CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Obtaining cDNA library

3.1.1.1 RNA Extraction

Green pit vipers (*Trimeresurus albolabris*), liquid nitrogen and Trizol reagent

3.1.1.2 mRNA Extraction

Poly AT tract mRNA Isolation System were purchased from Promega

3.1.1.3 cDNA Synthesis

Zap Express® cDNA Synthesis Kit and Zap Express® cDNA Gigapack® III Gold Cloning Kit were purchased from BD

3.1.2 Obtaining Full Length cDNA

3.1.2.1 Rapid amplification of cDNA Ends (RACE)

We use SMART™ RACE cDNA Amplification Kit purchased from CLONTECH Laboratories, Inc.

3.1.2.2 Gene Specific Primers (GSP)

Synthetic oligonucleotides were purchased from BGM

Name	Sequence	Description
T7	5′- GTAATACGACTCACTATAGGGC -3′	Sequencing primer from T7 promoter
SP6	5′-ACTCAAGCTATGCATCCAAC -3′	Sequencing primer from SP6 promoter
PUC/M 13 forward	5′-GTTTTCCCAGTGACGAC–3′	Sequencing primer from PUC/M13 forward promoter
PUC/M 13 reverse	5′-CAGGAAACAGCTACTATGAC–3′	Sequencing primer from PUC/M13 reverse promoter
Nup	5′-AAGCAGTGGTATCAACGCAGAGT–3′	Sequencing primer from Nup promoter
Serine F61	5′-GCGAATTCGTCATTGGAGGTGATGAATGC –3′	1^{st}5′-RACE PCR
KNBJ 1	5′-GTCTCTGATCCACGATGGCCAAGAATTT –3′	1^{st}5′-RACE PCR
KNBJ 27	5′-TGGGACATGATCCCTGCAGCACTA –3′	2^{nd}5′-RACE PCR

Serine F80	5′-GCGAATTCCAAAAATCTTCTGAACTGGTC -3′	N-terminal GSP for amplification of Albofibrase with *Eco*R I recognition site
Serine CLSR	5′-TTTCTAGAGCCGAGAGGCAAGTTGCATC -3′	C-terminal GSP for Albofibrase with 6xHis and *Eco*R I recognition site
5′-*AOX1*	5′- GACTGGTTCCAATTGACAAGC -3′	*pPICZ* sequencing primer
3′-*AOX1*	5′- GCAAATGGCATTCTGACATCC -3′	*pPICZ* sequencing primer
α-Factor	5′- TACTATTGCCAGCATTGCTGC -3′	*pPICZ* sequencing primer

Table 1 Oligonucleotides primers and their descriptions.

3.1.2.3 DNA Extraction and Purification from gel slice

QIAquick® Gel Extraction Kit was purchased from QIAGEN Inc., U.S.A.

Wizard® SV Gel and PCR Clean-Up System was purchased from Promega, U.S.A.

3.1.2.4 Cloning of RACE Products

pGEM®-T Easy Vector System II was purchased from Promega, U.S.A. It contains *Eschericia coli*, JM 109 strain, pGEM®-T Easy Vector, T4 DNA Ligase and 2x Rapid Ligation Buffer.

Isopropyl-β-D-Thiogalactopyranoside (IPTG), Dioxane-Free, Formula weight 238.3 was purchased from Promega, U.S.A.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 100 mg was purchased from Promega, U.S.A.

3.1.2.5 Enzymes

Tag DNA polymerase	(Invitrogen™ life Technologies)
T4 DNA Ligase	(Promega)
*Eco*R I	(Sigma)
Xba I	(Promega)
Sac I	(Pharmacia Biotech)

3.1.2.6 DNA Sequencing

We use ABI PRISM® BigDye® Terminator V.3.1 Cycle Sequencing Kit purchased from AB Applied Biosystems, U.S.A.

3.1.3 Expression of Albofibrase in *Pichia pastoris*

3.1.3.1 Polymerase Chain Reaction

Gene specific primers were purchased from Biogenomed.

3.1.3.2 Pichia expression system

EasySelect™ *Pichia* Expression Kit Version G, 122701, was purchased from Invitrogen™ life technologies.

3.1.3.3 Proteins Detection

3.1.3.3.1 Sodiumdodesylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Mini-Protein 3 Electrophoresis apparatus was purchased from Bio-Rad Laboratories, Ltd.

Prestained Protein Marker, Broad Range (Premixed Format) was purchased from New England BioLabs Inc.

Coomassie Brilliant Blue R-250 was purchased from USB, U.S.A.

3.1.3.3.2 Western Blotting Hybridization

Trans-Blot® SD semi-dry electrophoretic transfer cell was purchased from Bio-Rad Laboratories, Ltd.

Polyvinylidene difluoride (PVDF) membrane 0.45 µm was purchased from Bio-active Co., Ltd.

Mouse Anti-His antibody was purchased from Amersham Biosciences, Ltd.

Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP was purchased from Dako Cytomation, Denmark.

3, 3′-Diaminobenzidine (DAB) tetrahydrochloride was purchased from PIERCE Biotechnology.

3.1.3.4 Protein Purification

Protein purification system using Immobilized Metal Affinity Chromatography (IMAC). Talon Super-flow Metal Affinity Resin was purchased from BD Biosciences.

3.1.3.5 Concentration of Protein

Vivascience ultrafiltration was purchased from Vivascience Sartorius Group.

3.1.3.6 Protein Quantitative Assay

Micro BCA™ Protein Assay Reagent Kit was purchased from PIERCE Biotechnology.

3.1.4 Chromogenic assay

Chromogenic substrates were purchased from Sigma, U.S.A.

3.1.5 Enzyme Activity Assay

3.1.5.1 Thrombin clotting activity

Thrombin was purchased from Sigma, U.S.A.

3.1.5.2 Fibrino (geno) lytic activity assay

Fibrinogen was purchased from Sigma, U.S.A.

3.1.5.3 Plasminogen activator activity assay

Plasminogen was purchased from Sigma, U.S.A.

3.1.5.4 Anticoagulant activity

Heparin ,and APTT and PT reagents were purchased from Sigma, U.S.A.

3.1.5.5 Corrected anticoagulant activity

Heparin ,and APTT and PT reagents were purchased from Sigma, U.S.A. Antivenom of green pit viper were purchased from Saovabha memorial institute Bangkok Thailand.

3.1.5.6 Fibrin plate

Fibrinogen was purchased from Sigma, U.S.A. Thrombin was purchased from Sigma, U.S.A.

3.1.5.7 Factor II, V and X assays

Factor II, V and X were purchased from Sigma, U.S.A.

3.1.5.8 Platelet aggregation assays

Collagen was purchased from Sigma, U.S.A.

3.1.5.9 Fibrinogen

Fibrinogen was purchased from Sigma, U.S.A.

3.2 Methods

3.2.1 Obtaining cDNA library

3.2.1.1 Venom gland dissection

Venom glands of *T. albolabris* were dissected from anesthesized snakes, by Dr. Lawan, and immediately frozen in liquid nitrogen until use.

3.2.1.2 RNA extraction

Venom glands were ground frozen in liquid nitrogen. Total RNA was isolated using TRIzol LS reagent (Gibco BRL, Grand Island, NY, USA). RNA was purified from protein using chloroform and concentrated by absolute ethanol precipitation.

3.2.1.3 mRNA construction

Total RNA was then purified to obtain mRNA by Poly AT Tract system (Promega, Madison, WI) using magnetic beads coated with poly T. The system was used a biotinylated oligo (dT) primer to hybridize at high efficiency in solution to the 3'poly (A) region present in mRNA. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA is eluted from the solid phase by the simple addition of ribonuclease-free deionized water.

3.2.1.4 cDNA library construction

The poly $(A)^+$ RNA was used as a substrate to synthesize double-stranded cDNA. cDNA was synthesized by ZAP express cDNA synthesis Kit

(Stratagene, La Jolla, CA), ligated to the vector arms and fractionated for sizes over 600 bp using a drip columm. cDNA synthesis kit used a hybrid oligo (dT) linker-primer that contains an *Xho* I restriction site. Messenger RNA is primed in the first-strand synthesis with the linker-primer and is transcribed using MMLV-RT and 5-methyl dCTP. The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA and protects the cDNA from digestion with certain restriction endonucleases such as *Xho* I. Therefore, on *Xho* I restriction sites in the cDNA, only the unmethylated site within the linker-primer is cleaved. During second-strand synthesis, RNase H nicks the RNA bound to the frist-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I. DNA polymerase I "nick-translates" these RNA fragments into second-strand cDNA. The second-strand nucleotide mixture has been supplemented with dCTP to reduce the probability of 5-methyl dCTP became incorporated in the second strand. The sertriction sites in the linker-primer will be susceptible to restriction enzyme digestion. Recombinant cDNA ligation was packed to packaging extract to generate the infectious phage particles by ZAP express cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA). The phagemid was excised from XL1-BLUE MRF' bacteria that co-infected cDNA phage library and ExAssist helper phage. The phagemid was transformed into XLOLR bacteria. Plasmid DNA was isolated from bacteria cultures by the alkaline mini-prep method and double digested with *Eco* RI and *Xba* I to verity the presence of inserts.

3.2.2 Obtaining full length snake venom Albofibrase cDNA

Green pit viper venom gland library has been prepared. Serine protease partial sequence have been cloned from green pit viper, called clone 080 KNBJ.

3.2.2.1 5′-RACE

5′-RACE was used for generating full length cDNA. The templates of 5′-RACE-PCR are prepared by reverse transcriptase polymerase chain reaction

(RT-PCR) using the joint action of the SMART IIA™ oligonucleotide and PowerScript™, a variant of MMLV reverse transcriptase, reverse transcription. For preparation of 5′-RACE-Ready cDNA, we synthesized the first strand cDNA using poly A^+ RNA in the reaction as followed. Firstly, 500 ng of poly A^+ RNA from venom gland of *Trimeresurus albolabris*, 1 µl of 5′-CDS primer, 1 µl of SMART IIA oligonucleotide and sterile H_2O are combined to a final volume of 5 µl. It was, then, incubated at 70 °C for 2 minutes. When reach the given time, the reaction tube was kept on ice for 2 minutes. After that, the following reagents are added to the reaction; 2 µl of 5X first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 30 mM $MgCl_2$), 1 µl of DTT (20 mM), 1 µl of dNTP Mix (10 mM each), and 1 µl of PowerScript Reverse Transcriptase. The tube is then incubated at 42 °C for 1.5 hours in an air incubator. Finally, the first-strand reaction solution is diluted with Tris-EDTA buffer (10 mM Tris-KOH pH 8.5, 1 mM EDTA) and heated at 72 °C for 7 minutes.

At this point, we have 5′-RACE-Ready cDNA template. The SMART IIA™ oligonucleotide is incorporated in its 5′end. 5′-RACE is carried out by using the SMART RACE cDNA amplification Kit with gene specific primers based on nucleotide sequence derived from the primary library. The Tm should be between 60 – 70 °C. Firstly, PCR Master mix is prepared by combining 27 µl of PCR-Grade Water, 5 µl of 10X Advantage 2 PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 µl of dNTP Mix (10 mM each), and 1 µl of 50X Advantage 2 Polymerase. Secondly, 10 µl of 5′-RACE-Ready cDNA, 5 µl of 10X Universal Primer A Mix (UPM) that is complementary to SMART IIA oligonucleotide, and 10 µl of 10 pM Gene specific primer for clone 035 are then added to 34 µl of PCR Master mix as described. We used PE GeneAmp Systems 2400 thermal cycler for amplifying 5′-RACE fragments using 40 cycles with the temperature cycling

parameters: 94 °C for 5 seconds of denaturation, 68 °C for 10 seconds of annealing and 72 °C for 3 minutes of extension.

3.2.2.2 DNA Extraction and Purification from Gel Slice

After amplification of 5′-RACE, the RACE products is electrophoresed on 1.2 % agarose gel. A band of DNA is excised from agarose gel with a sterile blade. The RACE products are purified by the NucleoTrap™ Gel Extraction Kit. Two volumes of NT 1 Buffer to one volume of gel are added and the tube is placed in 50 °C water bath incubator. After agarose gel is completely dissolved, the tube is centrifuged at 20,000 x g for 30 seconds and supernatant is discarded. Then, 500 μl the NT 2 Buffer is added, mixed, and centrifuged at 20,000 x g for 30 seconds. The supernatant is discarded, repeat this step once. 500 μl the NT 3 Buffer is then added and centrifuged at 20,000 x g for 30 seconds. The supernatant is discarded, this step is repeated once and the pellet is air-dried. Finally, EB buffer is added to elute DNA before centrifugation at 20,000 x g for 10 minutes. After that, DNA is precipitated using 0.3 M sodium acetate in 100% ethanol before centrifugation at 20,000 x g for 10 minutes. The supernatant is discarded. The pellet is washed with 1 ml of 70 % ethanol, and centrifuged at 20,000 x g for 10 minutes. The supernatant is discarded. The dry pellet is dissolved with TE buffer pH 8.0 (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) or distilled water, and stored at –20 °C until use.

3.2.2.3 Cloning of RACE Products

3.2.2.3.1 Ligation of RACE Products into pGEM®-T easy Vector.

After the 5′-RACE products are purified by the NucleoTrap Gel Extraction Kit, the 5′-RACE products are cloned into pGEM®-T easy Vector. The ligation procedure is carried out in a 10 μl. The ligation reaction mixture contains 5

μl of 2X Rapid Ligation Buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG), 50 ng of pGEM®-T easy Vector, 3 Weiss units of T4 DNA Ligase and an appropriate amount of A-tailing PCR products that optimized from the insert: vector ratio of 3:1 by using the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1} = \text{ng of insert}$$

Subsequentily, deionized water is added to a final volume of 10 μl. Finally, the ligation reaction is mixed by pipetting and incubated at 4 °C for 16 – 18 hours.

3.2.2.3.2 Transformation to *E. Coli*, JM 109

10 μl ligation reaction is added to a sterile falcon tube Cat.#2059 on ice. JM 109 competent cells that is placed on ice until just thawed, are then mixed with DNA by gently flicking. Subsequently, 50 μl of competent cells are carefully transferred into falcon tube and gently mixed and placed on ice for 20 minutes. The reaction tube is then subjected to heat-shock for 40 – 50 seconds in a water bath at exactly 42 °C and immediately returned to ice for 2 minutes. The transformed cells are mixed with 450 μl of SOC medium and incubated at 37 °C for 1.5 hours with shaking at 150 rpm. Finally, 500 μl of the transformed cells are plated on LB agar plate with 100 μg/ml ampicillin supplemented with 100 mM IPTG and 50 μg/ml of X-gal for blue/white screening. The plate is incubated at 37 °C for 16 – 24 hours.

3.2.2.3.3 Preparation of plasmid DNA by Alkaline Lysis: Minipreparation

Each colony of transformed bacteria is inoculated in 3 ml of LB broth containing 100 μg/ml of ampicillin. The culture is incubated overnight at 37 °C with shaking at 250 rpm. The culture cells are poured into 1.5 ml microcentrifuge

tube and centrifuged at 20,000 x g for 10 minutes. An aliquot of the original culture is stored at –70 °C in 50 % glycerol. After centrifugation, supernatant is removed by aspiration and the pellet is resuspended in 500 μl of cold STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA pH 8.0) and repeated centrifugation. The bacterial pellet is lysed in 100 μl of ice-cold Alkaline lysis Solution I (50 mM Glucose, 25mM Tris-HCL pH 8.0, and 10 mM EDTA pH 8.0) and vigorously vortexed. Subsequently, 200 μl of freshly prepared Alkaline lysis Solution II (0.2 N NaOH, 1% w/v SDS) was added to bacterial suspension. The tube is closed and mixed by gently inversion five times. The tube is stored on ice for 10 – 30 minutes. After that, 150 μl of ice-cold Alkaline lysis Solution III (5 M Potassium acetate, glacial acetic acid, and H_2O) is added and mixed. The tube is stored on ice for 3 – 5 minutes. The bacterial lysate tube is centrifuged at 20,000 x g for 10 minutes. The supernatant is transferred to a fresh tube. Then, an equal volume of phenol: chloroform is added. The tube is mixed by vortexing and then centrifuged at 20,000 x g for 10 minutes. The aqueous upper layer is transfered to a fresh tube. Finally, plasmid DNA is recovered by precipitation from the supernatant by adding 2 volumes of 100 % ethanol. The solution is mixed by vortexing and centrifuged at 20,000 x g for 10 minutes. The supernatant is removed by gentle aspiration. The tube is stood in an inverted position on a paper to allow all of the fluid to drain away. Then, pellet is washed with 70 % ethanol and invert the tube several times. The tube is centrifuged at 20,000 x g for 10 minutes to recover the DNA. The supernatant is removed from the tube and open the tube at room temperature to allow ethanol evaporation. Finally, the pellet is dissolved with 50 μl of TE buffer pH 8.0. The DNA solution is mixed and stored at –20 °C.

3.2.2.3.4 Restriction Endonuclease and Electrophoresis

Approximately 500 ng of plasmid DNA is digested with 5 units of *Eco*R I according to manufacturer's protocol (Sigma) with 1 μl of 10X Buffer

(300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP) and 0.1 mg/ml BSA. The digestion reaction is incubated overnight at 37 °C. After digestion, the reaction is electrophoresed on 1.5 % gel. Clones containing the insert of interest are selected for sequencing.

3.2.2.3.5 DNA Sequencing

The sequencing is performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The PCR reaction is carried out in a 10 μl containing 4 μl of terminator ready reaction mix (AmpliTag DNA polymerase and FS with thermostable pyrophosphatase), 1 pM sequencing primer (T7 or SP6) and 1 μg DNA template. After incubation at 95 °C for 30 seconds, amplification is carried out for 25 cycles of the following thermal cycling parameters: 95 °C for 10 seconds of denaturation, 50 °C for 5 seconds of annealing, and 60 °C for 4 minutes of extension. The DNA is then precipitated by 95 % ethanol and 3.8 M sodium acetate pH 8.0. The solution is centrifuge at 25,000 x g for 20 minutes and the supernatant is removed by pipetting. The pellet is then washed with 1 ml of 70 % ethanol, and centrifuge tube at 25,000 x g for 8 minutes. Then the supernatant is removed. The pellet is dried in heated incubator at 95 °C for 2 minutes. Finally, the DNA pellet is resuspended in 10 μl. Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

3.2.2.3.6 Alignment and Computational Searching Sequences Analysis

The nucleotide sequences and their conceptual translation obtained from the clones of interest are compared against nucleotide or protein sequences in online databases using BLAST N (Basic Local Alignment Search Tool) program via the World Wide Web. Alignments of sequence are made using CLUSTALW multiple sequence alignment program.

3.2.3 Expression of Albofibrase in *Pichia pastoris*

3.2.3.1 Amplification of Albofibrase by PCR

PCR is used to amplify the cDNA fragment encoding Albofibrase domain. Two primers, SerineF80 and SerineCLSR, are used to amplified the Albofibrase. The *Eco*RI and six histidine residues are incorporated into the forward primer for facilitating purification and detection. The *Xba*I recognition site and UAA stop codon are incorporated into the reverse primer. The PCR reaction is carried out in a 50 μl containing 10X PCR buffer (100mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 1.25 units of Taq DNA polymerase (Pharmacia), 10 pM of each primer, 25 mM $MgCl_2$, 25 mM of each dNTPs, and 200 ng DNA template. After incubation at 94 °C for 5 minutes, amplification is carried out for 30 cycles with the following temperature cycling parameters: 94 °C for 1 minute of denaturation, 53 °C for 1 minute of annealing, 72 °C for 1 minute of extension and a final extension at 72 °C for 5 minutes. The PCR products is electrophoresed in 1.2 % agarose gel. Then, gel is extracted and purified as described in Section 3.2.2.2, the DNA is subcloned into pGEM®-T vector and transformed to *E. coli*, JM109 as described in Section 3.2.2.3.1 and 3.2.2.3.2 Plasmid clones are purified and restriction digested to verify the presence of inserts as described in Section 3.2.2.3.3 and 3.2.2.3.4.

3.2.3.2 Digestion Plasmid DNA and Expression Vector

After the plasmid clone is confirmed by sequencing the insert, plasmid DNA and expression vector, pPICZαA, are digested with *Eco*R I and *Xba* I, respectively. The digestion reaction is electrophoresed in 1.2 % agarose gel. Then, gel is extracted and purified as described in Section 3.2.1.2. After that, the DNA is precipitated by 0.3 M sodium acetate in 90 % ethanol . Then, the solution is centrifuged at 25,000 x g for 20 minutes. The pellet is washed by 1 ml of 70 %

ethanol, and centrifuged at 25,000 x g for 10 minutes. The pellet is then dried and dissolved in sterile distilled water.

3.2.3.3 Ligation of Albofibrase into *pPICZαA* Vector

Albofibrase were ligated with *pPICZαA* Vector. Optimize an appropriate amount of plasmid DNA and pPICZαA vector as described before. The ligation reaction is carried out in a 10 μl. The ligation reaction mixture contains 3 μl of 2X Rapid Ligation Buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol), 1 μl of pPICZαA vector, 5 μl of digested Albofibrase construct plasmid DNA, and 3 Weiss unit of T4 DNA Ligase. The ligation reaction is incubated at 4 °C overnight.

3.2.3.4 Transformation of Ligated product into *E. coli,* JM109

After ligation were finished, we transformed into ***E. coli.*** Transformation is performed by heat shock method. The procedure is described in Section 3.2.1.3.2. After transformation, plate 500 μl of the transformation is mixed onto Low Salt LB plate with 25 μg/ml Zeocin™ and incubated at 37 °C, overnight. After that, transformants are isolated and analyzed for the presence and correct orientation of insert. Zeocin™-resistant colonies are picked, inoculated into 3 ml of Low Salt LB medium with 25 μg/ml Zeocin™ and inoculated overnight at 37 °C with shaking. The plasmid DNA is isolated by miniprep for restriction analysis and sequenced as procedure described in Section 3.2.1.3.3, 3.2.1.3.4 and 3.2.1.3.5, respectively.

3.2.3.5 Linearization of the Plasmid DNA

Before transformation into *Pichia pastoris*, we prepared 5 – 10 μg of plasmid DNA by minipreparation and linearized with the restriction enzyme, which

cut one time in the 5′-*AOX 1* region of pPICZαA. 14 μl of plasmid DNA is mixed with 2 μl of 10X Buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), 0.1 mg/ml BSA and 1 unit of *Sac* I. The reaction is incubated at 37 °C for 16-18 hours. Aliquot of reaction is electrophoresed to verify complete linearization. The reaction is then inactivated using heat at 65 °C for 20 minutes. Then, plasmid DNA is precipitated by 2.5 volumes of 100 % ethanol and 1/10 volume of 3 M sodium acetate. Subsequently, the solution is centrifuged and pellet washed with 80 % ethanol, air-dried and resuspended in 5 μl sterile deionized water, and stored at –20 °C until use.

3.2.3.6 Preparation of *Pichia* for Transformation

Pichia were prepared to competent cells before transformation. Competent *Pichia* cells are prepared by chemical method as provided with the *Pichia* EasyComp™ Kit. *Pichia pastoris*, X-33 strain, is streaked on Yeast Extract Peptone (YEPD) plate to grow as single colonies. The YEPD plate is incubated at 30 °C for 2 days. After that, a single colony is inoculated in 10 ml of YEPD and incubated overnight at 30 °C in a shaking incubator (250 – 300 rpm). Yeast cells from overnight culture is then diluted to an OD_{600} of 0.1 – 0.2 in 10 ml of YEPD and allowed to grow at 30 °C in a shaking incubator until the OD_{600} reaching 0.6 – 1.0. Subsequently, the cells are centrifuged at 500 x g for 5 minutes at room temperature. The cell pellet is washed in 10 ml of Solution I (ethylene glycol and DMSO) and centrifuged at 500 x g for 5 minutes at room temperature. Finally, the pellet is resuspended in 1 ml of Solution I containing ethylene glycol and DMSO. Competent cells are aliquoted in to 50 μl in 1.5 ml sterile microcentrifuge tubes and kept in -80 °C freezer.

3.2.3.7 Transformation of the Linearized Plasmid DNA into *Pichia pastoris*, X-33

The linearized DNA plasmid with *Pichia* were transformed into yeast. The transformation is performed using the *Pichia* EasyComp™ Kit from Invitrogen. Solutions II and III are stored at room temperature before use. The 50 μl of competent cells are thawed at room temperature for each reaction. 3 μg of the linearized plasmid DNA is placed to the competent cells. Then, 1 ml of Solution II (PEG solution) is added to the DNA/cell mixture and mixed by vortexing or flicking the tube. After that, the transformation reaction is incubated at 30 °C for 1 hour in a water bath. The tube is vortexed every 15 minutes. Subsequently, the transformation reaction is subjected to heat shock at 42 °C for 10 minutes in water bath. The transformed cells are splited into 2 microcentrifuge tubes. Add 1 ml of YPD medium to each tube, incubate the transformed cells at 30 °C for 1 hour to allow expression of Zeocin™ resistance. After that, the transformed cells are centrifuged at 500 x g for 5 minutes at room temperature, resuspended in 500 μl of Solution III (Salt solution) and combined into one tube. The transformed cells are then centrifuged at 500 x g for 5 minutes at room temperature, and resuspended in 100 to 150 μl of Solution III. Finally, the transformed solution is plated on YPDS plate with 100 μg/ml Zeocin™ and incubated for 3 to 10 days at 30 °C.

3.2.3.8 PCR Analysis of *Pichia* Integrants

Genomic DNA is isolated from 6 to 10 *Pichia* clones using the protocol from the EasySelect™ *Pichia* Expression Kit manual. Amplification of Albofibrase gene is carried out with 5′ *AOX1* and 3′ *AOX1* primer including in the kit. PCR reaction is carried out in 50 μl reaction containing 10X PCR buffer (100mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 25 mM $MgCl_2$, 25 mM dNTPs, 0.2 pM of both 5′ *AOX1* and 3′ *AOX1* primers, and 200 ng genomic DNA. The PCR reaction is incubated at 95 °C for 5 minutes. Then, 1.25 units of

Tag DNA polymerase is added to the reaction. After that, amplification is carried out for 35 cycles with the following temperature cycling parameters: 95 °C for 1 minute of denaturation, 53 °C for 45 seconds of annealing, 72 °C for 1 minute of extension and final extension at 72 °C for 7 minutes. Recombinant plasmids with and without insert are used as positive and negative controls, respectively. PCR products are analyzed on 1.5 % gel electrophoresis.

3.2.3.9 Small-Scale of Expression of Recombinant *Pichia*

The purpose of this procedure is to determine the optimal method and conditions for expression of recombinant *Pichia* before starting large-scale expression. Firstly, a single colony is inoculated in 25 ml of BMGY in a 250 ml baffled flask and incubated at 30 °C in a shaking incubator until culture reaches an OD_{600} equal 2 to 6. Then, cell pellets are harvested by centrifugation at 500 x g for 5 minutes at room temperature and resuspended to an OD_{600} of 1.0 in BMMY. The culture is then placed in a 1 liter baffled flask covered with two layers of sterile gauze and returned to incubator for continue growth. The methanol concentration is maintained at 0.5 % (v/v) every 24 hours for optimal induction during the entire expression period. At 24, 48, 72, and 96 hours, an aliquot of 10 ml of the expression culture is transferred to a 15 tube. The tubes are centrifuged at 25,000 x g for 10 minutes. The supernatant is transferred to a separate tube and concentrated. Both supernatant and cell pellets are analyzed for protein expression by Coomassie stained SDS-PAGE .

3.2.3.10 Large-Scale of Expression of Recombinant Pichia

A single colony is inoculated in 10 ml of BMGY in a 250 ml baffled flask, and incubated at 30 °C in a shaking incubator for 16 – 18 hours or until culture reach an OD_{600} equal 2 to 6. Subsequently, 10 ml of culture is inoculated in

100 ml of BMGY in a 500 ml baffled flask and grow at 30 °C with shaking until the culture reaches an OD_{600} equal 2 to 6. After that, the cells are collected by centrifugation at 500 x g for 5 minutes at room temperature. To induce expression, supernatant is discarded and cell pellet is resuspended to an OD_{600} of 1.0 in BMMY medium. Then, the culture is aliquoted into several 4 liters baffled flask, covered with two layers of sterile gauze and continued to grow at 30 °C with shaking. The methanol concentration is maintained at 0.5 % (v/v) every 24 hours for induction expression until the time reach 96 hours. After that, the supernatant and cell pellets are separated by centrifuging at 25,000 x g for 10 minutes at room temperature. The cells are stored at –80 °C and the supernatant is concentrated by centrifuging concentrator.

3.2.3.11 Concentration of Proteins

The supernatant is separated by centrifugation and concentrated by ultrafiltration using Vivaspin concentrator that have MWCO of 5,000 Da. The supernatant is poured into the concentrator at maximum volume, and then the concentrator was placed in 50 ml centrifuge tube. Subsequently, the assembled concentrator was centrifuged at 25,000 x g for 40 minutes. The remaining sample from the bottom of the concentrated pocket is recovered with a pipette.

3.2.4 Purification of Recombinant Proteins

Recombinant disintegrin was purified according to protocol from BD TALON™ Metal Affinity Resins User Manual (BD Biosciences). All steps are carried out at 4 °C. The concentrated media is chromatographed on a cobalt-based immobilized metal affinity column (BioLogig™ LP System, Bio-Rad Laboratories, Ltd.). The column is equilibrated and washed with 50 mM sodium phosphate pH 8.0, 300 mM NaCl. The column is eluted with 50 mM sodium phosphate pH 5.0,

300 mM NaCl into 30 fractions. Absorbancy of each fractions are monitored at 280 nm. The appropriate fractions are analyzed by Coomassie-stained SDS-PAGE, pooled and concentrated using ultrafiltration, and diluted with 1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_4) and stored at –20 °C.

3.2.5 Protein Detection

3.2.5.1 Sodiumdodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brillient Blue Staining

12 % of resolving gel and 5 % of stacking acrylamide gel containing 10% SDS are freshly prepared. After gel setting, the recombinant protein is mixed with ¼ volume of 2X sample buffer (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 200 mM with or without β-mercaptoethanol) and then denatured at 95 °C for 10 minutes and loaded into gel slots. Electrophoresis is performed at 125 volts for 90 minutes in 1X running buffer, pH 8.3 (0.25 M Tris-HCl, 1.92 M glycine, 1 % w/v SDS). After electrophoresis, the gel is soaked in Coomassie Brillient Blue Solution for 30 minutes with gentle agitation. After the staining solution is removed, the destaining solution (10% glacial acetic acid, 30% methanol) is added and incubated for 2 – 3 hours. The destaining solution is changed 3 to 4 times during incubation

3.2.5.2 Western Blotting Hybridization

After SDS-PAGE is used to separate the proteins by size, the proteins are transferred from polyacrylamide gel to PVDF membrane using electrophoresis. For transferring of proteins from 10 % or 13 % gels to PVDF membrane, semi-dry transfer is used. The polyacrylamide gel and PVDF membrane are soaked in transfer buffer for 20 minutes. Both of equilibrated gel and wetted membrane are sandwiched between sheets of transfer buffer-soaked thick filter papers, and then are place on Trans-Blot® SD cell. The proteins are transferred at

20 voltage for 40 minutes. When finished, the blotted membrane is immediately placed into the blocking solution (5 % v/v Non-fat Dry Milk in 1X PBS buffer, pH 7.4) for 1 hour at room temperature with gentle agitation and, then washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. The membrane is incubated with 1:3,000 dilution of Anti-His Antibody in blocking buffer for 1 hour at room temperature with gentle agitation. The membrane is, then, washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. After that, the membrane is incubated with 1:1,000 dilution of Horse radish peroxidase-conjugated rabbit Anti-Mouse IgG:HRP in blocking buffer for 2 hours at room temperature with gentle agitation. The membrane is, then, washed as described previously. For developing the blot, the membrane is soaked in the visualizing solution (1.66 mM 3, 3′-diaminobenzidine (DAB) tetrahydrochloride, 0.04 % $NiCl_2$ and 3 % H_2O_2). The reaction is allowed to occur in the dark for 5 minutes. Finally, the solution is removed and the reaction is stopped with H_2O and let the membrane dry overnight.

3.2.6 Quantitative Assay for Recombinant Proteins

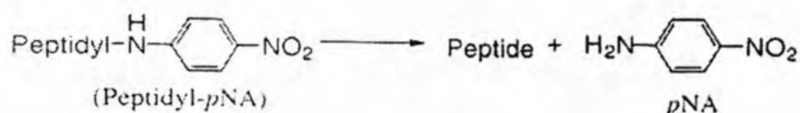
Protein concentration is determined using Micro BCA™ Protein Assay Reagent Kit (Pierce). The method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^+ that is formed when Cu^{2+} is reduced by protein in an alkaline environment. The bovine serum albumin standards (BSA) are diluted into 6 dilutions (0.025 – 0.1 mg/ml). Then fresh working reagent is prepared by mixing 25 parts of Micro BCA™ Reagent MA containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH and 24 parts Reagent MB containing 4% bicinchoninic acid in water with 1 part of Reagent MC containing 4% cupric sulfate, pentahydrate in water. 150 µl of each standard or the sample solution replicate are pipetted into microplate wells and 150 µl of the working reagent is added to each well and mixed. The plate is covered and incubated at 37

°C for 2 hours. The reaction is then measured the absorbance at 570 nm on a plate reader.

3.2.7 Testing activity Assay

3.2.7.1 Chromogenic assay

Peptidly-pNA substrates were used in chromogenic assay. Serine protease (albofibrase) with limited specificity hydrolyzes a peptidly-pNA substrates, releasing p-nitroaniline (pNA) as follows:



Proteolytic activities of 3.1 μM albofibrase were measured according to Lottenberg *et al.* (1981). The substrates included S-3013 (Bz-DL-Arg-pNA), S-3015 (Bz-Tyr-pNA), and S-3125 (Boc-Leu-Ser-Thr-Arg-pNA). They were from Sigma (St Louis, USA). The initial rate of increase in the pNA concentration can be monitored photometrically at 405 nm. Spectrophotometer was set at 405 nm at 25 °C. Pipette 940 μl of buffer and 30 μl of substrate stock solution into the cuvette. Incubate in the spectrophotometer for 3 min add 30 μl of enzyme solution (albofibrase or trypsin as positive control). Record the increase of the absorbption intensity for 3 min. Calculate the amount of pNA released using the following equation.

Amount (μmol) of pNA released/min = 0.302 x dA μmol / min

3.2.7.2 Thrombin cloting activity

Clotting time was determined by mixing 20 μl of albofibrase with 100 μl thrombin solution (15 U/ml) and 100 μl normal human plasma that were

incubated in waterbath at 37°C. After 3 minutes incubation, (100 μl $CaCl_2$) were added and the clotting time was measured.

3.2.7.3 Fibrinogenolytic activity assay

Albofibrase (0.658 μg/ml) and human fibrinogen (5mg/ml) were dissolved in 50 mM Tris HCl buffer, pH 7.8 (containing 0.1 M NaCl) and incubated at 37°C. At various time intervals, 20 μl of the incubation solution was pipetted into a small test tube to which 20 μl of solvent containing 2% sodium dodecyl sulfate and 2% β-mercaptoethanol was added. The fibrinogen degradation products at each time point was then checked on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.7.4 Plasminogen activator activity assay

The reaction was performed using human plasminogen (0.1 U/ml, Sigma) with albofibrase to provide the final concentrations ranging from 0.3 to 2.4nM. After 10 min at 37°C, 20 μl aliquots were taken, mixed with 180 μl of chromogenic substrate S-2251 (1 mM) subtrate in buffer, and incubated for 10 min to assay for the amidolytic activity of the active form of plasminogen, plasmin. The same procedure was applied for u-PA for calibrating the unit activity. The increase in absorbance at 405 nm during a 10 min incubation period was defined as ΔO.D.

3.2.7.5 Anticoagulant activity

To test the anticoagulant effect of albofibrase on (1:9) citrated normal human plasma, various concentrations of albofibrase are added to citrated plasma and incubated for varying time. Prothrombin Time (PT) and activated partial thromboplastin Time (aPTT) were used to monitoring anticoagulant activity of albofibrase, aPTT and PT will be determined compared with the effects of heparin.

3.2.7.5.1 Prothrombin Time (PT)

Prothrombin Time were used to check albofibrase for an anticoagulant effect on human plasma in the extrinsic pathway. The calcium in whole

blood was bound by sodium citrate preventing coagulation. Tissue thromboplastin, to which calcium has been added, was mixed with the plasma, and the clotting time note. 100 µl of (1:9) citrated normal human plasma were mixedwith albofibrase (2.3 nM) and 200 µl thromboplastin calcium mixture and incubated at 37°C for 3 minutes. After 3 minute incubation, 100 µl $CaCl_2$ were added and the clotting time was measured.

3.2.7.5.2 Activated partial thromboplastin Time (aPTT)

An activated partial thromboplastin Time (aPTT) were used to check albofibrase for an anticoagulant effect on human plasma the intrinsic pathway. The plasma after centrifugation contains all intrinsic coagulation factors, except calcium and platelets. Calcium, a phospholipids substitute for platelets (partial thromboplastin) were added to the plasma. The time required for the plasma to clot is the activated partial thromboplastin time. 100 µl thromboplastin, 200 µl (1:9) citrated normal human plasma and 25 µl albofibrase (2.3 nM) were incubated at 37°C for 3 minutes. After 3 minutes incubation 100 µl $CaCl_2$ was added and the clotting time was measured.

3.2.7.6 Correction of anticoagulant activity

Antivenom of green pit viper were purchased from Saovabha memorial institute Bangkok Thailand. Antivenom was co-incubated with plasma and albofibrase for 180 minutes Subsequently. PT and APTT were preformed as described in 3.2.7.5.

3.2.7.7 Fibrin plate assay

Fibrin plate assay was to show fibrinolytic activity of albofibrase. The fibrin plate assay was performed with 1% fibrinogen in 1% agarose and 200 µl of 50 units thrombin boiled to dissolve and kept at 56°C in water bath until used. Defrost 2 ml 1% fibrinogen at 37°C in 8 ml fibrin buffer (mM Tris, mM NaCl,pH 7.8) and 200 µl 50 units thrombin were mixed gently and then was poured on Petri dish. 2.3 nM Albofibrase (25 µl) was placed on the fibrin surface and incubated at 37°C for 48 hours. Fibrinolytic activity was assessed by the production of clear zone.

3.2.7.8 Factor II, V and X assays

Dilution of normal plasma and albofibrase were incubated at 37°C, 180 minutes and then 100 µl factor deficiency II, V, X was add with 100 µl $CaCl_2$. The clotting time was calculated from standard factor.

3.2.7.9 Platelet Aggregation Assay

Platelet aggregation assay is performed using a Helenna Aggregometer. Venous blood (9 parts) from a healthy donor who has not received any medication for at least 2 weeks is collected in 3.2 % sodium citrate (1 part). The whole blood is centrifuged at 1,000 x g for 10 minutes to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP) is prepared from the remaining whole blood by centrifuging at 3,500 x g for 10 minutes. PRP is diluted to 250×10^9 platelets/L with PPP. Platelet aggregation is initiated by adding albofibrase, or collagen (2 mg/ml) that was used as positive control. Light transmittance is recorded and the maximum aggregation response is obtained. The maximal aggregation of collagen is given a value of 100 % aggregation.

3.2.7.10 Fibrinogen

The assay is based on the clotting time of the mixture of diluted plasma and a standardized thrombin solution. The recorded clotting time depend on the plasma concentrations of functional fibrinogen. The fibrinogen values in g/L are obtained by comparing the clotting times of unknown plasma samples with serial dilutions of reference plasma with known fibrinogen concentration.