Fisheries Science 61(5), 787-790 (1995)

Immunoglobulin in Oocytes, Fertilized Eggs, and Yolk Sac Larvae of Red Sea Bream

Grigorios Kanlis,*1 Yuzuru Suzuki,*2 Masaru Tauchi,*3 Takeshi Numata,*3 Yoshioki Shirojo,*3 and Fumio Takashima*1

*1Department of Aquatic Biosciences, Tokyo University of Fisheries, Konan, Minato, Tokyo 108, Japan
*2Department of Fisheries, Faculty of Agriculture, The University of Tokyo, Yayoi, Bunkyo, Tokyo 113, Japan
*3Kanagawa Prefectural Fishery Experimental Station, Misaki, Miura, Kanagawa 238-02, Japan

(Received September 8, 1994)

Immunoglobulins in the oocytes, fertilized eggs, and yolk sac larvae of red sea bream were successfully detected by SDS-PAGE and Western blotting, using rabbit antibody against red sea bream blood IgM. Those immunoglobulins appeared structurally identical to the blood IgM of the mother fish, based on the same molecular weight of their H and L chains to those found for the blood IgM, 78 kDa and 26 kDa respectively. Referring to the oocytic immunoglobulin, this appeared in the early stage of vitellogenesis before the spawning season and was also present in reproductively active ovaries during spawning. Those observations indicate that immunoglobulin existing in the mother's blood can be transferred to its oocytes and subsequently to eggs and yolk sac larvae in red sea bream.

Key words: immunoglobulin, red sea bream, oocyte, egg, yolk sac larva

The red sea bream *Pagrus major* is a finfish of high commercial interest for the intensive aquaculture in the sea. The technique for its industrial rearing has been extensively studied and good farming conditions for both its broodstock fish and larvae have been established. However, problems on larval diseases during the first life stage have not yet been resolved and the knowledge on the immunity of eggs and larvae is still limited in this marine species.

The lymphoid organogenesis in red sea bream has been studied by histological methods by Chantanachookhin *et al.*[1] They suggested that no immune mechanism is developed during the larval period. The question arises as how then does the red sea bream larvae defend from infection prior to the development of their own immune system. Transfer of immunity from mother fish to them could be a possible answer.

Recent studies have demonstrated that antibody activity was present in fish ovaries and suggested the possibility of passive immunity from mother to the eggs. Bly *et al.*[2] immunized plaice against sheep red blood cells and detected the existence of an antibody against the sheep erythrocytes in ovarian extracts. Hayman and Lobb,[3] using monoclonal and polyclonal antibody procedures, determined that in channel catfish immunoglobulin was present in egg proteins obtained from 2 to 3 day-old fertilized eggs; and localized the immunoglobulin within the external membranes (eggshell) and the yolk of the egg. Fuda *et al.*[4] detected in the egg yolk of chum salmon an IgM-like protein which was antigenically identical with serum IgM. Castillo *et al.*[5] observed lymphocytes exhibiting cytoplasmatic and surface immunoglobulin in rainbow trout embryos at 12 and 8 days before hatching respectively; thus unfertilized eggs contained detectable amounts of immunoglobulin. Avtalion and Mor[6] immunized tilapia and demonstrated that extracts obtained from 0 to 9 day-old embryos possessed antibody activity that was blocked by the addition of homologous antigen. Suzuki *et al.*[7] identified a low molecular weight immunoglobulin in the ovaries and eggs of carp. With this background, it was important to determine if the female spawner could provide immunity to its eggs and larvae in red sea bream.

Investigations on red sea bream were also worth of attention due to the spawning characteristics of this marine teleost. Matsuyama *et al.*[8] determined that the red sea bream is a multiple spawner, having a diurnal ovarian maturation rhythm, and the rapid growth of oocytes takes place a few hours before spawning. Only one investigation on a similar spawning fish, the plaice,[2] has been reported so far suggesting a possibility of maternal immune activity to the eggs, although the existence of the IgM molecule was not discussed.

In our study, the red sea bream oocytes, eggs, and larvae were detected for the presence of IgM and its structure was examined in relationship to the immunoglobulin existing in blood. Thus, aspects for the immunoglobulin accumulation in the oocytes are discussed.

Materials and Methods

Source of Materials

Adult red sea breams were kept under natural conditions in a rearing pond at Kanagawa Prefectural Fishery Experimental Station. Blood plasma used for IgM purification and the development of techniques for IgM analysis was selected from 0.5-3.0 kg weight male individuals.

Before (in April) and during the spawning season (in June), five females weighing 0.7–4.7 kg were sacrificed. Their blood was taken from the caudal vasculature with a heparinized syringe and a needle, and the blood plasma was collected by centrifugation at 1,630 × g for 15 min at 4°C. Ovaries were removed from the fish and their oocytes were suspended in 0.1 M phosphate buffered saline (PBS, pH 7.2) by using forceps. After removal of the coexisting masses of ovarian tissue from the suspension, the oocytes were extensively washed three times in PBS and stored at −80°C until use. During the spawning season, naturally spawned eggs were collected from a broodstock pond and incubated in a plastic tank. Eggs right after fertilization and one day post hatching larvae were sampled and stored at −80°C until use.

The ovarian maturity of all tested fish was investigated by light microscopy. In this, ovaries collected both before and during the spawning season were sectioned at 8 μm and stained with Mayer's hematoxyline-eosine. Further, the oocyte maturation stage was determined according to Matsuyama *et al.*[8]

Purification and Characterization of Red Sea Bream Blood IgM

Using high-performance liquid chromatography, blood plasma of male fish was percolated through a gel filtration column (TOSOH G4000SW$_{XL}$) previously equilibrated with 0.05 M phosphate buffered saline (pH 7.0) and monitored at 280 nm. The immunoglobulin fraction was collected and subsequently applied to a column of protein A Sepharose CL-4B (Pharmacia) previously equilibrated with a binding buffer (3 M NaCl, 1.5 M glycine, pH 9.0). After washing the column with the same buffer, the absorbed protein fraction was eluted with citric acid (pH 3), collected and dialyzed against 0.01 M phosphate buffered saline (pH 7.4). This protein preparation was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie R250. Further, the molecular weight of the heavy and light chains of the purified immunoglobulin was determined by SDS-PAGE low molecular weight standards (Bio-Rad) according to the manufacturer's instructions.

Antibody against Red Sea Bream Blood IgM

Rabbits were injected in the inguinal lymph nodes with purified red sea bream immunoglobulin emulsified 1:1 in Freund's complete adjuvant. The injected immunoglobulin was at a dose of 100 μg/rabbit. Booster subcutaneous injections with the same antigen and Freund's incomplete adjuvant (1:1) were given 15 and 30 days later. Rabbit blood serum was collected two weeks after the last injection and the immunospecificity of the raised antibody was tested by the Ouchterlony's double immunodiffusion test. In this, purified red sea bream blood IgM was served as a standard antibody. Rabbit IgG was purified from the antiserum by applying it to a column of protein A Sepharose CL-4B (Pharmacia), after Suzuki *et al.*[7] The IgG fraction was then collected and dialyzed against 0.01 M sodium bicarbonate buffer (pH 8.5). Further, the rabbit IgG at a concentration of 2.3 mg/ml was reacted with biotin (long arm) N hydroxysuccinimide ester (BNHS; Vector Lab. Inc.) at an aliquot of the biotinylating reagent equal to 1/10 the weight of the protein labeled. Two hours later the reaction was stopped by adding glycine and the biotinylated protein was dialyzed against 0.1 M phosphate buffered saline (pH 7.2).

Detection of the Immunoglobulin in Red Sea Bream Plasma, Fertilized Eggs, Yolk Sack Larvae, and Oocytes

A technique applied by Hayman and Lobb[3] for fractionation of immunoglobulin from the channel catfish eggs was successfully adapted in our study. In this, 10 grams of red sea bream eggs, larvae, and oocytes were separately homogenized in 10 ml of 0.1 M Tris buffered saline containing 0.14 M NaCl, 1 mM EDTA (TBS-EDTA, pH 7.4). Further, each homogenate was centrifuged at 58,700 × g for 20 min and the supernatant was collected. The remaining pellet was suspended in 10 ml TBS-EDTA and centrifuged. The pellet was resuspended in 10 ml TBS-EDTA and centrifuged again. The corresponding supernatants were pooled and added with 3 ml of 10% dextran sulfate in TBS and sequentially 7.5 ml of 1 M calcium chloride. After stirring for 30 min at room temperature, the precipitated lipids and lipoproteins were sedimented at 58,700 × g for 15 min and the pellet discarded. The supernatant was added with an equal volume of saturated ammonium sulfate and stirred for an additional 30 min at room temperature. Following centrifugation, the ammonium sulfate pellet was dissolved in 1 ml of 20 mM Tris buffer (pH 8.0) and dialyzed extensively in the same buffer at 4°C. The insoluble material were removed by centrifugation and the protein preparation was ready for use.

Red sea bream plasma, purified blood IgM, protein preparations of fertilized eggs, yolk sac larvae, and oocytes were separated by SDS-PAGE; and the proteins were electrophoretically transferred to a Millipore membrane (PVDF). The membrane was rinsed with a blocking reagent (Block Ace: Yukijirushi Co. Ltd.) for 30 min at room temperature and consequently treated with biotinylated anti-red sea bream immunoglobulin-rabbit IgG (diluted × 800 in TBS-Tween). Further, it was reacted with peroxidase conjugated avidinbiotin complex (Vectastain Elite ABC kit, Vector Lab. Inc.) for 90 min and the antigen-antibody complex was visualized in diaminobenzidine (DAB) solution.

Results

Characterization of Red Sea Bream Blood IgM

Figure 1 shows the elution pattern during gel filtration combined with a HPLC in which the IgM appears at 7.4 to 8.4 minutes. Figure 2 shows the pattern of protein A affinity chromatography. The IgM was purified by this process and the molecular weights of the H and L chains were determined to be 78 and 26 kDa respectively (Fig. 3 left). A minor band of 74 kDa was also detected.

Antibody against Red Sea Bream Blood IgM

Figure 3 right shows the Western blotting patterns using antibody against blood IgM. The antibody has enough specificity since the highest intensity of staining was found in the immunoglobulin H and L chains than the other proteins of the blood plasma. However, minor bands between the H and L chains were also coupled with this antibody.

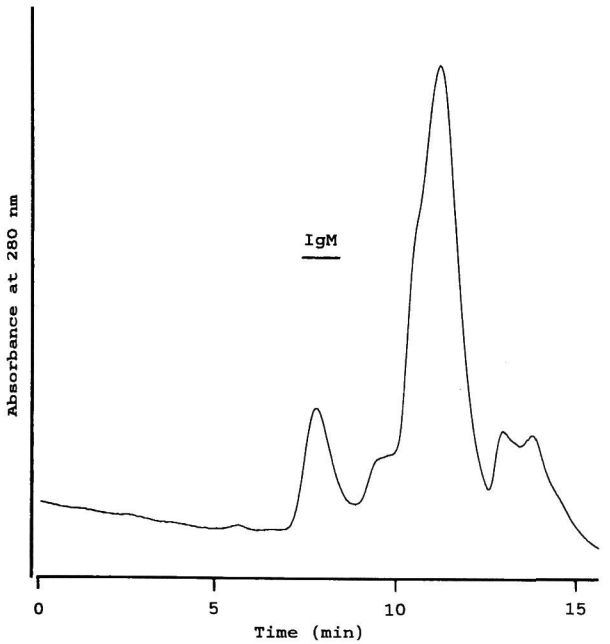

Fig. 1. High performance liquid chromatogram of red sea bream blood plasma using TOSOH G4000SW$_{XL}$.
The immunoglobulin fraction eluted at 7.4–8.4 min was collected.

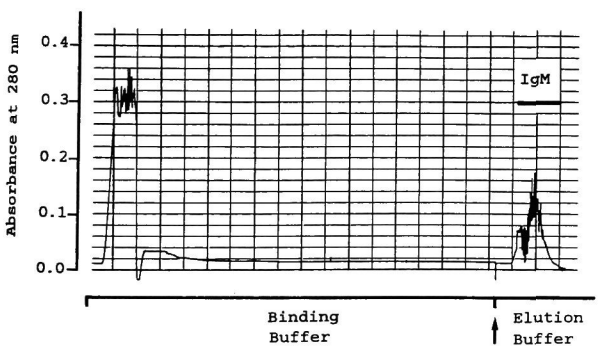

Fig. 2. Protein A Sepharose CL-4B (Pharmasia) affinity chromatogram of red sea beam IgM.
The immunoglobulin fraction was eluted just after changing the binding buffer to elution buffer (arrow).

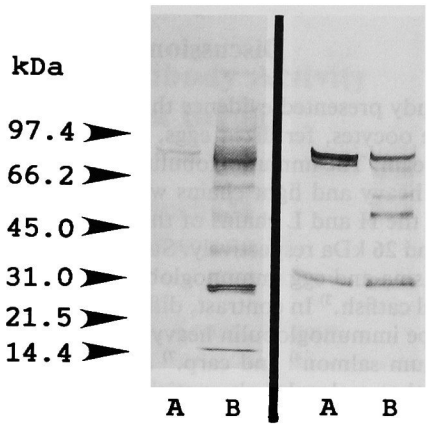

Fig. 3. SDS-PAGE and Western blotting analysis of red sea bream blood IgM and blood plasma.
Left; Coomassie brilliant blue staining. Right; Immunostaining using anti-red sea bream IgM antibody. Lane A, purified blood IgM; Lane B, blood plasma proteins diluted × 100 in PBS.

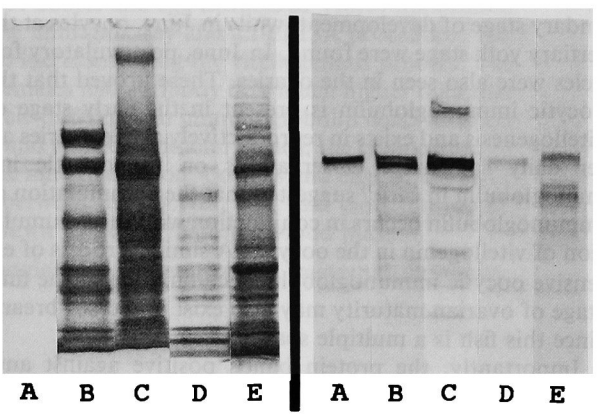

Fig. 4. SDS-PAGE and Western dlotting analysis of red sea bream blood IgM, fertilized egg, yolk sac larva, and oocytes.
Left; Coomassie brilliant blue staining. Right; Immunostaining using anti-red sea bream IgM antibody. Lane A, purified blood IgM; Lane B, egg extract; Lane C, larval extract; Lane D, oocytic extract obtained from ovary collected before the spawning season; Lane E, oocytic extract obtained from ovary collected during the spawning season.

Presence of Immunoglobulin in Red Sea Bream Fertilized Eggs and Yolk Sac Larvae

Immunoglobulin was clearly detected in the eggs and larvae (Fig. 4B and C). Furthermore, the relative position of the H and L chains were similar to those found for the blood IgM. However, many protein bands positive against anti-IgM antibody other than the H and L chains were also seen, especially in the yolk sac larvae.

Presence of Immunoglobulin in Oocytes

Before the spawning season (in April), the most advanced oocytes in the ovaries were in the secondary yolk stage of development, according to histological observations. Further, in the spawning season (in June) the fish had ovaries containing postovulatory follicles and oocytes in the primary, secondary and tertiary yolk stages. No ovulated eggs were found in the ovaries showing that the fish examined in June had already spawned.

Immunoglobulin was detected by Western blotting in ovaries having their most advanced oocytes at the secondary yolk stage of development before the spawning season (Fig. 4D). Immunoglobulin was also detected in ovaries containing vitellogenic oocytes at the primary, secondary, and tertiary developmental stages during the spawning season (Fig. 4E). The heavy and light chains of the oocytic immunoglobulin were also identified at the same relative position as the H and L chains described for the blood IgM. The small amount of protein bands positive against anti-IgM antibody other than the H and L chains were also detected.

Discussion

This study presented evidence that immunoglobulin exists in the oocytes, fertilized eggs, and yolk sac larvae of red sea bream. The immunoglobulin was found to be composed of heavy and light chains with identical molecular weight as the H and L chains of the blood IgM in mother fish, 78 and 26 kDa respectively. Such a similarity between blood plasma and egg immunoglobulin was also observed in channel catfish.[3] In contrast, different molecular weight of egg type immunoglobulin heavy chain has been reported for chum salmon[4] and carp.[7] A possible explanation proposes that molecular alternation may occur during the immunoglobulin accumulation in the ovary in some fish species, while no changes occur in red sea bream.

The Western blotting analysis documented that the immunoglobulin was present in the red sea bream oocytes not only during the spawning but also before the spawning season, in June and April respectively. Taking into consideration the oocytic composition of the examined ovaries, the most advanced oocytes in April were at the secondary stage of development, while in June, oocytes at the tertiary yolk stage were found. In June, postovulatory follicles were also seen in the ovaries. These proved that the oocytic immunoglobulin is present in the early stage of vitellogenesis and exists in reproductively active ovaries after daily spawning. Observations on the oocytic immunoglobulin in carp[7] suggested that the accumulation of immunoglobulin occurs in conjunction with the accumulation of vitellogenin in the oocytes. A similar process of extensive oocytic immunoglobulin accumulation at the final stage of ovarian maturity may also exist in red sea bream, since this fish is a multiple spawner.

Importantly, the protein bands positive against anti-IgM antibody other than the H and L chains were increased according to the stage of development from oocyte to egg in red sea bream. This may be caused by the fragmentation of the oocytic immunoglobulin by proteolytic enzyme, which is known to be activated prior to ovulation.

We succeeded to detect immunoglobulins in the oocytes, eggs, and larvae, by using an antibody which has enough affinity and specificity against the blood IgM. This was shown by the fact that the heavy and light bands were clearly detected among blood plasma proteins on Western blotting. However, in red sea bream blood, a minor heavy chain was also seen. Immunoglobulin heavy chains of lower molecular weight have been also reported in the plasma of chum salmon,[4] sheepshead,[9] and channel catfish.[10] Thus, the presence of low molecular weight heavy chains in fish blood are not unusual.

Another technical achievement of this study is the partial fractionation of immunoglobulin contained in the red sea bream oocytic, egg, and larval extracts. This was based on a procedure reported for the catfish egg IgM.[3] In this manner, the lipovitellin, which was considered to be the major component in the tested extracts, was precipitated by dextran sulphate and calcium chloride; and we attained preparations containing immunoglobulin that was clearly visualized by Western blotting.

Investigation on the lymphoid organogenesis in red sea bream indicated that the first appearance of morphologically mature lymphocytes occurs at the age of 23 days post hatch.[1] Therefore, it is reasonable to consider that the immunoglobulin contained in the eggs and yolk sac larvae originated from the mother fish. As a consequence, it is quite possible that this putative maternal immunoglobulin may have a protective role during the early stage of life in red sea bream. Further research on the IgM levels and the antibody activity in eggs and larvae of vaccinated mothers is required and the significance of maternal immunity should be established.

Acknowledgments The authors sincerely thank Dr. Carlos Strussmann, Department of Aquatic Biosciences, Tokyo University of Fisheries, for reviewing this manuscript. This study was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, and Culture.

References

1) C. Chantanachookhin, T. Seikai, and M. Tanaka: Comparative study of the ontogeny of the lymphoid organs in three species of marine fish. *Aquaculture*, **99**, 143–155 (1991).
2) J. Bly, A. Grimm, and I. Morris: Transfer of passive immunity from mother to young in a teleost fish: Haemagglutinating activity in the serum and eggs of plaice, *Pleuronectes platessa L. Comp. Biochem. Physiol.*, **84A**, 309–313 (1986).
3) R. Hayman and C. Lobb: Immunoglobulin in the eggs of the channel catfish (*Ictalurus punctatus*). *Dev. Comp. Immunol.*, **17**, 241–248 (1993).
4) H. Fuda, A. Hara, F. Yamazaki, and K. Kobayashi: A peculiar immunoglobulin M (IgM) identified in eggs of chum salmon (*Oncorhynchus keta*). *Dev. Comp. Immunol.*, **16**, 415–423 (1992).
5) A. Castillo, C. Sanchez, J. Dominguez, S. Kaattari, and A. Villena: Ontogeny of IgM and IgM-bearing cells in rainbow trout. *Dev. Comp. Immunol.*, **17**, 419–424 (1993).
6) R. Avtalion and A. Mor: Transfer of antibody activity from immunized mother to embryo in tilapias. *J. Fish Biol.*, **37**, 249–255 (1990).
7) Y. Suzuki, M. Orito, K. Furukawa, and K. Aida: Existence of low molecular weight immunoglobulin M in carp eggs. *Fisheries Sci.*, **60**, 159–162 (1994).
8) M. Matsuyama, S. Adachi, Y. Nagahama, and S. Matsuura: Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red sea bream, *Pagrus major*, during the spawning season. *Aquaculture*, **73**, 357–372 (1988).
9) C. J. Lobb and L. W. Clem: Phylogeny of immunoglobulin structure and function-X. Humoral immunoglobulins of the sheepshead (*Archosargus probatocephalus*). *Dev. Comp. Immunol.*, **5**, 271–282 (1981).
10) R. C. Lobb: Structural diversity of channel catfish immunoglobulins. *Vet. Immunol. Immunopathol.*, **12**, 7–12 (1986).