Separation and in vivo analysis of two extracellular proteases and the T-hemolysin from Aeromonas salmonicida

D. D. Rockey, J. L. Fryer, J. S. Rohovec

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331, USA

ABSTRACT: A procedure using DEAE cellulose and hydroxylapetite was developed for the separation of Aeromonas salmonicida P1 protease, P2 protease, and trout cell-specific hemolysin (T-lysin) from supernatants of broth cultures. The different proteases were demonstrated using protease inhibitors, substrate specificities, and polyacrylamide gel electrophoresis with protease substrates in the gels. Isolated P1 protease and T-lysin were shown to act independently in the complete lysis of rainbow trout erythrocytes in vitro. The T-lysin acted on the outer membrane and P1 protease acted on the nuclear membrane. Analysis of cell-free exudate from lesions of trout infected with A. salmonicida by injection demonstrated that more than one protease was also present in vivo. P1 protease was present in both lesions and in culture supernatants, but P2 protease was detected only in culture supernatants. No T-lysin activity was detected in cell-free exudate from lesions caused by A. salmonicida infection.

INTRODUCTION

Aeromonas salmonicida, the causative agent of fish furunculosis, inflicts serious losses in hatchery-reared salmon and trout in many parts of the world (McCarthy & Roberts 1980). Although efficacious vaccines have been developed for other, similar, gram-negative fish pathogens, commercial furunculosis vaccines have only recently been made available, and documentation of efficacy is absent in the literature (Michel 1985). Therefore, virulence factors of this organism have been the object of much research. Previous work has included investigations of cell-associated and secreted components of the bacterium. An outer membrane protein matrix, the A layer, has been directly associated with virulence, and the role of lipopolysaccharide in bacterial survival has also been addressed (Udey & Fryer 1978, Munn et al. 1982). Non-cellular factors secreted by A. salmonicida have been shown to be directly responsible for many of the clinical signs associated with the disease (Ellis et al. 1981). Secreted factors which have been investigated include proteases, hemolysins, and a leukocidin. Several authors have reported purification of these secreted proteins, as well as their individual and interactive characteristics (Fuller et al. 1977, Sheeran & Smith 1981, Titball & Munn 1981, 1983, 1985, Hastings & Ellis 1985, Fyfe et al. 1987).

Investigation of Aeromonas salmonicida proteases began with Dahle (1971) who reported characteristics of proteases of A. salmonicida and A. hydrophila. Subsequent research described a variety of proteolytic factors. The major secreted protease is a serine protease of molecular weight 70 000 with activity against casein and gelatin (Tajima et al. 1983, Fyfe et al. 1986). Sheeran & Smith (1981) reported 2 activities in culture supernatants of A. salmonicida one of which (P1) was the major serine protease; the other was an ethylene diamine tetraacetic acid (EDTA) sensitive protease (P2). P1 protease was active against casein and gelatin, while P2 protease had activity against gelatin but not casein. Other authors have reported a low molecular weight protease and a fibrinolytic protease with molecular weight 87 500 (Shieh & MacLean 1975, Mellergaard 1983). The presence of alternate proteases was disputed by Hastings & Ellis (1983) based on results associating all gelatinolytic activity with a single isoelectric point in preparative isoelectric focusing. This suggested that a single component accounted for the activity.

In order to facilitate analysis of secreted virulence factors of Aeromonas salmonicida, we have developed
a single technique for the separation of P1 protease, P2 protease, and T-lysin from culture supernatants. Using this technique, we demonstrated that multiple proteases are present, and have separated these activities. The relationship between the T-lysin and P1 protease in the complete lysis of trout RBC was also investigated in vitro. In addition, proteolytic and hemolytic activities were investigated in vivo. It was shown that more than one protease was present in lesions caused by injection of the microorganism, but in vivo hemolytic activity was not detected.

MATERIALS AND METHODS

Bacterial strains. Experiments were conducted with a recent isolate of Aeromonas salmonicida (RC1) from diseased spring chinook salmon Oncorhynchus tshawytscha at the Round Butte Salmon Hatchery, Madras, Oregon, USA. Comparisons for strain variation were conducted with strain SS70 (Evans & Fryer 1978). Cultures were stored on brain heart infusion (BHI) agar (Difco) at 4°C and grown for harvest in 500 ml BHI broth cultures at 17°C for 48 h with shaking.

Separation of virulence factors. Forty-eight hour broth cultures of Aeromonas salmonicida were pelleted at 2500 x g for 15 min and the supernatant removed and recentrifuged. The resulting supernatant was transferred to a flask on ice and powdered NH₄SO₄ (Mallinckrodt, GenAR grade) was added over a 10 min period with constant stirring to 45% saturation (32 g 100 ml⁻¹ supernatant). Fifty µl of 1 N NaOH were added for every 10 g NH₄SO₄. The solution was stirred on ice for 20 min and then centrifuged for 20 min at 10,000 x g. The pellet was dissolved in water, filter-sterilized, and extensively dialysed against 20 mM Tris pH 7.9 at 4°C. The supernatant from the previous centrifugation was concentrated by increasing the NH₄SO₄ concentration to 65% saturation (45.8 g 100 ml⁻¹ supernatant) and repeating the pelleting and dialysis. After dialysis, ion exchange chromatography using DEAE cellulose (Whatman DE23) was performed and fractions were eluted using step-gradients of 0.1, 0.15, 0.2, and 0.35 M NaCl in 20 mM Tris pH 7.9. Fractions containing proteolytic enzymes or hemolytic activity against trout red blood cells (T-lysin) were collected and dialysed into a 1 mM potassium phosphate buffer, pH 7.0. These samples were chromatographed on a hydroxylapatite column (HAP, Bio-Rad Laboratories) and step-eluted at 0.01, 0.1, and 0.5 M phosphate. The T-lysin was further purified on Sepharose 6B (Pharmacia) if necessary to remove contaminating proteolytic activity as described by Titball & Munn (1981).

Protease assays. Proteolytic activity was qualitatively assayed using a 10% gelatin overlay on 1% skim milk agar in Petri dishes. Seven µl of sample was injected through both layers with a micropipettor. Plates were incubated 3 h at 18°C before reading. Preparations with both caseinolytic and gelatinolytic activity cleared a zone in the skim milk and liquified the gelatin. Preparations with only gelatinolytic activity liquifed the gelatin and left the skim milk turbid.

Quantitative protease assays were conducted using a modification of the Lowry method of protein quantification adopted for detection of digested proteins (McDonald & Chen 1965). The assays utilized gelatin as the substrate at a final concentration of 0.2% in phosphate-buffered saline (PBS, 0.85% NaCl, 10 mM PO₄, pH 7.0). Twenty µl of enzyme were added to 400 µl of the gelatin solution and incubated for 40 min at room temperature before addition of 750 µl of 30% trichloroacetic acid (TCA). After a 15 min incubation on ice, precipitable material was pelleted in a microcentrifuge and 1 ml of the supernatant transferred to another tube. This was adjusted to neutral pH and subjected to a Lowry assay. Reactions were allowed to develop for 1 h. Optical density (OD) of each sample was determined at 525 nm on a Spectronic 20 spectrophotometer (Bausch and Lomb). One unit of enzyme was defined by an increase of 0.010 D in this assay.

Protease inhibition. Differential inhibition of the proteases was demonstrated by 2 methods. The first was to add a 0.1 M suspension of phenyl methyl sulfonyl fluoride (PMSF Sigma) in 50% isopropanol to equal volumes of the protease preparations. The other was inhibition with EDTA at a final concentration of 50 mM EDTA, pH 8.0. These techniques selectively inhibit 2 different classes of proteases, serine proteases and metalloproteases, respectively (Powers & Harper 1986a, b). In both cases, enzyme was incubated with inhibitor for 5 min before the quantitative protease assay. Negative controls were made by adding enzyme and inhibitor to the reactions after addition of TCA.

Gel electrophoresis. Purity of the various preparations was determined by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schleif & Wensink (1981). Gels were silver stained using the Bio-Rad Laboratories silver stain kit (Cat. #161-0443, Bio-Rad Laboratories).

Analysis of proteases by substrate gel electrophoresis followed the protocol described by Heussen & Dowdle (1980) with modifications. Gelatin or iso-casein (Difco) was added to 12% resolving gels at 0.01% from 10% stocks made in water (G-PAGE or C-PAGE respectively). No substrate was added to the stacking gels. Samples were mixed with equal volumes of tracking dye (5% SDS, 2% sucrose, 8 µg ml⁻¹ phenol red) and were not boiled before electrophoresis. Electrophoresis
was conducted on a Bio-Rad mini-Protean II apparatus according to the manufacturer’s instructions. After electrophoresis, gels were incubated in 2.5% TritonX-100 (Sigma) in water for 30 min to remove SDS and then in 0.1M glycine-HCl pH 8.3 for 2 h. Gels were stained with coomassie blue as described by Schleif & Wensink (1981). All electrophoresis and incubations were at room temperature. The effect of the protease inhibitors PMSF (10mM) and EDTA (25mM) was determined by adding the inhibitors to each incubation step in G-PAGE.

In vivo sample analysis. Lesions were induced in coho salmon with *Aeromonas salmonicida* by injecting $10^3$ colony forming units in 10μl of PBS into the body musculature adjacent to the dorsal fin. The tissue surrounding the injection site was removed aseptically from 2 fish on the 4th day post-injection, and pooled. A liquid fraction from this tissue was harvested by centrifugation (12,000 × g) and prepared for G-PAGE analysis. Negative controls were prepared with identical methods from PBS-injected fish.

Preparation of red blood cells (RBC). Four different species of salmonids were used in this study: chinook salmon with *Aeromonas salmonicida* and cutthroat *Salmo clarki* and rainbow trout *Salmo gairdneri*. Blood was harvested from fish anesthetized with benzocaine by inserting a heparin-treated hypodermic needle into the caudal vein behind the anal fin. Blood was removed and kept in heparin (Sigma grade 1, 150 IU 10ml⁻¹ whole blood) for 20 min on ice then centrifuged at 800 × g for 10 min and the plasma discarded. The cells were washed once in PBS and then resuspended in 2 volumes of PBS. The suspension was left on ice for 4 to 16 h for separation of cell types; the leukocyte layer was then removed with a transfer pipet.

Preparation of blood cell nuclei. Red blood cell outer membranes were lysed non-enzymatically in one of 3 ways. First, using a modification of the method described by Bayne et al. (1986), RBC were treated with 0.1% Nonidet P 40 (NP40, Sigma) at a ratio of 50 μl packed cells to 1 ml of the NP40 solution. Cells were incubated for 5 min and then diluted 1/10 in sterile PBS. The second lysis technique osmotically lysed the cells by lowering the salt concentration on a blood cell suspension. Cells were exposed for 5 min to dilutions of PBS in deionized water, centrifuged, and resuspended in PBS. The suspension was left on ice for 4 to 16 h for separation of cell types; the leukocyte layer was then removed with a transfer pipet.

Preparation of blood cell nuclei. Red blood cell outer membranes were lysed non-enzymatically in one of 3 ways. First, using a modification of the method described by Bayne et al. (1986), RBC were treated with 0.1% Nonidet P 40 (NP40, Sigma) at a ratio of 50 μl packed cells to 1 ml of the NP40 solution. Cells were incubated for 5 min and then diluted 1/10 in sterile PBS. The second lysis technique osmotically lysed the cells by lowering the salt concentration on a blood cell suspension. Cells were exposed for 5 min to dilutions of PBS in deionized water, centrifuged, and resuspended in PBS. The suspension was left on ice for 4 to 16 h for separation of cell types; the leukocyte layer was then removed with a transfer pipet.

Hemolysin assays. One hundred μl of ca $2.5 \times 10^6$ RBC/ml PBS were placed in each well of a PBS-rinsed Microtiter plate and 10μl of each column fraction were added. Wells were examined for evidence of lysis with an inverted microscope at 250× over a 90 min period. Units of T-lysin present in culture supernatants were determined by log2 dilutions of supernatant in PBS. An equal volume (50μl) of RBC was added to each well and the plate was incubated at 18°C. Wells were examined for lysis for 20 min and the titer was reported as the reciprocal of the highest initial dilution that had 90% lysed cells.

Detailed examination and photography at 1000× were accomplished with wet mounts of cells and nuclei preparations. Nuclear lysis was observed microscopically by adding 10μl P1 protease (2 units μl⁻¹ PBS) or bovine chymotrypsin (Sigma, 1 mg ml⁻¹ PBS) to the edge of a wet mount containing nuclei preparations.

RESULTS

Separation of protease and T-lysin

Fig. 1 shows a schematic representation of the protocol used to isolate proteases and T-lysin from *Aeromonas salmonicida* culture supernatants. Two

![Fig. 1. Flow diagram of protocol used to isolate secreted virulence factors from *Aeromonas salmonicida* culture supernatants](image)

NH₄SO₄ concentrations, 0 to 45% and 45 to 65%, resulted in enrichment of the proteases and T-lysin, respectively, although all activities were found in both concentrations. During separations, the P1 protease was eluted in 2 major steps from the DEAE column — 0.1 and 0.15 M NaCl. Fractions eluting at 0.15 M NaCl were the source of the P1 activity for further purification. P1 activity also eluted with the P2 activity at 0.35 M NaCl, but this contamination was removed on
the HAP column. P1 activity eluted from the HAP column at 0.1 M PO4 and P2 activity eluted at 0.01 M PO4. Hemolysin eluted from the DEAE column at 0.35 M NaCl and subsequently from the HAP column at 0.1 M PO4. Any P1 protease contamination was removed using Sepharose 6B (Titball & Munn 1981). This separation scheme resulted in P1 purified to near homogeneity as determined by silver-stained SDS-PAGE gels, and P2 free of contaminating P1 and T-lysin (Fig. 2). The T-lysin preparations resulted in 3 bands on SDS-PAGE gels (Fig. 3) and were free of detectable contaminating proteolytic activity. No difference was observed in the column separations using A. salmonicida strain SS70.

Protease substrate specificity and sensitivity to inhibitors

The 2-layer substrate plates differentiated substrate specificities and demonstrated that purified preparations of P1 were both gelatinolytic and caseinolytic, while P2 was only gelatinolytic. Before separation on HAP, the protease assays of the peaks eluting at 0.15 M and 0.35 M NaCl from the DEAE columns were both caseinolytic and gelatinolytic in qualitative assays. However, distinctions were evident in the quantitative assays in the presence of inhibitor. The activity present in the 0.15 M peak was sensitive to PMSF while activity in the 0.35 M peak contained a PMSF-resistant component (Fig. 4). After separation on HAP, protease sensitivities were conducted using both PMSF and EDTA as inhibitors. Isolated P1 was shown to be completely sensitive to PMSF while P2 had limited sensitivity (Fig. 5). The profile was reversed with EDTA; P2 was sensitive while P1 was insensitive.

Substrate gel electrophoresis

Samples of culture supernatant and the P1 and P2 proteases were subjected to substrate gel electrophoresis (Fig. 6). Supernatants were shown to have...
more than one gelatinolytic component and a single caseinolytic component. Molecular weight standards are included for reference but are not indicative of actual protein molecular weight because of the incomplete denaturing electrophoretic conditions. P1 was shown, in these gels, to be a single factor while P2 preparations consisted principally of a lower molecular weight protein, with 2 higher molecular weight components having low activity in these conditions. Sensitivity to PMSF and EDTA was determined for the purified preparations. Gelatin-PAGE demonstrated that P1 was sensitive to PMSF and insensitive to EDTA, while P2 proteases were insensitive to PMSF and sensitive to EDTA (not shown).

The smearing visible at the top of our gels was also reported by Huessen & Dowdle (1980). In their work, this was eliminated by electrophoresis at 4°C. We could not remove the smearing with this treatment. The source of the smearing was shown to be digestion of substrate by the P1 enzyme during electrophoresis (not shown).

Electrophoretic analysis demonstrated that multiple proteolytic components were also present in samples taken from lesions of fish infected with Aeromonas salmonicida, but the only band that was present in G-PAGE of both culture supernatants and lesions was the P1 protease band (Fig. 7). No lower molecular weight P2 protease band was seen in G-PAGE of lesions. All of the proteases detected in material from lesions were PMSF-sensitive and EDTA-resistant.

Hemolysin and P1 protease interaction

After separation of the proteases and T-lysin, we investigated the interaction of T-lysin and P1 protease in the complete lysis of trout RBC to define the role of each activity in the lytic process. This was facilitated by differential lysis of the RBC outer membrane using non-enzymatic means. The outer membrane of trout RBC was lysed with no apparent effect on the nuclear membrane when subjected to NP40, osmotic, or T-lysin mediated lysis (Fig. 8). Blood cells from all species tested responded similarly to NP40 lysis. Osmotic lysis...
of rainbow trout RBC outer membranes required 30 to 40% PBS in water (0.25 or 0.34% NaCl). Cutthroat trout and coho salmon RBC had similar osmotic sensitivities, but the chinook salmon RBC completely lysed at these PBS concentrations. Chinook salmon RBC required 50% PBS (0.43% NaCl) for selective outer membrane lysis. In each species tested, RBC with the outer membrane lysed, and subsequently treated with P1 protease, lost their nuclear integrity. In such cells, contrast differences between the nucleus and empty cytoplasm disappeared. Similar results were obtained with RBC having spontaneously lysed outer membranes (not shown). These results indicated that T-lysin was not necessary for nuclear lysis. Protease-dependent nuclear lysis of NP40- or saline-treated RBC was visualized using microscopy. Lysis was indicated by nuclear expansion to fill the empty, but intact cytoplasmic membrane. The nuclei were apparently under positive internal pressure.

As reported elsewhere (Titball & Munn 1983), complete lysis of RBC did not specifically require the P1 protease of *Aeromonas salmonicida*. Bovine chymo-
trypsin also facilitated complete lysis of the nuclei. PMSF inhibited the lytic action of all nuclear lysis by P1 or chymotrypsin. PMSF had no effect on the lysis of outer membranes in the presence of P1, indicating that P1 did not contribute to outer membrane lysis. P2 preparations did not lyse RBC nuclei.

Hemolytic activity in infected tissues was compared to activity in culture supernatants. While T-lysin activity in supernatants ranged from 128 to 1024 units, no hemolytic activity was observed in cell-free exudates from lesions.

**DISCUSSION**

There are several reports in the literature describing individual purifications and analyses of potential virulence factors from *Aeromonas salmonicida*. In this work a protocol for the separation of 3 previously identified factors from a single culture supernatant was developed. Using the described methods, 2 proteases, P1 and P2, and the T-lysin were partially purified and separated from one another for analysis. The P1 protease was purified to near homogeneity and contaminating activities were removed from both the P2 and T-lysin preparations.

The presence of P1 and P2 proteases in culture supernatants of *Aeromonas salmonicida* was demonstrated by different elution profiles from hydroxylationite, substrate specificity, and sensitivity to inhibitors. These data support the results of Sheeran & Smith (1981) who documented 2 proteases in culture supernatants. However, by using C-PAGE and C-PAGE, we have also demonstrated that culture supernatants of *A. salmonicida* contained more than 2 gelatinases. All except P1 protease are EDTA-sensitive and only P1 can digest both casein and gelatin. Investigators not detecting these different factors were apparently limited by the sensitivity of their assays or by substrates used in detection.

Separation of these factors also facilitated analysis of the in vitro relationship between P1 protease and T-lysin in the complete lysis of trout RBC. Selective lysis of outer membranes using NP40 and decreased salinity was also used in these experiments. We determined that T-lysin lyses the outer membranes of trout RBC and P1 protease lyses the nuclear membrane. The functions are independent of one another. This relationship was first investigated by Titball & Munn (1983) and our results agree with the sequence of events they proposed. These results suggested that if enzymatic lysis of RBC is a component of pathology it is solely attributable to the T-lysin, because lysis of the outer membrane is sufficient to destroy the function of the cell.

Substrate gel electrophoresis and T-lysin activity assays were also used to investigate activities associated with these factors in coho salmon experimentally infected with *Aeromonas salmonicida*. Although fish infected experimentally had more than one gelatinolytic component, P1 protease was the only protease detected in lesions which was produced by the bacterium in culture supernatants. No low molecular weight P2 protease was detected in lesions. Gelatin-PAGE demonstrated that proteases found in lesions were PMSF-sensitive and EDTA-resistant. Non-P1 proteases in culture supernatants were EDTA-sensitive and PMSF-resistant. The non-P1 proteases detected in lesions were possibly host proteases activated by P1 protease or otherwise liberated during the course of infection, but their source was not determined. Proteases other than P1 produced by *A. salmonicida* may have been present in lesions, but they were not within our limits of detection. This information suggested that P1 protease is the major proteolytic factor produced by *A. salmonicida* that is responsible for tissue destruction and lesion formation. Other investigators using alternate methods have come to the same conclusion (Sakai 1985, Fyfe et al. 1986). There is evidence, however, that *A. salmonicida* isolates lacking detectable caseinase activity (P1) can induce pathology and mortality in fish (Tajima et al. 1987). These infections do not involve lesion formation. Future research should address the potential role of non-P1 proteases in infection.

Attempts to detect hemolytic activity in cell-free exudate from lesions were unsuccessful. This suggested that released T-lysin was either not present, or was incapable of RBC lysis in vivo. A recent report by Fyfe et al. (1988) indirectly associated T-lysin with specific tissue damage after injection with P1 protease into Atlantic salmon. They did not, however, associate the T-lysin with lysis of RBC. Their work supports our conclusion that the major target tissue for T-lysin in vivo is not the erythrocyte.

**Acknowledgements** Principal funding for this research was received from USDA Science and Education grant no. 85-CRSR-2-2578. We thank M. Beer for technical assistance, S. L. Kaattari for critical review of the manuscript, and T. Curry and C. Pelroy for typing the manuscript. Oregon Agriculture Experiment Station Technical Paper No. 6360.

**LITERATURE CITED**


Ellis, A. E., Hastings, T. S., Munro, A. L. S. (1981). The role of...
Aeromonas salmonicida extracellular products in the pathology of furunculosis. J Fish Dis 4: 41–51
Michel, C. (1985). Failure of anti-furunculosis vaccination of rainbow trout (Salmo gairdnerii Richardson) using extra-cellular products of *Aeromonas salmonicida* as an immunogen. Fish Pathol. 20: 445–451

Responsible Subject Editor: Dr T Evelyn; accepted for printing on September 6, 1988