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The role of domain E in the activity of *Bacillus macerans* cyclodextrin glucanotransferase

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Hai-yin Chang

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This is to certify that the Doctoral dissertation of

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DEDICATION

To my parents, Kuang-han Chang and Tzu-ying Hsu
For their love and encouragement

To my husband, Sige Zou
For his love and caring

To my lovely daughter, Iris Rui-shi Zou
For bringing me the happiness

TABLE OF CONTENTS

ABSTRACT

Cyclodextrin glucanotransferase (CGT) is an industrial enzyme used for the production of cyclodextrins (CDs). CGT folds into five domains A, B, C, D and E. Domain E has the ability to bind starch and cyclodextrins (CDs) and is considered the starch-binding domain of CGT. Two maltose binding sites (MBS1 and MBS2) have been identified in domain E. Domain E participates in the formation of a deep groove which is located on the protein surface of CGT and extends from the active site to MBS2. To investigate the function of domain E in the catalysis of CGT, CGT mutants were constructed using a CGT from *Bacillus macerans*.

The truncated CGT (CGTΔE), was constructed by deleting domain E. In the chimeric CGT (CGT-SBD), domain E was replaced with the starch binding domain (SBD) of glucoamylase I (GAI), which has a similar structure and function to domain E. The modified CGT (CGT+6) was constructed by inserting six amino acids between domains D and E. The six amino acids were inserted as a pseudo linker in order to perturb the alignment of the groove by increasing the distance between domains D and E.

CGTΔE exhibited no detectable activity, whereas CGT-SBD had very low cyclization, starch-degrading, and coupling activities. CGT+6 exhibited about 50-60% of the wild type CGT (WT-CGT) activity, and its thermostability was lower than that of WT-CGT. The K_m value for the cyclization activity of CGT+6 was about 2.6 fold greater than that of WT-CGT. The efficiency of the cyclization reaction (k_{cat} /K_m) was also significantly reduced.

The effect of Tyr634 residue, located in the MBS2, on substrate binding was also investigated. The Tyr634 residue of MBS2, which has a strong hydrophobic interaction with bound maltose, was replaced with glycine (Y634G) and phenylalanine (Y634F), respectively. The interaction of β-galactosidase-domain E fusion proteins, β–Gal-E(WT) and β–Gal-E(Y634G), with immobilized α-cyclodextrin was studied to probe the binding behavior of the Y634G mutant without the interference of the active site.

The overall properties of mutant Y634F were similar to that of WT-CGT. The thermostability of the Y634G mutant was significantly lower than that of WT-CGT, Y634F, and CGT+6. Starch substrate provided little stabilization to Y634G indicating that the aromatic side chains of Tyr634 or Phe634 participated in binding soluble starch.

Using starch as a substrate, the K_m value of Y634G for the cyclization reaction was about 1.4 times greater than that of WT-CGT, indicating that this mutant had a reduced affinity for the substrate. β–Gal-E(Y634G) also showed weaker interaction with α-CD-Sepharose than β–Gal-E(WT), which is consistent with the greater K_m value of Y634G mutant compared with WT-CGT.

Based on the results of this study, it can be concluded that domain E cooperates with the catalytic domain in binding long substrates. The groove, extending from the active site to domain E, can be considered as an extension of the active site cleft which assists in the binding and/or directing the binding of long substrates in the active site. Thus, the proper orientation and the optimal distance

of domain E from the catalytic domain is important for maintaining the integrity of the groove structure and effective binding of starch during the cyclization reaction. The apparent perturbations of CGT structure caused by the Tyr to Gly substitution (Y634G) and by inserting 6 amino acids between domains D and E affected the enzyme thermostability, suggesting that domain E is an integral part of the CGT structure.

CHAPTER 1. GENERAL INTRODUCTION

CGT is an important industrial enzyme

Cyclodextrin glucanotransferase [1, 4-α-D-glucan 4-α-D-(1, 4-α-D-glucano)-transferase (cyclizing), EC 2.4.1.19, CGTase] produces cyclodextrins (CDs) from starch. Cyclodextrins (CDs) are cyclic, non-reducing oligosaccharides which consist of three kinds of molecules, α-CD, β-CD and γ-CD, containing six, seven, or eight glucopyranose units, respectively. The hydrophobic cavity of CDs can entrap small hydrophobic molecules and can form inclusion complexes with the trapped molecules (Schmid, 1989; Tao, 1991). Therefore, CDs are widely used in many industries, including the pharmaceutical, food, agricultural, chemical and cosmetics industries (Szejtli,1988). For example, CDs are used as micro-encapsulators and as stabilizers for light- and oxygen-sensitive reagents, and are used to improve the water solubility of water-insoluble reagents. In the food industry, they can be used to remove unpleasant smells and improve taste (Schmid, 1989). In addition, CDs can reduce the cholesterol level in food (Schlimme et al, 1991; Dalemans, 1994; Yen et al., 1995). These applications of CDs show that CGT is a valuable industrial enzyme.

Limitations of CGT for industrial applications

Most industrial applications require only one molecular form of cyclodextrin. All known cyclodextrin glucanotransferases (CGTs), however, produce three kinds of CDs (α-, β-, and γ-CD) in the same reaction. Separation of these CDs is a difficult

but necessary process which dramatically increases the cost of CD production. Moreover, the yield of CDs is severely reduced by product inhibition (Tomita et al., 1990; Lee et al., 1992; Kim et al., 1992). A modified CGT is highly desirable, which can produce CDs with a specific ring size, has no product inhibition and is stable at extreme temperatures and pHs. Designing such an enzyme requires detailed knowledge of CGT. This includes information on the three-dimensional structure, catalytic mechanism, and substrate binding patterns of this enzyme.

CGT catalyzes three types of reactions

CGT is present in a variety of bacteria, including *Bacillus, Klebsiella and Micrococcus* (Schmid, 1989). CGT is a monomeric extracellular protein with a molecular weight of approximately 74 kDa which corresponds to about 680 amino acid residues. This enzyme catalyzes at least three different reactions, intramolecular transglycosylation (cyclization reaction), intermolecular transglycosylation (coupling and disproportionation reaction) and hydrolysis (Amylase Research Society of Japan, 1988).

The most important activity of CGT is cyclization activity which produces CDs from starch.

$$\text{starch} \rightarrow \alpha, \beta, \gamma\text{-CD}$$

In the presence of suitable acceptors (ex: short chain acceptor smaller than maltoheptaose), CGT catalyzes intermolecular transglycosylation, including coupling reaction

$$c(G)_g + (G)_x \rightarrow (G)_{g+x}$$

and disproportionation reaction (Bovetto et al., 1992a).

$$(G)_x + (G)_y \rightarrow (G)_{x-a} + (G)_{y+a}$$

Where $c(G)_g$ is cyclodextrin, $(G)_x$, $(G)_{g+x}$, $(G)_y$, $(G)_{x-a}$, and $(G)_{y+a}$ are 1, 4-α–D-glucopyranosyl chains with x, g+x, y, x-a, and y+a D-glucopyranosyl residues. In addition, CGT has a weak hydrolytic activity toward starch and cyclodextrins (Nakamura et al., 1994; Bovetto, L. et al., 1992b).

Different CGTs produce the three cyclodextrins with a specific product distributions for each enzyme. CGTs have been classified as three types, α–, β–, and γ–CGT, based on which major type of cyclodextrin is produced initially (Tao, 1991).

Product distribution can be influenced by a number of factors. In general, the larger the substrate, the faster CDs are generated. For example, starch is the best substrate, while maltose is a poor substrate, and glucose cannot serve as a substrate for CGT (Bovetto et al., 1992a). Interestingly, reaction time is also an influencing factor. The distribution of CDs in the later stage of the reation differs from that in the early stage (Amylase Research Society of Japan, 1988). The presence of surfacants in the reaction mixture can induce conformation changes of the substrate and alter the product distribution. For example, sodium lauryl sulfate (SLS) can stimulate the formation of α-CD, triton (isooctylphenylpolyoxyethylene) can enhance the formation of β-CD and sodium acetate can increase the production of γ-CD (Amylase Research Society of Japan, 1988).

CGT folds into five domains

CGT of *Bacillus circulans* strain 8 consists of 684 amino acid residues which fold into five domains, A ,B, C, D and E (Fig. 1). The enzyme active site is located in domain A, which forms a $(\beta\alpha)_8$-barrel (TIM barrel). The catalytic residues in the active site of CGT from *Bacillus circulans* strain 251 are Asp229, Glu257, and Asp328. Domain B consists of approximately 55 residues which are inserted as a loop after the third β–strand of domain A. Domain C, which consists of approximately 90 residues, forms β-sheets and folds into a Greek-key motif. Domain D, with about 80 residues, adapts a β-sheet structure with an immunoglobulin folding pattern. Domain E, with roughly 95 residues, folds into a different β-sheet motif (Hofmann et al., 1989; Klein et al., 1991; Kubota et al., 1991; Jespersen et al., 1991). On the surface of CGT, a broad depression (groove) extends from the active site in domain A to domain B and domain E (Hofmann et al., 1989; Lawson et al., 1994).

Domains A, B, and C of CGT share structure and sequence similarities with α-amylase

α-Amylase hydrolyzes the α-1, 4-glucosidic bonds of starch and generates linear products. The folding patterns of domains A, B and C in CGT are similar to α–amylase (Fig. 2). Both contain a $(\beta\alpha)_8$-barrel in the catalytic domain and have a

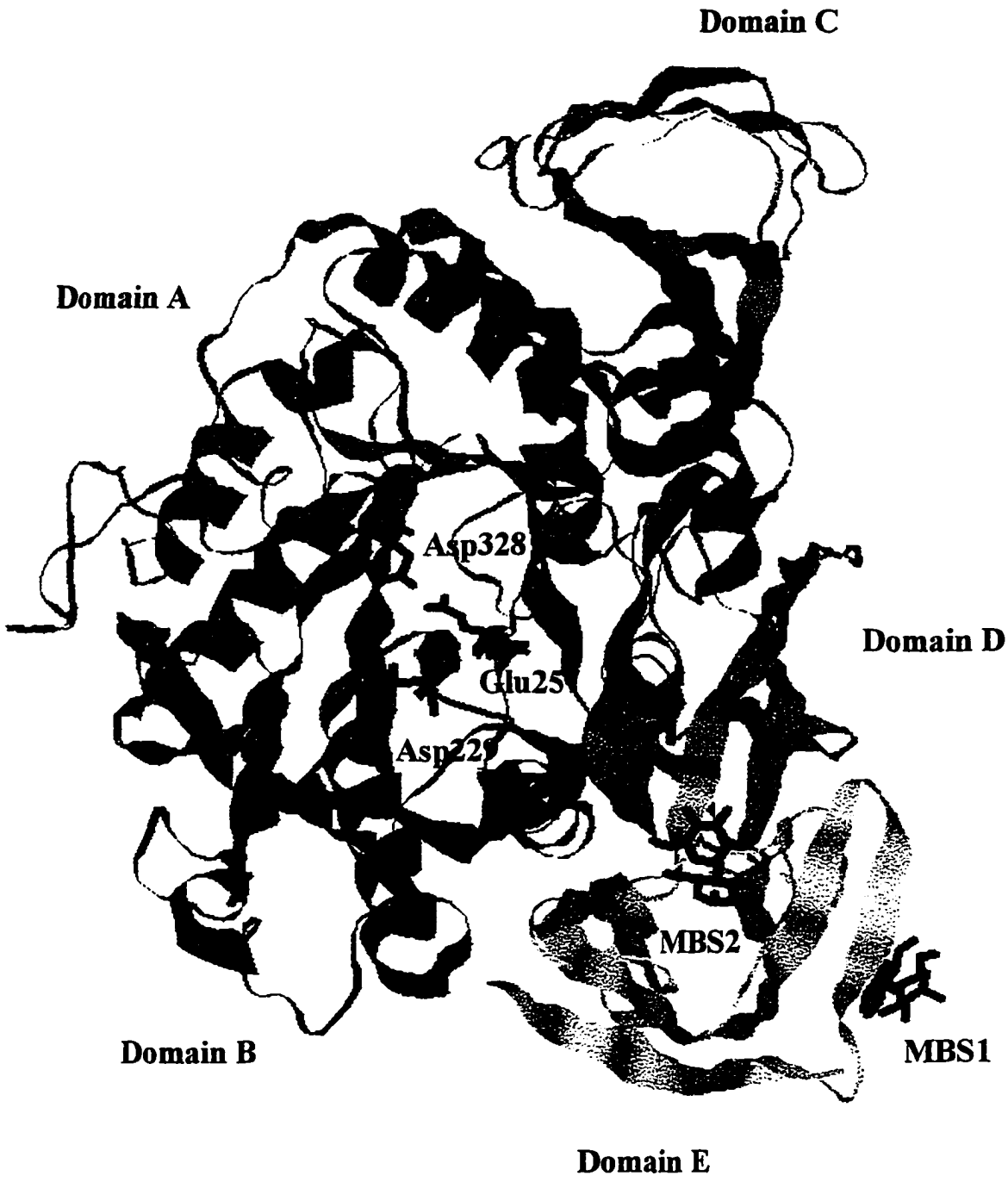

Figure 1. The three-dimensional structure of cyclodextrin glucanotransferase from *Bacillus circulans* strain 251. Asp229, Glu257 and Asp328 are catalytic residues. MBS1, MBS2: first and second maltose binding sites.

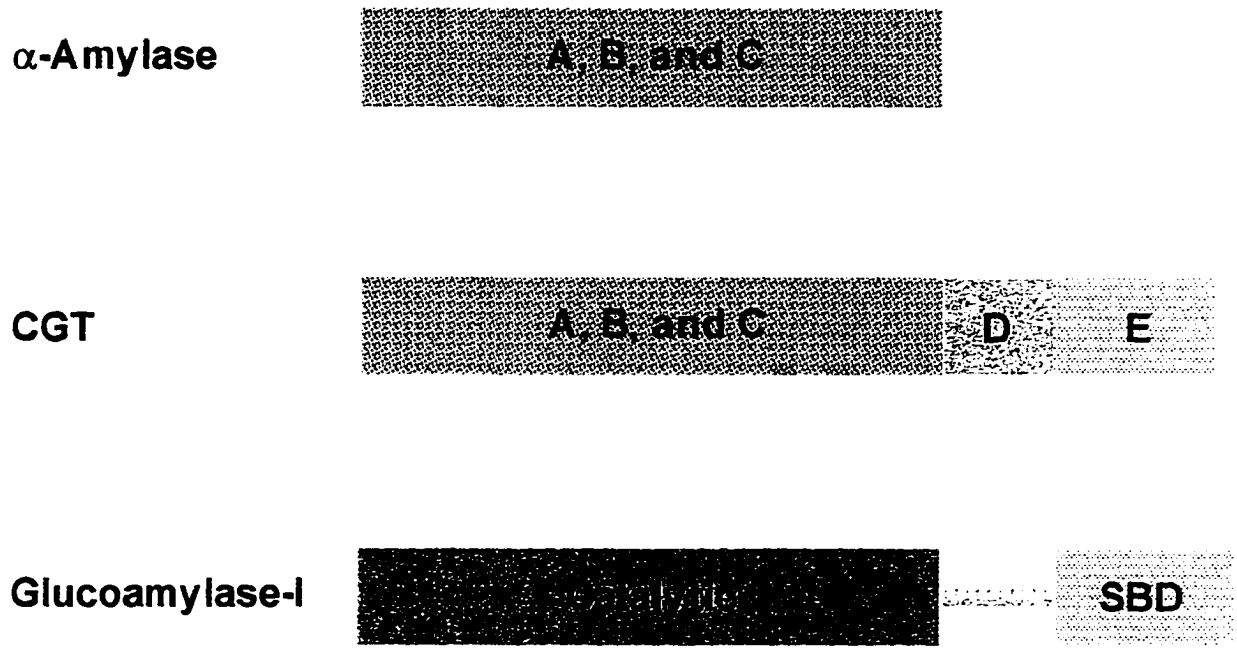

Figure 2. The folding pattern of CGT is shown as an α-amylase with two additional domains, D and E. Domain E and the starch binding domain (SBD) of glucoamylase I have similar structure and functions, but are connected to their respective catalytic domains in different patterns.

small domain inserted into the $(\beta\alpha)_8$ barrel as a loop. Four highly conserved regions have been found in the catalytic domains of these two enzymes (Macgreor et al., 1989; Nakamura et al., 1992; Jespersen et al., 1991; Binder et al.,1986; Kimura et al.,1987).

The amino acid sequence similarities between CGT and α-amylase suggest that they are related to each other. α-Amylase, however, is a hydrolase and CGT is a transferase which transfers groups or makes cyclic molecules. In terms of structure, the folding pattern of CGT is similar to α-amylase with two additional domains D and E, added to the C-terminus (Hofmann et al, 1989).

Domain E of CGT shares structure and sequence similarity with the starch-binding domain of glucoamylase I

Glucoamylase I (GAI) hydrolyzes soluble starch and releases β-D-glucose from the non-reducing end. The starch-binding domain (SBD) of GAI is essential for binding raw starch granules in order to facilitate their digestion by the catalytic domain (Svensson et al., 1986; Takahashi et al., 1985; Ueda, 1981; Dalmia et al., 1991). The starch binding domain can serve as an independent domain, which binds to raw starch granules. This was demonstrated by studying the fusions of SBD with β-galactosidase (Chen et al., 1991). From the sequence comparison, the raw-starch-binding region appears to exist in several starch-degrading enzymes, including CGT (Svensson et al., 1989).

The amino acid compositions of SBD from GAI and domain E from CGT have

approximately 40% identity and 60% similarity. The function of the domain E of CGT, however, is not completely clear. The study of the fusion proteins in which domain E of CGT from *Bacillus macerans* was fused to the C-terminus of β–galactosidase indicate that domain E is an independently functional domain that can bind to raw starch granule and cross-linked amylose. These results further suggest that domain E of CGT and the SBD of GAI share similar carbohydrate-binding abilities (Dalmia et al., 1995).

Despite their functional similarity, CGT and GAI adapt different three-dimensional structures. In *Aspergillus niger*, SBD is connected to the catalytic domain of GAI by a highly O-glycosylated linker (Svensson et al., 1983; Gunnarsson et al., 1984) (Fig. 2). This linker separates the catalytic domain and SBD by a fixed distance and has a random-coil structure which can be described as a semi-rigid rod (Williamson et al., 1992). The linker apparently provides the SBD with mobility and increases its freedom in approaching the surface of a starch granule. In CGT, however, domain E is close to the active site and is connected to the catalytic domain by domain D. Since domain D has a well-defined structure and is less mobile, domain E is probably rigidly held in the compacted globular protein.

The catalytic residues of CGT include two aspartic acids and one glutamic acid

Since domains A, B and C of CGT and α-amylase have a similar $(\beta\alpha)_8$ barrel structure, they are thought to share similar catalytic mechanisms. Based on amino

acid substitution analysis, Asp229, Glu257, and Asp328 residues of the CGT from alkalophilic *Bacillus* have been shown to be the catalytic residues (Nakamura, A. et al., 1992), which are conserved in α-amylase. Modeling studies of the three-dimensional structure of *Bacillus circulans var. alkalophilus* CGT suggest that Glu257 is more protonated than Asp328 (Demchuk et al., 1992). As shown in the crystal structure of CGT from *Bacillus circulans* strain 251, Glu257 is surrounded by hydrophobic residues (Phe259, Phe283 and Leu281), and Asp328 and Asp229 are in a more polar environment. This further suggests that Glu257 is protonated (Lawson et al., 1994). The detailed structure of CGT from *Bacillus circulans* strain 251 complexed with acarbose in the active site indicates that Glu257 acts as the proton donor to initiate the reaction, Asp229 serves as a general base or nucleophile to stabilize the oxocarbonium intermediate, and Asp328 is involved in substrate binding. In addition, Asp328 may play a role in increasing the pK_a of Glu257 by maintaining the carboxylate group of Glu257 that is protonated at higher pHs (Strokopytov et al., 1995).

The active site of CGT determines product specificity

Although mechanisms of substrate cleavage are relatively clear for CGT, the mechanism of the cyclization reaction is not well understood. After the substrate is cleaved, the nonreducing end part of the substrate remains in the active site and the reducing end part leaves. The nonreducing end of the remaining oligosaccharide must bend into the active site to be close enough to its reducing end for the

cyclization reaction to occur. Two models have been proposed to explain how CGT binds starch and catalyzes the cyclization reaction.

Phe191 of CGT from *Bacillus stearothermophilus* is thought to be capable of forming an inclusion complex with amylose and cyclodextrin (Kubota et al., 1991). Amino acid residues which function similarly to Phe191 are present in β-amylase (Mikami et al., 1993) and domain E of CGT. As shown in the crystal structure of CGT from *Bacillus circulans* strain 251, the side chain of Leu600 in the second maltose binding site can penetrate into the center cavity of α-CD (Knegtel et al., 1995).

Based on the above observations, the first model proposes that amylose is bound to the active site of CGT in a helical conformation. Amylose can form an inclusion complex with the Tyr195 residue of CGT from alkalophilic *Bacillus* sp. 1011 (corresponding to Phe191 in CGT from *Bacillus stearothermophilus*). After the α-1, 4-glucosidic bond of amylose is cleaved by the catalytic residues of CGT, the reducing side of the substrate is released from CGT, and the non-reducing side stays in the active site. When the nonreducing end is bent close to the catalytic residues, a cyclic molecule is produced by intramolecular transglycosylation. If an acceptor other than the reducing end of the bound substrate takes over the place, intermolecular transglycosylation will occur (Nakamura et al., 1994). This model further suggests that the ring size of CDs is controlled by the size of the side chain of the amino acid which can form an inclusion complex with amylose. Consistent with this model, when the Tyr188 of CGT from *Bacillus ohbensis* is replaced with

tryptophan, which has a larger side chain than tyrosine, the production of a larger CD, γ-CD, was doubled (Sin et al., 1994).

The second model suggests that a curved conformation of amylose is induced by the arrangement of subsite structure in the active site when the amylose is bound to the active site of CGT (Bender, 1990; Klein et al., 1992; Strokopytov et al., 1996). Evidence supporting this model comes from the crystal structure of CGT complexed with maltononaose. The glucose residues bound in the active site are arranged in a bent conformation (Strokopytov et al., 1996). This second model implies that the ring size of CDs depends on the subsite structure in the active site and the number of glucose units bound to the active site. This model predicts that making a larger CD requires more glucose units bound to the active site. Theoretically, by introducing steric blockage or changing the number of hydrogen bonds in the subsites, the number of glucose units bound to the active site can be manipulated to produce desired CDs (Strokopytov et al., 1996).

Two maltose binding sites are present in domain E of CGT

Two maltose binding sites were observed on the protein surface of the crystal structure of CGT from *Bacillus circulans strain 251* (Lawson et al., 1994). This crystal structure provides excellent details for further investigating the role of domain E. The first maltose binding site (MBS1) is away from the active site and is easily accessed by the substrates from the outer environment. The second maltose binding site (MBS2) is located at the opening of the deep groove which extends

from the active site of domain A to domain E (Fig. 3). The maltose molecules make hydrophobic contacts and hydrogen bonds with the enzyme. The hydrophobic interaction is achieved by stacking the glucose ring of maltose on the aromatic ring of an amino acid residue of the protein. Trp616 and Trp662 at MBS1 and Tyr633 at MBS2 (Fig. 4) are the residues that are involved in the hydrophobic interaction. In addition, MBS1 and MBS2 form hydrogen bonds with the hydroxyl groups of maltose. The amino acid residues involved in the hydrogen bonds are Trp616, Ser382, Asn667, Lys651 and Glu663 at MBS1, and Gln628, Asn627, Asn603, Gly601, Ala599, Thr598 at MBS2 (Lawson et al., 1994).

Domain E of CGT is involved in substrate binding and product inhibition

Since MBS2 is located in the opening of the deep groove on the protein surface, which extends from the active site of domain A to domain E, domain E is speculated to be important in binding large substrates, such as amylose. By fitting in the groove, amylose appears to serve as a bridge connecting the active site and domain E and stabilizes the structure of CGT (Fujiwara, S. et al., 1992a). Considering the orientation of the maltose bound in MBS2 (Lawson et al., 1994) and the acarbose bound in the active site (Strokopytov et al., 1995), amylose fits in the groove with its non-reducing end toward the active site and the reducing end toward domain E. Based on the modeling study of Lawson et al. (1994), 9 to 10 glucose residues can be accommodated in the groove from the active site (catalytic residues) to MBS2, and 15 to 20 glucose residues can be fit between the active site

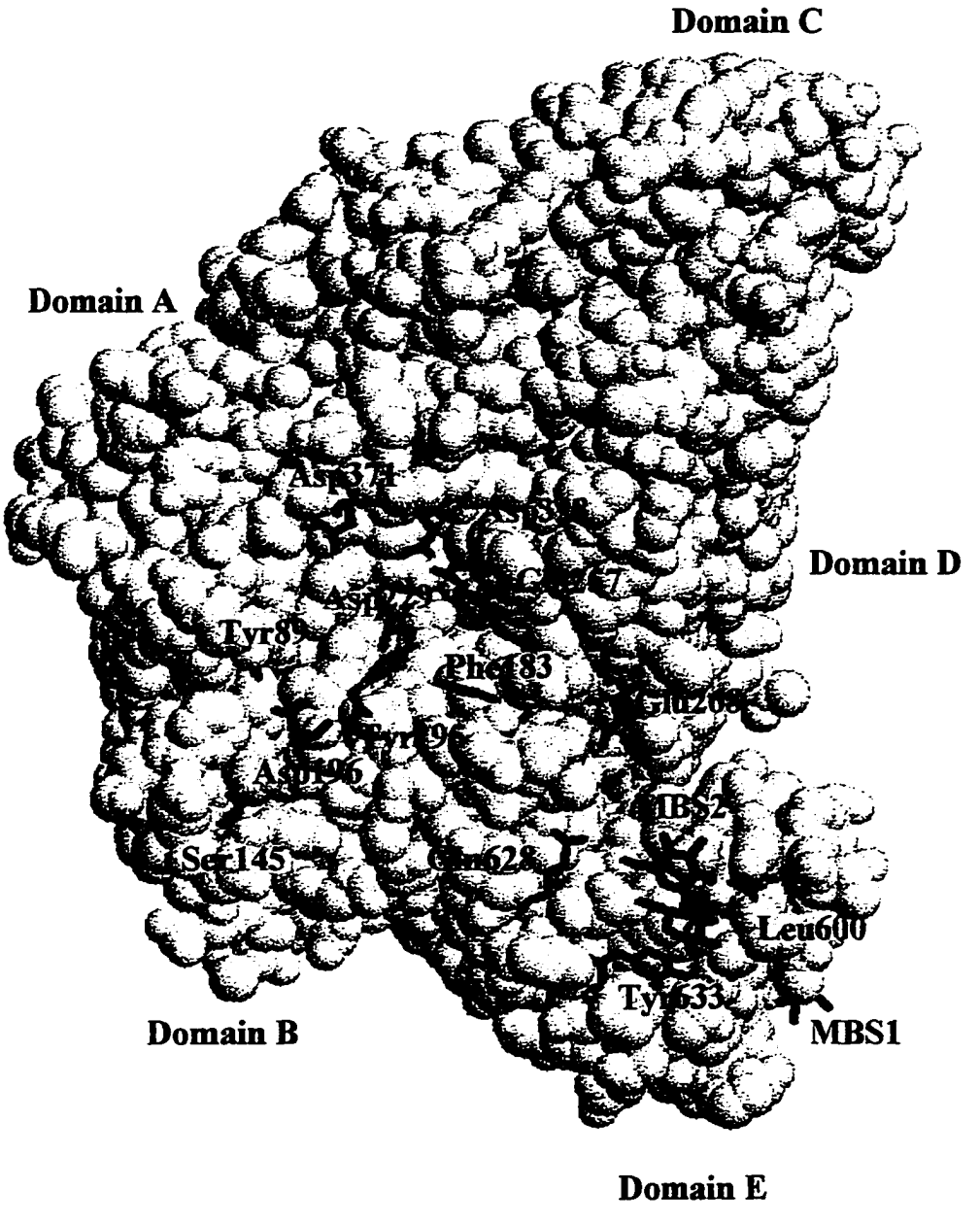

Figure 3. The putative substrate binding groove (Glu268, Glu257, Asp371, Tyr89, Phe183, Gln628, Tyr633, Leu600) in CGT from *Bacillus circulans* strain 251 which extends from the active site (Glu257) to the second maltose binding site (Tyr633) of domain E (Lawson et al., 1994). Asp328, Glu257 and Asp229 are catalytic residues. Leu600 and Tyr195 are proposed to penetrate into the center cavity of cyclodextrin. MBS1, MBS2: first and second maltose binding sites.

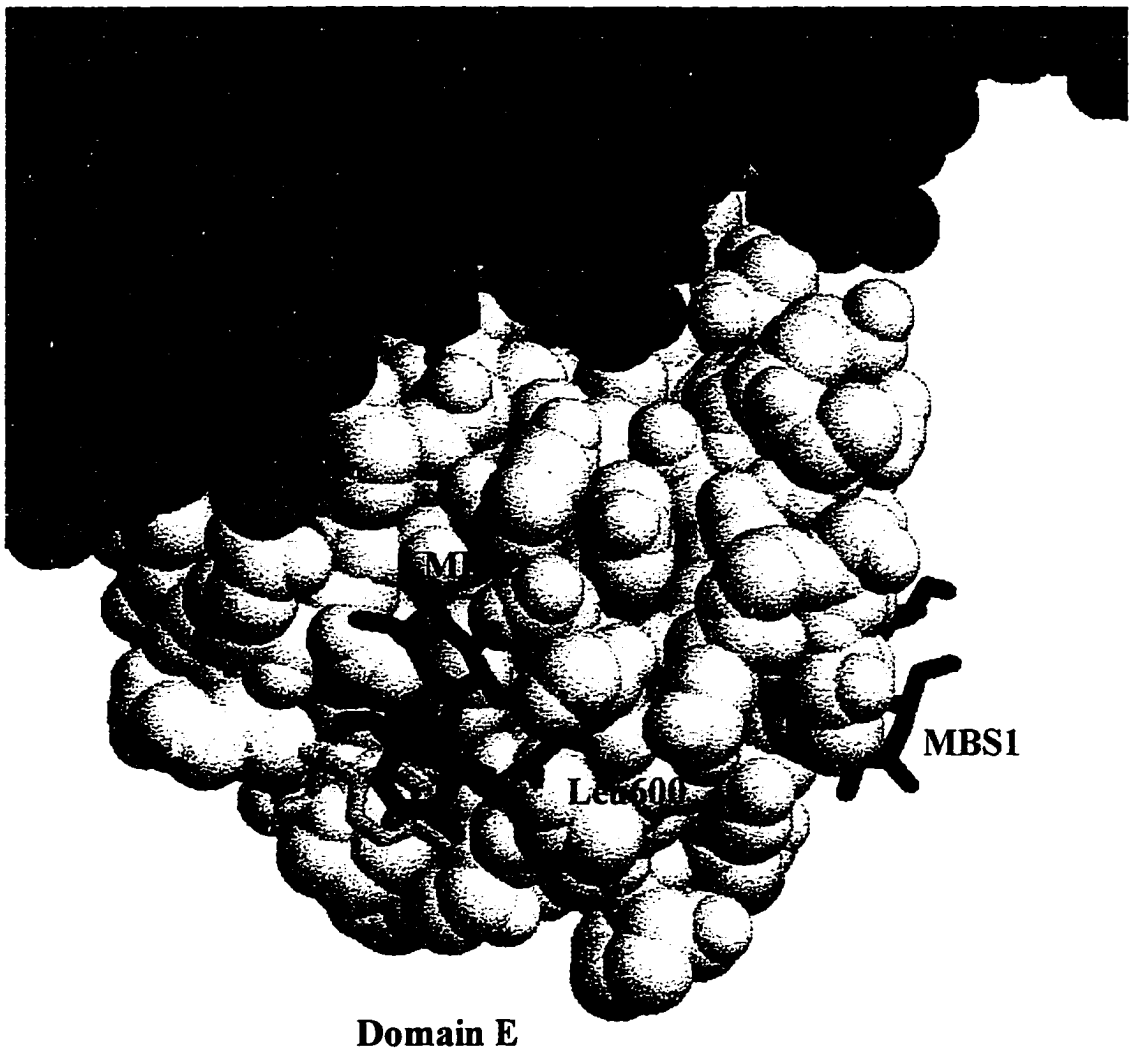

Figure 4. Second maltose binding site of domain E in CGT from *Bacillus circulans* strain 251. Tyr633 of domain E has hydrophobic contacts with maltose by stacking the aromatic ring of Tyr633 with the glucose ring of maltose (lawson et al., 1994). Leu600 was proposed to penetrate into the cavity of the bound cyclodextrn (Knegtel et al., 1995).

and MBS1. The crystal structure of CGT complexed with maltononaose (G9) shows that the reducing end of maltononaose points to MBS2. If maltopentaose (G5) is bound in MBS2 with the nonreducing end toward the active site, the G9 can be easily extended to meet G5 by adding 4 to 5 glucose residues between the G9 and G5 (Strokopytov et al., 1995). This suggests that MBS2 is involved in the binding of large substrates and may help guide amylose into the active site. A similar modeling study indicates that the nonreducing end of G3 bound in MSB1 can not be directed to the reducing end of G9 (Strokopytov et al., 1995). Thus MBS1 cannot bind directly to amylose extended from the active site.

One of the limitations of CGT in industrial applications is that its activity is sensitive to product inhibition, which leads to a low productivity of CDs from starch (Tomita et al., 1990; Lee et al., 1992; Kim et al., 1992). Because domain E enhances the binding of long substrates, it is hypothesized that domain E also participates in the product inhibition. Several lines of evidence support this hypothesis. First, binding of SBD (the analog to domain E) to raw starch granules is competitively inhibited by β-CD with a significant inhibition constant (K_i) of 11 μM (Belshaw et al., 1991). Second, the crystal structure of CGT from *Bacillus circulans* strain 251 complexed with α-CD, shows that two α-CDs instead of two maltose molecules are bound in MBS1 and MBS2 of domain E (Knegtel et al., 1995). The later study provides structural information for rational explanation of the involvement of domain E in product inhibition. Third, in the amino acid replacement studies of domain E, Tyr633 of MBS2 and Trp616 and Trp662 of MBS1 were replaced with

alanines using site-directed mutagenesis. The results suggest that MBS1 is mainly involved in binding raw starch granules and MBS2 is involved in leading the starch chain into the catalytic site (Penninga et al., 1996). This is consistent with the relative location of MBS1 and MBS2 on the protein surface. MBS1, which is on the protein surface, can be accessed by molecules in the outer environment, such as raw starch granules, more easily than MBS2 can, which is located in the groove. These observations suggest that the product inhibition of CGT is caused by the binding of cyclodextrin to MBS2 which competes with the binding of starch.

The function of domain E of CGT remains to be clarified

To study the role of domain E on CGT activities, mutants with C-terminal deletions were generated. Specifically, three mutants were created from alkalophilic *Bacillus* sp.#1011 CGT, named alkΔ10, alkΔ13 and alkΔ323. These mutants contained a deletion of 10, 13 and 323 amino acids from the C-terminus of the enzyme, respectively. AlkΔ323 had no detectable activities. AlkΔ10 and alkΔ13 retained starch hydrolytic activities, but had altered cyclization activities. The later two mutants produced more glucose, more maltooligosaccharide and more α-CD instead of β-CD, compared with the wild type CGT (Kimura et al., 1989). This suggests that domain E in CGT does not affect starch-hydrolytic activity, but is involved in the cyclization reaction and pH stability.

In the work by Hellman et al. (1990), 36, 84, 125 and 225 amino acids were removed from the C-terminus of CGT from *Bacillus circulans* var. Alkalophilus.

These mutants were named cirΔ36, cirΔ84, cirΔ125, and cirΔ225, respectively. The starch-hydrolytic and cyclization activities of cirΔ36 and cirΔ84 were only 0.5% of the wild-type CGT. CirΔ125 and cirΔ225 had no detectable activities. In addition, alkaline phosphatase from *E. coli* was fused to the C-terminus of cirΔ36 (cirΔ36AP). CirΔ36AP increased the activities of cirΔ36 to 2% of the wild type (Hellman et al., 1990). These results suggest that domain E is involved in both starch-hydrolytic and cyclization activities, which are not consistent with analysis of alkalophilic *Bacillus* CGT.

Interestingly, 90 amino acids were deleted from the C-terminus of CGT from *Klebsiella pneumoniae* strain M5al. The activities of truncated CGT did not show significant difference from that of the wild type (Bender, 1990). This suggests domain E is not involved in the catalytic activity of CGT. One explanation for this controversial result is that the CGT from *Klebsiella pneumoniae* strain M5al may be a different type of enzyme. The amino acid sequence of CGT from this strain shares only approximately 30% amino acid similarity with other CGTs, while other CGTs share 50 to 70% similarity.

Besides the deletion studies, two series of chimeric proteins were constructed to investigate catalytic mechanisms of CGT. In one set of experiments, 12 chimeric CGTs were constructed by combining different enzymes from *alkalophilic Bacillus* sp. strain No. 38-2 and *alkalophilic Bacillus* sp. strain No. 17-1. The results indicate that the N-terminus of CGT is important for thermostability. Both the N- and C-terminus of CGT influenced the pH-activity profile (Kaneko, T. et al., 1989). In

addition, the N- and C-terminus were involved in product specificity of CDs (Kaneko, T. et al., 1990). In the other set of experiments, 7 chimeric CGTs were engineered. These CGTs were from *B. stearothermophilus* NO2 and *B. macerans* IFO 3490. This study, on the other hand, suggests that the N-terminus of CGT is related to cyclization activity of CGT (Fujiwara et al., 1992b). Therefore, whether the domain E is involved in the catalysis of CGT is still a controversy to be resolved.

Dissertation organization

In chapter 2 of my dissertation, I describe the importance of domain E on the activity of CGT. The characterization of three mutant CGTs of *Bacillus macerans* are described. The results suggest that domain E needs to be properly oriented and maintained at a fixed distance from the catalytic domain in order to cooperate with the active site in binding large substrates such as soluble starch. Chapter 3 describes the importance of the second maltose binding site (MBS2) in the catalysis of CGT. The findings from the amino acid replacement (site-directed mutagenesis) in MBS2 suggest that the Tyr634 residue of MBS2 is important for substrate binding and domain E participates in the cyclization reaction of CGT by binding large substrates. Chapter 4 gives the general conclusions drawn from my dissertation research.

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CHAPTER 2. THE IMPORTANCE OF THE STARCH-BINDING DOMAIN ON THE ACTIVITY AND STABILITY OF *BACILLUS MACERANS* CYCLODEXTRIN GLUCANOTRANSFERASE

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ABSTRACT

To understand the role of domain E in intra- and intermolecular transglycosylation reactions catalyzed by cyclodextrin glucanotransferase (CGT), three mutants of *Bacillus macerans* CGT were constructed. In the truncated CGT (CGTΔE), part of the groove was removed by deleting domain E. The chimeric CGT (CGT-SBD), was constructed by replacing domain E with the starch-binding domain (SBD) of glucoamylase I (GAI) which has a similar structure and function as domain E. The modified CGT (CGT+6) was made by inserting six amino acids between domains D and E. These six amino acids were inserted as a pseudo linker to affect the alignment of the groove by increasing the distance between domains D and E and the flexibility of domain E.

The results showed no detectable activity of CGTΔE. The purified CGT-SBD had about 0.03% of the WT-CGT coupling, starch-hydrolyzing and cyclization activities. The purified CGT+6 had about 50-60% of the cyclization, coupling and starch-hydrolyzing activities of WT-CGT. The thermostability of CGT+6 was less

than that of WT-CGT. The K_m value for cyclization activity of CGT+6 using starch as substrate was about 2.6 fold of that of WT-CGT. The efficiency of the cyclization reaction of CGT+6 also decreased significantly.

The results indicate that domain E is important for the activity of CGT. Maintaining the integrity of the groove structure for proper fitting of starch molecules appears to be important. Apparently, domain E needs to be properly oriented and at a fixed distance from the catalytic domain to maintain an efficient cooperation with the active site.

INTRODUCTION

Cyclodextrin glucanotransferase (EC 2.4.1.19) is an industrially important enzyme for producing cyclodextrins (CDs). Cyclodextrins (α-, β-, and γ-CD) can form inclusion complexes with various molecules and have increasing applications in food, agricultural, chemical, pharmaceutical and cosmetics industries (Schmid, 1989). CGT produces cyclodextrins from starch by a cyclization (intramolecular transglycosylation) reaction. Besides the cyclization reaction, in the presence of a suitable acceptor, CGT also catalyzes coupling and disproportionation (intermolecular transglycosylation) reactions. CGTs exhibit a weak hydrolytic activity with soluble starch and cyclodextrin substrates (Amylase Research Society of Japan, 1988; Bovetto et al., 1992).

The three-dimensional structure of CGT from *Bacillus circulans* strain 8 (Hofmann et al., 1989; Klein et al., 1991; Klein et al., 1992), *Bacillus circulans* strain

251 (Lawson et al., 1994), and *Bacillus stearothermophilus* (Kubota et al., 1991) reveals that the protein consists of five domains: A, B, C, D, and E. Domain E, which was identified as the raw-starch-binding region in CGT (Svensson et al., 1989), shares 60% amino acid similarity with the starch binding domain (SBD) of glucoamylase I (GAI). The folding pattern of CGT is similar to α-amylase with two additional domains, D and E (Binder et al., 1986; Macgregor et al., 1989; Jespersen et al., 1991). A deep groove structure was observed on the protein surface of CGT (Hofmann et al., 1989). Domain E participates in the formation of the groove which spans between the active site located in domain A and one of the maltose binding sites in domain E (Hofmann et al., 1989; Lawson et al., 1994). Two maltose binding sites (MBS1 and MBS2) were identified in domain E from the X-ray structure of CGT of *Bacillus circulans* strain 251 complexed with maltose. MBS2 is located at the opening of the groove, and MBS1 is near Trp616 and Trp662 which is farther away from the active site than MBS2 (Lawson et al., 1994). Although named the maltose-binding site, it has been found that both sites can bind α-cyclodextrin (Knegtel et al., 1995). Binding of a cyclodextrin molecule at MBS2 apparently interferes with the binding of soluble starch in the groove and binding at MBS1 interferes with raw-starch adsorption of CGT (Knegtel et al., 1995; Penninga et al., 1996).

The previous attempts to show the effect of domain E in the various activities of CGT were inconclusive and even contradictory. The conclusions from C-terminal deletion studies were that domain E was important only for cyclization activity (Kimura et al., 1989), that both starch-hydrolyzing and cyclization activities were

affected by deletions in domain E (Hellman et al., 1990), and in the case of *Klebsiela pneumoniae*, the deletion of the 90 C-terminal residues did not have any affect on the activity (Bender, 1990).

Two series of chimeric CGTs were constructed by combining together different parts of CGT genes from different sources. The first study showed that the N-terminus was important for thermostability, and both N- and C-terminus were related to the product specificity (Kaneko et al., 1989, 1990). The second study showed that the N-terminus is related to cyclization activity (Fujiwara et al., 1992b).

To elucidate the importance of domain E for the groove integrity and the functioning of a *Bacillus macerans* CGT, three CGT mutants were constructed. In the first construct, a truncated CGT (CGTΔE), part of the groove was destroyed by removing the entire domain E. In the second construct, designated chimeric CGT (CGTSBD), domain E was replaced by the starch binding domain (SBD) of glucoamylase I (GAI) of *Aspergillus niger* whose three-dimensional structure and function is similar to the domain E of CGT (Dalmia et al., 1995; Svensson et al., 1989). In the last construct, a modified CGT (CGT+6), six amino acids were inserted between domains D and E to create a short linker. The objective of the insertion study was to affect the integrity of the groove structure by changing the distance and/or orientation of domain E relative to domain A.

MATERIALS AND METHODS

Strains and plasmids

E. coli strains XL1blue (Stratagene, La Jolla, CA) and Novablue (Novagen, Madison, WI) were used for general DNA manipulation. The pT7Blue T-vector for cloning of PCR fragments was purchased from Novagen. pKK223-3 (Pharmacia Piscataway, NJ) and pET-21(+) (Novagen) vectors were used for overexpression of CGT in *E. coli* JM105 and BL21(DE3), respectively. Plasmid pLCGT1 (Lee et al., 1994), containing 2.3 Kb of the *Bacillus macerans* CGT gene in vector pCRII (Invitrogen, San Diego, CA), was obtained from Dr. B. Tao of Purdue University. The sequence of the *Bacillus macerans* CGT gene was described by Takano et al., 1986. Plasmid pREI containing the 2.1Kb glucoamylase I (GAI) gene from *Aspergillus niger* in pBS+ vector (Stratagene) was obtained from Dr. C. Ford of Iowa State University (Evans et al., 1990; Svensson et al., 1983).

General DNA manipulation was done by the standard methods described in Maniatis et al., 1989. *E. coli* was transformed using electroporation according to Ausubel et al., 1987. Small and large scale plasmid preparations were performed using Wizard Minipreps and Qiagen-tip kits purchased from Promega (Madison, WI) and Qiagen (Chatsworth, CA). Restriction endonucleases, ligase and Taq polymerase were purchased from Promega. Alkaline phosphatase and Klenow fragment polymerase were purchased from Pharmacia. Geneclean kit used for purifying DNA recovered from cells were purchased from Bio101 (Vista, CA).

Construction and manipulation of CGT genes

To construct a plasmid for overexpression of the *B. macerans* CGT gene in *E. coli*, an EcoRI DNA fragment (2.28 kb) containing the CGT gene from plasmid pLCGT1 was subcloned into pKK223-3 to form plasmid pHC1 (Figure 1). To construct the truncated CGT (CGTΔE) with a deletion of domain E (Thr584-Asn687), a universal primer and a 41-mer were used to amplify the CGT gene in pLCGT1 by polymerase chain reaction (PCR) (Figure 2). The primers were synthesized by the Nucleic Acid Facility of Iowa State University and DNA International Inc.. The DNA sequence of the universal primer was 5'GTAAAACGACGGCCAGT 3'; and the 41-mer primer was 5'GAGCTCCCGGGTTAGCTAGCCAGTACATTGAAGCTTTTGAA 3'. The DNA sequence of the 41-mer primer corresponded to the DNA sequence encoding the region between domains D and E of the CGT protein (Phe577-Leu583). The PCR product was about 1.86 kb and encoded domains A through D. The 41-mer primer was designed to generate a NheI restriction site, a stop codon and a SmaI restriction site at the 3' end (corresponding to Leu583) of the PCR fragment (Figure 2). The 1.86 kb PCR fragment was cloned into pT7Blue T-vector (Novagen), and designated as pHCPCR. To create an expression plasmid for the truncated CGT gene (CGTΔE), plasmid pHC1 DNA was digested with BstXI and SmaI. The large fragment (6.11 kb) containing vector pKK223-3 and part of the CGT gene (corresponding to Ser1-Ile470) was ligated with the 0.34 kb BstXI-SmaI DNA fragment (corresponding to Thr471-Leu583) of the 1.86 kb PCR fragment from pHCPCR to construct plasmid pHC2. Plasmid pHC2 contained the pKK223-3

vector, part of the CGT gene encoding domains A to D, and an NheI site and stop codon at the 3'-end of the truncated CGT gene (Figure 2). The NheI site in pHC2 was used as a convenient restriction site for constructing chimeric and modified CGTs.

To create the chimeric CGT (CGT-SBD), a NheI-PstI fragment (0.5 kb) from plasmid pREI, containing the sequence of the starch-binding-domain of GAI, was inserted into NheI-PstI site of plasmid pHC2. The resulting plasmid was named pHC3 (Figure 3).

A modified CGT with a six-amino-acid insertion (CGT+6) between domains D and E was constructed as follows (Figure 4). A 0.4 kb HindIII fragment was isolated from pHC1 and the cohesive ends of the HindIII fragment were partially filled in with dGTP and dATP. The modified 0.4 kb HindIII fragment from pHC1 encoded domain E, five amino acids (Ser579-Leu583) of domain D, and one amino acid (Ala) derived from modifying the 5'-end of the HindIII fragment. Plasmid pHC2 DNA was digested with NheI and subsequently partially filled in with dCTP and dTTP to be compatible with the above modified HindIII ends. The modified pHC1 HindIII fragment was inserted into the modified NheI site of pHC2 to create pHC4 (Figure 4).

DNA sequencing was done by the Nucleic Acid Facility of Iowa State University to confirm that the DNA sequences of pHC1, pHC2, pHC3 and pHC4 were correct. The domain organization of the CGT constructs are shown in Figure 5.

In order to obtain higher expression, CGTs were also subcloned into a pET-21(+) vector. To subclone wild type CGT, a BamHI-SalI DNA fragment (2.28 kb) from plasmid pLCGT1 was cloned into pET-21(+) vector, and designated as pHC1W. To subclone the truncated CGT, an EcoRI-SalI DNA fragment (1.86 kb) from plasmid pHC2 was cloned into vector pET-21(+), and designated as pHC2T. To subclone the modified CGT, a SalI-BamHI DNA fragment (2.3kb) from pHC4 was cloned into vector pET-21(+), and designated as pHC4I. To subclone the chimeric CGT, since there was no convenient restriction site availble, different construction schemes were applied. Plasmid pHC4I DNA was digested with SalI, and the cohesive ends were partially filled in with dCTP and dTTP. The modified SalI fragment (7.67 kb) was then digested with NheI to obtain 7.23 kb DNA fragment containing part of CGT (encoding domains A to D). Plasmid pREI DNA was digested with BamHI, and a 0.5 kb BamHI DNA fragment which contained the starch binding domain of GAI was isolated. The cohesive ends of the 0.5 kb BamHI DNA fragment were partially filled in with dGTP and dATP and then digested with NheI. The modified NheI-BamHI DNA fragment (0.5 kb) was ligated with the 7.23 kb DNA fragment from pHC4I to form pHC3C.

Media

LB-amp plates (75 μg/mL ampicillin, 1.5% agar, 1% trypton, 0.5% yeast extract, and 0.5% NaCl, pH7.2) were used for the selection of *E. coli* transformants. X-gal-IPTG plates (LB agar plates containing 50μg/ml ampicillin, 15μg/ml

tetracycline, 70μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), and 80μM IPTG (isopropylthio-β-D-galactoside) were used for screening of PCR recombinants. LB-amp-starch plates (LB-amp agar plates containing 1% soluble starch) were used for plate-enzyme assays to detect the starch-hydrolyzing activity of CGT. LB-amp (75μg/mL) medium was used to cultivate *E. coli* cells for enzyme purification.

Production and purification of CGT

E. coli BL21(DE3) cells containing plasmids pHC1W, pHC2T, pHC3C, or pHC4I were grown in 1L of LB-amp medium at 37 °C. The expression of CGTs was induced with 0.3mM IPTG added at an A_{600} of 0.6. After induction, the cells were incubated at 25 °C for more than 3h. Cells were harvested by centrifugation (5,000xg, 10 min), and then broken by two passes through a French Press at a pressure of 4,000 psi. The soluble crude enzyme was separated from the cell debris by centrifugation (10,000xg, 10min). Affinity chromatography was performed according to Laszlo at al., 1981. The enzyme solution was applied to an α-CD-Sepharose 6B column. After washing the column with more than 10 column volumes of column buffer (1mM NaN_3, 50mM acetate, pH 5.5), the CGT was eluted with 12mM α-CD solution. Epoxy-activated Sepharose 6B was purchased from Pharmacia and the α-CD was coupled to Sepharose 6B to obtain α-CD-Sepharose 6B according to the protocol suggested by Pharmacia.

Enzymatic assays

The cyclization activity of CGT was assayed by using the methylorange method (Lejeune et al., 1989). The enzyme reaction was performed at 25 °C and pH 6.0. The reaction mixture (1 mL) contained 1% soluble starch, 35μM methyl orange in 50mM phosphate buffer (pH 6.0) and 0.2-0.5 μg/mL of CGT. The enzymatic reaction was quenched by addition of 6M HCl to a final concentration of 0.3M. The reaction mixture was incubated at 16 °C for 30min to develop the methyl orange-α-CD complex, and then the absorbance at 505nm was measured. One unit of cyclization activity was defined as the amount of enzyme which catalyzes the formation of 1μmole α-CD per minute under the assay conditions.

The starch-hydrolyzing activity of CGT was measured by a modification of the blue value method (Fuwa, 1954 and Kaneko et al., 1989). The reaction was performed at 25°C in 50 mM phosphate buffer of pH 6.0 using 0.2% soluble starch (Fisher) as a substrate and 0.03-0.08 μg of CGT. To stop the enzymatic reaction, 0.5 mL of 1M acetic acid solution was added to the reaction mixture (0.35 mL). The iodine-starch complex (blue color) was developed by adding 0.5 mL of 0.02% I_2 /0.2% KI solution to the reaction mixture. The reaction mixture was diluted with distilled water to a final volume of 7 mL and the absorbance at 660 nm was measured. One unit of starch-hydrolyzing activity was defined as the amount of enzyme which caused a 10% per minute decrease in the absorbance at 660 nm.

The coupling activity of CGT was assayed using a modification of the method described by Thoma et al., 1965. The reaction mixture (1mL) containing 0.1-0.4 μg

of CGT, 50 μg (2.55 U) of glucoamylase (*Aspergillus niger*; Sigma), 24 mM α-CD, and 100 mM methyl-α-D-glucoside in 50 mM phosphate buffer of pH 6.0 was incubated at 25°C. The enzymatic reaction was stop by boiling the reaction mixture for 10 minutes, and the concentration of glucose in the reaction mixture was determined by using the glucose assay kit from Sigma (Bergmeyer et al., 1974). One unit of coupling activity was defined as the amount of enzyme which catalyzed the transfer of 1 μmole α-CD to methyl-α-D-glucoside per minute under the assay conditions.

In all enzymatic assays, reaction rates were determined by taking at least eight aliquots at equally spaced time intervals. The initial rate of the reaction was estimated by a linear regression of the data points in the linear region of the kinetic curve.

Product specificity of CGT was measured by HPLC. The reaction mixture containing 5% soluble starch, 1.5 mM $CaCl_2$, in 50mM phosphate buffer, pH 6.0, was incubated with 0.2 U/mL (cyclization activity) of CGT in a total volume of 5 ml at 25°C. Aliquots (0.45 mL) were withdrawn at different time points and heated in a boiling water bath for 10min to stop the reaction. The concentration of α-, β-, and γ-CDs in the reaction mixture was determined by HPLC on a Spherisorb-NH_2 column (Phase Separations Inc., Norwalk, CT) equipped with a RI detector (ERC-7512, Erma Inc.). The mobile phase was 65% aqueous acetonitrile at a flow rate of 1 ml/min.

Activation energy

The cyclization and starch-hydrolyzing activities of CGT were determined at different temperatures using the methylorange and blue value methods as mentioned above. The initial rates of the reaction at different temperatures were determined as close to zero time as possible. The activation energy was estimated from the reaction rate at different temperature using equation 1.

$$k = A\, e^{-E_a / RT} \qquad \text{(Eq. 1)}$$

Where k is the rate constant, A is the Arrhenius constant, R is the universal gas constant, T is absolute temperature, and E_a is the activation energy in kJ/mol·K (Palmer, 1991; Maheshwari, 1995).

Thermostability determination

The thermostability of CGT was determined by incubating enzyme aliquots (1800 units/mL based on starch-degrading activity) for 10 minutes at different temperatures in 50 mM sodium phosphate buffer (pH 6.0) containing 1.2 mM $CaCl_2$, then the enzymes were placed on ice immediately after the incubation. The residual activity of starch-hydrolytic activity of CGT was determined in the same day of incubation at 25°C, pH6.0 as mentioned above. To determine the effect of maltose and starch on the enzyme stability, the thermostability experiments were repeated in the presence of 1% (w/v) of either starch or maltose in the reaction mixture.

Determination of kinetic parameters

The kinetic parameters for the cyclization reaction of CGT were estimated by measuring the formation of α-CD from soluble starch with the methylorange method. Conditions for all the kinetic experiments were 25°C and pH 6.0 in 50mM phosphate buffer. The soluble starch concentrations ranged from 0.1 to 20 g/L and the enzyme concentrations from 0.12 to 0.5 μg/ml. The initial reaction rates from the kinetic runs were fitted to the Michaelis-Menten equation (Fersht, 1985; Eisenthal et al, 1992) by using Sigma Plot program from Jandel Scientific (San Rafael, CA). Eight or more reaction rates at different substrate concentrations were used to calculate the kinetic parameters V_{max} and K_m. All kinetic experiments were repeated at least three times. The k_{cat} (the turnover number) values were calculated by dividing V_{max} by the enzyme concentration.

RESULTS

Expression and purification of CGTs

A starch-plate assay was used to examine the expression of CGTs in *E. coli*. *E. coli* JM105 carrying plasmids, pHC1, pHC2, pHC3 or pHC4 was plated on a LB-amp-starch plate (1% starch) and incubated overnight at 37 °C. The formation of halos was examined by exposing the plate to I_2 vapor. The halos which appeared around the colonies indicated that starch was degraded by CGT activity (Figure 6). *E. coli* JM105 carrying pHC1, which codes for WT-CGT, showed a large halo and served as a positive control. *E. coli* JM105 carrying pHC4 which encodes CGT+6

also formed a large halo. The results indicated that WT-CGT and CGT+6 were expressed in *E. coli* and were active. No halos were observed around the colonies of *E. coli* JM105 carrying pHC2 and pHC3, suggesting that either the expression levels of CGTΔE and CGT-SBD were too low to be detected or that the expressed proteins were inactive.

Because CGTΔE and CGT-SBD did not show activity on the starch plate their expression was further investigated by using SDS-PAGE and Western blot assays. A crude cell extract of *E. coli* BL21(DE3) with pHC2T was loaded in lane 4 (Figures 7a, 7b), whereas crude cell extract of a negative control (untransformed *E. coli* BL21(DE3)) was loaded in lane 3 (Figure 7a, 7b). A protein band with molecular weight of about 60 kDa appeared on both SDS-PAGE (Figure 7a) and the Western blot (Figure 7b) indicating the production of CGTΔE. The migration distance difference between the WT-CGT band (lane 2) and that of CGTΔE (lane 4) corresponds to the size of domain E. A crude extract of *E. coli* BL21(DE3) containing the CGT-SBD encoding plasmid (pHC3C) was loaded in lane 3 (Figures 8a and 8b). A protein band of similar molecular weight as the WT-CGT band (Figure 8a, lane 1) was detected in lane 3 of the SDS-PAGE gel indicating expression of CGT-SBD in *E. coli.* To confirm that the produced protein contained the SBD of GAI, anti-GAI polyclonal antibodies were used to detect the expressed protein (Figure 8b).

Crude extracts of WT-CGT and CGT mutants were loaded on α-CD-Sepharose 6B column for affinity purification. Judging from the SDS-PAGE and

Western blot data, lane 5 in Figure 7, CGTΔE did not bind to α-CD-Sepharose 6B. The elution of the resin with 12mM α-CD did not produce any detectable protein or CGT activity. CGT-SBD was purified by affinity chromatography on an α-CD-Sepharose 6B column (lane 4, Figure 8). The multiple-band pattern on the Western blot (Figure 8b, lane4) indicated a proteolytic degradation of CGT-SBD similar to that observed with β-Gal-SBD fusion proteins (Dalmia et al. 1995). Because the SBD interacts specifically with α-CD, the affinity purification on the α-CD Sepharose column apparently enriched the crude CGT-SBD sample in proteolytic fragments containing the intact SBD domain (compare lanes 3 and 4). No further proteolytic degradation of CGT-SBD was detected after storing the purified protein at 4°C for one week. WT-CGT and CGT+6 were purified from the crude cell extracts by affinity chromatography on α-CD Sepharose 6B and appeared as single protein bands on the SDS-PAGE and Western blot (data not shown).

Activity and stability of purified CGT mutants

Because CGTΔE in the crude cell extract did not have any detectable activity and could not be purified by affinity chromatography, further characterization of the mutant was not performed.

The thermostabilities of purified WT-CGT and CGT+6 were compared in the presence and absence of substrate molecules, starch and maltose. Soluble starch is a long molecule (DP>100) which can bridge the active site in domain A and the maltose binding site of domain E (Fujiwara et al., 1992a). Maltose (DP=2) is not

long enough to extend from the active site to domain E and served as a negative control. The effect of maltose or soluble starch addition on the thermostability of purified WT-CGT and CGT+6 is given in Figure 9. In general, the denaturation curve profiles indicate that CGT+6 is less thermostable than WT-CGT. The activity of CGT+6 in the absence of substrate started to decline at 35°C whereas that of WT-CGT declined at 50°C. The temperature corresponding to 50% residual activity ($T_{50\%}$) was about 57°C for WT-CGT and about 47°C for CGT+6. The addition of maltose had a slight stabilization effect, whereas soluble starch had a significant effect on stability of both WT-CGT and CGT+6. The presence of starch had a more pronounced effect on the initial temperature of the residual activity decline for CGT+6 than for WT-CGT but a greater overall stabilizing effect was observed with WT-CGT than with CGT+6. The slope of the denaturation curve of WT-CGT was more shallow than the curve of CGT+6. Compared with the respective controls, the $T_{50\%}$ for WT-CGT increased by approximately 13°C and that for CGT+6 by about 6°C.

The cyclization activities of purified WT-CGT and CGT+6 were measured at different temperatures. As shown in Figure 10 the temperature-activity curves of WT-CGT and CGT+6 had similar patterns, but the specific activities of CGT+6 at all temperatures were smaller than that of WT-CGT. The activation energy of cyclization and starch-hydrolyzing reactions of WT-CGT and CGT+6 is shown in Table 1. The energy of activation values were analyzed by using χ^2 test (Steel, et al., 1980) and showed no significant difference. The same activation energies

suggest that the insertion of six amino acids in CGT did not change the rate limiting step of the cyclization and starch-hydrolyzing ractions.

The starch-hydrolyzing, cyclization, and coupling activities of purified CGT-SBD and CGT+6 were compared to that of WT-CGT at 25°C and pH 6.0 (Table 2). As shown in Table 2, CGT+6 had 54, 59, and 61% of the starch-hydrolyzing, cyclization, and coupling activity of WT-CGT, respectively. The results suggest that the insertion of six amino acids in the WT-CGT affected the three activities of CGT+6 in a similar way. When incubated with 0.2% soluble starch CGT-SBD displayed very low starch-hydrolyzing activity and the change in the absorbance at 660 nm was difficult to measure accurately. Cyclization and coupling activity of CGT-SBD were approximately 0.03% of the respective activities of WT-CGT (Table 2).

The kinetic parameters of CGT+6

Because *Bacillus macerans* CGT produces mainly α-CD in the early stage of the cyclization reaction, kinetic parameters were estimated by measuring the initial rates of α-CD formation. The cyclization reaction was performed by using soluble starch as substrate at 25°C. The estimated K_m value of WT-CGT was about 2.6 times smaller than that of CGT+6 suggesting a significant decrease in affinity for the soluble starch molecules (Table 3). The k_{cat} value of CGT+6 was half of the k_{cat} value of WT-CGT. The k_{cat}/K_m value of CGT+6 was even lower, about 20% of the value of WT-CGT. The lower k_{cat}/K_m value indicates that the efficiency of the

cyclization reaction catalyzed by CGT+6 was significantly reduced by inserting the six amino acid residues between the domains D and E.

Product specificity of CGT+6

The amounts of α-, β-, and γ-CDs produced from soluble starch by WT-CGT and CGT+6 were assayed by HPLC. The time-course of cyclodextrin production by CGTs is shown in Fig. 11. WT-CGT and CGT+6 produced mainly α-CD at the initial stage of the reaction. The α-CD production gradually decreased whereas the amount of β-CD increased with the progression of the reaction. The kinetics of CD production were the same for the mutant and the wild-type enzyme. In conclusion, no significant difference of product distribution was measured between WT-CGT and CGT+6.

DISCUSSION

CGTΔE was produced in *E. coli* but no measurable activity in the crude cell extract was detected. The data is compatible with previous studies which showed that C-terminal deletion of CGT led to lower activity or no activity at all (Kimura et al, 1989; Hellman et al., 1990). The CGTΔE mutant did not bind to α-CD-Sepharose suggesting that either immobilized α-CD was not accessible for binding in the active site or major perturbation of the active site structure had occurred because of the lack of domain E. Removal of domain E probably interrupted the groove that extends between the active site and the MBS2 in domain E. Another possibility is

that loss of domain E led to improper folding of the molecule. Since no major degradation of CGTΔE was observed by Western Blot (Figure 7b), improper folding of CGTΔE is unlikely.

CGT-SBD was purified by affinity chromatography based on the interaction between the SBD and α-CD indicating proper folding and functioning of the SBD. The partial degradation of the CGT-SBD observed on the Western blot (Figure 8b) has also been previously observed with β-Gal-SBD fusion (Chen et al., 1991; Dalmia et al., 1995). A greater number of protein bands appeared in lane 4 in Figure 8b (purified sample) than in lane 3 (crude extract) apparently due to preferential column binding of degradation fragments carrying the intact SBD. The purified enzyme had all three activities but at a very low level. Domain E of CGT and the SBD of GAI have been shown to be independent domains with similar functions (Chen et al., 1991; Dalmia et al., 1995) and structures. However, the specific activity of CGT-SBD was only 0.026% of WT-CGT, indicating domain E is an integral part of the CGT protein structure, and that replacing it with a similar starch binding domain (SBD) is not sufficient for the recovery of activity. 104 amino acids from the C-terminus of CGT were deleted in CGT-SBD. The fused SBD in CGT-SBD is slightly larger and consists of 120 amino acids (Figure 5). Domain E was reported to cooperate with the active site in binding starch (Fujiwara, S. et al., 1992a; Penninga et al., 1996). Even though the SBD is capable of binding starch, the additional 16 amino acids may affect the orientation and alignment of MBS2 with active site leading to low CGT activities.

The thermostability of CGT+6 increased in the presence of starch (Figure 9b), indicating that domain E is functional in binding to starch. The comparison of the denaturation curve slopes in the presence of starch (Figure 9) suggest that binding of soluble starch had a greater stabilizing affect for WT-CGT than for CGT+6. Insertion of six amino acids apparently affected soluble starch binding. The K_m value of CGT+6 was 2.6 fold of that of WT-CGT, indicating less affinity of CGT+6 for starch. The k_{cat}/K_m value of CGT+6 was only 20% of that of WT-CGT. This suggests that the catalytic efficiency of the cyclization reaction was significantly decreased. Even though the starch binding was changed in CGT+6, the product distribution patterns of WT-CGT and CGT+6 were similar. This implies that domain E does not affect the product specificity. The result agrees with the active site studies of CGT, which proposed that the reaction specificity was determined by the catalytic domain (Strokopytov et al., 1996).

The modeling study by Strokopytov et al. (1996) suggested that part of the oligosaccharide chain needs to adapt a bent conformation, when amylose is bound into the deep groove. A similar turn of the oligosaccharide chain also was observed when a maltononaose was bound in the active site. Several residues in the groove were assumed to form hydrogen bonds (Strokopytov et al., 1996), or have major interaction (Lawson et al., 1994) with bound amylose. We propose that the groove running from the catalytic residues to MBS2 of domain E has similar subsite structure allowing the binding of oligosaccharides. According to this hypothesis, each subsite in the groove binds a single glucose residue of the bound amylose and

the groove can be considered as an extension of the active site. The seven subsites preceeding the catalytic residues plus the subsites which extend from the catalytic residues to MBS2 could be viewed by this model as an intact long binding site for starch molecules. Therefore, to maintain the continuity of the groove subsites, domain E should be well-positioned and relatively immobile. In CGT-SBD, the starch chain bound to the SBD might not be properly positioned to lead to the active site due to the mobility of the SBD and the lack of integrity of the groove. This would explain the very low CGT activities of CGT-SBD. The insertion of a six-amino-acid linker between domains D and E may have led to the "misalignment" of MBS2 and the rest of the groove resulting in a weaker binding of starch. This would explain the higher K_m value and decreased catalytic efficiency of CGT+6. Our findings support the hypothesis that domain E has to be positioned at the optimal distance and/or orientation with respect to the active site, as in the WT-CGT, to allow for optimal binding of the soluble starch substrate in the groove. Our results also suggest that domain E is important for maintaining the integrity of the groove structure.

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Table 1. Activation energy of starch-hydrolysis and cyclization reactions

Enzyme	Starch hydrolysis E_a (kJ/mol·K)	Cyclization E_a (kJ/mol·K)
WT-CGT	43	44
CGT+6	40	46

Table 2. The specific enzyme activities of CGTs

Enzyme	Starch hydrolysis (units/mg)	Cyclization (units/mg)	Coupling (units/mg)
WT-CGT	6700±100	118±3	206±7
CGT+6	3600±70	70±2	125±3
CGT-SBD	ND[a]	0.031	0.054±0.003

[a] ND, not determined

Table 3. The kinetic parameters of CGTs

Enzyme	K_m (g/L)	k_{cat} (sec^{-1})	k_{cat} / K_m ($sec^{-1}g^{-1}L$)
WT-CGT	0.26±0.03	192±4	750±80
CGT+6	0.66±0.08	102±3	160±20

FIGURE LEGENDS

Figure 1. Construction of pHC1.

Figure 2. Construction of pHC2.

Figure 3. Construction of pHC3.

Figure 4. Construction of pHC4.

Figure 5. Schematic representation of CGT constructs. CGT was divided into five domains A, B, C, D and E, according to the 3-D structure of CGT from *Bacillus circulans*. CGT, cyclodextrin glucanotransferase; SBD, starch-binding domain of glucoamylase I.

Figure 6. Halos formed by *E. coli* transformants on starch plate. Each transformant was plated onto a LB-amp-starch plate (1% starch) and incubated overnight at 37 °C. After incubation, the plates were exposed to I_2 vapor to examine the formation of halos. (A) *E. coli* JM105 carrying pHC1 (WT-CGT); (B) *E. coli* JM105 carrying pHC2 (CGTΔE); (C) *E. coli* JM105 carrying pHC3 (CGT-SBD); (D) *E. coli* JM105 carrying pHC4 (CGT+6).

Figure 7. Expression of truncated CGT (CGTΔE). (a) SDS-PAGE (8.5%), (b) Western Blot (anti-CGT). Lane 1, molecular weight standards. Lane 2, purified WT-CGT. Lane 3, control, crude extract of BL21(DE3) cell without plasmid. Lane 4, cell crude extract contained CGTΔE. Lane 5, flow-through fraction. The cell crude

extract of CGTΔE was loaded to α–CD-Sepharose 6B column. The crude extract which was collected after passage through the column still contained CGTΔE.

Figure 8. Expression of chimeric CGT (CGT-SBD). (a) SDS-PAGE (8.5%), (b) Western Blot (anti-GAI). Lane 1, purified WT-CGT. Lane 2, molecular weight standards. Lane 3, cell crude extract contained CGT-SBD. Lane 4, purified CGT-SBD. Lane 5, molecular weight standards for Western blot.

Figure 9. Thermostability of CGTs. The thermostability of CGT was assayed at different temperatures. Starch and maltose were added to the reaction mixture to investigate the effect of substrate stabilization. CGT in 50 mM sodium phosphate buffer (pH 6.0) containing 1.2mM $CaCl_2$ (1800 units/mL based on starch-degrading activity) was incubated for 10 minutes at different temperatures. The residual activity of starch-hydrolytic activity of CGT was determined at 25°C, pH6.0. The experimental errors of all points are less than 10%. (a) WT-CGT, (b) CGT+6. ● , CGT ; ■ , CGT with maltose addition (1% w/v) ; ▲ , CGT with starch addition (1% w/v).

Figure 10. Cyclization activity of CGTs at different temperatures. The cyclization activities of WT-CGT and CGT+6 were determined at different temperatures using the methylorange method. Soluble starch was used as substrate. ● , WT-CGT; ■ , CGT+6.

Figure 11. Production of α-, β-, and γ- cyclodextrins from soluble starch. The reaction mixture containing CGT (0.2 units/mL, cyclization activity), 5% soluble starch, 1.5 mM $CaCl_2$, 50mM phosphate buffer, pH 6.0, was incubated at 25°C with a total volume of 5 ml. Aliquots (0.45 mL) were withdrawn at different time points and heated in a boiling water bath for 10min to stop the reaction. The concentration of α-, β-, and γ-CDs in the reaction mixture was determined by HPLC. (a) WT-CGT, (b) CGT+6. O , αCD; □ , βCD; Δ, γCD.

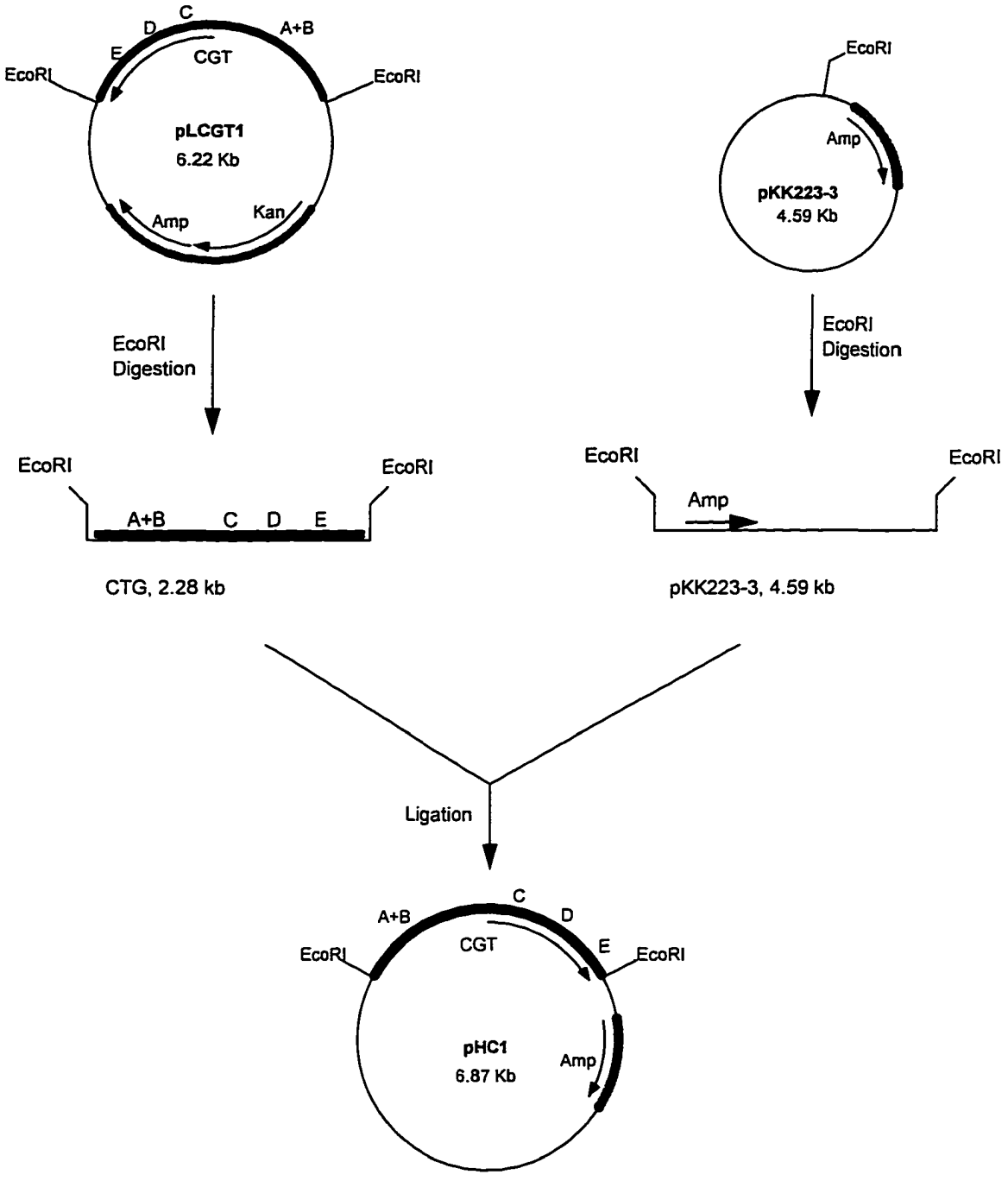
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Figure 1

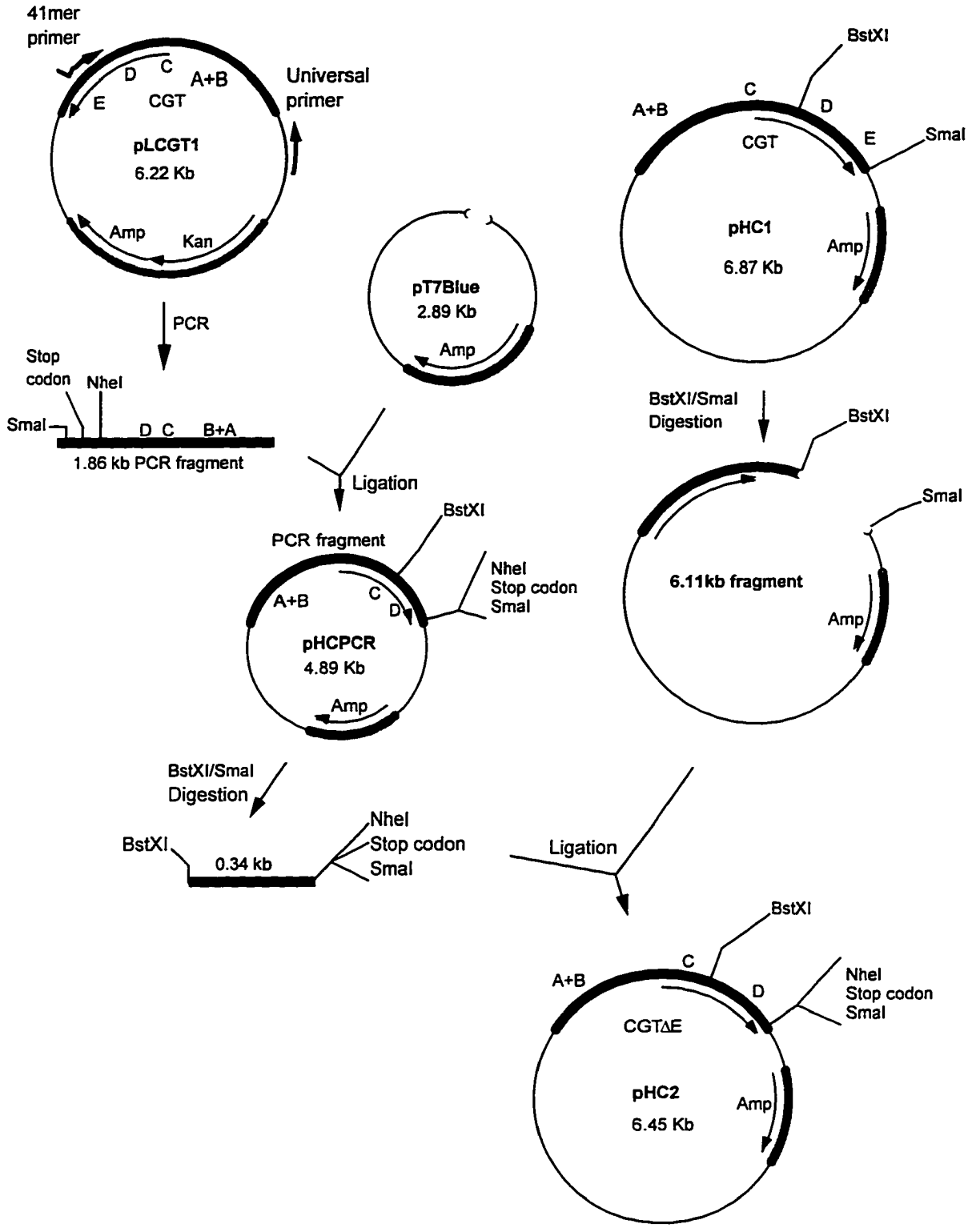
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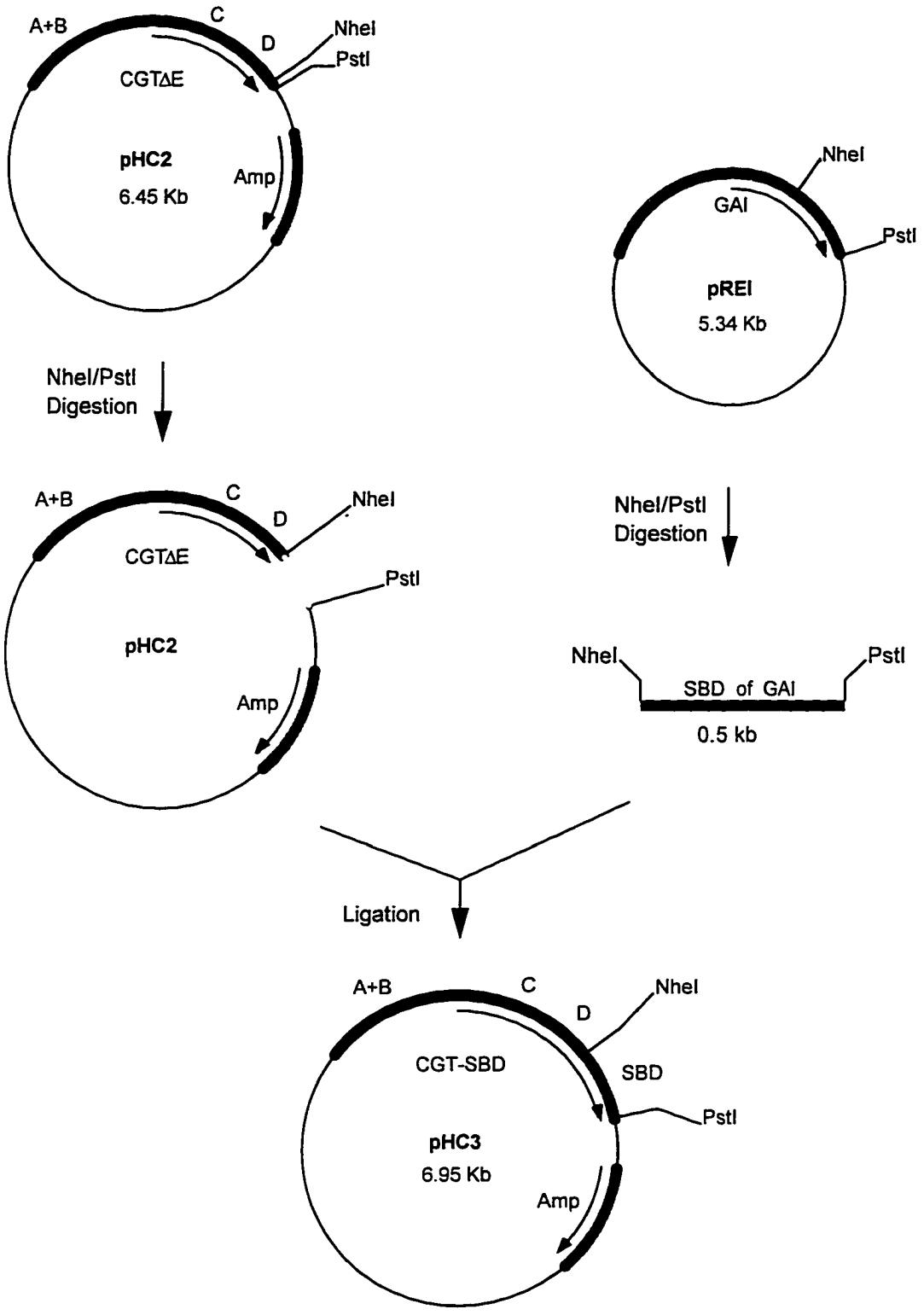
Figure 2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SBD
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6.95 Kb
Amp

Figure 3

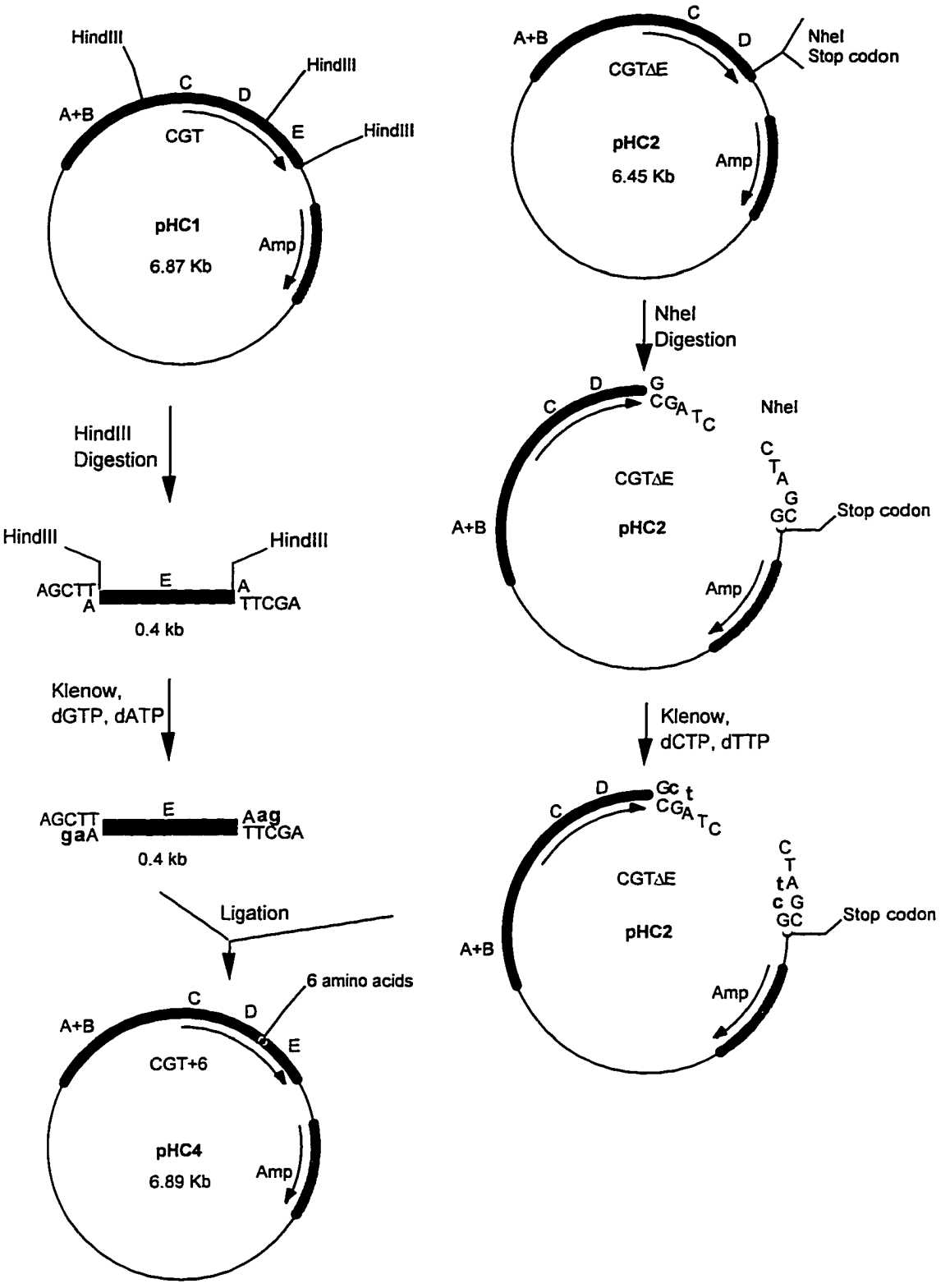

HindIII
HindIII
HindIII
A+B
C
D
E
CGT
pHC1
6.87 Kb
Amp
HindIII
Digestion
HindIII
HindIII
AGCTT
A
E
A
TTCGA
0.4 kb
Klenow,
dGTP, dATP
AGCTT
gaA
E
Aag
TTCGA
0.4 kb
Ligation
6 amino acids
A+B
C
D
E
CGT+6
pHC4
6.89 Kb
Amp
A+B
C
D
CGTΔE
pHC2
6.45 Kb
Amp
NheI
Stop codon
NheI
Digestion
A+B
C
D
G
CGA TC
CGTΔE
pHC2
Amp
NheI
C
TA
G
GC
Stop codon
Klenow,
dCTP, dTTP
A+B
C
D
Gc t
CGA TC
CGTΔE
pHC2
Amp
C
T
tA
c G
GC
Stop codon

Figure 4

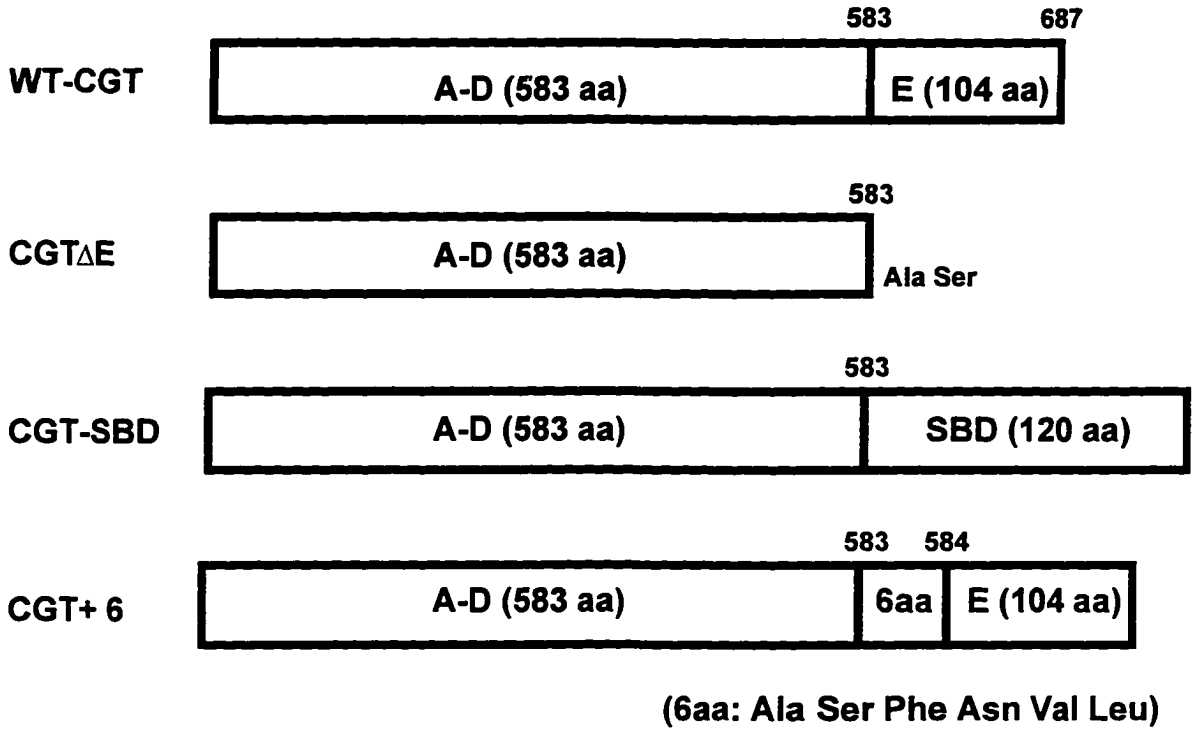

583
687
WT-CGT
A-D (583 aa)
E (104 aa)
583
CGTΔE
A-D (583 aa)
Ala Ser
583
CGT-SBD
A-D (583 aa)
SBD (120 aa)
583
584
CGT+ 6
A-D (583 aa)
6aa
E (104 aa)
(6aa: Ala Ser Phe Asn Val Leu)

Figure 5

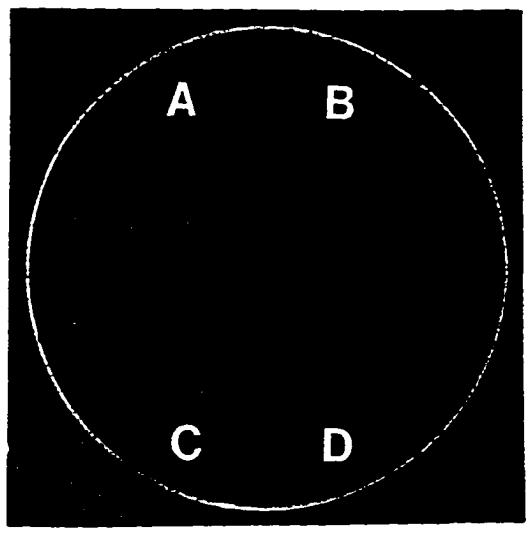


Figure 6

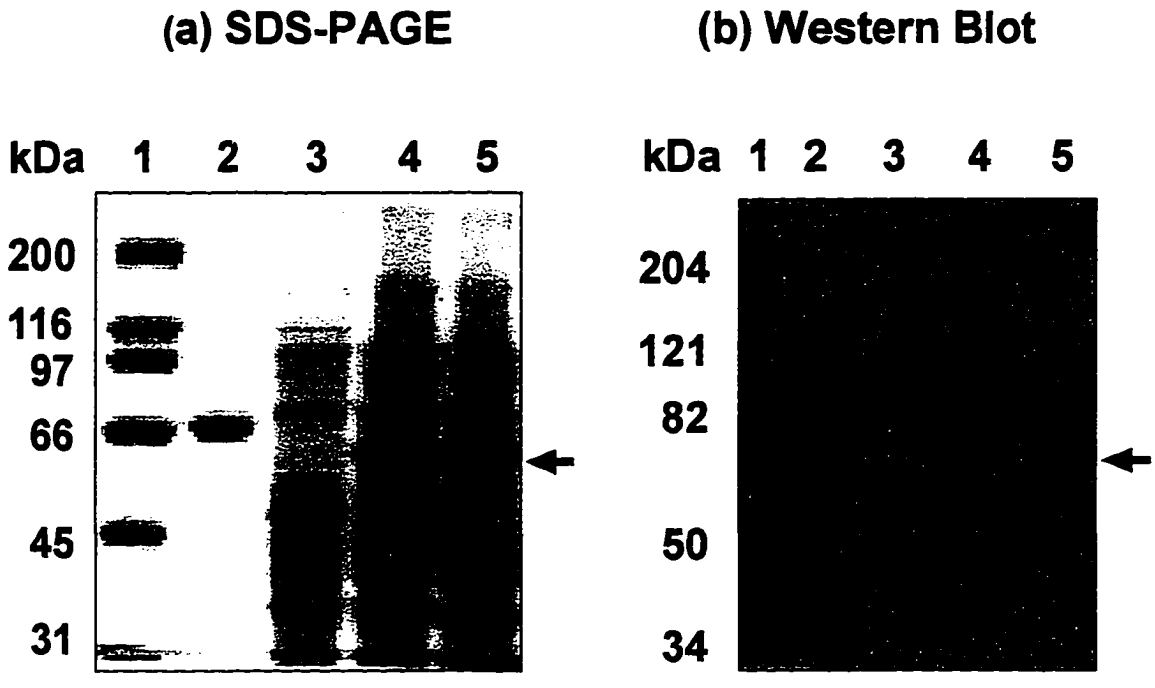

(a) SDS-PAGE
(b) Western Blot
kDa 1 2 3 4 5
200
116
97
66
45
31
kDa 1 2 3 4 5
204
121
82
50
34

Figure 7

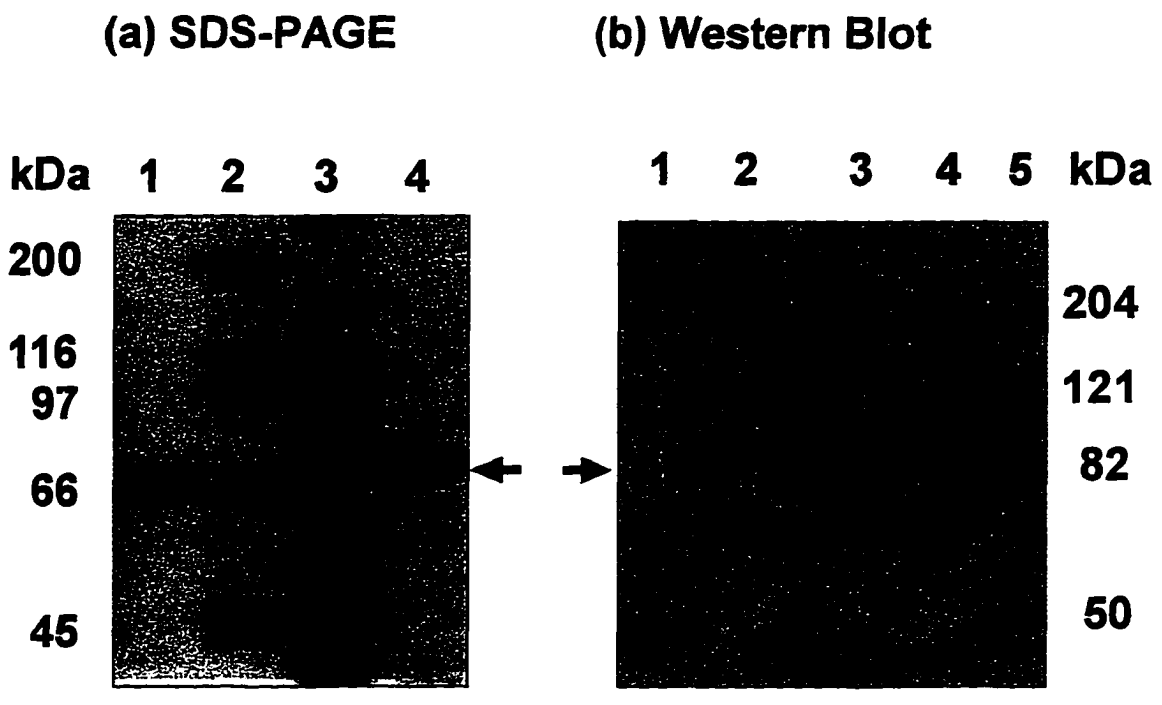
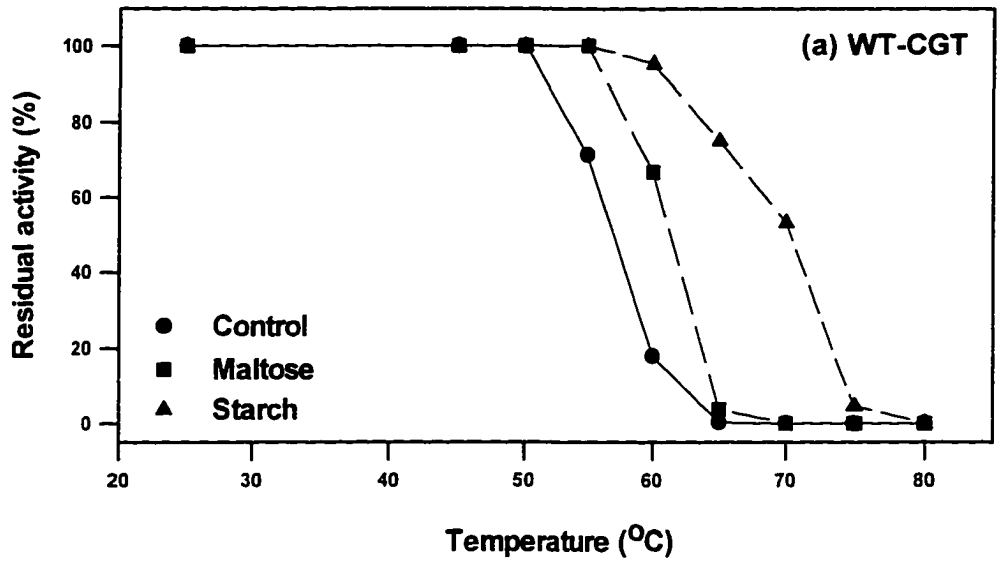

(a) SDS-PAGE
(b) Western Blot
kDa
1
2
3
4
200
116
97
66
45
1
2
3
4
5
kDa
204
121
82
50

Figure 8


(a) WT-CGT
Residual activity (%)
100
80
60
40
20
0
Control
Maltose
Starch
20
30
40
50
60
70
80
Temperature (°C)

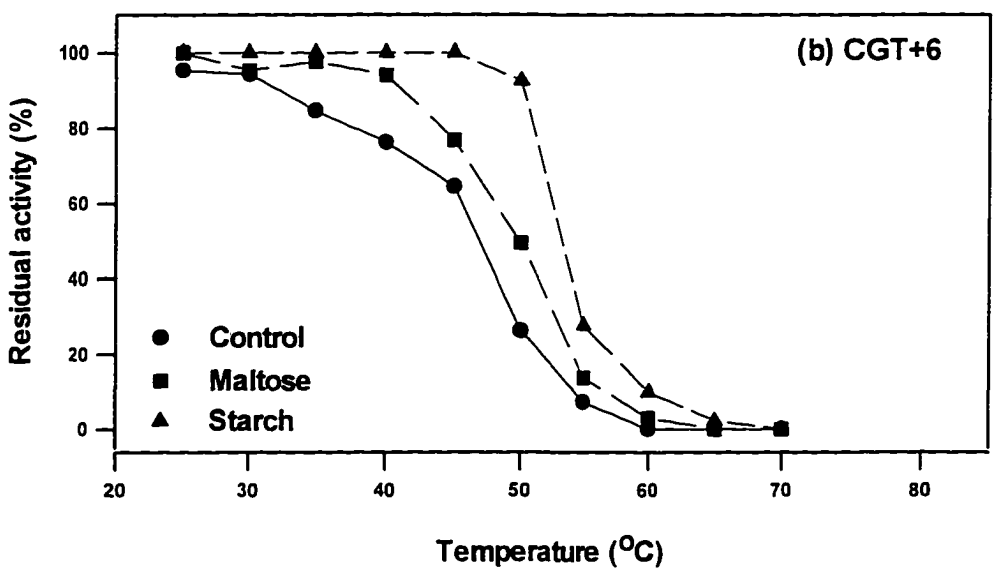

(b) CGT+6
Residual activity (%)
100
80
60
40
20
0
Control
Maltose
Starch
20
30
40
50
60
70
80
Temperature (°C)

Figure 9

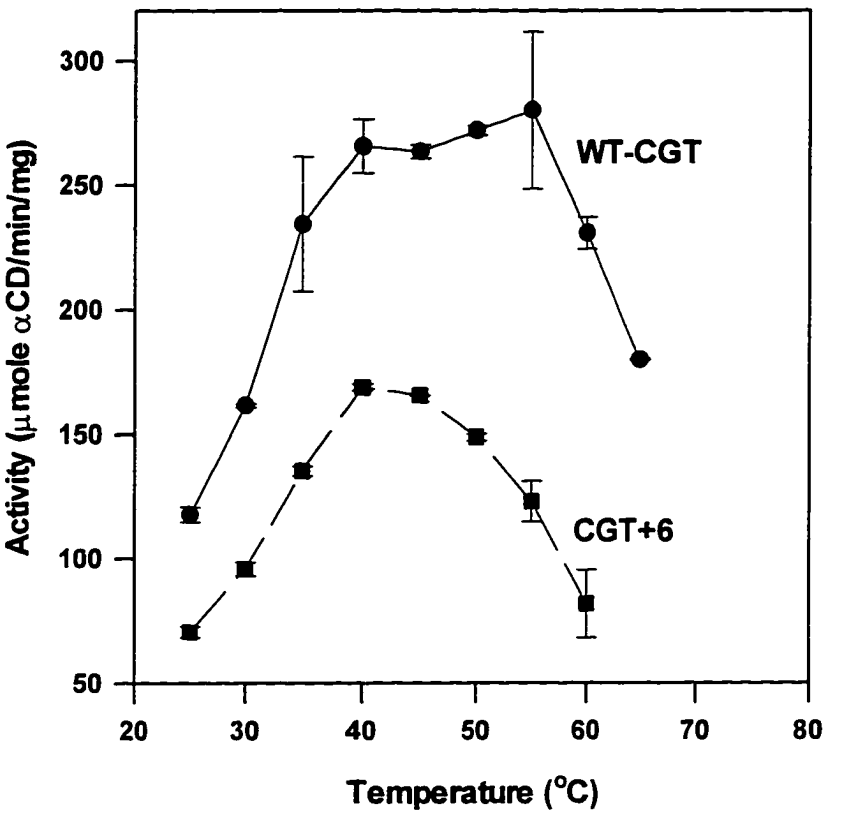

300
250
200
150
100
50
20
30
40
50
60
70
80
Activity (μmole αCD/min/mg)
Temperature (°C)
WT-CGT
CGT+6

Figure 10

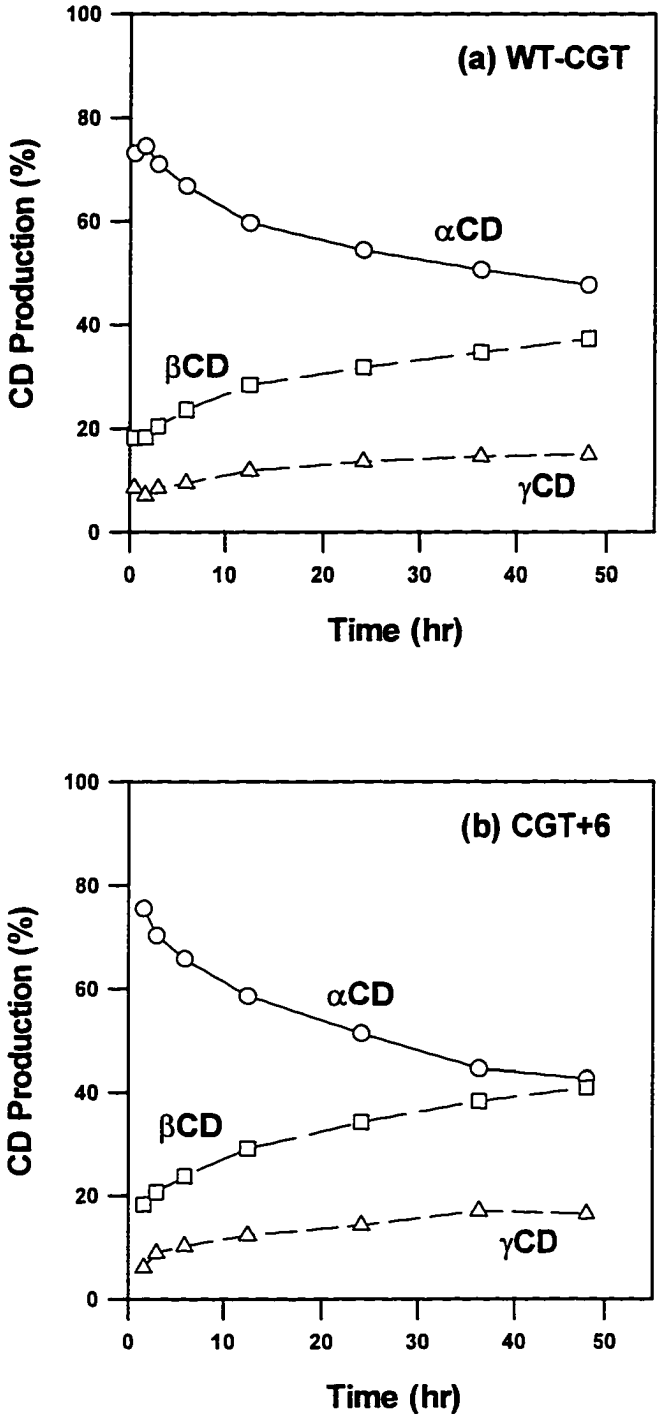

(a) WT-CGT
αCD
βCD
γCD
CD Production (%)
Time (hr)
0
20
40
60
80
100
0
10
20
30
40
50
(b) CGT+6
αCD
βCD
γCD
CD Production (%)
Time (hr)

Figure 11

CHAPTER 3. SITE-DIRECTED MUTAGENESIS IN THE MALTOSE BINDING SITE OF DOMAIN E OF CYCLODEXTRIN GLUCANOTRANSFERASE FROM *BACILLUS MACERANS*

A manuscript to be submitted to Applied Biochemistry and Biotechnology

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ABSTRACT

The goal of this study was to investigate the function of the second maltose binding site (MBS2) of domain E of cyclodextrin glucanotransferase (CGT) from *Bacillus macerans* and, in particular, the role of the Tyr634 residue of the MBS2 in the interaction with CGT substrates. The MBS2 is located in the opening of a deep groove structure which was observed in the protein surface of CGT and extends from the active site to domain E. To reveal the function of MBS2 in the catalysis of CGT, Tyr634 which has strong hydrophobic interactions with maltose was replaced with glycine (Y634G) and phenylalanine (Y634F), respectively. In addition, two β-galactosidase-domain E fusion proteins, β–Gal-E(WT) and β–Gal-E(Y634G) were constructed and studied to understand the binding behavior of domain E without the interference of the active site.

The stability and specific activity of mutant Y634F was similar to that of WT-CGT. Starch provided little thermal stabilization to Y634G, suggesting that the aromatic side chain of Tyr634 is important in the binding of starch. The K_m value of

Y634G for the cyclization reaction with starch as a substrate was about 1.4 fold greater than that of WT-CGT indicating reduced affinity for the substrate. β–Gal-E(Y634G) showed less interaction with α–CD-Sepharose, indicating that Tyr634 also is important for the binding of α–CD. The reduced binding of α–CD in Y634G may have reduced product inhibition, resulting in a slightly higher k_{cat} value.

INTRODUCTION

Cyclodextrin glucanotransferase (EC 2.4.1.19) is an industrially important enzyme which produces cyclodextrins (CDs) from starch. CDs are cyclic, nonreducing oligosaccharides which consist of six (α-CD), seven (β-CD), or eight (γ-CD) α-1, 4-linked D-glucopyranosyl residues (Schmid, 1989). The hydrophobic cavity of CD can entrap different organic molecules to form inclusion complexes. Since the formation of inclusion complexes alters the chemical and physical properties of trapped molecules, CDs have increasing applications in pharmaceutical, food, agricultural and chemical industries.

Cyclodextrin glucanotransferase (CGT) produces CDs from starch by an intramolecular transglycosylation reaction (cyclization). In addition, CGT catalyzes intermolecular transglycosylation (coupling and disproportionation reactions) and hydrolytic reactions (Kitahata, 1988). CGTs can be distinguished as α-, β-, or γ-CGT according to the main cyclodextrin produced in the early stage of the cyclization reaction. The CGT of *Bacillus macerans* has been classified as α-CGT.

Three-dimensional structures of several CGTs showed that these enzymes consist of five domains A, B, C, D, and E (Hofmann et al., 1989; Kubota et al., 1991; Lawson et al., 1994). The folding pattern of domains A, B, and C of CGT is similar to that of α-amylase. Four conserved amino acid regions have also been identified in CGT and α-amylase (Macgreor et al., 1989; Binder et al., 1986; Kimura et al., 1987). Sequence comparisons revealed that domain E has 60% amino acid similarity with the starch binding domain (SBD) of *Aspergillus* glucoamylase I (Svensson et al., 1989). Dalmia et al. (1995) confirmed that domain E is an independent starch-binding domain similar to SBD from *Aspergillus* glucoamylase I, suggesting that domain E is a raw-starch binding region. The chain fold of CGT can be considered as α-amylase with two additional domains D and E.

Two maltose binding sites (MBS1 and MBS2) were detected in domain E of CGT (Lawson et al., 1994). The second maltose binding site (MBS2) is located in the opening of a deep groove which extends from domain E to the active site. The first maltose binding site (MBS1) is away from the active site and the groove. X-ray structure studies revealed that α-cyclodextrin, as well as maltose, can bind to MBS1 and MBS2 (Knegtel et al., 1995). A modeling study from the same group suggested that linear starch molecules can fit into the groove (Strokopytov et al., 1996). Fujiwara et al.(1992) proposed that the linear starch molecule serves as a bridge connecting the active site and domain E, resulting in a stabilized CGT-starch structure.

Several C-terminal deletion studies were performed to investigate the role of domain E in CGT function. One study suggested that domain E does not affect the catalysis of CGT (Bender, 1990). Another study suggested that Domain E participated in the cyclization activity, but not in starch-hydrolytic activity (Kimura et al., 1989). The study of Hellman et al.(1990) showed domain E can affect both cyclization and starch-hydrolytic activity.

Because MBS2 is part of the groove which leads to the active site, suggesting its importance in the catalysis of CGT, we have chosen to probe the effect of modifying the tyrosine residue (Tyr634) in MBS2 of CGT from *Bacillus macerans*. Because the phenyl ring of Tyr634 has hydrophobic contacts with maltose (Lawson et al., 1994), this residue was substituted by glycine (Y634G) to eliminate the hydrophobic interaction. In addition, the Tyr634 residue was replaced by phenylalanine (Y634F) which has a smaller side chain than tyrosine. The mutated CGTs were used to elucidate the importance of MBS2 in the catalysis of CGT.

In order to study the binding behavior of domain E without interference from the active site, domain E was isolated and fused to the C-terminus of β-galactosidase (βGal-E). The interaction of the two fusion proteins, βGal-E(WT) and βGal-E(Y634G), with α–CD-Sepharose were studied.

MATERIALS AND METHODS

Strains and plasmids

E. coli strains XL1blue (Stratagene, La Jolla, CA) and Novablue (Novagen, Madison, WI) were used for general DNA manipulation. *E. coli* strain BL21(DE3) (Novagen) was used for the production of CGT. *E. coli* strain TG1 was used for the production of β-galactosidase-domain E fusion proteins. Plasmid pLCGT1 (Lee et al., 1994), containing 2.3 Kb CGT gene of *Bacillus macerans* in pCRII vector (Invitrogen, San Diego, CA), was obtained from Dr. B. Tao of Purdue University. The pET-21(+) vector for overexpression of CGTs in *E. coli* was purchased from Novagen. The sequence of CGT gene was reported by Takano et al. (1986).

DNA manipulation and transformation experiments

General DNA manipulations were performed by using standard methods described in Maniatis et al., 1989. *E. coli* was transformed using electroporation according to Aubusel et al., 1987. Small and large scale plasmid preparations were performed using Wizard Minipreps and Qiagen-tip purchased from Promega (Madison, WI) and Qiagen (Chatsworth, CA). Restriction endonuclease and ligase were purchased from Promega and the Geneclean kit from Bio101 (Vista, CA).

To construct a plasmid for overexpression of *Bacillus macerans* CGT gene in *E. coli*, a BamH1-Sal1 DNA fragment (2.28 kb) containing CGT gene from plasmid pLCGT1 was subcloned into pET-21(+) vector to form plasmid pHC1W.

Media

The LB-amp plates (75 μg/ml ampicillin, 1.5% agar, 1% trypton, 0.5% yeast extract, and 0.5% NaCl, pH7.2) were used for the selection of *E. coli* transformants. The LB-amp-starch plates (LB-amp agar plates containing 1% soluble starch) were used assays to detect the starch-hydrolyzing activity of CGT. LB-amp medium was also used to cultivate *E. coli* cells for enzyme purification.

Site-directed mutagenesis

Because the three-dimensional (3D) structure of CGT from *Bacillus macerans* is not available, the structure of CGT from *Bacillus circulans* strain No. 8 (Hofmann et al., 1989) was used as a guide in selecting the appropriate residues for mutation. Amino acid sequence comparison showed 65.0% identity and 75.7% similarity between CGTs from *Bacillus macerans* and *Bacillus circulans* strain No. 8. By using amino acid sequence alignment, we determined that the Tyr633 residue in the second maltose binding site of CGT from *Bacillus circulans* strain 251 corresponded to Tyr634 of CGT from *Bacillus macerans*.

Site-directed mutagenesis was performed according to Kunkel et al. (1985, 1987), using the muta-gene phagemid kit (version 2) purchased from Bio-Rad (Hercules, CA). The 30-mer oligonucleotide, 5'-AATACCAGGACGGGCCCTTGGCGATCACCT-3' (underline denotes the mutation), was used to replace Tyr634 in domain E of CGT with a Gly residue (Y634G). The 30-mer oligonucleotide 5'-AATACCAGGACGGGAACTTGGCGATCACCT-3'

(underline denotes the mutation), was used to replace Tyr634 with a Phe residue (Y634F). The oligonucleotide was synthesized by Gibco BRL (Gaithersburg, MD). Single-stranded DNA was prepared using pHC1W. The mutated Nco1-Sal1 DNA fragment (0.65 kb) was inserted into the corresponding sites of pHC1W after removing the original Nco1-Sal1 DNA fragment. Mutated plasmids pHCYG and pHCYF were transformed into *E. coli* BL21(DE3) for overexpression. To verify the presence of the mutation, DNA sequence analysis was performed by the Nucleic Acid Facility of Iowa State University.

Construction of β-galactosidase-domain E fusion proteins

The plasmid pBKD1, which encodes a β-galactosidase-domain E fusion protein (β–gal-E(WT)), was constructed previously in our lab (Dalmia et al., 1995). The fusion protein (β–gal-E(Y634G)), containing the mutated domain E (Y634G) was constructed using the same clonig strategy as described by Dalmia et al. (1995) and the resulting plasmid was designated as pHCYGf. The DNA sequence was determined by the Nucleic Acid Facility of Iowa State University to verify that the sequence was correct.

Production and purification of CGTs and β–gal fusion proteins

E. coli BL21(DE3) cells containing plasmids pHC1W, pHCYG, or pHCYF and *E. coli* TG1 cells containing plasmids pBKD1 or pHCYGf were grown in 1L of LB-amp medium at 37 ^{o}C. The expression of desired enzymes was induced by adding

0.4mM IPTG when the OD_{600} reached 0.6. After the induction, the cells were incubated at 25 ^{o}C for 4h and then harvested by centrifugation for 10 min at 5,000g. The cell pellet of CGTs was resuspended in 15 ml of lysis buffer, containing 50mM acetate, 5mM $CaCl_2$ and 5mM benzamidine, pH 5.5. The cell pellet of the β–gal fusion proteins were resuspended in 50 mM Tris-HCl, pH 7.0, containing 10mM $MgCl_2$, 10 mM β-mercaptoethanol, 5mM EDTA and 5mM benzamidine. The cells were broken by a French press in two passes at 4,000 psi, and 0.6 mL of 100 mM PMSF was added as a protein inhibitor immediately after the disruption of cells. The soluble crude extract was separated from the cell debris by centrifugation at 10,000g for 10min. The CGTs and β–gal fusions were purified from the crude extract solution by affinity chromatography, using an α-CD-Sepharose 6B column according to the methods of Laszlo et al. (1981). The enzyme was eluted with 12 mM α-CD and extensively dialyzed to remove the α-CD. Epoxy-activated Sepharose 6B was purchased from Pharmacia. The α-CD-Sepharose 6B was prepared according to the protocol from Pharmacia.

Analytical assays

Because the CGT of *Bacillus macerans* produces initially α-CD from starch, the cyclization activity of CGT was measured using the methylorange method of Lejeune et al., 1989. The enzyme reaction was performed at 25 ^{o}C and pH 6.0. The reaction mixture (1ml) contained CGT, 1% soluble starch, and 35μM

methylorange in 50mM phosphate buffer, pH 6.0. To stop the reaction, 50 μL of 6M HCl was added to a final concentration of 0.3M. The reaction mixture was incubated at 16 ^{o}C for 30min to stabilize the α-CD-methylorange complex and the absorbance at 505 nm was measured. One unit (U) of cyclization activity was defined as the amount of enzyme which catalyzes the formation of 1μmole α-CD per minute under the assay condition.

The starch-hydrolyzing activity was measured by modifying the method of Fuwa (1954) and Kaneko et al. (1989). The reaction was performed at 25^{o}C with 0.3 ml of 0.2% starch in 50mM phosphate buffer (pH6.0) as substrate. The reaction was stop by adding 0.5 ml of 1M acetic acid. The iodine-starch complex was developed by adding 0.5 ml of 0.02% I_2 / 0.2% KI solution. Distilled water was added to a final volume of 7 ml and the absorbance at 660 nm was measured. One unit of starch-hydrolyzing activity was defined as the amount of enzyme which reduced the initial absorbance of the iodine color by 10% per minute.

The coupling reaction (transglycosylation) of CGT was measured according to Thoma et al. (1965) with modification. 24 mM α-CD and 100 mM methyl-α-D-glucoside were used as substrate, and the reaction was performed in 50 mM phosphate buffer, pH 6.0 at 25° C. In this reaction, CGT transfers a cyclodextrin to methyl-α-D-glucoside to generate a linear oligosaccharide. Glucoamylase (2.55U, Sigma) was added to the reaction mixture to degrade the produced linear oligosaccharide to glucose. The amount of glucose in the reaction mixture was

determined using a glucose assay kit from Sigma (enzymatic assay, oxidase-peroxidase-o-dianisidine) (Bergmeyer et al., 1974). One unit of coupling activity of CGT was defined as the amount of enzyme which transferred 1 μmole of α-CD to methyl-α-D-glucoside per minute under the assay condition.

The protein concentration of the crude extract and purified enzyme was determined using the Bio-Rad protein assay (Bradford, 1976). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by using 8.5% gels and protein bands were stained with Coomassie Brilliant blue.

The product specificity of CGT was monitored by HPLC. The reaction mixture contained 5% soluble starch, 1.5 mM $CaCl_2$, and 50mM phosphate buffer (pH 6.0) incubated with 0.2 units/mL (cyclization activity) of CGT in a total volume of 5 ml at 25°C. Samples were withdrawn and heated in a boiling water bath for 10 min to stop the reaction. The concentration of α-, β-, and γ-CD in the reaction mixture was measured by HPLC on a Spherisorb-NH_2 column (Phase Separations Inc, Norwalk, CT) equipped with a RI detector (Erma RI detector, ERC-7512, Erma Inc). The mobile phase was 65% aqueous acetonitrile at a flow rate of 1 ml/min.

The activity of β-galactosidase was assayed according to Dalmia et al. (1995). The hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) by β-galactosidase was monitored by the changes of absorbance at 420 nm. One unit of β-galactosidase activity was defined as the amount of enzyme which hydrolyzed 1 nmole of ONPG per minute in the assay condition.

Thermostability of CGTs

The thermostability of CGT was determined by incubating enzyme aliquots (1800 units/mL based on starch-degrading activity) for 10 minutes at different temperatures in 50 mM sodium phosphate buffer (pH 6.0) containing 1.2 mM $CaCl_2$. The enzyme samples were placed on ice immediately after the incubation, and the residual starch-hydrolytic activity of CGT was determined the same day at 25°C and pH6.0 as described above. To determine the affect of maltose and starch on the enzyme stability, the thermostability experiments were repeated in the presence of 1% (w/v) of either starch or maltose in the reaction mixture.

Activation energy

The cyclization and starch-hydrolyzing activities of CGT were determined at different temperatures using the methylorange and blue value methods as mentioned above. The initial rates of the reaction at different temperatures were determined as close to zero time as possible. The activation energy was estimated from the reaction rate at different temperature using equation 1.

$$k = A \cdot e^{-E_a / RT} \qquad \text{(Eq. 1)}$$

Where k is the rate constant, A is the Arrhenius constant, R is the universal gas constant, T is the absolute temperature, and E_a is the activation energy in kJ/mol·K (Palmer, 1991; Maheshwari, 1995).

Determination of kinetic parameters

The kinetic parameters for the cyclization reaction of CGT were estimated by measuring the formation of α-CD from soluble starch with the methylorange method. All kinetic experiments were performed at 25°C and pH 6.0 in 50mM phosphate buffer. The soluble starch concentrations ranged from 0.1 to 20 g/L and the enzyme concentrations from 0.12 to 0.5 μg/ml. The initial reaction rates from the kinetic runs were fitted to the Michaelis-Menten equation (Fersht, 1985; Eisenthal et al, 1992) by using Sigma Plot program from Jandel Scientific (San Rafael, CA). Eight or more reaction rates at different substrate concentrations were used to calculate the kinetic parameters V_{max} and K_m. All kinetic experiments were repeated at least three times. The k_{cat} (the turnover number) values were calculated by dividing V_{max} by the enzyme concentration.

The change of activation energy $\Delta(\Delta G)$ for the cyclization reaction was calculated by using equation 2.

$$\Delta(\Delta G) = -RT \ln[(k_{cat} / K_m)_{mutant} / (k_{cat} / K_m)_{wild\ type}] \qquad \text{(Eq. 2)}$$

Where k_{cat} / K_m refers to the catalytic efficiency of the enzyme (Wilkinson et al., 1983; Nakamura et al. 1993), R is the gas constant (8.315 J mol^{-1} deg^{-1}) and K is the absolute temperature (K= °C+273.15).

Binding and elution of fusion proteins

The elution of fusion proteins was investigated by using α-CD affinity column. A 20 mL aliquot, containing$1.94x10^3$ units/mL of the purified fusion protein, was

loaded on the α-CD-Sepharose 6B column at a flow rate of 0.24mL/min. The bound fusion protein was eluted by washing the column with a 0-25 mM gradient of α-CD solution (total volume of 100mL). The eluted protein was collected in tubes (25 drops, about 1.5 mL, per tube) and the β-galactosidase activity in each tube was assayed.

RESULTS

Production and purification of CGTs and fusion proteins

WT-CGT, Y634F and Y634G were produced in *E. coli* BL21(DE3) and the fusion proteins were produced in *E. coli* TG1. These proteins were purified using affinity chromatography on α-CD-Sepharose 6B. The purity of CGTs was examined using SDS-PAGE (8.5%). WT-CGT, Y634F and Y634G were detected as single protein bands on SDS-PAGE with estimated molecular weights of 74kDa (data not show). The fusion proteins were also examined by SDS-PAGE, and their molecular weights were about 128 kDa (Figure 1a). The presence of fused domain E was confirmed by Western Blot using anti-CGT antibody as probe (Figure 1b).

Thermostabilities of WT-CGT, Y634F, and Y634G

The effect of temperature on the thermostabilities of WT-CGT, Y634F and Y634G in the presence and absence of substrate is summarized in Figure 2. WT-CGT and Y634F are more thermostable than Y634G. The temperature resulting in 50% inactivation ($T_{50\%}$) of WT-CGT in the absence of substrate was about 57°C,

which was 8°C higher than that of Y634G ($T_{50\%}$=49°C). The $T_{50\%}$ of 54°C for Y634F was similar to that of WT-CGT. Because Y634G was less thermostable, all the enzymatic assays were performed at 25°C lasting less than 30 minutes.

The results of these assays indicate that Tyr634 affects the thermostability of CGT. When Tyr634 was replaced with Phe (Y634F), which has a smaller hydrophobic side chain than Tyr, the thermostability of CGT decreased slightly. When the same residue was replaced with Gly, which does not have hydrophobic side chain, the stability of CGT decreased significantly more. This suggested that the interactions between Tyr634 and the other residues in the protein are important for thermostability of CGT.

Starch was proposed to increase the stability of CGT by forming a stabilizing bridge between the active site and domain E of CGT (Fujiwara et al., 1992). Maltose is too short to extend from the active site to domain E and had insignificant effect on Y634G. Soluble starch, however, when added to the enzyme mixture, dramatically increased the thermostabilities of WT-CGT and Y634F, but did not have substantial stabilization effect on Y634G. These results suggest that by replacing Tyr634 with glycine, starch molecules could not effectively bridge the catalytic domain and domain E, and that Tyr634 at MBS2 is important in the binding of large substrates like starch to CGT.

Activities of CGTs at different temperatures

The effect of temperature on CGT activity was examined. The cyclization activity was assayed at different temperatures using the methylorange method. As shown in Figure 3, the temperature of maximum cyclization activity for WT-CGT under the assay conditions was about 50-55^{o}C and it was similar to that for Y634F. Y634G, however, showed maximum cyclization activity at 35^{o}C. The pattern of temperature-activity curve of Y634G is totally different from those of WT-CGT and Y634F. The activity of Y634G was sensitive to the increase of temperature which reflect the decreased thermostability of Y634G.

The activation energy of cyclization and starch-hydrolyzing reactions of WT-CGT, Y634G, Y634F were shown in Table 1. The data was analyzed using χ^2 test (Steel, et al., 1980) by comparing WT-CGT and mutants and it showed no significant difference. It indicated that the mutations of Tyr634 did not change the rate limiting step of cyclization and starch-hydrolyzing reactions.

The specific activities of CGTs

The starch-hydrolyzing, cyclization and coupling reactions of WT-CGT, Y634F and Y634G were performed at 25°C and at pH6.0. The results are summarized in Table 2. WT-CGT and Y634F have similar specific activities for all three reactions. Specific activity of Y634G is approximately 20% more than those of WT-CGT and Y634F.

The kinetic parameters of the cyclization activity of CGT

The kinetic parameters of cyclizaton activity of WT-CGT and Y634G were estimated by using starch as a substrate at 25°C and at pH 6.0. Since the specific activities and stability of Y634F are similar to those of WT-CGT, we did not determine the kinetic parameters of Y634F. The methylorange method was used to monitor the production of α-CD which was mainly produced by CGT of *Bacillus macerans* in the initial stage of the cyclization reaction. The K_m value of Y634G was 0.36 g/L which was about 1.4 fold greater than that of WT-CGT (0.26 g/L) (Table 3), indicating that Y634G had a lower affinity for soluble starch than WT-CGT. When Tyr634 was replaced with glycine, this hydrophobic interaction would have been eliminated, resulting in decreased binding of CGT to large substrates like starch. The k_{cat} value of Y634G is slightly higher than that of WT-CGT, whereas the k_{cat}/K_m value of Y634G is 20% less than that of WT-CGT. The k_{cat} and k_{cat}/K_m of Y634G did not show substantial difference compared to WT-CGT. This indicated that Tyr634 may not be actively involved in the catalysis of CGT. The change in the free energy of activation, $\Delta(\Delta G)$, for the cyclization reaction catalyzed by Y634G was about 0.52 kJ/mol. The $\Delta(\Delta G)$ value is small, suggesting that the change of the binding strength of enzyme-substrate complex in the transition state is negligible. Therefore, Tyr634 does not seem to participate in the stabilization of enzyme-substrate complex in the transition state.

Cyclodextrin production of CGTs

The cyclodextrin production of CGTs using soluble starch as substrate as a function of time was assayed at 25°C by HPLC. The productivity of α-, β-and γ- CDs is shown in Figure 4. WT-CGT, Y634F and Y634G produced mainly α-CD at the initial stage of the reaction. The α-CD production gradually decreased and β-CD production increased with the progression of the reaction. The product profiles of WT-CGT, Y634F and Y634G did not show a significant difference. The results suggest that the amino acid replacement of Tyr634 at MBS2 in domain E does not affect the specificity of cyclodextrin production.

Elution of fusion proteins from α-CD-Sepharose 6B

Purified fusion proteins were absorbed onto α-CD-Sepharose 6B column and were eluted by using a linear gradient of 0-25 mM α-CD solution. The profile of the protein elution is shown in Figure 5. The concentration of α-CD required to elute β–Gal-E(Y634G) was two fold lower than that of β–Gal-E(WT). The β–Gal-E(WT) peak eluted with 7mM α-CD, whereas β–Gal-E(Y634G) eluted with 3mM α-CD. The results indicate that the binding strength of β–Gal-E(Y634G) to α-CD-Sepharose 6B is significantly lower than that of β–Gal-E(WT), and that Tyr634 is important for the binding of α-cyclodextrin in MBS2.

DISCUSSION

The objective of this study was to investigate the function of Tyr 634 in *Bacillus macerans* CGT on the binding of soluble starch and CDs in MBS2 and consequently in the catalysis of CGT. The previous investigations of the X-ray structure of *Bacillus circulans* CGT complexed with maltose (Lawson et al., 1994) and maltononaose (Strokopytov et al., 1996) demonstrated the existence of a long groove on the surface of CGT protein extending from the active site toward the second maltose binding site (MBS2) near Tyr 634 (Tyr 633 in *Bacillus cirulans* numbering). α-CD and maltose bind in MBS2 by making hydrophobic contact by stacking the apolar side of a glucosyl unit on the aromatic ring of Tyr 634 (Knegtel et al., 1995; Lawson et al., 1994). Because the maltose bound in MBS2 and the maltononaose bound in the active site have the same orientation (Strokopytov et al., 1996), it has been proposed that the MBS2 and the active site cleft can be regarded as extended binding site for a high molecular weight substrate such as soluble starch.

The function of Tyr 634 was investigated by replacing Tyr 634 with Phe which has a smaller aromatic side chain than Tyr, and with Gly which does not have an aromatic side chain. The characterization of the Y634F and Y634G mutants and their comparison with WT-CGT properties allows us to further shed light on the role of Tyr 634 interaction with substrate molecules.

Results from the thermostability investigation shown in Figure 2 demonstrated that maltose and starch had a stabilization effect on WT-CGT and

Y634F but a negligible one on Y634G. The binding of starch had significantly greater effect than maltose on the enzyme stability which could be explained by the bridging effect of long starch molecules connecting to active site and domain E, leading to a stabilized starch-CGT structure. The reduced starch stabilization effect of Y634G indicated that the aromatic side chain of Tyr634 is important in the interaction with starch. As would be expected, the substitution of Tyr with Gly also affected the binding of maltose to MBS2 resulting in a negligible stabilization of the Y634G mutant compared with WT-CGT and Y634F. The Y634F mutant was very similar to WT-CGT in terms of stability and specific activity (Table 2 and Figure 3) which suggests that the hydrophobic interactions between the glucosyl unit of substrates and the aromatic ring of the Phe residue is as strong as with the Tyr residue.

The K_m value of Y634G for the cyclization reaction using starch as substrate was about 1.4 fold greater than that of WT-CGT (Table 3) indicating reduced affinity for starch. This result is consistent with the reduced starch-stabilizing effect we detected with Y634G as well as with the study of Penninga et al. (1996). They found that replacing of Tyr633 (*Bacillus circulans* numbering) with Ala led to a three-fold increase of K_{50} (equivalent to the dissociation constant) which was obtained from the investigation of the effect of β–CD in cyclization reaction.

To confirm that the Tyr to Gly mutation also affected the interaction with α-CD, we examined the elution profile of a β–Gal-E(WT) fusion protein and an identical fusion protein with a Y634G mutation in the fused E domain, β–Gal-

E(Y634G) (Figure 5). The use of β-Gal fusions eliminated the potential binding interference from the active site of CGT. Since the only difference was a single mutation in the MBS2, the significantly different elution profile (different concentration profile of the α-CD ligand) indicates that the Tyr634 residue of MBS2 is also important for the binding of α-CD, as suggested by Knegtel et al. (1995). The mutation in MBS2 did not totally eliminate the ability of βGal-E(Y634G) to interact with the α-CD-Sepharose column because the other starch-binding site (MBS1) in domain E is also capable of binding α-CD (Knegtel et al. 1995).

The specific activities for the three reactions catalyzed by CGT (Table 2), revealed that WT-CGT and Y634F had similar activities and, surprisingly, that the Y634G mutant had a greater specific activity than the other two enzymes. This trend was confirmed by measuring the kinetic parameters for the cyclization reaction (Table 3). The k_{cat} value of Y634G was slightly higher than that of WT-CGT. A similar effect was observed by Penninga et al.(1996) with their Y633A mutant (*Bacillus circulans*). From measuring the product inhibition of β-CD on the cyclization reaction of Y633A, they concluded that β-CD binding at MBS2 non-competitively inhibits WT-CGT cyclization activity. Their results suggested that the groove which extends from active site to MBS2 can be considered as one binding site for large substrates, and the binding of CD at MBS2 can interfere and compete with the binding of large substrates to the groove. Because we have shown that Y634G has a weaker affinity for α-CD than WT-CGT, the reaction rate in Y634G

may have increased due to the reduced product inhibition resulting in a higher k_{cat} value.

Significantly lower thermostability of the Y634G mutant is related to the added flexibility (additional degree of freedom) provided by the Gly residue. The perturbation in the domain E structure by the Tyr to Gly substitution, which caused a loss in thermostability of CGT, suggests that domain E is an integral part of the CGT structure.

Even though the binding strength of starch and α-CD to Y634G was altered, no effects on product distribution were seen as shown in Figure 4. These results suggest that domain E does not determine the product specificity of the enzyme, which is consistent with the proposal that active site of CGT determines the product distribution (Nakamura et al., 1994; Strokopytov et al., 1996).

In conclusion, domain E participates in the cyclization reaction of CGT by binding to large substrates in the ground state.

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Table 1. Activation energy

Enzyme	Starch hydrolysis E_a (kJ/mol·K)	Cyclization E_a (kJ/mol·K)
WT-CGT	43	44
Y634F	46	39
Y634G	42	50

Table 2. Specific activities of CGTs

Enzyme	Starch hydrolysis (U/mg)	Cyclization (U/mg)	Coupling (U/mg)
WT-CGT	6700±100	118±3	206±7
Y634F	6800±300	116±8	207±3
Y634G	8400±100	146±4	227±9

Table 3. Kinetic parameters of CGTs

Enzyme	K_m (g/L)	k_{cat} (sec^{-1})	k_{cat} / K_m ($sec^{-1}g^{-1}L$)
WT-CGT	0.26±0.03	192±4	750±80
Y634G	0.36±0.04	219±4	610±70

FIGURE LEGENDS

Figure 1. Expression of β-galactosidase-domain E fusions. (a) SDS-PAGE (8.5%), (b) Western Blot (anti-CGT). Lane 1, purified βGal-E(WT). Lane 2, purified β–Gal-E(Y634G). Lane 3, molecular weight standards.

Figure 2. Thermostability of CGTs. The thermostability of CGT was assayed at different temperatures. Starch and maltose were added to the reaction mixture to investigate the effect of substrate stabilization. CGT in 50 mM sodium phosphate buffer (pH 6.0) containing 1.2mM $CaCl_2$ (1800 units/mL based on starch-hydrolytic activity) was incubated for 10 minutes at different temperatures. The residual activity of starch-hydrolytic activity of CGT was determined at 25°C, pH6.0. The experimental errors of all points were less than 10%. (a) WT-CGT, (b) Y634F, (c) Y634G. ● , CGT ; ■ , CGT with maltose addition (1% w/v) ; ▲ , CGT with starch addition (1% w/v).

Figure 3. Cyclization activity of CGTs at different temperatures. The cyclization activities of WT-CGT, Y634F and Y634G were determined at different temperatures using methylorange method and soluble starch was used as substrate. ● , WT-CGT; ■ , Y634F; ▲ , Y634G.

Figure 4. Production of α-, β-, and γ- cyclodextrins from soluble starch. The reaction mixture containing CGT (0.2 units/mL, cyclization activity), 5% soluble starch, 1.5 mM $CaCl_2$, 50mM phosphate buffer, pH 6.0, was incubated at 25°C with a total volume of 5 ml. Aliquots (0.45 mL) were withdrawn at different time points and heated in a boiling water bath for 10min to stop the reaction. The concentration of α-, β-, and γ-CDs in the reaction mixture was determined by HPLC. (a) WT-CGT, (b) Y634F, (c) Y634G. O , α–CD; □ , β–CD; Δ, γ–CD.

Figure 5. Binding and elution of fusion proteins. 20 mL of the purified fusion protein ($1.94x10^3$ unit/mL) was loaded to a α-CD-Sepharose 6B column at the flow rate of 0.24mL/min. The bound fusion protein was eluted by washing the column with a 0-25 mM gradient of α-CD solution (total volume of 100mL). (a) β–Gal-E(WT), (b) βGal-E(Y634G). Δ, α–CD ; ●, β–Gal-E(WT) ; ♦, β–Gal-E(Y634G).

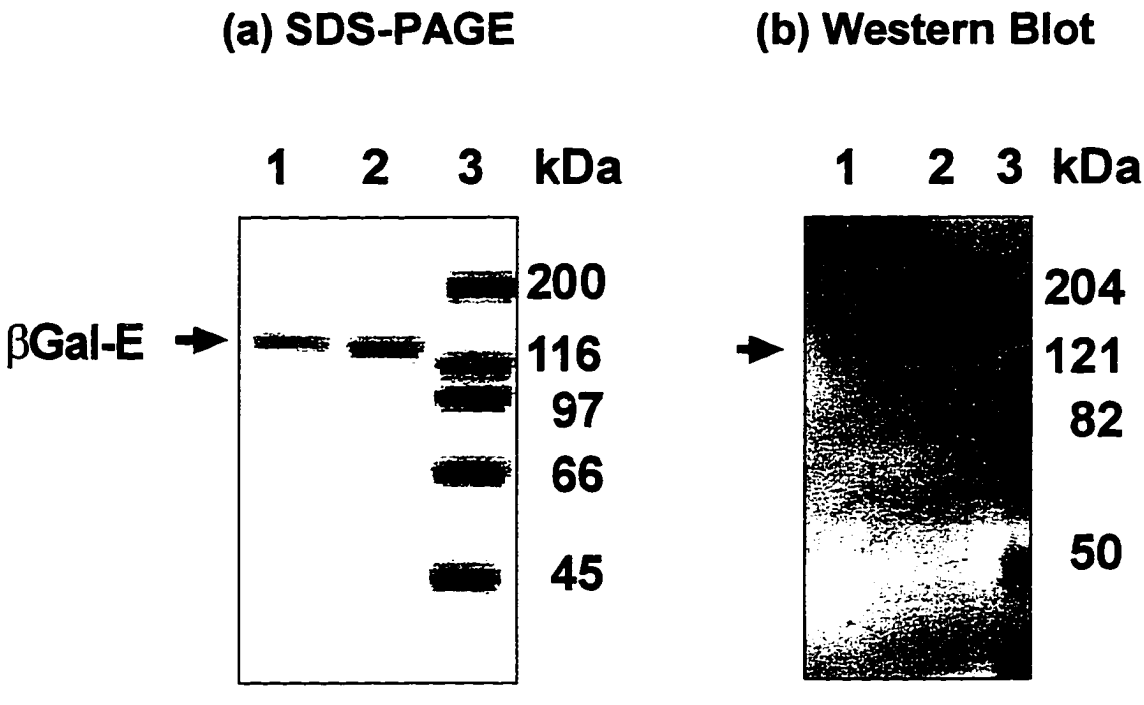
(a) SDS-PAGE
(b) Western Blot
1 2 3 kDa
1 2 3 kDa
βGal-E
200
116
97
66
45
204
121
82
50

Figure 1

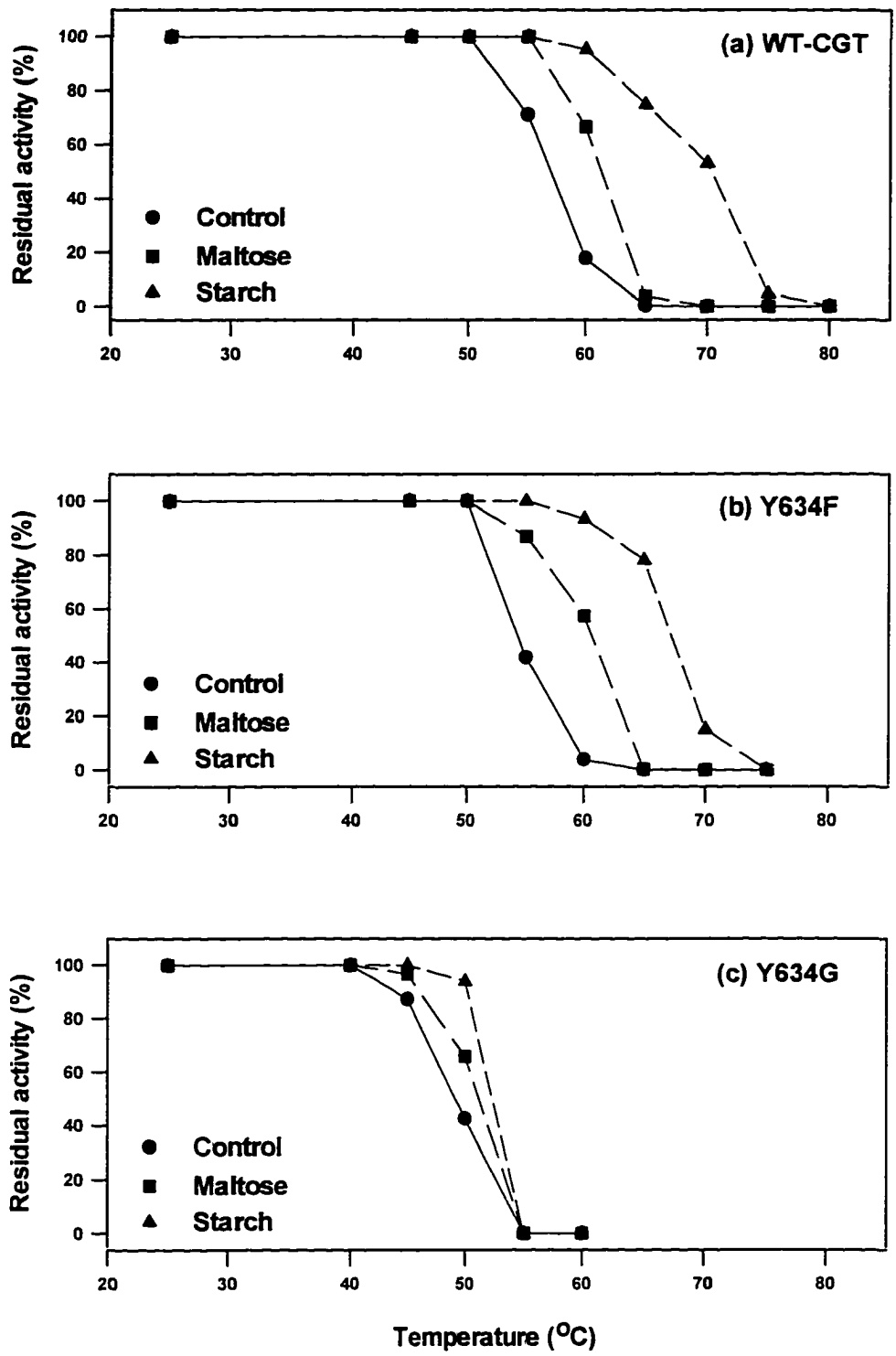
(a) WT-CGT
(b) Y634F
(c) Y634G
Control
Maltose
Starch
Residual activity (%)
Temperature (°C)
0
20
40
60
80
100
20
30
40
50
60
70
80

Figure 2

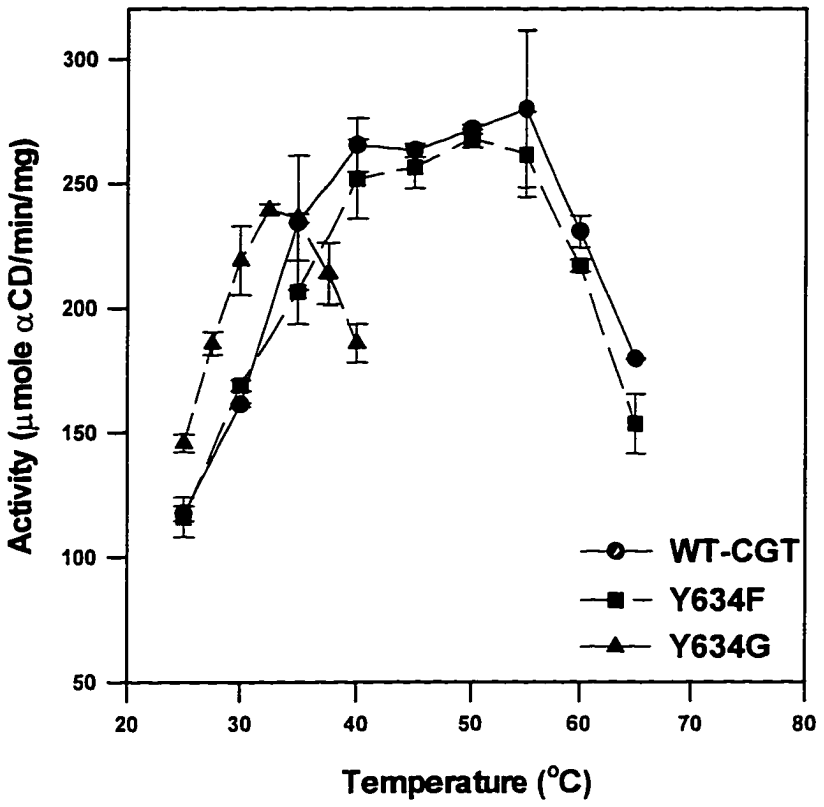
Activity (μmole αCD/min/mg)
300
250
200
150
100
50
20
30
40
50
60
70
80
Temperature (°C)
WT-CGT
Y634F
Y634G

Figure 3

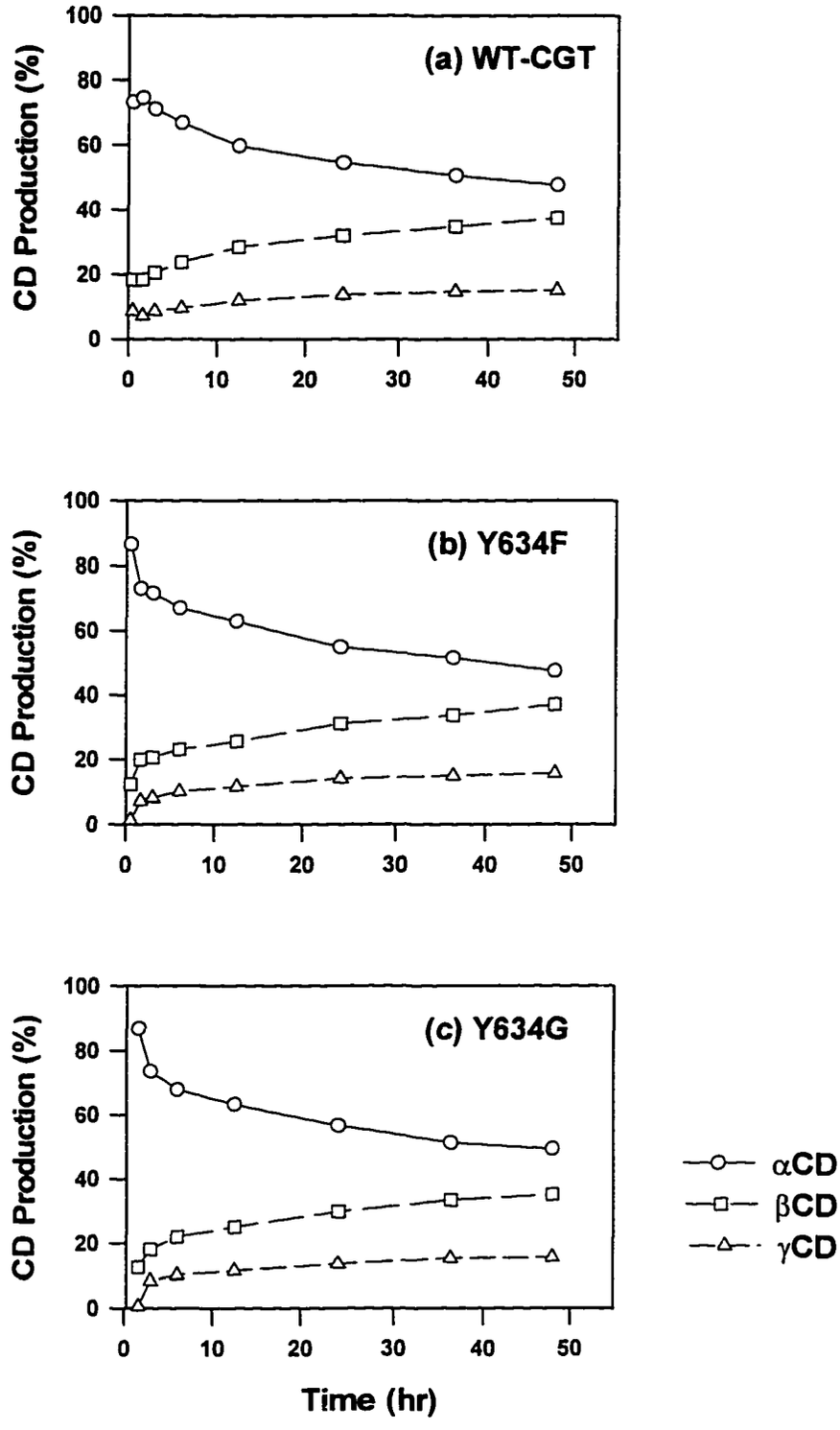

Figure 4

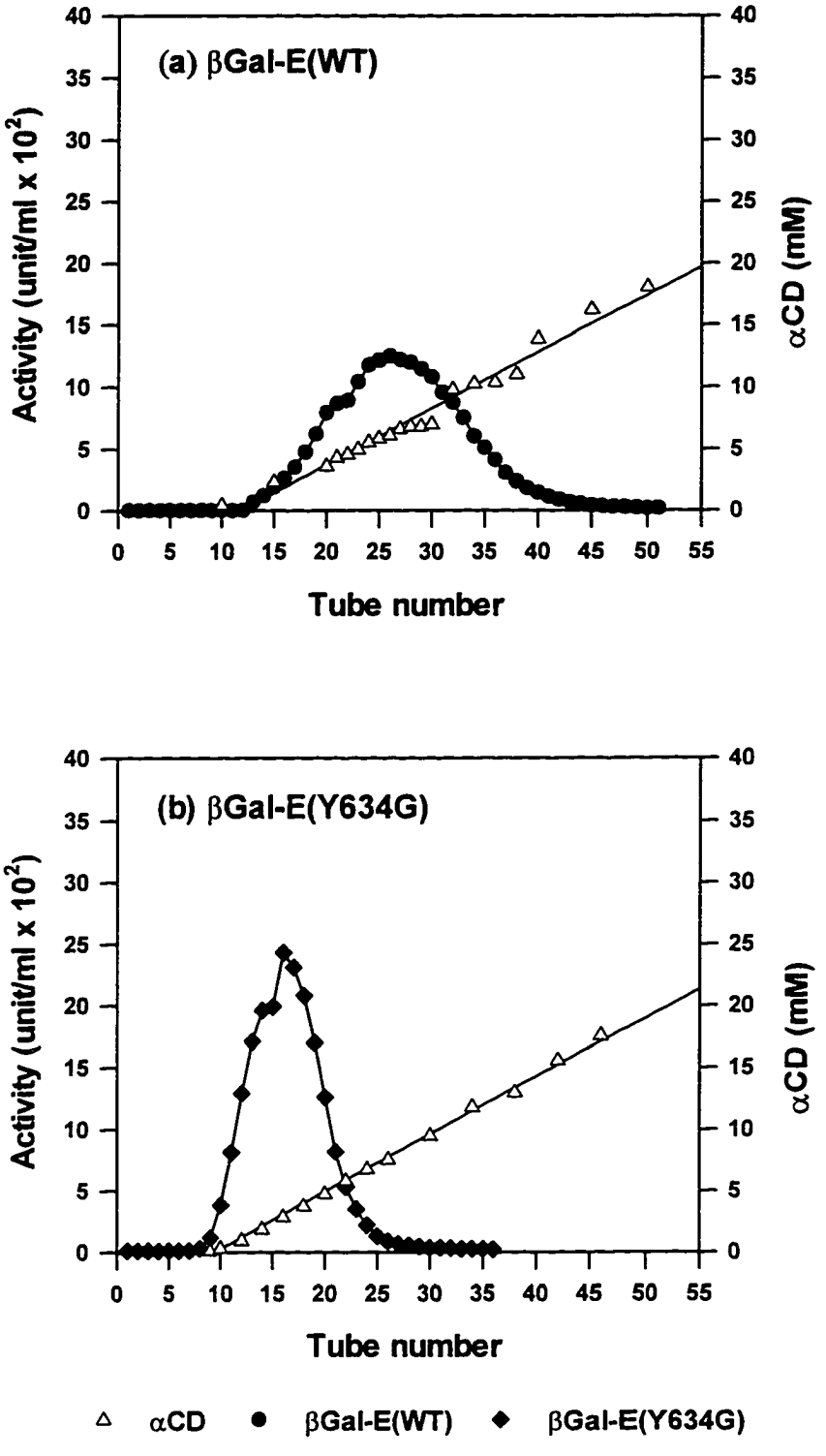
(a) βGal-E(WT)
Activity (unit/ml x 10²)
αCD (mM)
Tube number
(b) βGal-E(Y634G)
Activity (unit/ml x 10²)
αCD (mM)
Tube number
△ αCD ● βGal-E(WT) ◆ βGal-E(Y634G)

Figure 5

CHAPTER 4. GENERAL CONCLUSIONS

The three-dimensional structure of CGT revealed that CGT consists of five domains A, B, C, D and E. Domain E was identified as a starch binding domain (SBD), and its function in CGT activities is unknown. In my dissertation research, I investigated the role of domain E in CGT catalysis, and determined that domain E is important in the substrate binding and in cooperation with the catalytic domain.

Domain E is important for maintaining the integrity of the groove structure for proper fitting of starch molecules

The groove which extends from the active site to domain E has been considered as an extension of active site for binding large substrates. To maintain the integrity of the groove, domain E should be properly oriented and relatively immobile. When domain E was replaced with the starch binding domain (SBD) of glucoamylase I, the starch chain bound to SBD could not be properly positioned to lead to the active site due to the mobility of the SBD and the lack of integrity of the groove. A six-amino-acid linker was inserted between domains D and E, which led to the "misalignment" of the MBS2 and the rest of the groove and resulted in a weaker binding to starch. Domain E has to be positioned at an optimal distance and/or orientation with respect to the active site in the WT-CGT to allow the optimal binding of soluble starch substrate in the groove. Domain E is also important for maintaining the integrity of the groove structure.

Domain E participates in the cyclization reaction of CGT by binding to large substrates

The lowered starch stabilization of Y634G indicated that the aromatic side chain of Tyr634 is important in binding to starch. The K_m value of Y634G for the cyclization reaction using starch as a substrate was about 1.4 fold greater than that of WT-CGT indicating reduced affinity for starch. The significantly different elution profiles between βGal-E(WT) and βGal-E(Y634G) indicated that the Tyr634 residue of MBS2 is important for the binding of α-CD. The groove which extends from the active site to MBS2 is hypothesized to be an extended binding site for large substrates. The binding of CD at MBS2 apparently interferes with the binding of starch in the groove. The k_{cat} value of Y634G was slightly higher than that of WT-CGT. This indicated that the reaction rate of Y634G probably increased due to reduced product inhibition. In general, domain E apparently participates in the cyclization reaction of CGT by binding large substrates and directing them to the active site.

APPENDIX

Starch-Binding Domain of *Aspergillus* Glucoamylase-I

Interaction with β-Cyclodextrin and Maltoheptaose[a]

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INTRODUCTION

Glucoamylase I (GAI) from *Aspergillus* species has two functional domains: a catalytic domain (amino acids 1–440) and a COOH-terminal starch-binding domain (SBD) (amino acids 512–616).[1,2] The two domains are linked by highly *O*-glycosylated linker (amino acids 441–511).[3,4] Although recent data indicated that SBD provides the starch-binding capacity of GAI,[5–9] participation of *O*-glycosylated linker in the starch binding had also been observed.[10,11]

To demonstrate the independence and to determine the minimum functional length of the SBD, β-galactosidase fusion proteins containing various sizes of SBD of *Aspergillus* GAI were constructed. All β-galactosidase fusion proteins adsorbed to granular starch because of the presence of SBD.[7,8] The β-galactosidase fusion protein containing amino acids 498–616 of glucoamylase I (BSB119) had the strongest adsorption to granular starch. BSB119 also interacted with α-, β-, and γ-cyclodextrin, maltooligosaccharides, and acarbose.[12] The binding constants for these sugars could not be determined because of the partial degradation of the SBD, which produced heterologous fusion proteins.[7,8,12]

To overcome the problems associated with the β-galactosidase fusion protein system, we fused the starch-binding domain to the COOH-terminus of maltose-binding protein (MBP) flanked by a factor X_a cleavage site.[9] Functional starch-binding fragments were isolated by cleaving them from the respective MBP-fusion proteins by using factor X_a.

This article discusses the interactions of starch-binding peptides (SBD106 and SBD122) with β-cyclodextrin and maltoheptaose.

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MATERIALS AND METHODS

Production of the SBD Peptides

Starch-binding peptides, SBD106 and SBD122, which consist, respectively, of residues 511–616 and 495–616 of *Aspergillus* GAI, were produced in *Escherichia coli* as described by Kusnadi *et al.*[9] SBD-encoding gene fragments were fused to the 3' end of *E. coli malE* gene encoding maltose-binding protein. The purified fusion proteins were digested with factor X_a (a specific protease), and SBD fragments were separated from MBP affinity chromatography on cross-linked amylose. The specific elution of SBD peptides from cross-linked amylose with 500 mM maltose served as a proof of their starch-binding functionality. The concentration of the purified starch-binding peptides was calculated from the protein absorption at 280 nm by using a molar extinction coefficient of 30600 M^{-1} cm^{-1}.[13]

Characterization of SBD122

Intramolecular Disulfide Bond in SBD122

To confirm the presence of an intramolecular disulfide bond in SBD122, the factor X_a digest of MBP-SBD was analyzed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (0.1% SDS–12% PAGE) in the presence and absence of 5% (vol/vol) 2-mercaptoethanol (2-ME), following the method of Allore and Barber.[14] Protein bands were stained with silver nitrate.

Determination of Sulfhydryl Groups

The content of sulfhydryl groups was determined by titration with DTNB (5,5'-dithiobis(2-nitrobenzoic acid)).[15] SBD122 (10–20 μM) was assayed in 0.08 M sodium phosphate buffer of pH 8 containing 0.5 mg/ml EDTA. To determine the total protein sulfhydryl, SBD122 was titrated with DTNB after protein denaturation in 2% SDS.[16] The available sulfhydryl was determined by the same method, but without previous sample denaturation. Freshly prepared L-cysteine solution was used as standard solution.

Amino Acid Composition

About 1 nmol of the purified SBD domain was electrophoresed on a 13.5% polyacrylamide gel[17] and then electroblotted to a polyvynilidene fluoride membrane (Bio-Rad, Richmond, CA). Amino acid analysis of the protein bound on the dried membrane was performed by the Iowa State University Protein Facility (Ames, IA). The on-membrane amino acid analysis was performed using norleucine as an internal standard. The protein band was hydrolyzed with 6 N HCl at 165°C for 45 min. The free amino acids were derivatized under basic conditions with phenyl isothiocyanate in an Applied Biosystems model 420H derivatizer and separated on a narrow-bore C_{18} column. The phenylthiocarbamyl chromophore was detected at 254 nm.

NH_2-Terminus Sequencing

The NH_2-terminus sequence of SBD122 was determined by the Edman degradation at Iowa State University Protein Facility. An Applied Biosystems model 477A protein sequencer and a 120A PTH amino acid analyzer were used.

Difference Spectroscopy

Ligand-induced perturbation of absorption spectra was determined at ambient temperature (20 ± 2°C), using double-chamber cuvettes with light paths of 4.375 mm. Absorption spectra were recorded on a Cary Model 1501 spectrophotometer modified by On-Line Instrument Systems (Bogart, Georgia) for direct digital recording of spectra. The sample and reference cells containing starch-binding peptide (30–70 μM, in 5 mM sodium acetate, pH 4.5) and the buffer in the respective chambers were scanned five times at 315 to 245 nm to obtain the baseline. The ligand (0–3 mM) was added in aliquots ranging from 1 to 15 μl. For each ligand concentration, five spectra were recorded from 315 to 245 nm. Each spectrum consisted of 180 data points, and each point was the average of nine readings. The spectra were averaged, corrected for the dilution factor and baseline drift, and then smoothed by using the 17-point smoothing procedure. The difference in absorbance (ΔA) between the complex and the free SBD fragment was calculated as $\Delta A_{286} = [(A_{286} - A_{280}) + (A_{286} - A_{290})]/2$, where A_{280}, A_{286}, and A_{290} are the absorbance at 280, 286, and 290 nm, respectively.[18]

The dissociation constants of SBD106 and SBD122 from titrations with β-cyclodextrin and maltoheptaose were estimated by fitting the experimental data to the Scatchard equation[19]:

$$b/f = -(1/K_d) \cdot b + (1/K_d) \cdot e_o \qquad (1)$$

where b, f, and e_0 are the concentrations of bound ligand, free ligand, and total binding sites, respectively. Assuming that one mole of ligand binds to one mole of SBD106 and SBD122, the concentration of the bound ligand was calculated as $b = (\Delta A/\Delta A_{max})e$, where ΔA and ΔA_{max} are the difference and maximum difference in the absorbance, and e is the concentration of the SBD peptide.[20]

RESULTS AND DISCUSSION

Characterization of SBD122

According to the amino acid sequence of GAI, SBD122 contains two cysteine residues (Cys-509 and Cys-604), which presumably form a disulfide bond.[21] To confirm the presence of this disulfide bond, we analyzed the migration pattern of the proteolytic digest of MBP-SBD122 fusion protein. The apparent molecular weight of SBD122 in the presence (+) and absence (−) of reducing agent (FIG. 1, lanes 2 and 7) was different because, upon reduction, SBD122 (lane 2) unfolds, which results in a longer effective chain length.[14] The nonreduced MBP-SBD122 (lane 8) also migrated slightly farther than the reduced sample (lane 3), whereas MBP bands (lanes 4 and 9) were not affected by the reducing environment because MBP does not contain cysteine residues. Therefore, the migration pattern of the various bands given in FIGURE 1 are consistent with the hypothesis of disulfide bonding between Cys-509

and Cys-604 in SBD122. We measured the free sulfhydryl content (total and available) of SBD122 and estimated that about 10% of SBD122 molecules did not form a disulfide bond, probably because of misfolding or partial degradation.

Amino Acid Analysis

The amino acid composition of purified SBD122 peptide given in TABLE 1 is in a good agreement with expected composition of the 495–616 amino acid segment of GAI. The numbers of Ser and Thr residues were lower than expected because of the instability of these two amino acids during acid hydrolysis. Gln and Asn could not be detected because both amino acids were converted to the corresponding carboxylic

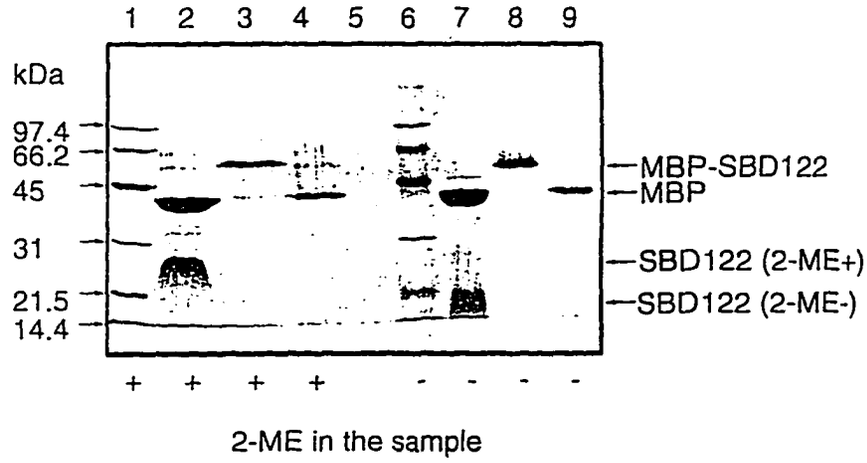

FIGURE 1. Gel electrophoresis of factor X_a digest of MBP-SBD122 fusion protein under reducing and nonreducing conditions. Protein samples in lanes 1 through 4 were reduced with 5% (vol/vol) 2-ME (+) before electrophoresis, and protein samples in lanes 6 through 9 were without 2-ME (−). Lanes 1 and 6 contain molecular weight standard. Lanes 2 and 7 contain purified MBP-SBD122 digested with factor X_a. Lanes 3 and 8 contain purified MBP-SBD122. Lanes 4 and 9 contain purified MBP. Lane 5 was not used. (Digestion and purification methods of MBP-SBD122 were reported by Kusnadi *et al.*[9])

acids. Thus, the resulting Asp and Glu were the sum of Asp and Asn (Asx) and of Glu and Gln (Glx), respectively. The number of Trp and Cys residues could not be determined by this method because they were destroyed during acid hydrolysis. The amino acid analysis of SBD106 was also in agreement with the expected amino acid content (TABLE 1), and the results of both analyses confirmed that we have purified the correct size of starch-binding peptides.

NH_2-Terminus Sequencing

From the NH_2-terminus sequencing of SBD122, we confirmed that the first 23 amino acids of SBD122 are the same as residues 495–517 of glucoamylase I

TABLE 1. Amino Acid Compositions of SBD106 and SBD122

	SBD106		SBD122	
Amino Acid	Expected	Determined	Expected	Determined
Asx	11	8	11	10
Glx	10	8	10	10
Ser	11	10	17	14
Gly	6	8	6	7
Arg	3	3	3	3
Thr	17	12	23	19
Ala	8	8	10	10
Pro	5	4	5	4
Tyr	6	5	6	6
Val	8	8	8	8
Ile	5	5	5	5
Leu	6	6	6	6
Phe	3	3	3	3
Lys	2	2	3	3
Cys	1	ND[a]	2	ND
Trp	4	ND	4	ND

[a]ND: not determined.

(495–ATASLTSTSTSSTSCTTPTAVAV–517), indicating that no vector-derived sequence remained attached to the starch-binding domain after factor X_a digestion. Although we only performed NH_2-terminus sequencing on SBD122, we believe that factor X_a also cleaved SBD106 from the maltose-binding protein at the junction, leaving no vector-derived sequence on both starch-binding fragments.

Interaction of Starch-Binding Peptides with β-Cyclodextrin

Although we characterized only SBD122, UV difference spectroscopy was performed with both SBD106 and SBD122. SBD106 does not contain Cys-509 and therefore cannot form an intramolecular disulfide bond. Because SBD106 retained its starch-binding activity,[9] we decided to estimate SBD106 interactions with the two soluble ligands and to compare them with those of SBD122.

The typical UV spectra of the SBD peptides induced by titration with β-cyclodextrin (FIG. 2) show four characteristic peaks, with absorbance maxima at 278 nm, 286 nm, 294 nm, and 303 nm. The peak observed at 278 nm suggests that one or more Tyr residues were affected by the binding of β-cyclodextrin to SBD.[22] The ligand-induced perturbation changes in the region of 280 to 295 nm are characteristic of the red-shifted absorbance bands of indole chromophore of one or more Trp residues as a result of their transfer to a less polar environment upon binding.[23] The ligand-induced perturbation changes near 300 nm are also characteristic for the indole chromophore because of changes in the electrostatic environment of the chromophore.[23] Because β-cyclodextrin is not a charged molecule, it could be speculated that the conformational change of SBD that occurred upon binding of β-cyclodextrin changed the electrostatic environment of the Trp residue(s) in the starch-binding domain.[24] Similar UV difference spectra were also observed when *Aspergillus* GAI[22] and a GAI fragment (amino acids 471–616)[18] were perturbed by β-cyclodextrin. Previous chemical modification of Trp residues of GAI[25] and COOH-terminal deletion studies[7] indicated that Trp-590 and Trp-615 participated in the starch

adsorption. Therefore, the UV spectra of SBD106 and SBD122 probably reflect the perturbation of these two residues upon β-cyclodextrin binding.

The maximum change of ΔA was observed at 286 nm (FIG. 2), and the dissociation constants of β-cyclodextrin with the two SBD peptides were calculated on the basis of ΔA_{286} values. ΔA_{286} curves of SBD106 and SBD122 plotted against β-cyclodextrin concentration increased hyperbolically with the β-cyclodextrin concentration (FIG. 3). The K_d values calculated by using Equation 1 are given in TABLE 2. The K_d value of SBD106 is slightly higher than the two values of K_d measured with SBD122, which were obtained by titrating two separate preparations of SBD122. The important conclusion from dissociation constants of β-cyclodextrin is that SBD106, although lacking a disulfide bond, is fully functional and has an affinity for β-cyclodextrin similar to that of SBD122.

Other studies have reported that β-cyclodextrin binds to the SBD of the native *Aspergillus* GAI with K_d values of 5.6 μM[26] and 19 μM,[22] and to COOH-terminal fragments of GAI with K_d ranging from 1.7 to 3.3 μM.[27] A possible explanation for the higher K_d values of SBD106 and SBD122 than the reported ones is the difference in the protein glycosylation; native GAI- and GAI-derived fragments are glycosylated, whereas the SBD peptides produced in *E. coli* are not. In light of the Williamson and co-workers article,[28] which showed that *O*-glycosylation of GAI residues 499 through 508 is important for the stability of the GAI derived starch-binding fragments, it could be speculated that SBD106 and SBD122 peptides have a different dynamics because of the lack of glycosylation.

Interaction of Starch-Binding Peptides with Maltoheptaose

In general, the maltoheptaose-induced UV difference spectra of SBD106 and SBD122 were very similar to those induced by β-cyclodextrin. The spectra show

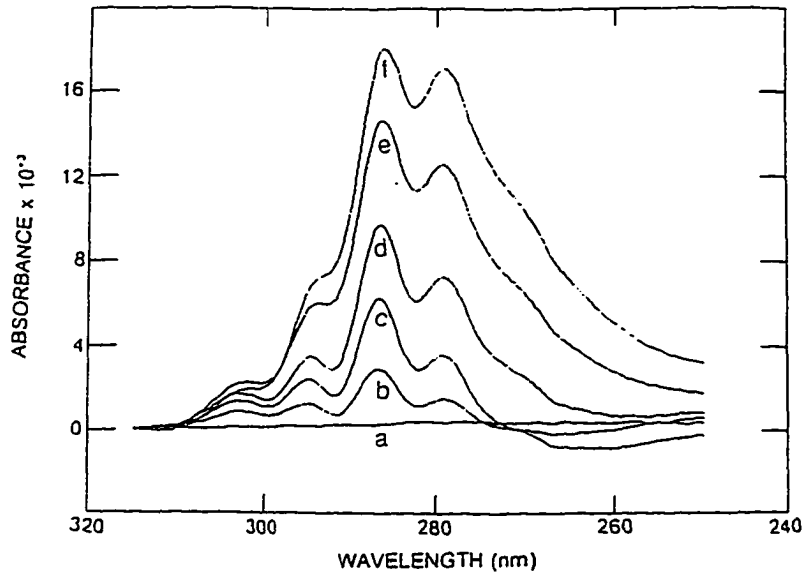

FIGURE 2. Ultraviolet difference spectra of the SBD induced by β-cyclodextrin. (a) 0, (b) 39.8, (c) 79.4, (d) 183, (e) 297, and (f) 554 μM β-cyclodextrin.

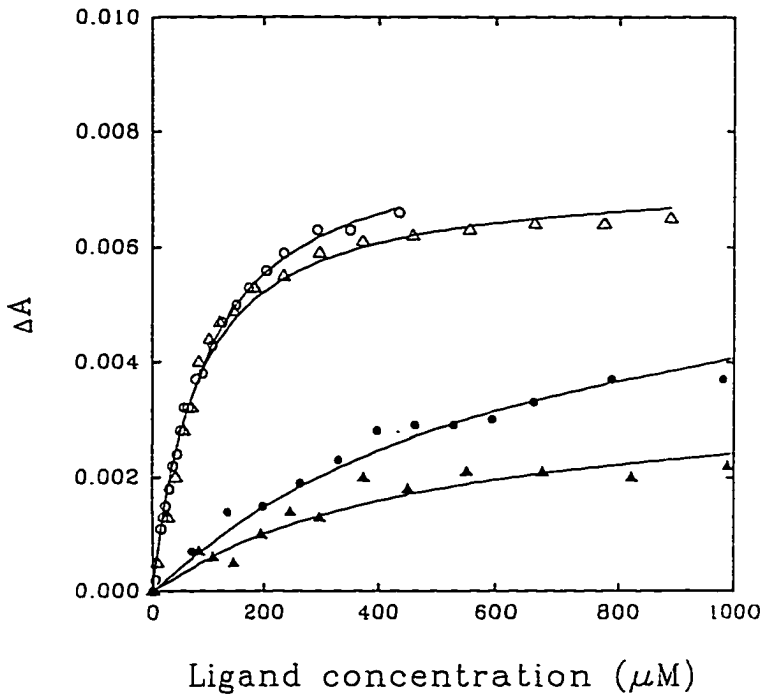

FIGURE 3. Titration of SBD peptides with β-cyclodextrin and maltoheptaose. Dependence of ΔA on ligand concentrations. (O) Titration of 74.3 μM SBD106 with β-cyclodextrin; (△) titration of 77.4 μM SBD122 with β-cyclodextrin; (●) titration of 64.2 μM SBD106 with maltoheptaose; (▲) titration of 55.6 μM SBD122 with maltoheptaose.

peaks at 285 nm and 293 nm and a shoulder near 303 nm (FIG. 4). The shoulder at 303 nm suggests that the electrostatic component of interaction in the vicinity of one or more Trp residue(s) was weaker with maltoheptaose than with by β-cyclodextrin.

The maximum change of ΔA occurred at 285 nm (FIG. 4). The titration curves of ΔA_{285} of SBD106 and SBD122 against maltoheptaose concentrations are shown in FIGURE 3. As with β-cyclodextrin, ΔA_{285} increased hyperbolically with the maltoheptaose concentrations. K_d values of SBD–maltoheptaose complexes were calculated by using the Scatchard equation (Eq. 1). Dissociation constants of maltoheptaose binding to SBD106 and SBD122 were not significantly different (TABLE 2) and were

TABLE 2. Dissociation Constant (K_d) for β-Cyclodextrin and Maltoheptaose Binding to Starch-Binding Domain (SBD)

	β-Cyclodextrin		Maltoheptaose	
	SBD106	SBD122	SBD106	SBD122
K_d (μM)	34 ± 5[a]	26 ± 4 21 ± 2	570 ± 50	500 ± 10

[a]Standard error of linear regression.

approximately 20 times greater than the corresponding K_d values of β-cyclodextrin binding. The dissociation constant of maltoheptaose that we have obtained is similar to the K_d value (0.38 mM) measured by Belshaw and Williamson[29] with the GAI-derived starch-binding fragment.

The weaker interaction of maltoheptaose than of β-cyclodextrin with the SBD peptides demonstrates the difference of the conformational structure of the ligands; β-cyclodextrin is a cyclic glucose heptamer, whereas maltoheptaose (also a glucose heptamer) is a random coil molecule. The conformation of β-cyclodextrin resembles the helical structure of starch molecules, which is a native substrate for the SBD of GAI; therefore, stronger interaction of β-cyclodextrin than maltoheptaose with the SBD can be expected.

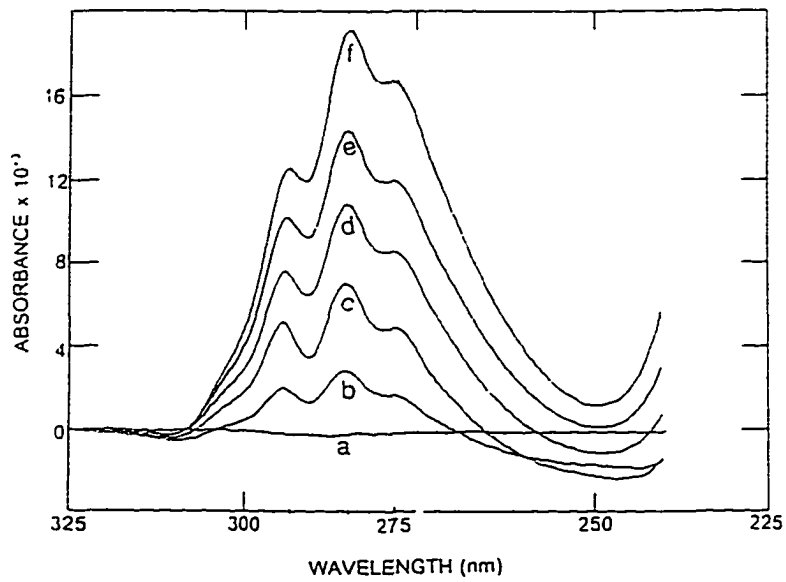

FIGURE 4. Ultraviolet difference spectra of SBD induced by maltoheptaose. (a) 0, (b) 133, (c) 462, (d) 658, (e) 980, and (f) 1675 μM maltoheptaose.

In conclusion, we found in this study that (1) the starch-binding domain is an independent domain that interacts with starch-like ligands (cross-linked amylose, maltoheptaose, and β-cyclodextrin) like the native SBD in GAI, (2) the intramolecular disulfide bonding (SBD106) is not required for the domain functioning, and (3) *O*-glycosylation is not critical for the functioning of the starch-binding domain, but may affect the conformation and dynamics of the domain.

SUMMARY

The characterization is reported of two peptide fragments (SBD106 and SBD122) containing the starch-binding domain (SBD) of *Aspergillus* sp. glucoamylase I. The

starch-binding peptides were produced in *Escherichia coli* as fusion proteins of the maltose-binding protein (MBP). SBD106 (11.9 kDa) and SBD122 (13.8 kDa) were purified from the factor X_a digest of MBP fusion proteins. The amino acid compositions were similar to those deduced from their amino acid sequences.

The interactions of β-cyclodextrin and maltoheptaose with purified SBD peptides were investigated by UV difference spectroscopy. SBD106 and SBD122 bound specifically β-cyclodextrin with a dissociation constant (K_d) of 34 μM and 23.5 μM, respectively. Maltoheptaose binding to SBD106 and SBD122 was weaker than that of β-cyclodextrin; dissociation constants were 0.57 and 0.50 mM, respectively. The results indicate that the intramolecular disulfide bonding is not required for the domain functioning and that *O*-glycosylation is not critical for the functioning of the starch-binding domain, but may affect its conformation and dynamics.

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