Research article

Extracellular Proteinases of Mamiensis avidus Causing Scuticociliatosis are Potential Virulence Factors

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ABSTRACT—Mamiensis avidus is the causative agent of scuticociliatosis in various marine fish species. The virulence factors of the parasite have not been identified, so far. In this study, we examined M. avidus extracellular proteinases (ECPs) as potential virulence factors, using culture supernatants as an ECPs source. We investigated the substrate specificity of ECPs using artificial peptides, and the cytotoxicity of the ECPs was examined using CHSE-214 cells. To elucidate the role of ECPs in ciliate growth, M. avidus was cultured on CHSE-214 cells in the presence of proteinase inhibitors. We detected proteinase activities from the supernatant of M. avidus. Viable CHSE-214 cells decreased significantly in number, when incubated in a medium supplemented with the culture supernatant of M. avidus. The growth of ciliates on CHSE-214 cells was delayed in the presence of PMSF (serine proteinase inhibitor) and E-64 (cysteine proteinase inhibitor). These results suggested that the culture supernatant contained ECPs showing cytotoxicity, and the proteinases facilitated nutrient uptake by the ciliates. Thus, ECPs may be responsible for virulence factors of M. avidus.

Key words: scuticociliatosis, Mamiensis avidus, extracellular proteinase, virulence factor

Scuticociliatosis is one of the most serious diseases in cultured fish species, causing economic losses in marine aquaculture worldwide. A number of scuticociliate species have been detected and/or isolated from diseased fishes, including Uronema marinum from aquarium fish, Philasterides dicentrarchi from sea bass Dicentrarchus labrax, turbot Scophthalmus maximus, the sea dragons Phycodurus eques and Phyllopteryx taeniatus (Cheung et al., 1980; Dragesco et al., 1995; Iglesias et al., 2001; Rossteuscher et al., 2008). Additionally, U. nigricans, Tetrahymena corlissi and Anophyroides haemophilus were reported in southern bluefin tuna Thunnus maccoyii, guppy Poecilia reticulata and American lobster Homarus americanus, respectively (Cawthorn et al., 1996; Munday et al., 1997; Imai et al., 2000). The ciliates are endoparasites that generate a systemic infection, invading internal organs such as the brain, liver, intestine and other tissues (Jung et al., 2007). The typical symptoms of scuticociliatosis are bleached spots, ulceration and hemorrhages on the skin.

Scuticociliatosis has also been observed in Japanese flounder Paralichthys olivaceus, one of the main cultured fish species in Japan and Korea. In addition to an unidentified scuticociliate, U. marinum, Pseudocohnilembus persalinus and Mamiensis avidus (syn. Philasterides dicentrarchi) have been reported as causative agents of the disease in both countries (Yoshinaga and Nakazoe, 1993; Jee et al., 2001; Kim et al., 2004a, 2004b; Jung et al., 2005). However, we demonstrated that M. avidus is the primary etiologic agent of the disease based on the results of experimental infections with four scuticociliate species: U. marinum, P. persalinus, P. hargisi and M. avidus (Song et al., 2009a). Among M. avidus isolates, we identified three serotypes based on the results of immobilization assays and western blotting: serotype I for the IyoI, JF05To, RF05To and SK05Kyo isolates; serotype II for the Nakajima isolate; and serotype III for the Mie0301 isolate (Song et al., 2009b). These serotypes corresponded to the cox1-genotypes of the strains but did not reflect their geographic origins, host species, or pathogenicity (Jung et al., 2011). Recently, it is revealed that the serotype-specific polypeptides of three serotypes were ciliary membrane proteins (Motokawa et al., 2018).
Regarding the outbreak of the disease, Takagishi et al. (2009) reported that lower-salinity conditions increased mortalities in challenged fish by experimental infection. As indicated above, the morphological, taxonomical, and genetic characteristics of scuticociliates have been extensively investigated, but the virulence factors of the ciliates have yet to be conclusively identified. Generally, proteinases are thought to play key roles in the infection process of protozoan parasites such as Tritrichomonas foetus, Tetrahymena spp. and Leishmania spp. (Leibowitz et al., 2009; Olivier et al., 2012; Tolbert et al., 2014). In M. avidus isolated from turbot in Spain, which was originally described as Philasterides dicentrarchi and later synonymized to M. avidus, intracellular proteinases and extracellular proteinases (ECPs) were also detected by Paramá et al. (2004). They reported that the intracellular proteinases and ECPs of M. avidus were almost similar to each other in terms of their electrophoretic profiles in gelatin-SDS-PAGE. Based on the similarity between intracellular and extracellular proteinases, they carried out the study with the assumption that intracellular proteinases and ECPs were identical. For example, Paramá et al. (2007a, 2007b) demonstrated chemotaxis inhibition and apoptosis induction by the proteinases in turbot proinflammatory leukocytes, using purified proteinases from homogenates of the ciliate (intracellular proteinases). However, it seemed that a slight difference was present between the electrophoretic profiles in substrate-SDS-PAGE between the intracellular proteinases and ECPs (Paramá et al., 2004), and the detailed substrate specificity of ECPs has been unclear. Moreover, cytotoxicity mediated by ECPs has not been directly demonstrated. In this study, therefore, we investigated the substrate specificity of ECPs derived from M. avidus using a peptide analogue 4-methylcoumaryl-7-amide (MCA) substrate assay and assessed the cytotoxicity of the ECPs to Chinook salmon embryo cells (CHSE-214). Moreover, in order to understand the role of the ECPs in ciliate growth, M. avidus was cultured on CHSE-214 cells in a medium supplemented with proteinase inhibitors.

Materials and Methods

Parasites

Three different serotypes of M. avidus (lyol, Nakajima and Mio0301) isolated from Japanese flounder during scuticociliatosis outbreaks at different farms in Japan (Song et al., 2009b) were used in this study.

Cell culture

CHSE-214 cells were maintained at 20°C in Eagle’s minimum essential medium (MEM, Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (abbreviated as MEM-FBS hereafter) in 25-cm² culture flasks. The CHSE-214 cell line was used to maintain the above-mentioned ciliate isolates.

MCA substrate assay

For MCA substrate assays, Escherichia coli JM109 cells were employed as feed for the ciliates to avoid proteinase contamination from FBS. The bacterium was cultured for 12 h at 37°C in Luria-Bertani broth medium supplemented with 1% glucose. The cultured bacterium (6.6 × 10⁸ colony forming units [CFU]/mL) was then autoclaved, washed twice by centrifugation at 1,800 × g for 10 min, and finally resuspended in MEM unsupplemented with FBS (abbreviated as MEM-N hereafter). Ciliates cultured on CHSE-214 cells were centrifuged at 1,000 × g for 5 min and then washed with MEM-N three times to remove the FBS. For each isolate, the ciliates were resuspended in MEM-N to a density of 3.0 × 10⁶ ciliates/mL. A total of 3.0 × 10⁵ ciliates and 1.0 × 10¹⁰ CFU of autoclaved E. coli were cultured with 15 mL of MEM-N in 75-cm² culture flasks at 20°C. After 6 days, the culture medium was centrifuged at 430 × g for 5 min, and the culture supernatant of M. avidus (abbreviated as M. avidus-SUP hereafter) was collected. We confirmed that this centrifugation did not crush the ciliate cells. In order to completely remove the ciliates, this step was repeated. Finally, the M. avidus-SUP was centrifuged at 2,330 × g for 5 min to remove E. coli cell debris. The M. avidus-SUPs were diluted with MEM-N in order to equalize the proteinase concentration based on the number of ciliates of each isolate and then used for the MCA substrate assay.

The proteolytic activity of the M. avidus-SUP was assayed using 19 types of peptide analogue MCA substrates (Peptide Institute) (Table 1). Each substrate was dissolved in dimethyl sulfoxide (DMSO) prior to use. All substrates were prepared as 1 mM stock solutions in DMSO. Incubation tubes were filled with 100-μL aliquots of the substrate stock solution and stored at −20°C until use. The assay was conducted as reported previously (Obayashi and Suzuki, 2005, 2008). For assays, 900 μL of the M. avidus-SUP was added to each tube to yield a final substrate concentration of 100 μM; the concentration was previously confirmed as the saturation level for Z-Phe-Arg-MCA using a proteinase sample from the Nakajima isolate (data not shown). Of the 19 substrates examined using the MCA assay, the supernatant of the Nakajima isolate exhibited the highest proteolytic activity against this substrate. Sample and substrate mixtures were incubated at 20°C for 2 h, before and after which the fluorescence of the hydrolytic product, 7-amino-4-methylcoumarin (AMC), was measured using a spectrofluorometer (F-2500, Hitachi) at excitation/emission wavelengths of 380/460 nm. After subtracting the blank fluorescence intensity of each proteinase sample (measured without substrate), the concentration of AMC in the incubation tube was calculated using a calibration curve obtained from several
concentrations of standard AMC. For the control, the supernatant of a culture without M. avidus was assayed using the same method described above and used to determine the level of non-enzymatically produced AMC. The substrate hydrolysis rates were estimated from the change in the concentration of AMC in the incubation tube after the concentration of non-enzymatically produced AMC was subtracted.

Cytotoxicity of the culture supernatant of M. avidus

*Miamiensis avidus* Iyo I isolate was cultured on CHSE-214 cells. The *M. avidus*-SUP collected as described above. An aliquot of the *M. avidus*-SUP was heated for 5 min to prepare heat-killed control of *M. avidus*-SUP. As a control, the cytoplasm solution of CHSE-214 cells was collected by sonication for 3 min in MEM-N on ice using a Sonifier 250 (Branson) operated at 30% duty cycle and output 3. The samples were concentrated by 15 times using Amicon Ultra-10K (Millipore).

To test the cytotoxicity of *M. avidus*-SUP, CHSE-214 cells were placed in a 24-well plate and cultured at 20°C in four different conditions: culture in MEM-FBS (no treatment control) and cultures in MEM-FBS supplemented (100 μL/well) with cytoplasm solution of CHSE-214, with *M. avidus*-SUP and with heated-*M. avidus*-SUP. After 12 h incubation, the culture supernatant was gently removed and the cells were treated with trypsin for 1 min. The cells in each well were then suspended with 1 mL of MEM-N and enumerated using a OneCell Counter (Bio-Medical Science). The experiments were carried out in triplicate using different initial cell densities of CHSE-214 cells (3.1 × 10⁵, 4.9 × 10⁵ and 1.6 × 10⁶ cells). The data are expressed as relative values to initial (pre-treated) cell numbers. Results were analyzed using Two-way ANOVA followed by the Tukey-Kramer multiple comparison tests, and differences were considered significant at *p* < 0.05.

Growth of M. avidus on CHSE-214 cells with proteinase inhibitor

In order to examine the importance of the ECPs in breaking food down into nutrients utilisable for ciliate growth, we cultured the ciliates on CHSE-214 cells in media with or without proteinase inhibitors. *Miamiensis avidus* Iyo I cells were cultured, washed, and resuspended in MEM-FBS to a density of 1.0 × 10⁴ cells/mL. A proteinase inhibitor cocktail containing aprotinin, bestatin, E-64, leupeptin and pepstatin A (Sigma-Aldrich) was diluted 300- or 900-fold with DMSO as recommended by the manufacturer. Next, 50 μL of ciliate suspension (2.0 × 10⁵ cells) and inhibitor was added to CHSE-214 cells cultured in MEM-FBS in a 25-cm² flask. As controls, the same volume of MEM-N or DMSO was added to the cells. At 30 h after inoculation, the same volume of proteinase inhibitor was added again. The ciliates were cultured until 114 h, with the number of ciliates determined using a OneCell Counter at 54, 66, 78, 90 and 114 h. Experiments were carried out in triplicate. Data are given as the mean ± S.D.

To reveal proteinase types which contribute to the digestion of CHSE-214 cells, five individual proteinase inhibitors were employed in inhibitor assay: phenylmethane sulfonyl fluoride (PMSF; serine proteinases inhibitor,
1 mM final conc.), leupeptin (cysteine and serine proteinases inhibitor, 20 $\mu$m final conc.), E-64 (cysteine proteinases inhibitor, 50 $\mu$m final conc.), pepstatin A (aspartic proteinase inhibitor, 10 $\mu$m final conc.) and EDTA (metallo-proteinases inhibitor, 1 mM final conc.). These were purchased from Sigma-Aldrich. Each proteinase inhibitor was dissolved with distilled water or ethanol as recommended by the manufacturer. Experiments were carried out by the same procedure as above.

Prior to analysis, the cell number was logarithmically transformed. Significant differences in the number between each inhibitor treatment and respective solvent control were tested by Mann-Whitney U test.

Results

Substrate specificity of the culture supernatant of M. avidus

The results of MCA substrate assays are shown in Fig. 1. Several substrates were hydrolyzed by the M. avidus-SUP, and the enzymatic hydrolysis pattern was similar among the three different serotypes of M. avidus described in our previous study (Song et al., 2009b). Among the tested substrates, 5 substrates, namely Z-Phe-Arg-MCA, Boc-Leu-Thr-Arg-MCA, Boc-Phe-Ser-Arg-MCA, Boc-Val-Leu-Lys-MCA and Boc-Glu-Lys-Lys-MCA, were readily hydrolyzed by these three M. avidus-SUPs, whereas low or no hydrolytic activity was detected for substrates of aminopeptidase and elastase. The highest proteinase activity was detected against Z-Phe-Arg-MCA (substrate for cathepsin B/L, plasma kallikrein and Arg-Gingipain). The substrates Boc-Glu-Lys-Lys-MCA and Boc-Val-Leu-Lys-MCA for plasmin were also well hydrolyzed by the M. avidus-SUPs.

Cytotoxicity of the culture supernatant of M. avidus

At 12 h post-treatment, the cell numbers in the no treatment control, and treatments with cytoplasm solution of CHSE-214, M. avidus-SUP and heated-M. avidus-SUP groups were 78%, 78%, 62% and 78% compared with pretreatment cell numbers, respectively (Fig. 2). The number of viable cells in the M. avidus-SUP treatment group was significantly lower than those in the control and the other treatments.

Effect of proteinase inhibitor on M. avidus cultured on CHSE-214 cells

At 30 h post-infection (hpi), CHSE-214 cells were lysed by histophagous M. avidus in the control and solvent-control groups (Fig. 3A and B), whereas cell lysis was inhibited in the presence of proteinase inhibitor cocktail (Fig. 3C and D). Most cells were lysed by the ciliates at 66 hpi in all groups (data not shown).

Ciliate growth was monitored until 114 hpi. At 54 hpi, the density of ciliates in the negative control, solvent control and low-dose proteinase inhibitor cocktail groups was $3.15 \times 10^4$, $2.38 \times 10^4$ and $1.99 \times 10^4$ cells/mL, respectively (Fig. 4). At this time point, the ciliate density in the high-dose proteinase inhibitor cocktail group could not be determined because of the low number of ciliates.
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The maximum density in the negative control, solvent control and low- and high-dose inhibitor-treated groups was $49.6 \times 10^4$ cells/mL at 114 hpi, $60.4 \times 10^4$ cells/mL at 66 hpi, $42.8 \times 10^4$ cells/mL at 66 hpi and $35.1 \times 10^4$ cells/mL at 114 hpi, respectively. The numbers of ciliates were significantly lower with high dose of proteinase inhibitor cocktail at 78, 90 and 114 hpi compared to the solvent control.

In assays using each individual inhibitor, the mean cell numbers at 72 hpi in PMSF, leupeptin and E-64 were $(4.2 \pm 1.2) \times 10^3$, $(6.7 \pm 2.4) \times 10^2$ and $(4.2 \pm 1.2) \times 10^3$ cells/mL, respectively, whereas that in their solvent control, solvent control, and low- and high-dose inhibitor-treated groups was $49.6 \times 10^4$ cells/mL at 114 hpi, $60.4 \times 10^4$ cells/mL at 66 hpi, $42.8 \times 10^4$ cells/mL at 66 hpi and $35.1 \times 10^4$ cells/mL at 114 hpi, respectively.

Fig. 2. Percent change of cell numbers at 12 h in control (no treatment), cytoplasm solution of CHSE-214 cells, culture supernatant of M. avidus and heated-culture supernatant of M. avidus treatment. The cell numbers were compared with that of pretreatment cells. Asterisk indicates a significant difference ($p < 0.05$) in multiple comparison test.

Fig. 3. CHSE-214 cells at 30 h post-infection with M. avidus Iyo I isolate. A: negative control, B: solvent (DMSO) control, C: low dose of protease inhibitor, D: high dose of protease inhibitor. There are many ciliates on the cells. Lysed cell spots are shown by dot circle. Scale bar = 100 μm.
control (0.1% ethanol) was $(12 \pm 2.4) \times 10^3$ cells/mL (Fig. 5). The numbers in pepstatin A and its control (1% ethanol) were $(4.2 \pm 1.2) \times 10^3$ and $(3.3 \pm 1.2) \times 10^3$ cells/mL, while those in EDTA and its control (0% ethanol) were $(4.0 \pm 2.5) \times 10^3$ and $(8.3 \pm 2.4) \times 10^3$ cells/mL, respectively. Comparing to respective solvent controls, the cell numbers of *M. avidus* in PMSF (serine proteinases inhibitor) and E-64 inhibitor (cysteine proteinases inhibitor) treatments were significantly lower ($p < 0.05$) at 72 hpi. The number of *M. avidus* in leupeptin (cysteine and serine proteinases inhibitor) was slightly lower than that in control, although the difference was not
Extracellular proteinases of *Miamiensis avidus* cause a systemic infection (Jung et al., 2007; Moustafa et al., 2010), the cathepsin-like proteinase(s) detected in this study might play roles in the degradation of host tissues and evasion of the host immune response.

We also examined whether *M. avidus*-SUP was cytotoxic, using CHSE-214 cells as model host fish cells. Our results showed that the proportion of intact CHSE-214 cells treated with *M. avidus*-SUP decreased significantly, in comparison with untreated control cells and cells treated with heated-*M. avidus*-SUP (Fig. 2). The cytotoxicity and proteolytic activities in the *M. avidus*-SUP suggested ECPs were responsible for cell lysis. To our knowledge, this is the first report demonstrating direct cytotoxic effects of ciliate ECPs on live cells, although histolytic activity of intracellular proteinases isolated from *M. avidus* has been reported so far (Paramá et al., 2004). In order to investigate the role of the ECPs during natural infection, we then cultured ciliates on CHSE-214 cells with and without proteinase inhibitors. As a result, with the proteinase inhibitor cocktail, PMSF (serine proteinase inhibitor) or E-64 (cysteine protease inhibitor), lysis of CHSE-214 cells and growth of ciliates were significantly inhibited (Figs. 4 and 5). With leupeptin (cysteine and serine proteinases inhibitor), growth of ciliates seemed to be inhibited, however, the difference was not statistically significant. The discrepancy between the result with leupeptin and those with PMSF and E-64 might be due to their mechanism of inhibition; PMSF and E-64 inhibit cysteine/serine proteinases by binding directly to their active site, whereas leupeptin is a competitive transition state inhibitor and the inhibition is not effective with excess substrates. In these growth experiments with proteinase inhibitors, it remained a possibility that the inhibition of growth of *M. avidus* resulted from inhibition of intracellular proteinases which were required to their cell growth or cell division. However, it was more likely that inhibition of ECPs caused growth inhibition of *M. avidus*, considering the consistent results of MCA assay (Fig. 1) and cytotoxicity (Fig. 2) of the *M. avidus*-SUP. All of these results suggest that the cysteine and serine-proteinase in ECPs are necessary for lysis of host cells and nutrient uptake by *M. avidus*, indicating that the ECPs are ciliate virulence factors.

Two of the highly hydrolyzed substrates by *M. avidus*-SUP in our experiment (Fig. 1 and Table 1), Boc-Glu-Lys-Lys-MCA and Boc-Val-Leu-Lys-MCA, are substrates for plasmin, a proteinase known to degrade thrombus for vascular patency (Castellino and Ploplis, 2005). As a part of the general host immune response, the blood clotting system is initiated to trap pathogens invading the blood vessels. As *M. avidus* causes a systemic infection via the circulation (Jung et al., 2005; Moustafa et al., 2010), blood clots could serve as obstacles to the spread of infection. The proteinases...
detected in the present study appear to play an important role in evading these obstacles via degradation of thrombi. Although no reports have described plasmin in scuticociliates, further investigation of these proteinases is worthwhile in order to clarify the details of the infection process of *M. avidus*.

In conclusion, we detected similar extracellular proteinase activities in culture supernatants of three different serotypes of *M. avidus*. These *M. avidus*-SUPs readily hydrolyzed substrates for cathepsin B/L and plasmin in particular, suggesting that ECPs of *M. avidus* might play important roles in the degradation of host proteins and the destruction of fibrin clots to facilitate invasion via the blood vessels. The *M. avidus*-SUPs were also cytotoxic in vitro and ECPs contained in the supernatant were likely involved in the cytotoxicity. The growth of ciliates on CHSE-214 cells was delayed in the presence of proteinase inhibitor cocktail, PMSF and E-64, suggesting that the serine and cysteine-proteinases facilitated nutrient uptake by the ciliates. Our findings thus suggest that the ECPs we described are virulence factors of *M. avidus*.

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**References**


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