Effect of iron on the proteolytic activity of live *Uronema marinum* (Ciliata: Scuticociliatida) measured by fluorescence polarization

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Effect of iron on the extracellular proteolytic activity of live *Uronema marinum* was determined by fluorescence polarization (FP) method. Supplementation of 0.5 and 5.0 μM iron significantly increased caseinolytic activity of live *U. marinum*. In contrast, supplementation of 50 μM iron showed no significant differences in FP values compared to the control. The present result suggests that iron in cultured water or skin tissue of olive flounder may influence on the penetration and establishment of *U. marinum*, correlating with modulation of extracellular protease activity of the ciliates.

**Key Words**: *Uronema marinum*, Protease, Iron, Fluorescence polarization

Several scuticociliate species previously regarded as being environmental scavengers, are being recognized as important opportunistic pathogens in marine fish world-widely (Thompson and Moewus, 1964; Cheung et al., 1980; Yoshinaga and Nakazoe, 1993; Dyková and Figueras, 1994; Dragesco et al., 1995; Gill and Callinan, 1997; Munday et al., 1997; Sterud et al., 2000; Iglesias et al., 2001, 2003). In Korea, scuticociliatosis has been one of the major culprits of severe losses of cultured olive flounder, *Paralichthys olivaceus* (Jee et al. 2001).

In the pathogenesis of parasitic diseases, proteases have been shown to play important roles in host tissue invasion, digestion of host proteins, and protection against immunological attacks by the host (McKerrow, 1989). As scuticociliates have a high potential for invading systemically and destroying fish tissues (Cheung et al., 1980; Sterud et al., 2000; Jee et al., 2001), proteases excreted by the parasite may play a major role in invasion of host tissues.

Although the infection route of scuticociliates is not clear, epithelium of gill and/or skin is supposed as a route of infection (Sterud et al., 2000), and iron participates in skin tissue damage through diverse pathological processes (Yeoh-Ellerton and Stacey, 2003). In the present study, we investigated the effect of iron on the proteolytic activity of live *Uronema marinum*, which was identified according to Kim et al. (2004), using fluorescence polarization (FP) assay.

The proteolytic activity of the live *U. marinum* was detected by incubating 5 × 10⁴ cells of the parasite in 10 μl PBS with 10 μl of 10 μg/ml of fluorescein isothiocyanate (FITC)-casein (Sigma) and 180 μl of PBS (pH 7.0) in a black 96-well plate (Greiner Bio-One Inc., USA) at 25°C. FeSO₄ was diluted with PBS (pH 7.0) and the final concentrations in 200 μl of a total reaction volume were 0.5, 5.0 and 50 μM. Wells not supplemented with iron were used as controls. In addition, wells with FITC-casein and each concentration of iron without the parasite were used to confirm whether iron influ-
ence on the fluorescence polarization (FP) value, and wells containing only 200 μL of PBS were designated as blanks.

The FP was measured in a Polarion instrument (TECAN Austria GmbH, Austria). The excitation wavelength was 485 nm and the emission wavelength was 535 nm. All assays were done in triplicate. Readings were automatically recorded at 0, 15, 30, 45, 60, 75 and 90 min in millipolarization units (mP).

The polarization is defined in Eq. (1) where \( I_{vv} \) is the fluorescence intensity when both the excitation and emission polarizers are parallel and \( I_{vh} \) is the fluorescence intensity when the excitation and emission polarizers are perpendicular.

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P = \frac{(I_{vv} - G \cdot I_{vh})}{(I_{vv} + G \cdot I_{vh})}
\]  

The instrument factor, \( G \), was set such that a 1 nM solution of FITC yielded a polarization value of 20 mP.

The Student’s \( t \)-test was used to determine the statistical difference between each concentration of iron and each relative control. Results were considered significant if \( P < 0.05 \).

The effect of iron on the proteolytic activity of live \textit{Uronema marinum} is shown in Fig. 1. Supplementa- 

The gradual drop in fluorescence polarization (FP) values with increase of incubation time indicates that the FITC-casein was cleaved by increasing excreted protease concentrations from live \textit{Uronema marinum}. Fluorescence polarization (FP) assay has the advantage of providing measurements of substrate hydrolysis by live parasites in real-time, which reflect actual conditions more precisely than other protease activity assays including radioactive counting, spectrophotometric or spectrofluorometric analysis and gel electrophoresis. Because measurements were taken in real-time, the progress of the reaction was followed both kinetically and at a single point. Additionally, FP assay is homogeneous and, as such, do not require separations, precipitations, or transfers of reaction mixture (Bolger and Checovich, 1994).

Caseinolytic activity of \textit{U. marinum}, in the present results, was significantly increased by iron supplementation. This result suggests that iron participates in the induction of metalloprotease expression. Iron is an essential nutrient for fish as well as microorganisms and the ability of pathogens to infect a host depends on its availability. Maintaining low concentrations of free iron in mucus membranes and in other tissues is thought to be one of the primary nonspecific host defenses against pathogens (Lall, 2000). In humans, iron level increased by skin lesions or ulcers (Yeoh-Ellerton and Stacey, 2003). Recently, Iglesias et al. (2003) reported that experimental infection of turbot by \textit{Philasterides dicentrarchi}, a scuticociliate
species, could be achieved by marking small scrape lesions on the gills and skin, and infection did not occur if the fish were not previously lesioned. Therefore, free iron in cultured water or skin of olive flounder may participates in the infection of *U. marinum* through modulation of extracellular protease activity.

**Acknowledgements**

This study was supported by a grant from the Ministry of Maritime Affairs and Fisheries, Republic of Korea.

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