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Studies on the production and purification of an extracellular protease from a nonpigmenting *Serration* sp.

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Nonpigmenting *Serratia* sp. 에서 균체의 단백질 분해효소의 생성과 정제에 관한 연구

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Cultivation conditions for the production of extracellular alkaline protease by a nonpigmentation *Serratia* sp. and purification of the enzyme were studied. The maximum enzyme level was obtained at the beginning of stationary phase when the organism was cultured on brain heart infusion medium at 25°C under aeration (gyratory shaking, 180 cycles/min). The enzyme was purified about 100 fold with 16.5% yield by ammonium sulfate precipitation, ammonium sulfate fractionation followed by DEAE-cellulose chromatography (1st and 2nd). The purified enzyme moved as a single symmetrical peak in the analytical ultracentrifuge. The enzyme demonstrated its maximum activity at pH 8.5-9.0 and 40°C when vitamin-free casein was used as a substrate.

The enterobacterial genus *Serratia* is composed of several species, all of which produce one to four extracellular protease[1-4]. Purification and characterization of *Serratia marcescens* proteases have been the subject of interesting research area[3-5] because of the possible involvement of the protease in many serratia-induced diseases in humans[4-8] and known pathogenicity in a variety of insects[10]. Usually, nonpigmented *Serratia* strains from various disease lesions were becoming threat in modern medicine[4-8], whereas the alkaline protease from pigmented *Serratia marcescens* ATCC 25419 has been shown to be the causative agent for pathogenicity in the boll weevil[10]. It has also been reported that the protease produced by pigmented *Serratia* strains that are pathogenic for insects are very similar[3].

Thus, it seems likely that characterization of proteases from various pigmented and nonpigmented *Serratia* strains isolated from different habitats might contribute not only to an understanding of taxonomic relationships among *Serratia* strains but also to the determination of the role of *Serratia* proteases in the pathogenesis.

This paper describes the culture conditions for an alkaline protease production from a nonpigmenting *Serratia* strain isolated from soil and a simple purification method for characterization of the enzyme.

Materials and Methods

Chemicals

Organic chemicals used were as follows: DEAE cellulose (Schleicher and Schuell Co.); tris (hydroxy-methyl) aminomethane (Sigma Chemical Co.); Brain heart infusion, gelatin (Difco Laboratories); vitamin-free casein (Nutritional

Biochemical Corporation). All salts and reagents were of analytical grade.

Organism and Maintenance

Serratia sp. strain LW-1 was obtained from Dr. H.D. Braymer of Louisiana State University. The organism was originally isolated from soil and identified as a non-pigmenting strain of *Serratia* producing a large amount of protease. The organism was maintained on nutrient agar slants at 4°C. All growth studies and production of crude enzyme were performed using brain-heart infusion broth.

Crude enzyme production

A loopful of the stock culture was transferred into 25 ml of the medium (BHI). These cells were grown with constant shaking (180 cycle/min) on a gyratory shaker at 25°C. After 24 hours of incubation, about 0.5 ml of the culture was transferred into 5 two-liter baffle flasks each containing one-liter of BHI medium. The cells were then grown at 25°C with constant shaking (180 cycle/min) for 76 to 80 hours. The cells were removed by centrifugation at 10,000 g for 20 min. The supernatant was pooled and stored at 4°C.

Enzymatic assays and Protein Determination

A quantitative assay was performed according to the method of Kunitz[11] with a slight modification. One ml of 1% vitamin free casein in 0.05 M sodium phosphate buffer (pH 7.5) was brought to 30°C in a water bath, and then 1.0 ml of enzyme solution was added. The reaction mixture was incubated for 20 min at 30°C. Three ml of 50% trichloroacetic acid was added to stop the reaction and to precipitate the undigested substrate. The undigested substrate was removed by centrifugation at 25,000 g for 15 min and absorbance of the supernatant was measured at 280 nm. One unit of activity was defined as a change of 0.1 absorbance unit per 20 min. The specific activity was expressed as the number of units per milligram of protein. A qualitative assay for location of proteolytic activity fractions eluted from column chromatography was carried out by gelatin spot assay method. A 3% gelatin solution was brought to boiling to dissolve gelatin and 0.3-0.5 ml of the gelatin solution was placed in each hole of the spot assay plate. One drop (0.05 ml) of enzyme solution was added to the assay plate and the mixture was incubated at room temperature for 20-30 min. The liquefaction of the gelatin after the assay plate was placed in a refrigerator for several hours indicated the presence of protease.

Protein was determined by the method of Lowry et al.[12]. A rough calculation of protein concentration was done by measuring optical density at 280nm ($E^{1mg/ml}_{280nm,\ 1cm} = 2.25$)[13]. This procedure was useful for the rapid determination of protein in column chromatography fractions.

Enzyme Purification

Solid ammonium sulfate was added slowly to the culture supernatant fluids (5 *l*), with constant stirring, to a final concentration of 80% saturation. After stirring continuously for one-half hour, the solution was allowed to precipitate for 12 h. The suspension was then centrifuged at 12,000 g for 20 min. The resulting precipitate was collected and dissolved in a minimal volume of cold 0.05 M phosphate buffer (pH 6.8). The preparation was dialyzed for 16 hours against four changes of the same buffer. After dialysis, the dialysate was diluted with 0.05 M phosphate buffer (pH 6.8) to protein concentration of 20 mg/ml, and the sample was subjected to ammonium sulfate fractionation. In this step 30-50% ammonium sulfate precipitate was collected and redissolved in a minimal volume of 0.05 M phosphate buffer (pH 6.8). The ammonium sulfate fractionated enzyme was dialyzed for 16 hours against four changes of 0.05 M phosphate buffer (pH 8.0). The dialyzed enzyme solution was loaded to a DEAE cellulose column preequilibrated with 0.05 M phosphate (pH 8.0). After washing to column with the same buffer to remove nonabsorbed protein, the column was then eluted with a linear gradient consisting of one column volume of the equilibrating buffer containing 0.1 M KCl and one column volume of the same buffer containing 0.4 M KCl. Ten ml fractions were collected and spot assayed. Two sizes of col-

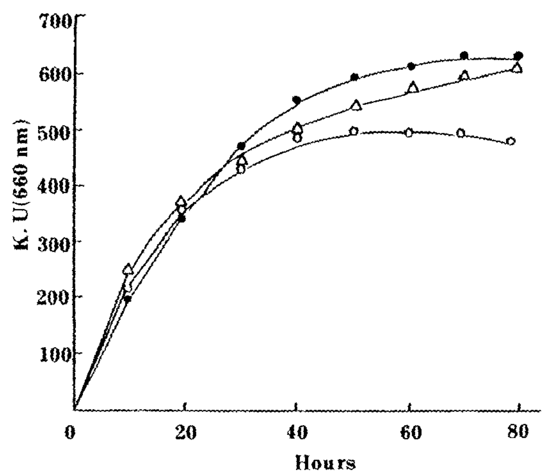

Fig. 1. Growth curves of the nonpigmenting *Serratia* sp. LW-1 at different temperatures.

●—● 25℃, △—△ 30℃, ○—○ 37℃. The organism was grown on BHI medium with shaking (180 cycles/min). Growth was masured by using a Klett-Summerson colorimeter (filter #66).

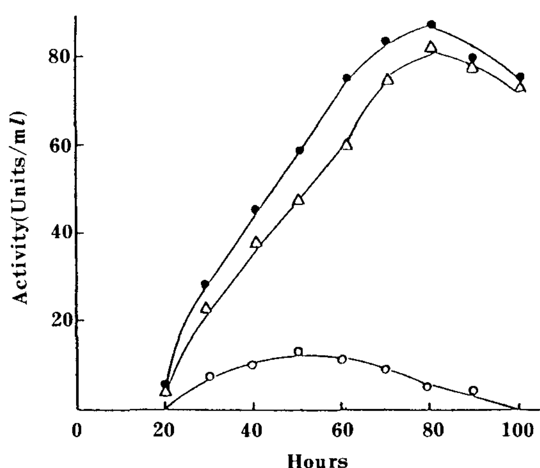

Fig. 2. Production of the protease by *Serratia* sp. LW-1 at various temperatures.

●—● 25℃, △—△ 30℃, ○—○ 37℃. Growth conditions were the same as those described in Fig. 1. Activity was expressed as units per m*l*.

umns, 5 ×45 cm and 2.5×30 cm, were used. Unless otherwise stated, all procedures were done at approximately 4°C.

Analytical Ultracentrifugation

Analytical ultracentrifugation was performed at 56,000 RPM in a double sector cell using the Schlieren optical system in a Beckman Spinco Model E analytical ultracentrifuge. Timing was begun at 2/3 maximum speed (37,000 RPM) and photographs were taken at 8 min intevals. For the preparation of sample a 10 mg/ml solution of enzyme was dialyzed intensively against two changes of sodium phosphate buffer, pH 8.0, containing 0.1 M NaCl.

Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis[18]. The protein samples (200 μg) in 40% sucrose solution was placed on the top of upper gel. Electrophoresis was performed in glycine buffer, pH 9.3, for 45 min. Gels were stained with 1% Amido Schwartz in 7% acetic acid and destained with 7% acetic acid.

Results and Discussion

Cultivation conditions for the protease production

In order to find conditions where *Serratia* sp. Strain LW-1 exhibit maximum level of protease production, growth experiments were performed in brain heart infusion medium. The growth was followed by measuring the increase in optical density using a Klett-Summerson colorimeter (filter # 66).

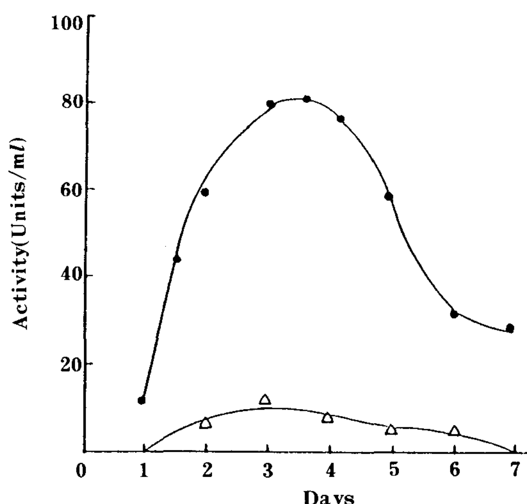

Fig. 3. Effect of aeration(●—●) and lack of aeration(△—△) on the production of protease.

The *Serratia* strain. was grown at 25℃ on BHI medium. Aeration w as performed on a gyrotary Aeration was performed on a gyrotary shaker with 180 cycles/min. Activity was expressed as units per m*l*.

Table 1. Effect of gelatin on the production of *Serratia* sp. protease on BHI medium at 25°C.

Conditions	Incubation Time	Activity* (unit) Gelatin	No Gelatin
aeration	24	9.0	5.0
	48	42.9	42.0
	72	58.0	59.0
	96	85.0	76.0
	120	79.0	45.0
	144	54.0	29.0
no aeration	24	0	1.0
	48	2.5	5.0
	72	15.0	12.0
	96	8.5	8.0
	120	8.0	9.0
	144	5.0	4.0

*One unit of activity was expressed as a change of 0.1 unit absorbance at 280 nm per m*l* of culture supernatant.

As shown in Fig. 1, the optimum temperature for growth was around 25°C rather than 30°C or 37°C even though the initial growth rate (0-20 h) was a little higher at 30°C and

Table 2. Purification of protease from *Serratia* sp. LW-1.

Step	Protein (mg)	Specific Activity[2]	Total Units of Activity[1]	Recovery	Fold Increase
Culture supernatant	63,000	2.5	157,500	100	1.0
30-55% $(NH_4)_2SO_4$ precipitation	1.152	87	100,200	63	35.0
First DEAE-cellulose chromatography	310	253	78,400	49.8	101
Second DEAE-cellulose	97	269	26,100	16.6	108

[1]One unit of activity was defined as a change of 0.1 absorbance unit at 280 nm.
[2]The specific activity was expressed as the number of units activity per milligram of protein.

37°C than at 25°C. The organism usually entered its stationary phase within 70 hours at 25°C and 30°C.

The production of the protease was correlated wtih growth conditions. The organism began to produce the protease in appreciable amount at mid logarithmic phase (20 hours) and the enzyme level reached maximum between the end of logarithmic phase and beginning of stationary phase, as shown in Fig. 2. The optimum temperature for enzyme synthesis was 25°C. At 37°C, the organism did not produce the protease in an appreciable amount. In addition to the effect of temperature, the production of the protease also depend on the rate of aeration during growth. The organism showed higher level of enzyme synthesis under aeration at 25°C as shown in Fig. 3.

Decedue et al.[3] reported that the culture supernatant fluid from a 36 hours culture of *Serratia marcescens* ATCC 25419 contained the highest level of protease when cells were grown on brain heart infusion medium. But the lag period and protease synthesis in the non-pigmenting *Serratia* strain was not clearly understood in the present study. Addition of 1% gelatin, which was known to induce extracellular proteases by various *Serratia marcescens* strains[3,14] growing on minimal medium, to BHI medium in the present investigation did not affect the duration of the lag period (Table 1). However, addition of 1% gelatin to BHI medium increased the production of protease even at the late stationary phase when the organism was grown under aeration.

Purification of the enzyme

A typical purification scheme can be seen in Table 2. The first step of the purification involved the precipitation of all desirable protein from culture supernatant prior to further precise fractionation. The 80% ammonium sulfate

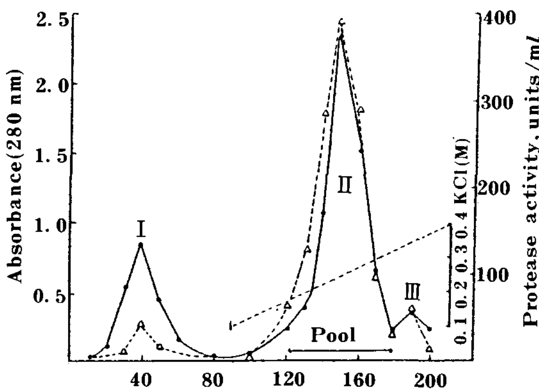

Fig. 4. Elution pattern of the protease of *Serratia* sp. from DEAE-cellulose column chromatography.

KCl gradient elution was performed employing 0.05 M potassium phosphate buffer, pH 8.0. ●—● O.D. at 280 nm △—△ protease activity, units/ml.

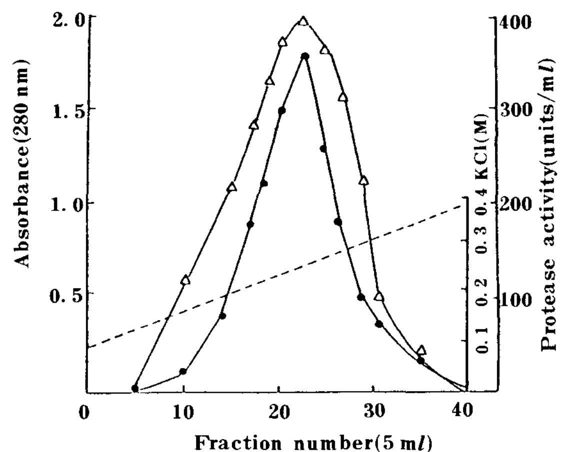

Fig. 5. Second DEAE-cellulose column chromatography of the protease.

About 100mg of the active fractions (peak II) from the first DEAE-cellulose column chromatography was layered on a 2.5×30 cm column. Sample volume: 15ml.

Fig. 6. Sedimentation pattern of the purified *Serratia* sp. protease in the analytical ultracentrifuge.
The photographs were taken at 8 minute intervals. Rotor speed: 56, 000 rpm. Temperature: 20℃. 10mg/ml solution of enzyme was dialyzed against sodium phosphate buffer, pH 8. 0, containing 0. 1 M NaCl prior to analysis. Sedimentation from right to left.

precipitate usually resulted in a 10 to 15 fold increase in specific activity with 69 to 75% recovery over the culture supernatant. The 30-55% ammonium sulfate fraction showed about 35-fold increase in the specific activity with 63% recovery of total activity. Fig. 4 represents the elution pattern of DEAE-cellulose chromatography when the 30-55% ammonium sulfate fraction was applied to the column. Two major protein peaks and one minor peak were detected as indicated in the figure. Peak I was non-absorbed washing fractions and peak II, III were eluted by the salt gradient. Using the gelating spot assay, all three peaks were found to possess proteolytic activity. By casein digestion assay, the peak II was found to contain the bluk of proteolytic activity and a total of 40-50% recovery and approximately 100-fold increase in specific activity of the culture supernatant could be obtained. This represents 3-4 fold increase of specific activity over the

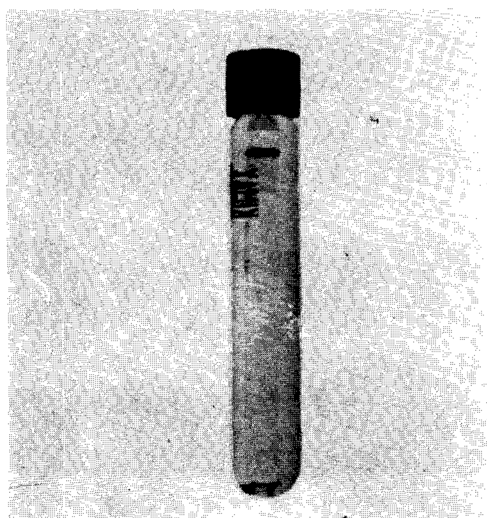

Fig. 7. Polyacrylamide gel electrophoresis of the purified protease preparation.

ammonium sulfate fractionated enzyme preparations. Rechromatography of the active fractions (peak II, fractions 130-170) of the first DEAE-cellulose chromatography resulted in only a slight increase in specific activity (Fig. 5) and a decrease in recovery. This result indicates that the use of the second DEAE-cellulose step is unnecessary for further purification.

Homogeneity of the purified protease

The sedimentation analysis of the protease on the Model E analytical ultracentrifuge revealved homogeneity of the enzyme preparations obtained from second DEAE-cellulose chromatography acitve fractions (Fig. 6). The enzyme sedimented at a uniform velocity as a single symmetrical

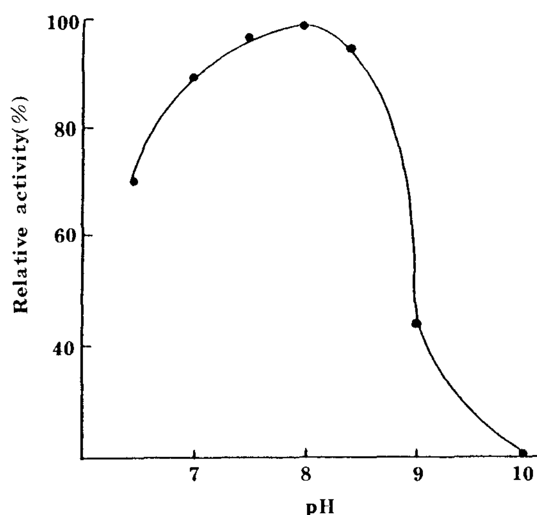

Fig. 8. Effect of pH on the activity of *Serratia* sp. protease.

Vitamin-free casein(1% w/v) was dissolved in 0.05 M potassium phosphate buffer and tris-HCl buffer, which had been adjusted to the desired pH. ●—● 0. 05M potassium phosphate buffer, △—△ 0. 05 M tris-HCl buffer.

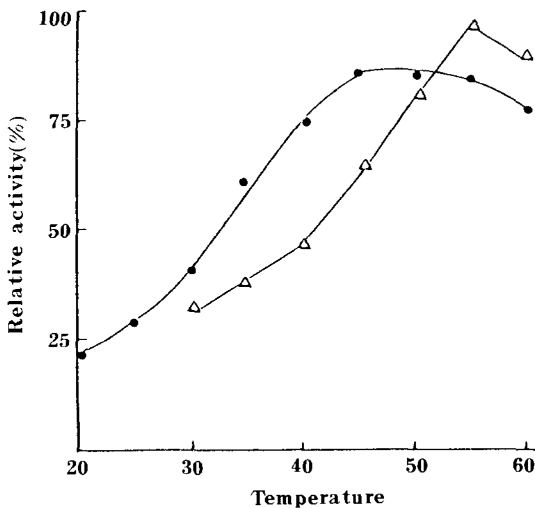

Fig. 9. Effect of temperature on the activity of *Serratia* sp. protease.

One m*l* of protease was incubated with one m*l* of vitamin-free casein (1% w/v) in 0.05 M potassium phosphate buffer for 25 minutes at desired temperatures.

peak indicating no gross contamination with other proteins. Electrophoresis resulted in one major band and one faint trace of minor band (Fig. 7). By this criterion, the enzyme preparation is more than 95% pure.

Effect of pH and temperature on the protease activity

Using 0.05 M tris-HCl buffer, the optimal pH of the purified enzyme for the protease activity was 9.5 (Fig. 8). In case of potassium phosphate buffer, the enzyme showed optimum pH range above pH 8.5. The pH optima for proteolytic activity of many serratial proteases have been reported to be about 9[3,15,16,17]. However, one major protease (56 K protease) of *Serratia marcescens* kums 3058 possessed a pH optimum of about 5, with gelatin as the substrate[4]. Neutral *Serratia marcescens* protease with the pH optimum range of 5.5 to 7.5 has also been reported[5]. Thus, the protease isolated in the present investigation could be classified as an alkaline *Serratia* protease.

The results of the optimum temperature study can be seen in Fig. 9. The optimum temperature for the protease activity was 40-45°C using vitamin-free casein as the substrate.

요 약

색소를 형성하지 못하는 ***Serratia*** sp. LW-1 균주의 균체의 protease 생성조건을 검토하고 효소를 간단한 방법으로 정제하였다. 효소 생산을 위한 최적온도는 brain heart infusion 배지에서 25 ℃ 이었으며, 배양후 76-80시간에 최고의 균체의 효소활성을 나타냈다. Aeration 효과는 5 *l* 용 flask 에 배지량을 1 *l* 주입하여 180 cycles/min으로 진탕배양 하였을 때가 aeration 하지 않았을 경우보다 약 8 배의 균체의 효소생산을 보였다.

효소의 정제는 ammonium sulfate 침전, ammonium sulfate 분별염석 및 두번의 DEAE-cellulose column 크로마토그라피에 의하여 수행하였으며, 정제된 효소는 정제도가 약 100배, 회수율이 16 % 이었다. 정제된 효소는 analytical ultracentrifuge pattern에서 단일 단백질로 나타났으며, 최고 활성을 나타내는 pH는 vitamin free casein을 substrate로 사용하였을 때 pH 8.5-9.5 이었고 최적온도는 40 ℃ 근처이었다.

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