CHAPTER IV

RESULTS AND DISCUSSION

4.1 Toxicity

4.1.1 Electrophoresis titration curve (ET)

The Electrophoresis titration profile of Malayan pit viper from Thailand and Vietnam are show on fig 4.1.

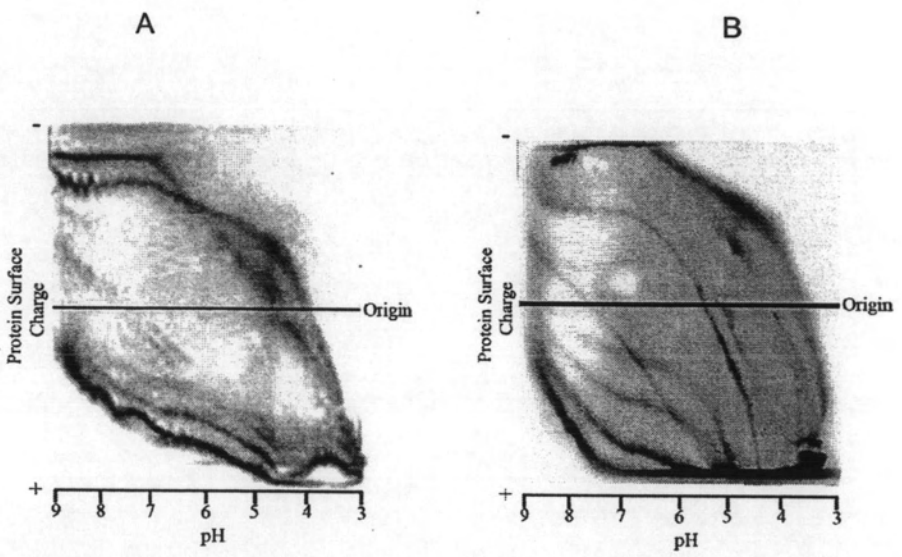


Fig. 4.1 Electrophoresis titration profile of Malayan pit viper from two sources Thailand (A) and Vietnam (B)

The electrophoresis profiles of Malayan pit viper venom from difference source are show difference pattern. The result confirms, this venom is containing with difference protein. Because of geological habitation are effects to protein synthesis.

4.1.2 Lethal dose (LD_{50})

Various concentrations of crude venom is injected into mice and observation on mice was done mice for 48 hours. The number of mice death is recorded on table 4.1. LD_{50} is calculated by prohibit analysis. As the result, LD_{50} of Malayan pit viper venom was 5.15 mg/kg

Table 4.1 Mice death report in 48 h

Concentration (mg/kg)	Mice death (0-24h)	Mice death (24-48h)	Total death (48h)
32	-	-	No injected[a]
16	8	0	8
8	6	2	8
4	1	0	1
2	0	0	0

[a] All of mices immediately die after injected with venom concentration of 16 mg/kg, therefore no injection at higher concentration was carried out.

Calculation

$$\text{Log } LD_{50} = \text{Log (100\%death)} - \frac{\text{LogDF}}{N} (\Sigma\text{Death-N/2})$$

Number of mice (N) = 8

Dilution factor (DF) = 2

Total mice death (ΣDeath) = 9

Concentration at 100%death (100%death) = 8 mg/kg

$$\text{Log } LD_{50} = \frac{\text{Log (100\%death)} - \text{LogDF} (\Sigma\text{Death-N/2})}{N}$$

$$= \text{Log8} - \frac{\text{Log2}}{8} (9\text{-}8/2)$$

$$= 0.9 - 0.188$$

$$\text{Log } LD_{50} = 0.712$$

$$LD_{50} = 5.15 \text{ mg/Kg}$$

4.1.3 Minimum active dose

Crude venom was tested for toxicity including fibrinolytic, hemorrhagic and gelatinase activities. The result was report by Minimum activity dose (table 4.2).

Table 4.2 Minimun active dose of crude venom

Activity	Minimun active dose (µg)	Unit
Fibrinolytic	13	MFD[a]
Hemorrhagic	15	MHD[b]
Gelatinase	10	MGD[c]

[a]Minimal amount of protein (µg) of crude venom to hydrolyze a 10 mm fibrinolytic spot.

[b]Minimal amount of protein (µg) of crude venom to hydrolyze a 10 mm hemorrhagic spot.

[c]Minimal amount of protein (µg) of crude venom to hydrolyze a 10 mm gelatinase spot.

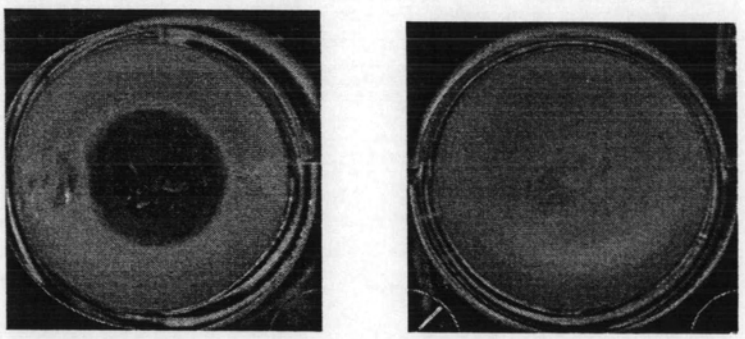
Minimum activity dose was used to determine the toxic of venom. Low level of Minimun active dose indicated the strong toxicity. As the result, the gelatinase activity is stronger than fibrinolytic and hemorrhagic activity.

4.2 Anti venom

Virginia opossum (*Didelphis virginiana*) serum was used as anti-toxin against crude Malayan pit viper venom. The activity of antihemorrhagic, antifibrinolytic and antigelatinase were reported.

4.2.1 Anti fibrinolytic activity

D. virginiana serum inhibited fibrinolytic activity from Malayan pit viper venom (Fig.4.2). As the result, 3.5 mg of serum could counteract 1 MFD of venom (13 µg) (Table 4.3).



A B

Fig. 4.2 Antifibrinolytic (A) Fibrinolytic activity from *C. rhodostoma* crude venom (MFD, 13 µg), (B) Inhibition of fibrinolytic activity from *C. rhodostoma* venom when incubated with *D. virginiana* crude serum

Table 4.3 Antifibrinolytic activity of *D. virginiana* serum against *C. rhodostoma* venom

Serum total protein (mg)[a]	1 MFD (µg)[b]	Fibrinolytic activity (mm)[c]	Specific activity (mm/µg)[d]
7.00	**13**	**0**	**0.00**
3.50	**13**	**0**	**0.00**
1.75	13	5	0.38
0.88	13	7	0.54
0.44	13	9	0.69
0.22	13	10	0.77
0.11	13	10	0.77
0.06	13	11	0.85
0.03	13	11	0.85

[a] The amount of protein (mg) mixed with 2 MFD.

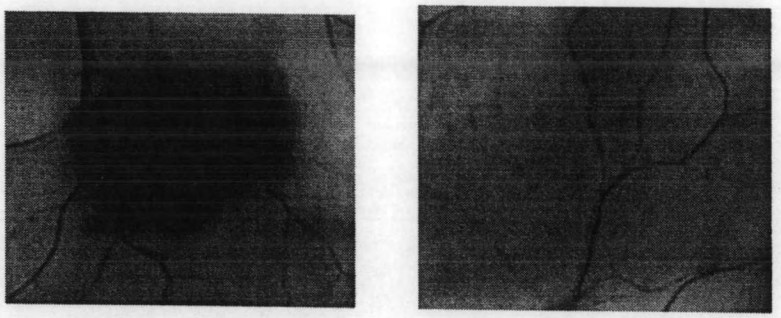
[b] Minimal amount of protein (µg) of crude venom to hydrolyze a 10 mm fibrinolytic spot.

[c] Diameter (mm) of the fibrinolytic spot induced by the serum/MFD mixture.

[d] The specific activity (mm/µg) is defined as the diameter (mm) of the fibrinolytic spot divided by 1 MFD.

4.2.2 Anti hemorrhagic activity

D. virginiana serum showed strong activity to inhibit hemorrhagic activity from Malayan pit viper venom. As the result, 0.88 mg of serum could counteract 1 MHD of venom (15 µg) (Table 4.3).



A B

Fig. 4.3 Antihemorrhagic activity (A) Hemorrhagic activity from crude *C. rhodostoma* crude venom (MHD, 15 µg) (B) Inhibition of hemorrhagic activity from *C. rhodostoma* venom when incubated with *D. virginiana* crude serum (0.88 mg of serum inhibited 1 MHD)

Table 4.4 Antihemorrhagic activity of *D. virginiana* serum against *C. rhodostoma* venom

Serum total protein (mg)[a]	1 MHD (µg)[b]	Hemorrhagic activity (mm)[c]	Specific activity (mm/µg)[d]
7.00	**15**	**0**	**0.00**
3.50	**15**	**0**	**0.00**
1.75	**15**	**0**	**0.00**
0.88	**15**	**0**	**0.00**
0.44	15	6	0.40
0.22	15	10	0.67
0.11	15	10	0.67
0.06	15	12	0.80
0.03	15	12	0.80

[a] The amount of protein (mg) mixed with 2 MHD.

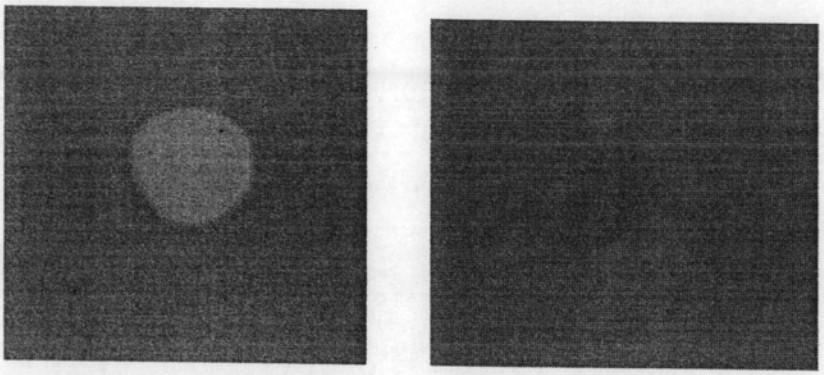
[b] Minimal amount of protein (µg) of crude venom to hydrolyze a 10 mm hemorrhagic spot.

[c] Diameter (mm) of the hemorrhagic spot induced by the serum/MHD mixture.

[d] The specific activity (mm/µg) is defined as the diameter (mm) of the hemorrhagic spot divided by 1 MHD.

4.2.3 Antigelatinase activity

D. virginiana serum showed strong activity to inhibit hemorrhagic activity from Malayan pit viper venom. As the result, 0.22 mg of serum could counteract 1 MGD of venom (10 µg) (table 4.5).



A B

Fig. 4.4 Antigelatinase (A) Gelatinase activity from *C. rhodostoma* crude venom (MGD, 10 µg) (B) Inhibition of gelatinase activity from crude venom when incubated with *D. virginiana* crude serum

Table 4.5 Antigelatinase activity of *D. virginiana* serum against *C. rhodostoma* venom

Serum total protein (mg)[a]	1 MGD (µg)[b]	Gelatinase activity (mm)[c]	Specific activity (mm/µg)[d]
7.00	10	0	0.0
3.50	10	0	0.0
1.75	10	0	0.0
0.88	10	0	0.0
0.44	10	0	0.0
0.22	10	0	0.0
0.11	10	5	0.5
0.06	10	10	1.0
0.03	10	11	1.1

[a] The amount of protein (mg) mixed with 2 MGD.

[b] Minimal amount of protein (µg) of crude venom to hydrolyze a 10 mm gelatinase spot.

[c] Diameter (mm) of the gelatinase spot induced by the serum/MGD mixture.

[d] The specific activity (mm/µg) is defined as the diameter (mm) of the gelatinase spot divided by 1 MGD.

4.2.4 Inhibition ratio

D. virginiana serum contained some chemicals that could counteract against fibrinolytic, hemorrhagic and gelatinase activity. The inhibition capacity was shown in table 4.6.

Table 4.6 Antiproteolytic efficacy of opossum crude serum against Malayan pit viper crude venom.

Antiproteolytic activity	Inhibition ratio (opossum serum : venom)[a]
Antihemorrhagic activity[b]	116.0 : 1
Antifibrinolytic activity[c]	269.0 : 1
Antigelatinase activity[d]	21.9 : 1

[a] The inhibition ratio is defined as ratio of opossum protein / proteolytic protein

[b] Opossum serum /venom 1 MHD

[c] Opossum serum /venom 1 MFD

[d] Opossum serum /venom 1 MGD

The Virginia opossum (*Didelphis virginiana*) is an animal which has a naturally resistance to the proteolytic effects of many snake venoms. This resistance was explained by the presence of soluble proteins in the sera, which neutralize the toxic effects of snake venoms (Jurgilas *et al.*, 2003; Perales *et al.*, 2005).

Fibrinolytic and hemorrhagic proteins are metalloproteinase enzymes that have a homologous sequence in the active site and rely on the coordination of a zinc atom by three invariant histidine residues located in a 10 residue segment that folds into the walls of the active site (Hite et al., 1992). The inhibitor of enzymatic activity by agents that chelate zinc supports the conclusion from atomic absorption spectroscopy showing one mole of zinc per mol of enzyme (Guan et al., 1991) and sequence analysis (Randolph et al., 1992) that shows the signature sequence of the metazincs (Bode et al., 1993; Bode et al., 1996; Stocker & Bode, 1995). Thus fibroblast is a zinc metalloproteinase and not a serine or cysteine proteinase (Swenson & Markland, 2005)

4.2.5 Toxicity of Malayan pit viper compare with North America snake

LD_{50} and MHD of Malayan pit viper were compared with North America snake (Table 4.7 and Table 4.8).

Table 4.7 LD_{50} of North America snake compared with Malayan pit viper (*C .rhodostoma*) (Sanchez *et al.*, 2003a)

Snake	LD_{50}(mg/kg)	Ratio[a]
C. s. scutulalus type A	0.47	10.96
C. h. horridus	0.53	9.72
C. h. atricuadatus	1.26	4.09
C. v. viridis	1.56	3.30
Sistrurus catenatus edwardsii	1.70	3.03
C. adamanteus	1.84	2.80
C. v. helleri	1.90	2.71
C. v. oreganos	2.10	2.45
S. c. tergiminus	2.10	2.45
A. p. leucostoma	2.75	1.87
C. m. molossus	4.84	1.06
C. atrox	5.10	1.01
C. s. scutulalus type B	5.10	1.01
C. rhodostoma	**5.15**	**1.00**
A. c. contortrix	5.20	0.99
A. c. laticinctus	6.80	0.76

[a] LD_{50} of *C. rhodostoma* / LD_{50} of this study

Table 4.8 MHD of North America compared with Malayan pit viper (*C. rhodostoma*) (Sanchez *et al.*, 2003a)

Snake	MHD(µg)	Ratio[a]
C. adamanteus	0.30	50.00
C. v. viridis	0.70	21.43
C. v. helleri	2.25	6.67
Sistrurus catenatus tergiminus	2.40	6.25
C. atrox	2.50	6.00
S. c. edwardsii	3.50	4.29
C. h. horridus	5.60	2.68
C. s. scutulalus –B	12.20	1.23
C. m. molossus	12.50	1.20
C. rhodostoma	**15.00**	**1.00**
A. p. leucostoma	29.00	0.52
C. h. atricaudatus	37.50	0.40
C. v. oreganos	43.00	0.35
A. c. laticinctus	67.00	0.22
A. c. contortrix	143.00	0.10
C. s. scutulatus –A	-	-

- No hemorrhagic activity

[a] MHD of *C. rhodostoma* / MHD of this study

As the result, Malayan pit viper showed lower toxicity than North America snake, by comparison of MHD and LD_{50}

4.3 Purification of fibrinolytic protein

4.3.1 HIC column

In the first step, Protein was purified by HIC columns and the chromatogram is shown in fig 4.5.

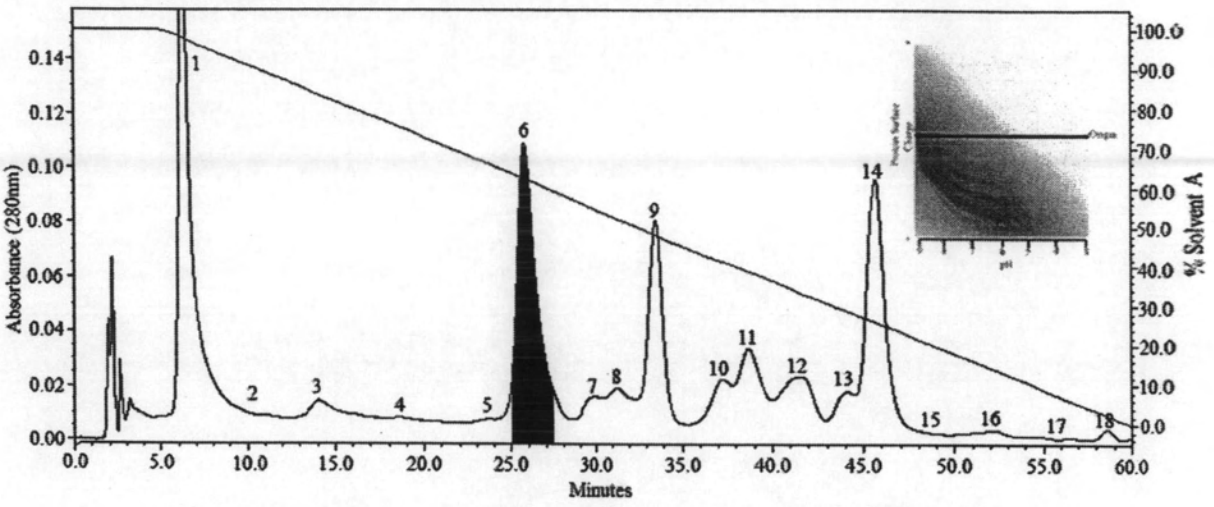


Fig. 4.5 Chromatogram from HIC column

Chromatogram from HIC column shows eighteen fractions. Each fraction was tested for biological activity. The result shown on table 4.9.

Table 4.9 Biological properties of fractions from HIC column

Fractions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Hemorrhagic Activity	-	1	-	1	1	-	-	-	-	2	3	2	-	-	-	-	-	-
Fibrinolytic Activity	-	-	-	-	-	3	1	1	1	-	-	-	-	-	-	-	-	-
Disintigrin Activity	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase Activity	-	1	-	1	-	3	-	-	-	2	3	2	-	-	-	-	-	-

- No activity, 1= weak activity, 2=mind activity, 3= strong activity

4.3.2 protein-pak™ 125 column

Fraction six from HIC column which showed strong fibrinolytic activity with out hemorrhagic activity was concentrated, desalted and injected into protein-pak™ 125 column. Chromatogram is shown in fig. 4.6 and biological activity of each fraction is shown in table 4.10.

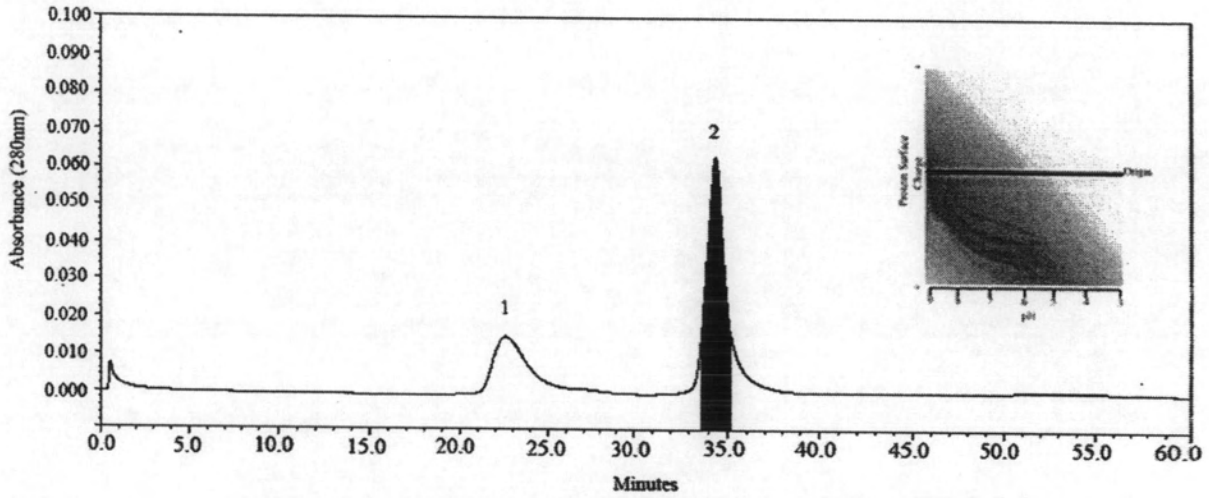


Fig. 4.6 Chromatogram from protein-pak™ 125 column

Table 4.10 Biological properties of fractions from size exclusion chromatography on Waters protein-pak™ 125 column

Fractions	1	2
Fibrinolytic activity	-	+
Gelatinase activity	-	+

4.3.3 protein-pak™60 column

Fraction two from protein-pak™125 column which showed strong fibrinolytic activity was concentrated, desalted and injected into protein-pak™60 column. Chromatogram is shown in fig. 4.7 and biological activity of each fraction is shown table 4.11.

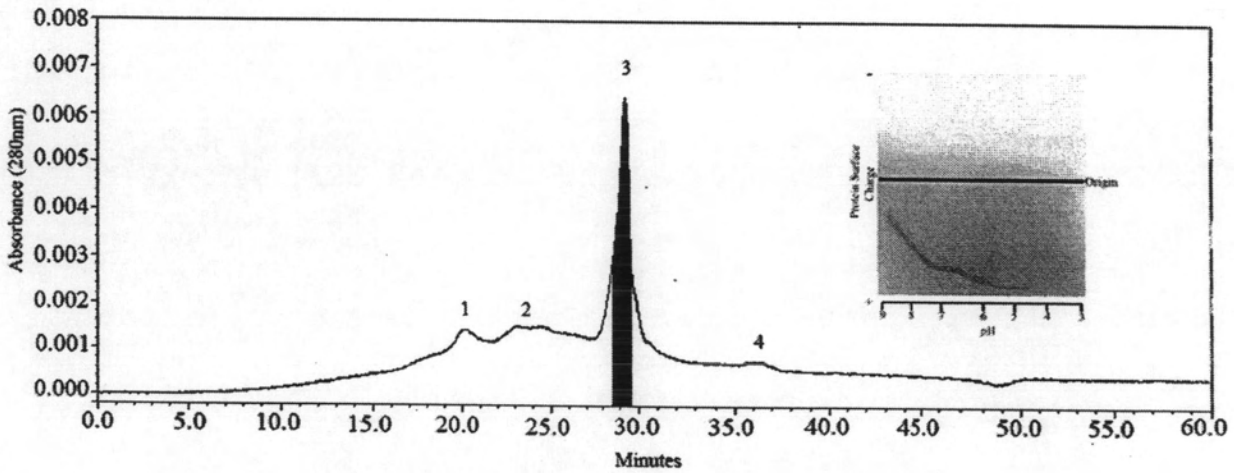


Fig. 4.7 Chromatogram from protein-pak™60 column

Table 4.11 Biological properties of fractions from protein-pak™60 column

Fractions	1	2	3	4
Fibrinolytic activity	-	-	+	-
Gelatinase activity	-	-	+	-

4.3.4 Purification conclusion

Protein was successful purified using three columns. The % recovery and specific activity of each purification step are show on table 4.12.

Table 4.12 Conclusion of HPLC separation of protein

Column	% recovery	Specific activity(mm/ μg)
HIC	13%	0.77
Protein-pak™ 125	45%	0.90
Protein-pak™ 60	30%	1.10

4.4 SDS gel electrophoresis

Fraction three from size exclusion protein-pak™ 60 column was applied to 12% SDS gel, and the electrophoresis pattern of SDS gel is shows in fig. 4.8.

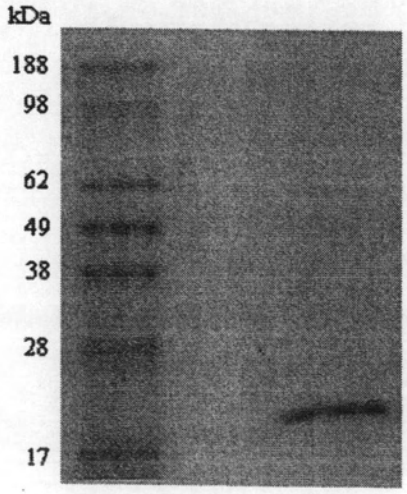


Fig. 4.8 Electrophoresis pattern of fraction three obtained from size exclusion protein-pak™ 60

4.5 Fibrinogenolytic activity

Fraction three from size exclusion protein-pak™ 60 column was mixed with human fibrinogen and incubated and subjected to 12% gel electrophoresis. The electrophoresis pattern is shown in fig 4.9. When compared with human fibrinogen, α band was absence on lane B. The result indicated that this protein digested the α chain of human fibrinogen, it was classified as α fibrinogenase.

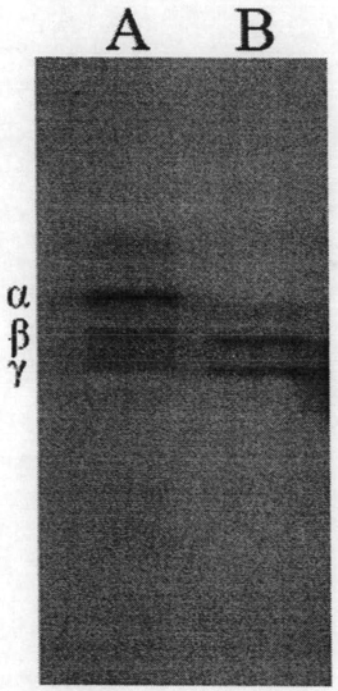


Fig. 4.9 12% SDS gel electrophoresis of fibrinogenolytic test (A) fibrinogen (B) fibrinogen incubate with fraction three from protein-pak™ 60 column

4.6 Insulin b chain digestion and anti insulin b chain digestion

The activity was determined by comparison of chromatogram from C18 column with control condition. The difference patterns indicated activity.

Control condition, insulin b chain was injected into C18 column. The result showd one main peak and two small peaks (Fig. 4.10).

Enzymatic condition, fraction three from size exclusion protein-pak™ 60 column was mixed with insulin b chain, incubated and injected into the same column. The result showd eight peaks which contain two main peaks and six small peaks (Fig. 4.11).

When compared chromatogram between two conditions, the enzyme conditions showd different pattern from the control condition. Thus it was suggested that the insulin b chain was digested by protein in fraction three from size exclusion protein-pak™ 60 column.

Inhibition condition, fraction three from size exclusion protein-pak™ 60 column was mixed with insulin b chain and opossum serum. Then incubated and injected into the C18 column. When compared with control condition, chromatogram from this condition showd insulin b chain peak (Peak no 5, Fig. 4.12) which indicated that the insulin b chain was not digested. The result supported the hypothesis is that enzyme was inhibited by opossum serum and it was classified as metalloproteinase.

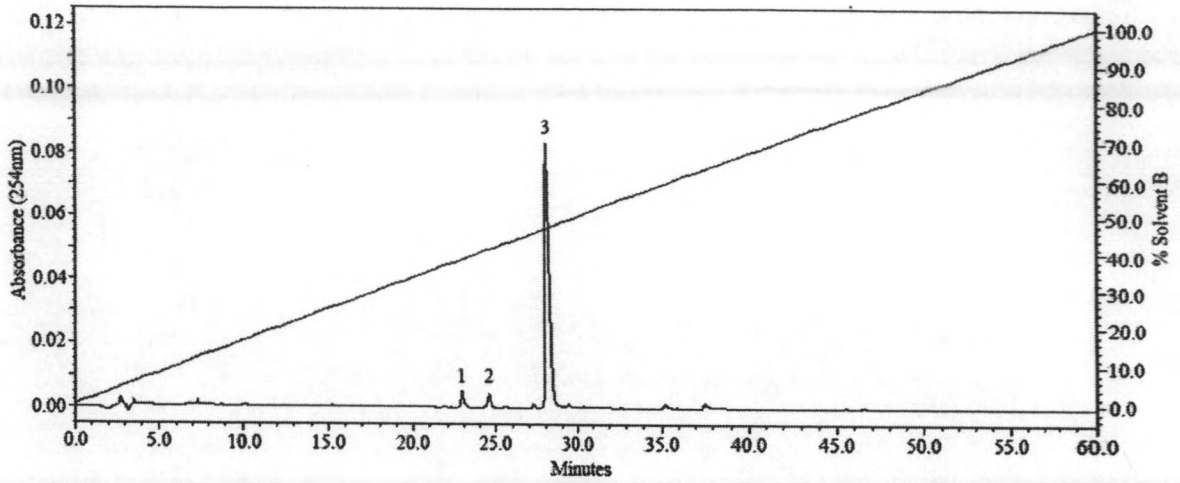


Fig 4.10 Chromatogram of control condition

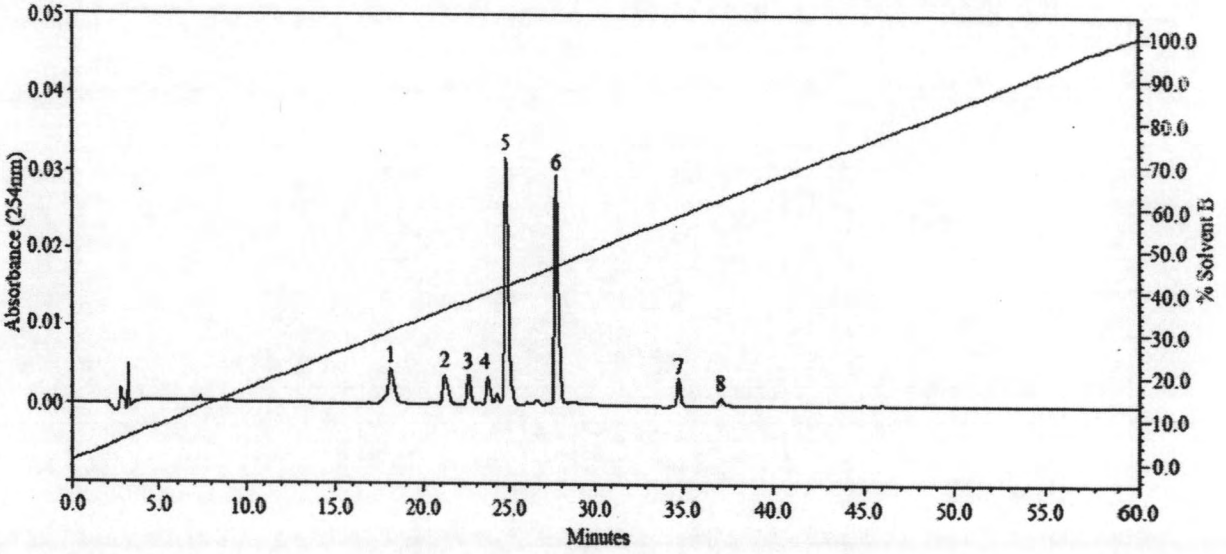


Fig 4.11 Chromatogram of enzymatic condition

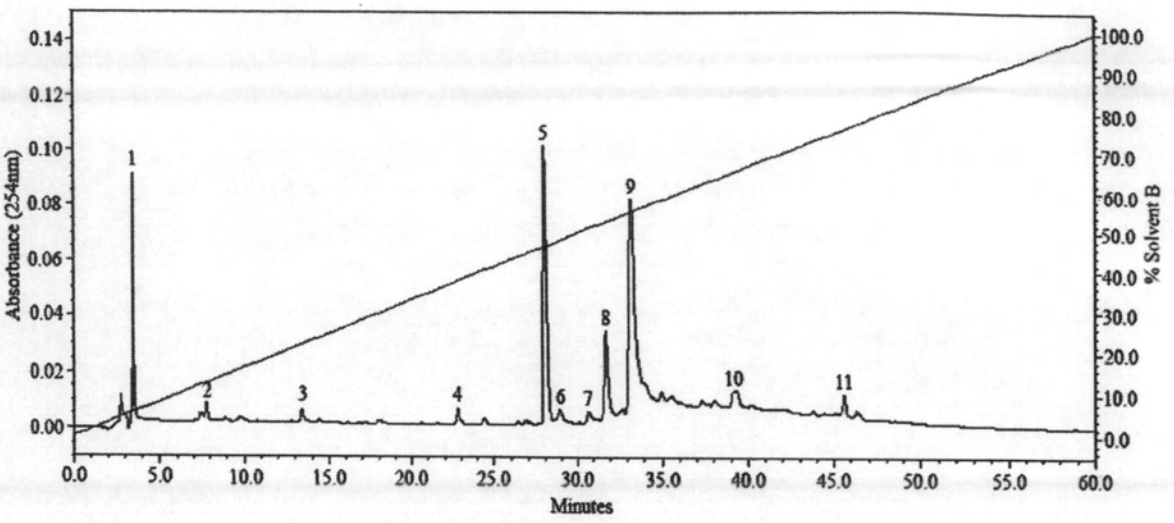


Fig 4.12 Chromatogram of inhibited condition

4.7 Mass spectrometry and computer analysis

Amino acid sequence was searched from protein database using information from mass spectroscopic analysis. The compatibles sequence was used to calculate protein property.

4.7.1 Amino acid sequence

VIGGDECNIN ERRFLVVAVY EGTNHTFICG GVLLIHPEWV ITAEHCARRR

MNLVFGMHRK SEKFDDEQER YPKKYFMRCN KTRTSWDEDI MLIRLNKPVN

NSEHIAPLSL PSNPPIVGSD CRVMGWGSIN RRIDVLSSDE PRCANINLHN

FTMCHGLFRK MPKKGRVLCA GDLRGRRDSC NSDSGGPLIC NEELHGIVAR

GPNPCAQPNK PALYTSIYDY RDWVNNVIAG NATCSP

4.7.2 Computer analysis of protein

Number of amino acid: 236

Molecular weight: 26701.5 Da

Theoretical PI: 8.61

Active site: His^{45}, Asp^{89} and Ser^{184}

Disulfide: 29-46, 121-190, 154-169 and 180-205

4.7.3 Predicted features

Disulfide	29 46	By similarity
Active site	45	Charge relay system (by similarity)
Active site	89	Charge relay system (by similarity)
Disulfide	21 190	By similarity
Disulfide	154 169	By similarity
Disulfide	180 205	By similarity
Active site	184	Charge relay system (by similarity)

4.7.4 Post translation modification

N-myristoylation	: 3-8	GGdeCN
	: 22-27	GTnhTF
	: 230-235	GNatCS
N-glycosylation	: 24-27	NHTF
	: 80-83	NKTR
	: 100-103	NNSE
	: 150-153	NFTM
	: 231-234	NATC
Protein kinase C phosphorylation	: 61-63	Sek
Tyrosine kinase phosphorylation	: 63-71 kfddEqery	

Casein kinase II phosphorylation	: 84-87	Tswd
	: 85-88	SwdE
	: 137-140	SsdE
	: 216-219	SiyD
Amidation	: 174-177	rGRR
cAMP- and cGMP- dependent protein kinase phosphorylation		
	: 176-179	RRdS

4.7.5 Three dimension model

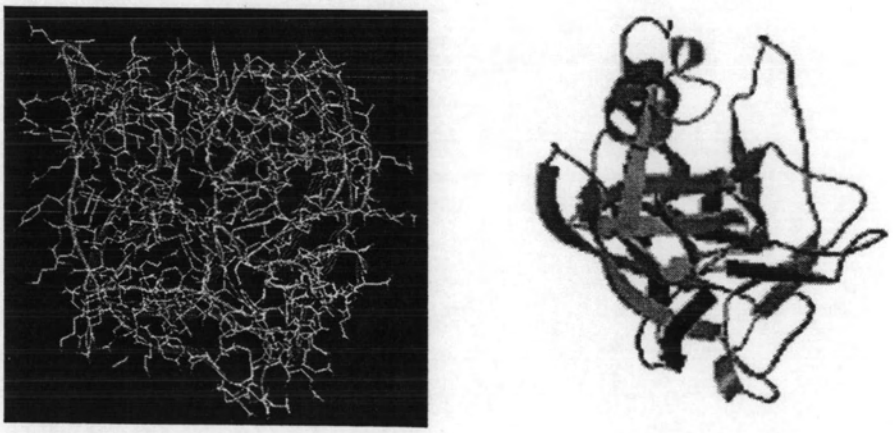


Fig. 4.13 Three dimension model of fibrinolytic protein from Malayan pit viper