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Relationship between Sporulation and Synthesis of Alkaline Protease in *Streptomyces* sp.

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방선균 일주에서 포자형성과 호알칼리성 단백질 분해효소의 생합성과의 관계성

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ABSTRACT: The aims of the present studies were to understand the physiolosical and genetic characters of *Streptomyces* sp. isolated from soil. It revealed that *Streptomyces* sp. SMF301 had very fast growth rate and produced extracellular protease and heavily sporulated on rich media. It also showed β-lactamase activity and pigment production. Nonsporulating mutants were isolated after NTG or acriflavin treatment and their characters were compared with the parent strain. It was found that the mutants obtained by acriflavin treatment and their characters were compared with the parent strain. It was found that the mutants obtained by acriflavin treatment lost the pigment formation and β-lactamase production. Protease activity of the mutant was lowered and the pH optimum was changed toward neutral. It was found that the changes were resulted from the reduction of alkaline protease biosynthesis in the bald mutant. Therefore it is considered that sporulation, pigment formation, β-lactamase production, and alkaline protease production in *Streptomyces* sp. might be controlled with a closely related relationship.

KEY WORDS □ ***Streptomyces*, sporulation, alkaline protease**

Streptomyces have been well recognized as industrially important microorganisms to produce large number of antibiotics and enzymes (Chater, *et al.*, 1982). The genus has been interested also to study on cellular differentiation because it produces aerial mycelia and spores (Chater, *et al.*, 1973: Hopwood *et al.*, 1973: Kalakoutskii *et al.*, 1976). It has been reported that some typical characters of *Streptomyces* such as melanin pigment production (Shaw & Piwowarski, 1977), fertility (Friend *et al.*, 1978), cell differentiation (Pogell, 1979), antibiotic resistance (Shaw & Piwowarski, 1977) and antibiotic production (Hopwood, 1978) were lost by the treatment of curing agents. Thus it was thought that extrachromosomal DNA in conjunction with the chromosomal DNA, might play important roles in the expression of these characters. It was reported also that protease might be involved in the formation of spore in *Bacilus* spp. (Dancer *et al.*, 1975: Burnett *et al.*, 1986), *Saccharomyces cerevisiae* (Betz, 1977), and *Streptomyces* sp. (Prikladnaya *et al.*, 1982).

In this study, it was contemplated to investigate the relationships between those characters observed in *Streptomyces* spp. As a first step a strain of *Streptomyces* sp. isolated from Korean soil was selected, because it had very rapid growth and showed heavy spore formation on rich media. Sporeless mutants (bald mutant) were obtained from the wild strain by treating with acriflavin.

Then physiological differences between the wild and the mutant strains were compared in order to elucidate the mechanisms involved in spore formation and cell differentiations.

METHODS

Organism and culture media

Streptomyces sp. SMF 301 isolated from soil was used in the presented study. The strain was maintained in YEME medium (Chater *et al.*, 1982). Protease producing culture medium (fermentation medium) was consisted of 2% soluble starch, 1% skim milk, 0.2% yeast extract, 0.1% KH_2PO_4, 0.3% K_2HPO_4, 0.01% $MnCl_26H_2O$, 0.01% $MgSO_47H_2O$, 0.02% $CaCl_22H_2O$, 0.025% $FeCl_2$ $6H_2O$, 0.02% Na_2CO_3. Media pH was adjusted to 7.2 before sterilization.

Culture conditions

The spore suspensions from the stock culture was inoculated to 50 m*l* of fermentation medium contained in baffled flasks. Cells cultured at a reciprocal shaking incubator for 3 days at 30 °C were transfered to 2.5 liter of fermentation medium for the production of protease. Batch culture was conducted in a 3.0 liter fermentor (C.F. model, Chemap). Culture pH was maintained at 7.0 and temperature was controlled to 30 °C. Dissolved oxygen tension was maintained above 50% of saturation by monitoring agitation and aeration.

Determination of protease activity

After cultivation, the cells and other debris were removed by centrifugation at 4000g for 30 min. The supernatant was then used as a crude enzyme solution. The activity of protease was assayed by the measurement of tyrosine liberated from casein 0.5 m*l* of enzyme solution was mixed with 2.5 m*l* of 0.6% (w/v) Hammarsten casein dissolved in 0.05M sodium borate buffer (pH 9.0). After 10 min incubation at 30 °C, 2.5 m*l* of TCA solution (consisting of 0.1M trichloroacetic acid, 0.22M sodium acetate and 0.33M acetic acid) was added. The mixture was incubated for 30 min at 30 °C, and then filtered. The tyrosine concentration was determined by measuring the absorbance of the filtrate at 275 nm. One unit of the enzyme was defined as the amount of the enzyme that released absorbance value equivalent to μg of tyrosine per min at 30 °C. Protein concentration was serum albumin as a standard protein.

Purification of proteases

Protein fractionation was carried out by ammonium sulphate precipitation and the fractions precipitated between 45% (w/v) to 85% (w/v) saturation were collected and used for further purification. The precipitated proteins were dissolved in 0.02M Tris-HCl (pH 7.5) and dialyzed against the same buffer. The dialysate was applied to a column (2.5 × 110 cm) of Sephadex G-75-50 equilibrated with 0.02M Tris-HCl buffer (pH 7.5), and 5 m*l* fractions were collected. The active fractions were pooled and applied to DEAE-Sephadex A-50 column (2.7 × 50 cm) equilibrated with the same buffer system used for the gel filtration. After washing the column with the same buffer, a linear gradient of NaCl from 0 to 0.4N in the buffer was applied, and 10 m*l* fraction was collected.

Determination of resistance to various antibiotics

The stock culture was inoculated into the YEME medium containing different concentrations of various antibiotics. The growth was determined after shaking incubation at 30 °C for 49 hours. The activity of β-lactamase was tested using the colorimetric method described by Sykes *et al.*(1979).

Mutagenesis

Spore suspensions were added to the 1 m*l* of 0.05 M TM buffer (0.05M Tris, 0.05M Maleic acid, pH 9.0) containing 1 mg of NTG. After incubation for 60 min at 30 °C, the culture was diluted and plated on the fermentation medium plate. After 7 days cultivation at 30 °C colonies which do not produce spores were isolated. Mutagenesis using acriflavin was carried out as follow; Spore suspensions were inoculated into YEME media containing 15 μg/m*l* acriflavin. After incubation for 48 hours at 30 °C, the culture was inoculated into the same media containing 100 μg/m*l* of acriflavin. After cultivation for 54 hours at 30 °C, the culture was diluted and plated on the plate of fermentation medium. None-spore

forming colonies were picked up and used for further experiments.

RESULTS AND DISCUSSION

Characters of wild strain of *Streptomyces* sp.

The isolated strain, SMF 301, grew very fast and formed abundant spores when it was grown on rich media (Figure 1). The strain showed the typical characters of *Streptomyces* spp. and was tentatively identified as *Streptomyces* sp. (Pye, 1986). It was found that the wild strain was very sensitive to 5 μg/m*l* of thiostrepton, tetracycline, and rifamycin. However it showed resistances to 1.2 mg/m*l* of ampicillin and 0.5 mg/m*l* of penicillin G. Based upon the colorimetric method to detect β-lactamase, it was clear that this strain produced β-lactamase (Figure 2).

Fig. 1. *Culture of Streptomyces spp. on YEME medium containing skim milk for 10 days at 30°C.*
Strain (SMF 301) grew faster and produced more extra-cellular protease and spores than other strains.

One of the most distinct character of the strain (SMF 301) was that it produced large amount of extracellular protease on the medium containing skim milk (Figure 1). When the strain was cultivated in fermentation medium, the activity of protease in the submerged culture supernatant was 112.7 units per mg of protein. It was found that the protease could be fractionated from the culture broth by salting out with ammonium sulphate from 45% to 85% saturation. The total activity of protease recovered by the fractionation was 64.2% of the total activity. It was about 4 fold

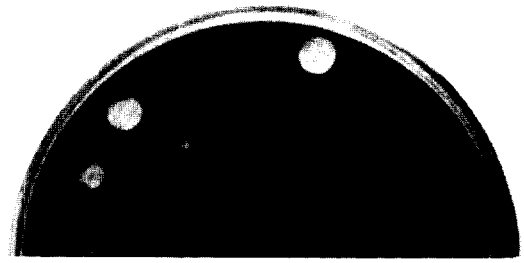

Fig. 2. *Detection of β-lactamase produced from the wild strain (SMF 301) of Streptomyces sp. Clear zone indicated that penicillin G pregnated into the medium was hydrolyzed by β-lactamase produced.*

enrichment in specific activity compared to the culture broth.

Gel filtation on Sephadex G-75-50 resulted in 41.8% recovery yield in terms of activity and the purification fold was 6.2. It was calculated that the protease concentration was about 25% to the total protein eluted from the gel filtration. Anion-exchange chromatography using DEAE-Sephadex A-50 was followed after the gel-filtration. As shown in Figure 3, it was very interesting to note that protease activities revealed in two parts.

Characters of the enzymes were determined and it was clear that the optimum pH of the enzyme I was 9.0 and that of the enzyme II was 7.0

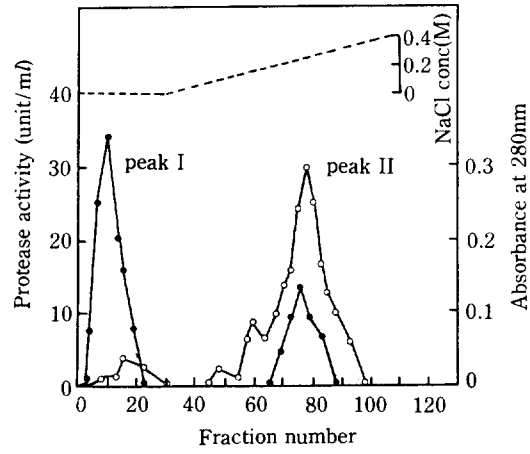

Fig. 3. *Anion-exchange chromatography of the extracellular protease produced by a wild strain (SMF 301) of Streptomyces sp. DEAE-Sephadex A-50 was used as an exchanger.*
(●); protease activity, (○); absorbance at 280 nm.

(Figure 4). Hence it was considered that the enzyme I was alkaline protease and the enzyme II was neutral protease.

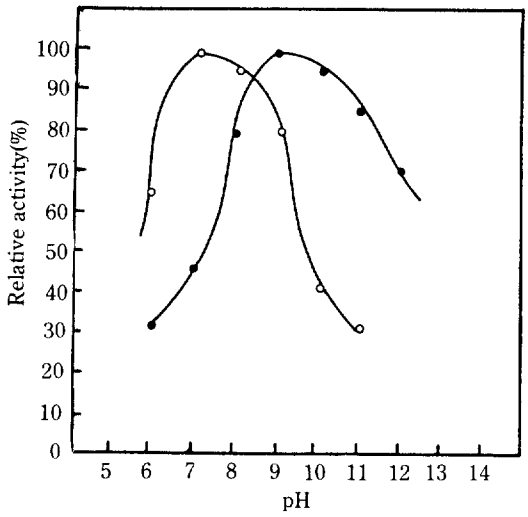

Fig. 4. *Effects of environmental pH on the activity of the purified proteases.*
(●); protease collected from peak I, (○); protease collected from peak II.

Isolation of bald mutants

It was attempted to isolate bald mutants from the wild strain, which produces abundant spore and β-lactamase, by treating with NTG and acriflavin. The frequency to obtain bald mutants were shown in Table 1. It was clear that acriflavin was more effective mutagen that NTG in obtaining bald mutants. Due to the bare colonies with tight structure, the mutants were considered as bald mutants. It was observed that extracellular protease activity was apparently reduced in the bald mutants as shown in Figure 5. It was found also that the pigment formation, earth smell production, and β-lactamase production observed in the parent strain were lost concomitantly in the bald mutants. Although it was evident that extracellular protease activity, pigment formation, β-lactamase production, and cell differentiation were affected to change by a single step mutagenesis, it was not well understood what was happened in the mutagenesis and what was the regulatory mechanism involved in the pleiotropic changes. It

Table 1. *Mutation frequency of Streptomyces sp. SMF301 after treatment of acriflavin(AF) and NTG*

	Lactamase⁻	Sporulation⁻
NTG	7.0×10^{-2}	1.2×10^{-2}
AF	1.9×10^{-1}	6.3×10^{-2}
Control	5.4×10^{-3}	10^{-3}

has been thought that plasmid might be involved in the sporulation, pigment formation, and β-lactamase production in *Streptomyces* sp. But some other possible mechanisms, such as, involvement of transposible elements or deletion in chromosome (Schrempf, 1981), could not be excluded. The elucidation of mechanism for the pleiotropic changes observed in *Streptomyces* sp. SMF 301, is subjected to next experiments.

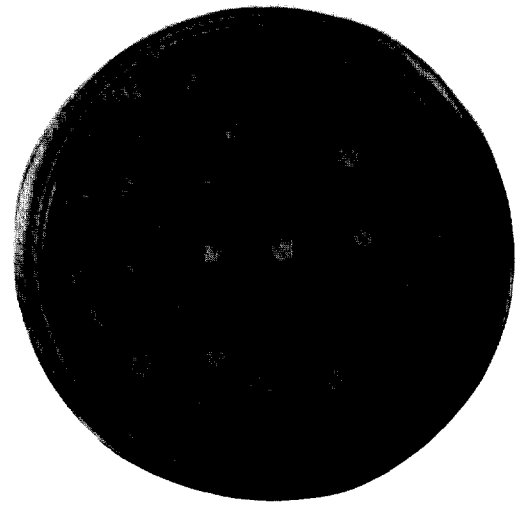

Fig. 5. *Comparisions between the wild strain and bald mutants of Streptomyces sp. Wild strain produced extra-cellular protease and white spores but bald mutants did not produced extracellular protease and spores.*

Comparisons between the wild strain and the bald mutants

It was contemplated to compare the wild strain and the mutant with their extracellular protease activity. As shown in Table 2, it was evident that the bald mutants from acriflavin treatment showed very low activity compared to the wild strain.

Table 2. *Comparison in total protease activity between parent (301) and mutants (302-308) obtained from acriflavin treatment*

Strain	301	302	303	304	305	308
Relative protease activity (%)	100.0	41.8	24.5	24.5	30.9	19.9
optimal pH	9.0	8.0	8.0	7.8	8.0	7.8

The reaction mixture contained 0.5 m*l* of 3 day cultured broth and 2.5 m*l* of 0.6% casein dissolved in 0.05M Na-borate buffer, pH 9.0.

In addition, the optimal pH for the activity of protease produced by bald mutants was changed toward neutral pH, while that of wild strain was pH 9.0. The results indicated that there would be changes in the profiles of protease. This interpretation was confirmed by the analysis of the pattern of protease produced in the bald mutant. As shown in Figure 6 it was very clear that the activity of alkaline protease (Peak I) produced in one bald mutant (SMF 302) was reduced in some extent, while that of neutral protease was maintained relatively constant.

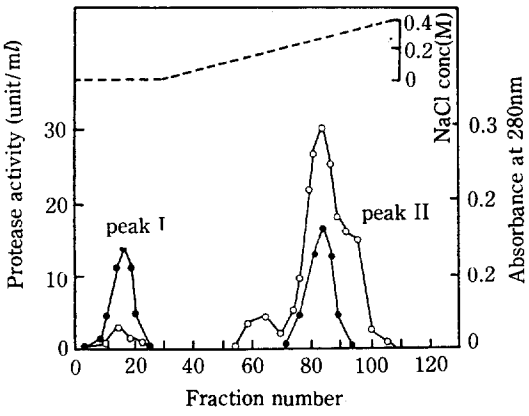

Fig. 6. *Anion-exchange chromatography of extra-cellular protease produced by a bald mutant (SMF 302) of Streptomyces sp. DEAE-Sephadex A-50 was used as an exchanger. (●); protease activity, (○); absorbance at 280 nm.*

The data shown in Fig. 3 and Fig. 6 were summarized at Table 3. It was evident that the shift of optimum pH from 9 to 8 and the reduction of the total protease activity in one bald mutant (SMF 302) compared to the wild strain (SMF 301) were resulted from the apparent reduction of the biosynthesis of alkaline protease. In wild strain (SMF 301) the maximum production of protease occurred in the early stage of stationary phase and sporulation was followed to after this stage.

Table 3. *Comparison of partially purified protease activity between parent(301) and bald mutant (302)*

Strain	Protease	Relative activity(%)
SMF301	Nuetral	100.0
	Alkaline	266.3
SMF302	Neutral	100.0
	Alkaline	71.1

It was reported that about 150 genes involved in the sporulation process of *Bacillus* spp. and that alkaline protease played an important role in the first stage of six sporulation stages (Errington *et al.*, 1985). The relationship between alkaline protease and sporulation was also proposed in yeast (Wolf *et al.*, 1979: Betz, 1977; Zubenko *et al.*, 1979(a),(b), and *Streptomyces* (Prikladnaya *et al.*, 1982).

From the results obtained in the current study, it was evident that alkaline protease activity was lowered in the non-sporulating mutant. It indicated that there was some close inter-relationship between alkaline protease activity and spore formation, in *Streptomyces* sp.

적 요

성장속도가 빠르고 포자형성이 우수한 放線菌 一株를 토양에서 분리한 뒤 분리균의 특성을 조사한 결과 세포외 단백질 분해효소가 중성과 알카리성의 두 종류가 생성되었으며 β-lactamase도 생성함을 알았다. 이 균주를 acriflarin 또는 NTG로서 처

리하여 얻은 변이주는 포자의 형성, β-lactamase의 생성 및 protease의 생합성 능력이 소실 또는 크게 저하되었다. 일단계의 변이주 취득에서 동시에 형질의 변화가 다양하게 나타난 원인을 규명한 결과 호알카리성 protease의 생합성이 크게 저하되었음을 알았다. 따라서 방선균에서 포자형성과 호알카리성 protease의 활성이 일정한 연관성이 있을 것으로 판단되었다.

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