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Purification and Characterization of Proteases from *Streptomyces* sp. SMF301

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Streptomyces sp. SMF 301에서 분리한 단백질 분해효소의 성질

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Procedure for the purification of protease from culture broth of *Streptomyces* sp. SMF 301 was developed. It was evident that the strain porduced two different proteases of which molecular weights were estimated to be 23,500 and 38,900 dalton. It was found that the optimum pH of the smaller was 9.0 and that of the larger was 7.0. The optimal temperature of the alkaline protease was 50°C and that of the neutral protease was much more stable than neutral protease at extreme condition viz. high temperature, and pH.

Proteases are extracellularly produced in most microorganisms. *Bacillus* spp. are the most widely used for the production of portease and the subtilisin produced by *Bacilus subtilis* has been extensively studed (3,6). *Streptomyces* spp. have been well recognized as industrially important microorganisms to produce extracellaular enzymes (2). It was reported that *Streptomyces* spp. produced neutral and alkaline proteases and that the alkaline protease having optimum pH near to 10-12 colud be very useful for the wide range of application (5,10, 11,14). In addition, the molecular roles of alkaline protease on the cell differentiation in *Streptomyces* spp. have been envisaged. However, the mechanisms have not been well understood. In these concepts, it was attempted to investigate the profiles of protease production in *Streptomyces* and to characterize the enzymes. As the first step, a strain of *Streptomyces* sp. isolated from soil was selected because it grew very fast and produced abundant spores when it grew on rich media. The characters of proteases produced in submerged culture were compared.

Materials and Methods

Bacterial strain and culture condition

Streptomyces sp. SMF 301 isolated from Korean soil was used. The strain was cultured at 30°C with shaking on a rich medium containing 2% soluble starch, 1% skim milk, 0.2% yeast extract, 0.1% KH_2PO_4, 0.34% K_2HPO_4, 0.01% $MnCl_2 \cdot 6H_2O$, 0.01% $MgSO_4$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.025% $FeCl_2 \cdot 6H_2O$, 0.02% Na_2CO_3, pH 7.0. After 36 hrs of cultivation, 100 m*l* of the seed culture was inoculated into 4 *l* of the same medium in a laboratory fermentor (Chemap CF) and cultured at 30°C for 72 hours. The crude enzyme solution was obtained by continuous centrifugation at 20.000g at 4°C.

Protease assay

The activity of alkaline protease and neutral protease was determined by measuring tyrosine concentration liberated from Hammarsten casein. Enzyme solution 0.5 m*l* was mixed with 2.5 m*l* of 0.6% Hammarsten casein dissolved in 0.05M K-phosphate buffer (pH 7.0) or 0.05M Na-borate buf-

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fer (pH 9.0). After 10 mins incubation at 30 °C, 2.5 m*l* of TCA solution (consisting of 0.11M trichloroacetic acid, 0.22M sodium acetate and 0.33M acetic acid) was added. The mixture was incubated for 30 mins at 30 °C, and then filtered. Enzyme activity was determined by measuring the absorbance of the filtrate at 275 nm. One unit of enzyme was defined as the amount of the enzyme that release μg of tyrosin per m*l* per min at 30 °C. Protein concentrations were determined by the method of Lowry *et al.*(8) using bovine serum albumin as a standard.

Purification of proteases

The culture broth was centrifuged at 5,000g for 10 min and the cell free broth was used as a crude enzyme solution. Ammonium sulfate was added slowly to the crude enzyme solution with gentle stirring to 45% saturation. After standing for 4 hrs at 4 °, the precipitates were discarded by centrifugation at 9,000 g for 30 mins, and then ammonium sulfate was added to the supernatant to final concentration of 85% saturation. After standing for 4 hrs at 4 °C, the precipitate was collected by centrifugation at 15,000g for 40 mins. It was dissolved in 0.02 M 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 0.02 M Tris-HCl buffer (pH 7.5), and 5 m*l* fraction was 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 0 to 0.5 N NaCl in the buffer was applied, and 10 m*l* fraction was collected. Second gel filtration was carried out by the same way. The active fractions of ion exchange were concentrated in amicon model DC-2 and concentrated enzyme solution was applied to Sephadex G-75-50 column (1.5 × 50 cm).

Molecular weight determination

SDS-polyacrylamide gel electrophoresis was performed by the Laemmli method(7). The concentration of running gel was 10% and that of stacking gel was 2.5% and the molecular weight markers were bovine serum albumin (66.000), eggalbumin (45.000), pepsin (34.700), trypsinogen (24.000) and β-lactoglobulin (18.400).

Determination of optimum pH and temperature

Protease preparations were added to 2.5 m*l* of substrate solution in the following buffer; 0.1M K_2HPO_4 buffer (pH 6.0-pH 7.0), 0.1M Tris-HCl buffer (pH 8.0-pH 9.0), 0.1M Na-borate buffer (pH 10.0-pH 12.0). After incubation at 30 °C for 30 mins, enzyme activity was measured. Optimum temperature was determined by varying the incubation temperature from 20 °C to 55 °C. The measurement was carried out using substrate in optimum reaction buffer.

Heat and pH stability

To determine heat stability, enzyme preparations were incubated at 4 °C, 30 °C, 40 °C, 50 °C, 60 °C, for 100 mins. At each 20 min, remaining enzyme activity was measured. To determine pH stability, enzyme dissolved in following buffer was incubated at optimum reaction temperature for 100 mins; 0.1M Na-acetate buffer (pH 3.0-pH 5.0). 0.1M K_2HPO_4 buffer (pH 6.0-pH 7.0), 0.1M Tris-HCl buffer (pH 8.0-pH 9.0), 0.1M Na-borate buffer (pH 10.0-pH 12.0).

Results and Discussion

Purification of extracellular proteases

The specific activity of proteases in the culture supernatant was 112.71 unit/mg. The crude enzyme solution was fractionated by salting out with ammonium sulfate. It was found that precipitates obtained from 45% to 85% of saturation gave to show protease activity. Gel filtration on Sephadex G-75-50 revealed that two adjacent peaks showed protease activity as shown in Fig. 1. Therefore it was necessary that the active fractions were subjected to further purification steps. As shown in Fig. 2, it was found that the two peaks appeared in the gel filotration were clearly seperated in the anion-exchange chromatography. It was thought that the two proteases might be different to each other. The summary of the purification procedures were shown in Table 1.

Molecular weight of the purified enzymes

SDS-polyacrylamide gel electrophoresis of the purified enzymes showed each single band as shown in Fig. 3A and Fig. 3B. It indicated that the enzymes obtained in those purification steps were

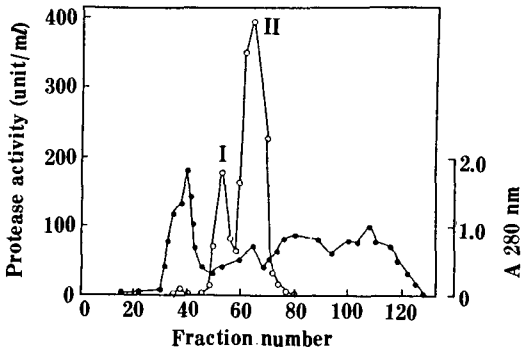

Fig. 1. Gel filtration of crude enzyme on Sephadex G-75-50 column.

Absorbance at 280 nm (-●-●-) was monitored and protease activity (-○-○-) was measured as described in the materials and methods sections

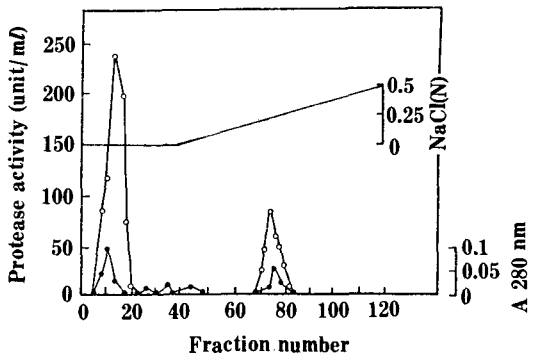

Fig. 2. Ion exchange chromatography of alkaline protease on DEAE-Sephadex A-50.

Protease peak II from gel filtration was applied to a column. Protein concentration (-●-●-) was indicated as absorbance at 280 nm, protease activity (-○-○-) was assayed at 0.05M Na-borate buffer, pH 9.0.

Table 1. Purification of *Streptomyces* sp. SMF 301 proteases

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Fold
Culture broth	9600	1082100	112.71	100	1
Ammonium sulfate Fractionation(45-85%)	1803.2	714320	392.11	66	3.5
Sephadex G-75-50 gel filtration					
protease I	155.35	108210	696.05	10	6.2
protease II	315.38	515515	1944.80	47	17.7
DEAE-Sephadex A-50 ion exchange					
protease I	43.78	75747	1730.10	7	15.4
protease II	162.89	444968	2731.67	41	24.3
Sephadex G-75-50 gel filtration					
protease I	20.68	57892	2788.45	5.3	24.7
protease II	66.95	236001	3525.1	21.8	31.3

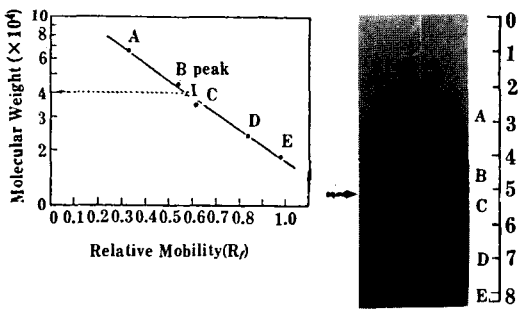

Fig. 3A. Molecular weight estimation of the purified neutral protease using SDS-polyacrylamide gel electrophoresis.

A; bovine albumin, B; egg albumin, C; pepsin, D; trypsinogen E; β-lactoglobulin, peak I; neutral protease.

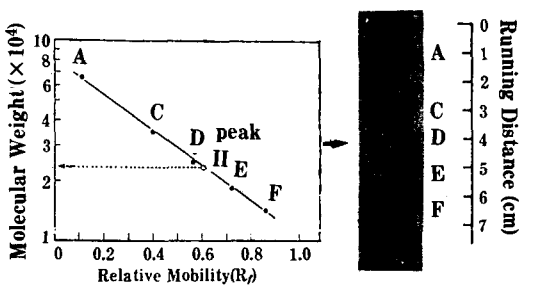

Fig. 3B. Molecular weight estimation of the purified alkaline protease using SDS-polyacrylamide gel electrophoresis.

A; bovine albumin, C; pepsin, D; trypsinogen, E; β-lactoglobulin, F; lysozyme, peak II; alkaline protease

electrophoretically homogeneous. The molecular weights of the protease I and protease II were found to be 38900 dalton for the protease I and 23500 dalton for the protease II. The molecular weight of protease I was similar to that of neutral protease from other source, such as *Bacillus stearothermophilus* (34000 dalton)(13). *Aspergillus oryzae* (37000 dalton)(9), *Saccharomycopsis lipolytica* (38500 dalton)(1), *Nocardia brasiliensis* (35000 dalton)(4). The molecular weight of protease II was similar to that of alkaline protease reported by Renko *et al.*(12).

Effects of pH and temperature on enzyme activity

The optimal pH of the two enzymes was determined and the data are shown in Fig. 4. It was clear that the optimum pH of the enzyme I was 7.0 and that of the enzyme II was 9.0. Hence it was considered that the enzyme I was neutral protease and the enzyme II was alkaline protease. Optimal temperatures for the enzyme activity of the both enzymes were determined and the data are shown in Fig. 5. The optimal temperature of the neutral protease was 35 °C and that of the alkaline protease was 50 °C.

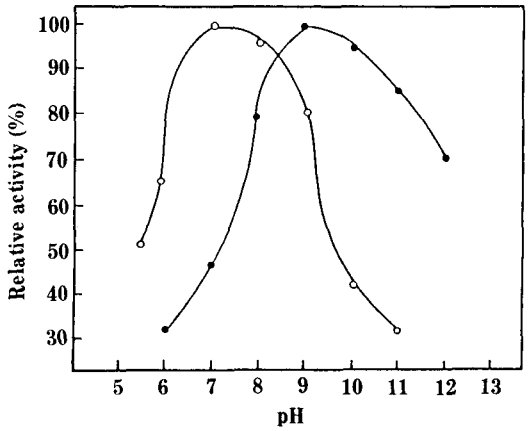

Fig. 4. Effect of pH on the activity of the purified protease collected from peak I (-○-○-), collected from peak II (-●-●-).

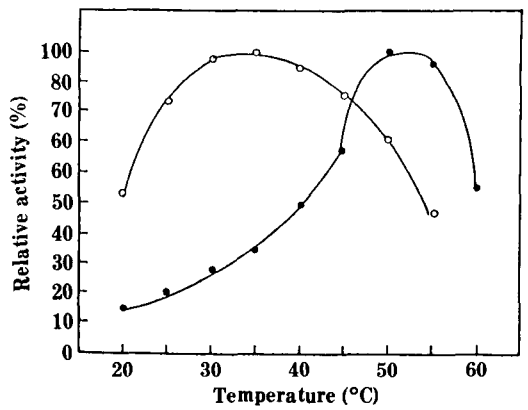

Fig. 5. Effect of themperature on the activity of the purified protease collected from peak I (-○-○-), collected from peak II (-●-●-).

Effects of pH and temperature on enzyme stability

The effects of pH on enzyme stability was examined, and the result is shown in Fig. 6. It was clear that the neutral protease (peak I) was much

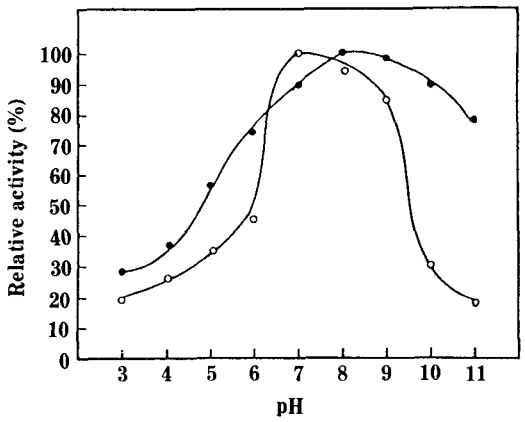

Fig. 6. Effect of pH on the stability of the purified protease.

neutral protease collected from peak I (-○-○-), alkaline protease collected from peak II (-●-●-).

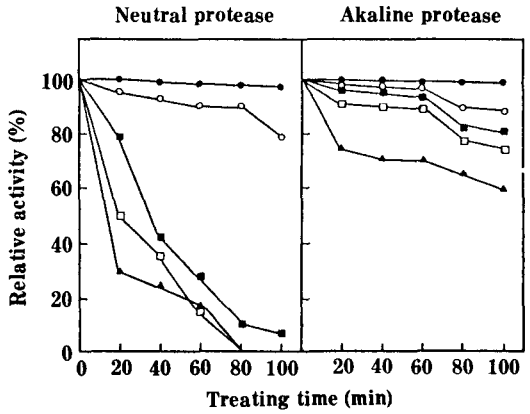

Fig. 7. Effect of temperature on the stability of the purified proteases.

4 °C(-●-), 30 °C(-○-), 40 °C(-■-), 50 °C(-□-), 60 °C(-▲-)

more stable in the pH range 7-9, and that the alkaline protease (peak II) was much more stable at the higher pH compared to the neutral protease. Thermal stability of the enzyme is shown in Fig. 7 and it was found that the neutral protease was very unstable at high temperature above 40℃, but that of the alkaline protease was very stable and showed relatively constant activities at 50℃.

Effect of substrate concentration on enzyme reaction

The comparisons of both proteases in terms of their affinity to the substrate(K_m) and maximum reaction rate(V_{max}) are shown in Fig. 8. With increasing the combination of Hammarsten casein, the rate of the reaction was increased and a typical Michaelis-Menten type substrate saturation pattern was obtained. The K_m values, where casein was used as a substrate, of the neutral protease and the alkaline protease were calculated as 0.54 g/*l* and 0.8 g/*l* respectively. The V_{max} values of the neutral protease was calculated as 12.4 μmole/min/mg protein and that of the alkaline protease was calculated as 15.1 μmole/min/mg protein.

요 약

방선균의 단백질 분해효소를 황산 암모늄분획, Sephadex G-75-50 gel filtration, DEAE-Sephadex A-50 ion-exchange chromatography, ultrafiltration 등의 과정을 통해 정제하였다. 염기성 단백질 분해효소의 분자량은 SDS 전기영동에 의해 23,500 dalton 이었으며 Hammarsten casein에 대한 Km값은 0.8g/*l*였고 이때 Vmax값은 15.1 μmole/min/mg 이었다. 효소반응 최적 pH는 9.0이었고 최적 반응온도는 50℃였다. pH에 대한 안정성은 9.0-10.0에서 최대로 안정하였고 50℃ 이상에서는 효소가 불활성화되었다. 중성단백질 분해효소의 분자량은 38900 dalton 이었으며 Hammarsten casein에 대한 Km값은 0.54g/*l*였고 이때 Vmax값은 12.4 μmole/min/mg이었다. 효소반응 최적 pH는 7.0이었고 최적 반응온도는 35℃였다. pH 7.0-9.0에서는 안정하였으나 40℃ 이상에서는 신속하게 불활성화되었다.

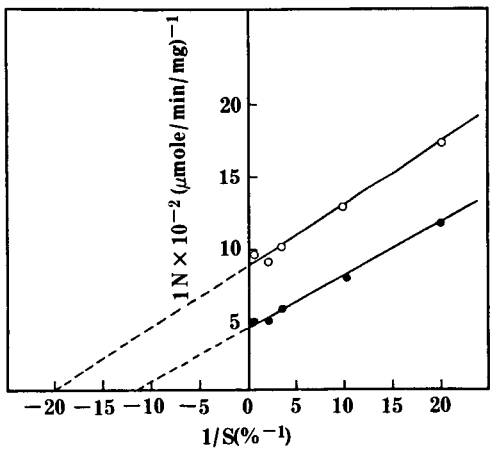

Fig. 8. Effect of Hammarsten casein concentration on the activity of purified neutral protease (-○-○-) and alkaline protease (-●-●-) with increasing Hammarsten casein concentration.

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