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Purification and Characterization of Extracellular Temperature-Stable Serine Protease from *Aeromonas hydrophila*

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Extracellular protease, from *Aeromonas hydrophila* Ni 39, was purified 16.7-fold to electrophoretic homogeneity with an overall yield of 19.9%, through a purification procedure of acetone precipitation, and Q Sepharose and Sephacryl S-200 chromatographies. The isoelectric point of the enzyme was 6.0 and the molecular mass, as determined by Sephacryl S-200 HR chromatography, was found to be about 102 kDa. SDS/PAGE revealed that the enzyme consisted of two subunits, with molecular masses of 65.9 kDa. Under standard assay conditions, the apparent K_m value of the enzyme toward casein was 0.32 mg/ml. About 90% of the proteolytic activity remained after heating at 60°C for 30 min. The highest rate of azocasein hydrolysis for the enzyme was reached at 60°C, and the optimum pH of the enzyme was 9.0. The enzyme was inhibited by the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), by about 87.9%, but not by E64, EDTA, pepstatin or 1,10-phenanthroline. The enzyme activity was inhibited slightly by Ca^{2+}, Mg^{2+} and Zn^{2+} ions.

Key words: *Aeromonas hydrophila,* characterization, extracellular protease, purification

Aeromonas hydrophila produces a wide range of proteases, which play an important role in the invasiveness and establishment of infections, by overcoming the initial host defenses, and by providing nutrients for cell proliferation (Hsu *et al.*, 1981; Leung and Stevenson, 1988; Pemberton *et al.,* 1997). There are many reports describing the numbers and different natures of proteases found in the culture supernatants of *A. hydrophila*. Some have reported finding the temperature-stable metalloprotease (TSMP) alone (Gross and Coles, 1969; Allan and Stevenson, 1981; Kanai and Wakabayashi, 1984), and others have described the TSMP to be one of the two produced, the other being the temperature-labile serine protease (TLSP) (Dahle, 1971; Nieto and Ellis, 1986; Leung and Stevenson, 1988). Rivero *et al.,* (1990) described another type of protease, which was stable to temperature and EDTA, along with the molecular biological properties of its structural gene. In addition, a novel zinc protease, which mimics the action of an isopeptidase on the γ-chain dimmers of cross-linked fibrin, was also purified and characterized. This was a 19 kDa protein, which was inhibited by 1,10-phenanthroline, but not by EDTA (Loewy *et al.*, 1993).

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In this paper, the purification and characterization of another temperature-stable extracellular protease from *A. hydrophila* Ni 39 is described.

Materials and Methods

Strain and growth conditions

The *Aeromonas hydrophila* Ni 39 was isolated from rotating activated *Bacillus* contactor (RABC), and used as a protease producer. The medium for this strain contained 1% skim milk in the nutrient broth, and was cultured in a 500 ml Erlenmeyer flask, 30°C, for 24 hr.

Chemicals

The Q Sepharose fast flow, Sephacryl S-200 HR and M_r markers for the gel filtration chromatography were purchased from Pharmacia Co. The isoelectric point markers, E64, EDTA, pepstatin, 1,10-phenanthroline and phenylmethylsulfonyl fluoride (PMSF), were purchased from Sigma Co., and the standard M_r proteins for the SDS-PAGE were obtained from Boehringer-Mannheim Co. All other chemicals used were of the highest quality generally available.

Enzyme assay

The enzyme activity was assayed using azocasein as the substrate, according to the method of Sarath *et al.*, (1989).

The reaction mixture consisted of 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0), containing 2.0% (w/v) azocasein and 0.15 ml of the enzyme solution. After incubating at 25°C for 15 min, the reaction was stopped by the addition of 1.2 ml of 10.0% (w/v) TCA, was incubated, at room temperature, for an additional 15 min and then the precipitate was removed by centrifugation at 8,000×g for 5 min. 1.4 ml of 1.0 M NaOH was added to 1.2 ml of the supernatant, and its absorbance measured at 440 nm. One unit of enzyme activity was expressed as giving an absorbance of 1.0, under the above condition. The protein concentrations were determined, according to the Bradford method (Bradford, 1976), using bovine serum albumin as the standard.

Enzyme purification

Unless otherwise stated, all procedures were performed at 4°C. The bacterial supernatant was centrifuged at 15,000 rpm for 30 min, with cold acetone (-10°C) added to give 70% saturation. The precipitated proteins were collected by centrifugation at 10,000 rpm for 10 min. After removing the acetone by nitrogen purging, the precipitate was redissolved in the minimum volume of 20 mM sodium phosphate buffer, pH 7.0 (buffer A). The redissolved protein was applied to a Q Sepharose column (2.8×15 cm), which had been equilibrated with buffer A. The column was subsequently washed with 100 ml of the equilibration buffer, and the enzyme fractions eluted with a linear concentration gradient of 0-0.2 M NaCl, in the same buffer, at a flow rate of 30 ml/h. The fractions containing protease activity were then pooled, concentrated and loaded onto a Sephacryl S-200 HR column (2.5×120 cm) equilibrated with buffer A. The enzyme was eluted with the same buffer at a flow rate of 30 ml/h and collected in 10 ml fractions. These were assayed for enzyme activity, and those containing the highest activity were collected. The homogeneity of the enzyme was confirmed by SDS-PAGE with the modified method of Laemmli (1970).

Molecular mass and isoelectric point determination

The native M_r of the purified protein was estimated by gel filtration chromatography, on a Sephacryl S-200 column. SDS-PAGE was performed on a 12.5% polyacrylamide gel. Coomassie brilliant blue G-250 was used for staining, according to the method of Neuhoff *et al.* (1988). The isoelectric point of the enzyme was determined on an isoelectric focusing gel (IEF-PAGE), with a pH gradient of 3 to 10 (125 by 65 mm, 0.4 mm thick; Bio-Rad, USA). The isoelectric point of the purified protease was determined by comparison with protein standard markers.

Temperature and pH optimum

The above assay, with azocasein as substrate, was also used to determine the optimum temperature of the *A. hydrophila* protease. To estimate the optimum pH of the enzyme, the activity was measured in 0.1 M citrate-phosphate buffer. All determinations were performed in duplicate.

Kinetic calculations

The K_m value was determined by measuring the initial velocity. All the kinetic studies were performed at least three times, and the data obtained fitted to a hyperbola, using the Michaelis-Menten equation. The best values were determined by a linear least-squares regression analysis.

Results and Discussion

Purification of protease

The enzyme was purified to homogeneity from the culture supernatant of *A. hydrophila* Ni 39, as summarized in Table 1. Two chromatographic steps were required, and at the end of the process the enzyme had been purified 16.7-fold, with a yield of 19.9%.

Characteristics of the enzyme

The purified enzyme was used for gel filtration chromatography, on Sephacryl S-200 HR, and the molecular mass of native enzyme was estimated as 102 kDa (Fig. 1). SDS electrophoresis of the enzyme produced a product with a molecular mass of 65.9 kDa (Fig. 2A). These results indicate that the enzyme may be a dimeric protein, similar to those described for the proteases of *A. hydrophila* ATCC 7966 and NRC 505. However, the native molecular mass of the enzyme was different from those of the strains ATCC 7966 and NRC 505, which had molecular masses of 145 and 54 kDa, respectively (Leung and Stevenson, 1988). The molecular mass and subunit composition of the extracellular protease varies between the *A. hydrophila* strains. The zinc and temperature-stable proteases of *A. hydrophila* were monomeric proteins with

Table 1. Purification profile of extracellular protease from *A. hydrophila* Ni 39

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture broth	65.58	194.8	0.34	100	1
Acetone precipitation	56.89	44.1	1.29	86.7	3.8
Q Sepharose™ fast flow	28.68	8.0	3.59	43.7	10.6
Sephacryl S-200 HR	13.06	2.3	5.68	19.9	16.7

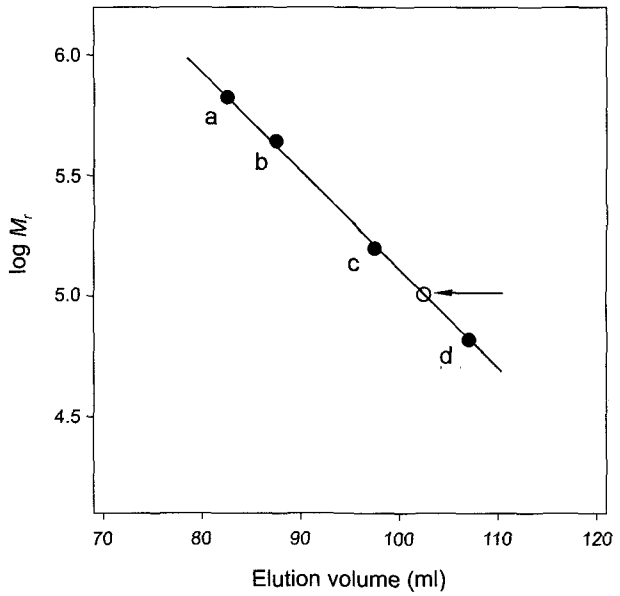

Fig. 1. Sephacryl S-200 HR chromatography of the purified protease from *A. hydrophila* Ni 39. The enzyme is indicated by the arrow. Thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and albumin (66 kDa) were used as molecular mass standards.

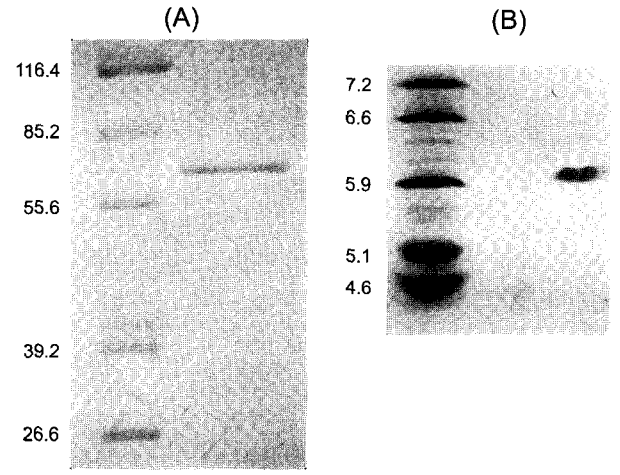

Fig. 2. SDS-PAGE (A) and analytical isoelectric focusing (B) of the protease purified from the culture supernatant of *A. hydrophila* Ni 39.

molecular masses of 19 and 38 kDa (Rivero *et al.*, 1990; Loewy *et al.*, 1993). The isoelectric point of the purified protease was 6.0 (Fig. 2B), which was unusual among proteases of *A. hydrophila*. *A. hydrophila* B_{51} produced at least three proteases, which had isoelectric point of 6.5-6.8, 7.0 and 8.3-8.5 (Nieto and Ellis, 1986). Thus, it is suggested that the amino acid composition, or sequence, of the extracellular protease of *A. hydrophila* Ni 39 is different from those of the other *A. hydrophila* proteases. The relationship between the enzyme activity and substrate concentration was of the Michaelis-Menten type. The apparent K_m value of the enzyme for casein, as determined from the Lineweaver-Burk plot, was estimated to be 0.32 mg/ml (Fig. 3).

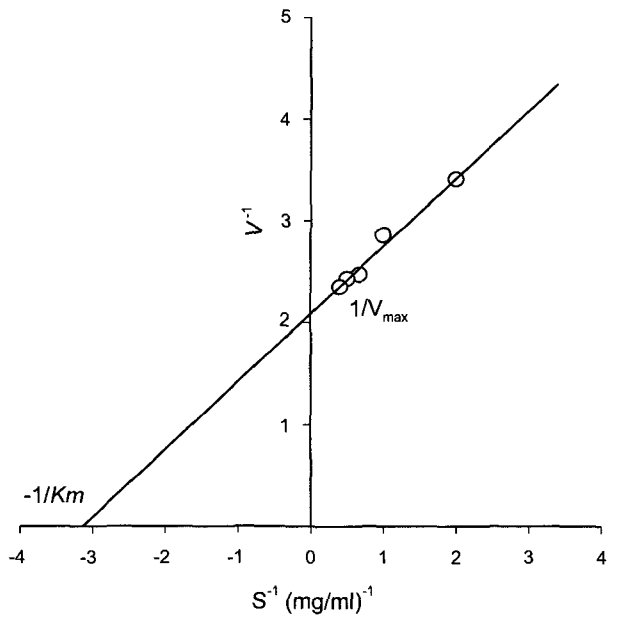

Fig. 3. The degradation kinetics of casein caused by the extracellular protease of *A. hydrophila* Ni 39. The estimated K_m value was 0.32 mg/ml.

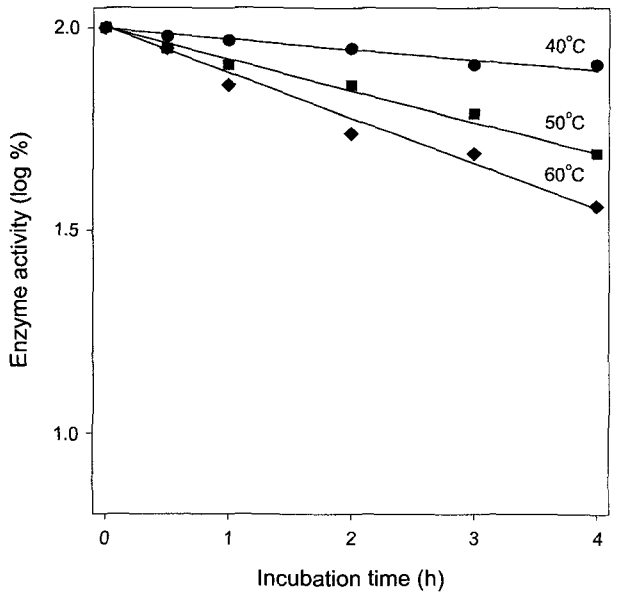

Fig. 4. The thermal stability of the purified protease. The stability of the enzyme was measured by incubating the enzyme at the defined temperature prior to assaying. After the addition of the substrate, the remaining activity was measured.

Effects of pH and temperature

The effect of temperature on the stability of enzyme was also measured. As shown in Fig. 4, the enzyme loses 63.2% of its activity when held at 60°C for 4 h. However, about 90% of the proteolytic activity remained after heating at 60°C for 30 min, which was similar to the results with the protease of *A. hydrophila* S02/2 (Rivero *et al.*, 1990). The effect of pH on enzyme activity was examined

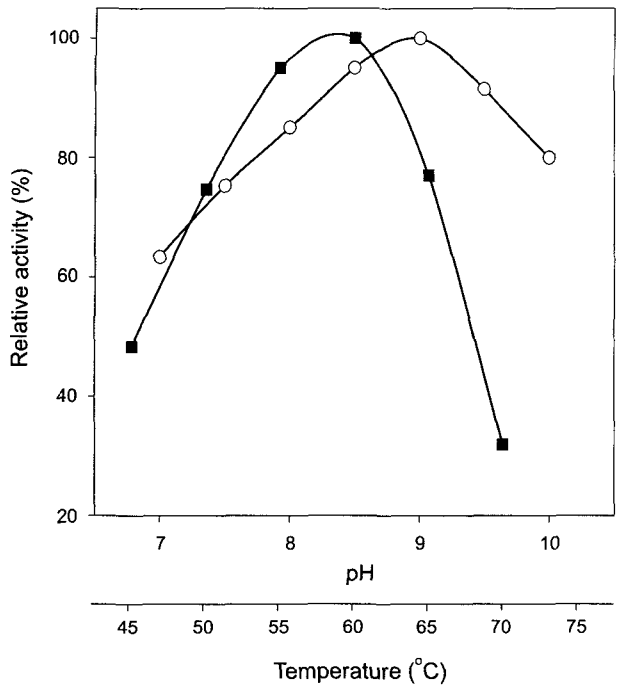

Fig. 5. The effects of pH (○) and temperature (■) on the protease activity. The enzyme activity was measured at various pH values, under standard assay conditions with 0.1 M citrate phosphate buffer, and the effect of temperature on the enzyme activity was assayed in 50 mM sodium phosphate buffer (pH 7.0) at the indicated temperatures.

Table 2. Effect of inhibitors on the proteolytic activity of the protease purified from the culture supernatant of *A. hydrophila* Ni 39

Treatment	Remaining activity (%)
None	100
E64 (2.5 mM)	99.1
EDTA (10 mM)	95.2
Pepstatin (2.5 μg/ml)	103
1, 10-Phenanthroline (2.5 mM)	95.2
PMSF (2.5 mM)	12.1

at pH values ranging from 7.0 to 10.0, with azocasein. The results showed that the protease was active over the pH range 8.0 to 10.0, but exhibited its maximum activity at pH 9.0 (Fig. 5). The enzyme was active at temperatures between 50 and 65°C, with an optimum activity at 60°C (Fig. 5).

Effects of inhibitors and metal ions

The effects of various inhibitors on the protease activity are summarized in Table 2. The extracellular protease of *A. hydrophila* Ni 39 was significantly inhibited by PMSF, which is a well-known inhibitor of serine protease. Other protease inhibitors, E64, EDTA, pepstatin and 1,10-phenanthroline, had no influence on the activity. Of the divalent ions tested, the protease activity was inhibited slightly by Ca^{2+}, Mg^{2+} and Zn^{2+} (Fig. 6). The effects of metal ions on protease activities differ from protease to

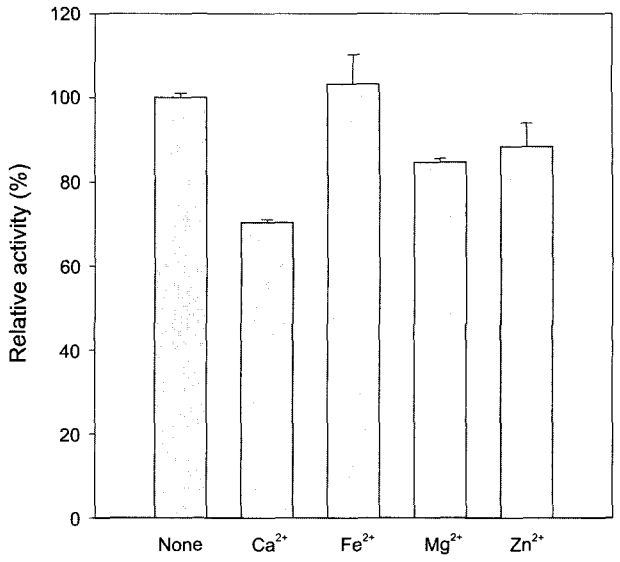

Fig. 6. The effect of divalent metal ions on the protease activity. The concentration of each metal ions used in the treatment was 0.1 mM.

protease. Ca^{2+}, Mg^{2+} and Na^{+} ions enhanced the activity of alkaline serine protease from *Bacillus pumilus*, but Cu^{2+} and Zn^{2+} ions caused slight inhibition (Huang *et al.* 2003). However, there was no inhibitory effect observed in the protease of *B. stearothermophilus* by Zn^{2+} (Kim *et al.*, 2002). All these results confirmed that the protease of *A. hydrophila* Ni 39 is a serine protease, and certain metal ions can promote its enzyme activity.

The results obtained during this work show that the protease of *A. hydrophila* Ni 39 presented quite different properties in relation to the inhibitors and heat stability, from those of the TSMP and TLSP purified from *A. hydrophila* NRC 505 and ATCC 7966 (Leung and Stevenson, 1988). The molecular mass of the purified enzyme was also different from the temperature and EDTA stable serine protease of *A. hydrophila* S02/2 (Rivero *et al.*, 1990). These results support the suggestions of Nieto and Ellis (1986), in that significant differences can be found in the characteristics of extracellular products and proteases isolated from different strains of *A. hydrophila*. We, therefore, suggest that this enzyme is a new member of the extracellular proteases of *A. hydrophila*, and may provide a useful material for further study.

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