CHAPTER III
MATERIALS AND METHODS

1. Chemicals

Mulberroside A (MuA) was obtained from Associate Professor Dr. Boonchoo Sritularuk (Pharmaceutical Sciences, Chulalongkorn University, Thailand), isolated from root bark of *M. alba* as described previously (Hirakura et al., 1986). The structure was confirmed by the comparison of nuclear magnetic resonance (NMR) data in the literature. Oxyresveratrol was obtained from Hangzhou Great Forest Biomedical (Hangzhou, China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants were purchased from Sigma (MO, USA). Peroxidase-labeled anti-rabbit IgG was purchased from MP Biomedicals (Ohio, USA). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-deoxynojirimycin (DNJ) were purchased from Wako (Osaka, Japan).

Murashige and skoog (MS) medium were identical to Murashige and Skoog 1996 (Appendix A). *N*-Phenyl-*N*'-1,2,3-thidiazol-5'-yl urea (Thidaizuron, TDZ) was purchased from Sigma-Aldrich (MO, USA). 1-Naphtalene acetic acid (NAA) was purchased from Fluka Chemical (Buchs, Switzerland).

Potato dextrose broth and Lysogeny broth (LB) were purchased from Becton, Dickinson and Company (Maryland, USA). Yeast extract (YE) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Methyl jasmonate (MJ) and salicylic acid (SA) were purchased from Wako Pure Chemical Industried, Ltd. (Osaka Japan). Chitosan (CT) was purchased from Fluka Chemical (Buchs, Switzerland).

1,1 Diphenyl-2-picrylhydrazyl (DPPH), *P*-nitrophenyl-α-*D*-glucopyranoside and L-DOPA were purchased from Tesque, Inc. (Kyoto, Japan). α-Glucosidase enzyme from microorganism was purchased from Toyobo, Ltd (Osaka, Japan). Tyrosinase enzyme from mushroom and Amberlite® XAD7HP were purchased from Sigma-Aldrich (MO, USA).

All other chemicals were standard commercial products of analytical grade.

2. The Production of MuA Polyclonal Antibody

2.1 Plant materials and sample preparation

Mulberry (*M. alba*) samples were collected from Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand (April 2011) and Graduate School of Pharmaceutical Sciences, Kyushu University, Japan (April 2012).

Cell suspension culture was obtained from leaf-derived callus of *M. alba* cultured on Murashige and Skoog (MS) liquid medium supplemented with 0.1 mg/l thidiazuron (TDZ) and 1 mg/l 1-naphthalene acetic acid (NAA). Root cultured was obtained from root of *M. alba* cultured on half strength MS liquid medium supplemented with 1 mg/l NAA.

Dried powdered of plant samples (10 mg) were weighed, extracted with 500 µl methanol and then sonicated for 15 min. The extracts were centrifuged at 3,000 rpm for 3 min to collect the supernatant. This extraction procedure was repeated three times. The combined extract were evaporated at 50°C and re-dissolved in 1 ml methanol. Consequently, sample solutions were diluted into appropriated concentration for MuA determination by ELISA and HPLC.

2.2 Synthesis of MuA-conjugates

The MuA-BSA conjugate was synthesized by the periodate oxidation method (Figure 6). MuA (4 mg) in 0.4 ml dimethyl sulfoxide (DMSO) was slowly added to 0.6 ml of aqueous solution containing sodium periodate (4 mg), and then stirred at room temperature for 1 h. Next, BSA (5 mg) in 1 ml carbonate buffer (50 mM, pH 9.6) was added and stirred for 5 h. The reaction mixture was dialyzed with water (5 times), then lyophilized. Finally, MuA-BSA was obtained. The same method was used to synthesize MuA-OVA.

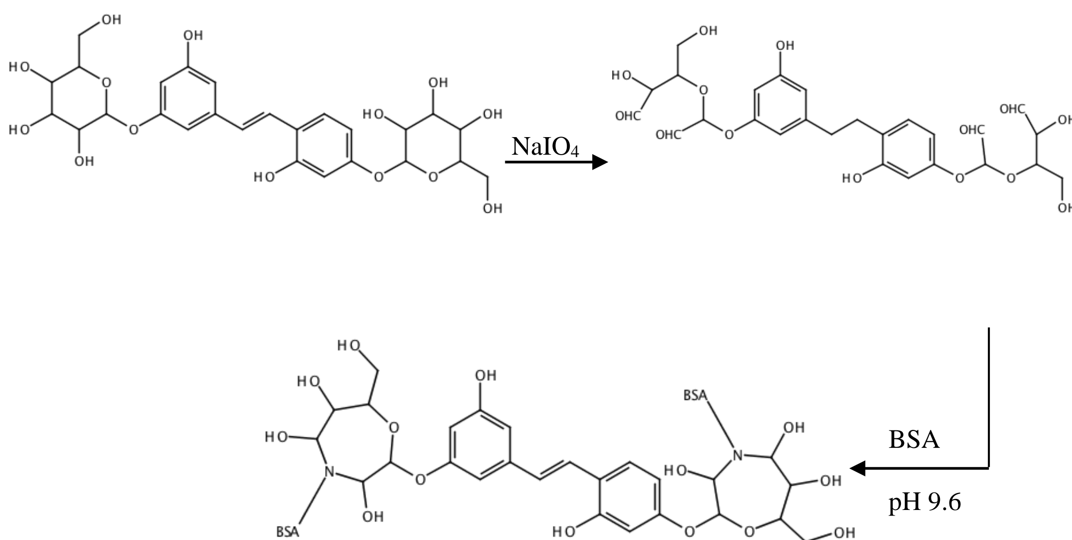

Figure 6 Periodate reaction of MuA-BSA conjugation

2.3 Determination for the hapten number of MuA–BSA

The hapten number in the MuA-BSA conjugate was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) similarly as previously described (Putalun et al., 2002). MuA-BSA conjugate (1-10 pmol) was mixed with a 3-10 folds molar excess of saturated sinapinic acid in an aqueous solution containing 30% of acetonitrile and 0.15% trifluoroacetic acid. The supernatant of mixture was subjected to the MALDI-TOF-MS (Autoflex III smartbeam; Bruker).

2.4 Immunization and purification

Female New Zealand White rabbits were obtained from National Animal Centre, Mahidol University, Nakhon Pathom, Thailand. Animal handing and the treatment protocol were conducted according to Khon Kaen University's Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethic Committee of Khon Kaen University (Record No. AEKKU 44/2555). The rabbits were immunized subcutaneously with 1 ml 1:1 emulsion mixture of MuA-BSA 0.6 mg in phosphate buffered saline (PBS) and Freund's complete adjuvant. After 4 weeks, second immunization was performed by same conjugate emulsified with Freund's incomplete adjuvant (intramuscular; I.M.) and final immunization was

carried out with the same emulsion mixture 2 weeks later. Seven days after the third immunization, rabbit serum was collected and stored at -20°C until used.

The serum was purified by using protein G FF column (Amersham Pharmacia Biotech, Uppsala, Sweden), washed with 20 mM phosphate buffer (pH 7). Bound IgG was eluted with 100 mM citrate buffer (pH 2.7). The eluted IgG was neutralized with 1 M Tris–HCl buffer (pH 9), then dialyzed with water and lyophilized.

2.5 Indirect ELISA

96-well immunoplate (Maxisorb Nunc, Roskilde, Denmark) were coated with 1 µg/ml MuA-OVA in carbonate buffer (100 µl/well), and then treated with 300 µl PBS containing 1% of gelatin for 1 h to reduce non-specific adsorption. The plate was washed three times with PBS containing of 0.05 % Tween 20 (TPBS), then reacted with 100 µl of PAb against MuA solution for 1 h. The plate was washed three times with TPBS, and then incubated with 100 µl of a 1,000 fold dilution of peroxidase-labeled anti-rabbit IgG for 1 h. After washing the plate three times with TPBS, 100 µl of substrate solution [0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/ml ABTS] was added to each well and incubated for 15 min. The absorbance at 405 nm was measured using a microplate reader (Model 550 Microplate Reader BioRad Laboratories, CA, USA). All reactions were incubated at 37°C.

2.6 Indirect competitive ELISA

After coating 96-well immunoplate with MuA-OVA, then it was treated with PBS containing 1% of gelatin to reduce non-specific adsorption. Various concentrations of MuA or samples (50 µl) dissolved with 20% methanol were incubated with 50 µl of PAb solution for 1 h. The plate was washed three times with TPBS, and then incubated with 100 µl of 1,000 fold dilution of peroxidase-labeled anti-rabbit IgG solution for 1 h. After washing the plate three times with TPBS, 100 µl of substrate solution was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm.

2.7 Method validation

The reactivity of PAb was determined by indirect ELISA. The ELISA method was validated for sensitivity, precision, specificity and accuracy. The indirect competitive ELISA was performed to determine the competition when using different concentrations of MuA to evaluate the sensitivity and measurement range. Intra-assay

precision was evaluated by the variation of the measurement of standard MuA from well to well (n=5) and inter-assay precision was obtained from the variation from different plates (n=5) expressed as relative standard deviation (RSD%). The specificity of the assay was evaluated by a cross reactivity experiment of PAb against various structure related compounds. The method accuracy was examined by a recovery experiment. MuA (0-150 µg/ml) were added to a dried powder of *M. alba* root bark, extracted and then analyzed by the developed indirect competitive ELISA.

2.8 HPLC analysis

HPLC analysis was performed using a PerkinElmer Series 200 LC pump with PerkinElmer 785A UV/VIS detector. RP-18 column (LiChroCART®, 125 × 4 mm, 5 µm particle size; Merck, Germany) was used as stationary phase. The mobile phase consisted of 18% aqueous methanol containing 0.1% acetic acid with flow rate 1 ml/min. The detector wavelength was 320 nm (Piao et al., 2010). Each sample was examined in triplicate.

3. *In Vitro* Cultures of *M. alba*

3.1 Callus and root induction

To obtain the callus cultures, *M. alba* leaves were rinsed with tap water, then sterilized with 20 and 10% sodium hypochlorite for 15 min, respectively. After that the sterilized leaves were washed with sterilized distilled water several times, then cut into small pieces. Finally, the small piece leaves were plated on MS medium with 0.1 mg/l TDZ and 1 mg/l NAA. After 4 weeks the leaf-derived callus was transferred on liquid MS medium with 0.1 mg/l TDZ and 1 mg/l NAA.

Young twigs and fibrous roots of *M. alba* were sterilized with the same method. After that, the explants were washed with sterilized distilled water several times and then plated on MS medium. The root cultures were obtained by cutting the regenerated roots and transferred on half strength MS liquid medium supplemented with 1 mg/l NAA.

The *in vitro* cultures of *M. alba* were maintained at 25±1°C under 16 h light/day and shaked at 100 rpm on gyratory shaker.

3.2 Growth rates study

For studies on growth rates and MuA production, *in vitro* cultures of *M. alba* were subcultured several times. Cell suspension and root cultures in 125 ml flasks containing 30 ml liquid medium were harvested every weeks. The biomass of samples were measured as fresh weight and then dried in hot air oven at 50°C for 2 days. The dried samples were grounded and prepared the same as dried powdered as previous described (2.1). Each liquid medium sample was mixed and collected at -20°C until used. The MuA contents from samples were determined by indirect competitive ELISA. Each experiment was done in triplicate.

3.3 Elicitor preparation

Yeast extract (YE) was weighed, then dissolved in distilled water and adjust to pH 5.5. After that the yeast extracted solution was autoclaved. Methyl jasmonate (MJ) was dissolved in a few drops of absolute ethanol follow by diluting with deionized water. The MJ solution was adjusted to pH 5.5 and then filter-sterilized. Chitosan (CT) was dissolved in glacial acetic acid to final concentration 2% (v/v) by adding drop wise at 60°C in 15 min, diluted with deionized water, adjust to pH 5.5 and autoclaved, Salicylic acid (SA) was filter-sterilized before used. The elicitors were added to *M. alba in vitro* cultures (YE 0.5, 1 and 2 mg/ml; MJ 50, 100 and 200 µM CT 10, 20 and 50 mg/l; SA 50, 100 and 200 µM) on days 18, 19 or 20 after subcultured.

Microorganisms with different characteristic isolated from *M. alba* were picked and subcultured at least three times to obtained single strain. Isolated microorganisms were transferred onto liquid potato dextrose broth (PDB) or lysogeny broth (LB). Seven days old microbes were harvested, and then centrifuged at 10,000 rpm. Supernatant were removed and replaced with distilled water. Microbial suspension solutions were autoclaved and centrifuged at 10,000 rpm. Final concentrations of sterilized supernatants were measured by phenol-sulfuric method (Fan et al., 2011) and then adjusted with sterilized distilled water to 12.0±2.0 µmol/ml glucose equivalent. The water extracts were stored at 4°C until used as elicitors.

3.4 Phenol-sulfuric method

50 µl glucose (10-100 nmol/well) or sample was added to each wells, follow by 150 µl concentrated sulfuric acid and 30 µl 5% phenol, respectively. Next, the

microplate was incubated at 90°C for 5 min and then absorbance was measured at 490 nm (n=5) (Masuko, 2005).

3.5 Effects of cell density

Effects of cell density study was started by transferring 1, 3, 6, 9, 12 and 15 (±1%) g fresh weights of cell cultures into 30 ml fresh medium supplemented with 0.1 mg/l TDZ and 1 mg/l NAA. After three weeks, callus and mediums were harvested, the samples were weighted and determined MuA accumulation. Fresh weights (FW) and dry weight per fresh weight (DW/FW) ratio were calculated as following equation.

$$\text{FW ratio} = \frac{\text{Final fresh weight of biomass}}{\text{Initial fresh weight of biomass}}$$

$$\text{DW/FW ratio} = \frac{\text{Final dry weight of biomass}}{\text{Initial fresh weight of biomass}}$$

3.6 Preparation of samples for indirect competitive ELISA

Standard MuA and sample extracts were adjusted with distilled water or methanol to obtain standard or sample in 20% methanol for indirect competitive ELISA. 20% Methanol was used as blank. The serial dilutions of samples were obtained by addition of 20% methanol to appropriate concentration.

To determine extracellular MuA concentrations in *in vitro* cultures medium, standard MuA in methanol was diluted with medium to 20% methanol. 20% Methanol in medium was used as negative control. Medium samples were prepared by addition of methanol to 20% methanol in sample and then serially diluted with 20% methanol in medium.

4. Evaluation of *In Vitro* Cultures and Microorganism Isolated From *M. alba* for Alpha-Glucosidase Inhibitor Activity and Anti-Tyrosinase Activity

4.1 Isolation of microorganism from *M. alba*

M. alba twigs and fibrous roots were rinsed with tap water, dipped in 10% sodium hypochlorite (10 min), 70% ethanol for 30 seconds and washed with sterilized distilled water several times. Surfaces of explants were removed by cutting under

sterile conditions. Small pieces of surface sterilized explants were plated on PDB and LB. Different characteristic microorganism were selected, and then subcultured at least three times to obtain single strain. Isolated microorganisms were transferred onto liquid potato dextrose (PDB) or lysogeny broth (LB).

Effective stains were identified by FASMAC Co., Ltd. (Japan) using partial 16s rDNA, 28s rRNA or D2 rRNA sequencing analysis.

4.2 Crude medium extraction

Various liquid medium samples of 7 days old microorganism extracts were obtained by partition with ethyl acetate using separatory funnel, then it was evaporated at 45 °C to obtain dried ethyl acetate crude extracts.

Water part of *Trichoderma* sp. from partition was applied to XAD-7 resin column and washed with distilled water to remove highly water soluble compound and reduced medium volume, and then the selected parts were eluted with methanol. Methanol was removed from aqueous extracts by evaporated at 45°C to obtain dried extract of the water part.

Mycelium of *Trichoderma* sp. was extracted with methanol and then evaporated at 45°C. All of samples were redissolved with methanol to 10 mg crude extract/ml.

4.3 Alpha-glucosidase inhibitor activity

α-Glucosidase enzyme (100 µl, 0.7 units/ml) was mixed with 10 µl methanol or samples and incubated at 37°C for 10 min in 96-well plate. After that, 20 mM *p*-nitrophenyl-α-*D*-glucopyranoside in 0.1 M PBS (pH 6.8) were added, mixed and incubated at 37°C for 15 min. The reaction was stopped by adding sodium carbonate. Produced p-nitrophenol was measured by microplate reader at absorbance 405 nm (modified from Pujiyunto et al., 2012). DNJ was used as positive control. The percentage of α-glucosidase inhibitor activity was calculated as the following equation.

$$\% \text{ Inhibition} = 100 \times [(A-B)-(C-D)]/(A-B)$$

A = absorbance of negative control (methanol with enzyme)

B = absorbance of background (methanol without enzyme)

C = absorbance of sample (with enzyme)

D = absorbance of sample (without enzyme)

4.4 Anti-tyrosinase activity

To determined anti-tyrosinase activity, 20 µl mushroom tyrosinase enzyme (31 unit/ml in 0.1 M PBS pH 6.8) or PBS were mixed with 20 µl methanol (50%) or samples in 50% methanol, and then PBS was added to 180 µl. The mixtures were incubated for 10 min and then 20 µl L-dopa (2 mg/ml) was added. After incubation at 37°C for 15 min, amount of dopachrome was measured at absorbance 490 nm. (modified from Kim et al., 2012). Oxyresveratrol was used as positive control. The percentage of tyrosinase inhibitor activity was calculated as following the equation.

$$\% \text{ Inhibition} = 100 \times [(A)-(B-C)]/(A)$$

A = absorbance of negative control (50% methanol with enzyme)

B = absorbance of sample (with enzyme)

C = absorbance of sample (without enzyme)

5. Statistical Analysis

One-way analysis of variance (ANOVA) was performed to check different biomass, activities and accumulations of MuA in samples and compared with Duncan at 0.05 level of significant.