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Proteolytic activity of *Aeromonas caviae*

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Aeromonas strains produce a variety of virulence factors including proteases. Studies on the kinetics of growth of *Aeromonas caviae* NRRL B-966 and its proteases suggest that the proteolytic activities are produced throughout the growth phase, with peak level occurring at stationary phase. *A. caviae* synthesize both intracellular and extracellular proteases with the latter account for major portion of the total activity. Optimum pH for the *A. caviae* proteolytic activity is at 7.0. *A. caviae* produces a thermoresistant protease, whose activity is dependent on Mg^{++} and Ca^{++} ions. Inhibition of proteolytic activity by phenyl methyl sulfonyl fluoride suggest the presence of a serine protease in *A. caviae*. Nitrogenous compounds enhance the proteolytic activity while carbohydrates tested in this study inhibit the activity.

Aeromonas caviae has been implicated in diarrhoeal cases of formula-fed children, intestinal and extra-intestinal infections and are isolated from aquatic ecosystems (ALTWEGG 1985, JANDA and BRENDEN 1987, ALTWEGG and GEISS 1989, NAMDARI and BOTTONE 1990a, SINGH and SANYAL 1992). The motile aeromonads as a group produce a large number of metabolic products while, *A. caviae* produces rather fewer than other biotypes including a heat stable cytotoxin, cytotonic enterotoxin, alpha or beta-haemolysin and haemagglutinin (JANDA 1985, STEWART *et al.* 1986, POTTOMSKI *et al.* 1987, CARELLO *et al.* 1988, NAMDARI and BOTTONE 1990b, 1991, MONFORT and BALEUX 1991, SINGH and SANYAL 1993, KARUNAKARAN and DEVI 1994a) which could be essential for its *in vivo* survival. *A. caviae* has also been reported to synthesize proteases (JANDA 1985), although a detailed examination is still lacking, and their involvement in multiplication and establishment of the pathogenicity of *A. caviae* is yet to be confirmed. Proteases of *A. salmonicida* and several other bacteria are reported to degrade connective tissue and immune components, simultaneously providing the bacteria with essential nutritional sources, and also evade host defense mechanisms (KILIAN 1981, SLOTS and GENCO 1984, SUNDQVIST *et al.* 1985, LEUNG and STEVENSON 1988, UITTO *et al.* 1988, WINKLER *et al.* 1988). *A. caviae* NRRL B-966 produces proteolytic and gelatinase activities on skim milk and gelatin agar plates respectively. This study was aimed towards the preliminary characterization of the proteolytic activity from *A. caviae*.

Materials and methods

Bacterial strain, culture condition and sample preparation: *Aeromonas caviae* NRRL B-966 (obtained from Northern Regional Research Laboratory, Peoria, IL, USA; LIU 1962) was routinely grown in Luria broth (LB; tryptone – 10, yeast extract – 5 and sodium chloride – 5, g/l, pH 7.2) or on LB agar plates containing either 5% (v/v) sheep red blood cells, 1.5% (w/v) skim milk or gelatin. Proteolytic activity was detected by the appearance of clearing zone around *A. caviae* colonies on skim milk agar plates, while gelatin degradation was assessed by congo red staining (CAPALASH *et al.* 1990).

Kinetics of growth and protease production were studied by growing *A. caviae*, aerobically at 37 °C in a flask containing 100 ml of LB with 5% (v/v) washed inoculum from an overnight culture. Cells were harvested every hour and the optical density at 660 nm determined. After centrifugation (12000 rev./min for 10 min) in a microcentrifuge, the supernate was collected, passed through 0.22 µm MILLIPORE

membrane filter and the culture filtrate were stored at −20 °C, till use. The cells were washed, suspended in sodium phosphate buffer (pH 7.0) and subjected to toluenization, osmotic shock or sonication (KARUNAKARAN and GUNASEKARAN 1992).

Protease assay: *A. caviae* proteolytic activity was assayed as described by LEUNG and STEVENSON (1988). Assay reaction mixture used for proteolytic activity contained 1.61 g of $Na_2HPO_4 \cdot 7\ H_2O$, 0.55 g of $NaH_2PO_4 \cdot H_2O$ and 0.07 g of KCl per litre (pH 7.2). Protease substrate, azocasein (SIGMA; 5 mg/ml) was prepared in the same buffer. Assay mixture containing 575 µl of the buffer, 100 µl of the substrate stock solution and 100 µl of the experimental samples (about 15 µl of protein), was thoroughly mixed in a microcentrifuge tube and incubated at 37 °C. Blank assay mixtures received assay buffer with substrate, LB (100 µl) and respective additives. After 3 h, 750 µl of 10% (w/v) trichloroacetic acid was added, mixed and incubated at room temperature. After 30 min, the tubes were centrifuged (12000 rev./min for 10 min) in a microcentrifuge. Equal volumes (750 µl) of the supernate and 1 M NaOH were mixed and absorbance read at 405 nm. The proteolytic activity was calculated as reported by SAKAI (1985) and expressed as Units/ml. Protein concentrations of the samples were determined by bicinchonic acid protein assay reagent (Pierce Chemical Company).

Influence of pH and various other agents on proteolytic activity: The effect of pH on the proteolytic activity was determined by growing *A. caviae* in LB of different initial pH ranges (5.0–10.0) as well as by using the above buffer of different pH ranges (5.0–10.0) in assay. The effects of divalent cations [Mg^{++} ($MgSO_4$) and Ca^{++} ($CaCl_2$)], ethylene diamine tetraacetic acid (EDTA), thiol compounds [L-cysteine, dithiothreitol (DTT), glutathione and 2-mercaptoethanol], and protease inhibitors Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) and phenyl methyl sulfonyl fluoride (PMSF) on proteolytic activity were determined by incorporating them into the protease assay. To study the effects of various nitrogenous compounds on the proteolytic activity, LB containing various concentrations of peptone, casein and NH_4Cl were used for growing *A. caviae*. The effects of various carbon sources on the proteolytic activity were studied by growing *A. caviae* in LB containing 1% (w/v) of glucose, fructose, sucrose, galactose, lactose or xylose.

Results and discussion

Protease production during the growth of A. caviae

In order to characterize the protease(s), the kinetics of the growth and proteolytic activity of *A. caviae* 966 were studied in LB (pH 7.2). During the course of aerobic growth of *A. caviae*, the pH of the media increased to 7.5–8.0. *A. caviae* synthesized both intracellular and extracellular proteolytic activities during the growth, with maximum proteolytic activity (specific activity 111.07 U/µg protein) occurring at stationary phase. Extracellular proteolytic activity comprised major portion (85%) of the total proteolytic activity as a consequence of growth of *A. caviae*. In order to determine whether extracellular activity is due to secretion, activities of alkaline phosphatase and β-galactosidase were also assayed from *A. caviae* samples; the former was found both in cells and culture supernatant like that of the proteolytic activity while the latter activity was found in association with the cells. There was no significant increase in A_{260} absorbing material (nucleic acid) in culture supernatant of *A. caviae* (data not shown). This suggest that extracellular proteolytic activity is resulted from secretion rather than osmotic shock of the cells. Extracellular proteases are also reported among strains of *A. hydrophila* and *A. salmonicida* (LEUNG and STEVENSON 1988). The time course of maximum protease production in *A. caviae* is similar to that for *A. hydrophila* LP50 (DENNIS and VEILLET-PONCET 1984).

Properties of A. caviae proteolytic activity

Effects of different initial pH of LB on the growth of *A. caviae* were studied. *A. caviae* exhibited maximum growth at pH 8.0. Acidic pH ranges 5.0 and 6.0 inhibited the growth by 92.5 and

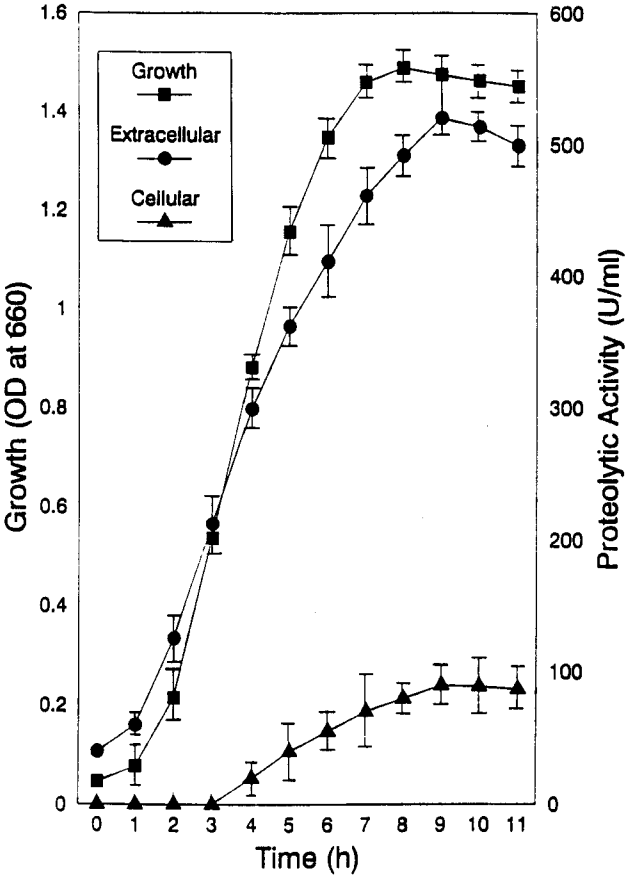

Fig. 1
Kinetics of growth of *A. caviae* and synthesis of proteolytic activity. *A. caviae* was grown in LB, at regular time intervals samples were collected, the turbidity of the culture and proteolytic activity were determined. Results represent the mean value ± SD of three different experiments in triplicate

14.4% (Fig. 2) and alkaline pH ranges 9.0 and 10.0 inhibited the growth by 10.6 and 92.4% respectively. *A. caviae* produced maximum proteolytic activity when the culture was grown at neutral pH. Growth of the culture at pH 6.0 decreased the proteolytic activity by 20.5%, and pH 8.0 and 9.0 inhibited the activity by 7.7 and 17.9% respectively.

Effect of the buffer with different pH on *A. caviae* proteolytic activity were also studied. At pH 7.0, maximum proteolytic activity was seen. The pH values 5.0 and 6.0 depressed the proteolytic activity by 39.4 and 24.2%, and alkaline pH values 8.0, 9.0 and 10.0 inhibited the activity by 6.1, 21.2 and 30.6%, respectively. This suggest that the optimum pH for *A. caviae* proteolytic activity is at 7.0 and shifting pH either towards acidic or alkaline inhibits the activity. This is contrary to the optimum pH (8.0) reported for hydrolysis of azocasein, azocoll or gelatin by an extracellular thermolabile serine protease of *A. hydrophila* (RIVERO *et al.* 1991).

Treatment of the *A. caviae* culture supernate at 60 °C for 15 min did not inhibit the proteolytic activity; however, at 100 °C for 5 min resulted in depression of the activity by 24%. Proteinase K treatment of the culture supernate completely destroyed the activity (Table 1) indicating that the proteolytic activity present in the supernatant is due to a thermoresistant protein.

The effects of divalent cation on *A. caviae* proteolytic activity are shown in Table 1. Addition of 10 mM Mg^{++} resulted in maximum enhancement of the activity (59%) and a concentration above 20 mM, inhibited the activity. Ca^{++} (1 mM) enhanced the activity by 37%. Addition of divalent cation chelator, EDTA partially inhibited the proteolytic activity. Similar effect was also seen with intracellular proteolytic activity of *A. caviae* (data not shown). This indicate that

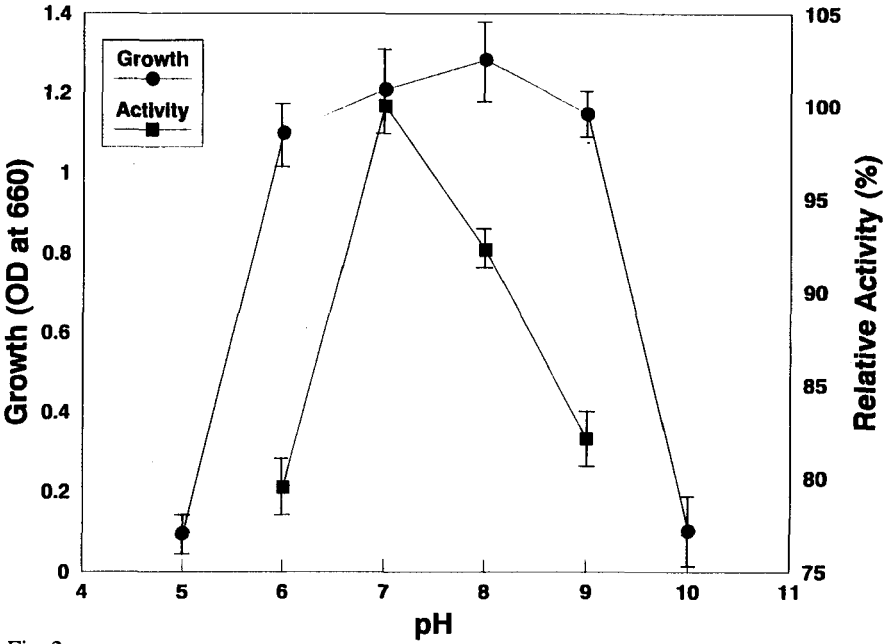

Fig. 2
Influence of pH on the growth of *A. caviae* and its proteolytic activity. *A. caviae* (same cell density) was grown in LB of different initial pH ranges (5–10) and the growth after 16 h and proteolytic activity were measured. Results represent the mean value ± SD of three different experiments in triplicate

divalent cations are required for the catalytic activity of *A. caviae* protease. To determine whether Mg^{++} and Ca^{++} enhanced the protease activity in heat treated (60 °C for 15 min) samples, the metal ions were added. The results (Table 1) indicate an enhancement (9–24%) of proteolytic activity of *A. caviae* with both the metal ions. Trypsin and collagenolytic activities of *Porphyromonas gingivalis*, and chymotrypsin-like activity of *Treponema denticola* are also enhanced by Ca^{++} and Mg^{++}, and are inhibited by EDTA (OHTA *et al.* 1986, UITTO *et al.* 1988, KATO *et al.* 1992, OTOGOTO and KURAMITSU 1993). The extracellular proteases of *A. hydrophila* B51, SO2/2 and D13 are reported to be heat resistant and are inhibited by EDTA (NIETO and ELLIS 1986, RIVERO *et al.* 1990).

The influence of thiol compounds on *A. caviae* proteolytic activity are shown in Table 2. Addition of 1 mM cysteine had no effect and at 10 mM concentration, slight increase in the activity was noticeable. Both dithiothreitol and glutathione at 1 mM, moderately enhanced the activity and a concentration of above 1 mM, appeared to be inhibitory. Mercaptoethanol inhibited the activity. Taken together, this suggest that addition of thiol compounds do not have any significant effect on proteolytic activity and *A. caviae* does not synthesize any thiol protease. While some thiol compounds (DTT and cysteine) and EDTA have been reported to inhibit extracellular proteases of *A. hydrophila* strains NRC505, SO2/2 and D13 (LEUNG and STEVENSON 1988, RIVERO *et al.* 1990).

Addition of protease inhibitor, TLCK had no effect on *A. caviae* proteolytic activity while serine protease inhibitor, PMSF inhibited the activity nearly to half of the original level (Table 2). Intracellular proteolytic activity is also inhibited by PMSF. This suggest that *A. caviae* also produces a serine protease. PMSF has been shown to inhibit several proteases of *A. hydrophila* (LEUNG and STEVENSON 1988, RIVERO *et al.* 1990, 1991), serine proteases of *Thermus aquaticus* (COWAN *et al.* 1987) and proteases of *T. denticola* (OHTA *et al.* 1986, UITTO *et al.* 1988).

Table 1
Influence of varius treatments on *A. caviae* proteolytic activity

Various treatments	Concentration	Activity (U/ml)*	Relative activity (%)
None added	–	337.7 ± 12.7	100.0
Mg^{++}	1 mM	506.5 ± 46.0	150.0
	10 mM	537.2 ± 15.3	159.1
	20 mM	463.3 ± 6.9	137.2
Ca^{++}	1 mM	475.8 ± 15.3	140.9
	10 mM	460.5 ± 61.5	136.4
EDTA	1 mM	225.3 ± 7.3	66.7
	10 mM	187.8 ± 9.8	55.6
Proteinase K	2 mg/ml, 37 °C	24.7 ± 1.3	7.3
60 °C for 15 min	–	274.2 ± 7.8	81.2
100 °C for 5 min	–	257.3 ± 6.1	76.2
60 °C for 15 min + Mg^{++}	1 mM	257.3 ± 32.2	76.2
	10 mM	289.4 ± 13.3	85.7
60 °C for 15 min + Ca^{++}	1 mM	273.2 ± 16.0	80.9
	10 mM	386.0 ± 9.4	114.3

* Activity represents the mean value ± SD of three experiments in triplicate

Influence of carbohydrates and nitrogenous compounds on A. caviae proteolytic activity

Growth of *A. caviae* in LB (initial pH 7.2) containing 1% (w/v) of glucose, fructose, galactose or sucrose resulted in subsequent drop in a final pH to 5.0–5.5, with a moderate inhibition of the growth of this bacterium and this is consistent with our previous report (KARUNAKARAN and DEVI 1994b). As a consequence of acidic pH, the proteolytic activity was inhibited (by 16–25%). Growth of *A. caviae* in LB containing 1% (w/v) of lactose or xylose did not alter the final pH as well as the growth; however, inhibited the proteolytic activity by 14–24%. The acidic pH is reported to be due to the catabolite repression of three enzymes of tricarboxylic acid (TCA) cycle and enhancement of pyruvate dehydrogenase, which ultimately lead to acetic acid synthesis as a major secondary catabolite, during the aerobic growth of *A. caviae* in glucose containing media (NAMDARI and CABELLI 1990).

Table 2
Influence of thiol compounds and protease inhibitors on *A. caviae* proteolytic activity

Various treatments	Concentration	Activity (U/ml)*	Relative activity (%)
None added	–	337.7 ± 12.7	100.0
L-Cysteine	1 mM	337.7 ± 13.4	100.0
	10 mM	394.4 ± 19.0	105.6
Dithiothreitol	1 mM	374.2 ± 4.7	110.8
	10 mM	164.8 ± 23.9	52.8
Glutathione	1 mM	375.2 ± 37.5	111.1
	10 mM	300.2 ± 7.1	88.9
Mercaptoethanol	0.2% (v/v)	303.3 ± 11.3	89.8
	0.4% (v/v)	262.1 ± 8.2	77.6
	0.6% (v/v)	234.4 ± 14.6	69.4
TLCK	1 mM	319.1 ± 19.3	94.5
	10 mM	356.6 ± 17.9	105.6
PMSF	1 mM	281.3 ± 9.7	83.3
	2 mM	178.3 ± 6.9	52.8

* Activity represents the mean value ± SD of three experiments in triplicate

Addition of 0.5% (w/v) of casein to the growth medium did not influence the proteolytic activity. Same concentration of peptone enhanced the activity by 54% and 1% (w/v) stimulated the activity by 115%. NH_4Cl also had a similar effect (109.2% at 0.5% concentration) on *A. caviae* proteolytic activity. This result is identical to the influence of nitrogenous compounds on the proteolytic activity of *Bacteroides fragilis* (GIBSON and MACFARLANE 1988).

These results, in conclusion, demonstrate that *A. caviae* produces more than one proteolytic activity; a thermoresistant metalloprotease and possibly a serine protease. Several other bacteria are reported to produce more than one protease (NIETO and ELLIS 1986, OHTA *et al.* 1986, GIBSON and MACFARLANE 1988, LEUNG and STEVENSON 1988, UITTO *et al.* 1988, KATO *et al.* 1992). Proteases are produced throughout the growth phase of *A. caviae* with maximum activity at stationary phase. Thermoresistant protease of *A. caviae* requires Mg^{++} and Ca^{++} for its activity while PMSF inhibits serine protease activity. Nitrogenous compounds, peptone and NH_4Cl also found to enhance the proteolytic activity. Since intracellular and extracellular proteolytic activities of *A. caviae* share similar properties, it appears that both the activities are due to same enzymes and extracellular activity could be due to secretion.

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