

Characters of Proteinase Inhibitor Isolated from *Streptomyces fradiae*

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Streptomyces fradiae 에서 분리한 단백질 분해효소저해물질의 특성

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ABSTRACT: The objective of the current study is to elucidate the biological roles of proteinase inhibitor in microorganisms. As the first step, a strain of *Streptomyces fradiae* was selected as a producer of extracellular proteinase inhibitor. The proteinase inhibitor was purified from culture broth through ultrafiltration, gel-filtration and ion-exchange chromatography. Molecular weight of the proteinase inhibitor was estimated to be 16,800 by SDS polyacrylamide gel electrophoresis. It was found that the proteinase inhibitor inhibited only alkaline serine proteinases such as subtilisin, α -chymotrypsin and Pronase E but not trypsin and other proteinases. The mode of inhibition against Pronase E with succinyl-phenylalanine-p-nitroanilide as a substrate was competitive.

KEY WORDS: Proteinase inhibitor, *Streptomyces fradiae*

It has been reported that proteinase inhibitors play very important roles in animals, plants and microorganisms (Umezawa and Aoyagi, 1983; Imada *et al.*, 1985; Barrett and Salvessen, 1986; Kundu and Sinha, 1989). Serine proteinase inhibitor (serpine) was reported to be a protein that functions in controlling the action of serine proteinase in many diverse physiological processes (Hill and Hastie, 1987). *Streptomyces subtilisin inhibitor* (SSI) was also a proteinaceous proteinase inhibitor (Murao *et al.*, 1972) that had very strong inhibitory activity against alkaline proteinases involved essentially in endogenous metabolism (Hill and Hastie, 1987; Obata *et al.*, 1989). Other proteinase inhibitor produced by *Coccidioides immitis* was thought to play important roles in regulation of the activity of the cell-wall-associated proteinase during sporulation (Yuan *et al.*, 1989). Therefore it was thought that the proteinase inhibitors were very useful in the elucidation of various biological processes, hence the three dimensional structure-function relationship

between proteinase and the inhibitor was well studied (Mitsui *et al.*, 1977; Obata *et al.*, 1989).

In this context, we are trying to investigate the biological roles of the proteinase inhibitor in *Streptomyces* spp. in conjunction with cell differentiation. As the first step, the characters of proteinase inhibitor isolated from *Streptomyces fradiae* were investigated.

MATERIALS AND METHODS

Microorganisms and maintenance

Streptomyces spp. collected from Korean Collection for Type Culture (KCTC) and those isolated from soil samples were used for the selection of microorganisms producing proteinase inhibitor. *Streptomyces* sp. SMF301 was used as a producer of extracellular proteinase for the selection of microorganisms producing proteinase inhibitor (Shin and Lee, 1986). All strains were stocked on rich media formulated as following; 1% glucose, 0.2% peptone,

0.3% yeast extract, 0.1% beef extract and 1.5% agar (pH 7.2 before sterilization).

Selection of microorganism producing proteinase inhibitor

Streptomyces sp. SMF301 was inoculated at the center of agar plate containing skim milk and incubated for 2 days at 30 °C as previous report (Jeong, 1988). Four agar pieces of proteinase inhibitor producing medium (3 mm in thickness and 5 mm in radius) were placed on the plate distanced 3 cm far from the center. Then the collected *Streptomyces* spp. were inoculated on the top surface of the agar pieces and incubated for 5 days at 30 °C. Microorganisms showing strong inhibition to the formation of clear zone, which was resulted from the hydrolysis of casein by the proteolytic enzyme, were selected as microorganisms producing proteinase inhibitor.

Culture conditions

The stock culture was transferred into a proteinase inhibitor producing medium formulated as following; 1% glucose, 2% peptone, 0.3% yeast extract, 0.3% NaCl, 0.1% KH₂PO₄, 0.34% K₂HPO₄, 0.02% FeSO₄·7H₂O, 0.02% ZnSO₄·7H₂O. The seed culture was carried out in a shaking incubator at 30 °C for 3 days and then inoculated into a fermentor (Chemap CF) with 5% inoculum size. Temperature and pH were maintained to 30 °C and pH7.0, respectively. Aeration was controlled to give 0.2 vvm and agitation speed was fixed at 300 rpm.

Analytical methods

Cell mass in the main culture broth was determined as dried cell weight (DCW) after drying at 80 °C for 24 hours. The concentration of glucose was estimated by the dinitrosalicylic acid method (Miller, 1959) and the concentration of protein was estimated by the Bradford method (Bradford, 1976). The activity of proteinase was estimated by measuring tyrosine liberated after hydrolysis of Hammerstein casein at 37 °C and pH 7.5 for 10 minutes. The 1 unit of proteinase was defined as the amount needed for the production of 1 μmol of tyrosine per minute.

The proteinase inhibitory activity was calculated by Equation 1 and 1 unit of inhibitory activity was defined as the amount of inhibitor needed for the 50% inhibition of 0.6 unit of proase E (from *Streptomyces griseus*, Sigma)

$$\left[1 - \frac{\text{proteinase activity with inhibitor}}{\text{proteinase activity without inhibitor}} \right] \times 100$$

Equation 1

Purification of proteinase inhibitor

After 60 hours culture, the culture broth was harvested by centrifugation (10,000 × g) and cell free culture broth was concentrated by ultrafiltration (NMWL 10,000 hollowfiber, Amicon DC-10) to give 1/5 volume. Cold acetone was added to the concentrated broth to make 40% (v/v) saturation and the precipitate formed was separated by centrifugation (10,000 × g, 15 min) at 4 °C. After centrifugation, cold acetone was added to the supernatant to make 70% saturation and then centrifuged (10,000 × g, 30 min). The precipitate collected from 40-70% acetone fraction was found to be active, hence, used further purification.

The acetone precipitate was resolved in 0.02 M NH₄-acetate buffer (pH 7.0) and loaded on ion exchange column (5 × 10 cm) packed with DEAE-Sephacel (Pharmacia) and washed with 2 volumes of the same buffer. The following elution was carried out with 0.02 M NH₄-acetate buffer with NaCl to make 0 to 0.75 M gradient. Elution rate was 60 ml/hr and fraction volume was 10 ml.

The active fractions were collected, dialysed, concentrated and loaded on Sephadex G-75 (Pharmacia) column (2.8 × 100 cm) equilibrated with 0.02 M NH₄-acetate buffer (pH 7.0) containing 0.1 M NaCl. The elution was carried out with the same buffer. The elution rate was 18 ml/hr and fraction volume was 10 ml. The active fractions were collected, concentrated and carried out chromatography on Sephadex G-50 gel column (2.8 × 90 cm) equilibrated with 0.02 M NH₄-acetate buffer (pH 7.0). The purity of the purified inhibitor was checked by the SDS-polyacrylamide gel electrophoresis described by Laemmli (1970).

RESULTS AND DISCUSSIONS

Selections microorganisms producing proteinase inhibitor

Over 500 strains of *Streptomyces* spp. obtained from KCTC and soil samples were tested to select strains producing proteinase inhibitors. As a result, *Streptomyces fradiae* NRRL2702 was found to produce extracellular proteinase inhibitor. Fig. 1 showed the typical pattern of competitive antimetabolic activity.

Batch culture

Batch culture data for the changes in the concentrations of glucose, biomass and proteinase inhibitor activity are shown in Fig. 2. The change of culture pH and dissolved oxygen tension are also shown. It

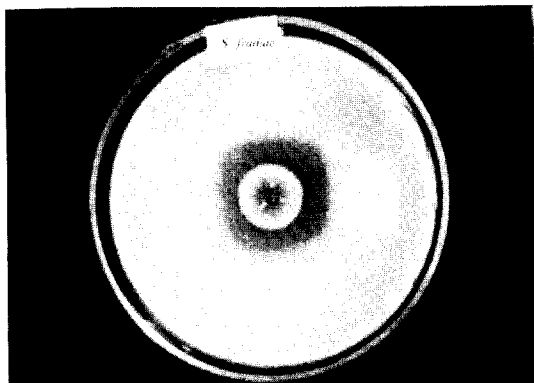


Fig. 1. Proteinase inhibition on skim milk agar. Center colony is SMF301 and four colonies around SMF301 are *S. fradiae*.

was found that the production of proteinase inhibitor was related very closely to the formation of biomass. Dissolved oxygen tension was reduced quickly as the glucose consumption was initiated and it was very interesting that the proteinase inhibitor biosynthesis was initiated when the DOT was relatively lower than 20%. It was therefore thought that more precise kinetic study was necessary to understand the relationship among substrate utilization, cell growth and the proteinase inhibitor production.

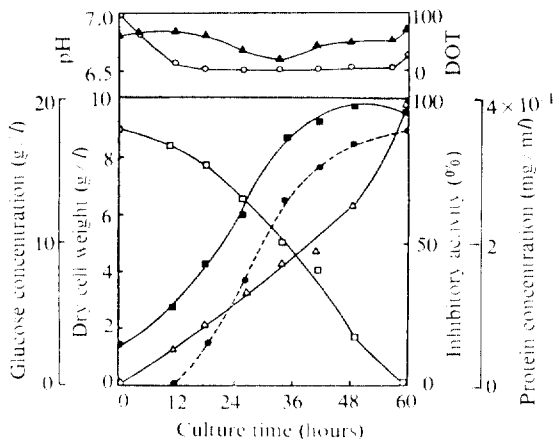


Fig. 2. The changes of glucose, dissolved oxygen tension, biomass, proteinase inhibitor, protein concentrations and pH in the batch culture of *Streptomyces fradiae*. Symbols: Glucose (—○—), D.O.T. (○—○), Biomass (■—■), Proteinase inhibitor (●—●), Protein (△—△), pH (▲—▲).

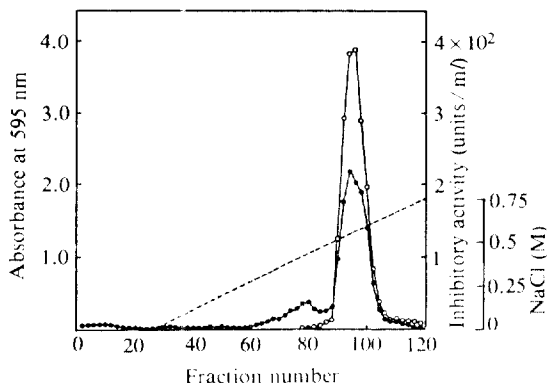


Fig. 3. Ion exchange chromatography of proteinase inhibitor on DEAE-Sephacel.

The sample solution was applied to the column (Φ 5 × 11 cm) equilibrated with 0.02 M ammonium acetate buffer (pH 7.5). The column was then washed with the same buffer and eluted with a 1000 ml/linear gradient of NaCl (0.0—0.75 M) in the same buffer. Fractions of 10 ml were collected at a flow rate of 60 ml/hr. Symbols: Protein (●—●), Inhibitory activity (○—○), and NaCl gradient (-----).

Purification of proteinase inhibitor

When the proteinase inhibitory activity reached maximum, the culture broth was harvested by centrifugation at 4°C and the free culture broth was concentrated by ultrafiltration to give 1/5 of the original volume. The concentrated broth was mixed with cold acetone and active fraction was obtained from 40% to 70% acetone saturation. DEAE-Sephacel ion-exchange chromatography of the active fraction was performed with NaCl gradient from 0 M at the beginning to 0.75 M at the end. As shown in Fig. 3, it was clear that the active proteinase inhibitor was begun to be eluted from NaCl 0.5 M. The active fraction was collected and NaCl was removed through counter-flow dialysis. Then, the active fraction was concentrated and gel filtration using Sephadex G-75 and Sephadex G-50 was performed. Fig. 4 and 5 show that the protein fraction and the proteinase inhibitory activity were closely related. The purification steps were checked with SDS-PAGE and the results are shown in Fig. 6. The results indicated that proteinase inhibitor obtained through the purification steps was pure and that the molecular weight was estimated to be 16,800. The purification fold and final yield were 3.1 and 28.1%, respectively (Table I).

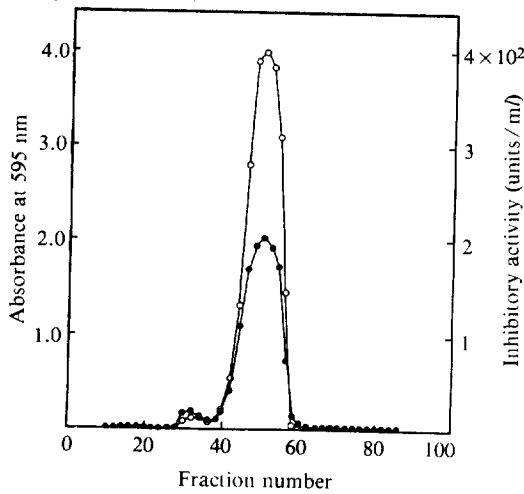


Fig. 4. Gel filtration of proteinase inhibitor on Sephadex G-75.

The sample solution was applied to the column ($\Phi 2.8 \times 100$ cm) equilibrated with 0.02 M ammonium acetate buffer (pH 7.0) containing 0.1 M NaCl and then eluted with the same buffer. The flow rate was 18 ml/hr and fractions of 6 ml were collected. Symbols: Protein (●—●), Inhibitory activity (○—○).

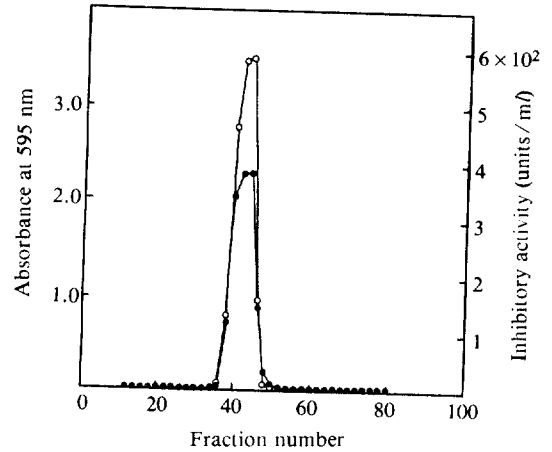


Fig. 5. Gel filtration of proteinase inhibitor on Sephadex G-50 after Sephadex G-75 gel filtration.

The sample solution was applied to the column ($\Phi 2.8 \times 90$ cm) equilibrated with 0.02 M ammonium acetate buffer and then eluted with the same buffer. The flow rate was 18 ml/hr and fractions of 6 ml were collected. Symbols: Protein (●—●), Inhibitory activity (○—○).

Characters of proteinase inhibitor

The inhibition spectrum of the purified proteinase inhibitor to various proteinases was tested. As shown in Table 2, it revealed that the proteinase inhibitor had inhibitory activities against only alkaline serine proteinases viz. α -chymotrypsin, subtilisin, and pronase E but did not inhibit the activities of cysteine proteinases, metallo proteinases, and aspartic proteinases. At present results, the inhibition specificity of the isolated inhibitor was not clear, but it was thought that the inhibitor might be synthesized for the specific regulation of alkaline proteinase activity in endogenous metabolism and cell differentiation.

In order to know the inhibition mode of the pro-

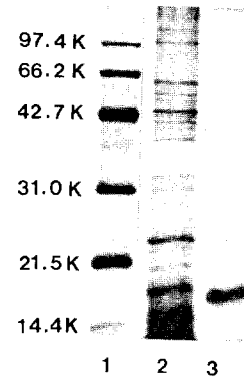


Fig. 6. SDS-polyacrylamide gel electrophoresis of proteinase inhibitor.

Lane 1: molecular weight marker

Lane 2: culture broth

Lane 3: purified proteinase inhibitor.

Table 1. Purification of proteinase inhibitor

| Purification step | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Recovery (%) | Fold |
|-----------------------|------------------------|--------------------|------------------------------|--------------|------|
| Culture broth | 316,800 | 4,620 | 68.6 | 100 | 1 |
| Ultrafiltration | 260,000 | 2,860 | 90.0 | 82.1 | 1.3 |
| Acetone fractionation | 171,136 | 1,121 | 152.6 | 54.0 | 2.2 |
| DEAE-Sephacel | 132,000 | 739 | 178.6 | 41.7 | 2.6 |
| Sephadex G-75 | 91,454 | 500 | 182.9 | 28.9 | 2.7 |
| Sephadex G-50 | 89,210 | 422 | 211.4 | 28.2 | 3.1 |

Table 2. Inhibitory spectrum of proteinase inhibitor produced by *Streptomyces fradiae* on various proteinase

| Proteinase | Substrate | Inhibition |
|--------------------|--------------|------------|
| Serine proteinase | | |
| Trypsin | casein | - |
| | bz-arg-4-na | - |
| -Chymotrypsin | casein | + |
| | suc-phe-4-na | + |
| Pronase E | casein | + |
| | suc-phe-4-na | + |
| Subtilisin | casein | + |
| Thiol proteinase | | |
| Papain | casein | - |
| Metallo proteinase | | |
| Thermolysin | casein | - |
| Acid proteinase | | |
| Pepsin | haemoglobin | - |
| Asp. saitoi | haemoglobin | - |

* + : More than 50%, - : less than 10% inhibition bz-arg-4-na: benzyl-arginine-*p*-nitroanilide suc-phe-4-na: succinyl-phenylalanine-*p*-nitroanilide.

teinase inhibitor, the activity of pronase E on a synthetic substrate, succinyl-phenylalanine-*p*-nitroanilide, was tested with different concentrations of proteinase inhibitor. The V_{max} and K_m values of the pronase E on the synthetic substrate were estimated to be 4.6 $\mu\text{mol/h}$ and 3.52 mM, respectively. As shown in Fig. 7, it was clear that the activity of the Pronase E was competitively inhibited as increasing the concentration of the inhibitor, therefore, it was concluded that the inhibition mode of the proteinase inhibitor was competitive. The inhibition constant (K_i) was calculated as 1.7 μM .

The proteinase inhibitor was highly thermostable

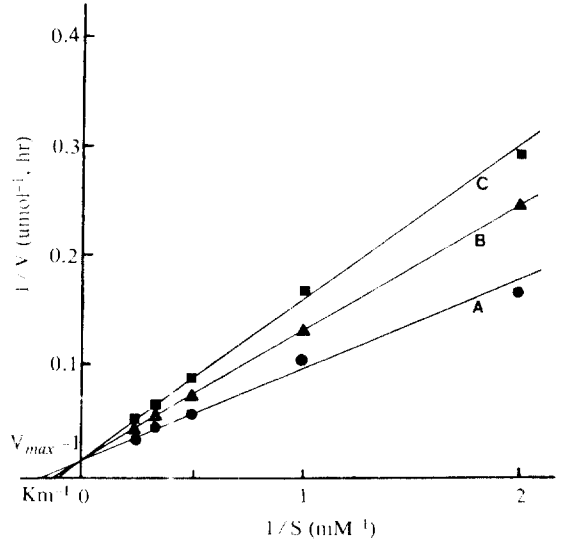


Fig. 7. Type of inhibition against pronase E by proteinase inhibitor Lineweaver-Burk plot of succinyl-phenylalanine-*p*-nitroanilide concentration against activity of pronase E in the presence and in the absence of the proteinase inhibitor.

A = in the absence of proteinase inhibitor
 B = in the presence of 12.5 $\mu\text{l/ml}$ inhibitor
 C = in the presence of 25.0 $\mu\text{l/ml}$ inhibitor

because it had 85% of initial inhibitory activity after boiling at 100°C for 2 hr.

From the presented results, we found that *Streptomyces fradiae* NRRL2702 produced proteinase inhibitor at very restricted culture condition, viz. limitation of dissolved oxygen tension. Furthermore it was evident that the strain produced proteinase instead of proteinase inhibitor at the other conditions. Although we did not elucidate the molecular mechanisms which can switch on synthesis either proteinase or the proteinase inhibitor, we have a certainty that the proteinase inhibitor play an important role in the diversity of physiological processes.

적 요

방선균의 세포분해와 관련된 연구의 일환으로 *Streptomyces fradiae* NRRL2702가 생성하는 단백질분해효소 저해물질을 분리정제하여 그 특성을 분석하였다. 즉 *S. fradiae*는 일반 환경 조건하에서 단백질분해효소를 생성하나 특정 조건하에서는 그 단백질의 활성을 저해하는 저해제를 생성함을 알았다. 이 저해제의 분자량은 16,800이며 serine proteinase의 일부만을 저해하는 특성이 있었다. Pronase E의 활성의 저해양상은 competitive inhibition 이었고 열에 대하여 매우 안정함을 알았다.

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