CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

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

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

Fraction collector: Model 2211 Pharmacia LKB, Sweden

Incubator: Haraeus, Germany

Incubator Shaker, Model SBS30, Control environment: Stuart scientific, USA

Luminescence specprometer: Perkin Elmer LS 55, UK

Peristaltic pump: Pharmacia LKB, Sweden

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

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

Viva flow, cut-off 10,000 daltons: Viva flow 50, Viva science, Germany

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

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

2.2 Chemicals

Acetonitrile (HPLC grade): BDH Laboratory Chemical-Division, England

Activated carbon NotriRO 0.8 pellet: Aldrich Chemical Company Ltd., USA

Acrylamide: Merck, USA

Ampholine pH 3.5-10.0 for IEF: Amersham Pharmacia Biotech: Sweden

Amylose EX-I: Hayashibara biochemical lab. Inc.: Japan

Bacto-peptone: Difco Laboratories, USA

Beef extract: Difco Laboratories, USA

Bio-Gel P-100 (medium): Bio-rad, USA

Blue dextran 2000: Pharmacia fine chemicals, Sweden

N-Bromosuccinimide: Sigma, USA

Cellobiose: Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

β-cyclodextrin: Nihon shokuhin kako company Ltd., Japan

α-, and γ–cyclodextrin: Sigma, USA

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

Diethylpyrocarbonate: Sigma, USA

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

N-Ethylmaleimide: Sigma, USA

Hydroxypropyl-β-cyclodextrin: Dexy pearl, Japan

Glycine: Sigma, USA

Glucose: APS finechem, Australia

Glucosyl-α- and β-cyclodextrin: Bioresearch Corporation of Yokohama, Japan

Iodoacetamide: Sigma, USA

Maltose: Sigma, USA

Maltotriose, Maltotetraose, Maltohexaose, Maltoheptaose: Sigma, USA

Maltopentaose: Wako, Japan

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

Phenolpthalein: BDH, England

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Pullulans: Wako, Japan

Standard molecular weight marker protein: Sigma, USA

Soluble starch, potato: Sigma, USA

Trichloroethylene: BDH, England

Trinitrobenzenesulfonic acid: Sigma, USA

Yeast extract: Scharlau, Spain

Other chemicals used were of reagent grade and were purchased from commercial sources. Corn starch (Maizena), cassava starch (dragon fish brand) were locally purchased.

2.3 Bacteria

Paenibacillus sp. RB01 was screened for CGTase activity by Tesana (2001), isolated from hot spring area at Ratchaburi province, Thailand.

2.4 Media preparation

2.4.1 Medium I

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

2.4.2 Cultivation medium (Horikoshi's medium)

Medium for enzyme production, slightly modified from Horikoshi (1971) (Rutchtorn, 1993), contained 1.0% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \bullet 7H_20$ and 0.75% Na_2CO_3. The pH of the medium was 10.1-10.2.

2.5 Cultivation of bacteria

2.5.1 Starter inoculum

Paenibacillus sp. RB01, isolated from hot spring area at Ratchaburi provinces (Tesana, 2001) were streaked on solid Medium I and incubated for 18 hours at 37 °C. Then one loop was put into liquid Medium I at 37 °C and grown until A_{660} reached 0.3-0.5.

2.5.2 Enzyme production

Starter inoculum (1.0%) was transferred into 200 ml Horikoshi's broth in 500 ml Erlenmeyer flask. Culture was harvested at 60 hours and cells were removed by centrifugation at 3,000 x g at 4 °C. Culture broth with crude CGTase was collected and kept at 4 °C for purification.

2.6 Purification of CGTase

CGTase was purified from the culture broth of *Paenibacillus* sp. RB01 by three steps of purification; starch adsorption, DEAE-cellulose chromatography column and Bio-Gel P-100 chromatography column.

2.6.1 Starch adsorption (Kato and Horikoshi, 1985, modified by Kuttiarcheewa, 1994)

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 3000 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 (2 x 125 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 water at 4 °C with 3 changes of water.

2.6.2 DEAE-cellulose chromatography

DEAE-cellulose was activated by washing sequentially with excess volume of 0.5 N HCl for 3 hrs followed by distilled water until pH was about 7.0 and then washed with 0.5 N NaOH for 3 hrs followed by distilled water to obtain pH about 8.0. The activated cellulose was equilibrated with Tris-HCl pH 8.0 (TB2). The prepared DEAE-cellulose was packed into the column (1.5 x 28 cm) and was equilibrated with TB2 at around 25 °C.

The dialyzed protein solution form section 2.6.1 was applied to DEAE-cellulose column. The unbound proteins were eluted from the column with the buffer. Normally, keep washing until the absorbance at 280 nm of eluant decreased to almost nil. After the column was washed thoroughly with the buffer TB2, the bounded proteins were eluted from the column with linear salt gradient of 0 to 0.3 M sodium chloride in the same buffer. The fractions of 4.0 ml were continuously collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.7.1. The fractions showing enzyme activity were pooled for further purification step.

2.6.3 Bio-Gel P-100 chromatography

Bio-Gel P-100 was swelled in distilled water for 24 hours at room temperature. The swelled gel was degassed and packed into a glass column (1.7 x 95 cm.) using peristalic pump at flow rate of 10 ml/hr. The Bio-Gel P-100 column (1.7 x 80 cm.) was equilibrated with elution buffer (50 mM Acetate buffer, pH 6.0) for 5 column volumes

at flow rate of 10 ml/hr to allow stabilization of bed volume of the column. The column was calibrated with molecular weight marker protein (Bovine serum albumin MW = 68 kDa, Ovalbumin MW = 43 kDa, Chymotrypsinogen A MW = 27 kDa, and Cytochrome C MW = 11.7 kDa) at flow rate of 10 ml/hr at around 25 °C. Blue dextran 2000 and potassium dichromate were used to determine the void volume and the total volume of the column. An aliquot (1 ml) of the concentrated enzyme from DEAE-cellulose column chromatography was loaded onto the column and eluted with elution buffer at a flow rate of 10 ml/hr. Fractions of 1 ml were collected. The elution profile was monitored for protein and dextrinizing activity as described in section 2.7.1.

2.7 Enzyme assay

CGTase activity was determined by assay of starch degrading (dextrinizing) activity, assay of CD product through the formation of CD-trichloethylene complex (CD-TCE), cyclization activity assay, and coupling activity assay.

2.7.1 Dextrinizing activity

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.

2.7.2 Cyclodextrin-trichloroethylene (CD-TCE) assay

Cyclodexrin-trichlroethylene (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 the term of 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.

2.7.3 Cyclization activity assay

Cyclization activity was determined by the phenolphthalein method of Goel and Nene (1995). To 1.25 ml of 6.0% soluble starch was added purified CGTase 0.25 ml. The reaction mixture was incubated for 30 minutes under 70 °C. Reaction was stopped by boiling for 5 minutes. To 1.0 ml of sample was incubated with 4.0 ml of phenolphthalein solution at 70 °C for 10 minutes. Absorption was measured at 550 nm and β-CD formed was calculated using the calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the corresponding condition.

Phenolphthalein stock 4 mM in ethanol; 125 mM Na_2CO_3 solution in distilled water; and 4 ml ethanol in 100 ml Na_2CO_3 solution just before starting the experiment. β-CD standard 0-2.5 mM was prepared.

2.7.4 Coupling activity assay

Coupling reaction were determined by incubating various concentration of α-, β- or γ–cyclodextrin or their derivatives as donor (0.5-15 mM) with 10 mM cellobiose as glucosyl acceptor at 55 °C. 50 mM acetate buffer pH 6.0 was added to make the total volume of reaction mixture 0.25 ml. Cyclodextrin and cellobiose were pre-incubated for 5 minutes at 55 °C. The reaction was started with 10 μl of 0.88 mg/ml of purified CGTase. Boiling for 5 minutes stopped the reaction. Subsequently, 0.2 units of *Aspergillus niger* glucoamylase (10 μl) was then added to convert linearized oligosaccharides to glucose. Measuring the released reducing sugars which monitored the amount of cyclodextrins degraded was by the dinitrosalicylic acid method.

2.8 Protein determination

Protein concentration was determined by the method of Bradford with bovine serum albumin as the standard.

2.9 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. 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 a 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.

2.10 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE was performed according to Bollag and Edelstein (1991), non-denaturing and denaturing gels, were employed for analysis of the purified protein. The gels were visualized by coomassie blue and Periodic acid-schiff (PAS) staining. For non-denaturing gel, dextrinizing acitivity staining was also undertaken.

2.10.1 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

Discontinuous PAGE was performed 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.

2.10.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing 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 8.0 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.

2.10.3 Detection of protein

2.10.3.1 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 unit gel background was clear.

2.10.3.2 Dextrinizing activity staining (slightly modified from the method of Kobayashi, *et al.*, 1978)

The running gel was soaked in 10 ml of substrate solution, containing 0.2% (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.

2.11 Characterization of the CGTase

2.11.1 Qualitative analysis by Periodic Acid-Schiff (PAS) staining (Zacharius et al., 1969)

The purified CGTase was analyzed on SDS-PAGE and native PAGE, then put the gel slab in 12.5% TCA for 30 minutes and then washed in distilled water for 15 minutes. Put the gel slab in 1% periodic in 3% acetic acid for 50 minutes. Next, the gel slab was washed with distilled water to remove the excess periodic. Then immersed the gel in Schiff's reagent and left in the dark at room temperature for 50 minutes. The excess dye was washed with 0.5% sodium metabisulfite for 10 minutes for 3 times.

Then washed with distilled water until the background was clear and stored the gel in 7% acetic acid (to prepare PAS staining solution, see Appendix 2).

2.11.2 Determination of the isoelectric point by isoelectric focusing polyacrylamide gel electrophoresis (IEF)

2.11.2.1 Preparation of gel support film

A few drops of water was pipetted onto the plate. The hydrophobic side of the gel support film was then placed against the plate and flatly rolled with a test tube to force excess water and bubbles. Subsequently, it was placed down on the casting tray with the gel support film facing down so that rest on the space bars.

2.11.2.2 Preparation of the gel

The gel solution composed of 30% acrylamide, 1% bis-acrylamide, 50% sucrose, 10% ammonium persulfate and TEMED (see Appendix 3) was carefully pipetted between the glass plate and casting tray with a smooth flow rate to prevent air bubbles. The gel was left about 45 minutes to allow polymerization, then lifted from the casting tray using spatula. The gel was fixed on the gel support film and ready for used.

2.11.2.3 Sample application and running the gel

The sample was loaded on a small piece of filter paper to allow its diffusion into the gel for 5 minutes and the filter paper was carefully removed from the gel. The gel with the adsorbed samples was turned upside-down and directly placed on top of the graphite electrodes. Focusing is carried out under constant voltage conditions in a stepwise fashion. The gel was firstly focused at 100 V for 15 minutes,

followed by an increase in voltage up to 200 V for 15 minutes and finally run at 450 V for an additional 60 minutes. After complete electrofocusing, the gel was stained. Standard protein markers with known pI's in the range 3-10 were run in parallel. The standards consist of amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydase B (5.85), human carbonic anhydase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsinogen (9.3). The pI's sample proteins were determined by the standard curve constructed from the pI's of the standard proteins and their migrating distance from cathode.

2.12 Optimum conditions for enzyme activity

2.12.1 Effect of pH

The purified CGTase was used to study the effect of pH on its activity. The enzyme was assayed as described in section 2.7.1-2.7.3. The 0.2 M buffer solution was in the pH range from 3.0 to 11.0. The buffer used were potassium acetate (pH 3.0-5.0), phosphate (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and Tris-Glycine NaOH (pH 9.0-11.0) (see Appendix 4)

2.12.2 Effect of temperature

The purified CGTase was incubated with 0.2% potato starch in phosphate buffer, pH 6.0 at various temperatures from 20-100 °C, for 10 minutes, then it was assayed activity as described in section 2.7.1.

2.13 Enzyme stability

2.13.1 Effect of pH

The stability of purified CGTase at different pHs was determined by incubating enzyme in various buffers of pH 5.0-10.0 at temperature which enzyme gave high activity (from 2.12.2) for 60 minutes (buffers used were the same as in 2.12.1). The enzyme activity was assayed at the optimal conditions obtained from 2.7.3.

2.13.2 Effect of temperature

Effect of temperature on the stability of enzyme was observed by incubating the enzyme at different temperatures in the range 45-70 °C, for 60 minutes. The cyclization activity was assayed at the optimal conditions obtained from 2.7.3.

2.13.3 Effect of substrate

The effect of substrate on enzyme stability was also studied by incubating the enzyme sample in the presence of final concentration of 0.2% soluble starch (potato) at 70 °C for 60 minutes. The enzyme activity was then assayed at the optimal conditions obtained from 2.7.3.

2.13.4 Effect of calcium and temperature upon long-term storage

The purified CGTase with and without calcium was kept at –20 and 4 °C for three weeks. The activity was then determined at time intervals by the method described in section 2.7.1.

2.14 Substrate specificity of CGTase

The cyclization activity of purified CGTase was determined as described in section 2.7.3 with varying substrates. Soluble starch, amylose, amylopectin, pullulans, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and dextrin were used at 6.0%, except for amylopectin of which 1.5% was used in acetate buffer pH 6.0.

2.15 Determination of kinetic parameters

Kinetic parameters of the coupling reaction were determined by incubated various concentration of α-, β- or γ–cyclodextrin and their derivatives as donor (0.5-15 mM) with 10 mM cellobiose as glucosyl acceptor at 55 °C. 50 mM acetate buffer pH 6.0 was added to the make the total volume of reaction mixture 0.25 ml. Cyclodextrin and cellobiose were pre-incubated for 5 minutes at 55 °C. The reaction was started with 10 ml of 0.88 mg/ml of purified CGTase. Boiling for 5 minutes stopped the reaction. Subsequently, 0.2 units 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 2.9. Kinetic parameter, K_m and V_{max}, were determined from the Michealis-Menten equation using nonlinear least square regression analysis of the EZ-FIT V1.1 Computer program.

2.16 Determination of cyclodextrin by High Performance Liquid Chromatography

The analysis of cyclodextrins by HPLC was performed as described by Ruchtorn (1993). The HPLC system was a Shimadzu LC-3A equipped with Lichrocart-NH_2 column (0.46 x 25 cm) and using Shimadzu RID-3A refractometer as

detector. For CD analysis, the reaction was performed by incubating 0.5 ml of enzyme sample with 1.5 ml of starch substrate (2.0 g% soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) at 40 °C for 24 hours. Boiling in water for 5 minutes stopped the reaction. After cooling the mixture was treated with 20 units of β–amylase at 25 °C for 3 hours, and the reaction was stopped by heating in boiling water. Prior to injection, the mixture was filtered through a 0.45 μm membrane filter. The mixture was injected 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 standard α-, β- and γ-CDs (20 μg/ml).

2.17 Determination of suitable concentration of reagent used in the modification of CGTase

2.17.1 Effect of modifying reagent concentration

2.17.1.1 Modification of carboxyl residues

Modification of carboxyl residues was carried out according to the method of Means and Feeney (1971). CGTase (30 μg/ml, 30 μl) was incubated with varying concentrations of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (5 mM) at 40 °C for 30 minutes. Total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.7.1.

2.17.1.2 Modification of histidine residues

Modification of histidine residues was carried out according to the method of Mile (1977). CGTase (30 μg/ml, 30 μl) was incubated with varying concentrations of diethylpyrocarbonate (DEP) (1.5 mM) at 40 °C for 30 minutes. Total

volume of reaction mixture was 60 µl. CGTase activity was then determined as described in section 2.7.1.

2.17.1.3 Modification of tryptophan residues

Modification of tryptophan residues was carried out according to the method of Lundblad (1991). CGTase (30 µg/ml, 30 µl) was incubated with varying concentrations of *N*-bromosuccinimide (NBS) (0.005 mM) at 40 °C for 30 minutes. Total volume of reaction mixture was 60 µl. CGTase activity was then determined as described in section 2.7.1.

2.17.1.4 Modification of tyrosine residues

Modification of tyrosine residues was carried out according to the method of Means and Feeney (1971). CGTase (30 µg/ml, 30 µl) was incubated with varying concentrations of *N*-acetylimidazole (1 mM) at 40 °C for 30 minutes. Total volume of reaction mixture was 60 µl. CGTase activity was then determined as described in section 2.7.1.

2.17.1.5 Modification of cysteine residues

Modification of cysteine residues was carried out according to the method of Means and Feeney (1971). CGTase (30 µg/ml, 30 µl) was incubated with varying concentrations of *N*-ethylmaleimide, iodoacetamide or dithiothreitol (1 mM) at 40 °C for 30 minutes. Total volume of reaction mixture was 60 µl. CGTase activity was then determined as described in section 2.7.1.

2.17.1.6 Modification of lysine residues

Modification of lysine residues was carried out according to the method of Means and Feeney (1971). CGTase (30 μg/ml, 30 μl) was incubated with varying concentrations of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (1 mM) at 40 °C for 30 minutes. Total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.7.1.

2.17.1.7 Modification of serine residues

Modification of serine residues was carried out according to the method of Wakayama (1996). CGTase (30 μg/ml, 30 μl) was incubated with varying concentrations of phenylmethylsulfonyl fluoride (PMSF) (1 mM) at 40 °C for 30 minutes. Total volume of reaction mixture was 60 μl. CGTase activity was then determined as described in section 2.7.1.

2.17.2 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 2.7.1.

2.18 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-, β-CD and γ-CD as protective substance. The CGTase (60 μg/ml, 15 μl) was preincubated with 20 mM of each substrate at 40 °C for 5 minutes prior to the addition of suitable concentration of

each modifying reagent. The total volume of reaction mixture was 60 μl. Then the residual CGTase activity was determined as described in section 2.7.1.

2.19 Measurement of fluorescence spectrum upon modification by NBS

To determine if tryptophan was located at active site of CGTase, fluorescence spectrum when CGTase was modified by NBS in the presence and the absence of β-CD was measured. To 50 μl of CGTase (0.15 mg/ml) was incubated with 0.1 M NBS for 5 minutes. In the protection condition, protection time with 1% and 2% β-CD was also 5 minutes. The different spectrum between two conditions was determined by spectrofluorometer. Excitation wavelength was at 280 nm while emission was performed in the range 290-450 nm (Yu *et al.*, 2001).

2.20 Urea-induced denaturation of CGTase

CGTase (0.15 mg/ml) was incubated with 0-10 M urea in acetate buffer pH 6.0 at 25 °C for 16 hours. Total reaction volume was 1.2 ml. Residual dextrinizing activity was then measured. Fluorescence measurements were also performed at 25 °C, excitation wavelength was 280 nm and emission wavelength was 290-450 nm. Native PAGE of the control and urea treated CGTase were compared.