Properties of the Proteinase from a Luminous Bacterium, *Vibrio splendidus* ATCC 33125

Shigeki FUKASAWA,*,[a] Ken TAKAGI[a] and Munetsugu KURATA[b]

Department of Biochemistry, Faculty of Science[a] and Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences,[b] Josai University, Sakado, Saitama 350-02, Japan. *Received June 10, 1988*

A proteinase was purified from the culture supernatant of a marine luminous bacterium, *Vibrio splendidus* ATCC 33125. The purified enzyme had a molecular weight of 60000. The enzyme was most active at pH 9.0 and 57 °C, and was stable below 45 °C. The enzyme activity was inhibited by phosphoramidon, ethylenediaminetetraacetic acid and orthophenanthroline. Metal ions such as Cu^{2+}, Hg^{2+} and Ni^{2+} also inhibited the activity. These results indicate that this enzyme is a metal-chelator-sensitive, alkaline proteinase.

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

Introduction

Production of extracellular proteinase by a marine luminous bacterium, *Vibrio splendidus* FLE-2 strain, and the purification and characterization of the enzyme have been reported previously.[1] The proteinase activity was also detected in the culture supernatant of *Vibrio splendidus* ATCC 33125, which is the Type strain of *V. splendidus* biotype I, although only a little or no activity was detected in that of other Type strains of luminous bacteria such as *Vibrio harveyi* ATCC 14126, *V. orientalis* ATCC 33934, *V. fischeri* ATCC 7744, *V. fischeri* ATCC 25918, *V. logei* ATCC 29985, *Photobacterium leiognathi* ATCC 25521 and *P. phosphoreum* ATCC 11040.

In this paper, we describe the purification and some properties of the proteinase from this luminous strain, *V. splendidus* ATCC 33125, and we compare the properties of this enzyme with those of the FLE-2 strain enzyme.

Materials and Methods

Materials *Vibrio splendidus* ATCC 33125 strain was obtained from The American Type Culture Collection (ATCC). All chemicals obtained were as described previously.[1]

Cultivation The cells, *V. splendidus* ATCC 33125, were inoculated into 500 ml flasks containing 120 ml of BGPY (basal glycerol peptone yeast extract) medium,[1] and cultivation was carried out at 23 °C for 48 h with shaking as previously described.[2] After cultivation, the cells were removed from the culture medium by centrifugation at $12000 \times g$ and the enzyme was purified from the supernatant.

Assay of Proteinase Activity The proteinase activity was measured according to the method of Hagihara *et al.*,[3] as described previously.[1] 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 280 nm-absorbing materials, calculated as tyrosine, per ml per min.

Determination of Protein Concentration The protein concentration was determined by the method of Lowry *et al.*,[4] with bovine serum albumin as a standard.

Purification of the Proteinase Ammonium sulfate was added to the culture supernatant (980 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 (780 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 0.4 M NaCl. The active fractions were combined and then applied on a Sephadex G-100 column (3.0×45 cm) equilibrated with the same buffer. The proteinase was eluted as a single peak and collected. DEAE-Sephacel and Sephadex G-100 chromatographies were carried out at 20 °C.

Electrophoresis Polyacrylamide disc gel electrophoresis (PAGE) was perfomed by the method of Davis,[5] as described previously.[1] Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn.[6] For the detection of the proteolytically active band, the gel was first soaked in 2% hemoglobin solution (pH 7.8) for 30 min in an ice bath, incubated for 15 min at 30 °C, and stained with 0.2% Coomassie brilliant blue in 12.5% trichloroacetic acid (TCA) solution. After destaining of the gel with the TCA solution, a clear zone showing the enzyme activity appeared on the gel.

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.[7]

Results and Discussion

The purification of the proteinase from *Vibrio splendidus*

TABLE I. Summary of Purification of the Proteinase from *Vibrio splendidus* ATCC 33125

Procedures	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery
Culture supernatant	216	17250	80	100
Ammonium sulfate precipitation	31	8890	287	52
DEAE-Sephacel	5.2	1870	360	11
Sephadex G-100	1.4	1840	1314	11

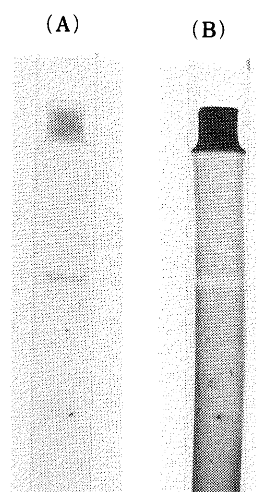

Fig. 1. Polyacrylamide Disc Gel Electrophoresis of the Purified Proteinase

Electrophoresis was performed using 10% gel. Protein was stained with Coomassie brilliant blue R-250 (A). For active staining (B), the clear zone on the gel showed the enzyme activity, as described under Materials and Methods.

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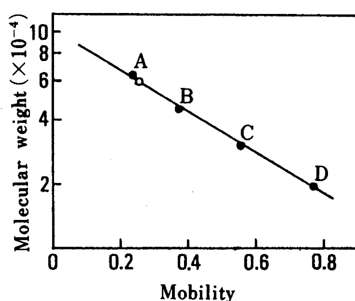

Fig. 2. Estimation of the Molecular Weight of the Purified Proteinase by SDS–Polyacrylamide Gel Electrophoresis

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

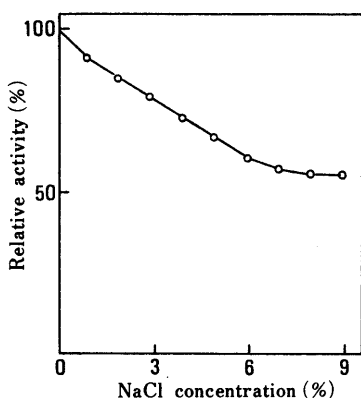

Fig. 3. Effects of the NaCl Concentration on the Purified Proteinase Activity

The NaCl concentration in the assay mixture was varied as indicated.

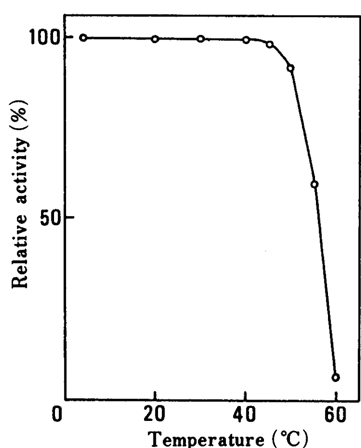

Fig. 4. Effects of Temperature on the Stability of the Purified Proteinase

The enzyme solution was kept for 30 min at various temperatures as indicated, and then the remaining activity was assayed.

ATCC 33125 is summarized in Table I. The purified proteinase had a specific activity of 1314 units per mg of protein, and showed 16-fold purification over the original culture supernatant, with an about 10% recovery. The purified enzyme gave a single protein band on PAGE, as shown in Fig. 1. The proteolytic activity on the gel was detected at the position corresponding to the protein band.

The molecular weight of the purified enzyme was estimated to be 60000 by SDS–PAGE, as shown in Fig. 2, and

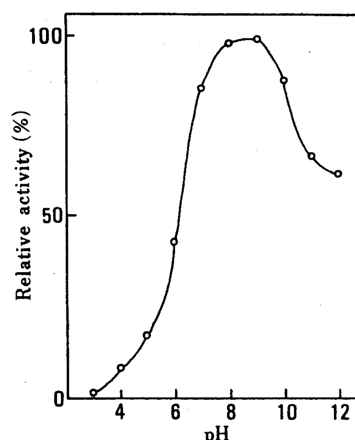

Fig. 5. Effects of pH on the Purified Proteinase Activity

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).

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

Inhibitors and chemicals	Concentration (mM)	Remaining activity (%)
Control	—	100
EDTA	1.0	11.5
	10.0	0.0
Orthophenanthroline	0.01	93.1
	0.1	38.1
	1.0	17.0
Phosphoramidon	0.1	0.6
Pepstatin A	0.1	106.0
Antipain	0.1	110.0
Phenylmethylsulfonylfluoride	1.0	110.0
p-Chloromercuribenzoic acid	1.0	117.0
2-Mercaptoethanol	1.0	95.2
$CuCl_2$	1.0	8.0
$HgCl_2$	1.0	9.6
$NiCl_2$	1.0	27.2
$MgCl_2$	1.0	99.2
$CaCl_2$	1.0	99.0

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, then the remaining activities were assayed and expressed as percentages of the control activity determined without an effector.

also estimated to be 60000 by Sephadex G-100 superfine gel filtration, indicating that the enzyme is a monomer. This value is slightly larger than that of the FLE-2 strain enzyme, which is also a monomer of 50000, and similar to that in the case of the Serratia sp. enzyme,[8] estimated to be 60000.

The enzyme exhibited higher activity at a lower NaCl concentration than the seawater concentration (3% NaCl), and the enzyme activity was suppressed at high NaCl concentration, that is, 125% and 70% of the original activity (at 3%) were seen at 0% and 9% NaCl, respectively (Fig. 3). The activity showed the same tendency as in the cases of FLE-2 and FLA-11 strains,[1,9] but the suppression of the activity at high NaCl concentration was lower than that of these strains, which showed approximately 50% of the original activities at 9% NaCl.

The proteinase activity was affected by temperature, owing to thermolability, above 55 °C, as shown in Fig. 4.

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This result showed that the proteinase of the ATCC 33125 strain is more thermostable than that of the FLE-2 strain. While, the enzyme reaction was carried out for 10 min at various temperatures to determine the effects of temperature on the proteinase activity. The maximum activity, which was 3-fold higher than that observed at 30°C, was seen at around 57°C.

The purified enzyme was most active at pH 9.0 and showed a somewhat broad pH optimum of from 7.0 to 10.0, as shown in Fig. 5.

The effects of various inhibitors and chemicals on the proteinase activity are shown in Table II. Pepstatin A, antipain, phenylmethylsulfonylfluoride, *p*-chloromercuribenzoic acid and mercaptoethanol 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 orthophenanthroline. Although metal ions such as Cu^{2+} and Hg^{2+} strongly inhibited the activity, Mg^{2+} and Ca^{2+} ions did not at all. Many kinds of metal-chelator-sensitive proteinases[10] and *Vibrio* proteinases[11,12] have been reported, and these results indicate that this enzyme is also a metal-chelator-sensitive, alkaline proteinase.

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