KOR. JOUR. MICROBIOL. September, 1990 p. 264-267 Vol. 28, No. 3

The Fermentation Kinetics of Protease Inhibitor Production by *Streptomyces fradiae*

Lee, Byong Kyu, Young Hwa Chung, and Kye Joon Lee
Department of Microbiology, Seoul National University, Seoul 151-742, Korea

*Streptomyces fradiae*에서 분리한 단백질 분해 효소 저해물질 생성의 동력학적 특성

이병규 · 정영화 · 이계준
서울대학교 자연과학대학 미생물학과

ABSTRACTS: The objectives of the current studies were to establish the optimal conditions for the production of extracellular protease inhibitor in a strain of *Streptomyces fradiae*. As results, it was found that cell specific growth rate was very critical for the production of protease inhibitor and the optimum specific growth rate was found to be 0.05 h^{-1}. Dissolved oxygen tension and pH were also important to regulate the inhibitor production. The inhibitory mode of the purified inhibitor to α-chymotrypsin was found to be competitive ($K_i = 5.5 \times 10^{-7}$ M). One mole of inhibitor could bind two moles of α-chymotrypsin and the complex has very low dissociation constant.

KEY WORDS □ Protease inhibitors, *Streptomyces fradiae*, Fermentation kinetics

A number of protease inhibitors have been discovered from various animal and plant tissues (Kaussell, 1970; Boison, 1983) as well as the culture filtrate of microorganisms (Umezawa, 1972; Imade *et al.*, 1985). It was reported that *Streptomyces* spp. produced different molecular weights of protease inhibitors in restricted culture conditions (Murao *et al.*, 1972; Umezawa, 1972; Oda *et al.*, 1989). Protease inhibitors play important physiological roles, such as controlling proteolysis and cell differentiation, however, the detailed physiological functions has not been well elucidated (Chung *et al.*, 1983; Yuan *et al.*, 1989; Laskowski *et al.*, 1982).

It was reported that a tylosin non-producing strain in *Streptomyces fradiae* produced an extracellular serine protease inhibitor in very restricted conditions and the physicochemical properties were characterized (Chung *et al.*, 1990). In the present study, environmental effects on the fermentation kinetics of the protease inhibitor production were examined and inhibition kinetics characters of the purified inhibitor were also evaluated.

MATERIALS AND METHODS

Microorganism and Media used.

One variant of *Streptomyces fradiae* NRRL 2702 was used throughout this work. The variant was isolated from the parent strain by single colony isolation and found that it lost the ability to produce tylosin. The maintenance of the strain and media compostions for seed culture and fermentation were followed the previous report (Chung *et al.*, 1990).

Culture conditions

One loopful spores and mycelia of the strain of *Streptomyces fradiae* were inoculated in 100 ml of seed medium in 500 ml baffled flask and cultured at 30 °C for 60 hours with a rotary shaking incubator (speed 200 rpm). Then the seed was inoculated into 2 liters culture medium in a 3 liter jar fermentor (Chemap CF). Temperature was controlled to 30 °C and agitation speed was maintained at 350 rpm. Co tinuous cultures were operated with changing feeding rate from 0.2 h^{-1} to 0.1 h^{-1}. The effects of dissolved

oxygen tension and pH on the protease inhibitor production was evaluated as well.

Analytical Methods

The concentrations of cells, glucose, and protein in the culture broth were analyzed by the methods described in previous report (Chung *et al.*, 1990). The activity of α-chymotrypsin was estimated as follows; 1.9 ml of α-chymotrypsin solution in 0.127 M triethanolamine (TEA) buffer (gave 0.6 unit/ml) were preincubated at 25 °C for 5 min. Then 1.0 ml of 2.5 mM succinyl-phenylalanine-p-nitroanilide (suc-phe-p-na) solution was added and reacted for 30 min. Optical density at 280 nm was measured immediately and again after 30 min. One unit of α-chymotrypsin was defined as the amount needed for the production of 1 μmol of p-nitroanilide per min. The inhibitory activity was calculated by the following equation; (A-B/A) × C × 100; where A was the activity of α-chymotrypsin without the inhibitor, B was the activity of α-chymotrypsin with the inhibitor, and C was the dilution factor of the sample. One unit of inhibitor was defined as the amount needed for the 50% inhibition of 1 unit of α-chymotrypsin.

RESULTS AND DISCUSSION

Kinetics of the protease inhibitor production

Data on the concentrations of biomass, residual glucose, and protease inhibitor at the steady-state are shown in Fig. 1A. The specific glucose uptake rates (Q_s) and protease inhibitor production rate (Q_p) are shown in Fig. 1B. It was clear that concentrations of glucose in the steady-state culture at different dilution rates were so high that it was thought that glucose was not limiting factor for the growth. It indicated that the cell growth was limited by other factor(s) which was fed also to the culture as the in-flow of medium.

It was interesting to note that the protease inhibitor was produced much more at the dilution rate of 0.05 h^{-1} which was considered as optimum dilution rate in terms of protease inhibitor production, although the biomass concentrations at different dilution rates were fairly constant. The specific protease inhibitor production rate (Q_p) was also maximum at the dilution rate of 0.05 h^{-1} whereas the specific glucose uptake rates (Q_s) was linearly increased as increasing dilution rates.

These results suggested that the protease inhibitor production was closely affected by cell growth rate which was influenced by the physicochemical environments of culture medium. Therefore, it was thought that elucidation of the factor(s) regulating the protease inhibitor production was the prime importance.

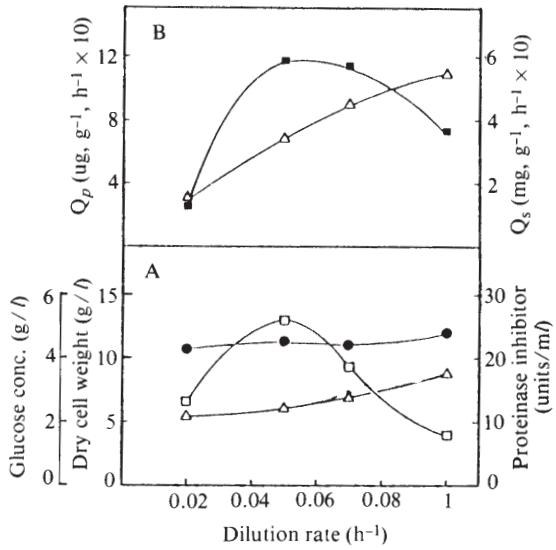

Fig. 1. *Effect of dilution rate on the protease inhibitor production kinetics.*
Protease inhibitor(□), glucose(△), dry cell weight (●), specific substrate uptake rate: Q_s(▲) and specific product formation rate: Q_p(■).

pH effect on protease inhibitor production

In order to evaluate the environmental factor regulating the protease inhibitor production, effect of pH on protease inhibitor production was evaluated at steady state in a continuous culture and the data obtained are shown in Fig. 2A. It was clear that protease inhibitor was produced more at neutral pH range (pH 6.7-7.3) compared to alkaline or acidic conditions, although cell concentrations were relatively constant through the different culture pH. However it was interesting to note that specific glucose consumption rates were higher at acidic or alkaline pH then those of the neutral culture condition (Fig. 2B). The results suggested that high glucose consumption rate at acidic or alkaline pH might be resulted from the increases in maintenance energy requirement.

Effects of dissolved oxygen tension on protease inhibitor production

In order to know the effect of dissolved oxygen tension (DOT) on the protease inhibitor production, DOT in continuous cultures were automatically

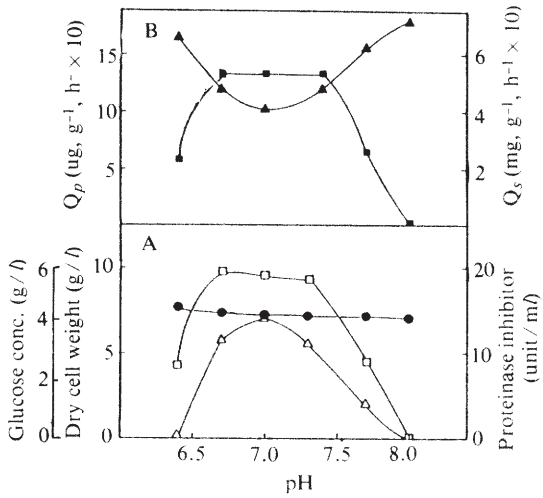

Fig. 2. *Effect of pH on the protease inhibitor production kinetics.*

Protease inhibitor(□), glucose(△), dry cell weight (●), specific substrate uptake rate: Q_s(▲), and specific product formation rate: Q_p(■).

maintained by feedback regulation of aeration. When aeration rate was controlled to 0.1 vvm, DOT in the culture broth was maintained 1.2%. Protease inhibitory activity of the culture broth was 66.5% (Table 1). However when aeration rate was increased to 0.6 vvm, DOT was increased to 36.3% and the inhibitor was not detected but protease was produced on the other hand. The results indicated that DOT in culture was very important to regulate the synthesis either protease or the inhibitor, although the molecular mechanisms were not elucidated.

Table 1. *Effect of aeration on protease inhibitor production in a chemost of S. fradiae* at dilution rate *0.05 h^{-1}*

Dissolved Oxygen Tension (%)	PI activity (unit/ml)	Protease (unit/ml)
1.2	26.5	0
36.3	0	11.3

Kinetics and stoichiometry and Inhibition

The activity of α-chymotrypsin on the synthetic substrate, suc-phe-p-nitroanilide, was tested with different concentration of the substrate. As shown in Fig. 3, the V_m and K_m values of the enzyme on the substrate were calculated as 850 μmol/min-mg and 2.27 mmol, respectively. It was clear that the inhibition mode of the inhibitor to α-chymotrypsin was competitive. In order to known the stoichiometry of the inhibitor to α-chymotrypsin, residual activities of α-chymotrypsin of the two molecules which were

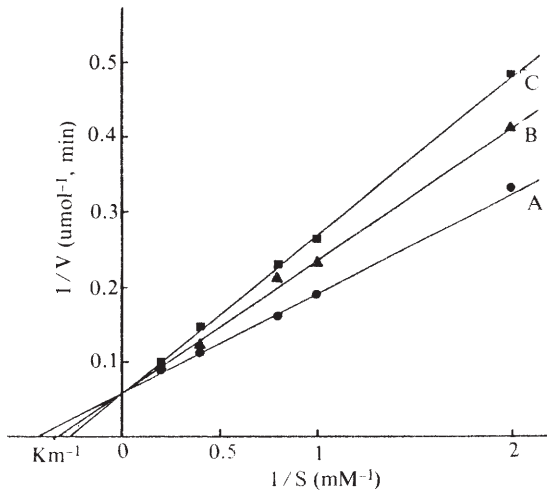

Fig. 3. *Inhibition mode of protease inhibitor against α-chymotrypsin.*

Lineweaver-Burk plot of succinyl-phenylalanine-*p*-nitroanilide concentration against activity of α-chymotrypsin in the presence and in the absence of the protease inhibitor.

A: in the absence of protease inhibitor.

B: in the presence of 13.5 μg/ml protease inhibitor.

C: in the presence of 27.0 μg/ml protease inhibitor.

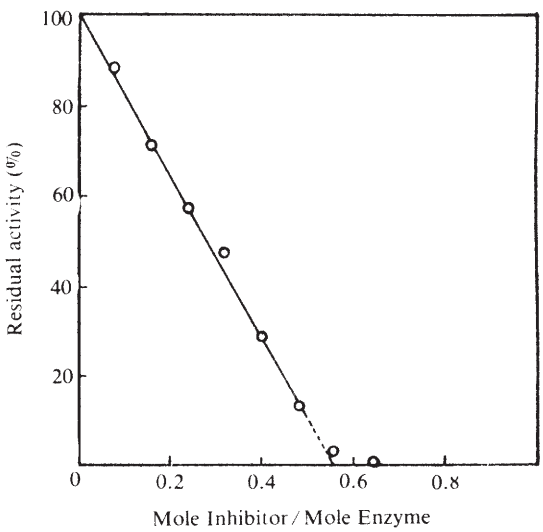

Fig. 4. *Determination of the stoichiometry of protease inhibitor binding to α-chymotrypsin.*

Teh initial "Burst" of *p*-nitroanilide release was measured at 405 nm and used to calculate the concentration of free α-chymotrypsin.

mixed with different molar ratio were measured according to slightly modified method of Zahanley *et al.* (1970). As shown in Fig. 4, it was found that the residual activity of α-chymotrypsin was linearly reduced as increasing the ratio of the inhibitor to the enzyme and eventually no activity was remained when the ratio was about 0.5. Therefore it was thought that one mole of inhibitor bound with two moles of α-chymotrypsin. The results indicated that one mole of the inhibitor had two binding sites for the chymotrypsin.

적 요

*Streptomyces fradiae*의 한 변이주로부터 균체외 단백질 분해효소 저해물질의 생성에 대한 발효동력학적 특성을 조사하였다. 그 결과 비증식속도 0.05 h $^{-1}$에서 저해물질의 생성이 가장 우수하였고 용존산소의 농도와 발효배양 pH가 동 저해물질 생합성에 가장 중요한 환경요인임을 알았다.

ACKNOWLEDGEMENT

The presented works were supported by a grant from the Korea Science and Engineering Foundation. The authors are very appreciating for the supports.

REFERENCES

1. **Anson, M.L.**, 1939. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.* **22**, 79-89.
2. **Boison, S.**, 1983. Proteinase inhibitors in cereals. Occurrence, properties, physiological role and nutritional influence. *Acta Agric. Scand.* **33**, 369-381.
3. **Chung, C.H., H.E. Ives, S. Almeda and A.L. Goldverg,** 1983. Purification from *Escherichia coli* of a periplasmic protein that is a potent inhibitor of pancreatic proteases. *J. Biol. Chem.* **258**, 11032-11038.
4. **Chung, Y.H., B.K. Lee and K.J. Lee, 1990. Characters of Proteinase Inhibitor Isolated from** *Streptomyces fradiae*. *Kor. Jour. Microbiol.* **28**, 65-70.
5. **Imada, C., M. Maeda and N. Taga,** 1985. Purification and characterization of protease inhibitor "Monastatin" from a marine *Alteromonas* sp. with reference to inhibition of the protease produced by bacterium pathogenic to fish. *Can. J. Microbial.* **31**, 1089-1094.
6. **Laskowski, M. Jr. and I. Kato,** 1980. Protein inhibitors of proteases. *Ann. Rev. Biochem.* **49**, 593-626.
7. **Murao, S. and S. Sato,** 1972. S-S1, a new alkaline proteinase inhibitor from *Streptomyces alboriseolus* S-3252. *Agric. Biol. Chem.* **36**, 160-163.
8. **Oda, K., Y. Fukuda, S. Murao, K. Uchida and M. Kainosho,** 1989. A novel proteinase inhibitor, tyrosatin, inhibiting some pepstatin-insensitive carboxyl proteinases. *Agric. Biol. Chem.* **53**, 405-415.
9. **Umezawa, H., 1972.** *Enzyme inhibitors of microbial origin.* University of Tokyo Press, pp. 1-100.
10. **Umezawa, H.**, 1982. Low-molecular-weight enzyme inhibitors a microbial origin. *Ann. Rev. Microbial.* **36**, 75-99.
11. **Winter, C.A., E.A. Riseley and G.W. Nuss,** 1962. Carageenin-induced edemas in hind paw of the rat as an assay for antiinflammatory drugs. *Proc. Soc. Exptl. Biol. Med.* **111**, 544-547.
12. **Yuan, L. and G.T. Cloe, 1989. Characterization of a proteinase inhibitor isolated from the fungal pathogen** *Coccidiodes immitis*. *Biochem. J.* **257**, 729-736.
13. **Zahanley, J.C. and J.C. Davis,** 1970. Determination of trypsin-inhibitor complex dissociation by use of the active site titrant, p-nirophenyl-p'-guanidino-benzoate. *Biochemistry* **9**, 1428-1433.

(Received August 2, 1990)
(Accepted August 30, 1990)