Properties of the Proteinase from a Luminous Bacterium, *Vibrio harveyi* Strain FLN-77

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A proteinase from the culture supernatant of marine luminous bacterium, *Vibrio harveyi* strain FLN-77, was purified. The purified enzyme had a molecular weight of 62000. The enzyme was most active at pH 8.0 and 55 °C, and was stable below 45 °C. The enzyme activity was completely inhibited by ethylendiaminetetra acetic acid, *ortho*-phenanthroline and phosphoramidon. Metal ions such as Cu^{2+}, Hg^{2+} and Ni^{2+} also inhibited the activity. These results indicated that this enzyme is a metal-chelator-sensitive, alkaline proteinase.

Keywords proteinase; luminous bacterium; marine bacterium; *Vibrio harveyi*

Introduction

In the previous paper,[1] we reported that a marine luminous bacterium, isolated from seawater at Chiba, could exhibit proteinase activity. We also identified it as *Vibrio harveyi*, and reported the purification and some properties of the proteinase from this FLA-11 strain. Since the proteinase activity in the culture supernatant with BGPY (basal glycerol peptone yeast extract) medium,[2] which is a nutritionally complete broth, was very low, we examined whether the enzyme activity from other *Vibrio harveyi* strains is also lower than that with BC (basal casein) medium,[1] which lacks carbon and energy sources other than the casein, or not. It was found that several strains, which were newly isolated and identified as *Vibrio harveyi*, exhibited high proteinase activity in the BGPY medium.

In this paper, we describe the identification of the proteolytic, marine luminous bacterium, FLN-77, which was isolated as one of one hundred and forty-one luminous strains (FLN-1 through FLN-141, free-living, series N) from surface coastal water at Kaichudoro, Okinawa, Japan, and the purification and some properties of the proteinase from this luminous strain. The enzyme was compared with that of the FLA-11 strain.

Materials and Methods

Isolation and Identification Seawater was obtained from surface coastal water at Kaichudoro, Okinawa prefecture, Japan, during September, 1987, and spread-plated (50 μl per plate) on MN (modified Nealson's) agar medium,[3,4] which is a nutritionally complete, seawater medium. Isolation of luminous strains was carried out according to the method described previously.[5] Identification was carried out according to the methods of Reichelt and Baumann,[6] Baumann and Baumann,[7] Yang *et al.*[8] and Baumann *et al.*,[9] as described previously.[1,2] The FLN-77 strain is a moderately luminous, motile gram-negative, straight 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 FLN-77 strain grows at 20, 30, 35 and 40 °C, but not at 4 °C. As sole carbon and energy sources, it utilizes maltose, cellobiose, D-gluconate, DL-lactate, D-α-alanine, acetate, propionate, L-tyrosine, pyruvate, α-ketoglutarate, L-proline, caprylate and pelargonate, but is unable to grow on D-glucuronate, mannitol, DL-β-hydroxybutyrate, sucrose and salicin. The FLN-77 strain produces amylase, gelatinase and lipase. These results indicate that the proteolytic, luminous bacterium, FLN-77, is a strain of *Vibrio harveyi*.

Chemicals All chemicals obtained were described previously.[1,2]

Cultivation The cells, FLN-77, were inoculated into 500 ml flasks containing 120 ml of BGPY medium,[2] and cultivation was carried out at 23 °C for 48 h with shaking as previously described.[10] After cultivation, the cells were removed from the culture medium by centrifugation at 12000 × *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.*,[11] as described previously.[2] 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 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.*,[12] with bovine serum albumin as a standard.

Purification of the Proteinase Ammonium sulfate was added to the culture supernatant (640 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 4 d. The dialyzate (914 ml) was mixed with diethylaminoethyl (DEAE)-Sephacel which had previously been equilibrated with 10 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 the applied to a Sephadex G-100 column (3.0 × 45 cm) equilibrated with the same buffer. The proteinase was eluted as a single peak and then collected. DEAE-Sephacel and Sephadex G-100 chromatographies were carried out at about 20 °C.

Electrophoresis Polyacrylamide disc gel electrophoresis (PAGE) was performed by the method of Davis,[13] as described previously.[1] Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn.[14]

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 × 95 cm) by the method of Determann and Michel.[15]

Results and Discussion

The purification of the proteinase from *V. harveyi* FLN-77 is summarized in Table I. The purified proteinase showed a single protein band on PAGE and SDS-PAGE. The proteinase had a specific activity of 1360 units per mg of protein, and showed 13-fold purification over the original culture supernatant, with about 10% recovery. The purified enzyme was a monomer with a molecular weight of 62000, as shown in Fig. 1. This is different from that of the *V. harveyi* FLA-11 enzyme, which had a molecular weight of 84000, being a tetramer of 21000-molecular-weight subunits.[1] The enzyme exhibited 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 42% of the original activity (at 3% NaCl) were seen at 0% and 9% NaCl, respectively. These results are similar to those in the case of the FLA-11 strain. The purified enzyme was most active at

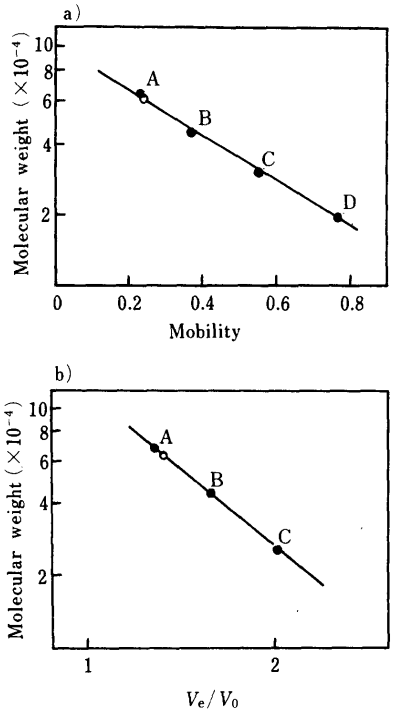

Fig. 1. Estimation of the Molecular Weight of the Purified Proteinase

a) SDS-polyacrylamide gel electrophoresis. 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). ○; the purified proteinase. b) Sephadex G-100 superfine gel filtration. The standard proteins used and their molecular weights were: A, bovine serum albumin (68000); B, hen egg albumin (45000); and C, chymotrypsinogen A (25000). ○; the purified proteinase.

pH 8.0 and 55 °C, and was stable below 45 °C. The proteinase of the FLN-77 strain, which is able to grow at 40 °C, is slightly more thermostable than that of the FLA-11 strain. The effects of various inhibitors and chemicals on the proteinase activity are shown in Table II. Pepstatin A, antipain, phenylmethylsulfonyl fluoride and *p*-chloromercuribenzoic acid did not inhibit the reaction of the proteinase, whereas the enzyme was inhibited by phosphoramidon and metal-chelating agents such as ethylenediaminetetraacetic acid (EDTA) and *ortho*-phenanthroline. Although some metal ions such as Cu^{2+}, Hg^{2+} and Ni^{2+} strongly inhibited the activity, Mg^{2+} and Ca^{2+} ions had no effect. In accordance with the classification of microbial proteinases by Morihara,[16] these results indicate that this enzyme is also a metal-chelator-sensitive, alkaline proteinase.

TABLE I. Summary of Purification of the Proteinase from *Vibrio harveyi* FLN-77

Procedures	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery
Culture supernatant	229	23200	101	100
Ammonium sulfate precipitation	44	14600	332	63
DEAE-Sephacel	2.8	3180	1140	14
Sephadex G-100	1.7	2310	1360	10

TABLE II. Effects of Various Inhibitors and Chemicals on the Proteinase Activity

Inhibitors and chemicals	Concentration (mM)	Remaining activity (%)
Control	—	100
EDTA	1.0	4.3
	10.0	0.0
ortho-Phenanthroline	0.1	7.5
	1.0	0.0
Phosphoramidon	0.1	3.7
Pepstatin A	0.1	99.7
Antipain	0.1	101
Phenylmethylsulfonyl fluoride	1.0	103
p-Chloromercuribenzoic acid	1.0	105
2-Mercaptoethanol	1.0	85.6
$CuCl_2$	1.0	4.5
$HgCl_2$	1.0	3.2
$NiCl_2$	1.0	7.5
$MgCl_2$	1.0	102
$CaCl_2$	1.0	104

The reaction mixture without casein 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 remaining activities were assayed and expressed as percentages of the control activity determined without effectors.

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