FRACTIONATION OF SAKISHIMA-HABU (Trimeresurus elegans) VENOM AND ITS TOXICITY.

(Lethal effects, hemorrhagic activity and determination of edema forming activity)

M. Yamakawa, M. Nozaki and Z. Hokama

INTRODUCTION

There are several species of poisonous sankes in the Ryukyu islands, and two of them are especially dangerous to human beings and domestic animals. One of the them, Habu (Trimeresurus flavoviridis), inhabits the main island of Okinawa and its neighboring islands, and the other, the Sakishima-habu (Trimeresurus elegans), is restricted to the Yaeyama islands. Both of them cause frequent serious and even lethal effects to man. The common symptom of snake bites by both species is severe local inflammation with hemorrhage, edema and necrosis. For the treatment of the patients bitten by Sakishima-habu, Habu antivenin derived from horses hyperimmunized with Habu venom has been used. As preliminary tests for the production of the specific antivenin for treating Sakishima-habu bites, fractionation and characterization of Sakishima-habu vemon were undertaken.

MATERIALS AND METHODS

Snake venom

The venom used was a dried and powdered pool of Sakishima-habu species (T, elegans), collected in the Yaeyama islands in 1965.

Estimation of protein

The protein content of venom solution was estimated by measuring ultraviolet absorption at 280 mμ in a 1 cm cell, based on the assumption that the absorbancy of 1 mg/ml solution of dried crude venom was 1.16

Fractionation

Step -1: 8.5 ml of a 10% solution of the crude venom were passed through Sephadex G 150 column (2.5 x 90 cm), equilibrated with 5 mM Tris-HCl buffer, PH 8.5, containing 0.15 M NaCl.
The flow rate was 20 ml/hr. and five milliliteres of fraction were collected.

Step -2: Further, fraction S_3 which prepared by step - 1, concentrated by lyophilization, dialyzed against 10 mM borax-HCl buffer (PH 9.0), and then centrifugated to remove insoluble precepitates. The dialysis residue containing 1500 mg of protein was applied onto a column (2.5 x 40 cm) of Amberlite CG 50 (Type -2).
Linear gradient elution was performed from 0 to 0.35 M of NaCl in the eluting solution. The flow rate was 15 ml/hr and ten milliliters of fraction were collected.

Determination of lethal toxicity

Lethal toxicity was assayed by intravenous injection into mice weighing 15-18g, with four doses of each venom solution graded with 1.4-fold intervals in the amount of 0.1 ml. Five animals were injected with each dose. Number of deaths and survivals in 3 days was checked and LD_{50} was calculated by the Reed-Muench method.

Determination of hemorrhagic activity

Hemorrhagic activity was determined by the method of Kondo et al. reported in 1960. Four doses of each venom solution graded with 3-fold intervals in the volume of 0.1 ml were injected intracutaneously into the depilated back skin of rabbits. Twenty-

four hours after injection rabbits were killed, and cross-diameters of each hemorrhagic spots were measured from the visceral side of the skin. Minimum hemorrhagic dose (MHD) was defined as the least quantity of venom causing a hemorrhagic spot of 10 mm in diameter within 24 hours after intracutaneous injection (Kondo et al., 1960).

Determination of edema-forming activity

Edema forming activity was assayed by inoculation to the mice foot pads. Mice weighing 15-18 g in groups of ten were injected into the right pads of mice with 3 to 4 doses of each venom solution graded with 3-fold intervals in the volume of 10 µl. Four hours after injection mice were killed by chloroform. Then, both legs were cut off with scissors almost at a right angle and weighed individually. The weight ratio of the injected leg to the healthy leg was calculated as a percentage, to express the severity of the edema as follows;

$$\text{Edema ratio } (Y\ \%) = \frac{\text{mg of right (edema) leg}}{\text{mg of left (normal) leg}} \times 100$$

The results were analysed statistically.

Antivenines

Two lots of Sakishima-habu antivenins, No 19 and No 20, were prepared and Habu antivenin lot 14 was also prepared from the plasma of horses hyperimmunized with crude Sakishima-habu venom and crude Habu venom respectvly. These antivenins were purified by ammonium sulfate and digested with pepsin and lyophilized.

Test Toxins

Three kinds of the partially purified venoms were used as test toxins; S_1, S_2 and S_3 prepared from crude Sakishima-habu venom. Test toxins HR-I and HR-II were prepared from crude Habu venom by passing through Sephadex G 100. And one test dose of each test toxins were used as shown in Table 3.

Titration of antilethal units

One test dose of each test toxins to titrate the antilethal potency was used as follows; S_1 = 3 LD_{50}. S_2= 5 LD_{50}, S_3 = 3 LD_{50} and RH-1 = 5 LD_{50}.

A proper quantity of test toxin was mixed with the serial dilution of antivenins graded with 1.25 fold intervals. Then, each mixtures containing one test dose per 0.2 ml were kept at room temparature for one hour and injected intravenously to mice in a volume of 0.2 ml. Number of deaths and survivals in 3 days was checked and ED_{50} of the antivenin was calculated by the Reed-Muench method.

One unit of antilethal potency is defined as the least amount of antivenin which neutralized one test dose of the toxin. The antilethal potency is expressed as units per ml of the antivenin.

Titration of antihemorrhagic units

One hundred MHD were used as one test dose of each toxins to titrate antihemorrhagic potency of the antivenins. A proper quantity of test toxin was mixed with the serial dilution of antivenin graded with 1.25 fold intervals. And each mixture containing one test dose per 0.2 ml reacted for about one hour at room temparature and injected intracutanously into depilated back skin of rabbits in a volume of 0.2 ml.

The hemorrhagic spots were measured at twenty four hours after injection. One unit of anti-hemorrhagic potency was calculated as a least quantity of antivenin which neutralized one test dose of the toxin at the end point of 10 mm diameter of hemorrhagic spots.

The antivenin potency is expressed as a unit per ml of the antivenin.

Titration of antiedema units

One test dose of each test toxins was used as;

S_2 = 40 MED, S_3 = 4 MED, HR-I = 40 MED and HR-II = 4 MED

A certein amounts of test toxin were mixed wixed with the

serial dilution of antivenin graded with 1.25 fold intervals. Then, each mixtures containing one test dose per 20 µl were kept at room temparature for one hour and injected to right foot pads of mice in a volume of 20 µl.

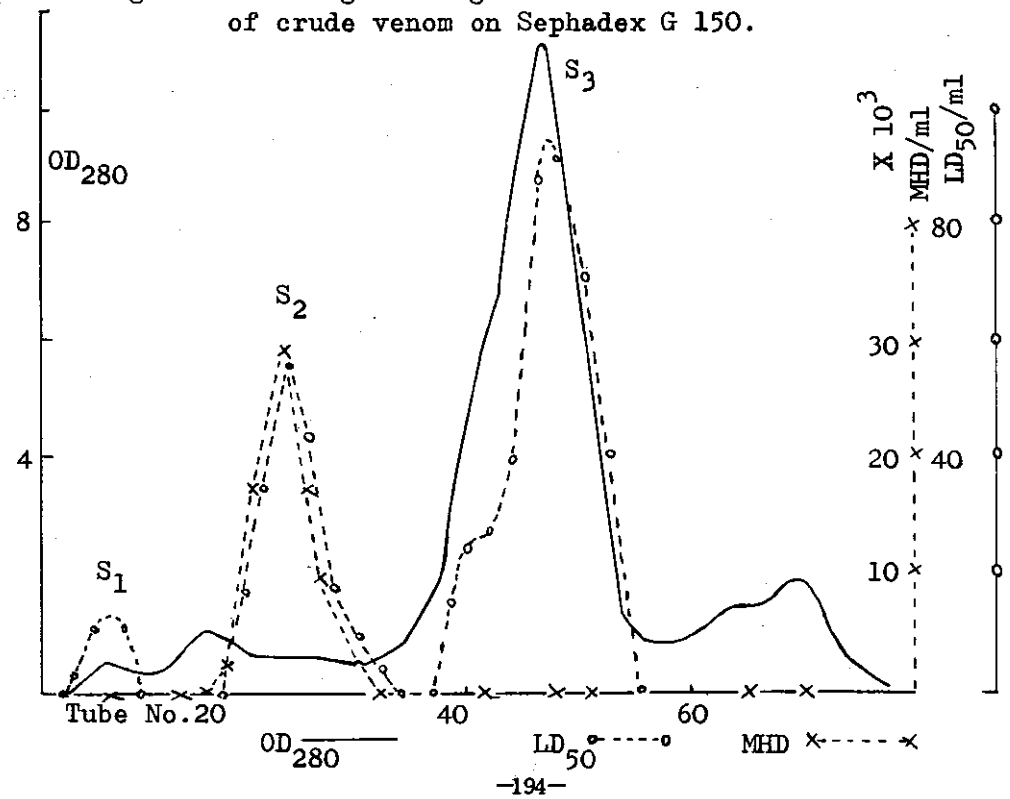
Both legs of mouse were cut off at four hours after injection and weighed immediatly. The weight ratio of the injected legs to the normal side was calculated as a percentage. One unit of anti-edema potency is defined as the least amount of antivenin which neutralizes one test dose of the toxin at the end point of 130% of edema ratio. The antiedema titer is expressed as units per ml of the antivenin.

RESULTS

Gel filtration of crude venom on Sephadex G 150

Eight fifty mg containg (710 mg of protein) of the crude Sakishima-habu venom were fractionated as a first step by passing through a Sephadex G 150 (Tris-HCl buffer, PH 8.5) and separated into three lethal frastions, S_1 S_2 and S_3. The diagram of gel filtration of crude venom is shown in Fig. I.

Fig. 1 The Diagram of gel filtration of crude venom on Sephadex G 150.



The total recoveries of lethal and hemorrhagic activities were 86% and 74% respectvly, as shown in Table 1. The specific activity of S_2 (Tube No 7 - 12), S_3 (No 38-54) was 2.4, 4.2 and 0.74 respectivly. The main part of the lethal toxicity was found in the fraction S_3 with the recovery rate of 70% against total. As shown in Table 1, the recoveries of lethal toxicity in S_1, S_2, and S_3 fractions were 5%, 21% and 60%, respectively. Thus, the ratio of recoveries of lethal toxicity in S_1, S_2, and S_3, was approximately 1: 4: 12. Characteristic lethal hemoptysis and pulmonary bleeding were observed in the mice injected intravenously with S_1 fraction.

Mice injected intravenously with S_2 fraction died. These mice showed general hemorrhage in the subcutaneous tessues, muscles and viscera, whereas neither hemoptysis nor systemic hemorrhage were resulted from intravenous injection with fraction S_3. The main hemorrhagic activity was found in fraction S_2 with the recovery of 74% assayed by the rabbits skin test.

Table 1. Recoveries in lethal and hemorrhagic activities.

	protein	lethal toxicity			hemorrhagic activity		
		LD_{50}	SP	reco.	MHD	SP	reco.
crude	715	80 μg	1	100%	0.6	1	100%
S_1	15	33.3	2.4	5			
S_2	35	19.1	4.2	21	0.04	15	74
S_3	579	107.8	0.74	60			
Total	629			86			74

SP : specific activity. reco. : recoveries
MHD : minimum hemorrhagic dose.

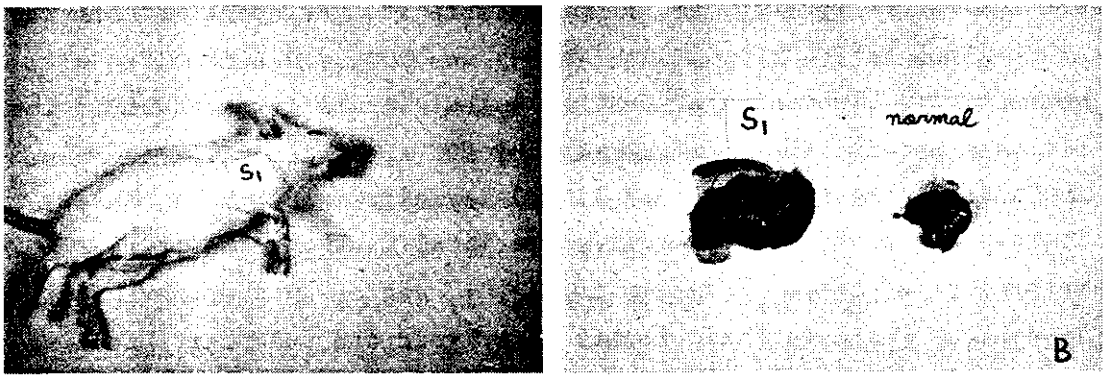


Photo. 1. Hemoptysis (A) and hemorrhage (B) of enlarged lungs of mouse after the intravenous injection of fraction S_1.

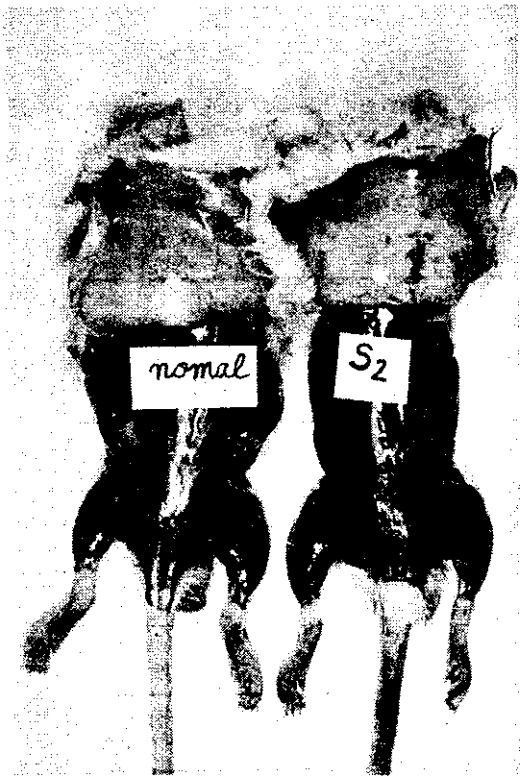


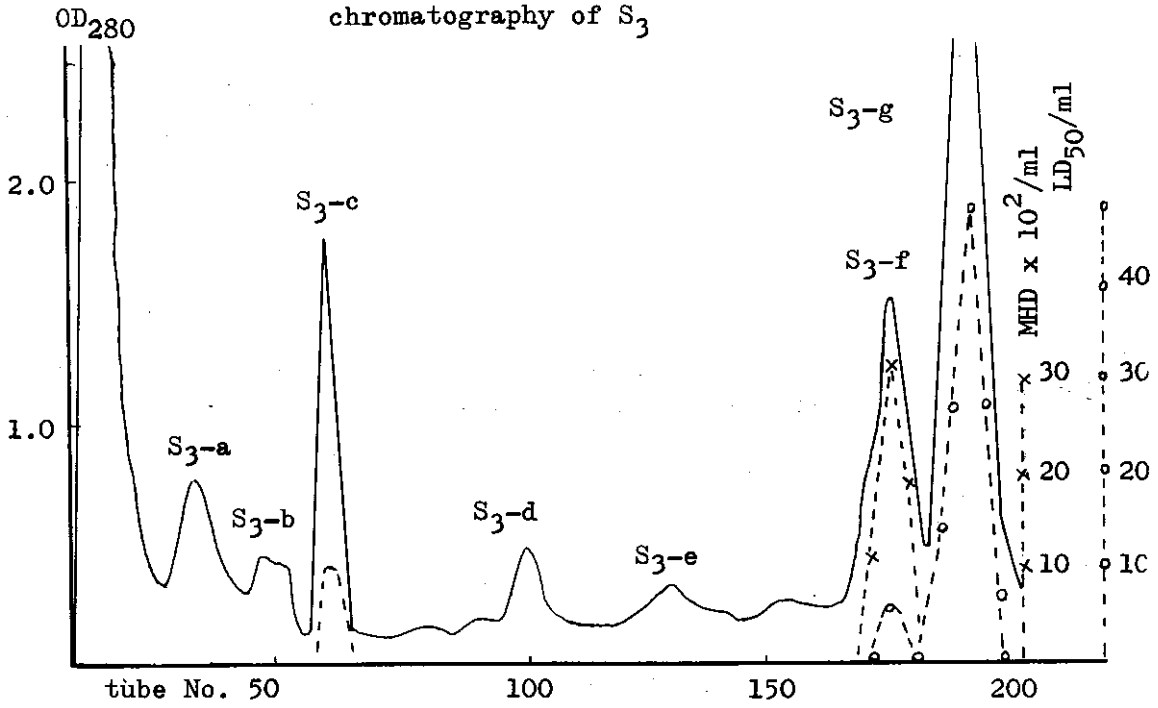
Photo. 2. Generalized hemorrhage of subcutaneous and muscle tissue by the intravenous injection of fraction S_2.

Photo. 3. Edema of mice legs with and without hemorrhage.

Amberlite CG 50 column chromalography of fraction S_3

Fraction S_3 contained a large amount of protein and induced unclear hemorrhagic spots on the enthickened rabbit skin at the step 1. So further purification of fraction S_3 seemed to be necessary. The elution diagram of the column chromatography of fraction S_3 is shown in fig. 2. The separated fractions were designated as S_{3-a}, S_{3-b}, and so on to S_{3-g}, in an order of elution.

Fig. 2 Amberlite CG-50 column chromatography of S_3



Fraction S_{3-f} induced clear hemorrhagic spots in the rabbit skin. There are at least tow kinds of hemorrhagic principles in the crude venom. As described above, the main hemorrhagic activity in the crude venom was found in fraction S_2, and a minor activity in fraction S_{3-f}. The main lethal toxicity was found in fraction S_{3-g}, which had no hemorrhagic effects at all. Thus the representative fractions of S_3 separated by chromatography were frations S_{3-f} and S_{3-g}.

Edema Forming activity of the crude venom and venom fractions.

Swelling cause by each venom developed quite quickly, and reached at the maximum severity within about 30 to 60 minutes after injection. As shown in Figure 3, the time-course curves of crude venom and venom fractions showed characteristic patterns. The mouse foot pads injected with fraction S_1 developed severe edema without hemorrhage in 30 to 60 minutes after the injection. They healed rapidly, in about 8 to 10 hours after the injection. One the other hand, crude venom and fraction S_2 caused intensive edema with severe hemorrhage on the foot pads, and these changes persisted for longer times. Fraction S_3 induced both edema and hemarrhage but to a lesser extent.

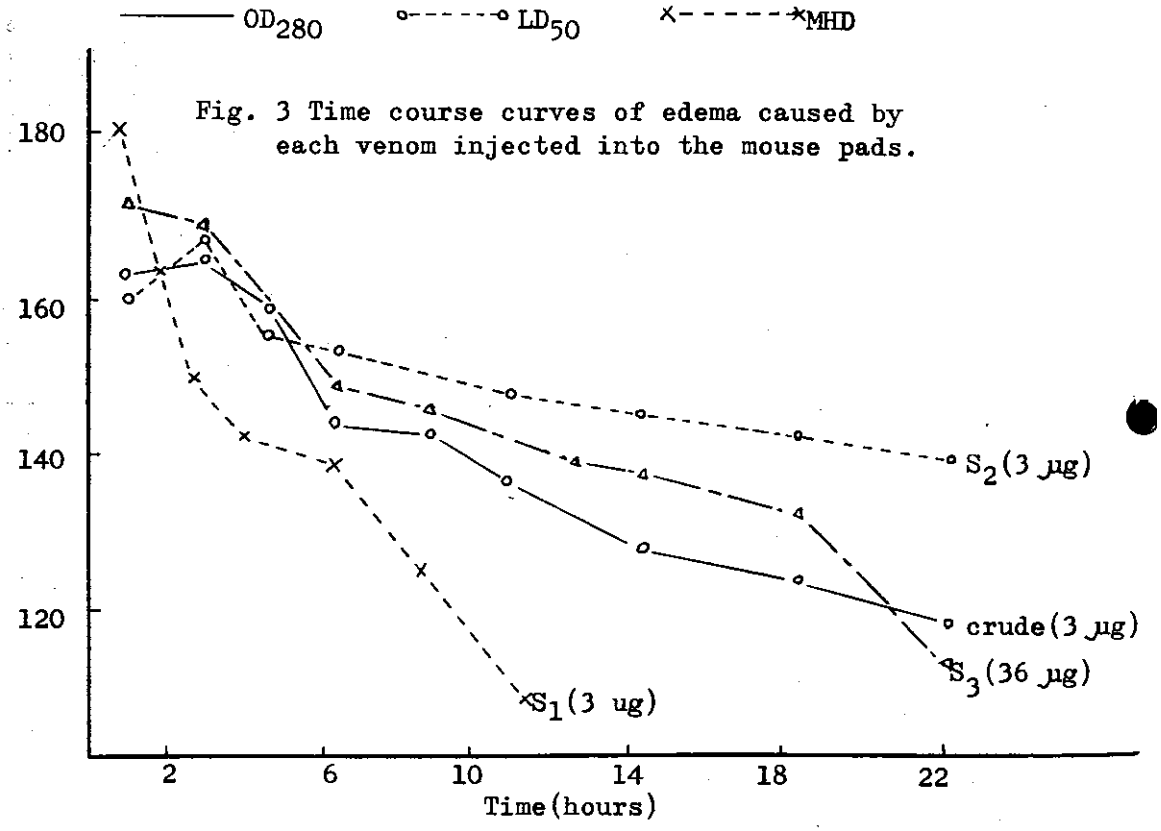


Fig. 3 Time course curves of edema caused by each venom injected into the mouse pads.

By statistical analysis of the results obtained, it was found that there is linearity and parallelism between the log dose of each venom and the edema ratio shown in Fig. 4. The common slope, $\bar{b}$ was 25.1, and common variance, S^2, was 43.3 at four hours' observation. The "minimum edema dose" was defined as" the least quantity of venom cousing 130% of the edema ratio" Thus, I MED of each venoms at 4 hours observation could be read from Fig. 4, or calculated by following equation ; $\bar{X} = \bar{X} - (\bar{Y} - 130) / \bar{b}$, where $\bar{X}$ are the mean log doses of venom and $\bar{Y}$ are the mean edema ratio of the venom, and $\bar{b}$ is the common slope. The edema-forming activities and relative potencies of both crude venom and venom fractions determind by this method are summarized in Table 2. A main part

Fig. 4 Dosage response lines of venoms.

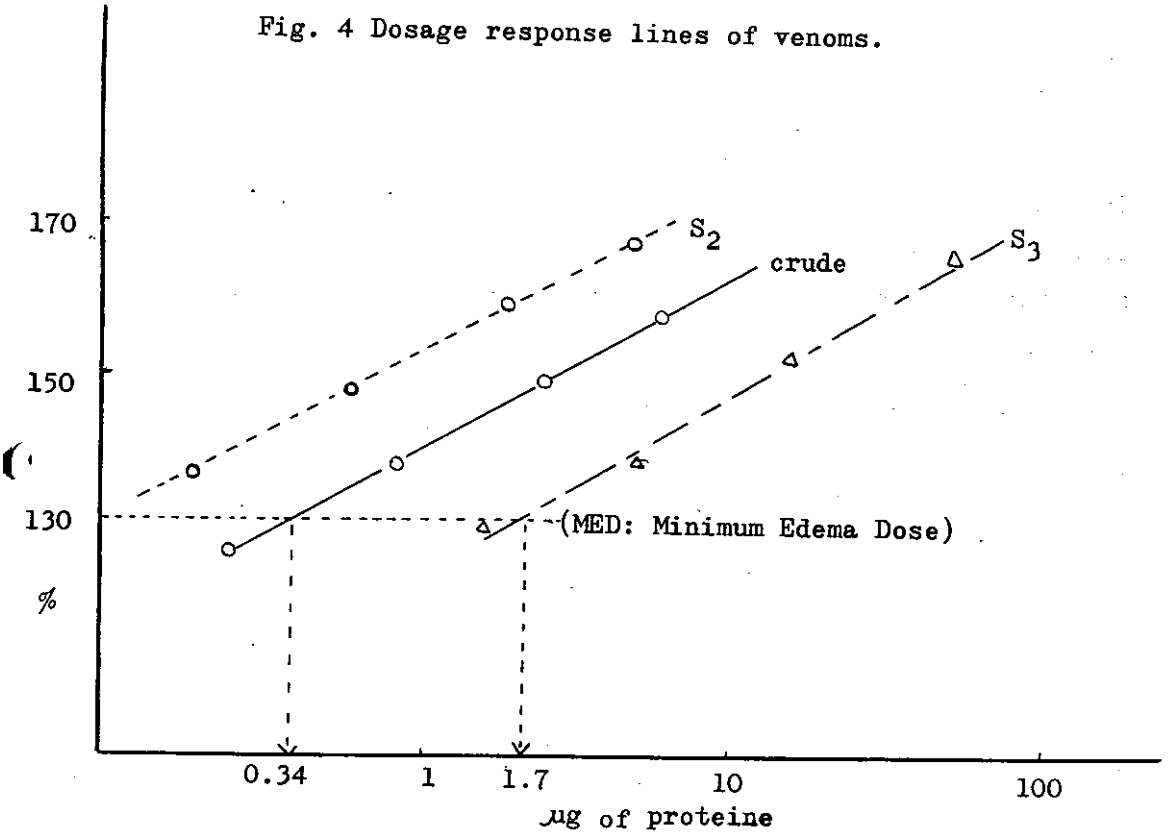


Table 2. The edema-forming activity and relative potency of crude venom and venom fractions.

	MED	Total MED	Recoveries	Relative potency
SC	0.34 μg (0.28-0.42)	21×10^5	100 %	1
S_2	0.1 (0.086-0.13)	9.5×10^5	44.8	3.3 (2.5-4.3)
S_3	1.74 (1.43-2.11)	3.2×10^5	14.6	0.2 (0.08-0.5)

of the edema forming activity in the crude venom was recovered in fraction S_2. The ratio of edema-forming activities recovered in S_2 and S_3 was appraximately 1: 0.35

Cross neutrarization test

On the basis of this study, tests for neutralization of each venom fraction with Habu and Sakishima-habu antivenins were performed with five test toxins; S_1, S_2, S_3, HR-I and HR-II. One test dose of each toxins are shown in Table 3 and the results are shown in Table 4. The titiers of antilethal - S_3 and antiedema - S_3 of

Table 3.
Test toxins : One test dose of each test toxins

		for anti-lethal	for anti-hemorrhage	for anti-edema
Samishima-Habu venom	S_1	3 LD_{50}		
	S_2	5 "	100 MHD	40 MED
	S_3	3 "		4 "
Habu venom	HR-1	5 "	100 "	40 "
	HR-II		13.5 "	4 "

MHD : Minimum Hemorrhagic Dose
MED : Minimum Edema Dose

Table 4.
Cross neutralization test of each venom fraction with Habu and Sakishima-habu antivenin. (units per ml)

	antilethal units				antihemorrhagic units			anti-edema units			
	SS_1	S_2	S_3	HR-1	S_2	HR-1	HR-II	S_2	S_3	HR-1	HR-II
No 19	11	21	11	<	400	55	240	125	80	<	<
No 20	33	42	<	10	500	155	175	143	<	100	<
Lot.14	13	14	<	63	215	400	350	89	<	317	300

<: was less than the detectable level. No 19 and No. 20 : Sakishima-habu antivenin. Lot. 14 : Habu-antivenin.

Sakishima-habu antivenin, No 19, were 11 u and 80 u respectivly, but the titers of antilethal-HR-I and antiedema-HR-I were lower than the detectable level. On the other hand, the titers of the antilethal-HR-I and antiedema-HR-I of Habu antivenin, lot 14, were 63 u and 317 u respectively, but antilethal-S_3 and antiedema-S_3 were lower than the detectable level. So, the anti-S_3, and anti-HR-I titers of thise antivenins showed reversed results each other.

Likewise, the titers of lot 14, No 19 and No 20, each determined with fraction S_2 and HR-I, showed a reversed connection; namely, Habu antivenin, lot 14, did not neutralize fraction S_3 and HR-I which represented the main part of the lethal, hemarrhagic and edema toxins of Habu venom was only partially neutralized by Sakishima-habu antivenins No 19 and No 20. Also, toxin HR-II was partially neutralized by Sakishima-habu antivenins. The antibody production against fraction S_3 was lesser amount than S_1 and S_2 in horses which immunized by crude Sakishima-habu venom. This experiment showed that an antivenin gave a higher potency when titrated with a homologous venom fraction than with a heterologous one.

SUMMARY

A crude Sakishima-habu venom solution was separated on Sedephadex G 150 into three lethal fraction S_1, S_2, and S_3. The ratio of recoveries of lethal toxicity in S_1, S_2 and S_3 was approximately 1; 4; 12.

Lethal hemoptysis and pulmonary bleeding occurred in mice injected intravenously with S_1 fraction, though no hemorrhage was resulted from intamuscular injection. The mouse foot pads injected with fraction S_1 developed severe edema without hemorrhage in 30 to 60 minuts after the injection, and healed quite quickly. Mice injected intravenously with fraction S_2 died from general hemorrhage, such as systemic hemorrhage of subcutaneous tissues, muscles and viscera, whereas neither hemoptysis nor systemic hemorrhage resulted from intravenous injection with fraction S_3 Fraction S_2 showed inensive hemarrhagic effect on the muscle of rabbits and mice by the intramuscuar injection, and on the rabbit skin injected intracutaneously. Fraction S_2 represented most important toxic portion of the crude Sakishima-habu venom. Further purification of fraction S_3 was performed by Amberlite CG 50 column chromatography and separated seven protein peaks, designated as S_{3-a}, S_{3-b}, and so on to S_{3-g}, A lesser extent of hemorrhage was observed in S_{3-f} injected intracutaneously on the rabbit skin. The main lethal toxicity and minor edema forming effect without hemorrhage were found in S_{3-g}.

Also, there are at least tow kinds of hemorrhagic principles in the crude Sakishima-habu venom, the main hemorrhagic activity was found in the fraction S_2.

Edema foming activity was assayed by injection to the foot pads of mice. The activity was expressed as a percentage calculated from the weight ratio of the injected legs to the healthy legs of mice. By the statistical analysis of the results obtained, it was elucidated that there are linearity and parallelism between log

dose of each venom and the edema ratio. The common slope $\bar{b}$ was 25.1, and the common variance S^2, was 43.3 at four hours' observation. The "Minimum Edema Dose" was defined as "the least quantity of venom causing 130% of the edema ratio"

One MED of the crude Sakishima-habu venom was resulted as 0.44 μg from this method.

Tests of neutralization of each venom fraction with Habu and Sakishima-habu antivenins were preformed. Habu antivenin did not neutralize fraction S_3 which represents main part of lethal toxicity of Sakishima-habu venom, and HR-I, which represented the main part of lethal and hemorrhagic toxins of Habu venom was only patially neutralized by Sakishima-habu antivenin.

Fraction S_2 which represented main part of hemarrhagic and edema forming activity was partially neutralized by Habu antivenin. From these results, we conclude that homologous antivenin should be used in the treatment of Sakishima-habu bite.

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