CHAPTER IV

DISCUSSION

4.1 Purification of CGTase

Many microorganisms capable of using starch as carbon and energy source are found in nature. Some of these microorganisms can produce extracellular CGTase to convert starch into cyclodextrins. By producing cyclodextrins, the organisms build up an external storage form of glucose, not accessible for most other organisms because they are not able to metabolize cyclodextrins. Alternatively, cyclodextrins may protect bacterial cells against toxic compounds in the enviroment by forming inclusion complexes as in biological waste water treatment, where the addition of small amount of β-cyclodextrin in activated sludge increases the tolerance level to toxic chemicals (Allegre and Deratani, 1994). Also the availability of compounds needed for growth may improve when present in an inclusion complex with cyclodextrins (Aeckersberg *et al.*, 1991). These alternatives, however, do not explain the presence of the specific uptake and degradation routes for cyclodextrins. Cyclodextrinase and transporter proteins for CDs were reported in *K. oxytoca*, *B. circulans* and some other strains and they were proposed to co-function in CD degradation (Tonkova, 1998). CD is channeled into the cells by transporters and it is metabolized to maltose or glucose which then becomes energy source of the organism. Studies on CGTase have been focussed due to its cyclization activity leading to CD formation since CD is known to be beneficial for various industrial users. CGTase from different bacterial strains differ especially in terms of optimum working conditions for maximum activity and the ratio of CD products formed (Matzusawa *et al.*, 1975; Depinto and Campbell, 1986; Tonkova, 1998). The search for CGTase is still attractive because it may possess an interesting or a better catalytic property. Our research group has been working on thermotolerant bacteria, which has an advantage on growth at higher temperature than

the group of mesophilic bacteria. A thermotolerant bacterium producing CGTase, *Paenibacillus* strain RB01, was screened from hot spring area in Ratchaburi province, Thailand. Optimization and partial purification were performed by Tesana (2001). The present work aims at purification and characterization of this enzyme.

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in biosciene and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contamination, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intended application (Queiroz *et al.*, 2001). The principle properties of enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites (Queiroz *et al.*, 2001 and Amersham Pharmacia Biotechnol, 1999). Most purification protocols require more than one step to achieve the desired level of product purity. Hence, the key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Amersham Pharmacia Biotechnol, 1999).

Consequently, to purify CGTase, the extracellular enzyme, the different separation techniques were evaluated. One common step in the purification processes was the use of starch adsorption (Kato and Horikoshi, 1985, modified by Kuttiarcheewa, 1994). Considering that the interaction between starch and the CGTase involves not only adsorption but also substrate-enzyme affinity type binding. In this research for the first step, CGTase was adsorbed by corn starch added to the crude enzyme and the adsorbed enzyme was eluted with the buffer containing maltose. To use the basic principle of substrate-enzyme specificity, maltose, the water-soluble substrate with lower affinity than starch competes for binding to CGTase thus the enzyme-substrate can be easily separated and present in soluble fraction. After this

step, the enzyme is purified to homogeneity with 29 purification fold and 72% yield. DEAE-cellulose, an anion exchanger, is the next step which separates proteins with differences in charge to give a very high resolution with high sample loading capacity. It popularity stems from the possibility of high resolution power, versatility, reproducibility and ease of performance (Eisenthal and Dason, 1992). Upon this step the CGTase was separated from other bulk proteins which constituted about 50% of total protein (Figure 9 and Table 7). The enzyme after concentration was subsequently transferred to the last column, which was a gel filtration column. The Bio-Gel P-100 was selected to be a supporting gel, which is made from acrylamide. Because of this enzyme using starch and oligosaccharide as a substrate, which can bind to the other kind of supporting gel, so that to use the acrylamide gel type is one way to overcome the drawback the retarding enzyme in the column. The chromatogram (Figure 9) shows that a peak of unwanted proteins (no activity) was clearly separated from the CGTase peak since it was eluted at around the void volume of the colume when compared to elution of standard proteins, CGTase was eluted close to ovalbumin and was calculated to have a molecular weight of 45 kDa.

Native and SDS-PAGE confirmed the purity of the enzyme. On native gel electrophoresis the enzyme showed 3 bands with amylolytic activity while on SDS-gel electrophoresis the enzyme showed only 1 band which suggested that this enzyme had multiple forms. The existence of isoform was previously reported in other strains (Nakamura and Horikoshi *et al.*, 1976; Mattson, *et al.*, 1990; Bovetto *et al.*, 1992; Abelyan *et al.*, 1994; Kaskangam, 1998). Whether the cause was by post-translational modification of a single protein or the existence of multiple genes for CGTase had to be found out (Prasong, 2002).

Most of the bacterial strains are known to produce other amylolytic enzymes besides CGTase (Volkova *et al.*, 2000). To ensure the existence of CGTase, the measurement of CD-TCE was performed in parallel to dextrinizing activity during purification process. We found that these two activities were well correlated at every

step of purification (Table 7). For the last step of Bio-Gel P-100, even if the fold of purification was increased but almost half of the recovery yields was reduced. However, the purification procedure of *Paenibacillus* RB01 CGTase in this study was considered appropriate with the acceptable yield and purification fold.

4.2 Characterization of purified CGTase

4.2.1 Molecular weight determination

The molecular weights of this enzyme was performed with two methods; native and denature condition by Bio-Gel P-100 column chromatography and SDS-PAGE, respectively. For Bio-Gel P-100 chromatography column; separation technique is based on exclusion effects. Particles are separated according to differences in size (shape) as they pass through a sieving medium (gel). The sieving is via, pores in the gel matrix which are comparable in size to the particles one wishes to separate. Bio-Gel P-100 is based on highly cross-linked porous acrylamide bead and free of charge (Bio-Rad Laboratory, 2000). While SDS-PAGE was technique of molecular sieving through polyacrylamide network and this technique was used to determine the subunit content of enzymes. The CGTase from RB01 was estimated to be 45 kDa by gel chromatography on a Bio-Gel column. While molecular weight determined by SDS-PAGE was 65 kDa . This result suggested that this CGTase is a monomeric enzyme. Elution volume of native form from Bio-Gel column was bigger than the expected volume might be due to protein interaction with gel matrix or extended shape of the protein. From the result of PAS staining, the CGTase was a kind of glycoprotein. In addition, the Sephacryl S-200 column showed the retardation of this enzyme which was then eluted at 43 kDa (data not shown). Previous reports on *Bacillus* sp. No 38-2, *Bacillus circulans* E 192 and *Bacillus* sp. A11 (Kaneko *et al.*, 1988; Villetto *et al.*, 1991; Techaiyakul, 1991) also showed similar results. Most of the reported CGTase are monomeric; those from *Bacillus* sp. ranging in molecular weight between 33 and

103 kDa while from *Micrococcus* sp. between 85-120 kDa. *B. agaradhaerens* LS-3C CGTase with the molecular weight of 110 kDa was the largest protein among the reported *Bacillus* CGTase (Martin and Kaul, 2002). However, there were reports that CGTase form *Bacillus macerans* IAM 1243 and *Bacillus megaterium* No.5 were dimeric proteins of identical subunit size of 66 and 145 kDa, respectively (Kitahata and Okada, 1974; Kobayashi *et al.*, 1978). The CGTase from *B. circulans* E 192 showed 2 isoenzymes with different subunits of 33.5 and 48.5 kDs, respectively (Bovetto *et al.*, 1992).

4.2.2 Carbohydrate determination

For qualitative analysis of glycoprotein, carbohydrate-specific periodic acid Schiff (PAS) staining method on polyacrylamide gel offers a good choice. The closed aldehyde groups in the conjugated polysaccharide were oxidized by periodic acid, permitting the fuchsin sulfite to stain the polysaccharide to the violet color. The result in this study showed that this each isoform of CGTase was a glycoprotein (Figure 15). Isoforms of CGTase from *Bacillus* sp. A11 were also reported to be a glycoprotein, each isoform has different carbohydrate content, determined by PAS staining and also phenol-sulfuric acid method (Kaskangam, 1998).

4.2.3 pI

The isoelectric points of these CGTase were determined by isoelectrofocusing gel in the ampholine pH range 3-10, comparing with standard pI markers (pI 3.5-9.3). The result showed that this enzyme had 2 major bands of 5.2 and 5.3 and 1 minor band of 5.1. This corresponded with many previous reports, such as *B. megaterium* which gave two fractions which were similar in enzymic properties but different isoelectric points (Kitahata *et al.*, 1974). The thermophilic anaerobic bacteria, *Thermoanaerobacterium thermosulfurigenes* EM1 revealed one major band on

Thermoanaerobacterium thermosulfurigenes EM1 revealed one major band on isoelectric fucusing with an isolectric point of 5.0 and three minor bands with isoelectric points of 4.3, 4.4 and 4.6 (Wind *et al.*, 1995). Kaskangam (1998) reported isoelectric points of isozyme from *Bacillus* sp. A11 were 4.73, 4.49, 4.40 and 4.31. Charge differences were detected by IEF may be arise from the carbohydrate content of glycoproteins or differences in a few amino acids in the polypeptide chain.

4.2.4 Effect of pH and temperature on enzyme activity and stability

Each enzyme has an optimum pH at which the rate of the reaction that it catalyzes is at its maximum. Slight shifts in the pH from the optimum value lead to a decrease in the reaction rate, maybe due to changes in the ionization of the of charged amino acid residues that function in the active site of the enzyme. Large shifts in pH may lead to denaturation of the enzyme because of interference with many weak noncovalent bonds maintaining its three-dimensional structure (Hames *et al.*, 1998 and Segal, 1976).

CGTase cyclizing activity from RB01 was most stable over pH range of 6.0-10.0 upon incubation at 55 °C, 70% activity was retained (Figure 20). According to the result, it suggests that this CGTase may be an alkalophilic enzyme. This conclusion was also related with the culturing pH of Horikoshi medium which was about 10.1-10.2. For temperature stability of RB01 CGTase, when the enzyme was incubated at temperature range of 45-65 °C for 1 hour, over 70% of activity was retained. Thermostability of an enzyme also depends on the presence of substrate. In the absence of substrate, the enzyme rapidly lost its activity at above 70 °C. On the other hand, the addition of substrate to the reaction resulted in a better stability at higher temperature as shown in Figure 21. The result was corresponded with those of Abelian *et al.* (1995) and Gawande *et al.* (1999) who reported that starch (substrate) enhanced the thermostability of CGTase from *Thermoanaerobic thermosulfurigenes* EM1 at 80-85 °C and *Bacillus firmus* at higher than 30 °C. Furthermore, other reports showed that

the stability of CGTase from *Bacillus* no A-40-2 at high temperature was increased when calcium ion was present (Horikoshi, 1971).

Although CGTase was capable to catalyze hydrolyzing and cyclizing reactions, the two activities occurred in different proportion. It depends on pH and temperature. For RB01, the optimum pH for dextrinizing, cyclizing and CD-TCE activity were 5.0, 7.0 and 7.0-9.0 and the temperature were 65 °C, 70 °C and 55 °C, respectively. Larsen *et al.* (1998) reported that the optimum pH and temperature of *Paenibacillus* sp. F8 on dextrinizing activity were 8.0 and 60 °C and cyclizing activity were 7.5 and 50 °C. CGTase from extremely thermophilic anaerobic archaeon strain B1001 had 110 °C and pH 5.0-5.5 for optimum starch degrading while for cyclodextrin synthesis the optimum were 90-100 °C and pH 5.0 (Tachibana, 1999).

4.2.5 Substrate specificity

In general, a substrate-binding site consists of an indentation or cleft on the surface of an enzyme molecule that is complementary in shape to the substrate (geometrical complementary). Moreover the amino acid residues that form the binding site are arranged to interact specifically with the substrate in an attractive manner (electronic complementary). Molecules that differ in shape or functional group distribution from the substrate cannot productively bind to the enzyme, that means they can not form enzyme-substrate complexes that lead to the formation of products (Voet and Voet, 1995). For CGTase, the conversion of substrate to CD was also affected by degree of starch hydrolysis; oligosaccharides with dextrin equivalent (DE) value in the range 1-22 were good for cyclizing reaction while most effective at DE value about 10. The conversion rate dropped sharply when DE of oligosaccharides was controlled to 13 and continued to decrease as DE increases over 20. The conversion was faster when there were more reducing end per mole of CGTase as starch was liquefied to shorter length. However, when liquefaction was allowed to proceed further

to produce oligosaccharides with <6 glucose units, resulted in poor conversion to CDs (Chung *et al.*, 1998).

The CGTase from thermotolerant *Paenibacillus* sp. RB01 showed conversion of amylose > starch > oligosaccharides. Longer chain oligosaccharides produced CDs more than short chain. The enzyme had specificity for substrates with α-1,4 glycosidic bond with minimum 3 glucose units, so glucose and maltose could not act as substrate. In addition, this CGTase could not use pullulans as substrate because its structure is linked by β-1,6 glycosidic bond. This finding agreed with CGTase mechanisms proposed in Table 5 (Szejtli, 1988) and corresponded with previous studies by Abelyan *et al.* (1994, 1995) on different bacterial CGTases. Those results showed that the maltooligosaccharides (MOS) with a degree of polymerization higher than seven glucose units acted as direct substrates in the cyclizing reaction. When the reaction mixture contained MOSs with a lower degree of polymerization than eight glucose residues as a substrate, in the initial reaction stage various linear MOSs were formed by the hydrolytic and intermolecular transglycosylation reaction. While low molecular MOSs were not direct substrates of CD formation. When their concentration in the reaction mixture was sufficient after the intermolecular transglycosylation, these low molecular MOSs were appropriate acceptors for the CGTase and a disproportionation reaction began. For other CGTase, the enzyme from *Bacillus* sp. ATCC21783 converted starch > amylose > glycogen > amylopectin β-limited dextrin to CDs (Nakamura and Horikoshi, 1976).

4.2.6 CD production

Partially purified enzyme from starch adsorption step produced a ratio of α- : β- : γ-CD as 1.0 : 5.4 : 1.2 (Tesana, 2001) while crude and purified CGTase in this study showed the average ratio of 1.0 : 1.8 : 0.4. The difference may arise from different buffer used in enzyme preparation. There was previous report by Volkova *et al.* (2000), that crude CGTase from *Bacillus* sp. 1070 produced 0.6 : 5.7 : 0.6 while

purified enzyme gave 0.6 : 9.0 : 0.0 of α- : β- : γ-CD. It was known that different ratio of CDs production may be performed by varying incubation time. Terada *et al.* (2001) once compared the cyclization reaction of three bacterial cyclomaltodextrin glucanotransferases. The results suggested that the larger cycloamyloses initially produced were converted into smaller cycloamyloses and finally into mainly α-, β- and γ-CD. These three enzymes also differed in their hydrolytic activities, which seemed to accelerate the conversion of larger cycloamyloses into smaller cycloamyloses. Furthermore, Bovetto *et al.* (1992) reported the production ratio of α- : β- : γ-CD by CGTase from *Bacillus macerans* E192.at initial was 1 : 7 : 2 while at the equilibrium condition the ratio was 3 : 3 : 1. Another report showed that the ratio of CDs produced by CGTase from *B. macerans* IFO 3490 was almost constant regardless of the pH range (4.0-8.5) of the reaction system (Kitahata and Okada, 1974).

4.3 Kinetics study

To determine the kinetic parameters by coupling reaction, a ring of CD molecule is opened and combined with a linear oligosaccharide chain to produce a longer linear oligosaccharide (Nakamura *et al.*, 1993). β-CD or its derivatives acts as donor and cellobiose as acceptor, a linear oligosaccharide generated then acts as a substrate, which is susceptible to hydrolytic cleavage by glucoamylase. The liberation of the reducing sugar measured by conventional method 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 amount of glucose in glucoamylase-treated reaction mixture. When kinetic parameters of purified CGTase from RB01 obtained in this study were compared with those of isoform I, the major isoform of CGTase of *Paenibacillus* sp. A11 (Prasong, 2002), both CGTase showed some similarity in which they used natural CD better than modified ones though the values were somewhat different A11.

4.4 Chemical modification of CGTase

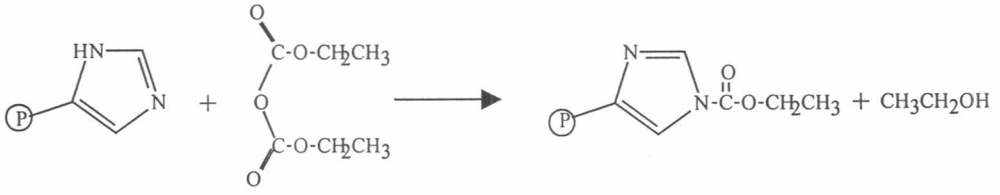
Several methods have been used to explore the molecular structure and the active site of enzymes. Among those general techniques, affinity labeling using substrate or substrate analogs, site-directed mutagenesis, X-ray crystallographic and diffraction studies or chemical modification have been reported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

Chemical modifications of reactive groups of enzyme have been used to identify the amino acid residues that are responsible for enzyme action (Mean and Feeney, 1971). With some modifying reagents, several different groups may be modified (Appendix 5). 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 (Mean 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 (Ohnishi *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. John *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 identification of essential amino acid residues at the active site of purified CGTase from thermotolerant *Paenibacillus* sp. RB01 was performed

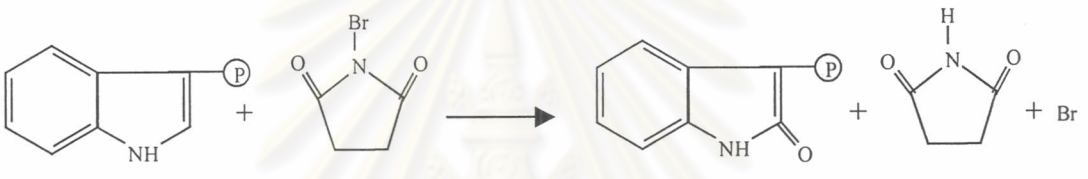
using chemical modification with group-specific reagents and substrate protection technique. Chemical modifications of seven different amino acid residues (carboxylic, histidine, tryptophan, tyrosine, cysteine, lysine and serine) of purified CGTase were determined under mild conditions. These amino acid residues have been selected because they are widely known as residues involved in enzyme catalysis, while some have been reported to be essential for CGTase of other sources (Mean and Feeney, 1971; Lundblad, 1991; Mattsson, 1992; Bender, 1991; Villette, 1993; Ohnishi, 1994).

The first step was to screen for essential amino acid residues that upon modification, the loss of purified CGTase activity was observed. Incubation of purified CGTase with series of covalent modifiers of amino acid residues at 1 mM concentration resulted in variable change in the catalytic ability of this enzyme. No inhibition of CGTase activities were observed in the modification of cysteines by *N*-ethylmaleimide (NEM), iodoacetamide (IAM), or dithiothretol (DTT), lysine residues by 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), serine residues by phenylmethylsulfonyl fluoride (PMSF) and tyrosine residues by *N*-acetylimidazole (NAI). Reaction under mild conditions with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), diethylpyrocarbonate (DEP), and *N*-bromosuccinimide (NBS), which were known to react specifically with carboxyl, histidine, and tryptophan residues, respectively (Reaction1, 2, and 3, respectively), resulted in extensive inhibition of purified CGTase. When analyzing this result considerately, DEP and NBS inhibited almost total activity of purified CGTase at only 1 mM (Figure 27) while EDC demonstrated partial inhibition. This suggests that histidine and tryptophan residues may be more essential for CGTase activities than carboxylic residues, because less concentration was used for inactivation. Since the experiment was followed by dextrinizing activity, thus these residues might be more important at the hydrolytic site. Whether or not they were important at the cyclization site remained to be further proved.



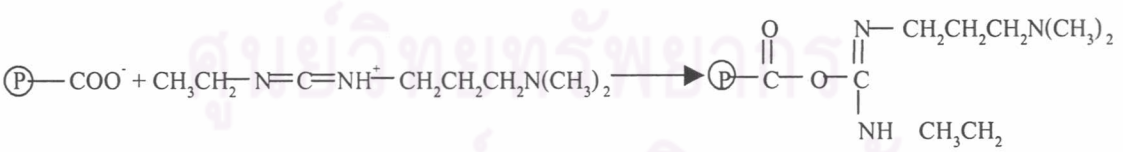
Reaction 1 Modification reaction of histidine residues in protein (P) with DEP

(Means and Feeney, 1971; Lundblad, 1991)



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

(Means and Feeney, 1971; Lundblad, 1991)



Reaction 3 Modification reaction of carboxyl residue in protein (P) with EDC

(Means and Feeney, 1971; Lundblad, 1991)

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 a1 were modified by DEP and proposed to be involved in the cyclization 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 were 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* was reported to be modified at tryptophan residues by NBS (Ohnishi *et al.*, 1992). The modification corresponded with inactivation of the CGTase catalysed hydrolysis of cyclomaltohexaose (cG_6). Researchers in our group reported that CGTase and its isoform I from *Bacillus circulans* A11 were inactivated by DEP, NBS, EDC, and NAI at 1 mM (Tongsima, 1998 and Kaulpiboon, 2000). CGTase of *Paenibacillus* sp. RB01 in this study was thus different from that of *Bacillus circulans* A11 since it was not inactivated by NAI.

The second step was to prove that the amino acid residues involved in purified CGTase activities were or were not at the active site. The inactivation of enzyme by chemical modification does not directly indicate that a specific residue is present at the active site. Protection of the enzyme activity by substrate, competitive inhibitor, end product, or related compounds has been used to confirm the presence of specific residues at the active site. If activity is retained following modification in the presence of substrate (or other protective substrates) 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-, and γ-CD were used as protective substances. And the experiment was performed by measuring purified CGTase inactivation by group-

specific reagents in the presence or the absence of protective substances. The result in Figures 33, 36 and 39 showed that the loss of purified CGTase activities were partially or totally reduced in the presence of protective substance. For histidine modification, it was interested because there are several reports that histidine was at the active site of CGTase from *Bacillus* sp. In this experiment the result when added α-, β- or γ-CD, significant level of CGTase activity was retained which indicated the presence of histidine at the active site of RB01 CGTase. Carboxyl residues were also involved at the active site since they were partially protected by CDs. Trytophan modification was especially interested because least concentration of modifier (0.005 mM NBS) was used for total inhibition of enzyme activity. And protection by β-CD was much better than other CDs. This suggest that tryptophan should be more important at the active site of this CGTase which produced more β-CD than other CDs. Moreover, we confirm that tryptophan was present at the active site by following fluorescence emission of CGTase when modified with NBS in the presence and absence of β-CD. For control (NBS only), there was no fluorescence emission observed at 310-360 nm (data not shown). The result shows that tryptophan was protected by β-CD, since the fluorescence emission spectrum returned to resemble the control pattern both for the maximum emission wavelength and fluorescence intensity. The wavelength of modified enzyme was shifted to shorter wavelength suggested that CGTase had conformational change and tryptophan was surrounded in a more hydrophobic environment than unmodified enzyme (Ruan, 1999). Hence, these experiments could lead to the interpretation that histidine, tryptophan and carboxyl residues were involved at the active site of purified CGTase but different in degree of the importance to enzyme catalysis. For other CGTase, Mattsson *et al.*(1992) reported that CGTase from *Bacillus circulans* var. alkalophilus (ATCC 21783) was protected against DEP-inactivation by α-, and β-CD suggesting that the modified histidine residues were 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. Trytophan was protected against NBS by glucose and the maltosaccharides G2-G4, which indicated tryptophan to be located at the substrate binding site of CGTase from *Bacillus strearothermophilus* (Ohnishi *et al.*, 1992). Tongsima, (1998) and Kaulpiboon, (2000) reported the loss of CGTase activities after the modifications of histidine, tryptophan, tyrosine, and carboxylic amino acids were reduced in the presence of protective substances suggesting the location at or near the active site of these residues.

4.5 Urea-induced denaturation of CGTase

The chaotropic agents such as 6 M guanidinium chloride (GdmCl) or 8 M urea, was used to denature proteins by disrupting the water structure around the protein. Usually the protein becomes unfolded in the presence of these reagents. In this experiment the enzyme was incubated with 0-10 M urea and following the fluorescence emission spectrum of tryptophan was followed. Exitation was at 280 nm while emission was from 320-380 nm. For control (urea only), there was no fluorescence emission observed at around 320-380 nm. The spectrum of purified CGTase was shifted to longer wavelength when urea was added. This suggests the tryptophan residue was in a less hydrophobic environment, which was usually at the surface of a protein (Ruan, 2002). Concommitanlty with the shift in fluorescence spectrum, the enzyme lost its dextrinizing activity. However, when native PAGE was used to analyze the isoform pattern using activity staining, we found no different in the pattern between control and urea-treated CGTase. This result was different from pullulanase study (Renz et al., 1998) which showed that multiple form of pullulanase was arise from different teriary structure of protein, as proved by urea-induced denaturation experiment.