การบ่งชี้ฮิสติดีนที่สำคัญในไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสไอโซฟอร์ม 1 จาก *Bacillus circulans* A11

นางสาวจารุณี ควรพิบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IDENTIFICATION OF ESSENTIAL HISTIDINES IN CYCLODEXTRIN GLYCOSYLTRANSFERASE ISOFORM 1 FROM *Bacillus circulans* A11

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A Thesis Submitted in Partial Fulfillment of the Requirements
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จารุณี ควรพิบูลย์ : การบ่งชี้ฮิสติดีนที่สำคัญในไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสไอโซฟอร์ม 1 จาก *Bacillus circulans* A11 (IDENTIFICATION OF ESSENTIAL HISTIDINES IN CYCLODEXTRIN GLYCOSYLTRANSFERASE ISOFORM 1 FROM *Bacillus circulans* A11) อ. ที่ปรึกษา : รศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, 131 หน้า. ISBN 974-346-387-9.

งานวิจัยในส่วนต้นต้องการเปรียบเทียบกรดอะมิโนสำคัญแต่ละไอโซฟอร์มของ CGTase จาก *Bacillus circulans* A11 โดยเตรียมไอโซฟอร์มบริสุทธิ์ 4 รูปแบบด้วยเทคนิค preparative gel electrophoresis แล้วใช้สารดัดแปลงกรดอะมิโนทำให้แอคติวิตีของไอโซฟอร์มลดลงหรือสูญเสีย พบว่ากรดอะมิโนสำคัญที่เหมือนกัน คือ ฮิสติดีน ทริปโตเฟน ไทโรซีน และกรดอะมิโนในกลุ่มคาร์บอกซิลิก ส่วนที่ต่างกัน คือ ไอโซฟอร์ม 2 และ 4 มีเซรีน ไอโซฟอร์ม 3 มีไลซีน เมื่อทำการป้องกันบริเวณเร่งของแต่ละไอโซฟอร์มด้วย 25 mM methyl-β-CD ก่อนการดัดแปลงกรดอะมิโน พบว่า กรดอะมิโนที่มีส่วนเกี่ยวข้องอยู่ในบริเวณเร่งของแต่ละไอโซฟอร์ม คือ ฮิสติดีน ทริปโตเฟน ไทโรซีน และกรดอะมิโนในกลุ่มคาร์บอกซิลิก รวมทั้งเซรีนในไอโซฟอร์ม 2 และ ไลซีนในไอโซฟอร์ม 3 ขั้นตอนที่สอง คือ ศึกษาความสำคัญ จำนวน และตำแหน่งฮิสติดีนที่อยู่ในบริเวณเร่งของไอโซฟอร์ม 1 การใช้สารดัดแปลงฮิสติดีน diethylpyrocarbonate (DEP) ที่ pH 6.0 อุณหภูมิ 40°ซ พบว่าความเข้มข้นที่เหมาะสมของ DEP คือ 0.325 มิลลิโมลาร์ และระยะเวลาที่เหมาะสมในการบ่มไอโซฟอร์ม 1 กับ DEP เท่ากับ 5 นาที ตามลำดับ ซึ่งจะทำให้แอคติวิตีของไอโซฟอร์มสูญเสียไปทั้งหมด ผลการศึกษาจลนพลศาสตร์ของการยับยั้งไอโซไซม์ 1 พบว่ามีค่าอัตราเร็วคงที่ของปฏิกิริยาการยับยั้งเชิงอันดับ 2 เท่ากับ 29.5 $M^{-1}s^{-1}$ และอัตราส่วนของสารยับยั้งต่อไอโซฟอร์ม 1 ในหน่วยโมลเป็น 1 : 1 นอกจากนี้ ยังพบว่า methyl-β-CD สามารถป้องกันกรดอะมิโนฮิสติดีนในไอโซฟอร์ม 1 ได้ 2 ตำแหน่ง เมื่อย่อยไอโซฟอร์ม 1 ด้วยทริปซิน แล้วนำเปปไทด์ที่ถูกย่อยมาแยกและวิเคราะห์โดยเทคนิค HPLC พบพีคที่สำคัญ 2 พีคที่ R_t เท่ากับ 11.348 และ 40.934 นาที จากการหามวลของสายเปปไทด์ทั้งสอง โดยแมสสเปกโตรเมตรีพบว่ามีค่าเท่ากับ 5,723 และ 2,540 ดาลตัน ตามลำดับ เมื่อวิเคราะห์ลำดับกรดอะมิโนที่ปลาย N ที่ R_t เท่ากับ 11.348 นาที ได้เป็น F A Q K และที่ R_t เท่ากับ 40.934 นาที ได้เป็น V I I D F A P N H T ซึ่งเมื่อตรวจสอบกับลำดับกรดอะมิโนของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสทำให้คาดได้ว่าฮิสติดีนที่ตำแหน่ง 140 และ 327 เป็นฮิสติดีนสำคัญที่บริเวณเร่งของไอโซฟอร์ม 1

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

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

ปีการศึกษา....2543.....**ลายมือชื่ออาจารย์ที่ปรึกษาร่วม**.....-.....

4172559623 : MAJOR BIOCHEMISTRY
KEY WORD : CYCLODEXTRIN GLYCOSYLTRANSFERASE / ACTIVE SITE / ISOFORM
JARUNEE KAULPIBOON : IDENTIFICATION OF ESSENTIAL HISTIDINES IN CYCLODEXTRIN GLYCOSYLTRANSFERASE ISOFORM 1 FROM *Bacillus circulans* A11. THESIS ADVISOR : ASSOS. PROF. PIAMSOOK PONGSAWASDI, Ph.D. 131 pp. ISBN 974-346-387-9.

The initial phase of this work is to compare essential amino acid residues in each isoform of CGTase from *Bacillus circulans* A11. The isoform were purified by preparative gel electrophoresis. Modification with certain group-specific reagents and measurement of the loss in isoform activities were performed. It was found that amino acid residues which were essential for all 4 isoforms were histidine, tryptophan, tyrosine, and carboxylic amino acids. Different residues identified to be essential for each isoform were : isoforms 2 and 4, serine ; isoform 3, lysine. From substrate protection experiment, by adding 25 mM methyl-β-CD prior to modification, amino acid residues which were found to be at the active site of each isoform were histidine, tryptophan, tyrosine, and carboxylic amino acids. In addition to these residues, serine in isoform 2 and lysine in isoform 3 were also protected by the substrate suggesting their presence at the active site. The second phase of this work is to characterize and determine the number and position of essential histidine(s) at the active site of isoform 1. When inactivation by diethylpyrocarbonate (DEP) was performed at pH 6.0, 40 °C, the suitable concentration of DEP was 0.325 mM, and the suitable incubation time was 5 minutes. Inactivation kinetics of isoform 1 with DEP resulted in the second-order rate constant ($k_{inactivation}$) of 29.5 $M^{-1}s^{-1}$. The ratio of DEP to isoform 1 (in mole unit) was 1 : 1. Moreover, it was found that methyl-β-CD protects two histidine residues of isoform 1. When isoform 1 was digested by trypsin, peptides resulting from enzymatic cleavage were separated by HPLC. It was observed that peptides of interest were those with R_t = 11.348 and 40.934 minutes. For the peak eluting at 11.348 minutes, mass spectrometry reveals the size M_r of 5,723 Da and the N-terminal sequence was F A Q K. While the peak eluting at 40.934 minutes, the size M_r of 2,540 Da was obtained and the N-terminal sequence was V I I D F A P N H T. When the data from peptide analysis was checked with the sequence of CGTase, it could be predicted that His-140 and His-327 were essential histidines in the active site of isoform 1.

Department…….Biochemistry…..……**Student' s signature**…..……..
Field of study……..Biochemistry…..…….**Advisor's signature**…….……...……
Academic year……...2000…..……...**Co-advisor's signature**…….-…..…….…..

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ABBREVIATIONS

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

CHAPTER I

INTRODUCTION

Cyclodextrins : Characteristics and applications

Cyclodextrins (CDs), also known as Schardinger dextrins, are a group of homologous oligosaccharides, obtained from starch by the action of cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19). They have a closed ring structure of six, seven or eight glucose units linked by α-1,4-glucosidic bonds, which are known as α-, β- or γ-CDs, respectively (French and Rundle, 1942; Freudenberg and Cramer, 1948) (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 bridges (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.

Several cyclodextrin derivatives have been developed through chemical or enzymatic means in order to obtain CDs with specific desirable properties. Some of those are methylated, hydroxypropylated and glycosylated at the hydroxyl groups, resulting in higher solubility than parent CD's. In addition, CD-polymers which are linked cyclodextrins, are often used as stationary phase in various liquid chromatography system (Casu and Roggiani, 1979; Ensuiko, 1994; Yamamoto *et al*., 1990). These modified CDs, in addition to their native or parental CDs (the α-, β-, γ-CD) offer wider range of properties to be selected as the suitable host molecules. Currently available cyclodextrins are listed in Table 2.

Due to their structural characteristics, cyclodextrins can accommodate various organic or inorganic molecules to form soluble or insoluble inclusion

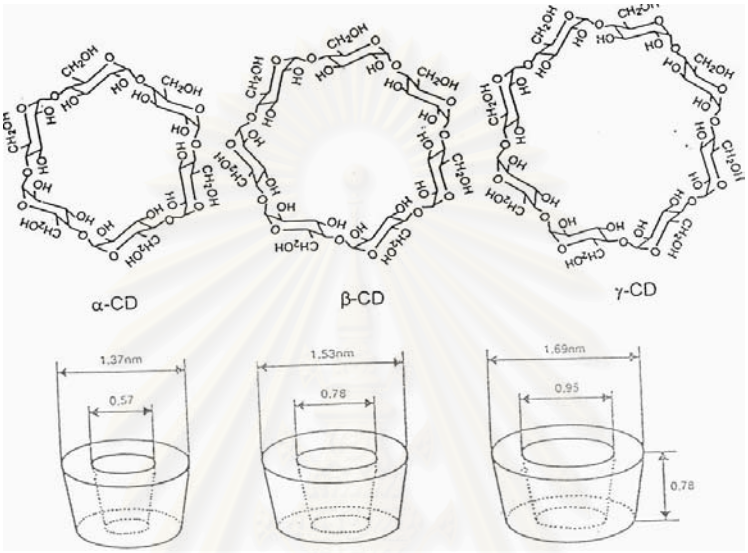


Figure 1. Structure and molecular dimension of cyclodextrins
(French and Rundle, 1942)

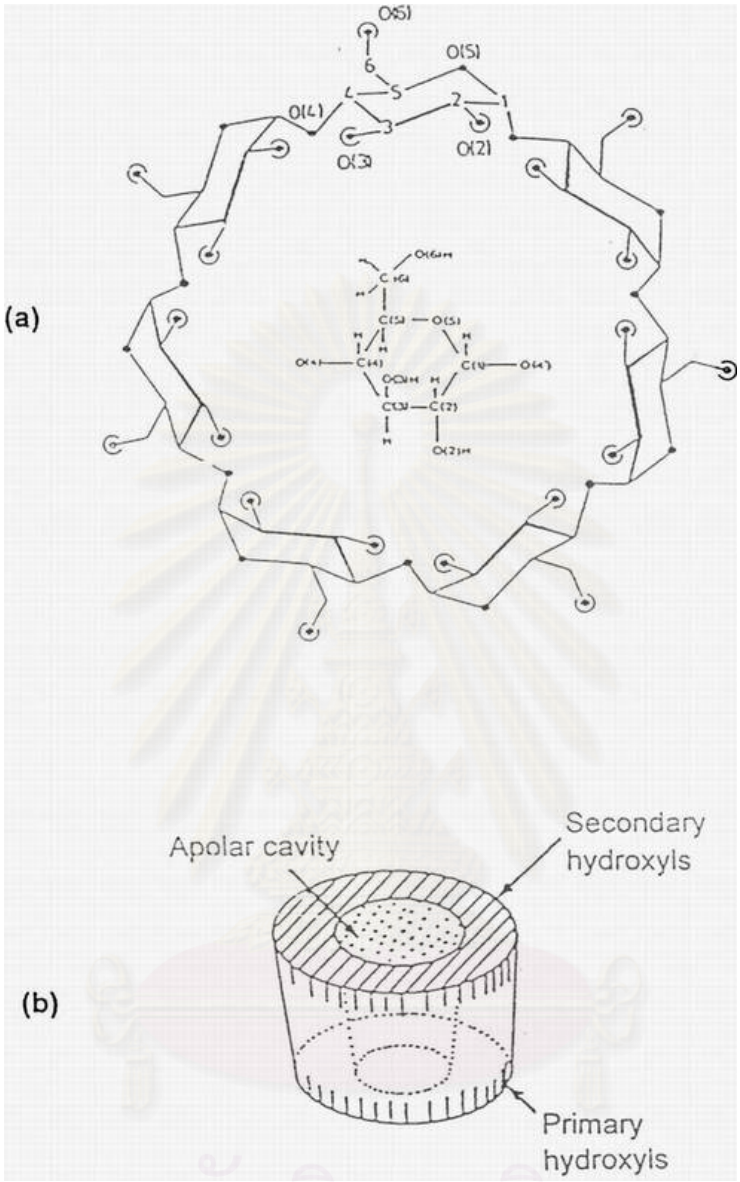


Figure 2. Structure of β-cyclodextrin (Bender, 1986 ; Szejtli, 1990)

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

(b) Functional structure scheme

Table 1. Properties of cyclodextrins (Szejtli, 1988)

Properties	α-CD	β-CD	γ-CD
Number of glucose unit	6	7	8
Molecular weight	973	1135	1297
Cavity diameter, A°	5-6	7-8	9-10
Cavity depth, A°	7-8	7-8	7-8
Crystal form (water)	Needle	Prism	Prism
Solubility, g/100 ml H_2O, 25 °C	14.5	1.85	23.2

Table 2. Classification of cyclodextrin derivatives (Ensuiko, 1993)

Parent CD	**Modified CD**		
	Substituted CD	**Branched CD**	**CD polymers**
α-, β-, γ-CD	Methylated CD - dimethylated - trimethylated Ethylated CD - diethylated - triethylated Hydroxyalkalated CD - 2-hydroxyethylated - 2-hydroxypropylated -3-hydroxypropylated	Homogeneous branched CD - glucosyl - maltosyl Heterogeneous branched CD - galactosyl - mannosyl - maltosyl	- Cross-linked CDs - Matrix coupled CDs

complexes. The inclusion complex is held together by non covalent bonding forces such as hydrophobic interaction, Van der Waal forces, London dispersion forces and hydrogen bonding (Komiyama and Bender, 1984). The binding of organic or inorganic molecules (Guest) within the cyclodextrins (Host) 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 as shown in Figure 3 (Amaizo, 1993).

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, alterration of the chemical reactivity, modification of liquid substances to powders, or reduction of undesirable smell or taste in products e.g. foodstuffs are among those known useful properties of CDs (Schmid, 1989). Therefore, cyclodextrins are increasingly used in industrial and research application (Table 3).

Cyclodextrin producing enzymes

Starch can be degraded to CDs by the action of cyclodextrin glycosyltransferase [1,4-α-D-glucan: 1,4-α-D-glucopyranosyl transferase, EC 2.4.1.19, CGTase]. The CGTase enzyme degrades starch by catalyzing cyclization, coupling and disproportionation reactions as shown below:

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

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

Where G_n and G_m are 1,4-α-D-glucopyranosyl chains with "n" and "m" residues; x is a part of the 1,4-α-D-glucopyranosyl chain, and cG_x is a symbol for CDs (Starnes R.L. *et al*., 1990). These mechanisms are shown in Figure 4.

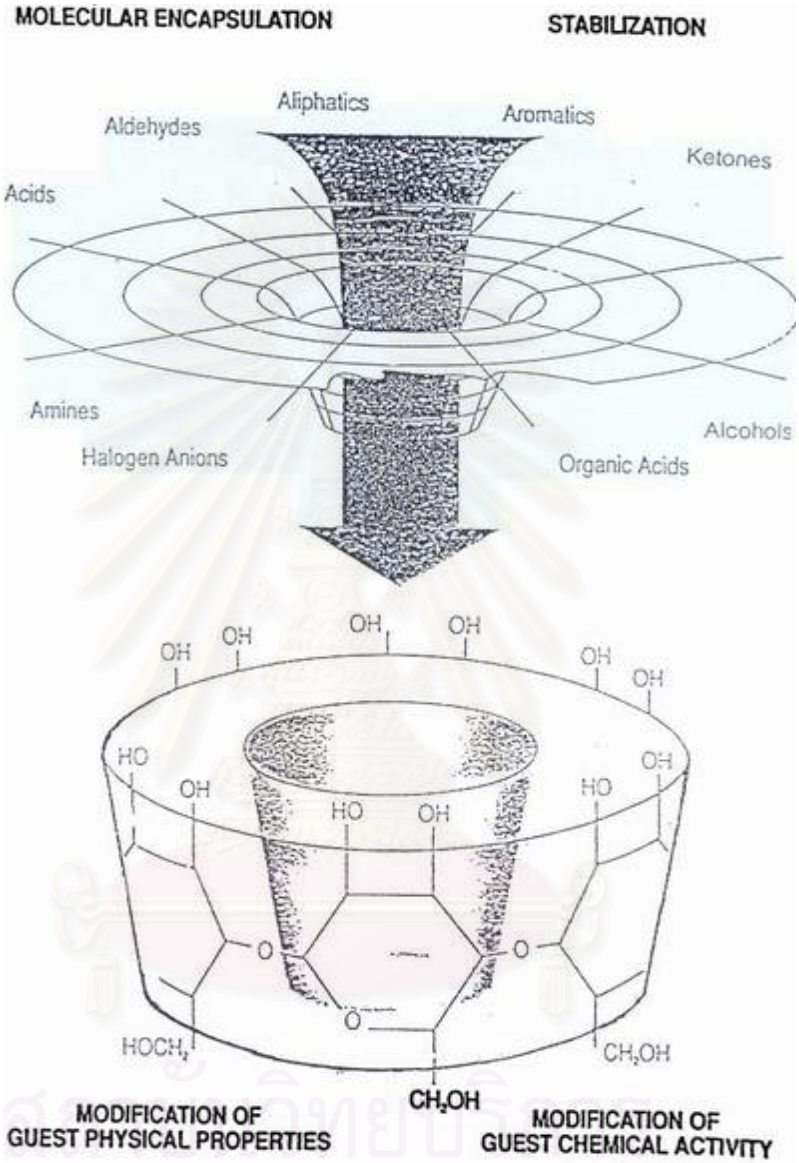


Figure 3. Beneficial modification of guest molecules by cyclodextrin (Amaizo, 1993)

Table 3. Industrial applications of cyclodextrins

(Horikoshi, 1982; Bender, 1986; Szejtli and Pagington, 1991)

Use	Guest compounds and end products
Foods	
1) Emulsification	Eggless mayonnaise, seasoning oil, Whipping cream, etc.
2) Increase of foaming power	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 hygroscopicity	Powder flavour products
6) Elimination of unpleasant tastes	Juice, milk, casein, ginseng, propylene glycol
7) Elimination of cholesterol	Egg yolk, milk, butter
8) Reduction of odour	Mutton, fish, soybean
Cosmetics and toiletries	
1) Color masking and control	Fluorescein, bath agents
2) Stabilization 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

Table 3. Industrial applications of cyclodextrins (continued)

Use	Guest compounds and end products
Pharmaceuticals	
1) Increase of solubility	Prostaglandin, phenobarbital, chloramphenicol
2) Taste masking	Prostaglandin
3) Powdering (nonvolatile)	Nitroglycerin, clofibrate
4) Stabilization (UV, thermal)	Prostaglandin, vitamins
5) Decrease of irritation	Cu-alcanolamine complex
6) Enhancement bioavailability	Barbiturate, flufenamic acid, digoxin
7) Reduction of systemic toxicity	2-amino, 4-methyl-phosphonobutyric acid
Agriculture	
1) Stabilization of volatility	Tobacco aroma
2) Stabilization of nutrient	Animal-feed
3) Improvement of palatability	Bone-powder, microbial cell-mass
Pesticides	
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, etc.
Plastics	
Stabilization of colors and flavors	Colors, flavors
Others	Adhesives

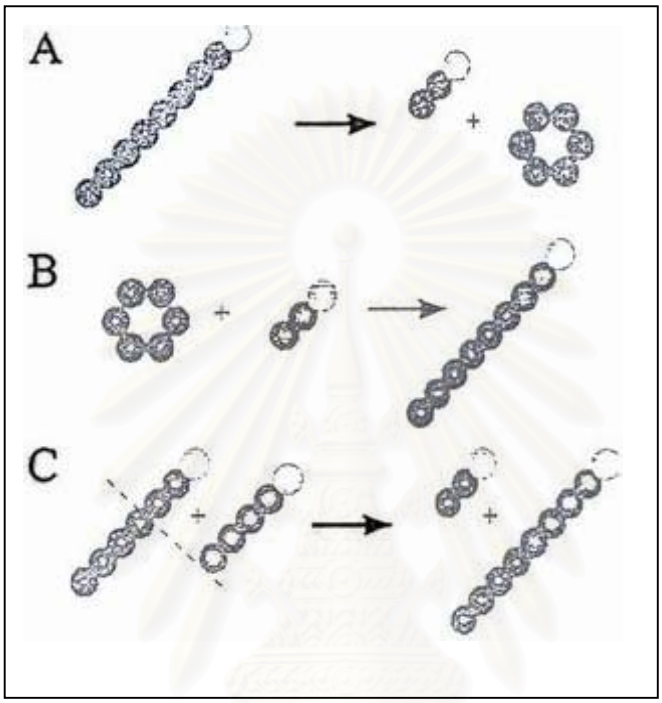


Figure 4. Schematic representation of the CGTase catalyzed reactions.

The circles represent glucose residues; the white circles indicate the reducing end sugars. (A) cyclization, (B) coupling and (C) disproportionation (Bart A. *et al.*, 2000)

The cyclization reaction produces cyclodextrins. These cyclized molecules have neither a non-reducing nor reducing end-group. Cyclization is a single substrate reaction with an affinity for the high molecular mass substrate. The coupling reaction is the reverse of the cyclization reaction and involves two substrates, which are combined to produce one product. Coupling (cleavage of an α-glycosidic bond in a cyclodextrin ring and transfer of the resulting linear malto-oligosaccharide to an acceptor substrate) proceeds according to a random-order ternary complex mechanism. Disproportionation (cleavage of a α-glycosidic bond of a linear malto-oligosaccharide and transfer of one part to an acceptor substrate), also a bisubstrate reaction, is proved to follow the ping-pong type of mechanism. The proposed model of the event taking place in the CGTase-catalyzed reaction was as shown in Figure 5 (Bart A. *et al*., 2000).

CGTase is produced by various microorganisms, for example *Klebsiella pneumoniae* M5 al (Bender, 1982), *Micrococcus* sp. (Yagi *et al.*, 1986) and 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 formed (Horikoshi, 1988). The enzymes from different sources show different characteristics such as working pH, temperature, and molecular weight. Each CGTase enzyme yields different ratio of cyclodextrin products for example, the CGTase of *B. macerans* produced α-, β-, and γ-CD in relative amount of 2.7:1.0:1.0 (Depinto and Campbell, 1968), while the CGTase of Alkalophilic *Bacillus* no. 38-2 and *B. circulans* produced CDs in relative amount of 1.0:11.0:1.5 (Matzuzawa *et al.*, 1975) and 1.0:10.0:1.0 (Pongsawasdi and Yagisawa, 1987), respectively. The CGTase of *Bacillus fermus* 290-3 was known to produce γ-CD in the initial phase of the enzyme production (Englbrecht *et al.*, 1990).

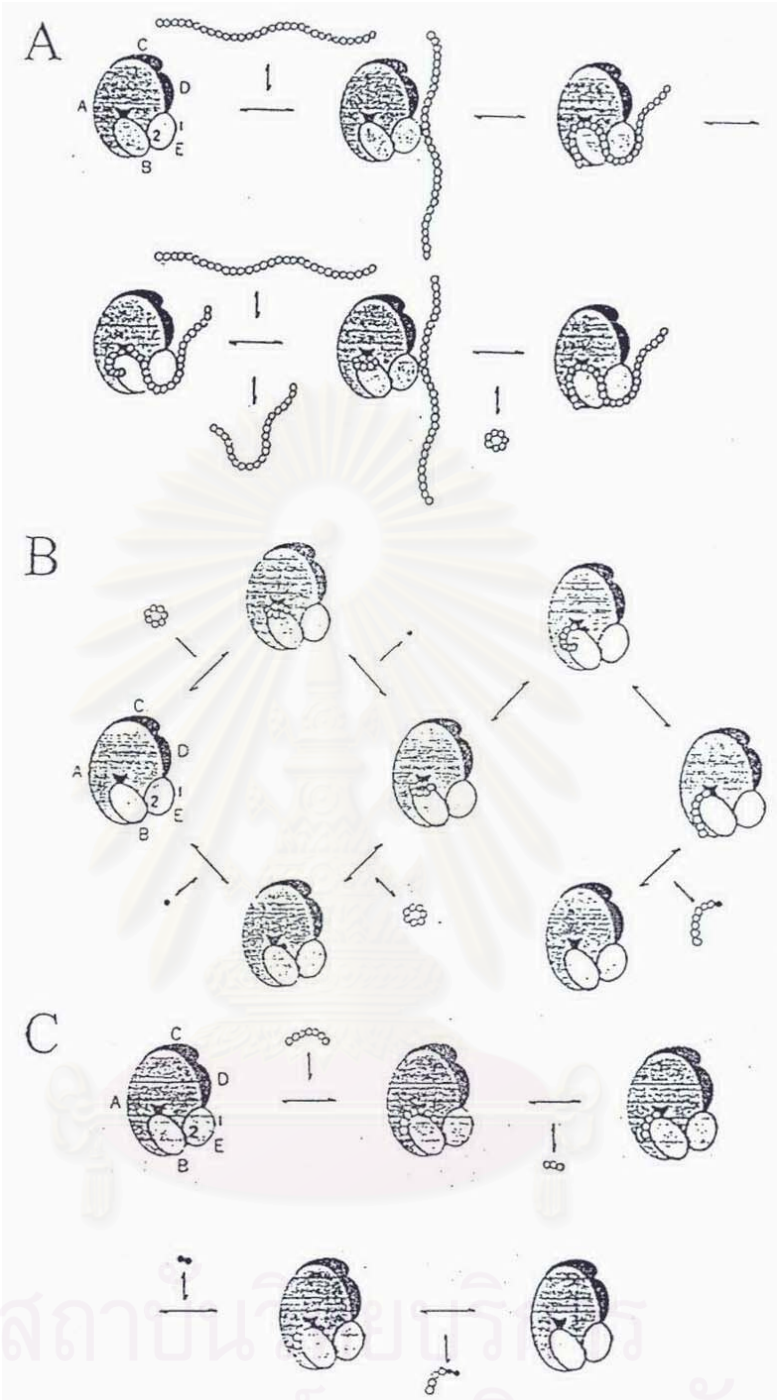


Figure 5. Proposed model of the events taking place in the CGTase-catalyzed reactions. (Bart A. *et al*., 2000)

(A) Cyclization. (B) Coupling. (C) Disproportionation. The different CGTase domains are indicated (A, B, C, D, and E). 1 and 2 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 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., 1977
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, 1982
Micrococcus sp.	β-CD	6.2	ND	139,000	ND	Yagi *et al.,* 1980
Bacillus circulans A11						Kaskangam, 1998
Isozyme 1	β-CD	6.0-7.0	40	72,000	4.73	
Isozyme 2	β-CD	6.0-7.0	40	72,000	4.49	
Isozyme 3	β-CD	6.0	50	72,000	4.40	
Isozyme 4	β-CD	7.0	50-60	72,000	4.31	

ND = Not Determined

Purification methods for CGTase

The study on physical and biochemical properties of CGTase required separation technique to purify 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* NO2 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 successfully purified CGTase from *Bacillus circulans* A11 by immunoaffinity chromatography using anti-CGTase linked to CNBr-activated Sepharose 4B.

Multiple forms of CGTase

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, 1984). 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) (Nagamura and Horikoshi *et al.,*1976).

The CGTase from alkalophilic *Bacillus* (ATCC 21783) was purified to near homogeneity by a two-step procedure involving affinity chromatography and high-performance anion exchange 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, from another report on isolation of CGTase from the same strain but using isoelectric focusing in immobilized pH gradients, it was found 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 the N-terminus of these CGTase subforms were determined and reported to be the same (Ala-Pro-Asp-Thr-Ser) (Mattsson, Meklin and Korpela, 1990). CGTase from *B. circulans* E192 was purified by FPLC on a 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 the two isozymes was found (Bovetto *et al*., 1992).

In 1998, Kaskangam attempted to purify CGTase isozymes isolated from *Bacillus circulans* A11 by preparative gel electrophoresis. Five isozymes (bands 1, 2, 3, 4 and 5) were separated by this technique. Band 1, 2, 3 and 4 can produce α-,β- and γ-CD while band 5 cannot produce CD. The results showed that some physicochemical and biochemical properties of each isozyme i.e. pI, product ratio, and carbohydrate content were different (Table 5) (Kaskangam, 1998).

The active site of CGTase

The three-dimensional structure of a few CGTases from x-ray crystallographic technique showed that CGTase consisted of five domains, labels A to E (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 and E domains remain 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

Table 5. Summarization of properties of CGTase isozymes from *Bacillus circulans* A11 (Kaskangam, 1998).

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

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

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 domain, but α-amylase lacks the additional domains D and E that are unique for CGTase. Several papers reported sequence similarities between CGTase and α-amylase, with four highly conserved regions and designated as regions A, B, B′, and C, respectively (Figure 6.) (Kimura *et al.,* 1987). The catalytic domains of both enzymes are folded into $(\beta/\alpha)_8$ barrel structures, and the four conserved regions within this domain constitute the active center (Klein and Schulz, 1991). These regions have also been found in other amylolytic enzymes such as isoamylase, pullulanase, and branching enzyme.

Three-dimensional structures of CGTase from *Bacillus circulans* and *Bacillus stearothermophilus* have also been reported by other research groups (Klein and Schulz, 1991 ; Kubota *et al.,*1991). The chain folds of both CGTases are similar. The NH_2-terminal 400 amino acids of CGTases are folded into barrel structures, which closely resemble those of α-amylases and which also contain the conserved regions. The catalytic residues of α-amylase have been proposed from their three dimensional structures. Although it has been proposed that the catalysts are different pairs among the three α-amylases (Taka- amylase A, porcine pancreatic α-amylase, and acid α-amylase from *Aspergillus niger*), they are restricted within the three conserved acidic amino acid residues away on the COOH-terminal side of the B-region (designated as the B′-region); and aspartate in the C-region. The one glutamate and the two aspartate residues in the B-, B′-, and C-region are found in all amylolytic enzymes possessing the conserved regions. Site-directed mutagenesis studies have shown that any one of these residues is essential for catalysis in α-amylase, neopullulanase, and CGTase (Nakamura *et al.,* 1993). These findings suggest that the catalytic mechanisms among these amylolytic enzymes are similar and that the variations in substrate specificity and products can be ascribed to the relationships between their similar catalytic centers and different subsite structures. It is thought that α-amylase catalyzes the

hydrolysis of the α-1,4-glucosidic bond by acid-base catalysis as proposed for lysozyme cleavage of the glucosidic bond is catalyzed via an oxocarbonium ion intermediate.

Studies on amino acid compositions were determined directly from amino acid analyzer (Schmid *et al.,* 1988; Takano *et al.,* 1986; Kimura *et al.,* 1987). Asx (aspartic and asparagine) was the most (100-120 residues) in CGTase (Bovetto *et al.,* 1992). Cysteine could not be detected in CGTases produced from *Bacillus circulans* E192 and *Bacillus macerans* IAM 1243 while a few could be detected in CGTases from other microorganisms. Laloknam (1997) reported that when the purified CGTase from *Bacillus circulans* A11 was determined for its amino acid composition, 40 mol % of the content was non-polar amino acids while aspartic acid, asparagine, glutamic, and glutamine was rather high (25 mol %). Only 5 mol % was aromatic amino acids: phenylalanine, tryptophan, and tyrosine. Sulfur-containing amino acids: cystein and methionine were low. In 1998, the amino acid composition of CGTase isozymes (band 1-5) from *Bacillus circulans* A11 were reported by Kaskangam. It was found that 40-50 mol% of the content were non-polar amino acids, while 50-60 mol% were polar amino acids. Acidic amino acids : aspartic acid and glutamic acid were rather high in band 2-5 (22 mol%) while about 15 % was found in band 1. Methionine, which is sulfur-containing amino acid was low. Each band had some difference in their amino acid composition, such as threonine, which were rather high in band 1, 2, 3, and 5 but not found in band 4. Histidine and proline which can be detected in small amount in bands 1, 2, 3, and 5, was rather high in band 4 (Table 6).

From modeling studies and alignments of amino acid sequences, it was suggested that a residue 221 (*Thermoanaerobacterium* numbering) was present in CGTase at a dominant position in the center of the active-site cleft. In most CGTases the residue was an aromatic amino acid (Tyr or Phe), whereas in α-amylases the residue was much smaller (Gly, Ser, or Val). Penninga *et al.* (1995) constructed mutants of *Bacillus circulans* strain 251 CGTase in which this aromatic residue had been replaced by a non aromatic residue. This results in a switch-over to synthesis of linear maltooligosaccharides from starch, which indicated that the aromatic amino acid residue at this position was of crucial importance for an efficient cyclization

Table 6. Amino acid composition of CGTase isozymes from *Bacillus circulans* A11 (Kaskangam, 1998).

Amino acid	Content (mol%)					
	P*	Band 1	Band 2	Band 3	Band 4	Band 5
Asp	14.35	10.04	13.25	13.15	12.24	14.63
Glu	10.04	4.79	8.90	9.96	10.33	7.59
Ser	5.43	6.45	5.95	5.85	**	5.90
Gly	8.90	**	**	**	**	**
His	1.29	2.11	1.64	3.96	7.79	5.42
Arg	2.87	4.80	3.53	3.78	8.62	3.89
Thr	8.17	10.09	10.43	9.98	**	9.89
Ala	9.63	15.41	11.74	11.39	8.42	11.15
Pro	5.49	7.41	6.17	6.55	11.37	6.51
Tyr	2.94	2.93	3.76	3.41	6.66	3.51
Val	8.64	9.43	10.02	9.04	10.71	8.41
Met	1.11	2.16	1.40	1.10	1.34	1.37
Cys2	Not determined					
Ile	4.91	7.85	6.63	5.67	4.54	6.56
Leu	6.32	6.53	6.18	6.49	8.31	6.56
Trp	Not determined					
Phe	4.31	5.53	5.62	4.42	4.30	4.38
Lys	5.59	4.46	4.77	5.26	5.38	4.30

P* = Purified enzymes from DEAE column by Laloknam (1997) analyzed in parallel

** = Not calculated because of glycine contamination in the enzyme sample

Figure 6. Alignment of amino acid sequences of bacterial CGTases

(Wind *et al.*, 1995).

Figure 6. Alignment of amino acid sequences of bacterial CGTases

(continued)

reaction. The alignments of amino acid sequences of bacterial CGTases was shown in Figure 6. (Wind *et al.,* 1995).

Modification of amino acids with chemical reagents has been used to identify the active site of CGTase from *Bacillus stearotheromophilus*. Tryptophan 97 was found to carry an essential role in the binding of maltotriose (Ohnishi *et al.,* 1994). Chemical modification studies of CGTases from *Bacillus circulans* var. *alkalpophilus* and *Bacillus circulans* E192 with the use of group - specific reagents suggested the presence of histidine at or near the active site (Mattson *et al.,* 1992; Villette *et al.,* 1992). Chemical modification studies of CGTases from *Klebsiella pneumoniae* and *Bacillus circulans* with diethylpyrocarbonate have suggested that at least one histidine residue is important for the cyclization reaction (Bender, 1991; Mattson *et al.,* 1992). Three-dimensional structure and site-directed mutagenesis were reported to be used in identification of amino acid residues at the active site of CGTase from *Bacillus* sp. 1011 which is an alkalophilic microorganism. Histidine 327 was found to be important for catalysis over an alkaline pH range, whereas histidine 140 and 233 were found to be important for substrate binding (Nakamura *et al.,* 1993). The structure of some CGTases has been studied by X-ray diffraction. Aspartic acid 229, glutamic acid 257, and aspartic acid 328 constituted the catalytic residues in CGTases from *Bacillus circulans* and *Bacillus circulans* strain 251. It was concluded that glutamic acid 257 acts as the proton donor in the reaction, whereas aspartic acid 328 is involved in the binding of the substrate and helps to elevate the pKa of glutamic acid 257 through a direct hydrogen bond to this residue that exist only when no substrate or inhibitor is present (Klein *et al.,* 1992 ; Lawson *et al.,* 1994 ; Strokopytov *et al.,* 1995). X-ray diffraction studies and site-directed mutagenesis experiments have also identified aspartic acid 255, glutamic acid 283, and aspartic acid 354 as catalytic residues in *Thermoanaerobacterium* CGTase. These residues were found to be conserved in the amino acid sequence of the *Thermoanaerobacterium* CGTase (Wind *et al.,* 1995). Chemical modification and substrate protection techniques of CGTase from *Bacillus circulans* A11 suggested that carboxyl (aspartic and glutamic acid), histidine, tryptophan, and tyrosine residues were located at the enzyme active site (Tongsima, 1998). Table 7 summarizes the amino acids involved in the active sites of CGTases.

Table 7. Summary of amino acid residues involved in the active sites of CGTases

Source	Amino acid residues at the active site	Reference
Bacillus circulans strain 251	Asp 229, Glu 257, Asp 328	Klein *et al.*, 1992 and Strokopytov, 1995
Bacillus circulans strain 251	Tyr 195	Penninga *et al*., 1995
Bacillus circulans E192	Tyr	Villette *et al*., 1993
Bacillus sp.1011	His 327, His 233, His 140	Nakamura *et al*., 1993, 1994
Bacillus stearothermophilus	Trp 97	Ohnishi *et al*., 1994
Thermoanaerobacterium Thermosulfurigenes EM 1	Asp 255, Glu 283, Asp 354	Wind *et al*., 1995
Bacillus circulans A11	Asp, Glu, His, Trp, Tyr	Tongsima, 1998

This research is a continual part of the work of the cyclodextrin research group at the Department of Biochemistry, Faculty of Science, Chulalongkorn University. The CGTase of *Bacillus circulans* A11, a strain isolated from South-East Asian soil and screened by Pongsawasdi and Yagisawa, 1987, was purified and characterized (Techaiyakul, 1991; Rojtinnakorn, 1994). Specific antibody against CGTase was prepared (Rojtinnakorn, 1994) and was used in immunoaffinity purification of the enzyme (Kim, 1996). Rutchtorn (1993) optimized CGTase production in a 5 litre-fermenter. Batchwise and continuous production of cyclodextrins using immobilized CGTase were reported (Rutchtorn, 1993; Kuttiarcheewa, 1994). Malai (1995) studied the production of cyclodextrins from rice starch by using free CGTase. Siripornadulsil (1992), Vittayakitsirikul (1995), and Boonchai (1996) reported on molecular cloning techniques, gene expression, mapping and partial nucleotide sequence determination. Laloknam (1997) synthesized oligonucleotide probes for detecting CGTase gene and Jantarama (1997) demonstrated the use of chemical and UV mutagenesis of *Bacillus circulans* A11 for the production of higher CGTase activity. Tongsima (1998) reported on the active site of CGTase and Kaskangam (1998) isolated and charaterized CGTase isozymes. The gene coding for CGTase has been recently cloned and sequenced (Rimphanitchayakit, 2000). As we are not certain how many gene, we will call isoform in this work.

Characterization of the active site of CGTase isoforms will be the first focal point of this work in the attempt to further differentiate each isoform. The study will also cover characterization of isoform 1, the major CGTase isoform, by identification of essential histidine at its active site. These experiments will lead to more insight into the understanding of the multiple forms of the enzyme and the ability to compare with other CGTase isoforms/isozymes. For industrial application, these information may enable selection of production of certain kind or certain ratio of CD as desired.

The objective of this research

1. To determine essential amino acid residues of each CGTase isoform of *Bacillus circulans* A11

2. To study inactivation kinetics of CGTase isoform 1 with a histidine-modifying agent

3. To identify the position of essential histidine residues at the active site of CGTase isoform 1

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Amino acid analyzer : Waters Picotag™ system, Waters 510 HPLC, Picotag™ (0.39x30 cm) : Waters company,U.S.A.

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

Autopipette : Pipetman, Gilson, France

C_{18} column : Model LUNA 5 μ size 250 x 4.6 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

Desalting column : Model HiTrap® (Sephadex G-25 Superfine, 20x50 mm), Pharmacia Biotech, Sweden

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

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

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

Fraction collector : Model 2211 Pharmacia LKB, Sweden

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

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

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

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

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

Mass spectrometer : TOF ESI-MS system, micromass, UK.

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

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

Spectrophotometer UV-240, Shimadzu, Japan, 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, Shaking : Heto lab Equipment, Denmark

2.2 Chemicals

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

N-acetylimidazole : Sigma, U.S.A.

Acrylamide : Merck, U.S.A.

N-bromosuccinimide : Sigma, U.S.A.

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

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

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

Diethylpyrocarbonate : Sigma, U.S.A.

DL-dithiothreitol : Sigma, U.S.A.

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

N-Ethylmaleimide : Sigma, U.S.A.

Glycine : Sigma, U.S.A.

Iodoacetamide : Sigma, U.S.A.

D(+)-maltose monohydrate : Fluka, Switzerland

Methylorange : BDH, England

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

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

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

Phenylmethylsulfonyl fluoride : Sigma, U.S.A.

Soluble strach (Potato) : Fluka A.G. Buchs S.G., Switzerland

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

Trifluoroacetic acid : BDH, England

2,4,6-Trinitrobenzenesulfonic acid : 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

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

2.4 Media preparation

2.4.1 Medium I

Medium I, consisted of 0.5 % beef extract, 1.0 % polypeptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble strach (Fluka) 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 °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 strach, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7\ H_2O$ and 0.75% Na_2CO_3 with starting pH of 10. Medium was steriled as above.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

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

2.5.2 Enzyme production

Starter *Bacillus circulans* A11 was transferred into 100 ml Horikoshi's broth in 500 ml Erlenmeyer flask with 1% inoculum and cultivated at 37 °C with 250 rpm rotary shaking. Culture was harvested after 72 hours and cells were removed by

centrifugation at 3,000 rpm for 30 minutes at 4 °C. Culture broth with crude CGTase was collected and kept at 4 °C for purification.

2.6 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 °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 °C for 24 hours. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in the dark. The activity was expressed in term of 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 Purification of CGTase

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

Corn starch (local grade) was oven dried at 120 °C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude broth to 5% (W/V) concentration. After 3 hours of continuous stirring at 4 °C, the starch cake was collected by centrifugation at 5,000 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 (3x150 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.

2.9 Isolation of CGTase isoforms

The concentrated partially purified enzyme (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 a flow rate of 1ml/min. Fractions of 2.5 ml were collected and measured for A_{280} and dextrinizing activity to identify the CGTase isoforms. Every 5 fractions were run on slab gels and stained for dextrinizing activity. Then the fractions that gave the electrophoretic band with the same mobility were pooled for further studies.

2.10 Purity of CGTase isoforms

2.10.1 Polyacrylamide Gel Electrophoresis (PAGE)

Non–denaturing gel electrophoresis was employed for analysis of the purified isoforms. Electrophoresis conditions, protein and activity staining were as described below.

2.10.1.1 Non–denaturing gel electrophoresis

Discontinuous PAGE was performed on slab gels (10x8x0.75 cm) of 7.5% (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.

2.10.1.2 Detection of proteins

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

2.10.1.2.1 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 background was clear.

2.10.1.2.2 Dextrinizing activity staining (slightly modified from the 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 °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 background indicates starch degrading activity of CGTase isoforms.

2.11 Effect of group-specific reagents on CGTase isoform activities

2.11.1 Modification of cysteine residues

Modification of cysteine residues was carried out according to the method of Means and Feeney (1971). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with *N*-ethylmaleimide (NEM), iodoacetamide (IAM) or dithiothreitol (DTT) at 40 °C for 30 minutes. The final concentration of the enzyme and NEM, IAM, or DTT were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.11.2 Modification of lysine residues

Modification of lysine residues was carried out according to the method of Means and Feeney (1971). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) at 40 °C for 30 minutes. The final concentration of the enzyme and TNBS were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.11.3 Modification of serine residues

Modification of serine residues was carried out according to the method of Wakayama *et al*. (1996). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with phenylmethylsulfonyl fluoride (PMSF) at 40 °C for 30 minutes. The final concentration of the enzyme and PMSF were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.11.4 Modification of carboxyl residues

Modification of carboxyl residues was carried out according to the method of Means and Feeney (1971). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at 40 °C for 30 minutes. The final concentration of the enzyme and EDC were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.11.5 Modification of tyrosine residues

Modification of tyrosine residues was carried out according to the method of Means and Feeney (1971). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with *N*-acetylimidazole (NAI) at 40 °C for 30 minutes. The final concentration of the enzyme and NAI were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.11.6 Modification of tryptophan residues

Modification of tryptophan residues was carried out according to the method of Lundblad (1991). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with *N*-bromosuccinimide (NBS) at 40 °C for 30 minutes. The final concentration of the enzyme and NBS were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.11.7 Modification of histidine residues

Modification of histidine residues was carried out according to the method of Miles (1977). Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with diethylpyrocarbonate (DEP) at 40 °C for 30 minutes. The final concentration of the enzyme and DEP were 25 μg/ml and 1mM, respectively. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each

isoform. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1.

2.12 Identification of essential amino acid residues at the catalytic site of CGTase isoforms.

2.12.1 For histidine residues

2.12.1.1 Determination of suitable DEP concentration

Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was incubated with varying concentrations of DEP (0-2 mM) at 40 °C, for 30 minutes. The final concentration of the enzyme in 60-300 μl total reaction volume was 25 μg/ml. After the incubation, CGTase isoform activity was measured as described in Section 2.6.1. The suitable concentration is the minimum concentration of DEP that laeds to the maximum inactivation of the enzyme.

2.12.1.2 Determination of suitable inactivation time

After the suitable concentration of DEP was chosen, the treated time was varied from 0-30 minutes and CGTase isoform activity was measured as described in Section 2.6.1. The suitable inactivation time is the incubation time at which about 50 % dextrinizing activity was retained.

2.12.1.3 Histidine modification and substrate protection

The experiment was performed by incubating suitable concentration of DEP for suitable time period with the CGTase isoforms in the presence or the absence of β-, methyl-β- or hydroxypropyl-β-CD as protective substance. Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was preincubated with 25 mM of each substrate at 40 °C for 5 minutes prior to the addition of suitable concentration of diethylpyrocarbonate (DEP) (25 mM of substrate was used according to Tongsima, 1998). The final concentration of each isoform was 25 μg/ml. The total volume of the reaction mixture was 60-300 μl depending on the original concentration of each isoform. After incubation with DEP for 3-7 minutes, the residual CGTase isoform

activity was determined as described in Section 2.6.1. Isoform 5 was not used in this experiment because it had no CGTase activity as reported by Kaskangam, 1998.

2.12.2 For other amino acid residues

Suitable concentration of group-specific reagents and incubation time were not determined for each isoform. The concentration used for NBS and NAI were 0.05 and 30 mM, respectively, according to Tongsima (1998). For other group-specific reagents, 1 mM concentration was used. Incubation time with group-specific reagents was 30 minutes for all reactions.

The experiment was performed by incubating group-specific reagents with the CGTase isoforms in the presence or the absence of methyl-β-CD as protective substance. Each CGTase isoform in 50 mM acetate buffer, pH 6.0, was preincubated with 25 mM of methyl-β-CD at 40 °C for 5 minutes prior to the addition of group-specific reagents. The final concentration of each isoform was 25 μg/ml. The total volume of the reaction mixture was 60-300 μl. After incubation with DEP for 30 minutes, the residual CGTase isoform activity was determined as described in Section 2.6.1. Isoform 5 was not used in this experiment because it had no CGTase activity as reported by Kaskangam, 1998.

2.13 Determination of the number of essential histidine residues of CGTase isoform 1

To measure the number of DEP-modified histidine residues of CGTase isoform 1 in the presence and the absence of a protective substance, the differential increase in the absorbance at 246 nm between the two conditions were followed (Wakayama *et al*., 1996). The reaction was performed in a 1-cm-pathlength quartz cuvette, consisting of 0.325 mM DEP and 0.05 g/l CGTase isoform 1 in the presence or the absence of 25 mM Methyl-β-CD. Protection time was 5 minutes while modification time was 30 minutes as in 2.11. The cuvette was put into the sample chamber of a spectrophotometer and the temperature inside was set at 40 °C. The absorbance at 246 nm was then recorded.

The number (n) of modified – histidine residues was calculated, using the equation :

$$n = \frac{A_{246}\ nm \times M_r}{\varepsilon_{246} \times C}$$

Where M_r is molecular weight of CGTase isoform 1 which is 72,000 (Kaskangam, 1998), ε_{246} of *N* – carbethoxyhistidine is 3,200 M^{-1} cm^{-1} (Wakayama, 1996) and C is the CGTase isoform 1 concentration (g/l).

2.14 Inactivation kinetics of CGTase isoform 1 with DEP

The CGTase isoform 1 in 50 mM acetate buffer, pH 6.0 (50 μg/ml, 30μl) was incubated with varying concentrations of DEP (0 –1.0 mM) at 40 °C. Time of incubation was varied from 0-10 minutes. The pseudo first-order rate constant ($k_{inactivation}$) was calculated from the slopes of the plot between the logarithm of relative residual activity and time of modification at different reagent concentrations. The secondary plot between the pseudo first-order rate constants and various concentrations of DEP gave the order of the reaction and second-order rate constant. The ratio of DEP : isoform 1 was then determined from the slope of the logarithmic plot between the pseudo first-order rate constant ($k_{inactivation}$) and DEP concentration.

2.15 Digestion of isoform 1 and separation of peptides

Three forms of isoform 1(native, DEP-modified, and protected forms) were digested by trypsin at a ratio of 1 : 80 (W/W) in 0.2 M Tris-HCl buffer, pH 8.0 for 18 hours at 37 °C according to the method of Delferge, D. *et al* (1997). The 1.5 ml total reaction volume was consisted of CGTase isoform 1 (240 μg/ml, 1 ml) and 0.5 ml of buffer (for native form) or 50 mM DEP (modified form) or 4 M methyl-β-CD and 50 mM DEP (protected form). Then desalting column was used to separate isoform 1 from other reagents in the reaction mixture. Desalting column is packed with the well-known size exclusion matrix Sephadex G-25 Superfine. The fractionation range for globular proteins is between 1,000-5,000 Da. The maximum recommended flow rate is 15 ml/min and recommended sample volume is 0.1-1.5 ml. Equilibration of the column was performed by passing 25 ml of 50 mM acetate buffer pH 6.0 at the

approximate flow rate between 1-10 ml/min. The digestion mixture (1.5 ml) was injected onto the desalting column through a syringe and elution was made by equilibrating buffer. Fractions containing isoform 1 were collected and pooled for further lyophilization.

For peptide separation, isoform 1 was dissolved with 60 μl of ultra pure water before filtration with 0.22 μm filter to remove particulate materials. Peptides resulting from enzymatic cleavage were then separated by reversed phase HPLC on a C_{18} column (size 250 x 4.6 mm) previously equilibrated with solvent A. Solvent A was 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 the mixture of 0.1% (V/V) trifluoroacetic acid in water and acetronitrile in the ratio of 1:3.

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100.0	0.0
5.0	90.0	10.0
20.0	80.0	20.0
60.0	50.0	50.0
90.0	0.0	100.0
120.0	0.0	100.0
140.0	100.0	0.0

Elution of HPLC was carried out at a flow rate of 1 ml/min. Detection of peptides was performed at 210 and 246 nm. Injecting the ultra pure water alone under the same chromatographic conditions was also performed as control. Eluted peptides were collected and either frozen at –80 °C or lyophilized for further analysis.

2.16 Mass analysis of peptides

Peptides of interest were dissolved in acetronitrile/0.02% formic acid in water (50:50) and their mass were determined by mass spectrometer. A mass spectrometer is an analytical device that determines the molecular mass of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). The molecular mass was calculated by (Siuzdak G.,1996)

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

The sample of interest from HPLC column (60 µl of dissolved peptides) was introduced into Mass Spectrometer : TOF ESI-MS system (of NSTDA, the National Science and Technology Development Agency) by electrospray ionization method. The mechanism of ionization was protonation. The ions were detected by time-of-flight mass analysis and the m/z ratio was determined with the on-line analyzer.

2.17 Determination of N-terminal sequence of peptides

Interested peptide peaks from HPLC were concentrated using Speedvac concentrator centrifuge before sending to NSTDA or Kobe University for amino acid sequencing. Amino acid residues were analyzed from the N-terminus by Edman degradation reaction (Waters, 1988). At NSTDA, peptide samples were hydrolyzed with 6 M HCl containing 1% phenol (V/V) in evacuated tubes at 110°C for 1 hour per 1 cycle in the Waters Pico-Tag Workstation after they were derivatized with phenylisothiocyanate (PITC). The hydrolyzed sample was then dried under vacuum and resuspended in 100 µl Picotag™ sample diluent. The amino acid mixtures obtained were analyzed on a Picotag™ column (0.39x30 cm) of the Waters 510 HPLC.

CHAPTER III
RESULTS

3.1 Partial purification of CGTase

Cells were removed from the culture broth of *Bacillus circulans* A11 by centrifugation at 3,000 rpm, at 4 °C for 30 minutes, and the supernatant was used for fractionation of extracellular CGTase by starch adsorption method as reported by Malai (1995). Crude CGTase was purified 47-fold with a yield of 96%. The specific activity of the partially purified enzyme was 2,182 units/mg protein (Table 8). Purity of the enzyme was confirmed by non-denaturing polyacrylamide gel electrophoresis followed by Coomassie blue staining (Figure 7). Later, the enzyme was concentrated by ultrafiltration and dialyzed with 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5 (TB_1). The preparation was subjected to preparative gel electrophoresis to separate CGTase isoforms according to the method of Kaskangam (1998).

3.2 Isolation of CGTase isoforms

The concentrated partial purified enzyme was loaded onto a discontinuous preparative polyacrylamide gel electrophoresis, which was performed on a Model 491 Prep cell (Figure 8). When the dye front reached the bottom of the gel, the fractionated enzyme was eluted by Tris-glycine buffer, pH 8.3. Figure 9 shows the elution profile of CGTase from preparative gel. CGTase was eluted between fractions 1 - 140. 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: fraction 1 to 18 (band 5), fraction 20 to 43 (band 4), fraction 45 to 70 (band 3), fraction 73 to 85 (band 2),and fraction 110 to 140 (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 11). Purification of each band was summarized in Table 8. The major band observed was band 1 which contained 9.6 % of the total activity

Table 8. Purification of CGTase from *Bacillus circulans* A11

Purification Step	Volume (ml)	Total Activity (Unit)*	Activity (U/ml)	Total protein (mg)	Specific Activity (Unit/mg)	Purification (fold)	Yield (%)	CD-TCE (2^n)
Crude enzyme	2,400	$12.5x10^4$	52	2,712	46	1	100	2^8
Starch Adsorption	500	$12.0x10^4$	240	55	2,182	47	96	2^9
Ultrafiltration	40	$64.8x10^3$	1,620	24	2,700	58	52	2^{15}
Preparative gel electrophoresis								
Band 1	55	$11.9x10^3$	217	2.59	4,608	100	9.6	
Band 2	14	$52.5x10^2$	375	1.17	3,070	67	4.2	
Band 3	35	$11.6x10^2$	33	0.70	1,650	36	0.9	
Band 4	16	$9.1x10^1$	5.7	0.16	570	12.4	0.07	
Band 5	4	$1.16x10^1$	2.9	0.14	82.9	1.8	0.009	

* dextrinizing activity

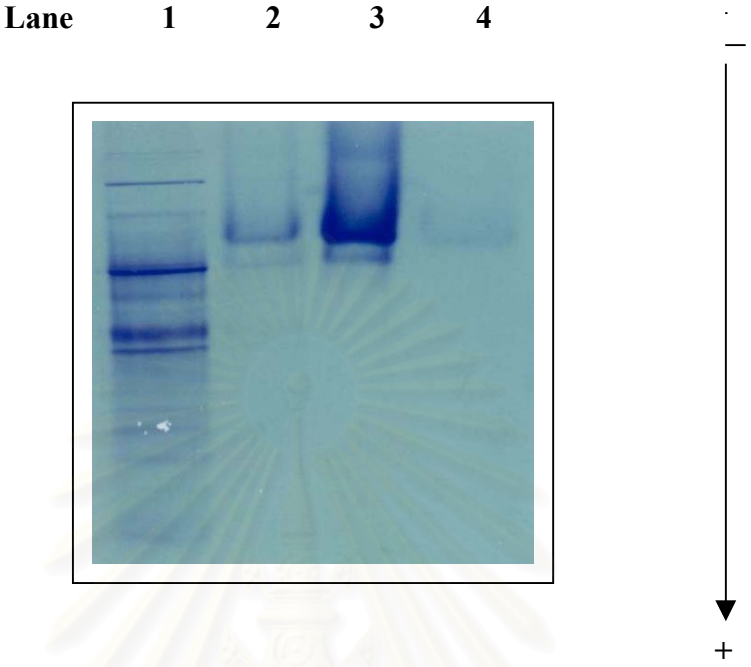


Figure 7. Non - denaturing PAGE of CGTase from different steps of purification

Coomassie blue staining

Lane 1. : Crude enzyme (20µg)

2. : Starch adsorbed enzyme (20µg)

3. : After ultrafiltration (25µg)

4. : Band 1 from preparative gel electrophoresis (2µg)

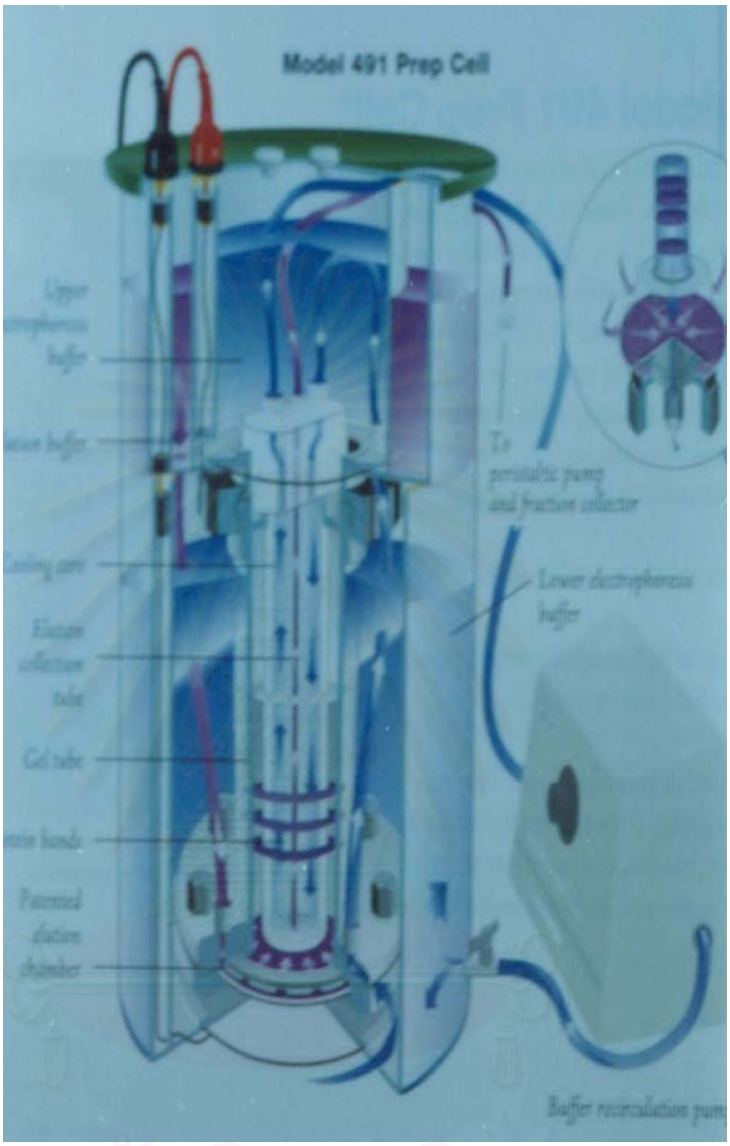


Figure 8. Preparative gel electrophoresis unit.

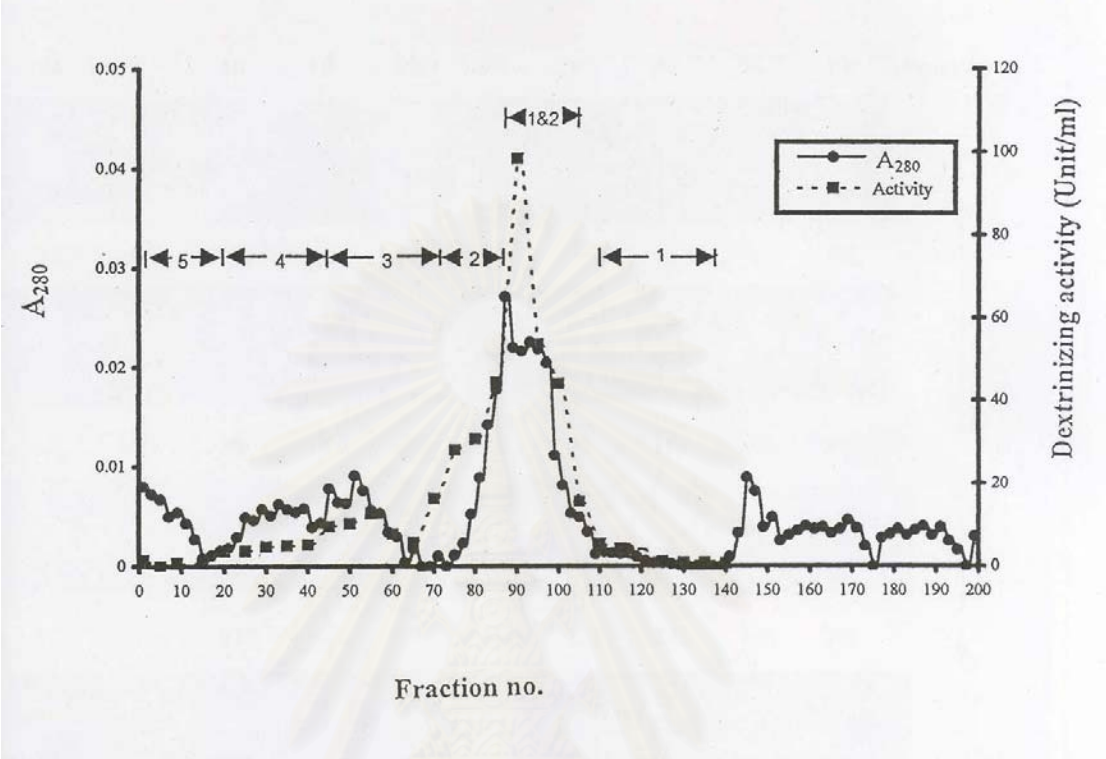


Figure 9. Elution profile of CGTase isoforms from preparative gel electrophoresis

F 1-18 = band 5 F 20-43 = band 4 F 45-70 = band 3

F 73-85 = band 2 F 87-107 = band 1+2 F 110-140 = band 1

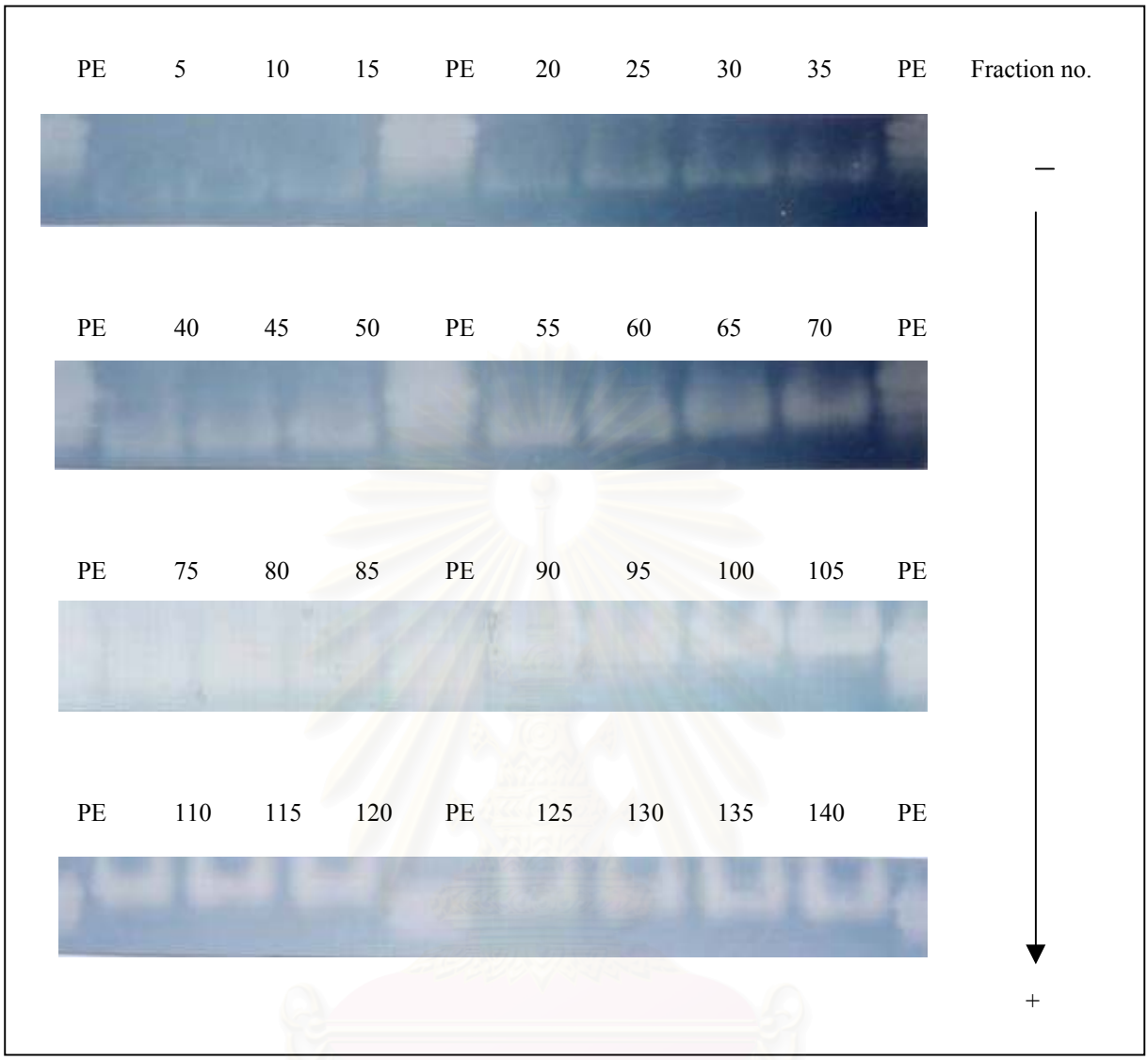


Figure 10. Non-denaturing PAGE pattern of every 5 fractions of CGTase isoforms eluted from preparative gel electrophoresis.

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

PE = Partially purified enzyme

F 1-18	= band 5	F 20-43	= band 4	F 45-70	= band 3
F 73-85	= band 2	F 87-107	= band 1+2	F 110-140	= band 1

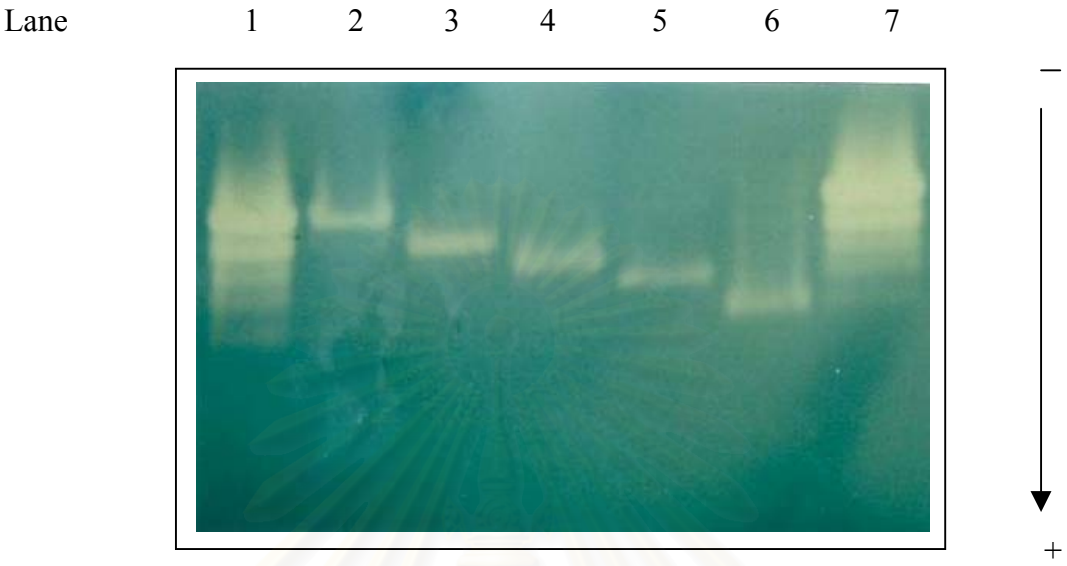


Figure 11. Non-denaturing PAGE of the CGTase bands 1 to 5 obtained from preparative gel electrophoresis

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

Lane 1,7. : Partially purified enzyme (Starch-adsorbed fraction)

2. : Band 1

3. : Band 2

4. : Band 3

5. : Band 4

6. : Band 5

and was purified upto 100 folds. Band 1-4, as proved by phenolpthalein-methyl orange staning, were CGTase while band 5 was not (Kaskangam, 1998). Hence, bands 1-4, namely CGTase isoform 1-4, were further compared for their active site residues 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 Chemical modification of CGTase isoforms

The initial phase of this study is to determine essential amino acid residues in each isoform by modification with certain group-specific reagents and following the loss in isoform activity. The reactions were performed by incubating CGTase isoforms with 1.0 mM of each modifying reagent at 40 °C, pH 6.0 for 30 minutes and the residual isoform activity was determined as described in Section 2.6.1. The results (Table 9) showed that NEM, IAM, and DTT had no effect on any of the CGTase isoforms since almost all activities retained after incubation with these reagents. This indicates that cysteine, a main residue modified by these three reagents, was not important for the activity of all isoforms. DEP which mainly modifies histidine, was very effective in inactivation of isoforms 1, 3, and 4, whereas NBS which modifies tryptophan, was very effective for all isoforms, especially the isoforms 1 and 2. EDC which interacts with carboxylic amino acid residues, demonstrated similar degree of inactivation for all isoforms. TNBS is known to be highly selective reagent for the modification of lysine residues. Activities of isoforms 1, 2 and 4 were not inhibited while isoform 3 was profoundly inactivated. PMSF which modifies serine residues, was not important for the activity of isoforms 1 and 3 but was important for the activities of isoforms 2 and 4. Finally, NAI which interacts with tyrosine, partially inhibited the activities of isoforms 1 and 3. For band 5 which was not the CGTase isoform, the partial loss of activity was observed when modified with TNBS, EDC, DEP, NBS and NAI.

Table 9. Effect of various group – specific reagents on the activity of CGTase isoforms

Group – specific reagents (1mM)*	Amino acid involved	% Residual dextrinizing activity of each isoform				
		1	2	3	4	5
None	-	100	100	100	100	100
N-ethylmaleimide (NEM)	Cysteine	99.8	99.1	99.5	91.8	100
Iodoacetamide (IAM)	Cysteine	97.6	100	99.1	93.6	99.5
Dithiothreitol (DTT)	Cysteine	98.1	100	99.3	91.7	97.1
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	Lysine	100	92.2	68.5	92.8	58.4
Phenylmethylsulfonyl fluoride (PMSF)	Serine	100	60.5	100	78.4	100
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	Carboxylic amino acids	67.1	75.0	43.6	69.4	61.8
Diethylpyrocarbonate (DEP)	Histidine	0	41.1	0	13.6	68.7
N-bromosuccinimide (NBS)	Tryptophan	0	0	11.4	6.5	12.6
N-acetylimidazole (NAI)	Tyrosine	69.9	100	89.5	100	67.4

*Incubation with 25μg/ml CGTase isoform at 40°C, pH 6.0 for 30 minutes (condition as described in Section 2.11)

3.3.1 Modification and protection of lysine residues

ε-NH_2 group of lysine residues of CGTase isoforms were modified by TNBS as described in Section 2.11.2. When isoform 3 was modified with 1 mM TNBS at pH 6.0, 40 °C for 30 minutes, 31.5 percents of the activity was lost, while isoforms 1, 2 and 4 were not inactivated (Table 9). To determine if lysine residue was involved at the active site of isoform 3, modification by TNBS in the presence or the absence of a protective substance was compared. 25 mM Methyl-β-CD was chosen as the protective substance as described in Section 2.12.2. In the presence of 25 mM methyl-β-CD, the loss of isoform 3 activity was significantly reduced. Only 10 % of the activity loss was observed (Table 10). This result indicates that methy-β-CD could protect essential lysine residue(s) of isoform 3 from modification by TNBS.

3.3.2 Modification and protection of serine residues

Serine residues of CGTase isoforms were modified by PMSF as described in Section 2.11.3. The result in Table 9 shows that, when modified with 1 mM PMSF at pH 6.0, 40 °C for 30 minutes, 39.5 and 21.6 percents of the activities of isoforms 2 and 4 were lost, while the activities of isoforms 1 and 3 were not affected. To determine if serine residue was involved at the active site of isoforms 2 and 4, modification by PMSF in the presence or the absence of a protective substance was compared. 25 mM Methyl-β-CD was chosen as the protective substance as described in Section 2.12.2. In the presence of 25 mM methyl-β-CD, the loss of isoform 2 activity was reduced. Approximately 20 % of the isoform 2 activity was increased in the presence of substrate. On the other hand, no change in the activity loss of isoform 4 when modified with PMSF in the presence or absence of 25 mM methyl-β-CD (Table 11). This result indicates that methy-β-CD could protect essential serine residue(s) in isoform 2 but not in isoform 4, from modification by PMSF.

Table 10. Effect of substrate on the inactivation of dextrinizing activity of CGTase isoform 3 by TNBS

Compound added	% Relative activity		
	I	II	Average
1) None	100	100	100
2) 25 mM Methyl-β-CD	96.0	99.0	97.5
3) 25 mM Methyl-β-CD, then 1 mM TNBS	89.7	89.9	89.8
4) 1 mM TNBS	70.4	67.1	68.8

Numbers under I and II are duplicate values

Table 11. Effect of substrate on the inactivation of dextrinizing activities of CGTase isoforms 2 and 4 by PMSF

Compound added	% Relative activity		
	I	II	Average
1) None			
-Isoform 2	100	100	100
-Isoform 4	100	100	100
2) 25 mM Methyl-β-CD			
-Isoform 2	94.0	91.7	92.9
-Isoform 4	92.4	94.0	93.2
3) 25 mM Methyl-β-CD, then 1 mM PMSF			
-Isoform 2	83.0	84.5	83.8
-Isoform 4	80.2	80.3	80.3
4) 1 mM PMSF			
-Isoform 2	61.8	64.7	63.3
-Isoform 4	78.1	79.4	78.8

Numbers under I and II are duplicate values

3.3.3 Modification and protection of carboxyl residues

Modification of carboxyl groups of carboxylic amino acids with EDC was performed as described in Section 2.11.4. Incubation with 1 mM EDC at pH 6.0, 40 ° C for 30 minutes led to inactivation of all isoforms (Table 9 and Table 12). The loss of isoform 1, 2, 3 and 4 activities were 41.7, 29.0, 41.5, and 32.7 %, respectively. To determine if carboxylic amino acid residue was involved at the active site of all isoforms, modification by EDC in the presence or the absence of a protective substance was compared. 25 mM Methyl-β-CD was chosen as the protective substance as described in Section 2.12.2. When each isoform was preincubated with 25 mM methyl-β-CD prior to EDC modification, the loss of activity was partially reduced. Only 8-17 % of isoform activities were protected by 25 mM methyl-β-CD. It was observed that substrate protection was not pronounced since only little of the activity was increased in the presence of substrate.

3.3.4 Modification and protection of tyrosine residues

Tyrosine residues of CGTase isoforms were modified by NAI as described in Section 2.11.5. Incubation with 30 mM NAI at pH 6.0, 40 °C for 30 minutes led to inactivation of all isoforms (Table 13) while at 1 mM NAI, only isoforms 1 and 3 were inactivated (Table 9). It should be pointed out here that NAI was especially effective in the inactivation of isoforms 1 and 3. The loss of activities of isoforms 1, 2, 3 and 4 were 79.0, 22.9, 61.6 and 51.4 %, respectively. To determine if tyrosine was involved at the active site of all isoforms, modification by NAI in the presence or the absence of a protective substance was compared. 25 mM Methyl-β-CD was chosen as the protective substance as described in Section 2.12.2. When each isoform was preincubated with 25 mM methyl-β-CD prior to NAI-modification, the loss of activity was partially reduced. Approximately 13-17 % of isoforms 2, 3 and 4 activites were protected by 25 mM methyl-β-CD while the protection in isoform 1 was significantly increased to 46 percents.

Table 12. Effect of substrate on the inactivation of dextrinizing activities of CGTase isoforms by EDC

Compound added	% Relative activity		
	I	II	Average
1) None			
-Isoform 1	100	100	100
-Isoform 2	100	100	100
-Isoform 3	100	100	100
-Isoform 4	100	100	100
2) 25 mM Methyl-β-CD			
-Isoform 1	88.9	84.5	86.7
-Isoform 2	94.0	94.4	94.2
-Isoform 3	83.7	80.3	82.0
-Isoform 4	94.8	95.7	95.3
3) 25 mM Methyl-β-CD, then 1 mM EDC			
-Isoform 1	78.3	72.1	75.2
-Isoform 2	80.4	81.0	80.7
-Isoform 3	72.7	70.4	71.6
-Isoform 4	71.3	79.8	75.6
4) 1 mM EDC			
-Isoform 1	59.9	56.6	58.3
-Isoform 2	70.0	72.0	71.0
-Isoform 3	64.1	52.8	58.5
-Isoform 4	63.6	71.0	67.3

Numbers under I and II are duplicate values

Table 13. Effect of substrate on the inactivation of dextrinizing activities of CGTase isoforms by NAI

Compound added	% Relative activity		
	I	II	Average
1) None			
-Isoform 1	100	100	100
-Isoform 2	100	100	100
-Isoform 3	100	100	100
-Isoform 4	100	100	100
2) 25 mM Methyl-β-CD			
-Isoform 1	97.6	100	98.8
-Isoform 2	95.7	94.8	95.3
-Isoform 3	97.6	96.6	97.1
-Isoform 4	97.7	93.5	95.6
3) 25 mM Methyl-β-CD, then 30 mM NAI			
-Isoform 1	69.4	64.5	67.0
-Isoform 2	90.0	89.2	89.6
-Isoform 3	54.2	55.2	54.7
-Isoform 4	62.4	65.2	63.8
4) 30 mM NAI			
-Isoform 1	20.6	21.3	21.0
-Isoform 2	77.6	76.5	77.1
-Isoform 3	38.8	37.9	38.4
-Isoform 4	47.2	50.0	48.6

Numbers under I and II are duplicate values

3.3.5 Modification and protection of tryptophan residues

Tryptophan residues of CGTase isoforms were modified by NBS as described in Section 2.11.6. Since 1 mM NBS inhibited all or almost all activities of CGTase isoforms (Table 9), the concentration of NBS was reduced to 0.05 mM NBS which was suitable for the mixed enzyme form (Tongsima, 1998). Incubation with 0.05 mM NBS at pH 6.0, 40 °C for 30 minutes led to inactivation of all isoforms (Table 14). The loss of activities of isoforms 1, 2, 3 and 4 were 89.7, 92.0, 74.9 and 85.9 %, respectively. To determine if tryptophan residue was involved at the active site of all isoforms, modification by NBS in the presence or the absence of a protective substrate was compared. 25 mM Methyl-β-CD was chosen as the protective substance as described in Section 2.12.2. When each isoform was preincubated with 25 mM methyl-β-CD prior to NBS-modification, the loss of activity was significantly reduced. Approximately 60-68 % of isoforms 1 and 2 activites were protected by 25 mM methyl-β-CD while the protection in isoforms 3 and 4 were only 17 and 29 % , respectively.

3.3.6 Modification and protection of histidine residues

The objective of this research was to identify the position of essential histidine residue(s) at the active site of CGTase isoform 1. Hence in this study, the experiment involving histidine modification was performed in more details. The suitable concentration and incubation time of DEP were initially determined in order to find the optimal mild conditions for chemical modification of each isoform. In addition, the experiments to confirm the modification of histidine and to determine the number of histidine residues in isoform 1 which were modified by DEP were also performed.

Isoform 1

Histidine residues of isoform 1 were modified by various concentrations of DEP as described in Section 2.11.7. CGTase isoform 1 activity was found to decrease with increasing DEP concentrations (Figure 12). At 0.325 mM DEP, isoform 1 activity was completely lost. Figure 13 showed that after 5 minutes of incubation, 50 percents of dextrinizing activity was lost whereas total activity loss was observed at

Table 14. Effect of substrate on the inactivation of dextrinizing activities of CGTase isoforms by NBS

Compound added	% Relative activity		
	I	II	Average
1) None			
-Isoform 1	100	100	100
-Isoform 2	100	100	100
-Isoform 3	100	100	100
-Isoform 4	100	100	100
2) 25 mM Methyl-β-CD			
-Isoform 1	94.4	96.1	95.3
-Isoform 2	95.6	95.8	95.7
-Isoform 3	93.7	94.4	94.1
-Isoform 4	91.4	93.0	92.2
3) 25 mM Methyl-β-CD, then 0.05 mM NBS			
-Isoform 1	73.4	70.3	71.9
-Isoform 2	73.2	78.6	75.9
-Isoform 3	40.9	42.3	41.6
-Isoform 4	42.2	44.6	43.4
4) 0.05 mM NBS			
-Isoform 1	8.6	11.9	10.3
-Isoform 2	8.9	7.1	8.0
-Isoform 3	23.4	26.8	25.1
-Isoform 4	11.5	16.6	14.1

Numbers under I and II are duplicate values

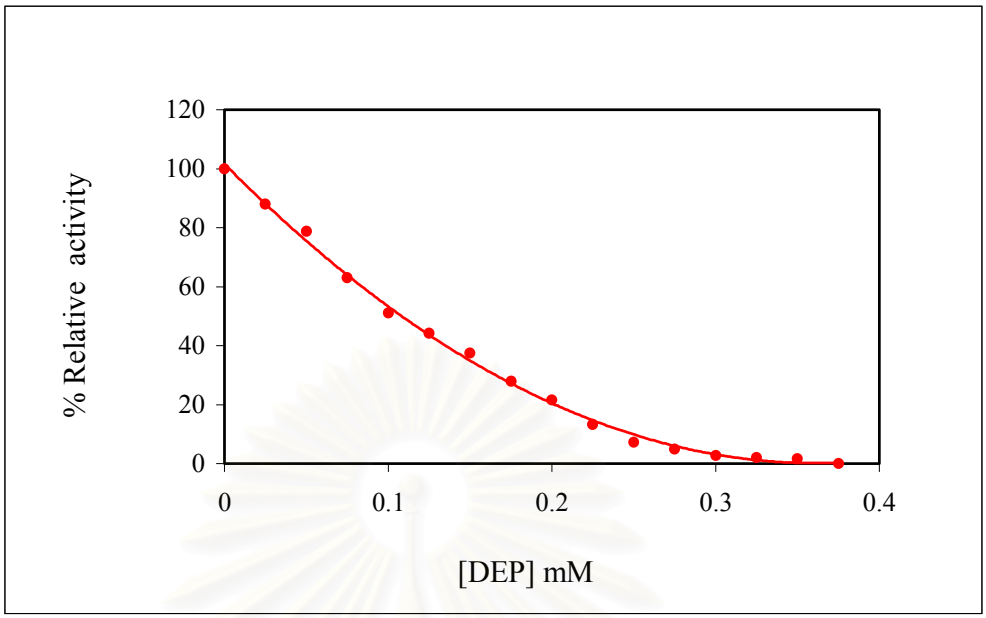


Figure 12. Effect of DEP on CGTase isoform 1 activity

CGTase isoform 1 was inactivated with varying concentrations of DEP at 40 °C for 30 minutes, as described in Section 2.11.7. After the incubation, the enzyme activity was determined as described in Section 2.6.1.

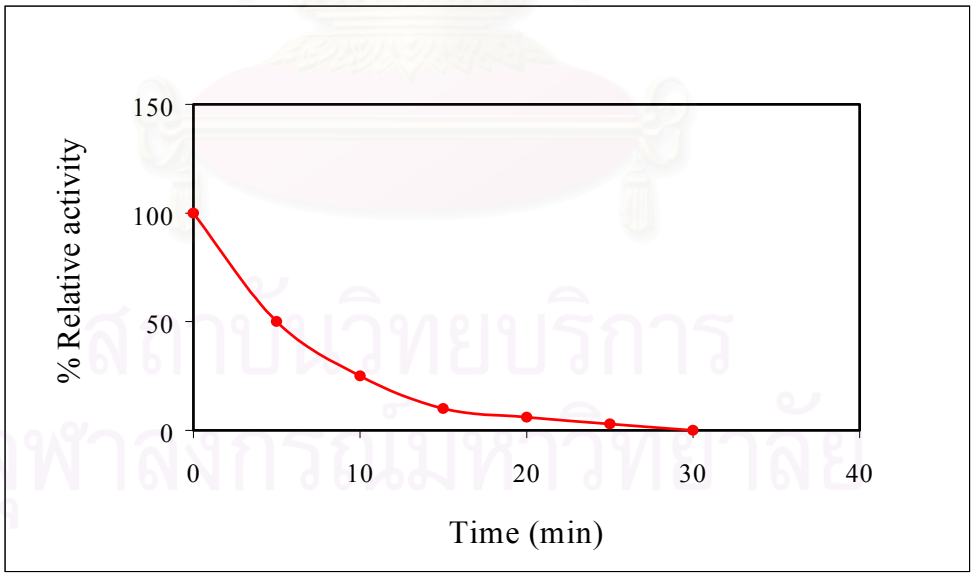


Figure 13. Inactivation of CGTase isoform 1 by 0.325 mM DEP

CGTase isoform 1 was inactivated with 0.325 mM DEP at 40 °C, then the activity remained was assayed at time intervals according to the methods as described in Section 2.11.7 and 2.6.1.

30 minutes. The suitable incubation time of the enzyme (the time period for which approximately 50 percents of activity was remained) with DEP was thus 5 minutes.

To determine whether histidine residue was involved at the active site of isoform 1, modification by DEP in the presence or the absence of substrate was compared. β-CD, methyl-β-CD or hydroxypropyl-β-CD were used as protective substrates. Four different conditions : 1. isoform 1 alone, 2. isoform 1 incubated with each substrate, 3. isoform 1 preincubated with each substrate then modified by DEP, and 4. isoform 1 modified by DEP, were compared. After the reaction, isoform 1 activity was then determined by dextrinizing activity assay method. Table 15 shows that modification by 0.325 mM DEP led to 50.4 % loss of isoform 1 activity. When isoform 1 preincubated with each substrate was modified by DEP, the loss of isoform 1 activity was partially reduced. In the presence of β-CD, methyl-β-CD or hydroxypropyl-β-CD, the activity loss were 29, 32,and 26 %, respectively.

The specificity of the DEP-modified reaction for histidine residue was shown by the correlation between the loss of isoform 1 activity and the increase in the absorbance at 246 nm, which indicates the formation of *N*-carbethoxyhistidine (Wakayama *et al*., 1996). Absorption spectra of before and after modification with DEP were shown in Figure 14. The spectrum of isoform 1 revaled the highest peak at about 280 nm, a typical of usual protein spectrum. When isoform 1 was modified with DEP, the absorption spectra at 246 nm demonstrated the significant increase with increasing time of modification.

The number of histidine residues which were modified by 0.325 mM DEP were determined from the increase in the absorbance at 246 nm, using an extinction coefficient of 3,200 $M^{-1}cm^{-1}$ as described in Section 2.13. The result in Table 16 shows that 8.5 histidine residues per mole isoform 1 were modified by 0.325 mM DEP when the enzyme was incubated with DEP only. The number of histidine residues decreased to 6.5 in the presence of methyl-β-CD, as protective substrate. Hence, methyl-β-CD could protect per mole of isoform 1, two histidine residues from modification by DEP.

Table 15. Effect of substrate on the inactivation of dextrinizing activity of CGTase isoform 1 by DEP

Compound added	% Relative activity		
	I	II	Average
1) None	100	100	100
2) 25 mM β-CD	98.3	98.0	98.2
25 mM Hydroxypropyl-β-CD	94.7	97.9	96.3
25 mM Methyl-β-CD	99.5	97.6	98.6
3) 25 mM β-CD, then 0.325 mM DEP	67.7	74.3	71.0
25 mM Hydroxypropyl-β-CD, then 0.325 mM DEP	69.0	68.0	68.5
25 mM Methyl-β-CD, then 0.325 mM DEP	77.5	70.8	74.2
4) 0.325 mM DEP	50.3	48.9	49.6

Numbers under I and II are duplicate values

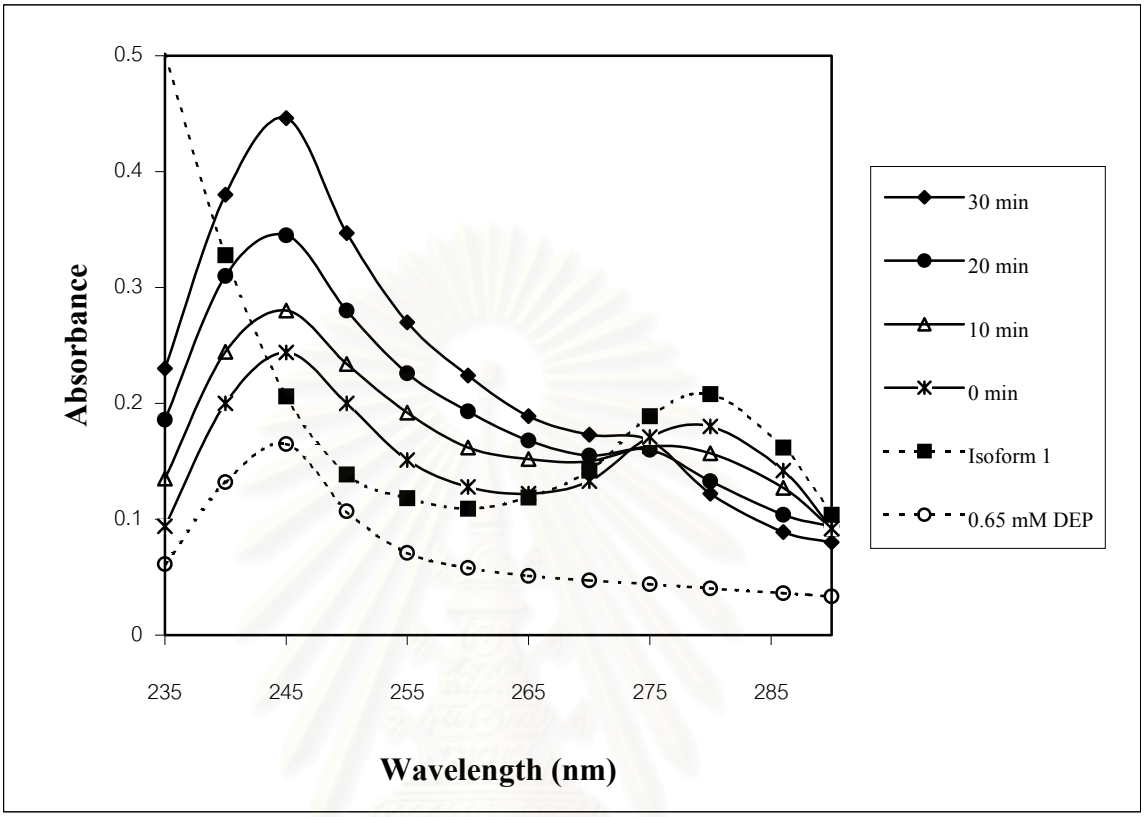


Figure 14. Absorption spectra of CGTase isoform 1 before and after modification with DEP

For control, CGTase isoform 1 (100 μg/ml) or DEP 0.65 mM were separately scanned. After 0-30 mintues of the modification, the modified CGTase isoform 1 from each incubation time was scanned at the same wavelength range.

Table 16. Number of histidine residues of CGTase isoform 1 modified by DEP in the presence and the absence of a protective substance

Protective substance	n^*	Protected residues per mole of isoform 1
None	8.5	0
Methyl-β-CD	6.5	2.0

n^* = number of modified histidine residues per mole of CGTase isoform 1

Isoform 2

Histidine residues of isoform 2 were modified by various concentrations of DEP as described in Section 2.11.7. CGTase isoform 2 activity was found to decrease with increasing DEP concentrations (Figure 15). At 2 mM DEP, isoform 2 activity was completely lost. Figure 16 shows that after 3 minutes of incubation, 50 percents of dextrinizing activity was lost whereas total activity loss was observed at 30 minutes. The suitable incubation time of the enzyme with DEP was thus 3 minutes.

To determine whether histidine residue was involved at the active site of isoform 2, modification by DEP in the presence or the absence of substrate was compared. Methyl-β-CD was used as protective substrate since it gave best protection for isoform 1. Four different conditions :1. isoform 2 alone, 2. isoform 2 incubated with methyl-β-CD, 3. isoform 2 preincubated with methyl-β-CD then modified by DEP, and 4. isoform 2 modified by DEP, were compared. After the reaction, isoform 2 activity was then determined by dextrinizing activity assay method. Table 17 shows that modification by 2 mM DEP led to 49.1 % loss of isoform 2 activity. When isoform 2 preincubated with methyl-β-CD was modified by DEP, the loss of isoform 2 activity was partially reduced. It was found that approximately 16 percents of isoform 2 activity was protected by 25 mM methy-β-CD.

Isoform 3

Histidine residues of isoform 3 were modified by various concentrations of DEP as described in Section 2.11.7. CGTase isoform 3 activity was found to decrease with increasing DEP concentrations (Figure 17). At 0.75 mM DEP, isoform 3 activity was completely lost. Figure 18 shows that after 3 minutes of incubation, 50 percents of dextrinizing activity was lost whereas total activity loss was observed at 30 minutes. The suitable incubation time of the enzyme with DEP was thus 3 minutes.

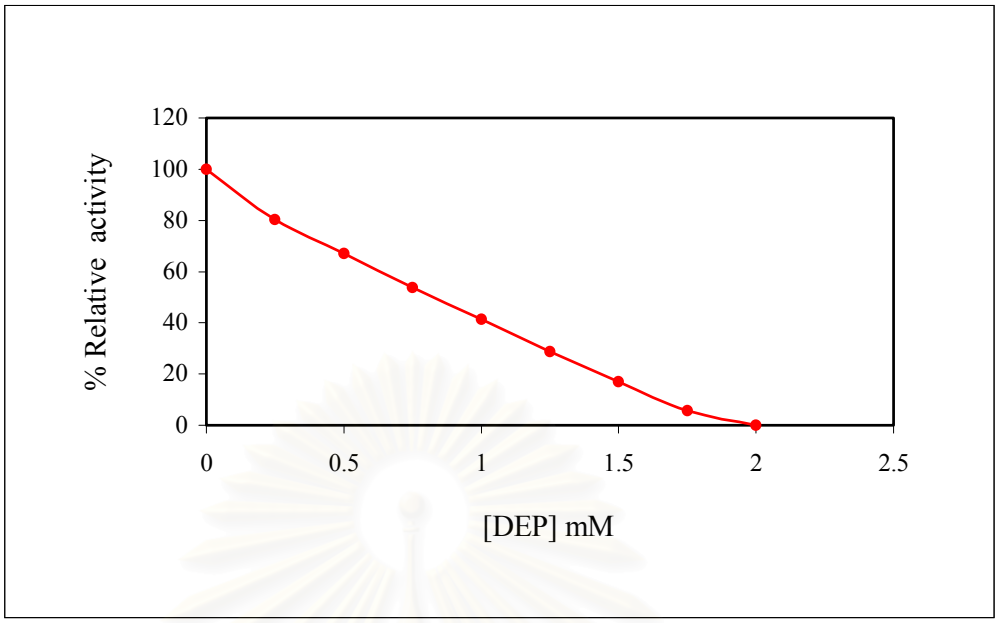


Figure 15. Effect of DEP on CGTase isoform 2 activity

CGTase isoform 2 was inactivated with varying concentrations of DEP at 40 °C for 30 minutes, as described in Section 2.11.7. After the incubation, the enzyme activity was determined as described in Section 2.6.1.

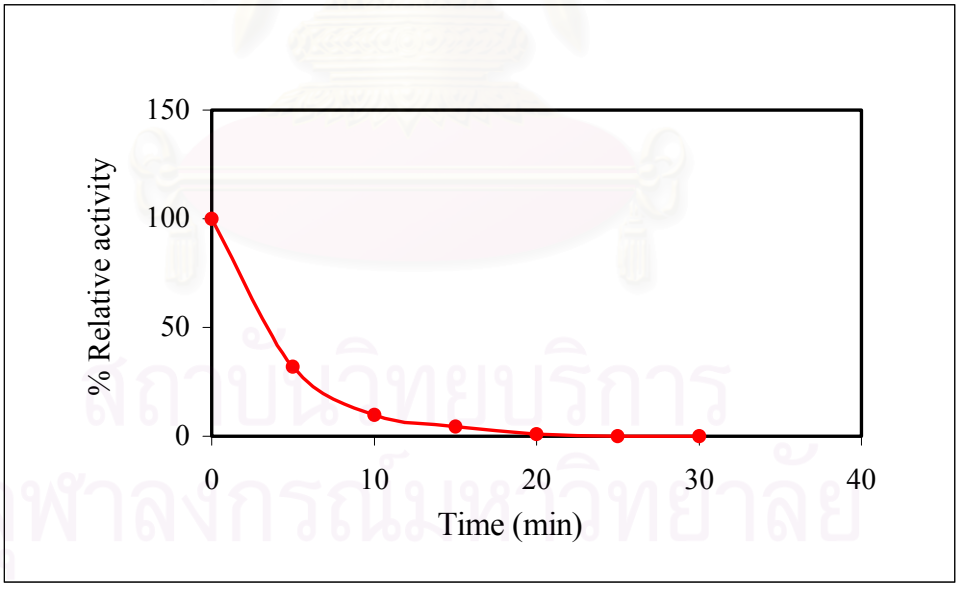


Figure 16. Inactivation of CGTase isoform 2 by 2 mM DEP

CGTase isoform 2 was inactivated with 2 mM DEP at 40 °C, then the activity remained was assayed at time intervals according to the methods as described in Section 2.11.7 and 2.6.1.

Table 17. Effect of substrate on the inactivation of dextrinizing activity of CGTase isoform 2 by DEP

Compound added	% Relative activity		
	I	II	Average
1) None	100	100	100
2) 25 mM Methyl-β-CD	94.0	90.4	92.2
3) 25 mM Methyl-β-CD, then 2 mM DEP	66.3	68.1	67.2
4) 2 mM DEP	48.3	53.4	50.9

Numbers I and II duplicate values

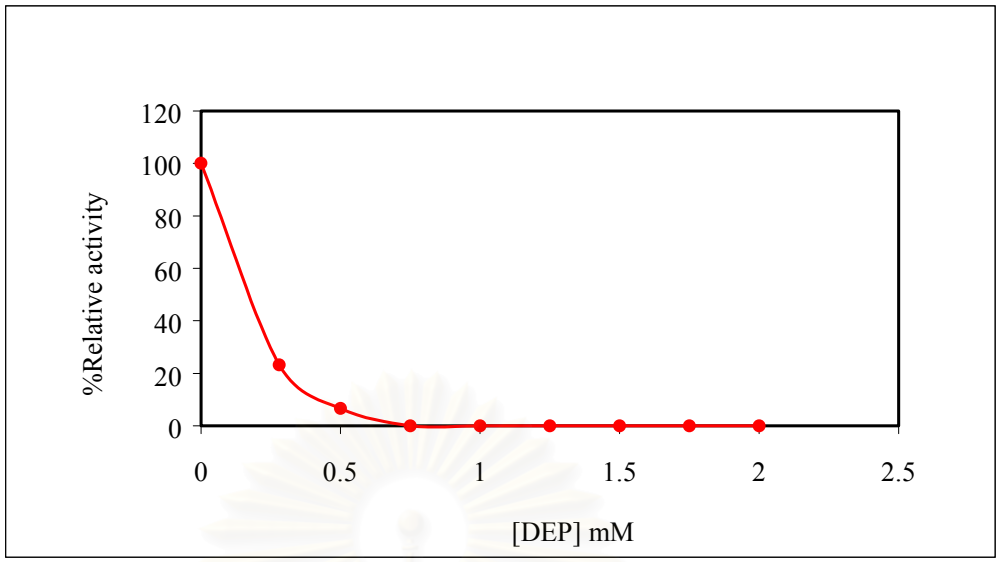


Figure 17. Effect of DEP on CGTase isoform 3 activity

CGTase isoform 3 was inactivated with varying concentrations of DEP at 40 °C for 30 minutes, as described in Section 2.11.7. After the incubation, the enzyme activity was determined as described in Section 2.6.1.

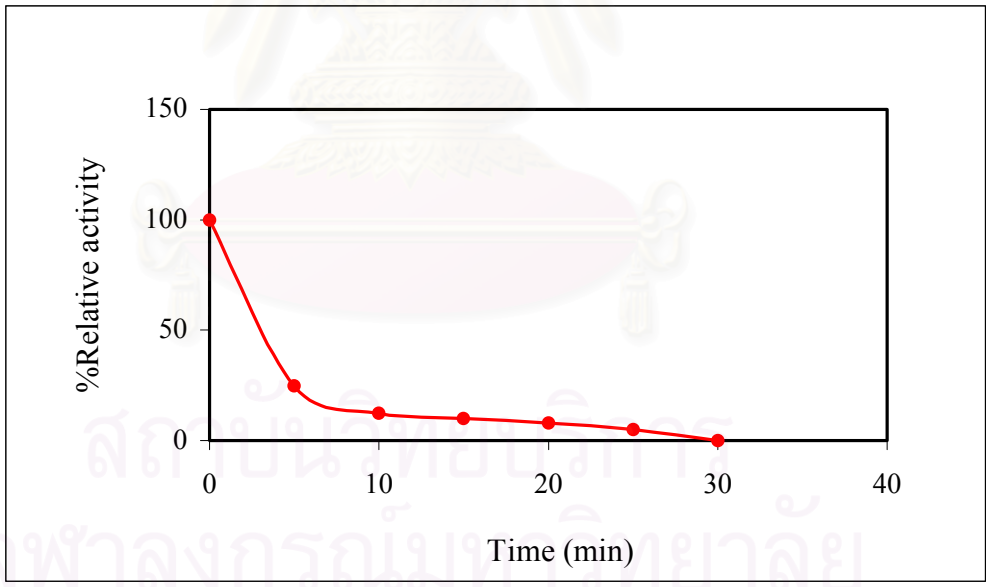


Figure 18. Inactivation of CGTase isoform 3 by 0.75 mM DEP

CGTase isoform 3 was inactivated with 0.75 mM DEP at 40 °C, then the activity remained was assayed at time intervals according to the methods as described in Section 2.11.7 and 2.6.1.

To determine whether histidine residue was involved at the active site of isoform 3, modification by DEP in the presence or the absence of substrate was compared. Methyl-β-CD was used as protective substrate. Four different conditions : 1. isoform 3 alone, 2. isoform 3 incubated with methyl-β-CD, 3. isoform 3 preincubated with methyl-β-CD then modified by DEP, and 4. isoform 3 modified by DEP, were compared. After the reaction, isoform 3 activity was then determined by dextrinizing activity assay method. Table 18 shows that modification by 0.75 mM DEP led to 50 % loss of isoform 3 activity. When isoform 3 preincubated with methyl-β-CD was modified by DEP, the loss of isoform 3 activity was partially reduced. It was observed that approximately 42 percents of isoform 3 activity was protected by 25 mM methyl-β-CD.

Isoform 4

Histidine residues of isoform 4 were modified by various concentrations of DEP as described in Section 2.11.7. CGTase isoform 4 activity was found to decrease with increasing DEP concentrations (Figure 19). At 2 mM DEP, isoform 4 activity was completely lost. Figure 20 shows that after 7 minutes of incubation, 50 percents of dextrinizing activity was lost whereas total activity loss was observed at 30 minutes. The suitable incubation time of the enzyme with DEP was thus 7 minutes.

To determine whether histidine residue was involved at the active site of isoform 4, modification by DEP in the presence or the absence of substrate was compared. Methyl-β-CD was used as protective substrate. Four different conditions : 1. isoform 4 alone, 2. isoform 4 incubated with methyl-β-CD, 3. isoform 4 preincubated with methyl-β-CD then modified by DEP, and 4. isoform 4 modified by DEP, were compared. After the reaction, isoform 4 activity was then determined by dextrinizing activity assay method. Table 19 shows that modification by 2 mM DEP led to 59 % loss of isoform 4 activity. When isoform 4 preincubated with methyl-β-CD substrate was modified by DEP, the loss of isoform 4 activity was partially reduced. It was observed that approximately 35 percents of isoform 4 activity was protected by 25 mM methyl-β-CD.

Table 18. Effect of substrate on the inactivation of dextrinizing activity of CGTase isoform 3 by DEP

Compound added	% Relative activity		
	I	II	Average
1) None	100	100	100
2) 25 mM Methyl-β-CD	100	98.3	99.2
3) 25 mM Methyl-β-CD, then 0.75 mM DEP	93.5	88.7	91.1
4) 0.75 mM DEP	50.0	48.9	49.5

Numbers I and II duplicate values

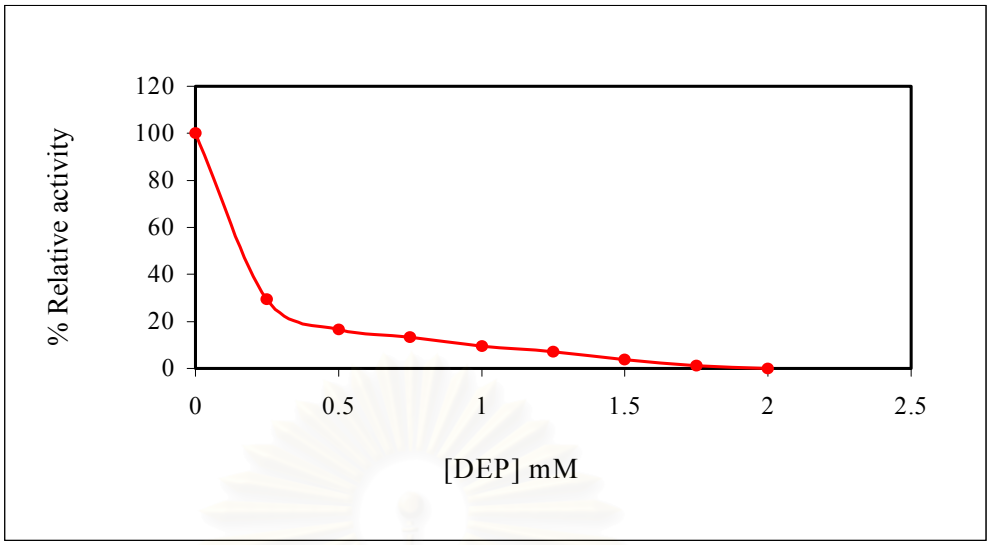


Figure 19. Effect of DEP on CGTase isoform 4 activity

CGTase isoform 4 was inactivated with varying concentrations of DEP at 40 °C for 30 minutes, as described in Section 2.11.7. After the incubation, the enzyme activity was determined as described in Section 2.6.1.

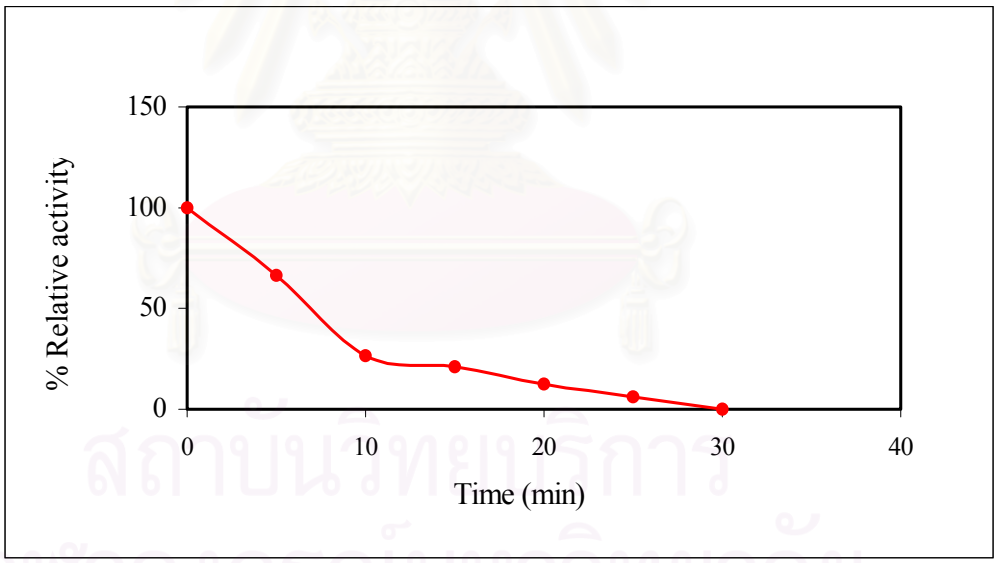


Figure 20. Inactivation of CGTase isoform 4 by 2 mM DEP

CGTase isoform 4 was inactivated with 0.75 mM DEP at 40 °C, then the activity remained was assayed at time intervals according to the methods as described in Section 2.11.7 and 2.6.1.

Table 19. Effect of substrate on the inactivation of dextrinizing activity of CGTase isoform 4 by DEP

Compound added	% Relative activity		
	I	II	Average
1) None	100	100	100
2) 25 mM Methyl-β-CD	100	100	100
3) 25 mM Methyl-β-CD, then 2 mM DEP	78.9	74.2	76.6
4) 2 mM DEP	42.1	40.9	41.5

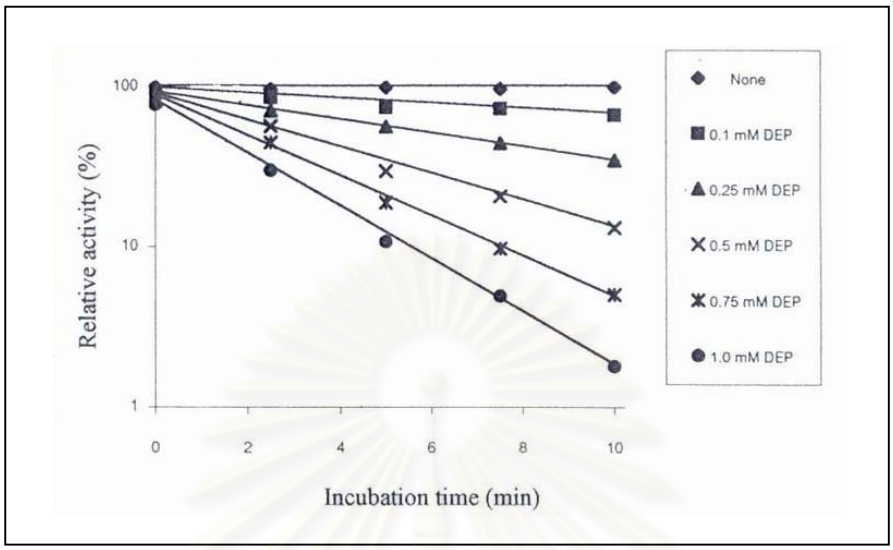
Numbers I and II duplicate values

3.4 Inactivation kinetics of CGTase isoform 1 with DEP

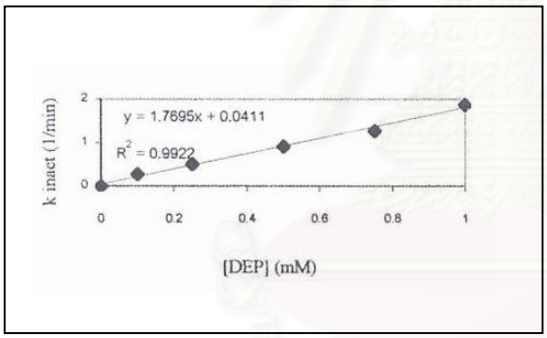
Incubation of CGTase isoform 1 with varying concentrations of DEP (0-1.0 mM) at pH 6.0, 40 °C resulted in a time-dependent loss of CGTase isoform 1 activity (Figure 21A), suggesting the modification of histidine located at or near the enzyme active site. When CGTase isoform 1 was incubated with 1.0 mM DEP for 5 min, the residual activity was 10%. At 10 min, only 2 % of the activity was remained. And at every concentrations of DEP, the loss of activities were linear with incubation time indicating pseudo first-order kinetics of inactivation. The pseudo first-order rate constant ($k_{inactivation}$) of this inactivation kinetics could be determined from the slope of the plot between the logarithm of relative residual activity (%) versus time at different DEP concentrations (Figure 21A).

When the pseudo first-order rate constants were plotted against [DEP], the straight line which indicates linear correlation was obtained (Figure 21B). This result leads to the indication that the chemical modification of CGTase by DEP is the result of a simple bimolecular reaction. The second-order rate constant obtained was 29.5 M^{-1} s^{-1}. For determining moles of DEP per mole of CGTase isoform 1, the plot of log k_{inact} versus log [DEP] was performed (Figure 21C). An apparent reaction order of 0.84 suggesting that inactivation results from the reaction of 1 mol of DEP with 1 mol of isoform 1.

A



B



C

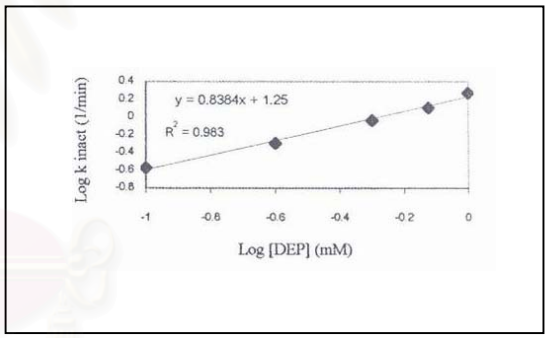


Figure 21. Inactivation of CGTase isoform 1 with DEP.

A, CGTase isoform 1 was incubated with DEP at 0 mM(◆), 0.10 mM(■), 0.25 mM(▲), 0.50 mM(×), 0.75 mM(✳), 1.00 mM(●) for 0-10 min. The pseudo first-order rate constants of inactivation k_{inact} were obtained from the slopes of straight lines. B, determination of the second-order rate constant of inactivation. C, determination of apparent order of reaction with respect to reagent concentration.

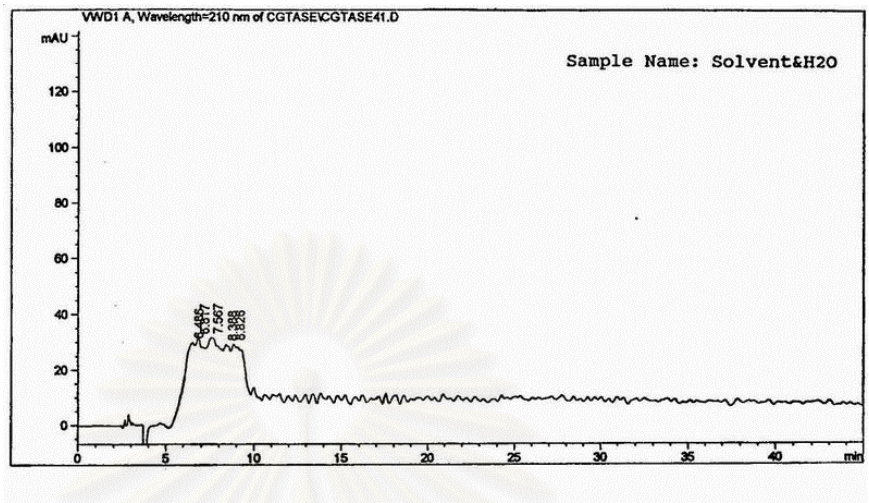
3.5 Digestion of isoform 1 and separation of peptides

In the attempt to localize essential histidine(s) in CGTase isoform 1, three forms of isoform 1: native, DEP-modified, and the methyl-β-CD protected prior to DEP–modified enzyme, were digested by trypsin at a ratio of 1 : 80 (w/w) for 18 hours, at pH 8.0, 37 °C (Delforge, D. *et al*, 1997). The digested mixtures were passed through desalting column to separate isoform 1 from other reagents in the reaction. Peptides resulting from enzymatic cleavage were then separated by HPLC on a C_{18} reversed phase column. The HPLC profiles of peptides were shown in Figures 22 and 23. Detection of peptides was performed at 210 nm (Figure 22) while detection of modified-histidine products was followed at 246 nm (Figure 23). Figures 22A and 23A show profiles of blank condition at wavelength 210 and 246 nm, respectively. Blank condition was performed by injecting only ultrapure water under the same chromatographic conditions as the sample. The broad peak at 210 nm during 6-10 minutes (Figure 22A) and the peaks from 2-4 minutes at 246 nm should be solvent peaks. Figures 22B, C, D and 23B, C, D were profiles of native, DEP-modified and the methyl-β-CD protected prior to DEP–modified forms, respectively. When these profiles were compared, it was found that the peaks eluting at R_t 11.348 and 40.934 minutes (Figure 22C) appeared when isoform 1 was modified with DEP, while the peaks at R_t 8.696 and 15.489 minutes (Figure 22B) disappeared. The comparison of Figure 22D with 22C confirmed the importance of these peaks, the peak eluting at R_t 11.241 minutes was decreased and R_t 40.934 minutes disappeared when isoform 1 was protected with methyl-β-CD prior to DEP-modified, while the peaks at R_t 8.628 and 15.705 minutes increased in Figure 22D. The appearance and disappearance of these peaks were confirmed at 246 nm (Figure 23), which was a specific wavelength for following the modification with DEP. The comparison of profile showed that the peaks eluting at R_t 11.412 and 40.865 minutes (Figure 23C) appeared when isoform 1 were modified with DEP, while these peaks were not seen in Figure 23B. When comparing Figure 23D with 23C, the peaks eluting at R_t 11.241 and 40.865 minutes decreased when isoform 1 was protected with methyl-β-CD prior to DEP-modified (we considered there was some minor shift in retention time of the same peak in different HPLC profiles, as summarized in Table20). From the result obtained, peptides from peaks at R_t 11.384 and 40.934 minutes (as of Figure 22 C) should

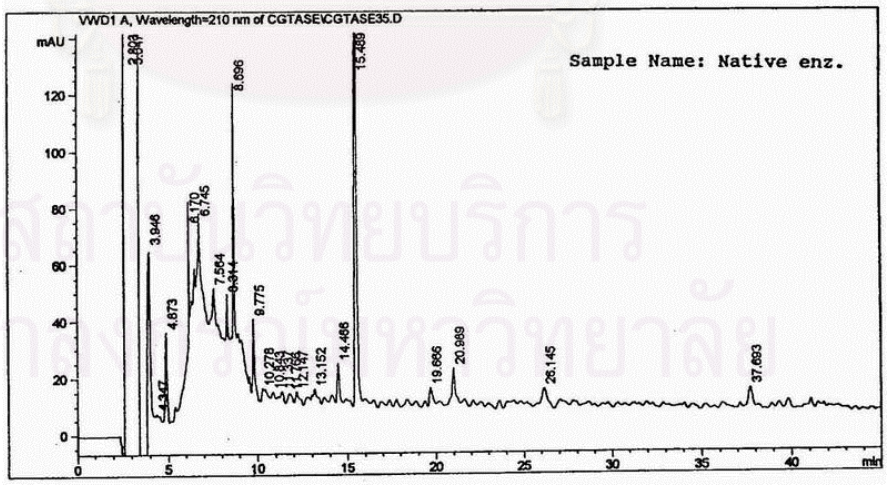
Figure 22. HPLC chromatograms at 210 nm of the peptides of CGTase isoform 1 resulting from digestion with trypsin

A reversed phase C_{18} column (250x4.6 mm) was used. The mobile phase was the gradient of 0.1 % TFA in 75 % acetonitrile as indicated in Methods Section 2.15. The flow rate was at 1 ml/min.

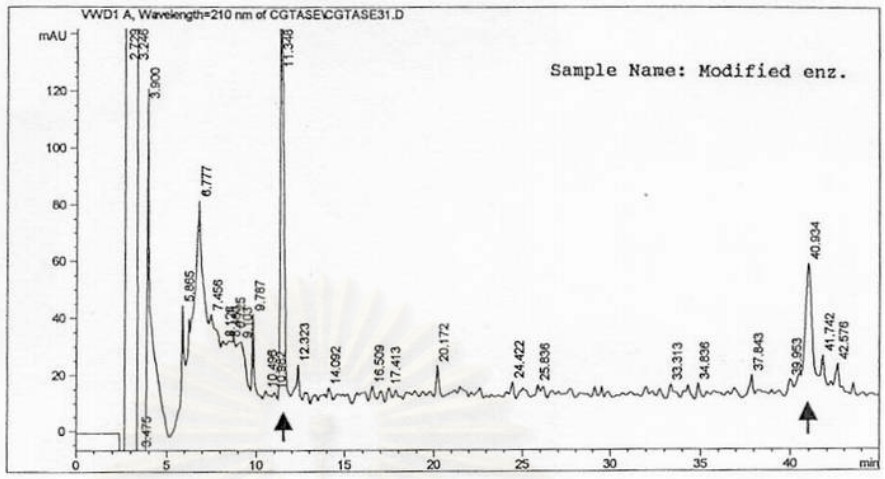
A. blank, injecting ultrapure water
B. digested products of native isoform 1
C. digested products of DEP-modified isoform 1
D. digested products of isoform 1 protected by methyl-β-CD prior to DEP-modification



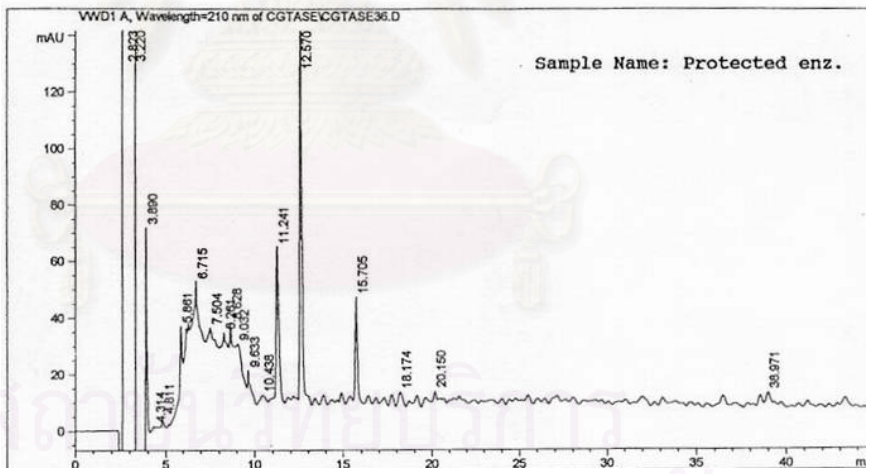
A. blank, injecting ultrapure water



B. digested products of native isoform 1



C. digested products of DEP-modified isoform 1. The arrows indicate peptides of interest.

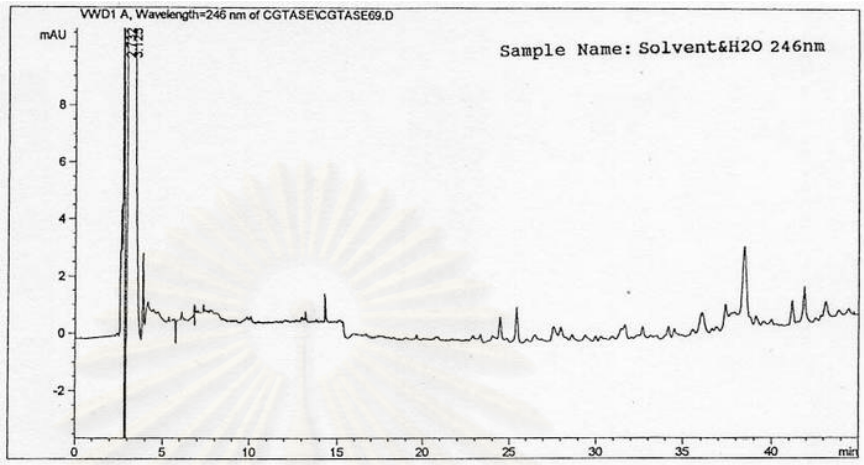


D. digested products of the methyl-β-CD-protected isoform 1

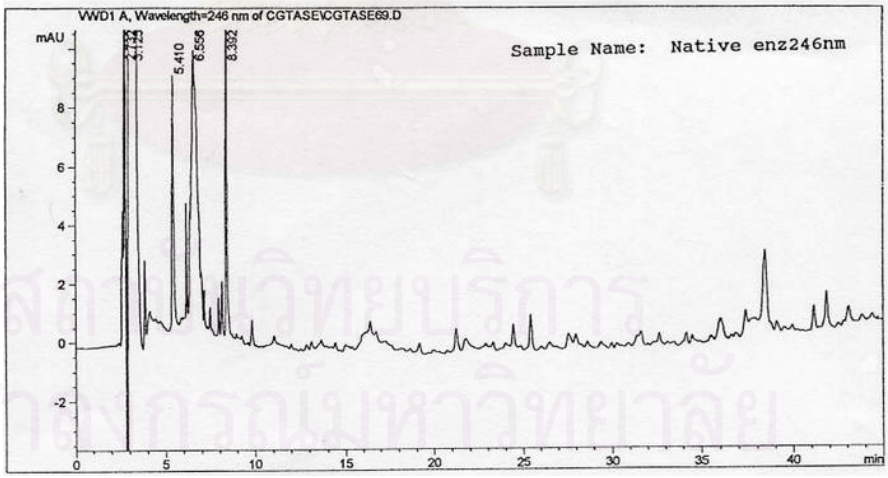
Figure 23. HPLC chromatograms at 246 nm of the peptides of CGTase isoform 1 resulting from digestion with trypsin

Experimental conditions were as described in Figure 22.

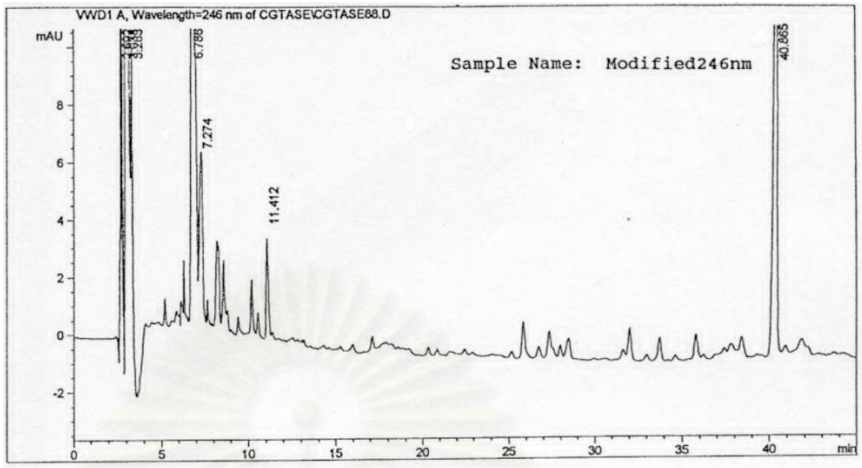
A. blank, injecting ultrapure water
B. digested products of native isoform 1
C. digested products of DEP-modified isoform 1
D. digested products of isoform 1 protected by methyl-β-CD prior to DEP-modification



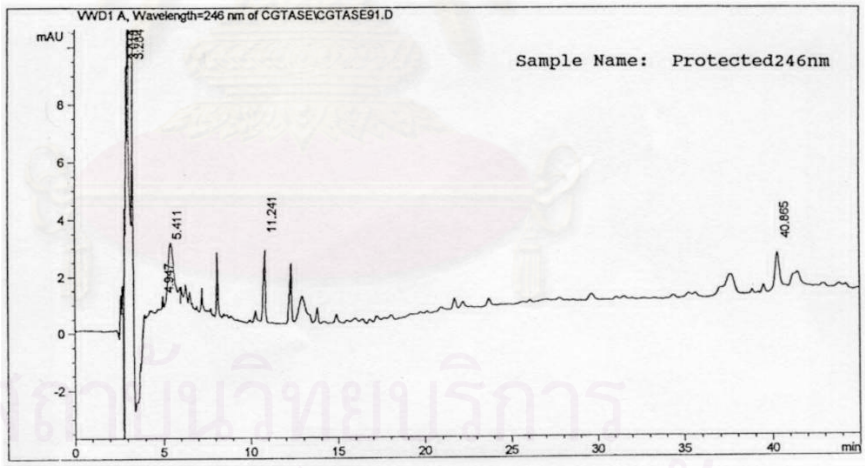
A. blank, injecting ultrapure water



B. digested products of native isoform 1



C. digested products of DEP-modified isoform 1



D. digested products of the methyl-β-CD-protected isoform 1

Table 20. Summarization of peptide peaks of interest from different HPLC profiles

Figure	R_t of peptide peaks (minutes)			
	1st peak	2nd peak	3rd peak	4th peak
22B	8.696	-	15.489	-
22C	Disappeared	11.348	Disappeared	40.934
22D	8.628 ↑	11.241 ↓	15.705 ↑	Disappeared
23B	8.392	-	-	-
23C	Decreased	11.412	-	40.865
23D	-	11.241 ↓	-	40.865 ↓

↑ = increased ↓ = decreased - = not seen

contain essential histidines since they were confirmed by absorbance at 246 nm (Table 20). These peaks were then pooled and submitted to mass determination and N-terminal sequence determination for further characterization.

3.6 Mass analysis of peptides

The peaks eluting at R_t 11.348 and 40.934 minutes were dissolved in acetonitrile / 0.02 % formic acid in water (50:50) and their mass spectra were as shown in Figures 24 and 25. The calculated mass determined (M_r) according to Methods Section 2.16, for the peaks at R_t 11.348 and 40.934 minutes were 5,732 and 2,540 daltons, which were approximately equivalent to 47 and 25 amino acid residues, respectively.

3.7 Determination of N-terminal sequence of peptides

The amino acid sequence of the N-terminus of the two peptides of interest, the R_t 11.348 and 40.934 minutes peaks, were determined by automated Edman degradation using the peptide sequencer. For the peak eluting at R_t 11.348 minutes, the sequence F A Q K could be identified (Figuse 26). And for the peak eluting at 40.934 minutes, the determined sequence was V I I D F A P N *H T (Figure 27).

3.8 Localization of essential histidines

The amino acid sequence of CGTase from *B. circulans* A11 decoded from nucleotide sequence was aligned with CGTase from other species and strains of *Bacillus* as shown in Figure 28. Four regions with conserve sequences were observed, and within these, three were found to contain histidine residues. When information on the size from mass spectra, and the N-terminal sequence of the two peptides were gathered with the knowledge on the action of trypsin which is known to cleave the C-terminal of lysine and arginine was collectively analyzed, the position of essential histidine from the peak of R_t 40.934 minutes was localized at His-140. In Figure 28, the positions of trypsin cleavage to generate that peptide of approximate size of 25 amino acids were shown by the arrows " a " and " a′ ". Trypsin cleaved at the C-terminal of Lys-131 and Arg-156. In this peptide, histidine (His-140) was at position 9 from the N-terminus. For peptide peak at R_t 11.348

minutes with the N-terminal sequence F A Q K, the positions of trypsin cleavage (“ b “ and “ b′ ” (Figure 28) were at Arg-284 and Arg-331 and this peptide contained exactly 47 residues as equal to the number estimated from mass determination. One histidine (His-327) was found at position 43 from the N-terminus.

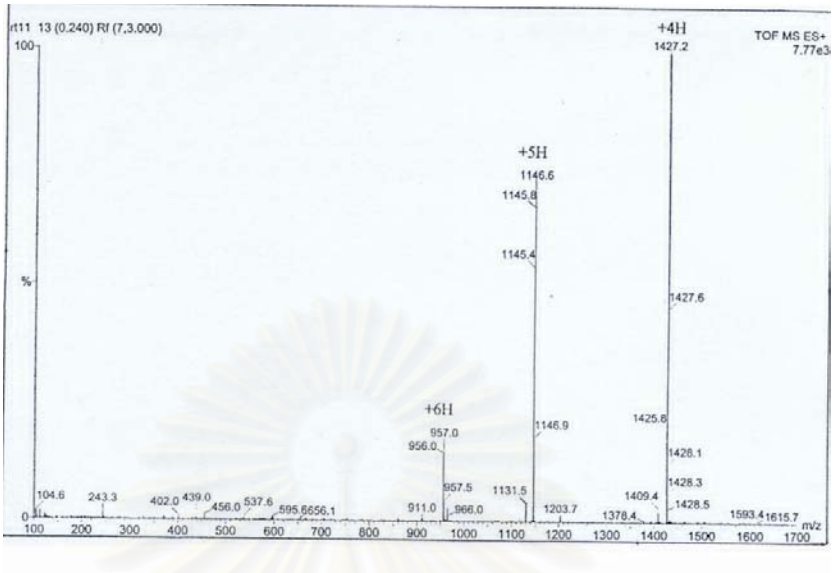


Figure 24. An electrospray mass spectrum of the peptide at R_t 11.3 minutes

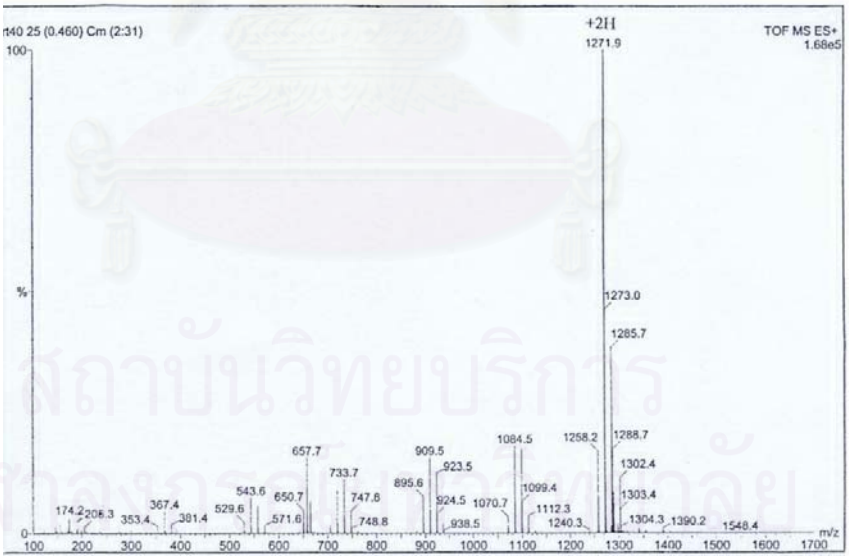
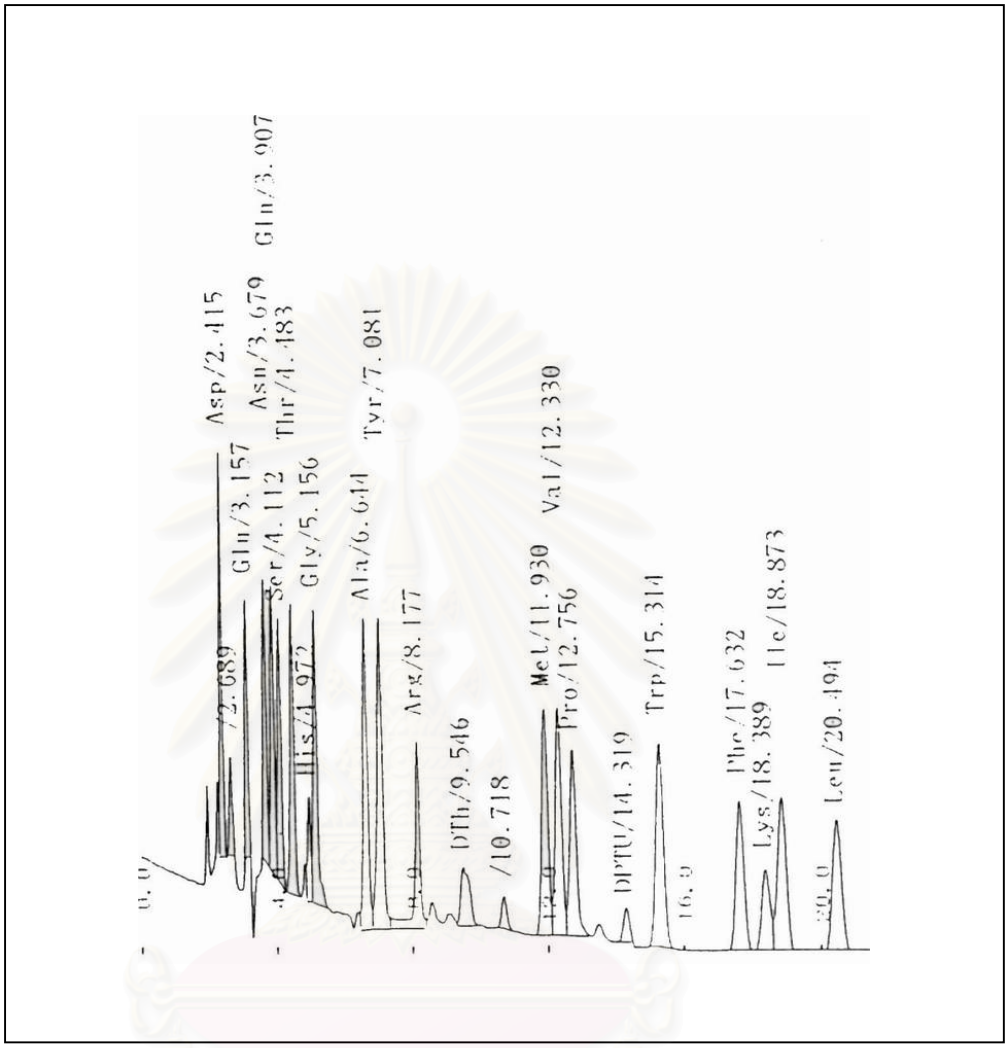


Figure 25. An electrospray mass spectrum of the peptide at R_t 40.9 minutes

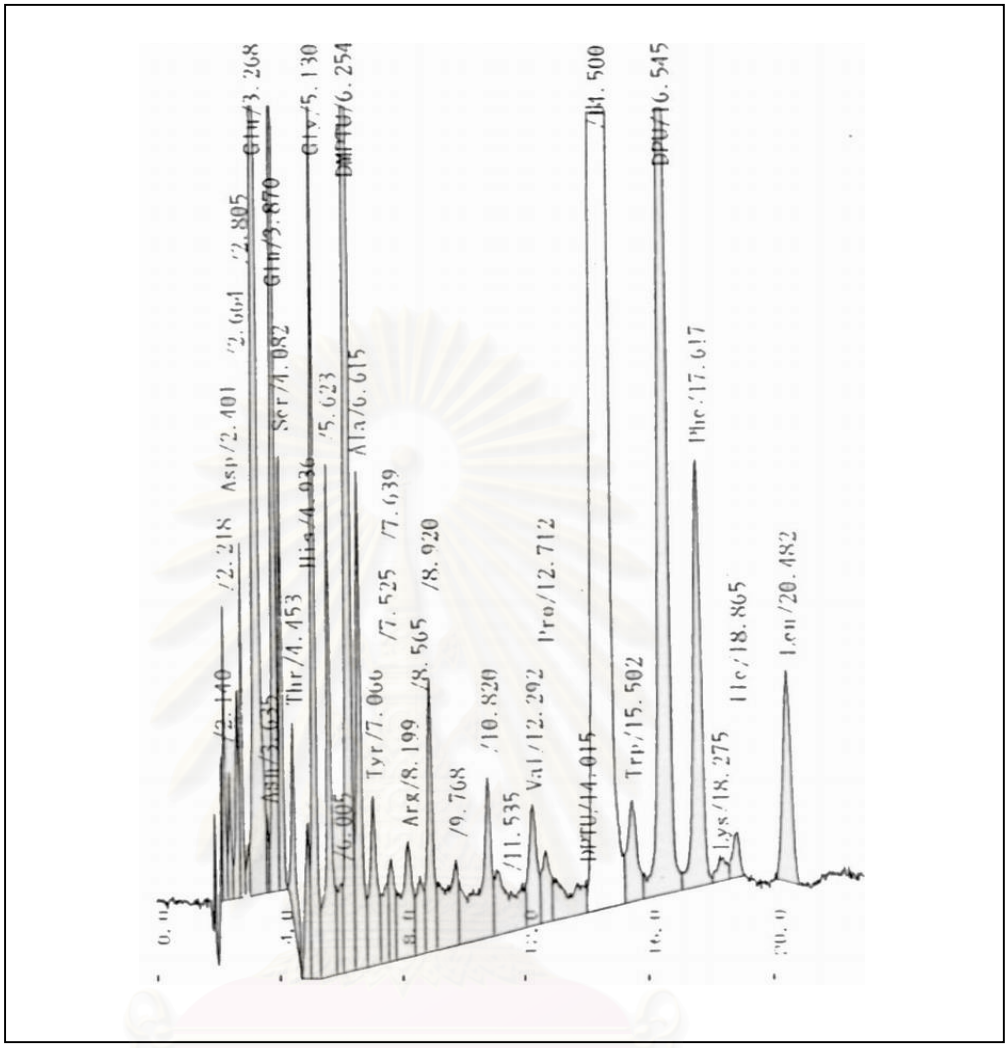
Figure 26. Chromatograms of N-terminal amino acids of the peptide at R_t 11.348 minutes from a Chromatopac C-R7A peptide sequencer

The amino acid identified in each cycle was indicated by the arrow.

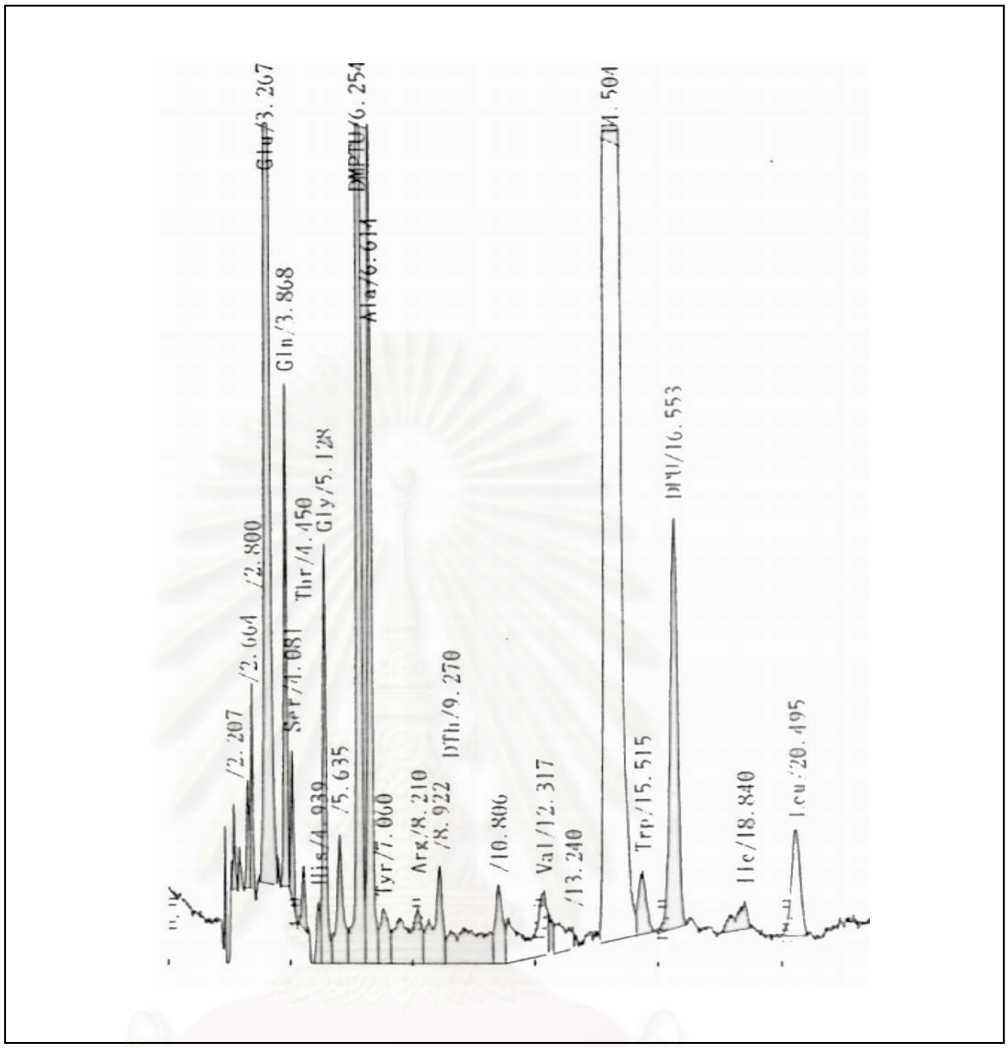
A. Standard amino acids
B. Cycle 1 (Residue 1)
C. Cycle 2 (Residue 2)
D. Cycle 3 (Residue 3)
E. Cycle 4 (Residue 4)



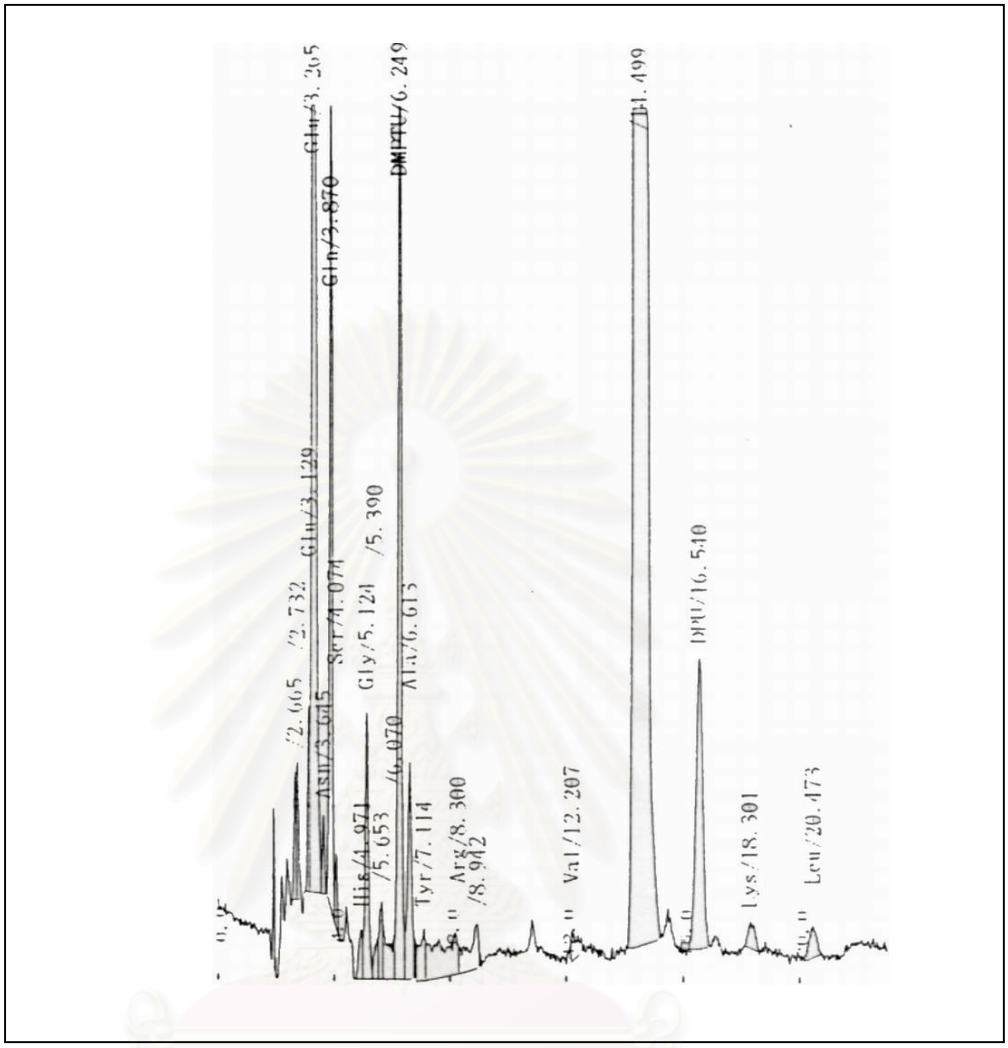
A. Standard amino acids



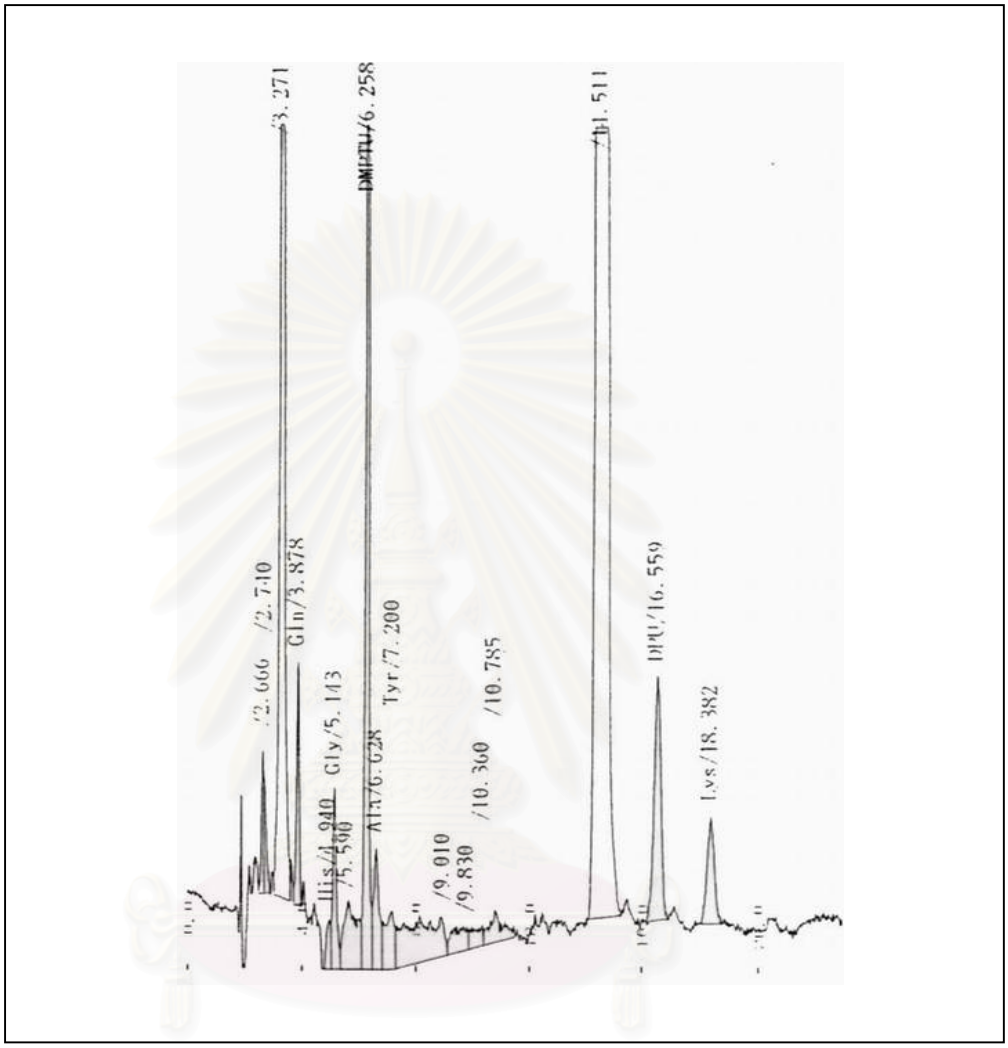
B. Residue 1



C. Residue 2



D. Residue 3

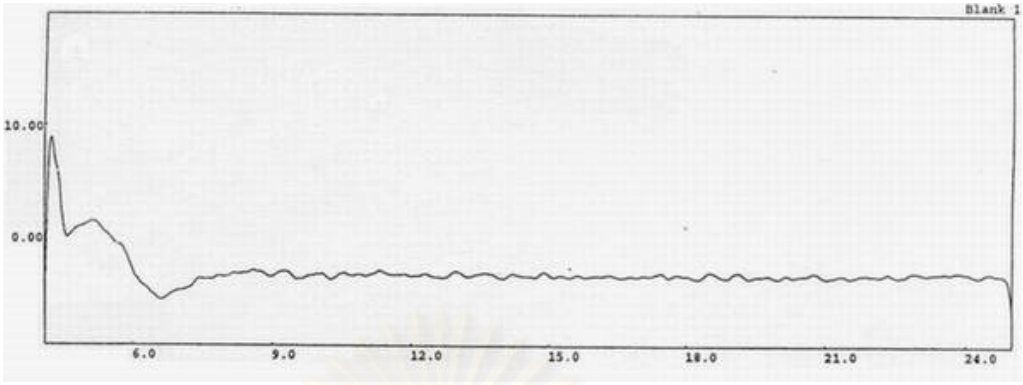


E. Residue 4

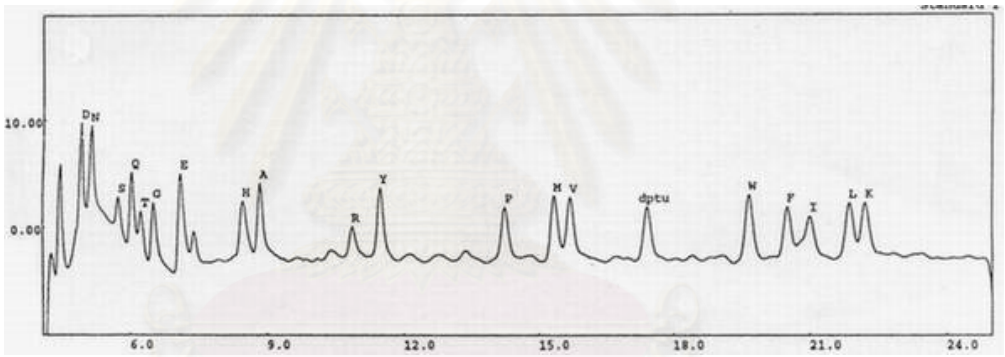
Figure 27. Chromatograms of N-terminal amino acids of the peptide at R_t 40.934 minutes from a Waters 510 HPLC peptide sequencer

The amino acid mixtures obtained were analyzed on a Picotag™ column (0.39x30 cm). The amino acid identified in each cycle was indicated by the arrow.

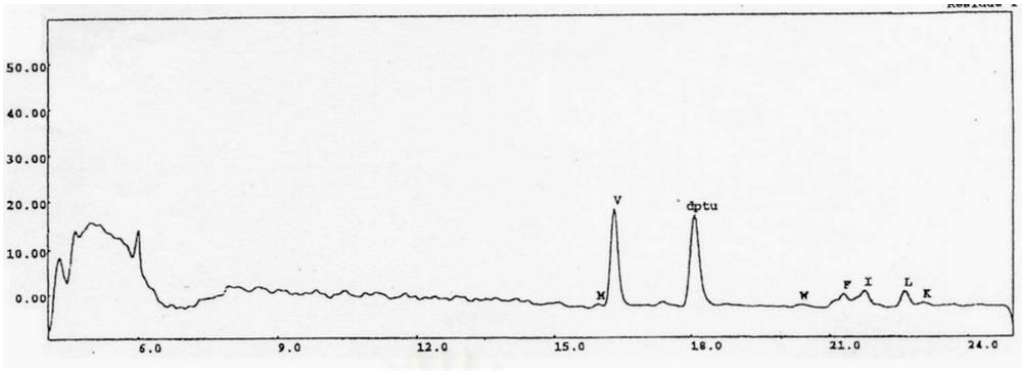
A. Blank
B. Standard amino acids
C. Cycle 1 (Residue 1)
D. Cycle 2 (Residue 2)
E. Cycle 3 (Residue 3)
F. Cycle 4 (Residue 4)
G. Cycle 5 (Residue 5)
H. Cycle 6 (Residue 6)
I. Cycle 7 (Residue 7)
J. Cycle 8 (Residue 8)
K. Cycle 9 (Residue 9)
L. Cycle10 (Residue 10)



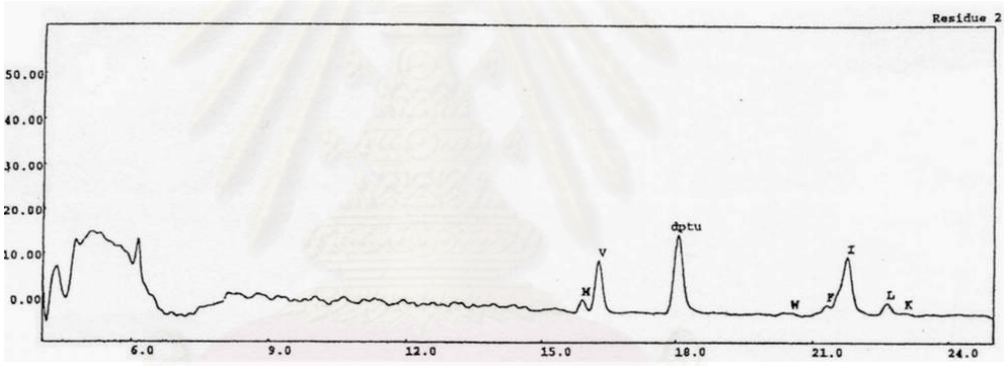
A. **Blank**



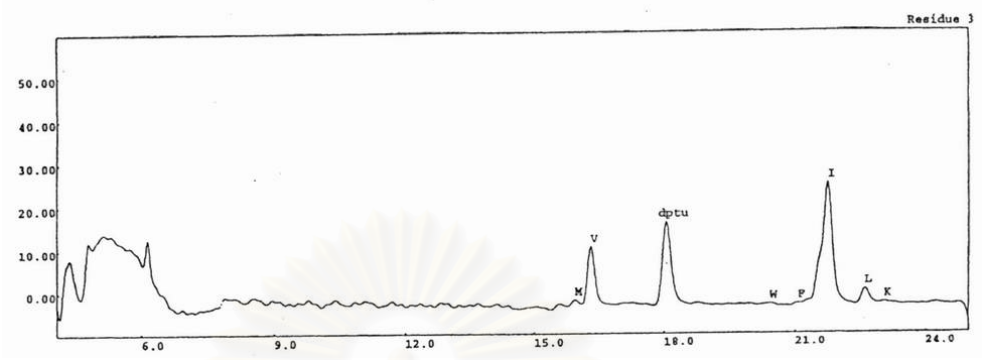
B. **Standard amino acids**



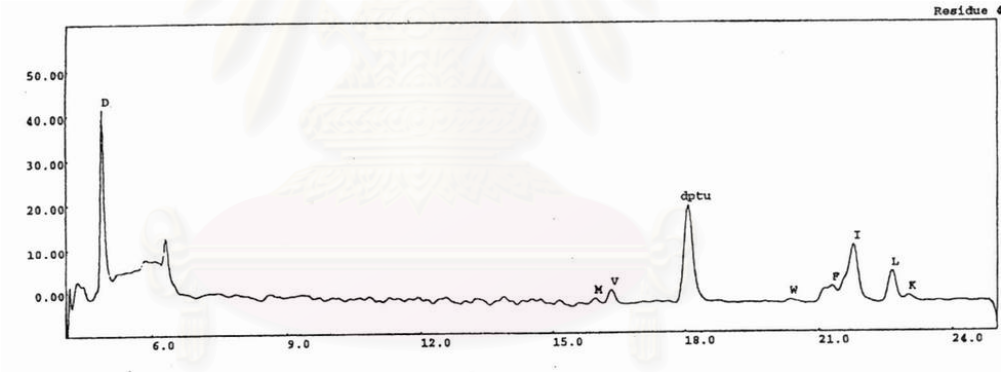
C. Residue 1



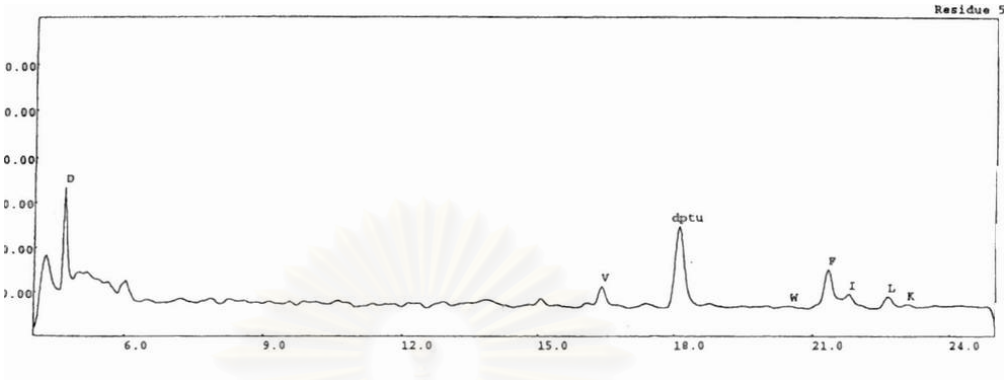
D. Residue 2



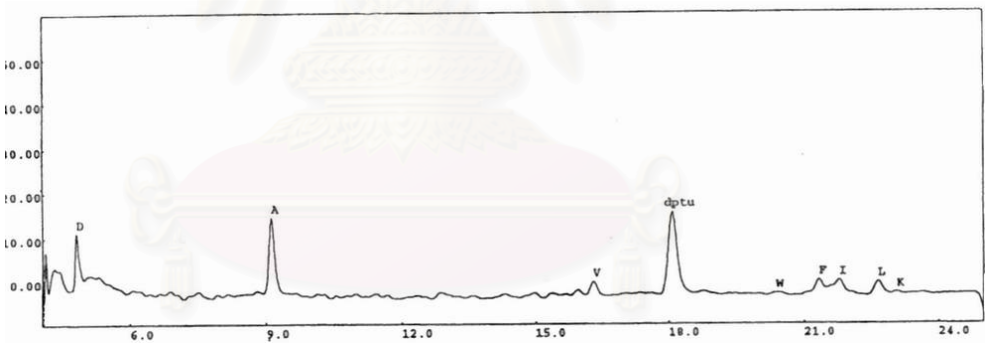
E. Residue 3



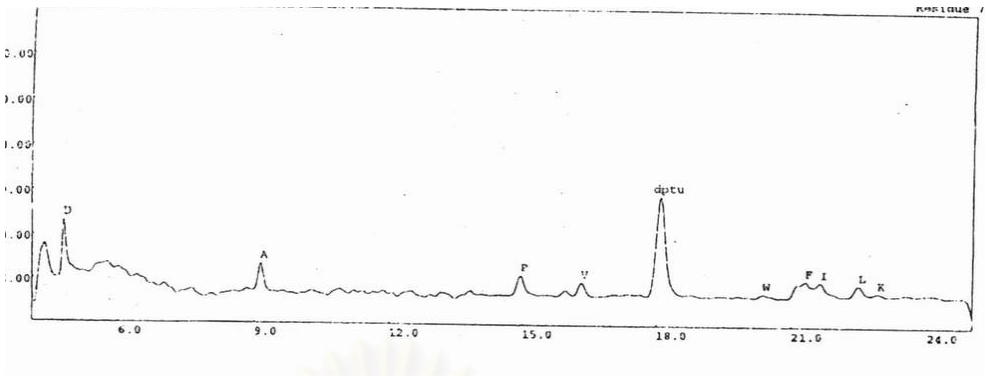
F. Residue 4



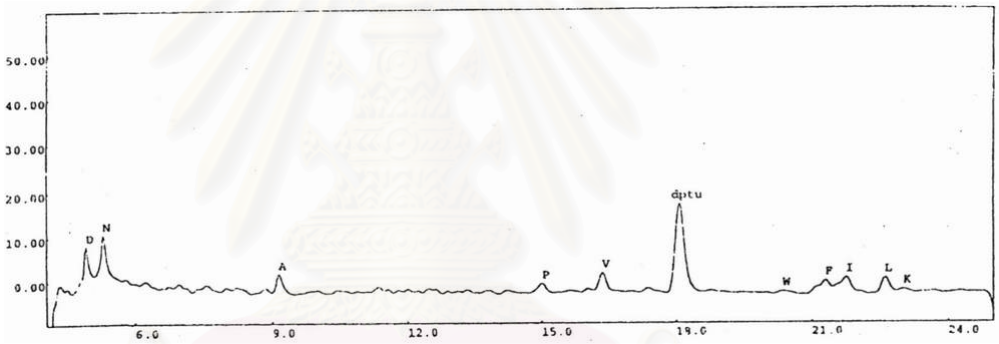
G. Residue 5



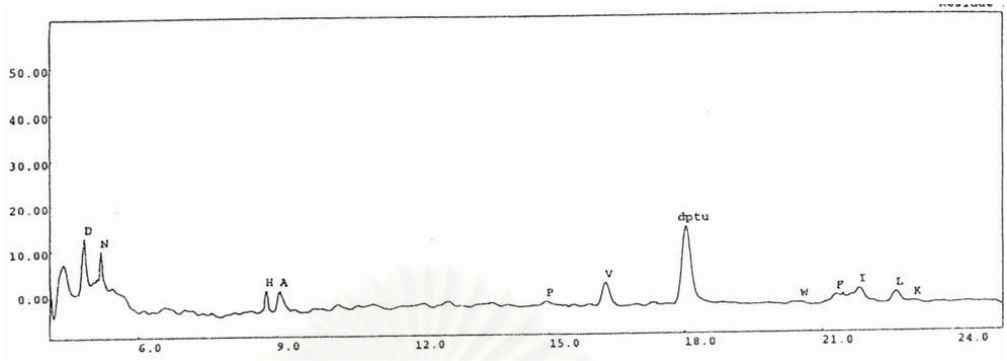
H. Residue 6



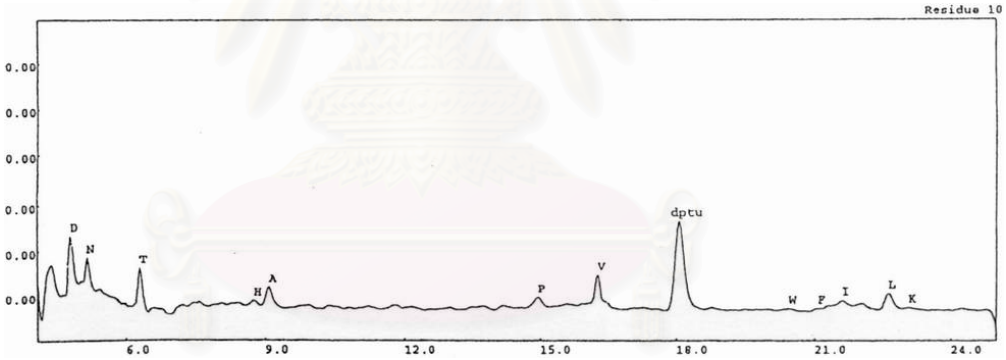
I. **Residue 7**



J. **Residue 8**



K. Residue 9



L. Residue 10

```
B.circulans A11   1 APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYC 50
B.sp 1011         1 APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGTCTNLRLYC 50
B.circulans 251   1 APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGTCTNLRLYC 50
B.circulans 8     1 DPDTAVTNKQSFSTDVIYQVFTDRFLDGNPSNNPTGAAYDATCSNLKLYC 50

B.circulans A11  51GGDWQGIINKINDGYLTGMGITAIWISQPVENIYSVINYSGVHNTAYHGY 100
B.SP 1011        51GGDWQGIINKINDGYLTGMGITAIWISQPVENIYSVINYSGVNNTAYHGY 100
B.circulans 251  51GGDWQGIINKINDGYLTGMGVTAIWISQPVENIYSIINYSGVNNTAYHGY 100
B.circulans 8    51GGDWQGIINKINDNYFSDIGVTALWISQPVENIFATINYSGVTNTAYHGY 100

                                                  a ↓
B.circulans A11 101WARDFKKTNPAYGTMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPS 150
B.SP 1011       101WARDFKKTNPAYGTMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPS 150
B.circulans 251 101WARDFKKTNPAYGTIQDFKNLIAAAHAKNIKVIIDFAPNHTSPASSDQPS 150
B.circulans 8   101WARDFKKTNPYFGTMQDFKNLITTAHAKGIKIVIDFAPNHTSPAMETDTS 150

                  a ↓
B.circulans A11 151FAENGRLYDNGNLLGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADL 200
B.SP 1011       151FAENGRLYDNGNLLGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADL 200
B.circulans 251 151FAENGRLYDNGTLLGGYTNDTQNLFHHNGGTDFSTTENGIYKNLYDLADL 200
B.circulans 8   151FAENGRLYDNGTLVGGYTNDTNGYFHHNGGSDFSSLENGIYKNLYDLADF 200
```

Figure 28. Alignment of amino acid sequences of *Bacillus* CGTases. Black highlighting indicates the conserved sequences. The arrows a, a′, b and b′ are the sites of tryptic cleavage which generate the two peptides of interest. *Bacillus circulans* A11 (Rimphanitchayakit, 2000) ; *Bacillus* sp. 1011 (Kimura *et al*., 1987) ; *Bacillus circulans* 251 (Penninga *et al.,* 1995) ; *Bacillus circulans* 8 (Nitschke *et al*., 1990)

```
B.circulans A11201NHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINNYK 250
B.SP 1011     201NHNNSSVDVYLKDAIKMWLDLGVDGIRVDAVKHMPFGWQKSFMATINNYK 250
B.circulans 251201NHNNSTVDVYLKDAIKMWLDLGIDGIRMDAVKHMPFGWQKSFMAAVNNYK 250
B.circulans 8 201 NHNNATIDKYFKDAIKLWLDMGVDGIRVDAVKHMPLGWQKSWMSSIYAHK 250

                                                    b↓
B.circulans A11251PVFTFGEWFLGVNEISPEYHQFANESGMSLLDFRFAQKARQVFRDNTDNM 300
B.SP 1011     251PVFTFGEWFLGVNEISPEYHQFANESGMSLLDFRFAQKARQVFRDNTDNM 300
B.circulans 251251PVFTFGEWFLGVNEVSPENHKFANESGMSLLDFRFAQKVRQVFRDNTDNM 300
B.circulans 8  251PVFTFGEWFLGSAASDADNTDFANKSGMSLLDFRFNSAVRNVFRDNTSNM 300

                                                b′↓
B.circulans A11301YGLKAMLEGSEVDYAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTL 350
B.SP 1011     301YGLKAMLEGSEVDYAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTL 350
B.circulans 251301YGLKAMLEGSAADYAQVDDQVTFIDNHDMERFHASNANRRKLEQALAFTL 350
B.circulans 8  301YALDSMINSTATDYNQVNDQVTFIDNHDMDRFKTSAVNNRRLEQALAFTL 350


B.circulans A11351TSRGVPAIYYGSEQYMSGGNDPDNRARIPSFSTTTTAYQVIQKLAPLRKS 400
B.SP 1011     351TSRGVPAIYYGSEQYMSGGNDPDNRARLPSFSTTTTAYQVIQKLAPLRKS 400
B.circulans 251351TSRGVPAIYYGTEQYMSGGTDPDNRARIPSFSTSTTAYQVIQKLAPLRKC 400
B.circulans 8  351TSRGVPAIYYGTEQYLTGNGDPDNRAKMPSFSKSTTAFNVISKLAPLRKS 400


B.circulans A11401NPAIAYGSTQERWINNDVIIYERKFGNNVAVVAINRNNNTPASITGLVTS 450
B.SP 1011     401NPAIAYGSTHERWINNDVIIYERKFGNNVAVVAINRNNNTPASITGLVTS 450
B.circulans 251401NPAIAYGSTQERWINNDVLIYERKFGSNVAVVAVNRNLNAPASISGLVTS 450
B.circulans 8  401NPAIAYGSTQQRWINNDVYVYERKFGKSVAVVAVNRNLSTSASITGLVTS 450
```

Figure 28. (continue) Alignment of amino acid sequences of *Bacillus* CGTases. Black highlighting indicates the conserved sequences.

```
B.circulans A11 451LPQGSYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATAPI 500
B.SP 1011       451LRRASYNDVLGGILNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATTPI 500
B.circulans 251 451LPQGSYNDVLGGLLNGNTLSVGSGGAASNFTLAAGGTAVWQYTAATATPT 500
B.circulans 8   451LPTGSYNDVLGGVLNGNNITS-TNGSINNFTLAAGATAVWQYTTAETTPT 500

B.circulans A11 501IGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQ 550
B.SP 1011       501IGNVGPMMAKPGVTITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQ 550
B.circulans 251 501IGHVGPMMAKPGVTITIDGRGFGSSKGTVYFGTTAVSGADITSWEDTQIK 550
B.circulans 8   501IGHVGPVMGKPGNVVTIDGRGFGSTKGTVYFGTTAVTGAAITSWEDTQIK 550

B.circulans A11 551VKIPAVPGGIYDIRVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTAL 600
B.SP 1011       551VKIPAVPGGIYDIRVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTAL 600
B.circulans 251 551VKIPAVAGGNYNIKVANAAGTASNVYDNFEVLSGDQVSVRFVVNNATTAL 600
B.circulans 8   551VTIPSVAAGNYAVKVA-ASGVNSNAYNNFTILTGDQVTVRFVVNNASTTL 600

B.circulans A11 601GQNVFLTGNVSELGNWDPNNAIGPMYNQVVYQYPTWYYDVSVPAGQTIEF 650
B.SP 1011       601GQNVFLTGNVSELGNWDPNNAIGPMYNQVVYQYPTWYYDVSVPAGQTIEF 650
B.circulans 251 601GQNVYLTGSVSELGNWDPAKAIGPMYNQVVYQYPNWYYDVSVPAGKTIEF 650
B.circulans 8   601GQNLYLTGNVAELGNWSTSTAIGPAFNQVIHQYPTWYYDVSVPAGKQLEF 650

B.circulans A11 651KFIKKQGSTVTWEGGANRTFTTPTSGTATMNVNWQP              686
B.SP 1011       651KFIKKQGSTVTWEGGANRTFTTPTSGTATVNVNWQP              686
B.circulans 251 651KFLKKQGSTVTWEGGSNHTFTAPSSGTATINVNWQP              686
B.circulans 8   651KFFKKNGSTITWESGSNHTFTTPASGTATVTVNWQ-              686
```

Figure 28. (continue) Alignment of amino acid sequences of *Bacillus* CGTases. Black highlighting indicates the conserved sequences.

CHAPTER IV

DISCUSSION

4.1 Purification and separation of isoforms of CGTase

Bacillus circulans A11 was screened for high CGTase producing-activity 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. In the initial step, CGTase was adsorbed by starch and the adsorbed enzyme was eluted with buffer containing maltose. Immunoaffinity column chromatography or ammonium sulfate precipitation and chromatography by DEAE-cellulose were usually performed to further purify the enzyme (Kim, 1996 ; Laloknam, 1997 and Tongsima, 1998). The purified enzyme demonstrated the same pattern on SDS-PAGE. It appeared as one band of protein with estimated molecular weight of 72,000 daltons (Rojtinnakorn,1994 ; Kim, 1996 ; and Tongsima, 1998). This CGTase was proved to be a single polypeptide chain by gel filtration (Techaiyakul, 1991). However, 2 major and 2-3 minor bands of protein at the purified enzyme level were usually observed on non-denaturing PAGE analysis (Rojtinnakorn, 1994 ; Laloknam, 1997 ; Tongsima, 1998). Rojtinnakorn (1994) reported that our CGTase from *Bacillus circulans* A11 could be fractionated on chromatofocusing column into 3-4 fractions, with pIs range from 4.40 to 4.90. The attempt to isolate these isoforms was accomplished by Kaskangam (1998) using preparative gel electrophoresis. In this work, isoform separation was performed using the procedure described (Kaskangam, 1998). The enzyme was partially purified by corn starch adsorption in the first step. The result obtained was similar to Kaskangam (1998), in that the loss of CGTase at the adsorption step (activity remained in the supernatant after adsorption) was found to be approximately 4 % while no activity was detected in the washing buffer. After CGTase was eluted by maltose, it was partially purified to 47 folds and the specific activity was 2,182 units/ mg protein with 96 % recovery (Table 8). When CGTase was further purified and concentrated by ultrafiltration technique, it was purified to 58

folds and the specific activity of 2,700 units/ mg protein with 52 % recovery. The final step of purification involves a preparative gel electrophoresis to isolate the isoforms according to their size, shape and charge characteristics. After elution of all protein bands from the gel, every other 5 fractions containing dextrinizing activity were analyzed by non-denaturing PAGE, then the fractions which gave the same band, named as bands 1,2,3,4 and 5 were pooled (Figure 10). Through this step, CGTase isoforms were purified from 2 to 100 folds (Table 8). The purity of CGTase at each purification step was observed on non-denaturing PAGE (Figure 7). When compared the overall result of purification with that reported by Kaskangam (1998), the specific activity of each purified isoform was about the same while the yield was higher in our study. For isoform 1 which is the major isoform, the yield was upto 10 %.

The pooled fractions were analyzed again by non-denaturing PAGE and activity stain was performed (Figure 11). It was found that each band was successfully separated. The result of Kaskangam (1998) showed that bands 1 to 4 exhibited both dextrinizing and CD-forming activities, while band 5 exhibited only dextrinizing activity. Hence 4 isoforms of CGTase of *Bacillus circulans* A11 was reported. Their isoelectric points were estimated by isoelectrofocusing gel to be 4.73, 4.49, 4.40 and 4.31, respectively. When bands 1 to 5 were analyzed by SDS-PAGE, a single protein band of molecular weight about 72,000 daltons were detected in all samples. Their carbohydrate contents were determined by phenol-sulfuric acid method to be 20.5, 18.7, 14.4 and 46.7% (w/w), respectively. Some physical and biochemical properties were analyzed. Their pH optima were 6.0-7.0, 6.0-7.0, 6.0 and 7.0, temperature optima were 40°C, 40°C, 50°C and 50-60°C and the ratio of α-, β-, and γ-cyclodextrins produced were determined as 10:18:5, 9:18:5, 5:18:5 and 5:18:7 for bands 1,2,3 and 4, respectively. Study on their amino acid compositions showed significant difference on the content of glutamine, histidine, alanine and proline (Kaskangam, 1998).

CGTase isoforms/isozymes have been previously reported. The isolation methods which depend on their charge characteristics and specificity towards the ligand such as high-performance anion exchange chromatography (Makela *et al.*,

1988), isoelectric focusing (Mattsson, Meklin and Korpela, 1990), FPLC on a mono Q column (Bovetto *et al.*, 1992) and affinity chromatography on a β-CD polymer (Abelyan *et al.*, 1994) were used. Makela and his group (1988) showed that the isoelectric points of CGTase fractions from an alkalophilic *Bacillus* strain ATCC 21783 were in the range between 4.55-4.90. The CGTases from *Bacillus circulans* E 192 showed 2 isozymes with different subunits of 33,500 and 48,500 daltons, respectively (Bovetto *et al*, 1992). CGTase isozymes (2-4 subforms) of *Bacillus* strains (INMIA-T6, INMIA-T42, and INMIA-A7/1) had molecular weights varied over the range from 25,000 to 50,000 daltons, while amino acid compositions and the CD-product ratios obtained were shown to be different in each subform (Abelyan *et al*, 1994).

4.2 Information on the active site of CGTase isoform

4.2.1 Chemical modification of CGTase isoform

In this work, the identification of essential amino acid residue(s) at the active site of CGTase isoforms from *Bacillus circulans* A11 was performed using chemical modification with group-specific reagents and substrate protection technique. Chemical modifications of seven different amino acid residues (carboxylic, histidine, tryptophan, tyrosine, cysteine, lysine and serine) of CGTase isoforms were determined under mild conditions. These amino acid residues have been selected because they are widely known as residues involved in enzyme catalysis, while some have been reported to be essential for CGTases of other sources (Means and Feeney, 1971 ; Lundblad, 1991 : Mattsson, 1992 : Bender, 1991 : Villette, 1993 : Ohnishi, 1992).

The first step was to screen for essential amino acid residues that upon modification, the loss of each isoform activity was observed. Incubation of purified CGTase isoforms from *Bacillus circulans* A11 with a series of covalent modifiers of amino acid residues at 1mM concentration resulted in variable changes in the catalytic ability of this enzyme. No inhibition of CGTase isoform activities were observed in the modification of cysteines by *N*-ethylmaleimide (NEM), iodoacetamide (IAM), or dithiothreitol (DTT) in all 4 isoforms, lysines by 2, 4, 6-trinitrobenzenesulfonic acid

(TNBS) in isoforms 1, 2, and 4, serine residues by phenylmethylsulfonyl fluoride (PMSF) in isoforms 1 and 3, and tyrosine by *N*-acetylimidazole (NAI) in isoforms 2 and 4. Reactions under mild conditions with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), diethylpyrocarbonate (DEP), and *N*-bromosuccinimide (NBS), which were known to react specifically with carboxyl, histidine, and tryptophan residues, respectively, resulted in extensive inhibition of all 4 isoform activities (Table 9). Among the three modifiers, DEP inhibited total activities of isoforms 1 and 3 while NBS inhibited total activities of isoforms 1 and 2. This suggests the crucial importance of histidine and tryptophan in those isoforms mentioned. Very high inhibition was also observed for DEP modification in isoform 4 and NBS modification in isoform 3 and 4. Partial inhibition was observed for EDC modification in all isoforms, and also for TNBS modification in isoform 3, PMSF modification in isoform 2 and 4, and NAI modification in isoform 1 and 3. When concentration of NAI was increased to 30 mM (Table 13), all 4 isoform activities were inhibited. It should be noted that the isoforms 1 and 3 were more sensitive to NAI inhibition than isoforms 2 and 4. It may be concluded here that amino acid residues which were essential for all 4 isoforms were histidine, tryptophan, tyrosine and carboxylic amino acids. Different residues identified to be essential for each isoform were : isoforms 2 and 4, serine ; isoform 3, lysine. When analyzing our results considerately, DEP and NBS inhibited total or almost total activities of CGTase isoforms at only 1 mM concentration (Table 9) while EDC demonstrated partial inhibition and NAI was equally effective at 30 mM.This suggests that histidine and tryptophan residues may be more essential for CGTase isoform activities than carboxylic or tyrosine residues, because they used less concentration for inactivation. In addition, isoforms 2, 3, and 4 were distinct since isoform 3 contained essential lysine while isoforms 2 and 4 contained essential serine. However, lysine and serine in these isoforms were not very much essential because only partial inhibition was observed. The result in this study was corresponded to that reported by Tongsima (1998). She found that essential amino acid residues of whole enzyme (mixed isoforms) of CGTase from *Bacillus circulans* A11 were histidine, tryptophan, tyrosine, and carboxylic residues. CGTase activity of whole enzyme was totally inhibited by 1 mM DEP and NBS, and partially inhibited by 1 mM EDC and NAI, but not inhibited by NEM, IAM, DTT, TNBS, and PMSF. When compared the

activity loss after modification by DEP, NBS, EDC and NAI, the result of whole enzyme was closed to that of isoform 1 in our study. This is not surprising since isoform 1 is the major isoform of the enzyme.

When compared to other studies, these important amino acid residues for our CGTase have been demonstrated elsewhere as essential residues for other CGTases as well. Bender (1991) reported that histidine residues of CGTase from *Bacillus circulans* strain 8 and *Klebsiella oxytoca* M5 al were modified by DEP and proposed to be involved in the cyclizing reaction. In 1992, Mattsson *et al.* reported the inhibition of CGTase from *Bacillus circulans* var. *alkalophilus* (ATCC 21783) by 0.25 mM DEP and EDC at 6,770 mol/mol CGTase which resulted in almost total loss of cyclizing activity indicating that histidine and carboxylic residues were involved in the catalytic activity. Villette *et al.* (1993) reported the modification of tyrosine residues of CGTase from *Bacillus circulans* E192 with tetranitromethane. The results suggested that the nitration of the CGTase induced a decrease in the catalytic properties of the enzyme. CGTase from *Bacillus stearothermophilus* was reported to be modified at tryptophan residues by NBS (Onishi *et al* ., 1992). The modification corresponded with inactivation of the CGTase catalysed hydrolysis of cyclomaltohexaose (cG_6).

4.2.2 Substrate protection

The second step was to prove that the amino acid residues involved in CGTase isoform activities were or were not at the active site. The inactivation of enzyme by chemical modification does not directly indicate that a specific residue is present at the active site. Protection of the enzyme activity by substrate, competitive inhibitor, end product, or related compounds has been used to confirm the presence of specific residues at the active site. If activity is retained following modification in the presence of substrate (or other protective substances) but is lost in its absence, it is usually assumed that a group in the active site has been protected (Means and Feeney, 1971). In this work, β-, hydroxypropyl-β, and methyl-β-CD were used as protective substances. And the experiment was performed by measuring CGTase isoform inactivation by group-specific reagents in the presence or the absence of protective substances. The results in Table 10, 11, 12, 13, 14, 15, 17, 18, and 19 showed that the

loss of CGTase isoform activities were partially or totally reduced in the presence of protective substance. Lysine modification in isoform 3 was almost totally protected by 25 mM methyl-β-CD (activity loss ≤ 10 %), while serine modification in isoform 2 was less protected (activity loss ≤ 16 %). Carboxyl modification of all 4 isoforms were partially protected by 25 mM methyl-β-CD (activity loss ≤ 20-30 %), protection in isoforms 1 and 3 were higher than in isoforms 2 and 4. Tyrosine and tryptophan modifications were also partially protected. It is interesting to point out here that protection in isoform 1 from tyrosine modification and in isoforms 1 and 2 from tryptophan modifications were significantly pronounced. Upto 62-68% protection was observed. For histidine modification, it was especially interested because there are several reports that histidine was at the active site of CGTases from *Bacillus* sp. So we did the experiment on histidine modification in more details than other chemical modifications. The suitable concentration of DEP and incubation time were found for each isoform (Figure 12, 13, 15, 16, 17, 18, 19 and 20) before substrate protection experiment. It was found that histidine modification in isoform 1 was partially protected by 25 mM β-, hydroxypropyl-β-, or methyl-β-CD (activity loss ≤ 20-30 %). The reaction of DEP with imidazole groups of histidine residues in isoform 1 was accompanied by an increase in absorbance at 246 nm, an indicative of the formation of *N*–carbethoxyhistidine (Figure 14). Moreover, the number of histidine residues of isoform 1 which were modified and protected by methyl-β-CD was estimated. The results in Table 16 indicates that 25 mM methyl-β-CD protects two histidine residues which suggests the presence of two histidines at isoform 1 active site. This result is the same as that reported for the whole enzyme (Tongsima, 1998). For histidine modification in isoform 3, the activity was almost totally protected by 25 mM methyl-β-CD (activity loss ≤ 9 %), while histidine modification in isoforms 2 and 4 were partially protected (activity loss ≤ 24-33 %). Differences in activities in the presence and absence of the substrate in this experiments could lead to the interpretation that histidine, tryptophan, tyrosine and carboxyl residues are involved at the active site of all 4 isoforms in addition to serine in isoform 2 and lysine in isoform 3, but with difference in degree of importance to enzyme catalysis.

When compared to other CGTases, Mattsson *et al.* (1992) reported that CGTase from *Bacillus circulans* var. alkalophilus (ATCC 21783) was protected

against inactivation by α-, and β-CD suggesting that the modified histidine residues were at or near the active site. The study by Villette *et al.* (1992) showed that CGTase activity was retained when chemical modification with DEP occurred in the presence of 5 mM acarbose (uncompetitive inhibitor of CGTase) or 5 mM salicin (competitive inhibitor of CGTase). These inhibitors protected one of the two faster reacting histidine residues in the active site, with a 49.7 % recovery of residual activity. Trp was protected against NBS by glucose and the maltosaccharides G_2-G_4, which indicates Trp to be located at the substrate binding site of CGTase from *Bacillus stearothermophilus* (Ohnishi *et al.*, 1992). Wakayama *et a.*, 1996 reported that DEP-inactivated *N*-acyl-D-glutamate amidohydrolase from *Pseudomonas* sp. 5f-1 showed different spectrum with a peak at 246 nm characteristic of mono-*N*-carbethoxylation of histidine residue. Using an extinction coefficient of 3,200 M^{-1} cm^{-1} , the number of essential histidine was calculated to be 1.1 per mole of the enzyme. Treatment of DEP-inactivated enzyme with NH_2OH restored about 67% of the initial activity after 60 minutes of incubation which confirmed that the target of DEP modification was histidine and histidine was essential for the enzyme activity.

When specificity of chemical modification by group-specific reagents is concerned, mild and suitable incubation conditions have to be used. In addition to modifier concentration and inactivation time, pH of the reaction is also of importance. For the effect of pH on the modification of CGTase isoforms, Tongsima (1998) reported that EDC modification was effective in the pH range of 5.0-6.0. For DEP, NBS and NAI, the optimum pH range were 5.5-7.5, 5.0-5.5, and 5.0-7.0, respectively. Treatment with NBS is usually performed in acetate or formate buffer at pH 4 or lower, but it can also be frequently done at pH closer to neutral. Higher pH has an advantage in that little or no peptide bond cleavage takes place. Also higher pH is usually more selective (Means and Feeney, 1971). Delferge *et al.* (1997) reported that the TNBS works effectively in the pH range of 7.5-8.0, while PMSF works effectively in the pH range of 6.0-8.0. From these information, the purified CGTase isoforms in 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$ were used in the modification of all residues in this work. Because pH 6.0 was optimum for dextrinizing activity assay, though not all modifications work best at this pH.

4.3 Inactivation kinetics of CGTase isoform 1 with DEP

Incubation of CGTase isoform 1 with varying concentrations of DEP resulted in a time-dependent loss of CGTase isoform 1 activity, suggesting the modification of histidine residue(s) located at or near the CGTase isoform 1 active site. Plots of the logarithm of remaining activity versus time at different reagent concentrations indicated in each case pseudo first-order kinetics (Figure 21A). A straight line was also observed for the plot of pseudo first-order rate constants ($k_{inactivation}$) versus varying concentrations of DEP (Figure 21B), suggesting that chemical modification is the result of a simple bimolecular reaction. This simply means reaction rate was dependent on both the enzyme and DEP concentrations. The second-order rate constant obtained for the modification by DEP was calculated from the slope of Figure 21B to be 29.5 M^{-1} s^{-1}. This value shows a rather high reactivity of DEP towards the active-site histidine residue(s). Plotting of log $k_{inactivation}$ versus log of DEP concentration yields an apparent reaction order of 0.84 for DEP, indicating that inactivation results from the reaction of approximately 1 mol of DEP with 1 mol of CGTase isoform 1 subunit (Figure 21C). This result was consistent with the result from chemical modification and substrate protection experiment, which suggested two histidine residues at the active site of isoform 1(Table 16). Two essential histidines were also confirmed by analysis of tryptic peptides of CGTase isoform 1 (Result Section 3.5).

No inactivation kinetics has been previously reported for any CGTase. For other enzymes, the study by Delforge *et al.*, 1997 showed that incubation of L-alanine dehydrogenase with varying concentrations of TNBS and SPDP resulted in a time-dependent loss of enzyme activity, which suggested the presence of lysine at or near the enzyme active site. The second-order rate constants ($k_{inactivation}$) obtained for the modification by TNBS and SPDP were 0.57 and 60.6 M^{-1} s^{-1}, respectively. SPDP was shown to react with lysine at a faster rate than TNBS. Hirano, N. *et al.* (1991) reported that the incubation of inorganic pyrophosphatase by DEP at 25 °C resulted in rapid loss of activity. The semi-logarithmic plot of residual activity versus time was straight line, indicating that the inactivation following the first-order kinetics. The inactivation of the enzyme N-acyl-D-glutamate amidohydrolase (D-AGase) from

Pseudomonas sp. 5f-1 was dependent on incubation time and reagent concentration when incubating with DEP(Wakayama *et al.*, 1996). The inactivation of the enzyme with DEP proceeded with pseudo-first order kinetics. The pseudo-first order rate constant (k_{obs}) for DEP was 0.12 min^{-1}. The double logarithmic plot of pseudo-first order rate constant versus DEP concentration gave a slope value of 1.15, indicating that inactivation results from the reaction of approximately 1 mol of DEP with 1 mol of enzyme subunit.

4.4 Localization of essential histidines at the active site of CGTase isoform 1

CGTase isoform 1 was digested by trypsin at C-terminal of arginine or lysine according to Section 2.15. From the known sequence of *Bacillus circulans* A11 CGTase reported by Rimphanitchayakit (2000), if trypsin cleaved at all positions of C-terminal of arginines or lysines, approximately 40 peptides should be obtained. HPLC profile of peptides resulted from tryptic cleavage of isoform 1(Figure 22B.) shows as many as 40 peaks but most of them were small peaks. Only about 10 peaks had significant amounts of peptide concentration as estimated from high absorbance values. This means that about one-fourth of the cleavage sites was constantly cleaved. While other sites were scarcely digested. From separation principle of reverse phase column, the peaks eluted earlier should be more polar than those came out later.

From the comparison of HPLC profiles of tryptic digests of native isoform 1, DEP-modified, and methyl-β-CD protected prior to DEP- modified form , as monitored by absorbance at 210 nm for peptides and 246 nm for modified-histidine (N-carbethoxyhistidine), the peaks at R_t 11.384 and 40.934 minutes were selected for further analysis since they showed significant difference in the peak height among Figure 22 B,C and D. And this difference was confirmed in Figure 23 B,C and D. It may be proposed that the peaks at R_t 8.696 and 15.489 minutes in native isoform 1 (Figure 22 B) which disappeared upon DEP-modification (Figure 22 C) became the peaks at R_t 11.348 and 40.934 minutes. This propose is possible since DEP-modification led to the increase of hydrophobicity of the peptide (Means and Feency,

1971), and the peaks at R_t 11.348 and 40.934 minutes are less polar than the peaks at R_t 8.696 and 15.489 mintues, respectively.

The mass M_r determined for the peptide peaks eluting at R_t 11.348 and 40.934 minutes were 5,732 and 2,540 daltons, respectively. The number of amino acids in the two peptides were then estimated to be about 47 and 25 residues, using the assumption that average molecular mass of amino acids was in the range of 100-120 daltons (Voet, 1990). When the N-terminus sequences of both peptides were determined, the sequence F A Q K and V I I D F A P N H T could be identified. These data were then fitted with the amino acid sequence of CGTase (Figure 28), and the tryptic cleavage sites at C-terminus of Arg-284 and Arg-331 were identified for the peptide of R_t 11.348 mintues, of which His-327, the only histidine, was localized. For the peptide peak at R_t 40.934 minutes, the cleavage sites at Lys-131 and Arg-156 were identified, and only one histidine (His-140) was found at position 9 from the N-terminus. Counting from the sequence in Figure 28, the peptides of R_t11.348 and 40.934 minutes consisted of 47 and 26 amino acid residues which are equal or almost equal to the size estimated from mass spectrometry.

CGTase sequences which have been reported in several papers demonstrated four conserved regions designated as regions A, B, B′, and C, respectively. Three conserved regions contained histidine residues as indicated by black highlighting (Table 21). It should be noted that similar pattern of conserved histidine residues was demonstrated in *K. pneumoniae* and many species of *Bacillus*. From the three dimensional structure of *B. stearothermophilus* CGTase, His-140 and His-327 bound the same glucose unit of amylose, whereas His-233 bound a different one (Kubota *et al.*, 1991). In *B. circulans* strain 251 CGTase, the ∈–nitrogen of His-327 was hydrogen bonded to the carboxylate of Glu-257, a putative proton donor of this CGTase (Klein *et al.*, 1992). The hydrogen bond between His-327 and Glu-257 may be important for catalysis of CGTase. Nakamura (1993) reported that the three histidine residues (His-140, His-233, and His-327) of CGTase from alkalophilic *Bacillus* sp. 1011 were essential histidine residues in the active center of the enzyme. His-327 was important for catalysis over an alkaline pH range, because the decrease of the activity was observed when replacement of His-327 by asparagine residue was

performed. For His-140 and His-233, different roles in catalysis could not be clarified. Most of studies on identification of essential residues at the active site of CGTases used x-ray diffraction or site-directed mutagenesis. The use of peptide mapping as in this work is not seen in any references so far reported. Two essential histidines at the active site of CGTase isoform 1, identified as His-140 and His-327 were corresponded to those residues of CGTases from other species (Table 21). However, the position number of His may be identical or slightly shift depending on how close our enzyme is to other CGTases. Another essential histidine, His-233, which were reported in many other CGTases, was not identified in this study of *Bacillus circulans* A11 CGTase isoform 1. His-233 may not be at the active site of our enzyme or the techniques of chemical modification and substrate protection could not identify this residue. Another alternative is that the peptide containing this residue may be one of those small peaks (Figure 22 B) due to inconsistency of trypsin digestion at the cleavage site which gave rise to the peptide. When the peptide peak was small, it was impossible to see differences of the peak among the native, the modified, and the protected form of the enzyme. To prove the importance of His-233, the use of other hydrolytic enzymes with different cleavage sites may be tried. The technique of site-directed mutagenesis may also be used for further identification.

Table 21. Comparison of amino acid sequences of the conserved regions (A,B,B', and C) of CGTases. Amino acids are numbered from the NH_2-terminus of enzyme. Black highlighting indicates the conserved histidine residues.

Enzyme	A-region	B-region	B'-region	C-region	Reference
CGTase					
B.circulans A11	^{135}DFAPNH		^{257}EWFL	^{323}FIDNHD	Rimphanitchayakit, 2000
Bacillus sp.#1011	^{135}DFAPNH...^{225}GIRVDAVKH...		^{257}EWFL....	^{323}FIDNHD	Kimura *et al.*, 1987
B. stearothermophilus	^{131}DFAPNH...^{221}GIRMDAVKH...		^{253}EWFL....	^{319}FIDNHD	Sakai *et al.*, 1987
B. macerans	^{135}DFAPNH...^{225}GIRFDAVKH...		^{258}EWFL....	^{324}FIDNHD	Sakai *et al.*, 1987
B. circulans 8	^{135}DFAPNH...^{225}GIRVDAVKH...		^{257}EWFL....	^{323}FIDNHD	Nitschke *et al.*, 1990
K. pneumoniae M5a1	^{130}DYAPNH...^{219}AIRIDAIKH...		^{257}EWFG....	^{328}FMDNHD	Binder *et al.*, 1986

CHAPTER V

CONCLUSION

1. Purifed CGTase isoforms from *Bacillus circulans* A11 were prepared by preparative gel electrophoresis. Purified isoform 1 was obtained with 100 folds purification with a 9.6 % yield and specific activity of 4,608 units/mg protein.

2. Activities of four isoforms of CGTase were lost after modifications of histidine, tryptophan, tyrosine, and carboxylic amino acids, while modification of cysteine did not affect all isoforms. Serine modification had an effect on isoforms 2 and 4 activities while isoform 3 activity was lost upon lysine modification.

3. The loss of activities of all isoforms after the modifications of histidine, tryptophan, tyrosine, and carboxylic amino acids were reduced in the presence of protective substances methyl-β-CD suggesting the location at or near the active site of these residues.

4. The loss of isoform 2 and isoform 3 activities after the modifications of serine and lysine, respectively, were reduced in the presence of methyl-β-CD.

5. Two histidines of isoform 1 were protected from DEP modification by methyl-β-CD, a substrate of CGTase.

6. Inactivation kinetics of isoform 1 with DEP resulted in a simple bimolecular reaction with pseudo-first order kinetics. The second-order rate constant ($k_{inactivation}$) was 29.5 $M^{-1} s^{-1}$. The mole ratio of DEP to isoform 1 was 1 : 1.

7. The peptide peak from trypsin digestion which contained essential histidine residues were eluted from HPLC C_{18} – reverse phase column at R_t 11.348 and 40.934 minutes. Their mass (M_r) were 5,732 and 2,540 daltons.

8. The N-terminal sequences of F A Q K and V I I D F A P N H T could be identified for the peptide peaks at R_t 11.348 and 40.934 minutes, respectively.

9. The positions of essential histidine residues in the active site of CGTase isoform1 were His-140 and His-327.

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06	g

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

2. Non- denaturing PAGE

7.0 % Seperating gel

30 % acrylamideml 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

5.0 % Stacking gel

30 % acrylamideml 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 addedto four parts of sample.

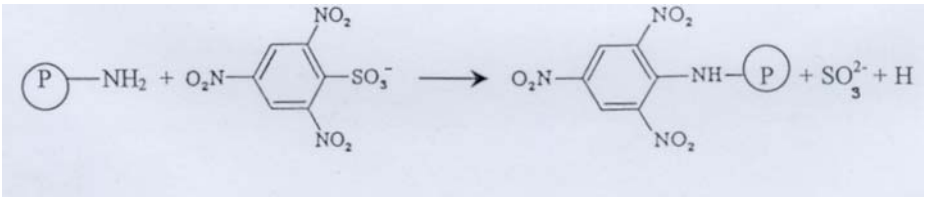
Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	ml

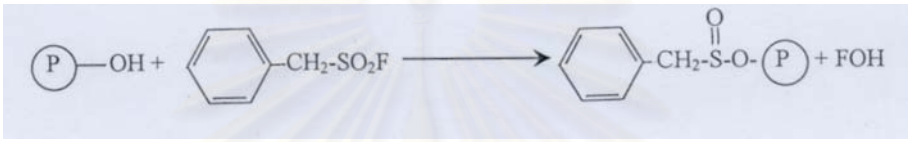
Dissolved in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3).

APPENDIX B : Modification reaction of group-specific reagents



Reaction 1 Modification of DEP with histidine residue in protein (P)

(Means and Feeney, 1971 ; Lundblad, 1991)

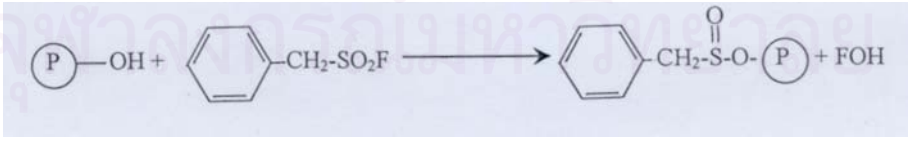


Reaction 2 Modification of NBS with tryptophan residue in protein (P)

(Means and Feeney, 1971 ; Lundblad, 1991)

Reaction 3 Modification of NAI with tyrosine residue in protein (P)

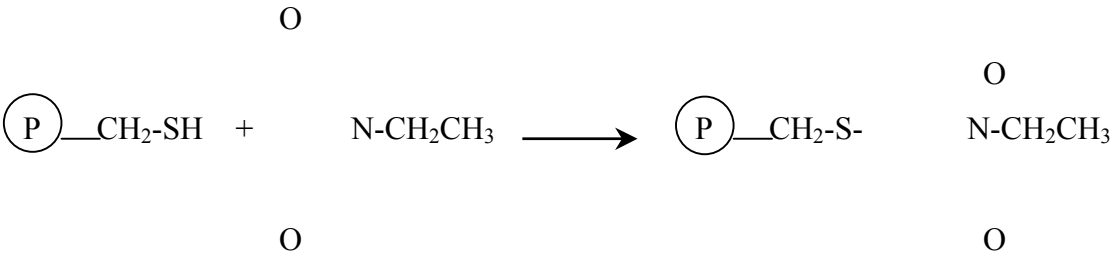
(Means and Feeney, 1971 ; Lundblad, 1991)



Reaction 4 Modification of EDC with carboxyl residue in protein (P)

(Means and Feeney, 1971 ; Lundblad, 1991)

APPENDIX B : Modification reaction of group-specific reagents (continued)

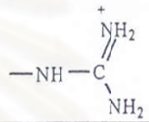
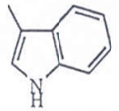


Reaction 5 Modification reaction of NEM with cysteine residue in protein (P)
(Means and Feeney, 1971 ; Lundblad, 1991)

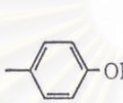
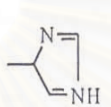
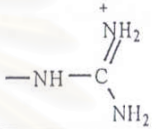
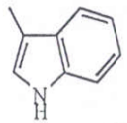
Reaction 6 Modification reaction of TNBS with lysine residue in protein (P)
(Means and Feeney, 1971 ; Lundblad, 1991)

Reaction 7 Modification reaction of PMSF with serine residue in protein (P)
(Means and Feeney, 1971 ; Lundblad, 1991)

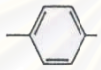
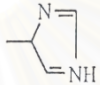
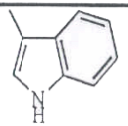
APPENDIX C : Reactivities of amino acid side chains (Means and Feeney, 1971)

Reagent	—NH_2	—SH				—COOH		—S—S—	—S—CH_3
Acetic anhydride	+++	+++[b]	+++[c]	+++[b]	-	-	-	-	-
N-acetylimidazole	±	+++[b]	+++[c]	+++[b]	-	-	-	-	-
acrylonitrile	±	+++	-	-	-	-	-	-	-
Aldehyde/ $NaBH_4$	+++	-	-	-	-	-	-	-	-
N-bromosuccinimide	-	+++	++	+	-	-	+++	-	-
N-carboxyanhydrides	+++	-	-	-	-	-	-	-	-
Cyanate	+++	+++[b]	++[b]	+[b]	-	+[b]	-	-	-
Cyanogen bromide	-	+	-	-	-	-	-	-	+++
1,2-cyclohexanedione	±	-	-	-	+++	-	-	-	-
Diacetyl trimer	+	-	-	-	+++	-	-	-	-
Diazoacetates	-	++	-	-	-	+++	-	-	-
Diazonium salts	+++	+	+++	+++	+	-	+	-	-
Diethylpyrocarbonate	+++	-	-	+++[c]	-	-	-	-	-
Diketone	+++[c]	-	+	-	-	-	-	-	-
Dinitrofluorobenzene	+++	+++	++	++	-	-	-	-	-
5,5'-dithiobis (2-nitrobenzoic acid)	-	+++[c]	-	-	-	-	-	-	-
Ethyleneimine	-	+++	-	-	-	-	-	-	+

APPENDIX C : Reactivities of amino acid side chains (continued)

Reagent	—NH_2	—SH				—COOH		—S—S—	—S—CH_3
N-ethylmaleimide	±	+++	-	-	-	-	-	-	-
Ethyl thiotrifluoacetate	+++[b]	-	-	-	-	-	-	-	-
Formaldehyde	+++	+++	+++	+++	+	-	+	-	-
glyoxal	++	-	-	-	+++	-	-	-	-
Haloacetates	+	+++	-	+	-	-	-	-	+
Hydrogen peroxide	-	+++	-	-	-	-	+	+	+++
2-hydroxy-5-nitrobenzyl bromide	-	++	-	-	-	-	+++	-	-
Iodine	-	+++	+++	+++	-	-	-	-	-
0-iodosobenzoate	-	+++	-	-	-	-	-	-	-
Maleic anhydride	+++[c]	++[c]	++[b]	++[b]	-	-	-	-	-
p-mercuribenzoate	-	+++	-	-	-	-	-	-	-
Methanol/ HCl	-	-	-	-	-	+++	-	-	-
2-methoxy-5-nitrotropone	+++[c]	-	-	-	-	-	-	-	-
Methyl acetimidate	+++	-	-	-	-	-	-	-	-
O-methylisourea	+++	-	-	-	-	-	-	-	-
Nitrous acid	+++	+++	±	-	-	-	-	+	-
Performic acid	-	+++	-	-	-	-	++	+++	+++

APPENDIX C : Reactivities of amino acid side chains (continued)

Reagent	—NH_2	—SH				—COOH		—S—S—	—S—CH_3
Phenylglyoxal	++	-	-	-	+++	-	-	-	-
Photooxidation	-	+++	++	+++	-	-	+++	±	+++
Sodium borohydride	-	+++[b]	++[b]	++[b]	-	-	-	-	-
Succinic anhydride	+++	+++	-	-	-	-	+++	-	-
Sulfite	-	+++	+++	+++	-	-	-	-	-
Sulfonyl halides	+++	+++	+++	-	-	-	+	-	+
Tetranitromethane	-	+++	+++	-	-	-	+	-	+
Tetrathionate	-	+++	-	-	-	-	-	-	-
Thiols	-	-	-	-	-	-	-	+++	-
Trinitrobenzenesulfonic acid	+++	++[b]	-	-	-	-	-	-	-
Water-soluble carbodiimide and nucleophile	±	±	±	-	-	+++	-	-	-

[a] -, +, ++, and +++ indicate relative reactivities; ±, ++, and +++ likewise indicate relative reactivities which may or may not be attained depending on the condition used.

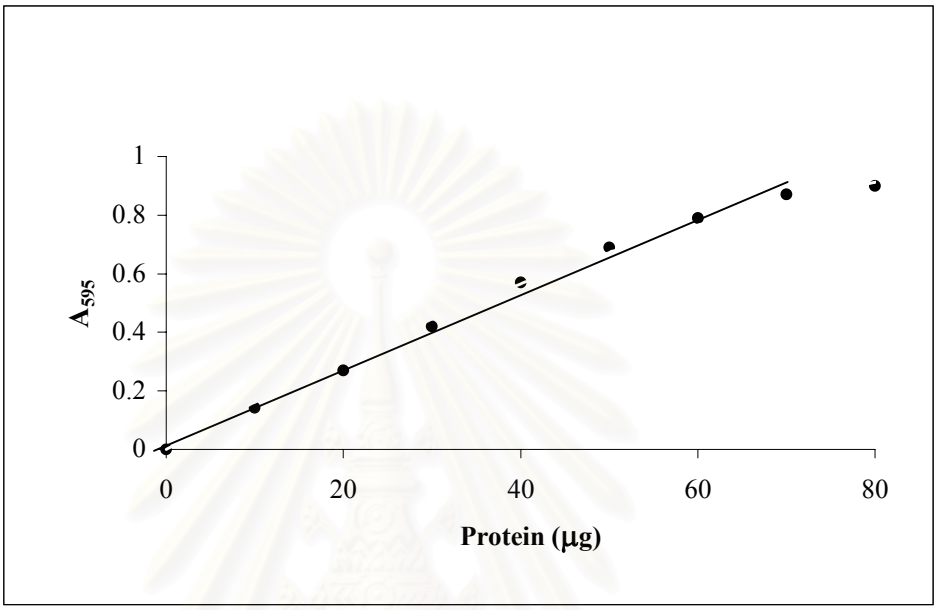
[b] Spontaneously reversible under the reaction conditions or upon dilution, regenerating original group.

[c] Easily reversible, regenerating original group.

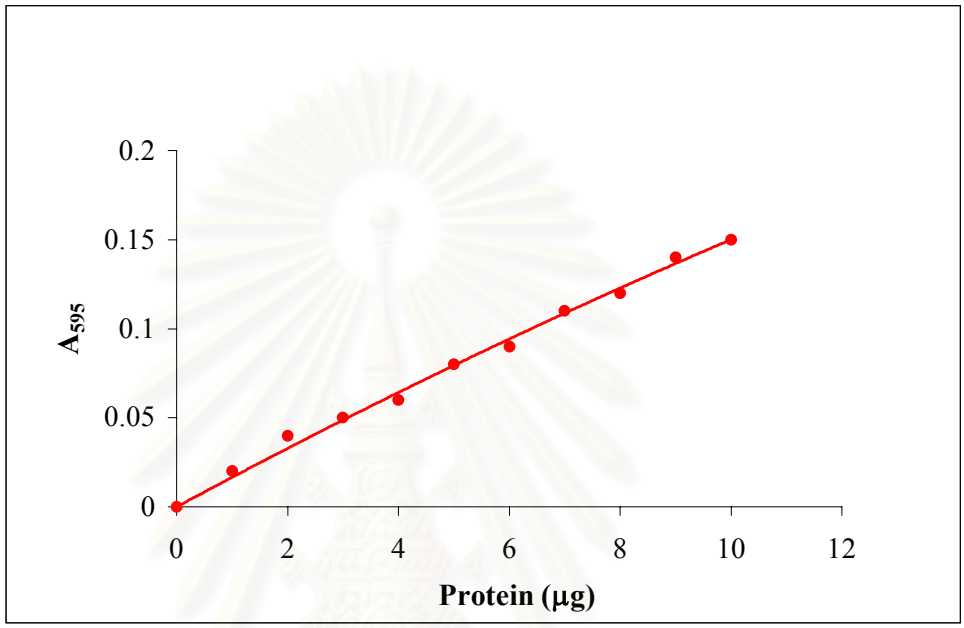
APPENDIX D : Abbreviation for amino acid residues (Voet, 1990)

Amino acid	3 Letter-Abbreviation	1 Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspatic acid	Asp	D
Cystein	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown	-	X

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



APPENDIX F : Standard curve for microprotein determination by Bradford's method



BIOGRAPHY

Miss Jarunee Kaulpiboon born on February 3, 1977. She graduated with the Bachelor Degree of Science in Biochemistry from Chulalongkorn University in 1998 and continued studying for Master in Biochemistry Program.