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*Detection of extracellular protease activity of Aeromonas hydrophila strains isolated from MAS diseased carp**

Badanie aktywności pozakomórkowych proteaz *Aeromonas hydrophila* izolowanych od karpi chorych na MAS

Key words: fish, *Aeromonas*, proteases
Słowa kluczowe: ryby, *Aeromonas*, proteazy

INTRODUCTION

Aeromonas infections are probably the most common bacterial disease diagnosed in cultured warmwater fish. Some factors, usually stress, has caused the fish to become more susceptible to the bacteria. Common sources of stress are poor water quality, overcrowding, or rough handling.

Aeromonas hydrophila is a Gram-negative bacterium that is causing concern as a poikilothermic host's pathogen (2). Various toxic substances produced by *Aeromonas hydrophila* have been described, including haemolysins, aerolysin, cytotoxin, enterotoxin and proteases. Such bacterial factors could be expected to play a role in the fish disease processes called motile aeromonad septicaemia (MAS). Proteases produced by *A. hydrophila* have been implicated in the spoilage of meats and fish and could have a toxic effect on their own (4, 8, 23).

An understanding of *A. hydrophila* extracellular proteases is of major importance in revealing the nature of virulence factors. The aim of the present study was to compare the extracellular protease activity produced by a collection of different *A. hydrophila* strains.

MATERIAL AND METHODS

The *A. hydrophila* strains used were originally received from MAS diseased carp. The strain was isolated on TSA with ampicillin. Trypticase soy broth (TSB) and trypticase soy agar (TSA) were prepared according to the manufacturer's instructions. For routine use, the organisms were

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maintained on TSA slants, stored at 4°C after 24 h of incubation at 28°C. Separate stocks were frozen at -70°C in TSB with 20% (v/v) glycerol. The supernatant of bacterial culture was obtained by centrifugation of the culture at 10.000 g for 20 min.

The caseinase activity was determined by the azocasein procedure described by Leung and Stevenson (15), with slight Mateos et al. modifications (17). The reaction mixture consisted of 0.1 ml of a 10% (w/v) Azocasein solution (Sigma), 0.1 ml of supernatant fluid sample and 2.3 ml of 0.1 mol l^{-1} sodium phosphate buffer, pH 7.2 and was incubated at 28°C for 4 h. The reaction was stopped with 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) and after 30 min at room temperature, the precipitate was removed by centrifugation. Equal volumes of supernatant fluid and NaOH 1 mol l^{-1} were mixed and absorbance was read at 450 nm. TCA was added to the blanks before incubation.

The elastase activity of the supernatant fluids was quantified by the spectrophotometric method of Bjorn et al. (3), with Congo-red-labelled elastin (Sigma) as a substrate. Gelatinase detection was performed by a radial diffusion method as described by Palmer (20) with Gudmundsdöttir (10) modifications. A total of 20 µl of EXP was placed in 4 mm wells cut in agarose gels (1% w/v in PBS) supplemented with 3% gelatin (Sigma) and incubated at 28°C for 24 h. After incubation the plates were immersed in a saturated ammonium sulphate solution at 70°C to precipitate unhydrolysed gelatin. The diameter of the zone of proteolysis around inoculated wells was measured against a black background. Proteolytic activity was determined from a standard curve using trypsin (bovine pancreas type III, Sigma). One unit of gelatinase activity was defined as equivalent to that of 1 µg of trypsin.

The Bradford method was used to determine total protein concentration of the ECP. A 0.5 ml sample of ECP was added to 0.5 ml Coomassie blue-G reagent (1% brilliant blue G, 5% ethanol and 10% phosphoric acid). Absorbance was read at 540 nm and protein concentrations were calculated by regression analysis with bovine serum albumin standards.

Standard SDS-PAGE were performed in miniformat gels using the Laemmli buffer system (14). Proteolytic activity was visualised in 12% SDS-PAGE by incorporating 0.5% sodium caseinate or gelatine. Electrophoresis was performed at 4°C and 100 V. To remove SDS the gels were washed in Triton x 100 (2.5%) for 2 h at room temperature and shaken. They were then incubated at 28°C overnight in glycine (0.1 mol l^{-1}, pH 8), for the proteolysis to take place and were finally stained with Coomassie Brilliant Blue R-250. Protease activity was visible as clear bands on a blue background. The relative migration distance was compared with a standard set of molecular weight markers to yield apparent molecular-weight values.

RESULTS AND DISCUSSION

The taxonomy of the genus *Aeromonas* has expanded in recent years, primarily because of the description of nine new mesophilic species. Motile aeromonads isolated from fish had been assigned to Popoff's species (*A. hydrophila, A. caviae, A. sobria*), or simply reported as *Aeromonas* species and *A. hydrophila* complex (21). *A. hydrophila* produced extracellular substances capable of causing pathological effects when injected into fish. In our studies, intramuscular injections of 0.1 ml $fish^{-1}$ of the *A. hydrophila* supernatant fluids produced haemorrhaging in some internal organs and external muscle lesions, disease

signs which are associated with motile aeromonad infections. Except F-4/95, F-5/95 (caused no skin lesions), strains, FP-1/93, FP-2/93, FP-9/95, FP-3/94, FP-7/95 were pathogenic for carp.

Extracellular products were proved to be important factors in the development of MAS caused by *A. hydrophila*. A l l a n and S t e v e n s o n (1) found indirect evidence of a correlation between haemolysin production and toxicity to fish. T h u n e et al. (22) suggested that proteases were insufficient to cause toxic effects in trout. W a k a b a y a s h i et al. (24) and H s u et al. (13) have demonstrated that the proteolytic activity of elastase and caseinase are associated with the virulence of *A. hydrophila* complex strains. The role of the *A. hydrophila* proteases in the mechanism of pathogenesis is still controversial (5, 16, 18). All of them found differences in the kind of proteolytic activities detected in the extracellular products, depending on the cultural conditions and on the origin of the used strain. In our study, ECPs of pathogenic FP-1/93, FP-2/93, FP-9/95, FP-3/94, FP-7/95 strains were more active concerning caseinase, elastase and gelatinase effects than not pathogenic F-4/95 and F-5/95 strains (Table 1).

Table 1. Proteolytic activity of extracellular products of *A. hydrophila* strains isolated from MAS diseased carp
Aktywność proteolityczna pozakomórkowych produktów szczepów *A. hydrophila* wyizolowanych od karpii chorych na MAS

Strains Szczepy	Activity at 28°C (units) Aktywność przy 28°C (jednostki)			Protein content (mg ml^{-1}) Zawartość białka (mg ml^{-1})
	caseinase*	elastase**	gelatinase‡	
A. hydrophila FP-1/93	22.3	7.4	5.6	0.22
A. hydrophila FP-2/93	20.7	8.3	4.6	0.24
A. hydrophila FP-9/95	19.8	6.7	5.5	0.19
A. hydrophila FP-3/94	19.6	5.5	4.8	0.18
A. hydrophila FP-7/95	23.5	8.7	5.6	0.19
A. hydrophila F-4/95	3.6	4.3	1.5	0.18
A. hydrophila F-5/95	2.5	3.7	1.3	0.17

* A unit of caseinolytic activity was defined as the enzyme activity in a 0.1 ml volume of sample that produced an increase in the absorbance of 0.1 at 450 nm.

** Elastase activity unit is expressed as the activity contained in 1 ml of supernatant fluid that increased absorbance by 0.1 at 495 nm.

‡ One unit of gelatinase activity was defined as equivalent to that of 1 µg of trypsin.

*Jednostkę aktywności kazeinolitycznej definiowano jako aktywność enzymatyczną w 0,1 ml objętości próbki, której absorbancja wzrastała o 0,1 przy 450 nm.

**Jednostkę aktywności elastazowej przedstawiono jako aktywność 1 ml supernatanu, której absorbancja wzrosła o 0,1 przy 495 nm.

‡Jednostkę aktywności żelatynazowej definiowano jako odpowiednik 1 µg trypsyny.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) is a reproducible and rapid method for quantifying, comparing and characterising proteins. This method separates proteins based primarily on their molecular weights (14). Polyacrylamide gels are formed by the copolymerization of acrylamide monomers with a cross-linking agent to form a three-dimensional lattice. The best and most commonly used crosslinking agent for the majority of PAGE applications is *N,N'*-methylene bisacrylamide. Gel polymerization is usually initiated with ammonium persulphate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) (11). Since first proposed by H e u s s e n and D o w d l e (12), substrate containing polyacrylamide gel electrophoresis has proven to be a valuable method for dissecting the proteolytic systems of many organisms.

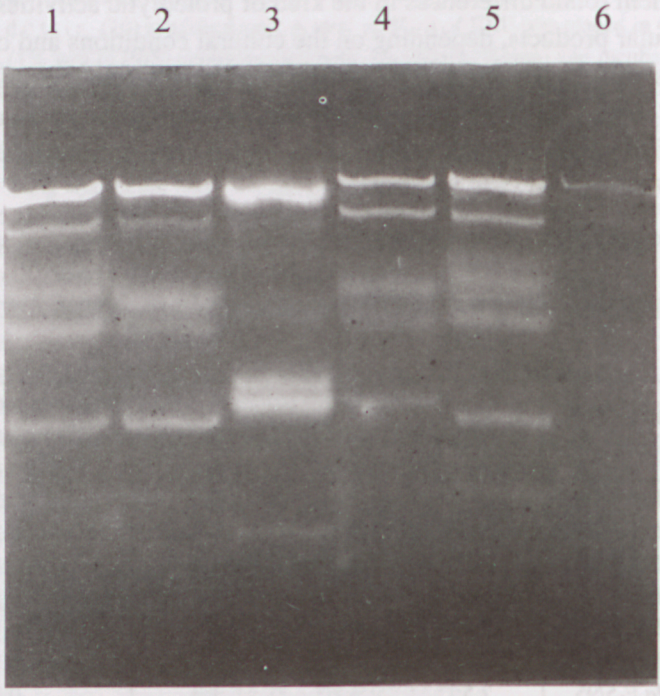

Fig. 1. Gelatinase electrophoretic profiles in culture supernatant fluids of *A. hydrophila* strains, observed by gelatin/SDS-PAGE. Lane 1 – FP-1/93; lane 2 – FP-2/93; lane 3 – FP-9/95; lane 4 – FP-3/93; lane 5 – FP-7/95; lane 6 – F-4/95

Elektroforetyczne profile żelatynaz w supernatantach szczepów *A. hydrophila*, badanych metodą: żelatyna/SDS-PAGE. Ścieżka 1 – FP-1/93; ścieżka 2 – FP-2/93; ścieżka 3 – FP-9/95; ścieżka 4 – FP-3/93; ścieżka 5 – FP-7/95; ścieżka 6 – F-4/95

For a qualitative analysis of the proteasic content of the *A. hydrophila* supernatant fluids were processed by sodium caseinate or gelatine – SDS-PAGE. Figure 1 and 2 show that different electrophoretic patterns were established on the

basis of the different migration of protease bands observed in the gels. In our study, strains FP-1/93, FP-2/93, FP-9/95, FP-3/95, FP-7-95 and F-4/95 produced 8, 7, 5, 7, 9, 1 gelatinases, and 6, 5, 5, 3, 7, 0 caseinases respectively (Table 2 and 3).

Extracellular factors have received considerable attention as possible protective antigens against furunculosis. Active immunization with crude ECP and protease have been reported (7, 19). 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 (6, 9).

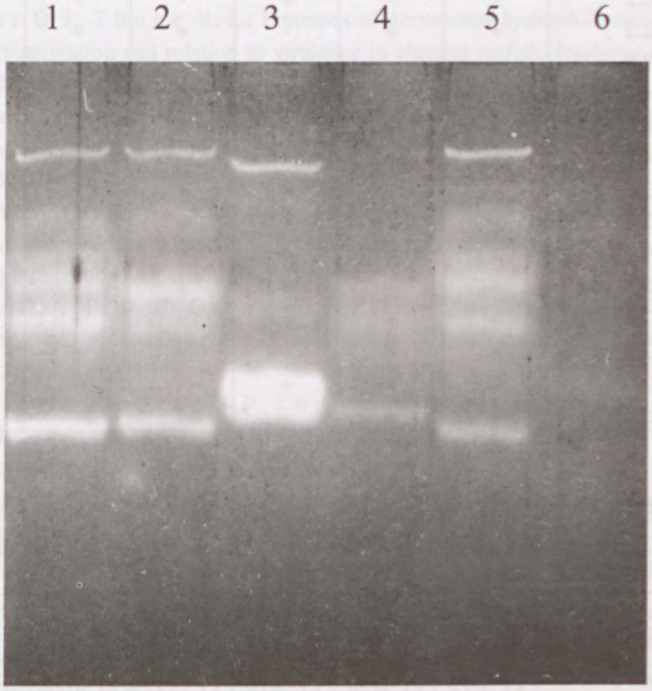

Fig. 2. Caseinase electrophoretic profiles in culture supernatant fluids of *A. hydrophila* strains, observed by sodium caseinate/SDS-PAGE. Lane 1 – FP-1/93; lane 2 – FP-2/93; lane 3 – FP-9/95; lane 4 – FP-3/93; lane 5 – FP-7/95; lane 6 – F-4/95

Elektroforetyczne profile kazeinaz w supernatantach szczepów *A. hydrophila*, badanych metodą: kazeina/SDS-PAGE. Ścieżka 1 – FP-1/93; ścieżka 2 – FP-2/93; ścieżka 3 – FP-9/95; ścieżka 4 – FP-3/93; ścieżka 5 – FP-7/95; ścieżka 6 – F-4/95

In summary, a positive relationship between strain pathogenicity and biological activities was observed in *A. hydrophila* isolated from carp, showing that their pathogenesis is multifactorial. These factors are present in pathogenic strains but not in those which only group avirulent strains. Proteases may play a role in spread of the disease. The exact mechanisms by which aeromonads cause disease in fish or other animals are still unknown.

Table 2. Characterisation of gelatinases produced by *A. hydrophila* strains
Charakterystyka żelatynaz wytwarzanych przez szczepy *A. hydrophila*

Molecular masses (kDa) Masy cząsteczkowe	*A. hydrophila* strains Szczepy *A. hydrophila*					
	FP-1/93	FP-2/93	FP-9/95	FP-3/93	FP-7/95	F-4/95
84.2	+	+	-	+	+	+
80.2	-	-	+	-	-	-
62.2	+	+	-	+	+	-
49.6	+	-	-	-	+	-
44.8	-	-	-	+	-	-
44.7	+	-	-	+	+	-
44.0	-	+	-	-	+	-
43.6	-	-	+	+	+	-
42.7	+	+	-	+	+	-
39.9	-	-	+	-	-	-
38.8	-	-	+	+	-	-
37.8	+	+	-	-	+	-
33.7	+	+	-	-	+	-
32.0	-	-	+	-	-	-
25.4	+	+	-			

Table 3. Characterisation of caseinases produced by *A. hydrophila* strains
Charakterystyka kazeinaz wytwarzanych przez szczepy *A. hydrophila*

Molecular masses (kDa) Masy cząsteczkowe	*A. hydrophila* strains Szczepy *A. hydrophila*					
	FP-1/93	FP-2/93	FP-9/95	FP-3/93	FP-7/95	F-4/95
100.0	+	+	-	-	+	-
95.7	-	-	+	-	-	-
79.8	-	-	-	-	+	-
66.0	-	-	+	-	-	-
63.4	+	+	-	-	+	-
56.7	+	-	-	-	+	-
51.7	+	+	-	+	+	-
49.2	-	-	+	-	-	-
46.8	-	-	+	-	-	-
44.6	+	+	-	+	+	-
33.1	-	-	+	-	-	-
30.0	-	-	-	+	-	-
26.3	+	+	-	-	+	-

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SUMMARY

Aeromonads are widely associated with fish and other poikilothermic organisms where they occur as members of the normal microbial flora as well as primary or secondary pathogens. The production of extracellular proteases by strains of *A. hydrophila* isolated from MAS diseased carp was determined. ECPs of pathogenic FP-1/93, FP-2/93, FP-9/95, FP-3/94, FP-7/95 strains were more active concerning caseinase, elastase and gelatinase effects than non-pathogenic F-4/95 and F-5/95 strains.

These findings indicate differences between pathogenic and non-pathogenic *A. hydrophila* strains with respect to the expression of proteases, and shows that they play a leading role in the pathogenicity of *A. hydrophila* for carp.

STRESZCZENIE

Bakterie z rodzaju *Aeromonas* są szeroko rozpowszechnione w przyrodzie i mogą być patogenne dla ryb i innych niższych kręgowców. W niniejszej pracy badano aktywność proteaz zewnątrzkomórkowych szczepów *A. hydrophila* izolowanych z klinicznych przypadków MAS karpi. Notowano wyższą aktywność kazeinazową, elastazową i żelatynazową u badanych patogennych szczepów *A. hydrophila* (FP-1/93, FP-2/93, FP-9/95, FP-3/94, FP-7/95) i niską aktywność szczepów niepatogennych (F-4/95, F-5/95).

Wykazanie, że szczepy patogenne posiadają wyższą aktywność proteolityczną, może świadczyć o znaczącej roli proteaz pozakomórkowych *A. hydrophila* w patogenezie MAS.