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HEMORRHAGIC AND PROTEOLYTIC ACTIVITIES OF THAILAND SNAKE VENOMS*

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Abstract—Venoms of six common Thailand snakes, *Agkistrodon rhodostoma* (Malayan pit viper), *Trimeresurus popiorum* (*Green pit viper*), *Vipera russellii siamensis* (Thailand Russell's viper), *Ophiophagus hannah* (King cobra), *Bungarus fasciatus* (Banded krait), and *Naja naja siamensis* (Thailand cobra) were investigated for hemorrhagic and proteolytic enzyme activities. Hemorrhage was induced experimentally under rabbit skin by injecting venom subcutaneously. Proteolytic activities of venoms were determined using the substrates casein, *p*-toluene sulfonyl-L-arginine methyl ester (TAME), *N*-benzoyl-L-arginine ethyl ester (BAEE), and *N*-benzoyl-L-tyrosine ethyl ester (BTEE). The possible relationship between hemorrhagic activity and proteolytic activities was discussed.

IN THAILAND there are six common poisonous snakes—*Naja naja siamensis* (Thailand cobra), *Ophiophagus hannah* (King cobra), *Bungarus fasciatus* (Banded krait), *Vipera russellii siamensis* (Thailand Russell's viper), *Agkistrodon rhodostoma* (Malayan pit viper), and *Trimeresurus popiorum* (Green pit viper). It is known from clinical observations that *A. rhodostoma* and *T. popiorum* cause hemorrhage on envenomation.

It has been suggested by a number of investigators that venom proteolytic enzymes are at least partly responsible for hemorrhage on snake envenomation.[1–4] Flowers,[5] and Goucher and Flowers[6] observed a direct relationship between hemorrhagic activity and venom proteolytic activity. They observed that EDTA reduced both hemorrhage and the protease activity of *Agkistrodon piscivorus piscivorus* (Eastern cottonmouth) venom.

In many instances, snakebites of *A. rhodostoma* and *T. popiorum* cause hemorrhaging which is not prevented by the use of antivenin. Therefore, it is important to get a better understanding of the factors responsible for the hemorrhaging caused by these snake venoms and of their possible relationship to proteolytic enzyme activities.

In order to understand more clearly the hemorrhagic properties of Thailand snake venoms, hemorrhage was induced experimentally in rabbits and the proteolytic enzyme activities were measured quantitatively. One naturally occurring substrate, casein, and three synthetic substrates, TAME (*p*-toluene sulfonyl-L-arginine methyl

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ester), BAEE (*N*-benzoyl-L-arginine ethyl ester), and BTEE (*N*-benzoyl-L-tyrosine ethyl ester) were employed for proteolytic enzyme activity determinations.

METHODS

Materials

Venoms were collected in the Queen Saovabha Memorial Institute (Pasteur Institute), Bangkok, Thailand. Snakes, kept in the snake farm, were milked and the venom was lyophilized whenever needed.

TAME, BAEE, and BTEE were purchased from Calbiochem. Casein was the product of Amend Drug and Chemical Co.

Hemorrhagic tests

Hemorrhagic activities of the venoms were determined by the methods of Kondo *et al.*[7] and of Goucher and Flowers.[6] Seven areas of 40 cm^2 were marked on depilated skin of rabbits weighing 3·7 kg and 100 μg venom in 0·2 ml of 0·9% saline was injected subcutaneously in the center of each area. Saline solution without venom was also injected as a control. After 48 hr the animals were sacrificed and the skin was removed. The hemorrhagic area was examined and photographed immediately. For *V. russellii siamensis* venom, Swiss white mice weighing 16 g were also injected subcutaneously for hemorrhagic tests with different venom doses.

Proteolytic enzyme activity

The rates of hydrolysis of TAME, BAEE, and BTEE were determined by the spectrophotometric method of Hummel[8, 9] and of Schwert and Takenaka.[10] To 3·0 ml substrate in 1-cm^2 quartz cells, 0·1 ml of venom solution was added and the change in absorbance followed. Substrate solutions were as follows: TAME, $1{\cdot}4 \times 10^{-3}$ M in 0·05 M phosphate buffer, pH 8·0; BAEE, $8{\cdot}2 \times 10^{-4}$ M in 0·067 M phosphate buffer, pH 7·0; BTEE, $5{\cdot}0 \times 10^{-4}$ M in 0·05 M phosphate buffer, pH 7·8, in 25·6% methyl alcohol. Rates of hydrolysis of TAME, BAEE, and BTEE were followed on a Beckman model DB ultraviolet spectrophotometer at wavelengths of 247 mμ, 253 mμ, and 256 mμ respectively. Enzymatic activity was calculated from the linear portion of the curve by the following equation:

$$\text{activity (units/mg)} = \frac{(\text{absorbance change/min})\ 1000}{\text{mg of venom}}.$$

A modified method of Kunitz[11] was used to detect hydrolysis of casein. One ml of 1% casein (0·1 M phosphate buffer, pH 7·0) was incubated with 0·5 ml venom solution. At different time intervals, the reactions were stopped by the addition of 2·0 ml of 5% trichloroacetic acid. After standing 30 min, the tubes were centrifuged in a Servall centrifuge for 15 min. The absorbance of the supernatant was read at 280 mμ. Enzymatic activity was calculated by the same equation used for TAME, BAEE, and BTEE.

RESULTS

Of the six venoms investigated, only *A. rhodostoma* and *T. popiorum* showed noticeable hemorrhagic activity after subsutaneous injection of each venom (Fig. 1).

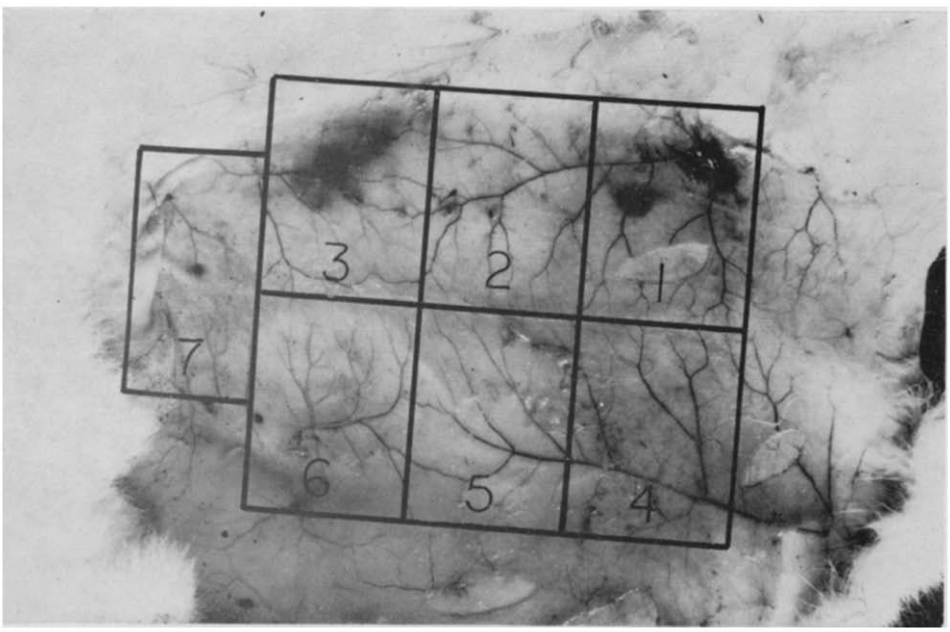

FIG. 1. Hemorrhagic activities of Thailand snake venoms. (1) *A. rhodostoma*, (2) *O. hannah*, (3) *T. popiorum*, (4) *N. naja siamensis*, (5) *V. russellii siamensis*, (6) *B. fasciatus*, (7) 0·9% saline solution. Note the hemorrhagic lesions due to injection of *A. rhodostoma* and *T. popiorum* venoms.

It is known from clinical observations that *O. hannah*, *N. naja siamensis*, *B. fasciatus*, and *V. russellii siamensis* do not produce hemorrhagic symptoms after envenomation. In the present investigation, no hemorrhage was detected after injection of these venoms, a finding which is in close agreement with clinical observation.

In order to determine venom proteolytic activities, the effects of substrate concentrations were studied (Fig. 2).

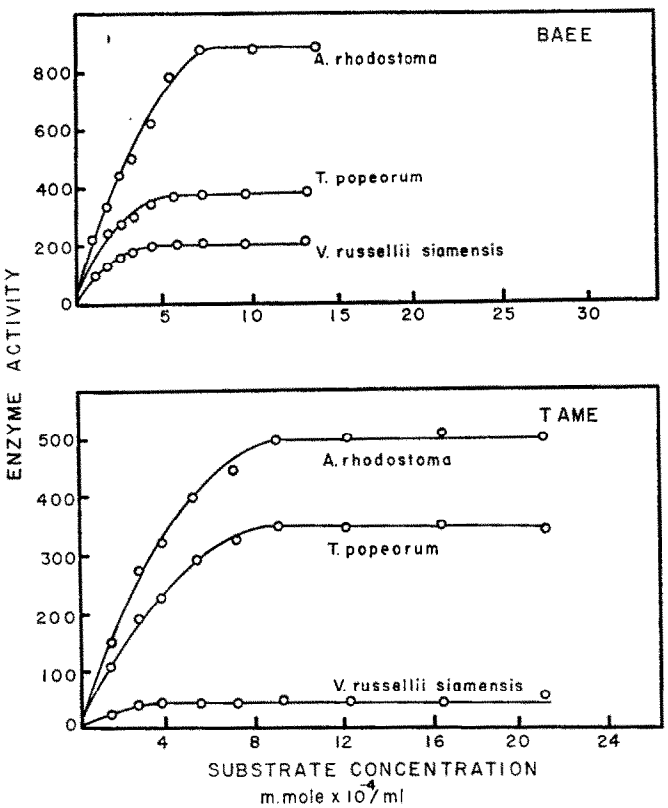

FIG. 2. Effect of substrate concentrations on the rates of hydrolysis. Hydrolysis of TAME was carried out in 0·05 M phosphate buffer, pH 8·0; BAEE was made in 0·067 M phosphate buffer, pH 7·0. Enzyme activities are defined in the text.

At high substrate concentrations (BAEE $> 7{\cdot}5 \times 10^{-4}$ M; TAME $> 1{\cdot}35 \times 10^{-3}$ M), the reaction velocity followed that of zero-order kinetics. For subsequent enzyme activity determinations, BAEE concentrations of $8{\cdot}2 \times 10^{-4}$ M and TAME concentrations of $1{\cdot}4 \times 10^{-4}$ M were used in order to give maximum turnover numbers. A high concentration of casein, 1%, was found to give maximum velocity.

Hydrolysis of TAME, BAEE, and casein was followed at definite time intervals and the linear portions of the curves were used, which gave zero-order kinetics for the calculation of enzyme activities (Fig. 3).

Proteolytic activities of each venom with TAME, BAEE, casein, and BTEE are summarized in Table 1. Venoms of *A. rhodostoma* and *T. popiorum* hydrolyzed TAME rather rapidly. *V. russellii siamensis* venom gave relatively low enzymatic activity.

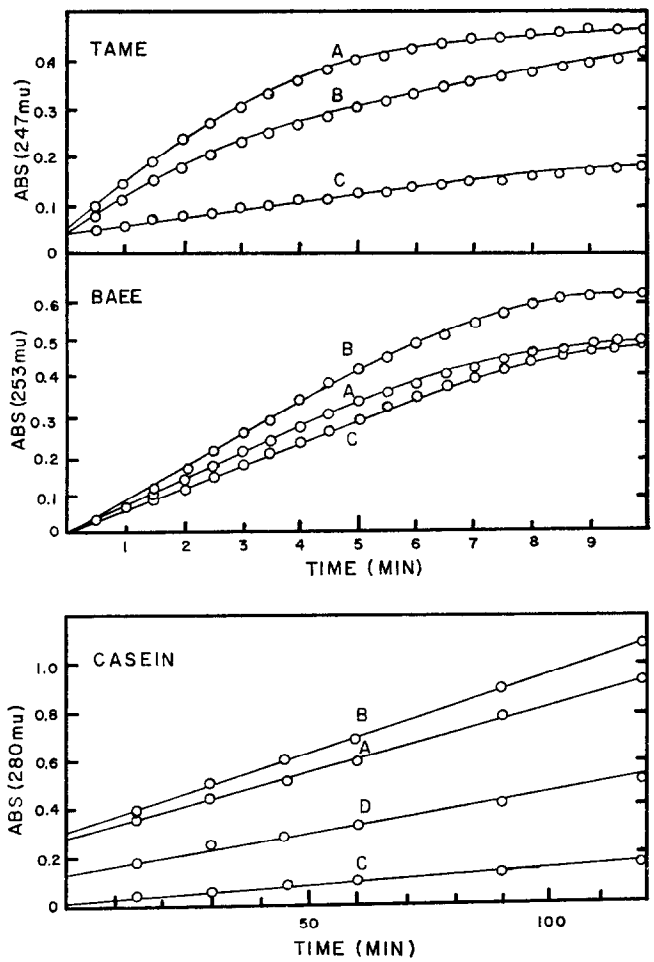

Fig. 3. Rates of hydrolysis of TAME, BAEE, and casein by snake venoms as measured by increase of absorbance with time. (A) *A. rhodostoma* venom: 0·2 mg, 0·05 mg, and 0·25 mg were used for TAME, BAEE, and casein respectively. (B) *T. popiorum* venom: 0·2 mg, 0·2 mg and 1·0 mg were used respectively. (C) *V. russellii siamensis* venom: 0·2 mg, 0·2 mg, and 1·0 mg were used respectively. (D) *O. hannah* venom: 1·0 mg was used for casein.

Table 1. Proteolytic enzyne activities of Thailand snake venoms with TAME, BAEE, casein, and BTEE as substrates*

Venoms	Substrates			
	TAME	BAEE	Casein	BTEE
A. rhodostoma (Malayan pit viper)	500	880	22	0
T. popiorum (Green pit viper)	350	390	7	0
V. russellii siamensis (Thailand Russell's viper)	50	220	1	0
O. hannah (King cobra)	0	86	3	0
B. fasciatus (Banded krait)	0	0	0	0
N. naja siamensis (Thailand cobra)	0	0	0	0

* Enzyme activities are defined in the text.

Similar results were obtained for the substrate BAEE with the exception of *O. hannah* which also slightly hydrolyzed this substrate. Venoms of *B. fasciatus* and *N. naja siamensis* did not hydrolyze the substrates TAME, BAEE, and casein. Casein was hydrolyzed only slightly by the venoms of *V. russellii* and *O. hannah*. The substrate BTEE, which is specific for chymotrypsin, was not hydrolyzed by any of the venoms investigated.

DISCUSSION

In this investigation, two venoms (*A. rhodostoma* and *T. popiorum*) produced pronounced hemorrhaging when injected into rabbits. These two venoms also exhibited markedly higher proteolytic enzymatic activity towards the substrates, TAME, BAEE, and casein. From these results, it appears that there is a correlation between the hemorrhagic activity and venom proteolytic activity.

Venom of *V. russellii siamensis* produced very weak activity toward TAME and casein; however, no detectable hemorrhagic symptoms could be found on rabbit skins. In order to clarify this problem, hemorrhagic tests were made on this venom under different conditions, When the amount of *V. russellii siamensis* venom was doubled (200 μg), the rabbit died within 12 hr. The skin was depilated immediately and examined, but no hemorrhage was observed. A very high dosage of the venom (10–20 μg venom/mouse) was injected subcutaneously into mice and the depilated skin was examined for hemorrhage. The mice died within 3 hr but no hemorrhagic area was observed, although there was some hypermia and dilation of blood vessels at the site of injection.

O. hannah venom did not hydrolyze TAME but did show weak activity toward BAEE and casein; however, no hemorrhaging was observed.

Maeno *et al.*,[2] working on fractionated venom, were able to show that the fraction which contained the proteolytic enzyme activity also produced hemorrhaging when injected into guinea pigs.

Among the three substrates tested, it appears that the activity toward TAME has the most direct correlation to hemorrhagic activity. Although correlations can be drawn between BAEE, casein, and hemorrhagic activity, the results are not as clear-cut as with TAME.

Venoms of *V. russellii siamensis* and *O. hannah* showed some proteolytic activities but did not produce visible hemorrhage. This tends to suggest that some other factors necessary for producing hemorrhage, in addition to proteolytic enzymes, are missing. Although venom proteolytic enzymes are generally believed to be the factor responsible for hemorrhage, this might be an oversimplification. Enzymes such as lipase, phospholipase, and lipoprotein lipase might well be involved as possible contributing factors for hemorrhage.

REFERENCES

1. H. L. Stahnke, F. M. Allen, R. V. Horan and J. H. Tenery, *Am. J. trop. Med. Hyg.* **6**, 323 (1957).
2. H. Maeno, S. Mitsuhashi, Y. Sawai and T. Okonogi, *Jap. J. Microbiol.* **3**, 131 (1959).
3. I. Sadaaki, T. Omori, G. Ohsima and T. Suzuki, *J. Biochem., Tokyo*, **57**, 392 (1965).
4. A. Ohsaka, H. Ikezawa, H. Kondo and N. Uchida, *Br. J. exp. Path.* **41**, 478 (1960).
5. H. H. Flowers, *Toxicon* **1**, 131 (1963).
6. C. R. Goucher and H. H. Flowers, *Toxicon* **2**, 139 (1964).

7. H. Kondo, S. Kondo, H. Ikezawa and R. Murata, *Jap. J. med. Sci. Trans. Abstr.* **13**, 43 (1960).
8. B. C. W. Hummel, *Can. J. Biochem. Physiol.* **37**, 1393 (1959).
9. A. T. Tu, A. Chua and G. P. James, *Toxic. appl. Pharmac.* **8**, 218 (1966).
10. G. W. Schwert and Y. Takenaka, *Biochim. biophys. Acta* **16**, 570 (1955).
11. M. Kunitz, *J. gen. Physiol.* **30**, 291 (1947).