Some properties of a protease inhibitor produced by *Bacillus cereus*

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Introduction

Presence of substances showing inhibitory activity on various proteases has been demonstrated in a variety of biological materials such as potatoes[1], beans[2], pancreas[3], colostrum[4] and eggs[5]. In particular, there have been many reports about trypsin inhibitors from the biological materials[1)~5)] and most of these inhibitors were peptides with molecular weight above 5000 and with lysyl or arginyl residue as an active site[6].

In 1962, HÖYEN *et al.*[7] published that *Clostridum botulinum* produced dialysable and heat stable trypsin inhibitors. Since then several protease inhibitors have been isolated from culture broth of fungi and actinomycetes. SHIMADA and MATSUSHIMA[8),9)] reported that many strains of moulds produced protease inhibitors which were kinds of polyacid consisting of L-malic acid. UMEZAWA *et al.* isolated trypsin-[10], chymotrypsin-[11] and pepsin-inhibitors[12),13)] which were peptides with small molecular weight from actinomycetes.

So far as the authors know, protease inhibitors produced by bacteria, however, have not been reported with a few exceptions of trypsin inhibitors from *Cl. botulinum*[7] and *Bacillus sp.*[14]. It seems very probable, therefore, that other many bacteria would produce various kinds of protease inhibitors.

From the point of view, the authors screened a trypsin inhibitor producing bacteria from a large number of soil bacteria, and obtained a strain which produced a trypsin inhibitor in culture filtrate.

In the present paper, screening, classification and cultural conditions of the strain were described, and purification and some properties of the produced inhibitor were investigated.

Materials and Methods

Isolation of microorganisms :

Received April 30, 1976

Samples of soil were suspended each in sterile water, and 1 ml of the suspension was spread in a Petri dish with Medium-1 which contained 3.0 % peptone, 0.3 % glucose, 0.3 % meat extracts, 0.1 % NaCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$ and 2.0 % agar (pH 7.0). This plate was incubated at 30 C for 48 hrs. Bacteria developed were streaked on slant Medium-1. After aboundant growth of the culture at 30 C, the tubes were stored at 5 C. One hundred and forty-two strains of bacteria were isolated.

Cultivation for screening test of trypsin inhibitor producing bacteria :

The bacteria isolated were inoculated in test tubes (2 × 18 cm) with 10 ml of agar free Medium-1. These cultures were cultivated at 30 C for 3 days with shaking (140 r. p. m). After the cultivation, each culture broth was filtered with Toyo No. 5B filter paper, and the filtrate was used for inhibitory assay.

Assay for the trypsin inhibiting activity :

The substrate employed for the assay of the proteolytic activity[15] was 4 % casein dissolved in 0.1 M phosphate buffer, pH 7.6. Trypsin (2000 E/G, Merck) was dissolved to 0.3 % in the same buffer.

A half millilitres of the filtrate of culture broth were pre-incubated at 37 C for 10 min with 0.5 ml of the trypsin solution and 1.5 ml of 0.1 M phosphate buffer, pH 7.6. Then, the mixture was incubated with 0.5 ml of the substrate solution at 37 C for 10 min and the reaction was stopped by addition of 3.0 ml of 5 % trichloroacetic acid solution (TCA). After 20 minutes' incubation at the same temperature, the reaction mixture was filtered with Toyo No. 5B filter paper. To 1 ml of the filtrate were added 5 ml of 0.55 M Na_2CO_3 and 1 ml of FOLIN's reagent diluted 3-fold with distilled water, and the mixture was held at 37 C for 30 min. The absorbance of the mixture was determined at 660 nm (Hitachi spectrophotomater Model 101).

In a blank sample the trypsin solution was inactivated by adding 3.0 ml of 5 % TCA prior to addition of the substrate solution, and in place of the filtrate of culture broth distilled water was used as the standard reaction.

The inhibiting activity was expressed as the percentage inhibition compared with the control activity using the following equation;

$$\text{Inhibiting activity } (\%) = \frac{100\{(S - B) - (T - B')\}}{S - B}$$

where S, B, T and B', respectively, were the absorbances at 660 nm of the standard reaction, blank sample for the standard reaction, test reaction and blank sample for the test reaction.

Identification of bacteria isolated :

Bacteria isolated were grown in test tubes with a number of different media that are commonly used in the study of bacteria. The types of cell, formation

of spore, biochemical characters on various media were noted and classified according to "the 7th eddition of Bergey's Manual of Determinative Bacteriology".

Preparation of insolubilised trypsin :

Nine grams of CNBr activated Sepharose 4B (Pharmacia Fine Chemicals) were washed with 2 l of 10^{-3} M HCl on a glass filter. Three hundred milligrams of trypsin (Boehringer Manheim) to be attached to the activated Sepharose 4B was dissolved in 200 ml of 0.1 M borate buffer containing 0.5 M NaCl, pH 8.3. The activated Sepharose was added to the trypsin solution with stirring. The mixture was stirred for 3 hrs at room temperature, then placed on a glass filter and washed with 1 l of the same buffer. The trypsin-Sepharose 4 B (insolubilised trypsin) prepared above was immersed in 200 ml of 1 M ethanolamine, pH 8.0 for 2 hrs, then placed on a glass filter and washed stepwise with 2 l of 0.1 M borate buffer containing 0.5 M NaCl, pH 8.3 and 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0. The insolubilised trypsin was washed again with the same borate buffer until pH of the effluent was constant, and stored in a refrigerator at 4 C.

Results and Discussion

1. Results of screening test

Five strains (No. 203, 603, 610, 615 and 702) which produced trypsin inhibitors in culture broth were selected from 142 strains of the isolated bacteria. Among these 5 strains, No. 702 was seemed to have the most strong producing ability of the inhibitor, therefore, was used for the experiment.

2. Taxonomical studies of No. 702

Morphological, cultural and physiological characters of No. 702 were shown in Table 1. Making reference these bacteriological characters to the Bergey's Manual, the authors determined that No. 702 belonged to *Bacillus cereus*. For example, No. 702 was rod-shaped with short chains, Gram-positive, catalase-positive, acethylmethylcarbinol-positive, aerobic, amino acids necessary for growth, fermentations of various sugars and so forth.

3. Examination on the conditions of cultivation

1) Source of carbon

In order to determine suitable carbon-source for the production of the inhibitor, various carbon compounds were added to Medium-1 which contained no both agar and glucose. One millilitre of pre-culture, which was cultivated in agar free Medium-1 at 30 C for 24 hrs with shaking, was inoculated in 100 ml of test media in 500 ml fiasks and cultured at 30 C with shaking. Each culture was stopped with 24 hrs intervals for 4 days and cetrifuged at 3000 r. p. m for 20 min.

Table 1 Morphological and physiological characters of No. 702.

Cell ; Rod, 0.8 to 1.0 by 2.0 to 3.0 microns with squre end, in short chains
Spore ; One to 1.5 microns, ellipsoidal, central or paracentral
Agar colonies ; Large, rough, flat, irregular with whiplike out-growth, whitish dull with characteristic mottled appearance
Agar slants ; Growth aboudant, rough, opaque, whitish, non-adherent, spreading, edge irregular with whiplike out-growth, covered with film
Glucose agar slants ; Identical with agar slants except growth more aboundant and spr-eading than agar slants
Glucose nitrate agar slants ; Non growth, amino acids necessary for growth
Broth ; Heavy, uniform turbidity with soft, easily dispersed sediment, without ring pellicle
Potato ; Growth aboundant, thick, spreading, soft, creamy white
Litomus milk ; Rapid peptonization, without coagulation
Gelatin stability ; Rapid liquefaction
Gram ; Positive
Mobility ; Positive
Production of catalase ; Positive
Methyl red test ; Positive
Acethylmethylcarbinol test ; Positive
Hydrolysis of starch ; Positive
Reduction of nitrate ; Positive
Demand of oxgen ; Positive (aerobic)
Fermentations of sugars ; Positive-glucose, sucrose and starch, Negative-lactose and mannitol

The supernatant diluted 10-fold with distilled water was used for the demonstration of trypsin inhibiting activity.

The effect of carbon-sources on the production of the inhibitor was shown in Table 2. From the Table, it could be noted that the production of the inhibitor was markedly affected by these carbon-sources and that the suitable concentration of glucose was 1.5-3.0 % (w/w). Fermentative carbohydrates such as sucrose and

Table 2 Effect of carbon-sources for the production of the inhibitor.

Carbon source	Content (%)*	Cultivation time (hr)	Cell yield**	24 hr. and Final pH	Inhibitory activity (%)
O	—	48	2.6	7.8, 8.5	13
Glucose	0.3	24	5.1	7.7, 7.7	30
Glucose	1.5	48	6.0	6.0, 7.8	60
Glucose	3.0	72	9.0	6.0, 7.5	61
Glucose	4.5	72	4.0	6.0, 4.9	27
Sodium citrate	1.5	24	2.9	7.8, 7.8	19
Sucrose	1.5	48	7.8	6.1, 7.5	61
Starch	1.5	48	6.5	6.1, 7.7	57
Lactose	1.5	48	2.7	7.8, 8.2	15

* Nutrient broth consists of peptone 3.0%, meat extract 0.3%, NaCl 0.1% KH_2PO_4 0.1% and $MgSO_4$ $7H_2O$ 0.05% (pH 7.0).

** Wet wt. g/100 ml

starch were very effective as much as glucose for the production of the inhibitor. On the other hand, lactose, which was non-fermentative sugar for this strain, and sodium citrate were not so effective.

2) Source of nitrogen

The effect of nitrogen sources was tested by addition of several kinds of nitrogen-sources to the medium containing 1.5 % glucose, 0.1 % NaCl, 0.1 % KH_2PO_4 and 0.05 % $MgSO_4 \cdot 7H_2O$ (pH 7.0). The methods of cultivation and sampling were essentially same as those described in item 1).

The effect of nitrogen-sources on the production of the inhibitor was shown in Table 3. The production of the inhibitor was affected markedly by concentration of peptone. Inhibiting activity increased progressively with the concentration of peptone and the optimum concentration was found to be 4.5 %. Suitable nitrogen-source for the production of the inhibitor was the mixture of 3.0 % peptone and 1.5 % casamino acid (casein hydrolyzates). On the other hand, inorganic nitrogen-source was not suitable for the production of the inhibitor.

Table 3 Effect of nitrogen-sources for the production of the inhibitor.

Nitrogen source*	Content (%)**	Cultivauion time (hr)	Cell yield***	Final pH	Inhibitory activity (%)
Ammo. sul.	1.0	—	—	—	—
Peptone	0.5	—	—	—	—
Peptone	1.5	24	3.8	6.6	16
Peptone	3.0	48	5.7	7.6	59
Peptone	4.5	48	6.7	7.7	69
Casamino acid	4.5	48	4.0	7.3	72
Meat ex.	4.5	48	6.0	7.5	46
Pep : Casamino aeid	3.0 : 1.5	48	6.0	7.3	75
Pep : Meat ex.	3.0 : 1.5	48	6.9	7.6	68
Pep : Casamino acid : Meat ex.	1.5 : 1.5 : 1.5	48	6.0	7.5	66

* Ammo. sul. : Ammonium sulfate, Casamino acid : Casein-hydrolyzates Meat ex. : Meat extract, Pep : Peptone

** Nutrient broth consists of glucose 1.5 %, NaCl 0.1%, KH_2PO_4 0.1 % and $MgSO_4 \cdot 7H_2O$ 0.05 % (pH 7.0).

*** Wet wt. g/100 ml

3) Effect of various salts

The effect of various salts was tested by addition of several kinds of salts to the medium containing 1.5 % glucose, 3.0 % peptone and 1.5 % casamino acid (pH 7.0). Significant increment on production of the inhibitor was not recognized by the addition of any kinds of salts tested, and it was concluded that the addition of salts was not necessary for the production of the inhibitor.

4. Purification of the inhibitor

According to the results mentioned above, the optimum condition for the

production of the inhibitor was determined as follows :

Medium composition; Glucose 1.5 %, Peptone 3.0 % and Casamino acid 1.5 %,

Initial pH 7.0, Temperature; 30 C, Shaking condition; 140 r. p. m (100 ml medium in a 500 ml flask)

No. 702 strain was cultivated under the optimum condition, and inhibiting activity, pH of the broth and cell yield (wet weight, g/100 ml) after varing cultivation time were tested.

The time cource of cultivation and production of the inhibitor were shown in Fig. 1. From this Figure, it was noted that the dominant inhibitor production

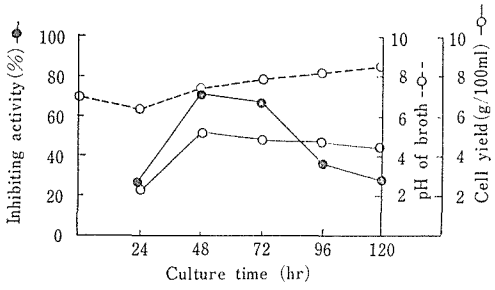


Fig. 1 Cnanges of inhibiting activity, pH of the broth and cell yield during cultivation at 30 C.

was generally occured in 48 hrs. Therefore, the culture broth was taken at 48 hrs and centrifuged at 3000 r. p. m for 20 min. pH of the cell-free supernatant solution was adjusted to 8.0. Active carbon was added to the solution with stirring. After 30 min the solution was filtered with Toyo No. 2 filter paper. The active carbon was washed with 0.1 N-HCl. Then, the inhibitor adsorbed on the active carbon was extracted with 0.5 N-HCl : 99 % ethanol solution (1 : 4). pH of the extracted solution was adjusted to 6.0. The solution was filtered with Toyo No. 2 filter paper and the filtrate was concentrated in vacuo at 37 C. Deep brown syrup was obtained.

The deep brown syrup was dissolved in 99 % ethanol and filtered with Toyo No. 2 filter paper. The precipitate was dissolved again in 99 % ethanol. The solution was filtered in the same way. The first and the second filtrates were pooled, and dried up in vacuo at 37 C. Brown powder was obtained.

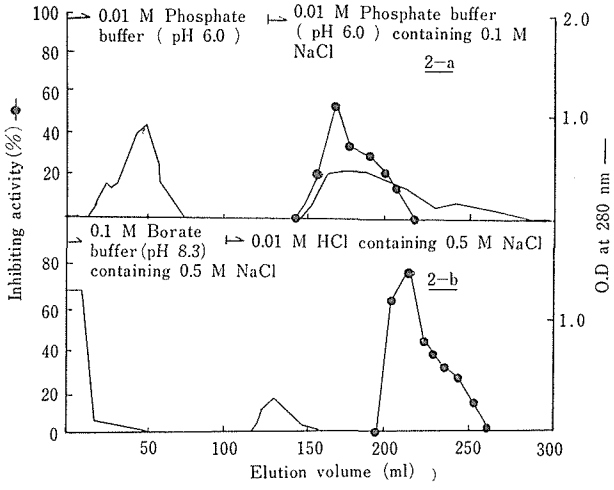
The powder was dissolved in distilled water. The solution was applied to a Sephadex G-10 column equilibrated with distilled water and eluted with the same water. The inhibitor eluted after the void volume, and the active fractions were pooled and concentrated in vacuo at 37 C.

The concentrated solution was applied to a DEAE cellulose column equilibrated

with 0.01 M phosphate buffer, pH 7.8 and eluted with the same buffer. The inhibitor did not adsorbed on the DEAE cellulose, and the active fractions were pooled and concentrated in vacuo at 37 C. Yellow syrup was obtained.

The yellow syrup was dissolved in 99 % ethanol and desalted with a Sephadex LH-20 column chromatography (3 × 45 cm, developed with 99 % ethanol). The desalted inhibitor solution was dried up in vacuo at 37 C. The inhibitor was dissolved in 0.01 M phosphate buffer, pH 6.0. The solution was applied to a CM Sephadex C-25 column equilibrated with the same buffer, and developed stepwise with both 0.01 M phosphate buffer, pH 6.0 and the same buffer containing 0.1 M NaCl. A typical chromatogram was shown in Fig. 2-a. The inhibitor was adsorbed on the CM Sephadex C-25 with 0.01 M phosphate buffer, pH 6.0 and eluted wifh the same buffer containing 0.1 M NaCl. The active fractions were pooled and concentrated in vacuo at 37 C. Light yellow syrup was obtained.

The syrup was desalted with a Sephadex LH-20 in the same way described above. The inhibitor was dissolved in 0.1 M borate buffer containing 0.5 M NaCl, pH 8.3. The solution was applied to the insolubilised trypsin column equilibrated with the same buffer, and developed stepwise with both the same buffer and 0.01 M HCl. A typical chromatogram was shown in Fig. 2-b. The inhibitor was adsorbed on the insolubilised trypsin with 0.1 M borate buffer and eluted with 0.01 M HCl. The active fractions were pooled and dried up in vacuo at 37 C. White powder was obtained.



2-a ; Column : 1.5 × 30 cm, Speed : 50 ml/hr
2-b ; Column : 1.6 × 10 cm, Speed : 90 ml/hr

Fig. 2 Elution patterns of the inhibitor on CM Sephadex C-25 (2-a) and trypsin-Sepharose 4B column (2-b).

The powder obtained was dissolved in 99 % ethanol and desalted with Sephadex LH-20 in the same way described above. The desalted inhibitor solution was freeze-dried. White powder was obtained.

The above purification procedure was summarized schematically in Table 4.

Table 4 Scheme of purification of the inhibitor.

Culture broth
↓ centrifuged
Supernatant
↓ adsorbed in active carbon, extracted with 0.5 N-HCl : ethanol (1 : 4)
Extracted solution
↓ concentrated at 37 C in vacuo, extracted with ethanol
Extracted solution
↓ dried up at 37 C in vacuo, dissolved in distilled water and applied to Sephadex G-10 column
Active fraction
↓ concentrated at 37 C in vacuo and applied to DEAE cellulose column
Active fraction
↓ concentrated at 37 C in vacuo, desalted with Sephadex LH-20 column chromatography and applied to CM Sephadex C-25 column
Active fraction
↓ concentrated at 37 C in vacuo, desalted with Sephadex LH-20 column chromatography and applied to insolubilised trypsin column
Active fraction
↓ dried up at 37 C in vacuo, desalted with Sephadex LH-20 column chromatography and freeze-dried
Purified inhibitor

5. Some properties of the purified inhibitor

1) Molecular weight

Molecular weight of the inhibitor was speculated below 500 from the results of gel filtration with Sephadex G-10 and membrane filter (Amicon, UM 05).

2) Heat stability

pH of the inhibitor solution was adjusted to various values ranged from 1 to 13 and heated in boiling water for 20 min. After the treatment the residual trypsin inhibiting activity was measured. The inhibitor was quite stable at pH's from 1 to 3 and relatively stable at pH's from 4 to 9.

3) Solubility on various organic solvents

The inhibitor was soluble in methanol, ethanol, and buthanol, and insoluble in ethylacetate, benzene, acetone, ether and chloroform.

4) Inhibition against various proteases

Inhibiting test against various proteases was carried out at optimum pH of each protease. The results were shown in Table 5. From this Table, it is noted that papain was inhibited as well as trypsin, and α-chymotrypsin, pepsin, Pronase and

Table 5 Effect of the inhibitor on various proteases.

Protease	pH of reaction	Inhibition*
Trypsin	7.6	+
α-Chymotrypsin	7.6	−
Pepsin	1.6	−
Pronase	7.0	−
Nagase	9.0	−
Papain	7.0	+

* + ; inhibited, − ; not inhibited

Nagase were not inhibited. On the other hand, the inhibiting pattern against trypsin was seemed to be competitive inhibition.

5) Antibiotic activity

The inhibitor had not antibiotic activity against *Bacillus cereus* IFO 3091, *B. subtilis* IFO 3007, *Aerobactor aerogenus* IFO 3319*, *Corynebacterium equi* 7 AM 1038, *Esherishia coli* K-12, *Aspergillus oryzae* chosen B, *Candida muscorum* and *Rhodotorula mucilaginosa.*

ARIMA *et al.*[14] reported a trypsin inhibitor from *Bacillus sp.* However, it was quite different from the inhibitor obtained in this experiment, because the former was a protein with molecular weight above 10000 and the later was a heat stable substance with molecular weight below 500.

On the other hand, the inhibitor which we obtained in this experiment considerably resembles to leupeptins produced by actinomycetes[10], that is, their molecular weight and their inhibition spectra aginst various proteases were found to be essentially the same.

Recently, leupeptins were attempted to be used for the medical supplies because they inhibited the kinin-formation, turmorigenesis, and etc. If the inhibitor isolated by us is improved to be similar biological activities with those of leupeptins, *B. cereus* will be better source for the isolation of leupeptins-like protease inhibitor, because the cultivation time of *B. cereus* for the production of the inhibitor is generally shorter than that of actinomycetes[17].

**Aerobactor aerogenus* : This bacterium is now called *Enterobacter aerogenus*[10].

Acknowledgement

The authors wish to express their thanks to Dr. A. Hosono for his kind suggestions during this work.

A part of this work presented at the Annual Meeting of Agricultural Chemical Society of Japan at Tokyo, April 2, 1973.

Summary

For the purpose to obtain a bacterium which is capable of producing a trypsin inhibitor, screening test was carried out.

1) Culture filtrate of No. 702 strain showed the strong inhibiting activity. Studing of morphological and physiological characteristics of the bacterium, the authors found that the strain belonged to *Bacillus cereus*.

2) The strain produced the inhibitor maximally, when it was cultivated at 30 C in a medium containing 3.0 % peptone, 1.5 % casamino acid and 1.5 % glucose (pH 7.0) for 48 hrs.

3) The inhibitor was purified with several column chromatography. Molecular weight of the purified inhibitor was below 500.

4) The inhibitor inhibited papain as well as trypsin but not α-chymotrypsin, pepsin, Nagase and Pronase, and had not antibiotic activity against various microorganisms. The pattern of inhibition against trypsin was seemed to be competitive inhibition.

5) The inhibitor obtained in this experiment was quite different in its properties from a protease inhibitor produced by *Bacillus sp.* which had been reported by ARIMA *et al.* and found to rather resemble to those of leupeptins produced by actinomycetes.

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Bacillus cereus の生産するプロテアーゼインヒビターの諸性質

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要　　約

土壌を分離源として得た142株から，トリプシンインヒビター生産性細菌1株を得た。その菌株の同定，さらに至適培養条件，生産インヒビターの精製法およびその性質について検討を行なった。結果は次の如く要約される。

1　本菌株は *Bacillus cereus* と同定した。

2　この*B. cereus* はグルコース1.5%，ペプトン3.0%およびカザミノ酸1.5%（初発pH7.0）よりなる培地100mlを500ml容フラスコで30°C，48時間の振盪培養（140r. p. m）において最もインヒビター生産が高い。

3　本菌株により生産されたトリプシンインヒビターは，その培養ロ液より活性炭処理，エタノール抽出さらに Sephaclex G-10，DEAE セルロース，CM Sephadex C-25 および不溶性トリプシンを用いたアフィニティーなどのカラムクロマトグラフィーにより精製した。

4　この精製したインヒビターは分子量500以下の物質で，メタノール，エタノールおよびブタノールに可溶，酢酸エチル，ベンゼン，アセトン，エーテルおよびクロロホルムに不溶であった。また，20分間の煮沸に対して，pH 1～3で安定，pH 4～9で比較的安定であった。

5　本インヒビターはさらに，トリプシンの他にパパインを活性阻害し，α-キモトリプシン，ペプシン，プロナーゼおよびナガーゼを阻害しなかった。また，各種細菌，カビ，酵母に対する抗菌作用は認められなかった。なお，トリプシン阻害は拮抗型阻害と推定した。

6　以上の結果から，本菌株の生産するプロテアーゼインヒビターは，既報の *Bacillus* 属生産蛋白態トリプシンインヒビターとは明らかに異なり，むしろ放線菌の培養ロ液より得られたロイペプチンと類似した点が多い。