ลักษณะสมบัติและการโคลนยีนของเอนไซม์ไซโคลเดกซ์ทริเนส จาก *Paenibacillus* sp. A11

นางสาวจารุณี ควรพิบูลย์

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CHARACTERIZATION AND GENE CLONING OF CYCLODEXTRINASE FROM *Paenibacillus* sp. A11

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จารุณี ควรพิบูลย์ : ลักษณะสมบัติและการโคลนยีนของเอนไซม์ไซโคลเดกซ์ทริเนส จาก *Paenibacillus* sp. A11 (CHARACTERIZATION AND GENE CLONING OF CYCLODEXTRINASE FROM *Paenibacillus* sp. A11) อ. ที่ปรึกษา : รศ. ดร. เปี่ยมสุข พงษ์สวัสดิ์, อ.ที่ปรึกษาร่วม : ผศ. ดร. วิเชียร ริมพณิชยกิจ, 251 หน้า. ISBN 974-17-4003-4

การจัดจำแนกสายพันธุ์แบคทีเรียโดยการวิเคราะห์ลำดับยีน 16S rRNA ร่วมกับการวิเคราะห์องค์ประกอบของเบสของ DNA และกรดไขมันของเซลล์ ได้ผลว่า ควรจัด *Bacillus circulans* A11 ให้เป็นจีนัสใหม่ คือ *Paenibacillus* sp. A11 ระดับความคล้ายคลึงของยีน 16S rRNA ระหว่างสายพันธุ์ A11 กับ *Paenibacillus* species อยู่ในช่วง 90-99 เปอร์เซ็นต์ ในขณะที่ระดับความคล้ายคลึงกับ *Bacillus circulans* มีค่าเพียง 86 เปอร์เซ็นต์ กรดไขมันหลักของเซลล์ คือ anteiso-$C_{15:0}$ (59.3 เปอร์เซ็นต์) จำนวนของนิวคลีโอไทด์เบสใน DNA คิดเป็นเปอร์เซ็นต์ของกวานีนกับไซโทซีนรวมกันได้เท่ากับ 50.3 ผลการศึกษาบนอาหารแข็งเฉพาะที่ประกอบด้วย β-CD และ phenolphthalein พิสูจน์ได้ว่าแบคทีเรียสายพันธุ์ A11 สามารถผลิตเอนไซม์ไซโคลเดกซ์ทริเนส (CDase) ได้ เมื่อนำเอนไซม์มาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตและแยกโดยโครมาโทกราฟีคอลัมน์ DEAE-Sephadex A-50 และคอลัมน์ Phenyl-Sepharose CL 4B พบว่า เอนไซม์บริสุทธิ์เพิ่มขึ้น 22 เท่า ให้ผลผลิต 28 เปอร์เซ็นต์ และมีแอคติวิตีจำเพาะ 133 ยูนิต/มิลลิกรัมโปรตีน เอนไซม์บริสุทธิ์แสดงแถบโปรตีน 1 แถบบน non-denaturing และ SDS-PAGE จากการศึกษาโดย gel filtration ร่วมกับ SDS-PAGE พบว่าเอนไซม์ประกอบด้วย 1 หน่วยย่อยที่มีน้ำหนักโมเลกุล 80 กิโลดาลตัน pH และ อุณหภูมิที่เหมาะสมในการทำงานของเอนไซม์ คือ 7.0 และ 40 องศาเซลเซียส ตามลำดับ จุดไอโซอิเล็กตริคของเอนไซม์เท่ากับ 5.4 การหาลำดับกรดอะมิโนที่ด้านปลายอะมิโน 15 ตัวแรก พบว่าเป็น M F L E A V Y H R P R K N W S เมื่อเปรียบเทียบความสามารถในการเร่งปฏิกิริยาของ CDase ต่อสับสเตรทชนิดต่างๆ พบว่า เอนไซม์มีความจำเพาะสูงมากต่อ β-CD โดยเอนไซม์มีความจำเพาะต่อหน่วยกลูโคส α-1,4 และอัตราการย่อยสับสเตรทขึ้นกับขนาดของโอลิโกแซคคาไรด์ ผลิตภัณฑ์หลักที่ได้จากสับสเตรทวงปิดหรือสายตรง คือ มอลโตส ค่า k_{cat}/K_m สำหรับ α-, β- และ γ-CD เท่ากับ 1.41×10^5, 8.28×10^5 และ 3.73×10^5 โมลาร์$^{-1}$นาที$^{-1}$ ตามลำดับ การตรวจหากรดอะมิโนจำเป็นของเอนไซม์ด้วยวิธีการดัดแปลงทางเคมี พบว่า เมื่อดัดแปลง ทริปโตเฟน และ ฮิสติดีน ด้วย *N*-bromosuccinimide และ diethylpyrocarbonate ตามลำดับ ที่ความเข้มข้น 1 มิลลิโมลาร์ เป็นเวลา 30 นาที มีผลให้เอนไซม์สูญเสียแอคติวิตีทั้งหมด สำหรับทริปโตเฟน พบว่าเป็นกรดอะมิโนที่สำคัญในบริเวณเร่งของเอนไซม์โดยการศึกษาโดยเทคนิคการป้องกันเอนไซม์ด้วยสับสเตรท จากการโคลนยีน CDase เข้าสู่ *E. coli* พบว่า open reading frame ของยีน CDase มีขนาด 1,959 คู่เบส แปลรหัสเป็นกรดอะมิโนได้ 653 ตัว การแสดงออกของ CDase ใน *E. coli* (pJK 555) โดยใช้ pUC18 เวคเตอร์ ถูกเหนี่ยวนำด้วยการเลี้ยงโคลนเป็นเวลา 24 ชั่วโมง และพบว่าอาหารที่มีซอร์บิทอล ความเข้มข้น 0.5 โมลาร์ มีผลเพิ่มการแสดงออกของ CDase ประมาณ 3 เท่าควบคู่ไปกับการเจริญของเซลล์ที่เพิ่มขึ้น หลังจากนั้นได้ทำเอนไซม์จากโคลน (pJK 555) ให้บริสุทธิ์ พบว่า เอนไซม์ CDase จาก *E. coli* (pJK 555) มีคุณสมบัติทางชีวเคมีไม่ต่างจากเอนไซม์จากสายพันธ์ตั้งต้น *Paenibacillus* sp. A11

สาขาวิชา…….วิทยาศาสตร์ชีวภาพ……....ลายมือชื่อนิสิต…..……

ปีการศึกษา……...2546……....ลายมือชื่ออาจารย์ที่ปรึกษา……..……

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……...……..……

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KEY WORD : CYCLODEXTRINASE / CYCLODEXTRIN METABOLISM / *Paenibacillus* sp.

JARUNEE KAULPIBOON : CHARACTERIZATION AND GENE CLONING OF CYCLODEXTRINASE FROM *Paenibacillus* sp. A11. THESIS ADVISOR : ASSOC. PROF. PIAMSOOK PONGSAWASDI, Ph.D., THESIS CO-ADVISOR : ASSIST. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., 251 pp. ISBN 974-17-4003-4

Bacillus circulans A11 isolated from South-East Asian soil was reclassified as *Paenibacillus* sp. A11 using 16S rRNA gene sequence, G+C content and cellular fatty acid composition analyses. Levels of similarity of 16S rRNA gene between strain A11 and the *Paenibacillus* species were 90-99%, while similarity with *Bacillus circulans* was only 86%. The major cellular fatty acid was anteiso-$C_{15:0}$ which accounted for 59.3% of total cellular fatty acids and the G+C content was 50.3 mol%. This bacterium was proved to possess cyclodextrinase (CDase) activity which could be detected on a specific screening medium containing β-CD and phenolphthalein. The CDase was purified approximately 22 folds with 28% recovery to a specific activity of 133 units/mg protein by 40-60% ammonium sulfate precipitation, DEAE Sephadex A-50, and Phenyl Sepharose CL-4B chromatography. It was proved to be homogeneous by non-denaturing and SDS-PAGE. The enzyme was a single polypeptide with a molecular weight of 80 kDa as determined by gel filtration and SDS-PAGE. The optimum pH and temperature for activity of the purified enzyme were pH 7.0 and 40^{o}C. The enzyme had isoelectric point of 5.4. N-Terminal sequence was M F L E A V Y H R P R K N W S. When relative hydrolytic activity of the CDase on different substrates were compared, it was found that high specificity was exerted by β-CD. The enzyme recognizes α-1,4-glucose units and the hydrolysis depends on the size of oligosaccharides. The major product from α-1,4-glucan substrates, either cyclic or linear, was maltose. The k_{cat}/K_m values for α-, β- and γ-CD were $1.41x10^5$, $8.28x10^5$ and $3.73x10^5$ $M^{-1}min^{-1}$. The enzyme activity was completely inactivated by 1 mM *N*-bromosuccinimide and diethylpyrocarbonate suggesting the crucial importance of Trp and His for its catalytic activity. Essential Trp was confirmed to be at enzyme active site by substrate protection experiment. In this study, the CDase gene coding for this enzyme was cloned into *E. coli*. The open reading frame of CDase gene was 1,959 bp encoding CDase of 653 amino acid residues. Expression of *Paenibacillus* sp. A11 CDase gene using pUC 18 vector in *Escherichia coli* JM 109 was induced by adding 0.5 M sorbitol as an osmolyte in the culturing medium. After 24 h induction, the formation of insoluble CDase was prevented while a three-fold increase in cytoplasmic CDase activity was achieved in parallel with increase in cell growth. The recombinant CDase protein was successfully purified and characterized. Biochemical properties of the CDases from *Paenibacillus* sp. A11 and *E. coli* transformant (pJK 555) were almost identical.

Field of study……Biological…Science…..Student's signature…..……..……..

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ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
CD	cyclodextrin
CDase	cyclodextrinase
CGTase	cyclodextrin glycosyltransferase
cm	centimeter
°C	degree Celsius
Da	dalton
DEAE	diethylaminoethyl
DEP	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
et al.	Et. Alii (latin), and others
Fig.	figure
g	gram
h	hour
IPTG	isopropylthiogalactoside
kb	kilobase
k_{cat}	catalytic constant
k_{cat}/ K_m	specific constant
K_m	Michaelis constant
l	litre

μg	microgram
μl	microlitre
M	molar
mA	milliampere
ml	millilitre
mM	millimolar
min	minute
mol	mole
MW	molecular weight
NBS	*N*-Bromosuccinimide
nm	nanometer
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PG	phenylglyoxal
pI	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography
V	volt
V_{max}	maximal velocity
V/V	volume by volume
W/V	weight by volume

CHAPTER I

INTRODUCTION

Starch metabolic pathway

Some microorganisms can utilize starch as a sole carbon and energy source. Mode of starch utilization has been proposed via two metabolic pathways. The first pathway involves extracellular degradation of starch into linear maltodextrins by hydrolysis of the α-1,6-glycosidic bonds through the action of pullulanase (EC 3.2.1.41) or/and isoamylase (EC 3.2.1.68) and subsequent cleavage of the α-1,4-glycosidic linkages by α-, β-amylase (EC 3.2.1.1; 3.2.1.2) or/and disproportionation activity of the cyclodextrin glycosyltransferase (EC 2.4.1.19). Maltodextrins are transported and assimilated via a binding protein-dependent ABC transporter and intracellular hydrolytic enzymes. The second pathway, a novel starch degradation pathway, involves an extracellular cyclodextrin glycosyltransferase (CGTase: EC 2.4.1.19) which degrades starch into α-, β- and γ-cyclodextrins (CDs). Evidence has been presented that the cyclodextrins are transported into the cytoplasm via a specific system and that they are metabolized inside the cell by another enzyme called cyclodextrinase (CDase: EC 3.2.1.54). Cyclodextrins are hydrolyzed by cyclodextrinase to form linear malto-oligosaccharides which then enter the maltose degradation pathway (Figure 1 and 2) (Fiedler *et al*., 1996; Ohdan *et al*., 2000; Hashimoto *et al*., 2001).

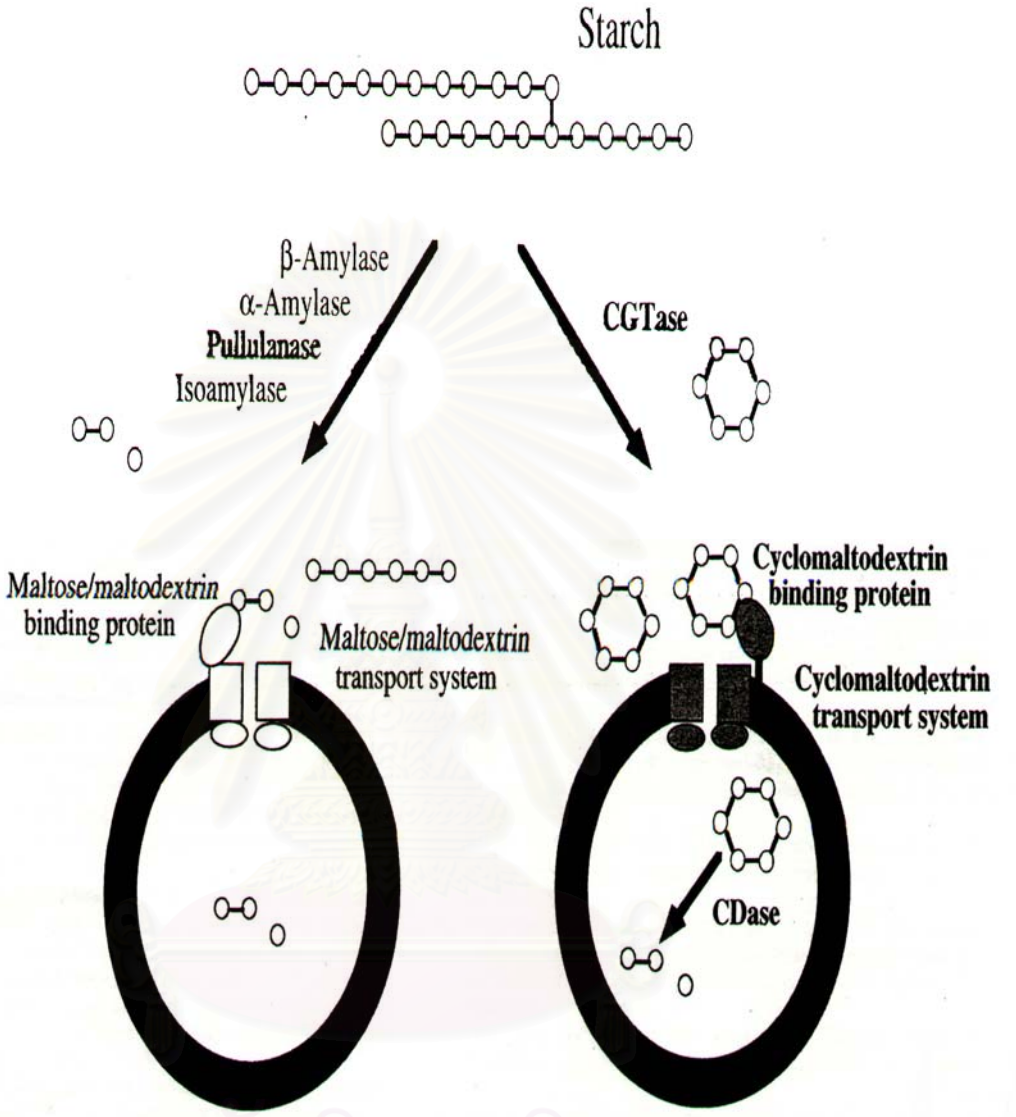

Figure 1 Proposed model for degradation of starch via the maltose-maltodextrin and the CD pathway of *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001)

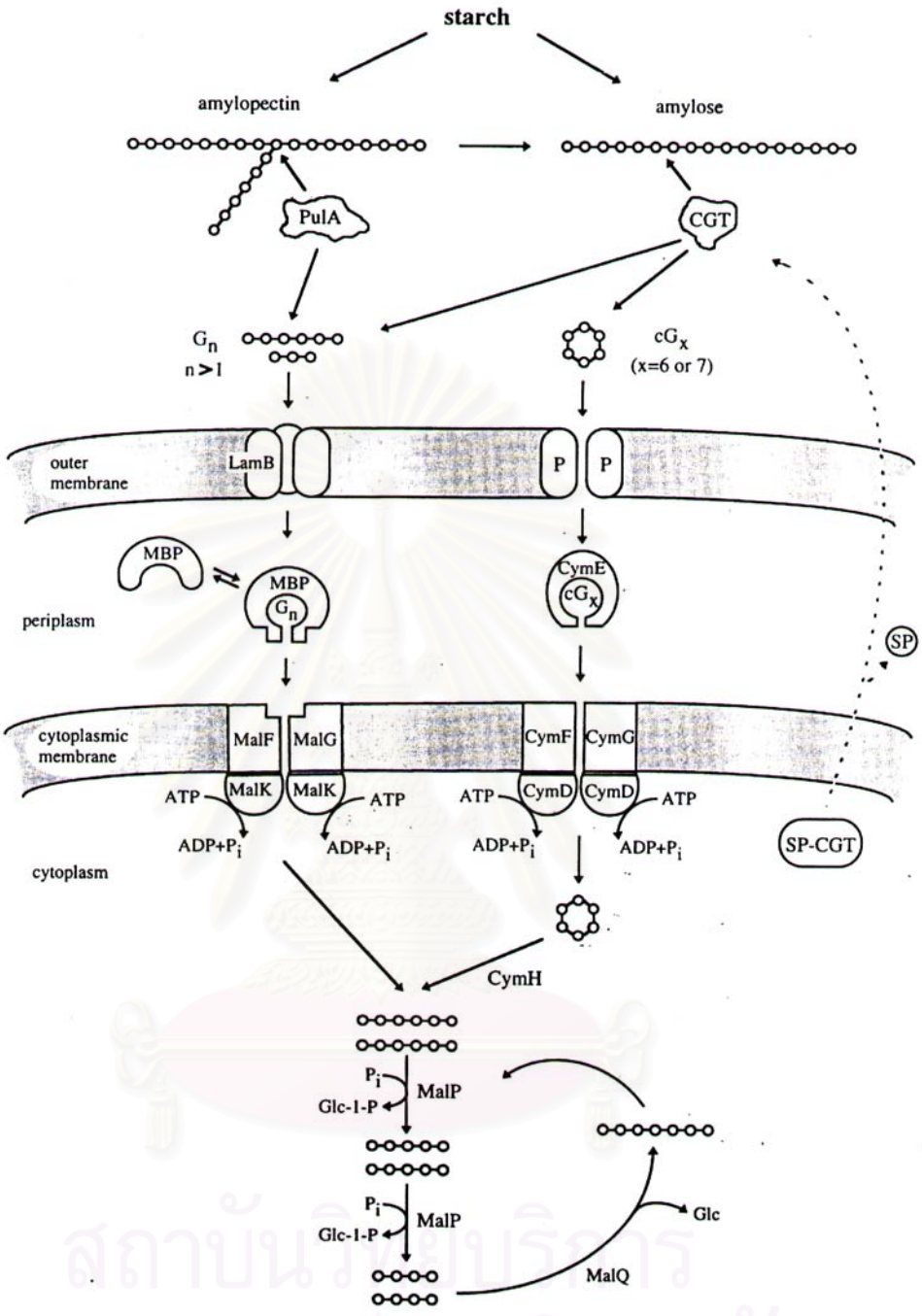

Figure 2 Proposed model for degradation of starch via the maltose-maltodextrin and the CD pathway of *Klebsiella oxytoca* M5a1 (Fiedler *et al*., 1996)

PulA = Pullulanase A
MBP = Maltose-binding protein
SP = Signal peptide
CymE = CD-binding protein
CGT = Cyclodextrin glycosyltransferase
CymD, F, G = Transport protein for CD
MalF,G,K = Transport protein for maltose
CymH = CDase

The unique starch metabolic pathway via CDs (CDs metabolism) may be more advantageous for microorganisms growing in environment because synthesis of CDs allows effective and competitive exploitation of starch utilization as a carbon source. CDs are not hydrolyzed by exo-type amylases such as glucoamylases and β-amylases because CDs have non-reducing ends. The raw starch-binding domain of exo-type amylases exhibits binding affinity not only to raw starch but also to α-, β-, and γ-CDs, although the enzyme cannot hydrolyze CDs. Thus, the activity of the amylolytic enzymes such as glucoamylase will be competitively inhibited by binding of CDs to the raw starch binding site of the enzyme (Fukuda *et al*., 1992; Goto *et al*., 1994). In addition, cyclodextrin binding protein (CBP) binds not only to CDs but also to linear maltodextrins. The first pathway is normally found in amylolytic microorganisms but the second pathway, CDs metabolism, is specially found in some microorganisms. The gene cluster encoding extracellular CGTase, the CBP-dependent ABC transport system for CD, and intracellular CDase would provide benefits to the cell in the exploitation of carbon sources from environment where starch substrates are in CD forms (Hashimoto *et al*., 2001).

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). Their major forms 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; Peninga, 1996; Bart *et al*., 2000) (Figure 3).

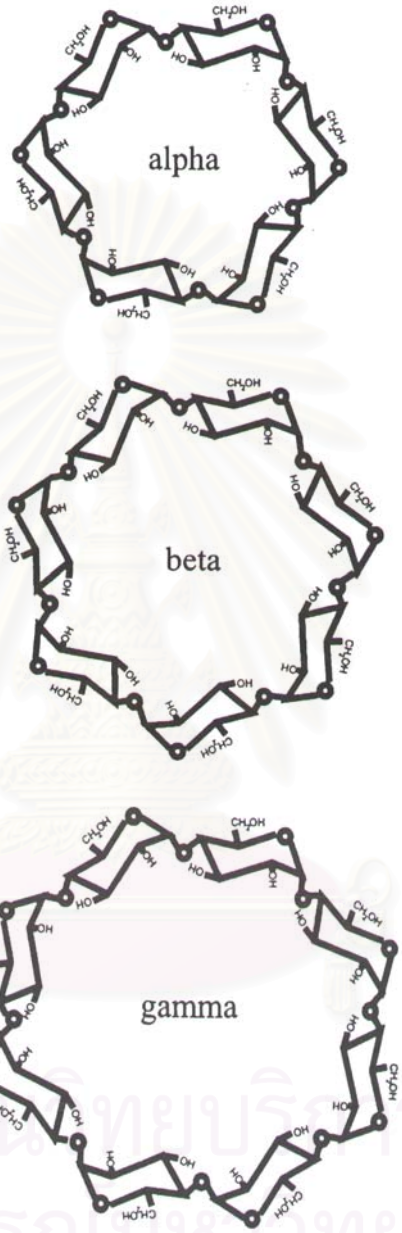

Figure 3 Chemical structure of cyclodextrins (Bart *et al*., 2000)

α-, β-, and γ-cyclodextrins

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, 1980, 1984; Bender, 1986) as shown in Figure 4A. Some physical properties of CDs are summarized in Table 1 (Szejtli, 1982; Uekama and Irie, 1987). Among the CD's, γ-CD has the biggest cavity and is 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 systems (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 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

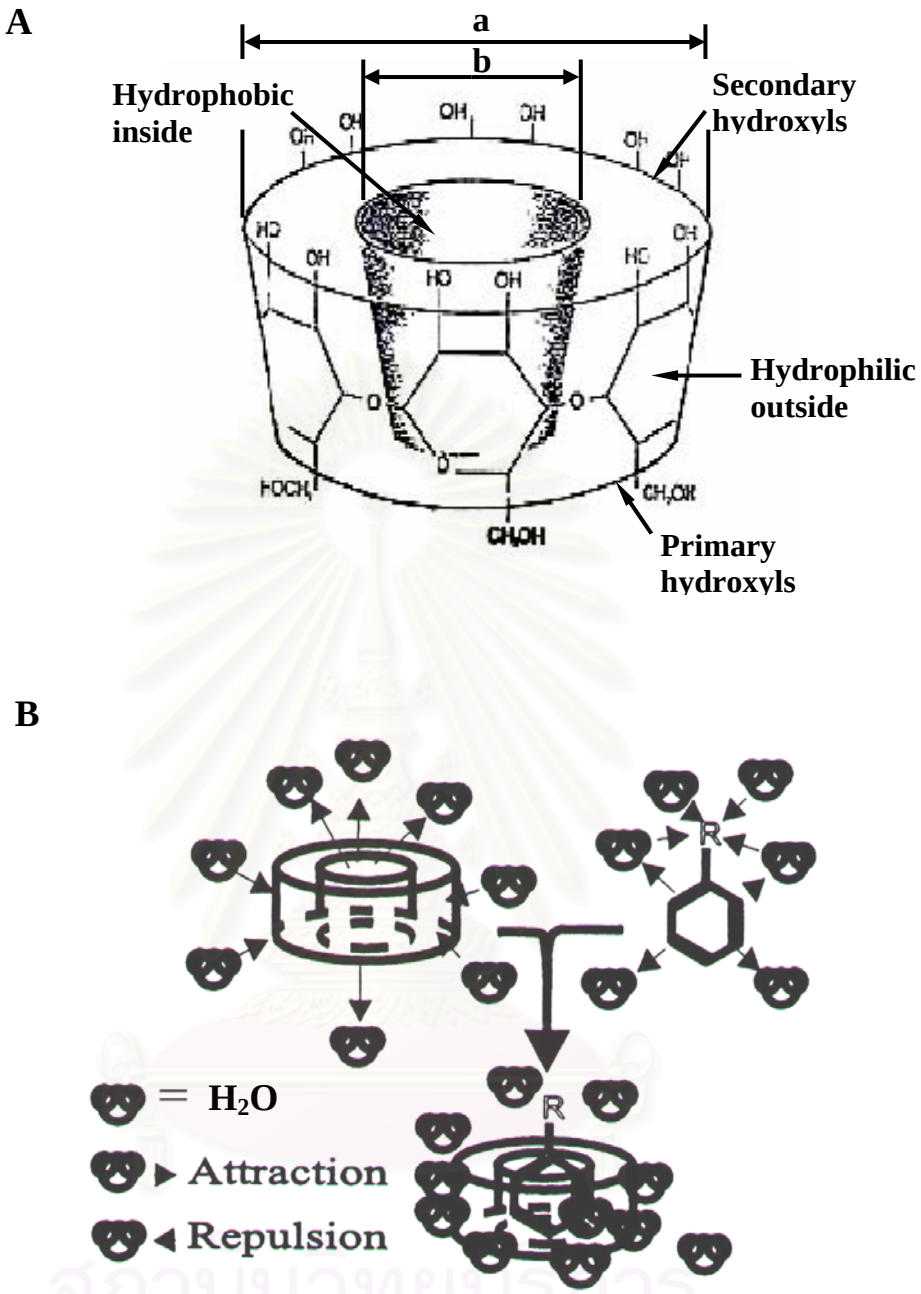

Figure 4 Structure and properties of cyclodextrin (Saenger, 1980, 1984; Bender, 1986)

The molecular dimension structure and properties of cyclodextrins (for sizes of a and b, see Table 1) (A); Formation of inclusion complex of a cyclodextrin with a hydrophobic guest molecule (B)

Table 1 Properties of cyclodextrins (Szejtli, 1982; Uekama and Irie, 1987)

Properties	α-CD	β-CD	γ-CD
Number of glucose unit	6	7	8
Molecular weight (g/mol)	972	1135	1297
Outer diameter (a) (A°)	14.6	15.4	17.5
Inner diameter (b) (A°)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (A°)	7.9	7.9	7.9
Crystal form (water)	Needle	Prism	Prism
Solubility in water at 25 ^{o}C (% w/v)	14.5	1.85	23.2

Table 2. Classification of cyclodextrin derivatives (Ensuiko, 1994)

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

ease of assembly and disassembly. Potential guests which can be encapsulated in cyclodextrins, a typical pattern of complexation is shown in Figure 4B (Bart *et al.*, 2000).

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). Cyclodextrins are hence increasingly used for various industrial and research applications (Table 3).

Cyclodextrin producing enzyme

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, disproportionation, and hydrolysis reactions as shown below:

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

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

Table 3. Industrial applications of cyclodextrins

(Horikoshi, 1982; Bender, 1986; Schmid, 1989; 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

$$G_n \xrightarrow{\text{hydrolysis}} G_{(n-x)} + G_x$$

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 *et al*., 1990). These mechanisms are shown in Figure 5.

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 reaction cleaves an α-glucosidic bond in a cyclodextrin ring and transfers the linear malto-oligosaccharide to an acceptor substrate, resulting in a longer linear oligosaccharide. Disproportionation reaction transfers one part of a donor oligosaccharide substrate to an acceptor oligosaccharide. Hydrolysis reaction hydrolyzes α-1,4-glycosidic bonds of the glucan or oligosaccharide substrate. For most CGTases, hydrolysis activity is relatively low when compared to other glycohydrolases.

CGTase is produced by various microorganisms, for example *Klebsiella pneumoniae* M5al (Bender, 1977), *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-CGTase, 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 *Bacillus macerans* produced α-, β-,

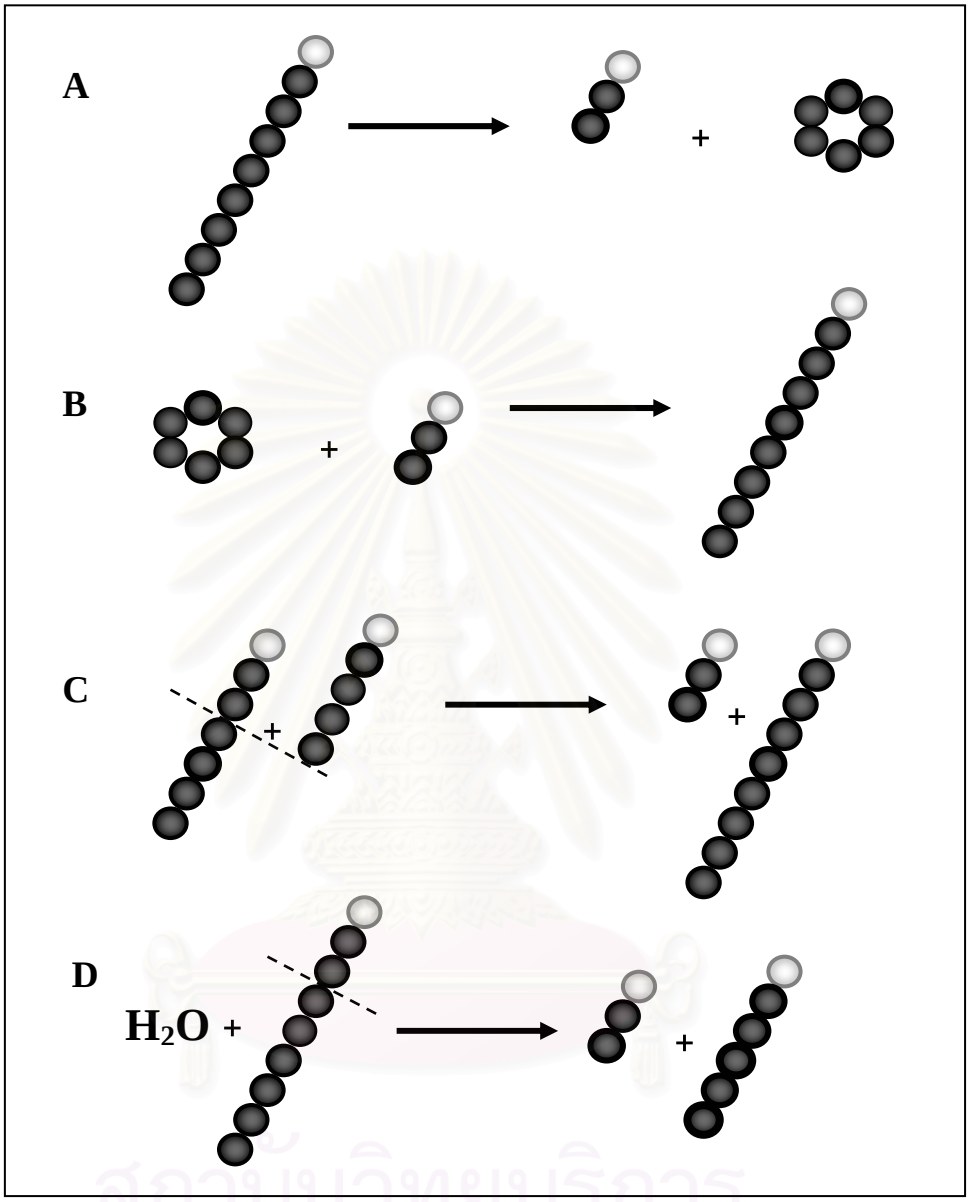

Figure 5 Schematic representation of the CGTase-catalyzed reaction (Bart *et al.*, 2000)

The circles represent glucose residues; the white circles indicate the reducing end sugars. Cyclization (A), coupling (B), disproportionation (C) and hydrolysis (D).

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	Stavn and Granum, 1979
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 M5al	α-CD	6.0-7.2	ND	68,000	4.8	Bender, 1977
Micrococcus sp.	β-CD	6.2	ND	139,000	ND	Yagi *et al.*, 1986
Bacillus circulans A11						Kaskangam, 1998
Isoform 1	β-CD	6.0-7.0	40	72,000	4.73	
Isoform 2	β-CD	6.0-7.0	40	72,000	4.49	
Isoform 3	β-CD	6.0	50	72,000	4.40	
Isoform 4	β-CD	7.0	50-60	72,000	4.31	

ND = Not Determined

and γ-CD in relative amount of 2.7: 1.0: 1.0 (Stavn and Granum, 1979), while the CGTase of alkalophilic *Bacillus* no. 38-2 and *Bacillus circulans* produced CDs in relative amount of 1.0: 11.0: 1.5 (Matzuzawa *et al.*, 1975) and 1.0: 4.1: 1.1 (Rojtinnakorn *et al*., 2001), respectively. The CGTase of *Bacillus firmus* 290-3 was known to produce γ-CD in the initial phase of enzyme production (Englbrecht *et al.*, 1990).

Several CGTases were purified and characterized (Nakamura and Horikoshi, 1976; Kitahata *et al.*, 1974; Matzuzawa *et al.*, 1975; Stavn and Granum, 1979; Kobayashi *et al.*, 1978; Laszlo *et al*., 1981; Jeang *et al.*, 1992; Kim, 1996; Chung *et al*., 1998; Spiridonova, 1998). The three-dimensional structure of CGTases were reported for *Bacillus circulans* 251, *Thermoanaerobacterium thermosulfurigenes* EM1, and alkalophilic *Bacillus* sp. 1011 (Lawson *et al*., 1994; Knegtel *et al*., 1996; Harata *et al*., 1996). Numerous works on gene cloning and overexpression of CGTase gene have been reported. For example, the CGTase gene from *Klebsiella pneumoniae* and alkalophilic *Bacillus* sp. 1-1 was each placed under *lac* promotor and was cloned into mutant *Escherichia coli*. The expression of the gene was higher than wild type, and when the gene was cloned into *Bacillus subtilis*, the activity was even higher than in *Escherichia coli* (Bender, 1986; Schmid, 1989). The CGTase from alkalophilic *Bacillus* sp. no. 38-2 was cloned into *Escherichia coli* and *Bacillus subtilis* and it was found that *Escherichia coli* mutant gave higher activity than *Bacillus subtilis* mutant (Kaneko *et al*., 1988). The CGTase gene from *Bacillus circulans* ATCC 21783 was inserted into pUB 110 and was cloned into *Bacillus subtilis.* The expression of the gene was very low. The gene was then placed under the control of α-amylase promotor, which resulted in 100 fold increase in activity (Paloheimo *et al*., 1992). Rimphanitchayakit *et al.* (2000) reported cloning and sequencing of a 7.9 kb

chromosomal DNA fragment containing the 2.1 kb β-CGTase gene from *Bacillus circulans* A11 (GenBank accession no. AF302787). Two reading frames encoding for ABC type transporters (Fieldler *et al*., 1996) were also found in the 7.9 kb CGTase-containing DNA fragment. These two proteins may be involved in the CD uptake in strain A11.

Cyclodextrin degrading enzyme

More attention was given to CGTase studies on account of its CD-forming activity. Until recently, interest in CDase has been intensified for the better understanding of CD metabolism. Mode of CD metabolism has been proposed in *Klebsiella oxytoca* 5a1 (Fiedler *et al*., 1996), alkalophilic *Bacillus* sp. A2-5a (Ohdan *et al*., 2000) and *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001). When the organisms are grown on starch as sole carbon source, they produce extracellular CGTase which degrades starch into CDs. Evidence has been presented that the CDs are transported into the cytoplasm via a specific system and that they are hydrolyzed inside the cell by CDase to form linear malto-oligosaccharides to be used as energy source.

The increasing use of CDs in the food and drug industries makes knowledge of the enzymatic degradation of these compounds essential. Cyclodextrinase (CDase: EC 3.2.1.54) is a member of the α-amylase family of glycosyl hydrolases (family 13), which hydrolyses cyclodextrins much faster than polysaccharides such as starch and amylopectin (Depinto and Campbell, 1968), and thus can be clearly distinguished from α-amylase (Ohnishi, 1971). Furthermore, CDase does not synthesize any cyclodextrins from starch, which makes it significantly different from cyclodextrin glycosyltransferase

(EC 2.4.1.19, CGTase), which synthesizes cyclodextrin from starch. The CDase-catalyzed reaction is shown in Figure 6.

Purification methods and some characterizations for CDase

A cyclodextrinase was first separated from *Bacillus macerans* (DePinto and Campbell, 1968), and so far not more than ten CDase enzymes have been reported. They were from *Bacillus coagulans* (Kitahata *et al*., 1983), *Clostridium thermohydrosulfuricum* 39E (Saha and Zeikus, 1990), alkalophilic *Bacillus* sp. (Yoshida *et al*., 1991), *Bacillus sphaericus* (Oguma *et al*., 1990; Glavin *et al*., 1994), *Klebsiella oxytoca* (Feederle *et al*., 1995), *Bacillus stearothermophilus* HY-1 (Yang *et al*., 1996) and *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001). Typical methods reported for CDase purification include crude extraction by Triton® X-100 or sonication, ammonium sulfate precipitation in the range of 40-60%, ion exchange chromatography (mostly by DEAE ion exchange column), hydrophobic chromatography (mostly by Phenyl-Sepharose CL-4B column) and gel filtration chromatography (Kitahata *et al*., 1983; Yoshida *et al*., 1991; Feederle *et al*., 1995; Kim *et al*., 1998). Characterization of each CDase was performed and summarized (Table 5). CDases from three thermophilic bacteria, *Clostridium thermohydrosulfuricum* 39E, *Bacillus stearothermophilus* HY-1, and *Thermococcus* sp. B1001, have been studied. Their optimum temperatures were 55-95^{o}C. Most CDases work best at neutral pH. The enzymes from most organisms are monomeric with a molecular weight of 61-69 kDa. *Thermococcus* sp. B1001 and *Bacillus sphaericus* ATCC 7055 CDases are of higher molecular weight, being 79 and 91-95 kDa, respectively. Few CDases are dimer, the enzymes from *Bacillus sphaericus*

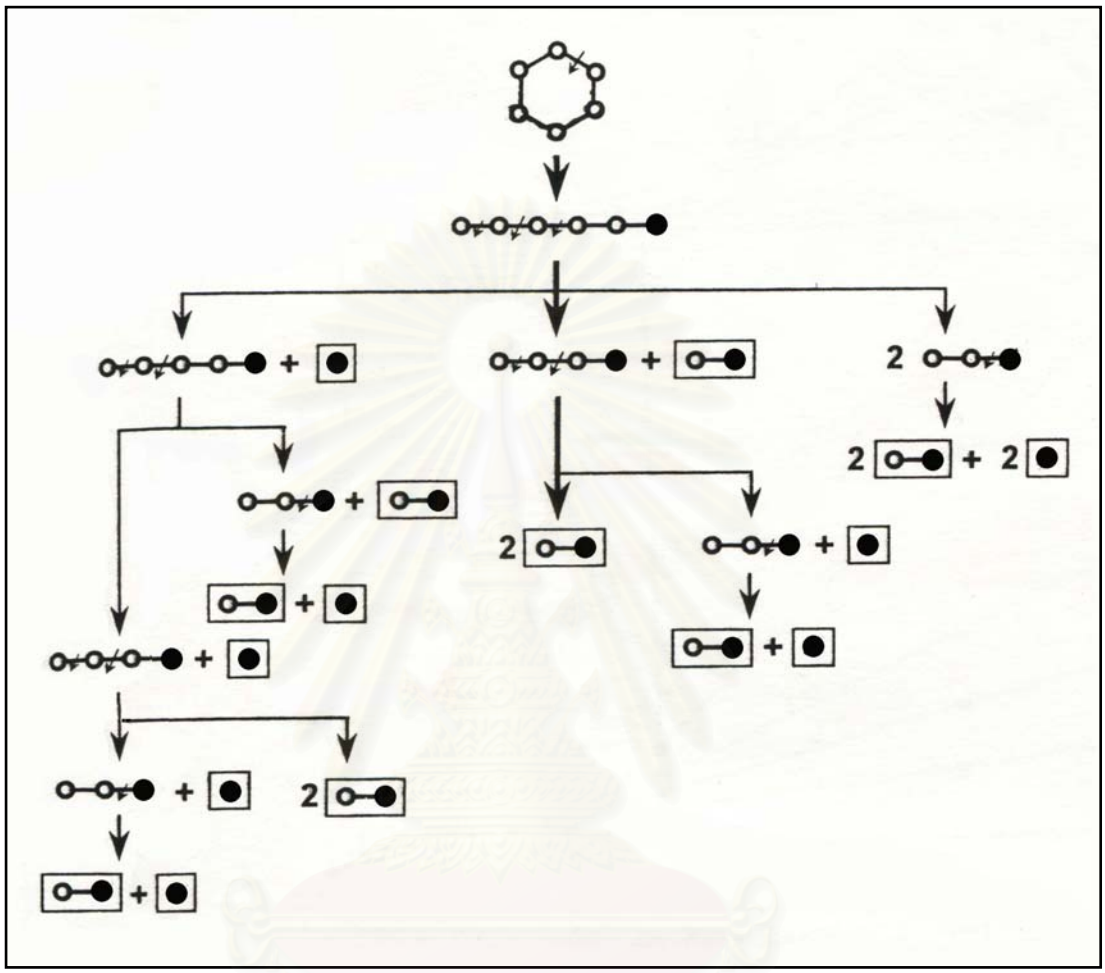

Figure 6 Schematic representation of the I-5 CDase-catalyzed reaction (Kim *et al*., 2000)

The circles represent glucose residues; the black circles indicate the reducing end sugar. The final product of I-5 CDase, glucose and maltose, was shown in the rectangle.

Table 5 Properties of Cyclodextrinase

Organism	Dominant Product	Optimum pH	Optimum Temperature (°C)	MW (dalton)	pI	References
Bacillus macerans	ND	6.2-6.4	30-40	67,000	ND	DePinto and Campbell, 1968
Bacillus coagulans	G_2	6.2	50	62,000	5.0	Kitahata *et al.*, 1983
Clostridium thermohydrosulfuricum 39E	G_2	6.5	65	68,000	ND	Saha and Zeikus, 1990
alkalophilic *Bacillus* sp. No.199	G_2	6.0	50	126,000	4.2	Yoshida *et al.*, 1991
alkalophilic *Bacillus* sp. I-5	G_2	ND	40-50	63,000	ND	Kim *et al.*, 1998
Bacillus sphaericus E-244	G_2&G_1	8.0	40	144,000	5.8	Oguma *et al.*, 1990
Bacillus sphaericus ATCC 7055	G_2&G_1	6.0-6.5	40	91,200-95,000	5.3	Galvin, 1994
Bacillus stearothermophilus HY-1	G_2	6.2	55	61,000	5.0	Yang *et al.*, 1996
Klebsiella oxytoca M5a1	G_2&G_3	7.0	23	69,000	ND	Feederle *et al.*, 1995
Thermococcus sp. B1001	G_2&G_1	5.5	95	79,000	ND	Hashimoto *et al.*, 2001

ND = Not Determined

E-244 and alkalophilic *Bacillus* sp. No. 199 consisted of two subunits, with the molecular weights of 144,000 and 126,000 Da, respectively. Most CDases produce maltose or maltose plus glucose as dominant end products. Only CDase from *Klebsiella oxytoca* M5a1 produces maltose and maltotriose as the dominant end products.

Substrate specificity and catalytic properties for CDase

Generally, CDase has high activity on CDs, maltooligosaccharides, soluble starch and pullulan, respectively, which makes it different from other enzymes in the α-amylase family (glycoside hydrolase family 13). CDases catalyze cleavage of α-1,4 glycosidic linkages, resulting in a series of various oligosaccharides (Park *et al*., 2000). Podkovyrov *et al.* (1993) demonstrated that single mutants at the catalytic Asp 325, Glu 354 and Asp 421 in CDase of *Thermoanaerobacter ethanolicus* 39E (old name as *Clostridium thermohydrosulfuricum* 39E) were completely inactive, comfirming the important role of these residues in catalysis. When *Bacillus* sp. I-5 CDase was mutated by changing Val 380 to Thr and Ile 388 to Glu (the two residues are localized between the third and fourth conserved region), the decrease in CD-degrading activity and the increase in hydrolysis of starch were observed (Park *et al.*, 2000).

Cyclodextrinase gene

From molecular cloning and sequencing analyses of CDase genes, only seven CDase genes have been reported. Nucleotide and amino acid sequences of CDases from *Clostridium thermohydrosulfuricum* 39E (Podkovyrov and Zeikus, 1992),

Bacillus sphaericus E-244 (Oguma *et al.*, 1993), *Klebsiella oxytoca* M5a1 (Fiedler *et al.*, 1996), alkalophilic *Bacillus* sp. I-5 (Kim *et al.*, 1998), alkalophilic *Bacillus* sp. A2-5a (Ohdan *et al*, 2000), *Alicyclobacilius acidocaldarius* (Matzke *et al*., 2000) and *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001) are known. From those publications, there are only three publications which reported on the cluster of CDase gene (Feederle *et al.*, 1996; Ohdan *et al.*, 2000; Hashimoto *et al.*, 2001). A 14.3 kb DNA fragment from a gram-negative bacterium of *Klebsiella oxytoca* M5a1 (Fiedler *et al.*, 1996) has been cloned and sequence analyzed. It contains eleven genes organized in two divergently oriented clusters separated by a non-coding region of 419 bp (Figure 7). These genes are proved by product analysis to be CDase (*CymH*), CGTase (*Cgt*) and cyclodextrin binding protein genes (*CymD*, *CymE*, *CymF* and *CymG*). Sequence analysis of CDase and CGTase showed high homology with other CDases and CGTases reported. For cyclodextrin binding protein genes, they were shown to have high homology with moltose/maltodextrin binding proteins (ABC transport system), a group of protein responsible for transport of maltose/maltodextrin in *Escherichia coli*. In another gram-negative bacterium, the hyperthermophilic archaeon *Thermococcus* sp. B1001, Tachibana *et al.* (1999) firstly purified and characterized CGTase. Then, Hashimoto *et al.* (2001) found a cluster of five genes in the same orientation, including a gene homolog encoding CDase involved in the degradation of CDs (*cgtB*), the gene encoding CGTase (*cgtA*), a gene homolog for a CD-binding protein (CBP) (*cgtC*), and a putative CBP-dependent ABC transporter involved in uptake of CDs (*cgtD* and *cgtE*) (Figure 8). In a gram-positive bacterium, the alkalophilic *Bacillus* sp. A2-5a (Ohdan *et al.*, 2000), it was reported that CDase, CGTase and putative cyclodextrin binding protein genes are close and aligned in the same direction in a DNA fragment of about 14.1 kb (Figure 9). Fiedler *et al.* (1996),

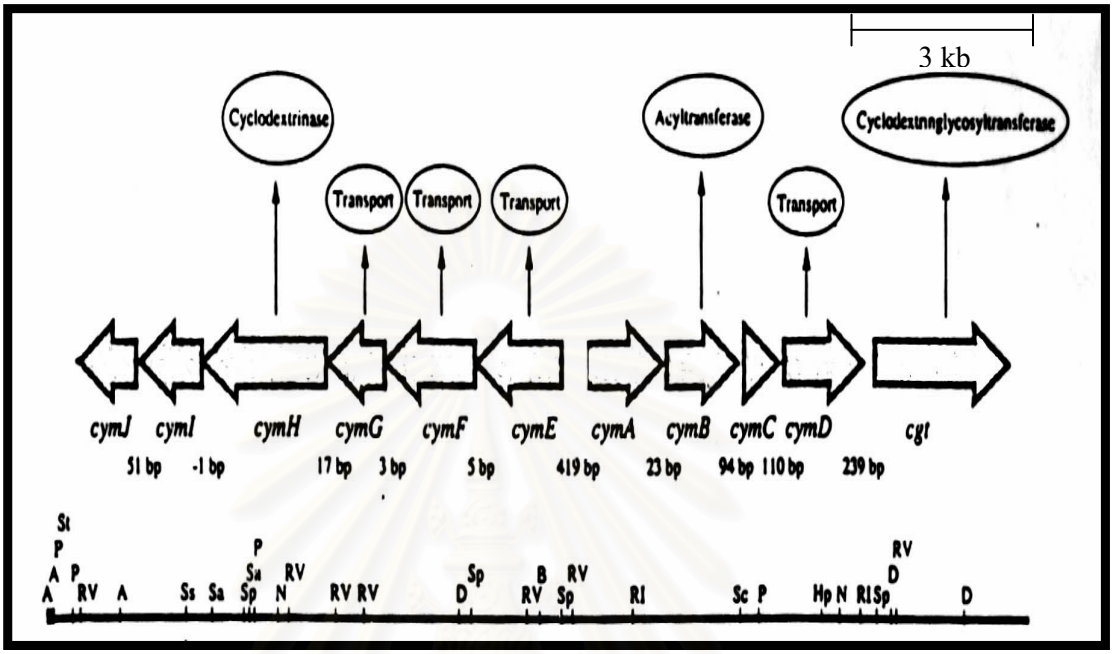

Figure 7 Physical map and gene organization of the CD metabolism in *Klebsiella oxytoca* M5a1 chromosome (Fiedler *et al.*, 1996)

The big arrows indicate size and orientation of each gene. The base-pair numbers written below the gene designations indicate the length of the respective intergenic regions. The black bar of the physical map denotes the sequenced region. Abbreviations for restriction sites: A, *Ava* I; B, *Bam* HI; D, *Dra* III; H, *Hin*d III; Hp, *Hpa* I; N, *Nhe* I; P, *Pst* I; RI, *Eco* RI; RV, *Eco* RV; Sa, *Sal* I; Sc, *Sca* I; Sp, *Sph* I; St, *Stu* I.

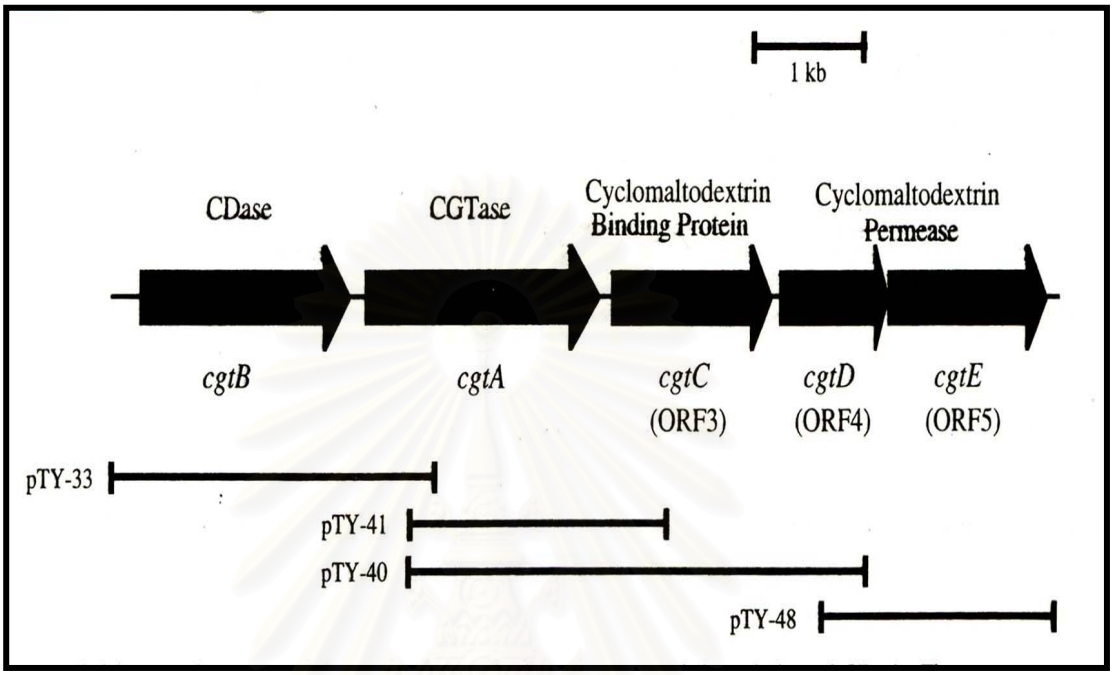

Figure 8 Structure of the gene cluster containing genes for synthesis, transport and degradation of CDs in *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001)

Arrows show the localization of each gene and the orientation of the coding sequences. pTY-33, pTY-40, pTY-41 and pTY-48 indicate the regions cloned by the respective plasmids.

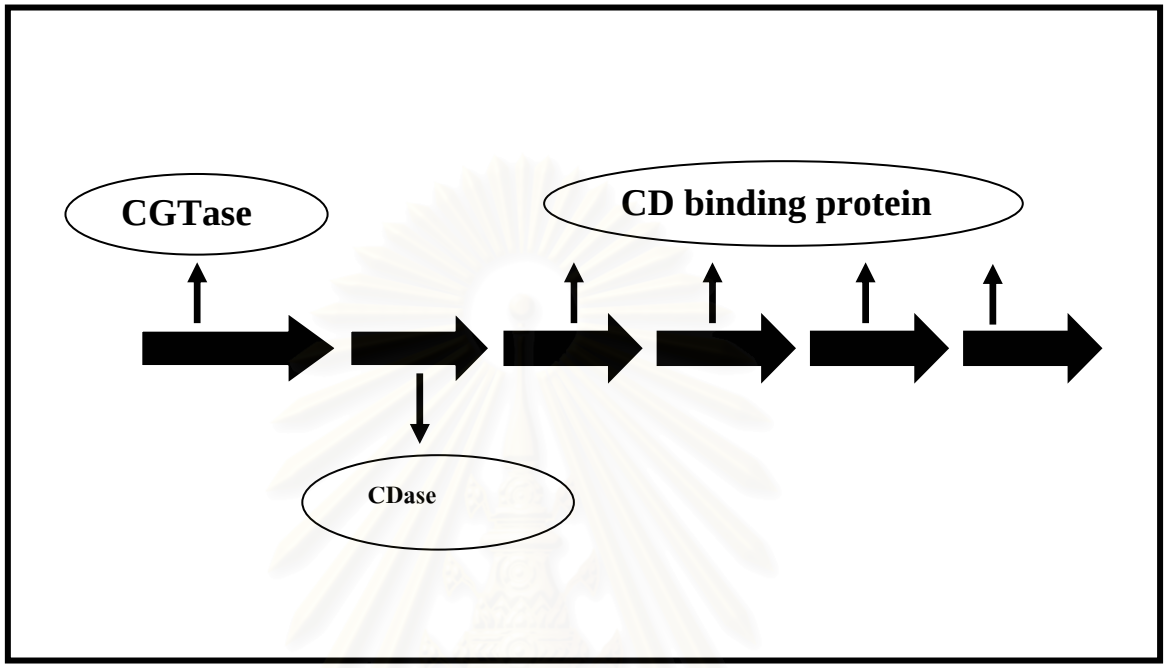

Figure 9 Structure of the gene cluster containing genes for synthesis, transport and degradation of CDs in alkalophilic *Bacillus* sp. A2-5a (Ohdan *et al.*, 2000)

The big arrows show the localization of each gene and the orientation of the coding sequences.

Ohdan *et al.* (2000), and Hashimoto *et al.* (2001) suggested that in other gram-positive or negative bacteria which can utilize or produce cyclodextrins, there may also exist a novel starch-degradation pathway which involves the extracellular conversion of starch into CDs by CGTase, the uptake of CDs by specific uptake system and the intracellular linearization by a CDase.

Comparison of CDase amino acid sequences

The deduced amino acid sequence of CDases were compared (Figure 10). The four highly conserved regions were found. These regions were also present in other members of the α-amylase family; α-glucosidases, pullulanases, isoamylases, and CGTases (Svensson, 1994). An amino acid sequence alignment showing these four conserved regions for diverse members of the α-amylase family is presented in Figure 11. All four regions contain completely invariant amino acid residues within the α-amylase family and the functions of most of these have been elucidated by X-ray crystallography, site-directed mutagenesis, and chemical modification of various members of this family. These residues are directly involved in catalysis, either through substrate binding, bond cleavage, transition state stabilization or as ligands of a calcium binding site present near the active site. For instance, three carboxylic acid groups, one glutamic acid and two aspartic acid residues, were found to be essential for catalytic activity in α-amylase, CGTase and CDase. The amino acids are equivalent to Asp 206, Glu 230 and Asp 297 in α-amylase from *Aspergillus oryzae* (Matsuura *et al.*, 1984), Asp 229, Glu 268 and Asp 328 in CGTase from *Bacillus circulans* (Klein *et al.*, 1992; Strokopytov *et al.*, 1995) and Asp 325, Glu 354 and Asp

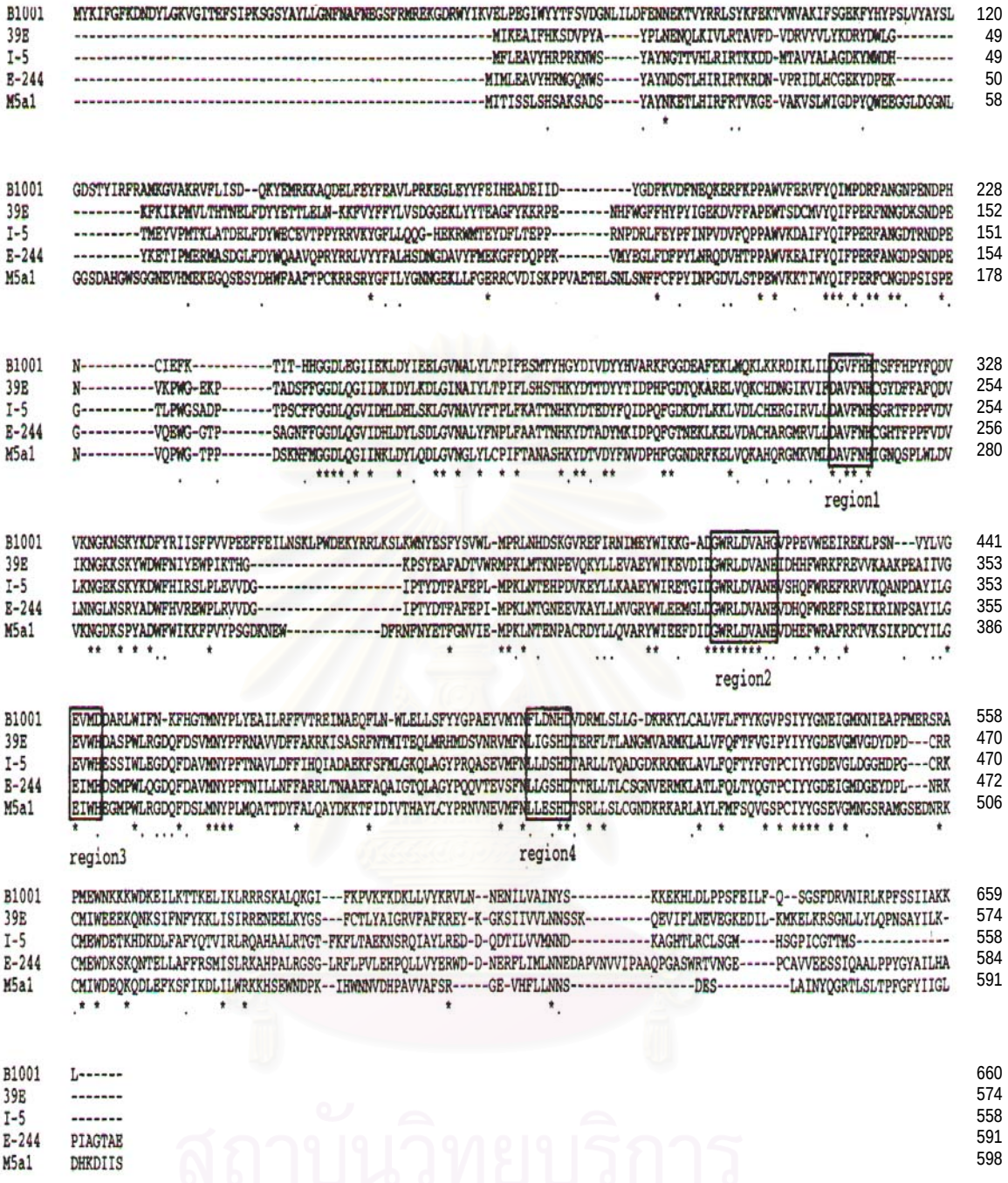

Figure 10 Comparison of the deduced amino acid sequences for the CDase Proteins

The four conserved regions among the α-amylase family enzymes are boxed. Asterisks represent identical amino acid residues in five polypeptides and dots indicate similar amino acid residues. Polypeptides: B1001, *Thermococcus* sp. B1001 (AB034969); 39E, *Clostridium thermohydrosulfuricum* 39E (M88602); I-5, alkalophilic *Bacillus* I-5 (U49646); E-244, *Bacillus sphaericus* E-244 (X62576) and M5a1, *Klebsiella oxytoca* (X86014).

			1		2
CD	*Clostridium thermohydrosulfuricum*	238	DAVFNH	320	DGWRLDVANE
CD	Alkalophilic *Bacillus sp. I-5*	238	DAVFNH	320	DGWRLDVANE
APL	*Clostridium thermohydrosulfuricum*	488	DAVFNH	594	DGWRLDVANE
NPL	*Bacillus stearothermophilus*	242	DAVFNH	323	DGWRLDVANE
AMY	*Aspergillus* oryzae	117	DVVANH	201	DGLRIDTVKH
AMY	*Bacillus amyloliquefaciens*	98	DVVLNH	226	DGFRIDAAKH
AMY	*Bacillus licheniformis*	129	DVVINH	255	DGFRLDAVKH
AMY	*Bacillus stearothermophilus*	101	DVVFDH	230	DGFRLDAVKH
ISO	*Pseudomonas amyloderamosa*	291	DVVYNH	369	DGFRFDLASV
CGT	*Bacillus* sp.	135	DFAPNH	224	DGIRVDAVKH
PUL	*Klebsiella aerogenes*	600	DVVYNH	670	DGFRFDLMGY

			3		4
CD	*Clostridium thermohydrosulfuricum*	354	EVWH	416	LIGSHD
CD	Alkalophilic *Bacillus sp. I-5*	354	EVWH	416	LLDSHD
APL	*Clostridium thermohydrosulfuricum*	627	ENWN	699	LLGSHD
NPL	*Bacillus stearothermophilus*	357	EIWH	419	LLGSHD
AMY	*Aspergillus* oryzae	230	EVLD	292	FVENHD
AMY	*Bacillus amyloliquefaciens*	261	EYWQ	323	FVENHD
AMY	*Bacillus licheniformis*	289	EYWQ	352	FVDNHD
AMY	*Bacillus stearothermophilus*	264	EYWS	326	FVDNHD
ISO	*Pseudomonas amyloderamosa*	454	EWSV	502	FIDVHD
CGT	*Bacillus* sp.	268	EYHQ	323	FIDNHD
PUL	*Klebsiella aerogenes*	704	EGWD	827	YVSKHD

Figure 11 Similarities in the amino acid sequences of CDase and various amylolytic enzymes (Nakajima *et al*., 1986; Podkovyrov and Zeikus, 1992; Kim *et al*., 1998; Bart *et al*., 2000)

Enzymes are abbreviated as follows; CD, CDase; APL, α-amylase-pullulanase; NPL, neopullulanase; AMY, α-amylase; ISO, isoamylase; CGT, CGTase; PUL, pullulanase. Numbering starts from the first amino acid of the mature proteins. Numbered bars above the sequences denote highly conserved regions among α-amylase family. Catalytic and substrate-binding residues proposed for CDase are shaded and underlined, respectively.

421 in CDase from *Clostridium thermohydrosulfuricum* 39E (Podkovyrov *et al.*, 1993). Two conserved histidine residues, His 140 and His 327 (CGTase numbering), are involved in substrate binding and transition state stabilization (Lawson *et al*., 1994; Strokopytov *et al.*, 1996). A third histidine, present only in some α-amylase and CGTase (His 233, CGTase numbering), is involved in substrate binding and acts as a calcium-ligand with its carbonyl oxygen (Lawson *et al*., 1994; Strokopytov *et al.*, 1996). For CDases, chemical modification could indicate that His residue is important for enzyme acitivity (Glavin *et al.*, 1994; Yang *et al*., 1996). In CGTase of *Bacillus circulans* 251 (Uitdehaag *et al*., 1999), Arg 227 was also proved to be important for the orientation of the Asp 229 nucleophile whereas the importance of Arg in CDase had never been reported.

Three-dimensional structure of CDase

α-Amylase family generally consists of three structural domains, A, B, and C. CGTase shows a similar domain organization with two additional domains, D and E (Figure 12). Domain A contains a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices. This is called the $(\beta/\alpha)_8$-TIM-barrel catalytic domain (Janecek, 1994). Domain B contributes to substrate binding. Domain C has an antiparallel β-sandwich fold. For the three-dimensional structure of CDase, only two structures from alkalophilic *Bacillus* sp. I-5 CDase (PDB. code 1EA9) (Lee *et al.*, 2002) and *Flavobacterium* sp. no. 92 CDase (PDB. code 1H3G) (Fritzsche *et al*., 2003) were proposed with the X-ray diffraction technique. The structure of CDase contains a distinct N-terminal domain in addition to a central $(\beta/\alpha)_8$-barrel domain

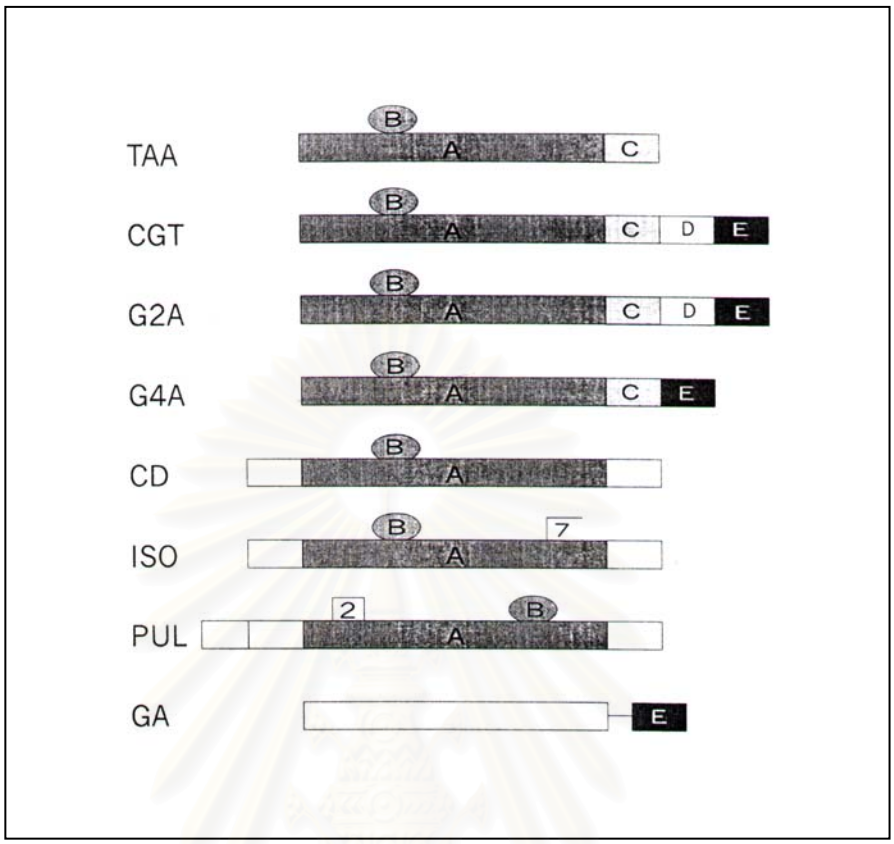

Figure 12 **Domain level organization of starch-degrading enzymes.** (Jespersen *et al*., 1991)

Enzymes are abbreviated as follows; CGT, CGTase from *Bacillus circulans*; G2A, maltogenic α-amylase from *Bacillus stearothermophilus*; G4A, maltotetraose forming α-amylase from *Pseudomonas stutzeri*; TAA, α-amylase from *Aspergillus oryzae* (Taka-amylase A); CD, cyclodextrinase from *Klebsiella oxytoca*; ISO, isoamylase from *Pseudomonas amyloderamosa*; PUL, pullulanase from *Klebsiella aerogenes*; GA, glucoamylase (family 15 of glycosyl hydrolases) from *Aspergillus niger*.

and a C-terminal domain. The N-terminal (residues 1-123) and C-terminal (residues 505-583) domains are composed exclusively of β-strands (Figure 13A). Lee *et al*. (2002) demonstrated that the N-terminal domain residue Trp 47 and the central domain residue Phe 288 provide a catalytically important interaction with CDs, whereas they provide a mere interaction with soluble starch (Figure 13B). In addition, the presence of hydrophobic residues Trp 47 and Phe 288 at CDase active site caused the active site pocket narrow and deep which is more suitable for binding with CDs than with soluble starch. It should be noted that Trp 47 and Phe 288 are invariant in the CD degrading enzymes and that could be used for distinguishing CDases from other amylolytic enzymes.

Our research interest

Our research group has been working on CGTase from *Bacillus circulans* A11. Purification, characterization, modification of CGTase enzyme and molecular cloning, expression, mutation, sequence analysis of CGTase gene were studied (Rutchtorn *et al*., 1994; Rimphanitchayakit *et al*., 2000; Rojtinnakorn *et al*., 2001; Keadsin, 2003; Kaulpiboon and Pongsawasdi, 2003). Since the organism can utilize starch and produce cyclodextrin, we are interested in investigating its starch metabolic pathway. The enzyme CDase and its encoding gene would be our target. In the present work, the existence and localization of CDase in the cell were investigated. A specific screening system was developed for detecting CDase activity. Then, purification and characterization of the CDase, and cloning and characterization of the CDase gene were our focus. Purification and characterization of the enzyme were

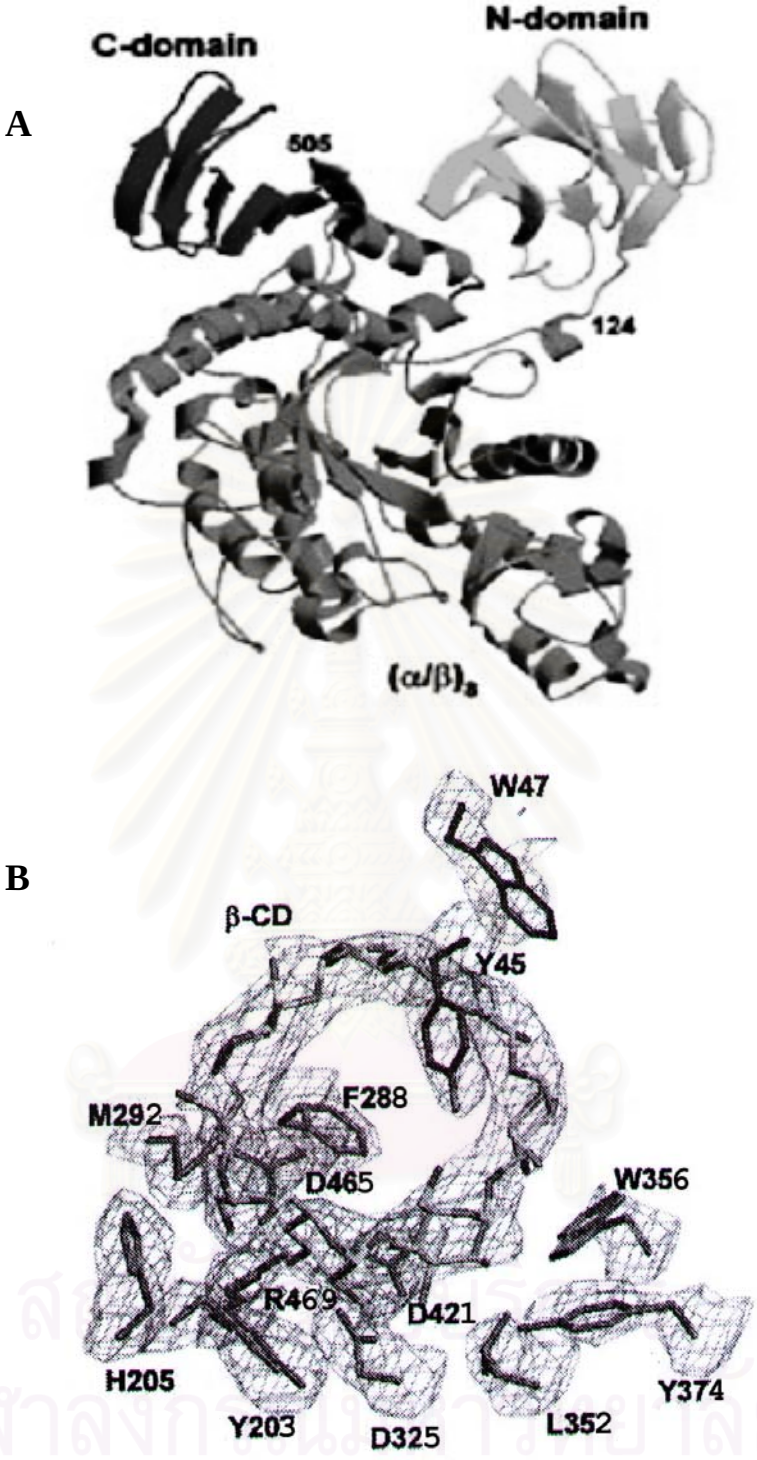

Figure 13 Structure of CDase from alkalophilic *Bacillus* sp. I-5 (A) and stereo view of the binding of β-CD to the active site of CDase (B) (Lee *et al*., 2002)

performed using various techniques specialized for proteins and enzymes. For gene isolation, single-stranded oligonucleotide primers were designed from the conserved sequence of previously reported CDase genes. DNA fragment was amplified by PCR technique and identified by using southern blot and colony hybridization. Gene characterization by construction of restriction map and DNA sequencing were studied. In addition, purification and characterization of the recombinant enzyme were performed and compared with A11 CDase. These results will lead to more information concerning with the ability to use starch as C-source and the metabolism of cyclodextrins of this microorganism.

The microorganism in this study, was originally identified as *Bacillus circulans* A11, using morphological and biochemical characterization (Appendix A). From scaning and transmission electron microscopy, A11 cells were rod-shaped and could produce endospore (Appendix B). From recent development in the molecular biological method, the genus *Bacillus* could be separated into several phylogenetically distinct genera by using 16S rRNA gene sequencing such as *Alicyclobacillus, Aneurinibacillus*, *Brevibacillus, Halobacillus* and *Paenibacillus* (Shida *et al*., 1997). Members of the genus *Paenibacillus* are facultatively anaerobic organisms that produce spores in definitely swollen sporangia and have G + C contents ranging from 45 to 54 mol%. Cells are rods measuring approximately 0.6-0.9 by 3.0-6.0 μm which have bigger size than bacteria in the genus *Bacillus* and motile by means of peritrichous flagella. Colonies are flat, smooth, circular, entire and yellowish gray. Some organisms in the *Paenibacillus* genus excrete diverse assortments of intracellular or extracellular polysaccharide-hydrolyzing enzymes (Yoon *et al*., 1998). The major cellular fatty acid is anteiso $C_{15:0}$ and the major quinone is menaquinone 7. To clarify the taxonomy of the strain A11, we determined 16S rRNA gene

sequencing, phylogenetic relationship, cellular fatty acid compositions and G+C content in the first step of this research.

The objectives of this research

1. To reidentify the bacterium *Bacillus circulans* A11

2. To purify and characterize CDase from strain A11

3. To clone the CDase gene from strain A11 into *Escherichia coli* JM 109

4. To express and characterize the CDase gene in the recombinant strain

5. To compare the purified CDase from the original strain with that of the recombinant strain

CHAPTER II

MATERIALS AND METHODS

2.1 Materials and Equipments

Amino acid analyzer: Model L-8500 Hitachi, Hitachi column (4.6x60 mm), Hitachi, Tokyo, Japan

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

Autopipette: Pipetman, Gilson, France

Camera: Model K1000 Pentax, Japan

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

Conductivity meter: Model CDM83, Radiometer, Denmark

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

Dialysis tubing: Sigma, U.S.A.

Electrophoresis unit: Model 111 mini IEF Cell, Bio-Rad Applied Biosystem company, U.S.A.

Electrophoresis unit: Model Mini-protein II Cell, Bio-Rad Applied Biosystem company, U.S.A.

Gas chromatography: Model GC-14A, Shimadzu, Japan

GC Capillary column: Model BP-20 size 30 m x 0.33 mm., SGE International Pty. Ltd., U.S.A.

GeneAmp PCR System: Model 2400, PERKIN-ELMER, U.S.A.

Gene Pulser™: Bio-Rad, U.S.A.

Fluorescence spectrophotometer: Model LS 55, PERKIN-ELMER, UK.

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

Fraction collector: Model 2211 Pharmacia LKB, Sweden

High Performance Liquid Chromatography: Model RID-10A, Shimadzu, Japan

HPLC Spherisorb 10NH_2 column: Model LUNA 5 μ size 250 x 4.6 mm., Phenomenex, U.S.A.

Hybond™-N^+ membrane: Amersham Pharmacia Biotech, Sweden

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

Incubator shaker, controlled environment: Psyco-therm, New Brunswick Scientific Co., U.S.A.

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

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

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

Microcentrifuge tube 1.5 ml: Bioactive, Thailand

Mini trans-blot electrophoresis transfer cell: Bio-Rad Applied Biosystem company, U.S.A.

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Power supply: Model W375, Heat systems-ultrasonics, U.S.A.

PVDF membrane: Amersham Pharmacia Biotech, Sweden

Sonicator: Model W375, Heat systems-ultrasonics, U.S.A.

Spectrophotometer UV-240: Model DU Series 650, Beckman, U.S.A.

Thin-wall microcentrifuge tube 0.2 ml: Axygen Hayward, U.S.A.

TLC plates: Silica gel 60 size 20 x 20 cm., Merck, Germany

Ultrasonic homogenizer: Model SONOPULS, Bandelin, Germany

UV transluminator: Model 2011 Macrovue, SanGabriel California, 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.

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

Aquasorb: Fluka, Switzerland

Ammonium sulphate: Merck, Germany

Ampicillin: Sigma, U.S.A.

Bovine serum albumin (BSA): Sigma, U.S.A.

5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal): Sigma, 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.

α-, β-, γ-Cyclodextrins: Sigma, U.S.A.

DEAE-Sephadex A50: Sigma, U.S.A.

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Diethylpyrocarbonate: Sigma, U.S.A.

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

DNA marker: Lamda (λ) DNA digest with *Hin*d III: GIBCOBRL, U.S.A.

ECL™ direct nucleic acid labeling and detection system: Amersham Pharmacia Biotech, Sweden

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

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

Glycine: Sigma, U.S.A.

Instant methanolic HCl kit: Alltech, U.S.A.

Iodoacetamide: Sigma, U.S.A.

Isopropyl-1-thio-β-D-galactopyranoside (IPTG): Sigma, U.S.A.

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

14-Methylpentadecanoic acid methyl ester: Sigma, U.S.A.

12-Methyltetradecanoic acid methyl ester: Sigma, U.S.A.

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

Palmitic acid methyl ester: Sigma, U.S.A.

Phenol: BDH, England

Phenolphthalein: BDH, England

Phenylglyoxal: Sigma, U.S.A.

Phenylmethylsulfonyl fluoride: Sigma, U.S.A.

Phenyl-Sepharose CL-4B: Amersham Pharmacia Biotech, Sweden

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

Protein molecular weight standards (MW 14,300-200,000): GibcoBRL, U.S.A.

QIAquick Gel Extraction Kit: QIAGEN, Germany

QIAquick Plasmid Extraction Kit: QIAGEN, Germany

Sephadex G-100: Pharmacia, U.S.A.

Sephadex G-200: Pharmacia, U.S.A.

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate (SDS): Boehringer Manheim, Germany

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

D-Sorbitol: Fluka AG, Switzerland

2,4,6-Trinitrobenzenesulfonic acid: Sigma, U.S.A.

Tryptic soy broth: DIFCO, U.S.A.

Tryptone: DIFCO, U.S.A.

Yeast extract: DIFCO, U.S.A.

2.3 Enzymes and Restriction enzymes

DNA Polymerase I (Klenow): New England Biolabs, Inc., U.S.A.

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction enzymes: GIBCOBRL, U.S.A. ; Takara, Japan and New England Biolabs, Inc., U.S.A.

RNase: Sigma, U.S.A.

Taq DNA polymerase: Pacific Science, France

T4 DNA ligase: New England Biolabs, Inc., U.S.A.

Glucomannan were from Dr. Masaru Iizuka, Enzyme Chemistry Laboratory, Osaka City University, Japan. The other common chemicals were reagent grade from Aldrich, U.S.A.; BDH, England; Fluka, Switzerland; Merck, Germany and Sigma, U.S.A.

2.4 Bacterial strains and Plasmids

The bacterial strain A11 (originally identified as *Bacillus circulans* A11), isolated from South-East Asian soil, was screened for CGTase activity by Pongsawasdi and Yagisawa (1987).

Escherichia coli strain XL-1-Blue with genotype recA1, relA1, endA1, gyrA96, thi-1, hsdR17, supE44, lac[*F′*, proAB, lac/^{9}ZΔM15Tn10(Tet^{r})] and strain JM 109 with genotype *F′* [traD36, proAB, laclqZM15], λ^{-}, endA1, gyrA96, hsdR17(r_{K-} m_{K+}), mcrB^{+}, recA1, relA1, Δ(lac-proAB), thi, supE44 were purchased from GIBCOBRL, U.S.A.

Plasmids pGEM® T-Easy and pUC 18 were purchased from Promega, U.S.A and Amersham Pharmacia Biotech, Sweden, respectively.

2.5 Media Preparation

2.5.1 Screening medium

A screening medium for the cyclodextrinase-producing bacteria contained 0.5% β-CD, 0.002% phenolphthalein, 0.5% yeast extract, 0.5% polypeptone, 0.1%

K_2HPO_4, 0.02% $MgSO_4$.$7H_2O$, 0.75% Na_2CO_3 and 1.5% agar with pH adjusted to 10. Growing temperature was at 37^oC.

2.5.2 Medium I (Pongsawasdi and Yagisawa, 1987)

Medium I, medium for starter, consisted of 0.5 % beef extract, 1.0 % polypeptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble starch (Fluka). The pH was adjusted to 7.2 with 1N HCl. For solid medium, 1.5% agar was added.

2.5.3 Horikoshi medium (modified from Horikoshi's medium by Rutchtorn, 1993).

Medium for enzyme production, slightly modified from Horikoshi (1971), contained 1.0% β-cyclodextrin, 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.

2.5.4 Luria-Bertani (LB) medium (Maniatis *et al*., 1982)

LB consisted of 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl. pH was adjusted to pH 7.2-7.4 with NaOH or HCl. When solid medium was required, agar was added to the final concentration of 1.5%. Ampicillin (100 μg/ml) was used when needed.

2.5.5 Terrific medium (Maniatis *et al*., 1982)

Terrific medium was used for culture the transformant cells, consisted of 1.2% tryptone, 2.4% yeast extract, 0.23% KH_2PO_4 and 1.25% K_2HPO_4.

2.5.6 Tryptic soy broth (Komagata and Suzuki, 1987)

Tryptic soy broth was used for culturing cells for cellular fatty acids extraction, comprising of 3% (w/v) tryptic soy broth (Difco Laboratories).

All mediums above were steriled by autoclaving at 121 °C for 15 min.

2.6 Screening for CDase activity

A11 was screened for CDase activity by growing on a specific selective medium as described in section 2.5.1. The medium was composed of β-CD-phenolphthalein complex which was colorless at pH 10. One loopful of the strain was then streaked onto the agar plate. After incubation at 37^{o}C for 2 days, if bacterial colonies on the screening medium produced the enzyme CDase, phenolphthalein included in the β-CD molecules would be released. A pink color around the colony would be observed. A positive colony on a screening medium was then characterized to identify bacterial type.

2.7 Identification of the bacterium producing CDase

2.7.1 Amplification of 16S rRNA gene fragment

A11 was grown on LB medium at 37^{o}C, pH 7.2 for 24 h and its chromosomal DNA was extracted as described previously (Sambrook and Russell, 1989). Specific DNA fragment was amplified from A11 chromosomal DNA using the forward primer pA 5′AGAGTTTGATCCTGGCTCAG3′ and the reverse primer pH′ 5′ AAGGAGGTGATCCAGCCGCA3′ (Edwards *et al*., 1989). The temperature program for each PCR cycle was 95°C for 1 min., 50 °C for 2 min. and 72°C for 3 min. Thirty cycles were run with 2.5 Units of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$, 100 pmoles of each primer and 1 μg of A11 chromosomal DNA. PCR product was checked by agarose gel electrophoresis and was ligated with pGEM® T-Easy vector at 16°C for 20 h. After that, recombinant plasmid (pJK 1.5) was transformed onto *E. coli* host cells and was grown at 37 °C in LB medium containing 100 μg ampicillin/ml. Plasmid DNA (pJK 1.5) was extracted and rechecked for size of insert from PCR product before sequencing.

2.7.2 Sequencing of 16S rRNA gene

Plasmid DNA (pJK 1.5) was sent for sequencing at the Bioservice Unit (BSU), Thailand. Sequencing primers were pA 5′AGAGTTTGATCCTGGCTCAG3′, pD 5′ CAGCAGCCGCGGTAATAATAC3′ and pF 5′CATGGCTGTCGTCAGCTCGT3′ (Edwards *et al.*, 1989). The 16S rRNA gene sequence was compared and aligned using BLAST and Clustal W software (Altschul *et al*, 1997; Thompson *et al.*, 1994).

Reference sequences were obtained from the GenBank, EMBL, DDBJ and PDB database. Percent homology obtained from comparison of the data with the reference base sequence of 16S rRNA gene of different bacterial strains leads to indication of the strain.

2.7.3 Phylogenetic analysis

The 16S rRNA gene sequence of strain A11 was aligned with the sequences of other *Paenibacillus* species and some rod-shaped, endospore-forming bacteria using the Tree View Program of Clustal W software (Thompson *et al.*, 1994). Other reference sequences, obtained from the GenBank database, had the following accession numbers : X60632 (*Paenibacillus polymyxa*); D78318 (*Paenibacillus azotofixans*); D78476 (*Paenibacillus peoriae*); D78319 (*Paenibacillus macerans*); X77846 (*Paenibacillus durum*); D78473 (*Paenibacillus lautus* NRRL NRS-666^{T}); D85609 (*Paenibacillus lautus* NRRL B-377); D85394 (*Paenibacillus lautus* NRRL B-379); D78470 (*Paenibacillus glucanolyticus* DSM 5162^{T}); D88514 (*Paenibacillus glucanolyticus* DSM 5188); D85395 (*Paenibacillus chibensis*); X60625 (*Paenibacillus macquariensis*); X60630 (*Paenibacillus pabuli*); D85396 (*Paenibacillus amylolyticus*); D85397 (*Paenibacillus illinoisensis*); D78317 (*Paenibacillus alvei*); U49247 (*Paenibacillus apiarius*); D78475 (*Paenibacillus thiaminolyticus*); D78466 (*Paenibacillus curdlanolyticus*); D78471 (*Paenibacillus kobensis*); D78320 (*Paenibacillus validus*); D82064 (*Paenibacillus chondroitinus*); D78465 (*Paenibacillus aliginolyticus*); X60636 (*Paenibacillus larvae* subsp. *pulvifaciens*); X60619 (*Paenibacillus larvae* subsp. *larvae*); X60646 (*Bacillus subtilis*); D16266 (*Bacillus cereus*); D78312 (*Bacillus circulans*); X60629 (*Bacillus*

megaterium); D78313 (*Bacillus coagulans*); X62174 (*Halobacillus halophilus*); D82065 (*Amphibacillus xylanus*); D78455 (*Aneurinibacillus aneurinolyticus*); D78457 (*Brevibacillus brevis*); X60742 (*Alicyclobacillus acidocaldarius*); AF021924 (*Paenibacillus campinasensis* 324); AB073187 (*Paenibacillus campinasensis* JCM11200); AB043866 (*Paenibacillus* sp. 38-2); AY071857 (*Bacillus licheniformis* PR-1) and *Paenibacillus* sp. RB01 (Tessana, 2001). The 16S rRNA gene similarity values were calculated from the alignments and the evolutionary distances were calculated using the CLUSTAL W (Thompson *et al.*, 1994). A bootstrap analysis with 1,000 replications for evaluating the topology of the phylogenetic tree was performed with the CLUSTAL W. The Genbank accession number for the 16S rRNA gene sequence of strain A11 is AY237109.

2.7.4 Cellular fatty acid composition

A11 was cultivated overnight in tryptic soy broth at 37^{o}C. Cells were removed by centrifugation at 10,000 xg for 15 min at 4^{o}C. Approximately 20 mg of freeze-dried cells were placed in a Teflon-lined, screw-capped tube with 2 ml of 5% anhydrous methanolic HCl at 100^{o}C for 3 h. After cooling, 1 ml of water was added, and fatty acid methyl esters were extracted three times with 3 ml of n-hexane. The n-hexane fraction was washed with an equal volume of water. The upper n-hexane phase containing the cellular fatty acid methyl esters was transferred to a dry test tube and dehydrated with anhydrous Na_2SO_4. Fatty acid methyl esters were concentrated and applied to gas chromatography. For separation of fatty acid methyl esters, a capillary column BP-20 (30 m x 0.33 mm) was used. The chromatographic conditions were as follows; injection temperature : 250 ^{o}C; carrier gas : helium; column oven :

initial temperature 170 ^{o}C for 4 min, increased from 170 ^{o}C to 240 ^{o}C at flow rate of 5 ^{o}C/min and held at 240 ^{o}C for 10 min. The spectra were recorded by flame ionization detector at 270 ^{o}C (Komagata and Suzuki, 1987).

2.7.5 Measurement of G+C content by melting temperature

Bacteria grown to the logarithmic phase were harvested by centrifugation. Cells were lysed and DNA was isolated using the procedure of Maniatis *et al*. (1982). The solution of A11 chromosomal DNA in saline citrate buffer, pH 7.0 was measured for A_{260} at various temperatures from 25 to 98^{o}C. The relative absorbance was calculated from dividing the absorbance at each temperature with the absorbance at room temperature. The relative absorbance was plotted against temperature. Temperature that coincides with half of the maximal relative absorbance is the melting temperature (T_m). The base composition of DNA was calculated from its thermal denaturation temperature. *Bacillus subtilis* ATCC 6633 was used as the reference strain. The G+C content in mole percent of native DNA was calculated using the following equation of De Ley, 1970.

$$\text{Mol \% (G + C)} = 2.44\,(T_m - 69.4)$$

2.8 CDase production

The strain A11 was grown in a 250 ml-shaking flask containing 50 ml of Medium I at 37^{o}C, for 4-6 h or until A_{420} reached 0.3-0.5 unit (Pongsawasdi and Yagisawa, 1987). One percent of culture in Medium I was transferred onto Horikoshi

medium containing 1% β-CD and cultivated for 48 h at 250 rpm and 37^{o}C. Cells (around 40 g wet weight) were harvested by centrifugation at 8,000 xg for 15 min at 4^{o}C and washed twice in 10 mM phosphate buffer, pH 6.5. Cells were then resuspended in cold extraction buffer (10 mM phosphate buffer, pH 6.5 containing 1 mM DTT, 1 mM EDTA and 1 mM PMSF) and sonicated with SONOPULS ultrasonic homogenizer (6 mm diameter-stepped microtip, 40% amplitude, Bandelin, Germany) for 6 cycles of 5 min sonicate and 5 min pulse. The homogenate was centrifuged at 100,000 xg for 1 h. The supernatant was used as crude CDase preparation.

2.9 Purification of CDase

All operations were performed at 4^{o}C. The buffer A used in all steps was 10 mM phosphate buffer, pH 7.5 containing 1 mM DTT, 1 mM EDTA and 10% glycerol. The purification procedure for CDase was as follows.

2.9.1 Ammonium sulfate precipitation

The precipitation of crude CDase was performed by slowly added solid ammonium sulfate to 40% saturation with gentle stirring. After 6 h, the supernatant was collected by centrifugation at 27,000 xg for 30 min and then brought to final concentration of 60% ammonium sulfate saturated. The solution was left for 6 h at 4 ^{o}C with continuous stirring and was subsequently centrifuged at 27,000 xg for 30 min. The precipitate was dissolved in buffer A. The protein solution was dialyzed against the same buffer at least 3 h for 3 times before determination of CDase activity and protein concentration as described in section 2.10 and 2.11, respectively.

2.9.2 DEAE-Sephadex A50 column chromatography

DEAE-Sephadex A50 was activated by washing with 0.5 N sodium hydroxide for 2-3 times before rewashing with deionized water until the pH reached 7.0. The active resin was resuspended in buffer A and packed into a 1.7x45 cm column. Equilibration of the column with the same buffer for 5-10 column volumes at flow rate of 30 ml/h was performed.

The dialyzed protein solution from section 2.9.1 was applied onto the DEAE-Sephadex A50 column. The unbound proteins were eluted from the column with buffer A. The bound proteins were then eluted from the column with a linear 200 ml salt gradient of 0 to 0.5 M sodium chloride in buffer A at a flow rate of 30 ml/h. Fractions of 3 ml were collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in the section 2.10. The active fractions were pooled, concentrated using aquasorb and then dialyzed against buffer A.

2.9.3 Phenyl-Sepharose CL-4B column chromatography

Phenyl-Sepharose CL-4B was activated by washing with 0.5 N sodium hydroxide for 2-3 times before rewashing with deionized water until the pH reached 7.0. The active resin was resuspended in buffer A containing 1 M ammonium sulfate and packed into a 1.7x10 cm column. Equilibration of the column with the same buffer for 5-10 column volumes at flow rate of 20 ml/h was performed.

Ammonium sulfate was slowly added to the pooled enzyme fraction from section 2.9.2 until final concentration was at 1 M. The solution was stirred gently for at least 30 min. The protein solution was then applied onto the equilibrated column at flow rate of 20 ml/h. The proteins were washed with the same buffer for 30 min and then eluted with a linear 160 ml gradient decreasing the ammonium sulfate concentration from 1 to 0 M. Finally, the column was rinsed with 80 ml of buffer A. Fractions of 3 ml were collected. The absorbance at 280 nm was measured. The enzyme activity was determined as described in the section 2.10. The active fractions were pooled, concentrated using aquasorb and dialyzed against buffer A.

2.10 CDase assay

CDase activity was assayed in 0.5 ml reaction mixture that contained 1%β-CD (w/v) in 0.1 M phosphate buffer, pH 7.0 and appropriately diluted enzyme. After incubation at 40^{o}C for 30 min, the reducing sugar formed was measured by using the 3,5 dinitrosalicylic acid method.

One unit (U) of CDase was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose in 1 min per ml of reaction at 40^{o}C.

Dinitrosalicylic acid method (Bernfeld, 1955)

Glucose standard (0-10 mM) was prepared. Then, 0.5 ml of standard glucose or sample was mixed with 0.5 ml of dinitrosalicylic acid reagent (prepared as described below). The solution was heated for 5 min in a boiling water bath, then the tubes were cooled in a pan of cold water for 5 min. The mixture was adjusted to a

final volume of 5 ml with distilled water. After mixing, the absorbance at 540 nm was recorded. The quantity of reducing sugar in sample was determined from standard curve of glucose.

Dinitrosalicylic acid reagent was consisted of dinitrosalicylic acid (5 g), 2 N NaOH (100 ml), and potassium sodium tartrate (150 g) in final volume of 500 ml.

2.11 Protein Determination

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

One hundred microlitres of sample was mixed with 1 ml of protein reagent and left for 15 min 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.

2.12 Polyacrylamide Gel Electrophoresis (PAGE)

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

2.12.1 Non–denaturing PAGE

Discontinuous PAGE was performed according to Weber and Osborn (1975) 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 (see Appendix C). The

electrophoresis was run from cathode towards the anode at constant current of 20 mA per slab at room temperature in a Midget LKB 2001 Gel Electrophoresis unit.

2.12.2 SDS-PAGE

The denaturing gel was performed according to Weber and Osborn (1975). The gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels and Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as electrode buffer (see Appendix C). Samples to be analyzed were treated with sample buffer and boiled for 5 min prior to application to the gel. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

2.12.3 Detection of proteins

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

2.12.3.1 Coomassie blue staining

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

2.12.3.2 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 0.1 M phosphate buffer pH 7.0, at 40 °C for 30 min. 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 band on the blue background indicates starch degrading activity of CDase enzyme.

2.13 Characterization of purified CDase from the strain A11

2.13.1 Molecular weight determination of native CDase by gel filtration on Sephadex G-100 column

Sephadex G-100 column (1.7x80 cm) was equilibrated with buffer A containing 0.1 M sodium chloride at 4 ^{o}C for 5 column volumes at the flow rate of 12 ml/h to allow stabilization of bed volume of the column. An aliquot of the concentrated enzyme solution from Phenyl Sepharose CL-4B column was applied to the Sephadex G-100 column and enzyme was then eluted with buffer A containing 0.1 M sodium chloride. Fractions of 2 ml were collected. The elution profile was monitored for protein and enzyme activity as previously described.

The elution volume (V_e) of enzyme was compared with standard protein molecular weight markers; phosphorylase b (97,000 Da), bovine serum albumin (67,000 Da), chymotrypsinogen (27,000 Da) and cytochrome C (12,500 Da). Blue

dextran 2000 and potassium dichromate were used to determine the position of the void volume (V_o) and the total bed volume (V_t), respectively. The partition coefficient (K_{av}) values for each standard protein marker calculated from $(V_e-V_o)/(V_t-V_o)$ were plotted against log molecular weight of each protein to obtain a calibration curve. The K_{av} of the enzyme was calculated and used to determine its native molecular weight from the calibration curve.

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

2.13.2.1 Preparation of gel support film

A few drops of water was pipetted onto the glass plate. The hydrophilic side (lower side) of the gel support film was then placed against the glass plate and flatly rolled the upper side with a test tube to force excess water and bubbles. Subsequently, put the glass plate with gel support film into the casting tray with the gel support film side facing down so that it was rest on the space bars.

2.13.2.2 Preparation of the gel

The gel solution composed of 30% acrylamide, 1% bis-acrylamide, 50% sucrose, 10% ammonium persulfate and TEMED (see Appendix D) was carefully pipetted between the glass plate and casting tray with a smooth flow rate to prevent air bubbles. The gel was left overnight to allow polymerization, then lifted

from the casting tray using spatula. The gel was fixed on the gel support film and ready for use.

2.13.2.3 Sample application and running the gel

The sample was loaded on a small piece of filter paper to allow its diffusion into the gel for 5 min and the filter paper was carefully removed from the gel. The gel with the adsorbed samples was turned upside-down and directly placed on top of the graphite electrodes. Focusing is carried out under constant voltage conditions in a stepwise fashion. The gel was firstly focused at 100 V for 15 min, followed by an increase in voltage up to 200 V for 15 min and finally run at 450 V for an additional 60 min. After complete electrofocusing, the gel was stained for 2 h and then destained with destaining solution I and destaining solution II, respectively (see Appendix D). Standard protein markers with known pI in the range 3-10 were run in parallel. The standards consist of amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-basic band (8.65) and trypsinogen (9.3). The pI of sample protein was determined by the standard curve constructed from the pI of standard proteins versus their migrating distances from cathode.

2.13.3 Determination of N-terminal amino acid sequence

2.13.3.1 Protein immobilization

Proteins on SDS-PAGE were transferred onto PVDF membrane. The membrane was wetted with methanol for a few seconds, then placed in a dish containing electroblotting buffer (20% methanol contained 25 mM Tris-glycine buffer, pH 8.3) for 5 min. The SDS-gel was sandwiched between pre-wet membrane, three layers of Whatman No. 1 paper, and sponges, was assembled on a BioRad Mini Trans-Blot Electrophoretic Transfer Cell and run at constant voltage of 50 volts at room temperature for 90 min. PVDF membrane was then removed from transblotting sandwich and rinsed with deionized water prior to the staining.

2.13.3.2 Staining and destaining of the membrane

The PVDF membrane was saturated with 100% methanol for a few seconds and stained with 0.1% Coomassie blue R-250 in 40% methanol, 10% acetic acid. The PVDF membrane was removed from staining solution and destained with 50% methanol. The membrane was rinsed extensively with deionized water. The protein bands of interest were excised and stored at –20 ^{o}C until N-terminal amino acid sequencing was performed.

2.13.3.3 N-terminal amino acid sequencing

Amino acid sequencing was performed by Edman degradation on an Applied Biosystems 476A Protein Sequencer with an on-line phenylthiohydantoin (PTH)-derivative analyzer, at the Protein and Proteomics Centre, The National University of Singapore.

2.13.4 Amino acid composition of purified CDase

Amino acid composition of purified CDase was determined using a Hitachi amino acid analyzer (Model L8500) at the Enzyme Chemistry Laboratory, Osaka City University, Japan. The enzyme sample was hydrolyzed with 6 M HCl in evacuated tubes at 110°C for 24, 48, 72 h in the Hitachi-Tag Workstation. The hydrolyzed sample was then dried under vacuum and resuspended in 100 μl Hitachitag™ sample diluent. The amino acid mixtures obtained were analyzed on a Hitachi Custom Ion Exchange Resin column (4.6x60 mm). The reaction formed a blue ninhydrin-amino acid derivative detectable at 570 nm for most residues and at 440 nm for the ninhydrin-proline derivative. The amino acid composition was then calculated by Maxima 820 program.

2.13.5 Effect of pH on CDase activity

The purified CDase was incubated with β-CD at different pHs and the enzyme was assayed as described in section 2.10. The 0.1 M of acetate, phosphate, Tris-HCl and glycine-NaOH were used as reaction buffers for pH 3.0-5.0, 5.0-7.0, 7.0-9.0 and

9.0-11.0, respectively. The result was expressed as a percentage of the relative activity. The pH at which maximum activity was observed was set as 100%.

2.13.6 Effect of temperature on CDase activity

The purified CDase was assayed by the method as described in section 2.10 at various temperatures from 0 to 100 ^{o}C. The result was expressed as a percentage of the relative activity. The temperature at which maximum activity was observed was set as 100%.

2.13.7 Effect of pH on CDase stability

The purified CDase was incubated at 4 ^{o}C for 24 h in 10 mM buffers at various pHs. An aliquot of the enzyme solution was withdrawn and the remaining activity was assayed as described in section 2.10. The buffers (10 mM) used were as in 2.13.6. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100%.

2.13.8 Effect of temperature on CDase stability

The thermostability of the enzyme was investigated over the range of 40-60^{o}C. The purified enzyme in 10 mM potassium phosphate buffer, pH 7.5 was preincubated at temperature 40, 50, and 60 ^{o}C for 0 to 60 min, then the residual activity was assayed as described in section 2.10. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100%.

2.13.9 Effect of temperature upon long-term storage

The purified CDase was kept at 30, 4, -20 and –80 ^{o}C for five weeks. The residual activity was determined by the method as described in section 2.10. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100%.

2.13.10 Substrate specificity of CDase

Various substrates at 1% (w/v) concentration were incubated with CDase (5 μl, 0.6 mg/ml) in 0.1 M phosphate buffer, pH 7.0 at 40 ^{o}C for 30 min. Total reaction volume was 0.5 ml. The enzyme was assayed as described in section 2.10. The result was expressed as a percentage of the relative activity and the maximum activity was set as 100%.

2.13.11 High performance liquid chromatography (HPLC)

The purified CDase (0.5 ml, 0.6 mg/ml) was incubated with 2.5 ml of various substrates at 2% w/v in 0.1 M phosphate buffer, pH 7.0 at 40^{o}C for 24 h. The reaction was stopped by boiling for 5 min. Hydrolytic products were filtered through a 0.45 μm membrane and analyzed by HPLC using Spherisorb 10 NH_2 column (4.6x250 mm) (Phenomenex, USA) and the refractive index detector (Shimadzu model RID-10A). The column was eluted by 70% acetonitrile with the flow rate of 1.0 ml min^{-1} at room temperature. The oligosaccaride peaks were identified by comparing the retention times with standards.

For time course, hydrolysis of 2% (w/v) β-CD by CDase was performed in 0.1 M phosphate buffer, pH 7.0 at 40^{o}C for 0.5, 1, 3, 6, 12, 18 and 24 h. The reaction was stopped by boiling for 5 min and then analyzed by HPLC.

2. 13.12 Thin layer chromatography (TLC) (Ammar *et al*., 2002)

TLC was run on silica gel (Kiesel Gel 60, Merck), and was developed at ambient temperature for 5 h. with the solvent system of 1-propanol : ethylacetate : water (7 : 1 : 2). Spots were visualized by dipping the plate in a solution of ethanol : sulfuric acid (9 : 1), drying, and heating at 110^{o}C for 15 min.

The hydrolytic products were prepared as mentioned in 2.13.11. After stop reaction, the reaction mixture was lyophilized and dissolved with 1 ml of deionized water before run on TLC plate.

2.13.13 Nuclear magnetic resonance (NMR) (Ammar *et al*., 2002)

Structural identification of different hydrolytic products produced by CDase was performed by ^{1}H-NMR with a Varian-UNITY plus 500 NMR spectrometer operating at 500 MHz in D_2O at ambient temperature, at the Enzyme Chemistry Laboratory, Osaka City University, Japan. Chemical shifts were measured with sodium-4,4-dimethyl-4-sila-pentane sulfonite (DSS) as an internal standard.

The hydrolytic products were prepared as described in 2.13.12. The dried sample was dissolved with 1 ml of D_2O containing 0.1% DSS, then analyzed by NMR.

2.13.14 Kinetic studies of CDase

Initial velocity studies for the hydrolysis reaction were carried out under the standard reaction condition as described in section 2.10. The reaction time of 10 min was used to incubate between enzyme with CD substrates. Up to a reaction time of 20 min, the only product seen was linear maltoheptaose when β-CD was used as substrate. The concentrations of CD substrates were varied from 0.5-16 mM. The Lineweaver-Burk of initial velocity against CD concentration was plotted and kinetic parameters were then determined using the EnzFitter program, version 2.0.14.0 (Biosoft).

2.13.15 Effect of metal ions, protective chemicals and saccharides on CDase activity

The purified CDase (30 μl, 0.6 mg/ml) was incubated in 10 mM phosphate buffer, pH 7.5 at 40^{o}C for 30 min in the presence of various metal ions, protective chemicals and saccharides at the final concentration of 10 mM. Total incubation volume was 60 μl and 20 μl was withdrawn for the assay of residual activity by using the 3,5 dinitrosalicylic acid method as described. The residual activity was compared with the control condition and reported as a percentage of the relative activity.

2.13.16 Enzyme inhibition by acarbose

CDase (15 μl, 0.6 mg/ml) in 10 mM phosphate buffer, pH 7.5 was mixed with various concentrations of β-CD and a fixed concentration of acarbose in a final

volume of 0.5 ml. The reaction mixture was incubated at 40 ^{o}C for 30 min. The residual activity was assayed as described in section 2.10. The velocity (v) was determined and expressed as unit of enzyme.

From all data, the plots of reciprocal initial velocities ($1/v_o$) against reciprocal substrate concentrations (1/[S]) at several concentrations of inhibitor were made for kinetic mechanism analysis. The inhibition constant (K_i) was calculated using the equation (Palmer, 1981) :

$$\text{Slope of competitive inhibitor} = \{ 1+\frac{[I]}{K_i} \} \frac{K_m}{V_{max}}$$

Where [I] is inhibitor concentration (mM), K_m is Michaelis-Menten constant (mM) and V_{max} is maximum velocity (μmole/min).

2.13.17 Effect of group-specific reagents on CDase activity

To investigate the active site residue of this enzyme, various group-specific reagents were tested for their abilities to inactivate the CDase activity. CDase in 10 mM phosphate buffer, pH 7.5, was incubated with reagents (Means and Feeney, 1971; Miles, 1977; Lundblad, 1991; Wakayama *et al*., 1996) at 40 °C for 30 min. The final concentration of the enzyme and reagents were 0.3 mg/ml and 5 mM, respectively. Only for the reagents, *N*-bromosuccinimide (NBS) and diethylpyrocarbonate (DEP), 0.1 and 1 mM final concentrations were used. The total volume of the reaction mixture was 60 μl. After incubation, samples (20 μl) were withdrawn and the residual activities were measured as described in section 2.10.

2.13.18 Protection of inactivation by substrate

To investigate that the tryptophan residue(s) is located at or near the active site of the enzyme, the experiment was performed by incubating 1 mM NBS with CDase in the presence or the absence of β-CD as protective substance. CDase in 10 mM phosphate buffer, pH 7.5, was preincubated with 1% or 2% (w/v) β-CD at 40 °C for 5 min prior to the addition of 1 mM NBS. The final concentration of CDase was 0.15 mg/ml. The total volume of the reaction mixture was 2.4 ml. After incubation with NBS for 5 min, the residual activity was measured as described in 2.10. The fluorescence emission spectrum of the CDase in different modification conditions were determined by using Fluorescence Spectrophotometer Model LS 55 (Perkin Elmer, UK). The excitation wavelength was fixed at 280 nm.

2.14 Cloning of cyclodextrinase gene from *Paenibacillus* sp. A11 into *Escherichia coli* cells

2.14.1 Extraction of chromosomal DNA of *Paenibacillus* sp. A11

A single colony of *Paenibacillus* sp. A11 grown on the screening medium was cultured in 10 ml LB broth at 37 ^{o}C with 250 rpm shaking for an overnight. The culture was transferred to 1.5 ml microcentrifuge tube, centrifuged at 10,000 xg for 5 min, discarded supernatant and resuspended cells with 100 μl SET buffer (20% sucrose, 50 mM Tris-HCl pH 7.6 and 50 mM EDTA). The cell suspension was added with 400 μl SET buffer containing 5 mg/ml lysozyme, incubated at 37 ^{o}C for 1 h.

Then 5 μl of 10% (w/v) SDS was added followed by 3 μl of 20 mg/ml proteinase K, gently mixing by inverting the tube, and incubated at 50 ^{o}C for an overnight.

The cell debris was separated from the supernatant by centrifugation at 12,000 xg for 10 min. After that, 50 μl of 3 M sodium acetate was added and gently mixed followed by an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and gently mixed. The mixture was separated into two phases by centrifugation at 12,000 xg for 10 min. The upper liquid phase was transferred to a new tube. This process was repeated several times until no white precipitate of protein was observed between the two phases.

After the upper aqueous phase was transferred to a new tube and added 2.5 volume of absolute ethanol at room temperature and mixed by inversion, genomic DNA was collected by centrifugation at 12,000 xg for 10 min. The supernatant was discarded and the genomic DNA precipitate was washed with 70% ethanol, air-dried, dissolved in 100 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and incubated at 37 ^{o}C overnight. Store the genomic DNA at 4 ^{o}C.

The purity of DNA was calculated from the absorbance at 260 nm divided by the absorbance at 280 nm (A_{260}/A_{280}). The DNA with the ratio range between 1.8-2 was good for further used.

2.14.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze the various sizes of DNA fragment, PCR product and plasmid. The size was estimated from standard curve of DNA markers, fragments of Lambda DNA digested with *Hin*d III range 23-0.6 kb and

100bp ladder range 1.5-0.1 kb. The standard curve was plotted between logarithmic fragment sizes and their relative mobilities.

The concentration of agarose gel used depends on the size of the DNA fragment to be separated. Generally, 0.7-1.5% gel in 1xTAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) was used.

DNA solution was mixed with 10-20% (v/v) of 6x loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 15% (w/v) Ficoll 400 in distilled water]. The mixture was loaded into the well of the submerged gel in electrophoretic chamber filled with 1xTAE. Electrophoresis was carried out at constant 50-100 volts.The duration of running depended on the size of DNA.

Generally, the gel was run until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 μg/ml in distilled water) for 3-5 min and then destained with distilled water 2-3 times. The DNA was visualized on an UV transilluminator and photographed.

2.14.3 PCR amplification of partial CDase gene

The nucleotide sequences of CDase genes from *Clostridium thermohydrosulfuricum* 39E (Saha and Zeikus, 1990), *Bacillus sphaericus* (Oguma *et al.*, 1990), alkalophilic *Bacillus* sp. I-5 (Kim *et al.*, 1998), *Alicyclobacillus acidocaldarius* (Matzke *et al.*, 2000) were downloaded from the GenBank and aligned using Clustal W software (Thompson *et al.*, 1994). Four oligonucleotides of various lengths were designed from the homologous sequences as follows;

Forward primer

Primer 3 5′GAY GGN TGG MGN YNT GAY GT 3′

Primer N 5′TAT CAR ATW TTY CCT GAD CGS TT 3′

Reverse primer

Primer 4 5′ GN TCN CAY GAY CAN GCN MGN 3′

Primer C 5′GAY GCG GTS ATG AAY TAT CC 3′

Abbreviations : **Y** = C,T; **R** = A,G; **M** = A,C; **W** = A,T; **S** = C, G; **D** = A,G,T; **N** = A,G,C,T

They were synthesized by the Bioservice Unit (BSU), Thailand and used as PCR primers. The PCR reaction was carried out in 25 μl in the presence of 0.5 μg of chromosomal DNA from *Paenibacillus* sp. A11 and 1 μM of each selected primer. The PCR started with a cycle of 94^{o}C for 6 min, 45 ^{o}C for 2 min and 72 ^{o}C for 3 min, followed by 25 cycles of 94^{o}C for 1 min, 45 ^{o}C for 2 min and 72 ^{o}C for 3 min and ended with a cycle of 94^{o}C for 1 min, 45 ^{o}C for 2 min and 72 ^{o}C for 13 min. The amplified products were visualized under UV illumination after electrophoresis on a 1.5% SeaKem®LE agarose gel in the presence of ethidium bromide. A PCR product was ligated with pGEM® T-Easy vector and transformed into *E. coli* XL-1 blue. The nucleotide sequence of partial CDase gene was determined by using M13 forward and reverse primers. The PCR product was also used as probe for the screening of CDase gene.

2.14.4 Labeling of the DNA probe

The 3-C PCR product from 2.14.3 was labeled with marker using the DNA-labeling kit and used as a probe for Southern blot and colony hybridization. The principle of the DNA-labeling kit is based on enhanced chemiluminescence which is outlined in Figure 14. First of the DNA probe labeling, the 10 ng/μl (25μl) of the 3-C PCR product was denatured by heating for 5 min in a boiling water bath and immediately cooling on ice for 5 min. After quick spin, the DNA sample was added the equal volume of DNA labeling reagent (25μl) and glutaraldehyde solution (25μl). The reaction mixture was incubated at 37 ^{o}C for 10-30 min. If not used immediately, the probe could be held on ice for a short period, for example 10-15 min.

2.14.5 Preparation of chromosomal DNA fragments for cloning

The 10 μg of chromosomal DNA per reaction was digested with various restriction enzymes to completion. The digests were subjected to 0.7% agarose gel electrophoresis and the DNA fragments were Southern blotted onto a nylon membrane for hybridization with the 3-C PCR probe as followed process;

2.14.5.1 Southern blot hybridization (Southern, 1975)

A blotted membrane was put in hybridization buffer (see Appendix E) and prehybridized at 42 ^{o}C for 1 h. The labeled probe from section 2.15.4 was added and incubated overnight at 42 ^{o}C. After, the hybridized nylon membrane was washed 2 times with the primary wash buffer (see Appendix E) at 42 ^{o}C for 20 min and

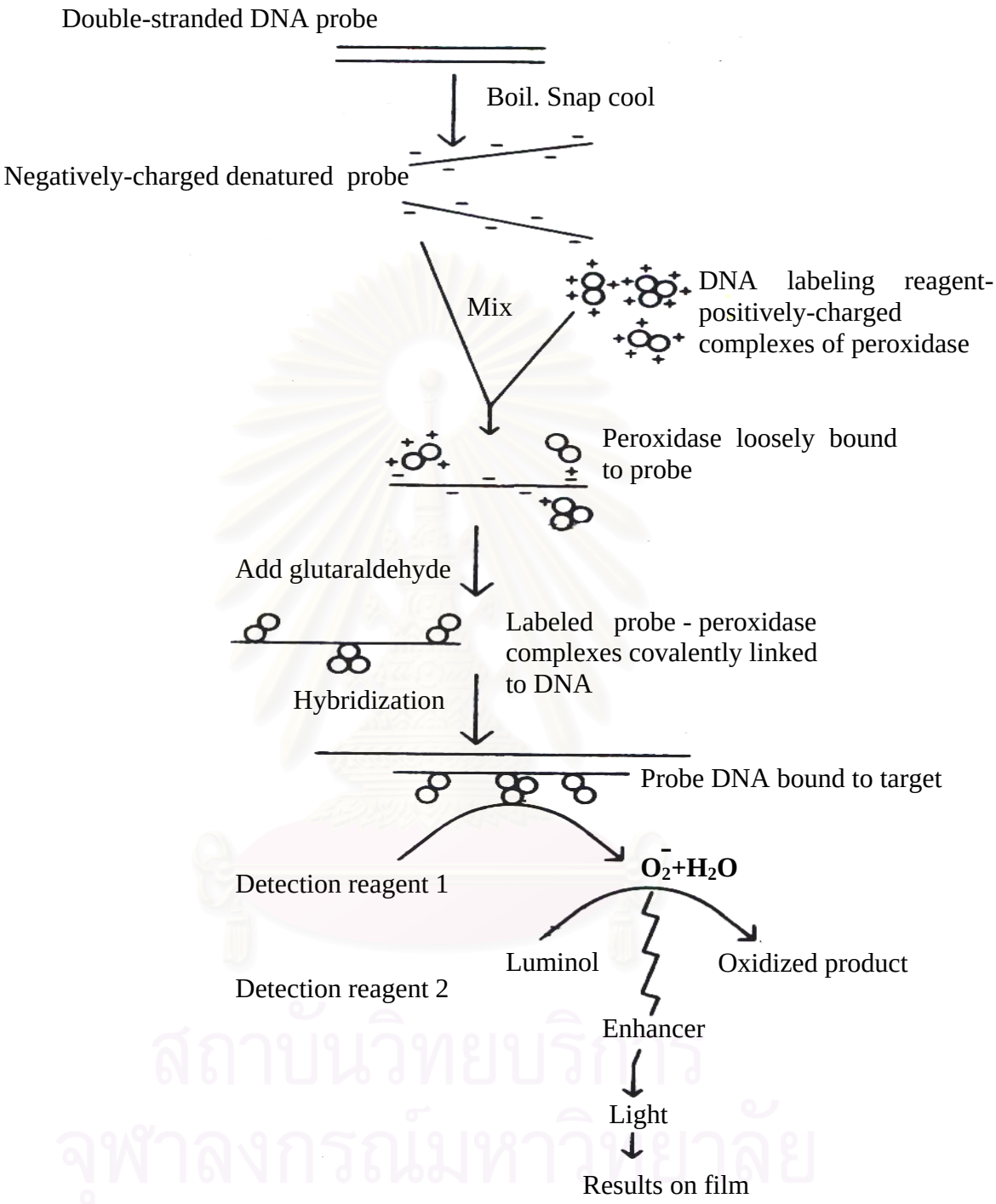

Figure 14 Principle of the ECL™ direct nucleic acid labeling and detection system

further washed 2 times with the fresh secondary wash buffer (see Appendix E) at room temperature for 5 min. The hybridized nylon membrane was drained the excess secondary wash buffer and put on a sheet of Saran Wrap with DNA side up. The detection reagent was directly added onto the hybridized membrane and incubated for 1 min at room temperature. The excess detection reagent was drained and the hybridized membrane was wrapped in Saran Wrap and subjected to autoradiography.

2.14.5.2 Autoradiography

All procedures for autoradiography was performed in a dark room. The hybridized DNA side was closely placed with a sheet of autoradiographic film (HyperfilmMP, Amersham) in the film cassette for 30-60 min at room temperature. The film was then removed and developed by using developer and fixer. The hybridization signal was seen on the film.

The hybridization signal revealed the locations of the DNA fragments containing the CDase gene in the agarose gel. An agarose gel piece at about the size where the hybridized DNA fragment located was excised. The DNA fragments were eluted and ligated with pUC 18 before transformed into *E. coli* JM 109 using conventional DNA cloning technique (Maniatis *et al*., 1982).

2.14.6 Plasmid DNA preparation by alkaline extraction

Plasmid DNA vector, pUC 18 was extracted by alkaline lysis method (Maniatis *et al*., 1982).

A single colony of *E. coli* JM 109 containing pUC 18 was inoculated into 1.5 ml of terrific broth, containing ampicillin 100 μg/ml and incubated at 37 ^{o}C with 250 rpm shaking overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 8,000 xg for 1 min. The supernatant was discarded, then 100 μl of solution I [25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose and 0.5% (w/v) lysozyme] was added, mixed by vigorous vortexing and placed on ice for 15 min. Two hundred microliters of freshly prepared solution II [0.2 N NaOH and 1% (w/v) SDS] was added for cell lysis and DNA denaturation, mixed gently by inverting the tube, and incubated on ice for 10 min.

The mixture was added with 150 μl of solution III (3M sodium acetate, pH 4.8) for renaturation, mixed gently and placed on ice for 30 min. The tube was centrifuged at 12,000 xg for 10 min to separate the pellet. The supernatant was transferred into a new 1.5 ml microcentrifuge tube, added an equal volume of a solution containing phenol : chloroform : isoamyl alcohol (25:24:1, v/v/v), mixed and centrifuged at 12,000 xg for 10 min. The upper aqueous phase was transferred to a new tube.

The plasmid DNA was precipitated by adding 2.5 volumes of absolute ethanol then mixed well and kept at –80 ^{o}C for least 10 min. The mixture was centrifuged at 12,000 xg for 10 min. The plasmid DNA was washed with 70% (v/v) ethanol, evaporated trace ethanol at 50 ^{o}C and then dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0) containing 20 μg/ml RNase A and incubated at 37 ^{o}C for overnight. Store the plasmid DNA at –20 ^{o}C.

The purity of plasmid DNA was calculated from the absorbance at 260 nm divided by absorbance at 280 nm (A_{260}/A_{280}). The plasmid with the ratio range between 1.8-2 was good for further used.

2.14.7 Ligation

The pUC 18, digested with *Sma* I and dephosphorylated with bacterial alkaline phosphatase, was used as vector. The DNA ligation mixture contained 100 ng of vector, 500 ng of the 5 kb *Pvu* II-digested DNA fragment from the section 2.14.5, 1x ligation buffer [50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (w/v) polyethylene glycol-8000], and 5 units of T4 DNA ligase in a total volume of 20 μl. The reaction was incubated at 16 ^{o}C for 16-24 h (Sambrook *et al*., 1989). The ligation mixture was stored at –20 ^{o}C until used.

2.14.8 Competent cell preparation

The competent *E. coli* strain JM 109 and XL-1 Blue were prepared according to the method of Sambrook *et al.* (1989). A single colony of *E. coli* JM 109 was cultured as the starter in 12 ml of LB broth and incubated at 37 ^{o}C with 250 rpm shaking for overnight. One percent of starter was inoculated into 200 ml LB broth in 1,000 ml Erlenmeyer flask and cultivated at 37 ^{o}C with 250 rpm shaking for about 3 h until the absorbance at 600 or 650 nm (A_{600} or A_{650}) of the cells reached 0.5-0.6 or 0.4-0.5, respectively.

The cells were chilled on ice for 15-30 min and harvested by centrifugation at 8,000 xg for 15 min at 4 ^{o}C. The supernatant was removed as much as possible. The cell pellet was washed with 800 ml of cold steriled water, resuspended by gently mixing and centrifuged at 8,000 xg for 15 min at 4 ^{o}C. The supernatant was discarded. The pellet was then wash and centrifuged with 400 ml of cold steriled water, followed by 20 ml of ice cold steriled 10% (v/v) glycerol, and finally resuspended in a final

volume of 1.6-2.0 ml ice cold steriled 10% (v/v) glycerol. This cell suspension was divided into 40 μl aliquots and stored at –80 ^{o}C until used. Usually, these competents were good for at least 6 months under these conditions.

2.14.9 Electrotransformation

The competent cells were thawed on ice. Fourty microlitres of the cell suspension was mixed with 1 μl of the DNA ligation mixture from section 2.14.7, mixed well and placed on ice for 1 min. The mixture of competent cell and DNA was electroporated in a cold 1 cm cuvette with apparatus setting as follows; 25 μF, 200 Ω of the pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were immediately resuspended with 1 ml of SOC medium [2.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.06% (w/v) NaCl, 0.02% (w/v) KCl, 0.2% (w/v) $MgCl_2$, 0.12% (w/v) $MgSO_4$ and 9% (w/v) glucose] and transferred to a sterile test tube. The cell suspension was incubated at 37 ^{o}C with shaking at 250 rpm for 1 h. All of the cells in the suspension was spread on the LB agar plates, containing 25 μl of 100 mg/ml ampicillin, 30 μl of 25 mg/ml iso-1-thio-β-D-galactopyranoside (IPTG) and 30 μl of 25 mg/ml chloro-3-indolyl-β-D-galactopyranoside (X-gal), and incubated at 37 ^{o}C for an overnight.

The positive transformant with insertion was screened for plasmid harboring CDase gene by colony hybridization as described below.

2.14.10 Colony hybridization

The selective recombinant colonies were transferred to a nylon membrane by placing the nylon membrane on the surface of agar plate in contact with the bacterial colonies until it was completely wetted. Marked the membrane and plate in two or three asymmetric locations with black drawing ink to ensure correct orientation of the colonies. The membrane was placed with colony side up for 5-30 min on the three sheets of Whatman 3MM paper which was saturated with 0.4 N NaOH while the plate was continuously incubated at 37 ^{o}C for an overnight. The nylon membrane was immersed in 400 ml of 0.4 N NaOH for 5 min. Then, the cellular debris was removed by strongly rubbing the membrane to avoid non-specific binding of DNA-labeled probe. The cleaned nylon membrane was then hybridized with the 3-C PCR probe as described in section 2.14.5.1.

2.15 Characterization of the cloned CDase gene

2.15.1 Mapping of recombinant plasmid containing CDase gene

A plasmid pJK 555 was extracted from the positive colony from section 2.14.10 according to the method as described in section 2.14.6. The map of the plasmid containing CDase gene was constructed using single and double digestion of the plasmid with restriction enzymes *Eco*R I, *Sac* I, *Kpn* I, *Hin*d III, *Sma* I, *Pst* I, *Sal* I and *Xho* I using the manufacturers's recommended conditions (New England BioLabs). DNA fragments were separated on 1.2% agarose gel electrophoresis at 100

volts in TAE buffer. Standard marker λ/*Hin*d III, 100 bp ladder and pUC 18 linear form were used.

2.15.2 DNA sequencing

For sequencing, plasmid DNA (pJK 555) was further purified by using QIAquick Plasmid Extraction kit (QIAGEN, Germany). DNA sequencing was performed sequentially by first using M13 forward, M13 reverse, 3 and C primers. The obtained DNA sequences were then used to design additional primers for further DNA sequencing. The designed primers were H1, H2, T1, T2, T3 and T4 as shown below;

Primer H1	5′ GCC TTC GGG ATC ATT GCG GG 3′
Primer H2	5′ GTC CTC GGT CGC TTT AG 3′
Primer T1	5′ GGA ATG GGA CGA GAC GAA GC 3′
Primer T2	5′ TTG ACC GTG AAG CTC CC 3′
Primer T3	5′ GGG GAT GCC ATG ACG GT 3′
Primer T4	5′ CTT GAG GTG ATC GGG TG 3′

2.15.3 The three-dimensional (3D) structure of CDase

The 3D structure of CDase was modeled by Swiss-Model ver 36 (Peitsch, 1996) from the deduced amino acid sequence obtained.

2.15.4 Expression of recombinant CDase

E. coli JM109 containing the pJK 555 plasmid was aerobically cultured in LB medium containing 100 μg/ml ampicilin either in the presence or absence of IPTG for 24 h at 250 rpm and 37 ^{o}C. After that, cells were centrifuged at 8,000 xg for 15 min, washed twice with 10 mM phosphate buffer, pH 7.0, resuspended in cold extraction buffer (10 mM phosphate buffer, pH 7.0 containing 1 mM DTT, 1 mM EDTA and 1 mM PMSF) and sonicated with SONOPULS ultrasonic homogenizer (3 mm diameter-stepped microtip, 15% amplitude, Bandelin, Germany) for 2 cycles of 5 min sonication with 5 min pulse. The homogenate was centrifuged at 100,000 xg for 1 h. CDase activity and protein determination were then measured in the supernate and also the pellet fractions as described in the section 2.10 and 2.11. CDase activity of pJK 555 plasmid was compared with those of other transformants used as positive and negative controls. Protein staining of all transformants was performed as described in section 2.12.3.1.

2.15.5 Stability of CDase gene in *E. coli* JM 109

The pJK 555 transformant was aerobically cultured in LB medium containing 100 μg/ml ampicilin for 24 h at 250 rpm and 37 ^{o}C. Cells were harvested by centrifugation. CDase activity and protein determination were measured as described in the section 2.10 and 2.11. The experiment with pJK 555 transformant was continued for another 6 times.

2.16 Effect of polyols on CDase expression

E. coli JM 109 containing the pJK 555 plasmid was cultured in 100 ml of LB medium with or without 0.5 M of each polyol in 500 ml Erlenmeyer flasks. The culture was shaken at 250 rpm, 37^{o}C for 24 h. The best polyol was chosen according to its ability in CDase overproduction. Its concentration in the medium was varied and cell growth and CDase activity were followed. Comparing CDase protein in the medium with or without polyol was performed on SDS-PAGE (Weber and Osborn, 1975).

2.17 Subcloning of pJK 555 transformant

In an attempt to improve the expression level, the size of cloned DNA was reduced. The 7.5 kb pJK 555 was digested with *Cla*I and *Kpn*I restriction enzymes and treated with Klenow. The 1.7 kb DNA fragment was removed. The 5.8 kb fragment was religated with T4 DNA polymerase and retranformed into *E. coli* JM 109 host cell. The resulting construct was designated as pJK 556. The crude CDase was prepared from the pJK 556 clone, culturing with 0.5 M sorbitol. The activity was measured and compared with that of pJK 555 clone and the original strain A11.

2.18 Overexpression and extraction of recombinant CDase

E. coli JM 109 (pJK 555) was grown in 2 litres of LB medium containing 0.5 M sorbitol at 37^{o}C for 24 h. Cells (around 12 g wet weight) were harvested by centrifugation at 8,000 xg for 15 min at 4^{o}C and washed twice in 10 mM phosphate buffer, pH 6.5. Cells were then resuspended in cold extraction buffer (10 mM

phosphate buffer, pH 6.5 containing 1 mM DTT, 1 mM EDTA and 1 mM PMSF) and sonicated with SONOPULS ultrasonic homogenizer (3 mm diameter-stepped microtip, 15% amplitude, Bandelin, Germany) for 5 cycles of 5 min sonicate and 5 min pulse. The homogenate was centrifuged at 100,000 xg for 1 h. The supernatant was used as crude CDase preparation. CDase activity was assayed by using the 3,5 dinitrosalicylic acid method (Bernfeld, 1955) as described in section 2.10. The amount of protein was determined by the Coomassie blue method (Bradford, 1976), using bovine serum albumin (BSA) as standard as described in section 2.11.

2.19 Purification of recombinant CDase

Preparation of crude recombinant enzyme and purification procedures were the same as described for original enzyme from the strain A11 (section 2.9) except that DEAE-Sephadex A50 was replaced by DEAE-Toyopearl 650M column.

2.20 Characterization of the purified recombinant CDase from *E. coli* JM 109

Investigation on main characters of the purified recombinant enzyme was performed using the same methods as mentioned for the original enzyme (section 2.13).

CHAPTER III

RESUTLS

Part I : BACTERIAL IDENTIFICATION

A bacterium in this study was isolated from South-East Asian soil and was originally classified as *Bacillus circulans* A11 by using morphological and biochemical characterization (Pongsawasdi and Yagisawa, 1987). To clarify the taxonomy of the strain A11, we analyzed 16S rRNA gene, cellular fatty acid composition and G+C content. Identification by 16S rRNA gene fragment amplification is the more recent technique widely used to confirm bacterial classification.

3.1 Analysis of 16S rRNA gene

3.1.1 Amplification of 16S rRNA gene fragment

Chromosomal DNA of A11 was extracted and checked by agarose gel electrophoresis and spectrophotometry ($A_{260/280}$) as mentioned in section 2.14.1 and 2.14.2. DNA obtained was larger than 23.1 kb and the absorbance ratio was 1.90. From the gel pattern and the absorbance ratio, the extracted DNA was good enough for amplification. After amplification using specific primer for 16S rRNA gene (section 2.7.1), PCR product of 1.5 kb was obtained (Figure 15). This gene amplification product was then ligated with pGEM® T-Easy vector and transformed into *E. coli*. The resulting plasmid was called pJK 1.5 which was extracted and rechecked for size of PCR inserted fragment before sequencing.

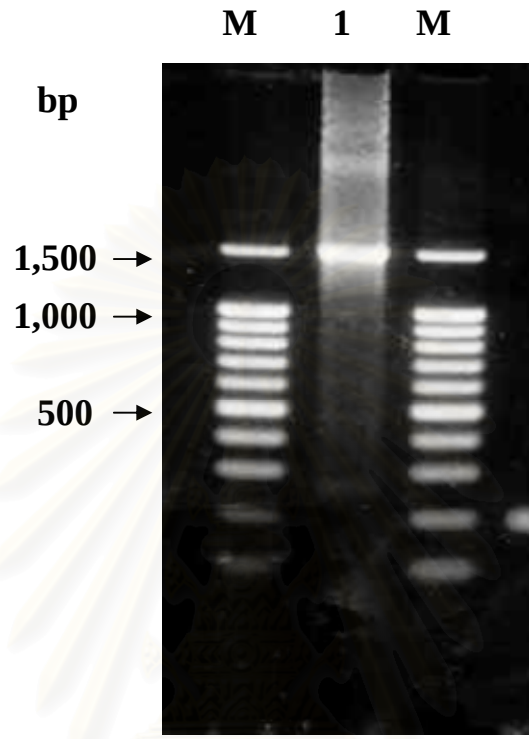

Figure 15 16S rRNA gene amplification product from the strain A11

Lane M = 100 bp marker

Lane 1 = 16S rRNA PCR product using genomic DNA of the strain A11 as a template

3.1.2 Sequencing of 16S rRNA gene

The 16S rRNA gene sequence was run by 3 primers (pA, pD and pF as described in section 2.7.2). Each primer gave more than 500 bp sequence. All sequences were extended and searched for overlapping region. The resulted sequence was 1,524 bp as shown in Figure 16 (GenBank accession no. AY237109). The alignment of this 16S rRNA gene of A11 was blasted with those deposited in the GenBank, EMBL, DDBJ and PDB databases. The result indicated that A11 showed 99% homology with *Paenibacillus campinasensis* JCM 11200 (AB073187), 97% homology with *Paenibacillus* sp. 38-2 (AB043866), and 86% homology with *Bacillus circulans* IAM 12462 (D78312) (Figure 17).

3.1.3 Phylogenetic analysis

The phylogenetic tree (Figure 18) constructed from the sequence data shows that strain A11 appeared within the evolutionary radiation encompassing the genus *Paenibacillus* and occupied a distinct phylogenetic position within the genus. Levels of 16S rRNA gene similarity between strain A11 and the *Paenibacillus* species were 90-99%. The highest 16S rRNA gene sequence similarity of 99% was observed among strain A11, *Paenibacillus campinasensis* JCM 11200 (AB073187), *Paenibacillus campinasensis* 324 (AF021924), and *Paenibacillus* sp. RB01 (Solos, 2001). The phylogenetic study clearly established that strain A11 was rather a *Paenibacillus* than a *Bacillus* species.

```
                                                                  pA
                                                  AGAGT TTGATCCTGG CTCAG-----
   ] GACAACGCTG GCGGCGTGCC TAATACATGC AAGTCGAGCG GAATCGATGG AGTGCTTGCA
  61 CTCCTGAGAT TTAGCGGCGG ACGGGTGAGT AACACGTAGG CAACCTGCCC TCAAGACTGG
 121 GATAACTACC GGAAACGGTA GCTAATACCG GATAGGATAT TTGGCTGCAT GGCCGGATAT
 181 GGAAAGGCGG AGCAATCTGT CACTTGAGGA TGGGCCTGCG GCGCATTAGC TAGTTGGTGG
 241 GGTAACGGCC TACCAAGGCG ACGATGCGTA GCCGACCTGA GAGGGTGAAC GGCCACACTG
 301 GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT AGGGAATCTT CCGCAATGGA
 361 CGAAAGTCTG ACGGAGCAAC GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG
 421 TTGCCAGGGA AGAACGCCAG AGAGAGTAAC TGCTCTTTGG GTAACGGTAC CTGAGAAGAA
                                      pD
 481 AGCCCCGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT AGGGGGCAAG CGTTGTCCGG
 541 AATTATTGGG CGTAAAGCGC GCGCAGGCGG TTCTTTAAGT CTGGTGTTTA AACCCGGAGC
 601 TTAACTTCGG GACGCACTGG AAACTGGGGA ACTTGAGTGC AGAAGAAGAG AGTGGAATTC
 661 CACGTGTAGC GGTGAAATGC GTAGATATGT GGAGGAACAC CAGTGGCGAA GGCGACTCTC
 721 TGGGCTGTAA CTGACGCTGA GGCGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
 781 GTAGTCCACG CCGTAAACGA TGAATGCTAG GTGTTAGGGG TTTCGATACC CTTGGTGCCG
 841 AAGTTAACAC ATTAAGCATT CCGCCTGGGG AGTACGGTCG CAAGACTGAA ACTCAAAGGA
 901 ATTGACGGGG ACCCGCACAA GCAGTGGAGT ATGTGGTTTA ATTCGAAGCA ACGCGAAGAA
 961 CCTTACCAGG TCTTGACATC CCTCTGACCC CTCTAGAGAT AGAGGTTTCC TTCGGGACAG
                                      pF
1021 AGGAGACAGG TGGTGCATGG TTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC
1081 GTTACGAGCG CAACCCTTGA TCTTAGTTGC CAGCACGTAA TGGTGGGCAC TCTAAGGTGA
1141 CTGCCGGTGA CAAACCGGAG GAAGGTGGGG ATGACGTCAA ATCATCATGC CCCTTATGAC
1201 CTGGGCTACA CACGTACTAC AATGGCTGGT ACAACGGGAA GCGAAGCCGC GAGGTGGAGC
1261 CAATCCTAAA AAGCCAGTCT CAGTTCGGAT TGCAGGCTGC AACTCGCCTG CATGAAGTCG
1321 GAATTGCTAG TAATCGCGGA TCAGCATGCC GCGGTGAATA CGTTCCCGGG TCTTGTACAC
1381 ACCGCCCGTC ACACCACGAG AGTTTACAAC ACCCGAAGTC GGTGGGGTAA CCGCAAGGAG
1441 CCAGCCGCCG AAGGTGGGGT AGATGATTGG GGTGAAGTCG TAACAAGGTA GCCGTATCGG
                   pH
1501 AAGGTGCGGC TGGATCACCT CCTT
```

Forward primer	pA	5′ AGAGTTTGATCCTGGCTCAG	3′
	pD	5′ CAGCAGCCGCGGTAATAC	3′
	pF	5′ CATGGTTGTCGTCAGCTCGT	3′
Reverse primer	pH	5′ TGCGGCTGGATCACCTCCTT	3′

Figure 16 Nucleotide sequence of 16S rRNA gene of strain A11

The primer used for amplifying and sequencing are shaded and underlined, respectively.

```
CLUSTAL W (1.81) multiple sequence alignment
Seq1            ------------------------------------------------------------
Seq2            ------------------------------------------------------------
Seq3            AGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAGAGTTTGATCCTGGCTCAG 60
Seq4            ------------------------------------GAGAGTTTGATCCTGGCTCAG 21

Seq1            -GACAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGC 59
Seq2            --CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGC 58
Seq3            GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGC 120
Seq4            GACGAACGCTGGCGGCGTGCCTNATACATGCAAGTCGAGCGGACTTTA---AAAGCTTGC 78
                    ****************** ******************** *  *   *  ******

Seq1            ACTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTG 119
Seq2            ACTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTG 118
Seq3            ACTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTG 180
Seq4            ---TNTTNAAGTNAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTG 135
                     *   * * ************************* ***********   *******

Seq1            GGATAACTACCGGAAACGGTAGCTAATACCGGATAGGATATTTGGCTGCATGGCCGGATA 179
Seq2            GGATAACTACCGGAAACGGTAGCTAATACCNGATAGGATATTTGGCTGCATGGCNGNATA 178
Seq3            GGATAACTACCGGAAACGGTAGCTAATACCGGATAGGATATTTGGCTGCATGGCCGAATA 240
Seq4            GGATAACTTCGGGAAACCGGAGCTAATACCGGATAATCCTTTTCCTCTCATGAGGAAAAG 195
                ******** * ****** * ********** ****     ***     ****     *

Seq1            TGGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTG 239
Seq2            TGGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTG 238
Seq3            TGGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTG 300
Seq4            CTGAAAGACGGTTTACGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTG 255
                  ***** ***   *  *********   ******** ********************

Seq1            GGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACT 299
Seq2            GGGTAANGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACT 298
Seq3            GGGTAATGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACT 360
Seq4            AGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACA 315
                 ***** ***  ************************************ *********

Seq1            GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG 359
Seq2            GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG 358
Seq3            GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG 420
Seq4            GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGNAGTAGGGAATCTTCCGCAATGG 375
                ************************************ ********************

Seq1            ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCT 419
Seq2            ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCT 418
Seq3            ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCT 480
Seq4            ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTNCGGATCGCAAAACTCT 435
                ************************************** ******* *** ****

Seq1            GTTGCCAGGGAAGAACGCCAGAGAGAGTAACTGCTCTTTGGGTAACGGTACCTGAGAAGA 479
Seq2            GTTGCCAGGGAAGAACGCCAGAGAGAGTAACTGCTCTTTGGGTGACGGTACCTGAGAAGA 478
Seq3            GTTGCCAGGGAAGAACGCCAGAGAGAGTAACTGCTCTTTGGGTGACGGTACCYGAGAAGA 540
Seq4            GTTGTTAGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGACGGTACCTAACCAGA 495
                ****  **********       ************  *    * ********  *  ***

Seq1            AAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCG 539
Seq2            AAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCG 538
Seq3            AAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCG 600
Seq4            AAGCCACGACTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCG 555
                ***** ** ********************************** ***************

Seq1            GAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAG 599
Seq2            GAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAG 598
Seq3            GAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAG 660
Seq4            GAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGG 615
                ****************************** ************ ***  ** ***   *

Seq1            CTTAACTTCGGGACG-CACTGGAAACTGGGGAACTTGAGTGCAGAAGAAGAGAGTGGAAT 658
Seq2            CTTAACTTCGGGACG-CACTGGAAACTGGGGNACTTGAGTGCAGAAGAGGAGAGTGGAAT 657
Seq3            CTTAACTTCGGGACG-CACTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAAT 719
Seq4            CTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGAAGAGTGGAAT 675
                ** ***  **   * ** ************ ***************  **********

Seq1            TCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTC 718
Seq2            TCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTC 717
Seq3            TCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTC 779
Seq4            TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTC 735
                ************************** ********************************
```

Figure 17 Alignment of 16S rRNA gene sequence of A11 (Seq1) with those of *Paenibacillus campinasensis* JCM 11200 (Seq2), *Paenibacillus* sp. 38-2 (Seq3) and *Bacillus circulans* IAM 12462 (Seq4)

* denote identical base

```
Seq1            TCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC 778
Seq2            TCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC 777
Seq3            TCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC 839
Seq4            TTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC 795
                * *** ****************************************************

Seq1            TGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGC 838
Seq2            TGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGC 837
Seq3            TGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGC 899
Seq4            TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGC 855
                ************************ ***** ******* ** **     ** ** ****

Seq1            CGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG 898
Seq2            CGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG 897
Seq3            CGAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG 959
Seq4            TGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAG 915
                 * **  *** ********* ****************** ****** **************

Seq1            GAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAG 958
Seq2            GAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAG 957
Seq3            GAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAG 1019
Seq4            GANTTGACGGGGGCCCGCACAAGCGGTGGNGCATGTGGTTTAATTCGAAGCAACGCGAAG 975
                ** ********* *********** **** * ****************************

Seq1            AACCTTACCAGGTCTTGACATCCCTCTGACCCCTCTAGAGATAGA--GGTTTCCTTCGGG 1016
Seq2            AACCTTACCAGGTCTTGACATCCCTTTGACCCCTCTAGAGATAGA--GGTTTCCTTCGGG 1015
Seq3            AACCTTACCAGGTCTTGACATCCCTCTGACCCCTCTAGAGATAGA--GGTTTCCTTCGGG 1077
Seq4            AACCTTACCAGGTCTTGACATCC-TCTGACACTCCTAGAGATAGGACGTCCCCCTTCGGG 1034
                ********************** * **** *  **********   *    ********

Seq1            ACAGAGGA-GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA 1075
Seq2            ACAGAGGA-GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA 1074
Seq3            ACAGAGGA-GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA 1136
Seq4            GGACAGAGTGACAGGTGGTGCATGGTTGTCGCCAGCTCGTGTCGTGAGATGTTGGGTTAA 1094
                  * **   ********************** ****************************

Seq1            GTCCCGTTACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGTAATGGTGGGCACTCTAA 1135
Seq2            GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGTGAAGGTGGGCACTCTAA 1134
Seq3            GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGTCATGGTGGGCACTCTAA 1196
Seq4            GTCCCGCAACGAGCGCAACCCNGGATCTTAGTTGGCAGCATTCAGT--TGGGCACTCTAA 1152
                ******  *************  ************ *****        ************

Seq1            GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT 1195
Seq2            GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT 1194
Seq3            GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT 1256
Seq4            GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT 1212
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Seq1            ATGACCTGGGCTACACACGTACTACAATGGCTGGTACAACGGGAAGCGAAGCCGCGAGGT 1255
Seq2            ATGACCTGGGCTACACACGTACTACAATGGCTGGTACAACGGGAAGCGAAGCCGCGAGGT 1254
Seq3            ATGACCTGGGCTACACACGTACTACAATGGCTGGTACAACGGGAAGCGAAGCCGCGAGGT 1316
Seq4            ATGACCTGGGCTACACACGTGCTACAATGGGTGGTACAAAGGGNAGNAAAACCGCGAGGT 1272
                ********************* ********* ******** *** **  ** *********

Seq1            GGAGCCAATCCTAAAAAGCCAGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGA 1315
Seq2            GGAGCCAATCCTAAAAAGCCAGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGA 1314
Seq3            GGAGCCAATCCTAAAAAGCCAGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGA 1376
Seq4            CGAGCAAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGA 1332
                 **** ***** * *** *** *************** *************** *****

Seq1            AGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTG 1375
Seq2            AGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTG 1374
Seq3            AGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTG 1436
Seq4            AGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATNCGTTCCCGGGCCTTG 1392
                **  ***** ********************************** ********** ****

Seq1            TACACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCGCA 1435
Seq2            TACACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCGCA 1434
Seq3            TACACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCGCA 1496
Seq4            TNCACACCGCCCGTCACACCACGAGAGTTTGTNACACCC---------------------- 1431
                * ************************** ******

Seq1            AGGAGCCAGCCGCCGAAGGTGGGGTAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGT 1495
Seq2            AGGAGCCAGCCGCCGAAGGTGGGGTAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGT 1494
Seq3            AGGAGCCAGCCGCCGAAGGTGGGGTAGATGATTGGGGTGAAGTCGTAACAAGGTAACCAA 1556
Seq4            ------------------------------------------------------------

Seq1            ATCGGAAGGTGCGGCTGGATCACCTCCTT 1524
Seq2            ATCGGAAGG-------------------- 1503
Seq3            GCCGAATTCC------------------- 1566
Seq4            ----------------------------
```

Figure 17 (Continued) Alignment of 16S rRNA gene sequence of A11 (Seq1) with those of *Paenibacillus campinasensis* JCM 11200 (Seq2), *Paenibacillus* sp. 38-2 (Seq3) and *Bacillus circulans* IAM 12462 (Seq4)

* denote identical base

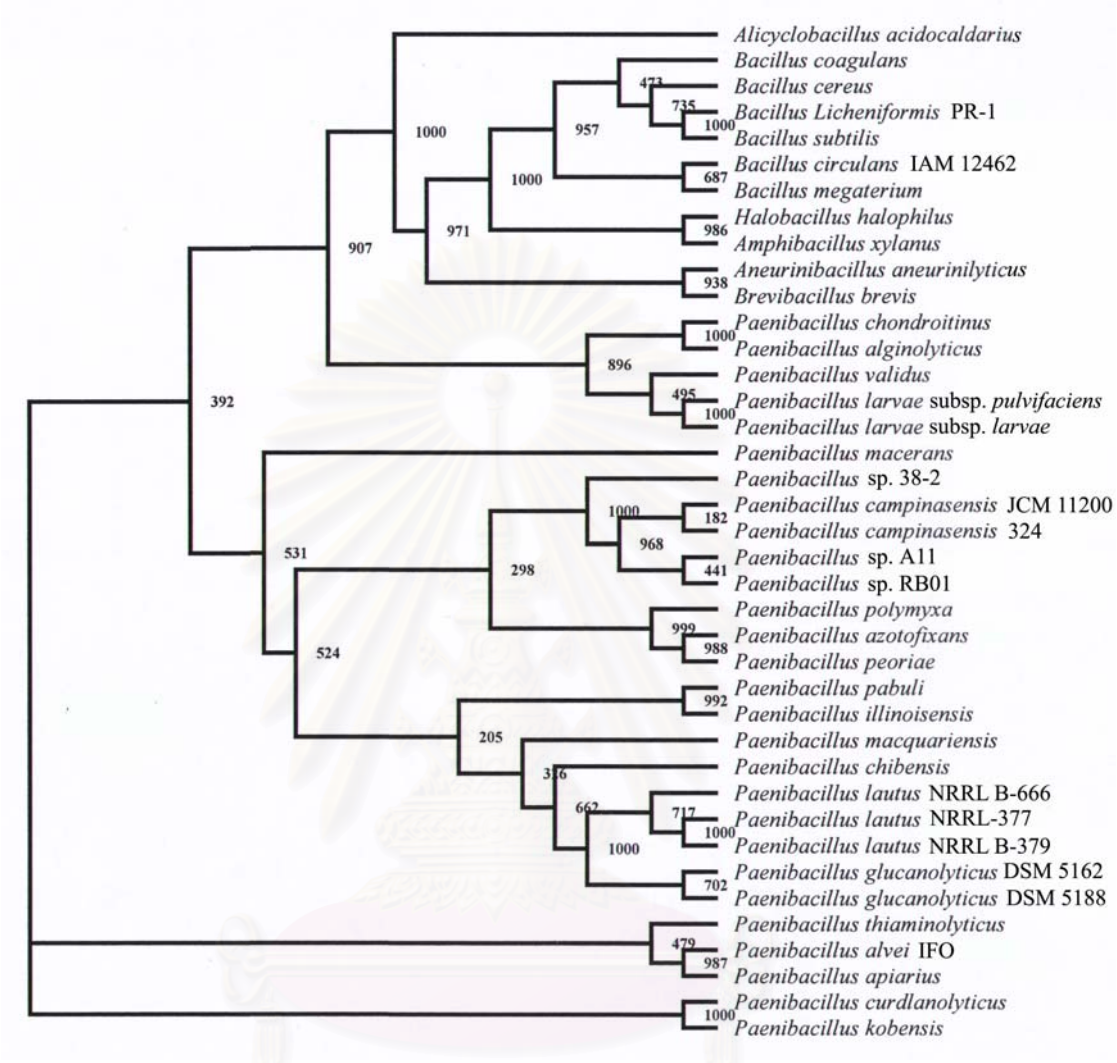


Figure 18 Phylogenetic tree showing the position of strain A11 in relation to *Paenibacillus* species and some rod-shaped, endospore-forming bacteria based on 16S rRNA gene sequences.

Bootstrap values are indicated.

3.2 Cellular fatty acid composition analysis

Extraction of fatty acids of bacterial cells was performed as described (section 2.7.4). Cellular fatty acids of the strain A11, *Paenibacillus* sp. RB01 and *Bacillus licheniformis* PR-1 were analyzed by GC (Figure 19). Saturated iso- and anteiso-branched acid of $C_{15:0}$, saturated straight-chain acid of $C_{16:0}$ and saturated iso-branched acid of $C_{16:0}$ were found as major components of the three strains. In the strain A11, anteiso-acid of $C_{15:0}$ (59.3%) was predominant fatty acid, followed by straight-chain acid of $C_{16:0}$ (18.3%), iso-acid of $C_{15:0}$ (12.0%) and iso-acid of $C_{16:0}$ (10.4%). The pattern of fatty acids was very similar to that in the strain RB01. Different pattern was observed in *Bacillus licheniformis* PR-1, the iso-acids of $C_{15:0}$ (51.6%) was predominant with smaller amounts of anteiso-acid of $C_{15:0}$ (30.9%).

3.3 Determination of G+C content

G+C content was determined using the measurement of melting temperature of the DNA. The average T_m values from triplicate experiments were 90 ^oC for A11 and 87 ^oC for *Bacillus subtilis* ATCC 6633 (using as the type strain) (Figure 20). The G+C content calculated from the T_m values by the equation of De Ley were 50.3 mol % and 42.9 mol% for A11 and *Bacillus subtilis* ATCC 6633, respectively. The G+C content of strain A11 was within the range found for the members of the genus *Paenibacilllus* (Shida *et al*., 1997).

The results from analyses of 16S rRNA gene, cellular fatty acid composition and G+C content showed that the strain A11 could be reclassified from the genus *Bacillus* into the genus *Paenibacillus*.

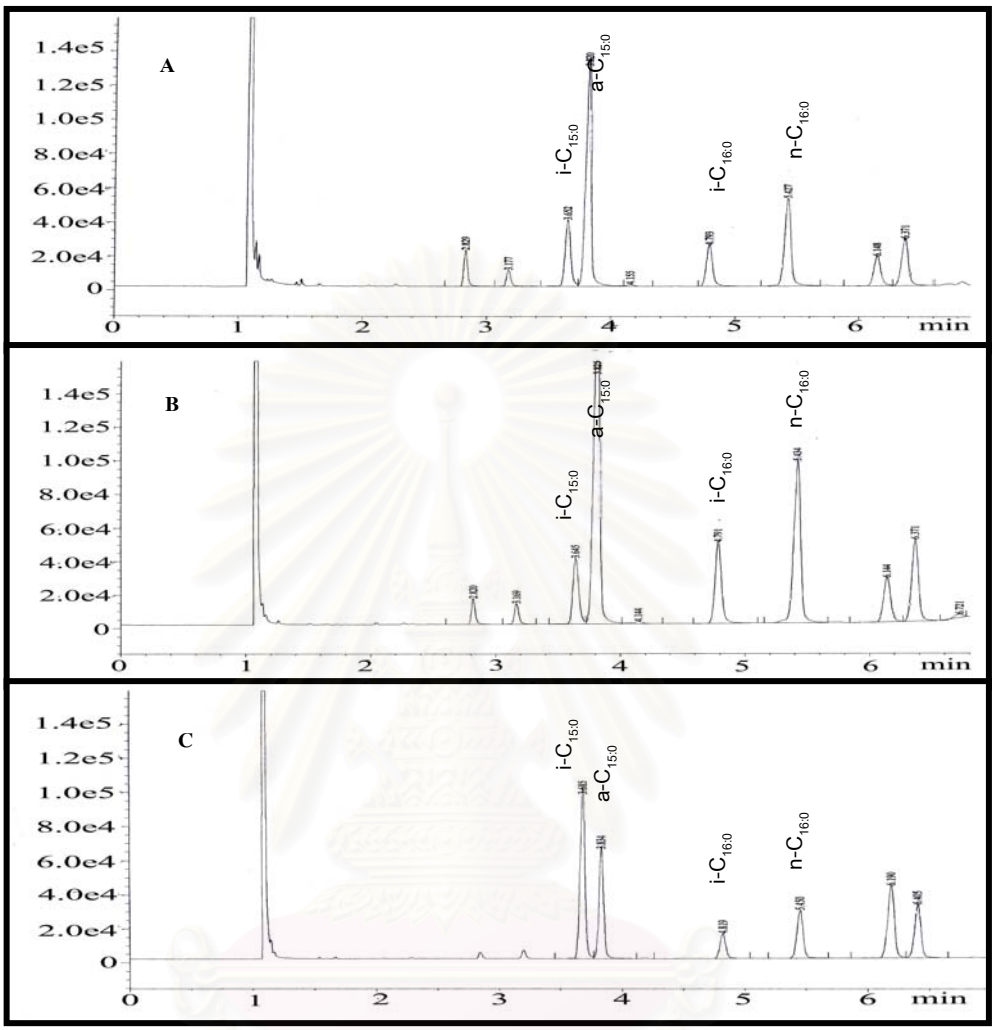


Figure 19 Gas chromatograms of cellular fatty acids of strain A11 (A), *Paenibacillus* sp. RB01 (B) and *Bacillus licheniformis* PR-1 (C)

i-$C_{15:0}$, Iso-branched acid of 13-methyltetradecanoic acid; a-$C_{15:0}$, Anteiso-branched acid of 12-methyltetradecanoic acid; n-$C_{16:0}$, Straight-chain acid of n-hexadecanoic acid, and i-$C_{16:0}$, Iso-branched acid of 14-methylpentadecanoic acid

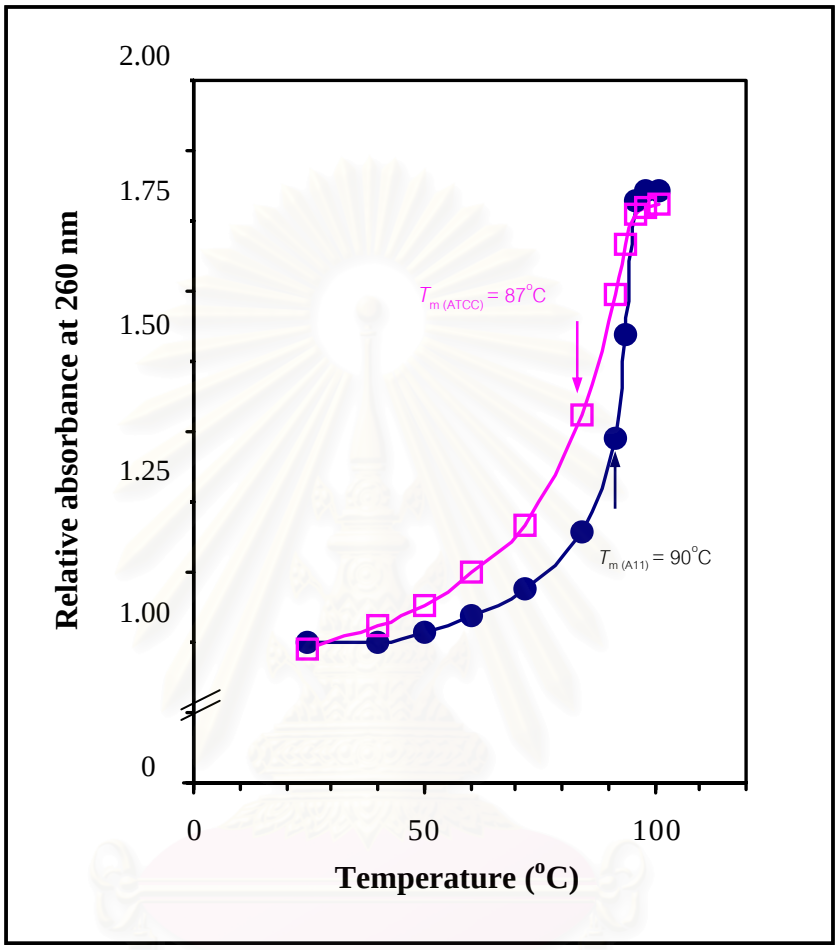


Figure 20 Melting curve of strain A11 (●) and *Bacillus subtilis* ATCC 6633 (□) chromosomal DNA

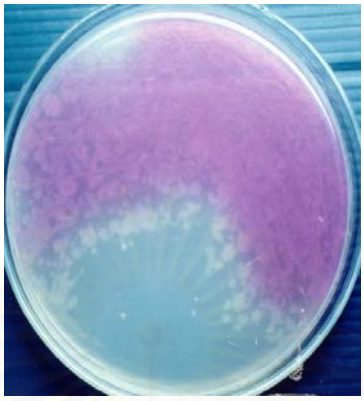
Part II : CDase FROM *PAENIBACILLUS* SP. A11

Paenibacillus sp. A11 was able to produce CDs by the action of CGTase on starch. The *Paenibacillus* sp. A11 CGTase, CDs-producing enzyme, was previously isolated and studied for biochemical properties by our research group (Rojtinnakorn *et al*., 2001). Recently, the novel starch degradation pathway via CD metabolism, including the extracellular conversion of starch into CDs by CGTase and uptake of the CDs by a specific system followed by intracellular linearization by a CDase, was proposed in a gram-negative *Klebsiella oxytoca* M5a1 and a gram-positive *Bacillus* sp. A2-5a (Ohdan *et al*., 2000). To prove existence of CD metabolism in our *Paenibacillus* sp. A11, the CD-degrading enzyme (CDase) activity was determined.

3.4 CDase production from *Paenibacillus* sp. A11

Using the specific screening medium developed in this study (section 2.5.1 and 2.6), pink color which indicates the presence of CDase activity was observed around the colonies of *Paenibacillus* sp. A11 while the control strain, *Bacillus subtilis* TISTR 25, did not produce any color (Figure 21). The color was supposed to be resulted from the release of phenolphthalein from the β-CD inclusion complex when the CD was uptaken by the cells and hydrolyzed by the intracellular CDase. A positive colony was then used for further experiment.

A



B

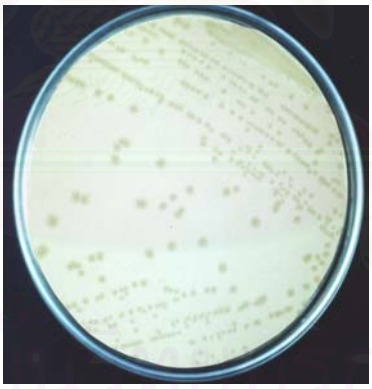


Figure 21 Expression of CDase activity from *Paenibacillus* sp. A11 on the specific screening medium

(A) *Paenibacillus* sp. A11

(B) *Bacillus subtilis* TISTR 25 (control strain)

3.5 Purification of CDase from *Paenibacillus* sp. A11

3.5.1 Preparation of crude enzyme solution

Crude CDase was prepared from 40 g (wet weight) of *Paenibacillus* sp. A11, which was cultivated from 8 litres of the modified Horikoshi medium containing 1% β-CD as described in section 2.5.3 and 2.8. Crude enzyme solution contained 680 mg protein with 4,300 units of CDase activity in total volume of 100 ml. Thus, the specific activity of the enzyme in the crude preparation was 6 units/mg protein (Table 6).

3.5.2 Ammonium sulfate precipitation

Crude enzyme solution was further purified by ammonium sulfate precipitation as mentioned in section 2.9.1. To determine the suitable ammonium sulfate concentration for precipitation of CDase, preliminary experiment was performed by a stepwise increase at 20% increment from 0 to 80%. Most of enzyme activity was detected in the 20-40 and 40-60% fractions with the highest activity in 40-60% fraction. Therefore, to harvest the most of enzyme, protein fractionation was carried out in the range of 40-60% saturated ammonium sulfate precipitation. The protein remained was 58 mg with enzyme activity yielded at 1,860 units (about 43% yield from crude enzyme). The specific activity of the enzyme from this step was 32 units/mg protein (Table 6).

Table 6 Purification of CDase from *Paenibacillus* sp. A11

Purification step	Volume (ml)	Total activity (U)	Activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	100	4300	43	680	6	1	100
40-60% $(NH_4)_2SO_4$	15	1860	124	58	32	5	43
DEAE-Sephadex A50	10	1340	134	35	38	6	31
Phenyl-Sepharose CL-4B	15	1200	80	9	133	22	28

3.5.3 DEAE Sephadex A50 column chromatography

The enzyme precipitate from 40-60% saturated ammonium sulfate was dissolved and dialyzed against 10 mM phosphate buffer, pH 7.5 containing 1 mM DTT, 1 mM EDTA and 10% glycerol (buffer A). The enzyme solution was applied onto DEAE Sephadex A50 column as described in section 2.9.2. The chromatographic profile is shown in Figure 22. The unbound proteins were eluted from the column with buffer A. The bound proteins were then eluted with linear salt gradient of 0 to 0.5 M sodium chloride in buffer A. The enzyme was eluted at 0.35 M sodium chloride solution as indicated in the profile. The fractions with CDase activity were pooled, concentrated by aquasorb to reduce enzyme volume and dialyzed against buffer A. The protein remained from this step was 35 mg with 1,340 activity units of enzyme and the specific activity of 38 units/mg protein (Table 6). The enzyme was 6 fold purified and the yield was about 31% compared with crude enzyme.

3.5.4 Phenyl-Sepharose CL-4B column chromatography

The pooled active fraction from DEAE Sephadex A50 column was further purified by Phenyl-Sepharose CL-4B column as described in section 2.9.3.The chromatographic profile is shown in Figure 23. The unbound proteins were eluted from the column with buffer A containing 1 M ammonium sulfate. The other proteins, which bound to the column, were eluted with a decreasing concentration of ammonium sulfate linear gradient from 1 to 0 M in buffer A. The enzyme was

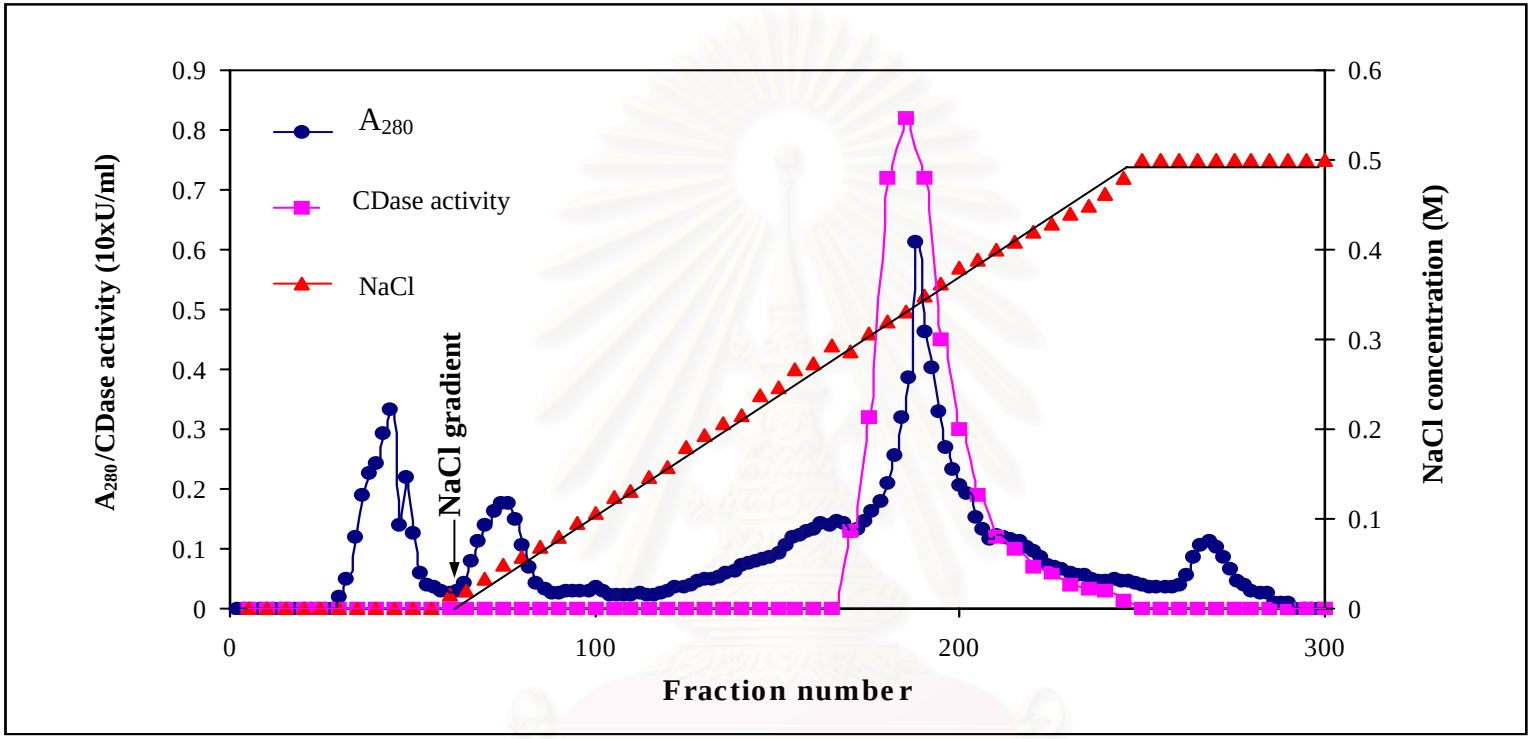


Figure 22 Purification of Cyclodextrinase from *Paenibacillus* sp. A11 by DEAE-Sephadex A50 column

The enzyme solution was applied to DEAE-Sephadex A50 column (1.7 X 45 cm) and washed with 10 mM potassium phosphate buffer, pH 7.5 containing 1 mM DTT, 1 mM EDTA and 10% glycerol until A_{280} decreased to baseline. Elution of bound proteins was performed by 0-0.5 M NaCl in the same buffer at the flow rate of 30 ml/h. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 165 to 250 was pooled.

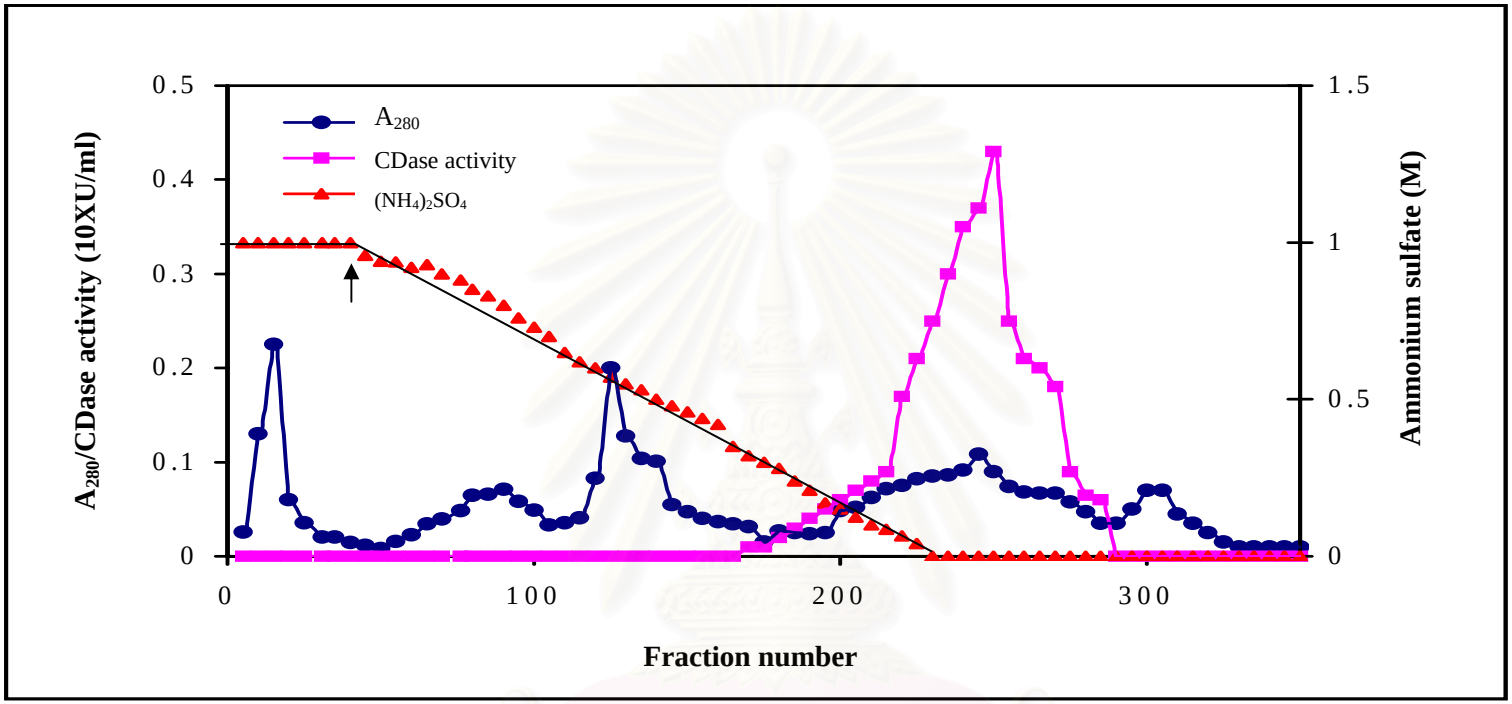


Figure 23 Purification of Cyclodextrinase from *Paenibacillus* sp. A11 by Phenyl-Sepharose CL-4B column

The enzyme solution was applied to Phenyl-Sepharose CL-4B column (1.7 X 10 cm) and washed with 10 mM potassium phosphate buffer, pH 7.5 containing 1M ammonium sulfate, 1 mM DTT, 1 mM EDTA and 10% glycerol until A_{280} decreased to baseline. Elution of bound proteins was then performed by a decreasing linear gradient of ammonium sulfate from 1 to 0 M at the flow rate of 20 ml/h. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 175 to 290 was pooled.

eluted at about 0 M ammonium sulfate concentration as indicated in the profile. The active fractions were pooled, concentrated by aquasorb and dialyzed against buffer A. This final step yielded the enzyme with 9 mg protein, 1,200 activity units and the specific activity of 133 units/mg protein. The enzyme was purified to 22 fold with about 28% yield (Table 6). The enzyme from this step was kept as aliquot at 4 ^{o}C for further characterization experiments.

3.6 Determination of enzyme purity and protein pattern on non-denaturing and SDS-PAGE

The enzyme from each step of purification was analyzed for purity and protein pattern by non-denaturing (Figure 24) and SDS-PAGE (Figure 25) as described in section 2.12.1 and 2.12.2. Both non-denaturing and SDS gels were stained for protein with Coomassie blue. On non-denaturing PAGE, the amylolytic activity staining was also performed to compare with protein staining. The enzyme in lane 4 (Figure 24) showed a single protein band on native gel which corresponded with its activity by amylolytic activity staining, indicating the purity of the enzyme from Phenyl-Sepharose CL-4B. The result was also confirmed by SDS-PAGE analysis (Figure 25).

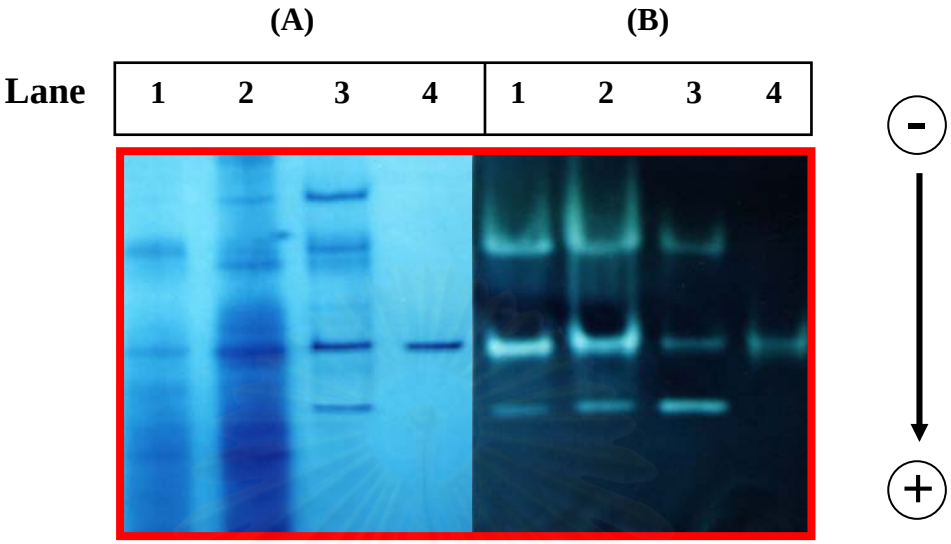


Figure 24 Non-denaturing PAGE analysis of *Paenibacillus* sp. A11 CDase in each step of purification on a 7.5% acrylamide gel

(A) Coomassie blue staining

Lane 1 : Crude enzyme (25 μg protein)

Lane 2 : 40-60% ammonium sulfate precipitation (25 μg protein)

Lane 3 : DEAE-Sephadex A50 (25 μg protein)

Lane 4 : Phenyl-Sepharose CL-4B (10 μg protein)

(B) Amylolytic activity staining

Lane 1-4, as in A, 0.2 units of dextrinizing activity was loaded to each well

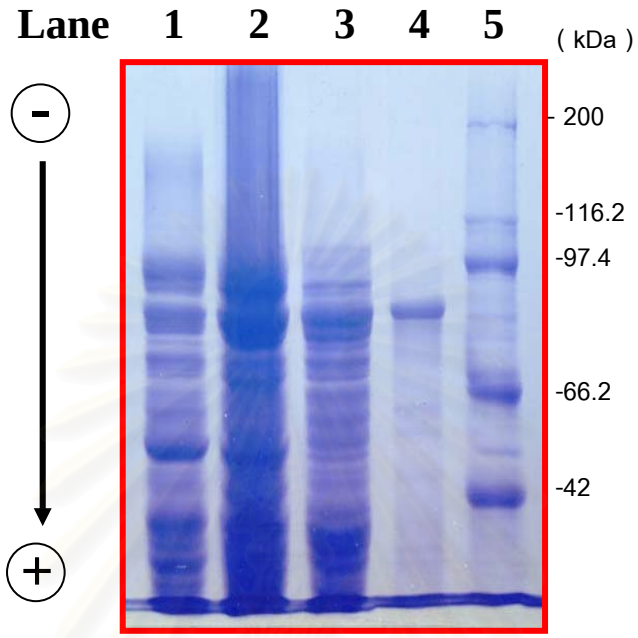


Figure 25 SDS-PAGE analysis of *Paenibacillus* sp. A11 CDase in each step of purification on a 7.5% acrylamide gel

Lane 1 : Crude enzyme (35 μg protein)

Lane 2 : 40-60% ammonium sulfate precipitation (35 μg protein)

Lane 3 : DEAE-Sephadex A50 (35 μg protein)

Lane 4 : Phenyl-Sepharose CL-4B (15 μg protein)

Lane 5 : Protein molecular weight markers

[myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45 kDa)]

3.7 Characterization of cyclodextrinase from *Paenibacillus* sp. A11

3.7.1 Molecular weight determination of CDase

The native molecular weight of CDase was determined from molecular weight calibration curve obtained from chomatography of standard proteins on Sephadex G-100 column as mentioned in section 2.13.1. The enzyme was found to have the native molecular weight of 80,000 Da (Figure 26 and 27). The molecular weight of CDase subunit was also determined by SDS-PAGE which included a series of standard proteins in the run (Figure 28 and 29). From the mobility on SDS-PAGE, the molecular weight of the enzyme was 80,000 Da which coincided with the native molecular weight from Sephadex G-100 column. These results indicate that the enzyme is monomeric.

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

Purified CDase was analyzed for its isoelectric point by separation on IEF gel electrophoresis, comparing to standard pI markers. Ampholyte pH range 3.0-10.0 was used and relative mobility againt pI was plotted. Figure 30 shows one band of CDase protein on ampholyte gel. Calibration curve of standard pI markers on polyacrylamide mini gel system in Figure 31 demonstrates that the pI value of CDase was 5.4.

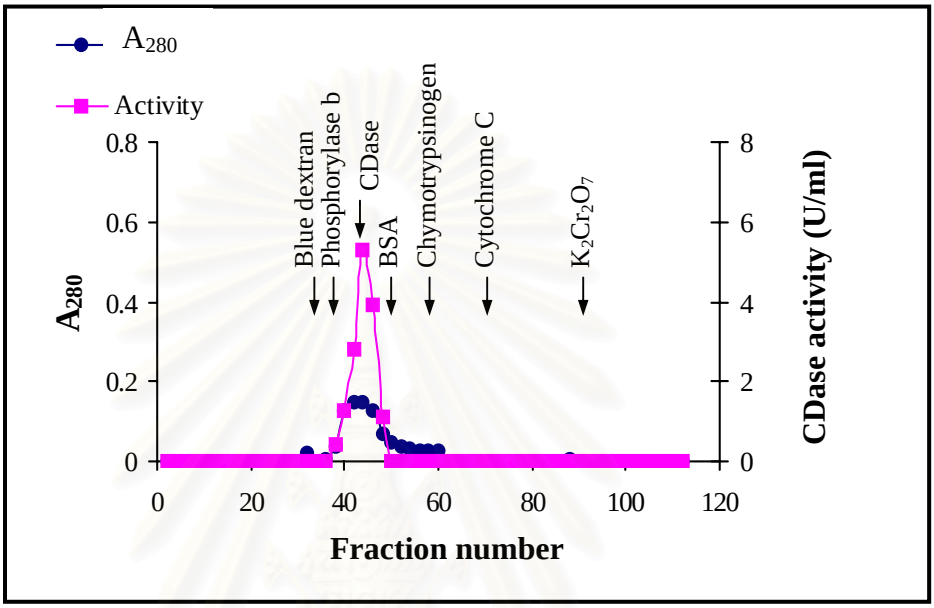


Figure 26 **Molecular weight determination of CDase from *Paenibacillus* sp. A11 by Sephadex G-100 column**

The enzyme solution was applied to Sephadex G-100 column (1.7 X 80 cm) and eluted with 10 mM potassium phosphate buffer, pH 7.5 containing 0.1 M NaCl at the flow rate of 12 ml/h. Fractions of 2 ml were collected. Positions of markers and CDase were shown.

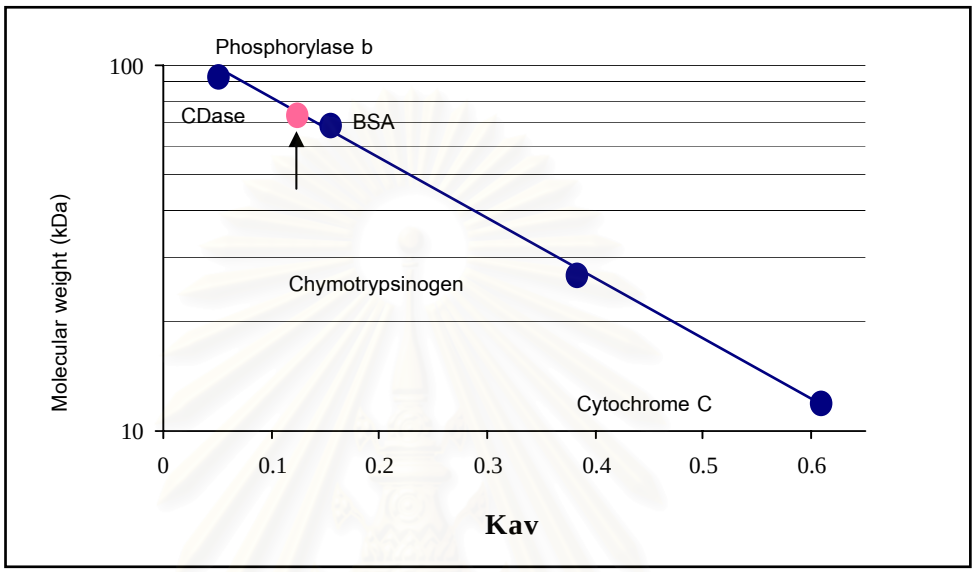


Figure 27 Calibration curve for native molecular weight of CDase from *Paenibacillus* sp. A11 determined by gel filtration chromatography on Sephadex G-100 column

Phosphorylase b	(MW 97,000 Da)
Bovine serum albumin	(MW 67,000 Da)
Chymotrypsinogen	(MW 27,000 Da)
Cytochrome C	(MW 12,500 Da)

Arrow indicates the K_{av} of CDase.

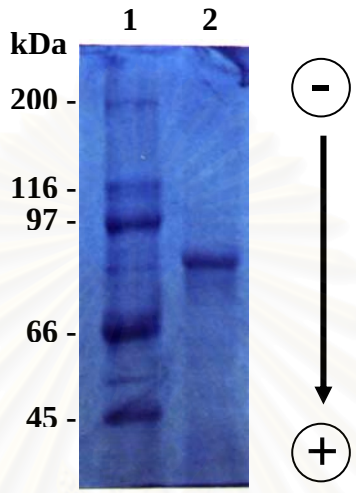


Figure 28 SDS-polyacrylamide gel electrophoresis of CDase from *Paenibacillus* sp. A11

Lane 1 : Protein molecular weight markers

[myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45 kDa)]

Lane 2 : Purified CDase (10 μg protein)

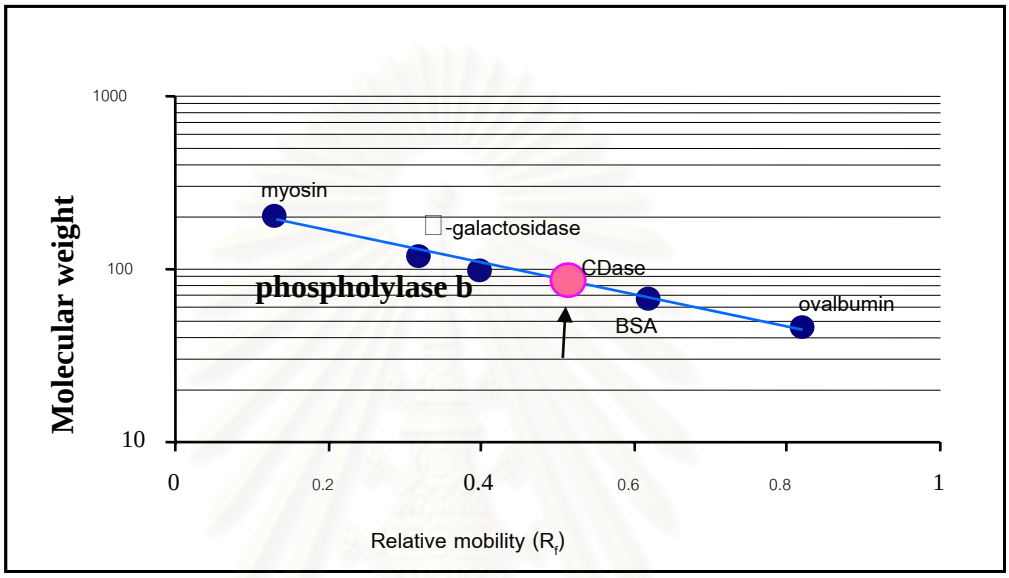


Figure 29 Calibration curve for molecular weight of CDase subunit from *Paenibacillus* sp. A11 on SDS-polyacrylamide gel electrophoresis

Myosin	(MW 200,000 Da)
β-galactosidase	(MW 116,250 Da)
Phosphorylase b	(MW 97,400 Da)
Bovine serum albumin	(MW 66,200 Da)
Ovalbumin	(MW 45,000 Da)

Arrow indicates the R_f of CDase.

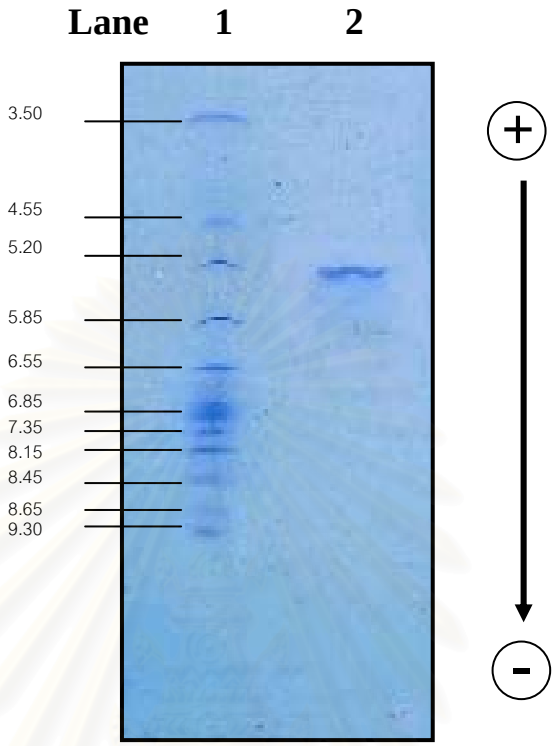


Figure 30 Isoelectric focusing gel with ampholyte solution (pH 3.0-10.0) of purified CDase

Lane 1 : Standard pI markers

amyloglucosidase	(pI 3.50)
soybean trypsin inhibitor	(pI 4.55)
β-lactoglobulin	(pI 5.20)
bovine carbonic anhydrase B	(pI 5.85)
human carbonic anhydrase B	(pI 6.55)
myogloblin-acidic band	(pI 6.85)
myoglobin - basic band	(pI 7.35)
lentil lectin - acidic band	(pI 8.15)
lentil lectin - middle band	(pI 8.45)
lentil lectin - basic band	(pI 8.65)
trypsinogen	(pI 9.30)

Lane 2 : Purified CDase

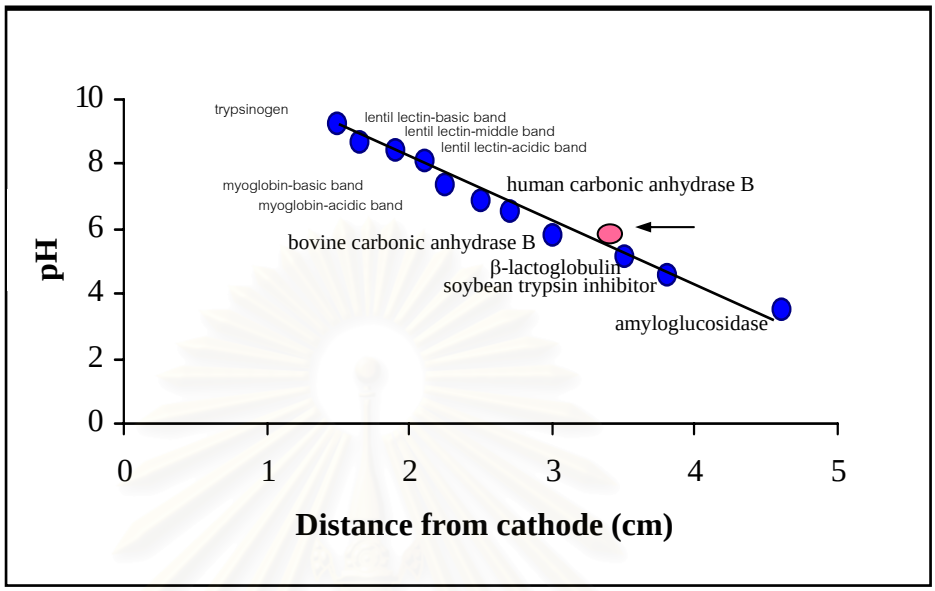


Figure 31 Calibration curve for pI determination on polyacrylamide mini gel system

amyloglucosidase	(pI 3.50)
soybean trypsin inhibitor	(pI 4.55)
β-lactoglobulin	(pI 5.20)
bovine carbonic anhydrase B	(pI 5.85)
human carbonic anhydrase B	(pI 6.55)
myogloblin-acidic band	(pI 6.85)
myoglobin - basic band	(pI 7.35)
lentil lectin - acidic band	(pI 8.15)
lentil lectin - middle band	(pI 8.45)
lentil lectin - basic band	(pI 8.65)
trypsinogen	(pI 9.30)

Arrow indicates the position of CDase.

3.7.3 Determination of N-terminal amino acid sequence

Twenty μg protein of the purified CDase was separated by SDS-PAGE. The SDS polyacrylamide gel was removed from the electrophoresis cell and soaked in electroblotting buffer for 5 min. The electroblotting was performed with the same buffer under a constant voltage of 50 volts at room temperature for 90 min as mentioned in section 2.13.3.1. After electroblotting, the protein band on the PVDF membrane was briefly stained with Coomassie blue and destained with 50% methanol until protein band appeared (Figure 32). The band of CDase was excised and subjected for sequencing.

The sequencing procedure for determination of the N-terminal amino acid residues was as described in section 2.13.3.3. The twenty cycles were analyzed on an Applied Biosystems 476A Protein Sequencer. Twenty amino acids, aspartic (D), asparagine (N), serine (S), glutamine (Q), threonine (T), glycine (G), glutamic acid (E), histidine (H), alanine(A), arginine (R), tyrosine (Y), proline (P), methionine (M), valine (V), tryptophan (W), phenylalanine (F), isoleucine (I), lysine (K), leucine (L) and cystein (C) were used as sequence standards and dptu (diphenylthiourea) and dpu (diphenylurea) peaks were used as reference peaks. The profiles of standard amino acids and profiles of amino acid sequence of CDase were shown in Appendix F.

The N-terminal amino acid sequence of the purified CDase was determined by automated Edman degradation up to 15 residues to be M F L E A V Y H R P R K N W S.

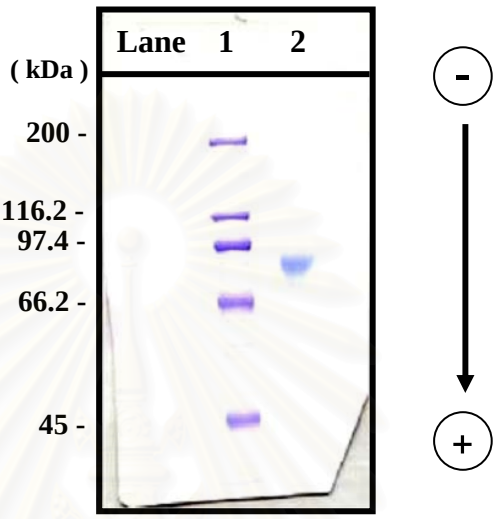


Figure 32 SDS-PAGE of CDase from *Paenibacillus* sp. A11 after electroblotted onto PVDF membrane

Lane 1 : Protein molecular weight markers

[myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45 kDa)]

Lane 2 : Purified CDase

3.7.4 Amino acid composition of purified CDase

The purified CDase from Phenyl-Sepharose CL-4B column was hydrolyzed with 6 M HCl at 110 ^{o}C for 24, 48, and 72 h in vacuum. The hydrolysates were analyzed with a Hitachi L-8500 Amino acid Analyzer. Chromatograms of various standard amino acids and enzyme sample were shown in Appendix G. The amino acid composition of the purified CDase of *Paenibacillus* sp. A11 was presented in Table 7. The result shows that CDase was rich in Asx and Glx while contained few amounts of Met, Phe and His, respectively. For Cys and Trp, they could not be detected under the experimental condition.

3.7.5 Effect of pH on activity and stability of CDase

The optimum pH of the enzyme was determined as mentioned in section 2.13.5. Activities at different pHs are shown in Figure 33. In this study, the 0.1 M of acetate, phosphate, tris-HCl and glycine-NaOH were used as reaction buffers for pH 3.0-5.0, 5.0-7.0, 7.0-9.0 and 9.0-11.0, respectively. No buffer effect was observed at all pH ranges. Optimum pH of this CDase was at pH 7.0. The enzyme showed 40-60% of the activity at pH 5.0-6.0 and 8.0-9.0 while no activity was observed at pH below 4.0 or above 11.0.

The pH stability of the enzyme was measured by incubating the enzyme at 4 ^{o}C for 24 h in 10 mM buffers, pHs from 3.0 to 12.0 before the residual enzyme activity was measured as described in section 2.10. The enzyme showed high stability over a pH range of 6.0 to 10.0 (Figure 34).

Table 7 Amino acid composition of purified CDase

Amino acid	Content (mol %)		
	24 h*	**48 h***	**72 h***
Asx**	11.6	11.4	11.6
Thr	5.0	4.9	4.7
Ser	4.6	4.5	4.0
Glx***	15.4	15.1	15.2
Gly	6.2	6.3	6.3
Ala	6.9	6.9	6.8
Cys	Not determined		
Val	6.8	6.8	7.2
Met	2.9	3.2	3.6
Ile	5.2	4.8	5.4
Leu	7.8	7.6	7.7
Tyr	4.5	4.7	4.4
Phe	3.4	3.2	3.1
Lys	4.7	4.6	4.6
His	2.0	2.0	2.0
Trp	Not determined		
Arg	6.1	6.0	6.0
Pro	6.9	8.0	7.4

* Duration of acid hydrolysis

** Asx = aspartic acid + asparagine

*** Glx = glutamic acid + glutamine

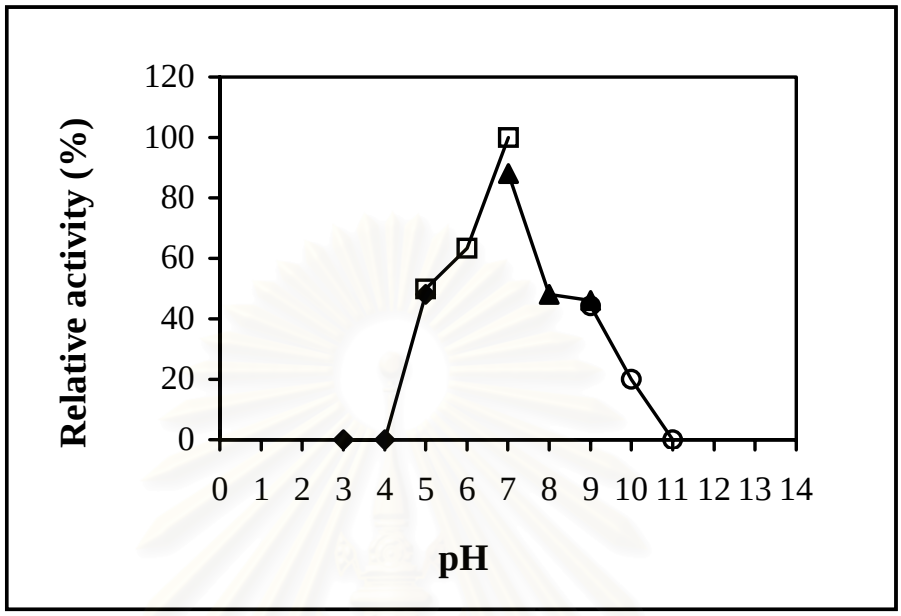


Figure 33 Effect of pH on cyclodextrinase activity

Buffers used: 0.1 M acetate buffer, pH 3.0-5.0 (◆); 0.1 M K-phosphate buffer, pH 5.0-7.0 (□); 0.1 M Tris-HCl buffer, pH 7.0-9.0 (▲) and 0.1 M glycine-NaOH buffer, pH 9.0-11.0 (○). The highest CDase activity was defined as 100%.

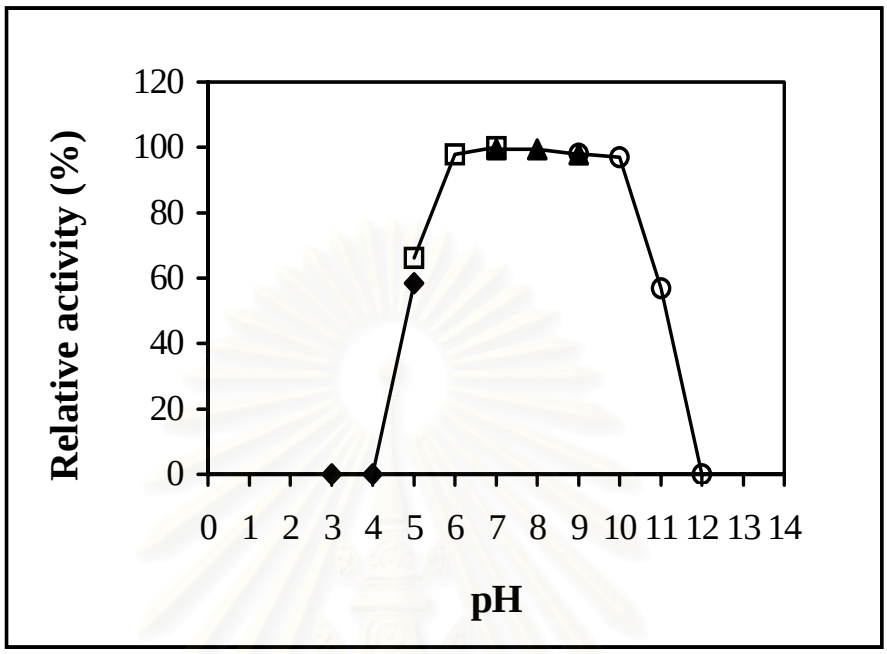


Figure 34 Effect of pH on cyclodextrinase stability

The enzyme was incubated in 0.01 M acetate buffer, pH 3.0-5.0 (◆); 0.01 M K-phosphate buffer, pH 5.0-7.0 (□); 0.01 M Tris-HCl buffer, pH 7.0-9.0 (▲) and 0.01 M glycine-NaOH buffer, pH 9.0-12.0 (○) at 4 ^{o}C for 24 h. The residual activity was measured as described in section 2.10. The highest CDase activity was defined as 100%.

3.7.6 Effect of temperature on activity and stability of CDase

The optimum temperature of the enzyme was investigated by incubating the reaction mixture at various temperatures as mentioned in section 2.13.6. The enzyme showed the highest activity at 40 ^{o}C which was defined as 100% activity. At 60 and 70 ^{o}C, 70 and 45 percents of the activity still remained (Figure 35).

The effect of temperature on stability of the CDase was investigated by preincubation at 40 to 60 ^{o}C for 0 to 60 min followed by measurement of residual CDase activity under condition as described in section 2.10. Total enzyme activity remained when CDase was incubated at 40 ^{o}C for 60 min (Figure 36). Heating over 50 ^{o}C for 10 min led to gradual inactivation. It was found that 5 mM $CaCl_2$ helps to stabilize CDase, 50% residual activity was retained when enzyme was incubated at 60 ^{o}C for 60 min. In the absence of $CaCl_2$, only 20% of the activity remained.

The effect of temperature upon long-term storage of CDase was also determined. The purified enzyme was stored at temperature 30, 4, -20 and –80 ^{o}C for 5 weeks. The sample was withdrawn every week for measuring enzyme activity by reducing sugar assay. There was no loss in enzyme activity after stored at –80 ^{o}C for 5 weeks, while almost 20% of the activity was lost when stored at –20 ^{o}C (Figure 37). Significant decrease in activity was observed upon 1 week storage at 4 ^{o}C and 30 ^{o}C. While after 5 weeks, only half and trace amount of activity remained, respectively.

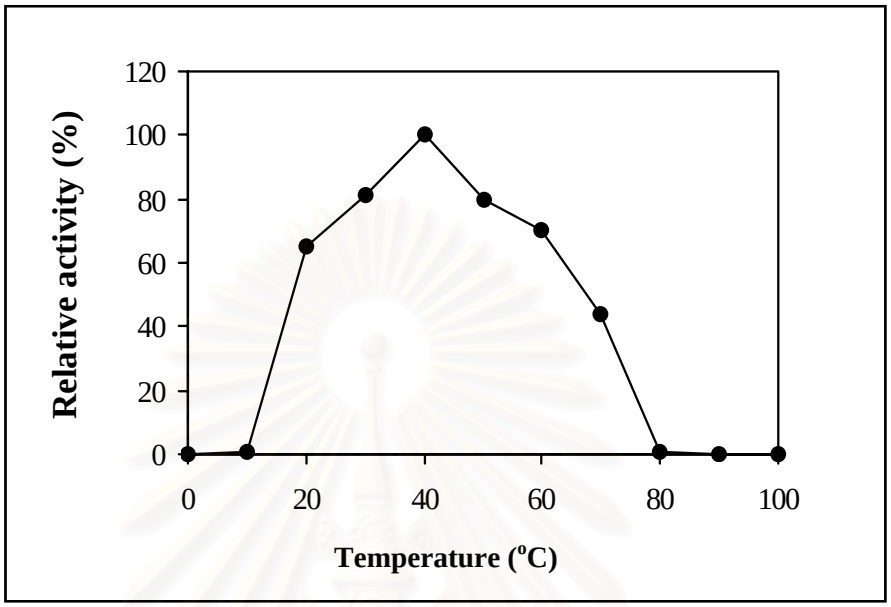


Figure 35 Effect of temperature on cyclodextrinase activity

The CDase activity was measured at various temperatures as described in section 2.13.6. The highest CDase activity was defined as 100%.

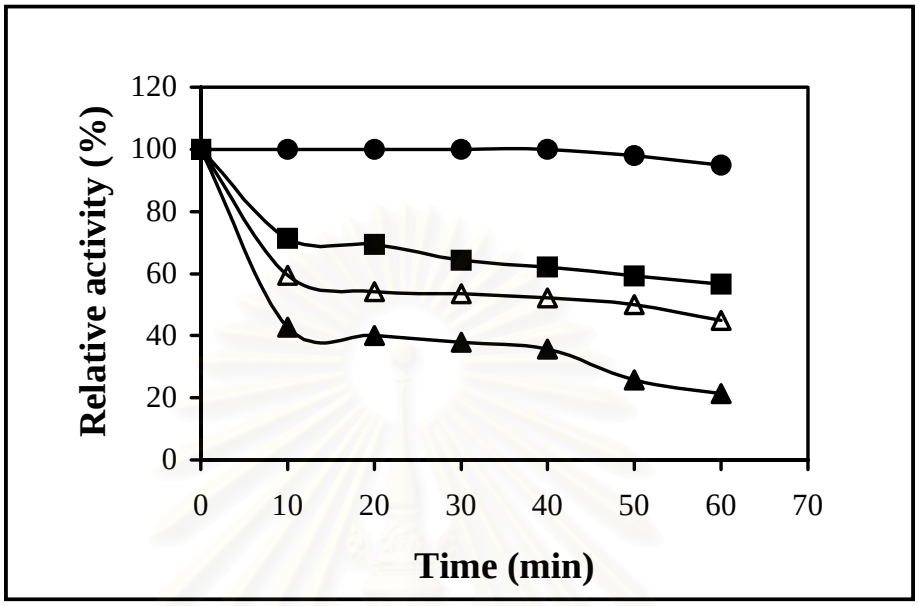


Figure 36 Effect of temperature on CDase stability

The enzyme was preincubated at temperature 40 ^{o}C (●), 50 ^{o}C (■), 60 ^{o}C (▲) and 60 ^{o}C in the presence of 5 mM $CaCl_2$ (Δ) for 0-60 min, followed by measuring the residual CDase activity as mentioned in section 2.10. The highest CDase activity was defined as 100%.

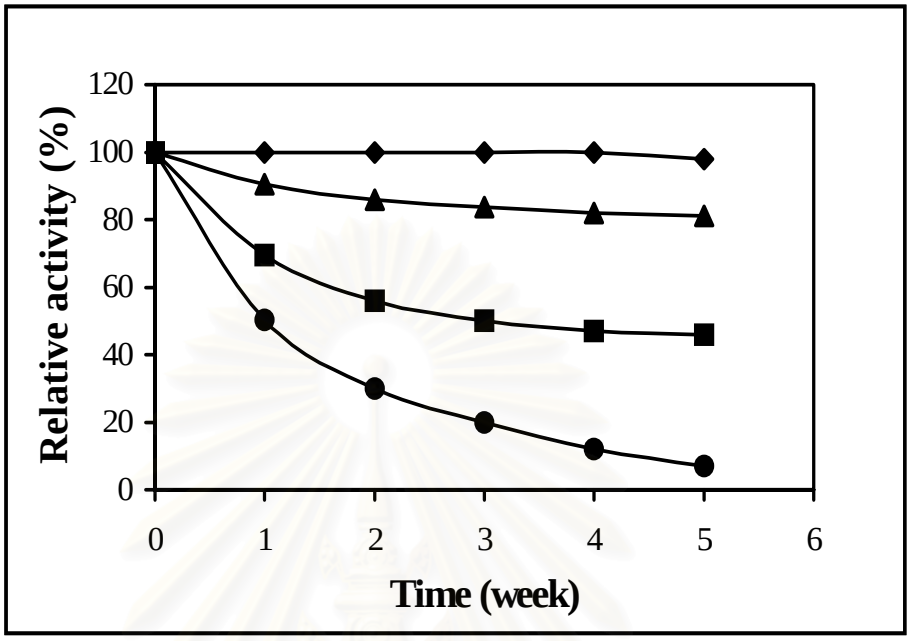


Figure 37 Effect of temperature upon long-term storage

The purified enzyme was stored at temperature 30 ^{o}C (●), 4 ^{o}C (■), -20 ^{o}C (▲) and –80 ^{o}C (◆) for 5 weeks before measuring enzyme activity by reducing sugar assay as described in section 2.10. The highest CDase activity was defined as 100%.

3.7.7 Substrate specificity of CDase

The determination of substrate specificity was performed as described in section 2.13.10. The CDase of *Paenibacillus* sp. A11 had high specificity towards β-CD (Table 8). Hydrolytic activities of 20-60% relative to that of β-CD were observed when substrates γ-CD, α-CD, maltoheptaose, maltohexaose, G1-β-CD, G2-β-CD, dextrin, G1-α-CD and G2-α-CD were used. Lower activity (8-18%) was observed with maltopentaose, maltotetraose, maltotriose, soluble starch as well as amylose while trace hydrolysis (2-3% hydrolytic activity) of maltose, trehalose, amylopectin, and pullulan could be detected. Substrates that could not be hydrolyzed include oligosaccharides such as cellobiose, lactose, melibiose, sucrose, leucrose, levanbiose, raffinose and polysaccharides such as glycogen, dextran, glucomannan and levan.

3.7.8 Product analysis by High performance liquid chromatography (HPLC), Thin layer chromatography (TLC), and Nuclear magnetic resonance (NMR)

The end-products after 24 h incubation of the enzyme with various substrates were investigated and analyzed by HPLC (Table 8). Each substrate was degraded to give reducing oligosaccharides with maltose and glucose as the dominant products.

Time course hydrolysis of β-CD initially produced maltoheptaose and maltose as main products (Table 9). The products of the initial phase were subsequently broken down and the final end products at 24 h were maltose, glucose, maltotriose and maltotetraose at the ratio of 1.0 : 0.5 : 0.5 : 0.1. β-CD, the best substrate for A11 CDase, was completely hydrolyzed after 12 h as shown in Table 9.

Table 8 Substrate specificity and end-products of cyclodextrinase

Substrate* (1%,w/v)	Relative activity (%)	End-product ** (relative mole ratio) G1 : G2 : G3 : G4 : G5
β-Cyclodextrin	100	0.5 : 1.0 : 0.5 : 0.1 : 0.0
γ-Cyclodextrin	59	0.7 : 1.0 : 0.1 : 0.4 : 0.0
α-Cyclodextrin	53	0.6 : 1.0 : 0.2 : 0.5 : 0.0
G1-β-Cyclodextrin	32	0.7 : 1.0 : 0.2 : 0.5 : 0.1
G2-β-Cyclodextrin	30	0.6 : 1.0 : 0.0 : 0.2 : 0.2
G1-α-Cyclodextrin	24	0.8 : 1.0 : 0.6 : 0.4 : 0.0
G2-α-Cyclodextrin	19	0.6 : 1.0 : 0.3 : 0.3 : 0.2
Maltotriose (G3)	11	0.7 : 1.0 : 0.4 : 0.0 : 0.0
Maltotetraose (G4)	12	0.6 : 1.0 : 0.1 : 0.1 : 0.0
Maltopentaose (G5)	18	0.6 : 1.0 : 0.3 : 0.1 : 0.0
Maltohexaose (G6)	33	0.7 : 1.0 : 0.1 : 0.1 : 0.0
Maltoheptaose(G7)	41	0.4 : 1.0 : 0.2 : 0.2 : 0.1
Dextrin	29	0.4 : 1.0 : 0.5 : 0.4 : 0.3

*Amylose and soluble starch were less hydrolyzed (8-9% relative activity) while very small amounts of maltose, trehalose, amylopectin and pullulan (2-3% relative activity) could be hydrolyzed. Substrates that were not hydrolyzed include cellobiose, lactose, melibiose, sucrose, leucrose, levanbiose, raffinose, glycogen, dextran, glucomannan and levan.

**After 24 h incubation, the end-products were analyzed by HPLC.

Table 9 Time course of hydrolytic products by *Paenibacillus* sp. A11 CDase

Substrate (2%,w/v)	Time (h)	β-CD*	Hydrolytic product (%, w/w)						
			G1	G2	G3	G4	G5	G6	G7
β-CD	0	100	0	0	0	0	0	0	0
	0.5	86.2	0	4.5	2.1	1.4	0	0.1	5.7
	1	73.5	4.6	9.4	4.0	2.0	0	1.3	5.2
	3	29.2	9.5	31.7	15.8	6.4	0	2.2	5.2
	6	18.3	12.6	36.6	18.7	9.5	0	0.8	3.5
	12	2.9	20.8	51.6	16.0	8.1	0	0.6	0
	18	0	22.0	52.1	14.3	7.7	2.9	0.8	0.2
	24	0	23.2	49.0	22.9	4.9	0	0	0

*β-CD remained.

G1; Glucose, G2; Maltose, G3; maltotriose, G4; Maltotetraose, G5; Maltopentaose, G6; Maltohexaose, G7; Maltoheptaose

The dominant-end products obtained from hydrolysis for 24 h by CDase of various substrates was also determined by TLC (Figure 38). All substrates except melibiose (lane 5) and raffinose (lane 6) could be hydrolyzed to maltose and glucose as main products. More maltose was found in most cases. α and γ-CDs gave also significant amount of maltotriose. Trace amount of glucose was formed when maltose (lane 7) and trehalose (lane 8) were used as substrate. Raffinose and melibiose could not be hydrolyzed (compare lane 5 to lane D and lane 6 to lane C). These results were corresponded with the HPLC result.

When analyzed by ^{1}H-NMR, CDase (0.5 ml, 0.6 mg/ml) was incubated with 2.5 ml of α-, β-, γ-CDs, maltose, trehalose or soluble starch at 2% w/v concentration in 0.1 M phosphate buffer, pH 7.0 at 40^{o}C for 24 h. The products from each substrate except trehalose were identified as saccharides having reducing end sugars with α 1,4-link which was shown as prominent peak at the chemical shift of around 5.35 ppm (Figure 39). For trehalose with α 1,1 glucosidic linkage, hydrolysis was weakly performed by CDase (Frame E).

Time course hydrolysis of β-CD initially produced less amount of α and β reducing end sugars (Figure 40). Sugars with β anomer were produced more than the α one. The products with α 1,4-link at around 5.38 ppm were obviously seen at 0.25 h and highest at 24 h. The β-CD substrate, the peak at the chemical shift of around 4.95 ppm, was completely hydrolyzed at 24 h (Frame A).

Figure 38 Thin layer chromatogram of the hydrolysis products produced by *Paenibacillus* sp. A11 CDase

Reactions were performed as described under experimental procedures (section 2.12.13). Standard used in each lane was : lane A; from top G1, G2, G3 and G4, respectively, lane B; from top α-CD, β-CD and γ-CD, respectively, lane C; raffinose, lane D; melibiose, lane E; fructose, lane F; galactose. Substrates (2% w/v) used in the hydrolyzed reaction of CDase were : lane 1; α-CD, lane 2; β-CD, lane 3; γ-CD, lane 4; soluble starch, lane 5; melibiose, lane 6; raffinose, lane 7; maltose, lane 8; trehalose. G1; glucose, G2; maltose, G3; maltotriose and G4; maltotetraose.

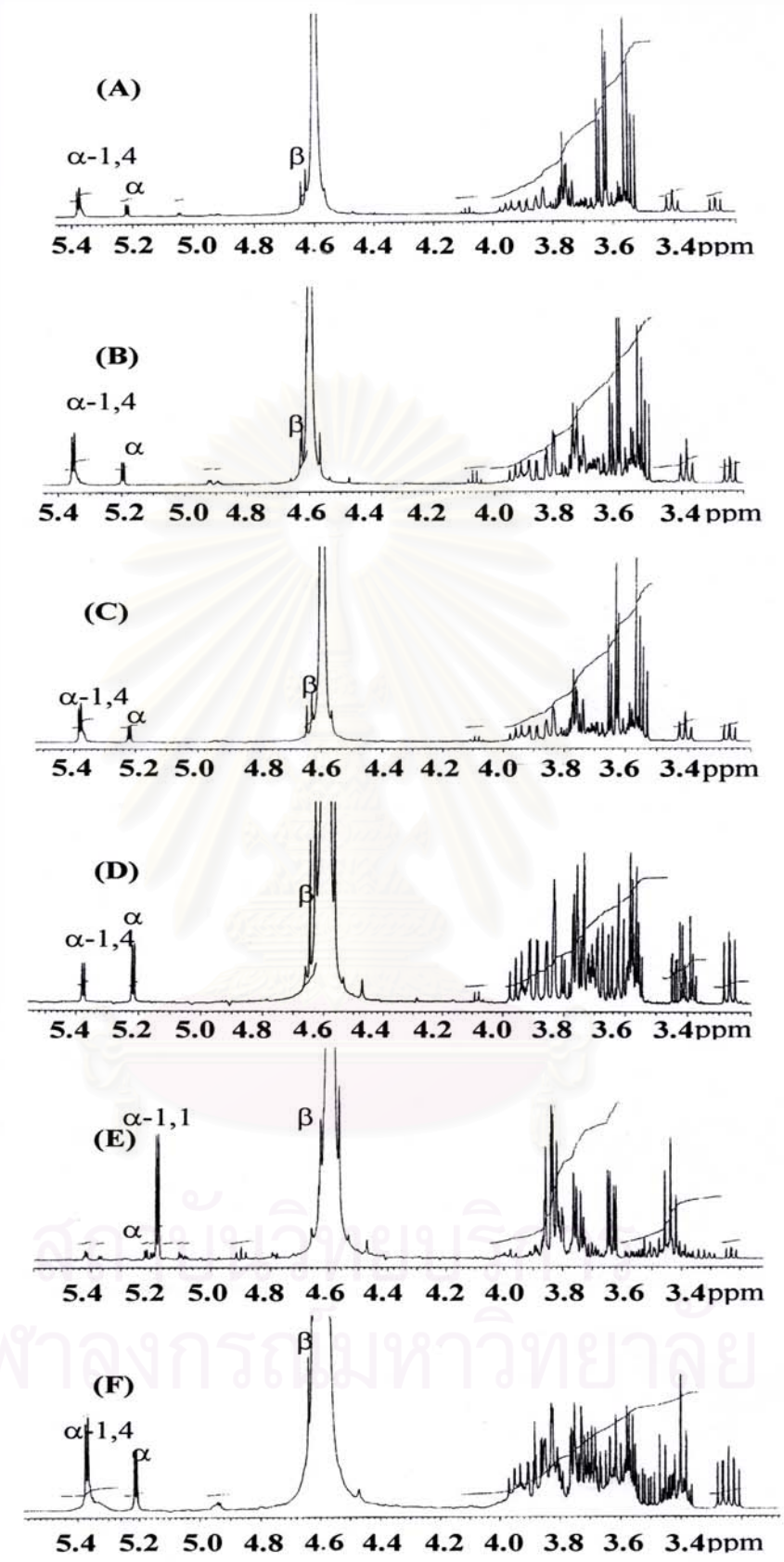


Figure 39 ^{1}H-NMR of the products catalyzed by CDase from α-CD (A), β-CD (B), γ-CD (C), maltose (D), trehalose (E) and soluble starch (F) at 40^{o}C for 24 h.

The ^{1}H-NMR analysis was conducted at 40^{o}C in D_2O and DSS as internal standard.

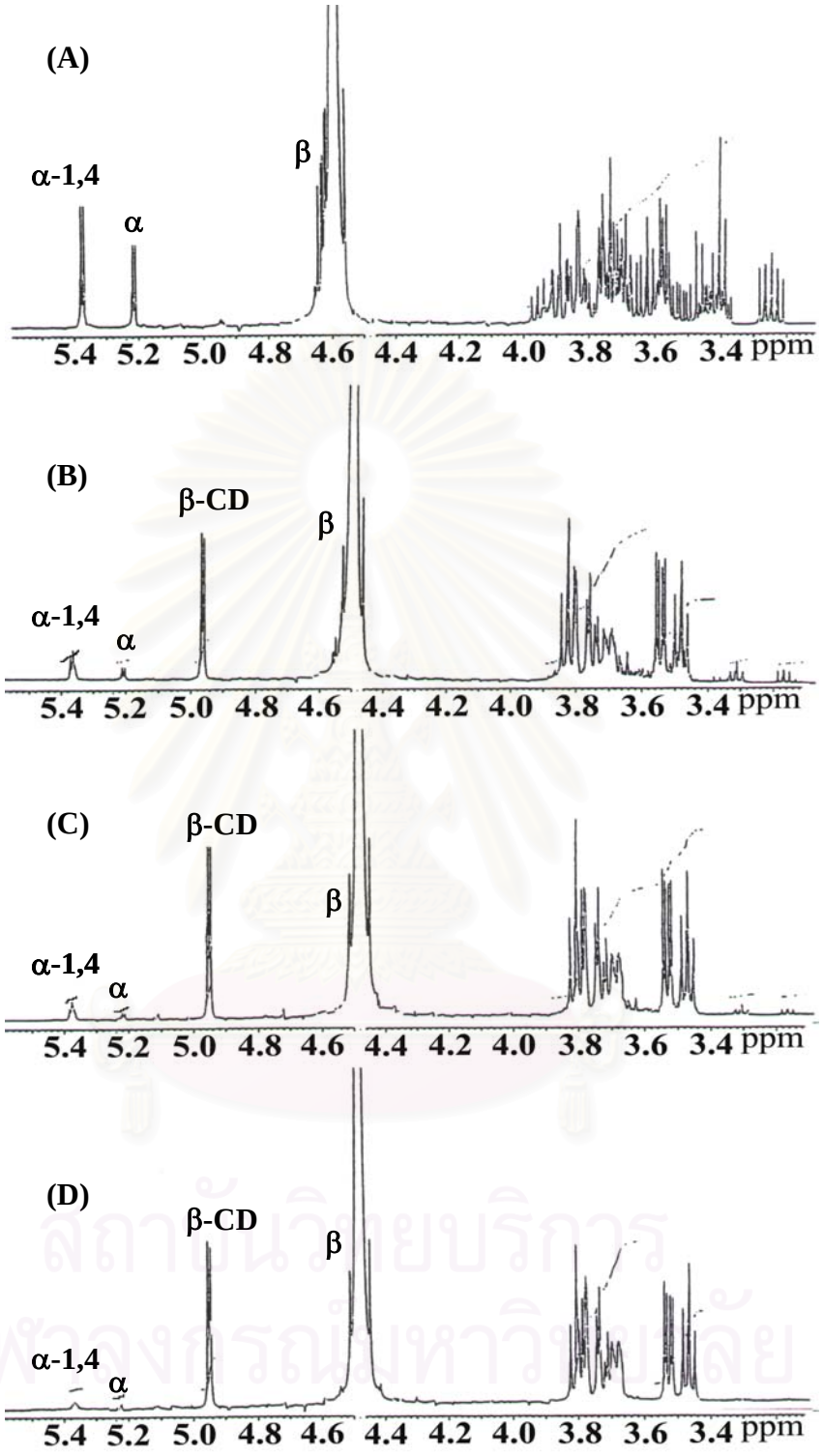


Figure 40 ^{1}H-NMR of the products upon time course hydrolysis of β-CD by CDase at 40^{o}C.

Hydrolysis time was 24 (A), 1 (B), 0.5 (C) and 0.25 (D) h, respectively. The ^{1}H-NMR analysis was conducted at 40^{o}C in D_2O and DSS as internal standard.

3.7.9 Kinetic studies of CDase

The typical Lineweaver-Burk plot was shown for β-CD substrate (Figure 41). Kinetic parameters of CDase with α-, β-, and γ-CDs as substrates were summarized in Table 10. The K_m, k_{cat} and k_{cat}/K_m values showed that the strongest binder, β-CD, was the best substrate with the highest turnover number and catalytic efficiency. Whereas the bigger ring, the γ-CD, was significantly better than the small ring of α-CD when catalytic efficiency, k_{cat}/K_m, was compared.

3.7.10 Effects of metal ions, protective chemicals and saccharides on CDase activity

The effects of metal ions, protective chemicals and saccharides on CDase activity are summarized in Table 11. The enzyme was completely inhibited by 10 mM concentration of Hg^{2+}, Ag^{+}, Cu^{2+} while Mn^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} showed some moderate inhibition. No inhibition was observed with Ba^{2+}, K^{+}, Na^{+}, and Li^{+}. The saccharides, glucose, maltose, cellobiose, lactose and dextran had no effect on enzyme activity. Among the saccharides tested, acarbose significantly inhibited CDase. Chemicals often used as protective substance for enzyme such as β-mercaptoethanol, EDTA or used as fungicide during enzyme purification (NaN_3) had no effect on the activity of this enzyme. Of all the chloride salts, only Ca^{2+} was shown to be enzyme activator.

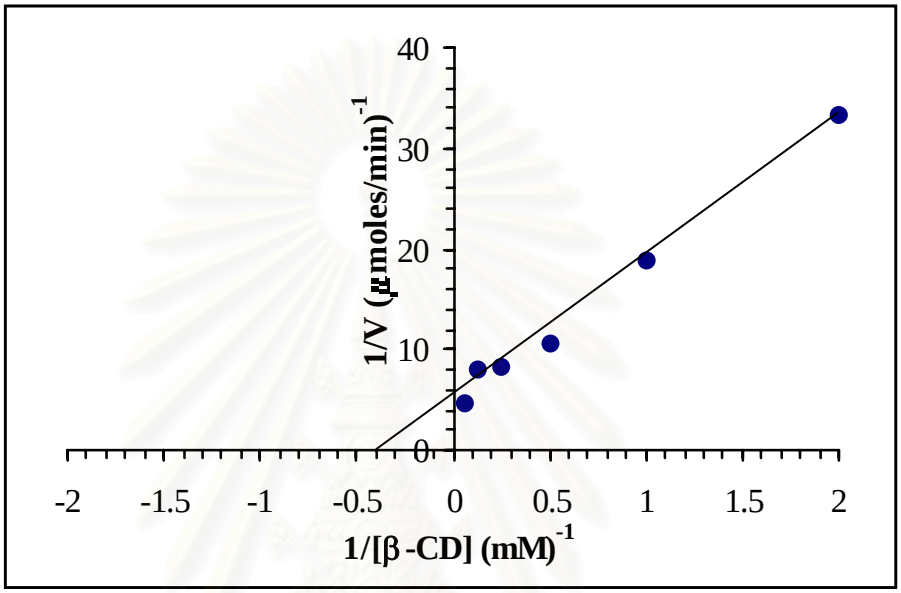


Figure 41 Lineweaver-Burk plot of CDase with β-CD as substrate

CDase (15 μl, 0.6 mg/ml) was incubated with 0.5-16 mM β-CD in 0.1 M phosphate buffer, pH 7.0 at 40^{o}C for 15 min. The hydrolytic products were determined as described in section 2.10.

Table 10 Kinetic parameters of the A11 CDase with cyclodextrins substrate

Substrate	K_m (mM)	V_{max} (μmoles/min)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}min^{-1}$)
α-CD	5.66	0.09	800	1.41×10^5
β-CD	2.47	0.23	2044	8.28×10^5
γ-CD	3.10	0.13	1155	3.73×10^5

Table 11 Effects of metal ions, protective chemicals and saccharides on CDase activity

Compound*	Final concentration	Relative activity (%)
None	-	100
Metal ions		
$CaCl_2$	10 mM	113
$BaCl_2$	10 mM	105
KCl	10 mM	99
NaCl	10 mM	97
LiCl	10 mM	93
$MnCl_2$	10 mM	67
$MgCl_2$	10 mM	65
$FeSO_4$	10 mM	32
$ZnCl_2$	10 mM	22
$CuSO_4$	10 mM	0
$AgNO_3$	10 mM	0
$HgCl_2$	10 mM	0
Protective chemicals		
β-mercaptoethanol	10 mM	105
EDTA	10 mM	100
NaN_3	10 mM	97
Saccharides		
Cellobiose	10 mM	100
Glucose	10 mM	95
Maltose	10 mM	95
Lactose	10 mM	96
Dextran	1 mg/ml	93
Acarbose	5 mM	9.8
	10 mM	0

*Incubation with 0.3 mg/ml enzyme at 40°C, pH 7.0 for 30 min.

As acarbose, a pseudotetrasaccharide, was shown to have a pronounce inhibition on CDase, inhibition kinetics was performed. A Lineweaver-Burk plot of the initial velocities versus variable concentrations of the inhibitor at fixed different concentrations of β-CD substrate is shown in Figure 42. It was found that acarbose was a strong competitive inhibitor for *Paenbacillus* sp. A11 CDase with a K_i value of 2.59×10^{-3} mM.

3.7.12 Effect of group-specific reagents on CDase activity and substrate protection

Group-specific reagents for certain amino acid modification were used as a tool to identify essential residues important for enzyme activity. Those at the active site could also be suggested through substrate protection experiment. The result in Table 12 showed that CDase activity was totally inactivated by 1 mM NBS and DEP while partial inactivation was observed when incubated with 5 mM IAM, EDC and PG at pH 7.5, 40^{o}C for 30 min. TNBS, PMSF and NAI at concentration of 5 mM had no effect on CDase activity whereas some activation was exhibited by reducing agent like DTT which suggests that purified CDase was not in a fully reduced state. And CDase activity was increased upon reduction of disulfide bond. These findings gave evidence the involvement of Trp, His, Cys, Carboxylic amino acids and Arg on CDase activity. Trp seemed to be the most important for CDase as observed by the lowest concentration of NBS used for effective enzyme inactivation. Substrate protection for Trp was then performed to identify its location on the enzyme. β-CD at concentrations of 1 and 2% (w/v) were added to the enzyme solution at 5 min prior to

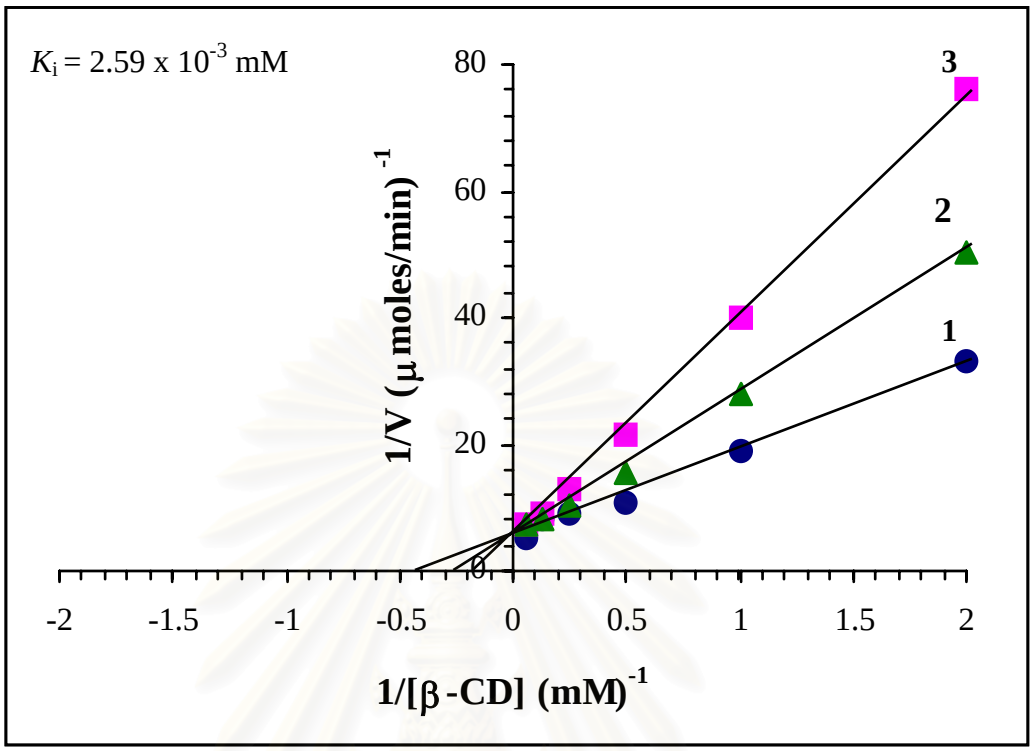


Figure 42 CDase inhibition pattern by acarbose

A Lineweaver-Burk plot for the *Paenibacillus* sp. A11 CDase reaction with various concentrations of acarbose as followed; no inhibitor (line 1), 2.5x10^{-3} mM acarbose (line 2) and 5.0x10^{-3} mM acarbose (line 3).

Table 12 Effect of various group-specific reagents on CDase activity

Reagent*	Concentration (mM)	Amino acid involved	Residual activity (%)
None	-	-	100
Iodoacetamide (IAM)	5	Cysteine	51
Dithiothreitol (DTT)	5	Cystine	113
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	5	Lysine	96
Phenylmethylsulfonyl fluoride (PMSF)	5	Serine	93
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	5	Carboxylic amino acids	63
Diethylpyrocarbonate (DEP)	5	Histidine	0
	1		0
	0.1		71
N-bromosuccinimide (NBS)	5	Tryptophan	0
	1		0
	0.1		11
N-acetylimidazole (NAI)	5	Tyrosine	100
Phenylglyoxal (PG)	5	Arginine	40

*Incubation with 0.3 mg/ml enzyme at 40°C, pH 7.0 for 30 min.

the adding of 1 mM NBS. It was found that 40 and 65% of the enzyme activity was protected by 1 and 2% β-CD, respectively (Table 13). Protection of Trp by β-CD was confirmed by comparing the fluorescence emission spectra of the enzyme. The fluorescence emission spectrum of native CDase (line 1) showed a peak at 350 nm, which is characteristic for Trp (Figure 43) (Yang *et al*., 1996; Yu *et al*., 2001). This characteristic peak disappeared in the modified enzyme (line 4) but regained in the protected conditions (line 2 and 3). These results indicate that the essential Trp was located at the active site of the enzyme.

Table 13 Effect of β-cyclodextrin substrate on the inactivation of hydrolytic activity of CDase by NBS

Compound added*	% Relative activity
1) None	100
2) 1% β-CD	98.6
2% β-CD	97.8
3) 1% β-CD, then 1mM NBS	46.6
2% β-CD, then 1mM NBS	71.7
4) 1 mM NBS	6.7

* CDase (0.15 mg/ml) was incubated with various compounds according to the above table at 40 ^{o}C for 5 min. The residual activity was measured as described in section 2.10. The highest activity was defined as 100%.

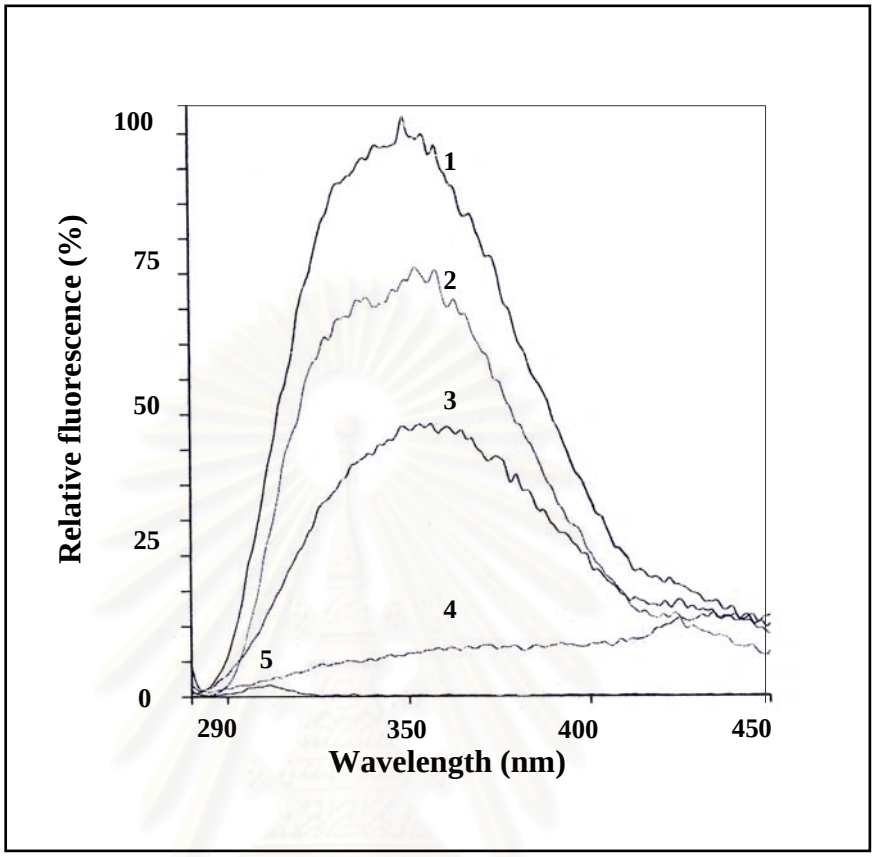


Figure 43 Fluorescence emission spectrum of CDase

Native CDase (1), protected enzyme with 2% (w/v) β-CD (2), protected enzyme with 1% (w/v) β-CD (3), 1 mM NBS modified enzyme (4) and 1 mM NBS (5)

Part III : CLONING, CHARACTERIZATION, AND EXPRESSION OF CDase GENE

To characterize CDase gene, we performed cloning and sequencing of the structural gene coding for CDase from *Paenibacillus* sp. A11. Cloning was by an *Escherichia coli* plasmid vector (pUC 18), and analysis of the CDase gene product expressed in the *E. coli* strain JM 109 was performed. In addition, we tried to overproduce the CDase in *E. coli* by controlling the culture condition and subcloning. We demonstrated that addition of sorbitol to the growth medium increased the growth of recombinant *E. coli* cells and prevented the aggregation of CDase, resulting in higher specific CDase activity in cell-free extract.

3.8 Cloning of CDase gene from *Paenibacillus* sp. A11

3.8.1 PCR amplification of the partial CDase gene

From amino acid and nucleotide sequence comparison, four oligonucleotides were designed from the conserved amino acid sequences of CDase genes and used as PCR primers. The annealing sites and the sequences of oligonucleotides are shown in Figure 44A and B. They were used to amplify the CDase gene segment from the chromosomal DNA of *Paenibacillus* sp. A11. Only one band of about 150 bp was observed when the primer pair 3-C was used while the other primer pairs did not give any PCR bands of expected sizes (Figure 44C). To ascertain that the 3-C PCR product contained part of the CDase gene, it was eluted from the agarose gel, cloned into the pGEM® T-Easy vector and sequenced. The resulting plasmid was named pJK 167.

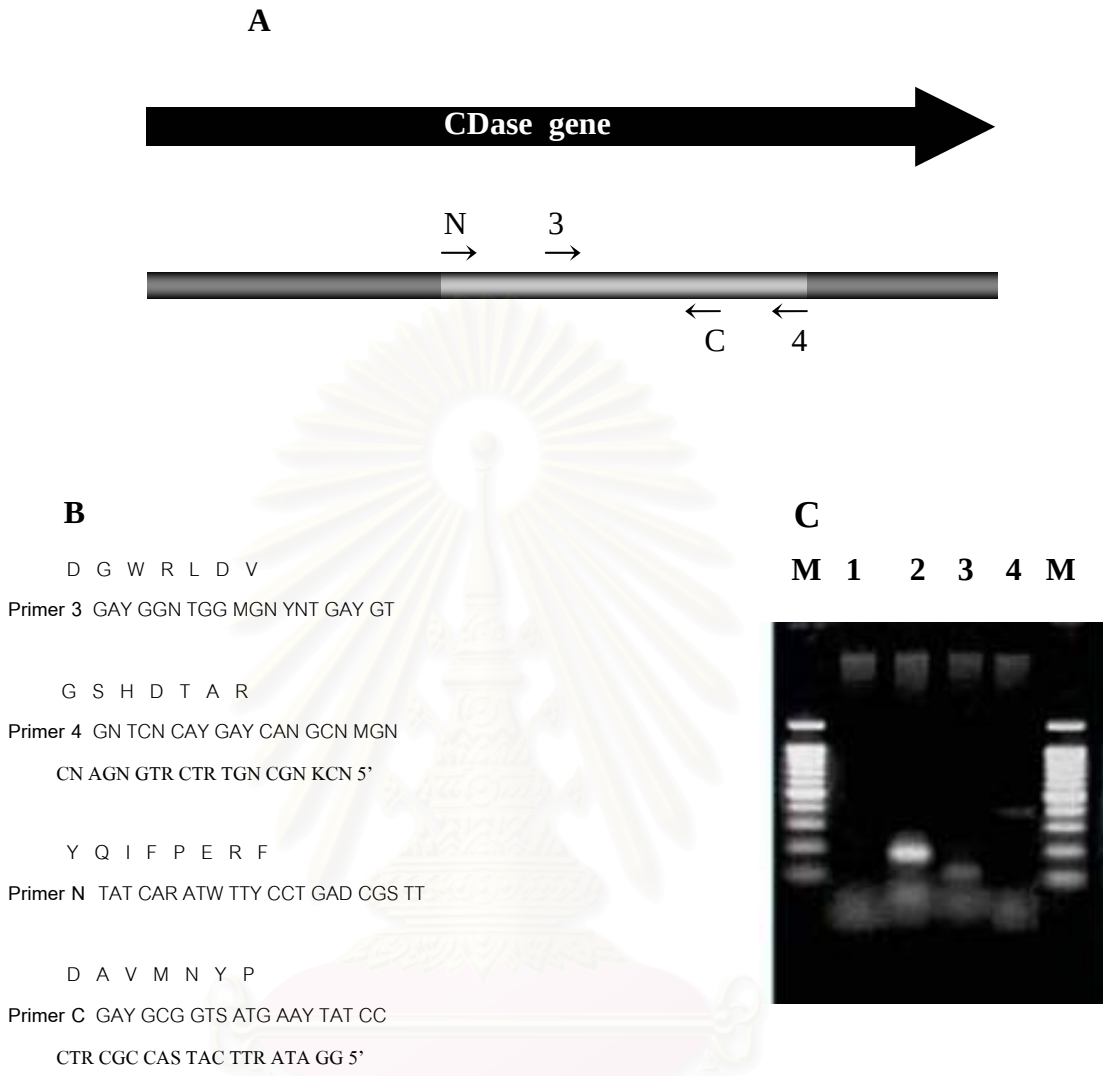


Figure 44 PCR of CDase gene segments.

(A) The drawing shows the approximate locations of PCR primer annealing sites.

(B) Oligonucleotide primers designed from the conserved nucleotide sequences. N = any, Y = C or T, R = A or G, M = A or C, K = G or T, D = A or G or T, and H = A or C or T

(C) The PCR products were amplified from the reactions using the indicated primer pairs as followed, lane 1; 3-4 primer pairs, lane 2; 3-C primer pairs; lane 3; N-C primer pairs and lane 4; N-4 primer pairs. Lane M represents the 100 bp ladder.

The DNA sequence of 3-C PCR product was shown to be part of the CDase gene (underlined sequence in Figure 49) when compared with the CDase genes deposited in the GenBank. The 3-C PCR product was then used as a probe for the screening of CDase gene.

3.8.2 Preparation of chromosomal DNA fragments for cloning

Chromosomal DNA of *Paenibacillus* sp. A11 was digested with *Bgl* I, *Cla* I, *Hin*d III, *Nde* I, *Pvu* II, *Sac* I, *Sal* I and *Kpn* I and electrophoresed in a 0.7% agarose gel. The DNA fragments were then visualized by ethidium bromide staining (Figure 45A) and Southern blotted onto a Hybond^{TM} - N^{+} membrane. Upon hybridization using the 3-C PCR probe, one band for each restriction enzyme digestion was observed (Figure 45B). The *Pvu* II fragment in lane 5 was chosen for DNA cloning because its size was the smallest but big enough to accommodate the CDase gene.

3.8.3 DNA cloning and colony hybridization

Chromosomal DNA was then completely digested with *Pvu* II and subjected to 0.7% agarose gel electrophoresis. The DNA fragments about the size of the *Pvu* II hybridization band were eluted from the gel and ligated with *Sma* I-digested and phosphatased pUC 18. The ligation mixtures were transformed into the JM109 by electroporation. Transformants were screened with colony hybridization using the 3-C PCR probe. One positive clone was obtained (Figure 46). Plasmid from this clone, was called pJK 555.

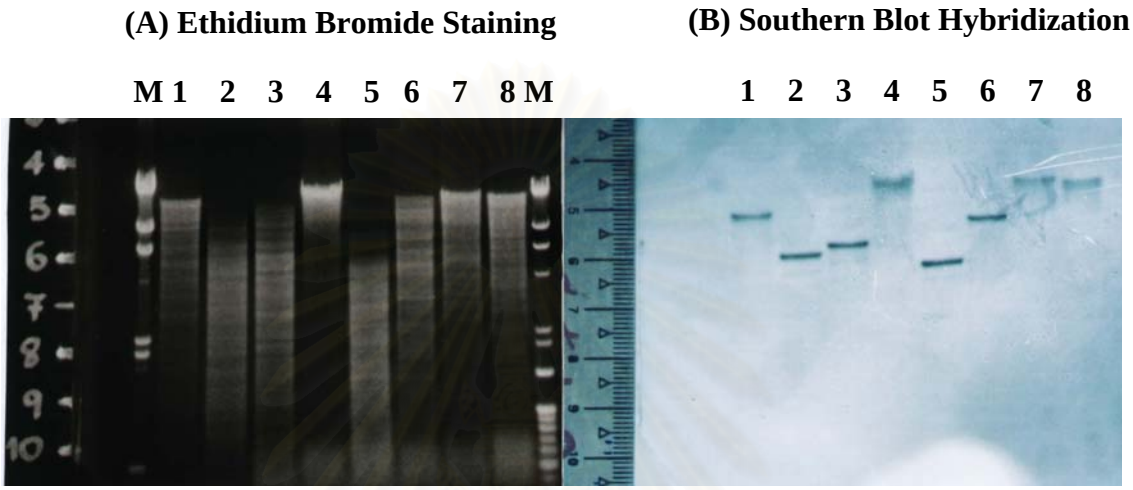


Figure 45 Ethidium bromide staining and Southern blot hybridization of chromosomal DNA from *Paenibacillus* sp. A11 digested with *Bgl* I (lane 1), *Cla* I (lane 2), *Hin*d III (lane 3), *Nde* I (lane 4), *Pvu* II (lane 5), *Sac*I (lane 6), *Sal* I (lane 7) and *Kpn* I (lane 8). Lanes M are marker lanes of λ / *Hin*d III ± 100 bp ladder.

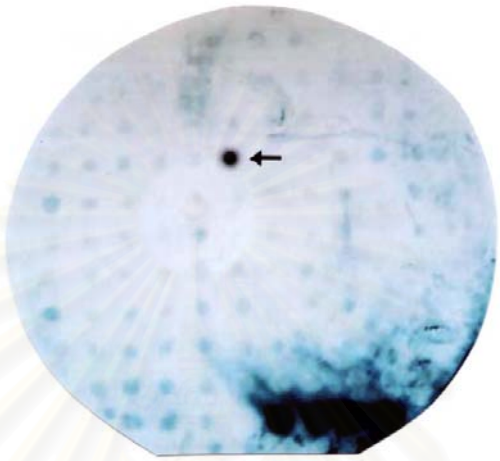


Figure 46 Colony hybridization screening with the 3-C PCR product as a probe.

The arrow indicates pJK 555 transformant. The top row is pNeg transformants (negative control) which was 5 kb DNA fragment without CDase gene inserted on pUC 18. The right end-bottom row is pJK 167 transformants (positive control) which was 167 bp partial CDase gene inserted on pGEM®T-Easy.

3.9 Characterization of CDase gene

3.9.1 Mapping of recombinant plasmid containing CDase gene

The pJK 555 plasmid was prepared and digested with various restriction enzymes. The results of single and double digested with restriction enzymes are shown in Figure 47 and the restriction map was constructed as shown in Figure 48. The size of pJK 555 insert fragment was 4.9 kb. The CDase gene was located by the restriction map and DNA sequencing to the left half of the insert away from the *lac* promoter and its orientation was opposite to that of the *lac* Z′ gene in pUC 18.

3.9.2 DNA sequencing of CDase gene

The DNA sequencing was done step by step. The sequences obtained using the primers M13 forward, M13 reverse, 3 and C were used to design additional primers in order to sequence the whole insert. Sequencing primers H1, H2, T1, T2, T3 and T4 were used for the sequencing of pJK 555 insert. Their annealing sites are shown in Figure 48. A total of 4,932 bp DNA sequence was obtained and submitted to the GenBank (accession No. AY205309). Part of the sequence with CDase gene was shown in Figure 49. The CDase gene encoded a protein of 653 amino acids with a deduced molecular weight of 75,546. A putative promoter and a Shine-Dalgarno sequence (SD) could be located upstream of the gene.

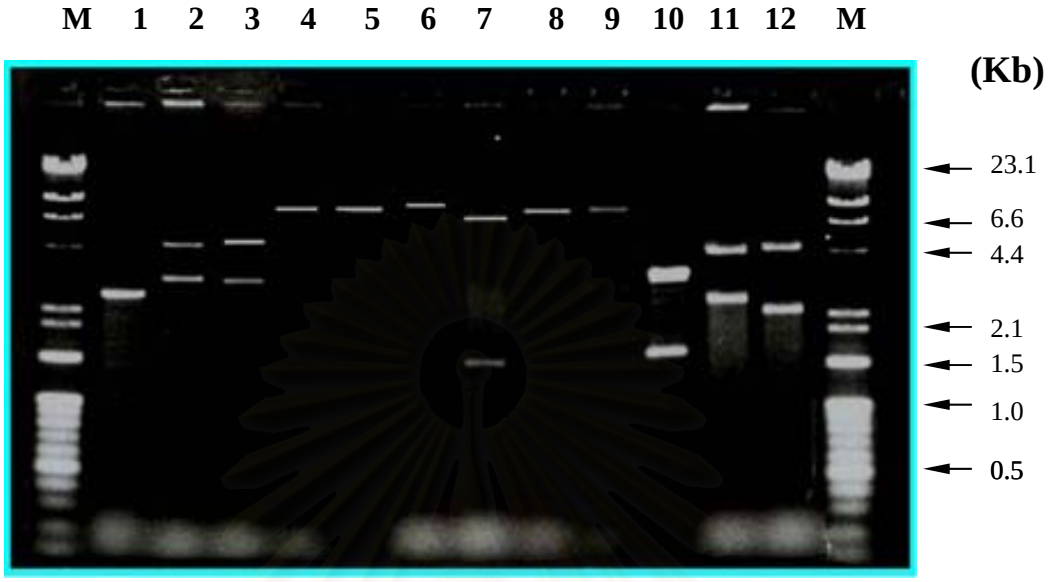


Figure 47 Mapping of pJK 555 with various restriction enzymes

Lane M = Standard DNA marker λ/*Hin*d III, 100 bp marker

Lane 1 = pUC 18/*Eco*R I

Lane 2-9 = Single digested with *Eco*R I, *Sac* I, *Kpn* I, *Hin*d III, *Sma* I, *pst* I, *Sa*l I and *Xho* I, respectively

Lane 10-12 = Double digested with *Sac* I- *Eco*R I, *Hin*d III- *Sma* I and *Hin*d III- *Xho* I, respectively

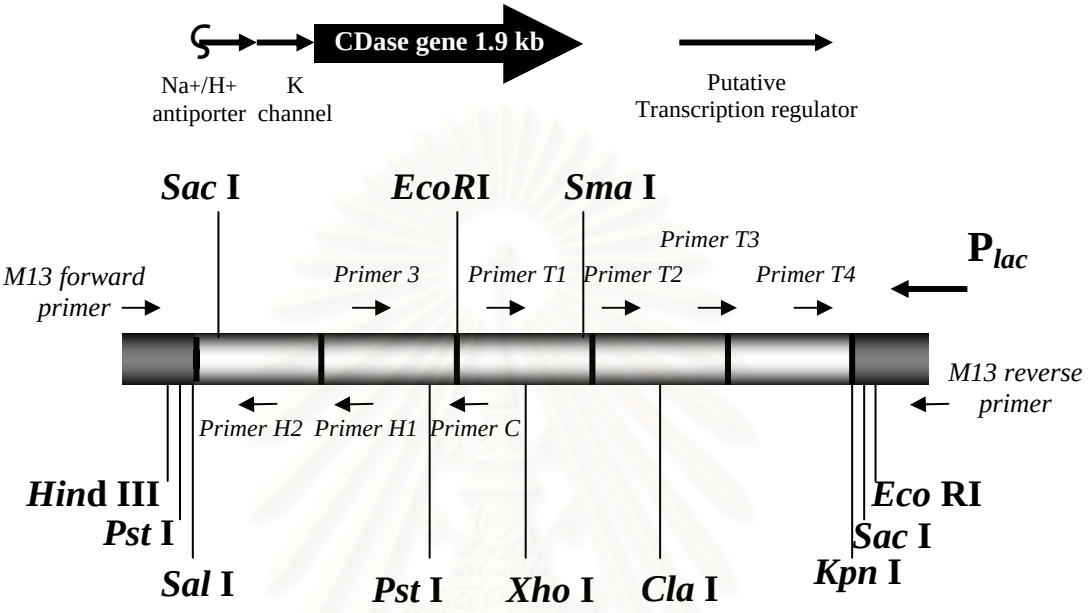


Figure 48 Restriction map of inserted fragment in pUC 18

The top arrow indicates orientation and size of CDase gene in pUC 18 including a partial Na^+/H^+ antiporter, a K channel and a putative transcription regulator. The small arrows locate the primers used for insert-pJK 555 plasmid sequencing.

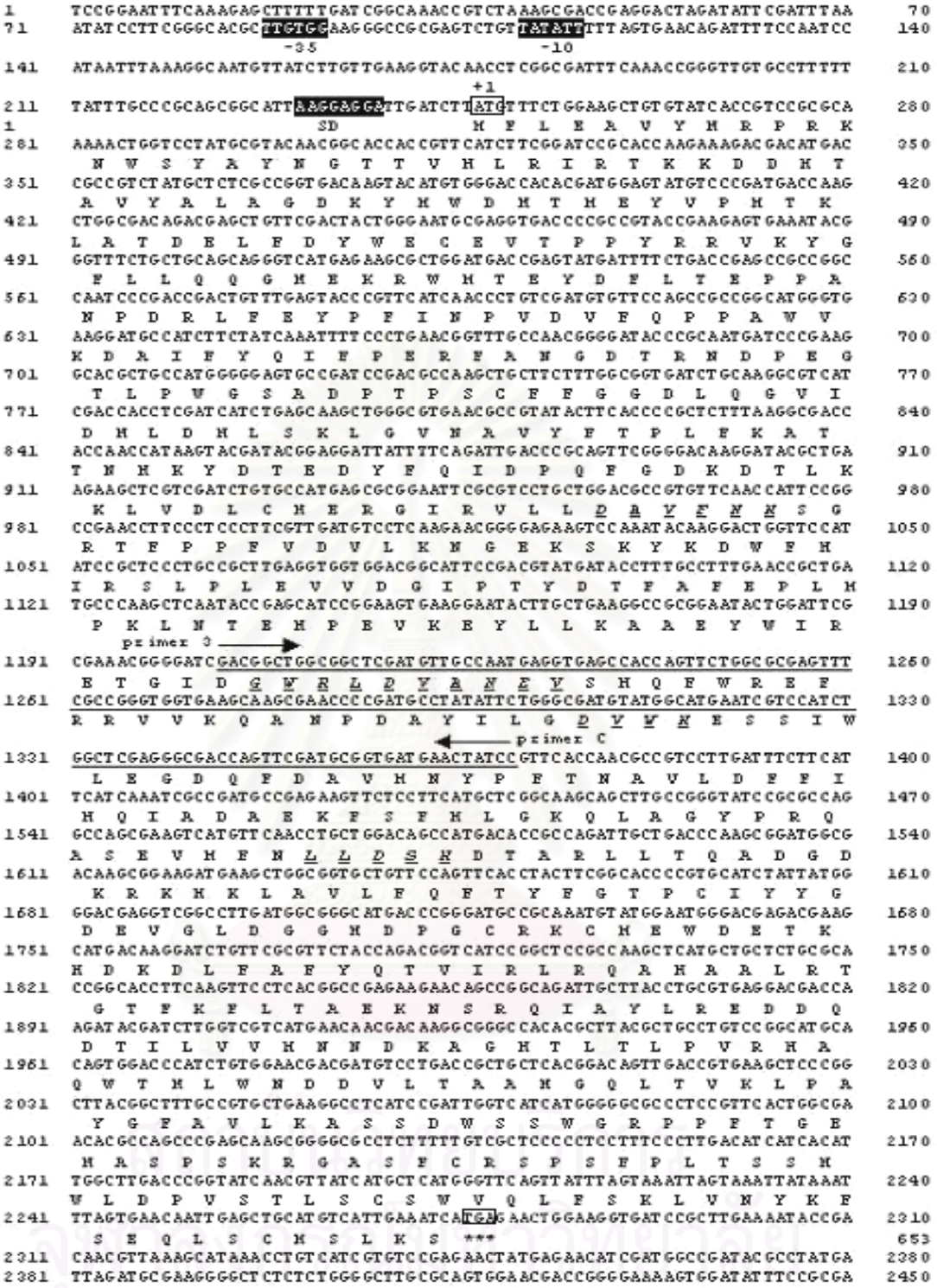


Figure 49 Nucleotide sequence of the DNA fragment containing the CDase gene from *Paenibacillus* sp. A11.

The start and stop codons are boxed. Putative promoter element and Shine-Dalgarno sequence are shaded. Arrows indicate priming sites for primers 3 and C. The sequence of 3-C PCR product is underlined. The conserved regions of starch-degraded enzymes are shown as italic letters with the broken underline.

3.9.3 The three-dimentional (3D) structure of CDase

The 3D structure which was modeled by Swiss-Model using the deduced amino acid sequence consisted of 3 domains; N-terminal, catalytic and carboxyl-terminal domains (Figure 50). The structure of catalytic domain is an 8 stranded alpha/beta barrel containing the active site, interrupted by N-terminal domain protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal beta barrel domain.

3.9.4 Expression of CDase gene in *Escherichia coli*

The *E. coli* strain JM109 carrying pJK 555 was aerobically cultured in LB broth at 37 ^{o}C for 24 h. The cells were lyzed by sonication and the crude lysate was prepared and assayed for CDase activity. As compared to that of *Paenibacillus* sp. A11 at maximum growth, the production of CDase for the pJK 555 clone was somewhat higher (Table 14). This is judged from the specific activity of the pJK 555 clone which was about 1.3 fold higher than that of *Paenibacillus* sp. A11. The protein was also analyzed by using SDS-PAGE. As shown in Figure 51A, one of the main translation products from pJK 555 clone (lane 2-3) with the same mobility as purified CDase from *Paenibacillus* sp. A11 (lane 1) was observed while it was absent from the control cell lysates and pellet fractions (lane 4-6).

To test whether the protein band was indeed CDase, activity staining in native gel was performed (Figure 51C). Although protein staining (Figure 51B) did not give well-resolved protein bands, the activity bands from *Paenibacillus* sp. A11 and

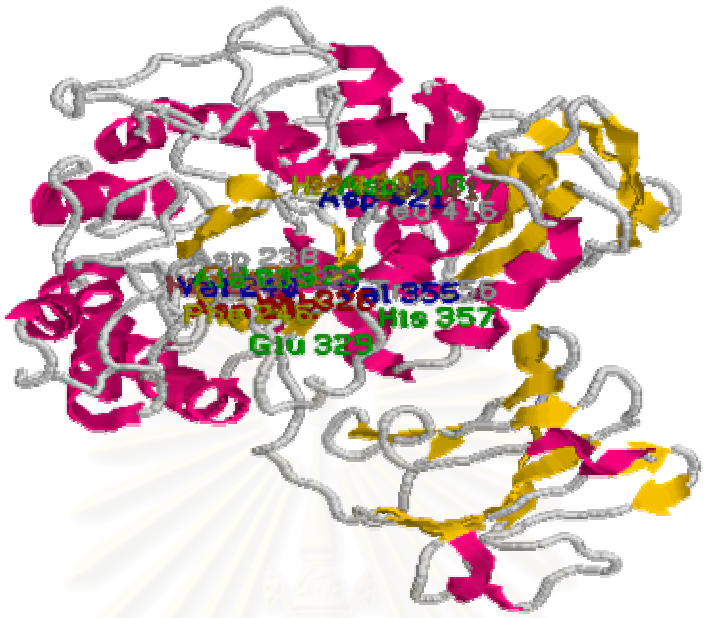


Figure 50 The 3D structure of CDase from *Paenibacillus* sp. A11.

Modeled by using Swiss-Model ver. 36 and displayed by RasWin Molecular graphics (Rasmol) ver. 2.6. β-sheet is in yellow color, α-helix in pink color and turns in white color. The upper part of the structure is catalytic domain whereas the right-lower part is N-terminal domain. The C-terminal domain is behind the catalytic domain.

Table 14 Comparison of CDase expressed by *E. coli* transformant with that of *Paenibacillus* sp. A11

Strain	CDase activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Paenibacillus sp. A11*	43.0	6.8	6.3
E. coli JM 109**	0	1.3	0
E. coli JM 109 (pUC 18)**	0	1.6	0
E. coli JM 109 (pNeg)**	0	1.3	0
E. coli JM 109 (pJK 555)**	14.3	1.7	8.4

* The CDase specific activity was determined from cells cultured for 48 h in Horikoshi medium containing 1% β-cyclodextrin.

** The CDase specific activities were determined from cells cultured for 24 h in LB medium containing 100 μg/ml ampicillin.

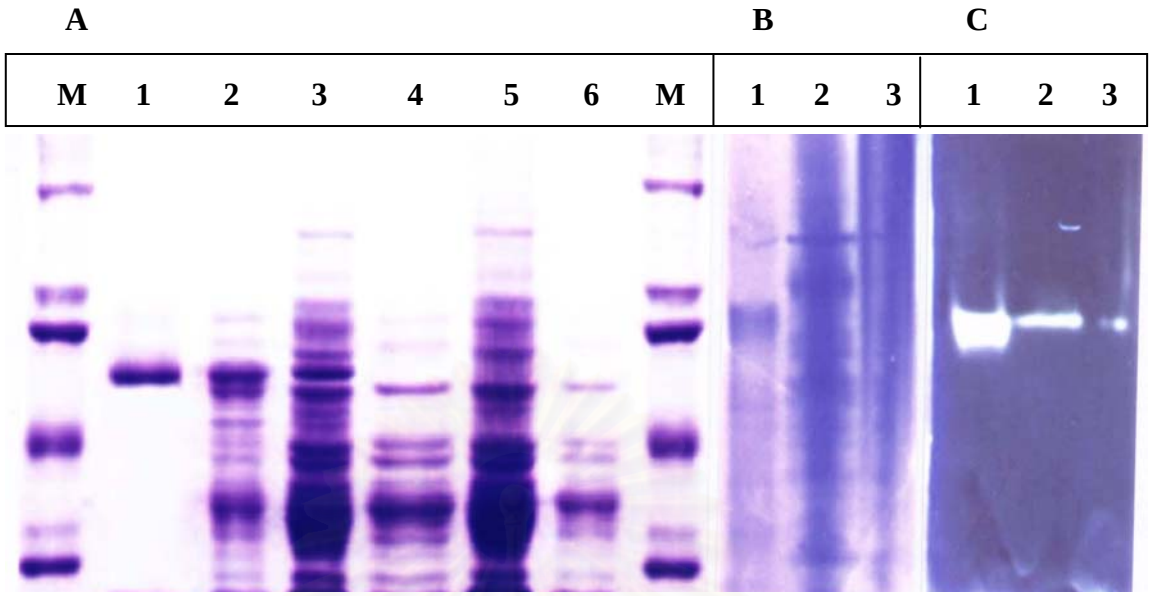


Figure 51 Detection and size estimation of CDase enzyme on polyacrylamide gel electrophoresis (PAGE)

(A) SDS-PAGE (Coomassie blue staining) of enzyme from various sources; Lane M : standard molecular weight marker; Lane 1 : purified CDase from *Paenibacillus* sp. A11; Lane 2 : crude intracellular proteins from *E. coli* (pJK 555); Lane 3 : crude proteins from the pellet fraction of *E. coli* (pJK 555); Lane 4 : crude intracellular proteins from *E. coli* (pUC 18); Lane 5 : crude proteins from the pellet fraction of *E. coli* (pUC 18); Lane 6 : crude intracellular proteins from *E. coli* containing pUC18 with no CDase insert

(B) Non-denaturing PAGE (Coomassie blue staining) Lane 1-3, as in A

(C) Non-denaturing PAGE (Amylolytic activity staining by iodine solution) Lane 1-3, as in A

transformant crude lysate were at the same position in the gel (lane 1-3, Figure 51C). The cloned CDase had the same molecular weight as that from *Paenibacillus* sp. A11 which was estimated from the SDS-PAGE to be 80,000. Production of CDase in JM109 (pJK 555) was not stimulated by the addition of IPTG since the activity could be equally observed either in the presence or absence of IPTG. The cloned CDase gene was, therefore, equipped with its own promoter that was active in *E. coli* host cells.

3.9.5 Stability of CDase gene in *E. coli* JM 109

The pJK 555 transformant was streaked on LB agar plate containing 100 μg/ml ampicillin, IPTG and X-gal. After colony was obtained, restreak was repeated for ten times. Cells obtained each time were inoculated in LB broth containing 100 μg/ml ampicillin, IPTG and X-gal for 24 h at 37 ^{o}C. Then they were harvested by centrifugation and CDase activity was measured. It was found that CDase activity could be measured in culturing pJK 555 clone after ten times of subcloning.

3.10 Effect of polyols on expression of CDase in *E. coli*

Several polyols were studied for their effects on the expression of *Paenibacillus* sp. A11 CDase in *E. coli* as described in section 2.16. The result showed that glycerol, mannitol and inositol did not change the level of enzyme production. Sorbitol, an osmotic stabilizer, was the only polyol which could increase CDase production. Its optimum effective concentration was evaluated in cells grown

at 37^{o}C. As shown in Table 15, sorbitol gave positive effect on the production of soluble CDase in *E. coli* harboring pJK 555. The maximum induction of soluble CDase, the three-fold increase in activity, could be achieved with 0.5 M sorbitol.

We further investigated the effect of sorbitol on the production of soluble CDase enzyme in cells. Sorbitol enforced the production of soluble CDase concurrently with cell growth. As shown in Figure 52A and 52B, after 8 h culturing, the cell growth and CDase activity were significantly higher when sorbitol was present in the culture medium.

The effect of sorbitol on prevention of the insoluble CDase was demonstrated by SDS-PAGE. After 24 h culture in LB medium under different conditions, the amount of CDase protein (80 kDa) was compared. When the cells were cultured at 37^{o}C in the presence of sorbitol, a small amount of insoluble CDase protein was observed, while the soluble CDase from cytoplasmic fraction was mainly detected (Figure 53B). An opposite result was observed when cells were grown in the medium without sorbitol (Figure 53A).

Table 15 Effect of sorbitol concentration on CDase production

Sorbitol (M)	CDase activity (U/ml)
0	14.0
0.1	27.3
0.2	33.5
0.3	36.4
0.4	40.7
0.5	45.6
0.6	39.1
0.7	32.3

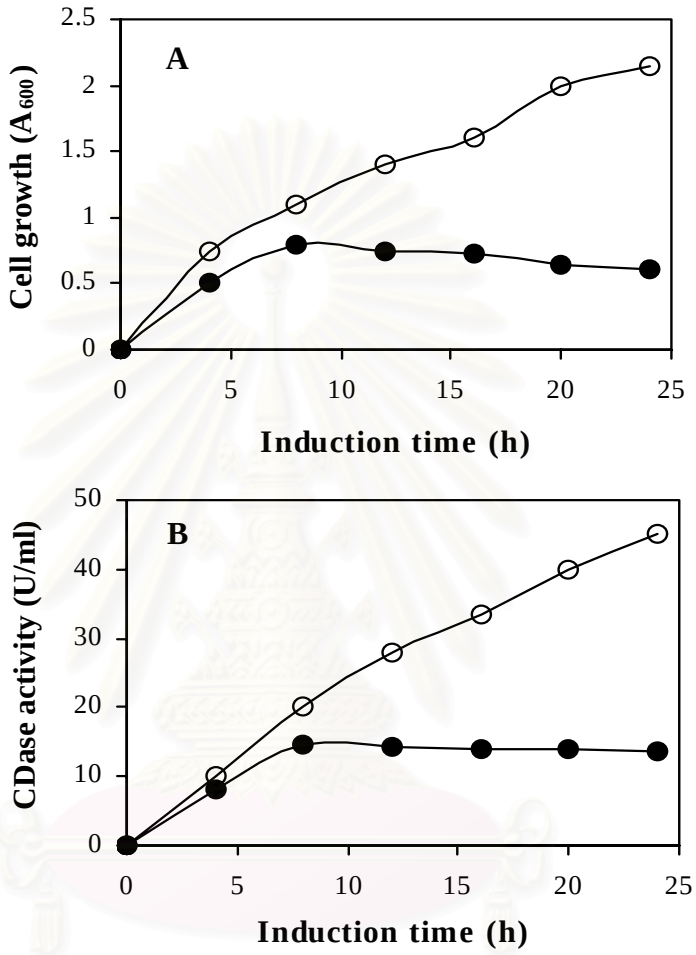


Figure 52 Effect of sorbitol on cell growth and soluble CDase production

E. coli (pJK 555) cells were cultured in the LB medium with (o) and without (•) 0.5 M sorbitol. Cell growth (A) and CDase activity (B).

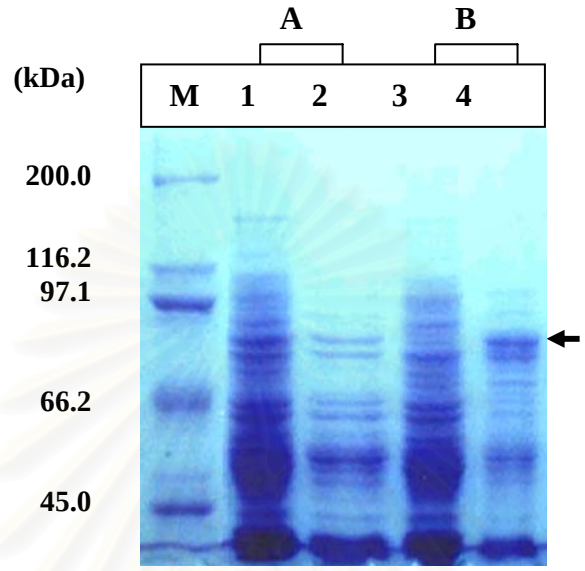


Figure 53 SDS-PAGE analysis of sorbitol effect.

(A) and (B) show induction results at 37°C in LB medium without and with 0.5 M sorbitol, respectively. Lanes 1 and 3; insoluble fraction, lanes 2 and 4; soluble fraction, lane M; molecular weight marker proteins. Arrow indicates induced CDase.

3.11 Subcloning of pJK 555 transformant

The 7.5 kb pJK 555 was digested with *Cla* I and *Kpn* I restriction enzymes and treated with Klenow fragment (Figure 54). The 1.7 kb DNA fragment was removed. The residual 5.8 kb DNA fragment was religated with T4 DNA polymerase and subcloned into *E. coli* JM 109 host cells. The resulting plasmid was named pJK 556. The restriction map of the pJK 556 was shown in Figure 55. CDase activity of *E. coli* (pJK 556) was compared with that of *E. coli* (pJK 555) and *Paenibacillus* sp. A11 under the best condition in cell culturing. The results are shown in Table 16. The subcloned transformant, pJK 556, gave the highest CDase activity of 69.2 U/ml which was about 1.5 and 1.6 fold higher than of *E. coli* (pJK 555) and *Paenibacillus* sp. A11, respectively. When comparing the specific activity of CDase, pJK 556 gave 2.4 fold higher than that of the original strain.

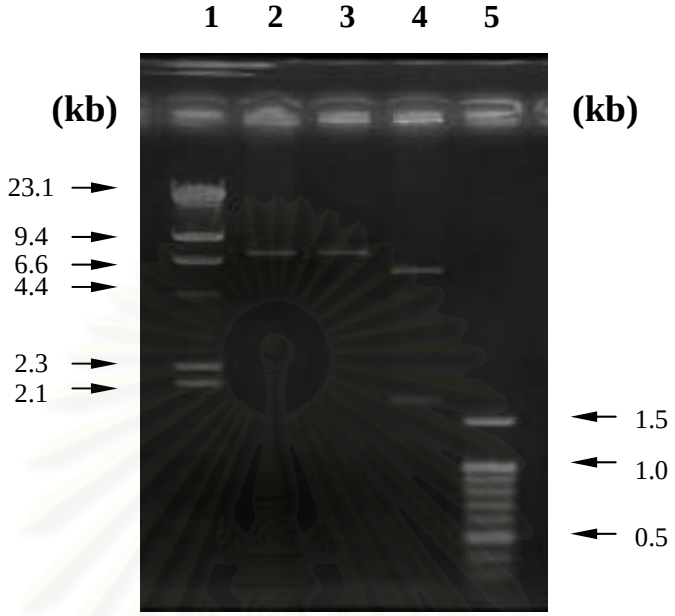


Figure 54 Subcloning of pJK 555 transformant

Lane 1 = λ/*Hin*d III marker

Lane 2 = pJK555/*Cla* I

Lane 3 = pJK555/*Kpn* I

Lane 4 = pJK555/*Cla* I-*Kpn* I

Lane 5 = 100 bp marker

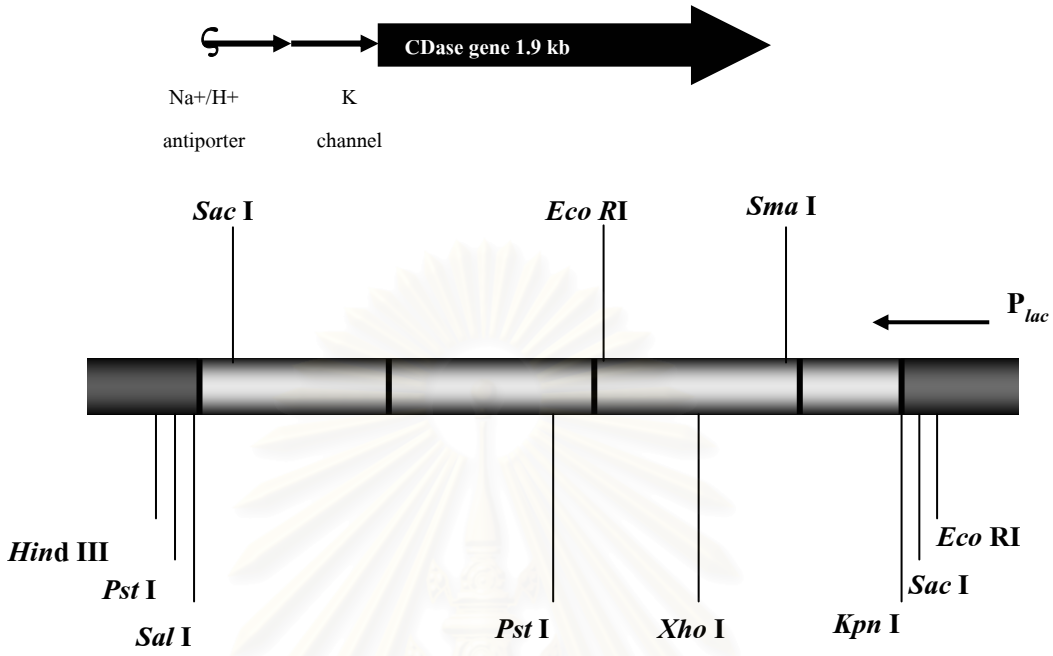


Figure 55 Restriction map of 3.2 kb inserted fragment in pUC 18

The top arrow indicates orientation and size of CDase gene in pUC 18 including a partial Na^+/H^+ antiporter and a K channel. The vertical lines indicate various restriction sites on pJK 556.

Table 16 Comparison of CDase expressed by *E.coli* harboring pJK 555, pJK 556 and *Paenibacillus* sp. A11

Strain	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
Paenibacillus sp. A11*	43.0	6.8	6.3
E.coli (pJK 555)**	45.0	4.4	10.2
E.coli (pJK 556)**	69.2	4.5	15.4

* The CDase specific activity was determined from cells cultured for 48 h in Horikoshi medium containing 1% β-cyclodextrin.

** The CDase specific activities were determined from cells cultured for 24 h in LB medium containing 100 μg/ml ampicillin and 0.5 M sorbitol.

Part IV : THE RECOMBINANT CDase

To compare the *E. coli* (pJK 555) CDase with the *Paenibacillus* sp. A11 CDase, the purification and main characterization of the recombinant CDase were performed.

3.12 Purification of the recombinant CDase

The recombinant CDase from *E. coli* (pJK 555) was successively purified after the following steps; cell disruption by ultrasonication, precipitation with ammonium sulfate, column chromatography on DEAE-Toyopearl 650M and chromatography on Phenyl-Sepharose CL-4B. The chromatographic profiles of DEAE-Toyopearl 650M and Phenyl-Sepharose CL-4B columns are shown in Figure 56 and 57, respectively. The enzyme was purified about 14-fold with a 31% yield. The specific activity was 141 unit/mg protein (Table 17). The purified enzyme showed a single band on native gel upon both protein and activity staining (Figure 58).

3.13 Characterization of the purified recombinant CDase from *E. coli* JM 109

3.13.1 Molecular weight determination

The molecular weight of the enzyme was 80 kDa as determined by SDS-PAGE (Figure 59). Gel filtration on a Sephadex G-200 column of the native CDase resulted in the estimation of the same molecular weight (Figure 60 and 61). These results indicate that the enzyme was a monomer with the molecular mass of 80 kDa.

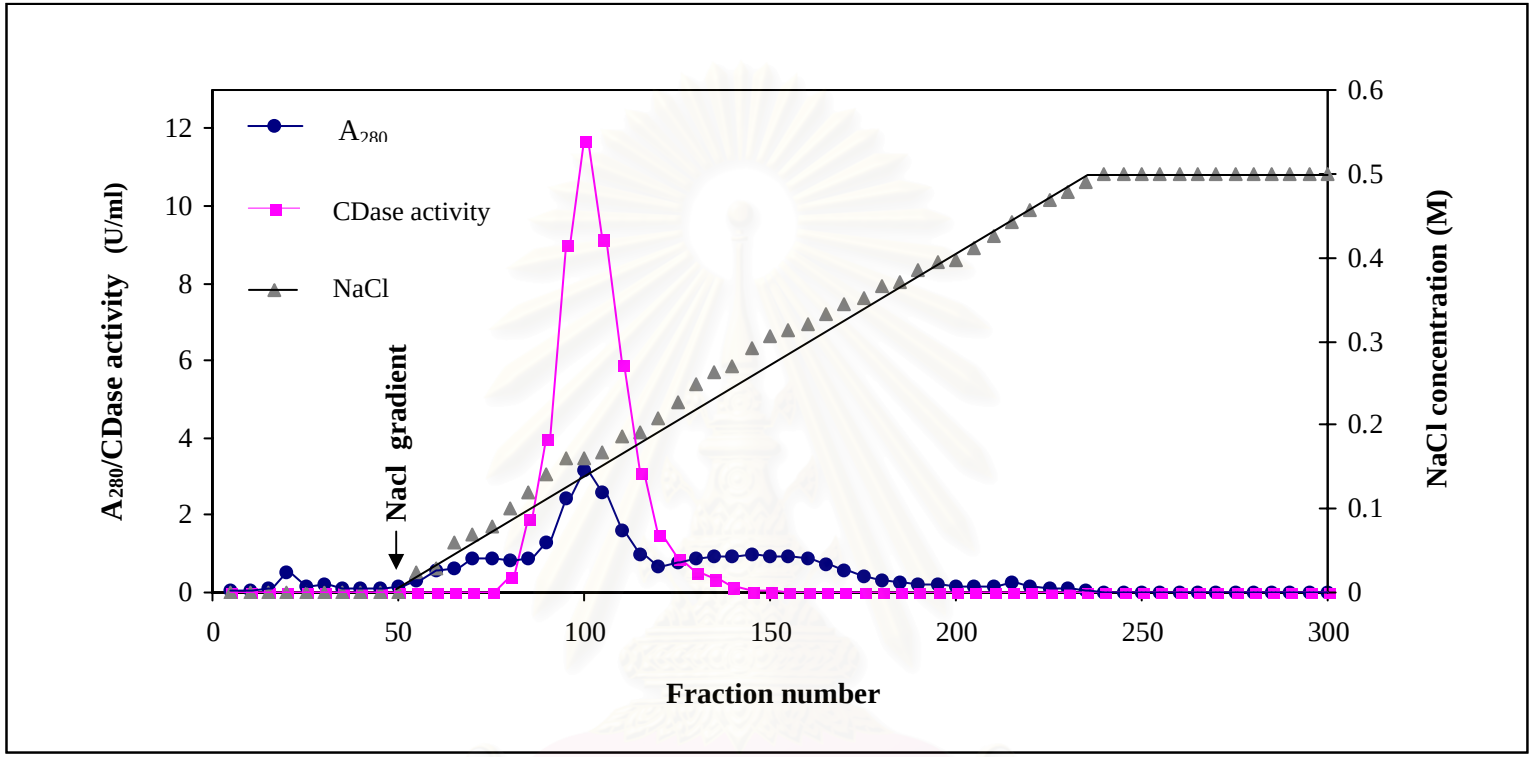


Figure 56 Purification of Cyclodextrinase from *E. coli* (pJK 555) by DEAE-Toyopearl 650M column

The enzyme solution was applied to DEAE-Toyopearl 650M column (1.7 X 17 cm) and washed with 10 mM potassium phosphate buffer, pH 7.5 containing 1 mM DTT, 1 mM EDTA and 10% glycerol until A_{280} decreased to baseline. Elution of bound proteins was performed by 0-0.5 M NaCl in the same buffer at the flow rate of 30 ml/h. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 80 to 125 was pooled.

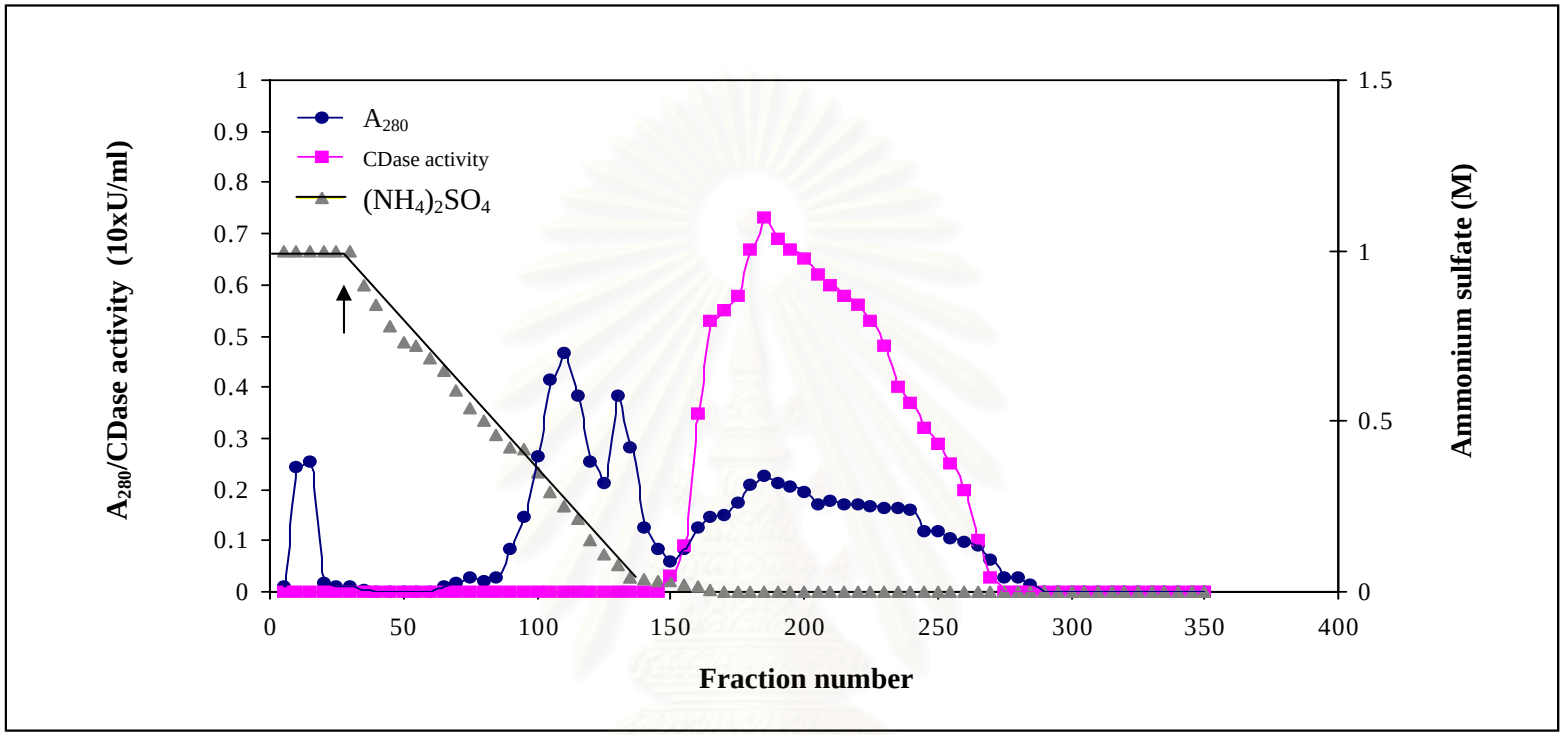


Figure 57 Purification of Cyclodextrinase from *E. coli* (pJK 555) by Phenyl-Sepharose CL-4B column

The enzyme solution was applied to Phenyl-Sepharose CL-4B column (1.7 X 10 cm) and washed with 10 mM potassium phosphate buffer, pH 7.5 containing 1M ammonium sulfate, 1 mM DTT, 1 mM EDTA and 10% glycerol until A_{280} decreased to base line. Elution of bound proteins was then performed by a linear gradient decreasing the ammonium sulfate from 1 to 0 M at the flow rate of 20 ml/h. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 150 to 275 was pooled.

Table 17 Purification of CDase from *E. coli* (pJK 555)

Purification step	Volume (ml)	Total activity (U)	Activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	25	1125	45	110	10.2	1	100
40-60% $(NH_4)_2SO_4$	10	750	75	43	17.4	1.7	67
DEAE-Toyopearl 650M	20	680	34	8	85	8.3	60
Phenyl-Sepharose CL-4B	5	352	70	2.5	141	13.8	31

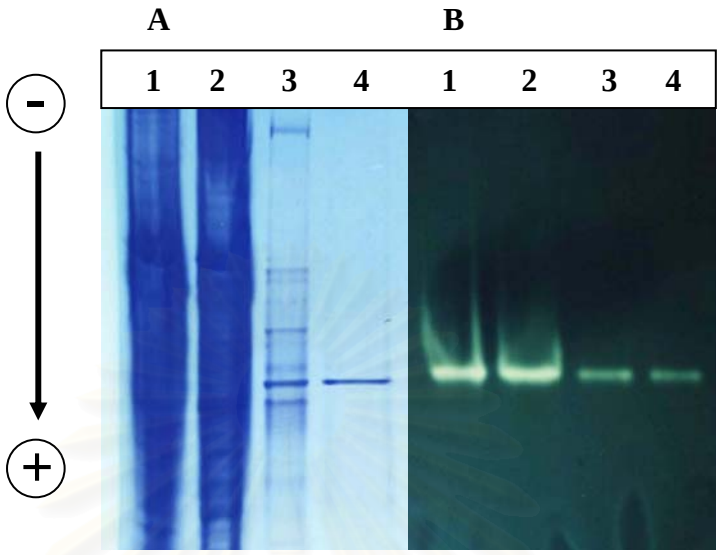


Figure 58 Non-denaturing PAGE analysis of *E. coli* (pJK 555) CDase in each step of purification on a 7.5% acrylamide gel.

(A) Coomassie blue staining

Lane 1 : crude enzyme (30 μg protein)

Lane 2 : enzyme from 40-60% ammonium sulfate precipitation (30 μg protein)

Lane 3 : enzyme from DEAE-Toyopearl 650M (15 μg protein)

Lane 4 : enzyme from Phenyl-Sepharose CL-4B (10 μg protein)

(B) Amylolytic activity staining

Lane 1-4, as in A, 0.2 units of dextrinizing activity was loaded to each well

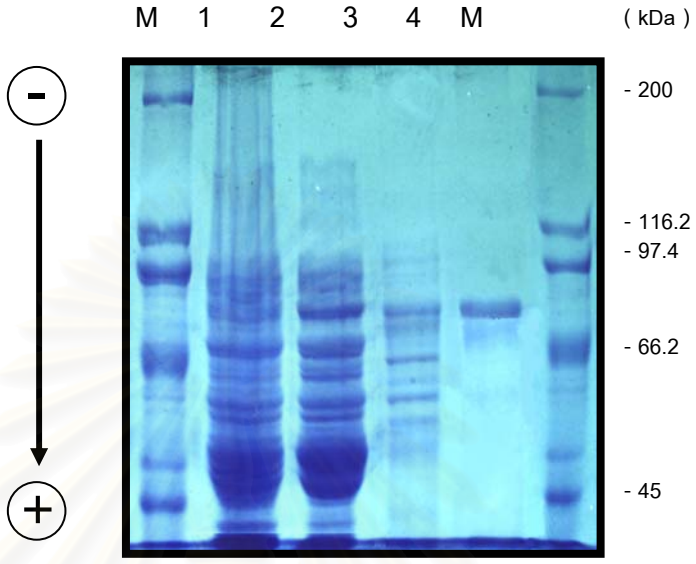


Figure 59 SDS-PAGE of *E. coli* (pJK 555) CDase from different steps of purification

Coomassie blue staining

Lane M : Protein molecular weight markers

Lane 1 : Crude enzyme (30 μg protein)

Lane 2 : enzyme from $(NH_4)_2SO_4$ Fraction (30 μg protein)

Lane 3 : enzyme from DEAE-Toyoperl (15 μg protein)

Lane 4 : enzyme from Phenyl Sepharose CL 4B (10 μg protein)

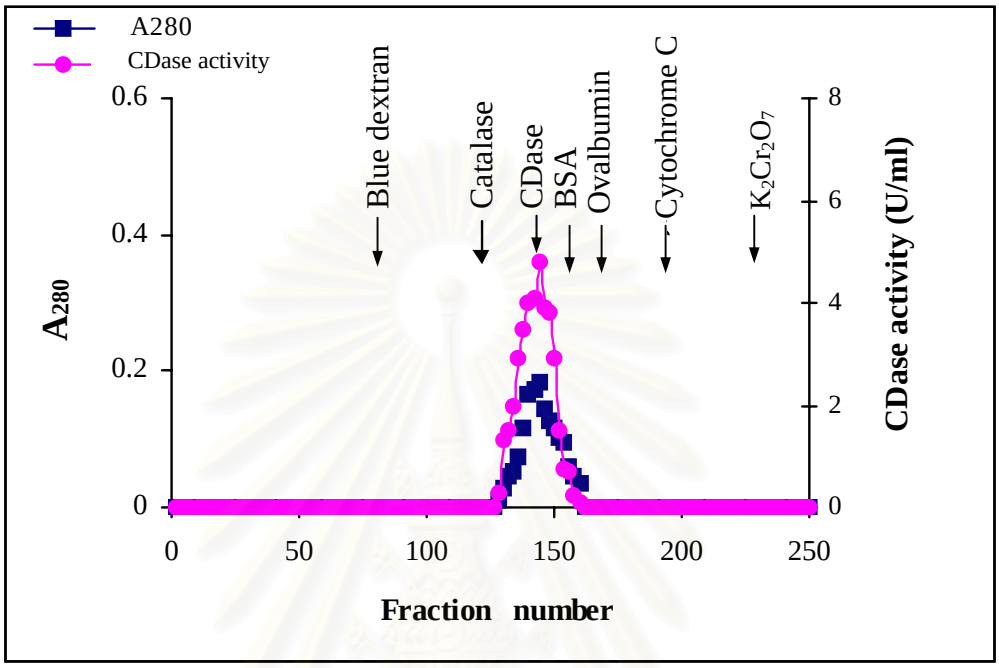


Figure 60 Molecular weight of CDase from *E. coli* (pJK 555) by Sephadex G-200 column

The enzyme solution was applied to Sephadex G-200 column (2.4 X 80 cm) and eluted with 10 mM potassium phosphate buffer, pH 7.5 containing 0.1 M NaCl at the flow rate of 20 ml/h. Fractions of 2 ml were collected. Positions of markers and *E. coli* (pJK 555) CDase are shown.

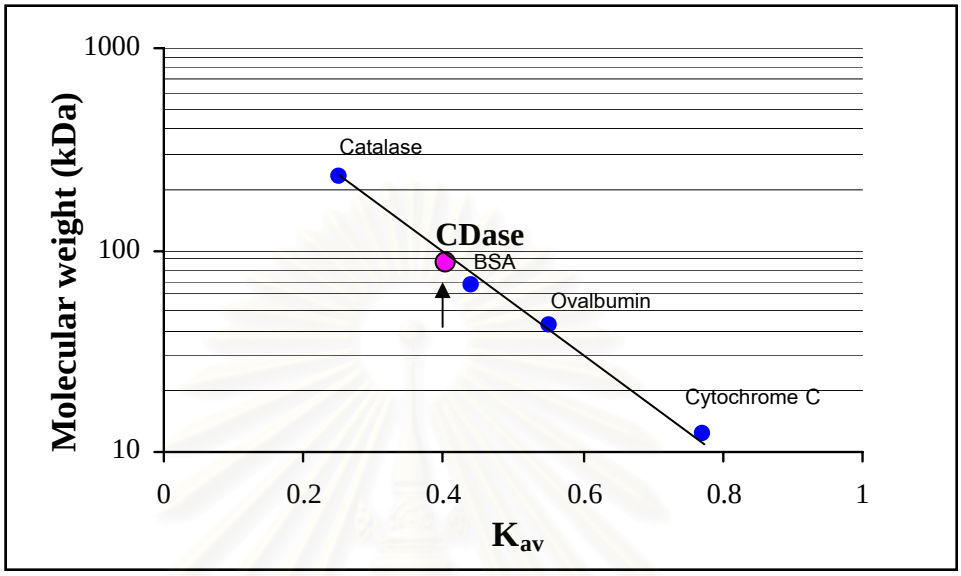


Figure 61 Calibration curve for native molecular weight of CDase from *E. coli* (pJK 555) determined by gel filtration chromatography on Sephadex G-200 column

Catalase	(MW 232,000 Da)
Bovine serum albumin	(MW 67,000 Da)
Ovalbumin	(MW 43,000 Da)
Cytochrome C	(MW 12,500 Da)

Arrow indicates the K_{av} of CDase.

3.13.2 Effect of pH and temperature on CDase activity

The effects of pH and temperature on the enzyme activity were examined (Figure 62A and B); the optimum pH and temperature were 7.0 and 40 ^{o}C, respectively. The enzyme showed 20-60% of the activity at pH 5.0-6.5 and 8.0-9.0 while very low or no activity was observed at pH below 5.0 or above 10.0. Upon incubation of enzyme with 1% (w/v) β-CD substrate at 50 ^{o}C for 30 min, 80 percents of the activity still remained. The recombinant CDase activity was inactivated at temperature lower than 10 or higher than 80 ^{o}C.

3.13.3 Substrate specificity and hydrolysis products

The recombinant CDase had high specificity towards β-CD, followed by γ-CD and α-CD, respectively. Hydrolytic activities of 20-45% relative to that of β-CD were observed when substrates maltopentaose, maltohexaose and maltoheptaose were used. Lower activity (10-14%) was observed with maltotriose, maltotetraose, and soluble starch while trace hydrolysis (3% hydrolytic activity) of pullulan could be detected (Table 18). In addition, this recombinant enzyme could not hydrolyze glycogen.

The end-products after 24 h incubation of the enzyme with various substrates were investigated and analyzed by TLC (Figure 63) and HPLC (Table 18). Each substrate was degraded to give reducing oligosaccharides with maltose and glucose as the dominant products. More maltose was observed in all cases while α-CD, β-CD, and soluble starch also gave certain amount of maltotriose.

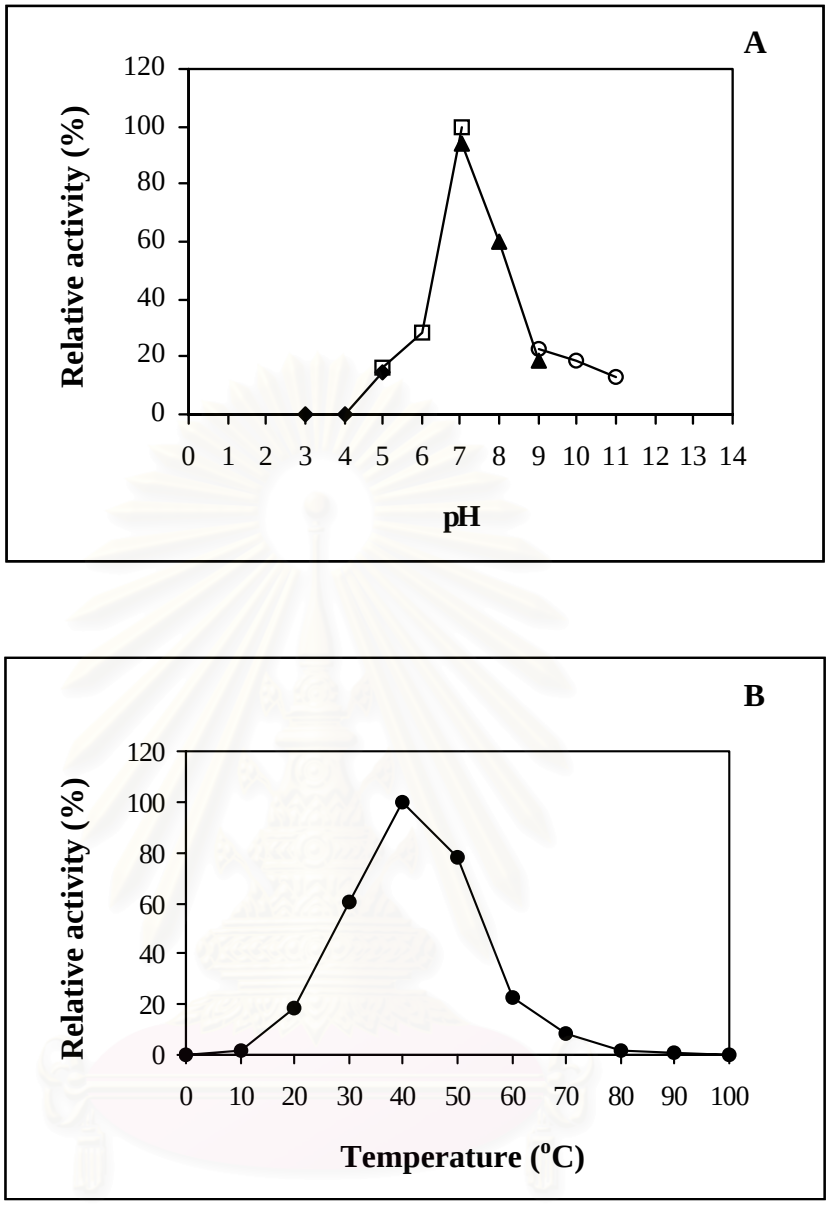


Figure 62 Effects of pH (A) and temperature (B) on enzyme activity.

Buffers used: (◆) 0.1 M acetate buffer, pH 3.0-5.0; (□) 0.1 M K-phosphate buffer, pH 5.0-7.0; (▲) 0.1 M Tris-HCl buffer, pH 7.0-9.0, and (○) 0.1 M glycine-NaOH buffer, pH 9.0-11.0.

Table 18 Substrate specificity and end-products of the recombinant cyclodextrinase

Substrate* (1%,w/v)	Relative activity (%)	End-product ** (relative mole ratio) G1 : G2 : G3 : G4 : G5
β-Cyclodextrin	100	0.7 : 1.0 : 0.3 : 0.1 : 0.0
γ-Cyclodextrin	72	0.7 : 1.0 : 0.1 : 0.1 : 0.1
α-Cyclodextrin	57	0.6 : 1.0 : 0.3 : 0.1 : 0.0
Maltotriose (G3)	11	0.7 : 1.0 : 0.2 : 0.0 : 0.0
Maltotetraose (G4)	14	0.6 : 1.0 : 0.1 : 0.1 : 0.0
Maltopentaose (G5)	22	0.6 : 1.0 : 0.3 : 0.1 : 0.0
Maltohexaose (G6)	35	0.7 : 1.0 : 0.1 : 0.1 : 0.0
Maltoheptaose(G7)	43	0.6 : 1.0 : 0.1 : 0.1 : 0.1

*soluble starch was less hydrolyzed (9% relative activity) while very small amount of pullulan (3% relative activity) could be hydrolyzed. Glycogen was the substrate that could not be hydrolyzed.

**after 24 h incubation

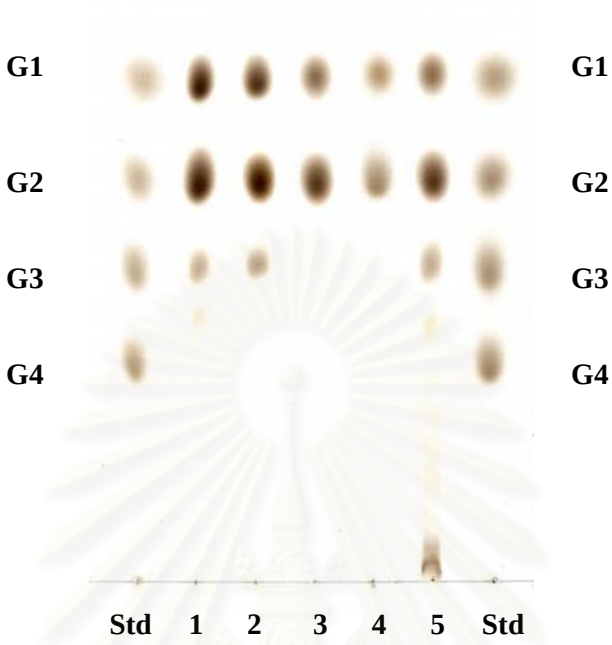


Figure 63 Thin layer chromatogram of the hydrolysis products produced by *E. coli* (pJK 555) CDase

Reactions were performed as described under experimental procedures. Lane 1; α-CD, Lane 2; β-CD, Lane 3; γ-CD, Lane 4; maltoheptaose and Lane 5; soluble starch. G1; glucose, G2; maltose, G3; maltotriose, G4; maltotetraose.

3.13.4 Kinetic studies of recombinant CDase

Kinetic parameters of the recombinant CDase with α-, β-, and γ-CDs as substrates were shown in Table 19. The recombinant CDase showed the greatest K_m, k_{cat} and k_{cat}/K_m values for β-CD. Whereas the bigger ring, the γ-CD, was significantly better than the small ring of α-CD when catalytic efficiency, k_{cat}/K_m, was compared.

3.13.5 Effects of metal ions, protective chemicals and saccharides on the activity of recombinant CDase

The effects of selected metal ions, protective chemicals and saccharides on CDase activity were summarized in Table 20. The enzyme was completely inhibited by 10 mM concentration of $Hg^{2+}$and Ag^{+} but moderately inhibited by Zn^{2+}. Mg^{2+} showed small inhibition while no inhibition was observed with Ca^{2+}, K^{2+}, Li^{+}, Na^{+}, and Ba^{2+}. Protective substance for enzyme such as β-mercaptoethanol and EDTA had no effect on the activity of this enzyme. The saccharides, glucose and maltose had no effect on enzyme activity, while acarbose, a pseudotetrasaccharide, significantly inhibited this CDase.

3.13.6 Effect of group-specific reagents on the activity of recombinant CDase

The result in Table 21 showed that recombinant CDase activity was totally inactivated by 1 mM NBS and DEP while partial inactivation was observed when

Table 19 Kinetic parameters of the recombinant CDase with cyclodextrins substrate

Substrate	K_m (mM)	V_{max} (μmoles/min)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}min^{-1}$)
α-CD	5.0	0.17	1500	3.0×10^5
β-CD	2.5	0.25	2200	8.8×10^5
γ-CD	2.9	0.18	1600	5.5×10^5

Table 20 Effects of metal ions, protective chemicals and saccharides on the activity of recombinant CDase

Compound*	Final concentration	Relative activity (%)
None	-	100
Metal ions		
$CaCl_2$	10 mM	100
KCl	10 mM	94
LiCl	10 mM	90
NaCl	10 mM	92
$BaCl_2$	10 mM	88
$MgCl_2$	10 mM	80
$ZnCl_2$	10 mM	37
$AgNO_3$	10 mM	0
$HgCl_2$	10 mM	0
Protective chemicals		
β-mercaptoethanol	10 mM	107
EDTA	10 mM	101
Saccharides		
Glucose	10 mM	98
Maltose	10 mM	95
Acarbose	5 mM	9.4
	10 mM	0

*Incubation with 0.15 mg/ml enzyme at 40°C, pH 7.5 for 30 min.

Table 21 Effect of various group-specific reagents on the activity of recombinant CDase

Reagent*	Concentration (mM)	Amino acid involved	Residual activity (%)
None	-	-	100
Iodoacetamide (IAM)	5	Cysteine	71
Dithiothreitol (DTT)	5	Cystine	115
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	5	Lysine	97
Phenylmethylsulfonyl fluoride (PMSF)	5	Serine	94
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	5	Carboxylic amino acids	76
Diethylpyrocarbonate (DEP)	5	Histidine	0
	1		0
	0.1		63
N-bromosuccinimide (NBS)	5	Tryptophan	0
	1		0
	0.1		5
N-acetylimidazole (NAI)	5	Tyrosine	99
Phenylglyoxal (PG)	5	Arginine	62

*Incubation with 0.15 mg/ml enzyme at 40°C, pH 7.5 for 30 min.

incubated with 5 mM IAM, EDC and PG at pH 7.5, 40^{o}C for 30 min. TNBS, PMSF and NAI at concentration of 5 mM had no inactivation effect. A reducing agent like DTT increased enzyme activity which suggests that reduction of disulfide bond was more or less important for this enzyme. The involvement of Trp, His, Cys, Carboxylic amino acids and Arg on the activity of enzyme could be then proposed.

3.13.7 Comparison of biochemical properties of the CDases from *Paenibacillus* sp. A11 and the *E. coli* transformant

The recombinant CDase was purified to a specific activity of 141 unit/mg protein, representing a 14-fold purification. It was a single polypeptide with M_r of 80 kDa which was consistent with the apparent molecular weight of *Paenibacillus* sp. A11 CDase. The optimum pH and temperature of CDases from the both sources were 7.0 and 40 ^{o}C. Other determined biochemical properties of the cloned enzyme were almost identical to those of the authentic one (Table 22). The substrate specificity of *E. coli* (pJK 555) CDase was very similar to that of *Paenibacillus* sp. A11. The best substrate for the recombinant and authentic CDases was β-CD. In addition, CDase activity from the *E. coli* transformant and *Paenibacillus* sp. A11 was strongly inhibited by acarbose.

Table 22 Comparison of biochemical properties of the CDases from *Paenibacillus* sp. A11 and the *E. coli* transformant

Parameter	*Paenibacillus* sp. A11	*E. coli* JM 109 (pJK 555)
Relative molecular weight (kDa)		
Sephadex G-200	80	80
SDS-PAGE	80	80
Specific activity (U/mg)	133	141
Optimum pH	7.0	7.0
Optimum temperature (^{o}C)	40	40
k_{cat}/K_m for β-CD ($M^{-1}min^{-1}$)	$8.2x10^5$	$8.8x10^5$
Dominant product	G2, G1	G2, G1
Activator	DTT	DTT
Inactivator	NBS, DEP, IAM, EDC, PG	NBS, DEP, IAM, EDC, PG
Inhibitor	Ag^+, Hg^{2+}, Zn^{2+} Acarbose	Ag^+, Hg^{2+}, Zn^{2+} Acarbose

CHAPTER IV

DISCUSSION

The novel starch degradation pathway via CDs metabolism, which includes the extracellular conversion of starch into CDs by CGTase, the uptake of CDs by a specific system, and the intracellular linearization by a CDase, was proposed in *Klebsiella oxytoca* M5a1, alkalophilic *Bacillus* sp. A2-5a and *Thermococcus* sp. B1001 (Figure 1-2) (Fiedler *et al*., 1996; Ohdan *et al*., 2000; Hashimoto *et al*., 2001). The genes responsible for CDs metabolism were found to be clustered on chromosomes of these organisms, though the pattern and the direction of the genes were different (Figure 7-9). Until now, no other organisms possessing both CGTase and CDase for the synthesis and degradation of CDs have been reported.

Our research group isolated *Bacillus circulans* A11 (reidentified as *Paenibacillus* sp. A11 in this study) CGTase that catalyzed predominantly the formation of β-CD, with smaller amounts of α- and γ-CDs, from starch (Kim, 1996; Kaskangam, 1998; Rojtinnakorn *et al*., 2001). The gene encoding for this CGTase has been cloned and sequenced (Rimphanitchayakit *et al*., 2000). Discovery of CGTase implied that the novel starch degradation pathway may also exist in this gram-positive organism. Possible CDase and putative CD binding protein genes should be found in the flanking region of the CGTase gene of strain A11 as what had occurred in *Klebsiella oxytoca* M5a1, alkalophilic *Bacillus* sp. A2-5a and *Thermococcus* sp. B1001 (Fiedler *et al*., 1996; Ohdan *et al*., 2000; Hashimoto *et al*., 2001). In this study, we focus on the catabolic pathway of CDs, involving CDase and its encoding gene which has never been reported in the genus *Paenibacillus*.

Part I : BACTERIAL IDENTIFICATION

The bacterial screening was initially carried out by Pongsawasdi and Yagisawa (1987) for the purpose of finding microorganisms that could effectively produce cyclodextrins from starch. From the screening result, the strain A11 which was isolated from South-East Asian soil, showed the CGTase (CD-forming) activity. After the CGTase research in strain A11, CDase activity was our next focus.

Previously, the strain A11 has been classified as *Bacillus circulans* A11 by using morphological and biochemical characterization (Pongsawasdi and Yagisawa, 1987). Bacterial identification by 16S rRNA gene fragment amplification has been recently reported as a more powerful technique (Edwards *et al*., 1989; Ash *et al*., 1993; Yoon *et al*., 1998; Sripo *et al*., 2002). Reidentification of the strain A11 using 16S rRNA gene analysis, cellular fatty acid composition and G+C content was performed to establish its correct taxonomic position.

Those bacteria with rod-shaped and endospore have generally been assigned to the genus *Bacillus*, a systematically diverse taxon (Claus and Berkeley, 1986). 16S rRNA oligonucleotide cataloguing (Fox *et al*., 1977; Stackebrandt and Goebel, 1994) and more recently comprehensive 16S rRNA gene sequence analysis have shown that the genus *Bacillus* is phylogenetically very heterogeneous, at least 10 phylogenetic groups have been identified in the genus *Bacillus* (Teather and Wood, 1982; Wallace and Brammall, 1985; Ash *et al*., 1991; Farrow *et al*., 1992; Ash *et al*., 1993). With the development in the molecular biology technique, five of the groups have been reclassified as the new genera *Alicyclobacillus*, *Paenibacillus*, *Halobacillus*, *Brevibacillus* and *Aneurinibacillus* (Shida *et al*., 1997).

In this study, the forward primer pA (AGA GTT TGA TCC TGG CTC AG) and reverse primer pH (AAG GAG GTGATC CAG CCG CA) were used to amplify the gene. Then primers pA: AGA GTT TGA TCC TGG CTC AG, pD: CAG CAG CCG CGG TAA TAA TAC and pF: CAT GGC TGT CGT CAG CTC GT were used for sequencing. Edwards *et al*. (1989) had used these primers to determine the nucleotide sequence of 16S rRNA gene of *Mycobacterium kansasii*, which was found to be 98.7% homologous to that of *Mycobacterium bovis* BCG. This is the first report on a contiguous sequence information of an entire amplified gene spanning 1.5 kb without any subcloning procedures. These primers have been used to identify various bacteria due to the conserved sequence property of 16S rRNA gene (Ash *et al*., 1991 Yoon *et al*., 1998; Sripo *et al*., 2002). After 16S rRNA gene of A11 was amplified, PCR product of 1.5 kb was sequenced. The 16S rRNA sequence of A11 was 1,524 bp and showed 99% homology with *Paenibacillus campinasensis* 324 and *Paenibacillus campinasensis* JCM 11200. Phylogenetic analysis of 16S rRNA sequence indicated that A11 was closely related to the *Paenibacillus* species, especially *Paenibacillus campinasensis*. Levels of similarity of 16S rRNA gene between strain A11 and the *Paenibacillus* species were 90-99%, while similarity with *Bacillus circulans* IAM 12462 was only 86%. So, the phylogenetic study from 16S rRNA gene analysis clearly establishes that strain A11 is a *Paenibacillus* species.

When cellular fatty acid of strain A11 was investigated, the major form was anteiso-$C_{15:0}$ (Figure 19) which was the characteristic of the genus *Paenibacillus* (Ash *et al*., 1991; Yoon *et al*., 1998). For the genus *Bacillus*, the main cellular fatty acid was variable (Komagata and Suzuki, 1987; Shida *et al.*, 1997). Usually, cellular fatty acid composition of bacteria in the same species is similar while the different value is observed in comparison with that of other species or genera (Shida *et al.*, 1997).

Therefore, cellular fatty acid composition is also used as informative tools for bacterial identification. For example, cellular fatty acids of *Paenibacillus azotofixans* strain NRRL B-14372 and NRRL B-14359 contained a large amount of anteiso-$C_{15:0}$ of 60.5 and 59.3%, respectively whereas for *Paenibacillus macerans* JCM 2500, the major anteiso-$C_{15:0}$ was determined to be 36.1% of total cellular fatty acids. Importantly, cellular fatty acid components of a bacterium essentially are not changed by cultural conditions, but the composition in quantity is affected. Thus, in the determination of cellular fatty acid composition, cultural conditions need to be controlled.

Genetic analysis was performed to measure the G+C content of strain A11 through the melting temperature method. That the %G+C value is very important for the identification and classification of bacteria has been established by Bolozersky and Spirin (1960). The G+C content of strain A11 was 50.3 mol%, a level included within the range found in the members of the genus *Paenibacillus* (Shida *et al*., 1997). Generally, G+C content of the genus *Paenibacillus* was between 45 and 54 mol%.

From the data obtained, strain A11 should be reclassified as *Paenibacillus* sp. A11. Although A11 was 99% similarity to *Paenibacillus campinasensis*, we could not place A11 as *Paenibacillus campinasensis* due to phenotypic uniqueness imparted by the alkaline-tolerant bacterium and some biochemical difference. Our strain was able to grow at pH 7.0 as well as pH 10.0 while could not resist higher than 3% NaCl which made A11 distinct from *Paenibacillus campinasensis* (Techaiyakul, 1991; Rutchtorn, 1994; Rojtinnakorn *et al*., 2001). Alkaliphilic *Paenibacillus campinasensis* 324, originally identified as *Bacillus firmus*, cannot grow at pH 7.0 but grows in the range of pH 7.5-10.5; optimum pH is 10.0 (Yoon *et al.*, 1998). In addition, one of the

main characteristics of *Paenibacillus campinasensis* 324 is its ability to grow in the presence of 7% NaCl.

Part II : CDase FROM *Paenibacillus* sp. A11

CDase production

As a first approach for CDase production, the CDase activity was followed using a specific agar medium which was developed in the present study. The specific agar medium which contained β-CD and phenolphthalein was colorless at culturing pH 10.0 due to the formation of β-CD-phenolphthalein inclusion complex (Mäkelä *et al*., 1988). When the organism grew and produced intracellular CDase, pink color was observed only around the colonies. The color was resulted from the release of phenolphthalein from β-CD-phenolphthalein complex when the CD was uptaken by the cells to be hydrolyzed by intracellular CDase. This medium resembles that reported for screening of CGTase activity by Park *et al.* (1989) except that soluble starch was replaced by β-CD and only the dye phenolphthalein was used in the present study. The medium was proved to be effective for screening CDase. The negative control, alkalophilic *Bacillus subtilis* TISTR 25 could grow on this medium but could not produce pink color around the TISTR 25 colonies.

Enzyme induction is defined as a relative increase in the rate of synthesis of a specific enzyme resulting from exposure to an inducer (Wang *et al*., 1979). The addition of enzyme inducers is usually very effective for maximize harvestable enzyme levels. α-, β-, and γ-CDs, soluble starch and starch syrup have been reported about their CDase inducible property, for example, the addition of 1.0% soluble

starch, 2.0% starch syrup and 0.5% β-CD to the medium promoted the production of CDases in alkalophilic *Bacillus* sp. 199 (Yoshida *et al*., 1991), *Bacillus coagulans* (Kitahata *et al.*, 1983) and *Bacillus sphearicus* ATCC 7055 (Galvin *et al*., 1994), respectively. The CDase reported in this study, was an inducible enzyme. It was found that using the modified Horikoshi medium (Horikoshi, 1971; Rutchtorn, 1993) with β-CD at the concentration of 2.0%, the optimal induction for CDase production of our strain, *Paenibacillus* sp. A11, was obtained.

Bacterial growth rates and enzyme production are greatly influenced by pH of the environment, growth temperature and culturing time. Most of bacteria producing CDase except *Clostridium thermohydrosulfuricum* 39E (Saha and Zeikus, 1990) and *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001), are mesophiles (30-40^{o}C) and have been reported to grow well in the similar conditions as *Paenibacillus* sp. A11. The optimal conditions for culturing *Paenibacillus* sp. A11 to produce CDase were found to be 37^{o}C, pH 10.0 and 48 h., respectively. Usual conditions for other CDase-producing bacteria were in the range of pH 7.0-7.5, 30-40^{o}C and 24-48 h. (DePinto and Campbell, 1968; Oguma *et al.*, 1990; Galvin *et al.*, 1994; Kim *et al*., 1998).

Purification of CDase

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intended application. The principal properties of

enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites. Most purification protocols require more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of step required (Janson and Ryden, 1998).

The first step in the purification of a protein is the preparation of an extract containing the protein in a soluble form and extraction procedures should be selected according to the source of the protein. In this work, CDase, an intracellular enzyme, was extracted from *Paenibacillus* sp. A11, a gram-positive bacterium, of which the major component of the cell wall is peptidoglycan (40-90%) together with teichoic acids, teichuronic acids and other carbohydrates (Voet and Voet, 1995). The cell wall is responsible for strength, rigidity as well as shape and is the major barrier to release of any intracellular proteins. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification. In the preliminary trial, triton X-100 was used. It was found that triton X-100 could extract cells to yield CDase in high level but it was subsequently caused difficulty in purification because of its interference. For this reason, ultrasonication was used in this work for cell breakage by cavitation and shear forces. Phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA) were used in the extraction buffer as serine protease inhibitor and metalloprotease inhibitor, respectively, because the control of metabolic regulation mechanisms is lost when the

cell is disrupted (Bollag and Edelstein, 1991). Thus, the desired protein may be degraded by intrinsic catabolic enzymes such as as proteolytic enzymes. In addition, the protein will encounter an oxidizing environment after disruption that may cause inactivation, denaturation or aggregation. Addition of a reagent containing a thiol group such as dithiothreitol (DTT) and also a chelating agent such as EDTA to chelate metal ions in the extraction buffer will minimize the oxidation damage (Bollag and Edelstein, 1991). Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation of protein. To maximize recovery of active enzyme, the extract and equipment were pre-chilled and several pauses of disruption used because short interval of disruption will also minimize foaming and shearing, thereby minimizing denaturation.

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols (Bollag and Edelstein, 1991). Ammonium sulfate was the salt of choice and was used in this work because it combined many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price. In the 40-60% ammonium sulfate precipitation step, about more than half of other proteins was removed but about half of the total enzyme activity was lost. The loss of significant portion of CDase activity may be caused by the removal of some factors important for stabilizing the enzyme activity.

Most of purification schemes involve some forms of chromatography, which has become an essential tool in every laboratory where protein purification is needed. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity (Janson and Ryden, 1998). DEAE-Sephadax A50 is an anion exchanger and was used in the purification of CDase from other sources (Kitahata *et al*., 1983; Yoshida *et al*., 1991; Podkovyrov

and Zeikus, 1992; Kim *et al*., 1998). Its popularity stems from the possibility of high resolving power, versatility, reproducibility and ease of performance. This column contributed greatly to the purification procedures, with less loss of CDase activity and about 40% of the other bulk proteins were eliminated. The result from chromatogram (Figure 22) also indicates that net charge of the enzyme in working buffer, 10 mM phosphate buffer pH 7.5, was negative because our enzyme could bind to the column. This implies that pI of A11 CDase was less than pH 7.5.

In purification of CDases, several purification methods have been employed including hydrophobic interaction chromatography (HIC) (Glavin *et al*., 1994; Feederle *et al*., 1995). HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar regions on the surface of proteins (Queiroz *et al*., 2001). Phenyl-Sepharose CL-4B is the resin with the chemically bonding phenyl groups on the surface of Sepharose. Prior to HIC column, the sample ionic strength should be adjusted with salt to increase hydrophobicity of protein (Queiroz *et al*., 2001). Thus, care must be taken in the aspect of the salt concentration used for adsorption. It should be lower than the concentration used in precipitation of our enzyme and the salt concentration of 1M was used for adsorption in this work. The elution of proteins is achieved by decreasing the salt concentration in order to increasing hydrophobicity. The different salts give rise to differences in the strength between proteins and the HIC adsorbent. Ammonium sulfate was chosen in this study because of its effective property in promoting hydrophobic interactions and it was widely used in HIC (Glavin *et al*., 1994; Feederle *et al*., 1995; Queiroz *et al*., 2001).

In our Phenyl-Sepharose CL-4B column, a gradient elution by decreasing concentration of ammonium sulfate from 1 M to 0 M was successfully used for specific separation of CDase from unwanted proteins. The success in using this column was judged by the homogeneity of CDase according to gel electrophoresis. At the last step of purification, it was found that even though some of CDase activity was lost but about 74% of the other proteins was also eliminated. For this HIC column, A11 CDase was eluted by 10 mM phosphate buffer pH 7.5 without ammonium sulfate. The result suggests that this enzyme is a relatively strong hydrophobic protein.

Some purification guidebooks suggest that HIC is ideal for used immediately after salt precipitation where the ionic strength of the sample will enhance hydrophobic interaction and also for avoiding the desalting step (Janson and Ryden, 1998; Queiroz *et al*., 2001). Preliminary experiment was performed by direct application of the enzyme solution from ammonium sulfate precipitation step to Phenyl-Sepharose CL-4B column. It was found that even though great removal of other proteins was observed but elution time was too long which caused the unacceptable great loss of CDase activity. The purification of A11 CDase using ammonium sulfate precipitation followed by Phenyl-Sepharose CL-4B and DEAE-Sephadax A50 column also did not lead to the desired level of enzyme purity since more than one band on native gel electrophoresis was observed.

In conclusion, effective purification of *Paenibacillus* sp. A11 CDase was by cell extraction, ammonium sulfate precipitation, followed by DEAE-Sephadex A50 and Phenyl-Sepharose CL-4B column, respectively. These procedures gave acceptable yield and purification fold (Table 6) in addition to high purity of CDase (Figure 24-25).

Not so many CDases were purified. Galvin *et al.* (1994) successfully purified CDase from *Bacillus sphearicus* ATCC 7055, passing cell extraction by Triton X®-100, extracti-Gel D, Q-Sepharose fast flow, Phenyl-Sepharose CL-4B and Superose-12 gel filtration. Such purification process gave a single CDase band on SDS-PAGE and preparative isoelectric focusing. In 1995, CDase protein was purified from *E. coli* strain JM 109 carrying plasmid pCYMH by Feederle *et al*., the process involved ammonium sulfate fractionation, and five column-chromatographic steps, namely ion-exchange chromatography on Q-Sepharose, Phenyl-Sepharose hydrophobic interaction chromatography, gel filtration through a Superdex 200 column, ion-exchange chromatography on a Mono-Q-HR 5/5 column, and a final Phenyl-Superose chromatography. A symmetrical peak consisting solely of the 69-kDa CDase protein was eluted from the final column and demonstrated a single CDase band on SDS-PAGE. Yang *et al.* (1996) achieved on purification of CDase from *Bacillus stearothermophilus* HY-1 to homogeneity by ammonium sulfate precipitation, DEAE-cellulose, hydroxypatite, Sephadex G-150 and α-CD-AH-Sepharose 4B, respectively. Efficient purification of CDase clone from alkalophilic *Bacillus* sp. I-5 into *Escherichia coli* was achieved on 50% ammonium sulfate precipitation and ion exchange fast performance liquid chromatography using Q-Sepharose, Mono-Q, and DEAE-8HR column, respectively. The purification procedure gave 11-fold enrichment of the enzyme activity and an overall yield of the purified enzyme was 13.6%. CDase purification in our study gave higher yield than those previously reported and we are the first to report CDase in the genus *Paenibacillus*.

Characterization of CDase from *Paenibacillus* sp. A11

Size of enzyme

The molecular weight of the enzyme was determined in the native and denatured conditions by Sephadex G-100 column chromatography and SDS-PAGE, respectively. The molecular weight of A11 CDase was estimated to be 80 kDa by SDS-PAGE. Gel filtration gave the same molecular weight suggesting the protein to be a monomer. Most of the reported CDases are monomeric; ranging in molecular weight between 60-90 kDa (Saha and Zeikus, 1990; Podkovyrov and Zeikus, 1992; Galvin *et al*., 1994; Feederle *et al.*, 1995; Yang *et al*., 1996; Kim *et al*., 1998; Hashimoto *et al*., 2001). Only the CDases produced by alkalophilic *Bacillus sphaericus* E-244 and *Bacillus* sp. 199 were found to be dimeric proteins of identical subunit size of 72 and 67 kDa, respectively (Oguma *et al*., 1990; Yoshida *et al*., 1991).

N-terminal, amino acid composition, and active site residues

The N-terminal amino acid sequence of the purified CDase from *Paenibacillus* sp. A11 was determined by automated Edman degradation. The sequence of fifteen residues at the N-terminus was M F L E A V Y H R P R K N W S which was identical to CDase of alkalophilic *Bacillus* sp. I-5 (Kim *et al*., 1998), while 65-70% sequence identity was found with CDases of *Bacillus* sphaericus E-244 (Oguma *et al*., 1993), and alkalophilic *Bacillus* sp. A2-5a (Ohdan *et al*., 2000). When compared with the N-terminus of CDases from *Alicyclobacillus acidocaldarius* (Matzke *et al*., 2000),

and *Clostridium thermohydrosulfuricum* 39E (Podkovyrov and Zeikus, 1992), only 30% sequence identity was observed. No homology was found with N-terminal sequence of CDases from *Klebsiella oxytoca* M5a1 (Fiedler *et al*., 1996), and *Thermococcus* sp. B1001 (Hashimoto *et al*., 2001). From the N-terminal sequence comparison, CDase from *Paenibacillus* sp. A11 was more similar to alkalophilic *Bacillus* sp. CDases than CDases which were produced from other genera such as *Clostridium, Klebsiella* and *Thermococcus*.

From the amino acid composition of the purified CDase of *Paenibacillus* sp. A11 (Table 7), the CDase was rich in Asx and Glx (11.6 and 15.4 mole%, respectively) while contained few amounts of Met, Phe and His, respectively. For Cys and Trp, they could not be detected under the experimental condition with the hydrolysis by 6 M HCl. In the determination of Cys, the sample had to be reacted with performic acid before the addition of 6 M HCl. For Trp, the protein should be hydrolyzed in either mercaptoethanesulfonic acid (MESA) or methanesulfonic acid (MSA) instead of 6 M HCl (Anders, 2002). As this is the first report of CDase amino acid composition, comparison with other CDases amino acid composition was not possible. Thus, A11 amino acid composition was compared with its deduced amino acid sequence obtained from CDase gene (through the ProtParam tool Program, Available from ExPASy.com). It was found that both sequences were similar (Table 23).

Several methods for investigation of amino acid residues which are essential for function or structure of protein such as affinity labeling with their substrate or substrate analog, X-ray crystallography, site-directed mutagenesis or chemical modification have been reported (Means and Feeney, 1971; Lundblad, 1991; Saha and Zeikus, 1990; Yoshida *et al*., 1991; Yang *et al*., 1996; Podkovyrov and Zeikus, 1992).

Table 23 Comparison of amino acid composition of *Paenibacillus* sp. A11 CDase with its deduced amino acid sequence obtained from CDase gene

Amino acid	Content (mol %)	
	***Paenibacillus* sp. A11**	**Deduced amino acid**
Asx*	11.6	11.3
Thr	5.0	4.6
Ser	4.6	5.1
Glx**	15.4	14.5
Gly	6.2	5.5
Ala	6.9	6.7
Cys	Not determined	1.4
Val	6.8	6.1
Met	2.9	2.1
Ile	5.2	4.9
Leu	7.8	7.2
Tyr	4.5	4.4
Phe	3.4	3.7
Lys	4.7	5.0
His	2.0	3.7
Trp	Not determined	2.9
Arg	6.1	5.1
Pro	6.9	5.8

* Asx = aspartic acid + asparagine

** Glx = glutamic acid + glutamine

Chemical modification using group-specific reagent is one of the most useful method for identifying the functional groups of a protein. The principle is that if an amino acid side chain involved in the catalytic activity is chemically modified, the enzyme will be inactivated (Means and Feeney, 1971). The modification reaction of these reagents is shown in Appendix H and the specificity of reagents used to chemically modify proteins is shown in Appendix I.

In this work, the screening of essential amino acid residues of the enzyme, which was the initial phase of chemical modification study, was investigated. Ten different amino acid residues tested were cysteine, cystine, lysine, serine, carboxylic amino acids, histidine, tryptophan, tyrosine and arginine. These amino acid residues have been selected because they are widely known as residues usually involved in enzyme catalysis (Means and Feeney, 1971). Incubation of purified enzyme with a series of group-specific modifying agents at 5 mM concentration resulted in variable changes in the catalytic ability of this enzyme. No inhibition of the *Paenibacillus* sp. A11 CDase activity was observed in the modification of lysine by 2, 4, 6 – trinitrobenzenesulfonic acid (TNBS), serine by phenylmethylsulfonyl fluoride (PMSF), and tyrosine by *N*-acetylimidazole (NAI). It may be assumed that lysine, serine, and tyrosine are not the important residues involved in enzyme catalytic activity. Very strong inhibition was observed in the modification of tryptophan by *N*-bromosuccinimide (NBS) and histidine by diethylpyrocarbonate (DEP). Moreover, the modification with iodoacetamide (IAM), 1-ethyl-3-(3-dimethylaminopropyl) arbodiimide and phenylglyoxal (PG) which were known to react specifically with cysteine, carboxylic amino acids (Asp and Glu) and arginine, respectively, resulted in extensive inhibition of CDase activity. Thus the indole group of tryptophan, imidazole group of histidine, sulfhydryl group of cysteine, carboxylic group of

aspartic/glutamic, and guanidinium group of arginine are all likely involved with CDase activity as the essential residues but different in degree of the importance on enzyme catalysis. Moreover, activation of this CDase activity was exhibited by reducing agent like dithiothreitol (DTT), which suggests that the reduced form of CDase was more active and the purified CDase we obtained was not in a fully reduced state.

The importance of tryptophan, histidine, cysteine and carboxylic amino acids on CDase activity was previously reported in other CDases (Saha and Zeikus, 1990; Yoshida *et al*., 1991; Yang *et al*., 1996; Podkovyrov and Zeikus, 1992; Kim *et al*., 1998), while the importance of arginine has never been reported. It is possible that arginine might be essential for CDase because it was found at the conserved sequence of CDases (Podkovyrov and Zeikus, 1992; Kim *et al*., 1998). For cysteine, Saha and Zeikus (1990), Yoshida *et al*. (1991) and Kim *et al*. (1998) had reported that CDase was a sulfhydryl enzyme because the enzyme activity was inhibited by thiol reagents such as PCMB and IAM. NBS and DEP which modified tryptophan and histidine have been reported to inactivate CDases from *Bacillus stearothermophilus* HY-1 (Yang *et al*., 1996) and *Clostridium thermohydrosulfuricum* 39E (Podkovyrov and Zeikus, 1992). Based on the results from site-directed, aspartic acid (325), glutamic acid- (354), and aspartic acid (421) of CDase from *Thermoanaerbacter ethanolicus* 39E have been reported to directly involve at the catalytic site. Single mutants at these three residues were completely inactive, confirming their important role in catalysis (Podkovyrov *et al*., 1993).

The inactivation of enzyme by chemical modification does not directly indicate that a specific residue is present at the active site. To prove that the amino acid residues involved in CDase activity are located within active site or not, the

substrate protection experiment is necessary and should be performed to confirm the presence of specific residues at the active site. If an interesting amino acid residue can be protected by substrate, the loss of activity will be less than that of without substrate protection. It means that the amino acid residue is in the active site of the enzyme (Means and Feeeney, 1971). For the A11 CDase, tryptophan modification was especially interested because least concentration of modifier (0.1 mM NBS) was used for almost total inhibition of enzyme activity, so, it was chosen for substrate protection study.

The experiment was performed by measuring purified CDase inactivation by NBS in the presence or the absence of β-CD as protective substance. The result in Table 13 showed that the loss of purified CDase activity was reduced in the presence of protective substance. This result suggests that tryptophan was essential amino acid at the active site of A11 CDase. Moreover, we confirm that tryptophan was present at the active site by following fluorescence emission of CDase when modified with NBS in the presence and absence of β-CD. The result shows that tryptophan was protected by β-CD, since the fluorescence emission spectrum returned to resemble the control pattern (Figure 43, line 1) in the maximum emission wavelength and the fluorescence intensity. Hence, tryptophan was confirmed to be one essential amino acid at the active site of A11 CDase. For similar study, Yang *et al.* (1996) reported that CDase from *Bacillus stearothermophilus* HY-1 was protected against NBS-inactivation by α-CD suggesting that the modified tryptophan residues were located at or near the active site of the enzyme.

Isoelectric points, optimum pH and temperature

The isoelectric point of A11 CDase was determined by isoelectrofocusing gel in the ampholine pH range 3-10, comparing with standard pI marker (pI 3.5-9.3). The enzyme showed one band at pH 5.4. This corresponded with many previous reports, such as CDases of *Bacillus sphaericus* ATCC 7055 and *Bacillus circulans* 109 which had one major band with isoelectric point of 5.3 and 5.0, respectively (Galvin *et al*., 1994; Kitahata *et al*., 1983). The lower isoelectric point was observed in alkalophilic *Bacillus* sp. 199 CDase with an isoelectric point of 4.2 (Yoshida *et al*., 1991).

CDase from *Paenibacillus* sp. A11 showed the optimum pH of 7.0, corresponding with the optimum pH of other CDases such as *Bacillus macerans* (DePinto and Campbell, 1968) and *Klebsiella oxytoca* M5a1 (Feederla *et al*., 1995). The CDase from *Bacillus sphaercus* E-244 (Oguma *et al*., 1990) had higher optimum pH at 8.0. From our result, the buffer effect was observed. Tris-HCl buffer was not suitable for the *Paenibacillus* sp. A11 CDase since low enzyme activity was observed (Figure 33). Potassium phosphate buffer was more appropriate and has been chosen to use widely as an assay and purified buffer for CDases (DePinto and Campbell, 1968; Kitahata *et al*., 1983; Feederla *et al*., 1995; Yoshida *et al*., 1991).

CDase from *Paenibacillus* sp. A11 was stable in a wide pH range of 6.0-10.0 upon incubation at 4^{o}C for 24 h which was similar to alkalophilic *Bacillus* sp. 199 CDase (Yoshida *et al*., 1991) but different from that of *Bacillus coagulans* (Kitahata *et al*., 1983) which was only stable in the pH range of 6.0-7.3.

The optimum temperature of CDase from *Paenibacillus* sp. A11 was 40^{o}C. For other CDases the range of optimum temperature was about 35-65^{o}C which was within the same range with our finding. The optimum temperature for CDases from

Bacteroides ovatus 3524 (Antenucci and Palmer, 1984), *Bacillus coagulans* (Kitahata *et al*., 1983), *Bacillus sphaericus* (Galvin *et al.*, 1994), alkalophilic *Bacillus* sp. I-5 (Kim *et al*., 1998), *Alicyclobacillus acidocaldarius* (Matzke *et al*., 2000), and *Clostridium thermohydrosulfuricum* 39E (Podkovyrov and Zeikus, 1992) were 37, 50,40, 50, 55 and 65 ^{o}C, respectively. The unique optimum temperature of 23^{o}C was observed with *Klebsiella oxytoca* M5a1 CDase (Feederla *et al*., 1995) while for *Thermococcus* sp. B1001, it was as high as 95^{o}C (Hashimoto *et al*., 2001).

Kinetics, effect of chemicals, substrate specificity, and product formation

To determine the kinetic parameters of CDase, parental CDs were used as substrate. A ring of CDs molecule was opened and reducing sugars were determined by 3,5-dinitrosalicylic acid reaction (Bernfeld, 1955). Table 24 summarizes kinetic parameters of purified CDase from A11 obtained in this study and compared with CDase from alkalophilic *Bacillus* sp. I-5 (Kim *et al*., 2000). Substrate binding affinity (K_m) and the rate of CD ring-opening (k_{cat}) of both enzymes, *Paenibacillus* sp. A11 and alkalophilic *Bacillus* sp. I-5 CDases, showed the highest efficiency in hydrolysis on β-CD, followed by γ- and α-CDs, respectively. On the other hand, different result was observed with *Bacillus coagulans* and *Klebsiella oxytoca* M5a1 CDases (Kitahata *et al*., 1983; Feederla *et al*., 1995). The substrate binding affinity (K_m) and reaction velocity (V_{max}) values of *Bacillus coagulans* and *Klebsiella oxytoca* M5a1 CDases for CDs decreased with increasing of glucose units. Thus, the strongest binder of *Bacillus coagulans* and *Klebsiella oxytoca* M5a1 CDases, γ-CD, was the substrate with the smallest turnover number (k_{cat}).

Table 24 Comparison of kinetic parameters of purified CDase from *Paenibacillus* sp. A11 and alkalophilic *Bacillus* sp. I-5

substrate	*Paenibacillus* sp. A11		alkalophilic *Bacillus* sp. I-5*	
	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
α-CD	5.66	800	1.23	590
β-CD	2.47	2044	0.83	1897
γ-CD	3.10	1155	0.92	1802

***** (Kim *et al*., 2000)

When CDase from *Paenibacillus* sp. A11 was tested for the effects of metal ions, protective chemicals and saccharides on enzyme activity, it was found that the enzyme was inhibited by metal ions such as Hg^{2+}, Ag^{+}, Cu^{2+}, Zn^{2+} and Fe^{2+} but stimulated by Ca^{2+}. The response of A11 CDase to metal ions seemed to follow a similar pattern to those of other CDases (Yoshida *et al*., 1991; Yang *et al*., 1996; Podkovyrov and Zeikus, 1992; Kim *et al*., 1998). The chemicals often used as protective substance for enzyme such as β-mercaptoethanol, EDTA or used as fungicide during enzyme purification (NaN_3) had no effect on the activity of this enzyme. The saccharides, glucose and maltose, the final main products of CDase had no effect on CDase activity while acarbose (Figure 64), a pseudotetrasaccharide, significantly inhibited activity of this CDase. The K_i value of A11 CDase on acarbose was 2.59 x 10^{-3} mM. Kim *et al.* (2000) reported that acarbose was also a strong competitive inhibitor for *Bacillus* sp. I-5 CDase with a K_i value of 1.24 x 10^{-3} mM.

From the experiment investigated on the substrate specificity of CDase, the A11 CDase showed a relatively high rate of hydrolysis for the CDs substrate. β-CD was best hydrolyzed, followed by γ-CD and α-CD. The pattern was similar to the action of *Bacillus sphaericus* ATCC 7055 and *Bacillus* sp. I-5 CDases (Galvin *et al.*, 1994; Kim *et al*., 1998). However, CDase of *Bacillus sphaericus* ATCC 7055 was different from our enzyme and that of *Bacillus* sp. I-5 in the relative hydrolytic activities among the three CD substrates. Different result was reported with CDases from alkalophilic *Bacillus* sp. 199 and *Klebsiella oxytoca* M5a1 (Yoshida *et al*., 1991; Feederle *et al*., 1995), where α-CD was rapidly hydrolyzed at two and three times higher rate than β-CD and γ-CD, respectively. Our study firstly showed that having glucosyl or maltosyl attached to β-CD resulted in significant decrease of

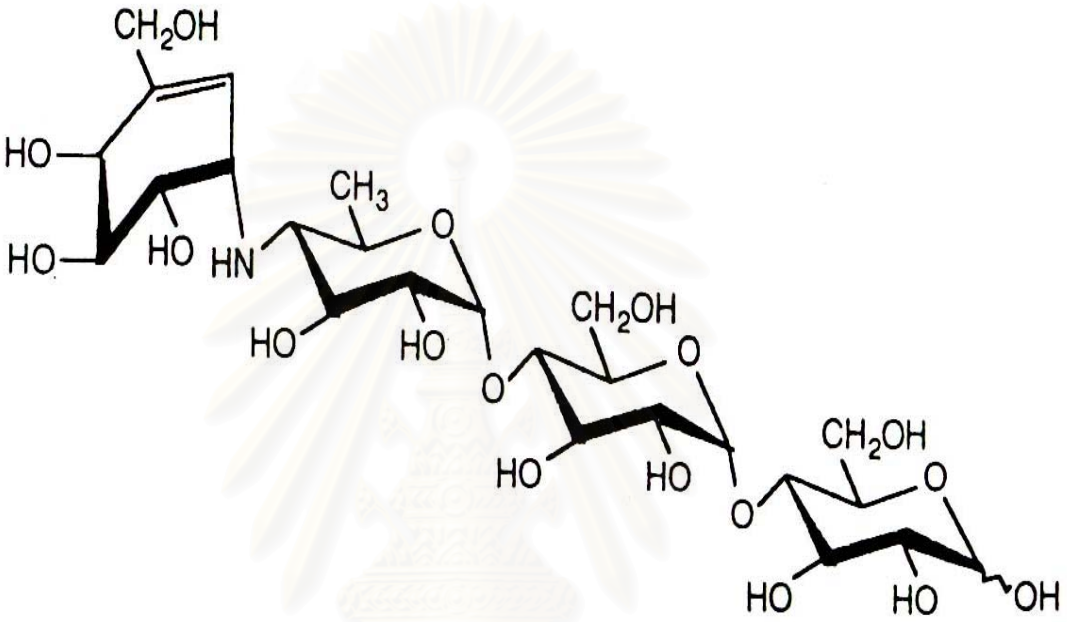


Figure 64 Structure of acarbose

substrate ability. Low to moderate hydrolysis activity was observed with linear oligosaccharides, G3-G7 and dextrin. Polysaccharides such as amylose and starch were less hydrolyzed while branched or heteropolysaccharides could not be hydrolyzed. In addition, di- or trisaccharides which were composed of other monomers besides glucose or other linkages besides α-1,4 such as lactose (Gal-β1,4-Glc), melibiose (Gal-α1,6-Glc), sucrose (Glc-α1,2-Fru), leucrose (Glc-α1,5-Fru_p), levanbiose (Fru-β2,6-Fru) and raffinose (Gal-α1,6-Glc-α1,2-Fru) were not hydrolyzed. Specificity towards α-1,4 linkage was best demonstrated by the inability of CDase to hydrolyze cellobiose (Glc-β1,4-Glc). CDase of A11 was different from other CDases in its ability to hydrolyse maltose and trehalose (Glc-α1,1-Glc), although, with very low hydrolytic activity. These overall results suggest that A11 CDase had high specificity with cyclic-oligosaccharides and appropriate size linear oligosaccharides, comprising of glucose units linked by the α-1,4 glycosidic bond. In addition, α-1,1 glycosidic bond could also be slowly hydrolyzed.

The dominant-end products obtained from hydrolysis by CDase of various glucan substrates with α-1,4 linkages as determined by TLC and HPLC were maltose with less amounts of glucose, maltotriose and maltotetraose which was similar to products from hydrolysis of other CDases (Saha and Zeikus, 1990; Oguma *et al*., 1990; Yoshida *et al*., 1991; Galvin *et al*., 1994). In contrast, final products of *Klebsiella oxytoca* M5a1 CDase were only maltose and maltotriose. In the present study, the structure of products from A11 CDase was confirmed by NMR. This was the first report using NMR technique to resolve the structure of CDase products. Hydrolytic product of A11 CDase was analyzed by NMR revealed that they were reducing end sugars, comprising of α-1,4 glycosidic bond.

Part III : CLONING, CHARACTERIZATION, AND EXPRESSION OF CDase GENE

Cloning of CDase gene

Primer 3 5'-GAY-GGN-TGG-MGN-YNT-GAY-GT-3' and primer C 5'-GAY-GCG-GTS-ATG-AAY-TAT-CC-3', designed from the conserved sequences of CDase genes, were used as PCR primers to amplify the CDase gene segment from *Paenibacillus* sp. A11 chromosomal DNA. After amplification, the PCR product obtained was then used as a probe for the screening of the complete CDase gene using ECL^{TM} direct nucleic acid labeling and detection kit. During the cloning, chromosomal DNA extracted from *Paenibacillus* sp. A11 was completely digested with several restriction enzymes and the DNA fragments containing CDase gene were detected. *Pvu* II was chosen as a restriction enzyme of choice because this restriction site was not found inside other CDase genes (Podkovyrov and Zeikus, 1992; Fiedler *et al*., 1996; Kim *et al*., 1998). Southern blot hybridization revealed a 4.9 kb *Pvu* II fragment which was subsequently ligated with *Sma* I-digested pUC18 and transformed into *E. coli* strain JM 109. The screening of the recombinant DNA containing CDase gene from *Paenibacillus* sp. A11 was successfully performed by colony hybridization with a specific CDase probe as described above. From approximately 600 transformant colonies, only one positive clone that showed high signal with a CDase probe was found. This clone was called pJK 555 which contained 7.5 kb plasmid, and it was further used for characterization of A11 CDase gene.

Characterization of CDase gene

By using a probe specific for CDase, a chromosomal DNA fragment of 4,932 bp was cloned and sequenced. A CDase open reading frame of 1,962 bp was identified, and encoded 653 amino acid residues. The CDase gene was expressible in the *E. coli* JM109 host cells independent of the vector *lac* promoter indicating that the CDase gene, together with its own promoter region, was contained entirely within the cloned DNA fragment.

Amino acid sequence alignment among the CDases from the *Paenibacillus* sp. A11 and other bacteria revealed the four highly conserved regions known to be present in various amylolytic enzymes (Figure 65). The CDase from *Paenibacillus* sp. A11 showed high homology to those from *Bacillus* sp. (Cho *et al*., PDB accession no. 1EA9) and alkalophilic *Bacillus* sp. I-5 (Kim *et al*., 1998). However, the CDase from *Paenibacillus* sp. A11 contained a carboxy-terminal extension of 70 and 95 amino acid residues longer than Cho's and strain I-5 CDases, respectively. The apparent size of the enzyme from strain A11 was determined by SDS-PAGE to be about 80 kDa whereas that of strain I-5 was 63 kDa, which agreed well with the length of the peptides decoded from their nucleotide sequences. In fact, comparison of nucleotide sequences between the CDase genes of strain I-5 and strain A11 indicated a frame-shift mutation at the carboxy-terminal nucleotide sequence resulting in different carboxy-terminal ends (data not shown). We thus proposed that the carboxy-terminal extension of strain A11 was not essential for the CDase activity. This is in accordance with the observation of Lee, *et al.* (2002) that a mutant CDase lacking the carboxy-terminal domain was still active. The three-dimensional structure modeling of

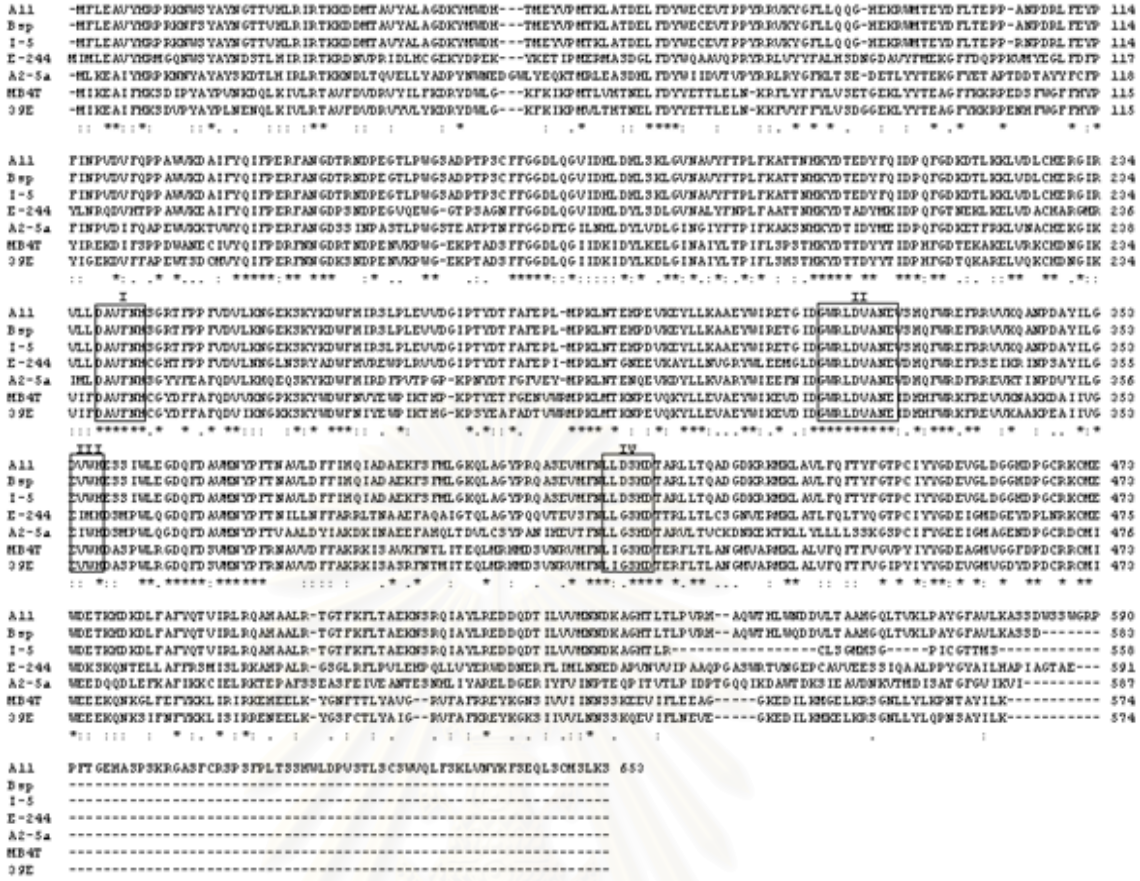


Figure 65 Comparison of amino acid sequences among *Paenibacillus* sp. A11 CDase and CDases from other bacteria.

Bacteria are abbreviated as follows; A11, *Paenibacillus* sp. A11; Bsp, Cho's *Bacillus* sp.; I-5, alkalophilic *Bacillus* sp. I-5; E-244, *Bacillus* sphaericus E-244; A2-5a, *Bacillus* sp. A2-5a; MB4T, *Thermoanaerobacter tengcongensis* MB4T; 39E, *Clostridium thermohydrosulfuricum* 39E. Numbering starts from the first amino acid of the mature proteins. The four conserved sequences among amylolytic enzymes are boxed.

Paenibacillus sp. A11 CDase using the program Swiss-Model ver 36 (Peitsh, 1996) revealed 3 structural domains, the N-terminal, the central $(\kappa/\kappa)_8$ barrel catalytic and carboxy-terminal domains. The carboxy-terminal extension formed additional β-sheets in the carboxy-terminal structure (Figure 50). CDase is classified as family 13 of the glycosyl hydrolases as well as α-amylase, maltogenic amylase, isoamylase, neopullulanase and CGTase. Comparison of the main-chain folding of the CDases with that of other enzymes in family 13 reveals very similar structure, except for the shape of the active site cleft and the N-terminal region. The N-terminal domain of CDase is approximately 130 residues longer, overhangs from the center of the $(\beta/\alpha)_8$ domain. The shape of the active site cleft is smaller than other enzymes in family 13 because most of amino acid residues were aromatic amino acid such as tyrosine, tryptophan and phenylalanine (Bart *et al*., 2000; Park *et al*., 2000; Lee *et al*., 2002).

In bacteria, genes involved in the metabolism of the certain substrates are often arranged into clusters or operons. The finding that several genes for cyclodextrin metabolism were clustered together in *Klebsiella oxytoca* M5a1 and *Thermococcus* sp. strain B1001 led to the hypothesis that there is a novel alternative metabolic pathway for starch degradation (Fieldler *et al*., 1996 ; Hashimoto *et al*., 2001). It is hypothesized that the bacteria secrete the extracellular CGTase to convert starch into cyclodextrins, which are transported into the cells by a specific uptake system (Pajatsch *et al*., 1999). The intracellular cyclodextrinase then hydrolyzes the cyclodextrins into simple sugars for use in cell metabolism. A similar cluster of CGTase, CDase and putative cyclodextrin binding protein was also found in *Bacillus* sp. A2-5a (Ohdan *et al*., 2000). This hypothesis is plausible and awaiting to be confirmed.

In this study, analysis of the region other than the CDase gene in the 4.9 kb DNA insert revealed 3 additional open reading frames, encoding a Na^+/H^+ antiporter, a K^+ channel and a putative transcription regulator, aligned in the same direction (Altschul *et al*., 1997). We previously reported cloning and sequencing of a 7.9 kb chromosomal DNA fragment containing the CGTase gene from *Paenibacillus* sp. A11 (GenBank accession no. AF302787). It was found that the two DNA inserts had no overlap region. The CDase and CGTase genes of our strain were not closely aligned as in alkalophilic *Bacillus* sp. A2-5a and *Thermococcus* sp. B1001 (Ohdan *et al*., 2000; Hashimoto *et al*., 2001); they were more than 3 kb apart. Whether the arrangement of the two genes in the chromosomal DNA of strain A11 was similar to that of *Klebsiella oxytoca* M5-a1 (Fiedler *et al*., 1996) remained to be confirmed. From the CDase screening experiment using phenolphthalein dye, it was certain that cyclodextrin is transported into the cell. A CD transport system should thus be present in strain A11. Two reading frames encoding for ABC type transporters (Fieldler *et al*., 1996) were found in the 7.9 kb CGTase-containing DNA fragment. These two proteins may be involved in the CD uptake in strain A11.

Expression of CDase in *E. coli* JM 109

When CDase was extracted from *E. coli* JM 109 harboring pJK 555 plasmid, we found that the CDase protein was localized mainly in the cell debris fraction while less amount was found in the soluble cytoplasmic fraction (Figure 53A). This was different from what observed in *Paenibacillus* sp. A11 where the CDase activity was only found in the cytoplasm. Similar result was frequently observed in the expression of several proteins in *E. coli* system (Kim *et al*., 1999; Hanning and Makrides, 1998;

Donovan *et al*., 1996). It is possible that the low solubility of the protein is caused by the expression of protein in different types of bacteria, i. e. *Paenibacillus* sp. A11 is a gram-positive bacterium while *E. coli* JM 109 is a gram-negative bacterium. The insoluble CDase problem was minimized by adding 0.5 M sorbitol as an osmotic stabilizer into the *E. coli* culturing LB medium in this study. We have demonstrated that sorbitol had positive effect on CDase overproduction of *E. coli* transformant in parallel with the increase of the cell growth. It was apparent that addition of sorbitol could prevent the formation of insoluble CDase aggregate, enforcing the correct folding to active soluble enzyme. Polyols, osmolytes, and some non-metabolizing sugars have already been reported to be important for enzyme activity and for the secondary/tertiary structures of some proteins including the prevention of protein aggregation (Lee and Lee, 1981). Bowden and Georgiou (1988) demonstrated that the addition of non-metabolizing sugars such as sucrose and raffinose to the growth medium prevented the aggregation of β-lactamase. In another study, some compatible osmolytes were found to be effective in producing soluble dimethylallyl pyrophosphate: 5'AMP transferase in the transformed *E. coli.* That is, large amounts of soluble and active protein were obtained by growing and inducing the cells under osmotic stress built up by sorbitol and glycyl betain (Blackwell and Horgan, 1991). Recently, Kim *et al.* (1999) found that mannitol, sorbitol, glycerol, erythritol, xylitol and arabitol could increase expression of soluble active CGTase from *Brevibacillus brevis* in *E. coli*.

In addition to adding of sorbitol into the *E. coli* culturing LB medium, subcloning of pJK 555 transformant, pJK 556, was also performed to increase the expression of CDase in *E. coli*. The result shows that CDase specific activity of pJK 556 transformant was around 1.5 time higher than that of pJK 555 transformant in

culturing with sorbitol (Table 16). When compared with the original *Paenibacillus* sp. A11 strain, it was found that the specific activity of the pJK 556 clone was about 2.4 time higher. The higher CDase expression of pJK 556 clone was resulted from the reduction of inserted DNA size.

Part IV : THE RECOMBINANT CDase

The purification and some characterization of the recombinant CDase from the *E. coli* (pJK 555) were performed in order to compare the identity with that of *Paenibacillus* sp. A11 CDase. The recombinant CDase was purified to a specific activity of 141 unit/mg protein, representing a 14-fold purification. When purification of recombinant and *Paenibacillus* sp. A11 CDase was compared (Table 25), the result was similar especially in the specific activity of the purified enzyme and the yield. The recombinant CDase was a single polypeptide with M_r of 80 kDa. The optimum pH and temperature of the cloned and authentic enzyme were both 7.0 and 40 ^oC. In addition, other biochemical properties of the cloned enzyme were similar to those of the authentic one (Table 22). The substrate specificity of *E. coli* (pJK 555) CDase was very similar to that of *Paenibacillus* sp. A11 and those of other strains (Podkovyrov and Zeikus, 1992; Oguma *et al.*, 1993; Kim *et al*., 1998). The best substrate for the recombinant CDase was β-CD. Polysaccharide such as soluble starch and pullulan were less hydrolyzed while more branched such as glycogen could not be hydrolyzed by this enzyme. Maltose was the main product of both CDases. And from all data, it could be concluded that the recombinant and original CDase were identical enzyme.

Table 25 Purification of CDases from *Paenibacillus* sp. A11 and the *E. coli* transformant

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Paenibacillus sp. A11*					
Crude enzyme	4300	680	6	1	100
40-60% $(NH_4)_2SO_4$	1860	58	32	5	43
DEAE-Sephadex A50	1340	35	38	6	31
Phenyl-Sepharose CL-4B	1200	9	133	22	28
E. coli JM 109 (pJK 555)**					
Crude enzyme	4500	440	10	1	100
40-60% $(NH_4)_2SO_4$	3000	172	17	2	67
DEAE-Toyopearl 650M	2720	32	85	9	60
Phenyl-Sepharose CL-4B	1408	10	141	14	31

* The A11 (40 g wet weight) was grown in 8L Horikoshi medium at 37 ^{o}C for 72 h.

** The pJK555 *E. coli* (50 g wet weight) was grown in 8L LB medium containing 100 μg/ml amplicillin and 0.5 M sorbitol at 37 ^{o}C for 24 h.

CHAPTER V

CONCLUSIONS

1. In the identification by 16S rRNA gene fragment amplification, strain A11 showed 90-99% homology with *Paenibacillus*. The major cellular fatty acid was anteiso-$_{C15:0}$ which accounted for 59.3% of total cellular fatty acids and the G+C content was 50.3 mol%. These data led to the reidentification of A11 as *Paenibacillus* sp. A11.
2. The presence of CDase activity in *Paenibacillus* sp. A11 was preliminary determined by using the specific screening medium containing phenolphthalein dye and β-CD developed in this study.
3. CDase from *Paenibacillus* sp. A11 was purified to homogeneity by 40-60% saturated ammonium sulfate precipitation, DEAE-Sephadax A50 and Phynyl-Sepharose CL 4B column chromatography with a 28 % yield, 22 purification folds and specific activity of 133 units/mg protein.
4. A11 CDase was a monomer with the molecular weight of 80 kDa. It was not a glycoprotein.
5. The isoelectric point was 5.4. The optimum pH and temperature were 7.0 and 40^{o}C. The enzyme was stable in a wide pH range of 6.0-10.0. The enzyme could be stored for one month at –80^{o}C without any loss of activity.
6. N-Terminal sequence as determined by amino acid sequencing, was M F L E A V Y H R P R K N W S.

7. A 11 CDase had high specificity towards cyclic-oligosaccharides and appropriate size linear oligosaccharides, comprising of glucose units linked by the κ-1,4 glycosidic bond.
8. The dominant end-products obtained were maltose with less amounts of glucose, maltotriose and maltotetraose.
9. Substrate binding affinity (K_m) and the rate of ring-opening (k_{cat}) showed the highest efficiency in hydrolysis on β-CD, followed by γ-CD and α-CD.
10. The enzyme was inhibited by Hg^{2+}, Ag^{+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and activated by Ca^{2+}.
11. Acarbose was a strong competitive inhibitor for A11 CDase with K_i value of 2.59 x 10^{-3} mM.
12. Trp, His, Cys, carboxylic amino acids and Arg were important residues on A11 CDase activity. Substrate protection by β-CD indicated that Trp was essential amino acid at the active site.
13. A11 CDase was unique in its ability to hydrolyze maltose and trehalose, though with very low hydrolytic activity.
14. The cloned CDase gene contained the open reading frame of 1,959 nucleotides coding for a 653 amino acid polypeptide. A putative promoter and a Shine-Dalgarno sequence (SD) could be located upstream of the gene.
15. The deduced amino acid sequence of this CDase showed about 97% homology with the CDase of alkalophilic *Bacillus* sp I-5. The difference was in the carboxy-terminal extension of 95 amino acid residues found in A11 CDase.
16. The CDase was overexpressed in *E.coli* in the presence of 0.5 M sorbitol in the culturing medium.

17. Subcloning of pJK 555 transformant by deletion of 1.7 kb fragment resulted in 2.4 times higher CDase expression than in the original strain.
18. The purified recombinant CDase was identical to the enzyme from the original strain, *Paenibacillus* sp. A11.

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APPENDICES

APPENDIX A: **Biochemical characteristics of *Bacillus circulans* A11**

Characteristics	Reaction
Gram reaction	+ve
Fermentative production of acid from:	
- glycerol	-
- erythritol	-
- D-arabinose	+
- L-arabinose	+
- ribose	+
- D-xylose	+
- L- xylose	-
- adonitol	-
- β-methyl-D-xyloside	+
- galactose	-
- glucose	+
- fructose	+
- mannose	+
- sorbose	-
- rhamnose	-
- dulcitol	-
- inositol	-
- mannitol	+
- sorbitol	-
- α-methyl-D-mannoside	-
- α-methyl-D-glucoside	-
- N-acetyl-glucosamine	-
- amygdalin	+
- arbutin	+
- esculin	+

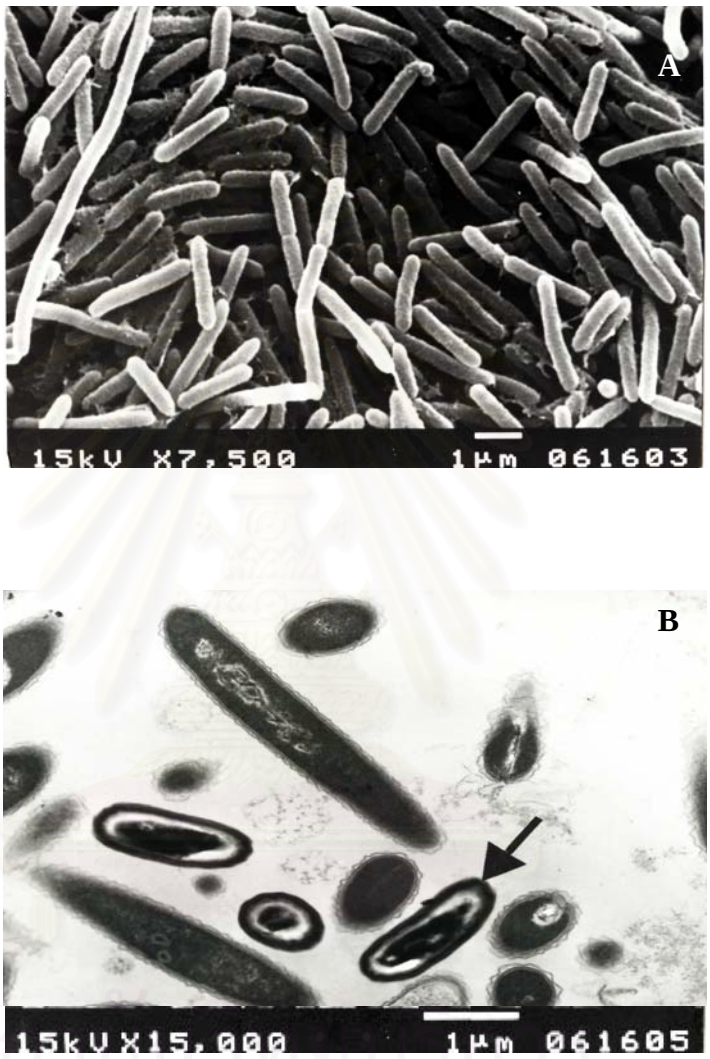
Remark: +ve = Gram positive bacteria
\+ = Positive reaction
\- = Negative reaction

APPENDIX A: **Biochemical characteristics of *Bacillus circulans* A11** (continued)

Characteristics	Reaction
Fermentative production of acid from:	
- salicin	+
- cellobiose	+
- maltose	+
- lactose	+
- melibiose	+
- sucrose	+
- trehalose	+
- inulin	-
- melezitose	-
- raffinose	+
- starch	+
- glycogen	+
- xylitol	-
- gentiobiose	+
- D-turanose	-
- D-lyxose	-
- D-tagatose	-
- D-fucose	-
- L-fucose	-
- D-arabitol	+
- L- arabitol	-
- gluconate	+
- 2-keto-gluconate	-
- 5-keto-gluconate	-
Cytochrome oxidase	+
Catalase	+

Remark: +ve = Gram positive bacteria
\+ = Positive reaction
\- = Negative reaction

APPENDIX B: **Morphology of *Bacillus circulans* A11 on Horikoshi medium for 72** (Juntarama, 1997)



Scanning electron micrograph (SEM) (A) and Transmission electron micrograph (TEM) (B). The arrow indicates endospore.

APPENDIX C: **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

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06	g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8	75	ml

10% SDS	4	ml
Distilled water	21	ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8	50	ml
10% SDS	4	ml
Distilled water	46	ml

2. Non- denaturing PAGE

7.5% Seperating gel

30% Acrylamide solution	2.5	ml
1.5 M Tris-HCl pH 8.8	2.5	ml
Distilled water	5.0	ml
10% $(NH_4)_2S_2O_8$	50	µl
TEMED	10	µl

5.0% Stacking gel

30% Acrylamide solution	0.67	ml
0.5 M Tris-HCl pH 6.8	1.0	ml
Distilled water	2.3	ml
10% $(NH_4)_2S_2O_8$	30	µl
TEMED	5.0	µl

5X Sample buffer

1 M Tris-HCl pH 6.8	3.1	ml
Glycerol	5.0	ml
1 % Bromophenol blue	0.5	ml
Distilled water	1.4	ml

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM Glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

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

3. SDS-PAGE

7.5% Seperating gel

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

5.0% Stacking gel

30% Acrylamide solution	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % $(NH_4)_2S_2O_8$	30	µl
TEMED	5.0	µl

5X Sample buffer

1 M Tris-HCl pH 6.8	0.6	ml
50% Glycerol	5.0	ml
10% SDS	2.0	ml

2-Mercaptoethanol	0.5	ml
1% Bromophenol blue	1.0	ml
Distilled water	0.9	ml

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM Glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g
SDS	1.0	g

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

APPENDIX D: Preparation for isoelectric focusing gel electrophoresis

Monomer-ampholyte solution

30% Acrylamide solution	0.9	ml
1.0% Bis-acrylamide solution	1.25	ml
Ampholyte pH 3-10	0.24	ml
Distilled water	1.39	ml
50% Sucrose	1.19	ml
TEMED	2	μl
0.02 M $(NH_4)_2 S_2O_8$	39.5	μl

Fixative solution, 100 ml

Sulfosalicylic acid	4	ml
Trichloroacetic acid	12.5	g
Methanol	30	ml

Immerse gels in this solution for 30 min.

Staining solution, 100 ml

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

Dissolve the $CuSO_4$ in water before adding the alcohol. Either dissolve the dye in alcohol or add it to the solution at the end. Immerse the gel in the stain for approximately 1-2 h.

Destaining solution

First destaining solution

Ethanol	12	ml
Acetic acid	7	ml
$CuSO_4$	0.5	g
Distilled water	81	ml

Dissolve the cupric sulfate in water before adding the alcohol. Immerse the gel in two or three changes of this solution until the background is nearly clear.

Second destaining solution

Ethanol	12	ml
Acetic acid	7	ml
Distilled water	81	ml

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

APPENDIX E: Preparation for Southern blot hybridization

Hybridization buffer

Stock ECL Gold hybridization buffer	100	ml
0.5 M NaCl	2.92	g
5% Blocking agent	5	g

Add NaCl, stir until completely dissolve. Gradually add blocking agent. Stir for 1-2 h at room temperature, then preheat to 42 ^{o}C for 0.5-1 h. with occasional mixing. Store at –15 to –30 ^{o}C for at least 3 months.

20X SSC, 500 ml

0.3 M Tri-sodium citrate	44.12	g
3 M NaCl	87.66	g

Adjust pH to 7.0 with HCl or NaOH.

Primary wash buffer

6 M Urea	360	g
0.4% SDS	4	g
20X SSC	25	ml

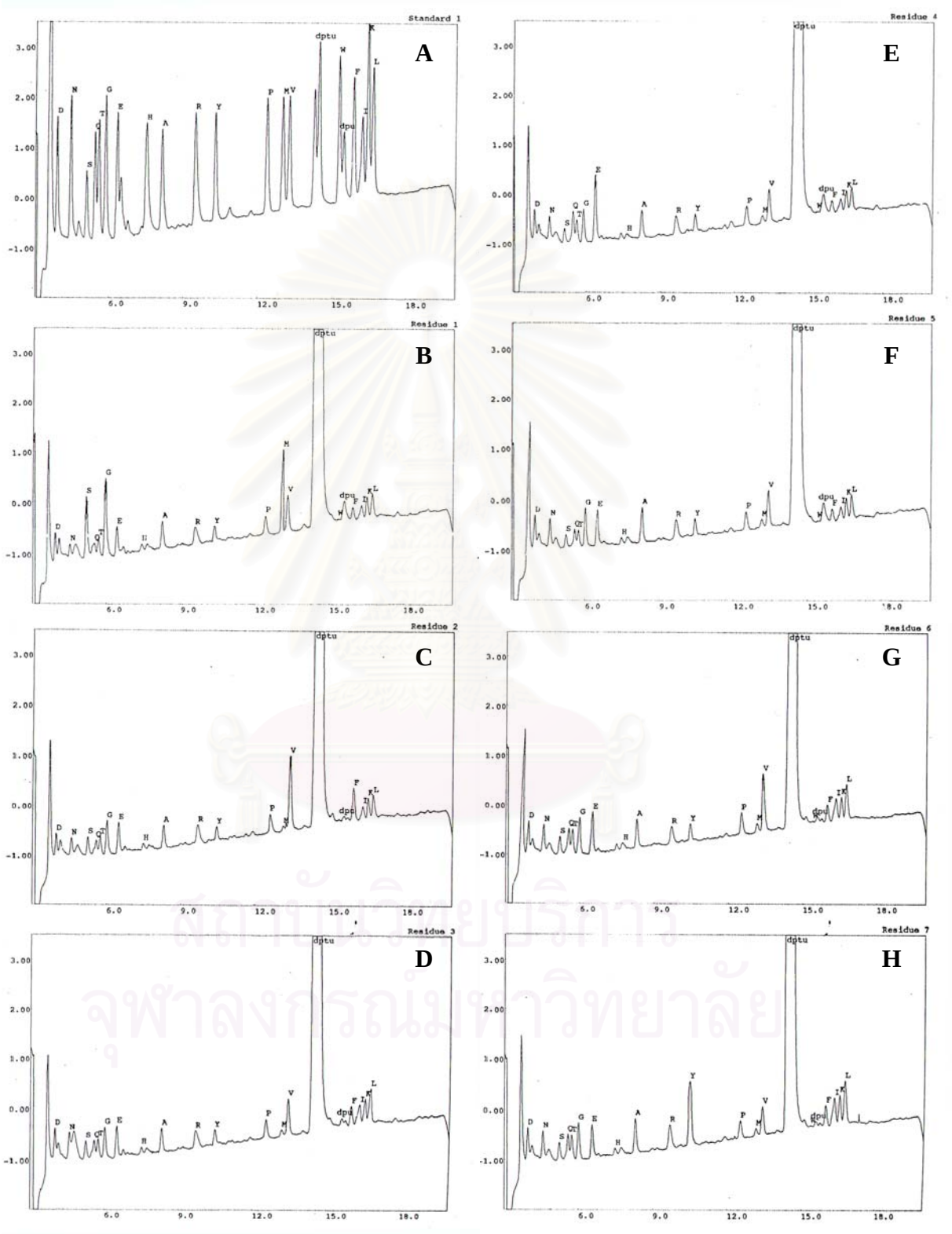
Make up to 1 litre. This can be kept for up to 3 months in a refrigerator at 2-8 ^{o}C.

Secondary wash buffer

20X SSC	100	ml

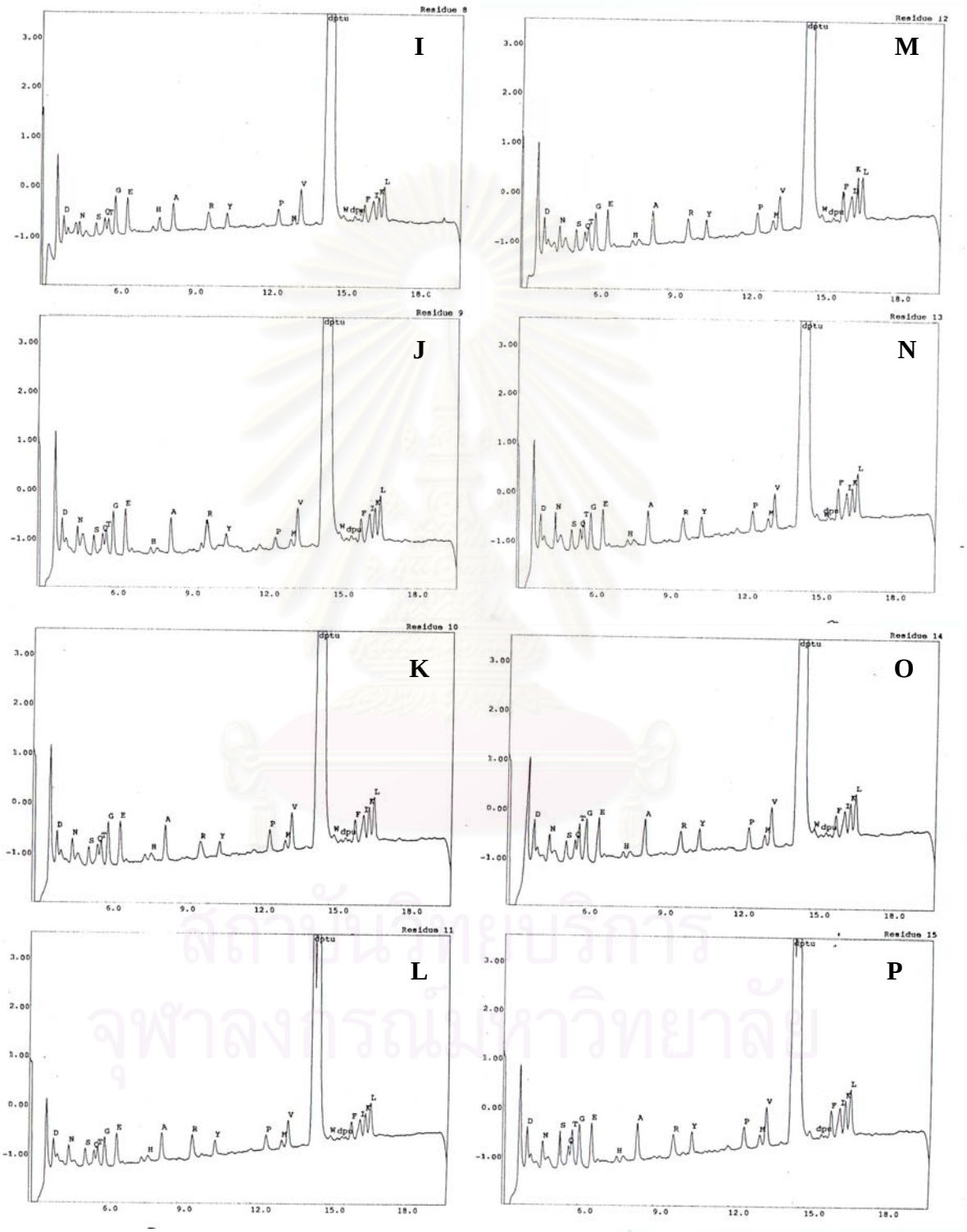
Make up to 1 litre. This can be kept for up to 3 months in a refrigerator at 2-8 ^{o}C.

APPENDIX F: Chromatograms of N-terminal amino acid sequencing of purified CDase from *Paenibacillus* sp. A11 using an applied Biosystems 476A protein sequencer



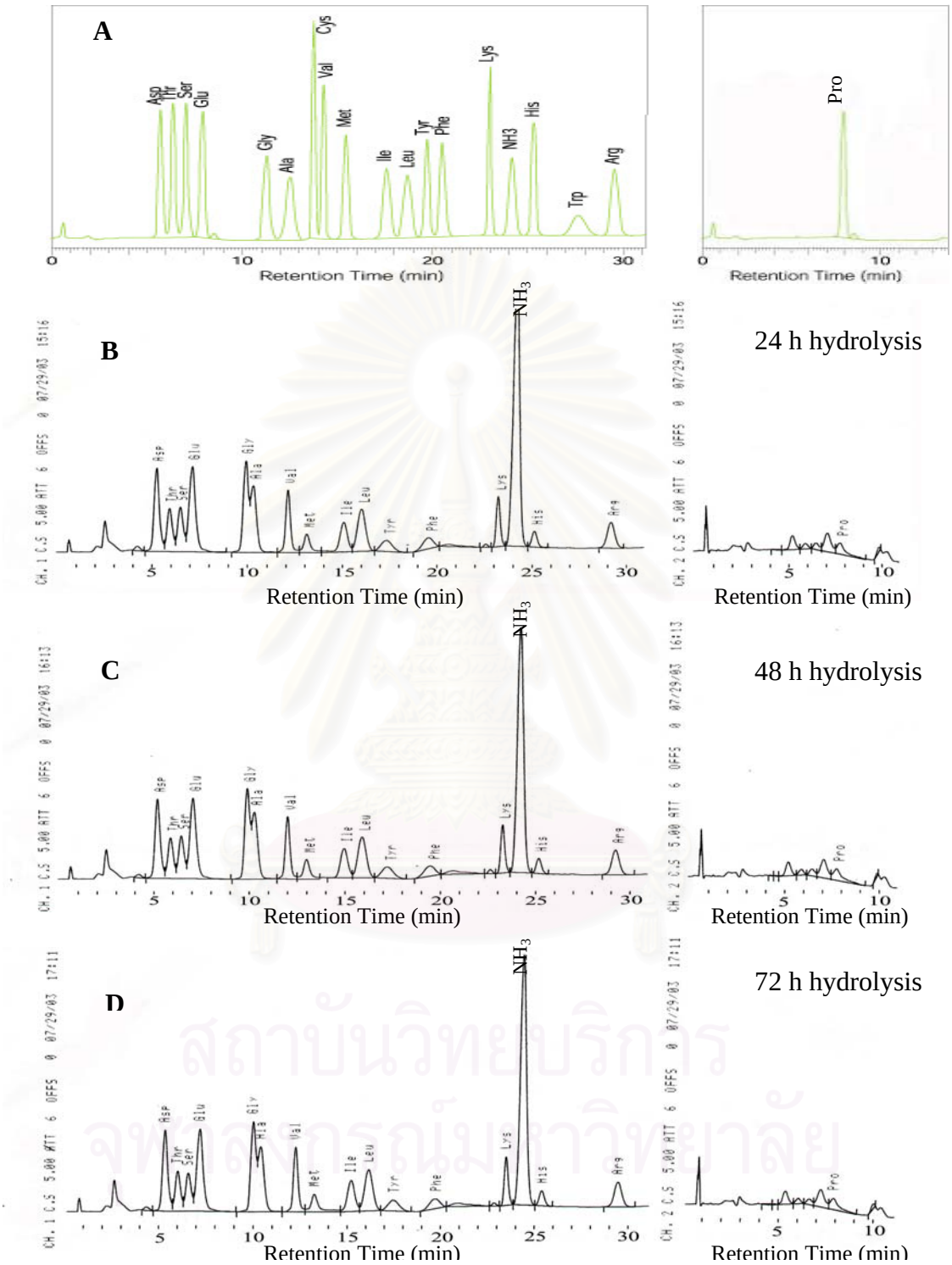
Standard amino acids (A), cycle 1 (B), cycle 2 (C), cycle 3 (D), cycle 4 (E), cycle 5 (F), cycle 6 (G) and cycle 7 (H)

APPENDIX F: Chromatograms of N-terminal amino acid sequencing of purified CDase from *Paenibacillus* sp. A11 using an applied Biosystems 476A protein sequencer (continued)



Cycle 8 (I), cycle 9 (J), cycle 10 (K), cycle 11 (L), cycle 12 (M), cycle 13 (N), cycle 14 (O) and cycle 15 (P)

APPENDIX G: Chromatograms of amino acid composition analysis

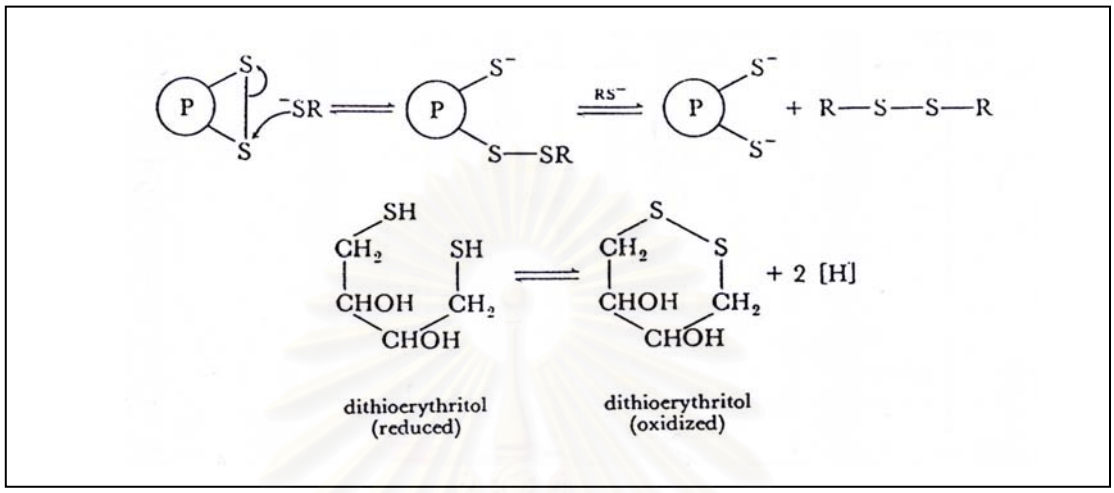


Standard amino acids (A) and acid hydrolysates of purified CDase (B-D) at different time of hydrolysis

APPENDIX H: Modification reaction of group-specific reagents

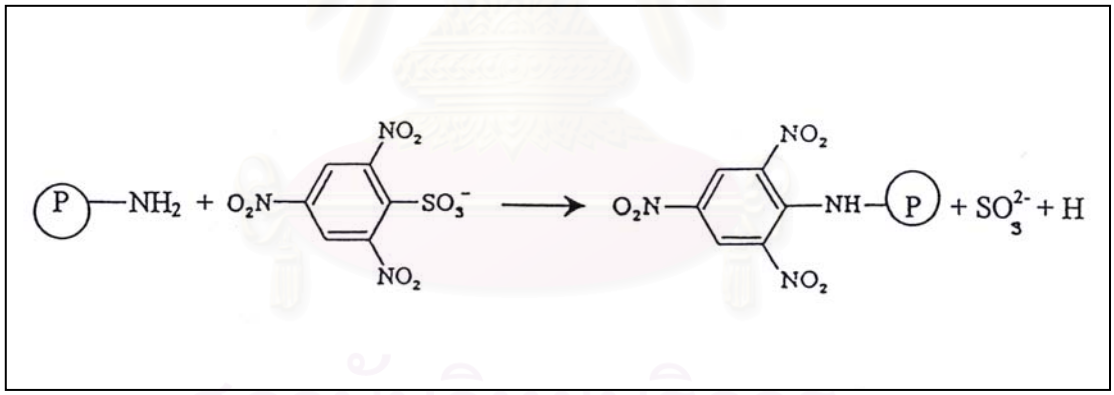
Reaction 1 Modification reaction of DTT with cystine residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)



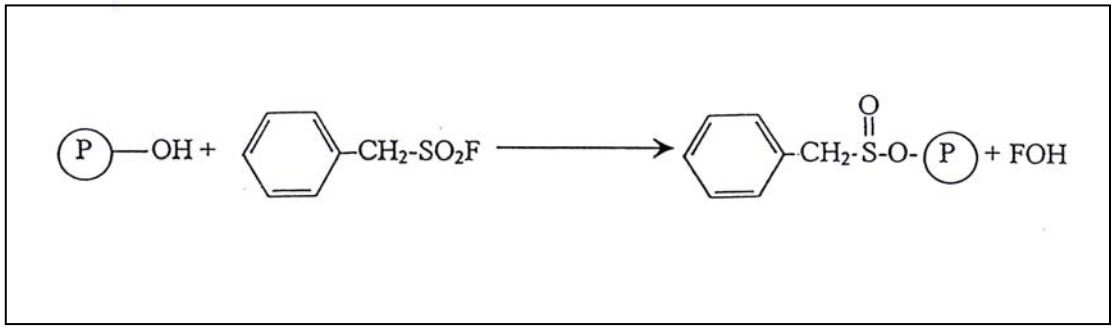
Reaction 2 Modification reaction of TNBS with lysine residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)



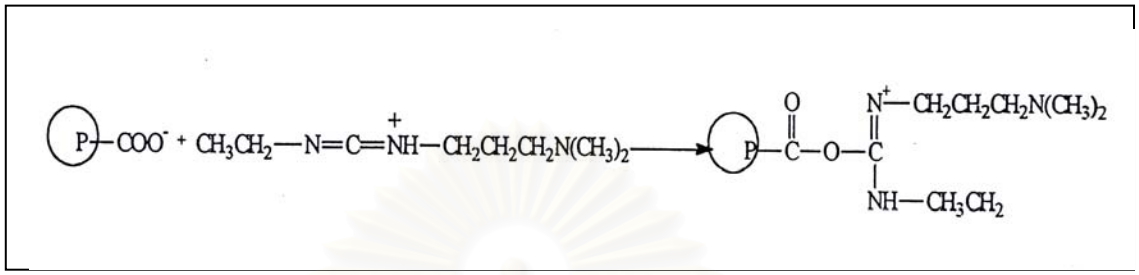
Reaction 3 Modification reaction of PMSF with serine residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)

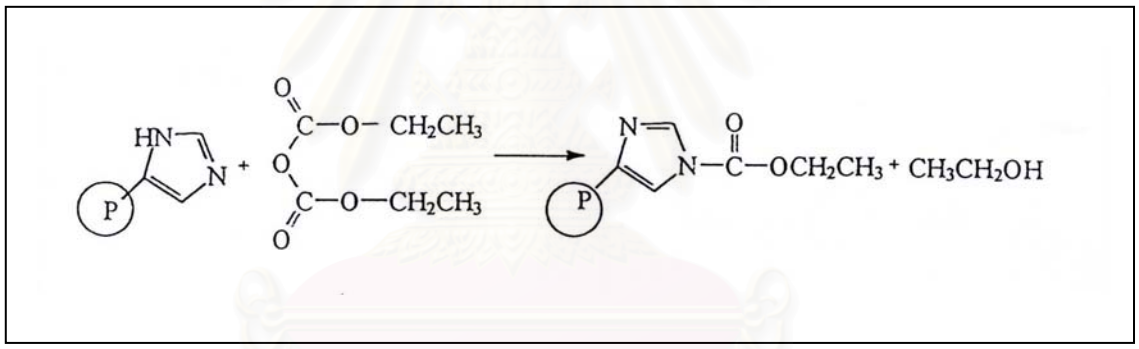


APPENDIX H: Modification reaction of group-specific reagents (continued)

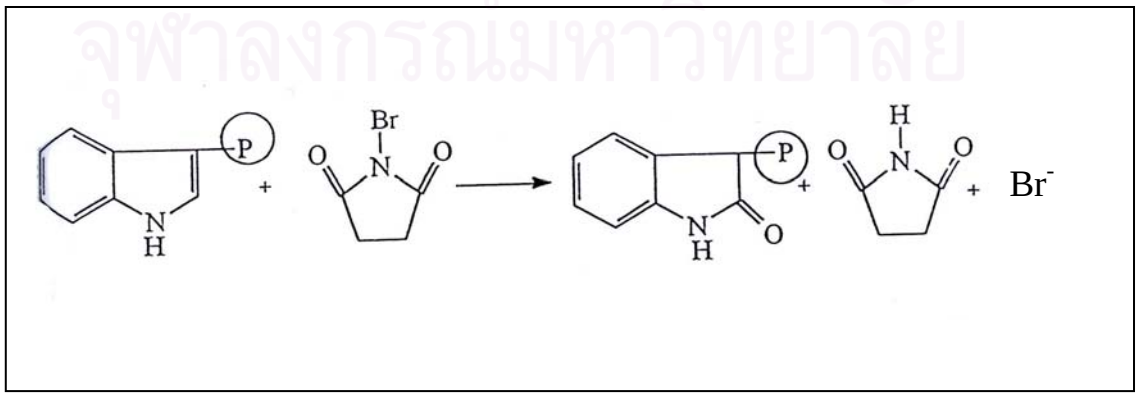
Reaction 4 Modification reaction of EDC with carboxyl residue in protein (P)
(Means and Feeney, 1971; Lundblad, 1991)



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

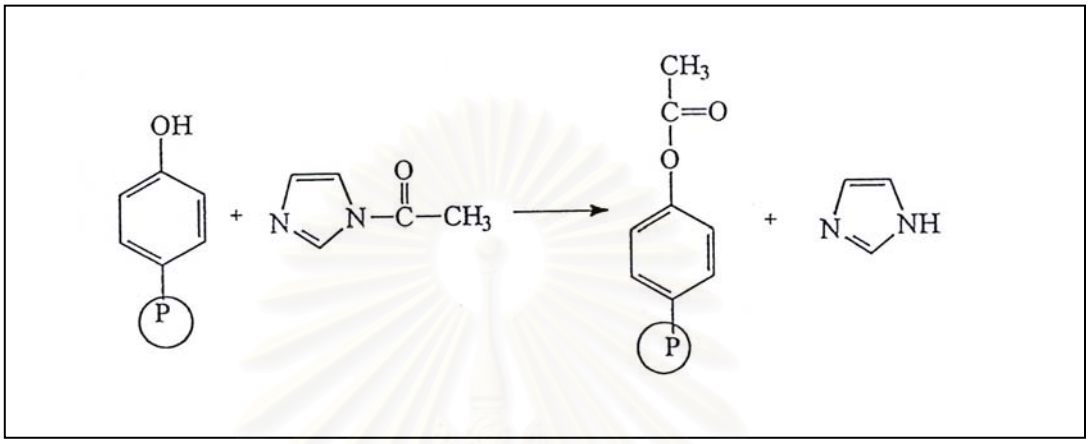


Reaction 6 Modification reaction of NBS with tryptophan residue in protein (P)
(Means and Feeney, 1971; Lundblad, 1991)

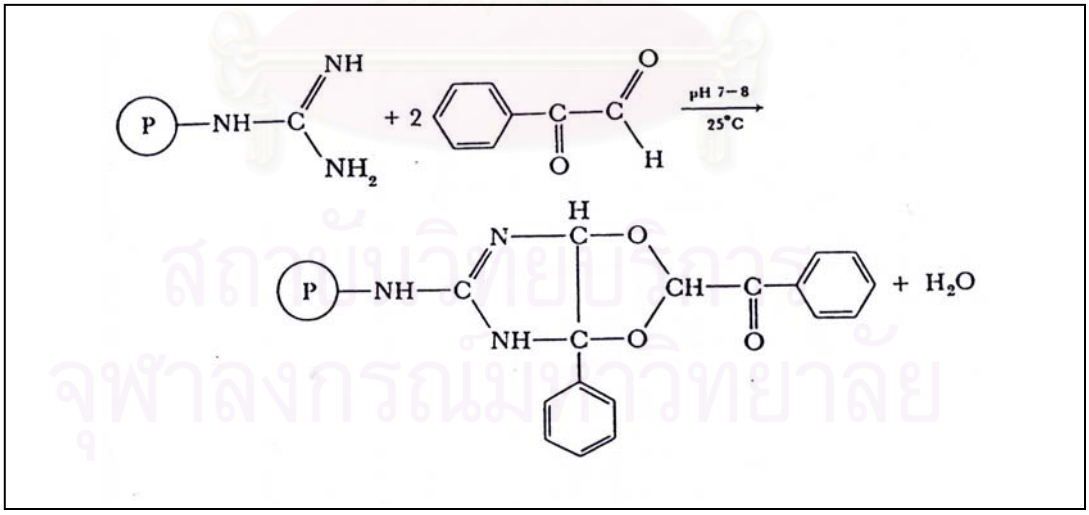


APPENDIX H: Modification reaction of group-specific reagents (continued)

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



Reaction 8 Modification of PG with arginine residue in protein (P)
(Means and Feeney, 1971; Lundblad, 1991)



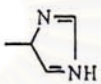
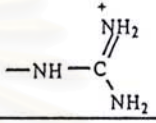
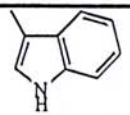
APPENDIX I: Reactivities of amino acid side chains (Means and Feeney, 1971)

Reagent	—NH_2	—SH	—OH	—NH	—NH—C(NH_2)(NH_2^+)	—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 I: Reactivities of amino acid side chains (continued) (Means and Feeney, 1971)

Reagent	—NH_2	—SH	—OH	—NH	—NH—C(NH_2^+)NH_2	—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 I: Reactivities of amino acid side chains (continued) (Means and Feeney, 1971)

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 reactivities which may or may not be attained depending on the condition used.

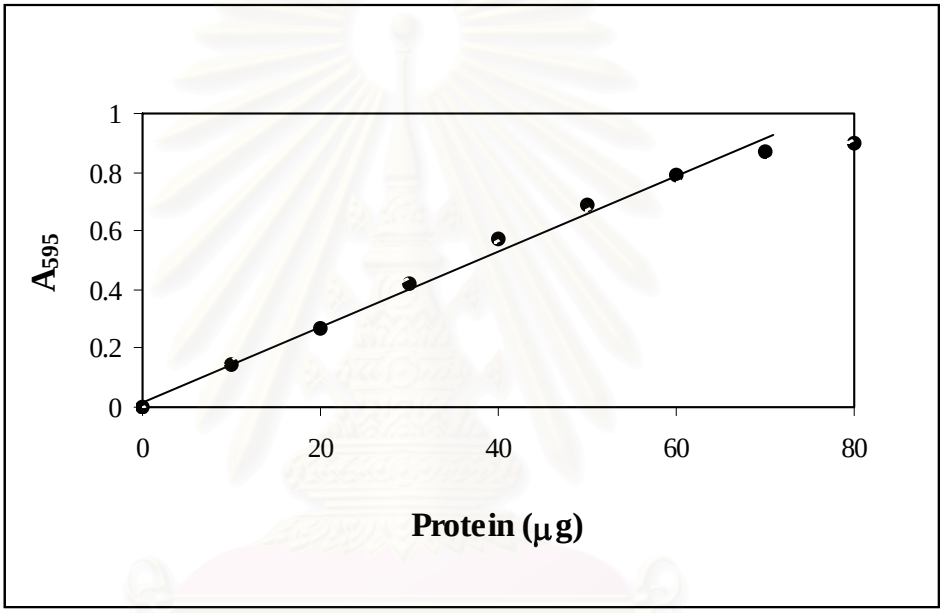
[b] Spontaneously reversible under the reaction conditions or upon dilution, regenerating original group.

[c] Easily reversible, regenerating original group.

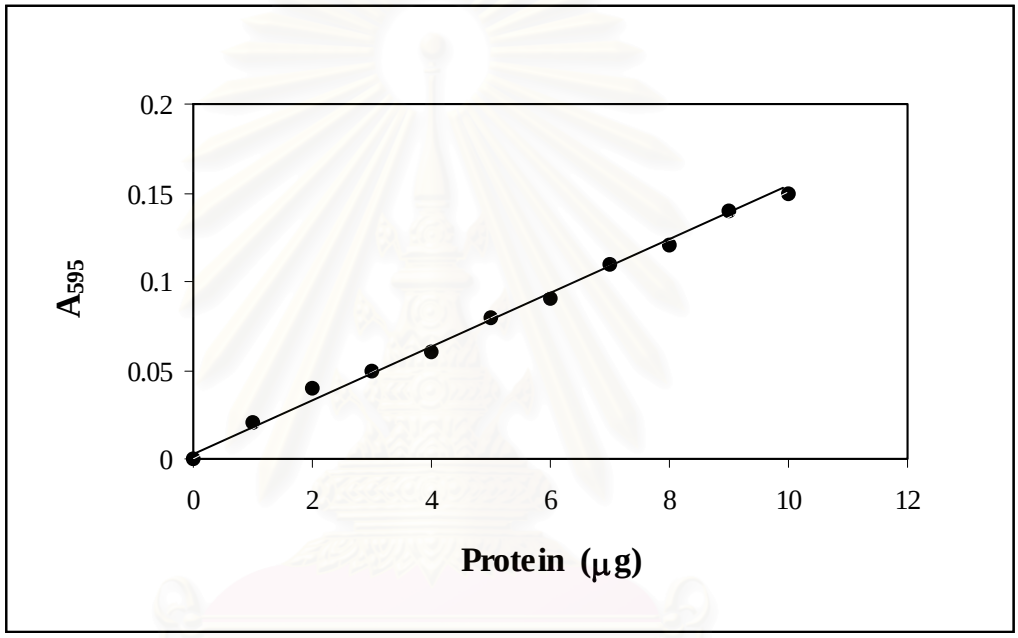
APPENDIX J: Abbreviation for amino acid residues (Voet, 1995)

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

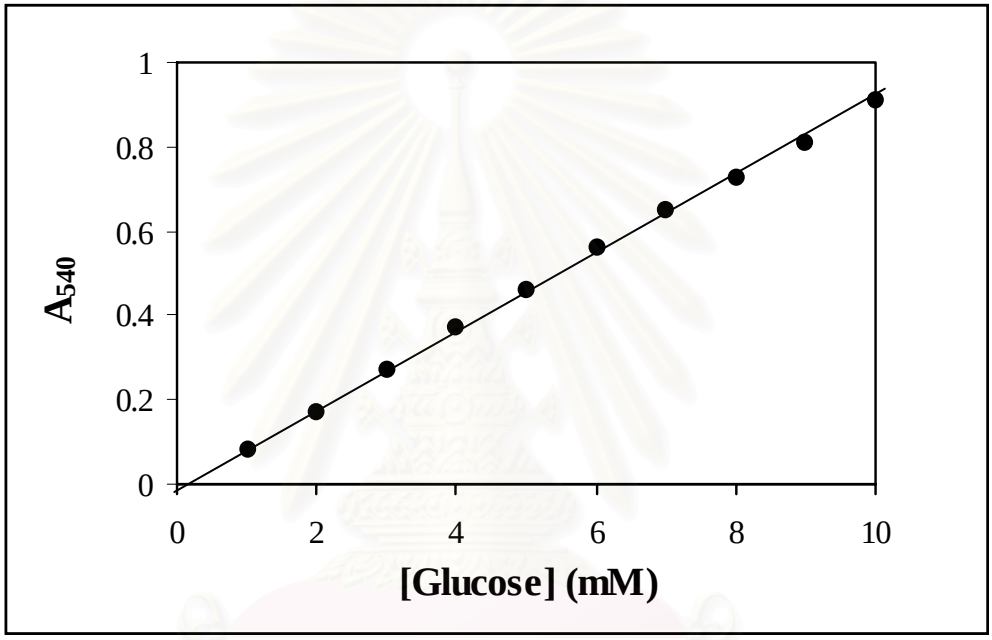
APPENDIX K: Standard curve for protein determination by Bradford's method



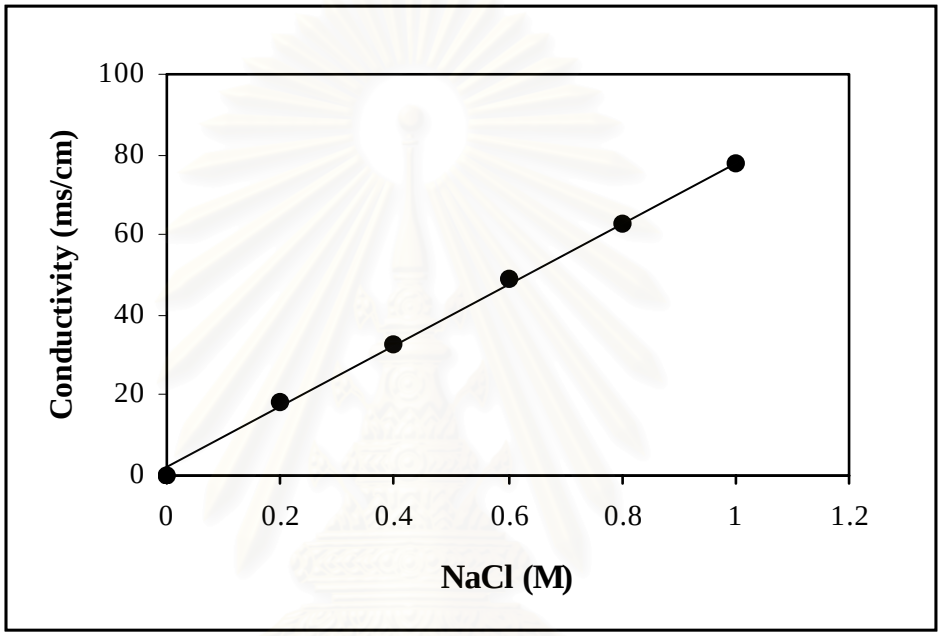
APPENDIX L: Standard curve for microprotein determination by Bradford's method



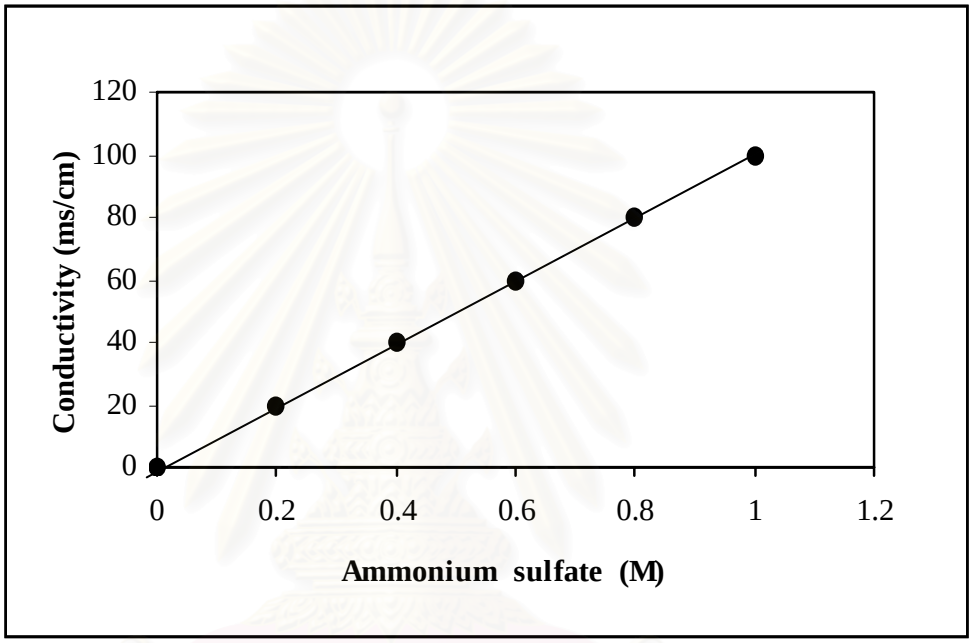
APPENDIX M: Standard curve of glucose by dinitrosalicylic acid method



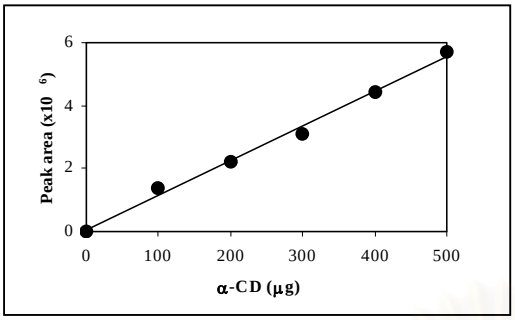
APPENDIX N: Standard curve for conductivity of sodium chloride

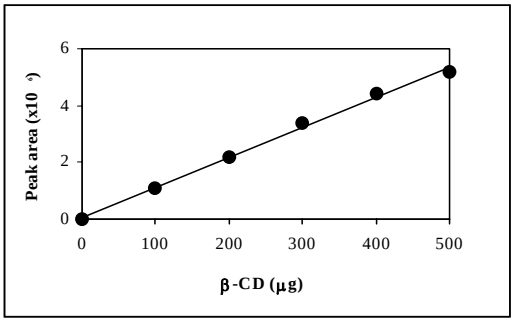


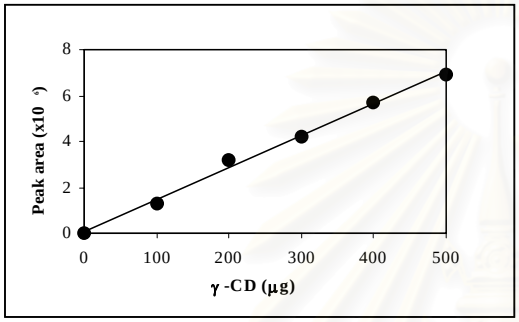
APPENDIX O: Standard curve for conductivity of ammonium sulfate

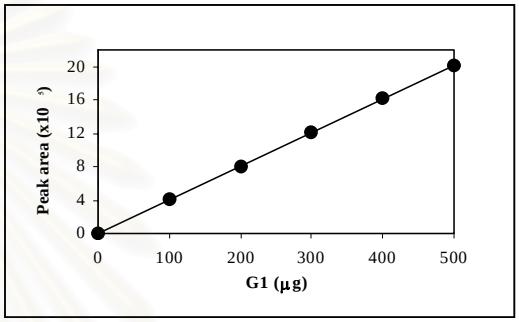


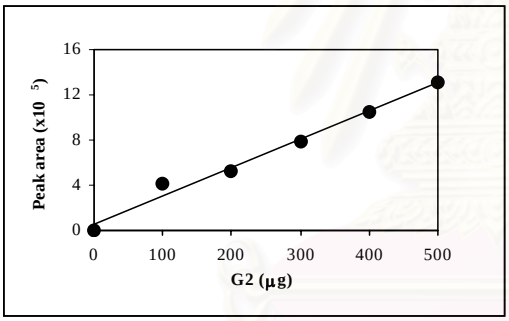
APPENDIX P: Standard curve of CDs and oligosaccharides by HPLC

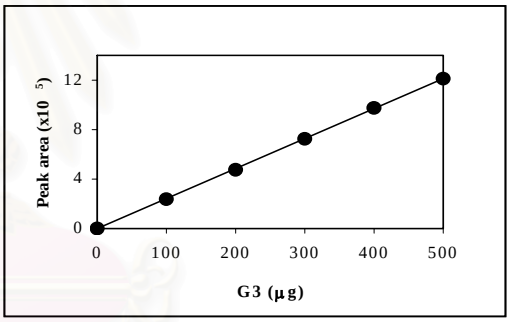


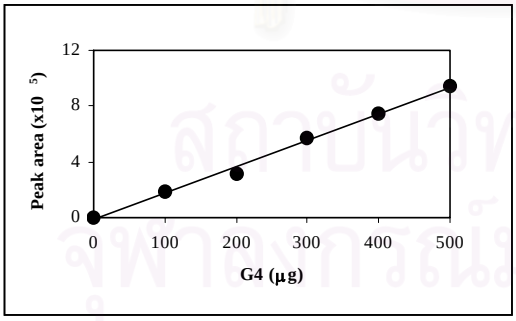


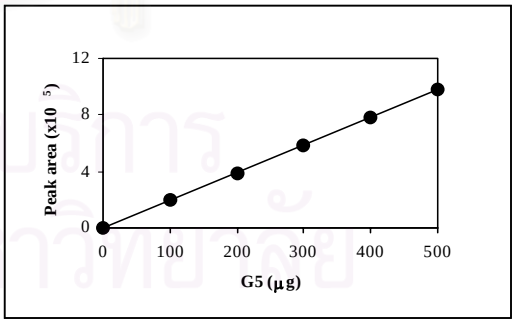


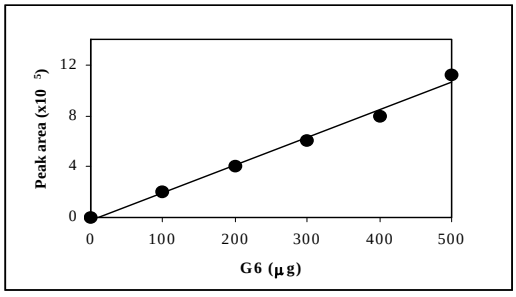


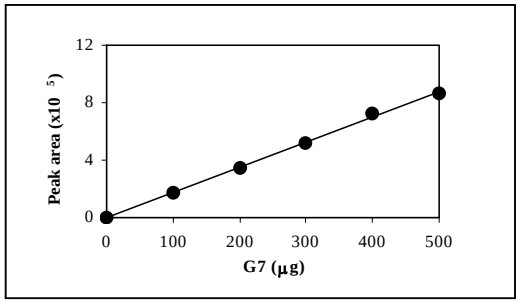




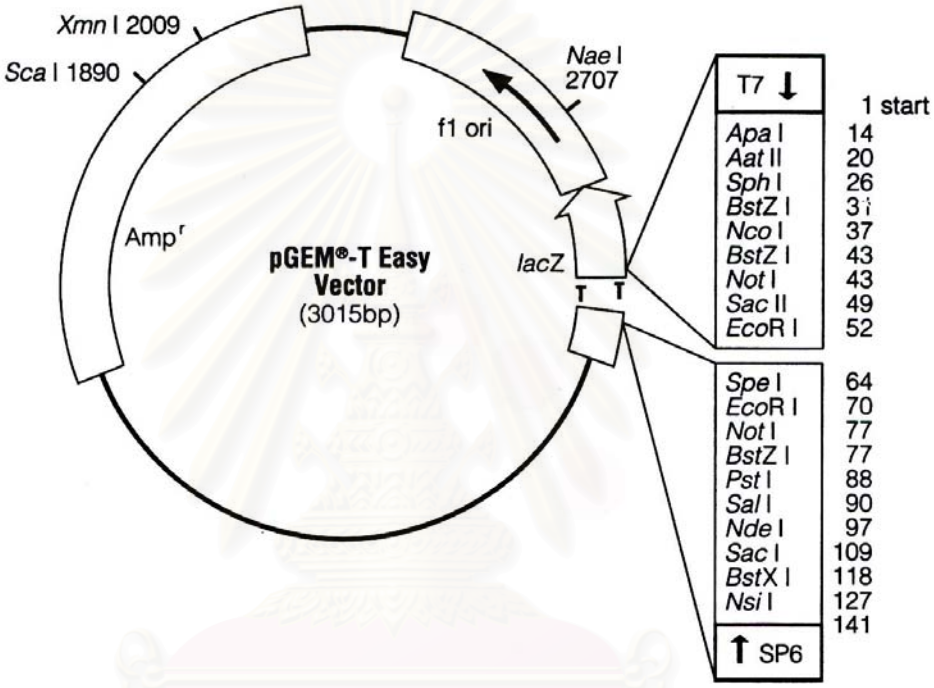




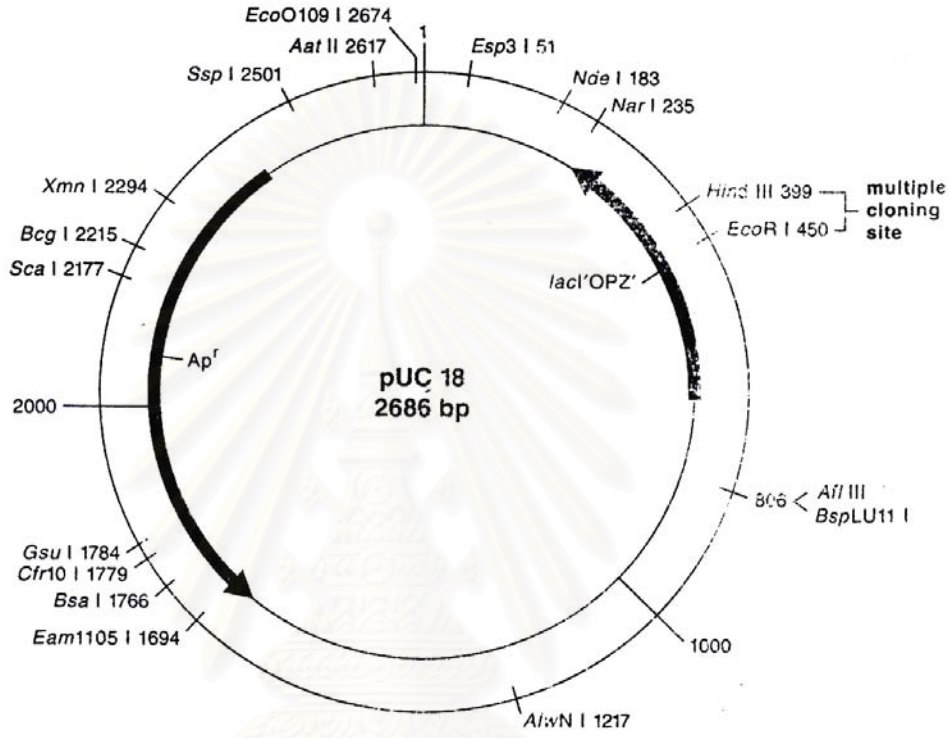




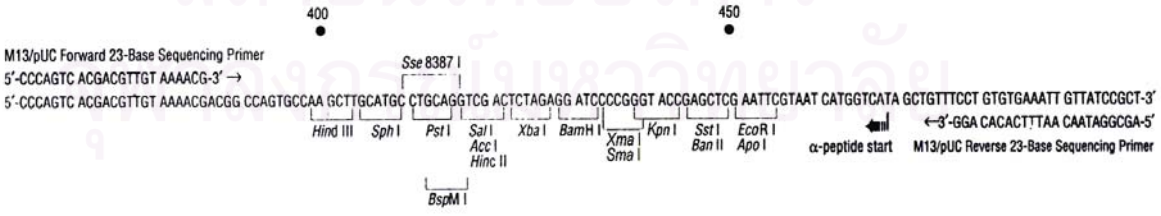
APPENDIX Q: Restriction map of Plasmid pGEM®-T Easy



APPENDIX R: Restriction map of Plasmid pUC 18



pUC18 multiple cloning site and primer binding regions: 364-500



BIOGRAPHY

Miss Jarunee Kaulpiboon was born on February 3, 1977 in Ratchaburee province, Thailand. She was a 1998 graduate from Chulalongkorn University with the Bachelor of Science in Biochemistry. She was then enrolled for Master of Science in Biochemistry at Chulalongkorn University during 1998-2000. After, she received her M.Sc. in 2000, she continued studying for the Degree of Philosophy of Science in Biological Science Program in the field of Cell and Molecular Biology, at the Faculty of Science, Chulalongkorn University in that year. She has publications :

1. Kaulpiboon, J., and Pongsawasdi, P. 2002. Molecular identification of *Paenibacillus* sp. A11 using 16S rRNA gene and preliminary investigation for cyclodextrinase production. In; Danvirutai, P. (ed.), Proceeding of the 14th annual meeting of the Thai Society for Biotechnology. Prathammakhant Printing, Khon Kaen. pp. 103-107.
2. Kaulpiboon, J., and Pongsawasdi, P. 2003. Identification of essential histidines in cyclodextrin glycosyltransferase isoform 1 from *Paenibacillus* sp. A11. *J. Biochem. Mol. Biol.* **36** : 409-416.
3. Kaulpiboon, J., and Pongsawasdi, P. 2003. Expression of cyclodextrinase gene from *Paenibacillus* sp. A11 in *Escherichia coli* and characterization of the purified cyclodextrinase. *J. Biochem. Mol. Biol.* (in press)