CHAPTER IV

DISCUSSION

In general, CGTases from various bacteria show a clear similarity in amino acid sequences, ranging from 47% to 99%. (van der Veen *et al.*, 2000c). From X-ray crystallography of CGTase, it has been known that all CGTases have similar three-dimensional structures. However, all wild type CGTases usually produce a mixture of α-, β- and γ-cyclodextrin in different ratios according to the source of CGTases. Thus, different CGTases should have subtle different activities that lead to different product specificity. It can be perceived that there should be local variation in the active sites or other sites involved in the activity of CGTases and hence their amino acid sequences. To elucidate the relationships between the sites involved on activity of CGTases and product specificity, a protein engineering approach is one of the many ways of studying. Protein engineering involves the design and construction of mutant proteins, usually by manipulation of their genes and requires a multi-disciplinary approach. It involves recombinant DNA techniques (gene cloning, gene expression and mutagenesis); biochemical techniques (protein purification and characterization), protein crystallography techniques (crystallization, soaking of crystals, X-ray analysis and determination of three-dimensional structures), computer graphics techniques (docking of molecules in the protein structures and homology modeling of protein) and identification of targets for protein mutagenesis (Penninga, 1996b).

In CGTases, it has been suggested that the size of the aromatic amino acid (Phe or Tyr), present in the center of the active site cleft of CGTases (detail in chapter I), influences the preferred cyclodextrin size. Sin *et al.*(1993) also proposed a mechanism in which the starch chain folded around this residue. Substitution of this amino acid by a tryptophan, Tyrl88Trp in the *B. ohbensis* CGTase (Sin *et al.*, 1994) and Tyrl95Trp in the *B. circulans* strain 8 CGTase (Parsiegla *et al.*,1998) indeed doubled the relative production of γ-cyclodextrin. However, several other Tyr188 mutations and the substitutions of Tyrl95 of the *B. circulans* 251 CGTase by other amino acids (Peninga *et al.*,1995) as well as the mutation Trp191Tyr at the similar position in the CGTase of *B. stearothermophilus* NO2 (Fujiwara *et al.*,1992) did not

support this proposed mechanism. Furthermore all natural α-, β- and γ-CGTases have Tyr or Phe at this position, indicating that this residue is not involved in the differences in product specificity. New insights from the refined X-ray structure of the CGTase from *B. circulans* strain 251 in complex with a maltononaose suggested that the specific sugar binding subsites (-6, -7 and -8) farther away from the catalytic site could be important for the enzyme's product specificity. It might also be possible to change the cyclodextrin ratios by altering the affinities at these specific sugar binding subsites (Strokopytov *et al.*, 1996).

As stated above that different types of CGTases should have slightly different active sites and sites involved in cyclization, there should be differences in amino acid sequences at certain parts of the structure. To determine these differences, four representative CGTases of the three types of CGTases, α-, β-, β/γ- and γ-CGTases, were chosen according to Takada *et al.*(2003) and their amino acid sequences were downloaded from the GenBank. As shown in Fig. 19, sequence comparison was performed using ClustalX software. Besides the several homologous sequences scattered all over the protein sequences, there are three major regions at positions 87-95, 141-152 and 532-536 (*B. circulans* A11 CGTase numbering) in γ-CGTases that are different from those of other typical CGTases. The first and second regions are located in domain A1 and B of CGTases that constitute the active site. These two sites are equivalent to subsites -3 and -7 proposed by Strokopytov *et al.*(1996). The third region is located in domain D of the CGTases, which the function is not known. The differences may involve in the proportion of cyclodextrins produced. To study their involvement in product specificity, the β-CGTase was mutated and genetically manipulated to contain the various combinations of all 3 mutated sites. The product ratios were determined thereafter.

To create the three mutations in the β-CGTase gene, three mutagenic primers were designed according to the amino acid regions in β-CGTase that are different from those of γ-CGTase. The primers introduced both base substitutions and deletions to change the three sites in β-CGTase in favor of the amino acid sequences in γ-CGTase (Fig. 20). The three mutated plasmids, pNan1, 2 and 3, containing the mutant sites I, II and III, respectively, were obtained as shown in Fig. 22. The mutation sites were subcloned into the wild-type CGTase gene replacing the equivalent sequences. Various mutant CGTases were constructed to contain all possible combinations of the

mutated sites. These resulted in pNan4, 5, 6, 7, 8, 9 and 10 which contained mutation sites I, II, III, I+II, I+III, II+III and I+II+III, respectively.

The three-dimensional structures of *B. circulans* A11 β-CGTase (wild type CGTase) and the mutant CGTases were predicted by using homology modeling (www.cbs.dtu.dk/services/CPHmodels). Since the amino acid sequence of CGTase from *B. circulans* A11 is about 98% homologous to that of CGTase from *Bacillus* sp. 1011 (PDB I.D. 1I75) that the x-ray crystallographic structure is already known. The predicted structure of the wild type CGTase from *B. circulans* A11 was compared to the crystal structure of CGTase from *Bacillus* sp. 1011. It is found that their overall structures are identical (Fig. 31). The predicted structures of wild type CGTase from *B. circulans* A11 and the mutant CGTase from pNan10 (Fig. 32) revealed that the mutation sites I and II are located on the surface of the enzyme and resides on the loop regions which upon deletion seems to have no effect on the folding of the main chain α-helices and β-sheets. The mutation in site III abolishes two sheets in domain D but has no influence on the active site structure. This may be the reason why all mutant CGTases retain their activity. It should be noted that site III resides on the far side of domain D, away from the active site and facing out into the surrounding of the enzyme.

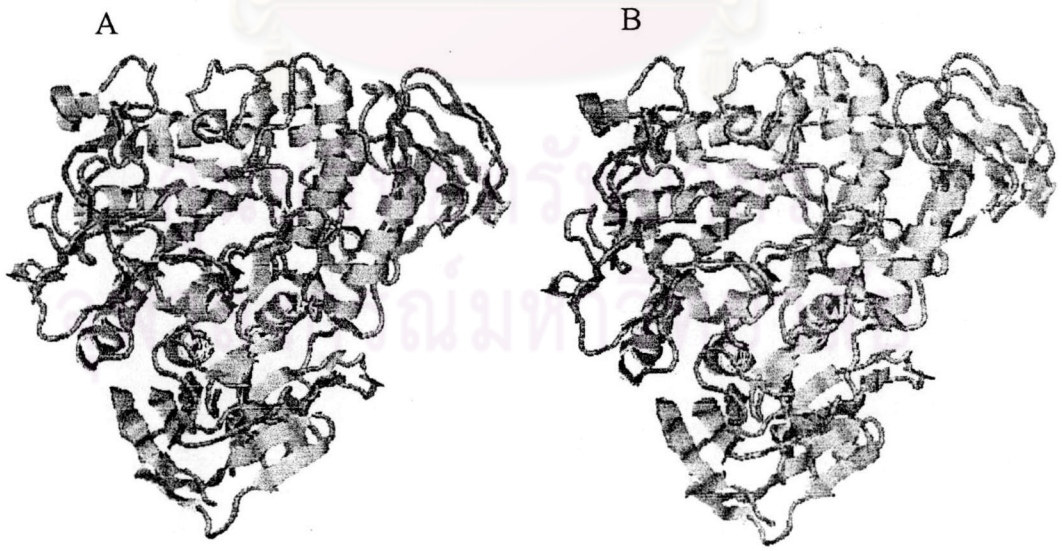

Figure.31 Comparison of three dimensional structures between CGTase from alkalophillic *Bacillus* sp.1011 (PDB I.D. 1I75)(A) and CGTase from *B.circulans* A11(B)

All the mutants exhibit the dextrinizing activity (Fig. 28-29) and cyclodextrin forming activity (Fig. 30). All but one mutant CGTases have decreased dextrinizing activity. Only the mutant CGTase from pNan7 has slightly increased dextrinizing activity. Mutation in site I has an effect on dextrinizing activity, probably due to change in the active site structure closed to the catalytic residues, Asp229 and Glu257. However, mutation in site II has no effect on dextrinizing activity. Surprisingly, mutation in site III also has moderate effect on dextrinizing activity although site III resides in domain D, which is farther away from the active site. It is difficult then to figure out the function of site II but it is certain that domain D contributes to the activity of enzyme. The combination of mutated sites I and II provides better dextrinizing activity than the wild type enzyme. This may be due to the compatibility of the two mutant sites in the active sites. Other combinations result in lower dextrinizing activity, which are the down mutation effects of either mutated site I or site III or both.

Van der Veen *et al.*, (2000b) proved the importance of the sugar binding subsites by site-directed mutagenesis of amino acid residues at subsites –3 (site I in this study) and –7 (site II in this study). They found that the mutagenesis of amino acid residues at subsites -3 and -7 effected the cyclodextrin ratios by increasing the proportion of α-cyclodextrin and decreasing that of β-cyclodextrin but had no effect on γ-cyclodextrin production. However, this mutation could not alter the major cyclodextrin produced; it was still β-cyclodextrin. Although other investigators had mutagenized other amino acid residues in subsite -3 and -7 regions of CGTases, the results were similar to that of van der Veen *et al.*, (2000b) as the cyclodextrin ratios could be altered but not the major cyclodextrin product (Table 4).

HPLC analyses of the cyclodextrin products from the CGTase reactions indicated that the mutant CGTases produced the cyclodextrins at various ratios that were different from that of wild type (Fig. 30 and Table 3). Mutation in site I in pNan4 resulted in an extraordinary increase in β-cyclodextrin production, up from about 64% to about 90%, while the production of α-cyclodextrin was decreased significantly and very little or no γ-cyclodextrin produced. The mutation in site I is equivalent to mutation in subsite -3. The site was deleted to reduce the size of the loop and the adjacent amino acid residues were changed in favor of the residues found in γ-CGTase(Fig.30). Therefore the geometry of the subsite –3 was changed. The change

in active site geometry may render the enzyme bound favorably to the maltoheptaose segment of the polysaccharide chain and thus produced favorably β-cyclodextrin. The deleted loop may provide flexibility in the binding of oligosaccharide chain making the wild type enzyme produces more α- and γ-cyclodextrins.

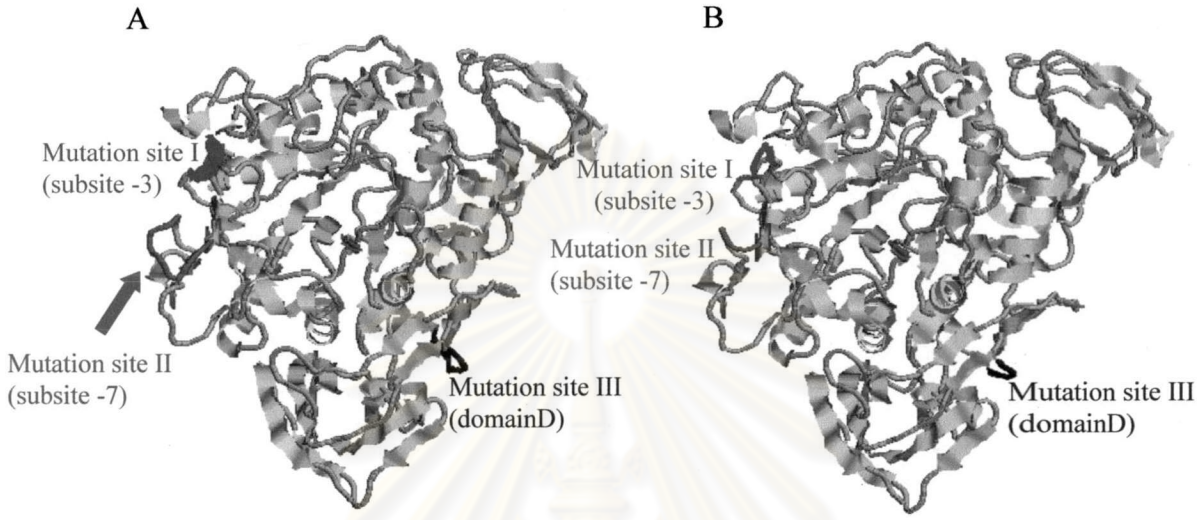

Figure. 32 Comparison of three dimensional structures between wild type CGTase.from *B. circulans* A11(A) and mutant CGTase from recombinant plasmid pNan10(B)

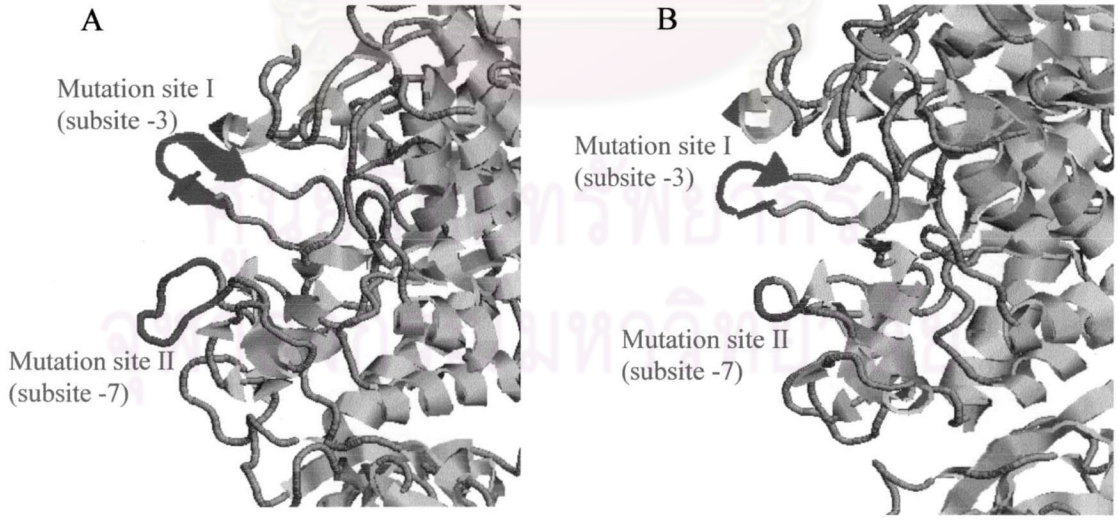

Figure.33 Comparison of the three dimensionanl structures of CGTase at subsite-3 and -7 between wild type CGTase(A) and mutant CGTase(B).

The mutation in site II of the CGTase in pNan5, almost doubled the production of γ-cyclodextrin while the productions of α- and β-cyclodextrins were slightly lower. The site II is equivalent to the subsite –7. Mutation in this site causes the reduction of the loop by six amino acid residues (SDDPSF)(Fig. 33) and amino acid substitution that replaces ASA with IDV. Takada *et al* (2003) had proposed that the γ-cyclization activity required more space for the glucosyl chain at subsite -7 (Fig.15)(Takada *et al.*, 2003) The hypothesis is based on the finding that a deletion mutant [(Δ145-151)D] of β-CGTase from *B. circulans* strain 8 produces greater amount of γ-cyclodextrin than the native enzyme.(Pasiegla, *et al* 1998). The result from pNan5 is in agreement with the above hypothesis. Although the production of γ-cyclodextrin is higher, the major product of pNan5 is still the β-cyclodextrin. We argue that more space in the binding subsite 7 is just one of the factors that govern the synthesis of γ-cyclodextrin. Other essential factors remain to be elucidated.

Table 4 Summary of CGTases mutagenesis that affected cyclodextrin products from previous studies

Mutagenes is residues	Domain locate	Effect on cyclodextrin ratios			Function	Source	Main product
		α	β	γ			
Y89D*[1]	A1	Increased	Decreased	Decreased	Cyclodextrin Product spcificity	*B.circulans* 251	β
Y89D/ S146P*[1]	A1, B	Increased	Decreased	Unaffected			
S146P*[1]	B	Increased	Decreased	Unaffected			
Y89S*[2]	A1	Unaffected	Unaffected	Unaffected		Alkalophilic *Bacillus* strain I-5	
Y89F*[2]	A1	Unaffected	Increased	Unaffected			
N94S*[2]	A1	Increased	Unaffected	Unaffected			
Δ84 carboxyl terminal amini acid*[3]	A1	Decreased	Decreased	Decreased	Substrate binding domain	*B. circulans* var alkalophilus	
Δ(141-151)D*[4]	B	Decreased	Decreased	Increased	May produce more space for the bound glycosyl chain	*B. circulans* strain 8	

*1 van der Veen *et al.*, 2000b, *2 Kim *et al.*, 1997.,*3 Hellman *et al.*, 1990 and*4Parsiegla *et al.*, 1998

The mutation in domain D in pNan6 significantly increased the production of β-cyclodextrin, while the production of α-cyclodextrin was decreased and the γ-cyclodextrin production was not affected. The mutation abolished two sheets in domain D(Fig.35) but had minor effect on the structure of the enzyme. Since the location of this site is far away from the active site and seem to have no influence on other domains, we cannot make any conclusion on the involvement of this site on cyclization. We do not know how the mutation at this site exerts its effect but we believe that domain D translates the effect of mutation through the adjacent domains C and E.

The presence of mutant site I in pNan7, besides the mutant site II, resulted in an increase production of β-cyclodextrin similar to the pNan4. The effect of mutant site II was shadowed by that of mutant site I since there was no production of γ-cyclodextrin. In the presence of the distant mutant site III as in pNan8, the effect of mutant site I diminished as the synthesis of γ-cyclodextrin was increased. This is also true with pNan10, which has all three mutant sites as we compare the result of pNan7 with that of pNan10. The distant mutant site III exerts its effect on the enzyme separately from that of mutant site I. The results indicate that the cyclodextrin production involved the other domains of enzyme as well not just the active site.

Mutation in site II resulted in an increase production of γ-cyclodextrin as seen in pNan5, 9 and 10. The results seem to indicate that γ-cyclodextrin synthesis requires space around the subsite –7 since the synthesis increases as the loop at subsite –7 is deleted. Although these results are in agreement with that of Parsiegla, *et al.*(1998), they also show that other factors may be involved. The mutation in pNan7 does not synthesis any γ-CD. In addition, Fig. 35 reveals that subsite -7 does not contact with finished product, γ-CD. Site II is considerately involved only during the bingding of substrate for cyclization. In addition to space, other interaction between the substrate and amino acid side chains at this subsite may be important factor involved in γ-CD production.

As mentioned above and from the results, we could propose a sliding model of the binding between CGTase and substrate, glycosyl chain. This model reveals that during the binding, the glycosyl chain slides through the groove from the substrate binding domains C and E pass to the catalytic domain from subsite +2 to subsite -7 (see Fig. 36 and 7). This model is supported by the fact that when the loop at subsite-7