CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave : Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette : Pipetman, Gilson, France

C_{18} column : Model LUNA 5 μ size 250 x 4.6 mm., Phenomenex, USA

Centrifuge, benchtop centrifuge : Model H 11n, Kokusan Enzinki Co. Ltd., Japan

Centrifuge, microcentrifuge high speed : Model MC-15A, Tomy Seiko Co. Ltd., Japan

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

Diaflo ultrafiltered : Stirred Ultrafiltration Cell 8050, Amicon W. R. Grance Cooperation, USA

High Performance Liquid Chromatography : Model Hewlett PACKARD series 1050, Japan

Incubator Shaker, Controlled environment : Psyco-therm, New Brunswick Scientific Co., USA

Incubator : Model OB-28L, Fisher Scientific Inc., USA

Incubator Shaker : Model G 76D New Brunswick Scientific Co., Inc. Edison, N. J. USA

Magnetic stirrer and heater : Model IKAMA® GRH, Janke & Kunkel Gmbh & Co. KG, Japan

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

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

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

Activated carbon : Aldrich, USA

Alumina : Sigma, USA

Aminopropyltriethoxysilane : Sigma, USA

L-Ascorbic acid-2-glucoside : Hayashibara Biochemical Laboratories, Japan

L-Ascorbic acid sodium salt : Sigma, USA

Chitosan was kindly presented by Dr. R. Pichayangkura, Chulalongkorn University

Coomassie brilliant blue G-250 : Sigma, USA

β-Cyclodextrin : Sigma, USA

Dialysis tubing : Sigma, USA

Glucoamylase : from *Aspergillus niger* 70.7 U/mg, Fluka, Switzerland

Glutaraldehyde : Sigma, USA

Glycine : Sigma, USA

Metaphosphoric acid : Merck, USA

Maltose monohydrate : Sigma, USA

Phenolphthalein : BDH, England

Silica Gel G6 : BDH, England

Soluble starch, potato : Sigma, USA

Thiourea : BDH, England

All other common chemicals were of analytical grade from Fluka, Sigma, Carlo or BDH laboratory Chemical. For raw rice starch (three heads elephant brand) and corn starch (Maizena) were locally purchased.

2.3 Bacteria

Paenibacillus sp. A11, isolated from Southeast Asian soil, was screened for CGTase activity by Pongsawasdi and Yagisawa (1987).

2.4 Media Preparation

2.4.1 Medium I

Medium I was consisted of 0.5% (w/v) beef extract, 1.0% (w/v) peptone, 0.2% (w/v) NaCl, 0.2% (w/v) yeast extract, and 1.0% (w/v) soluble starch and the pH was adjusted to 7.2. For solid medium, 1.5% (w/v) agar was added. The medium was then sterilized by autoclaving at 121°C for 15 minutes.

2.4.2 Cultivation medium (Horikoshi's medium)

Medium for enzyme production, slightly modified from Horikoshi (1971) (Rutchtorn, 1993), was composed of 1.0% (w/v) rice starch, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4.7H_2O$ and 0.75% (w/v) Na_2CO_3 with the pH of 10.1-10.2. The medium was sterilized as above.

2.5 Cultivation of bacteria and enzyme production

The *Paenibacillus* sp. A11, from glycerol stock, was streaked on medium I agar plate and incubated for 18 hours at 37°C. A colony of *Paenibacillus* sp. A11 was then picked and grown aerobically in 250 ml Erlenmeyer flask containing 50 ml of

sterile starter medium I. The flask was incubated at 37°C on a rotary shaker at 250 rpm until the absorbance at 420 nm reached 0.3-0.5 or about 4-6 hours.

The 1% (v/v) inoculum of the starter *Paenibacillus* sp. A11 prepared above was transferred into 500 ml Erlenmeyer flask containing 100 ml of sterile Horikoshi's medium and incubated at 37°C on a rotary shaker at 250 rpm for 72 hours. After that, cells were removed by centrifugation at 1380 *g* for 30 minutes at 4°C. Culture broth with crude CGTase was collected and kept at 4 °C for purification.

2.6 Purification of CGTase

CGTase was partially purified from the culture broth of *Paenibacillus* sp. A11 by starch adsorption method (Rutchtorn, 1993) (Figure 7).

Corn starch was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into the crude enzyme broth which was continuously stirred at 4°C for 3 hours to make 5 % (w/v) concentration. The starch cake was then collected by centrifugation at 3838 *g* for 30 minutes at 4°C and washed twice with 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.5. The adsorbed CGTase was eluted three times from the starch cake by stirring for 30 minutes with 80 ml of the same buffer containing 0.2 M maltose. CGTase eluted was recovered by centrifugation at 3838 *g* for 30 minutes at 4°C. The partially purified CGTase was concentrated by ultrafiltration using a 10 kDa molecular weight cut-off membrane and then dialyzed against 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 at 4°C. This enzyme was used for the preparation of the immobilized enzyme.

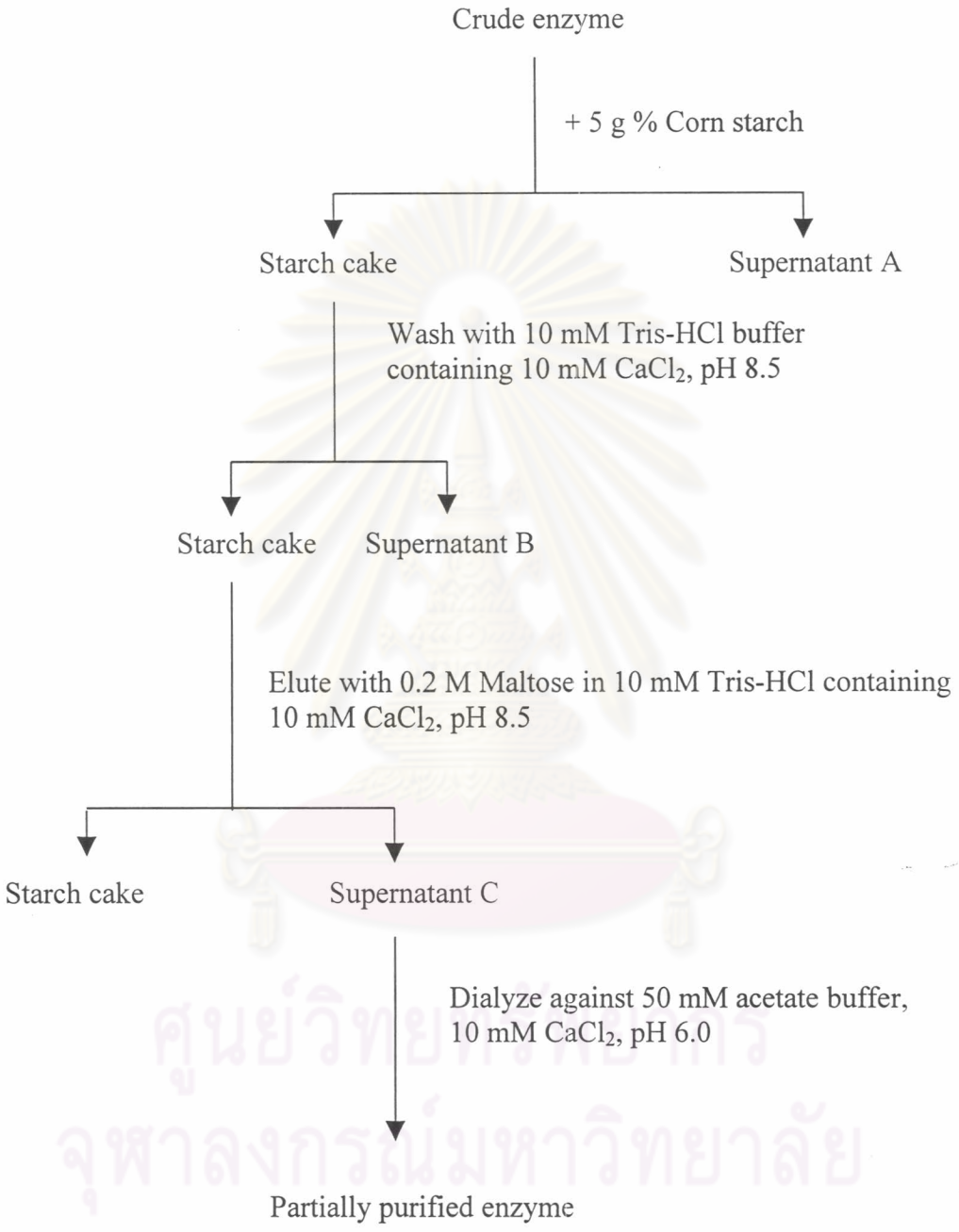

Figure 7. Schematic diagram illustrating the preparation of partial purified CGTase (Kuttiarcheewa, 1994).

2.7 Immobilization of CGTase

Several organic and inorganic supports including alumina, silica, activated carbon and chitosan were tested for covalent coupling of the enzyme to find out a suitable carrier for CGTase immobilization. The immobilization technique used was based on the silanization method described by Weetall (1976) with minor modification (Kuttiarcheewa, 1994).

2.7.1 Pretreatment of inorganic carrier

Before any inorganic support is silanized it should be cleaned to remove any adsorbed organic contaminants from the surface (Weetall, 1993). The supports were soaked in 5% (v/v) nitric acid solution at 100°C for 45 minutes followed by exhaustive washing with distilled water to remove any residual acid and dried at 110°C.

2.7.2 Activation of support material

Inorganic carriers (alumina and silica) were silanized with γ-aminopropyltriethoxysilane (APTS) and then coupled to glutaraldehyde (GA). One gram of clean inorganic support was activated through the contact with 10 ml of 2% (v/v) APTS in deionized water with gentle stirring for 3 hours at room temperature. The silanized carrier (alkylamine derivative) was removed by centrifugation 1380 *g* for 10 minutes at room temperature and washed thoroughly with deionized water. This was then further reacted with 10 ml of 0.25% (v/v) GA in 0.1 M phosphate buffer, pH 7.0 under mild agitation for 1 hour at room temperature. The excess GA was removed by centrifugation at 1380 *g* for 10 minutes at room temperature, and the carrier was washed with deionized water several times to remove residual GA.

2.7.3 Enzyme coupling

Ten units of CGTase solution was added into 1 g of activated carrier, from section 2.7.2, and the volume was adjusted to 5 ml with 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0. The mixture was gently stirred at 4°C for 12 hours. The unbound CGTase was removed by centrifugation at 1380 *g* for 10 minutes at 4°C. The carrier was successively washed (3 times) with 1 M NaCl in 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 and followed by successively washing (4 times) with the same buffer without salt until no activity or soluble protein was observed in the washing solutions. All eluates were collected and analyzed for the CGTase activities and protein contents as described in section 2.9 and 2.10. The covalently bound enzyme was stored in 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 at 4°C until further use.

2.8 Optimization of CGTase immobilization

The optimum conditions for covalent immobilization of CGTase were investigated by varying some of the conditions described in sections 2.7.2 and 2.7.3. The APTS concentration, ranging from 1-10% (v/v), and glutaraldehyde concentration, ranging from 0.1-2.5% (v/v), were tested. Various CGTase concentrations applied to the support were also investigated in the range of 2-20 units per gram support. The coupling time between the enzyme and the support was also examined during 2-12 hours. The immobilized enzyme activity and the protein content were determined by the methods mentioned in sections 2.9 and 2.10. The best condition yielding maximum activity retention of the immobilized CGTase was selected.

2.9 Enzyme assay

CGTase activity was measured as β-CD forming activity on the basis of its ability to form a stable, colorless inclusion complex with phenolphthalein (Nogrady *et al.* 1995).

To assay the activity of soluble CGTase, the enzyme solution was incubated with 1 ml of 4 % (w/v) soluble starch in 50 mM phosphate buffer, pH 6.0 at 60°C for 10 minutes. The reaction was stopped by the addition of 3.5 ml of 40 mM NaOH solution, and then 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 was added. The mixture was left to stand at room temperature for 15 minutes and the absorbance at 550 nm was measured. The concentration of β-CD was determined by the change in absorbance at 550 nm to a standard β-CD curve.

For assaying immobilized enzyme activity, 10 mg of immobilized enzyme was mixed with 1 ml of soluble starch 4 % (w/v) in 50 mM phosphate buffer, pH 6.0 and incubated at 60°C for 10 minutes with shaking. The reaction was stopped and supernatant was analyzed in the same way as in the case of soluble enzyme. For control tube, the reaction was stopped before the addition of enzyme sample.

One unit of enzyme activity (U) is defined as the amount of the enzyme forming one mg of β-CD per minute under the assay conditions.

2.10 Protein Determination

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

A 100 µl of sample was mixed with 1 ml of protein reagent and incubated for 5 minutes before measuring the absorbance at 595 nm. The protein content of the immobilized enzyme was estimated by subtracting the amount of unbound protein

determined in supernatant and washings after immobilization from the originally added protein.

One liter of Coomassie blue reagent was consisted of 100 mg Coomassie blue G-250, 50 ml of 95% ethanol, 100 ml of 85% H_3PO_4 and distilled water.

2.11 Calculation of the immobilization yield

The efficiency of immobilized enzyme was usually expressed as the activity retention after immobilization. The immobilization yield of the immobilized CGTase was calculated by equation as follows:

$$\text{Immobilization yield (\%)} = \frac{\text{Immobilized enzyme activity (U)} \times 100}{\text{Free enzyme activity used (U)} - \text{Unbound enzyme activity (U)}}$$

2.12 Characterization of the catalytic properties of the free and immobilized CGTase

The properties of immobilized CGTase were compared to those of the soluble enzyme when 4% (w/v) of starch solution was used as substrate under standard assay.

2.12.1 Effect of pH on the activity of free and immobilized CGTase

The effect of pH on the activity of free and immobilized CGTase was determined by using 4% (w/v) soluble starch in buffer solutions with pHs ranging from 4.0 to 11.0. Acetate buffer (50 mM, pH 4.0-5.5), phosphate buffer (50 mM, pH 6.0-7.5), Tris-HCl buffer (50 mM, pH 8.0-9.0), glycine-NaOH buffer (50 mM, pH 10.0-11.0) were used (Appendix A). The enzyme activity was measured by the method described in section 2.9.

2.12.2 pH stability

The effect of pH on the stability of the free and immobilized enzyme was examined after preincubating enzyme samples in buffers at different pH values (used the same buffer solution as described in section 2.12.1) at room temperature for 60 minutes. The residual activity was measured under the standard conditions as described in section 2.9.

2.12.3 Effect of temperature on the activity of free and immobilized CGTase

The temperature dependence on the activity of free and immobilized CGTase was determined in 50 mM phosphate buffer at pH 6.0. The activity was measured in the temperature ranging between 30 and 90°C by the method described in section 2.9. The activation energy (E_a) value has been calculated from the Arrhenius equation:

$$m = -E_a/2.303\ R$$

where m is the slope of the Arrhenius plot, E_a is the energy of activation, and R is the molar gas constant (R= 1.98 kcal/mol).

2.12.4 Thermal stability

The thermal stability of the immobilized enzyme was compared to that of the free enzyme. Both of the enzyme samples were incubated in 50 mM acetate buffer, pH 6.0 in the presence of 10 mM $CaCl_2$ at designated temperature (30-90°C) for 20 minutes. The residual activity was measured at 60°C under the standard condition as described in section 2.9. The residual activity was expressed relatively to the original activity at 60°C when the enzyme sample was not subjected to heat.

To investigate heat inactivation of the enzymes, both of the free and immobilized CGTase were suspended in 50 mM acetate buffer containing 10 mM

$CaCl_2$, pH 6.0 and incubated at 60°C for 1-8 hours. Samples were withdrawn periodically and the residual enzyme activity was measured at 60°C under the standard condition (section 2.9).

2.12.5 Kinetic determination

The kinetic parameters were determined by measuring the rate of β-CD formation using soluble starch as substrate as the method described in section 2.9. Soluble and immobilized CGTase were incubated with different concentrations of starch solution in 50 mM phosphate buffer (pH 6.0) ranging from 0.05-0.5 mg/ml for the free CGTase and from 25-40 mg/ml for the immobilized CGTase at 60°C for 10 minutes. The Michalelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) of both free and immobilized CGTase were determined by using the Lineweaver-Burk plots.

2.12.6 Storage stability

Both free and immobilized CGTase were stored in 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 at room temperature (about 27-30°C) and 4°C for 2 months. The remaining activity was measured every 5 days as described in section 2.9 and compared with the value at time zero.

2.13 Synthesis of 2-O-α-glucopyranosyl L-ascorbic acid with *Paenibacillus* sp. A11 CGTase

The enzyme reaction was performed by the method of Tanaka *et al.* (1991) with slight modifications. 2-O-α-Glucopyranosyl L-ascorbic acid (AA-2G) was synthesized in the reaction mixture containing 4% (w/v) β-CD, 4% (w/v) ascorbic acid sodium salt (AANa), 0.2% (w/v) thiourea, and 60 g (wet weight) of immobilized

CGTase or soluble CGTase (about 280 units) in a total volume of 20 ml of 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0. After 24-hour incubation at 40°C with continuous shaking at 150 rpm in the dark, a 200 µl-aliquot was withdrawn and mixed with 800 µl of 1.06% metaphosphoric acid to terminate the reaction and then centrifuged at 1380 *g* for 10 minutes to remove the immobilized enzyme. The amount of AA-2G in the reaction mixture was measured by high performance liquid chromatography (HPLC) as described in section 2.14.

2.14 Assay for AA-2G formation by high performance liquid chromatography (HPLC)

The AA-2G content in the reaction mixture was analyzed by HPLC according to the method described by Tanaka *et al.* (1991). The system was Hewlette PACKARD series 1050 equipped with UV spectrophotometric detector set at 240 nm and a C_{18} column (4.6 x 250 mm) was used. All the samples were previously filtered through a 0.45 µm membrane filter before injection. The mobile phase was 0.1 M potassium phosphate-phosphoric acid buffer (pH 2.0) and the flow rate of 0.3 ml/min was used. The amount of AA-2G formed was analyzed by comparing the retention times to that of the standard AA-2G and was then calculated on the basis of its standard curve of peak area.

The production yield of AA-2G was calculated as:

$$\text{Yield (\%)} = \frac{\text{the amount of AA-2G formed x 100}}{\text{the amount of AA added}}$$

2.15 Determination of suitable conditions for the production of AA-2G by immobilized CGTase

To determine the suitable conditions for the AA-2G formation by immobilized CGTase, the reaction conditions as described in 2.13 were examined by varying AANa concentrations, pH, temperature, and incubation time. The best condition yielding maximum amount of AA-2G formed by each parameter was then selected as the optimal conditions for the production of AA-2G. These conditions would then be used for further study in the reusability of immobilized CGTase for the production of AA-2G.

2.15.1 Effect of pH

To investigate the effect of pH on AA-2G synthesis, 60 g (wet weight) of immobilized CGTase (280 units) was incubated with 4% (w/v) β-CD, 4% (w/v) AANa, 0.2% (w/v) thiourea in 20 ml of 50 mM acetate buffer containing 10 mM $CaCl_2$ at different pH values (5.0, 5.5 and 6.0) at 40°C with shaking (150 rpm). The reaction was allowed to progress for 24 hours in the dark and then the amount of AA-2G content was determined by HPLC as described in section 2.14.

2.15.2 Effect of temperature

Suitable temperature for the enzymatic reaction of the AA-2G formation was determined. The immobilized CGTase (280 units) was incubated with the reaction mixture as described in section 2.13 at different temperatures (30°C, 40°C and 50°C). The amount of AA-2G content was determined by HPLC as described in section 2.14.

2.15.3 Effect of incubation time

The reaction mixture was prepared as previously described in section 2.13. The incubation time of CGTase catalyzed coupling reaction was varied form 0 to 48 hours. Samples were then taken at the designated time and 1.06% (w/v) metaphosphoric acid was added during the time-course of the reaction for the analysis of AA-2G. The amount of AA-2G content was determined by HPLC as described in section 2.14.

2.15.4 Effect of AA concentration

The concentration of AA as an acceptor was varied from 0.5-10 % (w/v). The reaction mixture was the same as 2.13. The AA-2G content in the reaction mixture after reaction was determined by HPLC as described in section 2.14.

2.16 Batch reusability of immobilized CGTase for AA-2G production

The reusability of immobilized CGTase on alumina for AA-2G production was studied in repeated batch in Erlenmeyer flask. Sixty grams (wet weight) of alumina immobilized CGTase containing approximately 280 units were incubated with 20 ml of 4% (w/v) β-CD and 2% (w/v) AANa in 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 with gentle shaking (150 rpm) at 40°C in the dark for 24 hours. After the end of each cycle, the reaction mixture was separated from immobilized CGTase by centrifugation (1380 *g*, 10 min) and assayed for AA-2G content by HPLC (section 2.14). The immobilized enzyme was successively washed 3 times with 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 5.0 and its activity was measured as described in section 2.9. The immobilized CGTase was then resuspended in 20 ml of freshly prepared substrate to start a new run. This treatment was repeated up to 3 times.