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Trypsin Inhibitor from *Streptomyces* sp. (Part 1) Isolation of microorganism and purification of the inhibitor

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Streptomyces 屬 菌株가 生成하는 Trypsin Inhibitor (第1報) 菌의 分離 및 沮害物質의 精製

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Abstract

One strain of *Streptomyces* sp. (AS-707) isolated from soil was found to produce a biologically active substance that showed a strong inhibitory activity against proteolytic enzymes viz. trypsin, papain, α-chymotrypsin, *Azotobacter* protease, and *Bacillus* protease.

The substance was separated from culture filtrate by ion exchange column chromatography using Amberlite IRC-50 and CM-cellulose column chromatography. It was found that the recovery yield was 26% as activity basis. The substance was stable in wide pH range from 2.0 to 12.0 at 37°C, but it was unstable in alkaline pH values at 60°C. The activity was thermostable to give 90% activity compared to the intact sample when it was treated at pH5.6 at 100°C for 2 hours.

Introduction

Substances inhibiting proteolytic enzymes have been demonstrated in a variety of biological materials. The first inhibitor known as bovine trypsin-kallikrein inhibitor was detected by Kunitz and Northrop(1). After this discovery many protease inhibitors have been found in various animals and plants, such as many organs of mammals, colostrums, eggs of certain birds, animal tissues and plasmas, soybean, limabean, potato, barly, maize, and wheat (2,3,4). The naturally occurring protease inhibitors of animals and plants origined have been reviewed by Vogel *et al*(2), Liener and Kakade(3) and Kassell(4).

Until recent years investigations on the inhibitor produced from microorganisms have been relatively few. It was first report about microbial inhibitor that Hoyen and Skulberg(5) reported the existence of trypsin inhibitors in culture supernatants of *Clostridium botulinum*, type A,B, and E. The distribution of inhibitor-producing strains among fungi

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has been studied by Shimada and Matsushima[6]. They examined 127 strains of *Aspergillus, Penicillium,* and *Rhizopus* and found 10 active producers. Most of these belong to the *Penicillium*. Brecher and Pugatch[7] reported the presence of a nondialyzable, heat-stable inhibitor against tryptic and chymotryptic activity in the 90,000xg supernatant fraction of sonicated *Escherichia coli* cells. Aoyagi *et al*[8] examined the antiplasmin activity of culture filtrates of *Actinomycetes* and named the inhibitor as leupeptins. Fossum[9] reported that trypsin, α-chymotrypsin, and a certain microbial extracellular proteases (but not ficin and papain) were inhibited by extracts from disintegrated cells of some gram-negative bacteria, including *Proteus mirabilis, Pr. vulgaris, E. coli,* a *Klebsiella* sp., *Serratia marcescens,* and *Pseudomonas aeruginosa*. Alkaline proteinase inhibitor (MAPI) produced by *Streptomyces* sp. WT-27 have been reported by Murao and Watanabe[10]. Oka *et al*[11] investigated ninhydrin negative peptidic trypsin inhibitor isolated from culture broth of *Streptomyces* sp..

For the potential applications of such inhibitors in medicine, biochemistry[12,13,14], we attempted to find an inhibitor against trypsin reaction that participate in the biological functions of the human body[15,16,17]. In this studies, a microbial inhibitor, produced by one *Streptomyces* sp., was obtained and its actions on varioos proteolytic enzymes, and its stabilities were investigated.

Materials and Methods

Isolation and screening of microorganism for trypsin inhibitor.

Samples of soil were suspended in saline, and one loop of this suspension was mixed with molten agar (1.8%) medium (pH 7.0) containing 1% glucose, 0.2% peptone and then poured and spread in Petri dish. The plates were allowed to incubate at 30°C until distinct clonies developed. Hence 350 strains of *Streptomyces* sp. were isolated. The isolated microorganisms were inoculated to the test tube (15 x 150mm) containing 5ml of the inhibitor production medium as shown in Table 1. These cultures were cultivated at 30°C for three or four days. After cultivation, the culture broth was filtrated with cotton, then the culture filtrate was subjected to further analyses.

Table 1. Composition of media

Medium for stock culture	
Potato extract*	20% (v/v)
glucose	1.0%
$NaNO_3$	0.1%
agar	1.8%
pH	7.0
Medium for inhibitor production	
glucose	2.0%
peptone	0.3%
$NaNO_3$	0.05%
K_2HPO_4	0.05%
$MgSO_47H_2O$	0.05%
NaCl	0.05%
pH	7.0

* 200g of potato in 1000ml of tap water was boiled for 30 min. After cooling, the broth was passed through cotton, and added to the medium.

Determinationof trypsin inhibitor activity

The inhibitory activity on trypsin was determined by measuring residual proteolytic activity of trypsin after preincubation the trypsin with the inhibitor solution. Modified Anson's method[18] was adopted for the measurement of residual trypsin activity. 1% solution of Hammarsten milk casein dissolved in M/15 phospho-borate buffer (pH 7.6) was used as substrate, and trypsin was dissolved to 0.1% in the same buffer. The mixtures composed of 0.5ml of buffer solution, 0.1ml of trypsin solution, and 0.1ml of inhibitor solution was incubated for

5 min at 37°C, and then 0.3ml of 1% casein solution was added. After incubation for 30 min at 37°C, enzyme reaction was stopped with 1ml of 0.44M trichloroacetic acid and incubated for 40 min. The reaction mixture was filtered with cotton. 1ml of the filtrate, 2.5ml of 0.55M Na_2CO_3 and 0.5ml of Folin's reagent (x3) were added in test tubes, then the mixture was reacted for 30 min at 37°C and optical density was measured at 660nm. The ratio of the inhibition was calculated with the following equation; the ratio of the inhibition (%),

$$I(\%) = 100\ (1-\frac{S-C_2}{C_1-C_2});$$ in this, C_1 stands for the absorbance without inhibitor, C_2 for the absorbance with inhibitor and S for the absorbance with both inhibitor and enzyme.

Inhibitory actions of the inhibitor against other proteolytic enzymes were also estimated by the same procedure of trypsin assay system described above, except buffer systems and enzyme concentrations. Papain (100μg/ml in M/15 phosphate buffer, pH 7.0), α-chymotrypsin (50μg/ml in M/15 phosphate buffer, pH 7.8), pepsin (100-500μg/ml in M/10 sodium acetate-HCl buffer, pH 2.0), snake venom proteases (100μg/ml in M/15 phosphate buffer, pH 7.0), and microbial proteases (cultured filtrate 0.1ml in M/15 phosphte buffer, pH 7.0) were mixed with inhibitor solution (100-200μg/ml) and preincubated for 5 min at 37°C, and then the residual enzyme activites were estimated.

Production and purification of the inhibitor

Selected strain *Streptomyces* sp. AS-707 was stocked in solid medium described in Table 1 and subcultured every month. For the production of the inhibitor, strain AS-707 was inoculated in 1000ml Erlenmeyer flask containing 200ml of medium described in Table 1. The flask were cultured at 35°C for three days as shaking cultures (reciprocal, 5cm,90 strokes/min).

Separation purification of the trypsin inhibitor from the culture broth was carried out with four steps as shown in Fig. 1.

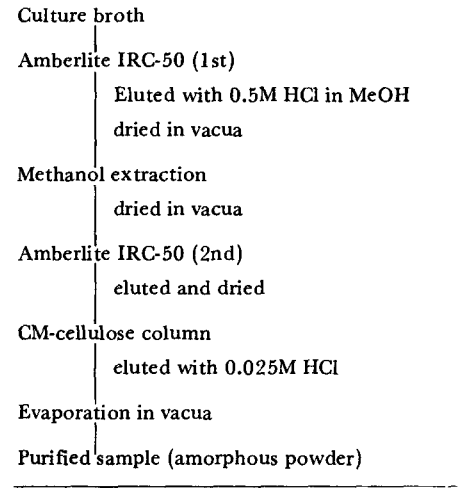

Fig. 1. Purification procedure of the inhibitor.

Results

Purification of the inhibitor

1) Amberlite IRC-50 column chromatography

Cultured filtrate (1000ml) was passed through a column (2.1 x 25cm) of Amberlite IRC-50 cation-exchanger resin. After washing the column with distilled water, pigmental substance was eluted with 200ml of 95% methanol and then effective substance was eluted with 100ml of 95% methanol containing 0.5N-HCl. The effluent was evaporated to be dryness in vacua.

2) Extraction with methanol

The evaporated active portion (3.4g of solid) obtained from Amberlite IRC-50 ion-exchanger was extracted with 25ml of 95% methanol (repeated 5 times with 5ml). The methanol extract was evaporated to dryness (1740mg) under reduced pressure.

3) Second IRC-50 column chromatography

Crude powder obtained above was dissolved in 5ml of distilled water and placed in refrigerator (-15°C). After 3-5 days, precipitate formed was discarded. Active supernatant was applied again to

the column of IRC-50 with the same way mentioned above (1st IRC-50). Active fraction was collected and evaporated to dryness in vacua.

4) CM-cellulose column chromatography

Twenty mg of active powder obtained from second IRC-50 was charged on a column (1.2 x 20cm). After washing the column with distilled water, the adsorbed inhibitor was eluted by the use of 0.025M HCl at a flow rate of 30ml per hour fractioning 3ml volume as shown in Fig. 2. The active fraction was evaporated, and the purified inhibitor was obtained with recovery of 26% of the activity of the culture filtrate. The detailed purification procedures are summarized in Table 2.

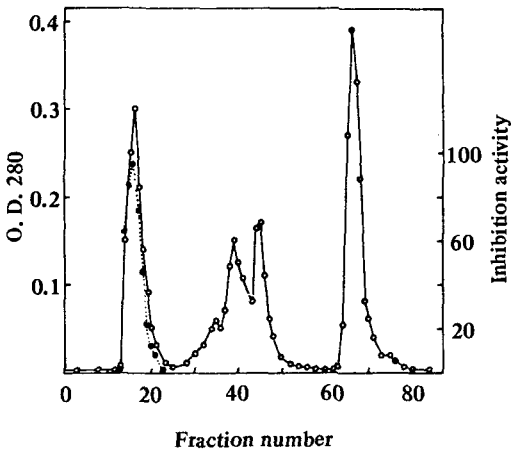

Fig. 2. Column chromatography of trypsin inhibitor on CM cellulose.

The column (1.2 x 20cm) was charged with 20mg (dissolved in 1ml of distilled water) of the active substance obtained from Amberlite IRC-50 cationexchanger and washed with distilled water. The adsorbed inhibitor was eluted with 0.025M HCl at a flow rate of 30ml per hour fractioning 3ml volume.

O—O: O.D. 280,

●···●: inhibition activity

Table 2. Summary of the purification of the inhibitor

Step	Amount	Total activity	Relative specific activity	Recovery (%)
Culture filtrate	1000ml	828	0.4	100
Amberlite IRC-50 (1st)	3400mg	625	1.8	75
Methanol extraction	1740mg	410	2.4	50
Amberlite IRC-50 (2nd)	450mg	284	6.3	34
CM-cellulose	135mg	213	16.0	26

Inhibitory activities of the purified inhibitor on various proteases

Because the inhibitor has an activity of inhibition against trypsin, the ability of the inhibitor to various other proteolytic enzymes was tested. From the result, shown in Table 3, papain, α-chymotrypsin, *Azotobacter vinelandi* protease, and *Bacillus subtilis* protease were also inhibited by the inhibitor but pepsin, snake venom proteases *(Agkistrodon blomhoffi, A. saxatilis, A. caliginosus)* and *Streptomyces* metal protease were not inhibited.

Stability of the inhibitor

1) Thermal stability

Five mg of the inhibitor was dissolved in 5ml of distilled water and the solution was heated for periods up to two hours in water bath at 100°C. As shown in Fig. 3, heat treatment of the inhibitor, even under the condition at 100°C for 120 min, did not cause a significant decrease in the inhibitory activity. Thus, this finding indicates that the inhibitor is a relatively thermostable substance.

2) pH stability

The stability of the inhibitor at various pH values between 2.0 to 12.0 was tested by incubation of the inhibitor solution (mg/ml) at 37°C and 60°C. After 60 min residual activity was estimated. As illustrat-

ed in Fig. 4, it was observed that the inhibitor was stable at wide pH range from 2.0 to 12.0, when it was treated at 37°C. However the inhibitor was stable at acidic condition but unstable at alkaline at 60°C.

Table 3. Effect of the inhibitor on the other proteases

Proteolytic enzyme	Inhibition	I (%)
Papain	+	84
α-chymotrypsin	+	73
Pepsin	-	0
Snake venom protease*		
Agkistrodon blomhoffi	-	0
Agkistrodon saxatilis	-	0
Agkistrodon caliginosus	-	0
Azotobacter vinelandi protease**	+	32
Bacillus subtilis protease**	+	52
Streptomyces metal protease***	-	0

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

** : Cultured broth was applied as enzyme solution.

*** : This protease was produced by *Streptomyces* sp. isolated from soil and the enzyme was inactivated by EDTA and reactivated by the addition of Co^{++} ions in the reaction mixture.

Inhibitory activity of the inhibitor was estimated by measuring residual enzyme activity after incubation of each enzyme with inhibitor. See "Materials and Methods" for detailed assay systems.

\+ : inhibited

\- : not inhibited

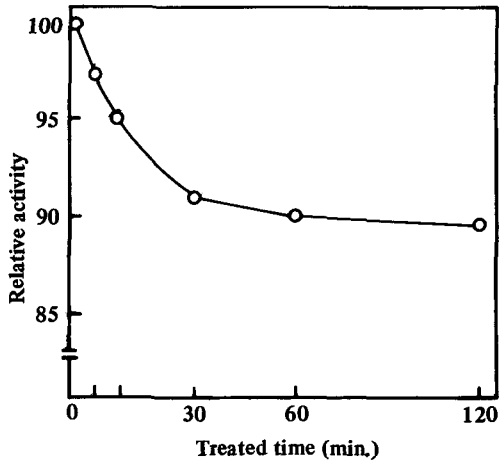

Fig. 3. Thermal stability of the inhibitor.
The inhibitor solution (mg/ml) (pH5.6) was pretreated at 100°C for the given time prior to determination of inhibition ratio. To the reaction mixture, 100μg of the pretreated inhibitor and 100μg of trypsin were added. Activity of the inhibitor not treated was set at 100.

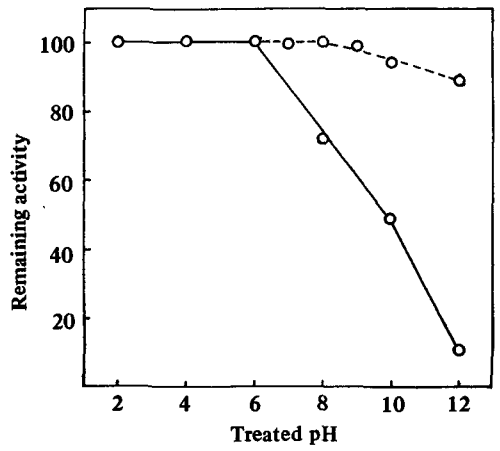

Fig. 4. pH stability of the inhibitor.
Inhibitor solution (mg/ml) was treated in the various pH ranged from 2.0 to 12.0 at 37°C and 60°C for 60 minutes. After treatment, residual activity was tested. To the reaction mixture, 100μg of the pretreated inhibitor and 100μg of trypsin were added. Activity remaining after treatment at pH 6.0 was set at 100.

○···○ : treated at 37℃, ○—○: treated at 60℃

Discussion

The availability of protease inhibitors from microorganism has been valuable for the study of proteases, and several applications within medicine and biochemistry have appeared (12-17).

We isolated one strain of *Streptomyces* sp. (AS-707) from soil. The microorganism was able to produce a biologically active substance that has a strong inhibitory activity against trypsin. The substance was obtained from culture filtrate by adsorption on Amberlite IRC-50 and chromatography on CM-cellulose column. Recovery (26%) for the inhibitor from *Streptomyces* sp. strain AS-707 lie at the same level as those reported by Oka *et al*(11), Kondo *et al*(19), and Torstensson(20).

The substance also has inhibitory activity on various proteolytic enzymes e.g. papain, α-chymotrypsin, and some microbial proteases such as *Azotobacter vinelandi* Protease and *Bacillus subtilis* protease, but not pepsin, snake venom proteases and *Streptomyces* metal protease. Among the peptidic inhibitors of microbial origin known at present, the inhibitor isolated in the current studies has similar inhibitory specificities to those of leupeptins(21) and antipain(21) isolated from culture filtrates of various species of *Streptomyces* except for the inhibitory actions against α-chymotrypsin. In addition, leupeptins and antipain did not inhibit α-chymotrypsin, whereas the inhibitor we had isolated had a potent inhibitory activity on α-chymotrypsin-hydrolysis of casein. As other protease inhibotors reported by Torstensson(20), Satoi(22), Murao *et al*(23), and Uyeda *et al*(24), the substance was stable against heat treatment and in wide pH range from 2.0 to 12.0.

要　約

Trypsin에 對한 強한 沮害物質을 生成하는 *Streptomyces*屬 菌株 AS-707을 토양으로부터 얻어 그 培養液에서 Trypsin inhibitor를 分離精製하여 沮害物質의 安定性과 여러가지의 protease에 對한 沮害性 與否를 검토한 결과는 다음과 같다.

培養液을 Amberlite IRC-50에 흡착,methanol 추출, 2次 Amberlite IRC-50, CM-cellulose column chromatography로 정제하여 active amorphous powder를 얻었는데 이 때의 收率은 26%였다. 分離精製된 物質은 trypsin以外에 papain, α-chymotrypsin, *Azotobacter vinelandi* protease와 *Bacillus subtilis* protease 等에 대해서도 沮害作用을 나타내었으며, 安定性은 비교적 커서 100℃에서 120分間 가열해도 残存活性이 약 90%였으며, pH處理에 대해서는 37℃에서 處理하면 酸에서 Alkali에 걸치는 대단히 넓은 pH범위(pH 2.0～12.0)에서 安定하였으나 60℃에서 처리하면 酸에서는 安定하였으나 Alkali에서는 不安定하였다.

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