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Inhibitory Substance Produced by *Aspergillus* sp. on the Snake Venom Proteinase
— Isolation of Microorganism and Biological Activities of the Inhibitor —

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Aspergillus 屬 菌株가 生成되는 蛇毒 Proteinase에 對한 沮害物質
— 菌의 分離 및 沮害物質의 生物學的 作用相 —

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***Aspergillus* sp. (MK-24) producing a biological active substance that inhibited the venom proteinase activity was isolated from soil. The substance also inhibited the activity of trypsin and coagulation of blood, but did not inhibit papain, α-chymotrypsin and pepsin. The substance was partially purified from culture filtrate by precipitaion with acetone, and by chromatography of DEAE-Sepadex A-50 column and Amberlite IRC-50 ion exchange. The inhibitory substance was stable in the wide pH range from 2.0 to 12.0 at 37°C, but not stable at 65°C in the alkaline pH. Only 12% of the activity was decreased by the heat treatment at 100°C for two hours. The inhibition on venom proteinase *(Agkistrodon bromohoffi brevicaudus)* was a mixed type. The inhibitory activity depended on the preincubation time and completely depressed by cupric, zinc and cobalt ions. The inhibition on the venom proteinase was appeared strongly on casein but not on ovalbumin or hemoglobin as a substrate.**

Approximately 300 species among nearly 2000 species of snake are known to be venomous (1-3). Thus many studies have been devoted to the inactivation and mechanism of snake venoms. For the inactivation of snake venoms, the immunological antiserum and chemical reagents such as steroid compounds, chelating compounds (7-10), formalin (11), were applied. But these chemicals are unspecific inhibitors. Recently a specific inhibitor (12, 13) was isolated from a *penicillum* sp. 175-68. In this work, another specific inhibitor was isolated from a *Aspergillus* sp. MK-24, and its stabilities and characteristics were investigated.

Materials and Methods

Isolation of the strain

One strain of *Aspergillus* sp. MK-24 was isolated from soil source and cultivated in a medium containing 2% glucose, 0.3% $NaNO_3$, 0.02% KCl, 0.02% K_2HPO_4, 0.02% $MgSO_4 7H_2O$, pH 5.0 for 7 days at 30°C

Preparation of the sample

Aspergillus sp. MK-24 was inoculated in 200 ml medium at 30°C for 14 days as standing culture. The culture fluid was precipitated by acetone. Next the precipitant was eluted through Activated carbon column, Sepadex G-25 column, DEAE-Sepadex A-50 column with 0.1 M-KCl and Amberite IRC-50. The inhibitor was recovered with methanol precipitation to make as an amorphous

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powder.

Vonom

The venoms used in this experiment were *Agkistrodon bromohoffi brevicaudus, Agkistrodon saxatilis, Agkistrodon calginosus, Agkistrodon halys bromohoffi and Trimeresurus flavoviridis.*

Three kinds of *Agkistrodon* venoms from south Korean snakes were collected by milking and desiccated in my laboratory and the last two venoms were obtained from the Japan Snake Institute, Gunma, Japan.

Enzymes and chemicals

Trypsin, α-chymotrypsin, papain, pepsin, ovalbumin and hemoglobin were purchased from Difco; α-amylase, β-amylase and Hammarsten milk casein were purchased from E. Merck Co. All the other chemicals were of analytical grade.

Determination of the inhibitory activity

Proteinase activity of snake venom was determined by Folin-Ciocalteu method (14). Mixture of 0.5 ml of M/15 phosphate buffer (pH 7.1), 0.1 ml of the sample solution (100μg), 0.3 ml of 1% casein solution and 0.1 ml of venom solution containing 100 μg of venom was incubated at 37°C for 60 min., and then added with 2.0 ml of 0.44 M TCA solution. The filtrate was reacted with Folin reagent to determine the absorbance at 660 nm. The ratio of inhibition (I) was expressed as follows; $(1-C_1-C_2)$ 100%.

C_1: O.D of the reaction solution from venom and substrate

C_2: O.D of the reaction solution from sample and substrate

S : O.D of the reaction solution from sample, venom and substrate

Inhibition against other proteolytic enzymes, papain (100-400 μg/ml), trypsin (100-400 μg/ml), pepsin (100-400 μg/ml), α-chymotrypsin (100-400 μg/ml) were also estimated by the same procedure. Inhibition against amylase was determined by Fuwa method (15) and chitinase and cellulase were determined by Somogyi-Nelson method (16).

Table 1. Effect of the inhibitor on the proteolytic enzymes and the other enzymes

enzyme	inhibition
α-Amylase	–
β-Amylase	–
T. viride cellulase*	–
Streptomyces chitinase	–
Papain	–
Pepsin	–
Trypsin	+
α-Chymotrypsin	–
Snake venom proteinase** from	
Agkistrodon bromohoffi	+
Agkistrodon saxatilis	+
Agkistrodon caliginosus	+
Agkistrodon halys bromohoffi	+
Trimeresurus flavoviridis	+

*Culture broth was applied as enzyme solution

**Dried snake venom without any purification was used as enzyme source

Inhibitory activity of the inhibitor was estimated by measuring residual enzyme activity after incubation of each enzyme with inhibitor.

+ : inhibited, – : not inhibited

Inhibition on blood coagulation

The effect of inhibitor on blood coagulation was determined by the method of Aoyagi (17).

Results and Discussion

Effect of the sample on the various enzymes

Because the sample has an inhibition against snake venom proteinase, the ability of the inhibitor to various other enzymes was tested. From the result shown in Table 1, snake venom proteinase *(Agkistrodon bromohoffi brevicaudus, A. saxatilis, A. Caliginous, A. halys bromohoffi, Trimeresurus flavoviridis)* and α-chymotrypsin were also inhibited, but α-amylase, β-amylase, papain, pepsin, *Streptomyces* chitinase and *T. viride* cellulase were not inhibited.

Effect of the sample concentrations

The effect of sample concentrations was investigated on the various venom proteinases. As illustrated in Fig. 1, the sample inhibited more on the

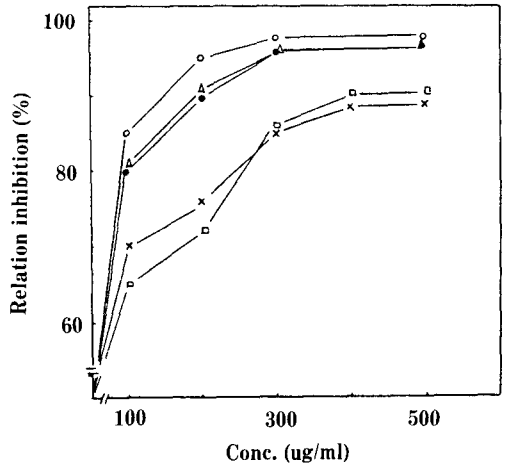

Fig. 1. The inhibitory action of the sample on several kinds of the snake venom proteinase.

The concentration of the venom proteinase in each reaction mixture was 100 ug/ml. Reaction mixture was incubated at 37C, for 40 minutes

○-○ : A.b.b. venom; ●-● : A.c venom

△-△ : A.h.b. venom, ×-× : A.s venom

□-□ : T.f. venom

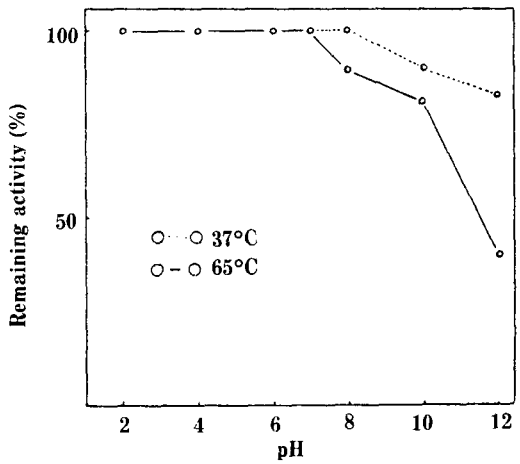

Fig. 2. pH stability of the inhibitor

Inhibitor solution (1 mg/ml) was treated in the various pH ranged from 2 to 12 at 37°C and 65°C for 60 minutes. To the reaction mixture, 100 ug of the pretreated inhibitor and 100 ug of venom (*A. bromohoffi brevicauds*) were added. Relative activity remaining at pH 7.0 was set at 100%.

venom of *A. bromohoffi brevicaudus, A. halys bromohoffi, A. caliginosus* than *A. saxatilis, T. flavovirids.*

pH stability

The residual activity of sample was estimated after 60 min of pretreatment between pH 2 and pH 12 at 37 °C or 60 °C. As illustrated in Fig. 2, it was observed that the inhibitor was stable at wide pH range from 2.0 to 12.0 at 37 °C, however the inhibitor was unstable on alkaline pH at 60 °C

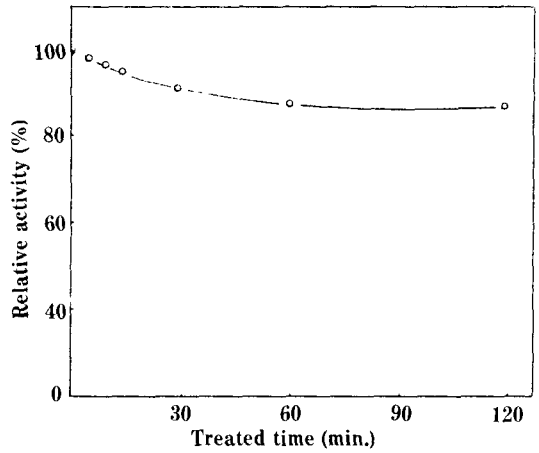

Fig. 3. Heat stability of the inhibitor.

The inhibitor solution (1 mg/ml) was pretreated at 100°C for the given time prior to determination of inhibition ratio. To the reaction mixture, 100 ug of the pretreated inhibitor and 100 ug of venom (*A. bromohoffi brevicauds*) were added. Relative activity of the inhibitor with no treatment was set at 100%.

Heat stability

5 mg of the inhibitor was dissolved in 5 ml of distilled water. It was heated for periods up to two hours in water bath.

As shown in Fig. 3, the heat treatment at 100 °C for 120 min did not cause a significant decrease in inhibitory activity.

Thus, it indicates that the inhibitor is a relative thermostable substance.

Type of inhibition

The inhibition type was examined by Linewaver-Burk plots.

The mixture containing 0.3 ml of buffer, 0.1 ml (100 μg) of venom proteinase *(A. bromohoffi brevicauds)* and 0.1 ml (100 μg) of the inhibitor was preincubated for 5 min at 37 °C. After addition of 0.5 ml of various concentrations of casein, the reaction mixture was incubated for 10 min at the same temperature.

Type of the inhibition was proved to be a mixed

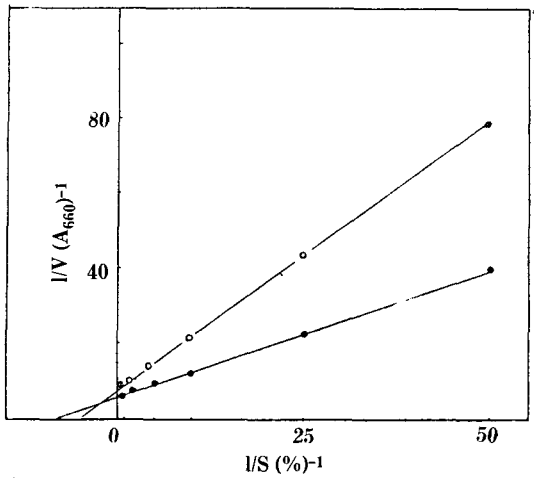

Fig. 4. Type of inhibition against venom proteinase by the inhibitor.

Lineweaver-Burk plots of casein concentration against activity of venom proteinase in the presence and absence of the inhibitor.

○ - ○ : presence of the inhibitor

● - ● : absence of the inhibitor (venom only)

type as illustrated in Fig. 4.

Effect of various substrates

The inhibition of the sample on the venom proteinase of *A. bromohoffi brevicaudus* and *A. caliginosus* was observed using several kinds of substrate, such as 0.5% casein, ovalbumin, and hemoglobin which was denatured by Anson's method (18). As illustrated in Table 3, the inhibition ratio in the substrate of casein was higher than that in the substrate of either ovalbumin or hemoglobin.

Table 2. Effect of substrates on inhibitory activity of the sample to the venom proteinase

venom	inhibition ratio (%)		
	casein	ovalbumin	hemoglobin
Agkistrodon bromohoffibrevicaudus	82	66	54
Agkistrodon caliginosus	74	4	53

Substrate concentration in the reaction mixture was 0.5% respectively. Hemoglobin and ovalbumin were denatured according to Anson's method. Venom 100 ug and inhibitor 100 ug were used.

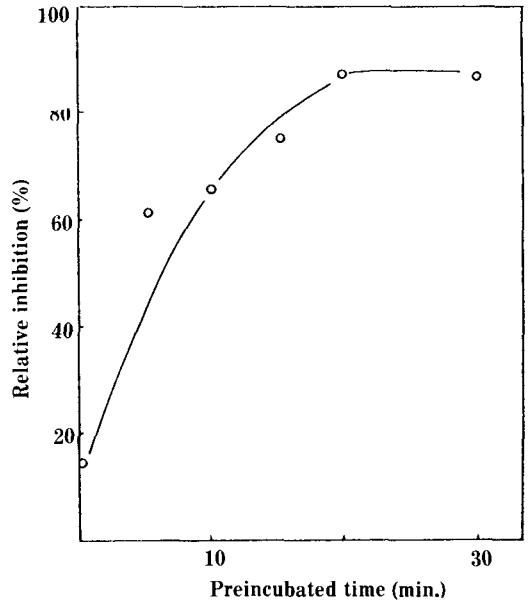

Fig. 5. Effect of preincubation time on the inhibitory activity of the sample to the venom proteinase.

Inhibitor (100 μg/ml) was allowed to react with *A. bromohoffi brevicaudus* venom (100 μg/ml) for various periods of time at 37°C and pH 7.0 prior to determination of inhibition ratio.

Effect of preincubation time

In order to find out the reaction rate between enzyme and inhibitor, we determined the inhibition ratio in conjunction with preincubation time. As illustrated in Fig. 5, the inhibition ratio was increased from 15% with no preincubation to 87% with 20 min of preincubation.

Effect of amino acids

In order to investigate the effect of amino acids on the inhibitory activity of the inhibitor to venom proteinase of *A. bromohoffi brevicaudus,* various kinds of L-amino acids were added to the reaction mixture at a concentration of 1 mM. The results are given in Table 3. All amino acids used did not affect the activity of inhibitor.

Effect of metal salt

In order to investigate the effect of metal salts on the inhibitory activity of the sample to venom proteinase of *A. bromohoffi brevicaudus,* various kinds of metal salts were added to the reaction mix-

Table 3. Effect of amino acid on the inhibitory activity of the sample

Amino acid	relative activity (%)
None	100
L-isoleucine	101
L-leucine	102
L-histidine	90
L-phenylalane	97
L-serine	102
L-proline	97
L-arginine	102
L-threonine	103
L-tyrosine	104
L-glutamine	94
L-aspartic acid	94
L-tryptophan	103
L-alanine	92
L-lysine	98
L-methionine	92
L-valine	100
L-asparagine	100
Glycine	95
L-cysteine	99

Each amino acid was in the reaction mixture with concentration of 1 mM. 100μg of the inhibitor and 100μg of venom were used. Relative activity without amino acid was set at 100.

Table 4. Effect of metal salt on the inhibitory activity of the sample

Metal salt	relative activity (%)
None	100
$MnSO_4$	61
$FeSO_4$	50
$MgSO_4$	90
$CuSO_4$	—
$ZnCl_2$	—
$CaCl_2$	98
$HgCl_2$	—
$CoCl_2$	—
$AgNO_3$	—
$Pb(NO_3)_2$	76

Concentration of added metal salt was 0.5 mM in the reaction mixture. Each 100 μg of inhibitor and venom (*A. bromohoffi brevicaudus*) was added. Relative activity without metal salt was at 100.

Table 5. Effect of the inhibitor on coagulation time of bovine blood

Reaction mixture	
buffer (M/5 Tris-HCl) (pH 7.6)	0.7 ml
blood*	1.0
inhibitor solution	0.2
$CaCl_2$ solution**	0.1
	at 37°C

*blood: mixture of bovine blood 9 volume and 4% Na-citrate 1 volume
**$CaCl_2$ solution: 1 M in M/5 Tris-HCl buffer

time / inhibitor	5	10	20	30
200 μg	−	−	+	+ +
100	−	−	+	+
50	−	−	+	+
25	−	−	+	+
12.5	−	+	+	+
6.25	−	+	+	+ +
3.1	±	+	+	+ +
1.6	+	+	+	+ +
0.8	+	+	+	+ +
°	+	+	+	+ +

(−) no coagulation, (+) a slight coagulation, (+) complete coagulation, (+ +) complete coagulation with shrinking of the clot and appearance of serum.

ture at a concentration of 0.5 mM. As resulted in Table 5, $CuSO_4$, $ZnCl_2$ and $CoCl_2$ exhibited the strong repressing effect to inhibitory activity of the sample.

Effect of the inhibitor on blood coagulation

Some proteolytic enzymes that relate to blood coagulation have been reported (19-22). we also investigated the effect of inhibitor on blood coagulation. It is intresting to note that the inhibitor produced by *Aspergillus* sp. MK-24 shows inhibition on coagulation of bovine blood. The results on bovine blood are given in Table 5. No coagulation was occurred in the reaction system containing the inhibitor less than 3.1 ug/2 ml in 5 min, however, coagulation was occurred even at a concentration of 200 ug/2 ml in 20 min.

요 약

Snake venom proteinase에 對한 沮害物質을 生成하는 Aspergillus 屬 菌株 MK-24를 土壤으로부터 얻어 그 培養液에서 沮害物質을 分離하여 Venom proteinase에 對한 作用樣相과 安定性에 對한 調査結果는 다음과 같다.

Glucose 2%, $NaNO_3$ 0.3%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02% 組成의 培地(pH 5.0)를 使用하여 30℃에서 7 日間 培養하여 얻은 培養液을 acetone 沈澱 활성탄, methanol 침전으로 無定形의 有効粉末을 얻었다. 이 物質은 A. b.b. venom proteinase에 對하여 1/2 培量에서 約 70% 沮害率을 나타냈으며 A. b.b. venom proteinase에 對한 沮害樣相은 混合形이었으며 enzyme-inhibitor complex를 形成하는데 20分 정도가 걸렸다. 反應液中에 Co^{++}, Zn^{++}, Cu^{++} 等이 存在하면 沮害作用이 完全히 抑制되었다. 沮害率은 使用한 基質의 種類에 따라 差異가 났다. 즉 casein을 使用했을 때는 hemoglobin이나 albumin 보다 沮害率이 높았다. 그리고 本 沮害物質은 snake venom proteinase 以外에 trypsin에 高濃度에서 약간의 沮害作用力을 나타냈으나 pepsin, α-chymotrypsin, papain 等과 炭水化物 加水分解酵素 等에는 沮害能이 없었고, 血液凝固에 대하여는 1.6 μg/2 ml 濃度 이상에서는 沮害作用을 나타내었다. 本 沮害物質은 熱이나 pH에 對한 安定性이 컸다. 즉, pH 처리에 대해서는 37℃에서 60分 處理로 酸이나 alkali에 對해서 대단히 넓은 범위에 걸쳐서 安定하였으며 65℃에서는 中性까지는 安定하였으나, pH 8 이상에서는 不安定하였고 熱처리에 대해서는 100℃에서 2時間 처리했을 때에도 殘存活性度가 約 90%로 매우 안정하였다.

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