# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Equipments

Autoclave: Model HV-110, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Balance: Model PB303-S, Mettler Toledo, Switzerland

Bandelin Sonoplus Sonicator: Model HD220, Bandelin Electronic, Germany

CentriVap Concentrator: Model 79700-01, Labconco Corporation, USA

Electrophoresis power supply: Model EC135-90, Thermo Scientific, USA

Electrophoresis Unit: Mini protein, Biorad, USA

Fraction collector: Model Frac-920, *Amersham* Biosciences, USA

HisTrap$^{FF}$TM column: GE Healthcare, UK

Hot plate stirrer: Fisher Scientific, UK

HPAEC DX600: Dionex Corporation, Sunnydale, USA

Column Dionex CarboPac® *PA1* column 4x250 mm, 4 µm, with a 4x50 mm guard

Pulse Amperometric Detector: Dionex Corporation, Sunnydale, USA

HPLC: Shimadzu Corporation, Japan

Rezex RSO-oligosaccharide $Ag^{+}$ column 200x10 mm, with a 60x10 mm guard

Phenomenex Incorporation, USA

Refractive index detector: RID-10A, Shimadzu Corporation, Japan

Incubator shaker: Model Innova 40, New Brunswick Scientific, USA

Lyophilizer: FreeZone Benchtop Freeze Dry System, Labconco Corporation, USA

Magnetic stirrer: Model AGE, VELP® Scientifica, Italy

Membrane filter: Whatman, England

Microcentrifuge: Model 5430R, Eppendorf, Germany

Oven: Thermotec2000, Contherm Scientific Company Limited, New Zealand

pH meter: MP220, Mettler Toledo, Switzerland

Refrigeration Centrifuge Beckman Coulter Avanti J-30I, Beckman Instrument, USA

Streamline Vertical Larminar Flow Cabinets: Model SCV-4A1, Streamline Laboratory, Singapore

UV-VIS spectrophotometer: Model GENESYS 10S, Thermo Scientific, USA

Vortex: Model K-550-GE, Scientific industry Incorporation, USA

Water bath: Memmert, Germany

Water bath shaking: Model SBS30, UK



## 2.2 Chemicals

Acrylamide: Merck, Germany

Agar: Scharlau, Spain

Ampicillin: Sigma, USA

D-(-)-Arabinose: BDH Chemical Limited, England

L-Ascorbic acid: Sigma, USA

Bovine serum albumin (BSA): Sigma, USA

Bromophenol blue: Merck, Germany

n-Butanol: Fisher Chemicals, UK

D-(+)-Cellobiose: Sigma, USA

Coomassie Brilliant Blue G-250: Sigma, USA

Coomassie Brilliant Blue R-250: Sigma, USA

Dialysis tube: Sigma, USA

Dipotassium hydrogen orthophosphate: Ajax Finechem, Australia

Epicatechin: Sigma, USA

Epigallocatechin gallate: Sigma, USA

Ethanol: Carlo Erba, Italy

Ethylacetate: Carlo Erba, Italy

Ethylenediaminetetraacetic acid (EDTA): Ajax Finechem, Australia

Fisetin: Sigma, USA

D-(-)-Fructose: Sigma, USA

L-Fucose: Sigma, USA



D-(+)-Galactose: Bio Basic Incorporation, Canada

D-(+)-Glucose: Fisher Chemicals, UK

Glycerol: Ajax Finechem, Australia

Glycine: Sigma, USA

Hesperidin: Sigma, USA

Hydrochloric acid: Carlo Erba, Italy

Imidazole: Fluka, Switzerland

Iodine: Baker Chemical, USA

Isopropyl-β-D-1-thiogalactopyranoside (IPTG): Sigma, USA

Lactose: Ajax Finechem, Australia

Maltoheptaose: Hayashibara Biochemical Laboratories Incorporation, Japan

Maltohexaose: Sigma, USA

Maltopentaose: Tokyo Chemical Industry Company Limited, Japan

Maltose: Conda, Spain

Maltotetraose: Hayashibara Biochemical Laboratories Incorporation, Japan

Maltotriose: Sigma, USA

D-(+)-Mannose: Sigma, USA

D-(+)-Melibiose: Tokyo Chemical Industry Company Limited, Japan

2-Mercaptoethanol: Bio Basic Incorporation, Canada

Methanol: Mallinckrodt, USA

Naringin: Sigma, USA

Palatinose: Sigma, USA

Phenol: Fisher Chemicals, UK

Phenylmethanesulfonyl fluoride: Sigma, USA



Phosphoric acid: Carlo Erba, Italy

Pinostrobin: Sigma, USA

Potassium dihydrogen phosphate: Ajax Finechem, Australia

Potassium iodide: Mallinckrodt, USA

Propanol: Feuergefährlichl, Germany

Pyridine: Carlo Erba, Italy

D-(+)-Raffinose: Nacalai Tesque Incorporation, Japan

L-Rhamnose: Sigma, USA

Rat intestine acetone powder: Sigma, USA

Ribose: Wako, Japan

Sodium acetate: BDH Chemical Limited, England

Sodium chloride: Ajax Finechem, Australia

Sodium dodecyl sulfate (SDS): Sigma, USA

Sodium hydroxide: Merck, Germany

Soluble potato starch: Scharlau, Spain

D-Sorbitol: Bio Basic Incorporation, Canada

Standard protein marker: Sigma, USA

Sucrose: Bio Basic Incorporation, Canada

Sulfuric acid: RCI Labscan limited, Thailand

N, N, N', N'-Tetramethylethylenediamine (TEMED): Carlo Erba, Italy

Tris (hydroxymethyl)-aminomethane: Carlo Erba, Italy

Tryptone: HiMedia, India

Xylitol: Bio Basic Incorporation, Canada

Yeast extract: Scharlau, Spain



### 2.3 Bacteria strain

AM gene from *C. glutamicum* ATCC 13032 was cloned and expressed in *E. coli* BL21(DE3) using pET-19b expression vector (Srisimarat *et al.* 2011).

### 2.4 Medium preparation

LB medium (Luria Bertani) was consisted of 0.5% yeast extract, 0.5% NaCl and 1.0% tryptone, all in w/v. The medium was then sterilized by autoclave at 121°C for 15 minutes.

### 2.5 Starter culture

*E. coli* was streaked on LB agar containing 100 µg/ml of ampicillin and incubated at 37 °C for 12 hours and then a single colony of *E. coli* was inoculated into LB broth and cultured at 37 °C for 14-18 hours with shaking.

### 2.6 Preparation and purification of enzyme

1% (v/v) of starter culture was transferred into LB medium containing 100

μg/ml of ampicillin and cultivated at the same condition as in starter culture until $O.D._{600}$ reached 0.4-0.6, then 0.4 mM IPTG was added to induce the enzyme production. After 2 hours of incubation, cells were collected by centrifugation at 4 °C, 3800×g for 15 minutes, washed by 0.85 % (w/v) NaCl (normal saline) and collected again by centrifugation at the same speed. Cell pellet was then resuspended by extraction buffer containing 100 mM PMSF, 0.5 M EDTA, 0.1% (v/v) mercaptoethanol and 50 mM phosphate buffer, pH 7.4, then centrifuged again to collect the cells. Cell pellet was weighed and dissolved by adding extraction buffer (2.5 ml of extraction buffer/1 g cell pellet). Intracellular crude enzyme was obtained by centrifugation at 4 °C, 18,000×g for 45 minutes to remove the cell debris after sonication and dialyzed against 20 mM phosphate buffer, pH 7.4. Finally, the enzyme was purified by HisTrap affinity column chromatography. 3 ml of crude enzyme was filtrated through a 0.45 μm filter membrane before applied onto the column, followed by washing with binding buffer containing 20 mM phosphate buffer, pH 7.4, 0.5 M NaCl and 20 mM imidazole at 4 °C with a flow rate of 1 ml/min. 3 ml of each fraction was collected to measure $A_{280}$ until absorbance was around zero. Then the enzyme was eluted with the elution buffer (500 mM imidazole in the washing buffer). The purified enzyme was collected and dialyzed against 20 mM phosphate buffer, pH 7.4 at 4°C. Then enzyme activity, purity, and concentration of protein were examined.



**2.7 Starch transglucosylation activity assay**

Starch transglucosylation activity was determined by iodine method measuring the decreasing of starch in the reaction. 100 μl of enzyme was incubated with 250 μl of 0.25% (w/v) soluble potato starch and 50 μl of 1% (w/v) maltose at 30

°C for 10 minutes and reaction was stopped by boiling for 10 minutes. After that, 100 µl of sample was removed to test with 1 ml of iodine solution (0.02% iodine in 0.2% potassium iodide, w/v) and $A_{600}$ was then immediately recorded (Park *et al.* 2007).

One unit of starch transglucosylation activity was defined as the amount of enzyme that produces 1% decrease in blue color of starch-iodine complex per minute.

**2.8 Determination of protein concentration**

Protein concentration was determined by Bradford method (Bradford 1976). 100 µl of enzyme was mixed with 1 ml of Bradford solution which consists of 0.1% (w/v) Coomassie Brilliant Blue G-250 in absolute ethanol with 85% (v/v) phosphoric acid. Then reaction was kept in the dark for 5 minutes and $A_{595}$ was measured. Protein concentration was analyzed compared with Bovine serum albumin (BSA) as a protein standard.



**2.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

The purity and molecular weight of enzyme were examined by SDS-PAGE (Bollag and Edlestein 1996). The casting frames (clamp two glass plates in the casting frames) were set on the casting stands and 7.5% separating gel solution comprising 40% acrylamide, 10% (w/v) SDS, Tris-HCl, pH 8.8, 10% (w/v) ammonium persulfate and 0.8 mg/ml TEMED was mixed and poured into the channel between two plates and wait until the gel was polymerized. Then 5% stacking gel solution containing the same solution as the separating gel but Tris-HCl, pH 8.8 was replaced by Tris-HCl, pH 6.8 was applied on the top of channel and the comb was immediately inserted. Sample/low molecular weight protein as a standard marker with SDS-dye was boiled for 5 minutes and loaded into the wells. The denatured protein ran from cathode (+)

to anode (-) in Tris-glycine buffer, pH 8.3 with constant current at 15 mA per slab gel.

Gel was stained by staining solution (1 liter consists of 1 g Commassie *Brilliant* Blue R-250 in absolute methanol and glacial acetic acid) for 2 hours with slow shaking. After removal of staining solution, the gel was destained by destaining solution until the background was clear.

**2.10 Determination of acceptor specificity and synthesis of glucoside products**

Three groups of acceptors were analyzed: short chain alcohols, flavonoids and saccharides. Short chain alcohols tested were consisted of methanol, ethanol, propanol and butanol. Various types of flavonoid were tested: hesperidin, naringin, pinostrobin, fisetin, epicatechin and epigallocatechin gallate. Saccharide group was classified into four subgroups: monosaccharides, this subgroup was divided into 4 types: hexose (glucose, mannose, galactose and fructose), deoxyhexose (fucose and rhamnose), pentose (arabinose and ribose) and monosaccharide derivatives (xylitol, sorbitol and ascorbic acid), disaccharides (maltose, cellobiose, sucrose, lactose, melibiose and palatinose), trisaccharides (maltotriose and raffinose) and oligosaccharides (maltotetraose, G4 to maltoheptaose, G7). The structure of acceptors used was as shown in Appendix F and G

Starch transglucosylation activity assay as described in 2.7 was performed to determine acceptor specificity for short chain alcohol and saccharide groups while flavonoids detection was by TLC. In the reaction mixture, soluble potato starch acts as a donor for various types of acceptors. For short chain alcohols and saccharides, 3 U/ml of enzyme solution was incubated with 0.2% (w/v) soluble potato starch and 1-30% (v/v) short chain alcohol or 2.5 mM saccharide acceptor at 30 °C for 10 minutes. The reaction was then stopped and analyzed as described. The relative activity was

calculated and the highest activity was set as 100%. After that, glucoside products from various acceptors were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) technique.

In order to investigate the effect of short chain alcohols on enzyme activity, 3 U/ml of enzyme solution was incubated with various concentrations of short chain alcohols (0, 1, 2, 3, 4, 5, 10, 20 and 30 %, v/v) in phosphate buffer, pH 6.0 at 30 °C for 60 minutes. Every 10 minutes, 100 µl of sample was then withdrawn for determination of starch transglucosylation activity by adding 1 ml of iodine solution (0.02% iodine in 0.2% potassium iodide, w/v) and $A_{600}$ was then recorded, immediately.



For flavonoids acceptor, 5 U/ml of enzyme was incubated with 10% (w/v) soluble potato starch and 0.05% (w/v) hesperidin or pinostrobin or fisetin, 2.5% (w/v) naringin, 0.5% (w/v) epicatechin or epigallocatechin gallate in phosphate buffer, pH 6.0 at 30°C for 24 hours. Glucoside products were analyzed by TLC.

### 2.11 TLC analysis

Glucoside products from saccharide acceptors were separated using butanol:pyridine:water (5:4:1 by volume) as a solvent system. 20 µl sample was spotted on silica gel 60 and this TLC plate was twice developed. After that, glucoside products were detected by spraying the mixture of concentrated sulfuric acid and absolute methanol (1:2 by volume) and TLC plate was heated at 110 °C for 20 minutes (Dawson *et al.* 1986).

Glucoside products from short chain alcohols or flavonoid acceptors were analyzed using ethylacetate:acetic acid:water (3:1:1 by volume) as a solvent system, then detected by the same spraying solvent and procedure as described above.

### 2.12 HPLC analysis

Glucoside products from saccharide acceptors were also analyzed by HPLC (Shimadzu) using Resex RSO-oligosaccharide $Ag^+$ column (200×10 mm) and refractive index detector (RID). Solution of glucoside products obtained before optimization was centrifuged at 4 °C, 18000×g for 45 minutes to remove the remain soluble potato starch in the reaction and filtrated through a 0.22 µm filter membrane before loading of 5 µl of sample onto the column. The glucoside products were eluted by filtrated ultrapure water at 80 °C with the flow rate of 0.2 ml/min. Each glucoside product was eluted at different retention time and the suitable acceptor was selected from the product yield obtained. Glucoside product yield was calculated from the equation:

$$\text{Yield (\%)} = \frac{\text{peak area of product}}{\text{peak area of acceptor at } t_0} \times 100$$

For the glucoside products obtained after optimization (see 2.13), the reaction mixture was further treated with 40 U/ml of glucoamylase from *Aspergillus niger* and incubated at 50 °C for 2 hours. After that, the reaction was stopped by boiling for 10 minutes. Then centrifuged, filtrated, and loading onto HPLC column as described aboved. The yield was then calculated as the equation where

peak area of product = peak area of acceptor after glucoamylase treatment – peak area of acceptor without glucoamylase treatment

### 2.13 Optimization of the synthesis of glucoside products

Optimization for the highest glucoside product yield was performed by varying four parameters: acceptor concentration, donor concentration, enzyme

concentration and incubation time.

**Effect of acceptor concentration**

In 600 µl of reaction mixture, 5% (w/v) soluble potato starch was incubated with 3 U/ml of enzyme and various acceptor concentrations (0, 1.0, 2.5, 5.0, 7.5, 10 and 15 mM) in 50 mM phosphate buffer, pH 6.0 at 30 °C for 24 hours. The reaction was then stopped by boiling for 10 minutes. Analysis of glucoside products was performed by HPLC.

**Effect of donor concentration**

Reaction mixture of 600 µl consisting of appropriate acceptor concentration with 3 U/ml enzyme and various soluble potato starch concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0 and 5.0 % w/v) in 50 mM phosphate buffer, pH 6.0 was incubated at 30 °C for 24 hours. The reaction was then stopped and analyzed as described above.



**Effect of enzyme concentration**

The optimal acceptor concentration was incubated with appropriate soluble potato starch concentration and various enzyme concentrations (0, 1.0, 3.0, 5.0, 7.0, 9.0 and 12.0 U/ml) in 50 mM phosphate buffer, pH 6.0 in a reaction mixture of 600 µl at 30 °C for 24 hours. The reaction was then stopped and analyzed as described above.

**Effect of incubation time**

Appropriate acceptor concentration was incubated with the optimal soluble potato starch concentration and suitable enzyme concentration in 50 mM phosphate

buffer, pH 6.0 in a reaction mixture of 600 µl at 30 °C for various times (0, 3, 6,12,18, 24, 30, 36 and 42 hours). The reaction was then stopped and analyzed as described above.

**2.14 Large scale preparation, product separation and identification**

The preparation scale of synthesis of glucoside products was enlarged from a reaction mixture of 600 µl (in 2.13) to 60 ml using optimized condition obtained. The glucoside products were then separated by a Biogel-P2 column (1.2 × 97cm). The remain soluble potato starch in the reaction mixture was removed by centrifugation at 4 °C, 18,000× g for 30 minutes before concentrated by CentriVap Concentrator at 45 °C and 2.5 ml of sample was applied onto Biogel-P2 column that was previously equilibrated with filtrated ultrapure water. Glucoside products were then eluted by that ultrapure water at room temperature with a flow rate of 8 ml/hr, Fractions of 1 ml were collected and sugar content was further analyzed by phenol-sulfuric acid method and identified by HPLC and HPAEC techniques.



**2.15 Phenol-sulfuric acid method**

Phenol-sulfuric acid method was performed to determine total carbohydrate in the reaction (Dubois *et al.* 1956). 100 µl of sample was added with 500 µl of 5% (w/v) phenol and 2 ml of concentrated sulfuric acid, mixed by vortex then let stand on ice for 10 minutes. After that, mixed again and stand at room temperature for 30 minutes before measuring $A_{490}$.

**2.16 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)**

HPAEC-PAD was examined to analyze carbohydrates with a CarboPac-PA1

column (4x250 mm) and the result was detected by PAD. Sample was filtrated through a 0.22 μm filter membrane before loading of 25 μl of sample onto the column. Then glucoside products were eluted by steps of gradient concentrations of NaOH and $CH_3COONa$ at 30°C with a flow rate of 1.0 ml/min. Firstly, the elution was by 150 mM NaOH at 1-5 minutes. After that, from 6-43 minutes, the glucoside products were eluted by 150 mM NaOH containing 600 mM $CH_3COONa$. Finally, for the last 2 minutes, 500 mM NaOH was used as elution buffer and glucoside products were detected and identified compared with standard G1-G7 by PAD. Then short chain palatinose glucosides (PG1-PG10) and long chain palatinose glucosides (PG1-15) were lyophilized.



### 2.17 Characterization of glucoside products

#### 2.17.1 Mass Spectrometry (MS)

The mass of PGs were analyzed by Time of Flight Mass Spectrometry (TOF-MS) with positive mode using the mixture of methanol and water (1:9 by volume) as solvent. TOF-MS was performed at the Central Instrument Facility, Mahidol University.

#### 2.17.2 Nuclear Magnetic Resonance (NMR)

5 mg of sample was dissolved in deuterated water ($D_2O$) and the linkage between palatinose and glucosyl unit was analyzed by $^1H$ NMR spectra. NMR analysis was performed at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

#### 2.17.3 Sweetness test

The sweetness of palatinose and glucoside products was measured in

comparison to sucrose as reference compound. Various concentrations (0, 5, 10, 15 and 20%, w/v) of sucrose were tested and brix values were measured by brix refractometer and the standard curve was plotted. The relative sweetness of 10% (w/v) palatinose, short chain PGs and long chain PGs was determined from sucrose standard curve.

% Brix (w/v) was defined as the amount in g of sucrose in 100 ml of solution (Wrolstad 2011).

### 2.17.4 Hygroscopic test

5 mg of palatinose, short chain PGs and long chain PGs were weighed in each preweighed tube, open the tube and put in growth chamber at 25 °C with 70% relative humidity. Then the weight of samples was recorded every 24 hours for one week.

Hygroscopic was defined as the increasing in weight from 2% to 15%.

### 2.17.5 Prebiotic activity

Prebiotic activity of palatinose, short chain PGs and long chain PGs were tested (Manderson *et al.* 2005). In the reaction of 1 ml, 0.5 ml of 100 mg/ml of rat intestinal enzyme in 25 mM acetated buffer, pH 6.0 was incubated with 5 mg palatinose, short chain PGs or long chain PGs at 37 °C for 1 hour and the reaction was stopped by boiling for 10 minutes. Then the products were analyzed by HPAEC-PAD.

### 2.17.6 Antibacterial activity

Two types of bacteria were tested: *E. coli* TOP10 and *Staphylococcus aureus*. The bacteria were spreaded on LB-agar plate containing 10% (w/v) palatinose, short

chain PGs or long chain PGs, then incubated at 37 °C for 16-18 hours. The clear zone was observed and recored. For *E. coli*, the positive control was LB-agar containing 100 µg/ml ampicillin while the negative control was LB-agar containing 100 µg/ml streptomycin. For *S.aureus*, the positive control was LB-agar containing ampicillin whereas the negative control was LB-agar containing 100 µg/ml kanamycin (Lalitha 2004).