

Chapter IV

Results

Crude venoms

Thrombin Activity

Both T. popeorum and C. rhodostoma venom possess coagulant action, thrombin activity, direct fibrinogen clotting to fibrin. Clots formed by the venom's action seemed to be more friable or dispersed and transparent than those formed by bovine thrombin. Their actions on fibrinogen solution (Table 1) and normal pool plasma (Table 2) were compared with thrombin (Table 3), as shown in Fig. 3 and 4.

They were concentration-dependent, higher concentrations of venom provided more rapid clotting time. On fibrinogen solution, T. popeorum venom gave mean clotting time of 261.2 sec with venom concentration of 20 $\mu\text{g/ml}$, and reduced to 107 sec with concentration of 2,000 $\mu\text{g/ml}$, whereas C. rhodostoma venom gave mean clotting time of 121.5 sec with venom concentration of 1 $\mu\text{g/ml}$, and reduced to the plateau level at 4.0 sec with 500 $\mu\text{g/ml}$ or more of venom (Fig. 3). Similar effects were observed on normal pool plasma, except that Malayan pit viper venom

Table 1. Thrombin Activities of T. popeorum and C. rhodostoma Venoms on Fibrinogen Solution (5 mg/ml)

Final venom concentration ($\mu\text{g/ml}$)	Clotting time (sec)					
	<u>T. popeorum</u>			<u>C. rhodostoma</u>		
	1	2	mean	1	2	mean
2000	10.4	11.0	10.7	4.0	4.0	4.0
1000	16.6	17.0	16.8	4.0	4.0	4.0
500	28.8	29.2	29.0	4.0	4.0	4.0
200	58.0	57.4	57.7	5.0	5.4	5.2
100	81.0	81.8	81.4	6.2	6.8	6.5
50	177.6	178.2	177.9	8.0	8.0	8.0
20	260.0	262.4	261.2	13.8	14.0	13.9
10				22.0	22.4	22.2
5				36.2	36.8	36.5
2.5				63.0	64.0	63.5
1				122.0	121.0	121.5

Table 2. Clotting Activities of T. popeorum and C. rhodostoma Venoms on Normal Pool plasma

Final venom concentration ($\mu\text{g/ml}$)	Clotting time (sec)					
	<u>T. popeorum</u>			<u>C. rhodostoma</u>		
	1	2	mean	1	2	mean
2000	22.0	21.6	21.8			
1000	36.0	36.0	36.0	28.0	28.4	28.2
500	56.4	58.0	57.2	19.0	19.0	19.0
200	110.2	110.6	110.4	11.6	11.4	11.5
100	240.0	242.2	241.1	9.9	9.4	9.7
50				13.0	13.8	13.4
20				22.0	22.0	22.0
10				35.8	34.6	35.2
5				50.0	50.4	50.2
2.5				75.8	76.4	76.1

Table 3. Coagulant Activities of Bovine Thrombin on Fibrinogen Solution (5 mg/ml) and Normal Pool Plasma

Final thrombin concentration (unit/ml)	Clotting time (sec)					
	fibrinogen solution			normal pool plasma		
	1	2	mean	1	2	mean
50	4.0	4.0	4.0	4.8	4.8	4.8
20	4.0	4.0	4.0	4.9	4.9	4.9
10	4.4	4.4	4.4	4.9	4.7	4.8
5.0	5.0	5.8	5.4	5.8	6.0	5.9
1.0	1.0	10.4	10.2	19.8	19.0	19.4
0.5	16.0	15.6	15.8	30.0	31.4	30.7
0.1	60.5	61.0	60.8	94.0	96.0	95.0

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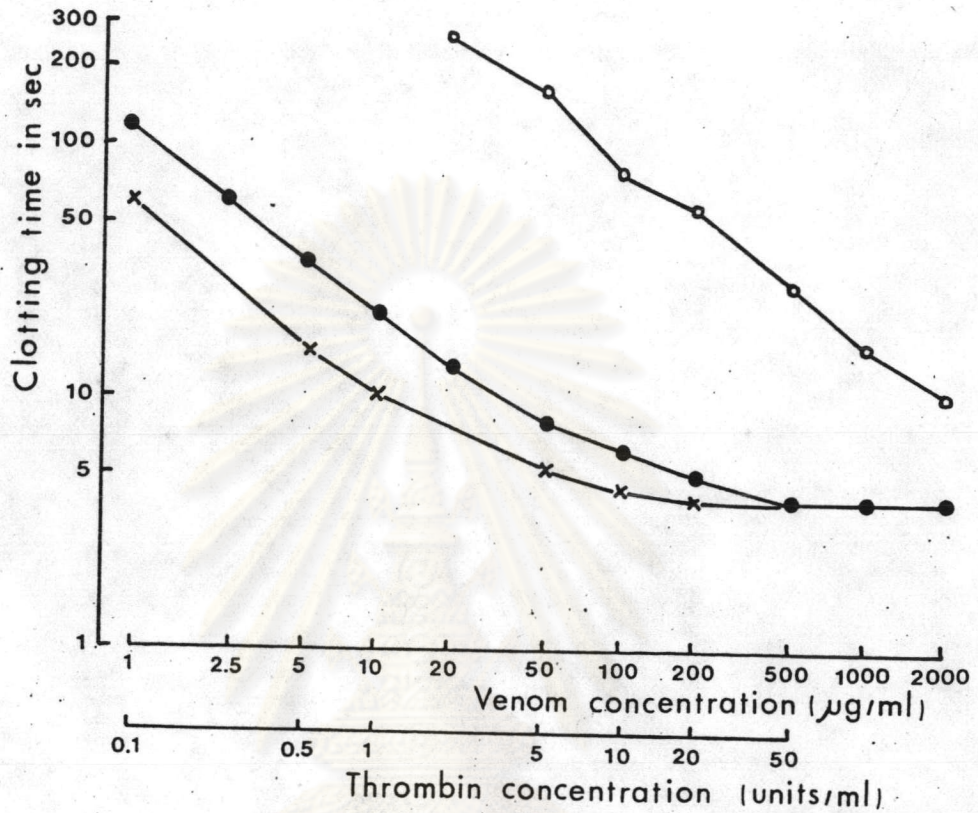


Figure 3. Thrombin activities of *T. popeorum* (○—○) and *C. rhodostoma* (●—●) venom on fibrinogen solution (5 mg/ml), compared with bovine thrombin (x—x).

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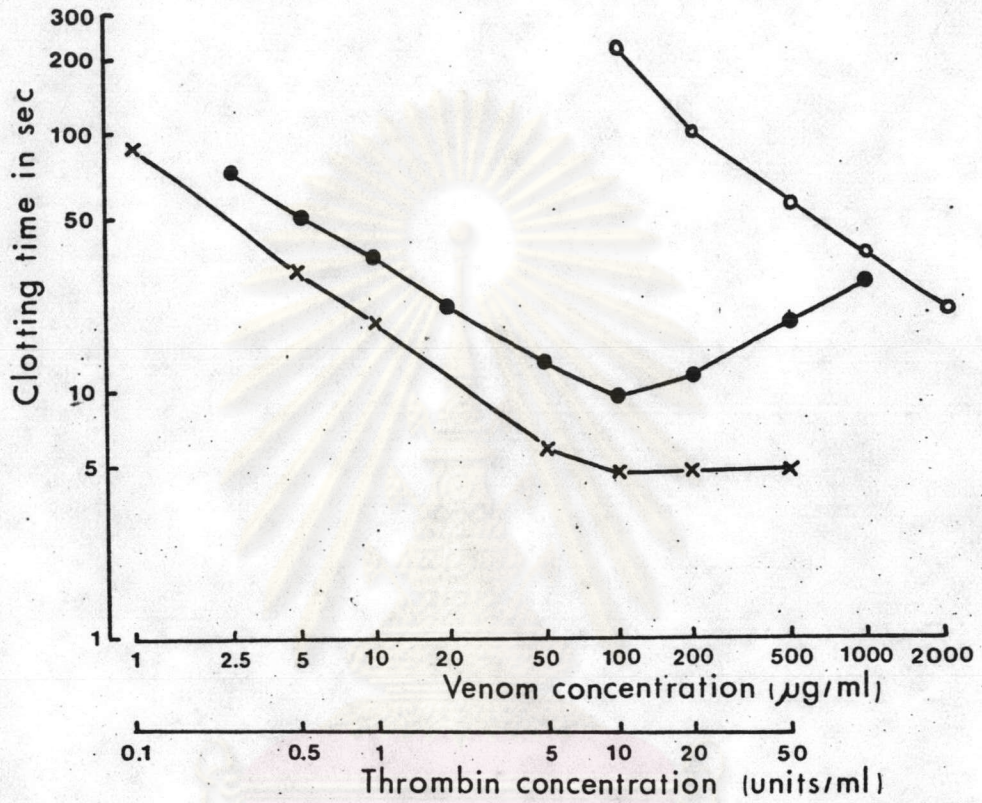


Figure 4. Coagulant activities of *T. popeorum* (○—○) and *C. rhodostoma* (●—●) venoms on normal pool plasma, compared with bovine thrombin (X—X).

produced more prolonged clotting time again from 9.7 sec to 11.5 sec or more with the venom concentration of more than 100 $\mu\text{g}/\text{ml}$. (Table 2, Fig. 4)

By parallel assay method approximately 3 mg of T. popeorum venom has an equivalent clotting activity of 1 N.I.H. unit of bovine thrombin, whereas only about 25 μg of whole venom for C. rhodostoma.

Fibrinolytic Activity

The fibrinolytic activities of T. popeorum and C. rhodostoma venoms were illustrated in Table 4, 5 and Fig. 5. Similar with thrombin activity, the fibrinolytic activities of both venoms were dose dependent. The mean lysed area were increased from 64 to 580 sq mm with T. popeorum venom concentration of 0.2 to 20 mg/ml, as from 62 to 888.5 sq mm with 25 to 1000 μg per ml of C. rhodostoma venom.

Direct Platelet Aggregating Activity

Normal platelet aggregation patterns with various inducers : ADP, adrenaline, thrombin and collagen were shown in Fig. 6.

No direct platelet aggregating effects were seen, neither with T. popeorum venom concentration of 1.0-50 μg

Table 4. Fibrinolytic Activity of *T. popeorum* Venom
(25 μ l) on Fibrin Plate

Venom concentration (mg/ml)	Lysis zone (sq.mm)		
	1	2	mean
20.0	600	560	580
10.0	462	460	461
5.0	312	320	316
1.0	144	144	144
0.5	90	90	90
0.2	64	64	64

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Table 5. Fibrinolytic Activity of *C. rhodostoma* Venom
(25 μ l) on Fibrin Plate

Venom concentration (μ g/ml)	Lysis zone (sq.mm)		
	1	2	mean
1000	881	896	888.5
500	462	484	473
200	315	300	307.5
100	144	144	144
50	100	110	105
25	64	60	62

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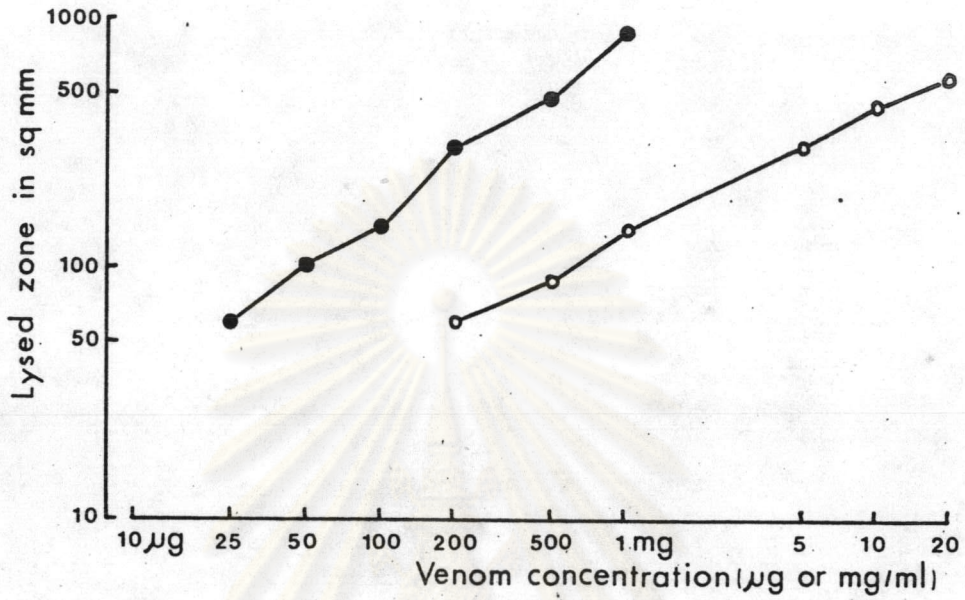


Figure 5. Fibrinolytic activities of *T. popeorum* (○—○) and *C. rhodostoma* (●—●) crude venom.

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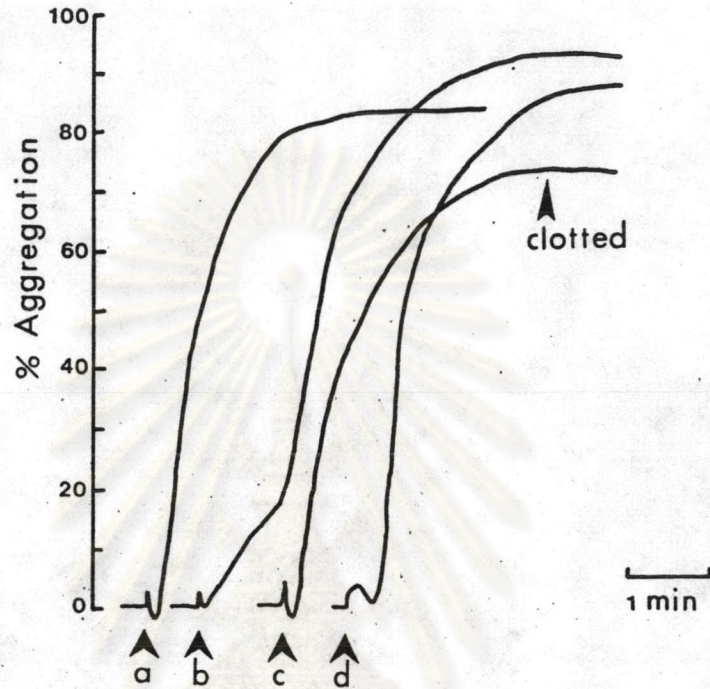


Figure 6. Platelet aggregation curves induced by various inducers.

Platelet aggregation was measured turbidimetrically and induced by (a) ADP $5 \mu\text{M}$, (b) adrenaline 0.1 mg/ml , (c) thrombin 0.25 unit/ml , and (d) collagen $100 \mu\text{g/ml}$. Inducers were added at \blacktriangle .

per ml (Fig. 7), nor with C. rhodostoma venom concentration of 1.0-10 $\mu\text{g/ml}$ (Fig. 9A) on human PRP.

Platelet Aggregation Inhibition

After PRP were pre-warmed at 37°C with 1.0-50 μg per ml of T. popeorum venom, no inhibition of ADP- or adrenaline-induced human platelet aggregation were observed (Fig. 8). Results obtained with thrombin and collagen induction were recognized similarly.

With 1.0-10 $\mu\text{g/ml}$ of C. rhodostoma, there seemed to be also no inhibition of ADP-induced platelet aggregation (Fig. 9B), same with adrenaline, thrombin and collagen. Limitation of tests happened with higher concentration of venom because they were interfered by fibrin clot.

Hemorrhagic Activity

Both T. popeorum and C. rhodostoma venoms have hemorrhagic activities (Fig. 10). Their response were dose related as shown in Table 6 and Fig. 11. The MHD of T. popeorum and C. rhodostoma venom were about 1.2 and 30 μg , or specific activities of hemorrhagic activity of venoms were 0.83 and 0.03 MHD per μg protein, respectively.

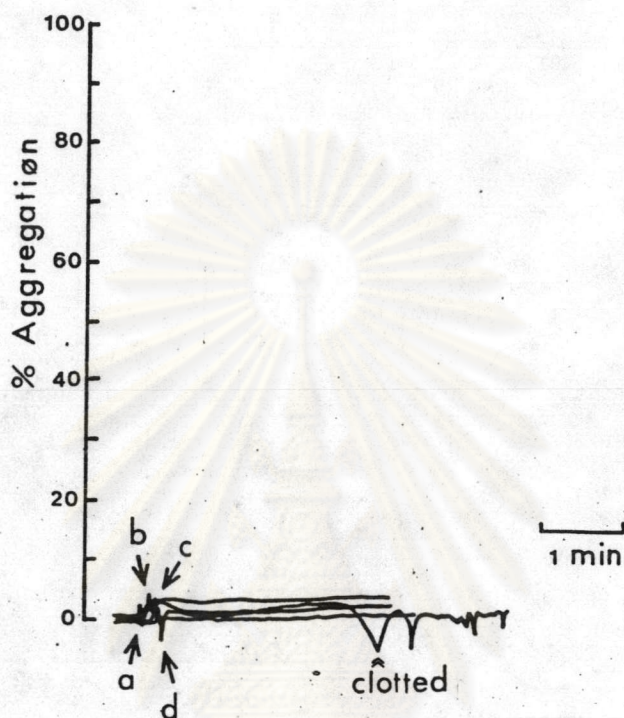


Figure 7. Effects of *T. popeorum* venom on human platelet aggregation.

The final concentrations ($\mu\text{g/ml}$) of the venom were (a) 5.0, (b) 10.0, (c) 20.0, and (d) 50.0.

The venoms were added at \uparrow .

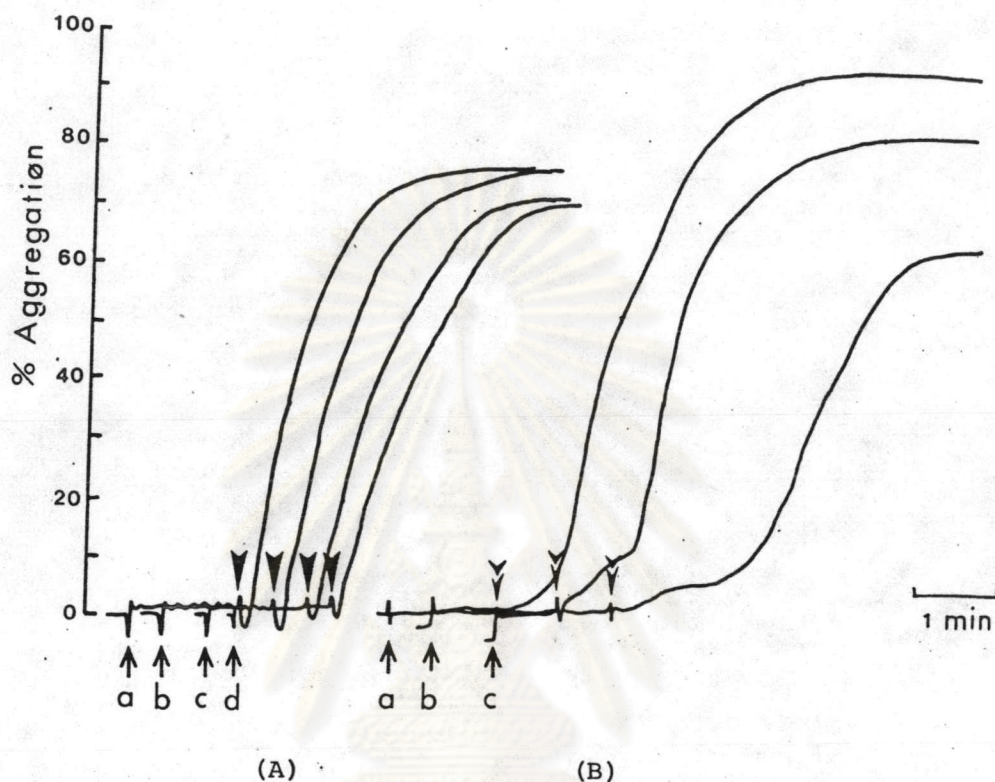


Figure 8. Inhibitory effects of *T. popeorum* venom on human platelet aggregation.

After PRP were pre-warmed with the venom at \uparrow , the final concentrations ($\mu\text{g}/\text{ml}$) of (a) 5.0, (b) 10.0, (c) 20.0, and (d) 50.0; platelet aggregation was induced by (A) ADP $5 \mu\text{M}$ at \blacktriangledown ; and (B) adrenaline $0.1 \text{ mg}/\text{ml}$ at \blacktriangledown .

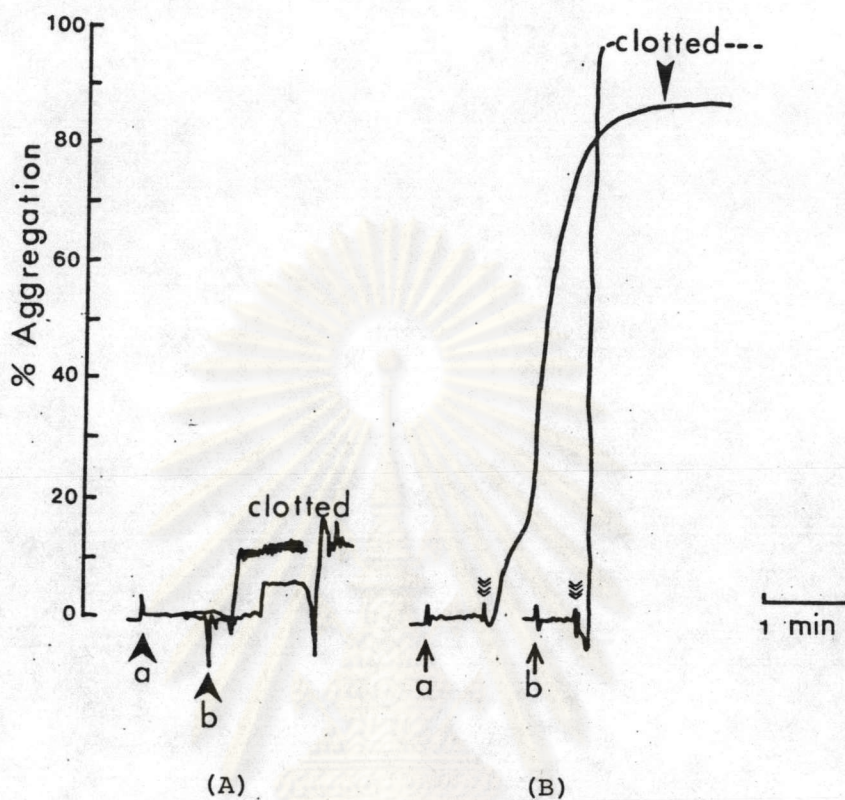


Figure 9. (A) Effect of *C. rhodostoma* venom on human platelet aggregation.

The final concentrations ($\mu\text{g/ml}$) of venom were (a) 5.0, and (b) 10.0.

The venoms were added at \blacktriangle .

(B) Inhibitory effect of *C. rhodostoma* venom tested on human platelet aggregation.

After PRP was pre-warmed with the venom at \blacktriangle (as A.), platelet aggregation was induced by ADP

$5 \mu\text{M}$ at \equiv .

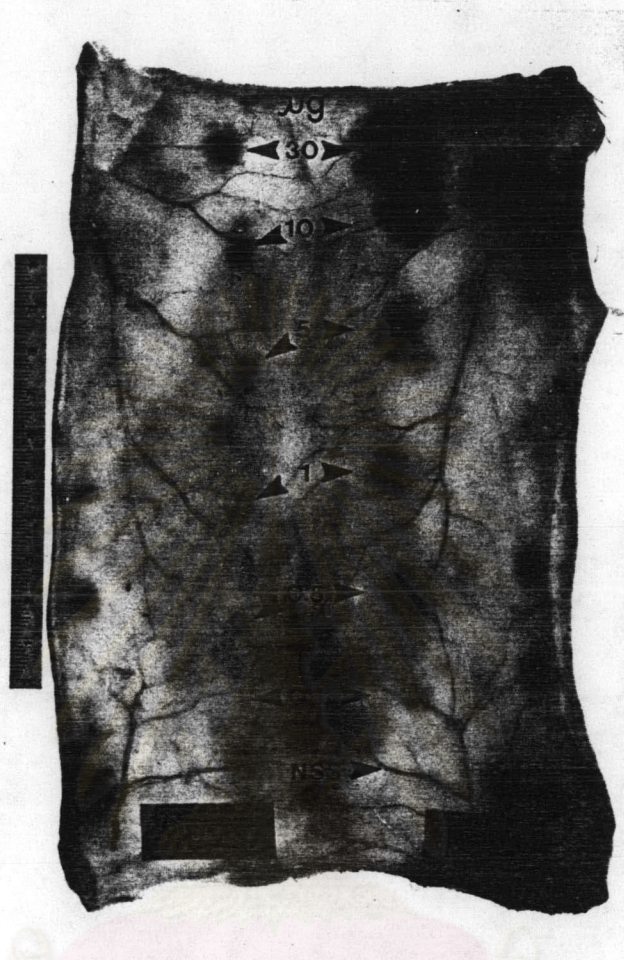


Figure 10. Venom-produced hemorrhage observed from visceral side of rabbit skin.

Various dilutions of 30, 10, 5, 1, 0.5 and 0.1 μg in 0.1 ml NSS, of *T. popeorum* (GPVV) and *C. rhodostoma* (MPVV) venoms were used. Normal saline solution (NSS) was injected as control.

Table 6. Hemorrhagic Activities of T. popeorum and C. rhodostoma crude venoms on rabbit skin

Venom amount (μg)	Diameter of hemorrhagic spot (mm)					
	<u>T. popeorum</u>			<u>C. rhodostoma</u>		
	1	2	mean	1	2	mean
30	21.0	22.0	21.5	9.0	9.0	9.0
20	17.5	17.0	17.3	6.0	6.0	6.0
5	15.0	13.0	14.0	4.0	4.5	4.3
1	8.5	9.0	8.8	2.5	2.5	2.5
0.5	5.0	5.0	5.0	1.0	1.0	1.0
0.1	0.0	0.0	0.0	0.0	0.0	0.0
NSS control	0.0	0.0	0.0	0.0	0.0	0.0

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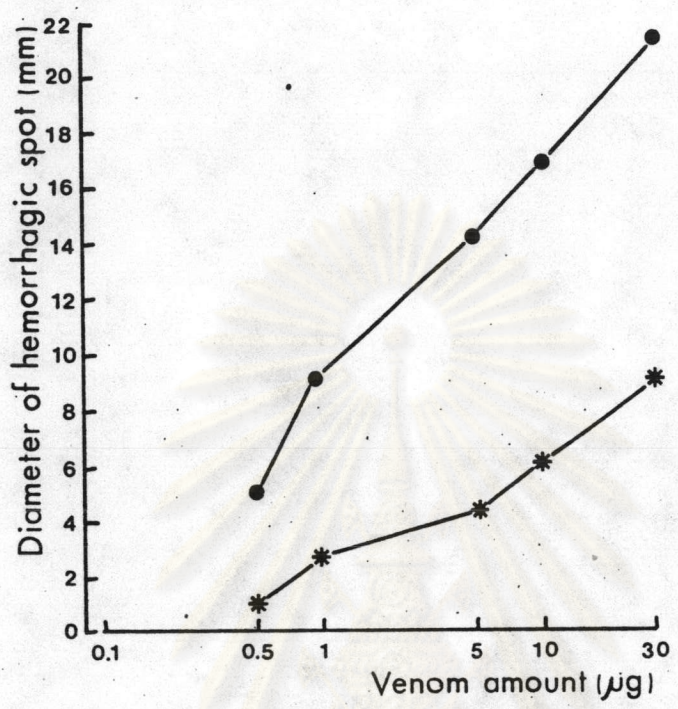


Figure 11. Dose-response curve of hemorrhage induced by *T. popeorum* (●—●) and *C. rhodostoma* (*—*) venom.

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Isolation and Fractionation of Venoms

Using DEAE-cellulose column chromatography, the *T. popeorum* venom was separated into nine fractions (Fig. 12). Two fractions were obtained by simple elution with 0.05 N ammonium acetate, pH 5.0, and the other six in the first stage gradient elution, none in the second stage.

In the same condition, the *C. rhodostoma* venom was separated into seven fractions (Fig. 13). Four fractions were eluted by simple elution and the other three in the first gradient stage, none in the second.

Thrombin-like Activity

Thrombin-like activity was recovered in fraction numbers II, V and VI of *T. popeorum* venom (Table 7), none in the rest; and in fraction number I, II, III, IV, V, VI of *C. rhodostoma* venom (Table 8.1 and 8.2), non in fraction VII.

For *T. popeorum* venom fractions containing thrombin-like activities, they were all more potent than the crude venom (Fig. 14). The fraction V appeared to exhibit the strongest action. About 100 μ g of this fraction has an equivalent activity to 1 NIH unit of thrombin.

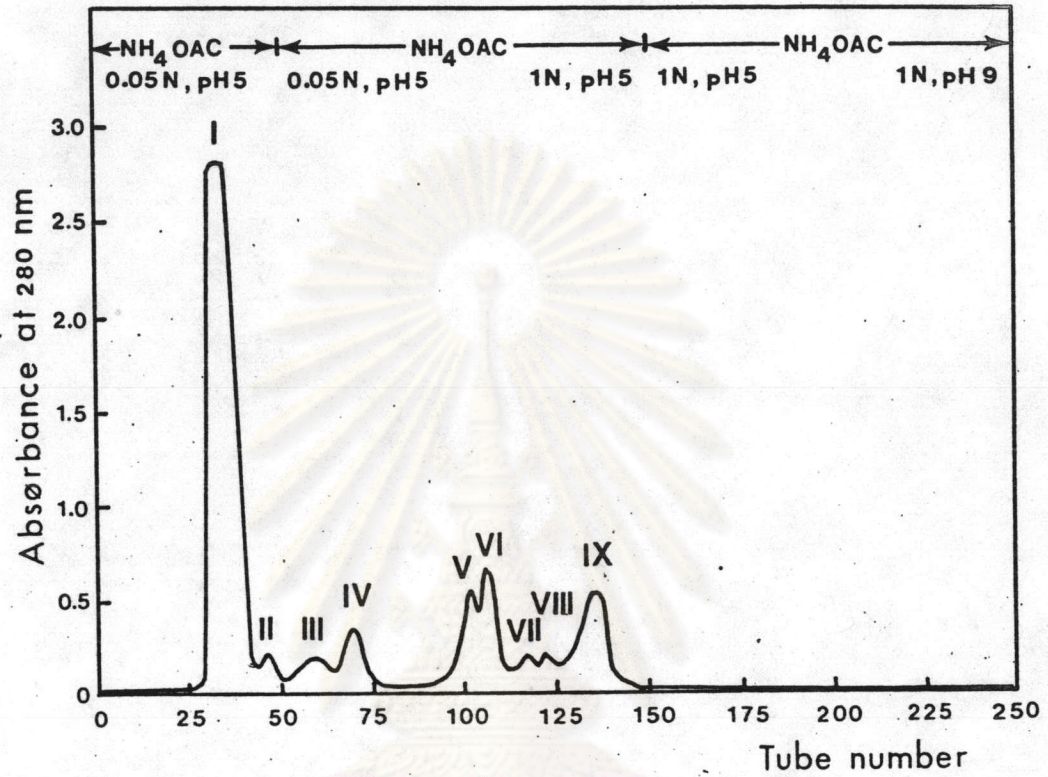


Figure 12. DEAE-cellulose (DE 52) column chromatography of
T. popeorum venom. Bed volume 250 ml.

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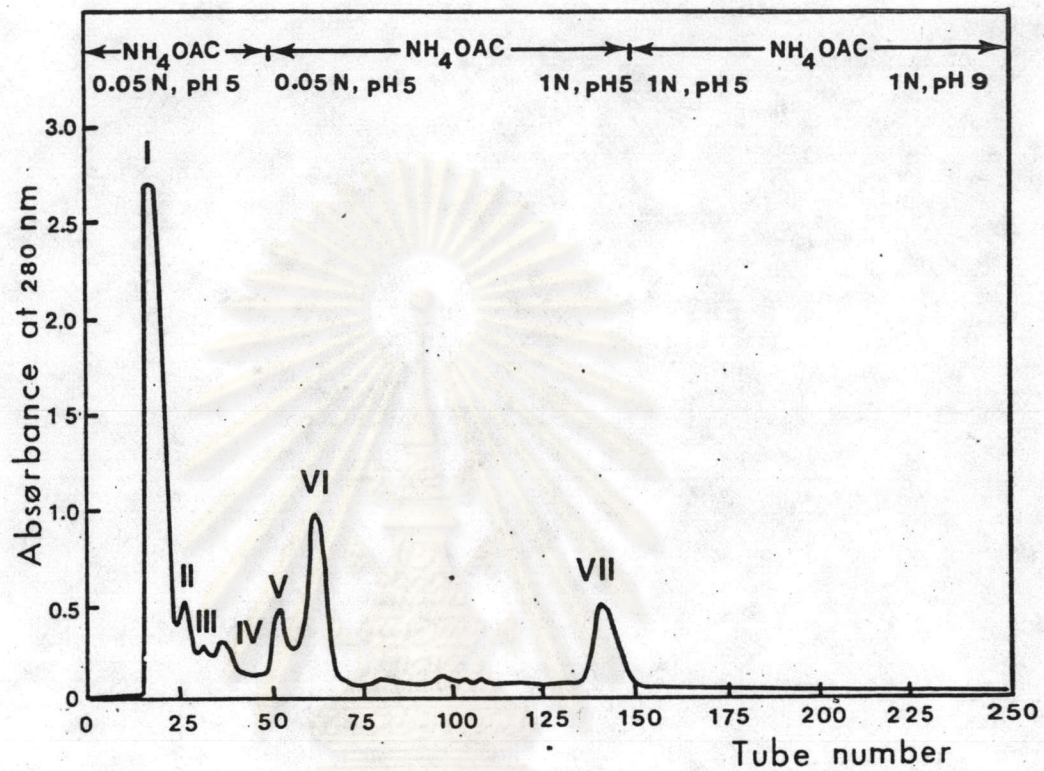


Figure 13. DEAE-cellulose (DE 52) column chromatography of *C. rhodostoma* venom. Bed volume 250 ml.

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Table 7. Thrombin Activities of *T. popeorum* venom fractions II, V, VI on fibrinogen solution (5 mg/ml)

Final venom concentration ($\mu\text{g/ml}$)	Clotting time (sec)								
	Fraction II			Fraction V			Fraction VI		
	1	2	mean	1	2	mean	1	2	mean
500				5.0	5.0	5.0			
200	34.0	34.6	34.3	7.2	7.0	7.1	30.0	28.6	29.3
100	55.0	53.0	54.0	9.8	10.2	10.0	44.0	46.0	45.0
50	90.0	90.0	90.0	15.4	16.0	15.7	76.0	76.0	76.0
20	170.0	170.8	170.4	32.2	32.8	32.5	142.0	141.0	141.5
10	272.0	270.0	271.0	49.0	49.0	49.0	229.0	228.0	228.5
5				84.0	83.4	83.7			
2.5				156.0	159.0	157.5			
1				275.0	275.0	275.0			

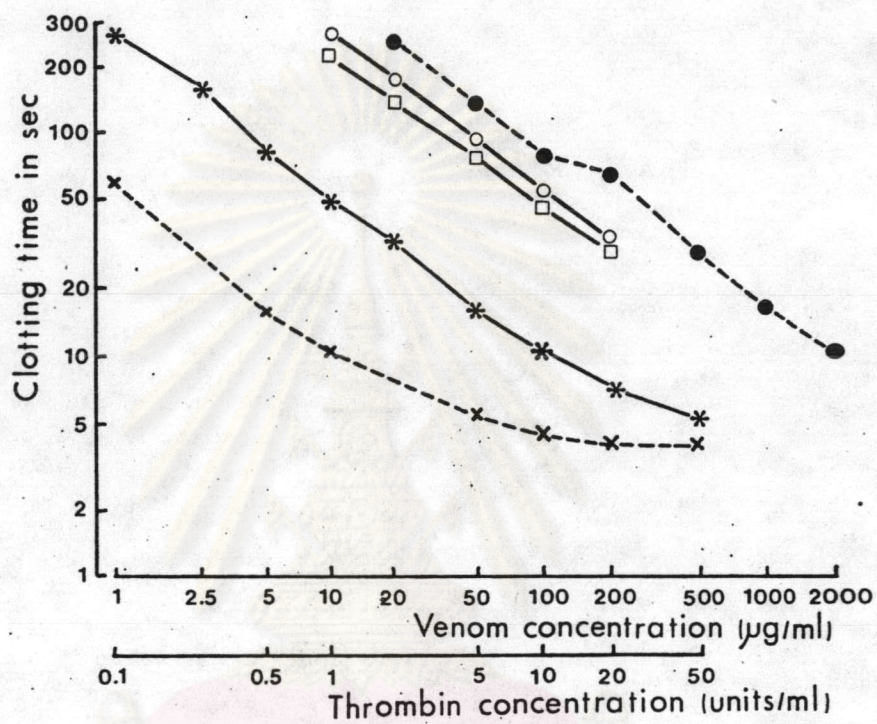


Figure 14. Thrombin activities of *T. popeorum* crude venom

(●---●), fraction II (○—○), fraction V (*—*),

and fraction VI (□—□), compared with bovine

thrombin (x---x).

Table 8.1 Thrombin Activities of *C. rhodostoma* venom fractions I, II, III on fibrinogen solution (5 mg/ml)

Final venom concentration ($\mu\text{g/ml}$)	Clotting time (sec)								
	Fraction I			Fraction II			Fraction III		
	1	2	mean	1	2	mean	1	2	mean
1000	6.0	6.0	6.0						
500	8.0	7.6	7.8	5.0	5.0	5.0			
200	13.2	12.3	12.8	6.6	6.0	6.3	5.8	5.8	5.8
100	22.2	21.0	21.6	9.0	9.0	9.0	7.0	7.2	7.1
50	32.4	32.0	32.2	14.6	14.8	14.7	10.6	11.0	10.8
20	56.5	61.5	59.0	28.0	29.0	28.5	21.0	20.6	20.8
10	102.6	99.4	101.0	46.8	46.0	46.4	35.0	35.2	35.1
5	172.4	168.0	170.2	70.6	70.6	70.6	56.0	56.2	56.1
2.5	307.8	304.6	306.2	130.0	133.0	131.5	90.4	91.0	90.7
1				250.0	248.0	249.0	175.0	176.0	175.5

Table 8.2 Thrombin Activities of *C. rhodostoma* venom fractions IV, V, VI on fibrinogen solution (5 mg/ml)

Final venom concentration ($\mu\text{g/ml}$)	Clotting time (sec)								
	Fraction IV			Fraction V			Fraction VI		
	1	2	mean	1	2	mean	1	2	mean
200	4.0	3.8	3.9	4.6	4.6	4.6	7.4	7.6	7.5
100	4.0	4.0	4.0	5.2	5.6	5.4	10.0	10.6	10.3
50	5.2	5.2	5.2	6.2	6.4	6.3	18.0	17.0	17.5
20	6.4	6.8	6.6	9.6	10.0	9.8	29.0	30.0	29.5
10	10.8	10.8	10.8	14.8	14.8	14.8	58.0	60.0	59.0
5	15.2	15.2	15.2	23.2	24.0	23.6	96.0	97.6	96.8
2.5	25.4	26.0	25.7	38.2	39.0	38.6	164.4	165.6	165.0
1	55.0	56.4	55.7	71.8	74.0	72.9	309.4	314.2	311.8

Concerning about *C. rhodostoma* venom, among fractions I, II, III, IV, V, VI, two of them : IV and V had stronger thrombin activity than the crude venom, and the rest four had weaker action, as shown in Fig. 15. The fraction IV has the most potent activity on fibrinogen, whereas fraction I has the least one. Compared with bovine thrombin, approximately 10 μ g of fraction IV has an equivalent activity of 1 N.I.H. unit.

Fibrinolytic Activity

The fibrinolytic activities were distributed in fractions I and V of *T. popeorum* venom. Fraction V lysed fibrin plate in mean areas of 64 to 225 sq mm with the fraction concentration of 100 μ g/ml to 1.5 mg/ml (Table 9). This activity was about two times higher than that of the crude venom (Fig. 16).

The fibrinolytic activity of *C. rhodostoma* venom can be demonstrated only in fraction I. Its action on fibrin plate was shown in Table 10, and compared with crude venom as Fig. 17.

Hemorrhagic activity

Of nine fractions of *T. popeorum* venom, the active components on hemorrhagic activity were found mainly in

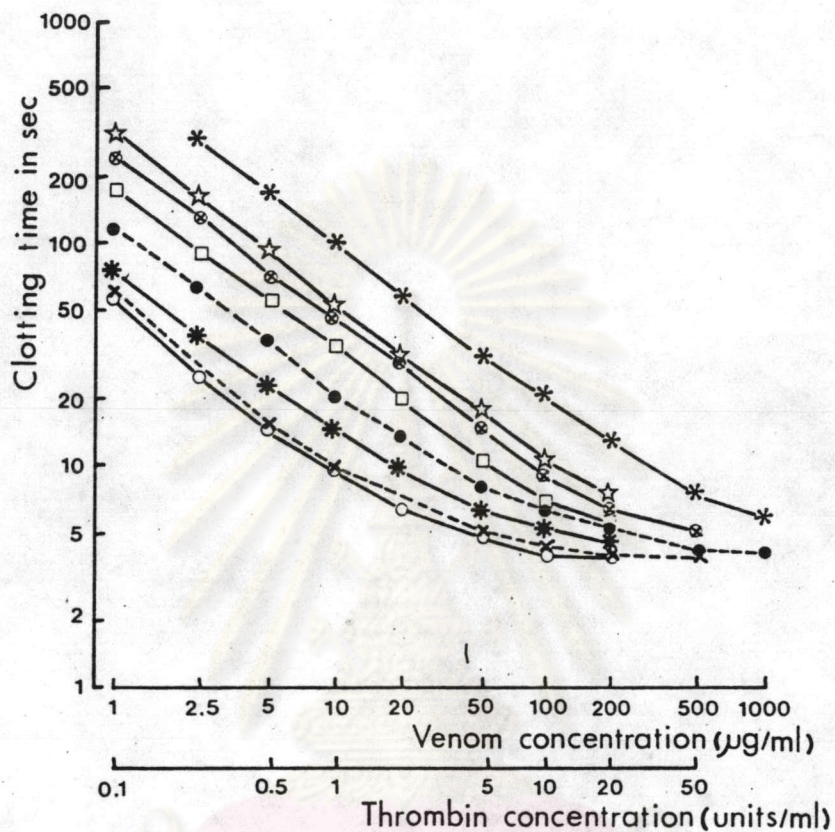


Figure 15. Thrombin activities of *C. rhodostoma* crude venom (●---●), fraction I (*---*), fraction II (⊗---⊗), fraction III (□---□), fraction IV (○---○), fraction V (*---*), and fraction VI (☆---☆), compared with bovine thrombin (X---X).

Table 9. Fibrinolytic Activities of *T. popeorum* Venom Fractions I and V on Fibrin Plate

Venom Concentration (per ml)	Lysed area (sq mm)					
	Fraction I			Fraction V		
	1	2	mean	1	2	mean
15.0 mg	414	400	407			
5.0	304	289	296			
2.5	225	210	217.5			
1.5				225	225	225
1.0	132	132	132	196	196	196
500 μ g	81	81	81	132	132	132
200				90	95	92.5
100				64	60	62

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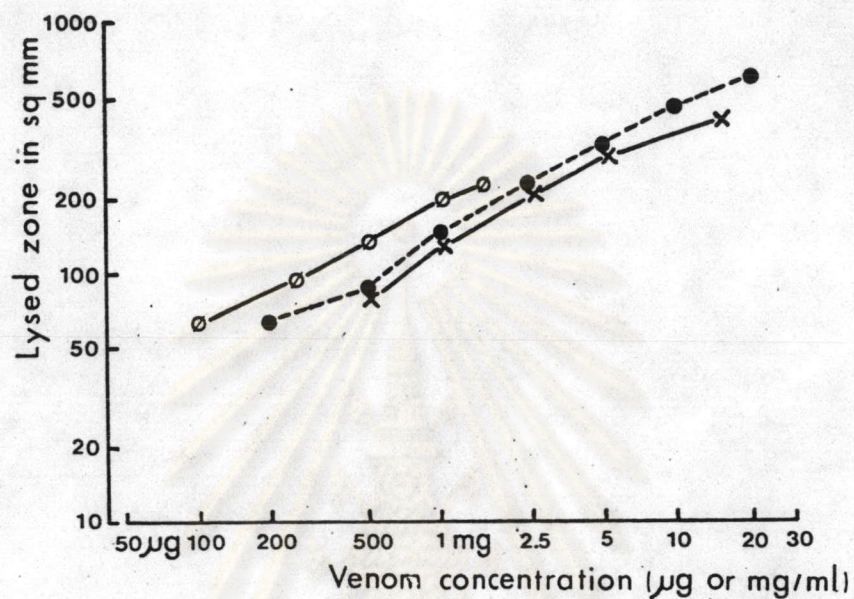


Figure 16. Fibrinolytic activities of *T. popeorum* crude venom (●---●), fraction I (x---x) and fraction V (○---○).

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Table 10. Fibrinolytic Activity of *C. rhodostoma* venom fraction I on fibrin plate.

Venom Concentration (per ml)	Lysed area (sq mm)		
	1	2	mean
5 mg	625	575	600
2	324	300	312
1	225	240	232.5
500 μ g	144	144	144
200	72	81	76.5
100	42	49	45.5

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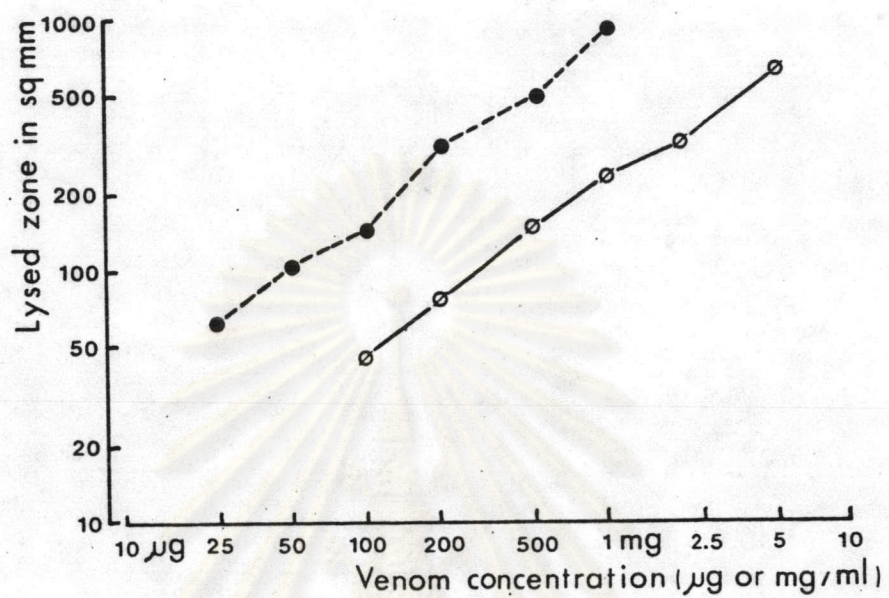


Figure 17. Fibrinolytic activities of *C. rhodostoma* crude venom (●---●) and fraction I (◊—◊).

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peaks I, III, VII, VIII, and weakly in peaks II and IV (Fig. 18). Most of the original hemorrhagic activity seemed to be concentrated in fraction I. The MHD of fractions I, III, VII, VIII were 1.05, 17.5, 40 and 2.8 μg ; as specific activities were 0.95, 0.06, 0.025 and 0.36 MHD per μg protein respectively.

For *C. rhodostoma* venom, The hemorrhatic activity was only present in the fraction I (Fig. 19). The MHD was approximately 40 μg , and specific activity was 0.025 MHD per μg protein

SDS-Polyacrylamide Gel Electrophoresis of Venoms

Electrophoresis on SDS-polyacrylamide gel (15%) at pH 8.3 of *T. popeorum*, *C. rhodostoma* crude venoms, and their fractions were demonstrated in Fig. 20 and 21, respectively.

T. popeorum Venom and its Fractions

After electrophoresis and staining. The main coagulant as well fibrinolytic fraction, venom peak V, appeared as eight bands (Fig. 20-e). Three bands with R_f of 0.50, 0.35 and 0.79, stained strongly; whereas the rests were less obvious. The second potent coagulant

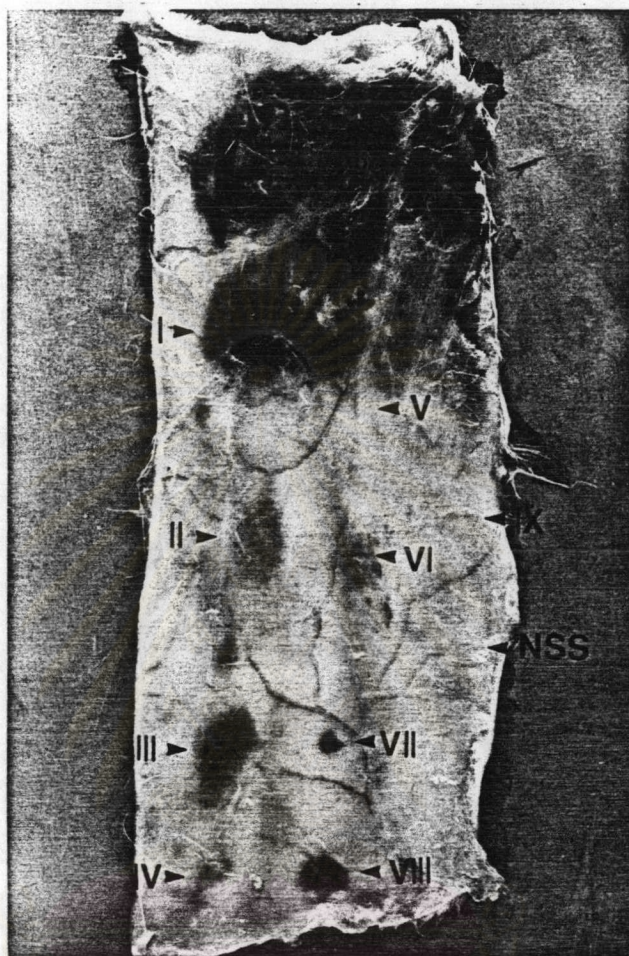


Figure 18. Hemorrhagic activities of venom fractions of *T. popeorum* on rabbit skin.

I - IX represent numbers of each fractions.

Normal saline solution (NSS) was used as control.



Figure 19. Hemorrhagic activities of venom fractions of

C. rhodostoma on rabbit skin.

I - VII represent numbers of each peaks.

Normal saline solution (NSS) was used as

control.

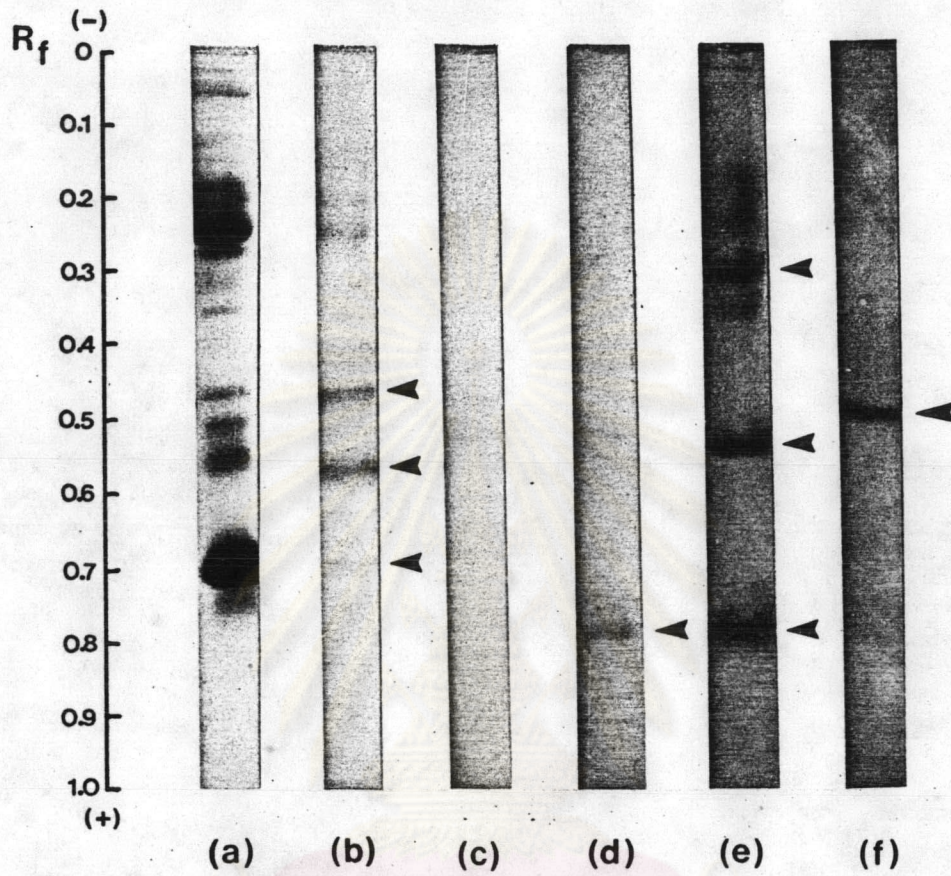


Figure 20. SDS-Polyacrylamide gel (15%) electrophoresis at pH 8.3 of *T. popeorum* crude venom (a), fraction I (b), fraction II (c), fraction III (d), fraction V (e), and fraction VI (f). R_f represents relative mobility ratio.

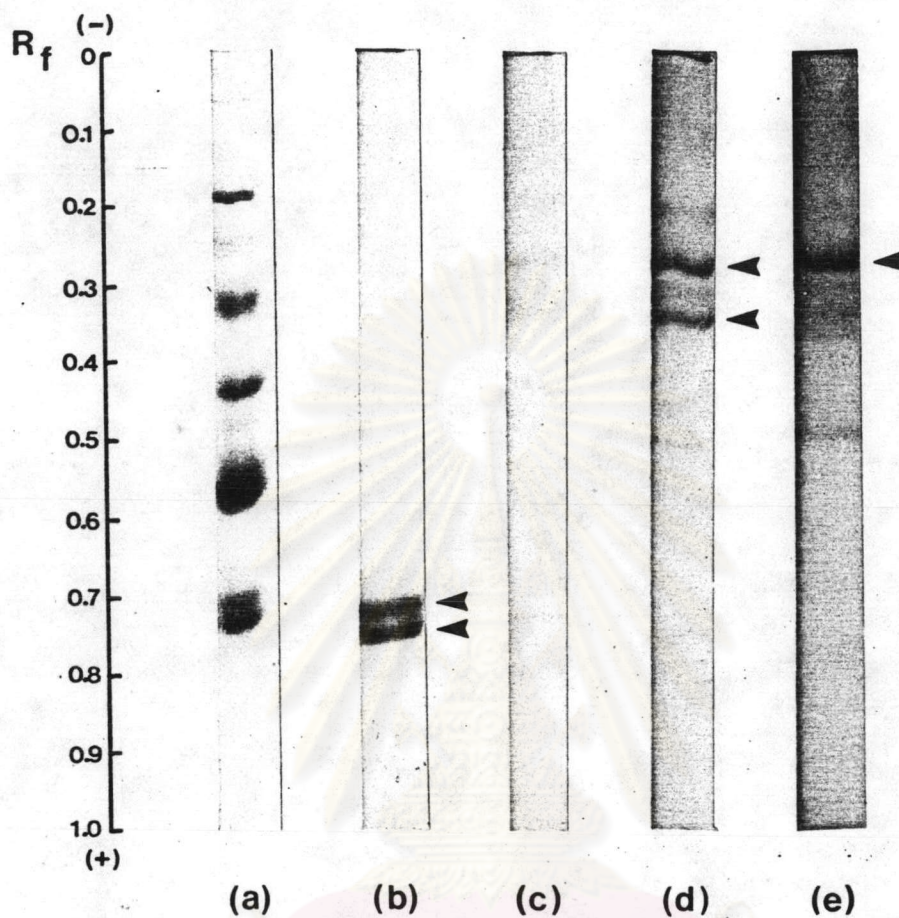


Figure 21. SDS-Polyacrylamide gel (15%) electrophoresis at pH 8.3 of the *C. rhodostoma* crude venom (a), fraction I (b), fraction II (c), fraction IV (d), and fraction V (e).

R_f represents relative mobility ratio.

fraction, peak VI, showed 90% of it in dense band with R_f 0.46 (Fig. 20-f).

The fraction I, potent hemorrhagic and less fibrinolytic activities, contained many different staining bands (Fig. 20-b). The more apparent ones were R_f of 0.46, 0.55 and 0.70 bands.

The gel patterns obtained for peak II and III were also shown in Fig. 20-c and 20-d.

C. rhodostoma Venom and its Fractions

The coagulant peak of C. rhodostoma venom, fraction IV. was run on 15% acrylamide gel electrophoresis as shown in Fig. 21-d. Two strongly staining bands were obvious, of which the R_f value were 0.27 and 0.35. The fraction V, which also possesses the strong thrombin-like activity, was separated to the pattern shown in Fig. 21-c. About 80% of that was concentrated in the dense band with R_f of 0.27, as seen in the peak IV subfraction.

The electrophoretic pattern of fraction I, containing both fibrinolytic and hemorrhagic activities, was demonstrated in Fig. 21-b. Two fast-running dark stained bands were striking present, taking about 90-95% of venom fraction content. They were characterized by R_f 0.71 and 0.75.