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CELLULAR AND MOLECULAR BIOLOGY

Acquirement of HRP conjunct IgG anti-IgMs from most widely cultured freshwater fishes in China and its immunoreactivity

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Abstract: Until now, custom-made or commercial polyclonal antibody against only one kind of fish IgM limited application of the antibody. During our research on development of vaccine against infection of *Clonorchis sinensis* (*C. sinensis*) in several kinds of fish, we were conscious of the urgency of secondary antibody to evaluate immune effect and screen *C. sinensis* infection with immunological technology instead of labor-intensive and time-consuming squash or artificial digestion of fish flesh. So that, we purified IgM of grass carp, bighead carp, crucian carp, common carp and tilapia which were widely cultured freshwater fishes in most areas of China. On this basis, we generated HRP-conjunct rabbit IgG anti-fish IgMs with high titers. IgM of other freshwater fishes including oshima, yellow catfish, bream, silver carp and so on could be recognized by the IgG sensitively. Additionally, The ELISA detection displayed that the IgG could be more specific and sensitive than custom-made rabbit IgG anti-grass carp IgM. The acquirement of HRP-conjunct rabbit IgG anti-fish IgMs was the cornerstone for studying the immune system of teleost fish, developing immunoassay methods and evaluation of fish vaccine with more convenience.

Key words: IgM, secondary antibody, IgG, freshwater fish, protein A chromatography, immunoreactivity.

INTRODUCTION

Immunoglobulins (Igs) play important roles in humoral adaptive immunity of vertebrate. Fish belong to a kind of lower vertebrate. It has been documented that Igs-centric specific immunologic mechanism first appeared in fish (Lobb & Clem 1981). Immunoglobulin M (IgM) from teleost, an analogous to IgG of mammal, is the predominant isotype found in serum and mucus (Solem & Stenvik 2006). Teleost IgM contributes to both innate and adaptive immunity in fish (Mashoof & Criscitiello 2016). It can mediate agglutination for phagocytosis and removing pathogen, and cellular cytotoxicity (Ye et al. 2013). Teleost IgM typically is expressed as a tetramer containing two heavy (H) chains with molecular weight (MW) of 70-81 kDa and two light (L) chains with MW of 22-32 kDa (Vesely et al. 2006a, Ye 2008).

On the one hand, IgM can be employed to investigate the immune response of immunized or infected teleost fishes, that will facilitate getting more massage about immune system and developing vaccine against infection of teleost fishes. On the other hand, fish diseases induced by infection of pathogens mainly identified by the appearances and behaviors of the fish, and pathogen detection by microscope,

although nucleic acid detection of pathogens by using polymerase chain reaction (PCR) was employed recently. These methods greatly depend on the professionals or equipment. Teleost serum contains natural IgM antibody before immunization, but antigen exposure with T cell helps drive release of specific tetrameric IgM (Boes 2000). It supports that detection of specific IgM induced by the pathogen theoretically is an alternatively method, but till now very few immune methods have been applied for diagnosis of infectious diseases in freshwater fish, especially parasite infection. Moreover, that secondary antibody against IgM from most species of teleost fishes for visualized detection with increasing sensitivity and signal amplification are unavailable may contribute to the disadvantageous situation of immune detection. The unavailability is partly due to that the IgMs still haven't been purified and characterized, especially IgM from some freshwater fish. IgM have been isolated and characterized in many commercially important aquaculture species such as Asian cat fish (Swain et al. 2004), Guppy (Lim et al. 2009), Indian major carps (Bag et al. 2009), Asian sea bass (Choudhury & Prasad 2011), and Wrasses (Bilal et al. 2016). However, IgMs from other teleost fishes still haven't been purified and completely characterized including grass carp (*ctenopharyngodon idella*), bighead carp (*Hypophthal michthysnobilis*), crucian carp (*Carassius carassius*), common carp (*Cyprinuscarpio*) and Tilapia (*Oreochromis niloticus*) which are known as the most widely cultured freshwater fishes in most areas of China (Song et al. 2017, Chiu et al. 2013).

Clonorchis sinensis (*C. sinensis*) is a fish-borne trematode resulting in infection of nearly 15 million people all over the world by digestion of infected raw or undercooked freshwater fish (Qian et al. 2016). In China, a total of 102 species of fish (59 genera in 15 families) and four species of shrimp are recognized as the second intermediate hosts. The infection of *C. sinensis* in freshwater fish mostly depends on microscope examination of *C. sinensis* metacercaria by squash or artificial digestion of fish flesh. To prevent against the infection of *C. sinensis*, grass carp, as the most common second intermediate host, were orally immunized with *Bacillus subtilis* spores displaying antigens from *C. sinensis* (recombinant *B. subtilis*) in our previous studies. In grass carp, the immune effects of recombinant *B. subtilis* were detected by rabbit anti-grass carp-IgM IgG (Sun et al. 2018, Tang et al. 2016). In challenge experiments, grass carp infected with *C. sinensis* were checked by squash or artificial digestion of fish flesh. It was labor-intensive and time-consuming. Considering that immunization of recombinant *B. subtilis* could be potentially applied to a variety of freshwater fish, it is urgent for us to obtain antibody against their IgMs for evaluation of immune effect and screening of *C. sinensis* infection.

Up to now, custom-made or commercial polyclonal antibody against fish IgM was usually generated in mammal by immunization of purified IgM from only one kind of fish that limited application of the antibody. We aim to generate universal antibody against IgMs of most cultured freshwater fish in China for further application in evaluation of immune response of freshwater fish and immunologic screening for infectious diseases of freshwater fish (including *C. sinensis* infection).

Proteins A and G from *Staphylococcus aureus* and *Streptococcus spp* are immunoglobulin binding proteins (Huse et al. 2002). Protein A chromatography is one of the most important methods for Igs purification due to its high purification efficiency (Bolton & Mehta 2016). Protein A chromatography for Igs purification from a number of teleost species have been

recently reported (Bromage et al. 2004). In mammals, different immunoglobulin isotypes have different binding preference to protein A as well as in teleost species based on the data from olive flounder, southern bluefin tuna and rainbow trout (Shin et al. 2007, Watts et al. 2001 a,b, Morrison & Nowak 2001). Recombinant Protein A (rProtein A) is based on DNA technology and enables efficient *in-situ* cleaning in industrial production of Igs purification. In addition, asparagine residues in protein A molecules have been selectively replaced to improve their stability in alkaline environment (Linhult et al. 2004).

The present study was designed to purify and characterize IgMs from grass carp, bighead carp, crucian carp, common carp and tilapia. On this basis, horseradish peroxidase (HRP) labelled mammalian antibody against IgMs from the five categories was generated and its immune reaction were checked.

MATERIALS AND METHODS

Ethics approval

Rabbits used in the study were purchased from Animal Center of Sun Yat-Sen University and raised carefully based on the National Institutes of Health on animal care and the ethical guidelines. All animal experiments were approved by Institutional Animal Care and Use Committee, Sun Yat-Sen University (Permit Numbers: SYXK (Guangdong) 2010–0107).

Sera collection

Healthy grass carp (1000 ± 20 g), bighead carp (6000 ± 20 g), crucian carp (600 ± 20 g), common carp (1000 ± 20 g) and Tilapia (600 ± 20 g) were kindly provided by Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences (Guangdong, China). Fifteen individuals were included in each category. Blood samples were collected from caudal vessel of the fishes, allowed to clot at room temperature for 1 h, and then stored at 4°C overnight. The sera were collected after centrifugation with 2000 g for 5 min at 4°C. Four to five healthy individuals of Japanese sea perch (*Lateolabrax japonocus*), sunfish (*Lepomis gibbosus*), Oshima (*Spinibarbus denticulatus*), mandarin fish (Siniperca chuatsi), Linnaeus (*Channa asiatica*), snakehead (*Ophiocephalus argus Cantor*), yellow catfish (*Pelteobagrus fulvidraco*), bream (*Parabramis pekinensis*), black pacu (*Piaractus brachypomus),* black bream *(Acanthopagrus latus),* and silver carp (*Hypophthalmichthys molitrix*) were purchased from Huangsha aquatic products market, Guangzhou, China. The sera samples were respectively collected with the same method mentioned above.

Purification and identification of Igs

The collected serum from fifteen individual in the same category were converged, diluted at 1:1 with the equilibrium buffer of phosphate buffered saline (20mM PBS, pH 8.0 with 3.0 M NaCl) and then subjected to rProtein A Sepharose FF packed column (Weishibohui Chromatography Technology Co. Ltd., Beijing, China) (Stec et al. 2004). In order to fully combine Igs to rProteinA in the column, the sample solutions were repeatedly subjected to the column three times at a flow rate of 1 ml/min and subsequently incubated in the column for 4 h at 4°C. Non-binding molecules were washed out with 10 columns volumes of the equilibrium buffer. The specific bindings were successively eluted with 6 column volumes of the elution buffer (0.1M glycine eluate, pH 2.5) and collected in aliquots of 1ml immediately neutralized with 65 µl neutralizing buffer-1M Tris-HCl, pH 9.0) (Kreutz et al. 2016). The purification was identified by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

as described before (Fernandez et al. 1998). The collected aliquots were subsequently pooled together, and dialyzed with PBS for 24 h by replacing one third of the dialysate every 6 h. Finally, the proteins were concentrated with polyethylene glycol (PEG20000) and store at -80 °C. Protein concentrations were determined by bicinchoninic acid (BCA) assay kit (GBCBIO Technologies, Guangzhou, China).

Acquirement of purified rabbit IgG anti-fish IgM

After identification, 40μg of purified IgM from each category were mixed and emulsified with Freund's complete adjuvant (Sigma, USA) at the volume ratio of 1:1. Each rabbit was injected in 8-10 sites subcutaneously. At a 2-week interval, the rabbits were subcutaneously boosted twice with half dose of the mixed IgMs emulsifying with Freund's incomplete adjuvant (Sigma, USA). At 6 and 7 weeks after the last injection, rabbit blood was collected by cardiac puncture under anesthesia and allowed to clot 1h at RT and left at 4°C overnight. The serum samples were then obtained by centrifugation at 2000 g for 20 min. Meanwhile, other rabbits were respectively immunized 200μg of purified IgM from single category with the same method. After determination of antibody titers, the sera from rabbit immunized with mix purified fish IgM were diluted at 1:1 with the equilibrium buffer (20 mM PBS, pH 7.4) and subjected to another rProtein A Sepharose FF packed column. The rabbit IgG was purified and identified as described above.

Labeling of rabbit IgG anti-fish IgM with HRP

HRP was conjugated to IgG purified from rabbit immunized mixed fish IgMs by means of the periodate method (Nakane & Kawaoi 1974). In brief, 4mg HRP (Roche, Switzerland) was dissolved in 1ml of distilled water in darkness. 1ml of distilled water containing 24.1mg sodium periodate was prepared. 200 μl of the sodium periodate solution was added to the prepared HRP solution, and then the container was kept on a stirrer for 20min at room temperature (RT) in darkness. The blend was dialyzed against sodium acetate buffer (1mM, pH 4.4) overnight at 4°C followed by the addition of 10μl of carbonate bicarbonate buffer (0.2 M, pH 9.5). The purified rabbit IgG anti-fish IgMs (4mg) was dialyzed against carbonate buffer (0.5M, pH 9.5) for 3 h, and then added to the active enzyme by gently stir at RT for 2 h. 10% thimerosal was added to the solution until its final concentration was 0.01%. Finally, glycerin was added to the solution in a volume ratio of 1:1 and stored at -20 ° C in the dark till used.

Enzyme linked immunosorbent assay (ELISA)

To detect the level of IgG anti-fish IgM in rabbit sera, 96-well plates were coated with the single IgM (5μg/ml) or the mixed IgMs (5 μg/ml) diluted in carbonate bicarbonate buffer (150 mM Na_2CO_3, 348 mM $NaHCO_3$, 30 mM NAN_3, pH 9.6) and incubated at 4°C overnight. The plates were washed with PBS with Tween-20 (PBST) three times at 5 min intervals followed by incubating at 37 °C with 100μl / well of blocking agent (5% skim milk in PBST) for 2h. After washing three times with PBST, 100μl of PBS with sera from immunized rabbit (at tiraes ratio dilution of 1:800, 1:1,600, 1:3,200 to 1:16382,400) were added to each well and reacted at 37 °C for 1h. Sera from preimmunized rabbit with the same dilutions as primary antibodies were employed to be negative controls. Each well was then reacted with 100μl of PBS including HRP labelled anti-rabbit IgG (1:2000 dilutions, Proteintech, USA) at 37 °C for 1h after washing with PBST. The reaction was developed color by using 100 μl of 3, 3', 5, 5'- tetramethylbenzidine (TMB, BD, USA) as a substrate and the absorbance was determined

at 450 nm after stopping the reaction using 50 µl of 2 M H_2SO_4.

To compare detection effect between rabbit IgG anti-fish IgMs we generated and commercial rabbit IgG anti-grass carp IgM, flat bottom 96-well plates were coated with 5 µg/ml/well of recombinant trichoplein keratin filament-binding protein of *C. sinensis* (r*Cs*TCHP). Sera from grass carp orally immunized with *B. subtilis* spores displaying *Cs*TCHP (*B.s-Cs*TCHP) (at tiraes ratio dilution of 1:100, 1:200, 1:400 to 1: 204,800) were employed as the primary antibody. HRP labelled rabbit IgG anti-fish IgMs (1:6000 dilutions) or commercial HRP labelled rabbit IgG anti-grass carp IgM (1:6000 dilutions, Zoonbio Biotechnology, China) was added as the second antibody. The process was same with that mentioned above.

Western blotting

Dithiothreitol (DTT) treated sera from sixteen categories of fishes (grass carp, crucian carp, bighead carp, common carp, tilapia, Japanese sea perch, sunfish, Oshima, mandarin fish, Linnaeus, snakehead, yellow catfish, bream, black pacu, black bream and silver carp) were respectively separated by 12% SDS-PAGE and electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, USA) in a Trans-Blot transfer cell (Bio-Rad, Hercules, USA) at 100 V for 1.5 h. The membrane was blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBST, pH 7.4) for 1h at RT. After washing with TBST three times, the membrane was incubated with prepared HRP conjugated rabbit IgG anti-fish IgMs (1:2000 dilution) at RT for 1h. Finally, detection was performed using chemiluminescence after washing the membrane 5 times.

RESULTS

Acquirement and quantification of purified fish IgM

After rProtein A Sepharose FF chromatography, the eluted Igs was analyzed by SDS-PAGE. Two main bands around 66kDa and 25kDa were observed in each sample, which were approximate to the molecular weight (MW) of H and L chain of fish IgM (Figure 1). After concentration with PEG20000, the final concentrations and quantities of purified IgM from each category were showed in Table I. The concentration of IgM from bighead carp was highest as well as its quantity. The total quantity of IgM from crucian carp or common carp was respectively only 2.856mg or 2.87mg, but it was enough for subsequent immunization.

Antibody titers in sera from rabbits immunized with purified fish IgM

6 weeks and 7 weeks after the first immunization, antibody titers of sera from immunized rabbit groups were detected by ELISA. Antibody titers in six immunized groups all peaked to 1:163,824,001 (Figure 2a-b) showing the high immunogenicity of the purified fish IgM.

Identification of rabbit IgG anti-fish IgMs

After rProtein A Sepharose FF chromatography of sera from mixed fish IgMs immunized rabbit and 12% SDS-PAGE, a dominant band about 50 kDa was observed indicating H chain of rabbit IgG. In addition, there were some fuzzy bands around 25 kDa which was MW of L chain (Figure 3). After dialyzed with PBS and treated with PEG20000, the final concentration of rabbit IgG anti-fish IgMs was 11 mg/ml. The total amount of 33 mg was extracted from 5 ml of rabbit serum.

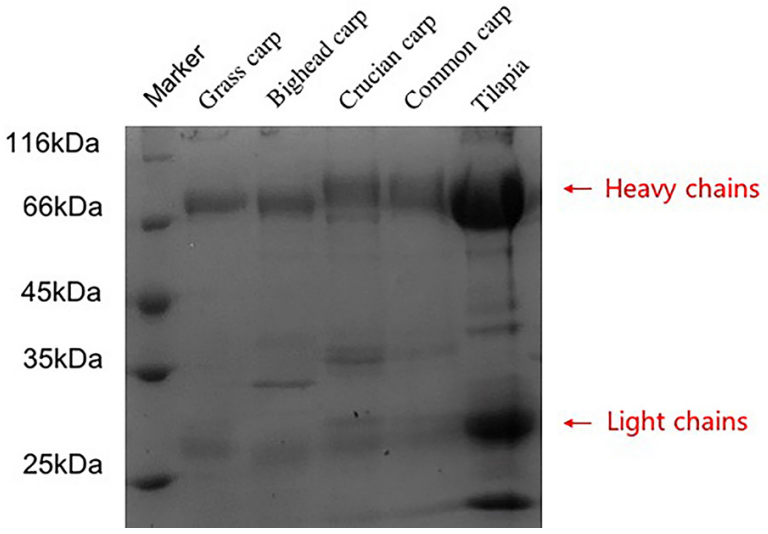

Figure 1. Purification of IgMs from fishes identified by SDS-PAGE. Lanes from left to right were MW marker (M), Grass carp, bighead carp, crucian carp, common carp, and tilapia.

Table I. The final concentration and quantity of purified IgM from five categories.

Categories	Final concentration (mg/ml)	Total quantity(mg)
Grass carp	0.56	3.136
Bighead carp	2.00	6.4
Crucian carp	0.68	2.856
Common carp	0.70	2.87
Tilapia	0.93	3.72

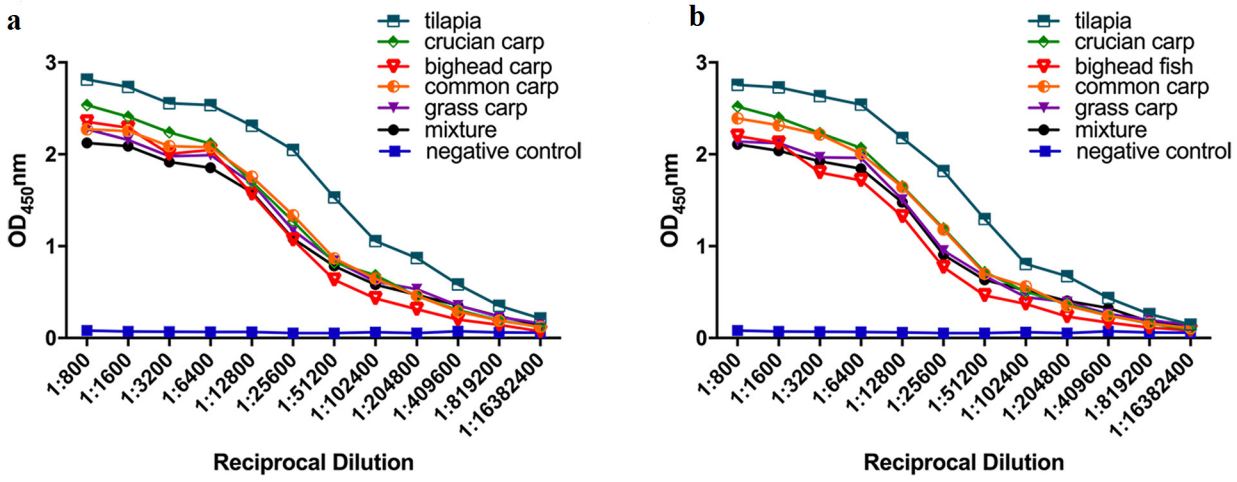

Figure 2. Antibody titers in sera from rabbits immunized with purified fish IgM by ELISA. Sera were collected 6 weeks **(a)** and 7 weeks **(b)** after the first immunization. Antibody titers of IgG induced by purified IgM from tilapia, crucian carp, bighead carp, common carp, grass carp or mixed IgM of the five categories (mixture) were assayed. Negative control denoted serum from preimmunized rabbit.

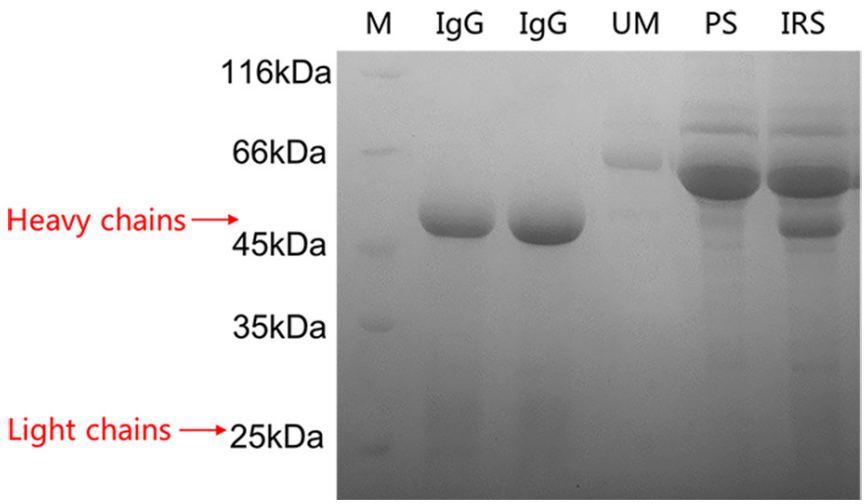

Figure 3. Identification of specific IgG anti-fish IgMs in rabbit serum by SDS-PAGE. Lanes from left to right were MW marker (M), specific IgG anti-fish IgM, specific IgG anti-fish IgM, non-specific binding proteins to rProtein A in packed column, immunized rabbit serum pass through the column and collected, immunized rabbit serum before chromatography.

Immunological reaction of rabbit IgG anti-fish IgMs with sera from different fishes by Western blotting

Dominant probed bands were observed in lanes of grass carp, bighead carp, crucian carp, common carp and tilapia respectively at MW around 70kDa indicating their H chains of IgM (Figure 4a) (Vesely et al. 2006b). Obvious immunoreactive bands appeared in lanes of oshima, yellow catfish, bream, black pacu, black bream and silver carp respectively at MW of 60-77kDa (Van Muiswinkel 1995). Light recognition bands could be observed in lanes of Japanese sea perch, sunfish, mandarin fish, Linnaeus and snakehead (Figure 4b).

Antibody titers detected by rabbit IgG anti-fish IgMs we generated or commercial rabbit IgG anti-grass carp IgM

8 weeks after the first immunization with heterogenous antigen, titers of specific IgM in sera from grass carp were detected with different second antibody by ELISA. By using rabbit IgG anti-fish IgMs we generated, specific IgM titer peaked to 1:204,800 which was similar with that detected with commercial rabbit IgG anti-grass carp IgM. The values of optical density at 450 nm (OD_{450nm}) by using rabbit IgG anti-fish IgMs were little higher than those by rabbit IgG anti-grass carp IgM at the same sera dilutions (Figure 5).

DISCUSSION

In the current study, we obtained purified IgMs of grass carp, bighead carp, crucian carp, common carp and tilapia which were most widely cultured freshwater fishes in most areas of china. On this basis, we generated HRP-conjunct rabbit IgG anti-fish IgMs with high antibody titers. IgM of other freshwater fishes including oshima, yellow catfish, bream, black pacu, black bream, silver carp and so on could be recognized by the IgG.

Both IgM of grass carp, bighead carp, crucian carp, common carp or tilapia and rabbit IgG anti-fish IgM were purified by rProtein A affinity chromatography in the present study. Compared with ion exchange chromatography, ammonium sulphate precipitation and other methods, rProtein A affinity chromatography is proved to be a simpler and faster one. In addition, the antibody possesses high purity and can maintain the immunological activity. Protein A from *Staphylococcus aureus* is a good ligand for antibody analysis by binding to Fc and Fab fragments of immunoglobulin (Sasso et al. 1991). After rProtein A Sepharose FF

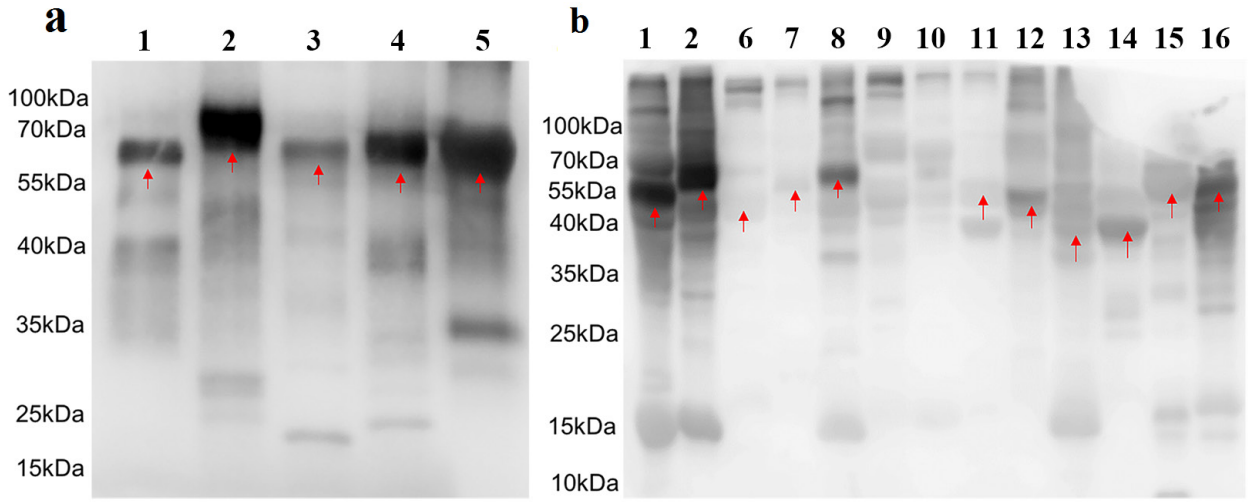

Figure 4. Sera from different fishes reacted with purified rabbit IgG anti-fish IgMs by western blotting. (a) Rabbit IgG anti-fish IgMs probed with sera from five categories of fishes from which fish Ig M were acquired. Lane 1: grass carp, Lane 2: crucian carp, Lane 3: bighead carp, Lane 4: common carp, and Lane 5: tilapia. (b) Rabbit IgG anti-fish IgMs reacted with sera from other 11 categories. Lane 6: Japanese sea perch, Lane 7: sunfish, Lane 8: Oshima, Lane 9: mandarin fish, Lane 10: Linnaeus, Lane 11: snakehead, Lane 12: yellow catfish, Lane 13: bream, Lane 14: black pacu, and Lane 15: black bream, Lane 16: silver carp. The arrows indicated H chains of them.

chromatography and SDS-PAGE assay, two main bands, around 66kDa and 25kDa, were observed in sample from grass carp, bighead carp, crucian carp, common carp or tilapia (Figure 1). The MWs were in reported range of H chain and L chain of IgMs from other teleost fishes (Rønneseth et al. 2015). It demonstrated that with higher salt concentration in equilibrium buffer (20mM PBS, pH 8.0, 3.0 M NaCl) IgMs of those fishes showed high binding affinity to protein A. The bands of L chains with lighter staining were unconcentrated in SDS-PAGE. It might due to easier degradation of the L chains.

The results of IgG titers in rabbit sera respectively induced by purified IgM from each fish category or mixed IgMs from five categories by ELISA showed that the titers in six immunized groups peaked to 1:163,824,001. Moreover, even though the immunized quantity of IgM only from one category (200μg) was 4 times than that in group immunized with mixed fish IgMs (40μg), the titers of specific IgG anti-mixed fish IgMs peaked to 1:163,824,001 (Figure 2a-b). It indicated the high immunogenicity of the purified fish IgMs. The titer usually can reach a relatively high level after that the animal model is immunized with heterologous protein three times at intervals of two weeks (Bag et al. 2009). About 30 ml to 40 ml blood can be obtained from rabbit by a direct heart puncture and about 15 ml to 20 ml serum can be collected finally. As for our study, to acquire more serum to generate more specific IgG anti-fish IgMs, we collected rabbit blood 6 weeks and 7 weeks after the first immunization taking into consideration that the rabbit should have enough time to recover and well survived. The results of ELISA showed that there was no significant difference in antibody titers between 6 weeks and 7 weeks after immunization. It indicated that this manner of serum collection could be used for harvest more specific IgG.

The rabbit IgG anti-mixed fish IgMs was then purified by rProtein A Sepharose FF chromatography. The SDS-PAGE results suggested that rabbit IgG could bind well to rProtein A in equilibrium buffer with normal salt concentration and well purified (Figure 3). The amount of rabbit IgG anti-fish IgMs extracted

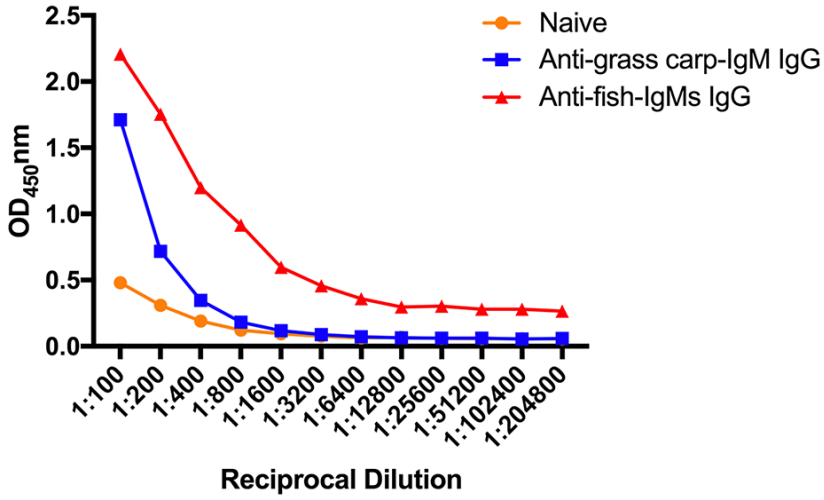

Figure 5. Detection of Specific IgM using rabbit IgG anti-fish IgMs we generated or commercial rabbit IgG anti-grass carp IgM by ELISA. Antibody titers of specific IgM induced by *B.s*-CotC-*Cs*TCHP were detected by using rabbit IgG anti-fish IgMs we generated or commercial rabbit IgG anti-grass carp IgM as second antibody, respectively. Naive indicated antibody titers of specific IgM in serum from preimmunized fish.

from only 5 ml of rabbit sera was considerable (up to 33mg). Intensive band of H chain of specific IgG was well presented around 50kDa in SDS-PAGE, while L chain about 25kDa was fuzzy (Figure 3). It might due to degradation of L chain and that the set voltage couldn't converge L chain with lower MW.

The immunoreaction of the purified rabbit IgG was checked by western blotting. The results demonstrated that the purified IgG could not only well recognize the revulsive IgM from grass carp, bighead carp, crucian carp, common carp or tilapia but also probed by IgM from Oshima, yellow catfish, bream, black pacu, black bream, or silver carp (Figure 4a-b). It indicated that purified IgG anti-mixed fish IgMs were generated successfully and the IgG could cross-react with IgM from other species. The conserved sequence in constant region of IgMs among different species was responsible for the cross reaction. The bands of L chains recognized by rabbit IgG anti-mixed fish IgMs were not obvious. It was documented that many polyclonal antibodies was produced only against H chain (Jirapongpairoj et al. 2017). The probed band of H chain of grass carp, bighead carp, crucian carp, common carp or tilapia was especially intensive. In immunolabeling, Fab domain of primary antibody binds to an antigen and exposes its Fc domain to secondary antibody. That is, secondary antibodies bind to H chains of primary antibodies. So that the rabbit IgG anti-fish IgMs as a secondary antibody might be used to assess, detect, and purify specific IgMs in western blotting assays, ELISA, radioimmunoassay (RIA), co-immunoprecipitation (co-IP) and so on.

In addition, we compared detection effects between rabbit IgG anti-fish IgMs we generated and commercial rabbit IgG anti-grass carp IgM by ELISA. By using both antibodies, titer of specific IgM in the immunized grass carp peaked to 1:204,800. But OD_{450nm} values by using the former were little higher than those by the latter at the same sera dilutions (Figure 5) showing the higher sensitivity and commercially application potential of the former. Polyclonal antibody is superior to monoclonal antibody when simultaneously detecting antigens with different epitopes, as polyclonal antibody can bind to more sites for better sensitivity (Lipman et al. 2005).

In summary, we purified IgM of grass carp, bighead carp, crucian carp, common carp and tilapia and obtained considerable amount of HRP-conjunct rabbit IgG anti-fish IgMs which could not only react with revulsive IgM from the

fives species but also probed with IgMs from other eleven species of teleost fish. The generated IgG with high purity would help us to evaluate immune effects elicited by candidates during the development of vaccine against *C. sinensis* infection in freshwater fish more conveniently. The acquirement of HRP-conjunct rabbit IgG anti-fish IgMs was the cornerstone for studying the immune system of teleost fish, developing immunoassay methods and evaluation of fish vaccine with more convenience.

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