CHAPTER I

INTRODUCTION

Cyclodextrins

Cyclodextrins (CDs) are consisted of anhydroglucose units joined together by α-1,4-glycosidic bonds into a ring molecule without any reducing end. The main CDs in the nature are α -,β -, and γ-CD in which the number of glucose residues are 6,7 and 8 units respectively as shown in Figure 1 (Szejtli, 1998). There are also reports of larger CDs (9-12 glucose units) (Endo et al., 1997) but they are not as well defined or studied. Within a cyclodextrin molecule, each D-glucopyranose residue assumes the C_1 (chair) conformation. The molecular geometry of a cyclodextrin can be described as a truncated cone or as a doughnut with hydrophilic outer surface and hydrophobic inner cavity. All secondary hydroxyl groups (C_2-OH and C_3-OH) are situated on the wider end of the cavity, whereas all primary hydroxyls (C_6-OH) are situated on the narrow end. The inside cavity is lined with C-H groups and glycosidic oxygen bridges. These orientations make cyclodextrin molecules hydrophilic on the outside and hydrophobic inside (Saenger, 1979,1982; Bender, 1986) as show in Figure 2.

The order of the solubility is β-CD<α-CD<γ-CD. The differences exist because different amounts of steric strain in the ring cause different amount of interaction of the hydroxyl groups in hydrogen bonding with each other. The C_2-OH group of one glucopyranoside unit can from a hydrogen bond with the C_3-OH group of the adjacent glucopyranose unit. In the β-CD molecule, a complete secondary belt is formed by these H bonds, therefore the β-CD is a rather rigid structure. This intramolecular hydrogen bond formation is probably the explanation for the observation that β-CD has the lowest water solubility of all CDs. The hydrogen-bond belt is incomplete in the α-CD molecule, because one glucopyranose unit is in a distorted position. Consequently, instead of the six possible H-bonds, only four can be established fully. The γ-CD is a non-coplanar, more flexible structure; therefore, it is the most soluble of the three CDs.

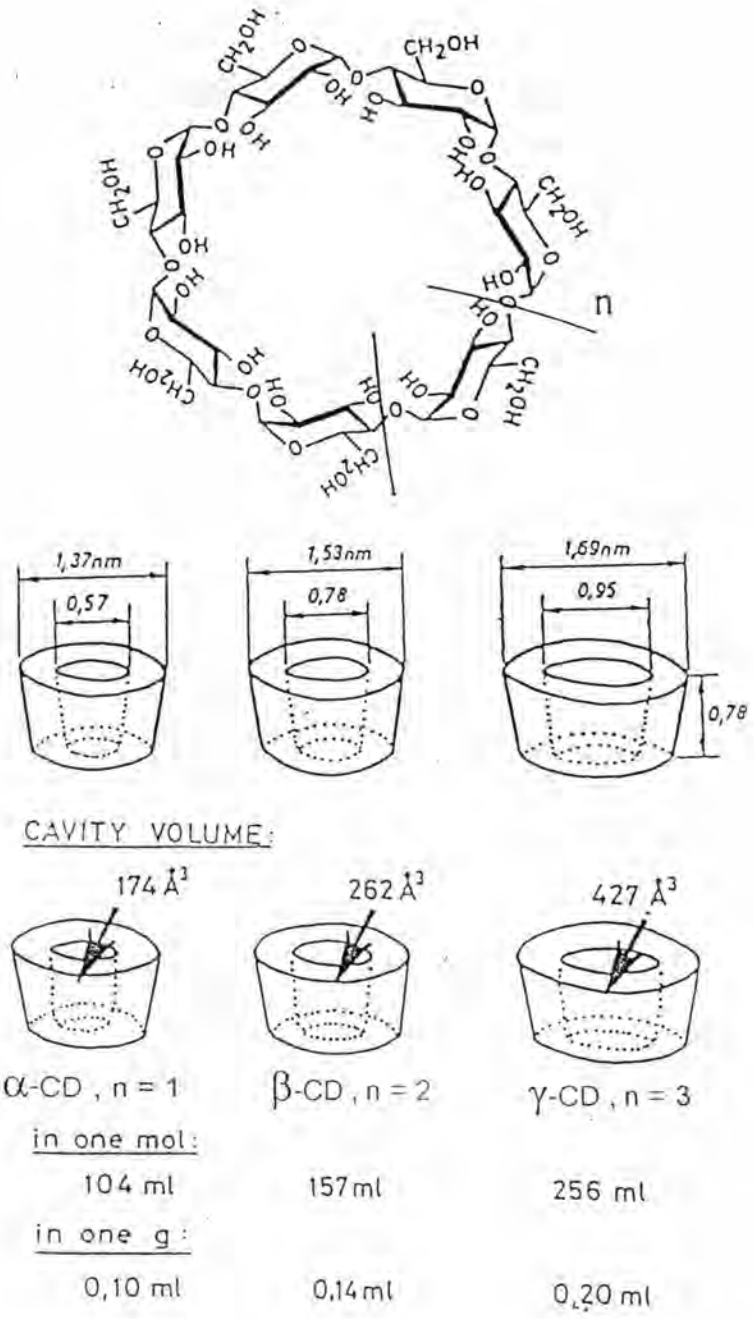
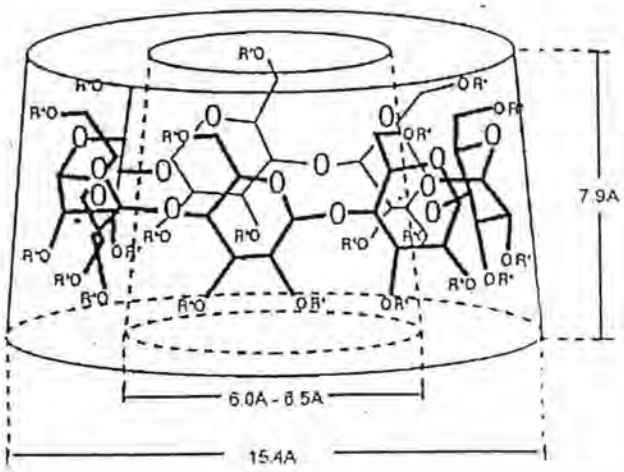
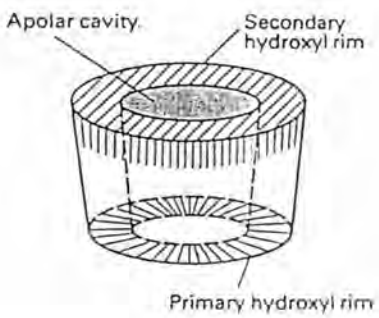


Figure 1. Structure and molecular dimension of cyclodextrins (Szejtli, 1998)



a



b

Figure 2 Structure of β-cyclodextrin chemical (Szejtli, 1990)

(a) Chemical structure

(b) Functional structure scheme

Some characteristics of cyclodextrins are summarized in Table 1.

Cyclodextrins are stable toward alkaline, even at elevated temperatures. The stability of cyclodextrins in alkaline solution is similar to cellulose. Cyclodextrins are hydrolyzed by strong acid such as hydrochloric acid or sulfuric acid (Szejtli, 1977). The thermal stability of cyclodextrins is far greater than that of common starch (Qi and Romberger, 1998). Cyclodextrins show varying degrees of resistance to hydrolysis by amylases (Saha and Zeikus, 1992). Since cyclodextrins do not have end groups, they are not hydrolyzed by glucoamylases or β-amylases, which require end groups for hydrolysis.

The physical and chemical properties of cyclodextrins are important for their ability to form complexes with various materials and hence, in the application of cyclodextrins. The most important property of inclusion complexes is that a "host" cyclodextrin can admit "guest" components into its cavity (Saenger, 1980; Bender, 1986). The host-guest 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). Guest molecules can fit entirely or at least partially into the cyclodextrin cavity in the solid state or in solution (Saenger, 1980). The potential guests which can be encapsulated in cyclodextrins are shown in Figure 3. They include straight or branched chain hydrocarbons chains, gases and some relatively polar compounds. The guest molecules must have dimensions compatible with the cavity in the CDs.

Complexation of guest molecule with a cyclodextrin can have one or more beneficial effect. They include increasing solubility of the guest, stabilization of the guest to prevent volatilization, reduction or elimination of lability due to heat or light, and prevention of chemical reactivity, and modification of liquid substances to powder. These benefits are of use in a wide variety of areas such as food, chemical, pharmaceutical, analytical, diagnostic and other industrial areas (Nagamoto, 1985). An excellent example to show the highly diverse application and of the distribution in

Table 1. Characteristics of Cyclodextrins (Szejtli, 1998)

	α-CD	β-CD	γ-CD
number of glucose unit	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100 ml at 25 °C)	14.4	1.85	23.20
Cavity dimensions			
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	427
Crystal form (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms
$[\alpha]_D$ 25 °C	150 ±0.5	162.5 ±0.5	177.4 ± 0.5
Approx. cavity volume			
in 1 mol CD (ml)	104	157	256
in 1 g CD (ml)	0.1	0.14	0.22
Melting range (°C)	255-260	255-265	240-245
Water of crystallization	10.2	13-15	8-18
Water molecules in cavity	6	11	17

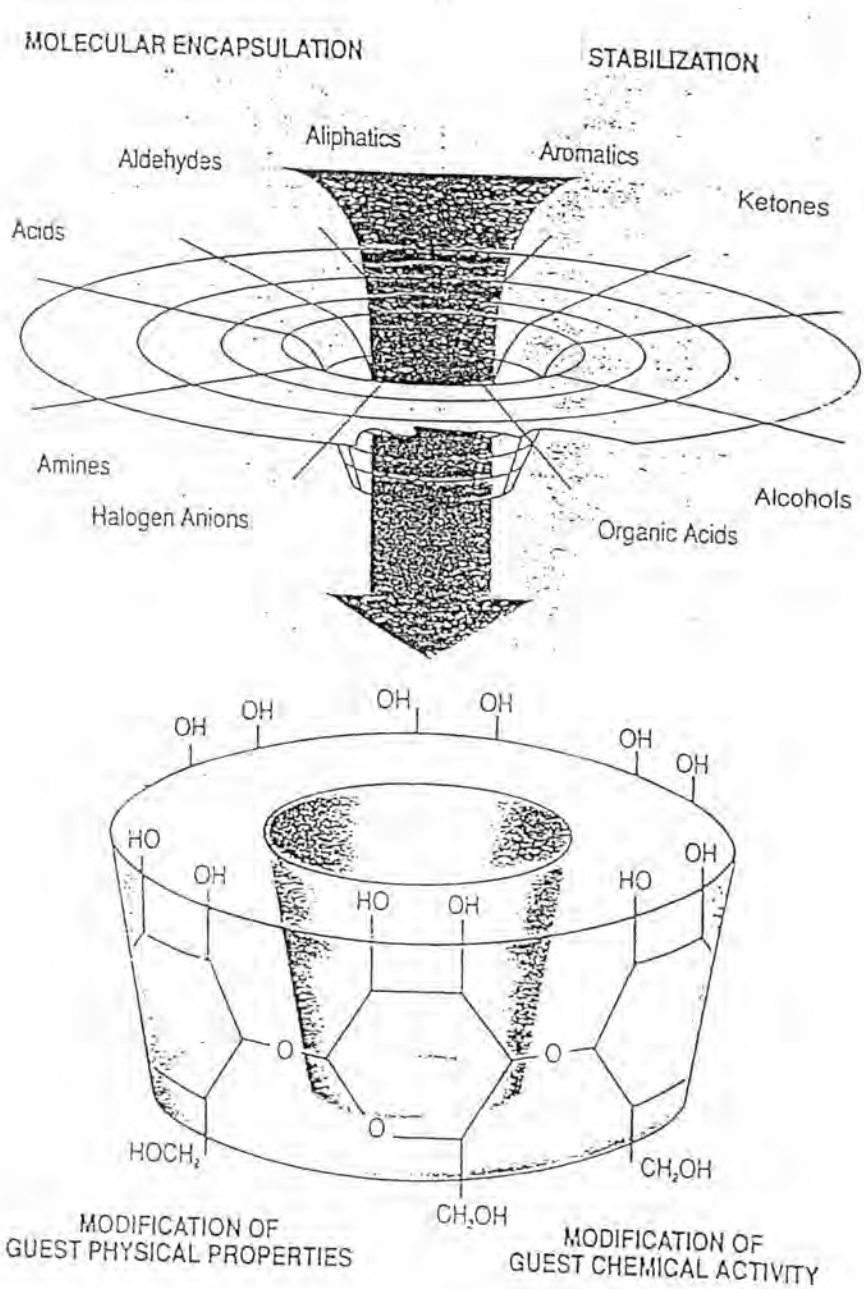


Figure 3 Inclusion complex formation between CDs and guest molecules leading to modification of guest physical and chemical properties.(Amaizo, 1993)

various sectors is shown in Figure 4 (Szejtli, 1998). The molecular inclusion complexation is the basis of increasing widespread application with cyclodextrins. The forecast for world market usage (all types of CDs) is shown in the Figure 5. (Strattan, 2000)

Cyclodextrins can be modified in order to alter their solubility behavior and complexation properties or to introduce groups with certain specific functions. Some of these are methylated, hydroxypropylated and glucosylated at the hydroxyl groups. CD-polymers (linked cyclodextrins) are often used to remove materials from aqueous sources, to release materials (Yamamoto et al., 1990), to entrap iodine from nuclear waste gas (Szente, Fenyves and Szejtli, 1999) and for chromatographic application.

Cyclodextrin production

Cyclodextrins can be produced from starch by bacterial enzyme called cyclodextrin glycosyltransferase (1,4-α-D-glucan:1,4-α-D-glucopyranosyl transferase, EC 2.4.1.19, CGTase). The enzyme is known to catalyze the degradation of starch and related α-(1-4) glucans to cyclodextrins by intramolecular transglycosylation (cyclization reaction). CGTase is produced by various microorganisms, for example, *Klebseilla pneumoniae* (Bender, 1982), *Brevibacterium* sp. (Mori et al., 1994) and mainly the *Bacillus* species, as listed in Table 2. The CGTase can be divided into three types, α -,β -, and γ-CD producing CGTase, according to the major type of CD formed (Horikoshi, 1988). The enzymes from different sources may show different characteristics, such as optimal pH, pI and molecular weight. Most CGTases produce all three kinds of CD but each CGTase enzyme yields different ratio of CDs, as listed in Table 3 (Kitahata, 1994). There was no report of CGTase origination from fungi and yeast.

CGTase catalyzes mainly three-tranglycosylation reaction: cyclization, coupling and disproportionation reactions (Kitahata and Okada, 1975). Recently, hydrolytic action was revealed by van der Veen, et al., (2000).

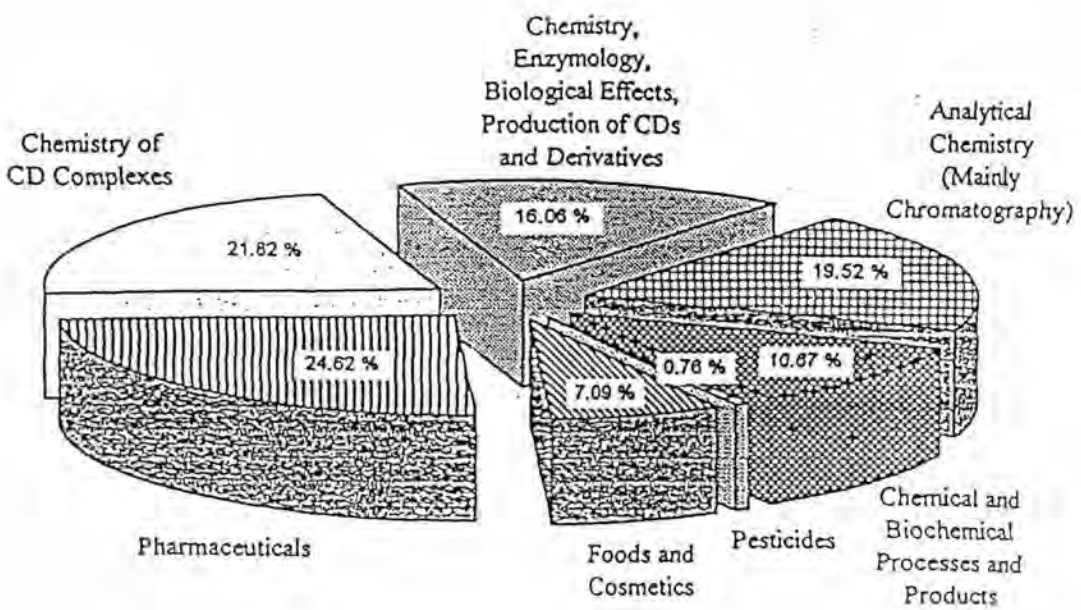


Figure 4 Distribution of the 1706 CD relevant abstracts published in 1996 by Cyclodextrin News. (Szejtli, 1998)

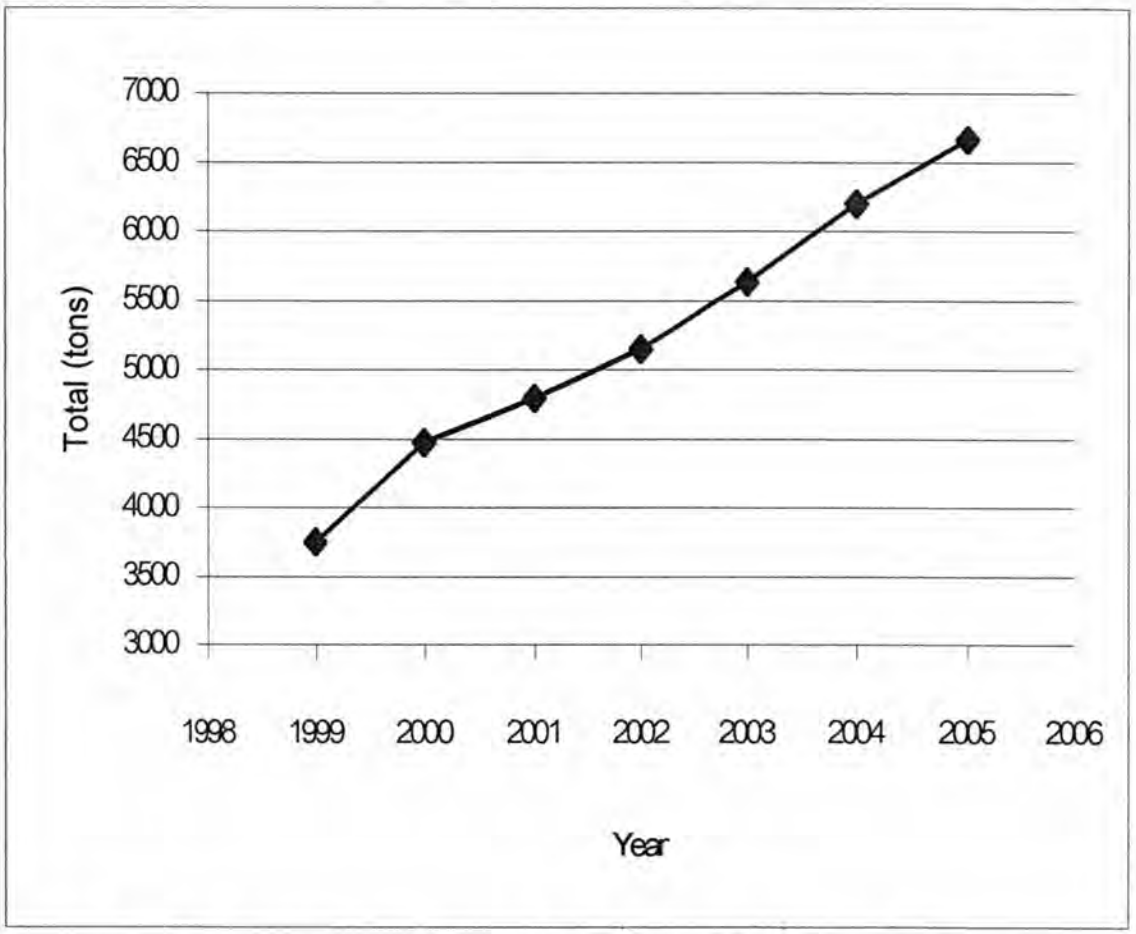


Figure 5 The world market usage of all CDs forecast (Strattan, 2000)

Table 2. Properties of cyclodextrin glycosyltransferase

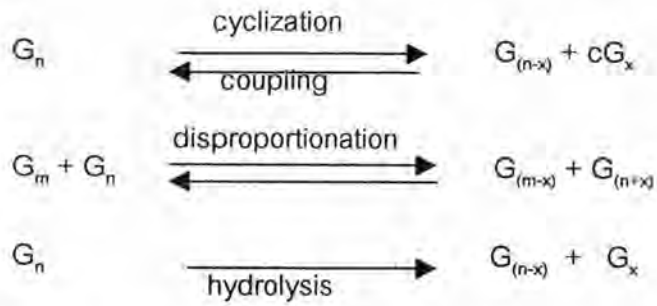
Organism	Predominant Product[a]	Optimum pH	Optimum temp(°C)	Molecular weight (dalton)	pI	Reference
Klebsiella pneumoniae M5al	α-CD	6.0-7. 2	N D	68,000	4.8	Bender, 1982
Bacillus stearothermophilus	α-CD	6. 0	N D	68,000	4.5	Kitahata and Okada, 1982
Bacillus macerans IFO3490	α-CD	5.0-5.7	55	65,000	4.6	Kitahata, and Okada, 1974
Bacillus megaterium	β-CD	5.0-5.7	55	ND	6.07	Kitahata, Tsuyama and Okada 1974
Micrococcus sp.	β-CD	5.8	55-65	88,000	4.2	Yagi, Iguchi and Juni, 1980
Bacillus circulans E192	β-CD	5.5	60	78,000	6.7-6.9	Bovetto et al., 1992
Paenibacillus sp. F8	β-CD	8.0-9.0	55	72,000	ND	Larson et al., 1998
Bacillus fermus/lentus 290-3	γ-CD	6-8	50	75,000	4.1	Englbrecht et al., 1990
Bacillus sp. AL-6	γ-CD	7.0-10.0	40	ND	ND	Fujita et al., 1990
Thermococus sp.	α-CD	5.0-5.5	90-110	83,000	ND	Tachibana et al., 1999
Bacillus stearothermophilus ET1	α-/β-CD	6.0	80	66,800	5.0	Chung et al., 1998

[a] Main CD produced in the initial phase of transfer reactions

ND, no data

Table 3. Ratio of α-, β- and γ-CD produced by various CGTases (Kitahata, 1994)

Enzyme source	α-CD : β-CD : γ-CD
Bacillus macerans	5.7 : 1.0 : 0.4
Bacillus megaterium	1.0 : 6.3 : 1.3
Bacillus circulans	1.0 : 6.4 : 1.4
Bacillus stearothermophilus	1.7 : 1.0 : 0.3
Klebsiella oxytoca	α-CD > β-CD > γ-CD
Bacillus sp. (alkalophilic)	
Acid	β-CD > α-CD, γ-CD
Neutral	β-CD > α-CD, γ-CD
Bacillus ohbensis	0 : 5.0 : 1.0
Bacillus sp. AL-6	0 : 1.0 : 2.7
Bacillus subtilis 313	γ-CD only
Bacillus firmus 290-3	γ-CD ≥ β-CD
Thermoanaerobacter sp.	β-CD > α-CD ≥ γ-CD
Brevibacterium sp.	0 : 6.5 : 16
Bacillus autolyticus	2.0 : 6.0 : 1.0
Bacillus coagulans	1.0 : 0.9 : 0.3



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 the symbol for CD. The rate of these reactions differs with the type of CGTase, kind of substrate, and condition of incubation.

The cyclization reaction is an intramolecular transglycosylation reaction, in which a linear oligosaccharide (α-1,4 glycosidic bond) chain is cleaved and the new reducing end sugar is transferred to the non-reducing end of another sugar residue of the same chain forming a cyclic compound. Coupling is the reverse reaction of cyclization. It involves cleavage of an α-1,4 glycosidic bond in the cyclodextrin ring and transfer of the resulting linear maltooligosaccharide to an acceptor substrate (such as sugar or linear oligosaccharides) resulting on the formation of a linear, longer oligosaccharide molecule. Disproportionation is the cleavage of an α-1,4 glycosidic bond of a linear maltooligosaccharides and a part of the linear maltooligosaccharides chain is transferred to another linear maltooligosaccharides chains. This reaction yields a mixture of smaller and longer maltooligosaccharides. Hydrolysis reaction is the cleavage of an α-1,4 glycosidic bond of a linear maltooligosaccharides and a part of the linear maltooligosaccharides chain is transferred to a water molecule, resulting in hydrolysis of the maltooligosaccharides. Only the hydrolysis reaction results in an increased number of reducing ends. (Figure 6) (van der Veen et al., 2000).

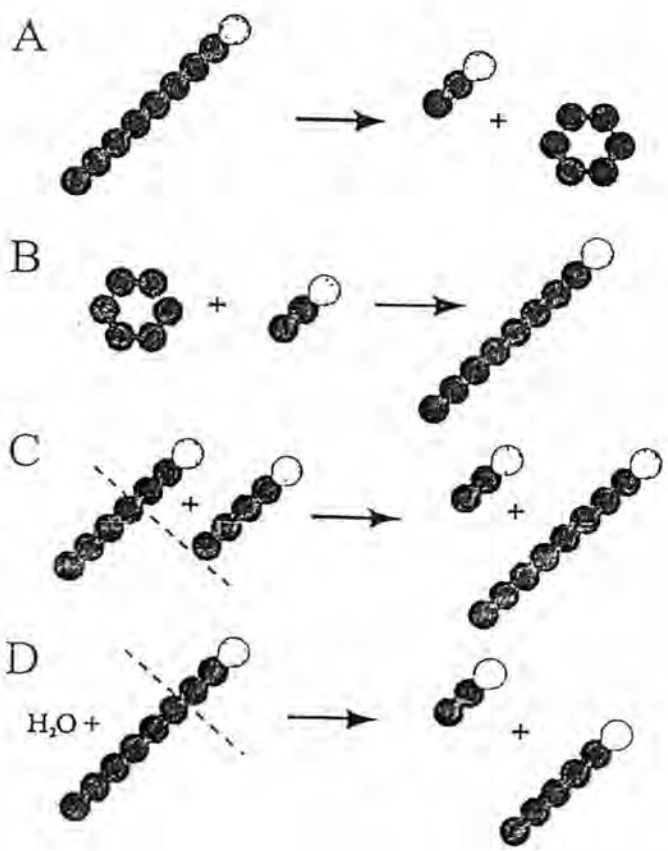


Figure 6. Schematic reprentation of the CGTase-catalyzed tranglycosylation reaction.
(A) Cyclization (B) Coupling (C) Disproportionation (D) Hydrolysis
● = glucose residues, O = reducing end sugar (van der Veen et al., 2000)

CGTase active site could be studied with the aid of linear oligosaccharides substrate bound at the active site. Figure 7 is derived from the x-ray crystallographic structure of the enzyme complexed with a maltononaose inhibitor (van der Veen et al., 2000). For this complex, either a cyclization or a disproportionation reaction can occur. In both cases the oligosaccharide chain is cleaved between subsites –1 and +1, and the glucose residue at subsite –1 is covalently linked to Asp229. Subsequently, the residues bound at subsites+1 and +2 have to make space for the non reducing end of the covalently linked maltoheptaose (cyclization), or an incoming acceptor substrate (disproportionation).

For cyclization, the non-reducing end of the chain serves as the acceptor, whereby the helical conformation of the substrate may be a prerequisite for the reaction (Bender, 1980). The cyclization and the disproportionation occur simultaneously with the same substrates suitable for cyclization. Cyclization can occur only if the acceptor binding-site is empty. (Bender, 1985). Disproportionation and coupling reactions need two oligosaccharides for reactions. The maximum cyclization rates are obtained with maltooligosaccharides having degree of polymerization (DP) between 16 and 80 (Bender, 1980). At high concentration of short maltooligosaccharides and sugar (such as α-methylglucosides, sucrose, cellobiose, maltobionic acid) they can serve as acceptor for the coupling reaction (Kitahata, Okada and Fukui, 1978). If the chain length is greater than 100 units, disproportionation reaction dominates. However, when the oligosaccharides are smaller than the binding site of CGTase, the oligosaccharides can be disproportionated in order to produce oligosaccharides having higher chain length suitable for cyclization (Bender, 1988).

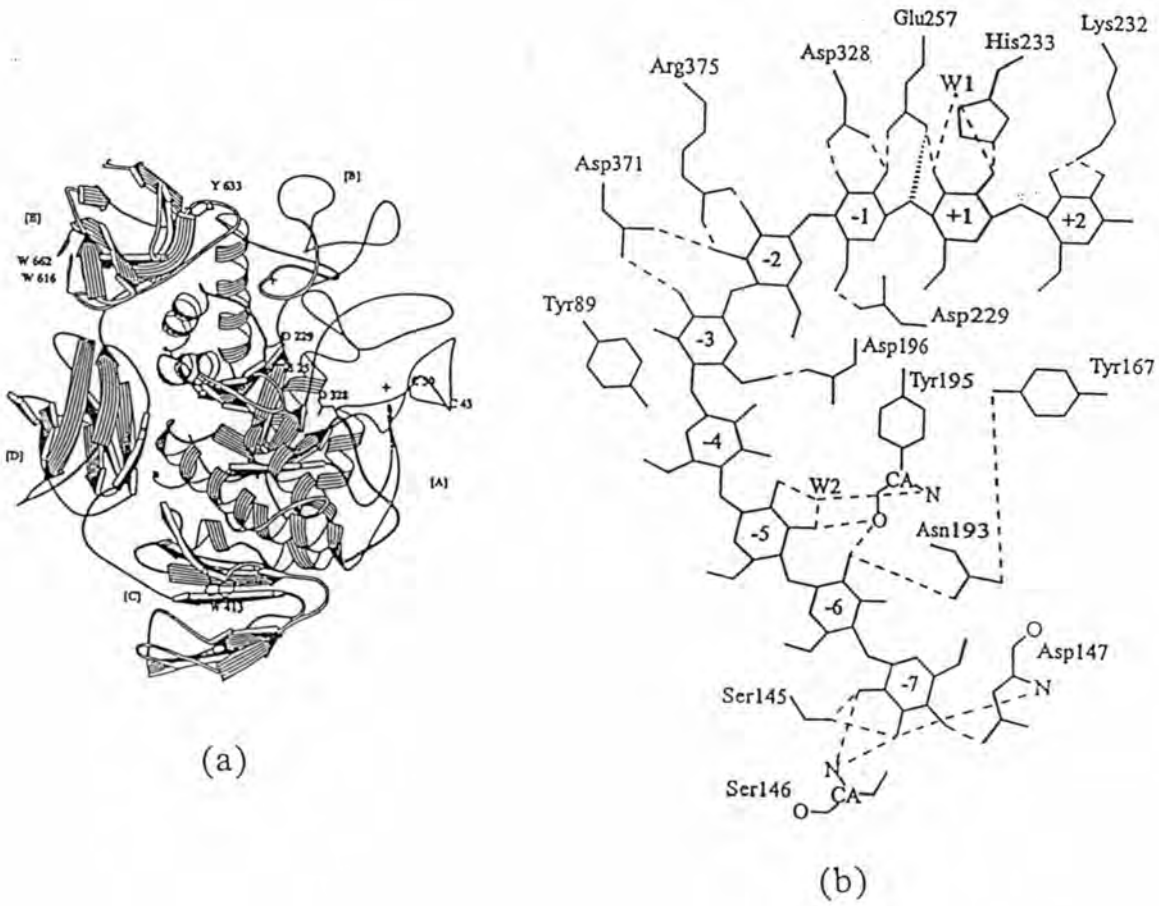


Figure 7. CGTase from *Bacillus circulans* 251

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

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

Although cyclodextrins have great potential use in industry, there are limitations because of the high cost of production. Many researchers attempted to reduce the cost on the production of cyclodexrins by several means such as screening for microorganisms which produce high cyclodextrin yield, modification of enzyme by genetic engineering (Fujiwara et al., 1992) or protein engineering (Pariegla, Schimidt and Schulz, 1998) to increase the yield of enzyme product, to produce specific type of CDs, or to improve stability of CGTase at extreme temperature or pH.

Production of cyclodextrins from starch generally consists of the following steps: 1) liquifaction (prehydrolysis of starch to maltodextrins) 2) enzymatic conversion of the maltodextrins to a mixture of cyclic and linear dextrins by CGTase 3) separation and purification of a cyclodextrin product from the reaction mixture. Each of these steps can affect the yield and type of CD produced.

In industry, starch concentration of 15-30g% is used (Bender, 1986) as starting material. Stirring of the suspension will more difficult and energy consuming with higher starch concentration due to increase viscosity. Therefore, starch is firstly modified by treatment with α-amylase (Horikushi, 1979) or thermostable CGTase (Horikoshi et al., 1982) to make more appropriate substrate for the enzyme and for economical purpose. Other enzymes such as isoamylase or pullulanase are added in simultaneous digestion of starch with CGTase (Nakamura and Horikoshi, 1977; Rendleman, 1997). Other pretreatments of starch for cyclodextrin production include sonication (Hitoshi et al., 1985), extrusion (Yashida, Nobuhiro and Kazumasa, 1989), milling (Lee and Kim, 1991).

Several methods for the production also exist, such as the use of immobilized CGTase or ultrafiltration process for continuous production. Membrane technology such as ultrafiltration membrane and reverse osmosis membrane were employed to increase the yield by reducing product inhibition (Hohse, Kaper and Wijpkema, 1984; Hashimoto et al., 1986). One of the methods to eliminate short oligosaccharides during

cyclodextrins production is by adding *Saccharomyces cerevisiae* together with CGTase in the bioreactor (Lima et al., 1998). Fermentation by *Saccharomyces cerevisiae* eliminates glucose and moltose while at the same time produces ethanol that complexes with CDs in the reactor mixture. Techniques of enzyme immobilization has been developed and investigated for the preparation of immobilized CGTase (Nakamura and Horokoshi, 1977; Kato and Horikoshi, 1984).

The distribution of α-, β- and γ-CD is highly dependent on the source of CGTase. Product distribution may be varied by the addition of specific precipitants. By addition of water-immiscible or miscible solvents known as complexing agent to a cyclization reaction, the equilibrium of enzymatic conversion can be shifted in favor of cyclodextrin production (French et al., 1949). Some well-known complexing agents are trichloroethylene, cyclohexane, toluene, bromobenzene, and long-chain aliphatic alcohol (Armbruster, 1988). For example, production cyclodextrin yield is 2 times in the presence of 10%(v/v) ethanol in the incubation mixture (Lee and Kim, 1991). In addition, long-chain ($>C_6$) primary aliphatic alcohols are known to form nearly insoluble inclusion complexes with cyclodextrins, especially with α-CD. 1-Decanol or n-decyl alcohol (FDA-approved, permitted by Health Council of Europe up to 5 ppm) was selected as the complexant for α-CD production (Flashel, Landert, Renken, 1982).

Normally, the enzymatic yield is a mixture of CDs products, linear oligosaccharides, and remaining starch. For purification of CDs, the linear oligosaccharides are digested by using amylases/glucoamylases (Horikoshi, 1975) or removal of CDs by precipitation with organic solvents such as methanol or ethanol. Final purification of CDs is performed by precipitation as insoluble clathrates, by chromatographic methods, and by crystallization (Bender, 1986).

Starch

Starch can be considered as condensation polymer of glucose, consisting of anhydroglucose units. The major components of starch are composed of amylose and amylopectin. Starches of different origin have different amylose to amylopectin ratios (Table 4). Amylose is the smaller of the two fractions (10^5 –10^6 Da; DP 500-5000) and possesses very few branches, 9-20 per molecule, with chain lengths of 4 to >100 glucose units. Amylopectin, the larger (10^7 –10^9 Da) fraction, is highly branched; 5% of its structure is α-1,6 branch links. Current models for amylopectin fine structure suggest two populations of chains A- and B-chains, which are present in almost equal proportions. A-chains are unbranched and attached to the molecule by a single linkage, whereas B-chains are branched and connected to two or more other chains. Each amylopectin molecule will also possess a single C-chain, which contains the sole reducing group (Oates, 1997).

Native granules have semi-crystalline structures (Figure 8). Granules contain between 15-45% crystalline material and yield X-ray diffraction patterns that correspond to one of two limiting polymers (A or B) or an intermediate form (C). Crystallinity occurs within the ordered arrays of amylopectin and is created by intertwining of chains with length greater than 10 glucose units forming a double helix. Double helices are packed in unit cells. The granule structure is due to amylopectin and based on a super helical structure in which the double helices wind around each other to from a super-helix. Amylose is arranged loosely within this structure (Oates, 1997).

Cassava starch

Cassava (*Manihot esculanta* Crantz) is a starch containing tuber like potato and is the most important food crop in tropical and sub-tropical regions of the world. Cassava tubers have entered the modern economic market and they are important

Table 4 Percent of amylose and amylopectin in various starch (Young, 1984)

Starch	Amylose(%)	Amylopectin(%)
Rice	18.5	81.5
Waxy	0	100
Wheat	28	72
Barley	22	78
Waxy	0	100
Oat	27	73
Corn (Zea mays)	28	72
Waxy	0.8	99.2
Tapioca	16.7	83.3
Potato	20	80
Sweet potato	17.8	82.2
Pea		
Smooth	35	65
Wrinkled	66	34

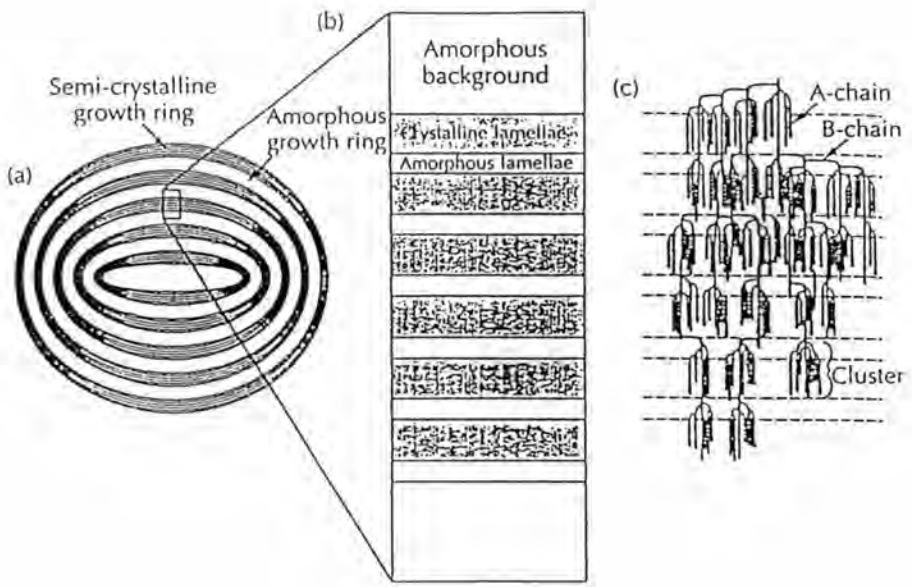


Figure 8 Schematic diagram of the structure of a starch granule. (a), Single granule comprising concentric rings, each containing stacks of amorphous and crystalline lamellae; (b), amorphous and crystalline lamellae; (c), chains of amylopectin arranged in a cluster structure. (Oates, 1997)

source of processed foods and feed products. Cassava starches and starch derivatives such as dextrin, glucose and high fructose syrup are the main products of the cassava agro industry. Cassava starch is used not only for the production of starch derivatives and food products but also for sizing paper and textiles and in the manufacture of adhesives and alcohol (Kay, 1987).

The starch granules are primarily composed of amylose and amylopectin. The amylose content of cassava starch ranges between 13.6 - 23.8% (Richard, Asaokao and Blanshard, 1991).

The native cassava starch granules are considerably irregular in shape with spherical, cap-shaped and truncated egg shaped and the ranges of granule size are 5-22 μm (Sriroth et al., 1999; Shonnon and Garwood 1984). The granule size of cassava starch in relation to the age of different cultivars of cassava starch has been studied by Moorthy and Ramanujam (1986) and Sriroth et al., (1999). The swelling power and %solubility are 71% and 48% (Leach, Macowen and Schoch, 1959). The range of pasting temperature of 6% cassava starch from our experiment is 69 –71 ^{o}C (Sriroth et al., 1999).

Cassava starch has good substrate potential for CGTase due to the high purity of starch and starch content. Bender (1984) reported that when 2 g% cassava starch was incubated with CGTase from *Klebsiella pneamoniae* M 5al for 48 hours in the presence of bromobenzene, the total cyclodextrin yield was 59.4% and the ratio of α: β: γ -CD was 1:10.6:3.9. Raja et al., (1990) demonstrated that five gram percent of cassava starch gave a yield of 8-10% β-CD in the absence of complexant. Adding bromobenzene, the yield of β-CD could be increased up to 25% (Raja and Ramakrishna, 1994). In addition, Bertolini, Cereda and Chuzel (1998) also reported a conversion of 46% by CGTase from *Bacillus macerans* using cassava starch as substrate.

One disadvantage of cassava for food consumption is that cassava contains cyanogenic glycosides on different level. However, the process for cassava starch production include washing, peeling, rasping, chipping, grating, crushing, milling and drying. It was discovered that through the process the cyanogenic glycosides from 91-1515 mg/kg fresh root to 0.0-11.3 mg/kg (O'Brien et al., 1992). The explanation is that during crushing, linamarase that is located in the cortex of the root are released and hydrolyzed linamarin to acetone and hydrogen cyanide. Hydrogen cyanide is heat labile and water-soluble. The fermentation or drying step can reduce cyanogenic glycoside level (Kemdirim et al., 1995).

Objective of the thesis

Cassava is one of the important food crops in the tropics, with global production now estimate 164 million ton per annum and is produced in Thailand about 20 million ton per annum (FAO, 1998). Approximately 70% of the cassava tubers are used in the processed cassava industries for cassava fibers and pellets (2.2 baht/kg), while the remaining 30% is used for flour and starch production (5 baht/kg). Mainly cassava pellet is used and exported for feed product. The price in the world market is remarkably low. Furthermore, European Union has set a quota per year for Thai cassava pellets. At present, the cost of cassava starch is $0.15/kg whereas the prices of CDs in bulk quantities are $70-180/kg for α-CD, $5-10/kg for β-CD, $99-900/kg for γ-CD and $12-495/kg for hydroxypropyl-β-CD) (Strattan, 2000). From these figures, to convert cassava starch into cyclodextrins could increase the value of cassava starch 33 to 6000 folds. Furthermore, local production of CD could promote the use of CD in many industrial sectors in Thailand and neighboring countries, which ultimately will lead to improve products which are more competitive in the world market.

The CGTase used in this experiment obtained from *Bacillus circulans* A11. The microorganism was screened from South-East Asian soil (Pongsawasdi and Yagisawa, 1987) and the enzyme is a β-CGTase. The present study aims to study and optimize the condition for the production of cyclodextrins from local cassava starch. The scope of the

study includes enhancement of cyclodextrin production by liquefaction of cassava starch with amylolytic enzymes; using complexing molecules; and to study the size of cassava hydrolysate suitable for cyclodextrin production.