Characteristics and Distribution of Extracellular Proteases from *Aeromonas hydrophila*

By K.-Y. LEUNG† AND R. M. W. STEVENSON*

Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

(Received 19 June 1987; revised 1 September 1987)

The major extracellular proteases of *Aeromonas hydrophila* NRC 505 and ATCC 7966 are a thermostable metallo-protease (TSMP) and a thermolabile serine-protease (TLSP), respectively. The purified enzymes differed in sensitivity to heating at 56 °C for 30 min, in response to the inhibitors EDTA and phenylmethylsulphonyl fluoride (PMSF), and were antigenically distinct. When crude extracellular products (ECP) of 47 strains of *A. hydrophila* were screened, 27 strains produced both TSMP and TLSP. TSMP was the only extracellular protease produced by 19 strains, whereas only *A. hydrophila* ATCC 7966 produced TLSP as its sole protease. This distribution of protease enzymes among strains explains conflicting reports from studies in which single strains of *A. hydrophila* were examined. The TLSP of *A. hydrophila* was similar to the protease of *A. salmonicida*. Either or both TSMP and TLSP were produced by some strains of *A. sobria* and *A. caviae*.

INTRODUCTION

Aeromonas hydrophila produces a variety of extracellular products (ECP), including toxins, haemolysins and proteases (Ljungh & Wadstrom, 1982), which may contribute to the virulence of this bacterium for fish and other hosts. The effects of proteases can include direct tissue damage, enhanced invasiveness or provision of nutrients, some of which have been suggested in the case of another fish pathogen, *Aeromonas salmonicida* (Sakai, 1985*a, b*). Other effects may be indirect, as, for example, by proteolytic activation of the aerolysin toxin precursor of *A. hydrophila* (Howard & Buckley, 1985).

We have been interested in defining the role of proteases in *A. hydrophila* infections of fish. A significant problem in such studies is the disparity between reports of the numbers and types of enzymes produced by *A. hydrophila*. For example, Gross & Coles (1969), Allan & Stevenson (1981) and Kanai & Wakabayashi (1984) identified one protease from isolates of *A. hydrophila*. Dahle (1971) purified two proteases from *A. hydrophila* ATCC 14715, whereas two and, subsequently, three proteases were reported from *A. hydrophila* AH22 by Thune *et al.* (1982) and Amborski *et al.* (1984). Denis & Veillet-Poncet (1984) found several proteases in strain P50. Moreover, Nieto & Ellis (1986) detected four or five zones of proteolytic activity with isoelectric focusing in crude extracts from strain B51. Unfortunately, these investigators worked on different strains of *A. hydrophila*, and in each case only a single strain was completely examined. The use of different media and growth conditions, and a variety of assay and purification procedures also make the literature reports very difficult to interpret and compare.

The objective in this work was to assess the extent of differences in the proteases produced by strains of *A. hydrophila*, by making direct comparisons on many isolates, grown under uniform

† Present address: Department of Microbiology and Immunology, University of Kentucky College of Medicine, Lexington, KY 40536-0084, USA.

Abbreviations: ECP, extracellular products; PMSF, phenylmethylsulphonyl fluoride; TLSP, thermolabile serine-protease; TSMP, thermostable metallo-protease.

cultural conditions. To provide a better basis for comparison of the proteases, a combination of inhibition patterns and thermostability characteristics was used (see Dahle, 1971; Thune *et al.*, 1982; Nieto & Ellis, 1986). In addition, it was recognized that serological comparisons were essential, which required purification of proteases. After initial characterization of purified enzymes, it was established that the serological and functional tests could be done on crude ECP, allowing the examination of enough strains to establish a general picture of protease enzyme distribution in motile aeromonads.

METHODS

Bacterial strains. Strain numbers for the 47 *A. hydrophila* strains and 29 other aeromonads examined are given in Table 1. The majority were fish isolates; others were derived from water, animal or human sources. Isolates were tested using standard biochemical tests, and their identities confirmed according to the results given by Popoff (1984). Strains were stored at 15 °C in tightly sealed slants of tryptic soy agar (TSA, Gibco). For protease production, cultures were grown at 25 °C on brain heart infusion-skim milk agar (Allan & Stevenson, 1981).

Protease assays. The buffer used for assays and all enzyme purification steps was 0.01 M-sodium phosphate, incorporating 0.5 mM-CaCl₂, at pH 7.2. During protease purifications, proteolytic activity was measured using Azocoll substrate (50–100 mesh) obtained from Calbiochem, following the procedure described by Allan & Stevenson (1981). Azocasein (Sigma) was a more convenient substrate for thermostability and inhibitor studies. Buffer (2.3 ml), enzyme sample (0.1 ml) and 0.1 ml Azocasein stock solution (10%, w/v) were added to each tube, and mixed. After 1 h incubation at 37 °C, the reaction was stopped by adding 2.5 ml 10% (w/v) TCA. After an additional 30 min at room temperature, the precipitate was removed by centrifugation at 10000 *g* for 10 min at 4 °C. Equal volumes (1 ml) of the supernate and 1 M-NaOH were mixed and the absorbance read at 450 nm on a Bausch & Lomb Spectronic 70 spectrophotometer. A blank was prepared using an enzyme sample inactivated by boiling for 10 min. Proteolytic activity is expressed as mg Azocasein hydrolysed h⁻¹.

Table 1. *Strains of Aeromonas examined*

Taxon	Source*	Strain designation
<i>A. hydrophila</i>	ATCC	7966 ^t , 9071, 14715
	CDC	3312.66, 3314.66, 3329.66, 4167.66, 5039.67, 1625.70, 1626.70, 1627.70, 1628.70, 1631.70, 3250.71, 3253.71, 5736.71, 232.76
	DMG	518, FORD, G55
	FHRL	1.19, 1.20, 1.23
	NCMB	1134
	NHW	76-388
	NRC	505
	NRS	35, TS-AH
	NZ	70-856, 70-2234, 70-4517, B59, EGG
	UM	Bal, Ba5, Ba6, Sau-1, TF7
	UV	18, 43, 53, 57, 67, 70, 93, 108, N35
<i>A. caviae</i>	CDC	3331.68, 3402.68, 1276.69, 1629.70
	ATCC	15467, 15468 ^t
	DMG	ONT396-4K
	HSC	G39
	UM	Pis-1
<i>A. sobria</i>	CDC	1624.70, 3251.71, 3252.71, 3254.71, 5732.71
	FHRL	1.40
	UV	66, 148, 151, 152, 153, 155, 162, 163, 174
	UM	OU2
<i>A. media</i>	ATCC	33907 ^t
<i>A. salmonicida</i>	OMNR	357, 426, As19

t, Type strain.

* Sources: ATCC, American Type Culture Collection, Rockville, Md., USA; CDC, Centers for Disease Control, Atlanta, Ga., USA; DMG, Department of Microbiology, University of Guelph, Ontario, Canada; FHRL, US National Fish Health Research Laboratory, Kearneysville, W. Va., USA; HSC, Hospital for Sick Children, Toronto, Canada; NRC, National Research Council, Ottawa, Canada; NRS, Pacific Biological Station, Nanaimo, BC, Canada; NZ, Fisheries Research Division, Wellington, New Zealand; UM, Université de Montreal, PQ, Canada; UV, University of Victoria, BC, Canada; NCMB, National Collection of Marine Bacteria, Aberdeen, UK; NHW, Department of National Health and Welfare, Ottawa, Canada; OMNR, Ontario Ministry of Natural Resources, Guelph, Canada.

Inhibitor and heat-treatment effects. The inhibitors used (all obtained from Sigma) and their final concentrations are noted later. From stock solutions, 0.1 ml samples of the inhibitors were added to the assay buffer and enzyme, and the mixture was pre-incubated at 22 °C for 30 min before addition of Azocasein. For heat inactivation, the enzyme preparation was held in a water bath at 56 °C for 30 min, followed by cooling to 4 °C before addition of Azocasein. The total assay volume in each case was 2.5 ml; all assays were done in duplicate.

ECP preparation. Brain heart infusion-skim milk agar (200 ml) was solidified in a 23 × 23 cm pan, then overlaid with a 24 × 24 cm sheet of sterile dialysis membrane with a molecular mass exclusion size of 12000–14000 (Spectrum Medical Industries). The membrane surface was inoculated by spreading with 1 ml of an 18 h bacterial culture. After 36 h at 25 °C, the growth was washed from the dialysis membrane with 20 ml buffer. Bacterial cells were removed by centrifugation for 30 min at 27000 *g* and the supernatant was filtered through a 0.45 µm filter (Gelman Sciences). This filtrate was the ECP preparation used in subsequent work.

Protease purification. A 20 ml volume of ECP was added to 50 ml DEAE-cellulose (Whatman; DE52) in a porcelain funnel and batch-eluted with 20 ml of buffer. Acetone (5 vols), pre-chilled to 20 °C, was added with slow stirring, and the precipitate collected by centrifugation at 12000 *g* for 10 min. The acetone was decanted and the pellet redissolved in 10 ml of buffer. This was applied to a 2.5 × 100 cm column of Sephacryl S-300 (Pharmacia), previously calibrated by using Blue Dextran and Pharmacia molecular mass markers. Eluted fractions were collected and assayed for protein (A_{280}) and proteolytic activity. Pooled fractions with proteolytic activity were concentrated by acetone precipitation. A sample (2 ml) of the redissolved precipitate was applied to a polyacrylamide gel for zone electrophoresis, using a 3% (w/v) spacing gel and a 5% (w/v) resolving gel, and 0.05 M-Tris/0.38 M-glycine buffer (pH 8.3). After electrophoresis, the gel was cut into horizontal strips of 2–3 mm. Each strip was allowed to elute into 5 ml of buffer for 18 h at 4 °C, then protease activity was located by Azocoll assay.

SDS-PAGE. Enzyme samples were denatured and run on SDS-PAGE, according to the procedure of Laemmli (1970). The proteins were silver-stained (Bio-Rad kit) and the molecular masses determined by comparison with sets of high and low molecular mass calibration markers (Pharmacia).

Serological tests. A sample of purified protease, containing 200–300 µg protein in 5 ml, was emulsified in an equal volume of Freund's complete adjuvant (Calbiochem). A New Zealand White rabbit was injected intramuscularly with 1 ml of the preparation. The injection regime was repeated at 1 week intervals for 4 weeks. Collected serum was tested by the precipitin ring reaction. For immunodiffusion assays, a 1% (w/v) agarose solution was made by melting in Bio-Rad immunoelectrophoresis buffer IV, pH 8.6, and 50 ml was cast onto a glass plate (15 × 20 cm). After wells were cut and samples added, the agarose plates were incubated at 37 °C in a humidity chamber for 24 h. In addition to the immunodiffusion assays illustrated, series of wells with the reference antigen flanking each sample were used to determine reactions of identity or partial identity.

RESULTS

Protease purification and characteristics

Approximately 10% of the original proteolytic activity was recovered from the ECP of *A. hydrophila* NRC 505 and ATCC 7966, with a purification of 26-fold for *A. hydrophila* NRC 505 and 43-fold for ATCC 7966. As DEAE-cellulose (anion exchange) chromatography resulted in a loss of 30% of the activity of the NRC 505 enzyme, with no increase in specific activity, the step was not used for purifying the enzyme of *A. hydrophila* ATCC 7966. All of the proteolytic activity, from either strain, was eluted from the Sephacryl S-300 column in association with the second of three protein peaks (Fig. 1). However, elution positions suggested enzymes of different molecular masses. The protease of *A. hydrophila* ATCC 7966 had an estimated molecular mass of 145 kDa (Fig. 1*a*), whereas that of NRC 505 was 54 kDa (Fig. 1*b*). Proteolytic fractions migrated very slowly in a non-denaturing polyacrylamide gel, remaining near the top of the gel. Significant activity was lost in zone electrophoresis (50 to 80%), but there was a compensating increase in specific activities. After this step, both protease preparations appeared as single protein bands in SDS-polyacrylamide gels (Fig. 2).

The SDS-PAGE bands again suggested that the proteases of the two strains differed in molecular mass: 35 kDa for the *A. hydrophila* NRC 505 enzyme, and 68 kDa for that of ATCC 7966 (Fig. 2). As the values from denaturing gels were about half those determined by gel filtration, both proteases may be dimers. Although the different molecular masses suggested different protease enzymes, substrate specificities did not appear to differ. The ECP of *A. hydrophila* NRC 505 and ATCC 7966 both hydrolysed a wide spectrum of proteins, including, in order of preference, casein, gelatin, haemoglobin and elastin.

Fig. 1. Elution of extracellular proteases of *A. hydrophila* from a Sephacryl S-300 column. The column was loaded with samples containing approximately 100 mg protein, which had been collected as an acetone-precipitate from the ECP of (a) *A. hydrophila* ATCC 7966 and (b) *A. hydrophila* NRC 505. Elution positions for molecular mass markers are indicated by arrows: thyroglobulin, 669 kDa; ferritin, 440 kDa; albumin, 67 kDa; ribonuclease A, 13.7 kDa; and Blue Dextran, 2000 kDa. Protease activity ($\blacktriangle$) was measured by the Azocoll assay, read as absorbance at 520 nm. Protein ($\circ$) was read as the absorbance of collected fractions at 280 nm.

Inhibitor and heat-stability studies

Purified protease from *A. hydrophila* NRC 505 was inhibited by the metal-ion chelators EDTA and 1,10-phenanthroline, and by the thiol compounds cysteine and DTT, but not by PMSF, a serine-protease inhibitor (Table 2). Approximately 75% of proteolytic activity remained after heating at 56 °C for 30 min. In contrast, purified protease from *A. hydrophila* ATCC 7966 was inhibited by PMSF but not by metal-ion chelators, and the activity was heat-labile (Table 2). Thus, the proteases of these two strains of *A. hydrophila* differed in activity as well as in size. The responses of unpurified proteolytic activities in the ECP of *A. hydrophila* NRC 505 and ATCC 7966 to inhibitors and heat-inactivation were the same as their respective purified counterparts (Table 2).

Serology

Antiserum raised against the protease from *A. hydrophila* ATCC 7966 produced a precipitin band with ECP from that strain, but not with the ECP produced by NRC 505 (Fig. 3A).

Fig. 2. SDS-PAGE of purified proteases of *A. hydrophila* strains NRC 505 and ATCC 7966. High molecular mass markers are in lane 1; ferritin (18.5 kDa), catalase (60 kDa) and albumin (67 kDa). Low molecular mass markers are in lane 2; phosphorylase B (94 kDa), albumin (67 kDa), carbonic anhydrase (30 kDa) and α -lactalbumin (14.4 kDa). Lane 3, protease purified from *A. hydrophila* ATCC 7966. Lane 4, protease purified from *A. hydrophila* NRC 505.

Similarly, antiserum to the protease from *A. hydrophila* NRC 505 reacted with ECP from its homologous strain, but not with the ECP produced by ATCC 7966 (Fig. 3B). No trace amounts of ATCC 7966 protease were detected in the ECP of *A. hydrophila* NRC 505, or vice versa, by immunodiffusion assay (Fig. 3), nor by rocket immunoelectrophoresis. The latter procedure was also used to determine that *A. hydrophila* ATCC 7966 and NRC 505 each produced only a single type of protease regardless of the stage of growth at which the ECP were collected (12–61 h at 25 °C).

Proteases from A. hydrophila Ba5

The ECP from a highly proteolytic strain, *A. hydrophila* Ba5, had a response pattern to inhibitors and heat-treatment that was a mixture of the characteristics of the ECP produced by *A. hydrophila* NRC 505 and ATCC 7966 (Table 2). In immunodiffusion tests, ECP from *A. hydrophila* Ba5 reacted with both anti-ATCC-7966-protease and anti-NRC-505-protease sera (Fig. 3A, B). With a mixture of both antisera, two distinct precipitin bands formed (Fig. 3C). Subsequently, one of the proteases was purified from the ECP of Ba5 on a single-step gelatin-agarose affinity column, eluted with 0.2 M-EDTA. This enzyme was similar to the NRC 505 enzyme in its response to inhibitors and heating (Table 2), and was antigenically identical. The

Fig. 3. Ouchterlony immunodiffusion of ECP with antisera raised against purified proteases. The centre wells contained 10 μ l of undiluted rabbit antiserum to the protease of *A. hydrophila* ATCC 7966 (A), the protease of *A. hydrophila* NRC 505 (B) or a mixture of equal volumes of the two sera (C). The outer wells contained 10 μ l samples of ECP from *A. hydrophila* NRC 505 (1), and *A. hydrophila* ATCC 7966 (2) or *A. hydrophila* Ba5 (3). The plate was incubated at 37 °C for 24 h.

Table 2. *Effects of inhibitors and heat-treatment on proteolytic activities of ECPs and purified enzymes from A. hydrophila NRC 505, ATCC 7966 and Ba5*

ECP or PP were pre-incubated with the inhibitors or substrates for 30 min before addition of Azocasein substrate. ECP are a cell-free filtrate, collected after growth of *A. hydrophila* for 36 h on brain heart infusion-skim milk agar; PP indicates proteases purified from *A. hydrophila* NRC 505 and ATCC 7966 by gel filtration and zone electrophoresis, and from *A. hydrophila* Ba5 by gelatin-agarose affinity chromatography.

Treatment	Proteolytic activity remaining (%) in:*					
	NRC 505		ATCC 7966		Ba5	
	ECP	PP	ECP	PP	ECP	PP
EDTA (10 mM)	2.3	4.2	90.3	98.8	41.3	4.5
1,10-Phenanthroline (10 mM)	8.3	5.3	91.9	97.6	32.4	5.6
Cysteine (5 mM)	36.4	8.3	69.4	72.1	73.8	18.2
DTT (10 mM)	4.6	4.2	41.9	58.1	28.7	12.1
Soy bean trypsin inhibitor (1 mg ml ⁻¹)	95.4	100.0	96.8	91.9	96.7	101.5
Lima bean trypsin inhibitor (1 mg ml ⁻¹)	95.4	100.0	96.8	97.7	100.0	98.5
ϵ -Aminocaproic acid (1 mg ml ⁻¹)	93.2	100.0	98.4	98.8	100.8	100.1
PMSF (1 mM)	97.0	94.7	4.2	1.2	71.7	118.6
56 °C, 30 min	63.6	75.0	2.7	5.8	50.0	83.6

* The remaining proteolytic activity is expressed as a percentage of the activity of an untreated sample on Azocasein substrate (1 h at 37 °C). Values are the mean of duplicate samples, with less than 5% deviation.

Fig. 4. Survey of TSMP and TLSP proteases in ECP of aeromonads by immunodiffusion. The top row of wells in each set contained 10 μ l of ECP, the lower wells contained 10 μ l of antisera, as follows: row A, anti-NRC-505-protease; row B, anti-ATCC-7966-protease; row C, mixture of the two antisera. Section (a) shows ECP from strains of *A. hydrophila* with only the TSMP: 1, NRC 505; 2, DMG 518; 3, NZ 70-4517; 4, FHRL 1.23; 5, FHRL 1.19; 6, TF7; 7, Ba6; 8, Sau-1. Section (b) strains have only TLSP: 1, *A. hydrophila* ATCC 7966; 2, *A. sobria* OU2; 3, *A. sobria* CDC 3252.71; 4, *A. salmonicida* OMNR 426; 5, *A. salmonicida* OMNR 357; 6, *A. salmonicida* As19. Section (c) strains produce both enzymes: 1, *A. hydrophila* ATCC 14715; 2, *A. hydrophila* FHRL 1.20; 3, *A. sobria* FHRL 1.40; 4, *A. hydrophila* Ba5; 5, *A. caviae* Pis-1; 6, *A. hydrophila* NHW 76-388; 7, *A. hydrophila* NRS 35; 8, *A. hydrophila* TS-AH.

second enzyme showed partial rather than complete identity with the ATCC 7966 protease (Fig. 3A, C).

Proteases of other strains of A. hydrophila

When ECP from a total of 47 strains of *A. hydrophila* were screened with the two antisera, 19 reacted with anti-NRC-505-protease serum but not with anti-ATCC-7966-protease serum (Fig. 4a). With the NRC 505 enzyme, all strains gave reactions of complete identity except *A. hydrophila* ATCC 9071, which had a reaction of partial identity. Proteolytic activities of the ECP from these strains were all sensitive to EDTA, resistant to PMSF, and relatively heat-stable (Table 3), as expected for the NRC 505 enzyme type. No other strain besides *A. hydrophila* ATCC 7966 produced ECP that reacted only with the anti-ATCC-7966-protease serum (Fig. 4b; Table 3). However, 27 strains of *A. hydrophila* gave a reaction with both antisera, and formed two precipitin bands with a mixture of the two sera (Fig. 4c). Of these strains, 10 produced an enzyme antigenically identical to that of ATCC 7966, while the others gave reactions of partial identity (Fig. 4c).

ECP from strains producing both enzymes gave intermediate responses to EDTA and PMSF inhibition and heat-inactivation of proteolytic activity (Table 3). The activity remaining after the treatments varied for different strains, depending upon the relative proportions of the two enzymes present. This was demonstrated by the responses of artificial mixtures of ECP from *A. hydrophila* ATCC 7966 and NRC 505, which produced mixed responses that were in proportion to the mixing ratios (Table 4). In addition, the relative amounts of the two proteases produced by different strains could be estimated serologically as there was a direct relationship between rocket height in immunoelectrophoresis and proteolytic activity for both enzymes. In tests on ECP from five strains that produced both enzymes, the relative proportions of the enzymes predicted by ratio of rocket heights and the responses to EDTA, PMSF and heating were relatively consistent with responses of the mixtures reported in Table 4 (data not shown).

Table 3. *Inhibitor and heat-stability of proteases in ECP of Aeromonas*

ECP were assayed for proteolytic activity on Azocasein substrate, at 37 °C for 1 h. Before the assay, preparations were pre-incubated with 10 mM-EDTA or 1 mM-PMSF for 30 min, or were heated at 56 °C for 30 min. Proteolytic activity remaining was calculated as a percentage of the activity of an untreated ECP sample. Values are the mean for all strains in the group, $\pm$ SD.

Taxon	Protease type*	No. of strains	Proteolytic activity remaining (%) after treatment with:		
			EDTA	PMSF	56 °C, 30 min
<i>A. hydrophila</i>	TSMP	19	10.7 $\pm$ 5.1	87.0 $\pm$ 7.8	62.6 $\pm$ 6.3
	TLSP	1	90.5	7.4	5.4
	Both	27	32.1 $\pm$ 14.9	65.1 $\pm$ 20.5	57.4 $\pm$ 16.0
<i>A. sobria</i>	TSMP	2	23.4 $\pm$ 0.5	77.9 $\pm$ 17.7	91.9 $\pm$ 9.2
	TLSP	2	91.6 $\pm$ 11.8	10.0 $\pm$ 6.2	22.6 $\pm$ 8.4
	Both	9	58.9 $\pm$ 14.0	28.9 $\pm$ 15.7	47.2 $\pm$ 22.0
<i>A. caviae</i>	TSP	2	50.5 $\pm$ 10.9	100.4 $\pm$ 0.6	85.8 $\pm$ 0.7
	Both	1	34.8	30.4	32.6
<i>A. salmonicida</i>	TLSP	3	87.9 $\pm$ 14.9	9.0 $\pm$ 5.5	15.7 $\pm$ 0.9

* Protease types in ECP were assigned on the basis of serological reactions with antisera against purified proteases: TSMP, ECP reacted with anti-NCR-505 serum; TLSP, ECP reacted with anti-ATCC-7966-serum; TSP, ECP did not give a reaction with either antisera.

Table 4. *Response to inhibitors and heat-treatment of proteases in mixtures of ECP from A. hydrophila ATCC 7966 and NRC 505*

Pre-treatments and determination of remaining activity were as described in Table 3.

Components of mixture (% v/v)*		Proteolytic activity remaining (%) after treatment with:		
NRC 505	ATCC 7966	EDTA	PMSF	56 °C, 30 min
100	0	3.6	89.0	64.6
90	10	17.1	67.0	58.5
80	20	29.4	64.7	55.3
67	33	36.1	59.0	51.8
50	50	47.2	49.4	40.4
33	67	57.1	35.7	32.1
20	80	67.5	30.0	30.0
10	90	75.8	15.2	18.2
0	100	92.8	3.6	5.4

* The ECP from cultures of *A. hydrophila* ATCC 7966 and NRC 505 were adjusted to equal concentrations of proteolytic activity (50 mg Azocasein hydrolysed h⁻¹).

Proteases of other aeromonads

The biochemical test reactions of the motile aeromonads selected for study indicated that 16 strains were *A. sobria* and 9 were *A. caviae*. Two strains of *A. sobria* produced only NRC-505-like proteases, which were EDTA-sensitive, PMSF-resistant and relatively heat-stable (Table 3). All but two other strains of *A. sobria* produced both enzymes (Table 3). The remaining two strains of *A. sobria*, OU2 and CDC 3252.71, were of interest because their ECP reacted only with anti-ATCC-7966 serum, but in a reaction of partial identity (Fig. 4a). Similarly, three strains of *A. salmonicida*, included for comparison, gave a reaction of partial identity with anti-ATCC-7966-protease, but no reaction with anti-NRC-505-protease (Fig. 4b). The precipitin bands of the strains of *A. salmonicida* and *A. sobria* joined, suggesting that they produced similar proteases that were antigenically related to that of *A. hydrophila* ATCC 7966. These enzymes resembled the ATCC 7966 enzyme in inhibitor and heat-treatment responses (Table 3).

No protease activity could be detected in the extracellular products of six strains of *A. caviae* nor of *A. media* ATCC 33907. One strain of *A. caviae*, UV66, produced both enzymes. Two other strains of *A. caviae*, CDC 3331.68 and CDC 3402.68, did not react with either antisera, but produced proteolytic activity in ECP that was moderately EDTA-resistant, PMSF-resistant and heat-stable (Table 3). This may be a third protease enzyme, of most significance in strains of *A. caviae*.

DISCUSSION

Two distinct types of extracellular proteases were produced by strains of *A. hydrophila*: a temperature-stable metallo-protease (TSMP) and a temperature-labile serine-protease (TLSP). These could be defined by a combination of the characteristics used by Thune *et al.* (1982) and Dahle (1971). The TSMP enzyme-type, produced by *A. hydrophila* NRC 505, was a metallo-protease, sensitive to EDTA, and stable on heating at 56 °C. In contrast, the extracellular protease of *A. hydrophila* ATCC 7966 was a serine-protease, sensitive to PMSF but not EDTA, and labile on heating at 56 °C for 30 min. In this study, it was demonstrated that the division, made on the basis of the functional criteria of inhibitors and heat-sensitivity, is consistent with antigenic characteristics of the two enzyme types.

Previous reports have described finding the TSMP alone (Gross & Coles, 1969; Allan & Stevenson, 1981; Denis & Veillet-Poncet, 1984; Kanai & Wakabayashi, 1984). Other studies have reported TSMP to be one of two proteases produced, the other being the TLSP (Dahle, 1971; Thune *et al.*, 1982; Nieto & Ellis, 1986). One evident source of the different results obtained in these studies is the initial choice of bacterial strain. The majority of the strains examined in this study, i.e. 27 of 47, produced both protease types, but many (19 of 47) produced only the TSMP. Production of TLSP as the only protease was rare in *A. hydrophila*, with the type strain, ATCC 7966, being the sole example. Thune *et al.* (1982) and Amborski *et al.* (1984) reported three enzymes, based on differences in heat-stability. Other than the distinctly different protease of two strains of *A. caviae*, we did not find evidence of another major extracellular protease in the strains studied. However, proteases with more specific substrate-specificities or induced by different growth conditions would not be detected in this study.

The TLSP of *A. hydrophila* is essentially the same as the major serine protease enzyme found in *A. salmonicida* (Fyfe *et al.*, 1987), based on serological reactions and response to inhibitors and heat. Thus, this study is in agreement with the results of Dahle (1971), who first reported the similarity. For all three strains of *A. salmonicida* and two of the strains of *A. sobria* tested, the TLSP appeared to be the single major protease. Reactions of partial identity, seen in the serological tests, suggest that there are two antigenically related forms of the TLSP. The response of ECP from strains producing this enzyme alone (*A. hydrophila* ATCC 7966 and three strains of *A. salmonicida*) do not suggest major differences in catalytic activity (Table 3), and the significance of the antigenic variation is not clear. The TSMP appeared less antigenically heterogeneous than the TLSP. Only *A. hydrophila* ATCC 9071 and two strains of *A. sobria*, FHRL1.40 and Pis-1, produced proteases which were partially rather than completely identical with the protease of NRC 505. Autolysis is a problem associated with studying protease enzymes, particularly in attempts to purify and characterize. This may be the source of some heterogeneity of serological reactions as well as variations in molecular sizes and numbers of enzyme forms reported in studies of both *A. hydrophila* (Nieto & Ellis, 1986) and *A. salmonicida* (Fyfe *et al.*, 1987). If the protease types of *A. hydrophila* had been designated on the basis of antigenicity alone, the variations indicated by reactions of partial identity would have suggested multiple forms. Protease typing using inhibitors and heat-stability in conjunction with serological studies allowed more useful categories of enzymes to be defined.

Because of autolysis and general loss of activity on purification, it was important that both the serological and functional tests could be done on crude ECP, with results consistent with those for purified enzymes. Previously, Thune *et al.* (1982) and Nieto & Ellis (1986) used the effect of inhibitors and heating on crude extracts as an estimate of the proportions of protease present; our serological and mixing experiments confirm the use of this approach. However, the response of ECP to inhibitors can be difficult to interpret with reliability.

This study clarifies the situation with regard to the number and types of extracellular proteolytic enzymes produced by strains of *A. hydrophila*. The functions of the enzymes, including their potential contributions to pathogenicity, remain undefined, and the question of why a strain might produce two forms of protease (Dahle, 1971) remains unanswered. Identification of the proteases produced by particular strains is an essential preliminary to answering such questions. ECP from *A. hydrophila* have been shown to have toxic effects when injected into fish (Allan & Stevenson, 1981; Thune *et al.*, 1982). However, Trust (1986) has emphasized that the contribution of an enzyme or toxin to virulence must be confirmed by studies with bacterial mutants, deficient in the specific exo-products. We have used the information obtained about protease distribution to select a virulent strain of *A. hydrophila* that produced only a single protease, and to produce protease-deficient mutants for virulence studies (Leung, 1987).

This research was supported by an Operating Grant from the Natural Sciences and Engineering Research Council of Canada to R. M. W. S. K.-Y. L. was a research student supported by a grant from the Ontario Ministry of Natural Resources. We are grateful to Dr R. J. Douglas and Dr J. Lam for helpful comments on the serological studies.

REFERENCES

- ALLAN, B. J. & STEVENSON, R. M. W. (1981). Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. *Canadian Journal of Microbiology* **27**, 1114–1122.
- AMBORSKI, R. L., BORALL, R. & THUNE, R. L. (1984). Effects of short-term storage on recovery of proteases from extracellular products of *Aeromonas hydrophila*. *Applied and Environmental Microbiology* **48**, 456–458.
- DAHLE, H. K. (1971). The purification and some properties of two *Aeromonas* proteinases. *Acta pathologica, microbiologica et immunologica scandinavica* **B79**, 726–738.
- DENIS, F. & VEILLET-PONCET, L. (1984). Purification partielle du système proteolytique extracellulaire de *Aeromonas hydrophila* LP50: étude comparative chromatographique et électrophorétique. *Annales de microbiologie* (Institut Pasteur) **135A**, 219–227.
- FYFE, L., COLEMAN, G. & MUNRO, A. L. S. (1987). Identification of major common extracellular proteins secreted by *Aeromonas salmonicida* isolated from diseased fish. *Applied and Environmental Microbiology* **53**, 722–726.
- GROSS, R. & COLES, N. W. (1969). A proteinase produced by *Aeromonas hydrophila*. *Australian Journal of Science* **31**, 330–331.
- HOWARD, S. P. & BUCKLEY, J. T. (1985). Activation of the hole-forming toxin aerolysin by extracellular processing. *Journal of Bacteriology* **163**, 336–340.
- KANAI, K. & WAKABAYASHI, H. (1984). Purification and some properties of proteases from *Aeromonas hydrophila*. *Bulletin of the Japanese Society of Scientific Fisheries* **40**, 1367–1374.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEUNG, K.-Y. (1987). *The role of proteases of Aeromonas hydrophila in infections of rainbow trout*. PhD thesis, University of Guelph, Ontario, Canada.
- LJUNGH, A. & WADSTROM, T. (1982). *Aeromonas* toxins. *Pharmacology and Therapeutics* **15**, 339–354.
- NIETO, T. P. & ELLIS, A. E. (1986). Characterization of extracellular metallo- and serine-proteases of *Aeromonas hydrophila* strain B₅₁. *Journal of General Microbiology* **132**, 1975–1979.
- POPOFF, M. (1984). *Aeromonas*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 545–548. Edited by N. R. Krieg. Baltimore: Williams & Wilkins.
- SAKAI, D. K. (1985a). Loss of virulence in a protease-deficient mutant of *Aeromonas salmonicida*. *Infection and Immunity* **48**, 146–152.
- SAKAI, D. K. (1985b). Significance of extracellular protease for growth of a heterotrophic bacterium, *Aeromonas salmonicida*. *Applied and Environmental Microbiology* **50**, 1031–1037.
- THUNE, R. L., GRAHAM, T. E., RIDDLE, L. M. & AMBORSKI, R. L. (1982). Extracellular proteases from *Aeromonas hydrophila*: partial purification and effects on age-0 channel catfish. *Transactions of the American Fisheries Society* **111**, 749–754.
- TRUST, T. J. (1986). Pathogenesis of infectious diseases of fish. *Annual Review of Microbiology* **40**, 479–502.