CHAPTER II

METERIALS AND METHODS

2.1 Equipments

Amino acid analyzer : Waters Picotag™ system, Waters 510 HPLC, Picotag™ column (0.39x30 cm) : Waters company, U.S.A.

Autoclave : Model HA-30, Hirayama Manufacturing Cooperation, Japan

Autopipette : Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge : Model J-21C, Beckman Instrument Inc., U.S.A.

Centrifuge, refrigerator microcentrifuge : Kubota 1300, Japan

Diaflo Ultrafilter : Stirred Ultrafiltration Cell 8050 Amicon W.R. Grace Cooperation, U.S.A.

Electrofocusing unit : model 111 MINI IEF Cell, Bio-Rad Applied Biosystem company, U.S.A.

Electrophoresis unit : 2050 MIDGET, LKB, Sweden

Fraction collector : model 2211, Pharmacia LKB, Sweden

Gas phase protein sequencer : Applied Biosystems model 476A, U.S.A.

High Performance Liquid Chromatography : Model LC 3A Shimadzu, Japan

Incubator : Haraeus, Germany

Incubator shaker, Controlled environment : Psyco-therm, New Brunswick Scientific Co., U.S.A.

Magnetic stirrer : 0188 GMS, Scientific Instrument Development and Service Center, Faculty of Science, Chulalongkorn University

Membrane filter : cellulose nitrate, pore size 0.45 μm, Whatman, Japan

Mini Trans-Blot Electrophoresis Transfer cell : Bio-Rad Applied Biosystem company, U.S.A.

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

Preparative gel electrophoresis unit : model 491 Prep Cell, Bio-Rad Applied Biosystem company, U.S.A.

Spectrophotometer UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.

Vortex : Model K-550-GE, Scientific Industries, U.S.A.

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

Water bath, Shaking : Heto Lab Equipment, Denmark

2.2 Chemicals

Acetonitrile (HPLC grade) : J.T. Baker Chemical, U.S.A.

Acrylmide : Merck, U.S.A.

Beta-amylase : Type 1-B from Sweet potato (A 7005), Sigma, U.S.A.

Coomasie brilliant blue G-250 : Sigma, U.S.A.

Coomasie brilliant blue R-250 : Sigma, U.S.A.

Dialysis tubing : Sigma, U.S.A.

Glycine : Sigma, U.S.A.

Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose : Sigma, U.S.A.

Methylorange : BDH, England

N,N′-methylene-bis-acrylamide : Sigma, U.S.A.

Nylon membrane : Biorad, U.S.A.

Phenolphthalein : BDH, England

Patato starch (soluble) : Sigma, U.S.A.

Soluble starch : Fluka A.G. Buchs S.G., Switzerland

Standard α-, β-, and γ-CD : Fluka A.G. Buchs S.G., Switzerland and Sigma, U.S.A.

Standard molecular weight marker proteins : Sigma, U.S.A.

Standard pI marker proteins : Pharmacia Biotech, U.S.A.

Trichloroethylene (TCE) : BDH Laboratory Chemical company, U.S.A.

Other common chemicals were obtained from Fluka or Sigma Raw rice Starch (three heads elephant brand) and corn starch (Maizena) were locally purchased.

2.3 Bacteria

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

2.4 Media Preparation

2.4.1 MEDIUM I

Medium I consisted of 0.5% beef extract, 1.0% polypeptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble starch (Fluka) was prepared and pH was adjusted to 7.2

with 1N HCl. For solid medium 1.5% agar was added. Medium was steriled by autoclaving at 121°C for 15 minutes.

2.4.2 Cultivation medium (modified from Horikoshi's medium) (Rutchtorn,1993).

Medium for enzyme production, slightly modified from Horikoshi (1971), contained 1.0% local grade of rice starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7\ H_2O$ and 0.75% Na_2CO_3 with starting pH of 10.1-10.2. Medium was steriled as above.

2.5 Cultivation of Bacteria

2.5.1 Starter inoculum

A colony of *Bacillus* sp. A11 was grown in 50 ml of starter medium I in 250 ml Erlenmeyer flask at 37°C with 250 rpm rotary shaking until A_{420} reached 0.3-0.5 or about 4-6 hours.

2.5.2 Enzyme production

Starter *Bacillus* sp. A11 was transferred into 100 ml Horikoshi's broth in 500 ml Erlenmeyer flask with 1% inoculum and cultivated at 37°C with 250 rpm rotary shaking. Culture was harvested after 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.

2.6 Enzyme assay

For this study, CGTase activity was determined by starch degrading (dextrinizing) activity assay and CD-forming activity (CD-trichloroethylene, CD-TCE) assay.

2.6.1 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 of starch substrate (0.2 g% potato starch 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 N 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 of each sample, HCl was added before the enzyme sample.

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

2.6.2 Cyclodextrin-Trichloroethylone (CD-TCE) assay

CD-forming activity of CGTase 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 sample and 2.5 ml of starch substrate (0.2% (w/v) potato starch 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 ($1:2^n$) which was the highest dilution that can produced observable CD-TCE precipitate lining between the upper starch solution layer and the lower TCE layer.

2.7 Protein Determination

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

One hundred microlitres of sample was mixed with 5 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, 10 ml of 85% H_3PO_4 and distilled water.

2.8 Purification of CGTase

CGTase was partially purified from the culture broth of *Bacillus* sp.A11 by starch adsorption method of Kato and Horikoshi (1984) with modification (Kuttiarcheewa, 1994) (Figure 6).

Corn Starch (local grade) was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude enzyme broth to

make 5 g% concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 5,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TBI). The adsorbed CGTase was eluted from the starch cake with TBI buffer containing 0.2 M maltose (3x150 ml for starting broth of 5 l), by stirring for 30 minutes. CGTase eluted was recovered by centrifugation at 5,000 rpm for 30 minutes. The partially purified CGTase was then concentrated approximately 100 times by ultrafiltration before loading onto a preparative gel electrophoresis Model 491 Prep cell.

2.9 Isolation of CGTase isozymes

The concentrated partially purified enzyme (5 mg protein) was loaded to a discontinuous preparative polyacrylamide gel electrophoresis, which was performed on Model 491 Prep cell (38 mm ϕ), with 7.5% separating gel and 5% stacking gel. Tris-glycine buffer, pH 8.3 was used as electrode buffer (see Appendix A). The electophoresis was run from cathode toward the anode at constant power of 12 W until the dye reached the bottom of the gel. Proteins were then eluted from the gel with electrode buffer at a flow rate of 1 ml/min. Fractions of 2.5 ml were collected and measured for A_{280} and dextrinizing activity. To identify the CGTase isozymes, every 5 fractions were run on slab gels and observed by dextrinizing activity staining. Then the fractions which gave the same band were pooled for further study.

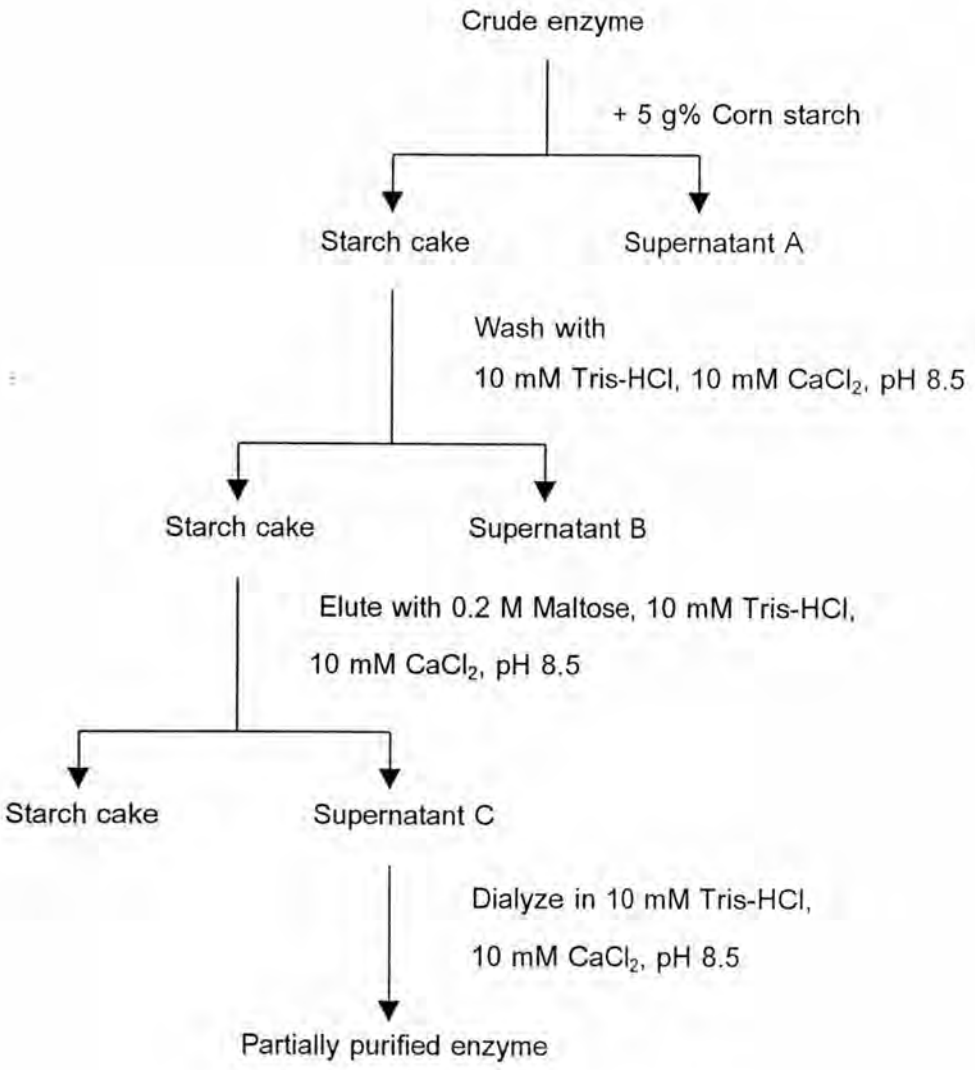

Figure 6. Flowchart for partial purification of CGTase.

2.10 Determination and characterization of CGTase isozymes

2.10.1 Polyacrylamide gel electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gel electrophoresis were employed for analysis of the purified isozymes. Electrophoresis conditions, protein and activity staining were as described below.

2.10.1.1 Non-denaturing polyacrylamide gel electrophoresis

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. The electrophoresis was run from cathode towards anode at constant current of 20 mA per slab at room temperature in a Midget LKB 2001 Gel Electrophoresis unit. The temperature was controlled at 10°C by LKB 2209 Multi-temperature thermostat water bath.

2.10.1.2 SDS- polyacrylamide gel electrophoresis

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 A). 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 Midget LKB 2001 Gel Electrophoresis unit from cathode towards anode.

2.10.1.3 Detection of proteins

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

2.10.1.3.1 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.

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

The 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. It 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.

2.10.1.3.3 Dye staining for cyclodextrin

The gel was stained by the method of Malai (1991) which modified from Park, *et al.* ,1989.

The basis of the staining is that CD produced by the CGTase can trap phenolphthalein dye in their cavities hence the dye become colorless.

The non-denaturing gel was soaked in 10 ml of phenolphthalein staining solution (2.0 g% potato starch, 0.03 g% phenolphthalein and 0.01 g% methyl orange solution of which pH was adjusted to 10.3 with 1.0% Na_2CO_3) at 40°C for 20 minutes. After leaving the gel to cool for 30 minutes, the yellow color of methyl orange appeared at the bands producing CD's whereas the background of the gel was red due to the color of methyl orange in alkaline condition.

2.10.2 Carbohydrate determination

2.10.2.1 Qualitative analysis by Periodic acid-Schiff (PAS) staining (Segrest and Jackson, 1972)

For PAS staining of glycoproteins, the discontinuous non-denaturing gel was fixed overnight in 100-200 ml of PAS fixative solution. The gel was then treated with the 0.7% periodic acid solution (covering the gel) for 2-3 hours, followed by treatment with 0.2% sodium metabisulfite for 2-3 hours with one change of solution after 30 minutes. The gel after clearing was soaked with Schiff reagent. Colour developed in 12-18 hours at room temperature. Thereafter, the gel should be stored at 4°C (to prepare PAS staining solution, see Appendix B)

2.10.2.2 Quantitative analysis by phenol-sulfulic acid method (Dubois, *et al.*, 1956)

The total carbohydrate content was determined by the phenol-sulfuric acid method : 0.5 ml of 5% phenol, 2.5 ml of concentrated H_2SO_4 and 0.5 ml of the sample solution were mixed. The absorbance was measured at 490 nm after 20 minutes incubation at room temperature.

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

2.10.3.1 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 they rest on the space bars.

2.10.3.2 Preparation of the gel

The gel solution composed of 30% acrylamide, 1% bis-acrylamide, 50% sucrose, 10% ammonium persulfate and TEMED (see Appendix C) 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.

2.10.3.3 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 5-7 were run in parallel. The standards consist of pepsinogen (2.80), amyloglucosidase (3.50), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85) and human carbonic anhydrase B (6.55). The pI's of sample proteins were determined by the standard curve constructed from the pI's of the standard proteins and their migrating distance from cathode.

2.10.4 Effects of pH and temperature on the isozyme activities

The isozyme activities were determined by the method as describe in 2.6.1 and 2.6.2 at pH range from 3.0 to 11.0 by changing the incubation buffers (0.2 g% potato starch in 0.2 M phosphate buffer, prepared by using potassium phosphate at different pK_a *i.e.* pK_{a1} 2.15 in the range of pH 3.0-4.0, pK_{a2} 7.20 in the range of pH 5.0-9.0 and pK_{a3} 12.38 in the range of pH 10.0-11.0) or change the incubation temperatures from 20 to 80°C.

2.10.5 Analysis of cyclodextrins by High Performance Liquid Chromatography

The sample solutions were analyzed for cyclodextrins by HPLC using Supelco-NH_2 column (0.46 x 25 cm) and detected by RI detector (Rutchtorn, 1992). To prepared the sample solution, the enzyme bands 1-5 (50 units of dextrinizing activity) were incubated with 2.5 ml of starch substrate (2.0 g% potato starch in water) at 40°C for 24 hours. The reaction was stopped by boiling in water for 5 minutes. After cooling, one half of the reaction mixtures of each isozyme was treated with 20 units of β-amylase at 25°C for an hour, and the reaction stopped by heating in boiling water bath. Prior to injection, the samples were filtered through 0.45 μm membrane filters. The eluant was a mixture of 75% acetonitrile and 25% water by volume, and the flow rate was 2 ml/minute. Cyclodextrins (α-, β- and γ-CD) were analyzed by comparing the retention times to those of standard CDs and quantitated. Cyclodextrins formed will be expressed as % conversation from starch.

$$\% \text{ Conversion} = \frac{\text{Concentration of cyclodextrins detected (g/l) x 100}}{\text{Concentration of starch substrate (g/l)}}$$

2.10.6 Amino acid analysis

Amino acid analysis of CGTase was determined using the method described (Waters, 1988). The enzyme samples were hydrolyzed with 6M HCl containing 1% phenol (v/v) in evacuated tubes at 110°C for 22 hours in the Waters Pico-Tag Workstation, after that they were derivatized with phenylisothiocyanate (PITC), dried under vacuum and resuspended in 100 μl Picotag™ sample diluent. The amino

acid mixtures obtained were analyzed on a Picotag™ column (0.39x30 cm) in a Waters 510 HPLC and the amino acid compositions were calculated by Maxima 820 program.