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Regulation of Extracellular Alkaline Protease Biosynthesis in a strain of *Streptomyces* sp.

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Streptomyces sp. 一株에서 균체외 호염기성 단백질분해 효소의 생합성 조절

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In fermentation studies it revealed that *Streptomyces sp.* SMF 301 started to synthesize extracellular alkaline protease from early exponential phase of cell growth. The biosynthesis of the alkaline protease was greatly induced by skim milk as a sole nitrogen source and further stimulation was observed under inorganic sulphur limited culture. However it was found that the biosynthesis was apparently repressed by NH_4^+ and free amino acids, specially by cysteine. It was considered that the strain SMF 301 of *Streptomyces sp.* would produce the alkaline protease for the uptake of sulphur compounds from protein contained in the culture broth.

Proteolytic enzymes are the most important enzymes used in various purposes and the marketing portion is about 70% of enzyme industry (Ward, 1983). A number of proteolytic enzymes were reported in terms of their reaction mechanisms or biological roles and also of their applications; (1976; Lgorov *et al.* 1980; Ward, 1983). Alkaline proteases are defined as the enzymes of which optimum pH for reaction were alkaline region. The enzymes have been reported to be produced extracellularly by *Bacillus subtilis* (Dancer, 1975), *Serratica spp.* (Bromake, 1975), *Aspergillus nidulans (Cohen, 1973), Pseudomonas spp.* (London, 1984), *Streptomyces spp.* (Renko *et al.*, Nakanishi, 1974;) and *Neurospora spp.* (Hanson, 1973). The alkaline protease produced from *Streptomyces sp* have been widly dispensed in anti-inflamatory agents (Nakanishi and Yamanoto, 1974).

Furtheremore biosynthetic regulatory mechanisms were also studied using those microorganisms and from those approaches it have been belived that the biosynthesis of the proteases were regulated by the nutritional and environmental conditions (Lasure, 1980; Yamada *et al.*, 1983; Cherdyntseva, 1983; Egorov 1983; Tsuchiya, 1984).

MATERIALS AND METHODS

Strain and maintenance

Alkaline protease producting strain SMF 301 of *Streptomyces sp.* was used in the present study. The strain was maintained on a slope of rich medium containing glucose 4%, beef extract 0.5%, peptone 0.5% and agar 1.5%.

Culture conditions

Spores from the stock culture were inoculate to the liquid rich medium contained in a flask and cultivated at a reciprocal shaking incubator for 3

days at 30°C. For the main cultures, a fermenter (6*l*, Microferm MF 207, New Burnswick Science) was used. Aeration rates were controlled to 1.2 VVM and agitation speed was 450 RPM. Temperature was maintained to 30°C and the composition of main culture medium was formulated as followed as; soluble starch 2.0%, skim milk 1%, KH_2PO_4 0.1%, K_2HPO_4 0.3%, $MnCl_2 \cdot 6H_2O$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.02%, $FeCl_2 \cdot 2H_2O$ 0.025%, and Na_2CO_3 0.02%. The initial pH was adjusted to 7.0.

Determination of Enzymes Activity

Cell free culture broth obtained from centrifugation (6.000 x g, 10 min) was used as a crude enzyme solution. 2.5ml of Hammarsten casein dissolved in 0.05M Na-borate buffer (pH 9.0) was mixed with 0.5ml of the crude enzyme solution and reacted at 30°C for 10 min, then stopped the enzyme reaction by the addition of trichloroacetic acid solution. The activity of the alkaline protease was determined by measuring optical density at 275 nm, and the enzyme activity unit was defined as μg of tyrosine liberated from casein during 1 min at 30°C.

Determination of biomass and protein and amino acids

The concentration of cell mass in the culture broth was measured as dried cell weight (D.C.W) after drying the washed cell at 100°C for 6 hours. Protein concentration was determined by the methods of Lowry et al with bovine serum albumin as a standard. Total concentration of soluble amino acids in the culture broth was estimated by the ninhydrin reaction.

Table 1. *Induction of alkaline protease by various nitrogenous compounds in* ***Streptomyces sp.***

Nitrogen Source		Protease Activity (unit ml^{-1})
Casein hydrolysate	1%	9.8
Milk casein	1%	14.0
Skim milk	1%	187.0
Soytone	1%	57.0
Soy been meal	1%	157.0

Basal medium: glucose 2.0%, KH_2PO_4 0.1%, K_2HPO_4 0.3%, $MnCl_2$ $6H_2O$ 0.01, $MgSO_4 \cdot 7H_2O$ 0.02%, $CaCl_2 \cdot 2H_2O$ 0.025%, $FeCl_2 \cdot 2H_2O$ 0.025%, Na_2CO_3 0.02%
Culture conditions: at 30℃, 72hr culture

RESULTS AND DISCUSSION

Effects of Nitrogen Compounds

The effects of various nitrogen compounds which were considered to be inducers for the biosyntheis of the alkaline protease were shown in Table 1. It was evident that unhydrolyzed proteineous compounds *viz.* skim milk and soy bean meal, were found to be better inducers than the hydrolyzed compounds. In order to confirm the inductive effect of skim milk, it was added during batch cultures in which ammonium sulphate was used as a nitrogen source. As shown in Figure 1, in a culture containing ammonium sulphate as a sole nitrogen source, the protease induction was not stimulated. However the addition of skim milk to the culture was apparenty to stimulate the pro-

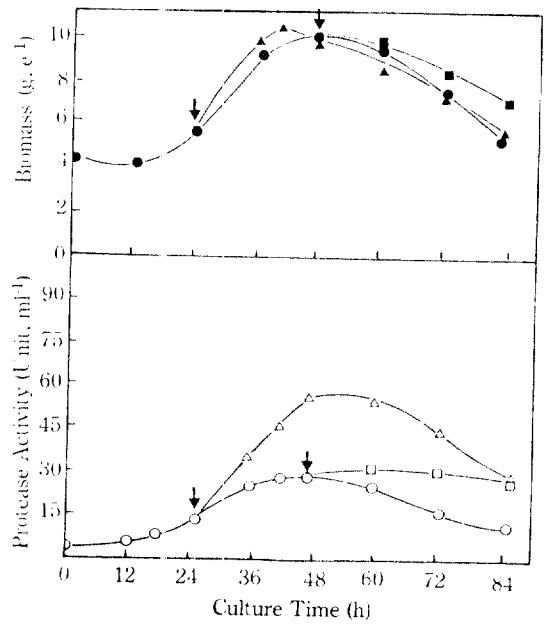

Fig. 1. *Induction of the biosynthesis of the alkaline protease by skim milk in the culture of Streptomyces sp.*
0.5% of skim milk was added to the culture containing of ammonium salt as a nitrogen source at the point of arrow.

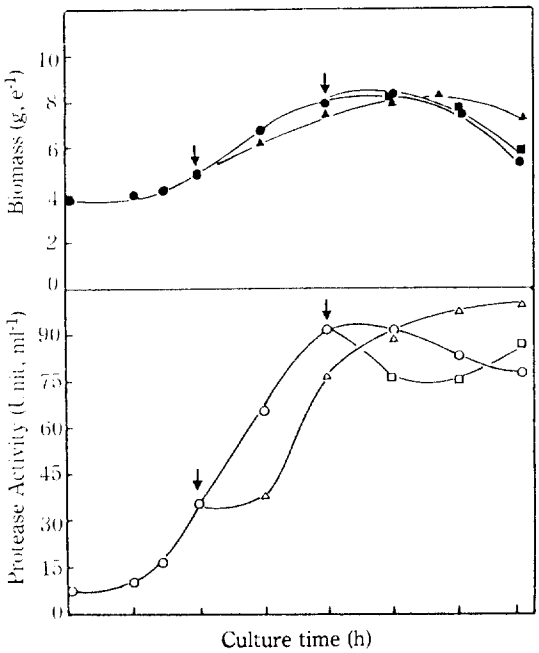

Fig. 2. *Effects of ammonium salts addition to the medium containing of skim milk on the biosynthesis of the alkaline proteases.*
0.05% of ammonium salt was added to the culture at the point indicated as arrow.

tease biosynthesis. It was very interest to note that addition of skim milk at begining of exponential growth phase was much more effective for the protease production than the addition at stationary growth phase.

Another attempt was done also to know the effect of ammonium sulphate on the induction of the protease biosynthesis. When 0.05% ammonium sulphate was added to the culture broth containing skim milk, biomass formation did not show any differences, but the protease biosynthesis very much depressed for a while until the added ammonium ion was depleted as shown in Figure 2. These results suggested that the biosynthesis of the protease was induced by the limitation of readly utilizable nitrogen sources in culture broth and that ammonium ion plays a great role in the induction of protease biosynthesis. Those observations were considered to be nitrogen catabolite repression (Cherdyntseva *et al.*, 1982 and Lausure, 1980).

Effects of Amino acids and Sulphate Compounds.

Effects of various amino acids on the induction of the protease were also estimated. When an individual amino acid was added to the culture broth at the middle of exponential growth phase the total activity of protease was varied with the addition of the indivisual amino acid. As shown in Table 2, most amino acids did not show any effects on the induction. However, leucine was the only amino acid a, which stimulated the protease production in contrast to that cysteine showed very strong repression on the protease biosynthesis. It was reported that leucine was a good inducer for the protease biosynthesis in the culture of *Serratia marsens* (Loriya *et al.*, 1977) and that most amino acids showed a strong catabolite repression in *Asp candidus* (Ivanitsa, *et al.*, 1977) and *B. subtilis* (Cherdyntseva, *et al.*, 1982) and *B. thuringiensis* (Egorov, 1983).

In order to know the effect of cysteine on the induction, it was added to the culture broth where containing 1% skim milk. When cysteine was added at the exponential phase the protease biosynthesis was repressed remarkablly and the microbial growth was retarded also to give longer stationary growth phase but the protease biosynthesis was repressed apparently. As shown in Figure 3 that cysteine concentrations in culture broth was very crucial for the biosynthesis of the

Table 2. *The effect of amino acids on the biosynthesis of the alkaline protease in Streptomyces sp.*

Amino acids	Protease Activity (unit ml^{-1})
Cysteine	41.7
Methionine	95.4
Lysine	100.0
Proline	150.4
Serine	152.8
Glutamate	150.4
Leucine	173.5
Control	147.1

0.5% skim milk was supplemented to the basal medium
0.5% of the individual amino acid was added to the 48hour culture broth Ptotease activites was measured with the 72 hour culture broth.

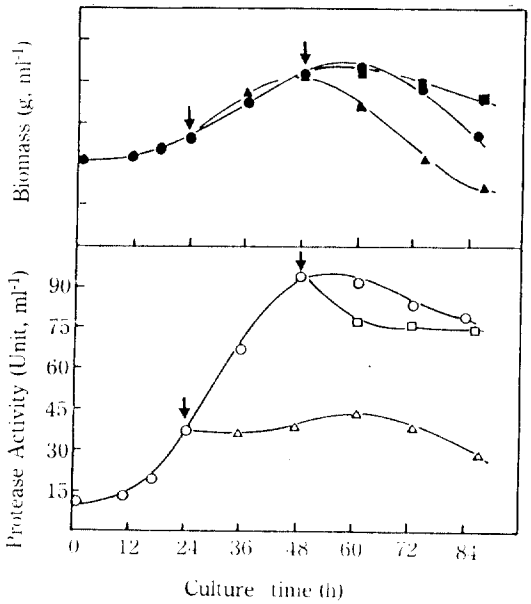

Fig. 3. *Effects of cysteine addition to skim milk medium on the biosynthesis of the alkaline proteases by Streptomyces sp..*
0.05% of cysteine was added at the point indicated as arrow.

extra-cellalar protease in *Streptomyces sp.* SMF 301. It has been reported that sulphur containing amino acids such as cystein and methionine were very strong repressors in the protease biosynthesis using *S. marscens* (Loriya *et al.*, 1977).

Batch cultures using basal medium replaced $MgSO_4$ with $MgCl_2$ were carried out in order to test the effect of inorganic sulphate on the induction of the protease biosynthesis. In these experiments the concentratins of Mg^{++} were not controlled to give constant level, but sulphate form was replaced with chloride form. As can be seen in Table 3. It was evident that Mg^{++} did not show any effects but the limitation of inorganic sulphate source gave rise to increase the protease biosynthesis. It was confirmed again that cysteine was very strong repressor in the alkaline protease biosynthesis using the isolated strain SMF 301 of *streptomyces sp.* These results suggested that the strain would excrete protease in order to harvest growth essential element(s), it could be sulphur, from proteins given to the culture broth.

Table 3. *Effects Organic/Introganic Sulphur sources on the biosynthesis of alkaline protease in Streptomyces sp.*

Sulphur sources		Protease activity (unit ml^{-1})
$MgSO_4$	0.1(%)	147.1
$MgCl_2$	0	
$MgSO_4$	0.05	149.1
$MgCl_2$	0.05	
$MgSO_4$	0	191.5
$MgCl_2$	0.1	
$MgCl_2$	0.05	22.6
Cysteine	0.05	

Culture conditions: at 30℃ 72hr culture in glucose-skim milk medium

Effect of carbon sources

The results obtained from batch cultures using various carbon sources were compared in Table 4. It was clear that solube starch was the best carbon/energy source for the biosynthesis of the protease. A further comparisons were made also with the results obtained from with and without addition of c-AMP to the culture containing glucose and soluble starch. As evident in Figure 4, better protease production was obtained from the culture of soluble starch while the microbial growth was lowered in some extended. The addition of c-AMP to the both cultures at the exponential growth

Table 4: *Effect of various carbon sources on the biosynthesis of alkaline protease in Streptomyces sp.*

Carbon sources		Protease activity (unit, ml^{-1})
Na-citrate	2 %	98.3
Na-acetate	2 %	98.5
Arabinose	2 %	40.0
Xylose	2 %	64.5
Glucose	2 %	67.1
Fructose	2 %	19.4
Sucrose	2 %	114.2
Maltose	2 %	36.5
Lactose	2 %	25.0
Soluble starch	2 %	146.7

Culture medium: 2 % of each carbon source was added to basal medium.

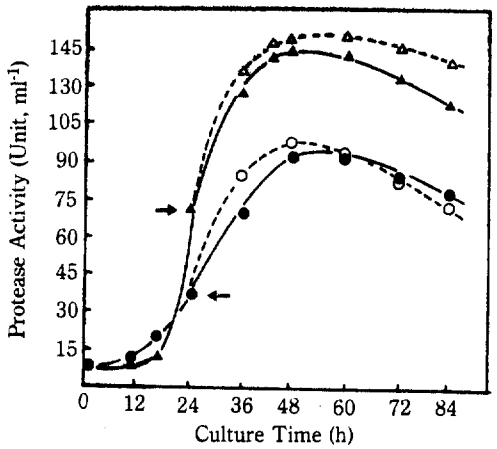

Fig. 4. *Effects of c-AMP addition to starch and glucose on the biosynthesis of alkaine protease in Streptomyces sp.*
50mM of c-AMP was added at the time as indicated by arrow
▲ : Protease activities in the culture broth of soluble starch
△ : In culture broth of soluble starch and c-AMP
● : In culture broth of glucose
○ : In culture broth of glucose and c-AMP

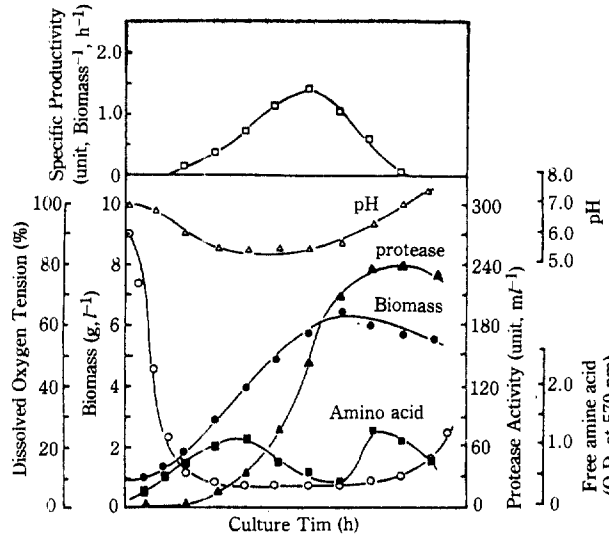

Fig. 5. *Changes in biomass (●), amino acids (■), protease (▲), dissolved oxygen tension (○), and pH (△) during a batch culture of Streptomyces sp.*

phase resulted in slight increase in the protease biosynthesis. It was not clear why the soluble starch was superior to glucose in the protease production, but it was well reported that carbon catabolite repression was evident at the protease biosynthesis in *B. thuringensis* (Egorov, *et al.*, 1982) and also in *Streptomyces sp.* (Disler, 1982,). Judging from those consideration it was believed that glucose exerted very strong catabolite repression on the biosynthesis of the alkaline protease, in the isolated strain SMF 301 of *Streptomyes sp.*

Batch culture data using Jar fermenter

Streptomyces sp. (SMF 301) was evaluated in the kinetic studies using the optimized fermentration, changes in pH, dissolved oxygen tension, and free amino acids concentration, and also the concentrations of the alkaline protease produced are shown in Figure 5. From the results it was apparent that protease biosynthesis was growth linked and also that the synthesis and excretion was initiated with the rapid decreases in dissolved oxygen tension. These data indicated that oxygen was an essential factor for the microbial growth and also for the protease production.

As the microbial growth initiated, free amino acids liberated from skim milk were accumulated in some extended. However when the microbial growth was in exponential growth phase and the specific protease formation rates were increased, the concentration of free amino acids were decreased. It was observed also that when the growth and protease formation were retarded the amino acids accumulation was restored. The results suggested that the biosynthesis of the protease was controlled apparently by the concentration of nitrogen sources which were uptaked readly. Data shown in Figure 1,2,3,4 indicated that the used stain SMF 301 of *Streptomyces sp.* would produce the alkaline protease in order to uptake nitrogen/sulphur compounds for the growth. The most effective compound was considered to be cysteine.

적 요

好 塩基性 단백질 분해효소를 생합성하는 放線菌 一株(SMF301)에서 동 효소의 생합성 조절을 검토한 결과 사용균주는 대수성장기의 초기에서 부터 효소를 생합성하여 배지내로 축적하였다. 효소의 생합성은 脱脂粉乳를 유일한 질소원으로 사용하는 배지에서 크게 유도됨을 알았고, 또한 無機硫黄化合物이 결핍된 상태에서 특히 촉진되었음을 관찰하였다. 한편 NH_4^+나 유리 아미노산을 배지내에 첨가해주면 효소 생합성이 확실히 저해되었는데 그중

에서도 cysteine이 첨가된 배지에서 효소의 생합성이 완벽하게 저해되는 것으로 보아 아마도 사용한 균주 SMF301은 배지내에 첨가해준 단백질을 분해해서 성장에 필요한 성분을 흡수하기 위하여 호염성 단백질분해 효소를 생합성하는 것으로 생각되어진다.

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