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Isolation of a new toxic protease from a strain of *Aeromonas salmonicida* subspecies *achromogenes*

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ABSTRACT: A new toxic protease was isolated from *Aeromonas salmonicida* subspecies *achromogenes* extracellular products (ECP). It was detected in 5 of 9 strains of the organism tested. The protease was purified by anion-exchange fast protein liquid chromatography (FPLC) on Mono Q HR 5/5, and FPLC gel filtration on Superose 12 HR 10/30. The LD_{50} of the purified protease for juvenile Atlantic salmon *Salmo salar* L. was 0.03 µg protein g^{-1} fish. The molecular weight of the protease was estimated to be 20 kD by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and it possessed 2 isoelectric points (pIs) of 6.2 and 6.4. The protease was completely inhibited by EDTA indicating it to be a metallo-protease.

INTRODUCTION

The Gram-negative bacterium *Aeromonas salmonicida* has been known as a fish pathogen for over 90 yr but its virulence mechanisms are still incompletely understood.

The species *Aeromonas salmonicida* is classified into 3 subspecies: *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *achromogenes* (Popoff 1984). Subspecies *salmonicida* is the causative agent of furunculosis in salmonid fish and is often referred to as the typical strain, with subspecies *masoucida* and *achromogenes* as the atypical strains, causing atypical furunculosis or ulcerative inflammations of skin and muscles (Austin & Austin 1987, Wichardt 1983). *A. salmonicida* subsp. *masoucida* has only been isolated from Japanese salmon (Popoff 1984), but subsp. *achromogenes* has been isolated both from salmonid and non-salmonid fish (Austin & Austin 1987).

The virulence mechanisms of *Aeromonas salmonicida* subsp. *salmonicida* have been investigated by many workers. The presence of an additional protein layer (A-protein) on the outer surface of the bacterium has been related to virulence (Udey & Fryer 1978, Trust et al. 1980, Kay et al. 1981, Evenberg & Lugtenberg 1982). However, some workers have described virulent strains lacking the A-layer (Johnson et al. 1985, Ward et al. 1985, Adams et al. 1988, Ellis et al. 1988a). Extracellular products (ECP) of the bacterium have been associated with the pathology of furunculosis and are lethal to salmonid fish when administered parenterally (Munro et al. 1980, Cipriano et al. 1981, Ellis et al. 1981). A variety of ECP activities of subsp. *salmonicida* have been described including glycerophospholipid-cholesterol acyltransferase (GCAT) (Buckley et al. 1982, Lee & Ellis 1990), a leucocytolysin (Fuller et al. 1977), haemolysins (Titball & Munn 1981, Hastings & Ellis 1985, Fyfe et al. 1987, Nomura et al. 1988, Lee & Ellis 1990) and proteases (Shieh & McLean 1975, Mellergaard 1983, Tajima et al. 1984, Hastings & Ellis 1985, Fyfe et al. 1986, Fyfe et al. 1987). Some strains have been reported as producing 2 distinct proteases, a caseinolytic serine protease (P1) and a gelatinolytic metallo-protease (P2) (Sheeran & Smith 1981, Rockey et al. 1988). The serine protease (molecular weight 70 kD) has been considered to be an important virulence factor of *A. salmonicida* (Sakai 1985a, b). It has been suggested that extracellular protease may be a protective antigen against furunculosis (Sakai 1985c, Shieh 1985, Ellis et al. 1988b, c, Hastings & Ellis 1988). A haemolytic toxin lethal to salmonid fish has been isolated from *A. salmonicida* subsp. *salmonicida* ECP (Nomura et al. 1988) and has been identified as GCAT complexed with lipopolysaccharide (Lee & Ellis 1990).

Little has been published regarding *Aeromonas salmonicida* subsp. *achromogenes* extracellular virulence

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factors. Pol et al. (1980) reported that crude ECP of one atypical strain was lethal for carp. Toxicity of ECP was heat-labile but the nature of the toxin(s) was not determined. Hastings & Ellis (1985) reported a difference in production of haemolytic and proteolytic activities by 5 isolates of *A. salmonicida* subsp. *salmonicida* and 1 isolate of *A. salmonicida* subsp. *achromogenes*. In contrast to the typical isolates, the *achromogenic* isolate did not produce detectable haemolysin and its caseinase had properties compatible with those of a metallo-protease. Evenberg et al. (1988) reported vaccination results where immunity against *A. salmonicida* subsp. *achromogenes* ECP was of prime importance, although the nature of the protective antigen(s) was not reported.

The present study deals with the purification and properties of a new lethal toxic proteolytic enzyme produced by *Aeromonas salmonicida* subsp. *achromogenes*.

MATERIALS AND METHODS

Bacterial strains. *Aeromonas salmonicida* strains used in this study are described in Table 1. The strains were classified as *A. salmonicida* subsp. *salmonicida* and *achromogenes* on the basis of biochemical reactions and antibiograms. All subsp. *achromogenes* strains used in the study were differentiated from subsp. *salmonicida* strains by the following characteristics: The former strains produced indole and acid from sucrose; failed to produce gas from glucose and to degrade aesculine; were resistant to the antibiotics ampicillin and cefaloridin; and showed no or delayed production of brown pigment.

The subsp. *salmonicida* strain used was nonaggregating, but all subsp. *achromogenes* isolates were autoaggregating.

Stock cultures were stored in tryptone soya broth (TSB, Oxoid) +10% glycerol at −20°C. Bacteria were routinely cultured on brain heart infusion agar (BHI, Oxoid +1% Oxoid bacteriological agar No. 1) at 22°C.

Extracellular products (ECP). ECP were produced by the cellophane overlay method as described by Munro et al. (1980) using BHIA at 22°C. After 48 h incubation, the culture was washed from the cellophane with a minimal volume of phosphate-buffered saline, PBS (Dulbecco's Ca^{++} and Mg^{++} free; Gibco) pH 7.2 and centrifuged. The supernatant (ECP) was filtered (Millipore, 0.22 µm) and stored at −20°C.

Protein determination. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Proteolytic assays. Proteolytic activity was determined in the following way: 2.4 ml PBS were added to 25 mg hide powder azure (HPA, Calbiochem). The enzyme (0.1 ml) was added and incubated at 37°C for 15 min. The reaction was stopped by the addition of 2.5 ml 10% (w/v) trichloroacetic acid (TCA). After centrifugation, the absorbance of the supernatant was measured at 595 nm (A_{595}) against substrate blanks. One unit of proteolytic activity was equivalent to a change of 0.01 A_{595} units.

Gelatinase and caseinase activities. Hydrolysis of gelatin and casein was detected by a radial diffusion method (Hastings & Ellis 1985). Caseinase and gelatinase activity was determined from a standard curve using trypsin (bovine pancreas type III, Sigma). One unit of caseinase or gelatinase activity was defined as that which produced a zone of clearing equal in area to that produced by 1 µg trypsin.

Haemolytic titration. Haemolytic activity was determined as previously described (Hastings & Ellis 1985) using rainbow trout, sheep, and horse erythrocytes. Blood was collected in Alsevers solution and the red blood cells (RBCs) were washed 3 times in PBS and resuspended to 1% (v/v) in PBS. Haemolytic activity was determined by diluting 100 µl of sample in 2-fold steps in PBS, adding 100 µl RBC suspension, and

Table 1. Bacterial strains investigated in this study

Isolate number	*A. salmonicida* subspecies	Host species	Country
MT004	*salmonicida*	Atlantic salmon *Salmo salar*	Scotland
Lon/82	*achromogenes*	Atlantic salmon *Salmo salar*	Iceland
265/87	*achromogenes*	Atlantic salmon *Salmo salar*	Iceland
FeII	*achromogenes*	Sea trout *Salmo trutta*	Faeroe Islands
23/87	*achromogenes*	Atlantic salmon *Salmo salar*	Iceland
17/87	*achromogenes*	Atlantic salmon *Salmo salar*	Iceland
Eldi/80	*achromogenes*	Atlantic salmon *Salmo salar*	Iceland
3.111	*achromogenes*	Goldfish *Carassius auratus*	USA
V75/93	*achromogenes*	Carp *Cyprinus carpio*	Yugoslavia
16/76	*achromogenes*	Rudd *Scardinius erythrophthalmus*	England

incubating at 22°C for 20 h. The dilution of sample which caused 50% haemolysis was defined to contain one haemolytic unit (HU).

Phospholipase assay. Phospholipase activity was determined by applying 20 µl sample into wells cut in 1% (w/v) agarose in PBS containing 1% L-α lecithin (Sigma) and incubating at 22°C for 48 h. Clearing around the well indicated a positive phospholipase reaction.

Cytotoxicity assay. Cytotoxic activity of ECP 265/87 and the toxic protease was determined using cultured rainbow trout gonad cells (RTG-2). Confluent cell monolayers were prepared in 96-well flat-bottom microtiter plates containing 200 µl Glasgow modification of Eagles medium (GMEM, Flow) supplemented with antibiotics, tryptose phosphate broth, 8 mM glutamine, and 10% foetal calf serum. Medium was aspirated from test wells containing RTG-2 cells. Half of the wells received 100 µl of GMEM medium +2% serum and the other half received GMEM without serum. Then 40 µl of enzyme solution, or PBS as a control, were added to wells and the reaction systems incubated at 15°C for 48 h. The cells were examined microscopically for cytotoxic effects.

Toxicity test. Lethal activity was estimated by intraperitoneal injection of juvenile Atlantic salmon *Salmo salar* L. (mean weight: 40 g) with 0.1 ml of protease, diluted in PBS. Control fish were injected with 0.1 ml PBS. Mortality was recorded after 24 h. LD_{50} was calculated by the method of Reed & Muench (1938).

Inhibition studies. The effects of ethylene diamine tetraacetic acid (EDTA) and phenyl methyl sulfonyl fluoride (PMSF) on the proteolytic activity were examined. Enzyme inhibition tests were performed by mixing 50 µl of purified enzyme and 50 µl freshly prepared 20 mM EDTA in PBS or 25 mM PMSF in propan-2-ol. Reaction mixtures were incubated at 22°C for 10 min prior to assay. Controls contained the solvents alone.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the molecular weight determination and to check the purity of fractions was carried out as described by Laemmli (1970), using 12.5% gels. Pharmacia low molecular weight (LMW) standards were used as marker proteins. Samples were incubated for 10 min at 100°C with sample buffer (1:2 v/v) comprised of 4% SDS and 5% 2-mercaptoethanol in Tris-HCl pH 8.0. Protein bands were visualized by silver staining using Bio-Rad silver stain kit according to the manufacturer's instructions.

Purification of a toxic protease. Strain 265/87 ECP (5 ml) was desalted on a Sephadex G-25M (Pharmacia PD-10) column. Proteins were eluted with distilled water and applied to a Mono Q HR 5/5 anion-exchange column (Pharmacia) in a Pharmacia fast protein liquid chromatography (FPLC) apparatus. Proteins were eluted using a gradient of 0 to 1 M NaCl in 20 mM Tris (hydroxymethyl) methylamine pH 7.7 at a flow rate of 1 ml min^{-1} and 1.0 ml fractions were collected. Elution profiles were recorded with an UV absorbance monitor at 280 nm (A_{280}). Total protein, toxicity, and proteolytic, haemolytic, cytotoxic and phospholipase activities were determined in peak fractions. Toxic fractions were examined by SDS-PAGE for protein purity.

Toxic fractions were run separately on a Superose 12 HR 10/30 gel filtration column (Pharmacia) in the FPLC system. Proteins were eluted with PBS pH 7.2 at a flow rate of 0.5 ml min^{-1}. Fractions (0.5 ml) were collected and tested for purity and activities as described above. The column was calibrated with the following molecular weight marker proteins: bovine serum albumin, soybean trypsin inhibitor, and α-lactalbumin under the same running conditions as described above.

Isoelectric focusing. Isoelectric focusing (IEF) was done in Pharmacia PhastSystem PhastGel IEF 3-9. Pharmacia standards pH 3 to 10 were used to determine the pH gradient. Gels were run for 500 Vh at 15°C and silver stained.

Preparation of rabbit antisera. Rabbit antiserum for formalin-inactivated ECP (f-ECP) of isolate 265/87 was prepared as follows: ECP was toxoided by adding formalin [to give a final concentration of 1.2% (w/v) formaldehyde] and incubating for 7 d at 22°C. A rabbit was injected subcutaneously with f-ECP (750 µg protein) emulsified with Freund's complete adjuvant (Difco). A booster injection of f-ECP (2 mg protein) without adjuvant was administered after 28 d. The rabbit was bled 20 d later and serum extracted and stored at −20°C (R-anti-f-ECP 265/87).

Western blotting. Samples were separated by SDS-PAGE in 4 to 30% gradient polyacrylamide gels (Pharmacia LKB) at 150 V for 170 min at 10°C. SDS-PAGE fractionated antigens were transferred to nitrocellulose by electrophoresis (30 V, 16 h). Transfer buffer was comprised of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol. Following transfer, nitrocellulose membranes were either stained for total protein using colloidal gold (Aurodye, Janssen) or blocked for 1 h with 3% gelatin prior to immunostaining. For Western blotting, gelatin-blocked membranes were washed (2 × 5 min) in tris-buffered saline pH 7.5 containing 0.05% Tween 20 (TTBS), and incubated for 1 h in control or immune rabbit serum diluted 1:1000. Antibody dilution buffer was TTBS containing 1% gelatin. After further washing in TTBS, membranes were incubated for 1 h with goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) diluted 1:3000. Bands were visualised by incubating membranes in 0.1 M carbonate buffer pH 9.8 containing 0.3 mg ml^{-1} *p*-nitro

blue tetrozolium chloride and 0.15 mg ml^{-1} 5-bromo-4-chloro-3 inoldyl phosphate toluidine salt.

Relationship between cultivation time and production of protease. One ml of a fresh culture of strain 265/87 was inoculated into 100 ml BHI broth in a 200 ml Erlenmeyer flask and incubated at 22 °C on a rotary shaker for 73 h. Samples were taken periodically during incubation. For colony-forming unit (cfu) counts, 0.1 ml samples of 10-fold serial dilutions were spread onto blood agar plates and incubated at 22 °C for 48 h. For detection of proteolytic activity 0.5 ml of the culture was centrifuged and proteolytic activity of the supernatant determined in a caseinase assay.

RESULTS

Purification of protease

ECP proteins eluted as several peaks in anion-exchange FPLC, with proteolytic activity eluting as a single peak between 0.09 and 0.20 M NaCl (Fig. 1). Peak fractions 10 and 11 were further separated by gel filtration FPLC. The elution profile revealed one major and one minor protein peak (Fig. 2). Proteolytic activity was detected only in the major peak with highest activity in fraction 33. Total recovery of protease activity following gel filtration was 11.7 % and the relative purification was 17-fold (Table 2).

Toxicity

Intraperitoneal injection of juvenile salmon with samples of elution peaks from FPLC anion-exchange column showed lethal toxicity only in fractions 9, 10 and 11 indicating coelution with proteolytic activity (Fig. 1). Protease-containing fractions 33/10 and 33/11 (fractions 33 from FPLC gel filtration) were lethal when injected into salmon (Table 3). The 24 h LD_{50} value of the protease was 0.03 µg protein g^{-1} fish.

Purity, molecular weight and isoelectric point of protease

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of fractions from the FPLC anion-exchange column showed that the lethal fractions 9, 10, and 11 all contained the same protein band but also other bands (Fig. 3). Gel filtration of fractions 10 and 11 produced one major peak with protease and another minor peak without detectable protease activity (Fig. 2). Analysis of protein fractions from the major peak by SDS-PAGE showed one protein band in fraction number 33 with a

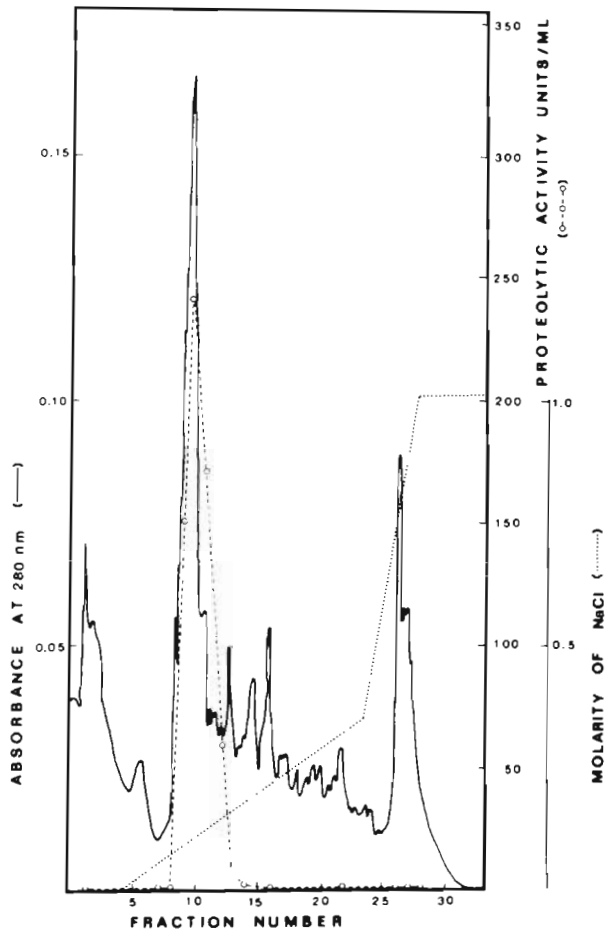

Fig. 1. *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87. Mono Q HR 5/5 FPLC anion-exchange chromatography of extracellular products (ECP)

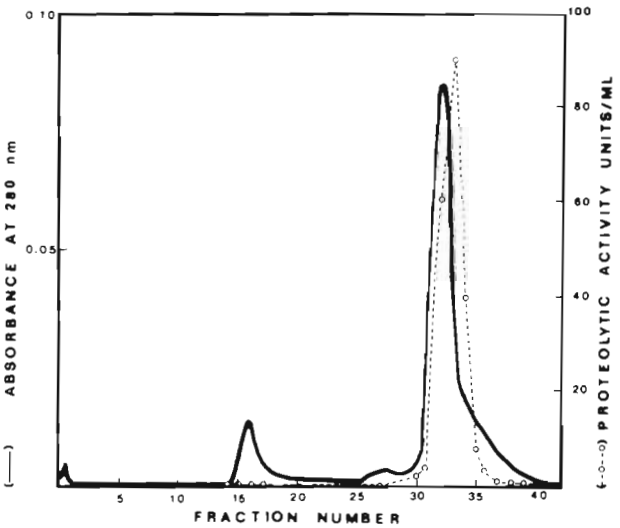

Fig. 2. *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87 Superose 12 HR 10/30 FPLC gel filtration chromatography of fraction 10 from Mono Q HR 5/5 FPLC anion-exchange chromatography of ECP

Table 2. Purification of *Aeromonas salmonicida* subsp. *achromogenes* toxic protease

Purification step	Fraction number	Volume (ml)	Total protein (mg)	Proteolytic units	Specific activity (units mg^{-1})	Recovery (%)	Relative purification
1. PD-10 gel filtration		7.0	2.625	770	293.3	100	1
2. Mono Q HR 5/5 anion-exchange	10 + 11	2.0	0.602	410	681.1	53.2	2.3
3. Sup. 12 HR 10/30 gel filtration	33/10[a] + 33/11	1.0	0.018	90	5000.0	11.7	17.0

[a] Gel filtration of fraction number 10 and 11 from anion-exchange chromatography

Table 3. *Salmo salar*. Lethal toxicity of *Aeromonas salmonicida* subsp. *achromogenes* protease to Atlantic salmon. Mean body weight of fish was 40 g

Dose µg protein $fish^{-1}$	No. of fish injected	Mortality within 24 h
3.60	2	2
2.40	5	5
1.80	5	4
0.90	5	1
0.45	5	0

LD_{50} = 0.03 µg g^{-1} fish

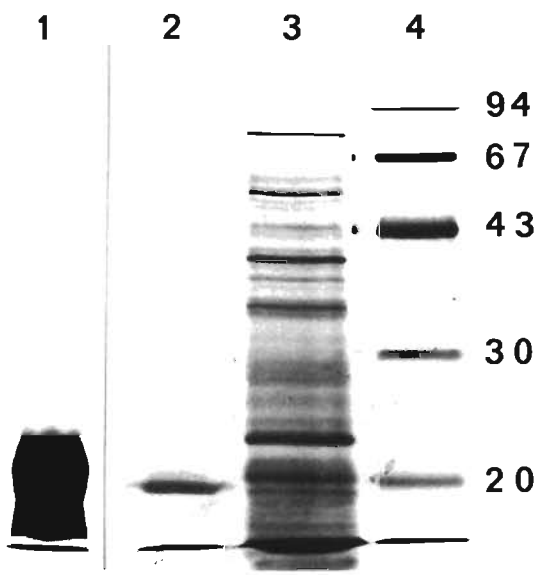

Fig. 3. *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87. Purification of extracellular protease, monitored by SDS-PAGE. Protein loadings are listed in parentheses. Lane 1: fraction 11 from ion-exchange chromatography (6.02 µg); lane 2: purified protease, fraction 33/11 following gel filtration (0.9 µg); lane 3: crude ECP (3.75 µg); lane 4: molecular weight marker proteins. Molecular weights are indicated in kD

MW of 20 kD. Two faint bands (not seen in fraction 11, from which fraction 33 originated) were also present in fraction 33 (Fig. 3). The same faint bands were seen in blank runs (data not shown), indicative of artifacts in the gel system previously seen in this MW range (68 kD, 54 kD) (Tasheva & Dessev 1983).

Calibration of the gel filtration column with molecular weight standards resulted in soybean trypsin inhibitor (MW 20 kD) eluting in fraction 27. The pure native protease was eluted in fraction 33, showing a molecular weight of less than 20 kD by gel filtration.

The results from the gel filtration coupled with SDS-PAGE indicate the protease to be a monomeric polypeptide in the native state. Fraction 33 showed both proteolytic activity and lethal toxicity (Fig. 2, Table 2), indicating a pure toxic protease.

Two isoelectric forms of the purified protease were detected by isoelectric focusing. The isoelectric points were pI 6.2 and 6.4 (Fig. 4).

Studies of protease activity

The protein concentration of fraction 33/11, the pure toxic protease, was 18 µg ml^{-1}. The proteolytic activity of the pure toxic protease was 90 units ml^{-1} in a HPA assay. Caseinase and gelatinase activities were determined to be 40 and 5 units ml^{-1}, respectively (Table 4a).

Neither the ECP from *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87 nor the pure toxic protease was haemolytic against rainbow trout, sheep, or horse erythrocytes and neither possessed phospholipase activity (ECP of *A. salmonicida* subsp. *salmonicida* strain MT004 was used as a positive control). Cytotoxic activity against rainbow trout gonad cells (RTG-2) was observed with ECP (HPA proteolytic activity, 170 units ml^{-1}) in GMEM medium without serum, but cells incubated with ECP in the presence of 2 % foetal calf serum or with purified protease (170 units ml^{-1}) (with and without calf serum) were unaffected. This indicates the

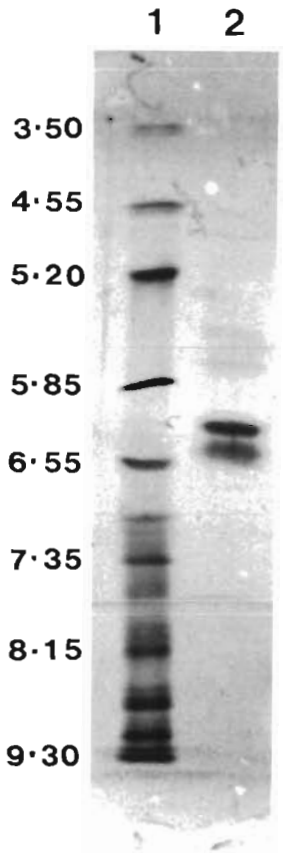

Fig. 4. *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87. Isoelectric focusing of extracellular protease. Lane 1: pI marker proteins; lane 2: purified protease

presence of a cytotoxin in ECP that is inhibited by serum. It also indicates that the protease was not cytotoxic.

Effect of inhibitors

Proteolytic activity of the toxic protease was completely inhibited by EDTA but PMSF had no effect, indicating this enzyme to be a metallo-protease (Table 4a). Inhibition of proteolytic activity of ECP from the different *Aeromonas salmonicida* isolates by EDTA and PMSF is shown in Table 4b. Only 0 to 32% of the protease activity in the ECP of atypical strains resisted treatment with EDTA but most of the protease activity (86 to 100%) resisted treatment with PMSF. Conversely, the proteolytic activity of the ECP of the typical strain (MT004) was mainly PMSF sensitive.

Relationship between cultivation time and production of protease

Protease activity was present in the supernatant from an early phase of exponential growth and increased at the same rate as the increase in cell numbers up to 24 h of culture. Thereafter, it increased at a slower rate than the cell counts (Fig. 5).

Western blotting

Antibody in R-anti-f-ECP 265/87 serum to the 20 kD band detected the band in the ECP of 5 of 9 *Aeromonas salmonicida* subsp. *achromogenes* isolates tested (Fig. 6, lanes 2, 3, 4, 6, and 7). All ECP's showing the 20 kD band possessed a metallo-protease activity (Table 4b). ECP of the typical strain MT004 possessing a high serine-protease activity (Table 4b) did not show a 20 kD band (Fig. 6, lane 1).

DISCUSSION

The extracellular protease isolated in this study from *Aeromonas salmonicida* subsp. *achromogenes* has not previously been described. The enzyme is caseinolytic and gelatinolytic, has a molecular weight of 20 kD and pIs of 6.2 and 6.4, and its activity is completely inhibited by the metal ion chelator EDTA, indicating it to be a metallo-protease. These properties distinguish it from the 2 extracellular proteases (P1 and P2) of *A. salmonicida* subsp. *salmonicida* which have previously been described. The P1 protease is a caseinolytic and gelatinolytic serine protease of molecular weight 70 kD and pI 5.6 (Tajima et al. 1984, Fyfe et al. 1986, Hastings & Ellis 1988, Price et al. 1989), whereas the P2 protease is a gelatinolytic (but not caseinolytic) metallo-protease

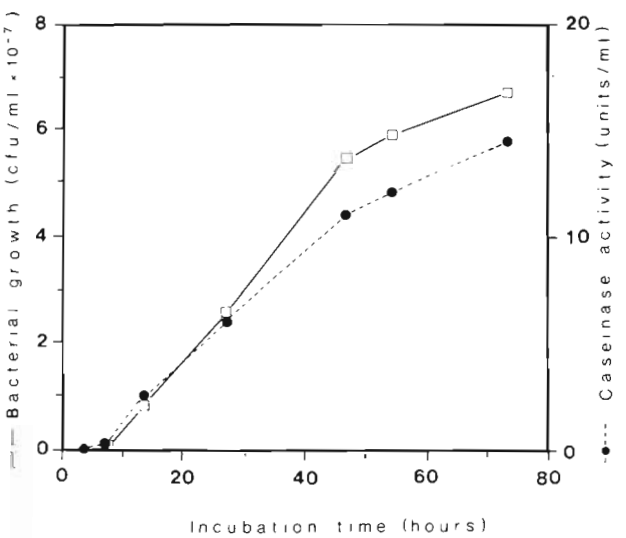

Fig. 5. *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87. Relationship between growth and the production of extracellular protease

Table 4a. Proteolytic activities of the purified 20 kD protease. EDTA: ethylene diamine tetraacetic acid; PMSF: phenyl methyl sulfonyl fluoride

Protein concentration ($\mu g\ ml^{-1}$)	Caseinase (units ml^{-1})	Gelatinase (units ml^{-1})	Proteolytic (HPA) (units mg^{-1} protein)	Percentage HPA activity remaining after treatment with:	
				EDTA	PMSF
18	40	5	90	0	100

Table 4b. Proteolytic activities of ECP from different *Aeromonas salmonicida* isolates

ECP number	Isolate number	Protein concentration ($\mu g\ ml^{-1}$)	Proteolytic activity[a] (units mg^{-1} protein)	Percentage activity remaining after treatment with	
				EDTA	PMSF
1	MT004	710	718	85	5
2	Lon/82	275	36	0	100
3	265/87	375	293	32	86
4	FeII	980	143	22	88
5	23/87	400	10	0	0
6	17/87	550	165	0	100
7	Eldi/80	500	6	0	100
8	3.111	600	0	0	0
9	V75/93	425	5	0	0
10	16/76	200	0	0	0

[a] Proteolytic activity in an HPA assay

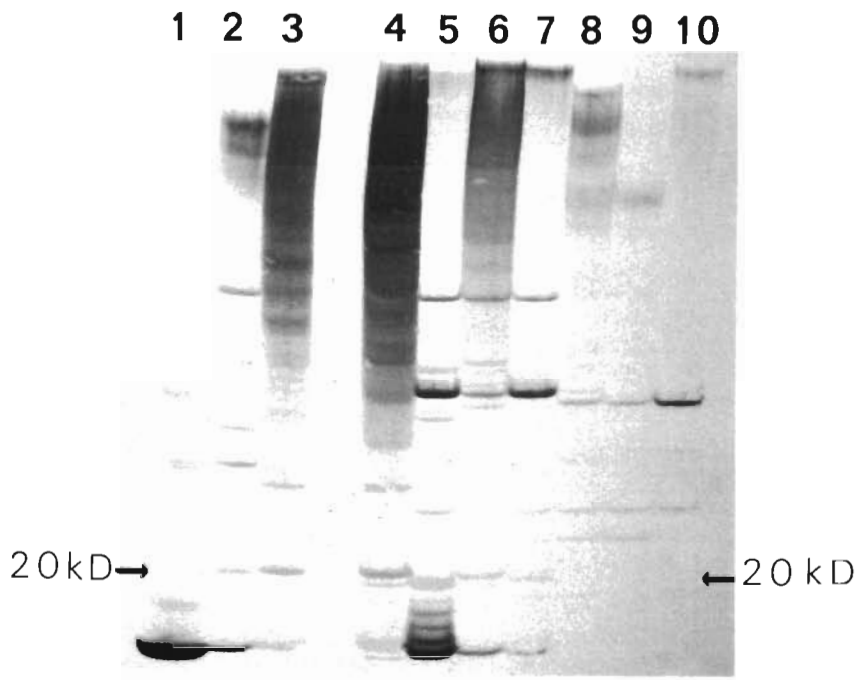

Fig. 6. Detection of antibody to *Aeromonas salmonicida* subsp. *achromogenes* extracellular 20 kD protease by Western blotting in ECP of: 1, MT004; 2, Lon/82; 3, 265/87; 4, FeII; 5, 23/87; 6, 17/87; 7, Eldi/80; 8, 3.111; 9, V75/93; 10, 16/76. The ECP were stained with rabbit antiserum to formalin-inactivated ECP of isolate 265/87. The 20 kD protein band is shown by an arrow

(Sheeran & Smith 1981, Rockey et al. 1988) with a molecular weight of ca 22 kD (Price et al. 1989).

Although the pathogenic role of the P2 protease is not known, the P1 protease is believed to play an important role in the pathogenesis of furunculosis (Sakai 1985a, b). The P1 protease is toxic for yamabe *Oncorhynchus masou* f. *ishikawi* with an LD_{50} of 1.52 $\mu g\ g^{-1}$ body weight by i.p. injection (Tajima et al. 1983), and for Atlantic salmon at a dose of 2.4 $\mu g\ g^{-1}$ body weight (Lee & Ellis 1989). However, the major

lethal toxin of *Aeromonas salmonicida* subsp. *salmonicida* appears to be glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide (GCAT/LPS) which has cytotoxic, haemolytic (T-lysin), and phospholipase activities (Lee & Ellis 1990). The LD_{50} of the GCAT/LPS for Atlantic salmon by i.p. injection is 0.045 $\mu g\ g^{-1}$ body weight (Lee & Ellis 1990).

Neither haemolytic nor phospholipase activity was detected in ECP of *Aeromonas salmonicida* subsp. *achromogenes* strain 265/87, but the purified extracellular metallo-protease isolated from the bacterium showed lethal toxicity for Atlantic salmon. The protease is a monomeric polypeptide in the native state with an LD_{50} of 0.03 $\mu g\ g^{-1}$ body weight, more lethally toxic than either the P1 protease or the GCAT/LPS toxin of *A. salmonicida* subsp. *salmonicida*. This finding, and the lack of lethal toxicity of non-proteolytic fractions of ECP, would suggest that the 20 kD protease is a major extracellular lethal toxic factor produced by this atypical strain.

In broth culture the protease appears early and the concentration is proportional to the cell number. It appears to be stable in the supernatant for at least 72 h of culture.

The mechanism of toxicity of the protease is not known. Although crude ECP of strain 265/87 was cytotoxic for RTG-2 cells, the purified protease did not show any detectable cytotoxicity. The absence of detectable haemolytic or phospholipase activity in the ECP suggests that the cytotoxin of strain 265/87 is not the same as that produced by *Aeromonas salmonicida* subsp. *salmonicida* (Lee & Ellis 1990).

Five *Aeromonas salmonicida* subsp. *achromogenes* strains (isolated from salmonid fish in Iceland and the Faroe Islands) produced the 20 kD protein, but this protein was not detected in ECP (possessing low or no proteolytic activity) of one Icelandic strain isolated from Atlantic salmon and 3 strains isolated from cyprinid fish in England, Yugoslavia and the USA. Inhibitor studies on proteolytic activity of strain 265/87 and FeII, both possessing the 20 kD band, showed significant inhibition by EDTA but the serine-protease inhibitor PMSF also had some effect. This would suggest the presence of a serine-protease in ECP besides the 20 kD metallo-protease. Proteolytic activity of the other 3 atypical strains (Lon/82, 17/87 and Eldi/80), possessing the 20 kD band, was completely inhibited by EDTA but PMSF had no effect. The close correlation between extracellular metallo-protease activity, and the presence of a 20 kD protein in the ECP, suggests that this toxin is produced by other atypical strains (Table 4b, Fig. 6). ECP of the one typical strain (MT004) tested, possessing high serine-protease activity, lacked the 20 kD protein. There is a lack of evidence in the literature of any *A. salmonicida* subsp. *salmonicida* strain producing an extracellular caseinolytic 20 kD metallo-protease and a well-defined strain of this organism (MT004) (Lee & Ellis 1990), tested in this study, did not. Therefore it is possible that *A. salmonicida* subsp. *salmonicida* does not produce this lethal 20 kD toxin. Further studies are, however, needed to confirm this. More studies are also needed to examine the frequency of this 20 kD toxic protease among different *A. salmonicida* subsp. *achromogenes* strains.

Extracellular factors of *A. salmonicida* subsp. *salmonicida* have received considerable attention as possible protective antigens against furunculosis. Active immunization with crude ECP and protease, and passive immunization with antibodies to these preparations, have been reported to protect salmonid fish against furunculosis (Cipriano 1982, Olivier et al. 1985, Shieh 1985, Ellis et al. 1988c). It has also been reported that toxoids of crude ECP of an atypical strain could protect carp against erythrodermatitis, though the nature of the protective antigen(s) was unknown (Evenberg et al. 1988). If anti-*A. salmonicida* vaccines are to be developed based on ECP components, it is important to know if the protective antigens are shared by typical and atypical strains.

The potential role of the toxic protease of *A. salmonicida* subsp. *achromogenes* as a protective antigen against atypical furunculosis is currently being investigated.

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