CHAPTER IV

RESULTS

4.1 cDNA library construction

4.1.1 Venom gland dissection

Trimeresurus albolabris were obtained from the central part of Thailand. Seven adult female anesthesized snakes were 112.6, 46.9, 116.8, 57.7, 68.3, 54.1 and 164.2 gram. The forteen venom glands weighing from 0.05 to 0.25 gram were dissected from anesthsized snakes by Dr. Lawan Chanhome, and immediately frozen in liquid nitrogen until use.

4.1.2 RNA extraction

Venom glands were ground frozen in liquid nitrogen. Total RNA was isolated using TRIzol LS reagent. The yield of total RNA was 1,211 μg.

4.1.2 mRNA construction

Total RNA was then purified to obtain mRNA by Poly AT Tract system. The yield of poly (A)+ RNA was 7.74 μg.

4.1.3 cDNA recombinants phagemid

The poly $(A)^+$ RNA was used as a substrate to synthesize double-stranded cDNA. cDNA was synthesized using ZAP express cDNA synthesis Kit and fractionated for sizes over 600 bp using gel filtration chromatography into 12 fractions. The first fraction was large cDNA molecules that was eluted frist followed by smaller cDNA and finally unincorporated nucleotides. The eluted fractions from the drip column representing the cDNA were shown in **figure 3**. The progression of the leading edge of the dye through the column will be used as a guideline to monitor collection for radioactivity detected by the handheld Geiger counter to verify that the cDNA had been recovered. The counts per second (cps)

for each fractions were recored. One fraction was composed of three drops. The cDNA fractions that were more than 600 bps in (size fraction number 1-5) were extracted using phenol-chloroform and precipitated with ethanol to recover the size-selected cDNA.

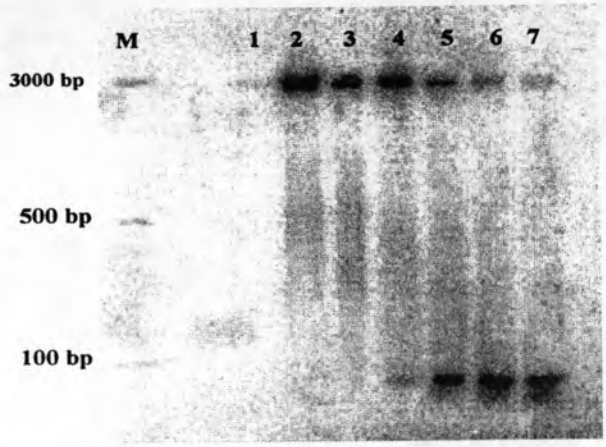


Figure 3. The cDNA fractions have been assessed for the presence of cDNA on a 5% nondenaturing acrylamide gel.

The cDNA concentration was determined using ethidium bromide plate assay. The concentration of cDNA was 25 ng/ µl compare with standard DNA concentrations **(figure 4)**. The yield of total cDNA was 250 µg.

cDNA was ligated to the Zap Express Vector. The recombinants had been packaged to the packaging extract. Bacteriophages were plated on bacterial lawn. The result was shown in **figure 5** demonstrating that plaques were clear zones on the top agar.

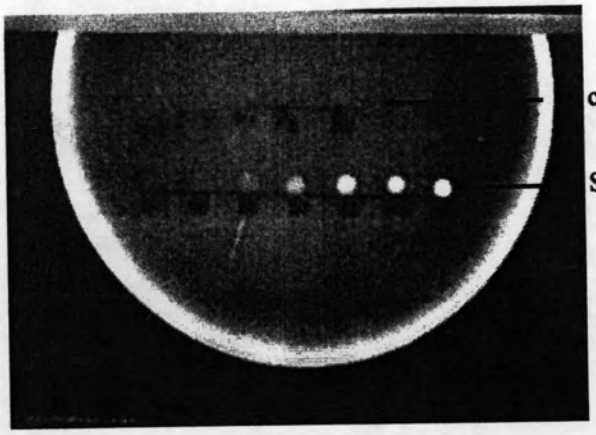


Figure 4. Ethidium bromide plate assay for cDNA concentration

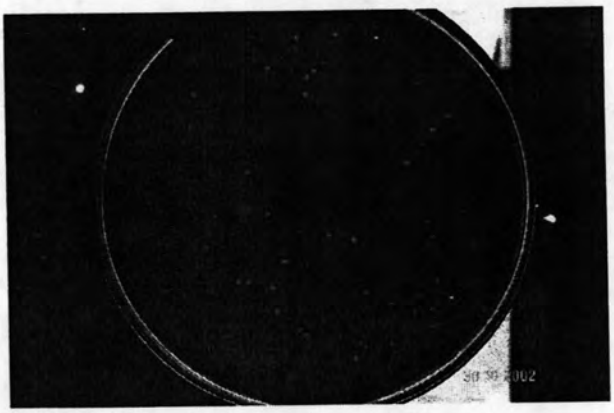


Figure 5. Bacteriophage plaques formation of *T. albolabris* venom gland library

4.1.4 cDNA containing plasmids

The in vivo excision of cloned inserts contained within the lamda vector to form a phagemid was performed within the lamda phage genome. The phage library was shown in **figure 5**. Each colony was picked. *E.coli* was infected with the phagemid. Recombinant plasmids DNA from excised colonies were used for

analysis for inserted DNA after restriction digestion (**figure 6**). The inserts were varying to sizes suggesting that they were different clones. The 140 recombinant plasmid clones were sequenced and gave rise to 11 unique clones of venom genes after analysis compared with GENBANK database using the BLAST N and CLUSTAL W program. The 11 inserted DNA in recombinant plasmids were the *T. albolabris* venom gland genes (**Table 2**).

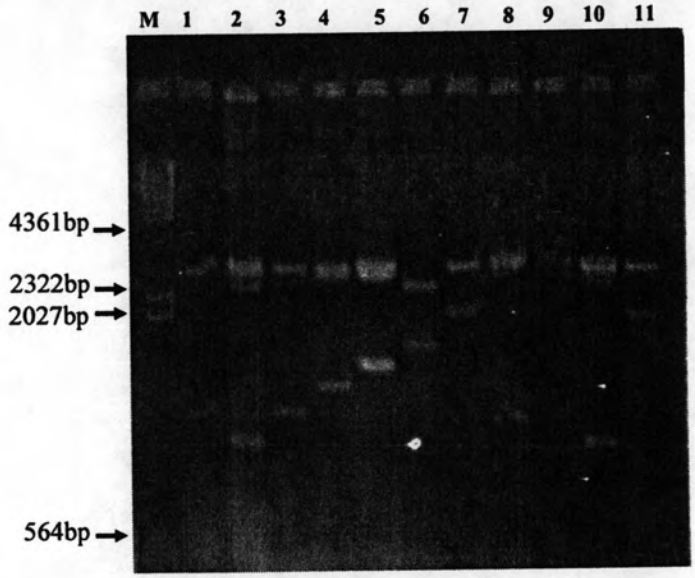


Figure 6. Digestion of recombinant plasmids from *T. albolabris* venom gland cDNA library

Clone Number	Length (bp)	Name
035	1200	Snake venom Metalloprotease (SVM)
036	600	Disulfide Cis Trans Isomerase
038	650	SVM
039	350	SVM
041	400	C-type lectin
051	300	C-type lectin
067	1300	Serine protease
070	500	Phospholipase A_2 (PL A_2)
096	1500	Cytochrome C oxidase
128	450	Ferrochelatase
129	850	PLA2 inhibitor (untranslated region)

Table 2. The11 Unique clones from primary library of *T. albolabris* venom gland.

C-type lectins contain amino acid sequence homology to the calcium regulatory domain of mammalian lectins. They comprise disulfide-linked αβ heterodimers. C-type lectins help platelet aggregation by targetting to von Willebrand factor, platelet glycoproteins and receptors.

Phospholipase A_2 (PLA_2) is a non-glycosylated protein that can induce a wide variety of pharmacological effects and interfere with physiological functions, e.g., endothelial injury, myotoxicity, thrombosis and hemostasis.

Snake venom metalloproteinases (SVM) are multi-domain proteins that compose of a catalytic domain and non–catalytic domains. The pro-region of the proteins prevent the catalytic activity of the metalloproteinase domain. SVMs are activated via cleavage of this region. SVMs show hemorrhagic activity. These

proteins cause hemorrhage by digestion of component of extracellular matrix protein and some blood coagulation factors, including von Willebrand factor enhancing their hemorrhagic effect.

Disulfide cis-trans isomerase, cytochrome C oxidase, ferrochelatase and PLA_2 inhibitor are unlikely to have activities on hemostatic system.

Serine proteases are the major active components of green pit viper. Serine proteases contained histidine, aspartate and serine residues on the catalytic sites. Serine proteases affect many coagulation proteins in mammalian plasma, such as fibrinogen, factor II, X and plasminogen activator in the fibrinolytic system. The serine protease clone was selected from first cDNA library to get complete mRNA sequence. 5′-RACE method was use. On the library screening, a 3′- untranslated region of a serine protease has been obtained and showed in **figure 7**. Therefore, a primer was designed for a 5′-RACE on additional cDNA material.

4.2 Cloning of Full Length Serine protease/Albofibrase from Green Pit Viper

4.2.1 The first 5′-RACE

From the primary cDNA library of *Trimeresurus albolabris* venom gland, one clone of partial cDNA of serine protease was obtained, designated clone number 067 serine protease (KNBJ). A primer was designed base on the sequence to clone the complete cDNA.

GCACGAGGATTTTTTGAATTATTTGGATTACAAATATCTATTTAATCGGGACTCTAAAGCTA
TTTGCAGAAAAGAAGAAAAGAAAAGAAGAGAAAAACTAAAAACTAAAATTCTTGGCCATC
GTGGATCAGAGACATGTAGAAAGAATATATCAAGAAAACATAAATACTCCACAGGAGGA
GATATATATTATACTGCAAATAATTCAAACAACAATGATTTTAAACCACAAAGAAATCCAAA
ATATCTTTGAAAATATAAATCAATCTATTTCCAAGTTAACTCTACAACAAAAATAATTCCCA
GAAAACAACAACAGAGAAAGAGCAGATAAAACAGATAAATGGATACAAAACTGAGAATA
GAAACTTAAAGATGGGACTTAACAAAACAGGAAACTGATTTAATCCCTATGGGGTAAAAA
AATCACAGTAATGGATAAACCATTTATCCTTAAACTGCAAATAATCTGAGACAGAAACAG
ATGGATAAATGGCTGTGAATGCAGGACTCGGGAGAAGAAAGATTTCACAACC

Figure 7. Partial cDNA of 067 serine protease was a 3′- untranslated region of a serine protease (obtained from the primary cDNA library)

The PCR product from the first 5′-RACE was estimated to be 1200 bps as showed in **figure 8**. The 5′-RACE products were subcloned into pGEM®-T easy vector and transformed to *Eschericia coli*, JM109. The positive clones were identified by blue-white color selection system shown in **Figure 9**. Plasmid clones were purified and digested with *Eco*R I to verify the presense of inserts. The digestion reactions were fractionated on gel electrophoresis to screen for clones containing inserts (**Figure 10**). After electrophoresis, the first 9 of 80 KNBJ clones all showed the presence of inserts. Subsequenctly, the plasmid-containing inserts were sequenced using ABI PRISM. The sequencing was performed in both orientations.

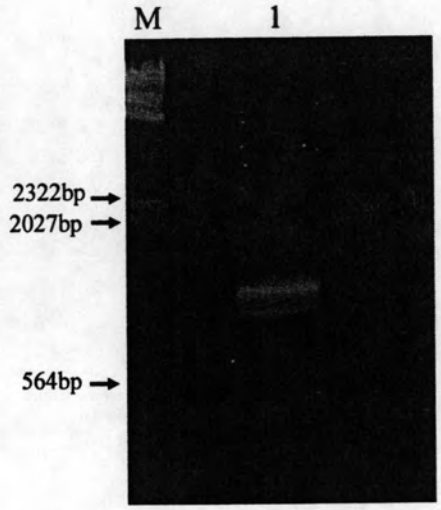


Figure 8. The First 5′-RACE PCR product of serine protease. M = molecular weight marker (λ*Hin*d III); Lane 1: 5′-RACE PCR product.

Figure 9. The colonies of recombinant serine protease cDNA from 5′-RACE.

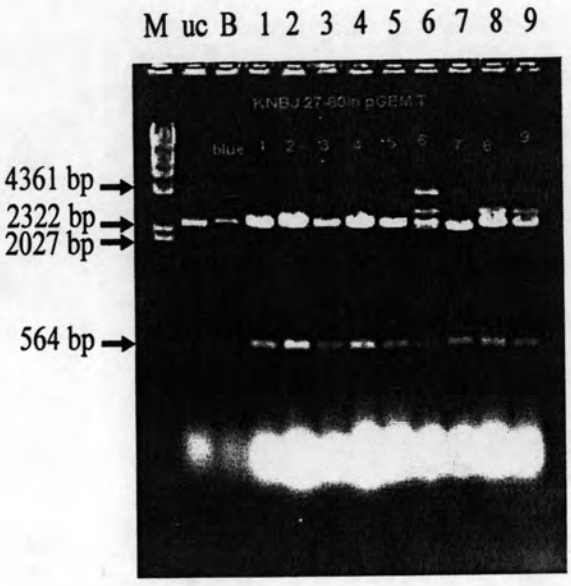


Figure 10. The *Eco*R I digestion clones of recombinant plasmid containing serine protease inserts. Clone 1-9 showed the presence of insert. M = molecular weight marker (λ *Hind* III). Uc = Un-cut plasmids. B = Digested blue colony (no insert).

4.1.2 Sequence Alignment and Computational Searching Analysis

The insert-positive clones were sequenced and analyzed compared with GENBANK database using the BLAST N and CLUSTAL W program. The nucleotide sequence of a serine protease was longer than that from the primary library but it was not a full length cDNA as shown in **figure 11**. Therefore, we designed another primer to repeat the 5′-RACE PCR.

CGGCCGCGAAATTCACTAGTGATTTGCGAACATGATCCCTGCAGCACTAT
TTTGAGCATTTCTTACTCCAAAGGAAGTCCTATTTGAGTCTAAAAAAAAG
TTTACTGCTTGGGAATATATTCCAAGGTAGTTGGATATGGTTAGGGATAT
AGAAGATATGTACTAATCTGCCCACATTTTCTCCTCTTGACTTTTTCAAAA
GTTTTCATGGGGGGCATGCCACAGTTGTATTTCCTGCAATAATGCTCTGG
ATCCAGTCAAGCATGATTGAAGACCTTGCCTGTAGAGGGCAACGCTTAC
GC

Figure 11. Partial sequence of the first 5′-RACE PCR product termed 061 serine protease.

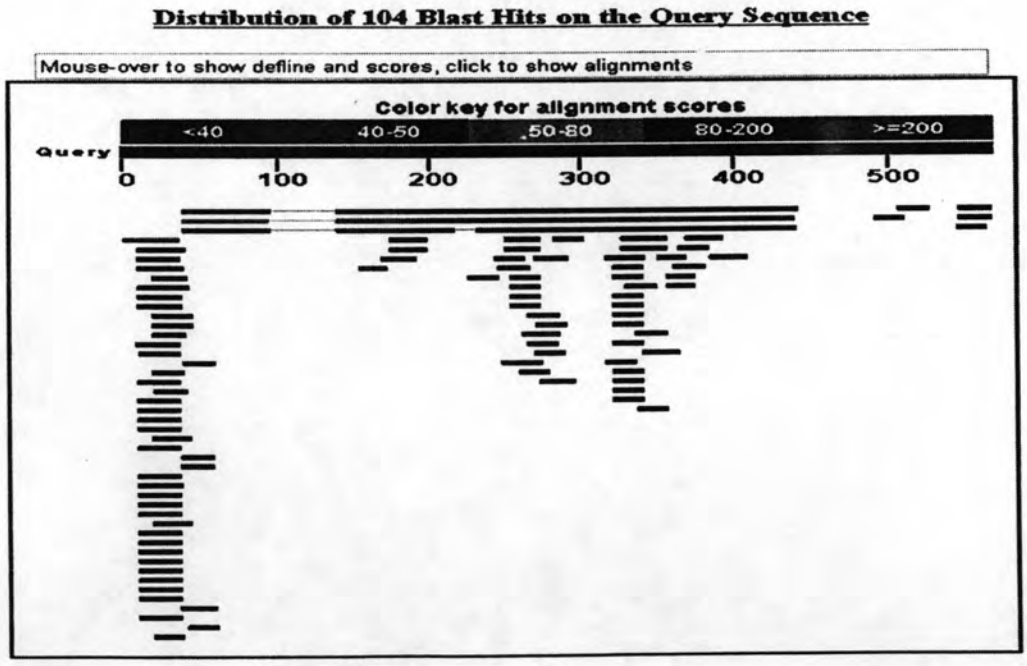


Figure 12. Distribution of 104 Blast hits of the query sequence of 061 serine protease

Sequences producing significant alignments:	(Bits)	Value
gi|2130945|dbj|AB004067.1| Bothrops jararaca mRNA for KN-BJ2, co	442	6e-121
gi|89892515|gb|DQ439973.1| Sistrurus catenatus edwardsi serin...	416	3e-113
gi|54650275|dbj|AB178322.1| Bothrops jararaca mRNA for hypoth...	321	1e-84
gi|56384959|gb|AY669323.1| Fenneropenaeus chinensis penaeidin mR	50.1	0.008
gi|10880174|emb|AL354943.9| Human DNA sequence from clone RP1...	48.1	0.033
gi|83745529|gb|AF368908.3| Glossina morsitans morsitans transfer	48.1	0.033
gi|3550650|emb|Y17051.1|SRFESTK Sycon raphanus mRNA Fes/FER r...	46.1	0.13
gi|18098531|gb|AC073647.9| Homo sapiens BAC clone RP11-707F14 fr	46.1	0.13
gi|32451341|emb|AL670689.7| Mouse DNA sequence from clone RP2...	46.1	0.13
gi|2921800|gb|AF045432.1|AF045432 Danio rerio stem cell leuke...	46.1	0.13
gi|9187132|emb|AL365345.1|HST000246 Homo sapiens mRNA full le...	46.1	0.13

Figure 13. Homology searching results of 061 Serine protease using BLAST N program.

4.1.3 The second 5′-RACE

The primer was designed complementary to the 5′end of the first 5′-RACE product to perform the second 5′-RACE to obtains full length gene. The 5′-RACE products were subcloned into pGEM® -T easy vector and transformed to *Escherichia coli*, JM109. The positive clones were identified by blue-white color selection system. Plasmid clones were purified and digested with *Eco*R I to verify the presense of inserts. The 1,200 bp digested products were fractionated on gel electrophoresis to screen for clones containing inserts (**Figure 14**). The first 11 of 20 KNBJ clones showed the presence of inserts. Subsequently, the plasmids containing inserts were sequenced in both orientations. The cDNA sequences were compared with GENBANK database using the BLAST N and CLUSTAL W program. The snake venom serine proteases from *T. albolabris* comprised thirteen

clones that could be classified into 4 groups (**Table 3**). they were closely homologous to enzymes with known functions from Chinese green tree viper (*Trimeresurus stejnegeri*). The first group that were named GPV-TL1 and GPV-TL2 was closely homologous to the thrombin-like enzyme (stejnobin) 92.3% and 94.2% identical residues, respectively . The second group was named albofibrase similar to a beta fibrinogenolytic protease (stejnofibrase2) (94.5% identical residues). The third group was named GPV-PA (94.5% identical residues) that was closely homologous to the plasminogen activator (TSV-PA). Amino acid sequence alignment of the four novel serine protease were showed in **(figure 16)**.

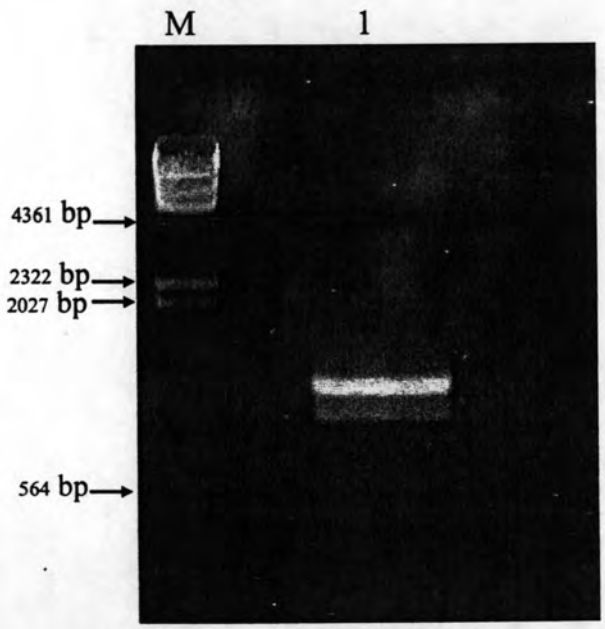


Figure 14. Second 5′-RACE PCR product of serine proteases. M = molecular weight marker (λ*Hin*d III); Lane 1: Second 5′-RACE PCR product.

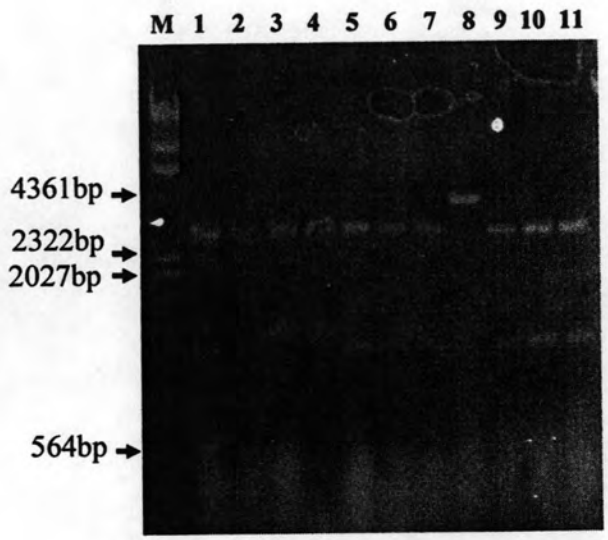


Figure 15. The *Eco*RI digested clones of plasmids containing inserts (clone 1 -11). The inserts were about 1200 bp. M = molecular weight marker (λ *Hin*d III).

	Amino acid	Homologous proteins	Identical residues (%)
GPV-TL1	**260**	**Stejnobin**	**92.3**
GPV-TL2	**260**	**Stejnobin**	**94.2**
Albofibrase	**258**	**Stejnofibrase2**	**94.5**
GPV-PA	**258**	**TSV-PA**	**94.5**

Table 3. Characteristics of four serine proteases from *T. albolabris* compared with those from *T. stejnegeri*

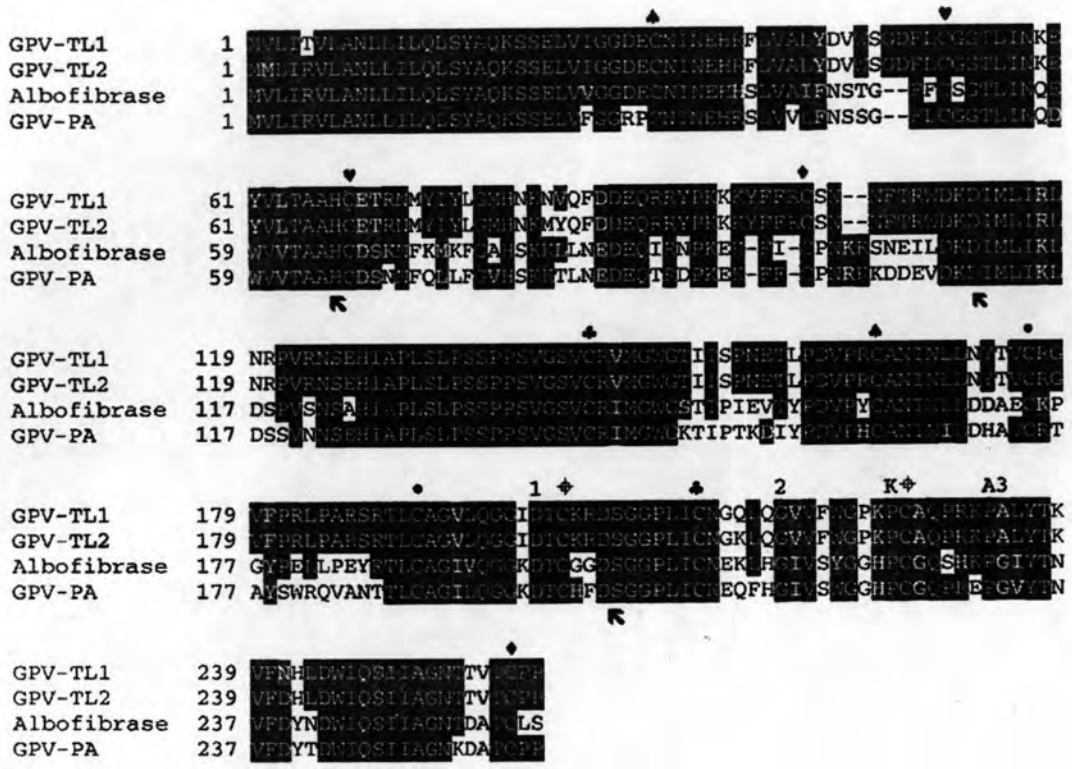


Figure 16. The four serine proteases from *T. albolabris* were aligned: GPV-TL1 and GPV-TL2 (thrombin-like enzymes), albofibrase (fibrinogenolytic enzyme) and GPV-PA (plasminogen activator). The number 1, 2 and 3 symbolize for the subsite1, 2 and 3. The alphabet K represents Pro219 (chymotrypsin numbering) similar to kallikein. The alphabet A symbolizes the position homologous to thrombin allosteric site. Arrowheads below the sequences show the catalytic triad, His57, Asp102 and Ser195. The other symbols are marked conserved Cys with similar symbols representing pairs of disulfide bond formation.

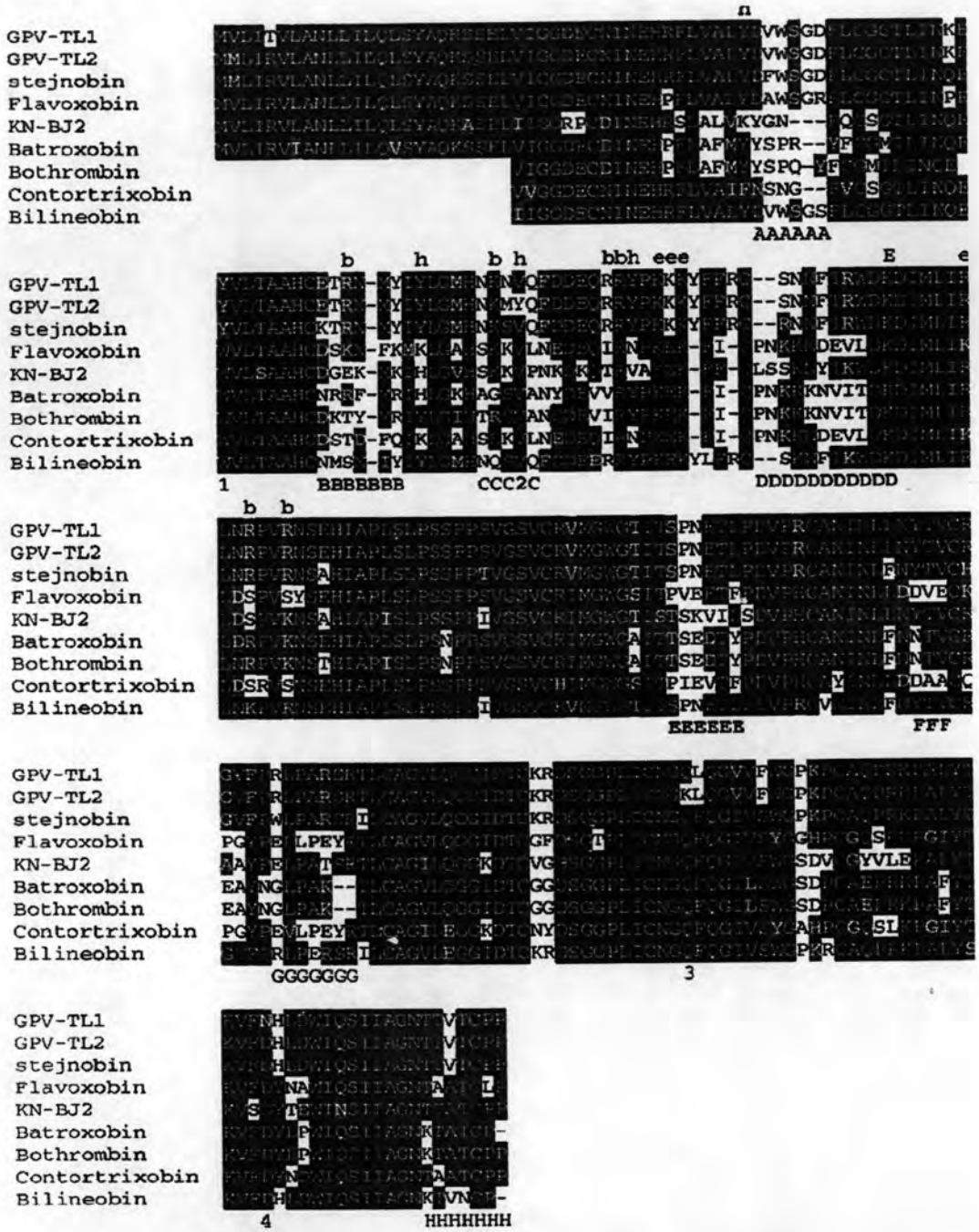


Figure 17. Multiple sequence alignment of thrombin-like serine proteases. Venombin A are GPV-TL-1 and GPV-TL-2 from *T. albolabris*, Stejnobin from *T. stejnegeri*, Flavoxobin from *T. flavovirides*, KN-BJ2 from *Bothrops jararaca*, Batroxobin from *B. atrox*, Bothrombin from *B. jararaca*, Venombin B is contortrixobin from *Agkistrodon contortrix* and Venombin AB is Bilineobin from *A. bilineatus*.

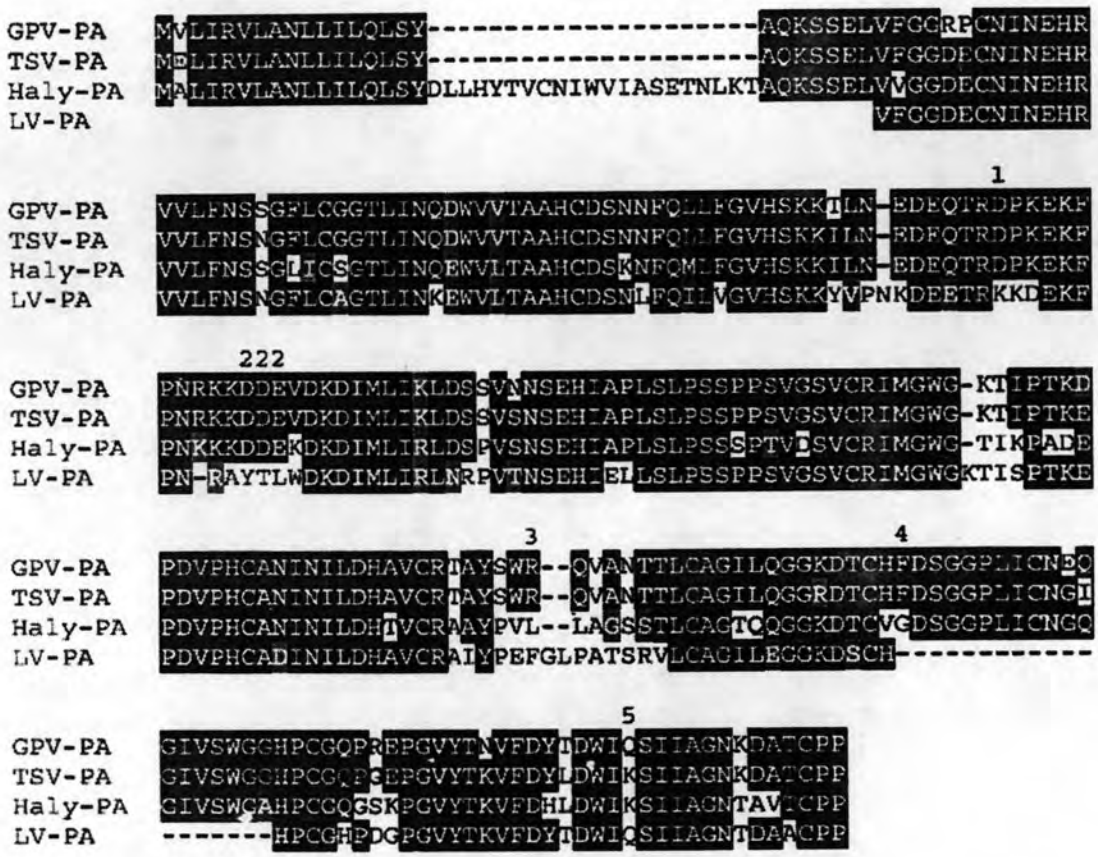


Figure 18. Multiple sequence alignment of snake venom plasminogen activator. GPA-PA is from this study. TSV-PA was from *T. stejnegeri*. Haly-PA was from *Gloydius halys* (Park et al., 1998) and LV-PA was from *Lachesis muta muta* (Sanchez et al., 2000). Number 1 denotes Asp 82 Number 2 represents the DDE motif. Number 3 marks Arg 174. Number 4 is on Phe 193. Finally, number 5 denotes Lys 240.

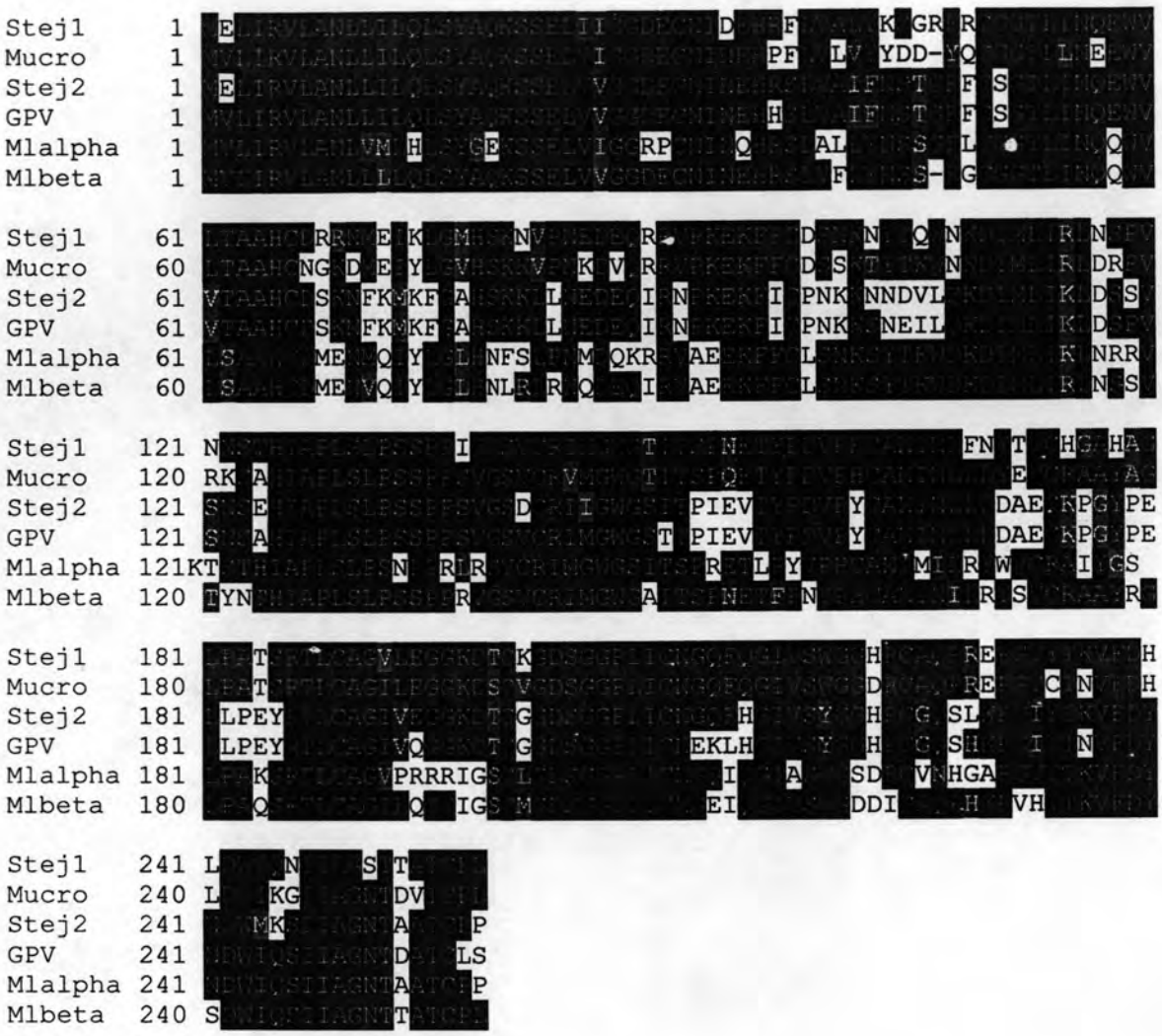


Figure 19. Multiple-sequence alignment of snake venom fibrinogenase.GPV is albofibrase. Stej1 and Stej2 are stejnefibrase1 (AAN52348.1) and stejnefibrase2 (AAN52349.1), respectively, from *Trimeresurus stejnegeri*. Mucro is mucrofibrase-1 (Q91507) from *Protobothrops mucrosquamatus*. Mlalpha and Mlbeta are *Macrovipera lebetina* alpha- fibrinogenase (AAM96674.1) and beta-fibrinogenase (AAM96700.1), respectively.

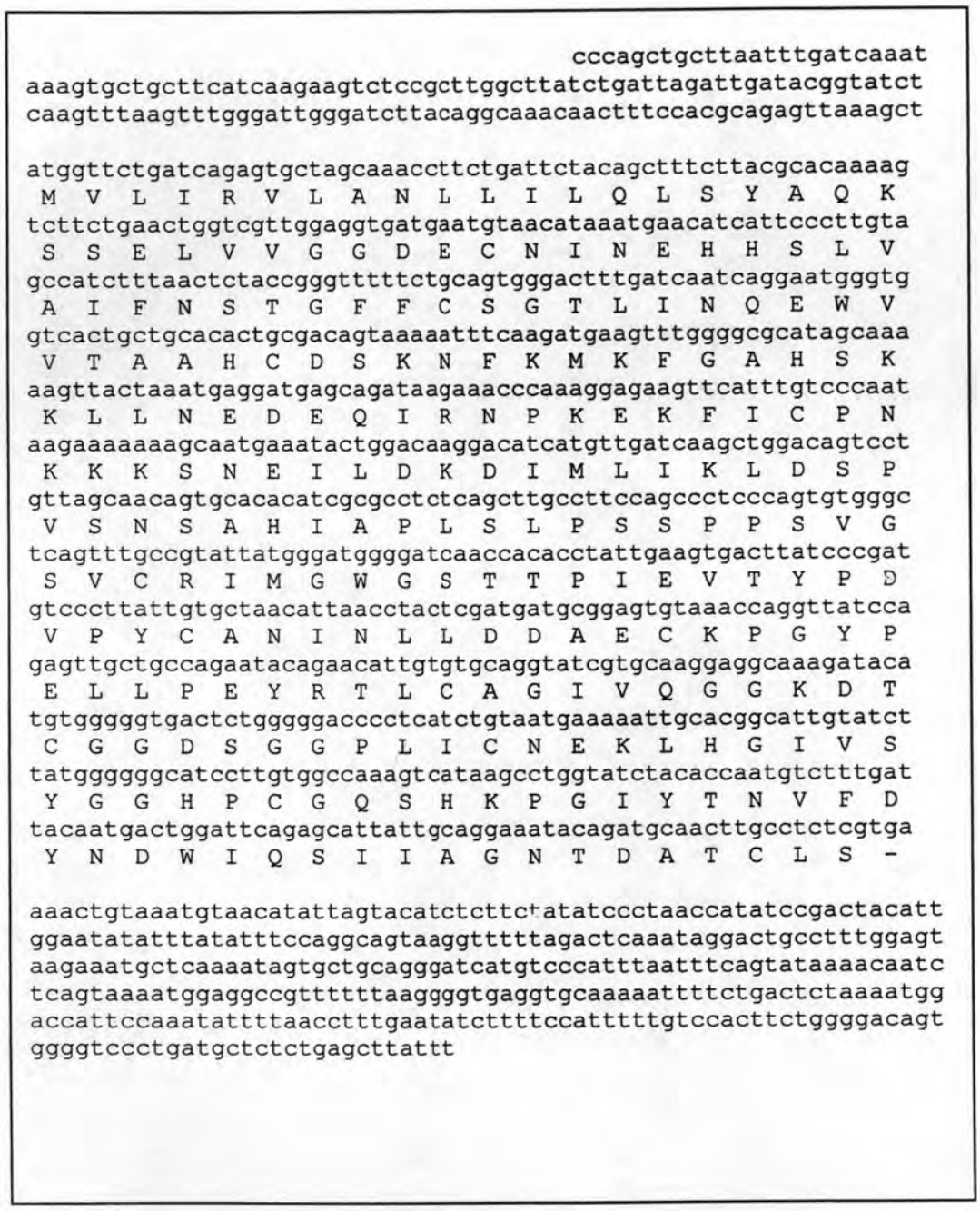


Figure 20. Complete cDNA and deduced amino acid sequences of snake venom serine protease (albofibrase) from *Trimeresurus albolabris*.

4.2 Expression of albofibrase in *Pichia pastoris*

4.2.1 Amplification of albofibrase by PCR

The cDNA of albofibrase excluding signal and pro-peptides was amplified by PCR with forward primer, SerineF80, that has an *Eco*R I recognition site and reverse primer, Serine CLSR , that has an *Xba* I recognition site. After electrophoresis, the PCR product size was approximately 1200 bp DNA in length (**Figure 21**).

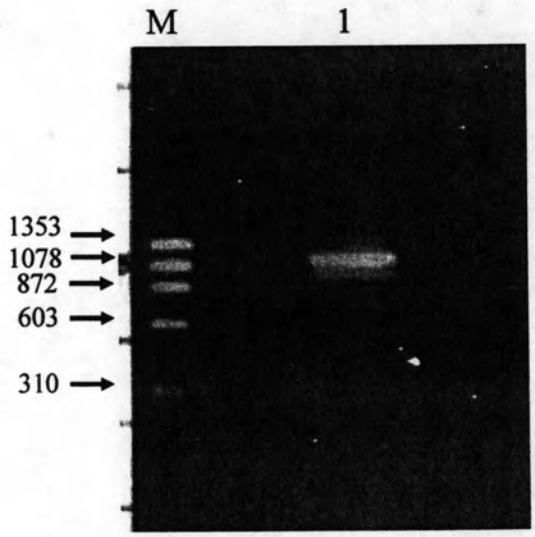


Figure 21. PCR product of albofibrase. M = Molecular weight marker lamda *Hin*dIII, Lane 1; PCR of albofibrase was approximately 1200 bp in length.

4.2.2 Ligation of albofibrase cDNA into pGEM® T-vector and Transformation of *E. coli*, JM109

The PCR product was extracted, purified and then cloned into pGEM® T-vector and transformed into *E. coli*, JM109. The positive clones were identified by blue-white color system (**Figure 22**). The white plasmid clones were isolated and digested with *Eco*R I to verify the presence of inserts (**Figure 23**) and then sequenced with T7 sequencing primer.

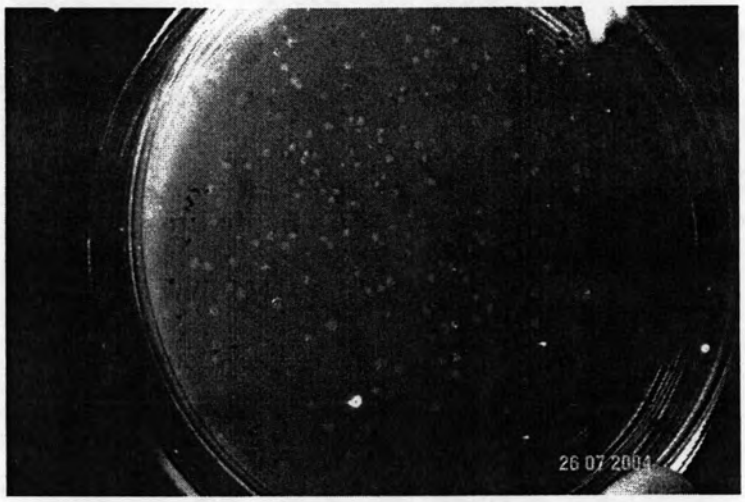


Figure 22. Albofibrase cDNA was transformed into *E. coli*, JM 109. The transformants were selected by a blue-white system.

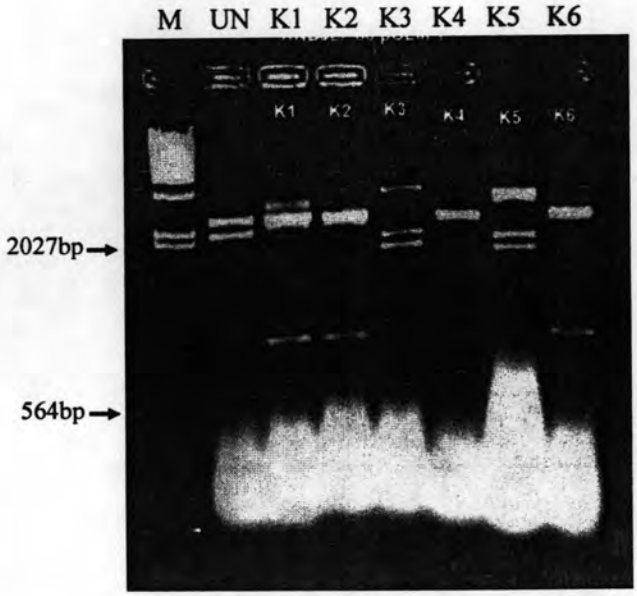


Figure 23. Albofibrase - transformed plasmid that represented the presence of inserts

4.2 Expression of Albofibrase in *Pichia pastoris*

4.2.1 Ligation of Albofibrase into pPICZαA and Transformation of *E. coli*, JM109

After the plasmid clone was confirmed by sequencing, plasmid DNA and expression vector, pPICZαA, were digested with *Eco*R I and *Xba* I. The digestion reaction were electrophoresed in 1.2 % agarose gel. After gel extraction and purification, the digestion product was cloned into the *Eco*R I and *Xba* I sites of expression vector, pPICZαA. The vector contained C-terminal histidine tag to facilitate proteins purification. The recombinant plasmid was transformed into *E. coli*, JM109, and the colonies were selected on agar plate with low-salt LB and 25 μg/ml Zeocin™. As a result, there were about 200 Zeocin™-resistant transformants. The recombinant plasmids were digested with *Eco*R I as shown in

Figure 24. The band of the correct clone was shifted after it was digested by *Eco*R I. The clones, which contained of insert, were about 3,900 bp in length. Finally, the correct in-frame clones were confirmed by sequencing.

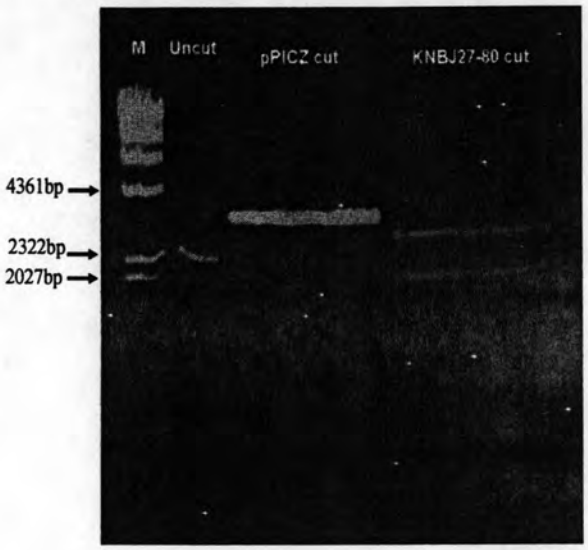


Figure 24. Electrophoresis of cut pPICZαA. M; Molecular weight marker λ *Hin* d III. KNBJ27-80 ; recombinant albofibrase plasmid digested with *Eco*R I and *Xba*I .

4.2.4 Transformation of Recombinant pPICZαA into *Pichia pastoris*, X-33

Prior to transformation into *Pichia pastoris*, recombinant pPICZαA was linearized with *Sac* I. After that, linearized recombinant pPICZαA was transformed into competent *Pichia* cells, X-33. Approximately 85 colonies transformants were found within 4 days as shown in **Figure 25**. PCR results revealed that albofibrase gene had been integrated into *Pichia* genome (**Figure 26**) and the PCR product was 1200 bp in length using 5′-*AOX1* primer.

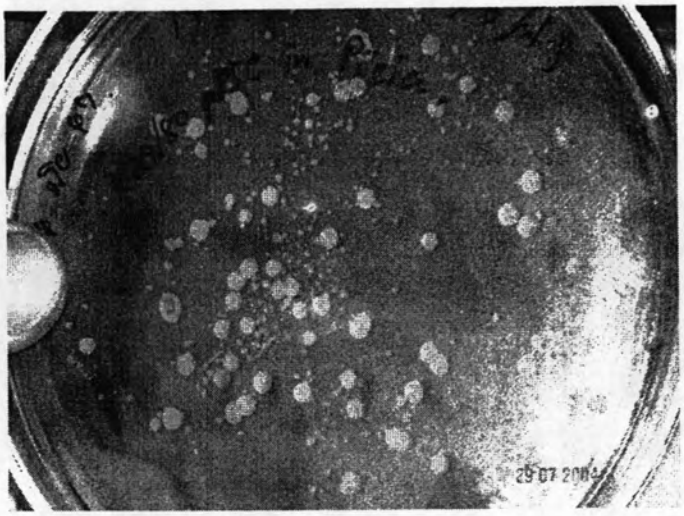


Figure 25. Recombinant albofibrase in *Pichia pastoris*, X-33, on YPDS (100 μg/ml Zeocin®)

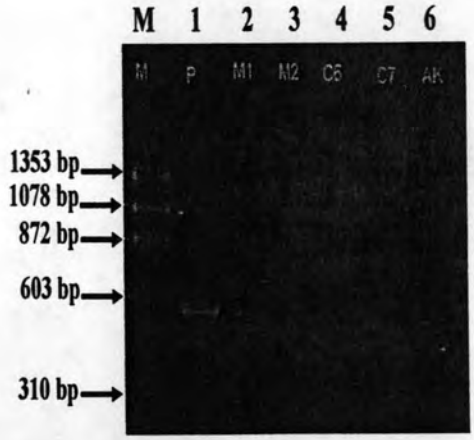
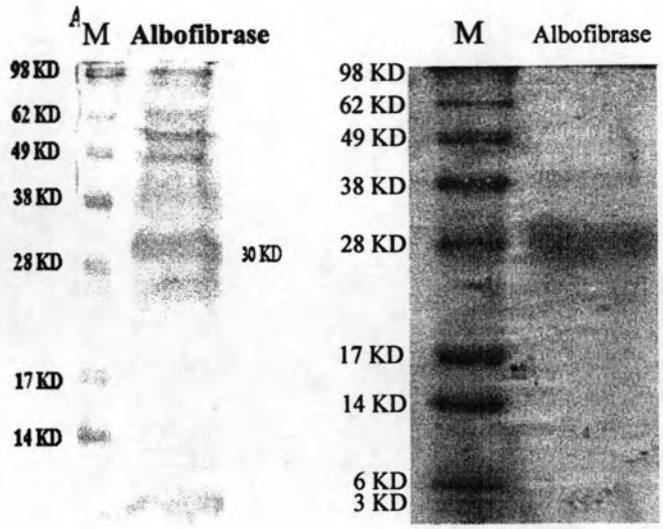


Figure 26. PCR analysis of *Pichia* integrants. M: molecular weight marker Phi X 174; Lane 1: pPICZαA vector; Lanes 2 and 3: recombinant *Pichia* with albofibrase and Lane 4-6: positive control: recombinant *Pichia* with other constructs.

4.2.5 Expression of Recombinant Albofibrase in *Pichia pastoris*, X-33 and Protein Purification

From the time-course experiments, the optimal incubation time for the maximum production of recombinant albofibrase was 96 hours. The apparent molecular mass of recombinant albofibrase was about 30 kDa in both the reduced and non-reduced condition on SDS-PAGE. For large scale expression, recombinant *Pichia* was expressed in 1.0 liter of BMMY and induced by 0.5 % v/v methanol. The concentrated media was chromatographed on a cobalt-based immobilized metal affinity column. The elution fractions of recombinant albofibrase were monitored by its absorbance at 280 nm. and analyzed by Coomassie-stained SDS-PAGE as shown in **Figure 27** and pooled into 15 ml. For the detection and confirmation of recombinant albofibrase protein, the pooled fraction was determined by Western blotting probed with anti-His antibody (**Figure 28**). The molecular mass of recombinant albofibrase was approximately 30 kDa.



A B

Figure 27. Coomassie-stained SDS-PAGE showed albofibrase protein expression. M: prestained protein marker, **Fig 27A**; Albofibrase before purification and **Fig 27B**; Albofibrase after purification by Magne-His.

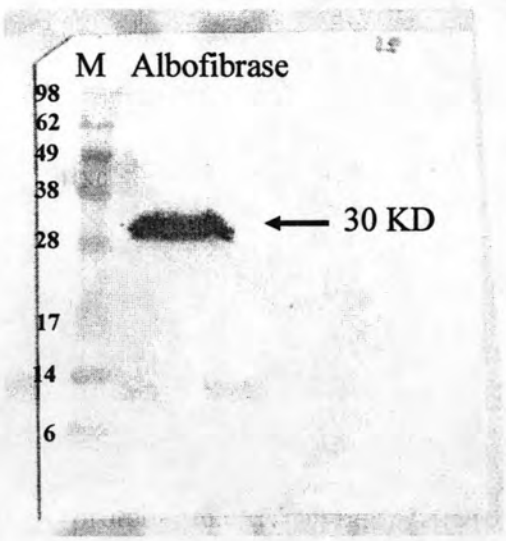


Figure 28. Western blot analysis of pooled fractions of albofibrase Lane 1; Prestained protein marker. Lane 2; Albofibrase probed with anti-His antibody.

4.2.6 Quantitative Assay of Purified Recombinant Protein

To determine the production level of purified recombinant albofibrase, the Micro BCA™ Protein Assay was used for measuring the protein concentration. Average absorbancy average of 0.426 at 570 nm at 1:10 dilution of duplicate protein sample was used to calculate protein concentration as 0.036 mg/ml (**Figure 29**). Therefore, the yield of recombinant albofibrase produced in *P. pastoris* was 0.658 mg/Liter of culture medium.

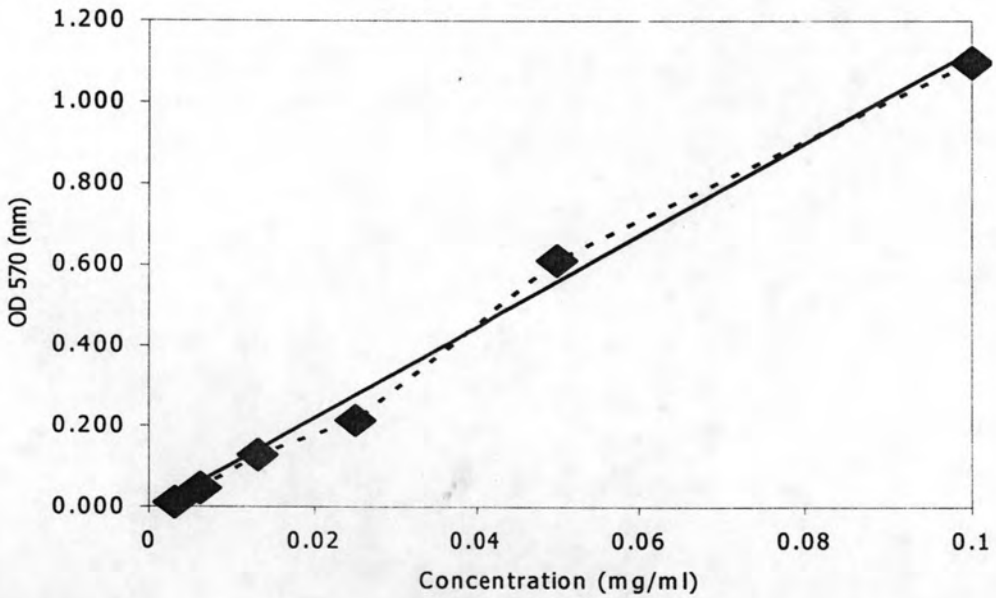


Figure 29. Standard curve of protein concentrations fitted by linear regression ($y = mx + b$, $m = 12.292$, $b = -0.005$, $r^2 = 0.994$)

4.3 Testing effects of albofibrase

4.3.1 The enzymatic activity

Substrates	Albofibrase pNA release / minute (nmol/min)	Trysin pNA release / minute (nmol/min)
S-3013	3.0	3.3
S-3015	0.0	0.0
S-3125	0.6	0.9

Table 4. The enzymatic activity of albofibrase on serine protease chromogenic substrates

Recombinant albofibrase was able to cleave serine protease substrates. Enzymatic activities represented by the rates of pNA release were shown in **Table 4.** It was active against a trypsin-like substrate (S-3013) and showed a weaker activity on an activated protein C substrate (S-3125). Both albofibrase and trypsin have no detectable activity against chymotrypsin substrate (S-3015).

4.3.2 Thrombin like clotting activity

The thrombin time tests the third stage of coagulation, the convestion of fibrinogen to fibrin. It measures the availability of functional fibrinogen. Albofibrase showed thrombin like effect on fibrinogen. The thrombin clotting time of albofibrase was 69 seconds. Therefore, 2.3 nM albofibrase was equivalent to 0.016 thrombin units as calculated from standard logarithmic curve of thrombin (**Figure 28**).

Number	Standard thrombin (unit)	Thrombin clotting time (seconds)
1	0.015	75
2	0.075	37
3	0.15	20
4	0.75	12
5	1.50	10

Table 5. Standard thrombin unit and thrombin clotting time

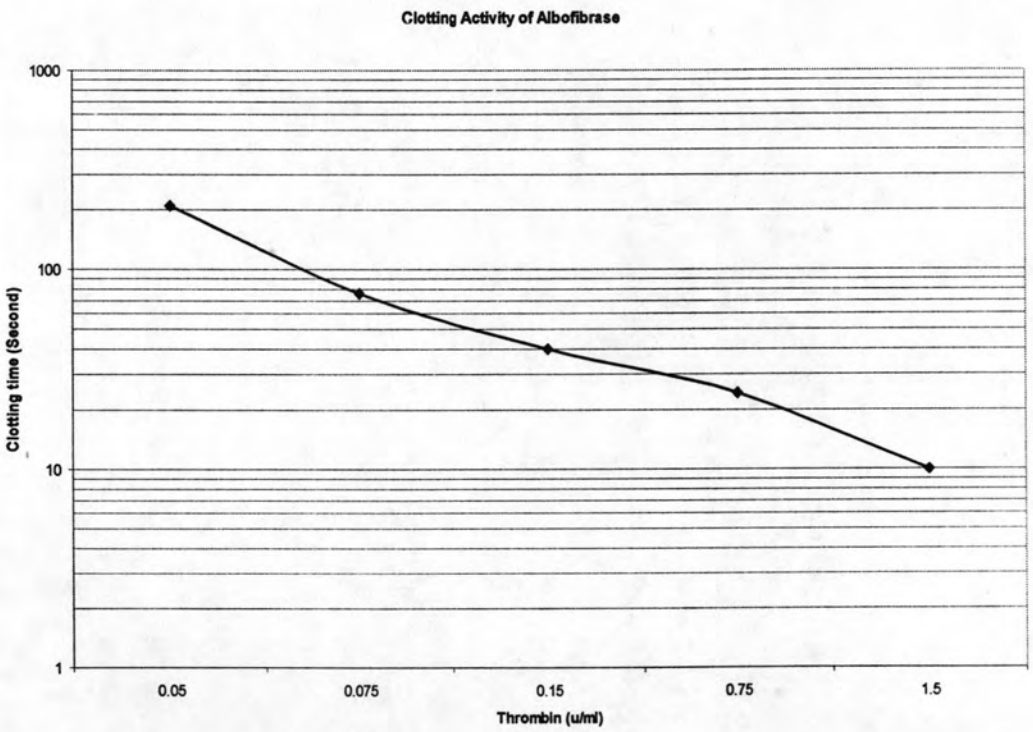


Figure 30. Standard logarithmic curve of thrombin unit versus thrombin clotting time. The clotting time of 2.3 nM albofibrase was 69 seconds equivalent to 0.016 units/ml thrombin.

4.3.3 Fibrinogenolytic activity

Albofibrase digested fibrinogen by cleavage of peptide bonds in the Aα-chain. The result was showed in **figure 31** (SDS-PAGE of fibrinogenolytic activity of albofibrase on fibrinogen). The Aα-chain of fibrinogen was initially cleaved by albofibrase at 180 minute incubation. The Bβ-chain and γ-chains of fibrinogen were not digested by albofibrase. Recombinant disintegrin a negative control could not cleaved any of α-, β- and γ-chains of fibrinogen.

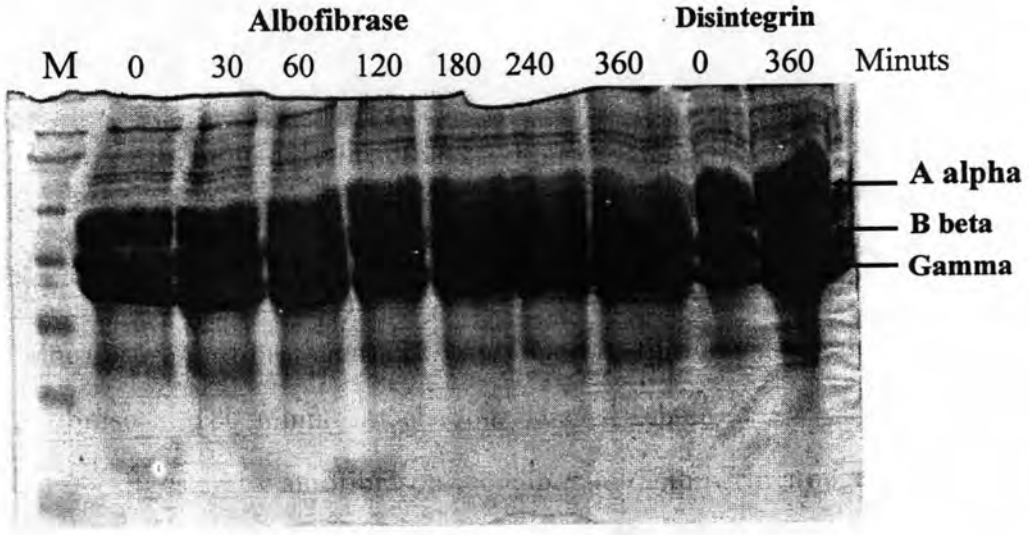


Figure 31. Fibrinogenolytic activity of serine protease (albofibrase) from *T. albolabris* on Aα-, Bβ- and γ-chain of fibrinogen. M = prestained protein marker. Incubation time of 0, 30, 60, 120, 180, 240 and 360 minutes for albofibrase with fibrinogen. Incubation time 0 and 360 minutes for disintegrin with fibrinogen.

4.3.4 Plasminogen activation assay

Plasminogen activator activity of albofibrase was modest with only 0.35 (0.67,0.15, 0.26 and 0.33) times the activity of control plasminogen activator (u-PA).

Concentration (nM) / O.D. at time	0.0	0.75	1.5	3.0
0 min	0.012	0.014	0.016	0.023
10 min	0.015	0.048	0.096	0.168
Delta O.D.	0.003	0.034	0.080	0.145

Table 6. Plasminogen activity of u-PA at various concentrations at 0 and 10 minutes

Concentration (nM) / O.D. at time	0.0	0.75	1.5	3.0
0 min	0.014	0.030	0.021	0.026
10 min	0.016	0.035	0.042	0.074
Delta O.D.	0.002	0.005	0.021	0.048

Table 7. Plasminogen activity of Abofibrase at various concentrations at 0 and 10 minutes

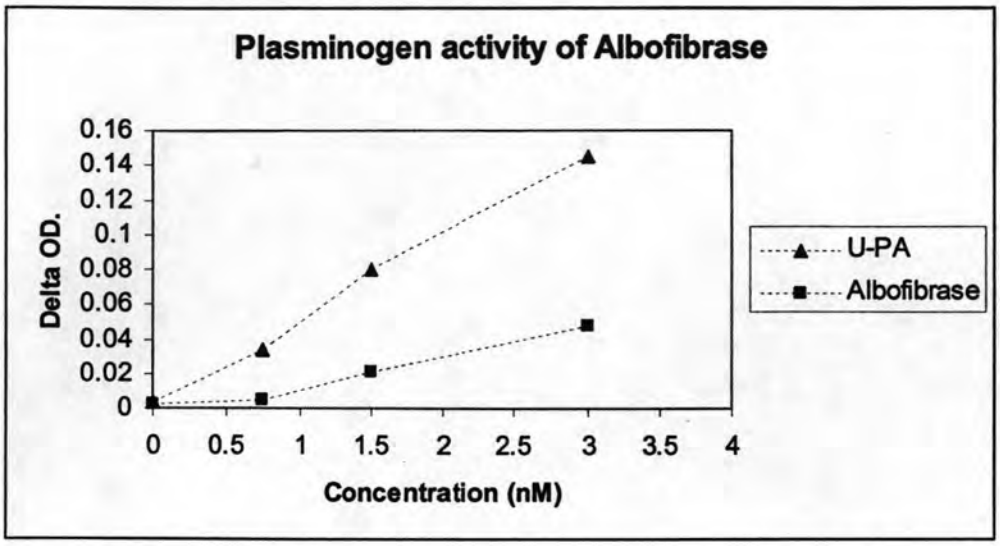


Figure 32. Plasminogen activation by Albofibrase. The delta OD represents absorbance changes Albofibrase added at the indicated concentrations time were measured for the absorbance (405 nanometre) at 0 minute and at 10 minutes. U-PA was used as standard and S2251 chromogenic substrate was used.

4.3.5 Anti-coagulant activity of Albofibrase

Albofibrase might be used as an anti-coagulant agent to protect the patients from stroke, coronary heart disease or disseminated intravascular coagulation (DIC). Prothrombin Time (PT) and activated partial thromboplastin time(PTT) were used to monitoring anti-coagulant activity of albofibrase.

The prothrombin time is a useful screening procedure for deficiencies in factor II, V, VII and X. This test was be used to follow the course of anticoagulation of albofibrase. In 1984, the international system for the standardization of the prothrombin time in anti-coagulant control based on International Normalized Ratios (INR) was recommened. The INR must be determined on the treatment and adjustment of the oral anticoagulant maintenance doses.

The activated partial thromboplastin time with albofibrase was represented as ratios with normal plasma without albofibrase.

The results showed that albofibrase could be anticoagulant at 30 minute or longer incubations as shown in **Table 7 and Figure 31-32.**

No	Plasma + 2.3nM Albofibrase (Time incubation)	INR	Ratio of aPTT with normal control
1	0 minute	1.07	1.00
2	30 minutes	1.32	1.20
3	60 minutes	1.41	1.22
4	120 minutes	1.61	1.32
5	180 minutes	1.68	1.35
6	240 minutes	1.73	1.37
7	360 minutes	1.82	1.34

Table 8. Normal human plasma and 2.3 nM albofibrase were incubated GPV venum at various concentrations

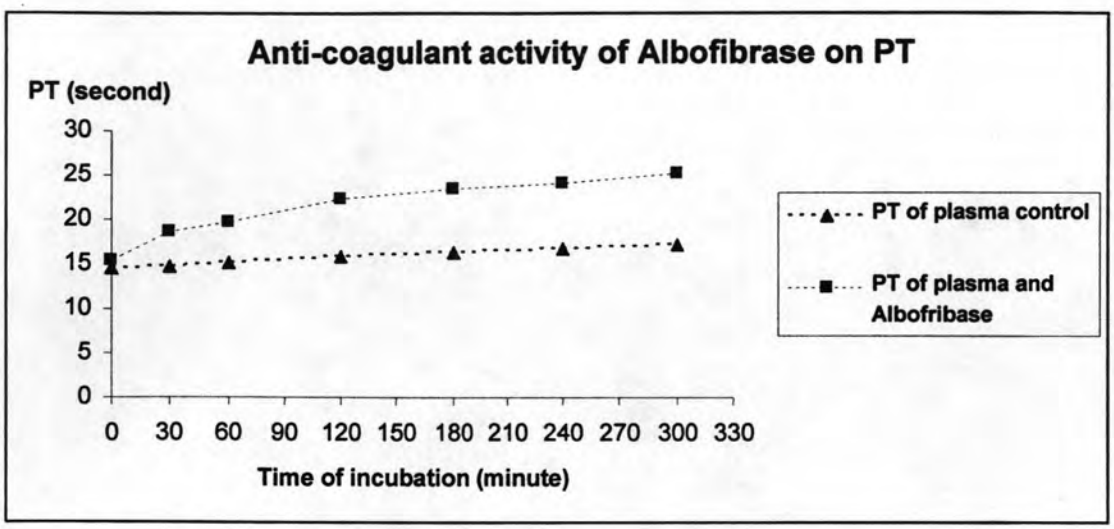


Figure 33. Comparation anti-coagulant activity of Albofibrase with normal human plasma in Prothrombin Time (PT) at various incubation time (minute)

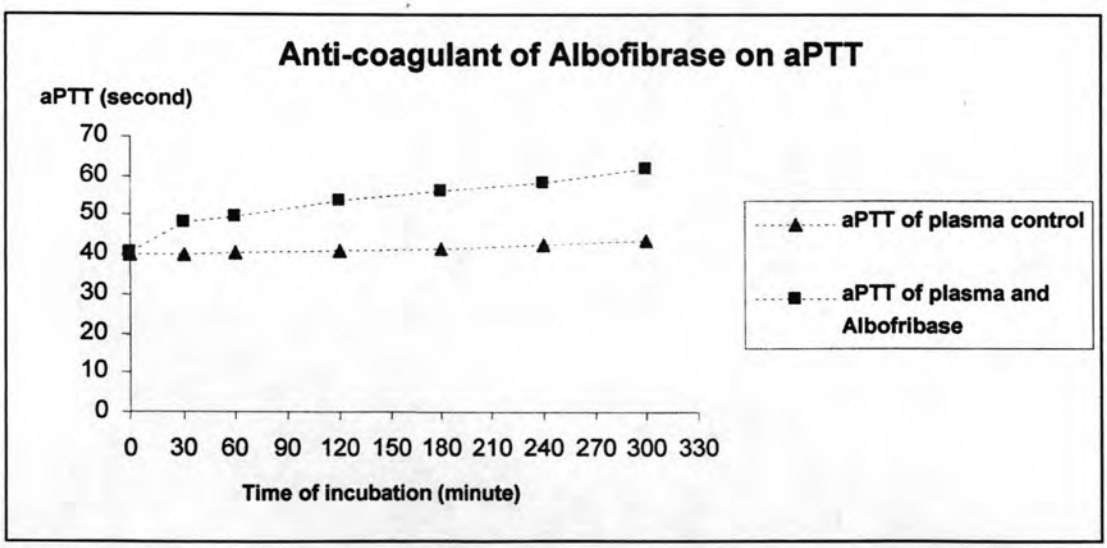


Figure 34. Comparision of anti-coagulant activity of Albofibrase with normal human plasma using partial thromboplastin time (aPTT) at various incubation time (minute)

4.3.6. Neutralization of anti-coagulant activity of Albofibrase with green pit viper antivenom

The co-incubation of 5.0 ng/ µl GPV antivenom could correct anti-coagulant of albofibrase on PT and aPTT. It was shown in **table 8.**

Number	GVP antivenom (ng/ µl)	PT (seconds)	INR	aPTT (seconds)
1	0	20.7	1.50	46.2
2	1.0	20.2	1.43	45.7
3	5.0	16.0	1.11	37.9
4	20.0	15.5	1.09	34.1

Table 9. Normal human plasma and 2.3 nM albofibrase were incubated with GPV Antivenom at various concentrations for 180 minuts before the tests

4.3.7Fibrin plate

Albofibrase could not digested fibrinogen on fibrin plate.

4.3.8. Factor II, V and X assays

Factor II, V and X assays of control plasma after incubation with albofibrase were normal. Factor II was 105 % (70-130), factor V was 72.7 % (60-130) and factor X was 103.4 % (70-120)

4.3.9. Platelet Aggregation Assay

Albofibrase could not induced platelet aggregation.

4.3.10. Fibrinogen

Albofibrase could decreased fibrinogen concentrations from 2.65 g/l to 2.10 g/l.