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Studies on the Microbial Pigment (Ⅳ)

—Isolation and Characterization of Protease—

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미생물의 색소에 관한 연구 (제 4 보)

—단백질 분해효소의 분리와 특성—

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ABSTRACT

In order to study on the pigment and protease of *Serratia marcescens*, the correlation between protease activity and pigment formation was investigated. The results are as follows;

(1) The protease activity exhibited two pH optima 6.0 and 7.5, respectively.

(2) The optimal temperature of proteolytic activity was 45°C.

With these-results, it is suggested that the proteolytic enzymes of *Serratia marcescens* is stable at neutral pH range and more active at the high temperature than that of other proteolytic enzymes.

INTRODUCTION

Serratia marcescens produces the red pigment, prodigiosin, as well as norprodigiosin and di-pyrolyl-di-pyromethane prodigiosin (Williams, 1973 : Ahn *et al.*, 1977). The optimal temperature for both of bacterial growth and pigmentation is 30°C and the optimal pH range for pigmentation is 5.0 (Ahn *et al.*, 1977).

The microbe is known to that they synthesizes and excretes extracellular protein into the surrounding medium with optimum action at pH 8.8 (Murakami *et al.*, 1969). The enzyme is not constitutive, but inducible, since it is formed only in the pressence of high molecular weight substrates, such as gelatin, albumin, and lactalbumin.

The induction of extracellular proteolytic enzymes by high molecular weight substrates is explained in two ways; either the protein itself is an inducer, and information on its presence in the medium is transmitted from the receptor sites on the cell surface along the membrane system into the cell, or the "basal" enzyme decomposes the protein of the medium to lower molecular weight compounds (peptides or amino acids), which play the

role of true inducers (Loriya *et al.*, 1977).

The production of extracellular proteolytic enzymes in culture filtrates of *Staphylococcus aureus* observed as early as 1916. However, it was only recently that attempts has been made to purify and characterize these proteases. But the properties of the proteolytic enzymes of *Serratia marcescens* were not completely investigated.

The purpose of this experiment was to study the synthesis of the extracellular protease, and to study some of the characteristics of the purified enzyme of *Serratia marcescens* in relation to pigmentation.

MATERIALS AND METHODS

1. Organism.

The *Serratia marcescens* strain P was used in these experiments.

2. Media and culture condition.

Modified nutrient broth media were used for determining the proteolytic enzyme. The medium was consisted of as the following; pepton 6g, and NaCl 8g in 1,000m*l* of distilled water. For stock, the liquid nutrient medium was consisted of as the following; beef extract 3g, peptone 5g, and NaCl 8g in 1,000m*l* of distilled water.

The inoculated medium was incubated at 30°C for 16hrs, and the inoculum size was as of 2.6×10^6 cells per milliliter.

3. Enzyme purification and assays.

Step 1. Enzyme preparation.

After 16hrs incubation, the culture were then chilled to about 4°C and centrifuged at 15,000rpm for 15 minute in a high speed refrigerated centrifuge

Step 2. Ammonium sulfate precipitation.

5 liters of centrifuged culture media were added with ammonum sulfate stirring for 10 hrs and the suspension was centrifuged at 15,000rpm for 15 minute. The supernatant was discarded and the dark brown precipitate was dissolved in 0.01M Tris-HCl buffer (pH 7.5). And then the solution was dialyzed with cellulose dialysis tube (pore size; 15Å) for 20hrs against the same buffer.

Step 3. Gel chromatography.

The dialyzed solution (35m*l*) were applied to 4.5×80cm column of Bio-Gel P-150 which had been equilibrated with 0.01M Tris-HCl buffer (pH 7.5). The flow rate was 30m*l* per hour and fractionation were collected 8m*l*, respectively.

Step 4. DEAE-cellulose chromatography.

The solution (40m*l*) from gel chromatography was directly applied to a column (2.5×35cm) of DEAE-cellulose, which had been equilibrated with 0.01M Tris-HCl buffer (pH 7.5). And then the solution was washed with potassium chloride as the concentration between 0.5 and 1.0M in linear gradient. The flow rate was 30m*l* per hour and fractionation was collected 10m*l*, respectively.

Step 5. Enzyme assays.

Proteolytic activity of *Serratia marcescens* was measured by the modified method of Kunitz (1947), with bovine serum albumin as the substate. The substrate and enzyme mixture was prepared 3m*l* and then incubated at 37°C. After 15 minute incubation, the reaction was stopped by addition of 3m*l* of 10% trich loroacetic acid. Standing for 15 minute, the contents in the tubes were centrifuged at 5,000rpm for 15 minute. And the enzyme activity in the supernatant was measured by spectrophotmeter (CE 272) at 280nm. One unit of enzyme activity is equivalent to the change of 0.01 OD per minute. And the specific activity of enzyme means the number of enzyme activity units per miligram of protein. To determine the amount of protein content, the purified enzyme was applied to the Lowry method (1951), using bovine serum albumin

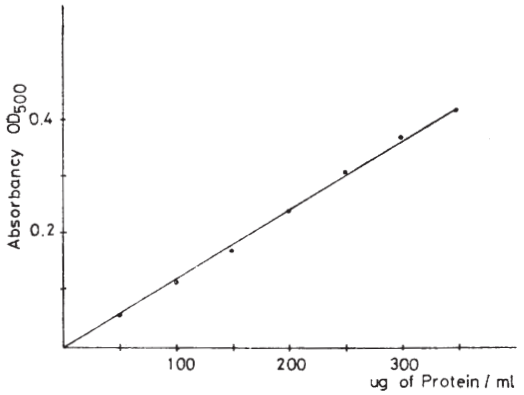

Fig .1 Standard curve for bovine serumm albumin by Lowry method

as standard protein (Fig. 1).

RESULTS AND DISCUSSIONS

1. Enzyme fraction and properties.

Referring to figure 2, the protease separated by gel chromatography seemed to consist of the different two enzymes. The one appeared at fraction from 42 to 50. The maximum activity of the two enzymes were determined as 422 and 318 unit, respectively.

Of the two kinds of enzyme, the high proteolytic enzyme (422 unit) was collected 40m*l* and applied to DEAE-cellulose chromatograpy for examining the properties of enzyme. (Fig. 2)

Determining the total protein in fraction, it

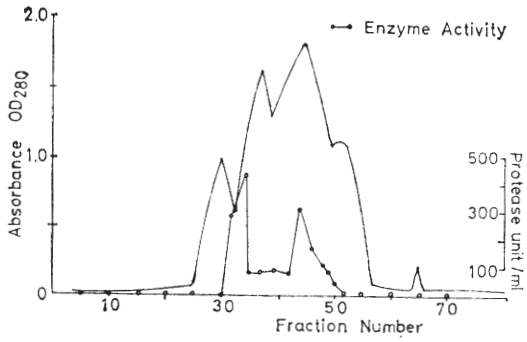

Fig. 2 Chromatography of *S. marcescens* on Bio-Gel

was revealed that the protein might be a sort of degradation of substrate media or that was originated from enzyme excretion.

Especially relationship between the amount of protein and maximum activity of enzyme fraction were not fully coincided with each other in the case of high proteolytic enzyme solution.

On the other hand, referring to figure 3, the high proteolytic enzyme solution in the experiment of gel chromatography was applied to DEAE-cellulose chromatography and known to that the fraction peak appeared only one at the number from 20 to 26. The maximum proteolytic activity of this fractionation was 335 unit (Fig. 3).

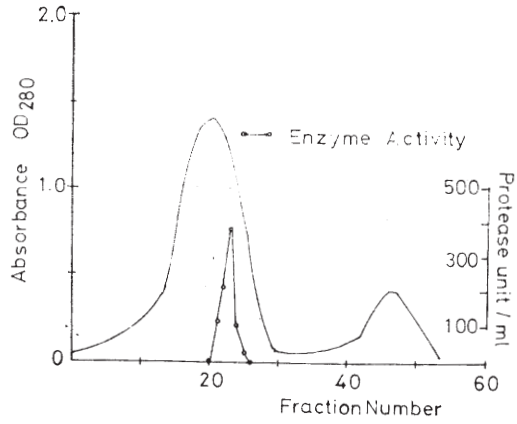

Fig. 3 Chromatograpy of *S. marcescens* on DEAE-Cellulose

In general, separation of enzyme may occur to addition of potassium chloride solution (0.5M) but separation of this enzyme occurred when the high concentration (0.5M to 1.0M) of potassium chloride solution be added to in linear gradient.

2. Effect of pH and temperature on proteolytic activity.

As shown in figure 4, this protease exhibited two pH optima that one is pH 6.0 and the other pH 7.5. It means that this enzyme

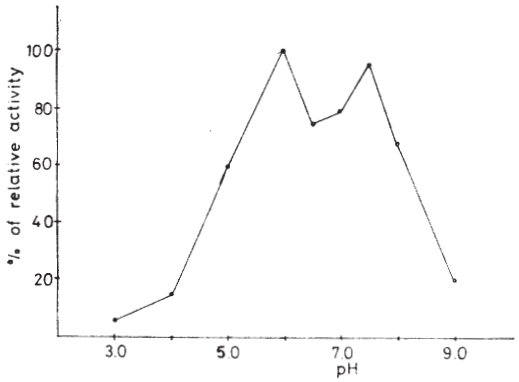

Fig. 4 Dependence of enzyme activity on pH

is stable at neutral pH range (Drapeau *et al.*, 1972; Setlow, 1976). (Fig. 4 5)

And the effect of temperature on proteolytic activity are shown in figure 5. The optimal temperature of proteolytic activity was 45°C. It is suggested that the enzyme is more active

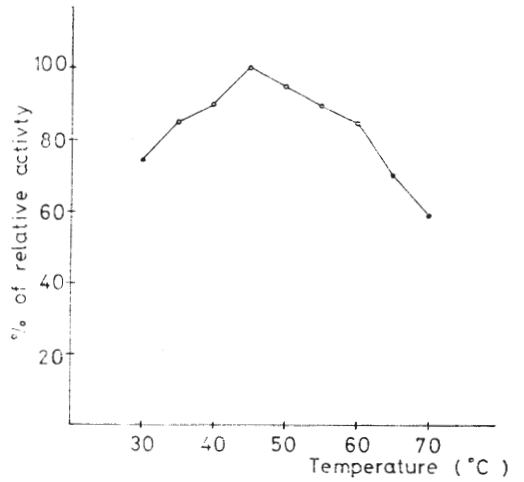

Fig. 5 Dependence of enzyme activity on temperature.

at the high temperature than that of the other enzymes (Drapeau, 1978; Drapeau *et al.*, 1972).

적 요

*Serratia marcescens*가 생성하는 단백질 분해효소를 연구하기 위하여 효소의 활성도를 규명한 결과는 다음과 같다.

(1) 단백질 분해효소의 활성은 pH 6.0과 pH 7.5의 두 범주에서 최대로 나타났다.

(2) 45°C에서 효소의 활성도가 가장 높았다. 이러한 결과로 미루어 볼 때 *Serratia marcescens*의 단백질 분해효소는 중성 pH 범위에서 안정된 효소활성을 갖는 것으로 생각할 수 있으며 다른 효소에 비해 비교적 높은 온도에서 활성도가 더욱 좋음을 알 수 있었다.

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