CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model J-21C, Beckman Instrument Inc, USA

Diaflo ultrafiltration: Stirrer Ultrafiltration Cell 8050, Amicon W.R. Grace Cooperation, USA

Electrophoresis unit: model Mini-protein II Cell, Bio-Rad, USA

Freeze-dryer: Stone Ridge, New York, USA

Fraction collector: model 2211 Pharmacia LKB, Sweden

Incubator: Haraeus, Germany

Incubator Shaker, Controlled environment: Phyco-Therm, New Brunswick Scientific Co., USA

Peristaltic pump: Pharmacia LKB, Sweden

pH meter : PHM 83 Autocal pH meter, Radiometer, Denmark

Plastic petri dish (60x15 mm): Costar, USA

Spectrophotometer UV-240, Shimadzu, Japan, and Du series 650, Beckman, USA

Vortex: Model K-550-GE, Scientific Industries, USA

Water bath: Charles Hearson Co., Ltd., England

Water bath, shaking: Heto lab Equipment, Denmark

2.2 Chemicals

N-acetylimidazole: Sigma, USA

Acrylamide: Merck, USA

Amyloglucosidase (Glucoamylase): from *Aspergillus niger* 70.7 u/mg, Fluka, Switzerland

N-bromosuccinimide: Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

Cyanogen bromide activated Sepharose 4B: Sigma, USA

α-, β-, and, γ-cyclodextrin : Sigma, USA

DEAE-cellulose resin: DE 32, Whatman Biosystems Ltd., England

Diethylpyrocarbonate: Sigma, USA

DL-dithiothreitol: Sigma, USA

Ethanolamine: BDH, England

1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide: Sigma, USA

N-Ethylmaleimide: Sigma, USA

Glycine: Sigma, USA

Iodoacetamide: Sigma, USA

D (+)-maltose monohydrate: Fluka, Switzerland

Maltotriose, maltotetraose: Sigma, USA

N,N′-methylene-bis-acrylamide : Sigma, USA

Noble agar: BBL, Becton, Dickinson and Company, USA

Phenolphthalein: BDH, England

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Standard molecular weight marker protein: Sigma, USA

Soluble starch, potato: Sigma, USA

Trichloroethylene: BDH, England

2,4,6-Trinitrobenzenesulfonic acid: Sigma, USA

The other common chemicals were of reagent grade. Raw rice starch (three-head elephant brand), corn starch (Maizena) were locally purchased.

2.3 Bacteria

Bacillus sp. A11, isolated from South-East Asian soil, was screened for CGTase activity by Pongsawasdi and Yagisawa (1987).

2.4 Preparation of anti-CGTase antibodies

Antibodies directed against purified CGTase from *Bacillus* sp. A11 was raised in rabbits by Rojtinnakorn (1994). The IgG fraction was isolated from the crude serum by ammonium sulfate precipitation and ion exchange chromatography on DEAE cellulose (Kim, 1996).

2.5 Media preparation

2.5.1 Medium I

Medium I was consisted of beef extract (0.5%), peptone (1.0%), NaCl (0.2%), yeast extract (0.2%) and soluble starch (1.0%) and then adjusted to pH 7.2 with 1 M NaOH. For solid medium, 1.5% agar was added.

2.5.2. Cultivation medium (Horikoshi's medium)

Medium for enzyme production, slightly modified from Horikoshi (1971) (Rutchtorn, 1993), contained local grade of rice starch (1.0%), peptone (0.5%), yeast extract (0.5%), K_2HPO_4 (0.1%), $MgSO_4.7H_2O$ (0.02%) and Na_2CO_3 (0.75%). The pH of the medium was 10.1-10.2.

2.6 Cultivation of bacteria

2.6.1 Starter inoculum

Bacillus sp. A11, after 18 hours inoculation at 37°C on solid medium I, was grown into 50 ml of starter medium I in 250 ml Erlenmeyer flask at 37°C until A_{420} reached 0.3-0.5 or about 3-5 hours.

2.6.2 Enzyme production

Starter *Bacillus* sp. A11 was transferred into 500 ml Horikoshi's medium in 1 l Erlenmeyer flask with 1% inoculum and cultivated at 37°C. Culture was harvested at 72 hours and cells were removed by centrifugation at 3,000 rpm for 30 minutes at 4°C. Culture broth with crude CGTase was collected and kept at 4°C for purification.

2.7 Purification of CGTase

CGTase was purified from the culture broth of *Bacillus* sp. A11 by techniques previously reported (Kim, 1996).

2.7.1 Starch adsorption

Corn starch was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude CGTase broth to make 5g% concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 3,000 rpm for 30 minutes and washed twice with 10 mM tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake with TB1 buffer containing 0.2 M maltose (2x125 ml for starting broth of 1 l), by stirring for 30 minutes. CGTase eluted was recovered by centrifugation at 3,000 rpm for 30 minutes. The solution was dialyzed against 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$, each time for at least 4 hours, at 4°C with 3 changes of buffer. The enzyme solution was concentrated by passing through an ultrafiltration membrane filter with molecular weight cut-off 10,000 daltons.

2.7.2 Immunoaffinity chromatography

Coupling of CNBr-activated Sepharose 4B was followed by those methods described (Pharmacia, 1979). CNBr-activated Sepharose 4B (1.43 g) was swollen by immersing in 50 ml of 1mM HCl for 15 minutes and washed on a sintered glass filter with 150 ml of the same solution. The gel was then washed with 25 ml of coupling

buffer (0.1 M $NaHCO_3$ buffer pH 8.3 containing 0.5 M NaCl) and immediately transferred to a solution of the ligand (10 ml of purified anti-CGTase). The mixture containing ligand and swollen gel was rotated end-over-end overnight at 4°C. The gel was transferred to 25 ml of 1 M ethanolamine pH 8.0 (blocking agent), rotated end-over-end for 2 hours at room temperature and removed the excess uncoupled ligand that remains after coupling by washing alternately with 25 ml of 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl and the same volume of 0.1 M $NaHCO_3$ buffer pH 8.3 containing 0.5 M NaCl three times. The gel was packed into a column of 0.8 x 4.5 cm and equilibrated with 50 mM acetate buffer pH 6.0 containing 0.5 mM NaCl at the flow rate of 6 ml/ hour.

The concentrated enzyme solution was applied on an affinity column chromatography at 4°C. The column was washed with 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl at 2 ml/ hour flow rate until A_{280} was negligible. Elution was performed by 3.5 M NaSCN in 50 mM NH_4OH pH 10.5 at room temperature and the flow rate of 6 ml/hour. Fractions of 2 ml were collected for measurement of A_{280} and dextrinizing activity. After collecting the active fractions, the enzyme solution was concentrated and dialyzed against 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$, each time for at least 4 hours, at 4°C with 3 changes of buffer.

2.8 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gels, were employed for analysis of the purified protein.

2.8.1 Non-denaturing polyacrylamide gel electrophoresis

Discontinuous PAGE was performed according to Rojtinnakorn (1994) on slab gels (10 x 8 x 0.75 cm), of 7.5% (w/v) separating gel, and 5:0% (w/v) stacking gels. Tris-glycine buffer pH 8.3 was used as electrode buffer (see Appendix 1). The

electrophoresis was run from cathode towards anode at constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit.

2.8.2 SDS-PAGE

The denaturing gel was performed according to Bollag and Edelstein (1991). The gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels and Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as electrode buffer (see Appendix 1). Samples to be analyzed were treated with sample buffer and boiled for 5 minutes prior to application to the gel. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

2.8.3 Detection of proteins

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining. For non-denaturing gel, dextrinizing activity staining was also undertaken.

2.8.3.1 Coomassie blue staining

Gels were stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for at least 2 hours. The slab gels were destained with a solution of 10% methanol and 10% acetic acid for 1-2 hours, followed by several changes of destaining solution until gel background was clear.

2.8.3.2 Dextrinizing activity staining

The running gel was soaked in 10 ml of substrate solution, containing 2.0% (w/v) potato starch in 0.2 M phosphate buffer pH 6.0, at 40°C for 10 minutes. The gel was then quickly rinsed several times with distilled water and 10 ml of I_2 staining reagent (0.2% I_2 in 2% KI) was added for color development at room

temperature. The clear zone on the blue background represents starch degrading activity of the protein.

2.9 Enzyme assay

For this study, CGTase activity was determined by assay of starch degrading (dextrinizing) activity, assay of CD product through the formation of CD-trichloroethylene complex (CD-TCE) and β-cyclodextrin-forming activity assay.

2.9.1 Dextrinizing activity assay

Dextrinizing activity of CGTase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (10-100 µl) was incubated with 0.3 ml starch substrate (0.2 g% soluble starch (potato) in 0.2 M phosphate buffer pH 6.0) at 40°C for 10 minutes. The reaction was stopped with 4 ml of 0.2 M HCl. Then 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, HCl was added before the enzyme sample.

One unit of enzyme was defined as the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described conditions.

2.9.2 Cyclodextrin-trichloroethylene (CD-TCE) assay

Cyclodextrin-trichloroethylene (CD-TCE) activity was determined by the method of Nomoto, *et al* (1986) with slight modification (Rojtinnakorn, 1994).

The enzyme sample was diluted in serial double dilution by 0.2 M phosphate buffer, pH 6.0. The reaction mixture, containing 0.5 ml of enzyme sample and 2.5 ml of starch substrate (2.0% w/v soluble starch (potato) in 0.2 M phosphate buffer, pH

6.0) was incubated at 40°C for 24 hours. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in the dark. The activity was expressed in term of the dilution limit ($1:2^n$), as the highest dilution that can produce observable CD-TCE precipitate between upper starch solution layer and lower TCE layer.

2.9.3 Cyclodextrin-forming activity assay

Cyclodextrin-forming activity was determined by the phenolphthalein method of Vikmon (1981) with slight modification. β-Cyclodextrin standard (0-2.5 mM) was prepared. Then 100 µl of standard or sample (β-cyclodextrin left after incubation of CGTase with β-cyclodextrin and maltotriose) was incubated with 2 ml of 75 µM phenolphthalein in 6 mM Na_2CO_3 and 2.9 ml of 6 mM Na_2CO_3 at 40°C for 20 minutes. The decrease in absorbance at 550 nm caused by complexing of the dye with β-cyclodextrin was calibrated.

2.10 Protein determination

Protein concentration was determined by the Coomassie blue micro method according to Bradford (1976), using bovine serum albumin as standard.

One hundred microlitres of sample was mixed with 1 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of 95% ethanol, 100 ml of 85% H_3PO_4 and distilled water.

2.11 Reducing sugar determination

Reducing sugar was determined by the method of Miller (1959). Glucose standard (0-50 mM) was prepared. Then 0.5 ml of standard glucose or sample was mixed with 0.5 ml of dinitrosalicylic acid reagent (prepared as described below). The solution was heated for 5 minutes in a boiling water bath, then the tubes were cooled

in a pan of cold water for 5 minutes. The mixture was adjusted to a final volume of 5 ml with distilled water. After mixing, the absorbance at 540 nm was recorded. The quantity of reducing sugar in sample was determined from standard curve of glucose.

Dinitrosalicylic acid reagent was consisted of dinitrosalicylic acid (5 g), 2 N NaOH (100 ml), potassium sodium tartrate (150 g) and adjusted volume to 500 ml with distilled water.

2.12 Kinetic parameters of the cyclodextrin-degrading activity

Cyclodextrin-degrading activity assay was determined by two methods.

2.12.1 Coupling activity assay (modified from Penninga *et al.* 1995)

Coupling activity was assayed with variable concentrations of β-cyclodextrin or derivatives of β-cyclodextrin plus 5 mM linear maltotriose (G_3) as substrate. Incubation with 10 μl of 0.3 mg/ml of purified CGTase at 40°C, for 5 minutes was performed. 50 mM acetate buffer pH 6.0 was added to make the total volume of reaction mixture 0.25 ml. β-Cyclodextrin disappearance was measured with phenolphthalein as described in section 2.9.3. One unit of activity was defined as the amount of enzyme coupling 1 μmole of β-cyclodextrin with G_3 per minute.

2.12.2 Cyclodextrin-degrading activity assay (Sin *et al.* 1994)

Kinetic parameters of the CD-degrading activity were determined at 40 °C using 10 μl of 0.3 mg/ml of the purified CGTase with 0.5-15 mM of α-, β-, or γ-cyclodextrin as donor and 5 mM maltotriose as acceptor. 50 mM acetate buffer pH 6.0 was added to make the total volume of reaction mixture 0.25 ml. 0.2 U of *Aspergillus niger* glucoamylase (10 μl) was then added to convert linearized oligosaccharides to glucose. Measuring the released reducing sugars monitored the amount of cyclodextrins degraded by the dinitrosalicylic acid method as described in section 2.11

K_m and V_{max} were determined from the Michaelis-Menten equation using nonlinear least square regression analysis of the EZ-FIT V1.1 Computer program

2.13 Determination of suitable conditions of reagents used in the modification of CGTase

2.13.1 Effect of modifying reagents concentration

2.13.1.1 Modification of carboxyl residues

Modification of carboxyl residues was carried out according to the method of Means and Feeney (1971). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0-6 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.1.2 Modification of histidine residues

Modification of histidine residues was carried out according to the method of Miles (1977). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of diethylpyrocarbonate (DEP) (0-0.25 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.1.3 Modification of tryptophan residues

Modification of tryptophan residues was carried out according to the method of Lundblad (1991). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of *N*-bromosuccinimide (NBS) (0-0.06 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.1.4 Modification of tyrosine residues

Modification of tyrosine residues was carried out according to the method of Means and Feeney (1971). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of *N*-acetylimidazole (0-30 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.1.5 Modification of cysteine residues

Modification of cysteine residues was carried out according to the method of Means and Feeney (1971). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of *N*-ethylmaleimide, iodoacetamide or dithiothreitol (0-100 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.1.6 Modification of lysine residues

Modification of lysine residues was carried out according to the method of Means and Feeney (1971). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (0-100 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.1.7 Modification of serine residues

Modification of serine residues was carried out according to the method of Wakayama *et al.* (1996). CGTase (40 μg/ml, 30 μl) was incubated with varying concentrations of phenylmethylsulfonyl fluoride (PMSF) (0-100 mM) at 40°C for 30 minutes. The total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.9.1.

2.13.2 Effect of incubation time on modified CGTase activity

After the optimum concentration of each modifying reagent was chosen, the treated time was varied from 0-30 minutes and CGTase activity was determined as described in section 2.9.1

2.13.3 Effect of pH on the modification of CGTase

To measure the effect of pH on the CGTase activity before and after modification by each modifying reagent, the enzyme was incubated at 40°C for 30 minutes at the indicated pH, then modifying reagent was added. The residual CGTase activity was determined as described in section 2.9.1.

2.14 Identification of amino acid residues involved in and present at the catalytic site of CGTase

The experiment was performed using suitable concentration and incubation time of each modifying reagent in the presence or absence of α-CD, β-CD, γ-CD, or maltotriose as protective substance. The CGTase (80 μg/ ml, 15 μl) was preincubated with each substrate at 40°C for 5 minutes prior to the addition of suitable concentration of each modifying reagent. The 20 mM substrate concentration has been determined to be suitable (data not shown). The total volume of reaction mixture was 60 μl. Then the residual CGTase activity was determined as described in section 2.9.1 or the reaction was dialyzed to remove excess reagent before determining the residual CGTase activity.

2.15 Determination of the number of essential residues

2.15.1 Determination of the number of histidine residues

To measure the number of histidine residues of CGTase modified by DEP in the presence and the absence of a protection substance (β-, or γ-CD), the differential increase in the absorbance at 246 nm between the two conditions was followed

(Wakayama *et al.*, 1996). The reaction was performed in a 1-cm-pathlength quartz cuvette, consisting of 0.225 mM DEP and 0.15 g/l CGTase in the presence or the absence of 20 mM β-CD or 20 mM γ-CD. The cuvette was then put into sample chamber of a spectrophotometer and the temperature inside was set at 40°C. The absorbance reading was performed at 5 min

The number (n) of modified-histidine residues was calculated, using the equation:

$$n = \frac{A_{246\ nm} \times Mr}{\varepsilon_{246} \times c}$$

where Mr is molecular weight of CGTase which is 72,000, ε_{246} of *N*-carbethoxy-histidine is $3.20 \times 10^3\ M^{-1}cm^{-1}$ and c is the CGTase concentration (g/l).

2.15.2 Determination of the number of tryptophan residues

To measure the number of tryptophane residues of CGTase modified by NBS in the presence and the absence of a protection substance (β-, or γ-CD), the differential decrease in the absorbance at 280 nm between the two conditions was followed (Lundbland, 1991). The reaction was performed in a 1-cm-pathlength quartz cuvette, consisting of 0.05 mM NBS and 0.15 g/l CGTase in the presence or the absence of 20 mM β-CD or 20 mM γ-CD. The cuvette was then put into sample chamber of a spectrophotometer and the temperature inside was set at 40°C. The absorbance reading was performed at 10 min

The number (n) of modified-tryptophan residues was calculated, using the equation:

$$n = \frac{A_{280\ nm} \times Mr}{\varepsilon_{280} \times c}$$

where Mr is molecular weight of CGTase which is 72,000, ε_{280} of *N*-acetyl-tryptophan is $4.00 \times 10^3\ M^{-1}cm^{-1}$ and c is the CGTase concentration (g/l).

2.15.3 Determination of the number of tyrosine residues

To measure the number of tyrosinee residues of CGTase modified by NAI in the presence and the absence of a protection substance (β-, or γ-CD), the differential decrease in the absorbance at 278 nm between the two conditions was followed (Means and Feeney, 1971). The reaction was performed in a 1-cm-pathlength quartz cuvette, consisting of 30 mM NAI and 0.15 g/l CGTase in the presence or the absence of 20 mM β-CD or 20 mM γ-CD. The cuvette was then put into sample chamber of a spectrophotometer and the temperature inside was set at 40°C. The absorbance reading was performed at 5 min.

The number (n) of modified-tyrosine residues was calculated, using the equation:

$$n = \frac{A_{278\ nm} \times Mr}{\varepsilon_{278} \times c}$$

where Mr is molecular weight of CGTase which is 72,000, ε_{278} of *O*-acetyltyrosine is $1.16 \times 10^3\ M^{-1}cm^{-1}$ and c is the CGTase concentration (g/l).

2.16 Effect of chemical modification on the structure and enzymatic properties of CGTase

This experiment is to determine the effect of chemical modification on electrophoretic mobility of CGTase. The enzyme after modification at suitable concentration and time was dialyzed to remove excess reagent and was concentrated by lyophilization. The modified CGTase was loaded onto a 7.5% non-denaturing polyacrylamide slab gel as described in section 2.8.1. After running, gels were stained for protein and CGTase activity.