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Extracellular protease production by *Moritella viscosa*

Wytwarzanie pozakomórkowych proteaz przez *Moritella viscosa*

Summary. *Moritella viscosa* (*Vibrio viscosus*) is the causative agent of winter ulcers in farmed salmonids, and bacteria have been isolated from various marine fish species. The protease activity at 6ºC and 16ºC of *M. viscosa* strain were studied. Substrate gel electrophoresis with gelatin and casein as the substrates showed that *M. viscosa* strain had two different proteinase bands, 73.5 and 67.5 kDa.

Key words: *Moritella viscosa*, winter ulcer, fish

INTRODUCTION

Moritella viscosa (*Vibrio viscosus*) is a psychrotrophic bacterium that has been found in winter ulcers in salmonids in Norway, Iceland, Scotland, Denmark, Ireland, and Canada [Lunder 1992, Lunder *et al.* 1995, 2000, Benediksdottir *et al.* 1998, Bruno *et al.* 1998, Lillehaug et al. 2003, Håstein et al. 2005], first described as *Vibrio viscosus* [Lunder *et al*. 2000]. Affected farmed fish, described by Lunder [1992], Benediksdottir *et al.* [1998] and Bruno *et al.* [1993] were lethargic, dark in colour, showed gill pallor, and exophthalmos. Extracellular, epidermal ulcers were recorded on the scale-covered areas and ranged in diameter from 0.5 to 2.0 cm. The ulcers were superficial or penetrated to the musculature. The disease occurs most frequently at low temperatures, thus explaining the name 'winter ulcer' ('vintersår' in Norwegian). In Norway the disease occurs mainly in February, March and April, and the fish usually recover when the water temperature rises above 8ºC. *Moritella viscosa* infections have been detected in various fish species in the field, including salmon, cod, plaice, rainbow trout, and lumpfish [Benediktsdottir *et al.* 2000, Lunder *et al.* 2000, Colquhoun *et al.* 2004, Gudmundsdóttir *et al.* 2006].

The aim of this study was to investigate the effect of incubation temperature upon the composition of extracellular proteases produced by *M. viscosa* strain.

MATERIAL AND METHODS

Bacteria and growth conditions. *Moritella viscosa* strain was isolated from Atlantic salmon (*Salmo salar*), and was kindly provided from the collection at the Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavik. The bacteria were cultured in tryptic soy agar containing 2% NaCl (TSA-2). The agar plates were incubated 48 hour at 16ºC. For production of extracellular proteases bacteria were grown in *V. viscosus* medium (VM) for 24 h at 6ºC and 18ºC under constant shaking (180 RPM). *Vibrio viscosus* medium: $MgSO_4 \cdot 7\ H_2O$ – 1 g/l, yeast extract – 20 g/l, NaCl – 12 g/l, KH_2PO_4 – 2 g/l, pH – 7,3.

Antibiotic susceptibility tests. The antibiotic susceptibility test was determined according to the procedure described by Ayres *et al*. [1999], with slight modifications. Briefly, antibiotic discs were purchased from Oxoid and consisted of amoxicilin 10 μg, oxolinic acid 2 μg, oxytetracycline 30 μg. Diagnostic sensitivity test agar (TSA-2) plates poured, and allowed to set. The cell suspension (0.25 ml) was pipeted onto the surface of each plate and spread evenly with a dry sterile swab. The inoculated plates were allowed to dry for 2 h, antibiotic discs placed onto the agar surface and the plates were incubated at 16ºC for 24 h and the zones of inhibition measured.

Biochemical tests. Biochemical testing was carried out using API Zym test strips (bioMérieux) at 18ºC and recorded after 72 h incubation.

Measurement of proteolytic activity of ECPs. Protein levels of ECPs solutions were determined using the Sigma protein assay kit with bovine albumin as standard.

Caseinase activity. The caseinase activity was determined by the azocasein procedure described by Leung and Stevenson [1988], with slight Mateos *et al.* [1993] and the authors' own modifications. Briefly, 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 20ºC for 24 h. The reaction was stopped with 2.5 ml of 10% (w/v) trichloroacetic acid (TCA), and after 30 min at room temperature, the precipitation 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 blank before incubation.

Elastase activity. The caseinase activity was determined by the azocasein procedure described by Bjorn *et al.* [1979], with slight modifications by Mateos *et al.* [1993] and our own. Briefly, one ml of culture supernatant fluids was added to 2 ml of Tris-maleate buffer (0.1 mol l^{-1}, pH 7.0) supplemented with $CaCl_2$ (0.001 mol l^{-1}) containing 10 mg of elactin-Congo red. The mixture was incubated at 20ºC for 24 h and the reaction was stopped by the addition of 2 ml of sodium phosphate buffer (0.7 mol l^{-1}, pH 6.0). The precipitate was removed by centrifugation. The blank consisted of 3 ml of the buffer containing 10 mg of elastin-Congo red. Elastase activity was determined by reading absorbance of the supernatant fluid at 495 nm.

SDS-PAGE. Proteolytic activity was visualized in 12% SDS-polyacrylamide gels [Laemmli 1970] by incorporating 0.1% sodium caseinate or gelatin, according to the method described by Mateos *et al.* [1993]. The SDS was removed by shaking the gels in 2.5% Triton X-100 (Sigma) for 1 h and then the gels were incubated in glycine 0.1 mol l^{-1} pH 8.3 overnight at 28ºC to allow proteolysis to take place. Protease activity was visible as clear bands on a blue background. The relative migration distance was compared with a standard set of low molecular-weight markers (BioRad), to yield apparent molecular-weight values.

RESULTS AND DISCUSSION

Moritella viscosa (previously *Vibrio viscosus*) has received increased attention as a cause of winter ulcer [Bruno *et al.* 1998, Benediktsdóttir *et al.* 2000, Lunder *et al.* 2000, Gudmundsdottir *et al.* 2006] in farmed salmonids, cod and various other marine fish species, in several countries around the North Atlantic [Colquhoun *et al.* 2004, Björnsdottir *et al.* 2004]. Winter ulcer is a disease affecting fish in sea water, most frequently during the winter months, when the temperature is below 10ºC [Larsen and Pedersen 1999]. In Norway the disease occurs mainly in February, March and April and the fish usually recover when the water temperature rises above 8ºC. Diseased fish show characteristic skin ulcers of varying size and depth. The mortality is usually low, from 0 to 10% during the outbreak, but the disease has economic significance due to lowered quality of the fish [Lunder 1992].

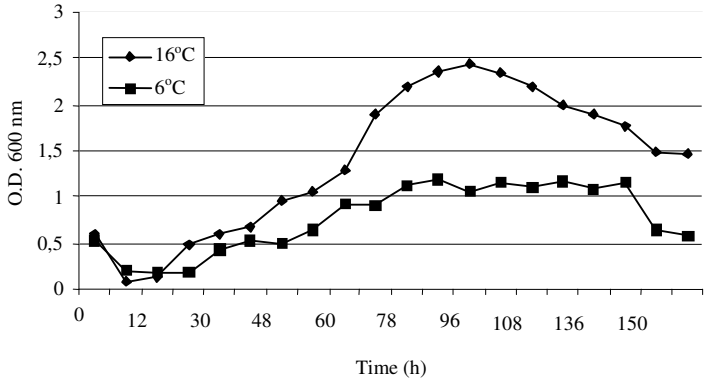

Fig. 1. Effect of temperature on growth of *Moritella viscosa* strain
Rys. 1.Wpływ temperatury na wzrost *Moritella viscosa*

The *M. viscosa* cultures were sensitive to amoxycilin, oxolinic acid and oxytetracycline. The biochemical properties of the bacterium corresponded with those described for a novel *Vibrio* sp. by Lunder [1992] named *Vibrio viscosus*, and Benediktsdottir *et al.* [2000]. Strains of *M. viscosa* are Gram-negative, short non-sporeforming rods that are motile by means of a single polar flagellum. The bacteria are oxidase and catalase positive, produced acid from glucose. They do not produce acid from D-arabinose, L-arabinose, cellobiose, glycerol, inositol, lactose, mannitol, melibiose, rhamnose, salicin, sorbitol, sucrose, and trehalose. *M. viscosa* strains are negative in arginine dihydrolase, ornithine decarboxylase and usually positive in lysine decarboxylase. Casein, gelatin, DNA, Tween 20, Tween 80 and starch are hydrolyzed, but neither alginate nor esculin are hydrolyzed. The Voges-Proscauer test is negative. The bacterium is susceptible to 2,4-diamino-6,7-diisopropylpteridine (vibriostatic agent, 0/129) [Lunder 1992, Bruno *et al.* 1998, Lunder *et al.* 2000, Benediktsdottir *et al.* 2000]. Moreover, the production of lysine decarboxylase and the production of acid from maltose and mannose are different, depending on subgroups [Benediktsdottir *et al.* 2000].

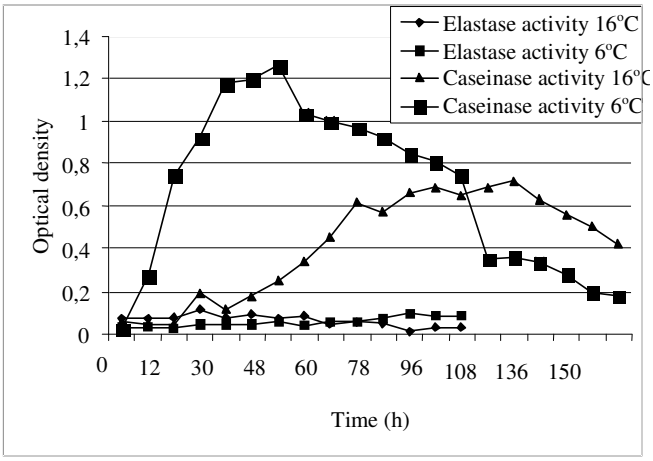

Fig. 2. Exoprotease production by *Moritella viscosa*
Rys. 2. Wytwarzanie proteaz przez *Moritella viscosa*

Table 1. Biochemical characteristics of *Moritella viscosa* using API Zym kit from bioMérieux. Results recorded at 18ºC after 72 h incubation
Tabela 1. Biochemiczna charakterystyka *Moritella viscosa* testem API Zym z firmy bioMérieux. Wyniki pochodzą z 18ºC po 72 godz. inkubacji

Enzyme	Activity mark of *Moritella viscosa* (at 18ºC/6ºC)*		
	Log phase	Stationary phase	Death phase
Phosphatase alcaline	0/3	0/5	0/5
Esterase (C 4)	1/1	2/1	2/1
Esterase Lipase (C 8)	3/3	4/3	4/3
Lipase (C 14)	2/0	2/1	1/1
Leucine arylamidase	4/1	5/1	4/3
Valine arylamidase	2/0	3/0	2/0
Cystine arylamidase	0/0	1/0	0/1
Trypsin	0/0	0/0	0/0
Chymotrypsin	1/1	2/1	2/1
Phosphatase acid	1/3	1/3	1/5
Naphthol-AS-BI-phosphohydrolase	1/1	1/1	1/1
α-galactosidase	0/0	0/0	0/0
β-galactosidase	0/0	0/0	0/2
β-glucuronidase	0/0	0/0	0/0
α-glucosidase	0/1	0/1	0/1
β-glucosidase	3/3	5/3	5/5
N-acetyl-β glucosaminidase	3/1	5/1	5/1
α-mannosidase	0/0	0/0	0/0
α-fucosidase	0/0	0/0	0/0

*Quantity of hydrolysed substrate: 0 – 0 nM (nanomole); 1 – 5 nM; 2 – 10 nM; 3 – 20 nM; 4 – 30 nM; 5 - > 40 nM

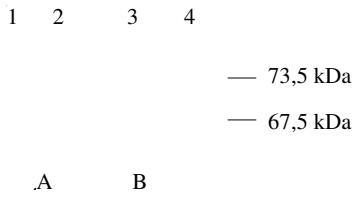

Fig. 3. Substrate SDS-PAGE analysis comparing extracellular products (ECPs) of *Moritella viscosa* measured in the middle of log phase: A – casein SDS-PAGE; B – gelatin SDS-PAGE; 1 and 3–6ºC; 2 and 4–16ºC
Rys. 3. Porównawcza analiza produktów zewnątrzkomórkowych (ECPs) *Moritella viscosa* mierzona w połowie fazy logarytmicznego wzrostu: A – kazeina SDS-Page; B – żelatyna SDS-PAGE; 1 i 3–6ºC; 2 i 4–16ºC

The observation that winter ulcer occurs mainly when water temperatures are low reflects the decreased reparative and regenerative ability that fish possesses at these temperatures [Roberts 1975]. It is well known that temperature is an important factor controlling the rate of development of microbial populations, a modulation of enzyme synthesis by the growth temperature having been observed in several microorganisms [Herendeen *et al.* 1979, Gügi *et al*. 1991, Mateos *et al.* 1993]. In our study, short, curved, Gram-negative staining rods were recorded on TSA-2 medium. The colonies were translucent, creamy yellow in appearance and viscous when touched with a loop. The bacteria were grown at 6ºC and 16ºC for 162 h. Figure 1 shows that the growth of bacteria was considerably slower at 6ºC than at 16ºC. The effect of incubation time and temperature on protease activity of *M. viscosa* ECPs is shown in Fig. 2. At 16ºC the bacterial growth reached its maximum effect after 96 h of incubation time. The lowest caseinase activity was observed in the supernatant fluid of culture grown at 16ºC for 124 h. Cultures grown at 6ºC showed that lowest bacterial growth, but highest caseinase activity reached its maximum after 48 h of incubation time were observed. Moreover, the elastase activities were not observed at either 6ºC or 16ºC. Biochemical properties of strain cultured at 6ºC and 16ºC (at log phase, stationary phase and death phase) are reported in Table 1.

Substrate gel electrophoresis with gelatin or casein as the substrates showed that *M. viscosa* strain produced two different bands showing proteolytic activity. Neither of them were affected by the treatment with β-mercaptoethanol. Substrate SDS-PAGE patterns of ECPs representative for all caseinase and gelatinase profiles are shown in Fig. 3. Substrate gel electrophoresis with gelatin and casein as the substrates showed that *M. viscosa* strain had two different proteinase bands, 73.5 kDa and 67.5 kDa. The influence of temperature of incubation on the types of produced proteases was scarce so all the proteases detected were produced at both 16ºC and 6ºC.

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Streszczenie. 'Winter ulcer' jest chorobą głównie ryb łososiowatych, wywoływaną przez bakterie *Moritella viscosa* (dawniej *Vibrio viscosus*). Chorobę notowano w Norwegii, Islandii, Szkocji, Danii, Irlandii i Kanadzie. Występuje w zimnych porach roku, gdy temperatura wody spada poniżej 10ºC. Śmiertelność ryb jest niewielka, ale ze względu na niższą jakość mięsa przynosi znaczne straty ekonomiczne. W niniejszej pracy zbadano wzrost bakterii *M. viscosa* w temperaturze 16ºC – optymalnej do wzrostu oraz w temperaturze 6ºC. Zbadano również aktywność kazeinazową i elastazową oraz wykonano analizę zymograficzną proteaz (SDS-PAGE), wykorzystując kazeinę i żelatynę jako substraty. Wykazano, że badany szczep wytwarza dwie proteazy o aktywności żelatynazowej i dwie proteazy o aktywności kazeinazowej.

Słowa kluczowe: *Moritella viscosa*, 'winter ulcer', ryby