CHAPTER III

MATERIALS AND METHODS

3.1 Equipment

Autoclave (Isuzu, Seisakushu Co., Ltd., Japan)
Autopipette (Pipetman, Gilson, France)
Dialysis bag (Snake Skin Dialysis Tubing, Pierce, USA)
Electrophoresis unit (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden)
Freeze dryer (Labconco, USA)
Hot air oven (Memmert, Germany)
IEF electrophoresis unit (Multiphor II, Amersham Pharmacia Biotech, Sweden)
Larminar Flow (Safety Lab, Asian Chemical and Engineering Co., Ltd., Thailand)
Liquid Column Chromatography (AKTA prime, Amersham Pharmacia Biotech, Sweden)
MALDI-TOF Mass spectrometer (Biflex, Bruker, Germany)
Microcentrifuge (Biofuge pico Heraeus, Kendro, Germany)
Orbital Shaker (Kika-Werke GMBH & Co., Germany)
pH meter (Denver Instrument, USA)
Pipette tips (Bioline, USA)
Power Supply (EPS 3500 XL, Pharmacia, England)
Refrigarated centrifuge (Himac CR20B2, HITACHI, Japan)
Sonicate (DHA-1000, Branson, USA)
Spectrophotometer (TECAN, Austria)
Speed vacuum centrifuge (Heto-Holten, Denmark)
Vortex mixer (Vortex-Genie2, Scientific Industries, USA)
Water Bath Shaking (Memmert, Germany)

3.2 Chemicals

Acetic acid (Merck Ag Darmstadt, Germany)
Acetone (Merck Ag Darmstadt, Germany)
Acrylamide (Plusone Pharmacia Biotech, Sweden)
Ammonium persulfate (Plusone Pharmacia Biotech, Sweden)

Ammonium sulfate (Merck Ag Darmstadt, Germany)

Avicel® PH 101 (Fluka, Switzerland)

Bovine serum albumin (Sigma, U.S.A.)

Bromophenol Blue (USB, U.S.A)

n-butanol (Fluka, Switzerland)

2-butanol (Fluka, Switzerland)

Calcium chloride (Merck Ag Darmstadt, Germany)

Carboxymethyl-cellulose (Fluka, Switzerland)

Cellobiose (Fluka, Switzerland)

Cellulose azure (Sigma, U.S.A)

Cobalt chloride (Fluka, Switzerland)

Coomassie Brilliant Blue G-250 (USB, U.S.A)

Congo red (Sigma, U.S.A)

Copper sulfate (Fluka, Switzerland)

Dipotassium hydrogen phosphate (Merck Ag Darmstadt, Germany)

6,7-Dihydroxycomarin-6-glucoside (Fluka, Switzerland)

Dimethyl sulfoxide (Fluka, Switzerland)

Dinitrosalicylic acid (Fluka, Switzerland)

DL-Dithiothreitol (Sigma, U.S.A)

Ethylenediaminetetraacetic acid (Merck Ag Darmstadt, Germany)

1-Ethyl-3-(3-dimethylaminoproplyl) carbodiimide (Sigma, U.S.A)

Ethanol (Merck Ag Darmstadt, Germany)

Gentiobiose (Sigma, U.S.A)

Glucono-δ-lactone (Sigma, U.S.A)

D-Glucose (Fluka, Switzerland)

Glycerol (Plusone Pharmacia Biotech, Sweden)

Glycinamide (Sigma, U.S.A)

Glycine (Plusone Pharmacia Biotech, Sweden)

Lactose (Fluka, Switzerland)

Laminaribiose (Sigma, U.S.A)

Magnesium sulfate (Fluka, Switzerland)

Maltose (Fluka, Switzerland)

Manganese Chloride (Fluka, Switzerland)

Mercury chloride (Merck Ag Darmstadt, Germany)

Methanol (Merck Ag Darmstadt, Germany)

2-mercaptoethanol (Merck Ag Darmstadt, Germany)

N,N'-methylene-bis-acrylamide (Plusone Pharmacia Biotech, Sweden)

4-methyllumberiferryl-β-glucoside (Sigma, U.S.A)

2-Methyl-1-propanol (Fluka, Switzerland)

2-Methyl-2-propanol (Fluka, Switzerland)

p-nitrophenol (Sigma, U.S.A)

o-nitrophenyl-β-D-galactopyranoside (Sigma, U.S.A)

o-nitrophenyl-β-D-glucopyranoside (Sigma, U.S.A)

p-nitrophenyl-β-D-glucopyranoside (Sigma, U.S.A)

p-nitrophenyl-β-D-xylopyranoside (Sigma, U.S.A)

Phenyl Sepharose Fast Flow (Plusone Pharmacia Biotech, Sweden)

n-propanol (Fluka, Switzerland)

2-propanol (Fluka, Switzerland)

Sodium acetate (Merck Ag Darmstadt, Germany)

Sodium chloride (Merck Ag Darmstadt, Germany)

Sodium dodecyl sulfate (Plusone Pharmacia Biotech, Sweden)

Sodium hydroxide (Merck Ag Darmstadt, Germany)

Sophorose (Sigma, U.S.A)

SP Sepharose Fast Flow (Plusone Pharmacia Biotech, Sweden)

Standard Molecular Weight Marker (Sigma, U.S.A)

Standard pI marker (Fluka, Switzerland)

Sucrose (Sigma, U.S.A)

Superdex 200 HR (Plusone Pharmacia Biotech, Sweden)

TEMED (Plusone Pharmacia Biotech, Sweden)

Tris (USB, U.S.A)

Zinc Sulfate (Merck Ag Darmstadt, Germany)

3.3 Organism

Daldinia eschscholzii, a wood-decaying ascomycete fungus obtained from the Royal Forest Department, Bangkok, Thailand, was used in this investigation. The fungus was maintained on potato dextrose agar (PDA) at room temperature with periodic transfer.

3.4 Identification of wood decaying fungi *D. eschscholzii*

3.4.1 Macroscopical features

Colony characteristic of specimens such as shape, size, color, margin, pigment, and others were studied using a stereomicroscope (Leica model MZ6).

3.4.2 Microscopical features

3.4.2.1 Preparation of specimen for light microscope

Material preparation for slide culture technique was done onto V-shaped glass rods on filter paper in Petri dishes. The material was sterilized in an autoclave. Potato Dextrose Agar was poured in to sterile Petri dishes and when set the agar was cut into 1 × 1 × 0.3 cm size, then aseptically put transferred on to the prepared glass slide and inoculated with the culture of *D. eschscholzii* on the middle of the four edges of agar, that the piece of agar was then covered with a cover slip and sterile distilled water added to maintain moisture inside the Petri dish. The slide cultures were incubated at room temperature (25-30°C) until the fungus grew onto the glass slide and cover slip.

Semipermanent slides (from slide cultures) for light microscopy were mounted in lactophenol-cotton blue for observation. The microscopical mycelial structure was examined using light microscope (Olympus CH2).

3.4.2.2 Preparation of ascospores for scanning electron microscope

The ascospores of *D. eschscholzii* were fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodelate buffer (pH 7.2) for 2 hrs. The samples were then dehydrated under the series of ethanol concentration (70-95%) within 15 minutes. The samples were critical point dried and coated with gold using a sputter coater model. Changes of each fine immersed in absolute ethanol for 30 minutes for each twice and observed and photographed with a JSM-5410 LV scanning electron microscope.

3.4.3 Technique for induction of teleomoph

The present study raises the possibility of inducing teleomorphs from pure cultures of anamorphs by inoculating fungi onto wood which is then incubated to produce the teleomorphs. Characteristically of the genus *Daldinia* is that it only produces anamorphic structures. In the absence of a mature teleomorph, it is rarely possible to identify these isolate to species and therefore a special technique was developed to induce teleomorph formation.

(*i*) The culture with young stromata and anamorphs were separated from the others using cultural morphology and characteristics of anamorphs following Thienhirun (1997).

(*ii*) The separated isolates were inoculated on to PDA in bottles and incubated for 10 days at room temperature.

(*iii*) Mango twigs of about 5 cm diameter were freshly cut into 15 cm long pieces. They were put in autoclavable bags and then autoclaved (121 °C for 1 hr). One piece of sterilized twig was put vertically on the surface of each culture in the bottle and the cultures were kept at room temperature for 1 month.

(*iv*) The twigs that were colonized by the fungus were transferred from the bottles to new sterile bottles with moist sand. The bottles were capped and kept at a room temperature for several months. Stromata production was observed at this stage. Teleomorph induction was performed under shade in moist conditions.

3.4.4 Molecular Identification

Genomic DNA was prepared from fresh mycelial cultures of *D. eschscholzii* and extracted with cetyltrimethylammonium bromide (CTAB) as described in Zhou *et al.* (1999). PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 50 μl which comprised approx. 100 ng genomic DNA, 1x PCR Master Mix (fermentas, Califonia, USA), and the primer ITS1f (Grades and Bruns, 1993) and ITS4 (White *et al.*, 1990). The amplification was performed in a thermocycler (TGradient; Biometra, Germany) with 94 °C for 5 min, followed by 38 cycles of 94 °C for 1 min, 51 °C for 1 min and 72°C for 1 min, with final extension of 72°C for 5 min. PCR Product was purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and sequenced externally by Macrogen (Seoul, Korea) using the same primer as for amplification.

The ITS sequence of *D. eschscholzii* was submitted to GenBank with accession number AB284189. This sequence was also searched or compared with other fungal sequences using BLAST 2.1 at the web site http://ncbi.nml.nih.gov/BLAST/Blast.cgi.

3.5 Wood decay activity by *D. eschscholzii*

Wood blocks measuring approximately 1.0 × 1.0 × 3.5 cm were prepared from *Tectona grandis* obtained from a wood fiber board market in Klong Toey, Bangkok. The wood was soaked for 48 hours in distilled water, which was changed regularly. This process has been shown to remove some of the extractives that may inhibit fungal growth. The wood blocks were then oven dried for two days at 60 °C and placed in the desiccator for two hours prior to weighing (dry wood mass before treatment). After weighing, the wood blocks were autoclaved at 121 °C for 15 minutes in 20 ml of distilled water prior to inoculation. Discs of fungal culture were transferred into the flask (containing 50 ml PDA). When the fungus grown on the agar, place sterilized wood blocks were placed on the agar in the same flask. After 12 weeks incubation, each wood block was collected, washed to removed mycelium and fruiting bodies, and oven dried for two days at 60 °C. The wood blocks were then stored in desiccator for two hours prior to weighing (dry wood mass after treatment). The percentage wood mass loss after 12 weeks incubation was calculated as follows:

$$\text{Percent wood mass loss} = 100 - \frac{(\text{Dry wood mass after treatment})}{\text{Dry wood mass before treatment}} \times 100$$

Photographs of wood blocks decayed by the fungus were taken using the scanning electron microscope. Before analysis, samples were dried in a desiccator, and coated with gold in a vacuum evaporator system.

3.6 Cellulolytic enzyme activities on solid media

3.6.1 Cellulolysis basal medium (CBM)

The use of solid media for the detection of cellulolytic enzymes suggested by Pointing (1999) was referred to as the basic method in the present investigation. It consist

of $C_4H_{12}N_2O_6$ 5.0 g/L, yeast extract 0.1 g/L, KH_2PO_4 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 2.0 g/L, $Ca_2Cl_2{\cdot}2H_2O$ 0.001 g/L. *D. eschscholzii* was screened for its ability to produce extracellular cellulolytic enzymes on solid media. The relative enzyme activity was estimated by measuring the migration of dye and the formation of a clear zone.

3.6.2 Cellulase (cellulose azure agar)

The CBM medium supplemented with 1.8% w/v agar was transferred in 10 ml aliquots to glass culture bottles. The medium was sterilized by autoclaving at 121 °C for 20 min, and allowed to solidify. Gently and carefully viscous 1.0 ml CBM medium supplemented with 1% w/v cellulose azure and 1.8% w/v agar were mixed and added to the surface of the solidified agar as an overlay. The bilayered medium was inoculated with discs of *D. eschscholzii* from PDA plates and incubated. Migration of dye into the clear lower layer indicated the presence of cellulose.

3.6.3 Endoglucanase (CMC agar)

The CBM medium was supplemented with 1% w/v low viscosity carboxymethyl-cellulose (CMC) and 1.8% w/v agar was added. The medium was sterilized by autoclaving at 121 °C for 20 min, and dispensed into Petri dishes, allowed to solidify and inoculated with discs of the test fungus and incubated. After growth for 5-10 days, the plates were flooded with 2% aqueous Congo red and allowed to stand for 15 minutes. The stain was washed from the agar surface with distilled water and the plates were then flooded with 1 M NaCl to destain for 15 minutes. The NaCl solution was then removed. CMC degradation around the colonies (as endoglucanase activity) appeard as a yellow-opaque area against a red color for the nondegraded CMC.

3.6.4 β-glucosidase (esculin agar)

The activity of β-glucosidase was detected by growing the test fungus on agar containing esculin (6,7-dihydroxycomarin-6-glucoside) as the sole carbon source. The CBM medium was supplemented with 0.5% esculin, 1.8% w/v agar. The medium was sterilized by autoclaving at 121 °C for 20 min. One ml of a sterile 2% w/v aqueous ferric sulfate solution was aseptically added for each 100 ml of CBM. The medium was

dispensed into Petri dishes, allowed to solidify, inoculated and incubated. A black color developed in the medium by the colonies producing β-glucosidase.

3.7 Culture conditions and production of β-glucosidase

The stock culture of *D. eschscholzii* was maintained on potato dextrose agar medium. Unless otherwise stated, actively growing fungal mycelium from a 7 day old culture were transferred to a 250-ml Erlenmeyer flask containing 100 ml of Mandel's medium (Mandels and Weber, 1969) composed of; urea 0.3 g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2.0 g/L, $CaCl_2{\cdot}2H_2O$ 0.4 g/L, $MgSO_4{\cdot}4H_2O$ 0.3 g/L, peptone 1.0 g/L, $FeSO_4{\cdot}7H_2O$ 5.0 mg/L, $MnSO_4{\cdot}4H_2O$ 1.6 mg/L, $ZnSO_4{\cdot}7H_2O$ 1.4 mg/L, $CoCl_2{\cdot}6H_2O$ 2.0 mg/L, and Tween 80 2.0 ml/L, pH 5.5 to which 1% (wt/vol) of various carbon sources such as Avicel® PH-101, Carboxymethyl-cellulose (CMC), Filter paper (Whatman No.1), and glucose were added. The medium was sterilized by autoclaving at 121 °C for 20 min. Inoculated flasks were incubated on a rotary shaker at 150 rpm at 25 °C for 14 days under natural light condition. The culture fluid was filtered through filter paper, and the supernatant fluid was used as the crude enzyme preparation.

3.8 Analytical method

3.8.1 Assay of β-glucosidase

β-glucosidase activity was determined by measuring the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (PNPG) as described previously (Cai *et al.*, 1998). The reaction mixture (1 ml) containing 5 mM in 0.1 M sodium acetate buffer (pH 5.0), and appropriately diluted enzyme solution. After incubation at 50 °C for 30 min, the reaction was stopped by adding 2.4 ml ice-cold 0.25 M Na_2CO_3, and the color formed was measured at 410 nm. One unit of β-glucosidase activity was defined as the amount of enzyme liberating 1 μmole of p-nitrophenol per min under the assay conditions. Specific activity is defined as the number of units per milligram of protein.

3.8.2 Protein determination

Protein concentrations in the enzyme preparations were determined by the method of Bradford (1976) with reference to a standard calibration curve for bovine

serum albumin (BSA). During the column chromatographic separations, the elution profiles of proteins were determined by measuring absorbance at 280 nm.

3.8.3 Reducing sugar content

Reducing sugar content was measured using dinitrosalicylic acid (DNS) with D-glucose as the standard. The reaction mixture (1 ml) containing 5 mM in 0.1 M sodium acetate buffer (pH 5.0), DNS reagent was mixed well with the sample to be tested. The mixture was heated of 90 °C for 10 min and then cooled. The resultant solution at 575 nm was determined.

3.9 Purification of β-glucosidase from *D. eschscholzii*

3.9.1 Step of purification

All of the procedures were performed at the 4 °C, unless otherwise stated.

(*i*) *$(NH_4)_2SO_4$ precipitation.* To 5 L of culture supernatant, $(NH_4)_2SO_4$ was added to give 80% saturation. After standing overnight, the precipitate formed was collected by centrifugation at 10,000 g for 20 min, dissolved in 20 mM sodium acetate buffer, pH 5.0. The dissolved sample was dialyzed against the same buffer and concentrated by lyophilization.

(*ii*) *Cation exchange chromatography.* The sample solution was applied on a column (1.6 × 10 cm) of SP Sepharose Fast Flow equilibrated with 20 mM sodium acetate buffer, pH 5.0. Elution was undertaken with the same buffer at a flow rate of 1.0 ml/min. A linear gradient of 0-1.0M NaCl in the same buffer was then applied. Fractions of 10.0 ml each were collected and assayed for β-glucosidase activity. The fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further purification.

(*iii*) *Hydrophobic interaction chromatography.* To the active fraction from the SP Sepharose Fast Flow, $(NH_4)_2SO_4$ was added to a concentration of 30%. The mixture was applied to a column (1.6 × 10 cm) of Phenyl Sepharose Fast Flow equilibrated with 30% $(NH_4)_2SO_4$ in 20 mM sodium acetate buffer, pH 5.0. The column was then eluted with a gradient of 30%-0% $(NH_4)_2SO_4$ in 20mM sodium acetate buffer, pH 5.0, at a flow rate of 1.0 ml/min. Fractions of 5.0 ml were collected and assayed for β-glucosidase

activity. The active fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further purification.

(iv) Gel filtration chromatography. The active fraction from Phenyl Sepharose Fast Flow was applied to a column (1.6 × 60 cm) of Superdex 200 HR equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 100 mM NaCl at a flow rate of 1.0ml/min. Fractions of 5.0 ml were collected and assayed for β-glucosidase activity. The active fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further analysis.

3.9.2 Determination of enzyme purity and protein pattern on native-PAGE

The enzyme from each step of purification was analyzed for its native protein pattern and purity according to the method of Bollag (Bollag *et al.*, 1996). Electrophoresis conditions, protein and activity staining were described below.

Non-denaturating gel electrophoresis. The gel was carried out with 7.5% separating gels, and 5.0% stacking gel. Tris-glycine buffer pH 8.3 was used as the electrode buffer. The electrophoresis was run from cathode toward anode at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. After electrophoresis, proteins in the gel were visualized by Coomassie Blue R-250 staining and activity staining.

(i) Coomassie blue staining. The gel was staining solution was (0.1% (w/v) Coomassie Blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol). Destaining was performed by immersing the gel in destaining solution (10% (v/v) acetic acid and 45% (v/v) methanol) followed by several changes of destaining solution until the background was clear.

(ii) Enzyme activity staining. After the electrophoresis was complete, the gel was washed with 0.1 M sodium acetate buffer, pH 5.0 for 3 times, and then stained for β-glucosidase activity using 2.0 mM 4-methyllumberiferryl-β-glucoside in 0.1 M sodium acetate buffer, pH 5.0 and visualized under UV light.

3.10 Biochemical characterization β-glucosidase from *D. eschscholzii*

3.10.1 Molecular weight determination

3.10.1.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The gel was prepared with 0.1% SDS in 12.5% separating gels and 5.0% stacking gels. Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli (1970). Samples to be analyzed were treated with sample buffer and boiled for 5 min before application to the gel. Electrophoresis was run from the cathode to anode at a constant current of 20mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie Blue R-250 (Laemmli, 1970).

3.10.1.2 Matrix-assisted laser desorption ionization time of flight

The purified β-glucosidase from a Superdex 200 HR column was analyzed. Desalting of enzyme was performed using a reusable reverse-phase cartridge. The desalted enzyme was dried using a freeze dryer. The dried enzyme was dissolved in 50% acetonitrile in trifluoroacetic acid. Mass spectra of β-glucosidase were acquired using a MALDI-TOF mass spectrometer operating in linear and reflectron modes.

3.10.2 Isoelectric-focusing PAGE

IEF-PAGE was performed on the Phast-System of Pharmacia LKB using a precast gel, PhastGel IEF 3-9. Isoelectric points were determined using standard *pI* markers.

3.10.3 Effect of temperature on β-glucosidase activity and stability

The optimum temperature for enzyme activity was determined by monitoring each activity at 0.1 M acetate sodium acetate buffer pH 5.0 at various temperatures from

30 °C to 80 °C. Stability was measured by incubating the enzyme in 0.1 M acetate buffer pH 5.0 for 30 min at temperatures from 30° to 80 °C. Following incubation, the enzyme solution was cooled, and the remaining activity was determined under standard enzyme assay conditions.

3.10.4 Effect of pH on β-glucosidase activity and stability

The optimum pH of activity was determined by monitoring each activity at 50 °C at various pH values ranging between 3.0 to 9.0. The following buffers were used: 0.1 M sodium acetate buffer (pH 3.0-6.0); 0.1 M phosphate buffer (pH 6.0-7.0) and 0.1 M Tris-HCl buffer (pH 7.0-9.0). The β-glucosidase stability was examined at the pH values 3.0-9.0. Enzyme samples were pre-incubated in the above-cited buffers at 30 °C for 1 h before adding the substrate. After adjustment of the pH the residual activity was determined under standard enzyme assay conditions.

3.10.5 Effect of metals and reagents

The effects of various metal ions and reagents at 1 mM on β-glucosidase activity were determined by preincubating the enzyme with the individual reagents in 0.1 M sodium acetate buffer pH 5.0 at 30 °C for 30 min. Activities were then measured at 50 °C in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was recorded as 100%.

3.10.6 Substrate specificity

Several α- and β-glucosides (20 mM), saccharides (1%, wt/vol), and arylglycosides (5 mM) were tested as substrates for the purified enzyme. The p-nitrophenol released was determined under standard enzyme assay conditions. The total amount of reducing sugars (expressed as equivalent glucose) in 1.0 ml supernatant was determined by the modified dinitrosalicyclic acid (DNS) method.

3.10.7 Effect of alcohols

The effects of methanol, ethanol, *n*-propanol, and *n*-butanol on the hydrolysis of PNPG were studied using the β-glucosidase assay.

3.11 Kinetic mechanism studies of β-glucosidase from *D. eschscholzii*

3.11.1 Determination of kinetic parameters

The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for β-glucosidase by incubating in 0.1 M sodium acetate buffer pH 5.0 at 50 °C with PNPG at concentrations ranging from 0.5-25 mM.. Values for K_m and V_{max} were determined from Lineweaver-Burk plots.

3.11.2 Inhibitory effect of glucose and glucono-δ-lactone

Inhibition by glucose and glucono-δ-lactone of β-glucosidase was determined in the presence of PNPG as the substrate. Inhibition constants (K_i) were determined from corresponding Lineweaver-Burk plots using standard linear regression techniques.

3.11.3 Identification of active-site residues by carboxyl group modification

3.11.3.1 Kinetic of β-glucosidase inactivation by EDC

Carboxy groups of purified β-glucosidase from *D. eschscholzii* were activated by EDC in the presence of glycinamide as a nucleophile. Glycinamide (1 M) was added to 2.0 ml (5.0 U/ml) of purified β-glucosidase solution and the pH was adjusted to 5.5 with 12 M NaOH. An aliquot was taken before the addition of EDC and the reaction was initiated by adding a fixed amount of EDC at 25 °C. Aliquots (200 μl) were withdrawn at intervals and added to 200 μl of 0.5 M sodium acetate buffer, pH 5.0, and containing 1 M hydroxylamine to quench the reaction. This procedure was repeated at different EDC concentrations (25-100 mM). The time course aliquots were assayed for β-glucosidase activity after 6 hours.

3.11.3.2 Kinetic of active-site protection by glucono-δ-lactone

Glycinamide (1 M) was added to 2.0 ml (5.0 U/ml) of purified β-glucosidase solution and the pH was adjusted 5.5 with 12 M NaOH. An aliquot was taken before the addition of EDC and then the reaction was initiated by adding 75 mM of

EDC. Aliquots (200 μl) were withdrawn at intervals and added to 200 μl of 0.5 M sodium acetate buffer, pH 5.0, and containing 1 M hydroxylamine to quench the reaction. This procedure was repeated at different concentrations of glucono-δ-lactone, which is the competitive inhibitor of β-glucosidase. The time course aliquots were assayed for β-glucosidase activity after 6 hours.

3.12 Identification of β-glucosidase from *D. eschscholzii*

The internal amino acid sequence of β-glucosidase was performed by in gel digestion of protein and sequencing of different peptides by mass spectrometry. The ion spectra were analyzed and sequence determined. The analysis was performed at the National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand. Sequence comparison of internal peptides of β-glucosidase with β-glucosidases family was performed using individual peptides. Peptide matching for sample mass spectra was based on an accuracy of ± 1 Da. Peptides. Masses were matched to the SWISSPROT.

3.13 Synthesis of alkyl-glucosides using β-glucosidase

3.13.1 Transglycosylation reaction

Synthesis of alkyl-β-glucoside was performed by adding a fixed amount of β-glucosidase 0.5 U/ml to a reaction mixture containing 20 mM PNPG (glucosyl-donor) and primary, secondary, tertiary-alcohol 50% v/v (glucosyl-acceptor) in 0.1 M sodium acetate buffer pH 5.0. The reaction was performed at 30 °C with shaking and started by adding the enzyme solution for 24 h, and reaction stopped by boiling.

3.13.2 TLC analysis and confirm structures of alkyl-β-glucosides products

For different alkyl-β-glucosides, the products were separated by thin-layer chromatography on silica gel 60 F_{254}. The TLC was developed using 2 solvent systems; 1) ethylacetatae, methanol, water in the ratio (16:6:1) two times for 8.5 cm, and 2) 2-propanol, ethanol, water in the ratio (5:1:2) two times for 3.5 cm. After finishing each round of development, the plates were dried at 80-90 °C for 5 min. The developed TLC was stained by 10% sulfuric acid in ethanol (v/v) and heating in a hot air oven at

125 °C for 5 min. For confirmation of product structures by ^1H NMR, the product band was scraped and eluted with developing solvent, and filtrated on No.1 filter paper. The filtrate was concentrated by rotary evaporation. The samples prepared on D_2O were analyzed by ^1H NMR