Humoral Immune Response of White-Spotted Char \textit{Salvelinus leucomaenis} to \textit{Aeromonas salmonicida} Extracellular Products

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White-spotted char \textit{Salvelinus leucomaenis} were immunized intramuscularly with \textit{Aeromonas salmonicida} extracellular products (ECP). By immunization two or three times at four week intervals, the serum agglutinating antibody titers at twelve weeks after the first immunization were raised ranging from 1:128 to 1:512 or from 1:512 to 1:4,096 respectively. Disc electrophoretic analysis did not show any difference in the protein composition between immunized and non-immunized fish sera. By immunodiffusion analysis, char anti-ECP sera formed a single precipitating line against ECP and salmolysin. The component protein of the char antisera, which reacted to ECP, also formed a single precipitating line against ECP in $\gamma$-globulin region by immunoelectrophoresis. The antiserum was separated into three fractions by gel filtration on Sepharose 6B, and antibody activity was found in early eluate of the second peak with $K_{av}$ of 0.4 to 0.6. The results indicate that salmolysin, which is included in ECP, possesses high immunogenicity to white-spotted char.

\textit{Aeromonas salmonicida}, the causative agent of furunculosis, infects many species of fish.1–5) The disease occurs particularly in cultured white-spotted char \textit{Salvelinus leucomaenis}, coho salmon \textit{Oncorhynchus kisutch} and rainbow trout \textit{O. mykiss} in Japan.

Damage caused by the disease on white-spotted char and coho salmon is more severe than that on rainbow trout due to the different sensitivity of those fish to \textit{A. salmonicida}.6–8) At present, the disease has been only controlled by chemotherapy. A vaccine against the disease is desirable because strains of \textit{A. salmonicida} exhibit plasmid-mediated drug resistance.9)

In order to produce an effective vaccine for the disease, fish immune responses against formalin-killed cells,10,11 endotoxin12 and extracellular products (ECP)13,14 have been investigated.

Recently, Kawahara and Nomura15 reported that \textit{A. salmonicida} salmolysin which is a hemolysin purified from the culture supernatant of the bacterium possessed immunogenicity to white-spotted char. The purification of salmolysin, however, would be too expensive to produce, and ECP or crude salmolysin therefore is expected as a reasonable immunogen to the fish.

This paper describes on the humoral immune response in white-spotted char which were injected with \textit{A. salmonicida} ECP.

Materials and Methods

Experimental Fish

Yearling white-spotted char weighing about 400 g were maintained in flowing water at 12–13°C from a gushing spring at Iwate Prefectural Institute of Inland-Water Fisheries. The fish were fed pellets daily at a rate of 1% of body weight per day throughout the experimental period.

Bacterium

\textit{A. salmonicida} A-5 was isolated from the kidney

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of diseased coho salmon which was obtained from a fish farm in Tamayama, Iwate Prefecture.

**Preparation of Extracellular Products**

The bacterium was subcultured on Tryptosoya agar (Nissui Pharmaceutical Co., Ltd., Tokyo) at 20°C for 72 h. The subcultured bacterium was inoculated into 100 ml of a liquid medium composed of isotonic peptone and yeast extract\(^\text{16}\) and incubated at 20°C for 16 h with shaking. Then, the culture fluid was transferred into 3 l of the same medium and was incubated at 20°C for 24 h with shaking. The culture fluid was centrifuged to remove the cells at 8,100 g for 60 min. Ammonium sulfate was slowly added to the supernatant with gentle stirring at a final concentration of 70% saturation. After being allowed to stand for 2 h, the precipitate was collected by centrifugation at 10,200 g for 30 min, dissolved in a small volume of 10 mM phosphate buffered saline (PBS) at pH 7.2, and dialyzed against PBS overnight at 4°C.

**Purification of Salmolysin**

All procedures for purification of *A. salmonicida* salmolysin were performed as previously described by Nomura *et al.*\(^\text{17}\) In brief, salmolysin was purified from the culture supernatant of the bacterium by ammonium sulfate precipitation, anion-exchange chromatography and gel filtration chromatography.

**Protein Determination**

Protein concentration was determined by the method of Lowry *et al.*\(^\text{18}\) using bovine serum albumin as a standard.

**Preparation of Rabbit Antisera**

ECP was adjusted to 1.8 mg/ml in PBS, and emulsified with Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, Michigan, U.S.A.) at a volume ratio of 1 : 1. The emulsion was injected into rabbits: intramuscularly into each nates (0.6 ml) and subcutaneously between the scapulae (0.8 ml). Booster were prepared and injected in the same manner at five-week intervals. Rabbits were bled weekly, sera obtained and stored at -20°C.

**Immunization of Fish**

ECP was adjusted to 1.8 mg/ml in PBS and emulsified with FCA at a volume ratio of 1 : 1. White-spotted char were intramuscularly injected with 100 μl of the emulsion. Booster were prepared and injected two or three times in the same manner at four-week intervals. Control fish were intramuscularly injected three times with 100 μl of a PBS-FCA emulsion at four-week intervals. The immunized and control fish were bled at two-week intervals, sera obtained and stored at -20°C.

**Titration of Antibody**

Passive hemagglutination test (PHAT) was performed to detect antibody titer by using a microtiter apparatus. The sheep red blood cells (Nippon Bio-Test Laboratories Inc., Tokyo) were washed with PBS three times by centrifugation at 500 × g for 5 min, resuspended in PBS containing 15 μg/ml tannic acid at a volume ratio of 1 : 5, and incubated at 37°C for 60 min. After incubation the blood cells were washed with PBS three times by centrifugation at 500 × g for 5 min, resuspended in PBS containing 1.1 mg/ml ECP, and incubated at 37°C for 3 h. After incubation, the blood cells were washed with PBS by centrifugation at 500 × g for 5 min and resuspended in PBS containing 1% normal rabbit serum at a volume ratio of 1 : 5. Twofold serial dilutions of the white-spotted char sera were made in the PBS containing 1% normal rabbit serum by using microtiter plates. Twenty-five microliters of the cell suspension were added to each well. The plates were incubated at room temperature for 2 h, and then hemagglutination was observed.

Immunodiffusion analysis\(^\text{19}\) was performed on agar plates, which were made in plastic petri dishes (48 × 8.5 mm, Millipore Corp., Bedford, Mass., U.S.A.) with 5 ml of 1.2% Purified agar (Oxoid Ltd., Basingstoke, Hampshire, England) in PBS containing 0.1% sodium azide. Antigens and antisera were placed in 5 mm wells and allowed to react in a moist chamber for 48 h at room temperature. The gels were washed with PBS containing 0.1% sodium azide, and then washed with distilled water. After washing, the gels were stained with 15 min with 1% amido black 10 B in 7% acetic acid, and destained with 7% acetic acid.

**Analyses of Serum Proteins**

Polyacrylamide gel disc electrophoresis (PAGE)\(^\text{20}\) was performed with 7.5% polyacrylamide gel at a constant 3 mA/disc until the tracking dye reached the bottom of the gel. The gel was stained with 0.05% Coomassie brilliant blue R-250 in methanol-acetic acid-water (9 : 2 : 9) and destained with methanol-acetic acid-water...
Immunoelectrophoresis was performed on microscope slides covered with 3 ml of 1.2% purified agar in PBS containing 0.1% sodium azide. Antigens were placed in each 2 mm well and subjected to electrophoresis at a constant 2.5 mA/cm for 45 min. Antisera were then added to the 2 mm wide trough, and precipitation arcs were allowed to form in a moist chamber for 48 h at room temperature. Washing, staining and destaining were performed as described in the explanation of immunodiffusion.

Gel filtration was performed as follows. One milliliter of white-spotted char anti-ECP serum was dialyzed against 20 mM Tris-hydrochloride buffer (THB) at pH 8.0 containing 2% sodium chloride and 0.1% sodium azide. The dialyzate was applied to the column (1.6 × 50 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with THB and eluted with THB at a flow rate of 12 ml/h, and 3 ml fractions of the eluate were collected. Then optical density of the resulting fractions at 280 nm was measured. By immunodiffusion, precipitating antibody titers of the fractions against ECP were assayed using twofold serial dilutions of the fractions.

Fig. 1. Antibody response of white-spotted char immunized two (▲) or three times (●) at four-week intervals with Aeromonas salmonicida extracellular products. 
P, pre-immunization; ■, control.

Fig. 2. Immunodiffusion analysis of white-spotted char antisera. Both outer wells; antisera at 0 (1, 2), 6 (3, 4) and 12 (5, 6) weeks after first immunization, of which char immunized three times at four week intervals.

Center well: Aeromonas salmonicida extracellular products (E) or purified salmolysin (S).
Table 1. Precipitating antibody activities in sera of white-spotted char immunized three times

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+, presence of precipitating line; -, absence of precipitating line.
Concentration of precipitin, ++ ++ > ++ ++ ++.
Boosted at 4 and 8 weeks after first immunization.

Table 2. Precipitating antibody activities in sera of white-spotted char immunized two times

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+, presence of precipitating line; -, absence of precipitating line.
Concentration of precipitin, ++ ++ > ++ ++ ++.
NT, not tested.
Boosted at 4 weeks after first immunization.

Results

In some white-spotted char immunized with *A. salmonicida* ECP-FCA emulsion, swelling was observed at injection site, but no mortality was found throughout the experimental period. Serum agglutinating antibody titers to ECP were raised in char which were immunized two or three times with ECP-FCA emulsion at four week intervals (Fig. 1), whereas anti-*A. salmonicida* ECP agglutinin titers were detectable in control fish sera. Primary antibody production was initiated within two weeks after the first antigen injection. At between 10 and 12 weeks after injection, agglutinin titers of char immunized three times were higher than that of char immunized two times. In the antisera of char immunized two times, maximum antibody titer was reached at 8 weeks.

Precipitating antibody was also detected by immunodiffusion analysis, only a single precipitating line was observed when the immunized char sera reacted to ECP or purified salmolysin (Fig. 2). The activity was initiated within the first two weeks and reached its highest level at 8 weeks.

Fig. 3. Comparison of immunodiffusion analysis between white-spotted char antisera and rabbit antisera against *Aeromonas salmonicida* extracellular products.
Outer wells; char antisera (1, 3, 5) and rabbit antisera (2, 4, 6).
Center well; extracellular products.
weeks after injection. In the sera of char immunized three or two times, the activity tended to increase until 12 or 10 weeks after immunization, respectively (Tables 1 and 2). Precipitating antibody activity to ECP was undetectable in control fish sera.

Immunoprecipitate patterns in rabbits and char anti-ECP sera are shown in Fig. 3. Precipitating antibodies to 7 ECP components were detected in rabbit anti-ECP sera, while the precipitating antibody to one ECP component was detected in char anti-ECP sera as mentioned above.

PAGE of char serum resulted in the bands shown in Fig. 4. Twenty-two components in char sera were detected. The banding patterns of serum from immunized fish proved no distinction from those of the non-immunized controls, following injection with ECP, the concentrations of components did not change until 12 weeks after immunization.

By immunoelectrophoretic analysis, 12 precipitation arcs were detected when char sera reacted to rabbit anti-char sera after electrophoresis (Fig. 5a). No significant differences
were found in the immunoelectrophoretic patterns of char sera taken at various times after immunization. In char antisera at 8, 10 and 12 weeks after the first immunization, only one precipitation arc was detected in $\beta$ to $\gamma$-globulin region of the gel when the sera reacted to ECP (Fig. 5b).

After gel filtration of whole char anti-ECP sera on Sepharose 6B, three protein peaks were revealed (Fig. 6). By immunodiffusion test, precipitating antibody activity was detected in the early eluate of second peak with $K_{av}$ of 0.4 to 0.6.

### Discussion

Serum agglutinating antibody titer determined by PHAT and precipitating antibody activity detected by immunodiffusion to *A. salmonicida* ECP were raised in white-spotted char which were immunized with ECP-FCA. The precipitating antibody activity was correlated to the agglutinating antibody titer, and precipitating antibodies were able to be detected in the sera which had more than 1:16 to 1:32 of agglutinating antibody titer because of the different sensitivity between the methods.

Hastings and Ellis\(^{14}\) reported that no antibodies to hemolysin and protease were found in rainbow trout immunized either with native ECP or formalin-inactivated ECP. Nomura et al.\(^{17}\) demonstrated that salmolysin possessed immunogenicity to rabbits. Kawahara and Nomura\(^{10}\) showed native and detoxified salmolysin possessed immunogenicity to white-spotted char.

In the present report, white-spotted char anti-ECP sera formed a single precipitating line against ECP or salmolysin. While rabbit anti-ECP sera formed 7 precipitating lines against ECP. These findings indicate that immunogenicity of ECP components is more intense in rabbits than in white-spotted char. ECP which contains salmolysin therefore is able to apply as immunogen to white-spotted char. The difference of the immunogenicity between salmolysin in white-spotted char and the hemolysin in rainbow trout may be due to the difference of antigen characters and species specificity with respect to the immune reactivity.

By immunoelectrophoretic analysis, goldfish *Carassius auratus* and carp *Cyprinus carpio* immunoglobulins have the mobility of a $\beta$-globulin.\(^{21}\) While immunoglobulins of rainbow trout,\(^{12}\) coho salmon,\(^{11}\) yellowtail *Seriola quinquergadiata*\(^{23}\) and masu salmon *O. masou*\(^{24}\) have the mobility of a $\beta$ or $\gamma$-globulin. In the results of present study, because white-spotted char anti-ECP sera formed a single precipitating line in $\beta$ to $\gamma$-globulin region against ECP by immunoelectrophoresis, the component in the antisera which responded to ECP seems to be immunoglobulin.

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### References