CHAPTER I

INTRODUCTION

1.1 Cyclodextrins (CD)

1.1.1 Structure and properties of CD

Cyclodextrins (Schardinger dextrins, Cycloamylose, Cyclomaltose or Cycloglucans) are the oligomers of anhydroglucose units join to form a ring structure with α–1,4 glycosidic bonds. The main CDs synthesized naturally by the cyclodextrin glycosyltransferase (CGTase) are composed of 6, 7 and 8 glucose units called α–(alpha), β-(beta) and γ–(gamma) cyclodextrin, respectively as shown in Figure 1 (Pulley and French, 1996). They have different physical properties as summarized in Table 1 (Saenger, 1982; Szejtli, T. 1988). The CDs are water-soluble. As a consequence of the 4C1 conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. The ring in reality, is a cylinder, or better said a conical cylinder, which is frequently characterized as a doughnut or wreath-shaped truncated cone. The hydrogen atoms and the glycosidic oxygen bridges line the cavity (Figure 2). The non-bonding electron pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity producing a high electron density there and lending to it some Lewis base characteristics.

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The C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the 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 glucogyranose unit is in a distorted

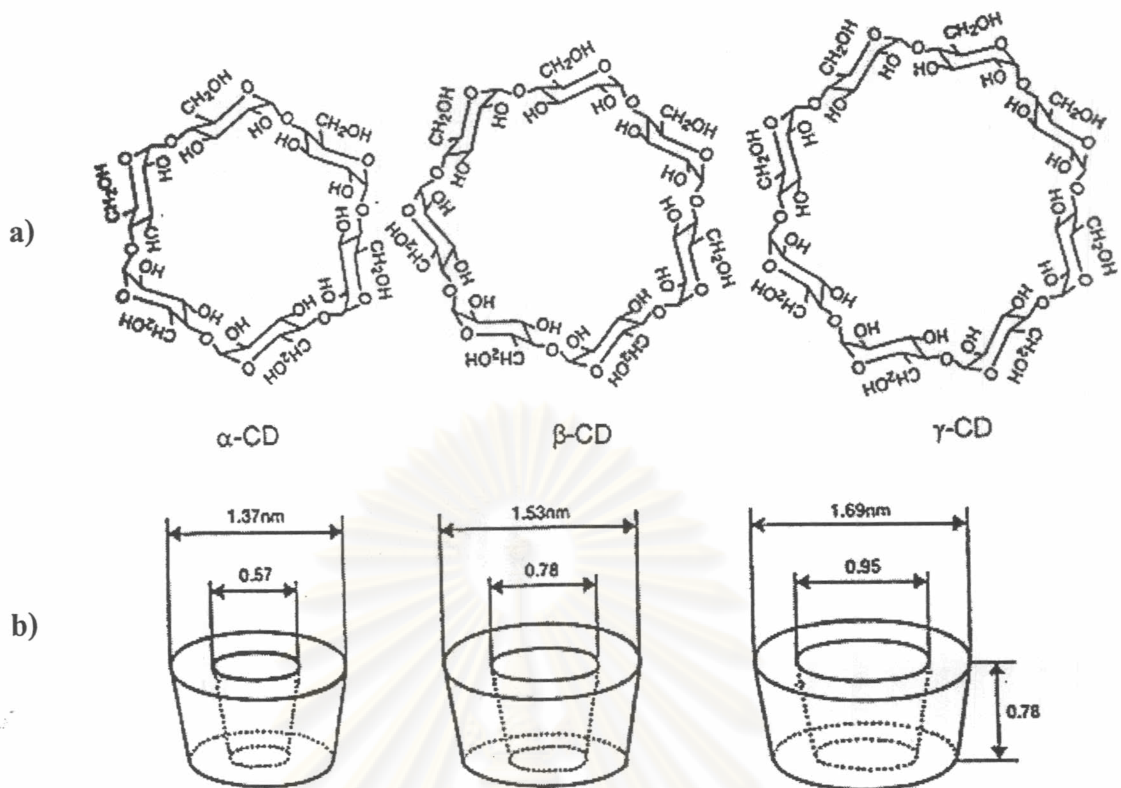

Figure 1 (a) Chemical structure of three kinds of CDs

(b) The molecular dimension structure of CDs (Szejtli, 1990)

Table 1 Approximate geometric dimensions and properties of α-, β-, and γ–CD molecules (Szejtli, 1998)

	α-CD	β-CD	γ-CD
Number of glucopyranose units	6	7	8
Molecular weight (g/mole)	972	1,135	1,297
Solubility in water at 25°C (%w/v)	14.5	1.85	23.2
Outer diameter (°A)	14.6	15.4	17.5
Inner diameter (°A)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (°A^3)	7.9	7.9	7.9
Approx. cavity volume 1 mol CD (ml)	174	262	427

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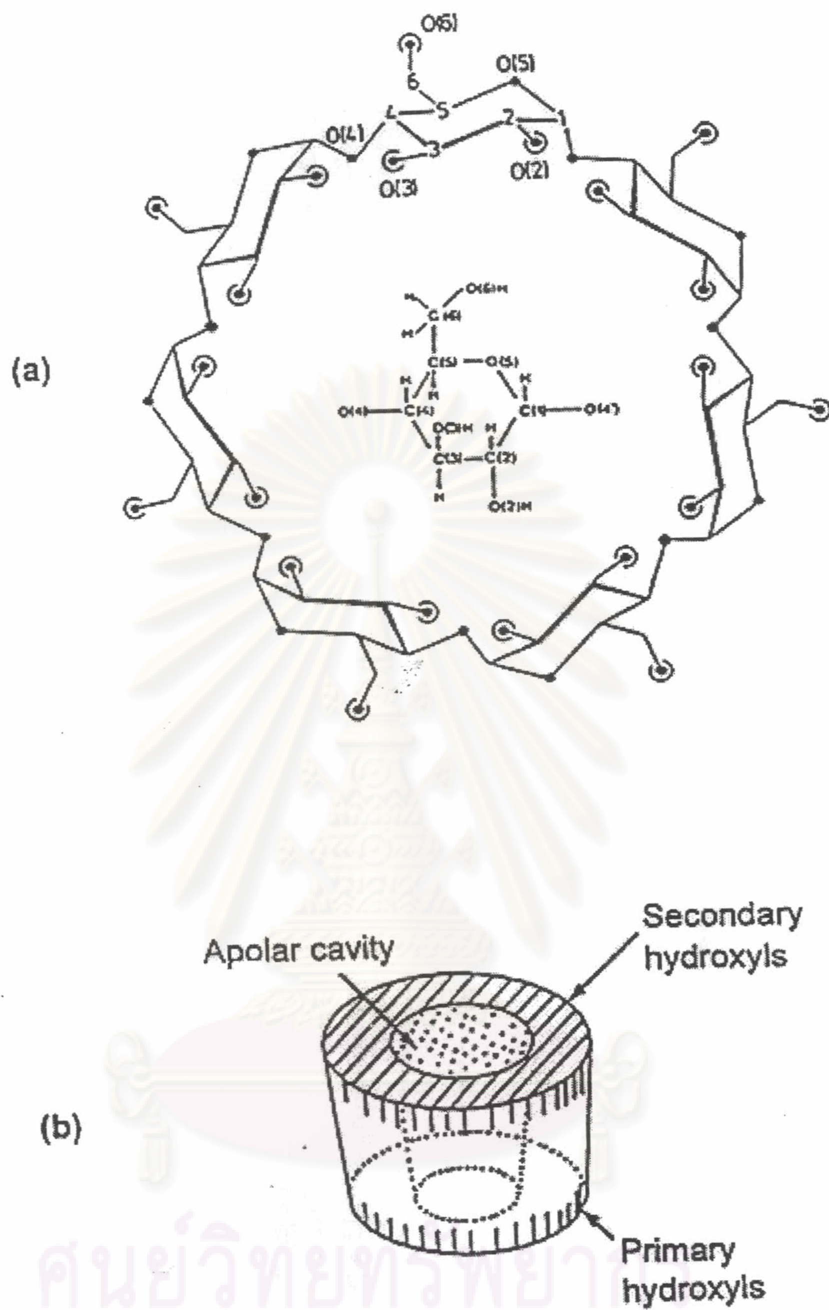

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Figure 2 Structure of β-cyclodextrin (Bender, 1986; Szejtli, 1990)

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

(b) Functional structure scheme

position. Consequently, instead of the six possible H-bonds, only four can be established fully. The γ-CD is a noncoplanar, more flexible structure; therefore, it is the most soluble of the three CDs. The doughnut shaped molecules have been investigated with the use of spectroscopic, kinetic, and crystallographic methods. X-ray crystal structure analysis of the larger δ-CD (composed 9 glucose units) was reported. Beyond that, the crystal structure of two larger members of the cyclodextrins have recently been published, namely that with 10 glucoses (ε-CD) and that with 14 glucoses in the ring (ι-CD). The crystal structure of an even larger cyclodextrin with 26 glucoses has been obtained. Whereas larger cyclodextrins with more than 100 glucoses in the ring and beyond have been prepared by the action of disproportionating enzyme on amylose, smaller molecules would be strically strained and are therefore not produced by the glucosyltransferase enzymes (Saenger *et al.*, 1998).

Several cyclodextrin derivatives have been developed through chemical or enzymatic means in order to obtain CDs with specific desirable properties. Examples are those with solubility better than parent compounds e.g. methylated, hydroxylpropylated, and maltosyl-cyclodextrins (substitution of the hydroxyl groups by methyl, hydroxypropyl, and maltosyl residues, respectively). Intensive research is expected in the area of chemical and enzymatic modification of CDs. Considering that CDs contain 18 (α-CD), 21 (β-CD) or 24 (γ-CD) substitutable hydroxyl groups, the number of possible derivative is unlimited. By 1997, the syntheses of more than 1500 derivatives have been published. The CD duplex homo- or heterodimers of CDs (constructed from identical or two different CDs) form complexes that are more stable by orders of magnitude than the single CD (Szejtli, 1998).

1.1.2 CD inclusion complexes

The glucose residues in the cyclodextrin rings are arranged in such manner that the secondary hydroxyl-groups (O2 and O3) are located on one edge of the ring and

the primary hydroxyl-groups (O6) on the other edge, resulting in torus shaped molecules. The apolar O3 and O5 hydrogens and ether-like oxygens are at the inside and the hydroxyl-groups at the outside of these molecules. This results in a molecule with a hydrophilic outside, which can dissolve in water, and an apolar cavity, which provide a hydrophobic matrix, enabling cyclodextrins to form "inclusion complexes" with a wide variety of hydrophobic "guest" molecules (Figure 3). Thus specific (α-, β-, or γ-)cyclodextrins are required for complexation of specific guest molecules. The dissolved cyclodextrin is the "host" molecule, and the "driving force" of the complex formation is the substitution of the high enthalpy water molecules by an appropriate guest molecule (Szejtli, 1998). One, two, or three cyclodextrin molecules contain one or more entrapped guest molecules. Most frequently the host : guest ratio is 1 : 1. This is the essence of "molecular encapsulation". The formed inclusion complexes can be isolated as stable crystalline substances. Upon dissolving these complexes, an equilibrium is established between dissociated and associated species, and this is expressed by the complex stability constant K_a. The association of the CD and guest (G) molecules, and the dissociation of the formed CD/guest complex is governed by a thermodynamic equilibrium.

$$CD + G \longleftrightarrow CD \bullet G$$

$$K_{1:1} = \frac{[CD \bullet G]}{[CD][G]}$$

Complexes can be formed either in solution or in the crystalline state and while water is typically the solvent of choice, inclusion complexation can be accomplished in co-solvent systems and with some non-aqueous solvents (Amaizo, 1993). Cyclodextrins are used to obtain certain benefits that result from complexation with the cyclodextrins. These include alteration of the solubility of the guest compound, stabilization against the effects of light, heat, and oxidation, masking of unwanted physiological effects, reduction of volatility, and others (van der Veen, 2000) (Table 2). Inclusion in cyclodextrins can markedly improve the chemical and physical of

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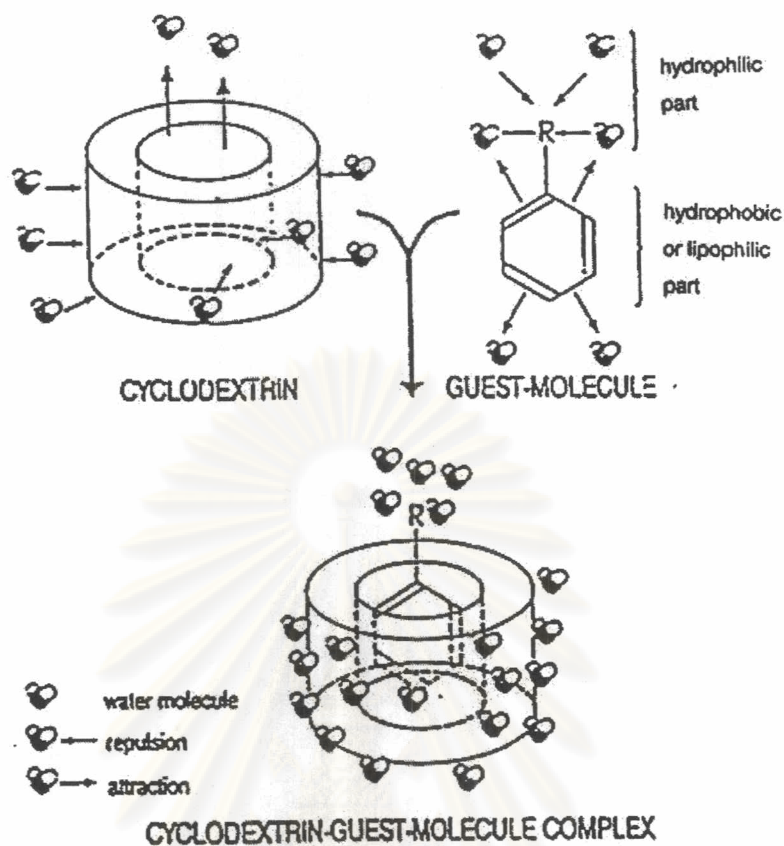

Figure 3 Guest orientation in CD-guest complex (Janssen, 1992)

Table 2 Possible effect of the formation of inclusion complexes on properties of guest molecules (van der Veen, 2000)

Stabilization of light- or oxygen-sensitive compounds
Stabilization of volatile compounds
Alteration of chemical reactivity
Improvement of solubility
Improvement of smell and taste
Modification of liquid compounds to powders

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properties guest molecules as they are temporarily docked or caged within the host cavity, giving rise to the following beneficial modifications of guest molecules (Figure 4). Table 3 lists selected products containing cyclodextrins. The applicationsof cyclodextrins have been increased rapidly in food, cosmetics, pharmaceutical, agrochemical, and plastic industries.

Cyclodextrins generally used in complex formation with industrial benefit are β-CD and their derivatives; hydroxypropyl-, methylated-, carboxymethyl-, and triacetyl-β-CD (Hedges, 1998). Large ringed cyclodextrins are inapplicable for various industrial used due to instability, they may have some unique characters in comparison with the conventional cyclodextrins. Elucidation of their structures and physicochemical properties may provide much information on a basic knowledge and development of oligosaccharides (Tomohoro *et al*, 1994).

1.1.3 Production Process of CD

In general, two different types of CD production processes can be disintiguished (Biwer *et al.*, 2002): In "Solvent Processes" an organic complexing agent precipitates one type of CD selectively and as such directs the enzyme reaction to produce mainly this type of CD. In the "Non-Solvent Processes" no complexing agent is added and therefore a mixture of different CDs is formed (Figure 5a and 5b, respectively). The ratio of CDs produced depends on the CGTase used and on the reaction conditions. Efficient production process using whole cell biotransformation are not known so far.

1.2 Cyclodextrin producing enzyme

1.2.1 Starch degrading enzymes

Many (micro) organisms capable of using starch as carbon and energy source are found in nature. Starch degradation, like starch synthesis, requires a whole range of

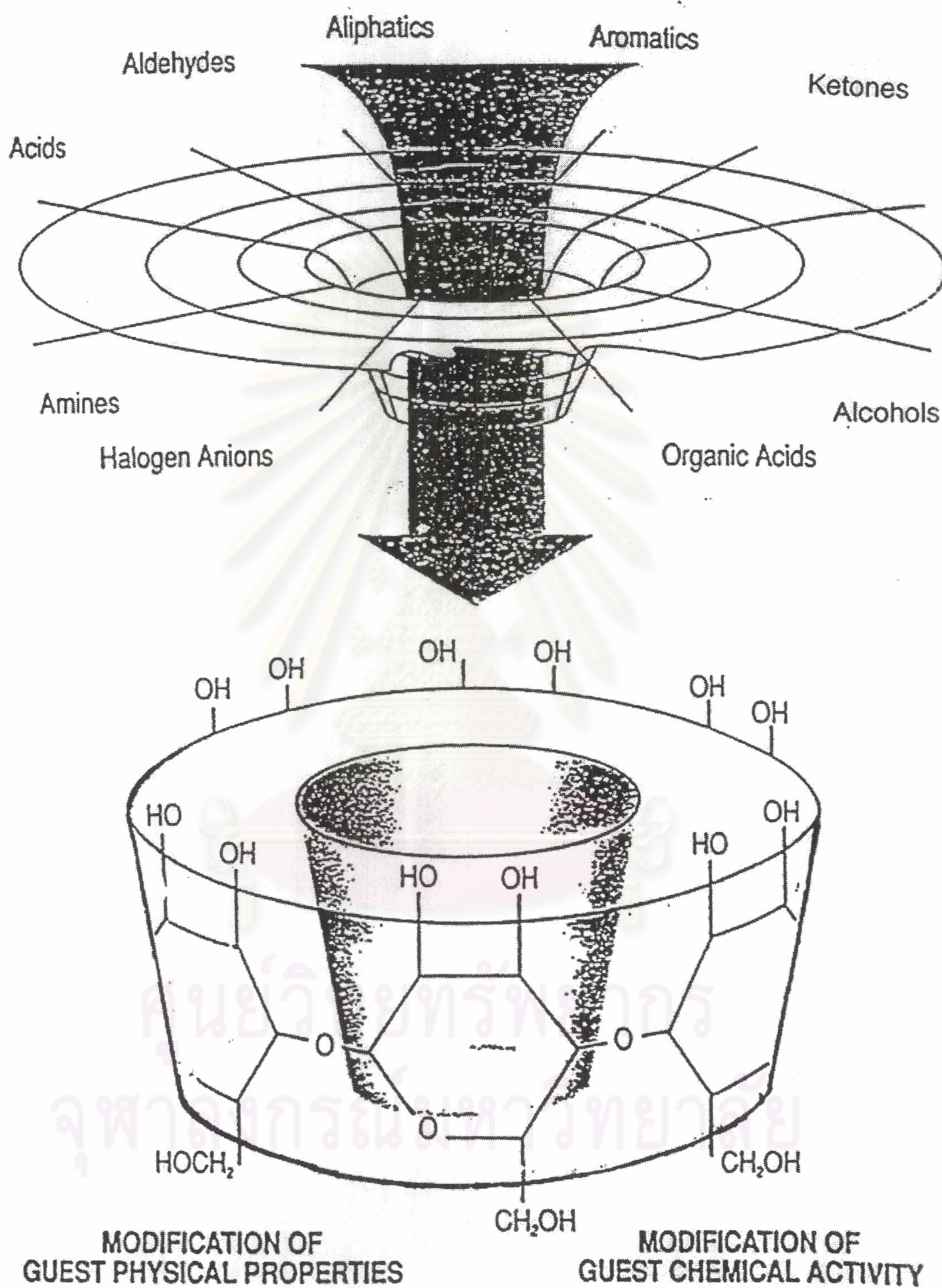

Figure 4 Beneficial modification of guest molecules by cyclodextrin (Amaizo, 1993)

Table 3 Selected applications of cyclodextrins (Hedges, 1998)

Industrial	Use	Industrial	Use
Food		**Pharmaceutical**	
Cinnamon-flavored apples	Stabilize flavor	Itraconazole	Increase solubility
Mint and green tea mints	Stabilize flavor	Piroxicam	Reduce irritation
Peppermint-flavor chewing gum	Flavor delivery	Garlic oil	Mask order
Mustard oil steak sauce	Improve solubility	Hydrocortisone	Increase solubility
Acetic acid	Convert to a powder	PGE_1	Increase stability
Aloe-containing beverage	Mask bitterness		
Water purifier	Absorb order	**Cosmetics and Personal care items**	
Lemon-flavored sugar	Flavor stabilization	Skin cleanser	Tocopherol carrier
		Artificial tanning Lotion	Stability, mask order
Miscellaneous		Powdered hair bleach	Stability
Laundry drier sheet	Fragrance control	Perfume	Prolonged release
Chromatography column	Separations	Cold cream	Solubility

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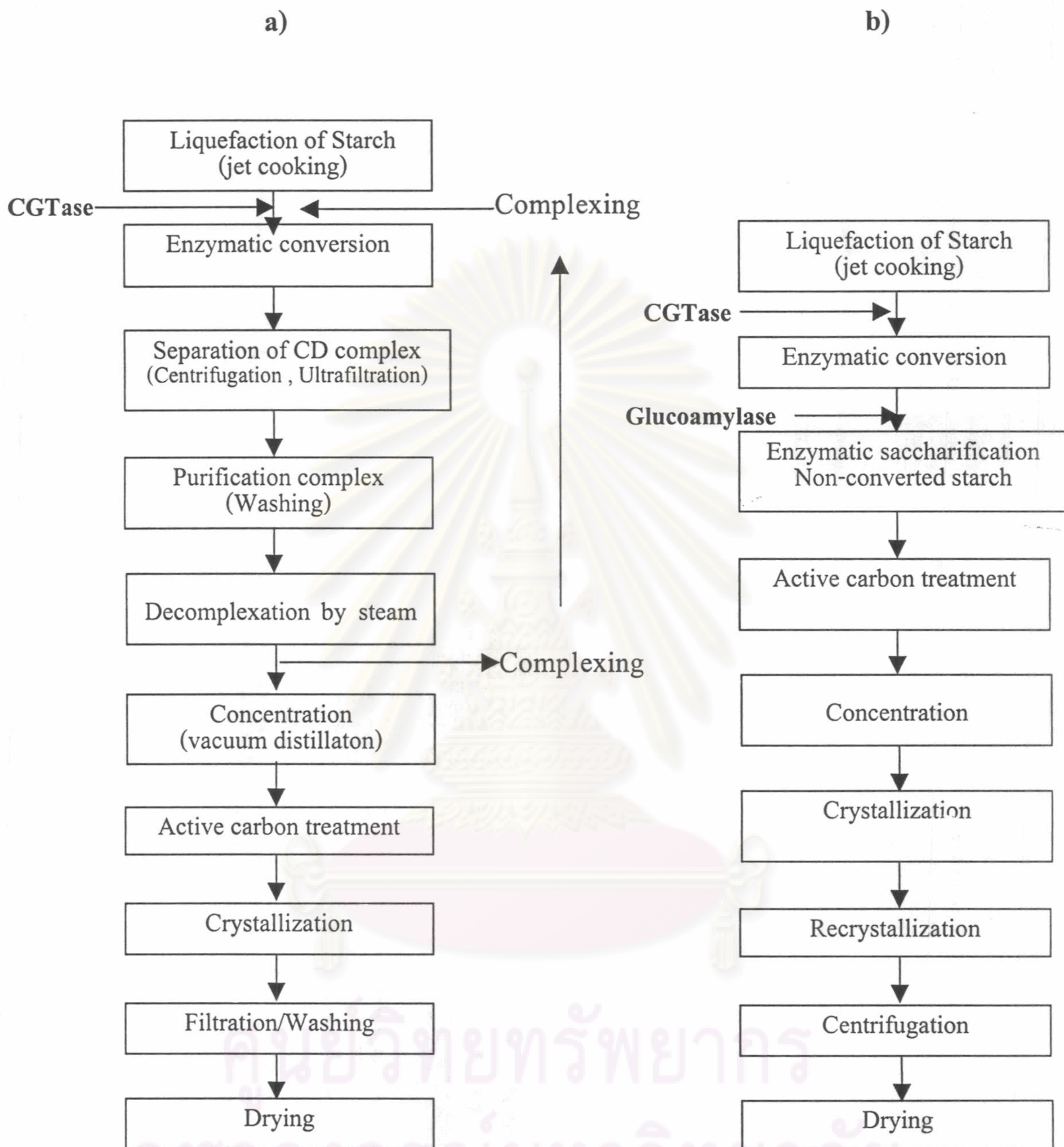

Figure 5 a) Solvent process and b) Non-Solvent process for cyclodextrin production. (here for β–CD production) (Schmid, 1996)

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enzymes. In order for organinsms to use the glucose of the starch granule as a growth substrate, the starch molecules need to be converted extracellularly into molecules suitable for uptake and further conversion by the cells. A whole range of starch degrading enzymes with specific activities has evolved in these organisms (van der Veen *et al.*, 2000) (Figure 6). Most enzymes are hydrolytic, cleaving the linkages in the starch molecules followed by the reaction of the cleavage product with water, resulting in a new reducing end. They can be roughly divided into amylases, hydrolyzing α(1-4) linkages, and debranching enzymes, hydrolyzing α(1-6) linkages.

1.2.2 Cyclodextrin glycosyltransferase (CGTase)

Cyclodextrin glycosyltransferase (CGTase, 4-α-D-glucan:1,4-α-glucanotransfease, CE 2.4.1.19) is a unique member of the α–amylase family of glycosylase with a low hydrolytic activity. CGTase is responsible for the conversion of starch and related α-1,4-glucans into CDs (Nagamura *et al.*, 1993; van der Veen *et al.*, 2000). The CGTase is an extracellular enzyme produced by a large number of miroorganisms including; i) aerobic mesophilic bacteria such as *Bacillus macerans, Bacillus megaterium, Klebsiella oxytoca, Klebsiella Pseudomonas, Micrococcus luteus;* ii) aerobic thermophilic *B. stearothermophilus;* iii) anaerobic thermophilic *Thermoanaerobacterium thermosulfurigenes;* iv) aerobic alkalophilic bacteria such as *B. circulans, Bacillus sp.* AL-6 ; and v) aerobic halophilic *Bacillus halophilus* (Tonkova,1998). Whereas α-amylase generally hydrolyzes α(1-4) glucosidic bonds (Figure 7a), CGTase mainly catalyzes transglycosylation reaction. Such reaction can be described as: $G(n) + G(m) \longrightarrow G(n-x) + G(m+x)$ in which G(n) is donor and G(m) the acceptor oligosaccharide consisting of n and m glucose residues, respectively. Disproportionation (Figure 7b) can be regarded as the default reaction, and is also catalyzed by several other members of the α–amylase family (e.g. 4-α-glucantransferase, EC 2. 4. 1. 25 (amylomaltase, disproportionating enzyme)). The

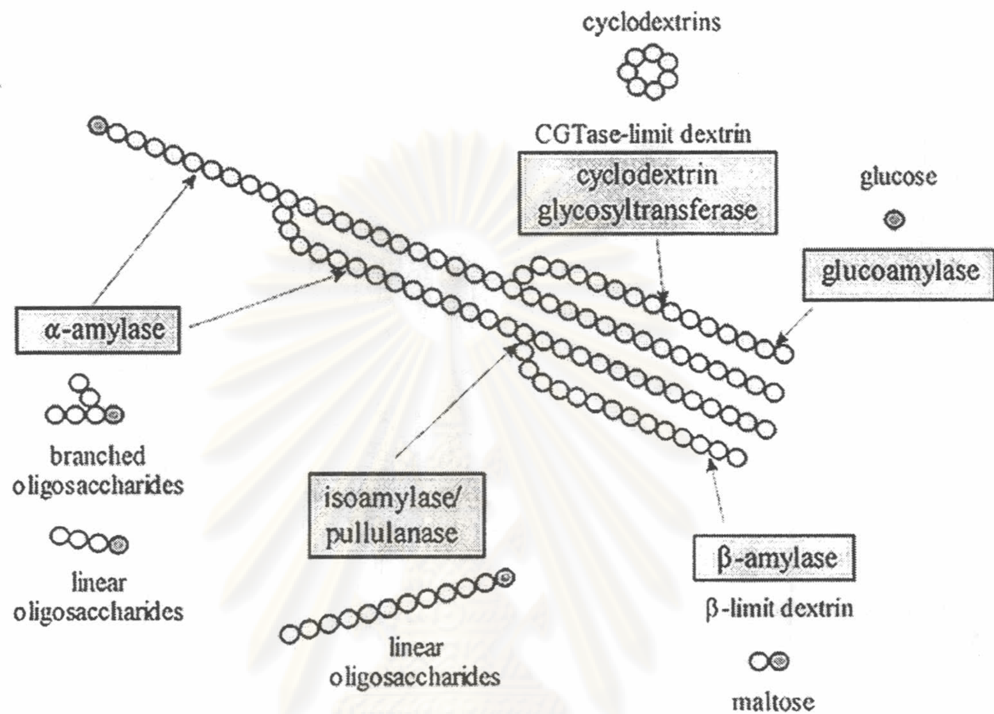

Figure 6 Action of enzymes involved in the degradation of starch

(●) Glucose molecules with a reducing end; (○) glucose molecules without a reducing end. Arrows indicate preferred cleaving points in the starch molecule (van der Veen *et al.*, 2000)

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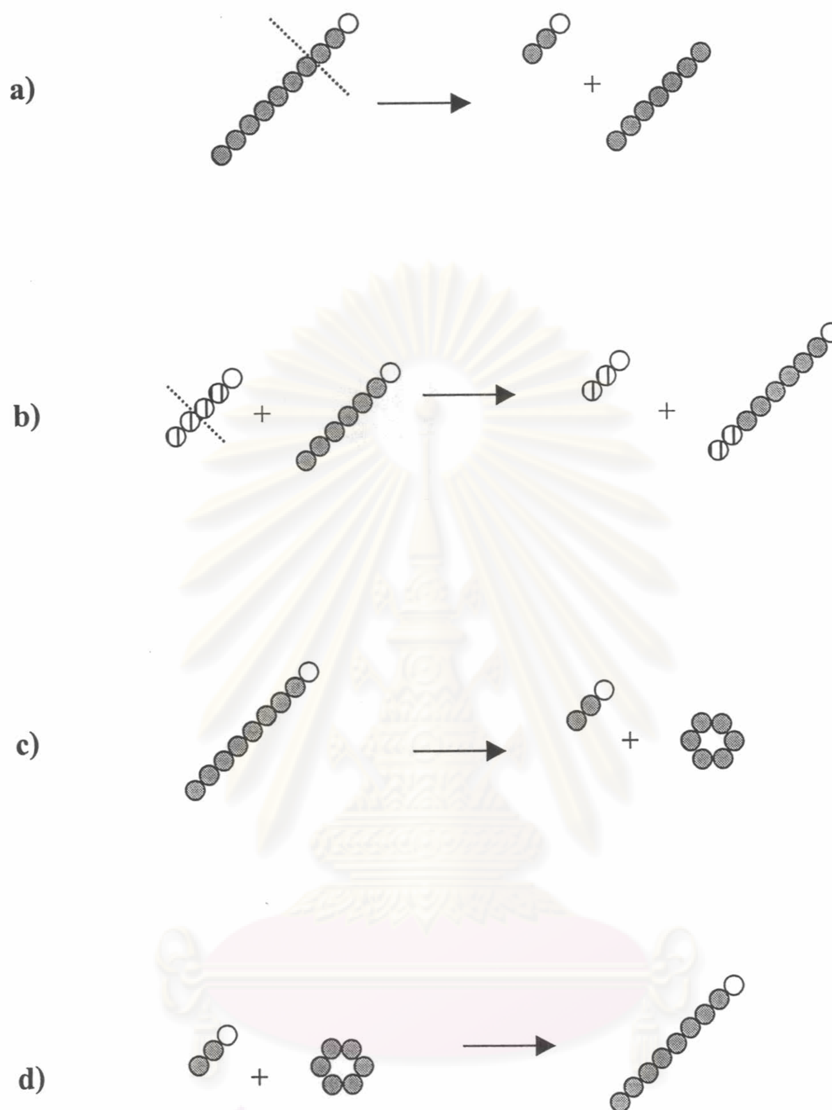

Figure 7 Schematic representation of the CGTase catalyzed reactions The circles represent glucose residues; the white circles indicate the reducing end sugars. (a): hydrolysis, (b): disproportionation, (c): cyclization, (d): coupling (van der Veen *et al.*, 2000)

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specific CGTase reaction is the cyclization reaction (Figure 7c) in which the part of the donor that has been cleaved off also acts as the acceptor, resulting in formation of a cyclodextrin, described as : G(n)⟶ cyclicG(x) + G(n-x). The reverse reaction is also catalyzed by this enzyme and is referred to as coupling reaction (Figure 7d). Table 4 summarizes the mechanisms of CGTase catalytic activity. It should be mentioned that the acceptor binding site of enzyme is not absolutely for glucose or malto oligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrates containing 16-80 glucopyranosyl residues. If chain length is greater than 100 units, disproportionation reaction dominates. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 5. Higher concentration of malto-oligosaccharides or glucose favours the reversed coupling reaction resulting in linear end products with negligible amount of CDs (Kitahara *et al*, 1978). The action of CGTase is different from that of other starch-degrading enzymes that the products are cyclic (non-reducing oligosaccharide). The CGTase may be grouped into three categories depending on the kind of CD mainly produced in the initial reaction. The enzymes produced from different sources show different properties, such as working pH and temperature, molecular weight and yield different ratio of CD products. (Table 6).

1.2.3 Three-dimension structure similarities in the α-amylase family

In contrast to a limited similarity in primary structure (<30%), the three-dimensional structures of α-amylases (Matsuura *et al*., 1984; Qian *et al*., 1994, and Kadziola *et al*., 1998) and CGTases (Klein and Schulz, 1991) are quite similar. α-Amylases generally consist of three structure domains, A, B, and C, while CGTases show a similar domain organization with two additional domains, D and E (Figure 8). Domain A contains a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices. This so-called $(\beta/\alpha)_8$-or Tim-barrel catalytic domain (Janecek, 1994) of 300 – 400 residues is present in all enzymes of the α-

Table 4 Summary of CGTase mechanisms (Okada and Kitahata, 1975)

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

Table 5 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
16-18	- good substrate for cyclization reaction
> 100	- good substrate for disproportionation

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Table 6 Some properties of bacterial CGTases (Tonkova, 1998)

Producer	Optimum pH	Optimum Temp(°C)	Molecular mass	Main CD produced	Reference
Bacillus macerans ATCC 8514	6.1-6.2	60 °C	139,000	-	De Pinto, 1968
Bacillus macerans IFO 3490	5.0-5.7	55 °C	-	α-CD	Kitahata, 1974
Bacillus macerans IAM 1243	-	-	74,000	α-CD	Takano, 1986
Bacillus megaterrium No5	5.0-5.7	55 °C	-	β-CD	Kitahata, 1974
Bacillus circutans var. *alkalophilus* ATCC 21783	4.5-4.7	45 °C	88,000	β-CD	Nakamura, 1976
Bacillus sp. AL-6 (alkalophilic strain)	7.0-10.0	60 °C	74,000	γ-CD	Fujita, 1990
Bacillus cereus NCIMB	5.0	40 °C	-	α-CD	Jamuna, 1993
Bacillus sp. INMIA T6 (thermophilic strain)	6.5	55 °C	38,000	α-CD	Abelian, 1994
Bacillus sp. INMIA T42 (thermophilic strain)	6.5	55 °C	35,000	β-CD	Abelian, 1994
Bacillus sp. INMIA A7/1 (alkalophilic strain)	6.0	50 °C	44,000	β-CD	Abelian, 1994
Bacillus sp. INMIA 1919	4.0	50°C	42,000	α-CD	Abelian, 1994
Bacillus halophilus INMIA 3849	7.0	60-62°C	71,000	β-CD	Abelian, 1995
Thermoanaerobacterium thermosulfurigene EM1	4.5-7.0	80-85 °C	68,000	β-CD	Wind, 1995
Bacillus ohbensis sp. nov. C-1400	5.0	55 °C	80,000	β-CD	Sin, 1991

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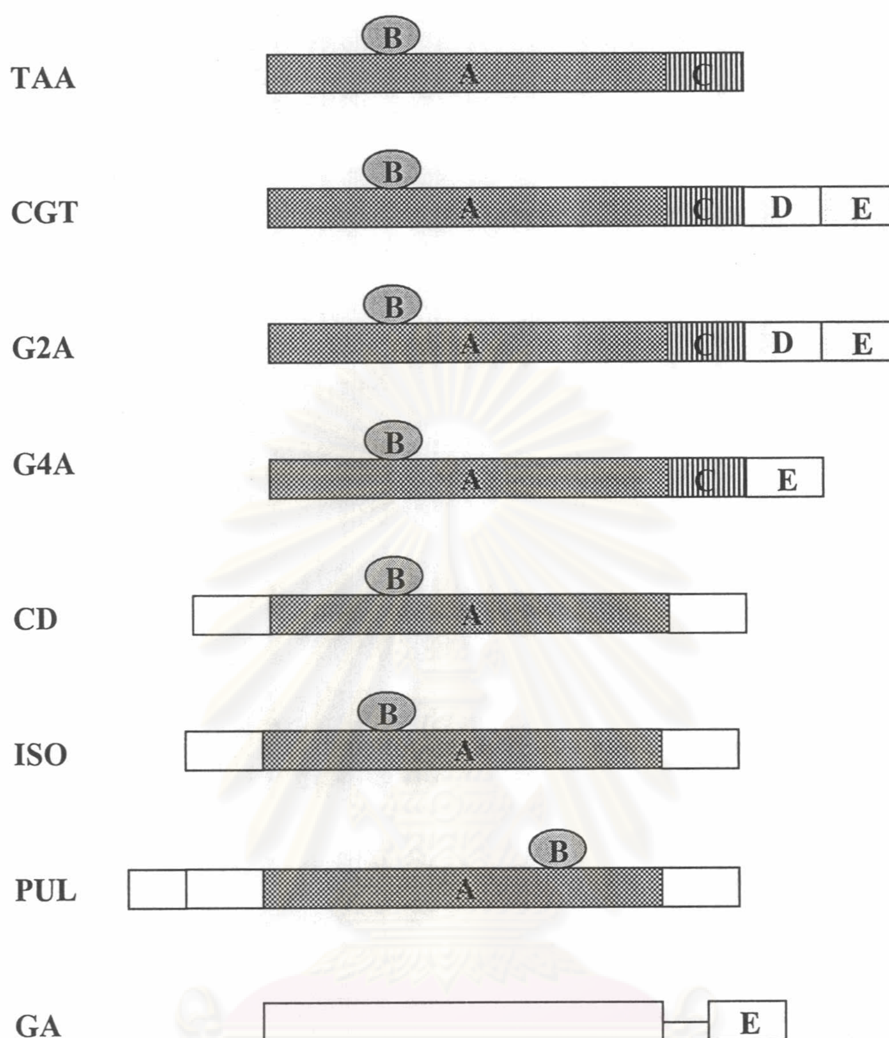

Figure 8 Domain level organization of starch degrading enzymes

TAA: α-amylase from *Aspergillus oryzae* (Taka-amylase A); CGT: CGTase from *Bacillus circulans*; G2A: maltogenic α-amylase from *Bacillus stearothermophilus*; G4A: maltotetraose forming α-amylase from *Pseudomonas stutzeri*; CD: cyclodextrinase from *Klebsiella ozytoca*; ISO: isoamylase from *Pseudomonas amyloderamosa*; PUL: pullulanase from *Klebsiella aerogenes*; GA: glucoamylase (family 15 of glycosylases) from *Aspergillus niger* (Jespersen *et al*., 1991 with modified by van der Veen, 2000)

amylase family. The catalytic and substrate binding residues conserved in the α-amylase family are located in loops at the C-termini of β-strands in domain A. The B-domain consists of 44-133 amino acid residues and contributes to substrate binding. The C-domain is approximately 100 amino acids long and has an antiparallel β-sandwich fold. Domain C of the CGTase from *B.circulans* strain 251 contains one of the maltose binding sites observed in the structure derived from maltose dependent crystals (Lawson *et al.*, 1994). This maltose binding site was found to be involved in raw starch binding (Penninga *et al.*, 1996), suggesting a role of the C-domain in substrate binding. Some authors suggest that this domain is involved in bond specificity, in hydrolyzing or forming α-1,6-bonds (e.g. pullulanase, isoamylase, branching enzyme). The D-domain, consisting of approximately 90 amino acids with an immunoglobulin fold, is almost exclusively found in CGTase and has an unknown function. The E-domain, following the D-domain in CGTase is more widespread in starch degrading enzymes. Besides in the α-amylase family, where it is found as the C-terminal domain when present, it is also found in glucoamylase (family 15 of glucosylase), where it is attached to the C- or N- terminus of the catalytic domain via a glycosylated linker. The enzyme glucoamylase is not in the same α-amylase family and has different core structure except for the similar E-domain. It consists of approximately 110 amino acids and was found to be responsible for the adsorption onto granular starch. 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 catalyzes other activities, including the reconstitution of an α–1,4-glycosidic linkage for cyclizing the maltooligosaccharide produced (Kimura *et al.*, 1987).

1.2.4 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 or affinity resin (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 this strain produces mainly α-CD, the affinity method using α-CD attached to resin is appropriate only for this CGTase. While β-CD has been suggested to serve as the affinity ligand for β-CD-producing enzymes (Bender, 1986; Chung *et al*., 1998). Separation of isoforms or multiple forms of CGTase were also reported using HPLC on anion exchange chromatography (Makela *et al*., 1988), isoelectric focusing in immobilized pH gradients (Mattsson *et al*., 1990), FPLC on a Mono Q column (Bovetto *et al*., 1992) and affinity chromatography on a β-CD polymer (Abelyan *et al*., 1994). In addition, immunoaffinity chromatography was reported to be an efficient purification method for CGTase from *Bacillus* sp. A11 (Tongsima, 1998). The isolation of this CGTase isozymes was performed by preparative gel electrophoresis (Kaskangam, 1998).

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1.2.5 Research for overproduction and application of CGTase

Gene cloning and overexpression of CGTase gene, not only provides satisfactory CD 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 a1 (Binder *et al.,* 1986) were cloned and expressed in *E. coli* and *Bacillus subtilis.*

Various studies have been emphasized on improvement of CD productions. Development of cultivation for CD over-production was carried out under optimized culture condition and with complex nutrient media. A need for thermostable or thermotolerant CGTase which gives high CD yield has been recognized. CGTase from an alkalophilic *Bacillus* strain no. 38-2 (ATC 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 reduced the production cost (Nakamura and Horikoshi, 1976; Kato and Horikoshi, 1984; Yang and Su, 1989). Protein engineering site-directed mutagenesis and gene cloning are also used to increase the yield of enzyme product. These techniques are not only based partly on an assumption of lower production costs, but also on the trends towards greater acceptability of CDs (Schmid, 1989).

1.2.6 Shortcomings of industrial applications of CGTase

The conventional procedure for the production of CDs includes liquefaction of starch using a thermostable α-amylase at 105 °C. After the reaction mixture is allowed to cool to ~50 °C, it is treated with bacterial CGTases (Kitahata, 1974; Nakamura and Horikoshi, 1976; Bender, 1977; Kobayashi, 1978; Kitahata and Okada, 1982; Makela, 1988; Yagi, 1986), which have optimum catalytic reaction temperature in the range of 30-65 °C. Most bacteria that produce CGTase including the ones that are used in industries are mesophilic bacteria, most of them have the optimum temperature between 30-37 °C such as *Bacillus macerans* (Depinto, and Campbell, 1986), *Bacillus lentus* (Englbrecht, 1990), and Alkalophilic *Bacillus* sp. (Nakamura and Horikoshi, 1976). This conventional CD production process is inefficient in many aspects: first, α-amylase should be inactivated before the addition of CGTase; second, microbial contamination is possible: third, it is time consuming, conversion of starch to CDs

requires an extended reaction time before reasonable yields are achieved. Therefore, a CGTase with liquifying and cyclizing activities at high temperatures would be appropriate for efficient production of CDs.

In recent years, several processes using thermostable CGTase from extremophiles have been reported (Biwer *et al.*, 2002). Extremophiles are unique microorganisms that are adapted to survive in ecological niches such as high or low temperature, extremes of pH, high salt concentrations or high pressure. Various enzymes from thermophiles (grow above 50 °C) and hyperthermophiles (grow above 80 °C) have been purified, and their genes cloned and expressed in mesophilic hosts, e.g. *E. coli* and *B. subtilis.* As a general rule, they show extraordinary heat stability, and are resistant to chemical reagents, detergents, urea and guanidinium hydrochloride. Optimal reaction temperatures with thermostable CGTases are between 60 and 90 °C and are higher than in conventional processes where typical temperatures are around 20-40 °C. Benefits in performing processes at higher temperature include reduced risk of contamination, improved reaction rate of enzymatic conversion, lower viscosity and higher solubility of substrates. Furthermore, such CGTases can often be used for starch liquefaction. All CGTases can catalyze starch hydrolysis, but normal CGTases are not stable at the gelatinization temperature of starch (around 90 °C). Using thermostable CGTases eliminates the need for other enzymes like amylases. Thermostable CGTases are produced by, for instance, the anaerobic bacteria *Thermanaerobacter thermosulfurigenes* and *Anaerobranca gottschalkii* (Prowe and Antranikian, 2001). However, it is sometime wasteful in terms of energy to use microorganisms that can tolerate at 60 °C or higher for most of the production of enzymes in industries. In addition, inactivation of the enzyme upon the completion of the process would not be easy due to the thermostability of the enzyme. Therefore, it is advantage to perform overexpression of these thermostable enzymes in mesophilic hosts such as *Bacillus* sp., *yeast (Pichia* sp.), filamentous fungi or, more recently, *Staphylococcus* (Sturmfels *et al.*, 2001) and to optimize the cultivation process. Alternatively, searching for

thermotolerant microorganisms which can be grown at temperature between 30-50 $^{\circ}$C is quite interesting. It has been defined that thermotolerant microorganisms have evolved together with mesophiles but are able to adapt themselves to higher temperature than that of mesophiles. Moreover, the phylogenic evolution is also different from hyperthermophiles. Nevertheless, the production of CGTase by thermotolerant bacteria has not been at the focal point.

The cyclodextrin research group at the Department of Biochemistry, Faculty of Science, Chulalongkorn University has been working on β-CGTase of *Paenibacillus* sp. RB01, a thermotolerant strain which was first isolated from hot spring area of Ratchaburi province. CGTase from this bacterial strain was partially purified by starch adsorption and characterized for some properties by Tesana (2001). It was found that RB01 was grown and able to produce CGTase at temperature range of 30-45 $^{\circ}$C, while grown best at 37 $^{\circ}$C but exerted highest activity at 40 $^{\circ}$C. The optimum pH and temperature of the enzyme were 6.0 and 55 $^{\circ}$C, while the pH and temperature stability of the enzyme were 7.0 and 40 $^{\circ}$C, respectively. The molecular weight was estimated to be 65,000 Da by SDS-PAGE. The enzyme formed mainly β-cyclodextrin with small amounts of α- and γ- cyclodextrins. The ratio of α-, β- and γ-cyclodextrins was 1.0 : 5.4 : 1.2. The best condition for storing enzyme was –20 $^{\circ}$C. The work presents here is the continuous research which aims to purify this enzyme using chromatographic means. Characterization including kinetics and active site investigation of the purified enzyme will then be performed.

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1.3 Objective of this research

1. To purify CGTase from thermotolerant *Paenibacillus* sp. strain RB01
2. To perform biochemical characterization of the purified enzyme
3. To study kinetics and substrate specificity of the purified enzyme
4. To investigate the enzyme active site