Purification and Properties of a Proteinase from a Marine Luminous Bacterium, *Vibrio splendidus* Strain FLE-2

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A proteolytic, marine luminous bacterium was isolated from seawater and identified as *Vibrio splendidus* biotype I strain FLE-2. A proteinase from this strain, with a specific activity 21-fold higher than that of the culture supernatant, was purified to homogeneity. The purified enzyme had a molecular weight of 50,000. The enzyme was most active at pH 8.0 and 55°C, and stable below 35°C. The enzyme activity was completely inhibited by EDTA, orthophenanthroline and phosphoramidon. Also metal ions such as Cu^{2+} , Hg^{2+} , Ni^{2+} , Cd^{2+} and Co^{2+} inhibited the activity. These results indicate that this enzyme is a metal-chelater-sensitive, alkaline proteinase.

Bioluminescent bacteria are commonly found in the marine environment as free-living forms, saprophytes, parasites, commensal forms and light organ symbionts.^{1~3)} While much is known about their ecological distribution, their physiology and the control mechanisms of their luminescent systems,^{1~3)} production of extracellular enzymes has not been investigated except for the use of certain extracellular enzymes for their taxonomic identification.^{2~6)}

Many kinds of microbial proteinases have been reported,^{7,8)} and it is also known that numerous marine bacteria produce proteolytic enzymes,^{9~17)} but as yet little information is available on the proteinases of marine luminous bacteria.

Here we report the identification of a proteolytic, marine luminous bacterium, FLE-2, which was isolated from among two hundred and fifty-one luminous strains (FLE-1 through FLE-251; free-living, series E) obtained from surface coastal water at Teradomari, Niigata prefecture, Japan, and the purification and some properties of the proteinase from this luminous strain, since the proteinase activity

of the culture supernatant of the FLE-2 strain was 3.5-fold higher than those of *Vibrio splendidus* ATCC 33125 and other *V. splendidus* isolates.

MATERIALS AND METHODS

Isolation and identification. Seawater was obtained from surface coastal water at Teradomari, Niigata prefecture, Japan, during June, 1984, and spread-plated (50 μl per plate) on MN agar,^{18,19)} which is a nutritionally complete, seawater medium. Isolation of luminous strains was carried out according to the method described previously.²⁰⁾ Identification was carried out according to the methods of Reichelt and Baumann,⁴⁾ Baumann and Baumann,²⁾ Yang *et al.*⁵⁾ and Baumann *et al.*⁶⁾

Preparation of extracellular proteinase test medium. Extracellular proteinase activity was detected on milk seawater agar-seawater agar two layer plates. In preparing this test agar medium, the two layers were poured separately, in a similar manner to in the case of milk agar-marine agar two layer plates.²¹⁾ The bottom layer consisted of 300 ml of distilled water with 20 g of skim milk dissolved in it, and 700 ml of seawater containing 50 ml of 1 M Tris-HCl buffer (pH 7.8) and 15 g of agar. The two solutions were autoclaved separately (15 min at 121°C), cooled to 55°C and then mixed aseptically just before pouring. After the milk seawater layer had solidified, the

MN agar layer was poured on top.

Chemicals. Casein (Hammersten) was purchased from Merck. Sephadex G-100, Sephadex G-100 superfine, DEAE-Sephacel and molecular weight markers for SDS-PAGE were obtained from Pharmacia, and molecular weight markers for gel filtration from Boehringer Mannheim. Phosphoramidon, pepstatin A, leupeptin, antipain and chymostatin were obtained from the Protein Research Foundation. PMSF (phenylmethylsulfonyl-fluoride), PCMB (*p*-chloromercuribenzoate), IAA (iodoacetic acid) and TPCK (*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone) were obtained from Nakarai. DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) and other chemicals were obtained from Wako Pure Chemical Industries.

Cultivation. Cells, FLE-2, were inoculated into 500 ml-Sakaguchi flasks containing 120 ml of BGPY (basal glycerol peptone yeast extract) medium [500 ml of artificial seawater containing 35.1 g of NaCl, 1.5 g of KCl, 24.7 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2.9 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per liter; 444 ml of distilled water; 1 ml of 10% ammonium ferric citrate solution; 1 ml of 7.5% K_2HPO_4 solution; 5 ml of 20% NH_4Cl solution; 50 ml of 1 M Tris-HCl buffer (pH 7.8); 3 g of glycerol; 5 g of Bacto-peptone (Difco); and 3 g of yeast extract (Difco)]. Cultivation was carried out at 23°C for 24 hr with shaking as previously described.²²⁾ After cultivation, the cells were removed from the culture by centrifugation at $12,000 \times g$ and the enzyme was purified from the supernatant.

Assaying of proteinase activity. The proteinase activity was measured according to the method of Hagihara *et al.*²³⁾ 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 mixture was stirred vigorously and then centrifuged at $15,000 \times g$ for 5 min. The absorbance of the supernatant was measured at 280 nm with a Shimadzu UV-120-02 spectrophotometer. 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 liberated 1 μg of tyrosine per ml per minute.

Determination of protein concentration. The protein concentration was determined by the method of Lowry *et al.*²⁴⁾ with bovine serum albumin as a standard.

Purification of the proteinase. Ammonium sulfate was added to the culture supernatant (600 ml) to 100% saturation. The precipitate was dissolved in an aliquot of 20 mM Tris-HCl buffer (pH 7.8) and then dialyzed against 10 mM Tris-HCl buffer (pH 7.8) at 4°C for 3 days. The

dialyzate (300 ml) was mixed with DEAE-Sephacel which had been previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8). The enzyme was eluted with the same buffer containing 0.6 M NaCl. The active fractions were combined and then applied on a Sephadex G-100 column (3.0×46 cm) equilibrated with the same buffer. The proteinase was eluted as a single peak and then collected. The purified enzyme solution had a specific activity of 1888. DEAE-Sephacel and Sephadex G-100 chromatography were carried out at room temperature (20–23 °C).

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed by the method of Davis.²⁵⁾ Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn.²⁶⁾ After electrophoresis, the gel was sliced and the slices were chopped up in test tubes with the buffer. The eluates were assayed for proteinase activity.

Estimation of molecular weight. The molecular weight of the purified enzyme was estimated by SDS-PAGE and gel filtration on a column of Sephadex G-100 superfine (1.5×93 cm) by the method of Determann and Michel.²⁷⁾

RESULTS AND DISCUSSION

Proteolytic activity and identification

Extracellular proteinase activity was detected on milk seawater agar-seawater agar two layer plates, as shown in Fig. 1. The characteristics of the proteolytic, marine luminous bacterium, FLE-2, are shown in Table I and Fig. 2. The FLE-2 strain is a moderately luminous, motile gram-negative, curved rod. It has seathed polar flagella, does not accumulate

FIG. 1. Behavior of the Proteolytic, Luminous Bacterium, FLE-2, on a Milk Seawater Agar-Seawater Agar Two Layer Plate.

The inoculated agar medium was incubated at 25°C for 48 hr. Clear zones indicating proteolytic activity can be seen around colonies.

TABLE I. PHENOTYPIC CHARACTERIZATION OF THE PROTEOLYTIC, LUMINOUS BACTERIUM, FLE-2^a

Traits	FLE-2	Previously reported characterizations ^b					
		<i>Vibrio splendidus</i> biotype I	<i>Vibrio harveyi</i>	<i>Vibrio orientalis</i>	<i>Vibrio fischeri</i>	<i>Photobacterium phosphoreum</i>	<i>Photobacterium leiognathi</i>
Luminescence	+	+	+	+	+	+	+
Straight rods ^c	—	—	+	—	98	+	97
PHB-Accumulation ^d	—	—	—	+	—	+	+
Gas from D-glucose	—	—	—	—	—	94	39
Arginine dihydrolase	+	+	—	+	—	—	—
Growth at 4°C	+	25	—	+	—	95	—
20°C	+	+	+	+	+	+	+
30°C	+	+	+	+	92	85	+
35°C	—	75	+	+	67	—	93
Growth on single carbon and energy sources:							
Maltose	+	+	+	+	92	99	—
Cellobiose	+	+	+	+	92	—	—
D-Gluconate	+	25	99	+	—	86	+
D-Glucuronate	—	+	99	—	—	52	—
DL-Lactate	+	+	+	+	—	16	+
Mannitol	+	+	+	+	92	—	—
D- α -Alanine	+	+	99	+	—	—	—
DL- β -Hydroxybutyrate	—	—	—	+	—	—	—
Acetate	+	75	93	+	—	—	—
Propionate	+	+	55	—	—	—	—
L-Tyrosine	+	75	+	—	—	—	—
Pyruvate	+	+	+	+	—	—	97
α -Ketoglutarate	+	+	91	—	—	—	3
L-Proline	+	+	+	+	+	6	97
Sucrose	—	75	61	+	8	—	—
Salicin	—	—	55	—	42	—	—
Caprylate	—	—	67	—	—	—	—
Pelargonate	—	—	84	—	—	—	—
Production of extracellular enzymes:							
Amylase	+	+	+	+	—	—	—
Gelatinase	+	+	99	+	8	—	—
Lipase	+	+	+	+	92	—	80

^a +, all strains positive; —, all strains negative; numbers indicate % of positive strains.^b Data abstracted from Reichelt and Baumann,⁴⁾ Baumann and Baumann,²⁾ Yang *et al.*⁵⁾ and Baumann *et al.*,⁶⁾ and the references cited therein.^c +, straight rods; —, curved rods.^d Ability to accumulate poly- β -hydroxybutyrate as an intracellular reserve product.

poly- β -hydroxybutyrate, does not produce gas on fermentation of D-glucose and exhibits arginine dihydrolase activity. This strain grows at 4, 20 and 30°C, but not at 35°C. It utilizes a variety of compounds as sole carbon and energy sources, but is unable to grow on D-glucuronate, DL- β -hydroxybutyrate, sucrose, salicin, caprylate or pelargonate. This strain produces amylase, gelatin-

ase and lipase. These results indicate that the proteolytic, luminous bacterium, FLE-2, is *Vibrio splendidus* biotype I.

The production of extracellular proteinases by *V. splendidus* ATCC 33125 and other *V. splendidus* isolates in the FLE series was also observed with the use of this two layer test medium. The proteinase activity of the culture supernatant of the FLE-2 strain was 3.5-fold

TABLE II. SUMMARY OF PURIFICATION OF THE PROTEINASE FROM *Vibrio splendidus* FLE-2

Procedures	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture supernatant	405	36,780	91	100
Ammonium sulfate precipitation	25.5	18,510	726	50
DEAE-Sephacel	12.6	16,108	1,278	44
Sephadex G-100	5.7	10,760	1,888	29

FIG. 2. Electron Micrograph of the Proteolytic, Luminous Bacterium, FLE-2.

Negatively stained. The marker represents $1\mu\text{m}$ ($\times 8,000$).

higher than those of *V. splendidus* ATCC 33125 and the other *V. splendidus* strains.

Purification

The purification of the proteinase from *V. splendidus* FLE-2 is summarized in Table II. The purified proteinase showed 21-fold purification over the original culture supernatant, with an about 30% recovery. The purified enzyme showed a single protein band on disc gel electrophoresis, as shown in Fig. 3, and sodium dodecyl sulfate disc gel electrophoresis, which suggests the homogeneity of the preparation.

Molecular weight measurement

The molecular weights of various bacterial proteinases have been reported, *i. e.*, 34,000 for *Bacillus cereus*,²⁸⁾ 46,000 for *Pseudomonas maltophilia*,²⁹⁾ 49,000 for *Vibrio* B-30¹⁴⁾ and 60,000 for *Serratia* sp.³⁰⁾

FIG. 3. Polyacrylamide Disc Gel Electrophoresis of the Purified Proteinase.

Electrophoresis was performed using 7.5% gel as described under MATERIALS AND METHODS. Protein was stained with Coomassie brilliant blue R-250.

The molecular weight of the proteinase from *V. splendidus* FLE-2 was estimated to be 50,000 by SDS-PAGE, as shown in Fig. 4, and also estimated to be 50,000 by Sephadex G-100 superfine gel filtration.

Effects of the NaCl concentration on activity

Figure 5 shows the effects of the NaCl concentration on the enzyme activity. In the experiment, the NaCl concentration in the assay mixture was varied.

Although the *V. splendidus* FLE-2 strain was isolated from seawater, the proteinase

FIG. 4. Estimation of the Molecular Weight of the Purified Proteinase by SDS-Polyacrylamide Gel Electrophoresis.

The standard proteins used and their molecular weights were: A, bovine serum albumin (67,000); B, ovalbumin (43,000); C, carbonic anhydrase (30,000); D, soybean trypsin inhibitor (20,100).

FIG. 5. Effects of the NaCl Concentration on the Purified Proteinase Activity.

The NaCl concentration in the assay mixture was varied.

showed higher activity at low NaCl concentration than that at seawater concentration (3% NaCl), and the enzyme activity was suppressed at high NaCl concentration, that is, 130% and 50% of the original activity (at 3% NaCl) were seen at 0% and 9% NaCl, respectively.

FIG. 6. Effects of pH on the Purified Proteinase Activity.

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); 0.1 M Gly-NaOH (pH 9~12).

Effects of pH on activity

The purified enzyme was most active at pH 8.0, and showed a somewhat narrow pH optimum of from 7.0 to 9.0, as shown in Fig. 6. This value is similar to those of the alkaline proteinases of *Serratia marcescens*,⁹⁾ *Pseudomonas* sp.,¹⁶⁾ *Serratia* sp.³⁰⁾ and *Pseudomonas aeruginosa*.³¹⁾

Effects of temperature on stability and activity

The enzyme solution was kept for 30 min at various temperatures and then the remaining activity was assayed. The proteinase activity was affected by temperature, owing to thermolability, above 40°C, as shown in Fig. 7. The effects of temperature on the activity was also examined (Fig. 7). The maximum activity, which was 1.8-fold higher than that observed at 30°C, was seen around 55°C.

Effects of inhibitors and chemicals

For this assay system, the reaction mixture without casein as substrate was preincubated with individual effectors in 100 mM Tris-HCl buffer (pH 7.8), for 2 hr at 20°C, and the reaction was started by adding the casein

FIG. 7. Effects of Temperature on the Stability and Activity of the Purified Proteinase.

In the experiment on the effects of temperature on the stability (○—○), the enzyme solution was kept for 30 min at various temperatures and then the remaining activity was assayed. While, the enzyme reaction was carried out at various temperatures to determine the effects of temperature on the proteinase activity (●—●).

solution to the assay mixture after preincubation for 10 min at 30°C, and then the remaining activities were expressed as percentages of the control activity determined without effectors. The effects of various inhibitors and chemicals on the proteinase activity are shown in Table III. Pepstatin A, leupeptin, antipain and chymostatin did not inhibit the reaction, whereas the purified enzyme was inhibited by metal-chelating agents such as EDTA and orthophenanthroline, and phosphoramidon. PMSF, PCMB and IAA did not show any inhibitory effect, but dithiothreitol and thioglycol acid affected the reaction. Although some metal ions, such as Cu^{2+} , Ni^{2+} , Cd^{2+} and Co^{2+} , strongly inhibited the activity, the Mg^{2+} and Ca^{2+} ions did not at all.

These results indicate that this enzyme is a metal-chelator-sensitive, alkaline proteinase.

In the marine environment, luminous bacteria are generally present in the enteric tracts of many fishes and the surrounding water, as

TABLE III. EFFECTS OF VARIOUS INHIBITORS AND CHEMICALS ON THE PROTEINASE ACTIVITY

The enzyme was preincubated at 20°C for 2 hr with inhibitors and chemicals in 100 mM Tris-HCl buffer (pH 7.8), and then the remaining activity was assayed.

Inhibitors and chemicals	Concentration (mM)	Remaining activity (%)
Control	—	100.0
EDTA	1.0	24.0
	10.0	1.0
Orthophenanthroline	0.1	27.0
	1.0	0.0
Phosphoramidon	0.1	8.4
Pepstatin A	0.1	107.0
Leupeptin	0.1	110.0
Antipain	0.1	109.0
Chymostatin	0.1	108.0
PMSF	1.0	106.0
PCMB	1.0	118.0
IAA	1.0	102.0
DTNB	1.0	88.0
TPCK	1.0	95.0
2-Mercaptoethanol	1.0	88.0
Thioglycol acid	1.0	12.4
Dithiothreitol	1.0	55.2
CuCl_2	1.0	3.8
HgCl_2	1.0	58.0
NiCl_2	1.0	17.0
CdCl_2	1.0	39.0
CoCl_2	1.0	37.0
MgCl_2	1.0	104.0
CaCl_2^a	1.0	101.6

^a Preincubated and assayed in 100 mM HEPES buffer (pH 7.8).

reported Ruby and Morin.³²⁾ The concentration of luminous cells in the nutrient-rich enteric tract may be 10^5 to 10^6 times greater than in the surrounding water, and there is cycling of cells between the enteric contents and the planktonic population. In addition, it has been speculated by Hastings and Neelson³³⁾ that the luminous bacteria may contribute a service to the host by virtue of their production of chitinolytic enzymes.³⁴⁾ This may indicate the dynamics on ecology of the population of luminous bacteria.

As to the optimal activity (pH 7.0~9.0) and stability (below 35°C), the proteinase from the marine luminous bacterium, *V. splendidus*

FLE-2, is able to function in the marine environment. It may be considered that the proteinase also contributes to the population dynamics as described for chitinolytic enzymes.

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