CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cloning of P-II Snake venom metalloproteinase

3.1.1.1 Gene Specific Primers (GSP)

Synthetic oligonucleotides were purchased from Bio Geno Med company (BGM)

Table 1 Oligonucleotide primers and their descriptions.

Name	Sequence	Description
T7	5′- GTAATACGACTCACTATAGGGC -3′	Sequencing primer from T7 promoter
SP6	5′-ACTCAAGCTATGCATCCAAC –3′	Sequencing primer from SP6 promoter
SVM 038	5′-CGGAATTCCATCATCATCATCATC ATAGATTCATTGAGCTTGTCA TCATAATT- 3′	GSP* for PCR of the N-terminus of P-II SVM from metalloproteinase with 6-HIS -Tags and *E co R I* recognition site

Name	Sequence	Description
SVM 038 R-II	5′- GCTCTAGATTAGGA ATTTCTGGGACAGTAC–3′	GSP for PCR of the C-terminus of P-II SVM with stop codon and *Xba* I recognition site
5′- *AOX1*	5′- GACTGGTTCCAATTGACAAGC –3′	*Pichia* sequencing primer
3′- *AOX1*	5′- GCAAATGGCATTCTGACATCC –3′	*Pichia* sequencing primer

*GSP = Gene specific primer

3.1.1.2 DNA Extraction and Purification from gel slice

High Pure Plasmid Isolation kit was purchased from Roche Applied Science, Germany

High Pure PCR Product purification Kit (Gel Extraction Kit) was purchased from Roche Applied science, Germany

3.1.1.3 Cloning of SVM P-II 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.

3.1.1.4 Enzymes

Tag DNA polymerase (Clontech)

T4 DNA Ligase (Promega)

*Eco*R I (Promega)

Xba I (Promega)

Sac I (Promega)

3.1.1.5 DNA Sequencing

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

3.1.2 Expression of Disintegrins in *Pichia pastoris*

3.1.2.1 Polymerase Chain Reaction

Gene specific primers were purchased from Biogenomed.

3.1.2.2 Pichia expression system

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

3.1.2.3 Protein Detection

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 Bio-Rad Laboratories, Ltd.

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

3.1.2.4 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-Rad Laboratories, Ltd.

Mouse Anti-His antibody was purchased from Roche diagnostics, Germany.

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

ECL Plus Western blotting detection system was purchased from Amersham™ UK.

Hyperfilm™ ECL high performance chemiluminascence film was purchased from Amersham™ UK.

3.1.2.5 Protein Purification

MagneHIS™ protein Purification System was purchased from Promega , USA.

3.1.2.6 Concentration of Protein

Amicon ®Ultracentrifugal filter Devices Millipore was purchased from USA

3.1.2.7 Protein Quantitative Assay

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

3.1.3 Activity Assay

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

3.2 Methods

3.2.1 Expression of Disintegrin Domain in *Pichia pastoris*

3.2.1.1 Amplification of P-II SVMP by Polymerase Chain Reaction (PCR)

PCR was used to amplify the SVM 038 cDNA in pGEM®-T easy Vector which encodes the prodomain, metalloproteinase and disintegrin domain. Two primers, SVMR038 and SVMR038 R.2, were used to amplify these domains. The *Eco*RI and six histidine residues were incorporated into the forward primer for facilitating purification and detection. Six histidine tags were lies on the N-terminus because folding of C-terminus close to RGD sequence would affect the hemostatic activity of protein. The *Xba*I recognition site and UAA stop codon were incorporated into the reverse primer. The PCR reaction was 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 Tag DNA polymerase (Clontech), 10 pM of each primer, 25 mM $MgCl_2$, 25 mM of

each dNTPs, and 200 ng DNA template. After incubated at 95 °C for 10 minutes, amplification was carried out for 30 cycles with the following temperature cycling parameters: 95 °C for 30 seconds of denaturation, 66 °C for 30 seconds of annealing, 68 °C for 1.5 minute of extension and a final extension at 68 °C for 10 minutes. The PCR products were electrophoresed in 1.2 % agarose gel. Subsequently, gel was extracted and purified.

3.2.1.2 DNA Extraction and Purification from Gel Slice

After amplification, the products were electrophoresed on 1.2 % agarose gel. A band of DNA was excised from agarose gel with a sterile blade. The PCR products were purified by High Pure Plasmid Isolation kit (Roche) according to the manufacturer's instruction.

3.2.1.3 Cloning of PCR Products

3.2.1.3.1 Ligation of PCR Products into pGEM®-T Vector.

After the PCR products were purified by an extraction kit, the PCR products were cloned into pGEM®-T Vector. The ligation procedure was carried out in a 10 μl ligation reaction mixture containing 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 PCR products that optimized from the insert: vector ratio of 3:1 by using the following equation.

Subsequently, de-ionized water was added to a final volume of 10 μl. Finally, the ligation reaction was mixed by pipetting and incubated at 4 °C for 16 – 18 hours.

3.2.1.3.2 Transformation to E. Coli, JM 109

10 μl ligation reaction was added to a sterile falcon tube Cat. #2059 on ice. JM 109 competent cells that were placed on ice until just thawed were mixed with DNA by gently flicking. Subsequently, 50 μl of competent cells were carefully transferred into a falcon tube and gently mixed and placed on ice for 20 minutes. The

reaction tube was then subjected to heat-shock for 45 seconds in a water bath at exactly 42 °C and immediately returned to ice for 10 minutes. The transformed cells were 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 were plated on LB agar plate with 100 µg/ml amplicillin supplemented with 100 mM IPTG and 50 µg/ml of X-gal for blue/white screening. The plate was incubated at 37 °C for 16 – 24 hours.

3.2.1.3.3 Preparation of plasmid DNA by High Pure Plasmid Isolation kit

High Pure Plasmid Isolation kit (Roche) were used for extraction of plasmid pGEM®-T Vector in E. Coli, JM 109 .

3.2.1.3.4 Restriction Endonuclease and Electrophoresis

Approximately 500 ng of plasmid DNA was digested with 5 units of *Eco*R and *Xba* I according to manufacturer's protocol (Sigma), 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 was incubated overnight at 37 °C. After digestion, the reaction was electrophoresed on 1.5 % gel. Clones containing the insert of interest were selected for sequencing.

3.2.1.3.5 DNA Sequencing

The sequencing was performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The PCR reaction was 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) and 1 µg DNA template. After incubation at 95 °C for 30 seconds, amplification was 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 was then precipitated by 95 % ethanol and 3 M sodium acetate pH 8.0 on

4 °C. Then, the solution was centrifuge at 25,000 x g for 20 minutes and the supernatant was removed by pipetting. The pellet was then washed with 1 ml of 70 % ethanol, and centrifuge tube at 25,000 x g for 10 minutes. Then, the supernatant was removed. The pellet was dried in a heated incubator at 95 °C for 2 minutes. Finally, the DNA pellet was resuspended in 10 μl. Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

3.2.1.3.6 Alignment and Computational Searching Sequences Analysis

The nucleotide sequences and their conceptual translation obtained from the clones of interest were 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 were made using CLUSTALW multiple sequence alignment program.

3.2.1.4 Digestion of Plasmid DNA and Expression Vector

After the plasmid clone was confirmed by sequencing the insert, plasmid DNA and expression vector, pPICZαA, were digested with *Eco*R I and *Xba* I, respectively. The digestion reaction was electrophoresed in 1.2 % agarose gel. Then, gel was extracted and purified as described in Section 3.2.1.2. After that, the DNA was precipitated by 0.3 M sodium acetate in 90 % ethanol. Then, the solution was centrifuged at 25,000 x g for 20 minutes. The pellet was washed by 1 ml of 70 % ethanol, and centrifuged at 25,000 x g for 10 minutes. The pellet was then dried and dissolved in sterile distilled water.

3.2.1.5 *Ligation of SVMP into pPICZαA Vector*

An appropriate amount of plasmid DNA and pPICZαA vector had been calculated as described before. The ligation reaction was carried out in a 10 μl. The ligation reaction mixture contained 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 SVMP construct plasmid DNA,

and 3 Weiss unit of T4 DNA Ligase. The ligation reaction was incubated at 4 °C overnight.

3.2.1.6 Transformation of Ligated product into E. coli, JM109

Transformation was performed by heat shock method. The procedure was described before. After transformation, plate 500 µl of the transformation was mixed onto Low Salt LB plate with 25 µg/ml Zeocin™ and incubated at 37 °C, overnight. After that, colony transformants were isolated and analyzed for the presence and correct orientation of insert. Zeocin™-resistant colonies were 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 was isolated by High pure plasmid isolation kit for restriction analysis and sequenced.

3.2.1.7 Linearization of the Plasmid DNA

Prior to transformation into *Pichia pastoris*, we prepared 5 – 10 µg of plasmid DNA linearized with the restriction enzyme, which cut one time in the 5′-*AOX 1* region of pPICZαA. Fourteen µl of plasmid DNA was 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 was incubated at 37 °C for 16-18 hours. An aliquot of reaction was electrophoresed to verify complete linearization. The reaction was then inactivated using heat at 65 °C for 20 minutes. Then, plasmid DNA was precipitated by 2.5 volumes of 100 % ethanol and 1/10 volume of 3 M sodium acetate. Subsequently, the solution was centrifuged and pellet washed with 80 % ethanol, air-dried and resuspended in 5 µl sterile deionized water, and stored at –20 °C until use.

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

The transformation was performed using the *Pichia* EasyComp™ Kit from Invitrogen. Solutions II and III were stored at room temperature before use. The 50 µl of competent cells were thawed at room temperature for each reaction. Three

microgram of the linearized plasmid DNA was placed to the competent cells. Then, 1 ml of Solution II (PEG solution) was added to the DNA/cell mixture and mixed by vortexing or flicking the tube. After that, the transformation reaction was incubated at 30 °C for 1 hour in a water bath. The tube was vortexed every 15 minutes. Subsequently, the transformation reaction was subjected to heat shock at 42 °C for 10 minutes in water bath. The transformed cells were split into 2 microcentrifuge tubes. 1 ml of YPD medium was added to each tube. The transformed cells were incubated at 30 °C for 1 hour to allow expression of Zeocin™ resistance. After that, the transformed cells were 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 were, subsequently, 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 was plated on YPDS plate with 100 µg/ml Zeocin™ and incubated for 3 to 10 days at 30 °C.

3.2.1.9 Expression of Recombinant Protein in Pichia pastoris

A single colony was 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 to 2 to 6. Then, cell pellets were harvested by centrifugation at 500 x g for 5 minutes at room temperature and resuspended to an OD_{600} of 1.0 in BMMY. Subsequently, 10 ml of culture were inoculated in 100 ml of BMGY in a 1 liter baffled flask and grew at 30 °C with shaking cover the flask with two layers of sterile gauze and returned to incubator for continue growth. The methanol concentration was 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 the expression culture was transferred to Amicon 15 ml tube. The tubes were centrifuged at 25,000 x g for 10 minutes. The supernatant was transferred to a separate tube and concentrated.

3.2.1.10 Concentration of Proteins

The supernatant was separated by centrifugation and concentrated by ultrafiltration using AMICON™ concentrator that had MWCO of 10,000 Da. The

supernatant was poured into the concentrator at the maximum volume, and then the concentrator was placed in 4 ml centrifuge tube. Subsequently, the assembled concentrator was centrifuged at 25,000 x g for 30 minutes. The remaining sample from the bottom of the concentrated pocket was recovered with a pipette.

3.2.2 Purification of Recombinant Proteins

Recombinant of P-II SVMPs was purified according to protocol by adding 30µl MagneHis™ Ni-Particles to 200µl of cell lysate (equivalent to 1ml of culture) and mixing by pipetting up and down approximately 10 times and incubating for 2 minutes at room temperature. The tube was then placed in the appropriate magnetic stand for approximately 30 seconds to allow the MagneHis™ Ni-Particles to be captured by the magnet and the supernatant was remove with a pipette. After removal of the tube from the magnet, 150µl of MagneHis™ Binding/Wash Buffer was added to the MagneHis™ Ni-Particles and mix by pipetting. The tube was placed in the magnetic stand again for 30 seconds. After MagneHis™ Ni-Particles were captured by the magnet, supernatant was carefully removed with a pipette. This step was repeated twice. Finally, MagneHis™ Elution Buffer was added and mixed by pipetting and incubated for 1–2 minutes at room temperature. A magnetic stand was placed to allow the MagneHis™ Ni-Particles to be captured by the magnet and supernatant containing the purified protein was removed using a pipette. The samples were analyzed for expression of the fusion protein by SDS-PAGE or by functional assay or kept at -80 °C.

3.2.3 Protein Detection

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

10 % of resolving and 5 % of stacking acrylamide gel containing 10% SDS were freshly prepared. After gel setting, the recombinant protein was 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 was 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 was soaked in Coomassie Brillient Blue Solution for 30 minutes with gentle agitation. After the staining solution was removed, the destaining solution (10% glacial acetic acid, 30% methanol) was added and incubated for 2 – 3 hours. The destaining solution was changed 3 to 4 times during incubation

3.2.3.2 Western Blotting Hybridization

After SDS-PAGE was used to separate the proteins by size, the proteins were transferred from polyacrylamide gel to PVDF membrane using electrophoresis. For transferring of proteins from 10 % gels to PVDF membrane, semi-dry transfer was used. The polyacrylamide gel and PVDF membrane were soaked in transfer buffer for 20 minutes. Both equilibrated gel and wetted membrane were sandwiched between sheets of transfer buffer-soaked thick filter papers, and then were placed on Trans-Blot® SD cell. The proteins were transferred at 40 voltages for 40 minutes. When finished, the blotted membrane was 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 was incubated with 1:3,000 dilution of Anti-His Antibody in blocking buffer for 1 hour at room temperature with gentle agitation. The membrane was, then, washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. After that, the membrane was incubated with 1:1,000 dilution of Horse radish peroxidase (HRP) -conjugated rabbit Anti-Mouse IgG in blocking buffer for 2 hours at room temperature with gentle agitation. The membrane was then, washed as described previously. For developing the blot, the membrane was soaked in the visualizing solution (1.66 mM 3, 3′-diaminobenzidine (DAB) tetrahydrochloride, 0.04 % $NiCl_2$ and 3 % H_2O_2). The reaction was allowed to occur in the dark for 5 minutes. Finally, the solution was removed and the reaction was stopped with H_2O_2 and the membrane was let dry overnight.

3.2.4 Quantitative Assay for Recombinant Proteins

Protein concentration was determined using Micro BCA™ Protein Assay Reagent Kit (Pierce). The method utilized bicinchoninic acid (BCA) as the detection reagent for Cu^{+} that was formed when Cu^{2+} was reduced by protein in an alkaline environment. The bovine serum albumin standards (BSA) were diluted into 6 dilutions (0.025 – 0.1 mg/ml). Then fresh working reagent was 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 were pipetted into microplate wells and 150 µl of the working reagent was added to each well and mixed. The plate was covered and incubated at 37 °C for 2 hours. The reaction was then measured the absorbance at 570 nm on a plate reader.

3.2.5 Platelet Aggregation Assay

Platelet aggregation assay was performed using a Helenna Aggregometer. Venous blood (9 parts) from a healthy donor who had not received any medication for at least 2 weeks was collected in 3.2 % sodium citrate (1 part). The whole blood was centrifuged at 150 x g for 15 minutes to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP) was prepared from the remaining whole blood by centrifuging at 800 x g for 10 minutes. PRP was diluted to 250×10^{9} platelets/L with PPP. Different amount of recombinant P-II SVMPs were added to PRP and incubated at 37 °C for 10 minutes. Platelet aggregation was initiated by adding collagen (2 mg/ml). Light transmittance was recorded and the maximum aggregation response was obtained. The maximal aggregation in the absence of recombinant P-II SVM was given a value of 100 % aggregation.