Thermostable Proteinases from a Luminous Bacterium, *Vibrio logei* Strain CPM-D3. Purification and Some Properties

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Two proteinases (I and II) from an intestinal luminous bacterium, *Vibrio logei* strain CPM-D3, were purified. The molecular weights of the proteinases I and II were estimated to be 62000 and 54000, respectively. These enzymes were most active at pH 9.0 and 60 °C (proteinase I) and 50 °C (proteinase II), respectively. These enzyme activities were inhibited by orthophenanthroline, but not by ethylenediamine-tetraacetic acid and phosphoramidon. Metal ions such as Cu^{2+}, Hg^{2+} and Ni^{2+} also inhibited these enzyme activities. Both enzyme activities were not affected by heat treatment at 100 °C for 30 min.

Keywords proteinase; thermostable proteinase; luminous bacterium; intestinal bacterium; *Vibrio logei*

Introduction

Bioluminescent bacteria are commonly found in marine environments as free-living forms, saprophytes, parasites, commensal forms and light organ symbionts.[1-3] The purifications and characterizations of the extracellular proteinases from free-living luminous bacteria have been previously reported.[4-8]

Recently, the intestinal luminous bacteria (commensal forms) were isolated from the guts of fish, *Pagrus major*, and identified. In the process of examining the extracellular enzymes, proteinase activity was found in the culture of an intestinal luminous bacterium, *Vibrio logei* strain CPM-D3. After centrifugation of the culture, the proteinase activity was detected in cell suspension, but not in culture supernatant, and the proteinase was liberated from the cells by the addition of sodium deoxycholate solution. The CPM-D3 strain produced two proteinases, each of which had different electrophoretic mobilities. These two cell-associated proteinases were very thermostable, although the extracellular proteinase activities of luminous bacteria were unstable above 55 °C.[4-8]

In this paper, we describe the purification and some properties of these two proteinases.

Materials and Methods

Materials and Isolation Fish, *Pagrus major*, which were collected in Kagoshima (two fish) and Nagasaki (two fish in an ocean culture), were kindly donated by Mr. Ryoichi Satoh of Taiyo Central R & D Institutes, Taiyo Fishery Co., Ltd. These four fish were carried in an ice box to the laboratory within 24 h after being caught. After the dissection of the fish, the intestines were cut in lengths of one centimeter each and each intestinal fragment was crushed in 5 ml of sterile 3% NaCl solution with a homogenizer. The homogenate was spread on MN (modified Nealson's) agar medium,[9,10] which is a nutritionally complete, seawater medium. All colonies and luminous colonies that grew on MN agar medium at 20 °C for 48 h were counted and the isolation of luminous strains was carried out by the method previously described.[11] From these four fish, more than 230 strains of intestinal luminous bacteria were isolated and maintained.

Cultivation Cells were inoculated into 500 ml flasks containing 120 ml of BGPY (basal glycerol peptone yeast extract) broth,[4] which is a nutritionally complete, artificial seawater medium. Cultivation was carried out at 23 °C for 2 d with shaking as previously described.[12] The culture was inoculated into a 10 l glass bottle containing 8 l of BGPY broth and cultivation was carried out aseptically at 23 °C for 4 d with aeration.

Assay of Proteinase Activity The proteinase activity was measured according to the method of Hagihara *et al.*,[13] as described previously.[4] The assay mixture contained 0.25 ml of 2% casein (Hammersten) solution, 0.25 ml of 100 mM Tris–HCl buffer (pH 7.8) in 6% NaCl and 0.25 ml of enzyme solution. After incubation for 10 min at 30 °C, the reaction was stopped by the addition of 0.75 ml of 8.5% trichloroacetic acid. The absorbance of the supernatant of the mixture was measured at 280 nm. Blanks were prepared by adding the enzyme solution after adding the trichloroacetic acid solution. One unit of proteinase activity was defined as the amount of enzyme which librated 1 μg of 280 nm-absorbing materials, calculated as tyrosine, per ml per min. The cell-associated proteinase activity was assayed with cell suspension, which was prepared from the culture by centrifugation, instead of enzyme solution. For characterization of the purified proteinases, the assay mixture was incubated for 60 min at 30 °C.

Identification Identification was carried out according to the methods of Reichelt and Baumann,[14] Jensen *et al.*,[15] Baumann and Baumann,[2] Yang *et al.*[16] and Baumann *et al.*,[17] as described previously.[4,5] The CPM-D3 strain is a moderately luminous, motile gram-negative rod. It was negative for the traits of accumulation of poly-β-hydroxybutyrate, production of gas on fermentation of D-glucose and activity of arginine dihydrolase. The CPM-D3 strain grows at 4 and 20 °C, but not at 30 °C. As sole carbon and energy sources, it utilizes maltose, cellobiose, mannitol and L-proline, but is unable to grow on D-gluconate, D-glucuronate, DL-lactate, D-α-alanine, DL-β-hydroxybutyrate, acetate, propionate, L-tyrosine, α-ketoglutarate and sucrose. The CPM-D3 strain produces lipase, but does not produce amylase and gelatinase. These results indicate that the intestinal luminous bacterium, CPM-D3, is a strain of *Vibrio logei*.

Purification of the Proteinase The cells, CPM-D3, were harvested from the culture (13 l) by centrifugation at 12000 × *g* and suspended with 5 volumes of 100 mM Tris–HCl buffer (pH 7.8) containing 3% NaCl and 0.5% sodium deoxycholate. The cell suspension was vigorously stirred overnight at 4 °C and centrifuged at 12000 × *g*. The enzyme was purified from this supernatant (crude supernatant). Ammonium sulfate was added to the supernatant (120 ml) to 100% saturation. The precipitate was dissolved in an aliquot of 10 mM Tris–HCl buffer (pH 7.8) and then dialyzed against 5 mM Tris–HCl buffer (pH 7.8) at 4 °C for 5 d. The dialyzate (200 ml) was mixed with DEAE-Sephacel which had been previously equilibrated with 10 mM Tris–HCl buffer (pH 7.8). The enzymes were eluted with the same buffer containing 0.4 and 0.8 M NaCl, respectively, as in the case described on the proteinases from *Vibrio harveyi* strain FLN-108,[6] and designated as proteinases I and II. The active fractions of proteinases I and II were combined, separately, and then applied on a Sephadex G-100 column (3.0 × 45 cm) equilibrated with the same buffer. Proteinases I and II were eluted as single peaks and then collected. DEAE-Sephacel and Sephadex G-100 chromatographies were carried out at 20 and 4 °C, respectively.

Determination of Protein Concentration The protein concentration was determined by the method of Lowry *et al.*,[18] with bovine serum albumin as a standard.

Electrophoresis Polyacrylamide disc gel electrophoresis (PAGE) was performed by the method of Davis,[19] as previously described.[4] Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn.[20]

Measurement of Molecular Weight The molecular weights of the purified enzymes were estimated by SDS-PAGE and gel filtration on

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a Sephadex G-100 superfine column (1.5×95 cm) by the method of Determann and Michel.[21]

Chemicals All chemicals obtained were previously described.[4]

Results and Discussion

The purification of the proteinases from the intestinal luminous bacterium, *Vibrio logei* strain CPM-D3, is summarized in Table I. Proteinases I and II were eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 0.4 and 0.8 M NaCl, respectively, on a DEAE-Sephacel column (Fig. 1). The total activity of the dialyzate (ammonium sulfate precipitation) was higher than that of the crude supernatant, because the crude supernatant contained sodium deoxycholate and the proteinase activity was suppressed by the addition of sodium deoxycholate. The purified proteinases I and II had low specific activities such as 0.048 and 0.055 units per mg of protein, respectively, in comparison with those of the proteinases from *Vibrio splendidus* FLE-2 strain[4] and ATCC 33125 strain,[8] and *Vibrio harveyi* FLN-77 strain,[7] which had 1888, 1314 and 1360 units per mg of protein, respectively, using casein as the substrate.

The purified proteinases I and II showed single protein bands on PAGE (Fig. 2), and proteinase I showed lower electrophoretic mobility than proteinase II.

The molecular weights of the proteinases I and II were estimated to be 62000 and 54000 by SDS-PAGE, as shown in Fig. 3, and also estimated to be 62000 and 54000 by

TABLE I. Summary of Purification of the Proteinases from *Vibrio logei* CPM-D3

Procedures	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery
Crude supernatant	2900	50	0.017	100
Ammonium sulfate precipitation	2500	120	0.048	240
DEAE-Sephacel				
Proteinase I	250	8.0	0.032	16.0
Proteinase II	240	12.0	0.050	24.0
Sephadex G-100				
Proteinase I	100	4.8	0.048	9.6
Proteinase II	110	6.6	0.060	13.2

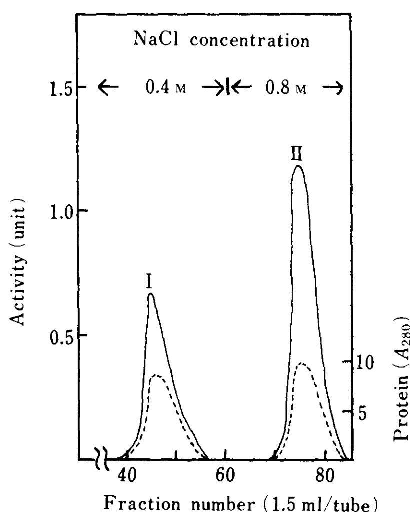

Fig. 1. Elution Profiles of Proteinases from *V. logei* CPM-D3 on a DEAE-Sephacel Column

The proteinases were eluted with 10 mM Tris-HCl buffer (pH 7.8) containing stepwise NaCl concentration. ——, proteinase activity; ------, protein (A_{280}); column size, 4×15 cm; flow rate 10 ml/h.

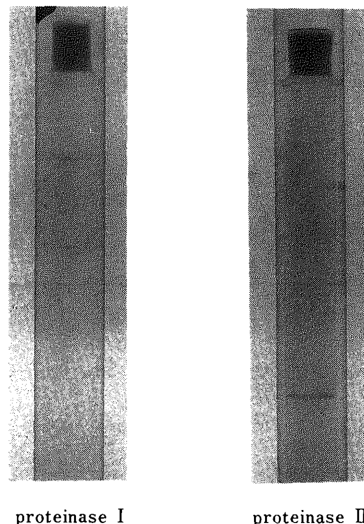

Fig. 2. PAGE of the Purified Proteinases I and II

Electrophoresis was done using 10% gel as described in Materials and Methods. Protein was stained with Coomassie brilliant blue R-250.

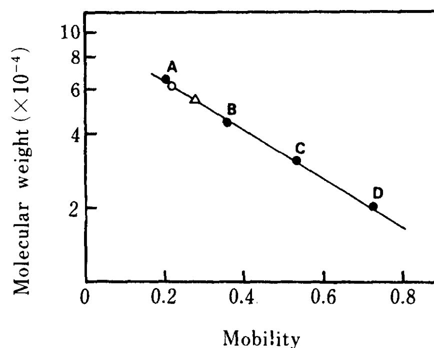

Fig. 3. Estimation of the Molecular Weights of the Purified Proteinases I and II by SDS-PAGE

The standard proteins used and their molecular weights were: A, bovine serum albumin (67000); B, ovalbumin (43000); C, carbonic anhydrase (30000); and D, soybean trypsin inhibitor (20100). ○, proteinase I; △, proteinase II.

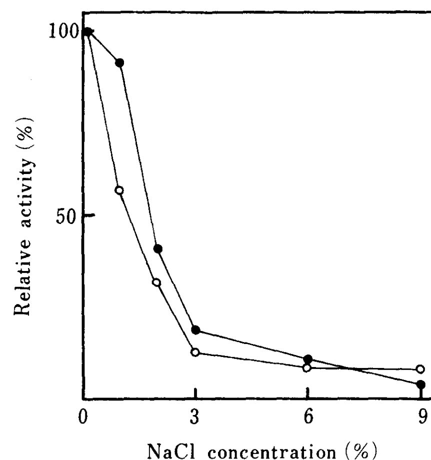

Fig. 4. Effect of NaCl Concentration on the Activity of the Purified Proteinases I and II

The NACl concentration in the assay mixture was varied as indicated. ○, proteinase I; ●, proteinase II.

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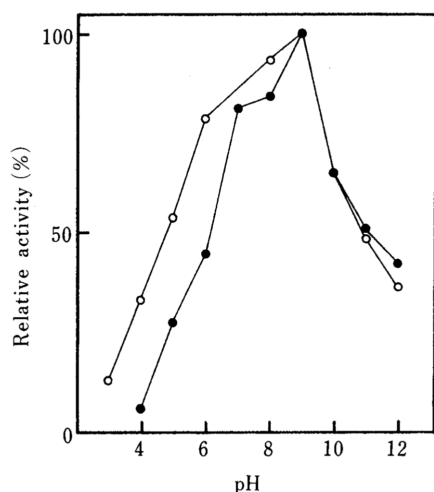

Fig. 5. Effect of pH on the Activity of the Purified Proteinases I and II

The buffers used were; 0.1 M Citrate–Na_2HPO_4 (pH 3—6); 0.1 M KH_2PO_4–Na_2HPO_4 (pH 6—8); 0.2 M Tris–HCl (pH 8—9); and 0.1 M Gly–NaOH (pH 9—12). ○, proteinase I; ●, proteinase II.

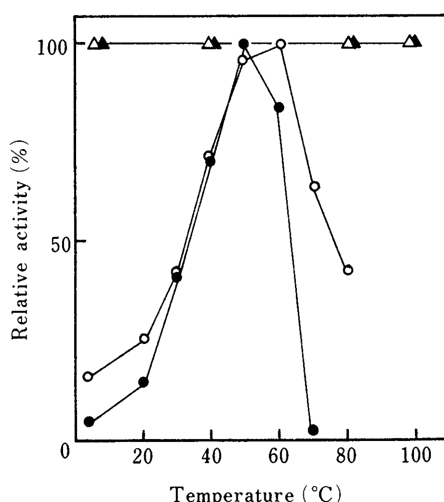

Fig. 6. Effect of Temperature on the Activity and Stability of the Purified Proteinases I and II

The enzyme reaction was carried out at various temperatures to determine the effect of temperature on the proteinase activity (○, proteinase I; ●, proteinase II). While, in the experiment on the effect of temperature on stability (△, proteinase I; ▲, proteinase II), the enzyme solution was kept for 60 min at 4 through 80 °C and for 30 min at 100 °C, and then the remaining activity was assayed.

Sephadex G-100 superfine gel filtration, indicating that the enzymes I and II are monomers.

The enzymes I and II exhibited higher activities at low NaCl concentrations than the seawater concentration (3% NaCl), that is, more than 8-fold and 5-fold increases of the activities at 3% NaCl were seen at 0% NaCl, respectively (Fig. 4). These activities of the cell-associated proteinases showed the same tendency as in the case of the extracellular proteinases from luminous bacteria.[4–8]

Both purified enzymes were most active at pH 9.0, as shown in Fig. 5. The proteinase I showed higher activity than the proteinase II at a lower pHs.

The effect of temperature on the activity was examined (Fig. 6). The maximum activities of the proteinases I and II, which were about 2.4-fold higher than that observed at 30 °C, were seen at 60 and 50 °C, respectively.

While, the effect of temperature on the stability of the purified proteinases was examined. As shown in Fig. 6, the purified proteinases I and II were very thermostable, although the proteinase activities of the free-living luminous bacteria, *V. splendidus*[4,8] and *V. harveyi*,[5–7] were unstable above 55 °C. Furthermore, 66% and 100% of the remaining activities were observed, after these enzyme solutions in sealed vials were autoclaved at 121 °C for 15 min.

Table II. Effects of Various Inhibitors and Chemicals on the Activities of Proteinases I and II

Inhibitors and chemicals	Concentration (mM)	Remaining activity (%)	
		Proteinase I	Proteinase II
Control	—	100	100
Ethylenediamine-tetraacetic acid	1.0	110	101
	10.0	104	117
Orthophenanthrolin	0.1	12.9	110
	1.0	10.8	0.0
Phosphoramidon	0.1	93.0	94.7
Pepstatin A	0.1	98.4	102
Antipain	0.1	96.2	104
Actinonin	0.1	110	109
Amastatin	0.1	107	110
Bestatin	0.1	108	104
Phenylmethyl-sulfonylfluoride	1.0	103	97.3
p-Chloromercuri-benzoic acid	1.0	204	111
$CuCl_2$	1.0	73.1	54.5
$HgCl_2$	1.0	13.4	14.9
$NiCl_2$	1.0	19.9	23.9
$MgCl_2$	1.0	82.9	87.5
$CaCl_2$	1.0	87.6	95.0

The reaction mixture without casein as a substrate was preincubated with individual effectors in 100 mM Tris–HCl buffer (pH 7.8) for 2 h at 20 °C and the reaction was started by adding the casein solution to the assay mixture after preincubation for 10 min at 30 °C. The mixture was incubated for 60 min at 30 °C and then the remaining activities were assayed and expressed as percentages of the control activity determined without an effector.

The effects of various inhibitors and chemicals on the proteinase activities are shown in Table II. Pepstatin A, antipain, actinonin, amastatin, bestatin, phenylmethylsulfonylfluoride and *p*-chloromercuribenzoic acid did not inhibit the activities of proteinases I and II. Although ethylenediaminetetraacetic acid and phosphoramidon inhibited the activities of the extracellular proteinases from the luminous bacteria,[5–8] both chemicals did not inhibit the activities of the proteinases I and II. These purified enzymes were inhibited by some metal ions such as Cu^{2+}, Hg^{2+} and Ni^{2+}. The proteinases I and II were inhibited by orthophenanthroline at concentrations of 0.1 and 1.0 mM, respectively. The activity of the proteinase I was stimulated by the addition of *p*-chloromercuribenzoic acid. It has also been shown on the activity of the proteinase I from *V. harveyi* strain FLN-108, as previously described.[6]

In accordance with the classification of microbial proteinases by Morihara,[22] it is considered that the proteinases I and II are alkaline proteinases, because the enzymes are most active at pH 9.0. Since the enzymes are inhibited by metal-chelating agents such as orthophenanthroline, but not by ethylenediaminetetraacetic acid, it might be necessary to examine the sensitivity of these activities against metal ions.

The hydrolytic actions by the purified proteinases I and II on various *p*-nitroanilide peptides were examined. Only

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a little hydrolysis of alanine-*p*-nitroanilide and leucine-*p*-nitroanilide by the proteinases I and II was observed, indicating that these synthetic *p*-nitroanilide derivatives were not suitable for the substrates. However, the proteinase I was active on dipeptides such as His–Leu, Gly–Leu and Leu–Gly, but not on Gly–Phe, Gly–Gly, Gly–Pro and Glu–Glu. On the other hand, the proteinase II was active on dipeptides such as His–Leu, Gly–Leu, Gly–Phe, Leu–Gly and Gly–Gly, but not on Gly–Pro and Glu–Glu. Although the purified proteinases I and II exhibited low activities on casein as the substrate, the rates of hydrolysis of these dipeptides by the proteinases I and II are higher than those by the extracellular proteinases from *V. splendidus* FLE-2[4] and ATCC 33125.[8] Experiments on the substrate specificities of these proteinases are in progress.

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