# Studies on the Production of Serratiopeptidase from Serratia Culture

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## 세라티아 배양에 의한 세라티오펩티다아제의 생산에 관한 연구

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**Abstract** — An anti-inflammatory agent, serratiopeptidase, was produced from the culture of the *Serratia marcescens*. The effects of carbon sources, nitrogen sources and inducers on the production were investigated. Citrate was found to be inhibitory in the production of serratiopeptidase. The enzyme was synthesized in the synthetic medium without inducers, albeit low level of synthesis. But the synthesis was increased by the addition of proteinaceous substrate and leucine. Induction of extracellular proteinase by its end-product was discovered, which is not common in the proteinase synthesis in the bacteria. By the glucose fed-batch culture, we found the possible catabolite repression on the production of serratiopeptidase.

Microbial proteases have been widely used as detergents, cheese and milk processors, meat tenderizers, and medicines (1-5). *Serratia marcescens* ATCC 21074, formerly designated as *Serratia piscatorum* or *Serratia* sp. E-15 strain, is known to produce an extracellular protease which has M.W. of 51kd. This protease, serratiopeptidase, is a metalloprotease which requires zinc atom as an essential moiety for activity and has been used as an antiinflammatory agent by virtue of its fibrinolytic character. The biochemical properties of this serratiopeptidase have been well characterized and now are believed to have its optimal temperature of 30℃ and pH of 9.0. The culture conditions for its production have also been studied to some extent (6-10). Here, we report some detailed studies on the factors affecting the production of serratiopeptidase. In this study, we investigated the effects of carbon sources, nitrogen sources, and inducers on the pro-

duction of serratiopeptidase. We also describe the result of fed-batch culture and the glucose effect on the serratiopeptidase production which is likely the catabolite repression of the enzyme synthesis.

## Materials and Methods

### Microorganisms used

*Serratia marcescens* ATCC 21074 was purchased from American Type Culture Collection(ATCC) and mutagenized with chemical mutagen, nitrosoguanidine (11). The mutant with the highest productivity of serratiopeptidase was selected and used in this study.

### Culture medium

The basal medium of Miyata (6) was used with slight modification. Its composition was as follows; $(NH_4)_2HPO_4$ (2 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $CaCl_2 \cdot H_2O$ (0.2 g/l), KCl (0.5 g/l), and NaCl(1 g/l). In order to elucidate the effect of inducers or nutrients, it was supplemented with appropriate amount of necessary compounds.

## Culture conditions

Cells were cultivated in a shaking water bath (Precision Sci. Inc.) at 30℃, initial pH 7.0, or a jar fermentor BIOSTAT E (B. Braun) at 30℃ with aeration rate of 1 vvm.

## Analytical procedures

Cell growth was estimated by measuring the optical density at 600 nm with appropriate dilution of the culture. Assay of the protease activity was carried out by the method of Prestidge *et al.* (12) using Azocasein as a substrate. Purified serratiopeptidase dissolved in 50 mM potassium phosphate buffer (pH 7.5) or centrifuged culture supernatant was used as enzyme source. Mixture of enzyme solution (0.3 m*l*) and 2% Azocasein solution (0.7 m*l*, pH 9.0, 50 mM Tris·Cl) was incubated at 40℃ for 30 min. The reaction was stopped by the addition of 7% perchloric acid (2 m*l*). The centrifuged supernatant (2 m*l*) was mixed with 10 N NaOH (0.3 m*l*) and the optical density of the solution was measured at 436 nm. One proteolytic activity unit (pu) of protease was arbitrarily determined as the amount of enzyme required to increase absorbance by 0.01 under the above conditions. Protein samples were analyzed by a SDS-discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemnli (13). For the preparation of samples for SDS-PAGE, culture broth was centrifuged and the protein was precipitated with trichloroacetic acid (TCA. final 10%). The precipitate was dissolved in distilled water. The pH of the suspension was adjusted to 9.0 with saturated Trizma base solution.

## Results and Discussion

### Identification of serratiopeptidase

Besides serratiopeptidase, *Serratia marcescens* ATCC 21074 also secretes other proteins into culture medium. In order to prove that the protease activity in culture supernatant is mainly due to serratiopeptidase, SDS-polyacrylamide gel electrophoresis and inhibition test with EDTA were carried out. As shown in Fig. 1, the major component of protein in culture supernatant is 51 kd in its MW which is identical to the serratiopptidase isolated
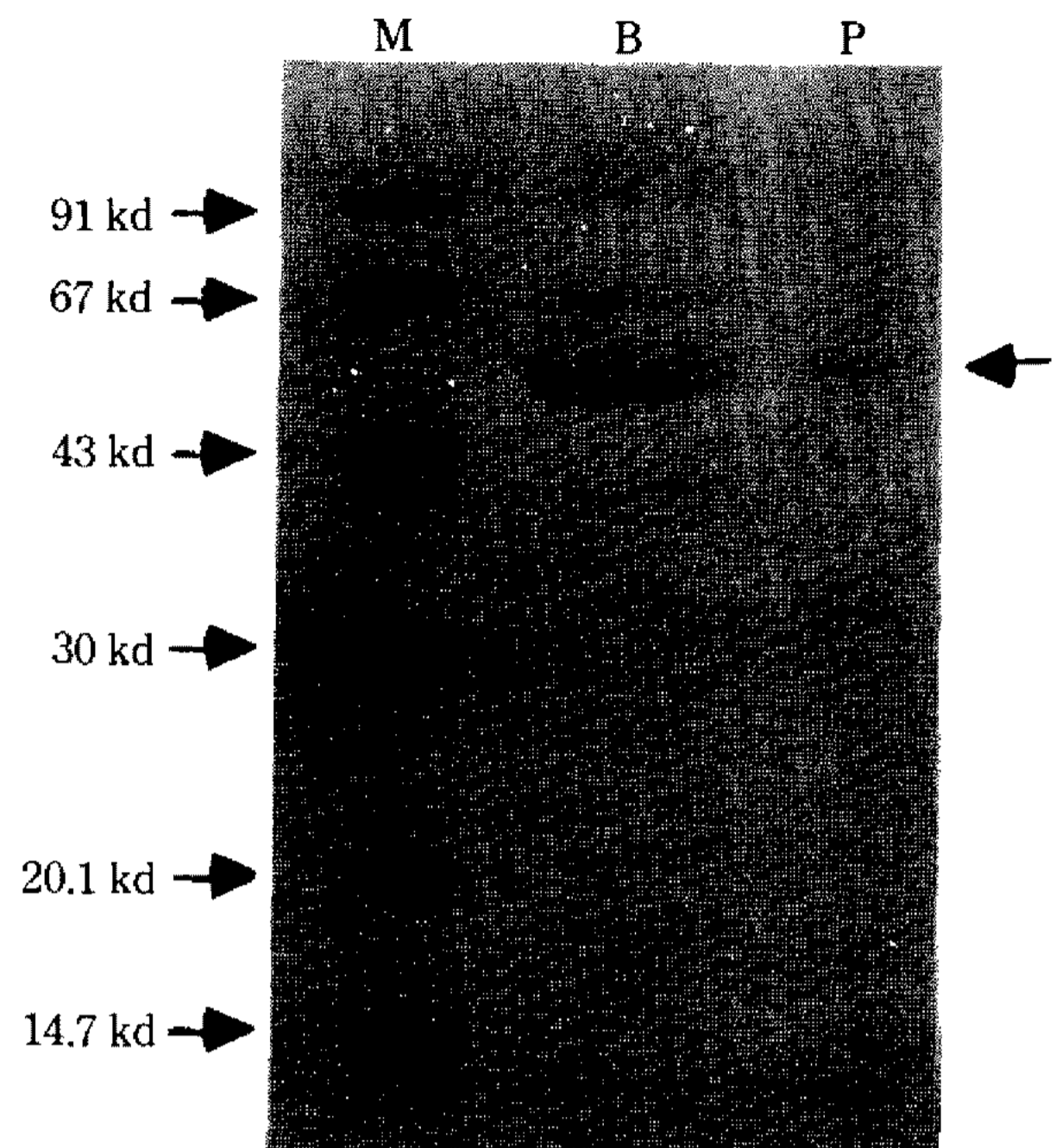


**Fig. 1. Analysis of the fermentation broth by SDS-polyacrylamide gel electrophoresis.**
The arrow indicates 51 kd protein, serratiopeptidase. Lane M: size marker; lane B: TCA-treated fermentation broth; lane P: purified serratiopeptidase from KOLON Pharm. Inc. tablet.

from serratiopeptidase tablet from KOLON Pharm. Inc. The protease activity in culture supernatant was completely removed by the treatment of EDTA (Data not shown), which is typical for the zinc containing protease, serratiopeptidase.

### Effect of carbon sources on the production of serratiopeptidase

In basal medium, various kinds of carbon sources were added (10 g/*l*) and the production of serratiopeptidase in the culture was examined. The effect of carbon sources on the growth and serratiopeptidase production is summarized in Table 1 and Fig. 2. Generally, the hexoses and organic acids showed relatively good effect on the growth of cells, while the disaccharides and sugar alcohols produced lower cell mass. The specific productivity of enzyme was almost same as in the case of hexoses, disaccharides, and sugar alcohols. Interestingly, organic acid carbon sources, citrate and acetic acid generated almost same amount of cell masses as in the case of hexoses but the specific productivities were much lower. Because the citrate or acetate did not

**Table 1. Effects of various carbon sources on the cell growth and the serratiopeptidase production**

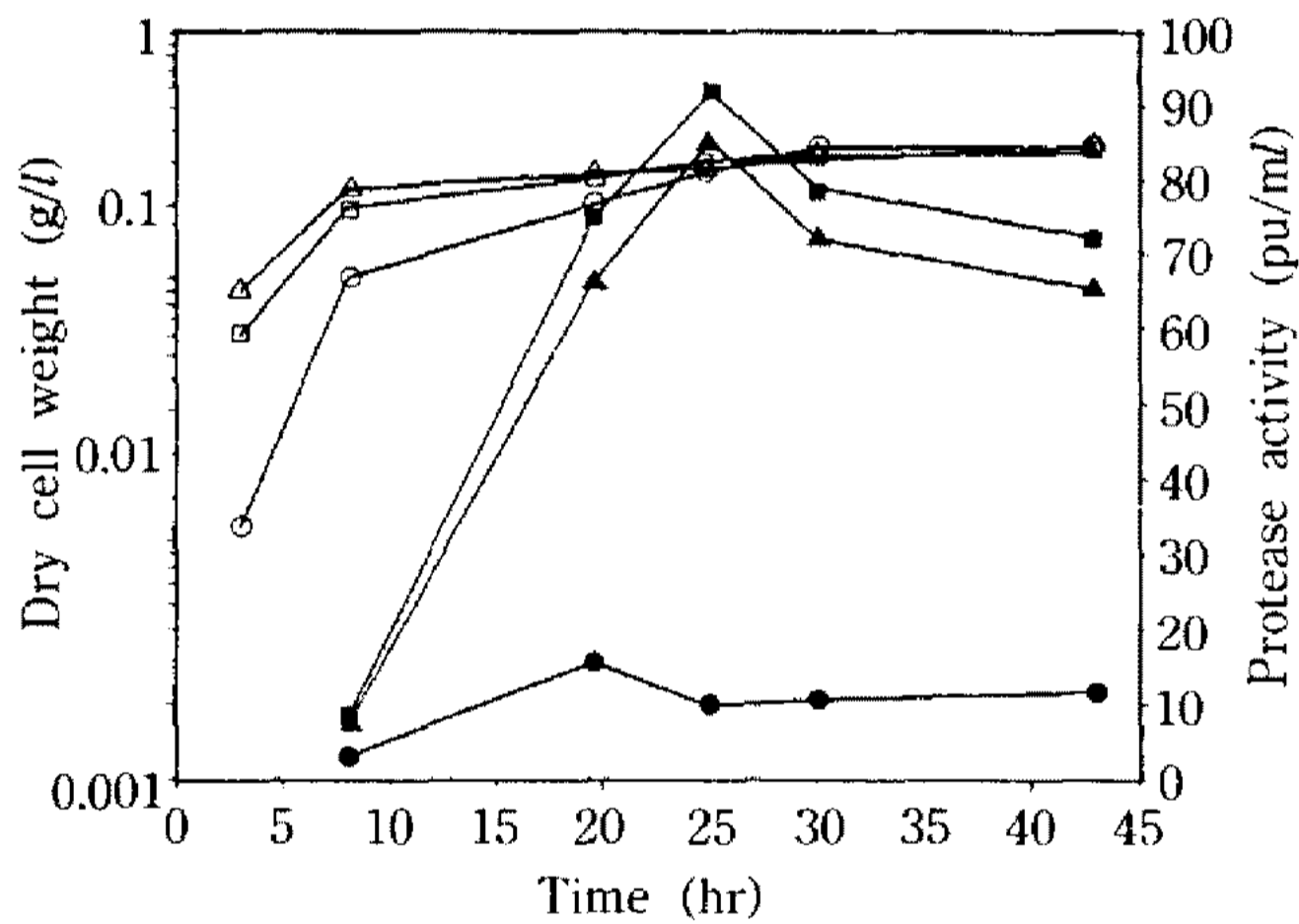| Carbon source | Protease production | Cell growth |
|---|---|---|
| Fructose | + + + | + + + |
| Glucose | + + + | + + + |
| Mannose | + + + | + + + |
| Sorbitol | + | + |
| Mannitol | + | + |
| Glycerol | + | + |
| Citrate | + | + + + |
| Acetic acid | + | + + + |
| Sucrose | + | + + |
| Maltose | + + | + |
| Lactose | + | + |



**Fig. 2. The cell growth and serratiopeptidase production with some utilizable carbon sources.**
Citrate generated almost the same amount of cell mass as in the case of hexose but the productivity was much lower. The closed symbols represent the protease activity and the open symbols cell growth.
— ■ — : glucose, — ▲ — : fructose, — ● — : citrate
— □ —          — △ —          — ○ —

influence on the activity of serratiopeptidase (14), the lower activity detected in the medium must be due to the decreased productivity of serratiopeptidase. This kind of phenomenon was once observed in the secretion of exoprotease in *Pseudomonas* (15), and it was suggested that organic acid or TCA cycle intermediates might inhibit the secretion of exoprotease from *Pseudomonas*. More detailed biochemical studies are thought to be necessary to identify the possible effect of the organic acids on the decrease of serratiopeptidase production.
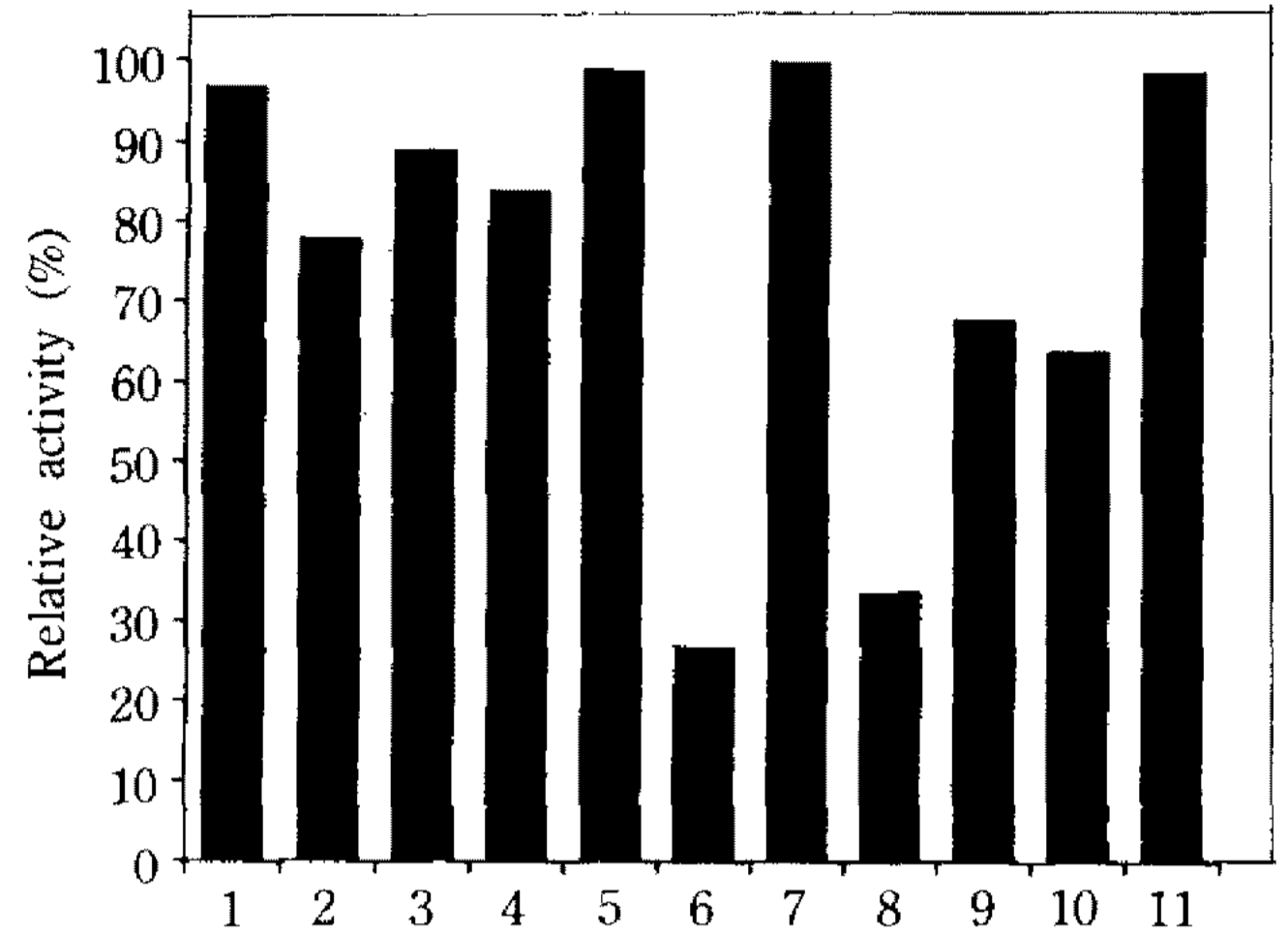


**Fig. 3. Effects of various organic nitrogen sources on the production of the serratiopeptidase.**
Soytone, casein, yeast extract and peptone were effective for the production of serratiopeptidase, while skim milk and casamino acid were shown to be relatively ineffective. 1: soytone, 2: tryptone, 3: tryptic soy broth, 4: casitone, 5: casein, 6: skim milk, 7: yeast extract, 8: casamino acid, 9: malt extract, 10: corn steep liquor, 11: peptone

## Effect of nitrogen sources on the production of serratiopeptidase

Studies on the selection of favorable carbon sources showed that the supplementation of only carbohydrates in the basal medium without proteinaceous substrates did not augment the production of both the cell mass and serratiopeptidase. Complex nitrogen sources were thought to be necessary for higher productivity and were added (10 g/l) in basal medium to investigate the effects on the enzyme production. Fig. 3 shows that soytone, casein, yeast extract, and peptone were effective for the production of enzyme, while skim milk and casamino acid were shown to be relatively ineffective. Cell growth was not specifically affected by particular nitrogen source but almost identical. The concentration of yeast extract was varied from 0 to 1.6% to examine the effect of concentration on the enzyme production. The optimal yeast extract concentration was 0.9% as shown in Fig. 4. At higher concentration of yeast extract no detectable increase in cell mass was observed but the serratiopeptidase production decreased and the reason for this inhibition has yet to be studied.
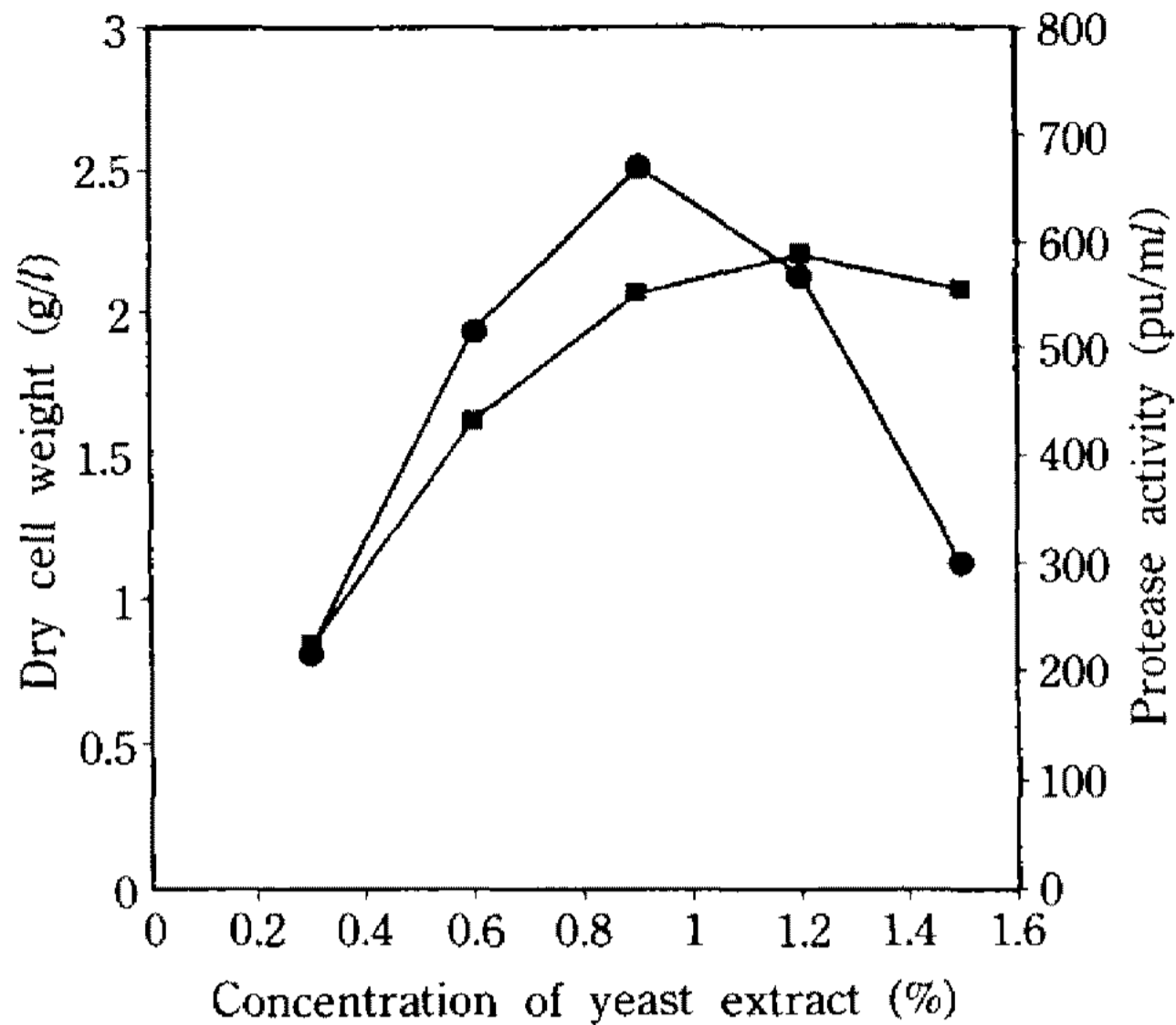
**Fig. 4. Effect of yeast extract concentration on the cell growth and serratiopeptidase production.**
The optimal concentration was 0.9% and the decrease in serratiopeptidase production was observed at higher concentration.
—●— : protease activity, —■— : cell growth



**Fig. 5. Effect of leucine concentration on the production of protease.**
The production of serratiopeptidase increased in accordance with the increase of leucine concentration but no increase in the production was observed at the concentration higher than 0.03%.

**Table 2. Effects of some inducers and nitrogen sources on the production of the serratiopeptidase**

| Nitrogen source added to basal medium (10 g/l) | Inducer (1 g/l) | Relative activity (%) |
|---|---|---|
| Yeast extract | no | 100 |
| | leucine | 142 |
| | gelatin | 46 |
| | casein | 66 |
| Casein | no | 100 |
| | leucine | 64 |
| Soytone | no | 100 |
| | leucine | 135 |

**Effect of inducers on the enzyme production**

The casein, leucine, and gelatin which had been assumed to be potential inducers of serratiopeptidase production were added and tested their induction capabilities. As demonstrated in Table 2, casein and gelatin were not effective but rather inhibitory in serratiopeptidase production in the presence of yeast extract in the medium. Leucine was shown to be effective for the induction in the medium enriched with yeast extract or soytone but it was inhibitory when using casein in the medium as a nitrogen source. The concentration of leucine in the medium supplemented with 9 g/l of yeast ext-
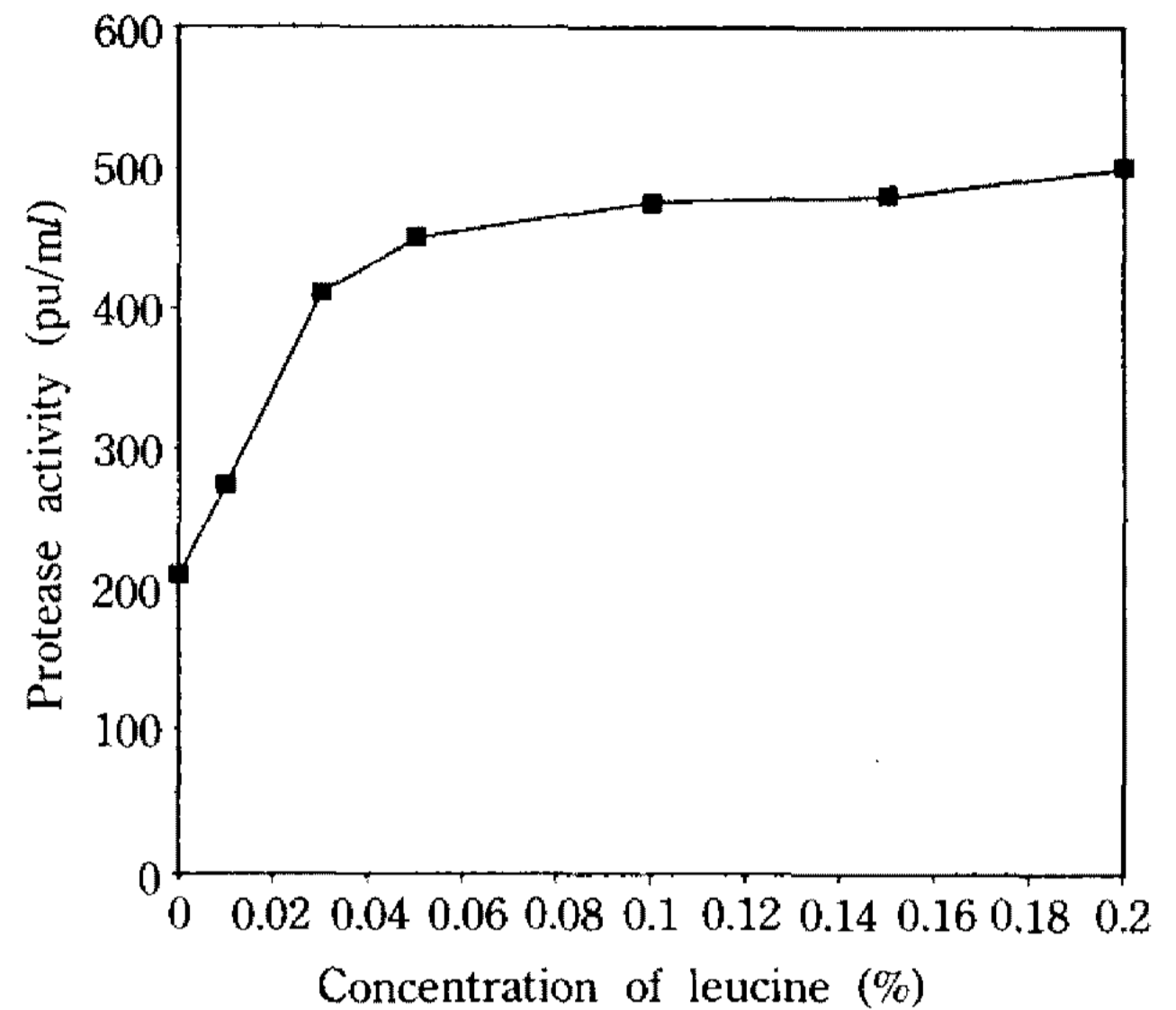
ract was varied from 0 to 0.3% to check the concentration effect on the cell growth and enzyme production. Growth of cells was not stimulated by leucine but production of serratiopeptidase increased in accordance with the increase of leucine concentration. When the concentration of leucine was higher than 0.03%, a slow increase of enzyme production was observed (Fig. 5).

**Batch fermentation**

Fermentation was carried out under the defined conditions. In comparison with the flask culture, the cultivation time to reach the stationary phase of cell growth in the fermenter was much shorter than that in the flask, but the amount of produced enzyme was nearly identical with each other (Fig. 6). The concentration of dissolved oxygen (DO) rapidly decreased and was exhausted after 3-4 hours from the start. Cells grown in yeast extract enriched medium started to synthesize serratiopeptidase from the late exponential growth phase and then the level sharply increased with time. When the cell growth entered into the stationary phase, the production of the serratiopeptidase also stopped.
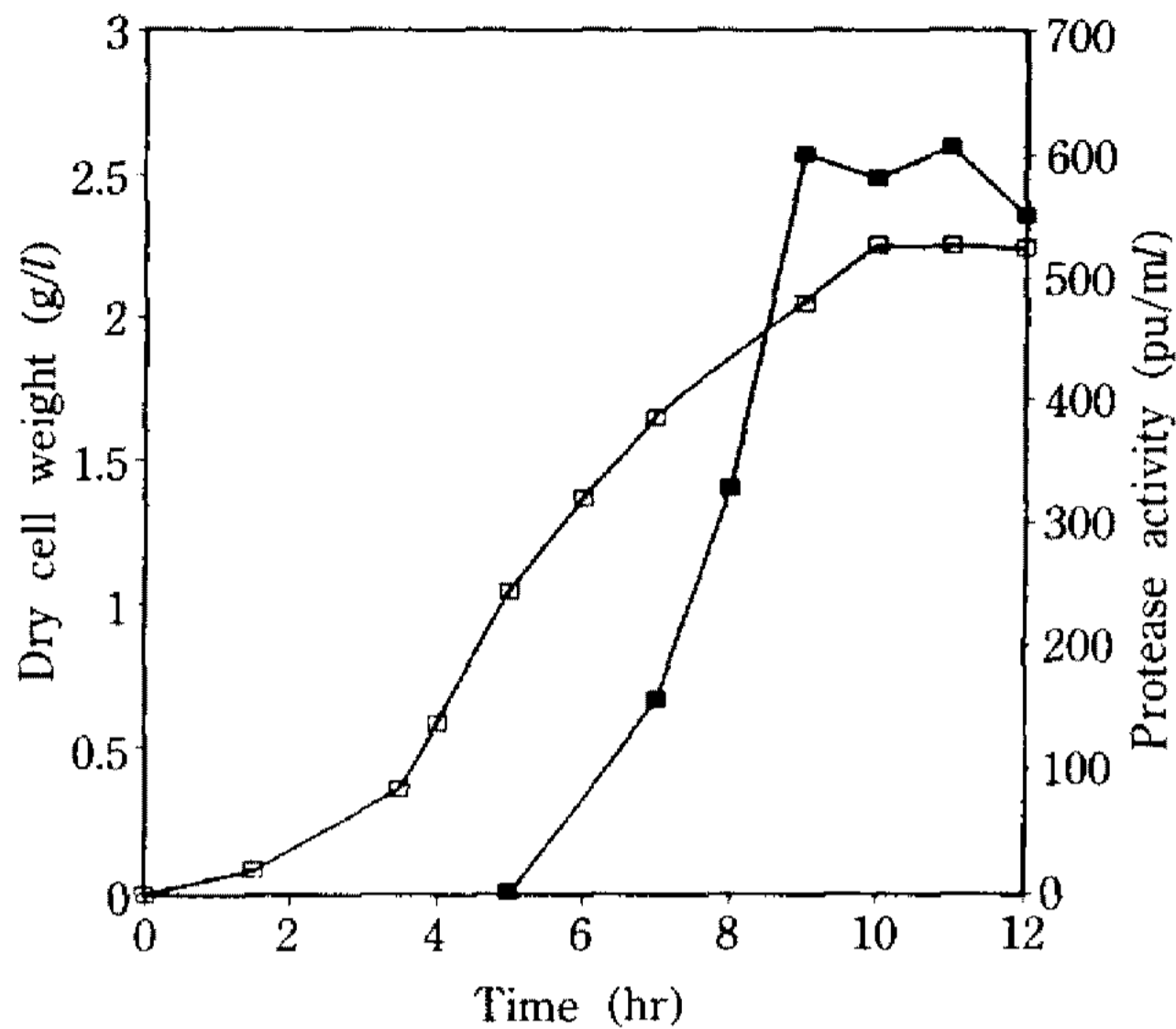
Fig. 6. Time course of the cell growth and serratiopeptidase production in the fermenter. The synthesis of serratiopeptidase started from the late exponential growth phase and stopped when the cell growth entered into the stationary phase.
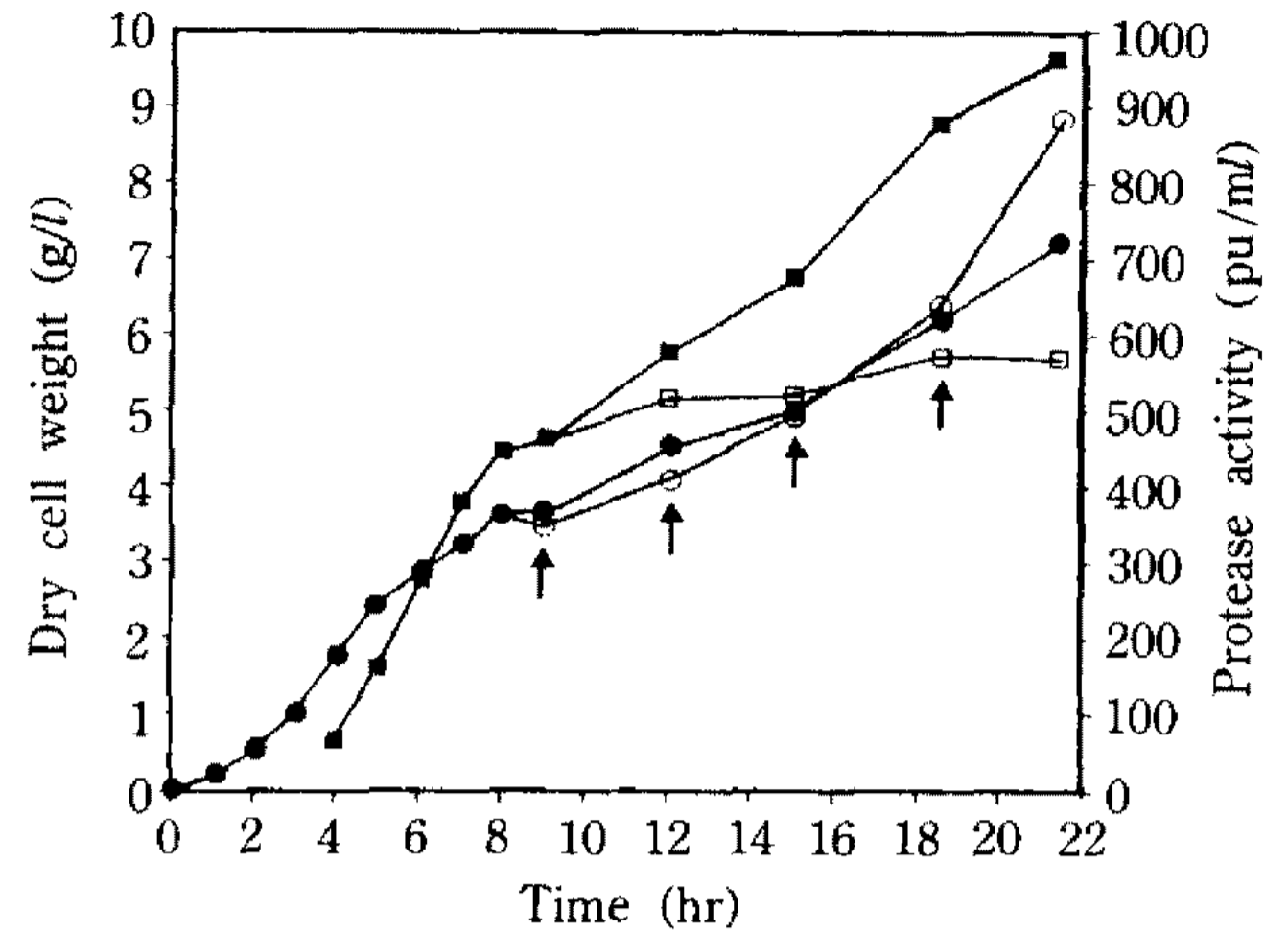
—■—: protease activity, —□—: cell growth



Fig. 7. Fed-batch cultivation with glucose or yeast extract.

The arrows indicate the points of medium feeding. Cell growth continuously increased with the feeding of glucose but no further increase in serratiopeptidase production was observed. The closed symbols represent yeast extract fed-batch and the open symbols glucose fed-batch.

—●—: cell growth, —■—: protease activity
—○—:            —□—:

## Fed-batch cultivation

Finally, we tried the fed-batch fermentation and found that the utilizable carbohydrate, glucose, exerted a repression of the synthesis of serratiopeptidase. Glucose was fed every two hours after the culture reached stationary phase. Fig. 7 illustrates the result of glucose fed-batch culture. Cell growth continuously increased in accordance with the feeding of glucose but no further increase in enzyme production was observed, while the feeding of yeast extract resulted in the continuous increase of both cell population and enzyme production. In 1977, Loria et al. (7) suggested that organic acids generated by glucose catabolism may interfere with the transport of inducers into cytoplasm finally causing the decrease in the production of exoprotease. Our result apparently coincides with their suggestion in that the level of enzyme production in glucose fed-batch culture did not increase. But we do not exclude the possibilities of catabolite repression by glucose. The enzyme was synthesized even in the absence of inducers in the medium but the synthesis was regulated by the high concentration of glucose or sped up by the inducers present in the culture medium, free amino acid leucine or oligopeptides generated by the activity of serratiopepti-

dase. The data presented above (Fig. 2, Table 1 and Fig. 7) suggest that the extracellular serratiopeptidase is produced constitutively and also is catabolite-repressible. This kind of phenomenon was detected by Hsu and Vaughn in Aeromonas liquefaciens (16). Their studies furnish reliable evidence that the synthesis of a constitutive enzyme is subject to catabolite repression. McDonald and Chambers (17) studied the regulation of proteinase formation in a species of Micrococcus and suggested partial constitutive synthesis of proteinase. The enzyme was synthesized constitutively in synthetic medium (without inducer) and induced by the addition of proteinaceous substrate in the culture. But as the increase of the concentration of catabolite sugar added in the medium, the production of proteinase was suppressed even in the presence of inducers. The regulation of serratiopeptidase production is deemed to be unique and intriguing. Induction in bacteria of extracellular proteinase by amino acid (i.e. by a type of end-product induction) does not appear to be popular. The synthesis of serratiopeptidase could be an ideal system for a more detailed investigation of the mechanism of catabolite repression or induction in the protease

production in Enterobacteriaceae *Serratia marcescens.*

## Acknowledgement

## 요 약

세라티아 균주의 배양으로부터 소염제로 사용되는 세라티오펩티다아제의 생산에 관한 연구를 수행하였다. 여러가지 탄소원, 질소원 및 유도제가 효소의 생산에 미치는 영향을 조사하였는데, 탄소원은 효소의 생산이나 세포성장에 좋지 못한 기질이었으며, 특히 citrate의 경우 균체성장량은 포도당과 거의 동일하였으나, 세라티오펩티다제의 생산에 저해효과가 있음이 밝혀졌다.

세라티오펩티다아제는 아미노산인 leucine을 첨가해 주었을 때 그 생산되는 양이 크게 향상되었으며 leucine의 최적 농도는 0.03%였다.

포도당을 기질로 한 유가배양시에 세라티오펩티다아제의 생산이 catabolite repression으로 생각되는 조절기작에 의하여 저해받았으나, yeast extract를 기질로 하였을 때는 저해현상은 나타나지 않았다.

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