การทำให้บริสุทธิ์และลักษณะสมบัติของไซโคลเดกซ์ทรินไกลโคซิลทรานเฟอเรสจากแบคทีเรียที่แยกได้จากดินบริเวณน้ำพุร้อน

นางสาวอำพรพันธุ์ ประนอมมิตร

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PURIFICATION AND CHARACTERIZATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM BACTERIA ISOLATED FROM HOT SPRING SOIL

Miss Ampornpun Pranommit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry

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อำพรพันธุ์ ประนอมมิตร : การทำให้บริสุทธิ์และลักษณะสมบัติของไซโคลเดกซ์ทรินไกลโคซิลทรานเฟอเรสจากแบคทีเรียที่แยกได้จากดินบริเวณน้ำพุร้อน. (PURIFICATION AND CHARACTERIZATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM BACTERIA ISOLATED FROM HOT SPRING SOIL) อ. ที่ปรึกษา : ผศ.ดร. พีรดา มงคลกุล, 138 หน้า. ISBN 974-17-0246-9.

แบคทีเรียที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานเฟอเรสถูกคัดแยกได้จากดินบริเวณบ่อน้ำร้อนแม่กาษา จังหวัดตาก เมื่อจำแนกสายพันธุ์แบคทีเรียที่ถูกคัดเลือกชนิดนี้โดยใช้ลักษณะทางชีวเคมีและการวิเคราะห์ยีน 16S rRNA พบว่าจัดอยู่ในจำพวก จีนัส *Paenibacillus* และใกล้เคียงกับ *Paenibacillus macerans* ภาวะที่เหมาะสมในการผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานเฟอเรสจากแบคทีเรียชนิดนี้คือ เลี้ยงในอาหารเลี้ยงเชื้อ Horikoshi ในพีเอช 10.0 ที่มีแคลเซียมคลอไรด์ความเข้มข้น 0.05 มิลลิโมลาร์ ที่ 37°ซ นาน 84 ชั่วโมง *Paenibacillus* T16 เริ่มผลิต CGTase เมื่อเข้าสู่การเจริญแบบ log และผลิตสูงสุดในช่วง stationary phase การเตรียมเอนไซม์ให้บริสุทธิ์ด้วยการดูดซับกับแป้งและบีตาไซโคลเดกซ์ทรินอัฟฟินีตีคอลัมน์พบว่าสามารถถูกทำให้บริสุทธิ์ได้ 100 เท่าโดยได้ผลผลิตเท่ากับ 21.2 เปอร์เซนต์และมีค่า dilution limit ของ CD-TCE เท่ากับ 1:2[11] เมื่อนำเอนไซม์มาวิเคราะห์ด้วยพอลิอะไครลาไมด์เจลอิเลคโตรโฟเรซิสในสภาวะเสียสภาพพบว่ามีน้ำหนักโมเลกุลเท่ากับ 76,000 ดาลตัน มีค่า pI เท่ากับ 5.20 และเมื่อนำมาย้อม Periodic acid-Schiff's reagent พบว่าเอนไซม์ดังกล่าวเป็นไกลโคโปรตีน ในการศึกษาลักษณะสมบัติเอนไซม์ชนิดนี้พบว่า ภาวะที่เหมาะสมในการเร่งปฏิกิริยาการย่อยแป้งคือ ที่ 65°ซ ในบัฟเฟอร์ทริส-ไฮโดรคลอไรด์ พีเอช 8.0 ที่มีแคลเซียมคลอไรด์ความเข้มข้น 10 มิลลิโมลาร์ นาน 10 นาที และสำหรับปฏิกิริยาการสร้างไซโคลเดกซ์ทริน (วัดด้วยวิธีฟีนอล์ฟธาลีน)คือ ที่ 75°ซ ในบัฟเฟอร์ทริส-ไฮโดรคลอไรด์ พีเอช 8.0 ที่มีแคลเซียมคลอไรด์ความเข้มข้น 15 มิลลิโมลาร์ นาน 15 นาที จากผลการวิจัยยังพบว่าเอนไซม์ชนิดนี้มีความเสถียรต่ออุณหภูมิถึง 60°ซ แต่เพิ่มขึ้นเป็น 70°ซ ในที่มีแคลเซียมคลอไรด์ความเข้มข้น 10 มิลลิโมลาร์ และเสถียรต่อพีเอชในช่วง 4.0-11.0 นอกจากนี้ยังได้ศึกษาถึงสัดส่วนของผลิตภัณฑ์ α-, β-, และ γ-cyclodextrin โดยเทคนิค HPLC พบว่ามีสัดส่วนเท่ากับ 0.74 : 1 : 0.27 เมื่อใช้ 2% แป้งเป็นสับสเตรท แคลเซียมคลอไรด์มีผลเพิ่มแอคติวิตีและความเสถียรของเอนไซม์ ส่วนอิออนของปรอท PMSF และ EDTA สามารถยับยั้งการทำงานของเอนไซม์อย่างมีนัยสำคัญ

ภาควิชา ..... ชีวเคมี.....	ลายมือชื่อนิสิต .....
สาขาวิชา .....ชีวเคมี.....	ลายมือชื่ออาจารย์ที่ปรึกษา .....
ปีการศึกษา .....2544.....	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....-.....

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KEY WORD: Cyclodextrin glycosyltransferase / genus *Paenibacillus*

AMPORNPUN PRANOMMIT : PURIFICATION AND CHARACTERIZATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM BACTERIA ISOLATED FROM HOT SPRING SOIL. THESIS ADVISOR : ASSIST. PROF. PEERADA MONGKOLKUL, Ph.D., 138 pp. ISBN 974-17-0246-9.

Hot spring soils from northern Thailand was screened for cyclodextrin glycosyltransferase (CGTase) producing bacteria. A bacterial isolate from Mae ka sa, Tak province was selected and identified, using biochemical characteristics and 16S rRNA gene analysis, to be Gram positive bacterium of the genus *Paenibacillus*, possibly *P. macerans*. The optimum conditions for bacterial growth and CGTase production were in Horikoshi liquid medium, pH 10.0 with 0.05 mM $CaCl_2$ at 37°C for 84 hours . CGTase production was initiated as cells entered log phase but the enzyme was highest at stationary phase. CGTase was purified by starch adsorption and β-CD affinity column to 100 folds with 21.2% yield. The dilution limit of the enzyme by CD-TCE precipitation was $1:2^{11}$. Its molecular weight on sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE) was 76,000 daltons. Its isoelectric point was estimated by isoelectrofocusing gel to be 5.20. It was proved to be a glycoprotein by Periodic acid-Schiff's staining on SDS-PAGE. The optimum conditions for dextrinizing and CD-forming (phenolphthalein method) activities were in Tris-HCl buffer (pH 8.0) with 10mM $CaCl_2$ at 65°C for 10 minutes and in Tris-HCl buffer (pH 8.0) with 15mM $CaCl_2$ at 75°C for 15 minutes, respectively. The thermostability of the enzyme was 60°C but could be enhanced to 70°C in the presence of 10mM $CaCl_2$ and was stable at pH 4.0-11.0. Analysis of its CD products by HPLC demonstrated that the ratio of α-, β-, and γ-cyclodextrin was 0.74 : 1 : 0.27 using 2% starch as substrate. Calcium chloride had stimulated and stabilizing effects on the enzyme. Mercuric ion, PMSF and EDTA inhibited the enzyme considerably.

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

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

Academic year……2001..…….. Co-advisor's signature……-……

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ABBREVIATION

A	absorbance
BSA	bovine serum albumin
bp	base pair
CD	cyclodextrin
CGTase	cyclodextrin glycosyltransterase
cm	centimeter
°C	degree Celsius
g	gram
hr	hour
l	litre
mA	milliampere
min	minute
µl	microlitre
ml	millilitre
mM	millimolar
M	molar
nm	nanometer
rpm	revolution per minute

CHAPTER I

INTRODUCTION

Cyclodextrins

Cyclodextrins (celluloseine, cycloamylose, cyclomaltose, Schardinger dextrin : CD) are a family of starch-derived cyclic compounds commonly containing six, seven or eight glucose residues per molecule, designated α-, β- and γ-CD, respectively. The glucose units are linked by α-(1,4)-glycosidic linkage and contain no reducing ends. (Figure 1) (French, *et al*., 1942, 1949; Schardinger, 1903, 1904). The ring shape of the CDs is a consequence of the C1-chair configuration of the D-glucopyranosyl units, and their 1,4-α-D-linkages. The overall appearance of the CD molecule is that of a truncated cone : the "wide" side is formed by secondary O(2)H and O(3)H hydroxyls, whereas primary O(6)H hydroxyls are located on the more closed side (Figure 2), rendering the molecule a hydrophilic outside. The cavity of the CDs is hydrophobic, with all the intramolecular hydrogen bonded between the secondary hydroxyl groups of neighboring D-glucopyranosyl units O(3)…O(2), contributing to the hydrophobicity and rigidity of the CD molecule.

Some physical properties of CDs are summarized in Table 1 (Szejtli, 1988). Among the CDs, γ-CD has the biggest cavity, the most flexible characteristics and is more soluble than α-CD and β-CD. The secondary hydroxyl groups of the nearby glucose unit in the molecule of β-CD can form seven hydrogen bonds, called the secondary belt, which gives β-CD the most stable but the lowest soluble form. A glucose unit of α-CD is in a distorted position and can form only four hydrogen bonds (Szejtli, 1988).

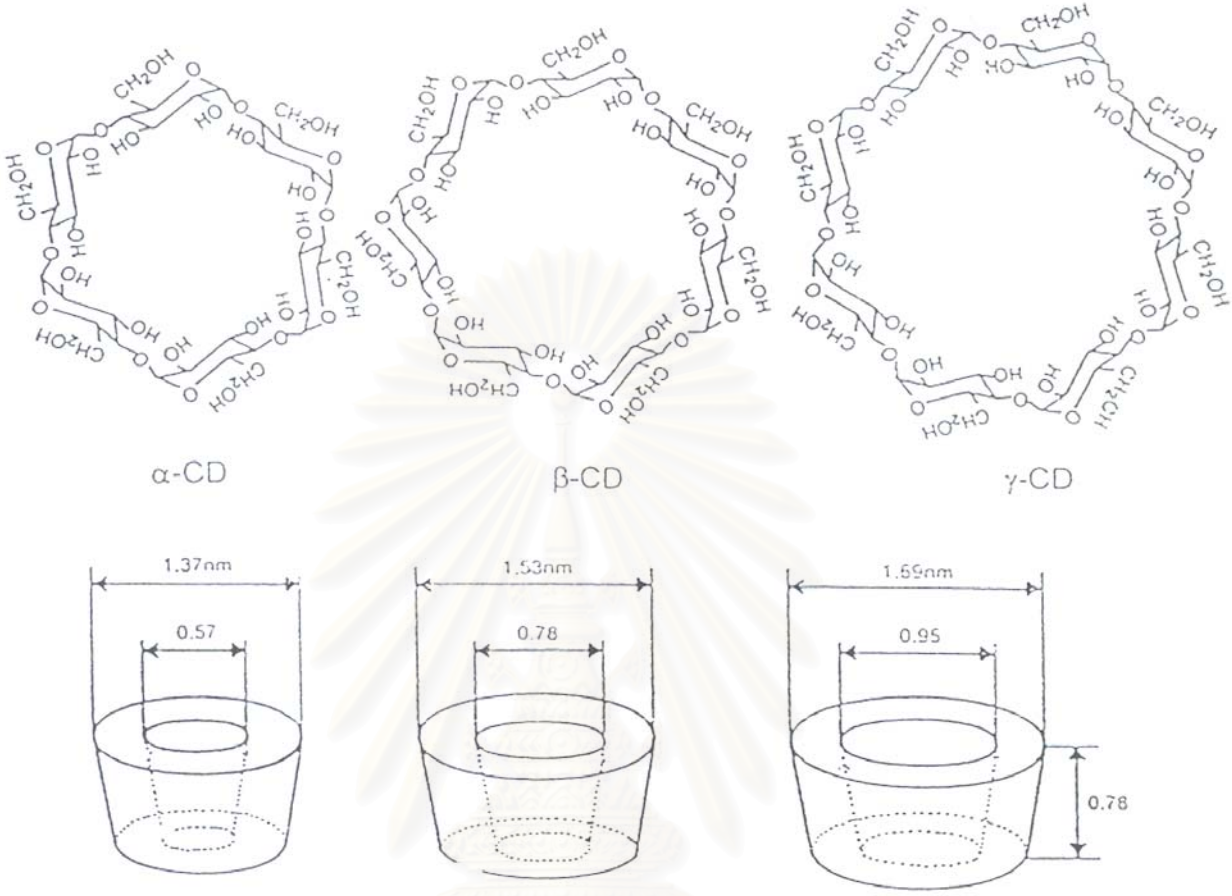

Figure 1 Structure and molecular dimension of cyclodextrin (CDs) (Szejtli, 1990)

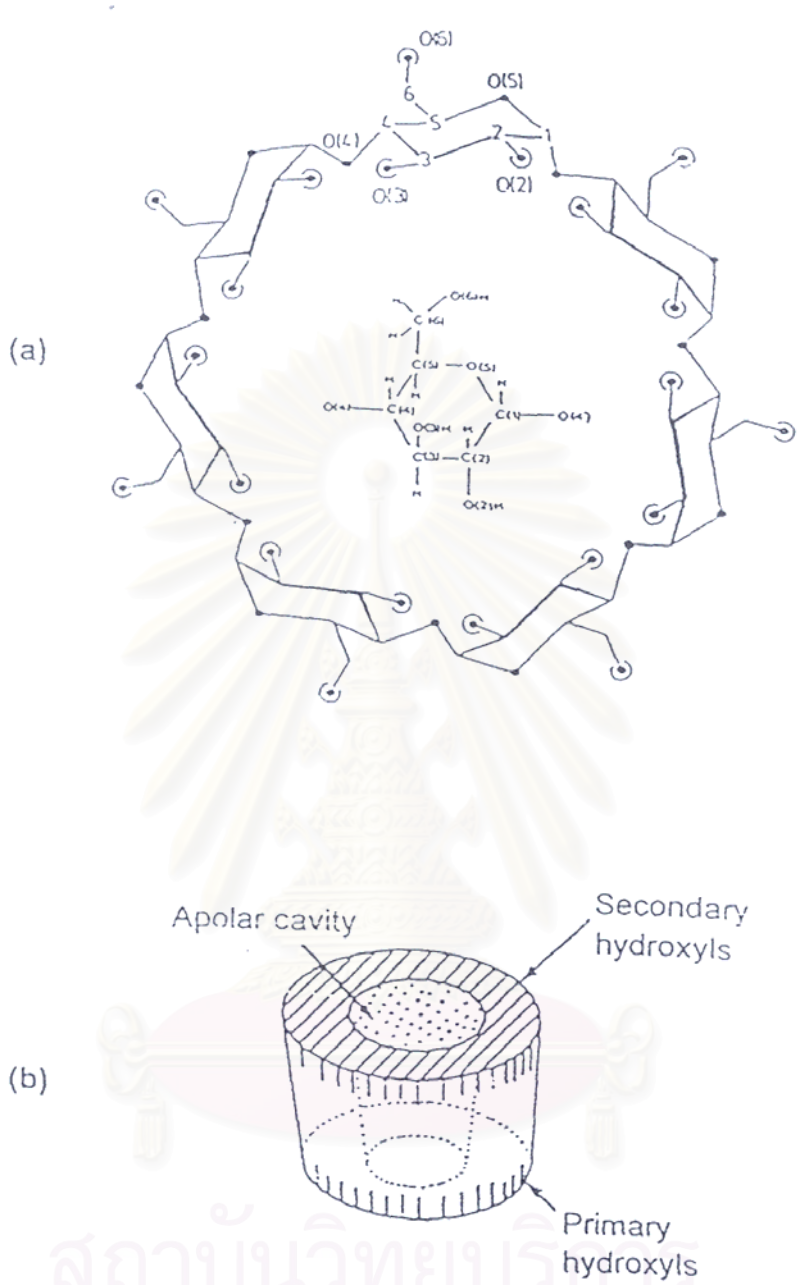

Figure 2 Structure of β-cyclodextrin (Bender, 1986; Szejtli, 1990)

(a) Chemical structure; O = oxygen atoms, ● = hydroxyl groups

(b) Functional structure scheme

Table 1 Characteristics of cyclodextrin (Saenger, 1982; Szejtle, 1988)

	α-CD	β-CD	γ-CD
Glucose monomers	6	7	8
Molecular weight	972	1,135	1,297
Cavity dimension			
Cavity diameter (A°)	4.7-5.3	6.0-6.5	7.5-8.3
Cavity depth (A°)	7.9±0.1	7.9±0.1	7.9±0.1
Cavity volume (A°)	174	262	472
Solubility in water (g/100ml at 25°C)	14.40	1.85	23.20
Crystal form (from water)	Hexagonal plates	Monoclinic parallelograms	Quadratic prisms
Melting range (°C)	255-260	255-265	240-245
Water of crystallization	10.2	13-15	8-18
Water molecules in cavity	6	11	17

Complex formation of cyclodextrin and guest molecule can markedly improve the chemical and physical properties of the guest molecule such as protection against oxidative degradation or destruction by UV light, improvement of the solubility of hydrophobic substances in aqeous solution, stabilization of volatile compounds, alternation of the chemical reactivity, modification of liquid substances to powder, or reduction of undesirable smell or taste in products, e.g., foodstuffs are among those known useful properties (Schmid, 1989). The applications of cyclodextrins as emulsifiers, antioxidants and stabilizing agents have rapidly increased in food, cosmetics, pharmaceutical, agrochemical and plastic industries (Nagamoto, 1985) (Table 2).

Several cyclodextrin derivatives have been developed through chemical or enzymatic means in order to obtained CDs with specific desirable properties. Some of those are methylated, hydroxypropylated and glycosylated at the hydroxyl groups, resulting in higher solubility than parental CDs. CD-polymers (linked cyclodextrins) are often used as stationary phase in various liquid chromatography system (Casu and Roggiani, 1979; Ensuiko, 1994; Yamamoto, *et al*., 1990). These modified CDs, in addition to their native or parental CDs (the α-, β- and γ-CD) offer wider range of properties to be selected as the suitable host molecules. Currently available modified cyclodextrins are listed in Table 3.

As cyclodextrins have been widely used in various industries since early 1970s, many countries (for example, Japan, Germany, France, Netherland, Denmark, Spain, Italy, Belgium, Hungary, USA and Taiwan) have approved the use of cyclodextrins (at different levels) in several industrial fields (Amaizo, 1991). The forecast for world market usage (all types of CDs) is shown in Figure 3.

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 hydroscopicity	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 odour	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

Table 2 Industrial applications of cyclodextrins (continued)

Use	Guest compound/end product
6.Reduction of irritation	Shampoo, cream, skin powder
7.Enhancement of attained concentration	Skin moisturizing lotion
8.Defoaming effect	Laundry
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, Cholramphenical
2.Taste masking	Prostaglandin
3.Powdering (non-volatile)	Nitroglycerin, elofibrate
4.Stabilization (UV, thermal)	Prostaglandin, vitamin
5.Decrease irritation	Cu-alcanomine complex, tiamulin
6.Enhancement of bioavailibility	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	
1.Catalyzation for reaction	Products of hydrolysis, substitution

Table 3 Classification of cyclodextrin derivatives (Ensuiko, 1993; Rajewski and Stella, 1996; Szejtli, 2000)

Parental CD	Modified CD		
	Substituted CD	Branched CD	CD polymers
α-, β-, γ-CD	Methylated CD -dimethylated -trimethylated -randomly methylated Ethylated -diethylated -triethylated Hydroxy alkylated -2-hydroxyethylated -2-hydroxypropylated -3-hydroxypropylated -hydroxybutenyl Sulfobutylether	Homogeneous branched CD -glucosyl -maltosyl Heterogeneous branched CD -galactosyl -mannosyl -maltosyl	Cross-linked CDs Matrix coupled CDs

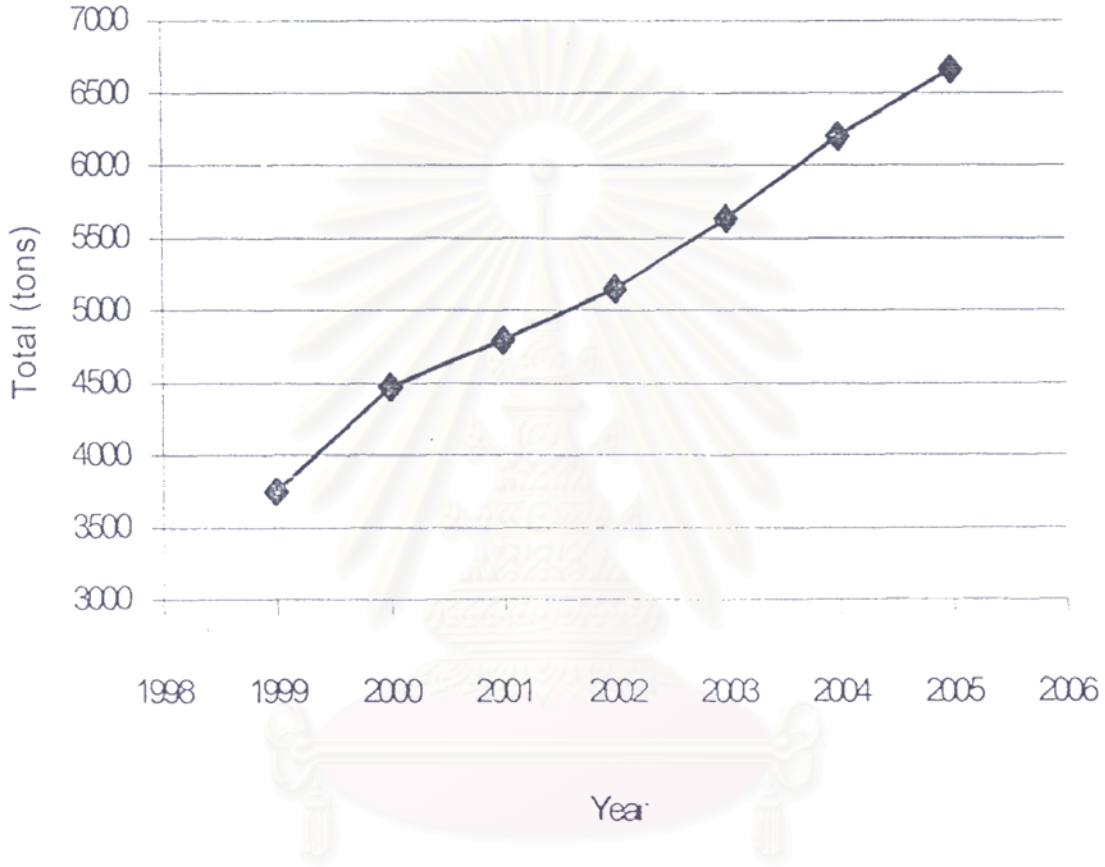

Figure 3 The world market usage of all CDs forecast (Strattan, 2000)

By way of general introduction, it can be said that chemical combinations in solution can exist in a number of forms including simple mixtures, salts or complexes. Different types of complexes are classified according to the nature of the complexants. These can be metal complexes, complexes of organic molecules, or structural complexes. Structure complexes can be distinguished by the type of associations which occur.

Inclusion complexes are cage-like molecular arrangements which hold other molecules. The included molecule is called the guest while the complexing agent is known as the host molecule. Complexation is a dynamic process. The interactions between host, guest, and solvent molecules are all involved in determining the nature of the complexes, if in fact they occur (Amaizo, 1993) (Figures 4 and 5).

Several major factors direct the formation and dissociation of cyclodextrin complexes. These forces are related primarily to molecular interactions and molecular size. There are several types of molecular interaction that affect complexation:

a) The most important molecular interaction is the hydrophobic attraction between a hydrophobic solute in aqueous solution and the hydrophobic cavity of the cyclodextrin.

b) The close proximity of the cavity walls permits increased Van Der Waals interactions between included guest molecules and the cyclodextrin.

c) Hydrogen bonding, when possible, can play a significant role in the stabilization of a guest molecule in the CD cavity.

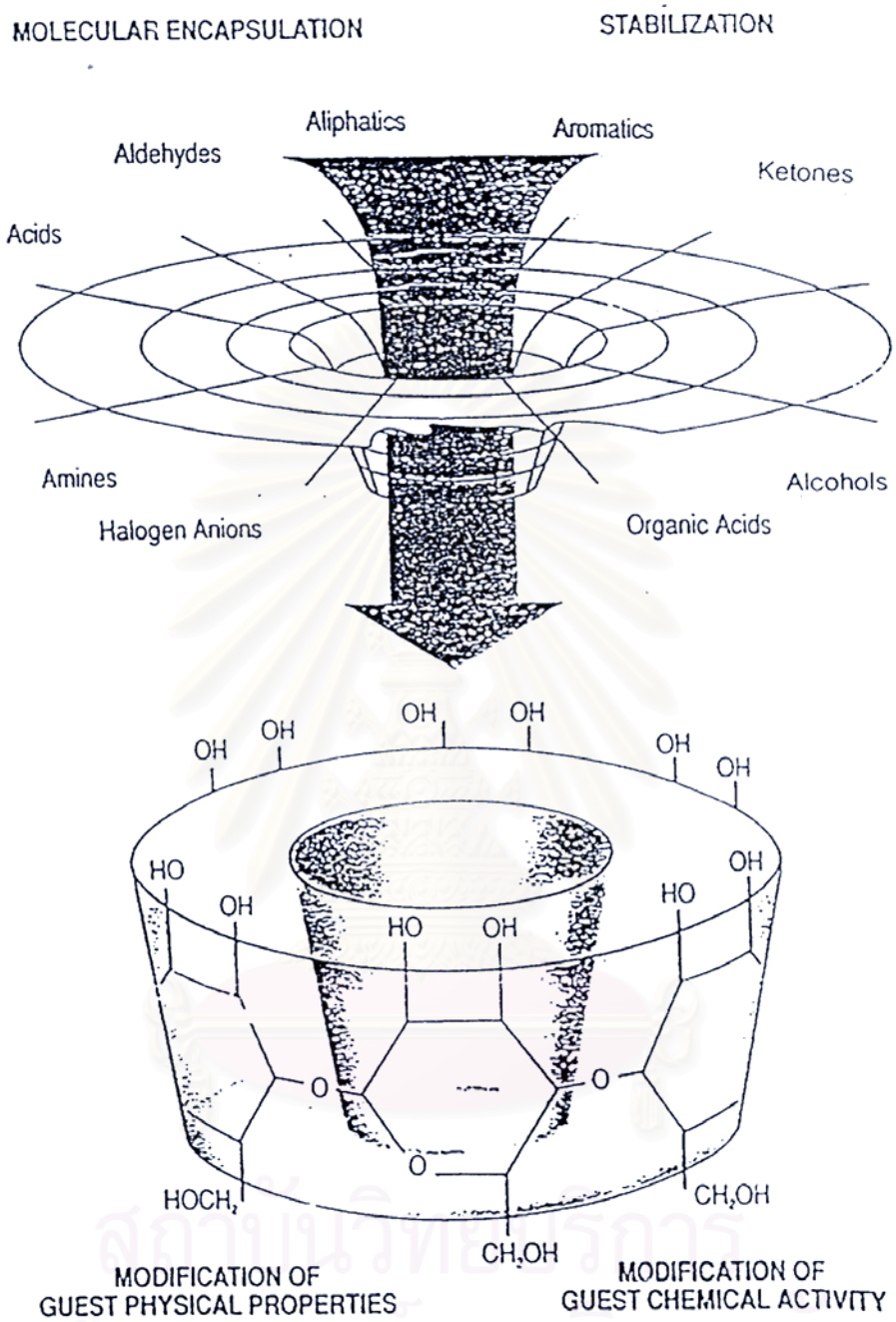

Figure 4 Inclusion complex formation between CDs and guest molecules leading to modification of guest physical and chemical properties. (Amaizo, 1993)

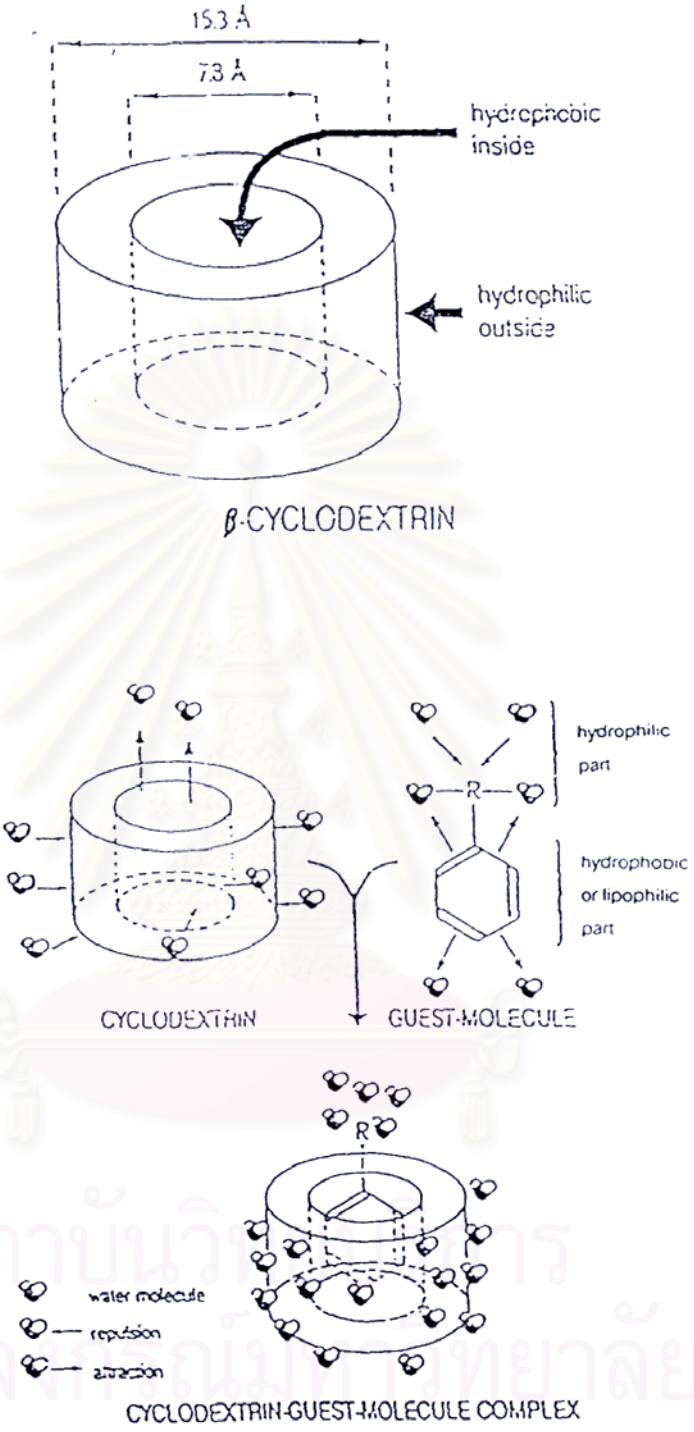

Figure 5 Orientation of guest molecule in CD-guest complex. (Janssen, 1992)

d) Another major driving force is the displacement of high energy water molecules from the cavity by complexation of a more hydrophobic guest. Release of water energy when it combines with the bulk water at a lower energy level can be a significant driving force for complexation. Even though the cavity has hydrophobic character, when cyclodextrin is crystallized from water, water is included in the cavity, though at a higher energy level. Up to 6 moles of water can be incorporated into cyclodextrin in this fashion.

e) One source of energy driving complexation is derived from a more stable guest conformation. For example, under alkaline conditions the red color of phenophthalein is bleached when it reverts to a more stable colorless form during cyclodextrin complexation (Millone, 1989).

Cyclodextrin producing enzymes

Cyclodextrin glycosyltransferase (1,4-α-D-glucan : 1,4-α-D-glucopyranosyltransferase, EC 2.4.1.19. CGTase) is known to catalyze degradation of starch to form cyclodextrins. This enzyme catalyzes three possible mechanisms : cyclization, coupling, and disproportionation reactions (Kitahata and Okada, 1975), as shown in the following equations:

$$G_n \underset{\text{coupling}}{\overset{\text{cyclization}}{\rightleftharpoons}} G_{(n-x)} + cG_x$$

$$G_m + G_n \xrightarrow{\text{disproportionation}} G_{(m-x)} + G_{(n+x)}$$

Where G_n and G_m are 1,4-α-D-glucopyranosyl chains with “n” and “m” glucose residues ; x is a part of the 1,4-α-D-glucopyranosyl chain and cG_x is a symbol for CDs.

In addition, the enzyme has a weak hydrolyzing activity (Bart, *et al*., 2000). These reactions are demonstrated in Figure 6.

The cyclization reaction is thought to be a special type of disproportionation, the non-reducing end of one chain itself serving as acceptor, whereas the helical conformation of substrate is thought to be a prerequisite for cyclization. Catalyzing the two-substrate reactions, disproportionation and coupling, the CGTases have been proposed to posses two binding sites for donor and acceptor, respectively. The acceptor binding site proved to be not specific for glucose or maltooligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrates containing 16-80 glucopyranosyl residues. If the chain length is greater than 100 units, disproportionation reaction dominate. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 4. Higher concentration of maltooligosaccharides or glucose favors the reversed coupling reaction resulting in linear products with negligible amount of cyclodextrins (Kitakata, Okada, and Fukai, 1978). The action of CGTase is different from that of other starch-degrading enzymes in that the products are cyclic and non-reducing.

The three-dimensional structure of CGTase from X-ray crystallographic technique showed that CGTase consisted of five domains, labeled A to E. A, B, and C domains are homologous to α-amylase. Compared of α-amylase, CGTase contains two additional domains (D and E). The E-domain of CGTase has been recognized as a putative raw (native) starch binding domain. β-cyclodextrin tightly binds to this E-domain, as observed in crystal structure, which may interfere with starch binding,

providing at least part of the explanation for the observed cyclodextrin product inhibition. Analysis of a maltose-dependent CGTase crystal structure revealed that each enzyme molecule contained three maltose molecules, situated at contact points between protein molecules in the crystal. Two of these maltoses were bound to specific sites in the E-domain, the third maltose was bound at the C-domain. The roles of the two maltose binding sites in the E-domain in raw starch binding, cyclization reaction kinetics, and product inhibition by β-cyclodextrin were studied by replacing Trp616 and Trp662 of maltose binding site 1 and Tyr633 of maltose binding site 2 by alanines using site-directed mutagenesis. Characterization of purified (mutant) CGTase shows that maltose binding site 1 is most important for raw starch binding whereas maltose binding site 2 is involved in guiding linear starch chains into the active site groove. Inhibition of the cyclization reaction by β-cyclodextrin is of a mixed type, which can be divided in competitive inhibition interfering with catalysis in the active site and non-competitive inhibition interfering with the function of maltose binding site 2 in the E-domain (Figure 7).

Model of mechanism for the cyclization has been postulated that CGTase binds eight to ten (or even more) glucose units of a starch molecule. The active site of CGTase thus consisted of eight to ten (or more) subsites. The reaction is an exoattack on glucose chains from the non-reducing ends. The resulting maltohexaose intermediate is bound to an aspartyl group of enzyme by ester bond. The non-reducing end of the maltohexaose subsequently binds to subsite two and new α-1,4-glycosidic bond is formed between glucose residues one and six of maltohexaose, as shown in Figure 7 (Bender, 1988).

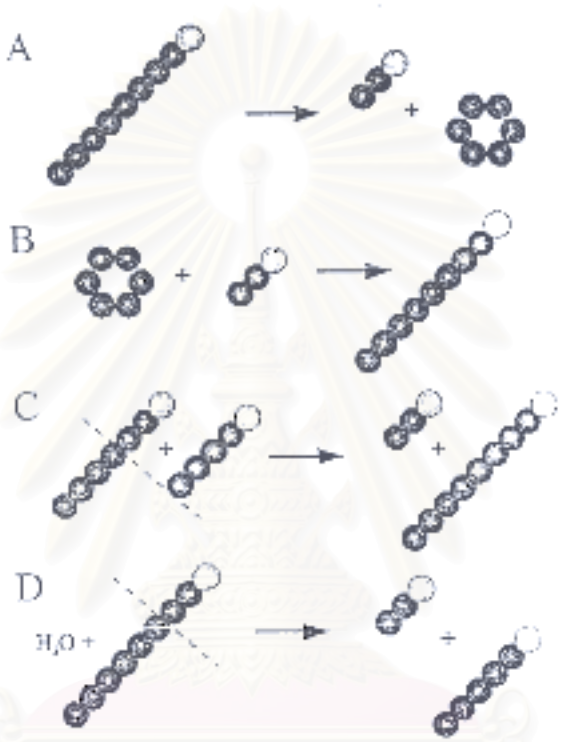

Figure 6 Schematic representation of the CGTase-catalyzed transglycosylation reactions. (A) cyclization; (B) coupling; (C) disproportionation; and (D) hydrolysis. The shaded circles represent glucose residues; the white circles indicate the reducing end glucoses. (Bart, 2000)

Table 4 Relationship between length of subsite and mechanism of CGTase (Szejtli, 1988)

Substrate chain length (residues)	Effect on mechanism of CGTase
1 (D-glucose)	-no catalysis
2-4	-inhibit initial reaction of cyclization
	-substrate for coupling reaction
5-14	-good substrate for coupling reaction
	-poor substrate for disproportionation reaction
16-80	-good substrate for cyclization reaction
> 100	-good substrate for disproportionation reaction

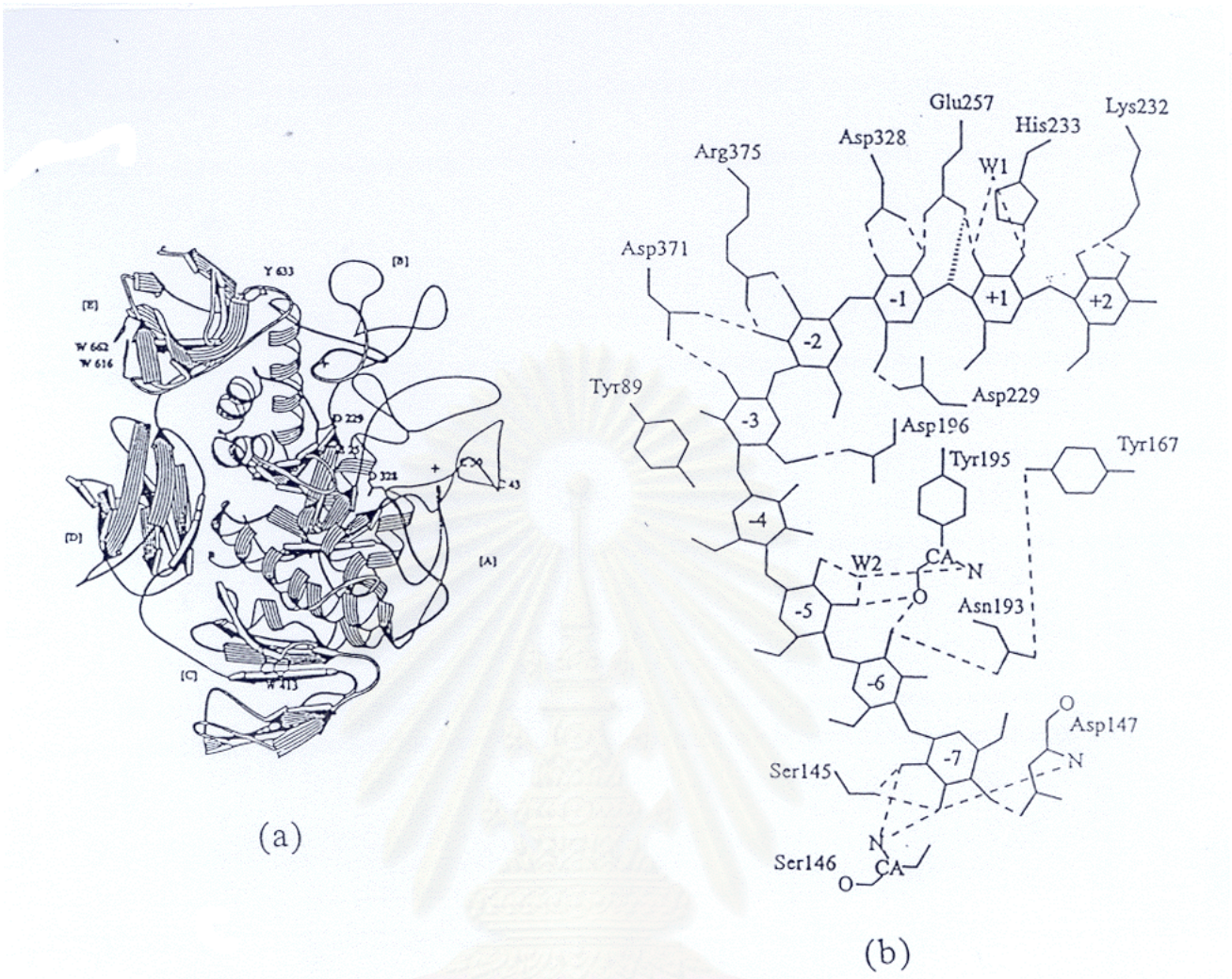

Figure 7 CGTase from *Bacillus circulans* 251

(a) Stereo ribbon drawing of CGTase (Lawson, *et al*., 1994)

(b) Schematic represent the active site (van der Veen, *et al*., 2000)

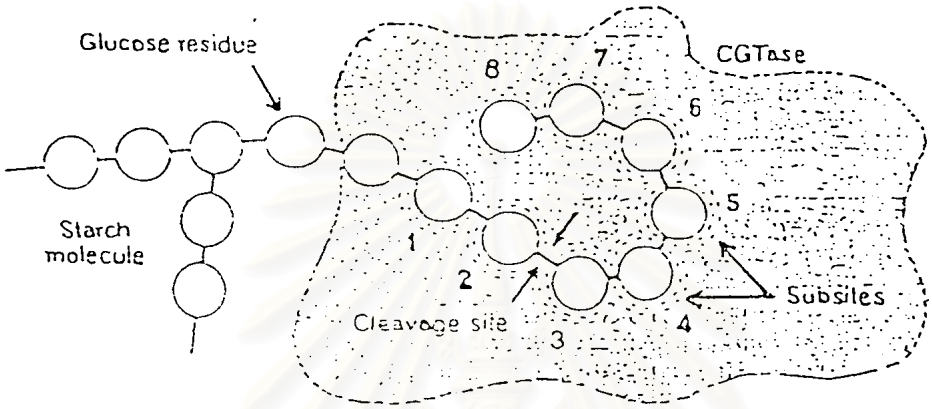

Figure 8 Model of CGTase mechanism from *Klebsiella pneumoniae* M5 al (Bender, 1988)

CGTase is produced by various microorganisms, for instance *Klebsiella pneumoniae* (Bender, 1977), *Brevibacterium* sp. (Mori *et al*., 1994), and mainly the *Bacillus* sp. (Bender, 1986 ; Komitani *et al*., 1993), as listed in Table 5. The CGTase can be devided into three types : α-, β-, and γ-, according to the major type of CD formed (Horikoshi, 1988). The enzymes from different sources show slightly different characteristics such as working pH, temperature, and molecular weight. Each CGTase enzyme yields different ratio of cyclodextrin products, for example, the CGTase of *B. macerans* produced α-, β-, and γ-CD in relative amount of 2.7:1.0:1.0 (Depinto and Campbell, 1968), while the CGTase of alkalophilic *Bacillus* no. 38-2 and *B. circulans* produced CDs in relative ratio of 1.0:11.0:1.5 (Matzuzawa, *et al*., 1975) and 1.0:10.0:1.0 (Pongsawasdi and Yagisawa, 1987), respectively. The *Bacillus fermusI lentus* 290-3 was known to produce γ-CGTase in the initial phase of the enzyme production (Englbrecht, *et al*., 1990)

Although the potential of cyclodextrins in industrial application is well known, the market for cyclodextrins is still limited due to high cost and the availability of α-, and γ-cyclodextrins. Various studies have been emphasized on improvement of cyclodextrin productions. Development of cultivation for cyclodextrin over-production was carried out under optimized culture condition and with complex nutrient media. A need for a thermostable or thermotorelant CGTase which gives high cyclodextrin yield has been recognized. CGTase from an alkalophilic *Bacillus* strain no.38-2 (ATCC 21783) was observed to provide these required properties (Horikoshi and Akiba, 1982). Immobilized CGTase can be utilized in several conversion cycles to steadily increase the volume of production, hence reduce the production cost (Nakamura and

Table 5 Properties of cyclodextrin glycosyltransferases

Organism	Predominant product	Optimum pH	Optimum temperature (°C)	MW (dalton)*	pI	References
Alkalophilic *Bacillus* sp. 38-2 Neutral CGTase	β-CD	7.0	50	88,000	ND	Larsen, *et al.*, 1998
Alkalophilic *Bacillus* sp. 38-2 Acidic CGTase	β-CD	4.5-4.7	45	88,000	ND	Larsen, *et al.*, 1998
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	75,000	4.6	Kitahata, *et al.*, 1974
Bacillus macerans IAM 1243	α-CD	5.5-7.5	60	74,000	ND	Kobayashi, *et al.*, 1978
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

ND = no data , * = by SDS-PAGE

Horikoshi, 1976; Kato and Horikoshi, 1984; Yang and Su, 1989). Protein engineering, site-directed mutagenesis and gene cloning were also used to increase the yield of enzyme product. These techniques were not only based partly on an assumption of lower production costs, but also on the trends towards greater acceptability of cyclodextrins (Schmid, 1989).

Gene cloning and over-expression of CGTase gene, not only provides satisfactory cyclodextrin production, but also provides more enzyme for studies on its structure and mechanism including determination of its nucleotide sequence. In such studies, the β-CD synthetase gene from an alkalophilic *Bacillus* sp. #1011 (Kimura *et al*., 1987, 1989, 1990), *Bacillus* sp. strain no. 38-2 (Kaneko *et al*., 1988) and *Klebsiella pneumoniae* M5 al (Binder, *et al*., 1986) were cloned and expressed in *E. coli* and *Bacillus subtillis.*

The conventional procedure for the production of CDs includes liquefaction of starch using a thermostable α-amylase at 105 to 110°C and pH 6.0 to 6.5. However, further α-amylase treatment reduces the CD yield, because the maltodextrins, oligosaccharides, and glucose formed during this process act as acceptors, so that the coupling reaction (the degradation of CD) is accelerated. Another problem relating to the pH of liquefaciton is the need to raise the pH of the 30 to 35% (wt/vol) starch slurry (the pH in general is 4.5 to 5.0) to pH 6.0 to 6.5. This pH adjustment require the costly addition of acid-neutralizing chemicals. Moreover, in the next CD formation step, the pH must be adjusted to optimum for CGTase. After the reaction mixture is allowed to cool to ≈ 50°C, it is treated with bacterial CGTase (Kitahata, *et al*.,1974; Nakauma and Horikoshi, 1976; Bender, 1977; Kobayashi, *et al*., 1978; Kitahata and Okada, 1982; Makela, *et al*., 1988; Yagi, *et al*., 1986), which have an optimum catalytic reaction temperature of 50-65°C. However, this procedure is inefficient in

many aspects : firstly, α-amylase should be inactivated before the addition of CGTase; secondly, microbial contamination is possible; thirdly, it is time consuming. Therefore, a CGTase with liquefying and cyclizing activities at high temperature would be appropriated for efficient production of CDs. Norman and Jorgensen (1992) reported a thermostable CGTase isolated from a thermophilic anaerobe, Thermoanaerobacter. This thermostable CGTase has a temperature optimum of 90°C and produced CDs more efficiently than the CGTase isolated from *Bacillus macerans* with a temperature optimum of 55°C (Yagi, *et al*., 1986). However, the producing organism, Thermoanaerobacter, is not presently included in those organisms that are generally reconized as being safe for the production of food or pharmaceutical products of their applications. Inactivation of the enzyme upon the completion of the process would not be easy due to the thermostability of the enzyme.

Purification methods for CGTase

The study on physical and biochemical properties of CGTase required separation techniques to purify the enzyme. Methods reported thus for CGTasse purification include precipitation with organic solvents or ammonium sulfate, starch adsorption , electrophoresis and chromatography on DEAE-cellulose (Nakamura and Horikoshi, 1976; Kitahata, *et al*., 1974; Matzuzawa, *et al*., 1975; Stavn and Granum, 1979; Kobayashi, *et al*., 1978). Very efficient purification of CGTase from *B. macerans* was achieved on α-CD-derivatized agarose (Laszlo, *et al*., 1981). Because CGTase from that strain produces mainly α-CD, the current view is that the α-CD ligand is appropriate only for this CGTase. Thus, β-CD has been suggested to serve as the affinity ligand for β-CD producing enzyme (Bender, 1986).

The cyclodextrin research group at the Department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on β-CGTase of *Bacillus circulans* A11, a strain isolated from South-East Asian soil (Pongsawasdi and Yagisawa, 1987). The enzyme was purified and characterized in its properties such as molecular weight, working pH and temperature and the enzyme activity on various substrates (Techaiyakul, 1991).Effect of some carbohydrates on the induction of CGTase to produce higher CD-products mainly γ-CD was also studied (Rattapat, 1996). The enzyme was purified by chromatofocusing column and analysis on native-PAGE suggested that it may compose of 4 isozymes with different isoelectric points in the range of 4.40-4.90 (Rojtinnakorn, 1994). Specific antibody against CGTase was prepared (Rojtinnakorn, 1994) and was used in enzyme purification through immunoaffinity column chromatography (Kim, 1996). Optimization of CGTase production in a 5 litre-fermenter and cyclodextrin production from rice starch by using immobilized CGTase in both batchwise and continuous processes and also free CGTase were studied (Rutchatorn, 1993; Kuttiarcheewa, 1994 and Malai, 1995). Siripornadulsil (1992), Vittayakitsirikul (1995) and Boonchai (1996) reported on molecular cloning techniques, gene expression, mapping and partial nucleotide sequence determination. Laloknum (1997) synthesized oligonucleotide probes for CGTase gene and Jantarama (1997) studied on the mutation of *Bacillus* sp. A11 for the production of higher CGTase activity. Kaskangam (1998) later isolated and characterized CGTase isozymes from this strain. Production of cyclodextrins from cassava starch was studied (Nilmanee, 2000) and Wongsangwattana (2000) reported on specificity of glycosyl acceptor in coupling and transglycosylation reactions of CGTase from *Bacillus circulans* A11. In addition reduction of naringin and limonin in

tangerine *Citrus reticulata*, Blanco juice with β-cyclodextrin polymer was also studied (Rodart, 2001).

In the hope to reduce the before mentioned problems in the process of CDs production, the thermostable CGTase-producing bacteria were, therefore, screened from the hot spring environments at northern Thailand. Conditions for growth and CGTase production were optimized. The enzyme was also purified and studied some of its physical and biochemical characteristics.

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., U.S.A.

Centrifuge, microcentrifuge high speed : Model MC-15A, Tomy Seiko Co. Ltd., Tokyo, Japan

Diaflo Ultrafilter : Stirred Ultrafiltration Cell 8050 Amicon W.R. Grace Cooperation , U.S.A.

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

Freeze-dryer : Stone Ridge, New York, U.S.A.

Fraction Collector : Model 2211 Pharmacia LKB, Sweden

Gene Amp PCR System : Model 2400 PERKIN ELMER, U.S.A

High Performance Liquid Chromatography : Model Hewlett PACKARD series 1050, Japan

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

Incubator shaker, controlled environment : Psycrotherm, New Brunswick Scientific Co., U.S.A

Incubator shaker : Model G76D New Brunswick Scientific Co., Inc. Edison, N.J. U.S.A.

Magnetic stirrer and heater : Model IKAMA R GRH, Janke&Kunkel Gmbh&Co.KG, Japan

Spectrophotometer : UV-240, Shimadzu, Japan and Jenway, England

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

Water bath : Charles Hearson Co.Ltd., England

Water bath, Shaking : Heto lab Equipment, Denmark

2.2 Chemicals

Acetonitrile (HPLC grade) : J.T. Baker Chemical, U.S.A.

Acrylamide : Merck, U.S.A.

Beta-amylase : Type 1-B from sweet potato (A7005), Sigma, U.S.A.

Coomassie Brilliant Blue G-250 : Sigma, U.S.A.

Coomassie Brilliant Blue R-250 : Sigma, U.S.A.

Glycine : Sigma, U.S.A.

Maltose, maltotriose, maltotetraose, maltoheptaose, maltohexaose, and maltoheptaose : Sigma, U.S.A.

Methyl orange : BDH, England

N,N′-methylene-bis-acrylamide : Sigma, U.S.A.

Noble agar : BBL, Becton, Dickinson and Company, U.S.A.

Phenolphthalein : Fluka A.G, Buchs S.G., Switzerland

Potato starch (soluble) : Sigma, U.S.A.

Soluble starch (potato) : Fluka A.G. Buchs S.G., Switzerland

Standard α-, β-, and γ-CD : Sigma, U.S.A.

Standard molecular weight marker proteins : Biorad, U.S.A.

Standard pI marker proteins : Sigma, U.S.A.

Trichloroethylene (TCE) : BDH Laboratory Chemical company, U.S.A.

The other common chemicals were of reagent grade. Corn starch (Maizena) were locally purchased.

2.3 Media preparation

2.3.1 Medium I

The medium to maintain bacterial culture was prepared as described by Pongsawasdi and Yagisawa (1987). Medium I consisted of 0.5% beef extract, 1.0% polypeptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble starch (Fluka) was prepared and adjusted to pH 7.2 with 1.0M HCl. For solid medium, 2.0% agar was added. Medium I was sterilized by autoclaving at 121°C for 15 minutes.

2.3.2 Cultivation Medium (modified from Horikoshi's medium)

Medium for enzyme induction, slightly modified from Horikoshi (1971), contained 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.75% $NaCO_3$ with starting pH of 10.0. For screening medium, 2.0% agar, 0.03% phenolphthalein and 0.01% methyl orange were added. The medium was sterilized before used.

2.4 Screening of bacterial strains

Soil samples were collected from hot spring environments in northern Thailand such as Chiang Rai, Chiang Mai, and Tak province. The samples were taken at various distances from the hot springs as shown in Appendix A.

2.4.1 Primary screening

A small amount of soil sample was suspended in sterilized water and was diluted in serial dilution from 10^{-1} to 10^{-6}. Subsequently, each dilution was spreaded onto Medium I agar plate. The culture was incubated at 37°C for 2-3 days. The strains tested positive for starch hydrolyzing activity on agar plate stained with iodine solution (0.2% (w/v) I_2 in 2.0% (w/v) KI) were selected and kept in Medium I slant at 4°C.

2.4.2 Secondary screening

Taguchi (1986) found that phenolphthalein was transformed into colorless dianion within the cavity of β-CD. To distinguish CGTase activity from the actions of acids produced by some microorganisms, methyl orange were also included in the method

The positive strains from 2.4.1 were further screened for CD-forming activity by the phenolphthalein test developed by Park (1989). The bacteria were streaked on Horikoshi's agar plate. The culture was incubated at 37°C for 2-3 days. The colony which was surrounded by appreciable yellowish halo zone was selected and maintained in Medium I slant at 4°C.

2.5 Identification of the CGTase producing bacteria

2.5.1 Biochemical test

The isolated strain was identified by biochemical characteristics at Thailand Institute of Scientific and Technological Research.

2.5.2 Analysis of the 16S rRNA gene

The 16S rRNA gene of the strain was amplified by PCR technique with forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' (primer A) and reverse primer 5'-AAGGAGGTGATCCAGCCGCA-3' (primer H). The amplified PCR product was sequenced by primer pA, pD, and pF. (Edwards, *et al*, 1989) The 16S rRNA gene sequence was then aligned with those sequences of representative group of *Paenibacillus* and *Bacillus* obtained from GenBank (http://www.ncbi.com).

2.6 Cultivation of bacteria

2.6.1 Starter inoculum

A colony of the bacteria was grown in 50 ml of starter Medium I in 250 ml Erlenmeyer flask at 37°C with 250 rpm rotary shaking until A_{660} reached 0.3-0.5 unit or about 8-10 hours.

2.6.2 Enzyme production

The starter bacteria was transferred into 100 ml Horikoshi's broth in 500 ml Erlenmeyer flask with 1% inoculum and cultivated at 37°C with 250 rpm rotary shaking. Culture was harvested after 84 hours and cells were removed by centrifugation at 5,000 rpm for 30 minutes at 4°C. Culture broth with crude CGTase was collected and kept at 4°C until purification.

2.6.3 Physiological characteristics of the bacteria

The physiological characteristics of the isolated bacteria was investigated for optimal growth and enzyme production condition. The incubation temperature, pH, and $CaCl_2$ concentration of Horikoshi's medium were varied. The growth temperature was tested for 37-60°C. The pH of the medium was varied from 6 to 10. The $CaCl_2$ concentration ranged from 0-1.0 mM $CaCl_2$. Samples were collected every 12 hours and the cell density was measured at A_{660}. The protein content and dextrinizing activity were also examined.

2.7 Enzyme assay

For this study, CGTase activity was routinely determined by starch degrading (dextrinizing) activity and CD-forming activity [phenolphthalein or CD-trichloroethylene (CD-TCE)] assays.

2.7.1 Dextrinizing activity assay

Dextrinizing activity of CGTase was measured by the modification methods of Fuwa (1954) and Techaiyakul (1991)

Enzyme sample (5-100 μl) was incubated with 0.3 ml of starch substrate (0.2%(w/v) soluble starch (potato) in 0.2M Tris-HCl buffer, pH 8.0 containing 10mM $CaCl_2$) at 65°C for 10 minutes. The reaction was stopped with 4 ml 0.2M 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 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 condition.

2.7.2 Phenolphthalein assay

CD-production of CGTase was determined by the method of Chung, *et al*. (1998).

The enzyme solution (100μl) was added to 1 ml of soluble starch solution (4% (w/v) in 50mM Tris-HCl buffer, pH 8.0 containing 15mM $CaCl_2$) and incubated at 75°C for 15 minutes. The reaction was stopped by adding 3.5 ml of 30mM NaOH. Then 0.5 ml of phenolphthalein solution (0.03% (w/v) in 5mM Na_2CO_3) was added to the reaction mixture and left to stand at room temperature for 15 minutes. The concentration of β-CD was calibrated by comparing the reduction in absorbance at 550 nm

with a standard β-CD curve. For control tube, NaOH was added before the enzyme sample.

One unit of enzyme activity was defined as the amount of enzyme producing 1 mg of β-CD per minute.

2.7.3 Cyclodextrin-trichloroethylene (CD-TCE) assay

CD-forming activity of CGTase was determined by the method of Nomoto, *et al*., (1986) with slight modification (Rojtinnakorn, 1994). This method is specifically measuring β-CD which is insoluble in TCE.

The enzyme sample was diluted in serial double dilution by 0.2M phosphate buffer, pH 6.0. The reaction mixture, containing 0.5 ml of sample and 2.5 ml of starch substrate (2.0% (w/v) soluble potato starch in 0.2M phosphate buffer, pH 6.0) was incubated at 40°C for 24 hours. Thereafter the mixture was vigorously mixed with 0.5 ml of trichlorothylene (TCE) and left overnight at room temperature in the dark. The activity was expressed in term of dilution limit ($1:2^n$) which was the highest dilution that still produce observable CD-TCE precipitate at the interphase between the upper starch solution layer and the lower TCE layer.

2.8 Protein determination

Protein concentration was determined by the Coomassie Blue 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 at 25°C 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, 10 ml of 85% H_3PO_4 and distilled water.

2.9 Purification of CGTase

CGTase was partially purified from the culture broth of the bacteria by starch adsorption method of Kato and Horikoshi (1984) with some modification (Kuttiarcheewa, 1994). It was further purified by the affinity chromatography method of Larsen, *et al*., (1998). All enzyme purification procedures were performed at 4°C.

2.9.1 Starch adsorption

Corn starch (local grade) was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude broth to make 5% (w/v) concentration. After 24 hours of continuous stirring at 4°C, the starch cake was collected by centrifugation at 8,000 rpm for 30 minutes and washed twice with 10mM Tris-HCl containing 10mM $CaCl_2$, pH 8.5. The adsorbed CGTase was eluted from the starch cake with the same buffer containing 0.2M maltose (3x150 ml for the culture broth of 5 L), by stirring for 30 minutes. The partially purified CGTase was then concentrated approximately 10 times by ultrafiltration (cut off M.W. = 30 kDa) before loading onto the affinity column.

2.9.2 Affinity chromatography (Larsen, *et al*., 1998)

Affinity gel was prepared by covalently linking β-CD to epoxy-activated Sepharose 6B. Four gram of freeze-dried epoxy activated Sepharose 6B was washed on a sintered glass filter with 800 ml of water. The gel was mixed with 1.2 g of β-CD in 24 ml of 0.1M NaOH at 45°C for 24 hours. Unreacted epoxy groups were blocked in 120 ml of 0.1M ethanolamine for 24 hours at 45°C and washed respectively with 400 ml each of 0.1M acetate buffer, pH 4.0, 0.1M NaCl, 0.1M NaOH, 0.5M NaCl, and 0.01M phosphate buffer, pH 9.0.

The affinity column was prepared by packing the matrix in a 10x200 mm column and equilibrated with 10 volumes of the 20mM phosphate buffer, pH 7.0. The crude enzyme solution was loaded onto the column and washed with 20mM phosphate buffer, pH 7.0 until the eluant showed no absorption of A_{280}. Contaminating proteins non-specifically adsorbed to the column were removed by washing with the same buffer containing 0.5M NaCl. The CGTase bound to the column was eluted with 20mM phosphate buffer, pH 7.0 containing 1% β-CD. The eluted fractions containing CGTase activity were then pooled. The flow rate of the affinity chromatography was 0.2 ml/min. Fractions of 2 ml were collected for measurement of A_{280} and dextrinizing activity.

2.10 Detection of the CGTase

2.10.1 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gel electrophoresis were employed for analysis of the purified enzyme. Electrophoresis conditions, protein and activity staining were as described below.

2.10.1.1 Non-denaturing gel electrophoresis

Discontinuous PAGE was performed on slab gels (10x8x0.75 cm) of 5.0% (w/v) stacking and 7.5% (w/v) separating gels. Tris-glycine buffer, pH 8.3 was used as electrode buffer. The electrophoresis was run from cathode towards the anode at constant current of 20 mA per slab at room temperature in a Midget LKB 2001 Gel Electrophoresis unit.

2.10.1.2 SDS-polyacrylamide gel electrophoresis

The denaturing gel was performed according to Bollag and Edelstein (1991). The gel system was 0.1% (w/v) SDS in 5.0% (w/v) stacking and 7.5% (w/v) separating gels and Tris-glycine buffer, pH 8.3, containing 0.1% SDS was used as electrode buffer (Appendix B). 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 20mA per slab at room temperature on a Midget LKB 2001 Gel Electrophoresis unit from cathode towards anode.

2.10.1.3 Detection of proteins

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining, whereas the enzyme was detected by activity staining for the dextrinzing activity or dye staining for the product cyclodextrin.

2.10.1.3.1 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 1 hour. The slab gels were destained with excessive solution of 10% methanol and 10% acetic acid for 3-4 hours until gel background was clear.

2.10.1.3.2 Dextrinzing activity staining (slightly modified from the method of Kobayashi, *et al*., 1978)

The gel was soaked for 10 minutes in 10 ml of substrate solution, containing 2.0% (w/v) potato starch in 0.2M phosphate buffer, pH 6.0, at room temperature. It was then quickly rinsed several times with distilled water to remove unbound starch before 10 ml of I_2 staining reagent (0.2% I_2 in 2.0% KI) was added for color development at room temperature. The clear zone on the dark blue background represents starch degrading activity of the enzyme.

For SDS-PAGE, the gel was washed with 1.0% Triton X-100 in 0.2M phosphate buffer, pH 6.0 at 37°C with gentle shaking for 3 hours before being soaked in the substrate.

2.10.1.3.3 Dye staining for cyclodextrin

The gel was stained by the method of Malai (1991) which was modified from Park, *et al*., (1989).

The basis of the staining is that β-CD produced by the CGTase can trap phenolphthalein dye in its cavities hence the dye become colorless. To clearly determine the color of the positive result, methyl orange was also used together in the method.

The non-denaturing gel was soaked in 10 ml of phenolphthalein staining solution (2.0g% potato starch, 0.03g% phenolphthalein, and 0.01g% methyl orange in 1.0% Na_2CO_3) at room temperature for 20 minutes. After leaving the gel to cool for 30 minutes, the yellow color of methyl orange appeared at the CD producing band whereas the background of the gel was red due to the color of methyl orange in alkaline condition.

2.11 Characterization of the CGTase

2.11.1 Carbohydrate determination

Qualitative analysis for the presence of carbohydrate in CGTase was performed by periodic acid-Schiff (PAS) staining (Zacharius and Zell, 1960)

For PAS staining of glycoprotein, the SDS gel was immersed in 12.5% trichloroacetic acid for 30 minutes and then rinsed lightly with distilled water. The gel was then treated with the 1.0% periodic acid solution (Appendix C) for 50 minutes followed by washing 6 times for 10 minutes each in 200 ml distilled water. After that

the gel was immersed in Schiff's reagent in dark for 50 minutes. The gel was then washed with freshly prepared 0.5% metabisulfite 3 times for 10 minutes each. Thereafter, the result immediately recorded or the gel should be stored in 3.0% acetic acid at 4°C.

2.11.2 Determination of the isoelectric point by isoelectric focusing polyacrylamide gel electrophoresis (IEF)

IEF-PAGE was performed on slab gel (10x8x0.75 cm), of 2.4% gel (Appendix D). The catholyte (20mM NaOH) was added to upper buffer and for lower buffer chamber was added by anolyte (10mM phosphoric acid). Focusing was carried out under constant voltage conditions in a stepwise fashion. The gel was firstly focused at 200V for 1.5 hours and then run at 400V for 1.5 hours. Current would be about 400 mA at the start and decrease during focusing. The temperature was controlled at 4°C by ice-water bath.

After complete electrofocusing, the gel was stained. Standard protein markers with known pI's in the range 3-10 were run together. The standards consist of amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), and trypsinogen (9.30). The pI's of sample proteins were determined by the standard curve constructed from the pI's of the standard proteins and their migrating distance from cathode.

2.11.3 Analysis of cyclodextrins by high performance liquid chromatography (HPLC)

The sample solution were analyzed for cyclodextrins by HPLC using Spherisorb 10 NH_2 column (0.46x25cm) and detected by RI detector. The eluant was a mixture of 75% acetonitrile and 25% water by volume, and the flow rate was 2 ml/min. To prepare the sample solution, the enzyme were firstly incubated with 2.5 ml of starch substrate (2.0g% potato starch in 0.2M phosphate buffer, pH 6.0) at 40°C for 24 hours. The reaction was stopped by boiling in water for 5 mintues. After cooling, the reaction mixtures was treated with 20 units of β-amylase at 25°C for 3 hours, and the reaction stopped by heating in boiling water bath. Prior to injection, the samples were filtered through 0.45 μm membrane filters. Cyclodextrin (α-, β-, and γ-CD) were analyzed by comparing the retention time with those of standard CDs and the amount was calculated from peak area.

2.11.4 Effects of temperature, pH, $CaCl_2$ and time on CGTase activity

The enzyme activity was determined by the methods described in 2.7.1 and 2.7.2. The incubation condition was varied as follows : temperature range from 0 to 100 °C, pH range from 3.0 to 11.0 (Appendix E), 0-20mM $CaCl_2$ concentration, and 0-30 minutes for incubation time.

2.11.5 Effects of metallic ions and chemicals on CGTase stability

The enzyme were incubated in various metallic ions and chemicals for 15 minutes at 0°C. Its remaining activity was determined by the method described in Section 2.7.1.

2.11.6 Effects of temperature on CGTase stability

The enzyme was incubated at various temperature, from 0 to 100°C, in phosphate buffer with or without 10mM $CaCl_2$ for 15 minutes. The activity was then determined by the methods described in Section 2.7.1 and 2.7.2.

2.11.7 Effects of pH on CGTase stability

The enzyme was kept in buffer solution of various pH, (pH 3.0 to 11.0) at 4°C for 24 hours. Its remaining activity was determined by the methods described in Section 2.7.1 and 2.7.2.

2.11.8 Effects of storage on the enzyme

The crude enzyme was kept at –20 and 4°C. It was then determined the activity by the method described in Section 2.7.1 every week. It was also measured the amounts of protein.

CHAPTER III

RESULTS

3.1 Isolation of CGTase producing bacteria

Soil samples from hot springs in northern Thailand were collected and screened for amylolytic bacteria (primary screening) by method as described in Section 2.4.1. The colony which produced clear zone (Figure 9) was kept in Medium I slant or tested for CGTase activity on the agar medium containing phenolphthalein-methyl orange. For colonies grown on the plate, 24 hours incubation at 37°C was sufficient for the appreciable yellowish zone around the colony (Figure 10). Of the 158 amylolytic colonies, only one colony exhibited CGTase activity (Table 6) and was chosen for further study. It was named as T16.

3.2 Identification of CGTase producing strain

3.2.1 Biochemical Test

The biochemical characterization of this bacterium were done by Thailand Institute of Scientific and Technological Research. The results in Table 7 suggested that the strain most closely resembled those of either *Bacillus macerans* or *Paenibacillus macerans.*

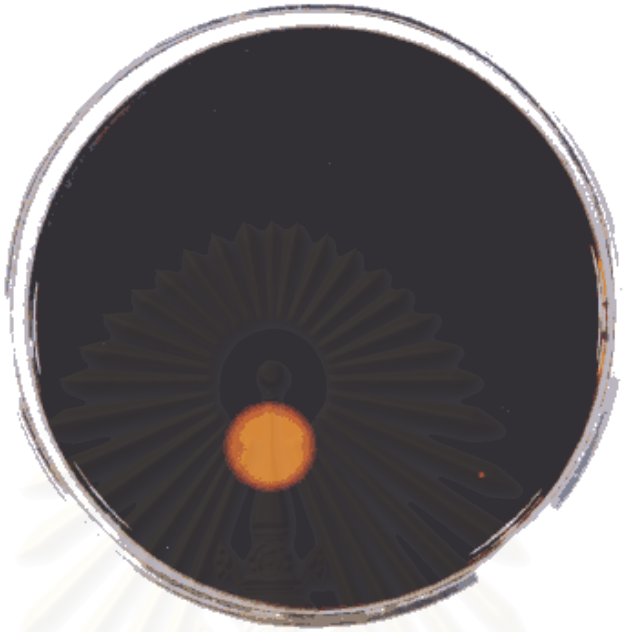

Figure 9 Halo zone of amylolytic bacteria on starch-containing agar medium (primary screening).

Bacterial cells were spreaded on the starch containing agar plate, followed by incubation for 24 hours at 37°C. The presence of clear zone after iodine staining indicated amylolytic activity. (Method in Section 2.4.1)

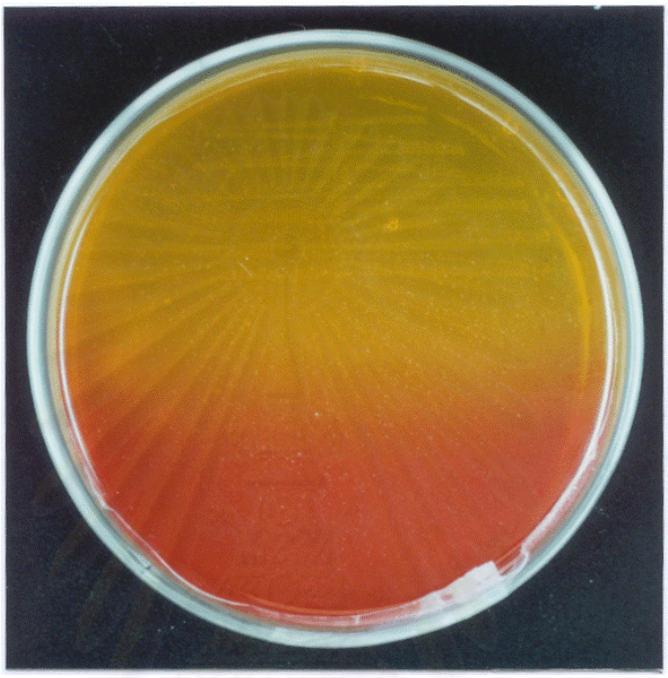

Figure 10 Representative of secondary screening of CGTase activity on phenolphthalein-methyl orange agar medium.

Bacterial cells were streaked on the plate, followed by incubation for 3 days at 37°C. The presence of yellow zone indicated CGTase activity. (Method in Section 2.4.2)

Table 6 Amylolytic and CD-producing bacteria from various sources.

Source of soil sample	Number of colonies	
	Amylolytic activity	CD-forming activity
Chiang Rai		
Huay sai kwao	2	-
Mae sa rauy	4	-
Tung tay wee	7	-
Chiang Mai		
Sun kum paeng	6	-
Tak		
Mae ka sa	139	1
Total	158	1

Table 7 Biochemical characteristics of the bacteria T16.

Characteristics	Reaction
Gram reaction	+ve
Fermentative production of acid from :	
-glycerol	-
-erythritol	-
-D-arabinose	+
-L-arabinose	+
-ribose	+
-D-xylose	+
-L-xylose	-
-adonitol	-
-β-methyl-D-xyloside	+
-galactose	+
-D-glucose	+
-D-fructose	+
-D-mannose	+
-L-sorbose	-
-rhamnose	-
-dulcitol	-

Table 7 (continued) biochemical characteristics of the bacteria T16.

Characteristics	Reaction
Fermentative production of acid from : (continued)	
-inositol	-
-mannitol	+
-sorbitol	-
-α-methyl-D-mannoside	-
-α-methyl-D-glucoside	+
-N-acetyl-glucosamine	+
-amygdaline	+
-arbutine	+
-esculine	+
-salicin	+
-cellobiose	+
-maltose	+
-lactose	+
-melibiose	+
-sucrose	+
-trehalose	+

Table 7 (continued) biochemical characteristics of the bacteria T16.

Characteristics	Reaction
Fermentation production of acid from : (continued)	
-inulin	-
-melezitose	-
-D-raffinose	+
-starch	+
-glycogen	+
-xylitol	-
-β-gentiobiose	+
-D-turanose	+
-D-lyxose	-
-D-tagatose	-
-D-fucose	-
-L-fucose	-
-D-arabitol	+
-L-arabitol	-
-gluconate	+
-2-keto-gluconate	-
-5-keto-gluconate	-

3.2.2 Analysis of the 16S rRNA gene

To further identify the bacterium isolate T16, analysis of the 16S rRNA gene was performed. Total genome DNA was extracted and the 16S rRNA gene of the strain was amplified by the method as described in Section 2.5.2. A 1,500 bp fragment was obtained from the PCR product (data not shown) and the sequence (1,203 bp) was determined by Bioservice Unit, National Science and Technology Development Agency (NSTDA) Thailand. The determined sequence is shown in Figure 11 and has been deposited in the NCBI Data Library. Comparison of this gene sequence with those of other Bacilli and Paenibacilli clearly demonstrated that it is similar to *Paenibacillus* sp. 38-2 (99%), *Paenibacillus macerans* (92%), and *Bacillus macerans* (90%). The 16S rRNA gene sequence of the strain was also aligned with *Paenibacillus* sp. 38-2 by CLUSTAL W software (http://www.ebi.ac.uk/clustalw/) (Figure 12). From the biochemical characteristics and 16S rRNA gene sequence, it clearly indicated that the isolate T16 belonged to the genus *Paenibacillus*, possibly *Paenibacillus macerans*. It will be named *Paenibacillus* sp. T16 in subsequently text.

3.3 Physiological characterization

Some physiological characteristics of *Paenibacillus* sp. T16 were investigated to study the optimal condition for growth and enzyme production as described in Section 2.6.

```
1                       AGAGTTTGAT  CCTGGCTCAG  ---------
AACGCTGGCG  GCGTGCCTAA  TACATGCAAG  TCGAGCGGAA  TCGATGGAGT
GCTTGCACTC  CTGAGATTTA  GCGGCGGACG  GGTGAGTAAC  ACGTAGGCAA
CCTGCCCTCA  AGACTGGGAT  AACTACCGGA  AACGGTAGCT  AATACCAGAT
AGGATATTTG  GCTGCATGGC  CGNATATGGA  AAGGCGGAGC  AATCTGTCAC
TTGAGGATGG  GCCTGCGGCG  CATTAGCTAG  TTGGTGGGGT  AATGGCCTAC
CAAGGCGACG  ATGCGTAGCC  GACCTGAGAG  GGTGAACGGC  CACACTGGGA
CTGAGACACG  GCCCAGACTC  CTACGGGAGG  CAGCAGTAGG  GAATCTTCCG
CAATGGACGA  AAGTCTGACG  GAGCAACGCC  GCGTGAGTGA  TGAAGGTTTT
CGGATCGTAA  AGCTCTGTTG  CCAGGGAAGA  ACGCCAGAGA  GAGTAACTGC
TCTTTGGGTG  ACGGTACCTG  AGAAGAAAGC  CCCGGCTAAC  TACGTGCCA-
CAGCCGCGGT  AATACGTAGG  GGGCAAGCGT  TGTCCGGAAT  TATTGGGCGT
AAAGCGCGCG  CAGGCGGTTC  TTTAAGTCTG  GTGTTTAAAC  CCGGAGCTTA
ACTTCGGGAC  GCACTGGAAA  CTGGGGAACT  TGAGTGCAGA  AGAGGAGAGT
GGAATTCCAC  GTGTAGCGGT  GAAATGCGTA  GATATGTGGA  GGAACACCAG
TGGCGAAGGC  GACTCTCTGG  GCTGTAACTG  ACGCTGAGGC  GCGAAAGCGT
GGGGAGCAAA  CAGGATTAGA  TACCCTGGTA  GTCCACGCCG  TAAACGATGA
ATGCTAGGTG  TTAGGGGTTT  CGATACCCTT  GGTGCCGAAG  TTAACACATT
AAGCATTCCG  CCTGGGGAGT  ACGGTCGCAA  GACTGAAACT  CAAAGGAATT
GACGGGGACC  CGCACAAGCA  GTGGAGTATG  TGGTTTAATT  CGAAGCAACG
CGAAGAACCT  TACCAGGTCT  TGACATCCCT  NTGACCCCTC  TAGAGATAGA
GGTTTCCTTC  GGGACAGANG  AAACAGGTGG  TGCATGGTTG  TCGTCAGCTC
GTGTCGTGAG  ATGTTGGGTT  AAGTCCCGCA  ACGAGCGCAA  CCCTTGATCT
TAGTTGCCAG  CACGTAATGG  TGGGCACTCT  AAGGTGACTG  CCGGTGACAA
ACCGGAGGAA  GGTGGGGATG  ACGTCAAATC  ATCATGCCCC  TTATNACCTG
GGCT 1203
```

Figure 11 Partial 16S rRNA gene sequence of the strain T16.

pA 5’-AGAGTTTGATCCTGGCTCAG-3’

pD 5’-CAGCAGCCGCGGTAATAC-3’

pF 5’-CATGGTTGTCGTCAGCTCGT-3’

```
T16   : 1    ACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGCA 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 62    ACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGCA 121


T16   : 61   CTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTGG 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 122   CTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTGG 181


T16   : 121  GATAACTACCGGAAACGGTAGCTAATACCAGATAGGATATTTGGCTGCATGGCCGNATAT 180
               |||||||||||||||||||||||||||| |||||||||||||||||||||||| ||||
38-2 : 182   GATAACTACCGGAAACGGTAGCTAATACCGGATAGGATATTTGGCTGCATGGCCGAATAT 241


T16   : 181  GGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGG 240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 242   GGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGG 301


T16   : 241  GGTAATGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTG 300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 302   GGTAATGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTG 361


T16   : 301  GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA 360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 362   GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA 421


T16   : 361  CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTG 420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 422   CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTG 481


T16   : 421  TTGCCAGGGAAGAACGCCAGAGAGAGTAACTGCTCTTTGGGTGACGGTACCTGAGAAGAA 480
               |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
38-2 : 482   TTGCCAGGGAAGAACGCCAGAGAGAGTAACTGCTCTTTGGGTGACGGTACCYGAGAAGAA 541


T16   : 481  AGCCCCGGCTAACTACGTGCCA-CAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGG 539
               |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
38-2 : 542   AGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGG 601


T16   : 540  AATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAGC 599
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 602   AATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAGC 661


T16   : 600  TTAACTTCGGGACGCACTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTC 659
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 662   TTAACTTCGGGACGCACTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTC 721


T16   : 660  CACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTC 719
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 722   CACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTC 781
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T16  : 720  TGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG 779
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 782  TGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG 841


T16  : 780  GTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCG 839
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 842  GTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCG 901


T16  : 840  AAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA 899
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 902  AAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA 961


T16  : 900  ATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAA 959
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 962  ATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAA 1021


T16  : 960  CCTTACCAGGTCTTGACATCCCTNTGACCCCTCTAGAGATAGAGGTTTCCTTCGGGACAG 1019
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
38-2 : 1022 CCTTACCAGGTCTTGACATCCCTCTGACCCCTCTAGAGATAGAGGTTTCCTTCGGGACAG 1081


T16  : 1020 ANGAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC 1079
            | || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
38-2 : 1082 AGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC 1141


T16  : 1080 GCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGTAATGGTGGGCACTCTAAGGTGA 1139
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
38-2 : 1142 GCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGTCATGGTGGGCACTCTAAGGTGA 1201


T16  : 1140 CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATNAC 1199
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
38-2 : 1202 CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGAC 1261


T16  : 1200 CTGGGCT 1206
            |||||||
38-2 : 1262 CTGGGCT 1268
```


Figure 12 Alignment of 16S rRNA gene sequence between *Paenibacillus* sp. 38-2 and T16.

3.3.1 Effects of temperature on growth and CGTase production of the bacteria

The bacteria could grow equally well in Horikoshi liquid medium from 37°C up to 50°C (Figure 13a). However, it could not grow at 55°C to 60°C. At 37°C, which is the optimum temperature for CGTase production, the cells began to produce the enzyme after 12 hours (entering log phase), reaching maximum activity (134.66 U/mg protein) at 84 hours and the dilution limit of CD-TCE assay was $1:2^6$. The enzyme production reached highest level while the cells was at mid stationary phase (Figure 13b). The activity of CGTase was much lower at 45°C and 50°C. At these temperatures, the enzyme levels declined after prolonged incubation period even though cell densities were the same. Subsequent experiments for CGTase production were, therefore, carried out at 37°C.

3.3.2 Effects of pH on growth and CGTase production of the bacteria

Figure 14a shows that *Paenibacillus* sp. T16 could grow at a wide pH range (6.0-10.0) with slight preference of alkaline pH. The optimum pH for CGTase production was at pH 10.0, initiating after 12 hours of incubation. Similar to Figure 13b, marked increase of the enzyme was noted after 36 hour, reaching maximum at 84 hour. The maximum activity at 84 hour was 137.25 U/mg protein and the dilution of CD-TCE was $1:2^6$. Less enzyme was observed at near neutral pH (6.0-7.0).

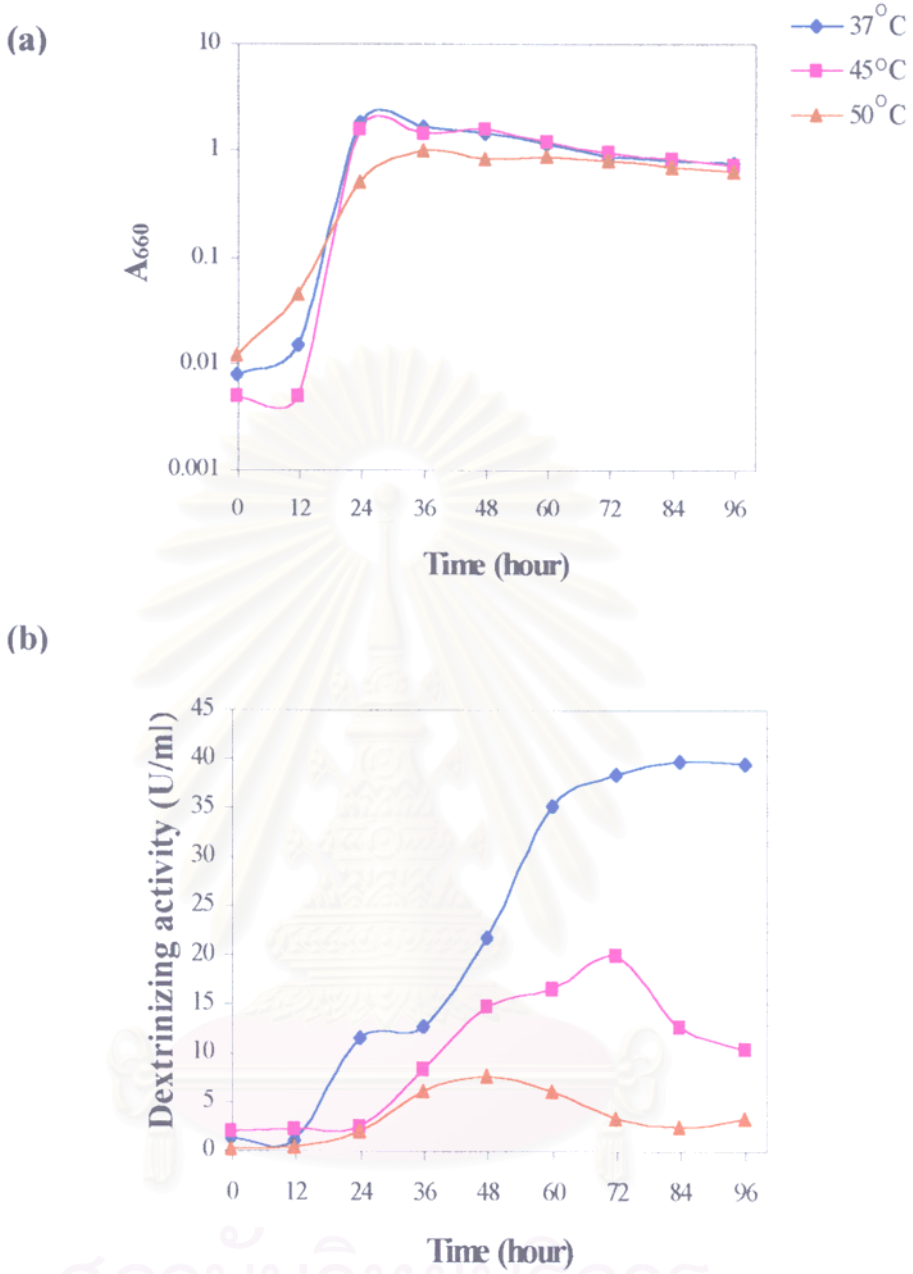

Figure 13 Effects of temperature on growth (a) and CGTase production (b) of *Paenibacillus* sp. T16.

The cells were cultured in Horikoshi liquid medium pH 10.0 at various temperature for 96 hours. (Method in Section 2.6.3)

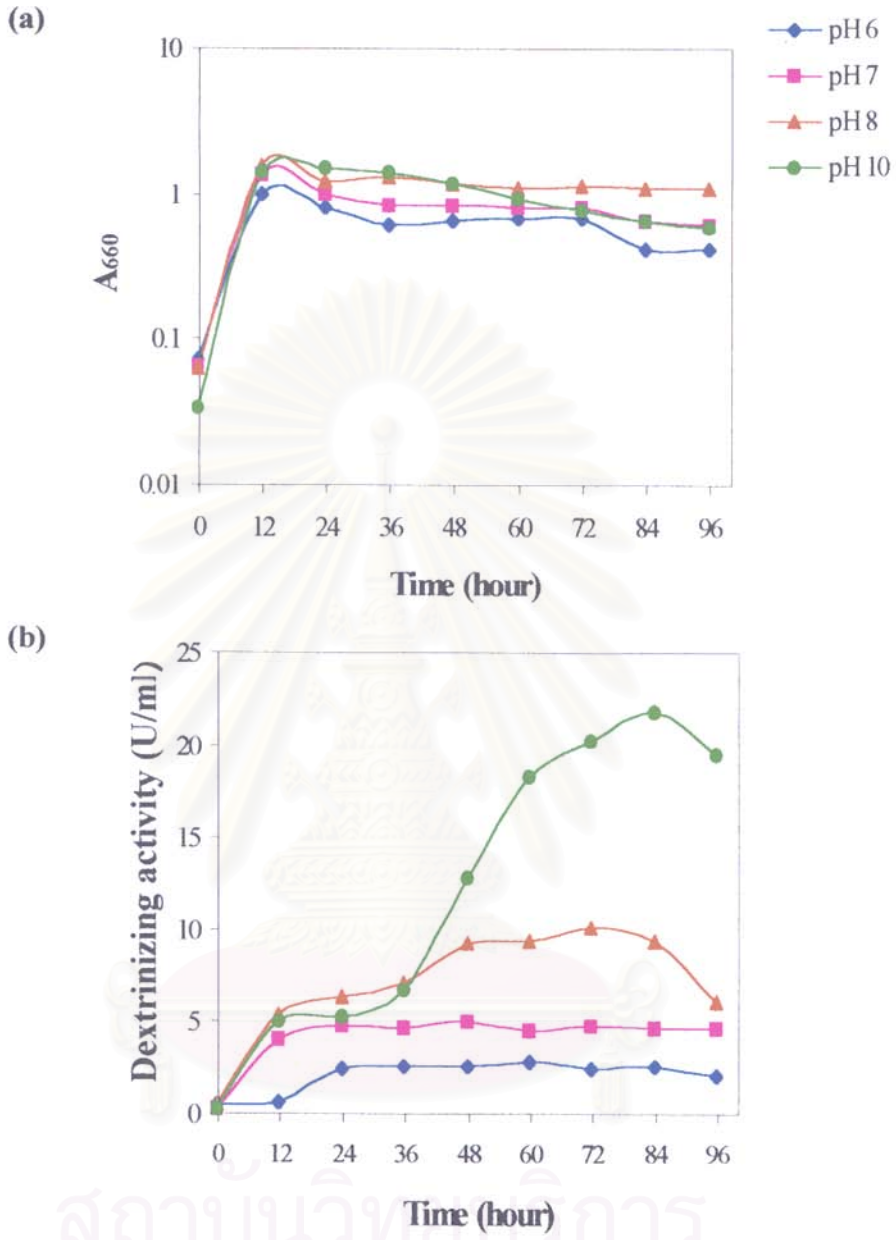

Figure 14 Effects of pH on growth (a) and CGTase production (b) of *Paenibacillus* sp. T16.

The cells were cultured in Horikoshi liquid medium at 37°C, at various pH for 96 hours. (Method in Section 2.6.3)

3.3.3 Effects of $CaCl_2$ on growth and CGTase production of the bacteria

$CaCl_2$ was reported to stabilize CGTase (Mori, 1994). To see if $CaCl_2$ has any effect on growth and CGTase production of the bacteria, the medium containing various amount of $CaCl_2$ was inoculated with *Paenibacillus* sp. T16. It was found that the enzyme activity was enhanced by $CaCl_2$ and the highest activity was designated at 0.05 mM $CaCl_2$ (Figure 15b). High $CaCl_2$ (1.0 mM) did not show any stimulation effect. On the contrary to CGTase production, the growth at all $CaCl_2$ concentration tested were not significantly different. The growth medium in subsequently experiments for CGTase production were, therefore, added with 0.05 mM $CaCl_2$.

3.4 Purification of CGTase

The bacteria were grown in Horikoshi medium containing 0.05 mM $CaCl_2$, pH 10.0, at 37°C for 84 hours. Crude enzyme was purified from the culture of *Paenibacillus* sp T16. By starch adsorption method as reported by Malai (1995) and affinity column chromatography. The details of the procedure was described in Section 2.9.2. The capacity of the β-CD : Sepharose 6B column was estimated as 1 mg protein/ 12 ml gel bed volume before loading of crude enzyme.

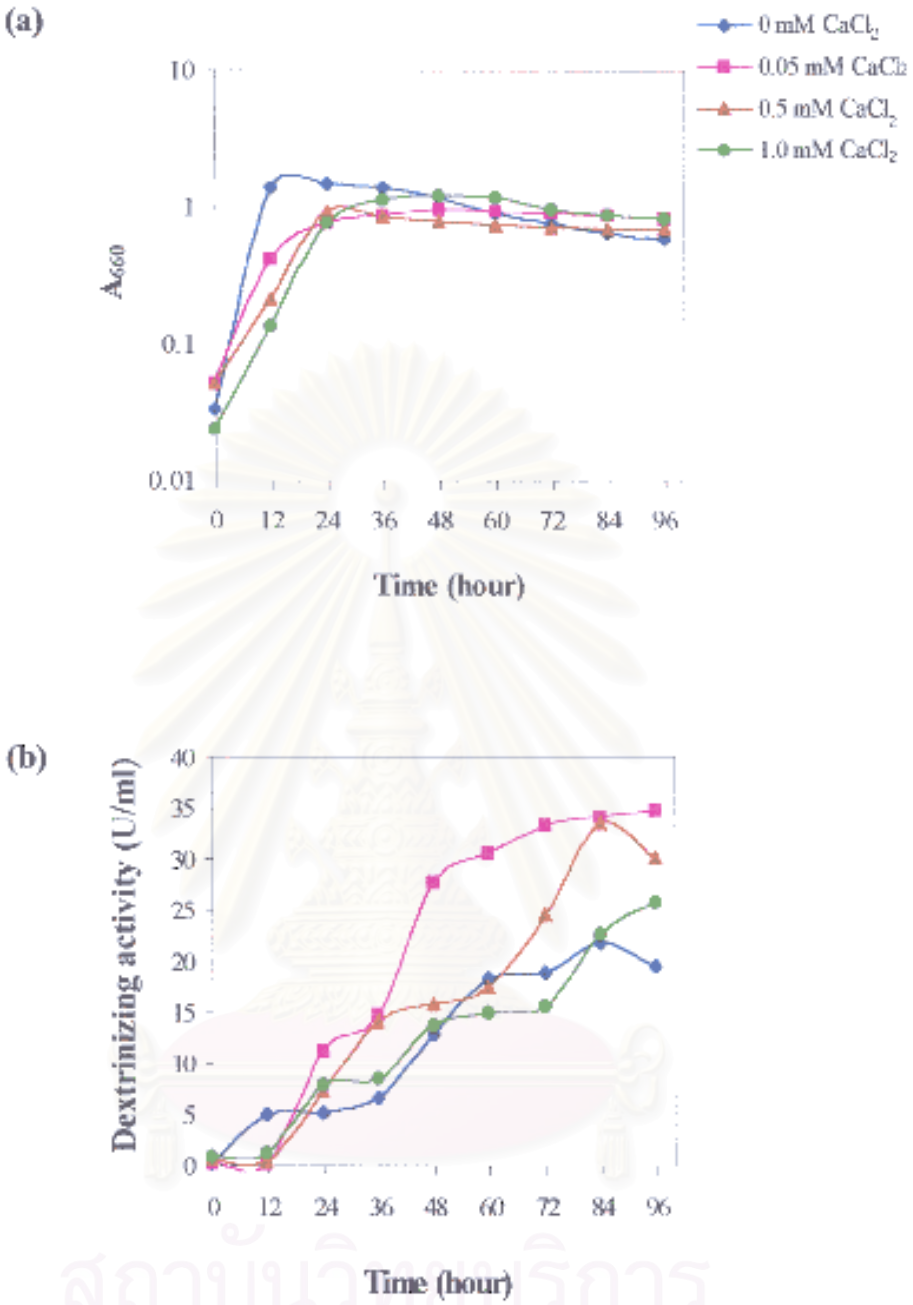

Figure 15 Effects of $CaCl_2$ on growth (a) and CGTase production (b) of *Paenibacillus* sp. T16. (Method in Section 2.6.3)

The cells were cultured in Horikoshi liquid medium at 37°C, pH 10.0 with and without $CaCl_2$ for 96 hours.

Figure 16 shows the elution profile of CGTase from the β-CD affinity column. One major protein peak without enzyme activity was washed off with the equilibrating buffer. CGTase was eluted between fractions 45-57. The highest dextrinizing activity was obtained at the same position as the protein peak in the column. The purification fold and recovery of CGTase obtained at each purification step are shown in Table 8. Specific activity expressed in the 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 purification step, a yield of 21.2% was obtained with 99.99 folds of purity. Figure 16 shows the purity of the enzyme preparation.

Activity staining of enzyme on native polyacrylamide gel showed that the major protein band from purification process corresponded with CGTase activity (Figure 17 a and c). However, 3-4 bands were observed with amylolytic activity (Figure 17b). All amylolytic activity bands gave only a single band in SDS-PAGE gel (Figure 18b). Activity staining with phenolphthalein-methyl orange could not be achieved in this SDS-PAGE system. The data also shows that a low molecular weight protein of more positive charge was eleminated through the process of affinity chromatography.

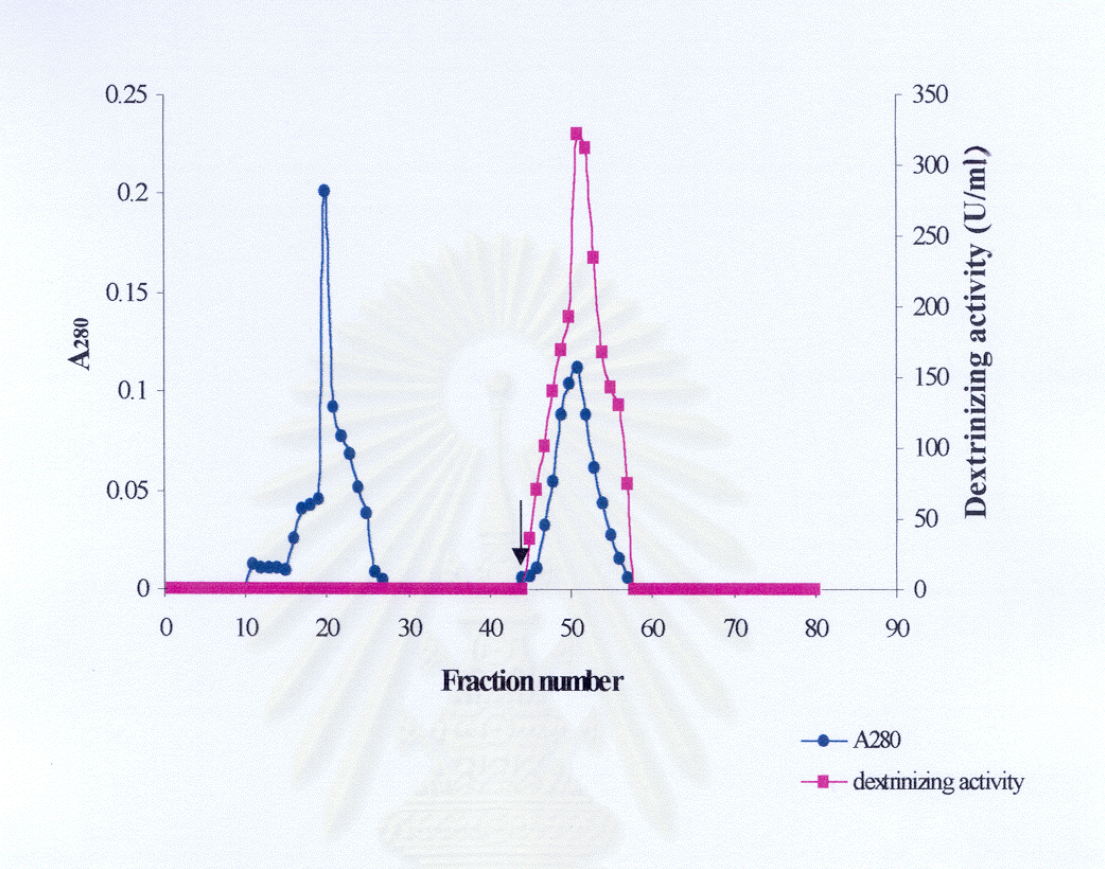

Figure 16 Purification of CGTase from *Paenibacillus* sp. T16 by β-CD affinity column chromatography

Concentrated CGTase solution from corn starch adsorption was applied onto β-CD affinity column (0.79 x 20 cm) and the column was washed with 20mM phosphate buffer pH 7.0 in 0.5M NaCl at the flow rate of 12 ml/hr. Elution was made by 1% β-CD in 20mM phosphate buffer pH 7.0 at the same flow rate (the arrow indicates where elution starts). Fraction sizes of 2 ml were collected.

Table 8 Purification of CGTase from *Paenibacillus* sp. T16

Purification step	Volume (ml)	Total activity (unit)*	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Crude enzyme	1,600	44,752	465.6	96.12	1	100
Starch adsorption	400	27,324	8.8	3,105	32.30	61.06
Ultrafiltration	20	28,482	3.8	7,495	77.98	63.64
β-CD affinity column	21	9,486	0.987	9,611	99.99	21.20

* Unit of dextrinizing activity

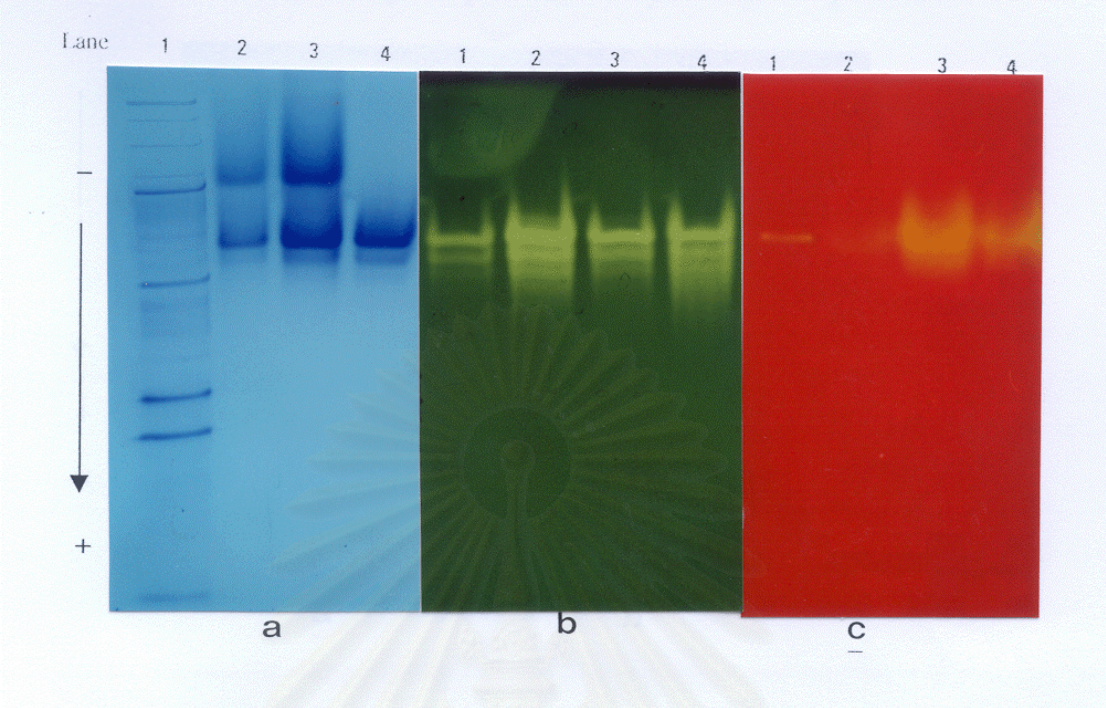

Figure 17 Non-denaturing PAGE (7.5% gel)of CGTase from different steps of purification.

Lane 1 : Crude enzyme (20 μg)

2 : Starch adsorbed enzyme (20 μg)

3 : Post-ultrafiltration enzyme (20 μg)

4 : Enzyme from β-CD affinity column (20 μg)

a : Coomassie Blue staining (Method in Section 2.10.1.3.1)

b : Amylolytic activity staining by iodine reagent (Method in Section 2.10.1.3.2)

Each well was loaded with protein which were adjusted to 0.2 units of dextrinizing activity.

c : Dye staining for cyclodextrin (Method in Section 2.10.1.3.3)

Each well was loaded with protein which were adjusted to 2.0 units of dextrinizing activity.

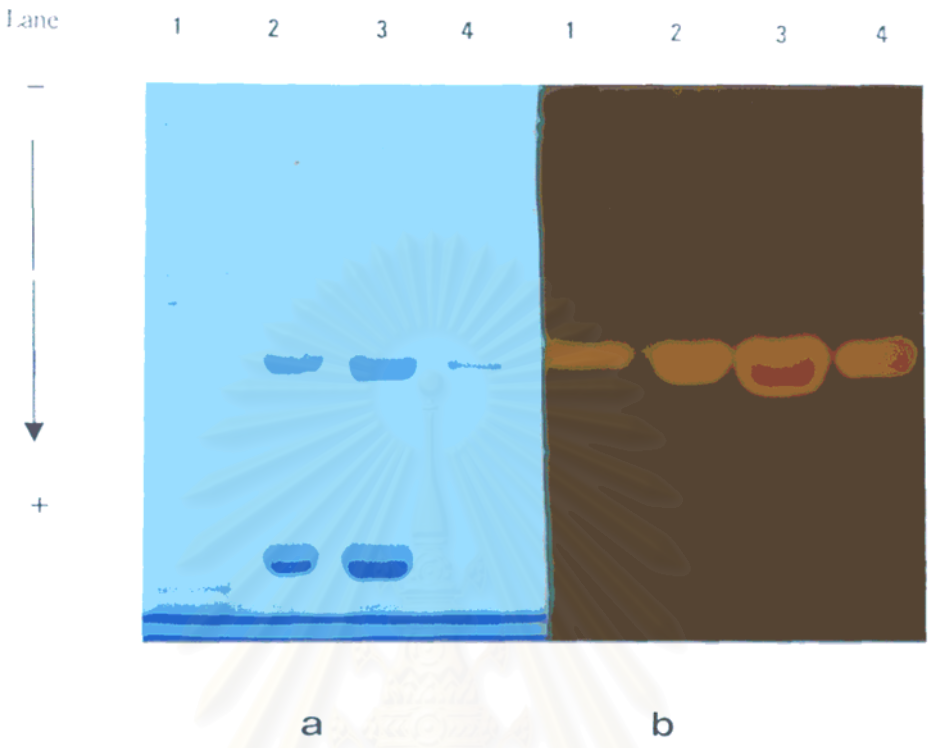

Figure 18 SDS-PAGE (7.5% gel) of CGTase from different steps of purification

Lane 1 : Crude enzyme (20 units)

2 : Starch adsorbed enzyme (20 units)

3 : Post-ultrafiltration enzyme (20 units)

4 : Enzyme from β-CD column (20 units)

a : Coomassie Blue staining, each well contained protein 20 µg (Method in Section 2.10.1.3.1).

b : Amylolytic activity staining by iodine reagent (Method in Section 2.10.1.3.2).

3.5 Characterization of the CGTase

3.5.1 Molecular weight determination by SDS-PAGE

In the SDS-PAGE (Figure 19), the molecular weight of the band which could be stained with I_2 reagent was determined from a calibration curve of standard proteins (Appendix F). The molecular weight of CGTase was estimated to be approximately 76,000 daltons.

3.5.2 Carbohydrate determination

Bands on SDS-PAGE was stained for glycoprotein by PAS method (Section 2.11.1). BSA was used as positive control, while haemoglobin was used as negative control. The band that was corresponded with CGTase from previous experiments gave a deep magenta color similar to BSA (Figure 20). This result suggests that CGTase may be a glycoprotein.

3.5.3 Determination of the isoeletric point

The purified enzyme solution was also analyzed for its isoeletric point by observing the migration on IEF gel electrophoresis (pH 3-10) as compared to standard pI markers (Appendix G). The purified enzyme from β-CD Sepharose column yielded 3 bands with pI 5.7, 5.2, and 4.77 respectively (Figure 21). The major band belonged to pI 5.2.

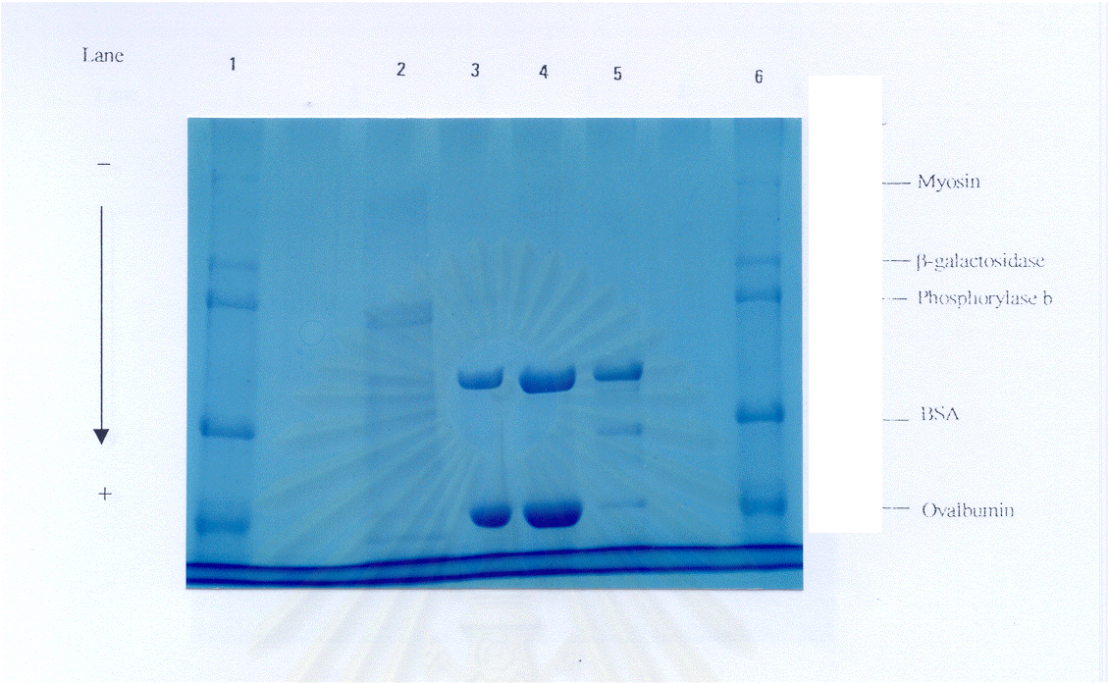

Figure 19 SDS-PAGE of CGTase from different steps of purification

Lane 1, 6 : Protein molecular weight marker proteins.

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 : Starch adsorbed enzyme (20 μg)

4 : Post-ultrafiltration enzyme (20 μg)

5 : Enzyme from β-CD affinity column (20 μg)

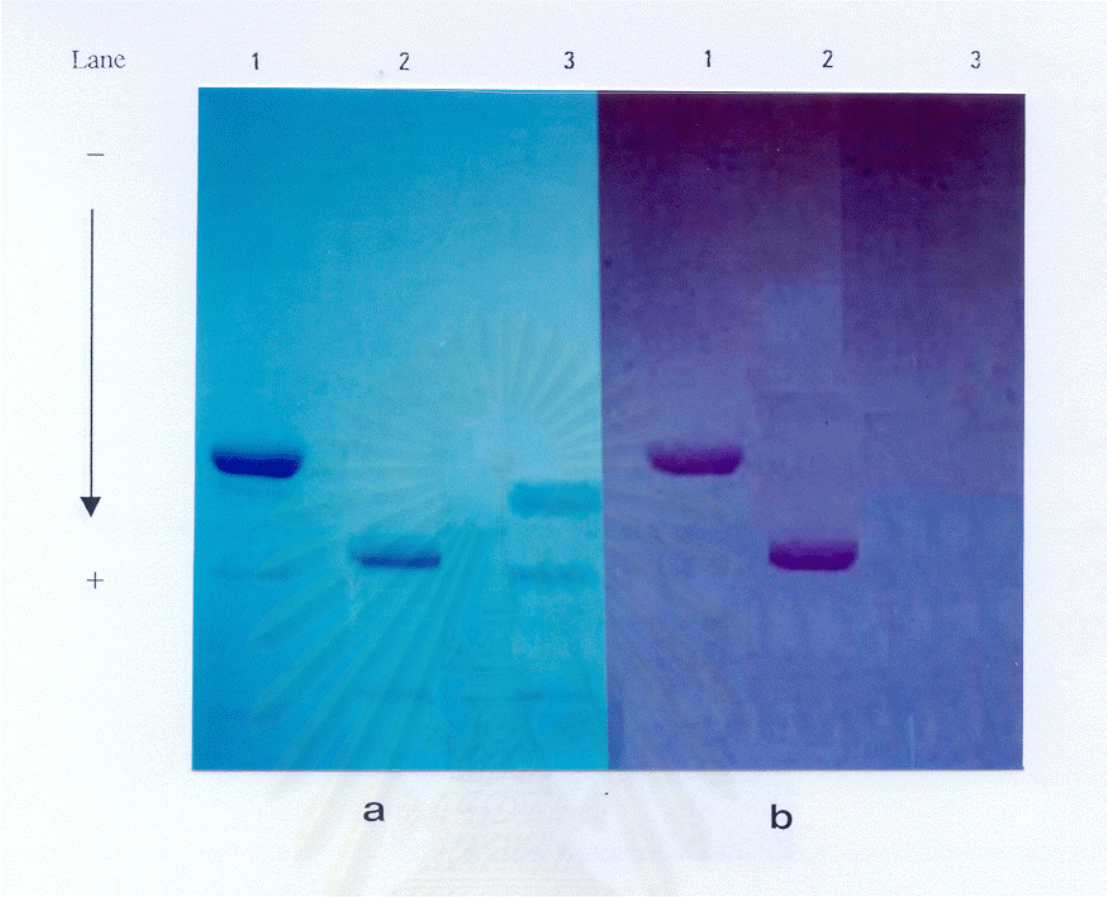

Figure 20 PAS stain of CGTase from β-CD affinity column. The enzyme preparation was run in SDS-PAGE.

Lane 1 : Enzyme from β-CD affinity column

2 : BSA (positive control)

3 : Haemoglobin (negative control)

a : Coomassie Blue staining for protein (20 μg protein of each)

b : PAS staining for glycoprotein (100 μg protein of each)

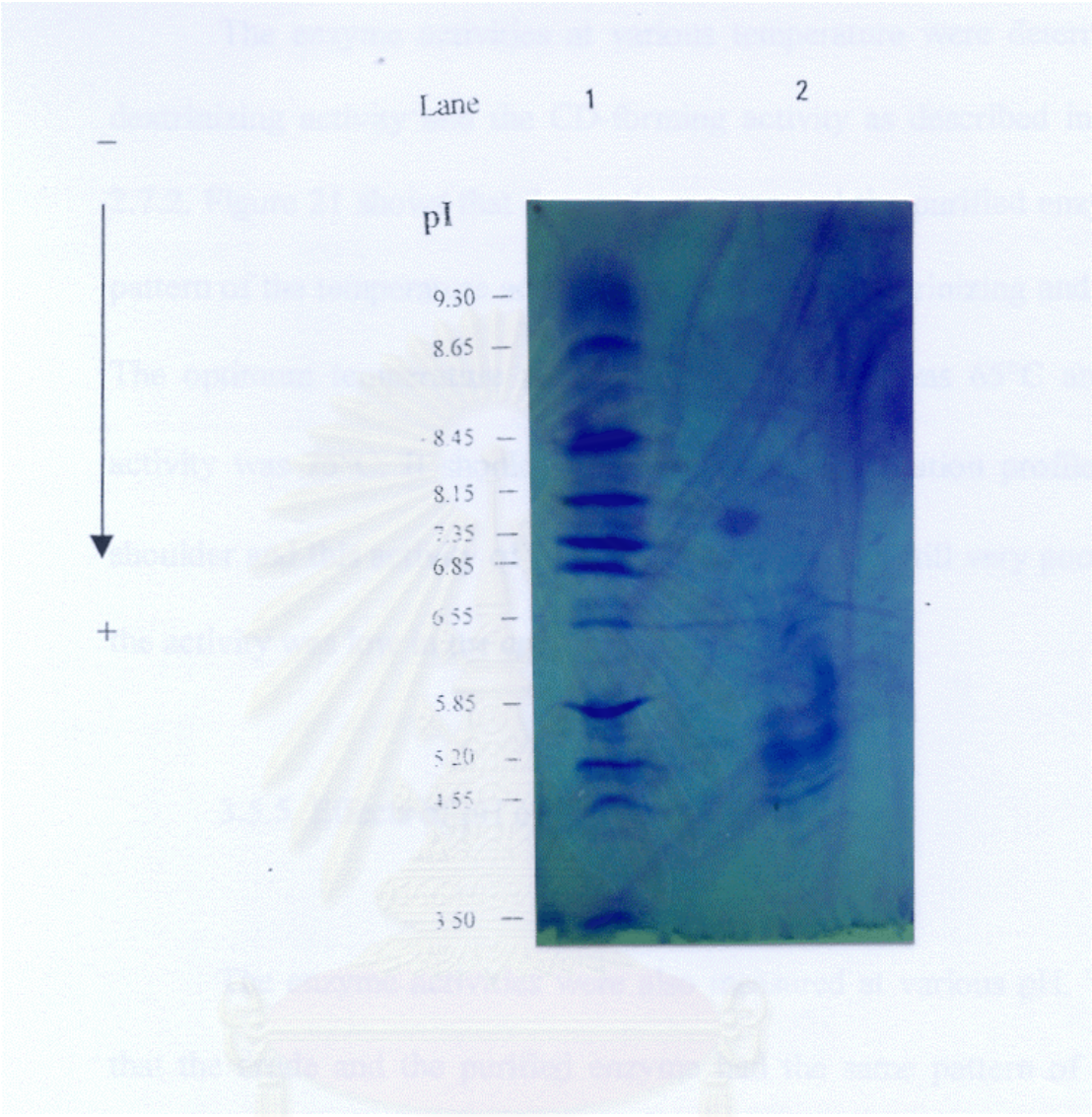

Figure 21 Isoeletrofocusing gel of CGTase using ampholyte pH 3-10. (Method in Section 2.11.2)

Lane 1 Standard pI marker proteins

2 Purified enzyme (50 μg)

3.5.4 Effects of temperature on CGTase activity

The enzyme activities at various temperature were determined for both the dextrinizing activity and the CD-forming activity as described in Section 2.7.1 and 2.7.2. Figure 22 shows that the crude enzyme and the purified enzyme gave the same pattern of the temperature activity profile for both dextrinizing and CGTase activities. The optimum temperature for dextrinizing activity was 65°C and the CD-forming activity was 75°C. It should be noted that the cyclization profile had a broad left-shoulder and this activity of the purified enzyme was still very good at 80°C although the activity was low in the crude enzyme fraction.

3.5.5 Effects of pH on CGTase activity

The enzyme activities were also measured at various pH. The results showed that the crude and the purified enzyme had the same pattern of pH-activity profile (Figure 23). The optimum pH for dextrinization and cyclization was 7.0-8.0 and 8.0, respectively. The enzyme lost activities at extreme acidic and alkaline pH. Figure 23-a and 23-b also showed that both Tris-HCl and phosphate buffer could be used equally well for dextrinization whereas Tris-HCl was the better buffer for cyclization.

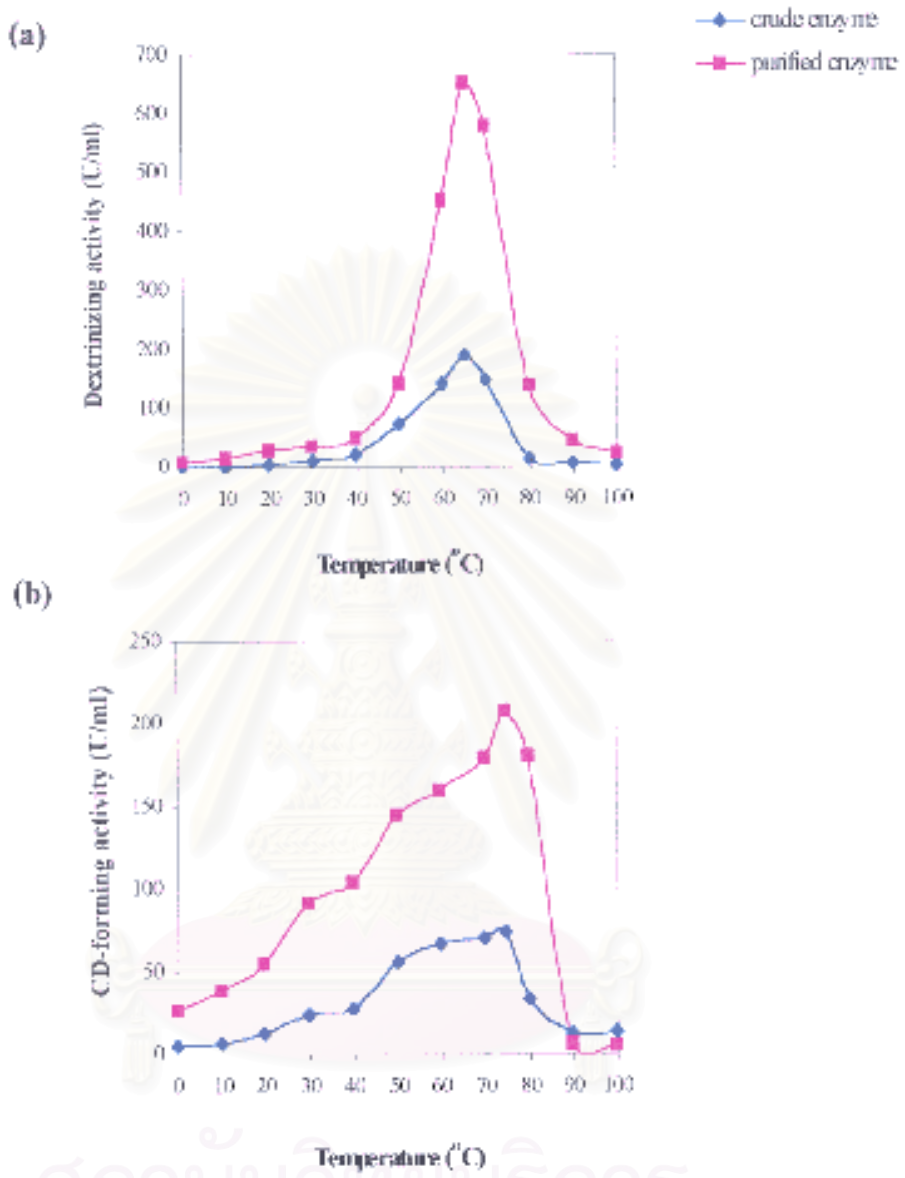

Figure 22 Effects of temperature on the activities of CGTase

The enzyme solution was incubated in 0.2M phosphate buffer (pH 6.0) for 10 minutes at various temperatures. (Method in Section 2.11.4)

a. Dextrinizing activity (iodine method)

b. CD-forming activity (phenolphthalein method)

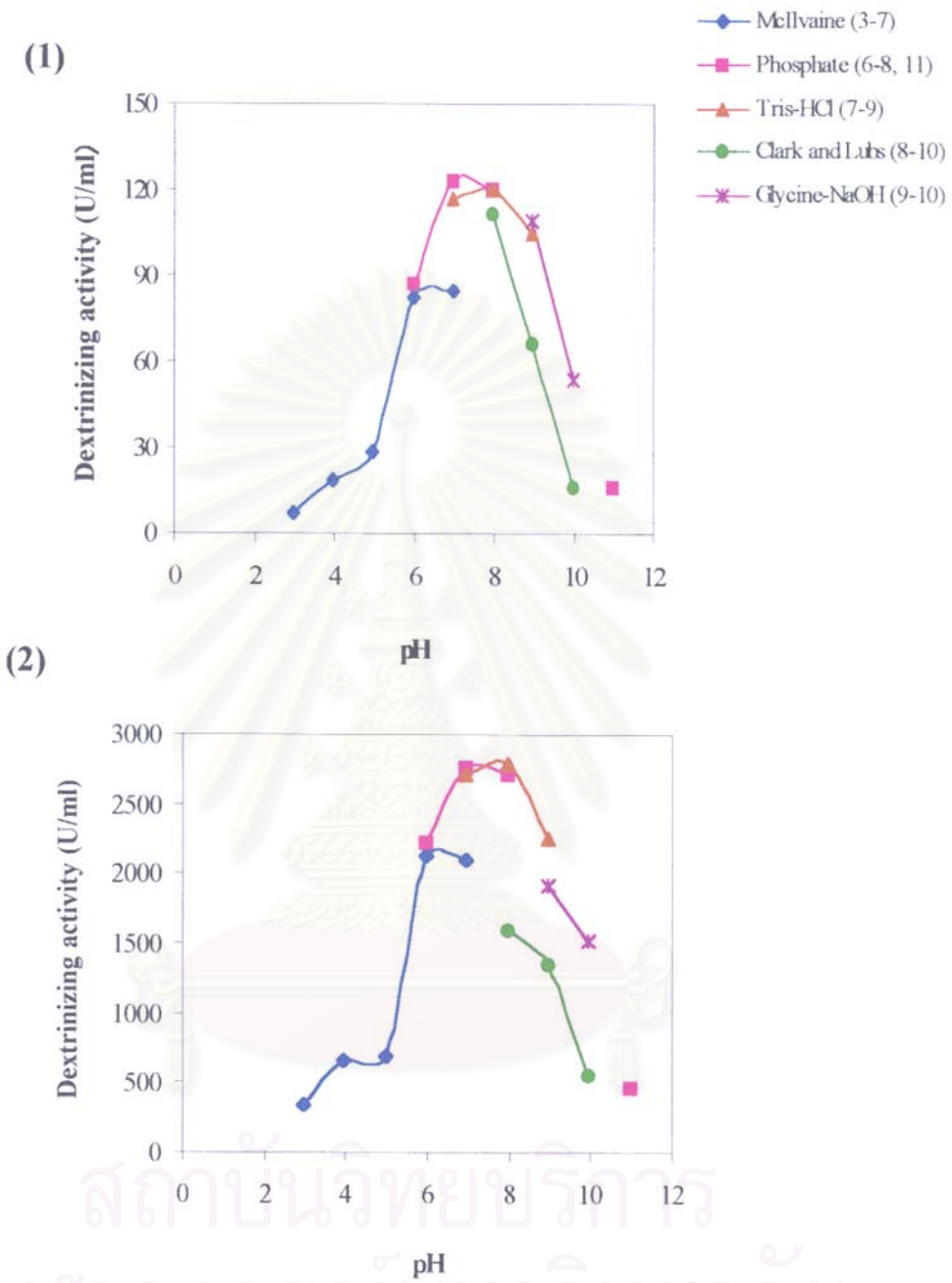

Figure 23-a Effects of pH on the dextrinizing activity of CGTase (1) crude enzyme (2) purified enzyme. The enzyme solution was incubated at 65°C in various pH (3-10) for 10 minutes. (Method in Section 2.11.4)

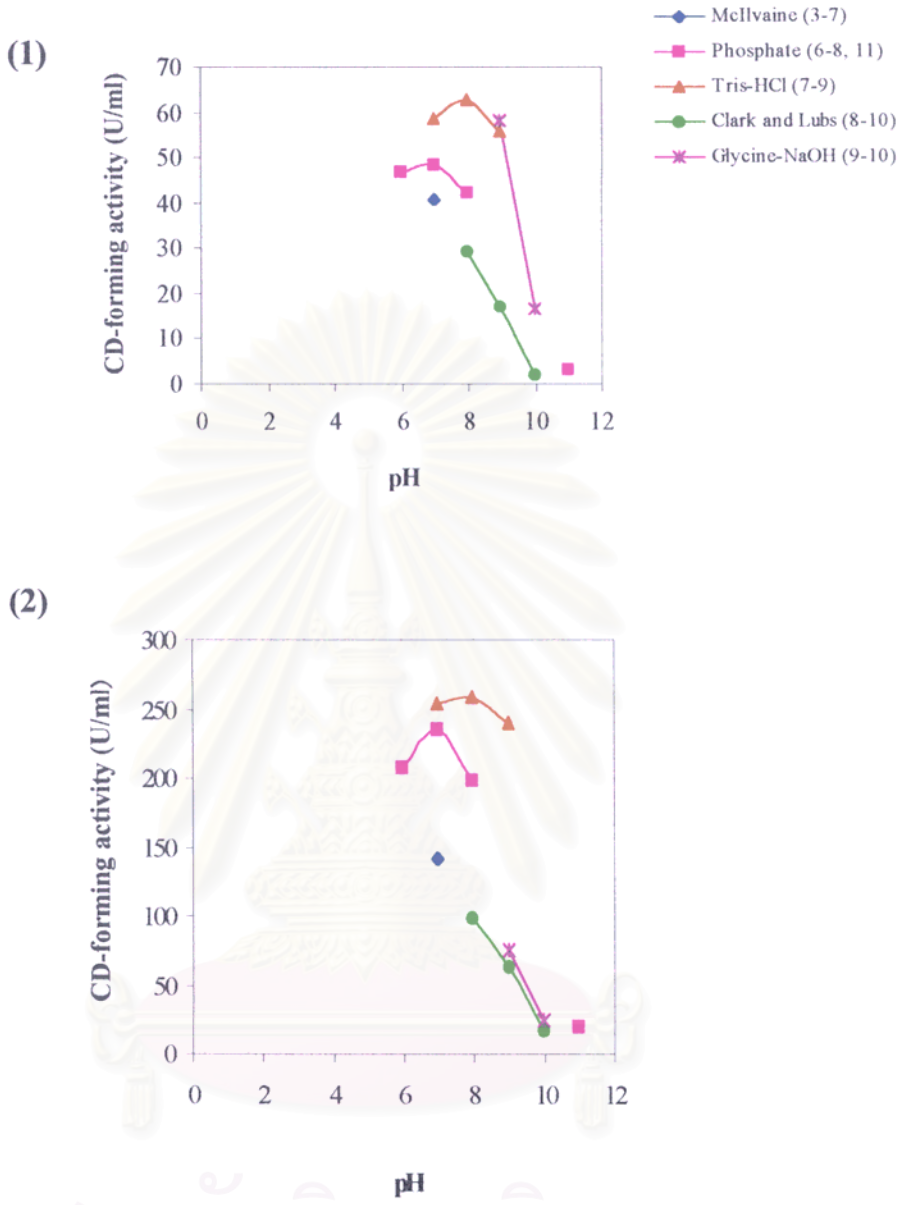

Figure 23-b Effects of pH on CGTase activities (phenolphthalein method) (1) crude enzyme (2) purified enzyme. The enzyme solution was incubated at 75°C in various pH (3-10) for 10 minutes. (Method in Section 2.11.4)

3.5.6 Effects of $CaCl_2$ on CGTase activity

The enzyme activities were determined in Tris-HCl buffer (pH 8.0) with different amount of $CaCl_2$. The results in Figure 23 showed that both dextrinizing and CGTase activities of the crude enzyme were not enhanced by $CaCl_2$. However, the dextrinization and cyclization of purified enzyme was enhanced by 10 and 15 mM $CaCl_2$, respectively as shown in Figure 24-a and 24-b.In addition, higher concentration of $CaCl_2$ tended to inhibit only the dextrinizing activity of the purified enzyme.

3.5.7 Effects of incubation time on CGTase activity

To assess the effect of incubation time on the enzyme at the calibrated optimum condition, the enzyme activities were measured by the method in Section 2.7.1 and 2.7.2 at different time (0-30 minutes). The results demonstrated that both dextrinizing and CGTase activities showed a time-dependent relationship (Figure 25-a and 25-b). A minimum of 15 minutes would be recommended for the condition used.

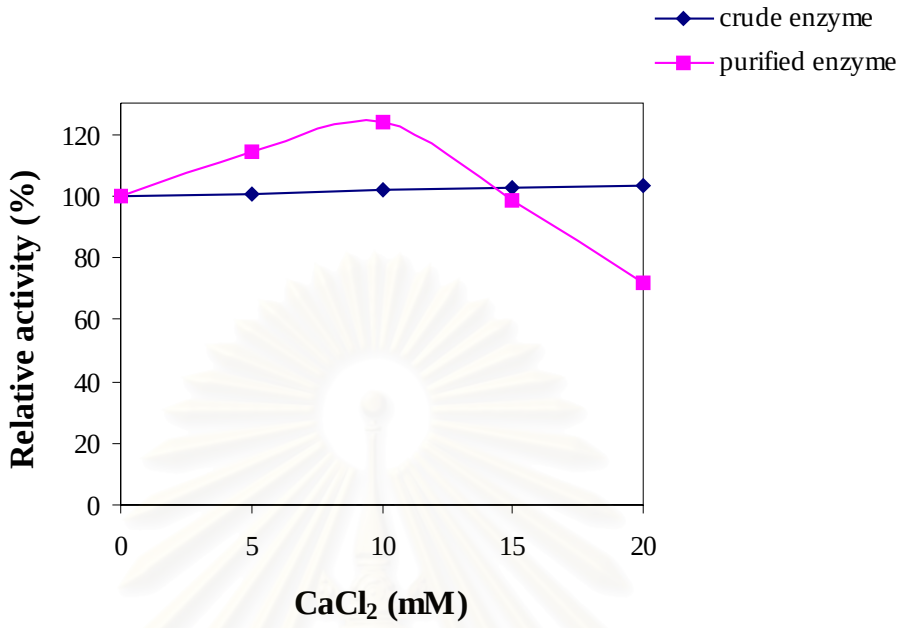

Figure 24-a Effects of $CaCl_2$ on the dextrinizing activity of CGTase. The enzyme solution was incubated at 65°C in 0.2M Tris-HCl buffer (pH 8.0) with various $CaCl_2$ for 10 minutes. (Method in Section 2.11.4)

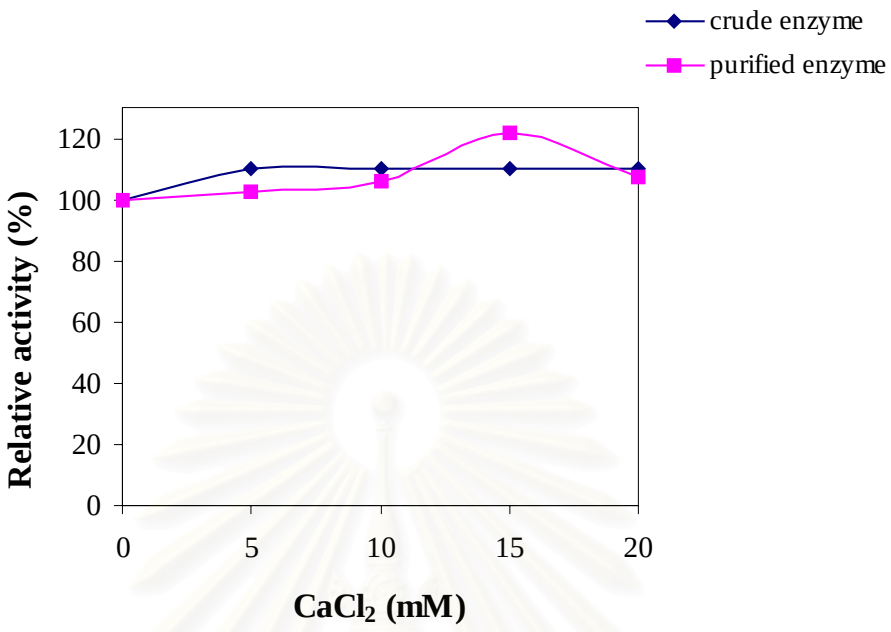

Figure 24-b Effects of $CaCl_2$ on the CGTase activity (phenolphthalein method). The enzyme solution was incubated at 75°C in 0.2M Tris-HCl buffer (pH 8.0) with various $CaCl_2$ for 10 minutes. (Method in Section 2.11.4)

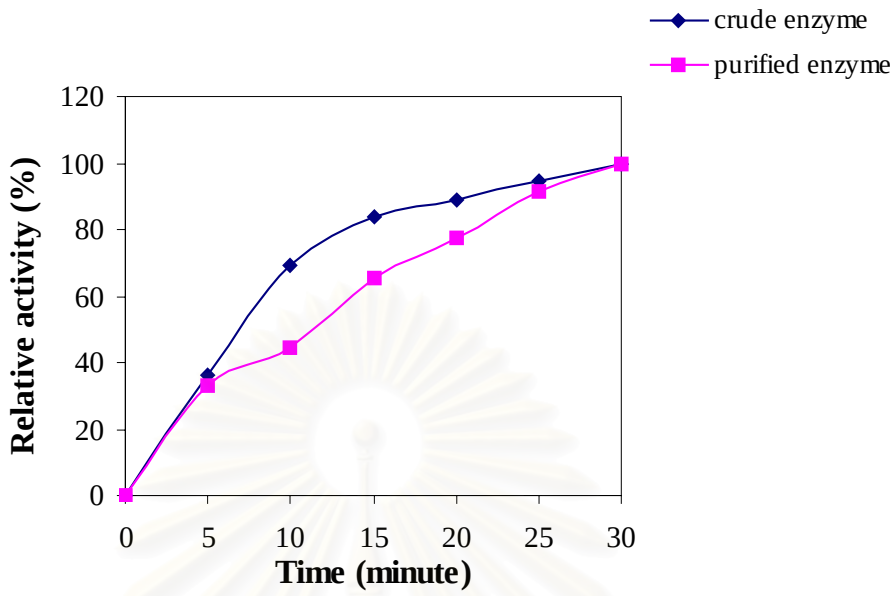

Figure 25-a Effects of incubation time on the dextrinizing activity of CGTase. The enzyme solution was incubated at 65°C in 0.2M Tris-HCl buffer (pH 8.0) with 10mM $CaCl_2$ for various time. (Method in Section 2.11.4)

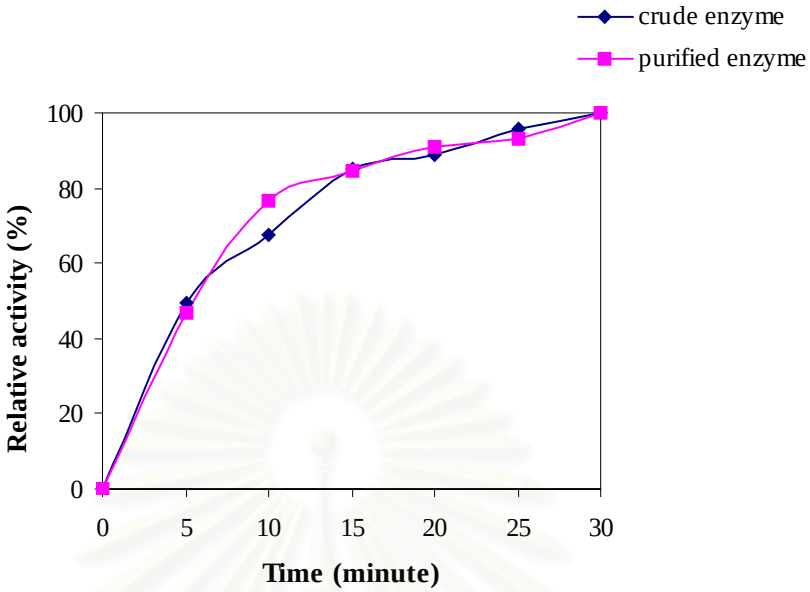

Figure 25-b Effects of incubation time on the CGTase activity (phenolphthalein method) The enzyme solution was incubated at 75°C in 0.2M Tris-HCl buffer (pH 8.0) with 15mM $CaCl_2$ for various time. (Method in Section 2.11.4)

3.5.8 Analysis of cyclodextrin products by HPLC

The aim of this experiment was to determine the types of CD products formed by the enzyme. Since the reaction products always contained linear molecules of different sizes, the reaction mixture was priorly treated with 20 units of β-amylase at 25°C for 3 hours as recommended by Laloknam (1997) to completely hydrolyze the linear chain. The CD standard and linear oligosaccharides were analyzed in Spherisorb 10 NH_2 by the method detailed in Section 2.11.3. They were firstly eluted by a mixture of 65% acetonitrile and 35% water (v/v) at the flow rate of 2 ml/min as the method described by Laloknam (1997) and are routinely used in our laboratory. The results showed that resolution of the peaks were not quite good (data not shown). The eluant was, therefore, modified to use a mixture of 75% acetonitrile and 25% water (v/v). It was found that separation of peaks was better under this condition.

The retention times of individual and mixture of standards α-, β-, and γ-CD were shown in Figure 26. Similarly, those of oligosaccharides G_1 to G_7 were shown in Figure 27. Table 9 summarizes both results. The CD samples at 24 hours showed a major CD peak with retention time corresponded to that of standard β-CD and the α : β : γ-CD ratio was 0.74 : 1 : 0.27. The kinetic of CD production from this result could be represented in Figure 29.

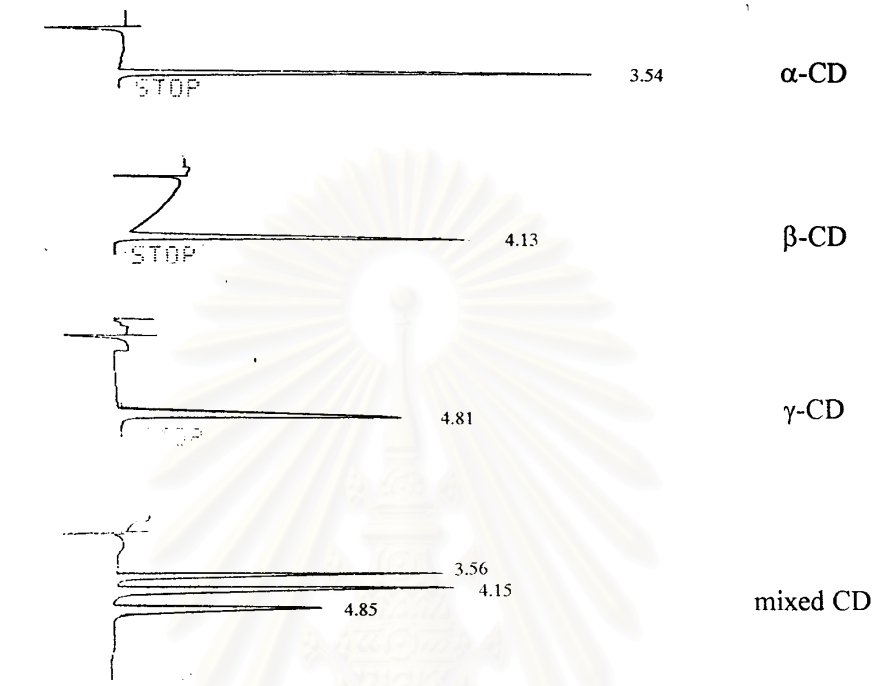

Figure 26 HPLC chromatograms of standard cyclodextrins. Spherisorb 10 NH_2 column was used. Acetonitrile : water (75:25) (v/v) was used as eluent at 2 ml/min flow rate.

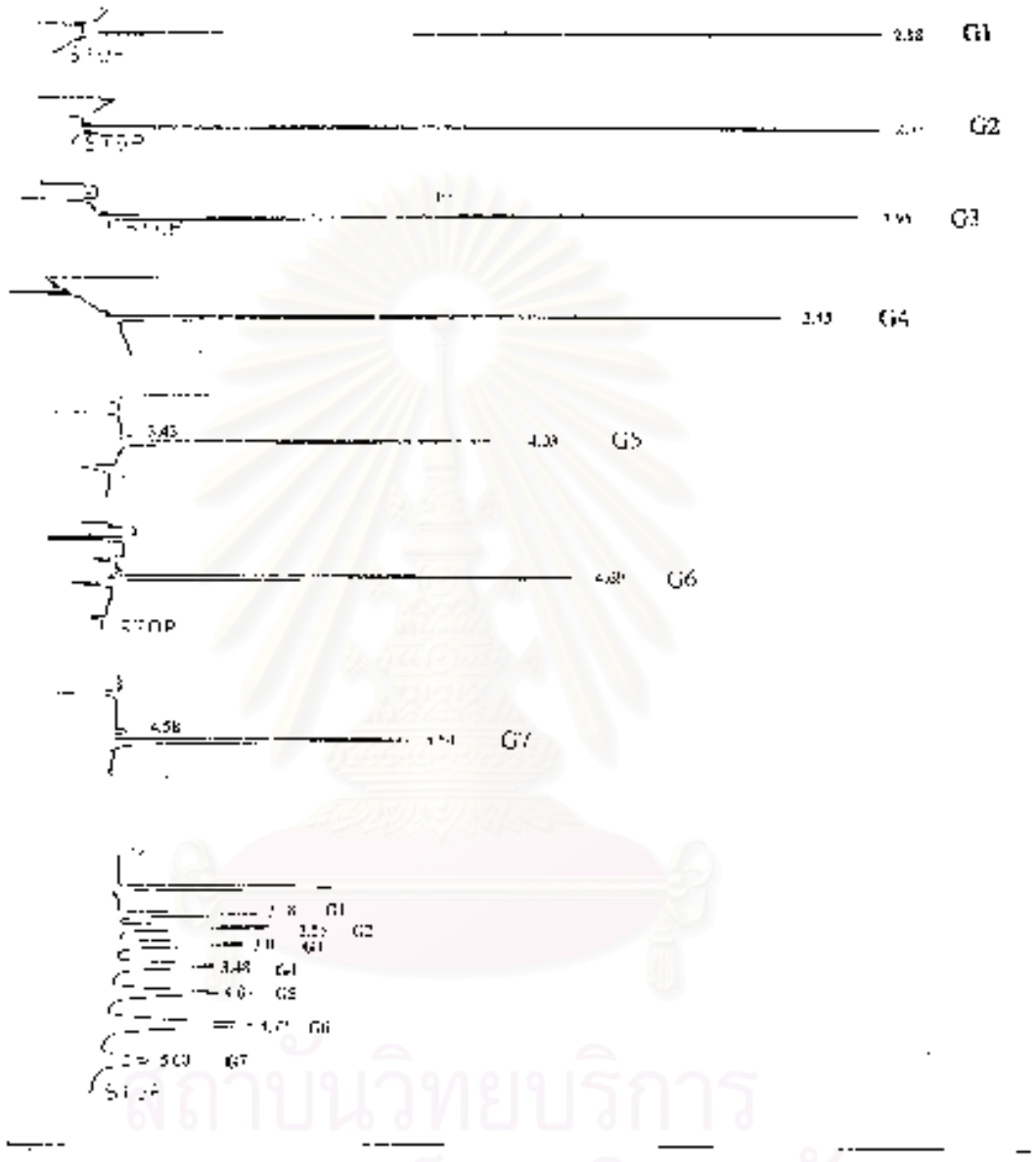

Figure 27 HPLC chromatograms of standard linear oligosaccharides. Spherisorb 10 NH_2 column was used. Acetonitrile : water (75:25) (v/v) was used as eluent at 2 ml/min flow rate.

Table 9 Retention time of individual standard CDs and linear oligosaccharides on HPLC Spherisorb 10 NH_2 column

Standard	Retention time (min)
Glucose (G1)	2.18
Maltose (G2)	2.53
Maltotriose (G3)	2.95
Maltotetraose (G4)	3.45
Maltopentaose (G5)	4.03
Maltohexaose (G6)	4.69
Maltoheptaose (G7)	5.54
α-CD	3.54
β-CD	4.13
γ-CD	4.81

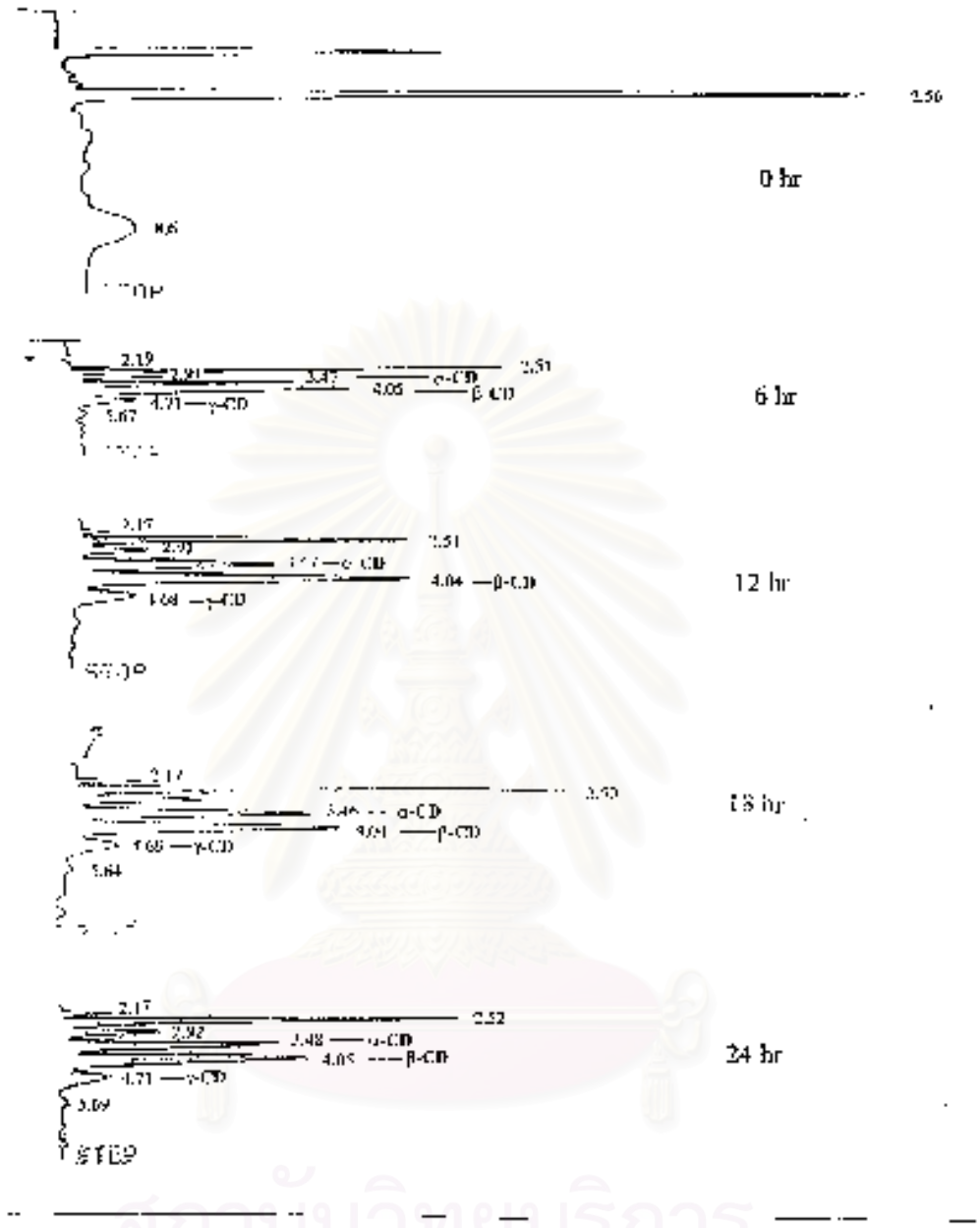

Figure 28 HPLC chromatograms of reaction products which were taken at every 6 hour of the incubation period. Spherisorb 10 NH_2 column was used. Acetonitrile : water (75:25) (v/v) was used as eluent at 2 ml/min flow rate.

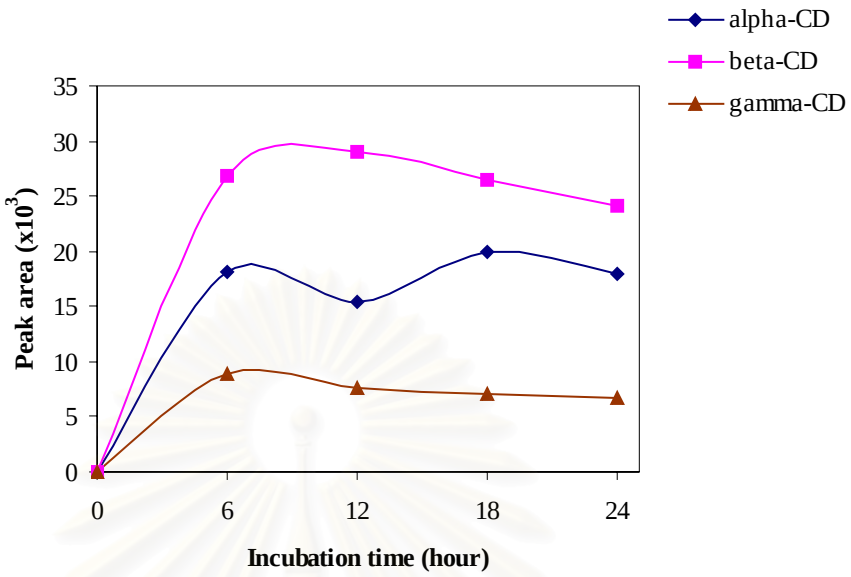

Figure 28 Kinetic of CD formation.

The enzyme solution was incubated with 2.0% soluble starch in 0.2M phosphate buffer, pH 6.0 for 24 hours. The reaction products which were taken at every 6 hours of incubation period were analyzed by HPLC. (Method in Section 2.11.3)

3.5.9 Stability to metal ions and chemicals

The dextrinizing activity was measured after incubating the enzyme in the presence of various metal ions and some chemicals at 0°C for 15 minutes. It was found that $HgCl_2$, EDTA, PMSF, KCl, and $CuSO_4$ reduced the enzyme activity. Other metal ions and reagents tested did not affect the enzyme activity significantly as shown in Table 10.

3.5.10 Thermal stability of the enzyme

The result in Figure 30 demonstrated that the dextrinizing activity of the enzyme was less stable than the cyclization activity. Dramatic lost of dextrinization occurred if the enzyme was incubated above 40°C for 15 minutes. CD-forming activity could withstand heat treatment for 15 minutes up to 60°C with little appreciable lost of activity. The enzyme was not stable above 70°C. Addition of $CaCl_2$ significantly stabilized the enzyme raising the stability to 70°C. $CaCl_2$ had more stabilizing effect on the dextrinizing activity.

3.5.11 pH stability of the enzyme

Both the dextrinizing activity and CD-forming activity of the enzyme were determined for pH stability. It was shown that the enzyme was relatively stable at 4.0 to 11.0 for both activities (Figure 31). The cyclization activity was more stable at pH 8.0-10.0.

Table 10 Effects of metal ions and chemicals on the enzyme stability

Metal ions and reagents (2mM)	Relative activity (%)
None	100
α-CD	113.3
γ-CD	109.4
NaCl	108.4
β-CD	105.5
$CaCl_2$	103
$MgSO_4$	101.6
$MgCl_2$	100
$NaNO_3$	98.5
$ZnSO_4$	96.4
$CuSO_4$	93.8
KCl	92.7
EDTA	87.4
$HgCl_2$	82.1
PMSF	81.8

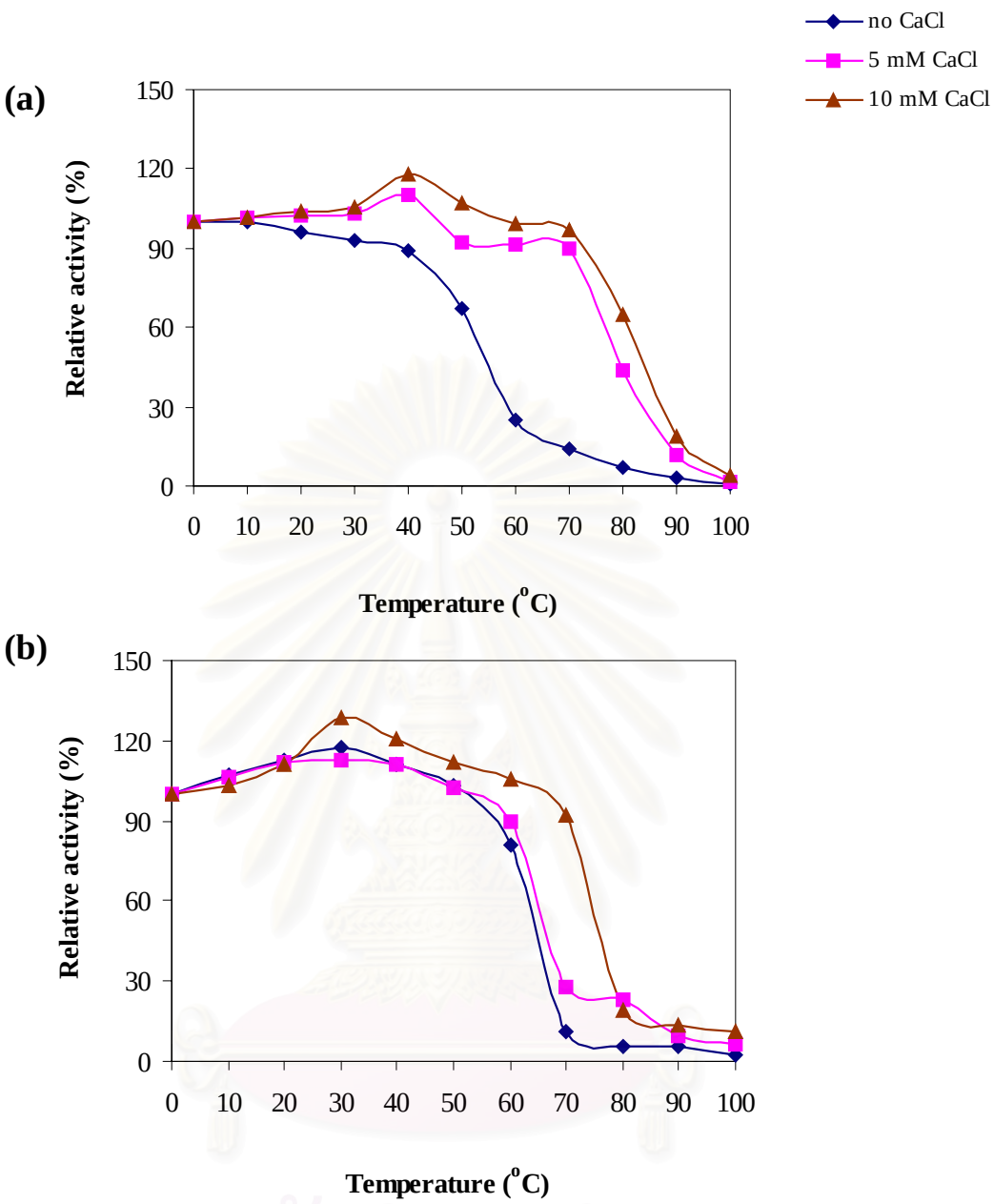

Figure 30 Thermostability of CGTase at various temperatures in 20mM phosphate buffer pH 7.0 with 0, 5, and 10 mM $CaCl_2$. The enzyme solution was kept at pH 7.0 for 15 minutes at different temperatures. After cooling, the remaining activity was assayed at the enzyme optimum condition. (Method in Section 2.11.5).

a. Dextrinizing activity

b. CD-forming activity

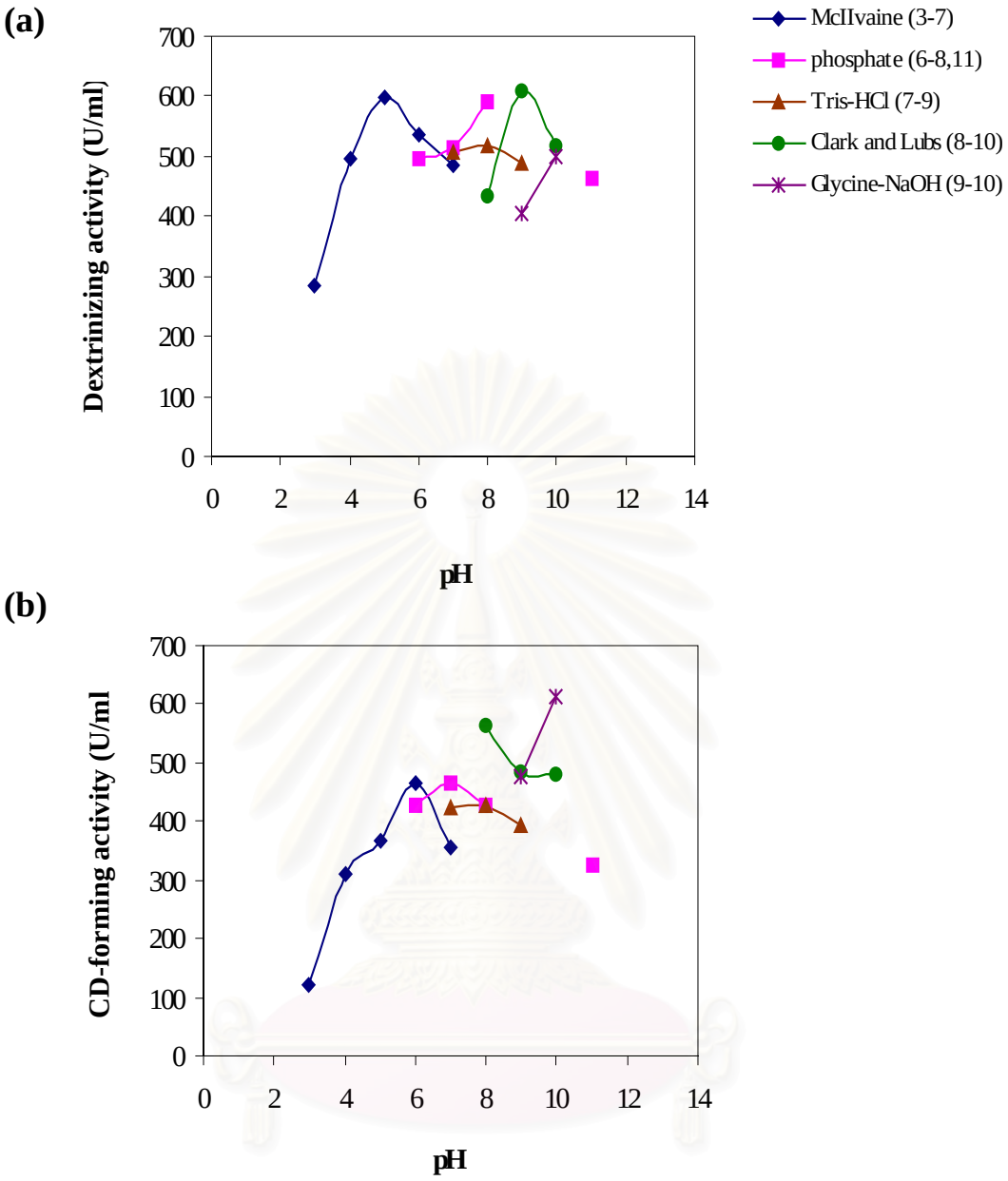

Figure 31 pH stability of CGTase at various pH

The enzyme solution was kept at 4°C for 24 hours in different pH. After that, the remaining activity was assayed at the enzyme optimum condition. (Method in Section 2.11.6)

a. Dextrinizing activity

b. CD-forming activity

3.5.12 Effects of storage condition on enzyme

In this experiment, prolonged enzyme storage at –20 and 4°C were compared. The crude enzyme was determined for its dextrinizing activity as described in Section 2.7.1 and the amount of protein were also measured by Bradford reagent. Gradual lost of activity was observed upon storage at both –20 and 4°C. Steeper lost occurred at higher temperature (4°C). Half of the activity was lost after 6 weeks at -20°C and 4 weeks at 4°C. The amount of protein shared the same trend of loss but to a lesser extent.

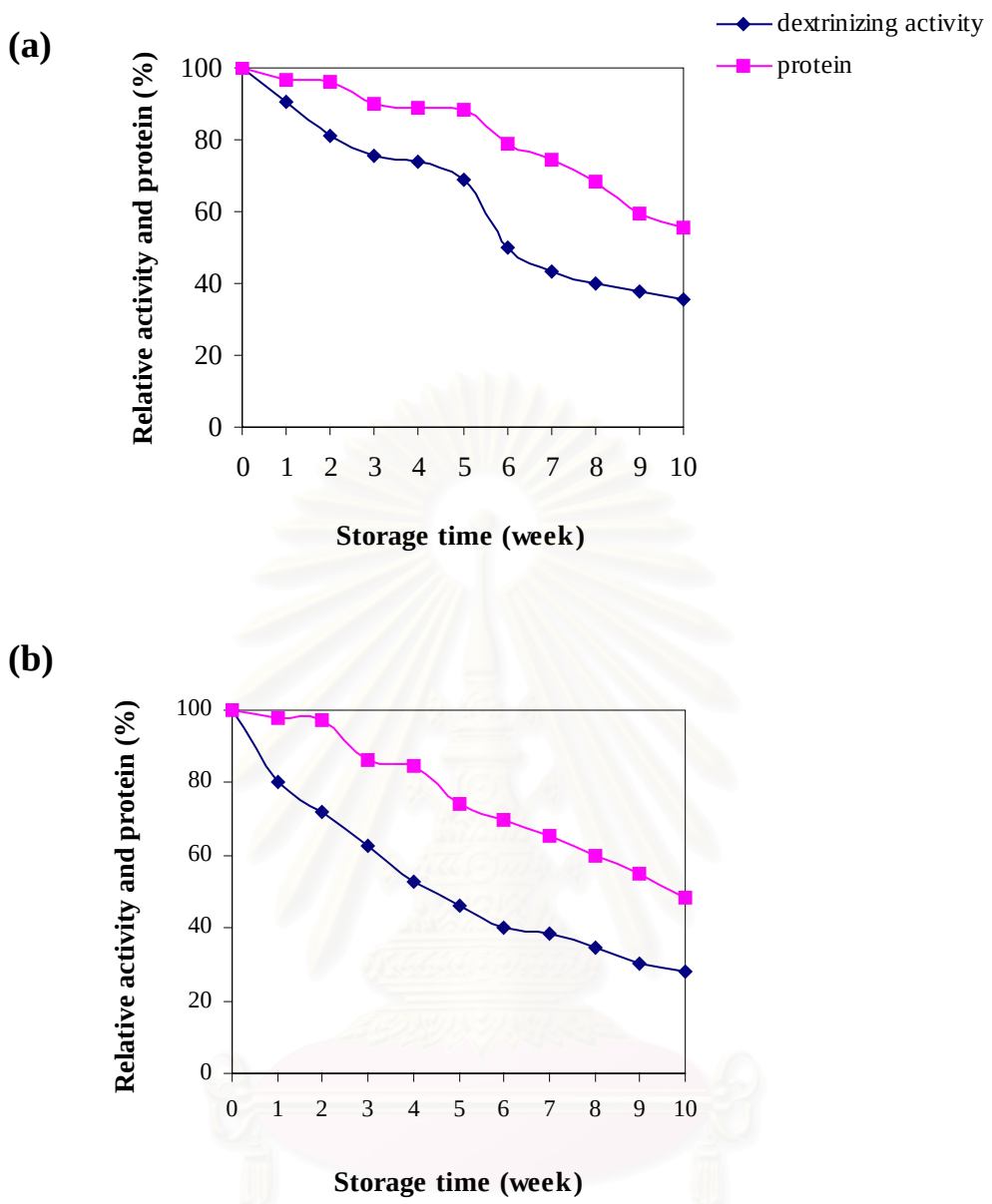

Figure 32 Effect of storage on the crude enzyme at –20°C and 4°C .

The crude enzyme was kept at –20 (a) and 4°C (b) in phosphate buffer (pH 7.0) at different time. The dextrinizing activity and protein content were then measured every week.

CHAPTER IV

DISCUSSION

The industrial utilization of enzymes particularly aimed at producing fine chemicals. It has several advantages over non-enzymatic processes. Biocatalysts are energy efficient and show high specificity and stereo selectivity. Biocatalyst has become more and more important in biotechnology research. Successful use of biocatalyst requires basic research on its structure, properties, mechanism of reaction, and working condition. The discovery of a variety of new and more active enzymes have led to expanded growth in the industrial enzyme market and progress in the field of biotechnology. Many of the newly developed enzymes have enhanced thermostability as compared to previously available enzymes. Interest in thermostable enzymes have grown, mainly due to the fact that most of the existing industrial enzyme processes are run at high temperatures using enzyme from mesophile sources. There are quite a few advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes. The main advantage is that as the temperature of the process is increased, the rate of reaction increases. The thermostable enzymes are also able to tolerate higher temperatures, which gives a longer half-life to the enzyme. The use of higher temperatures (above 60°C) also is inhibitory to microbial growth, decreasing the possibility of microbial contamination.

The use of high temperatures in industrial enzyme processes may also be useful in mixing, causing a decrease in the viscosity of liquids and may allow for higher concentrations of low solubility materials. The mass transfer rate is also increased at higher temperature as is the rate of many chemical reactions. The main application for thermostable enzyme has predominantly been starch liquefaction using

amylases from *B. licheniformis* and *B. stearothermophilus*, protease for food processing and detergents, production of cyclodextrins using cyclodextrin glycosyltransferase (CGTase) and biobleaching of wood pulps using xylanses (Zamost, 1991).

Most of the definition of thermostability relate to the inherent nature of the enzyme and take into consideration the source of the enzyme. It was found that thermophiles do not require or necessarily posses enzymes with higher activities when growing optimally at higher temperature. On the other hand, thermostable enzymes from mesophiles do exhibit increased reaction rate when utilized at high temperatures, although they simultaneously experience a higher denaturation rate. Since most industrial enzyme reactions are operated between 50-100°C, enzymes from thermophiles will have less of an increase in activity as compared to that from mesophiles between this temperature range. Thermostable enzyme from extreme thermophiles may have little value as far as increase in activity is concerned for moderately thermophile processes (Brock, 1986). In addition, many thermophiles are anaerobic pathogens and are not suitable for use in food or pharmaceutical industries.

For effective use of biocatalyst, the enzyme has to be produced in large quantity, be easily recoverable (preferably extracellular), and be produced by a microorganism with low cultivation cost. In starch industry, liquefaction of starch is a standard process. The primary liquefaction involves short-term jet cooking treatment at 105°C for 5 minutes. This is followed by dextrinization the starch slurry with α-amylase, usually at 95°C for 90 minutes. Thermostable α-amylase is of important used in this aspect. The product of α-amylase, maltose, could inhibit CD-production so the action of α-amylase must be limited or replaced. Using two enzyme systems in the bioreactor is time consuming and costly. The discoveries of many thermostable

CGTases (Wind, 1995; Chung, 1998; Tachibana, 1999) provide more efficient production of CD since they can be used in starch liquefaction as well as CD formation. Most of the thermostable CGTase are derived from anaerobic archeon, such as *Thermoanaerobacter* (Starnes, *et al*., 1991; Wind, *et al*., 1995 and Brunet, *et al*., 1998) or *Thermococcus* (Tachibana, *et al*., 1999). These organisms require high temperature and anaerobic condition for growth. In addition, precautions on toxin released by these organisms may hinder their use as CGTase producer. The objective of this study is to isolate bacteria which could grow at lower temperature and produce extracellular thermostable CGTase as a result, the screening in this work, therefore, done at 37°C.

It was expected that the hot spring environments might induce the bacteria to express thermostable enzyme. Therefore, to obtain the bacteria which produced thermostable CGTase, the soil samples were collected at hot spring environments in northern Thailand, such as Tak, Chiang Mai, and Chiang Rai. Many CGTase-producing microorganisms have been isolated on agar medium containing starch on which clear zone was formed upon staining with iodine reagent. In this screening method, however, CGTase activity was indistinguishable from amylase activity, so additional methods specific for CGTase activity such as HPLC or trichloroethylene method were necessary. In 1986 Taguchi reported that phenolphthalein was transformed into a colorless dianion within the cavity of β-CD. Basing on discovery, Park (1989) later developed a simple and rapid screening method by which CGTase can be specifically detected on the basis of the reduction of the color intensity of phenolphthalein under alkaline condition by β-CD product of CGTase. However, a reduction in the color intensity was not observed for α-CD since the complex of α-CD and phenolphthalein was not easily formed. Bromocresol green are generally

employed for detection of γ-CD (Larsen, 1998). In the present study, we used the modification method of Nomoto (1986) and Park (1989) to screen for the bacteria of interest. On primary screening 158 isolates showed amylase activity. Of these only one isolate from Mae ka sa hot spring in Tak province contained CGTase activity when further screened by the phenolphthalein-methyl orange method.

There are two principles in this study for identifying the bacteria namely phenotypic and phylogenetic characters. The first one rely on the phenotypic and biochemical characteristics, i.e. Gram staining, the utilization of various substrates as sole carbon sources for respiration. The work was done by Thailand Institute of Science and Technology Research and suggested that the isolate most closely resembled those of genus *Paenibacillus marcerans* or *Bacillus macerans*. The second principle was 16S rRNA gene analysis. The use of 16S rRNA gene sequences as molecular genetic criteria to characterize microorganisms has gained wide acceptance and is particular useful for phylogenetic analysis (Ash, *et al*., 1993 and Yoon, *et al*., 1998) and for characterization of an organism of uncertain affiliation. Furthermore, the variable domains at the 5' terminus of the 16S rRNA molecule is hypervariable and show considerable sequence diversity between closely related species (Woese, 1987) and can therefore be used as target sites for construction of taxon-specific probes for rapid identification of microorganisms. The technique rapidly provides complete, unambiguous and contiguous sequence determination of 16S rRNA gene (Edwards, 1989). For this study, 1,203 nucleotides of the 16S rRNA gene of the isolated strain was sequenced and aligned with reference strains representing the genus *Paenibacillus* and *Bacillus*. The result indicated that the strain was similar to the genus *Paenibacillus* sp.38-2 with 99% *Paenibacillus macerans* with 92%, and *Bacillus macerans* with 90% similarity. On the basis of both phenotypic and genotypic considerations strongly suggest that the CGTase producing isolate belongs and was named *Paenibacillus* sp.

T16 to the genus *Paenibacillus* rather than *Bacillus* (Figure 33). It is likely that it may be a *Paenibacillus macerans* is required, however, before such conclusion can be drawn. This bacteria was used for CGTase studies.

Paenibacillus is a *bacillus*, because it derives from this genus but is phylogenetically distinct. Member of the genus *Paenibacillus* are facultatively anaerobic organisms that produce spores in definitely swollen sporangia and have G+C contents ranging from 45 to 54 mol%. Some of these organisms excrete diverse assortments of extracellular polysaccharide-hydrolyzing enzymes. Interestingly, many recently described *Bacillus* species posses the general characteristics of the genus *Paenibacillus* (Shida, *et al*., 1997).

Paenibacillus sp. T16 is a Gram positive, alkalophilic bacteria that could grow well in wide pH range (6.0-10.0) (Figure 14). It is also a mesophile which could grow at 37-50°C, with 37°C being the optimum temperature (Figure 13). It could be explained in term of bacterial selection method that the screening method was done at 37°C from soil samples about 2 meters from hot spring area. The temperature of the soil could be around 37°C or little higher, but not as extreme as the hot spring water (70°C). $CaCl_2$ did not have any effect on growth.

CGTase production began as the cell entered log phase and the rate was greatest at mid-stationary phase (Figure 13). The result correlated well with an alkalophilic *Bacillus* spp. from deep sea mud sample (Georganta, *et al*., 1993). For CGTase preparation, the enzyme was usually collected at late stationary phase. Many

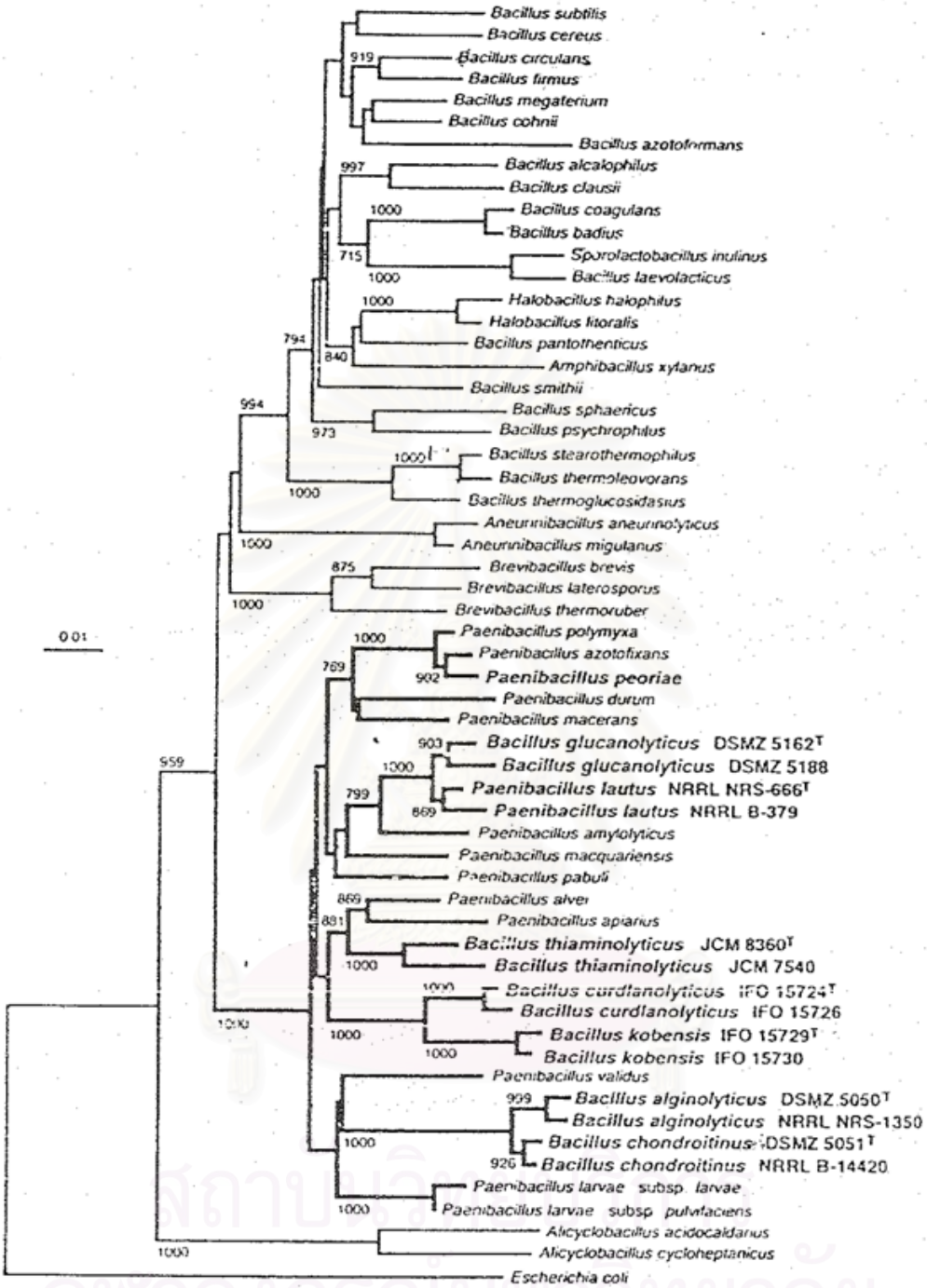

Figure 33 Phylogenetic relationship of *Paenibacillus* species and some aerobic bacteria based on 16S rRNA gene sequence. (Shida, *et al*., 1997)

spore-forming bacteria produced secondary metabolites or enzyme at this stage to gain advantage over other surrounding organisms. These were suggestions that CD-producing bacteria used CD as their carbohydrate reserve. Enzyme induction was best at pH 10, whereas near neutral pH (6.0-7.0) was not suitable for enzyme production. However, it was different from *B. amylolyticus* 11149 (Tomita, 1993) and *Thermococcus* sp. (Tachibana, 1999) which had optimum pH for growth and the enzyme production at 6.0 and 7.0, respectively.

In contrast to growth, 0.05 mM $CaCl_2$ stimulated CGTase activity significantly as compared to culture. It is position that Ca^{2+} may act by stabilizing the enzyme (Figure 24). At higher $CaCl_2$ concentration, turbidity of the culture medium was observed. This correlated with the finding that high $CaCl_2$ did not stimulate CGTase production. The turbidity may be arisen from precipitation between $CaCl_2$ and some constituents in the medium important for CGTase enhancement.

Purification of CGTase has been reported from *B. macerans*, *B. circulans*, and Alkalophilic *Bacillus* sp. (Kobayashi, 1978; Bender, *et al*., 1982 and Kometani, *et al*., 1994). One common step in the purification procedures reported by the different laboratories was the use of starch adsorption and the adsorbed enzyme was eluted with buffer containing maltose. The interaction between starch and the CGTase involves not only adsorption but also substrate-enzyme affinity type binding. Ammonium sulfate precipitation, chromatography on DEAE-cellulose (Laloknam, 1997), immunoaffinity column(Wongsangwattana, 2000), β-CD affinity column (Chung, 1998), and α-CD affinity column (Tachibana, 1999) were usually performed to further purity the enzyme. Affinity chromatography is a biospecific technique. This procedure takes advantage of one or more biological properties of the molecule being purified. These interactions may be due to the general properties of the molecule such as isoelectric point (pI), hydrophobicity, or size. The affinity chromatography permits

simple, effective, and rapid purification (Han and Tao, 1997). In this work, the purification was accomplished by β-CD affinity column chromatography, following the method reported by Larsen (1998).

The enzyme was partially purified by corn starch adsorption to 32.3 folds with 61.6% yield in the first step. Concentration by ultrafiltration with 30 kDa molecular weight cut off resulted in higher purification without yield loss, 77.98 folds with 63.6% yield. The final step of purification was done by β-CD affinity column chromatography. The adsorbed CGTase was detached with 1% β-CD in 20 mM phosphate buffer. The enzyme was purified to 99.99 folds with 21.2% yield, which corresponded to the increase in CD-product as determined by CD-TCE dilution limit (Table 8). Despite using β-CD affinity column chromatography, which has high specificity to CGTase, high amount of CGTase lost occurred in this step. It might be caused by low capacity of the column and degradation of the enzyme. It is noteworthy that due to the rather low column capacity (1 mg protein/12 ml gel bed volume), the enzyme preparation from previous step had to be divided into several portions for loading, and the purified enzyme were pooled for further studied. Since the capacity of the reused column were not calibrated, it was possible that some enzyme was lost in the process. In addition, numerous reports indicate the presence of extracellular protease in Gram positive bacteria. Steighardt and Kleine (1993) reported a mixture of protease in the bacterial culture of *B. marcerans* which produced CGTase. The result in SDS-PAGE also was suggestive of CGTase degradation during the purification process.

Another reason of using unsuitable eluant could be taken into account. Larsen (1998) and Volkova (2000) reported the use 20 mM of α-CD and 5 μmol of β-CD as eluant, respectively. Urea or ethanol could also used as eluant (Makela, 1988). In this study, 1% β-CD, which was successful in the report of Chung (1998), was employed.

Purity of the enzyme was demonstrated by native PAGE. Two major and two minor bands were observed through the amylolytic activity stain of the purified preparation. The result was similar to CGTase from *B. circulans* A11 (Kaskangam, 1998). Of these only one band which was corresponded to the major protein band showed CD-forming activity (Figure 17). The other amyloactive bands could be other starch hydrolyzing enzyme or may be CGTase isozyme which had very low activity and cannot be detected under the detection limit of phenolphthalein. In order to verify this assumption, an experiment was done by increasing the amount of CGTase in the hope to increase the activity of other bands. It caused overloading of the gel and smearing in the phenolphthalein staining (data not shown). Migration of different proteins in native PAGE could be reflected by their pI values. The purified protein from β-CD affinity column gave 3 bands with pI of 5.70, 5.20, and 4.77, respectively (Figure 21). The result further confirmed that the many amylolytic bands in Figure 16b are distinct proteins. Whether they are CGTase isozymes need further confirmation.

To investigate how many isozymes of the enzyme has, a discontinuous preparative polyacrylamide gel electrophoresis method could be used to separate each isozymes. The hypothesis can be confirmed by gel filtration column chromatography or by other undenaturating principles.

The pI's of CGTase from other species have been reported. The isoelectric points of CGTase fractions from an alkalophilic *Bacillus* strain ATCC 21783 were showed in the range between 4.55-4.90 as measured by ampholine electrofocusing and

by chromatofocusing (Makela, *et al*., 1988). Bovetto and his group (1992) studied on *Bacillus circulans* E192 and reported on two isozymes separated by FPLC on a mono Q column, the products of each isozyme from various substrates analyzed by HPLC were mainly β-CD and their isoelectric points were estimated as 6.7 and 6.9. Wind and his group (1995) showed that the isoelectric points of CGTase fraction from *Thermoanaerobacterium thermosulfurigenes* EM1 were in the range between 4.3-4.6 measured by the Pharmacia-LKB Phastsystem. It was reported that CGTase of *Bacillus circulans* A11 (Kaskangam, 1998) focused on the pI range between 4.23-4.73 with 5 bands positive for I_2 staining. The purified protein from affinity column gave pI of 5.70, 5.20, and 4.77. Comparing with the result from Figure 17 a and c, CGTase from *Paenibacillus* sp. T16 had pI of 5.20 (major protein band). It would be interesting to subject the IEF gel to activity staining by I_2 or phenolphthalein reagent to characterize the remaining whether all the bands appear on IEF gel are also CGTase isoforms.

In this study, a technique for detection of amylolytic activity in SDS-PAGE was developed. The molecular weight of purified CGTase was determined by SDS-PAGE. It showed that the product was not homogeneity and one intense protein band with an estimated molecular weight of 76,000 daltons. It falls in the range 60,000-140,000. It was near to CGTase from *B.circulans* A11 (72,000 daltons) and *B. macerans*, (74,000 daltons) (Kobayashi, 1978). The faint bands in lane 4 of Figure 18a might be degraded protein product. If the four amylolytic bands in native PAGE were indeed CGTase isozymes, these enzymes should have the same molecular weight. It could be expected that the enzyme has probably four isozymes with same molecular weight of each.

The CGTase from *B. circulans* E192 showed 2 isozymes with different subunits of 33,500 and 48,500 daltons, respectively (Bovetto, *et al*., 1992). On the other hand, there were reports that CGTase from *Bacillus macerans* IAM 1243 and *Bacillus megaterium* NO.5 were dimeric protein of identical subunit size of 66,000 and 145,000 daltons, respectively (Kitahata and Okada, 1974; Kobayashi, *et al*., 1978).

To determine whether the CGTase was a glycoproteins, qualitative analysis by carbohydrate specific periodic acid-Schiff (PAS) staining method for polyacrylamide gel offers a good choice. The periodic acid oxidizes the closed aldehyde groups in the polysaccharide conjugate, permitting the fuchsinsulfite to stain the polysaccharide constituents and giving the glycoprotein the magenta color. The result in this study showed that the protein is glycoprotein with comparison to BSA (positive control) and haemoglobin (negative control). The result is similar to the report of Kaskangam (1998). However, it was generally known that bacteria did not have post-translational modification such as glycosylation. Many of a glycoenzyme in bacteria are carbohydrate metabolizing enzyme, so the possibility of artefacts arising from catbohydrate contaminants should not be overlooked.

The cyclodextrin products of the enzyme were analyzed by HPLC as described in Section 2.11.3. The results displayed that *Paenibacillus* sp.T16 produced mainly β-CD. The ratio of the reaction products (α : β : γ-CD) at 24 hour incubation period of CGTase with starch were 0.74 : 1 : 0.27. This result was slightly different from CGTase from other *B. macerans* which produced mainly α-CD (Kitahata, *et al*., 1974; Kobayashi, *et al*., 1977; Depinto and Campell, 1986). Although β-CD was the major product, the enzyme surprisingly produce quite high α-CD when comparison with β-CGTase producer including *B. circulans* A11. However, inducing high α-CD production could be obtained through improving the incubating conditions of the CD-

TCE reaction such as type and amount of substrate, temperature, pH, and incubation time.

There were reports that CGTase initially synthesized α-CD which was later overcome by β-CD production (Fujiwara, 1992). To test this observation purified CGTase was incubated with starch and samples were taken every 6 hours and quantitated for CD products. It was observed that, in the condition studied, β-CD was the major product at all time and at the first six hours. The result is support by Figure 29.

Abelyan and his group (1994) reported the yields of CD-products obtained from several fractions of CGTase isolated from *Bacillus* strain INMIA-T42, INMIA-T6 and INMIA-A7/1. These fractions showed different product ratios which were also different from this report. Factors such as the kind of CGTase, the concentration of starch used and the technique employed for determining the percent formation of cyclodextrin may have contributed to the different values reported (Nakamura and Horikoshi, 1976).

As with *Bacillus* sp. A11 (Techaikul, 1991) there were some discrepancies in the biochemical characteristics of the amylolytic and cyclization activities of this CGTase, from both crude and purified preparation. The optimum temperature and pH for dextrinizing activity was 65°C and 7.0-8.0 respectively comparing to 75°C and pH 8.0 for CD-forming activity. There might be some inhibitors of the cyclization active site in the crude preparation since the purified enzyme had much greater activity at 80°C than the crude enzyme in which the activity was only marginal. The observation also demonstrated that the chemical species of buffer had greater effect on the latter activity. Such discrepancies are useful if one wishes to employ CGTase for starch liquefaction and CD production processes.

To explain the different behaviors, the reaction mechanism of CGTase has to be taken into account. CGTase is a subclass of α-amylase Family 13. CGTase will cleave the α-1,4-glucosidic bond of amylose in the same way as α-amylase does. Most of enzyme, except pepsin and alkaline phosphatase, can catalyze the reaction in the pH range of 4.0-10.0. At lower pH 4.0 and higher pH 10.0 the enzymes are irreversibly inhibited because their tertiary structure were demolished (Veerakalus, 1998)

Both enzymes have Asp and Glu as the reactive residues at the active site. CGTase contains additional by near aromatic residues such as Tyr and Phe. Hega and Vamane, (2000) proposed the catalytic mechanism of CGTase (*Bacillus* sp. #1011), Glu257 plays the acid/base catalyst, Asp229 the catalytic nucleophile and Asp328 stabilizes substrate binding and elevates the pKa of Glu257. His140 and His327 are in close contact with Glu257, Asp229 and Asp328, and they undergo distortion from the chair conformation of the sugar residue in subsite-1 (transition state) and the covalent bond between Asp229 and C1 of the sugar residue in subsite-1 in an intermediately complex (Figure 34). Moreover, the charged residues described above undergo catalysis with the help of Tyr100 (stacking interaction) and Arg227 (hydrogen bonds). Only histidine has a good buffering capacity at the pH range of 6.0-8.0 (Veerakalus,1998).

The transglycosylation reaction of CGTase is operated by a “ping-pong” mechanism (Nakamura, 1994). In this mechanism, the transglycosylation occurs after the reducing end of the cleaved amylose is released from the enzyme. Then the enzyme transform the newly formed reducing end of the substrate either to the nonreducing end of a separate linear acceptor molecule or glucose (the disproportionation reaction) or to its own nonreducing end (the cyclization reaction or CD synthesis reaction). The hydrolysis reaction (the starch degrading reaction) will

Figure 34 Stereoview of the structure of alkalophilic CGTase from *Bacillus* sp. 1011 complexed with 1-deoxynojirimycin. CGTase consists of five domains : A (1-406, blue), B (139-203, yellow), C (407-496, green), D (497-584, purple), E (585-686, light blue). Calcium ions are shown by magenta circles. Two 1-deoxynojirimycins (red) are located at the catalytic active site in domain A and the maltose binding site 1 in domain E. The structure was drawn using the program MOLSCRIPT. (Kraulis, 1991)

occur when this intermediate is attacked nucleophilically by a water molecule. For preferential CD synthesis, the efficient formation of the helical structure of amylose in the active site cleft of enzyme is required (Fujiwara, 1992 and Nakamura, 1994). In a crystal structure, amylose can occur as a single helix with six to eight glucose molecules in one helical turn (Kubik, 1996). The most widely accepted hypothesis describes amylose in solution having an interrupted coil-like structure composed of helical and nonhelical segments (Szejtli, 1991). Therefore, the formation of CD by CGTase can be explained as a consequence of a preferential helical structure of the amylose in solution. At different temperature the structure of amylose may alter and may result in a shift toward the CD-forming reaction.

Ca^{2+} stabilized a variety of enzymes including amylase and CGTase. Ca^{2+} also increased the heat stability and activity of the enzyme. In the presence of 10 mM $CaCl_2$, the crude enzyme from *Paenibacillus* sp. T16 could withstand temperature up to 70°C with only 10% loss of activity (Figure 30). Without $CaCl_2$, the enzyme was stable at ≤ 40°C for dextrinizing activity and up to 60°C for CD-forming activity. Interestingly, Ca^{2+} had more dramatic effect on dextrinizing activity of the enzyme. It was shown to stimulate α-amylase activity and stabilized the enzyme structure. The results were also supported by Wind, 1995; Chung, 1998; Larsen, 1998. Akimaru, *et al*., (1991) who observed that saturation of CGTase with Ca^{2+} resulted in an increased of heat stability and the optimum temperature of the enzyme shifted from 60 to 70°C. Mori (1994) suggested that the enzyme activity was enhanced by $CaCl_2$. However, Chung, *et al*. (1998) mentioned that $CaCl_2$ did not increase their enzyme activity but had stabilizing effect. For this work, both dextrinizing and CD-forming reaction were assayed in the presence of various concentration of $CaCl_2$. The results displayed that activities of crude CGTase were not enhanced by $CaCl_2$ (Figure 24 a and b). However,

$CaCl_2$ could enhance the purified CGTase activities. The stimulate level of $CaCl_2$ for dextrinizing activity was slightly higher than the CD-forming activity. It was invariably demonstrated in crystalized CGTase from various origins that Ca^{2+} is an inherent component of CGTase (Figure 34).

In this work, the effects of metal ions and several chemical reagents were studied. The results showed that the enzyme activity was slightly decreased by $HgCl_2$, EDTA, and PMSF about 20%. For other reagents, the enzyme activity was not or only slightly affected. It could be explained for Hg^{2+} ion that it is a heavy metal ion, which can precipitate and denature protein. For EDTA, it is metal ion chelator and could bind Ca^{2+} which is an important constituent of the CGTase. The enzyme activity was also decreased by PMSF. It can sulfonylate protein at the position of histidine and serine. The effects of metal ions and chemicals on CGTase varied. CGTase activity from *Bacillus autolyticus* 11149 was slightly inhibited by CDs and completely inhibited by Hg^{2+}. It was similar to CGTase activity from *Brevibacterium* sp. NO.9650 (Mori, 1994). Nomoto (1986) however, reported that PMSF, PCMB and EDTA did not inactivate the CGTase activity from alkalophilic Bacterium of Taiwan, but some metal ions such as Zn^{2+}, Co^{2+}, Fe^{2+} and Mg^{2+} showed strong inhibitory effects on the enzyme activity.

Many of the thermostable CGTase derived from *Thermoanaerobacterium* or *Thermococcus*. Chung, *et al*. isolated a *Bacillus stearothermophilus* ET1 (1998) which was also thermostable. These enzymes had high optimum temperature (80-100°C) and high thermostability, (80°C). *B.stearothermophilus* ET1 was stable at 60°C and increased to 80°C in the presence of $CaCl_2$ which is quite similar to *Paenibacillus* sp. T16. *Thermoanaerobacter* and *Thermococcus* are pathogen which may not be suitable for utilization but *Paenibacillus* was accepted by the Scientific Committee on Food,

European Commission Health & Consumers Protection Directorate-General as safe CD-producer. Therefore, *Paenibacillus* sp. T16 should be a good candidate for CD-producer.

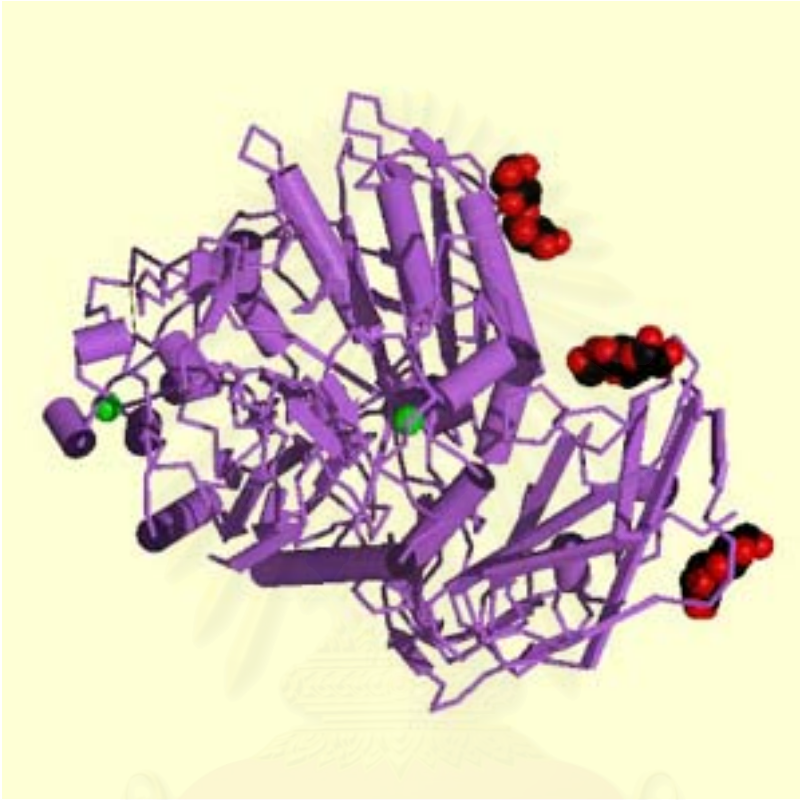

Figure 35 Secondary structure and ligands of CGTase from *Bacillus circulans* strain 251. (http://www.glycoforum.gr.jp/science/word/saccharide/SA-B03E.html)

Violet color = protein : 686 residues

Red color = maltose

Green color = calcium ions

CHAPTER V

CONCLUSION

1. A CGTase-producing bacteria from Mae Ka Sa hot spring, Tak province was identified by biochemical characteristics and 16S rRNA gene analysis. It was belonged to genus *Paenibacillus*, possibly *P. macerans*.
2. The optimum conditions for growth and CGTase production of the bacteria were in Horikoshi's medium at 37°C, pH 10.0 with 0.05 mM $CaCl_2$ for 84 hours. CGTase production was initiated at the beginning of log phase and has highest product at mid-stationary phase.
3. The optimum conditions for dextrinizing activity of CGTase from the bacteria were in 0.2 M Tris-HCl (pH 8.0) with 10 mM $CaCl_2$ at 65°C for 10 minutes. On the other hand, for optimum CD-forming activity (phenolphthalein method) occured in 0.2 M Tris-HCl (pH 8.0) and 15 mM $CaCl_2$ at 75°C for 15 minutes.
4. The CGTase was purified by β-CD affinity column. A yield of 21.2% was obtained with 99.99 folds purity. The dilution limit value of the CD-TCE reaction was $1:2^{11}$.
5. The enzyme was shown to be a glycoprotein. Its molecular weight on SDS-PAGE was 76,000 daltons, with pI value of 5.20.
6. The purified enzyme fraction contained 3 protein bands on IEF gel with pI 5.70, 5.20, and 4.77.
7. Four amylolytic forms were always present in all enzyme preparations with the major band exhibiting detectable CGTase activity.

8. The enzyme can produce mainly β-CD and quite high α-CD. The ration of α- : β- : γ-CD at 24 hours period was 0.74 : 1 : 0.27.
9. The CGTase was not stable in the presence of $HgCl_2$, EDTA, and PMSF.
10. Thermostability of CGTase were up to 70°C with 10 mM $CaCl_2$. It could be stable at pH 4.0-11.0.
11. The crude enzyme was better stored at –20°C than at 4°C. Appreciable lost was observed with storage time.

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APPENDICES

APPENDIX A : Sources of soil sample

Sources of soil sample	Physical character of sources			
	Temperature of water (°C)	pH of water	Distance from hot spring (m)	Depth from soil surface (cm)
Chiang Rai				
Huay sai kwao	50	7.0	1, 2	5
Mae sa ruay	70	7.6	0.5, 1, 2	5
Tung tay wee	60	7.4	1, 2	5
Chiang Mai				
Sum kum paeng	70	7.8	0.5, 1, 2, 5	5
Tak				
Mae ka sa	70	7.2	1, 2, 5	5

APPENDIX B : 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

Adjust 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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.20 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 1M HCl and adjusted volume to 100 ml with distilled water

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.10 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% sepatating 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	1.67	ml
0.5 M Tris-HCl pH 6.8	2.50	ml
distilled water	5.00	ml
10% $(NH_4)_2S_2O_8$	30	µl
TEMED	5	µl

Sample buffer

For analytical gel

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

distilled water	1.4	ml

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

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

3. 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 C : Preparation for Periodic acid-Schiff (PAS) staining solution

Fixative solution

Ethanol	200	ml
Glacial acetic acid	20	ml

Adjust volume to 500 ml with distilled water

Schiff's reagent

1. Dissolve 1 g of basic fuchsin in 200 ml of boiling distilled water. Stirred for 5 minutes and cool in ice-bath to 50°C (not below 40°C).
2. Then filtered and added 20 ml of 1N HCl. Mix, and cool to 25°C.
3. Add 1 g of sodium metabisulfite (fresh metabisulfite was required for optimum results). Mix and let sit overnight at 4°C in the dark.
4. Add 2 g of activated charcoal, shaked for 1 minute, filtered.
5. Stored in a brown bottle at 4°C.

0.1% Periodic acid solution

Periodic acid	1.0	g
3% Acetic acid	100	ml

0.5% Sodium metabisulfite

Sodium metabisulfite	0.5	g
3% Acetic acid	100	ml

APPENDIX D : Preparation for isoelectric focusing gel electrophoresis

Monomer-ampholyte solution

30% acrylamide solution	2	ml
Ampholyte pH 3-10	240	µl
Distilled water	9.7	ml
10% $(NH_4)_2S_2O_8$	50	µl
TEMED	5	µl

Fixative solution, 100 ml

Trichloroacetic acid	10	g

Immerse gels in this solution for 30 minutes.

Staining solution, 100 ml

Ethanol	27	ml
Acetic acid	10	ml
Coomassie brilliant blue R-250	0.04	g
$CuSO_4$	0.5	g
Distilled water	63	ml

Dissolve the $CuSO_4$ in water before adding the alcohol. Either dissolve the dye in alcohol or add it to the solution at the end.

Immerse the gel in the stain for approximately 0.5-1 hours.

Destaining solution

Ethanol	25	ml
Acetic acid	7	ml
Distilled water	68	ml

Immerse the gel in this solution to remove the last traces of stain and $CuSO_4$

APPENDIX E : Buffer preparation

1. Citric acid-Na_2HPO_4 (McIlvaine) buffer, pH 3.0-7.0 in 100 ml

Citric acid ($C_6H_8O_7.H_2O$, MW 210.14), Na_2HPO_4 (MW 141.98)

pH	Citric acid (g)	Na_2HPO_4 (g)
3	4.13	0.05
4	3.59	0.41
5	1.53	1.80
6	1.20	2.03
7	0.84	2.27

Adjust pH to required pH with 1 M HCl or 1 M NaOH and make volume to 100 ml with distilled water.

2. Phosphate buffer, pH 6.0-8.0, 11.0 in 100 ml

$Na_2HPO_4.2H_2O$ (MW 178.05), $NaH_2PO_4.H_2O$ (MW 138.01)

pH	$Na_2HPO_4.2H_2O$ (g)	$NaH_2PO_4.H_2O$ (g)
6	1.54	1.57
7	2.14	1.10
8	3.34	0.17

Adjust pH to required pH with 1 M HCl or 1 M NaOH and make volume to 100 ml with distilled water.

For pH 11.0, dissolve 0.14 g of $Na_2HPO_4.2H_2O$ in distilled water, then adjust pH with 1 M NaOH and make a volume to 100 ml

3. Tris (hydroxymethyl) aminomethane buffer, pH 7.0-9.0 in 100 ml

$C_4H_{11}NO_3$ (MW 121.14), HCl (MW 36.5)

pH	Tris (g)
7	0.18
8	1.10
9	2.16

Adjust pH to required pH with 1 M HCl and make volume to 100 ml with distilled water.

4. Clark and Lubs buffer, pH 8.0-10.0 in 100 ml

KCl (MW 74.55), H_3BO_3 (MW 61.84)

pH	KCl (g)	H_3BO_3 (g)
8	0.08	1.17
9	0.54	0.79
10	1.27	0.18

Adjust pH to required pH with 1 M NaOH and make volume to 100 ml with distilled water.

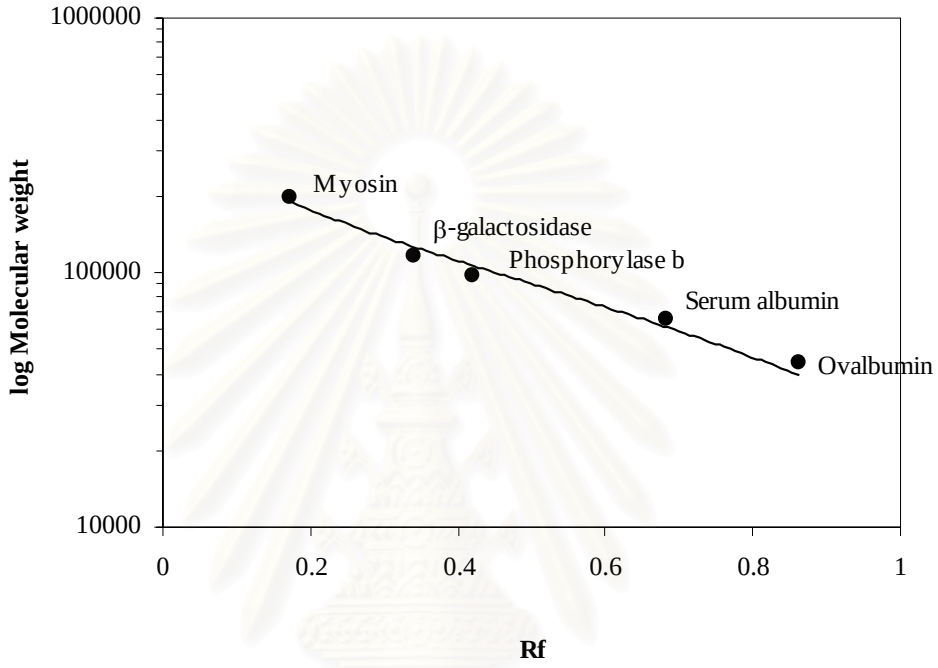
5. Glycine-NaOH buffer, pH 9.-10.0 in 100 ml

Glycine (MW 75.07)

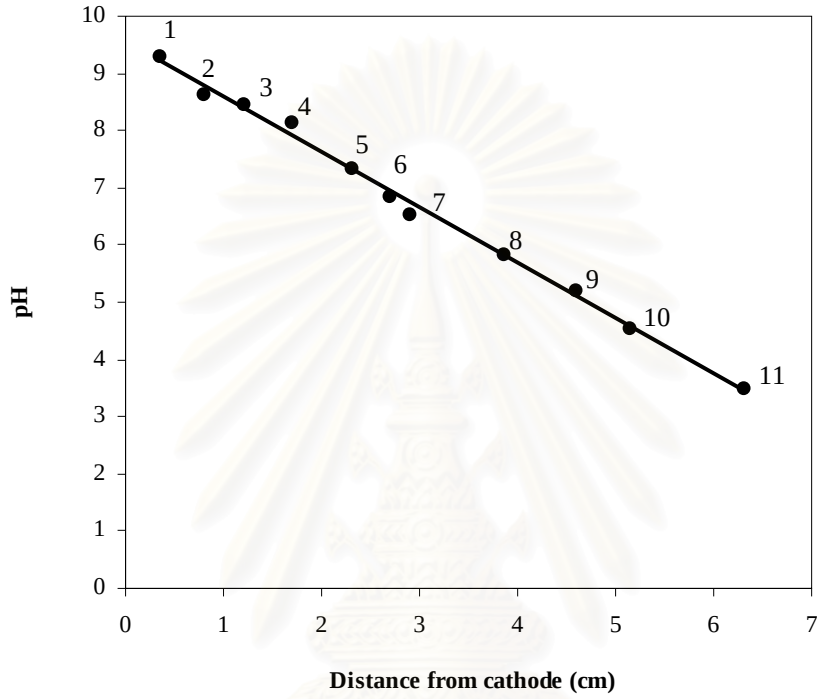
pH	Glycine (g)
9	0.22
10	0.94

Adjust pH to required pH with 1 M NaOH and make volume to 100 ml with distilled water.

APPENDIX F : Molecular weight calibration curve of standard protein on 7.5% SDS-PAGE



APPENDIX G : Standard pI calibration curve (pI 3-10)



1 - trypsinogen (9.30)

2 - lentil lectin-basic band (8.65)

3 - lentil lectin-middle band (8.45)

4 - lentil lectin-acidic band (8.15)

5 - myoglobin basic band (7.35)

6 - myoglobin acidic band (6.85)

7 - human carbonic anhydrase B (6.55)

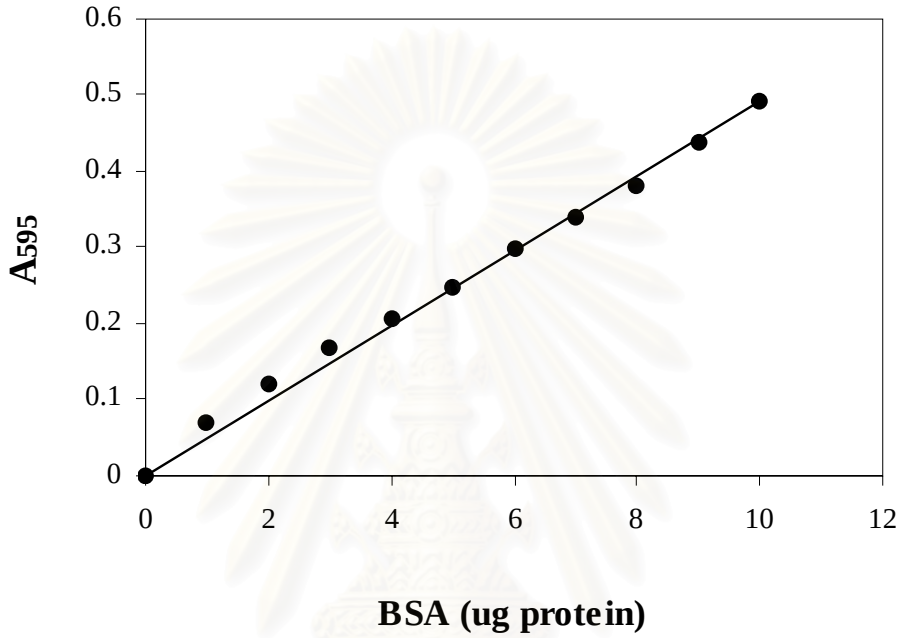
8 - bovine carbonic anhydrase B (5.85)

9 - β-lactoglobulin A (5.20)

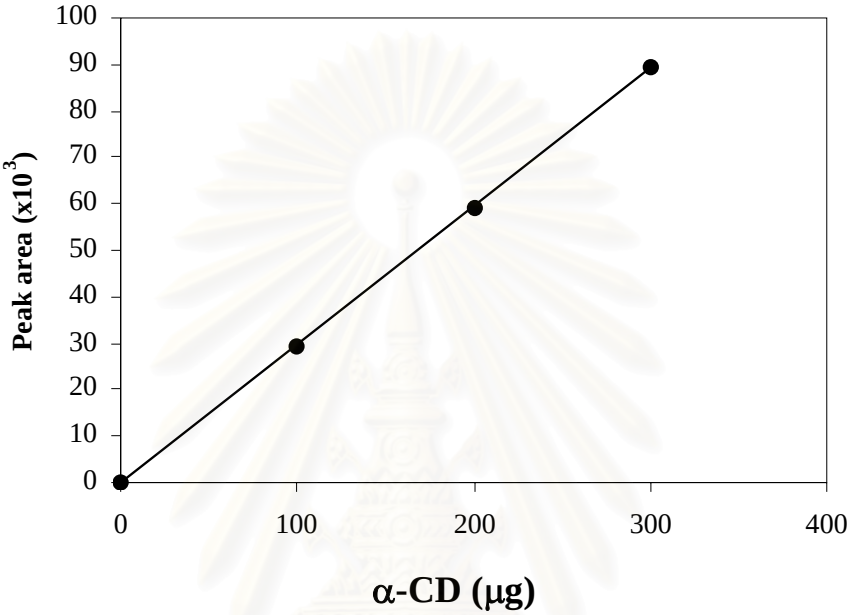
10 - soybean trypsin inhibitor (4.55)

11 - amyloglucosidase (3.50)

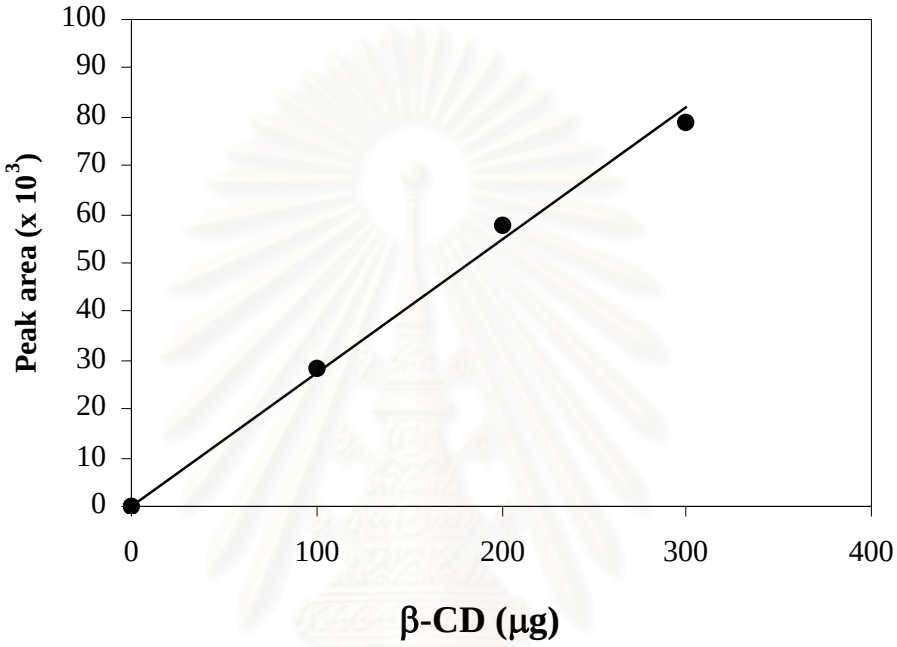
APPENDIX H : Standard curve for protein determination by Bradford's method



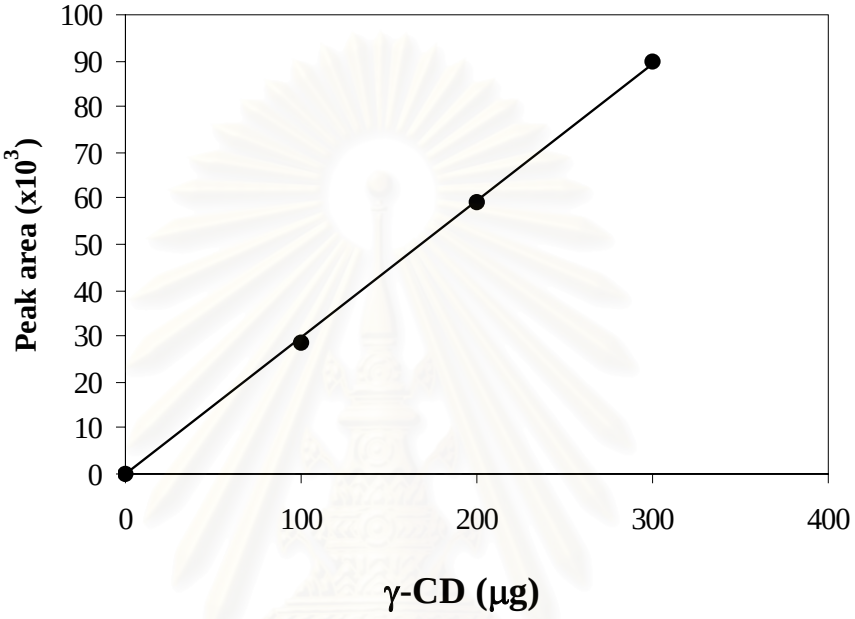
APPENDIX I : Standard curve of α-CD by HPLC method



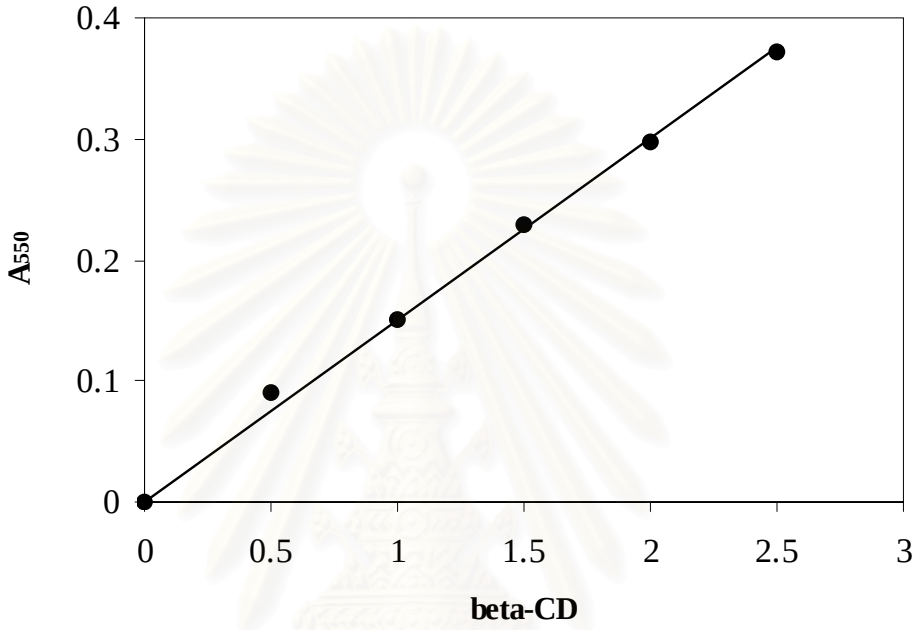
APPENDIX J : Standard curve of β-CD by HPLC method



APPENDIX K : Standard curve of γ-CD by HPLC method



APPENDIX L : Standard curve of β-CD by phenolphthalein method



BIOGRAPHY

Miss Ampornpun Pranommit was born on Sep. 25, 1977. She graduated with the Bachelor Degree of Science in General Science with second class honor from Kasetsart University in 1999 and continued studying for Master in Biochemistry Department, Chulalongkorn University.