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# The Role of Extracellular Protease Produced by *Vibrio anguillarum*

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The role of protease in the pathogenicity of *Vibrio anguillarum* in experimental infections in Japanese eel (*Anguilla japonica*) and ayu (*Plecoglossus altivelis*), and its protective immunogenicity were investigated.

It was confirmed antigenically that the pathogen produced the same protease in the experimentally infected fish as *in vitro*, though proteolytic activity was not detected. Pre-treatments with a sublethal dose of the protease by intramuscular injection in eels or by immersion in ayu enhanced the susceptibility of the fish to *V. anguillarum*. When an eel serum was treated *in vitro* with 10–50 μg/ml of protease, the bactericidal activity of the serum was completely reduced.

The protective immunogenicity of protease was demonstrated when the antigen was given to eels and ayu by injections. However, efficacy of the protease vaccine was overridden by its toxicity when ayu were vaccinated and challenged by immersion method.

*Vibrio anguillarum* is the causative agent of vibriosis, a fatal infectious disease of fishes in the world. Many workers have investigated on the disease and its causative agent, but the mechanisms of the pathogenicity of the pathogen has not been fully understood.

Various virulence factors such as extracellular and intracellular toxins (UMBREIT and TRIPP, 1975; MUNN, 1980; KODAMA *et al.*, 1985), plasmid mediating iron-sequestering system (CROSA, 1980), resistance to serum bactericidal activity (TRUST *et al.*, 1981) and adhesion to host tissues (HORNE and BAXENDALE, 1983) have been reported, and our previous studies revealed that *V. anguillarum* produced an extracellular toxin (INAMURA *et al.*, 1984) and its toxicity proved to consist in a protease (INAMURA *et al.*, 1985). The $LD_{50}$ value of the protease to goldfish and mouse was 1.7 and 1.6 μg protein/g body weight, respectively, and its molecular weight was estimated to be 36,000 by SDS-polyacrylamide gel electrophoresis.

In the present study, roles of the protease in the pathogenesis of *V. anguillarum* infection were investigated. Experiments were carried out to determine (i) whether *V. anguillarum* produces the above-mentioned protease in the tissues of experimentally infected fish as *in vitro*, (ii) predisposing effects of a sublethal dose of the protease on the susceptibility of the fish to experimental infection, and (iii) effects of the protease on the bactericidal action of eel serum. In addition, we examined the protective efficacy of the vaccination with *V. anguillarum* protease.

## Materials and Methods

### *Bacterial strains and preparation of protease*

A virulent strain of *V. anguillarum*, PT-81049 (serotype: J-O-1) isolated from a diseased ayu, was used in this study. An avirulent strain pOH-8304, which was isolated from a healthy larva of ayu and sensitive to serum killing, was used only in the experiment on the bactericidal assay of eel serum.

The extracellular products (ECP) of the strain PT-81049 were prepared from a cellophane plate culture (at 25°C for 24 h) on trypticase soy agar (BBL, NaCl 1%) and the protease was purified by the same procedures as reported previously (INAMURA *et al.*, 1985).

### *Fish and experimental condition*

Healthy Japanese eels (*Anguilla japonica*) (50–150 g) purchased from private eel farms and ayu (*Plecoglossus altivelis*) (5–15 g) raised from fingerlings in a pond in the campus were used in this

study. During the experimental periods, both fishes were kept at about 20°C and ayu were fed with commercial pellet but the eels were not.

*Detection of V. anguillarum protease in tissues*

Japanese eels were infected by intramuscular (IM) injection with *V. anguillarum* at $10^9$ CFU (colony forming unit)/100 g body weight and ayu were immersed in bacterial suspension of $10^6$ CFU/m*l* for 5 min. Immediately after the fish became moribund or dead, the spleen and injected or hemorrhagic site of the muscle including the skin were homogenized with 0.01 M phosphate-buffered saline (PBS, pH 7.0), and then centrifuged at 10,000 rpm (4°C) for 10 min. The supernatants obtained were assayed for proteolytic activity and submitted to precipitation test using rabbit anti-protease serum by the methods described previously (INAMURA *et al.*, 1985).

*Pre-treatment with sublethal dose of protease*

In order to examine predisposing effects of the protease, fish were pre-treated with sublethal doses of the enzyme by the following methods: IM-injection for eel (25 μg protein/100 g B.W.), intraperitoneal (IP) injection (1 μg/fish) or immersion (5 μg/m*l* in PBS, for 10 min) for ayu. Two hours after the pre-treatment, fish were submitted to bacterial challenge by either injection or immersion.

*Vaccination*

In addition to the protease, non-washed cells from a cellophane plate culture (at 25°C for 24 h) were used as immunogens. This cell suspension containing ECP was inactivated by formalin of 0.3%. Eels were immunized twice at a 2-week interval by IM-injections with the doses of 135 μg protein (cells) or 10 μg (protease)/100 g B.W., and ayu were immunized twice by IP-injections with cells (135 μg protein/fish) or protease (1 μg/fish). Immersion vaccination was also employed in ayu; 135 μg protein/m*l* (cells) or 4 μg protein/m*l* (protease). One week after the second injection or 2 weeks after immersion, fish were challenged with live bacteria by injection or immersion.

An additional immersion vaccination was conducted in ayu using washed and non-washed cells of the strain as immunogens. In this experiment, an immersion challenge was carried out 2 weeks after the immunization.

*Challenge tests*

The fishes pre-treated or vaccinated were challenged with *V. anguillarum* in the following way: eels were challenged by IM-injection of bacteria at $10^6$–$10^9$ CFU/100 g B.W. and ayu were challenged by IP-injection at $10^0$–$10^2$ CFU/fish or immersion for 5 min in the bacterial suspension ($10^3$–$10^5$ CFU/m*l*) with 1% NaCl.

After challenge, mortalities were monitored daily for 7 days, and dead fish were submitted to bacterial isolation.

*Bactericidal assay of eel serum*

Healthy Japanese eels were bled from the bulbus arteriosus. Blood samples were allowed to stand at room temperature for about 2 h, and then the serum was separated by centrifugation at 3,000 rpm (4°C) for 10 min. A sample of normal serum was pooled from 5 eels and used within 2 h.

A 0.5 m*l* of serum was mixed with the same volume of variously diluted protease solution at final protease concentrations of 0, 0.5, 5, 10 and 50 μg/m*l*, and kept at 25°C for 2 h. Thus treated serum or heat-inactivated serum (at 50°C for 30 min) were mixed with *V. anguillarum* cells at a final concentration of $10^5$ CFU/m*l* and incubated at 25°C for 6 h with gentle agitation. The viable cell number in the mixtures was determined by spread-plate count technique on nutrient agar.

## Results

*Detection of V. anguillarum protease from experimentally infected fish*

The production of protease by *V. anguillarum* was examined in the tissues of five eels and three ayu. No proteolytic activity was detected in the homogenates of the muscle and the spleen of any fish tested. However, only homogenates of the spleen from ayu formed a single precipitin line against rabbit anti-protease serum in Ouchterlony double diffusion test.

*Changes in the susceptibility by pre-treatment with protease*

In the IM-injection challenge tests of eels (Table 1), higher mortalities were always observed in the

**Table 1.** Predisposing effects of protease on the mortality of Japanese eels challenged by IM-injection with *V. anguillarum*

| Pre-treatment | Challenge dose* (CFU/100 g BW) | Mortality (dead/tested) | Mean time to death (days) |
|---|---|---|---|
| IM-injection of protease (25 μg/100 g BW) | $1.4\times10^{6}$ | 60% (3/5) | 2.8 |
| | $1.4\times10^{7}$ | 100 (5/5) | 2.4 |
| | $1.4\times10^{8}$ | 100 (5/5) | 1.8 |
| IM-injection of sterilized PBS | $1.4\times10^{6}$ | 0 (0/5) | — |
| | $1.4\times10^{7}$ | 0 (0/5) | — |
| | $1.4\times10^{8}$ | 60 (3/5) | 4.0 |

* Challenge tests were performed 2 h after pre-treatment. Water temperature: 20.1°C (19.5–21.3°C). Body weight of fish: 89 g (46–126 g).

**Table 2.** Predisposing effects of protease on the mortality of ayu challenged by IP-injection with *V. anguillarum*

| Pre-treatment | Challenge dose* (CFU/fish) | Mortality (dead/tested) | Mean time to death (days) |
|---|---|---|---|
| IP-injection of protease (1 μg/fish) | $4.3\times10^{0}$ | 40% ( 4/10) | 2.2 |
| | $4.3\times10^{1}$ | 40 ( 4/10) | 2.4 |
| | $4.3\times10^{2}$ | 100 (10/10) | 2.1 |
| IP-injection of sterilized PBS | $4.3\times10^{0}$ | 20 ( 2/10) | 2.7 |
| | $4.3\times10^{1}$ | 70 ( 7/10) | 2.6 |
| | $4.3\times10^{2}$ | 100 (10/10) | 2.3 |

* Challenge tests were performed 2 h after pre-treatment. Water temperature: 19.9°C (19.0–21.3°C). Body weight of fish: 7.1 g (4.1–15.3 g).

**Table 3.** Predisposing effects of protease on the mortality of ayu challenged by immersion with *V. anguillarum*

| Pre-treatment | Challenge dose* (CFU/m*l*) | Mortality (dead/tested) | Mean time to death (days) |
|---|---|---|---|
| Immersion in protease (5 μg/m*l* for 10 min) | $2.7\times10^{3}$ | 60% ( 6/10) | 4.3 |
| | $2.7\times10^{4}$ | 100 (10/10) | 2.3 |
| | $2.7\times10^{5}$ | 100 (10/10) | 2.3 |
| Immersion in PBS (for 10 min) | $2.7\times10^{3}$ | 0 ( 0/10) | — |
| | $2.7\times10^{4}$ | 70 ( 7/10) | 3.6 |
| | $2.7\times10^{5}$ | 100 (10/10) | 2.3 |

* Challenge tests were performed 2 h after pre-treatment. Water temperature: 19.6°C (19.0–20.3°C). Body weight of fish: 8.8 g (4.8–13.5 g).

eels pre-treated with protease than in corresponding groups without protease treatment. On the contrary, in the IP-injection challenge of ayu (Table 2), there were little differencies in mortality or time to death between protease-treated and PBS-treated groups. When ayu were pre-treated by immersion and challenged by immersion (Table 3), the mortalities were much higher in the protease-treated groups than in controls.

*Effects of protease on the bactericidal action of eel serum*

Results are shown in Table 4. Normal serum of Japanese eel had a bactericidal action against *V. anguillarum* strain pOH-8304, as shown by that the number of viable cells of the strain decreased from $10^{5}$ CFU/m*l* to less than 10 CFU/m*l*

**Table 4.** Effects of protease on the bactericidal action of Japanese eel serum to *V. anguillarum*

| Treatment of serum | Strain pOH-8304 CFU/m*l* after 6 h at 25°C (Initial conc.: $2.2\times10^{5}$) |
|---|---|
| Protease-treated (at 25°C for 2 h) | |
| 0 μg/m*l* | $<10^{1}$ |
| 0.5 | $<10^{1}$ |
| 5 | $4.1\times10^{4}$ |
| 10 | $1.4\times10^{7}$ |
| 50 | $1.6\times10^{7}$ |
| Heat-inactivated (at 50°C for 30 min) | $1.6\times10^{7}$ |

**Table 5.** Challenge of the vaccinated eels by IM-injection of *V. anguillarum*

| Immunized with | Mortality (dead/tested) [Mean time to death: days] Challenge dose (CFU/100 g BW) $1.8\times10^{8}$ | $1.8\times10^{9}$ |
|---|---|---|
| Non-washed cells | 0% ( 0/10) | 0% ( 0/10) |
| Protease | 45 ( 9/20) [4.5] | 100 (10/10) [3.1] |
| Control (PBS) | 100 (20/20) [2.1] | 100 (10/10) [1.7] |

Vaccination: fish (71 g) were immunized twice by IM-injections with non-washed cells (135 μg protein/100 g BW) or protease (10 μg protein/100 g BW) at 17.4°C.

Table 6. Challenge of the vaccinated ayu by immersion with *V. anguillarum*

| Immunization method | Immunized with | Challenge dose (CFU/m*l*) | Mortality (dead/tested) | Mean time to death (days) |
|---|---|---|---|---|
| IP-injection | Non-washed cells | $1.8\times10^5$ | 0% ( 0/25) | — |
| | Protease | | 0 ( 0/25) | — |
| | Control (PBS) | | 64 (16/25) | 2.1 |
| Immersion | Non-washed cells | $2.3\times10^5$ | 0% ( 0/27) | — |
| | Protease | | 37 (10/27) | 3.1 |
| | Control | | 4 ( 1/27) | 5.2 |

Vaccination: fish (6.9 g) were immunized twice by IP-injections with non-washed cells (135 μg protein/fish) or protease (1 μg/fish) at 20.1°C, or once by immersion with non-washed cells (135 μg protein/m*l*) or protease (4 μg protein/m*l*) at 19.8°C.

Table 7. Immersion vaccination of ayu with washed and non-washed cells of *V. anguillarum*

| Immunized with | Challenge dose (CFU/m*l*) | Mortality (dead/tested) |
|---|---|---|
| Non-washed cells | $3.7\times10^4$ | 0% ( 0/25) |
| | $3.0\times10^5$ | 0 ( 0/24) |
| Washed cells | $3.7\times10^4$ | 4 ( 1/25) |
| | $3.0\times10^5$ | 15 ( 3/20) |
| Control | $3.7\times10^4$ | 67 (16/24) |
| | $3.0\times10^5$ | 100 (24/24) |

Vaccination: fish (9.5 g) were immunized once by immersion for 10 min with non-washed (135 μg protein/m*l*) or washed cells (120 μg protein/m*l*) at 20.2°C.

in the non-treated serum after 6 h. The bactericidal activity was greatly reduced by pre-treatment with 10 or 50 μg/m*l* of protease, where the number of the bacteria increased and reached to the same levels ($10^7$ CFU/m*l*) as that in heat-inactivated serum.

*Protective immunogenicity of protease*

The results of active immunization in eels and ayu are shown in Tables 5 and 6, respectively. As shown in Table 5, high level of protection against *V. anguillarum* was shown in the eels vaccinated with non-washed cells by IM-injection. The eels vaccinated with protease by IM-injection also showed protective immunity though a significant difference in mortality was observed only in the groups challenged with the lower dose. When ayu were vaccinated by IP-injection with protease (Table 6), they showed high protections against experimental vibriosis as in the case with non-washed cells. However, when ayu were vaccinated with protease by immersion (4 μg/m*l* for 10 min), the vaccination brought about an adverse effect.

When the protective immunogenicity was compared between washed and non-washed cells of the strain, the latter provided ayu with higher protection (Table 7).

## Discussion

Recently various virulence factors of *Aeromonas salmonicida*, the causative agent of fish furunculosis, have been investigated and the roles of its protease in the pathogenesis of the infection were also examined (SAKAI, 1984, 1985b, c; TITBALL, 1985; FYFE *et al.*, 1986). These voluminous studies were thought to be carried out due to the incompletion of effective vaccine. On the contrary, successful developments in vaccination against vibriosis have led to insufficient studies on pathogenicity of *V. anguillarum*. The present study was performed to investigate the roles of *V. anguillarum* protease in the pathogenesis of vibriosis, following to our previous works where enzymatic properties of the protease were studied.

Although proteolytic activity was not detected in the tissues of infected fish, it was confirmed by antigenic examination that *V. anguillarum* produced the protease *in vivo*. SAKAI (1985b) reported that high proteolytic activity was detected in the muscle of sockeye salmon received IM-injection of virulent *A. salmonicida*. We suppose that the protease produced by *V. anguillarum in vivo* was dispersed so that the proteolytic activity could not be detected clearly.

As reviewed by INGRAM (1980), various humoral and mucous substances involved in natural defence mechanisms are present in fish. Among these mechanisms, the direct bactericidal reaction of complement in fish serum has been indicated to play an important role in the protection of non-immune fish (IIDA and WAKABAYASHI, 1983; SAKAI, 1983; NAKAI, 1985). In the present study, bactericidal activity of eel serum was decreased by the treatment with protease (Table 4). The same phenomenon was reported by SAKAI (1984), who demonstrated that hemolytic activity ($CH_{50}$) of rainbow trout serum was considerably reduced by treatment with *A. salmonicida* protease. The depletion of complement induced by the protease may play a part in increasing susceptibility of Japanese eel to *V. anguillarum* (Table 1). On the contrary, any marked increase in the mortality was not observed in the ayu which were treated by IP-injection of a sublethal dose of the protease prior to IP-injection challenge (Table 2). It was supposed that the $LD_{50}$ value of the used strain of *V. anguillarum* to ayu was too small to be lowered further by the protease pre-treatment.

The skin mucus also plays an important role in natural defence systems of fish. Recently, it was reported that the skin mucus of ayu contained lysozyme-like substance which showed bacteriolytic activity against *Micrococcus lysodeikticus* (ITAMI *et al.*, 1986). In our study, the susceptibility of ayu to *V. anguillarum* was enhanced by immersion in the protease solution (Table 3). The effects of protease on the mucus components were not investigated in this study, but it is possible that the protease of *V. anguillarum* may degrade such external defence systems of fish.

As to the protective immunogen of *V. anguillarum* vaccine, it has been suggested that heat-stable antigen or lipopolysaccharide (LPS) of bacterin would play the most important role in the protection against vibriosis (KUSUDA *et al.*, 1978; ITAMI and KUSUDA, 1980; KAWAI and KUSUDA, 1983). In the present study, the protease was confirmed to be another protective immunogen as in the case with *A. salmonicida* protease (SHIEH, 1984, 1985; SAKAI, 1985a). Although the immersion immunization with the protease in ayu resulted in an adverse effect due to its toxicity, fish should be immunized with vaccine including some amount of protease as well as cell components in order to attain the highest protection. Actually, non-washed cells harvested from a cellophane-plate culture gave better protection than washed cells in immersion vaccination of ayu (Table 7).

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