Characterization of Extracellular Proteases of *Aeromonas hydrophila*

A. C. PANSARE, N. F. LEWIS and V. VENUGOPAL*

Food Technology and Enzyme Engineering Division,
Bhabha Atomic Research Centre,
Bombay 400 085, India

Received December 10, 1985

Two endoproteases, proteinase-I and proteinase-II, and one aminopeptidase were isolated from the culture supernatant of *Aeromonas hydrophila* by ammonium sulfate precipitation, gradient elution on diethylaminoethyl-Sephadex A-50 and Sephadex G-75 column chromatography. The molecular weights of the proteinases and aminopeptidase ranged between 30,000 to 48,000. The proteinases had comparable pH (8.5) and temperature (48~50°C) optima. The aminopeptidase assayed using L-leucine-*p*-nitroanilide showed maximum activity at pH 8.0 and at a temperature of 70°C. The aminopeptidase was remarkably heat stable, while the proteinases were heat sensitive. Both the proteinases hydrolysed several proteins including fish myofibrillar proteins. Divalent cations like Fe^{2+} , Cu^{2+} and Zn^{2+} inhibited the activities of the two proteinases while Ca^{2+} and Mg^{2+} did not affect either the activities of the proteinases or that of the aminopeptidase. The aminopeptidase was inhibited by metal chelating agents as well as thiol reagents.

Aeromonas hydrophila, commonly found in stagnant water, forms part of the normal microflora of fish and other aquatic animals.^{1,2)} The bacterium has been recognized as an enteric pathogen^{3,4)} as well as an active spoiler of fish under refrigeration.^{5,6)} The pathogenicity and spoilage potential of *A. hydrophila* can be attributed to its ability to secrete several extracellular virulence factors including hemolysins, lipases and proteases.⁷⁾ Extracellular proteases from psychrotropic bacteria are also known to cause deterioration of flesh foods during refrigerated storage.⁸⁾ Attention has, therefore, been focused on regulation of the synthesis and the properties of extracellular proteases of spoilage microorganisms. O'Reilly and Day⁹⁾ showed that the synthesis of an extracellular protease by *A. hydrophila* was dependent on the pH, aeration and temperature of the growth medium. We recently observed that the extracellular protease synthesis by this bacterium did not require complex nitrogen compounds, but was supported by several individual amino acids,

while the enzyme was repressed by ammonium and glutamine.¹⁰⁾ We also found that the secretion of the enzyme was inhibited by certain food preservatives¹¹⁾ as well as by iron.¹²⁾ The present report pertains to the isolation and characterization of two extracellular proteinases and one aminopeptidase produced by *A. hydrophila*.

MATERIALS AND METHODS

Organism and growth conditions. *Aeromonas hydrophila* was isolated from Indian mackerel (*Rastrelliger kanagurta*). The bacterium was grown in 0.8% Nutrient Broth (Difco) and the active culture (18 hr) was inoculated at 1% level into one liter flasks, each containing 500 ml of 0.8% Nutrient broth. The flasks were incubated at 28°C on a rotary shaker for 24 hr after which the cultures were centrifuged at $5920 \times g$ for 10 min. The pooled culture supernatant (2500 ml) was used as the source of crude enzymes. The purification procedures were carried out at 0~2°C.

Lyophilization. The culture supernatant was lyophilized and the powder was dissolved in about 300 ml of 50 mM phosphate buffer, pH 7.5. Powdered ammonium sulfate

* To whom correspondence should be addressed.

was added with constant stirring to the crude enzyme preparation to 80% saturation. The mixture was allowed to stand overnight at 4°C and centrifuged at $5,920 \times g$ for 10 min, and then the precipitate was collected. The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.5 (50 ml), and then dialysed at 2°C for 24 hr against three changes of the buffer. The dialysed enzyme solution was centrifuged at $5,920 \times g$ for 10 min and the supernatant obtained was used for further purification.

DEAE-Sephadex A-50 column chromatography. The crude enzyme (10 ml) was applied to a DEAE-Sephadex A-50 column (21.5×1.8 cm) that had been equilibrated with 50 mM phosphate buffer, pH 7.5. The unbound proteins were eluted with the starting buffer after which a linear NaCl gradient (0 to 0.5 M) was applied, with elution at a rate of 12 ml per hour. Fractions (3 ml) were collected and examined for proteinase and aminopeptidase activities. The active fractions were pooled and dialysed against 50 mM phosphate buffer, pH 7.5, for 24 hr with two changes of the buffer, and then the dialysed enzyme was lyophilized.

Sephadex G-75 chromatography. The lyophilized enzyme was dissolved in 5 ml of 50 mM phosphate buffer, pH 7.5, and then applied to a Sephadex G-75 column (63×1.6 cm) equilibrated with the same buffer. Elution was carried out a flow rate of 15 ml per hour and fractions of 2.5 ml each were collected. The fractions containing the enzyme were pooled, concentrated by lyophilization and then taken up in 50 mM phosphate buffer, pH 7.5.

Proteinase assay. The assay system, containing 15 mg hemoglobin (Difco), 1.5 ml of 100 mM phosphate buffer, pH 7.5, and enzyme in a total volume of 2.0 ml, was incubated at 50°C for 30 min. The reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid (TCA). After 15 min, the supernatant was collected by filtration through Whatman No. 1 filter paper and tyrosine in the supernatant was determined as described earlier.⁸⁾ One unit of enzyme activity was expressed as μ mol tyrosine liberated per mg protein in 30 min.

Aminopeptidase assay. Aminopeptidase activity was determined using leucine *p*-nitroanilide as the substrate.¹³⁾ L-Leucine *p*-nitroanilide (29 mg) was dissolved in 8 ml of distilled water and 2 ml of dimethylsulfoxide. The stock solution was diluted 1:10 with distilled water before the assay. The assay system, containing 0.3 ml of this solution, 2 ml of 50 mM Tris-HCl buffer, pH 8.0, and enzyme in a total volume of 3.0 ml, was incubated at 70°C for 10 min. The reaction was stopped with 1 ml of 1% mercuric chloride and then the color was measured at 405 μ m. One unit of aminopeptidase activity was defined as 1 μ mol of *p*-nitroaniline formed per mg of enzyme during the incubation.

Molecular weight determination. The molecular weights of the proteases were determined from the elution profiles of the enzymes on a 63×1.6 cm Sephadex G-100 column. The column was calibrated using proteins of known mol. wt., namely lysozyme (12,000), pepsin (35,000) and bovine serum albumin (69,000). The void volume was determined with blue dextran.

Effect of pH on enzyme activity. The assay system for proteinase contained 15 mg hemoglobin in 1.5 ml each of the following buffers at 100 mM concentration: citrate-phosphate buffer (pH 3.0 to 5.0), potassium phosphate buffer (pH 6 to 8.0), boric acid-borax buffer (pH 8.5) and glycine-NaOH buffer (pH 9.0 to 10.0). For aminopeptidase activity, 100 mM Tris-HCl was used at pH values ranging from 7.0 to 9.0.

Effect of temperature on enzyme activity. The assay system, containing hemoglobin or L-leucine-*p*-nitroanilide, was preincubated for 30 min at temperatures in the range of 0 to 80°C before the enzyme was added. The enzyme activity was determined as described above.

Heat and radiation stability. The enzyme solution containing 60~90 μ g/ml protein in 50 mM phosphate buffer, pH 7.5, was subjected to heat treatment at 65°C or 70°C for periods ranging from 0 to 90 min, or subjected to radiation treatment at doses ranging from 0 to 400 krad in a ⁶⁰Co Gamma Cell (BARC model; dose rate, 115 krad per hour). After the heat or radiation treatment, the residual enzyme activity was determined as described above.

Inhibitor studies. The enzyme solution, containing 60~90 μ g protein per ml protein, was dialysed against 50 mM Tris-HCl buffer, pH 7.5, for 4 hr. The dialysed enzyme was incubated for 15 min at room temperature with the following compounds at 1 mM concentration: ethylenediamine tetraacetic acid (EDTA), dipyrindyl, *o*-phenanthroline, iodoacetamide, cysteine, dithioerythritol, *p*-chloromercuribenzoate and phenylmethyl sulfonylfluoride. After treatment with the inhibitors, the proteinase activity was determined with bovine serum albumin in 25 mM Tris-HCl buffer, pH 7.5, and the aminopeptidase activity was determined with L-leucine-*p*-nitroanilide, as described earlier.

Effects of divalent metal ions. Solutions of divalent metal salts, viz., $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, ZnCl_2 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ at 1 mM concentration, were each preincubated with the enzyme protein (100 μ g) for 15 min at room temperature prior to determining the enzyme activity.

Substrate specificity. The proteinase activity was determined using various proteins as substrates, namely, hemoglobin, lysozyme, ribonuclease, myoglobin and fish myofibrillar proteins. The fish myofibrillar proteins were

prepared as described by Venugopal *et al.*⁸⁾

Protein concentration was determined by the method of Lowry *et al.*¹⁴⁾ using bovine serum albumin as the standard.

RESULTS

The results of typical purification of the proteinases are summarised in Table I. Cell free supernatant (2.5 liters) was used as the starting material. Preliminary experiments showed that the protein content of the dialysed cell free supernatant, as determined by Lowry's method,¹⁴⁾ was about 980 μ g per ml. The culture supernatant was concentrated by

lyophilization prior to ammonium sulfate precipitation. The ammonium sulfate precipitate obtained was dissolved in a minimum volume of 50 mM phosphate buffer, pH 7.5, and then dialysed against the above buffer. The dialysed enzyme was then absorbed on a DEAE-Sephadex A-50 column under the chromatographic conditions employed. Two enzymes showing proteinase activities were found to be eluted with the NaCl gradient. Proteinase-I was eluted at approximately 0.13 M NaCl and Proteinase-II at 0.27 M NaCl. The aminopeptidase was eluted at 0.3 M NaCl (Fig. 1). The proteinases and aminopeptidase were sepa-

TABLE I. PURIFICATION OF *Aeromonas hydrophila* PROTEINASES

Purification step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold purification
Culture filtrate	2500	3750	2450	1.5	1
80% Ammonium sulphate fraction	18	900	293	3	2
DEAE-Sephadex A-50 chromatography					
Proteinase-I	6	162	6	27.5	18
Proteinase-II	8	232	12	19	12
Sephadex G-75 chromatography					
Proteinase-I	26.5	156	1.6	108	67
Proteinase-II	28	220	2.6	89	60

FIG. 1. Elution Profiles of *A. hydrophila* Proteases on a DEAE-Sephadex A-50 Column. A, proteinase-I; B, proteinase-II; C, aminopeptidase. The details are given in the text.

TABLE II. PURIFICATION OF *Aeromonas hydrophila* AMINOPEPTIDASE

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold purification
Culture filtrate	2500	4400	690	0.4	1
80% Ammonium sulphate fraction	12	402	32.4	0.52	1.3
DEAE-Sephadex A-50 chromatography	12	14.5	174	12	30
Sephadex G-100 chromatography	12	0.24	15	62	155

FIG. 2. Thermal Inactivation Profiles of *A. hydrophila* Proteases.

●, proteinase-I; ○, proteinase-II; ▲, aminopeptidase.

rately purified starting from separate culture filtrates. The proteinase rich fractions from DEAE-Sephadex A-50 chromatography were pooled, dialysed against the above phosphate buffer and then lyophilized. The enzyme preparations in 5 to 6 ml of 50 mM phosphate buffer, pH 7.5, were charged separately on a column of Sephadex G-75. Both proteinases were eluted as single symmetrical peaks, but showed different elution profiles. Proteinase-I was eluted at an elution volume of 62 ml while proteinase-II was eluted at 45 ml on a Sephadex G-75 column. The aminopeptidase was eluted at 70 ml on Sephadex G-100. The extents of purification obtained were 60~67 fold for the proteinases (Table I) and 155 fold for the aminopeptidase (Table II). The low level of

protein in the culture supernatant could partially account for the comparatively low levels of purification of the enzymes.

The molecular weights of the enzymes, as determined by gel filtration, were about 40,000 for proteinase-I, 48,000 for proteinase-II and 30,500 for the aminopeptidase.

Both the proteinases were found to have a pH optimum of 8.5. Enzymes I and II showed more than 50% of their maximum activities in the pH ranges of 6 to 10 and 7.5 to 10, respectively. The aminopeptidase showed 70% activity in the pH range of 7.5 to 8.5, the maximum being at pH 8.0.

The optimum temperature of proteinase-I was found to be 50°C and that of proteinase-II to be 48°C. Proteinase-I showed approximate-

ly 66% of the maximum activity at 65°C and about 50% activity at 37°C, while proteinase-II retained 50% of its activity at 23°C and about 26% at 65°C. The aminopeptidase, on the other hand, showed maximum activity at 70°C.

Figure 2 shows the thermal inactivation profiles for the proteinases. After heat treatment at 65°C for half an hour, more than 30% of the proteinase-II activity remained. At the end of 1 hr at 65°C only 8.5% of the enzyme activity remained. Proteinase-II was comparatively more temperature labile, 90% of the activity being lost after 5 min treatment at 65°C. In contrast, the aminopeptidase was remarkably heat stable. Thus, as shown in Fig. 2, after heat treatment at 70°C for 1 hr only 25% of the activity was lost.

The effect of gamma radiation on the enzymes is shown in Fig. 3. While 50% activity of proteinase-I remained after exposure to a radiation dose of about 125 krad, only about 13% of the maximum activity remained after subjecting the enzyme to a dose of 370 krad. Proteinase-II was found to be more sensitive to gamma radiation; a dose of 25 krad resulted in a 50% loss in maximum activity while exposure to 75 krad destroyed about 95% of its activity. The aminopeptidase was also similarly sensitive to radiation, losing about 65% of its activity upon exposure to a dose of 115 krad (Fig. 3).

Table III shows the specific activities of proteinases-I and II with respect to different substrates. Both the enzymes had broad substrate specificities. Proteinase-I was most active against fish myofibrillar proteins, followed by myoglobin and ribonuclease. Proteinase-II was found to be most active against ribonuclease followed by myofibrillar proteins. Lysozyme and myoglobin showed about 56% and 62% of the proteinase-II activity with respect to ribonuclease, while bovine serum albumin was least susceptible to proteinase-II.

Table IV presents the activities of proteinases-I and -II in the presence of different metals. The activities of both proteinase-I and proteinase-II were inhibited by Fe^{2+} , Cu^{2+}

FIG. 3. Radiation Inactivation Profiles of *A. hydrophila* Proteases.

●, proteinase-I; ●, proteinase-II; ×, aminopeptidase.

TABLE III. ACTIVITIES OF PROTEINASES I AND II AGAINST VARIOUS PROTEINS

Substrate	Proteinase-I	Proteinase-II
Hemoglobin	250	96
Myoglobin	267	70
Myofibrillar protein fraction from fish	328	104
Ribonuclease	260	113
Lysozyme	155	64
Bovine serum albumin	177	42

The values are expressed as μmol tyrosine liberated by mg enzyme in 30 min.

and Zn^{2+} , while both enzymes were unaffected by Ca^{2+} or Mg^{2+} . Aminopeptidase activity was lost in presence of Mn^{2+} but not in that of Zn^{2+} .

Table IV also presents the effects of various inhibitors on the enzymes. Chelating compounds like EDTA and *o*-phenanthroline exhibited high inhibitory activity toward proteinase-I. Parachloromercuribenzoate did not show any significant inhibition of either proteinase. The serine protease inhibitor, phenylmethyl sulfonylfluoride (PMSF), was comparatively more inhibitory against proteinase-II. The aminopeptidase activity was significantly lost in the presence of EDTA and 1,10-phenanthroline, and also in the presence of thiol reagents like cysteine and dithioeryth-

TABLE IV. EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF *A. hydrophila* PROTEASES

Compounds tested	Final Concentration (mm)	% Relative activity		
		Proteinase-I	Proteinase-II	Aminopeptidase
None	0	100	100	100
FeSO ₄	1	23	0	90
MnCl ₂	1	55	113	33
CuCl ₂	1	17	30	—
CaCl ₂	1	100	150	88
MgCl ₂	1	96	117	107
ZnCl ₂	1	16	10	105
Ethylenediamine tetracetic acid	1	40	44	53
1,10-Phenanthroline	1	44	69	0
α,α' -Dipyridyl	1	—	—	65
<i>p</i> -Chloromercuribenzoate	1	73	100	65
Iodoacetamide	1	32	94	100
Phenylmethylsulfonylfluoride	1	59	34	57
Cysteine	1	—	—	80
Cysteine	5	—	—	35
Dithioerythritol	1	—	—	15
Dithioerythritol	5	—	—	10

—, not tested.

reitol.

DISCUSSION

A. hydrophila produces at least three extra-cellular proteases including two types of proteinases and one aminopeptidase, which were isolated and characterised. Proteinase-I, which was eluted at a salt concentration of 0.13 M from a DEAE-Sephadex A-50 column, had a molecular weight of about 40,000, while proteinase-II, eluted at a salt concentration of 0.27 M, had a mol. wt. of about 48,000. The aminopeptidase was co-eluted with proteinase-II at a salt concentration of about 0.3 M. The enzyme had a mol. wt. 30,500 and was optimally active at pH 8.0 and at a temperature of 70°C. Both the proteinases had a pH optimum of about 8.5 and were appreciably active over the pH range of 6 to 10. The enzymes were sensitive to metal chelating agents. Several proteases produced by Gram-negative bacteria have been shown to possess alkaline pH optima and to be sensitive to metal chelating compounds.¹⁵⁾ In these respects, the proteinases of *A. hydrophila* resemble alkaline pro-

teases which are sensitive to metal chelating compounds. According to Morihara,¹⁵⁾ such proteases also possess broad substrate specificities. Thus, the present proteinases were also found to act on several proteins.

Proteinase-II differed from proteinase-I in certain respects. It had a higher molecular weight and exhibited higher sensitivities towards heat and radiation treatment. Proteinase-II showed a lower electrophoretic mobility than proteinase I (data not shown in results). Proteinase-II was comparatively more sensitive towards inhibition by the serine-protease inhibitor, phenylmethyl sulfonylfluoride, suggesting it to be a serine-protease. Both the enzymes were inhibited by metals like Fe²⁺, Cu²⁺ and Zn²⁺, but not by Ca²⁺ or Mg²⁺, even at 10 mm level. Dahle¹⁶⁾ has reported the purification and properties of two proteases from *Aeromonas liquefaciens*. He observed that both the enzymes appreciably differed from one another with respect to their pH optima, thermoresistance and sensitivities towards certain inhibitors. The properties of the two proteinases from *A. hydrophila* are comparable with those of the extracellular proteases of

other organisms belonging to the same genus. Thus the extracellular protease produced by *Aeromonas salmonicida*¹⁷⁾ could be similar to proteinase-I of *A. hydrophila*. The *A. salmonicida* proteinase has a pH optimum of 9.0 and a temperature optimum of 48°C, and is insensitive to inhibition by parachloromercuribenzoate.¹⁷⁾ The optimum pH values of the two proteinases of *A. hydrophila* were also within the pH range, 8~11, reported for another *Aeromonas* protease by Shieh and Maclean.¹⁸⁾ The enzyme from *A. salmonicida* has a mol. wt. of 11,000, which is much smaller than those found for the *A. hydrophila* proteinases. In contrast, the *A. proteolytica* extracellular protease has neutral pH optimum and a molecular weight of 34,800.¹⁹⁾

A. hydrophila also produced an extracellular aminopeptidase, which was remarkably thermostable. Heat treatment at 70°C for 1 hr caused loss of only about 25% of its original activity. Furthermore, the enzyme also required a temperature as high as 70°C for maximum activity. The radiation sensitivity of the aminopeptidase was, however, comparable to those of the proteinases. The enzyme was inhibited by metal chelators and thiol reagents, suggesting it to be a metalloenzyme. Prescott *et al.*²⁰⁾ reported that the *Aeromonas proteolytica* extracellular aminopeptidase was a zinc-containing enzyme. The *A. proteolytica* aminopeptidase was also optimally active at 70°C and had a mol. wt. of 29,500.

Several bacteria have been recognized to release aminopeptidases during growth and the pathogenicity of many of these organisms has been attributed to their aminopeptidase activity.²¹⁾ The pathogenicity and spoilage potential of *A. hydrophila* could be correlated to

the ability of the bacterium to secrete endoproteases as well as an aminopeptidase during growth.

REFERENCES

- 1) T. C. Hazen, C. B. Flermans, R. P. Hirsch and G. W. Esch, *Appl. Env. Microbiol.*, **36**, 731 (1978).
- 2) J. B. Kaper, H. Lockman and R. R. Colewell, *J. Appl. Bacteriol.*, **50**, 359 (1981).
- 3) B. Austin and D. A. Austin, *J. Appl. Bacteriol.*, **58**, 483 (1985).
- 4) E. Annapurna and S. C. Sanyal, *J. Med. Microbiol.*, **10**, 317 (1977).
- 5) P. Lerke, L. Farber and R. Adams, *Appl. Microbiol.*, **13**, 625 (1965).
- 6) N. F. Lewis, M. D. Alur and U. S. Kumta, *Ind. J. Exp. Biol.*, **9**, 45 (1971).
- 7) A. Ljung and T. J. Wadstrom, *Toxicol. Toxins Rev.*, **1**, 257 (1982-83).
- 8) V. Venugopal, M. D. Alur and N. F. Lewis, *J. Food Sci.*, **48**, 671 (1983).
- 9) T. O'Reilly and D. F. Day, *Appl. Env. Microbiol.*, **45**, 1132 (1983).
- 10) A. C. Pansare, V. Venugopal and N. F. Lewis, *J. Appl. Bacteriol.*, **58**, 101 (1985).
- 11) V. Venugopal, A. C. Pansare and N. F. Lewis, *J. Food Sci.*, **49**, 1078 (1984).
- 12) A. C. Pansare, V. Venugopal and N. F. Lewis, *Agric. Biol. Chem.*, **49**, 2451 (1985).
- 13) H. Ansari and L. Stevens, *J. Gen. Microbiol.*, **129**, 1637 (1983).
- 14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 15) K. Morihara, *Adv. Enzymol.*, **41**, 179 (1974).
- 16) H. K. Dahle, *Acta Path. Microbiol. Scand. Sec. B.*, **79**, 726 (1971).
- 17) S. Mellegaard, *J. Appl. Bacteriol.*, **54**, 289 (1983).
- 18) H. S. Shieh and J. R. Maclean, *Int. J. Biochem.*, **6**, 563 (1975).
- 19) S. H. Wilkes and J. M. Prescott, *Methods in Enzymol.*, **45**, 404 (1976).
- 20) J. M. Prescott, S. H. Wilkes, F. W. Wagner and K. J. Wilson, *J. Biol. Chem.*, **246**, 1756 (1971).
- 21) R. R. Watson, *Methods in Enzymol.*, **9**, 1 (1976).