イトウの血清イムノグロブリンM(IgM):精製,血中濃度測定ならびにSalmincola stellatusに対する特異IgMの産生

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Serum Immunoglobulin M (IgM) in Sakhalin Taimen (*Hucho perryi*): Purification, Characterization, Circulating Levels, and Specific IgM Production by the Parasitic *Salmincola stellatus*

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Abstract: Immunoglobulin M (IgM) was purified from the serum of Sakhalin taimen (*Hucho perryi*) by salting-out, ion-exchange chromatography on DEAE-cellulose, and gel filtration on Sepharose 6B. The intact, tetrameric taimen IgM has a mass of 750 kDa with molecular architecture typical of IgM from other salmonids. The molecular weights of heavy (μ) chain and light chains (L) of the IgM monomer were estimated to be 68 kDa and 23 kDa, respectively. Purified taimen IgM was used to raise a specific rabbit antiserum and to develop a single radial immunodiffusion assay for measuring circulating IgM levels. The serum IgM levels in captive, immature or maturing female taimen varied between 1 and 5 mg/m*l*, showing seasonal changes regardless of fish age, with relatively low levels in spring and conversely high levels in autumn. Production of specific serum IgM to a parasite, *Salmincola stellatus*, was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The parasitized taimen serum could react with protein components of aqueous extracts from the parasite that were blotted on a nitrocellulose membrane after SDS-PAGE, but normal taimen serum did not, indicating that the parasitized fish produced the specific IgM to *S. stellatus* in the serum.

Key Words: Sakhalin taimen; Immunoglobulin M; Purification; Immunoassay; Parasite; *Salmincola stellatus*

Introduction

Many studies on fish immunobiology have dealt with the phylogeny of immunoglobulin structure and function coupled with investigations into the factors affecting the immune response after antigenic stimulation[1,2]. Fish immunoglobulin has been considered to be equivalent to mammalian immunoglobulin M (IgM) class with respect to numerous features of biochemical architecture. These include polymerization of several identical monomeric subunits, each made up of dual heavy (μ) and light (L) chains covalently linked together to form typical Fab and Fc functional domains[3]. Although the most of the IgM generally exists in tetrameric form in teleosts, pentamer and monomer forms of IgM also have been reported[3].

In addition to these studies, serum (plasma) IgM levels have been measured for various fish species: the nurse shark, *Ginglymosstoma cirra-*

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tum[4], paddlefish, *Polyodon spathula*[5,6], gar, *Lepisosteus osseus*[7], carp, *Cyprinus carpio*[8,9], gold fish, *Carassius auratus* and tench, *Tinca tinca*[9], yellowtail, *Seriola quinqueradiata*[10], channel catfish, *Ictalurus puncutatus*[11], turbot, *Scophthalmus maximus*[12], Atlantic cod, *Gadus morhua* L.[13,14], Pacific herring, *Clupea pallasi*[15], and for salmonids, including coho salmon, *Oncorhynchus kisutch*[16], brown trout, *Salmo trutta*[17], chum salmon, *Oncorhynchus keta*[18,19], rainbow trout, *Oncorhynchus mykiss*[20-22], and masu salmon, *Oncorhynchus masou*[23].

Sakhalin taimen (*Hucho perryi*) is an ecologically important salmonid species and is recently depleted in streams from over fishing, loss of spawning grounds and water pollution in Japan. Although there are ecological studies[24-26], and physiological studies[27-32], no humoral pathological study has been conducted in this species. More detailed knowledge of the immunobiology of taimen, including mechanisms of humoral immunity, is essential to develop strategies for maintaining fish health in captive broodstock. The present study describes purification, characterization, and changes in concentration of serum IgM for taimen to obtain basic information on the immune system of this species.

Furthermore, a preliminary data with regard to diagnosis of parasitism is provided in the present paper. In July 1994, we found that *Salmincola stellatus* was parasitic on captive taimen reared at Nanae Fish Culture Experimental Station, Hokkaido University, Hokkaido, Japan. The majority of the host fish (>95%) died due to poor appetite caused by the parasitism. The parasite seemed to be transferred to our station when we introduced 10 individuals of wild taimen from Syumarinai Lake, Hokkaido, Japan. This experience lead us to test an immunobiochemical method using antiserum raised to taimen IgM to detect the parasities in future introductions of wild fish to the station.

Materials and Methods

Experimental animals and blood

Sakhalin taimen were reared at Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University. To observe seasonal and maturational changes of serum IgM levels, four-year-old immature fish (n=3) were sampled monthly during January 1993 to January 1994 (4-year-old group). Five-year-old mature fish (n=5-7) were also sampled each month from November 1992 to May 1994 (5-year-old group). Blood samples were collected from their caudal vessels and allowed to stand at 4°C for several hours. Serum was then separated by centrifugation at 2,000×*g* for 5 min and stored at -30°C.

For purification of taimen IgM, serum was collected from several fish, including immature and mature individuals of both sexes (n=10), and pooled.

Salmincola stellatus (n>100) was collected from taimen's buccal cavity in July 1994, and serum samples of parasitized taimen also were collected for immunoblotting analysis. *S. stellatus* was mixed with equal volume of 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl and 0.1% NaN_3 (w/v), and homogenized in a glass tissue grinder at 4°C. The homogenate was centrifuged at 10,000×*g* for 10 min to remove insoluble materials. The supernatant was collected as aqueous extracts of *S. stellatus*.

Purification of serum IgM

Purification of taimen IgM was carried out according to our previous reports on chum salmon and masu salmon[18,23,33]. Fractions from each purification step were checked by double immunodiffusion using antiserum raised against chum salmon IgM (anti-chum IgM).

Preparation of antisera

Polyvalent antiserum to taimen serum proteins (anti-taimen serum proteins) and anti-chum IgM used in the present study were the same as described in our previous study[23,29]. Antiserum against taimen IgM (anti-taimen IgM) was obtained from rabbit immunized with 1 m*l* of solution containing 1 mg of purified IgM mixed with an equal volume of Freund's complete adjuvant. The rabbit received four injections at 7 day intervals.

Electrophoresis and immunological procedure

Immunoelectrophoresis and double immunodiffusion were conducted in 1% agarose gels using barbital buffer (pH 8.6) according to our routine procedure[31].

Single radial immunodiffusion (SRID) was carried out using antiserum against taimen IgM according to the procedure of Mancini *et al.*[35]. The protein concentration of the purified IgM was measured using Bio-Rad Protein Assay kit (Bio-Rad, USA), with immunoglobulin G (IgG) as a reference standard.

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 3%, 12.5% homologous gel and 5-25% gradient gel was carried out as described in our previous study[18,23,33,34]. Gels were stained with 0.1% Coomassie Brilliant Blue R 250 in a solution of 40% ethanol/10% acetic acid/50% distilled water.

Immunoblot analysis of parasite-induced specific IgM

Aqueous extracts of *S. stellatus* were electroblotted onto a nitrocellulose (NC) membrane (Bio-Rad) by the method of Towbin *et al.*[36] after SDS-PAGE (12.5% homologous gel) under reducing conditions. Before blotting, the gel and NC membrane were dipped briefly in transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol). Transfer of the proteins was carried out at 4 mA/cm^2 for 15 min using a transfer chamber (Nippon EIDO, Japan). After blotting, the NC membrane was incubated for 1.5 hr at room temperature with 5% skim milk solution in 0.02 M Tris-HCl buffered saline, pH 7.5 (TBS) to reduce non-specific binding. After blocking, the membrane was incubated in a 1:25 dilution of taimen serum (serum from parasitized or normal taimen) in TBS for 1hr at room temperature. The NC membrane was washed 2 times for 5 min with TBS containing 0.05% Tween 20 (TBS-T) and 2 times for 5 min with TBS. After washing, the membrane was incubated in a 1:1,000 dilution of anti-taimen IgM in TBS for 2 hr. After washing as described above, 1:2,500 dilution of secondary antibody solution (goat anti-rabbit IgG horseradish peroxidase conjugate; Bio-Rad, USA) was added and incubated in TBS for 1hr at room temperature. After washing the membrane as described above, immunoreactive bands on the membrane were developed using HRP Color Development Reagent (Bio-Rad, USA).

Results

Purification of Sakhalin taimen IgM

Sakhalin taimen serum (100 m*l*) was precipitated by addition of ammonium sulfate at 50% saturation. The precipitate, collected after centrifugation at 10,000× *g* for 10 min, was dissolved in 15 mM Tris-HCl buffer, pH 8.0 and dialyzed against the same buffer. The dialysate was fractionated on a DEAE-cellulose (DE-52, Whatman) column equilibrated with Tris-HCl buffer. Although taimen IgM was detected in all fractions eluted from 0.1 M to 0.4 M NaCl by double immunodiffusion using anti-chum IgM, it was mainly eluted at 0.1 M of NaCl (data not shown).

The 0.1 M-NaCl fraction was applied to gel filtration on Sepharose 6B. As shown in Fig. 1A, the elution pattern showed a single major protein peak with three shoulders following void volume. By the double immunodiffusion assay, taimen IgM was detected in shoulder 1 (data not shown) and therefore these fractions were pooled and then applied to rechromatography on Sepharose 6B. Elution pattern of the rechromatography showed a major symmetrical peak with apparent molecular mass of ~750 kDa (Fig. 1B), and the peak fraction was designated purified Sakhalin taimen IgM.

Purity of Sakhalin taimen IgM

The immunoelectrophoretic pattern of purified taimen IgM is shown in Fig. 2. The taimen IgM gave a single precipitin arc against anti-taimen serum proteins and anti-chum IgM, indicating that the purified taimen IgM was antigenically homogenous.

Specificity of anti-taimen IgM

The anti-taimen IgM used in immunoelectrophoresis revealed a single arc to taimen whole taimen serum and purified taimen IgM,

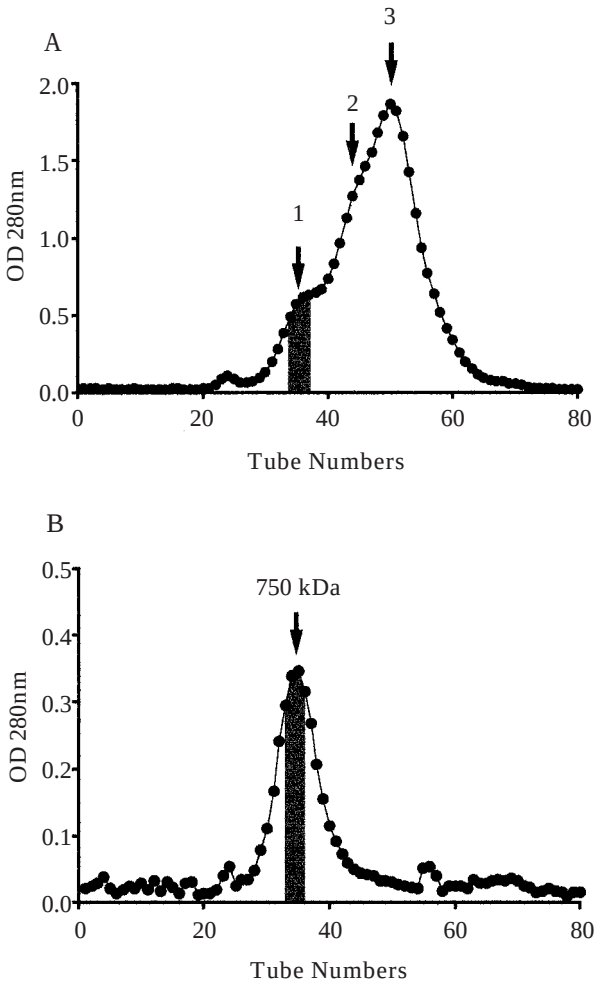

Fig. 1. Elution pattern of gel filtration on Sepharose 6B of the taimen IgM fraction eluted from the DEAE-cellulose chromatography (A). The shoulder indicated by arrow 1 was pooled (represented by shadow) and applied for rechromatography on the same Sepharose 6B column (B). Peak fractions (tube numbers 33-36 in graph B) were collected at a volume of 2.3 m*l*/tube with a flow rate of 12.5 m*l*/hr.

Fig. 2. Imunoelectrophoresis of purified Sakhalin taimen IgM. 1: taimen serum, 2: purified taimen IgM; a: antiserum to chum salmon IgM, b: polyvalent antiserum against taimen serum proteins.

Fig. 3. Immunoelectrophoresis using specific antiserum to taimen IgM (b) and antiserum to whole serum proteins (a). 1: taimen serum, 2: purified taimen IgM.

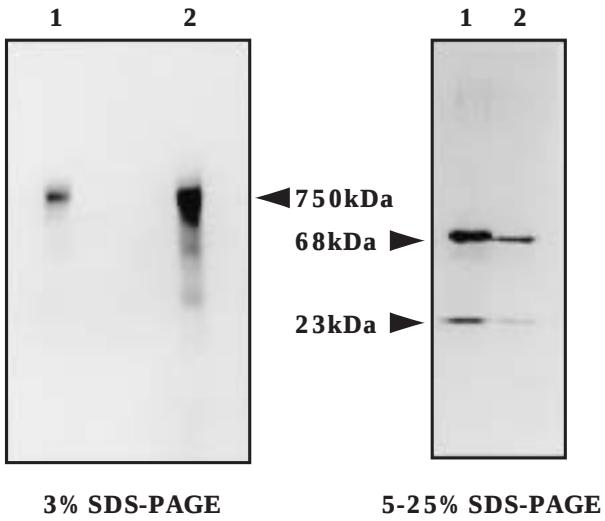

Fig. 4. Patterns of SDS-PAGE of serum IgM purified from chum salmon (1) and taimen (2). Samples were applied to 3% gel under non-reducing conditions or a 5-22.5% gradient gel under reducing conditions, respectively.

respectively (Fig. 3), indicating that the antiserum is mono-specific to taimen IgM.

Biochemical characteristics of taimen IgM

The intact molecular mass of intact taimen IgM estimated by gel filtration on Sepharose 6B was ~750 kDa. Banding patterns of purified taimen and chum salmon IgM after SDS-PAGE are shown in Fig. 4. On 3% gel run under non-reducing condition, taimen IgM gave one main band with an apparent molecular mass of 750 kDa and three minor bands. Under reducing conditions, taimen IgM showed two main bands corresponding to 68 kDa and 23 kDa, as chum

salmon IgM did.

Development of SRID for serum IgM of Sakhalin taimen

Purified IgM standards (25-400μg/m*l*) were added to SRID plates containing anti-taimen IgM at various concentrations ranging from 0.5% to 2.0% (v/v) to determine the optimal conditions for the assay. Consequently, the standard curve on the SRID plate containing 1.25% (v/v) antiserum concentration showed the best result (data not shown).

Seasonal changes in serum IgM of Sakhalin taimen

Changes of serum IgM levels in 4 and 5-year-old fish for a year were measured by the SRID as above developed (Fig. 5). IgM was detected throughout the experimental period, and the concentration ranged from approximately 1 mg/m*l* to 5 mg/m*l*. Concentration of serum IgM dropped in spring (March-May) and rose during late summer and autumn (August-October) regardless of the age-group of the fish.

Detection of specific IgM to S. stellatus in taimen serum

Aqueous extracts of *S. stellatus* were applied to SDS-PAGE and immunoblotting, which was primarily based on the interaction between protein components of *S. stellatus* and specific IgM in the serum (Fig. 6). All protein bands on SDS-PAGE were recognized by serum of parasitized taimen on the immunoblot, while serum of normal (non-parasitized) taimen showed no immunoreactivity to *S. stellatus* extracts.

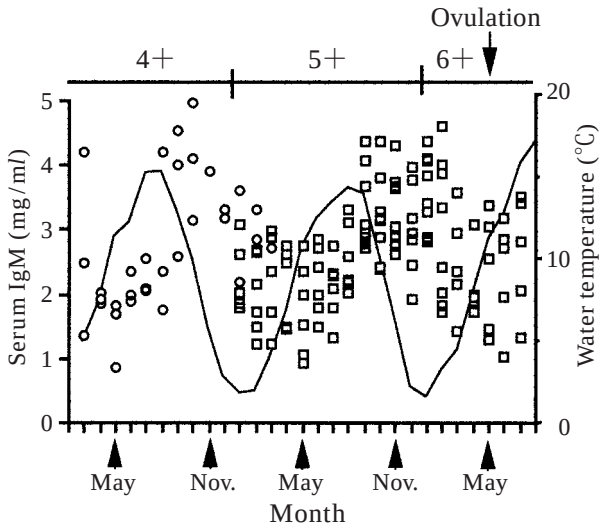

Fig. 5. Seasonal IgM levels in taimen. Open circles and squares represent individuals from 4-year-old and 5-year-old age groups, respectively and line represents water temperature.

Discussion

Previously, we have reported the purification of masu salmon IgM by a combination of salting-out, ion-exchange chromatography, and gel filtration[33,34]. In the present study, purification of the Sakhalin taimen IgM resulted in the isolation of single class of antibodies, based on the result of immunoelectrophoresis using anti-chum IgM and gel filtration on Sepharose 6B. Conversely, the antiserum raised against the purified IgM preparation gave rise to a single precipitin arc against the taimen whole serum, suggesting that the IgM preparation was antigenically pure. Thus, it was shown that this purification method is useful for IgM from other salmonids.

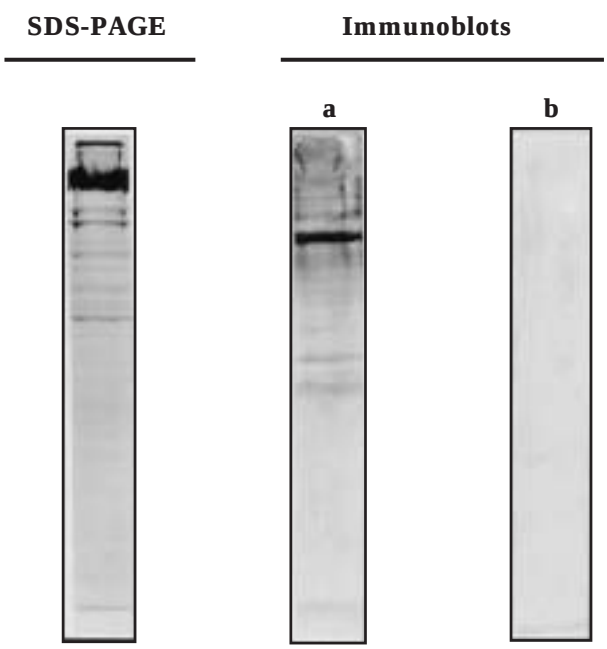

Fig. 6. SDS-PAGE and corresponding immunoblotting of aqueous extracts of *Salmincola stellatus*. Serum from parasitized (a) and non-parasitized (b) taimen were incubated for 1 hr with the transferred nitrocellulose membrane. Antiserum against taimen IgM was used to detect a specific serum IgM to the parasite extracts.

In mammals, IgM is known to have an apparent molecular weight of ~900 kDa and to consist of five disulfide-linked 7S subunits, each of which contains two heavy (μ chain, 70 kDa) and two light (23 kDa) polypeptide chains, while in most of the teleosts, the immunoglobulin has a tetrameric structure and belongs to the IgM class because it possesses a heavy chain (μ chain) of ~70 kDa[3]. In the present study, the molecular mass of taimen IgM estimated by gel filtration and 3% gel was estimated to be ~750 kDa, considerably lower than that of human IgM but close to that of other salmonid IgM[18,33,34]. The peptide subunits of taimen IgM were revealed as two distinct bands corresponding to the heavy (μ) chain and L chain of the IgM monomer by SDS-PAGE. The molecular weights of the μ- and L-chains were calculated to be 68 kDa and 23 kDa, respectively. These molecular masses estimated for the intact IgM and its subunits support the tetrameric structure of taimen IgM, in agreement with that of other teleosts. Concentrations of serum IgM has been measured in different fish species using SRID and enzyme immunoassay (EIA)[23]. Total IgM levels in blood varies among species, by age, and by environmental conditions. In the present study, IgM was detected in taimen serum throughout the period of the experiment by the SRID. The maximum levels of IgM (approximately 5 mg/m*l*) were higher than those in masu salmon and chum salmon[18,23], but lower than that in brown trout (1 year old: 7.3 mg/m*l*)[38]. Serum IgM levels appeared at low levels in spring (in May, ~1 mg/m*l*) and conversely at high levels in autumn regardless of age-group, indicating that the IgM level is affected by seasonal factors rather than maturational factors because similar changes were found in both immature (4-year-old group) and maturing (5-year-old group) fish. However, these seasonal changes of serum IgM levels do not correlate with changes in water temperature[39,40]. The water temperatures in our station are the lowest in January (~4°C) and the highest in August (~17°C), and ~10°C in both May and October. Seasonal changes in the humoral immune response for rainbow trout and scorpion fish, *Sebasiscus marmoratus*, have been reported when fish were reared at a condition of constant water temperature. These results and the present study indicate that temperature may not be a major environmental factor in production of IgM for these species. In contrast, masu salmon did not show such a seasonal change of serum IgM levels[23]. Several factors that affect production of IgM have been reported in various teleosts, and include water temperature, body size[13,14], smoltification[41], growth hormone, cortisol, testosterone[21,22], external antigenic stimulation, and immunological maturation[1]. In the present study, the changes of serum IgM levels in taimen are not likely dependent on water temperature, age, body size, or hormones (e. g. estrogen) involved in maturation. Further environmental and physiological study is needed to explain this phenomenon.

Artificial fertilization of Sakhalin taimen reared at our station is usually carried out in May, and the first ovulation of this species occurs at 6-7 year of age[29]. From the time of ovulation and thereafter, it was often observed that the fish died after spawning due to infection. High levels of stress associated with handling for the artificial spawning and low levels of serum IgM may lead to the unhealthy condition of captive taimen after spawning.

Salmincola stellatus, a species of parasitic copepoda, appears to be host-specific to Sakhalin taimen[42]. We found that *S. stellatus* parasitized the floor and roof of the host's buccal cavity, injecting its attachment organs called "bulla" into the skin of fish, as reported by Nagasawa and Urawa[43]. In the present study, serum of parasitized taimen contained antibodies against all protein bands of the parasite extracts, as revealed by immunoblotting, but normal serum did not, indicating that specific IgM was produced in the serum of taimen by parasitism by *S. stellatus*. Detection of specific IgM in the serum by EIA has been developed to diagnose infectious diseases caused by bacteria or virus in fish[44-50]. Further investigation is needed to verify whether the parasitism-induced IgM is specific to *S. stellatus* or it crossreacts with pro-

tein components of other parasitic with non-parasitic copepods. However, the immunoblotting method developed in this study can provide an effective and easy method for the diagnosis prediction of the parasitism. Recently, we developed an immunochromatography assay for detection of teleost vitellogenin that is known as a useful biomarker to assess a contamination of estrogenic substances in aquatic environment (A. Hara, unpublished data). The immunochromatography can detect the serum vitellogenin within 20 min and the sensitivity is similar to or higher than the EIA. Development of immunochromatography for detecting a serum specific IgM is possible based on the result of immunoblotting in the present study, and may be useful a simple technique to evaluate parasitism rather than the standard EIA.

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イトウの血清イムノグロブリンM（IgM）：精製，血中濃度測定ならびに *Salmincola stellatus* に対する特異IgMの産生

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免疫グロブリンM（IgM）を，イトウ（*Hucho perryi*）血清より精製した。イトウIgMは，他のサケ科魚類のIgMと同様に分子量75万の4量体構造を示した。血清IgM量は，1 mg/m*l* から5 mg/m*l* の範囲で推移し，年令群（成熟度）および水温には依存せず，春期に低く秋期に高い傾向を示した。さらにイムノブロット法により，*Salmincola stellatus* の寄生を受けたイトウ血中に，寄生虫への特異抗体を検出した。