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Studies on the Proteases of Marine Bacteria—III

Purification and Some Enzymatic Properties of Proteases

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Abstract

Four components with casein hydrolysing activity were found in extracellular protease produced by a marine *Pseudomonas* sp. I-6. Each protease component was fractionated by use of DEAE-Sephadex A-50, Sephadex G-100 and polyacrylamide gel electrophoresis. Protease activity was determined by the digestion of casein in the reaction mixture for fractions obtained on column chromatography and on a casein agar plate for polyacrylamide gels, respectively. The method of a casein agar plate was found to be convenient to detect the protease activity on the gel.

Protease fractions were alkaline proteases of which optimal pHs were from pH 10.0 to pH 11.0. Proteases F-IIa and F-IIb were very low in activity without Ca^{++} ion but reactivated with the addition of Ca^{++} ion remarkably, whereas the activities of F-Ia and F-Ib were unaffected or slightly affected by Ca^{++} ion. Proteases of this strain were stabilized to heat by Ca^{++} ion but no substantial decrease in activity was observed during 6 hr incubation at 25 C regardless of the absence of Ca^{++} ion.

Numerous proteases of terrestrial and marine bacteria have been purified and characterized[1-3]. Kato et al[4] showed that a marine bacterium *Pseudomonas* sp. No. 548 produced at least four kinds of extracellular protease and that all four enzymes were inactivated by EDTA and three enzymes were stabilized by Ca^{++} ion.

In a preceding paper[5] it was shown that the supplement of Ca^{++} ion to a basal suspending medium was indispensable to the production of extracellular protease in marine isolates.

To determine the effect of cations on enzyme proteins an investigation on purification and some enzymatic properties of a marine isolate *Pseudomonas* sp. I-6 has now been undertaken.

Materials and Methods

Organism. A marine isolate, *Pseudomonas* sp. I-6 was used in this work.

Medium and cultural conditions. A modification of the medium of ZoBell 2216E contained polypeptone (0.5%) yeast extract (0.1%) and Herbst's artificial sea water (ASW). Cultures (250 ml of medium in 500 ml flasks) were incubated on a

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shaker at 25 C until early stationary phase (24 hr) and harvested by centrifugation.

Enzyme assay. The assay method of protease activity was the same as described previously[5].

Protein concentration. The protein was determined spectrophotometrically by measuring its absorbance at 280 nm.

Acrylamide gel electrophoresis. Gel electrophoresis was performed by the method of Davis (pH 9.4 gel)[6]. After electrophoresis of enzyme preparations the gel was divided lengthwise to two parts with a razor knife. One of them was stained with amino black. The other was put on a casein agar plate and incubated for 6–12 hr at 30 C. A casein agar plate was made from 0.6% casein (Hammarsten) and 1.0% agar mixture in 0.05 sodium carbonate buffer (pH 10.0) and dried at 37 C overnight.

Results

Purification of proteases

Step 1. Acetone precipitation. Cold acetone was added to culture supernatant (about 1 l) to 70% (v/v). After standing in a cold room for one hour the precipitate was collected by filtration on a Buchner funnel and dissolved in 0.05 M Tris-HCl buffer (pH 7.6, about 30 ml) containing 2 mM $CaCl_2$. Insoluble materials were removed by filtration and the filtrate was dialyzed for 24 hr against the same buffer using cellulose tubing (Visking co. type 18/32).

Step 2. DEAE-Sephadex A–50 column chromatography. The dialyzed solution was applied to a DEAE-Sephadex A–50 column (1.5 × 25 cm) which had been equilibrated with 0.05 M Tris-HCl (pH 7.6) containing 2 mM $CaCl_2$. The proteases adsorbed were eluted with a linear gradient increase in sodium chloride concentration at a flow rate of 20 ml per hr and 5 ml fractions were collected. The reservoir contained 250 ml of 0.05 M Tris-HCl buffer containing 0.7 M sodium chloride and 2 mM $CaCl_2$ and the mixing chamber 250 ml of the same buffer without sodium chloride. A typical chromatogram is shown in Fig. 1. Two active peaks appeared in which the front and rear peaks were designated as F–I and F–II, respectively. The active fractions were combined and precipitated by the addition of solid ammonium sulfate to a final concentration of 0.80 saturation. The precipitate was collected by centrifugation, dissoleved in the same buffer and dialyzed overnight against the buffer.

Step 3. Sephadex G–100 filtration. The dialyzed solutions were then passed through a column of Sephadex G–100 (2.5 × 75 cm) and the buffer was allowed to flow at a rate of 16 ml/hr. The elution patterns on Sephadex G–100 of Fractions I and II are illustrated in Figs. 2 and 3, respectively. Enzyme preparations of both Fractions I and II were separated to two fractions with protease activity on a column of Sephadex G–100. The results obtained in these experiments indicate that pro-

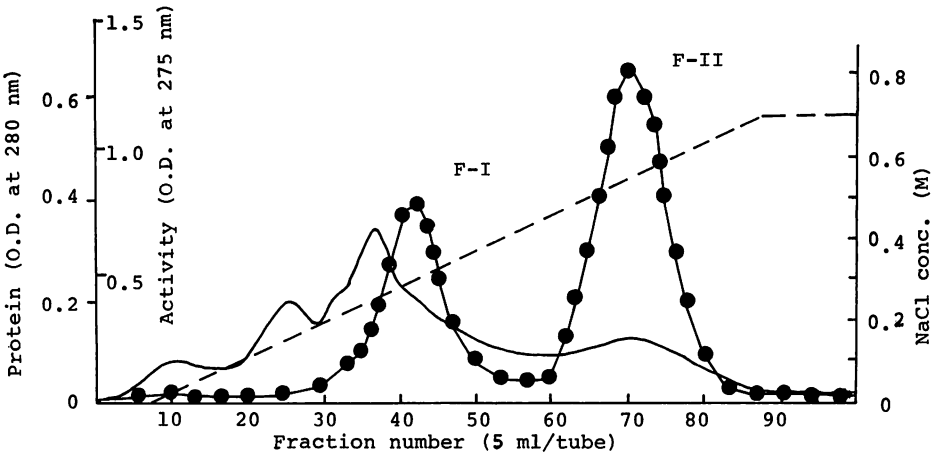

Fig. 1. Chromatography of Protease on DEAE-Sephadex A-50.

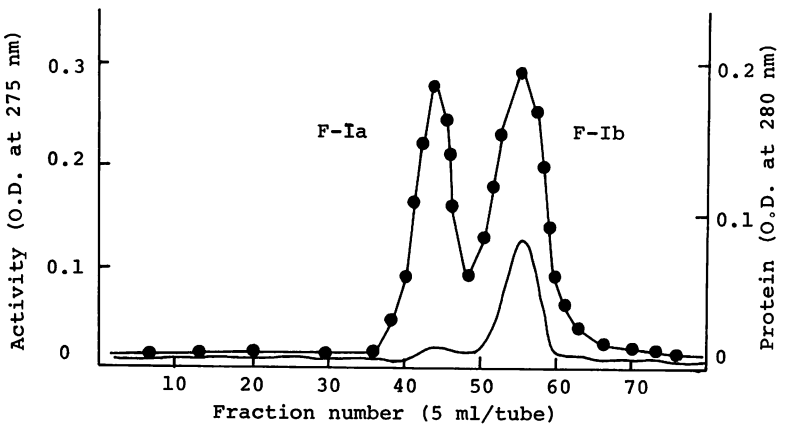

Fig. 2. Chromatography of Fraction I on Sephadex G-100.

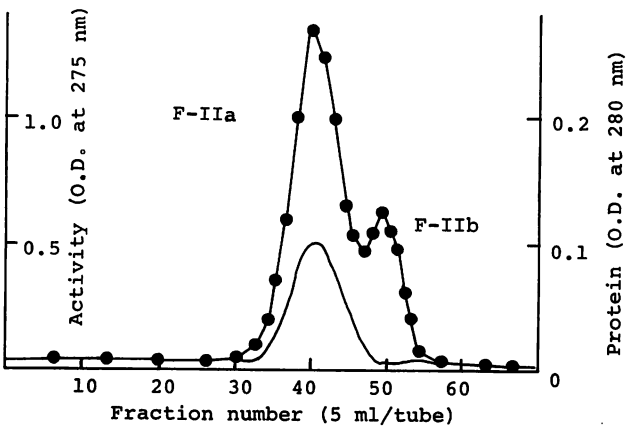

Fig. 3. Chromatography of Fraction II on Sephadex G-100.

tease activity of this strain is fractionated to at least four components, which are designated as F–Ia, F–Ib, F–IIa and F–IIb.

Acrylamice gel electrophoresis. Acrylamide gel electrophoresis was performed with each enzyme preparation and protease activity was detected on a casein-agar plate according to materials and methods. As shown in Fig. 4, crude enzyme preparation had four components and each enzyme preparation gave a mixture composed of a major band and one or two minor bands with protease activity. Unfor-

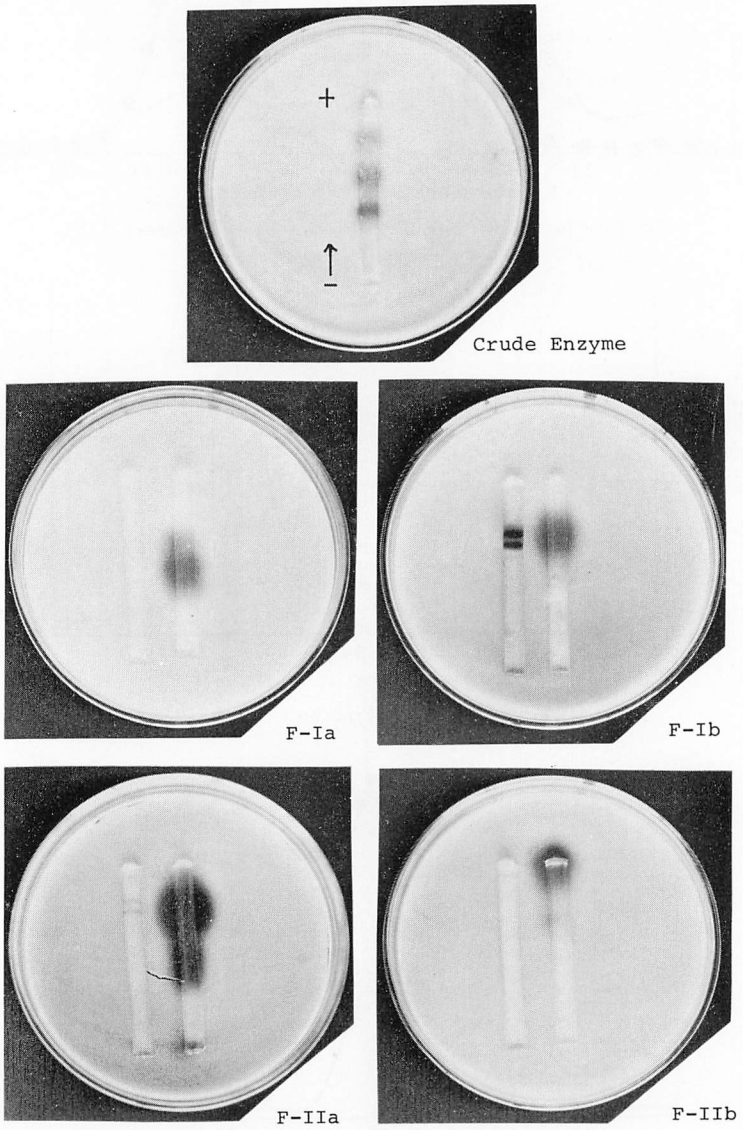

Fig. 4. Polyacrylamide Gel Electrophoresis of Enzyme Preparations. The gels were put on casein agar plates and incubated at 30 C.

tunately, the enzyme preparations which were completely pure were not obtained by the present procedure of purification.

Thermal stability of enzyme activity

The enzyme solutions in 0.05 M Tris-HCl buffer (pH 7.6) with or without the supplement of Ca^{++} ion were incubated at various temperatures for 10 min followed by rapid cooling. The remaining activities were assayed under standard conditions. As shown in Fig. 5, F–I preparation was somewhat more stable than F–II to temperature. Almost complete inactivation of F–II was observed at 65 C, while most of F–I activity remained at this temperature after ten min, and thermal stability of F–I increased by the addition of Ca^{++} ion. The result was shown on thermal stability of F–II with Ca^{++} and was not without Ca^{++} ion because in the absence of Ca^{++} ion the activity of F–II decreased markedly.

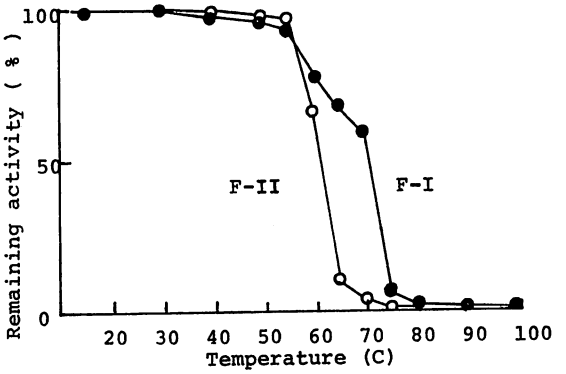

Fig. 5. Effect of Temperature on the Stability of Proteases. Enzyme solutions in 0.05 M Tris-HCI buffer (pH 7.6) with 0.002 M $CaCl_2$ were incubated at the temperature indicated for 10 min. The remaining activity was determined.

Effect of pH on enzyme activity

Fig. 6 illustrates the effect of pH on the activity of F–I and F–II preparations. Both enzyme preparations showed pH optima at the alkaline side. Optimal activities of enzyme preparations F–I and F–II were at pH 10.0 and pH 11.0, respectively.

Effect of cations and some reagents

Tables 1 and 2 summarize the effect of various cations, EDTA and DFP on the activities of enzyme preparations. The enzyme preparations used in this experiment had been dialyzed against 0.05 M Tris buffer for 24 hr, which was changed one time or three times during dialysis. After enzyme preparations were incubated at 30 C for 1 hr with the addition of various cations (final conc. 1 mM), the remaining activities were determined. Among cations tested heavy metal ions, such as Zn^{++}, Cu^{++} and Hg^{++}, were inhibitory to the enzyme activities. DFP (diisopropylfluorophosphate,

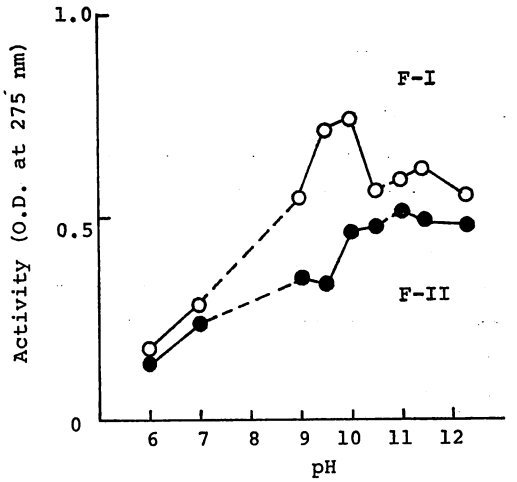

Fig. 6. Effect of pH on Proteolytic Activity. The buffer solutions used were 0.1 M Na_2HPO_4–KH_2PO_4 (pH 6.0–7.0), 0.1 M $NaHCO_3$–Na_2CO_3 (pH 9.0–10.5) and 0.1 M glycine-NaOH (pH 11.0–12.5).

Table 1. Effect of Chemicals on Protease Activity. The enzyme preparations were used after dialyzed for 24 hr. The outer fluid was not changed during dialysis.

Chemical	Conc.	Remaining activity (%) F–I	F–II
None		100	100
KCl	1 mM	101	89
LiCl	1	99	92
$AgNO_3$	1	76	69
$MgCl_2$	1	93	52
$MnCl_2$	1	95	89
$ZnCl_2$	1	40	26
$CuSO_4$	1	56	27
$HgCl_2$	1	23	11
$CaCl_2$	1	85	306
EDTA	1	78	85
〃	10	40	19
DFP	1	43	11
〃	10	24	12

serine enzyme inhibitor) and EDTA (ethylenediaminetetraacetate, metal chelating agent) inactivated both enzyme preparations F–I and F–II and more inhibitory effect was observed on F–II than on F–I. When the dialysis solution was changed 3 times, enzyme preparations F–IIa and F–IIb lost protease activities almost completely and reactivated markedly with the addition of Ca^{++} ion in contrast to the case of

Table 2. Effect of Chemicals on Protease Activity. In this experiment the enzyme preparations were used after the outer fluid of dialysis was changed three times.

Chemical	Conc.	Remaining activity (O. D. at 275 nm) F–Ia	F–Ib	F–IIa	F–IIb
None		0.68	0.36	0.19	0.03
$CaCl_2$	1 mM	0.64	0.39	1.08	0.60
EDTA	1	0.70	—	—	—
〃	10	0.70	0.21	—	—
DFP	1	0.07	0.16	—	—
〃	10	0.01	0.17	—	—

F–Ia and F–Ib which decreased less in protease activity after exhaustive dialysis.

Enzyme stability

In a previous paper it was demonstrated that protease activity of this strain was not observed after incubation for 6 hr in a medium without the supplement of Ca^{++} ion. To approach the possibilities that enzyme proteins were not released at all into the medium or that enzyme proteins were unstable and lost activity rapidly in the absence of Ca^{++} ion, stabilities of enzyme activities during 6 hr incubation period were examined. After being dialysed thoroughly against 0.05 M Tris-HCl buffer, enzyme preparations F–I and F–II were incubated for 6 hr at 12 C or 25 C with or without the addition of Ca^{++} ion (final conc. 5 mM) and assayed for remaining activity. When remaining activity was estimated, Ca^{++} ion was added to enzyme solutions to which Ca^{++} ion had not been added during incubation. As shown in Table 3, no substantial decrease in enzyme activity was observed after 6 hr incubation both

Table 3. Enzyme Stability in Buffer Solution with or without Ca^{++} Ion. Enzyme preparations were incubated for 6 hr at 12 C or 25 C and assayed for remaining activity.

Time	Addition	Remaining activity (%) F–I 12 C	F–I 25 C	F–II 12 C	F–II 25 C
0	−Ca	90%		18	
	+Ca	100		100	
6 hr	−Ca	93	90	19	16
	+Ca	95	90	95	96
Post incubation[a)]	+Ca	100	95	104	103

a) Enzyme preparations were incubated in 0.05 M Tris-HCl buffer (pH 7.6) without Ca^{++} ion and supplemented with $CaCl_2$ (5 mM) after incubation.

at 12 C and 25 C regardless of the absence of Ca^{++} ion. In the case of F–II, protease activity was very low without Ca^{++} ion but the addition of Ca^{++} ion after 6 hr incubation reactivated the enzyme activity.

Discussion

Four components with casein hydrolysing activity were demonstrated in supernatant medium from a marine *Pseudomonas* sp. I–6, by use of two steps of column chromatography with DEAE-Sephadex A–50 and Sephadex G–100 or acrylamide gel-electrophoreses. To examine the purity of enzyme preparations samples of each step in purification were subjected to electrophoresis in polyacrylamide gel and incubated on a casein agar plate to detect protease activities. The method of casein agar plates used in this experiment was found be very convenient to detect protease activity and check the purity of the samples. This method may be able to be applied to various enzymes which hydrolyse macromolecular substances.

The protease fractions obtained were alkaline proteases of which the optimum pHs were from pH 10.0 to pH 11.0. Fractions F–IIa and F–IIb were reactivated markedly and became more stable to heat by the addition of Ca^{++} ion. On the other hand, Fraction F–Ia was not affected and F–Ib was slightly affected by the presence of Ca^{++} ion. Fractions F–I and F–II obtained on DEAE-Sephadex A–50 column chromatography did not decrease in activity substantially during 6 hr incubation in buffer solution at 25 C. F–II was especially low in activity without Ca^{++} but the addition of Ca^{++} ion after incubation reactivated its activity remarkably. These results suggest the possibility that proteases were not produced by the cells suspended in the medium without Ca^{++} rather than the possibility that proteases produced into the medium lost their activities in the absence of Ca^{++} ion during 6 hr incubation. Namely Ca^{++} ion is suggested to be an indispensable factor which controls the synthesis and secretion of extracellular proteases of this strain.

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