CHAPTER III

RESULTS

3.1 Purification of CGTase

Crude CGTase was purified by two successive steps of corn starch adsorption and immunoaffinity column chromatography. Figure 7 shows the elution profile from immunoaffinity column chromatography. One major protein peak with no enzyme activity was washed off with the equilibrated buffer. CGTase was eluted as a single peak between fraction 20 and 24. The highest dextrinizing activity was obtained at the same position as the protein peak. Tailing of enzyme activity was observed from fraction 25-35. The enzyme fraction (20-23) was pooled and concentrated for further studies, so called 'purified CGTase'.

The purification fold and recovery of CGTase obtained at each step are shown in Table 7. Specific activity expressed in terms of dextrinizing activity per mg protein was increased through each step. These corresponded to the increase in CD-product, which was determined by CD-TCE dilution limit. After the final step of purification, a yield of 36% was obtained with 144 folds of purify. Purified CGTase showed one intense protein band on SDS-polyacrylamide gel (Figure 8) and two major protein bands and one faint band, all with corresponded amylolytic activity, on native polyacrylamide gel (Figure 9).

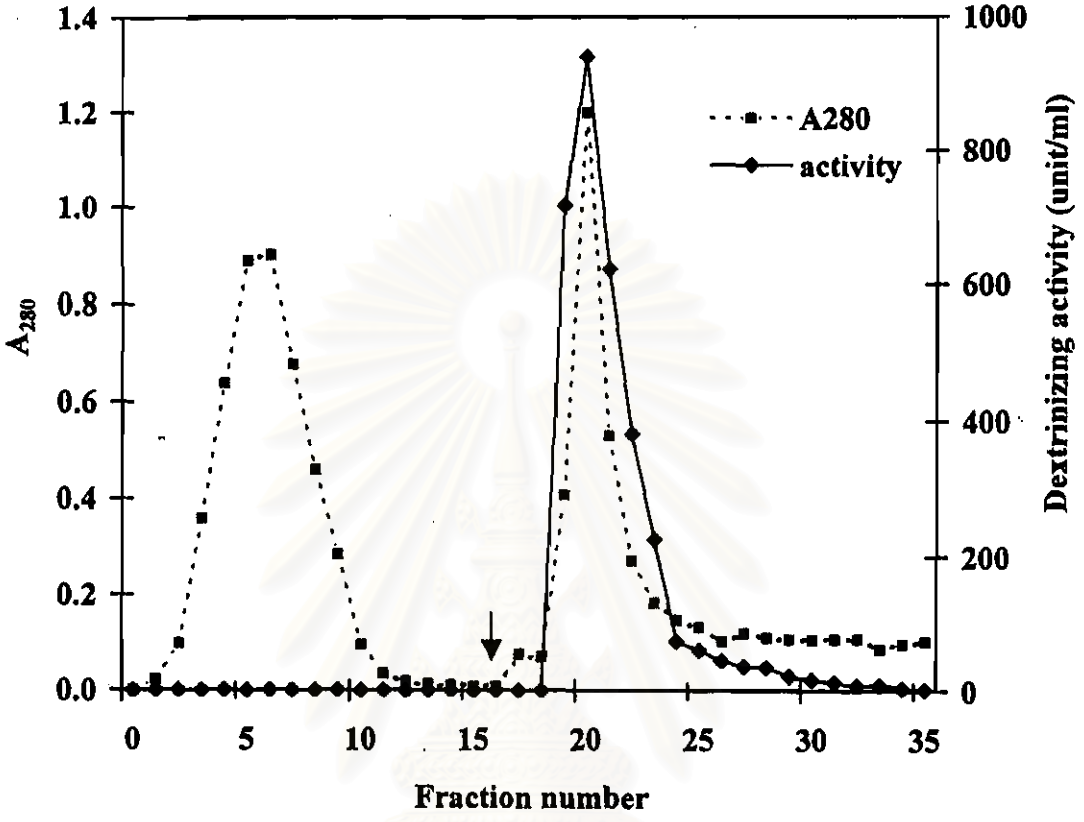


Figure 7 Purification of CGTase from *Bacillus* sp. A11 by immunoaffinity column chromatography

Concentrated CGTase solution from corn starch adsorption was applied onto immunoaffinity column chromatography (0.8 x 4.5 cm) and washed with 50 mM acetate buffer pH 6.0 containing 0.5 mM NaCl at the flow rate of 2 ml/ hr. Elution was made by 3.5 M NaSCN in 50 mM NH_4OH, pH 10.5 at the flow rate of 6 ml/ hr. (the arrow indicates where elution starts)

Table 7 Purification of CGTase from *Bacillus* sp. A11

Step	Volume (ml)	Total activity (unit) x 10^3	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)	CD-TCE (2^n)
Crude enzyme	4,000	61.0	2,450	24.9	1	100	2^4
Corn starch adsorption	18.0	39.8	16.5	2,412	97	65	2^{10}
Immuno-affinity column	18.5	21.8	6.1	3,574	144	36	2^{11}

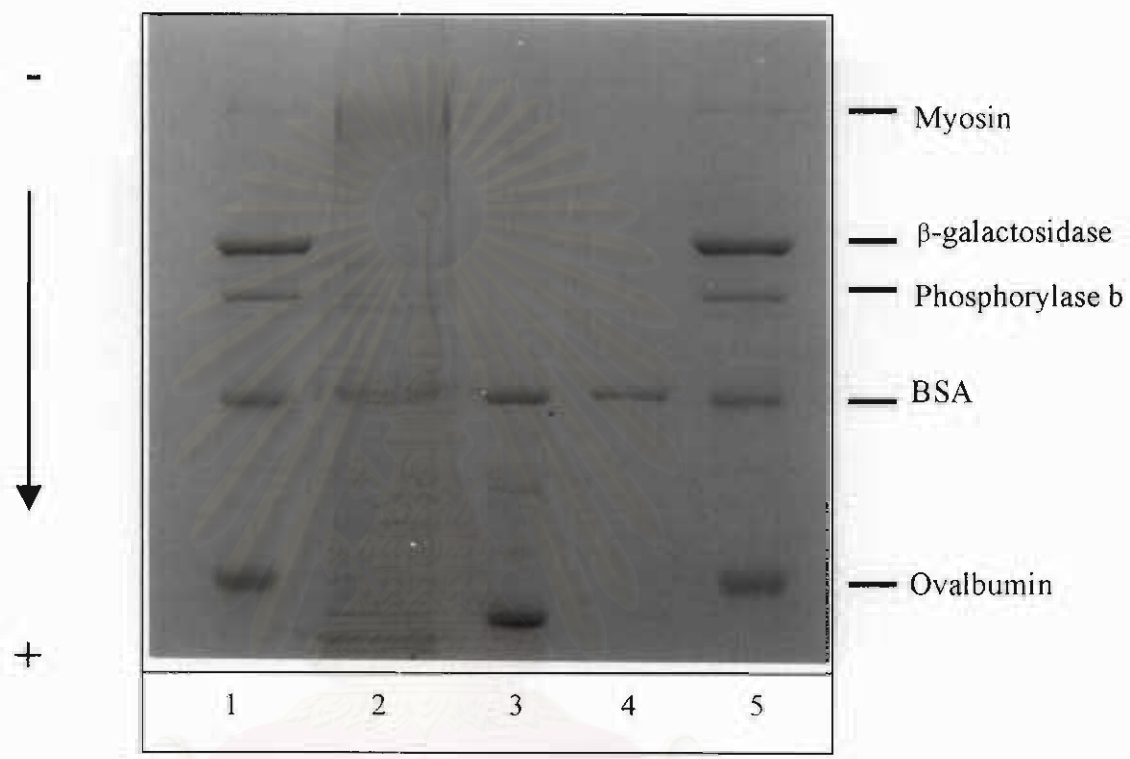


Figure 8 SDS-PAGE of CGTase from different steps of purification

Lane 1, 5 : Protein molecular weight markers
[myosin (200 kD), β-galactosidase (116.2 kD), phosphorylase b (97.1 kD), BSA (66.2 kD), and ovalbumin (45 kD)]

2 : Crude enzyme (20 μg)

3 : Starch adsorbed enzyme (20 μg)

4 : Immunoaffinity-purified enzyme (5 μg)

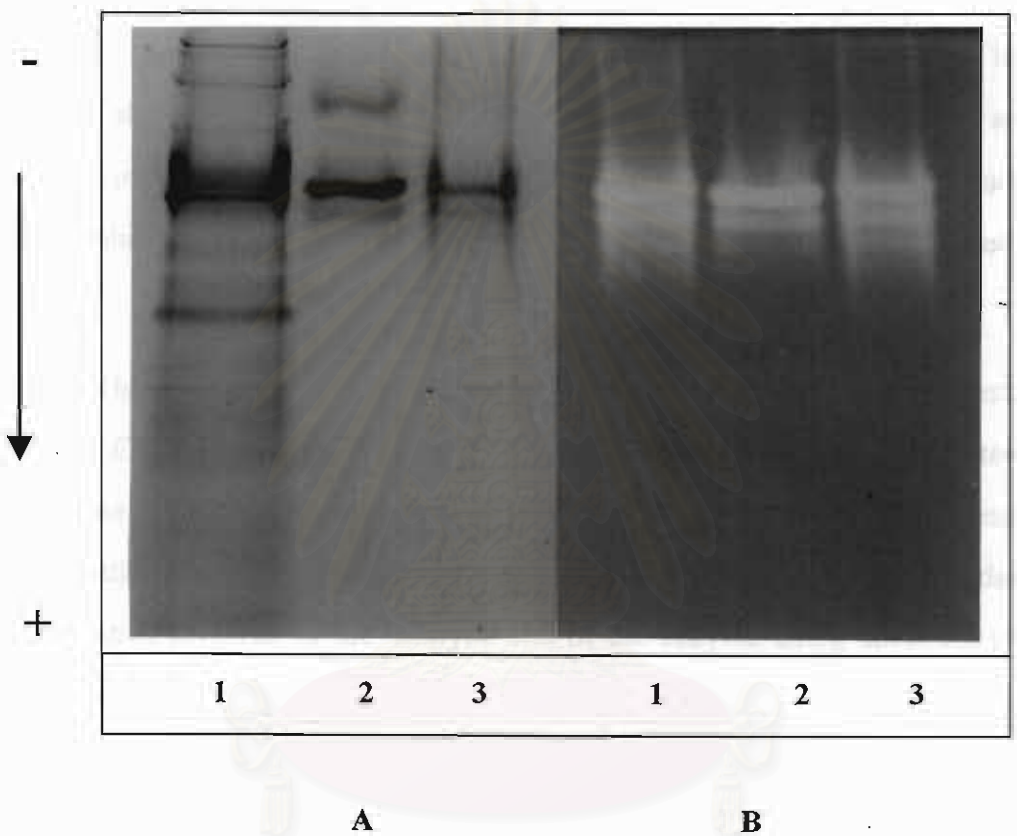


Figure 9 Non-denaturing PAGE of CGTase from different steps of purification

A : Coomassie blue staining

Lane 1 : Crude enzyme (20 μg)

2 : Starch adsorbed enzyme (20 μg)

3 : Immunoaffinity-purified enzyme (5 μg)

B : Amylolytic activity staining by iodine solution

[Lane 1-3, as in A, 0.2 units of dextrinizing activity was loaded to each well]

3.2 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. The residual enzyme activity was then determined as described in section 2.9.1. Table 8 shows that CGTase activity was totally inhibited by DEP and NBS, partially inhibited by EDC and NAI (33 and 15% inhibition, respectively), and hardly or not inhibited by NEM, IAM, DTT, TNBS, and PMSF (93-100% residual activity detected).

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 the enzyme. Then the suitable conditions were used to identify the amino acids involved in the catalytic site of the enzyme using substrate protection technique.

To determine the suitable concentration of modifying reagent used in the 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 2.9.1. The suitable concentration is the minimum concentration of the reagent that leads to the 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 2.13.2. The suitable incubation time is the incubation time at which about 50% dextrinizing activity was left.

Table 8 Effect of various group-specific reagents on CGTase activity

Modifying reagents (1 mM)	% Residual dextrinizing activity
None	100.0
N-ethylmaleimide (NEM)	96.1
Iodoacetamide (IAM)	99.3
Dithiothreitol (DTT)	92.6
Trinitrobenzenesulfonic acid (TNBS)	100.0
Phenylmethylsulfonyl fluoride (PMSF)	100.0
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	67.3
Diethylpyrocarbonate (DEP)	0
N-bromosuccinimide (NBS)	0
N-acetylimidazole (NAI)	85.0

3.2.1 Modification of carboxyl residues

Carboxyl residues of CGTase were modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as described in section 2.13.1.1. At 5 mM or higher concentrations of EDC, CGTase activity was completely lost (Figure 10). Thus, the suitable concentration of EDC was 5 mM. Figure 11 shows that after 5 minutes of incubation, dextrinizing activity of CGTase was decreased about 40% whereas total activity lost 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-, γ-CD or maltotriose 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 2.9.1. Table 9 shows that modification by 5 mM EDC led to 40% loss of CGTase activity. When CGTase preincubated with each substrate was modified by EDC (condition 3), the loss of CGTase activities were significantly reduced in the presence of β-CD, maltotriose, and α-CD. In the presence of γ-CD, about 12% of CGTase activity was lost.

To further determine if any carboxyl residue was present at the catalytic site of CGTase, modification was carried out in four different conditions as the previous experiment but after the reaction, dialysis was performed to remove excess reagent prior to the measurement of residual CGTase activity as described in section 2.9.1. The results were summarized in Table 10. The modification by EDC resulted in 54% loss of CGTase activity. When CGTase preincubated with each substrate was modified by EDC, the loss of CGTase activities were significantly reduced

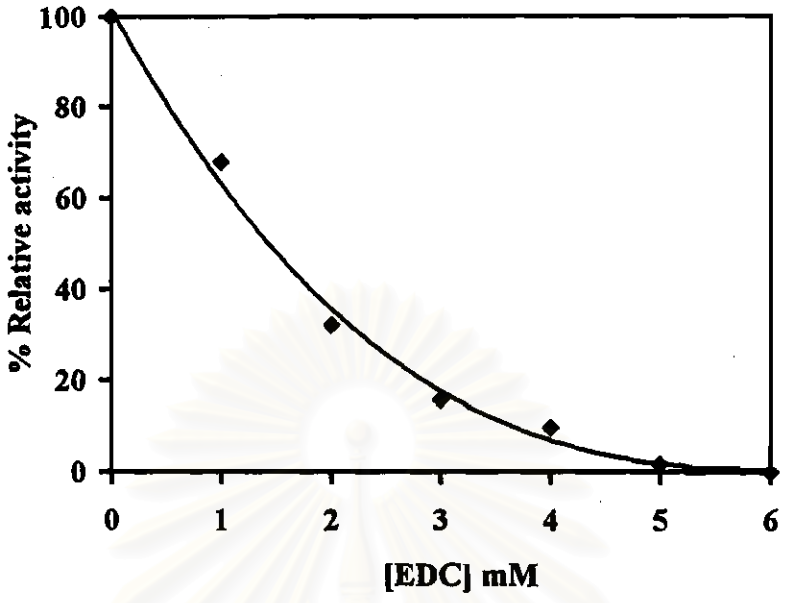


Figure10 Effect of EDC on CGTase activity

CGTase was incubated with varying concentrations of EDC at 40°C for 30 minutes according to the method as described in section 2.13.1.1. After the incubation, CGTase activity was determined as described in section 2.9.1

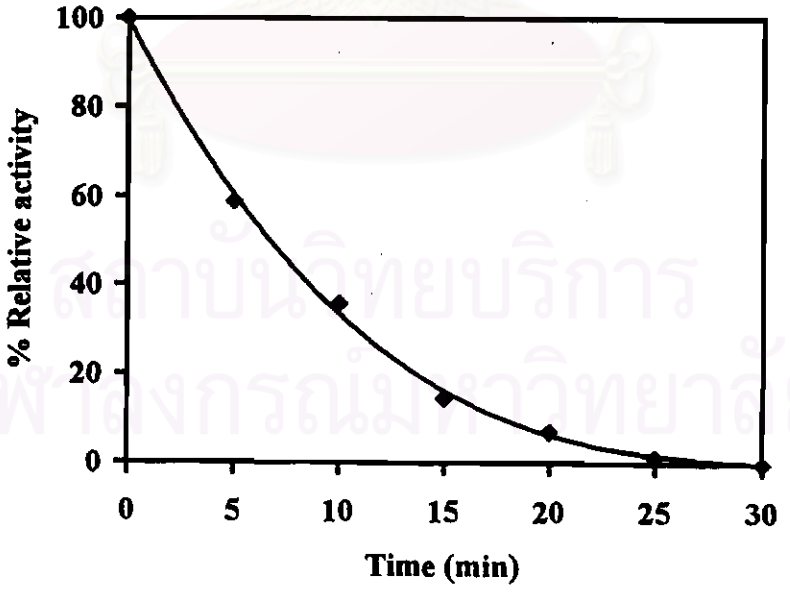


Figure 11 Inactivation of CGTase activity by 5 mM EDC

CGTase was incubated with 5 mM EDC at 40°C at various times according to the method as described in section 2.13.1. CGTase activity was determined as described in section 2.9.1

Table 9 Effect of substrate on the inactivation of dextrinizing activity of CGTase by EDC

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	100.0	100.0
20 mM β-CD	100.0	99.5	99.8
20 mM γ-CD	98.8	99.6	99.2
20 mM maltotriose	100.0	100.0	100.0
3) 20 mM α-CD + 5 mM EDC	92.0	96.0	94.0
20 mM β-CD + 5 mM EDC	97.1	99.6	98.4
20 mM γ-CD + 5 mM EDC	85.2	90.0	87.6
20 mM maltotriose + 5 mM EDC	99.0	93.2	96.1
4) 5 mM EDC	60.1	60.4	60.2

Table 10 Residual CGTase activity of EDC-modified enzyme in the presence and the absence of a protective substance

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	93.4	96.7
20 mM β-CD	98.3	94.4	96.4
20 mM γ-CD	96.9	100.0	98.4
20 mM maltotriose	98.7	100.0	99.4
3) 20 mM α-CD + 5 mM EDC	94.6	89.9	92.2
20 mM β-CD + 5 mM EDC	96.6	91.3	94.0
20 mM γ-CD + 5 mM EDC	85.9	81.2	83.6
20 mM maltotriose + 5 mM EDC	91.2	87.1	89.2
4) 5 mM EDC	44.8	47.4	46.1

after dialysis (condition 3). In the presence of β-CD and α-CD, CGTase activities were hardly lost (only 2-5%), while in the presence of maltotriose or γ-CD, higher loss were observed (10 and 15%, respectively). The loss of activity due to the modification of carboxyl residues was significantly reduced (39-52%) in the presence of CGTase substrate.

3.2.2 Modification of histidine residues

Histidine residues of CGTase were modified by diethylpyrocarbonate (DEP) as described in section 2.13.1.2. CGTase activity was found to decrease with increasing DEP concentrations (Figure 12). At 0.225 mM DEP, CGTase activity was completely lost. Thus, the suitable concentration of DEP was 0.225 mM. Figure 13 shows that after 5 minutes of incubation, dextrinizing activity was decreased 43% whereas total activity loss was observed at 30 minutes. The suitable incubation time of the enzyme with DEP was thus 5 minutes.

To determine if histidine residue was involved at the catalytic site of CGTase, modifications by DEP in the presence or the absence of substrate were compared. α-, β-, γ-CD or maltotriose 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 DEP, and 4. CGTase modified by DEP, were compared. After the reaction, CGTase activities were then determined as described in section 2.9.1. Table 11 shows that modification by 0.225 mM DEP led to 51% loss of CGTase activity. When CGTase preincubated with each substrate was modified by DEP (condition 3), the loss of CGTase activities were partially reduced. In the presence of α-, β-, γ-CD, and maltotriose, the activities loss were 38, 30, 29, and 34%, respectively.

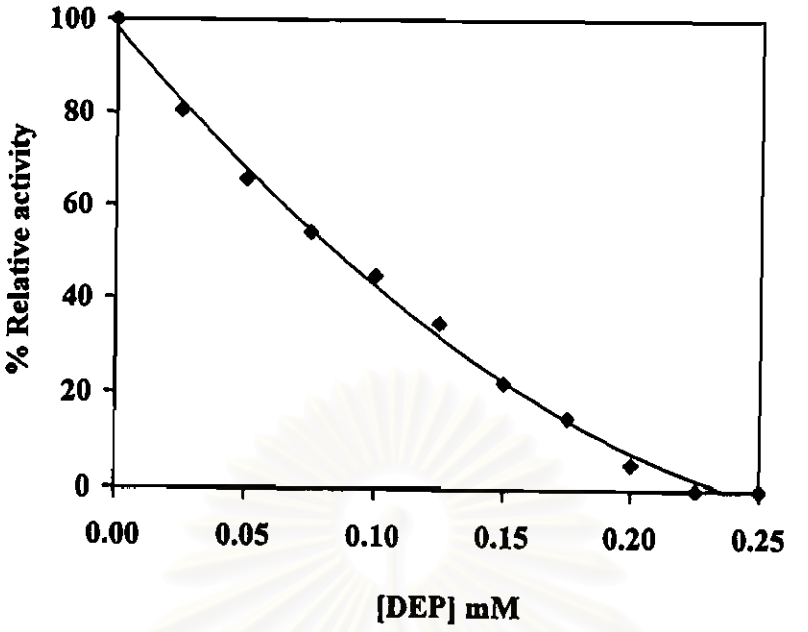


Figure 12 Effect of DEP on CGTase activity

CGTase was incubated with varying concentrations of DEP at 40°C for 30 minutes according to the method as described in section 2.13.1.2. After the incubation, CGTase activity was determined as described in section 2.9.1

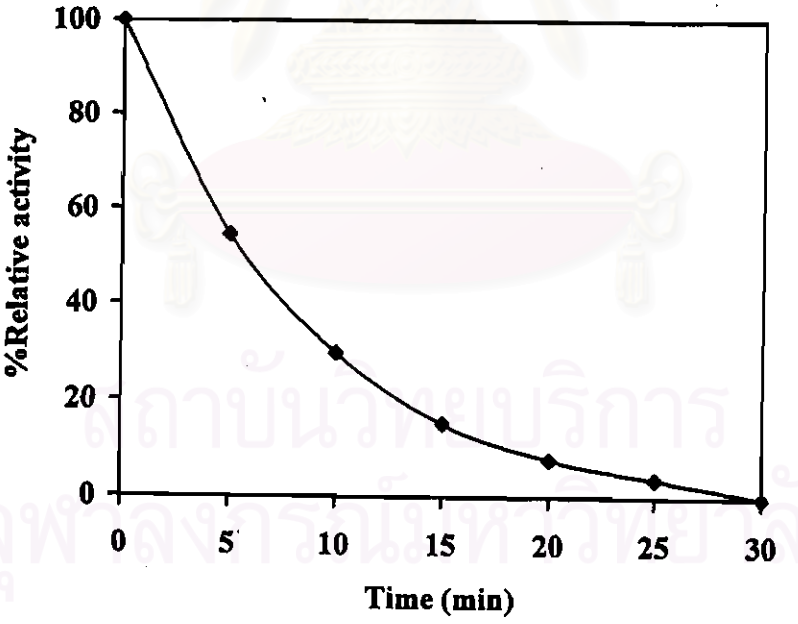


Figure 13 Inactivation of CGTase activity by 0.225 mM DEP

CGTase was incubated with 0.225 mM DEP at 40°C at various times according to the method as described in section 2.13.2. CGTase activity was determined as described in section 2.9.1

Table 11 Effect of substrate on the inactivation of dextrinizing activity of CGTase by DEP

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	100.0	100.0
20 mM β-CD	100.0	97.4	98.7
20 mM γ-CD	99.7	96.2	98.0
20 mM maltotriose	100.0	100.0	100.0
3) 20 mM α-CD + 0.225 mM DEP	60.8	63.7	62.2
20 mM β-CD + 0.225 mM DEP	68.0	68.3	68.2
20 mM γ-CD + 0.225 mM DEP	66.3	71.2	68.8
20 mM maltotriose + 0.225 mM DEP	68.1	64.8	66.4
4) 0.225 mM DEP	49.0	48.1	48.6

Table 12 Residual CGTase activity of DEP-modified enzyme in the presence and the absence of a protective substance

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	93.4	96.7
20 mM β-CD	98.3	94.4	96.4
20 mM γ-CD	96.9	100.0	98.4
20 mM maltotriose	98.7	100.0	99.4
3) 20 mM α-CD + 0.225 mM DEP	46.3	45.5	45.9
20 mM β-CD + 0.225 mM DEP	63.6	64.8	64.2
20 mM γ-CD + 0.225 mM DEP	67.5	64.5	66.0
20 mM maltotriose + 0.225 mM DEP	52.6	54.7	53.6
4) 0.225 mM DEP	34.0	37.4	35.7

To further determine if any histidine residue was present at the catalytic site of CGTase, modification was carried out in four different conditions as the previous experiment but after the reaction, dialysis was performed to remove excess reagent prior to the measurement of residual CGTase activity as described in section 2.9.1. The results were summarized in Table 12. The modification by DEP resulted in 64% loss of CGTase activity. When CGTase preincubated with each substrate was modified by DEP, the loss of CGTase activities were partially reduced after dialysis (condition 3). In the presence of β-CD or γ-CD, 32% of CGTase activities were lost, while in the presence of maltotriose or α-CD, higher loss were observed (46 and 51%, respectively). The loss of activity due to the modification of histidine residues was obviously reduced (13-32%) in the presence of substrate.

The specificity of the DEP-modified reaction for histidine residue was supported by correlation between the loss of enzyme activity and the increase in the absorbance at 246 nm, indicative of the formation of *N*-carbethoxyhistidine (Wakayama *et al.*, 1996). Absorption spectra of CGTase before and after modification with DEP were shown in Figure 14. The spectrum of CGTase shows the highest peak at about 280 nm (usual protein peak). When CGTase was modified with DEP, the absorption spectra at 246 nm after 30 minutes of modification were significantly increased.

The number of histidine residues which were modified by 0.225 mM DEP were determined from the increase in the absorbance at 246 nm, using an extinction coefficient of 3.20×10^3 $M^{-1}cm^{-1}$ as described in section 2.15.1. Table 13 shows that 7.8 histidine residues per mole CGTase were modified by 0.225 mM DEP when the enzyme was incubated with DEP only. The number decreased to 5.9 and 5.6 in the presence of β-CD and γ-CD, respectively, as protective substance. Hence, β-CD or γ-CD could protect per mole of enzyme, two histidine residues from modification by DEP.

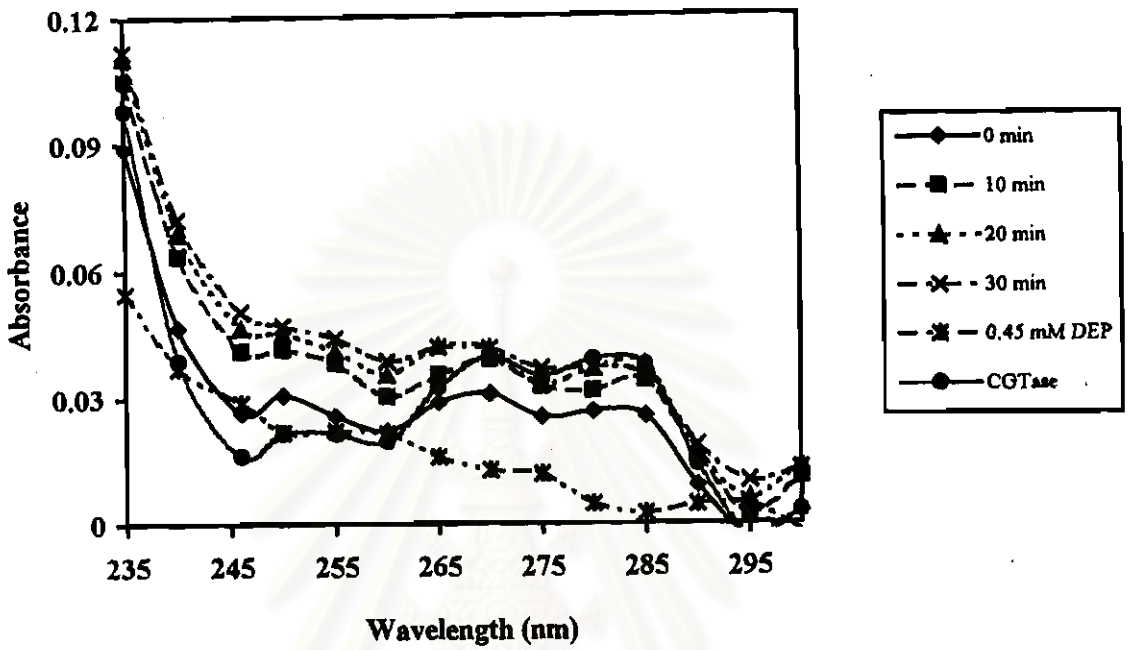


Figure 14 Absorption spectra of CGTase before and after modification with DEP
For control, CGTase or DEP were separately scanned. After 0-30 minutes of the modification, the modified CGTase from each incubation time was scanned at the same wavelength.

Table 13 Number of histidine residues of CGTase modified by DEP in the presence and the absence of a protective substance

Protective substance	n	Protected residues per mole of enzyme
None	7.8	0
β-CD	5.9	1.9
γ-CD	5.6	2.2

n = number of modified histidine residues per mole CGTase

3.2.3 Modification of tryptophan residues

Tryptophan residues of CGTase were modified by *N*-bromosuccinimide (NBS) as described in section 2.13.1.3. CGTase activity was found to decrease with increasing NBS concentrations. At 0.06 mM NBS, CGTase activity was completely lost (Figure 15). Thus, the suitable concentration of NBS was 0.06 mM. Figure 16 shows that 0.05 mM NBS was chosen to determine the suitable incubation time because if modified by 0.06 mM NBS, the enzyme activity was completely lost even at zero time. When 0.05 mM NBS was used, after 10 minutes of incubation, dextrinizing activity of CGTase was decreased about 50% and reaching a plateau at 20-25 minutes. The suitable incubation time of NBS was thus 10 minutes.

To determine if tryptophan residue was involved at the catalytic site of CGTase, modifications by NBS in the presence or the absence of substrate were compared. α-, β-, γ-CD or maltotriose 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 2.9.1. Table 14 shows that modification by 0.05 NBS led to 45% loss of CGTase activity. When CGTase preincubated with each substrate was modified by NBS (condition 3), the loss of CGTase activities were negligible in the presence of γ-CD and significantly reduced when protected by β-CD (6.5% loss of CGTase activity). Less protection (10-15% loss of CGTase activity) against inactivation was achieved by α-CD or maltotriose.

To further determine if any tryptophan residue was present at the catalytic site of CGTase, modification was carried out in four different conditions as the previous experiment but after the reaction, dialysis was performed to remove excess reagent prior to the measurement of residual CGTase activity as described in section 2.9.1. The results were summarized in Table 15. The modification by NBS resulted in 68%

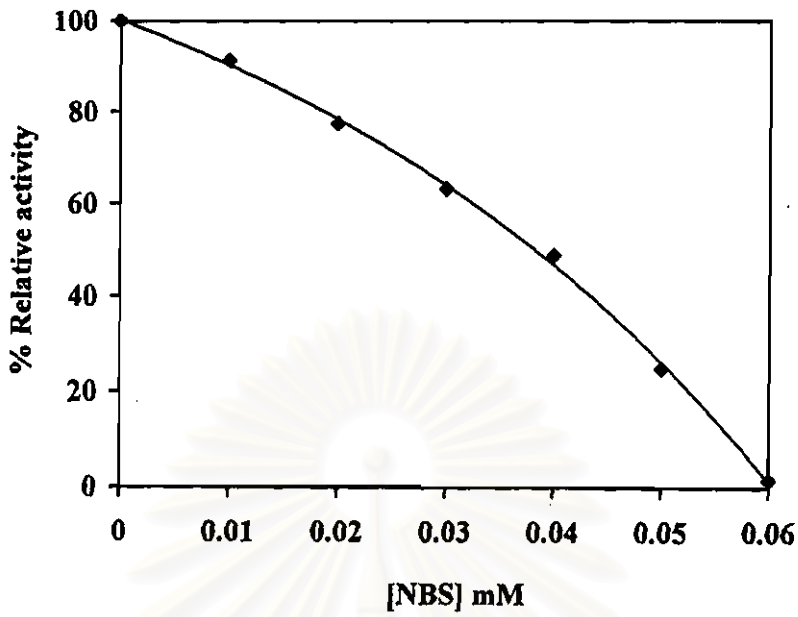


Figure 15 Effect of NBS on CGTase activity

CGTase was incubated with varying concentration of NBS at 40°C for 30 minutes according to the method as described in section 2.13.1.3. After the incubation, CGTase activity was determined as described in section 2.9.1.

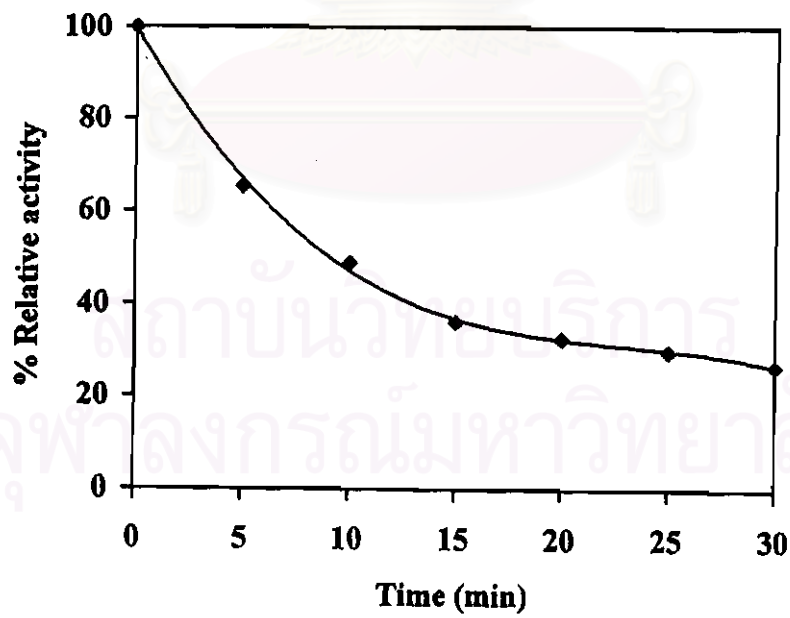


Figure 16 Inactivation of CGTase activity by 0.05 mM NBS

CGTase was incubated with 0.05 mM NBS at 40°C at various times according to the method as described in section 2.13.3. CGTase activity was determined as described in section 2.9.1.

Table 14 Effect of substrate on the inactivation of dextrinizing activity of CGTase by NBS

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	100.0	100.0
20 mM β-CD	95.8	100.0	97.9
20 mM γ-CD	95.6	99.4	97.5
20 mM maltotriose	100.0	100.0	100.0
3) 20 mM α-CD + 0.05 mM NBS	86.8	93.0	89.9
20 mM β-CD + 0.05 mM NBS	93.0	89.7	91.4
20 mM γ-CD + 0.05 mM NBS	98.4	96.3	97.4
20 mM maltotriose + 0.05 mM NBS	87.2	82.9	85.0
4) 0.05 mM NBS	51.6	57.7	54.6

Table 15 Residual CGTase activity of NBS-modified enzyme in the presence and the absence of a protective substance

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	93.4	96.7
20 mM β-CD	98.3	94.4	96.4
20 mM γ-CD	96.9	100.0	98.4
20 mM maltotriose	98.7	100.0	99.4
3) 20 mM α-CD + 0.05 mM NBS	61.0	58.3	59.4
20 mM β-CD + 0.05 mM NBS	76.3	79.2	77.7
20 mM γ-CD + 0.05 mM NBS	96.9	100.0	98.4
20 mM maltotriose + 0.05 mM NBS	62.4	62.7	62.6
4) 0.05 mM NBS	29.3	33.8	31.6

loss of CGTase activity. When CGTase preincubated with each substrate was modified by NBS, the loss of CGTase activities were reduced at different extent after dialysis (condition 3). CGTase was totally protected against inactivation by γ-CD but partially protected by β-CD (19% loss of CGTase activity). In the presence of maltotriose or α-CD, higher loss was observed (37%). The loss of activity due to the modification of tryptophane residues was significantly reduced (31-68%) in the presence of substrate.

The specificity of the NBS-modified reaction for tryptophan residue was supported by correlation between the loss of enzyme activity and the decrease in the absorbance at 280 nm, indicative of the formation of *N*-acetyltryptophan (Lundbland, 1991). Absorption spectra of CGTase before and after modification with NBS were shown in Figure 17. The spectrum of CGTase shows the highest peak at 270-280 nm. The 240-300 nm spectrum shows an important change in absorbance at 280 nm for the 30 minute-modified CGTase in comparison with that of the zero minute-modified enzyme.

The number of tryptophan residues which were modified by 0.05 mM NBS were determined from the decrease in the absorbance at 280 nm, using an extinction coefficient of 4.00×10^3 $M^{-1}cm^{-1}$ as described in section 2.15.2. Table 16 shows that 1.8 tryptophan residues per mole CGTase were modified by 0.05 mM NBS when the enzyme was incubated with NBS only. The number decreased to 0.5 and 0.4 in the presence of β-CD and γ-CD, respectively, as protective substance. Hence, β-CD or γ-CD could protect per mole of enzyme, one tryptophan residue from modification by NBS.

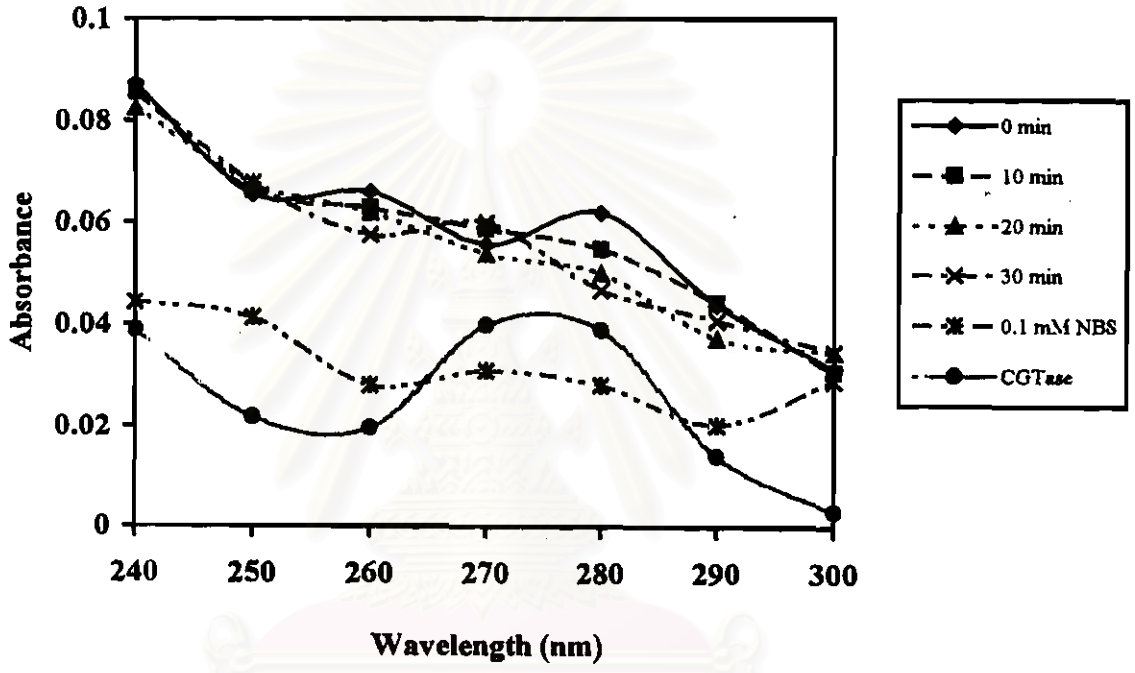


Figure 17 Absorption spectra of CGTase before and after modification with NBS For control, CGTase or NBS were separately scanned. After 0-30 minutes of the modification, the modified CGTase from each incubation time was scanned at the same wavelength.

Table 16 Number of tryptophan residues of CGTase modified by NBS in the presence and the absence of a protective substance

Protective substance	n	Protected residues per mole of enzyme
None	1.8	0
β-CD	0.5	1.3
γ-CD	0.4	1.4

n = number of modified tryptophan residues per mole CGTase

3.2.4 Modification of tyrosine residues

Tyrosine residues of CGTase were modified by *N*-acetylimidazole (NAI) as described in section 2.13.1.4. CGTase activity was found to decrease with increasing NAI concentrations. At 30 mM NAI, CGTase activity was completely lost (Figure 18). Thus, the suitable concentration of NAI was 30 mM. Figure 19 shows that after 5 minutes of incubation, dextrinizing activity of CGTase was decrease about 50% whereas almost total activity lost was observed at 30 minutes. The suitable incubation time of NAI was thus 5 minutes.

To determine if tyrosine residue was involved at the catalytic site of CGTase, modifications by NAI in the presence or the absence of substrate were compared. α-, β-, γ-CD or maltotriose 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 NAI, and 4. CGTase modified by NAI, were compared. After the reaction, CGTase activities were then determined as described in section 2.9.1. Table 17 shows that modification by 30 mM NAI led to 50% loss of CGTase activity. When CGTase preincubated with each substrate was modified by NAI (condition 3), the loss of CGTase activities were negligible in the presence of maltotriose, or β-CD (0.2-1% loss of CGTase activities, respectively). In the presence of α-CD or γ-CD, 14-20% of CGTase activities was lost.

To further determine if any tyrosine residue was present at the catalytic site of CGTase, modification was carried out in four different conditions as the previous experiment but after the reaction, dialysis was performed to remove excess reagent prior to the measurement of residual CGTase activity as described in section 2.9.1. The results were summarized in Table 18. The modification by NAI resulted in 65% loss of CGTase activity. When CGTase preincubated with each substrate was modified by NAI, the loss of CGTase activities were partially reduced after dialysis (condition 3). In the presence of α-CD, β-CD, γ-CD or maltotriose, 15-30% of

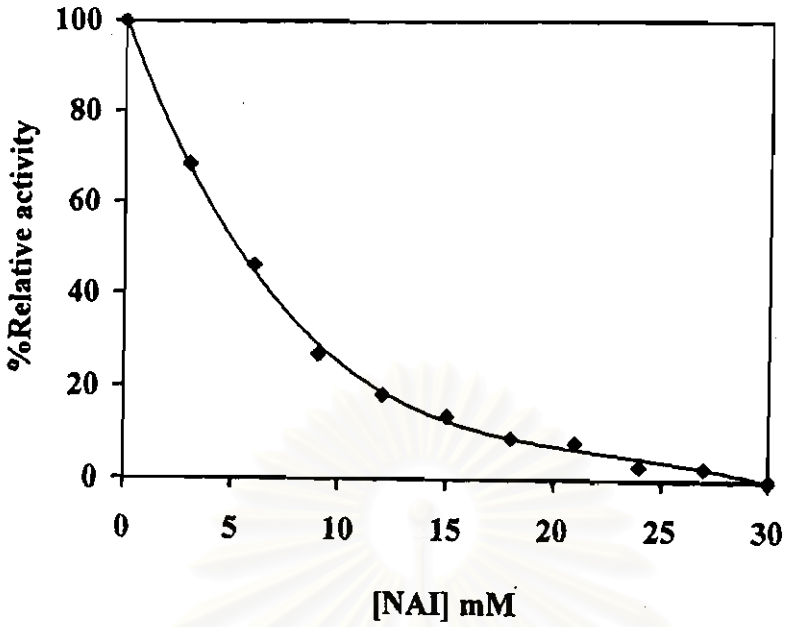


Figure 18 Effect of NAI on CGTase activity

CGTase was incubated with varying concentrations of NAI at 40°C for 30 minutes accordind to the method as described in section 2.13.1.4. After the reaction CGTase activity was determined as described in section 2.9.1.

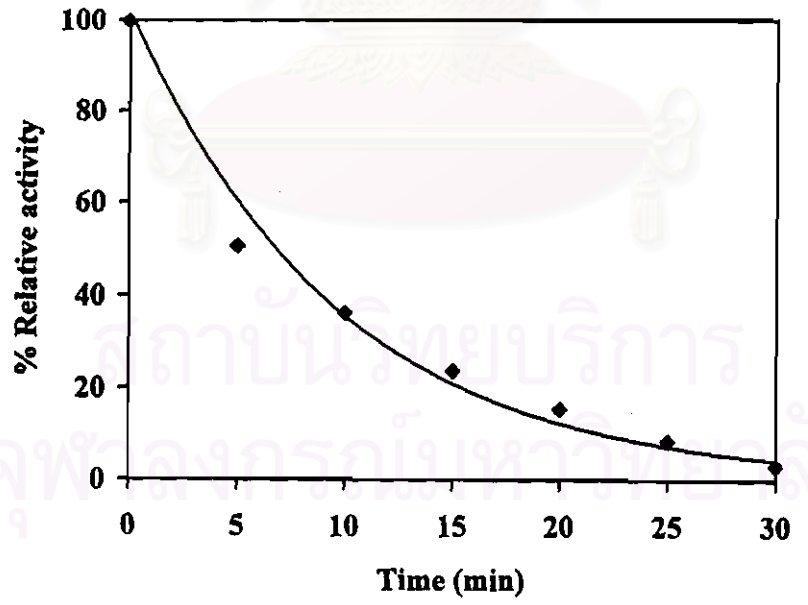


Figure 19 Inactivation of CGTase activity by 30 mM NAI

CGTase was incubated with 30 mM NAI at 40°C at various times according to the method as described in section 2.13.4. CGTase activity was determined as described in section 2.9.1.

Table 17 Effect of substrate on the inactivation of dextrinizing activity of CGTase by NAI

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	96.0	100.0	98.0
20 mM β-CD	100.0	98.5	99.2
20 mM γ-CD	96.7	100.0	98.4
20 mM maltotriose	100.0	100.0	100.0
3) 20 mM α-CD + 30 mM NAI	80.8	87.4	84.1
20 mM β-CD + 30 mM NAI	97.8	100.0	98.2
20 mM γ-CD + 30 mM NAI	80.2	76.4	78.3
20 mM maltotriose + 30 mM NAI	100.0	99.5	99.8
4) 30 mM NAI	48.9	51.9	50.4

Table 18 Residual CGTase activity of NAI-modified enzyme in the presence and the absence of a protective substance

Compound added	Relative activity (%)		
	I	II	Average
1) None	100.0	100.0	100.0
2) 20 mM α-CD	100.0	93.4	96.7
20 mM β-CD	98.3	94.4	96.4
20 mM γ-CD	96.9	100.0	98.4
20 mM maltotriose	98.7	100.0	99.4
3) 20 mM α-CD + 30 mM NAI	64.1	71.2	67.6
20 mM β-CD + 30 mM NAI	77.2	85.9	81.6
20 mM γ-CD + 30 mM NAI	62.4	69.9	66.2
20 mM maltotriose + 30 mM NAI	85.6	82.1	83.8
4) 30 mM NAI	32.8	36.4	34.6

CGTase activities was lost. The loss of activity due to the modification of tyrosine residues was significantly reduced (35-50%) in the presence of substrate.

The specificity of the NAI-modified reaction for tyrosine residue was supported by correlation between the loss of enzyme activity and the decrease in the absorption at 278 nm, indicative of the formation of *O*-acetyltyrosine (Means and Feeney, 1971). Absorption spectra of CGTase before and after modification with NAI were shown in Figure 20. After 60 minutes of modification by NAI, the absorption spectrum was decreased at 260-280 nm in comparison with that of the initially modified enzyme.

The number of tyrosine residues which were modified by 30 mM NAI were determined from the decrease in the absorbance at 278 nm, using an extinction coefficient of 1.16×10^3 $M^{-1}cm^{-1}$ as described in section 2.15.3. Table 19 shows that 4.9 tyrosine residues per mole CGTase were modified by 30 mM NAI when the enzyme was incubated with NAI only. The number decreased to 2.8 and 3.4 in the presence of β-CD and γ-CD respectively, as protective substance. Hence, β-CD or γ-CD could protect per mole of enzyme, two tyrosine residues from modification by NAI.

3.2.5 Modification of cysteine residues

Cysteine residues of CGTase were modified by *N*-ethylmaleimide (NEM), iodoacetamide (IAM), and dithiothreitol (DTT) as described in section 2.13.1.5. The result in Table 8 shows that these reagents at 1 mM concentration had no effect on CGTase activity. We then checked if higher concentrations would lead to inactivation. Up to 100 mM concentration was tried. The result in Table 20 shows that these modifying reagents at any concentrations did not affect dextrinizing activity of CGTase.

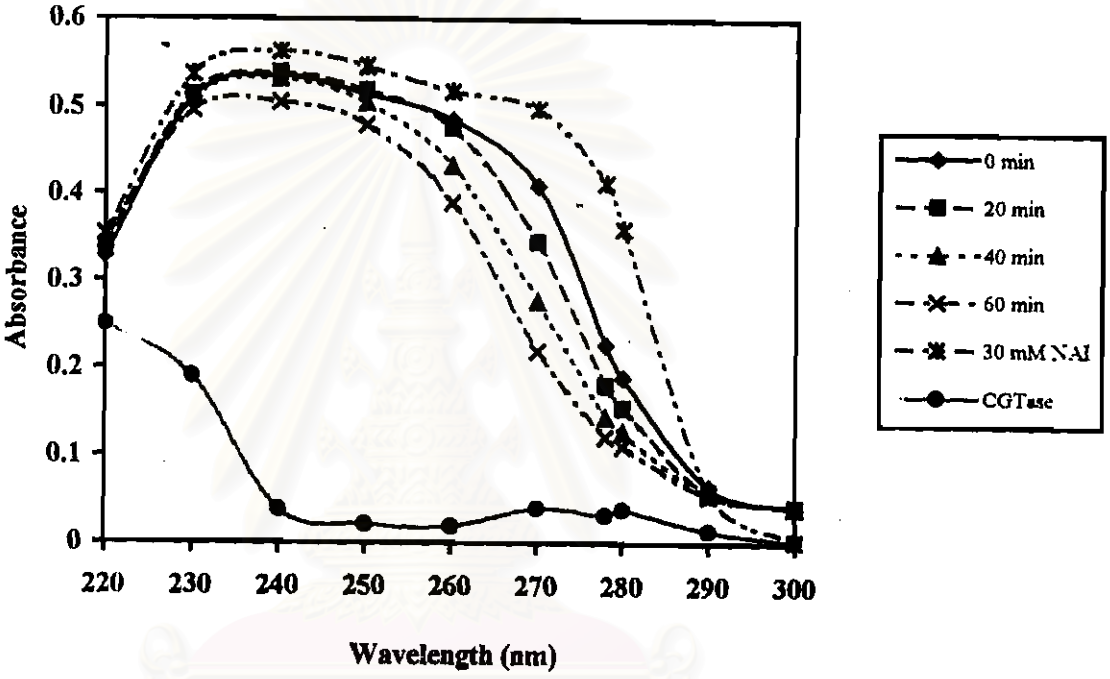


Figure 20 Absorption spectra of CGTase before and after modification with NAI
For control, CGTase or NAI were separately scanned. After 0-60 minutes of the modification, the modified CGTase from each incubation time was scanned at the same wavelength.

Table 19 Number of tyrosine residues of CGTase modified by NAI in the presence and the absence of a protective substance

Protective substance	n	Protected residues per mole of enzyme
None	4.9	0
β-CD	2.8	2.1
γ-CD	3.4	1.5

n = number of modified tyrosine residues per mole CGTase

3.2.6 Modification of lysine residues

Lysine residues of CGTase were modified by 2,4,6-trinitrobenzenesulfonic acid (TNBS) as described in section 2.13.1.6. The result in Table 8 shows that 1 mM TNBS had no effect on CGTase activity. We then checked if higher concentrations would lead to inactivation. Up to 100 mM concentration was tried. The result in Table 20 shows that this modifying reagent at any concentrations did not affect dextrinizing activity of CGTase.

3.2.7 Modification of serine residues

Serine residues of CGTase were modified by phenylmethylsulfonyl fluoride (PMSF) as described in section 2.13.1.7. The result in Table 8 shows that 1 mM PMSF had no effect on CGTase activity. We then checked if higher concentrations would lead to inactivation. Up to 100 mM concentration was tried. The result in Table 20 shows that this modifying reagent at any concentrations did not affect dextrinizing activity of CGTase.

3.3 Effect of pH on the modification of CGTase

To determine the effect of pH on the modification of CGTase, CGTase was incubated at indicated pH and CGTase activity was determined before and after modification of each modifying reagent as described in section 2.13.3. The results were summarized in Table 21. When CGTase incubated with acetate buffer pH 5.0,5.5, or 6.0 and modified with EDC, CGTase activity were lost by 65, 57, and 58%, respectively. Whereas other buffers led to less modification than acetate buffer (activity loss was lower than 39%). The result shows that EDC modification works effectively in the pH range of 5.0-6.0.

The modification of CGTase by DEP at various pH resulted in similar loss of CGTase activity when used acetate buffer pH 5.0-6.0 or phosphate buffer pH 6.0-7.5

Table 20 Effect of various concentrations of chemical modifying agents on CGTase activity

Modifying reagent	% Relative activity			
	0.1 mM	1.0 mM	10 mM	100 mM
None	100.0	100.0	100.0	100.0
NEM	96.2	96.1	98.8	93.8
IAM	95.7	99.3	100.0	94.7
DTT	91.5	92.6	91.6	91.5
TNBS	100.0	100.0	95.7	n.d.
PMSF	99.8	100.0	100.0	99.5

n.d. = not determined

Table 21 Effect of pH on the modification of CGTase

Buffer, pH	% Relative residual activity			
	EDC	DEP	NBS	NAI
Acetate 5.0	34.9	42.5	21.6	42.2
Acetate 5.5	43.2	44.4	24.1	41.1
Acetate 6.0	42.2	42.1	41.8	41.2
Phosphate 6.0	60.9	49.4	42.3	40.4
Phosphate 6.5	72.8	46.3	42.3	43.5
Phosphate 7.0	71.0	48.0	51.8	40.6
Phosphate 7.5	77.0	48.8	67.0	47.4
Tris 7.5	78.6	59.9	80.1	50.1
Tris 8.0	77.0	65.5	80.0	55.8
Tris 8.5	77.6	63.5	80.3	59.0
Tris 9.0	85.7	66.7	83.6	58.1

* Each value was relative to the activity when only CGTase was incubated, in the absence of chemical modifiers

(activity loss was 52-58%) while less loss was observed when tris buffer pH 7.5-9.0 was used (activity loss was lower than 40%). DEP modification thus works effectively in the pH range of 5.0-7.5.

For NBS, CGTase activity was lost 76-78% when acetate buffers pH 5.0-5.5 was used. For acetate buffer pH 6.0 and phosphate buffer pH 6.0-6.5, CGTase activity loss was less (only 58% loss). Less loss was observed when pH was 7.0 or above. The result indicated that NBS modification works best at pH 5.0-5.5.

The modification of CGTase by NAI resulted in 60% loss of CGTase activity when acetate buffer pH 5.0-6.0 or phosphate buffer pH 6.0-7.0 was used. Less loss (activity loss lower than 53%) was observed at pH 7.5 or above. NAI modification thus works effectively in the pH range of 5.0-7.0.

3.4 Effect of chemical modifications on the structure and enzymatic properties of CGTase

To study the effect of chemical modification on the structure and enzymatic properties of CGTase, the enzyme after each modification reaction was investigated by the non-denaturing polyacrylamide gel electrophoresis in Tris-glycine pH 8.3 as described in section 2.15. Duplicates of the non-denaturing PAGE were separately stained for protein and dextrinizing activity (Figure 21). The enzyme prior to modification showed two major protein bands with one faint band (labeled 'a', 'b', and 'c', respectively) as indicated in lane 1 and 6 of the gel. For activity staining, the unmodified enzyme showed three major bands (bands 'a', 'b', and 'c') which corresponded to their cognate protein bands and one faint band (labeled 'd') which did not show up in protein staining. The modification of carboxyl groups of CGTase by 5 mM EDC (lane2) resulted in the modified enzyme, which moved slower than the unmodified enzyme in both protein and activity stains. An extra band above label 'a' (lane 2A and 2B) was observed. When modification of histidine with 0.225 mM DEP

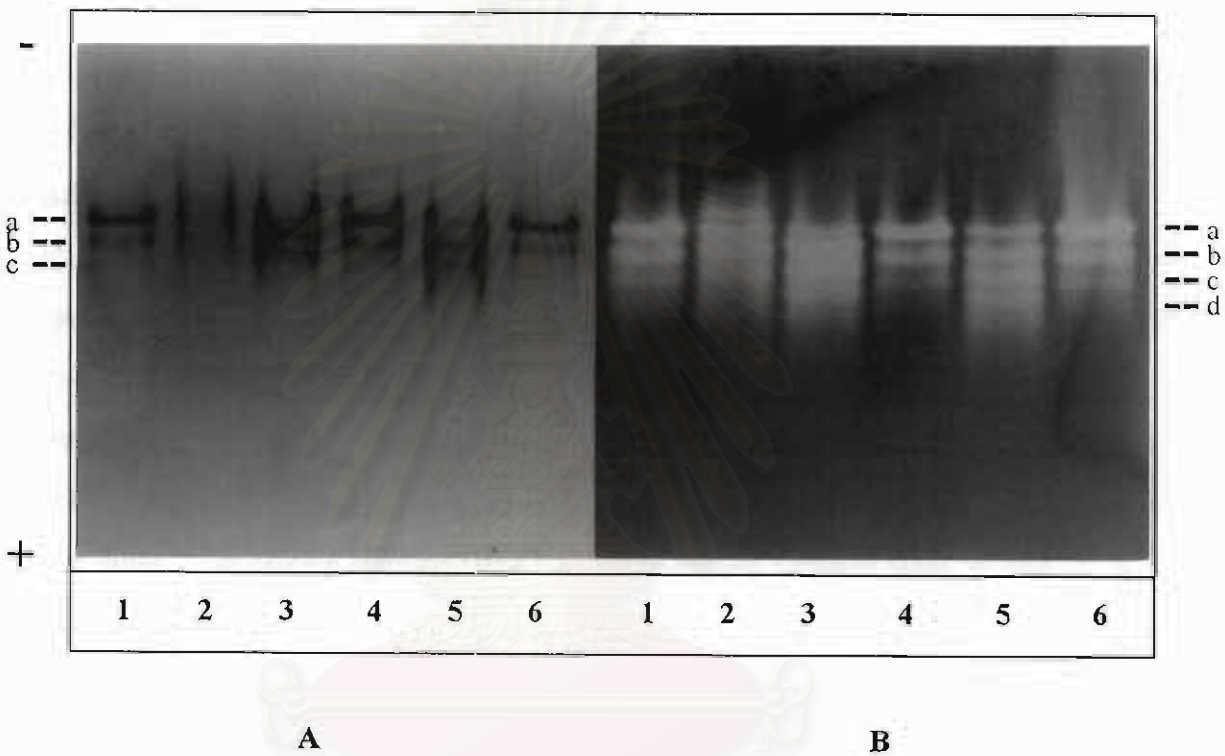


Figure 21 Non-denaturing PAGE of CGTase modified by different modifying reagents

A : Coomassie blue staining

Lane 1, 6 : Control (purified CGTase, without modification (5 μg))

2 : Purified CGTase, modified by 5 mM EDC (5 μg)

3 : Purified CGTase, modified by 0.225 mM DEP (5 μg)

4 : Purified CGTase, modified by 0.05 mM NBS (5 μg)

5 : Purified CGTase, modified by 30 mM NAI (5 μg)

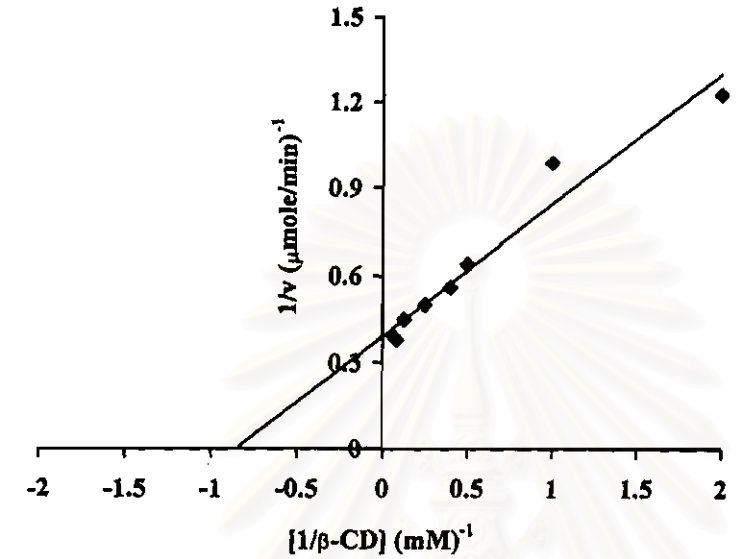
B : Amylolytic activity staining by iodine solution

[Lane 1-6, as in A, 0.2 units of dextrinizing activity was loaded to each well]

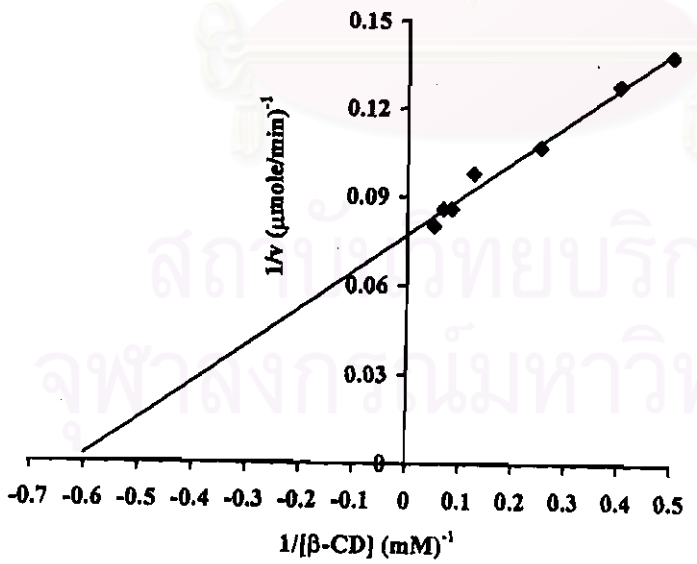
was analyzed, the result showed that the modified enzyme maintained the native enzyme and activity pattern (intensity of bands and distance between bands) but moved faster than the unmodified enzyme (lane 3). Band 'a' of the unmodified enzyme maintained 'a' position while bands 'b' and 'c' did not clearly show up in protein staining. Bands 'a' and 'b' of the modified enzyme moved between label 'a' and 'b', and between label 'c' and 'd', respectively and band 'c' moved under label 'c' for protein staining (lane 3A). For dextrinizing activity staining (lane 3B), the result was similar to protein staining (lane 3A), with band 'c' moved between label 'c' and 'd', while band 'd' moved under label 'd' position. The same result was observed in modification of tyrosine with 30 mM NAI (lane 5). Band 'a' of the unmodified enzyme maintained 'a' position, but the modified portion moved between label 'a' and 'b' (nearer to label 'b' than that of the modification of histidine by DEP). For band 'b', it moved between label 'b' and 'c' (near label 'c'), and band 'c' moved under label 'c' in protein staining (lane 5A) while moved between label 'c' and 'd' in dextrinizing activity staining (lane 5B). Band 'd' moved under label 'd' (lane 5B). When tryptophan modification of CGTase was performed with 0.05 mM NBS, neither the pattern of protein bands nor activity bands were changed (lane 4A and 4B).

3.5 Kinetic parameters of CGTase for cyclodextrin substrates

Kinetic parameters for some substrates: α-, β-, and γ-cyclodextrin, derivatives of β-cyclodextrin or maltotriose were determined with the purified enzyme as described in section 2.12.1 and 2.12.2. Figure 22A and 22B show the Lineweaver-Burk plot from CD coupling activity and cyclodextrin degrading activity assays, respectively. These were typical plots for all substrates and the summarized result was shown in Table 22. For CD coupling activity, K_m and Vmax were obtained by using three different substrates, β-CD, maltosyl (G_2)-β-CD, and methyl-β-CD. Values obtained were 1.55, 1.60, and 1.94 mM, respectively, for K_m and 2.81, 2.50, and 1.40 μmoles/min, respectively for V_{max}. When cyclodextrin-degrading activity was



A)



B)

Figure 22 Lineweaver-Burk plot of CGTase with β-cyclodextrin as substrate
CGTase was incubated with 5 mM G_3 and various concentration of β-CD in 50 mM acetate buffer, pH 6.0 at 40°C for 5 minutes.

A) β-CD disappear was measured by phenolphthalein method as described in section 2.12.1 and 2.9.3

B) 0.2 unit of *Aspergillus niger* glucoamylase was added to convert linearized oligosaccharide to glucose. The amount of β-CD degraded was monitored by the dinitrosalicylic acid method as described in section 2.12.2 and 2.11.

Table 22 Kinetic parameters of CGTase for cyclodextrin substrates

Cyclodextrin substrate	Coupling activity		Cyclodextrin degrading activity	
	K_m (mM)	V_{max} (μmole/min)$^{+}$	K_m (mM)	V_{max} (μmole/min)$^{++}$
G_2-β-CD*	1.60 ± 0.36	2.50 ± 0.18	-	-
Methyl-β-CD*	1.94 ± 0.23	1.40 ± 0.06	-	-
β-CD*	1.55 ± 0.20	2.81 ± 0.10	1.69 ± 0.18	13.12 ± 0.31
α-CD*	-	-	3.16 ± 0.24	58.96 ± 4.42
γ-CD*	-	-	1.42 ± 0.43	8.61 ± 0.96
Maltotriose**	-	-	91.63 ±25.40	10.69 ± 1.90

- = not detect

* = vary donor cyclodextrin, fix acceptor maltotriose

** = vary acceptor maltotriose , fix donor cyclodextrin

\+ = μmoles of β-CD or derivatives coupled to G_3 per minute

++ = μmoles of glucose formed per minute

followed, K_m value for β-CD was 1.69 mM which was closed to 1.55 mM obtained by coupling activity assay. γ-CD demonstrated the lowest K_m (similar to β-CD) while maltotriose binds poorly to the enzyme. The enzyme-catalyzing rate was best when α-CD was the substrate.