#### CHAPTER I

### INTRODUCTION



# **Cyclodextrins**

Cyclodextrins (Celluloseine, cycloamyloses, cyclomaltoses, Schardinger dextrins: CDs) are cyclic, non-reducing oligosaccharides composed of glucose units linked by  $\alpha$ -1,4-glycosidic bonds. The main CDs synthesized naturally by the cyclodextrin glycosyltransferase (CGTase) are built up from six to eight glucopyranose units, known as  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins, respectively, as shown in Figure 1 (Schardinger, 1903,1904; French *et al.*,1942; Pulley and French, 1961). Some physical properties of cyclodextrins are summarized in Table 1 (Szejtli, 1988). The cyclodextrins are water-soluble. This fundamental characteristic derives from the location of all free hydroxyl groups of each successive glucose unit on rims of these doughnut-shaped molecules. The C6 primary hydroxyls on the narrower side and the C2 and C3 secondary hydroxyls occupying the wider sides. These two hydrophilic planes thus confer hydrophilicity upon the molecule, while the inside cavity of CDs is relatively hydrophobic because it is lined with C-H groups and glycosidic oxygen bridges (Saenger, 1979, 1982) (Figure 2).

The most important characteristic of cyclodextrins is their ability to form three-dimensional inclusion complexes with a wide variety of suitable size "Guest" molecules, including these molecules wholly or partially within the central cavity of the "Host" cyclodextrins (Bender, 1986). The inclusion complex is held together by non-covalent bonding forces such as hydrophobic interaction, Van der Waals forces, London dispersion forces, and hydrogen bonding. The guests which can be encapsulated in cyclodextrins, include such compounds as straight or branched chain

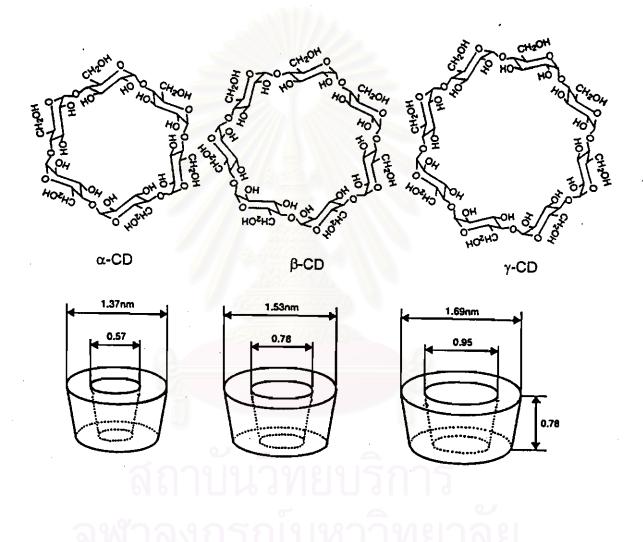
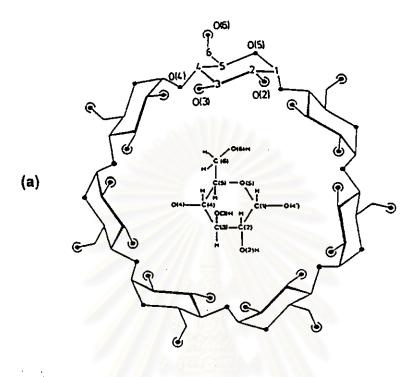


Figure 1 Structure and molecular dimension of cyclodextrins (CDs) (Szejtli, 1990)



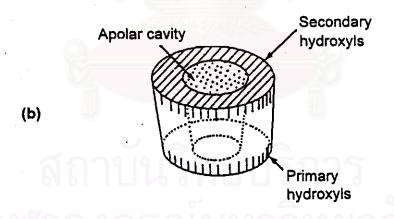


Figure 2 Structure of β-cyclodextrin (Bender, 1986; Szejtli, 1990)

- (a) Chemical structure; o = oxygen atoms, = hydroxyl groups
- (b) Functional structure scheme

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

|   | 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 ambient temp. | 14.40         | 1.85           | 23.20         |
| Cavity dimensions                               |               |                |               |
| Cavity diameter (A°)                            | 4.7-5.3       | 6.0-6.5        | 7.5-8.3       |
| Cavity depth (A°)                               | $7.9 \pm 0.1$ | $7.9 \pm 0.1$  | $7.9 \pm 0.1$ |
| Cavity volume                                   |               |                |               |
| $(A^{\circ})^3$                                 | 174           | 262            | 472           |
| ml per moi                                      | 104           | 157            | 256           |
| ml per g  | 0.10          | 0.14           | 0.20          |
| Crystal forms (from water)                      | Hexagonal     | Monoclinic     | Quadratic     |
|   | plates        | parallelograms | prisms        |
| Hydrolysis by A. oryzae α-amylase               | Negligible    | Slow           | Rapid         |
| Partial molar volume                            | 611.4         | 703.8          | 801.2         |

hydrocarbons, gasses, and some relative polar compounds, as shown in Figure 3 (Amaizo, 1992) and Figure 4 (Janssen, 1992).

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, hydroxypropylated, and maltosyl-cyclodextrins (substitution of the hydroxyl groups by methyl, hydroxypropyl, and maltosyl residues, respectively). CD-polymers (linked cyclodextrins) are used often 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 ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD), can be chosen according to their properties to be used as the suitable host molecules.

Complex formation of cyclodextrins and guest molecules leads to the change in some physical or chemical properties of the guest molecules. Protection against oxidative degradation or destruction by UV light, improvement of solubility of hydrophobic substances in aqueous solution, stabilization of volatile compounds, alternation of the chemical reactivity, modification of liquid substances to powders, or reduction of undesirable smell or taste in products e.g. food stuffs, are among those known useful properties (Schmid, 1989). The applications of cyclodextrins as solubilizers, emulsifiers, antioxidants, stabilizing agents, and ingredient removers have been increased rapidly in food, cosmetics, pharmaceutical, agrochemical, and plastic industries (Table 2) (Nagamoto, 1985). Since early 1970s, many countries for example, Japan, Germany, France, Netherlands, Denmark, Spain, Italy, Belgium, Hungary, USA, and Taiwan, have approved the use of cyclodextrins (at different levels) in several fields of industries (Amaizo, 1991).

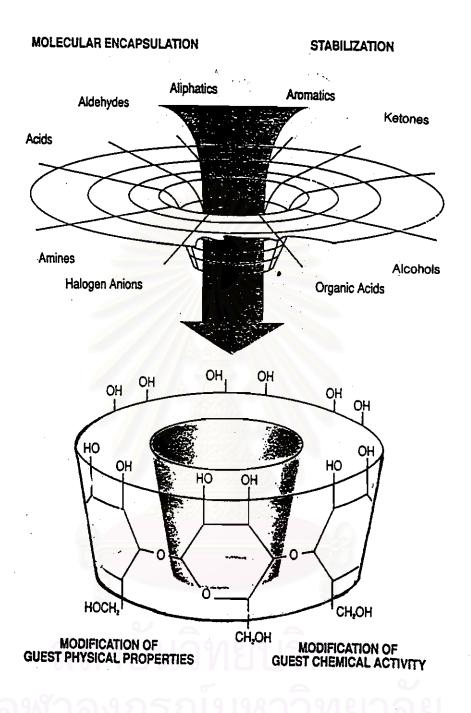
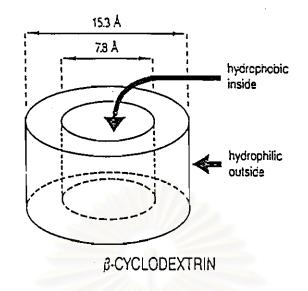


Figure 3 Beneficial modification of guest molecules by cyclodextrins (Amaizo, 1993)



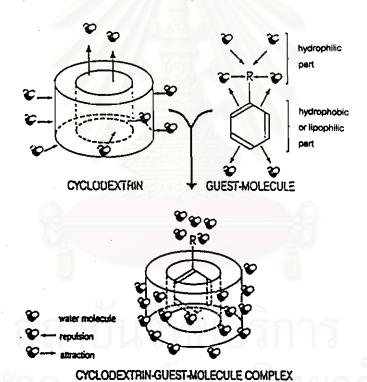


Figure 4 Guest orientation in CD-guest complex (Janssen, 1992)

Table 2 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          | Chewing gum flavor, biscuit flavor, seasoning  |  |  |
| 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 odor                     | Mutton, fish, soybean                          |  |  |
| 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 2 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                                     |

## Cyclodextrin producing enzymes

 $G_m + G_n$ 

Cyclodextrin glycosyltransferase  $(1,4-\alpha$ -D-glucan:  $1,4-\alpha$ -D-glucopyranosyl transferase, EC 2.4.1.19, CGTase) is known to catalyze the degradation of starch and related  $\alpha$ -(1-4) glucans to cyclodextrins. CGTase is produced by various microorganisms, for example, *Klebseilla pneumoniae* (Bender, 1977), *Brevibacterium* sp. (Mori et al., 1994) and mainly the *Bacillus* species, as listed in Table 3. The CGTase can be divided into three types,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -, according to the major type of CDs formed (Horikoshi, 1988). The enzymes from different sources show slightly different characteristics, such as working pH and molecular weight. Each CGTase enzyme yields different ratio of CD-product for example, the CGTase of *Bacillus macerans* produced  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD in relative amount of 2.7:1.0:1.0 (Depinto and Campbell, 1986), while the CGTase of alkalophilic *Bacillus* sp. No. 38-2 produced CDs in relative ratio of 1.0:11.0:1.5 (Matzuzawa et al., 1975). The CGTase of *Bacillus fermus* 290-3 was known to produce  $\gamma$ -CGTase in the initial phase of the enzyme production (Englbrecht et al., 1990).

CGTase catalyzes three possible mechanisms: cyclization, coupling and disproportionation reactions (Kitahata and Okada, 1975) as shown in the following equations:

$$G_n$$
 $G_{(n-x)} + cG_x$ 
 $Coupling$ 
 $Coupling$ 

 $G_{(m-x)} + G_{(n+x)}$ 

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

Table 3 Properties of cyclodextrin glycosyltransferases

| Organism                    | Predominant product | Optimum<br>pH | Optimum temperature | MW<br>(dalton) | pΙ   | Reference                  |
|-----------------------------|---------------------|---------------|---------------------|----------------|------|----------------------------|
| 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          |

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

| Reaction           | Action  |
|--------------------|---|
| Cyclization        | Starch cyclodextrin   |
| Coupling           | Cyclodextrin + glucose oligosaccharide                              |
| Disproportionation | (oligosaccharide) <sub>m</sub> + (oligosaccharide) <sub>n</sub>     |
|                    | (oligosaccharide) <sub>m-x</sub> + (oligosaccharide) <sub>n+x</sub> |

Table 5 Relationship between length of substrate and mechanism of CGTase (Szejtli, 1988)

| Substrate chainlenght (residues) | Effect on mechanism of CGTase                    |  |  |
|----------------------------------|--|--|--|
| 1(D-glucose)                     | - no catalysis                                   |  |  |
| 2-4                              | - inhibit initial reaction of cyclization        |  |  |
| 00,800000                        | - 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 |  |  |

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 substance is thought to be a prerequisite for cyclization. It should be mentioned that the acceptor binding sites of enzyme is not absolutely specific for glucose or malto-oligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrates containing 16-80 glucopyranosyl residues. If the chainlenght is greater than 100 units, disproportionation reaction dominates. This reaction occurs mainly at the beginning of the enzymatic reaction if long chain starch is used. The relationship between chainlenght of substrate and reaction of CGTase is summarized in Table 5. Higher concentration of malto-oligosaccharides 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.

Several methods have been used to study the molecular structure and the active center of enzyme. For example, affinity labeling using substrate or substrate analogs, chemical modification, site-directed mutagenesis or X-ray crystallographic and diffraction studies.

Model of mechanism for the cyclization has been postulated from the study of CGTase from Klebsiella pneumoniae M 5 al, that the enzyme binds eight to ten (or even more) glucose units of a starch molecule (Bender, 1988) (Figure 5). The active site of CGTase thus consisted of eight to ten (or more) subsites. The reaction is an exoattact 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 maltohexaose subsequently binds to subsite two and new  $\alpha$ -1,4-glycosidic bond is formed between glucose residues one and six of maltohexaose.

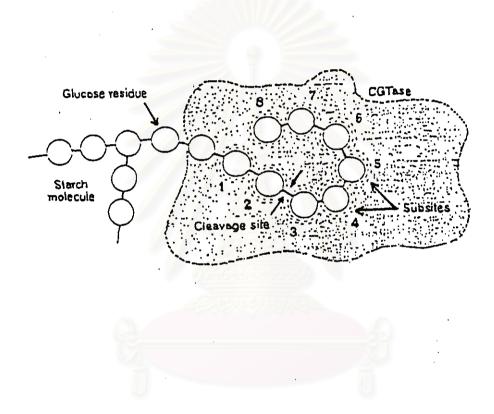


Figure 5 Model of CGTase mechanism from *Klebsiella pneumoniae* M 5 al (Bender, 1988)

The CGTase from *Bacillus circulans* strain 251 is consisted of a single polypeptide chain of 686 amino acids grouped in five distinct domains labeled A through E. Domain A, B, and C are structurally homologous to the equivalent domains of α-amylases. Domain E contains a raw starch-binding motif (Svensson *et al.*, 1989) and was found to bind two maltose molecules. A third maltose molecule is bound by the C-domain and is involved in crystal packing contacts between symmetry related molecules, but the precise functions of the D and E domains remain to be resolved.

In a computer modeled structure of CGTase from *Bacillus circulans* var. alkalophilus, the small domain B situates on the principal domain A, the structure of which closely resembles that of α-amylase and contains a groove with the catalytic site. The C, D, and E domains locate around domain A without any S-S bridged linkage to it. There is a high similarity in domain E among all of the CGTases and also some similarity with glucoamylases and other starch degrading enzymes. Domain E may be involved in catalysis and in binding the substrate leading to a stabilized structure of the enzyme due to a starch bridge fixed between domain A and E (Fugiwara et al., 1992).

CGTases genes from bacilli consist of 2,100-2,800 bases encoding the CGTases of 680-690 amino acids and have over 60% identity within their amino acid sequences. Although sequence similarities between CGTase and  $\alpha$ -amylase, which hydrolyze the  $\alpha$ -1,4-glycosidic bonds of starch, are usually below 25%, four highly conserved regions have been reported and designated as regions A, B, B', and C, respectively (Kimura *et al.*, 1987). Furthermore, the catalytic domains of both enzymes are folded into  $(\beta/\alpha)_8$  barrel structures, and the four conserved regions within this domain constitute the active center (Klein and Schulz, 1991). These regions have also been found in other amylolytic enzymes such as isoamylase, pullulanase, and branching enzyme. Based on the homology with the known structures of  $\alpha$ -amylase,

the active center of CGTase is proposed to locate at the C-terminal ends of the  $\beta$ -strands of the 'Tim-barrel' (Hofmann *et al.*, 1989). It has been proposed from the studied of CGTase of *Bacillus circulans* that the active center of CGTase is composed of tandem subsites as are those of  $\alpha$ -amylases and lysozymes and that it contains seven subsites (Klein *et al.*, 1992).

Three-dimensional structures of CGTases from Bacillus circulans and Bacillus stearothermophilus have been elucidated (Klein and Schultz, 1991; Kubota et al., 1991). The chain folds of both CGTases are similar. The NH<sub>2</sub>-terminal 400 amino acids of CGTases are folded into barrel structures, which closely resemble those of aamylases and which also contain the conserved regions. The catalytic residues of αamylase have been proposed from their three dimensional structures. Although it has been proposed that the catalysts are different pairs among the three α-amylases (Takaamylase A, porcine pancreatic α-amylase, and acid α-amylase from Aspergillus niger), they are restricted within the three conserved acidic amino acid residues away on the COOH-terminal side of the B-region (designated as the B'-region); and aspartate in the C-region. The one glutamate and the two aspartate residues in the B-, B'-, and C'-region are found in all amylolytic enzymes possessing the conserved regions. Site-directed mutagenesis studies have shown that any one of these residues is essential for catalysis in α-amylases, neopullulanase, and CGTase (Nakamura et al., 1993). These findings suggest that the catalytic mechanisms among these amylolytic enzymes are similar and that the variations in substrate specificity and products can be ascribed to the relationships between their similar catalytic centers and different subsite structures. It is thought that  $\alpha$ -amylase catalyzes the hydrolysis of the  $\alpha$ -1,4glucosidic bond by acid-base catalysis as proposed for lysozyme cleavage of the glucosidic bond is catalyzed via an oxocarbonium ion intermediate.

Modeling studies and alignments of amino acid sequences have suggested that a residue 221 (*Thermoanaerobacterium* numbering) is present in CGTases at a

dominant position in the center of the active-site cleft. In most CGTases the residue is an aromatic amino acid (Tyr or Phe), whereas in  $\alpha$ -amylases the residue is much smaller (Gly, Ser, or Val). Penninga et al. (1995) constructed mutants of Bacillus circulans strain 251 CGTase in which this aromatic residue had been replaced by a nonaromatic residue. This resulted in a switch-over to synthesis of linear maltooligosaccharides from starch, which indicated that the aromatic amino acid residue at this position is of crucial importance for an efficient cyclization reaction. The alignment of amino acid sequences of bacterial CGTases was shown in Figure 6 (Wind et al., 1995).

Studies on amino acid compositions were determined directly from amino acid analyzer (Schmid et al., 1988; Takano et al., 1986; Kimura et al., 1987). Asx (aspartic and asparagine) was the most (100-120 residues) in CGTase (Bovetto et al., 1992). Cysteine could not be detected in CGTases produced from Bacillus circulans and Bacillus macerans while a few could be detected in CGTases from other microorganisms. Modification of amino acids with chemical reagents has been used to identify the active site of CGTase from Bacillus stearotheromophilus. Tryptophan 97 was carried an essential role in the binding of maltotriose (Ohnishi et al., 1994). Chemical modification studies of CGTases from Bacillus circulans var. alkalophilus and Bacillus circulans E192 with the use of group-specific reagents suggested the presence of histidine at or near the active site (Mattsson et al, 1992; Villette et al, 1992). Site-directed mutagenesis was reported to be used in identification of amino acid residues at the active site of CGTase from Bacillus sp. 1011. Histidine 327 was found to be important for catalysis over an alkaline pH range (Nakamura et al, 1993) while the CGTase from Bacillus circulans strain 251 possessed the tyrosine 195 as an important residue for the cyclization reaction (Penninga et al, 1995). The structure of some CGTases has been studied by X-ray diffraction. Aspartic acid 229, glutamic acid 257, and aspartic acid 328 constituted the catalytic residues in CGTases from Bacillus circulans and Bacillus circulans strain 251. It was concluded that glutamic

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M----KKTF-KLILVLMLSLTLVFGL--TAPIQAASDTAVSNVVNYSTDV
                                                                                  43
T. thermosulfurigenes
                      M----KKFLKSTAALAVGLSLTFGLF--SPAQAAPDTSVSNKQNFSTDV
B. circulans strain 251
                                                                                  43
                      MFOMAKRVLLSTTLTFSLLAGSALPFLPASAIYADADTAVTNKONFSTDV
B. licheniformis
                      M----KRFMKLTAVWTLWLSLTLGLL--SPVHAAPDTSVSNKQNFSTDV
B. sp. 1011
                                                                                  43
                      M----KRFMKLTAVWTLWLSLTLGLL--SPVHAAPDTSVSNKQNFSTDV
B. sp. 38-2
                      MKNLT--VLLKT-----IPLALLLFILLSLPTAAQADVTNKVNYTRDV
B. ohbensis
                       IYQIVTDRFVDGNTSNNPTGDLYDPTHTSLKKYFGGDWQGIINKINDGYL
                                                                                   93
T. thermosulfurigenes
                       iyqiftdrfsdgnpannptgaafdgtctnlrlycggdwqgiinxindgyl
B. circulans strain 251
                       IYQVFTDRFLDGNPSNNPTGAAFDGTCSNLKLYCGGDWQGLVNKINDNYF 100
B. licheniformis
                       IYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINKINDGYL
                                                                                   93
B. sp. 1011
                       IYQIFTDRFSDGNPANNPTGAAPDGSCTNLRLYCGGDWQGIINKINDGYL
                                                                                   93
B. sp. 38-2
                       IYQIVTDRFSDGDPSNNPTGAIYSQDCSDLHKYCGGDWQGIIDKINDGYL
B. ohoensis
                       TGMGVTAIWISQPVENIYAVLPDSTFGG--STSYHG-WARDLRE-QSYFG 139
T. thermosulfurigenes
                       TGMGVTAIWISQPVENIYSII---NYSCVNNTAYHGYWARDFKKTNPAYG 140
B. circulans strain 251
                       SDLGVTALWISQPVENIFATI---NYSGVTNTAYHGYWARDFKKTNPYFG 147
B. licheniformis
                       TGMGITAIWISQPVENIYSVI---NYSGVNNTAYHGYWARDFKKTNPAYG 140
B. sp. 1011
                       TGMGITAIWISQPVENIYSVI---NYSGVHNTAYHGYWARDFKKTNPAYG 140
B. sp. 38-2
B. ohbensis
                       TDLGITAIWISQPVENVYALHP-SGY----TSYHGYWARDYKRTNPFYG 135
                                                   * *** ****
                       SFTDFQNLINTAHAHNIKVIIDFAPNHTSPASETDPTYAENGRLYDNGTL 189
T. thermosulfurigenes
                       TIADFONLIAAAHAKNIKVIIDFAPNHTSPASSDQPSFAENGRLYDNGTL 190
B. circulans strain 251
                       TMTDFQNLVTTAHAKGIKIIIDFAPNHTSPAMETDTSPAENGKLYDNGNL 197
B. licheniformis
                       TMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNL 190
B. sp. 1011
                       TMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNL 190
B. sp. 38-2
                       DFSDFDRLMDTAHSNGIKVIMDFTPNHSSPALETDPSYAENGAVYNDGVL 185
B. ohbensis
                                   ** ** ** ** ***
                                    Domain Al Domain B
                       LGGYTNDTNGYFHHYGGTDFSSYEDGIYRNLFDLADLNQONSTIDSYLKS 239
LGGYTNDTQNLFHHNGGTDFSTTENGIYKNLYDLADLNHNNSTVDVYLKD 240
VGGYTNDTNGYFHHNGGSDFSTLENGIYKNLYDLADLNHNNSTIDTYFKD 247
LGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADLNHNNSSVDVYLKD 240
LGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADLNHNNSSVDVYLKD 240
IGNYSNDPNNLFHHNGGTDFSSYEDSIYRNLYDLADYDLNNTVMDQYLKE 235
T. thermosulfurigenes
B. circulans strain 251
B. licheniformis
B. sp. 1011
B. sp. 38-2
B. ohbensis
                       Domain B Domain A2
                       AIKVWLD:GIDGIRLDAVKHMPPGWQKNFMDSILSYRPVFTPGEWFLGTN 289
T. thermosulfurigenes
B. circulans strain 251
                       AIKMWLDLGIDGIRMDAVKHMPPGWOKSPMAAVNNYKPVFTFGEWPLGVN 290
B. licheniformis
                       AIKLWLDMGVDGIRVDAVKHMPQGWQKNWMSSIYAHKPVPTFGEWFLGSA 297
B. sp. 1011
B. sp. 38-2
                       AIKMWLDLGVDGIRVDAVKHMPFGWQKSFMATINNYKPVFTFGEWFLGVN 290
                       AIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINNYKPVPNFGEWPLGVN 290
B. ohbensis
                       SIKLWLDKGIDGIRVDAVKHMSEGWQTSLMSDIYAHEPVFTFGEWFLGSG 285
                        ** *** * **** ***** *** *
                        EIDVNNTYFANESCMSLLDFRFSQKVRQVPRDNTDTMYGLDSMIQSTASD 339
 T. thermosulfurigenes
                        EVSPENHKFANESCMSLLDFRFAQKVRQVPRDNTDNMYGLKAMLEGSAAD 340
 B. circulans strain 251
                        APDADNTDFANESGMSLLDFRFNSAVRNVPRDNTSNMYALDSMLTATAAD 347
 B. licheniformis
 B. sp. 1011
                        EISPEYHOFANESCHSLLDFRFAQKARQVPRDNTDNMYGLKAMLEGSEVD 340
 B. sp. 38-2
                        EISPEYHOFANESGMSLLDFPFAQKARQVFRDNTDNMYGLKAMLEGSEVD 340
 B. ohbensis
                        EVDPQNHHFANESGMSLLDFQFGQTIRDVLMDGSSNWYDFNEMIASTEED 335
                                                    *.*. *
                                                                * . *. ..
                        IV
YNFINDMVTFIDNHDMDRFY-NGGSTRPVEQALAFTLTSRGVPAIYYGTV 388
 T. thermosulfurigenes
 B. circulans strain 251
                        YAQVDDQVTFIDNHDMERFHASNANRRKLEQALAFTLTSRGVPAIYYGTE 390
B. licheniformis
B. sp. 1011
B. sp. 38-2
B. ohbensis
                        YNQVNDQVTFIDNHDMDRFKTSAVNNRRLEQALAFTLTSRGVPAIYYGTE 397
                        YAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSE 390
                        YAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSE 390
                        YDEVIDOVTFIDNHDMSRFSFEQSSNRHTDIALAVLLTSRGVPTIYYGTE 385
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Figure 6 Alignment of amino acid sequences of bacterial CGTases

(Wind et al., 1995)

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-YDR-OWRPYNRAMMTSFNTSTTAYNVIKKLAPLRKSNPAIAYGTTQQRW 436
T. thermosulfurigenes
                      QYMSGGTDPDNRARIPSFSTSTTAYQVIQKLAPLRKCNPAIAYGSTQERW 440
B. circulans strain 251
                      QYLTGNGDPDNRGKMPSFSKSTTAFNVISKLAPLRKSNPAIAYGSTQQRW 447
B. licheniformis
B. sp. 1011
B. sp. 38-2
                      QYMSGGNDPDNRARLPSFSTTTTAYQVIQKLAPLRKSNPAIAYGSTHERW 440
                      QYMSGGNDPDNRARIPSFSTTTTAYQVIQKLAPLRKSNPAIAYGSTQERW 440
B. ohbensis
                      QYLTGGNDPENRKPMSDFDRTTNSYQIISTLASLRQNNPALGYGNTSERW 435
                                                      Domain A2 Domain C
T. thermosulfurigenes
                      INNDVYIYERKPGNNVALVAINRNLSTSYNITGLYTALPAGTYTDVLGGL 486
B. circulans strain 251
                      INNDVLIYERKPGSNVAVVAVNRNLNAPASISGLVTSLPQGSYNDVLGGL 490
B. licheniformis
                      INNDVYIYERKFGKSVAVVAVNRNLTTPTSITNLNTSLPSGTYTDVLGGV 497
B. sp. 1011
                      INNDVIIYERKPGNNVAVVAINRNMMTPASITGLVTSLRRASYNDVLGGI 490
B. sp. 38-2
                      INNOVIIYERKFGNNVAVVAINRNMNTPASITGLVTSLPOGSYNDVLGGI 490
B. ohbensis
                      INSDVYIYERSFGDSVVLTAVNSG-DTSYTINNLNTSLPQGQYTDELQQL 484
                      ** ** *** ** * * * * * *
                                                  . . .
                                                         * * *
                                                                  .
T. thermosulfurigenes
                      LNGNSISVASDGSVTPFTLSAGEVAVWQYVSSSNSPLIGHVGPTMTKAGQ 536
B. circulans strain 251
                      LNGNTLSVGSGGAASNFTLAAGGTAVWQYTAATATPTIGHVGPMMAKPGV 540
B. licheniformis
                      LNGNNIT-ESGGNISSFTLAAGATAVWQYTASETTPTIGHVCPVMGKPGN 546
B. sp. 1011
B. sp. 38-2
                      LNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATTPIIGNVGPMAKPGV 540
                      LNGNTLTVGAGGAASNFTLAPGGTAVWQYTTDATAPINGNVGPMMAKAGV 540
B. ohbensis
                      LDGNEITVNSNGAVDSFQLSANGVSVWQITEEHASPLIGHVGPMMGKHGN 534
                      *.** .. . * * * *..
                                          Domain C Domain D
T. thermosulfurigenes
                      TITIDGRGFGTTSGQVLFGSTAGT---IVSWDDTEVKVKVPSVTPGKYNI 583
                      TITIDGRGPGSSKGTVYPGTTAVSGADITSWEDTQIKVKIPAVAGGNYNI 590
VVTIDGRGPGSAKGTVYPGTTAVTGSAITSWEDTQIKVTIPPVAGGDYAV 596
B. circulans strain 251
B. licheniformis
B. sp. 1011
                      TITIDGRGFGSGKGTVYFGTTAVTGADIVAWEDTQIQVKIPAVPGGIYDI 590
                      TITIDGRA-SARQGTVYFGTTAVTGADIVAWEDTQIQVKILRVPGGIYDI 589
B. sp. 38-2
B. ohbensis
                      TVTITGEGFGDNEGSVLFDSDF---SDVLSWSDTKIEVSVPDVTAGHYDI 581
                                                  *
                       ** * . . * * * . .
T. thermosulfurigenes
                      slktsscatsntynniniltgnqicvrfvvnnastvygenvyltgnvael 633
                      KVANAAGTASNYYDNFEVLSGDQVSVRFVVNNATTALGQNVYLTGSVSEL 640
B. circulans strain 251
B. licheniformis
                      kva-angvnsnayndftilsgdqvsvrfvinnattalgeniyltgnvsel 645
B. sp. 1011
B. sp. 38-2
                      RVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTALGQNVFLTGNVSEL 640
                      RVANAAGAASNIYDNFEVLTGDQVTVRFVINNATTALGQNVPLTGNVSEL 639
B. ohbensis
                      SVVNAGDSQSPTYDKFEVLTGDQVSIRFAVNNATTSLGTNLYMVGNVNEL 631
                            Domain D Domain E
                      GNWDTSKA-IGPMFNQVVYQYPTWYYDVSVPAGTTIQFKFIKNGNT-IT 681
T. thermosulfurigenes
B. circulans strain 251
                      Gnwdpaka-igpmyngvvygypnwyydvsvpacktiepkplkkggst-vt 688
B. licheniformis
                      Onwitgaasiopapnqvihayptwyydvsvpackqlepkppkkngat-it 694
B. sp. 1011
B. sp. 38-2
B. ohbensis
                      Gnwdpnna-igpmyngvvygyptwyydvsvpaggtiepkplkkggst-vt 688
                      GNWDPNNA-IGPMYNQVVYQYPTWYYDVSVPACQTIEFKFLKKQGST-VT 687
                      GNWDPDQA-IGPMFNQVMYQYPTWYYDISVPAEENLEYKFIKKDSSGNVV 680
                              * *** '***' ** ****'***
                                                            ******
                       WEGGSNHTYTVPSSSTGTVIVNWOO
T. thermosulfurigenes
                                                                              706
B. circulans strain 251
                      WEGGSNHTFTAPSSGTATINVNWQP
                                                                              713
B. licheniformis
B. sp. 1011
                       WEGGSNHTPTTPTSGTATVTINWQ
                                                                              718
                      WEGGANRTFTTPTSGTATVNVNWQP
                                                                              713
B. sp. 38-2
                      WEGGANRTFTTPTSGTATVNVNWQP
                                                                              712
B. ohbensis
                      WESGNNHTYTTPATGTDTVLVDWQ
                                                                              702
                       ** * * * * * * . . * * . . *
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Figure 6 Alignment of amino acid sequences of bacterial CGTases

(continued)

acid 257 acts as the proton donor in the reaction, whereas aspartic acid 328 is involved in the binding of the substrate and helps to elevate the pKa of glutamic acid 257 through a direct hydrogen bond to this residue that exists only when no substrate or inhibitor is present (Klein et al, 1992; Lawson et al, 1994; Strokopytov et al, 1995). X-ray studies and site-directed mutagenesis experiments have identified Asp-255, Glu-283, and Asp-354 (Thermoanaerobacterium numbering) as catalytic residues in CGTase. These residues were found to be conserved in the amino acid sequence of the Thermoanerobacterium CGTase (Wind et. al., 1995). Table 6 summarizes the amino acids involved in the active sites of CGTases.

This work is a continual part of the work of the cyclodextrin research group at the Biochemistry Department, Faculty of science, Chulalongkorn University. Our CGTase of Bacillus sp. All, screened from South-East Asian soil (Pongsawasdi and Yagisawa, 1987), was purified and characterized (Techaiyakul, 1991; Rojtinnakorn, 1994). Specific antibody against CGTase was prepared (Rojtinnakorn, 1994) and was used in enzyme purification through immunoaffinity column chromatography (Kim, 1996). Rutchtorn (1993) studied the optimization of CGTase production in 5 litrefermanter. Batchwise and continuous production of cyclodextrins using immobilized CGTase were reported (Rutchatorn, 1993; Kuttiarcheewa, 1994). Malai (1995) studied the production of cyclodextrins from rice starch by using free CGTase. Siripornadulsil (1992), Vittayakitsirikul (1995), and Boonchai (1996) reported on molecular cloning techniques, gene expression, mapping and partial nucleotide sequence determination. Laloknam (1997) synthesized oligonucleotide probes for CGTase gene and Jantarama (1997) studied the mutation of Bacillus sp. All for the production of higher CGTase activity. Studies for the information on active center of our CGTase will lead to more insight into the understanding of the enzyme and the ability to compare with other CGTases. Characterization of the specific amino acid residues participating in the catalytic reactions through chemical modification is our focal point for this research. The binding affinity and the catalytic rate of the reaction

Table 6 Summary of amino acid residues involved in the active sites of CGTases

| Source                        | Amino acid residues  at the active site | Reference                                  |
|-------------------------------|---|--|
| Bacillus circulans strain 251 | Asp 229, Glu 257, Asp 328               | Klein et al.,1992 and<br>Strokopytov, 1995 |
| Bacillus circulans var.       | His                                     | Mattsson et al,                            |
| alkalophilus                  |   | 1992,1995                                  |
| Bacillus circulans E192       | Tyr                                     | Villette et al, 1993                       |
| Bacillus circulans strain 251 | Tyr 195                                 | Penninga et al, 1995                       |
| Bacillus sp. 1011             | His 327, Tyr 195                        | ·Nakamura <i>et al</i> ,<br>1993,1994      |
| Bacillus stearothermophilus   | Trp 97                                  | Ohnishi et al, 1994                        |
| Thermoanaerobacterium         | Asp 255, Glu 283, Asp 354               | Wind et. al., 1995                         |
| thermosulfurigenes EM 1       |   | •  |



between substrates and the enzyme are also determined for more information on the active center of the enzyme.

# The objective of this research

- 1. To determine the effect of chemical modification on CGTase activity
- 2. To identify amino acid residues involved in and present at the active site of CGTase
- 3. To determine the effect of chemical modification on the structure and enzymatic properties of CGTase
- 4. To determine the kinetics for cyclodextrin substrates of CGTase

