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〈NOTE〉

Real Time Measurement of Protease Activity of Live *Uronema marinum* (Ciliata: Scuticociliatida) by Fluorescence Polarization Assay

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Proteolytic activity of live *Uronema marium* was analyzed by fluorescence polarization (FP) technique. Protease activity was measured by a decrease in FP value using fluorescein isothiocynate (FITC)-casein as a protein substrate. The results demonstrated an inverse linear relationship between fluorescence polarization (FP) values and live ciliate concentration over the range 1×10^4 to 2×10^5 cells/well. However, the FP values of $10\sim10^3$ live parasites were not different significantly from that of control. Time-dependent decrease in FP value was shown in the wells containing live *U. marinum*. In the present study, FP assay had the benefit to provide measurements of substrate hydrolysis by live parasites in real-time, and did not require separations, precipitations, or transfers of reaction mixture.

Key words: *Uronema marinum, Paralichthys olivaceus,* Proteases, Fluorescence polarization

Several scuticociliate species belonging to the genera *Uronema, Miamiensis* and *Philasterides* are facultative histophagous parasites in marine fish (Thompson & Moewus, 1964; Cheung et al., 1980; Yoshinaga & Nakazoe, 1993; Dykova & Figueras, 1994; Dragesco et al., 1995; Gill & Callinan, 1997; Munday et al., 1997; Sterud et al., 2000; Iglesias et al., 2001) The recent outbreaks of scuticociliatosis by *U. marinum* have resulted in severe losses of cultured olive flounder, *Paralichthys olivaceus,* in Korea (Jee et al., 2001).

Proteases are expressed in various cells and tissues of numerous species of living organisms. In the pathogenesis of parasitic diseases, proteases have been shown to play important roles in the facilitation of host tissue invasion, digestion of host proteins, and protection against immunological attacks by the host (McKerrow, 1989; 1993). As *U. marinum* has 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 would play a major role in invasion to host's tissues.

Fluorescence polarization (FP) is based on the principle that a fluorescent-labeled compound excited by plane polarized light will emit polarized fluorescent light into the same plane if there is minimal molecular movement between excitation and emission times. The smaller the molecular mass of the labeled molecule, the greater the motion will be and the lower the emitted fluorescence polarization value. Cleavage of a large fluorescently labeled substrate molecules results in a protease concentration dependent decrease in fluorescence polarization as the more mobile protease digestion products are released, and rapidly rotate to emit fluorescence out of the excitation plane of polariza-tion. Therefore, the decrease in fluorescence polarization value is a measure of proteolytic activity. In the present study, the proteolytic activity of live *U. marium* was analyzed in real time by fluorescence polarization technique.

U. marinium isolated from the brain of infected olive flounders (*P. olivaceus*) was cultured in Eagle's

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minimum essential medium (MEM; Sigma Chemical Co., USA) containing 10% fetal bovine serum (FBS; Sigma), 200 units/mL of penicillin G (Sigma), and 200 units/mL of streptomycin (Sigma) at 20℃. Parasites harvested from the cultures were washed 3 times in phosphate buffered saline (PBS) containing penicillin G and streptomycin by centrifugation at 1,000×g for 5 min at 4℃. The proteolytic activity of the live parasite was detected by incubating 1×10^1 to 2×10^5 cells of the parasite in 10 μL PBS with 10 μL of 10 μg/mL of fluorescein isothiocynate (FITC)-casein (Sigma) and 180 μL of PBS (pH 7.0) in a black 96-well plate (Greiner Bio-One Inc., USA) at 25℃. Wells without live parasites were used as controls and wells with only PBS were designated as blanks. The fluorescence polarization was measured in the 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, 30, 60 and 90 min in millipolarization units (mP).

The polarization (P) is defined as 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.

$$P=(I_{vv}-G\cdot I_{vh})/(I_{vv}+G\cdot I_{vh}) \quad (1)$$

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 control and experimental assays. Results were considered significant if $P<0.05$.

The results demonstrated an inverse linear relationship between fluorescence polarization (FP) values and live ciliate concentration over the range $1\times10^4\sim2\times10^5$/well (Fig. 1). However, the FP values of $10\sim10^3$ live parasites were not different significantly from that of control. The longer incubation time elicited the greater decrease of FP values.

Although Shade et al. (1996) used FP assay to measure proteolytic activity of whole bacteria, *Porphyromonas gingivalis*, so far as we know, there have been no reports on the measurement of proteolytic activity of protozoans by FP assay. No statistical differences in FP values by adding live ciliates at initial time indicated that the movement of the ciliates in the well of microplate did not interfere with the measurements. Time-dependent decrease of FP values indicated that proteases were excreted continuously by the ciliates.

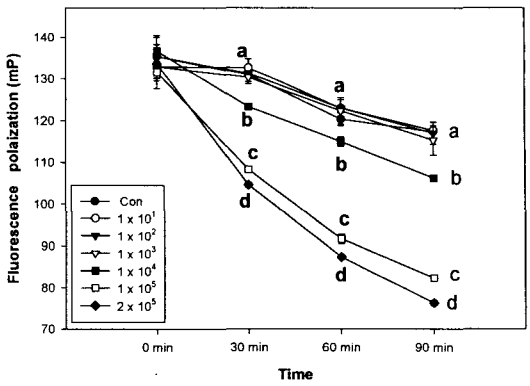

Fig. 1. Protease activity of live *U. marinum* at different cell numbers as measured by fluorescence polarization assay. Proteolytic activity was detected using FITC-casein as a substrate at pH 7. Points represent mean values ± standard deviation. Different letters represent significant difference at $P<0.05$.

In comparison to other assays of protease activity including radioactive counting, spectrophotometric or spectrofluormetric analysis and gel electrophoresis, FP assay has the benefit to provide measurements of substrate hydrolysis by live parasites in real-time. 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 & Checovich, 1994).

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