CHAPTER III

EXPERIMENTAL

3.1 Material

3.1.1 Fungi

Isolated fungi were obtained from stock cultures of Research Centre of Bioorganic Chemistry (RCBC) Chulalongkorn University, Bangkok, Thailand.

3.1.2 Plant material

Fresh potatoes used for fungal media were purchased from Lotus Supermarket Rama III Bangkok, Thailand.

3.1.3 Starch sources

Corn starch (Knorr, Unilever Thai Holding.,Ltd.), tapioca starch (Baiyoke, Bangkok Inter food co.,Ltd0), rice Starch, sticky rice starch (Thai Better foods co.,Ltd) and wheat starch were purchased from Lotus Supermarket Rama III Bangkok, Thailand. Soluble starch was obtained from Merck (Germany)

3.1.4 Culture media

Starch agar was used for screening endophytic fungi. Potato dextrose agar (PDA) was used for the endophytic fungi isolation and observation of isolated endophytic fungi. Starch was used for cultivation method. The media formulas were shown in Appendix B

3.1.5 Chemical and Reagents

Acetic acid: Merck Ag Darmstadt, Germany

Acrylamide: Amersham pharmacia biotech, Sweden

Ammonium persulfate: Plusone pharmacia biotech, Sweden

Bovine serum albumin: Sigma, St. Louis, MO, USA

Bromophenol Blue: USB, U.S.A

Coomassie brilliant blue: USB, U.S.A

Dinitrosalicylic acid: Fluka, Germany

Ethanol: Merck Ag Darmstadt, Germany

Formic acid: Merck Ag Darmstadt, Germany

Glycine: USB, U.S.A

Iodine: Merck Ag Darmstadt, Germany

Maltose: Ajax Fine chem., Australia

Methanol: Merck Ag Darmstadt, Germany

N, N'- methylenebisacrylamide; Plusone pharmacia biotech, Sweden

Phenol: Merck Ag Darmstadt, Germany

Phosphoric acid:

Piperazine: Aldrich

Potassium iodide: Fluka, Germany

Potassiumdihydogen phosphate

Potassium sodium tartrate: Fluka, Germany

SDS (Sodium Dodecyl Sulfate): USB, U.S.A

Sodium hydroxide: Merck Ag Darmstadt, Germany

Sodium sulfite: Fluka, Germany

Trichloroacetic acid: BHD

Trifluoroacetic acid: Fluka, Germany

Tris: USB, U.S.A

α-Cyano-4-hydroxycinnamic acid: Sigma Chemical, U.S.A

3.1.6 Apparatus and Instruments

Aquasil C_{18} column (Thermo electron, USA.).

Autoclave (Isuzu, Seisakushu co., LTD, Japan)

Autopipette: Pipetman, Gilson, France

Desalting Cartridge (Protein macrotrap,Michrom BioResource Inc.)

Dialysis bag (SnakeSkin Dialysis Tubing, Pierce, USA.)

Freeze-dryer (Labconco, USA)

Hot Air oven (Memmert, Germany)

Laminar Flow (SAFETY LAB, Asian Chemical and Engineering co., LTD, Thailand)

Liquid Column Chromatography (AKTA prime, Amersham pharmacia biotech wikstroms, Sweden)

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometer (BIFLEX Bruker, Germany)

Microcentrifuge: Biofuge pico Heraeus, Kendro, Germany

Micropipette (Pipetteman, Gilson, France.)

Orbital Shaker (Kika-Werke GMBH&Co., Germany).

pH meter (Denver Instrument USA. system).

Pipette tips (Bioline USA.)

Power Supply: EPS 301, Amersham pharmacia biotech, Sweden

Refrigarated centrifuge: Hettich, Germany

SDS-PAGE Hoefer^{TM} miniVE (minivertical), 8×9 cm gels. (Amersham pharmacia biotech Uppsala Sweden).

Shaker 200 rpm

Spectrophotometer (TECAN, Austria)

Speed vacuum (MAXI dry plus, Heto vacuum centrifuge, Denmark)

Sonicate (DHA-100; Branson, U.S.A.)

Vortex mixer (Vortex-genie2, Sciencetific Industries.)

Water Bath Shaking (Memmert, Germany)

3.2 Screening amylase activity of endophytic fungi

Twenty five isolated of endophytic fungi were obtained from stock cultures of Research Centre of bioorganic Chemistry (RCBC), Chulalongkorn University. The fungal cultures were grown on a screening starch agar plate (Appendix B). After that, the cultures were incubated at room temperature for 8-10 days. The media were flooded with iodine solution (0.1% I_2 and 1% KI) for 15 sec and the iodine solution was removed. Clear zone were observed around the growing colony and the colonies which have a largest clear zone were isolated for further studied [18].

3.3 Identification of endophytic fungi

3.3.1 Morphological identification

3.3.1.2 Preparation of slide culture

Colony characteristics of endophytic fungus isolated EF6, for example, shape size color, margin, pigment and others were studies. The detail of preparation of slide culture was described below.

Slide cultures are made by setting up a small petri dish moist chamber containing round paper towel into the bottom of a sterile petri dish and placed a v-shaped piece of glass tubing or straw resting on several layers of paper towel. A sterile block of agar medium about 1 cm square is placed on a flame-sterilized microscope slide and the slide is then set in the moist chamber on the tubing (Figure 3.1). With a sterile inoculating needle or sterile teasing needle, inoculate a small fragment of the fungus to the four edges of each block. Place a steriled cover slip over each block and apply slight pressure to ensure adherence. Pipette a small amount of water into the bottom of the petri dish to saturate the paper towel. And then close plate and incubate at room temperature (or 30°C). After, growth visually appears. The cover slip was collected and then stained with a small drop of lactophenol cotton blue examined under microscope. The remaining block can be left intact for further incubation if necessary [48].

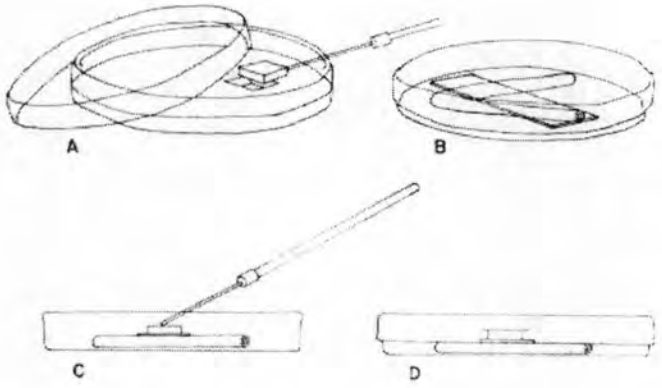

Figure 3.1 Slide culture techniques. A block of sterile agar is cut out of a Petri dish (A) and is placed upon a sterile slide resting on a bent glass tube within a sterile Petri dish (B). A few spores of a fungus are inoculated at the edges of the sterile agar block (C) and topped with a cover-glass (D) for incubation. A disc of moist filter-paper in the dish maintains humidity for the culture.

3.3.2 Molecular Identification

3.3.2.1 Preparation of DNA endophytic fungus isolated EF6 analysis

Genomic DNA was prepared from the fresh mycelial culture of isolated EF6 and extracted with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999) [49]. 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 [50] and ITS4 [51]. 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 endophytic fungus EF6 was submitted to GenBank with accession number AB274832. 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.

3.4 Growth measurement of fungal isolated EF6

The stock culture was maintained on Potato Dextrose Agar (PDA). Endophytic fungi were grown in starch broth containing, 2 g KH_2PO_4, 6 g NH_4NO_3, 0.1 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$ and 20 g soluble starch per liter of distilled water. The starch broth medium was steriled at 121°C for 15 min. A 250 ml Erlenmeyer flask containing 100 ml of the broth medium was inoculated with 3 hyphal disc (0.7 cm disc) using sterile cork-borer from stock culture and incubated at room temperature on a rotary shaker at 200 rpm. The growth of culture expressed in dry weight of mycelium was measured by harvesting the mycelium at regular interval of 24 h, filtering through Whatman filter paper No. 1 and washing three times with distilled water. The dry weight was determined after drying at 50°C to a constant dry weight overnight. The amount of amylase activity was determined using the method as describes below.

3.5 Cultivation method of fungal isolated EF6

For the production of enzyme, three pieces of hyphal disc using sterile cork-borer were transferred from stock culture to 250 ml Erlenmeyer flask containing 100 ml of the sterile broth medium. The medium was composed of 2 g KH_2PO_4, 6 g NH_4NO_3, 0.1 g $MgSO_4.7H_2O$, 0.01 g $FeSo_4.7H_2O$ and 20 g soluble starch per liter of distilled water. The incubation was carried out at room temperature on a rotary shaker at 200 rpm for 8 day. After the cultivation was completed, the culture was filtrated through a Whatman filter paper No.1 and the filtrate was used as the crude enzyme.

3.6 Amylase activity of fungal isolated EF6

Amylase activity was assayed by measuring the reducing sugar released during the reaction, using soluble starch as substrate. The reaction mixture consisted 1% (w/v) soluble starch in 0.02M piperazine buffer pH 5.5 0.1 ml and 0.1 ml of enzyme solution. After 15 min of incubation at 50°C, the reaction was stopped by placing in boiling water for 5 min and the amount of reducing sugar was measured by the dinitrosalicylic acid method of Miller (1959) as describe below. One unit of enzyme activity was defined as the amount of enzyme that released 1μmol reducing sugar per minute under relevant condition.

3.7 Determination of reducing sugar

The reducing sugar was determined by dinitrosalicylic acid method. The technique was prepared by using maltose to generate a standard curve. 0.1 ml of DNS reagent was added to 0.1 ml of sample in eppendorf. The mixture was placed in boil water for 10-15 min to develop the red-brown color. 30 μl of 40% potassium sodium tartrate solution was added to stabilize the color. The reaction was cooled at room temperature and measured the absorbance with a spectrophotometer at 575 nm [52].

3.8 Protein determination

Protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as standard calibration curve. For the quantitative of the protein in this research, micro assay method using a microplate reader has been used. Pipette 10 μl of protein sample into 96 well plates and add 200 μl of the Bradford working solution (Appendix B, 5). Measuring absorbance at 595 nm monitored protein. The concentration of the sample is obtained from a standard curve obtained by using known concentration of standard protein [53].

3.9 Enzyme purification from fungal isolated EF6

3.9.1 Precipitation with $(NH_4)_2SO_4$

The crude enzyme solution was obtained from cultivation broth after removal of mycelia by filtration was precipitated with $(NH_4)_2SO_4$ at 30%, 60% and 90% saturation and kept at 4°C overnight. The precipitated protein were collected by centrifugation at 4,000 rpm 15 min and dissolved in minimal amount of distilled water, dialyzed against three changes of distilled water and the insoluble residue was removed by centrifugation [53].

3.9.2 Ion Exchanges Chromatography: Q Sepharose

All steps were carried out at 4°C. The dialyzed enzyme solution was applied onto Q Sepharose column (1.6x10 cm) (Amersham Biosciences, Uppsala, Sweden) and equilibrated with 0.02M piperazine buffer pH 5.5 at a flow rate 1 ml/ min. The bound proteins were eluted with a NaCl salt gradient (0-0.5M) in the same buffer. Fraction of 10 ml were collected and assayed for amylase activity. The active fractions were pooled and concentrated maintained in a cold room. The protein absorption was monitored at 280 nm.

3.9.3 Gel Filtration : Superdex 75

The fraction containing enzyme activity from Q sepharose and dialyzed against with 0.02M piperazine buffer pH5.5 was applied on to a Superdex 75 Gel filtration column (1.6x60 cm) (Amersham Biosciences, Uppsala, Sweden), equilibrated and eluted by the same buffer. Elution was done at a flow rate of 0.5 ml/min and fractions of 10 ml were collected and assayed for amylase activity. The active fractions were pooled and concentrated. The protein absorption was monitored at 280 nm.

3.10 Characteristic of enzyme

3.10.1 Determination of amylase molecular weight

Amylase molecular weight was measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis 15%T, 3%C (SDS-PAGE) by the method of Laemmli method [53].

(a) Ensure that all gel cassettes, spacers, comb, and buffer chambers are clean. Always wear gloves while assembling the equipment and casting the gels. The separating gel is cast first and then overlaid with the stacking gel.

(b) Set up the casting apparatus and prepare the solution (Appendix B) for separating gel then allowed gel to polymerize by adding 10% ammonium persulfate and TEMED.

(c) Transfer the solution into the gel cassette and determine the gel volume to provide sufficient height of the separating gel ensuring that sufficient space (1-2 cm) below the comb is available for the stacking gel. Overlay the solution with water to exclude air and allow the gel to polymerize for 30-60 min.

(d) Rinse the top of separating gel with water and dry of residual liquid with a clean paper towel. Pour the stacking gel solution (Appendix B, Table 3) on top of separating gel, carefully insert and align the comb ensuring that no air bubbles are trapped under the teeth. Allow the gel polymerize for at least 30 min.

(e) Four parts of samples were mixed with one part of reducing buffer (Appendix B) in a 0.5 ml eppendorf, and heat in boiling water-bath for 5 min. Then cool to ambient temperature.

(f) Gently removed the comb from the stacking gel and loaded the samples and protein standard (molecular mass range 14.4 to 97.0 kDa) into the well using the gel loading tip.

(g) Attach the gel cassette to power supply. The lower electrode chamber is connected to the anode and the upper one to cathode in SDS-PAGE. The electrophoresis was carried out with electrophoresis buffer (Appendix B), 280V and

20 mA for one gel and then was terminated when the dye front reached to the bottom of the gel.

(h) Stain the electrophoresis gel by soaking in the Coomassie blue staining solution with agitation for 30 min and destain using methanol and acetic acid (see in Appendix B) with the several change until the background is sufficiently clear.

3.10.2 Native-PAGE

The method of Native-PAGE is the same with SDS-PAGE but different reagent (Appendix B). For Native–PAGE, the sample was mixed with the sample buffer not containing SDS or mercaptoethanol and was not heated. The gel from Native-PAGE was soaked in 1% soluble starch with gentle agitation at 50°C for 30 min. After that, removed soluble starch and flooded the gel with iodine solution [53,54].

3.10.3 Determination optimum pH of enzyme activity

The optimum pH of the reaction on hydrolysis of soluble starch was carried out in 0.02 M Sodium acetate buffer pH 3.0-6.0 ; 0.02 M phosphate buffer pH 5.0-7.0 and 0.02 M Tris-HCl buffer pH 7.0-9.0. The reaction mixtures containing 1 ml of 1% soluble starch in each buffer with 1 ml enzyme solution added were incubated at 50°C for 10 min. The reaction was stopped by placing them in boiling water for 5 min. The amount of sugar was expressed as reducing sugar determined using the method as describe above.

3.10.4 Determination optimum temperature of enzyme activity

The optimum temperature on enzyme activity was studies at different temperature 30, 40, 50, 60, 70, 80 and 90°C. The reaction mixtures containing 1 ml of 1% soluble starch in 0.02 M piperazine with 1 ml enzyme solution added were incubated at each temperature. Thermostability was measured by incubating the enzyme in 0.02 M piperazine buffer pH 5.5 for 30 min at 30, 40, 50, 60, 70, 80 and 90°C. After incubating, enzyme solution was cooled and the remaining activity was determined under standard assay conditions as described in section 3.6.

3.10.5 Effect of metal ion on enzyme activity

Metal ions $AgNO_2$, $AlK(SO_4)_2$, $BaCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$, $MgCl_2$, $MnCl_2$, NaCl, N_2O_6Sr and $ZnSO_4$ were dissolved in 0.02 M piperazine buffer pH 5.5. The effects of various metal ions at 10 mM on enzyme activity were determined by assaying the enzyme with the individual metal ions under the standard assay condition. The activity assayed in the absence of metal ions was taken as 100%.

3.10.6 Substrate specificity of enzyme activity

The effects of different raw starches as substrate were determined by the reaction of enzyme preparation in the presence of various raw starches (soluble starch, corn, tapioca, wheat, rice, sticky rice starch). One millitre of enzyme was added to 1 ml of 1% (w/v) raw starches in 0.02 M piperazine buffer pH 5.5 and incubated at 50°C for 10 min. The reaction was stopped by placing them in boiling water for 5 min. The amount of reducing sugar was assayed by standard procedure as described earlier. The control was reaction mixture with soluble starch.

3.10.7 Kinetic properties of amylase

Kinetic constant as K_m and V_{max} were measured by estimating hydrolysis with starch. Innitial velocity studies were carried out under the standard condition as described in section 3.6. The concentrations of soluble starch substrate were varied from 0.2-20 mg. The Lineweaver-Burk of initial velocity against substrate concentration was ploted.

3.11 Protein Identification of amylase

3.11.1 Trypsin In-Gel Digestion

The protein band of interest was excised from SDS-PAGE and place into 1.5 ml microcentrifuge tube. Add 200 µl of water (5 min, 2 times) for wash the gel. Remove the destain from gel by soaking in the 50% ACN/0.1 M NH_4HCO_3 with the gentle agitation for 20 min. The liquid was removed, and repeat this step until blue color is washed out. Dehydrate gel by using speedvac for 15 min or until gel shrink. Rehydrate gel in excess with 10 mM DTT/ 0.1 M NH_4HCO_3/EDTA and incubated for 45 min at 60°C to reduce the protein. Removed DTT solution and add 100 µl of 55 mM iodoacetamide (IAA) /0.1 mM NH_4HCO_3. Incubate at room temperature in the dark place for 30 min. Remove IAA and wash the gel in excess 0.05 M Tris-HCl pH 8.5/50% ACN. Dried gel by using speedvac and rehydrate gel particle in the 180 µl digestion buffer (containing 100 µl of 0.1 M Tris-HCl, pH 8.5, 2 µl of 100 mM $CaCl_2$, 20 µl of ACN and 78 µl of distilled water) and 20 µl of trypsin solution and incubate for overnight at 37°C. After overnight, add 100 µl of 2% TFA incubate for 30 min 60°C keep the digest solution supernatant into clean 1.5 ml microcentrifuge tube. Add 30 µl of digestion buffer to the containing the gel incubate for 10 min at 30°C and sonicate 5 min. The liquid was collected and combine in before microcentrifuge tube containing supernatant. After that add 30 µl of ACN/digestion buffer (1:1), incubate for 10 min at 30°C and sonicate 5 min. Combine the liquid in prior tube and add 5% formic/ACN in gel 30 µl for 10 min at 30°C and sonicate 5 min. Pool all supernatant in tube and dry in vacuum centrifuge [55].

3.12 Sample preparation for MALDI-TOF

Two microlittre of sample was mixed with 2 µl of CCA solution (10 mg α-cyano-4-hydroxy-cinnamic acid (CCA) in 50% ACN/ 0.1% TFA) in eppendrof 0.5 ml and spotted on the target plate. Myoglobin (1 mg/ml), Angiotensin II (1 mg/ml) and Bovine serum albumin (1 mg/ml) were used as external calibration for protein molecular mass.