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Identification of Major Common Extracellular Proteins Secreted by *Aeromonas salmonicida* Strains Isolated from Diseased Fish

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Ten different strains of *Aeromonas salmonicida* that were isolated from diseased fish were grown under identical conditions (24 h at 25°C) in 3% (wt/vol) tryptone soya broth medium supplemented with vitamins and inorganic ions. In each case the extracellular proteins that were formed were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and it was found that there were two significant common components, one with a molecular weight of 70,000 and the other with a weight of 56,000. Application of enzyme purification techniques to the supernatant fraction proteins of a culture of one of the strains resulted in the isolation of a 70-kilodalton (kDa) component, which was found to be a serine protease, and a 56-kDa component, which was hemolytic to trout erythrocytes. Rocket immunoelectrophoresis with rabbit antibodies to the isolated protease and hemolysin showed the same antigenic components in the supernatant fractions of all the cultures. These activities were assayed, and protease activity was found to vary by a factor of three, from 59 to 195 U/ml, while the range of hemolytic activity was over a narrow band, from 28 to 43 U/ml. There was an inconsistency between the immunoelectrophoretic and direct assay data in only one case. This indicated the presence of additional hemolytic activity, in addition to the 56-kDa component. The detection of large amounts of the same protease and hemolysin, two potent degradative activities, in a random series of strains of *A. salmonicida* suggests that they may be obligatory virulence factors in the development of furunculosis.

Aeromonas salmonicida, the causative agent of furunculosis in salmonid fish, is a gram-negative, exoprotein-secreting organism which Mackie et al. (9) showed to possess a very active proteolytic action. Subsequent in vitro studies revealed other identifiable components of the extracellular proteins that have been claimed as virulence factors (13). These include, in addition to various proteases (4, 12, 15–17; A. Finley, PhD. thesis, University of Nottingham, United Kingdom, 1983), hemolysins (6, 18, 19), lipid-metabolizing enzymes (3; T. S. Hastings, A. E. Ellis, and A. L. S. Munro, Soc. Gen. Microbiol. Q. 8:254, 1981), and a leukocytolytic factor (5, 7).

The inability to demonstrate any one of these activities among the extracellular proteins produced in vitro by a particular strain of *A. salmonicida* does not exclude the possibility of its production by other strains. Thus, the claims of workers with one strain which cannot be reproduced in another are not necessarily at conflict but simply reflect the individual patterns of activity of the different strains under investigation.

With this in mind, it was considered to be of interest to examine a number of strains of *A. salmonicida* isolated from diseased fish and to compare the extracellular protein patterns produced under identical conditions with a view to identifying common components which may be potential virulence factors in the development of furunculosis. The characterization of such components will be usefully applied to simplifying, without loss of pathogenicity, a crude extracellular protein preparation that is known to be lethal to salmonid fish.

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MATERIALS AND METHODS

Organisms. *A. salmonicida* Unilever 2862 was provided by Unilever Research Laboratories, Colworth House, Sharnbrook, Bedford, United Kingdom. All other strains were donated by the Ministry of Agriculture, Food and Fisheries, Fish Diseases Laboratory, Weymouth, Dorset, United Kingdom.

Growth conditions. Cultures were grown at 25°C with shaking in a medium containing tryptone soya broth (3% [wt/vol]; Oxoid Ltd., London, England), trace metal ions, vitamins, and 0.8 mM $MgSO_4$, as described by Abbas-Ali and Coleman (1). Batches (50 ml) of the medium contained in 250-ml conical flasks were loop inoculated and incubated in a cooled incubator (Orbital; Gallenkamp & Co. Ltd., Loughborough, United Kingdom).

Protease activity. Protease activity was assayed by the method essentially used by Rappaport et al. (14). One unit of protease activity was defined as the amount of enzyme that hydrolyzed 1 mg of soluble casein, under the standard conditions of the assay procedure, that is, in 30 min at 37°C and at an optimum pH of 9.0, to the stage at which it gave no precipitate with trichloroacetic acid reagent.

Hemolysin activity. Hemolytic activity was determined by the method described by Bernheimer and Schwartz (2), which was modified by the use of trout erythrocytes, which required an increased isotonicity to the equivalent of 0.98% (wt/vol) NaCl, and the addition of 20 μg of DNase (type I; Sigma Chemical Co. Ltd.) to each assay.

SDS-polyacrylamide gel electrophoresis. The method described by Laemmli (8) was used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and 1-ml samples of 24-h stationary-phase culture supernatant fractions were prepared for electrophoresis as described by Mackman and

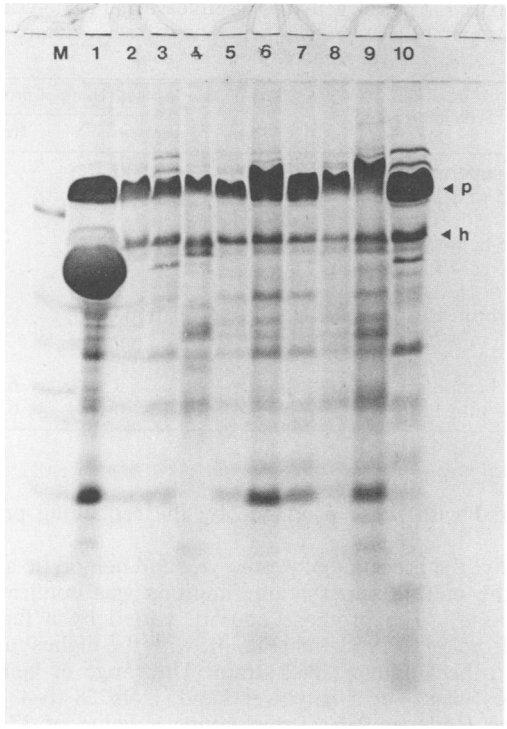

FIG. 1. SDS-polyacrylamide gel electrophoresis patterns of the extracellular proteins of a number of different strains of *A. salmonicida*. Lanes 1 to 9, Extracellular proteins present in the culture supernatant fractions from a number of strains of the organism obtained from the Ministry of Agriculture, Food and Fisheries, Fish Diseases Laboratory; lane 10, the pattern produced by the Unilever 2862 strain. Molecular weight marker proteins (MW-SDS-70L; Sigma) are separated in lane M.

Holland (10). The resulting preparations (50 μg of each) were run in parallel with a prepared mixture of molecular weight marker proteins (MW-SDS-70L; Sigma). Individual lanes of the SDS-polyacrylamide gels, in which the protein bands were visualized by staining with Coomassie brilliant blue R (Sigma), were scanned with an electrophoresis–thin-layer chromatographic densitometer (Quick Scan R & D; Helena Laboratories, Beaumont, Tex.) at a wavelength of 600 nm, a slit width of 0.2 mm, and a gain setting of 600, operating at 300 V.

Preparation of protease. Preparation of protease was carried out as described by Finley (Ph.D. thesis). Thus, the material obtained after desalting with Sephadex G25-150 (Sigma) and after lyophilizing 20 ml of the *A. salmonicida* culture supernatant fraction was dissolved in 2.5 ml of 20 mM phosphate buffer (pH 7.0). The preparation was loaded onto a DEAE–Sephadex A-50 (Sigma) column (1.6 by 24 cm) which was equilibrated with three column volumes of the same 20 mM phosphate buffer (pH 7.0). After the sample was loaded, the column was washed with six column volumes of the same phosphate buffer. The column was then eluted with a linear gradient of increasing molarity of phosphate buffer (pH 7.0). Protease was eluted as a single peak over a phosphate buffer concentration range of 110 to 170 mM.

Preparation of hemolysin. Ammonium sulfate was added to the supernatant fraction from a 24-h culture of *A. salmonicida* Unilever 2862 to 70% of saturation (470 g/liter). The preparation was allowed to stand overnight at 4°C, after which the precipitated protein was collected by centrifuging at 10,000 × *g* for 15 min. The protein from 4 liters of the culture supernatant fraction was dissolved in 20 ml of 25 mM imidazole hydrochloride buffer (pH 7.4). The solution was dialyzed twice overnight at 4°C against 3 liters of the same buffer. The dialyzed preparation was then loaded onto a chromatofocusing column (1 by 50 cm; Polybuffer Exchange; PBE 94; Pharmacia Ltd.) which was equilibrated with 25 mM imidazole hydrochloride buffer (pH 7.4). The column was eluted with Polybuffer 74 (Pharmacia Ltd.) at pH 4, and hemolytic activity was detected in the fraction which emerged at pH 6.4. SDS-polyacrylamide gel electrophoresis showed the presence of two components, one of 20 kilodaltons (kDa) and the other of 56 kDa, which were separated by gel filtration on a Sephadex G50-150 (1 by 30 cm; Sigma) column. The 56-kDa protein was shown to be the hemolysin. Sufficient detail is given above to prepare the hemolysin; however, a more detailed description of the preparation and properties of the isolated hemolysin will be published elsewhere.

Immunological procedures. Rocket immunoelectrophoresis and immunodiffusion experiments were carried out by the standard techniques described by Mayer and Walker (11).

RESULTS

Ten different strains of *A. salmonicida*, all of which were isolated from diseased fish and one of which (Unilever 2862 strain) was the subject of previous studies, were grown under identical conditions for 24 h at 25°C in the presence of air in a 3% (wt/vol) tryptone soya broth medium supplemented with vitamins and inorganic ions. Samples of the culture supernatant fractions were taken, and the extracellular proteins were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1). It can be seen that while different exoprotein patterns were produced by the various strains examined, they all had two major common components, designated p and h. The very intense p band ran in a position corresponding to a molecular weight higher than that of the 66,000-molecular-weight marker protein (66K marker protein), while the less intense h band ran between the 45K and 66K marker proteins.

More precise values for the molecular weights of the p and h components were obtained by measuring their R_f values from Fig. 1, together with those of the marker proteins in lane M. The molecular weights of the marker proteins were plotted on a logarithmic scale against their relative mobilities (R_fs) when a linear relationship was obtained. Marking the relative mobilities of the p and h bands on this graph gave corresponding molecular weights of 70,000 and 56,000, respectively.

Detailed studies have been carried out previously on the Unilever 2862 strain and its extracellular proteins (Fig. 1, lane 10). During the course of this work, the 70-kDa component was isolated (Finley, Ph.D. thesis) and identified as a serine protease. More recent studies on the same organism resulted in the isolation of the 56-kDa component, which was found to be hemolytic to trout erythrocytes but was without effect on rabbit, sheep, horse, human, and hen erythrocytes. The SDS-polyacrylamide gel electrophoretic mobilities of isolated protease and a mixture of hemolysin-protease relative to the crude extracellular protein preparation from which they were derived are shown in the densitometer tracings in Fig. 2, together with a series of molecular weight marker proteins. The specific activities of both hemolysin and protease in the various preparations shown in Fig. 2

were determined. The results in Table 1 confirm the distribution of these activities, and the effect of phenylmethylsulfonyl fluoride (PMSF) demonstrates the presence of serine protease.

The data presented so far show that each bacterial strain that was examined has two major components in common which have the same molecular weight as a serine protease and a hemolysin isolated from strain Unilever 2862. To demonstrate that these same proteins occurred in all the other strains, antibodies were raised in a rabbit to a mixture of the two, that is, the preparation used for Fig. 2b. The resulting antibody preparation was used in an immunological study of all the strains. In an Ouchterlony double immunodiffusion experiment, samples of 24-h supernatant fractions from all the cultures were compared by using the antibody preparation in the center well. Precipitin lines of identity were obtained with all the supernatant fractions and a mixture of protease-hemolysin. This observation was supported by rocket immunoelectrophoresis (Fig. 3). The same pattern was obtained in every case with a low-migrating protease and a high-migrating hemolysin. However, the hemolysin rocket in lane 4 (Fig. 3) was at a very low level compared with those produced by the remaining preparations.

TABLE 1. Specific activities of protease and hemolysin in various extracellular protein preparations from a culture of *A. salmonicida* Unilever 2862

Protein preparation	Sp act (U/mg of protein) of:	
	Protease	Hemolysin
Culture supernatant fraction (24 h)	1,530	500
With 5 mM PMSF	0	500
Protease-hemolysin	1,340	940
With 5 mM PMSF	0	940
Isolated protease	1,560	0
With 5 mM PMSF	0	0
Isolated hemolysin	0	6,550
With 5 mM PMSF	0	6,550

Finally, the presence of proteolytic and hemolytic activity in all the culture supernatant fractions was confirmed by direct assay. The protease activity varied by a factor of three, from 59 to 195 U/ml (Fig. 4), with the highest activity being in the Unilever 2862 strain. The range of hemolytic activities was over a narrower band, from 28 to 43 U/ml, with the Unilever 2862 strain giving a value of 37 U/ml. These results were consistent with the heights of the rockets shown in Fig. 3 and the intensities of the corresponding bands in Fig. 1 in all but one case. The anomaly was related to the hemolytic activity in strain 4 (Fig. 4), of 28 U/ml, which was close to the value of 31 U/ml in strain 2 (Fig. 4). Hemolysin rockets similar in height might have been expected for the two strains (Fig. 3); however, this correspondence was observed only after a fivefold concentration of strain 4. This suggests that in strain 4 (Fig. 4) there was additional activity that was immunologically different from that of the 56-kDa hemolysin.

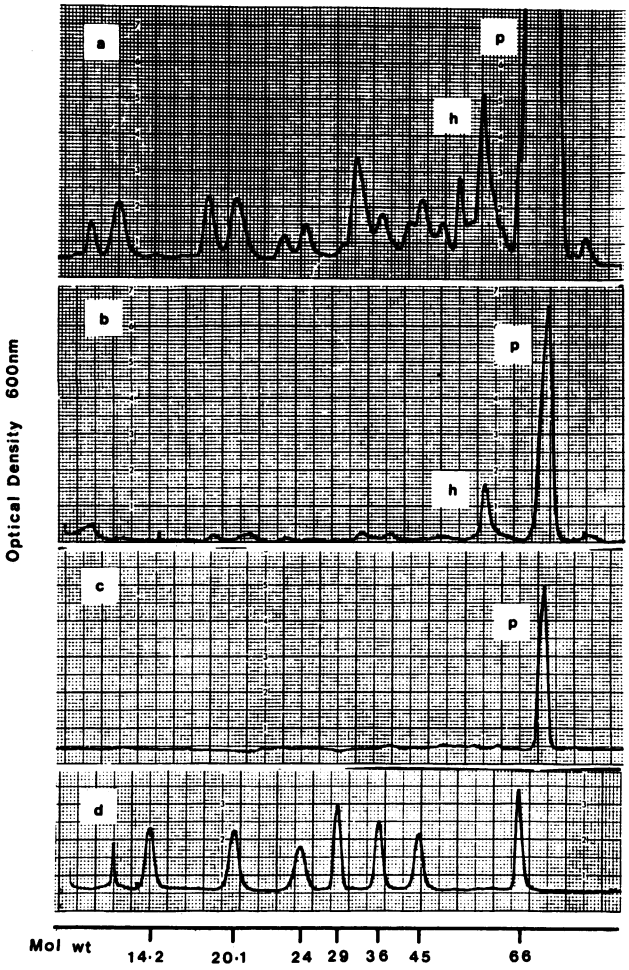

FIG. 2. Densitometer tracings of SDS-polyacrylamide gel electrophoresis patterns of the extracellular proteins from a 24-h culture of *A. salmonicida* Unilever 2862 (a), protease p plus hemolysin h (b), and isolated protease p from the same organism (c), together with molecular weight marker proteins (MW-SDS-70L; Sigma) (d), the molecular weights (Mol wt) of which are indicated in thousands.

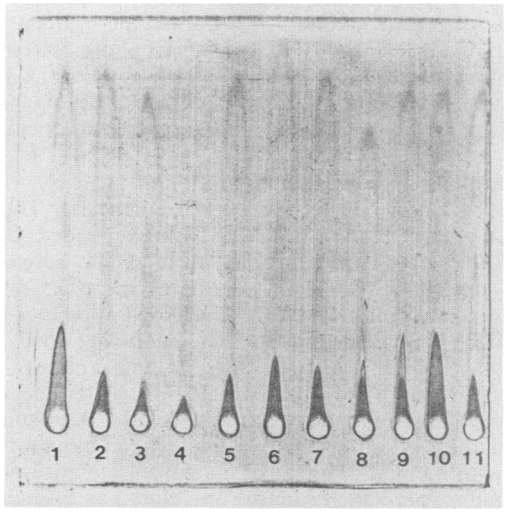

FIG. 3. Rocket immunoelectrophoresis of 24-h culture supernatant fractions from 10 strains of *A. salmonicida* (lanes are as described in the legend to Fig. 1) and a mixture of isolated protease and hemolysin (lane 11). A rabbit antibody preparation raised against a mixture of extracellular protease-hemolysin isolated from a culture of the Unilever 2862 strain of the organism was used.

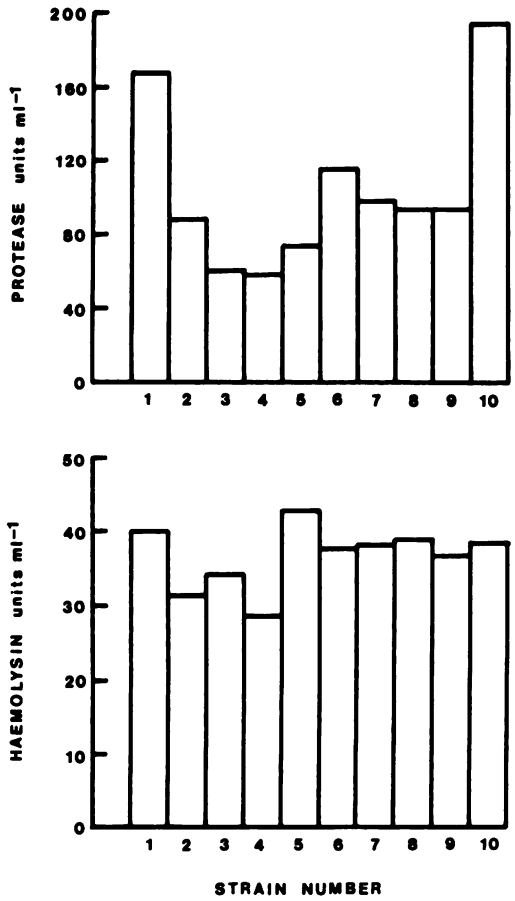

FIG. 4. Histograms showing protease and hemolysin levels in the supernatant fractions of 24-h cultures of a number of *A. salmonicida* strains. Strains are numbered in accordance with the lanes in Fig. 1 (see Fig. 1 legend for descriptions).

DISCUSSION

It is of considerable interest to compare the results of this study with those available in the literature.

Various investigators have studied the extracellular proteolytic activity of *A. salmonicida*, and claims have been made of PMSF-sensitive serine proteases with molecular weights of 11,000 (16), 70,000 (Finley, Ph.D. thesis), 71,000 (17), and 87,500 (12). Sheeran and Smith (15) identified two proteolytic activities, one a serine protease and the other an EDTA-inhibited metalloprotein, but they did not determine molecular weights. On the other hand, Dahle (4) detected a protease with a molecular weight of 43,600 without attempting to classify it.

The absence of 11- and 87.5-kDa bands in Fig. 1 suggests that the proteases described by Shieh and MacLean (16) and Mellergaard (12), respectively, do not occur in any of the strains examined. By contrast, there is a component in lanes 5, 6, 7, and 9 of Fig. 1 which could correspond to the 43.6-kDa protease described by Dahle (4). The most obvious extracellular component of all the strains, however, was the PMSF-sensitive protease activity at 70 kDa which has a pH optimum of 9.0 and is active against casein and collagen (Finley, Ph.D. thesis). It is clear that this is the same enzyme as that described by Tajima et al. (17) with a reported molecular weight of 71,000.

Karlsson (6) demonstrated the formation of a hemolysin among the extracellular products of *A. salmonicida* which has been studied in considerable detail by Titball and Munn (18). Thus, two hemolytic activities were identified: one that lysed trout erythrocytes, T-lysin, and another that was specific for horse erythrocytes, H-lysin. The latter was found to be synthesized as an inactive 42.3-kDa precursor which, on proteolytic cleavage, yielded an active 29.5-kDa hemolysin. This activity was subsequently purified and shown to be lethal to cultured trout leukocytes and gonad cells, in addition to lysing horse erythrocytes; a molecular weight of 25,900 has been assigned to the purified material (19).

In relation to the hemolytic activity of culture supernatant fractions, although produced by all the strains examined, a 56-kDa hemolysin has not been reported previously, but the possibility that it could correspond to the hemolysin described by Karlsson (6) is not excluded. The hemolysins observed by Titball and Munn (18) may be less widely distributed because there is little evidence of 29.5-kDa material in any of the lanes in Fig. 1. Although it has not been identified as H-lysin in this study, however, 25.9-kDa polypeptide may be present in some of the preparations shown in Fig. 1.

A leukocytolytic factor was demonstrated by Klontz et al. (7) and later identified by Fuller et al. (5) as a glycoprotein with a molecular weight in the range of 100,000 to 300,000. Such an activity may be present because material in this molecular weight range was observed in half of the preparations shown in Fig. 1.

It has been claimed that phospholipase is present among the extracellular products of *A. salmonicida* (Hastings et al., Soc. Gen. Microbiol. Q., 1981); however, the only specific lipid-metabolizing enzyme identified to date is a glycerophospholipid-cholesterol acyltransferase, which has a molecular weight of 23,600 (3).

No systematic experimental search was made for the presence of lipid-metabolizing enzymes, including glycerophospholipid-cholesterol acyltransferase with a molecular weight of 23,600 (3), in this study, although there is an indication of a minor 24-kDa component in most, but not all, of the preparations shown in Fig. 1.

While the major common components revealed in this study were the 70-kDa protease and the 56-kDa hemolysin, other activities that are not significant in every strain of the organism may, nevertheless, have roles, albeit of lesser importance, in determining the virulence of *A. salmonicida* during the development of furunculosis.

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