CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cloning of Snake venom serine proteinase

Synthetic oligonucleotide gene specific primers (GSPs) were purchased from Bio Geno Med (BGM).

Table 1 Oligonucleotides and their descriptions.

Name	Sequence	Description
T7	5′- GTAATACGACTCACTATAGGGC -3′	Sequencing primer from T7 promoter
SP6	5′-ACTCAAGCTATGCATCCAAC –3′	Sequencing primer from SP6 promoter
GPVPAF	5'-TGA AGA ATT CCT GGT CTT TGG AGG TCG TCC ATG TAACAT AAA TGC CCA T-3'	GSP for PCR of the N-terminus of serine protease domain with 6xHis and *Eco*R I recognition site
GPVPAR	5'-TTC ATC TAG ACC CGG GGG GCA GGT TCG ATC TTT ATT TCC-3'	GSP for PCR of the C-terminus of serine protease domain with stop codon and *Xba* I recognition site

Table 1 Oligonucleotides and their descriptions. (Cont.)

Name	Sequence	Description
5′- *AOX1*	5′- GACTGGTTCCAATTGACAAGC –3′	*Pichia* sequencing primer
3′- *AOX1*	5′- GCAAATGGCATTCTGACATCC –3′	*Pichia* sequencing primer
α- Factor	5′- TACTATTGCCAGCATTGCTGC –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 Snake Venom Serine proteinase Products

pGEM® -T Easy Vector System II was purchased from Promega, U.S.A. The kit 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	(Invitrogen™ Life technologies)
T4 DNA Ligase	(Promega)
*Eco*R I	(Sigma)
Xba I	(Promega)
Sac I	(Pharmacia Biotech)

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 Serine protease 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 Proteins Detection

Sodium Dodesyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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

Pre-stained 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.

3.1.2.4 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-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.

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

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

MagneHis™ Protein Purification System was purchased from Promaga, USA

3.1.2.6 Concentration of Protein

Amicon ®Ultracentrifugal Filter Devices was purchased from Millipore, USA

3.1.2.7 Protein Quantitative Assay

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

3.1.3 Activity Assay

Chromogenic substrate (Sigma)

Urokinase Plasminogen Activator (Sigma)

Human plasminogen (Sigma)

3.2 Methods

3.2.1 Expression of GPV-PA in *Pichia pastoris*

3.2.1.1Amplification of GPV-PA by Polymerase ChainReaction (PCR)

PCR was used to amplify the cDNA fragment encoding the serine proteinase domain. Two primers, GPVPAF and GPVPAR, were used to amplify the serine proteinase. The *Eco*RI and six histidine residues were incorporated into the forward primer for facilitating purification and detection. 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 (Clonetech), 10 pM of each primer, 25 mM $MgCl_2$, 25 mM of each dNTPs, and 200 ng DNA template. After incubation 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, the DNA was extracted and purified from the gel.

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 manufacture'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 product was purified by an extraction kit, it was 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 Vector, 3 Weiss units of T4 DNA ligase and an appropriate amount of the PCR product that was optimized from the insert: vector ratio of 3:1.

Subsequently, de-ionized water was added to the 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 into E. Coli, JM 109

The 10-µl ligation reaction was added to 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, 50µl of competent cells were carefully transferred into 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 hour with shaking at 150 rpm. Finally, 500 µl of the transformed cells were 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 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 were digested with 5 units of EcoR I and Xba I according to manufacturer's protocol (Sigma), 1 µl of 10X Buffer (300 mM Tris – HCl pH 7.8, 100 nM $MgCl_2$, 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 inserts were selected for sequencing.

3.2.1.3.4 DNA sequencing

The sequencing was performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The PCR reaction was carried out in a 10 µl reaction containing 4 µl of the terminator ready reaction mix (Amplitag DNA polymerase and FS with thermostable pyrophosphophatase), 1 pM sequencing primer (T7) and 1µg 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 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 re-suspended in 10 µl. Template Suppression Reagent (Pekin-Elmer) and loaded to the ABI PRISM sequencer.

3.2.1.3.5 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 available

in the World Wide Web. An alignment of sequence were made using Genious™ program.

3.2.1.4 Digestion 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 is 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 GPV-PA into pPICZαA Vector

Appropriate amounts of plasmid DNA and pPICZαA vector were optimized as described before. The ligation reaction was carried out in a 10-µl reaction. The ligation reaction mixture contained 3 µl of 2xRapid 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 construct plasmid DNA, and 3 Weiss units 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 the heat shock method. The procedure was described in Section 3.2.1.3.2. 500 µl of the transformation were mixed and plated onto Low Salt LB plate with 25 µg/ml Zeocin™ and incubated at 37 °C, overnight. After that, transformants were isolated and analyzed for the presence and the correct orientation of the insert. Zeocin™-resistant colonies were picked, inoculated into 3 ml of Low Salt LB medium with 25 µg/ml Zeocin™ and

incubated overnight at 37 °C with shaking. The plasmid DNA was isolated by the Miniprep 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 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 were 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 the pellet was washed with 80 % ethanol, air-dried and re-suspended in 5 μl sterile de-ionized 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. 3 μg of the linearized plasmid DNA were placed with 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. Add 1 ml of YPD medium to each tube and incubated the transformed cells 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, re-suspended in 500 µl of Solution III (Salt solution) and combined into one tube. The transformed cells were then centrifuged at 500 x g for 5 minutes at room temperature, and re-suspended 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 pastroris

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 reached an OD_{600} between 2 to 6. Subsequently, 10 ml of culture were inoculated in 100 ml of BMGY in a 500 ml baffled flask and grown at 30 °C with shaking until the culture reached an OD_{600} of 2 to 6. After that, the cells were collected by centrifugation at 500 x g for 5 minutes at room temperature. To induce expression, supernatant was discarded and the cell pellet was resuspended to an OD_{600} of 1.0 in BMMY medium. Then, the culture was aliquoted into several 4 liters baffled flasks covered with two layers of sterile gauze. They continued to grow at 30 °C with shaking. The methanol concentration was 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 were separated by centrifuging at 25,000 x g for 10 minutes at room temperature. The cells were stored at –80 °C and the supernatant was concentrated by a centrifuging concentrator.

3.2.1.10 Concentration of Proteins

The supernatant was separated by centrifugation and concentrated by ultrafiltration using Vivaspin concentrator that have MWCO of 5,000 Da. The supernatant was 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 was recovered using a pipette.

3.2.2 Purification of Recombinant Proteins

Recombinant serine proteinase was purified according to protocol from MagneHis™ Ni-particles from 200 µl of the concentrate (equivalent to 1 ml of culture). The solution was mixed 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 removed with a pipette. After removal of the tube from the magnet, 150 µl of MagneHis™ binding/ wash buffer were added to the MagneHis™ Ni-particles and mixed by pipetting. Then, it 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 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 until tests.

3.2.3 Protein Detection

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

The 10% resolving and 5% stacking acrylamide gels 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), 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, the proteins were transferred to PVDF membrane using electroblotting in the semi-dry system. The polyacrylamide gel and PVDF membrane were soaked in a transfer buffer for 20 minutes. Both of equilibrated gel and wetted membrane were sandwiched between sheets of transfer buffer-soaked thick filter papers and then placed on Trans-Blot® SD cell. The proteins were transferred at 40 volts 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, subsequently, 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-conjugated rabbit Anti-Mouse IgG:HRP in blocking buffer for 2 hours at room temperature with gentle agitation and 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 and let the membrane 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 were 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 Plasminogen activator activity assay

The reaction was performed using human plasminogen (0.1 U/ml, Sigma) with serine protease protein 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 plasminogen activating

activity. The increase in absorbance at 405 nm during a 10 min incubation period was defined as ΔO.D.

3.2.6 Platelet Aggregation Assay

Platelet aggregation assay is performed using a Helenna Aggregometer. Venous blood (9 parts) from 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. Different amount of recombinant disintegrins are added to PRP and incubated at 37 $^{\circ}$C for 10 minutes. Platelet aggregation is initiated by adding collagen (2 mg/ml). Light transmittance is recorded and the maximum aggregation response is obtained. The maximal aggregation in the absence of recombinant serine protease is given a value of 100 % aggregation