CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Screening of Serine beta-fibrinogenase clones from ZAP Express cDNA Library

1.1.1 ZAP Express cDNA Library of Russell's viper venom Glands

ZAP Express cDNA library of Russell's viper venom glands used in this study was obtained from our previous study (Nuchprayoon *et al.*, 2001). ZAP Express vector map is shown in **Figure 4**.

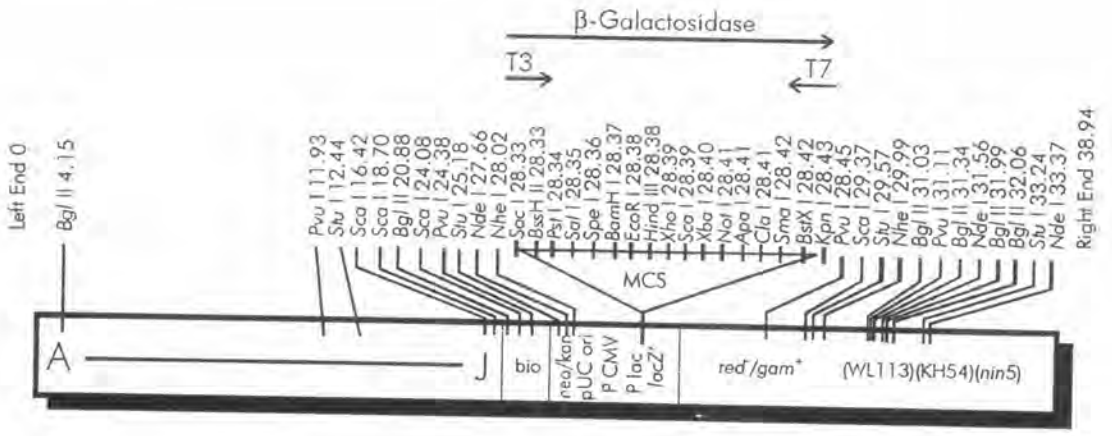

Figure 4 Map of the ZAP Express vector.

1.1.2 Plaque Lift Hybridization and Detection System

Hybond-N, Nylon membrane (Phamacia Biotech AB, USA) and North2South® Direct HRP Labeling and Detection Kit (PIERCE, USA) were used in the plague lift hybridization analysis.

1.1.3 The pBK-CMV Phagemid Vector containing partial Serine beta-fibrinogenase cDNA sequences from the Expressed Sequences Taqs (ESTs) study.

The pBK-CMV Phagemid Vector containing partial Serine beta-fibrinogenase cDNA sequences was selected from the Expressed Sequences Taqs (ESTs) in our previous studies. The ESTs study was used ExAssist helper phage generating the pBK-CMV phagemid vector provided in ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene). Map of pBK-CMV phagemid vector is shown in **Figure 5**.

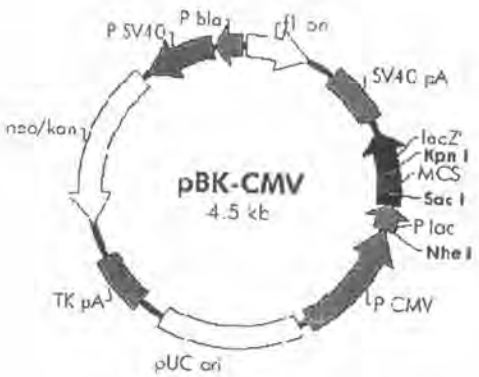

Figure 5 Map of the pBK-CMV vector.

1.1.4 Genotypes of *Escherichia coli* Strain

XL1-Blue MRF' strain; Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gryA96 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10(Tet^{r})].

The *E. coli* strain, XL1-Blue MRF', was used as a bacterial host for propagating the pBK-CMV phagemid. The XL1-Blue MRF′ (Minus Restriction) strain is a restriction minus (McrA–, McrCB–, McrF–, Mrr–, HsdR–) derivative of Stratagene's XL1-Blue strain. XL1-Blue MRF′ cells are deficient in all known restriction systems [*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173*], and are endonuclease (*endA*), and recombination (*recA*) deficient. The *hsdR* mutation prevents the cleavage of cloned DNA by the *Eco*K endonuclease system, and the *recA* mutation helps ensure insert stability. The *endA1* mutation greatly improves the quality of miniprep DNA. XL1-Blue MRF' cells contain the *lacI*q*ZΔM15* gene on the F′ episome, allowing bluewhite screening.

1.1.5 Enzymes

Table 3 Restriction enzymes with their recognition sites, recommended buffer and manufacturer.

Enzymes	Recognition sequence	Buffer	Manufacturer
Xho I	C^TCGAG	NEBuffer 2	New England Biolabs
*Eco*R I	G^AATTC	NEBuffer 2	New England Biolabs
*Bam*H I	G^GATCC	NEBuffer 2	New England Biolabs

Note ^ represents the cleavage sites of restriction enzymes.

1.2 Molecular cloning of Full length Serine beta-fibrinogenase homolog

1.2.1 Obtaining cDNA encoding mature sequences by Reverse transcription-polymerase chain reaction (RT-PCR) is amplify

SuperScript ONE-STEP™ RT-PCR System (Invitrogen™ Life Technologies) was used for cDNA synthesis. The system consists of two major components: RT/ *Taq* Mix and 2X Reaction Mix. The RT/ *Taq* Mix contain a mixture of SuperScript™ II Reverse Transcriptase and *Taq* DNA Polymerase for optimal cDNA synthesis and PCR amplification.

1.2.1.1 Subcloning in Plasmid *E. coli* Expression Vector

pTrcHis A vector (Invitrogen™ Life Technologies) and pET32a(+) vector (Novagen, Madison, WI, USA) were used for expression of recombinant proteins in *E. coli*. The pTrcHis A vector is designed to offer enhanced translation initiation and high-level expression in *E. coli*. Features of the pTrcHis A vector are including *trc-lac* promoter (the high-level regulated transcription promoter contains the -35 region of the *trp* promoter together with the -10 region of the *lac* promoter), T7*lac* promoter for high-level, IPTG-inducible expression of the gene of interest in *E. coli*, N-terminal fusion tags for detection and purification of recombinant fusion proteins, protease recognition site for cleavage of the fusion tag from the recombinant protein of interest, *lacI* gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter, kanamycin and ampicillin antibiotic resistance marker for selection in *E. coli*, and pBR322 origin for low-copy replication and maintenance in *E. coli*. The physical map of pTrcHis A and pET32a(+) are shown in **Figure 6.**

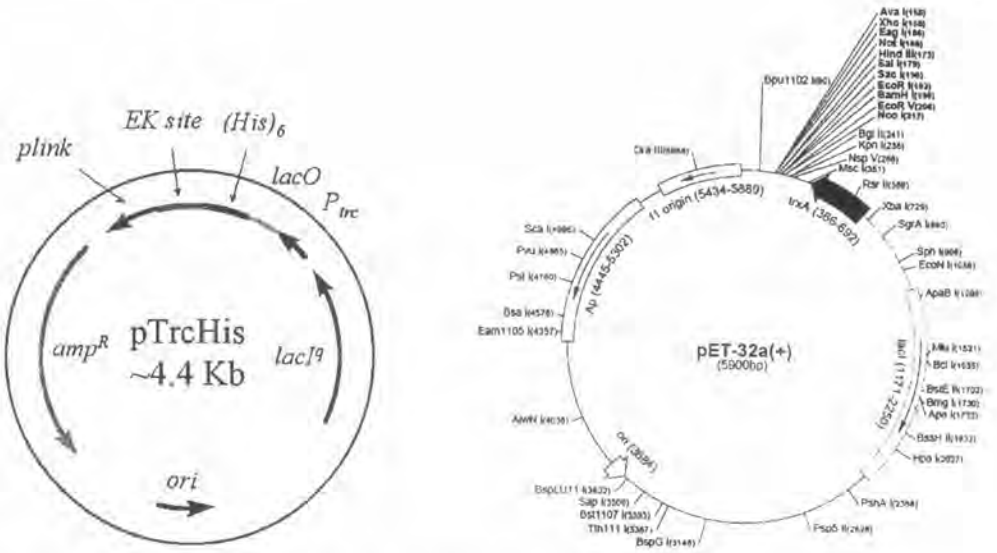

Figure 6 Map of the pTrcHisA and pET-32a(+) expression vector.

1.2.1.2 Enzymes

***Taq* DNA polymerase** (Promega, WI, USA)

T4 DNA ligase (USB, OH, USA) was used in the ligation into the plasmid vector.

***Eco*R I and *Bam*H I** (New England Biolabs, Beverly, MA, USA) The recognition sites, and recommended buffer were listed in Table 3.

1.2.1.3 DNA Purification from Gel Slice

QIAquick® Gel Extraction Kit (QIAGEN Inc., USA) was used for isolating DNA fragments from agarose gel.

1.2.1.4 Genotypes of *Escherichia coli* Strain

DH5α strain; supE44 ΔlacU169 (phi 80 lacZ ΔM15) hsdR17 recA1 endA gyrA96 thi-1 relA1.

TOP10 strain; mcrA Δ(mrr-hsdRMS-mcrBC) phi 80 Δlac ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu) 7697 galU galK λ^- rpsL endA1 mupG.

1.2.2 Obtaining Full Length cDNA 5' Rapid Amplification of cDNA Ends (5'RACE)

5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 Kit (Invitrogen™ Life Technologies) was used to complete the cDNA sequence.

1.2.2.1 Gene Specific Primers (GSP) or Primers

The oligonucleotides (or Primers) were synthesized, and purchased from BGM, BKK, Thailand. The details of oligonucleotide sequences are shown in **Table 4**.

Table 4 Oligonucleotides and their descriptions.

Name	Sequence	Description
T7	5′- GTAATACGACTCACTATAGGGC -3′	Sequencing primer
T_3	5'-AATTAACCCTCACTAAAGGG-3'	Sequencing primer
SBF-F	5'- CGGGATCCGTCDTTGGAGGTSRTSMRTG -3'	RT-PCR
SBF-R	5'- GGAATTCCGGGGGACAAGTCACATTTG -3'	RT-PCR
SP6	5′-ACTCAAGCTATGCATCCAAC –3′	Sequencing primer
SBF-GSP1	5′- AATGTTCGCACAATGAG -3′	5′-RACE
SBF-GSP2	5′- GTGATTGCGCCCCATCCCATAATACG -3′	5′-RACE
SBF-GSP3	5′- CACTGGGAGGGCTGGAAGGCAAGCT -3′	5′-RACE

1.2.2.2 DNA Extraction and Purification from gel slice

QIAquick® Gel Extraction Kit (QIAGEN Inc., USA) was used for isolating DNA fragments from agarose gel.

1.2.2.3 Cloning of PCR Products

pGEM®-T Easy Vector System II (Promega, WI, USA) contains *Escherichia 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 (Promega, WI, USA).

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Promega, WI, USA).

1.2.2.4 DNA Sequencing Reaction

ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit Version 2.0 (Applied Biosystems, USA).

1.3 Chemicals

All other chemicals used in this work were either analytical or molecular biology grades purchased from many suppliers (Sigma; BIO-RAD; Phamacia Biotech AB; USB; Scharlau Chemie, S.A., and Merck).

2. Methods

2.1 Screening of Serine beta-fibrinogenase Clones from ZAP Express cDNA Library

2.1.1 Plaque-lift Hybridization

2.1.1.1 Plating

The host bacteria, XL1-Blue MRF' cells, was prepared as follows. A single colony was inoculated into 3 ml of LB broth (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, pH 7.0) with 10 mM $MgSO_4$ and 0.2% w/v maltose, incubated with shaking at 37°C overnight or to an OD_{600} of 0.5-1.0. Then, the cells were spun at 600 rpm for 2 min and supernatant was discarded. The cells were diluted to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$. The lambda phage cDNA library was diluted in SM buffer (50 mM Tris-HCl pH 7.5, -NaCl, -$MgSO_4 \cdot 7H_2O$, 0.01% gelatin) and added the equivalent of 50,000 pfu/plate to 200 µl of host cells at an OD_{600} of 0.5. The bacteria and phage mixture were then incubated at 37°C for 15 min to allow the phage to attach to the cells. Three ml of NZY top agar (0.5% w/v NaCl, 0.2% w/v $MgSO_4 \cdot 7H_2O$, 0.5% w/v yeast extract, 1% NZ amine (casein hydrolysate), 0.7% w/v agarose, pH 7.5) was added into the bacteria and phage mixture and immediately pored onto an NZY agar plate (0.5% w/v NaCl, 0.2% w/v $MgSO_4 \cdot 7H_2O$, 0.5% w/v yeast extract, 1% NZ amine (casein hydrolysate), 1.5% w/v agar, pH 7.5). The plate was incubated overnight at 37°C.

2.1.1.2 Lifting

After plating, the plate was chilled for 2 hours at 4°C to prevent the NZY top agar from sticking to the nitrocellulose membrane. The nitrocellulose membrane was placed onto the NZY agar plate for 2 min to allow the transfer of the phage particles to the membrane. Both of the membrane and plate were marked same position to return collect the interested clones. Following lifting step, the membrane was denatured and neutralized by submerge into a denaturation solution (1.5 M NaCl

and 0.5 M NaOH) for 2 min, and a neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl, pH 8.0) for 5 min, respectively. The membrane was briefly submerged in a 0.2 M Tris-HCl, pH 7.5 and 2X SSC buffer solution (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 30 sec and blotted on a Whatman™ 3MM paper for 1-2 min. Finally, the membrane was baked in the oven at 80°C for 1.5-2 hours to crosslink the DNA to the membrane and stored at 4°C.

2.1.1.3. Probe Labeling

The RVV141-Serine beta-fibrinogenase homolog DNA fragment[40] which isolated from previous work and shared homology to Serine beta-fibrinogenase of *Macrovipera Lebetina* venom (Levantine viper), was prepared from PCR amplification was labeled by North2South® Direct HRP Labeling and Detection Kit as follows. One hundred ng of PLA_2 DNA fragments in 10 µl water were denatured at 95 °C for 5 min and snapped cool for 5 min. Ten µl of North2South® Direct Stabilized HRP Label and 10 µl of North2South® Direct Reaction Buffer were added. After incubation at 37°C for 30 min, 30 µl of North2South® Direct Enzyme Stabilization Solution was added and mixed. The probe concentration was approximate1.67 ng/µl.

2.1.1.4. Hybridization

Hybridization of the Serine beta-fibrinogenase homolog-cDNA on the membrane with a DNA probe was conducted by North2South® Direct HRP Labeling and Detection Kit under the following condition.

To pre-hybridization, equal volume of North2South® Direct Hybridization Buffer Component 1 and 2 were combined in a clean plastic plate at least 0.1 ml per cm^2 membrane. The hybridization solution was incubated at 55°C in a hybridization oven (HYBAID). After warming the hybridization solution at least 5

min, the blot-membrane was placed and pre-hybridized with gentle rotation for at least 15 min. Following the pre-hybridization step, about 5-10 ng of HRP-labeled DNA probe per ml of hybridization solution was added and incubated 1-4 hours with gentle rotation at 55°C. After hybridization, the membrane was washed 3 times with 0.5 ml per cm^2 membrane washing buffer 1 (2X SSC, 0.1% SDS) at 55°C for 5 min per each wash, performed same process with washing buffer 2 (2X SSC) at room temperature, and proceed to detection step.

2.1.1.5 Detection

The membrane was placed in a clean plastic bag containing equal volume of the North2South® Luminol/Enhancer Solution and North2South® Stable Peroxide Solution, incubated for 5 min at room temperature. After development, the solution was removed and the membrane was transferred and sealed in a new plastic bag. The chemiluminescent signals were detected by exposure to the X-ray film for 1 min.

2.1.1.6 Double Strand Phagemid DNA Extraction by Alkaline Lysis Method

A single colony of bacteria was inoculated in 3 ml NZY broth and incubated at 37°C with 200 rpm shaking for 16-20 hr. The cells were harvested in 1.5 ml microcentrifuge tubes by centrifugation at 5,000 rpm for 3 min and then resuspended in 100 µl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) with 20 µg/ml RNase A. To the cell suspension, 200 µl of freshly prepared solution II (1% SDS, 0.2 N NaOH) was added and incubated on ice for 5 min. Then, 150 µl of solution III (3 M potassium acetate, 11.5% glacial acetic acid) was added to the mixture and incubated on ice for 5 min. The mixture was pellet by centrifugation at 10,000 rpm for 10 min. The supernatant was decanted to a new tube. The phagemid DNA was recovered from the supernatant by adding 7/10 volume of isopropanol and

standing in room temperature for 10 min. The content was centrifuged at 12,000 rpm for 10 min. The pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 rpm for 5 min and dried at room temperature. The DNA pellet was resuspended in 30 µl of TE buffer pH 7.5 (10 mM Tris HCl, pH 7.5; 1 mM EDTA) or sterile water.

2.1.2 Digestion of Restriction Endonucleases and Analysis

About 500 ng of phagemid DNA was double-digested with 5 units of *Eco*R I and *Xho* I according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided), 1X BSA and sterile distilled water to a final volume of 20 µl. The digestion was incubated at 37°C for 3 hours. After digestion, digested phagemids were fractionated on 1.2% agarose gel electrophoresis. Clones which containing of about 0.8 kb insertions, the expected size of patail Serine beta-fibrinogenase cDNA sequence, were selected for sequencing.

2.1.3 Sequencing

The PCR sequencing was performed by using BigDye™ Terminator Cycle Sequencing Ready Reaction kit. The PCR reaction was carried out in a 10 µl reaction containing 4.0 µl of terminator ready reaction mix, 3.2 pmol of sequencing primer and 500 ng DNA template. After incubation at 95°C for 2 min, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10 sec of denaturation, 50°C for 5 sec of annealing and 60°C for 4 min of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and incubated at -20°C for 1 hour. After centrifugation at 12,000 rpm for 10 min, the pellet was washed with 500 µl of 70% ethanol and air dried. The DNA pellet was resuspended in 10 µl Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

2.2 Molecular cloning of Full length Serine beta-fibrinogenase homolog

2.2.1 cDNA encoding mature sequence of Serine beta-fibrinogenase homolog was obtained by Reverse transcription-polymerase chain reaction (RT-PCR)

A pair primers are SBF-F and SBF-R , the Degenerate forward primer, SBF-F was designed by aligned consensus more than 20 highest homology of Serine beta-fibrinogenase homolog sequence because of snake venom serine protease group have signal peptide region highly conserve .Reasonable SBF-F was designed from conserved sequences among the Serine beta-fibrinogenase downstream the signaling sequence. SBF-R (reverse primer) was designed from 3'end of coding sequence of serine beta-fibrinogenase homolog (RVV141). These primers also contain some restriction recognition sites at their 5' end (PLAF: *Bam*H I, PLAR: *Eco*R I) for facilitating the ligation to cloning vector. After cDNA encoding Serine beta-fibrinogenase was amplified by one step RT-PCR.

The one step RT-PCR reaction was carried out in a 50 μl reaction containing 2X Reaction Mix, 1 μl of RT/ Platinum *Taq* Mix, 1 μM of each primer, and 200 ng RNA template. Perform 1 cycle of: 50 °C for 30 min 94°C for 2 min for cDNA synthesis and pre-denaturation. After incubation at 94°C for 2 min, amplification was carried out for 5 cycles with the following temperature cycling parameters: 94°C for 15 sec of denaturation, 45°C for 30 sec of annealing and 72°C for 45 sec of extension. Then, 30 cycles of the following temperature cycling parameters were performed: 94°C for 15 sec of denaturation, 60°C for 30 sec of annealing and 72°C for 30 sec of extension. The final amplification cycle included an addition of a 7 min extension at 72°C.

2.2.1.1 Ligation into pGEM-T Vector

The ligation procedure was carried out in a 10 µl reaction 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 A-tailing PCR products that optimized from the insert: vector ratio of 3:1 using the following equation.

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

Subsequently, deionized 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.

2.2.1.2 Transformation to E. Coli, *JM 109*

The 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 then mixed with DNA by gently flicking. Subsequently, 50 µl 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 40 – 50 seconds in a water bath at exactly 42 °C and immediately returned to ice for 2 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.

2.2.1.3 Restriction Endonuclease and Electrophoresis

Amount of 10 White colonies were selected to preparation of plasmid DNA by alkaline lysis minipreparation. Approximately 500 ng of plasmid DNA was digested with 5 units of *Eco*R I according to manufacturer's protocol (New England Biolabs), 1X reaction *Eco*R I buffer (provided) and sterile distilled water to a final volume of 20 µl. The digestion was incubated at 37°C for 3 hours. After digestion, the reaction was electrophoresed on 1.5 % gel. Clones containing the insert of interest were selected for sequencing.

Finally, the nucleotide sequences were compared with GENBANK database by using BLAST N program via the World Wide Web and alignments by using CLUSTAL X multiple alignment program.

2.2.2 Subcloning to Expression Vector

Plasmid DNA of pGEM-T easy Vector containing Serine beta-fibrinogenase homolog was selected for one clone. About 500 ng of plasmid DNA was double-digested with 5 units of *Eco*R I and *BamH* I according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided), 1X BSA and sterile distilled water to a final volume of 50 µl. The digestion was incubated at 37°C overnight. After digestion, digested phagemids were fractionated on 1.2% agarose gel electrophoresis. Clones which containing of about 0.7 kb insertions.

2.2.2.1 DNA Purification from Gel Slice

After Plasmid DNA was double-digested with enzyme . It was purified by QIAquick® Gel Extraction Kit in order to remove contamination from plasmids DNA vector. A band of the DNA fragment of interest was excised from agarose gel with a clean, sharp razor blade. Three volumes of Buffer QG to one volume of gel (100 mg ~ 100 µl) was added and the tube was placed in a 50°C water bath incubator.

After agarose gel was completely dissolved, one gel volume of isopropanol was added, mixed and applied to the QIAquick column. After centrifugation at 10,000 xg for 1 min, the flow-through solution was discarded. The DNA fragments was washed with Buffer PE and centrifuged for 1 min. Buffer EB (10 mM Tris-HCl, pH 8.5) or distilled water was added to elute DNA and was then centrifuged for 1 min, stored at -20°C.

2.2.2.2 Ligation of PCR Products into Plasmid Vector

After *Bam*H I and *Eco*R I-digestion of pTrcHisA vector, pET32a(+) vector and Serine beta-fibrinogenase homolog DNA, the digested DNA was purified from gel slice by QIAquick® Gel Extraction Kit as described in section 2.2.2.2 and ligation was proceeded under as follows. The ligation reaction was carried out in a 10 µl reaction each mixture containing Expression vector and Serine beta-fibrinogenase homolog DNA in the molar ratio 1:3, 2 units of T4 DNA ligase and 1X buffer. An appropriate amount of sterile water was added to make the 10 µl final volume. The pTrcHisA vector and pET32a(+) are approximate 4.4 kb, 5.9 kb respectively, and supplied at 50 ng/µl.

The ligation reaction was carried out at 16°C for 16-18 hours and the ligation products were used to transform *E. coli* competent cells prepared by $CaCl_2$ method.

2.2.2.3 Preparation of *E. coli* Competent Cells by $CaCl_2$ Method

A single colony of *E. coli* was inoculated into 3 ml of LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hours. The overnight culture was diluted 1:100 into 100 ml of new LB broth and incubated at 37°C until an OD_{600} of 0.4-0.5. The cell culture was chilled on ice for 10 min prior to pipetting the 10 ml aliquots of the culture into 15 ml sterile polypropylene tubes. The cells were pelleted

by centrifugation at 4,000 rpm for 10 min at 4°C. After that, the pellet was suspended in 5 ml of ice-cold 0.1 M $MgCl_2$, centrifuged, resuspended in 5 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 30 min to establish competency. Finally, after centrifugation, the pellet was resuspended in 750 µl of 15% v/v glycerol and 0.1 M $CaCl_2$. The cells were kept in 200 µl aliquots at -80°C until required.

2.2.2.4 Transformation of *E. coli* Competent Cells

Two hundred µl of *E. coli* competent cells were mixed with 2 µl of ligation products and immediately placed on ice for 30 min. The cells were subjected to heat-shock at 42°C for 45 sec and placed on ice for an additional 3 min. The transformed cells were mixed with 800 µl of LB broth and incubated at 37°C for 1 hour with shaking at 200 rpm. Finally, 100 µl of the transformed culture was spread on a LB agar plate containing 50 µg/ml ampicillin and incubated at 37°C overnight.

After transformation, couple expression vector containing serine beta-fibrinogenase homolog DNA were extracted by alkaline lysis method as described in section 2.1.2 Then, PCR sequencing as described in section 2.1.3 was proceeded to ensure the correct PLA_2 sequences.

2.2.3 Obtaining full length Serine beta-fibrinogenase homolog cDNA by 5' Rapid Amplification of cDNA Ends (5'RACE)

The partial cDNA sequences have been previously prepared from RT-PCR. 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 Invitrogen™ Life Technologies, a variant of MMLV reverse transcriptase, reverse transcription. For preparation of 5′-RACE-Ready

cDNA, we synthesized the first strand cDNA using mRNA in the reaction as followed. Firstly, 500 ng of mRNA from venom gland of *Daboia russellii siamensis*, 1 µl of SBF-GSP1 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. Subsequently, the reaction tube was kept on ice for 2 minutes. After that, the following reagents were 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 was then incubated at 42 °C for 1.5 hours. Finally, the first-strand reaction solution was 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 templates with the SMART IIA™ oligonucleotide incorporated in its 5′end. 5′-RACE was carried out using the SMART RACE cDNA amplification Kit with gene specific primers based on nucleotide sequences derived from the primary library. The calculate Tm should be between 60–70 °C. Firstly, PCR Master mix was 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 was complementary to the SMART IIA oligonucleotide, and 10 µl of 10 pM Gene specific primer for clone 041 or 051 were then added to 34 µl of PCR Master mix as described. We used PE GeneAmp Systems 2400 thermal cycle for amplifying 5′-RACE fragments using 40 cycles with following 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.

2.2.3.1 DNA Extraction and Purification from Gel Slice

After amplification of 5′-RACE, the RACE products was electrophoresed on 1.2 % agarose gel. A band of DNA was excised from an agarose gel using a sterile blade. The RACE products were purified by QIAquick® Gel Extraction Kit.

Ligation of 5'RACE Products into pGEM®-T easy Vector .Ligation product were Transformation to *E.coli* cell. Plasmid DNA containing insert DNA was performed by Alkaline Lysis Minipreparation. And then Restriction Endonuclease and analyze with Electrophoresis. DNA purification was cleaned up using QIAquick PCR purification kit and then sequencing DNA Sequencing Plasmid DNA from Alkaline Lysis Minipreparation method

2.3 Bioinformatics and Computational Searching sequence 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 and BLAST P (Basic Local Alignment Search Tool) program via the World Wide Web. Multi-sequences alignment was performed with CLUSTALX and phylogenetic tree was constructed with MEGA 3.1 using the Neighbor-Joining (NJ) method.