STRUCTURAL ANALYSIS OF CYCLODEXTRIN GLYCOSYLTRANSFERASE ISOFORMS FROM *Paenibacillus* sp. A11

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การวิเคราะห์โครงสร้างของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสไอโซฟอร์ม จาก *Paenibacillus* sp. A11

นางสาววนิดา ประสงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	Structural analysis of cyclodextrin glycosyltransferase isoforms from *Paenibacillus* sp. A11
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วนิดา ประสงค์ : การวิเคราะห์โครงสร้างของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสไอโซฟอร์มจาก *Paenibacillus* sp. A11 (STRUCTURAL ANALYSIS OF CYCLODEXTRIN GLYCOSYLTRANSFERASE ISOFORMS FROM *Paenibacillus* sp. A11) อ.ที่ปรึกษา : รศ.ดร.เปี่ยมสุข พงษ์สวัสดิ์, อ.ที่ปรึกษาร่วม : ผศ.ดร.วิเชียร ริมพณิชยกิจ, 130 หน้า, ISBN 974-17-1137-9

งานวิจัยนี้มีวัตถุประสงค์ในการศึกษาลักษณะโครงสร้างของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส (CGTase) ไอโซฟอร์มจาก *Paenibacillus* sp. A11 เพื่อนำข้อมูลมาประกอบการวิเคราะห์การเกิดหลายรูปแบบของเอนไซม์ เมื่อทำการแยกไอโซฟอร์มทั้ง 4 ของ CGTase จาก *Paenibacillus* sp. A11 ด้วยเทคนิค preparative gel electrophoresis แล้วศึกษาผลของ post-translational modification ได้แก่ ไกลโคซิเลชัน ฟอสโฟริเลชัน การสร้างพันธะไดซัลไฟด์ และการดัดแปรหมู่คาร์บอกซิลิก พบว่าเมื่อทำปฏิกิริยา deglycosylation ด้วยเอนไซม์ PNGase F และ Endo H และด้วยสารเคมี trifluoromethanesulfonic acid (TFMS) สรุปได้ว่า deglycosylation ไม่มีผลต่อขนาดและประจุสุทธิของทุกไอโซฟอร์ม แต่มีผลต่อแอคติวิตีของไอโซฟอร์ม 3 และ 4 และเมื่อทำปฏิกิริยา dephosphorylation ด้วยเอนไซม์ alkaline phosphatase และตรวจสอบการเกิดพันธะไดซัลไฟด์โดยการใช้สารรีดิวซิ่ง พบว่า ทั้งสองกลไกไม่มีผลต่อการเกิดหลายรูปแบบของเอนไซม์ จากการเปลี่ยนแปลงหมู่โซ่ข้างกรดอะมิโนที่เป็นหมู่คาร์บอกซิลิกด้วยสารเปลี่ยน-แปลงกลุ่มจำเพาะชนิด 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) ได้ผลซึ่งคาดว่ากลไก amidation/deamidation มีผลต่อการเปลี่ยนประจุสุทธิในโครงสร้างของทุกไอโซฟอร์ม ในผลการทดลองส่วนที่สอง ทำการทดลองเพื่อวิเคราะห์ว่าหลายรูปแบบของ CGTase เป็นโปรตีนเดียวกันหรือไม่ เมื่อวิเคราะห์เพปไทด์ที่เกิดจาก การย่อยด้วยเอนไซม์ทริปซินโดยคอลัมน์ HPLC (C_{18}) แบบ reverse phase ได้ผลว่าจำนวนและชนิดของเพปไทด์ของทุกไอโซฟอร์มไม่มีความแตกต่างกัน แล้วตรวจสอบกรดอะมิโนที่เป็นองค์ประกอบ พบว่า องค์ประกอบกรดอะมิโนของทุก ไอโซฟอร์มไม่แตกต่างกันอย่างชัดเจน ในการตรวจสอบองค์ประกอบโปรตีนโดยคอลัมน์ HPLC (C_4) แบบ reverse phase พบว่า ทุกไอโซฟอร์มประกอบด้วยพีคหลัก 2 พีคเหมือนกัน คือที่ R_t 10.924-10.954 และ 11.951-11.595 นาที เมื่อวิเคราะห์ลำดับกรดอะมิโนที่ปลาย N ของทุกไอโซฟอร์ม พบว่า มีลำดับกรดอะมิโน 5 ตัวแรก (APDTS) เหมือนกับเอนไซม์ในรูป unfractionate ผลจากการวิเคราะห์พอลิอะคริลาไมด์เจลอิเลคโทรโฟริซิสสองไดเมนชัน สนับสนุนว่า ทุกไอโซฟอร์มเกิดจากโปรตีนเดียวกัน นอกจากนี้ในการเปรียบเทียบ CGTase ที่ผลิตจาก *E.coli* ที่ได้รับยีน CGTase จากสายพันธุ์ *Paenibacillus* sp. A11 กับเอนไซม์ของสายพันธุ์เดิม พบว่ามีรูปแบบการเกิดหลายไอโซฟอร์มเหมือนกัน จากข้อมูลทั้งหมดอาจสรุปได้ว่า หลายรูปแบบของ CGTase เกิดจาก โปรตีนชนิดเดียวที่มีการเปลี่ยนแปลงหลังการถอดรหัสและแปรรหัสจากยีน เมื่อศึกษาจลนพลศาสตร์ของไอโซฟอร์ม 1 พบว่า α-CD เป็นสับสเตรทที่ดีที่สุดในปฏิกิริยา coupling reaction (k_{cat}/K_m 3368 $\pm$ 300 $mM^{-1}.min^{-1}$) และ maltohexaose เป็นสับสเตรทที่ดีที่สุดในปฏิกิริยา cyclization reaction (k_{cat}/K_m 107 $\pm$ 5 $mM^{-1}.min^{-1}$)

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The aim of this work was to determine the structure of cyclodextrin glycosyltransferase (CGTase) isoforms from *Paenibacillus* sp. A11, the information obtained will be used for analysis of the formation of multiple forms of the enzyme. Four CGTase isoforms of *Paenibacillus* sp. A11 were isolated by preparative gel electrophoresis. For the effect of post-translational modification on structure and activity of CGTase isoforms, glycosylation, phosphorylation, disulfide bond formation, and modification of carboxyl residues were studied. When enzymatic deglycosylation by the enzyme PNGase F and Endo H and chemical deglycosylation by trifluoromethanesulfonic acid (TFMS) were performed, it was found that deglycosylation, if occurred, had no effect on the size and net charge of all isoforms but exerted some effect on activity of isoforms 3 and 4. When dephosphorylation by alkaline phosphatase and investigation of disulfide bond formaion by using reducing agents were performed, it was found that both mechanisms, if occurred, had no effect on isoform pattern. Modification of carboxyl side chain with a group specific reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) suggests that amidation/deamidation had an effect on net charge of all CGTase isoforms. In the second part, the experiments were performed to analyze whether multiple forms of CGTase was the same protein. When peptides from tryptic digestion were analyzed by reverse phase HPLC on a C_{18} column, the number and type of peptides from all isoforms were not significantly different. Amino acid composition of all isoforms was also similar. When protein constituent in each isoform was determined by reverse phase HPLC on a C_4 column, it was found that all isoforms consisted of two major forms of protein, those having R_t 10.924-10.954 and 11.951-11.595 min). When N-terminus sequence was analyzed, the first five residues of all isoforms obtained were APDTS which were the same as those of unfractionated enzyme. The result from two dimensional PAGE supports that all isoforms were rooted from a single protein. In addition, comparison of CGTase from *E. coli* harbouring CGTase gene of *Paenibacillus* sp. A11 with enzyme from original strain indicated that isoform pattern was very similar. The overall data from this work suggests that multiple forms of CGTase was the result of post-translational modification of the transcribed and translated form of the enzyme. When kinetic parameters of isoform 1 were determined, α-CD was the best substrate for coupling reaction (k_{cat}/K_m 3368 $\pm$ 300 $min^{-1}.mM^{-1}$) and maltohexaose was the best substrate for cyclization reaction (k_{cat}/K_m 107 $\pm$ 5 $min^{-1}.mM^{-1}$).

Department/Program.....Biochemistry..... Student's signature.....
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ABBREVIATION

A	absorbance
BSA	bovine serum albumin
CD	cyclodextrin
CGTase	cyclodextrin glycosyltransferase
cm	centimeter
^{o}C	degree Celsius
Da	dalton
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

1.1 Cyclodextrins

1.1.1 Molecular Characteristics of CDs

Cyclodextrins (cycloamyloses, cyclomaltaoses, Schardinger dextrins, CDs) are cyclic, non-reducing maltooligosaccharides produced from starch and related compounds by the cyclodextrin glycosyltransferases (CGTases) of certain bacteria. The main CDs are composed of six, seven, or eight 1,4-α-D-linked glucopyranose residues(α-, β-, or γ- cyclodextrins, respectively), as shown in Figure 1.

The most stable three dimensional molecular configuration for these non-reducing cyclic oligosaccharides takes the form of a truncated cone with the upper (larger) and lower (smaller) opening of the cone presenting C2 and C3 secondary and C6 primary hydroxyl groups, respectively. These orientations make cyclodextrin molecules hydrophilic on the outside, and the hydrophobic inside cavity is lined with C-H groups and glycosidic oxygen brides (Saenger, 1979, 1982; Bender, 1986) as shown in Figure 2. Some physical properties of CDs are summarized in Table 1 (Szejtli, 1988). Among the CD's, γ-CD has the biggest cavity and the most soluble.

The most important property of cyclodextrins is their ability to form inclusion compound. CD as a "host" component can encapsulate so called "guest" molecule of appropiate size, shape and polarity, into its cavity with noncovalent bonding forces such as hydrophobic interaction, Van deer Waal forces, London dispersion forces and hydrogen bonding (Komiyama and Bender, 1984). The binding are not fixed or permanent, but rather is governed by a dynamic equilibrium and thereby affording an ease of assembly and disassembly. Potential guests which can be encapsulated in cyclodextrins are shown in Figure 3 (Amaizo, 1993).

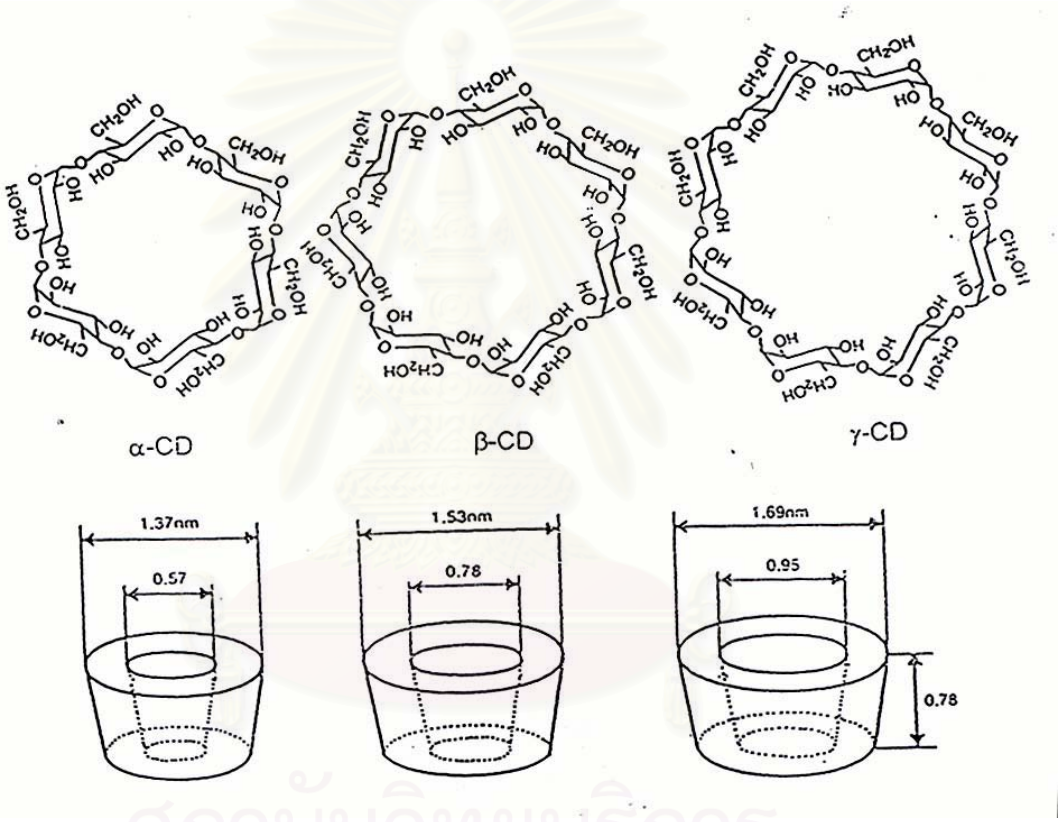


Figure 1. Structure and molecular dimension of cyclodextrins

(French and Rundle, 1942)

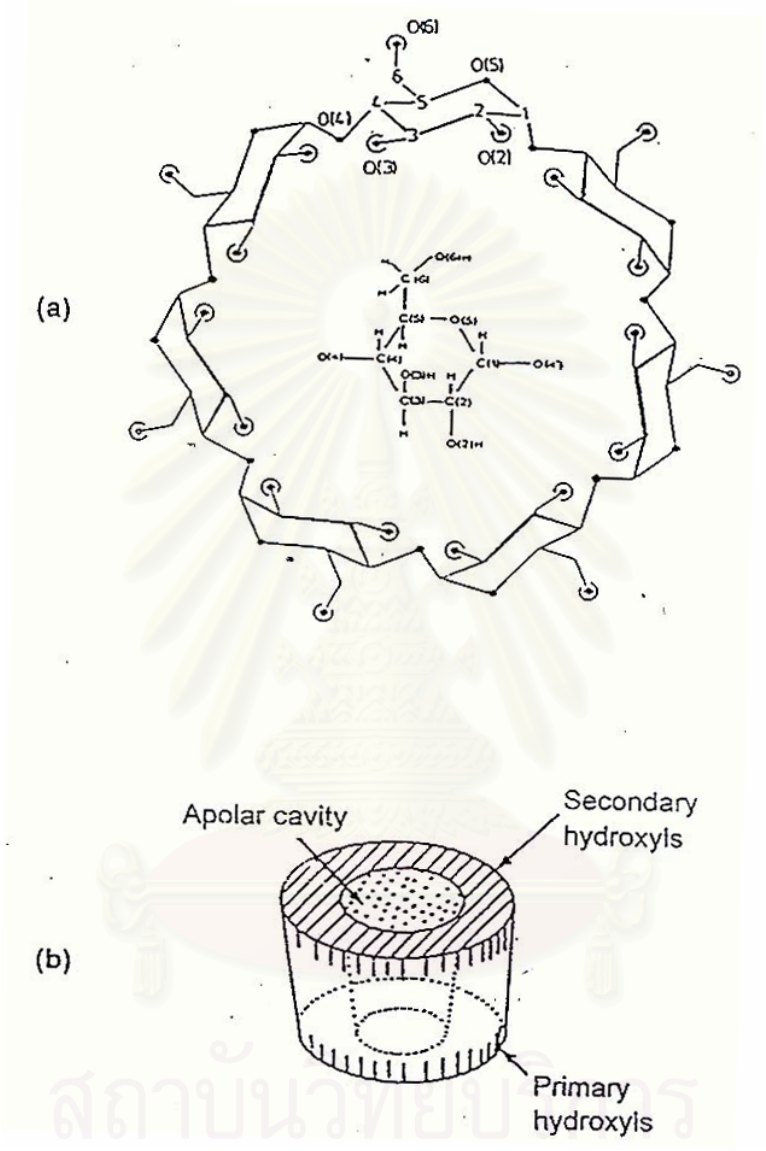


Figure 2. Structure of β-cyclodextrin (Bender, 1986; Szejtli, 1990)

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

(b) Functional structure scheme

Table 1. Physical properties of cyclodextrins (Saenger, 1982; Szejtli, 1988)

Property	α-CD	β-CD	γ-CD
Number of glucose unit	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100ml) at ambient temp.	14.40	1.85	23.20
Cavity diameter (^{o}A)	4.7-5.3	6.0-6.5	7.5-8.3
Cavity depth (^{o}A)	7.9±0.1	7.9±0.1	7.9±0.1
Cavity volume (^{o}A)^{3}	174	262	472
Crystal forms (from water)	hexagonal plates	monocyclic paralellograms	quadratic prism

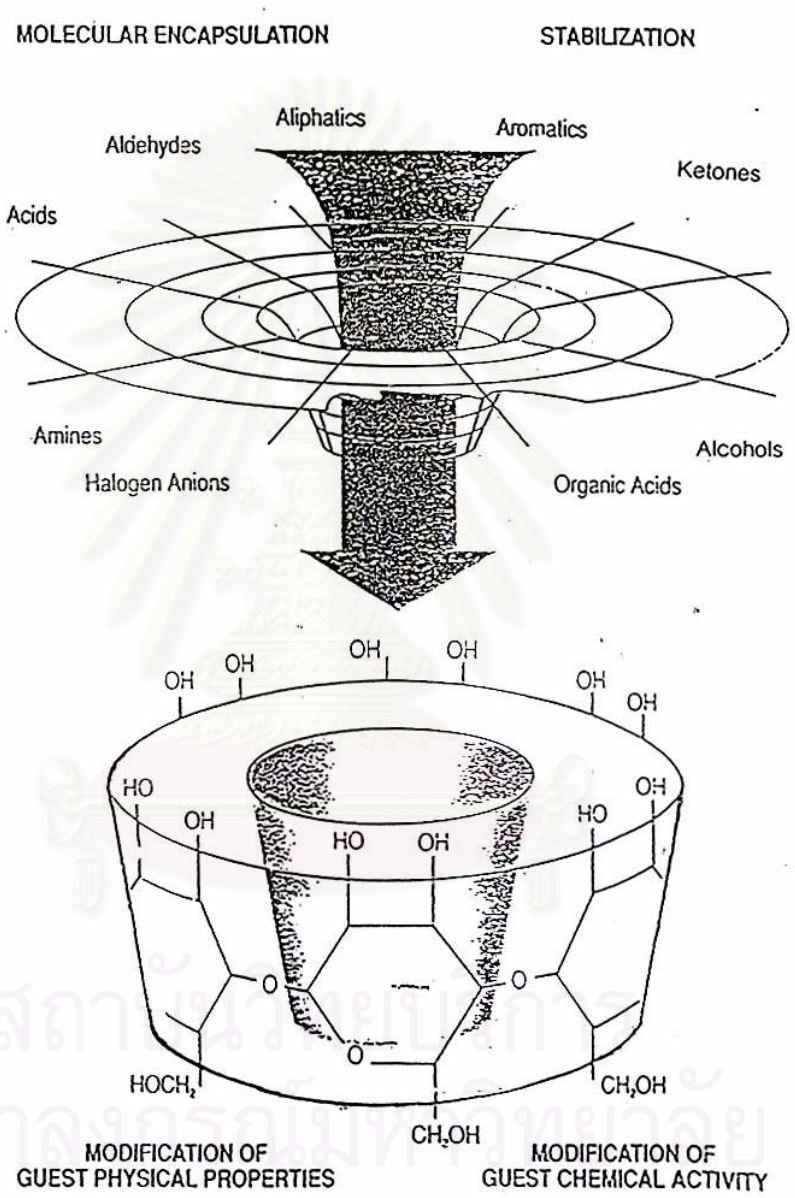


Figure 3. Beneficial modification of guest molecules by cyclodextrin

(Amaizo, 1993)

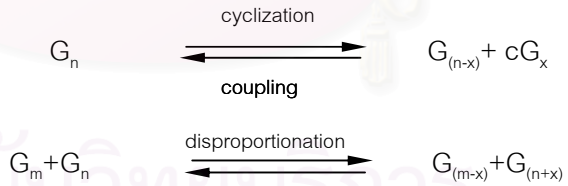
1.1.2 Application of CDs

Complex formation of cyclodextrins and guest molecules leads to the change in the physical or chemical properties of guest molecules. Protection against oxidative degradation or destruction by UV light, improvement of the solubility of hydrophobic substances in aqueous solution, stabilization of volatile compounds, alteration of undesirable smell or taste in products e.g. foodstuffs, are among those known useful properties of CDs (Schmid, 1989). At present, cyclodextrins are increasingly used in industrial and research applications (Table 2).

1.2 Cyclodextrin producing enzymes

1.2.1 Properties of CGTase

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a starch degrading enzyme belonging to the important α -amylase family (family 13) of glycosyl hydrolases(Henrissat, 1991). The formation of cyclodextrins by CGTase proceeds through three transglycosylation reactions : cyclization, coupling and disproportionation as shown below :



Where $G_n$and G_m are 1,4-α-D-glucopyranosyl chains with “n” and “m” residues; x is a part of 1,4-α-D-glucopyranosyl chains, and cG_x is a symbol for CDs (Starnes R.L.*et al*. 1990).

A scheme of the CGTase-catalyzed transglycosylation reactions is as depicted in Figure 4 (Bart *et al*., 2000). Cyclization (cleavage of an α - glycosidic bond in amylose or starch and subsequent formation of a cyclodextrin) is a single-substrate reaction with an affinity for the high molecular mass substrate. Coupling (cleavage of an

Table 2. Applications of cyclodextrins

(Fromming, 1981; Horikoshi, 1982; Bender, 1986; Szejtli, 1988).

Use	Guest compounds and end products
Foods	
1) Emulsification	Eggless mayonnaise, seasoning oil, whipping cream, etc.
2) Increase of foaming powder	Egg white (freeze-dry), hotcake-mix, cake-mix, etc.
3) Stabilization of flavors and seasonings	Chewing gum flavor, biscuit flavor, powdered seasoning, 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 odor	Mutton, fish, soybean
Coosmatics and toiletries	
1) Color masking and control	Fluorescein, bath agents
2) Stability of fragrances	Menthol
3) Stabilization	Chalcone, dihydrochalcone(toothpaste), Perfume
4) Preventing inflammation of skin	Skin lotion, sun block cream
5) Deodorant	Mouth wash, in refrigerator
6) Reduction of irritation	Shampoo, cream, skin powder
7) Enhancement of attained concentration	Skin moisturizing lotion
8) Defoaming effect	Laundry liquid
Pharmaceuticals	
1) Increase of solubility	Prostaglandin, phenolbarbital, chloramphenicol
2) Taste masking	Prostaglandin
3) Powdering (non-volatile)	Nitroglycerin, clofibrate
4) Stabilization (UV, thermal)	Prostaglandin, vitamins
5) Decrease of irritation	Cu-alcanolamine complex
6) Enhancement bioavailabiity	Barbiturate, flufenamic aci, digoxin
7) Reduction of systematic toxicity	2-amino, 4-methyl-phophonobutyric acid

Table 2. Applications of cyclodextrins (continued)

Use	Guest compounds and end products
Agriculture	
1) Stabilization of volatility	Tobacco aroma
2) Stabilization of nutrient	Animal-feed
3) Improvement of palatability	Bone-powder, microbial cell-mass
Pestisides	
1) Stabilization (UV, thermal)	Pyrethrins, pyrethroids, isoprenoid
2) Powdering (non-volatile)	DDVP and other phosphorous pesticides
Chemical technology	
Catalyzation for reaction	Products of hydrolysis, substitution, Diels-Alder reaction, stereospecific reaction, ect.
Plastics	
Stabilization of colors and flavors	Colors, Flavors
Others	Adhesives

α -glycosidic bond in a cyclodextrin ring and transfer of the resulting linear malto-oligosaccharide to an accepter substrate) proceeds according to a random ternary complex mechanism. Disproportionation (cleavage of an α -glycosidic bond of a linear malto-oligosaccharide to an acceptor substrate) proceeds according to a ping-pong mechanism, The proposed model of the events taking place in the CGTase – catalyzed reactions is shown in Figure 5.

The cyclization 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. It should be mentioned that the acceptor binding sites of enzyme are not absolutely specific for glucose or malto-oligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrates containing 16-80 glucopyranosyl residues. If chain length is greater than 100 units, disproportionation reaction dominates. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 3. High concentration of malto-oligosaccharides or glucose favours the reversed coupling reaction resulting in linear end products with negligible amount of CDs (Kitahara *et al*., 1978). The action of CGTase is different from that of other starch-degrading enzymes in that the products are cyclic and non-reducing.

CGTase is an extracellular enzyme produced by a variety of bacteria, mainly the *Bacillus* sp., as listed in Table 4. The CGTase can be divided into three types (α-, β- and γ-) according to the major type of CD product formed (Horikoshi, 1988). The enzymes from different sources show different characteristics such as working pH, temperature, pI and molecular weight. Each CGTase enzyme yields different ratio of CD-product, i.e. *B.macerans* and Alkalophilic *Bacillus* 38-2 CGTase produce α-, β- and γ-CDs in relative amounts of 2.7:1.0:1.0 (Depinto and Campbell, 1968) and 1:11.5:1.5 (Nakamura and Horikoshi, 1976), respectively, while the CGTase of *B.fermus* 290-3 is known to produce γ-CD in the initial phase of the enzyme production (Englbrecht *et al*., 1990).

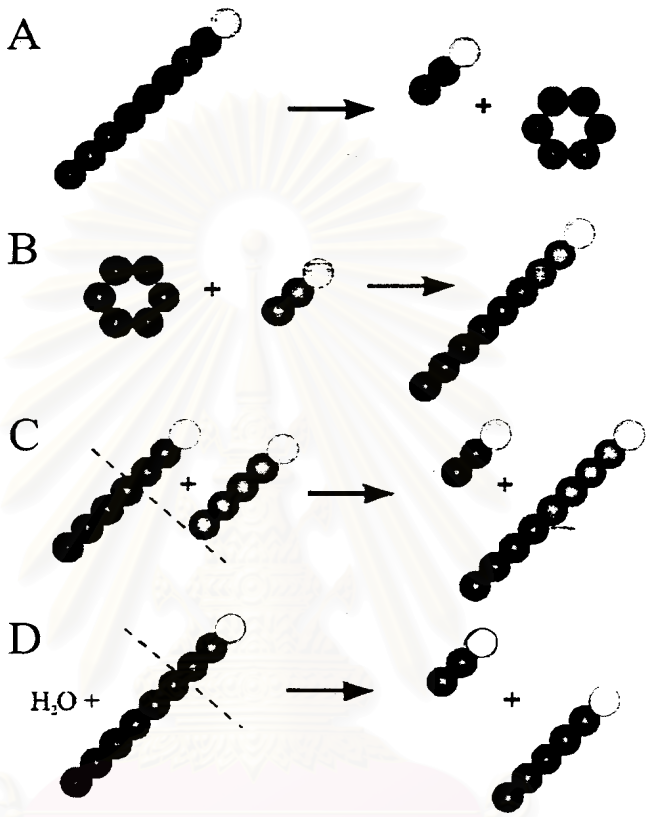


Figure 4. Schematic representation of the CGTase-catalyzed transglycosylation reactions. (Bart *et al*., 2000)

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

● = glucose residues; o = the reducing end glucoses.

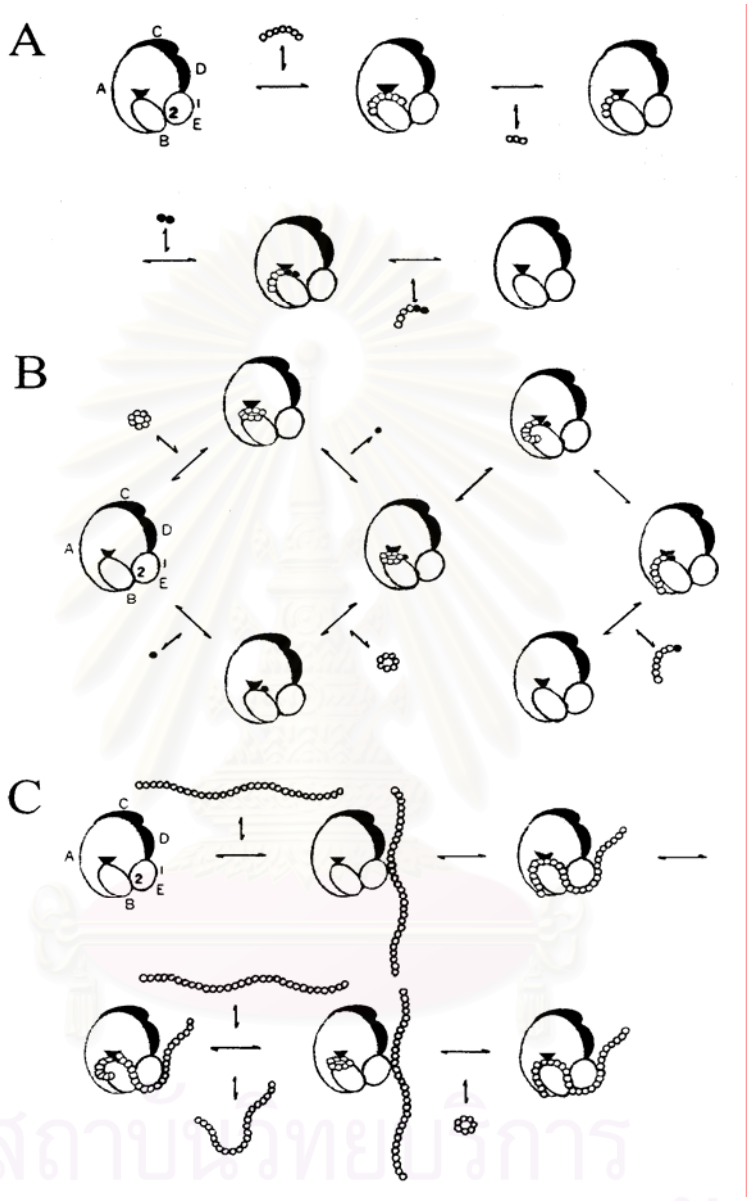


Figure 5. Proposed model of the events taking place in the CGTase-catalyzed reactions. (Bart *et al*., 2000)

(A) Disproprtionation, (B) coupling, (C) cyclization. The different CGTase domains are indicated (A, B, C, D, and E), 1 and 2 indicate the maltose binding sites on the E-domain. The triangle indicates the cleavage site in the active site. Circles represent glucose residues; acceptor residues are represented in black.

Table 3. Relationship between length of substrate and mechanisms of CGTase (Szejtli, 1988).

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

Table 4. Properties of cyclodextrin glycosyltransferases

Organism	Predominant Product	Optimum pH	Optimum Temperature (°C)	MW (dalton)	pI	References
Alkalophilic *Bacillus* 17-1	β-CD	6.0	ND	74,000	ND	Yamamoto *et al.*, 1972
Bacillus fermus 290-3	γ-CD	6.0-8.0	50	75,000	4.1	Englbrecht *et al.*, 1990
Bacillus macerans IFO 3490	α-CD	5.0-5.7	55	5,000	4.6	Kitahata *et al.*, 1974
Bacillus macerans IAM 1243	α-CD	5.5-7.5	60	145,000	ND	Kobayashi et al, 1978
Bacillus macerans ATCC 8514	α-CD	6.2	ND	139,000	ND	Depinto and Campbell, 1986
Bacillus megaterium	β-CD	5.0-5.7	55	ND	6.07	Kitahata and Okada, 1975
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. 1977
Micrococcus sp.	β-CD	6.2	ND	139,000	ND	Yagi *et al.*, 1980
Bacillus circulans A11						Kaskangam, 1998
Isoform1	β-CD	6.0-7.0	40	72,000	4.73	
Isoform2	β-CD	6.0-7.0	40	72,000	4.49	
Isoform3	β-CD	6.0	50	72,000	4.40	
Isoform4	β-CD	7.0	50-60	72,000	4.31	

ND = Not Determined

1.2.2 Purification of CGTase

The study on physical and biochemical properties of CGTase requires separation technique to purify the enzyme. Methods reported thus far for CGTase purification include precipitation with organic solvents or ammonium sulfate, adsorption onto starch, 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 α-CGTase from *B. macerans* was achieved on α-CD-derivatized agarose (Laszlo *et al*., 1981). The β-CD affinity column was prepared for purification of CGTase from *B. stearothermophilus* ET1 (Chung *et al*., 1998). Spiridonova (1998) reported that CGTase enzyme from *B. stearothermophilus* No.2 was purified by ion exchange high performance liquid chromatography using a protein-Pak DEAE-8HR column. Active fractions of CGTase were collected after elution using a NaCl gradient from 0 to 0.5 M. In 1996, Kim succesfully purified CGTase from *Paenibacillus sp. A11* by immunoaffinity chromatography using anti-CGTase linked to CNBr-activated Sepharose 4B.

1.2.3 Three-dimensional structure and active site of CGTase

The three-dimension structure of CGTase from x-ray crystallographic technique showed that CGTase consisted of five domains (Figure 5). The experiment of Svensson *et al*. (1989) demonstrated that the CGTase from *Bacillus circulans* strain 251 is consisted of a single polypeptide chain of 686 amino acids grouped in five distinct domains labeled A through E. Domain E contains a raw starch-binding motif and was found to bind two maltose molecules. A third maltose molecule is bound by the C-domain and is involved in crystal packing contact between symmetry-related molecules, but the precise functions of D domain remains to be resolved. In a computer model structure of CGTase from *Bacillus circulans* var. *alkalophilus*, the small domain B situates on the principal domain A and contains a groove with the catalytic site. The C, D, and E domains locate around domain A without any S-S bridged linkage to it. There is a high similarity in domain E among all of the CGTases and also some similarity with

glucoamylases. Domain E may be involved in catalysis and in binding the substrate leading to a stabilized structure of enzyme due to a starch bridge fixed between domain A and E (Fugiwara *et al*., 1992).

CGTase structure is related to α-amylase at A, B, and C domains, but α-amylase lacks the additional domains D and E that are unique for CGTase. Although both enzyme families show a low overall degree of similarity in their amino acid sequence, the N-terminal domain of CGTase contains three highly conserved regions which presents and constitutes the active centers of α-amylase (Kimura *et al*., 1987). These regions have also been found in other amylolytic enzymes such as isoamylase (Amamura *et al*., 1986), pullulanase (Katsuraki *et al*, 1987), and α-glucosidases (James *et al*., 1987). In contrast, the C-terminal region of CGTase was completely different from those of α-amylase. This region would contain an extra 200 to 250 amino acids (which consists of D and E domains) in addition to the polypeptide exhibiting the amylase activity. This findings suggest that CGTase may consist of two protein domains, the one in the N-terminal side cleaves the α-1,4-glucosidic bond in starch, and the other in the C-terminal side catalyzed other activities, including the reconstitution of an α-1,4-glucosidic linkage for cyclizing the maltooligosaccharide produced (Kimura *et al*., 1987).

1.2.4 Multiple forms of CGTase

A few reports on CGTase isoform were published (Table 5). Isoforms are defined as enzymes that catalyze the same reaction but have different properties such as kinetics and catalytics. They are, however, encoded by a single gene which makes isoforms different from isozymes (Voet *et. al*., 1999). At early stages of the study by isoelectric focusing, CGTase from alkalophilic *Bacillus* was separated into a few active bands. Because of this, an attractive possibility was that CGTases of various bacteria actually are mixtures of α-, β- and γ- CD producing individual enzymes. Some support for such speculations has been provided by a report claiming the development of a CGTase producing only γ- CD (Kato and Horikoshi, 1986). In addition to possible

differences in specificities, CGTase from alkalophilic *Bacillus* has been reported to contain three isozymes possessing markedly different pH optima (4.6, 7.0, and 9.5 respectively) (Nakamura *et al*., 1981).

The CGTase from alkalophilic *Bacillus* (ATCC 21783) was purified to near homogeneity by a two-step procedure involving affinity chromatography and anion exchange high performance liquid chromatography. The latter method produced several fractions with different pI in the range of 4.55-4.90 but their properties such as activity levels and product compositions were identical under various reaction conditions (Makela *et al*., 1988). In 1990, another reference on isolation of CGTase from the same strain but using isoelectric focusing in immobilized pH gradients reported that the enzyme could be resolved into more than 6 subforms, a major one with pI 4.97 and the others between pH 4.75-4.99. Five amino acids at N- terminus of these CGTase subforms were determined and reported to be the same (Ala-Pro-Asp-Thr-Ser) (Mattsson, Meklin and Korpela, 1990). In 1992, CGTase from *B. circulans* E192 was purified by FPLC on Mono Q column. Two isozymes were separated and their isoelectric points were estimated as 6.7 and 6.9 with amino acid compositions of 705 and 716 residues, respectively. No difference in the sequence of 30 amino acid residues at the N-terminus of two isozymes was found (Bovetto *et al*., 1992). In 1994, Abelyan *et al.* isolated CGTase isoforms from *Bacillus* sp. INMIA-T6, -T42 and –A7/1 into 2, 4 and 2 isoforms, respectively. Amino acid composition and CD product ratio were slightly different. These works hence showed variable differences between isoforms reported. In our research group, the CGTase from *Paenibacillus sp. A11* was separated to 3-4 isoforms by chromatofocusing column (Rojtinnakorn, 1994). However, the isoforms were not well separated by the condition used in this column. In 1998, Kaskangam was able to isolate the four CGTase isoforms by preparative gel electrophoresis. Each isoform had some different physicochemical and biochemical properties especially pI, amino acid composition, product ratio, and carbohydrate content (Table 6), though they showed the same molecular weight of 72,000 on SDS-PAGE. By chemical modification and substrate protection experiment, Kualpiboon (2000) reported that some essential

Table 5. Multiple forms of CGTases

Source	Number of forms	Characteristics	References
Alkalophilic *Bacillus*	3	pH optima 4.6, 7.0, 9.5	Nakamura and Horikoshi *et al*., 1976
Alkalophilic *Bacillus*	6*	pI 4.97 (major form), other between 4.75-4.99, five amino acids at N-T are all the same	Mattsson *et al*., 1990
B. circulans E192	2*	pI 6.7 and 6.9, number of amino acids 705 and 716, 30 amino acids at N-T are the same	Bovetto *et al*., 1992
Bacillus sp. INMIA T6 *Bacillus* sp. INMIA T42 *Bacillus* sp. INMIA A7/1	2* 4* 2*	different sizes, pH optimum, amino acid composition, and CD production ratio	Abelyan *et al*., 1994

* isoform separation was reported

Table 6. Summarization of properties of CGTase isoforms from *Paenibacillus* sp. A11 (Kaskangam, 1998).

Band no.	MW (kDa)	pI	Optimum pH	Optimum temp. (°C)	Product ratio (α:β:γ-CD)	CHO content (μg/μg protein)
1	72	4.73	6.0-7.0	40	10:18:5	0.205
2	72	4.49	6.0-7.0	40	9:18:5	0.187
3	72	4.40	6.0	50	5:18:5	0.144
4	72	4.31	7.0	50-60	5:18:7	0.467

CHO = Carbohydrate, analyzed by the phenol-sulfuric acid method

Figure 6. Alignment of amino acid sequences of *Bacillus* CGTase. Black highlighting indicates the conserved sequences. ***Paenibacillus*** sp. A11 (Rimphanitchayakit, 2000); *Bacillus* sp. 1011 (Kimura *et al*., 1987); *Bacillus circulans* 251 (Penninga *et al*., 1995); *Bacillus circulans* 8 (Nitschke *et al*., 1990).

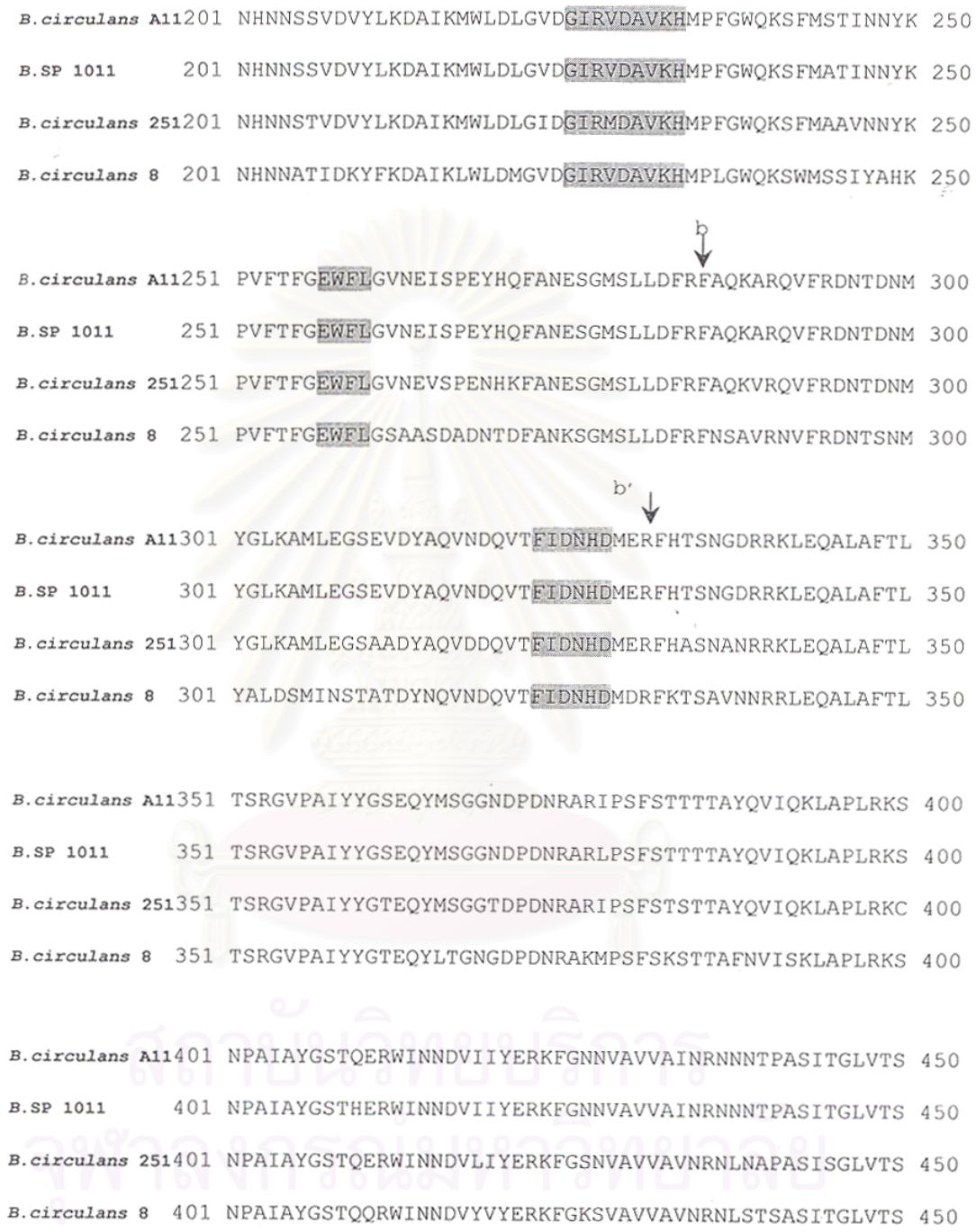


Figure 6. (continued) Alignment of amino acid sequences of *Bacillus* CGTase.

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B.circulans A11451 LPQGSYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATAPI 500
B.SP 1011     451 LRRASYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATTPI 500
B.circulans 251451 LPQGSYNDVLGGLLNGNTLSVGSGGAASNFTLAAGGTAVWQYTAATATPT 500
B.circulans 8  451 LPTGSYNDVLGGVLNGNNITS-TNGSINNFTLAAGATAVWQYTTAETTPT 500

B.circulans A11501 IGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQ 550
B.SP 1011     501 IGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQ 550
B.circulans 251501 IGHVGPMMAKPGVTITIDGRGFGSSKGTVYFGTTAVSGADITSWEDTQIK 550
B.circulans 8  501 IGHVGPVMGKPGNVVTIDGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIK 550

B.circulans A11551 VKIPAVPGGIYDIRVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTAL 600
B.SP 1011     551 VKIPAVPGGIYDIRVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTAL 600
B.circulans 251551 VKIPAVAGGNYNIKVANAAGTASNVYDNFEVLSGDQVSVRFVVNNATTAL 600
B.circulans 8  551 VTIPSVAAGNYAVKVA-ASGVNSNAYNNFTILTGDQVTVRFVVNNASTTL 600

B.circulans A11601 GQNVFLTGNVSELGNWDPNNAIGPMYNQVVYQYPTWYYDVSVPAGQTIEF 650
B.SP 1011     601 GQNVFLTGNVSELGNWDPNNAIGPMYNQVVYQYPTWYYDVSVPAGQTIEF 650
B.circulans 251601 GQNVYLTGSVSELGNWDPAKAIGPMYNQVVYQYPNWYYDVSVPAGKTIEF 650
B.circulans 8  601 GQNLYLTGNVAELGNWSTSTAIGPAFNQVIHQYPTWYYDVSVPAGKQLEF 650

B.circulans A11651 KFIKKQGSTVTWEGGANRTFTTPTSGTATMNVNWQP 686
B.SP 1011     651 KFIKKQGSTVTWEGGANRTFTTPTSGTATVNVNWQP 686
B.circulans 251651 KFLKKQGSTVTWEGGSNHTFTAPSSGTATINVNWQP 686
B.circulans 8  651 KFFKKNGSTITWESGSNHTFTTPASGTATVTVNWQ- 686
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Figure 6. (continue) Alignment of amino acid sequences of *Bacillus* CGTase.

amino acids at the active site of each isoform might be different. However, when the gene coding for this CGTase has been cloned and sequenced (Figure 6) (Rimphanichayakit, 2000), only one structural gene was observed.

This research aims to prove the cause of the isoform pattern of CGTase from *Paenibacillus* sp. A11. The study will analyze the importance of post-translational modification i.e. glycosylation or phosphorylation on structure and activity of isoforms. Glycosylation was concerned since this CGTase was found to be a glycoprotein (Tachaiyakul, 1991; Kaskangam, 1998), moreover, the carbohydrate content of each isoform was different as determined by phenol-sulfuric method (Kaskangam, 1998). However, glycosylation was reported to be rare in prokaryotes (Nelson and Cox, 2000). While in lower eukaryotes, this mechanism certainly exists. Stratilova *et al*. (1998) reported that glycosylation had effect on isoform pattern of *Aspergillus* polygalacturonase. Padmajanti *et al.* (2000) reported that deglycosylation of recombinant isopullulanase (rec-IPU) from *Aspergillus oryzae* M-2-3 by endoglycosidase H (Endo H) had effect on structure and activity. Molecular weight of rec-IPU decreased and activity was 65% of the original activity. For other post-translational modifications which might lead to difference in net charge of each isoform, phosphorylation is interesting since protein phosphorylation/ dephosphorylation catalyzed by protein kinases and phosphatases have been revealed to be widespread among prokaryotes (Nelson and Cox, 2000). Phosphorylation, methylation, adenylation and uridylylation are means of covalent modification on biomolecules which are found in *E.coli* (White, 2000) while glycosylation has not been reported.

In the attempt to clarify whether or not the CGTase is expressed from one gene, in addition to investigation on possible post-translational modification, this work will also determine the N-terminal amino acids and the peptide map of each isoform. Comparison of isoform pattern from the wild type with the recombinant *E.coli* harbouring CGTase gene will be analyzed. These experiments will lead to more insight into the understanding of the multiple forms of the enzyme.

Objectives

The objectives of this research is to prove the cause of the isoform pattern of CGTase from *Paenibacillus* sp. A11. The work focuses at

1. Comparison of isoform pattern of CGTase from *Paenibacillus* sp. A11 and *Escherichia coli* harboring CGTase gene
2. The investigation of the effect of post-translational modification on structure and activity of CGTase
3. Kinetic study of CGTase isoforms
4. Analysis of N-terminus amino acid of CGTase isoform
5. Determination of digested peptides of CGTase isoform

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave : Model HA-30, Hirayama Manufacturing Cooperation, Japan

Autopipette : Pipetman, Gilson, France

C_{18} column : Model LUNA 5 μ size 250x4.6 mm. Phenomenex, U.S.A.

C_4 column : Model Vydac 5 μ size 250x4.6 mm. Phenomenex, U.S.A.

Centrifuge, concentrator centrifuge (Speedvac) : Model UNIVAPO 100 H, N.Y.R., Thailand

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.

Electroblotting Unit : Model Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad, U.S.A.

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

Fraction collector : Model 2211 Pharmacia LKB, Sweden

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

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

Incubator shaker, Controlled environment : Psyco-therm, 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® GRH, Janke&Kunkel Gmbh&Co.KG, Japan

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

pH meter : PHM83 Autocal pH meter, Radiometer, Denmark

Preparative gel electrophoresis unit : Model 491 Prep Cell, Bio-Rad Applied Biosystem company, U.S.A.

Spectrophotometer JENWAY 6400, UK and Du series 1050, BECKMAN, U.S.A.

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

Water bath : Charles Hearson Co. Ltd., England

Water bath shaker : Heto Lab Equipment, Denmark

2.2 Chemicals

Acetonitrile (HPLC grade) : BDH Laboratory Chemical-Division, England

Acrylamide : Merck, U.S.A.

Alkaline phosphatase : Sigma, U.S.A.

Amylase : Sigma, U.S.A.

Cellobiose: Sigma, U.S.A.

Coomassie brilliant blue G-250 : Sigma, U.S.A.

Coomassie brilliant blue R-250 : Sigma, U.S.A.

α-cyclodextrin : Sigma, U.S.A.

γ-cyclodextrin : Sigma, U.S.A.

β-cyclodextrin

Methyl-, Maltosyl- and Hydroxypropyl- cyclodextrin : Sigma, U.S.A.

Endoglycosidase H : Sigma, U.S.A.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide : Sigma, U.S.A.

Glucose : Sigma, U.S.A.

Glycine : Sigma, U.S.A.

Maltotriose, maltotetraose, matopentaose, maltohexaose, maltoheptaose: Sigma, U.S.A.

D(+)-maltose monohydrate : Fluka, Switzerland

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

Peptide-N-glycosidase F : Boehringer Mannheim, Germany

Potato starch (Potato) : Fluka, Switzerland

Soluble starch : BDH, U.S.A.

Trichloroethylene (TCE) : BDH, U.S.A.

Trifluoroacetic acid (TFA) : Fluka, Switzerland

Trifluoromethanesulfonic acid (TFMS) : Sigma, U.S.A.

Trypsin : Sigma, U.S.A.

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

2.3 Bacteria

2.3.1 *Bacillus circulans* A11, isolated from South-East Asian soil, was screened for CGTase activity by Pongsawasdi and Yagisawa (1987). This strain was reclassified as *Paenibacillus* sp. A11 by analysis of 16S rRNA gene (Kualpiboon, 2001)

2.3.2 *Escherichia coli*, harbouring cyclodextrin glycosyltransferase gene of *Paenibacillus* sp. A11, was prepared by Rimpanitchayakit (2000).

2.4 Media preparation

2.4.1 Medium I

Medium I, consisted of 0.5% beef extract, 1.0% polypeptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble starch was prepared and adjusted to pH 7.2 with 1N HCl. For solid medium, 1.5% agar was added. Medium I was steriled by autoclaving at 121 ^{o}C for 15 minutes.

2.4.2 Cultivation medium (modified from Horikoshi' s medium by Rutchtorn, 1993)

Medium for enzyme production, slightly modified from Horikoshi (1971), contained 1.0% local grade of rice starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.75% Na_2CO_3 with starting pH 10. Medium was steriled as above.

2.4.3 Luria-Bertani (LB) medium

LB medium consisted of 1% Bacto tryptone, 0.5% yeast extract and 0.5% NaCl was prepared and adjusted to pH 7.4 with NaOH. For solid medium, 1.5% agar was added. Medium was sterilized by autoclaving at 121 ^{o}C for 15 minutes.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

2.5.1 A colony of *Paenibacillus* sp. A11 was grown in 30 ml of starter Medium I in 250 ml Erlenmeyer flask at 37 ^{o}C with 250 rpm rotary shaking until A_{420} reached 0.3-0.5 unit or about 4-6 hours.

2.5.2 A colony of *E. coli* harbouring CGTase gene was grown in 30 ml of LB medium in 250 ml Erlenmeyer flask at 37 ^{o}C with 250 rpm rotary shaking for overnight.

2.5.2 Enzyme production

2.5.1 Starter *Paenibacillus* sp. A11 was transferred into 100 ml Horikoshi's broth in 500 ml Erlenmeyer flask with 1% inoculum and cultivated at 37 ^{o}C with 250 rpm rotary shaking. Culture was harvested after 72 hours and cells were removed by centrifugation at 3,000 rpm for 30 minutes at 4 ^{o}C. Crude CGTase was collected and kept at 4 ^{o}C for purification.

2.5.2 Starter *E.coli* harbouring CGTase gene was transferred into 100 ml LB medium in 500 ml Erlenmeyer flask with 1% inoculum and cultivated at 37 ^{o}C with 250 rpm rotary shaking. Culture was harvested after 18 hours and cells were removed by centrifugation at 3,000 rpm for 30 minutes at 4 ^{o}C. Crude CGTase was collected and kept at 4 ^{o}C for purification.

2.6 Enzyme assay

For this study, CGTase activity was determined by starch degrading (dextrinizing) activity assay and CD-forming activity (CD-trichloroethylene, CD-TCE) assay.

2.6.1 Dextrinizing activity assay

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

Sample (5-100 μl) was incubated with 0.3 ml of starch substrate (0.2% w/v soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) at 40 ^{o}C for 10 minutes. The reaction was stopped with 4 ml of 0.2 N HCl. Then 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube of each sample, 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.

2.6.2 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).

The enzyme sample was diluted in serial double dilution by 0.2 M phosphate buffer, pH 6.0. The reaction mixture, containing 0.5 ml of sample and 2.5 ml of starch substrate (2.0% w/v soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) was incubated at 40 ^{o}C for 24 hours. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in dark. The activity was expressed in term of dilution limit (1 : 2^{n}) which was the highest dilution that can

produce observable CD-TCE precipitate lining between the upper starch solution layer and the lower TCE layer.

2.7 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 5 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of 95%ethanol, 10 ml of 85% H_3PO_4 and distilled water.

2.8 Carbohydrate determination

The total carbohydrate content was determined by the phenol-sulfuric acid method according to Dubois (1959) using glucose as standard. The reaction mixture, containing 0.5 ml of the sample, 0.3 ml of 5% phenol and 2.0 ml of concentrated H_2SO_4 were mixed. The absorbance was measured at 484 nm after 30 minutes incubation at room temperature.

2.9 Reducing-sugar determination

Reducing sugar was determined by the method of Miller (1959). Glucose standard (0.5 ml of 0-10 mM) was prepared. Then 0.5 ml of dinitrosalicylic acid reagent (prepared as described below) was added. The solution was heated for 5 minutes in boiling water bath, then the tubes were cooled in a bowl of cold water for 5 minutes. The mixture was adjusted to a final volume of 5 ml with distilled water. After mixing, the absorbance at 540 nm was recorded. The quantity of reducing sugar in the sample was determined from the standard curve of glucose.

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

2.10 Purification of CGTase

CGTase was partially purified from the culture broth of *Paenibacillus* sp. A11 and *E.coli* by starch adsorption method of Kato and Horikoshi (1984) with modification (Kuttiarcheewa, 1994).

Corn starch (local grade) was oven dried at 120^{o}C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude broth to 5%(w/v) concentration. After 3 hours of continuous stirring at 4 ^{o}C, the starch cake was collected by centrifugation at 3,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB_1). The adsorbed CGTase was eluted from the starch cake with TB_1 buffer containing 0.2 M maltose (3 x 150 ml for the culture broth of 5 L), by stirring for 30 minutes. The partially purified CGTase was then concentrated by ultrafiltration before loading on a preparative gel electrophoresis Model 491 Prep cell.

2.11 Isolation of CGTase isoforms

The concentrated partially purified enzyme from *Paenibacillus* sp. A11 (5 mg protein) was loaded to a discontinuous preparative polyacrylamide gel electrophoresis (7.5% separating and 5% stacking gel), which was performed on Model 491 Prep cell (38 mm ϕ). Tris-glycine buffer, pH 8.0 was used as electrode buffer (see Appendix A). The electrophoresis was run from cathode towards the anode at constant power of 12 W until the dye reached the bottom of the gel. Proteins were then eluted from the gel with electrode buffer at flow rate of 1 ml/min. Fractions of 2.5 ml were collected and measured for A_{280} and dextrinizing activity. Then the fractions that gave the electrophoretic band with the same mobility were pooled for further studies.

2.12 Purity of CGTase isoforms

2.12.1 Polyacrylamide Gel Electrophoresis (PAGE)

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

2.12.1.1 Non-denaturing polyacrylamide gel electrophoresis

Discontinuous PAGE was performed on slab gel (10x8x0.075 cm) of 7.0%(w/v) separating and 5.0% (w/v) stacking 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.

2.12.1.2 SDS- polyacrylamide gel electrophoresis

The denaturing gel was performed according to Bollag and Edelstein (1991). The gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels and Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as electrode buffer (see Appendix A). Samples to be analyzed were treated with sample buffer and boiled for 5 minutes prior to application to the gel. 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.

2.12.1.3 Detection of proteins

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining and dextrinizing activity staining.

2.12.1.3.1 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 2 hours. The slab gels were destained

with a solution of 10% methanol and 10% acetic acid for 1-2 hours until gel blackground was clear.

2.12.1.3.2 Dextrinizing activity staining (slightly modified from method of Kobayashi *et al*., 1978)

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

2.13 Analysis of cyclodextrins by High Performance Liquid Chromatography

The sample solutions were analyzed for cyclodextrins by HPLC using Supelco-NH_2 column (0.46 x 25 cm) and detected by RI detector (Rutchtorn, 1993). To prepare the sample solution, the partially purified enzyme (50 units of dextrinizing activity) was incubated with 2.5 ml of starch substrate (2.0 g% potato starch in 0.2 M phosphate buffer, pH 6.0) at 40 ^{o}C for 24 hours. The reaction was stopped by boiling for 5 minutes. After cooling, the reaction mixture was treated with 20 units of β-amylase at 25 ^{o}C for 2 hours, and the reaction was stopped by heating in boiling water bath. Prior to injection, the samples were filtered through 0.45 μ membrane filters. The eluant was a mixture of 75% acetonitrile and 25% water by volume, and the flow rate was 2 ml/minute. Cyclodextrins (α-, β-, and γ- CD) were analyzed by comparing the retention times to those of standard CDs, which was composed of α-, β-, and γ- CD mixture (20 mg/ml). For quantitative analysis, peak area corresponded with each cyclodextrin was determined from standard curve.

2.14 Investigation of CGTase isoforms by deglycosylation reaction

2.14.1 Enzymatic deglycosylation

2.14.1.1 Deglycosylation by endoglycosidase H (Endo H)

Reactions of 100 μl contained 40 μg of CGTase and 10 μl of 1 mU/μl Endo H in 50 mM sodium acetate buffer, pH 5.5. Incubation was performed at 37°C for 24 hours. The results were followed by non-denaturing and SDS - PAGE, and also measuring of dextrinizing activity and carbohydrate content. Ovalbumin and invertase, the O-linked glycoproteins, were used as positive control (Padmajunti *et al*., 2000).

2.14.1.2 Deglycosylation by peptide : N-glycosidase F (PNGase F)

CGTase were prepared in 2 conditions : 1) native CGTase, reactions of 100 μl contained 50 μg of CGTase with variable amounts of PNGase F, 60-6,000 mU/ml in 0.25 M sodium phosphate buffer (pH8.6) containing 10 mM β-mercaptoethanol and 2) denatured CGTase 50 mg/ml CGTase in 10%SDS, boiled for 5 minutes before reaction, reactions of 100 μl contained 50 μg of denatured CGTase with 6,000 mU/ml PNGase F in 0.5 M sodium phosphate buffer (pH 7.2) containing 50 mM EDTA, 5% β-mercaptoethanol and 2.5% triton X-100. Incubation was performed at 37 °C for 18 hours. The results were followed by non-denaturing and SDS-PAGE, and measuring of carbohydrate content. Human transferrin (HTF), the N-linked glycoprotein, was used as positive control. (Tarentino *et al*., 1985)

2.14.2 Chemical deglycosylation by Trifluoromethanesulfonic acid (TFMS)

The deglycosylation procedure was as follows : 10 micrograms of CGTase was dissolved in 100 μl of distilled water and 20 μl of 20% TFMS was mixed. Incubation was performed in ice-bath for 0, 1, 2, 3, 4 and 5 hours. Reaction mixture was neutralized with 100 μl of 60% pyridine followed by dialysis against distilled water for 3 hours. The results were followed by SDS–PAGE and measuring of carbohydrate content.

2.15 Investigation of CGTase isoforms by dephosphorylation reaction (slightly modified from method of Marushige and Marushige, 1975)

Four purified CGTase isoforms were incubated with *E.coli* alkaline phosphatase (25 units/mg) in 70 mM Tris- HCl pH 8.0 with phosphatase : CGTase ratio of 1 : 10 (by wt.) at 37 ^{o}C for 2 hrs. The reaction was stopped by adding 20% cold TCA and protein precipitation was allowed to occur at 0 ^{o}C for 15-30 minutes. Then solution was centrifuged at 12,000 g for 45 minutes, the protein precipitates were subjected to non-denaturing gel electrophoresis. Ovalbumin, the phosphoprotein, was used as positive control.

2.16 Modification of carboxyl residues of CGTase isoforms

Modification of carboxyl residues was carried out according to the method of Means and Feeney (1971). Each CGTase isoform (1 dextrinizing activity unit) in 50 mM acetate buffer, pH 6.0, was incubated with 5 mM 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) at 40 ^{o}C for 2 hours. The total volume of reaction mixture was 60 μl. After the incubation, the treated CGTase was compared with control (untreated) on non-denaturing PAGE. CGTase isoform activity staining was detected as described in Section 2.10.1.2.2.

2.17 Digestion of CGTase isoforms and Separation of peptides (Delferge *et al.*, 1997)

For peptide digestion, two hundred micrograms of four purified CGTase isoforms which were separated by preparative gel electrophoresis were lyophilized and 60 μl of 8M Urea was added. Reaction mixture was incubated at 37 ^{0}C for 1 hour. Then 180 μl of 0.2 M Tris-HCl buffer, pH 8.0, 25 μl of 0.1 mg/ml trypsin (a ratio of trypsin : CGTase is 1:80 w/w) were added and incubation was performed at 37 ^{0}C for 18 hours. The sample was then lyophilized.

For peptide separation, digested CGTase isoforms were dissolved with 60 μl of ultrapure water before filtration with 0.22 micron filter to remove particulate materials.

Peptides resulting from enzymatic cleavage were then separated by reversed phase HPLC on C_{18} column (250 x 4.6 mm) previously equilibrated with solvent A (0.1% (v/v) trifluoroacetic acid in water). Elution was made by mixing solvent A and B at the indicate proportion and time as shown below. Solvent B was 0.1% (v/v) trifluoroacetic acid in 75%(v/v) acetonitrile. Elution of HPLC was carried out at a flow rate of 1 ml/min. Detection of peptides was performed at 210 nm.

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
5	95	5
30	80	20
35	0	100
40	100	0

2.18 Analysis of amino acid composition

Fifity micrograms of four purified CGTase isoforms were sent to the Department of Food Engineering, King Mongkut's University of Tecnology Thonburi for analyzing amino acid composition. Amino acid content was determined using the method described (Water, 1995). The enzyme samples were hydrolyzed with 6 N HCl in evacuated tubes at 110 $^{\circ}$C for 24 hours. After that they were derivatized with 6-amino quinolyl-N-hydroxy succinimidyl carbamate (AQC) and analyzed by reverse phase HPLC on Nova-Pak^{TM} C_{18}, 4 microns and detected by fluorescent detector (395 nm). The amino acid compositions were calculated by Millennium Version 2.15 program.

2.19 Amino acid sequence determination

A hundred micrograms of partially purified enzyme was separated into 4 isoforms on non – denaturing PAGE and transferred onto PVDF membrane. The membrane was wetted with methanol for a few seconds, then placed in a dish

containing blotting buffer (10% methanol containing 25 mM Tris-glycine buffer, pH 8.3) for 5 minutes. The gel was sandwiched between pre-wet membrane (Whatman No.1 paper) and sponges, was assembled on a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell and run at a constant voltage of 50 volts at room temperature for 90 minutes. PVDF membrane was then removed from transblotting sandwich, rinsed with D.I. water and saturated with 100% methanol for a few seconds prior to staining with 0.1% Coomassie blue R-250 in 40% methanol, 10% acetic acid. The PVDF membrane was removed from staining solution and destained with 50% methanol. The membrane was rinsed extensively with D.I. water. The protein bands of interest were excised and dried at room temperature. The PVDF membrane was sent to analyze N-terminal amino acid by Edman degradation method at the Department of Biological Science, National University of Singapore, Singapore.

2.20 Analysis of CGTase isoforms by High Performance Liquid Chromatography

Four purified CGTase isoforms which were separated by preparative gel electrophoresis were dialyzed against distilled water at 4 $^{\circ}$C overnight. Each isoform (20-100 μg) was lyophilized and 100 μl of ultrapure water was added before filtration with 0.22 micron filter to remove particulate materials. After that they were analyzed by reverse phase HPLC on Vydac™ C_4 column (250 x 4.6 mm) previously equilibrated with solvent A (0.1% (v/v) trifluoroacetic acid in water). Elution was made by mixing solvent A and B at the indicate proportion and time as shown below. Solvent B was 0.1% (v/v) trifluoroacetic acid in 60% (v/v) acetonitrile. Elution of HPLC was carried out at a flow rate of 1 ml/min. Detection of enzyme was performed at 220 nm.

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
5	100	0
30	0	100
35	0	100
45	100	0

2.21 Analysis of CGTase isoforms by two dimensional polyacrylamide gel electrophoresis

The partially purified CGTase (20 μg, 5 units) was loaded as 2 lanes on SDS-PAGE. After the run, the gel was removed from the electrophoresis cell and washed with 1.0% Triton X-100 in 0.2 phosphate buffer, pH 6.0 at 37 °C with gentle shaking for 3 hours. Then it was washed in water for several times. After that, the first lane was used for determining the position of CGTase by activity staining method. The second lane, was cut at the same position of the activity band appeared on the first gel and transferred to non-denaturing PAGE. After electrophoresis, detection of enzyme was then performed by activity staining method.

2.22 Kinetic study of CGTase isoform 1

Kinetic parameters of CGTase isoform were measured by incubating appropiately diluted enzyme at 40 °C with substrate solutions in 50 mM acetate buffer, pH 6.0. For cyclodextrin substrate (α-, β- and γ-CD) and some derivatives (methyl-, hydroxypropyl- and maltosyl- β-CD), the parameters were determined by coupling reaction. For oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose), cyclization reaction were used.

2.22.1 Coupling reaction

Various concentrations of cyclodextrins or derivatives (0 - 1 mM) were used as donor substrate in the reaction while 10 mM cellobiose was used as acceptor substrate. Cyclodextrin and cellobiose were pre-incubated in 50 mM acetate buffer, pH 6.0 (total volume of 0.25 ml) for 10 minutes at 40 °C before the reaction was started with the enzyme (10 units of dextrinizing activity) and incubation was proceeded for 5 minutes. The reaction was stopped by boiling for 5 minutes. Subsequently, 0.2 unit of *Aspergillus niger* glucoamylase was added to convert linear oligosaccharides to single glucose residues at 40 °C for 20 minutes. The glucose concentration was determined by the dinitrosalicylic acid method as described in section 2.9. Kinetic parameters, K_m and

V_{max}, were determined from the Michealis-Menten equation using nonlinear least square regression analysis of the EZ-FIT V1.1 Computer program.

2.22.2 Cyclization reaction

Various concentrations of oligosaccharides (0 - 1 mM) were pre-incubated in 50 mM acetate buffer, pH 6.0 (total volume of 0.25 ml) for 10 minutes at 40 ^{o}C before the reaction was started with the enzyme (10 units of dextrinizing activity) and the incubation was proceeded for 5 minutes. The reaction was stopped by boiling for 5 minutes. Subsequently, 0.2 unit of *Aspergillus niger* glucoamylase was added to convert linear oligosaccharides to single glucose residues at 40 ^{o}C for 20 minutes. The glucose concentration was determined by the dinitrosalicylic acid method as described in section 2.9. Kinetic parameters, K_m and V_{max}, were determined from the Michealis-Menten equation using nonlinear least square regression analysis of the EZ-FIT V1.1 Computer program.

CHAPTER III

RESULTS

3.1 Partial purification and comparison of CGTases

Crude CGTases from *Paenibacillus* sp. A11 and *E.coli* harbouring CGTase gene were partially purified by starch adsorption method as reported by Malai (1995). The yields of the enzymes were 87 and 52 % with purification of 45 and 69 folds, respectively (Tables 7 and 8). The enzymes were then concentrated by ultrafiltration and dialyzed with 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5 (TB_1). After this step, the yields of 58 and 39 % were obtained with 64 and 82 folds purification, respectively. The specific activity of the *Paenibacillus* sp. A11 enzyme was 1,335 while that of the *E.coli* was 2,214 U/mg of protein. Purity of the enzyme was confirmed by non-denaturing polyacrylamide gel electrophoresis followed by Coomassie blue staining.

Coomassie staining of the enzyme on non-denaturing PAGE (Figure 7A) showed that both *Paenibacillus* sp. A11 and *E.coli* crude enzymes gave several protein bands. However, only one major band with one faint band were observed with both enzymes after starch adsorption and ultrafiltration steps. Activity staining of enzyme on non-denaturing PAGE showed that the major protein band from purification process corresponded with CGTase activity (Figure 7A and 7B). CGTase from *Paenibacillus* sp. A11 gave 4-5 activity bands while CGTase from *E.coli* gave 3-4 bands. Two major bands of approximate equal intensity were observed in both cases while minor bands were more intense in *Paenibacillus* sp. A11 enzyme. Figure 8 showed Coomassie staining of enzyme on SDS-PAGE. Both crude enzymes gave several protein bands with similar and different mobility and intensity. The starch adsorbed and ultrafiltrated enzyme from *E.coli* showed a single band of 72,000 kDa. While starch adsorbed and ultrafiltrated enzyme from *Paenibacillus* sp. A11 gave one major band which was similar to *E.coli* and two faint bands below.

To determine the CD products formed by the two enzymes, analysis was made by HPLC. The reaction was composed of equal amounts of partially purified enzyme which were incubated with 2% soluble starch at 40 °C for 24 hours. The reaction products were treated with 20 units of β-amylase at 25 °C for 2 hours. This can differentiate the linear (i.e. G_4-G_7) from the cyclic oligosaccharides by hydrolysis of long chain oligosaccharides to G_2-G_3. The retention times of the reaction products were compared with standard CDs. The retention times of standard α-, β- and γ-CDs were 5.18, 6.50 and 8.18 minutes, respectively (Figure 9). The β-amylase treated samples from both enzymes showed similar major peaks with retention time corresponded to the standard CDs and an extra peak at R_t 2.64 -2.93 minutes which was not observed in the untreated samples. This extra peak should be short chain oligosaccharide products from β-amylase treatment. The ratios of α- : β- : γ-CD products from *Paenibacillus* sp. A11 and *E.coli* CGTase were 2 : 7 : 1 and 2 : 8 : 1, for treated samples.

3.2 Isolation of CGTase isoforms

The partially purified preparation of CGTase from *Paenibacillus* sp. A11 was subjected to preparative gel electrophoresis to separate CGTase isoforms according to the method of Kaskangam (1998). Figure 10 shows the elution profile, CGTase was eluted between fractions 80 - 220. The highest dextrinizing activity was obtained in the major protein peak. Samples from every 5 fractions eluted were subjected to discontinuous gel electrophoresis under non-denaturing condition and stained for dextrinizing activity, as shown in Figure 10. Selected fractions that yield high purity of each band on non- denaturing gel were pooled : fractions 80 to 90 (band 5), fractions 95 to 105 (band 4), fractions 115 to 135 (band 3), fractions 145 to 170 (band 2) and fractions 195 to 220 (band 1). The pooled fractions (band 1 to 5, respectively) were analyzed again by non-denaturing PAGE and activity staining. It was found that bands 1-5 all exhibited dextrinizing activity (Figure 12). Purification of each band was summarized in Table 7. The major band observed was band 1 which contained 6.8% of the total activity and was purified upto 90 folds. Bands 1-4,as proved by phenolphthalein

Table 7. Purification of CGTase from *Paenibacillus* sp. A11.

Purification Step	Volume (ml)	Total Activity (Units)*	Total Protein (mg)	Specific Activity (Units/mg)	Purification (fold)	Yield (%)
Crude enzyme	1000	27410	1340	21	1	100
Starch adsorption	100	23777	25	951	45	87
Ultrafiltration	30	16024	12	1335	64	58
Preparative gel Electrophoresis						
Band 1	20	1855	0.98	1893	90	6.8
Band 2	5	673	0.52	1302	62	2.5
Band 3	5	230	0.24	955	46	0.8
Band 4	5	90	0.15	600	29	0.3
Band 5	6	18	0.06	293	14	0.07

*dextrinizing activity

Table 8. Purification of CGTase from the recombinant *E.coli*.

Purification Step	Volume (ml)	Total Activity (Units)*	Total Protein (mg)	Specific Activity (Units/mg)	Purification (fold)	Yield (%)
Crude enzyme	1000	53700	1983	27	1	100
Starch adsorption	100	27914	15	1861	69	52
Ultrafiltration	30	20808	9.4	2214	82	39

*dextrinizing activity

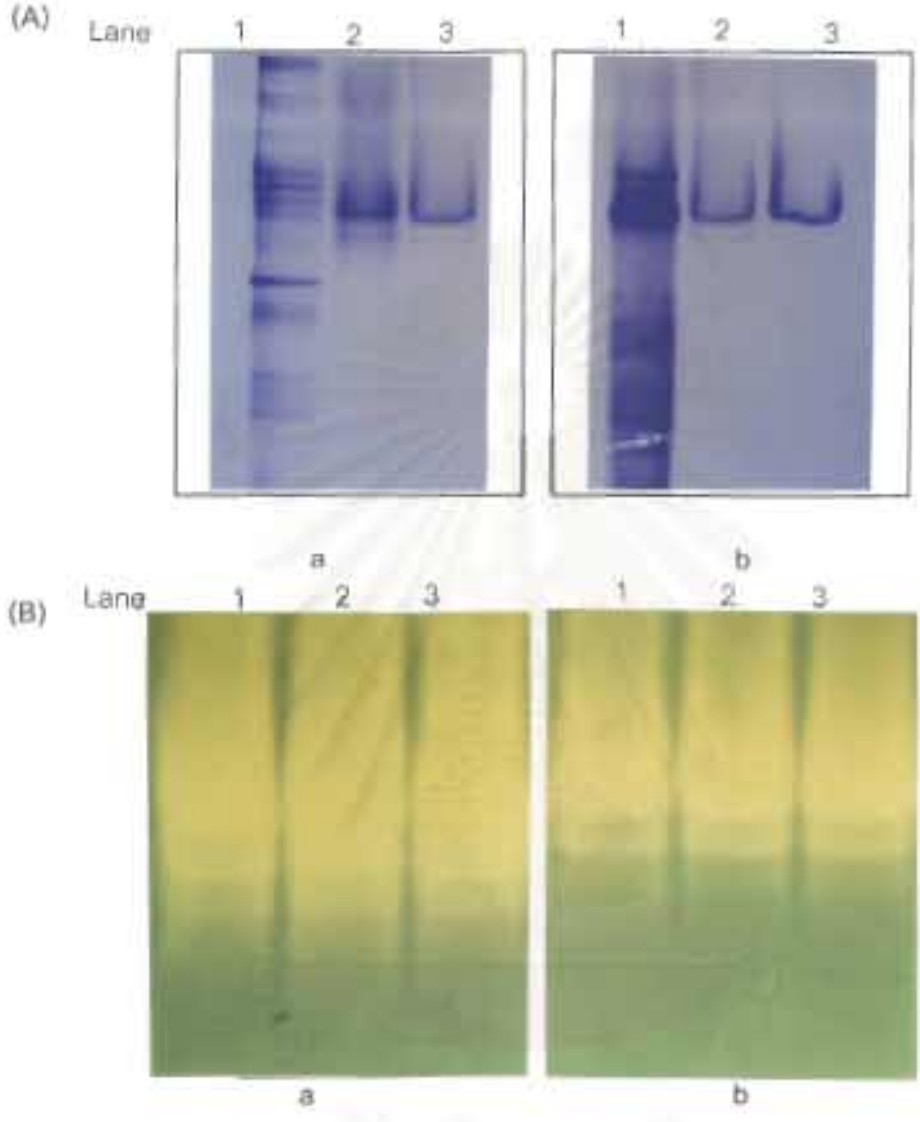


Figure 7. Non-denaturing PAGE of CGTase from different steps of purification

Lane 1 Crude enzyme

Lane 2 Starch adsorbed enzyme

Lane 3 Ultrafiltrated enzyme

(A) Coomassie Blue Staining [Lane 1-3 (a) 27, 15 and 15 µg and (b) 46, 15 and 15 µg, respectively]

(B) Amylolytic Activity Staining [0.2 dextrinizing activity units was loaded to each lane]

(a) CGTase from *Paenibacillus* sp. A11

(b) CGTase from *E.coli*

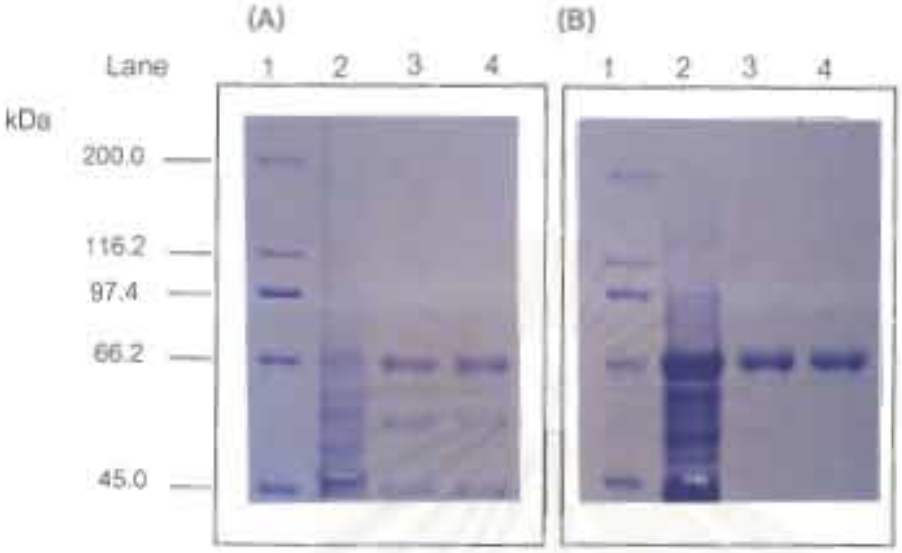


Figure 8. SDS-PAGE of CGTase from different steps of purification

(A) CGTase from *Paenibacillus* sp. A11 (B) CGTase from *E.coli*

Lane 1 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

Lane 2 Crude enzyme [(A) 27 and (B) 46 µg]

Lane 3 Starch adsorbed enzyme [(A) 15 and (B) 15 µg]

Lane 4 Ultrafiltrated enzyme [(A) 15 and (B) 15 µg]

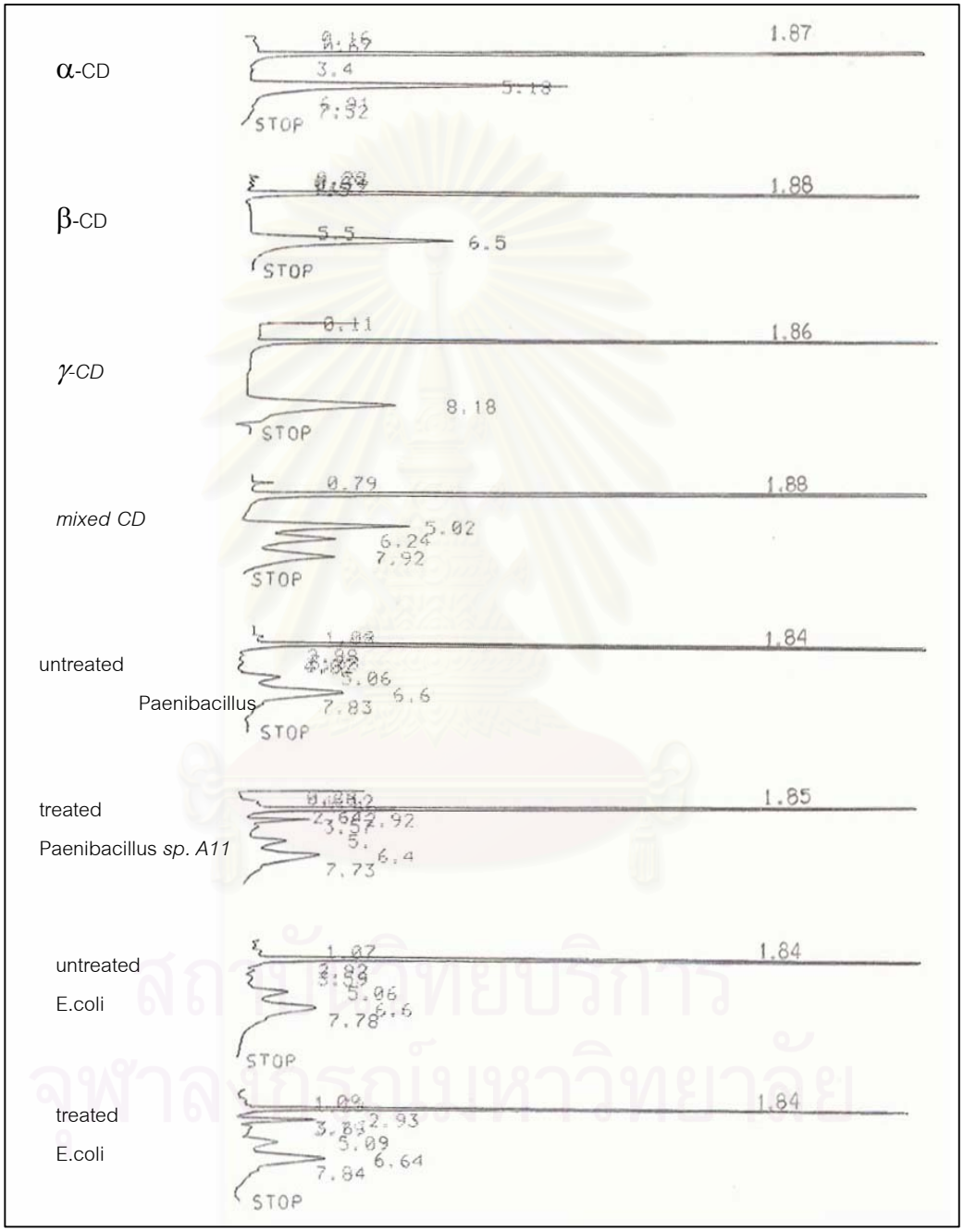


Figure 9. HPLC chromatograms of standard cyclodextrins and reaction products from CGTase catalysis. The untreated and β-amylase treated samples were compared. Supelco-NH_2 column was used. Acetonitrile : water (75:25) (v/v) was used as eluent at 2 ml/min flow rate.

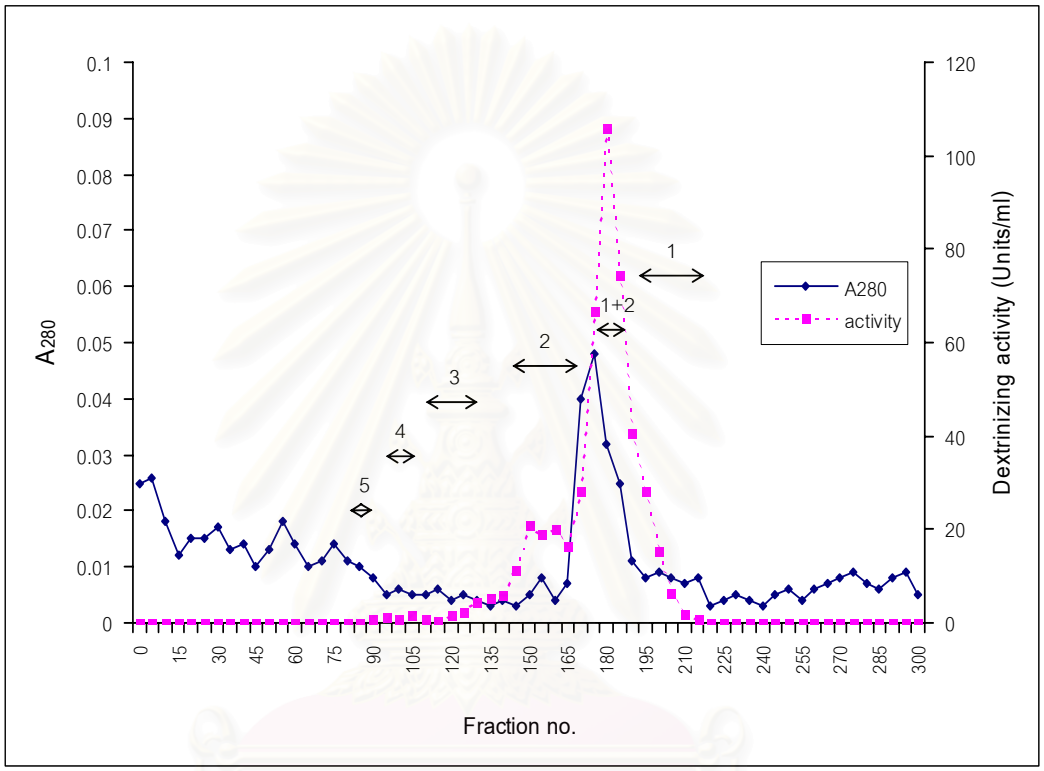


Figure 10. Elution profile of CGTase isoforms from preparative gel electrophoresis

Partially purified enzyme from *Paenibacillus* sp. A11 was loaded onto the separation system, and elution was as described in section 2.11.

F 80-90 = band 5 F 95-105 = band 4 F 115-135 = band 3

F 145-170 = band 2 F 175-190 = band 1+2 F 195-220 = band 1

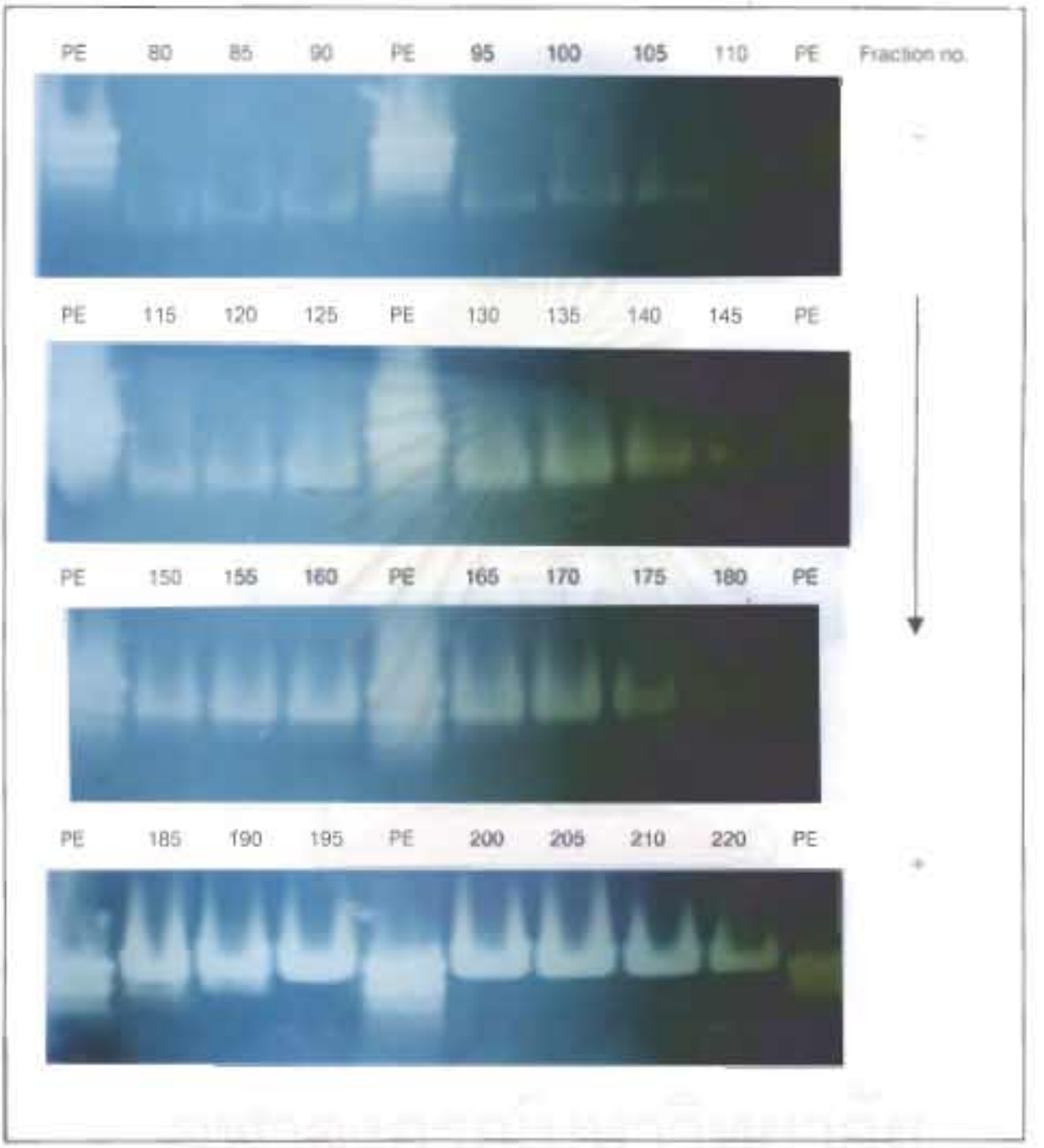


Figure 11. Non-denaturing PAGE pattern of every 5 fractions from preparative gel electrophoresis.

Staining was performed by amylolytic activity method (0.2 units of dextrinizing activity).

PE = Partially purified enzyme

F 80-90 = band 5 F 95-105 = band 4 F 115-135 = band 3

F 145-170 = band 2 F 175-190 = band 1+2 F 195-220 = band 1

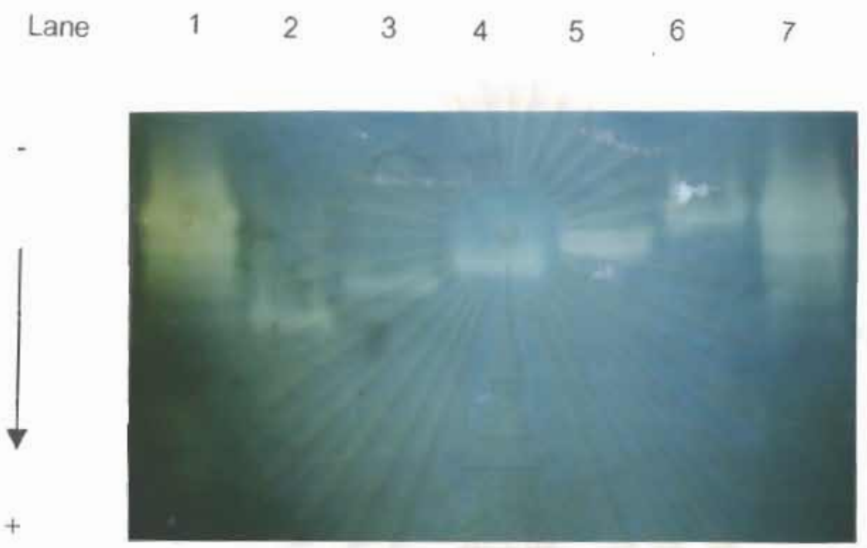


Figure 12. Non-denaturing PAGE of the activity bands 1 to 5 obtained from preparative gel electrophoresis.

Amylolytic activity staining by iodine solution (0.2 units of dextrinizing activity)

Lane 1, 7	Partially purified enzyme (Starch-adsorbed fraction)
Lane 2	Band 5
Lane 3	Band 4
Lane 4	Band 3
Lane 5	Band 2
Lane 6	Band 1

-methylorange staining, were CGTase while band 5 was not (Kaskangam, 1998). Hence, bands 1-4, namely CGTase isoforms 1 - 4, were further analyzed for their structure in this study. The pooled fractions were dialyzed against 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 and concentrated for further study.

3.3 Effect of Post-translational modification on CGTase

3.3.1 Experiment on deglycosylation

The effect of glycosylation on structure and activity of CGTase was investigated by performing deglycosylation experiments and following the change in the enzyme structure and activity when compared to that of the control. Two types of enzyme which cleave N-linked oligosaccharide : PNGase F and Endo H were used. The CGTase used was in the form of partially purified enzyme (PE) and separated isoform. The results were followed by measurement of CGTase activity and investigation of structure on non-denaturing and SDS-PAGE. Comparison was made between the control and the PNGase F or Endo H treated condition.

For investigation of O-linked glycosylation, trifluoromethanesulfonic acid (TFMS) which cleaves all O- and N-linked oligosaccharides was used. Partially purified CGTase (PE) and CGTase isoforms were incubated with TFMS. CGTase pattern was observed on SDS-PAGE and carbohydrate content was determined by phenol-sulfuric method.

3.3.1.1 Effect of PNGase F

After CGTase was treated with PNGase F, it was found that PNGase F had no effect on activity of PE, isoforms 1 and 2 (Table 9) while the activities of isoforms 3 and 4 decreased by 26-28%. When the protein pattern on non-denaturing PAGE was compared, 6,000 mU/ml PNGase F had no effect on mobility of denatured (by boiling) and nondenatured PE (Figure 13 – compared lane 3 and 4 and lane 7 and 8). This suggests that denatured and nondenatured CGTase gave the same result for PNGase F treatment. Therefore only the nondenatured form of CGTase isoforms was used in the

following studies. It was found that PNGase F also had no effect on mobility of each isoform (Figure 15 – compared lane 3 and 4 for isoform 1, lane 5 and 6 for isoform 2, lane 7 and 8 for isoform 3 and lane 9 and 10 for isoform 4). When analyzed by SDS-PAGE, also no change in mobilities of PE and CGTase isoforms were observed (Figures 14 and 16). In this experiment, human transferrin (HTF) was used as positive control. When HTF, both denatured and nondenatured, was treated with PNGase F at 3,000 mU/ml which was half of that treated with CGTase, change in mobility was found on SDS-PAGE, but not on non-denaturing PAGE. When denatured and nondenatured HTF were compared, they gave the same result on both SDS-PAGE and non-denaturing PAGE.

3.3.1.2 Effect of Endo H

After CGTase was treated with 100 mU/ml Endo H, it was found that Endo H had no effect on the activities of PE, isoforms 1, 2 and 4 (Table 10) while the activity of isoform 3 decreased by 17%. When the protein pattern on non-denaturing PAGE was compared, the mobilities of PE and all isoforms did not change after Endo H treatment (Figure 17 – compared lane 1 and 2 for isoform 1, lane 3 and 4 for isoform 2, lane 5 and 6 for isoform 3, lane 7 and 8 for isoform 4 and lane 9 and 10 for PE). When analyzed by SDS-PAGE, Endo H also had no effect on mobilities of all forms of CGTase tested (Figure 18 – compared lane 2 and 3 for isoform 1, lane 4 and 5 for isoform 2, lane 6 and 7 for isoform 3, lane 8 and 9 for isoform 4 and lane 10 and 11 for PE). Invertase and ovalbumin were used as positive control in this experiment. It was found that 50-200 mU/ml Endo H had no effect on mobility of invertase (both denatured and nondenatured forms) when analyzed by SDS-PAGE (Figure 19). When ovalbumin was used, no change was observed on non-denaturing PAGE (Figure 20) whereas significant change was found on SDS-PAGE even at 100 mU/ml Endo H (Figure 21 - compared lane 2 and 3, 4 and lane 5 and 6, 7). When denatured and nondenatured ovalbumin were compared, they gave the same result on both SDS-PAGE and non-denaturing PAGE.

Table 9. Dextrinizing activity of CGTase after deglycosylation by PNGase F

Sample	PNGase F (mU/ml)	Dextrinizing activity (U/ml)		
		control	treatment	%decrease
PE	3,000	708.2	705.2	-
Isoform 1	6,000	180.5	183.6	-
Isoform 2	6,000	61.9	60.1	-
Isoform3	6,000	70.0	49.9	28.7
Isoform 4	6,000	31.7	23.4	26.2

The activity values are averaged from two separate measurements

PE : partially purified CGTase

Table 10. Dextrinizing activity of CGTase after deglycosylation by Endo H

Sample	Endo H (mU/ml)	Dextrinizing activity (U/ml)		
		control	treatment	%decrease
PE	100	803.4	808.8	-
Isoform 1	100	350.4	348.9	-
Isoform 2	100	115.6	117.3	-
Isoform 3	100	79.5	66.0	17.0
Isoform 4	100	44.3	45.0	-

The activity values are averaged from two separate measurements

PE : partially purified CGTase

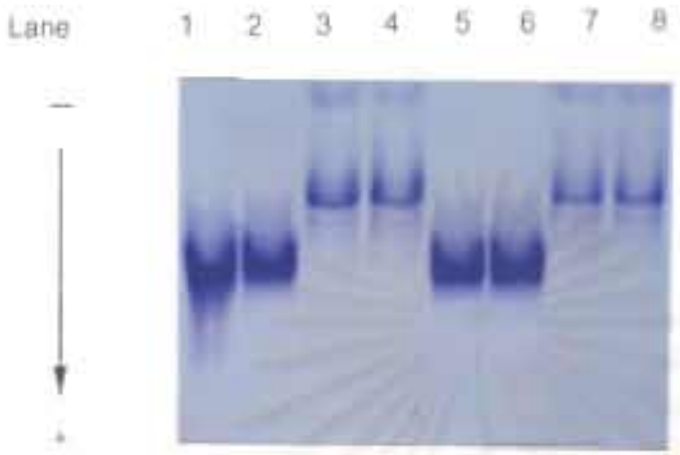


Figure 13. Non-denaturing PAGE of human-transferrin and CGTase (PE) treated with PNGase F

lane 1 nondenatured human-transferrin (positive control)

lane 2 nondenatured human-transferrin treated with PNGase F 3000 mU/ml

lane 3 nondenatured PE

lane 4 nondenatured PE treated with PNGase F 6000 mU/ml

lane 5 denatured human-transferrin (positive control)

lane 6 denatured human-transferrin treated with PNGase F 3000 mU/ml

lane 7 denatured PE

lane 8 denatured PE treated with PNGase F 6000 mU/ml

PE = partially purified enzyme

10 µg protein was loaded to each lane.

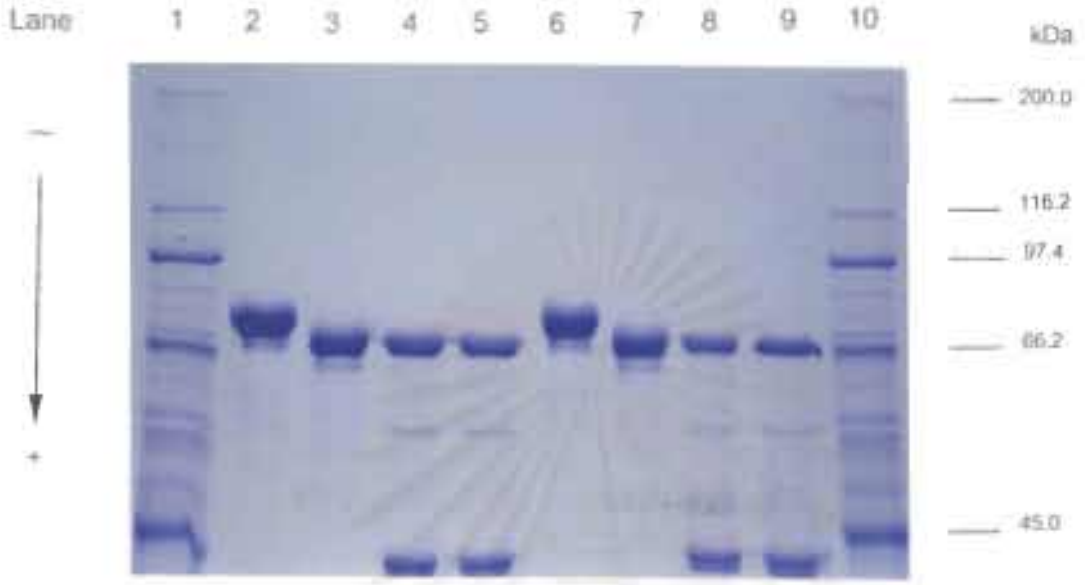


Figure 14. SDS-PAGE of human-transferrin and CGTase (PE) treated with PNGase F

lane 1,10 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

lane 2 nondenatured human-transferrin (positive control)

lane 3 nondenatured human-transferrin treated with PNGase F 3000 mU/ml

lane 4 nondenatured PE

lane 5 nondenatured PE treated with PNGase F 6000 mU/ml

lane 6 denatured human-transferrin (positive control)

lane 7 denatured human-transferrin treated with PNGase F 3000 mU/ml

lane 8 denatured PE

lane 9 denatured PE treated with PNGase F 6000 mU/ml

PE = partially purified enzyme

10 µg protein was loaded to each lane.

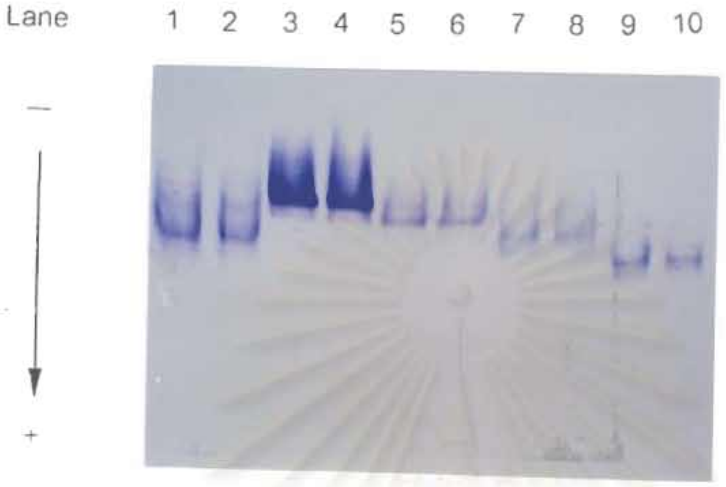


Figure 15. Non-denaturing PAGE of human-transferrin and CGTase isoforms treated with PNGase F

lane 1 nondenatured human-transferrin (positive control)

lane 2 nondenatured human-transferrin treated with PNGase F 3000 mU/ml

lane 3 nondenatured isoform 1

lane 4 nondenatured isoform 1 treated with PNGase F 6000 mU/ml

lane 5 nondenatured isoform 2

lane 6 nondenatured isoform 2 treated with PNGase F 6000 mU/ml

lane 7 nondenatured isoform 3

lane 8 nondenatured isoform 3 treated with PNGase F 6000 mU/ml

lane 9 nondenatured isoform 4

lane 10 nondenatured isoform 4 treated with PNGase F 6000 mU/ml

5 µg protein was loaded to each lane.

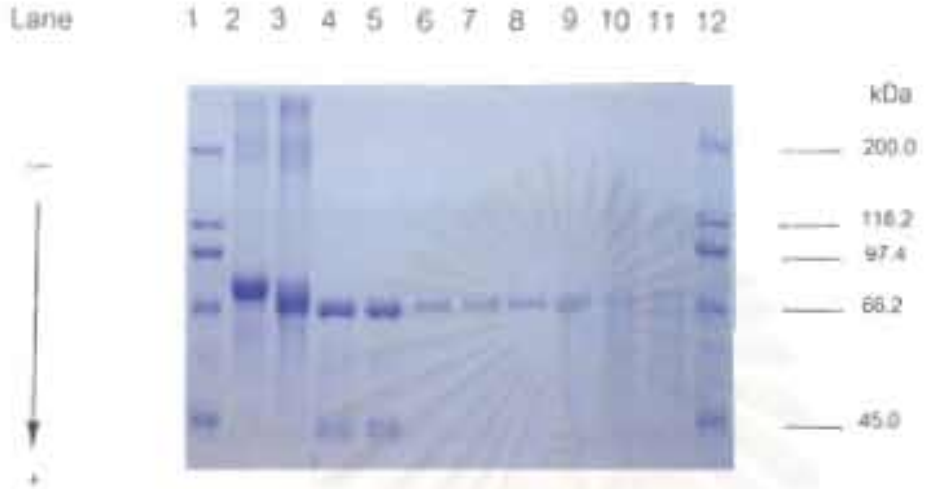


Figure 16. SDS-PAGE of human-transferrin and CGTase isoforms treated with PNGase F

lane 1,12 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

lane 2 nondenatured human-transferrin (positive control)

lane 3 nondenatured human-transferrin treated with PNGase F 3000 mU/ml

lane 4 nondenatured isoform 1

lane 5 nondenatured isoform 1 treated with PNGase F 6000 mU/ml

lane 6 nondenatured isoform 2

lane 7 nondenatured isoform 2 treated with PNGase F 6000 mU/ml

lane 8 nondenatured isoform 3

lane 9 nondenatured isoform 3 treated with PNGase F 6000 mU/ml

lane 10 nondenatured isoform 4

lane 11 nondenatured isoform 4 treated with PNGase F 6000 mU/ml

5 μg protein was loaded to each lane.

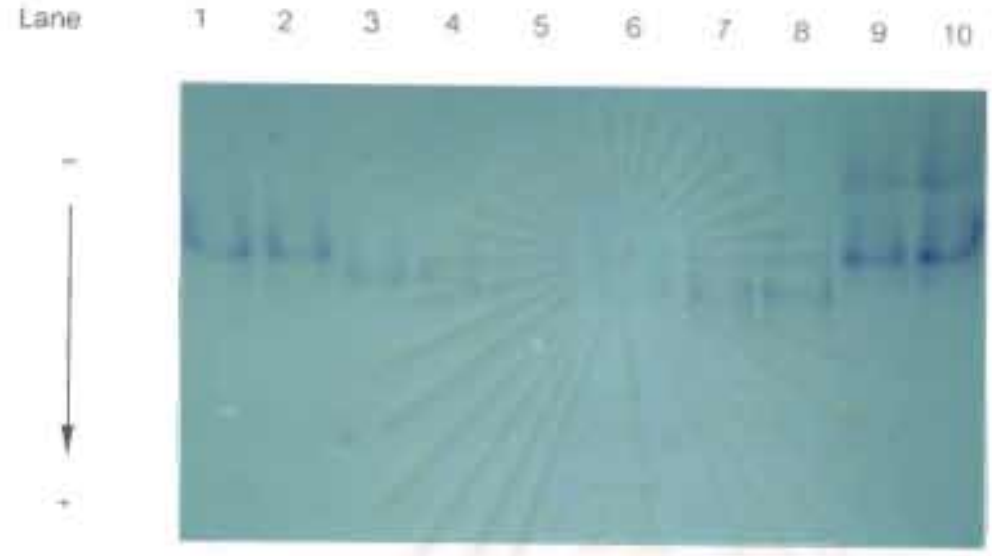


Figure 17. Non-denaturing PAGE of CGTase treated with 100 mU/ml Endo H

lane 1 isoform 1 (5 µg)
lane 2 treated isoform 1 (5 µg)
lane 3 isoform 2 (5 µg)
lane 4 treated isoform 2 (5 µg)
lane 5 isoform 3 (5 µg)
lane 6 treated isoform 3 (5 µg)
lane 7 isoform 4 (5 µg)
lane 8 treated isoform 4 (5 µg)
lane 9 PE (10 µg)
lane 10 treated PE (10 µg)

PE = partially purified enzyme

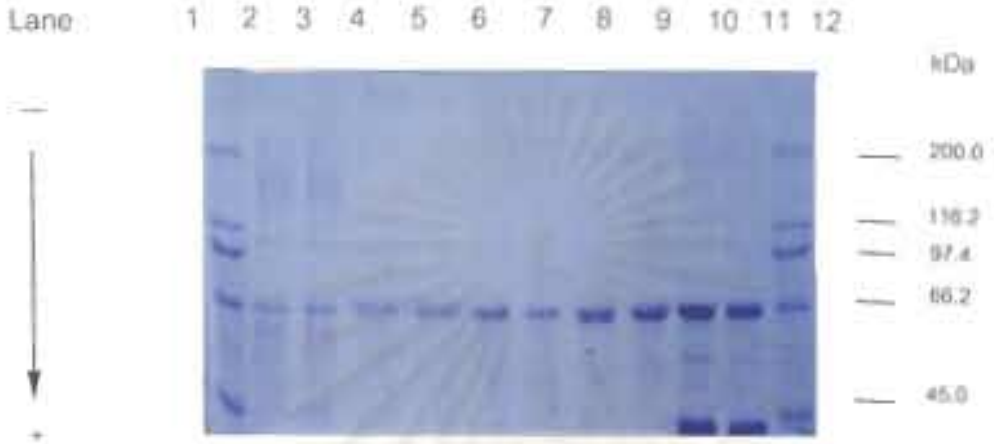


Figure 18. SDS-PAGE of CGTase treated with 100 mU/ml Endo H

lane 1,12 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

lane 2 isoform 1 (5 µg)

lane 3 treated isoform 1 (5 µg)

lane 4 isoform 2 (5 µg)

lane 5 treated isoform 2 (5 µg)

lane 6 isoform 3 (5 µg)

lane 7 treated isoform 3 (5 µg)

lane 8 isoform 4 (5 µg)

lane 9 treated isoform 4 (5 µg)

lane 10 PE (10 µg)

lane 11 treated PE (10 µg)

PE = partially purified enzyme

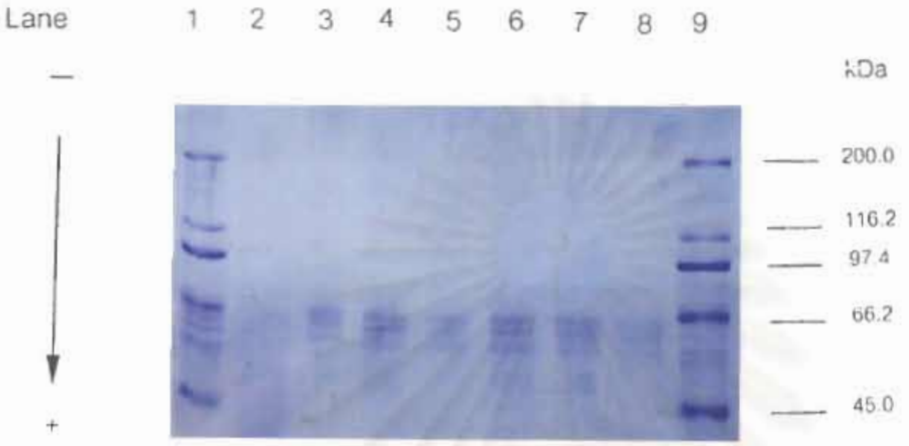


Figure 19. SDS-PAGE of invertase treated with Endo H

lane 1,9 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

lane 2 invertase

lane 3 nondenatured invertase treated with Endo H 50 mU/ml

lane 4 nondenatured invertase treated with Endo H 100 mU/ml

lane 5 nondenatured invertase treated with Endo H 200 mU/ml

lane 6 denatured invertase treated with Endo H 50 mU/ml

lane 7 denatured invertase treated with Endo H 100 mU/ml

lane 8 denatured invertase treated with Endo H 200 mU/ml

10 μg protein was loaded to each lane.

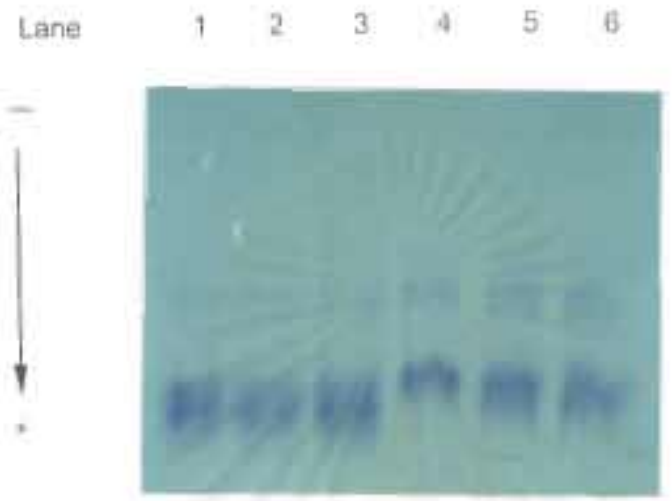


Figure 20. Non-denaturing PAGE of ovalbumin treated with Endo H

lane 1 nondenatured ovalbumin

lane 2 nondenatured ovalbumin treated with Endo H 100 mU/ml

lane 3 nondenatured ovalbumin treated with Endo H 500 mU/ml

lane 4 denatured ovalbumin

lane 5 denatured ovalbumin treated with Endo H 100 mU/ml

lane 6 denatured ovalbumin treated with Endo H 500 mU/ml

10 µg protein was loaded to each lane

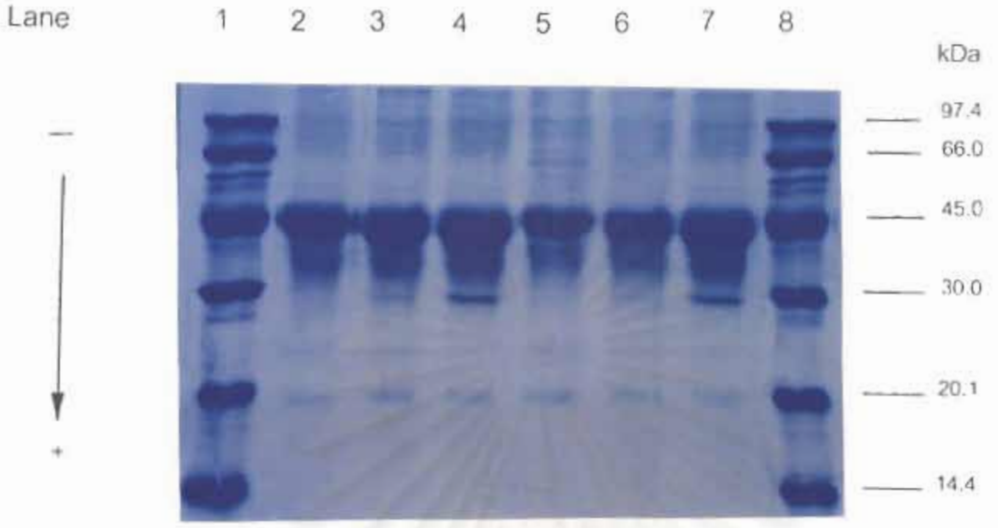


Figure 21. SDS-PAGE of ovalbumin treated with Endo H

lane 1,8 Protein molecular weight marker [phosphorylase b (97.4 kDa), albumin 66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)]

lane 2 nondenatured ovalbumin

lane 3 nondenatured ovalbumin treated with Endo H 100 mU/ml

lane 4 nondenatured ovalbumin treated with Endo H 500 mU/ml

lane 5 denatured ovalbumin

lane 6 denatured ovalbumin treated with Endo H 100 mU/ml

lane 7 denatured ovalbumin treated with Endo H 500 mU/ml

10 μg protein was loaded to each lane.

3.3.1.3 Effect of TFMS

When CGTase was treated with TFMS and the protein pattern on SDS-PAGE was analyzed, it was found that TFMS had no effect on CGTase (Figure 22 – compared lane 2 and 3 for PE, lane 4 and 5 for isoform 1, lane 6 and 7 for isoform 2, lane 8 and 9 for isoform 3 and lane 10 and 11 for isoform 4). In this experiment, two types of protein which had O-linked oligosasccharides, peroxidase (partially purified form) from cassava root and royal jelly protein (highly purified form) were used as positive control. No difference on mobilities of royal jelly protein after treated with TFMS for 0, 2, 4 and 24 hours were observed (Figure 23). The same result was also found with cassava root peroxidase.

When no change in protein mobility was observed, even with the positive control, sugar content before and after treatment was then determined. It was found that sugar content of peroxidase and royal jelly protein decreased by 40 and 20 %, respectively, after incubation with TFMS for 4 hours (Figure 24) while no significant change was observed in all forms of CGTase (Table 11).

3.3.2 Experiments on dephosphorylation

Investigation of the effect of phosphorylation on structure of CGTase was performed by carrying out dephosphorylation reactions using *E.coli* alkaline phosphatase. The CGTase used was in the form of partially purified enzyme (PE) and separated isoform. The result was followed by analysis on non-denaturing PAGE. Comparison was made between the control and the phosphatase treated.

Ovalbumin was used as positive control. When ovalbumin was treated with alkaline phosphatase at various ratios of phosphatase : ovalbumin (1:20, 1:10 and 1:5 (by wt.)), change in mobility was found with the ratio 1:10 and 1:5 on non-denaturing PAGE but not on SDS-PAGE. This suggested that dephosphorylation of ovalbumin by alkaline phosphatase at the minimum ratio of 1:10 (by wt.) could be detected by charge difference from the phosphorylated form when analyzed by non-denaturing PAGE. So

the ratio of 1:10 (by wt.) was used for CGTase treatment and the reaction followed by non-denaturing PAGE.

When CGTase was treated with alkaline phosphatase and the protein pattern on non-denaturing PAGE was analyzed, it was found that alkaline phosphatase had no effect on CGTase (Figure 27 - compared lane 1 and 2 for isoform 1, lane 3 and 4 for isoform 2, lane 5 and 6 for isoform 3, and lane 7 and 8 for isoform 4). This suggests that dephosphorylation of CGTase, if occurred, had no significant effect on charge and size of the protein. There was also possibility that no dephosphorylation occurred when CGTase was treated with alkaline phosphatase.

3.3.3 Disulfide bond formation

Whether the disulfide bond formation, either within the same CGTase molecule or between different CGTase molecule, was the cause of isoform pattern could be proved by comparing structure of partially purified enzyme (PE) in the absence or presence of a reducing agent, β-mercaptoethanol or DTT. The experiment was performed by incubation of the enzyme with 5% β-mercaptoethanol and 5 mM DTT, then the results were followed by non-denaturing and SDS-PAGE. It was found that mobility of protein bands was not change on both PAGE (Figure 28).

3.3.4 Modification of carboxyl residues

Since CGTase contained high Asp/Asn and Glu/Gln (Table 14) content, charge of carboxylic group to amide group or vice versa is possible as post-translational modification. However, to perform the experiment to change carboxylic group to amide group or amide group to carboxylic group is difficult because no commercial enzyme was available and chemical treatment was not specific only for these residues. The indirect way was performed by blocking carboxylic group with a group-specific reagent, EDC. All isoforms of CGTase were modified by 5 mM EDC and investigated by the non-denaturing polyacrylamide gel electrophoresis. The gel was stained for dextrinizing activity (Figure 29). The partially purified enzyme prior to modification showed three

major bands and one faint band (labeled a, b, c, and d, respectively) as indicated in lane 1. After modification, an extra band above label "a" (lane 2) was observed. Unmodified isoforms 1 to 4 showed one band each at the position of a, b, c and d, respectively (lane 3, 5, 7 and 9, respectively). While the modified isoforms moved slower than the unmodified, in every cases (lane 4, 6, 8, and 10). This result indicates the importance of changes in net charge by modification of carboxyl residues on enzyme mobility on non-denaturing PAGE. In addition, carboxylic groups modification had no effect on activity of each isoform as evidenced by positive activity staining in every cases.

3.4 Digestion of CGTase isoforms and separation of peptides

To investigate whether CGTase isoforms were rooted from one gene or had the same amino acid sequence, comparison of the peptides resulted from isoforms digestion were made. Four CGTase isoforms were treated with trypsin and then the peptides were investigated by reverse phase C_{18} HPLC column as described in section 2.17. The results (Figure 30 a-d) showed peaks of digested peptides of isoforms 1 to 4, respectively. When the peaks recorded within 30 minutes were compared, all profiles showed four high peaks (about 400-1000 milli absorbance unit, mAU) at the retention time (R_t) of 2.8 to 4.3 minutes. These peaks should be solvent peaks. All isoforms exerted 12 peptide peaks in the range of 10-100 mAU at R_t 6.4-27.6 minutes, most of them had similar R_t while some had different peak height. Peak height level from +1 to +5 was assigned and compared as shown in Table 12.

A noticeable peak of isoform 3 at R_t 10.8 minutes with peak height level +4 was observed while only small peak (not over 10 mAU) was observed at the same retention time in the other isoforms. In addition, when recording time was extended to 40 minutes, all isoforms showed similar broad peak in the range of 34-36 minutes and a high peak at 36.6 minutes (Figure 30 –e) with the absorbance about 300-400 mAU. These results suggest that the isoforms were very similar or even identical in terms of amino acid sequence.

Figure 22. SDS-PAGE of CGTase treated with TFMS

lane 1,12 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

lane 2 PE (10 μg)

lane 3 treated PE (10 μg)

lane 4 isoform 1 (5 μg)

lane 5 treated isoform 1 (5 μg)

lane 6 isoform 2 (5 μg)

lane 7 treated isoform 2 (5 μg)

lane 8 isoform 3 (5 μg)

lane 9 treated isoform 3 (5 μg)

lane 10 isoform 4 (5 μg)

lane 11 treated isoform 4 (5 μg)

PE = partially purified enzyme

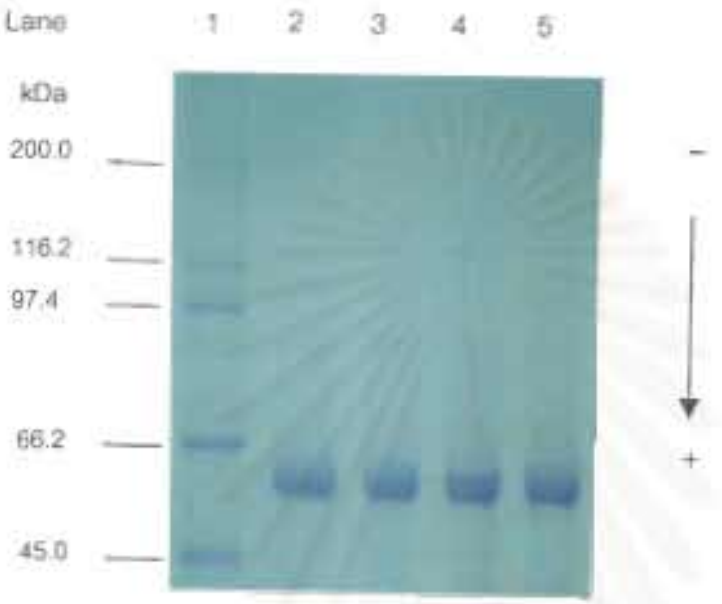


Figure 23. SDS-PAGE of royal jelly protein treated with TFMS

lane 1 Protein molecular weight marker [myosin (200.0 kDa, β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa)]

lane 2 royal jelly protein

lane 3 treated royal jelly protein (2 hr)

lane 4 treated royal jelly protein (4 hr)

lane 5 treated royal jelly protein (24 hr)

5 µg protein was loaded to each lane

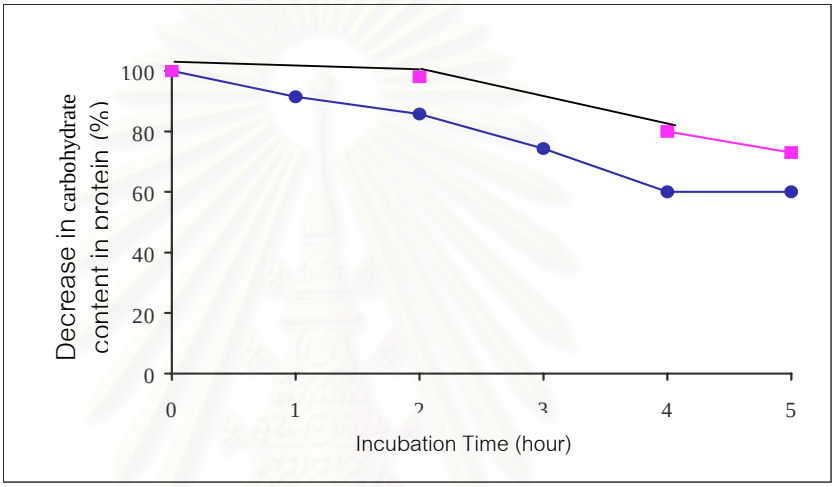


Figure 24. Decrease in carbohydrate content in proteins after incubation with TFMS for different time period.

Peroxidase Royal jelly protein

Table 11. Carbohydrate content of CGTase treated by TFMS

CGTase	Carbohydrate content (mg/mg protein)		
	0 h	5 h	24 h
PE	1.80	1.58	1.83
Isoform 1	0.88	1.01	0.93
Isoform 2	0.85	0.96	0.79
Isoform 3	2.27	2.01	2.06
Isoform 4	2.64	3.21	3.14

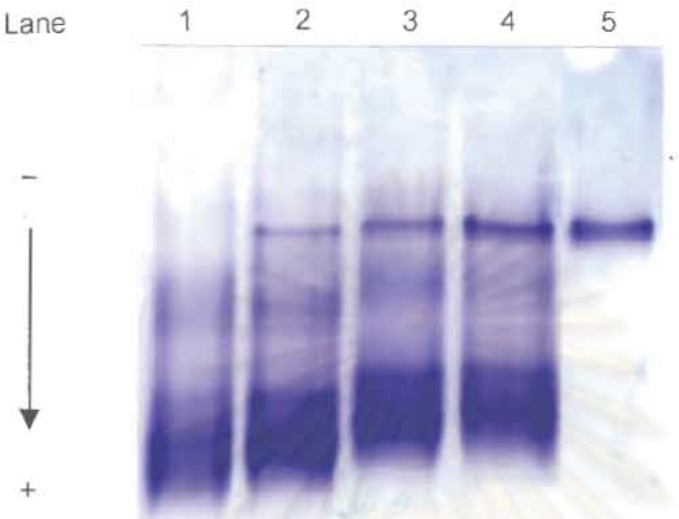


Figure 25. Non-denaturing PAGE* of ovalbumin treated with alkaline phosphatase.

lane 1 ovalbumin

lane 2 treated ovalbumin (ratio of 1:20 by wt.)

lane 3 treated ovalbumin (ratio of 1:10 by wt.)

lane 4 treated ovalbumin (ratio of 1:5 by wt.)

lane 5 alkaline phosphatase

*12% Separating gel was used

10 μg protein was loaded to each lane.

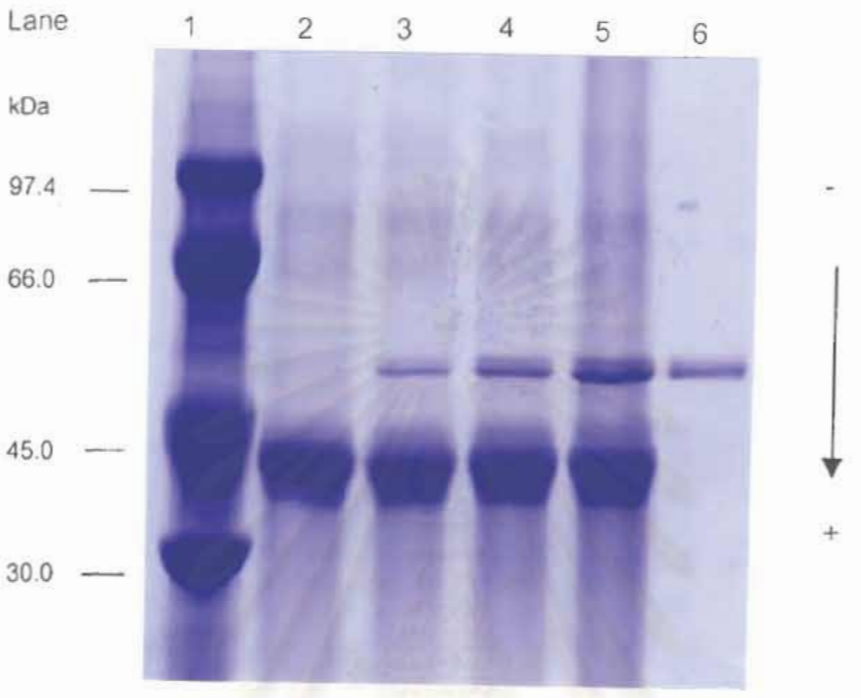


Figure 26. SDS-PAGE* of ovalbumin treated with alkaline phosphatase

lane 1 Protein molecular weight marker [phosphorylase b (97.4 kDa), albumin 66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)]

lane 2 ovalbumin

lane 3 treated ovalbumin (ratio of 1:20 by wt.)

lane 4 treated ovalbumin (ratio of 1:10 by wt.)

lane 5 treated ovalbumin (ratio of 1:5 by wt.)

lane 6 alkaline phosphatase

* 12% Separation gel was used.

10 μg protein was loaded to each lane.

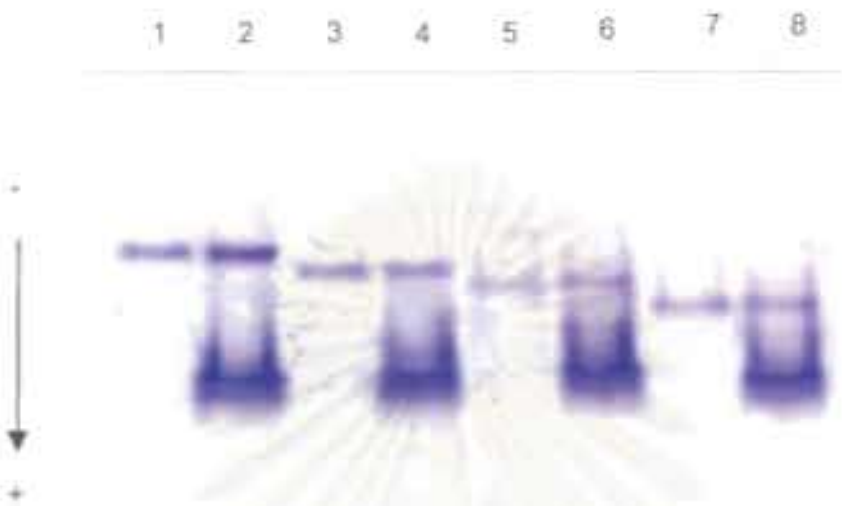


Figure 27. Non-denaturing PAGE of CGTase treated with alkaline phosphatase at alkaline phosphatase : CGTase ratio of 1:10 by wt.

lane 1 isoform 1

lane 2 treated isoform 1

lane 3 isoform 2

lane 4 treated isoform 2

lane 5 isoform 3

lane 6 treated isoform 3

lane 7 isoform 4

lane 8 treated isoform 4

7% Separating gel was used

5 µg protein was loaded to each lane.

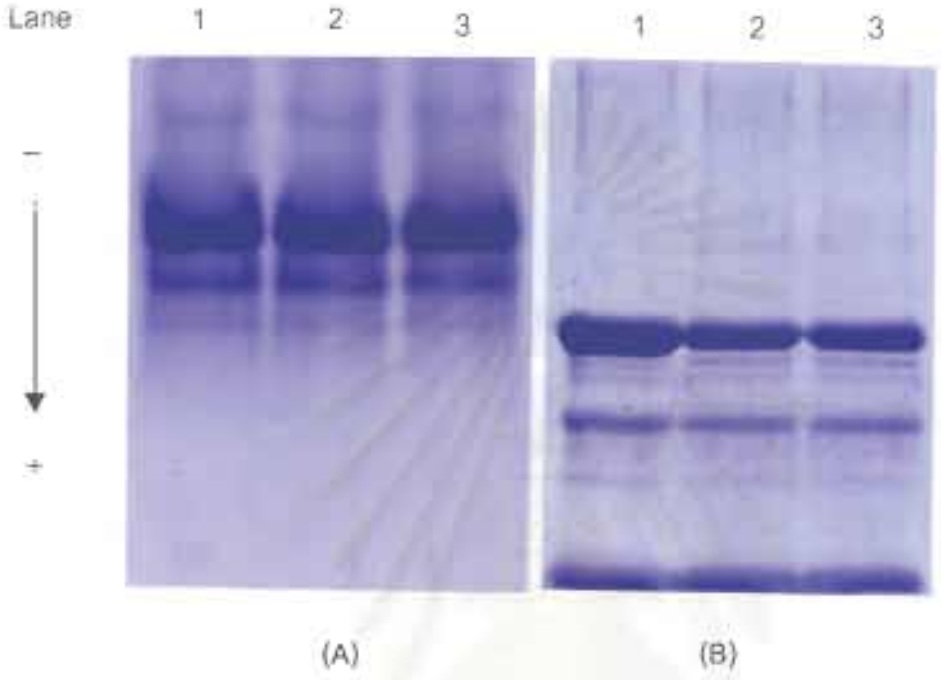


Figure 28. Non-denaturing (A) and SDS-PAGE (B) of CGTase (10 μg protein) treated with 5% β-mercaptoethanol and 5 mM DTT.

Lane 1	PE
Lane 2	PE treated with β-mercaptoethanol
Lane 3	PE treated with DTT

PE : partially purified enzyme

10 μg protein was loaded to each lane.

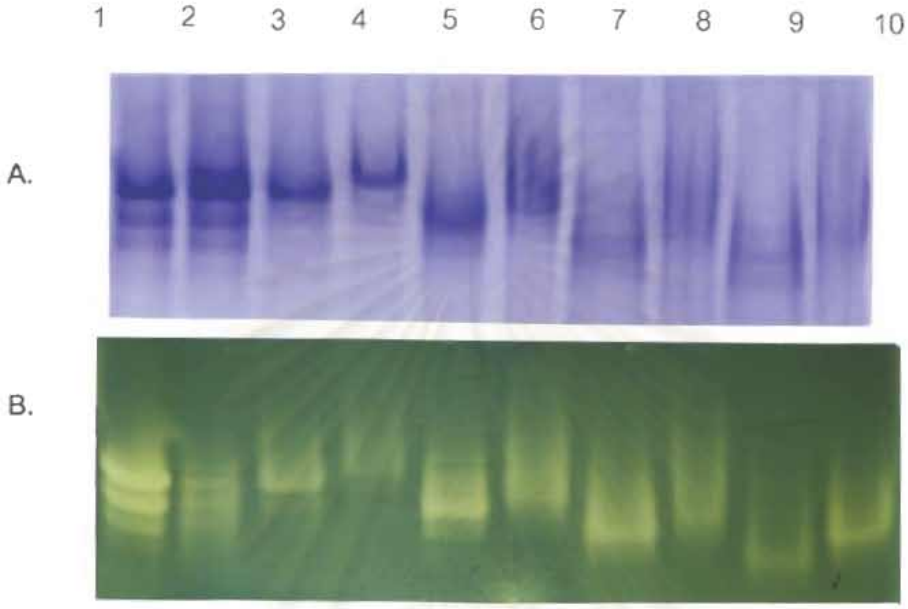


Figure 29. Non-denaturing PAGE of CGTase modified by 5 mM EDC.

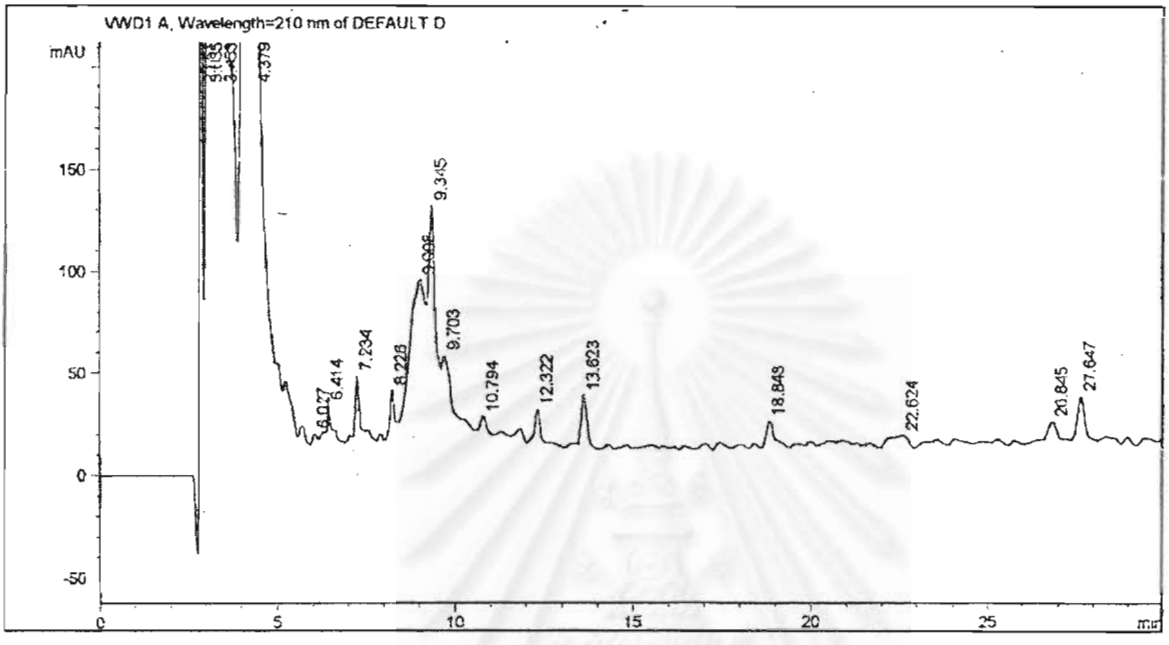
Lane 1	PE
Lane 2	modified PE
Lane 3	isoform 1
Lane 4	modified isoform 1
Lane 5	isoform 2
Lane 6	modified isoform 2
Lane 7	isoform 3
Lane 8	modified isoform 3
Lane 9	isoform 4
Lane 10	modified isoform 4

(A) Coomassie Blue Staining (5 μg protein was loaded to each lane)

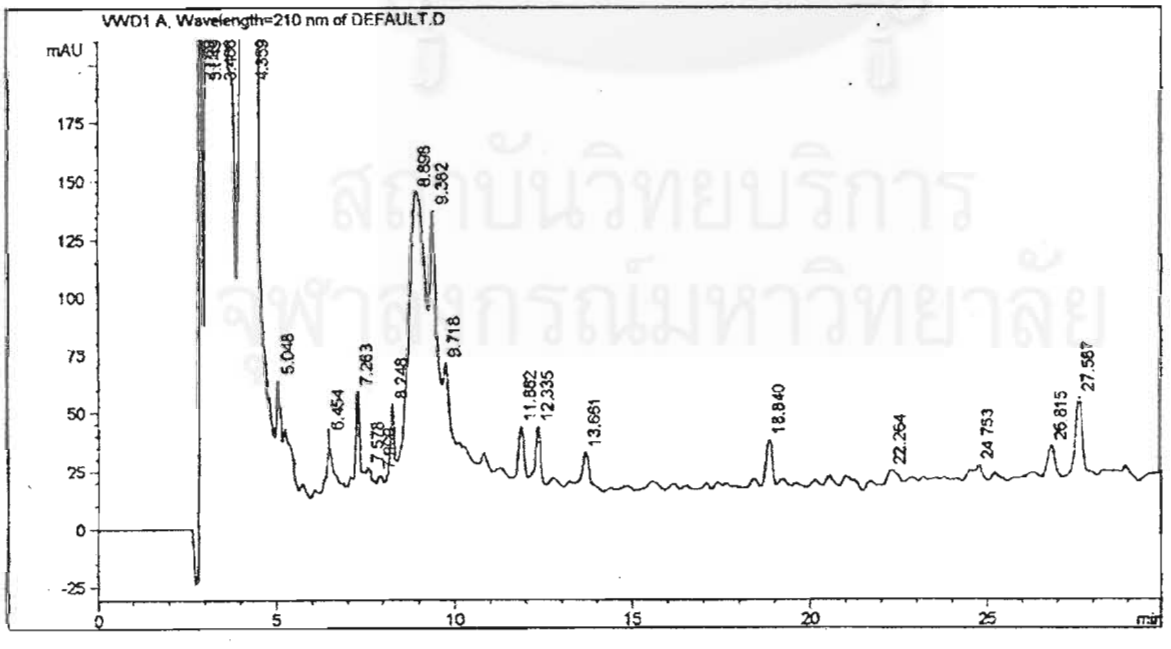
(B) Amylolytic activity staining (0.2 units of dextrinizing activity).

Figure 30. HPLC chromatograms from reverse phase C_{18} column of the peptides of CGTase isoforms resulting from digestion with trypsin

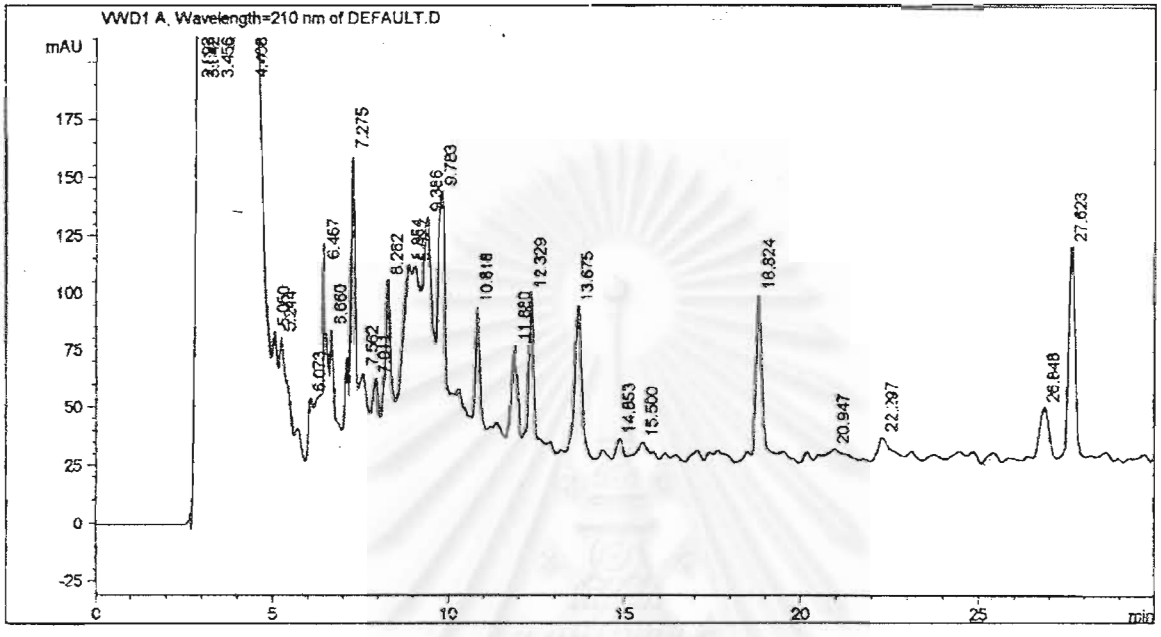
a-d; digested products of isoforms 1 to 4, respectively. (recording time 30 min)
e; digested products of isoform 3 (recording time 40 min)



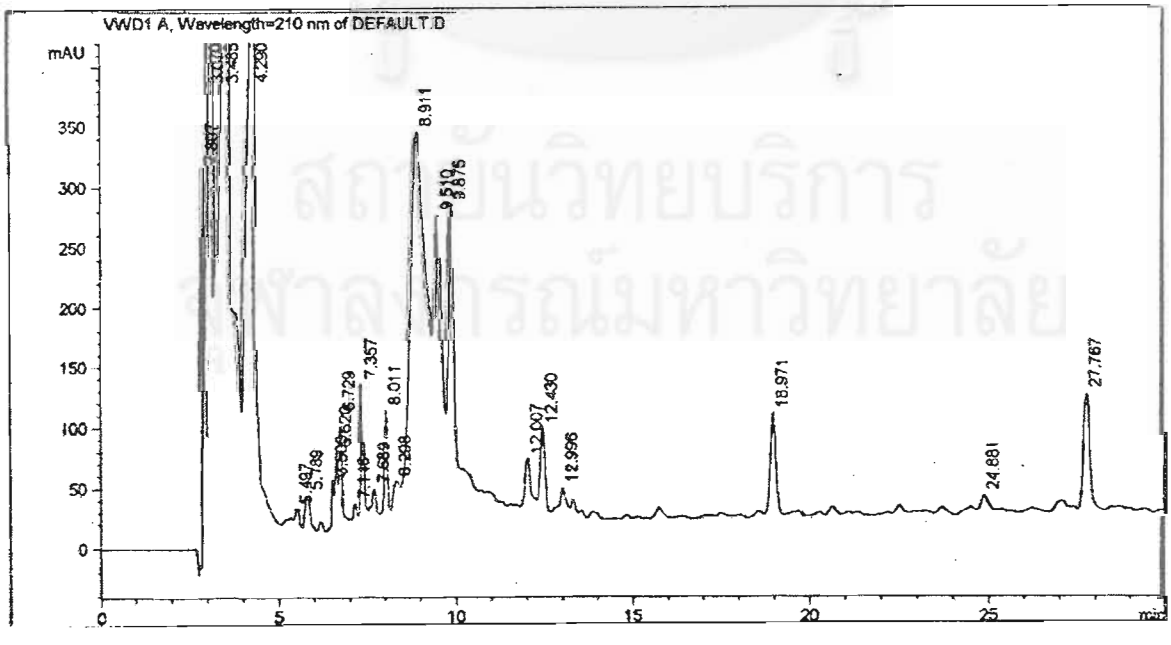
a. digested products of isoforms 1 (recording time 30 min)



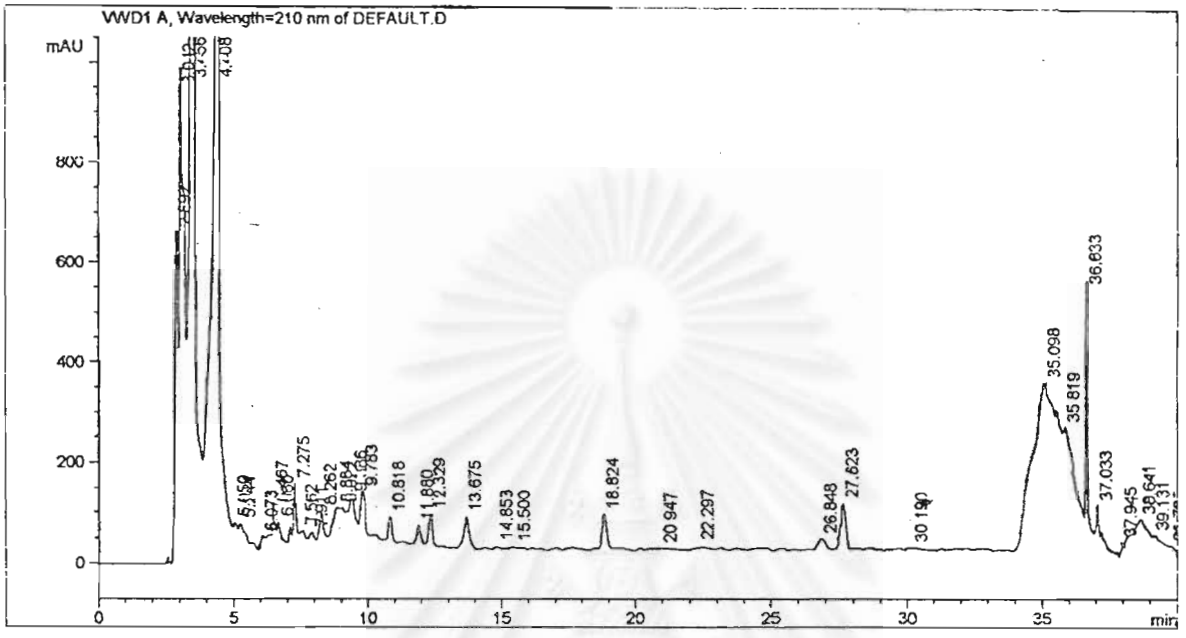
b. digested products of isoforms 2 (recording time 30 min)



c. digested products of isoforms 3 (recording time 30 min)



d. digested products of isoforms 4 (recording time 30 min)



e. digested products of isoforms 3 (recording time 40 min)

Table 12. Summary of notable peptide peaks from digested products of CGTase isoforms

Isoform	R_t of peptide peaks (minutes)												
1	6.4	7.2	8.2	9.0	9.3	9.7		10.7	12.3	13.6	18.8	26.8	27.6
	+3	+3	+3	+4	+5	+3		↓	+2	+3	+2	+1	+3
2	6.4	7.2	8.2	8.8	9.3	9.7		11.8	12.3	13.6	18.8	26.8	27.5
	+2	+2	+2	+5	+4	+3		+3	+3	+2	+3	+2	+3
3	6.4	7.2	8.2	9.0	9.3	9.7	10.8	11.8	12.3	13.6	18.8	26.8	27.6
	+4	+5	+4	+4	+4	+4	+4	+3	+4	+4	+4	+3	+5
4	6.7	7.3	8.0	8.9	9.5	9.8		12.0	12.4	13.2	18.9	27.0	27.7
	+3	+3	+3	+5	+4	+4		+1	+2	↓	+3	↓	+3

Number under R_t values indicate peak height, highest at +5 to lowest at +1

↓ means very low peak height, lower than 10 mAU

3.5 Analysis of CGTase isoforms by HPLC

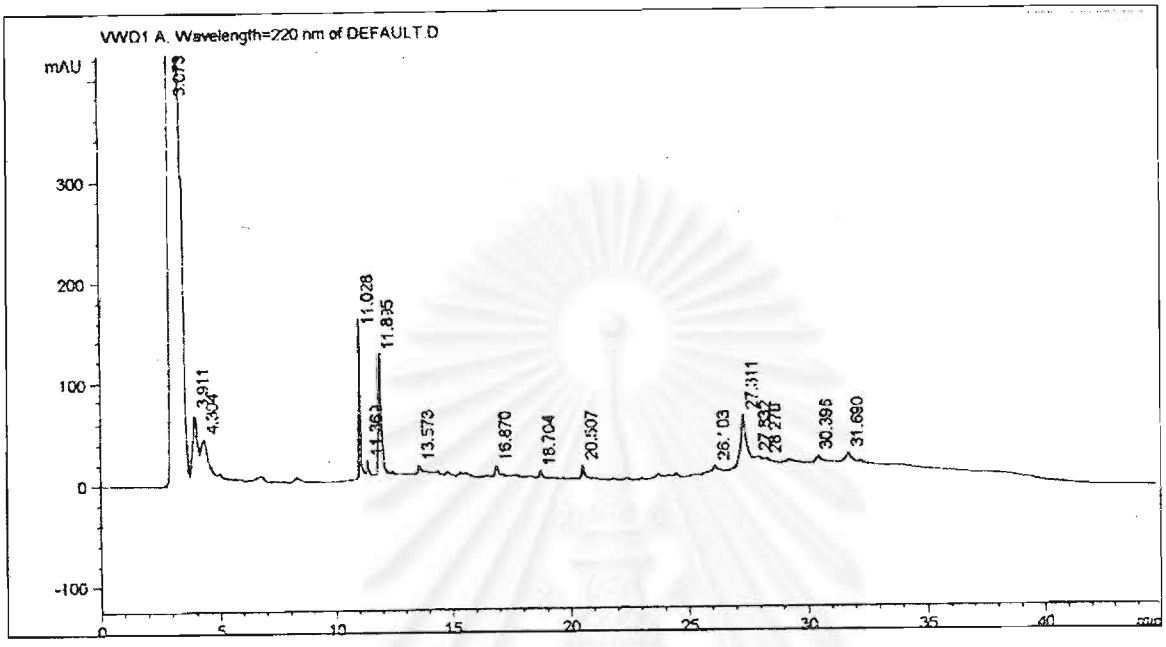
To analyze the native intact form of CGTase isoforms which were separated by preparative gel electrophoresis, each isoform was separated by HPLC on a C_4 reversed phase column. The HPLC profiles of the enzymes were shown in Figure 31. Detection of protein constituents in CGTase isoforms was performed at 220 nm. All profiles showed peaks at retention time (R_t) of 3.0 to 4.3 minutes which should be solvent peaks. When these profiles were compared, it was found that all profiles exerted 2 main peaks eluting at retention time of 10 to12 minutes. There were some minor shift in retention time of the same peak in different HPLC profiles. These peaks were R_t 11.028 and 11.859 minutes for isoform 1, R_t 10.943 and 11.951 minutes for isoform 2, R_t 10.954 and 11.955 minutes for isoform 3 and R_t 10.924 and 11.959 minutes for isoform 4. The peak height of R_t 11.028 minutes was higher than R_t 11.895 minutes for isoform 1 while the peak height of R_t 11.951-11.959 minutes was higher than R_t 10.924-10.954 minutes for other isoforms. Peak area ratios of these two peaks in each isoform were shown in Table 13. Isoforms 2, 3 and 4 contained the two peaks with similar ratio of 1 : 4.66 – 1 : 5.08. While isoform 1 was different, it was consisted of 1 : 1.94 peak I : peak II.

3.6 Amino acid composition of CGTase isoforms

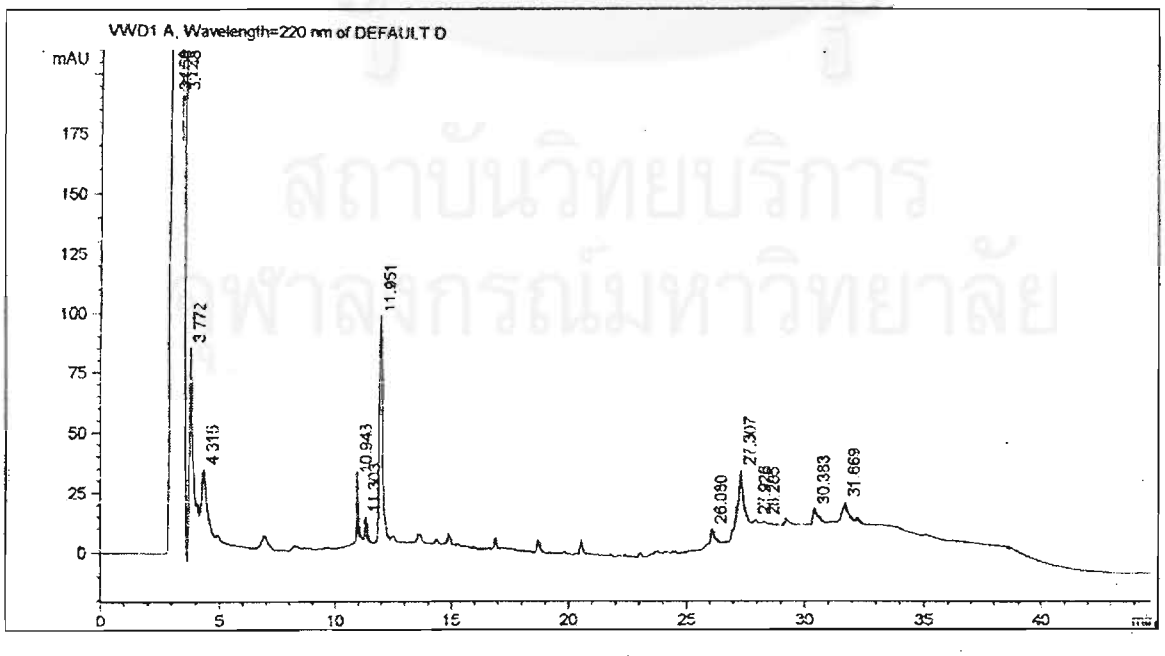
Four CGTase isoforms which were obtained from preparative gel electrophoresis were dialyzed against distilled water to remove Tris-glycine buffer, the electrode buffer for electrophoresis. The enzymes were hydrolyzed with 6N HCl at 110 $^{\circ}$C for 24 hours and were derivatized with 6-amino quinolyl-N-hydroxy succinimidyl carbamate. In this method, tryptophan and cysteine could not be detected. The amino acid mixtures obtained were analyzed on reverse phase HPLC on Nova-Pak™ C_{18} and calculated for their amino acid compositions. Chromatograms of various amino acid standards and enzyme samples were shown in Appendix F. The amino acid compositions of isoforms 1 to 4 were presented in Table 14. The result showed that their amino acid compositions were slightly different. The lowest difference was in Thr content (0.5 mol% among all

Figure 31. HPLC chromatograms from reverse phase C_4 column of protein constituents in CGTase isoform.

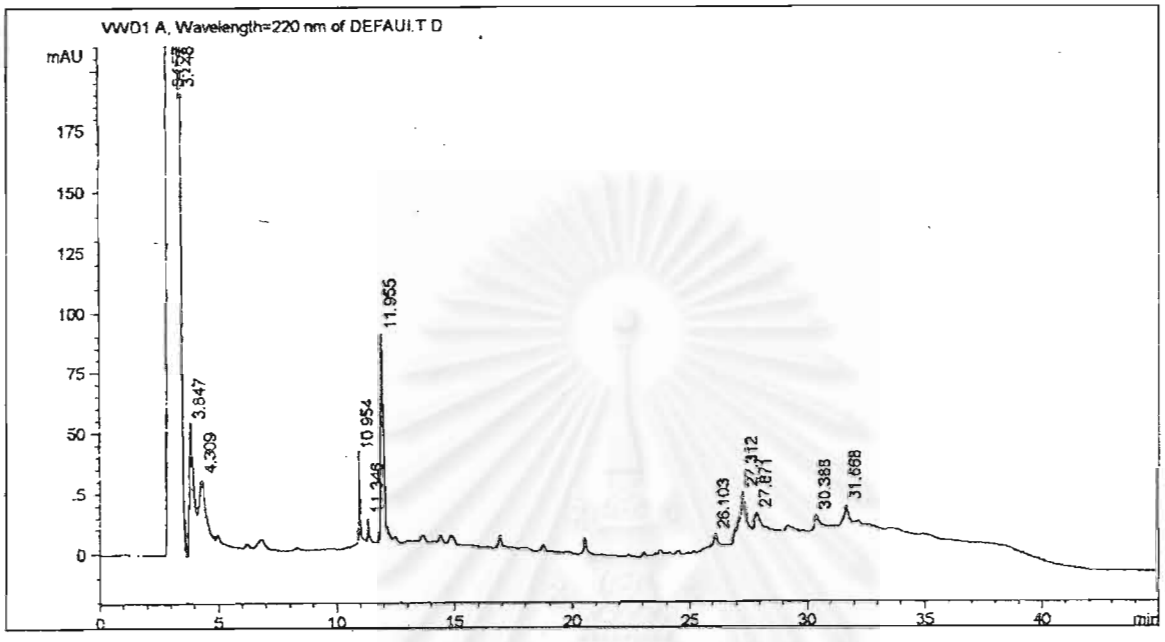
a-d; protein constituents of isoform 1 to 4, respectively. (recording time 40 min)



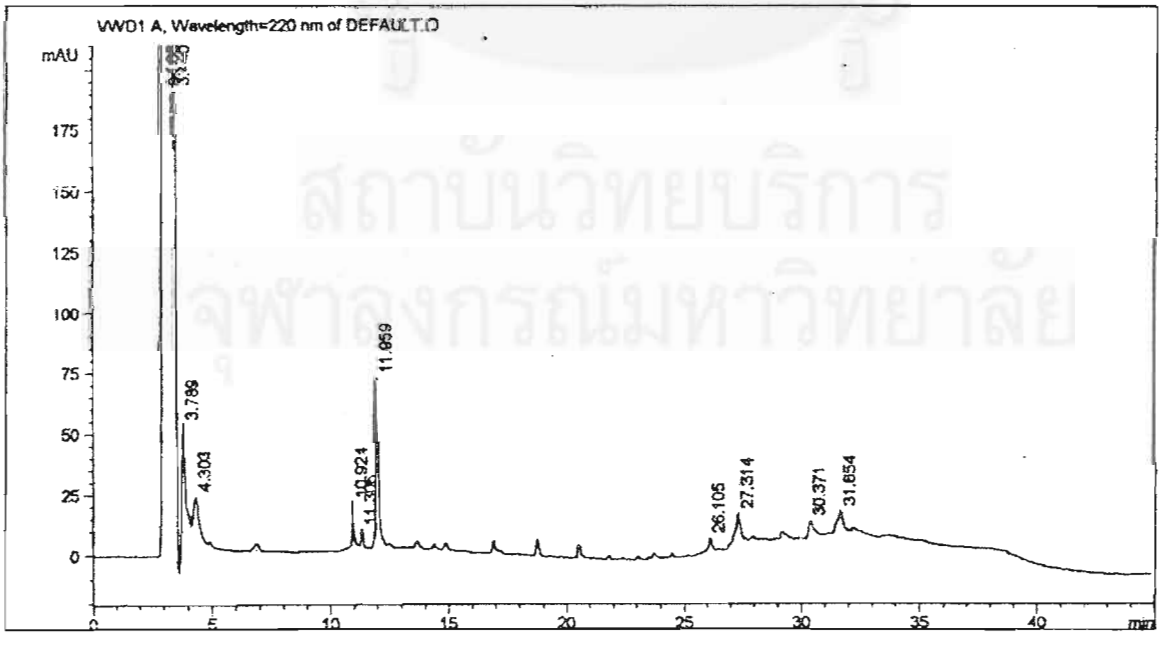
a. isoform 1



b. isoform 2



c. isoform 3



d. isoform 4

Table 13. Ratio of major peaks from analysis of CGTase isoforms by reverse phase C_4 column.

Isoform	R_t as min/(peak area)		Peak area ratio
	peak I	peak II	I : II
1	11.028	11.895	1 : 1.94
	(1.5520)	(3.0039)	
2	10.943	11.951	1 : 4.66
	(0.6774)	(3.1544)	
3	10.954	11.955	1 : 5.08
	(0.5422)	(2.7552)	
4	10.924	11.959	1 : 4.84
	(0.5728)	(2.7558)	

Table 14. Amino acid composition of CGTase isoforms.

Amino acid	Content (mol %)			
	Isoform1	Isoform2	Isoform3	Isoform4
Asp	8.78	10.92	9.02	9.56
Ser	9.22	7.19	8.39	5.96
Glu	13.52	14.00	16.42	12.25
Gly	15.45	11.86	14.07	21.46
His	3.59	2.24	2.18	3.09
Arg	4.55	4.89	4.29	4.04
Thr	6.25	6.65	6.15	6.35
Ala	10.31	10.43	10.62	8.50
Pro	5.00	5.42	4.68	4.85
Tyr	2.07	2.41	1.90	1.72
Val	5.60	6.63	6.65	6.83
Met	0	0	0	0
Lys	3.08	3.49	3.68	3.73
Ile	3.66	4.01	3.12	3.08
Leu	5.82	6.19	6.09	5.63
Phe	3.10	3.65	2.73	2.95

isoforms) while the highest difference was in Glu/Gln content (4.1 mol%). These difference might arise from degree of purity of the isoforms or different methods and instruments used for composition analysis.

3.7 N-terminal sequence of CGTase isoform

The partially purified CGTase after starch adsorption and ultrafiltration was separated into four isoforms by non-denaturing polyacrylamide gel electrophoresis. Then the gel was removed from the electrophoresis cell and soaked in electroblotting buffer for 5 minutes. The electroblotting was performed with the same buffer under a constant voltage current of 50 volts at room temperature for 90 minutes. After electroblotting, the protein bands in the PVDF membrane was briefly stained with Coomassie blue and destaining with 50% methanol until protein bands appeared. The four bands of CGTase isoforms was excised and subjected for sequencing.

The N-terminal amino acid sequences of the four isoforms were shown in Table 15. The sequence profiles were shown in Appendix G. Isoforms 1 and 2 were consisted of more than 1 amino acid residue at almost all positions. At certain positions, e.g. residue no. 1-2 in isoform 1 or residue no. 1-4 in isoform 2, the amino acid found were of 2-3 types which belonged to the major sequences. This results suggest that isoforms 1 and 2 were consisted of 2-3 types of protein with different N-terminal sequences. It was observed that the four N-terminal amino acid residues of isoform 1 were very closed to that of isoform 2. For isoforms 3 and 4, one major amino acid sequence was found. When compared isoforms 3 and 4 with isoforms 1 and 2, very similar amino acid sequence was observed.

Since obscure result was obtained in the determination of N-terminal sequence from the sample prepared by blotting the isoform bands from non-denaturing gel when partially purified (unfractionated enzyme) was used, the experiment was performed by another way to check reproducibility of the result. N-terminal sequence was determined again but this time the sample was prepared by blotting each fractionated isoform

Table 15. N-terminal amino acid residues of CGTase isoforms

Residue	CGTase Isoforms			
number	1	2	3	4
1	Asp, Gly, Ala(Leu)	Asp,Leu(Gly,Ala)	Gly, Leu (Asp,Ala)	Leu (Gln, Gly)
2	Thr, Pro, Val (Phe)	Ala, Asn, Val	Asn (Pro, Val)	Ala (Asn, Val)
3	Thr (Lys, Val)	Thr, Gln, Lys(Val)	Lys	Gln (Lys, Thr)
4	Leu (Asn, Phe)	Leu, Pro, Asn	Thr (Asn, Leu)	Asn (Pro)
5	Thr (Lys, Val)		Lys, Thr(Val)	Lys (Thr)
6	Val		Val (Lys)	Val Pro
7	Gln (Lys, Glu)			
8	Gln, Glu, Val(Asn)			
9	Lys, Val			
10	Phe			

Residues in brackets are of minor sequences

(obtained from preparative gel electrophoresis) from SDS polyacrylamide gel. The amino acid sequence found was A P D T S V S N K for isoform 1 and A P D T S for isoforms 2, 3 and 4.

3.8 Analysis of CGTase isoforms by two dimensional polyacrylamide gel electrophoresis

To investigate whether CGTase isoforms were rooted from a single form, two dimensional polyacylamide gel electrophoresis was performed. The partially purified enzyme was denatured in sample buffer before subjected to SDS-PAGE. After that the denatured enzyme was renatured when the gel was washed with Triton X-100 in 0.2 M phosphate buffer, pH 6.0. A slice of SDS polyacrylamide gel containing renatured enzyme was transferred to non-denaturing PAGE. With amylolytic activity staining, one intense band was observed from the 1^{st} dimension SDS-PAGE while four activity bands was observed in the 2^{nd} dimension non-denaturing PAGE (Figure 32). The result shows that the denatured form of partially purified enzyme can be renatured to four isoforms.

3.9 Kinetic parameters of CGTase isoform 1

Kinetic parameters for some substrates : α-, β- and γ- cyclodextrins, derivatives of β-cyclodextrin, and oligosaccharides (G_3-G_7) were determined with the purified CGTase isoform 1 as described in section 2.12.1 and 2.15.2. Figure 32 showed the Lineweaver-Burk plot of CGTase isoform1 with β- cyclodextrin as substrate. This pattern was typical plots for all substrates and the summarized result was shown in Table 16.

For CD and derivatives, K_m and V_{max} were obtained by using variable concentrations of six different substrates (α- CD, β- CD, γ- CD, maltosyl (G_2)- β-CD, methyl-β-CD and hydroxylpropyl-β-CD) at 10 mM cellobiose. Values obtained for different variable substrates were 0.063 ± 0.01, 0.10 ± 0.01, 0.12 ± 0.02, 0.19 ± 0.01, 0.16 ± 0.04 and 0.07 ± 0.02 mM for K_m and 35.14 ± 1.92, 14.00 ± 2.68, 25.86 ± 4.43, 15.70 ± 1.97, 18.02 ± 0.37 and 7.15 ± 2.28 μmoles/min for V_{max}, respectively, . α- CD demonstrated the lowest K_m and the highest V_{max}. In addition, the values of K_m/V_{max} and

k_{cat}/K_m were also determined. Values obtained were 558 ± 50, 140 ± 12, 216 ± 10, 83 ± 6, 113 ± 21 and 102 ± 28 μmoles.min^{-1}.mM^{-1} for K_m/V_{max} and 3368 ± 300, 845 ± 70, 1301 ± 50, 499 ± 35, 680 ± 125 and 617 ± 169 mM^{-1}min^{-1} for k_{cat}/K_m, respectively. α- CD demonstrated the highest V_{max}/K_m and k_{cat}/K_m. This result suggests that the enzyme-catalyzing rate was the best when α- CD was the substrate for coupling reaction.

For oligosaccharide substrates, K_m and V_{max} were obtained by using five different substrates (maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose). K_m and V_{max} cannot be determined when maltotriose was used as substrate. Other values obtained for different substrates were 1.76 ± 0.23, 1.89 ± 0.96, 2.90 ± 0.47 and 3.74 ± 0.26 mM for K_m and 31.28 ± 2.48, 62.12 ± 0.89, 121.23 ± 40.44 and 134.24 ± 1.05 μmoles/min for V_{max} respectively. In addition, the values of V_{max}/K_m and k_{cat}/K_m were also determined. Values obtained were 18 ± 0.8, 33 ± 10.8, 42 ± 6.2 and 36 ± 2.1 μmoles.min^{-1}.mM^{-1} for V_{max}/K_m and 107 ± 5, 198 ± 15, 252 ± 30 and 217 ± 13 min^{-1}.mM^{-1} for k_{cat}/K_m, respectively. Maltohexaose demonstrated the highest V_{max}/K_m and k_{cat}/K_m. This result suggests that the enzyme-catalyzing rate was the best when maltohexaose was the substrate for cyclization reaction.

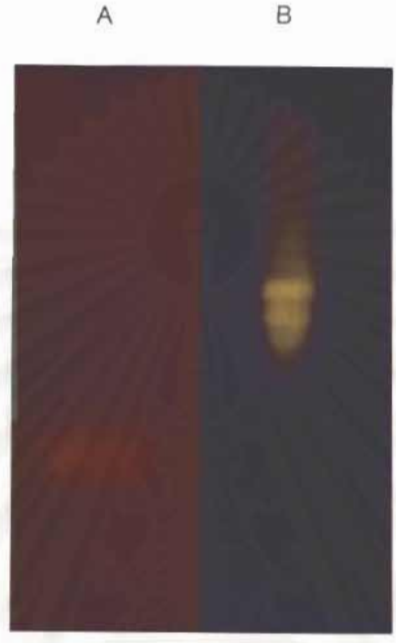


Figure 32. Two dimensional polyacrylamide gel electrophoresis of CGTase. Amylolytic activity staining was performed by using iodine solution.

A. SDS-PAGE (1st dimension)

B. Non-denaturing PAGE (2nd dimension)

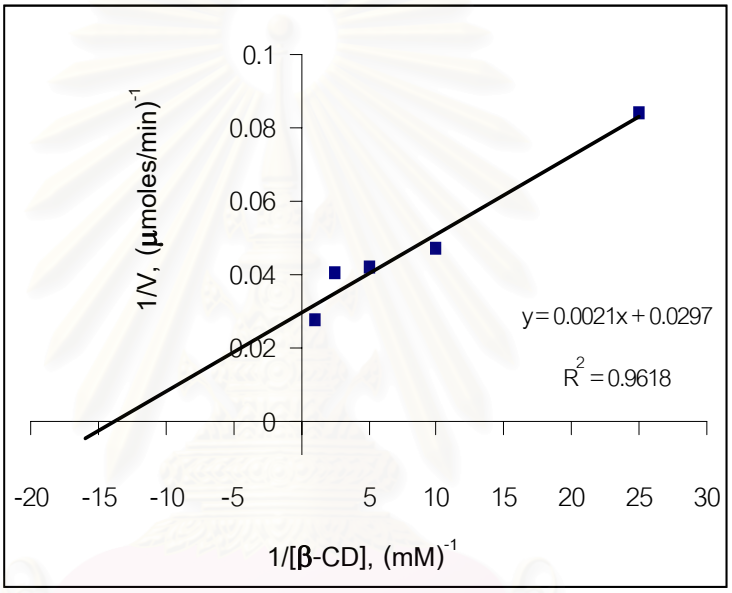


Figure 33. Lineweaver-Burk plot of CGTase isoform 1 with β- cyclodextrin as substrate. CGTase was incubated with 10 mM cellobiose and various concentration of β- cyclodextrin in 50 mM acetate buffer, pH 6.0 at 40 ^{o}C for 5 minutes.

Table 16. Kinetic parameters of CGTase isoform 1.

Substrate	K_m (mM)	V_{max} (μmoles /min)	V_{max}/K_m ($\mu moles.min^{-1}.mM^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}.min^{-1}$)	K_m [a] (mM)	V_{max} [a] (μmoles/min)	V_{max}/K_m [a] ($\mu moles.min^{-1}.mM^{-1}$)	V_{max}/K_m [b] ($\mu moles.min^{-1}.mM^{-1}$)
For coupling reaction									
- α- CD	0.063 ± 0.01	35.14 ± 1.92	558 ± 50	212 ± 21	3368 ± 300	3.16 ± 0.24	58.96 ± 4.42	18.66 ± 0.02	-
- β- CD	0.10 ± 0.01	14.00 ± 2.68	140 ± 12	85 ± 16	845 ± 70	1.69 ± 0.18	13.12 ± 0.31	7.76 ± 0.58	32.66 ± 1.72
- γ- CD	0.12 ± 0.02	25.86 ± 4.43	216 ± 10	156 ± 27	1301 ± 50	1.42 ± 0.43	8.61 ± 0.96	6.06 ± 0.89	-
- maltosyl - β- CD	0.19 ± 0.01	15.70 ± 1.97	83 ± 6	95 ± 12	499 ± 35	1.60 ± 0.36	2.50 ± 0.18	1.56 ± 0.20	-
- methyl- β- CD	0.16 ± 0.04	18.02 ± 0.37	113 ± 21	109 ± 20	680 ± 125	1.94 ± 0.23	1.40 ± 0.06	0.72 ± 0.05	-
- hydroxypropyl- β- CD	0.07 ± 0.02	7.15 ± 2.28	102 ± 28	43 ± 8	617 ± 169	-	-	-	-
For cyclization reaction									
- maltotriose	ND	ND	ND	ND	ND	-	-	-	-
- maltotetraose	1.76 ± 0.23	31.28 ± 2.48	18 ± 0.8	189 ± 15	107 ± 5	-	-	-	-
- maltopentaose	1.89 ± 0.96	62.12 ± 0.89	33 ± 10.8	375 ± 5	198 ± 15	-	-	-	-
- maltohexaose	2.90 ± 0.47	121.23 ± 40.44	42 ± 6.2	732 ± 24	252 ± 30	-	-	-	-
- maltoheptaose	3.74 ± 0.26	134.24 ± 1.05	36 ± 2.1	811 ± 6	217 ± 13	-	-	-	-

* = μmoles of reducing sugar (measured as glucose) formed

[a] Values obtained with unfractionated purified CGTase, 5 mM maltotriose was used as glycosyl acceptor, as reported by Tongsima (1998)

[b] Values obtained with unfractionated purified CGTase, 5 mM cellobiose was used as glycosyl acceptor, as reported by Wongsangwattana (2000)

ND = could not be determined -- = not determined

CHAPTER IV

DISCUSSION

CGTase from *Paenibacillus* sp. A11 was firstly screened from South-East Asian soil by Pongsawasdi and Yagisawa (1987). The CGTase produced was extracellular enzyme with β-CD as the main product (Techaiyakul, 1991). The enzyme was purified and characterized by our research group in the Department of Biochemistry. CGTase isoform preparation was obtained in three steps of purification consisting of corn starch adsorption, ultrafiltration and preparative gel electrophoresis, as previously reported by Kaskangam (1998) and Kaulpiboon (2000). The existence of isoform was also reported in other strains (Nakamura and Horikoshi *et al*., 1976; Mattsson *et al*., 1990; Bovetto *et al*., 1992; Abelyan *et al*., 1994). In this work, by preparative gel electrophoresis, four CGTase isoforms were successfully isolated. They were of the same size as suggested by the same mobility on SDS-PAGE (Kaskangam, 1998). But when analyzed by non-denaturing PAGE, four different bands were observed with activity staining (Figure 12) which suggested that each isoform differed mainly by charge characteristics. When protein staining was performed, one major (isoform 1) with 3 minor forms (isoforms 2, 3 and 4) were always found. The present study was designed to gather information for the understanding of the cause of isoform pattern of this CGTase. Whether the cause was by post-translational modification of a single protein or the existence of multiple genes for CGTase had to be found out.

The effects of post-translational modification, especially glycosylation, phosphorylation, disulfide bond formation, and modification of amino acids with carboxylic side chain on isoform structure and activity were investigated, to point out possibility of post-translational modification as the cause of isoform pattern. Glycosylation and interchain disulfide bond formation, if occurred, should result in the increase in the size of protein. A change in charge might also occur if conformation change followed these modifications. Ser/Thr phosphorylation and modification of

carboxyl residues would result in significant change in net charge by observing mobility on non-denaturing PAGE, which may help explain the emerging isoforms. In addition, experiments were also performed to prove whether or not the isoforms were different proteins (expressed from more than one genes). These experiments include comparison of CGTase from *Paenibacillus* sp. A11 and *E.coli* harbouring CGTase gene in order to determine that isoform pattern existed only in *Paenibacillus* sp. A11 or even in the recombinant strain. Analysis of digested peptides of each isoform, N-terminal amino acid sequence and determination of amino acid composition were carried out. Kinetic study was also performed to find whether there was the difference in substrate binding affinity and catalytic rate of the isoform.

Studies on post-translational modification

The effect of glycosylation on activity and structure of CGTase isoforms

Glycosylation is a concern because previous data showed that each isoform had different carbohydrate content (Kaskangam, 1998). In addition, CGTase is an extracellular enzyme which is typically modified after translation for processing through plasma membrane and preparing for its functional role. Through information on glycosylation in prokaryotes is rare, a few were reported (Nelson and Cox, 2000).

In the experiment, two types of deglycosylated enzyme; PNGase F and Endo H; were used. Endo H which cleaves high mannose and hybrid oligosaccharides from intact glycoprotein as shown in Figure 33 was used for deglycosylation of CGTase. Dextrinizing activity of the treated enzyme (PE, isoform 1, isoform 2 and isoform 4) did not change except for isoform 3 activity which was decreased by 17 % (Table 10). No difference in band migration of PE and four isoforms were found when analyzed by non-denaturing and SDS-PAGE systems (Figure 17 and 18). Trimble and Maley (1984) reported that invertase which was used as positive control (also in our study) was deglycosylated by Endo H and mobility on PAGE was changed. In this study, either nondenatured or denatured invertase did not change in mobility on PAGE after treated with Endo H (Figure 20), even though the denatured protein which had better

susceptibility to Endo H than native protein was used (Tarentino, 1985). Ovalbumin which had similar N-linked oligosaccharide as invertase was then tried. It was found that mobility of nondenatured and denatured ovalbumin on SDS-PAGE were changed after Endo H treatment. This means that when ovalbumin was degylcosylated, the protein was smaller in size which could be detected by faster migration on SDS gel (Figure 21).

PNGase F which hydrolyzed all asparagine-linked glycans (Figure 33) can rapidly hydrolyze high-mannose or complex multibranched oligosaccharide chains including tri- and tetraantennary structures while Endo H does not hydrolyze tri- and tetraantennary carbohydrate chains. The result obtained was similar to Endo H. Only the activities of isoforms 3 and 4 decreased by 26-28 % (Table 9). The results suggested that structure of each isoform might be somewhat different so susceptibility to PNGase F was different. PE consisted of four isoforms with different content (isoform 1>2>3>4) so effect on PE (mixed isoforms) was similar to those of isoforms 1 and 2 which were major forms of PE.

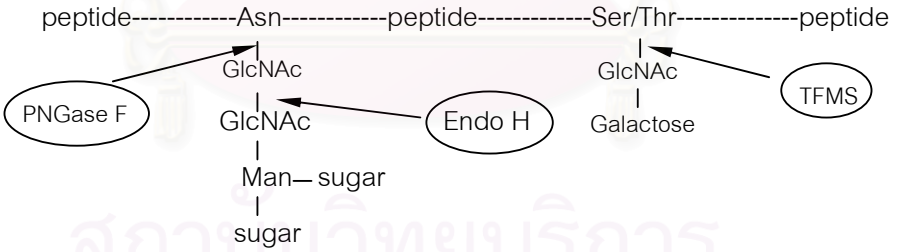


Figure 34. Action of PNGase F, Endo H and TFMS

When analyzed by polyacrylamide gel electrophoresis, the results (Figure 13 - 16) showed that deglycosylation with PNGase F had no significant effect on structure of CGTase, both of PE and isoform, even PNGase F used was as high as 6,000 mU/ml. For human transferrin (HTF), which was used as positive control, deglycosylation with 3,000 mU/ml PNGase F resulted in changes in molecular size on SDS-PAGE,(Figure 14 and

16). Denatured form of CGTase were also used for treatment with PNGase F and the result was not different from nondenatured protein.

Deglycosylation by trifluoromethanesulfonic acid (TFMS) which cleaves the O-linked oligosaccharide (Figure 33) was also used. It was found that TFMS had no effect on mobility of CGTase (both of PE and isoform) on SDS-PAGE. Mobility of the two positive control, partially purified peroxidase from cassava root and purified royal jelly protein, did not change as well. The result of peroxidase was not the same as that reported by Sornwattana (2001) eventhough the same condition was used. We then determine carbohydrate content of the proteins after incubation with TFMS for different time period. It was found that carbohydrate content of peroxidase and royal jelly protein decreased by 40 and 20 %, respectively after 4 hours incubation while that of CGTase (both PE and isoform) was not changed (Table 11). The result suggested that CGTase had no O-glycosylation or the extent of O-glycosylation was not much as compared to the whole protein, then change in carbohydrate content was so little that cannot be observed by phenol-sulfuric acid assay.

From these results, it can be concluded that glycosylation/deglycosylation, if occurred, had no effect on the isoform pattern of CGTase. Study on glycosylation of CGTase had not been previously reported but the effect of glycosylation on isoform pattern of polygalacturonase and isopullulanase from fungi (Stratilova *et al*., 1998 and Padmajunti *et al*., 2000) and of peroxidase from plant (Sornwattana, 2001) were repoted.

The effect of phosphorylation on structure of CGTase isoforms

Phosphorylation is a concern because it is post-translational modification which has been revealed to be widespread among prokaryotes (Nelson and Cox, 2000). In this experiment, *E.coli* alkaline phosphatase was used for dephosphorylation and ovalbumin was used as positive control since it is a known phosphoprotein. It was clearly shown on non-denaturing PAGE that dephosphorylated ovalbumin moved slower towards the anode, the protein was less negative (Figure 25). When CGTase isoforms

were treated with alkaline phosphatase, no difference in band migration of four CGTase isoforms were found when analyzed by non-denaturing PAGE (Figure 27). The result suggested that phosphorylation/dephosphorylation, if occurred, had no effect on isoform pattern.

The effect of disulfide bond formation on structure of CGTase

Disulfide bond formation is one important post-translational mechanism which usually results in changes in the structure and may be activity of the enzyme or even stability. When CGTase was incubated in the absence and presence of reducing agent, such as β-mercaptoethanol or DTT, and the results were followed by non-denaturing and SDS- PAGE (Figure 28). The result showed that reduction of disulfide(-S-S-) to sulfidryl (-SH) group in CGTase, if occurred, had no effect on the isoform pattern. When analyzing the amino acid composition of CGTase from the deduced amino acid sequence, only 2 Cys were found at position 43 and 50 of the whole 686 residues of mature CGTase. It was unlikely that internal disulfide bond formation occurred since only molecular size of 72 KDa was found in the reduced and oxidized conditions. Interaction disulfide bond, however, might occur, but did not result in net charge difference which could be detected on non-denaturing PAGE.

The effect of modification of carboxyl residues on activity and structure of CGTase isoforms

This experiment was performed to check whether the change in number of charged carboxyl residues will result in mobility change which could be detected on non-denaturing PAGE. From activity staining, the unmodified partially purified enzyme showed three major bands and one faint band. The modification of carboxyl group by 5 mM EDC, decreased the negative charge as shown in Figure 29, was confirmed by an extra band (above label “a”) which migrated slower than the unmodified bands. This result was similar to that reported by Tongsima (1998).

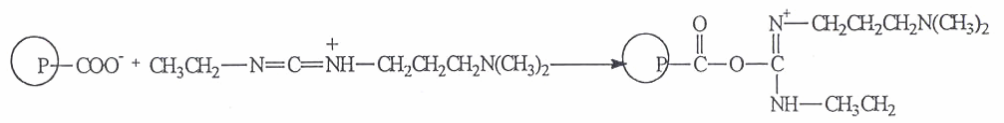


Figure 35. Modification reaction of EDC with carboxyl residue in protein (P)

(Means and Freeney, 1971; Landblad, 1991)

For CGTase isoform, the result was similar to partially purified enzyme. All modified isoforms migrated slower than the unmodified isoforms (Figure 29). This observation suggests that carboxyl residues of each isoform leading to change in the mobility on the gel when modified by EDC.

The modification by group-specific reagent, EDC, as performed in this study, could be used to propose the importance of amidation (amide formation) or deamidation on the structure of CGTase isoforms. Since CGTase contained high Asp/Asn and Glu/Gln (approximate 25 mole%), enzymatic interconversion between carboxylic and amide group could be the probable cause of isoform pattern.

Comparison of CGTase from *Paenibacillus* sp. A11 and *E.coli*

E.coli harbouring CGTase gene of *Paenibacillus* sp. A11 was prepared by Rimphanitchayakit (2000). It was grown in LB medium at 37 ^{o}C, 250 rpm for 18 hours while *Paenibacillus* sp. A11 was grown in Horikoshi's medium at 37 ^{o}C, 250 rpm for 72 hours. *E.coli* CGTase produced was found as extracellular enzyme with high activity. Both enzymes were partially purified by starch adsorption, then further purified and concentrated by ultrafiltration technique. The protein pattern of both CGTases on non-denaturing PAGE at each step of purification (Figure 7A) showed that crude enzyme from *E.coli* gave more bands with higher intensity suggesting more proteins were secreted. While the starch adsorbed and ultrafiltrated enzyme from both preparations gave only 2-3 protein bands (1 major and 1-2 minor bands) which were CGTase with the same mobility since they corresponded to activity staining (Figure 7B). Activity

staining of enzyme showed that both gave 3-4 bands (2 major and 1-2 minor bands) while *Paenibacillus* sp. A11 CGTase minor bands were more obviously detected. For CD product ratio formed by these enzymes as analyzed by HPLC, the α : β : γ- CD ratio of *Paenibacillus* sp. A11 and *E.coli* CGTase were 2 : 7 : 1 and 2 : 8 : 1 respectively, for β-amylase treated sample.

The results suggested that CGTase from *Paenibacillus* sp. A11 and *E.coli* harbouring CGTase gene of *Paenibacillus* sp. A11 was not different. Cloning and sequencing of CGTase gene from *E.coli* gave no clue of multiple genes (Rimphanitchayakit, 2000). Thus, CGTase isoforms of *Paenibacillus* sp. A11 should be transcribed and translated from a single gene because we can observe multiple bands of crude CGTase of *E.coli* on non-denaturing PAGE by amylolytic activity staining. In addition, *E.coli* was cultured in LB medium (without rice starch), hence carbohydrate contaminants (as existed in Horikoshi's medium for *Paenibacillus* sp. A11) should not be the cause of isoform pattern.

Analysis of digested peptides of CGTase isoform

Peptides from tryptic digest were separated on C_{18} reverse phase column with elution by solvent A and B as described in section 2.17. Solvent A (0.1% TFA in water) had more polarity than solvent B (0.1%TFA in 75 % acetonitrile). In this sysytem, polarity gradually decreased so the prior eluting peaks (low R_t) were more polar than latter peaks (high R_t). As the result, peaks eluting at R_t 2.8 to 4.3 minutes were solvent peaks and broad peaks eluting at R_t 34 to 36 minutes should be all peptide peaks which were low in polarity. Broad peaks obtained might be due to inappropiate condition used because solvent B to A ratio was rapidly increased in the range of 30 to 35 minutes. This should be solved by decreasing solvent B to A ratio after 30 minutes. Peak height more than 10 mAU was analyzed as shown in Table 12. Approximate twelve peptide peaks were noticeable within 30 minutes elution time. The peaks obtained was not different in terms of R_t for each isoform. Only peak height which indicates quantity of peptide was different. The result suggested that all isoforms should be the same protein with the

same amino acid sequences since peptide constituents were not different. However, side chain of some amino acids (not lysine and arginine) may be changed by post-translational modification. Trypsin hydrolyzed peptide bond at certain lysines and arginines from the C-terminal (Nelson and Cox, 2000) of all isoforms so peak eluting at the same R_t were obtained. However, difference in peak height observed at some peaks may be caused by difference in protein folding, so some position was more sensitive for trypsin digestion than others.

Amino acid composition of CGTase isoform from *Paenibacillus* sp. A11

Amino acid composition of each CGTase isoform was compared in Table 14. For all isoforms, 46-53 mol% of the content was non-polar amino acids, while 47-52 mol% was polar amino acids. Only 4.7-6.1 mol% was aromatic amino acids : phenylalanine and tyrosine (Trp could not be detected by the method analyzed). It was found that amino acid composition of each isoform was not much different. The lowest difference was in Thr content (only 0.5 mol% among all isoforms) while the highest difference was in Glu/Gln content (4.1 mol%). The result was in the same trend as that reported by Kaskangam (1998), though some values were different which might arise from enzyme purity and different analysis method.

Table 17. Comparison of amino acid composition of CGTase isoforms with the unfractionated enzyme from *Paenibacillus* sp. A11.

Amino acid	Content (mol%)									
	D[a]	P[b]	Isoform1	Isoform 2	Isoform 3	Isoform 4	Band 1*	Band2*	Band3*	Band4*
Asx	15.7	14.4	8.8	10.9	9.0	9.6	10.0	13.3	13.2	12.2
Thr	8.7	8.2	6.3	6.7	6.2	6.4	10.1	10.4	10.0	-
Ser	5.7	5.4	9.2	7.2	8.4	6.0	6.5	6.0	5.9	-
Glx	6.9	10.0	13.5	14.0	16.4	12.3	4.8	8.9	10.0	10.3
Pro	4.4	5.5	5.0	5.4	6.7	4.9	7.4	6.2	6.6	11.4
Gly	9.8	8.9	15.5	11.9	14.1	21.5	-	-	-	-
Ala	8.0	9.6	10.3	10.4	10.6	8.5	15.4	11.7	11.4	8.4
Cys	0.3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Val	6.9	8.6	5.6	6.6	6.7	6.8	9.4	10.0	9.0	10.7
Met	2.0	1.1	0	0	0	0	2.2	1.4	1.1	1.3
Ile	6.6	4.9	3.7	4.0	3.1	3.1	7.9	6.6	5.7	4.5
Leu	5.0	6.3	5.8	6.2	6.1	5.6	6.5	6.2	6.5	8.3
Tyr	5.0	2.9	2.1	2.4	2.0	1.7	2.9	3.8	3.4	6.7
Phe	4.8	4.3	3.1	3.7	2.7	3.0	5.5	5.6	4.4	4.3
Lys	3.5	5.6	3.1	3.5	3.7	3.7	4.5	4.8	5.3	5.4
Trp	1.9	ND	ND	ND	ND	ND	ND	ND	ND	ND
His	1.7	1.3	3.6	2.2	2.2	3.1	2.1	1.6	4.0	7.8
Arg	3.2	2.9	4.6	4.9	4.3	4.0	4.8	3.5	3.8	8.6

[a] Deduced amino acid sequence from CGTase gene sequence (Rimphanitchayakit 2000)

[b] Purified enzyme (unfractionated form) (Laloknam, 1997)

* CGTase isoforms reported by Kaskangam (1998)

ND = Not determined

N-terminal amino acid sequence and protein constituents of CGTase isoform

When CGTase isoforms was prepared by blotting each band from non-denaturing PAGE pattern of PE, then all forms were sent for N-terminal sequence. It was found that more than one sequence was obtained in all isoforms which suggests the mixture of at least two protein populations in each isoform. When the N-terminal amino acid sequence were analyzed, it was observed that all isoforms had two types of N-terminal amino acid sequence. They were Gly/Leu-Asn-Lys-Leu (isoform 3) and Leu-Ala-Gln-Asn (isoform 4). Both sequences were observed in isoform 2 whereas isoform 1 was unclear because 2-3 types of major sequences and 1-2 types of minor sequences were obtained. However, it might be assumed that isoform 1 consisted of these two major sequences as similar to isoform 2 and this two sequences were CGTase while other sequences in isoform 1 were or were not CGTase.

The N-terminal amino acid sequence obtained cannot lead to clear conclusion whether all isoforms were the same protein. Each isoform seemed to contain more than one protein population, so we decided to checked on protein constituents using HPLC. C_4 reverse phase column was used. Each isoform which was separated by preparative gel elctrophoresis was injected to C_4 column, eluted by gradient of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 60% acetonitrile) as shown in section 2.20. Figure 31 showed the profile of four CGTase isoforms. All isoforms yielded 2 major peaks eluting at R_t 10.9-11.0 (Peak I) and 11.8-11.9 minutes (Peak II). Difference in peak ratio of this 2 peaks was observed (Table 13). These should be the cause of net charge difference for each isoform so that mobility on non-denaturing PAGE was different. Both peaks should be CGTase, however, it is not clear whether they are the same or different protein. The result of N-terminal amino acid sequence analysis favors the different protein suggestion. Confirmation should then be made by analysis of N-terminal amino acid sequence of peak I and II.

The N-terminal sequence of each isoform obtained from the unfractionated sample blotted off the native gel was different from that reported by Laloknam (1997) and Rimphanitchayakit (2000). Laloknam reported that the first five amino acids, unfractionated CGTase were Ala-Pro-Asp-Thr-Ser, which were identical to those of CGTases produced from, *Bacillus* sp. strain 1011, *Bacillus* sp. strain 38-2 and *Bacillus* sp. strain 17-2. Rimphanitchayakit (2000) reported that the deduced amino acid sequence of CGTase from *Paenibacillus* sp. A11was highly similar to that of *Bacillus* sp. strain 1011. The homology was about 98%. And the N-terminal sequence was the same as that of Laloknam. In determining N-terminal residues, Laloknam blotted the unfractionated CGTase from SDS-PAGE.

When the four purified isoforms was prepared by preparative gel electrophoresis, then run on SDS-PAGE and blotted on PVDF membrane, and sent for N-terminal sequence. The first five amino acids obtained for all isoforms were A P D T S which were identical to that reported by Laloknam (for unfractionated CGTase) and Rimphanitchayakit (for deduced sequence). The difference obtained when sequencing two preparations of isoforms, blotting from non-denaturing PAGE pattern of unfractionated CGTase and blotting from each purified isoform run on SDS-PAGE, should be due to purity of the preparations In addition, treatment with SDS and β-mercaptoethanol might reduce complexity of constituents in the molecule. The identical N-terminal sequence of the four isoforms obtained in this study thus supports that all CGTase isoforms of this *Paenibacillus* sp. A11 were the same protein. Their difference, then, should arise from post-translational modification event.

Analysis of CGTase isoforms by two dimensional polyacrylamide gel electrophoresis

The partially purified enzyme and all isoforms gave a single band of protein with the same molecular weight on SDS-PAGE while partially purified enzyme gave 4 bands of isoform on non-denaturing PAGE, when run on one dimension gel. In this study, when denatured form of partially purified enzyme on SDS-PAGE (1^{st} dimension) was renatured, it showed four activity bands of isoforms 1-4 on non-denaturing PAGE (2^{nd}

dimension) (Figure 32). From the result, it confirms that CGTase isoforms were rooted from a single protein. The cause of isoform pattern, should be arised from post-translational modification, e.g. from amidation/deamidation as proposed earlier in this study or from different folding of a single form.

Kinetic study of CGTase isoform 1

In the proposal, kinetic study of each isoform was proposed. But after the finding that each isoform was consisted of at least two major constituents (Figure 31), it was then no point to use this experiment to try to compare substrate binding affinity and catalysis rate of the isoform. However, since isoform 1 was the major isoform, kinetic study of it would give good information as representative of CGTase of *Paenibacillus* sp. A11.

For this work, kinetic parameters were determined by cyclodextrin-degrading activity assay method. Coupling and cyclization reactions were studied. Coupling is the reaction where a ring of cyclodextrin molecule is opened and combined with a linear oligosaccharide chain to produce a longer linear oligosacchride (Nakamura *et al*., 1993). β-CD or its derivatives acts as donor and cellobiose as acceptor. After coupling of cyclodextrin to a nonreducing end of the acceptor, a linear oligosaccharide generated acts as a substrate, which is susceptible to hydrolytic cleavage by glucoamylase. The liberation of the reducing sugar measured by conventional method gives the procedure the ease and convenience of routine sugar analysis, dinitrosalicylic acid method (Miller, 1959). The activities were calculated from the consumed amounts of cyclodextrin calibrated from the amounts of glucose in glucoamylase-treated reaction mixtures. The result showed that α- CD demonstrated the highest V_{max}/K_m and k_{cat}/K_m. The value of k_{cat}/K_m for α- CD was about 4 and 3 times higher than that for β- and γ- CD, respectively. This means that α- CD had the highest specificity for coupling reaction of isoform 1. Our results correspond to the result of Tongsima (1998), who reported that the valua of V_{max}/K_m for α- CD was the highest. However, K_m for isoform 1 for α- CD was lower than those for β- and γ- CD , while it was vice versa for the unfractionated enzyme (Table16). The nature and environment of substrate binding site of CGTase of

Paenibacillus sp. A11 makes it fit very well with 7-8 residues of cyclized glucose for efficient catalytic reaction to proceed. It may be assumeded that the whole enzyme and fractionated enzyme might be somewhat different. Immunoaffinity-purified CGTase was used in the study of Tongsima (1998). While isoform 1 (fractionated enzyme) was used in this study. In addition, Tongsima (1998) used 5mM G_3 as glycosyl acceptor while we used 10 mM cellobiose. When V_{max}/K_m for β- CD was compared with the result reportes by Wongsangwattana (2000) who also used celloboise as acceptor but at 5 mM concentration, the value for isoform 1 was about 4 times higher then that of unfractionated enzyme.

For cyclization reaction, G_3-G_7 were used as substrate. After cyclization linear oligosaccharides remained was determined by dinitrosalicylic acid method. The result showed that maltohexaose demonstrated the highest V_{max}/K_m and k_{cat}/K_m and the values of K_m/V_{max} and k_{cat}/K_m for maltoheptaose were very closely to that of maltohexaose. While the one demonstrated the lowest V_{max}/K_m and k_{cat}/K_m was maltotetraose. Maltotriose can not be used as substrate. This suggests that maltohexaose and maltoheptaose were good substrate for cyclization. Comparison of k_{cat}/K_m values of all substrates tested suggests that long chain oligosaccharides had higher efficiency for catalysis than the short chain oligosaccharides.

Overall conclusion

From all the information obtained, the isoform pattern of CGTase from *Paenibacillus* sp. A11 should arise from post-translational modification of the transcribed and translated form of the enzyme. The result of N-terminal sequence determination which shows sequence identity of the first five residues of all isoforms supports the one protein hypothesis of CGTase. We proposed amidation/deamidation as the main post-translational mechanism CGTase encountered. Each isoform might consist of only 2 forms of the enzyme with different composition ratio as evidenced by the result on fractionation of the isoform using C_4 reverse phase HPLC. And each form or each constituent was resulted from amidation/deamidation reaction on Asp/Asn or

Glu/Gln. The major isoform (isoform1) should consist of more deamidated form (less carboxylic group, more amide group) while the other isoforms were modified by deamidation and resulted in more carboxylic group.

CHAPTER V
CONCLUSION

1. CGTase from *E.coli* harbouring CGTase gene from *Paenibacillus* sp. A11 was similar to CGTase of the original strain in the isoform pattern and product formation.
2. Deglycosylation by enzymes (PNGase F and Endo H) and chemicals (TFMS), if occurred, had no effect on size and net charge of all isoforms but exerted some effect on activity of isoforms 3 and 4.
3. Dephosphorylation by alkaline phosphatase, if occurred, had no effect on net charge of all CGTase isoforms.
4. By treatment with β-mercaptoethanol and DTT, structure of all isoforms on SDS- and non-denaturing PAGE did not change suggesting that disulfide bond formation had no influence on the isoform pattern.
5. When carboxyl residues of the isoforms were modified with EDC, significant change in net charge was observed with all isoforms.
6. The number and type of peptides from tryptic digestion of all isoforms were not signicantly different. And so were the amino acid compositions. These findings support that all isoforms were the same protein.
7. All isoforms composed of two types of protein constiuent, those having the R_t10.924-10.954 and 11.951-11.959 min from C_4 column.
8. The first five N-terminal amino acid sequence of all isoforms were APDTS which were the same as that of unfractionated enzyme.
9. For coupling reaction of isoform 1, α-CD was the best substrate. For cyclization, maltohexaose was the best substrate while maltotriose could not act as substrate for cyclization.
10. Multiple forms of CGTase was probably the result of post-translational modification of the transcribed and translated enzyme.

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APPENDICES

APPENDIX A : Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

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

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

Adjusted volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

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

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06	g

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

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

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

Solution B (SDS-PAGE)

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

Solution C (SDS-PAGE)

1 M Tris-HCl pH 8.8	50	ml
10% SDS	4	ml
distilled water	46	ml

2. Non-denaturing PAGE

7.0% Separating gel

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

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

5.0% Stacking gel

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

Sample buffer

For analytical gel

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

For preparative gel

0.5 M Tris-HCl pH 6.8	1.0	ml
glycerol	0.8	ml
0.5% bromophenol blue	0.4	ml

distilled water	5.8	ml

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

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

3. 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 volumn to 1 litre with distilled water (pH should be approximately 8.3).

4. Coomassie blue staining

Staining solution, 100 ml

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

Destaining solution, 100 ml

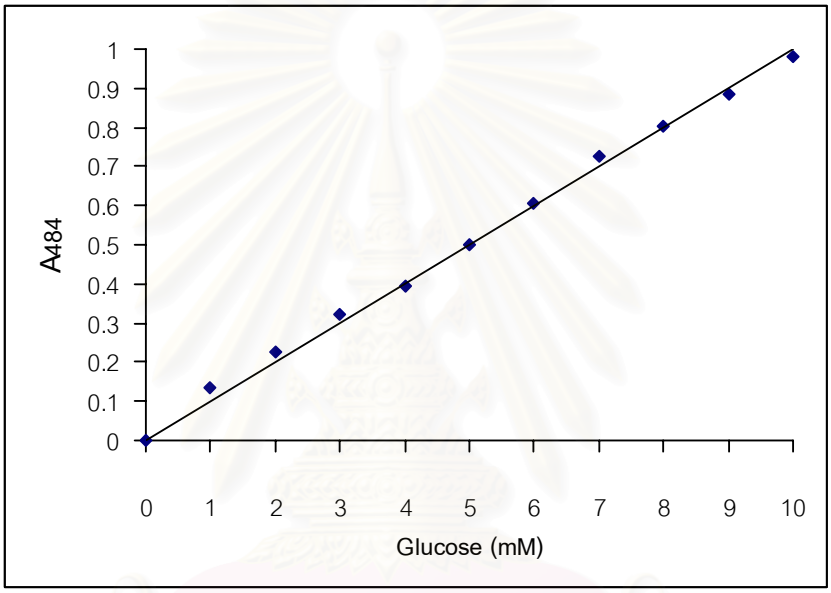
methanol	10	ml
acetic acid	10	ml
distilled water	80	ml

APPENDIX B : One and three letter symbols for the amino acids[a] (Voet, 1995)

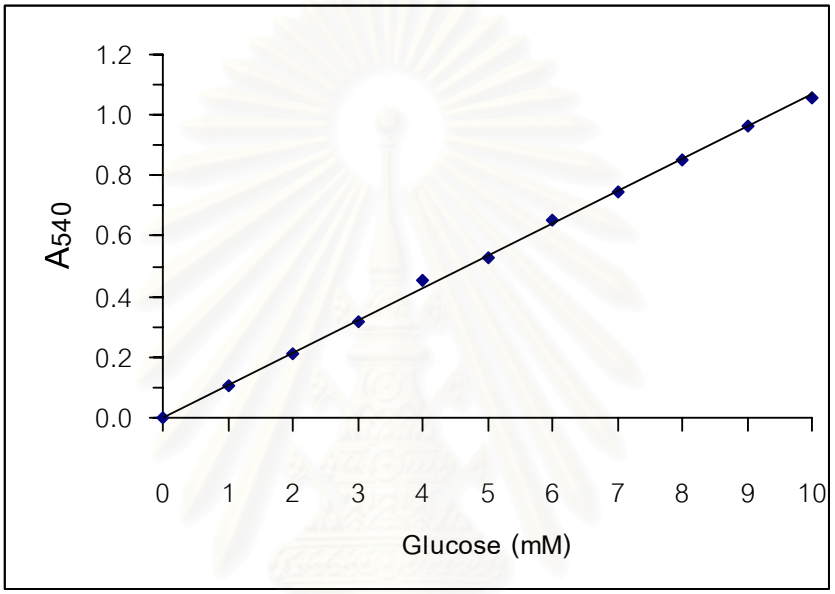
A	Ala	Alanine	M	Met	Methionine
B	Asx	Asparagine or aspatic acid	N	Asn	Asparagine
C	Cys	Cystein	P	Pro	Proline
D	Asp	Aspatic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Gly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophan
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamine or glutamic acid

[a] The one letter symbol for an undetermined or nonstandard amino acid is X.

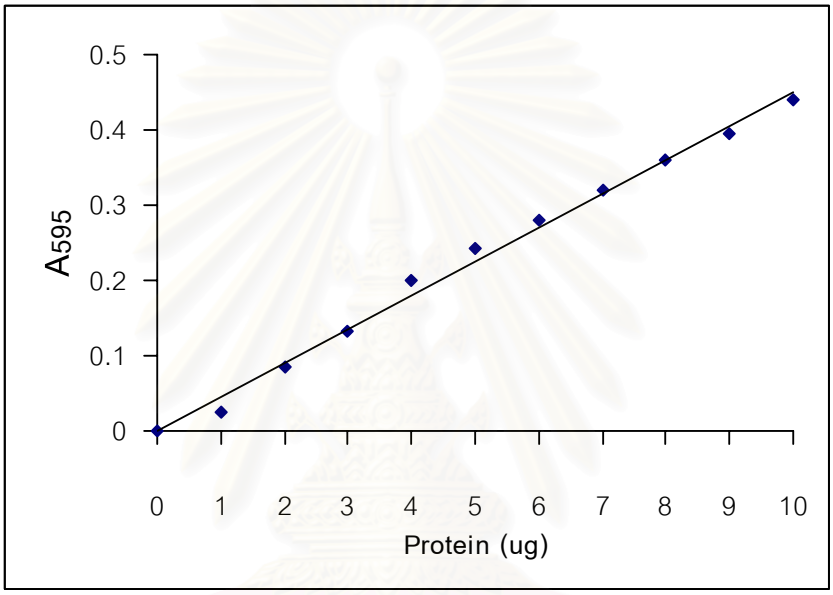
APPENDIX C : Standard curve for carbohydrate determination by Phenol-sulfuric method



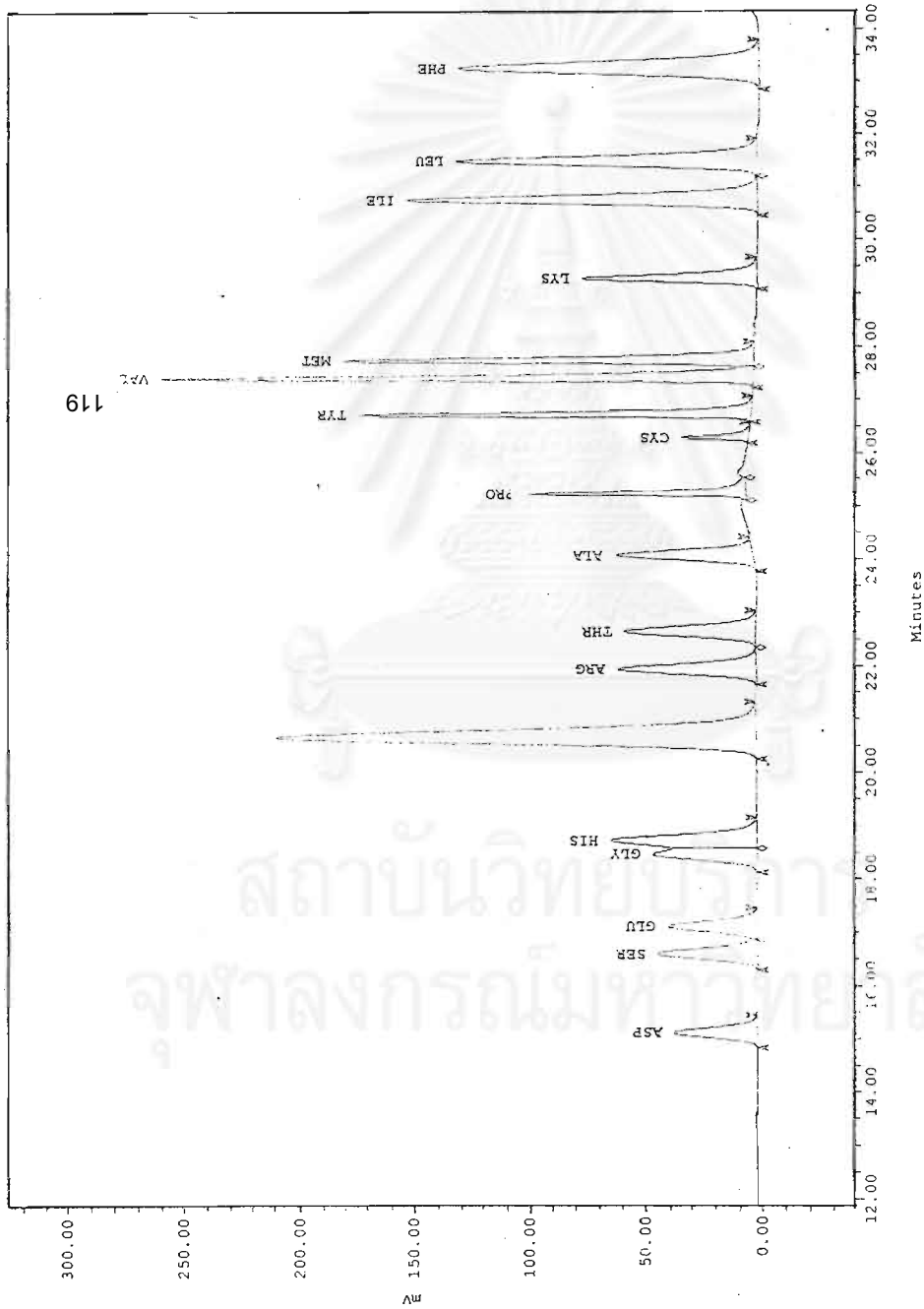
APPENDIX D : Standard curve for glucose determination by DNS method



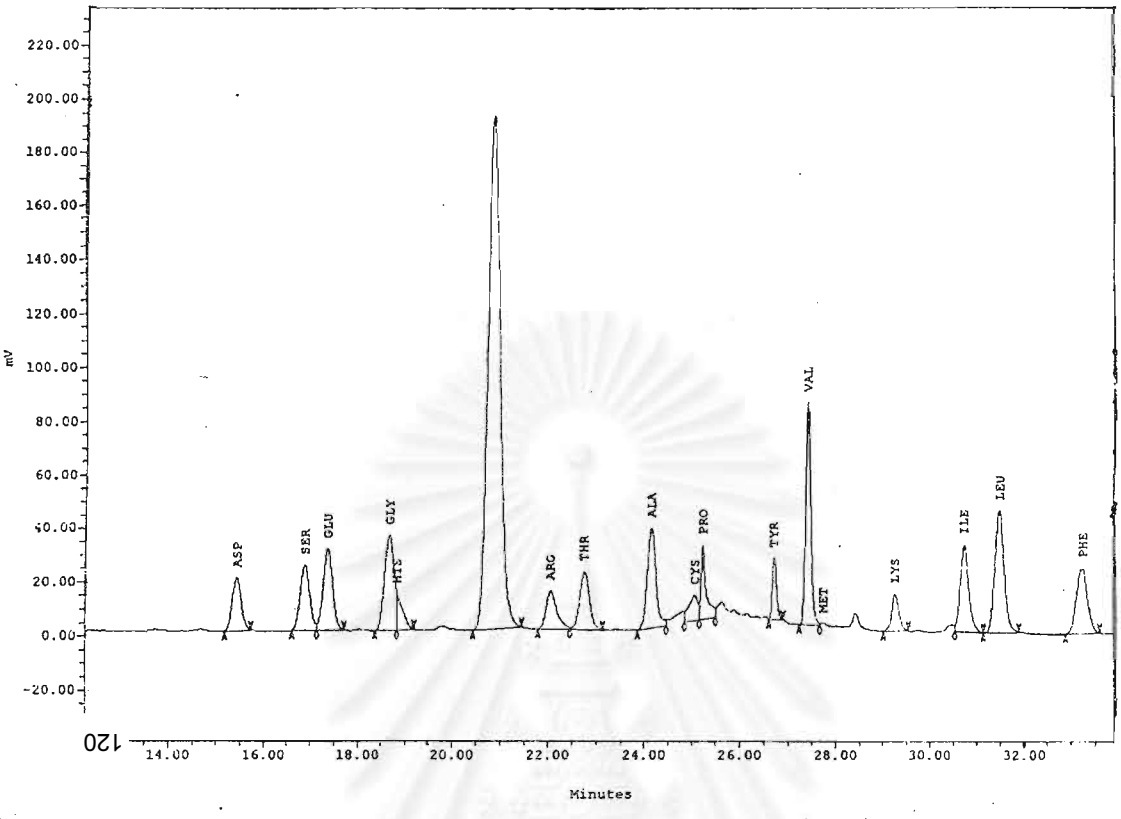
APPENDIX E : Standard curve for protein determination by Bradford's method



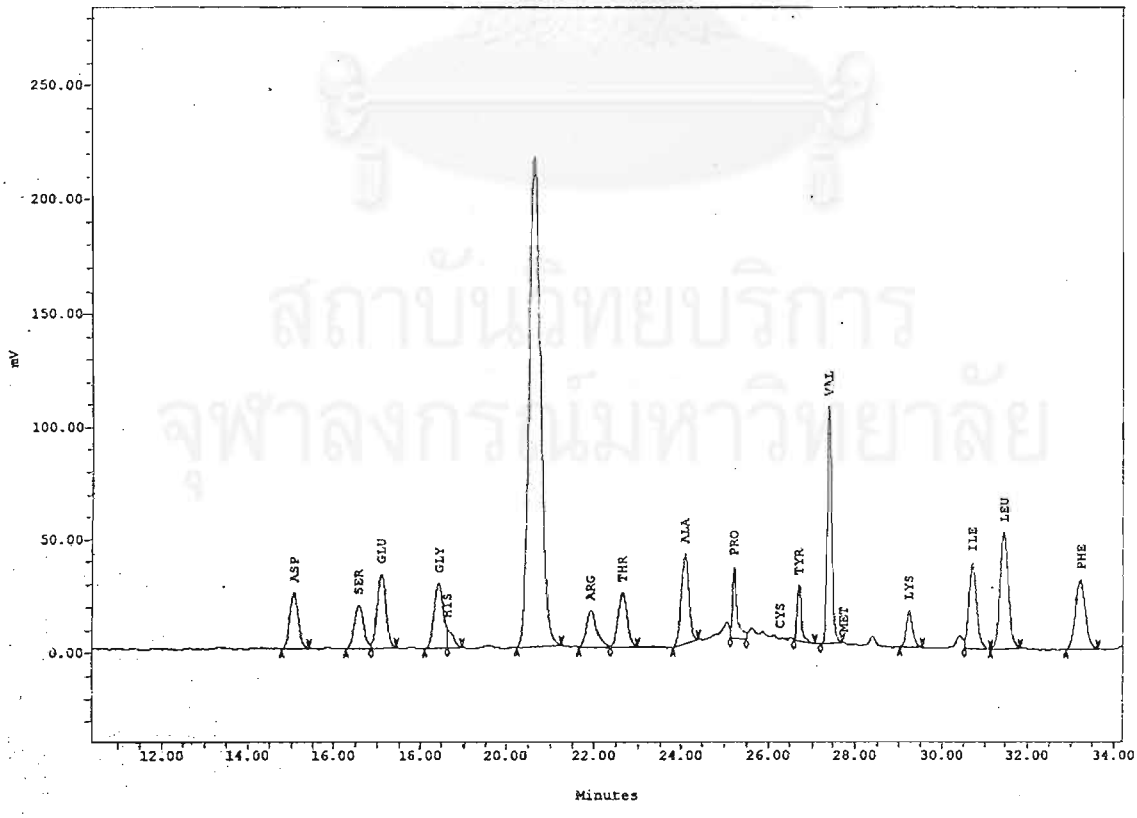
APPENDIX F : Chromatograms of amino acid composition from amino acid analyzer



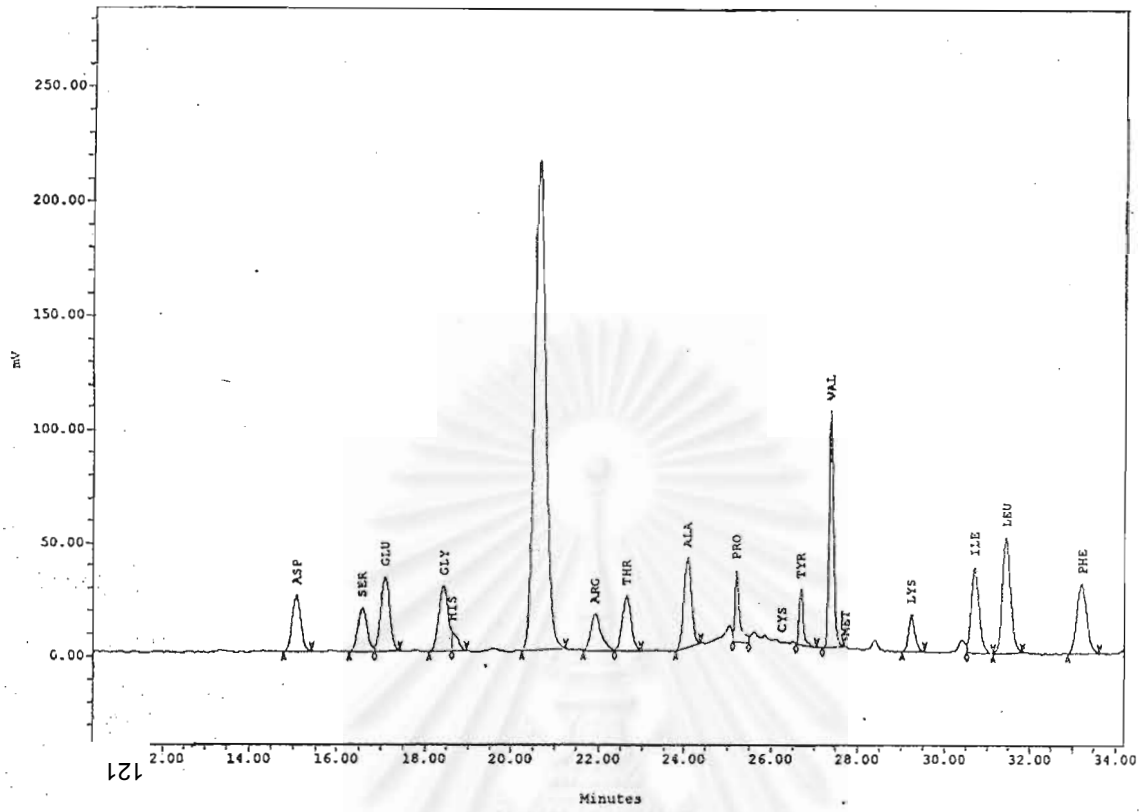
(a) amino acid standard



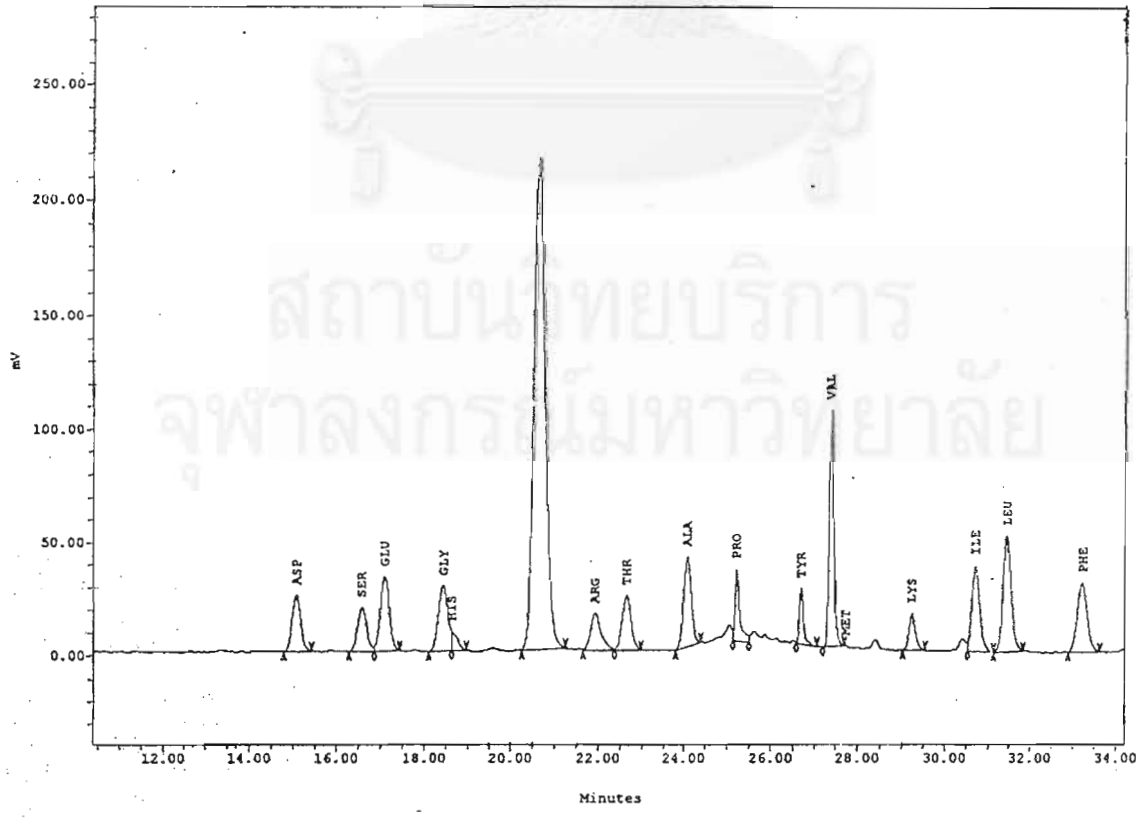
(b) isoform 1



(c) isoform 2

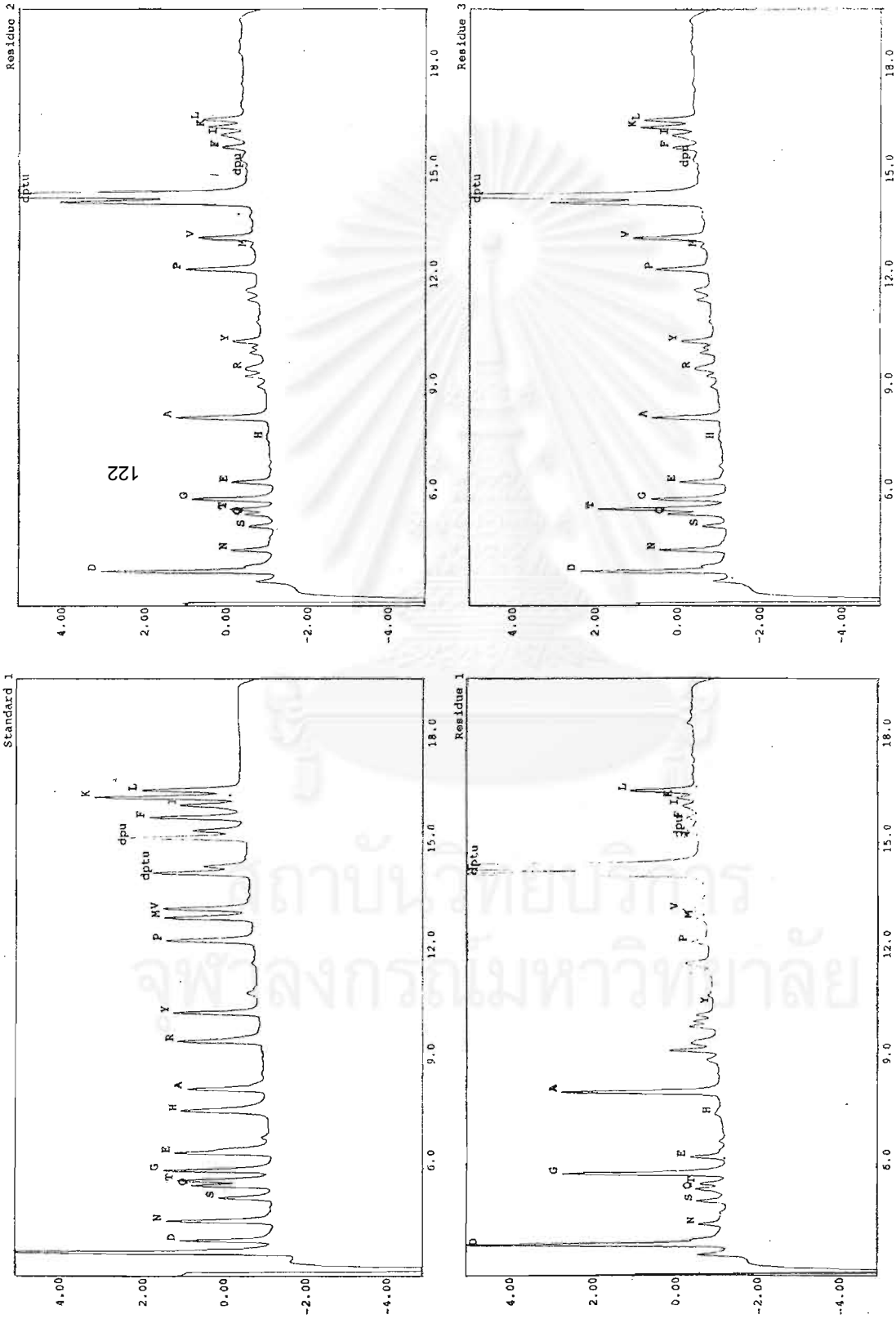


(d) isoform 3

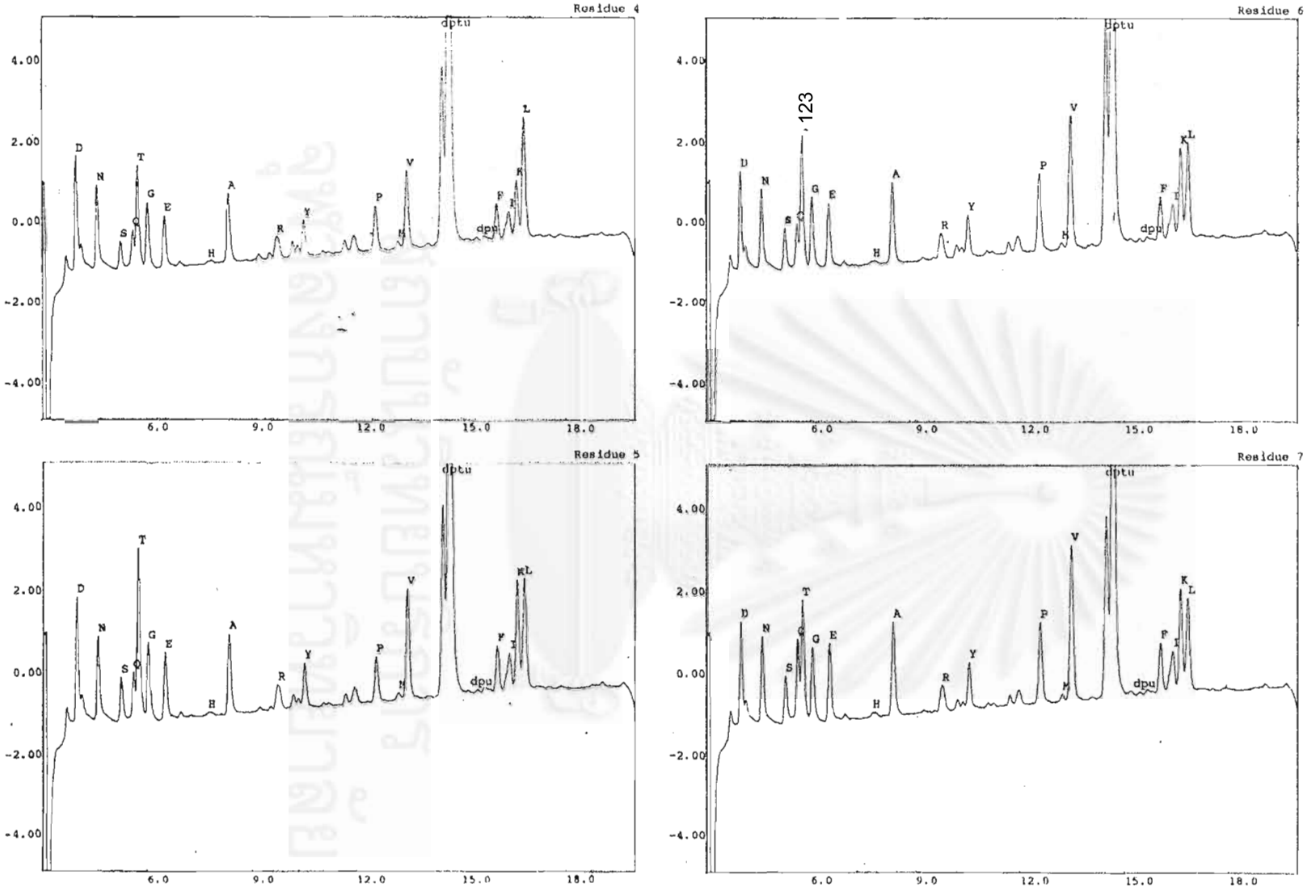


(e) isoform 4

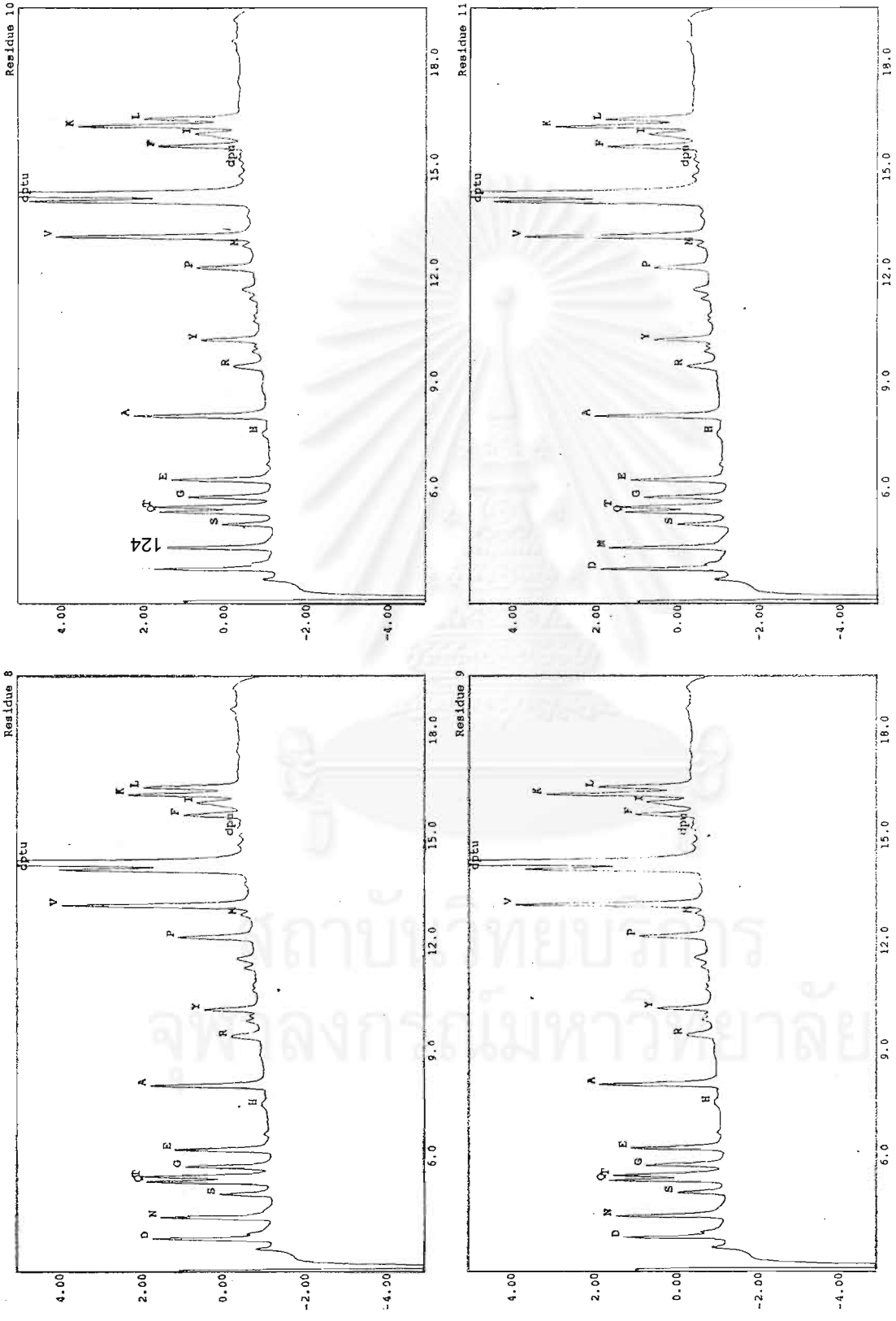
APPENDIX G : Chromatograms of N-terminal amino acid sequence of CGTase isoforms



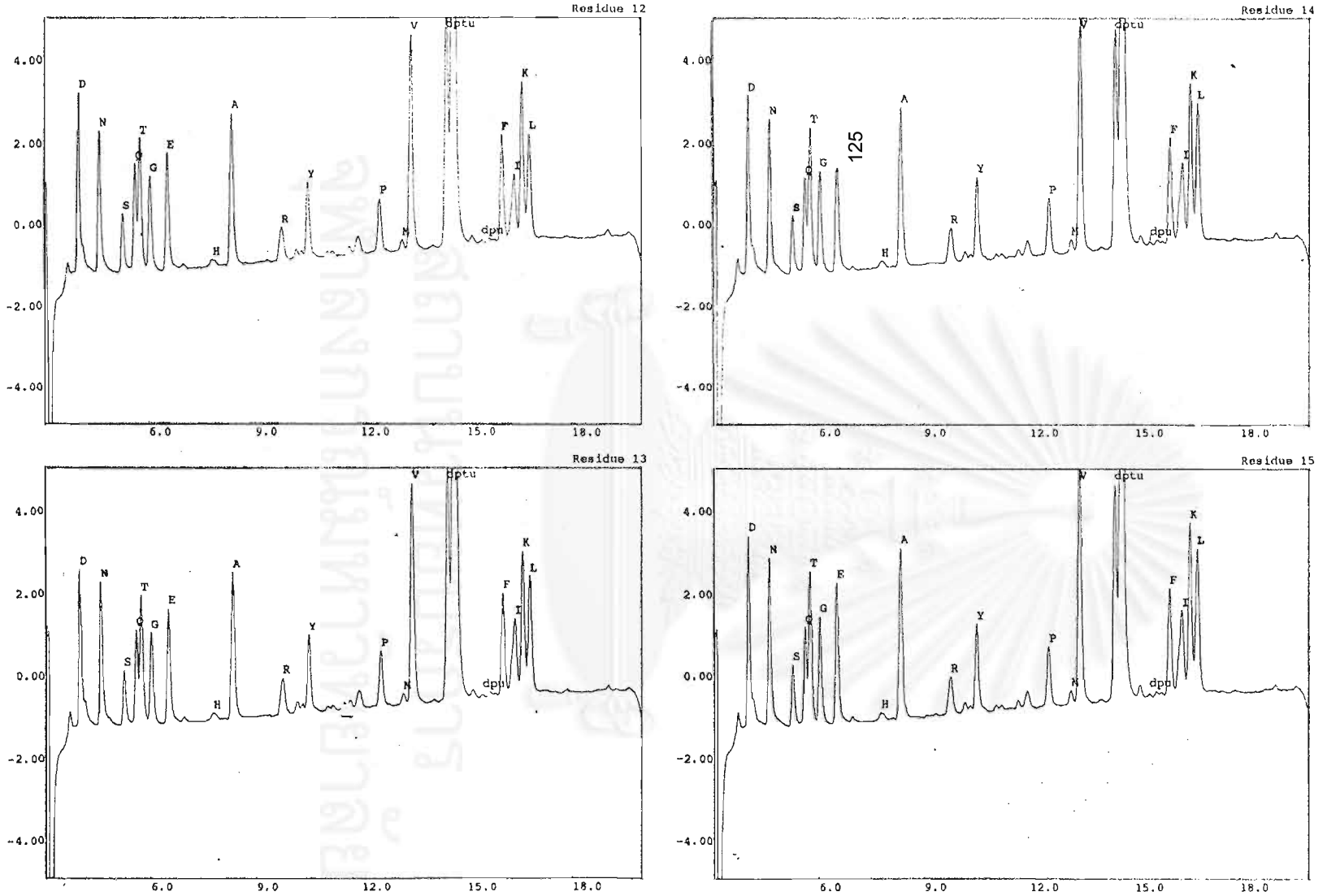
(a) isoform 1



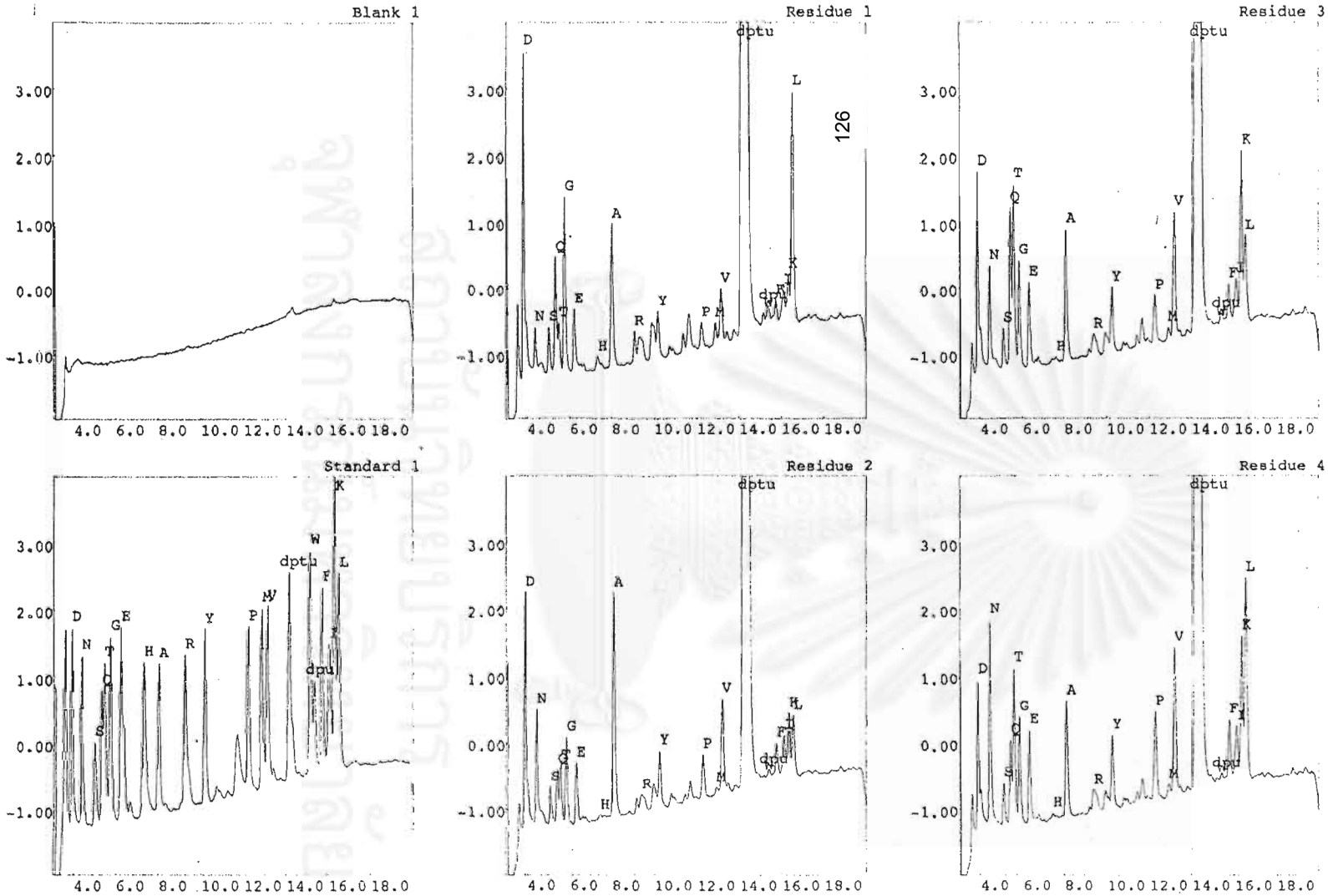
(a) isoform 1 (continued)



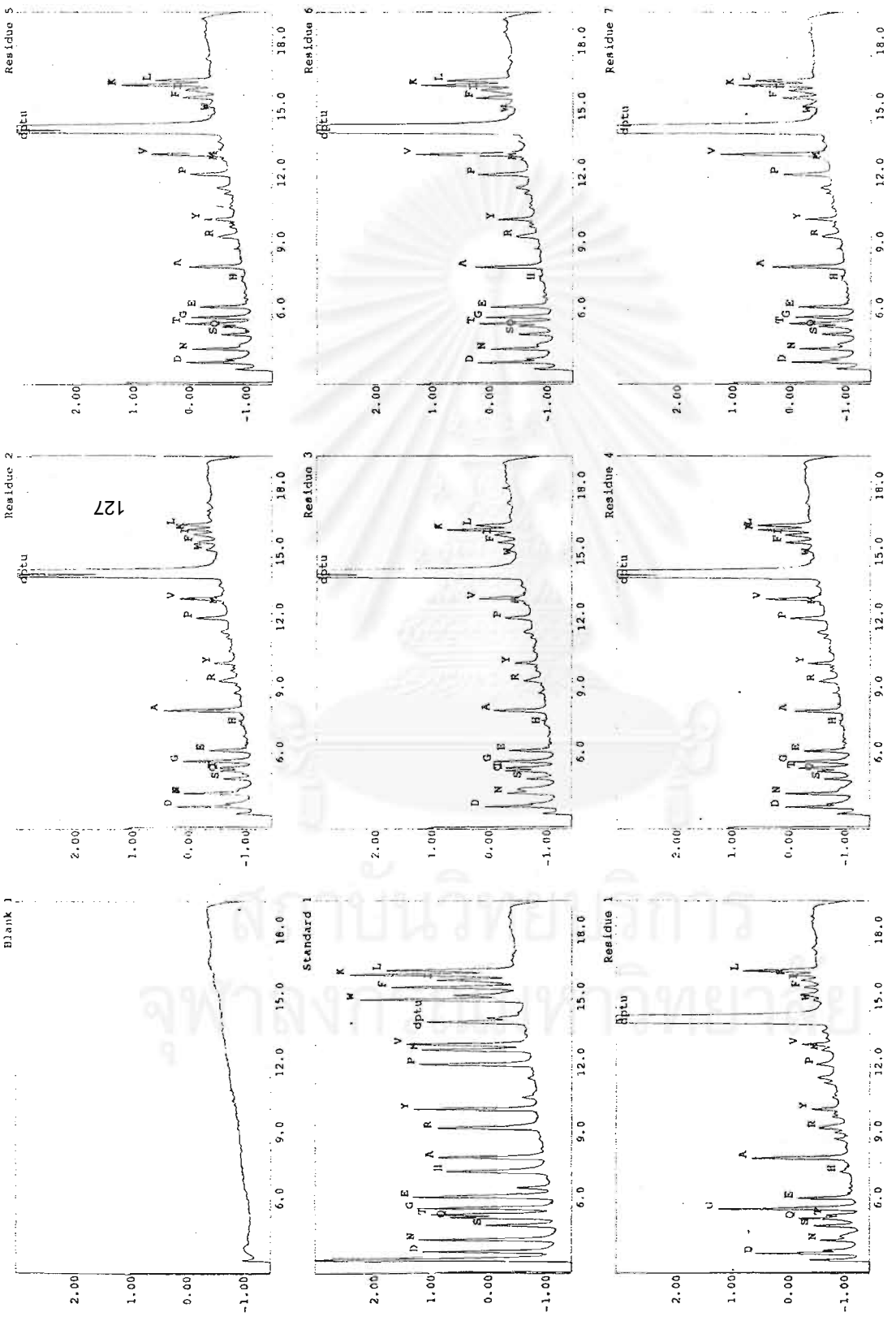
(a) isoform 1 (continued)



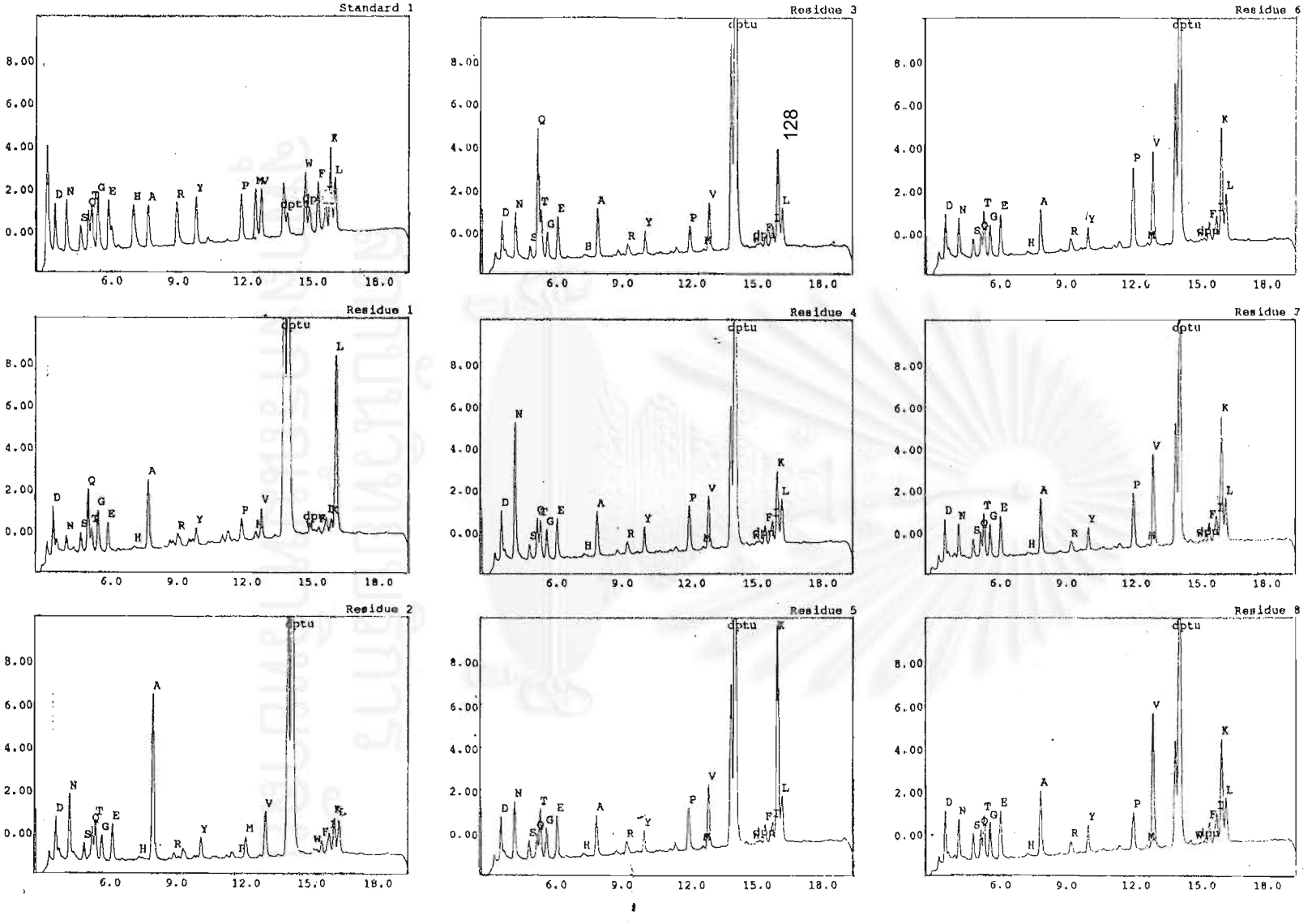
(a) isoform 1 (continued)


Blank 1
Residue 1
Residue 3
Standard 1
Residue 2
Residue 4
dptu
dpu
3.00
2.00
1.00
0.00
-1.00
4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0

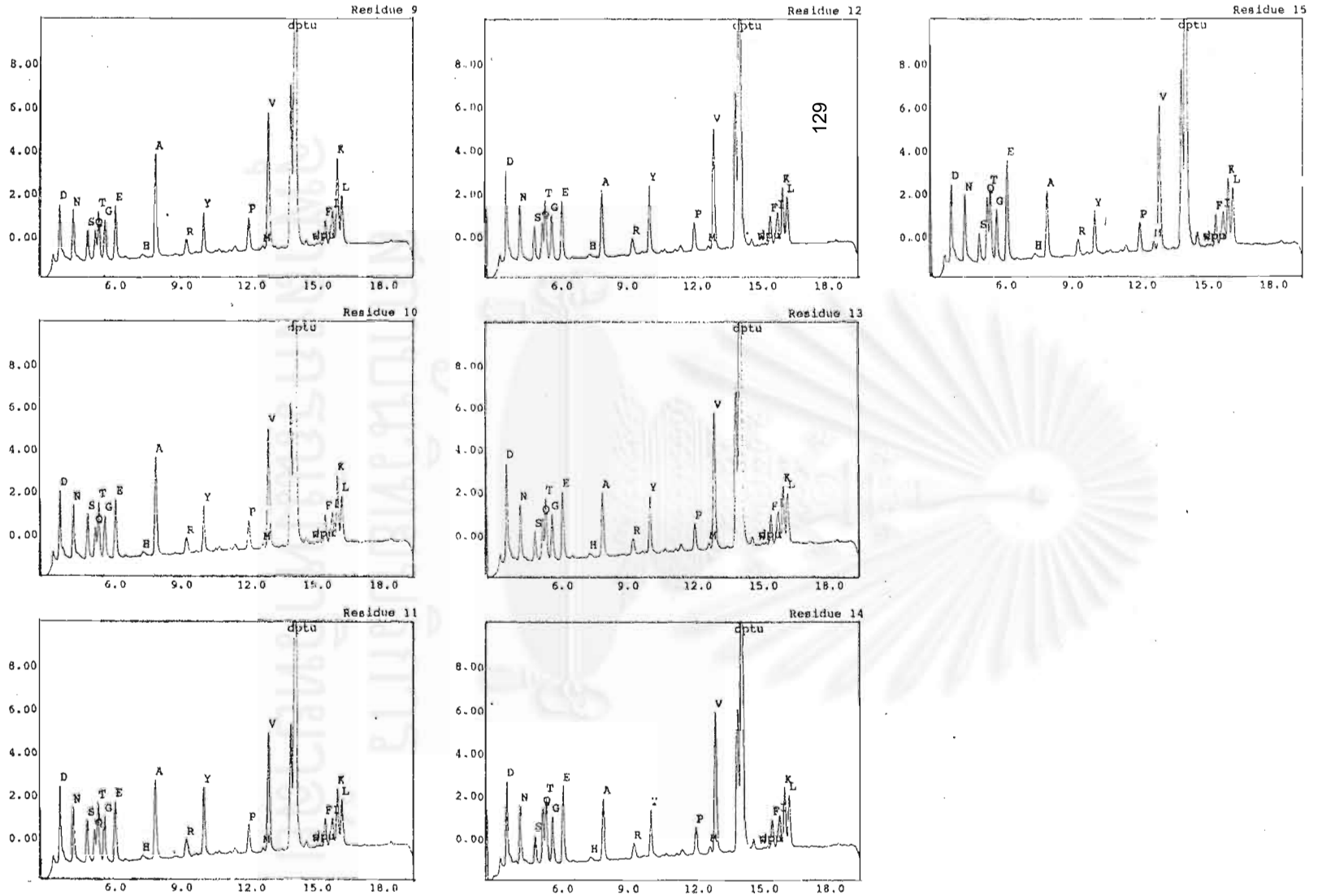
(b) isoform 2



(c) isoform 3


Standard 1
Residue 3
Residue 6
Residue 1
Residue 4
Residue 7
Residue 2
Residue 5
Residue 8
dptu
128

(d) isoform 4



(d) isoform 4 (continued)

BIOGRAPHY

Miss Wanida Prasong was born on August 17, 1977. She graduated with the Bachelor Degree of Science in Agro-Industrial Technology with first class honor from King Mongkut's Institute of Technology North Bangkok in 1999 and continued studying for Master in Biochemistrty Department, Chulalongkorn University.