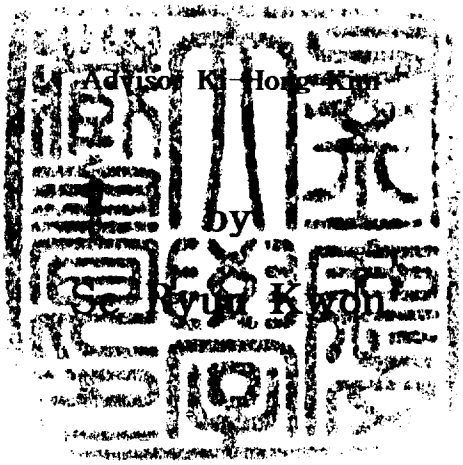
The infection of *Uronema marinum* (Ciliata: Scuticociliatida) and non-specific immune responses of olive flounder, *Paralichthys olivaceus*

*Uronema marinum*의 감염과 넙치, *Paralichthys olivaceus*의 면역반응



Department of Fish Pathology

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The infection of *Uronema marinum* (Ciliata: Scuticociliatida) and non-specific immune responses of olive flounder, *Paralichthys olivaceus*

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Abstracts

Relationships between non-specific immune responses of olive flounder (*Paralichthys olivaceus*) against *Uronema marinum* and escaping abilities of the scuticociliate against host immune responses were investigated. To evaluate effects of the parasite on the host's cellular immune response, live parasites and excretory-secretory (ES) products of the ciliates were co-incubated with olive flounder phagocytes, and measured respiratory burst activity of phagocytes using chemiluminescence (CL) assay. Effect of stress-mediated suppression of non-specific humoral immune responses of olive flounder including serum lysozyme activity, alternative complement haemolytic activities (ACH_{50}) and serum parasiticidal activity was analyzed and compared with those of rockfish (*Sebastes schlegeli*). Proteases and antioxidative enzymes of the ciliate were analyzed as evasive and/or virulent factors against host reactions. Live

scuticociliates and ES products had a negative and dose dependent effects on CL responses of olive flounder. ES products lysed the host phagocytes and showed considerably high activities of superoxide dismutase (SOD) and catalase. Parasiticidal activity of olive flounder serum was significantly lower than that of rockfish serum. The parasiticidal activity of olive flounder serum was highly suppressed by acute stress. Serum lysozyme and alternative complement activities, also, much lowered by stress. The optimal conditions of *U. marinum* proteases were pH 5.0~7.0 and 30~40℃. From the results by gelatine SDS-PAGE and colorimetric measurements, 152 kDa band was metalloprotease and 40 kDa band was cysteine protease because these were abolished or become weaker when treated with EDTA and E-64, respectively. The proteolytic activity of *U. marinum* was widely different between long-term culture (LTC) and short-term culture (STC). E-64 inhibited proteolytic activity of both LTC and STC in similar rates. However, inhibition of proteolytic activity by EDTA in STC was higher than that in LTC. Protease activity of *U. marinum* was inhibited by co-incubation with serum of olive flounder and rockfish. Rock fish serum showed higher inhibition of the ciliate proteolytic activity than olive flounder serum. The present results suggest that *U. marinum* can resist to the host phagocyte's oxidative stress by scavenging ROIs with antioxidant enzymes and can establish infection by destruction or suppression of the host's immune factor with various proteolytic enzymes. Therefore, further researches on the improvement of innate immunity of olive flounder and the development of specific inhibitors of the ciliate's enzymes including proteases and antioxidatives are needed to control scuticociliatosis effectively.

Key words: *Uronema marinum*, *Paralichthys olivaceus*, non-specific immune responses, chemiluminescence, parasiticidal activity, antioxidative enzymes, proteases

Introduction

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 and Nakazoe, 1993; Dykova and Figueras, 1994; Dragesco *et al.*, 1995; Gill and Callinan, 1997; Munday *et al.*, 1997; Sterud *et al.*, 2000; Iglesias *et al.*, 2001). These ciliates are characterized by their high potential for invading systemically and destroying fish tissues, leading to high mortalities in cultured fish.

In Korea, scuticociliatosis is a serious problem in culturing olive flounder *Paralichthys olivaceus*, and the causative agent is identified as *Uronema marinum* by morphological characteristics (Jee *et al.*, 2001). Although there have been some reports on the in vitro killing of the scuticociliates by several chemotherapeutics (Yoshimizu *et al.*, 1993; Mark *et al.*, 1996; Choi *et al.*, 1997; Alastair *et al.*, 1999), any effective control measures in vivo or in culturing fields have not been developed. To develop an efficient controlling measure on scuticociliatosis, knowledges on the factors related to the infection of the ciliates including immunological responses of the host against the ciliates and escaping ability of the parasite from host's responses are essential. However, little information is available on the infection mechanism of the scuticociliate. Therefore, in the present study, interactions between fish non-specific immune responses and infectivity of *U. marinum* were investigated.

Systemic infection of scuticociliates in fish associates with inflammatory cellular infiltrate consisting of macrophages, lymphocytes and granulocytes at the site of infection (Munday *et al.*, 1997; Sterud *et al.*, 2000; Iglesias *et al.*, 2001; Jee *et al.*, 2001). However, very little is known about the evasive mechanisms of the parasite in escaping the hosts cellular defense mechanisms. Activation of the respiratory burst of granulocytes and macrophages by invading microorganisms is a key first line cellular defence against infection (Secombes, 1996). When

vertebrate macrophages are stimulated by foreign particles or organisms, stimulation of NADPH oxidase and activation of the hexose monophosphate pathway occurs. This process is accompanied by production of toxic reactive oxygen intermediates (ROI) such as OH , H_2O_2, O_2, and O_2 , which are involved in cellular killing of pathogens. Also, H_2O_2, along with myeloperoxidase and halide ions, results in the formation of hypohalites and singlet oxygen, which are microbicidal. Failure to generate this response leads to persistent life-threatening infection. In *Ichthyophthirius multifiliis*, Cross and Matthews (1993) reported that leukocytes are generally degraded within the necrotic tissue layer surrounding the trophonts, presumably due to lytic enzymes and/or metabolites released by the parasite. Survival and establishment of systemic infections of scuticociliates in spite of intimate contact with cellular immune components of their hosts, also, suggests that scuticociliates are pro-active in disrupting the initiation or expression of host's cellular immune responses. Therefore, in this study, the protective mechanism of *U. marinum* against the toxic reactive oxygen intermediates (ROIs) produced by respiratory burst of olive flounder phagocytes was investigated.

Fish can counteract against parasite infections by a number of non-specific humoral immune reactions. The complement is an important part of the humoral defence system against pathogens. It has been known that teleosts complement possess both classical and alternative pathway activity, and can form a terminal membrane attack complex (MAC) to cause lysis of pathogens. According to the results of Sigh and Buchmann (2001), theronts of *I. multifiliis* were immobilized and lysed by non-immune fish serum through activation of alternative complement pathway. Of the humoral immune factors, lysozyme is the most abundant and widespread enzyme, and splits the exposed peptidoglycan wall of susceptible bacteria (Roitt, 1997). Thus, in this study, alternative complement activity (ACH_{50}), lysozyme activity, and parasiticidal activity of olive flounder serum were investigated in relation to stress, and were compared with rockfish (*Sebastes schlegeli*), which is resistant comparatively against scuticociliates.

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). The presence of proteases is a very common finding in crude extracts or excretory-secretary products of protozoan and metazoan species (Bolla and Weinstein, 1990; Deguercy *et al.*, 1990; Bozner and Demes, 1991; Hunter *et al.*, 1992; Armas-Serra *et al.*, 1993). However, few report is known about the proteolytic enzymes of *U. marinum*. Zuo and Woo (1997a) reported that the pathogenic strain of *Cryptobia salmositica* has both cysteine protease and metalloprotease, but the parasite loses its metalloprotease as it becomes avirulent during in vitro culture (Zuo and Woo, 1997a). The α2 macroglobulin is a protease inhibitor found at relatively high concentration in plasma. It is capable of inhibiting most proteases regardless of their class or substrate specificity (Barrett *et al.*, 1981; Swotter-Jensen *et al.*, 1989; Chu and Piazzo, 1994; Feinman, 1994). Inhibition is result of a conformational change in the inhibitor which traps the protease in a cage-like structure. The conformational change is induced after cleavage of the inhibitor's bait region by the target protease. The bait region is approximately a 30-residue segment of sequence susceptible to cleavage by most proteases. There was the report suggesting that the metalloprotease secreted by *C. salmositica* was neutralized by α2 macroglobulin in the blood of fish (Zuo and Woo, 1997c). And α2 macroglobulin in rainbow trout (*Oncorhynchus mykiss*) and brook charr (*Salvelinus fontinalis*) inhibited *C. salmositica* proteases under in vitro conditions (Zuo and Woo, 1997a). Therefore, in this study, general characteristics of *U. marinum* proteases according to pH, temperature, inhibition profiles and pattern of bands on substrate SDS-PAGE were examined in order to find the invasion and escaping ability of parasite. Furthermore, the difference in proteolytic activity between a short-term culture (STC) and a long-term culture (LTC) of *U. marinum* was investigated, and in vitro neutralization of *U. marinum* proteases by the serum of olive flounder was compared with that of rockfish.

Materials and Methods

1. Fish

Olive flounder (*Paralichthys olivaceus*) weighting 110 ± 8 g and rockfish (*Sebastes schlegeli*) weighting 104.2 ± 29 g were obtained from a local fish farm and maintained in 0.5 ton fiberglass tanks supplied with fresh seawater (salinity 33‰) everyday at 20℃ prior to the experiment. They were fed daily with a dry commercial pelleted diet.

2. Collection of serum

Olive flounder and rockfish were anaesthetized in MS222. The blood sample was withdrawn from the caudal vein with non-heparinized syringe and immediately centrifuged at 2,500 g for 5 min at 4℃. For analysis of non-specific humoral immunity, serum was separated in small aliquots and stored at -70℃ until used.

3. Isolation and culture of *Uronema marinum*

U. marinium was isolated from the brain of an infected olive flounder (*Paralichthys olivaceus*). According to the aim of the experiments, the isolated ciliates were cultured in different environments and/or culture periods. In this study, the following three kinds of cultures were prepared; long-term culture (LTC) was cultured ciliates in egles minimum essential medium (MEM; Sigma) containing 200 units/㎖ of penicillin G (Sigma) and 200 units/㎖ of streptomycin (Sigma) at 20℃ for about 2 years, short-term culture (STC) of the ciliates was obtained by culturing at 20℃ in Hank's balanced salt solution (HBSS, Sigma)

containing brain slices of olive flounder within 1 week and mid-term culture (MTC) was cultured ciliates at 20℃ in MEM for about 5 months.

4. Effects of *U. marinum* on the Chemiluminescent (CL) response of phagocytes

4.1. Preparation of parasite antigen

The MTC was harvested and washed 3 times in HBSS containing the same concentration as above of penicillin and streptomycin by centrifugation at 1,000g for 5min at 4℃. Excretory-secretory (ES) products were collected by incubation of the washed ciliates in HBSS at 20℃ for 24h. The incubation medium was centrifuged at 1,000 g for 10 min at 4℃ and the supernatant was concentrated using Centricon-10 (AMICON, Beverly, MA, USA) with a size exclusion of 10 kDa. Protein concentration was determined by the method of bicinchoninic acid assay (BCA, Smith *et al.*, 1985). The concentrated ES products were stored in aliquots at -70℃. The live ciliates used in CL assay was enumerated with a hemocytometer and diluted in HBSS to be a proper number for the experiment.

4.2. Chemiluminescence (CL) assay

Olive flounder was anaesthetized with tricaine methanesulfonate (MS222; Sigma) and sacrificed to separate the kidney. And then the kidney was passed through a 100㎛ nylon mesh using HBSS containing heparin (10 units/㎖ Sigma), penicillin (100 units/㎖) and streptomycin (100 units/㎖). The resulting cell suspension was placed on 34/51% percoll density gradient and centrifuged at 500g for 30min at 4℃. The interphase was collected and the phagocytes including neutrophils and macrophages were washed 3 times in HBSS. The cell viability was examined with trypan blue exclusion and was evaluated to be greater than 95%. The all number of phagocytes were adjusted to 1×10^6/㎖

HBSS.

The reactive oxygen intermediates (ROIs) produced by stimulated phagocytes were quantified using an automatic photoluminometer (Bio-Orbit 1251, Finland). Each test cuvette contained 0.7㎖ of luminol (Sigma) made according to the method of Scott and Klesius (1981), 0.4㎖ of phagocytes suspension, and 0.4 ㎖ of live parasites at 4:1, 2:1 and 1:1 phagocytes:parasites ratios or 0.4 ㎖ of ES products containing 0.3, 0.15 and 0.075 ㎎ protein/㎖ HBSS. In control cuvettes, 0.4 ㎖ HBSS was added instead of live parasites or ES products. To confirm the direct stimulatory effects of the parasites antigens on phagocytes, CL responses were measured for 20 min prior to adding boiled zymosan without opsonization, then, 0.3 ㎖ zymosan was added to each cuvette. The measurements of each triplicated sample were made for 100 min and the light emission was recorded as mV. All assays were done in triplicate.

After CL assay, the viability and lysis of phagocytes in each cuvette were examined by trypan blue exclusion test.

5. Determination of superoxide dismutase (SOD) and catalase activity of ES products and somatic extract

SOD was determined by the method of McCord and Fridovich (1969) based on the inhibition of the reduction of ferricytochrome C in the presence of O_2 generation by superoxide dismutase. The reaction mixture consisted of 0.1 ㎖ of 0.3 mM ferricytochrome C (Sigma), 0.1 ㎖ of 1.5 mM xanthine (Sigma), 2.7 ㎖ of 0.05 M potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA. The reaction was initiated by the addition of 0.1 ㎖ of xanthine oxidase. The cuvettes were incubated in spectrophotometer at 20°C for 5 min and measured the increase in absorbance at 550 nm for 3 min. Several dilutions of xanthine oxidase were used to produce a rate of reduction of ferricytochrome C at 550 nm of 0.025 absorbance unit/min. Addition of 0.1 ㎖ of different dilutions of SOD (Sigma) to the incubation mixture yielded an inhibition of ferricytochrome

C reduction. Under these conditions, the amount of SOD (or ES products) required to inhibit the rate of reduction of cytochrome C by 50% is defined as 1 unit of activity.

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 1.95 ㎖ of 0.05 M phosphate buffer (pH 7.0), 1 ㎖ of 0.02 M hydrogen peroxide (Sigma) and 0.05 ㎖ of ES products in a final volume of 3 ㎖. Change in absorbance was recorded at 240 nm for 5 min. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/㎎ protein.

6. The activity of alternative complement pathway (ACH_{50})

The activity of alternative complement pathway was assayed with small modification based on method of Sirois *et al.* (1999). Complement activity was measured in serum by a colorimetric haemolytic assay using isolated erythrocytes from rabbit. Briefly rabbit blood was withdrawn from an ear vein into a anticoagulant buffer (ACD; 3.15% sodium citrate and 2.45% glucose, pH 7.4) treated syringe. Blood was centrifuged at 500 g for 10 min and erythrocytes were isolated, washed with ACD and finally washed twice with gelatine veronal buffer solution (GVBS) supplemented with EGTA (GVBS-EGTA: 7 mM Sodium barbital, 0.15 mM NaCl, 10 mM $MgCl_2$, 0.15 mM $CaCl_2$, 10 mM EGTA, 0.1% gelatine, pH 7.4). The cells were resuspended in GVBS-EGTA to concentration of 5×10^8 cells/㎖. The assay was performed with 30 ㎕ of the erythrocyte suspension which were incubated with 100 ㎕ of serum dilution in GVBS-EGTA (1:4, 1:8, 1:16, 1:50, 1:64, 1:80, 1:100, 1:128, 1:512). Samples were incubated for 45 min at 20℃. At the end of incubation time, 30 ㎕ of 0.2 M EDTA were added to stop the reaction and samples were centrifuged for 3 min at 1,600 g. An aliquot of 100 ㎕ of the supernatant was taken and the absorbance of free haemoglobin was measured at 405 nm on a Bio-kinetics readers (EL 312e, Bio-Tek instruments). Negative controls were done by adding 30 ㎕ of 0.2 M EDTA before the erythrocyte suspension. Spontaneous lysis (0%) and total lysis

(100%) were also done by incubating 30 ㎕ erythrocytes with 130 ㎕ of GVBS-EGTA or 130 ㎕ water, respectively.

Lysis percentage (Y) was calculated for each dilution of plasma as:

$$Y = \frac{ODsample - OD(-)control}{OD100\%\,lysis - OD0\%\,lysis} \times 100$$

For the calculation of complement concentration giving rise to 50% of lysis via the alternative pathway (1 ACH_{50} unit), results were modified by Klerx *et al.* (1983) as a plot of:

log(Y/100-Y) vs. log(plasma dilution)

Complement activity (ACH_{50}) is given in U/㎖ and calculated as follows:

$ACH_{50} = 10^{1+P}$

Where P is graphically determined as the point of intersection with the x axis.

7. Lysozyme activity

The lysozyme activity of serum was determined by a turbidimetric method (Ellis, 1990). A suspension of *Micrococcus lysodeikticus* (0.2㎎/㎖ phosphate buffered saline, pH 6.0) is mixed with 100㎕ of serum to give a final volume of 2㎖. The reaction is carried out at 25℃ and the absorbancy was read at 0.5min and 4.5min intervals at 530nm. The unit of lysozyme activity was defined as the amount of lysozyme that caused a decrease in absorbancy of 0.001/min.

8. Parasiticidal activity

The parasite killing activity of serum was assessed in the following manner. Plasma was diluted in HBSS (pH 7.4) as follows; 1:16, 1:32, 1:40, 1:64, 1:80, 1:128, 1:160, 1:256, 1:512. The diluted serum was mixed with live parasite (MTC) suspension (10^4 cell/㎖) at 10:1 mixing ratio. The mixture was incubated at room temperature for 2 days. Lysed or immobilized parasites were detected by the dissected microscopy during the incubation period.

To compare the parasiticidal activity according to fish species, the parasiticidal activity of rockfish, *Sebastes schlegeli* highly resistant to scuticociliates was assayed as the same regime of olive flounder serum.

9. Effects of stress on non-specific immune response of olive flounder

For this experiment, the fish were divided into two groups of five fish each (stressed group and control group) and maintained in 200 ℓ fiberglass reinforced plastic tanks prior to the experiment. Stressed fish were quickly netted and exposed to air for 1min at 10min intervals. After ten times treatments, stressed and non-stressed fish were sacrificed and used to collect the plasma.

Non-specific humoral immunity including the lysozyme, ACH_{50}, parasiticidal activities were estimated as mentioned above. And then, comparison between stressed group and non-stressed group was performed. Plasma glucose as an indicator of stress response was also measured using a glucose oxidase/peroxidase enzymatic assay kit (Sigma). Plasma was mixed with potassium phosphate buffer containing glucose oxidase. Glucose solution (2 ㎎/㎖) was also mixed with the buffer containing glucose oxidase as a standard. After incubation at 20℃ for 10 min, absorbance was detected at 500 nm against the buffer as blank. The concentration of glucose was determined as follow:

$$\text{Glucose (㎎/㎗)} = \frac{A_{sample}}{A_{standard}} \times 200$$

10. General characteristics of *U. marinum* proteases

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

The MTC was used in this experiment. When a large number of the parasites were obtained, a suspension of parasite pellet in distilled water containing 0.2% (v/v) Triton X-100 was sonicated at 30sec intervals for 1.5min on ice to lyse all the cells. After centrifugation at 1,000g for 5min at 4℃, the

supernatant was used as the cell lysate (crude extract). Protein concentration of parasite lysates was measured by the BCA (bicinchoninic acid) assay (Smith *et al.*, 1985) using bovine serum albumin as reference.

10.2. Protease assay

The characterization of proteolytic activity in the parasite lysate was detected using azocasein as the substrate. The assay conditions were slightly modified from Coomb's method (1982). All assays were done in triplicate. Briefly, 100㎕ of the sample (1 ㎎/㎖) was incubated with 100㎖ of azocasein (10 ㎎/㎖) and 0.6㎖ of glycine or HEPES buffer (0.1 M, various pH) at 25℃ for 24h. After incubation, 0.75㎖ of 5% trichloroacetic acid (TCA) was then added to terminate the reaction. After centrifugation at 13,000g for 5min, the insoluble material was removed and the dye released was determined colometrially at 366nm against the blank that was the same incubation solution but with distilled water instead of protease sample). The activity was given in units 1㎍ substrate protein hydrolyzed per hour ㎎ protein of the lysates. An increase of 1 absorbance unit represented the hydrolysis of 0.4㎎ azocasein.

10.3. Effects of pH and temperature on proteolytic activity

The optimal pH of the parasite lysate was assayed using azocasein as the substrate in the following buffer systems: 0.1 M glycine buffer (pH 3.0~7.0), 0.1 M HEPES (pH 7.0~9.0). Each mixture was incubated at 25℃ and 37℃ for 24 h, then the dye released was detected as previously mentioned.

The proteolytic activity of parasite lysate was determined at various temperature, 10℃, 20℃, 30℃ and 40℃ using azocasein as a substrate at pH 5.0 and 7.0.

10.4. Inhibitory effects of various inhibitors

The effects of trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), pepstatin A, phenyl-methanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) on the protease activity were examined. Protease inhibitors were assayed using azocasein and gelatine as a substrates in colormetric assay and substrate SDS-PAGE, respectively. The parasite lysate was mixed with each inhibitor at 37℃ for 30 min 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. After the mixtures were incubated at 37℃ for 24 h, 5% TCA was added, then centrifuged. The dye released was determined at 366 nm. The level of inhibition was expressed as a percentage of the activity remaining (with inhibitor) of the control activity (without inhibitor).

In gelatin SDS-PAGE, samples were mixed with inhibitors at 37℃ for 30 min and then electrophoresed. After then, gel was incubated in 2.5% Triton X-100, and developed with 0.1 M glycine buffer (pH 5.0). The clear bands were detected by staining with Coomassie blue R-250.

10.5. Substrate 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. 10 $\mu\ell$ of protease sample (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 according to the method of Zuo and Woo (1998). Electrophoresis was performed at constant current of 15 mA/gel at 4℃ until bromophenol blue ran at the bottom of the gel (takes about 3 h). After electrophoresis the gel was immersed in 2.5% (v/v) Triton X-100 for 1 h at 4℃

to remove the SDS and washed three times with glycine buffer (0.1 M, pH 5.0). Protease bands were developed by immersion of gels in glycine buffer at 37℃ for about 12 h. Zones of proteolysis appeared as clear bands against a blue background after the staining with Coomassie Blue R-250.

11. Comparison of proteolytic activity between different cultures

In this assay, LTC and STC were used. The collected parasite pellets (number of parasites, 2×10^7) in distilled water containing 0.2% (v/v) Triton X-100 were sonicated at 30sec intervals for 1.5min on ice to lyse all the cells. After centrifugation at 1,000g for 5min at 4℃, the supernatant was used as the cell lysate (crude protease extract).

Proteolytic activities of LTC and STC were determined on optimal condition; 20℃ and 30℃, pH 5 and pH 7. Azocasein was used as a substrate. All assays were done in triplicate. Inhibitory effects of E-64, pepstatin A, PMSF and EDTA on the proteolytic activity were examined. The concentration of each reagent was presented in Table 2. The parasite lysate was mixed with each inhibitor at 37℃ for 30min prior to adding substrate. Appropriate control was preincubated with distilled water. After the mixtures were incubated at 37℃ for 24h, 5% TCA was added, then centrifuged. The dye released was determined at 366nm. The level of inhibition was expressed as a percentage of the activity remaining (with inhibitor) of the control activity (without inhibitor).

12. Effect of fish serum on parasite proteases

Olive flounder and rockfish were sacrificed to obtain the serum. Protein concentration of collected serum was measured by the BCA assay. Each serum was separated in small aliquots and stored at -70℃ until used.

To determine the protease inhibitory activity of fish serum, 100 ㎕ serum (100 ㎍/㎖) was preincubated with the parasite lysate at room temperature for

10 min. Appropriate control was preincubated with distilled water. Azocasein as a substrate and 0.1 M glycine buffer (pH 5.0) were added. After the mixtures were incubated at 37℃ for 24h, 5 % TCA was added, then centrifuged. The dye released was determined at 366 nm. The level of inhibition was expressed as a percentage of the activity remaining (with serum) of the control activity (without serum).

13. Statistical analysis

One-way analysis of variance (ANOVA), followed by the Tukey multiple comparisons test was employed to evaluate the level of significance in CL response and protease activity. The Student's *t*-test was used to determine statistical differences in other assays. The results were considered significant when $P<0.05$.

Results

1. CL responses and viability of olive flounder phagocytes by live *U. marinum* and it's ES products

Live *U. marinum* inhibited CL responses dose-dependently of zymosan-stimulated phagocytes (Fig. 1). Significant reduction in CL response was observed compared with controls when phagocytes were incubated with live *U. marinum*. However, there was no statistical significance in suppression of the CL response of phagocytes incubated with the ciliates at 4:1 and 2:1 ratios. Phagocytes incubated with the ciliates at 1:1 ratio showed significantly lower CL response than phagocytes incubated with the ciliates at 2:1 and 4:1 ratios. ES products from *U. marinum* similarly inhibited CL responses of zymosan-stimulated phagocytes in a dose dependent manner (Fig. 1). The reduction of CL response was significantly higher in phagocytes exposed to 0.15 and 0.3 ㎎/㎖ of ES products as compared to live ciliates or controls. Stimulation of phagocytes with live *U. marinum* or ES products alone did not elicit any CL responses (data not shown).

After CL assay, the number of phagocytes showing viability was significantly reduced in the cells incubated with live *U. marinum* at 2:1 and 1:1 phagocytes: ciliates ratios or ES products with 0.3 ㎎ protein/㎖^{1} when compared to control (Fig. 2). Lysis of phagocytes by exposure to ES products was observed.

2. Superoxide dismutase and catalase activity of ES products from *U. marinum*

ES products from *U. marinum* showed considerably high antioxidative enzyme activities. SOD activity of ES products was 332.84 ± 52.50 units mg^{-1} protein (mean ± SD, assayed in triplicate). Catalase activity in enzyme solutions prepared from ES products was 565.13 ± 8.08 μM H_2O_2 consumed min^{-1} mg^{-1} protein (mean ± SD, assayed in triplicate).

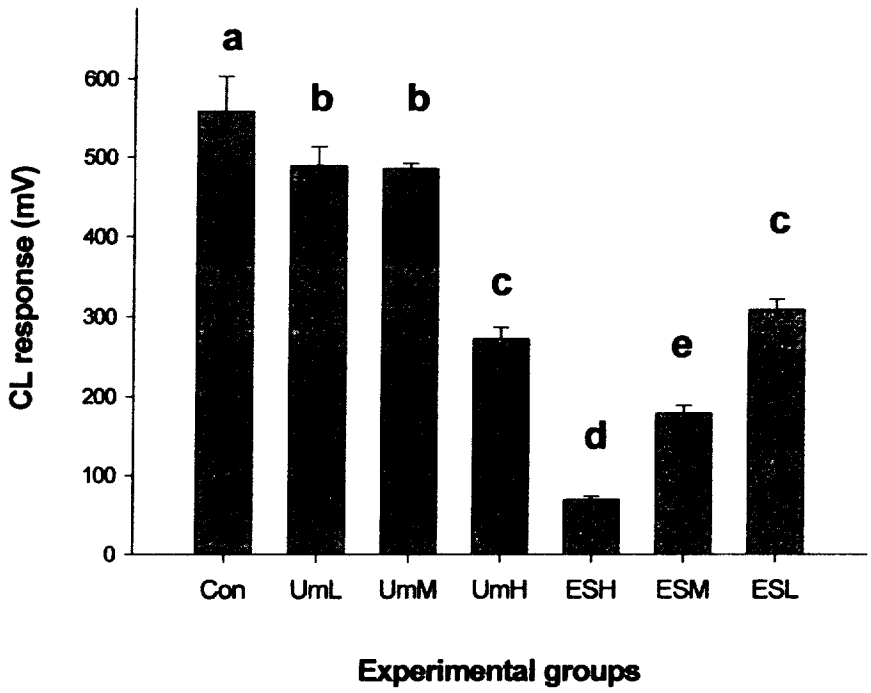

Fig. 1. Inhibition of zymosan induced CL response in olive flounder phagocytes by live *U. marinum* at 4:1 (UmL), 2:1 (UmM), 1:1 (UmH) phagocytes:ciliates ratios, or 0.3 (ESH), 0.15 (ESM), and 0.075 (ESL) ㎎ protein/㎖ HBSS of excretory-secretory (ES) products. In control (Con), 0.4 ㎖ HBSS was added instead of live parasites or ES products. The mean peak value of CL response and standard deviation are shown. The different letters above the bars denote statistically significant difference ($P<0.05$).

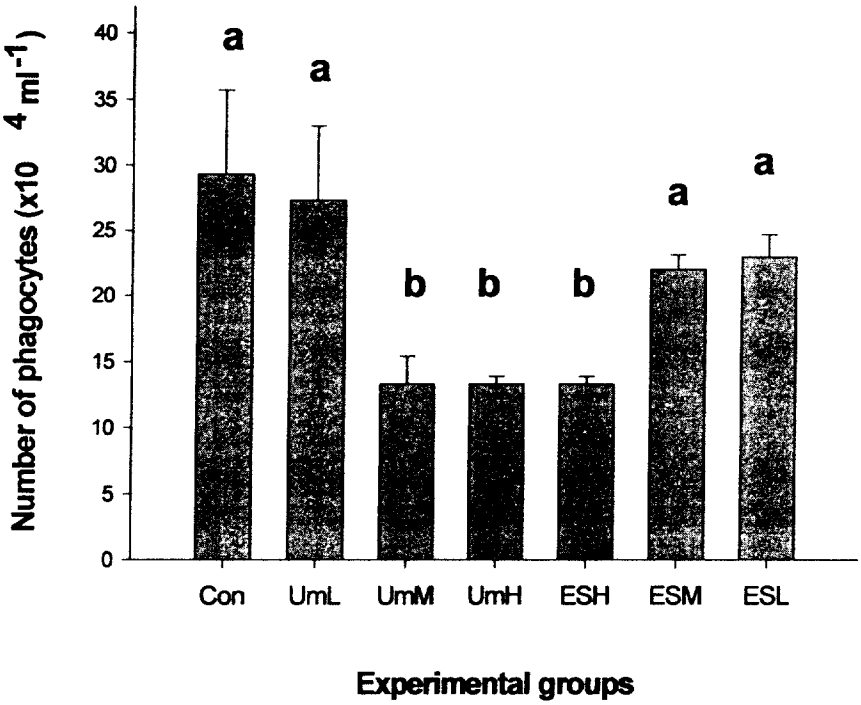

Fig. 2. The number of olive flounder phagocytes showing viability after CL assay. Phagocytes were exposed to live *U. marinum* at 4:1 (UmL), 2:1 (UmM), 1:1 (UmH) phagocytes:ciliates ratios, or 0.3 (ESH), 0.15 (ESM), and 0.075 (ESL) ㎎ protein/㎖ HBSS of excretory-secretory (ES) products. In control (Con), 0.4 ㎖ HBSS was added instead of live parasites or ES products. The mean value of phagocytes number and standard deviation are shown. The different letters above the bars denote statistically significant difference ($P<0.05$).

3. Non-specific humoral immune response of olive flounder

Lysozyme and alternative complement activities of olive flounder were 2±0.56 and 102.49±18.04, respectively (Fig. 3). The parasiticidal activity was represented as the maximal diluted ratio of serum to kill the ciliates. The activity was significantly higher in rockfish than olive flounder (Fig. 4).

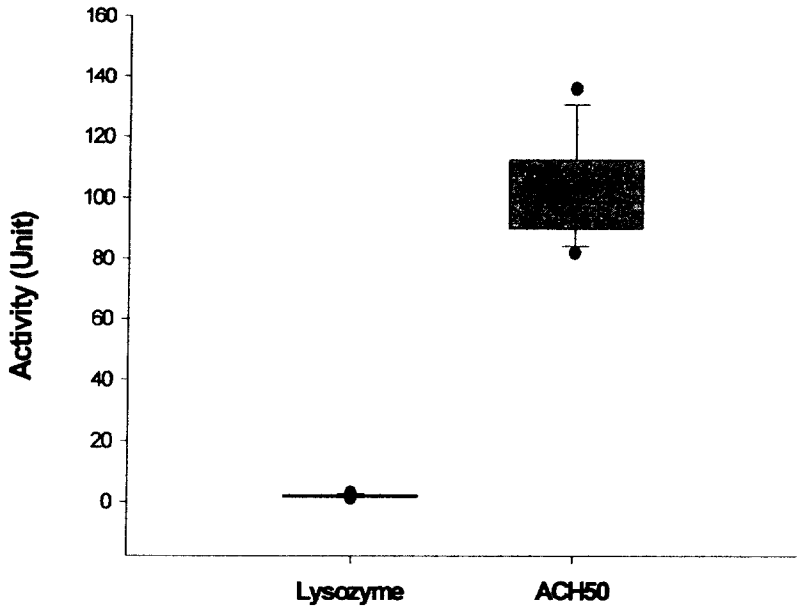

Fig. 3. Non-specific humoral immune response of olive flounder. Lysozyme activity and alternative complement activity (ACH_{50}) of olive flounder was given in Unit.

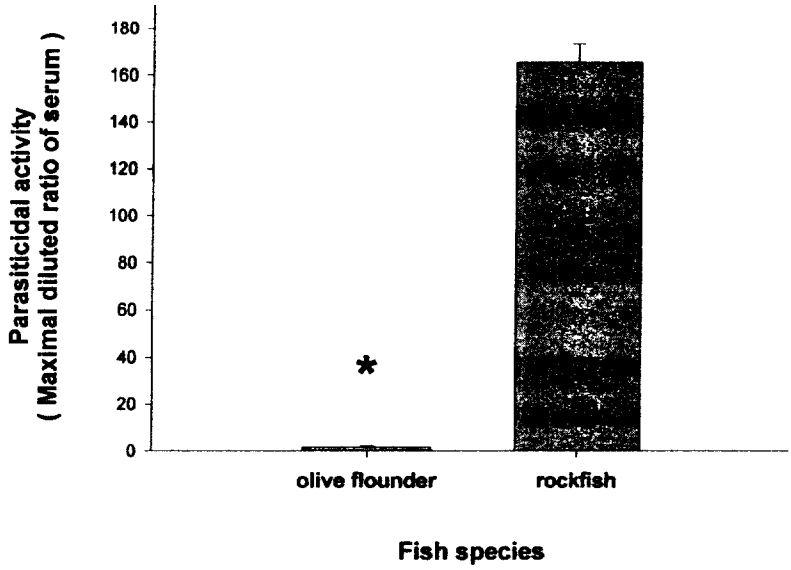

Fig 4. Parasiticidal activity of olive flounder compared with rockfish. The activity was expressed in serum diluted ratio.

* Significantly ($P<0.05$) lower than the rockfish.

4. Effects of stress on humoral immunity of olive flounder

The mean values of lysozyme and parasiticidal activity and ACH_{50} were higher in non stressed fish than stressed fish, but significant difference was not found (Table 1). In addition, glucose level of stressed fish was much higher than that of non stressed fish, but was not statistically significant.

Table 1. Effects of stress on humoral immunity of olive flounder. Each value is mean ± standard error from 5 independent determinations. There was no significant difference between two groups.

	Stressed fish	Control fish
Lysozyme activity (Unit)	1.45±0.22	2.4±1.82
ACH50 (Unit)	100.86±9.67	139.41±29.8
Parasiticidal activity (Serum diluted ratio)	128±19.6	224±39.19
Glucose (㎎/㎗)	13.02±9.08	5.87±1.82

5. Effects of pH and temperature on proteolytic activity

No proteolytic activities were shown in pH 3 and pH 4 at 25℃ and 37℃. High protease activities of parasite lysates were observed over a wide range of pH 5.0~7.0 at 25℃ and 37℃ and maximal activities was shown in pH 5.0 and pH 7.0 at 37℃ (Fig. 5). However, the proteolytic activities in pH 8.0 and pH 9.0 were lower than the activities in pH 5.0 ~pH 7.0. Maximal activities of the parasite lysate was showed at 30℃ in pH 5.0 and pH 7.0 (Fig. 6). The proteolytic activities were increased according to a rise of temperature but decreased at 40℃ than 30℃, indicating that the protease was heat labile.

6. Inhibitory effects of various inhibitors

Proteases were further defined by their sensitivity to inhibitors. The activities were assayed in optimal condition (pH 5.0 and pH 7.0 at 37℃) and the results were presented in Fig. 7. Among the protease inhibitors, E-64 was the most effective while EDTA inhibited a less extent, indicating that the parasite lysate is abound in the cysteine protease. Inhibition by serine protease inhibitor PMSF was minimal in pH 7.0.

7. Gelatine SDS-PAGE

The proteases in the *U. marinum* lysate were detected using gelatine SDS-PAGE. Four protease bands (152, 97, 67, and 40 kDa) were found (Fig. 8). 152 kDa and 40 kDa protease bands were identified as a metalloprotease and cysteine protease, respectively, because they showed the abolishment of clear band when treated with EDTA and E-64, respectively. Pepstatin and PMSF were not shown any effect.

8. Comparison of protease activities between two cultures

The activities of LTC and STC were measured in optimal conditions (pH 5.0 and pH 7.0, 20℃ and 30℃). Azocasein was used as a substrate. The activity of STC was significantly higher than that of LTC ($P<0.05$) in all experimental conditions (Fig. 9). Difference of activity was much higher at 30℃ than 20℃. The highest activity was appeared in STC in pH 7.0 at 30℃.

9. Inhibitory effects of protease inhibitors and fish serum against the proteolytic activities of two cultures

Effects of inhibitors and fish serum on the activities of LTC and STC were shown in Table 3. Proteolytic activities in STC were significantly inhibited by all inhibitors and rockfish serum, but significantly difference was not found in inhibition by olive flounder serum suggesting that fish serum dose not affect the activities of short-term cultured scuticociliates. On the other hand, the activities of LTC were significantly inhibited by only pepstatin, 20 μM E-64, rockfish plasma and olive flounder serum. Besides, EDTA was more of an activator than an inhibitor, namely, proteolytic activity by EDTA did not inhibited. This indicates that metalloprotease was only present in the STC.

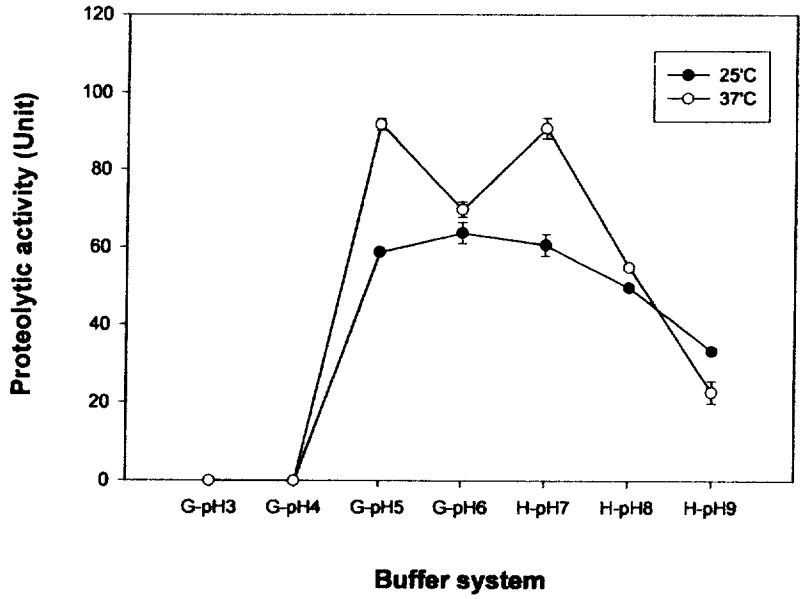

Fig. 5. Effects of pH on proteolytic activities of *U. marinum* lysate. The buffers used were 0.1 M Glycine buffer (pH 5.0 to 7.0) and 0.1 M HEPES buffer (pH 7.0 to pH 9.0). AZC was used as substrate. Each point is mean ± standard deviation from 3 independent determinations. Proteolytic activity is given in units (μg substrate hydrolyzed per hour per mg protein of enzyme).

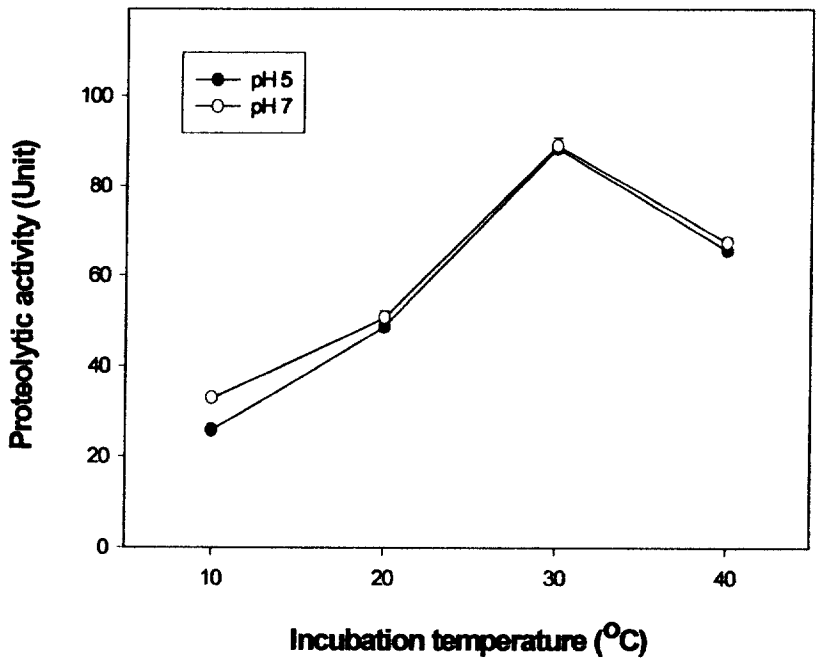

Fig. 7. Effects of temperature on proteolytic activity. AZC was used as substrate (10~40℃). Each point is mean ± standard deviation from 3 independent determinations. Proteolytic activity is given in units (㎍ substrate hydrolyzed per hour per mg protein of enzyme).

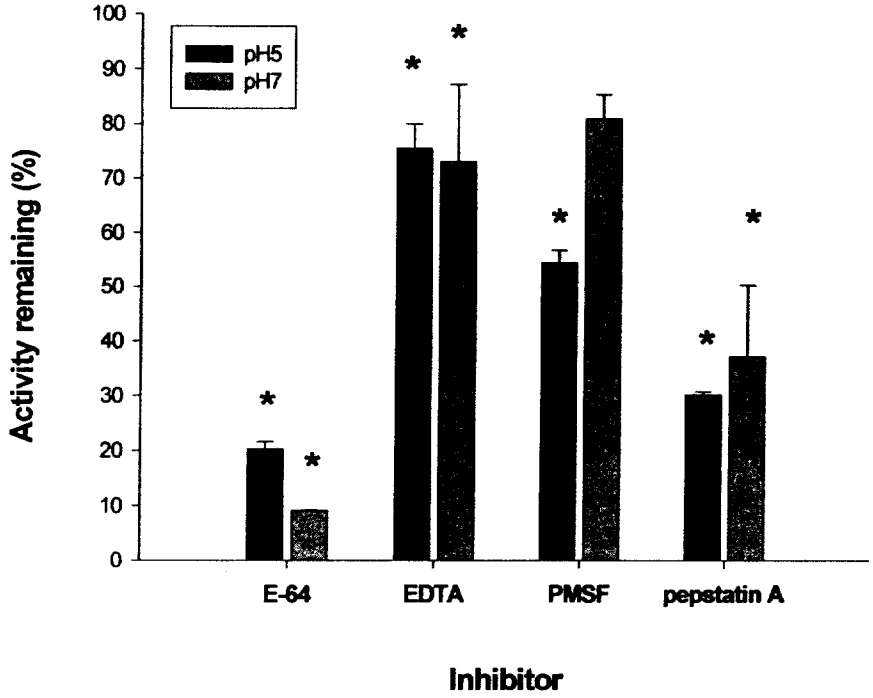

Fig. 8. Inhibitory effects of protease inhibitors on proteolytic activity. Inhibitory effects were assayed using azocasein as a substrates. 0.1 M glycine buffer (pH 5.0) and 0.1 M glycine buffer (pH 7.0) were used. The level of inhibition was expressed as a percentage of the activity remaining (%) of control activity.

*Significantly ($P<0.05$) lower than the control

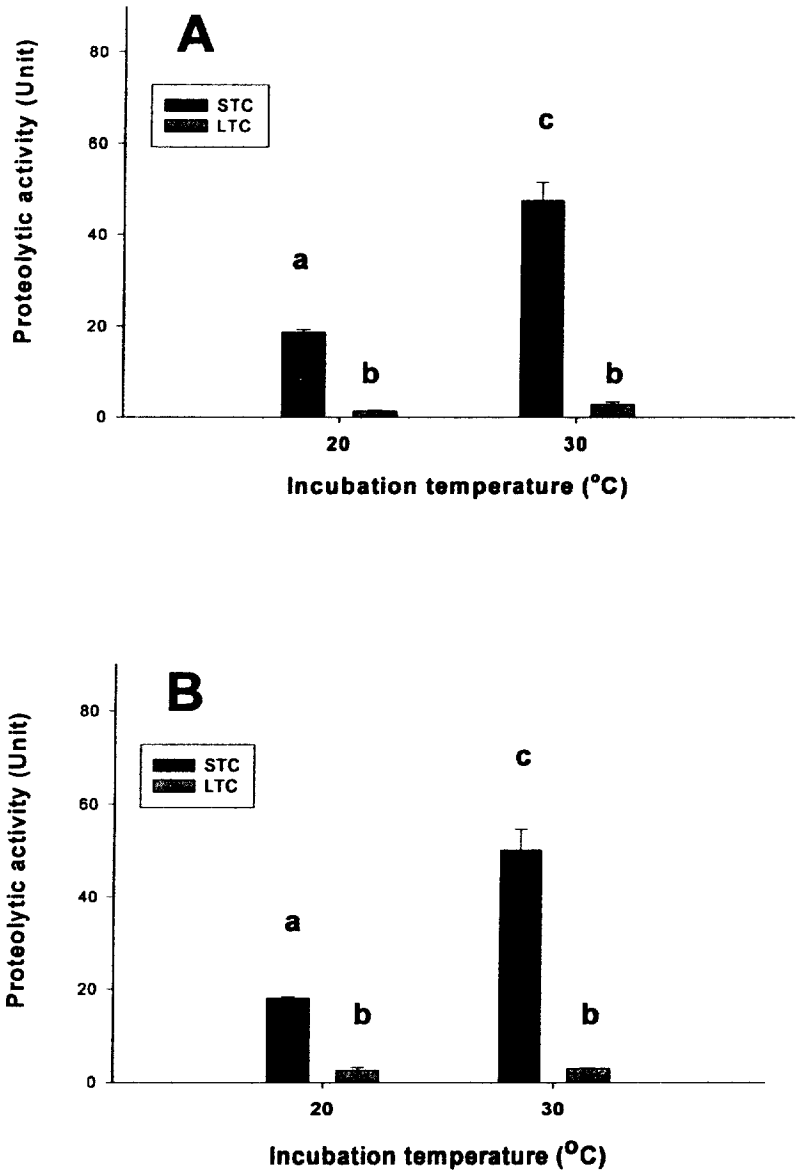

Fig. 9. Differences between LTC and STC on proteolytic activity. The activities were given in units (μg substrate hydrolyzed per hour per mg protein of parasite lysate). Each value is mean ± standard deviation from 3 independent determinations. **A**. pH 5.0; **B**. pH 7.0. The different letters above the bars denote statistically significant difference ($P<0.05$).

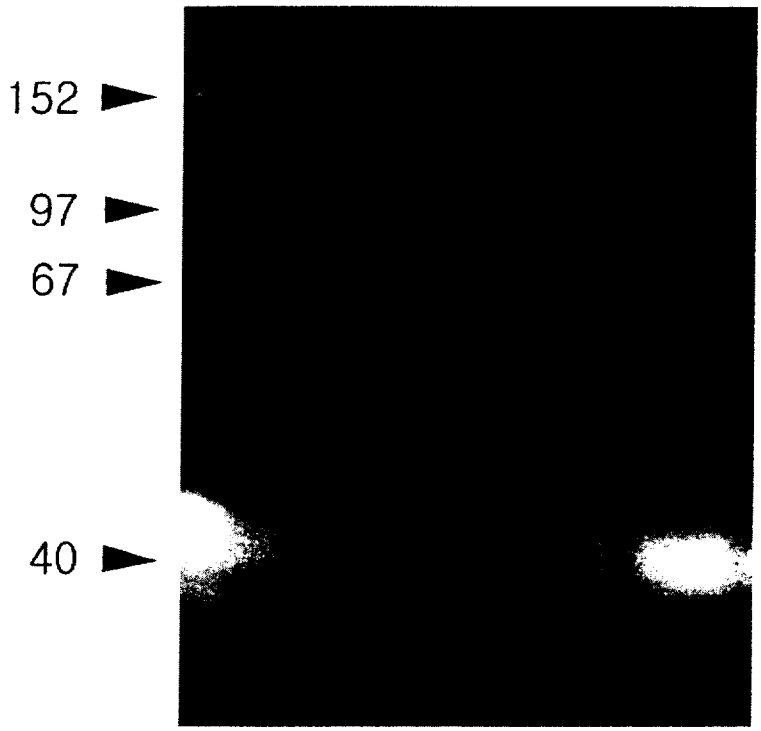

Fig. 10. Proteases of *U. marinum* detected using gelatine SDS-PAGE. Lane 1, the sample only; Lane 2, the sample incubated with E-64; Lane 3, the sample incubated with EDTA; Lane 4, the sample incubated with pepstatin A; Lane 5, the sample incubated with PMSF. Molecular masses (kDa) of major protease bands are indicated by arrowheads.

Table 2. Effects of protease inhibitors and fish plasma on proteolytic activities of LTC (long-term culture) and STC (short-term culture)

Inhibitor class	Inhibitor	Activity remaining (%)	
		STC	LTC
Cysteine protease	E-64		
	10μM	67.72±2.81*	99.96±36.72
	20μM	63.8±3.73*	63.21±5.09*
Metallo protease	EDTA		
	2mM	94.9±2.96*	168.78±15.47**
	8mM	89.81±4.14*	334.18±14.1**
Aspartic protease	Pepstatin A		
	50μM	45.54±10.27*	36.75±14.18*
	100μM	36.11±3.77*	22.04±4.43*
Serine protease	PMSF		
	1mM	71.04±1.09*	120.54±22.2**
	2mM	49.43±1.09*	92.61±11.67
Serum	olive flounder	99.74±5.06	90.3±3.86*
(100μg/mℓ)	rockfish	77.38±2.25*	74.97±11.67*

*Significantly lower ($P<0.05$) than the control (without inhibitors or serum)

**Significantly higher ($P<0.05$) than the control

Activity remaining is expressed as a percentage of the control value; mean values±S.D. from the triplicate

Discussion

In this study, live *Uronema marinum* and ES products had a negative and dose-dependent effect on luminol-enhanced chemiluminescent response of zymosan-stimulated phagocytes of olive flounder. Similar results were reported by Arbo *et al.* (1990) that live *Entamoeba histolytica*, an extracellular protozoan parasite that is capable of invading the intestinal mucosa and spread to other organs of human, inhibits the respiratory burst of human polymorphonuclear leukocytes in a dose-dependent manner. Disruption of the respiratory burst of effector cells has been well documented in several intracellular protozoan parasites (Hall and Joiner, 1991; Panaro *et al.*, 1996; Jain *et al.*, 1996; Kim *et al.*, 1998; Anderson, 1999). Significant reduction in numbers of viable phagocytes after incubation with live ciliates and ES products compared with controls and lysis of phagocytes by ES products indicate that *U. marinum* secrets cytotoxic substances, which can lyse olive flounder phagocytes. Cross and Matthews (1993) reported that carp leukocytes come into close contact with *Ichthyophthirius multifiliis* trophozoites are generally degraded, presumably due to lytic enzymes and/or metabolites released by the parasite. Cell infiltration around invading *E. histolytica* also leads to rapid lysis of inflammatry cells followed by tissue necrosis (Martínez-Palomo *et al.*, 1985; Shibayama *et al.*, 1997). The presence of superoxide dismutase and catalase activities in ES products of *U. marinum* indicate that these antioxidative enzymes play a role in quenching ROIs, which results in significantly diminished CL responses of zymosan-stimulated phagocytes. Toxic superoxide anion (O_2) formed by the respiratory burst of phagocytes would be detoxified by enzymatic dismutation with SOD to H_2O_2, which would be then reduced to H_2O by catalase. Therefore, *U. marinum* can protect themselves against toxic oxygen metabolites through secretion of antioxidant enzymes, thus enabling the parasite to survive in the

host.

In this study, the parasiticidal activity of rockfish serum was much higher than that of olive flounder serum. There were many reports on the presence of differences in immune capacity against a pathogen among fish species. Kim *et al.* (2000a) reported differences in larvicidal activity of serum and chemiluminescent response of phagocytes in carp (*Cyprinus carpio*), crucian carp (*Carassius auratus*) and false dace (*Pseudorasbora parva*) against excysted metacercariae of *Clonorchis sinensis*. According to the results of Shaw *et al.* (2001), macrophages of Atlantic salmon (*Salmo salar* L.), which are resistant to the microsporidian parasite, *Loma salmonae*, had a significantly higher phagocytic index than those of chinook salmon (*Oncorhynchus tshawytscha*), a susceptible species. Although the factors associated with producing difference between olive flounder and rockfish in parasiticidal activity of plasma are not known, higher inhibition activity of rockfish serum than olive flounder against the ciliate's protease activity might contribute partly to the difference of *U. marinum* killing activity.

In the present study, the parasiticidal activity of olive flounder serum was highly suppressed by acute stress. Lysozyme and alternative complement activities, also, much lowered by stress. The complement system is an important part of the organism's defence against microorganisms. Rainbow trout (*Oncorhynchus mykiss*) complement possesses both classical and alternative pathway activity and contains components which are structurally and functionally similar to mammalian C3 and C5. Teleost complement forms a terminal membrane attack complex (MAC) to produce lysis. It also has an opsonizing function in phagocytosis. Several studies have been made on the effects of stress on immunity of fish, and stress-mediated suppression of immune function, generally, results in increase of disease susceptibility (Khansari *et al.*, 1990; Cohen *et al.*, 1991; Kort, 1994). Stress has also been shown to reduce lysozyme activity in fish (Mock and Peters, 1990).

The existence of proteolytic activity in crude extract of *Uronema marinum*

was elucidated in this study. The optimal conditions of *U. marinum* proteases were pH 5.0~7.0 and 30~40℃. From the results by gelatine SDS-PAGE and colorimetric measurements, 152 kDa band was metalloprotease and 40, 67, 97 kDa bands were cysteine protease because these were abolished or become weaker when treated with EDTA (former) and E-64 (later). Although the 152 kDa metalloprotease was surely disappeared by EDTA in gelatine SDS-PAGE, the proteolytic activity was hardly inhibited by EDTA in colorimetric measurement. That is because EDTA, inhibitor of metalloprotease, is also an activator of cysteine protease. Therefore, the total remaining proteolytic activities were not reduced by EDTA in colorimetric assay.

In this study, the proteolytic activity of *U. marinum* was widely different between LTC and STC. E-64 inhibited proteolytic activity of both LTC and STC in similar rates. However, inhibition of proteolytic activity by EDTA in STC was higher than that in LTC. There were many investigations on cysteine proteases and metalloproteases in relation to diseases (Woo and Wehnert, 1983; Bouvier *et al.*, 1990; Munoz *et al.*, 1990; Woo and Li, 1990; Bonaldo *et al.*, 1991; Woo and Thomas, 1991; Etges, 1992; North *et al.*, 1992; Harth *et al.*, 1993; Bahmanrokh and Woo, 1997; Zuo and Woo, 1997a). Several authors had previously suggested that the secreted metalloprotease contributes to the formation of histopathogenic lesions (Bahmanrokh and Woo, 1994; Zuo and Woo, 1997a) and to the direct transmission of *Cryptobia salmositica*, a haemoflagellate of fish, between fish (Woo and Wehnert, 1983). Moreover, there was many other reports about function of metalloprotease which helps *Leishmania* to invade host macrophages by cleaving the host complement factor (Bouvier *et al.*, 1990; Etges, 1992), or degrades host collagen in *Entamoeba histolytica*, suggesting that metalloprotease plays an important role in the invasion of host tissue (Munoz *et al.*, 1982). Zuo and Woo (1997b) reported that cysteine protease was found in pathogenic and nonpathogenic *Cryptobia* spp., but metalloprotease was present only in the pathogenic strain of *C. salmositica*. Furthermore, the loss of virulence of pathogenic *C. salmositica* by 1 year of serial in vitro cultivation

was closely related with the loss of metalloprotease activity (Woo and Li, 1990; Zuo and Woo, 1997). Therefore, no significant loss of metalloprotease in STC but a significant loss of the protease in LTC in this results suggest that the infection potential of *U. marinum* might be reduced by a long-term in vitro culture. However, it was not clear whether STC of *U. marinum* showing high metalloprotease activity is an infectious form or pathogenic form, since challenge test with *U. marinum* had not been done successfully yet. Therefore, further investigation on challenge test connected with parasite proteases should be conducted.

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 protease is important in intracellular protein catabolism, e.g. in digesting exogenous host proteins to obtain free amino acids for protein synthesis. Hence in this study, the stable level of cysteine protease in both STC and LTC of *U. marinum* indicates that this protease is involved in the metabolism of the ciliate.

The α2 macroglobulin, a major natural antiproteases in mammalian blood, has also been detected in fish blood (Zuo and Woo, 1997b, c). The α2 macroglobulin inhibits non-specifically all four major classes of proteases. In the present study, protease activity of *U. marinum* was inhibited by co-incubation with serum of olive flounder and rockfish. These inhibition predominated in rockfish serum, while effect to STC in olive flounder serum was minor. This finding was consistent with the report on *C. salmositica* that the metalloprotease secreted by *C. salmositica* was neutralized by α2 macroglobulin in the blood of fish under in vitro (Zuo and Woo, 1997b) and in vivo (Zuo and Woo, 1997c) conditions.

In conclusion, *U. marinum* can resist to the host phagocyte's oxidative stress by scavenging ROIs with antioxidant enzymes and can establish infection by destruction or suppression of the host's immune factor with various

proteolytic enzymes. Therefore, further researches on the improvement of innate immunity of olive flounder and specific inhibitors of the ciliate's enzymes including proteses and antioxidatives are needed to control scuticociliatosis effectively.

*Uronema marinum*의 감염과 넙치, *Paralichthys olivaceus*의 면역반응

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Abstracts

양식 넙치에 스쿠티카충을 일으키는 *Uronema marinum*에 대한 넙치의 비특이적 면역 반응 및 숙주의 면역 반응에 대한 스쿠티카충의 회피 능력 간의 관계를 조사하였다. 숙주의 세포성 면역반응에 대한 충의 영향을 밝히기 위하여, 살아있는 충과 충의 분비배설 (excretory-secretory; ES) 산물을 넙치 식세포와 함께 배양하고 chemiluminescence (CL) 반응을 측정하였다. 스트레스가 넙치 혈청의 라이소자임, 보체 대체 경로 활성 및 혈청의 살충능력 등을 포함한 비특이적 체액성 면역 반응에 미치는 영향을 분석하였으며, 넙치의 이러한 비특이적 면역 반응을 조피볼락과 비교하였다. 또한 숙주 면역 반응에 대한 방어 인자로서 충의 protease와 항산화 효소를 분석하였다. 실험 결과, 살아있는 충과 ES 산물은 농도 의존적인 방식으로 넙치의 CL 반응을 억제시켰다. ES 산물은 숙주의 식세포를 일부 용해시켰으며, 또한 높은 superoxide dismutase (SOD)와 catalase 활성을 보였다. 스트레스는 넙치 혈청의 lysozyme과 보체 대체 경로 활성 및 살충능을 감소시켰으며, 넙치 혈청의 살충 활성은 조피볼락에 비해 유의적으로 낮았다. *U. marinum* protease의 최적 조건

은 pH 5.0~7.0 이며 30~40℃ 이었다. gelatine SDS-PAGE와 비색 반응 측정에서 EDTA와 E-64를 처리했을 때 band가 약해지거나 소멸하는 것으로서 152 kDa의 band는 metalloprotease이며, 40, 67, 97 kDa의 band는 cysteine protease 임을 알 수 있었다. *U. marinum*의 단기 배양층 (STC)과 장기 배양층 (LTC)은 protease 활성에서 차이가 있었다. E-64에 의한 protease 활성의 억제 효과는 STC와 LTC에서 비슷한 양상이었으나, 반면 EDTA에 의한 억제효과는 LTC에 비해 STC에서 더 높게 나타났다. *U. marinum*의 protease 활성은 넙치와 조피볼락의 혈청과 배양했을 때 억제되었는데, 조피볼락의 억제 활성이 넙치의 것보다 높은 것으로 나타났다. 이상의 결과로부터 *U. marinum*은 SOD 및 catalase와 같은 항산화 효소를 사용하여 숙주 식세포에서 생성되는 산소래디칼 (reactive oxygen intermediates; ROIs)을 중화시킴으로서 숙주 식세포에 의한 산화적 스트레스에 대해 저항하고 또한 다양한 proteolytic enzyme으로 숙주의 비특이적 면역 인자를 파괴하거나 억제시키는 것으로 사료된다. 따라서 스쿠티카증에 대해 효율적으로 대처하기 위해서는 넙치의 선천적 면역능을 개선시키는 것과 충의 protease와 항산화 효소에 대한 특이적인 inhibitor를 개발하는 것에 대한 연구가 진행되어야 할 것이다.

Key words: *Uronema marinum*, *Paralichthys olivaceus*, non-specific immune responses, chemiluminescence, parasiticidal activity, antioxidative enzymes, proteases

감사의 글

우선 제가 이 자리에 오기까지 이끌어 주신 하느님과 먼 곳에서 저를 지켜보시곤 자랑스러워 하실 아버지와 그리고 큰 딸을 항상 믿어 주심으로서 용기와 사랑을 심어주셨던 어머님께 감사드리며 영광을 돌립니다. 고집스럽고 권위적인 장녀를 항상 잘 따라주었던 계영이와 태원이에게도 고맙다는 말을 전하고 싶습니다.

아무것도 모르던 코흘리개 새내기 때부터 석사 학위 수여를 앞둔 지금까지 저를 사랑으로 돌보아 주시고, 다방면의 지식을 쌓게 해 주신 지도 교수 김기홍 교수님께 진심어린 감사를 드립니다. 아울러 선생님의 기대에 답해드리지 못한 점이 마음 아픕니다. 그리고 저에게 어병학에 대한 지식을 자상하게 일깨워 주셨던 박수일 교수님과 바쁘신 와중에도 제 논문에 관심을 가져주신 정준기 교수님께도 감사 드립니다. 학사 4년과 석사 2년 동안 저에게 많은 지식과 학문에 대한 흥미를 느끼게 해주셨던 정현도 교수님, 허민도 교수님, 강주찬 교수님께도 감사 말씀 드립니다.

실험실 생활을 5년 넘게 해오면서 저는 좋은 인연을 많이 만났습니다. 7호관 시절을 함께 했던 은석이 선배, 주원이 선배, 희탁이 선배, 명덕이 선배, 주은이 언니와 그때부터 지금까지 실험실의 큰 기둥으로서 든든하게 자리를 지키고 있는 재범이 선배와 경진이 선배, 그리고 박사과정, 어촌지도사 그리고 예비엄마의 역할을 잘 해내고 계신 윤정이 언니, 이 분들과의 만남은 제게 행운이었습니다. 실험실을 이끌어 가면서 석사과정 2년차로 한창 바쁘실 천수 선배, 찬휘 선배, 재혁이 선배와 학부생 같지 않은 우리방의 학부생들인 은혜, 별님, 성미, 지영, 도선, 영숙, 현진이에게도 감사드립니다. 지금은 다른 곳에 있지만 실험실에 있는 동안 수고가 많았던 미자, 경이와 실험실에서의 소중한 우리 동기들 은미와 은진이도 잊지 못합니다. 대학 4년을 함께 했던 친구 민정이와 우리 96 동기들, 효주, 성애, 재영, 안은, 민아, 정희, 희정, 은임, 현자, 희원, 선영, 상환 오빠, 영주, 도원, 재훈, 호열, 순범, 신후, 이 분들에게도 힘이 되어줘서 감사하다고 전하고 싶습니다. 함께 석사 학위를 준비하면서 서로의 고충을 나눠가질 줄 알았던 정다운 동기들, 희정이와 은영이에 수고했다는 말과 함께 감사 드립니다. 석우 선배에게도 감히 수고하셨다고 전해 드리고 싶습니다. 형길이 선배와 승호 선배도 좋은 결과가 있기

를 기원합니다. 제 논문에 대해 많은 조언을 해 주었던 정수 선배에게 정말 고맙다는 말을 전하고 싶습니다. 후배에게 모범이 되셨던 덕찬이 선배, 수미 언니, 규석이 선배, 자근이 선배, 정훈이 선배, 민진이 선배에게도 감사 드립니다.
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너무나도 감사드리고 싶은 분들이 많습니다. 행여나 제가 어느 분의 이름을 빠뜨리지는 않았나 걱정도 됩니다. 제가 미쳐 감사드리지 못한 모든 분들께도 감사의 말씀을 전하고 싶습니다.
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