ความจำเพาะของตัวรับหมู่ไกลโคซิลในปฏิกิริยาคู่ควบและปฏิกิริยาทรานสไกลโคซิเลชันของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจาก *Bacillus circulans* A11

นางสาว วรรณนภา วงศ์แสงวัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SPECIFICITY OF GLYCOSYL ACCEPTOR IN COUPLING AND TRANSGLYCOSYLATION REACTIONS OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Bacillus circulans* A11

Miss Wannapa Wongsangwattana

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for the Degree of Master of Science in Biochemistry

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วรรณนภา วงศ์แสงวัฒนา : ความจำเพาะของตัวรับหมู่ไกลโคซิลในปฏิกิริยาคู่ควบและปฏิกิริยาทรานสไกลโคซิเลชันของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส จาก *Bacillus circulans* A11 (SPECIFICITY OF GLYCOSYL ACCEPTOR IN COUPLING AND TRANSGLYCOSYLATION REACTIONS OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Bacillus circulans* A11) อ.ที่ปรึกษา : รศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, 135 หน้า. ISBN 974-13-0740-13

ไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส ที่ผลิตได้จาก *Bacillus circulans* A11 สามารถเร่งปฏิกิริยาคู่ควบ (coupling) และปฏิกิริยาการโยกย้ายหมู่ไกลโคซิล (transglycosylation) จากคุณสมบัติดังกล่าวจึงทำให้สามารถผลิตโอลิโกแซคาไรด์ที่มีปลายเปิดและไกลโคไซด์ที่น่าสนใจและมีประโยชน์ ได้ทำการศึกษาการเกิดปฏิกิริยาโดยใช้เบต้าไซโคลเดกซ์ทรินทำหน้าที่เป็นตัวให้หมู่ไกลโคซิล และใช้ตัวรับชนิดต่างๆ จากการทดลองทางจลนพลศาสตร์พบว่า โมเลกุลของตัวรับไม่ว่าจะเป็นแซคคาไรด์ หรืออนุพันธ์ของมัน หรือกลูโคไซด์ในธรรมชาติที่มีการจัดเรียงของหมู่ไฮดรอกซิลในตำแหน่ง 2-, 3-, และ 4-ที่เหมือนกันและมี 6 คาร์บอนในโมเลกุลน้ำตาล เช่น กลูโคส ซอร์โบส กรดแอสคอร์บิค แลคโตส เซลโลไบโอส และเฮสเพอริดีน ทำหน้าที่เป็นตัวรับที่ดี ในกลุ่มตัวรับที่ให้โอลิโกแซคคาไรด์ที่มีโซ่ตรงแบบปลายเปิด พบว่าเซลโลไบโอสมีค่า V_{max}/ K_m สูงที่สุดจึงทำหน้าที่เป็นตัวรับที่ดีที่สุด นอกจากการศึกษาทางจลนพลศาสตร์แล้ว ยังมีการคำนวณปริมาณของตัวรับที่ถูกใช้ในปฏิกิริยา (transglycosylation yield) วัดโดยเทคนิค HPLC ซึ่งใช้บอกความสามารถของการเกิดปฏิกิริยาได้ จากการเปรียบเทียบค่า transglycosylation yield ของปฏิกิริยาคู่ควบและปฏิกิริยาการโยกย้ายหมู่ไกลโคซิลของแซคคาไรด์ที่ทำหน้าที่เป็นตัวรับที่ดี พบว่า เมื่อใช้เซลโลไบโอส ซอร์โบส และกลูโคส เป็นตัวรับไกลโคซิลจากเบต้าไซโคลเดกซ์ทริน ได้ค่า transglycosylation yield 78, 57 และ 54% ตามลำดับ และเมื่อใช้แป้งเป็นตัวให้หมู่ไกลโคซิล พบว่า ตัวรับซอร์โบส และ กรดแอสคอร์บิค ให้ค่า transglycosylation yield มากที่สุด คือ 63 และ 57% ตามลำดับ ผลสรุปคือ เซลโลไบโอสทำหน้าที่เป็นตัวรับที่ดีที่สุดในปฏิกิริยาคู่ควบที่มีเบต้าไซโคลเดกซ์ทรินทำหน้าที่เป็นตัวให้ ในขณะที่ซอร์โบสทำหน้าที่เป็นตัวรับที่ดีที่สุด ในปฏิกิริยาการโยกย้ายหมู่ไกลโคซิลที่มีแป้งเป็นตัวให้หมู่ไกลโคซิล

จากการศึกษาสภาวะที่เหมาะสมสำหรับปฏิกิริยาคู่ควบ โดยมีเบต้าไซโคลเดกซ์ทรินทำหน้าที่เป็นตัวให้ และเซลโลไบโอสซึ่งพบว่าเป็นตัวรับที่ดีที่สุดทำหน้าที่เป็นตัวรับ เมื่อใช้เบต้าไซโคลเดกซ์ทริน 2.0% เซลโลไบโอส 0.5% และ CGTase 16 ยูนิต บ่มที่ พีเอช 6.0 อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 2 ชั่วโมง จากสภาวะนี้จะให้ค่า transglycosylation yield 78% และให้ผลิตภัณฑ์ 2 ชนิด แยกได้ด้วย HPLC ที่ R_t 3.81 และ 4.42 นาที ในอัตรา ส่วน 1:1 โดยการคำนวณจากพื้นที่ใต้พีก ในการพิสูจน์ชนิดและโครงสร้างของผลิตภัณฑ์ ได้ทำการเก็บแยกผลิตภัณฑ์ทั้ง 2 โดยตรวจสอบจากพีก HPLC แล้วหามวลโมเลกุลและโครงสร้างโดยการใช้เทคนิค MS และ NMR พบว่าผลิตภัณฑ์ทั้งสองชนิดมีมวลโมเลกุล 504 และ 666 ดาลตัน โดยโครงสร้างทั้งสองน่าจะเป็นแซคคาไรด์ 3 หน่วยและแซคคาไรด์ 4 หน่วยของกลูโคสเรียงต่อกันเป็น glc(α1$\rightarrow$4)glc(β1$\rightarrow$4)glc และ glc(α1$\rightarrow$4)glc(α1$\rightarrow$4)glc(β1$\rightarrow$4)glc ตามลำดับ

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD : CYCLODEXTRIN GLYCLOSYLTRANSFERASE / COUPLING / OLIGOSACCHARIDE

WANNAPA WONGSANGWATTANA : SPECIFICITY OF GLYCOSYL ACCEPTOR IN COUPLING AND TRANSGLYCOSYLATION REACTIONS OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Bacillus circulans* A11. THESIS ADVISOR : ASSOC. PROF. PIAMSOOK PONGSAWASDI, Ph. D. 135 pp. ISBN 974-13-0740-13.

Cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* A11 is able to catalyze coupling and transglycosylation reactions. Characteristics of the intra-, and inter-molecular transglycosylations are the ability to form interesting and useful linear opened-chain oligosaccharides (LOCO) and glycosides. The CGTase-catalyzed coupling reaction using β-CD as glycosyl donor and various acceptors were analyzed. When kinetic parameters were determined, the result shows that efficienct acceptors, saccharides or their derivatives or natural glycosides composed of the same configuration of C2-, C3-, and C4-OH as D-glucopyranoside with six carbon units sugar monomer such as glucose, sorbose, ascorbic acid, lactose, cellobiose, and hesperidin. Among LOCO-forming acceptors, cellobiose was the best acceptor according to its highest V_{max}/ K_m values. Besides kinetic parameters, the transglycosylation yield which measured the consumption of acceptor by HPLC technique can also be used to follow how well the reaction proceeds. The transglycosylation yield of the coupling and transglycosylation reactions using β-CD or soluble starch donor with the good acceptor candidates judging from kinetic values were compared. When cellobiose, sorbose, and glucose were used as glycosyl acceptor with β-CD donor, the transglycosylation yield of 78, 57, and 54% were obtained. When glycosyl transfer was from soluble starch, good acceptors were sorbose and ascorbic acid with the transglycosylation yield of 63 and 57%, respectively. It was thus concluded that cellobiose was the best acceptor when using β-CD as glycosyl donor in the coupling reaction whereas sorbose was the best when soluble starch was used as glycosyl donor in the transglycosylation reactions.

The optimal condition for coupling reaction using cellobiose as the glycosyl acceptor with β-CD donor was also determined. The condition was performed with 2% β-CD, 0.5% cellobiose, incubated with 16 U of CGTase at pH 6.0, 30°C for 2 hrs. The transglycosylation yield obtained was 78%, with two main transfer products, PC1 and PC2, observed at R_t of 3.81 and 4.42 min, respectively. The product ratio was 1:1 as determined by peak area. In the identification of the type and the structure of the transfer products, each was collected from the separate peak of HPLC chromatogram and subjected to mass spectrometer and NMR analyses. The molecular mass of PC1 and PC2, the two transfer products were 504 and 666 daltons. The structures suggested by NMR were a trisaccharide of the structure glc(α1→4)glc(β1→4)glc and a tetrasaccharide of the structure glc(α1→4)glc(α1→4)glc(β1→4)glc.

Department Biochemistry ..... Student's signature W. Wongsangwattana

Field of study Biochemistry ..... Advisor's signature P. Pongsawasdi

Academic year 2000 ..... Co-advisor signature .....

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ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
CD	cyclodextrin
CGTase	cyclodextrin glycosyltransferase
cm	centimeter
°C	degree Celsius
g	gram
hr	hour
l	litre
mA	milliampare
min	minute
µl	microlitre
ml	millilitre
mM	millimolar
M	molar
nm	nanometer
rpm	revolution per minute
LOCO	linear opened-chain oligosaccharide
R_t	retention time

CHARTER I

INTRODUCTION

Cyclodextrins

Cyclodextrins (cycloamyloses, cyclomaltaoses, CDs) as they are known today, were called cellulosine or Schardinger dextrins. They are cyclic, non-reducing oligosaccharides composed of glucose units linked by α-1,4-glycosidic bonds. The main CDs produced from starch and related compounds by the action of cyclodextrin glycosyltransferase (CGTase) are built up from six to eight glucopyranose units, known as α-, β-, and γ-cyclodextrins, respectively, as shown in Figure1 (Schardinger, 1903, 1904; French *et al.*, 1942; Pully and French, 1961). Some physical properties of cyclodextrins are summarized in Table 1 (Szejtli, 1988). The cyclodextrins are water-soluble. This fundamental characteristic derives from the location of all free hydroxyl groups of each sucessive glucose unit on rims of these doughnut-shape molecules. The ring shape of the CDs is the consequnce of the chair C1-chair conformation of the D-glucopyranosyl units, and their 1,4-α-D-linkages. The overall appearance of the CD molecules is that of a truncated cone: the "wide" side is formed by secondary C2 and C3 hydroxyls, whereas primary C6 hydroxyls are located on the more close side, rendering the molecule hydrophilic outside. The cavity of the CDs is hydrophobic, because it is lined by C-H groups and glycosidic oxygen bridges (Saenger, 1971, 1982) (Figure 2).

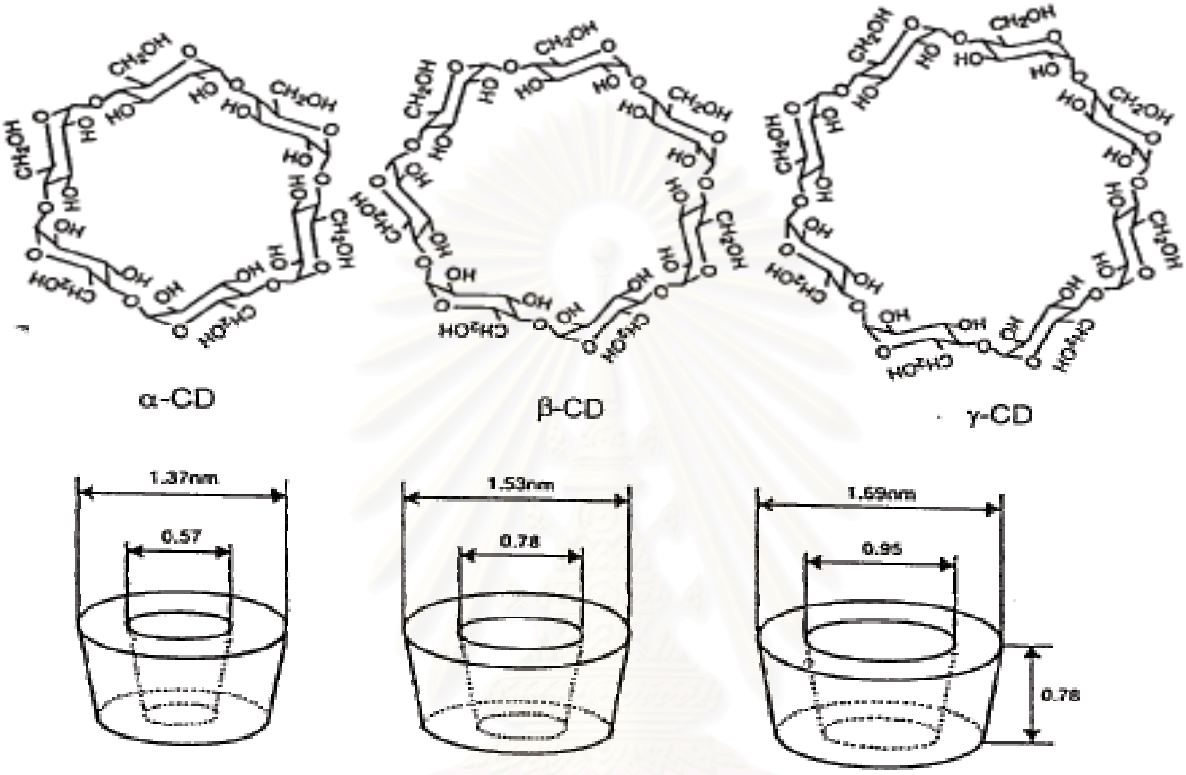


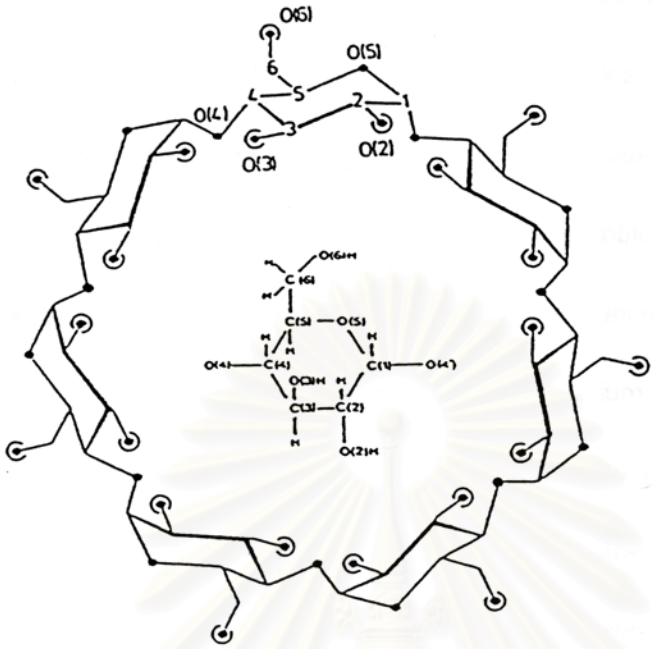
Figure 1 Structure and molecular dimension of cyclodextrins (CDs)

(Szejtli, 1990)

Table 1 Characteristics of cyclodextrins (Saenger,1982 ; Szejtli, 1988)

	α-CD	**β-CD**	**γ-CD**
Number of glucose unit	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 ml) at Ambient temp.	14.40	1.85	23.20
Cavity dimensions			
Cavity diameter (A^{o})	4.7-5.3	6.0-6.5	7.5-8.3
Cavity depth (A^{o})	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Cavity volume			
$(A^{o})^{3}$	174	262	472
ml per mol	104	157	256
ml per g	0.10	0.14	0.20
Crystal forms (from water)	Hexagonal plates	Monoclinic parallelograms	Quadratic prisms
Hydrolysis by *A. oryzae* α-amylase	Negligible	Slow	Rapid
Partial molar volume	611.4	703.8	801.2

(a)



(b)

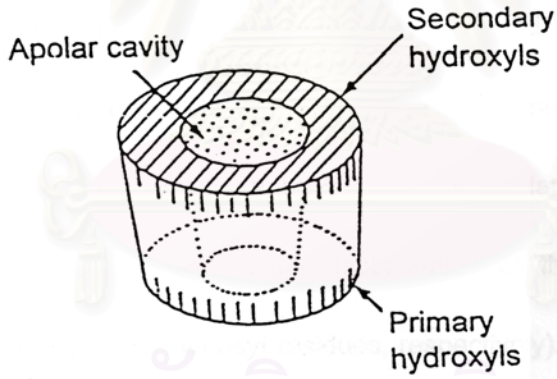


Figure 2 Structure of β-cyclodextrin (Bender, 1986; Szejtli, 1990)

(a) Chemical structure; o = oxygen atoms • = hydroxyl groups

(b) Functional structure scheme

One of the most important characteristics of the cyclodextrins is their ability to form (crystalline) inclusion complex, in which various guest molecules of suitable size are included within the cavity. (Bender, 1986) It is the interplay of the atomic (Van der Waals), thermodynamic (hydrogen bonding), and solvent (hydrophobic) force that accounts for the stable complexes that may be formed with chemical substances while in the apolar environment of the CD cavity. The complex exists in an equilibrium dependent upon the concentration of the CD, the guest chemicals and the water. The guests which can be encapsulated in cyclodextrins, included such compounds as straight or branch chain hydrocarbons, gases, and some relative polar compounds, as shown in Figure 3 (Amaizo, 1992) and Figure 4 (Janssen, 1992).

Several cyclodextrin derivatives have been developed through chemical or enzymatic means in order to obtain CDs with specific desirable properties. Examples are those with solubility better than parent compounds e.g. methylated, hydroxypropylated, maltosyl-cyclodextrins, and glucosyl-cyclodextrin (substitution of the hydroxyl groups by methyl, hydroxypropyl, maltosyl, and glucosyl residues, respectively). In the methylated CD the cavity is blocked on the sides of primary hydroxyl groups by a hydrophobic bottom which contributes to complex stability. CD-polymers (cross-linked cyclodextrins) are often used as stationary phase in various liquid chromatography system (Casu and Ruggiani, 1979; Ensuiko, 1994; Yamamoto *et al*, 1990). These modified CDs, in addition to their native or parent CDs (α-, β-, and γ-CD), can be chosen according to their properties to be used as the suitable host molecules.

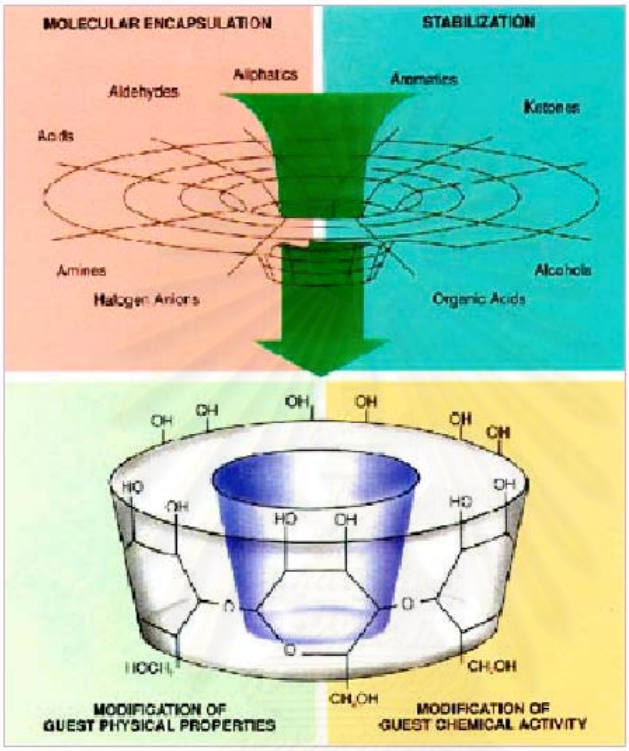


Figure 3 Beneficial modification of guest molecules by cyclodextrins (Amaizo, 1993)

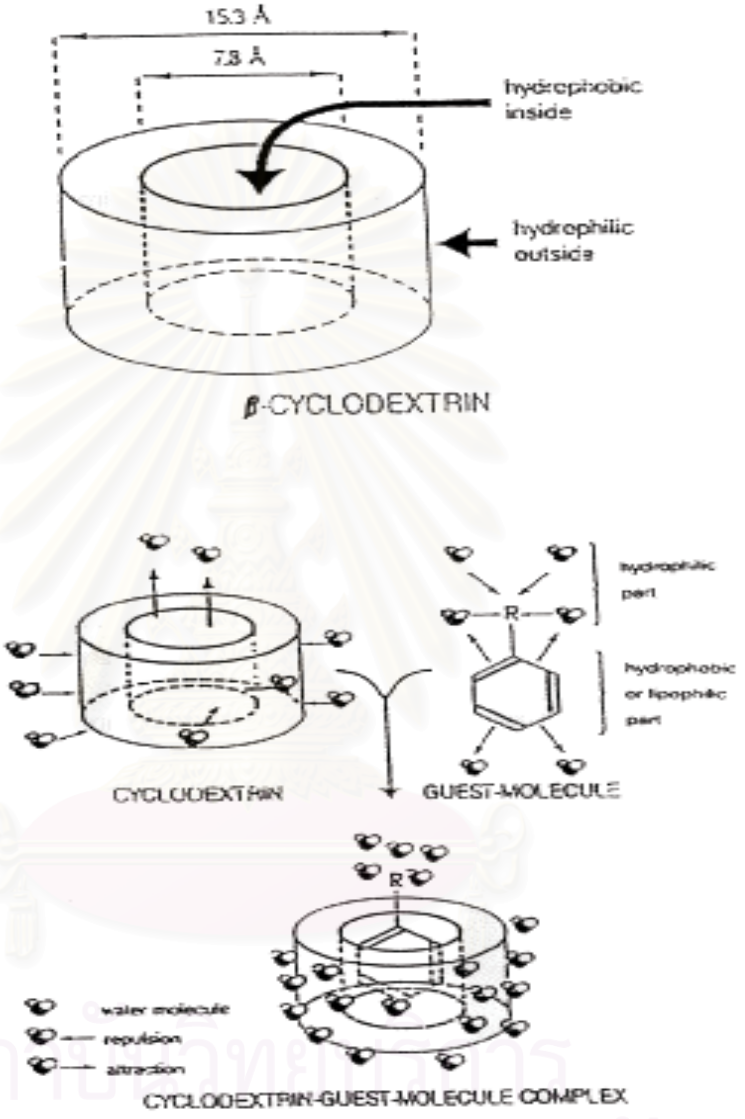


Figure 4 Guest orientation in CD-guest complex (Janssen, 1992)

Complex formation of cyclodextrin and guest molecules leads to the change in some physical or chemical properties of the guest molecules.Stabilization of the light- or oxygen-sensitive substances or labile compounds, solubilization of non-soluble components, modification of the chemical activity of the guest molecules, fixation of very volatile substances, modification of the physiochemical properties of the guest molecules such as conversion of viscous or oily compounds into powders, or reduction of unpleasent tastes in products e.g. food stuffs, are among those known useful properties (Schmid, 1989; Shiraishi, 1989). The applications of cyclodextrins as solubilizers, emulsifiers, antioxidants, stabilizing agent, and ingredient removers have been increased rapidly in food, cosmetics, pharmaceutical, agrochemical, and plastic industries (Table 2) (Nagamoto, 1985). Since early 1970s, many countries for example, Japan, Germany, France, Netherlands, Denmark, Spain, Italy, Belgium, Hungary, USA, and Taiwan, have approved the use of cyclodextrins (at different levels) in several fields of industries (Amaizo, 1991).

Cyclodextrin producing enzyme

Cyclodextrin glycosyltransferase (1,4-α-D-glucan: 1,4-α-D-glucopyranosyl transferase, EC 2.4.1.19 , CGTase) is known to catalyze the degradation of starch and related α-(1,4) glucans to cyclodextrins. CGTase is produced by various microorganisms, for example, *Klebseilla pneumoniae* (Bender, 1977), *Brevibacterium* sp.(Mori *et al*.,1994) and mainly the *Bacillus* species, as listed in Table 3. The CGTase can be divided into three types, α-, β-, and γ-, according to the major type of CDs formed (Horikoshi, 1988). The enzyme from different sources show slightly different

Table 2 Industrial applications of cyclodextrins (Horikoshi, 1982; Bender, 1986**;** Szejtli and Pagington, 1991)

Use	Guest compound/ end product
Food	
1.Emulsification	Eggless mayonnaise, seasoning oil, whipping cream, etc.
2.Increase of forming powder	Egg white (freeze-dry), hotcake-mix, cake-mix, etc.
3.Stabilization of flavors and seasoning	Chewing gum flavor, biscuit flavor, seasoning powder, instant noodles, seasoning paste, etc.
4.Taste masking	Meat paste
5.Reduction of hygroscopicity	Powder flavor products
6.Elimination of unpleasant tastes	Juice, milk, casein, ginseng, propylene glycol
7.Elimination of cholesterol	Egg yolk, milk, butter
8.Reduction of odor	Mutton, fish, soybean
Cosmetics and toiletries	
1.Color masking and control	Fluorescein, bath agent
2.Stabilization of fragrance	Menthol
3.Stabilization	Chalcone, dihydrochalcone (toothpaste), Perfume
4.Preventing inflammation of skin	Skin lotion, sun block cream
5.Deodorant	Mouth wash, refrigerator
6.Reduction of irritation	Shampoo, cream, skin powder
7.Enhancement of attained concentration	Skin moisturizing lotion
8.Defoaming effect	Laundry

Table 2 Industrial applications of cyclodextrins (continued)

Use	Guest compound/ end product
Agriculture	
1.Stabilization of volatility	Tobacco aroma
2.Stabilization of nutrient	Animal-feed
3.Improvement of palatability	Bone-powder, microbial cell-mass
Pharmaceuticals	
1.Increase of solubility	Prostaglandin, phenobarbital, chloramphenical
2.Taste masking	Prostaglandin
3.Powdering (non-volatile)	Nitroglycerin, clofibrate
4.Stabilization (UV, thermal)	Prostaglandin, vitamin
5.Decrease irritation	Cu-alcanomine complex, tiamulin
6.Enhancement of bioavailability	Barbiturate, flufenamic acid, digixin
7.Reduction of systemic toxicity	2-amino, 4-methyl-phosphynobutyric acid
Pesticides	
1.Stabilization (UV, thermal)	Pyrethrins, pyretenoids, isoprenoids
2.Powdering (non-volatile)	DDVP and other organic phosphorus Pesticides
Chemical technology	
Catalyzation for reaction	Products of hydrolysis, substitution, Diels-Alder reaction, stereospecific reaction, etc.
Plastic	
Stabilization	Colors, flavors
Others	Adhesives

Table 3 Properties of cyclodextrin glycosyltransferases

Organism	Predominant product	Optimum pH	Optimum temperature (°C)	MW (dalton)	pI	Reference
Alkalophilic *Bacillus* 17-1	β-CD	6.0	ND	74,000	ND	Yamamoto *et al.*, 1972
Bacillus fermus/lentus	γ-CD	6.0-8.0	50	75,000	4.1	Englbrecht *et al.*, 1990
Bacillus macerans IFO 3490	α-CD	5.0-5.7	55	5,000	4.6	Kitahata *et al.*, 1974
Bacillus macerans IAM 1243	α-CD	5.5-7.5	60	145,000	ND	Kobayashi *et al.*, 1977
Bacillus macerans ATCC 8514	α-CD	6.2	ND	139,300	ND	Deprito and Campbell, 1986
Bacillus megaterium	β-CD	5.0-5.7	55	ND	6.07	Kitahata and Okada, 1974
Bacillus stearothermophilus	α-CD	6.0	ND	68,000	4.5	Kitahata and Okada, 1982
Klebsiella pneumoniae M5 al	α-CD	6.0-7.2	ND	68,000	4.8	Bender, 1982
Micrococcus sp.	β-CD	6.2	ND	139,300	ND	Yagi *et al.*, 1980

ND = no data

pH and molecular weight. Each CGTase enzyme yields different ratio of CD-products (Table 4).

CGTase catalyzes three transglycosylation reactions :cyclization, coupling and disproportionation. Cyclization, a single substrate reaction, with an affinity for the high molecular mass substrate, is the formation of cyclodextrin through an intramolecular transglycosylation reaction. Coupling, the reverse reaction, is the bisubstrate reaction, in which a cyclodextrin ring is cleaved and transferred to a linear acceptor substrate, and proceeds according to a random-order ternary complex mechanism. Disproportionation is the major transferase reaction, in which a linear malto-oligosaccharide is cleaved and transferred to the linear acceptor substrate, also a bisubstrate reaction, and proved to follow the ping-pong type of mechanism. The proposed model of the event taking place in the CGTase-catalyzed reaction was shown in Figure 5 .In addition, the enzyme has weak hydrolyzing activity (Figure 6)(Bart A. *et al*., 2000). These mechanisms are summarized in Table 5.

Studies on transglycosylation reactions of some CGTases have been reported, though not as extensive and well-known as other sugar transferases such as glucosidases and mannosidases (Takana, *et.al*., 1991). For CGTase, transglycosylation occurs when either starch or cyclodextrin is used as glycosyl donor. However, to be more specific, the term “coupling”, a type of transglycosylation, is often referred when the glycosyl donor used is the cyclodextrin. For acceptor compounds, various saccharides and glucosides, for example glucose, maltose and hesperidin have been identified.

Table 4 Ratio of α-, β-, and γ-CDs produced by various CGTases

(Yamamoto T. *et al.* ,1994)

Enzyme source	α-CD	:	β-CD	:	γ-CD
B.macerans	5.7	:	1.0	:	0.4
B.megaterium	1.0	:	6.3	:	1.3
B.circulans	1.0	:	6.4	:	1.4
B.sterothermophilus	1.7	:	1.0	:	0.3
Klebsiella oxytoca	α-CD	>	β-CD	,	γ-CD
Bacillus sp.(alkalophilic)					
Acid	α-CD	>	β-CD	,	γ-CD
Neutral	α-CD	>	β-CD	,	γ-CD
B.ohbensis	0	:	5.0	:	1.0
Bacillus sp.AI 6	0	:	1.0	:	2.7

Table 5 Summarization of CGTase mechanisms (Okada and Kitahata, 1975)

Reaction	Action
Cyclization	Starch ⟶ cyclodextrin
Coupling	Cyclodextrin + glucose ⟶ oligosaccharide
Disproportionation	$(\text{oligosaccharide})_m + (\text{oligosaccharide})_n \longrightarrow (\text{oligosaccharide})_{m-x} + (\text{oligosaccharide})_{n+x}$

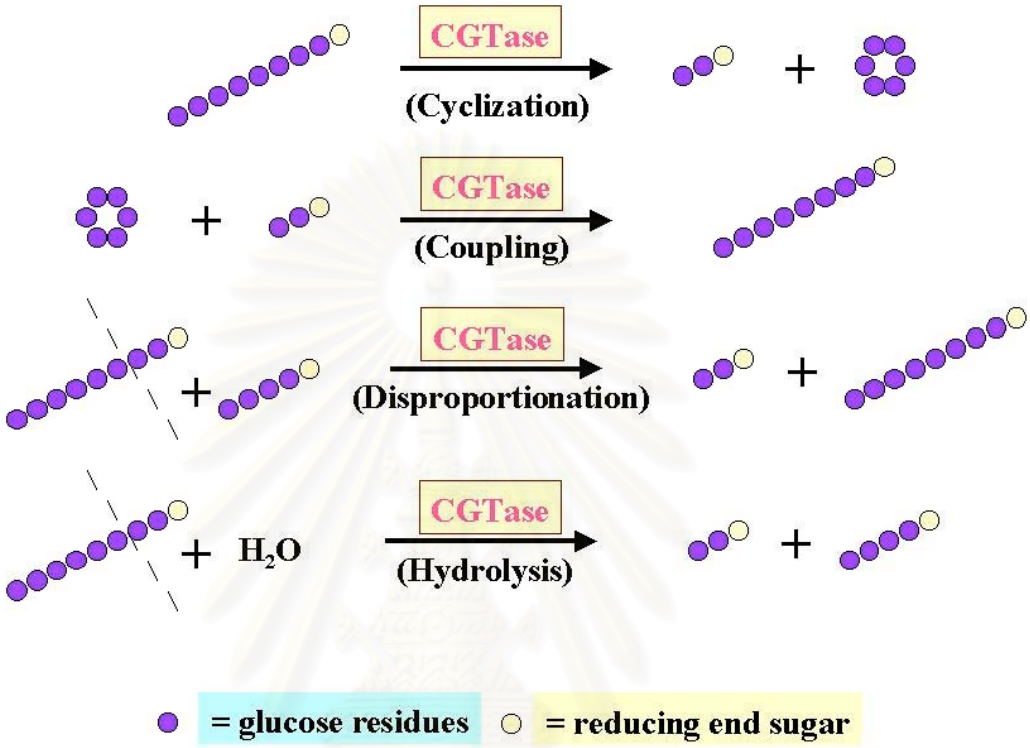


Figure 5 The CGTase-catalyzed transglycosylation reactions.

(A) Cyclization (B) Coupling (C) Disproportionation and (D) Hydrolysis.

The blue circles represent glucose residue: the yellow circles indicate the reducing end glucose. (Bart A. *et al*., 2000)

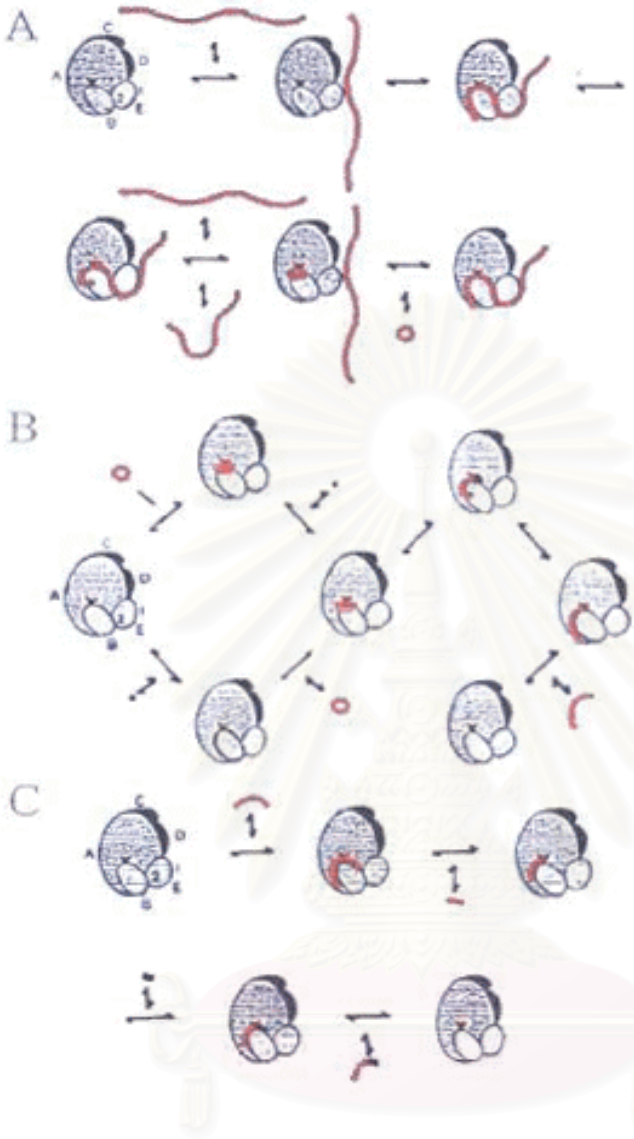


Figure 6 Proposed model of the event taking place in the CGTase-catalyzed reactions.

(Bart A. *et. al.*,2000)

(A) Cyclization. (B) Coupling. (C) Disproportionation. The different CGTase domains are indicated (A, B, C, D,and E). 1 and 2 indicated the maltose binding sites on the E-domain. The triangle indicated the cleavage site in the active site. Small red circles represent glucose residues : acceptor residues are represented in small black circles.

Kitahata (1976) investigated the acceptor specificity of the transglycosylation of CGTase from *Bacillus megaterium* strain No.5 using different sugars and sugar alcohols. It was shown that L-sorbose, D-xylose, and D-galactose, which contain configuration or structural changes relative to the D-glucopyranose unit at position other than position 1, were efficient acceptors in the transglycosylation when soluble starch was used as the glycosyl donor. In 1992 Kitahata *et.al.* reported that *Bacillus stearothermophilus* CGTase had a wider acceptor specificity than *Bacillus macerans* CGTase and produced different types of transfer products from various acceptors such as D-galactose, D-mannose, D-fructose, D- and L-arabinose, D- and L-fucose, L-rhamnose, D-glucosamine, and lactose, while these sugars were inefficient acceptors for *Bacillus macerans.* In 1991 Abe proposed from the work with CGTase of *Bacillus macerans* that CGTase transfers residues of malto-oligosaccharides to the non-reducing-end glucose residue of the acceptors to form α-1,4-glucans and the requirement for an acceptor of the intermolecular transfer reaction is the pyranose structure having the same configuration of the free C2-, C3-, and C4-hydroxyl groups as D-glucopyranose. Nakamura *et. al.* (1994) proved that the same configuration of free hydroxyl groups with those of D-glucopyranose at C2, C3, and C4 positions were required for the acceptors used by CGTase from alkalophilic *Bacillus* sp.. The structure around C6 on acceptors was not essential for acceptor function, but it was recognized by CGTase. And at least two glucopyranosyl rings were recognized by the acceptor binding sites.

In addition to the linear opened-chain-oligosaccharide transglycosylated products, different glycosides can also be formed by coupling or transglycosylation reaction of CGTase. In 1991 Aga *et.al.* reported that CGTase from *Bacillus stearothermophilus*

catalyzed the transglycosylation from α-cyclodextrin glycosyl donor to L-ascorbic acid acceptor and formed 2-O-α-D-glucopyranosyl L-ascorbic acid which was a useful glycoside. CGTase from an alkalophilic *Bacillus* species produced hesperidin monoglucoside and a series of its oligosaccharides by the transglycosylation reaction with hesperidin (a flavonoid in citrus) as an acceptor and soluble starch as a donor (Kometani *et.al.*, 1994). In 1996 they also reported that CGTase from an alkalophilic *Bacillus* species catalyzed the glycosyl transfer from β-CD and soluble starch to neohesperidin and naringin, thus formed neohesperidin glycosides and naringin glycosides, respectively. It was also reported that *Bacillus stearothermophilus* CGTase catalyzed the transglycosylation reaction that used dextrin as glycosyl donor and rutin as the acceptor to form a glycosylated rutin product (Suzuki, 1991).

It is well recognized that transglycosylation often results in new properties of glycosylated compounds. The transglycosylated opened-chain-saccharide or glycoside products that have mono- or oligo-saccharide residues linked to their parent acceptor molecules usually show significantly improved functionalities such as improved sweetness, increased water solubility, increased stability against chemical or stimulating growth of Bifidobacteria, (Park *et. al.*, 1997). Kobayashi and Abe (1984) reported that glucosyl (α1→ 6) -α-CD was more soluble in water than the parent α-CD. From the coupling reaction of ascorbic acid and α-cyclodextrin by CGTase, glycosyl ascorbic acid is reported to be more stable to oxidation than its parent ascorbic acid because of glucosylation at C2-OH of ascorbic acid (Kurimoto *et al.*, 1997). The water solubility of hesperidin glycosides, a transglycosylation product of hesperidin and β-CD, was 300 times higher than hesperidin. The yellow crocin, from fruits of *Gardenia jasminoides*, was reported to be more stabilized

against ultraviolet radiation in the glycoside form (Kometani *et. al.*, 1994). In 1996 Kometani *et. al.* also reported that the solubility of neohesperidin glycoside in water was 1,500 times higher than that of neohesperidin. In addition, the bitterness of neohesperidin glycoside was 10 times less than its parent neohesperidin.

The present thesis is a continual part of the work of the cyclodextrin research group at the Biochemistry Department, Faculty of Science, Chulalongkorn University. The CGTase of *Bacillus circulans* A11, screened from South-East Asian soil (Pongsawasdi and Yagisawa, 1987), was purified and characterized (Techaiyakul, 1991; Rojtinnakorn, 1994). Specific antibody against CGTase was prepared (Rojtinnakorn, 1994) and was used in purification through immunoaffinity column chromatography (Kim, 1996). Tongsima (1998) reported on the essential amino acid at the active site of CGTase and Kaskangam (1998) isolated and characterized CGTase isoforms. The gene coding for CGTase has been cloned and sequenced (Rimphanichayakit, 2000) and Kualpiboon (2000) identified essential histidines in CGTase isoform 1 from *Bacillus circulans* A11. For this study, since the advantage of coupling and transglycosylation reactions of CGTase in the production of interesting oligosaccharides or the formation of glycosides of better quality has been evidenced, the focus will be on the investigation of the acceptor specificity of our CGTase enzyme.

The objective of this research

1. To determine the specificity of glycosyl acceptor in coupling and transglycosylation reactions.

2. To choose the suitable acceptor for the production of linear opened-chain oligosaccharides.
3. To find the optimum conditions in the CGTase-catalyzed coupling reaction using the suitable acceptor chosen.
4. To isolate and characterize the transfer products from the reaction.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

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

Diaflo ultrafiltration: Stirrer Ultrafiltration Cell 8050, Amicon W.R. Grace Cooperation, USA

Electrophoresis unit: model Mini-protein II Cell, Bio-Rad, USA

Freeze-dryer: Stone Ridge, New York, USA

Fraction collector: model 2211 Pharmacia LKB, Sweden

High Performance Liquid Chromatography: Model LC 31 Shimadzu, Japan & Applied Biosystems PTH

Incubator: Haraeus, Germany

Incubator Shaker, Controlled environment: Psyco-therm, New Brunswick Scientific Co., USA

Magnetic stirrer: 0188 GMS, Scientific Instrument Development and Service Center, Faculty of Science, Chulalongkorn University

Peristaltic pump: Pharmacia LKB, Sweden

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

Plastic petri dish (60x15 mm): Costar, USA

Spectrophotometer UV-240, Shimadzu, Japan, and Du series 650, Beckman, USA

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

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

Water bath, shaking: Heto lab Equipment, Denmark

2.2 Chemicals

N-acetyl-D-glucosamine: Sigma, USA

Acetronitrile (HPLC grade) : J.T. Baker Chemical, USA

Acrylamide: Merck, USA

Amyloglucosidase (Glucoamylase): from *Aspergillus niger* 70.7 u/mg, Fluka, Switzerland

Arabinose: BDH, England

Ascorbic acid: Sigma, USA

Butanol: Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

Cellobiose: Sigma, USA

Cholesterol: Sigma, USA

Cyanogen bromide activated Sepharose 4B: Sigma, USA

β-Cyclodextrin : Sigma, USA

DEAE-cellulose resin: DE 32, Whatman Biosystems Ltd., England

Decanol: BDH, England

2-Deoxy-D-glucose: Sigma, USA

Dialysis tubing: Sigma, USA

3,5-Dinitrosalicylic acid: Sigma, USA

Ethanolamine: BDH, England

Fructose: Sigma, USA

Fucose: Sigma, USA

Galactose: BDH, England

Geraniol: Sigma, USA

Gluconic acid: Sigma, USA

D (+) glucose: Sigma, USA

Glycine: Sigma, USA

Hesperidin: Sigma, USA

Hexanol: Sigma, USA

Hydroquinone: Sigma, USA

Myo-inositol: Sigma, USA

Isomaltose: Sigma, USA

Lactose: BDH, England

D (+)-maltose monohydrate: Fluka, Switzerland

Mannitol: Sigma, USA

D (+) mannose: Sigma, USA

N,N´-methylene-bis-acrylamide : Sigma, USA

Naringin: Sigma, USA

Noble agar: BBL, Becton, Dickinson and Company, USA

Octanol: Fluka, Swittzerland

Pentanol: Sigma, USA

Phenolphthalein: BDH, England

Potassium sodium tartrate: Sigma, USA

Resorcinol: Sigma, USA

Rhamnose: Sigma, USA

Ribose: Sigma, USA

Rutin: Sigma, USA

Salicin: Sigma, USA

Sorbitol: Sigma, USA

Sorbose: Sigma, USA

Standard molecular weight marker protein: Sigma, USA

Soluble starch, potato: Sigma, USA

Trichloroethylene: BDH, England

Thymol: Sigma, USA

Xylose: Sigma, USA

The other common chemicals were of reagent grade. Raw rice starch (three-head elephant brand), corn starch (Maizena) were locally purchased.

2.3 Bacteria

Bacillus circulans A11, isolated from South-East Asian soil, was screened for CGTase activity by Pongsawasdi and Yagisawa (1987).

2.4 Media preparation

2.4.1 Medium I

Medium I was consisted of beef extract (0.5%), peptone (1.0%), NaCl (0.2%), yeast extract (0.2%) and soluble starch (1.0%) and the pH was adjusted to 7.2 with 1 M NaOH. For solid medium, 1.5 % agar was added.

2.4.2. Cultivation medium (Horikoshi's medium)

Medium for enzyme production, slightly modified from Horikoshi (1971) (Rutchtorn, 1993), contained local grade of rice starch (1.0%), peptone (0.5%), yeast extract (0.5%), K_2HPO_4 (0.1%), $MgSO_4.7H_2O$ (0.02%) and Na_2CO_3 (0.75%). The pH of the medium was 10.1-10.2.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

Bacillus circulans A11, after 18 hours inoculation at 37°C on solid medium I, was grown in 50 ml of starter Medium I in 250 ml Erlenmeyer flask at 37°C until A_{420} reached 0.3-0.5 or about 3-5 hours.

2.5.2 Enzyme production

Starter *Bacillus circulans* A11 was transferred into 500 ml of Horikoshi's medium in 1 litre Erlenmeyer flask with 1% inoculum and cultivated at 37°C. Culture was

harvested at 72 hours and cells were removed by centrifugation at 3,000 rpm for 30 minutes at 4°C. Culture broth with crude CGTase was collected and kept at 4°C for purification.

2.6 Preparation of anti-CGTase antibodies

Antibodies directed against purified CGTase from *Bacillus circulans* A11 was raised in rabbits by Rojtinnakorn (1994). The serum was kept frozen at –80 °C. The IgG fraction was isolated from the crude serum by ammonium sulfate precipitation and ion exchange chromatography on DEAE cellulose following the method previously reported (Kim, 1996).

2.7 Purification of CGTase

CGTase was purified from the culture broth of *Bacillus circulans* A11 using the techniques previously reported (Kim, 1996).

2.7.1 Starch adsorption

Corn starch was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude CGTase broth to make 5 g% concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 3,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake with TB1 buffer containing 0.2 M maltose (2x125 ml for starting broth of 1 litre), by stirring for 30 minutes. Eluted CGTase was recovered by centrifugation at 3,000 rpm for 30 minutes. The solution was dialyzed against 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$, each time for at least 4 hours, at 4°C with 3 changes of buffer. The enzyme solution

was concentrated by passing through an ultrafiltration membrane filter with molecular weight cut-off 10,000 daltons.

2.7.2 Immunoaffinity chromatography

Coupling of CNBr - activated Sepharose 4B was followed by those methods described (Pharmacia, 1979). CNBr-activated Sepharose 4B (1.43 g) was swollen by immersing in 50 ml of 1mM HCl for 15 minutes and washed on a sintered glass filter with 150 ml of the same solution. The gel was then washed with 25 ml of coupling buffer (0.1 M $NaHCO_3$ buffer pH 8.3 containing 0.5 M NaCl) and immediately transferred to a solution of the ligand (10 ml of purified anti-CGTase). The mixture containing ligand and swollen gel was rotated end-over-end overnight at 4^{o}C. The gel was transferred to 25 ml of 1 M ethanolamine pH 8.0 (blocking agent), rotated end-over-end for 2 hours at room temperature and removed the excess uncoupled ligand that remains after coupling by washing alternately with 25 ml of 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl and the same volume of 0.1 M $NaHCO_3$ buffer pH 8.3 containing 0.5 M NaCl three times. The gel was packed into a column of 0.8 x 4.5 cm and equilibrated with 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl at the flow rate of 6 ml / hour.

The concentrated enzyme solution was applied on an immunoaffinity column chromatography at 4^{o}C. The column was washed with 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl at the flow rate 2 ml / hour until A_{280} was negligible. Elution was performed by 3.5 M NaSCN in 50 mM NH_4OH pH 10.5 at room temperature (approximately 25 ^{o}C) and the flow rate at 6 ml/hour. Fractions of 2 ml were collected for measurement of

A_{280} and dextrinizing activity. After collecting the active fractions, the enzyme solution was concentrated and dialyzed against 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$, each time for at least 4 hours, at 4^{o}C with 3 changes of buffer.

2.8 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gels, were employed for analysis of the purified protein.

2.8.1 Non-denaturing polyacrylamide gel electrophoresis

Discontinuous PAGE was performed according to Rojtinnakorn (1994) on slab gels (10 x 8 x 0.75 cm), of 7.5% (w/v) separating gel, and 5.0% (w/v) stacking gels. Tris-glycine buffer pH 8.3 was used as electrode buffer (see Appendix 1). The electrophoresis was run from cathode towards anode at constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit.

2.8.2 SDS-PAGE

The denaturing gel was performed according to Bollag and Edelstein (1991). The gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels and Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as electrode buffer (see Appendix 1). Samples to be analyzed were treated with sample buffer and boiled for 5 minutes prior to application to the gel. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

2.8.3 Detection of proteins

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining. For non-denaturing gel, dextrinizing activity staining was also undertaken.

2.8.3.1 Coomassie blue staining

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 slab gels were destained with a solution of 10% methanol and 10% acetic acid for 1-2 hours, followed by several changes of destaining solution until gel background was clear.

2.8.3.2 Dextrinizing activity staining

The running gel was soaked in 10 ml of substrate solution, containing 2.0% (w/v) soluble starch (potato) in 0.2 M phosphate buffer pH 6.0, at 40^{o}C for 10 minutes. The gel was then quickly rinsed several times with distilled water and 10 ml of I_2 staining reagent (0.2% I_2 in 2% KI) was added for color development at room temperature. The clear zone on the blue background represents starch degrading activity of the protein.

2.9 Enzyme assay

For this study, CGTase activity was determined by different assays depending on the experiment. For purification, assay of starch degrading (dextrinizing) activity and assay of CD product through the formation of CD-trichloroethylene complex (CD-TCE) were made.

For the studies on the effect of acceptors on transglycosylation reaction , CD-degrading activity and coupling activity assays were used.

2.9.1 Dextrinizing activity assay

Dextrinizing activity of CGTase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (10-100 μl) was incubated with 0.3 ml of starch substrate (0.2 g%) soluble starch (potato) in 0.2 M phosphate buffer pH 6.0) at 40°C for 10 minutes. The reaction was stopped with 4 ml of 0.2 M 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. For a control tube, HCl was added before the enzyme sample.

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 conditions.

2.9.2 Cyclodextrin-trichloroethylene (CD-TCE) assay

Cyclodextrin-trichloroethylene (CD-TCE) activity was determined by the method of Nomoto *et al.* (1986) with slight modification (Rojtinnakorn, 1994).

The enzyme sample was diluted in serial double dilution by 0.2 M phosphate buffer, pH 6.0. The reaction mixture, containing 0.5 ml of enzyme sample and 2.5 ml of

starch substrate (2.0% w/v soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) was incubated at 40°C for 24 hours. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in the dark. The activity was expressed in term of the dilution limit ($1:2^n$), as the highest dilution that can produce observable CD-TCE precipitate between upper starch solution layer and lower TCE layer.

2.9.3 Cyclodextrin-degrading activity assay (Sin *et al*. 1994)

The CD-degrading activity was determined at 40 °C using 10 μl of 0.3 mg/ml of the purified CGTase with 10 mM of β-cyclodextrin as donor and variable concentrations of the acceptor (1-20 mM). 50 mM acetate buffer pH 6.0 was added to make the total volume of 0.25 ml. After that 0.002 U of *Aspergillus niger* glucoamylase (10 μ l of 0.2 U/ml enzyme dissolved in water) was then added to convert linearized oligosaccharides to glucose. Measuring the released reducing sugars which monitored the amounts of cyclodextrin degraded was performed by the dinitrosalicylic acid method as described in section 2.11.

2.9.4 Coupling activity assay (modified from Penning *et al*. 1995)

Coupling activity was assayed with 10 mM of β-cyclodextrin plus variable concentrations of acceptor (1-20 mM). Incubation with 10 μl of 0.3 mg/ml of purified CGTase at 40°C, for 5 minutes was performed. 50 mM acetate buffer pH 6.0 was added to make the total volume of 0.25 ml. β-cyclodextrin disappearance was measured by the phenolphthalein method of Vikmon (1981) with slight modification. The method was

as following : 100 μl of β-Cyclodextrin standard (0-2.5 mM) or sample (β-cyclodextrin left after incubation of CGTase with β-cyclodextrin and acceptor) was incubated with 2 ml of 75 μM phenolphthalein in 6 mM Na_2CO_3 and 2.9 ml of 6 mM Na_2CO_3 at 40°C for 20 minutes. The decrease in absorbance at 550 nm caused by complexing of the dye with β-cyclodextrin was measured. Conversion of ΔA_{550} to μmoles β-cyclodextrin was performed using the β-cyclodextrin phenolphthalein standard curve.

2.10 Protein determination

Protein concentration was determined by the Coomassie blue micro method according to Bradford (1976), using bovine serum albumin as standard.

One hundred microlitres of sample was mixed with 1 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of 95% ethanol, 100 ml of 85% H_3PO_4 and distilled water.

2.11 Reducing sugar determination

Reducing sugar was determined by the method of Miller (1959). Glucose standard (0-50 mM) was prepared. Then 0.5 ml of standard glucose or sample was mixed with 0.5 ml of dinitrosalicylic acid reagent (prepared as described below). The solution was heated for 5 minutes in a boiling water bath, then the tubes were cooled in a bowl of cold water for 5 minutes. The mixture was adjusted to a final volume of 5 ml with distilled water.

After mixing, the absorbance at 540 nm was recorded. The quantity of reducing sugar in the sample was determined from the standard curve of glucose.

Dinitrosalicylic acid reagent was consisted of dinitrosalicylic acid (5 g), 2N NaOH (100 ml), and potassium sodium tartrate (150 g) in the total volume of 500 ml adjusted by using distilled water.

2.12 Effect of acceptors which form linear opened-chain oligosaccharides on the CGTase catalyzed coupling and transglycosylation reactions

Various saccharides including monosaccharides and disaccharides were compared for their ability as glycosyl acceptors for coupling and both inter- and intra- molecular transglycosylation reactions. The glycosyl donor used was β -cyclodextrin (for coupling) and soluble starch (for transglycosylation). When using β-CD, a final concentration of 10 mM which was approximately six times higher than its K_m values (with G_3 acceptor) was used (Tongsima, 1998). For all reactions, β -cyclodextrin was incubated with variable amounts of different acceptors (1-20 mM) and the reactions were followed by measuring the increase in reducing sugars obtained from the opening of CD ring and transglycosylation using the CD-degrading activity assay as described in 2.9.3. Kinetic parameters, K_m and V_{max}, were determined from the Michaelis-Menten equation using nonlinear least regression analysis of the EZ-FIT V1.1 computer program.

In addition to using kinetic values to compare ability of acceptors, transglycosylation yield was another parameter which was compared. Both β-CD and soluble starch were used as glycosyl donors. Only good acceptors judging from kinetic values were used in this

experiment. The reaction condition was as optimized in section 2.15 with either 2% soluble starch or β-CD as glycosyl donor. Concentration of acceptors used was 0.5%. The decrease in substrates and the formation of products were determined by HPLC as described in section 2.15. Transglycosylation yield of the reactions was calculated as mentioned in section 2.16. The ratio of the transfer products was determined by comparing peak areas from HPLC profiles.

2.13 Effect of acceptors which form glycosides on the CGTase catalyzed coupling reaction

Various compounds which form glycosides upon addition of glycosyl residues were compared for their ability as glycosyl acceptors for intermolecular transglycosylation reaction. These compounds were those with hydroxyl groups e.g. alcohols (both aliphatic and aromatic), cholesterol, carbohydrate derivatives such as ascorbic acid, and some natural glycosides such as hesperidin, rutin, etc. The glycosyl donor used was β -cyclodextrin at the final concentration of 10 mM which was approximately six times higher than its K_m values when G_3 was used as the acceptor (Tongsima, 1998). For all reactions, β-cyclodextrin was incubated with variable amounts of different acceptors (1-20 mM) and the reactions were followed by either measuring the increase in reducing sugars or disappearance of β-cyclodextrin substrate. Measuring the increase in reducing sugars obtained from the opening of the CD ring and transglycosylation was by the CD-degrading activity assay as described in 2.9.3. while measuring the disappearance of β-cyclodextrin substrate was performed by the phenolphthalein method as described in 2.9.4. Kinetic

parameters, K_m and V_{max} were determined from the Michaelis-Menten equation using nonlinear least square analysis of the EZ-FIT V1.1 computer program.

2.14 Effect of acceptor on the affinity constant of β-CD in the CGTase catalyzed coupling reaction

β-CD was compared for it ability as glycosyl donor for coupling and intermolecular transglycosylation reactions with some small saccharide acceptors. For all reactions, a glycosyl acceptor at 5 mM final concentration (Tongsima, 1998) was incubated with variable amounts of β-CD (1-30 mM) and the reactions were followed by measuring the increase in reducing sugars obtained from the opening of CD ring and the transglycosylation using the CD-degrading activity assay as described in 2.9.3. Kinetic parameters, K_m and V_{max} were determined from the Michaelis-Menten equation using nonlinear least regression analysis of the EZ-FIT V1.1 computer program.

2.15 Determination of suitable conditions for the production of linear opened-chain-oligosaccharides from the most suitable carbohydrate acceptor

From the experiments on the effect of the acceptors on the CGTase catalyzed-coupling and transglycosylation performed in section 2.12 and 2.13, the most suitable carbohydrate acceptor which produced linear opened-chain-oligosaccharides was chosen. The criteria of chosen was by comparing kinetic parameters, K_m and V_{max}. Acceptors with low K_m and high V_{max} values were grouped as good candidates for further studies. Other than kinetic parameters, tranglycosylation yield was another parameter which was used in

acceptor selection. Only one acceptor was selected for further study in the production of interesting linear opened-chain oligosaccharides in this work. For glycosyl donor, β-CD was chosen because the transglycosylation yield of β-CD was higher than that of soluble starch for all acceptors tested.

2.15.1 Effect of incubation time

The incubation time of the CGTase catalyzed-coupling reaction was varied from 0-24 hours. The reaction mixture was consisted of 2% β-cyclodextrin, 2% glycosyl acceptor, and 10.72 U of CGTase. 50 mM acetate buffer pH 6.0 was added to make the total volume of 0.25 ml. Incubation was made at 40°C. The decrease in substrates and the formation of products were determined by HPLC as described in section 2.16.

2.15.2 Effect of acceptor concentration

After the optimum incubation time was determined, the concentration of the acceptor was varied from 0-5%. The reaction mixture was the same as in section 2.15.1 except the acceptor concentration was varied and the incubation time was fixed at the suitable time obtained. The decrease in substrates and the formation of products were determined by HPLC as described in section 2.16.

2.15.3 Effect of CGTase concentration

After the optimum incubated time and acceptor concentration were determined, the concentration of CGTase was varied from 1,072-6,432 U/g β-CD

(5-32 U/ 0.25 ml of the reaction mixture). The reaction mixture was the same as in section 2.15.2 except the CGTase concentration was varied, and the incubation time and acceptor concentration were fixed at the suitable time and acceptor concentration obtained. The decrease in substrates and the formation of products were determined by HPLC as described in section 2.16.

2.15.4 Effect of pH

Determination of the suitable pH for the reaction of the oligosacchride production was performed by varying the pH of the reaction buffer, from pH 3.5 to 9.5. The enzyme CGTase was dialyzed against each buffer before adding to the reaction mixture which was the same as mentioned in 2.15.3 except that when making total volume to 0.25 ml, various buffer : 50 mM acetate buffer from pH 3.5 to 5.5 and 50 mM phosphate buffer from pH 6.5 to 9.5 were used. The decrease in substrates and the formation of products were determined by HPLC as described in section 2.16.

2.15.5 Effect of temperature

Determination of suitable temperature for the reaction of the oligosaccharide production was performed by varying the temperature of the reaction from 30 to 80°C. The reaction mixture was the same as mentioned in 2.15.4 except the incubation temperature was varied. The decrease in substrates and the formation of products were determined by HPLC as described in section 2.16.

2.16 Analysis of the reaction mixture by High Performance Liquid Chromatography

The sample solutions were analyzed for residual amounts of cyclodextrin and the selected acceptor, and the transfer products formed by HPLC using Spherisorb-NH_2 column (0.46 x 25 cm) and detected by RI detector (Rutchtorn,1992). Prior to injection, the samples were filtered through 0.45 μm membrane filter. The eluent was a mixture of 75 % acetonitrile and 25 % water by volume, and the flow rate was 2 ml/minute. The amount of degraded substrates and products forming were analyzed by comparing the retention times to those of standard β-CD and the suitable acceptor. The transglycosylation yield and degradation of β-CD were calculated as

$$\text{The transglycosylation yield (\%)} = \frac{\text{the disappearance of the amount of acceptor x 100}}{\text{the initial amount of acceptor}}$$

$$\text{The degradation of β-CD (\%)} = \frac{\text{the disappearance of the amount of β-CD x 100}}{\text{the initial amount of β-CD}}$$

2.17 Isolation of the transfer products

To identify the transfer products, production of linear opened-chain oligosaccharides were performed using the condition optimized in section 2.15 but in a larger scale. The reaction mixture was consisted of 2% β-CD as glycosyl donor, 0.5% of the suitable glycosyl acceptor, and 3,200 U of CGTase. 50 mM acetate buffer was added to make the total volume of 50 ml. After the reaction, the reaction mixture was subjected to HPLC. Each

transfer product was collected as separate pool corresponded to separate HPLC peak observed.

2.18 Characterization of the transfer products

2.18.1 Mass analysis

Each transfer product was determined for its size by mass spectrometer. The sample collected from separated HPLC peak was directly subjected to mass spectrometer. A mass spectrometer is an analysis device that determines the molecular mass of chemical compound by separating molecular ions according to their mass-to-charge ratio (m/z). The molecular mass was calculated by (Siuzdak,1996)

(molecular mass – number of protons) / charge = mass-to-charge ratio (m/z)

The sample of interest from HPLC column was introduced into Mass spectrometer : TOF ESI-MS system (of NSTDA, the National Science and Technology Development Agency) by electrospray ionization method. The mechanism of ionization was protonation. The ions were detected by time-of-fight mass analysis and the m/z ratio was determined with the on-line analyzer.

2.18.2 ^{1}H and ^{13}C-NMR analysis

The samples of interest (transfer products) from HPLC column were concentrated by lyophilization, and dissolved in D_2O (30 mg of sample dissolved in 0.8 ml of D_2O). They then were introduced into the ^{1}H and ^{13}C-NMR spectrometer for analysis of

the structures. ^{1}H and ^{13}C-NMR spectra were recorded with a JNM-A500 (500 MHz) spectrometer.

CHAPTER III

RESULTS

3.1 Purification of CGTase

Crude CGTase was purified by corn starch adsorption and immunoaffinity column chromatography. Figure 7 shows the elution profile from immunoaffinity column chromatography. One major protein peak with no enzyme activity was washed off with the equilibrating buffer. CGTase was eluted as a single peak between fractions 20 to 23. The highest dextrinizing activity was obtained at the same position as the protein peak. Tailing of the enzyme activity was observed from fraction 24-32. The enzyme fraction (fractions 20-22) was pooled and concentrated for further study, so called " purified CGTase".

The purification fold and recovery of CGTase obtained at each step are shown in Table 6. Specific activity expressed in term of dextrinizing activity per mg protein was increased through each step. These corresponded to the increased in the CD-product, which was determined by CD-TCE dilution limit. After the final step of the purification, a yield of 33 % was obtained with 100 folds of purity. Purified CGTase showed one intense protein band on SDS-polyacrylamide gel (Figure 8) and two major protein bands and one to two faint bands, all with corresponded amylolytic activity, on native polyacrylamide gel (Figure 9).

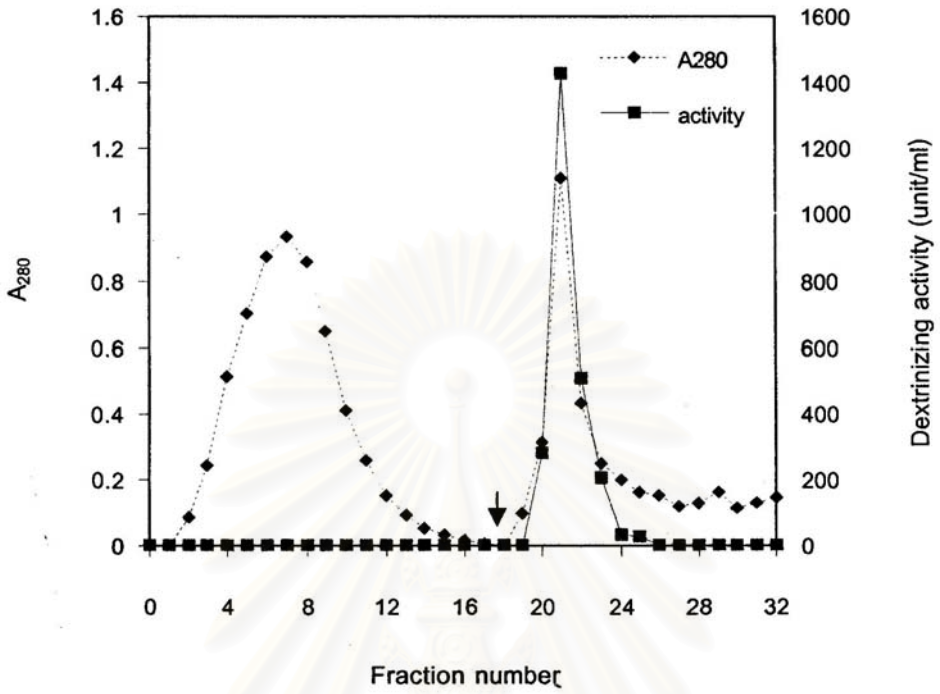


Figure 7 Purification of CGTase from *Bacillus circulans* A11 by immunoaffinity column chromatography

Concentrated CGTase solution from corn starch adsorption was applied onto immunoaffinity column chromatography (0.8x4.5 cm) and washed with 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl at the flow rate of 2 ml/hr. Elution was made by 3.5 M NaSCN in 50 mM NH_4OH, pH 10.5 at the flow rate of 6 ml/hr. (the arrow indicates where elution starts)

Table 6 Purification of CGTase from *Bacillus circulans* A11

Step	Volume (ml)	Total Activity (unit) x 10^3	Total Protein (mg)	Specific Activity (unit/mg)	Purification Fold	Yield (%)	CD-TCE (2^n)
Crude enzyme	5,000	76.4	3,600	21.2	1	100	2^5
Corn starch adsorption	1,500	74.5	59.3	1,256	59	98	2^6
Ultrafiltration	94	61.8	39.7	1,557	73	81	2^8
Immunoaffinity column	42	25.3	11.9	2,126	100	33	2^{10}

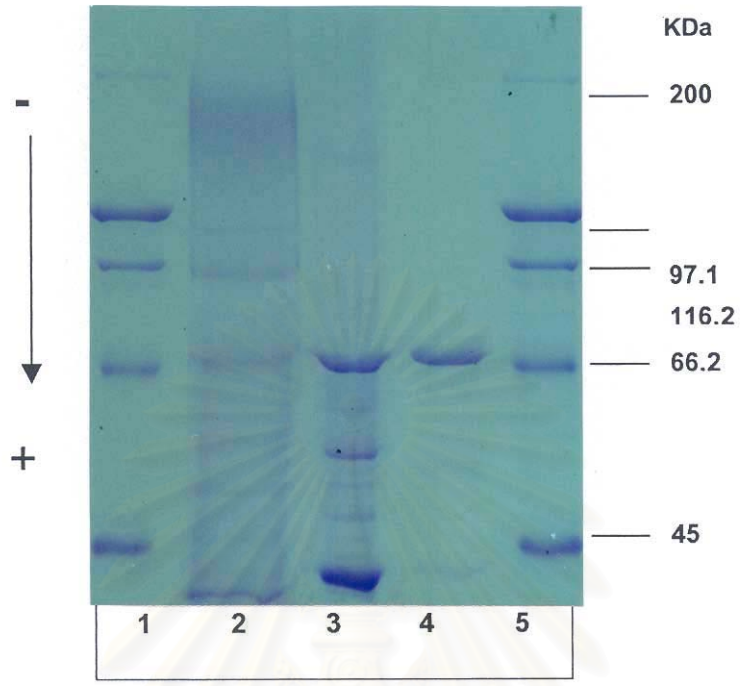


Figure 8 SDS-PAGE of CGTase from different steps of purification

Lane 1,5 : Protein molecular weight markers

[myosin (200 KDa), β-galactosidase (116.2 KDa),

phosphorylase b (97.1 KDa), BSA (66.2 KDa), and

ovalbumin (45 KDa)]

2 : Crude enzyme (20 μg)

3 : Concentrated starch adsorbed enzyme (20 μg)

4 : Immunoaffinity-purified enzyme (5 μg)

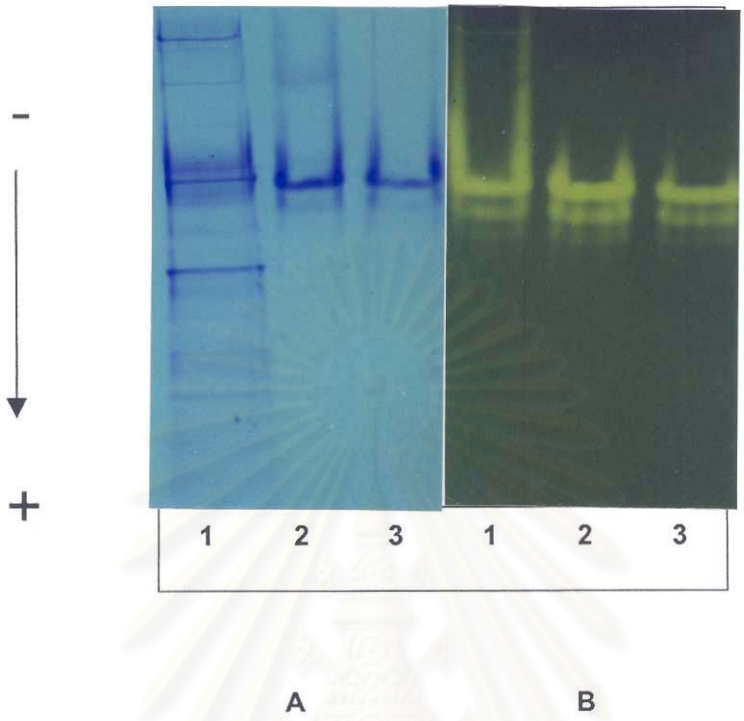


Figure 9 Non-denaturing PAGE of CGTase from different steps of purification

A : Coomassie blue staining

Lane 1 : Crude enzyme (20 μg)

Lane 2 : Concentrated starch adsorbed enzyme (20 μg)

Lane 3 : Immunoaffinity-purified enzyme (5 μg)

B : Amylolytic activity staining by iodine solution

[Lane 1-3, as in A, 0.2 units of dextrinizing activity was loaded to each well]

3.2 Specificity of glycosyl acceptors in the CGTase catalyzed coupling and transglycosylation reactions

The specificity of CGTase from *Bacillus circulans* A11 towards acceptors were determined by following how well the coupling and transglycosylation reaction proceeds. The kinetic parameters K_m and V_{max} values when acceptor was varied and donor was fixed were compared among different types of acceptor. β-CD or soluble starch was used as the glycosyl donor.

3.2.1 Acceptors which form linear opened-chain oligosaccharides

The specificity of CGTase on the coupling reaction towards linear opened-chain oligosaccharide (LOCO) acceptors was examined. Different groups of these types of acceptor used in this study were monosaccharides, monosaccharide derivatives, and disaccharides. All of them were carbohydrates. The glycosyl donor was β-CD and its degradation was measured by the cyclodextrin degrading activity assay. Figure 10 shows the Lineweaver-Burk plot when cellobiose was used as the glycosyl acceptor. This pattern was typical for the plots of all acceptors. (see Appendix 10). K_m and V_{max} values of all acceptors were determined and shown in Table 7.

Monsaccharides that have 6 carbons linked to form pyranose structure were divided into aldoses and ketoses. For aldoses, K_m values were 6.05±0.99, 10.50±8.30, 12.62±2.44, and 18.19±9.94 mM and V_{max} values were 0.36±0.02, 0.08±0.03, 0.08±0.01, and 0.27±0.08 μmoles/min for glucose, mannose, galactose, and 2-deoxy-glucose, respectively. Among the four aldoses, glucose and 2-deoxy-glucose were good acceptors because they

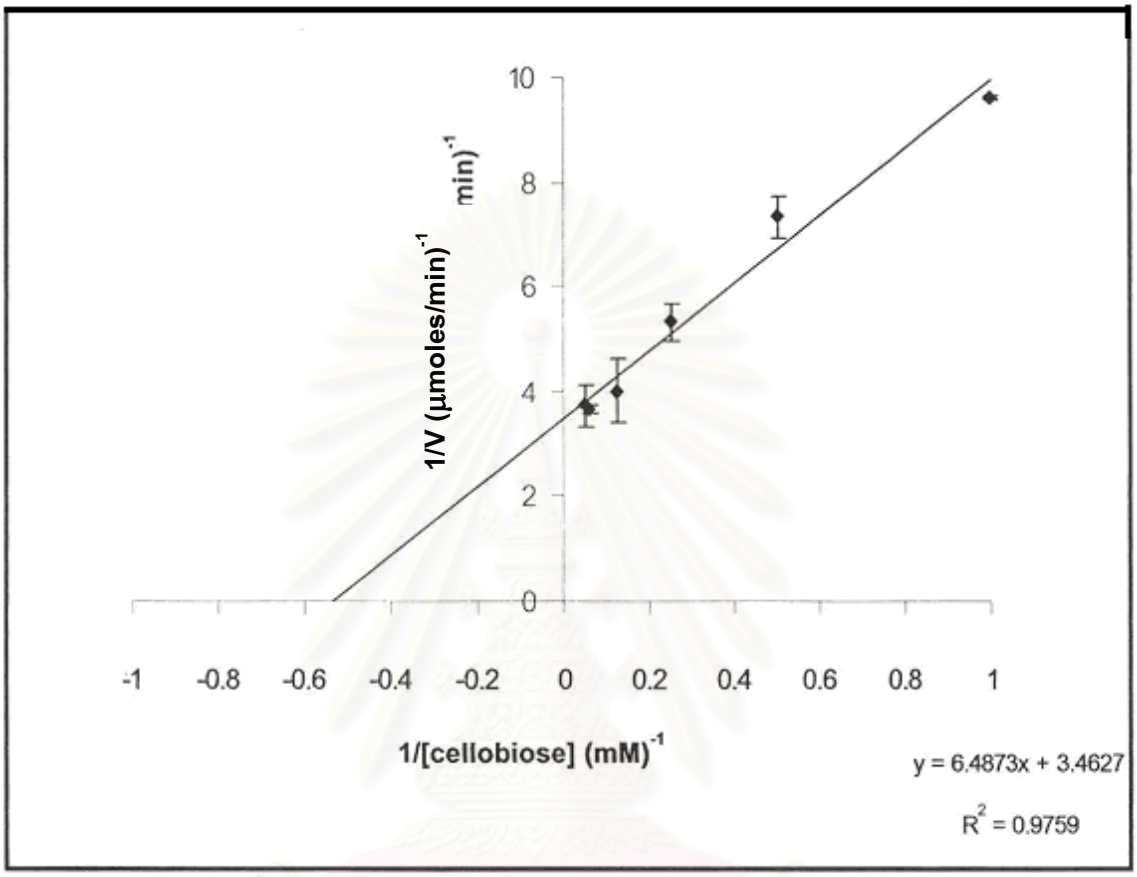


Figure 10 Lineweaver-Burk plot of the rate of CGTase-catalyzed coupling reaction at different concentrations of cellobiose acceptor

CGTase was incubated with 10 mM of β-CD and various concentrations of cellobiose in 50 mM acetate buffer, pH 6.0 at 40°C for 5 minutes. 0.2 Unit of *Aspergillus niger* glucoamylase was added to convert linearized oligosaccharides to glucose. The amount of β-CD degraded was monitored as µmoles glucose formed using dinitrosalicylic acid method.

Table 7 Kinetic parameters of CGTase catalyzed-coupling reaction using β-CD and the acceptors which form linear opened-chain oligosaccharides as substrates

LOCO forming acceptors		K_m (mM)	V_{max} (μmoles[a]/min)	V_{max}/K_m[b]	k_{cat}[c]
Monosaccharide (C_6)					
Aldose	Glucose	6.05±0.99	0.36±0.02	0.060	2,880
	Mannose	10.50±8.30	0.08±0.03	0.008	640
	Galactose	12.62±2.44	0.08±0.01	0.006	640
	2-Deoxy-glucose	18.19±9.94	0.27±0.08	0.015	2,160
Ketose	Fructose	50.05±56.57	0.26±0.22	0.005	2,080
	Sorbose	3.83±0.81	0.33±0.03	0.086	2,640
Monosaccharide	Rhamnose	4.70±1.44	0.06±0.01	0.013	480
Derivative (C_6)	NAG	4.66±1.94	0.15±0.02	0.032	1,200
	Fucose	29.77±18.57	0.29±0.12	0.010	2,320
	Gluconic acid	0.69±0.17	0.05±0.00	0.079	400
	Inositol	0.40±0.06	0.04±0.00	0.100	320
	Mannitol	1.15±0.40	0.03±0.00	0.026	240
	Sorbitol	0.77±0.17	0.05±0.00	0.065	400
	Ascorbic acid	18.90±10.69	0.34±0.11	0.018	2,720

In each reaction, 10.72 U of CGTase was used.

[a] = μmoles of reducing sugar (measured as glucose) formed

[b] = (μmoles/min) / mM

[c] = $mole.min^{-1}(mole\ enzyme)^{-1}$

Values of K_m and V_{max} are averaged from two separate determinations.

Table 7 (continue) Kinetic parameters of CGTase catalyzed-coupling reaction using β-CD and the acceptors which form linear opened-chain oligosaccharides as substrates

LOCO-forming acceptors		K_m (mM)	V_{max} (μmoles[a]/min)	V_{max}/K_m[b]	k_{cat}[c]
Monosaccharide (C_5)	Ribose	181.89±228.38	0.10±0.11	0.001	800
	Arabinose	2.43±1.66	0.09±0.02	0.037	720
	Xylose	1.59±0.70	0.03±0.00	0.019	240
Disaccharide	Maltose	6.19±2.07	0.09±0.01	0.010	720
	Lactose	10.11±3.01	0.36±0.05	0.036	2,880
	Trehalose	1.00±0.18	0.05±0.00	0.050	400
	Cellobiose	2.27±2.83	0.31±0.01	0.137	2,480
	Isomaltose	2.63±1.33	0.11±0.02	0.042	800

In each reaction, 10.72 U of CGTase was used.

[a] = μmoles of reducing sugar (measured as glucose) formed

[b] = (μmoles/min) / mM

[c] = $mole.min^{-1}(mole\ enzyme)^{-1}$

Values of K_m and V_{max} are averaged from two separate determinations.

possesed high V_{max}/ K_m and k_{cat}. For ketoses, the result demonstrated that sorbose which gave higher V_{max}/ K_m and k_{cat} value was the better acceptor when compared to fructose. Other C_6 glycosyl acceptors were monosaccharide derivatives including N-acetyl-D-glucosamine (NAG), gluconic acid, deoxy glucose e.g. fucose, rhamnose and sugars alcohols e.g. inositol, mannitol and sorbitol. Ascorbic acid is also in this group but with the furanose structure (see Appendix 10). It was found that although inositol, gluconic acid, sorbitol, mannitol and NAG had high V_{max}/ K_m values but they possesed rather low k_{cat}. While ascorbic acid and fucose had lower V_{max}/ K_m but higher k_{cat} values. Monosaccharides that have 5 carbons such as ribose, arabinose, and xylose were investigated. k_{cat} values were rather low which suggested that they could not act as good acceptors. In addition, the K_m for ribose was exceptionally high. When disaccharides were used as the glycosyl acceptor, K_m values of maltose, lactose, trehalose, cellobiose, and isomaltose obtained were 6.19±2.07, 10.11±3.01, 1.00±0.19, 2.27±2.83, and 2.63±1.33 mM while V_{max} values were 0.09±0.01, 0.36±0.05, 0.05±0.00, 0.31±0.01, and 0.11±0.02 μmoles/min, respectively. Only lactose and CB had high k_{cat}. The result suggested that lactose and cellobiose were the good acceptor. The disaccharide sucrose was also tested but the reaction did not proceed at the experimental conditions used though up to 20 mM sucrose was used. Judging from kinetic values, those acceptors which gave high V_{max}/ K_m and high k_{cat} values were considered as good acceptors. From these results, it can be concluded that among these acceptors which form linear opened-chain oligosaccharides upon coupling with β-CD, glucose, sorbose, ascorbic acid, lactose and cellobiose were the good candidates. Although glucose (an aldose) was one of the good acceptors, but the transfer products produced by the coupling reaction would be maltooligosaccharides of α 1-4 linkage which

are not new or interesting products. In the case of ascorbic acid, it showed rather high k_m. When considered sorbose (a ketose), lactose (a disaccharide with structure gal β(1-4) glc) or cellobiose (a disaccharide with structure glc β(1-4) glc) as glycosyl acceptors, the linear opened-chain oligosaccharide transfer products were more interesting. Cellobiose was then chosen as the most suitable acceptor for further work due to its higher V_{max}/ K_m value.

3.2.2 Acceptors which form glycosides

The acceptor specificities of CGTase towards glycosides and alcohols when β-CD was used as the glycosyl donor were examined. The reaction was performed as described in section 2.13 and either by the CD coupling activity or the CD degrading activity assay. The Lineweaver-Burk plot of all acceptors were similar to that of the linear opened-chain oligosaccharide-forming acceptors (see Appendix 9)

For glycoside acceptors, hesperidin, naringin (the flavonoid from citrus fruit), rutin, and salicin were investigated. From kinetic parameters, V_{max}/ K_m and k_{cat} values shown in Table 8, hesperidin was found to be the best acceptor in this group. Naringin, and rutin could also act as the glycosyl acceptor, but not as good. Salicin could not be used as glycosyl acceptor in the experimental conditions used since the CGTase-catalyzed glycosyl transfer reaction could not be measured.

Acceptors without carbohydrate moiety such as alcohols were also investigated. Linear alcohols, from butanol to decanol and geraniol (see structure in Appendix 10) were determined. It was found that all linear alcohols could not act as glycosyl acceptor. For cyclic alcohols, such as hydroquinone, thymol, resorcinol, glycosyl

Table 8 Kinetic parameters of CGTase catalyzed-coupling reaction using β-CD and the acceptors which form glycosides as substrates

Glycosides-forming acceptor		K_m (mM)	V_{max} (μmoles[a]/min)	V_{max}/K_m[b]	k_{cat}[c]
Glycoside	Naringin	0.86±0.24	0.06±0.00	0.070	480
	Hesperidin	0.11±0.04	0.26±0.01	2.364	2,080
	Rutin	0.84±0.20	0.04±0.00	0.048	320
Alcohol					
Cyclic alcohol	Hydroquinone	2.27±0.38	0.02±0.00	0.009	160
	Resorcinol	0.33±0.08	0.04±0.00	0.120	320
	Thymol	1.06±0.19	0.04±0.00	0.038	320
Sterol	Cholesterol	0.10±0.06	0.38±0.04	3.800	3,040

In each reaction, 10.72 U of CGTase was used.

[a] = μmoles of reducing sugar (measured as glucose) formed when acceptors were glycosides

= μmoles of β-CD disappeared when acceptors were alcohols

[b] = (μmoles/min) / mM

[c] = $mole.min^{-1}(mole\ enzyme)^{-1}$

Values of K_m and V_{max} are averaged from two separate determinations

transfer reactions could proceed but at a very low speed. V_{max}/ K_m values were 0.009, 0.120, 0.038, and 3.800 (μmoles/min)/ mM and k_{cat} values were 160, 320, 320, and 3,040 mole/ min^{-1} (mole enzyme)$^{-1}$, respectively. However, cholesterol gave the highest V_{max}/ K_m and k_{cat} values. when compared with other alcohols with aromatic structure. In conclusion, cyclic alcohols could act as acceptor for the coupling reaction and cholesterol was the good acceptor in this group.

3.2.3 Comparison of transglycosylation yield using soluble starch or β-CD as glycosyl donor for the CGTase-catalyzed coupling and transglycosylation reactions

In addition to determining acceptor specificity through the comparison of kinetic parameters as described in 3.2.1 and 3.2.2, specificity was also judged by comparison of the transglycosylation yield. As transglycosylation yield is a parameter measuring the consumption of acceptor in the reaction (how well the reaction proceeds), this parameter was reported to be used in determining acceptor specificity (Park *et al*. 1998). In this experiment, both β-CD and soluble starch were used as glycosyl donors. Figure 11 compares the transglycosylation yield when different types of linear opened-chain oligosaccharide acceptors were used with either β-CD or soluble starch substrate. Only the acceptors which gave low K_m and high k_{cat} values from 3.2.1 were used. 2% of β-CD or soluble starch and 0.5% of acceptor were incubated with 16 U of CGTase at 30°C, pH 6.0 for 2 hrs. The HPLC profiles (see Appendix 11) of the reaction mixtures when using soluble starch as the glycosyl donor, at 0 hour indicated that CGTase catalyzed the cyclization reaction and produced β-CD peak at the retention time of 5.34-5.65 minutes. After 2 hours incubation, CGTase still catalyzed cyclization while transglycosylation reaction

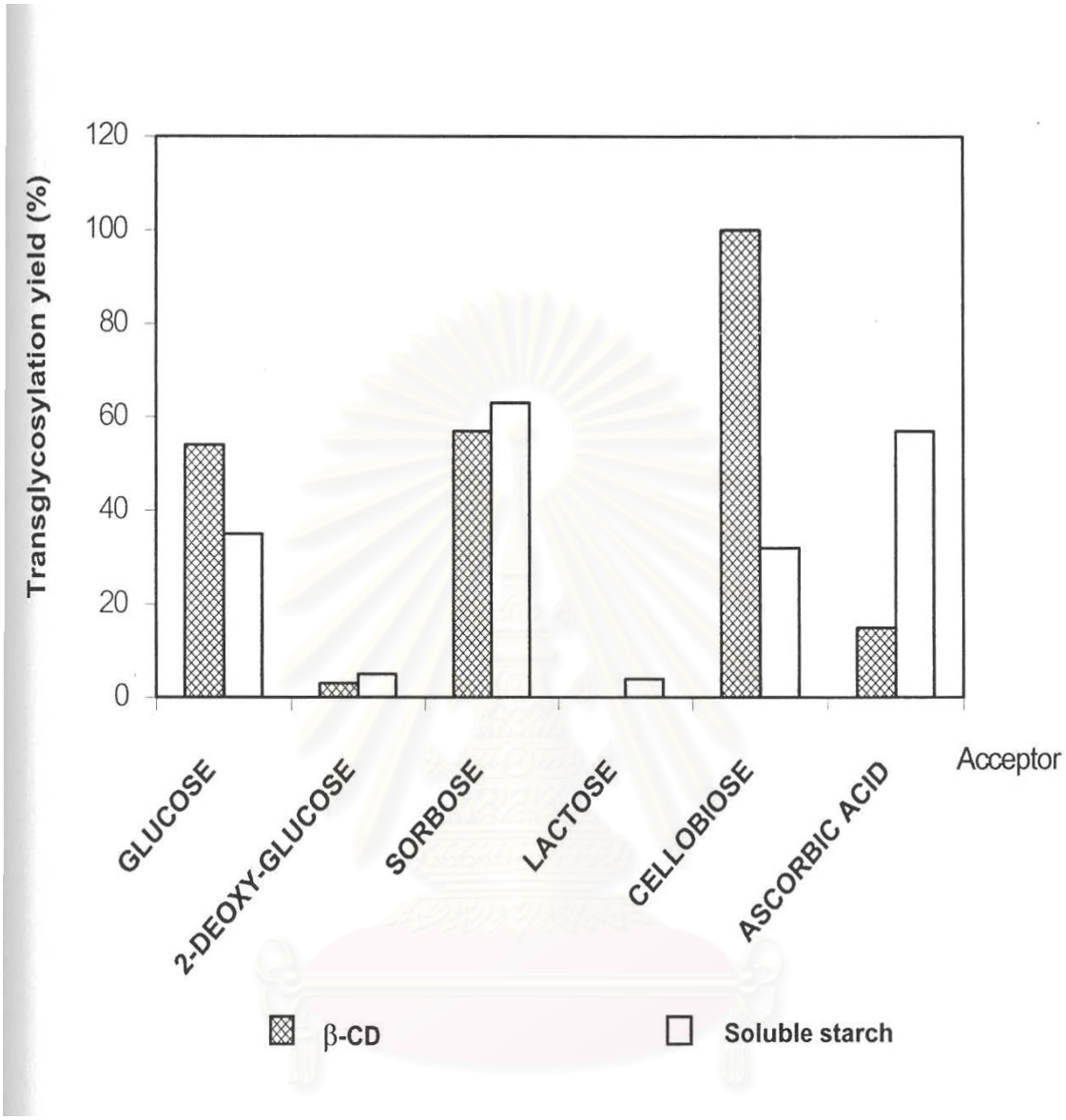


Figure 11 Transglycosylation yield of the CGTase-catalyzed coupling and transglycosylation reactions when using β-CD or soluble starch as the glycosyl donor with different types of acceptor

which produced oligosaccharide transfer products was noticable. Using HPLC profile of reaction mixture when using soluble starch and cellobiose as substrates, (Figure 12B) at 0 hour, the peak at the retention time of 3.27 were cellobiose and the one at 5.48 min should be β-CD product that was produced by the cyclization reaction. Two transfer products at the retention time of 3.71 and 4.54 were also determined. At 2 hrs, cellobiose peak at the retention time 3.27 was reduced and the peak of β-CD and two transfer products were increased. When using β-CD as the glycosyl donor for cellobiose in the coupling reaction, HPLC profile was as shown in Figure 12A. At 0 hr, two peaks at the retention time 3.27 and 5.94 were cellobiose and β-CD substrates. After 2 hrs, the peaks of two substrates were reduced and the two peaks of transfer products at the retention time 3.81 and 4.42 were produced. The transglycosylation yields of glucose, sorbose, cellobiose, and ascorbic acid were 54, 57, 78, and 15%, respectively when using β-CD as glycosyl donor (Figure 12 and Appendix 11). While the respective transglycosylation yields were 35, 63, 32, and 57% when using soluble starch as glycosyl donor. Transglycosyltion yields of 2-deoxy-glucose and lactose for both donors were very low. For fucose and NAG, transglycosylation yields could not be calculated since both acceptors were hardly lost during 2 hrs incubation (see Appendix 11). From this experiment, it can be concluded that cellobiose was the best acceptor for coupling reaction with β-CD while sorbose was the best acceptor in transglycosylation reaction with soluble starch as glycosyl donor.

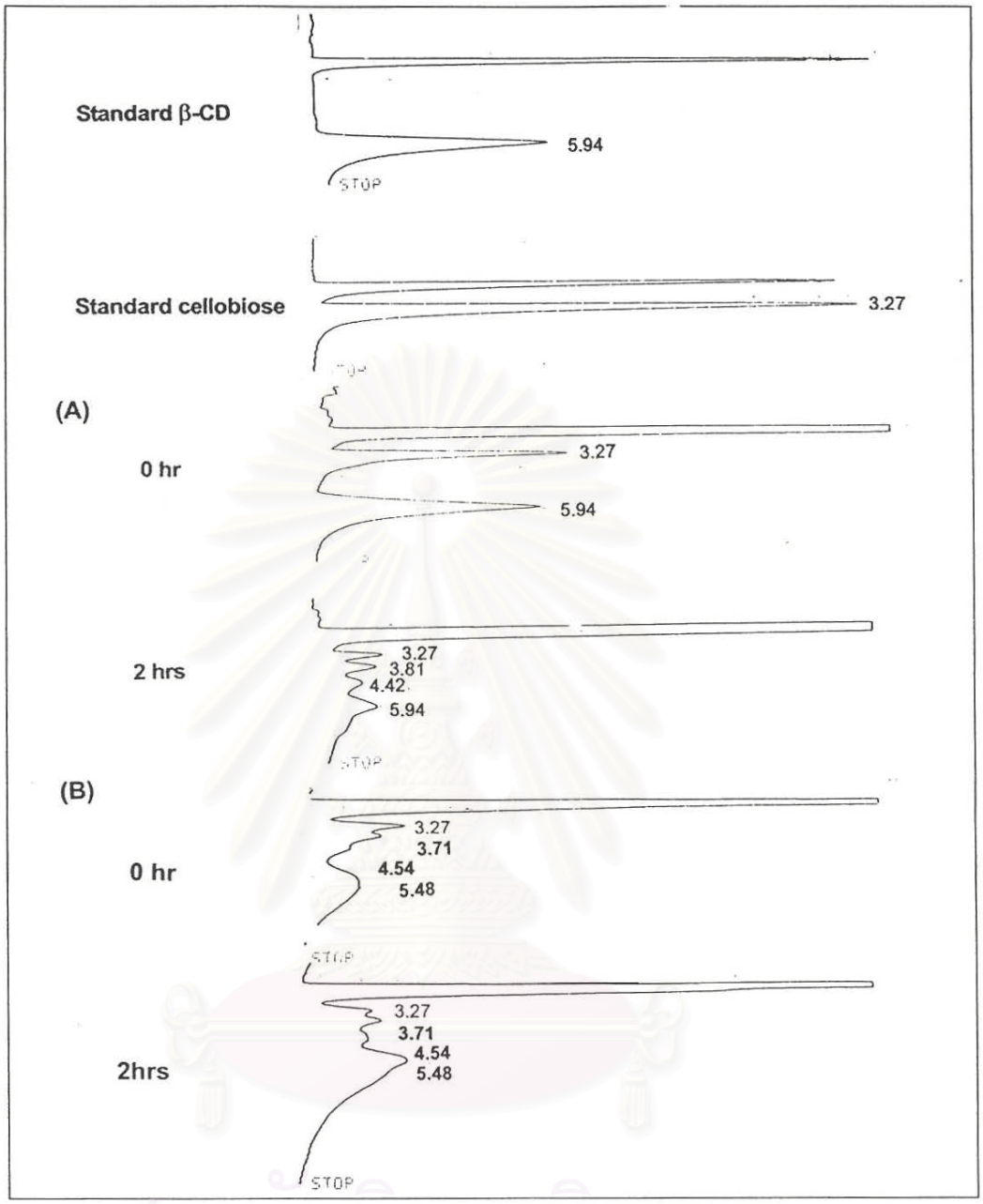


Figure 12 HPLC chromatograms of standard β-CD, standard cellobiose, and reaction mixtures of the CGTase-catalyzed coupling (A) and transglycosylation (B) reactions.

The reaction conditions were : 2.0% β-CD or soluble starch, 0.5% cellobiose, 16 U CGTase at 30 ^{o}C, pH 6.0 for 2 hrs. Spherisorb-NH_2 column was used. Acetronitrile : water (75 : 25) (v/v) was used as eluent at flow rate of 2 ml/min.

3.3 Analysis of reaction products of the CGTase-catalyzed coupling and transglycosylation reactions

When analyzed the transfer products obtained in these coupling and transglycosylation reactions using different glycosyl donors and acceptors, the HPLC profiles (Appendix 11) show different types and ratios of the products as judging by their retention times and peak areas. The data from the acceptors which gave high transglycosylation yield are summarized in Table 9. For glucose acceptor, three transfer products, PG1, PG2, and PG3, were detected with the ratio 1:1:1 with either β-CD or soluble starch was the glycosyl acceptor. For cellobiose, two transfer products, PC1 and PC2, were obtained. The product ratio was 3:1 with β-CD but 1:1 with soluble starch donor. For sorbose, in the case of β-CD substrate, four products, PS1 to PS4, were observed with the ratio of 1:1:3:1 while only one product was found when soluble starch was the glycosyl donor.

3.4 Effect of acceptors on the affinity constant of β-CD in the CGTase-catalyzed coupling reactions

To determine the effect of acceptors on the value of affinity constant of β-CD, three kinds of linear opened-chain oligosaccharides: glucose, sorbose, and cellobiose (which gave high V_{max}/ K_m and k_{cat} values and high transglycosylation yield as mentioned in sections 3.2.1 and 3.2.3), were used in this experiment. The concentrations of β-CD were varied from 1-30 mM when each glycosyl acceptor was fixed at the concentration of 5 mM in all reactions. The reaction was performed as described in section 2.14. K_m values of

Table 9 Ratio of the transfer products obtained in coupling and transglycosylation reactions

Donor	Acceptor	Transfer product	R_t (min)	Peak area ($x10^3$)	Product ratio
β-CD	Glucose	PG1	3.41	29.38	1:1:1
		PG2	3.95	28.62	
		PG3	5.16	28.71	
	Sorbose	PS1	3.05	17.20	1:1:3:1
		PS2	3.35	13.78	
		PS3	3.48	34.91	
		PS4	4.59	18.71	
	Cellobiose	PC1	3.54	28.49	3:1
		PC2	4.42	11.25	
Soluble starch	Glucose	PG1	3.37	22.12	1:1:1
		PG2	3.84	22.84	
		PG3	4.96	17.59	
	Sorbose	PS1	3.24	57.56	1
	Cellobiose	PC1	3.71	18.74	1:1
		PC2	4.54	21.32	

β-CD were 4.21±0.84, 1.27±0.33, and 4.86±0.37 mM and V_{max} values were 0.29±0.01, 0.22±0.01, and 0.47±0.01 μmoles/min for glucose, sorbose, and cellobiose, respectively. The k_{cat} value was the highest with cellobiose acceptor and the lowest with sorbose acceptor (Table 10).

3.5 Determination of suitable conditions for the production of linear opened-chain oligosaccharides from the most suitable carbohydrate acceptor

The suitable conditions for the production of linear opened-chain oligosaccharides were determined using β-CD as glycosyl donor and cellobiose as the acceptor. The reaction were performed described in section 2.15 and reaction products were analyzed by HPLC. The transglycosylation yield and degradation of β-CD were calculated from cellobiose consumption and disappearance of β-CD in the reaction, respectively (Section 2.16).

3.5.1 Effect of incubation time

Figure 14 illustrates the effect of the incubation time on HPLC profiles of the reaction mixture of the CGTase-catalyzed coupling reaction. The experimental conditions were 2% β-CD, 2% cellobiose incubated with 10.72 U of CGTase at 40°C, pH 6.0 at various time intervals from 0-24 hrs. At 0 hr, two peaks at retention time (R_t) 3.27 and 5.94 minutes were cellobiose and β-CD. From 0.5-24 hrs, cellobiose and β-CD peaks at R_t 3.27 and 5.94 minutes were reduced while two peaks of transfer products at R_t 3.81 and 4.42 minutes were increased as the incubation time increased. Figure 15 shows that the transglycosylation yield and degradation of β-CD were increased proportionally as the

Table 10 Kinetic parameters of the CGTase-catalyzed coupling reaction using the fixed amount of linear opened-chain oligosaccharide acceptors and variable amounts of β-CD as substrates

LOCO-forming Acceptors	K_m (mM)	V_{max} (μmoles[a]/min)	V_{max}/K_m	k_{cat}[c]
Glucose*	4.21±0.84	0.29±0.04	0.069	2,320
Sorbose*	1.27±0.33	0.22±0.01	0.173	1,760
Cellobiose*	4.68±0.37	0.47±0.01	0.100	3,760

In each reaction, 10.72 U of CGTase was used.

* = vary donor β-cyclodextrin (1-30 mM), fix glycosyl acceptor at 5 mM

[a] = μmoles of reducing sugar (measured as glucose) formed

[b] = (μmoles/min) / mM

[c] = $mole.min^{-1}(mole\ enzyme)^{-1}$

Values of K_m and V_{max} are averaged from two separate determinations.

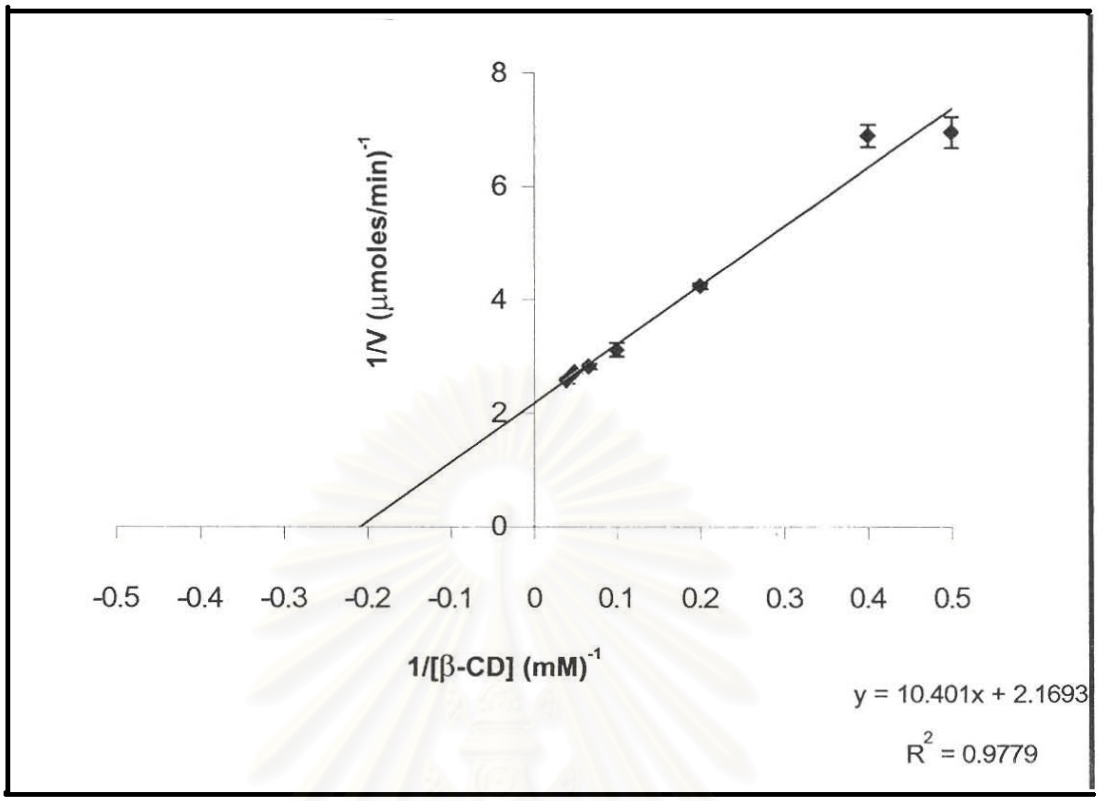


Figure 13 Lineweaver-Burk plot of the rate of CGTase-catalyzed coupling reaction at different concentrations of β-CD

CGTase was incubated with 5 mM of cellobiose and various concentrations of β-CD in 50 mM acetate buffer, pH 6.0 at 40°C for 5 minutes. 0.2 Unit of *Aspergillus niger* glucoamylase was added to convert linearized oligosaccharides to glucose. The amount of β-CD degraded was monitored as μmoles glucose formed using dinitrosalicylic acid method.

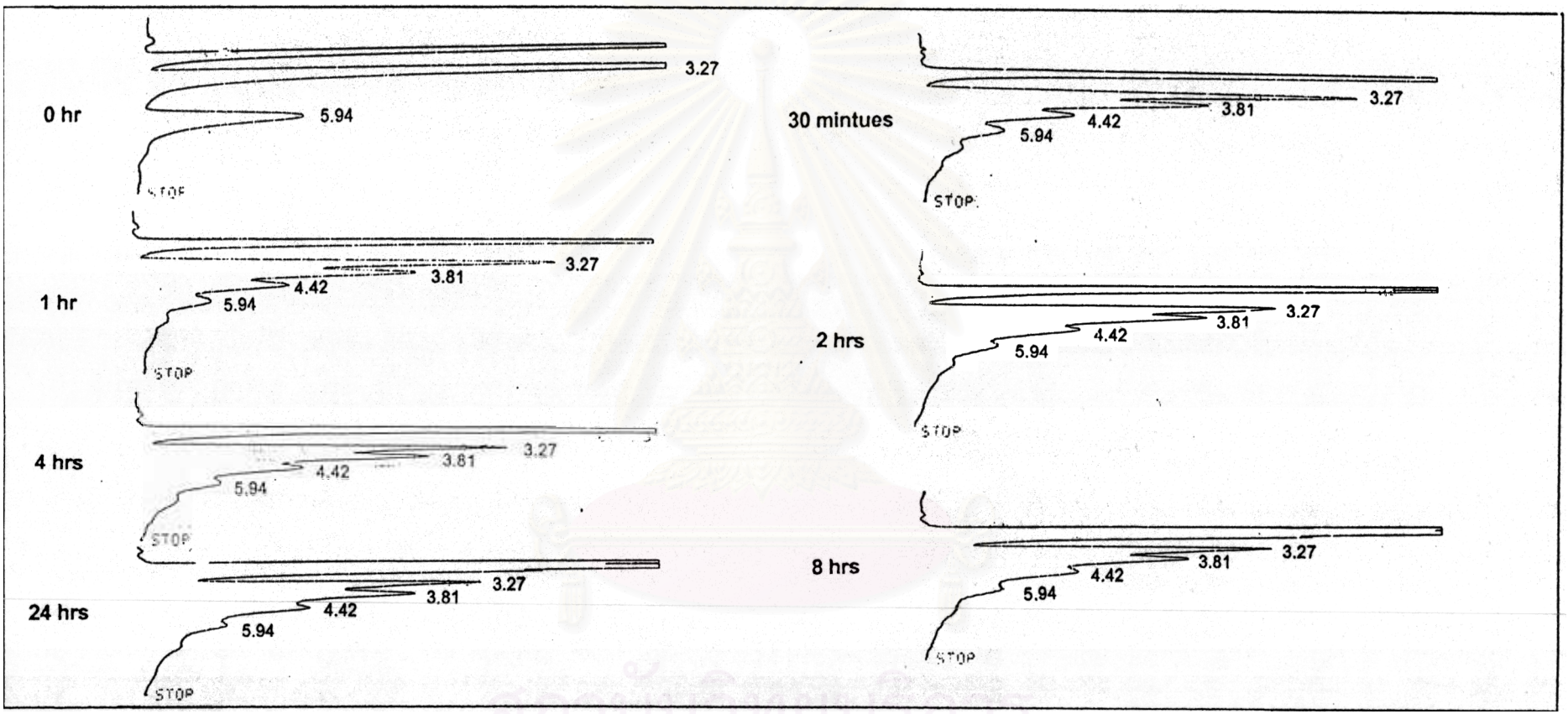


Figure 14 HPLC chromatograms of reaction mixtures of the CGTase-catalyzed coupling reaction at different incubation times

The reaction condition was 2.0% β-CD, 2.0% cellobiose, incubated with 10 U of CGTase, at pH 6.0, 40 ^{o} C, at various incubation times. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

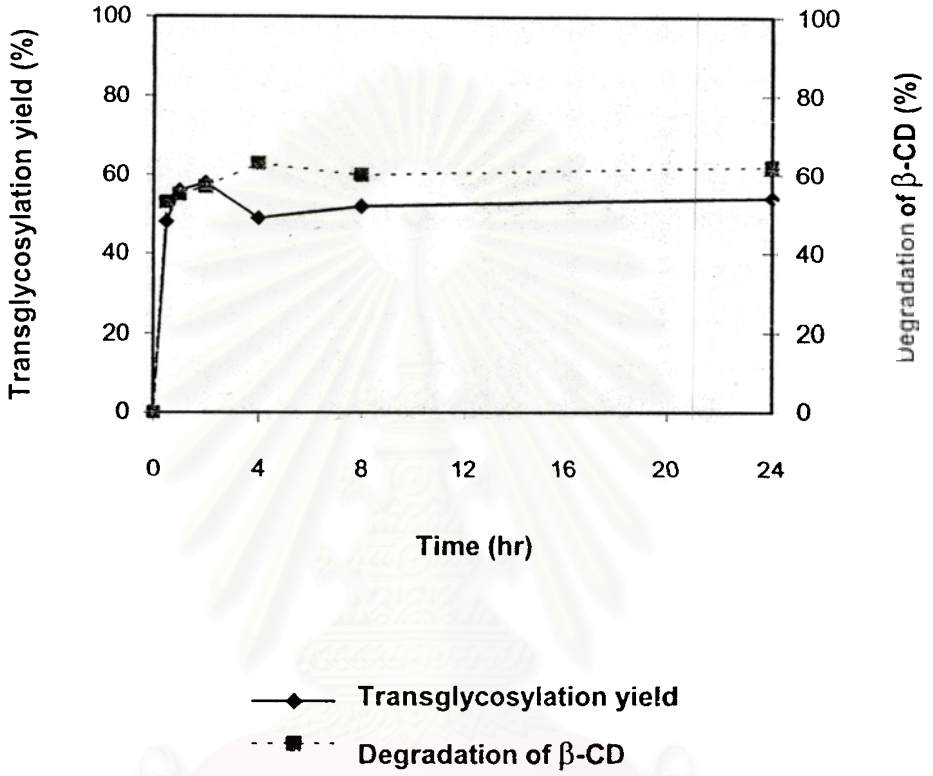


Figure 15 Effect of incubation time on the transglycosylation yield and degradation of β-CD in the CGTase catalyzed coupling reaction

incubation time increased from 0-2 hrs. From 2-24 hrs. incubation period, the two values were rather constant. The optimal incubation time thus determined to be 2 hrs.

3.5.2 Effect of cellobiose concentration

Figure 16 illustrates the effect of cellobiose concentration on HPLC profiles of the reaction mixture of the CGTase-catalyzed coupling reaction. β-CD was fixed at 2.0% and the amount of cellobiose was changed from 0.2 to 2.0%. Incubation was performed with 10.72 U of CGTase at the same conditions as in section 3.4.1. At 0 hr, HPLC profiles show peaks of cellobiose and β-CD at R_t 3.27 and 5.94 minutes, respectively. The height of cellobiose peak was increased as cellobiose concentration increased. After 2 hrs, both peaks were reduced while two peaks of the transfer products at R_t 3.81 and 4.42 minutes were increased. Figure 17 shows the transglycosylation yield and degradation of β-CD at different concentrations of cellobiose. The maximum transglycosylation yield was obtained at 0.25% cellobiose, However, the transglycosylation yield at 0.5% cellobiose was not much different from the maximum value while the degradation of β-CD was at maximum at this concentration. Thus, 0.5% was chosen as the optimal concentration of cellobiose. When cellobiose was increased from 0.5 to 2.0%, both the transglycosylation yield and degradation of β-CD were decreased.

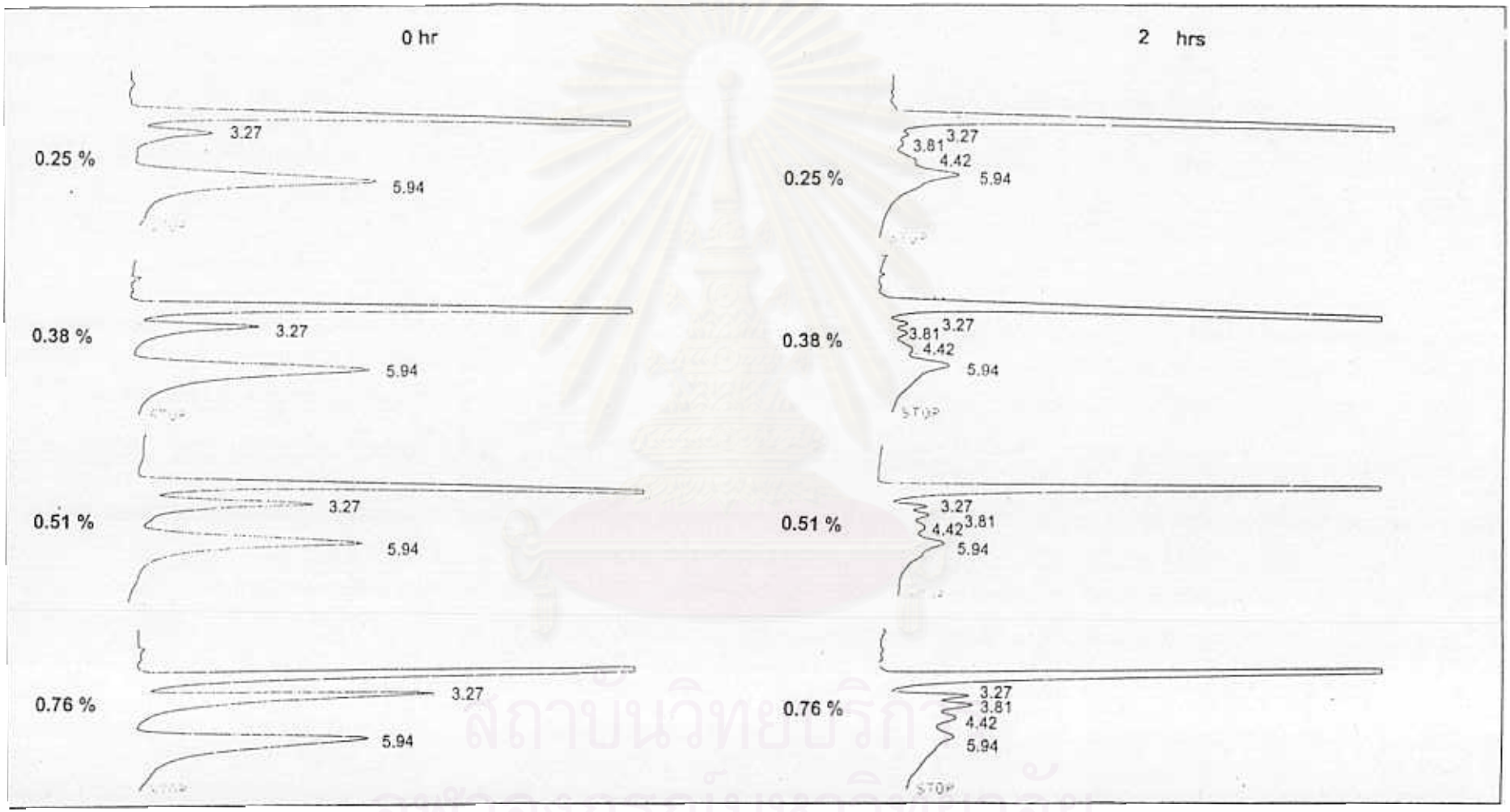


Figure 16 HPLC chromatograms of reaction mixtures of the CGTase-catalyzed coupling reaction at different concentrations of cellobiose at 0 and 2 hrs. incubation.

The reaction condition was 2.0% β-CD, various concentration of cellobiose, incubated with 10 U of CGTase, at pH 6.0, 40°C for 2 hrs. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

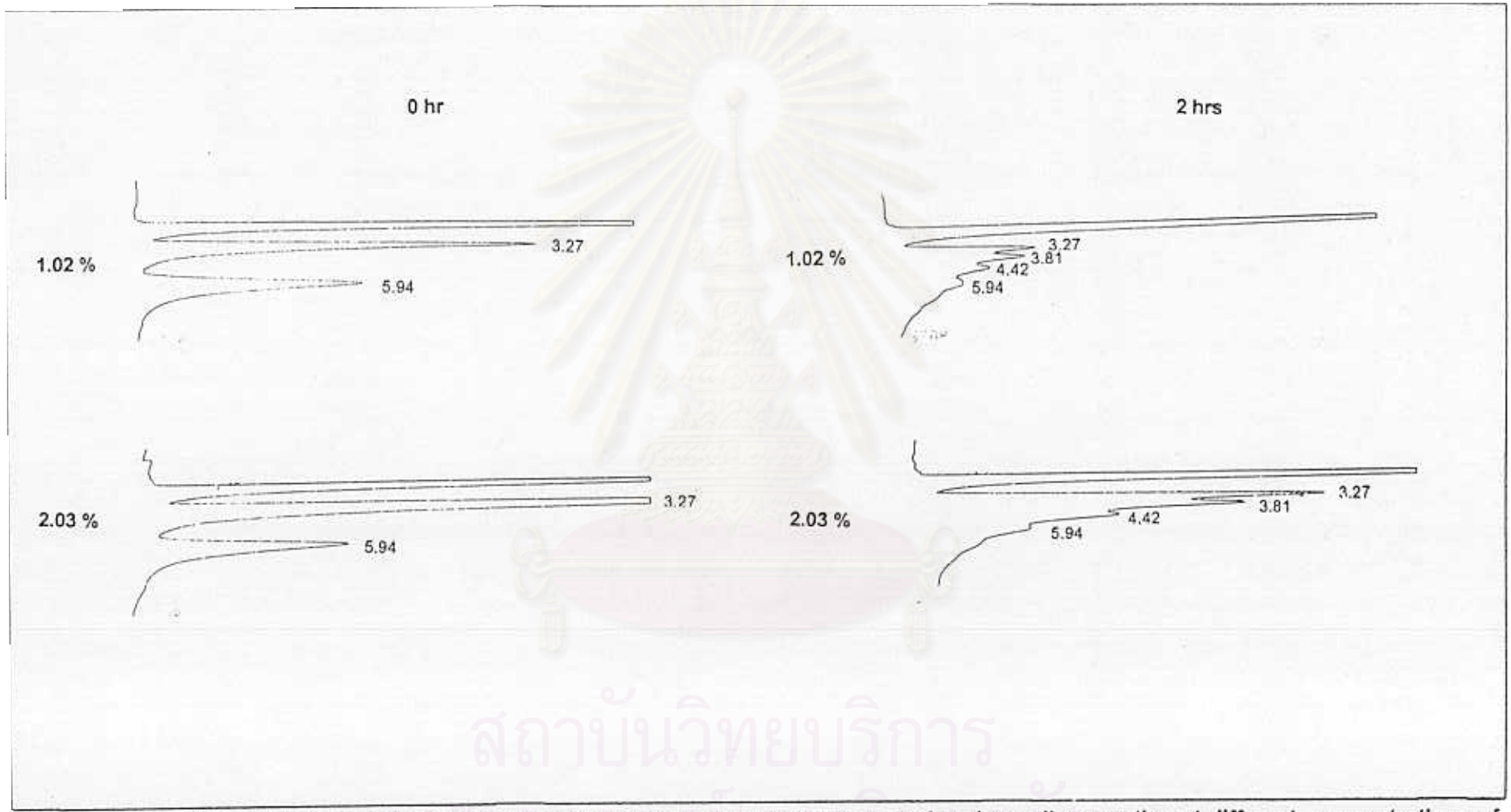


Figure 16 (Continue)HPLC chromatograms of reaction mixtures of the CGTase-catalyzed coupling reaction at different concentrations of cellobiose at 0 and 2 hrs. incubation.

The reaction condition was 2.0% β-CD, various concentration of cellobiose, incubated with 10 U of CGTase, at pH 6.0, 40°C for 2 hrs. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

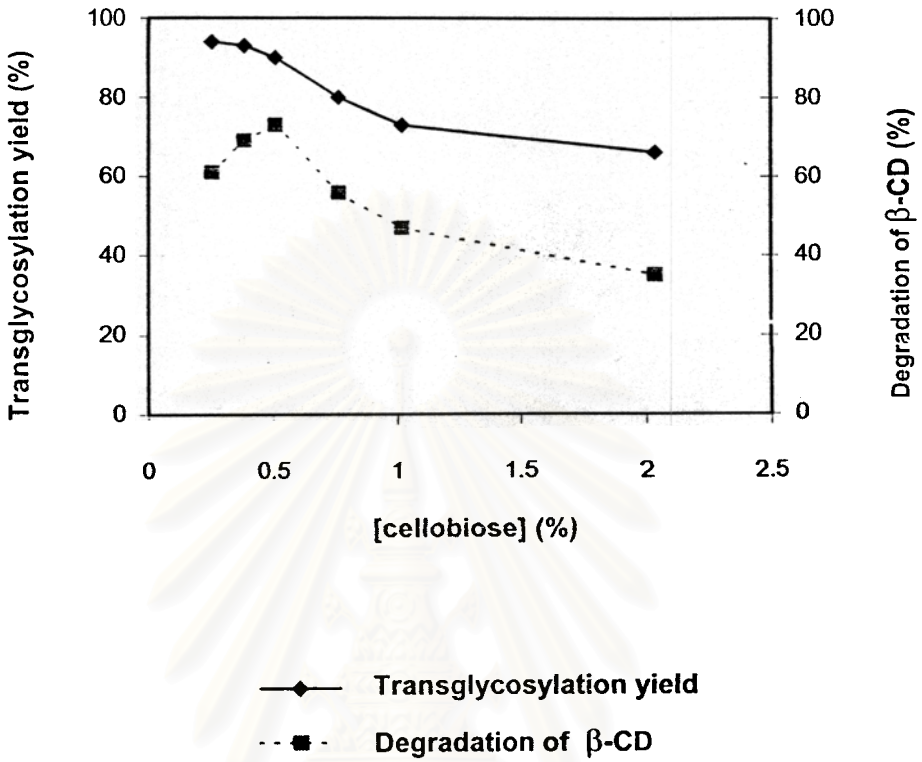


Figure 17 Effect of cellobiose concentration on the transglycosylation yield and degradation of β-CD in the CGTase catalyzed coupling reaction

3.5.3 Effect of CGTase concentration

Figure 18 shows the effect of the amount of CGTase on the HPLC profiles of the reaction mixture of CGTase-catalyzed coupling reaction. When adding CGTase from 5 to 32 U/0.25 ml reaction mixture (1,072 to 6,432 U/g β-CD), the residual amounts of β-CD (peak at R_t 5.94 minutes) and cellobiose (peak at R_t 3.27 minutes) and the amounts of transfer products (R_t 3.81 and 4.42 minutes) were not significantly altered. Figure 19 shows the transglycosylation yield of cellobiose and degradation of β-CD in the coupling reaction performed at different amounts of CGTase. The transglycosylation yield did not change as the amount of the enzyme increased while 15% increase in β-CD degradation was observed at 3,216 U/g β-CD and thereafter. Thus, 3,216 U/ g β-CD was chosen as the optimal amount of CGTase.

3.5.4 Effect of pH

Figure 20 shows the effect of pH on the HPLC profiles of the reaction mixture of the CGTase-catalyzed coupling reaction. At 2 hrs. incubation, when pH of the reaction was increased from 3.5 to 9.5, the amounts of cellobiose (peak at R_t 3.27 minutes) and β-CD (peak at R_t 5.94 minutes) were decreased while the two peaks of transfer products (peaks at R_t 3.81 and 4.42 minutes) were increased. It should be noticed that at 0 hr of pH 7.5 and 8.5, the CGTase could immediately catalyze coupling reaction and the transfer products were obviously seen. Figure 21 shows that the transglycosylation yield was increased when pH of the reaction was increased from 3.5 to 4.5. At pH 4.5 to 6.0 the transglycosylation yield was rather constant, then dropped at pH 6.5 and gradually

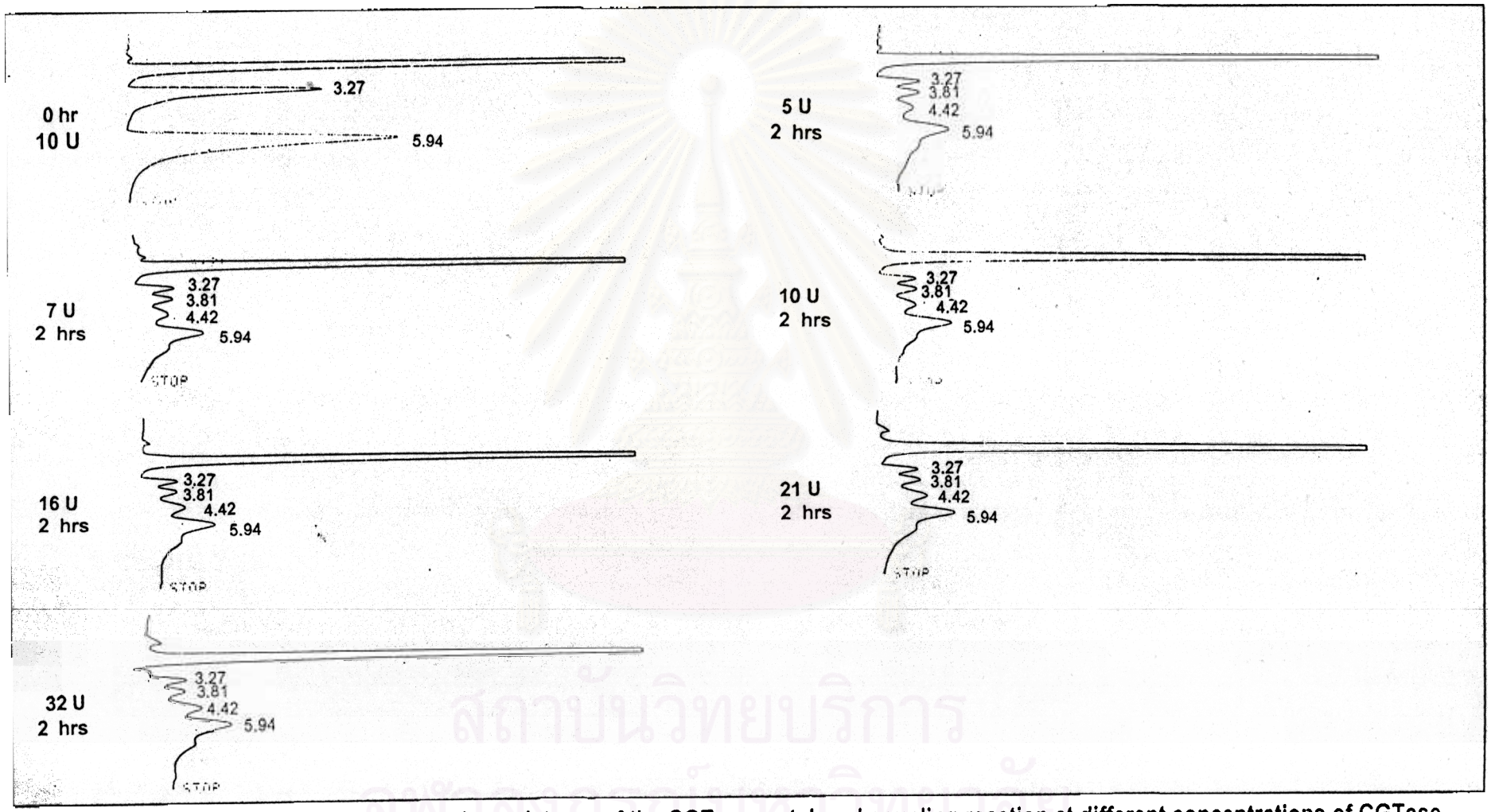


Figure 18 **HPLC chromatograms of reaction mixtures of the CGTase -catalyzed coupling reaction at different concentrations of CGTase**

The reaction condition was 2.0% β-CD, 0.5% cellobiose, incubated with various concentration of CGTase, at pH 6.0, 40°C for 2 hrs. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

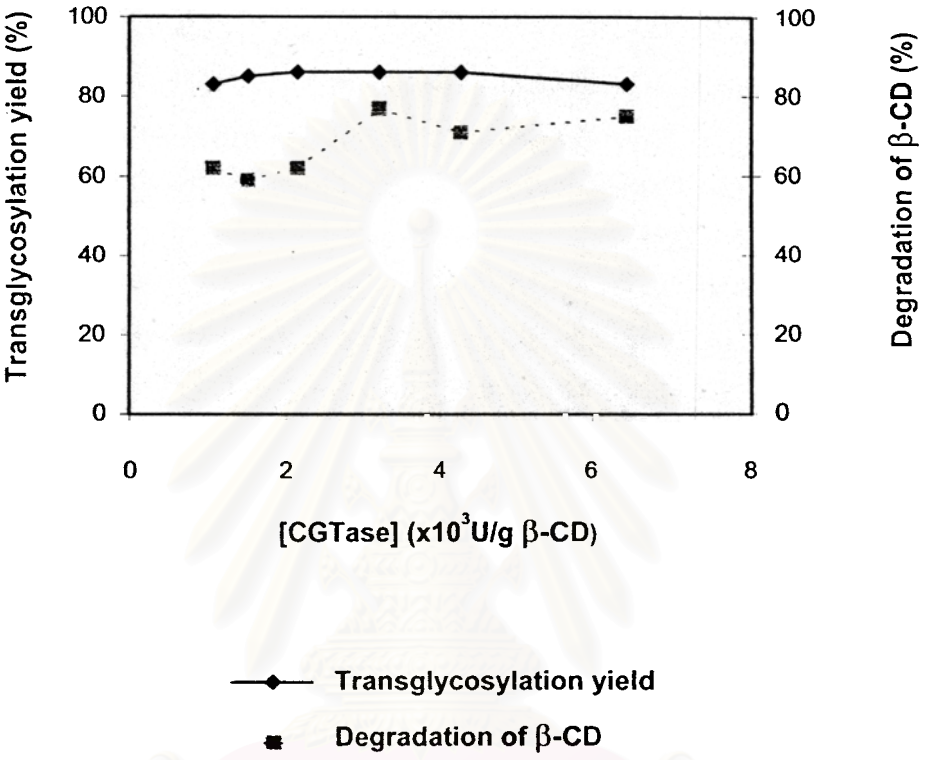


Figure 19 Effect of CGTase concentration on the transglycosylation yield and degradation of β-CD in the CGTase catalyzed coupling reaction

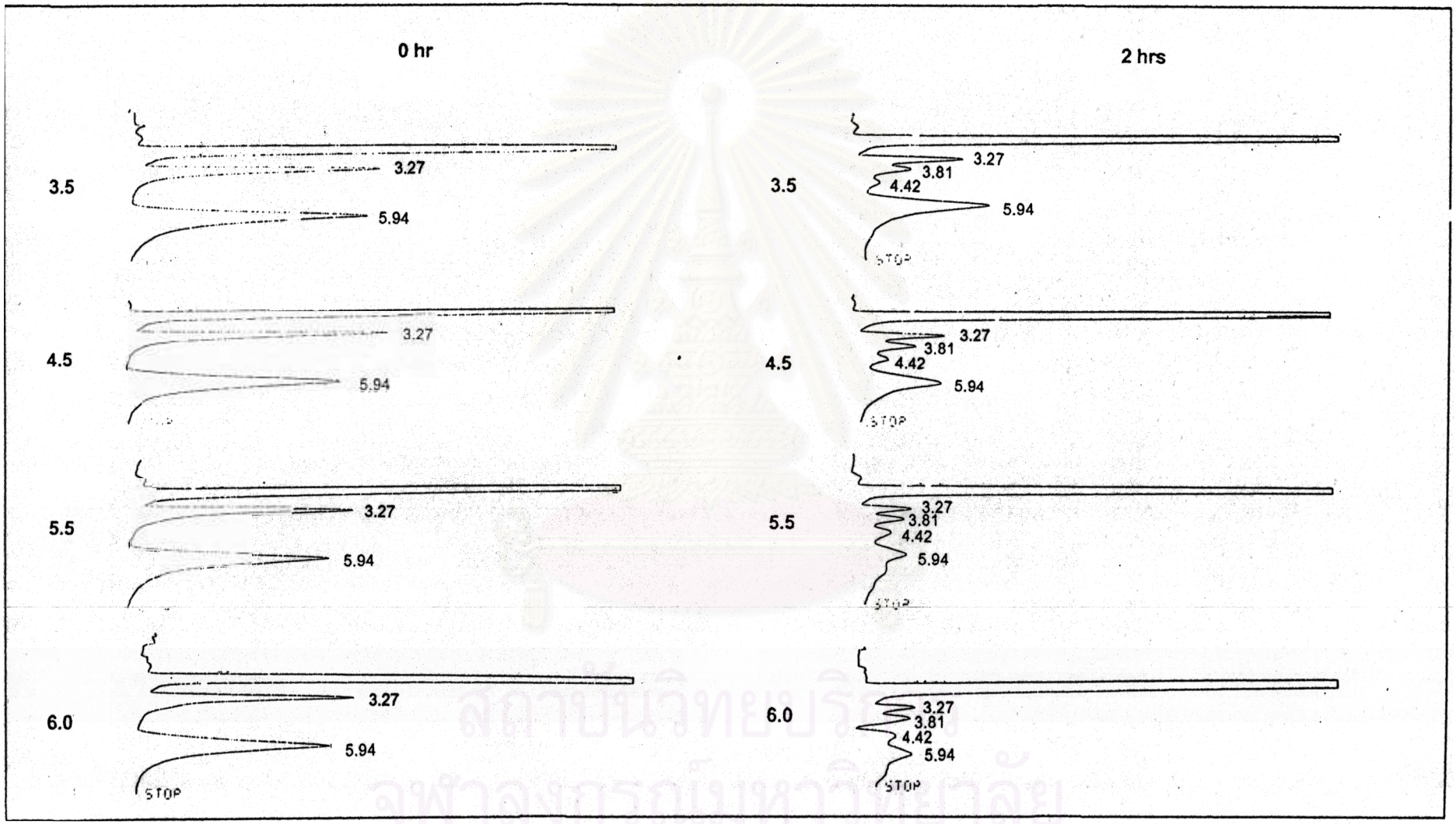


Figure 20 HPLC chromatograms of reaction mixtures of the CGTase-catalyzed coupling reaction at different pH

The reaction condition was 2.0% β-CD, 0.5% cellobiose, incubated with 16 U of CGTase, at various pH, 40°C for 2 hrs. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

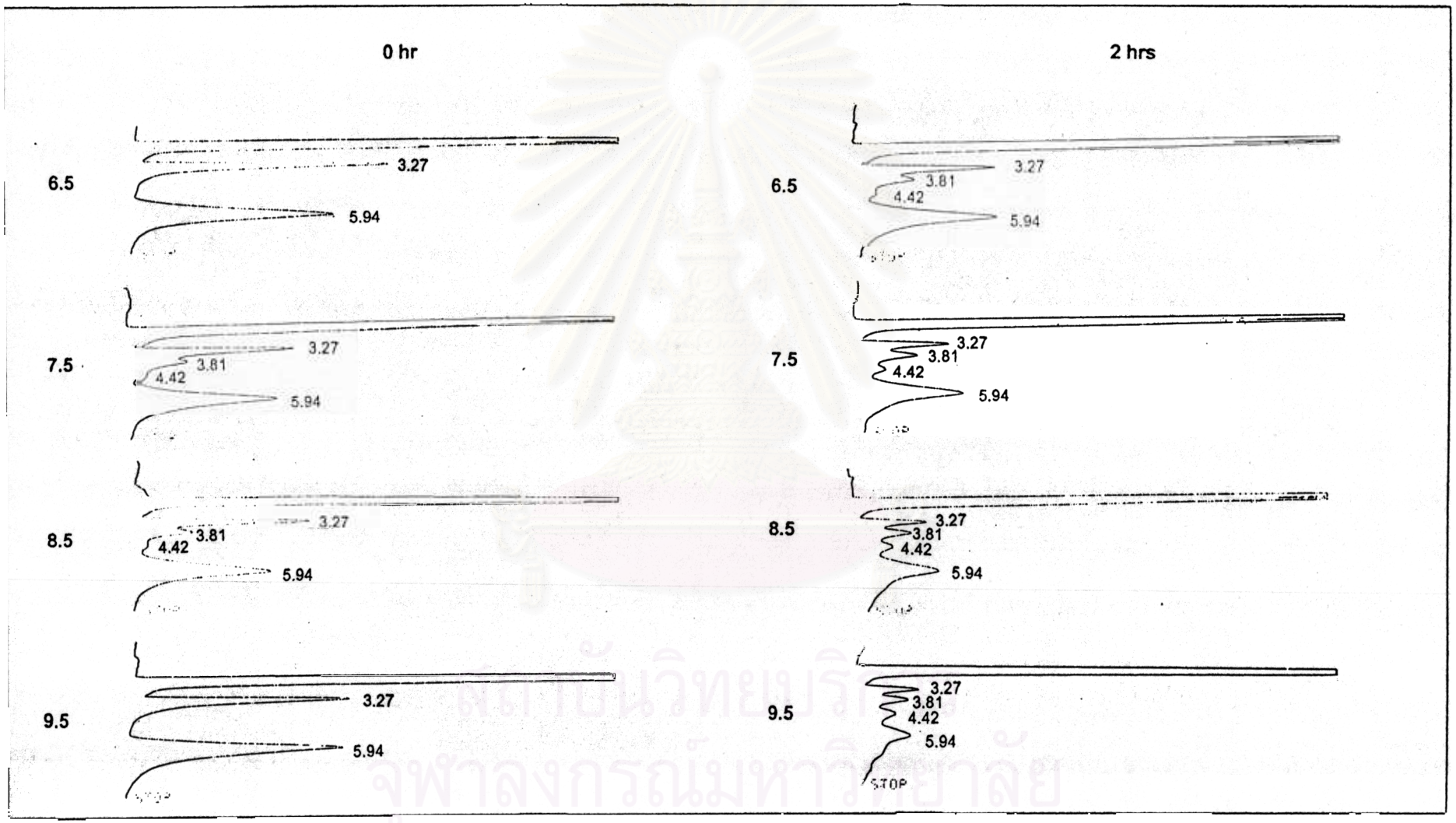


Figure 20 (Continue) HPLC chromatograms of reaction mixtures of the CGTase-catalyzed coupling reaction at different pH

The reaction condition was 2.0% β-CD, 0.5% cellobiose, incubated with 16 U of CGTase, at various pH, 40°C for 2 hrs. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

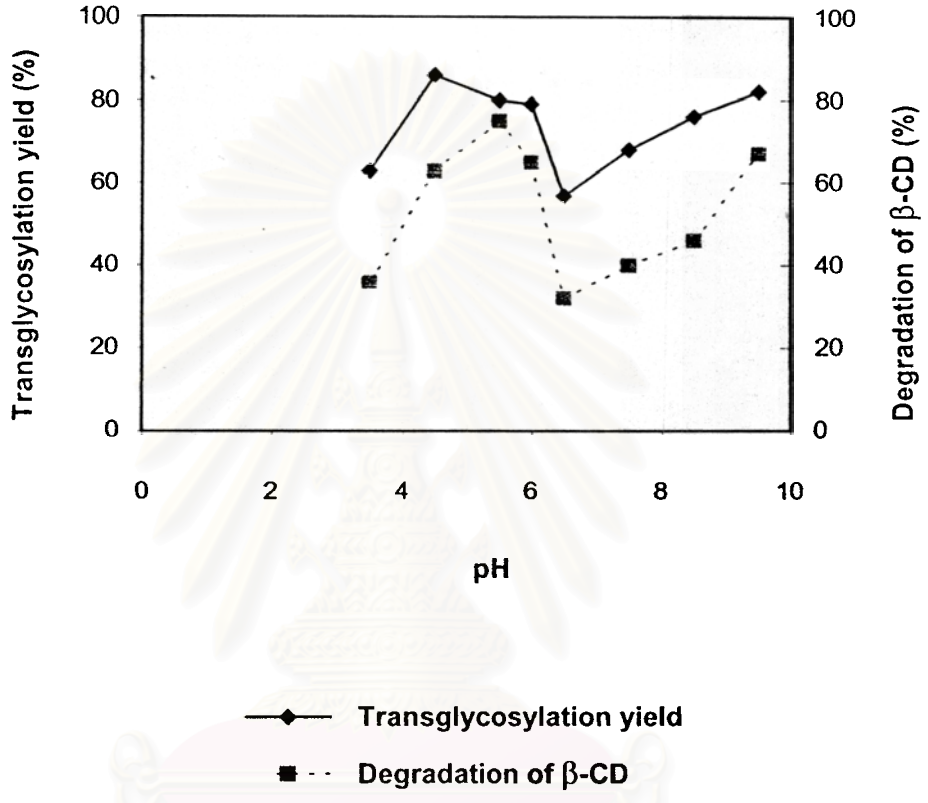


Figure 21 Effect of pH on the transglycosylation yield and degradation of β-CD in the CGTase catalyzed coupling reaction

increased thereafter (from pH 6.5 to 9.5). Degradation of β-CD pattern in related to change in pH was similar to transglycosylation yield pattern. pH 6.0 was chosen as the suitable pH due to high transglycosylation yield and also enzyme stability. In addition, the amount of transfer products formed was also high at this pH.

3.5.5 Effect of temperature

Figure 22 shows the effect of temperature on HPLC profiles of the reaction mixture of the CGTase-catalyzed coupling reaction. At 0 hr, cellobiose and β-CD peaks at R_t 3.27 and 5.94 minutes were high. From 30 to 70 °C, both cellobiose and β-CD were decreased and two main transfer products at R_t 3.81 and 4.42 minutes were observed. At 80°C, the peaks of the two substrates, cellobiose and β-CD were high and two main transfer products at R_t 3.81 and 4.42 minutes were decreased compared to those from 30 to 70°C, the result which indicated that the enzyme could not work well at this high temperature. Figure 23 shows that the transglycosylation yield did not show much change when temperature was increased from 30 to 80°C while degradation of β-CD were increased about 15% as temperature increased from 30 to 50°C. From 50 to 70 °C, degradation of β-CD was rather constant, but significantly dropped at 80°C. In addition, the amount of transfer products obtained at 30 °C was about the same as at those higher temperature. The optimal temperature of 30 °C was thus chosen from this experiment. From these suitable conditions, gave 78% of the transglycosylation yield.

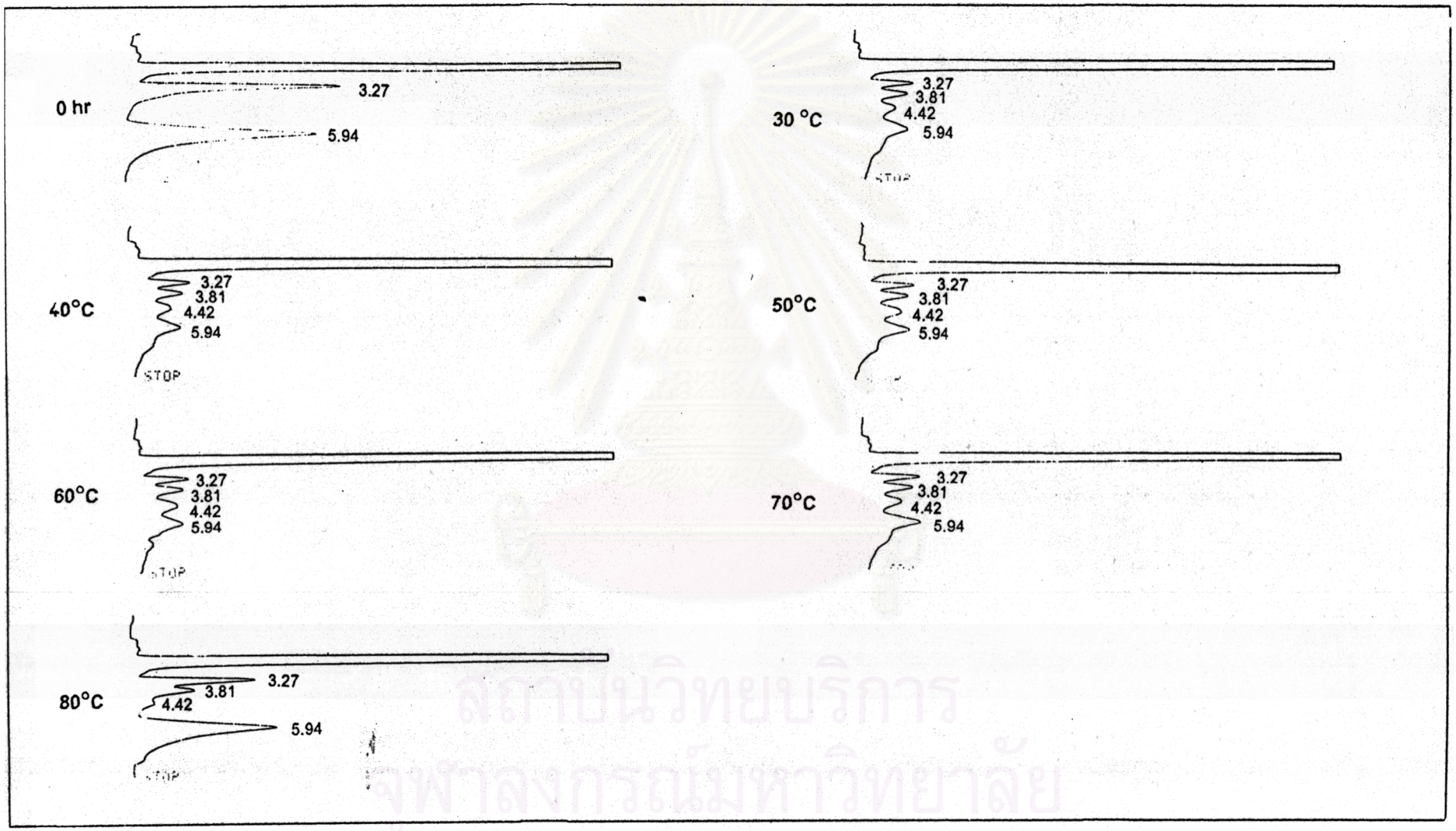


Figure 22 **HPLC chromatograms of reaction mixture of the CGTase catalyzed transglycosylation reaction at different temperatures**

The reaction condition was 2.0% β-CD, 0.5% cellobiose, incubated with 16 U of CGTase, at pH 6.0, 2 hrs at various temperatures. Spherisorb-NH_2 column was used. Acetonitrile : water (75 : 25) was used as eluent at the flow rate of 2 ml/min.

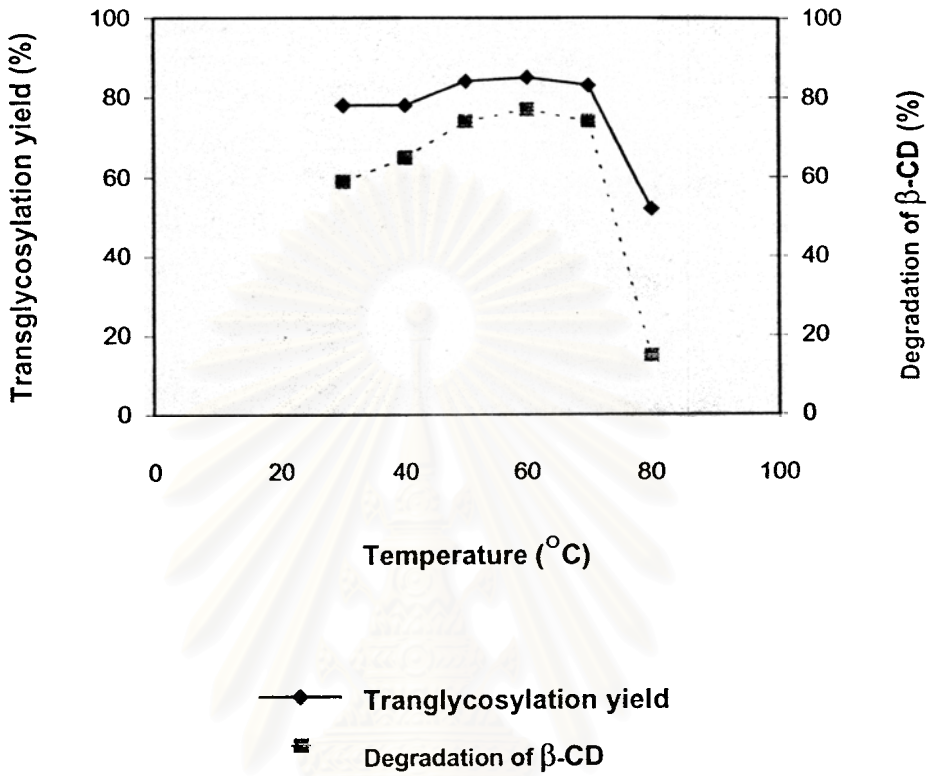


Figure 23 Effect of temperature on the transglycosylation yield and degradation of β-CD in the CGTase catalyzed coupling reaction

3.6 Comparison of the amounts of transfer products before and after optimization

To compare the transfer products of the CGTase-catalyzed coupling reaction when the reaction was performed by incubating 2% β-CD, 0.2% cellobiose, and 10.72 U of CGTase at 40°C, pH 6.0 for 5 minutes (before optimization) or 2% β-CD, 0.5% cellobiose, and 16 U of CGTase at 30°C, pH 6.0 for 2 hrs. (after optimization). The result showed that the productivity of the transfer product at R_t 3.81 minutes was 16 times and the transfer product at R_t 4.42 minutes was 18 times higher than those of the transfer products obtained before optimization (data not shown).

3.7 Isolation and identification of transfer products

To isolate and identify the transfer products of the CGTase-catalyzed coupling reaction when cellobiose was used as the acceptor, the reaction was performed using the suitable conditions found in 3.5, but at a larger scale volume of 50 ml. The conditions used were 2% β-CD, 0.5% cellobiose incubated with 3,200 U of CGTase were incubated. The transfer products were isolated by collecting samples from separated HPLC peaks. Two main different transfer products at the retention times of 3.81 (PC1) and 4.42 minutes (PC2) (similar to the peaks obtained in small preparation) were isolated (Figure 24). The ratio of PC1 to PC2 was 1:1 determined from the peak area. Identification of both transfer products was performed by MS and NMR analyses.

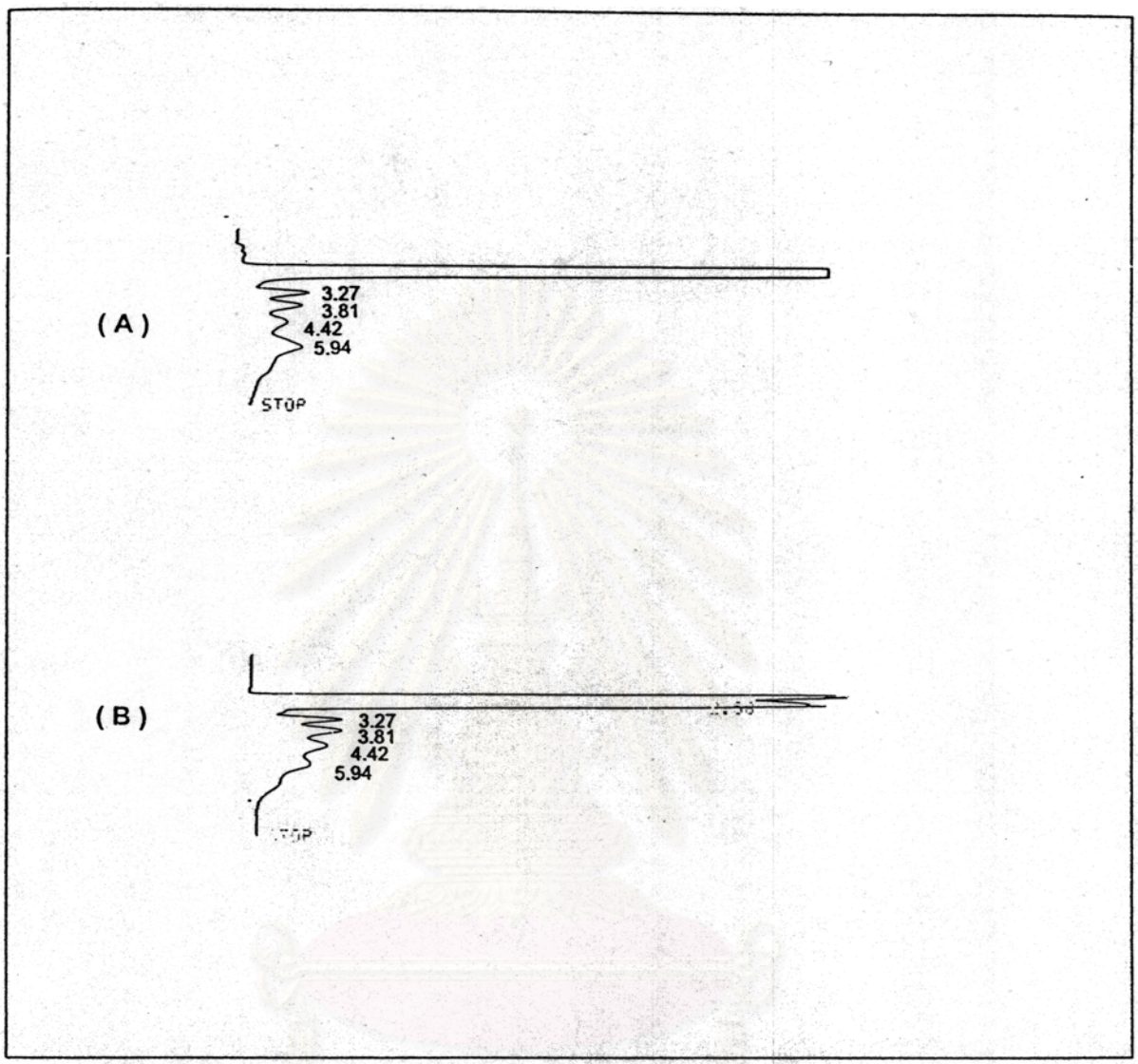


Figure 24 The HPLC profile of the reaction mixtures of the CGTase-catalyzed coupling reaction when (A) 0.25 ml or (B) 50 ml of the reaction mixture was used.

The reaction conditions were : 2.0% β-CD, 0.5% cellobiose, 16 U CGTase at 30 °C, pH 6.0 for 2 hrs. Spherisorb-NH_2 column was used. Acetronitrile : water (75 : 25) (v/v) was used as eluent at flow rate of 2 ml/min.

3.7.1 MS analysis

The molecular mass of the transfer products (PC1 and PC2) were estimated to be 504 and 666 daltons by mass spectrometer, which correspended to molecular ion at m/z of 503 and 665 ($[M-H]^-$), respectively (Figures 25 and 26).

3.7.2 ^{1}H and ^{13}C-NMR analysis

To investigate the structures of the two transfer products, NMR analysis was performed. The assignments of the signal observed in ^{1}H- and ^{13}C- NMR spectra were shown in Figures 27 and 28.

In ^{1}H-NMR spectrum, the pattern of the spectra of PC1 and PC2 was quite the same except for the higher peaks in PC2, which suggest the higher number of proton in PC2 for each functional group. The chemical shift values (δ) of the transfer products of 5.4 ppm corresponded to anomeric protons and δ about 4.0 to 3.2 ppm should be H-atoms in aromatic ring. While δ of 5.2 and 4.6 ppm were characterstics of β and α configuration, respectively.

In ^{13}C-NMR spectrum, the pattern of the spectra of PC1 and PC2 was more or less the same. The peak height in PC2 spectra was not as significantly higher than PC1 as observed in proton spectrum. The chemical shift (δ) of 102 and 104 ppm of transfer products were characteristics of α and β linkages, respectively. δ of 62 to 64 ppm was $-CH_2OH$ and δ of 71 to 82 ppm was of carbon atoms in aromatic ring.

From ^{1}H- and ^{13}C-NMR spectra and mass analysis, the transfer products (PC1 and PC2) should be trisaccharides and tetrasaccharides with α- and β- linkages in the molecules, respectively.

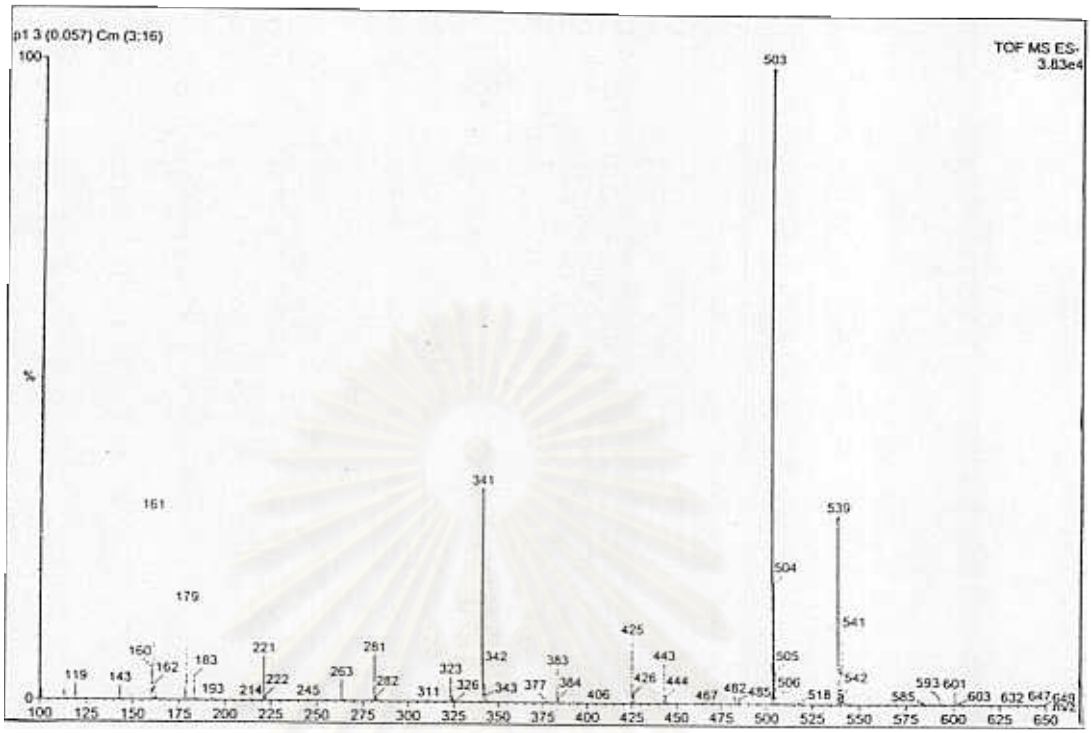


Figure 25 An electrospray mass spectrum of the transfer product (PC1) at R_t 3.81 minutes

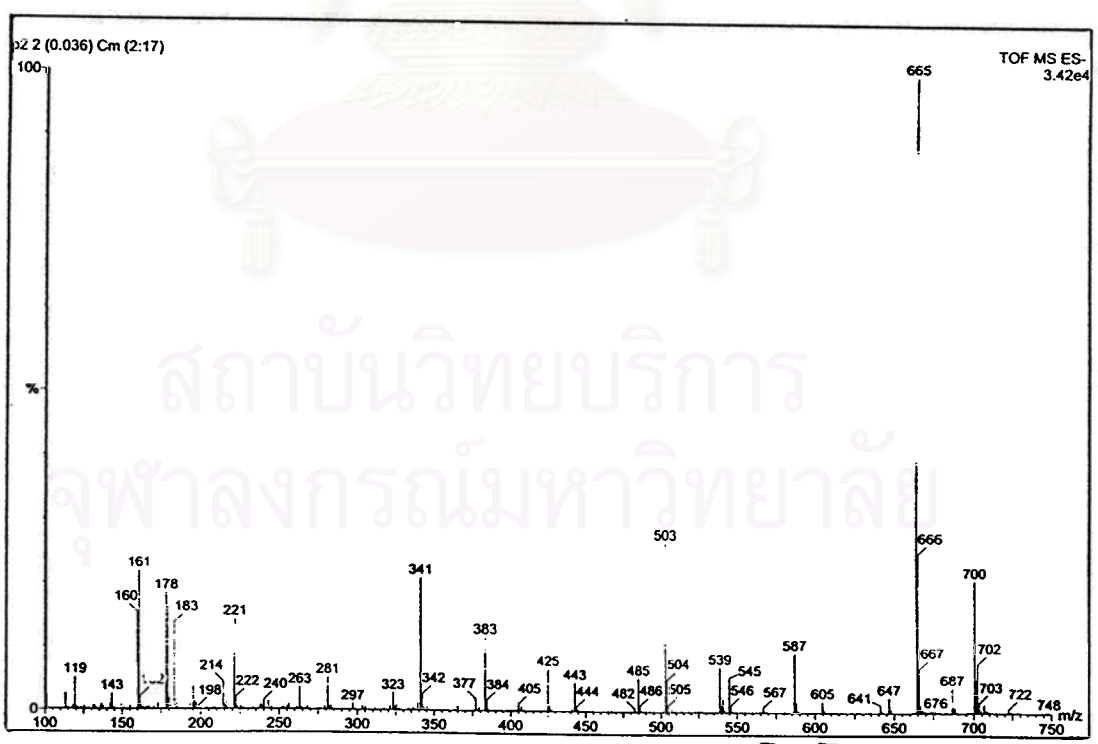


Figure 26 An electrospray mass spectrum of the transfer product (PC2) at R_t 4.42 minutes

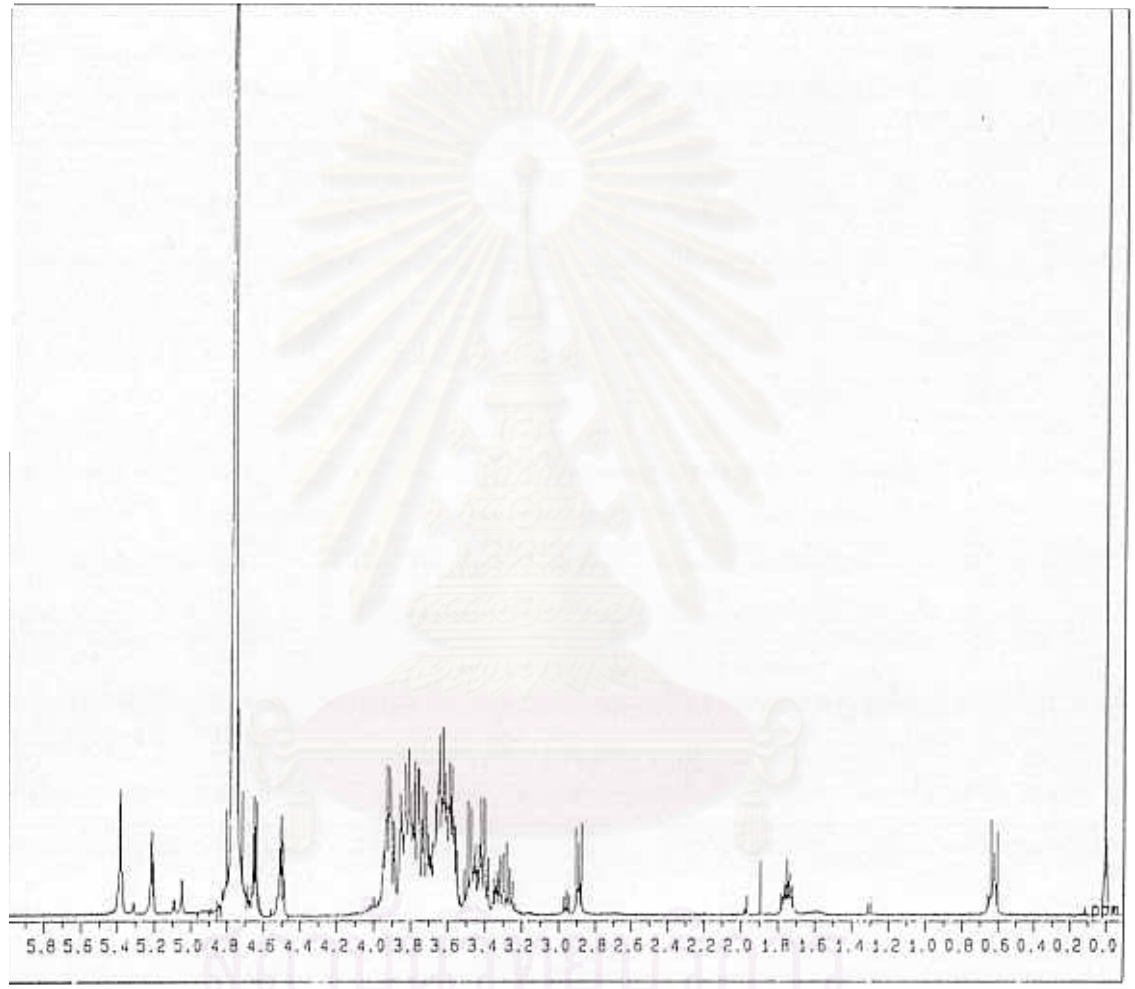


Figure 27 ^{1}H-NMR spectrum of transfer product (PC1) at the R_t 3.81 minutes

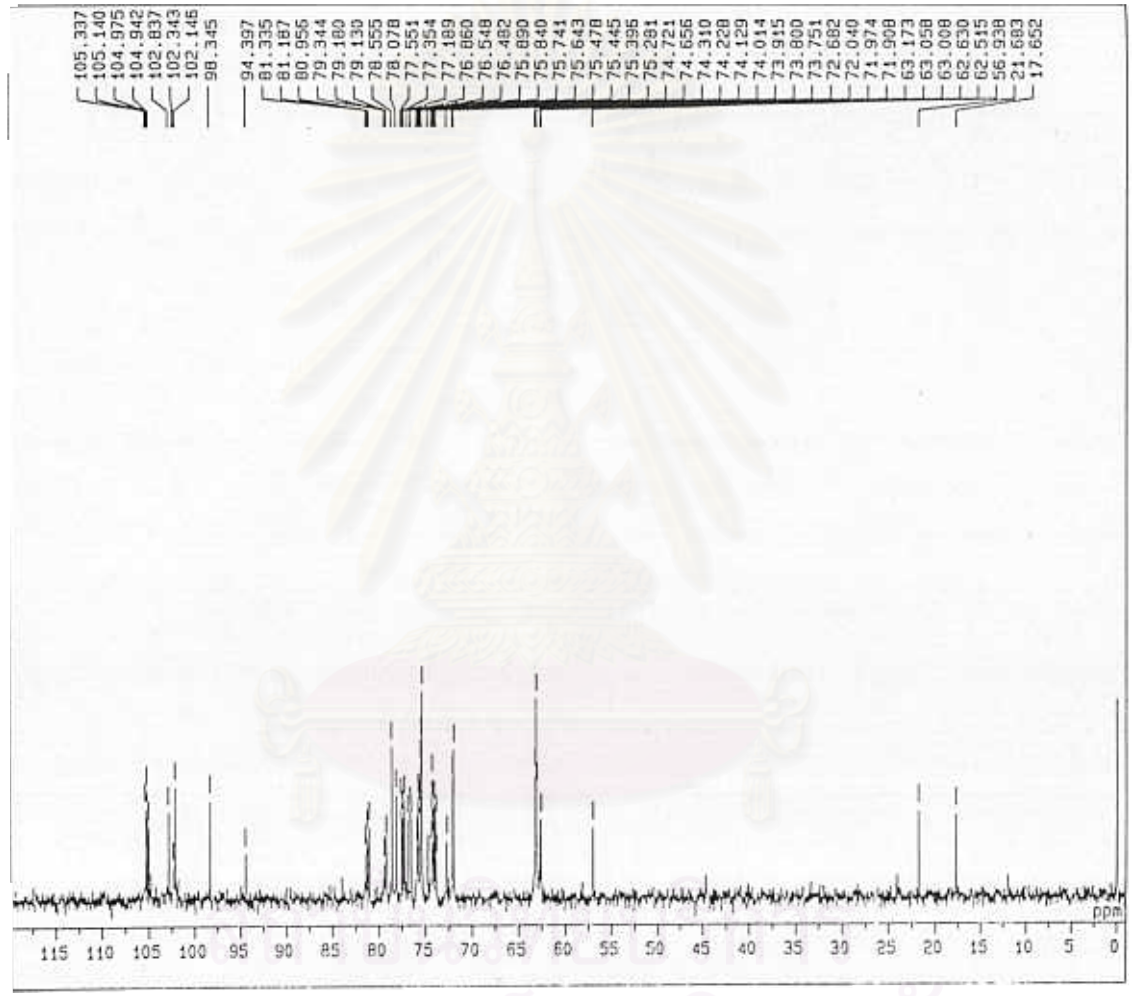


Figure 27 (Continue) ^{13}**C-NMR spectrum of transfer product (PC1) at the R_t 3.81 minutes**

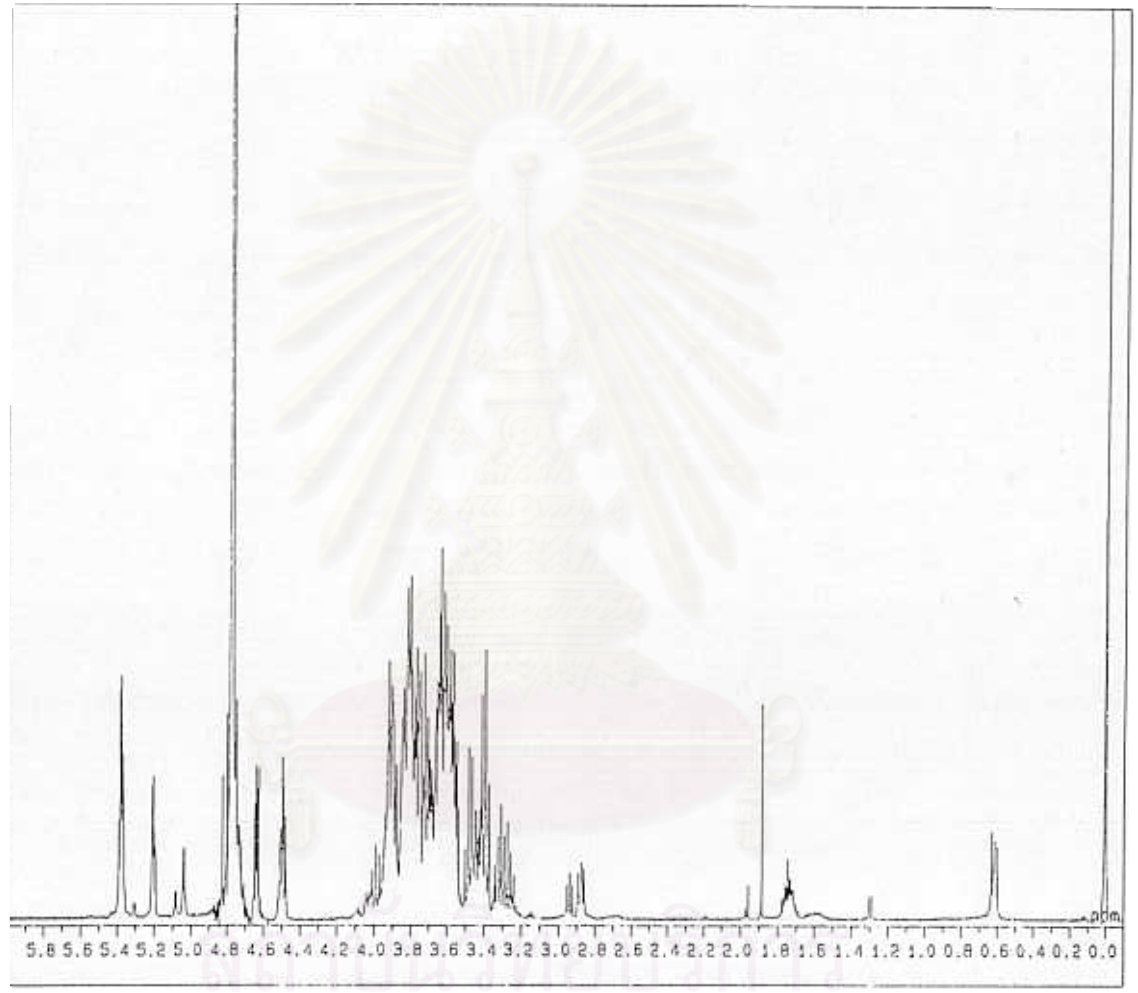


Figure 28 ^{1}H-NMR spectrum of the transfer product (PC2) at the R_t 4.42 minutes

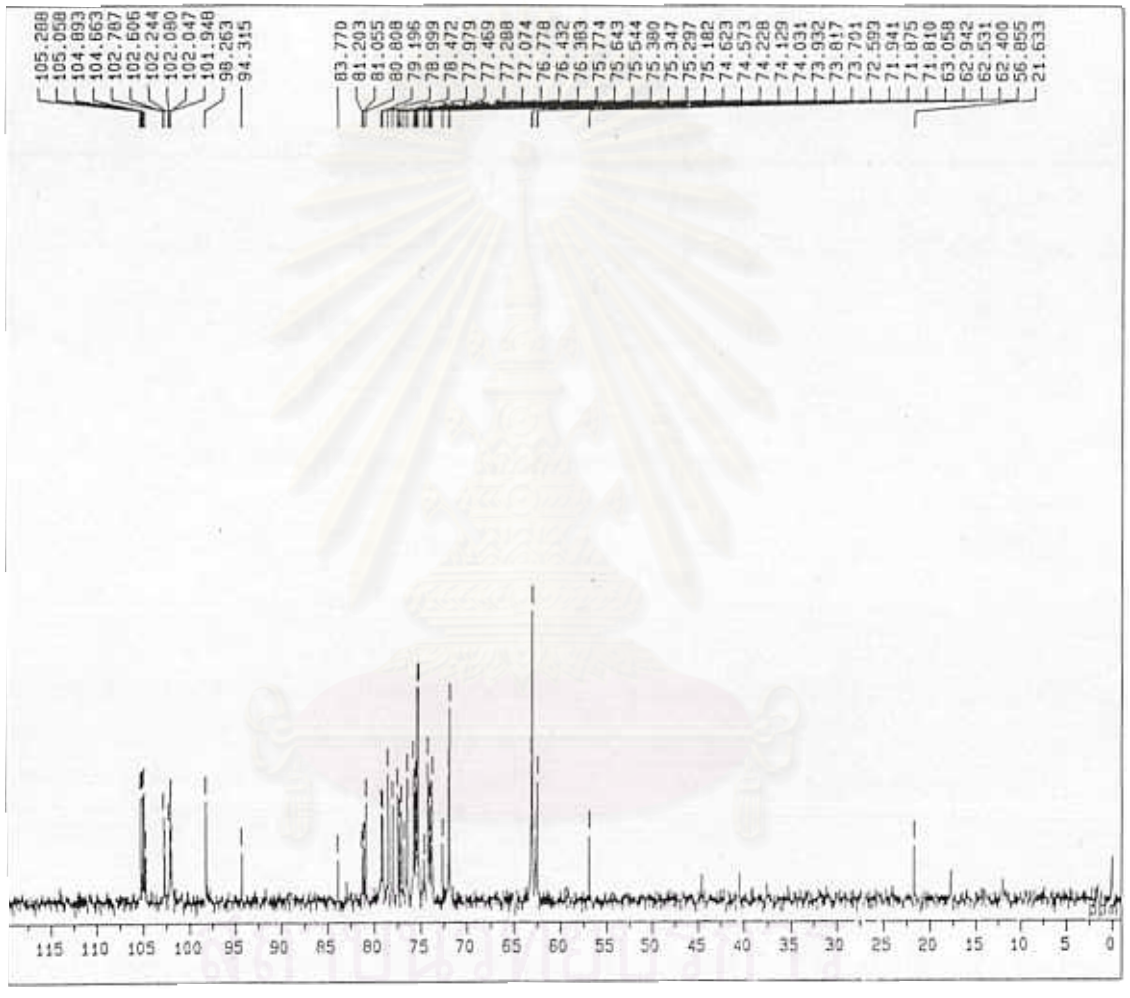


Figure 28 (Continue) ^{13}C-NMR spectrum of the transfer product (PC2) at the R_t 4.42 minutes

CHAPTER IV

DISCUSSION

4.1 Purification of CGTase

Bacillus circulans A11 was screened for CGTase producing activity by Pongsawasdi and Yagisawa (1987) and was purified from culture broth by our research group in the Department of Biochemistry (Techaiyakul, 1991 ; Rojtinnakorn, 1994 ; Kim,1996). The CGTase produced was the extracellular enzyme with β-CD as the main product (Techaiyakul, 1991). In this work, the purification was accomplished by immmunoaffinity column chromatography, following the methods reported by Kim (1996). The enzyme was partially purified by corn starch adsorption in the first step. The loss of activity in this step was approximately 2 % while no activity was detected in the washing buffer (Table 6). After CGTase was eluted by maltose, it was partially purified to 59 folds and specific activity was 1,256 unit/mg protein with 98 % recovery. When CGTase was further purified and concentrated by ultrafiltration technique, it was purified to 73 folds and the specific activity 1,557 unit/mg protein with 81 % recovery. The final step of purification was immunoaffinity column chromatography. After eluted CGTase by 3.5 M NaSCN in 50 mM NH_4OH, it was purified to 100 folds and the specific activity was 2,126 unit/mg protein with 33 % recovery (Table 6), which corresponded to the increase in CD-product, determined by CD -TCE dilution limit. This result is similar to that reported by Kim (1996) and Tongsima (1998), who demonstrated CGTase purification by immunoaffinity column chromatography. Purity of the enzyme was demonstrated by native PAGE. Two

major and two minor isoform patterns were observed through the amylolytic activity stain of the purified preparation (Figure 8), the same behavior as previously reported by Rojtinnakorn (1994) and Kaskangam (1998). The purified enzyme showed one intense protein band with an estimated molecular weight of 72,000 daltons which was identical to the previous reports. (Rojtinnakorn,1994 ; Kim, 1996 ; Tongsima,1998).

4.2 Specificity of glycosyl acceptors in the CGTase-catalyzed coupling and transglycosylation reactions

Cyclodextrin Glycosyltransferase (CGTase) converts starch to cyclodextrin by intramolecular transglycosylation. In the presence of suitable acceptors, the enzyme can also catalyze intermolecular transglycosylation where glycosyl residues are transferred, either from starch or β-CD to the acceptor molecules. The specificities of intramolecular transglycosylation of the CGTases have been well-recognized, and it has been reported that the ratio of α-, β-, and γ-CDs produced from starch differed depending mainly on the source of CGTases (Table 4). With respect to intermolecular transglycosylation, there were some reports from other CGTases (Kitahara *et al*., 1978; Takana *et al*., 1991; Suzuki *et al.*, 1991; Kometani *et al*., 1994; Kurimoto *et al*., 1997). The present work focuses mainly on the acceptor specificity of CGTase from *Bacillus circulans* A11.

4.2.1 Acceptors which form linear opened-chain oligosaccharides

Several methods have been used to determine acceptor specificity. Among those techniques, paper chromatography, thin-layer chromatography, High Performance Liquid Chromatography (HPLC), and kinetic parameters have been employed

(Kitahara *et al*.,1976, 1978, 1992; Kometani *et al*., 1994). In this work, kinetic parameters and HPLC methods were used. Kinetic parameters were measured in the initial phase where acceptors were screened for their potential activity. Only good candidates were then re-examined by the HPLC technique.

Kinetic parameters were determined by cyclodextrin-degrading activity assay, the system employed a coupled enzyme assay. After coupling of cyclodextrin to a non-reducing-end of the acceptor, a linear oligosaccharide generated act as a substrate, which is susceptible to hydrolytic cleavage by glucoamylase (to convert linearlized oligosaccharides to glucose). The liberation of the reducing sugar measured by conventional methods gives the procedure the ease and convenience of routine sugar analysis, dinitrosalicylic acid method (Miller, 1959). The activities were calculated from consumed amounts of cyclodextrin calibrated from the amounts of glucose in glucoamylase-treated reaction mixtures. In this experiment, β-CD, a good substrate for coupling activity reported by Tongsima (1998), was used as glycosyl donor. Different types of glycosyl acceptors including monosaccharides, monosaccharide derivatives, and disaccharides were tested for their acceptor capability. For monosaccharides including aldoses and ketoses (C_6 structure) and C_5 sugars, the result showed that glucose and sorbose demonstrated high V_{max}/K_m and high K_{cat} values whereas mannose, galactose, fructose, ribose, arabinose, and xylose demonstrated low values (Table 7). This suggests that glucose and sorbose were the good candidates but galactose, mannose, fructose, xylose, and arabinose were poor acceptors. When analyzing the structures of these monosaccharides (Appendix 10), the importance of pyranose ring with C_6 unit and the same configurations of C2-, C3-, and C4-hydroxyl groups as glucopyranose is evidenced. Glucose and sorbose had the same

structure and configuration except modification at C1 which had no effect on acceptor specificity. Change of configuration of C2- or C4-hydroxyl as in mannose or galactose caused serious impairment of the efficiency as acceptor, while modification at C2-OH to C2-H in 2-deoxy-glucose without the change in configuration resulted in less effect (K_m of 2-deoxy-glucose was 3 times higher than that of glucose while V_{max} was decreased only about 25% and K_{cat} was not much reduced). The requirement of six carbons was necessary as demonstrated by the result of xylose comparing with glucose, both molecules have the same structure and configuration except that xylose has no $-CH_2OH$ as C_6. The furanoside structure was not supportive to the acceptor efficiency as evidenced by the result of fructose and ribose. The K_m of ribose was exceptionally high which may be caused by the steric effect of C2- and C3-OH (both axial positions) when compared to fructose. When compared with previous work, Kitahata *et al.* (1978) reported that the requirement for an acceptor of the intermolecular transfer reaction catalyzed by *Bacillus megaterium* CGTase is the pyranose structure having the same configurations of the free C2-, C3-, and C4-hydroxyl groups as glucopyranose. In 1994, Nakamura *et al.* reported the same configuration of the hydroxyl groups with those of D-glucopyranose at C2-, C3-, and C4-positions were required for the acceptors used by CGTase from alkalophilic *Bacillus* sp.. The structure around C6- on acceptors was not essential for acceptor function, but it was recognized by CGTase. Kitahata (1978) also reported that change in the structure or configuration at the other C atoms does not much affect the efficiency of those sugars as acceptors. However, modification of one of the groups at C2-, C3-, or C4- position causes impairment in the efficiency as acceptor, as seen in 2-deoxy-glucose.

The result of monosaccharide derivatives including gluconic acid, deoxy sugars (fucose and rhamnose) and sugar alcohols (inositol, mannitol and sorbitol) showed that they were not good acceptors though some had high V_{max}/K_m but which low K_{cat} value. In this group, NAG yielded V_{max}/K_m and K_{cat} value which was half of that of glucose, again indicated that modification at C2-OH was not really serious. Ascorbic acid gave surprisingly high V_{max}, though with the furanoside structure. This might be the result of the reducing power of ascorbic acid, and the unique structure with double bond between C2- and C3- and the C=O at C1 position, or the interference of the ascorbic acid in enzyme assay using the dinitrosalicylic acid method. However, We did subtract the interaction of ascorbic acid with dinitrosalicylic acid by making control experiment. And when we determined transglycosylation yield, the result in section 3.2.3 confirmed that ascorbic acid was a good acceptor. In 1991 Aga *et al*. also reported that ascorbic acid which is quite different from the structures of effective acceptors was an efficient acceptor of transglycosylation by CGTase from *Bacillus stearothermophilus*.

For disaccharides, it was found that all those tested could be used as acceptors except sucrose that has the non-reducing-end and fructofuranosyl structure. Maltose, isomaltose, and trehalose were poor acceptors. Lactose was good while cellobiose was the best. When analyzing the structures, it can be interpreted that β linkage between C1 and C4 of glycosyl residues in the disaccharides was important for acceptor specificity. Maltose, isomaltose, trehalose, and sucrose, all posses α 1-4 linkage while cellobiose and lactose have the β-linkage. Nakamura *et al.* (1994) reported that the acceptor binding site of CGTase from alkalophilic *Bacillus* sp. can recognize at least two glucopyranose whereas

the fructofuranosyl ring of sucrose inhibits acceptor binding to the acceptor binding site of CGTase.

From our result, glucose, sorbose, ascorbic acid, cellobiose, and lactose were good acceptors as determined by kinetic parameters of the CGTase-catalyzed coupling reaction.

4.2.2 Acceptors which form glycosides

In this study, kinetic parameters were determined by two methods, coupling activity assay when acceptors were alcohols and cyclodextrin-degrading activity assay for glycoside acceptors. Tongsima (1998) reported that the two methods gave almost identical K_m values of β-CD when G_3 was used as the acceptor which confirmed the acceptable accuracy of both assays. Coupling activity were determined on the basis of the ability of β-CD to form a stable colorless inclusion complex with phenolphthalein (Vikmon, 1982). Acceptors used in the present study (alcohols and natural glycosides) will form glycosides upon coupling with β-CD. It was found that linear alcohols (butanol to decanol, and geraniol) could not be used as acceptors whereas cyclic alcohols (hydroquinone, resorcinol, and thymol) were poor acceptors (Table 8). However, cholesterol was found to be an efficient acceptor. It can be suggested here that CGTase from *Bacillus circulans* A11 had rather broad acceptor specificity in the CGTase-catalyzed coupling reaction since glycosyl group could be transferred to the acceptor molecules which had no carbohydrate moiety such as cholesterol. However, there has been no report with alcohol acceptor for other CGTases. When compared to other transferase enzymes, Sulistyo *et al.* (1995) reported that β- xylosidase from *Aspergillus pulverulentus* was shown to have broad acceptor specificity in transferring of xylosyl residues of xylooligosaccharides to various alcohol and

phenolic compound acceptors. In 1988 Shinoyama *et al*. reported that β-xylosidase from *Aspergillus niger* is effective in catalyzing transxylosylation from xylooligosaccharides to alcohols.

For acceptors as natural glycosides, hesperidin was a good acceptor (high V_{max}/K_m and high K_{cat} values) whereas naringin and rutin were not as good (moderate V_{max}/K_m and K_{cat} values) for coupling reaction (Table 8). While salicin could not act as glycosyl acceptor because of the configuration at C4 of glucosyl residue of salicin is modified (Appendix 10). Hesperidin had the same configuration of C2-, C3- and C4-OH as glucopyranose while naringin had different configuration. Rutin, though had the same C2-, C3-, and C4-OH configuration as glucose but was a poor acceptor which might be the result of steric effect within the molecule. Suzuki (1991) reported that *B. stearothermophilus* CGTase efficiently catalyzed the transfer reaction from dextrin (donor) to the C4 hydroxyl group of glucose residues in the rutinose moiety in rutin (acceptor). But the glucosyl residue in rutinose moiety in rutin was a very poor acceptor in the case of *B. macerans* CGTase in the transglycosylation reaction. In 1994 Kometani *et al.* reported that hesperidin was glycosylated by CGTase from an alkalophilic *Bacillus* sp. and rutin was glycosylated by CGTase from *Bacillus stearothermophilus*, and each monoglucoside was identified as 4^G-α-D-glucopyranosyl hesperidin and 4^G-α-D-glucopyranosyl rutin, respectively. On the other hand Kometani *et al.*(1996) identified that naringin monoglucoside formed by CGTase from *B. stearothermophilus* was 3^G-α-D-glucopyranosyl naringin. This suggested that the acceptor specificity of different CGTases might be different.

4.2.3 Comparison of transglycosylation yield using soluble starch or β-CD as glycosyl donor for the CGTase-catalyzed coupling and transglycosylation reaction

To compare the transglycosylation yield (calculated from the disappearance of acceptor) when different types of linear opened-chain oligosaccharide acceptors were used with either β-CD or soluble starch substrate in the CGTase-catalyzed coupling and transglycosylation reactions. For this work, eight glycosyl acceptors which gave low K_m and high V_{max} values (glucose, 2-deoxy-glucose, sorbose, lactose, cellobiose, NAG, fucose, and ascorbic acid) were used in the coupling and transglycosylation reactions. In HPLC profile analysis, when soluble starch was used as glycosyl donor, β-cyclodextrin, which was synthesized by intramolecular transglycosylation was also detected (Figure 12B). At time 0 min, the transfer product were also synthesized and it was proved that they were not the contaminants from soluble starch. This was confirmed by HPLC profile of control condition when soluble starch was injected. This means that CGTase simultaneously catalyzed transglycosylation and cyclization reactions. This corresponded to the result of Kitahara *et al*. (1992) who reported that the transglycosylation reaction of CGTase from *Bacillus circulans* and *Bacillus stearothermophilus* using soluble starch as donor, α-, β-, and γ-CDs were synthesized and could be detected by HPLC analysis. Baek *et al.* (1993) who studied the transglycosylation reaction of stevioside in an agitated bead reaction system using raw starch as the glucosyl donor, reported that the transglycosylation reaction occurred via two steps: firstly, the synthesis of CD from raw starch, and secondly, the transglycosylation of glucosyl residue from CD to the acceptor molecules. In 1996, Kometani *et al.* reported that CGTase from an alkalophilic *Bacillus* species produced neohesperidin monoglucoside and a

series of its maltooligoglucosides by transglycosylation with neohesperidin as an acceptor and soluble starch or β-CD as a donor. As a result, its amount with β-CD at pH 10 was about 7 times greater than that with soluble starch at pH 5.

In this work, when used β-CD as glycosyl donor, cellobiose, sorbose, and glucose were effective acceptors (transglycosylation yields of 78, 57, and 54%) whereas when soluble starch was used as glycosyl donor, sorbose and ascorbic acid (transglycosylation yields of 63 and 57%) were effective acceptors (Figure 12 and Appendix 11). For cellobiose, β-CD was especially better than soluble starch in acting as glycosyl donor. The situation was in contrast in the case of ascorbic acid where soluble starch was a better glycosyl donor. For 2-deoxy-glucose and lactose, transglycosylation yield were low which indicated that they were not as good acceptors. While fucose and NAG could not act as acceptors for both glycosyl donors. These results were in relative accordance with the results when kinetic parameters were examined. Park *et al*. (1997) also reported that most of transglycosylation yield of disaccharides showed much higher values compared to those of monosaccharides. This indicates that the CGTase has higher than affinity to disaccharides and the acceptor binding site of CGTase can recognized at least two glucopyranose molecule. In 1994, Nakamura *et al*. also observed that maltose and cellobiose showed higher acceptor specificity than glucose for the transglycosylation reaction of CGTase from *Bacillus stearothermophilus*. Thus, these previous reports showed similar results to the present study except for the high specificity of maltose found by Nakamura *et al*. (1994).

When compared the transfer products formed by the CGTase-catalyzed coupling and transglycosylation reactions using β-CD or soluble starch as glycosyl donor with different types of acceptors (low K_m and high V_{max} values), the results were summarized in Table 9. The same transfer products were observed from transglycosylation or coupling reaction (when either used soluble starch or β-CD as donor) with cellobiose or glucose as acceptor. The product ratio was the same for glucose; PG1:PG2:PG3 was 1:1:1 while PC1:PC2 from cellobiose acceptor was different. When soluble starch was used as donor, higher PC1:PC2 was obtained. The ratio was 3:1 when compared to 1:1 in the coupling reaction. Sorbose, on the other hand, gave four products with β-CD substrate while only one product peak was observed when soluble starch was used. From this result, various types of transfer products can be chosen by varying the acceptor while the preference on the amount of each product can be governed by the choice of glycosyl donor.

4.3 Effect of acceptor on the affinity constant of β-CD in the CGTase-catalyzed coupling reaction

For this work, kinetic parameters were determined by measuring cyclodextrin-degrading activity. β-CD was used as glycosyl donor and the effective acceptors such as glucose, sorbose, and cellobiose were used. The result showed that with sorbose acceptor, K_m for β-CD was lower than with glucose or cellobiose. When V_{max} of the reaction was compared, cellobiose acceptor gave the highest value when compared to glucose and sorbose. The result indicated that the acceptor had the effect on the binding of β-CD to the enzyme and the V_{max} of the CGTase-catalyzed coupling reaction. Cellobiose was the best acceptor in pairing with β-CD donor from judging from V_{max}/K_m value. Precautions should be

made here since the concentration of all acceptors used might not be at saturated concentration. However, the kinetic values shown here were in the same range as previous by reported. In 1998, Tongsima reported that K_m values were 3.16±0.24, 1.69±0.18, and 1.42±0.43 mM, respectively for α-, β-, and γ-CD for coupling reaction of CGTase from *Bacillus circulans* A11 when maltotriose was used as an acceptor. Other CGTases possessed either similar or different kinetic values from CGTase in this study. Nakamura *et al.* (1994) determined the Michaelis constant of CGTase from *Bacillus* sp. 1011. For β-cyclodextrin-forming activity using amylose as substrate, K_m value was 57.4 μM. By coupling activity assay with γ-CD as donor and methyl-α-D-glucoside as acceptor, K_m value was 16.9±1.4 mM. In 1994, Sin *et al.* reported the kinetic parameters of the CD-degrading activity of CGTase from *Bacillus ohbensis*, K_m values for α-, β-, and γ-CD were 2.4, 0.5, and 6.1 mM, respectively. In 2000, Bart *et al*. reported that K_m values for α-, β-, and γ-CD were 1.09±0.26, 8.5±2.2, and 15.7±6.1 mM and V_{max} values were 192±5.7, 294±7.6, and 150±9.0 U/mg, respectively for coupling reaction of CGTase from *Bacillus circulans* (strain 251) when methyl α-D-glucopyranoside was the acceptor molecule. In the absence of acceptor, K_m values of α-, β-, and γ-CD were 0.45±0.05, 18.1±1.4, and 16.6±3.0 mM, respectively.

4.4 Determination of suitable conditions for the production of linear opened-chain-oligosaccharides from the most suitable carbohydrate acceptor

To investigate the suitable conditions of CGTase-catalyzed coupling reaction, the transglycosylation yield (calculated from the disappearance of acceptor) and degradation of β-CD (calculated from the disappearance of β-CD) was monitored when several reaction

parameters were varied. The optimal incubation time was found to be 2 hrs. The result showed that though the incubation time was prolonged, the transglycosylation yield was rather constant. For the determination of the effect of cellobiose concentration on the CGTase-catalyzed coupling reaction, the results were summarized in Figure 16-17 that the transglycosylation yield was reduced when the concentration of cellobiose was increased. This suggests that at high concentration, cellobiose may inhibit the catalysis of CGTase on the coupling reaction and 0.5% cellobiose was suitable for coupling with β-CD. When the CGTase concentration was determined (Figure 18-19), the result showed that the transglycosyltion yield was rather constant though concentrations of CGTase were six times increased. The concentration range of CGTase used was high and the excess amount had no effect on transglycosylation yield. Lower concentration than 1,072 U/g β-CD should be tested. Park *et al*. (1997) reported that excess CGTase may induce unnecessary side reactions, such as, the hydrolysis of cyclodextrin or disproportionation reaction; therefore it cannot contribute to the increase in transglycosylation yield.

When pH of the CGTase-catalyzed coupling reaction was varied (Figure 20-21), the effective range was from pH 3.5-6.0. pH 6.0 was chosen as the suitable pH due to high transglycosylation yield and also enzyme stability. For our CGTase, the activity was significantly reduced at pH$<$5 and $\geq$ 9.0 (Techaiyakul, 1991). Optimum pH was depended on the enzyme and also the substrates. Takana *et al*. (1991) reported that the optimum pH for the transglycosylation reaction of CGTase from *Bacillus stearothermophilus* using α-CD as a substrate and ascorbic acid as an acceptor to produce ascorbic acid-2-*O*-α-D-glucoside was around 5.0. In 1994, Kometani *et al*. found that the formation of the hesperidin glycosides from soluble starch donor was more effective at alkaline pHs than at

neutral or acidic pHs, because of higher solubility of the acceptor. When the effect of temperature on the CGTase-catalyzed coupling reaction was determined (Figure 22-23), the result showed that the transglycosylation yield was rather constant when the temperature was increased from 30-70°C. Whereas at 80°C, the transglycosylation yield was decreased which should be due to denaturing of CGTase at high temperature and loss of CGTase activity. The temperature at 30°C was chosen as optimum.

4.5 Isolation and identification of transfer products

Two different transfer products were detected by HPLC technique from the CGTase-catalyzed coupling reaction with cellobiose acceptor. The result showed that after 2 hrs of incubation time, the peak height of the parent acceptor and donor molecules were reduced and the transfer products PC1 at the retention time 3.81 and PC2 at the retention time 4.42 were obtained (Figure 24). The transfer products should be linear opened-chain oligosaccharides that were produced by cleavage of cyclodextrin ring and transfer residues of maltooligosaccharides to the non-reducing-end glucose residue of acceptors to form α-1,4-glucans. This means that the transfer products should be (glycosyl)$_n$-α-1,4-linkage to cellobiose.

To isolate and identify the transfer products, the reaction mixture was separated by HPLC chromatography on Spherisorb-NH_2 column. Each of the transfer product fraction was collected, then analyzed of the molecular mass on mass spectrophotometer. The result in Figure 25-26 shows that the molecular mass of the transfer products (PC1 and PC2) were 504 and 666 daltons. Analysis of the structures of the transfer products using ^{1}H and ^{13}C-NMR was then performed (Figure 27-28). Both proton and carbon NMR spectra of PC1

and PC2 were very similar, with the characteristic peaks at the same chemical shift values. The difference was observed with the peak height, the peaks at 5.4 (anomeric proton), 5.2 (β-proton), 4.6 (α-proton), and 4.0-3.2 (aromatic ring proton) ppm in ^{1}H-NMR spectrum of PC2 were higher than those of PC1. The ^{13}C-NMR spectrum shows similar result, the peak height at 62-64 (-CH_2OH), 71-82 (aromatic carbon), 102 (α-carbon) and 104 (β-carbon) ppm were higher in PC2 than PC1 spectrum. The higher peak height indicated the higher number of proton and carbon for each functional group.

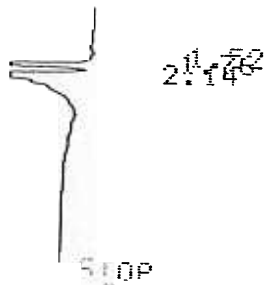
NMR spectra obtained suggested that both PC1 and PC2 were not pure.The purity was then checked by HPLC. Figure 29 shows HPLC profiles of PC1 and PC2 which were dissolved in D_2O (the same sample which were subjected for NMR analysis). HPLC trace confirmed that the transfer products were not pure. The peak area indicates that PC1 was about 80% pure (calculated from peak area at 3.57 min which was 80% of total products eluted at 3.57, 4.24, 4.90, and 5.38 min) and PC2 was only 30% purity (calculated from peak area at 4.10 min which was 30% of total products eluted at 3.54, 4.10 and 4.87 min). PC2 fraction seemed to be heavily contaminated by PC1. HPLC profiles show negative refractive index due to the presence of D_2O.

From the overall results of, it can be proposed that the transfer product PC1 was a trisaccharide of the structure glucose(α1→4)cellobiose (glc(α1→4)glc(β1→4)glc) with a molecular mass of 504. While PC2 was a tetrasaccharide of the structure glucose(α1→4) glucose(α1→4)cellobiose (glc(α1→4)glc(α1→4)glc(β1→4)glc) with a molecular mass of 666. The assignment for the attachment of glucose to cellobiose (in PC1) or glc-glc to cellobiose (in PC2) through the 1→4 are based on previous study by Abe (1991) which

proposed that CGTase transfers residues of maltooligosaccharide to the non-reducing end glucose residue of the acceptors to form α-1,4-glucans.

(A)

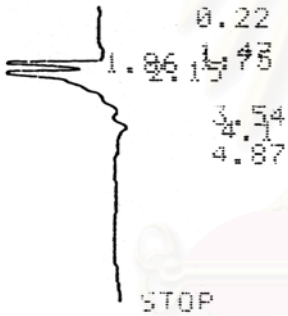
(A)



STOP

#	NAME	TIME	CONC	AREA
0		1.52	6.9864	5690
0		1.76	11.1939	9117
0		2.14	11.9205	9709
0		3.57	55.714	45378
0		4.24	6.7533	5500
0		4.9	6.7166	5470
M		5.38	0.715	582
	TOTAL		00.	81449

(B)



STOP

#	NAME	TIME	CONC	AREA
0		1.47	5.2372	3803
0		1.75	8.255	5995
0		1.86	5.4619	3967
0		2.15	10.3391	7509
0		3.54	47.7249	34662
0		4.1	19.7527	14346
0		4.87	3.2288	2345
	OTAL		99.9999	72630

Figure 29 Rechecking the purity of the transfer products by HPLC

(A) The transfer product at R_t 3.81 minutes (PC1)

(B) The transfer product at R_t 4.42 minutes (PC2)

CHAPTER V

CONCLUSION

1 CGTase from *Bacillus circulans* A11 was purified approximately 100 folds with a specific activity of 2,126 units/mg protein.

2 Among 22 acceptors which form linear opened-chain oligosaccharides, glucose, sorbose, and cellobiose were good acceptors as determined by kinetic parameters of the CGTase-catalyzed coupling reaction.

3 When transglycosylation yield was measured, cellobiose, sorbose, and glucose were effective acceptors which coupled with β-CD donor with the transglycosylation yield of 78, 57, and 54%, respectively. While soluble starch was used as glycosyl donor, sorbose, and ascorbic acid (transglycosylation yield of 63 and 57%) were effective acceptors. For cellobiose, β-CD was especially better than soluble starch whereas the result was vice versa with ascorbic acid. The types of transfer products can be chosen by varying the acceptor while the preference on the amount of each product can be governed by the choice of glycosyl donor.

4 Among acceptors which form glycosides, cholesterol, and hesperidin were good acceptors as determined by kinetic parameters of the CGTase-catalyzed coupling reaction. Aliphatic alcohols could not act as the glycosyl acceptor.

5 The best and most suitable linear opened-chain oligosaccharide forming acceptor was cellobiose.

6 Efficient acceptors require six carbons in the monosaccharide unit, with the pyronoside structure and configuration of C2-, C3-, and C4-hydroxyls as glucopyranose. The disaccharide with the β-linkage was supportive to the acceptor efficiency.

7 The acceptor had the effect on the binding of β-CD to the enzyme and the V_{max} of the CGTase-catalyzed coupling reaction.

8 The optimum conditions for coupling reaction that used cellobiose and β-CD as substrates were 2% β-CD, 0.5% cellobiose incubated with16 U of CGTase at pH 6.0, 30°C, for 2 hrs. This condition gave 78% transglycosylation yield and high production of two transfer products, PC1 and PC2, at the retention times of 3.81 and 4.42 min, respectively.

9 The molecular mass of the two transfer products were 504 and 666 daltons. The structures suggested by NMR analyses and mass spectra were a trisaccharide of glc(α1→4)glc(β1→4)glc and a tetrasaccharide of glc(α1→4)glc(α1→4)glc(β1→4)glc.

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APPENDICES

APPENDIX 1: Preparation for polyacrylamide gel electrophoresis

1) Stock reagents

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

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

Adjusted volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 g

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

Solution B (SDS PAGE)

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

Solution C (SDS PAGE)

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

2. Non-denaturing PAGE

7.5% Separating gel

30% acrylamide solution	2.5 ml
1.5 M Tris-HCl pH 8.8	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
0.5 M Tris-HCl pH 6.8	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	3.1 ml
glycerol	5.0 ml
1% bromophenol blue	0.5 ml
distilled water	1.4 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	3.0 g
Glycine	14.4 g

Dissolved in distilled water to 1 litre (final pH should be 8.8).

3. 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.

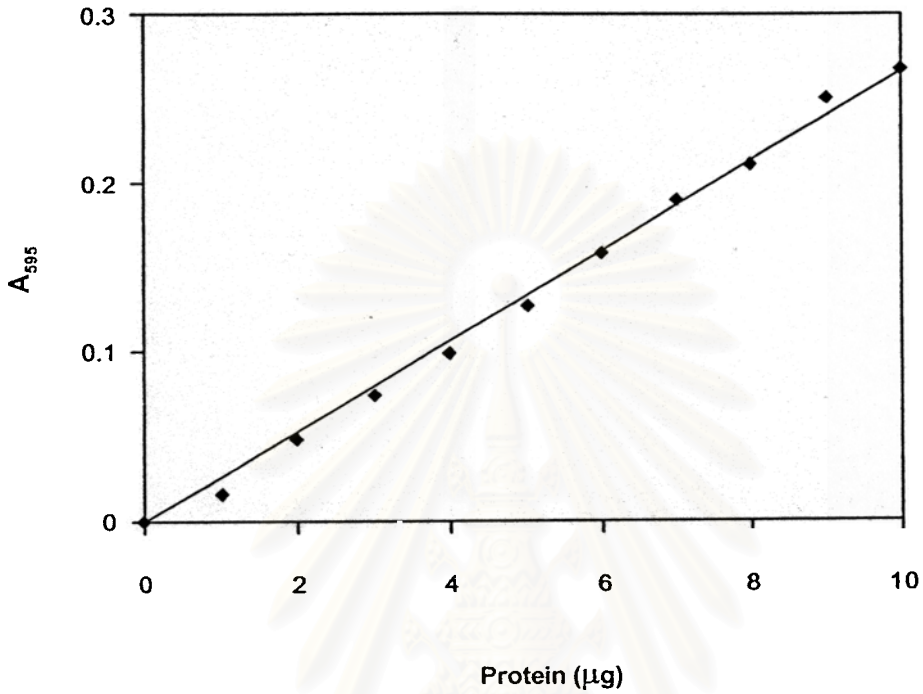
Electrophoresis buffer, 1 litre

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

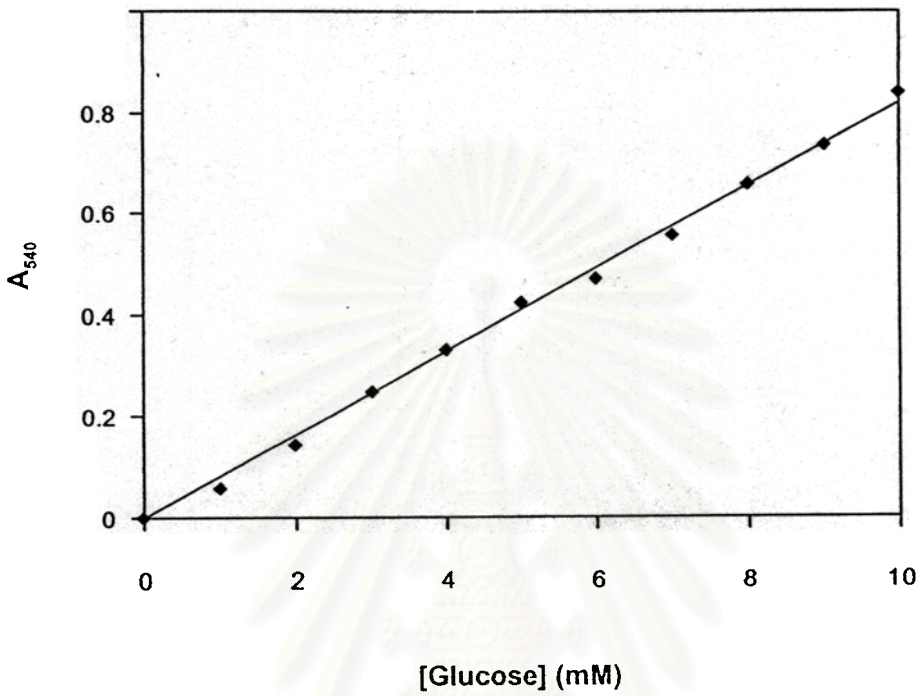
Adjusted volume to 1 litre with distilled water

(pH should be approximately 8.3).

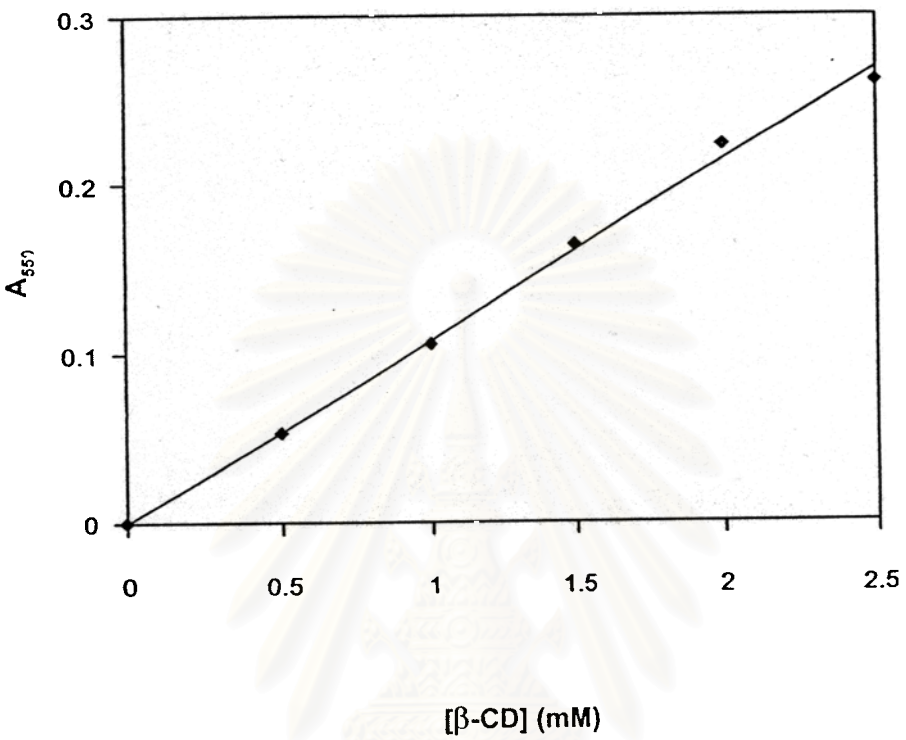
APPENDIX 2 : Standard curve for protein determination by Bradford's method



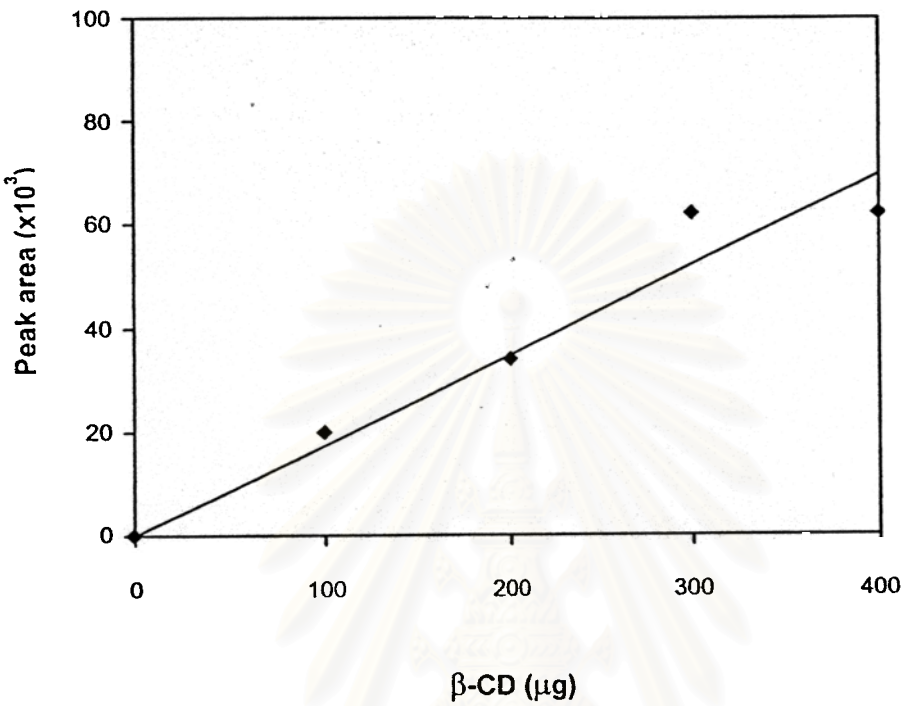
APPENDIX 3 : Standard curve of glucose by dinitrosalicylic acid method



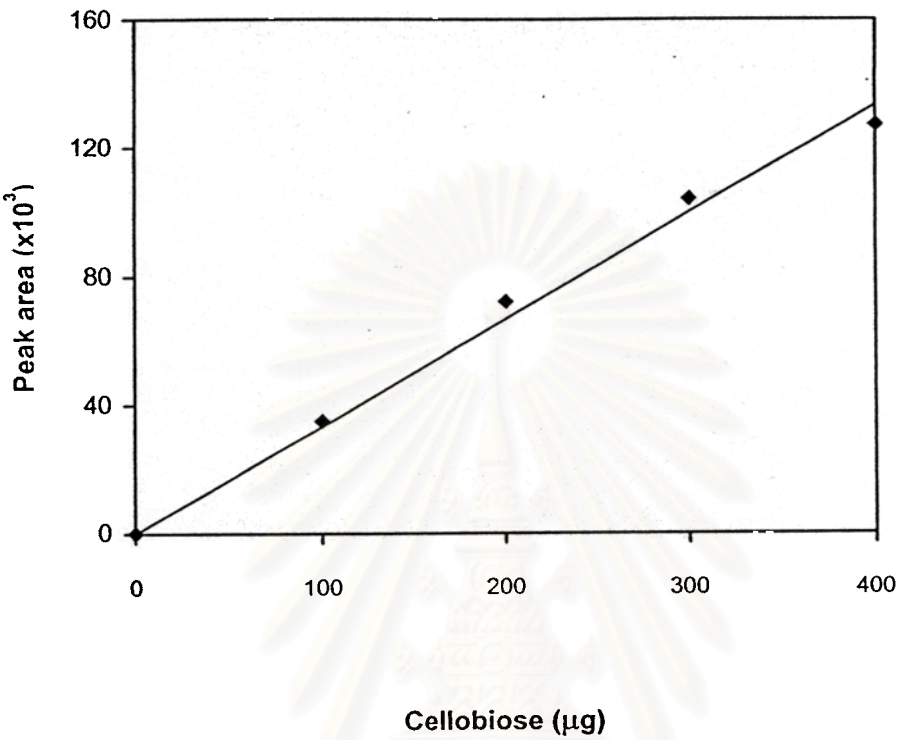
APPENDIX 4 : Standard curve of β-cyclodextrin by phenolphthalein method



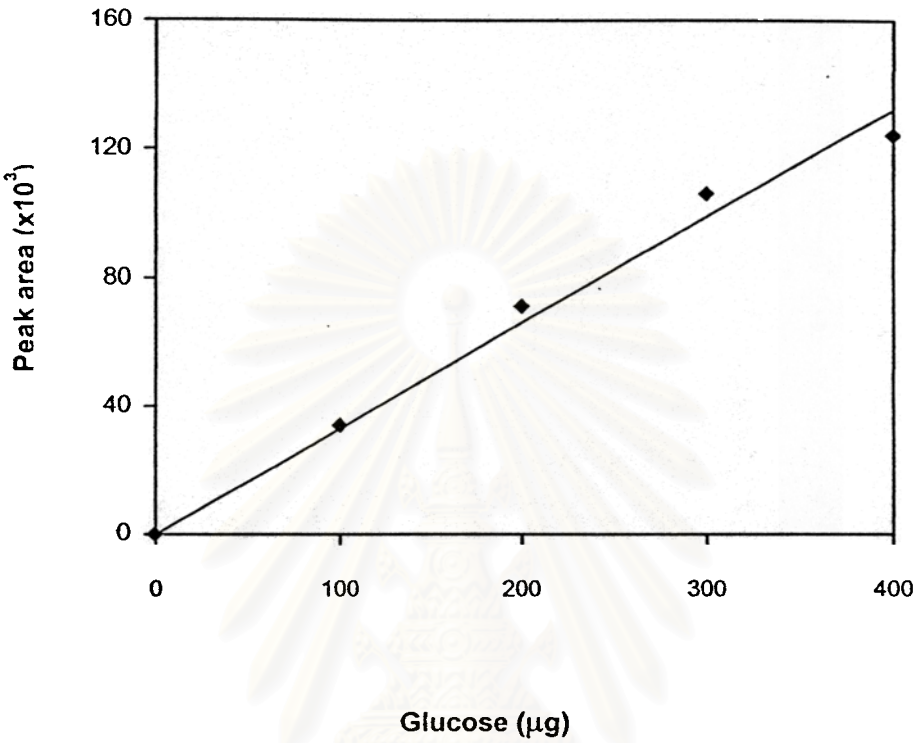
APPENDIX 5 : Standard curve of β-cyclodextrin by HPLC method



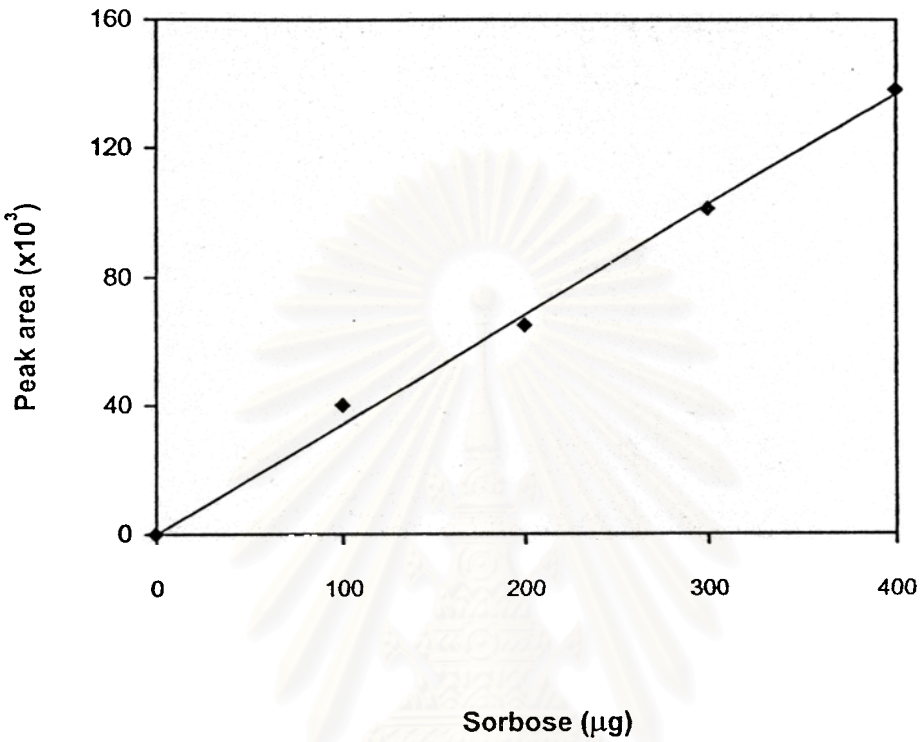
APPENDIX 6 : Standard curve of cellobiose by HPLC method



APPENDIX 7 : Standard curve of glucose by HPLC method

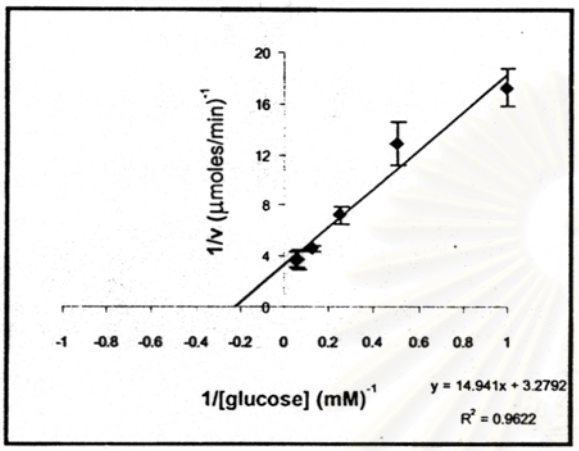


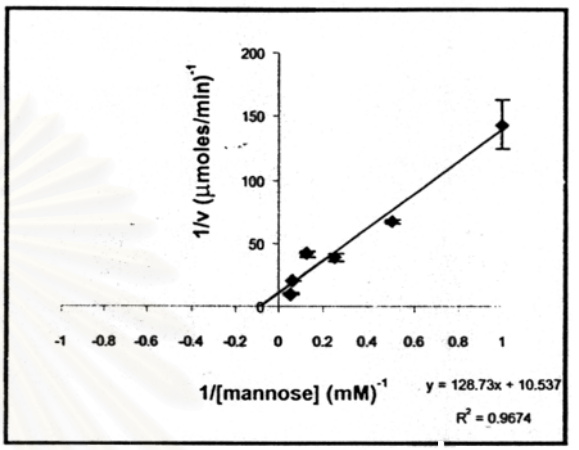
APPENDIX ฮ : Standard curve of sorbose by HPLC method

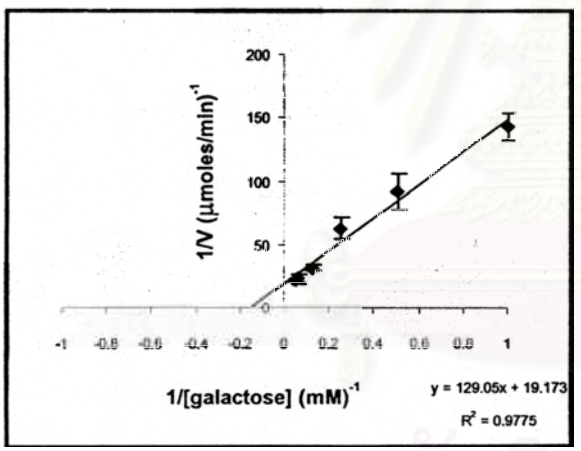


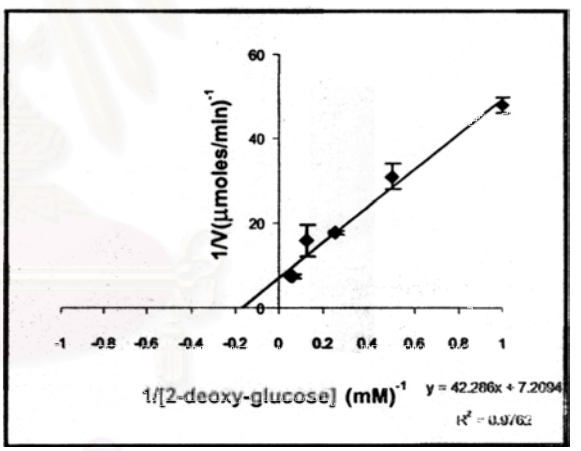
APPENDIX 9 : Lineweaver-Burk plot of CGTase with β-CD as glycosyl donor and various acceptors

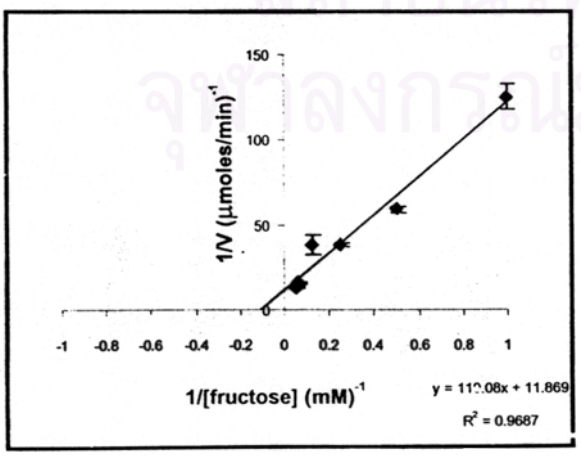
A) LOCO forming acceptors

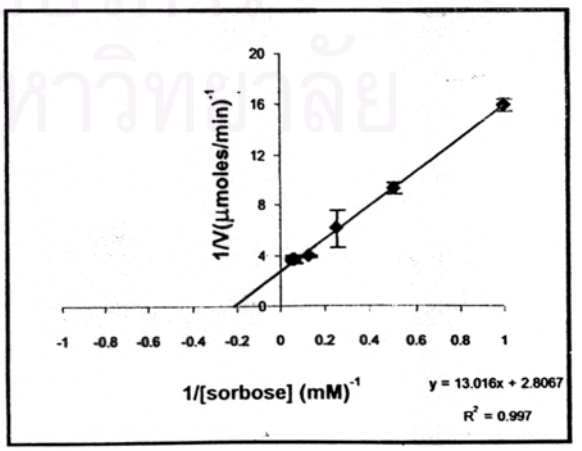




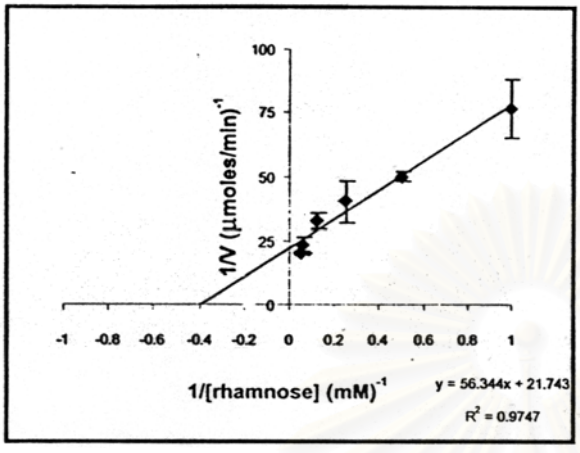


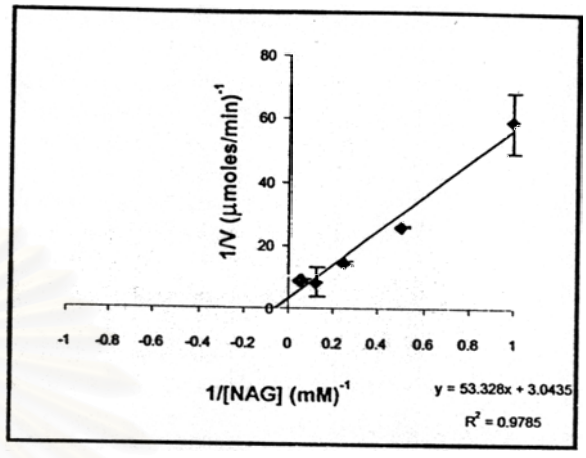


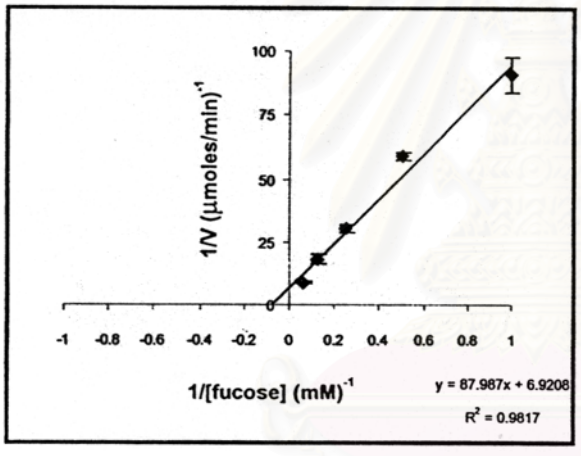


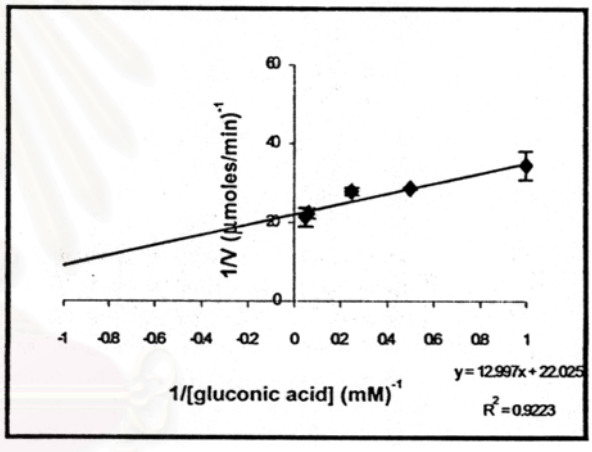


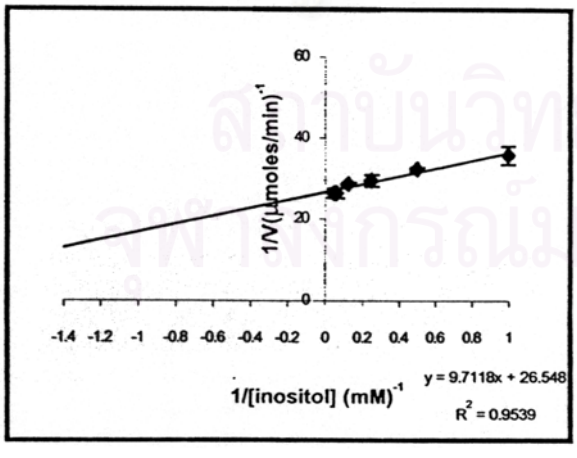
APPENDIX 9: Lineweaver-Burk plot of CGTase with β-CD as glycosyl donor and various acceptors (Continue)

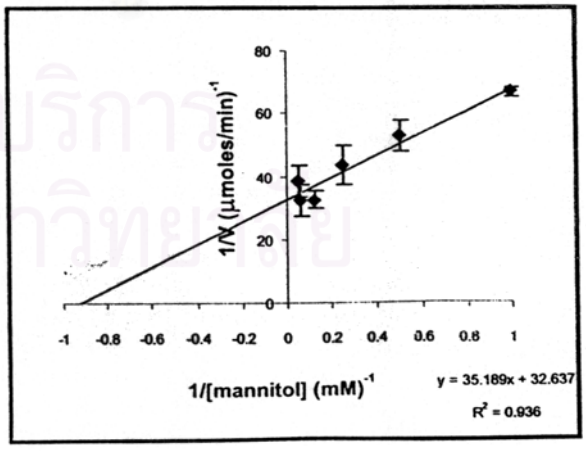




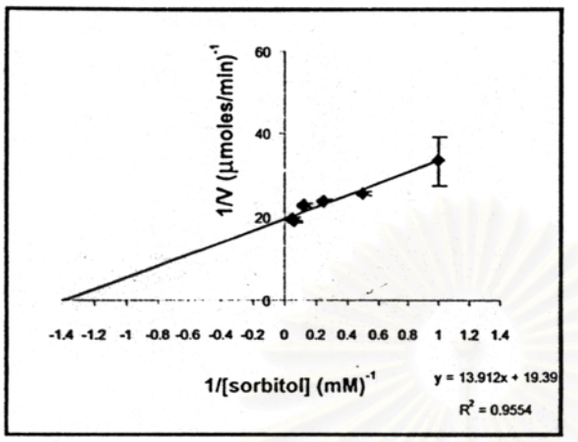


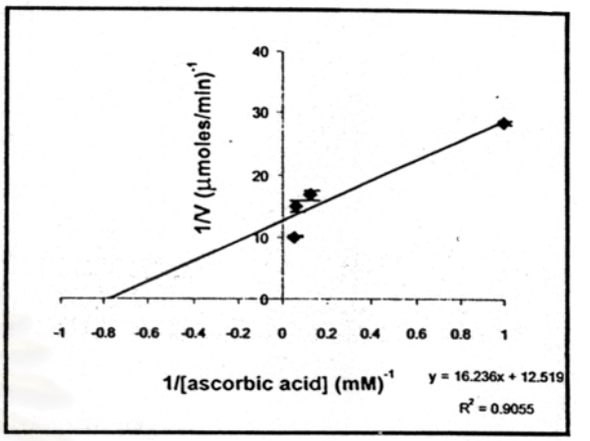


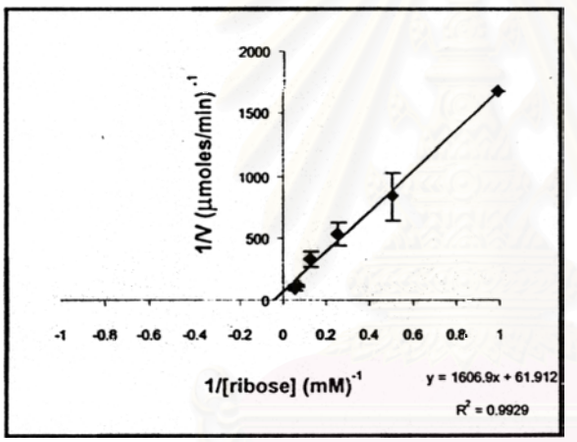


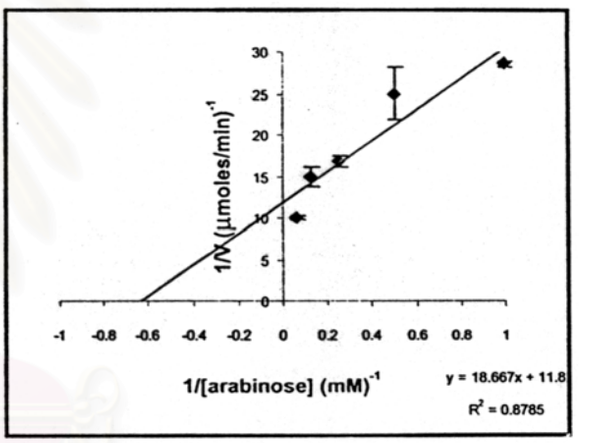


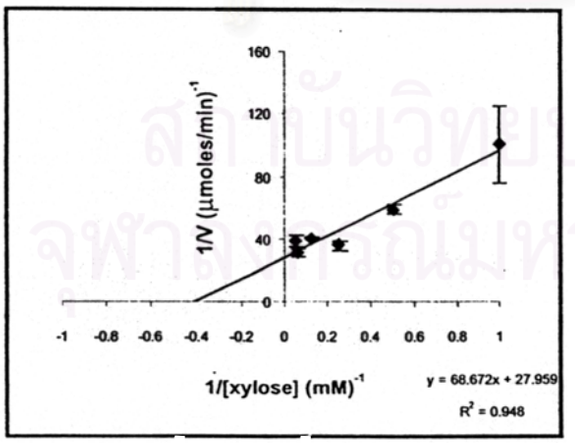
APPENDIX 9: Lineweaver-Burk plot of CGTase with β-CD as glycosyl donor and various acceptors (Continue)

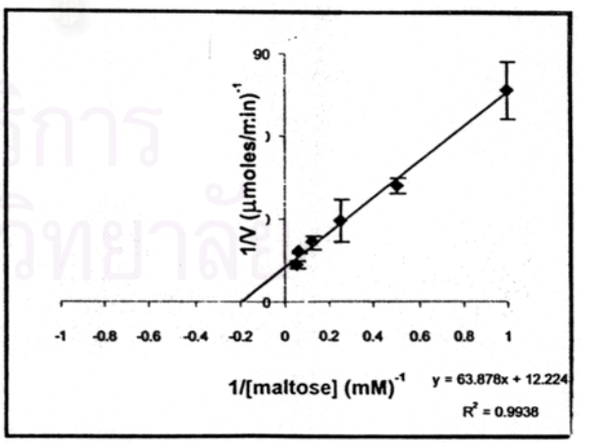




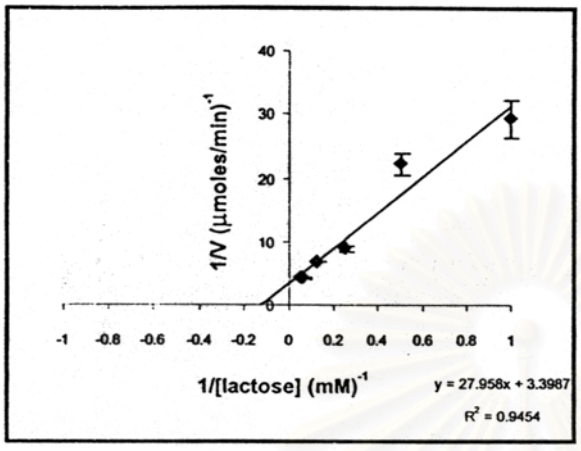


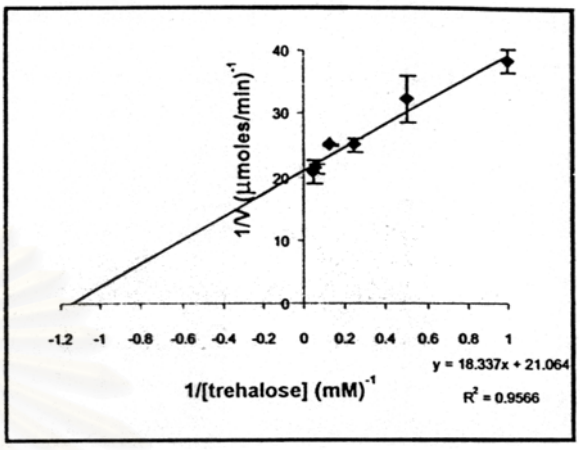


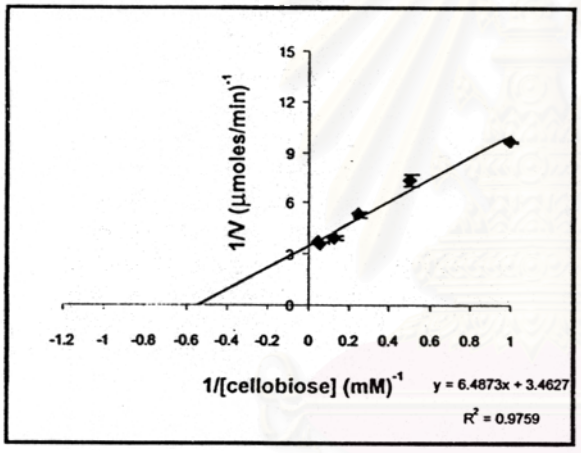


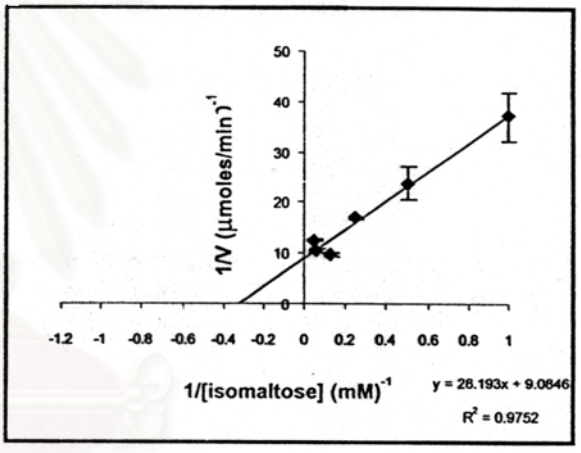


APPENDIX 9 : Lineweaver-Burk plot CGTase with β-CD as glycosyl donor and various acceptors (Continue)

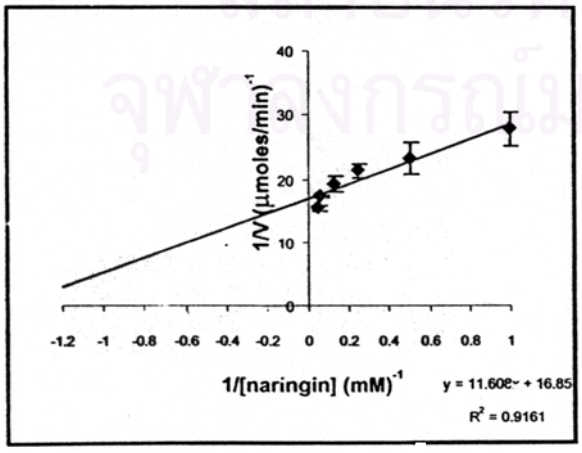


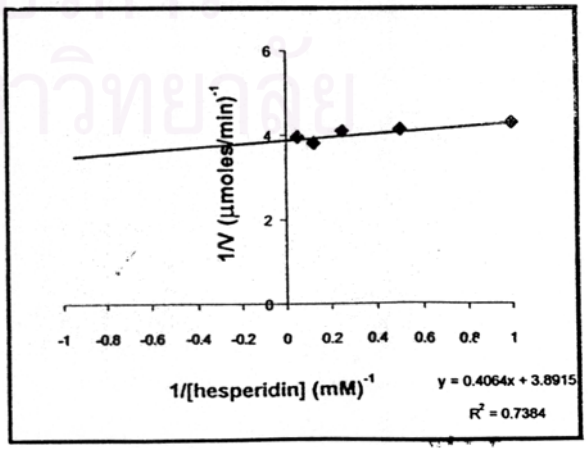




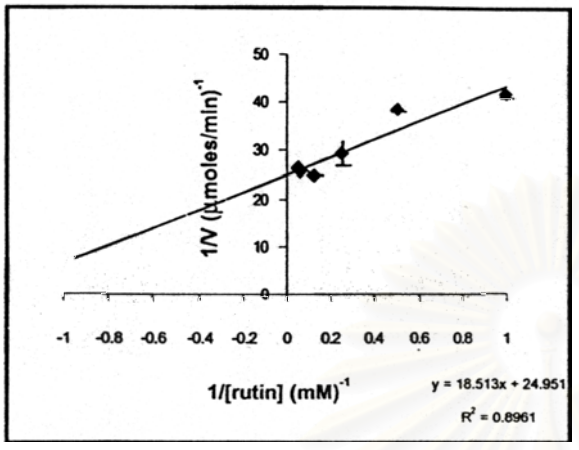


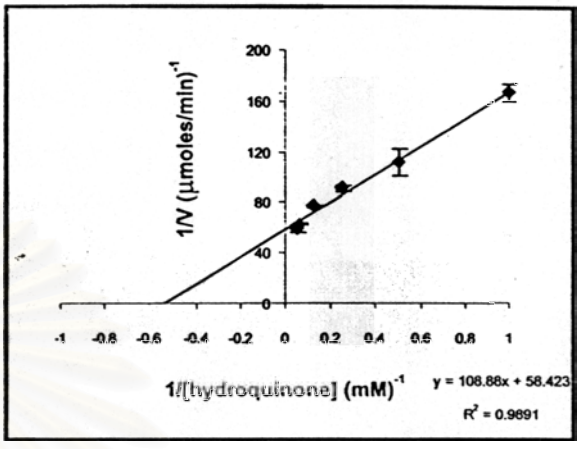
B) Glycoside forming acceptors

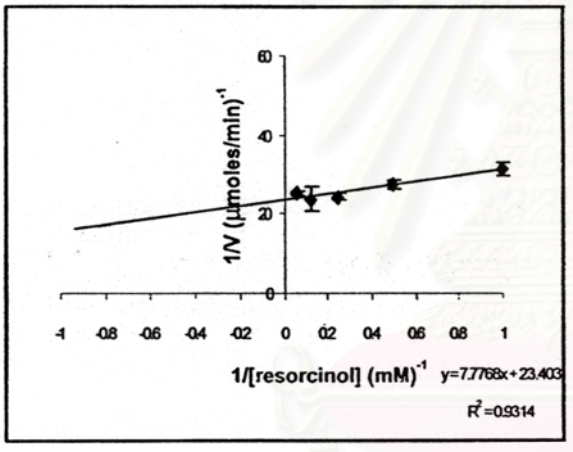


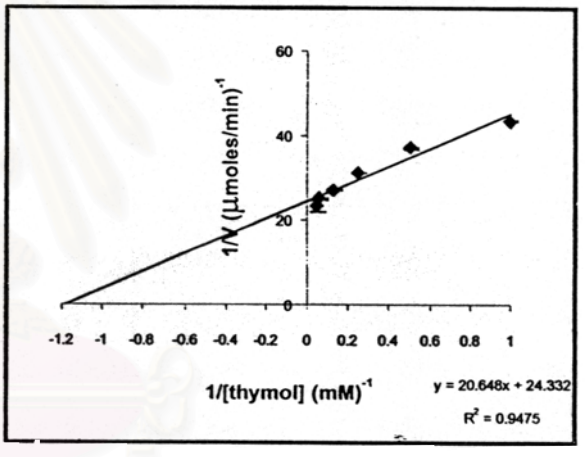


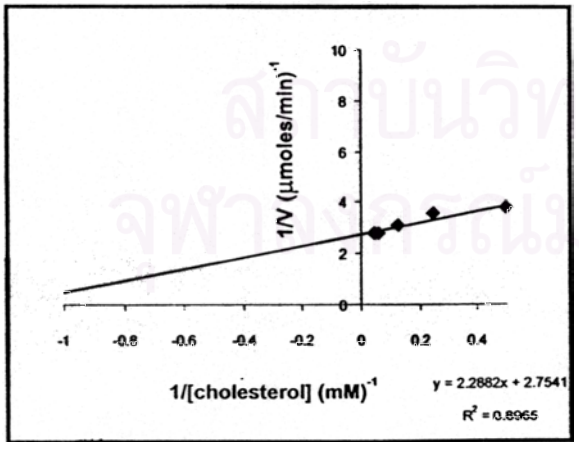
APPENDIX 9 : Lineweaver-Burk plot CGTase with β-CD as glycosyl donor and various acceptors (Continue)







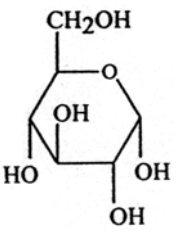




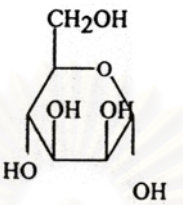
APPENDIX 10 : Structure of glycosyl acceptors

Monosaccharides :

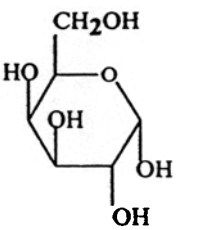
C_6



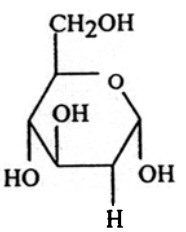
GLUCOSE



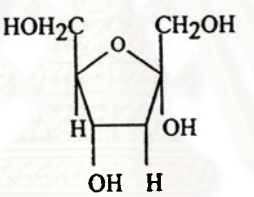
MANNOSE



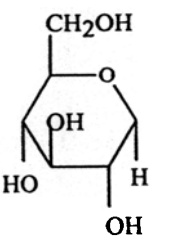
GALACTOSE



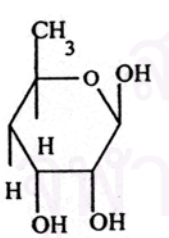
2-DEOXY-GLUCOSE



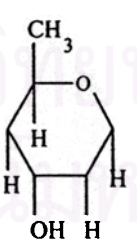
FRUCTOSE



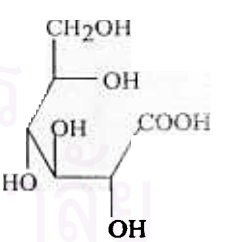
SORBOSE



RHAMNOSE



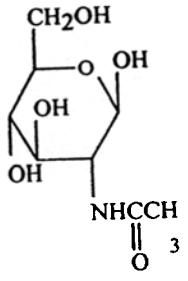
FUCOSE



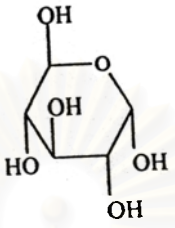
GLUCONIC ACID

APPENDIX 10 : Structure of glycosyl acceptors (Continue)

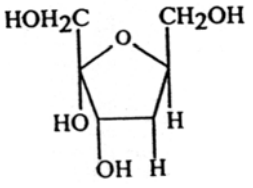
Monosaccharides :



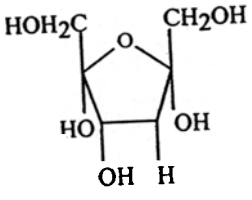
NAG



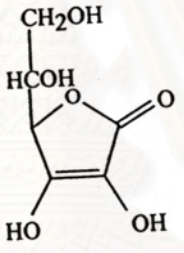
INOSITOL



MANNITOL

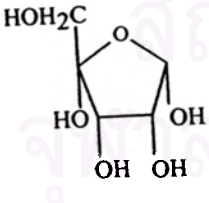


SORBITOL

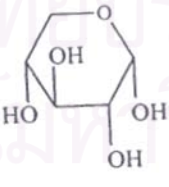


ASCORBIC ACID

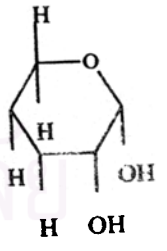
C_5:



RIBOSE



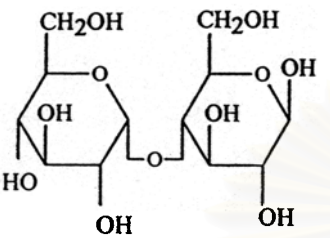
XYLOSE



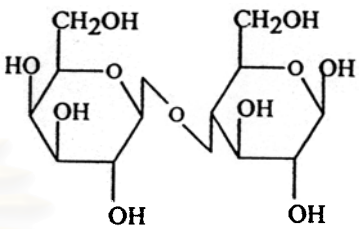
ARABINOSE

APPENDIX 10 : Structure of glycosyl acceptors (Continue)

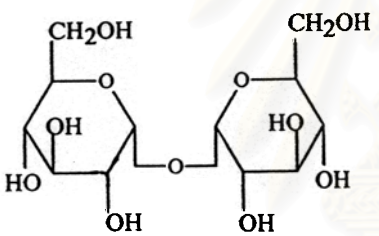
Disaccharides :



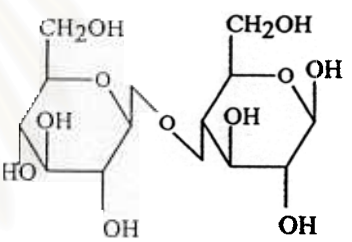
MALTOSE



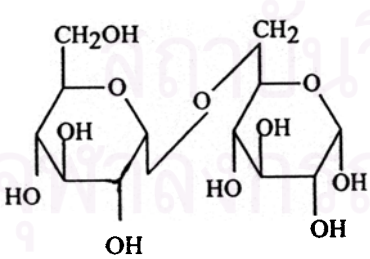
LACTOSE



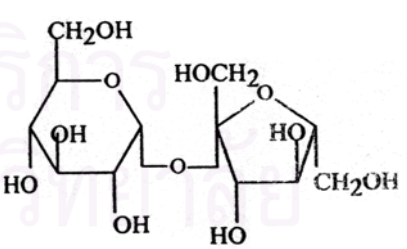
TREHALOSE



CELLOBIOSE



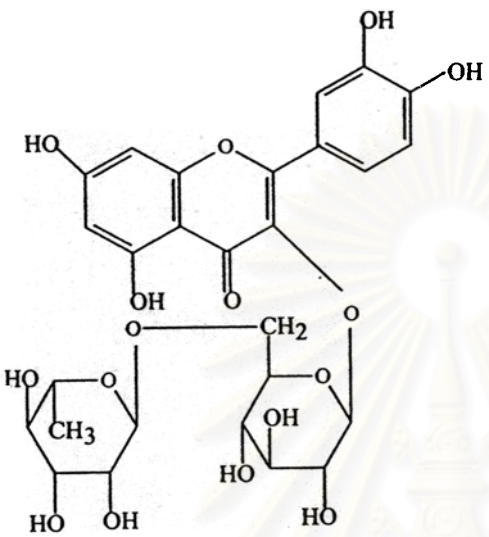
ISOMALTOSE



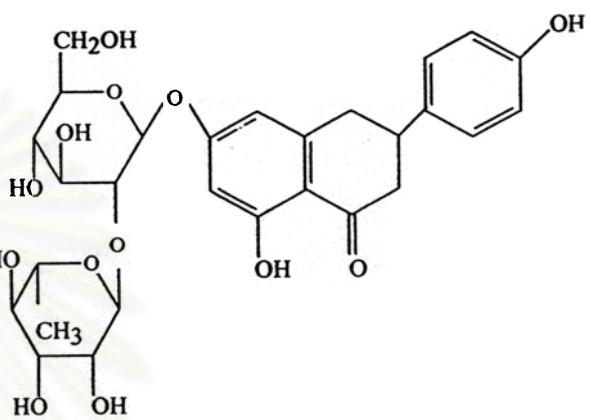
SUCROSE

APPENDIX 10 : Structure of glycosyl acceptors (Continue)

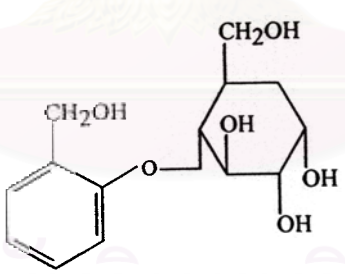
Glycosides :



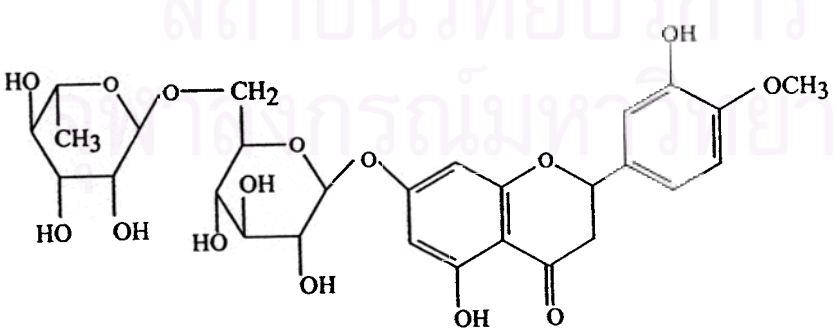
RUTIN



NARINGIN



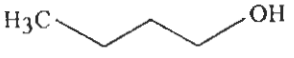
SALICIN



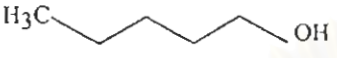
HESPERIDIN

APPENDIX 10 : Structure of glycosyl acceptors (Continue)

Alcohols :



BUTANOL

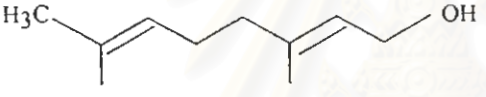


PENTANOL

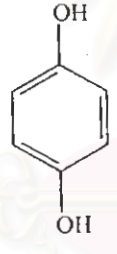
HEXANOL

OCTANOL

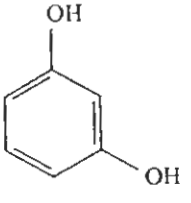
DECANOL



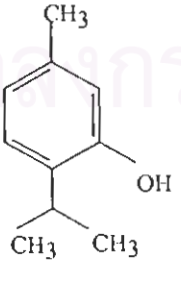
GERANIOL



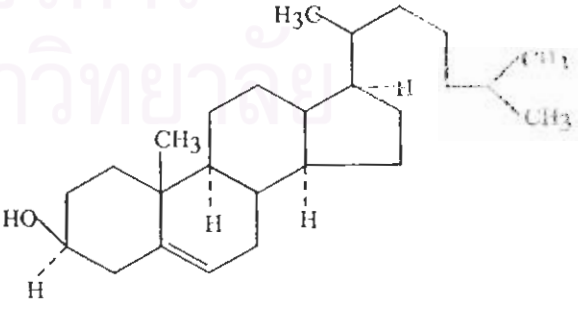
HYDROQUINONE



RESORCINOL

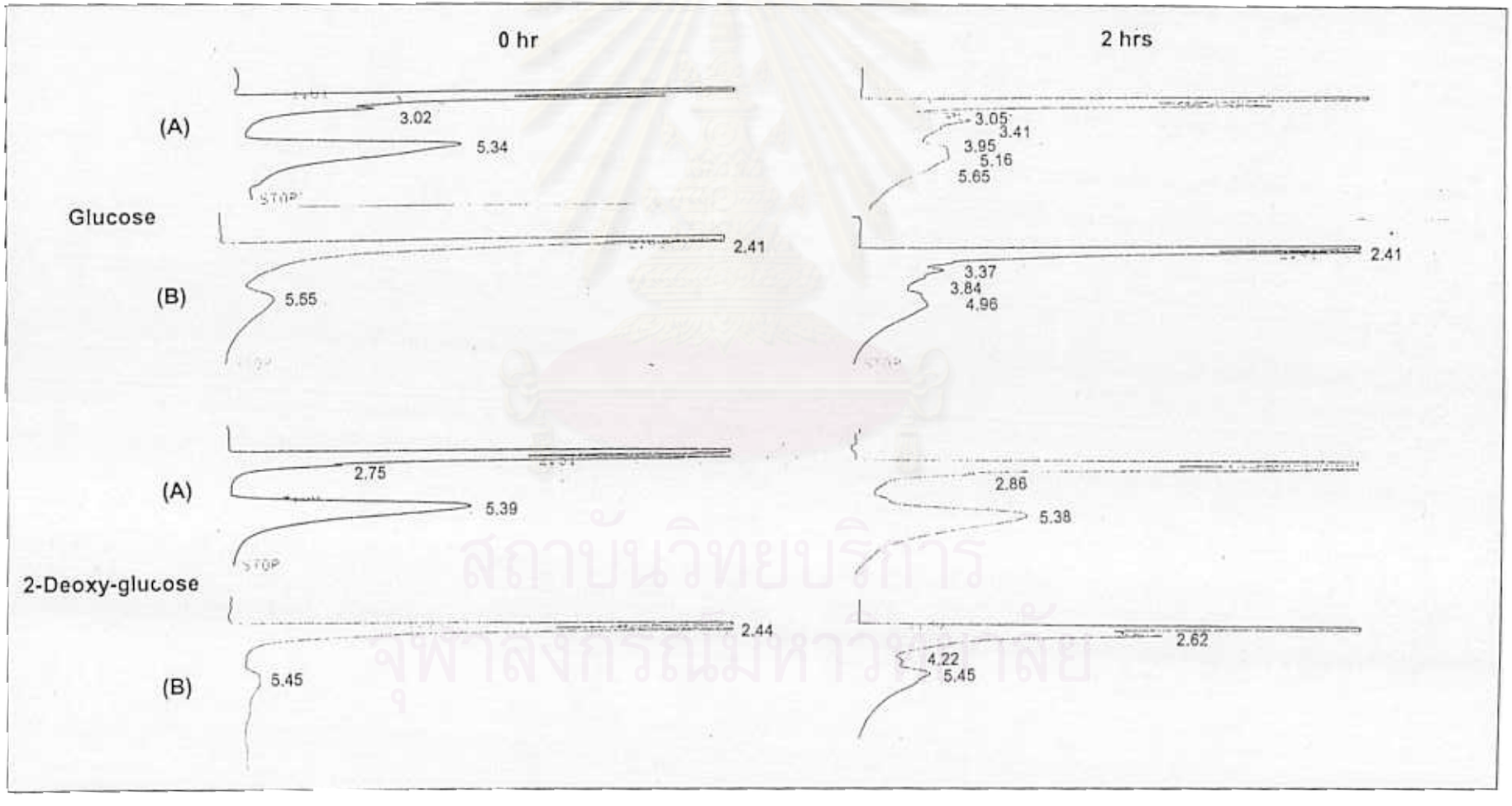


THYMOL

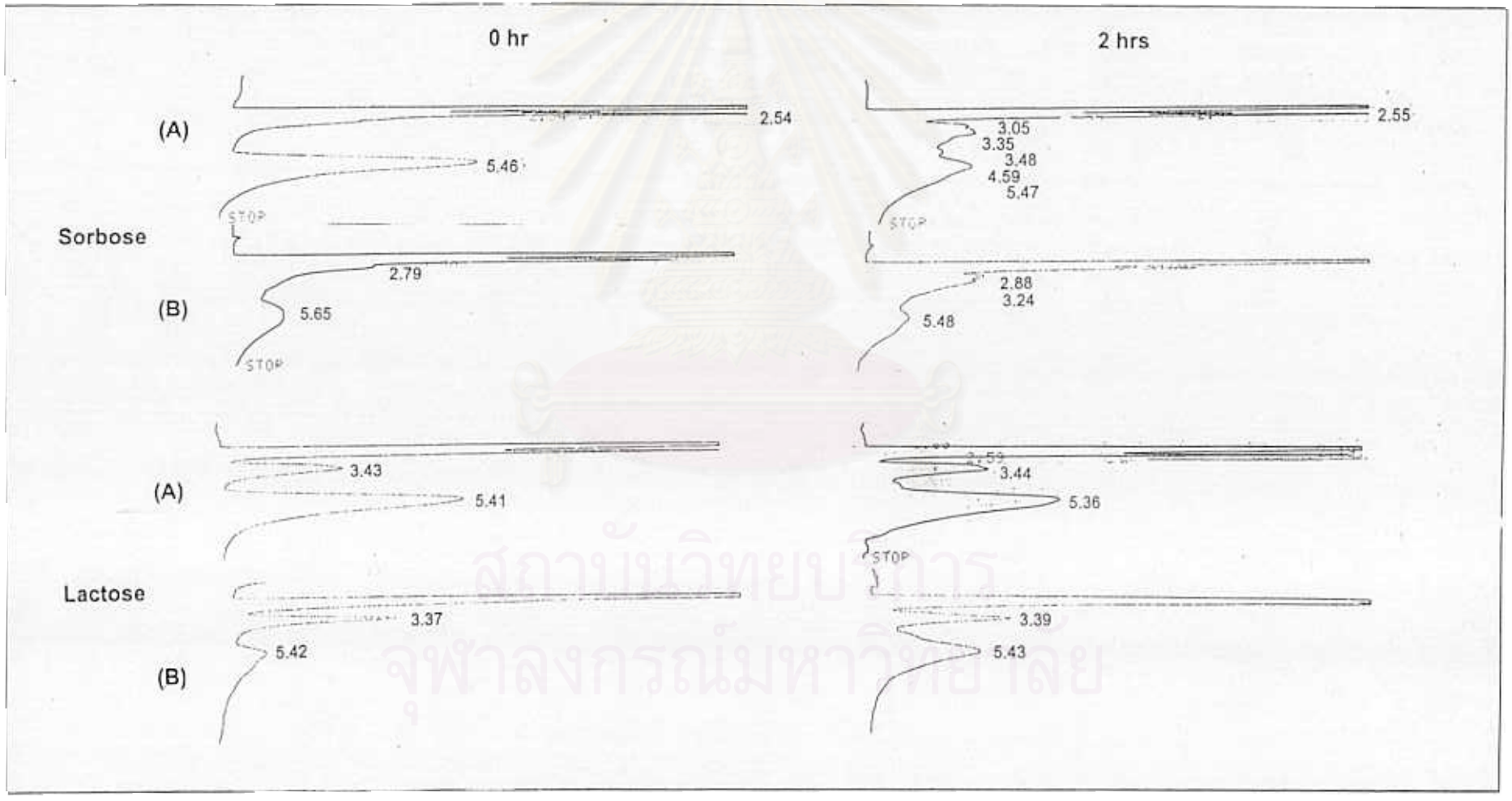


CHOLESTEROL

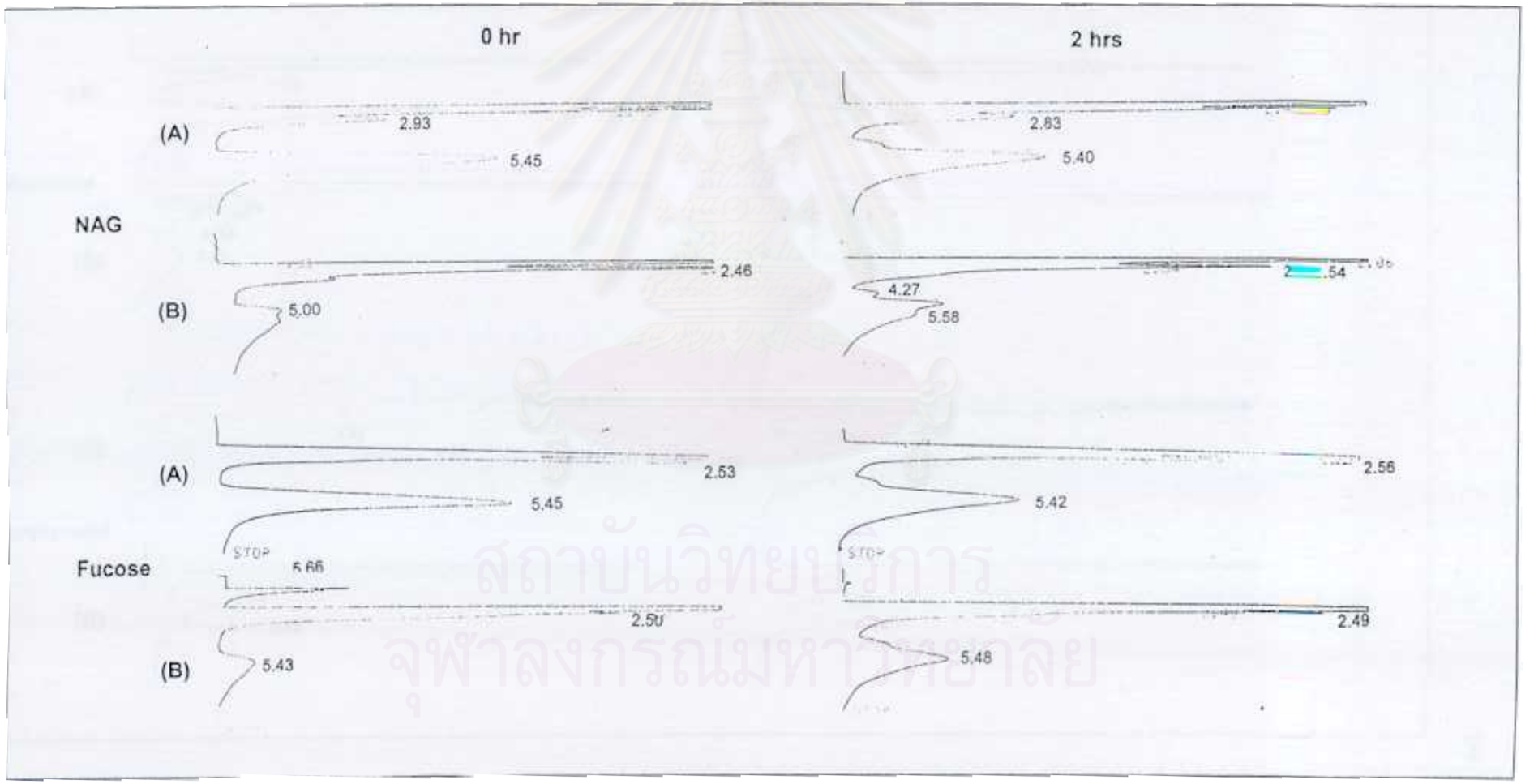
APPENDIX 11 HPLC chromatograms of reaction mixtures of CGTase-catalyzed coupling and transglycosylation reactions with different types of acceptor and (A) β-CD or (B) soluble starch as glycosyl donor



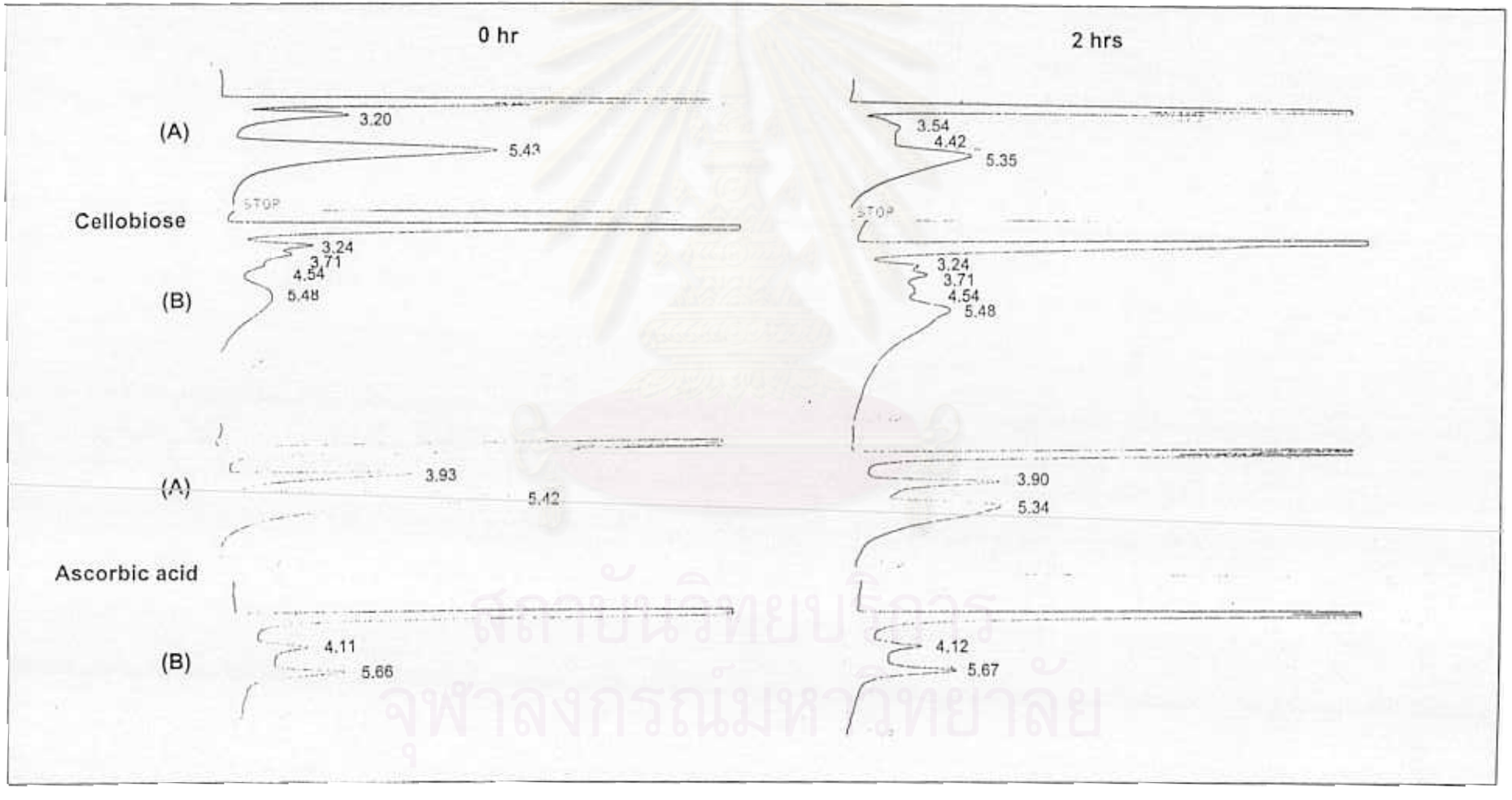
APPENDIX 11 : HPLC chromatograms of reaction mixtures of CGTase-catalyzed coupling and transglycosylation reactions with different types of acceptor and (A) β-CD or (B) soluble starch as glycosyl donor (Continue)



APPENDIX 11 : HPLC chromatograms of reaction mixtures of CGTase-catalyzed coupling and transglycosylation reactions with different types of acceptor and (A) β-CD or (B) soluble starch as glycosyl donor (Continue)



APPENDIX 11 : HPLC chromatograms of reaction mixtures of CGTase-catalyzed coupling and transglycosylation reactions with different types of acceptor and (A) β-CD or (B) soluble starch as glycosyl donor (Continue)



BIOGRAPHY

Miss Wannapa Wongsangwattana was born on June 10, 1974. She graduated with the Bachelor Degree of Science in Chemistry from Burapha University in 1995 and studying for Master in Biochemistry Program.