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Inhibitory Substance on the Snake Venoms Produced by *Penicillium* sp.

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蛇毒의 阻害物質에 關한 硏究

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Abstract

One strain of *Penicillium* sp. (175-66-B), isolated from soil, was able to produce a substance that has a strong inibition activity against the Agkistrodon and Trimeresurus venoms.

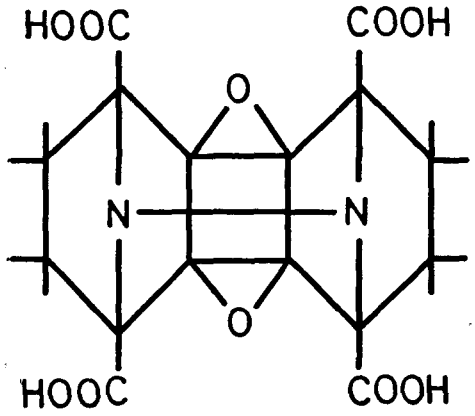
In this experiment, the chemical and biological properties of the sample were investigated. As an inhibitory substance, it was effective to the proteinase, hemorrhagic and lethal factors of Agkistrodon and Trimeresurus venoms, and also effective to several fractions of the proteinases and hemorrhagic factors of Agkistrodon halys blomhoffi venom. Moreover, in the addition of prednisolone, it was more effective for the cure of the mouse envenomated with the venom amount of two fold of MLD_{100}.

This substance was very stable to the acid, alkali and heat. Its melting point was high enough to sublime at 222°C without any decomposition. This sample was easily dissolved only in hot water, but not in several organic solvents except for a little dissolution in etate. It did not have the chelating activity. It had very strong specificity to the snake venoms. but its activity was depressed by the addition of zinc or cupric salts. This sample had no acute toxicity to the mouse.

Its chemical formula was $C_{16}H_{12}N_2O_{10}$ with the molecular weight of about 392. It has two epoxy groups and four carboxyl radicals, but amino, nitrite and nitrate radicals, unsaturated bonds and aromatic ring were not detected. Theuchemical configuration of this sample was suggested to be;

Introduction

Snake venoms are recognized as proteinous substance tohave enzymatic actions. For the inactivation of snake venoms, the physical and chemical methods are generally applied. In the chemical method for the inactivation of venoms, the distinct concepts arf the application of immunological an-



tiserum and chemical reagents. On the application of chemical reagents, many works have been carried out with DMSO[1,2,3,4], EDTA[1,56,7,8,9], DTP A[1,4], cystein[5,6] N-(2-hydroxylethylen) ethylendiaminetetraacetic acid[4], esterol succinate[10,11], dihydrothioctic acid[12,13,14,15], epsilon amino capric acid[16], tannic acid[17,18,19], alphathiolactyl glycin Na[6], glycyrrhizine[6,20], formalin [21,22,23,24], dexamethason[25], microbial inhibitor[26], BAL[26], thiabendazol[27], estriol-16, 17 disodium succinate[11], trasylol[28], cystein thioglycolate Na[15], heparin[28,29,30], isoxsupprin[34], ergosterols[31,32,33], and so on.

These kinds of chemicals act on the snake venoms as an inhibitor, but their actions are unspecific to venoms. Until now, there have not been any repots about the specific inhibitor. In our studies, a microbial substance, ISV-33, produced by one Penicillium sp. was isolated in crystal. Its actions on the proteinase, hemorrhagic factors and iethalcity of snake venoms and its biological and physico chemical properties were investigated. This substance had a strong specificity on snake venoms, Agkistrodon and Trimeresurus, *in vivo* and *in vitro*.

Meterials and methods

Isolation and cultivation of the microorganism

One strain of *Penicillium* sp. (175-68-B) was isolated from soil source and cultivated in a medium containing

glucose	3 %
$NaNO_3$	0.2%
K_2HPO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
KCl	0.05%
pH	6.8

for 8 days at 30°C.

Preparation of the sample

The cultured filtrate of *Penicillium* sp. was treated with the anion exchange resin of Dowex 60. The absorbed effective substance was eluted with 5 N-sodium hydroxide solution and the pH of the effluent was adjusted to 1.0 with concentrated hydrochloric acid. The acidic effluent was extracted with large volume of ethylacetate and the organic phase was evaporated in vacuum after drying over anhydrous sodium sulfate. The obtained yellowish solid substance was recrystalized in hot water for several times. Finally a needle-like fine crystal was obtained and this crystal gave single spot on paper chromatography and idetified as a carboxylic acid on many kinds of determination test. The preparation scheme of the sample was presented in Fig. 1.

Culture filtrate
↓
Dowex MS 60
↓
Eluted with 5N Na OH
↓
Effluent
↓ pH to 1.0 with HCl
Extracted with EtAc
↓
Dried over anhyd. Na_2SO_4
↓
Evaporated in vacua
↓
Crystallized in hot water
↓
Sample (ISV-33)

Fig. 1. Preparation Procedure of the Sample.

Venom

The venoms used in this experiment were Agkistrodon blomhoffi brevicaudus, Agkistrodon sxatilis, Agkistrodon calginosus, Agkistrodon halys blomhoffi (Japanese Mamushi), Trimeresurus (Okinawa Habu) and Trimeresurus okinavensis. The acetone dried venoms of Agkistrodon halys blom-

hoffi, Trimesurus flavoviridis and Trimeresurus okinauensis were obtained from The Japan Snake Institute and other 3 kinds of Agkisirodon venoms were collected in our laboratory by milking from the south Korean snakes and desiccated over sulfuric acid without any purification.

Fractionation of Agkistrodon halys blomhoffi venom

A 70mg of Japanese mamushi venom was dissolved in 1.5m*l* of 0.3% sodium chloride solution which was adjusted to pH 7.0 with sodium hydroxide and applied on a Sephadex G-100 column (1.8×140 cm). Elution was carried out with 0.3 % sodium chloride solution (pH 7.0). Elution speed was 1 m*l* per 4 minutes and fraction volume was 3 m*l*. And all operation was carried out at 25°C. The fractions were collected and than its optical density was measured at 280 nm. In the result, eight peaks were observed. Proteinase activity and hemorrhagic action of the peaks were determined as previously described in methods.

Substrate

Hammarstein milk casein(E. Merck), egg albumin and hemoglobin (Difco) were used in this experiment.

Determination of the inhibitory action of the sample on the venom proteinase

The proteinase activity of snake venom was determined by the Folin-Ciocalteu method[35] adopted for the measurement of aromatic amatic amino acid liberated by the proteinase reaction. The mixture was composed fo 1/15 M sodium phosphate buffer (pH 7.0) 0.5 m*l*, 1 % casein 0.3 m*l*, venom solution 0.1 m*l* (50-150 μg). After incubation for 30 minutes at 37°C. the amount of aromatic amino acid released in the reaction solution was determine spectro-photometrically at 660 nm after developing blue color with the Folin reagent. The inhibitory activity was determined by the addition of the sample solution 0.1 m*l* to the reaction mixture. The ratio of the inhition was estimated with the following equation: the ratio of the inhibition(%),

$$I=100\left((1-\frac{S-C_2}{C_1-C_2}\right).$$

C_1 : optical density of the reaction solution from venom and substrate

C_2 : optical density of the reaction solution from sample and substrate

S : optical density of the reaction solution from sample, venom and substrate

Inhibitory test of the hemorrhagic activity of venom

White rabbits weighing average 1.5 kg were subcutaneously injected with various amounts of venom and sample in 0.2 m*l* of solution of isotonic saline-buffer, after pretreating the venom-sample mixture for a given time at 37°C. The venom in the same saline-buffer solution (0.2 m*l*) was injected as a reference after pretreatment as mentioned above. The rabbits were sacrificed aften 2 hours, the skin was removed and measured with the diameter of each hemorrhagagic spot appearing at the inner side of the skin.

Determination of the inhibitory action of the sample on the venom lethalcity *in vitro*

One mg of the Agkistrodon blomhoffi brevicaudus venom and 0.5 mg of the sample were dissolved in 5 m*l* of 1/15 M phosphate buffer (pH7.0), and the mixture was incubated for 15 minutes at 37°C. The remaining lethal toxicity of thf venom was detected by the intraperitoneal administration to the mouse with each amount of 50 μg and 100 μg of the venom respectively. The sample-free venom solution was also administrated as a reference after treatment for 15 minutes at 37°C as above.

Determination of the inhibitory action of the sample on lethalcity of venom *in vivo*

In order to investigate the sample as an inhibitor to the snake venom *in vivo*, the mice (20-23 g) were artificially envenomated with each given amount of 30 μg to 100 μg of Agkistrodon blomhoffi brevicaudus venom. After 5 to 30 minutes of envenomation the sample wat injected and the resulting lethalcity was observed. The injected sample solution was prepared by dissolving 1 mg of sample and 55 μg of prednisolon in 0.3 m*l* of saline. The venom and sample were intraperitoneally administrated separately.

Paper chromatography

The purity of the sample was determined by the

paper chromatography. The developing solvent systems were the mixture of n-butanol:acetic acid: water(4 : 1 : 5, v/v) and n-butanal: formic acid: water (8 : 3 : 2, v/v). After development, the paper strips were sprayed with pH indicator(BPB) for spot detection.

Determination of molecular weight of the sample

Molecular weight of the sample was estimated by the method of Raoult's molecular elevation of boiling point. The elevation of boiling point of the aqueous solution (553 mg/25 *ml*) of the sample was determined and calculated by the Raoul's equation;

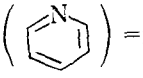
$$\Delta Ts = \lambda s \frac{W}{M}.$$

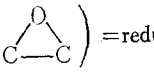
Determination of functional groups of the sample

The functional groups of the sample were determined by the following methods.[36,37]

Carboxy radical (−COOH)=iodide-iodate mixture test and sodium-bicarbonate mixing test

Amino radical ($-NH_2$)=ninhydrin test

Pyridine ring  =polymethine dyes formation test

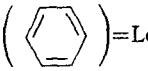
Epoxy group  =reduction of periodic acid process

Chlorine (Cl)=sodium fusion and silver nitrate test

Unsaturated bond=potassium permanganate absorption test

Nitroso group (N=O)=Lieberman test

Carbonyl group(C=O)=bisulfite interaction test

Aromatic ring  =Le Rosen test

IR, UV and NMR spectrum

Infrared spectrum was recorded with a Shimazu IR-430 spectrometer. Ultraviolet spectrum was recorded with a Hitachi-Elmer 137 UV-spectrophotometer. NMR spectrum was measured at 60 and 100 MHz with Japan Electron Optic by using DMSO as a solvent and TMS as a internal reference.

Preparation of derivatives of the sample

As the sample was found to be a carboxylic acid, the following derivatives were prepared.

A) Amide: The amide of the sample was prepared by the reflux of 300 mg of the sample with 14*ml* of thionylchloride for 2 hours and 40 minutes, then the reaction mixture was cooled in ice bath. The excess of thitonylchloride was decomposed by the addition of 60 *ml* of concentrated ammonium hydroxide at ice cold. The formed amide was collected by the filtration and dissolved in the ammonium hydroxide alkaline water and extracted with ethylacetate, and an amide crystal was obtained by the evaporation of this extract in vacuum, after drying over anhydrous sodium sulfate.

B) Methyl ester: The methyl ester of the sample was prepared by the reflux of 300mg of the sample and 1.7 *ml* of concentrated hydrochloric acid with 50 *ml* of dry methanol for 3 hours. After removing the excess of methanol by evaporation in vacuum, 10 *ml* of water was added to the oily residue. And then the pH of the aqueous phase adjusted to 9.0 with sodium hydroxide, the formed methyl ester was extracted with ether. The ether layer was separated and dried with anhydrous sodium sulfate, and the light brownish oily product of methylester was finally obtained by the evaporation of ether in vacuum.

Toxicity test of the sample

Albino mice weighing about 20 g were used in order to test the toxicity of the sample. The sample in the isotonic saline-buffer solution was injected intraperitoneally, and observed the acute aymptoms of the mice.

Results

Inhibitory action on the venom proteinase with various concentration of the sample

The Agkistrodon halys blomhoffi and Trimeresurus flavoviridis venoms were used in this experiment. The sample and venom mixture was preincubated for 5 minutes at 37°C, and then the casein solution was added to the mixture and incubated for 30 minutes. The reaction solution was colored by the Folin-Ciocalteu method. If the venom was treated with the sample in vitro the proteinase

Table 1. The Inhibitory Action of the Sample on Several Kinds of the Snake Venom Proteinases.

Venom	Inhibition ratio (%)
Trimeresurus flavoviridis	85
Trimeresurus okinavensis	88
Agkistrodon halys blomhoffi	87
Agkistrodon blomhoffi brevicaudus	90
Agkistrodon sxatilis	56
Agkistrodon caliginosus	91

Each concentration of the venom and the sample in the reaction solution were both 50 μg, and the incubation was held for 30 minutes at 37°C. The substrate was casein.

Effect of substrate concentration on the inhibitory activity of the sample to venom proteinase

The mixture of the sample and each concentration (0.1—2.0 %) of casein solution was preincubated for 5 minutes at 37°C, and then Agkistrodon blomhoffi brevicaudus venom was added to react with the mixture for 10 minutes. But each concentration of the substrate did not almost affect the ratio of the inhibitory activity of the sample to venom was used in this experiment.

Fractionation of the Japanese Mamushi venom

Fractionation of Agkistrodon halys blomhoffi (Japanese Mamushi) venom by Sephadex G-100 column chromatography resulted in eight peaks.

Table 2. The Fractionation of Agkistrodon halys blomhoffi Venom with Sephadex G-100 Column Chromatography

Peak No.	Activity	Total relative activity of proteinase	Hemorrhagic activity* (cm)	Inhibitionratio with sample	
				Proteinase** (%)	Hemorrhagic***
1	Proteinase hemorrhagic	48.0	3.0×1.5	100	0.3×0.2
2	Proteinase hemorrhagic	84.0	5.0×2.5	84	0.2×0.2
3	Hemorrhagic		0.5×1.0		—
4	Proteinase hemorrhagic	148.2	0.3×0.8	67	—
5	No any activity				
6	Proteinase	61.3		100	
7	No any activity				
8	No any activity				

* A 0.2 ml of the highest UV absorbing fraction was injected to the rabbit intracutaneously and the hemorrhagic spot was indicated.

** A 100 μg of the sample was added to the each reaction system.

*** The hemorrhagic fraction as above mentioned was treated with 300 μg of the sample, placed for 15 minutes at 30°C, and then injected.

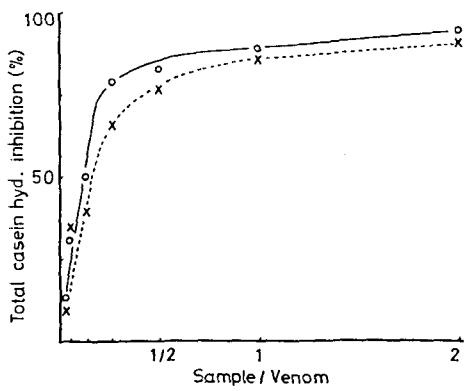


Fig. 2. The Inhibitory Effect of the Sample Concentration on the Venoms of Agkistrodon halys blomhoffi and Trime resurus flavovirdis.

The concentration of the venom in each reaction mixture was 50μg.

o—o; Agkistrodon halys blomhoffi: x—x; Trimeresurus flavoviridis

activity of the venom was remarkably decreased. The proteinase activity was found in the 1st, 2nd, 4th and 6th peak and a total of the relative activity of each peak was 48.0, 84.0, 148.2 and 61.3 respectively.

The inhibitory activity of the sample to the pro-

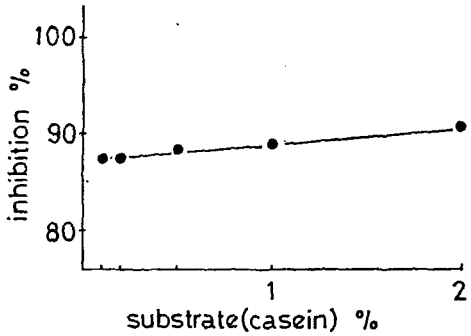


Fig. 3. Effect of Casein Concentration on the Inhibitory Activity of the Sample to Agkistrodon blomhoffi brevicaudus Venom Proteinase.

Each 150 μg of the sample and venom was added in the reaction mixture and the mixture was treated for 10 min. at 37°C.

teinase of each peak was investigated. Proteinase of the 2nd and 4th peak was inhibited 84% and 67% respectively but the 1st and 6th peaks were inhibited completely with the sample. The hemorrhagic activity was detected in the 1st, 2nd, 3rd and 4th peak, but compared with the 1st and 2nd peak, the 3rd and 4th peak were very weak. And the strong inhibitory activity of the sample to the hemorrhagic activity of each peak was obtained. The peaks 7th and 8th did not give any activity of proteinase or hemorrhage.

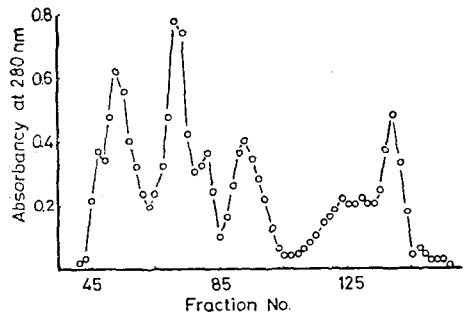


Fig. 4. The Fractionation of Agkistrodon halys blomhoffi Venom with Sephadex G-100 Column Chromatography.

A 70 mg of the venom was applied on 1.8×140 cm column and eluted with 0.3 % NaCl solution. Elution speed and fraction volume were 1 m*l* per 4 min. and 3 m*l* volume each.

Inhibitory activity of the sample on hemorrhagic action of venom

To investigate the inhibitory effect of the sample on the hemorrhagic action Agkistrodon halys blomhoffi, 0.1m*l* of sample solution (300μg) was added to 0.1 m*l* of venom solution (300 μg) then the mixed solution was preincubated for 0 to 20 minutes at 37°C, and then 0.2 m*l* of the treated solution was injected subcutaneously to white rabbit. And other hand, the venom solution without the addition of sample, treated as above was injected as a reference. Then, the diameter of the hemorrhagic spot appeared was measured after 4 hours of injection. As the result, the hemorrhagic activity of the venom was decreased by preincubation with the sample, and it was completely inactivated by 20-minute preincubation. Also, the inhibitory effect on hemorrhagic action was investigated to other snake venoms; Agkistrodon blomhoffi brevicaudus, Agkistrodon sxatilis and Agkistrodon calginosus. After 200 μg of venom and the same amount of sample were preincubated for 10 minutes at 37°C, their injection were performed as the case of Agkistrodon halys blomhoffi. The diameter of the hemorrhagic spot appeared was measured. The sample was effective to the inhibition of hemorrhagic action of Agkistrodon blomhoffi brevicaudus and Agkistrodon sxatilis, but not effective to the venom of Agkistrodon caliginosus.

Table 3. Inhibitory Activity of the Sampie on the Hemorrhagic Action of Agkistrodon halys blomhoffi Venom.

Reaction time	Venom only	Venom pulus sample
0 min.	4×4.5 cm	1.8×1.6 cm
5	—	1.3×1.5
10	3.8×3.7	1.2×1.4
15	—	1.2×1.0
20	3.2×3.5	0

Each 300 μg of venom and sample was used.

Inhibitory action of the sample on the lethalcity of the Agkistrodon blomhoffi brevicaudus venom in vivo

The sample solution mixed with prednisolone

Table 4. Inhibitory Activity of the Sample on the Hemorrhagic Action of Some Agkistrodon Venoms.

Venom	Venom only	Venom plus sample
Agkistrodon blomhoffi brevicaudus	9.0×9.0 cm	0.5×1.0 cm
Agkistrodon sxatilis	5.0×6.0	1.0×1.0
Agkistrodon caliginosus	9.0×6.0	8.0×4.0

Each 200 μg of venom and sample was used. Venom and venom-sample mixture were preincubated for 10 minutes ai 37°C.

(55 μg of prednisolone and 1 mg of sample/0.25 ml of saline). were administrated by intraperitonal injection to investigate the as an inhibitor to the lethalcity of Agkistrodon blomhoffi brevicaudus venom *in vivo*. In experiment, the mice were previously envenomated and after a given time the mixture of the sample and prednisolon was injected. And when only 50 μg of the venom was

Table 5 *In Vivo*, Inhibitory Action of the Sample on the Agkistrodon blomhoffi brevicaudus Venom

	Venom μg/0.2 ml	Only prednisolone 55 μg	Sample 1000 μg/0.25 ml Prednisolone 55 μg and	Injection interval (min.)	Result
Reference	30	—	—	—	X O O O O
	40	—	—	—	X X O O O
	50	—	—	—	X X X X X
	50	+	—	5	X X X X X
	100	—	—	—	X X X X X
Sample	50	—	+	5	O O O O O
	50	—	+	10	O X X X X
	50	—	+	30	X X O O O
	100	—	+	5	X O O O O
	100	—	+	10	X X X X X

In this experiment, 5 mice were used in each group. X ; dead in 8 hrs., O ; alive

Table 6. Inhibitory Action of the Sample on the Lethalcity of the Venoms with *in Vitro* Pretreatment. The venom solution and venom-sample mixing solution were pretreated for 15 minutes at 37°C, then injected to the mouse.

Venom	Reference group	Sample group	
	Venom 50 μg only	Venom 50 μg plus sample 25 μg	Venom 100 μg plus sample 50 μg
Agkistrodon blomhoffi brevicaudus	××××	○○○○	×○○○
Trimeresurus flavoviridis	××××	○○○○	×○○○

× ; dead within 10 hours
○ ; alive normally
In this experiment, 4 mice were used in each group.

Table 7. Effect of the Kinds of Substrates on Inhibitory Activity of the Sample to the Venom Proteinase. The concentration of casein, egg albumin and hemoglobin in the reaction were 0.6 %, 1.0 % and 0.9 % respectively.

Venom	Inhibition ratio (%)		
	Casein	Egg albumin	Hemoglobin
Agkistrodon blomhoffi brevicaudus	87	86	87
Trimeresurus flavoviridis	85	77	95

Each concentration of sample and venom in the reaction mixture was 255 μg, and reaction condition was for 30 minutes at 37°C.

Table 8. Effect of the Sample on the Enzymes not Related to the Venom

Enzyme	Related metal	Final concentration of sample ($\mu g/ml$)	Inhibition ratio(%)
Succinic dehydrogenase*	Fe	100	0
Catecholase**	Cu	23	8
Blood coagulating enxyme***	Ca	100	0
Bacterial protease****	Zn?	100	76.7
Trypsin	—	200	25
Alpha-chymotrypsin	—	50	0
Pepsi	—	400	0
Rhizopus gluc-amylase	—	50	0
Papain	—	200	(activated 200 %)

* ; Mouse liver cell was used as enzyme source.
** ; Fresh apple juice was applied as enzyme solution.
*** ; Coagulation time of rabbit blood was measured.
**** ; This protease was produced by *Streptomcyes* sp. isolated from soil in our laboratory and the enzyme was inactivated by EDTA and reactived by the addition of Zn ions in the reaction solution.

injected all mice were dead in 8 hours, but when the sample solution mixed with prednisolone was administrated after 5 minutes of envenomation, the all mice tested were survived and the detailed results were shown in table 5. Also, when the pretreated venom solution with the sample in test tube was applied to mouse, the lethalcity of the venom was decreased remarkably as shown in table 6.

Substrate effects on the inhibitory activity of the sample

Inhibitory action of the sample on the venom proteinase of Agkistrodon blomhoffi brevicaus and Trimeresurus flavoviridis was also observed using some kinds of substrates, such as 0.6 % casein, 1.0 % egg albumin and 0.9 % hemoglobin which was denatured by Anson method, the sample was not affected by any kind of substrates and the inhibition ratio on the venom of Agkistrodon blomhoffi brevicaudus was 87 %, 86 % and 87 %, and on the venom of Trimeresurus flavoviridis the inhibition ratio was 85 %, 77 % and 95 % respectively.

Effect of the sample on other enzymes

Because the sample acts as an inhibitor on the venom proteinase and hemorrhagic factor from various kind of snakes, the sample was applied as the inhibitor to other kinds of enzyme. The venoms used in this experiment contain some metals in itself. And on the other hand, the sample has a good probability to chelated with the metals. With these reasons, the metal-containing enzymes and the enzymes that need metal ions essentially on its reactions were also accepted to investigate the effect of the sample. On the obtained resultes, the enzyme containing metals in the molecule were not always inactivated with the sample. But a protease originated from bacteria, which inhibited by EDT A, was nactivated markedly by the enzyme, trypsin, ialpha-chymotrpsin, pepsin, succinic dehydrogenase, glucoamylase, catecholate, bacterial neutral protease and papain were submitted in this experiment.

Effect of metal salts on the activity of the sample

In order to investigate the effect of metal salts on the inhibitory activity of the sample to venom proteinase of Agkistrodon blomhoffi brevicaudus, varius kinds of metal salts were added to the reaction mixture at various concentration. The $PbCl_2$, CoAc, $MnCl_2$, $FeSO_4$, $AgNO_3$ and $CaCl_2$ did not affected the inhibitory activity of the sample but $ZnCl_2$ and $CuSO_4$ exhibited the strong repressing effect to the sample and CoAc only repressed at high concentration.

Thermal stability of the sample

A 8 mg of the sample was dissolved in 8 m*l* of

Table 9. Effect of Metal Salts on the Sample Activity

Salt \ M/S*	0	1/4	1/2	1
$AgNO_3$	85	60	60	60
$CaCl_2$		60	60	55
$CuSO_4$		20	20	15
$CoAc_2$		83	65	35
$FeSO_4$		85	85	90
$HgCl_2$		65	60	55
$MnCl_2$		77	77	65
$PbCl_2$		85	85	85
$ZnCl_2$		0	0	0
$ZnSO_4$		0	0	0

On the reaction, 100 μg of sample and 100 μg of Agkistrodon blomhoffi brevicaudus venom an a protenase were used, and the numbers of this table indicate the % of inhibition by the sample against the proteinase of venom withe presence of metal salt in the reaction system.

* ; $M/S=\frac{\text{amount of salt}}{\text{amount of sample}}$

M/15 phosphate buffer solution (pH 7.0) and the solution was treated at 100°C for a given time. After the treatment, the remaining avtivity of the sample solution was detected using the proteinase of the Agkistrodon blomhoffi brevicaudus venom. The activity was only decreased about 4 % by the heat treatment for 120 minutes against the untreated sample solution.

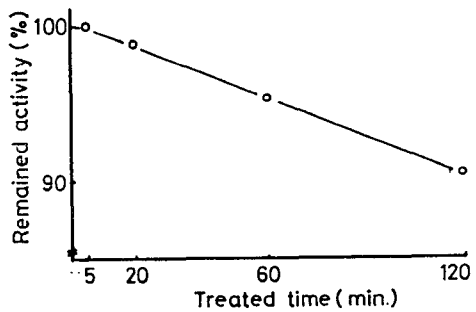


Fig. 5. Heat Stability of the Sample.
The sample solution (100 μg/m*l*) was pretreated at 100°C for the given time. To the reaction mixture, 100 μg of the pretreated sample and 75 μg of venom were added.

pH stability of the sample

The remaining activity was estimated, after the sample solution (500 μg/m*l*) was treated in pH range 7.0 to 13.0 for 60 minutes at 60°C. The actvity was decreased only 8 % at pH 13.0 against non-

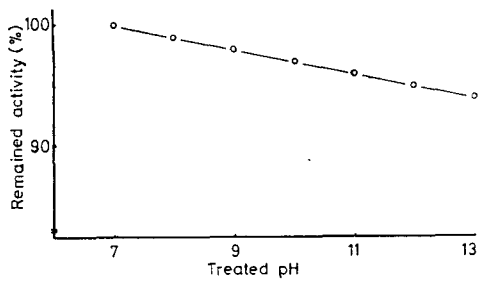


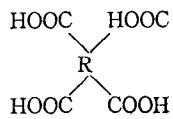
Fig. 6. pH Stability of the Sample.
The sample solution (100 μg/m*l*) was pretreated for 60 min. at 70°C in the given pH, and 100 μg of the sample and 75 μg of the venom were added to the reaction mixture.

treatment. The sample was very stable at the neutral and the alkaline condition. At the acidic condition, the sample was not examined because of the acidic substance in itself.

pH titration of the sample

A 22.0 mg of the sample was dissolved in 10 m*l* of hot water and this solution was titrated with N/50 sidium hydroxide solution using pH meter. The resulted titration curve indicated pK_1 and pK_2, as 3.3 and 5.1 respectively. In this titration, 22.0 mg of the sample was neutralized with 9.89 mg of sodium hydroxide. If the molecular weight of this sample is confirmed as 392.283 $-C_{16}H_{12}N_2O_{10}$, a 8.97 mg of sodium hydroxide will be consummed to neutralize 22.0 mg of this sample, and this value gives the 4 equivalent of carboxylic acid radical to be arranged on one molecule of the sample. Consequently, this sample was estimated as tetra carboxylic acid.

For example:



$R=C_{12}H_8N_2O_2$

In this titration curve, the 4 carboxylic radicals were conformed in two kinds of very similar structural conformation.

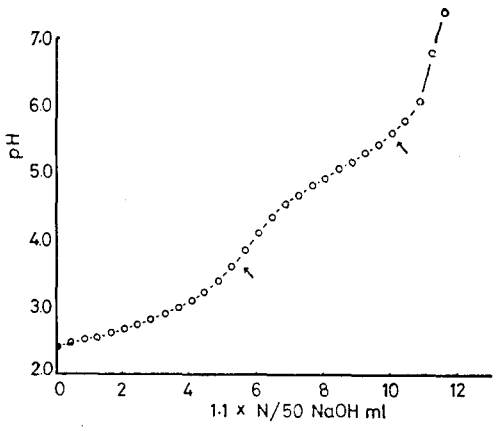


Fig. 7. pH Titration Curve of the Sample.
A 22 mg of the sample was titrated with N/50 sodium hydroxide solution and the obtained pK_1 and pK_2 were 3.3 and 5.1.

Paper chromatograpy

Paper chromatography was used for the determiantion of the samplepurity. Adequate amount of the sample was spotted on the Toyo filter paper No. 52, which washed twice with the developing solvent, and developed with the solvent system as previously described in methods. After dried completely, the paper gave only one spot on both solvent systems.

Table 10. The Rf Value of the Sample on Paper Chromatography

Sample	n-Butanol 4 v/v Acetic acid 1 Water 5	n-Butanol 8 v/v Formic acid 3 Water 2
Free acid	0.62	0.66
Na salt	0.47	0.74

Toyo filter paper No. 52 was used.

Melting poing of the sample

Melting point of the sample was determined using electro hot plate type equipment. The sample was sublimated at 222°C without any decomposition.

Chemical composition of the sample

The sample completely dried at 110°C was submitted to the chemical analysis. The obtaining results were C; 50.2, H; 3.18, N; 8.08. With this value the chemical formula was estimated as $(C_8 H_6 N O_5)n$. To obtain the compositional ratio, we analyzed the sample twice and in both case we obtained same results. And with the result of titration, the chemical formula of the sample was decided as $(C_8 H_6 NO_5)_2$.

Determination of molecular weight of the sample

In this experiment, the elevation of the boilling point was read as 0.03°C, and from this value the molecular weight was calculated as 383.413 by Rolt's equation. And the analyzed composition of the sample was given as $(C_8 H_6 N O_6)n$. On this chemical formula, the n was estimated as 2 by the detected molecular weight on the above experiment. The theoretical molecular weight as $C_{16} H_{12} N_2 O_{10}$ was 392.283.

Test of functional groups of the sample

Of the functional groups of the sample presence of carboxylic radical was revealed by the iodide-iodate mixture method and by the sodium bicarbonate mixing test according to the liberation of carbon dioxide gas by the bicarbonate alkaline soltion. Also we recognized the presence of epoxy group in the molecule of the sample by the reduction of periodic acid process. Then the amino radical, pyridine ring, aromatic ring, carbonyl group. nitroso group, unsaturated bond and chlorine element were not identified in the sample.

Table 11. The Functional Group of the Sample.

Carboxylical	+
Epoxy group	+
Amino radical	—
Pyridine ring	—
Aromaiic ring	—
Carbonyl group	—
Nitroso group	—
Unsaturated bond	—
Chlorine element	—

+ ; identified
— ; not identified

UV spectrum of the sample

The UV spectrum of the sample showed that the sample (in water) has a maximum absorption

at nm as shown in fig. 8.

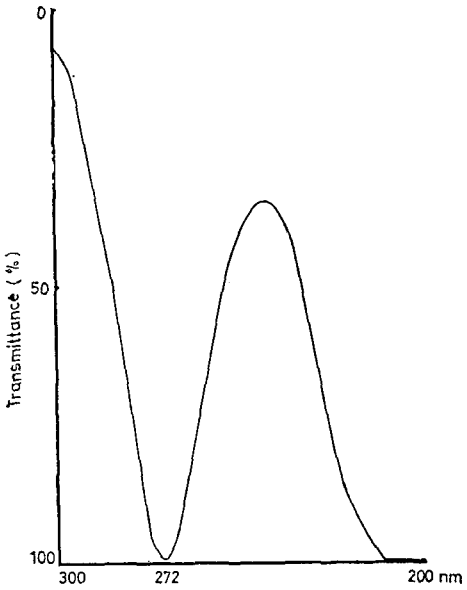


Fig. 8. The UV Spectrum of the Sampl.

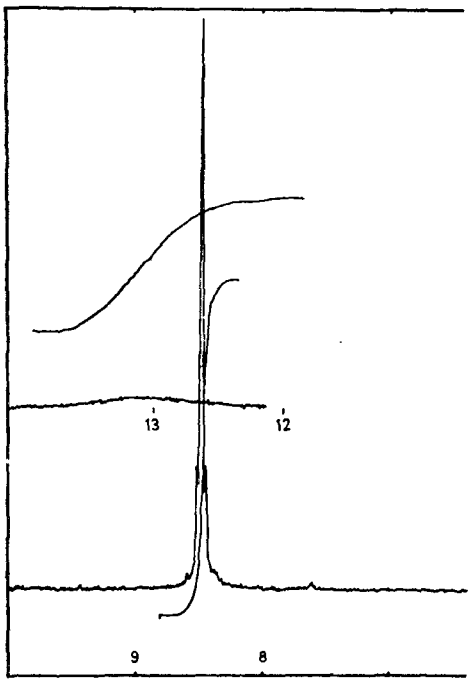


Fig. 9. The NMR Spectrum of the Sample.
The sample was measured at 100 MHz and DMSO and TMS were used as a solvent and internal reference.

NMR spectrum of the sample

On NMR spectrum, a broad carboxylic hydrogen stretch was observed at 13 ppm and a very strong

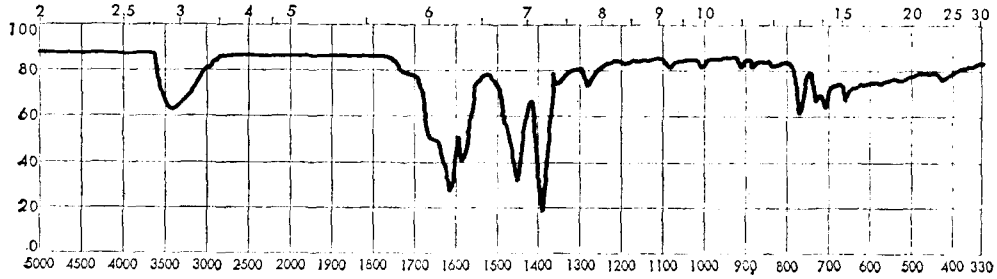


Fig. 10. The Infrared Spectrum of the Free Acid Type of Sample.

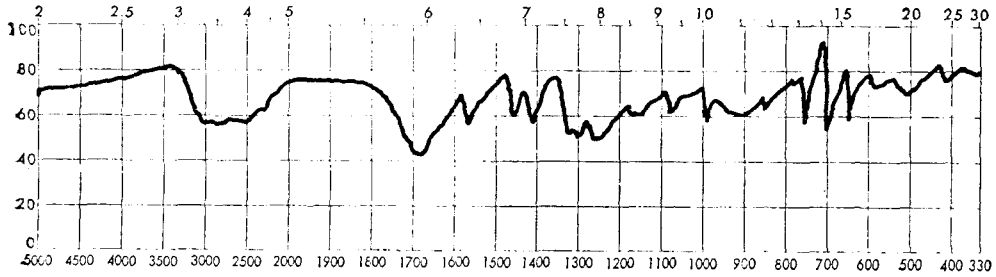


Fig. 11. The Infrared Spectrum of the Sodium Salt of Sample.

triplet stretch was appeared at 8.5 ppm but on this stretch we do not have any significant confidence. But the strength of hydrogenic proton ratio was detected to about 2 : 5 at 13 ppm and 8.5 ppm respectively, also the peak of 8.5 ppm indicated that the absolutely homogeneous type of hydrogens were formulated in the sample molecule except for carboxylic hydrogens.

Infrared spectrum of the sample

Infrared spetrum of the sample of the free acid exhibited the presence of the stretch of epoxy group (850, 1260), C—N—C—C (1150), cyclo hexane ring (1460), C=O (1280-1330, 1680), O-H from carboxylic and C-H (2500-3000), and C-O-C (920). The spectrum of sodium salt of the sample also indicated the presence of epoxy group, C—N—C—C, cyclo hexane ring and C-O-C stretches but the carboxylic O-H stretch was almost disappeared at 2500-3000 nm. The resultes gave the strong confidence about the chemical structure of the sample described in the abstract.

Inhibitory activity of the derivatives of sample on venom proteinase

The derivatives of the sample, such as methyl ester and amide did not have an inhibitory activity on the proteinase, hemorrhagic and lethal factors of the Agkistrodon venoms, but the sodium, copper and magnesium salts reacted as an inhibitor on the venoms as much as original free acid type of the sample. In this experiment, the obtained derivatives of the sample, methyl ester and amide were not identified chemically. Only we estimate the ester and amide to be formed during the preparation described in methods.

Toxicity of the sample

The mouse weighing 20 g was used to investigate the toxicity of the sample. we found that the intraperitoneal administration of 200 mg of the sample (as neutral salt) per kg of body weight did not cause any acute toxicity. In this experiment five mice were used.

Discussion

A biologically active substance which had a strong inhibitory activity on proteinase of Agkistrodon and Trimeresurus venoms was produced by one strain of *Penicillium* sp. isolated from soil. This substance also inhibited hemorrhagic factor of Agkistrodon halys blomhoffi, Agkistrodon blomhoffi brevicaudus and Agkistrodon caliginosus venoms at *in vivo* and *in vitro* reaction which distinguished this substance from other chemical inhibitors on its specificity.

In spite of many results obtained from various tests, the fine structure of the sample was not elucidated, but the sample seemed to have 4 carboxylic acid radicals of its molecte as shown in the abstract. Generally polycarboxylic acid is known to inhibit metal enzyme by chelating effect. We think that the venoms were inactivated by chelating with carboxylic acid of this substance, because snake venom contains metals. To investigate whether the inactivation of the venom was due to chelating of this substance or not, this substance was applied with some other enzyme -succinic dehydrogenase, catecholase, blood-coagulating enzyes as metal enzyme, and gluco-amylase, papain, pepsin as non-metal enzyme. But it chelated none of above enzymes. Therefore, we could think that this substance acted with snake venom proteinase, hemorrhagic and lethal factores, especially without any chelation.

In *vitro* this sample was very effective to detoxicate snake venom incubated with the sample for 15 minutes at 37°C, and also effective even in the sample administration after 5 to 30 minutes of envenomation of mouse. In our unpublished data, this substance showed *in vivo* detoxication of snake venom equivalent to five-fold of MLD_{100}.

In the sample injection, moreover, addition of prednisolone to the sample was more effective to detoxicateenvenamated mouse. Free acid type of the sample was more effective tha n sodium ro magnesium salt type *in vivo*. It is known that injected venom remained around the injected tissue area for a fairly long time; then, the free acid type of the sample was more difficult to spread than the salt type of the sample, so that long retention at high concentration around the injected area led to a strong inhibiton. The maximum in-

hibition ratio to the venom proteinase was obtained when the sample used was equivalent to one fourth to half amount of the venom *in vitro*. But to obtain the effective action of the sample on the lethalcity of the venom was required the venom was required the sample of 10 to 20 times of the venom amount *in vivo*.

The chemical formula of this substance was found to be $C_{16}H_{12}N_2O_{10}$ by the chemical experiments and its molecular weight of about 392 coincided with experimental calculation of boiling point elevation. One molecule of the sample was found to have four carboxylic acid radicals by alkaline titration, and the curve showed only pK_1 and pK_2. Therefore this sample was belived to be a dimer with 2 kinds of the carboxylic acid radicals on the conformation of the sample molecule; $(C_8H_6NO_5)n$, $n=2$.

The complete structure of the sample was almost impossible to elucidate with IR, UV, NMR and Mass spectra owing to its high molecular weight. As the results obtained from the above data, it is found not to be an aromatic compound or an unsaturated substance with less hydrogen than carbon as shown in the chemical formula. Consequently we could conclude that it was framed as cyclo hexane. Therefore it has no unsaturated bond. Its perfect structure will be reported later.

In order to investigate its chemical properties, various tests were also carried out. It is not decomposed at the temperature as high as as 200°C or more, but sublimed. And then its acivity was not lost even by heat treatment at 100°C in aqueous state. That showed it had a very stable molecular structure chemically, It was dissolved only in water, but hardly dissolved in organic solvents. But, even in water, it could dissolved at the boiling temperature, and its solubility decreased sharply at room temperature. So we could determine its molecular weight only by the method of boiling point elevation in water.

Its activity was much depressed by zinc and cupric ions. The *Penicillium* sp. producing this sample was isolated from soil to get an inhibitor on venom proteinase, necrotic, hemorrhagic and lethal factors. About 50 to 80 mg of the crystalline sample was obtained from one liter of culture broth. But this strain also has unstable producility as well as other microorganisms producing a biologically active substance. So we will have to pay much attention to the single spore isolation to keep its high producibility of this strain, for successive transplanting on various media may cause to decrease its producibity remarkably.

Acknowledgement

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要 約

蛇毒은 蛋白性物質이며 그 作用은 酵素的이다. 動物에 對한 强力한 作用은 毒蛇咬傷의 治療에 있어서 重要한 問題點을 가지고 있다.

蛇毒에 對한 抗血淸의 開發은 오래 前부터 이루어 졌으며 近年에 와서는 거의 完成된 狀態라고 생각한다.

그러나 이러한 형편에서도 抗血淸에 지니고 있는 몇가지의 缺點으로 因하여 有効한 化學治療劑의 開發이 要求되고 있다. 그러나 蛇毒의 多樣性을 考慮할 때 劃一的인 治療劑를 얻기란 매우 困難할 것으로 생각된다.

蛇毒을 化學物質로써 不活性化 시키려는 試圖는 많아서 chelate 性 物質 thio 化合物, 或種의 Amino acid, Tannic acid 등 蛋白質 凝固劑 등 試驗管內에서는 强力히 作用하는 物質이 많이 發見되었으나 이들의 作用은 蛇毒에 對해서 非特異的이며 또한 生體內에서의 作用이 弱하여 그의 利用에 많은 制限이 있다. 著者들은 微生物로부터 蛇毒에 對한 阻害物質의 檢索을 試圖했던바 *Penicillium* 屬의 한 菌株로 부터 有効한 物質을 얻을 수 있었으며 이것을 ISV-33 이라 命名하였다. 이 物質은 Agkistrodon 및 Trimeresurus 類 蛇毒의 Proteinase 와 出血

因子 및 致死因子에 잘 作用하여 이것들을 不活性化시키나 一般酵素에는 거의 作用하지 않았다.

이 物質은 蛇毒에 對해서 $\frac{1}{4}$~$\frac{1}{8}$ 量으로서 充分히 作用하나 그 自體의 毒性은 쥐를 對象으로 實驗했을 때 거의 나타나지 않는다. 또 이 物質은 蛇毒에 對해서 特異性이 强하며 *in vivo* 에서도 作用한다. 이 物質은 結晶性이며 熱水에는 容易하게 溶解하나 冷水나 各種 有機溶媒에는 거의 溶解하지 않으며 단지 Ethylacetate 에는 약간 溶解한다. mp는 222°C 에서 昇華하며 分子量 約 392 인 Tetra carboxylic acid로 同定되었다. 分子式은 $(C_8H_6NO_5)_2$ 라고 認定되며 이 物質은 Polycarboxylic acid 이므로 chelate 作用이 있을 可能性이 있으나 金屬酵素에 對해서는 一律的으로 作用하지 않음으로 蛇毒의 不活性化에 있어서 chelate 作用에 因한 것은 아니라고 생각된다.

그 構造式은 아직 確定하지는 못하였으나 지금까지의 實驗結果로서 cyclo hexane 을 骨格으로 하고 여기에 Epoxy group 와 carboxyl 基가 있는 것으로 推定된다. 또 이 物質은 酸, Alkali, 加熱등에 對해서 매우 安定한 것으로 나타났다.

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