CHAPTER IV

DISCUSSION

4.1 Purification of CGTase

CGTase from *Bacillus* sp. A11 was firstly screened from South-East Asian soil by Pongsawasdi and Yagisawa (1987) and was purified from the culture broth by our research group in the Department of Biochemistry (Techaiyakul, 1991; Rojtinnakorn, 1994; Kim, 1996). In this work, an essentially pure enzyme preparation was obtained in two steps of purification, corn starch adsorption and immunoaffinity column chromatography, previously reported by Kim (1996). The purified enzyme demonstrated the same pattern on PAGE. It appeared as one band with estimated molecular weight of 72,000 daltons, the same size as previously reported (Rojtinnakorn, 1994; Kim, 1996). Two major protein bands and one faint band was observed on non-denaturing PAGE, all these bands were positive when stained for amylolytic activity. The fourth activity band with the most negative charge could also be detected (Figure 9B, lane 3). Three protein bands observed on non-denaturing PAGE may be isozymes of 72,000 daltons or may be the result of different glycosylation state. This CGTase was proved to be a single polypeptide chain by gel filtration and SDS-PAGE techniques (Techaiyakul, 1991) which was similar to many CGTases such as those from *Bacillus* sp. No. 38-2 and *Bacillus circulans* E192 (Kaneko *et al.*, 1988; Villette *et al.*, 1991). However, Rojtinnakorn (1994) reported that our CGTase from *Bacillus* sp. A11 could be fractionated on chromatofocusing column, with pIs range of 4.40 to 4.90. The CGTases from *Bacillus circulans* var. Alkalophilus, *Bacillus circulans* E192, and *Bacillus* strains (INMIA-T6, INMIA-T42, and INMIA-A7/1) were reported to contain 2, 6, and 2-4 subforms, respectively (Mattsson *et al.*, 1990; Bovetto *et al.*, 1992; Abelyan *et al.*, 1994). There has been

some report that CGTases from *Bacillus macerans* IAM 1243 and *Bacillus megaterium* No. 5 were dimeric proteins of similar subunit size of 145,000 and 66,000 daltons, respectively (Kobayashi *et al.*, 1978; Kitahata *et al*, 1974).

When purification data was analyzed, the enzyme was purified to 144 folds and the specific activity was 3,574 units/ mg protein with 36% recovery. This result is similar to that reported by Kim (1996), who demonstrated that the enzyme could be purified to 155 folds with the specific activity of 3,302 units/ mg protein and 45% recovery.

4.2 Information on active site of CGTase

4.2.1Chemical modification of CGTase

Several methods have been used to explore the molecular structure and the active site of enzymes. Among those general techniques, affinity labeling using substrate or substrate analogs, site-directed mutagenesis, X-ray crystallographic and diffraction studies or chemical modification have been reported (Means and Feeney, 1971; Lundblad, 1991; Villette *et al.*, 1993; Nakamura *et al.*, 1993, 1994; Sin *et al.*, 1994; Strokopytov *et al.*, 1995).

Affinity labeling is a technique for the specific modification of an amino acid residue in a protein, which involves both the binding of the reagent (affinity label), on the basic of biological specificity and subsequent modification of an amino acid residue through the formation of a covalent bond (Means and Feeney, 1971; Lundblad, 1991). There are almost an infinite variety of compounds which can be used as affinity labels. First are derivatives that form relatively stable analogs/ homologs of enzyme intermediate. Second are the so-called K_s reagents, which are isosteric with respect to substrate or ligands and posses a relatively unreactive functional group. In another type of modification, the reactive species is generated after binding of the reagent to

the protein. An example of the use of affinity labeling is the use of toluenesulfonyl-phenyl-alaninechloromethyl ketone (TPCK) to effect a highly specific alkylation of an active-center histidine in α-chymotrypsin (Schoellmann and Shaw, 1963). To design a good and effective affinity label is the key step for this technique.

Site-directed mutagenesis, on the other hand, is based. on hybridizing a synthetic oligonucleotide, with a mismatch region in its center, to a single-stranded target DNA template and extending with T4 DNA polymerase to create a double-stranded structure (Leonard *et al.*, 1994). Nicks in the duplex are sealed by T4 DNA ligase, after which the duplex structure is transformed into *E. coli* host where DNA replication proceeds. This technique requires the known information on base sequence of the enzyme region to be studied. In practice, to select the suitable vector, the host, or the polymerase enzyme used for a good level of mutagenesis efficiency should be of important concern. In 1993 and 1994, Nakamura *et al.* reported the use of this technique to study three histidine and four aromatic residues in the active center of CGTase from *Bacillus* sp. 1011.

X-ray crystallography is a technique that directly images molecules (Voet and Voet, 1990). Interest in X-ray crystallographic studies of proteins has promoted active investigation of chemical methods to produce derivatives containing the necessary heavy atom. Isomorphic replacement of a small atom for a large atom is necessary for most X-ray studies (Means and Feeney, 1971). The heavy atom serves as a reference point around which the other atoms may be located. Complete assurance that conformational changes have not occurred is of great concerned. With many enzymes, the retention of enzymatic activity by heavy-atom-containing crystals has served to demonstrate some degree of the native structure. A relatively new approach to the introduction of heavy atoms into protein crystals utilizes covalent attachment to the protein of a heavy-atoms-containing or chelating group. Metal-ion-chelating groups such as 3-aminotyrosine and picolinamidine groups have been proposed to be

potentially useful for preparing heavy-metal-containing protein derivatives. There appears to be no great difficulty with this approach, except perhaps, in preparing the specifically modified derivatives and showing them to be isomorphous with the native protein. The potential of this procedure for crystallographic studies will depend upon whether such derivatives are found to be isomorphous with the unmodified proteins. The X-ray structure of hen egg white losozyme, which was elucidated by Phillips in 1965, was the second structure of a protein and the first of an enzyme to be determined at high resolution. In 1967, Blow elucidated the X-ray structure of bovine chymotrypsin, which folded into two domains, both of which have extensive regions of antiparallel β-sheets in barrel-like arrangement, but contain little helix (Means and Feeney, 1971).

Chemical modifications of reactive groups of enzymes have been used to identify the amino acid residues that are responsible for enzyme action (Means and Feeney, 1971). With some modifying reagents, several different groups may be modified (Appendix 2). Lack of specificity is not always a disadvantage. Surrounding side chains or the environment of proteins may affect a specific reaction condition. In addition, changes in conformation frequently occur, either due to the modified protein's new properties, or as a consequence of too harsh reaction conditions. Therefore, the reaction of the modifying reagents with proteins should be done under mild conditions to prevent non-specific reaction or protein denaturation (Lundblad, 1991). Differences in protein modifications, with and without a specific binding substance, may provide information about the amino acid residues at the binding site. For example, modifications of an enzyme with or without its specific inhibitor may suggest the amino acid residues being located at the active site. If enzyme activity is retained after modifications in the presence of inhibitor but is lost in its absence, it is usually assumed that inhibitor has protected a group in the active site (Means and Feeney, 1971; Lundblad, 1991). Chemical modification is a relatively easy method and a method of choice in active site study because it dose not require much preliminary

data of the enzymes and only a small amount of enzyme is used. Many examples can be cited. Tryptophan residues of glucoamylase from *Rhizopus niveus* by chemical modification with NBS and their involvement at enzyme active site were reported (Ohinishi *et al.*, 1983). In 1996, Wakayama studied chemical modification of histidine residue for the investigation of active site of *N*-acyl-D-glutamate amidohydrolase from *Pseudomonas* sp. 5f-1. Johns *et al.* (1997) studied the effect of covalent modification of tyrosine, arginine and lysine residues on coproporphyrinogen oxidase from chicken red blood cells.

In this work, the investigation of amino acid residues in the active site of CGTase of *Bacillus* sp. A11 have been performed using chemical modification with group-specific reagents. Chemical modifications of seven different amino acid residues (carboxylic, histidine, tryptophan, tyrosine, cysteine, lysine, and serine) of CGTase have been determined under mild conditions. These amino acid residues have been selected because they are widely known as residues involved in enzyme catalysis, while some have been reported to be essential for CGTases of other sources (Means and Feeney, 1971; Lundblad, 1991).

The first step was to screen the residues that upon modification, the loss in CGTase activity was observed. Pre-incubation of purified CGTase from *Bacillus* sp. A11 with a series of covalent modifiers of amino acid residues resulted in variable changes in the catalytic ability of this enzyme. Little inhibition of CGTase activity was seen in the modification of cysteines by *N*-ethylmaleimide (NEM), iodoacetamide (IAM), or dithiothreitol (DTT), lysines by 2,4,6-trinitrobenzenesulfonic acid (TNBS), and serine residues by phenylmethylsulfonyl fluoride (PMSF). Reactions under mild conditions with 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC), diethylpyro-carbonate (DEP), *N*-bromosuccinimide (NBS), and *N*-acetylimidazole (NAI), which were known to react specifically with carboxyl, histidine, tryptophan, and tyrosine residues, respectively, resulted in extensive inhibition of CGTase activity (Table 8).

Among the four modifiers, DEP and NBS totally inhibited the enzyme at 1 mM while EDC and NAI were equally effective at 5 mM (Figure 10) and 30 mM (Figure 18), respectively. It may be assumed here that histidine, tryptophan, carboxyl, and tyrosine were all likely to be involved in CGTase activities. When compared to other studies, these important amino acid residues for our CGTase have been demonstrated elsewhere as essential residues for other CGTases as well. Bender (1991) reported that histidine residues of CGTase from *Bacillus circulans* strain 8 and *Klebsiella oxytoca* M5 al were modified by DEP and proposed to be involved in the cyclizing reaction. In 1992, Mattsson *et al.* reported the inhibition of CGTase from *Bacillus circulans* var. *alkalophilus* (ATCC 21783) by 0.25 mM DEP and EDC at 6,770 mol/mol CGTase which resulted in almost total loss of cyclizing activity indicating that histidine and carboxylic residues are involved in the catalytic activity. Villette *et al.* (1993) reported the modification of tyrosine residues of CGTase from *Bacillus circulans* E192 with tetranitromethane. The results suggested that the nitration of the CGTase induced a decrease in the catalytic properties of the enzyme. CGTase from *Bacillus stearothermophilus* were reported to be modified at tryptophan residues by NBS (Onishi *et al.*, 1992). The modification corresponded with inactivation of the CGTase-catalysed hydrolysis of cyclomaltohexaose (cG_6).

The second step was to prove that the amino acid residues involved in CGTase activity were at the active site or not because the inactivation of enzymes by chemical modification does not directly indicate that a specific residue is present at the active site. Protection of enzyme by substrate, competitive inhibitor, end product, or related compounds has been used to selectively prevent the modification of active site residues in many enzymes. If enzymatic activity is retained following modification in the presence of substrate (or other protective substances) but is lost in its absence, it is usually assumed that a group in the active site has been protected (Means and Feeney, 1971). In this work, α-, β-, γ-CD, and maltotriose were used as protective substances. Two experimental approaches have been performed. The first was to measure enzyme

inactivation by group-specific reagents in the presence or the absence of protective substances. The results in Table 9, 11, 14, and 17 showed that the loss of CGTase activities were partially or totally reduced (activity loss 0-50%) in the presence of protective substance. Carboxyl modification was almost totally protected by all protective substances (activity loss ≤ 12%), while histidine modification was partially protected (activity loss 29-35%). Tryptophan modification was totally protected by γ-CD and almost totally protected by α-, β-CD and maltotriose (activity loss ≤ 15%). Tyrosine modification was totally protected by β-CD and maltotriose and partially protected by α-and γ-CD (activity loss 14-20%). The interpretation that carooxylic, histidine, tryptophan, and tyrosine residues are involved at the catalytic site of the enzyme can then be made. However, to confirm the presence of these residues at the active site, the second experimental approach was performed. After incubation of the enzyme with chemical modifiers in the presence or the absence of protective substance, dialysis was performed to remove excess reagents prior to the measurement of residual CGTase activity. The results (Table 10, 12, 15, and 18) were similar to the results from the first approach except that in most cases, the loss of CGTase activity was higher. Carboxyl modification was almost totally protected by α- and β-CD but partially protected by γ-CD and maltotriose (activity loss 10-15%), while histidine modification was partially protected (activity loss 32-50%). Tryptophan modification was totally protected by γ-CD and partially protected by α-, β-CD and maltotriose (activity loss 19-38%). Tyrosine modification was totally protected by β-CD and maltotriose and partially protected by α-and γ-CD (activity loss 15-32%). This may be the result of incomplete removal of the excess reagents by dialysis or the enzyme is exposed to the modifying reagent during dialysis for a longer time than the optimal time used in the inactivation experiment. These results, however, support the involvement of carboxylic, histidine, tryptophan, and tyrosine residues in the active site of CGTase.

When compared to other CGTases, Mattsson *et al.* (1992) reported that CGTase from *Bacillus circulans* var. alkalophilus (ATCC 21783) was protected against inactivation by α-, and β-CD suggesting that the modified histidine residues is at or near the active site. The study by Villette *et al.* (1992) showed that CGTase activity was retained when chemical modification with DEP occurred in the presence of 5 mM acarbose (uncompetitive inhibitor of CGTase) or 5 mM salicin (competitive inhibitor of CGTase). These inhibitors protected one of the two faster reacting histidine residues in the active site, with a 49.7% recovery of residual activity. Trp was protected against NBS by glucose and the maltosaccharides G_2-G_4, which indicates Trp to be located at the substrate binding site of CGTase from *Bacillus stearothermophilus* (Ohnishi *et al.*, 1992).

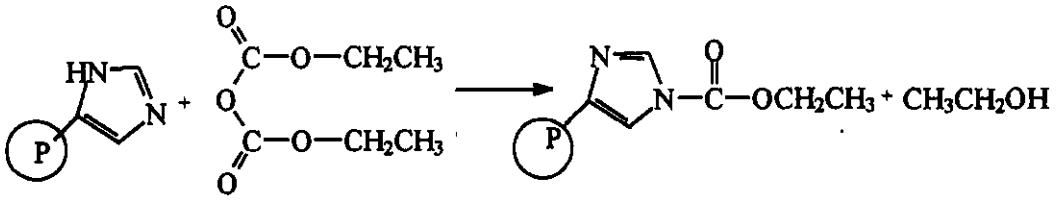
The third step of the chemical modification experiment was to estimate the number of amino acid residues which were protected by protective substances. Quantitative determination of essential groups can be done in a number of ways. The change in light absorption has been generally used to quantitatively determine content of some amino acid residues in an enzyme. The reaction of DEP with imidazole groups of proteins is accompanied by an increase in absorbance at 246 nm indicative of the formation of *N*-carbethoxyhistidine (Reaction 1). This can be used to quantitate the extent of reaction using a molar extinction coefficient of 3.20×10^3 $M^{-1}cm^{-1}$ (Wakayama *et al.*, 1996). The paper reported that 1.1 mole histidine was protected per mole *N*-acyl-D-glutamate amidohydrolase. Thus one histidine residue was involved at the active site. For tryptophan residues, the reaction with NBS is accompanied by a decrease in absorbance at 280 nm. These changes in absorption indicative of the formation of *N*-acetyltryptophan (Reaction 2) have been used to quantitatively determine tryptophan content of proteins using a molar extinction coefficient of 4.00×10^3 $M^{-1}cm^{-1}$ (Lundblad, 1991). By this method of determination, six tryptophan residues of egg white lysozyme were oxidized by NBS. In the case of tyrosine residues, acylation of tyrosine phenolic groups is distinguished from that of

amino groups by an increase in absorption at 278 nm accompanying the formation of *O*-acetyltyrosine (Reaction 3). This can be used to quantitate the extent of reaction using a molar extinction coefficient of 1.16 x 10^3 M^{-1} cm^{-1}. Examples of works reported are: NAI selectively acetylates six tyrosyl reduces in bovine carboxypeptidase A, seven tyrosyl residues in bovine pancreatic carboxypeptidase A, and two tyrosyl residues in pepsin. Such reactions are easily followed spectrophoto-metrically (Means and Feeney, 1971).

In the estimation of number of histidine, tryptophan, and tyrosine residues at the active site of CGTase of *Bacillus* sp. A11, β-CD and γ-CD were used to protect these residues from modification because the CGTase activity after protection with these substrates were more regained than when using other protective substances (α-CD or maltotriose) (Table 9-10, 11-12, 14-15, and 17-18). The results in Table 13, 16, and 19 indicated that β-CD or γ-CD protects two histidine residues, one tryptophan residue, and two tyrosine residues of CGTase, respectively and thus suggest the presence of these residues at the enzyme active site. However, this technique which follows the change in light absorbance cannot be applied for determination of number of essential carboxylic residues because the EDC-carboxyl complex dose not posses specific absorption at any wavelength.

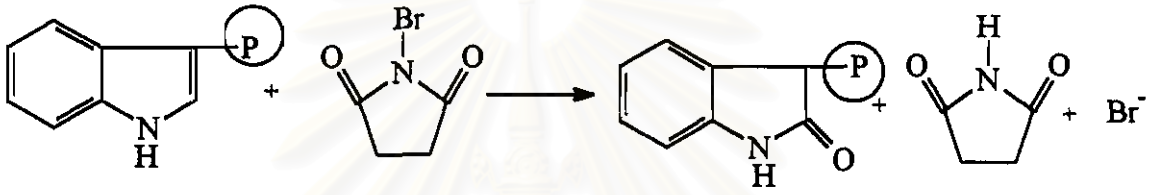
For other CGTases, not much work has been done on the quantitation of number of active residues at the active site. Bender (1991) reported that ethoxyformylation with DEP of ≈ 1.5 histidine residues per molecule of enzyme reduced the cyclising activity of both the α-CGTase from *Klebseilla pneumoniae* strains M5 al and β-CGTase from *Bacillus circulans* strains 8 by more than 90%. Pre-incubation with substrate protected the enzyme from ethoxyformylation.

The fourth step of the work was to determine the effect of chemical modification on the structure of CGTase. Using electrophoresis, the change in structure after chemical modification can be suggested. The enzyme prior to



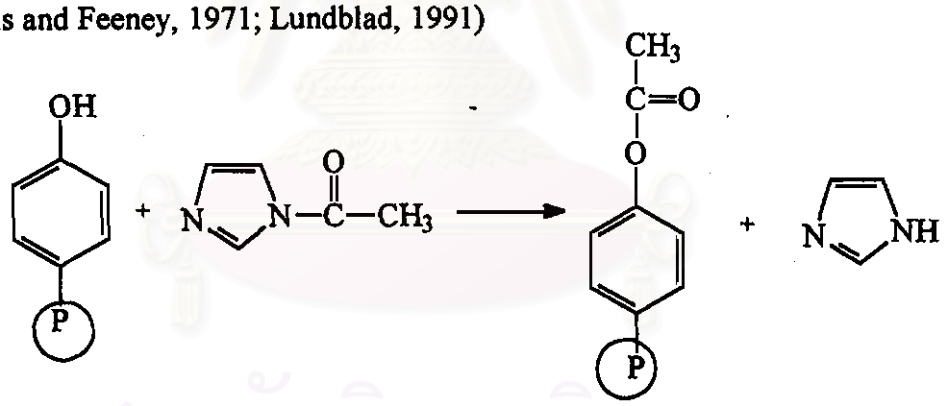
Reaction 1 Modification reaction of DEP with histidine residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)



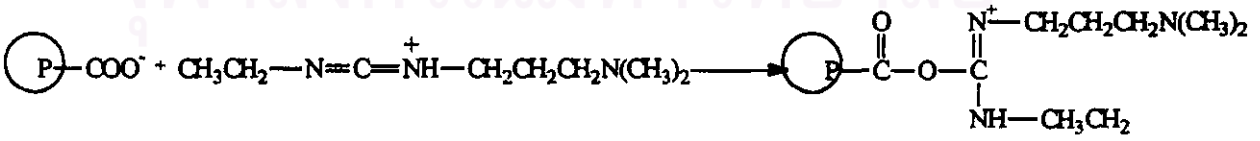
Reaction 2 Modification reaction of NBS with tryptophan residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)



Reaction 3 Modification reaction of NAI with tyrosine residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)



Reaction 4 Modification reaction of EDC with carboxyl residue in protein (P)

(Means and Feeney, 1971; Lundblad, 1991)

modification showed two major protein bands with one faint band (Figure 21A, lane 1 and 6). For activity staining, the unmodified enzyme showed three major bands which corresponded to their cognate protein bands and one faint band which did not show up in protein staining (Figure 21B, lane 1 and 6). The modification of carboxyl group by 5 mM EDC, decreases the negative charge as shown in Reaction 4, was confirmed by an extra band (above label a) which migrates slower than the unmodified bands (Figure 21, lane 2A and 2B). On the other hand, modification of histidine residues by DEP or modification of tyrosine residues by NAI should result in the modified enzyme with slightly more negative charge (Reaction 1 and 3). The modified bands moved faster than the unmodified bands in both protein and activity stained (Figure 21; lane3A, 3B, 5A, and 5B). In the case of modification of tryptophan residues by NBS, the net charge was not changed (Reaction 2), thus result in normal pattern like the unmodified enzyme in the electrophoresis (lane 4A, and 4B). From PAGE analysis of the enzyme after EDC, DEP, NAI, and NBS treatment, the modification of carboxyl, histidine, tyrosine, and tryptophan residues of CGTase were evidenced.

For the determination of the effect of pH on the modification of CGTase, the results were summarized in Table 21. It suggests that EDC modification was effective in the pH range of 5.0-6.0. Horinishi (1968) reported that the water-soluble carbodiimide (WSC)-promoted reaction between acetylglycine and glycine methyl ester has a broad flat optimum pH between 5 to 7. However, with proteins, the reaction appears to be best at the lower end of this range, between pH 4.5 and 5.0. For our CGTase, the activity was significantly reduced at pH < 5 (Techaiyakul, 1991), so we did not go beyond pH 5.0 when studied the effect of pH on the modification of enzyme. The modification of CGTase by DEP at various pHs resulted in similar loss of CGTase activity and works effectively in the pH range of 5.0-7.5. In the pH range of 5.5-7.5, DEP is reasonably specific for reaction with histidine residues, forming an *N*- carbethoxyhistidyl derivative, as shown in Reaction 1 (Means and Feeney, 1971). For NBS, the result indicated that NBS modification works best at pH 5.0-5.5.

Treatment with NBS is usually performed in acetate or formate buffer at pH 4 or lower, but it can also be frequently done at pH closer to neutral. Higher pH has an advantage in that little or no peptide bond cleavage takes place. Also higher pH is usually more selective (Means and Feeney, 1971). The modification of CGTase by NAI works effectively in the pH range of 5.0-7.0. Means and Feeney (1971) reported that NAI acetylates tyrosyl groups of proteins at pH 7.5 or below yielding an *O*-acetylated derivative, as shown in Reaction 3. In this work, the purified CGTases in 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$ were used in the modification of all residues because pH 6.0 was optimum for dextrinizing activity assay, though not all modifications work best at this pH.

When specificity of chemical modification is concerned, it is generally known that several different groups may be modified by some modifying reagents (Appendix 2). Carbodiimides react not only carboxylic acids but also with water, alcohols, amines, and many compounds with active hydrogen. The reaction of water-soluble carbodiimide (WSC) with phenolic groups of tyrosine residues is thought to give *O*-arylisoureas (Means and Feeney, 1971). These adducts are stable at neutral pH and are even moderately stable during acid hydrolysis of the protein, but they can be decomposed by treatment with hydroxylamine. Sulhydryl groups of proteins react similarly but the resulting product appears to be more stable and is not decomposed upon treatment with β-mercaptoethanol or ammonia. These two reactions appear to be possible for all proteins containing sulfhydryl groups or tyrosine phenolic groups and should be considered when interpreting the results of WSC-promoted reactions with such proteins. Several WSC's have been used for modification of proteins, 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-p-toluenesulfonate (CMC) and EDC are now commercially available and are the most widely used. 1-Benzyl-3-(3-dimethyl-aminopropyl) carbodiimide (BCD) is also frequently used (Hoare and Koshland, 1966). All three reagents react similarly although the smaller reagents might be expected to have greater access to partially buried carboxyl groups. This

expectation appeared correct in a recent study with tobacco mosaic virus protein, wherein up to three additional carboxyl groups could be modified with EDC, which did not react with CMC (Means and Feeney, 1971). For DEP, it hydrolyzes slowly in water, giving two equivalents each of ethanol and carbon dioxide (or bicarbonate ion). In the pH range from 5.5 to 7.5, DEP is reasonably specific for reaction with histidine residues. At pH 4.0 it reacts principally with the amino and imidazole groups of proteins. Phenolic and aliphatic hydroxyl groups are not affected, with the exception of certain active serine residues as present in α-chymotrypsin. No reaction with sulfhydryl groups has been detected. The reaction of DEP with bovine serum albumin at pH 7.0 appears to involve tryptophan residues (Means and Feeney, 1971). Several reports (Patchronik *et al.*, 1958; Ramachandran and Witkop, 1959; Viswanatha *et al.*, 1960) indicated NBS to be a useful reagent for selectively cleaving tryptophanyl peptide bonds in peptides and proteins. It also cleaves tyrosyl peptide bonds, but more slowly than those of tryptophan. Histidine residues undergo a similar reaction, but very much more slowly. NBS is a potent oxidizing agent. It oxidizes -SH groups more rapidly than its reaction with tryptophan. Methionine and cysteine can also be oxidized. Of the aromatic amino acids, tryptophan is most susceptible to NBS oxidation. The reaction is best performed in acidic media, usually in acetate or formate buffers at pH 4 or below but it can also be frequently done at pH closer to neutral. Like DEP, NAI reacts with both amino and tyrosyl groups of proteins, but is more selective for tyrosine (Means and Feeney, 1971).

Although the chemical modification of amino acid residues and the use of substrate or inhibitor to protect the residues at the active site may appear simple, there are limitations and error that should be noted. Each modifying reagent should be chosen for use under a mild condition so that loss of enzyme activity due to general drastic condition during modification is avoided. In addition, each reagent should be specific for an intended amino acid residue. To minimize any side reaction, a minimal concentration of the reagent needed for the maximal inactivation has to be

established. Using the optimal concentration of each reagent should improve the specificity towards a particular amino acid residue. In this study, minimal concentration and minimal reaction time have been used for modification by EDC, DEP, NBS, and NAI. Specificity also relies significantly on pH of the modification reaction as previously mentioned. In the dialysis experiment, the determination of residual activity after chemical modification may be subjected to some errors due to incomplete removal of the excess protective substance or modifying reagent by dialysis although a large volume of buffer is used and several changes are made. So a control has to be included. During dialysis, the enzyme is exposed to the modifying reagent for a long time, hence some enzyme denaturation or side reactions may occur. In addition, the increase in the volume of enzyme solution from dialysis may also introduce errors in the results. Finally, in the estimation of the number of essential amino acid residues at the active site of the enzyme, it has to be considered that the values were determined using extinction coefficients of the group-specific modified products from the references.

The result from all steps in chemical modification experiment suggests that the active site of CGTase of *Bacillus* sp. A11 consists of carboxylic, histidine, tryptophan, and tyrosine residues. However, CGTase from this strain could be separated into four isozymes by preparative gel electrophoresis (Kaskangam, 1998) while the unfractionated enzyme was used in this study. Therefore, the four essential residues found in this study may be located either in different isozymes or in all isozymes, which needs to be further proved. For other CGTases reported, their active sites comprise of either carboxylic and tryptophan, or histidine, or tyrosine (Table 6). When analyzing our results considerately, DEP and NBS totally inhibited CGTase activities at only 1 mM concentration (Table 8) while EDC and NAI were equally effective at 5 mM (Figure 10) and 30 mM (Figure 18), respectively. This suggests that histidine and tryptophan residues may be more essential for CGTase activities than carboxylic or tyrosine residues, because they used less concentration for

inactivation. For carboxylic residue, the result was not clear-cut since up to 5 mM concentration of EDC was required for total inactivation of CGTase activity, which may be due to the fact that the modification of carboxyl residues by EDC was not performed at the most efficient pH for modification. Higher concentration of NAI may suggest that tyrosine residues might exert its catalytic effect as a supporting role, secondary to other primary catalytic group such as histidine or tryptophan via ionic interaction. However, the ability of group-specific reagents to access their specific amino acid residues should be another parameter of concern in interpretation of essential residues. When substrate protection studied were analyzed, the loss of CGTase activities were totally or nearly totally regained in the presence of protective substance tested when CGTase was modified by either EDC or NBS. However, the loss of CGTase activities was partially regained when the enzyme was modified by NAI or DEP. This suggests that essential carboxylic and tryptophan residues may be localized at the center of the active site while histidine and tyrosine residues may be located at the active site rim of CGTase.

Further studies are needed to establish more information about amino acid sequence of CGTase from *Bacillus* sp. A11 or to make use of sequence of closely related CGTases in order to be able to estimate or determine the position of essential amino acid residues in the peptide sequence. Site-directed mutagenesis is then a method of choice for the exploration of structure-function relationships of amino acid residues at the active site of the enzymes (Nakamura *et al.*, 1993, 1994; Sin *et al.* 1994; Penninga *et al.*, 1995; Mattsson *et al.*, 1995).

4.2.2 Kinetic parameters of cyclodextrin substrate

For this work, kinetic parameters were determined by two methods, coupling activity assay and cyclodextrin-degrading activity assay. Coupling is the reaction where a ring of cyclodextrin molecule is opened and combined with a linear oligosaccharide chain to produce a longer linear oligosaccharide (Nakamura *et al.*,

1993). Coupling activity were determined on the basis of the ability of β-CD to form a stable colorless inclusion complex with phenolphthalein (Vikmon, 1982). In the present work, β-CD or derivative of β-CD acts as donor and maltotriose as acceptor. The result showed that β-CD was lower in K_m for coupling activity than their derivatives tested. This suggests that β-CD was a good substrate for coupling activity. For α-, γ-CD and maltotriose, their kinetic parameters were not determined by this method because they could not form inclusion complex with phenolphthalein.

For cyclodextrin-degrading activity assay, the system employs a coupled enzyme assay. After coupling of cyclodextrin to a nonreducing end of the acceptor, a linear oligosaccharide generated act as a substrate, which is susceptible to hydrolytic cleavage by glucoamylase. The liberation of the reducing sugar measured by conventional methods gives the procedure the ease and convenience of routine sugar analysis, dinitrosalicylic acid method (Miller, 1959). The activities were calculated from the consumed amounts of cyclodextrin calibrated from the amounts of glucose in glucoamylase-treated reaction mixtures. In this reaction, cyclodextrins were used as donor and maltotriose as acceptor. The results showed that γ-CD demonstrated the lowest K_m whereas the one demonstrated the highest was maltotriose. This means that γ-CD was a good substrate but maltotriose was very poor substrate for cyclodextrin degrading activity. The similar Km values of β-CD by the two methods confirm the acceptable accuracy of both assays. Comparison of K_m values of all substrates tested suggests that β- and γ-CD had higher affinity for substrate binding site than the smaller molecules (α-CD and maltotriose). These corresponded to the result of Horikoshi and Alkiba (1982), who reported the kinetic constants for cyclodextrins in coupling reaction of *Bacillus* sp. No. 38-2. Their K_m values were 5.88, 0.39, and 0.25 mM, respectively and V_{max} values were 133.3, 23.4, and 12.3 μ moles/ min, respectively for α-, β-, and γ-CD. Kitahata and Okada (1985) reported the kinetic parameters of the CGTase for hydrolysis of maltotriose from *Bacillus coagulans*, K_m value for maltotriose was 5.3 mM. Bender (1991) reported that the

Michaelis constants for cyclisation reaction of CGTases from *Bacillus circulans* strains 8 and *Klebsiella pneumoniae* strain M5 al. using G_8 and soluble starch as substrate were 1.735 x 10^{-4} M^{-1} and 0.163 g/l and 2.5 x 10^{-4} M and 0.286 g/l, respectively. In 1994, Sin *et al.* reported the kinetic parameters of the CD-degrading activity of CGTase from *Bacillus ohbensis*, K_m values for α-, β-, and γ-CD were 2.4, 0.5, and 6.1 mM, respectively. Nakamura *et al.* (1994) determined the Michaelis constant of CGTase from *Bacillus* sp. 1011. For β-cyclodextrin-forming activity using amylose as substrate, K_m value was 57.4 μM. Coupling activity assay using γ-CD as substrate, K_m was 0.12 mM and CD-hydrolyzing activity assay using β- and γ-CD as substrate, K_m values were 0.11 and 0.57 mM, respectively. In 1995 Lee and Tao, reported the kinetic constant for the product inhibition reaction of α-, β-, and γ-CD from *Bacillus macerans*, K_m values were 0.073, 0.052, and 0.047 mg/ml, respectively and V_{max} values 0.0051, 0.0046, and 0.0045 μmoles/min/mg, respectively.

Comparison of K_m values of all substrates used in the study indicates that β-and γ-CD were more suitable for substrate binding site than the smaller molecules, α-CD or linear smaller oligosaccharide, maltotriose. The nature and environment of substrate binding site of CGTase of *Bacillus* sp. A11 makes it fit very well with 7-8 residues of cyclized glucose for efficient catalytic reaction to proceed.