of a cyclodextrin with a guest molecule (Source : van der Veen *et al.,*2000d).

1.2. Applications of CGTase

CGTase can be used for the transfer of oligosaccharides from donor substrate such as cyclodextrin or starch to various acceptor molecules by the coupling and disproportionation reactions, resulting in the novel glycosylated compounds. A commercial application of this method is found in the glycosylation of the intense sweetener, stevioside. Glycosylation decreases bitterness and increases solubility of this compounds. CGTase can be applied in the processes for surface sizing or coating of paper, improving the writing quality of the paper and obtaining a glossy and well printable surface. CGTase can also be used in the preparation of doughs for baked products which comprises the incorporation of the CGTase into the dough to increase the volume of the baked product (van der Veen *et al*., 2000d, Sin *et al*., 1994).

2. Limitation of cyclodextrin production by CGTase

A major disadvantage of cyclodextrin production by CGTase is that all wild type CGTases usually produce a mixture of α- , β- and γ-cyclodextrin and are sensitive to product inhibition (van der Veen *et al*., 2000d,e). Two different industrial approaches are used to purify the produced cyclodextrins, solvent and non-solvent processes. The solvent process is the selective crystallization of complexes of cyclodextrins with organic solvents. The toluene and cyclohexane are commercially used for the complexation and selective precipitation of β- cyclodextrin. For α- cyclodextrin, 1-decanol can be used, but this compound is difficult to remove from aqueous solution because of its high boiling point (229 $^{\circ}$C). For γ-cyclodextrin, cyclododecanone can be used but this solvent is very expensive. Further, disadvantages of the use of organic solvents are their toxicity, which limits the applications to human consumption, their flammability and the need for a solvent recovery process (van der Veen *et al*., 2000d).

The non-solvent process was first developed for β-cyclodextrin production. Due to its low solubility, β-cyclodextrin can be easily purified by crystallization steps. The purification of α- and γ-cyclodextrin is achieved via complex and expensive chromatography with low yields and a wide range of by-products. Compared to the solvent process, a lower yield of enzyme reaction, a more complex purification process, ineffective crystallization step, a higher energy demand and a large number of by-products are the major disadvantages of the non-solvent process (Biwer *et al*., 2002).

Another disadvantage of CGTase is its low activity on native starch liquefied by α-amylase treatment or heating in water to weaken the hydrogen bonds away from starch molecules. The α-amylase used for liquefaction produces maltodextrins, which can act as acceptor molecules in the coupling reaction of CGTase, severely reducing the yield of cyclodextrins. However, many thermostable CGTases have been isolated and characterized from thermophilic bacteria. These CGTases are active and stable at high temperature, and are able to solubilize starch, thereby eliminate the need for α-amylase pretreatment.

3. CGTase catalysed reactions

The CGTases can catalyze 2 types of transglycosylation reaction and a weak hydrolysis reaction, though it mainly catalyses the former (Fig. 5 and 6).

(i) Intramolecular transglycosylation reaction is sometimes called cyclization. The reaction proceeds by cleaving a linear oligosaccharide chain and the transfer of reducing end sugar to the non-reducing end of the same oligosaccharide chain, which acts as the acceptor.

(ii) Intermolecular transglycosylation reaction can be divided into coupling and disproportionation reactions. Coupling reaction is the reverse reaction of cyclization, in which a cyclodextrin ring is cleaved and transferred to a linear acceptor oligosaccharide. The kinetic mechanism of coupling proceeds via random ternary complex mechanism (van der Veen *et al.*, 2000a). For disproportionation reaction, a linear oligosaccharide is cleaved and transferred to a linear acceptor oligosaccharide. The kinetic mechanism proceeds via ping-pong mechanism (Nakamura *et al.*, 1994b, van der Veen *et al.*, 2000a).

(iii) Hydrolysis is a weak reaction of CGTase, in which a water molecule acts as acceptor instead of a linear oligosaccharide.
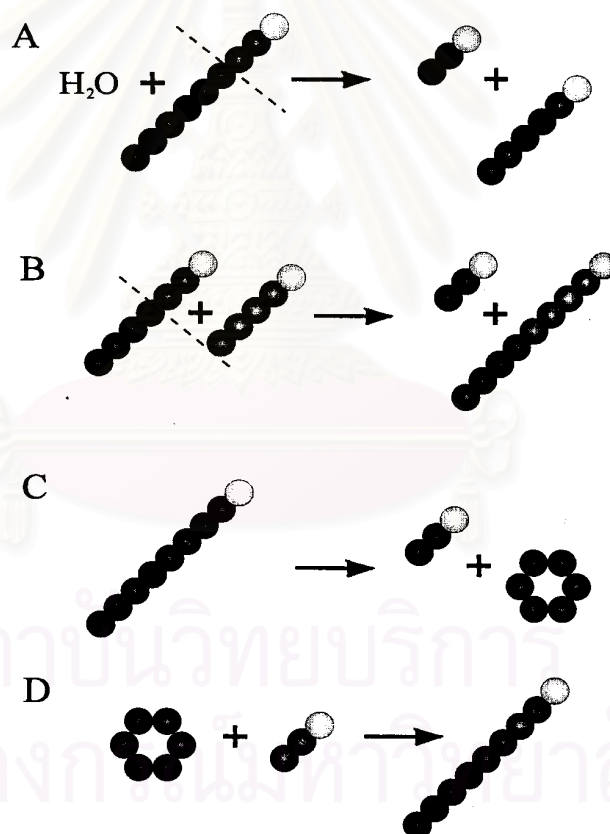
Figure 5 : Schematic representation of the CGTase-catalysed reaction. The circles represent glucose residues; the white circles indicate the reducing end sugars. (A) hydrolysis; (B) disproportionation; (C) cyclization; (D) coupling (Source : van der Veen *et al.,* 2000d)
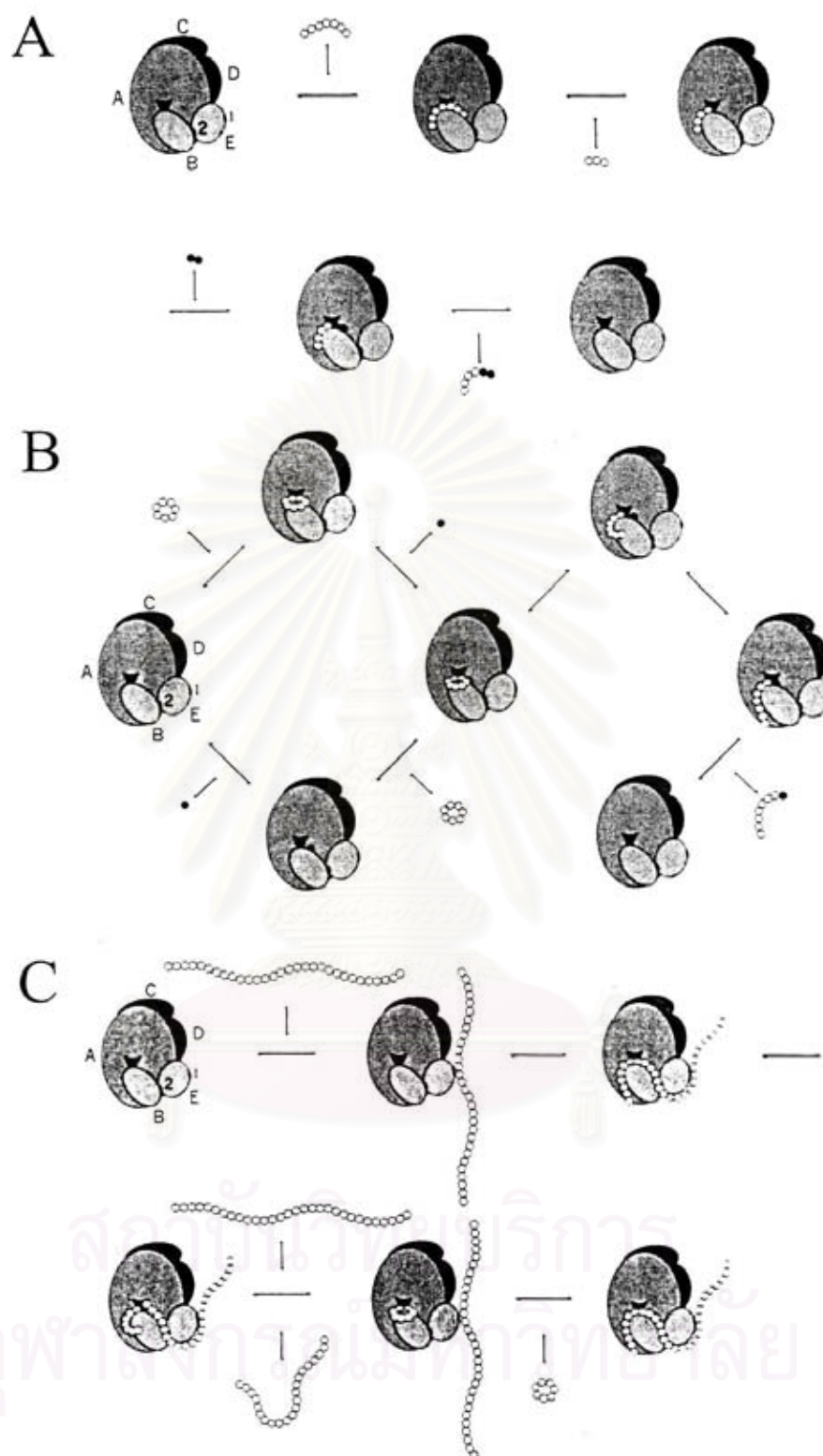
Figure 6 : Proposed model of the events taking place in the CGTase-catalysed reaction. (A) disproportionation; (B) coupling; (C) cyclization. The different CGTase domains are indicated A, B, C, D and E. The circles represent glucose residues; acceptor residues are represented in black

(Source : van der Veen *et al.,* 2000a).

4. CGTase and α-amylase family (family 13)

Many starch-processing enzymes, such as α-amylase, pullulanase, neopullulanase, isoamylase, α-glucosidase and CGTase belong to an α-amylase family (family 13), with the following four requirements (Kuriki and Imanaka, 1999).

(i) They act on α-glucosidic bonds.

(ii) They hydrolyze α-glucosidic linkages to produce α-anomeric mono- and oligosaccharide or to form α-glucosidic linkages by transglycosylation.

(iii) They have four highly conserved regions in their primary sequences which contain all the catalytic and most of the important substrate-binding sites (Fig. 7).

(iv) They have aspartate, glutamate and aspartate residues as catalytic residues in their conserved regions 2, 3 and 4 (Fig. 7).

Although enzymes belonging to this group show a wide diversity in reaction specificities, the relationship of specificities for the target linkage and the reactions can be easily summarized in Fig. 8.

Most α-amylases, which typically catalyze hydrolysis of α-1,4-glucosidic bonds, should be located in the upper left-hand corner. CGTase which catalyzes transglycosylation to form α-1,4-glucosidic linkages should be in the bottom left-hand corner. Pullulanase and isoamylase, which exclusively hydrolyze α-1,6-glucosidic linkages, should be in the upper right-hand corner. Branching enzymes, which exclusively catalyze transglycosylation to form α-1,6-glucisidic linkages, should be in the bottom right-hand corner. Neopullulanase, which catalyzes all four reaction, should be located in the center (Fig. 8).

All four highly conserved regions (Fig. 7) contain highly conserved invariant amino acid residues within the α-amylase family. These residues are directly involved in catalysis, either through substrate binding, bond cleavage, transition state stabilization or as ligands for the binding of a calcium ion. Three carboxylic acid groups from one glutamic acid and two aspartic acid residues, are found to be essential for catalytic activity in the α-amylase family. The amino acids are equivalent to Asp206, Glu230 and Asp297 in α-amylase from *Aspergillus oryzae* and Asp229, Glu257 and Asp328 in CGTase from *Bacillus* spp. (van der Veen, *et al*., 2000d,e). Two conserved histidine residues, His140 and His327 (*B. circulans* 251 CGTase numbering) are involved in substrate binding and transition state stabilization (van der Veen *et al*., 2000d,e). A histidine residue, His233 (*B. circulans* 251 CGTase numbering), present only in some α-amylases and CGTases is involved in substrate binding and acts as a calcium ligand with its carbonyl oxygen. Arg227 (*B. circulans* 251

CGTase numbering) is important for the orientation of a nucleophile (Asp229) (van der Veen *et al.*, 2000d,e). Asp135 (*B. circulans* 251 CGTase numbering) in conserved sequence region 1 is required for the proper conformation of several catalytic site residues and therefore for activity (Leemhuis *et al.*, 2003a)

The reactions catalysed by the enzymes belonging to the α-amylase family (family 13) proceed with the retention of the substrate's anomeric α-configuration (a covalent glycosyl-enzyme intermediate). Since each substitution at a chiral center results in inversion of configuration, catalysis must proceed through a double displacement reaction (Fig. 9), A carboxylate group of Asp as a nucleophile attacks C-1 and covalent bond is formed between the remaining glycosyl residue and the catalytic nucleophile via the transition state oxocarbonium ion, after Glu protonates the scissile bond and the substrate is cleaved. The formation of the glycosyl-enzyme complex involves the first anomer inversion ; the Walden inversion. Then, the hydroxyl ion of water or C4-OH at the non-reducing end of another oligosaccharide nucleophilically attacks the C-1 from the reverse side of the convalent bond of glycosyl-enzyme complex and finalizes the reaction via the transition state, in which Glu deprotonates the hydroxyl ion of water or C4-OH. The product has again α-(1,4) glycosidic linkage (Kuriki and Imanaka, 1999).

Enzyme	Origin	Region1	Region2	Region3	Region4
α-Amylase	*Aspergillus oryzae*	117DVVANH	202GLRIDTVKH	230EVLD	292FVENHD
CGTase	*Bacillus macerans*	135DFAPNH	225GIRFDAVKH	258EWFL	324FIDNHD
Pullulanase	*Klebsiella aerogenes*	600DVVYNH	671GFRFDLMGY	704EGWD	827YVSKHD
Isoamylase	*Pseudomonas amyloderamosa*	292DVVYNH	371GFRFDLASV	435EPWA	505FIDVHD
Branching enzyme	*Escherichia coli*	335DWVPGH	401ALRVDAVAS	458EEST	521LPLSHD
Neopullulanase	*Bacillus stearothermophilus*	242DAVFNH	324GWRLDVANE	357EIWH	419LLGSHD
α-Amylase-pullulanase	*Clostridium thermohydrosulfulcum*	488DGVFNH	594GWRLDVANE	627ENWN	699LLGSHD
α-Glucosidase	*Saccharomyces carlsbergensis*	106DLVINH	210GFRIDTAGL	276EVAH	344YIENHD
Clyclodextrinase	*Thermoanaerobacter ethanolicus*	238DAVFNH	321GWRLDVANE	354EVWH	416LIGSHD
Oligo-1,6-glucosidase	*Bacillus cereus*	98DLVVNH	195GFRMDVINF	255EMPG	324YWNNHD
Dextran glucosidase	*Streptococcus mutans*	98DLVVNH	190GFRMDVIDM	236ETWG	308FWNNHD
Amylomaltase	*Streptococcus pneumoniae*	224DMWAND	291IVRIDHFRG	332EELG	391YTGTHD
Glycogen debranching enzyme	*Human*	298DVVYNH	504GVRLDNCHS	534ELFT	603MDITHD

Figure 7 : Enzymes belonging to α-amylase family and the four conserved regions. Invariable three catalytic residues are indicated by open circles (Source : Kuriki and Imanaka, 1999).

α-1,4 ◄── Specificity for glucosidic linkage ──► α-1,6

Hydrolysis	α-amylase Bacterial saccharifying α-amylase	Amylopullulanase	Pullulanase Isoamylase
Fate of Reaction		Neopullulanase	
Transglyco-sylation	CGTase		Branching enzyme
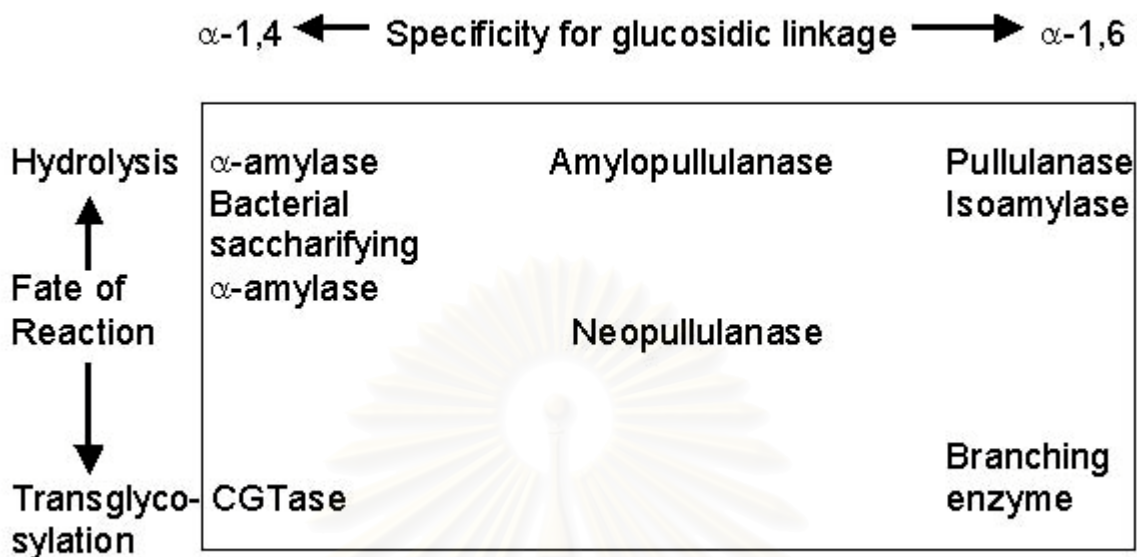
Figure 8 : Schematic representation of relationship of enzymes that belong to α- amylase family according to the types of reaction (Source : Kuriki and Imanaka, 1999).
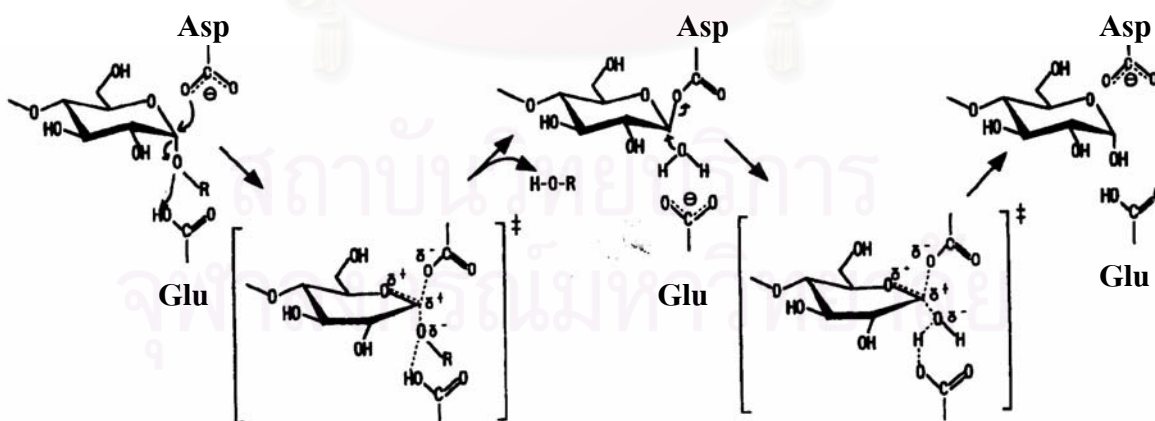
Figure 9 : Catalytic mechanism of anomer retaining enzyme via the formation of covalent glycosyl-enzyme complex (Source : Kuriki and Imanaka, 1999).

5. CGTase three dimensional structure

The increasing availability of X-ray crystallographic structures of CGTases, especially with inhibitors, substrates, substrate analogues or products bound at the active site, provides more insights into CGTase structure and the interaction between ligands and key functional amino acid residues during CGTase catalysis (Lawson *et al*., 1994, Penninga *et al*., 1995, Knegtel *et al.,* 1995, Strokopytov *et al*., 1996, Penninga *et al*., 1996a,b, Parsiegla *et al*., 1998, Uitdehaag *et al*., 1999 and 2000). CGTase consists of 5 domains (A-E). Domains A, B and C are quite similar to α-amylase and other enzymes in the α-amylase family. Domains A and B constitute the catalytic domain. Domain A consists of 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 is called $(\alpha/\beta)_8$ or TIM barrel (Fig. 10). Several prolines and glycines flanking loops connecting the β-strands and α-helices have been found to be highly conserved in the CGTases and enzymes in α-amylase family.

The catalytic and substrate binding residues are located in loops at the C-termini of β-strands in domain A. The loop between β-strand 3 and helix 3 of the catalytic domain is rather large and is regarded as domain B, which consists of 44-133 amino acid residues and contributes to substrate binding. The domain C is approximately 100 amino acids long and has an antiparallel β-sandwich fold. Domain C of the CGTase from *B. circulans* strain 251 contains one of the three maltose binding sites (Lawson *et al.,* 1994). Domain D, consisting of approximately 90 amino acids with an immunoglobulin fold, is almost exclusively found in CGTases and has an unknown function. Domain E, consists of approximately 110 amino acids and is found to be responsible for substrate binding, another two maltose binding sites (Penninga *et al*., 1998).

Analysis of the maltose-CGTase co-crystal structure reveals that each enzyme molecule contains three maltose molecules, situated at the contact points between protein molecules. Two of these maltoses are bound to specific sites in the E domain, the third maltose is bound at the C domain (Lawson *et al.,* 1994 and Penninga *et al*., 1996a,b). Penninga *et al*., (1996a) replaced Trp616 and Trp662 in the maltose binding site 1 and Tyr633 of maltose binding site 2 with alanine using site-directed mutagenesis. The results showed that maltose binding site 1 was the most important for raw starch binding, while maltose binding site 2 was involved in guiding the linear starch chain into the active site via a groove at the surface of CGTase. Further, the maltose binding site 1 and 2 on the CGTase E-domain were also found to interact strongly with cyclodextrins (Knegtel *et al*., 1995 and Penninga *et al*., 1996a).

Chang *et al*., (1998) constructed several mutants of *Paenibacillus macerans* CGTase E-domain. Removing the entire E-domain resulted in an inactive enzyme. Adding six amino acids between domain D and E caused a decrease in activity and thermostability. Replacing domain E with the similar starch binding domain from *Aspergillus awamori* glucoamylase I caused a drastic decrease in activity, indicating the necessity of correct alignment of bound substrate. Substituting Tyr634 in domain E with phenylalanine had very little effect on starch-hydrolyzing activities compared with that of the wild type enzyme. The result of this study indicated that domain E was important also for the stability and integrity of *P. macerans* CGTase in addition to for raw starch binding.
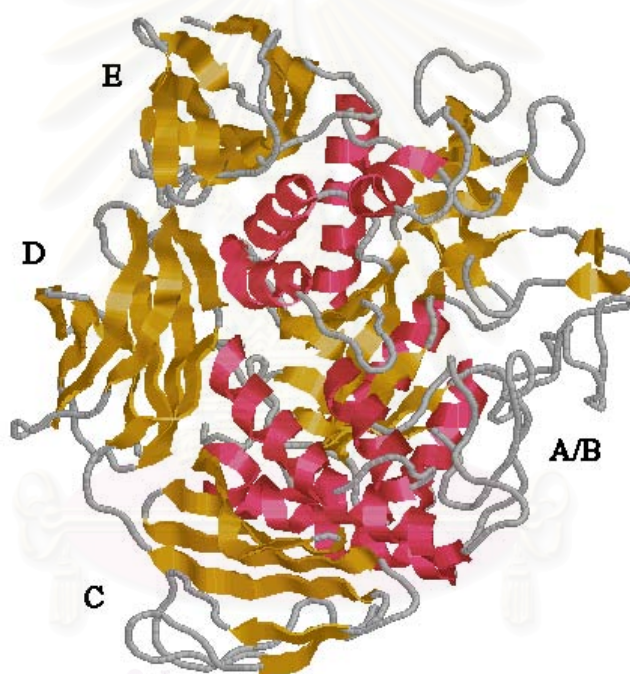
Figure 10 : A common three dimensional structure of CGTase (Source : www.rcsb.org).

6. Catalytic mechanism of CGTase

As a member of α-amylase family, CGTase uses the double displacement mechanism, the α-retaining mechanism (Fig. 11 and 13). After the binding of a starch chain at maltose binding site 1 (MBS 1) in domain E, then this binding is extended to the active site (domain A/B) via maltose binding site 2 (MBS 2). The CGTase cleaves an $\alpha(1,4)$ glycosidic bond in its substrate at subsite -1 and $+1$, and forms a covalent $\beta(1,4)$-linked glycosyl-enzyme intermediate. The $\alpha(1,4)$ glycosidic bond is reformed with an acceptor, which can be water or C4-OH of another sugar residue (van der

Veen *et al*.,2000a,d and e). CGTase is unique in its ability to use the free hydroxyl at the non-reducing end of the intermediate sugar chain as an acceptor, thus forming a cyclic oligosaccharides, cyclodextrins. Three amino acids in the active site play distinct role in catalysis. Glu257 acts as the acid/base catalyst, which protonates the glycosidic oxygen of the scissile bond in the first step, and then deprotonates the attacking OH group in the second step. Asp229 acts as the catalytic nucleophile, which attacks the sugar, forming the covalent intermediate. Asp328 stabilizes the substrate binding and elevates the pKa of Glu257 (Fig. 12)(Uitdehaag *et al*., 1999a).

The essential amino acid residues for cyclization are four aromatic amino acids, Phe183, Phe/Tyr195, Phe259 and Phe283, commonly located in the active site of CGTase (Fig. 14), but not in that of α-amylase. Phe259 and Phe183 at subsite +2 play a critical role in the binding of non-reducing end of starch to the acceptor site. Leemhuis *et al*. (2002b) and van der Veen *et al*., (2001) showed that both amino acids are essential for cyclization, coupling and disproportionation. Moreover, the hydrophobicity of Phe183 and Phe259 limits the hydrolyzing activity of the enzyme. When Phe183 and Phe259 are replaced by polar amino acid, the mutants have lower transglycosylation activity and higher hydrolysis activity (Nakamura *et al*., 1994a, van der Veen *et al*., 2001, Leemhuis *et al*.,2002b). Phe283 is important for the hydrophobic environment around Glu257, and hence participates in raising the pKa value of Glu257 (Penninga *et al*., 1996b). Phe/Tyr195 is in a dominant position in the center of the active site cleft of CGTase. It may influence the preferred cyclodextrin size. Substitution of this central amino acid by another amino acid, however, does not support this above notion. Furthermore, natural CGTases all have Tyr or Phe at this position, indicating that this residue is not involved in the differences in product specificity. Therefore, the function of this amino acid residue remains to be elucidated.

Terada *et al*. (1999) investigated the cyclization reactions of three CGTases from alkalophilic *Bacillus* sp. strain AZ-5a, *Paenibacillus macerans* and *Bacillus stearothermophilus*. They found that all three enzymes produced large cyclodextrins at the early stage of the reaction but these were subsequently converted into smaller cyclodextrins, and the rate of this conversion differed among the three enzymes. For this conversion, large cyclodextrin is first linearized by either a coupling or a hydrolytic reactions. The β-cyclodextrin and γ-cyclodextrin are extremely poor substrates for both reactions compared to the mixtures of larger cyclodextrins. Thus, among the initial cyclization products, larger cyclodextrins are selectively subjected to the linearization reactions, and the linear products are cyclized again into smaller cyclodextrins. Repetition of the cyclization reaction and the linearization reaction may be the principle mechanism whereby large cyclodextrins are converted into smaller cyclodextrins, resulting in the final equilibrium composition

of cyclodextrins (Fig. 15). The factors that directly determine the composition of cyclodextrins are the product specificity of the cyclization reaction and the substrate specificity of the linearization reaction, including the coupling and hydrolytic reactions of larger cyclodextrins.

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 in all three transglycosylation reactions (cyclization, coupling and disproportionation) but not in hydrolysis reaction. They found that Gly180 and Asn193 were important for the β- and γ-cyclization, while Gly179 was important for α-cyclization.
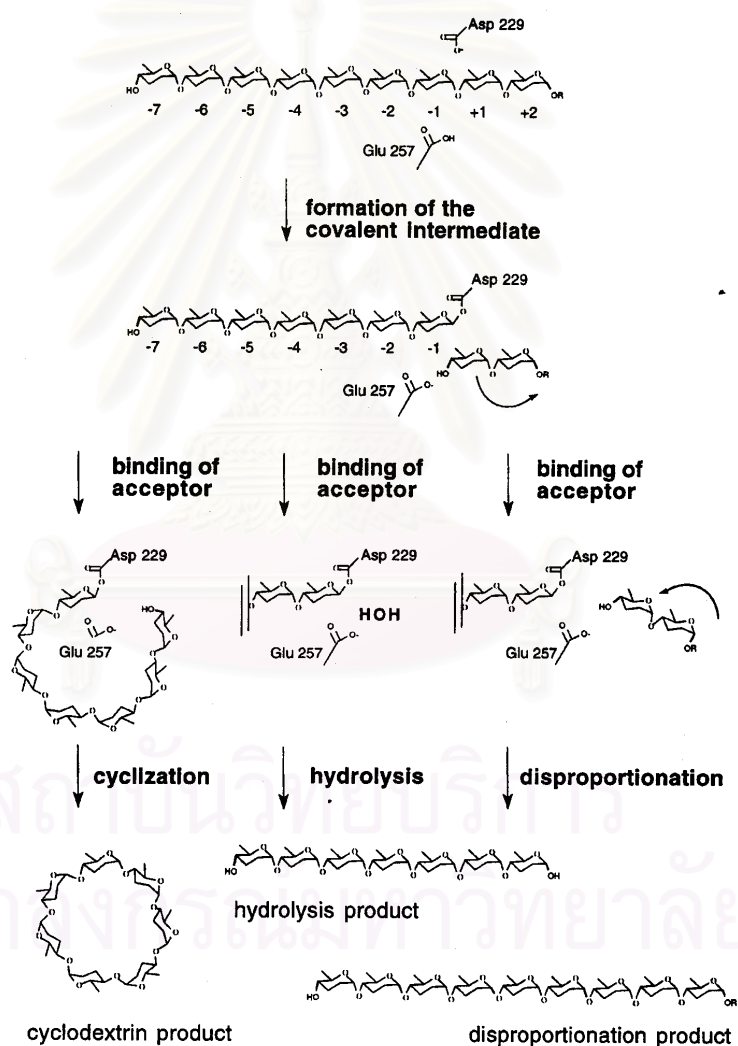
Figure 11 : The catalytic reaction of CGTase, which can be cyclization, disproportionation and hydrolysis (Source : Uitdehaag *et al.,*2000).
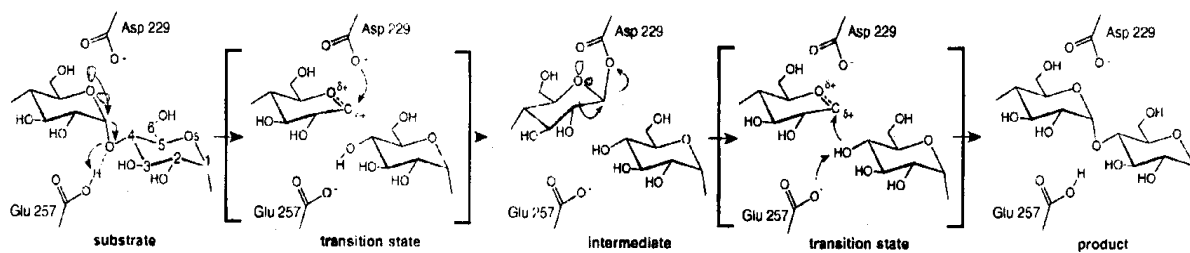
Figure 12 : Scheme of the CGTase reaction mechanism (Source : Uitdehaag *et al.,*1999a).
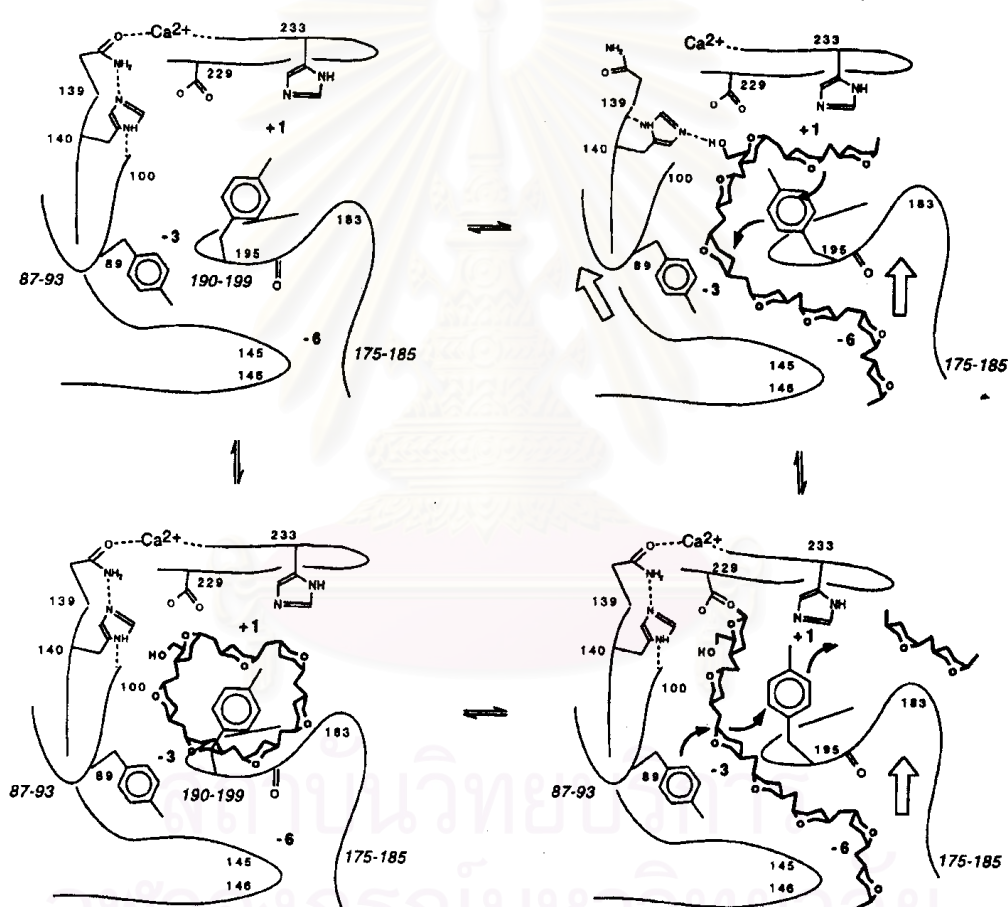
Figure 13 : Possible scenario for the structural rearrangements during the CGTase reaction cycle. The picture in clockwise order shows a cyclization reaction cycle, and in counterclockwise order a coupling reaction cycle. The hydrolysis follows top left to top right and to bottom right. The disproportionation follows bottom right to top right (Source : Uitdehaag *et al.,*2000).
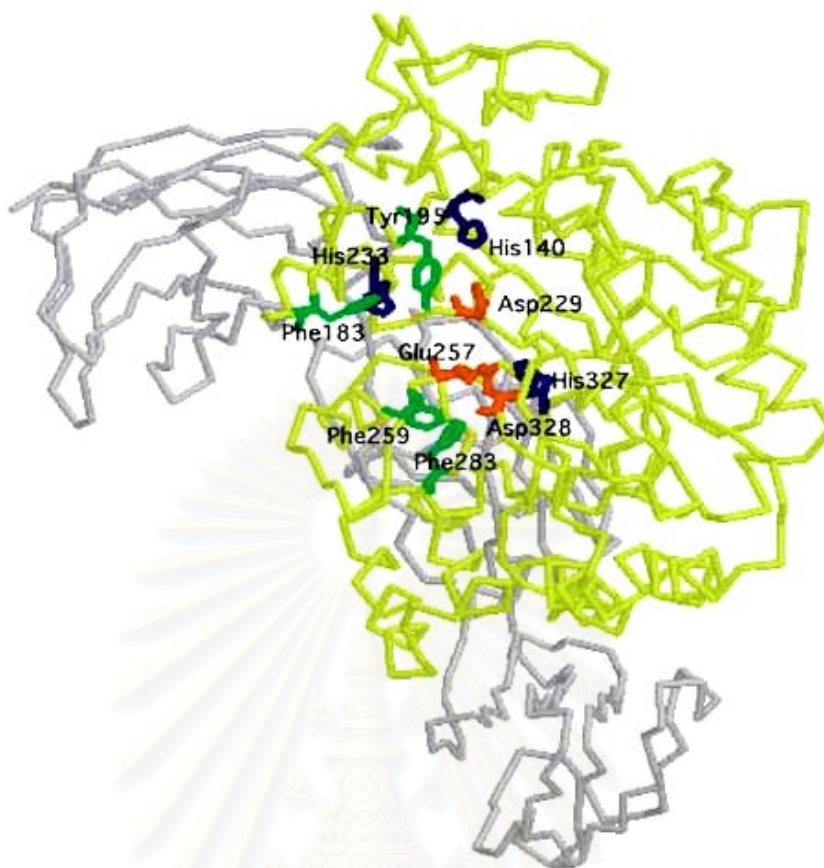
Figure 14 : Essential amino acid residues for cyclization reaction of CGTase (Source : www.glycoforum.gr.jp/science/word/saccharide/SA-B03E.html).
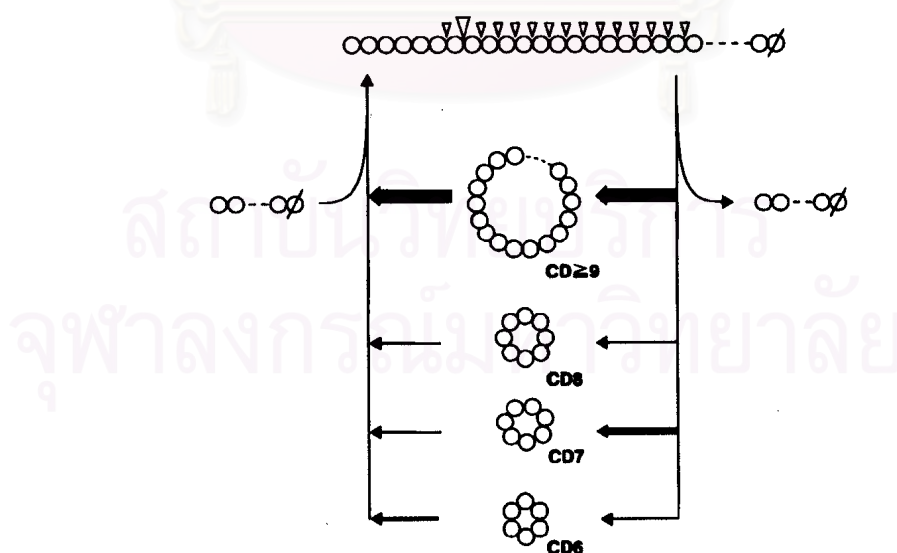
Figure 15 : Proposed model of the cyclization reaction of CGTase. The right and left arrows indicate cyclization and coupling reactions, respectively, and the relative width of each arrow represents the relative rate of the reaction (Source : Terada *et al.,* 2000).

7. Comparison of CGTase sequences

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 amino acid sequences, ranging from 47% to 99%. Figure 16 and Table 2 show the alignment of amino acid sequences of α-, β-, β/γ- and γ-CGTase from different sources. The first specific CGTase residues are found between β- strand 1 and α- helix 1, which consist of the stretches of amino acid residues 27-36 and 53 and are involved in calcium binding. Residue 47 is involved in binding (semi)cyclic oligosaccharides and is typically an Arg in β-CGTase, Lys in α-CGTase, Thr in β/γ- and γ-CGTases or His in the CGTase producing virtually no α -cyclodextrin (Fig. 16)(van der Veen *et al*., 2000d and Takada *et al*., 2003).

The amino acid sequences in the region 145–152 (subsite –7) of α- and β-CGTases, which are involved in product specificity and located in a loop at the start of the B-domain, are SSTDPSFA and SSDQPSFA, respectively. β/γ- and γ-CGTases completely lack the six amino acid residues in this region (D-I), see Fig. 16. This indicates that the absence of these amino acid residues may be necessary for a higher level of γ- cyclization activity; it provides more space for the bound glycosyl chain in this region (Takada *et al*., 2003). Asp196 and Asp371 are completely conserved in the four types of CGTases. It has been reported that both amino acid residues stabilize linear oligosaccharide chain (van der Veen *et al.*, 2000d). Another specific region are residues 87-94 in subsite -3. These residues show hydrophobic interactions with glucose unit bound in this region, and are remarkably different in 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. 16 and Table 2) (Takada *et al*., 2003).

```
Bmace    1:-SPDTSVNNKLNFSTDTVYQIVTDRFVDGNSANNPTGAAFSSDHSNLKLYFGGDWQGITNKINDGYLTGMGITALWISQP 79
Bc251    1:AP-DTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGTCTNLRLYCGGDWQGINKINDGYLTGMGVTAIWISQP 79
Bf290    1:——NENLDNV-NYAQEIIYQIVTDRFYDGDPTNNPEGTLFSPGCLDLTKYCGGDWQGVIEKIEDGYLPDMGITAIWISPP 76
Bcla     1:SNATNDLSNV-NYAEEVIYHIVTDRFKDGDPDNNPGGQLFSNGCSDLTKYCGGDWQGIIDEIESGYLPDMGITALWISPP 79
              *    . *.*.****  **. *** *  *.  .   *  *.*****.. .   *  *** **.**  *** *
              β1          Ca²⁺ binding site                            α1              β2
                                                                    I
Bmace   80:VENITAVINYSGVNNTAYHGYWPRDFKKTNAAFGSFTDFSNLIAAAHSHNIKVVMDFAPNHTNPASSTDPSFAENGALYN 159
Bc251   80:VENIYSIINYSGVNNTAYHGYWARDFKKTNPAYGTIADFQNLIAAAHAKNIKVIIDFAPNHTSPASSDQPSFAENGRLYD 159
Bf290   77:IENVMELHP-GGF——ASYHGYWGRDFKRTNPAFGSLADFSRLIETAHNHDIKVIIDFVPNHTSPVD——————IENGALYD 147
Bcla    80:VENVFDLHP-EGF——SSYHGYWARDFKKTNPFFGDFDDFSRLIETAHAHDIKVVIDFVPNHTSPVD——————IEDGALYD 150
              .  **         ***** ****. **..  **.  **  **  ***. ** ****. *        *. **.
              G-3loop                    α2              β3              G-7loop
                                                                          II
Bmace  160:NGTLLGKYSNDTAGLFHHNGGTDFSTTESGIYKNLYDLADINQNNNTIDSYLKESIQLWLNLGVDGIRFDAVKHMPQGWQ 239
Bc251  160:NGTLLGGYTNDTQNLFHHNGGTDFSTTENGIYKNLYDLADLNHNNSTVDVYLKDAIKMMLDLGIDGIRMDAVKHMPFGWQ 239
Bf290  148:NGRLVGHYSNDSEDYFYTNGGSDFSSYEDSIYRNLYDLASLNQQNSFIDRYLKESIQMMLDLGIDGIRVDAVAHMPVGWQ 227
Bcla   151:NGTLLGHYSTDANNYFYNYGGSDFSDYENSIYRNLYDLASLNQQHSFIDKYLKESIQLWLDTGIDGIRVDAVAHMPLGWQ 230
              **.*. *.. ** ***  *  ** ****** . *  * ***. .   ** ** *. ***  **  *** . ***
                                         Y195              α3              β4
                                 III
Bmace  240:KSYVSSIYSSANPVFTFGEWFLGPDEMTQDNINFANQSGMHLLDFAFAQEIREVFRDKSETMTDLNSVISSTGSSYNYIN 319
Bc251  240:KSFMAAVNNYK-PVFTFGEWFLGVNEVSPENHKFANESGMSLLDFRFAQKVRQVFRDNTDNMYGLKAMLEGSAADYAQVD 318
Bf290  228:KNFVSSIYDY-NPVFTFGEWFTGAGGSDEYHYFINN-SGMSALDFRYAQVVQDVLRNNDGTMYDLETVLRETESVYEKPQ 305
Bcla   231:KAFISSVYDY-NPVFTFGEWFTGAQGSNHYHHFVNN-SGMSALDFRYAQVAQDVLRNQKGTMHDIYDMLASTQLDYERPQ 308
              *. .... **********  * .  *  .*  ***. ***. **  * *  .. * . .
              α4          β5              α5        β6       α6
                     IV
Bmace  320:NMVTFIDNHDMDRFQQAGASTRPTEQALAVTLTSRGVPAIYYGTEQYMTGNGDPNNRGMMTGFDTNKTAYKVIKALAPLR 399
Bc251  319:DQVTFIDNHDMERFHASNANRRKLEQALAFTLTSRGVPAIYYGTEQYMSGGTDPDNRARIPSFSTSTTAYQVIQKLAPLR 398
Bf290  306:DQVTFIDNHDINRFSRNGHSTRTTDLGLAFLLTSRGVPTIYYGTEIYMTGDGDPDNRKMMNTFDQSTVAYQIIQQLSSLR 385
Bcla   309:DQVTFIDNHDIDRFTVEGRDTRTTDIGLAFLLTSRGVPAIYYGTENYMTGKGDPGNRKMMESFDQTTTAYQVIQKLAPLR 388
              .. ********  ** .   *.   **. ******* ****** **. *. ** **..  *.  ..**. *. *. **
              β7          α7              β8                      α8
Bmace  400:KSNPALAYGSTTQRWVNSDVYVYERKFGSNVALVAVNRSSTTAYPISGALTALPNGTYTDVLGGLLNGNSITVNGGTVS- 478
Bc251  399:KCNPAIAYGSTQERWINNDVLIYERKFGSNVAVVAVNRNLNAPASISGLVTSLPQGSYNDVLGGLLNGNTLSVGSGGAAS 478
Bf290  386:QENRAIAYGDTTERWINEDVFIYERSFNGEYALIAVNRSLNHSYQISSLVTDMPSQLYEDELSGLLDGQSITVDQNGSIQ 465
Bcla   389:QENKAVVYGSTKERWINDDVLIYERSFNGDYLLVAINKNVNQAYTISGLLTEMPAQVYHDVLDSLLDGQSLAVKENGTVD 468
              _   * *. **.*. *. ** ** .*** *     ...*.*.  . **.. * * * *.* .** *.  *   .
Bmace  479:NFTLAAGGTAVWQYTTTESSPI-IGNVGPTMGKPGNTITIDGRGFGTTKNKVTFGTTAVTGANIVSWEDTEIKVKVPNVA 557
Bc251  479:NFTLAAGGTAVWQYTAATATPT-IGHVGPMMAKPGVTITIDGRGFGSSKGTVYFGTTAVSGADITSWEDTQIKVKIPAVA 557
Bf290  466:PFLLAPGEVSVWQYSNGQNVAPE-GQIGPPIGKPGDEVRIDGSGFGSSTGDVSFAGSTMNV———LSWNDDTIIAELPEHN 542
Bcla   469:SFLLGPGEVSVWQHISESGSAPVIGQVGPPMGKPGDAVKISGSGFGSEPGTVYFRDTKIDV———LTWDDETIVITLPETL 545
              *.  *   ***.    * **  . .***   *.* *   . * *  * *.  **   * .
Bmace  558:AGNTAVTVTNAAGTTSAAFNNFNVLTADQVTVRFKVNNATTALGQNVVYLTGNVAELGNWTAANAIGPMYNQVEASYPTWY 637
Bc251  558:GGNYNIKVANAAGTASNVYDNFEVLSGDQVSVRFVVNNATTALGQNVVYLTGSVSELGNWDPAKAIGPMYNQVVYQYPNWY 637
Bf290  543:GGKNSVTVTTNSGESSNGYP-FELLTGLQTSVRFVVNQAETSVGENLYVVGDVPELGSWDPDKAIGPMFNQVLYSYPTWY 621
Bcla   546:GGKAQISVTNSDGVTSNGYD-FQLLTGKQESVRFVVDNAHTNYGENVVYLVGNVPELGNWNPADAIGPMFNQVVYSYPTWY 624
              . *   *.     *. *  * . ***. .  *** . *** * * ** *** *.  ***** *** .. ** **
Bmace  638:FDVSVPANTALQFKFIKVNGSTVT-WEGGNNHTFTSPSSGVATVTVDWQN 686
Bc251  638:YDVSVPAGKTIEFKFLKKQGSTVT-WEGGSNHTFTAPSSGTATINVNWQP 686
Bf290  622:YDVSVPANQDIEYKYIMKDQNGNVSWESGNNHIYRTPENSTGIVEVNFNQ 671
Bcla   625:YDVSVPADTALEFKFIIVDGNGNVTWESGGNHNYRVTSGSTDTVRVSFRR 674
              ******  ..*.  .    ** * **   ..  ...*
```

Figure 16 : Alignment of amino acid sequences of typical α-, β-, β/γ- , and γ-CGTases. Bmac, Bc251, Bf290 and Bcla indicate 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 2 Comparison of the amino acid residues around the active center in the four types of CGTases.

Residue No.	Residue in α-CGTase	Residue in β-CGTase	Residue in βγ-CGTase	Residue in γ-CGTase	Function in CGTase
Subsite +2					
183	F	F	F	F	Cyclization
232	K	K	<u>A</u>	<u>A</u>	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	<u>HP-GGF-</u>	HP-GGF-	Cyclization specificity
371	D	D	D	D	Cyclization
196	D	D	D	D	Cyclization
47	K	R	<u>T</u>	<u>T</u>	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	<u>D___I</u>	<u>D___I</u>	Cyclization Specificity
Central					
195	Y	Y/F	Y	Y	Cyclization
Ca^{2-} binding site					
32-36	NNPTG	NNPTG	NNP<u>E</u>G	NNP<u>Q</u>G	Ca^{2+} binding

(Source : Takada *et al.,* 2003, *Leemhuis *et al*., 2003b)

8. Cyclodextrin product specificity of CGTases

Kaneko *et al*., (1989 and 1990) constructed twelve chimeric CGTases from alkalophilic *Bacillus* sp. strain No. 38-2 and alkalophilic *Bacillus* sp. strain No. 17-1. Both types of CGTases produces β- cyclodextrin predominantly. They found that the N-terminal and the C-terminal segments were important for cyclodextrins production, heat stability and the pH activity profile.

Fujiwara *et al*. (1992a) constructed seven chimeric CGTases from *Geobacillus. stearothermophilus* NO2 and *P. macerans* IFO 3490, 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.

In the past, various site-directed mutagenesis affecting the product specificity of CGTases had been made on the aromatic amino acid reisidue 195 (Phe or Tyr), presented in a dominant position in the center of the active site cleft of the enzymes, because it had been suggested that the size of this aromatic amino acids might influence the preferred cyclodextrin size. Substitution of this amino acid by Tyr188Trp (equivalent to residue 195 in CGTase of *B. circulans* 251) in the *B. ohbensis* (Sin *et al.,*1994), Tyr195Leu in the *B. circulans* strain 251 (Penninga *et al*., 1995) and Tyr195Trp in the *B. circulans* strain 8 (Parsiegla *et al*., 1998) could improve product specificity to a limited extant with higher production of γ-cyclodextrin. However, several other Tyr188 and Tyr195 mutations by other amino acids as well as Phe191Tyr (equivalent to residue 195 in CGTase of *B. circulans* 251) in the CGTase of *G. stearothermophilus* NO2 did not support the importance of the central amino acid (Fujiwara *et al*., 1992b and van der Veen *et al*., 2000d). 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.

The study of the X-ray structure of the CGTase from *B. circulans* strain 251 in complex with a maltononaose inhibitor suggested that 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 cyclodextrin by altering the affinities for glucose residues at these sugar binding subsites (Fig. 17, Strokopytov *et al*., 1996). Subsites -6, -7 and -8 may be key sites for the product specificity. Uitdehaag *et al*. (2000) determined the X-ray structures of CGTase in complex with maltoheptaose for β-cyclodextrin and maltohexaose for α-cyclodextrin, they found that the conformations of maltoheptaose and maltohexaose were different at subsites -3, -6 and -7 (Fig. 18). The conformation differences at specific sugar binding subsites suggested that the determinants for cyclodextrin product 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.
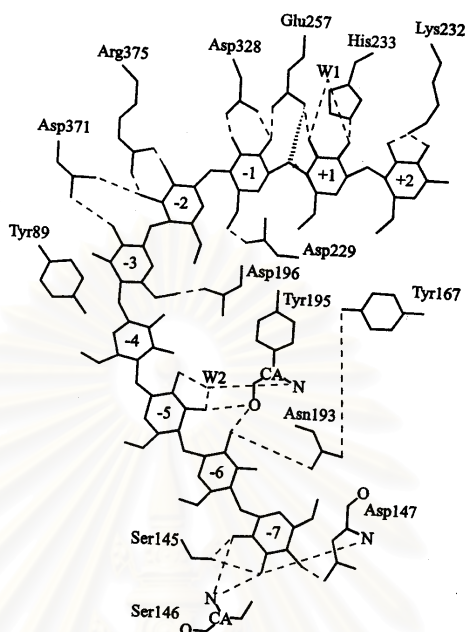
Figure 17 : Schematic representation of the hydrogen bonds between the *B. circulans* strain 251 CGTase and a maltononaose inhibitor bound at each subsites of the active site (Source : van der Veen *et al.*, 2000d).
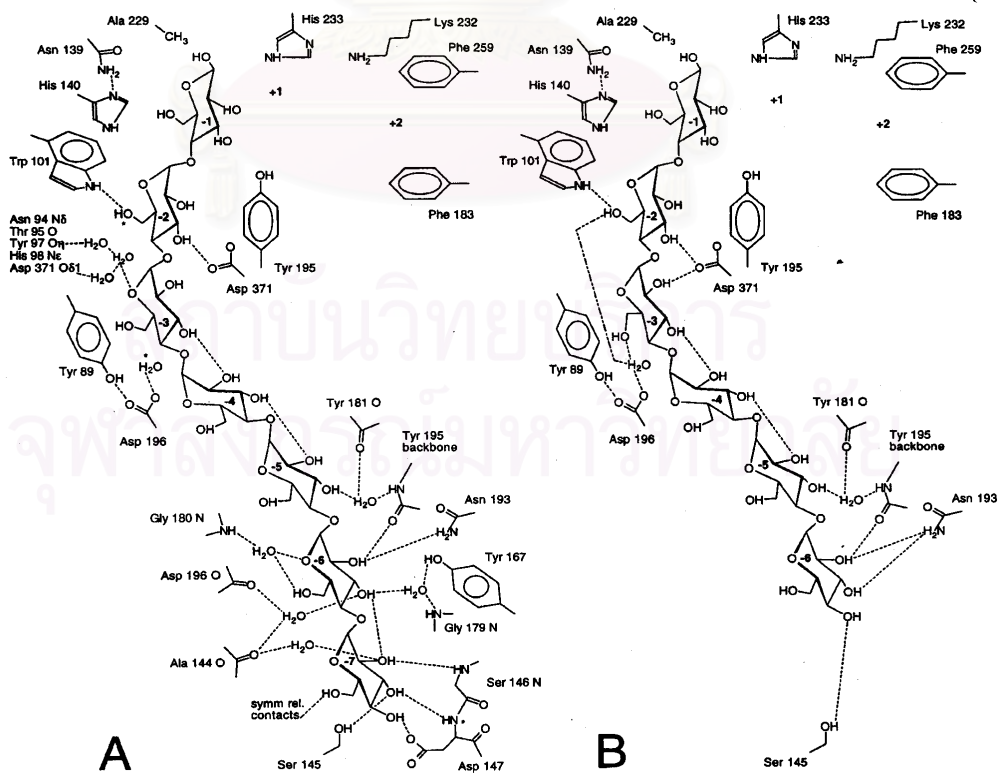
Figure 18 : Schematic representation of all interaction of (A) maltoheptaose, and (B) maltohexaose with the CGTase from *B. circulans* strain 251 (Source : Uitdehaag *et al.*, 2000).
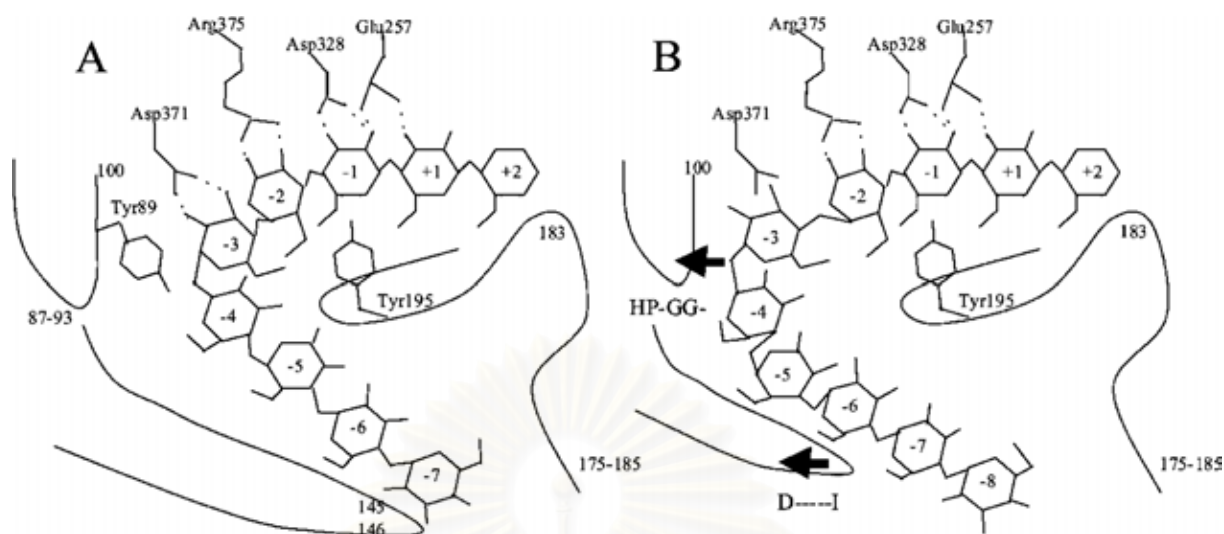
Figure 19 : Schematic structure of the substrate binding sites of CGTase. (A) β-CGTase, (B) γ-CGTase (Source : Takada *et al.,* 2003).

Parsiegla *et al*. (1998) constructed β-CGTase mutant from *B. circulans* strain 8 by replacing residues 145-151 with a single Asp(Δ(145-151)D), which removed most interactions at subsite -7. The mutant showed a reduced production of β-cyclodextrin and increased production of γ-cyclodextrin. The higher γ-cyclization activity of this mutant were explained as an opening 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 the stretch HTSPADAE similar to the stretch HTSPVDIE in the β/γ-CGTase from *B. firmus* strain 290-3 (Ebglbrecht *et al*., 1990) and γ-CGTase from *B. clarkii* 7364 (Takada *et al.,* 2003). This indicated that it might be necessary for a higher level of γ-cyclization activity to have more space for the bound glucosyl chain at subsite -7 (Fig. 19).

van der Veen *et al*. (2000b) constructed CGTase mutants from *B. circulans* strain 251 at subsite -3 (Tyr89Asp and Tyr89Gly), -7 (Ser146Pro) and a double mutant at subsites -3 and -7 (Tyr89Asp / Ser146Pro), using site-directed mutagenesis. They found that Tyr89Asp mutant produced more α-cyclodextrin while produced less both β- and γ-cyclodextrins. The Ser146Pro mutant produced more α- cyclodextrin, while β- and γ- cyclodextrin production was not effected. Mutation Tyr89Gly had no effect on α- , β- and γ- cyclodextrin production. The double mutant Tyr89Asp / Ser146Pro showed a two fold increase in the production of α- cyclodextrin, decrease in the production of β- cyclodextrin and had no effect on γ- cyclodextrin production. Although all CGTase

mutants from *B. circulans* strain 251 showed increase α-cyclodextrin production, the β- cyclodextrin was still the major product, suggesting that mutations at all these subsites were not sufficient to change the product specificity. There might be other parts of the CGTase that are essential for the product specificity other than subsites -3 and -7.

Our research group in the Department of Biochemistry have been working on β-CGTase of *B. circulans* A11. The enzyme was purified, characterized, cloned and sequenced under TJTTP-OECF project (Rojtinakorn, 1994; Tongsima, 1998; Kaskangam, 1998; Prasong, 2002 and Rimphanichayakit, 2000). The gene encodes a β-CGTase of 713 amino acid residues, including a signal peptide. The molecular weight is approximately 72 kDa. We obtained α-CGTase gene of *Paenibacillus macerans* IAM 1243 as a gift from Mr. Toshiya Takano. This gene encodes an α-CGTase of 712 amino acid residues, including a signal peptide with a molecular weight approximately 71 kDa (Takano *et al.*, 1986). From the amino acid sequence alignment, both β- and α-CGTases gene have an amino acid sequence similarity of 67% (Fig. 20). Different amino acids are located through out the polypeptide chain. It is interesting to determine which part of the enzymes is involved in different ratios of cyclodextrin production or product specificity.

The chimeric CGTases between β-CGTase gene from *B. circulans* A11 and α-CGTase gene from *P. macerans* IAM1243 were constructed by *in vivo* homologous recombination. The recombination machinery of *E. coli* cell could be used *in vivo* to obtain recombinant genes by crossing-over at any sites showing sufficient homology. It is possible to generate with minimal effort a variety of chimeras or hybrids not easily obtained by ordinary genetic engineering technique. The activities, cyclodextrin production of the chimeras are studies in order to determine the essential part of the enzyme that might be involved in different ratio of cyclodextrin production.

The objective.

To determine an essential part of CGTase amino acid sequence that might be involved in different ratio of cyclodextrin production.

```
BcA11  APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINK
Pmace  SPDTSVDNKVNFSTDVIYQIVTDRFADGDRTNNPAGDAFSGDRSNLKLYFGGDWQGIIDK
       :*****.** **********.****:**: :***:* **.*. :**.** ********.*
BcA11  INDGYLTGMGITAIWISQPVENIYSVINYSGVHNTAYHGYWARDFKKTNPAYGTMQDFKN
Pmace  INDGYLTGMGVTALWISQPVENITSVIKYSGVNNTSYHGYWARDFKQTNDAFGDFADFQN
       **********:**.********* ***.****:**.**********.** *:* : **.*
BcA11  LIDTAHAHNIKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNLLGGYTNDTQNLFHHYGG
Pmace  LIDTAHAHNIKVVIDFAPNHTSPADRDNPGFAENGALYDNGSLLGAYSNDTAGLFHHNGG
       ************.************. *.*.***** *****.***.*.*** .**** **
BcA11  TDFSTIENGIYKNLYDLADLNHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQK
Pmace  TDFSTIEDGIYKNLYDLADINHNNNAMDAYFKSAIDLWLGMGVDGIRFDAVKHMPFGWQK
       *******.***********.****.::*.*:*.**.:**.:*.****** ***********
BcA11  SFMSTINN-YKPVFTFGEWFLGVNEISPEYHQFANESGMSLLDFRFAQKARQVFRDNTDN
Pmace  SFVSSIYGGDHPVFTFGEWYLGADQTDGDNIKFANESGMNLLDFEYAQEVREVFRDKTET
       **:*:*  . :*********:**.:: . : :******* ****.:**:.*:****:*:.
BcA11  MYGLKAMLEGSEVDYAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIY
Pmace  MKDLYEVLASTESQYDYINNMVTFIDNHDMDRFQVAGSGTRATEQALALTLTSRGVPAIY
       * .* :* .:* :* :* :*: ********:**:.... * *****:***********
BcA11  YGSEQYMSGGNDPDNRARIPSFSTTTTAYQVIQKLAPLRKSNPAIAYGSTQERWINNDVI
Pmace  YGTEQYMTGDGDPNNRAMMTSFNTGTTAYKVIQALAPLRKSNPAIAYGTTTERWVNNDVL
       **:****:*..**.*** .:**.* ****.*** **************.* ***.****:
BcA11  IYERKFGNNVAVVAINRNMNTPASITGLVTSLPQGSYNDVLGGILNGNTLTVGAGGAASN
Pmace  IIERKFGSSAALVAINRNSSAAYPISGLLSSLPAGTYSDVLNGLLNGNSITVGSGGAVTN
       * *****...*.****** .:. .*:**:.*** *.*.***.*.****.:***.***.:*
BcA11  FTLAPGGTAVWQYTTDATAPIIGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGA
Pmace  FTLAAGGTAVWQYTAPETSPAIGNVGPTMGQPGNIVTIDGRGFGGTAGTVYFGTTAVTGS
       ****.*********: *.* ****** *..** :********. ************:
BcA11  DIVAWEDTQIQVKIPAVPGGIYDIRVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTA
Pmace  GIVSWEDTQIKAVIPKVAAGKTGVSVKTSSGTASNTFKSFNVLTGDQVTVRFLVNQANTN
       .**:******:. ** *..* .: * .::*.*** ...*.**********.:*:.*.*
BcA11  LGQNVFLTGNVSELGNWDPNNAIGPMYNQVVYQYPTWYYDVSVPAGQTIEFKFLKKQGST
Pmace  YGTNVYLVGNAAELGSWDPNKAIGPMYNQVIAKYPSWYYDVSVPAGTKLDFKFIKKGGGT
       * **:*.** .:***.****.*********: .**:**********. .::***.** *.*
BcA11  VTWEGGANRTFTTPTSGTATMNVNWQP
Pmace  VTWEGGGNHTYTTPASGVGTVTVDWQN
       ******.*:*:***.**..*:.*:.:**
```

Figure 20 : Alignment of the amino acid sequences of β-CGTase from *B. circulans* A11(BcA11) and α-CGTase from *P. macerans* IAM1243 (Pmace).

CHAPTER II

MATERIALS & METHODS

1. Equipments

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

Autopipette : Pipetman, Gilson, France.

Centrifuge : Refrigerated centrifuge : Model J-21C, Beckman Instrument Inc., U.S.A.

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

Electrophoresis Unit : Model Mini-protein II Cell, BioRad, U.S.A.

Incubator : Model OB-28L Fisher Scientific Inc., U.S.A.

Magnetic stirrer and heater : Model IKAMA$^{®}$ GRH, Janke & Kunkel Gmbh & Co.KG, Japan.

pH meter : PHM 83 Autocal pH meter, Radiometer, Denmark.

Membrane filter : cellulose nitrate, pore size 0.2 μ, Whatman, Japan.

Spectrophotometer : Jenway 6400, England.

Vortex : Model K 550-GE, Scientific Industries, U.S.A.

Water bath : Charles Hearson Co. Ltd., England.

U.V. transilluminator : 2011 MA Crovue, San Gabrial, U.S.A.

Transformation apparatus : Gene pulser™ : Biorad, U.S.A.

High performance liquid chromatography : Shimadzu, Japan.

Orbital shaker : Gallenkamp, Germany.

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

Microcentrifuge tubes 1.5 ml : Bioactive, Thailand.

2. Chemicals

Acetonitrile (HPLC grade), Labscan, Ireland.

Acrylamide, Merck, Germany.

α- , β- and γ- cyclodextrins, Sigma, U.S.A.

Glycine, Sigma, U.S.A.

D (+)-maltose monohydrate, Fluka, Switzerland.

N, N'-methyl-bis-acrylamide, Sigma, U.S.A.

Soluble starch, Sigma, U.S.A.

Tris-base, USB, U.S.A.

Boric acid, Merck, Germany.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Sodium dodecyl sulfate, Sigma, U.S.A.

Sodium hydroxide, Carlo Erba, Italy.

Hydrochloric acid, Lab Scan, Ireland.

Sodium chloride, Univar, Australia.

Glacial acetic acid, BDH, England.

Glucose, Sigma, U.S.A.

Calcium chloride, Merck, Germany.

Sodium citrate, Carlo Erba, Italy.

Di-Sodium hydrogenphosphate, Fluka, Switzerland.

Sodium dihydrogen orthophosphate, Carlo Erba, Italy.

Phenol, BDH, England.

Chloroform, Sigma, U.S.A.

Sodium carbonate, BDH, England.

Isopropyl-β-D-thiogalactopyranoside (IPTG), Serva, Heidelberg, Germany.

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), Sigma, U.S.A.

Agarose, SEAKEM LE Agarose, FMC Bioproducts, U.S.A.

Phenolpthalein, BDH, England.

Methyl orange, BDH, England.

Bromocresol green, Carlo Erba, Italy.

Bromophenol blue, Merck, Germany.

Glycerol, Scharlau, Spain.

Xylene cyanol FF, Sigma, U.S.A.

Absolute alcohol, Sigma, U.S.A.

Polyethylene glycol 8000, Fluka, Switzerland.

Qiaquick Gel Extraction Kit, Qiagen, Germany.

Iodine, Baker chemical, U.S.A.

Potassium iodide, Mallinckrodt, U.S.A.

Coomassie® brilliant blue R, Acros organics, Belgium.

Coomassie® brilliant blue G 250, Fluka, Switzerland.

Ethidium bromide, Sigma, U.S.A.

Ampicillin, Biobasic Inc, Thailand.

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

Bovine Serum Albumin (BSA), Sigma, U.S.A.

β-mercaptoethanol, Fluka, Swithzerland.

Yeast extract, Scharlau, Spain.

Tryptone, Merck, Germany.

Agar, Merck, Germany.

Methanol, Scharlau, Spain.

3. Bacterial strains

Escherichia coli JM109 (*F', traD36, proA$^+$, proB$^+$, lacI^q, lacZ ΔM15/recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB, mcrA*) was used for DNA manipulation.

Escherichia coli JC8679 (*F$^-$, thr-1, leu-6, thi1, lacY1, galK2, ara14, xy15, mtl1, proAZ, his4, argE3, str31, tsx33, supE44, λ^-, recB21, recC22, sbcΔ23*) was used for homologous recombination.

4. Plasmid vectors

4.1.   pUC119 for subcloning of recombination region from chimeric CGTases for DNA sequencing

4.2.   pVR300, a pUC119 containing the α-CGTase gene from *Paenibacillus macerans*

4.3.   pVR316, a pUC119 containing the β-CGTase gene from *B. circulans* A11

4.4.   pVR321, a pUC119 containing the β-CGTase gene from *B. circulans* A11 at 5' side of α-CGTase gene from *P. macerans*, used for homologous recombination.

4.5.   pVR388, a pUC119 containing the α-CGTase gene at 5' side of β-CGTase gene, used for homologous recombination.

5. Enzymes

5.1.   Restriction endonucleases

- *Eco*RI, *Nde*I, *Nco*I, *Bsr*GI, *Sph*I, *Hin*dIII, *Bst*EII, *Msc*I, *Kpn*I, *Bgl*II, *Bam*HI were purchased from New England Biolabs Inc., U.S.A.

- *Eco*RV, *Sac*II and *Eco*47III were purchased from Promega, Co. Ltd., U.S.A.

5.2. DNA ligase was purchased from SibEnzyme, Russia.

5.3. Glucoamylase was purchased from Fluka, Switzerland.

5.4. RNase A was purchased from Sigma, U.S.A.

6. Media preparation

6.1. Luria-Bertani broth (LB medium)

LB broth consists of 1% Bacto tryptone, 0.5% yeast extract and 0.5% NaCl, supplemented with 100 μg/ml ampicillin when needed.

6.2. LB-starch agar

LB-starch agar consists of 1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar and 1% soluble starch, supplemented with 100 μg/ml ampicillin when needed. LB-starch agar was used for the selection of chimeric CGTase.

6.3. LB-IPTG-X-gal agar

LB-IPTG-X-gal agar consists of 1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar, supplemented with 100 μg/ml ampicillin when needed. X-gal and IPTG are added to the final concentrations of 70 μg/ml and 80 μM, respectively. The agar was used for the screening of blue-white colonies.

7. General techniques in genetic engineering

7.1. Preparation of competent cells

A single colony of *E. coli* JM109 or JC8679 was cultured as a starter in 5 ml of LB-broth and incubated at 37 $^{\circ}$C with 250 rpm shaking for 24 hours. The starter was diluted in 500 ml of LB-broth, and the culture was incubated at 37 $^{\circ}$C with 250 rpm shaking until the optical density at 600 nm of the cells 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 4 $^{\circ}$C. 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 4°C. 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 1-2 ml of ice cold sterile 10% glycerol. The cell suspension was divided into 40 μl aliquots and store at -80°C until used.

7.2. Electroporation

The competent cells were thawed on ice. Fourty microlitres of the cell suspension were mixed with 1-2 μl of the ligation mixture or restriction digested 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 follows ; 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 37 °C for 1 hour with shaking at 250 rpm. One hundred microlitres of the cells in the media were spread on the LB-starch agar or LB-IPTG-X-gal agar for the selection of chimeric CGTase and the blue-white colony screening, respectively.

7.3. Plasmid preparation (Birnboim and Doly, 1979)

Plasmid harboring cells were cultured in LB broth (1.5 ml) and harvested by centrifugation at 5000 x g, 4 °C for 1 minute. The packed cells were resuspended in 100 μl of Solution I (25 mM Tris-HCl, pH 8.0, 10 mM Na$_2$EDTA and 50 mM glucose), mixed by vortexing well. 200 μl of Solution II (1% SDS, 0.2 N NaOH) was added, mixed by inversion and kept on ice for 5 minutes. The mixture was neutralized by adding 150 μl of Solution III (3 M sodium acetate, pH 5.2), mixed by inversion and kept on ice for 10 minutes. After centrifugation at 12,000 rpm for 10 minutes, 10 mg/ml of RNase A was added to the supernatant to give a final concentration of 10 μg/ml and incubated at 37 °C for 20 minutes. The supernatant was extracted with one volume of phenol:chloroform (1:1). Two volumes of absolute alcohol were added, mixed and stored at –20 °C for 30 minutes. The plasmid was pelleted by centrifugation at 12,000 rpm, washed with 70% ethanol and vacuum dried for 10 minutes. The pellet was dissolved in TE buffer.

For DNA sequencing, after vacuum dried for 10 minutes, the pellet was dissolved in 32 μl of deionized water. Then the plasmid was reprecipitated by first adding 8 μl of 4 M NaCl, then adding 40 μl of sterile 13% PEG-8000, mixed thoroughly and incubated on ice for 20 minutes. The plasmid was pelleted by centrifugation at 12,000 rpm, 4 °C for 15 minutes, washed with 70% ethanol, vacuum dried and resuspended in 20 μl distilled deionized water.

7.4. Agarose gel electrophoresis

To measure the size of DNA 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 1x 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 ethidium bromide solution (2.5 µg/ml) for 2-5 minutes, and the DNA bands were visualized under UV light from UV transluminator. The gels were photographed through a filter onto Kodak Tri-X pan 400 film. The sizes of DNA fragments were determined by comparing the relative mobilities with those of the standard DNA fragments (λ/*Hin*dIII marker).

7.5. DNA fragment extraction from agarose gel

QIAquick gel extraction kit was used for extracting DNA fragment from agarose gel, and performed according to the kit protocol. Briefly, DNA fragment was excised from an agarose gel, added 3 volume of buffer QG and incubated for 10 minutes at 50°C. After the gel slice has dissolved completely, the sample was applied to the QIAquick column, and centrifuged for 1 minute. The flow-through was discarded. Buffer QG was added and centrifuged for 1 minute. The column was washed twice with buffer PE and centrifuged for 1 minute. Finally, the elution buffer was added to the center of the QIAquick membrane to elute the DNA, the column was left stand for 1 minute, and then centrifuged for 1 minute.

8. Construction of pVR321 and pVR388

The pVR321 was previously constructed by Rimphanitchayakit, V. (unpublished data). In pVR321, the β-CGTase gene is located on the 5' side of the α-CGTase gene. To construct a plasmid with the α-CGTase gene on the 5' side of the β-CGTase gene, pVR300 was digested by *Bam*HI and *Kpn*I, and pVR 316 was digested by *Bgl*II and *Kpn*I. A *Bgl*II-*Kpn*I fragment (3.2 kb) from pVR316 containing the β-CGTase gene was inserted into the *Bam*HI-*Kpn*I site of pVR300 (Fig. 21). The resulting plasmid was pVR388 that had the α-CGTase gene on the 5' side of the β-CGTase gene. The maps of pVR321 and pVR388 are shown in Fig. 22.

9. Construction of chimeric CGTase from pVR321 and pVR388

pVR321 was digested by *Eco*RV or *Msc*I. The *Eco*RV or *Msc*I large fragments were gel purified with Qiaquick gel extraction kit. The eluted fragment was heated at 95 $^{\circ}$C, and slowly cooled to room temperature. The fragment was then transformed into *E. coli* JC8679 using electroporation. The transformed cells were grown in LB-starch agar containing 100 µg/ml of ampicillin at 37 $^{\circ}$C for 24 hours, The colonies of transformed cell were transfered to LB-broth containing 100 µg/ml of ampicillin and cultured at 37 $^{\circ}$C for 16 hours. Plasmid was prepared and retransformed into *E. coli*

JM109. The chimeric plasmid was prepared again. This was to ensure that only a single chimeric plasmid was isolated from each recombination event.

To construct chimeras from pVR388, the same procedure as in pVR321 was used but the plasmid was digested with *Bsr*GI and *Nco*I . The diagrams for the construction of chimeric CGTases are shown in Fig. 22.

10. Restriction mapping of chimeric CGTase plasmids to determine the regions of recombination

Chimeric CGTase plasmids obtained from pVR321 were digested with *Eco*RI, *Hin*dIII, *Msc*I, *Eco*RV, *Nde*I, *Nco*I, *Bst*EII, *Sac*II, *Sph*I, *Bgl*II, *Bsr*GI, *Hin*dIII + *Eco*RI and *Nde*I + *Eco*RI. Chimeric CGTase plasmids from pVR388 were digested with *Eco*RI, *Hin*dIII, *Msc*I, *Bsr*GI, *Eco*RV, *Nco*I, *Nde*I, *Sph*I, *Bam*HI, *Bst*EII, *Sac*II, *Hin*dIII + *Eco*RI and *Nde*I + *Eco*RI.

11. Subcloning of recombination regions from the chimeric CGTase plasmid for DNA sequencing

A chimeric CGTase plasmid series from pVR321, which had amylolytic activity, was digested with *Sph*I and *Hin*dIII. The *Sph*I – *Hin*dIII fragment with potential recombination site was ligated to *Sph*I – *Hin*dIII site of the pUC119. A chimeric CGTase plasmid series from pVR388 was digested with *Eco*RV and *Eco*RI . The *Eco*RV – *Eco*RI fragment with potential recombination site was ligated to *Sma*I – *Eco*RI site of the pUC119. The ligation reactions were incubated for 16-24 hours at 16°C.

The ligation products were transformed into *E. coli* JM109. The transformed cells were grown on LB-IPTG-X-gal agar containing 100 μg/ml of ampicillin at 37 $^{\circ}$C for 24 hours. The plasmid was prepared from white colony. The DNA sequencing was carried out by Bioservice Unit, Thailand.

12. Determination of an essential part of CGTase involved in the different ratios of cyclodextrin production

An essential part of chimeric CGTase structure that is involved in the different ratio of cyclodextrin production were determined by gene portion shuffling method. The *Nde*I-*Sma*I, *Nde*I-*Eco*47III and *Eco*47III-*Bam*HI fragments of chimeric CGTases (pVR 402) were selected to replace with the identical fragments from wild type CGTase (α-CGTase; pVR300) (Fig. 23). The ratios of cyclodextrin production were determined from the recombinant clones, pVR404, 405 and 406.
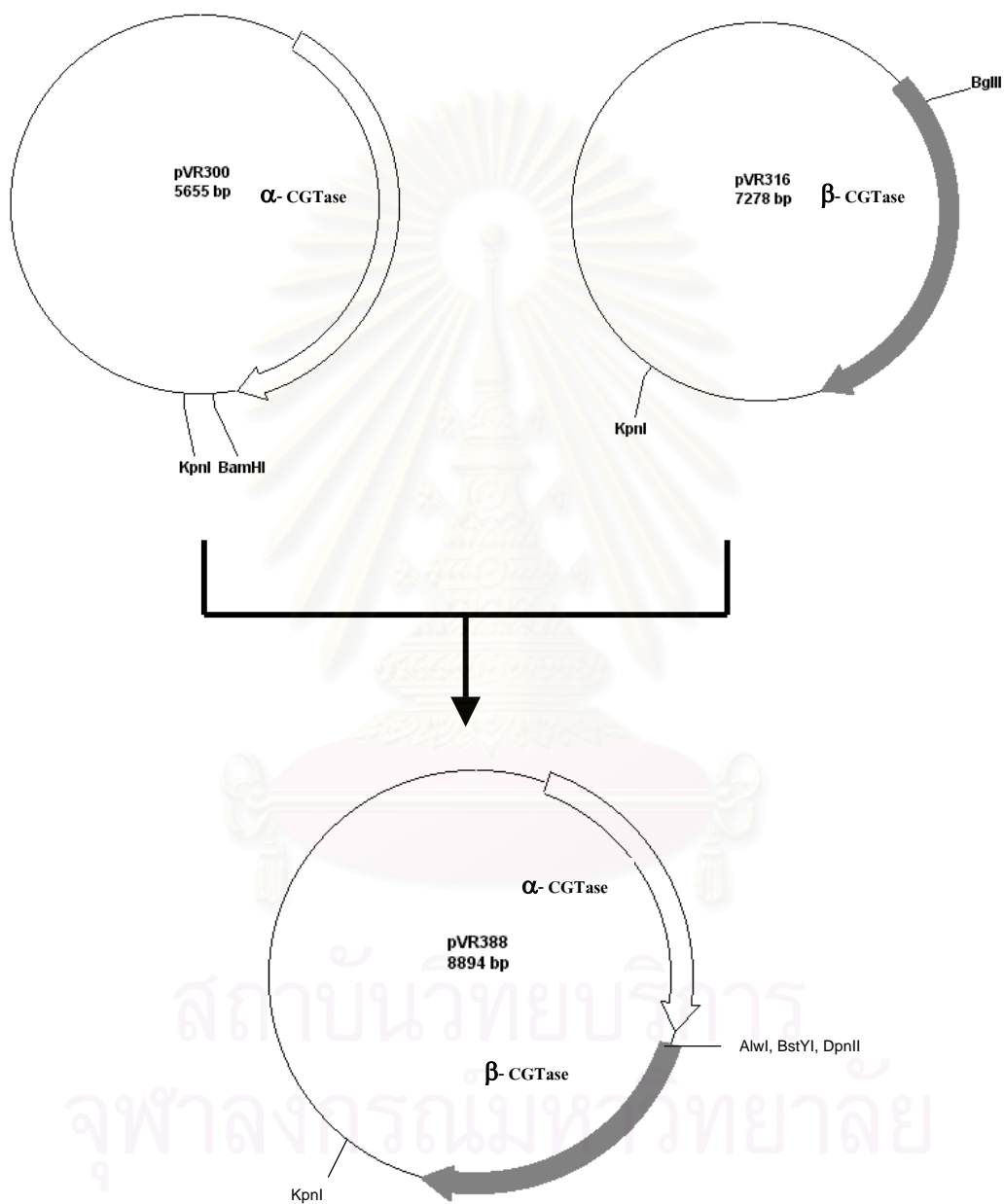
Figure 21 : The construction of pVR388 (bottom) from pVR300 (top left) and pVR316 (top right). The restriction sites for construction are indicated.
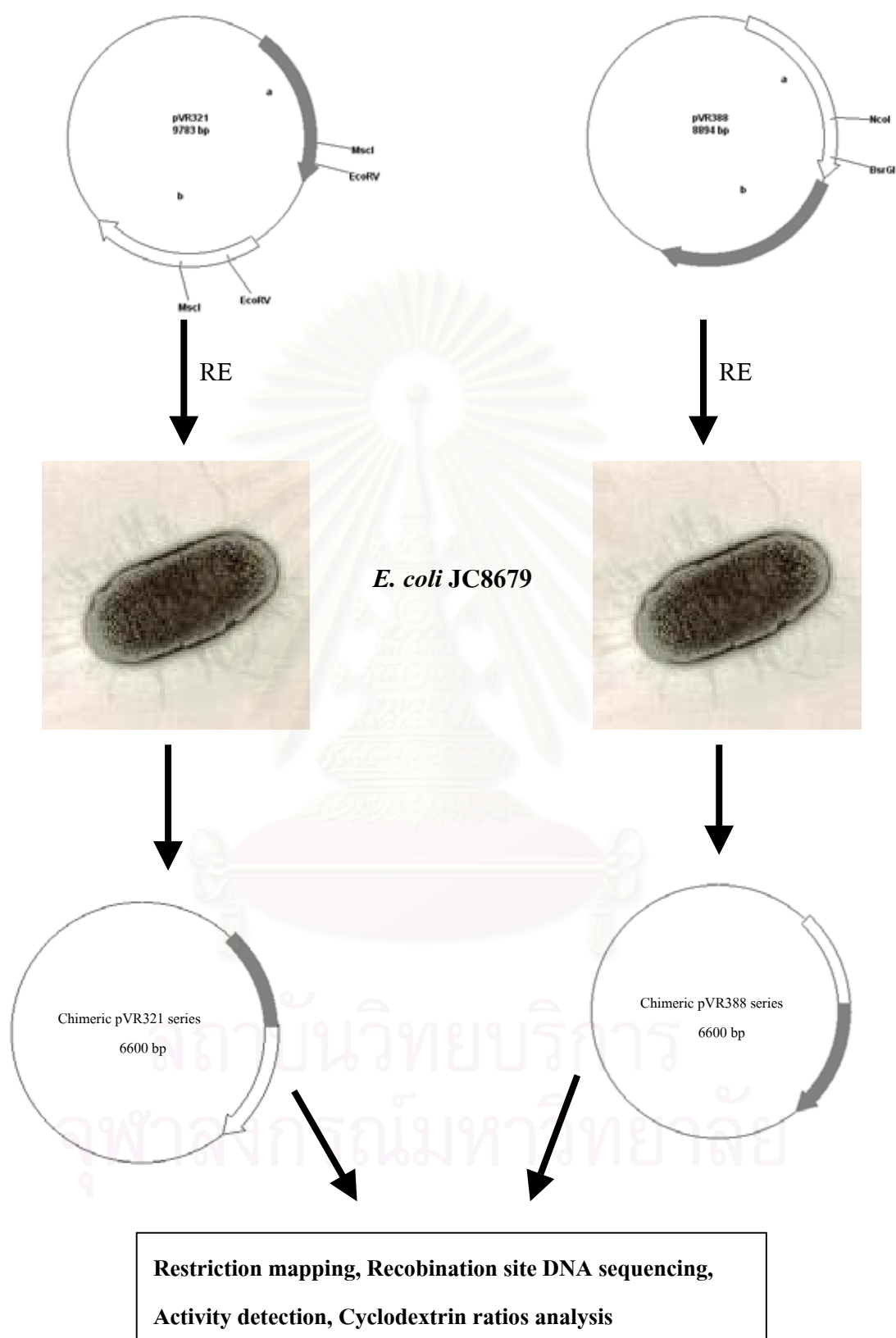
Figure 22 : The construction of chimeric CGTase by homologous recombination process.
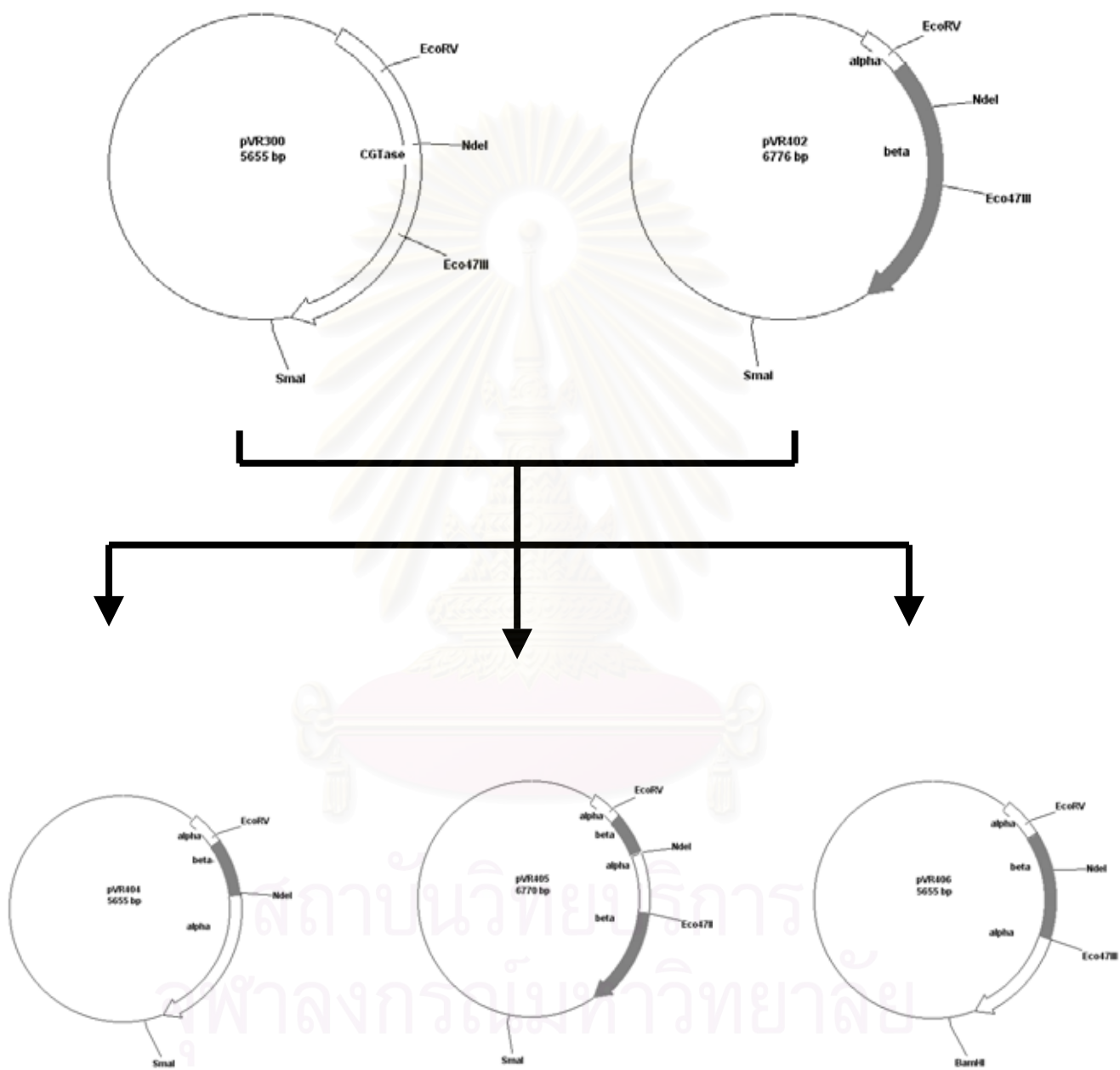
Figure 23 : The construction of pVR404, 405 and 406

13. Detection of chimeric CGTase activity

13.1. Dextrinizing activity

E. coli JM109 cells, containing the chimeric CGTase, were plated on LB-starch agar and were incubated at 37 $^{\circ}$C for 24 hours. The halo formation was observed after iodine solution (0.02% I_2 in 0.27% KI) was sprayed.

13.2. Cyclodextrins-forming activity

E. coli JM109 cells, containing the chimeric CGTase, were grown at 37 $^{\circ}$C for 16 hours in LB broth and pelleted. Supernatant (200 μl) was incubated with 0.5 ml of 1% soluble starch at 37 $^{\circ}$C for 10 minutes. The reaction was stopped by boiling for 10 minutes. The reaction was clarified by centrifugation at 12,000 rpm for 10 minutes and the supernatant was analyzed for α-, β- and γ-cyclodextrins. The activities were assayed by mixing 100 μl of supernate with methyl orange solution (35 μl of 1mM methyl orange and 865 μl of 50 mM phosphate buffer, pH 6) for α-cyclodextrin forming activity or phenolpthalein solution (2.9 ml of 6 mM sodium carbonate and 2 ml of 75 μM phenolpthalein in 6 mM sodium carbonate) for β-cyclodextrin forming activity or bromocresol green solution (100 μl of 5 mM bromocresol green and 2 ml of 0.2 M citrate buffer, pH 4.2) for γ-cyclodextrin forming activity. The reactions were incubated at room temperature for 15 minutes and then measured the absorbance at 505, 550 and 630 nm for α-, β- and γ-cyclodextrin forming activities, respectively. One unit of cyclodextrin forming activity (cyclization) was defined as the amount of enzyme that catalyzed the formation of 1 μmole of each cyclodextrin per minute under the assay condition.

13.3. HPLC analysis of cyclodextrin

The cyclodextrin samples were prepared as described in 13.2, but incubated at 37 $^{\circ}$C for 6-8 hours. After boiled, the mixture was treated for an overnight with about 30 U of glucoamylase to digest the remaining starch. After stopping the glucoamylase reaction by boiling for 10 minutes, the reaction was clarified by centrifugation. The cyclodextrin samples were filtered through 0.45 μ membrane filters. The samples were injected into HPLC using supeles-NH$_2$ column (0.46$\times$25 mm) and detected by RI detector. The eluent was 70% (v/v) acetonitrile in water and the flow rate was 1 ml/minute. α-, β- and γ-cyclodextrins were identified by comparing the retention times to those of standard mixture of α-, β- and γ-cyclodextrins (20 mg/ml each). For quantitive analysis, peak area corresponding to each cyclodextrin was used to calculate the cyclodextrin ratios.

14.   Production of CGTase

  14.1. Starter inoculum

    A single colony of *E. coli* JM109 harboring a chimeric or wild type CGTase gene was grown in 5 ml of LB broth at 37 $^{\circ}$C with shaking 250 rpm for 16 hours.

  14.2. Enzyme production

    Starter *E. coli* JM109 was transferred into 500 ml of LB broth and cultivated at 37 $^{\circ}$C with shaking 250 rpm for 16-24 hours. Cells were removed by centrifugation at 7,000 rpm for 15 minutes at 4 $^{\circ}$C. Crude enzyme was collected and kept at 4 $^{\circ}$C for purification.

15.   Purification of CGTase

  CGTase was purified from culture broth of *E. coli* JM109 harboring wild type and chimeric CGTases by starch adsorbtion method (Martins *et al*., 2002).

  Corn starch (local grade) was oven dried at 120 $^{\circ}$C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude broth to 5% (W/V) concentration. After 2 hours of continuous stirring at 4 $^{\circ}$C, the starch cake was collected by centrifugation at 10,000 rpm for 10 minutes and washed twice with cold sterile distilled water. The adsorbed CGTase was eluted from the starch cake with 10 mM Tris-HCl buffer, pH 8.0, containing 0.25 M maltose by stirring for 30-60 minutes. The purified CGTase was then concentrated by Vivaflow 50.

16. Purity of CGTase

  16.1. SDS-polyacrylamide gel electrophoresis

    Protein analysis was carried out using the SDS denaturing gel. Samples to be analyzed were treated with sample buffer and boiled for 5 minutes before loading into the gel. The electrophoresis was run from anode towards the cathode at constant current of 20 mA per slab at room temperature in a Midgel LKB 2001 gel electrophoresis unit.

  16.2. Non-denaturing polyacrylamide gel electrophoresis

    For activity staining of CGTase, PAGE without SDS was used. The electrophoresis was run from anode towards the cathode at constant current of 20 mA per slab at room temperature in a Midgel LKB 2001 gel electrophoresis unit.

16.3. Detection of proteins in polyacrylamide gels

16.3.1. Coomassie blue staining

The gels were stained with 0.1% (W/V) of Coomassie brilliant blue R-250 in 45% (V/V) methanol and 10 % (V/V) acetic acid for at least 2 hours. The gels were destained with a solution of 10% methanol and 10% acetic acid for 1-2 hours until the gel background was clear.

16.3.2. Dextrinizing activity staining

The gels were soaked in 10 ml of substrate solution, containing 2% (W/V) soluble starch (potato) in 0.2 M phosphate buffer pH 6.0, at 37 $^{\circ}$C for 10 minutes. They were then rinsed several times with distilled water, and 10 ml of iodine staining reagent (0.2% I_2 in 2% KI) was added for color development at room temperature. The clear band on the dark-blue background indicates dextrinizing activity of CGTase.

17. CGTase assay

17.1. Dextrinizing activity assay (Fuwa, 1954)

CGTase sample (100 μl) was incubated with 0.3 ml of 1% soluble starch in 0.2 M phosphate buffer, pH 6.0 at 37 $^{\circ}$C for 10 minutes. The reaction was stopped with 4 ml of 0.2 N HCl. Then 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured.

One unit of enzyme was defined as the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition.

17.2. Kinetic studies for cyclization activity

17.2.1. α-cyclization activity (Lejeune et al., 1989)

The reaction mixtures (1 ml), containing various concentrations of soluble starch, 35 μM methyl orange in 50 mM phosphate buffer (pH 6.0) were incubated with CGTases (0.036, 0.02 and 0.04 mg protein of CGTases from pVR316, 300 and 402) for 10 minutes at 37°C. The enzymatic reaction was stopped by the addition of 6 N HCl to final concentration of 0.3 N. The reaction mixture was incubated at 16 $^{\circ}$C for 30 min for the forming of methyl orange-α-CD complex, and then the absorbance at 505 nm was measured. A calibration curve was made using a

series of dilutions of 5 mM α-cyclodextrin in phosphate buffer with methyl orange at a final concentration of 0.035 mM.

One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of α-cyclodextrin per minute under the assay condition.

17.2.3. β–cyclization activity (Vikmon, 1981)

The reaction mixtures (5 ml) containing various concentrations of soluble starch was incubated with the enzymes (0.06, 0.084 and 0.07 mg protein of CGTases from pVR316, 300 and 402) for 10 minutes at 37 $^{\circ}$C. The reaction was stopped by boiling for 10 minutes. Subsequently, 2 ml of 75 μM phenolpthalein and 2 ml of 6 mM sodium carbonate were added. After standing at room temperature for 15 minutes, the absorbance at 550 nm was measured. A calibration curve was made using a series of dilutions of 5 mM β- cyclodextrin in sodium carbonate buffer with phenolpthalein at a final concentration of 30 μM.

One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of β-cyclodextrin per minute under the assay condition.

17.2.4. γ-cyclization activity (Kato and Horikoshi, 1984)

The reaction mixture (3 ml), containing various concentration of soluble starch, was incubated with the enzymes (0.12, 0.2 and 0.07 mg protein of CGTases from pVR316, 300 and 402) at 37 $^{\circ}$C for 10 minutes. The enzymatic reaction was stopped by boiling for 10 minutes. Subsequently, 100 μl of bromocresol green (5 mM) and 2 ml of 0.2 M citrate buffer (pH 4.2) was added. After incubated at room temperature for 15 minutes, the absorbance at 630 were measured comparing with a calibration curve that was made using a series of dilutions of 5 mM γ- cyclodextrin in citrate buffer (pH 4.2) with bromocresol green at a final concentration of 30 μM.

One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of γ-cyclodextrin per minute under the assay condition.

17.3. Protein determination (Lowry et al., 1951)

The protein sample was mixed with 2.5 ml of solution C (Appendix A), and stood at room temperature for 5-10 minutes. Then 0.25 ml of solution D (Folin- Ciocalteu phenol reagent) was added and mixed. After 20-30 minutes, the wavelength of 750 nm was measured. The protein concentration was calculated from a standard curve of bovine serum albumin.

CHAPTER III

RESULTS

1. Construction of pVR388

As shown in Fig. 21, pVR388 was constructed by the insertion of a *Bgl*II-*Kpn*I fragment of pVR316 (3.2 kb) into *Bam*HI-*Kpn*I sites of pVR300, resulting in a plasmid that had the α-CGTase gene on the 5' side of the β-CGTase gene. Fig. 24 shows a restriction digestion of pVR388 which confirms the correct construction of pVR388. This pVR388 was used as the second plasmid for the construction of the chimeric CGTases that had the α-CGTase sequence preceded the β-CGTase sequence. For the first plasmid, pVR321 had a β-CGTase gene on the 5' side of α-CGTase gene and was constructed previously by Rimphanitchayakit, V (unpublished). This plasmid was used for the construction of the chimeric CGTases that had the β-CGTase sequence preceded that of the α-CGTase sequence.

2. The chimeric CGTase series from pVR321 and pVR388

The chimeric CGTases were divided into 2 groups according to the parental plasmids used in the construction. The parental plasmids, pVR321 and pVR388, therefore, generated 2 series of chimeric CGTases, namely, pVR321 and pVR388 series.

The plasmids were digested with appropriate enzymes to inactivate both the α- and β-CGTase genes, leaving enough gene sequence for homologous recombination. Each of the linearized plasmids were transformed into *E. coli* JC8679. The plasmids, that recombined *in vivo*, rendered the transformed cells resistant to ampicillin. The plasmid isolated contained chimeric CGTases.

The plasmid pVR321 was digested with either *Eco*RV or *Msc*I. The *Eco*RV-digested pVR321 generated chimeric CGTases containing plasmids, designated as pVR327, 359, 389, 390, 391 and 392. The *Msc*I-digested pVR321 generated 4 plasmids, pVR393, 394, 395 and 396.

Seven chimeric CGTase plasmids were obtained from pVR388, double digested with *Nco*I and *Bsr*GI, and designated as pVR397, 398, 399, 400, 401, 402 and 403.

3. Restriction mapping of the recombination sites

The chimeric CGTase plasmids from pVR321 series were digested with *Nde*I, *Eco*RV, *Msc*I, *Sph*I, *Nco*I, *Bsr*GI, *Eco*RI, *Hin*dIII, *Sac*II, *Bgl*II, *Bst*EII, *Eco*RI+*Nde*I and *Eco*RI+*Hin*dIII to determine the locations of the recombination sites. Fig. 25, 26 and 27 showed the restriction patterns for pVR389,

393 and 395, pVR390 and 391, pVR327 and 396, respectively. The pVR359, 392 and 394 had unique restriction patterns (Fig. 28, 29 and 30). The restriction maps of all chimeric CGTases and the locations of recombination sites were summarized in Fig. 31.

As shown in Fig. 31, the recombination sites of pVR389, 393 and 395 were located between *Nde*I and *Sac*II sites. Those of pVR390 and 391 were found between *Eco*RI and *Nco*I sites. Those of pVR327 and 396 were found between *Nde*I and *Nco*I sites. That of pVR359 was located between *Msc*I and *Sac*II sites. pVR 394 had a deletion in the internal region between *Sph*I and *Hin*dIII sites while pVR392 had a gene deletion on the 5' side of the chimeric gene as well as part of the vector sequence.

The seven chimeric CGTase plasmids from pVR388 series were digested with *Nde*I, *Eco*RV, *Msc*I, *Nco*I, *Bsr*GI, *Sph*I, *Eco*RI, *Hin*dIII, *Sac*II, *Bst*EII, *Bam*HI, *Eco*RI+*Nde*I and *Eco*RI+*Hin*dIII. The pVR397, 398 and 401 had unique restriction patterns while pVR399 and 400 and pVR402 and 403 had the same restriction patterns (Fig. 32-36).The summary of the restriction maps and the locations of recombination sites were shown in Fig. 37. The recombination site of pVR397 was located between *Nde*I-*Bst*EII site. That of pVR398 was located between *Sac*II-*Eco*RV site. pVR399 and 400 had gene deletions within the recombination region between *Msc*I and *Eco*RI sites. That of pVR401 has a recombination site between *Msc*I-*Msc*I. Finally, those of pVR402 and 403 were found between *Eco*RV and *Sph*I sites.
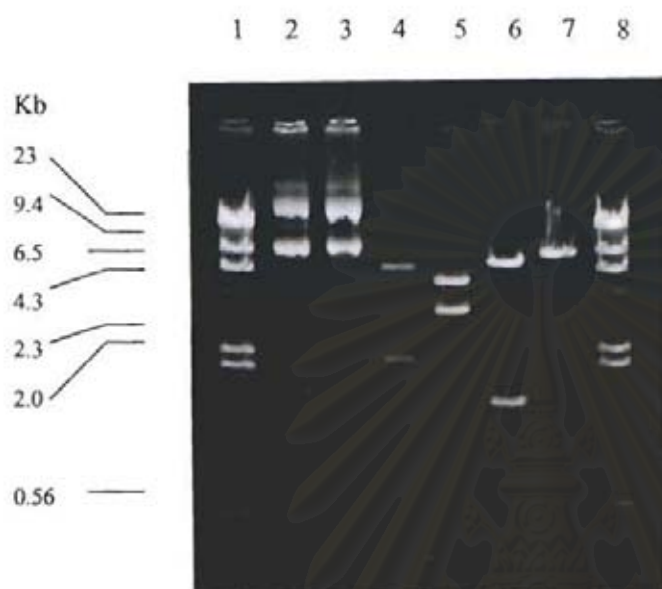
Figure 24 : Restriction digestion of pVR388. Lanes 1, 8 : λ/*Hin*dIII marker, lane 2 : undigested pVR388, lanes 3-7 : pVR388 digested with *Bgl*II, *Nde*I, *Eco*RV, *Bam*HI and *Nco*I, respectively.
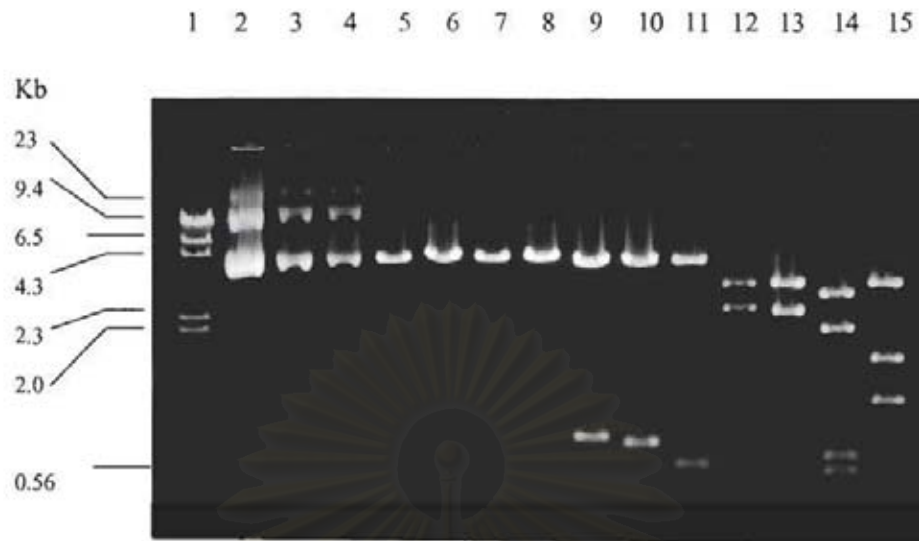
Figure 25 : Restriction pattern of pVR389. The pattern is the same for pVR393 and 395. Lane 1 : λ/*Hin*dIII marker ; lane 2 : undigested ; lanes 3-15 : digested with *Eco*RV, *Msc*I, *Nde*I, *Sph*I, *Nco*I, *Bsr*GI, *Bgl*II, *Bst*EII, *Sac*II, *Hin*dIII, *Eco*RI, *Hin*dIII+*Eco*RI and *Nde*I+*Eco*RI, respectively.

Figure 26 : Restriction pattern of pVR390. The pattern is the same for pVR391. Lane 1 : λ/*Hin*dIII marker, lane 2 : undigested, lanes 3-15 : digested with *Eco*RV, *Msc*I, *Nde*I, *Sph*I, *Nco*I, *Bsr*GI, *Bgl*II, *Bst*EII, *Sac*II, *Hin*dIII, *Eco*RI, *Hin*dIII+*Eco*RI and *Nde*I+*Eco*RI, respectively.
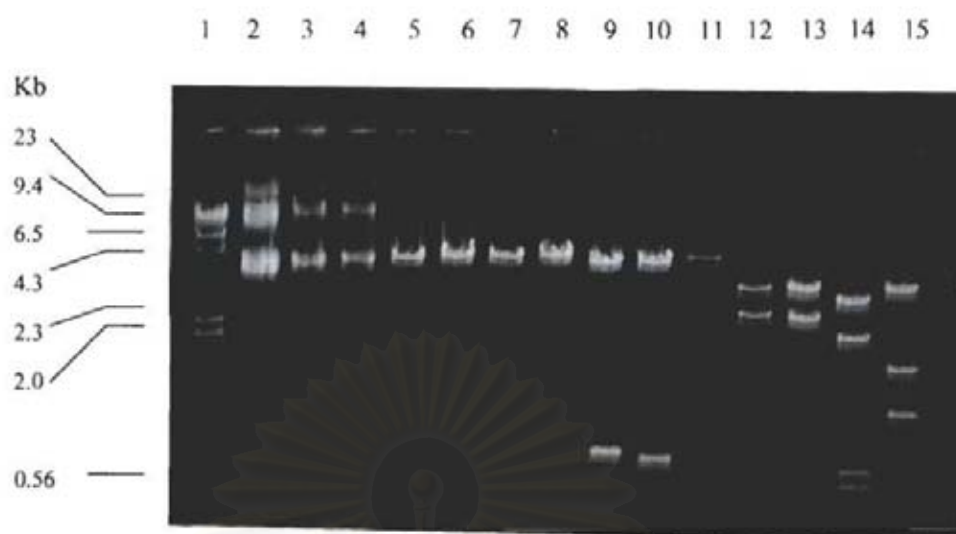
Figure 27 : Restriction pattern of pVR327. The pattern is the same for pVR396. Lane 1 : λ/*Hind*III marker, lane 2 : undigested, lanes 3-15 : digested with *Eco*RV, *Msc*I, *Nde*I, *Sph*I, *Nco*I, *Bsr*GI, *Bgl*II, *Bst*EII, *Sac*II, *Hind*III, *Eco*RI, *Hind*III+*Eco*RI and *Nde*I+*Eco*RI, respectively.
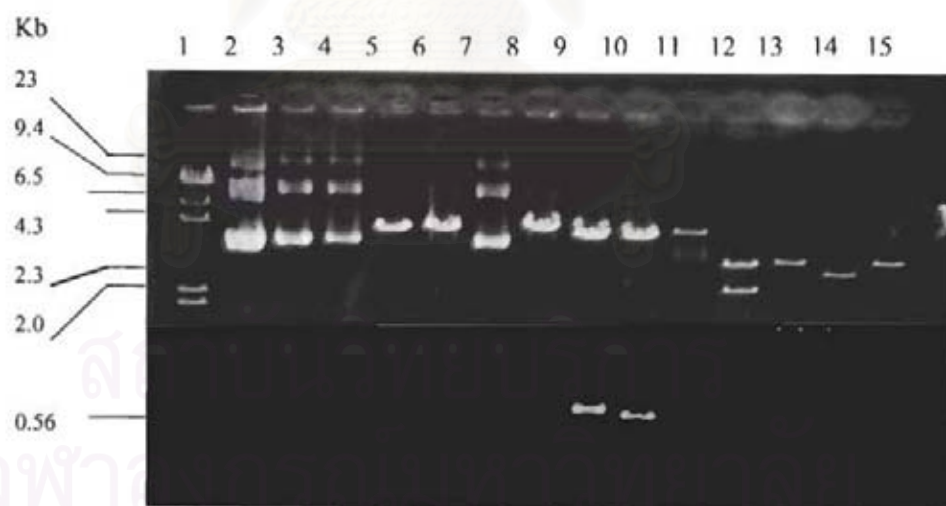
Figure 28 : Restriction pattern of pVR359. Lane 1 : λ/*Hind*III marker, lane 2 : undigested, lanes 3-15 : digested with *Eco*RV, *Msc*I, *Nde*I, *Sph*I, *Nco*I, *Bsr*GI, *Bgl*II, *Bst*EII, *Sac*II, *Hind*III, *Eco*RI, *Hind*III+*Eco*RI and *Nde*I+*Eco*RI, respectively.
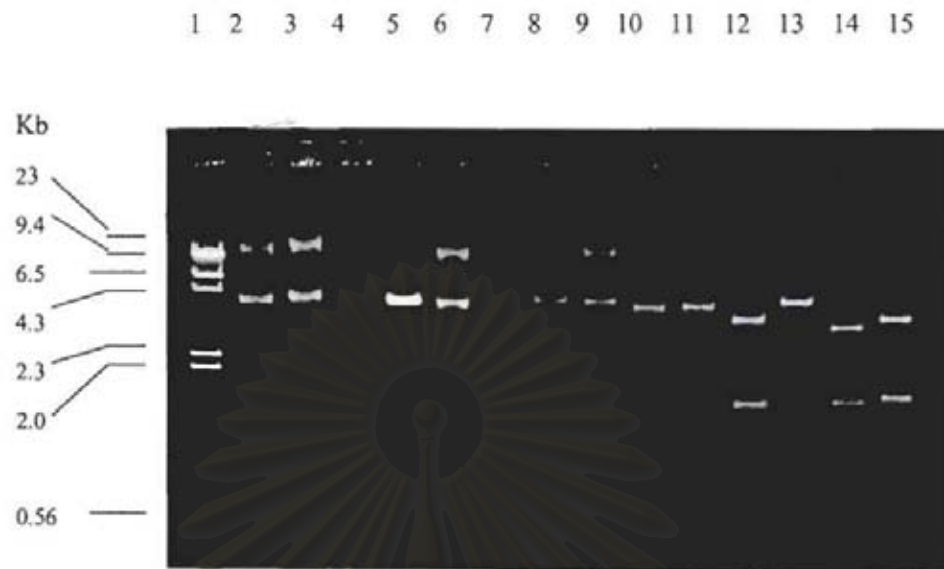
Figure 29 : Restriction pattern of pVR392. Lane 1 : λ/HindIII marker, lane 2 : undigested, lanes 3-15 : digested with EcoRV, MscI, NdeI, SphI, NcoI, BsrGI, BglII, BstEII, SacII, HindIII, EcoRI, HindIII+EcoRI and NdeI+EcoRI, respectively.

Figure 30 : Restriction pattern of pVR394. Lane 1 : λ/HindIII marker, lane 2 : undigested, lanes 3-15 : digested with EcoRV, MscI, NdeI, SphI, NcoI, BsrGI, BglII, BstEII, SacII, HindIII, EcoRI, HindIII+EcoRI and NdeI+EcoRI, respectively.
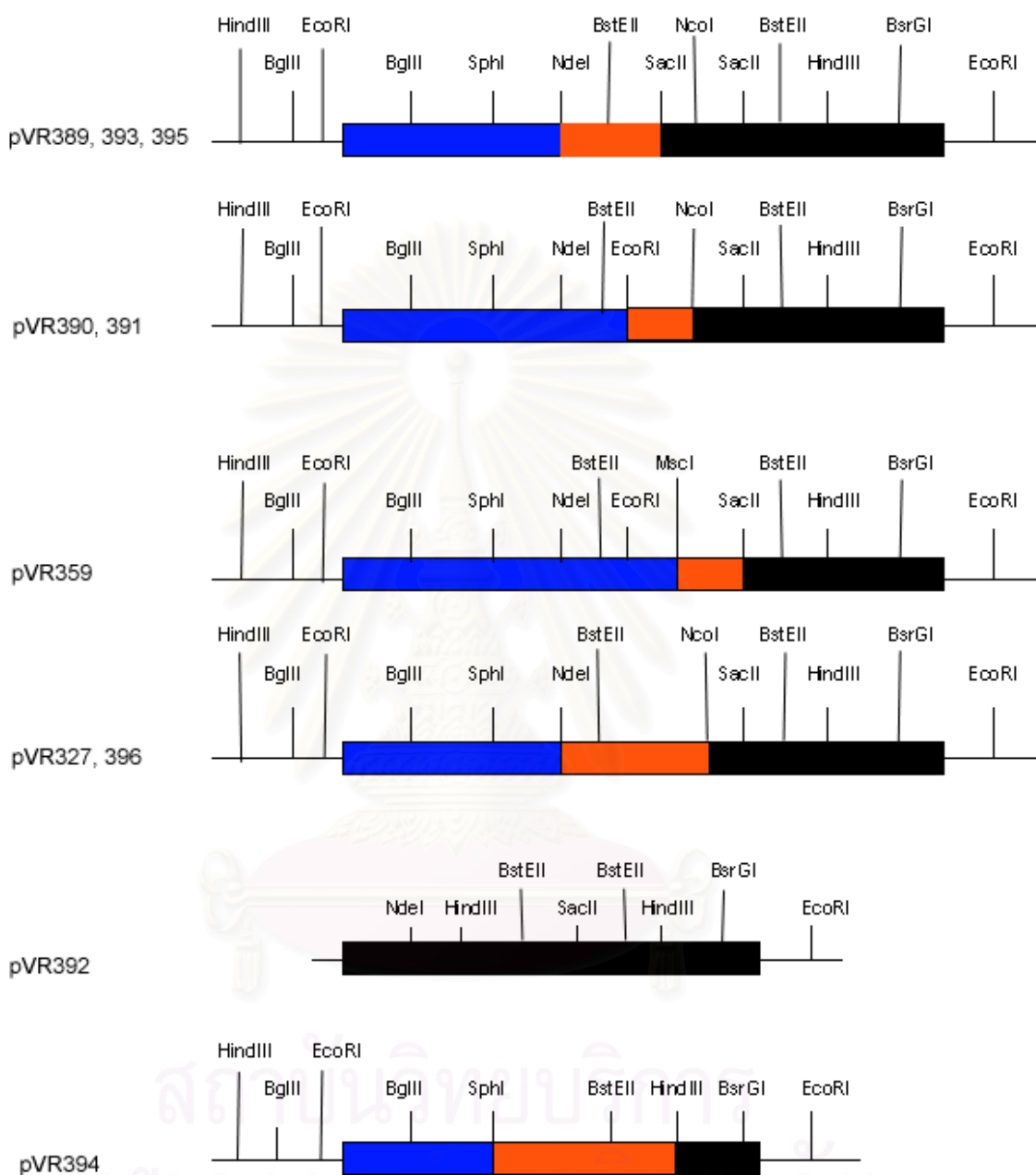
Figure 31 : Restriction maps of chimeric CGTases from pVR321 series. The blue box indicates β-CGTase gene, the black box indicates α-CGTase gene, and the red box indicates the location of recombination site.

Figure 32 : Restriction pattern of pVR397. Lane 1 : λ/*Hin*dIII marker, lane 2 : undigested, lanes 3-15 : digested with *Nco*I, *Bsr*GI, *Nde*I, *Msc*I, *Sac*II, *Hin*dIII, *Sph*I, *Bst*EII, *Eco*RV, *Bam*HI, *Eco*RI, *Eco*RI+*Hin*dIII and *Eco*RI+*Nde*I, respectively.

Figure 33 : Restriction pattern of pVR398. Lane 1 : λ/*Hin*dIII marker, lane 2 : undigested, lanes 3-15 : digested with *Nco*I, *Bsr*GI, *Nde*I, *Msc*I, *Sac*II, *Hin*dIII, *Sph*I, *Bst*EII, *Eco*RV, *Bam*HI, *Eco*RI, *Eco*RI+*Hin*dIII and *Eco*RI+*Nde*I, respectively.

Figure 34 : Restriction pattern of pVR399 as well as pVR400. Lane 1 : λ/HindIII marker. lane 2 : undigested, lanes 3-15 : digested with NcoI, BsrGI, NdeI, MscI, SacII, HindIII. SphI, BstEII, EcoRV, BamHI, EcoRI, EcoRI+HindIII and EcoRI+NdeI, respectively.
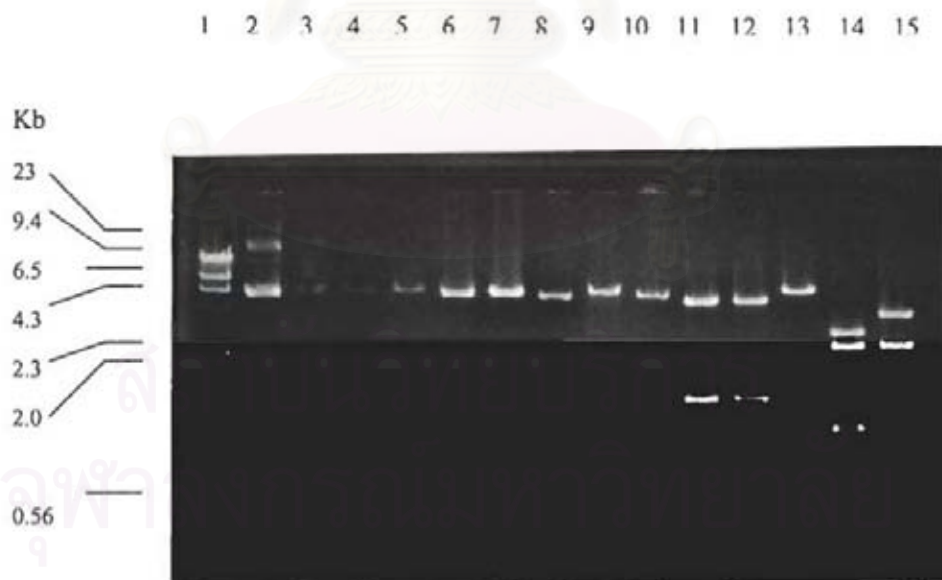
Figure 35 : Restriction pattern of pVR401. Lane 1 : λ/HindIII marker, lane 2 : undigested, lanes 3-15 : digested with NcoI, BsrGI, NdeI, MscI, SacII, HindIII, SphI, BstEII, EcoRV, BamHI, EcoRI, EcoRI+HindIII and EcoRI+NdeI, respectively.

Figure 36 : Restriction pattern of pVR402 as well as pVR403. Lane 1 : λ/*Hin*dIII marker, lane 2 : undigested, lanes 3-15 : digested with *Nco*I, *Bsr*GI, *Nde*I, *Msc*I, *Sac*II, *Hin*dIII, *Sph*I, *Bst*EII, *Eco*RV, *Bam*HI, *Eco*RI, *Eco*RI+*Hin*dIII and *Eco*RI+*Nde*I, respectively.
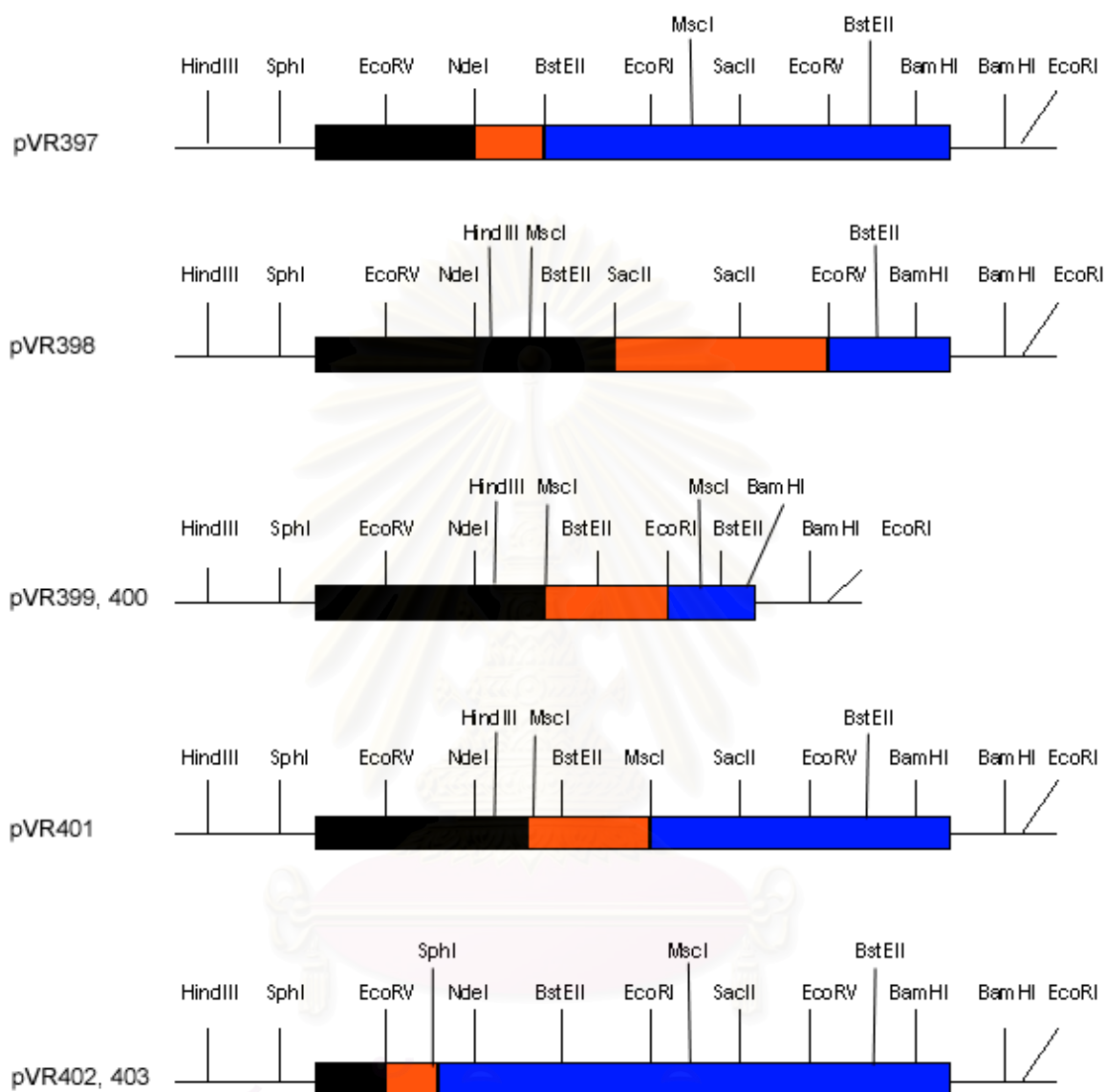
Figure 37 : Restriction maps of chimeric CGTases from pVR388 series. The black box indicates α-CGTase gene, the blue box indicates β-CGTase gene and The red box indicates the location of recombination site.

4. **The chimeric CGTase activities and cyclodextrin ratios**

E. coli JM109 containing the chimeric CGTase was tested for starch hydrolysis activity (dextrinizing) on LB-starch agar and cyclodextrin forming activity. Only six chimeras, pVR392, 394, 398, 399, 400 and 401, were tested negative, the others, pVR327, 359, 389, 390, 391, 393, 395, 396, 397, 401 and 403 were positive as shown in Fig. 38 and Table 3.

The cyclodextrin production was determined by using HPLC and assays for the cyclodextrin forming activity. All positive dextrinization chimeras from pVR321 and pVR388 series show the β-cyclodextrin forming activity higher than other cyclodextrin forming activity (Table 3). The cyclodextrin ratio was calculated from the peak areas of each cyclodextrin in HPLC profile. The results were shown in Fig. 39 and Table 3 for chimeric CGTases from pVR321 series and pVR388 series. The chimeric CGTases from pVR321 and pVR388 series can be divided into 2 groups, active and inactive for cyclodextrin production. The cyclodextrin producing group produced β-cyclodextrin as a major product for chimeric CGTases from pVR321 and 388 series, corresponding to their cyclodextrin forming activity assays (Table 3). The pVR327, 359, 390 and 391 had increased β- and γ-cyclodextrin production, while α-cyclodextrin production were decreased significantly. The pVR389 and 395 produced slightly lower β-cyclodextrin while produced slightly higher γ-cyclodextrin. The α-cyclodextrin production was not effected.

The pVR393 had increased γ-cyclodextrin production significantly while α-cyclodextrin production was not effected and β-cyclodextrin production was decreased. The pVR396, pVR402 and pVR403 produced the same proportion of β-cyclodextrin as the wild type β-CGTase (pVR316) although pVR402 and 403 derived their N-terminal half of A1 subdomain from α-CGTase (pVR300). The α-cyclodextrin production was slightly decreased while γ-cyclodextrin was slightly increased. Fig. 46 summarizes the characteristics and activities for all chimeric CGTases.

5. **DNA sequence determination of the recombination sites**

The precised recombination sites were determined by DNA sequencing. The DNA fragment, determined to contain the recombination site, was subcloned into a pUC119. The pVR327 and pVR359 were determined previously by Rimphanitchayakit, V. (unpublished), and other chimeras from pVR321 series were double-digested by *Hin*dIII and *Sph*I, and then the 1.1 kb DNA fragment were ligated into a pUC119 at the corresponding sites. The chimeras from pVR388 series were double-digested with

*Eco*RV and *Eco*RI and then the 1.2 kb DNA fragment were ligated into *Sma*I-*Eco*RI site of a pUC119. The results were shown in Fig. 40 and 41.

Fig. 42 summarized the recombination sites of all chimeric CGTases. pVR389 and 395 had the same recombination site in subdomain A2; the recombined sequence was GGTGACCTTCATCGACAA. The pVR393 and 396 had the recombination sites in subdomain A2 with the recombined sequences CTGACTTC and TACGGCA, respectively. The recombination site of pVR327 was located in domain C with the recombined sequence TGAACGCAAATTCGGCA. pVR390 and 391 had the recombination site within the same region in domain C with the recombined sequence GTATGGCAGTACACA. The pVR359 recombination site was found in domain D and the recombined sequence was ACGATTGACGGCCGCGGCTT. pVR397 had the recombination site in subdomain A2 around the *Nde*I restriction site and the recombined sequence was AAGCATATGCC. The pVR402 and 403 had the recombination site in the same region of subdomain A1 and the recombined sequence was CTTATCACGG.

6. **Determination of an essential part in CGTase involved in cyclodextrin specificity**

Because pVR402 gave a major product as β-cyclodextrin instead α-cyclodextrin while the N-terminal of the enzyme was α-CGTase, it was obvious that the N-terminal sequence had no influence on major cyclodextrin production. To determine the region of enzyme that had the influence on major cylodextrin production, the pVR404 was constructed by shuffling the *Nde*I-*Sma*I fragment of pVR402 with the corresponding fragment from pVR300 (α-CGTase). The resulting plasmid had subdomain A2, domains C, D and E of the β-CGTase replaced by subdomain A2, domains C, D and E from the α-CGTase. pVR405 was constructed by shuffling the *Nde*I-*Eco*47III fragment of pVR402 with the corresponding fragment from pVR300 ; the subdomain A2 of the β-CGTase replaced by subdomain A2 from the α-CGTase. The pVR406 was constructed by shuffling the *Eco*47III-*Bam*HI fragment of pVR402 with the corresponding fragment from pVR300 ; the domains C, D and E of the β-CGTase replaced by domains C, D and E from the α-CGTase .The constructed pVR404, 405 and 406 were analyzed as shown in Fig. 43.

The pVR404 was active in dextrinizing activity and cyclodextrin forming while pVR405 had weak dextrinizing activity and inactive in cyclodextrin production, and pVR406 had neither dextrinizing activity nor cyclodextrin production (Fig. 44 and 45). The HPLC analysis of cyclodextrin ratios from

pVR404 revealed that the major product was α-cyclodextrin although the proportion of β-cyclodextrin was increased as compared to wild type α-CGTase.

Table 3 : Summary of chimeric CGTases activities.

pVR	Dextrinizing activity	Cyclodextrin forming activity (U/ml crude)			Cyclodextrin ratios*[4]
		α-CD*[1]	β-CD*[2]	γ-CD*[3]	
316	+	275	1450	0.014	28 : 63 : 9
300	+	465	125	ND	73 : 23 : 4
327	+	100	1500	0.193	5 : 76 : 19
359	+	55	1560	0.07	11 : 72 : 17
389	+	375	1375	0.1	29 : 57 : 14
390	+	65	1475	0.038	8 : 75 : 17
391	+	80	1460	0.027	5 : 82 : 13
393	+	275	825	0.096	29 : 45 : 26
395	+	250	1150	0.1	32 : 58 : 10
396	+	140	1300	0.17	16 : 67 : 17
397	+(weak)	ND	ND	ND	No cyclodextrin production
402	+	375	1415	0.07	21 : 63 : 16
403	+	360	1400	0.096	19 : 69 : 12
392, 394, 398, 399, 400 and 401	-	ND	ND	ND	No cyclodextrin production

*[1] Methyl orange assay, *[2] Phenolpthalein assay, *[3] Bromocresol green assay, *[4] average of 2 separate determinations

ND = Can not determined
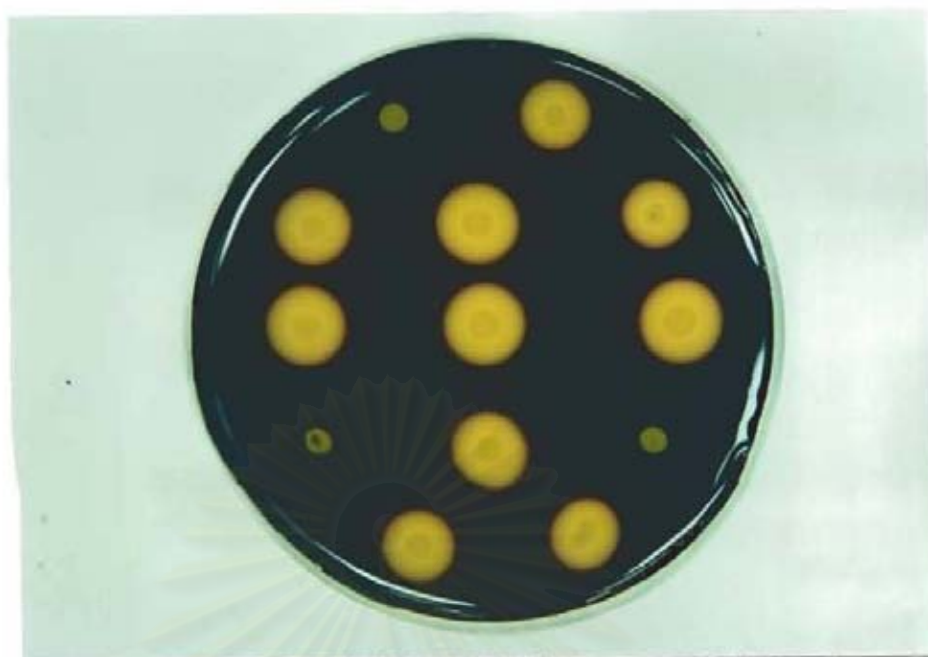
pVR321 series
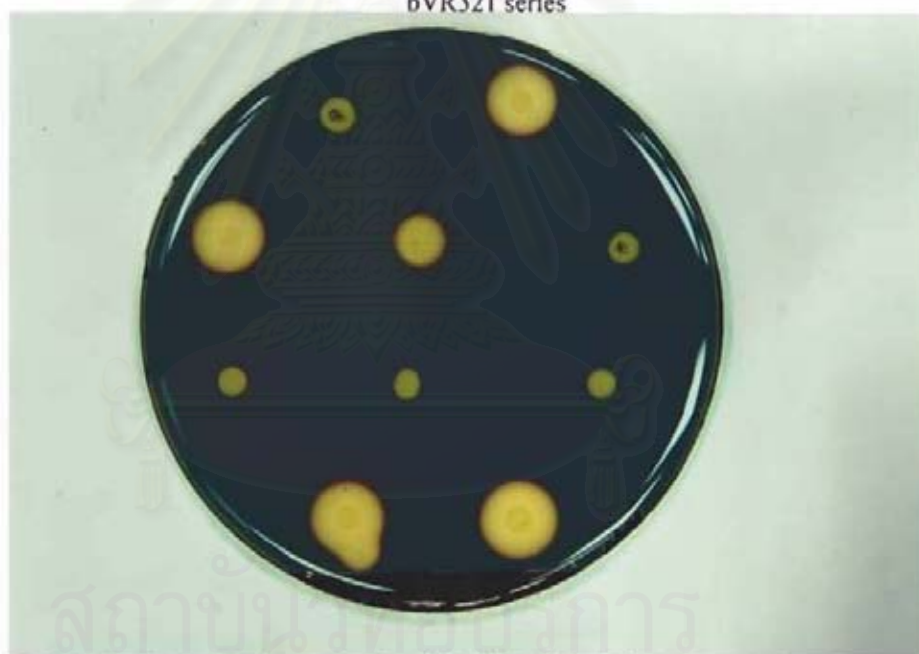
pVR388 series

Figure 38 : Iodine test for dextrinizing activity of chimeric CGTases from pVR321 series and pVR388 series. Clear zone surrounding the colonies indicates starch hydrolysis activity. Top picture were pVR321 series (left to right) ; row 1 : pUC119 and **pVR300 ; row 2** : pVR316, 327 and 359 ; row 3 : pVR389, 390 and 391 ; row 4 : pVR392, 393 and 394 ; row 5 : pVR395 and 396. Bottom picture were pVR388 series (left to right) ; row 1 : pUC119 and pVR300 ; row 2 : pVR316, 397 and 398 ; row 3 : pVR399, 400 and 401 ; row 4 : pVR402 and 403.

α β γ

Standard α-,β- and γ-cyclodextrins

pVR300

pVR316

pVR327

pVR359

pVR389
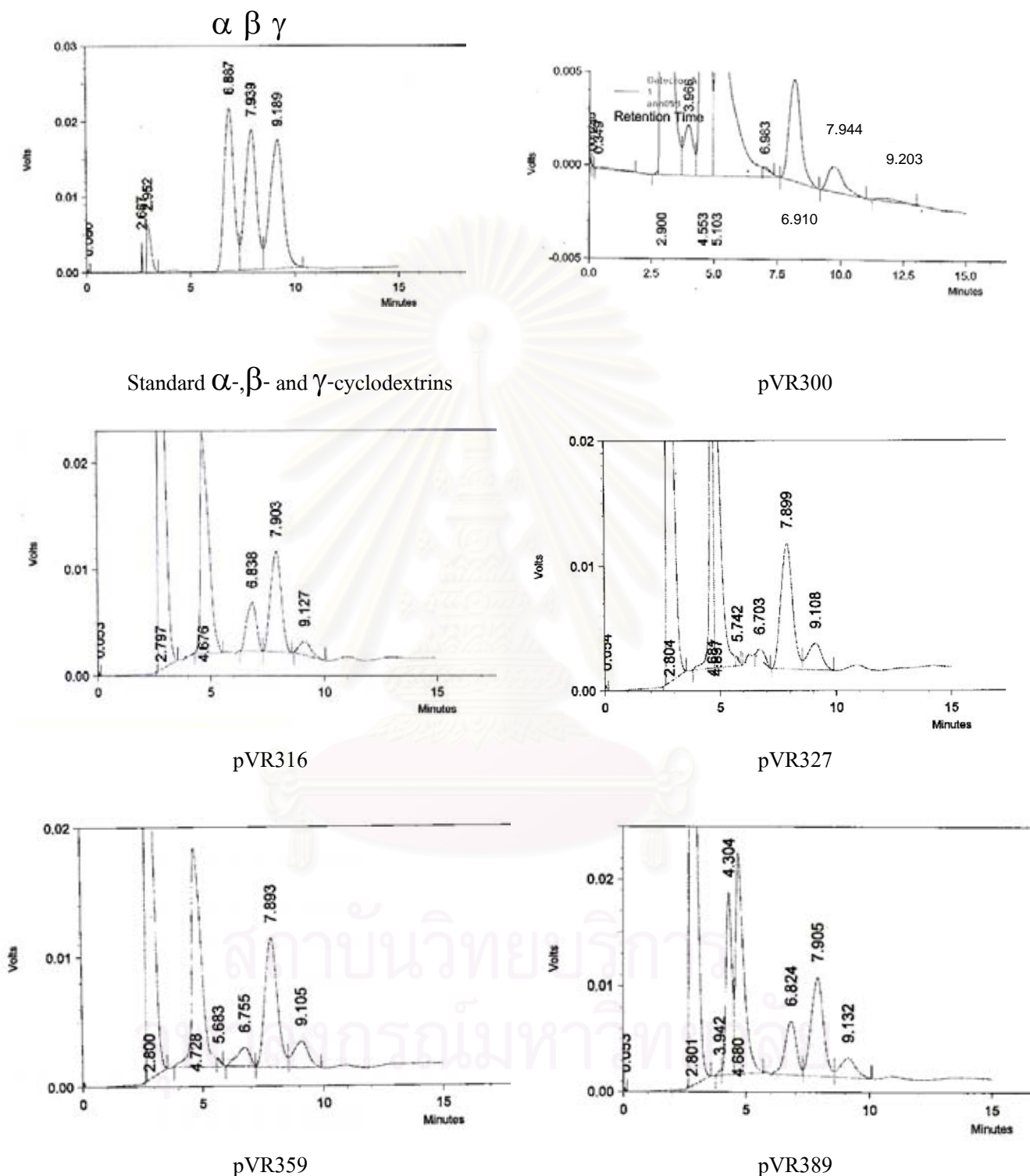
Figure 39 : HPLC profiles of cyclodextrins formed by the chimeric CGTases

pVR390

pVR391

pVR392,394,397,398,399,400 and 401

pVR393

pVR395

pVR396
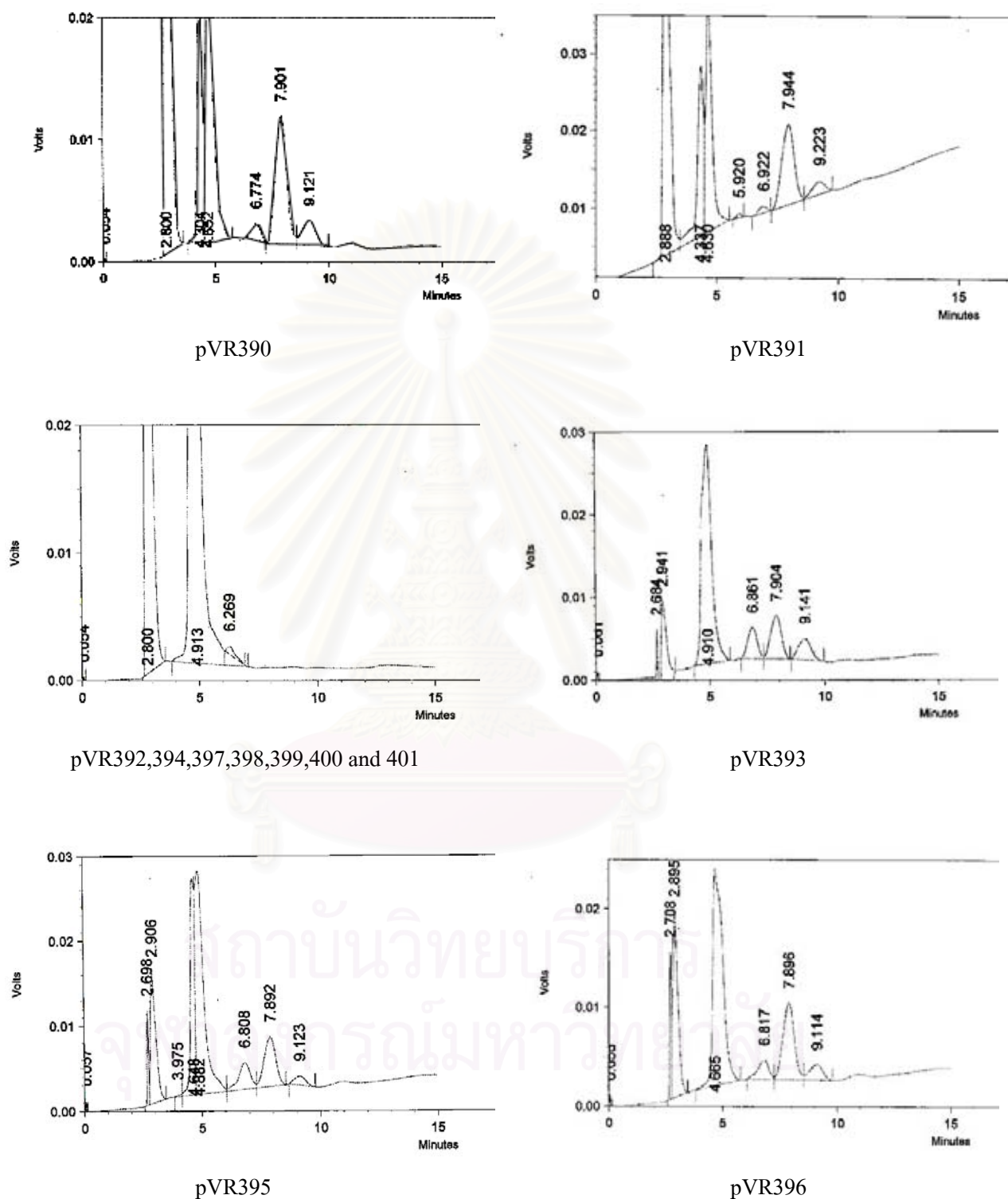
Figure 39 (continue) : HPLC profiles of cyclodextrins formed by chimeric CGTases
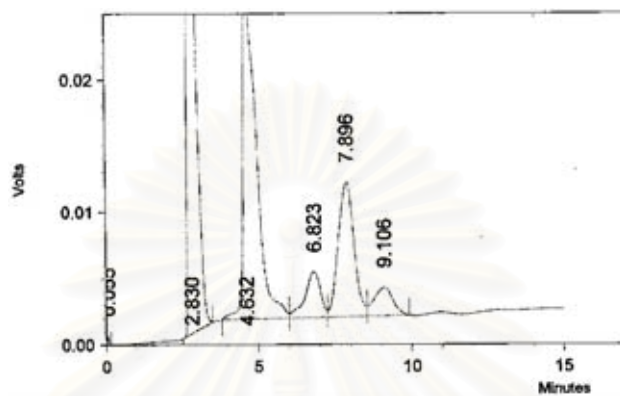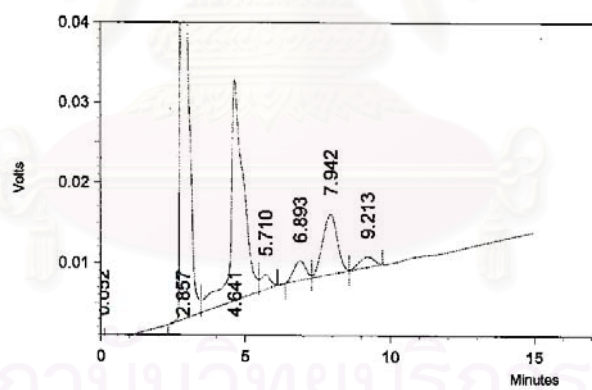
pVR402

pVR403

Figure 39 (continue) : HPLC profiles of cyclodextrins formed by chimeric CGTases

Figure 40 : Restriction analysis of subcloned chimeric pVR321 series for DNA sequencing. Lane 1 : λ/HindIII marker ; Lane 2 : undigested pUC119 ; Lane 3 : pUC119 digested with HindIII+SphI ; Lanes 4, 6, 8, 10, 12 and 14 were pVR389, 390, 391, 393, 395 and 396 digested with HindIII+SphI ; Lanes 5, 7, 9, 11, 13 and 15 were pVR389, 390, 391, 393, 395 and 396 digested with NdeI. The bands corresponding to 1.1 kb fragment are insertion fragments.

Figure 41 : Restriction analysis of subcloned chimeric pVR388 series for DNA sequencing. Lane 1 : λ/HindIII ; Lane 2 : undigested pUC119 ; Lane 3 : pUC119 digested with BamHI+EcoRI ; Lanes 4, 6 and 8 were pVR397, 402 and 403 digested with BamHI+EcoRI ; Lanes 5, 7 and 9 were pVR397, 402 and 403 digested with NdeI. The bands corresponding 1.2 kb fragment are insertion fragments.

```
                                     M  K  R  F  M  K  L  T  A  V  W  T  L  W  L
β-CGTase  ATGAGGAGGTATAGTATGAAAAGATTTATGAAACTAACAGCCGTATGGACACTCTGGTTA 60
                                 ******          ****        **      * * ** * * *
α-CGTase  --------------ATGAAATCGCGGTACAAACGTTTGACCTCCCTGGCGCTTTCGCTG 45
                                     M  K  S  R  Y  K  R  L  T  S  L  A  L  S  L

          S  L  T  L  G  L  L  S  P  V  H  A  P  D  T  S  V  S  N
β-CGTase  TCCCTCACGCTGGGCCTCTTGAGCCCGGTCCACGCAGCCCCGGATACCTCGGTATCCAAC 120
              *  ** **** * *       ** *     *** * ** *****     **    ****
α-CGTase  AGTATGGCGTTGGGGATTTCACTGCCCGCATGGGCATCACCCGATACGAGCGTGGACAAC 105
          S  M  A  L  G  I  S  L  P  A  W  A  S  P  D  T  S  V  D  N
                                                   BglII
          K  Q  N  F  S  T  D  V  I  Y  Q  I  F  T  D  R  F  S  D  G
β-CGTase  AAGCAGAATTTCAGCACGGATGTCATATATCAGATCTTCACCGACCGGTTCTCGGACGGC 180
          ***    ******** ***** ***** ******** * ******** *** *******
α-CGTase  AAGGTCAATTTCAGTACGGACGTCATCTATCAGATTGTGACCGACCGCTTCGCGGACGGG 165
          K  V  N  F  S  T  D  V  I  Y  Q  I  V  T  D  R  F  A  D  G

          N  P  A  N  N  P  T  G  A  A  F  D  G  S  C  T  N  L  R  L
β-CGTase  AATCCGGCCAACAATCCGACCGGCGCGGCATTTGACGGATCATGTACGAATCTTCGCTTA 240
          *    * * ********* * ** *  ** **    ***      * * *** *      *
α-CGTase  GACAGGACGAACAATCCGGCGGGGGATGCGTTCAGCGGCGACCGATCCAATTTGAAGCTC 225
          D  R  T  N  N  P  A  G  D  A  F  S  G  D  R  S  N  L  K  L

          Y  C  G  G  D  W  Q  G  I  I  N  K  I  N  D  G  Y  L  T  G
β-CGTase  TACTGCGGCGGCGACTGGCAAGGCATCATCAACAAAATCAACGACGGTTATTTGACCGGC 300
          ** * *** ** ******* ** ** *** **** ** ******************
α-CGTase  TATTTCGGGGGAGACTGGCAGGGGATTATCGACAAGATTAACGACGGTTATTTGACCGGC 285
          Y  F  G  G  D  W  Q  G  I  I  D  K  I  N  D  G  Y  L  T  G

          M  G  I  T  A  I  W  I  S  Q  P  V  E  N  I  Y  S  V  I  N
β-CGTase  ATGGGCATTACGGCCATCTGGATTTCACAGCCTGTCGAGAATATCTACAGCGTGATCAAC 360
          ****** * ** *** ******* ** ******* ** ****** *   *** *****
α-CGTase  ATGGGCGTCACCGCCCTCTGGATATCCCAGCCTGTGGAAAATATCACCTCCGTCATCAAG 345
          M  G  V  T  A  L  W  I  S  Q  P  V  E  N  I  T  S  V  I  K
                                EcoRV
          Y  S  G  V  H  N  T  A  Y  H  G  Y  W  A  R  D  F  K  K  T
β-CGTase  TACTCCGGCGTCCATAATACGGCTTATCACGGCTACTGGGCGCGGGACTTCAAGAAGACC 420
          ** ********   * ****** ********** ********* **** ** *** * ***
α-CGTase  TATTCCGGCGTTAACAATACGTCTTATCACGGTTACTGGGCGAGGGATTTTAAGCAAACC 405
          Y  S  G  V  N  N  T  S  Y  H  G  Y  W  A  R  D  F  K  Q  T
                                recombination site of pVR402 & 403
                                                            SphI
          N  P  A  Y  G  T  M  Q  D  F  K  N  L  I  D  T  A  H  A  H
β-CGTase  AATCCGGCCTACGGAACGATGCAGGACTTCAAAAACCTGATCGACACCGCGCATGCGCAT 480
          **    ** * ***      *   ** ** **** ***** ** ** ** ** ** ***
α-CGTase  AACGACGCTTTCGGGGATTTTGCCGATTTTCAAAATCTGATTGATACGGCTCACGCTCAT 465
          N  D  A  F  G  D  F  A  D  F  Q  N  L  I  D  T  A  H  A  H
```

Figure 42 : DNA sequence alignment between pVR316 (β-CGTase) and pVR300 (α-CGTase). The red alphabets indicate the recombination site of each chimeric CGTase from pVR321 series (top) and pVR388 series (bottom) that active on dextrinizing and cyclodextrin forming actvity, and the violet alphabets indicate the signal peptides.

```
                            Subdomain A1  ◄────►  Domain B
              N  I  K  V  I  I  D  F  A  P  N  H  T  S  P  A  S  S  D  D
β-CGTase  AACATAAAAGTCATCATCGACTTTGCACCGAACCATACATCTCCGGCTTCTTCGGATGAT  540
          *****  **  ***  *  ********  **  **  *****  **  ********       ***    *
α-CGTase  AACATCAAGGTCGTGATCGACTTCGCCCCCAACCACACGTCTCCGGCCGACAGGGACAAC  525
              N  I  K  V  V  I  D  F  A  P  N  H  T  S  P  A  D  R  D  N

              P  S  F  A  E  N  G  R  L  Y  D  N  G  N  L  L  G  G  Y  T
β-CGTase  CCTTCCTTTGCAGAGAACGGCCGCTTGTACGATAACGGCAACCTGCTCGGCGGATACACC  600
          **    ***  **  ********       ****  ********       **********    ****  *
α-CGTase  CCCGGCTTCGCCGAGAACGGTGCGCTGTATGATAACGGTTCGCTGCTCGGCGCCTACAGC  585
              P  G  F  A  E  N  G  A  L  Y  D  N  G  S  L  L  G  A  Y  S

              N  D  T  Q  N  L  F  H  H  Y  G  G  T  D  F  S  T  I  E  N
β-CGTase  AACGATACCCAAAATCTGTTCCACCATTATGGCGGCACGGATTTCTCCACCATTGAGAAC  660
          **  *****       **  *****  ***  *  **  **  **  *****  *****  *****    **
α-CGTase  AATGATACGGCCGGCCTTTTCCATCATAACGGGGGGACCGATTTTTCCACGATTGAAGAC  645
              N  D  T  A  G  L  F  H  H  N  G  G  T  D  F  S  T  I  E  D
                                            Domain B  ◄────►  Subdomain A2
              G  I  Y  K  N  L  Y  D  L  A  D  L  N  H  N  N  S  S  V  D
β-CGTase  GGCATTTATAAAAACCTGTACGATCTGGCTGACCTGAATCATAACAACAGCAGCGTCGAT  720
          **  *****  **  *****  *****  *****  ***  *  **  **********  *      *  **
α-CGTase  GGTATTTACAAGAACCTCTACGACCTGGCGGACATCAACCATAACAACAACGCTATGGAC  705
              G  I  Y  K  N  L  Y  D  L  A  D  I  N  H  N  N  N  A  M  D

              V  Y  L  K  D  A  I  K  M  W  L  D  L  G  V  D  G  I  R  V
β-CGTase  GTGTATCTGAAGGATGCCATCAAAATGTGGCTCGACCTCGGGGTTGACGGCATTCGCGTG  780
          *    ***  *  **       **  ***  *    *  *******  *  *  **  **  *****  *****   *
α-CGTase  GCTTATTTTAAAAGCGCTATCGACCTTTGGCTCGGCATGGGTGTGGACGGGATTCGTTTT  765
              A  Y  F  K  S  A  I  D  L  W  L  G  M  G  V  D  G  I  R  F
                      Eco47III
                      NdeI
              D  A  V  K  H  M  P  F  G  W  Q  K  S  F  M  S  T  I  N  N
β-CGTase  GACGCGGTCAAGCATATGCCATTCGGCTGGCAGAAGAGCTTTATGTCCACCATTAACAAC  840
          ********  ***********  ***********  **  *****    *  ***  *  ***  **    *
α-CGTase  GACGCGGTGAAGCATATGCCTTTCGGCTGGCAAAAAAAGCTTCGTTTCCTCGATTTACGGC  825
              D  A  V  K  H  M  P  F  G  W  Q  K  S  F  V  S  S  I  Y  G
              Recombination site of pVR397           HindIII

              Y  K     P  V  F  T  F  G  E  W  F  L  G  V  N  E  I  S  P
β-CGTase  TACAAGC---CGGTCTTCACCTTCGGCGAATGGTTCCTTGGCGTCAATGAGATTAGTCCG  897
          *  *  *      ****  **  **  *****  *******       *******    **  *  *
α-CGTase  GGCGATCATCCGGTATTTACGTTCGGGGAATGGTATCTTGGCGCGGATCAAACCGACGGA  885
              G  D  H  P  V  F  T  F  G  E  W  Y  L  G  A  D  Q  T  D  G

              E  Y  H  Q  F  A  N  E  S  G  M  S  L  L  D  F  R  F  A  Q
β-CGTase  GAATACCATCAATTCGCTAACGAGTCCGGGATGAGCCTGCTCGATTTCCGCTTTGCCCAG  957
          **    **    *  *******  *****       ********  ******  **  **      *    **  ***
α-CGTase  GACAACATTAAATTCGCCAACGAAAGCGGGATGAACCTGCTGGACTTTGAATACGCGCAG  945
              D  N  I  K  F  A  N  E  S  G  M  N  L  L  D  F  E  Y  A  Q
```

Figure 42 (continue).

```
                K   A   R   Q   V   F   R   D   N   T   D   N   M   Y   G   L   K   A   M   L
β-CGTase   AAGGCCCGGCAAGTGTTCAGGGACAACACCGACAATATGTACGGCCTGAAAGCGATGCTG 1017
           *  *    **  ******** ******* **  **  *   *** *  *   **    *  *  * *****
α-CGTase   GAAGTGCGCGAAGTGTTCCGGGACAAAACGGAAACGATGAAGGATCTCTATGAGGTGCTG 1005
                E   V   R   E   V   F   R   D   K   T   E   T   M   K   D   L   Y   E   V   L
```

```
                                                    Recombination site of pVR389 & 395
                E   G   S   E   V   D   Y   A   Q   V   N   D   Q   V   T   F   I   D   N   H
β-CGTase   GAGGGCTCTGAAGTAGACTATGCCCAGGTGAATGACCAGGTGACCTTCATCGACAATCAT 1077
            *    ** *  **      *  **  *  *  * **   *  ***************** ***
α-CGTase   GCCAGCACGGAGTCGCAATACGACTACATCAACAATATGGTGACCTTCATCGACAACCAT 1065
                A   S   T   E   S   Q   Y   D   Y   I   N   N   M   V   T   F   I   D   N   H
                MscI                                            BstEII
```

```
                D   M   E   R   F   H   T   S   N   G   D   R   R   K   L   E   Q   A   L   A
β-CGTase   GACATGGAGCGTTTCCACACCAGCAATGGCGACAGACGGAAGCTGGAGCAGGCGCTGGCC 1137
           ** ***** ** *****       *   *   **   *   ***   *   ***** *** ****
α-CGTase   GATATGGACCGGTTCCAGGTTGCCGGTTCCGGTACGCGGGCGACCGAGCAAGCGTTGGCG 1125
                D   M   D   R   F   Q   V   A   G   S   G   T   R   A   T   E   Q   A   L   A
```

```
             Recombination site of pVR393              recombination site of pVR396
                F   T   L   T   S   R   G   V   P   A   I   Y   Y   G   S   E   Q   Y   M   S
β-CGTase   TTTACCCTGACTTCACGCGGTGTGCCTGCCATCTATTACGGCAGCGAGCAGTATATGTCT 1197
            *  ** ******** ***** ***** ******* *******  ******** *** *
α-CGTase   CTGACGCTGACTTCCCGCGGCGTGCCAGCCATCTACTACGGCACGGAGCAGTACATGACC 1185
                L   T   L   T   S   R   G   V   P   A   I   Y   Y   G   T   E   Q   Y   M   T
                        SacII
```

```
                G   G   N   D   P   D   N   R   A   R   I   P   S   F   S   T   T   T   T   A
β-CGTase   GGCGGGAATGATCCGGACAACCGTGCTCGGATTCCTTCCTTCTCCACGACGACGACCGCA 1257
           ****     ** **  ******* **   *** * ** **    **   ****** **
α-CGTase   GGCGATGGCGACCCCAACAACCGGGCGATGATGACCTCGTTTAATACCGGGACGACGGCT 1245
                G   D   G   D   P   N   N   R   A   M   M   T   S   F   N   T   G   T   T   A
```

```
                                                    Domain A  ◄───►  Domain C
                Y   Q   V   I   Q   K   L   A   P   L   R   K   S   N   P   A   I   A   Y   G
β-CGTase   TATCAAGTCATCCAAAAGCTCGCTCCGCTCCGCAAATCCAACCCGGCCATCGCTTACGGT 1317
           *** **** ** **    * ** ***** ** ******** *************** **
α-CGTase   TATAAAGTGATTCAGGCATTGGCGCCGCTGCGTAAATCCAATCCGGCCATCGCTTATGGG 1305
                Y   K   V   I   Q   A   L   A   P   L   R   K   S   N   P   A   I   A   Y   G
```

```
                Eco47III              Recombination site of pVR327
                S   T   Q   E   R   W   I   N   N   D   V   I   I   Y   E   R   K   F   G   N
β-CGTase   TCCACACAGGAGCGCTGGATCAACAACGATGTGATCATCTATGAACGCAAATTCGGCAAT 1377
            *  **    ******** * *********** *  **   ****************
α-CGTase   ACGACGACAGAGCGCTGGGTTAACAACGATGTGTTGATTATTGAACGCAAATTCGGCAGC 1365
                T   T   T   E   R   W   V   N   N   D   V   L   I   I   E   R   K   F   G   S
```

```
                N   V   A   V   V   A   I   N   R   N   M   N   T   P   A   S   I   T   G   L
β-CGTase   AACGTGGCCGTTGTTGCCATTAACCGCAATATGAACACACCGGCTTCGATTACCGGCCTT 1437
            * **  **  *  ** **  ***** ** **   *   *  *   * ***** * ** **
α-CGTase   AGCGCCGCTTTGGTGGCGATTAATCGAAACTCGTCCGCCGCTTATCCGATTTCGGGTCTG 1425
                S   A   A   L   V   A   I   N   R   N   S   S   A   A   Y   P   I   S   G   L
```

Figure 42 (continue).

```
                                                                EcoRI
            V  T  S  L  P  Q  G  S  Y  N  D  V  L  G  G  I  L  N  G  N
β-CGTase GTCACTTCCCTCCCGCAGGGCAGCTATAACGATGTGCTCGGCGGAATTCTGAACGGCAAT 1497
         * * *** ** ***  ***** ***   ***** *  **** *  * *******
α-CGTase TTGAGTTCGCTGCCGGCGGGCACTTATTCGGATGTATTGAACGGACTCTTAAACGGCAAC 1485
            L  S  S  L  P  A  G  T  Y  S  D  V  L  N  G  L  L  N  G  N


            T  L  T  V  G  A  G  G  A  A  S  N  F  T  L  A  P  G  G  T
β-CGTase ACGCTAACCGTGGGTGCTGGCGGTGCAGCTTCCAACTTTACTTTGGCTCCTGGCGGCACT 1557
          *   * ********    ***** ** *  ********** ****  * ********
α-CGTase TCCATTACCGTGGGCAGCGGCGGCGCCGTCACCAACTTTACGCTGGCGGCCGGCGGCACG 1545
            S  I  T  V  G  S  G  G  A  V  T  N  F  T  L  A  A  G  G  T


                 Domain C ◄►  Domain D
               Recombination site of pVR390 & 391
            A  V  W  Q  Y  T  T  D  A  T  A  P  I  I  G  N  V  G  P  M
β-CGTase GCTGTATGGCAGTACACAACCGATGCCACAGCTCCGATCATCGGCAATGTCGGCCCGATG 1617
         ** ************** *   *  ** * ***  ********** ** ** *
α-CGTase GCGGTATGGCAGTACACAGCGCCGGAAACGTCGCCGGCGATCGGCAATGTGGGTCCCACC 1605
            A  V  W  Q  Y  T  A  P  E  T  S  P  A  I  G  N  V  G  P  T
                             Recombination site of pVR359      NcoI
            MscI                                     SacII
            M  A  K  P  G  V  T  I  T  I  D  G  R  G  F  G  S  G  K  G
β-CGTase ATGGCCAAGCCAGGGGTCACGATTACGATTGACGGCCGCGGCTTCGGCTCCGGCAAGGGA 1677
         **** * **** ***    *    * ******************* ***   *    ***
α-CGTase ATGGGCCAGCCGGGGAATATAGTGACGATTGACGGCCGCGGCTTTGGCGGCACGGCGGGC 1665
            M  G  Q  P  G  N  I  V  T  I  D  G  R  G  F  G  G  T  A  G


            T  V  Y  F  G  T  T  A  V  T  G  A  D  I  V  A  W  E  D  T
β-CGTase ACGGTTTACTTCGGTACAACGGCAGTCACTGGCGCGGACATCGTAGCTTGGGAAGATACA 1737
         ******** ***** ** ***** ** ** *** * * *******   ***** ** **
α-CGTase ACGGTTTATTTCGGGACGACGGCGGTGACCGGCTCCGGCATCGTAAGCTGGGAGGACACG 1725
            T  V  Y  F  G  T  T  A  V  T  G  S  G  I  V  S  W  E  D  T
                             BstEII


                                                EcoRV
            Q  I  Q  V  K  I  P  A  V  P  G  G  I  Y  D  I  R  V  A  N
β-CGTase CAAATCCAGGTGAAAATCCCTGCGGTCCCTGGCGGCATCTATGATATCAGAGTTGCCAAC 1797
         ** ** *** *  ** **   **** * * ****     *   *    ** *    *
α-CGTase CAGATTAAGGCGGTCATACCGAAGGTCGCGGCGGGCAAAACGGGCGTATCGGTCAAAACG 1785
            Q  I  K  A  V  I  P  K  V  A  A  G  K  T  G  V  S  V  K  T


                                                  Domain D ◄►  Domain E
            A  A  G  A  A  S  N  I  Y  D  N  F  E  V  L  T  G  D  Q  V
β-CGTase GCAGCCGGAGCAGCCAGCAACATCTACGACAATTTCGAGGTGCTGACCGGAGACCAGGTC 1857
          * **** * ******** *  * * * *  *** * ** ***** ** ** ******
α-CGTase TCGTCCGGCACCGCCAGCAATACATTCAAAAGCTTCAATGTACTGACGGGGGATCAGGTC 1845
            S  S  G  T  A  S  N  T  F  K  S  F  N  V  L  T  G  D  Q  V
            BstEII                    HindIII
            T  V  R  F  V  I  N  N  A  T  T  A  L  G  Q  N  V  F  L  T
β-CGTase ACCGTTCGGTTCGTAATCAACAATGCCACAACGGCGCTGGGACAGAATGTGTTCCTCACG 1917
         ** ** ** *** * **** * **** **    *** ***** *  **
α-CGTase ACGGTGCGTTTCCTGGTCAATCAAGCCAATACCAATTACGGAACAAATGTTTATCTTGTC 1905
            T  V  R  F  L  V  N  Q  A  N  T  N  Y  G  T  N  V  Y  L  V
```

Figure 42 (continue).

```
                                        BamHI
          G  N  V  S  E  L  G  N  W  D  P  N  N  A  I  G  P  M  Y  N
β-CGTase GGCAATGTCAGCGAGCTGGGCAACTGGGATCCGAACAACGCGATCGGCCCGATGTATAAT 1977
         *****  *  *  ******  ***  ******  ********  *****  **  ********  ***
α-CGTase GGCAACGCCGCCGAGCTCGGCTCCTGGGACCCGAACAAAGCGATTGGGCCGATGTACAAT 1965
          G  N  A  A  E  L  G  S  W  D  P  N  K  A  I  G  P  M  Y  N
                                                            BsrGI
          Q  V  V  Y  Q  Y  P  T  W  Y  Y  D  V  S  V  P  A  G  Q  T
β-CGTase CAGGTCGTCTACCAATACCCGACTTGGTATTATGATGTCAGCGTTCCGGCAGGCCAAACG 2037
         *****  **  *  *  ******  *  ********  ***********  *****  **     **  *
α-CGTase CAGGTGATCGCCAAGTACCCGTCCTGGTATTACGATGTCAGCGTGCCGGCGGGGACAAAG 2025
          Q  V  I  A  K  Y  P  S  W  Y  Y  D  V  S  V  P  A  G  T  K

          I  E  F  K  F  L  K  K  Q  G  S  T  V  T  W  E  G  G  A  N
β-CGTase ATTGAATTTAAATTCCTGAAAAAGCAAGGCTCCACCGTCACATGGGAAGGCGGCGCGAAT 2097
         *  **  ********  *  ******    ***    **  **  **  **********  *    **
α-CGTase CTGGATTTTAAATTTATTAAAAAGGGCGGCGGTACGGTGACTTGGGAAGGCGGGGGCAAC 2085
          L  D  F  K  F  I  K  K  G  G  G  T  V  T  W  E  G  G  G  N

          R  T  F  T  T  P  T  S  G  T  A  T  M  N  V  N  W  Q  P
β-CGTase CGCACCTTCACCACCCCAACCAGCGGCACGGCAACGATGAATGTGAACTGGCAGCCTTAA 2157
         *    **  *  ***  **  **    ********    *    ***  ***    ***  *******      ****
α-CGTase CATACGTACACGACGCCGGCCAGCGGCGTAGGGACGGTGACGGTGGACTGGCAAAATTAA 2145
          H  T  Y  T  T  P  A  S  G  V  G  T  V  T  V  D  W  Q  N
```

Figure 42 (continue).
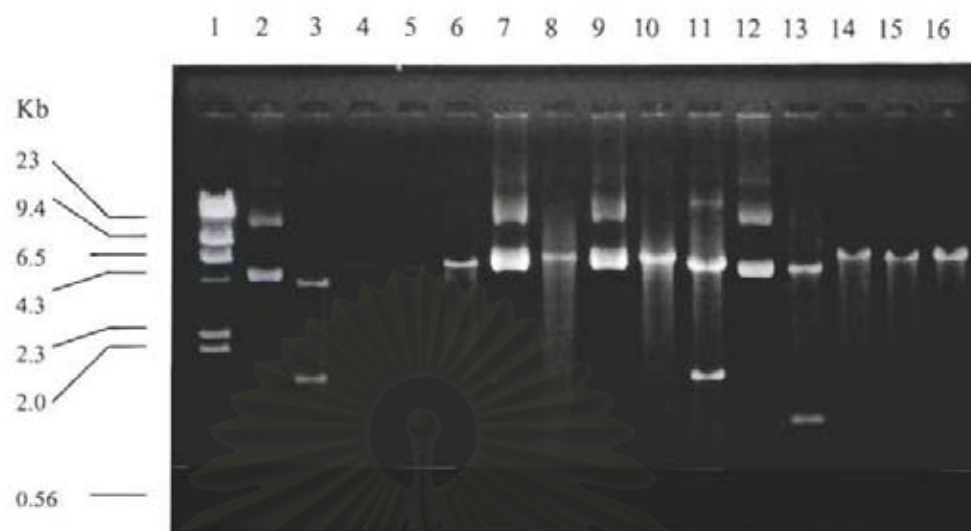
Figure 43 : Restriction analysis of pVR404, 405 and 406. Lane 1 : λ/HindIII marker, lane 2 : undigested pVR404, lanes 3-6 : pVR404 digested with NdeI+SmaI, NcoI, SacII and BamHI , respectively, lane 7 : undigested pVR405, lanes 8-11 : pVR405 digested with NdeI+Eco47III, NcoI, SacII and BamHI, respectively, lane 12 : undigested pVR406, lanes 13-16 : pVR406 digested with Eco47III+BamHI, NcoI, SacII and BamHI, respectively.

Figure 44 : Iodine test for dextrinizing activity of E. coli JM109 harboring pVR404 (top left) pVR405 (top right) and 406 (bottom).
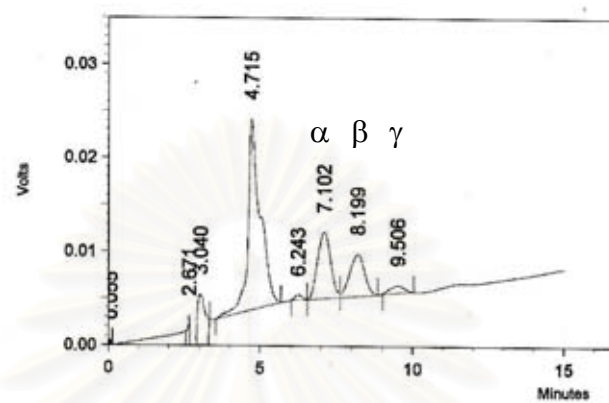
pVR404
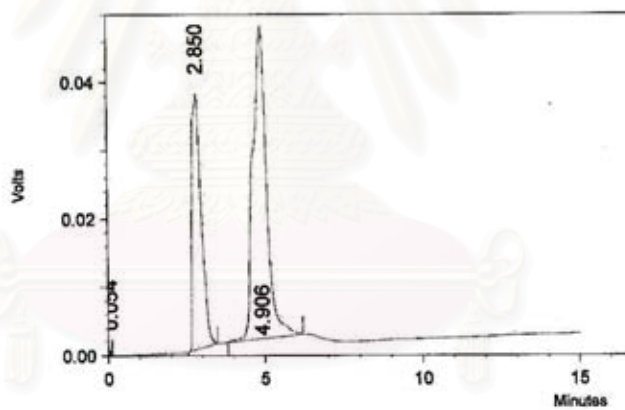
pVR405 and 406

Figure 45 : HPLC profiles of cyclodextrins formed by CGTases from pVR404, pVR405 and pVR406.
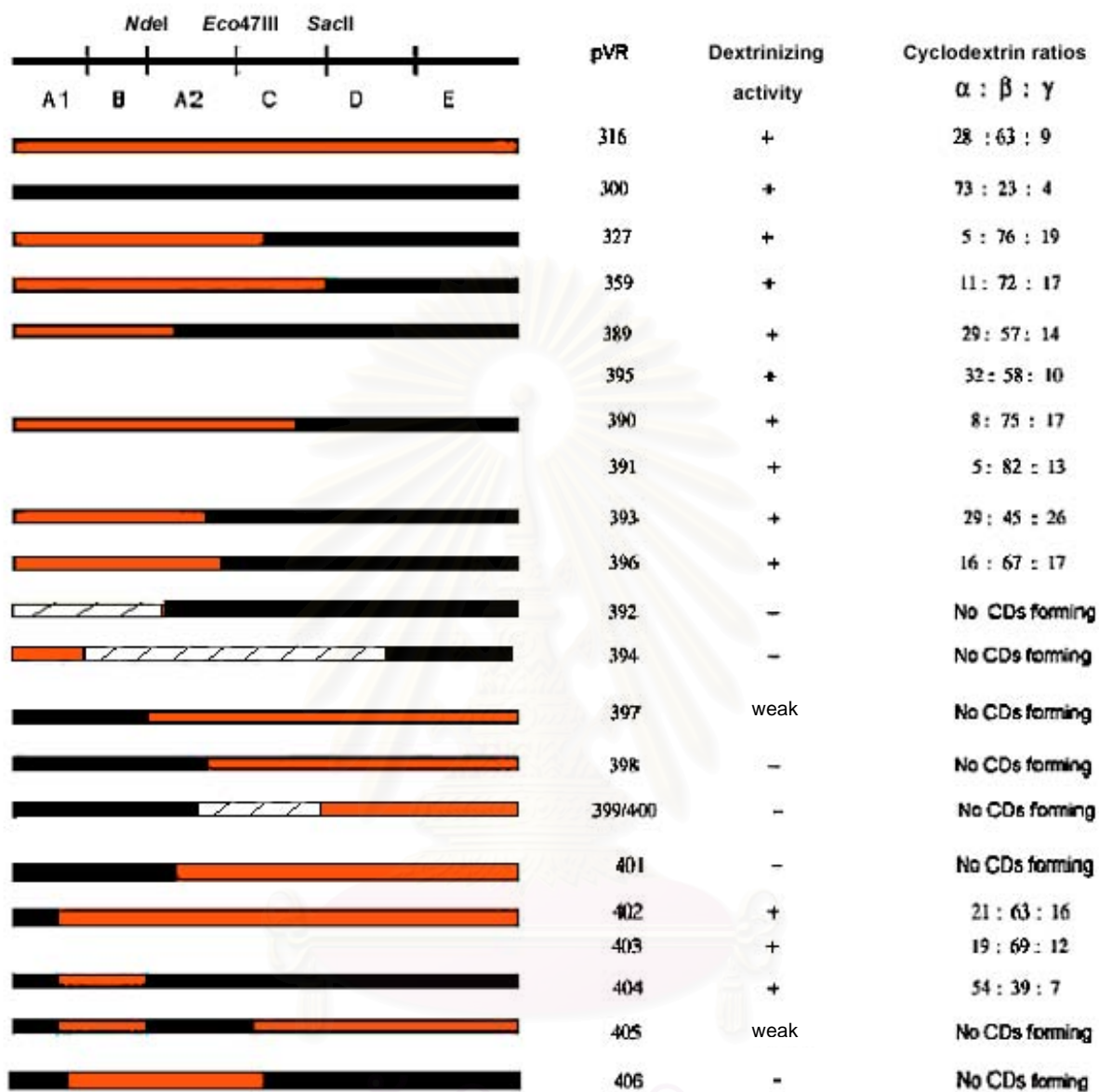
Figure 46 : Summary of the chimeric CGTases and their dextrinizing and cyclodextin ratios production. The red box represent the β-CGTase, the black box represent the α-CGTase and the dash box represent of the deletion region.

7. CGTase purification

        The chimaric CGTase from pVR402 was purified along with wild type CGTases from pVR300 and 316. The chimeric CGTase from pVR402 was chosen because it produced β-cyclodextrin as a major product though it contained an α-CGTase sequence at its N-terminal half of A1 subdomain. It produced slightly more γ-cyclodextrin and less α-cyclodextrin than that of wild type β-CGTase. It was expected that the kinetic parameters of the chimera should be similar to those of wild type β-CGTase .

        The chimeric (pVR402) and wild type CGTases (pVR300 and 316) were sufficiently purified by the starch adsorbtion as the sole purification step with 29% recovery and 4.2 fold purification for pVR316, 54.8% recovery and 10.5 fold purification for pVR300 and 21% recovery and 5.4 fold purification for pVR402 (Table 4).

        SDS-PAGE of the purified enzymes revealed one prominent band and the molecular weight of the pVR316, 300 and 402 were estimated to be 72 kDa (Fig. 47), and the activity stain of each CGTases revealed one band of activity (Fig. 48).

8. Kinetic study

        The purified CGTases were subjected to kenetics study. The reactions were carried out using soluble starch as substrate in the presence of various concentrations of enzymes. The products, α-, β- and γ-cyclodextrins, were determined separately as described in Materials and Methods. For the determination of α-cyclization, 0.036, 0.02 and 0.04 mg protein of pVR316, 300 and 402 were used. For β-cyclization, 0.06, 0.084 and 0.07 mg protein of pVR316, 300 and 402 were used. For γ-cyclization reaction, 0.12, 0.2 and 0.07 mg protein of pVR316, 300 and 402 were used. Fig. 49, 50 and 51 showed the Lineweaver-Burk plots of CGTases from pVR300, 316 and 402 for α-, β- and γ-cyclization reactions, respectively.

        Table 5 showed a summary of kinetic parameters obtained from the study of the α-, β- and γ-cyclization reactions. The kinetic values were obtained after normalized by dividing the velocity of each assay by milligram of protein of uses. The values obtained from α-cyclization reaction were 3.3, 1.1 and 2.7 mg/ml soluble starch for K_m and 12,500, 4,348 and 6,452 μmoles/min for V_{max}, and 462,962, 90,583 and 121,736 min^{-1} for K_{cat} of pVR300, 316 and 402, respectively. The values obtained from β-cyclization were 4.3, 4 and 6 mg/ml soluble starch for K_m and 6,060, 11,764 and 28,571 μ moles/min for V_{max} , and 72,143, 175,582 and 307,215 for min^{-1} for K_{cat} of pVR300, 316 and 402,

respectively. The values from γ-cyclization were 22, 20 and 26 mg/ml soluble starch for K_m and were 0.077, 0.166 and 0.285 μmoles/min for V_{max} , and 0.028, 0.104 and 3.06 min^{-1} for K_{cat} of pVR300, 316 and 402, respectively.

Table 4 : Purification of CGTases

pVR	Fraction	Volume (ml)	Dextrinizing activity		Total protein (mg)	Specific activity (U/mg)	% Yields	Purification fold
			Total Unit	U/ml				
316	crude	470	2068	4.4	2174	0.95	100	1
	Starch adsorbtion	125	604	4.83	150	4.0	29	4.2
300	crude	280	72.8	0.26	1332.8	0.055	100	1
	Starch adsorbtion	70	40	0.57	68.6	0.58	54.8	10.5
402	crude	280	196	0.7	1246	0.16	100	1
	Starch adsorbtion	50	41.5	0.83	48.3	0.86	21	5.4

Table 5 Kinetic parameters of CGTases from pVR300, 316 and 402 for α-, β- and γ-cyclization.

pVR	α- cyclization			β-cyclization			γ-cyclization		
	K_m (mg/ml)	V_{max} (μmoles/min)	K_{cat} (min^{-1})	K_m (mg/ml)	V_{max} (μmoles/min)	K_{cat} (min^{-1})	K_m (mg/ml)	V_{max} (μmoles/min)	K_{cat} (min^{-1})
300	3.3	12,500	462,962	4.3	6,060	72,143	22	0.077	0.028
316	1.1	4348	90,583	4	11,764	175,582	20	0.166	0.104
402	2.7	6452	121,736	6	28,571	307,215	26	0.285	3.06
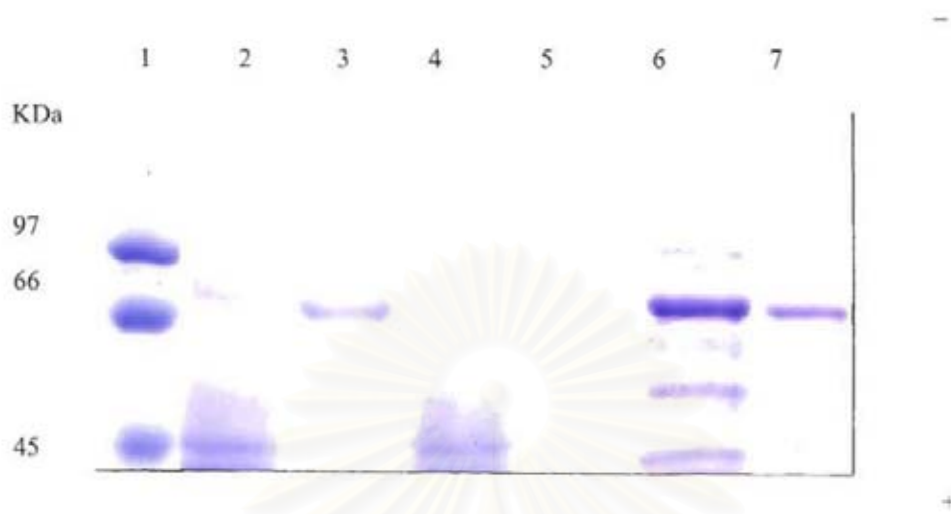
Figure 47 : SDS-PAGE analysis of purified CGTases from *E.coli* JM109 harbouring CGTase genes on 7.5% SDS-acrylamide gel. Lane 1 : Molecular weight marker, lanes 2, 4 and 6 : crude enzyme from pVR402, 316 and 300 (90 μg each), lanes 3, 5 and 7 : CGTases from pVR402, 316 and 300 eluted after starch adsorbtion (25 μg each).
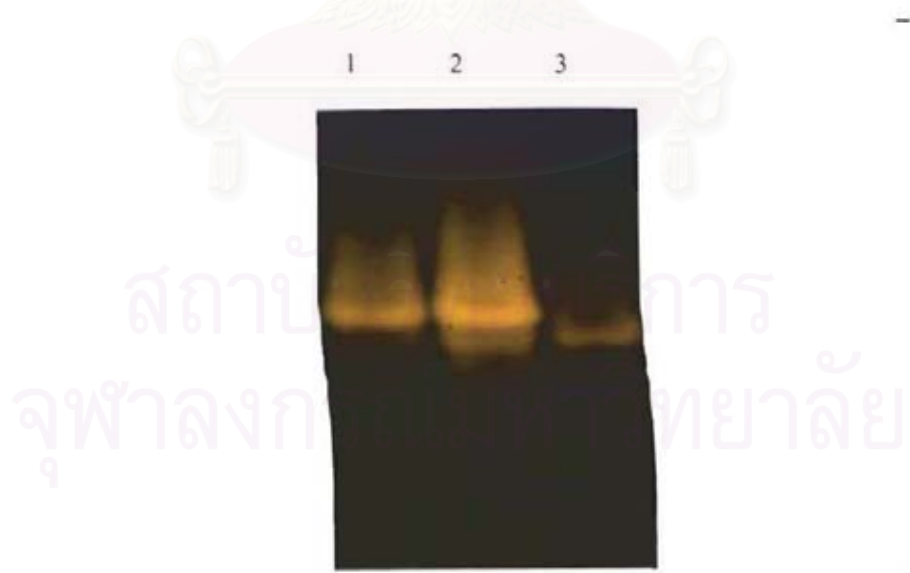
Figure 48 : Dextrinizing activity staining, Lane 1 : pVR402, Lane 2 : pVR316, Lane 3 : pVR300 (0.1 dextrinizing activity units were loaded to each lane)
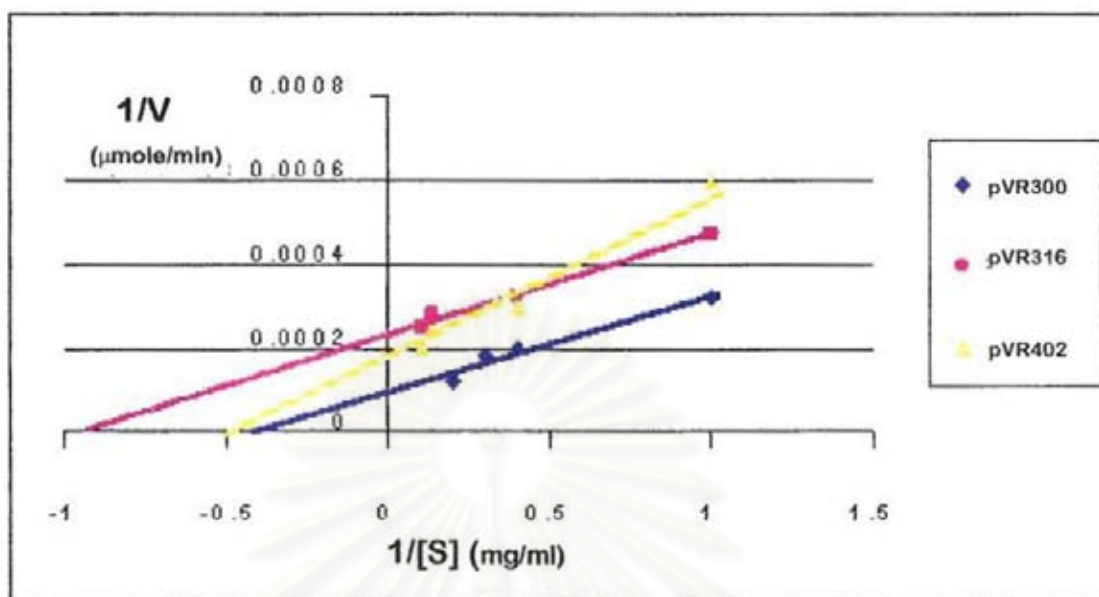
Figure 49 : Lineweaver-Burk plot of CGTases each with soluble starch as substrate for α-cyclization.
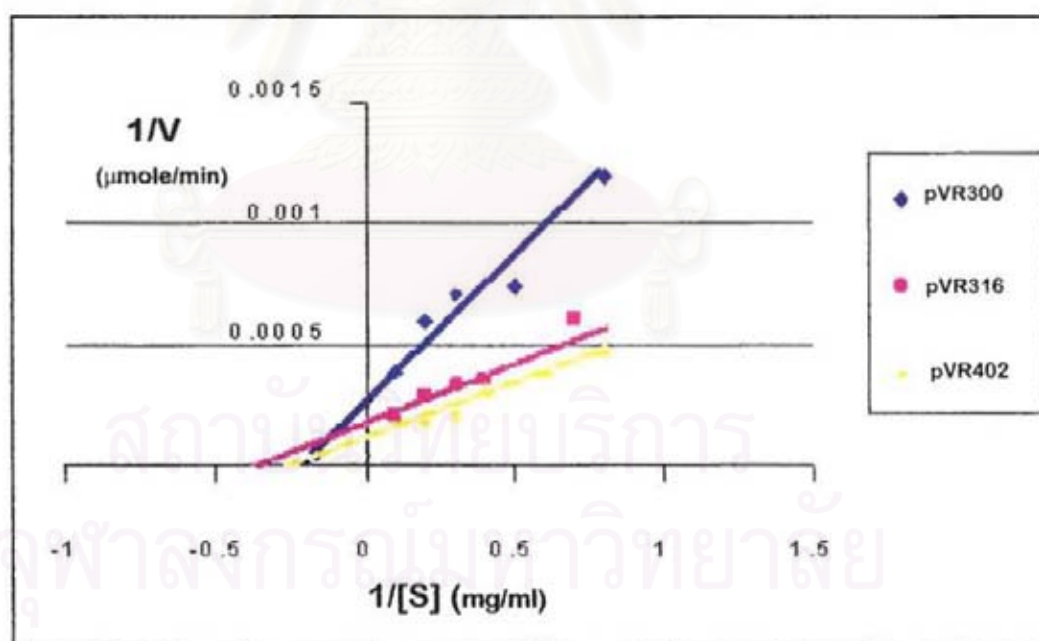
Figure 50 : Lineweaver-Burk plot of CGTases each with soluble starch as substrate for β-cyclization.
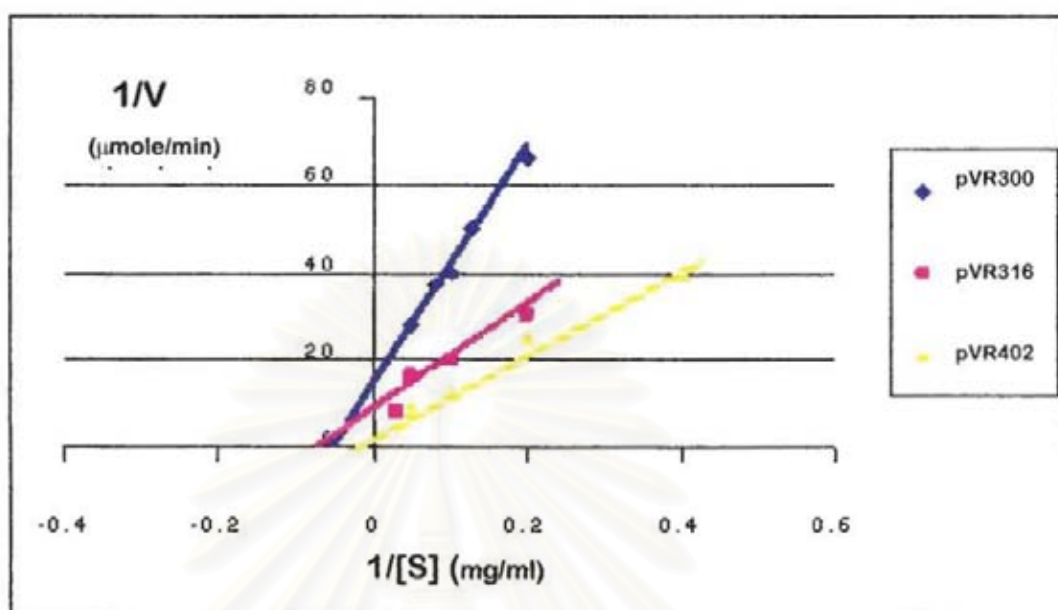
Figure 51 : Lineweaver-Burk plot of CGTases each with soluble starch as substrate for γ-cyclization.

CHAPTER IV

DISCUSSION

It has been well known that cyclodextrins (α-, β- and γ-CDs) are the valuable enzymatic conversion products from starch and related substances by cyclodextrin glucanotransferase (CGTase). They are useful carrier molecules for several applications in industries, *e.g.* pharmaceuticals, chemicals, food, agriculture and so on. For industrial application, β-cyclodextrin costs around US\$ 3-4/kg, α-cyclodextrins US\$ 20-25/kg and γ-cyclodextrins US\$ 80-100/kg. In 1999 global consumption was around 6,000 metric tons, with annual growth rate of 15-20% (Biwer *et al.*, 2002). Despite their wide uses in industries, the production of cyclodextrins suffers from several shortcomings that limit their applicability. All natural CGTases produce a mixture of α-, β- and γ-cyclodextrins. The cyclodextrins have to be purified by selective crystallization using expensive organic solvents that are sometimes difficult to remove and may be disadvantage for human consumption. A CGTase, which only produces a single type of cyclodextrin or increases the proportion of wanted cyclodextrin, is highly interesting for industry.

The protein engineering approach to improve the enzymatic product specificity or enzymatic properties is mainly based on site-directed mutagenesis or the shuffling of gene portions using genetic engineering techniques to generate chimeric proteins. A different approach is to generate recombinant genes by *in vivo* recombination between related CGTase sequences. This approach can potentially create a large array of novel protein variants not easily obtained by ordinary genetic engineering, since crossing over can occur within any regions of little homology. It is easy and inexpensive. The *in vivo* recombination for protein engineering had been successfully used in various proteins such as α-interferon (Weber and Weissman, 1983), insect toxin from *Bacillus thuringiensis* (Calogero *et al.*, 1992) and PQQ glucose dehydrogenase (Sode *et al.*, 1995 and Yoshida *et al.*, 1999).

In the past, various protein engineering techniques for the improvement of the product specificity and properties of CGTases had been made and are summarized in Table 6 (Kaneko *et al.*, 1989 and 1990, Kimura *et al.*, 1989, Hellman *et al.*, 1990, Fujiwara *et al.*, 1992b, Nakamura *et al.*, 1993 and 1994a, Sin *et al.*, 1994, Penninga *et al.*, 1995, Mattsson *et al.*, 1995, Wind *et al.*, 1998a and b, Parsiegla *et al.*, 1998, van der Veen *et al.*, 2000b and c, Yamamoto *et al.*, 2000, Leemhuis *et al.* 2002a and b). Most of the early mutations were based upon amino acid residue 195 (Tyr or Phe in CGTase numbering), an amino acid centrally located in the active site cleft. However, these mutations only improved product specificity to a limited extent and sometimes it was not successful. Until new insights from the refined X-ray structure

of the CGTase from *B. circulans* strain 251 in complex with a maltononaose was obtained, it was suggested that specific sugar binding subsites (-6, -7 and -8) further away from the catalytic site could be important for the enzyme's product specificity. It might be possible to change the cyclodextrin ratios by altering the affinities at these specific sugar binding subsites (Strokopytov *et al*., 1996). van der Veen *et al*., (2000b) proved the important of these sugar binding subsites by site-directed mutagenesis of amino acid residues at subsite -3 and -7. They found that the mutagenesis of amino acid residues at subsite -3 and -7 effected the cyclodextrin ratios by increased the proportion of α-cyclodextrin and decreased that of β-cyclodextrin but had no effect on γ-cyclodextrin production. However, this mutation could not alter the major cyclodextrin produced; it was still β-cyclodextrin. Although other investigators had mutagenized other amino acid residues in several regions of CGTases, the results were similarly to that of van der Veen *et al*., (2000b) as the cyclodextrin ratios could be altered but not the major cyclodextrin product (Table 6). Therefore, the other factors may play an important role in the cyclodextrin specificity and ratios, *i.e.*, domain-domain interaction in CGTase molecule.

The three dimensional structures of *B. circulans* A11 β-CGTase and *Paenibacillus macerans* α-CGTase were predicted by homology modeling using SWISS-MODEL (Fig. 51) (www. Expasy.ch/swissmod/SWISS-MODEL.html). Comparison of their overall structures revealed little difference in size though the structure of β-CGTase was less compact than α-CGTase. The compactness may influent the special arrangement of sugar binding subsites. Previous studies of the β-CGTases from *B. circulans* 8 and strain 251 revealed the 9 subsites, from +2 to -7 (Bender *et al*., 1990 and Strokopytov *et al.*, 1996). For the α-CGTase from *K. pneumoniae* and *P. macerans*, 9 subsites were also revealed but the predicted special arrangement was from +3 to -6 (Bender *et al*., 1990 and Abe *et al.*, 1991). Binding up to subsite +3 had also been observed in the structure of the α/β-CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1 complex with a maltohexaose inhibitor (Wind *et al*., 1998b). Although these studies emphasized on the catalytic domain A/B, other domains might play an important role as well in CGTase specificity.

This study is aimed to determine an essential part of CGTase that governs the cyclodextrin specificity and ratios. The α-CGTase from *P. macerans* IAM1243, which produces α-cyclodextrin as major product, and β-CGTase from *B. circulans* A11, which produces β-cyclodextrin as major product, are used as models. By shuffling the amino acid sequence between these 2 enzymes, a sequence, essential for their specificity, might be emerged. To randomly shuffling the sequence, *in vivo* homologous recombination using *E. coli* JC8679 was used. A plasmid contains a direct repeat of the 2 CGTases is
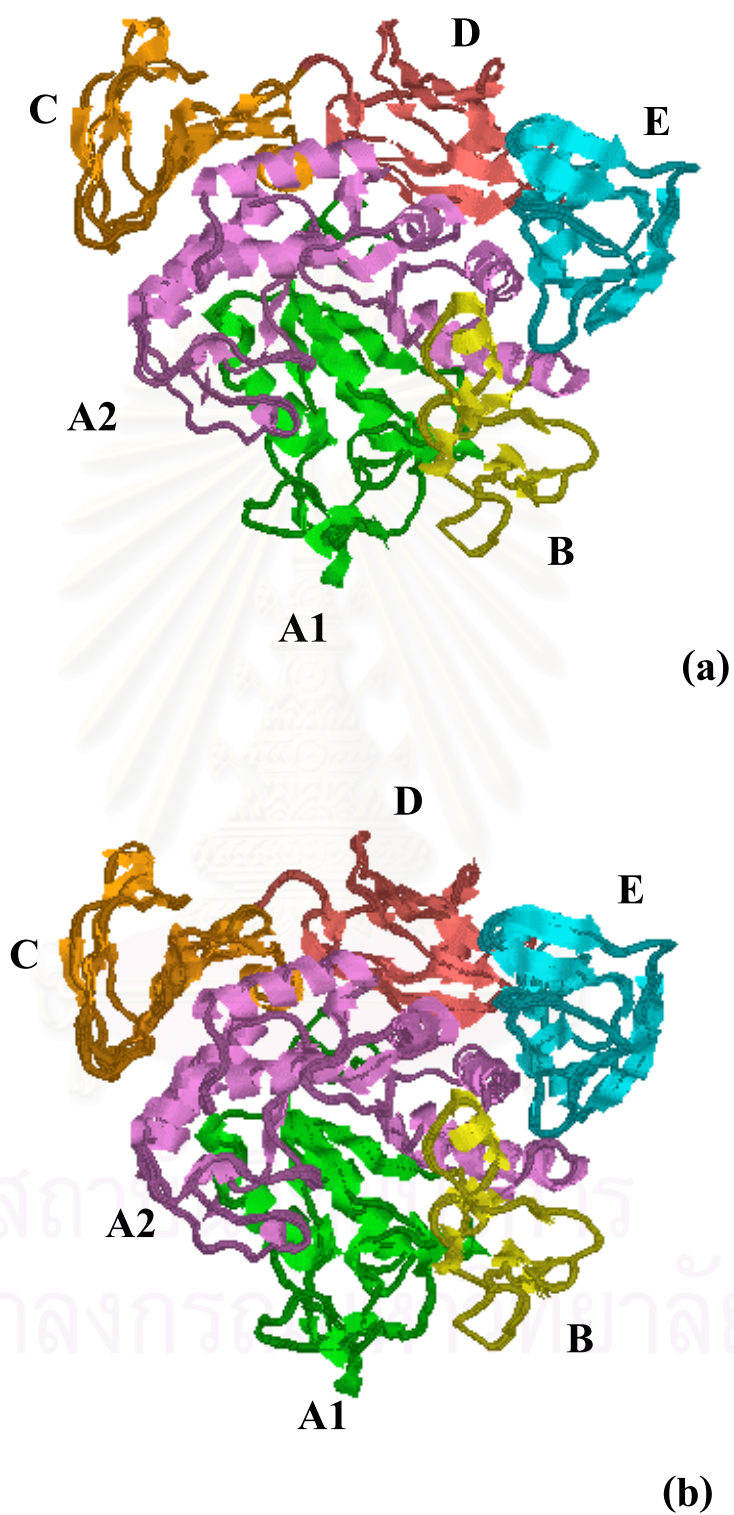
Figure 51 : Comparison of the three dimensional structures between β-CGTase from *B. circulans* A11 (a) and α-CGTase from *P. macerans* (b).

digested with appropriate restriction enzymes to inactivate the enzymes. After transformation, only the linearized plasmids, that can be recombined, survive and render the cells resistant to ampicillin. These clones produce chimeric CGTases with potentially different activities.

Using 2 plasmids with alternate direct repeats of α- and β-CGTases, 2 groups of chimeric CGTases were obtained, the pVR321 and pVR388 series. Several chimeric CGTases are inactive in cyclodextrin forming activity. These clones are pVR392, 394, 397, 398, 399, 400 and 401. The pVR392, 394, 399 and 400 loss their dextrinizing and cyclodextrin forming activities because they contain deletions in the CGTase genes from the recombination process. The pVR397 is inactive in cyclodextrin forming activity but weakly active in dextrinization. The chimeric CGTase from pVR397 may have different conformation of A/B domain that can not cyclize the oligosaccharide but still be able to cleave the saccharide. The pVR398 and 401 are inactive in dextrinization and cyclodextrin forming activity, owing to the frameshift in the open reading frame of CGTase genes and can not produce the correct enzymes.

The chimeric pVR321 series

The chimeric CGTases in pVR321 series that are active in dextrinization and cyclodextrin forming are from pVR327, 359, 389, 390, 391, 393, 395 and 396. They produce β-cyclodextrin as a major product. They can be divided into 4 groups according to their cyclodextrin productions. The first group consists of pVR327, 359, 390 and 391, which have their recombination sites located in the domains C and D (Fig. 46). The chimeras produce higher β-cyclodextrin, higher γ-cyclodextrin and lower α-cyclodextrin as compared to those of wild type β-CGTases. All of these chimeric CGTases contain the complete catalytic A/B domains from wild type β-CGTases. It is therefore not surprising that these chimeras produce β-cyclodextrin as major product. It is, however, interesting that they produce more β-, more γ- and less α-cyclodextrins. It seems to indicate that the other domains might play an important role in cyclodextrin forming activity. The domain C is also believed to play a role in localizing the enzyme on the substrate (Lawson *et al*., 1994 and Dauter *et al.*, 1999). It can be that the different geometric arrangement of domains in the chimeras affects the substrate binding subsites, which favors the bigger cyclodextrin products. The results also indicate the important of the A/B domains in product specificity; the chimeras are β-CGTases.

The second group consists of pVR389 and 395, which have their recombination sites around the middle of subdomain A2 (Fig. 46). Recombination in this group results in slightly decreased production of β-cyclodextrin and increased production of γ-cyclodextrin but has no effect on the

production of α-cyclodextrin. This group of chimeras in pVR321 series has the cyclodextrin ratio more or less similar to that of β-CGTase although most of the domains are from α-CGTase. The third group consists of a chimeric CGTase from pVR396 with the recombination site around the C-terminal end of subdomain A2 (Fig. 46). This chimeric produces more γ-, less α- and no effect on β-cyclodextrins. The final group consists of a chimeric CGTase from pVR393 with the recombination site around the C-terminal end of subdomain A2 (Fig. 46). This chimeric enzyme produces higher proportion of γ-cyclodextrin significantly, lower proportion of β-cyclodextrin and comparable proportion of α-cyclodextrin as compared to the β-CGTase. These 3 groups of chimeras are very interesting as they have their recombination sites in the catalytic A/B domains. They all have increased production of γ-cyclodextrin with no or slight effect on the production of α-cyclodextrin. The recombination however effects the production of β-cyclodextrin except that of pVR396. Drastic changes on the production of β- and γ-cyclodextrin are observed with pVR393. The results suggest that the amino acid region in subdomain A2 may have influence on the product specificity although the other domains (C and D) may also contribute to the product specificity as well.

The chimeric pVR388 series

The chimeric CGTases in pVR388 series are mostly inactive (pVR397, 398, 399, 400 and 401) except pVR402 and 403. The two chimeras have the same recombination sites at around the C-terminal half of subdomain A1 (Fig. 46). They produce β-cyclodextrin as a major product, although they are composed of the N-terminal half of subdomain A1 from α-CGTase. They also produce slightly increased proportion of γ-cyclodextrin but decreased proportion of α-cyclodextrin when compared to wild type α-CGTase. Interestingly, the two chimeras whose N-terminal regions about 100 amino acid residues derived from α-CGTase can produced the same proportion of β-cyclodextrins as the wild type β-CGTase. Therefore, the N-terminal half of subdomain A1 has no effect on the proportion of major cyclodextrin product. This may be due to the fact that most of other domains are derived from β-CGTase. It is however difficult to delineate how the N-terminal sequence from α-CGTase effects the proportion of α- and γ-cyclodextrins. Van der Veen *et al.* (2000) had reported the effect of mutation of Arg47 in subdomain A1 and suggested that the amino acid residue might involve in the stabilization of the transition state intermediate during catalysis. The R47Q mutant showed lower proportion of β-cyclodextrin similar to that of pVR402 and 403 (see Table 6).

To elucidate the effect of recombination in pVR402 and 403, the chimeric enzyme from pVR402 was purified and assayed to determine the kinetic parameters. Since the starch adsorption could

be sufficiently used for the purification, it was used as the sole of purification step. The V_{max}, K_m and K_{cat} were determined and summarize in Table 5 for α-, β- and γ-cyclization reactions. The cyclization reaction can be considered as 3 separate reactions occurred simultaneously. The CGTase from pVR300 should favor the α-cyclization reaction and those from pVR316 and 402 should both favor β-cyclization reaction. The results in Table 5 agree well with this notion. Comparison of the parameters in the same reaction among the CGTases indicate several interesting results. For the α-cyclization, the α-CGTase (pVR300) has the expected highest V_{max} and K_{cat} but the unexpected highest K_m. The β-CGTase (pVR316) had the lowest K_m as well as its V_{max} and K_{cat}. Although the β-CGTase has higher affinity for substrate in this reaction than the α-CGTase, it produces less α-cyclodextrin owing to its low catalytic rate. The chimeric CGTase (pVR402) seems to be more active than the β-CGTase with higher V_{max} and K_{cat} but it is less reactive owing to its low affinity for the substrate. Similar interpretation is also true for β- and γ-cyclization reactions. The results are more or less correlated with the cyclodextrin product ratios of these CGTases, particularly the proportion of γ-cyclodextrin. The chimeric CGTase produces higher proportion of γ-cyclodextrin, it should have higher reaction rate for γ-cyclization than the two wild type CGTases.

Determination of an essential part of chimeric CGTase that involves in product specificity

As described above, the chimeric CGTases from pVR402 and 403, whose N-terminal regions are derived from α-CGTase, produce β-cyclodextrin similar to that of β-CGTase. Therefore, the region for product specificity should reside in the β-CGTase sequence. To determine the location of this region, pVR402 was chosen for CGTase gene manipulation. Three more chimeric CGTases were constructed. By replacing the subdomain A2, domains C, D and E in pVR402 with those of wild type α-CGTase sequence, the resulting chimeric CGTase clone pVR404 was obtained. By replacing the subdomain A2 in pVR402 with that of wild type α-CGTase sequence, the resulting chimeric CGTase clone pVR405 was obtained. Finally, by replacing the domains C, D and E in pVR402 with that of wild type α-CGTase sequence, the resulting chimeric CGTase clone pVR406 was obtained (Fig. 46).

The chimeric CGTases from pVR405 and 406 are inactive in cyclodextrin forming activities and weakly or inactive in dextrinization, respectively. It is not known why the two clones are inactive. It is possible that there may be incompatibility among the domains in these chimeric enzymes leading to the loss of their activities, preventing them from secretion to the medium or causing rapid proteolysis in the cells (Fujiwara *et al.*, 1992). The chimeric pVR404, whose CGTase gene is largely similar to α-CGTase with the C-terminal half of subdomain A1 and domain B from β-CGTase, is active in dextrinization and cyclodextrin production. Interestingly, the chimeric pVR404 produces α-

cyclodextrin as a major product; it is reverted back to an α-CGTase. By comparing the results from pVR404 with those of pVR389, 395, 393 and 396, the subdomain A2 region most likely contains the determinant for CGTase product specificity.

According to the data from Rimphanitchayakit, V. (unplublished), several chimeric CGTases had been constructed. In particular, the 2 chimeras that subdomain A2, domains D and E and subdomain A2, domains C, D and E of β-CGTase from *B. circulans* A11 are replaced by the same fragments from α-CGTase from *P. macerans*. The 2 chimeras produce α-cyclodextrin in the same proportion as β-cyclodextrin, while the γ-cyclodextrin production is increased. These results also indicate the crucial role of subdomain A2 in product specificity. Yamamoto *et al*. (2000) suggested that Tyr267 in A2 subdomain of α-CGTase from *Thermococcus* sp. B1001 played a critical role in α-cyclodextrin specificity. The corresponding residue in α-CGTase from *P. macerans* is also Tyr (equivalent to Tyr260), while other CGTases have Phe (equivalent to Phe259 in *B. circulans* CGTase) at this position. Mutation at this site in α-CGTase of *Thermococcus* sp. B1001 from Tyr to Trp (Tyr267Trp) reduced the production of α- cyclodextrin.

When considered the amino acid sequence in the subdomain A2, the 4 stretches of amino acid sequences, residues 239-250 in the region between α4 and β5, residues 262-271 in the region between β5 and α5, residues 284-320 in the region between α6 and β7, and residues 333-342 in the region between β7 and α7 (*B. circulans* A11 β-CGTase numbering), are found to be highly variable among CGTases (Fig. 52). The amino acid residues in these regions may involve in the cyclodextrin product specificity.

```
                  NdeI site
                  231    239           250      259 262          271              284
                   |      |             |        |   |            |                |
Bf290_3  VAHMPVGW QKNFVSSIY------DYN PVFTFGEWFTG AGGSD-----EYHY FINNSGMSALDF RYAQVVQDVLRNNDG-
Bclar    VAHMPLGW QKAFISSVY------DYN PVFTFGEWFTG AQGSN-----HYHH FVNNSGMSALDF RYAQVAQDVLRNQKG-
BcA11    VKHMPFGW QKSFMSTIN------NYK PVFNFGEWFLG VNEISP----EYHQ FANESGMSLLDF RFAQKARQVFRDNTD-
Bc38_2   VKHMPFGW QKSFMSTIN------NYK PVFNFGEWFLG VNEISP----EYHQ FANESGMSLLDF PFAQKARQVFRDNTD-
Bc1011   VKHMPFGW QKSFMATIN------NYK PVFTFGEWFLG VNEISP----EYHQ FANESGMSLLDF RFAQKARQVFRDNTD-
Bc251    VKHMPFGW QKSFMAAVN------NYK PVFTFGEWFLG VNEVSP----ENHK FANESGMSLLDF RFAQKVRQVFRDNTD-
Bc8      VKHMPLGW QKSWMSSIY------AHK PVFTFGEWFLG SAASDA----DNTD FANKSGMSLLDF RFNSAVRNVFRDNTS-
Pmac2    VKQYPFGW QKSFVSSIYG-----GDH PVFTFGEWYLG ADQTDG----DNIK FANESGMNLLDF EYAQEVREVFRDKTE-
Pmac1    VKHMPFGW QKSFVSSIYG-----GDH PVFTFGEWYLG ADQTDG----DNIK FANESGMNLLDF EYAQEVREVFRDKTE-
TtEM1    VKHMPFGW QKNFMDSIL------SYR PVFTFGEWFLG TNEIDV---NNTY FANESGMSLLDF RFSQKVRQVFRDNTD-
Gsth     VKHMPFGW QKSFMDEVY------DYR PVFTFGEWFLS ENEVDS----NNHF FANESGMSLLDF RFGQKLRQVLRNNSD-
Bohb     VKHMSEGW QTSLMSDIY------AHE PVFTFGEWFLG SGEVDP----QNHH FANESGMSLLDQ FGQTIRDVFLMDGSS-
BKC201   VKHMSEGW QTSLMSEIY------SHK PVFTFGEWFLG SGEVDP----QNHH FANESGMSLLDF QFGQTIRNVLKDRTS-
Brev     VKHMSEGW QTSLMSDIY------TYK PVFTFGEWFLG TGEVDP----QNHH FANESGMSLLDF QFGQTIRSVLKDRTS-
Kleb     IKHMDKSF IQKWTSDIYDYSKSIGRE GFFFFGEWFGA SANTTTGVDGNAID YANTSGSALLDF GFRDTLERVLVGRSGN
Tkoda    VKHMELGW LETFYLRLY------SKG PLFIYGEYFT- PSLQKGD---DLYE FYRYSNVSPVLS IPIREDIVRIFAFFGG
TB1001   VKHLEPGW LKTYADYVY------ARK NVFMFGEWYQG FNDEMYW---DMVK FANYTGIG-LIN IPLQQVLVDVFAYDTK
          : :     .:            :              .* :**::          .    : . :.    :::           .
         _____                       _____             _____        _____
           α4                            β5                   α5   β6        α6
                      320            333                    342             359
                       |              |                      |               |
Bf290_3  TMYDLETVLRETESVYEKPQDQ VTFIDNHDINRF SRNGHS-------------------TRTT DLGLAFLLTSRGVPTIY
Bclar    TMHDIYDMLASTQLDYERPQDQ VTFIDNHDIDRF TVEGRD-------------------TRTT DIGLAFLLTSRGVPAIY
BcA11    NMYGLKAMLEGSEVDYAQVNDQ VTFIDNHDMERF HTSNGD------------------RRKL EQALAFTLTSRGVPAIY
Bc38_2   NMYGLKAMLEGSEVDYAQVNDQ VTFIDNHDMERF HTSNGD------------------RRKL EQALAFTLTSRGVPAIY
Bc1011   NMYGLKAMLEGSEVDYAQVNDQ VTFIDNHDMERF HTSNGD------------------RRKL EQALAFTLTSRGVPAIY
Bc251    NMYGLKAMLEGSAADYAQVDDQ VTFIDNHDMERF HASNAN------------------RRKL EQALAFTLTSRGVPAIY
Bc8      NMYALDSMINSTATDYNQVNDQ VTFIDNHDMDRF KTSAVN------------------NRRL EQALAFTLTSRGVPAIY
Pmac2    TMKDLYEVLASTESQYDYINNM VTFIDNHDMDRF QVAGSG-------------------TRAT EQALALTLTSRGVPAIY
Pmac1    TMKDLYEVLASTESQYDYINNM VTFIDNHDMDRF QVAGSG-------------------TRAT EQALALTLTSRGVPAIY
TtEM1    TMYGLDSMIQSTASDYNFINDM VTFIDNHDMDRF YNGGS--------------------TRPV EQALAFTLTSRGVPAIY
Gsth     DWYGFNQMIQDTASAYDEVIDQ VTFIDNHDMDRF MADEGD------------------PRKV DIALAVLLTSRGVPNIY
Bohb     NWYDFNEMIASTEEDYDEVIDQ VTFIDNHDMSRF SFEQSS-------------------NRHT DIALAVLLTSRGVPTIY
BKC201   NWYDFNEMITSTEKEYNEVIDQ VTFIDNHDMSRF SVGSSS-------------------NRQT DMALAVLLTSRGVPTIY
Brev     NWYDFNEMIKSTEKDYDEVIDQ VTFIDNHDMSRF SM-VVF-------------------NFQT DIALAVLLTSRGVPTIY
Kleb     TMKTLNSYLIKRQTVFTSDDWQ VVFMDNHDMARI GTALRSNATTFGPGNNETGGSQSEAFAQKRI DLGLVATMTVRGIPAIY
Tkoda    -LDKLSEELGDYYSHFVYPTKA VNFLDSHDLVRF LNAGDRKDE----------------IQRF HMALALTLTLPGIPVIY
TB1001   TMWDLESAVNKYTIDFMWQNKL TIFVDSHDVPRF LSLRN--------------------LIRF HQALAFVLTAPGIPIIY
          :     :        :       . *:*.**: *:                            . .*.  :*  *:* **
                                                                        Recombination site
                                                                            of pVR396
         _____                    _____               _____    _____
            β7                                                  α7            β8
```

Figure 53 : The amino acid alignment of CGTases in subdomain A2 region between *Nde*I site and before the recombination site of pVR396. The bold characters indicates variable regions. The number is corresponding to *B. circulans* A11 numbering. Bf : *Bacillus firmus*; Bclar : *B. clarkii*; Bc : *B. circulans*; Bohb : *B. ohbensis*; BKC201 : *Bacillus* sp. KC201; Pmac : *Paenibacillus macerans*; Brev : *Brevibacillus brevis*; TtEM1 : *Thermoanaerobacterium thermosulfurigenes* EM1; Gsth : *Geobacillus stearothermophilus*; Kleb : *Klebsiella pneumoniae*; Tkoda : *Thermococcus kodakaraensis*; TB1001 : *Thermococcus* sp. B1001.

Table 6 : Summary of CGTases mutagenesis that affected to cyclodextrin products from previous studies.

Amino acid residues	Mutagenesis residues	Domain locate	Effect on cyclodextrin ratios			Function	Source	Main Product	References
			α	β	γ				
Histidine	H140N	B	Decreased	Unaffected	Unaffected	Stabilization of the intermediate transition state and catalysis for an alkaline pH range for H327	*Bacillus* sp. 1011	β	Nakamura *et al.*, 1993
	H233N	A2	Not produced	Unaffected	Unaffected				
	H327N		Decreased	Unaffected	Unaffected				
	H140D	B	Decreased	Unaffected	Unaffected		*B. circulans* var alkalophilus	β	Mattsson *et al.*, 1995
	H233D	A2	Decreased	Unaffected	Unaffected				
	H327D		Unaffected	Unaffected	Unaffected				
	H177P	B	Decreased	Decreased	Unaffected	Thermal stability and the width of the pH optimum			
	H98D	A1	Unaffected	Unaffected	Unaffected	Stabilization of substrate binding subsite +2 and destabilizes the intermediate for cyclization			
Leucine	L194T	B	Decreased	Unaffected	Unaffected	Unknown	*Bacillus* sp. 8	β	Parsiegla *et al.*, 1998
Tryptophan	W191Y/F		Unaffected	Unaffected	Unaffected	Unknown	*Thermococcus* sp. B1001	α	Yamamoto *et al.*, 2000
Arginine	R47L	A1	Unaffected	Unaffected	Unaffected	Stabilizes the oligosaccharide chain in the transition state	*B. circulans* 251	β	van der Veen *et al.*, 2000c
	R47Q		Decreased	Increased	Unaffected				
Serine	S146P	B	Increased	Decreased	Unaffected	Cyclodextrin product specificity			van der Veen *et al.*, 2000b
Tyrosine	Y89D	A1	Incresed	Decresed	Decresed				
	Y89D/S146P	A1 and B	Incresed	Decreased	Unaffected				

Amino acid residues	Mutagenesis residues	Domain located	Effect on cyclodextrin ratios			Function	Source	Main product	Refferences
			α	β	γ				
Tyrosine	Y100W	A1	Unaffected	Unaffected	Unaffected	Unknown	Thermococcus sp. B1001	α	Yamamoto et al., 2000
	Y267F	A2	Unaffected	Unaffected	Unaffected	Important role in α-cyclodextrin formation			
	Y267W		Decreased	Unaffected	Unaffected				
	Y167F	B	Increased	Unaffected	Unaffected	Substrate binding subsite -6	B. circulans 251	β	Leemhuis et al., 2002a
	Y634F/G	E	Unaffected	Unaffected	Unaffected	Important for the stability and integrity of P. macerans CGTase	Paenibacillus. macerans	α	Change et al., 1998
	Y195L	B	Not produced	Increased	Increased	Might be involved in hydrophobic interaction with the carbohydrate residue, or in bending the non-reducing end towards the reducing end of the bound oligosaccharide, resulting in cyclodextrin formation, or might be keeping water from the active site, thus preventing hydrolysis	Bacillus sp. 1011	β	Nakamura et al., 1994a
	Y195F		Unaffected	Unaffected	Unaffected				
	Y195W		Decreased	Decreased	Increased		Bacillus sp. 8	β	Parsiegla et al., 1998
	Y195G/W/F		Unaffected	Unaffected	Unaffected		B. circulans 251	β	Penninga et al.,1995
	Y195L		Not produced	Increased	Unaffected				
	Y188W		Unaffected	Decreased	Increased		B. ohbensis	β (no α)	Sin et al., 1994
Phenylalanine	F191Y	B and A2	Decreased	Increased	Unaffected		G. stearothermophilus	α/β	Fujiwara et al., 1992b
	F191Y/F255Y		Decreased	Increased	Unaffected				
	F196GΔ'DE	B	Unaffected	Unaffected	Unaffected		Thermoanaerobacterium thermosulfurigenes EM1	β/α	Wind et al., 1998a
	F196G		Decreased	Decreased	Decreased				Leemhuis et al., 2002b
	F196G/F260N	B and A2	Decreased	Decreased	Decreased				

Amino acid residues	Mutagenesis residues	Domain located	Effect on cyclodextrin ratios			Function	Source	Main product	Refferences
			α	β	γ				
Phenylalanine	F283L	A2	Unaffected	Unaffected	Unaffected	Transition state stabilization	*Bacillus* sp. 1011	β	Nakamura *et al*., 1994a
	F183L	B	Decreased	Unaffected	Unaffected	Acceptor binding subsite +2 and limiting the hydrolytic activity			
	F259L	A2	Decreased	Unaffected	Unaffected				
	F184S	B	Unaffected	Unaffected	Unaffected		*T. thermosulfurigenes* EM1	β/α	Leemhuis *et al*., 2002b
	F260N/L/I/E/H/R	A2	Decreased	Decreased	Decreased				
	F184S/F260N	B and A2	Decreased	Decreased	Decreased				
	F284K	A2	Unaffected	Unaffected	Unaffected	Unknown			Wind *et al*., 1998b
Aspartate	D371R		Decreased	Increased	Increased	Hindering the maltohexaose conformation			
	D197H	B	Increased	Decreased	Decreased	Stabilization of the maltohexaose conformation			
Asparagine	N327D	A2	Unaffected	Unaffected	Unaffected	Unknown			
Glycine	N193G/L	B	Increased	Unaffected	Unaffected	Substrate binding subsite –6 and control the transferase activity	*B. circulans* 251	β	Leemhuis *et al*., 2002a
	G179L		Unaffected	Unaffected	Unaffected				
	G180L		Increased	Unaffected	Unaffected				
	G179L/G180L		Unaffected	Unaffected	Unaffected				
Alanine	A230V	A2	Decreased	Decreased	Decreased	Essential for transferase specificity at acceptor subsite+1 and restrict the hydrolytic activity			Leemhuis *et al*., 2003b

Amino acid residues	Mutagenesis residues	Domain located	Effect on cyclodextrin ratios			Function	Source	Main product	Refferences
			α	β	γ				
Deletion	Δ(145-151)D	B	Decreased	Decreased	Increased	May produce more space for the bound glycosyl chain	*Bacillus* sp. 8	β	Parsiegla *et al.*, 1998
	Δ36 Carboxyl terminal amino acid	E	Decreased	Decreased	Decreased	Substrate binding domain	*B. circulans* var alkalophilus	β	Hellman *et. al.*, 1990
	Δ84 Carboxyl terminal amino acid		Decreased	Decreased	Decreased				
	Δ125 Carboxyl terminal amino acid		Not produce	Not produce	Not produce				
	Δ225 Carboxyl terminal amino acid		Not produce	Not produce	Not produce				
	Δ10 & 13 Carboxyl terminal amino acid		Increased	Decreased	Decreased	Substrate binding domain	Alkalophilic *Bacillus* sp. 1011	β	Kimura *et al.,* 1989

CHAPTER V

CONCLUSION

1. Domains other than the catalytic A/B domains in CGTase also contribute to specificity and ratios of cyclodextrin production.

2. The recombination of α- and β-CGTase within the C and D domains decreases the production of α-cyclodextrin significantly and increases the production of β- and γ-cyclodextrins.

3. The recombination of α- and β-CGTase within the 3'-half of A2 subdomain has little effect on the production of α- and β-cyclodextrins while the proportion of γ-cyclodextrin is increased.

4. The N-terminal half of A1 subdomain has no influence on specificity of cyclodextrin.

5. The existence of 3'-half of A1 subdomain and B domain regions of β-CGTase in α-CGTase renders the CGTase produces more β- and less α-cyclodextrins although the major product is still α-cyclodextrin.

6. The subdomain A2 most likely contains the determinant for CGTase product specificity.

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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$_2$O	7.55 ml

1.2. Alkaline SDS solution : 10 ml

- 5 % SDS	2 ml
- 5 N NaOH	0.4 ml
- ddH$_2$O	7.6 ml

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

- NaOAc . 3H$_2$O	408.1 g
- ddH$_2$O	700 ml

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

2. OTHER REAGENTS FOR PREPARATION

2.1. RNase 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 to 100 $^{\circ}$C, 15 min and Cool slowly at room temperature, aliquot, store at -20 $^{\circ}$C.

2.2. 10% Glycerol

Glycerol	10 ml
Water	90 ml

2.3. 5x 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. 0.5M EDTA

EDTA	186.1 g
Water	1000 ml

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

2.5. 1M Tris-HCl

Tris-base	121.1 g
Water	1000 ml

* Adjust pH to 7-8 before adjust volume to 1 litre, 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 adjust volume to 100 ml

2.8. Loading buffer (For agarose gel electrophoresis)

Glycerol	20 ml
Bromphenol blue	4 mg
Water	80 ml

2.9. Lowry's solution

2.9.1. Solution A

$CuSO_4.5H_2O$	5 g
$Na_3C_6H_5O_7.2H_2O$	1 g

2.9.2. Solution B

Na_2CO3_3	20 g
NaOH	4 g

2.9.3. Solution C

Solution A	1 ml
Solution B	50 ml

2.9.4. Solution D

Folin-Ciocalteu phenol reagent	10 ml
Distilled water	10 ml

3. Preparation for polyacrylamide gel electrophoresis

3.1. Stock reagents

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

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

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 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 8.8	50 ml
10% SDS	4 ml
distilled water	46 ml

3.2. Non-denaturing PAGE

7.0% Separating gel

30% acrylamide solution	2.33 ml
1.5 M Tris-HCl pH 8.8	2.50 ml
distilled water	5.15 ml
10% $(NH_4)_2S_2O_8$	50 μl
TEMED	5 μl

For preparative gel, 25 μl of 10% $(NH_4)_2S_2O_8$ and 2.5 μl of TEMED were added

5% Stacking gel

30% acrylamide solution	1.67 ml
0.5 M Tris-HCl pH 6.8	2.50 ml
distilled water	5.80 ml
10% $(NH_4)_2S_2O_8$	50 μl
TEMED	10 μl

3.3. Sample buffer

For analytical gel

1 M Tris-HCl pH 6.8	3.1 ml
glycerol	5.0 ml
1% bromophenol blue	0.5 ml
distilled water	1.4 ml

For preparative gel

0.5 M Tris-HCl pH 6.8	10 ml
glycerol	0.8 ml
0.5% bromophenol blue	0.4 ml
distilled water	5.8 ml

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

Electrophoresis buffer, 1 litre (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

Dissolved in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3).

3.4. SDS-PAGE

7.5% Separating gel

30% acrylamide solution	2.5	ml
Solution B	2.5	ml
distilled water	5.0	ml
10% $(NH_4)_2S_2O_8$	50	μl
TEMED	10	μl

5.0% Stacking gel

30% acrylamide solution	0.67	ml
Solution C	1.0	ml
distilled water	2.3	ml
10% $(NH_4)_2S_2O_8$	30	μl
TEMED	5	μl

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

Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

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

3.5. Coomassie blue staining

Staining solution, 100 ml

Coomassie brilliant blue R-250	0.1 g	
methanol	45	ml
acetic acid	10	ml
distilled water	45	ml

Destaining solution, 100 ml

methanol	10	ml
acetic acid	10	ml
distilled water	80	ml

APPENDIX B

Standard curve for protein determination by Lowry's method
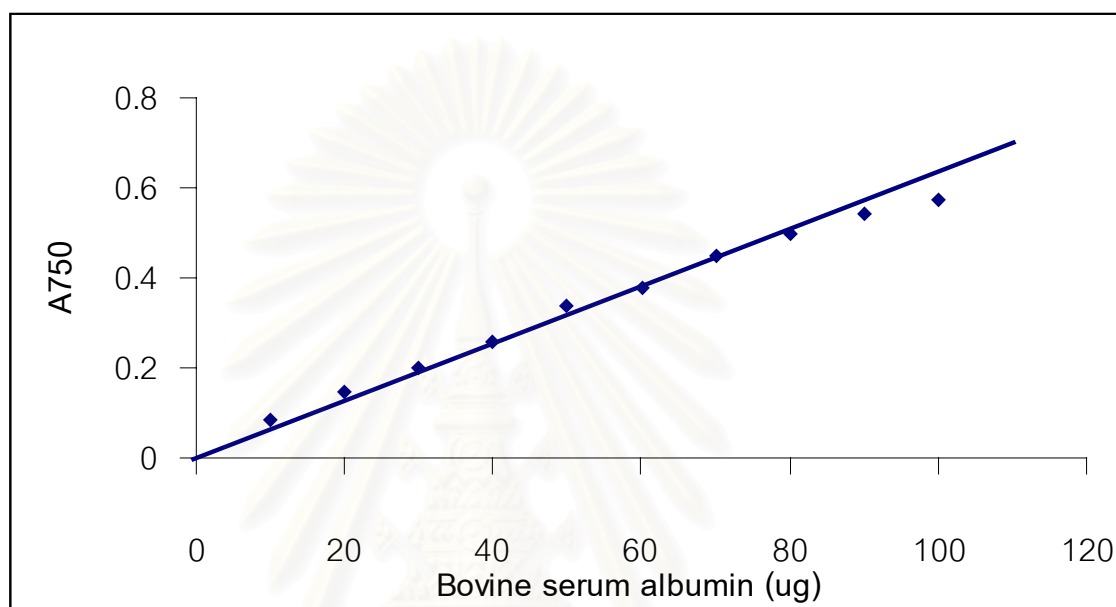
APPENDIX C

Standard curve for α-cyclodextrin determination by methyl orange assay
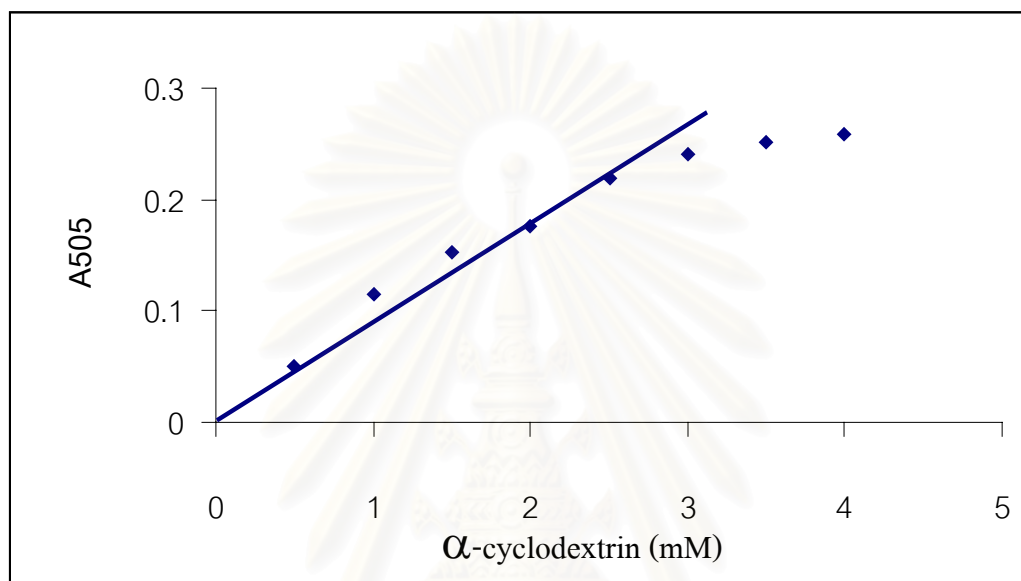
APPENDIX D

Standard curve for β-cyclodextrin determination by phenolpthalein assay
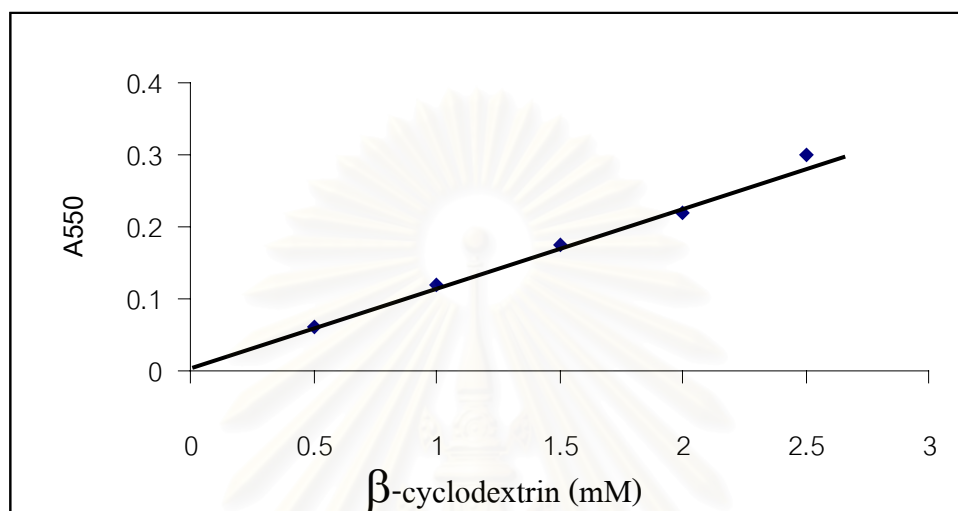
APPENDIX   E

Standard curve for γ-cyclodextrin determination by bromocresol green assay
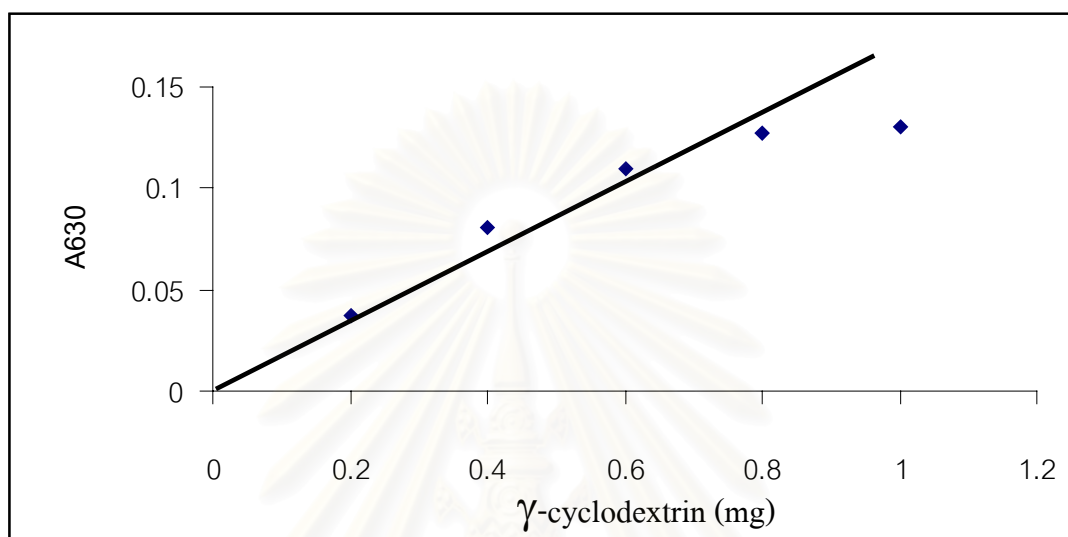
BIOGRAPHY

Mr. Anusak Keadsin was born on September 9, 1978. He graduated with the Bachelor Degree of Science in Applied Biology (Microbiology) from Suan Sunandha Rajabhat University in 1999. He has working at Department of Medical Science, Ministry of Publish Health. Then, continued studying for Master of Science in Biochemistry Program, Faculty of Science, Chulalongkorn University since 2001.