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A Proteinase Produced by *Pseudomonas aeruginosa* Isolated from *Chilo suppressalis*

Sir:

A crystalline proteinase was obtained from culture broth of *Pseudomonas aeruginosa* isolated from *Chilo suppressalis*.

Cultivation and purification were carried out as follows: *Ps. aeruginosa* was cultured in 2000 ml of glucose-bouillon medium.

The medium was contained in shaking flasks of 5000 ml volume, which were placed on a rotary shaker and incubated at 28~30°C for 2 days.

The culture broth was centrifuged at 11,000 rpm and the proteinase in the supernatant solution was purified by salting out with ammonium sulfate (0.65 saturation), dialysis (against 0.001 M tris buffer, pH 8.6 containing 10^{-3} M CaCl_2 for 16 hr at 5°C), acetone-calcium chloride treatment (2% CaCl_2 and 10% acetone; to remove the mucin-like substances) and precipitation by acetone (75%).

The precipitate obtained was dissolved into 0.05 M tris buffer pH 8.6 and dialyzed against 0.001 M CaCl_2 solution for 16 hr at 5°C. The

FIG. 1. Photograph ($\times 350$) of Crystalline Proteinase from *Ps. aeruginosa*.

FIG. 2. Electrophoretic Homogeneity of Crystalline Proteinase (tris-HCl buffer, pH 9.4).

FIG. 3. Optimum pH for Proteolytic Action of the Proteinase.

The reaction mixture contained 1 ml of 1% solution of casein at various pH values and 1 ml of 0.005% solution of the enzyme in various buffer solutions. Mixtures were kept for 20 min at 40°C.

After precipitation by 5% trichloroacetic acid solution, optical densities at 280 mμ of the supernatant solution were measured.

FIG. 4. Effect of pH on Stability of the Proteinase.

Buffer solutions used were: citrate buffer (pH 4.0~5.0); phosphate buffer (pH 6~8); glycine-sodium hydroxide buffer (pH 9~10); borate buffer (pH 10~12). To 0.8 ml of each buffer solution, 0.2 ml of a 0.1% solution of the enzyme (in distilled water) was added and the mixture was kept at 30°C for 20 hr. After adjusting the pH to 8.6, the final volume was made up to 5.0 ml. Proteolytic activity was determined before and after treatment, and the percentage of the activity remaining is indicated.

FIG. 5. Effect of Heat Treatment on Stability of the Proteinase.

To 0.8 ml of the 0.05 M tris buffer solution pH 8.6 which was preincubated at various temperatures for 10 min, 0.2 ml of 0.01% solution of the proteinase (in distilled water) was added and kept at the respective temperatures. After 10 min each solution was rapidly cooled. The remaining activity was determined and the percentage of the residual activity is indicated.

dialyzate was concentrated by lyophilization.

Needle-like crystals were formed, as shown in Fig. 1. After recrystallization the enzyme appeared to be homogeneous on disc electrophoresis, as shown in Fig. 2.

The nature of this enzyme is as follows: the optimum pH of the proteolytic activity is at pH 8~9 for casein as shown in Fig. 3.

Figures 4 and 5 show the effects of pH and heat treatment on the stability of this enzyme. These results show that this proteinase is stable within the pH range from 6 to 10 and is also stable for temperatures up to 65°C, unlike most proteinases.

Proteins such as casein, gelatin, egg albumin, haemoglobin were easily hydrolyzed by the enzyme. Elastin was also hydrolyzed to some extent, as shown in Fig. 6. Keratinase ac-

FIG. 6. Elastolytic Activity of Crystalline Enzyme.

Each reaction mixture contained 20 mg of orcein elastin, 3 ml of 0.05 M tris buffer pH 8.6 and various amounts of enzyme as shown in the figure. The reaction was carried out for 3 hr at 40°C and 2 ml of 0.5 M phosphate buffer pH 6 were added to the reaction mixture, which then was centrifuged. Optical density of the supernatant solution was read at 590 mμ.

tivity was determined by the partially modified method of K. Morihara *et al.*,¹⁾ that is, 50 mg of trypsin-treated wool was suspended in 5 ml of 0.05 M tris buffer, pH 8.6, containing 1 mg of enzyme. This mixture was kept for 16 hr at 30°C. The value 0.145, which is the in-

1) K. Morihara, H. Tsuzuki, T. Oka, H. Inoue and M. Ebata, *J. Biol. Chem.*, **240**, 3295 (1965).

crease in optical densities at 280 m μ , was obtained. The value 0.013, was obtained from the mixture of trypsin and wool, as determined under the conditions described above.

Neither collagenase activity nor peptidase activity on L-leucineamide were observed. However, poly-L-lysine was hydrolyzed into di-, tri-, tetra-lysine *etc.*

The metal-chelating agent, EDTA, and heavy metal ions such as Ag⁺, Cu²⁺, Hg²⁺ and Pb²⁺ inactivated enzymatic activity but the soybean trypsin inhibitor had no effect on the enzyme.

The molecular weight of this enzyme, as determined by gel filtration on Sephadex G-75, was about 24,000.

Morihara *et al.* reported that *Ps. aeruginosa* produced three kinds of proteinases, fractions I, II and III, and that fraction III proteinase was not produced in the natural medium.²⁾

The enzyme isolated by us seems to be most similar to the semialkaline proteinase (fraction II) designated by Morihara because of similarities in formation (even in the natural medium), in its stability for heat treatment and in its elastase activity.

However, the optimum pH for proteolytic

activity of this enzyme and fraction II are pH 8~9 and pH 7~8, respectively. The pH stability relationship of this enzyme also seems to differ from that of fraction II, *i.e.* this enzyme has no activity below pH 5, but fraction II retains about 70% activity even at pH 5.¹⁾

Wool keratin was not hydrolyzed by fraction II proteinase¹⁾ but was hydrolyzed to some extent by this enzyme. Its molecular weight also seems to be different. From these results this crystalline enzyme seems to be different from enzymes such as those investigated by K. Morihara *et al.*¹⁻³⁾

The elastase activity of this enzyme was observed to vary with successive inoculations and to decrease with dialysis.

Study of these interesting phenomena is now in progress.

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2) K. Morihara, *J. Bacteriol.*, **88**, 745 (1964).

3) K. Morihara, *Biochim. Biophys. Acta*, **73**, 113 (1963).