CHAPTER I

INTRODUCTION

Cyclodextrins

Cyclodextrins (celluloseine, cycloamylose, cyclomaltose, Schardinger dextrin : CD) are cyclic oligosaccharides consisting mainly of six, seven or eight D-glucopyranosyl units connected by α-(1,4) - glycosidic linkage, named as α-, β- and γ-CD, respectively (Figure 1) (Villiers, 1891; Schardinger, 1903, 1904; French, *et al.*, 1942, 1949; Pulley and French, 1961). The most stable three dimensional molecular configuration for these non-reducing cyclic oligosaccharides takes the form of a truncated cone with the upper (larger) and lower (smaller) opening of the cone presenting C2 and C3 secondary and C6 primary hydroxyl groups, respectively. These orientations make cyclodextrin molecules hydrophilic on the outside, and the hydrophobic inside cavity is lined with C-H groups and glycosidic oxygen bridges (Saenger, 1979,1982; Bender, 1986) as shown in Figure 2.

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

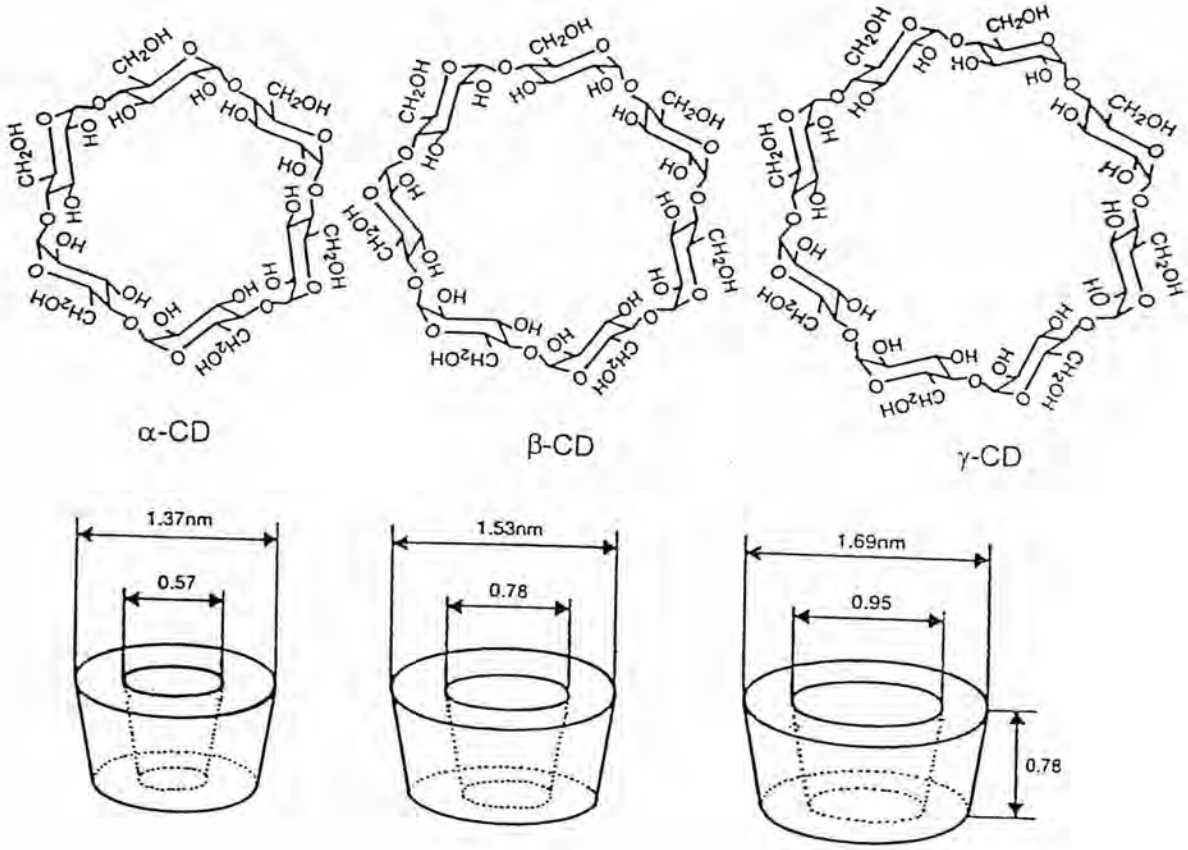
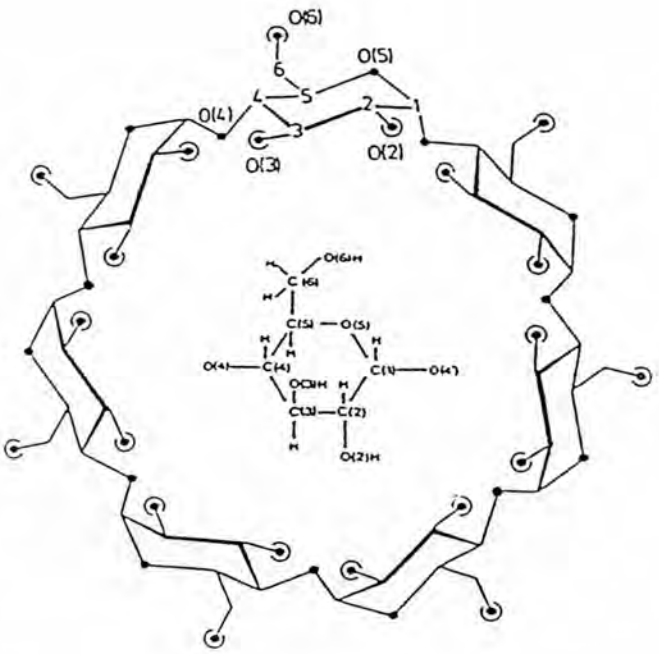


Figure 1. Structure and molecular dimension of cyclodextrins (CDs)

(Szejtli, 1990)

(a)



(b)

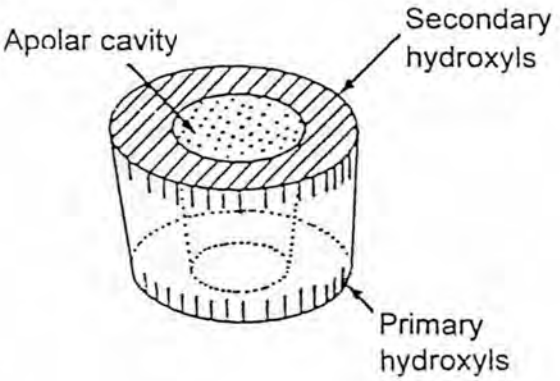


Figure 2. Structure of β-cyclodextrin (Bender, 1986; Szejtli, 1990)

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

(b) Functional structure scheme

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

	α-CD	β-CD	γ-CD
Number of glucose unit	6	7	8
Molecular weight	972	1,135	1,297
Cavity dimension			
Cavity diameter (A°)	4.7 - 5.3	6.0 – 6.5	7.5 - 8.3
Cavity depth (A°)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Cavity volume $(A°)^3$	174	262	472
Solubility in water (g/100 ml at 25°C)	14.40	1.85	23.20
Crystal form (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms

The cavity of cyclodextrins are filled with water molecules. It can also form three dimensional inclusion complex in the solid state or in solution by including a wide variety of suitable size "Guest" molecules wholly or partially within the central cavity of the "Host" cyclodextrin (Saenger, 1980; Bender, 1986). 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 "Guest" molecules within the "Host" cyclodextrin is not fixed or permanent, but rather is governed by a dynamic equilibrium and thereby affording an ease of assembly and disassembly. Binding strength depends on how well the Host-Guest molecules fit together geometrically and specific local interactions between their surface atoms. The potential guests which can be encapsulated in cyclodextrin as shown in Figure 3 (Amaizo, 1993) and Figure 4 (Janssen, 1992), include such compounds as straight or branched chain hydrocarbons, gasses, and some relatively polar compounds.

At first, β-CD is known to be more suitable for practical use because their inclusion complexes are easily prepared and more stable due to the size of the apolar cavity being optimum for a large variety of guest molecules (Horikoshi and Akiba, 1992; Horikoshi, 1979). In addition, it can be easily separated from the reaction mixture because of its low solubility in water. At present, γ-CD now is becoming an attractive molecule especially in pharmaceutical industry due to its higher solubility and bigger inner cavity. However, the production of γ-CD is still a problem because very few CGTases preferentially produced γ-CD have been reported (Englbrecht, *et al.*, 1990).

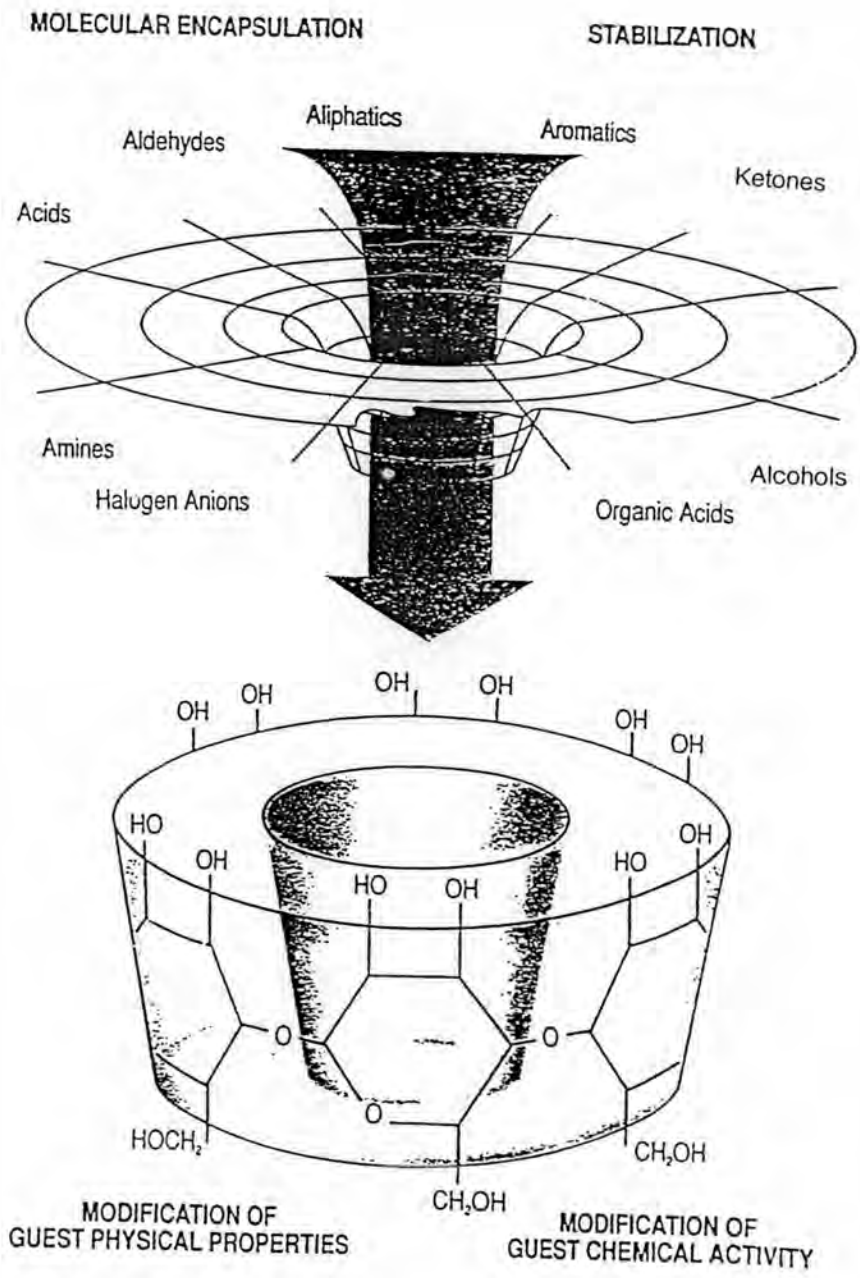


Figure 3. Inclusion complex formation between CDs and guest molecules leading to modification of guest physical and chemical properties.

(Amaizo, 1993)

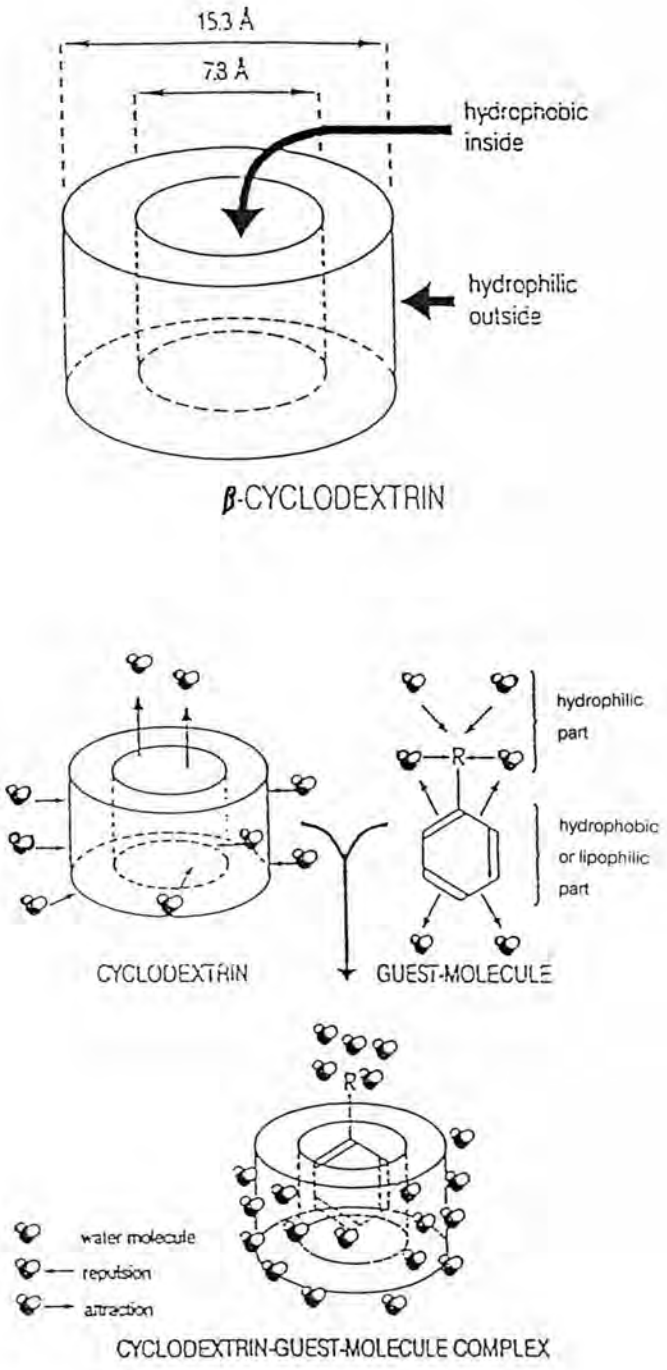


Figure 4. Orientation of guest molecule in CD-guest complex (Janssen, 1992)

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

Complex formation of cyclodextrins and guest molecules can markedly improve the chemical and physical properties of guest molecules such as protection against oxidative degradation or destruction by UV light, improvement of the solubility of hydrophobic substances in aqeous solution, stabilization of volatile compounds, alternation of the chemical reactivity, modification of liquid substances to powder, or reduction of undesirable smell or taste in products *e.g.* foodstuffs are among those known useful properties (Schmid, 1989). The applications of cyclodextrins as emulsifiers, antioxidants and stabilizing agents have rapidly increased in food, cosmetics, pharmaceutical, agrochemical and plastic industries (Nagamoto, 1985), see Table 3.

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

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

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

Table 3. Industrial applications of cyclodextrins (Horikoshi,1982 ; Bender, 1986 ; Szejtli and Pagington, 1991)

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

Table 3. Industrial applications of cyclodextrins (continued)

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

for cyclodextrins are growing, Table 4 shows a ten-fold increase in demand during 1989-1995.

Cyclodextrin producing enzymes

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

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

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

Where G_n and G_m are 1,4-α-D-glucopyranosyl chains with "n" and "m" glucose residues ; x is a part of the 1,4-α-D-glucopyranosyl chain and cG_x is a symbol for CDs. These machanisms are summarized in Table 5.

The cyclization reaction is thought to be a special type of disproportionation, the non-reducing end of one chain itself serving as acceptor, whereas the helical conformation of substrate is thought to be a prerequisite for cyclization. It should be mentioned that the acceptor binding site of the enzyme is not absolutely specific for glucose or maltooligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrate containing 16-80 glucopyranosyl residues. If the chain length

Table 4. World market of cyclodextrins

Application	Market (ton per year)	
	1989	1995
Pharmaceutical	50	2,000
Food	700	2,500
Cosmetics	50	500
Agriculture	10	100
Chemical industry (biotransformations, separation, catalysis)	30	300
Other purposes (*e.g.* diagnistics)	10	200

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

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

is greater than 100 units, disproportionation reaction dominates. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 6. Higher concentration of maltooligosaccharides or glucose favors the reversed coupling reaction resulting in linear end products with negligible amount of cyclodextrins (Kitahata, Okada, and Fukai, 1978). The action of CGTase is different from that of other starch-degrading enzymes in that the products are cyclic and non-reducing.

Model of mechanism for the cyclization has been postulated that CGTase binds eight to ten (or even more) glucose units of a starch molecule. The active site of CGTase thus consisted of eight to ten (or more) subsites. The reaction is an exoattack on glucose chains from the non-reducing ends. The resulting maltohexaose intermediate is bound to an aspartyl group of enzyme by ester bond. The non-reducing end of the maltohexaose subsequently binds to subsite two and new α-1,4-glycosidic bond is formed between glucose residues one and six of maltohexaose, as shown in Figure 5 (Bender, 1988).

About 20-30 residues of N-terminal amino acid sequences of CGTase were deduced from determination of nucleotide sequences and use of amino acid sequencer, (Makela, *et al.*, 1988 ; Hamamoto, *et al.*,1987). CGTase gene from various microorganisms were found consisting of 2,100-2,800 bases, encoding the CGTase of 680-690 amino acids and contained 60-80% homology in their amino acid sequences. Amino acid compositions were also determined directly from amino acid analyzer (Schmid, *et al.*, 1988; Takano, *et al.*, 1986; Kimura, *et al.*, 1987; and Binder, *et al.*, 1986).

Table 6. Relationship between length of substrate and mechanism of CGTase (Szejtli, 1988)

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

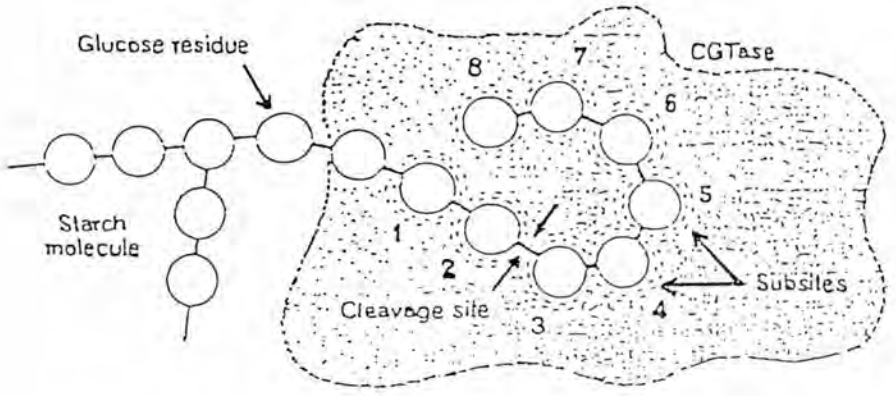


Figure 5. Model of CGTase mechanism from *Klebsiella pneumoniae* M 5 al

(Bender, 1988)

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

CGTase is produced by various microorganisms, for example *Klebsiella pneumoniae* (Bender, 1977), *Brevibacterium* sp. (Mori *et al.*, 1994), and mainly the *Bacillus* sp. (Bender, 1986 ; Komitani *et al.*, 1993) , as listed in Table 7(a-b). The CGTase can be devided into three types : α- , β- , and γ- ,according to the major type of CD formed (Horikoshi, 1988). The enzymes from different sources show slightly different characteristics such as working pH, temperature, and molecular weight.

Table 7-a. 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/lentus	γ-CD	6.0-8.0	50	75,000	4.1	Englbrecht, *et al.*, 1990
Bacillus macerans IFO 3490	α-CD	5.0-5.7	55	5,000	4.6	Kitahata, *et al.*, 1974
Bacillus macerans IAM 1243	α-CD	5.5-7.5	60	145,000	ND	Kobayashi, *et al.*, 1977
Bacillus macerans ATCC 8514	α-CD	6.2	ND	139,300	ND	Depinto and Campbell, 1986
Bacillus megaterium	β-CD	5.0-5.7	55	ND	6.07	Kitahata and Okada, 1974
Bacillus stearothermophilus	α-CD	6.0	ND	68,000	4.5	Kitahata and Okada, 1982
Klebsiella pneumoniae M5 al	α-CD	6.0-7.2	ND	68,000	4.8	Bender, 1982
Micrococcus sp.	β-CD	6.2	ND	139,300	ND	Yagi, *et al.*, 1980

ND = no data

Table 7-b. CGTase-producing bacteria (Bender, 1986)

Organism	Cultivation mode	mg CGTase/ litre culture filtrate[a]	References
Alkalophillic *Bacillus* 38-2	Batchwise	430	Horikoshi, Ando and Yoshida, 1982 ; Nakamura and Horikoshi, 1976
Bacillus circulans	Batchwise	100	Kitahata and Okada, 1982b
Bacillus macerans	Batchwise	360-480	Miskolci-Torok,*et al.*, 1980
Bacillus megaterium	Batchwise	260	Kitahata , Tsuyama and Okada, 1974
Bacillus ohbensis	Batchwise	24	Yagi and Iguchi, 1974
Bacillus stearothermophilus	Batchwise	ND	Kitahata and okada, 1982a, 1982b
Klebsiella pneumoniae M5 al	Continuous	120	Bender, 1977a, 1977b, 1982
Micrococcus sp.	Batchwise	199	Yagi, Kouno and Juni, 1980

[A] CGTase-protein was calculated from the enzyme activities

ND = no data

Each CGTase enzyme yields different ratio of cyclodextrin products for example, the CGTase of *B. macerans* produced α-, β-, and γ-CD in relative amount of 2.7:1.0:1.0 (Depinto and Campbell, 1968), while the CGTase of alkalophillic *Bacillus* no. 38-2 and *B. circulans* produced CDs in relative ratio of 1.0:11.0:1.5 (Matzuzawa, *et al.*, 1975) and 1.0:10.0:1.0 (Pongsawasdi and Yagisawa, 1987), respectively. The *Bacillus fermusl lentus* 290-3 was known to produce γ-CGTase in the initial phase of the enzyme production (Englbrecht, *et al.*,1990).

Although the potential of cyclodextrins in industrial application is well known, the market for cyclodextrins is still limited due to high cost and the availability of α-, β- and γ-cyclodextrins. Various studies have been emphasized on improvement of cyclodextrin productions. Development of cultivation for cyclodextrin over-production was carried out under optimized culture condition and with complex nutrient media. A need for a thermostable or thermotorelant CGTase which gives high cyclodextrin yield has been recognized. CGTase from an alkalophilic *Bacillus* strain no. 38-2 (ATCC 21783) was observed to provide these required properties (Horikoshi and Akiba, 1982). Immobilized CGTase can be utilized in several conversion cycles to steadily increase the volume of production, hence reduce the production cost (Nakamura and Horikoshi, 1976; Kato and Horikoshi, 1984; Yang and Su, 1989). Protein engineering, site-directed mutagenesis and gene cloning were also used to increase the yield of enzyme product. These techniques were not only based partly on an assumption of lower production costs, but also on the trends towards greater acceptability of cyclodextrins (Schmid, 1989).

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

Purification methods for CGTase

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

CGTase isozymes

At early stages of the study by isoelectric focusing, CGTase from alkalophillic *Bacillus* separated into a few active bands. Because of this, an attractive possibility was that CGTases of various bacteria actually are mixtures of α-,β- and γ-producing

individual enzymes. Some support for such speculations has been provided by a report claiming the development of a CGTase producing only γ-CD (Kato and Horikoshi, 1986). In addition to possible differences in specificities, CGTase from alkalophilic *Bacillus* has been reported to contain three isozymes possessing markedly different pH optima (4.6, 7.0 and 9.5, respectively) (Nagamura and Horikoshi, 1976; Horikoshi, *et al.*,1981).

The CGTase from alkalophilic *Bacillus* (ATCC 21783) was purified to near homogeneity by a two-step procedure involving affinity chromatography and high-performance anion exchange chromatography. The latter method produced several fractions with different pI in the range of 4.55-4.90 but their properties such as activity levels and product compositions were identical under various reaction conditions (Makela, *et al.*,1988). Similar results were reported on CGTase from *Bacillus circulans* var. *alkalophillus* (ATCC 21783) and *Bacillus circulans* E 192. CGTase from the strain ATCC 21783 fractionated by isoelectric focusing in immobilized pH gradients (IPG), could be resolved into more than 6 subforms, a major one with pI 4.97 and the others between pI 4.75-4.99. The 5 amino acids at the N-terminus of these CGTase subforms were determined and reported to be the same (Ala-Pro-Asp-Thr-Ser) (Mattsson, Meklin and Korpela, 1990). *B. circulans* E 192 CGTase was purified by FPLC on a Mono Q column. Two isozymes were separated and their isoelectric points were estimated as 6.7 and 6.9 with amino acid compositions of 705 and 716 residues, respectively, of the amino acid compositions with no difference of 30 amino acid sequence at the N-terminus (Bovetto, *et al.*,1992)

In 1994, Abelyan and his group attempted to purify CGTase isozymes isolated from *Bacillus* strains INMIA-T6, INMIA-T42 (thermophilic), INMIA-A7/1 (alkalophilic) and INMIA-1919 (mesophilic) by affinity chromatography on a β-CD polymer. The results showed that physicochemical and biochemical properties of each isozyme, including their amino acid composition were different (Tables 8,9) (Abelyan, *et al.*, 1994).

The cyclodextrin research group at the Department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on β-CGTase of *Bacillus* sp. A11, a strain isolated from South-East Asian soil (Pongsawasdi and Yagisawa, 1987). The enzyme was purified and characterized in it's properties such as molecular weight, working pH and temperature and the enzyme activity on various substrates (Techaiyakul, 1991). Effect of some carbohydrates on the induction of CGTase to produce higher CD-products mainly γ-CD was also studied (Rattapat, 1996). The enzyme was purified by chromatofocusing column and analysis on native-PAGE suggested that it may compose of 4 isozymes with different isoelectric points in the range of 4.40-4.90 (Rojtinnakorn, 1994). Specific antibody against CGTase was prepared (Rojtinnakorn, 1994) and was used in enzyme purification through immunoaffinity column chromatography (Kim, 1996). Optimization of CGTase production in a 5 litre-fermenter and cyclodextrin production from rice starch by using immobilized CGTase in both batchwise and continuous processes and also free CGTase were studied (Rutchatorn, 1993; Kuttiarcheewa , 1994 and Malai, 1995). Siripornadulsil (1992), Vittayakitsirikul (1995) and Boonchai (1996) reported on molecular cloning

Table 8. Some properties of CGTase isoenzymes (Abelyan, *et al.*, 1994)

Source	Molecular weight (kD)	pH optimum	pH stability	Thermal stability, °c	CD molar ratio (α/β/γ) [a]	Total starch-derived CD [a], %
INMIA-T42						
Fraction 1	35	6.5	6.0-10.0	75	2.9/3.5/1.0	62
Fraction 2	31	7.5	6.0-8.0	70	5.2/3.1/1.0	55
Fraction 3	28	6.5-7.0	5.5-8.5	60	4.8/3.5/1.0	60
Fraction 4	25	6.5	5.0-8.0	60	5.2/3.4/1.0	46
P[b]	69	6.5	6.0-9.0	70	1.4/3.1/1.0	55
INMIA-T6						
Fraction 1	38	6.5	5.0-9.5	60	4.8/3.8/1.0	55
Fraction 2	30	7.0	6.0-8.0	60	5.4/3.7/1.0	61
P[b]	68	7.0	5.5-9.0	60	4.4/3.7/1.0	53
INMIA-A7/1						
Fraction 1	50	7.0	5.0-8.0	65	1.0/27.0/4.0	64
Fraction 2	44	6.0	5.5-10.0	55	1.0/58.4/7.4	70
P[b]	93	6.5	5.0-8.0	65	1.0/3.7/1.2	56
INMIA-1919	42	4.0	5.0-8.0	50	4.7/2.3/1.0	3

[a] Reaction conditions : starch (10 mg/ml), enzyme (3.0 units), 50°c, 60 min, optimal pH.

[b] Intact enzyme

Table 9. Amino acid composition of CGTase isoenzymes (Abelyan, *et al.*, 1994)

Amino acid	Content, g per 100 g protein											
	INMIA-T42					INMIA-T6			INMIA-A7/1			INMIA-1919
	P[a]	F1	F2	F3	F4	P[a]	F1	F2	P[a]	F1	F2	
Aspartate	17.1	19.4	18.7	12.9	17.3	14.8	12.5	17.1	14.9	14.1	15.7	16.0
Threonine	6.3	5.1	4.3	7.3	8.5	4.7	4.3	5.0	5.0	5.5	4.4	11.3
Serine	10.4	13.5	12.0	9.0	8.1	5.1	7.1	3.1	9.8	8.8	10.8	10.1
Glutamine	9.4	9.6	10.3	9.0	8.7	11.9	9.0	15.2	11.6	12.7	10.5	8.2
Proline	2.6	2.2	3.6	1.4	3.3	5.1	5.1	5.1	2.5	2.7	2.2	4.1
Glycine	10.0	11.6	14.1	9.0	9.5	8.8	4.8	12.7	6.8	4.4	9.2	5.6
Alanine	8.4	8.7	6.8	10.4	7.6	7.9	8.2	7.6	5.9	7.1	4.7	5.0
Cystine/2	0.2	0	0.2	0	0.3	0	0	0	0	0	0	0
Valine	7.2	9.0	6.5	7.0	6.1	6.2	9.3	3.0	6.9	8.8	5.0	6.2
Methionine	1.8	0.3	0.4	1.8	0.7	2.5	3.3	1.7	1.4	1.4	1.5	1.5
Isoleucine	4.8	5.6	2.7	5.0	5.8	3.6	5.4	1.7	3.8	4.8	2.9	3.7
Leucine	5.9	6.7	4.7	7.9	4.4	5.5	8.0	3.0	6.6	8.8	4.4	3.7
Tyrosine	2.6	1.2	3.1	1.4	4.5	5.4	4.2	6.7	6.0	7.2	4.8	6.0
Phenylalanine	3.6	1.1	5.1	2.4	5.7	2.2	2.6	1.7	4.5	3.3	5.7	5.7
Lysine	3.4	1.6	1.2	6.8	3.8	4.4	4.7	4.0	4.4	4.2	4.6	2.4
Tryptophan	Not determined											
Histidine	3.9	2.6	3.4	6.3	3.2	8.3	7.9	8.7	3.7	1.3	6.0	4.5
Arginine	2.4	1.8	2.9	2.4	2.5	3.6	3.6	3.7	6.2	4.9	7.6	6.0

[a] Unfractionated enzyme

techniques, gene expression, mapping and partial nucleotide sequence determination. Laloknum (1997) synthesized oligonucleotide probes for CGTase gene and Jantarama (1997) studied on the mutation of *Bacillus* sp. A11 for the production of higher CGTase activity.

The present study aims at purification of the catalytically active subforms (or isozymes) of CGTase from *Bacillus* sp. A11, as detected on the non-denaturing slab gel, using preparative gel elctrophoresis technique. Physical and biochemical properties such as isoelectric points, carbohydrate contents, optimum pH and temperature including product ratio (α-:β-:γ-CD) will be characterized. Difference in any of these properties may add to the additional advantage in the industrial application of cyclodextrin or in the understanding of the mechanism of cyclodextrin produced by the enzyme CGTase.