CHAPTER III

RESULTS

3.1 Purification of CGTase

RB01 was cultivated in Horikoshi medium containing 1.0% soluble starch, at pH 10.0, 40 °C, 250 rpm for 60 hours which was optimum condition determined by Tesana (2001). Cells were removed from the culture by centrifugation (3000g) at 4 °C for 30 min, and the crude enzyme in the supernatant fraction was collected. Through the step of corn starch adsorption as described in section 2.6.1, the purification of CGTase was increased by 29 fold and 72% yield was obtained. The enzyme was then concentrated by ultrafiltration and dialyzed against 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5 (TB1). The enzyme had negative charge in this condition. Then concentrated CGTase solution was applied onto DEAE-cellulose chromatography. Figure 9 shows the elution profile from DEAE-cellulose column chromatography. Two overlapped protein peaks were eluted by NaCl gradient in the range of 0.05-0.25 M. The second peak which was eluted by 0.17 M NaCl contained dextrinizing activity. Fractions from number 130 to 150 were then pooled. For this step, the enzyme with 36.2 purification fold and 52% yield was obtained. Before the next Bio-Gel P-100 chromatography, the CGTase was concentrated by lyophilization to dryness. Figure 10 shows Bio-Gel P-100 chromatography column profile of CGTase which was well separated from other contaminated protein of larger size. And CGTase was the major protein eluted from this column. The overall purification steps of CGTase from *Paenibacillus* sp. strain RB01 are summarized in Table 7. The CGTase was purified to 47.5-fold with the yield of 35%. Specific dextrinizing activity was increased from 153 of the crude enzyme to 7,268 U/mg protein of the purified enzyme. This correlated to CD-TCE activity which was increased from $1:2^8$ to $1:2^{13}$.

Table 7 Purification of CGTase from *Paenibacillus* sp. strain RB01

Step	Volume (ml)	Total activity* ($Ux10^3$)	Total protein (mg)	Specific activity* (U/mg)	Purification Fold	Yield (%)	CD-TCE activity ($1:2^n$)
Crude	1,000	59.5	388.9	153	1	100	2^8
Starch adsorption	85	42.8	9.66	4,437	29	72	2^{12}
DEAE-cellulose	362	30.9	5.59	5,539	36.2	52	2^{13}
Bio-Gel P-100	34	20.8	2.86	7,268	47.5	35	2^{13}

* Dextrinizing activity

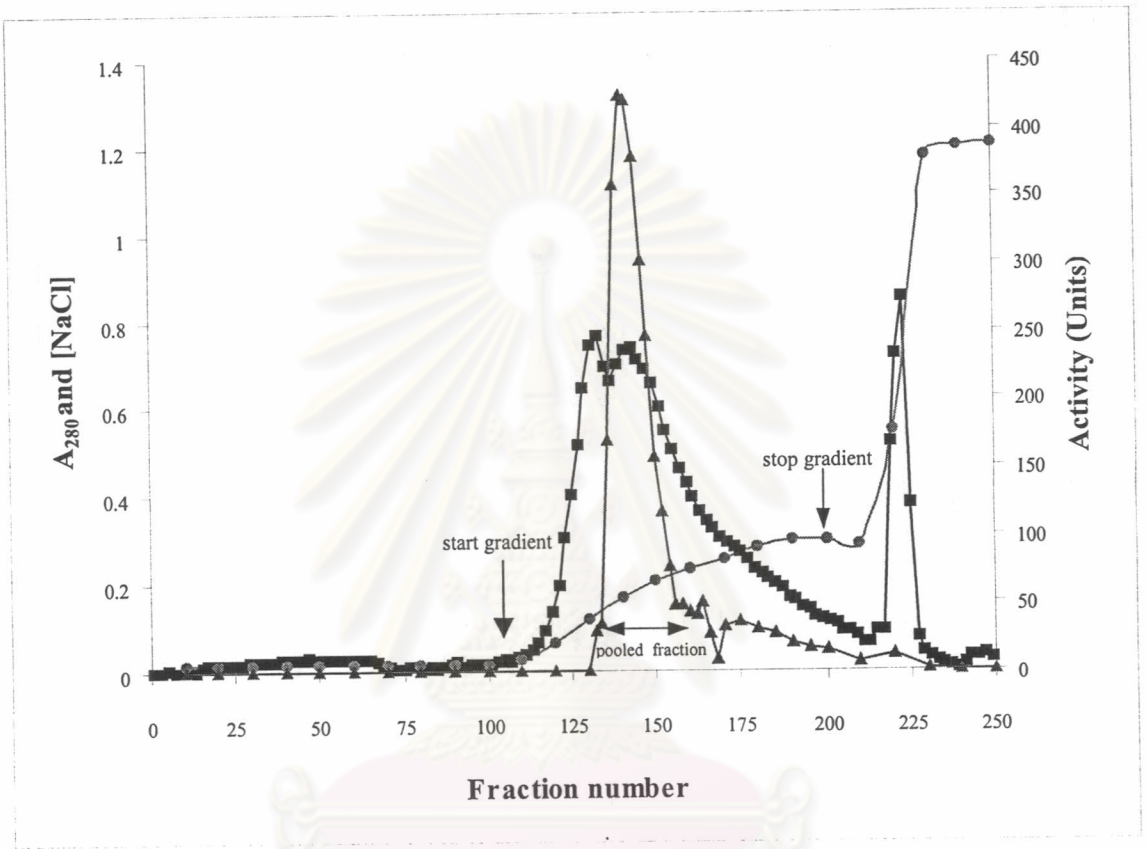


Figure 9 DEAE-cellulose column profile of CGTase separation at pH 8.5. Column size was 1.5 x 28 cm, elution was by 0 - 0.3 M NaCl in TB1 buffer. Fractions of 3 ml were collected. —■— A_{280}, —▲— Dextrinizing activity, —●— Conductivity

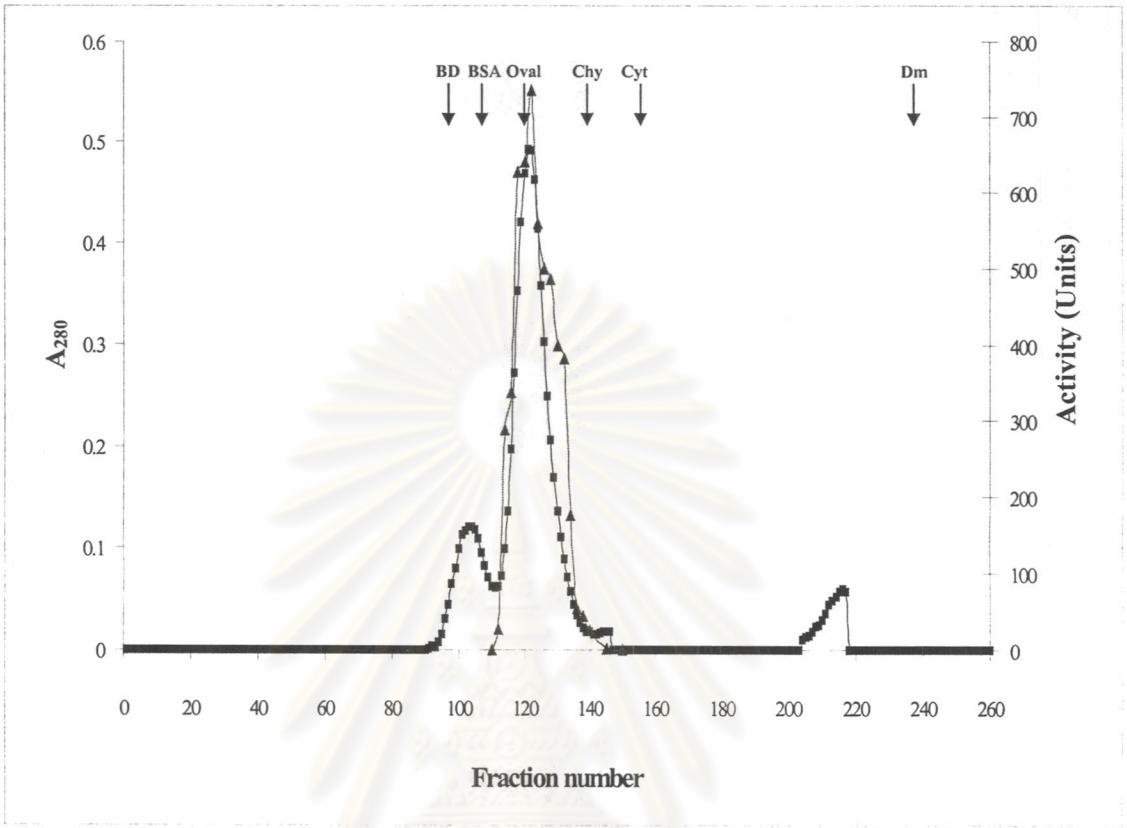


Figure 10 Bio-Gel P100 column profile of CGTase purification. Column size was 1.7 x 80 cm and TB1 buffer was used. Fractions of 2 ml were collected. —■— **A_{280},** —▲— **Dextrinizing activity**

BD = Blue dextran

BSA = Bovine serum albumin

Oval = Ovalbumin

Chy = Chymotrypsinogen A

Cyt = Cytochrome C

Dm = Dichromate

The purity of CGTase enzyme was determined by native polyacrylamide gel electrophoresis (Native-PAGE). Protein staining revealed that the enzyme was successfully purified since less protein bands were observed through purification step. In the purified preparation, the mobilities of the active bands as determined by dextrinizing activity staining (Figure 11b) coincided with those of 3 bands stained with Coomassie brilliant blue (Figure 11a). This suggests that the purified enzyme had 3 isoforms with different net charge and all forms showed dextrinizing activity. When sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme was performed, only one main band was observed from Coomassie brilliant blue staining of the gel (Figure 12, lane 5 and 6).

3.2 Characterization of purified CGTase

3.2.1 Molecular weight determination

By Bio-Gel P-100 column chromatography, the enzyme under native conditions gave a molecular weight of about 43.3 kDa (Figure 13). While the molecular weight of the denatured CGTase was estimated to be 65 kDa by SDS-PAGE. (Figure 14) The result suggests the protein to be a monomer.

3.2.2 Carbohydrate determination

Bands on SDS-PAGE and native PAGE were stained for glycoprotein by PAS method (Section 2.11.1). BSA was used as positive control, while haemoglobin was used as negative control. The CGTase bands, similar to BSA, gave a deep magenta color upon PAS staining (Figure 15). This result suggests that CGTase is a glycoprotein.

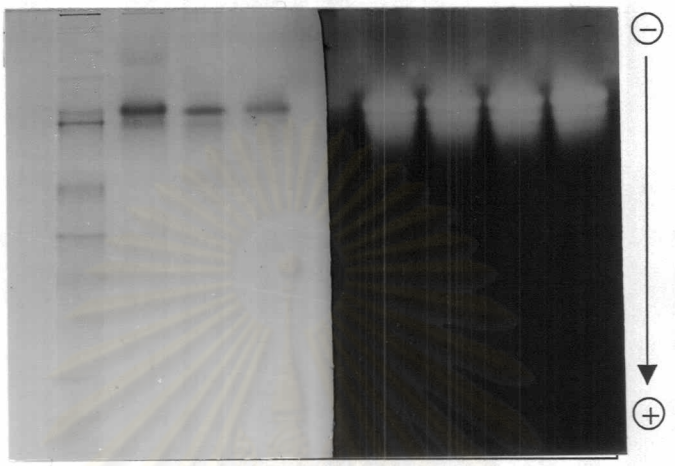


Figure 11 Non-denaturing PAGE of CGTase from different purification steps

a : Coomassie blue staining

Lane 1 : Crude enzyme (55 μg) Lane 3 : DEAE cellulose column (20 μg)

Lane 2 : Starch adsorbed enzyme (20 μg) Lane 4 : Bio-Gel P-100 (20 μg)

b : Dextrinizing acitivity staining, 0.2 units per each well

Lane 1 : Crude enzyme Lane 3 : DEAE cellulose column

Lane 2 : Starch adsorbed enzyme Lane 4 : Bio-Gel P-100

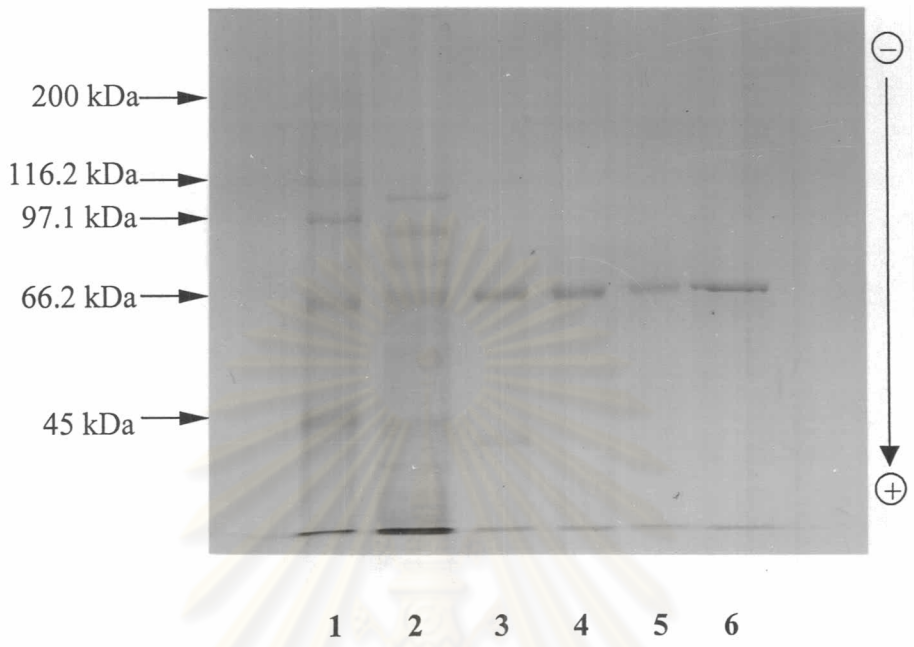


Figure 12 SDS-PAGE of CGTase from different purification steps

Lane 1 : Protein molecular weight markers [Myosin (200 kDa), β-galactosidase (116.2 kDa), Phosphorylase b (97.1 kDa), BSA (66.2 kDa) and Ovalbumin (45 kDa)]

Lane 2 : Crude enzyme (70 μg)

Lane 3 : Starch adsorbed enzyme (20 μg)

Lane 4 : DEAE-cellulose (20 μg)

Lane 5 : Bio-Gel P-100 (20 μg)

Lane 6 : Bio-Gel P-100 (20 μg)

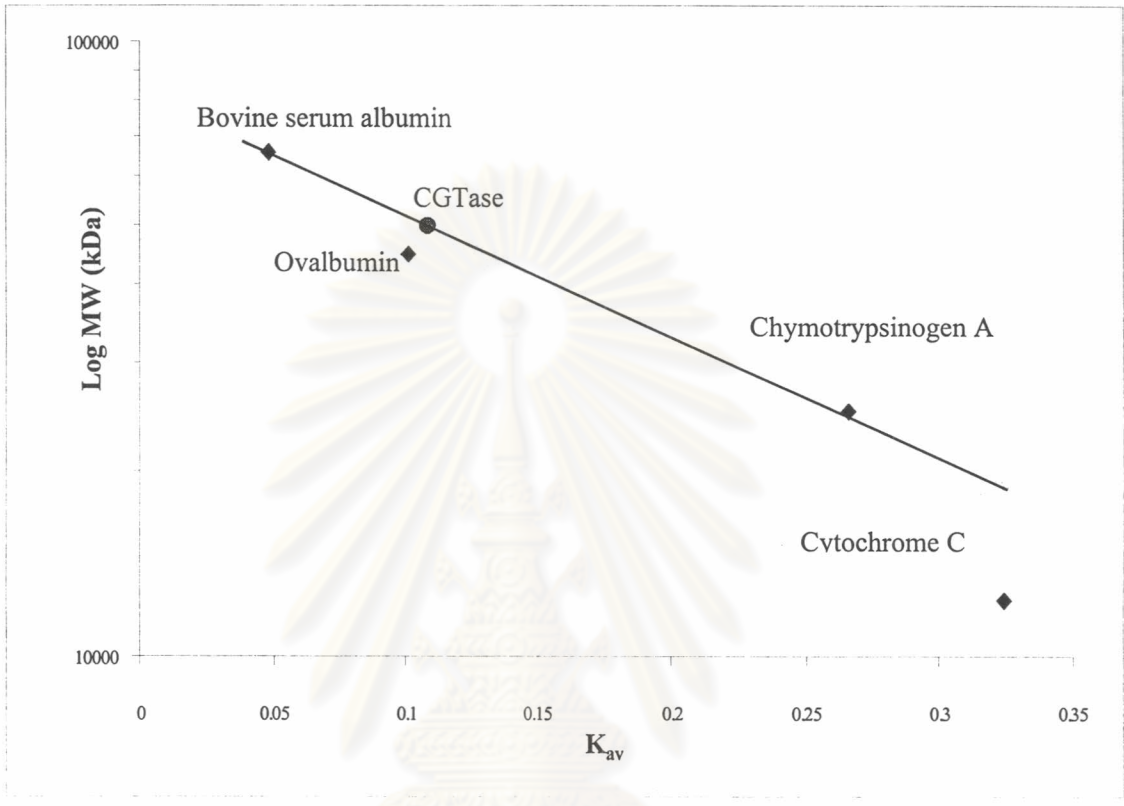


Figure 13 Standard curve of molecular weight and retention coefficient from Bio-Gel P-100

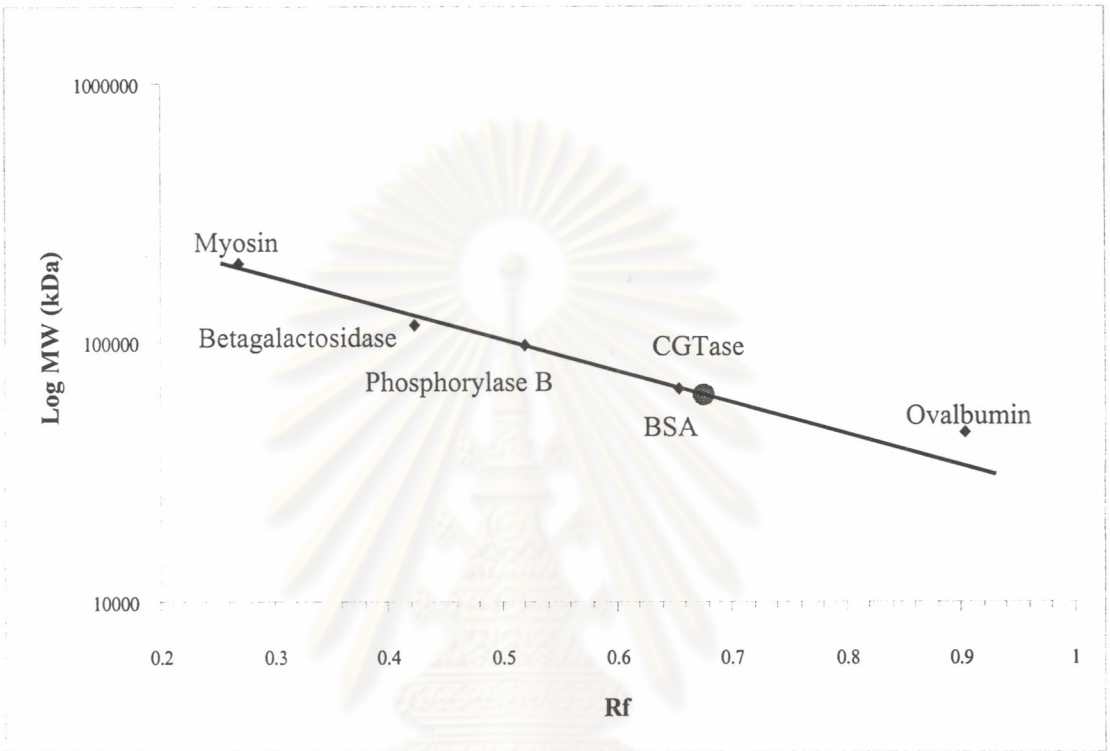


Figure 14 Standard curve of molecular weight and relative mobility from SDS-PAGE

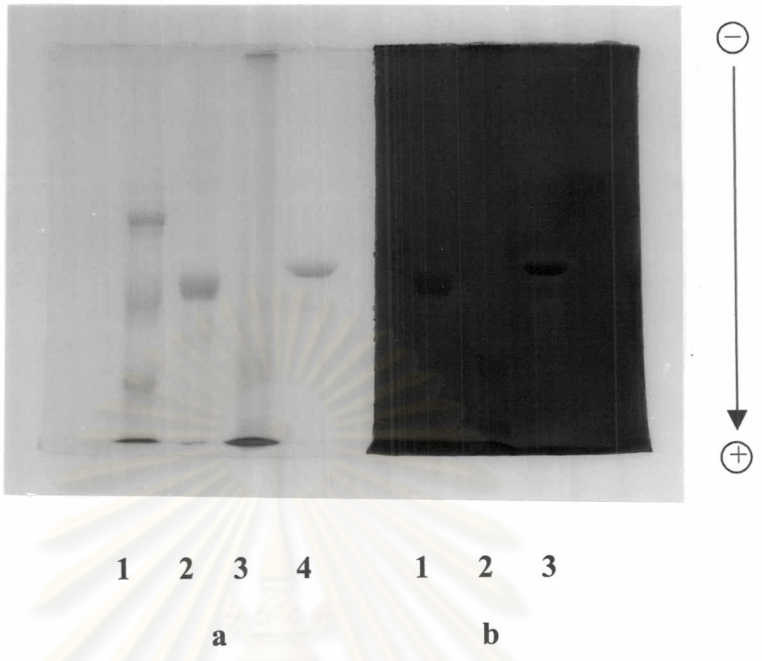


Figure 15 PAS staining of purified CGTase from RB01 on SDS-PAGE

The enzyme preparation was run on SDS-PAGE with protein markers [Phosphorylase B MW = 94 kDa, Bovine serum albumin MW = 67 kDa, Ovalbumin MW = 43 kDa, Carbonic anhydrase MW = 30 kDa, Soybean trypsin inhibitor MW = 20.1 kDa, and Lactabumin MW = 14.4 kDa]

a) Coomassie blue staining

Lane 1 = Protein marker

Lane 2 = BSA (10 μg)

Lane 3 = Hemoglobin (10 μg)

Lane 4 = Purified CGTase (5 μg)

b) PAS staining

Lane 1 = BSA (positive control) (20 μg)

Lane 2 = Hemoglobin (negative control) (20 μg)

Lane 3 = Purified CGTase (5 μg)

3.2.3 pI

Purified CGTase was analyzed for their isoelectric points by separation on IEF gel electrophoresis, comparing to standard pI markers. Ampholine pH range 3-10 was used and relative mobility against pI was plotted. Figure 16 showed 3 bands of CGTase on ampholine gel. Two major bands were found at pI 5.2 and 5.3 with one minor band at 5.1 (Figure 16 and 17).

3.2.4 Effect of pH on the enzyme activity

The effect of pH on the dextrinizing, cyclization and CD-TCE activity of the purified enzyme was performed as described in section 2.12.1. pH was varied from 3-11 at 40 °C. The optimum pH of dextrinizing activity was 5.0 in acetate buffer and cyclization activity was at 7.0 in Tris-HCl buffer while CD-TCE activity shows broad optimum pH (7.0 to 9.0) (Figure 18). For cyclization activity, the type of buffer was an important factor for the enzyme activity, since only about 75% of the optimal activity was observed in phosphate buffer pH 7.0 when compared to that in Tris-HCl at the same pH (Figure18).

3.2.5 Effect of temperature on the enzyme activity

The effect of temperature on the dextrinizing, cyclization and CD-TCE activity of the purified enzyme was performed in Tris-HCl at pH 7.0. Figure 19 shows the enzyme to be optimally active at 55 °C, 65 °C and 70 °C of CD-TCE, dextrinizing and cyclization activity, respectively. The enzyme activity was lost significantly when temperature was higher than 75 °C. At 80 °C the dextrinizing, cyclization and CD-TCE activity had only 6.0%, 15% and 45% relative activity compared with maximum activity. The enzyme was completely inactive at 100 °C.

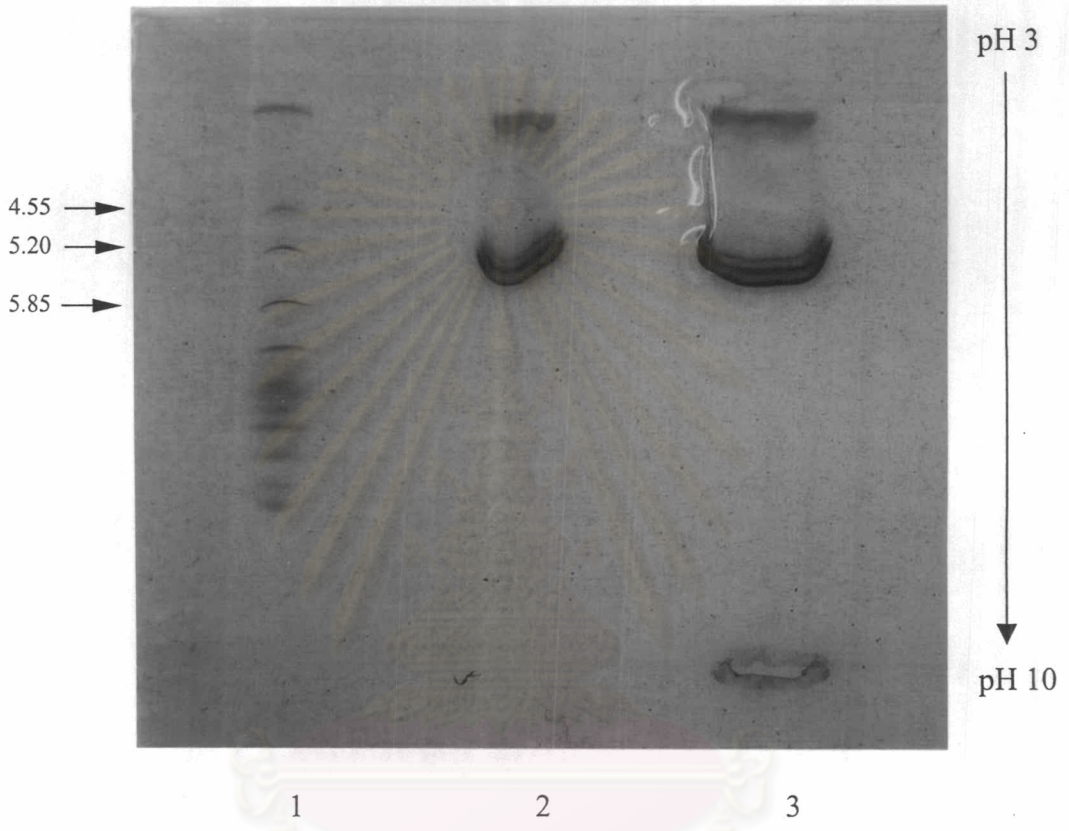


Figure 16 Isoelectrofocusing Gel with ampholine (pH 3-10) of purified CGTase

Lane 1 = Standard pI marker proteins

Lane 2 = Purified CGTase (5 μg)

Lane 3 = Purified CGTase (10 μg)

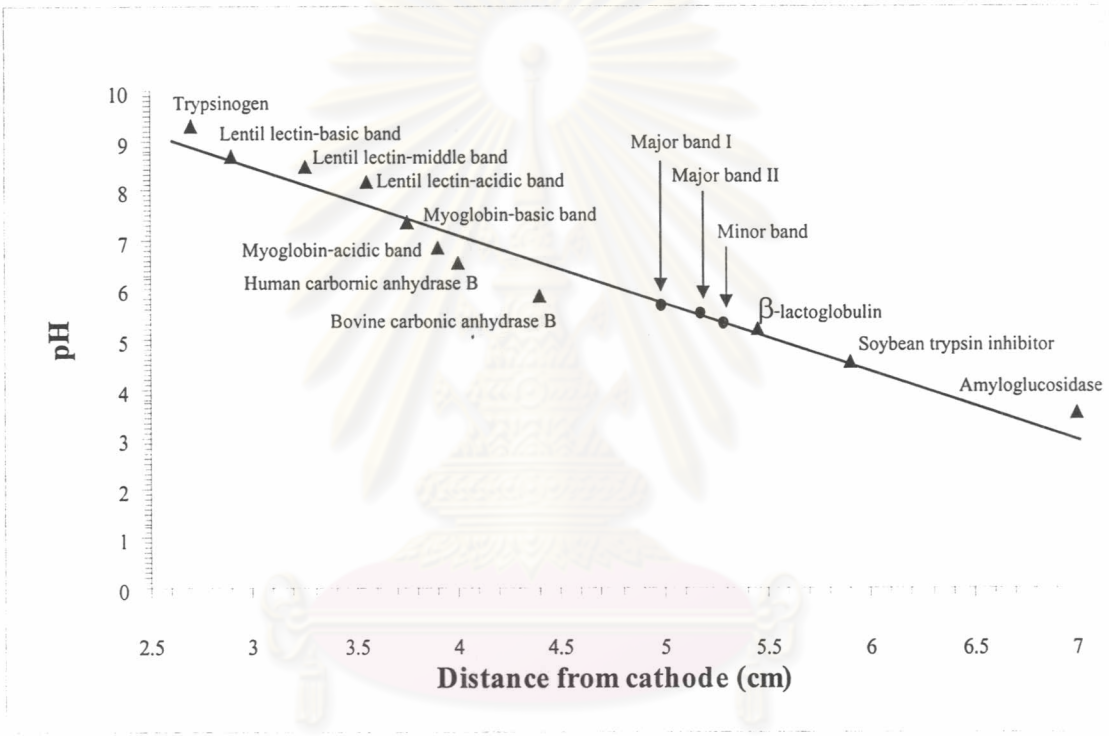


Figure 17 Standard curve of pI determination

▲ = Standard proteins

● = CGTase

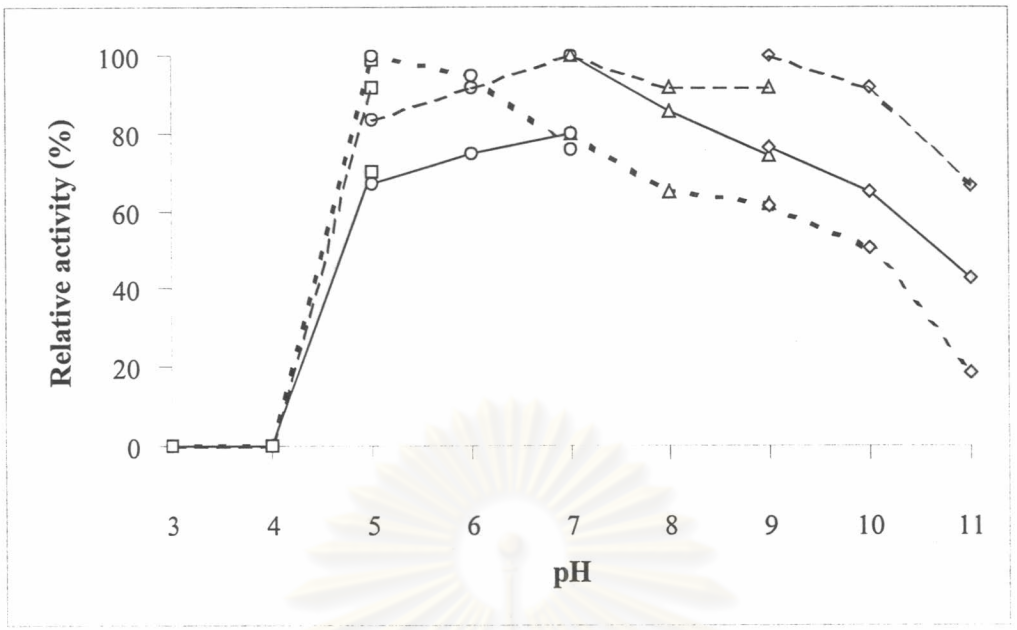


Figure 18 Optimum pH for dextrinizing activity (▪▪▪), cyclizing activity (—) and CD-TCE (– –)activity of CGTase

□ = Acetate buffer (pH 3-5) ○ = Phosphate buffer (pH 5-7)

△ = Tris-HCl buffer (pH 7-9) ◇ = Tris-Glycine (pH 9-11)

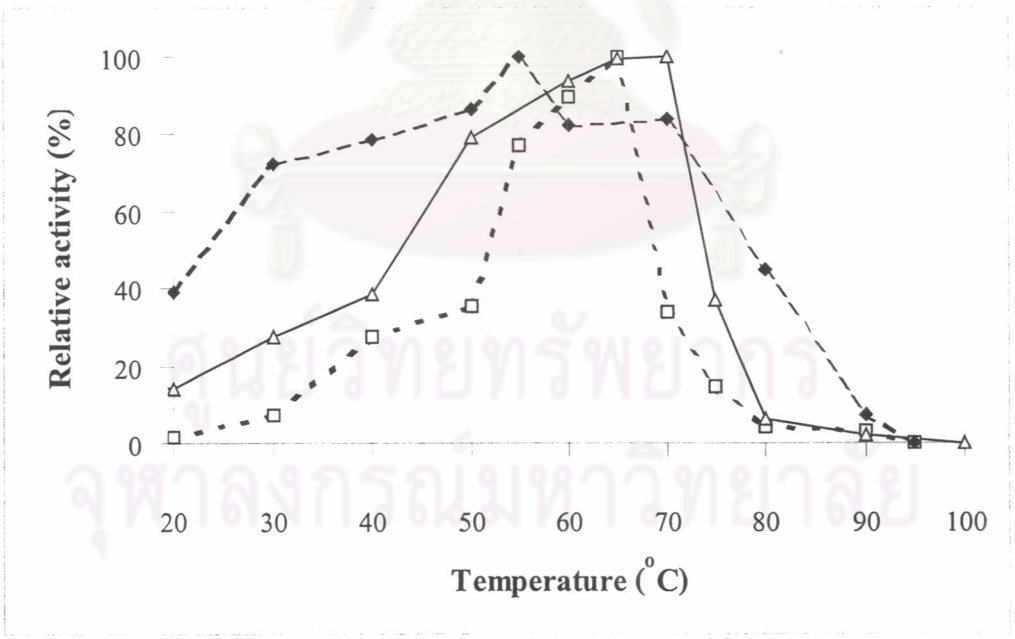


Figure 19 Optimum temperature for dextrinizing activity (▪□▪), cyclizing activity (–△–) and CD-TCE activity (-◆-) of CGTase at pH 7.0

3.2.6 pH stability of purified CGTase

The enzyme was incubated for 1 hr at 70 °C under various pH conditions prior to measurement of residual cyclization activity described in section 2.7.3. The enzyme retained more than 85% residual activity in a range of pH 7.0-9.0, while in phosphate buffer at pH 6.0 and Tris-glycine buffer at pH 10.0, the residual activity was about 70% and 60%, respectively. The enzyme was unstable in phosphate buffer at pH 5.0 since after 1 hr the activity retained was less than 10% (Figure 20).

3.2.7 Thermostability of purified CGTase

The effect of temperature on stability of the CGTase was also investigated by incubation of the enzyme for 1 hr at various temperatures followed by measurement of residual cyclization activity under condition described. The enzyme retained more than 80% activity in the temperature range of 45-60 °C. The enzyme was almost inactive at 65-70 °C when incubated for only 10 minutes. Figure 21 shows that at 70 °C in the presence of substrate (6% soluble starch), the CGTase was retained more than 80% residual activity at incubation time of 30 minutes.

3.2.8 Substrate specificity of CGTase

When different types of substrate were incubated with CGTase, and the cyclization activity was measured. Among various polysaccharides at 6% w/v, amylose showed the maximum activity while pullulans was the poorest substrate. Soluble starch was as almost good as amylose but dextrin was less catalyzed. Only 1.5% w/v concentration of amylopectin was used in this experiment because of its low solubility and about 80% relative activity compared to amylose was obtained. For oligosaccharides, G7 was as good as soluble starch. When comparing G3-G7, it was observed that the longer oligosaccharide, the higher the ability to being catalyzed by CGTase. While G1 and G2 could not act as the substrate as shown in Figure 22.

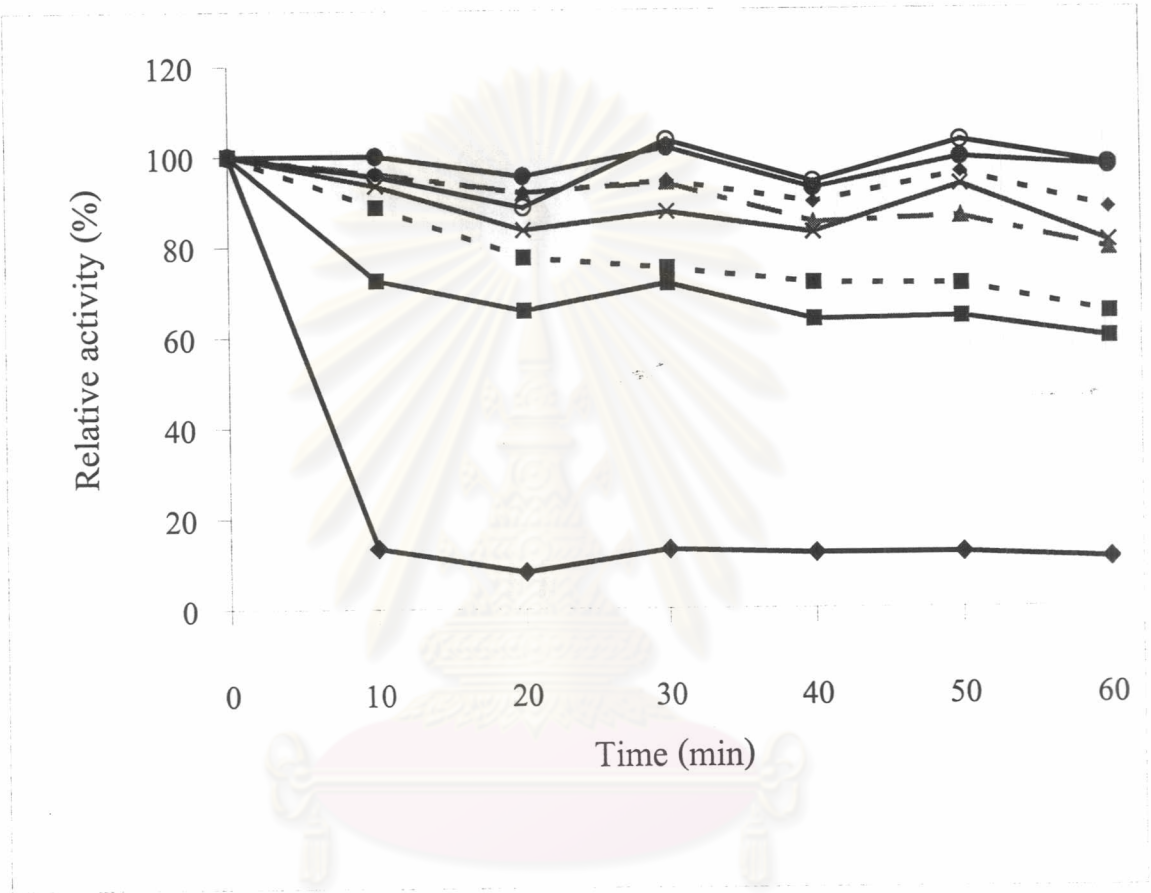


Figure 20 pH stability of cyclization activity of CGTase at 55 °C

Phosphate 5.0 Phosphate 6.0 Phosphate 7.0 Tris-HCl 7.0
Tris-HCl 8.0 Tris-HCl 9.0 Tris-Glycine 9.0 Tris-Glycine 10.0

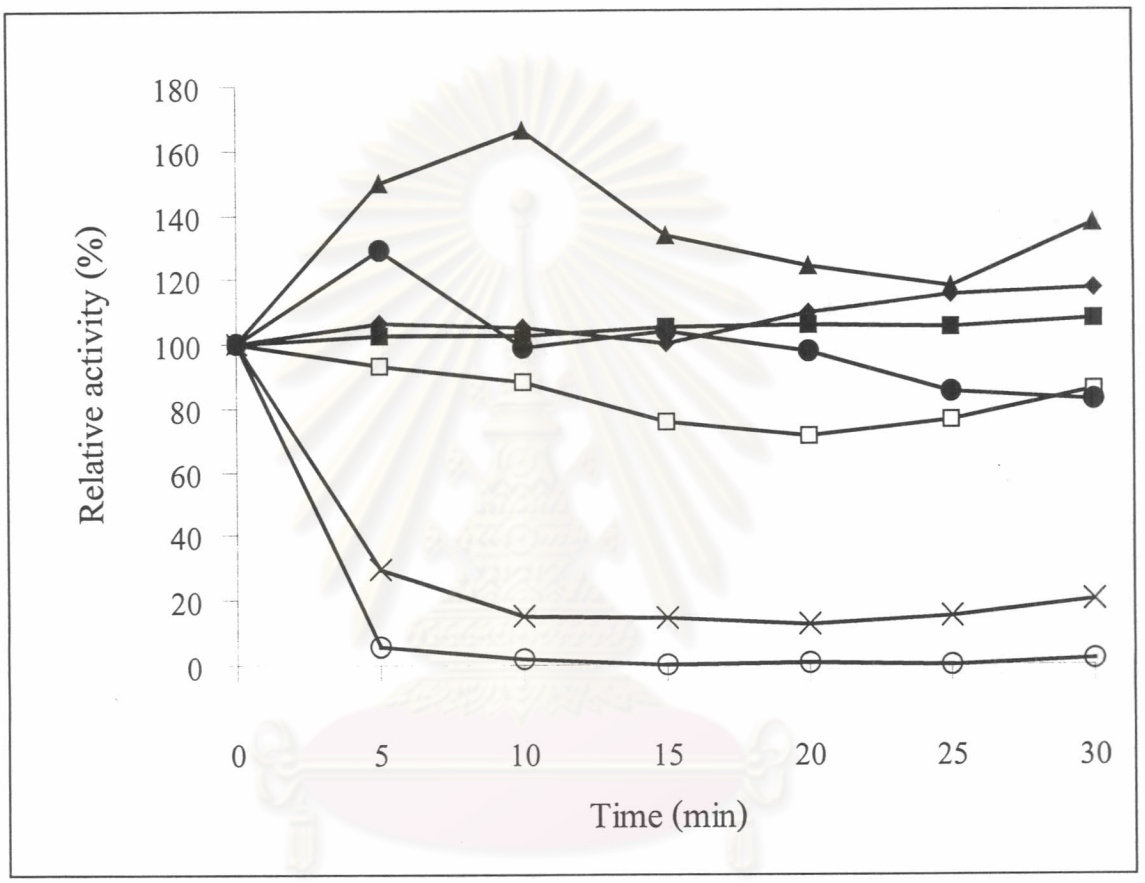


Figure 21 Thermostability of cyclization activity of CGTase at pH 7.0

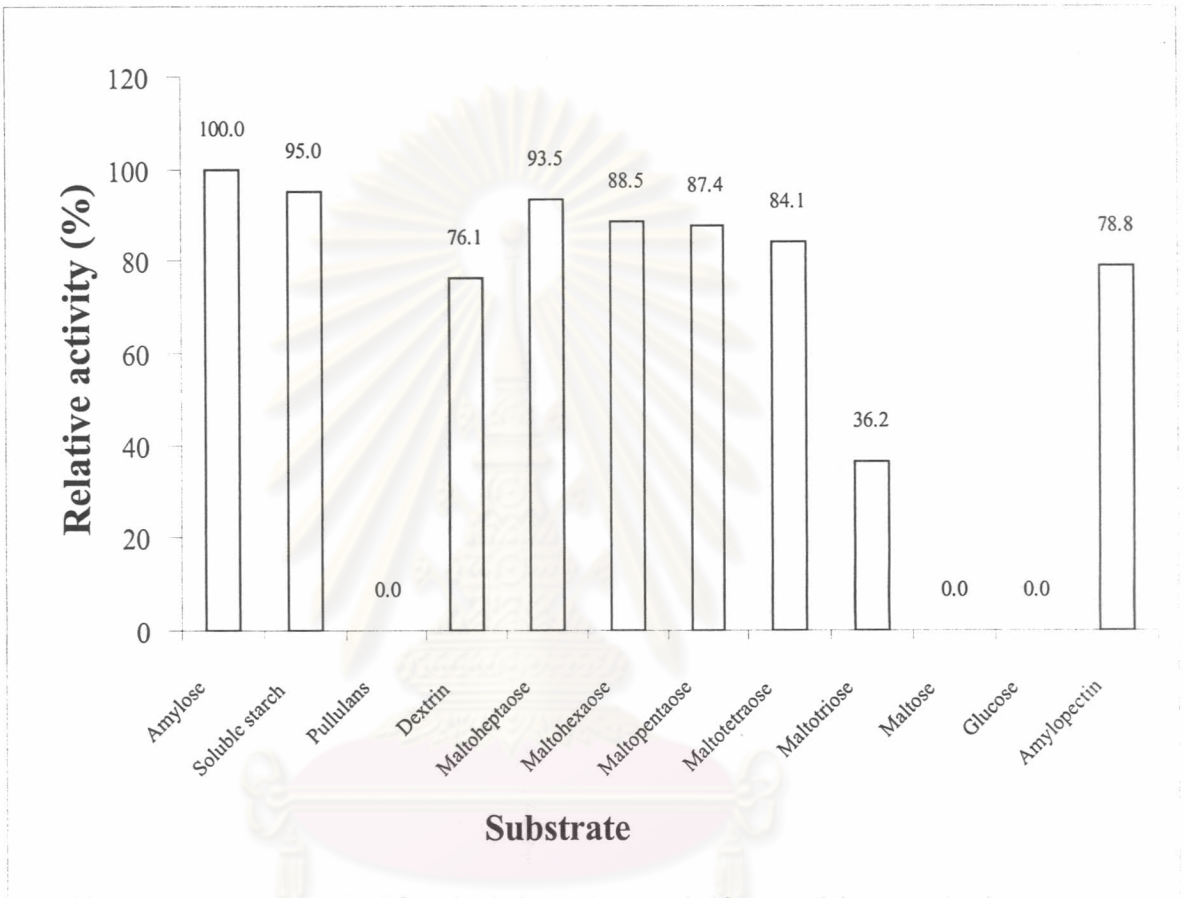


Figure 22 Substrate specificity of purified CGTase. Cyclization activity was measured in acetate buffer pH 6.0

3.2.9 Effect of calcium and temperature upon long-term storage

Enzyme samples (0.1 mg/ml in 0.2 M acetate buffer pH 6.0) were stored at 4 °C and –20 °C for 3 weeks. 10 mM $CaCl_2$ was also added in the enzyme sample to compare with control enzyme. Sample was withdrawn every week for measuring enzyme activity by dextrinizing activity assay. Figure 23 shows that the enzyme activity was reduced with time. $CaCl_2$ did not have an effect on stability at both storage temperatures. The activity after storing at 4 °C or –20 °C for 2 weeks was not different. But after 2 weeks, stability at –20 °C was better. The most suitable condition for storing enzyme was to keep the enzyme at –20 °C.

3.3 Kinetics Study

Kinetics of CGTase was determined by coupling reaction with α-, β-, γ-CD, glucosyl-α-CD, glucosyl-β-CD and hydroxypropyl-β-CD as donor substrates and 10 mM cellobiose as acceptor. Lineweaver-Burk plot of varying concentrations of β-CD was shown in Figure 24. This was the typical plot for all substrates and the summarized result was shown in Table 8. Hydroxypropyl-β-CD gave the highest K_m value while glucosyl-α-CD gave the lowest K_m value (3.962 and 0.951 mM, respectively). The V_{max} values for β- and γ-CD substrate were significantly higher than other substrates (165 and 167 μmoles min^{-1}, respectively) whereas glucosyl-α-CD and hydroxypropyl-β-CD gave the lowest (30 and 36 μmoles min^{-1}). The k_{cat}/K_m values showed similar trend as that of γ- > β- ≅ α-CD > modified CDs.

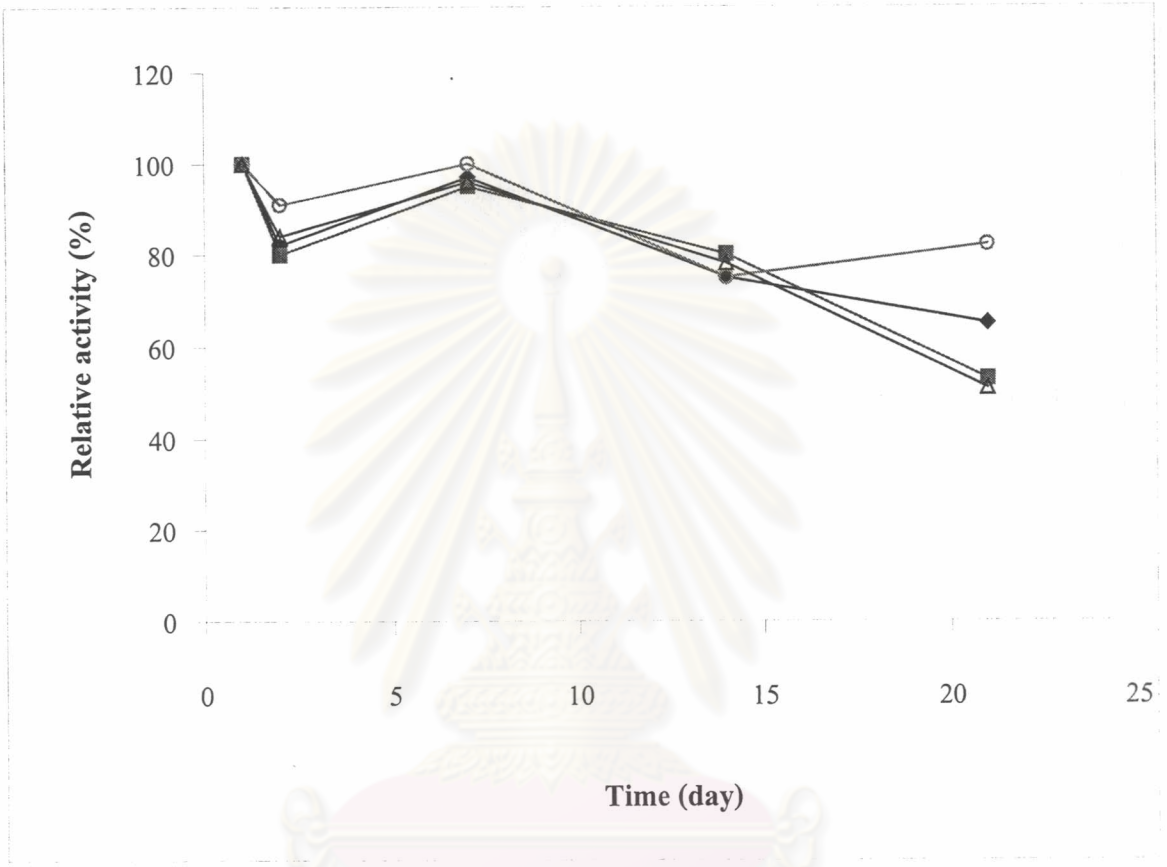


Figure 23 Long-term storage of CGTase at 4 °C and –20 °C with and without 10 mM $CaCl_2$

4 °C
-20 °C
4 °C with 10 mM $CaCl_2$
-20 °C with 10 mM $CaCl_2$

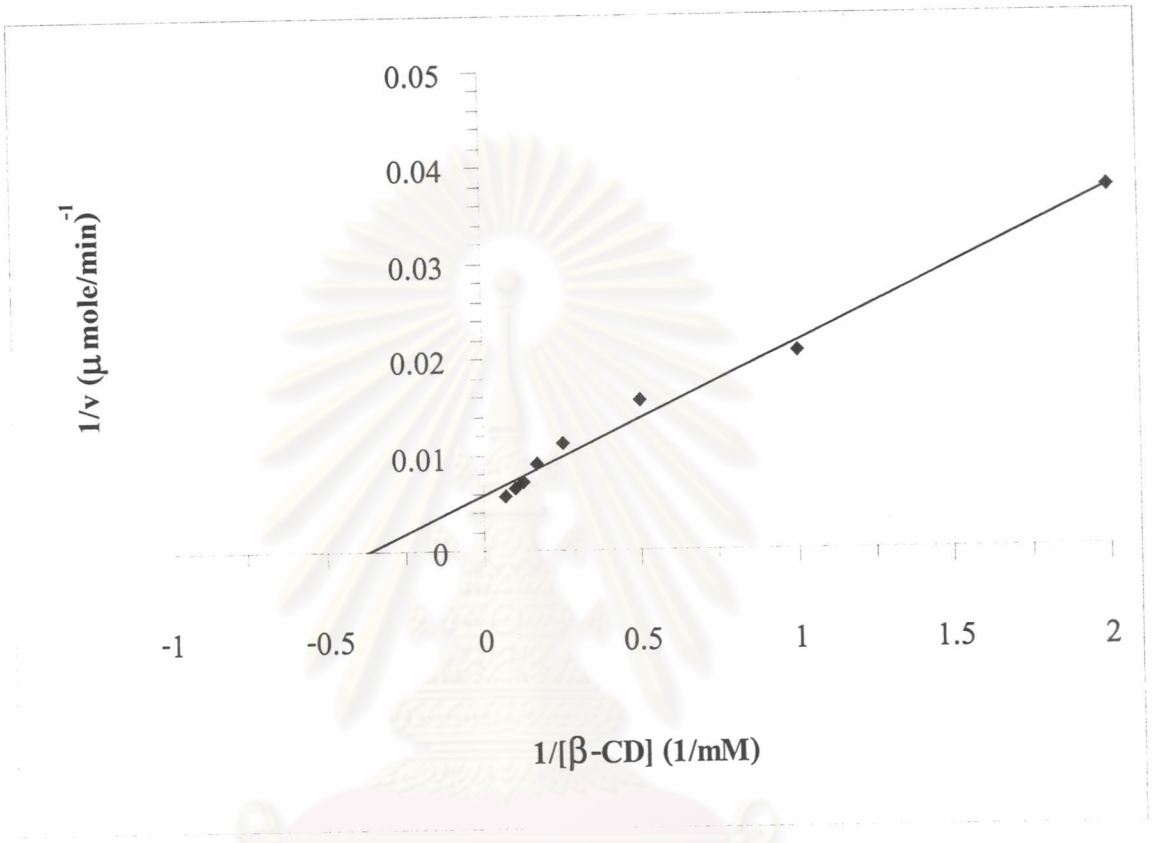


Figure 24 Lineweaver-Burk plot of CGTase with β–cyclodextrin as substrate

CGTase was incubated with 10 mM cellobiose and various concentrations of β–cyclodextrin in 50 mM acetate buffer, pH 6.0 at 40 °C for 5 minutes. 0.2 unit of *Aspergillus niger* glucoamylase was added to convert linearized oligsaccharides to glucose. The amount of β–CD degraded was monitored by the dinitrosalicylic acid method as described in section 2.9.

Table 8 Kinetic parameters of CGTase for different cyclodextrin substrates

Substrate	K_m (mM)	V_{max} (μmoles min^{-1})	V_{max}/K_m (μmoles $min^{-1}mM^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)
α-Cyclodextrin	1.398 ± 0.084	78.856 ± 6.573	56.406	4.480	3.205
β-Cyclodextrin	2.638 ± 0.227	165.706 ± 4.030	62.815	9.415	3.569
γ-Cyclodextrin	2.135 ± 0.381	167.249 ± 11.439	78.337	9.503	4.451
Glucosyl-α-Cyclodextrin	0.951 ± 0.233	30.439 ± 2.542	32.007	1.729	1.818
Glucosyl-β-Cyclodextrin	1.949 ± 0.201	85.012 ±1.826	43.618	4.830	2.478
Hydroxypropyl-β-Cyclodextrin	3.962 ± 0.285	36.170 ± 3.559	9.219	2.055	0.519

3.4 Product analysis by High Performance Liquid Chromatography

Crude and purified CGTase was incubated with soluble starch as described in methods. The reaction mixture which was passed through 0.45 μm membrane filter was injected to HPLC column and eluted with acetronitrile-water (70:30, v/v) using a flow rate of 1.6 ml/min. The CD peak was identified by comparing the retention time with that of standard α-,β- or γ-CDs (20 mg/ml). When β-amylase was added to hydrolyze linear oligosaccharides produced in the reaction mixture, the peaks at R_t of 4.68, 5.45 and 6.63 minutes were decreased. After conversion of the peak area to CD concentration, it was found that crude enzyme produced α- : β- : γ-CD in the ratio of 1.0 : 1.1 : 0.1, while purified enzyme produced similar ratio of 1.0 : 1.8 : 0.4 (Figure 25).

3.5 Chemical modification of CGTase

To determine the effect of group-specific reagents on enzyme activity, purified CGTase was incubated with 1.0 mM of each modifying reagent at 40 °C for 30 minutes in phosphate buffer pH 6.0. The residual enzyme activity was then determined as described in section 2.7.1. Enzyme activity was almost totally inhibited by NBS and DEP, while partially inhibited by EDC (38.6% residual activity). This result suggests the importance of trp, his, and carboxylic amino acids for enzyme catalytic activity. Other modifying reagents: NAI, NEM, IAM, DTT, TNBS and PMSF did not show any inhibition (Figure 26).

The modification of CGTase with different group-specific reagents, which affected CGTase activity, was then carried out in two steps. The first step was to determine the suitable concentration and incubation time of the reagent used in the modification of enzyme. Then the suitable conditions were used to identify the amino acids involved in the catalytic site of enzyme using substrate protection technique.

To determine the suitable concentration of modifying reagent used in the

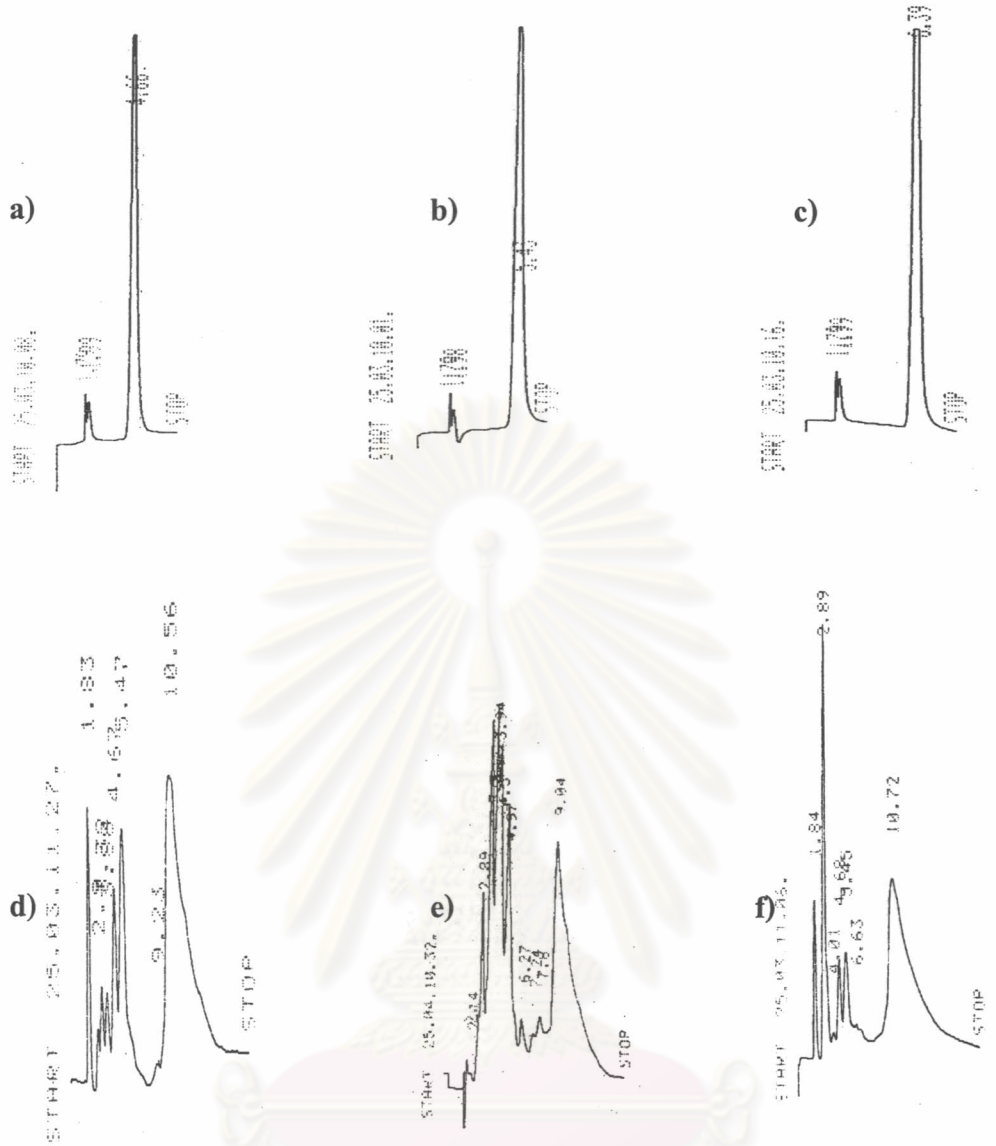


Figure 25 HPLC chromatogram of CDs produced by purified CGTase from RB01 and standard cyclodextrins (α-CD, β-CD and γ-CD). a-c) standard CD, d) CGTase without β-amylase, e) Crude CGTase with β-amylase, f) purified CGTase with β-amylase. Lichrocart-NH_2 column was used. Acetonitrile : water (70 : 30, v/v) was used as eluent at 1.6 ml/min flow rate.

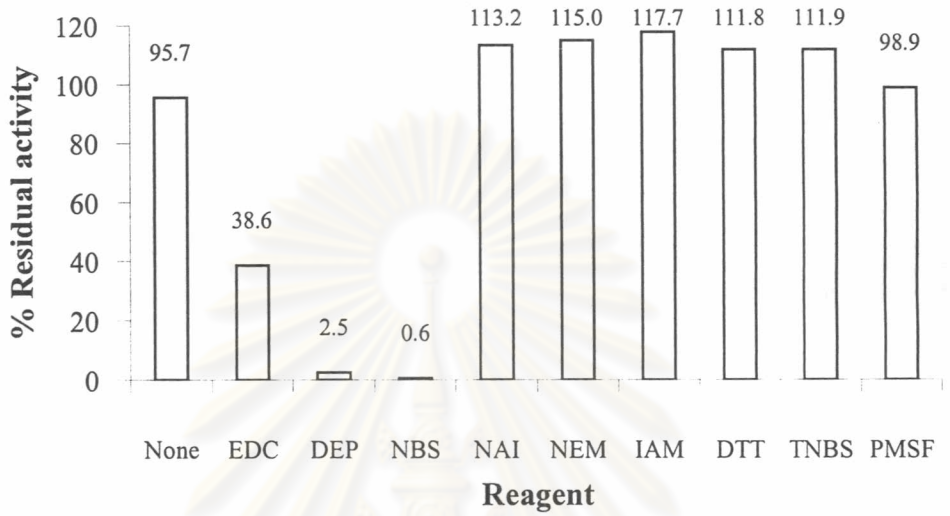


Figure 26 Effect of 1 mM various group-specific reagents on CGTase dextrinizing activity at pH 6.0

EDC = 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

DEP = Diethylpyrocarbonate

NBS = *N*-bromosuccinimide

NAI = *N*-acetylimidazole

NEM = *N*-ethylmaleimide

IAM = Iodoacetamide

DTT = Dithiothreitol

TNBS = Trinitrobenzenesulfonic acid

PMSF = Phenylmethylsulfonyl fluoride

modification, the enzyme was incubated with varying concentrations of the reagent at 40 °C, for 30 minutes. CGTase activity was then determined as described in section 2.7.1. The suitable concentration for modification is the minimum concentration of the reagent that leads to maximum inactivation of the enzyme.

To determine the suitable incubation time used in the modification, the suitable concentration of each modifying reagent was incubated with CGTase by varying time as described in section 2.17.2. The suitable incubation time is the incubation time at which about 50% dextrinizing activity was left.

3.5.1 Modification of carboxyl residues by EDC

Carboxyl residues of CGTase were modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as described in section 2.17.1.1. Figure 27 shows the effect of various concentrations of EDC on CGTase activity within 30 minutes incubation time. At 5 mM or higher concentrations of EDC, CGTase activity was completely lost. Thus, 5 mM EDC was chosen to be the suitable concentration. Figure 28 shows that after 5 minutes of incubation with 5 mM EDC, dextrinizing activity of CGTase was decreased about 50% whereas almost total activity loss was observed at 30 minutes. The suitable incubation time of the enzyme with EDC was thus 5 minutes.

To determine if carboxyl residue was involved at the catalytic site of CGTase, modifications by EDC in the presence or the absence of substrate were compared. α-, β- and γ–CD were used as protective substances. Four different conditions: 1. CGTase alone, 2. CGTase incubated with each substrate, 3. CGTase incubated with each substrate then modified by EDC, and 4. CGTase modified by EDC, were compared. After the reaction, CGTase activities were then determined as described in section 2.7.1. The loss of CGTase activity was about 60% when modified with 5 mM EDC. Relative activity was increased 20% when CGTase was preincubated with each substrate and then modified by EDC (Figure 29a, b and c).

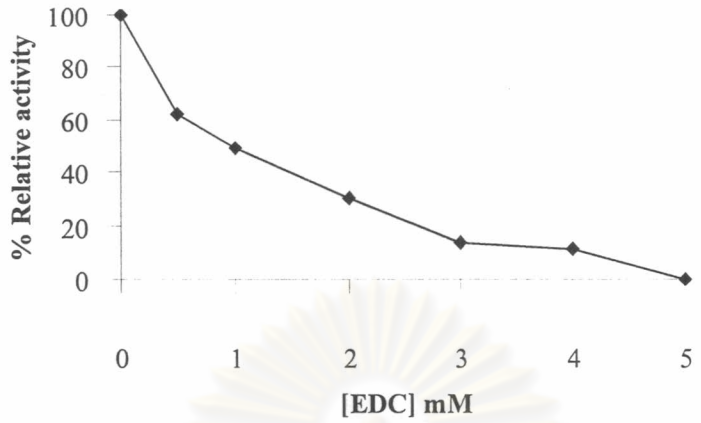


Figure 27 Effect of EDC on CGTase activity

CGTase was incubated with varying concentrations of EDC at 40 °C for 30 minutes according to the method as described in section 2.17.1.1. After the incubation, CGTase activity was determined as described in section 2.7.1.

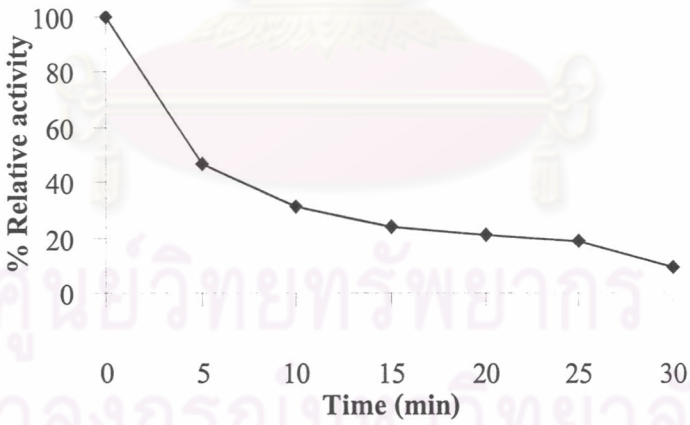


Figure 28 Inactivation of CGTase activity by 5 mM EDC

CGTase was incubated with 5 mM EDC at 40 °C at various times according to the method as described in section 2.17.2. CGTase activity was determined as described in section 2.7.1.

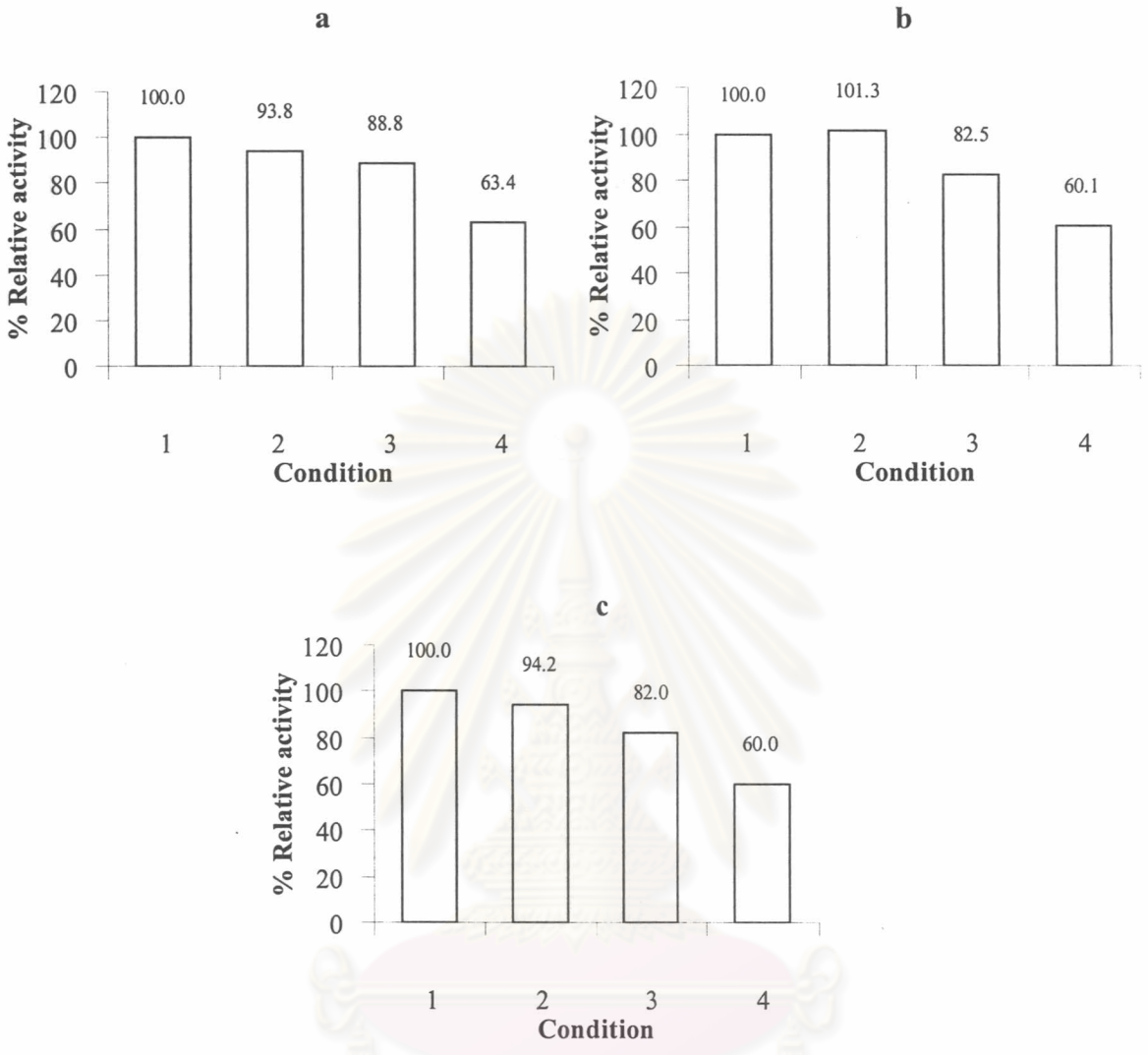


Figure 29 Residual CGTase activity of EDC-modified enzyme in the presence and absence of a protective substance; α–CD(a), β–CD (b) and γ–CD (c)

1 = CGTase alone

2 = CGTase incubated with 20 mM α-,β- or γ-CD

3 = CGTase incubated with 20 mM α-,β- or γ-CD then modified by 5 mM EDC

4 = CGTase modified by 5 mM EDC

3.5.2 Modification of histidine residues by DEP

Histidine residues of CGTase were modified by diethylpyrocarbonate (DEP) as described in section 2.17.1.2. CGTase activity was decreased with increasing DEP concentration (Figure 30). At 1.5 mM DEP, CGTase activity was completely lost. Thus, the suitable concentration of DEP was 1.5 mM. Figure 31 shows that after 5 minutes of incubation, dextrinizing activity was decreased 50%. Thus the suitable incubation time was 5 minutes.

To determine if histidine residue was involved at the catalytic site of CGTase, modification by DEP in the presence or the absence of substrate were compared. α-, β- and γ–CD were used as protective substances. Four different conditions: 1. CGTase alone, 2. CGTase incubated with each substrate, 3. CGTaseincubated with each substrate then modified by DEP, and 4. CGTase modified by DEP, were compared. After the reaction, CGTase activities were then determined as described in section 2.7.1. Figure 32 shows that modification by 1.5 mM DEP led to about 41% loss of CGTase activity. When CGTase preincubated with each substrate was modified by DEP (condition 3), the loss of CGTase activities were significantly reduced. In the presence of α-, β- and γ–CD, the activities loss were 8,0.2 and 11%, respectively.

3.5.3 Modification of tryptophan residues by NBS

Tryptophan residues of CGTase were modified by *N*-bromosuccinimide (NBS) as described in section 2.17.1.3. At 0.005 mM NBS, CGTase activity was completely lost (Figure 33). Thus, the suitable concentration of NBS was 0.005 mM. Figure 34 shows that after 5 minutes of incubation, dextrinizing activity was decreased about 50%. Thus the suitable incubation time was 4 minutes.

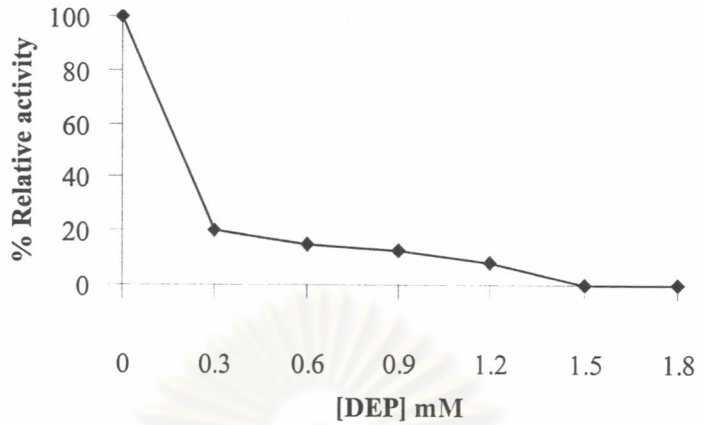


Figure 30 Effect of DEP on CGTase activity

CGTase was incubated with varying concentrations of DEP at 40 °C for 30 minutes according to the method as described in section 2.17.1.2. After the incubation, CGTase activity was determined as described in section 2.7.1.

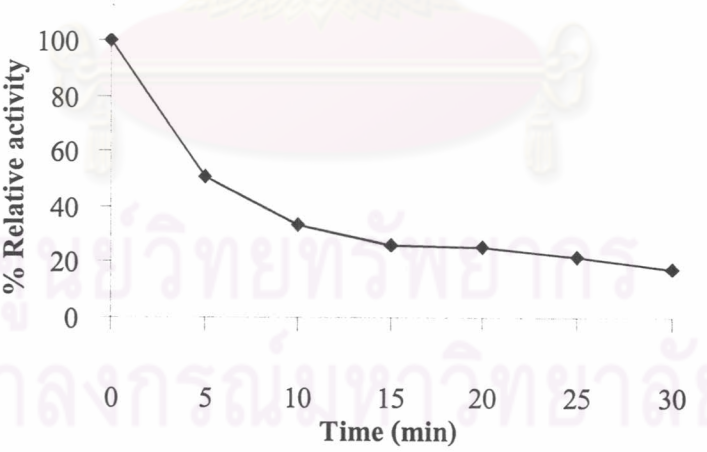


Figure 31 Inactivation of CGTase activity by 1.5 mM DEP

CGTase was incubated with 1.5 mM DEP at 40 °C at various times according to the method as described in section 2.17.2. CGTase activity was determined as described in section 2.7.1.

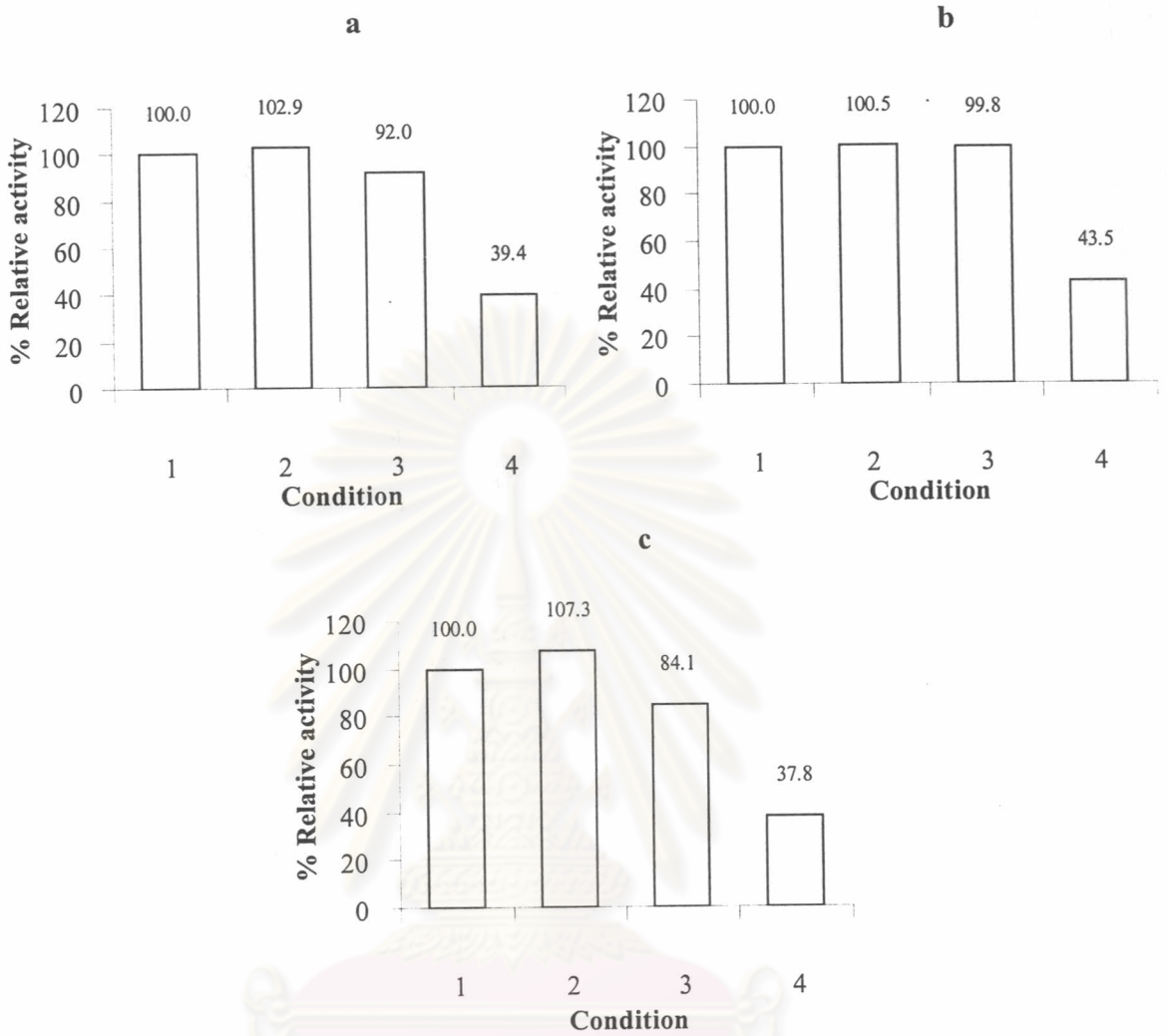


Figure 32 Residual CGTase activity of DEP-modified enzyme in the presence and absence of a protective substance; α–CD(a), β–CD (b) and γ–CD (c)

1 = CGTase alone

2 = CGTase incubated with 20 mM α-,β- or γ-CD

3 = CGTase incubated with 20 mM α-,β- or γ-CD then modified by 1.5 mM DEP

4 = CGTase modified by 1.5 mM DEP

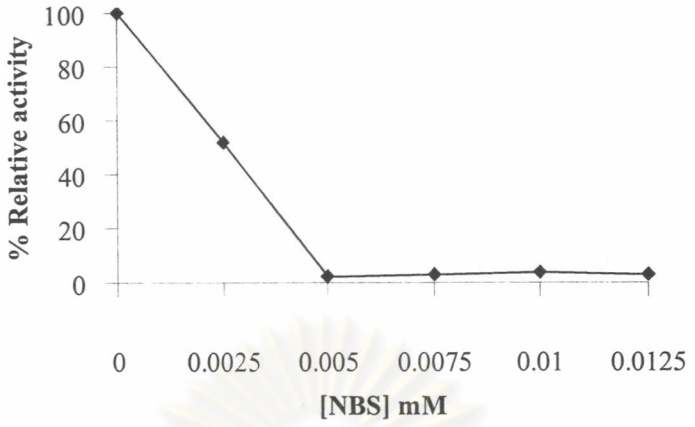


Figure 33 Effect of NBS on CGTase activity

CGTase was incubated with varying concentrations of NBS at 40 °C for 30 minutes according to the method as described in section 2.17.1.3. After the incubation, CGTase activity was determined as described in section 2.7.1.

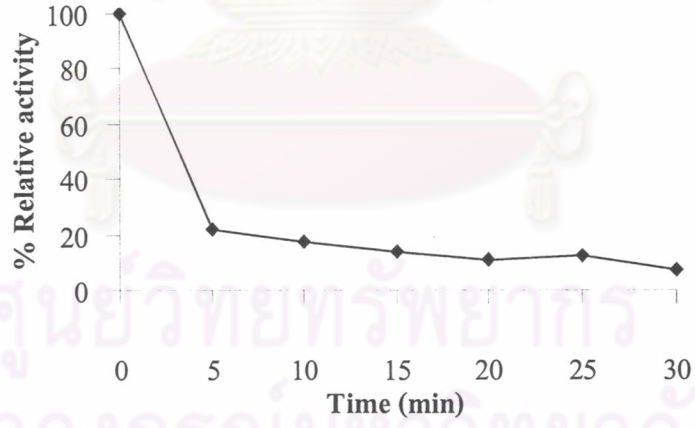


Figure 34 Inactivation of CGTase activity by 0.005 mM NBS

CGTase was incubated with 0.005 mM NBS at 40 °C at various times according to the method as described in section 2.17.2. CGTase activity was determined as described in section 2.7.1.

To determine if tryptophan residue was involved at the catalytic site of CGTase, modification by NBS in the presence or the absence of substrate were compared. α-, β- and γ–CD were used as protective substances. Four different conditions: 1. CGTase alone, 2. CGTase incubated with each substrate, 3. CGTase incubated with each substrate then modified by NBS, and 4. CGTase modified by NBS, were compared. After the reaction, CGTase activities were then determined as described in section 2.7.1. Figure 35 shows that when CGTase was preincubated with β-CD then modified by DEP (condition 3), the loss of CGTase activities were significantly reduced. In the other hand, in the presence of α- and γ–CD, change of CGTase activity could not be recovered.

3.6 Fluorescence emission spectrum upon modification by NBS

Since chemical modification experiment suggested tryptophan was the most important residue for CGTase activity. Fluorescence emission spectrum of CGTase due to tryptophan residues was followed. To confirm if any tryptophan residues were present at the catalytic site of CGTase, modification was carried out by comparison between four conditions. CGTase alone, CGTase incubated with 0.1 mM NBS for 5 minutes, CGTase pre-incubated with 1% and 2% β-CD for 5 minutes and then modified by 0.1 mM NBS 5 minutes. The fluoresence emission spectrum of CGTase modified with NBS was different from that of control CGTase. Maximum emission wavelength was shifted to a shorter wavelength and fluorescence intensity was significantly reduced. However, when CGTase was incubated with 1% or 2% β-CD prior to the modification with 0.1 mM NBS, the emission spectrum was gradually returned to the control pattern especially with the maximum emission wavelength as shown in Figure 36.

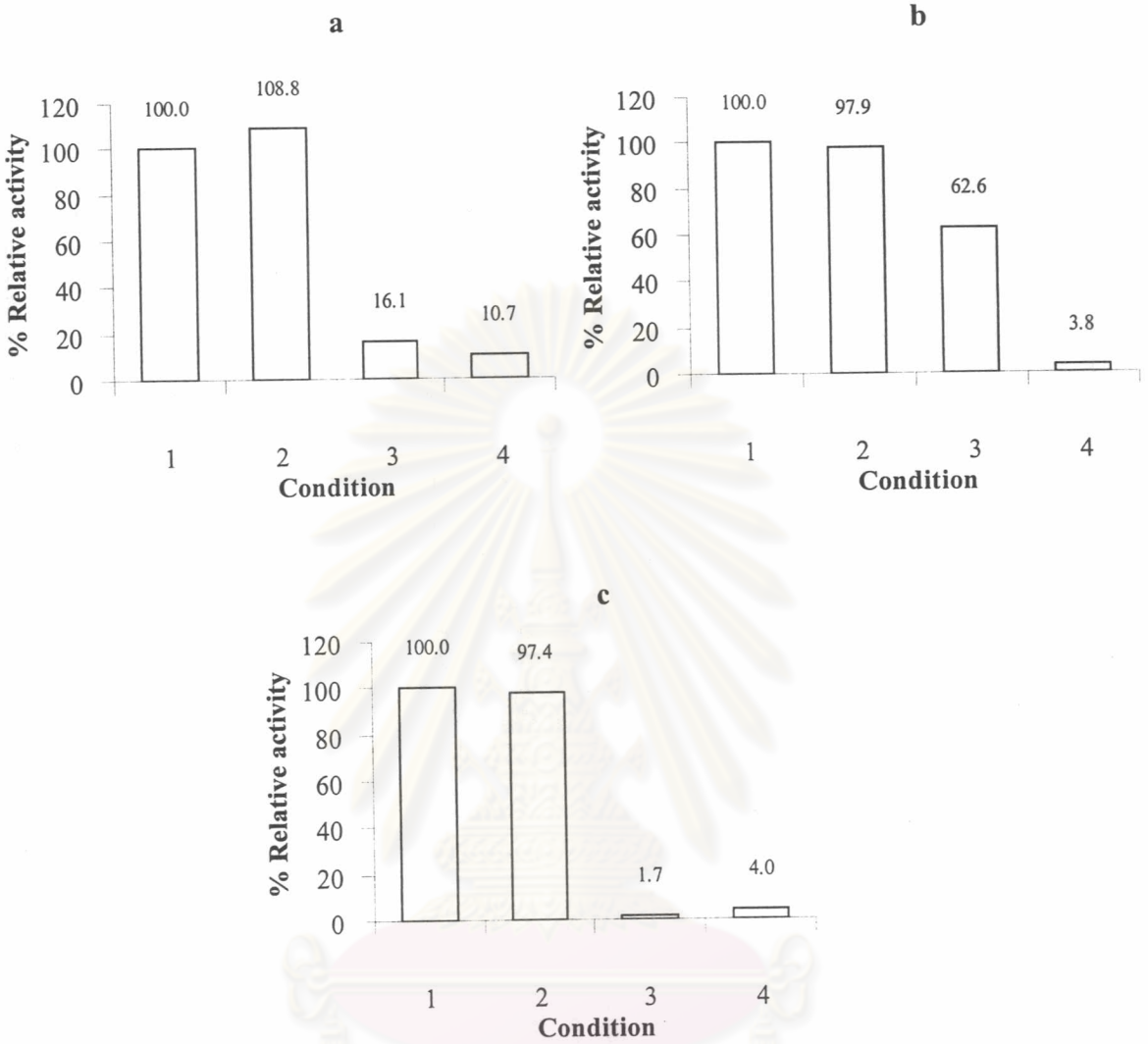


Figure 35 Residual CGTase activity of NBS-modified enzyme in the presence and absence of a protective substance; α–CD(a), β–CD (b) and γ–CD (c)

1 = CGTase alone

2 = CGTase incubated with 20 mM α-,β- or γ-CD

3 = CGTase incubated with 20 mM α-,β- or γ-CD then modified by 0.005 mM NBS

4 = CGTase modified by 0.005 mM NBS

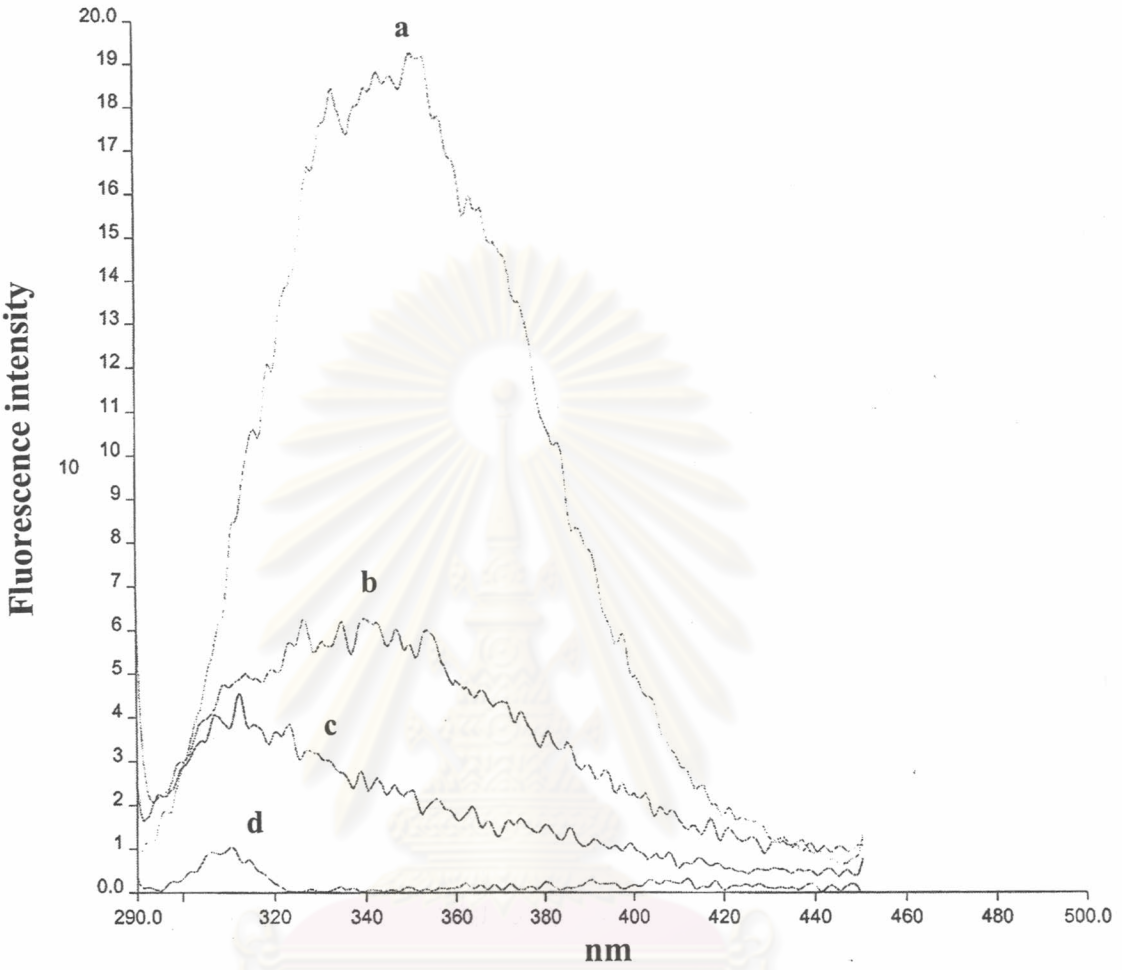


Figure 36 Fluorescence emission spectrum of CGTase before and after modification with NBS

For control, CGTase was scanned. After 5 minutes of the modification, the modified CGTase from each condition was scanned at the same wavelength.

a = CGTase alone (Control)

b = CGTase preincubated with 2% β-CD, then modified 0.1 mM NBS

c = CGTase preincubated with 1% β-CD, then modified 0.1 mM NBS

d = CGTase incubated with 0.1 mM NBS

3.7 Urea-induced denaturation of CGTase

To examine the relation of the tertiary structure of the protein and the formation of isomeric forms, CGTase was unfolded stepwise with urea and the degree of unfolding was assessed by following fluorescence emission. Figure 37 showed the change in emission spectrum of CGTase under urea-induced denaturation (0-10 M urea concentration). The maximum emission wavelength was shifted to longer wavelength when increased the concentration of urea. Fluorescence emission at 350 nm and % relative dextrinizing activity of CGTase at different concentration of urea were shown in Figure 38. The enzyme lost 5% of its activity at 2 M urea, 50% at 4 M, 60% at 6 M and all activity at 10 M urea. At 2 M and 5 M urea, transitions in fluorescence emission occurred concomitantly with loss of enzyme activity. When dextrinizing activity activity was checked on native PAGE (Figure 39), no difference in pattern of multiple forms was observed between control CGTase and CGTase treated with 3 M and 6 M urea.

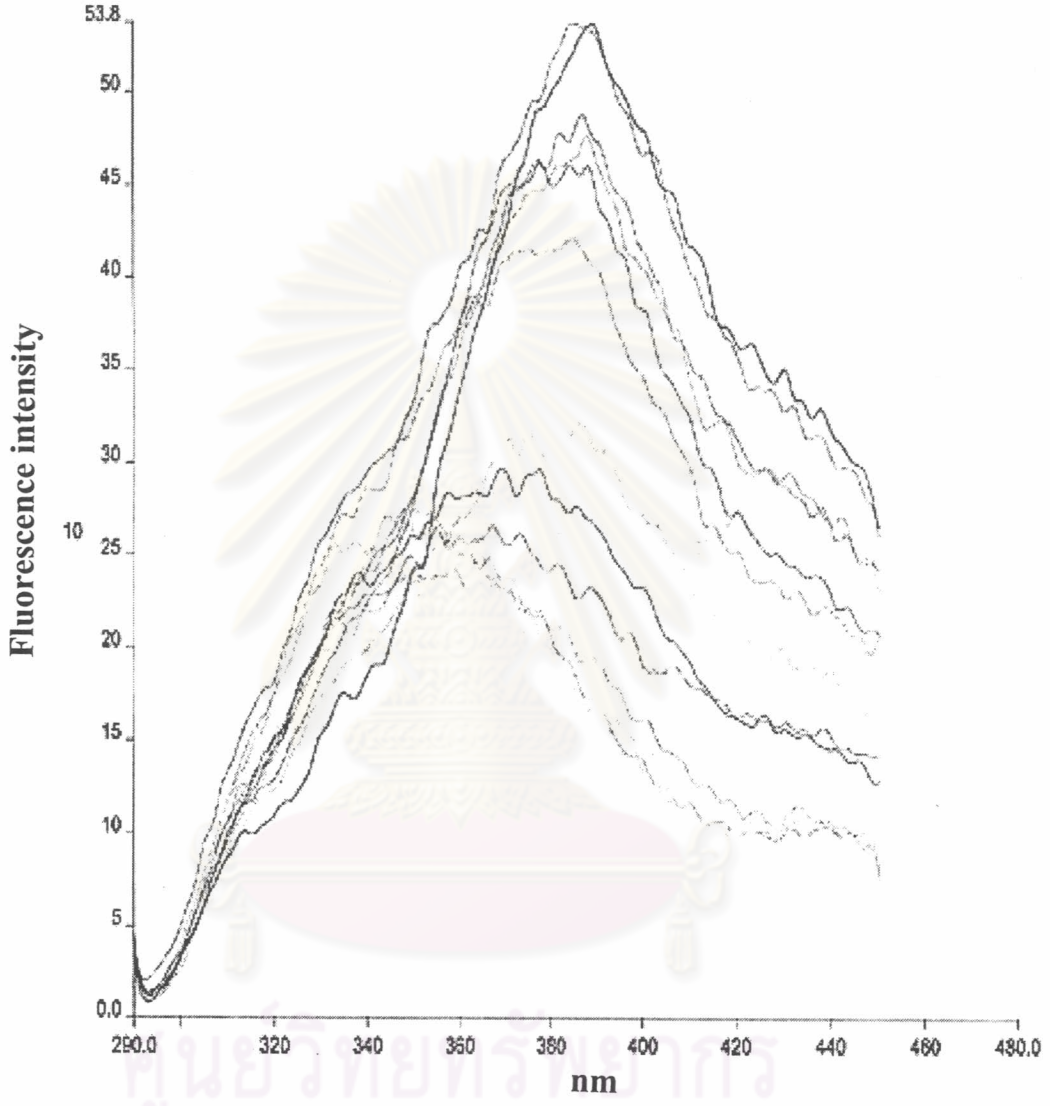


Figure 37 Fluorescence emission spectrum of CGTase when modified by urea 0-10 M at 25 °C.

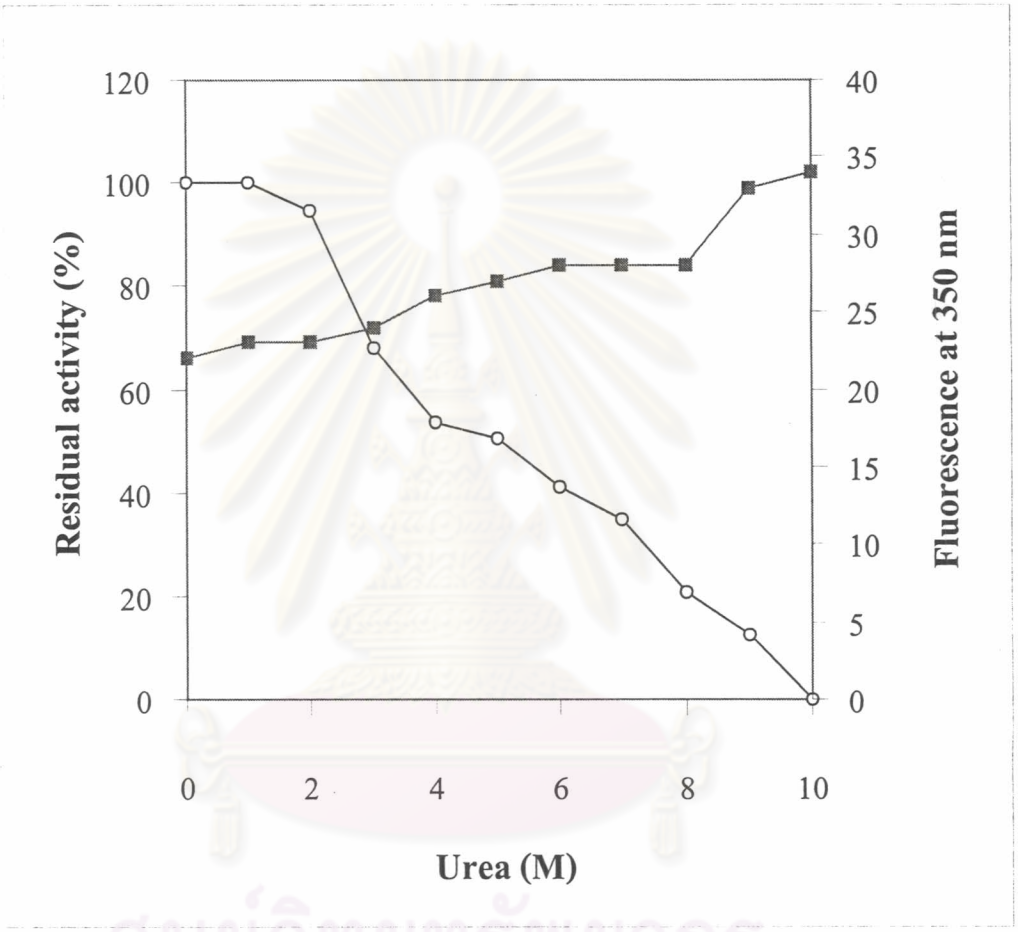
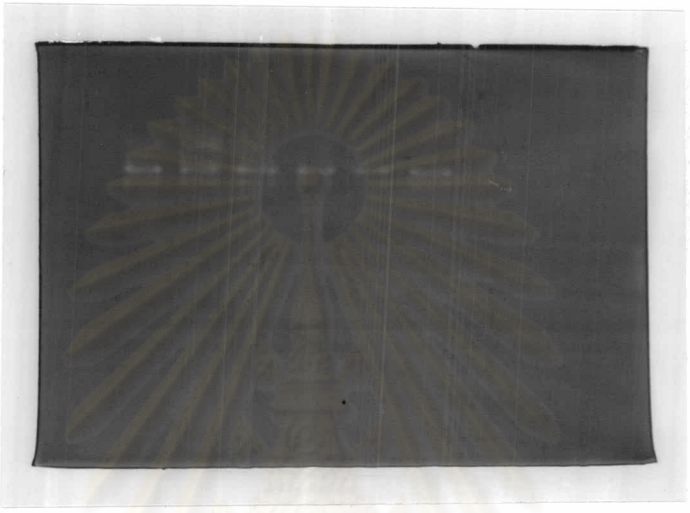


Figure 38 Fluoresence intensity at 350 nm and dextrinizing activity of CGTase after incubation for 16 hours at different urea concentration



1 2 3 4 5 6

Figure 39 Dextrinizing activity staining on native PAGE of urea-induced denaturation for 16 hours at 25 °C

Lane 1 and 2 = CGTase only

Lane 3 and 4 = CGTase with 3 M urea

Lane 5 and 6 = CGTase with 6 M urea