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# Protease Inhibitor Production using *Streptomyces* sp. SMF13

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**The aim of the current study is to evaluate the effects of medium compositions on the production of protease inhibitor in *Streptomyces* sp. SMF13. The production of protease inhibitor was countercurrently linked to extra-cellular protease, which were regulated by the culture conditions. Nitrogen source was the most critical ingredient affecting the production of protease inhibitor and protease. Carbon source was an important factor to determine the culture pH which affected very clearly the formation of protease and protease inhibitor. Inorganic phosphate inhibited the protease inhibitor production which was linked to the cell growth rate, although the optimal conditions for the production of protease inhibitor were not favouring to the cell growth.**

Microbial proteases and protease inhibitors have been interesting due to the biological roles in the producing microorganisms (1, 7, 8, 11, 15, 17). The most interesting function of microbial protease inhibitor is the regulation of various proteolytic enzymes involved in many diverse physiolgical processes, such as cell differentiation and cellular protein turnover (2, 4, 16, 20). The protease inhibitor produced by *Coccidioides immitis* played important roles in regulation of the activity of protease associated in cell wall during sporulation (21). It has been reported that *Streptomyces* polymorphism is closely related with the formation of proteases, of which activity may be regulated by the endogeneous inhibitors (3, 6). In addition, the protease inhibitors have been focussed as new and potential biological active substances (5, 13, 14, 18, 19).

The objective of the current study is to elucidate the biological roles of extracellular low molecular weight protease inhibitor in *Streptomyces* spp. As the first step, *Streptomyces* sp. SMF13 producing thiol protease inhibitor was isolated and the properies of inhibitor were characeterized (9). In this report, optimal culture conditions for the fermentation of the protease inhibitor were evaluated.

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Key words: Protease inhibitor, *Streptomyces*, fermentation kinetics, environmental condition

## MATERIALS AND METHODS

### Microorganism and Media Used

The microorganism used in this study was *Streptomyces* sp. SMF13 isolated from soil in Korea. The characters were reported in elsewhere (10). The strain was maintained by transferring to fresh stock culture media each month and storing at 10℃. Stock culture medium was prepared with 10 g glucose, 2 g peptone, 3 g yeast extract, 1 g beef extract, and 18 g agar in 1 liter of distilled water (pH 7.0). Seed culture medium was formulated with 30 g glucose, 18 g soytone, 3 g peptone, and 4 g $CaCO_3$ in 1 liter of distilled water (pH 7.0). Basal components of fermentation medium was followed as: 1 g $KH_2PO_4$, 3.4 g $K_2HPO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g $ZnCl_2$, 0.01 g $CuSO_4 \cdot 7H_2O$, 0.003 g $MnCl_2 \cdot 4H_2O$, 0.01 g $CaCl_2$, 0.03 g NaCl in 1 liter of distlled water (pH 7.0). Nitrogen and carbon sources in the fermentation medium were selected for the optimal production of protease inhibitor.

### Culture Conditions

A single colony of *Streptomyces* sp. SMF13 developed on the plate of the stock culture medium was transferred to 50 *ml* of the seed culture medium and incubated in a rotary shaking incubator (150 rpm) for 36 hours at 28℃. The seed culture was inoculated into 2.5 liters of the fermentation medium containing in vessels (5 li-

ters) of jar fermentors (Korea Fermentor Co. Ltd.). Temperature was controlled to 28℃ and culture pH was maintained to 7.0 by addition of 1 N HCl or 1 N NaOH automatically. Agitation and aeration were contolled for the optimal production of protease inhibitor.

**Analytical Methods**

Cell mass was determined as dried cell weight (DCW) after drying at 80℃ for 24 horus. The concentration of glucose was measured by the dinitrosalicylic acid method (12). The activity of protease was estimated by measuring tyrosine liberated after hydrolysis of Hammarsten casein at 37℃ and pH 7.5 phosphate buffer (0.05 M) for 15 minutes. The 1 unit of protease activity was defined as the amount needed for the production of 1 μg of tyrosine per minute. The protease inhibitor activity was calculated as follows:

%Inhibition $= 100 \times (A-B)/A$; where, A is the protease activity without inhibitor and B is the protease activity with the inhibitor. 1 unit of inhibitory activity was defined as the amount of inhibitor needed for the 50% inhibition of 3.6 units of papain (Sigma Co.).

## RESULTS AND DISCUSSION

### Effect of Substrate on Protease Inhibitor Production

The effect of carbon and energy sources on the protease inhibitor production was evaluated with various carbohydrates, as results, glucose was selected as the best substrate for the production of the protease inhibitor. The effect of the initial concentration of glucose on the protease inhibitor production was tested. As showed in Fig. 1, the protease inhibitor production was increased as the increasing glucose concentration up to 30 g/*l*. Further increases in the initial concentration of glucose accompanied the falling of pH which might be resulted from the accumulation of acidic metabolites produced from the glucose catabolism.

The effect of nitrogen source on the production of protease inhibitor was tested again, where 10 g/*l* of each nitrogen source was added as a sole nitrogen source to the basal fermentation medium containing 20 g/*l* of glucose. As showed in Table 1, peptone was found to be the choice of nitrogen source for the production of the protease inhibitor. However, it was very interesting to note that the protease inhibitor was not produced in the medium containing skim milk, but extra-cellular protease was produced on the other hand. In addition, it was found that relatively low level of both molecules were detected from the culture broth where nitrogen source was starved. The data indicated that the biosythesis of protease inhibitor and protease were inter-related as switching on/off system which was closely affected by external environments.

As shown in Fig. 2, biomass formation and protease inhibitor production were enhanced as increase in the concentrations of peptone. The specific productivity of the protease inhibitor production was clearly increased from 1% to 3% peptone and then leveled off thereafter. Final pH of the culture broth was slightly increased in proportional to peptone concentration. It suggested that ammonia ions liberated from the amino acids were accounted for the increase of the final pH of culture broth.

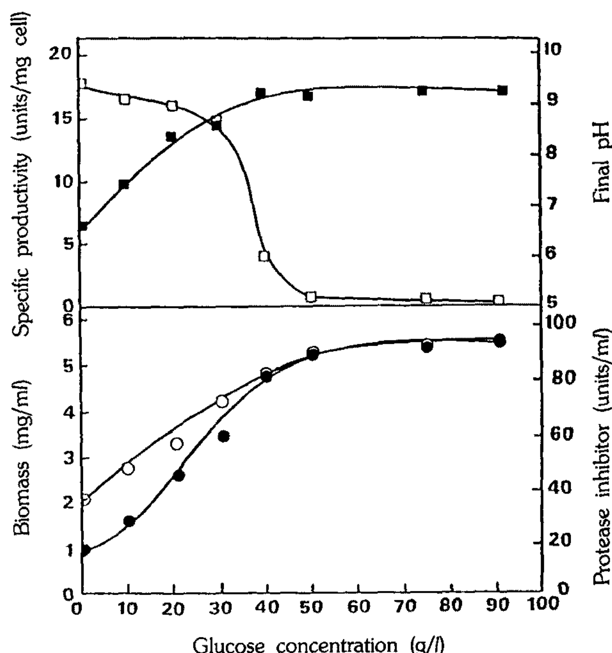


**Fig. 1. Effect of initial glucose concentration on the protease inhibitor production.**

The culture was carried out using the basal medium containing peptone 1% and supplemented with glucose as dictated. After 72 h culture, biomass (○), protease inhibitor (●), final pH (□), and specific productivity (■) were measured.

**Table 1. Effect of nitrogen sources on the production of protease inhibitor and protease in a batch culture using *Streptomyces* sp. SMF13.**

| Nitrogen source | Final pH | Cell growth (mg/m*l*) | Protease inhibitor (units/m*l*) | Protease (units/m*l*) |
|---|---|---|---|---|
| No addition | 7.25 | 0.92 | 3.5 | 24.83 |
| $(NH_4)_2SO_4$ | 4.37 | 5.53 | 1.0 | 0 |
| Peptone | 7.58 | 11.1 | 90.0 | 0 |
| Skim milk | 6.80 | **ND | 0.0 | 112.99 |

*Medium composition: glucose 2%, $KH_2PO_4$ 0.1%, $K_2HPO_4$ 0.34%, $FeSO_4 \cdot 7H_2O$ 0.001% $MgSO_4$ 0.03%, $ZnCl_2$ 0.001%, $CuSO_4 \cdot 7H_2O$ 0.001%, $MnCl_2 \cdot 4H_2O$ 0.0003%, $CaCl_2$ 0.001%, NaCl 0.03%

**ND: Not determined

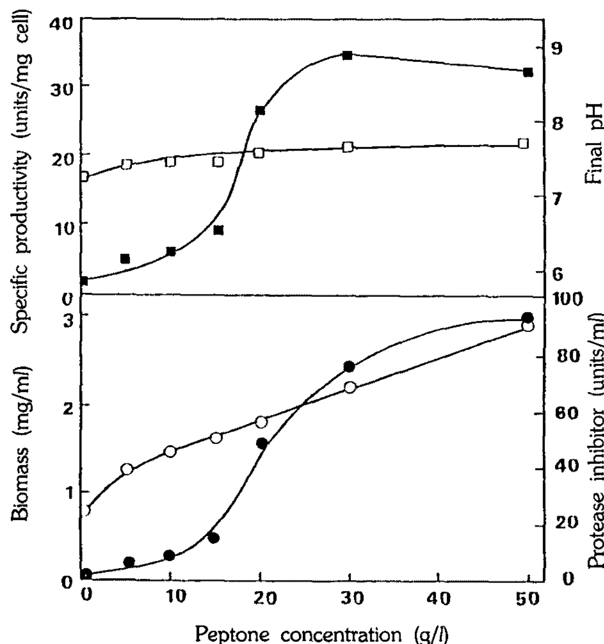


**Fig. 2. Effect of initial peptone concentration on the protease inhibitor production.**

The culture was carried out using the basal medium containing glucose 2% and supplemented with peptone as dictated. After 72 h culture, biomass (○), protease inhibitor (●), final pH (□), and specific productivity (■) were measured.

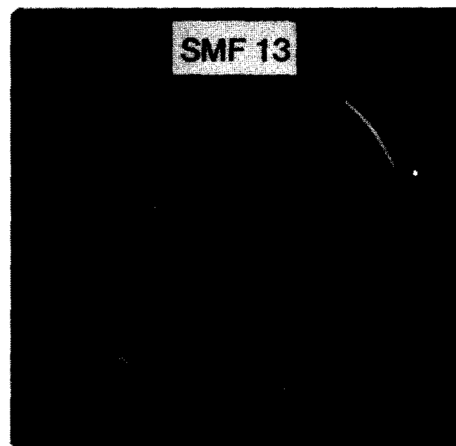


**Fig. 3. Self antimetabolic pattern between protease and protease inhibitor produced by *Streptomyces* sp. SMF13.**

*Streptomyces* sp. SMF13 was inoculated at the center of the plate containing protease production medium and incubated 2 days at 28℃. Again the strain was inoculated on agar pieces (8 mm diameter and 4 mm thickness) of protease inhibitor production medium and incubated 2 days at 28℃ in a humidified chamber. The 4 colonies (indicated as ↓) were loaded on a circle line aparted 3 cm from the center of the plate and incubated further 2 days at 28℃. A square formed between the colonies indicates that protease activity was inhibited by the inhibitor produced from 4 colonies.

## Extra-cellular Inhibition Pattern between Protease Inhibitor and Protese

It was noted that *Streptomyces* sp. SMF13 produced protease inhibitor and protease upon the culture conditions (Table 1), therefore it was necessary to test the inhibition pattern between the extra-cellular molecules. Fig. 3 shows an extra-cellular inhibition pattern between protease inhibitor produced on the solid fermentation medium containing peptone (indicated as ↓) and protease produced on the solid fermentation medium containing skim milk. It was very clear that the protease inhibitor inhibited the protease produced by the same strain at different culture conditions, which indicated that the protease inhibitor was an endogeneous inhibitor. From the data, it was concluded that the protease inhibitor might play important role(s) in post regulation of protease activity in *Streptomyces* spp.

## Effect of Initial pH on Protease Inhibitor Production

The effect of culture pH on the production of protease inhibitor and protease was evaluated. As results, it was found that optimal pH for the cell growth was ranged from 5 to 9 and the culture pH values were biologically adjusted to neutral pH during incubation, whatever the initial pH values were either acidic or alkaline. As showed in Fig. 4, it was clear that protease inhibitor production was increased in alkaline pH, whereas protease was produced more at neutral and acidic culture conditions. From the data obtained in the evaluation of the effects of pH and nutritional conditions, it was thought that the culture pH played a very important role in the synthesis of protease inhibitor and protease.

## Phosphate Inhibition on Protease Inhibitor Production

The effect of inorganic phosphate on protease inhibitor production was evaluated. As shown in Fig. 5, protease inhibitor production was produced at the phosphate limited culture condition and the production was severely inhibited by inorganic phosphate higher than 5 mM, although cell growth was increased. Fig. 6 Shows the effect of inorganic phosphate (25 mM) addition at different growth phases (4, 10, 22 h) in batch culture. It was very clear that the earlier addition of inorganic phosphate caused the more apparent inhibition of the protease inhibitor production. These results suggested that protease inhibitor production in *Streptomyces* sp. SMF13 was regulated by phosphate.

### Effect of Growth Rate on Protease Inhibitor Production

In order to elucidate the relationship between growth rate and protease inhibitor production, continuous culture was carried out with the medium which was optimized by the previous experiments. Steady-state concentrations of biomass, residual glucose, and protease inhibitor at the different dilution rates are shown in Fig. 7A. Because the steady-state concentrations of residual glu-

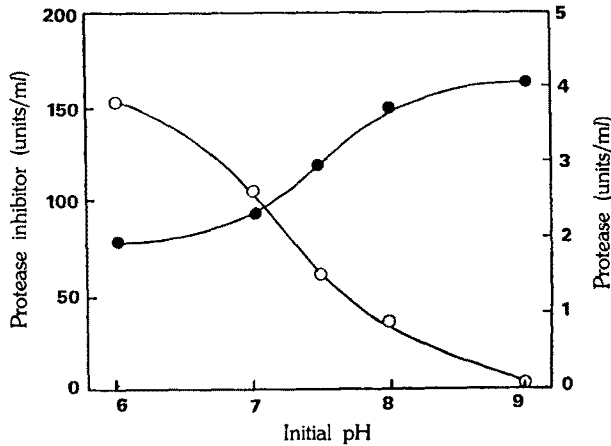


**Fig. 4. Effect of initial pH on the protease inhibitor production.**
The culture was carried out using protease inhibitor production medium and the initial pH was adjusted with NaOH and HCl. After 72 h culture, protease inhibitor (●) and protease (○) were measured.

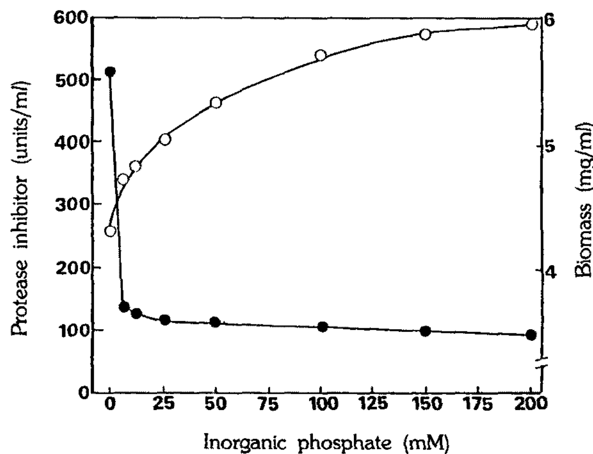


**Fig. 5. Effect of inorganic phosphate on the protease inhibitor production.**
The culture was carried out using protease inhibitor production medium containing various concentration of inorganic phosphate. After 72 h culture, protease inhibitor (●) and biomass (○) were measured.

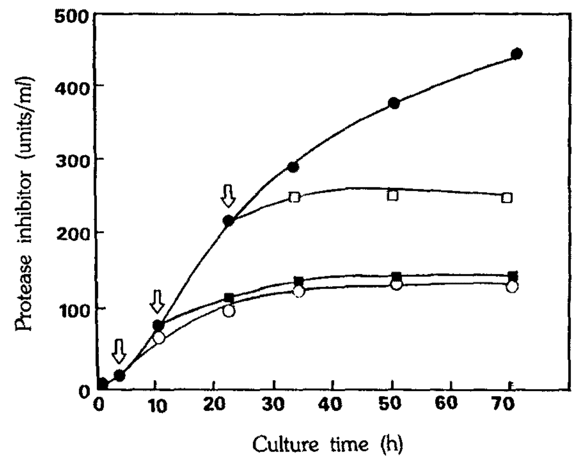


**Fig. 6. Effect of inorganic phosphate addition on the protease inhibitor production in a batch culture of *Streptomyces* sp. SMF13.**
25 mM of inorganic phosphate was added at 4, 10, and 22 h as indicated by arrowes (⇩).

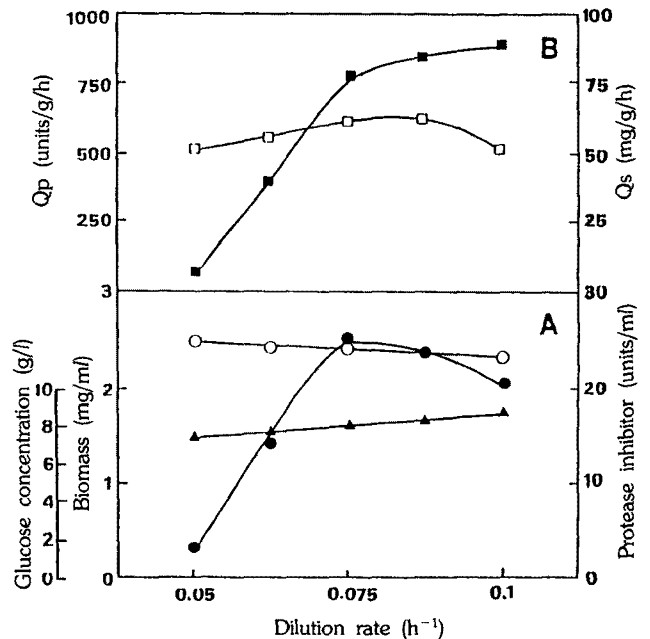


**Fig. 7. Effect of dilution rate on the protease inhibitor production kinetics.**
The continuous culture was carried out at 28℃, 250 rpm agitation, 0.4 vvm aeration, and pH 7.0. The fermentation media was glucose 1%, peptone 2%, yeast extract 0.3%, $KH_2PO_4$ 0.1%, and $K_2HPO_4$ 0.34%. The kinetic parameters such as protease inhibitor (●), residual glucose (▲) biomass (○), specific product formation rate: Qp (■), and specific glucose uptake rate: Qs (□) were measured.

cose were high at the different dilution rates, it was thought that cell growth was limited not by glucose but by other factor(s).

The specific protease inhibitor production rate ($q_p$) and the specific glucose uptake rate ($q_s$) were calculated and the data are showed in Fig. 7B. The results suggested that the production of the protease inhibitor was associated to the cell growth, which was also observed in batch cultures (9).

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