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Trypsin Inhibitor from *Streptomyces* sp. (Part 2) Biological Activities of the Inhibitor

Dong-Heui Yi* and Jung-Hwn Seu
Department of Agricultural Chemistry, College of Agriculture,
Kyungpook National University, Taegu, Korea
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Streptomyces 屬 菌株가 生成하는 Trypsin Inhibitor (第2報) 沮害物質의 生物學的 作用相

李東羲, 徐正塤
慶北大學校農科大學農化學科
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Abstract

Trypsin inhibitor produced by *Streptomyces* sp. was investigated its reactive characteristics against trypsin. The mode of inhibition against trypsin was mixed type of non-competitive and competitive with casein, and enzyme-inhibitor complex was formed rapidly. The inhibitory activity was increased by the addition of isoleucine and depressed by silver, mercuric or cupric ion. And when egg albumin or hemoglobin was used as substrate for trypsin, the inhibition ratio was changed. The inhibitor inhibited coagulation of blood of bovine.

Introduction

An inhibitor produced by a *Streptomyces* sp. strain was obtained. The purification, stabilities of the inhibitor was reported in a previous paper (1).

In this paper, the characteristics of reaction of the inhibitor to trypsin and effect on coagulation of blood are described.

Materials and Methods

Enzyme, substrates and other chemicals

Trypsin (20,000 units, from hog pancreas) was purchased from E. Merck Co., and inhibitor was prepared as described in a previous paper(1).

Hammarsten milk casein (E. Merck), egg albumin (Difco) and hemoglobin (Difco) were used as substrate for trypsin in the assay of trypsin inhibitor activity.

All other chemicals were of analytical grade.

Determination of trypsin inhibitor activity

Determination of trypsin inhibitor activity was made by measuring residual proteolytic activity of

*Present address: Dept. of Microbiological Technology, Kon-Kuk University, Seoul, Korea

trypsin after solution of trypsin was preincubated with the inhibitor solution in the method described previously[1]. The rate of tryptic hydrolysis of milk casein was determined at 37°C, pH 7.6 (M/15 phospho-borate buffer).

Method of testing for the effect of the inhibitor on blood coagulation.

The effect of inhibitor on blood coagulation was determined by the method of Aoyagi *et al*[2] with slight modification. A 0.7ml of M/5 Tris-HCl buffer (pH 7.6) and 1ml of mixture of fresh bovine blood and 4% sodium citrate (9:1, v/v) were placed in each test tube. Then 0.2ml of inhibitor solution and 0.1ml of 1M $CaCl_2$ in M/5 Tris-HCl buffer were added and the occurrence of coagulation was observed after 5, 10, 20, 30 min.

Results and Discussion

Effect of concentration of the inhibitor on the trypsin

The correlation between concentration of the inhibitor and the inhibition of trypsin was studied (Fig. 1). Trypsin was added in the reaction mixture at the concentration of 100μg/ml. It was found that the proteolytic activity of the trypsin was remarkably decreased, when the trypsin was treated with the inhibitor; 50% inhibition of the trypsin activity with 12.5μg/ml of inhibitor in the reaction mixture.

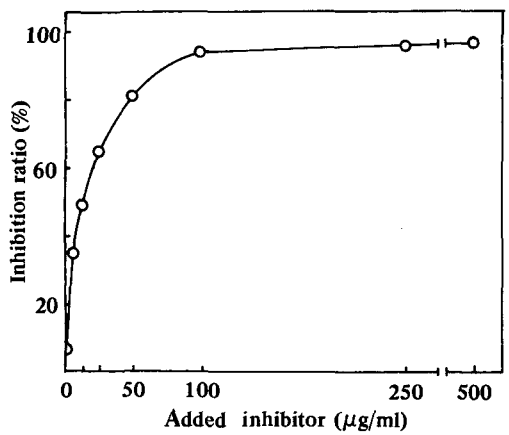

Fig. 1. Effect of concentration of the inhibitor on the trypsin.
The concentration of the trypsin in each reaction mixture was 100μg/ml. Reaction mixture was incubated at 37°C, for 30 minutes.

Type of inhibition

Type of the inhibition of trypsin by the inhibitor was examined by Lineweaver-Burk plots.

The mixture containing 0.1ml (100μg) of trypsin solution, 0.1ml (100μg) of the inhibitor solution and 0.3ml of buffer was preincubated for 5 min at 37°C. After addition of 0.5ml of various concentrations of casein, the reaction mixtures were incubated for 10 min at the same temperature.

As illustrated in Fig. 2, type of the inhibition of trypsin by the inhibitor was proved to be a mixed type. A similar tendency was observed when E-64, thiol protease inhibitor investigated by Inaba *et al*[3], was treated with squid cathepsin B_1, but leupeptins reported by Aoyagi *et al*[2,4] were competitive inhibitors to trypsin and papain, and Pepsta-

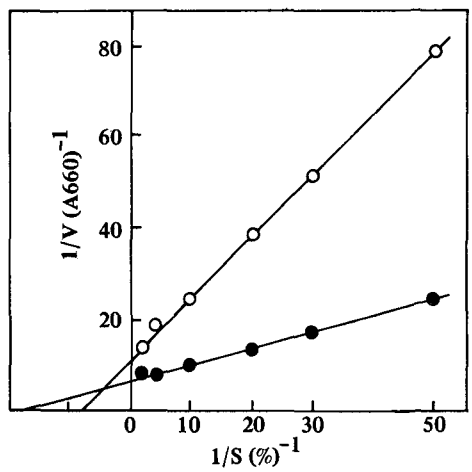

Fig. 2. Type of inhibition against trypsin by the inhibitor.
Lineweaver-Burk plots of casein concentration against activity of trypsin in the presence and in the absence of the inhibitor.
○ - ○ : presence of the inhibitor
● - ● : absence of the inhibitor (trypsin only)

tin[5], a pepsin inhibitor, and bestatin[6], an inhibitor of aminopeptidase B, also competitive.

Effect of substrate concentration on the inhibitory activity of the sample to trypsin

The mixture of the inhibitor and casein solution at various concentration from 0.025% to 1.0% was preincubated for 5 min at 37°C, and then trypsin was added to react with the mixture for 10 min. As shown in Fig. 3, the degree of inhibition was reduced as the concentration of substrate was increased. The result indicates that the inhibition appears to be a combination of non-competitive and competitive inhibition[7].

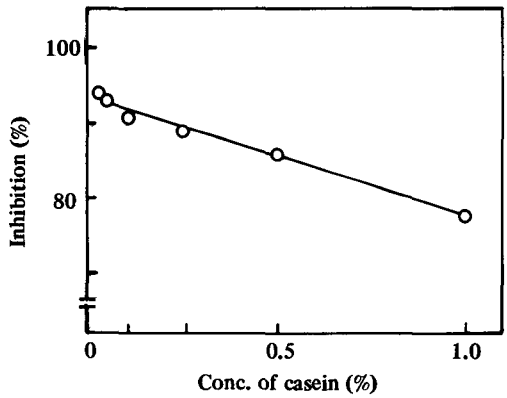

Fig. 3. Effect of substrate concentration on the inhibitory activity of the sample to trypsin. Final concentration of casein in the reaction mixture was 0.025% to 1.0%. A200 μg of the inhibitor and 200 μg of trypsin were added in the reaction mixture and the mixture was incubated for 10 minutes at 37°C.

Effect of preincubation time on the activity of inhibitor

In order to find out rate of the reaction between enzyme and inhibitor, we determined inhibition ratio in conjunction with preincubation time.

As illustrated in Fig. 4, the inhibition ratio was increased lineary ata very slow rate; 89% to 93% inhibition for preincubation time of 0 to 30 min, indicating that enzyme-inhibitor complex was formed rapidly. Among naturally occurring protease inhibitors known at present, two groups with different character of enzyme-inhibitor complex formation seem to exist, those which are able to form rapidly and those which form slowly. To the first group belong the protease inhibitor investigated by Matsushima and Shimada[8], and leupeptins[4]. But antipain[4], and a trypsin inhibitor studied by Ogiso *et al*[9] belongs to the second group.

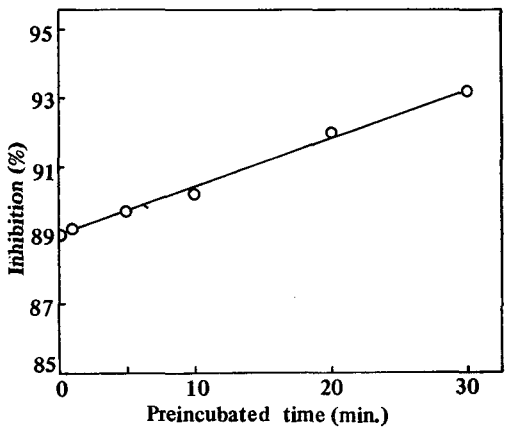

Fig. 4. Effect of preincubation time on the inhibitory activity of the sample to the trypsin. Inhibitor (100μg) was allowed to react with trypsin (100μg) for various periods of time at 37°C, pH 7,6 prior to determination of inhibition ratio.

Effect of the kind of substrate

Effect of the kind of substrate on inhibitory activity of the inhibitor to trypsin was investigated. The results with casein, and hemoglobin and egg albumin both denatured with urea in accordance with Anson's method[10], are given in Table 1. Inhibition ratio was varied with the kind of substrate; the inhibition ratio in the presence of egg albumin was higher than that in the presence of either casein or hemoglobin.

Effect of amino acid on the activity of the inhibitor

In order to investigate the effect of amino acid

Table 1. Effect of the kind of substrate on inhibitory activity of the sample to trypsin.

Substrate	Inhibition ratio(%)
Denatured hemoglobin	54
Denatured egg albumin	76
Milk casein	62

Substrate concentration in the reaction mixture was 0.3% respectively. Hemoglobin and egg albumin were denatured according to Anson's method. Trypsin 100μg and inhibitor 25μg were used.

on the inhibitory activity of the inhibitor to trypsin, various kinds of L-amino acids were added to the reaction mixture at concentration of 1mM. The results are given in Table 2. All amino acids that were added did not affect acitivity of the inhibitor, but L-isoleucine increased the inhibitory activity. As illustrated in Fig. 5, L-isoleucine increased inhibitory activity of the inhibitor to a maximum level of about 30%, when the amino acid was added in the concentration of 5mM and over.

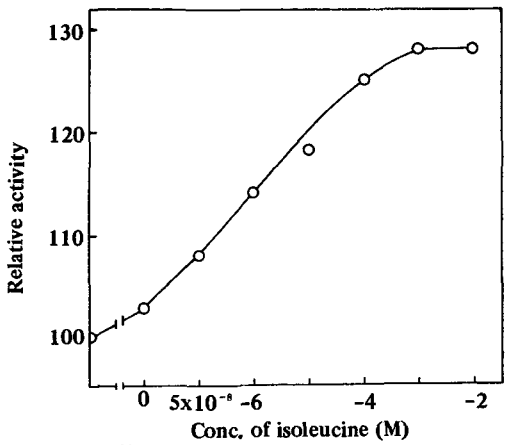

Fig. 5. Effect of L-isoleucine on the inhibitory activity of the sample.
L-isoleucine was added in the reaction mixture with various concentration from 5 x10^{-8} M to 5x10^{-2} M. Twenty five μg of the inhibitor and 100μg of trypsin were used. And activity not added isoleucine was set at 100.

Table 2. Effect of amino acid on the activity of the inhibitor.

Amino acid	Relative activity
none	100
L-alanine	101
L-arginine	100
L-leucine	100
L-isoleucine	118
L-methionine	101
L-tryptophan	105
L-lysine	102
L-aspartic acid	99
L-glutamic acid	100
L-glutamine	97
L-valine	96
L-threonine	102
L-serine	100
L-cysteine	103
L-phenylalanine	104
L-histidine	99
L-proline	104
L-tyrosine	106
glycine	93

Each amino acid was added in the reaction mixture with concentration of 1mM. Fifty μg of the inhibitor and 100μg of trypsin were used. Activity not added amino acid was set at 100.

Effect of metal salt on the inhibitory activity

Various metal salts were added to the reaction mixture at a concentration of 0.5mM in order to investigate the effect of metal salt on the activity of the inhibitor against trypsin. As resulted in Table 3, $MgSO_4$, $CoCl_2$, $CaCl_2$, $MnSO_4$, $FeSO_4$, $ZnSO_4$, and Pb-acetate did not affect the inhibitory activity, but Hg-acetate and $AgNO_3$ showed strong repressing effect to the inhibitor and $CuSO_4$ also repressed the inhibitor weakly.

Table 3. Effect of metal salt on the activity of the inhibitor.

Metal salt	Relative activity
None	100
$CuSO_4 \cdot 5H_2O$	86
$MgSO_4 \cdot 7H_2O$	101
$CoCl_2$	103
$CaCl_2$	101
$MnSO_4 \cdot 4\text{-}6H_2O$	99
$FeSO_4$	98
$ZnSO_4 \cdot 7H_2O$	101
Pb-acetate	95
Hg-acetate	53
$AgNO_3$	38

Concentration of added metal salt was 0.5mM in the reaction mixture. Each 100μg of inhibitor and trypsin was added. Activity not added metal salt was set at 100.

Effect of the inhibitor on blood coagulation

Some proteolytic enzymes that relate to blood coagulation have been reported[11,12]. So we investigated the effect of inhibitor on blood coagulation.

It is intresting to note that our inhibitor shows inhibition activity on coagulation of bovine blood. The results with bovine blood are given in Table 4. No coagulation occurred in the reaction system containing the mixture of the inhibitor less than 6.2 μg/2ml after 5 min, but after 30 min coagulation occurred even at concentration of 200μg/2ml. This tendency is similar to leupeptins, but different with heparin. In the case of heparin, when it is used at high concentration, no coagulation occurs even after a long time[13].

Table 4. Effect of the inhibitor on coagulation time of bovine blood.

Reation mixture	
buffer (M/5 Tris-HCl, pH 7.6)	0.7ml
bovine blood*	1.0
inhibitor solution	0.2
$CaCl_2$ solution**	0.1

* : blood; mixture of bovine blood 9 volume and 4% sodium citrate 1 volume

** : $CaCl_2$ solution; 1M in M/5 Tris-HCl buffer

time / I/mixture*	5	10	20	30min
200μg	−	−	−	+
100	−	−	+	+
50	−	±	+	+
25	−	+	+	+
12.5	−	+	+	+
6.2	±	+	+	++
3.1	+	+	+	++
1.6	+	+	+	++
0.8	+	+	++	++
0	+	++	++	++

* : Amount of added inhibitor in the reaction mixture

(−) means no coagulation,

(+) complete coagulation,

(±) a slight coagulation,

(++) complete coagulation with shrinking of the clot and appearance of serum.

要　　約

Streptomyces屬菌이 생산하는 trypsin inhibitor의 trypsin에 對한 反應性을 조사해 본 結果 本 沮害物質은 crystalline trypsin(20,000unit,hog pancreas)에 對하여 1/8量에서 約 50%의 沮害率을 나타내었으며 trypsin에 대한 沮害樣相은 mixed noncompetitive-competitive inhibition type이었으며 enzyme-inhibitor complex를 빨리 형성하는데 反應液中 isoleucine이 共存하면 活性이 증가되었으며 Ag^+, Hg^{++} 등의 金屬ion은 強하게 本 沮害物質의 作用을 抑制하였다. 沮害率은 사용한 기질의 종류에 따라 차이가 나서 albumin을 사용하였을 때는 casein이나 hemoglobin을 사용하였을 때보다 沮害率이 높았다. 그리고 血液의 응고에 대해서도 沮害作用을 나타내었다.

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