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# Proteases in Cell Lysate of *Uronema marinum* (Ciliata: Scuticociliatida), an Opportunistic Pathogen of Cultured Olive Flounder (*Paralichthys olivaceus*)

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The effects of pH, temperature and various inhibitors on the proteolytic activity of the cell lysate of *Uronema marium* were investigated using colorimetric and substrate gel electrophoretic methods. The cell lysate of *U. marinum* showed proteolytic activity over a wide range of pH, and pH optima ranged from pH 5 to 7. The proteolytic activity was increased according to a rise of temperature but decreased at 40℃. The proteolytic activity of the parasite lysate was significantly inhibited by protease inhibitors including trans-epoxysuccinyl -L-leucylamido-(4-guanidino) butane (E-64), pepstatin A, phenyl-methanesulfonyl fluoride (PMSF), and ethylenediamine-tetraacetic acid (EDTA). Preincubation of the lysate with E-64 showed the maximum inhibition of the caseionolytic activity. Four protease bands (152, 97, 67 and 40 kDa) were detected by gelatin SDS-PAGE. Significant inhibition of caseinolytic activity and complete abolition of a 152 kDa band in gelatin SDS-PAGE by EDTA indicated that the cell lysate of *U. marinum* had a metalloprotease. Another three proteolytic bands were inhibited by E64, a cysteine protease inhibitor. Preincubation of the cell lysate with pepstatin or PMSF had no effects on the protease bands.

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

## Introduction

Scuticociliatosis is defined as invasive external or systemic infection with the protozoan ciliates belonging to the genera *Uronema, Miamiensis* and *Philasterides*, and is being recognized as a fatal disease in marine fish (Thompson and Moewus, 1964; Cheung et al., 1980; Yoshinaga and Nakazoe, 1993; Dykova and Figueras, 1994; Dragesco et al., 1995; Gill and Calinan, 1997; Munday et al., 1997; Sterud et al., 2000; Iglesias et al., 2001). The recent outbreaks of scuticociliatosis by *Uronema marinum* have been resulted in severe losses of cultured olive flounder, *Paralichthys olivaceus*, in Korea (Jee et al., 2001).

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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).

Proteases are classified into four large groups including cysteine, serine, aspartic, and metallo- proteases. Among them, cysteine and metallo- proteases have been demonstrated to play potential roles in infection and invasion of pathogenic protozoans (Rosenthal, 1999). Although *U. marinum* has high potential for invading systemically and destroying fish tissues (Cheung et al., 1980; Sterud et al., 2000; Jee et al., 2001), little information is available concerning the proteases, which would be involved in

pathogenesis. Therefore, in this study, the effects of pH, temperature and various inhibitors on the proteolytic activity of the cell lysate of *U. marium* were investigated using colorimetric and substrate gel electrophoretic methods.

## Materials and Methods

### Isolation and culture of *Uronema marinum*

*U. marinium* isolated from the brain of infected olive flounders (*Paralichthys olivaceus*) was cultured in Eagle's 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℃ for 5 months.

### Preparation of cell lysate of *U. marinum*

Parasites harvested from the cultures were washed 3 times in Hank's balanced salt solution (HBSS, Sigma) containing penicillin G and streptomycin by centrifugation at 1,000×g for 5 min at 4℃. The suspension of washed parasites in HBSS containing 0.2% (v/v) Triton X-100 was sonicated for about 5 min on ice to lyse all the cells. After centrifugation at 10,000×g for 5 min at 4℃, the supernatant was used as the cell lysate (crude extract), and stored in aliquots at −70℃. Protein concentration in the cell lysate was determined by the bicinchonic acid assay, using bovine serum albumin as a standard (Smith et al., 1985).

### Protease assay

The proteolytic activity of the parasite lysate was detected using azocasein (Sigma) as the substrate. All assays were done in triplicate. Briefly, 100 μL of the cell lysate (1 mg protein $mL^{-1}$) was incubated with 100 μL of azocasein (100 mg $mL^{-1}$) and 0.6 mL of glycine or Hepes buffer for 24 hrs. Then, 0.75 mL of 5% trichloroacetic acid (TCA) was added to terminate the reaction. After centrifugation at 13,000 ×g for 5 min, the insoluble material was removed and the dye released was determined spectrophotometrically at 366 nm against the blank (the same incubation solution but with distilled water instead of cell lysate). Proteolytic activities were represented by the increase in absorbency.

### Effects of pH and temperature on proteolytic activity

Optimal pH of the proteases in parasite lysate was determined by measurement of the proteolytic activity using azocasein as the substrate over a pH range of 3.0~9.0 at 25℃. The buffers used were 0.1 M glycine buffer (pH 3.0~7.0) and 0.1 M Hepes buffer (pH 7.0~9.0). The optimal temperature for the proteolytic activity of parasite lysate was assayed at 10, 20, 30 and 40℃ using azocasein as the substrate at pH 5.0 and 7.0.

### Effects of inhibitors on proteolytic activity

The effects of inhibitors including trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64, Sigma), pepstatin A (Sigma), phenyl-methanesulfonyl fluoride (PMSF, Sigma), ethylenediaminetetraacetic acid (EDTA, Sigma) on the proteolytic activity of the parasite lysate were examined using azocasein as the substrate. The parasite lysate was incubated with each inhibitor for 30 min at 30℃ prior to adding substrate. The concentrations of inhibitors used for preincubation were 20 μM E-64, 2 mM EDTA, 0.1 mM pepstatin A, and 1 mM PMSF. Control was preincubated with distilled water. The level of inhibition was expressed as a percentage of the activity remaining (with inhibitor) of the control activity (without inhibitor).

### Substrate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The gels (10% separating gel and 5% stacking gel) used in SDS-PAGE were prepared as described by Laemmli (1970), except that the gelatin (0.1% w/v) as a protease substrate was incorporated into the separating gel. A 10 μL of the parasite lysate (100 μg of protein concentration) was mixed with SDS-sample buffer (0.5 M Tris-Cl, pH 6.8, 10% SDS, 20% glycerol and 0.02% bromophenol blue) without boiling. Following electrophoresis at 4℃, the gel was immersed in 2.5% (v/v) Triton X-100 for 1 hr to remove the SDS and rinsed three times in distilled water, and placed in glycine buffer (0.1 M, pH 5.5) for 12 hrs at 30℃. Following staining with Coomassie brilliant blue R-250 and several cycles of destaining with 40% methanol and 10% acetic acid, proteolytic activity toward gelatin is visible as a clear band on the Coomassie blue-stained back-

ground. Protease activity was inhibited by preincubating the lysate for 30 min at 30℃ with 20 $\mu$M E-64, 2 mM EDTA, 0.1 mM pepstatin A, and 1 mM PMSF. The molecular mass of an individual protease was determined from the standard separated in an adjacent lane.

### Statistical analysis

The Student's *t*-test was used to determine the statistical difference between control and experimental assays. Results were considered significant if $P<0.05$.

## Results

### Effects of pH and temperature on proteolytic activity

High proteolytic activities of the parasite lysate were observed over a range of pH 5.0~7.0 (Fig. 1). Proteolytic activities at pH 8.0 and pH 9.0 were lower than the activities at pH 5.0~7.0. No proteolytic activities were shown in pH 3 and pH 4. The proteolytic activity was increased according to a rise of temperature but decreased at 40℃ (Fig. 2).

### Inhibitory effects of various inhibitors

The proteolytic activity of the parasite lysate was significantly inhibited by all 4 protease inhibitors (except PMSF at pH 7) at both pH 5 and pH 7 (Fig. 3). Preincubation of the lysate with E-64 showed the maximum inhibition of the caseionolytic activity.

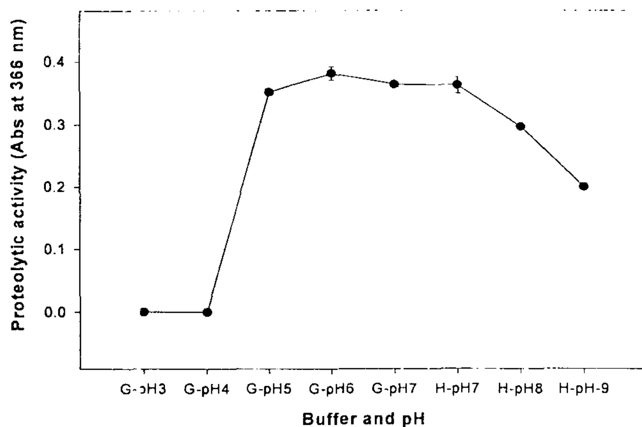


**Fig. 1.** **Effects of pH on the proteolytic activity of the *Uronema marinum* cell lysate. Proteolytic activity was detected using azocasein as the substrate in glycine buffer (G, pH 3~7) and Hepes buffer (H, pH 7~9) at 25℃. Points represent mean values ± standard deviation.**

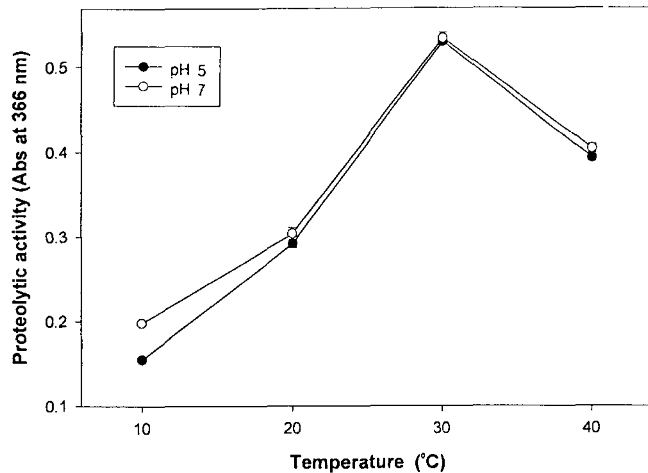


**Fig. 2.** **Effects of temperature on the proteolytic activity of the *Uronema marinum* cell lysate. Proteolytic activity was detected using azocasein as the substrate at pH 5 and pH 7. Points represent mean values ± standard deviation.**

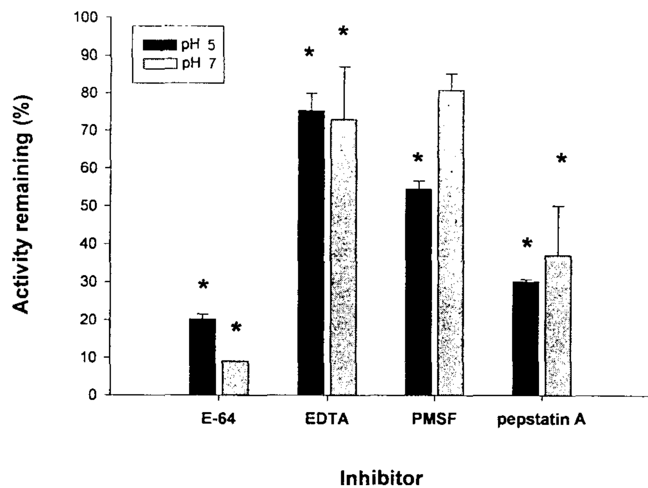


**Fig. 3.** **Effects of protease inhibitors on the proteolytic activity of the *Uronema marinum* cell lysate. Inhibitory effects were assayed using azocasein as the substrates. Activity remaining (with inhibitor) is expressed as a percentage of the control value (without inhibitor). Bars represent mean values ± standard deviation. [E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenyl-methanesulfonyl fluoride; *Significantly ($P<0.05$) lower than the control].**

### Gelatine SDS-PAGE

Four protease bands (152, 97, 67 and 40 kDa) were detected by gelatin SDS-PAGE (Fig. 4, lane A). The 152 kDa protease band was disappeared completely by preincubation of the cell lysate with EDTA (Fig. 4, lane C). The other three-protease bands (97, 67 and 40 kDa) were abolished by pretreatment of the lysate with E-64 (Fig. 4, lane B). Preincubation of the cell lysate with pepstatin or PMSF had no effects on the protease bands (Fig. 4, lanes C, D).

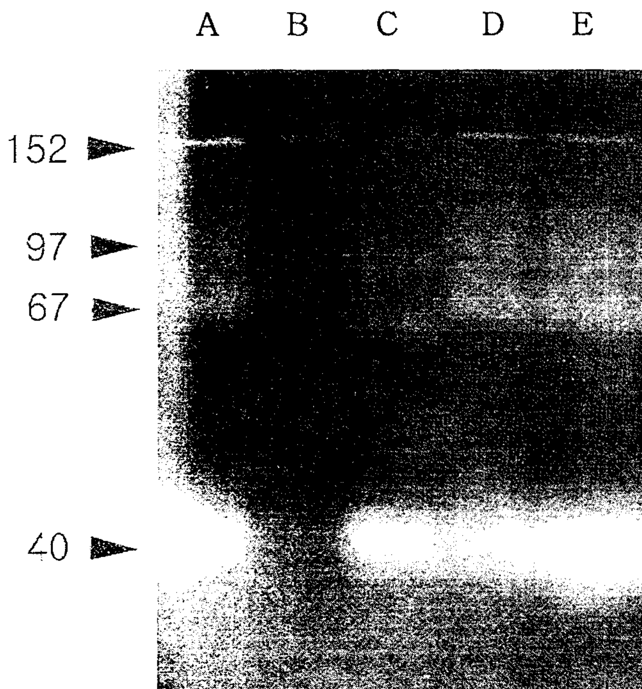


**Fig. 4. Gelatin SDS-PAGE of *Uronema marinum* cell lysate. The parasite lysate was preincubated in buffer alone (A lane) or with the protease inhibitors E-64 (B lane), EDTA (lane C), pepstatin A (lane D), or with PMSF (lane E). Molecular masses (kDa) of protease bands are indicated by arrowheads.**

## Discussion

In the present study, the cell lysate of *Uronema marinum* showed proteolytic activity over a wide range of pH, and pH optima ranged from pH 5 to 7. The decreased proteolytic activity at 40℃ indicated that the proteases were heat labile.

Significant inhibition of caseinolytic activity and complete abolition of a 152 kDa band in gelatin SDS-PAGE by EDTA in this study indicated that the cell lysate of *U. marinum* had a metalloprotease. Metalloprotease in some parasitic protozoa contributes to invasion and degradation of host tissues and thus plays an important role in the disease process (McKerrow et al., 1993). The functions of metalloproteases in pathogenesis have been well documented in *Cryptobia salmositica*, a haemoflagellate parasite of fish (Zuo and Woo, 1997a, b; 1998a, b; 2000).

In the present study, another three proteolytic bands (97, 67 and 40 kDa) were detectable by gelatin SDS-PAGE and were inhibited by E64, a cysteine protease inhibitor. The bands may represent multimers of the same enzyme or activity of several enzymes. Suzuki et al. (1998) detected two cysteine protease bands (28 and 48 kDa) in the cell lysate and only a 28 kDa cytein protease (tetrain) in the culture medium of *Tetrahymena pyriformis*, a facultative parasitic ciliate of freshwater fish. They suggested that tetrain was synthesized in the cells in a precursor form (48 kDa component), and then secreted into the culture medium as a mature form (28 kDa component). Cysteine proteases in parasitic protozoa have been assumed to have metabolic and physiologic roles (North, 1992). This protease has a function to degrade the intracellular protein and remodel the parasite during transformation between stages (Harth et al., 1993). Cysteine proteases are critical to host invasion in a number of human protozoan parasites including *Trypanosoma cruzi* (Franke de Cazzulo et al., 1994), *Plasmodium falciparum* (Mayer et al., 1991), *Cryptosporidium parvum* (Forney et al., 1996), and *Entamoeba histolytica* (Que and Reed, 2000).

In this study, inhibition of proteolytic activity of the parasite lysate by pepstatin and PMSF was observed in azocasein assays but not in gelatin SDS-PAGE gel assays. This may be due to loss of activity or assay insensitive under the conditions used.

As *Uronema marium* has high potential for invading systemically and destroying fish tissues, metallo- and cysteine proteases could be involved in the invasion and pathogenicity. Further research into the biochemical nature and the functions of the proteases in *U. marinum* may provide us with a better understanding of the disease process in diseases caused by scuticociliates.

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