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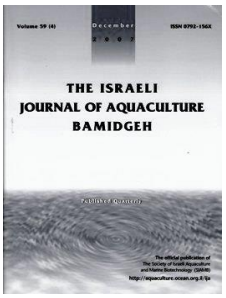

Ontogeny of Nile tilapia (*Oreochromis niloticus*) Immunoglobulin Type M Antibody Response

Natthaporn Kuendee[1], Sompong Klaynongsruang[1], Wandee Bunyatratchata[2], Bundit Tengjaroenkul[3], Kittipoj Ngamcharoen[4], Jureerut Daduang[5], Piti Ungarreevittaya[6] , Sakda Daduang[1, 7]*

[1]*Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand*

[2]*Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand*

[3] *Department of Medicine, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*

[4]*Charoen Pokphand Foods Public Company Limited, 47/45-47 Soi Chan 24, Khwaeng Thung-wat-don, Khet Sathon, Bangkok, 10120, Thailand*

[5]*Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, 40002, Thailand*

[6]*Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand*

[7]*Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand*

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* Corresponding author. Tel.:+66817686580, fax number:+6643-20237943, e-mail:sakdad@kku.ac.th

Abstract

Nile tilapia (*Oreochromis niloticus*, Cichlidae) are cultured worldwide, however, the study of humoral immunity in these fish has been neglected, leading to mismanagement of prevention of common diseases by vaccination. In this study we purified and characterized the Nile tilapia immunoglobulin type M (IgM). In addition, we have described the production of a mouse polyclonal antibody for the investigation of the onset of antibody responses. After one-step purification using protein G sepharose beads, SDS-PAGE, and mass fingerprint analysis we found that the heavy chain of Nile tilapia IgM was 70 kDa, whereas the light chain was 27 kDa. Western immunoblotting techniques using mouse anti-Nile tilapia IgM antibody, produced by intraperitoneal injection with purified Nile tilapia IgM for 3, periods with booster inoculations every 10 days, could effectively detect the onset of antibody responses in Nile tilapia sera at 42 days post-hatch.

Introduction

Nile tilapia (*Oreochromis niloticus*) is a relatively large freshwater cichlid fish. Due to its rapid growth and good taste, it is well-known as a low-cost protein source and is a commercially popular fish. Nile tilapia is cultured worldwide in countries such as Brazil, China, Egypt, India, Indonesia, Japan, Philippines, United States, and Thailand (Suanyuk et al., 2008) which produce more than 1 million tons annually. However, disease is one of the major problems as culture is primarily in floating baskets in rivers or canals where there is exposure to pathogenic microorganisms especially from *A. hydrophila* which is a common pathogen causing motile aeromonads disease (Tenjaroenkul et al., 2000). Vaccination is a logical choice for prevention, however basic information, including the ontogeny of the antibody response for Nile tilapia is still unknown, leading to mismanagement of vaccination.

To study the ontogeny of the antibody response, Nile tilapia immunoglobulin type M (IgM) was isolated, purified using protein G Sepharose, and confirmed by mass fingerprinting prior to the production of mouse anti-Nile tilapia IgM antisera. The serum was used for Western immunoblotting (WB) technique.

Materials and Methods

Fish cultivation and blood sampling. Nile tilapia at different ages: 35, 42, 56, 84, 112 and 140 days post hatch (dph), respectively raised in aerated earthen ponds (Thawatchai Farm, Muang District, Khon Kaen Province, Thailand) were fed with commercial pellet feed twice a day. Blood was drawn from the caudal vein and allowed to clot at room temperature for 1 h and at 4 °C overnight. After centrifugation at 3,000 ×g for 10 min, serum was taken and stored at -30 °C.

One-step purification of Nile tilapia Ig. Ig in the Nile tilapia sera was purified by protein G Sepharose beads. After 3 rinses with phosphate buffered saline (PBS) with 0.1% Triton X (PBST), the beads were incubated with sera at 4 °C for 1 h and washed 3 times with PBST. Ig was eluted by incubation with 0.1 mM Tris-glycine buffer, pH 3.0 for 5 min. Ig in the supernatant was retained after centrifugation at 3,000 ×g. Buffer was changed to Tris-HCl, pH 9.0 before storing the Ig at -20 °C.

Partial amino acid sequence determination. Protein samples excised from an SDS-PAGE profile were identified by the publicly available MASCOT program, which assigned peptides to the tandem mass spectrometry (MS/MS) spectra, and was used for statistical validation of the peptides

Production of mouse anti-Nile tilapia IgM antiserum. ICR mice were first immunized by intraperitoneal injection (i.p.) with 10 mg of purified Nile tilapia IgM in sterile PBS (pH 7.2) with an equal volume of complete Freund's adjuvant. Mice received booster inoculations every 10 days for 30 days with a mixture of purified IgM and an equal volume of incomplete Freund's adjuvant. Blood samples were collected using the retro-orbital plexus bleeding technique (Riley, 1960). Mouse sera were checked for antibody titer and specificity using ELISA and WB.

Immunological techniques. For ELISA, 96-well microtiter plates were coated with antigen and incubated, in sequence, with mouse anti-Nile tilapia IgM antisera diluted in TBST buffer, goat anti-mouse IgG linked with alkaline phosphatase diluted 1:5000 for 1 h at 37 °C, and p-nitrophenyl phosphate in substrate buffer with 3 to 6 washes before the next incubation. The yellowish product was measured at 405 nm. Data were analyzed and graphically presented using GraphPad Prism (version 6.01, Graph-Pad Software, Inc., La Jolla, CA, USA). For WB analysis, SDS-PAGE-resolved proteins were electrically transferred to nitrocellulose membranes (Al-Harbi et al., 2000).The membrane was incubated, in sequence, with mouse anti-Nile tilapia IgM antiserum diluted 1:1000 and goat anti mouse IgG conjugated with alkaline phosphatase diluted 1:5000, with 3 to 6 washes before the next incubation. Color bands were developed using an AP substrate kit (Bio-Rad, USA).

Results

Nile tilapia IgM. The purification of fish sera using protein G Sepharose beads revealed that the heavy chain of the Nile tilapia Ig was 70 kDa and the light chain was 27 kDa (Fig. 1).

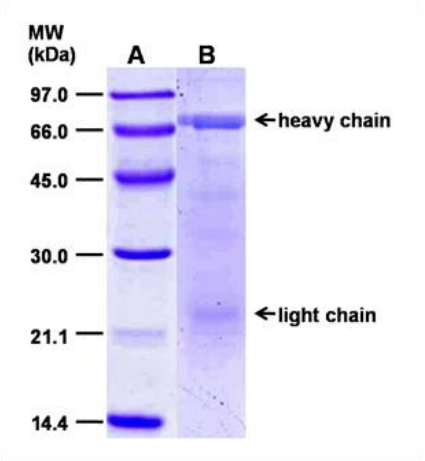

Fig. 1. Nile tilapia Ig purification. Whole fish serum was loaded into a protein G Sepharose column and eluted as described in the Materials and Methods before resolving using 13% SDS-PAGE. Lane A, protein maker; B, purified Ig.

The sequence KTEDSAVYYCAR was obtained from one dimensional mass spectrometry from the protein band of approximately 70 kDa (Table 1). The results from a best match score MS/MS database demonstrated that the protein was similar to the IgM heavy chain of 2 fish species, *Anarhichas minor* and *Oncorhynchus mykiss*.

Table 1. Mass spectrometry analysis of purified Nile tilapia IgM.

Protein Annotation	*Organism*	*Score*	*Peptide*
Ig heavy chain variable region	spotted wolffish (*Anarhichas minor*)	107	K.TEDSAVYYCAR.D R.QQLYLQMNSLK.T + Oxidation (M)
Ig heavy chain precursor V region	rainbow trout (*Oncorhynchus mykiss*)	104	K.LYLQMNSLK.S + Oxidation (M) K.SEDTAVYYCAR.S
IgM heavy chain membrane bound	rainbow trout (*Oncorhynchus mykiss*)	51	K.LYLQMNSLK.S + Oxidation (M) K.SEDTAVYYCAR.S

Mouse anti-Nile tilapia IgM antibody production and its cross-reaction. Antibody production of anti-Nile tilapia IgM was generated in ICR mice. The antibody titer performed using ELISA technique demonstrated that 6 mice had an antibody level after the third immunization of approximately $1{:}10^5$ (Fig. 2).

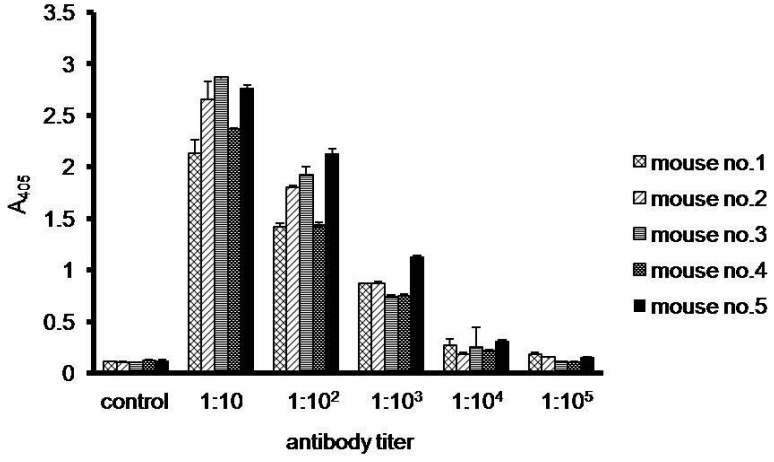

Fig. 2. Mouse anti-Nile tilapia IgM antiserum production. After 3 immunizations with purified IgM in 5 mice, antibody titers were checked by ELISA. Purified Nile tilapia IgM was fixed on microtiter plates and probed with ten-fold serially diluted mouse serum.

Sera from 4 species commonly cultivated as commercial products, i.e., the common swamp eel (*Monopterus albus*), frog (*Rana rugulosa*), striped snakehead (*Channa striata*) and catfish (*Clarias* sp.), were tested for a cross reaction to mouse anti-Nile tilapia IgM antiserum using ELISA (Fig. 3). The antiserum reacted with its own antigen, the Nile tilapia IgM with a titer at $1{:}5^5$ dilution. However, the anti-Nile tilapia IgM antiserum cross–reacted with the heavy chain of eel and striped snakehead fish Igs (at $1{:}5^2$ dilution) but not with those of the frog and catfish.

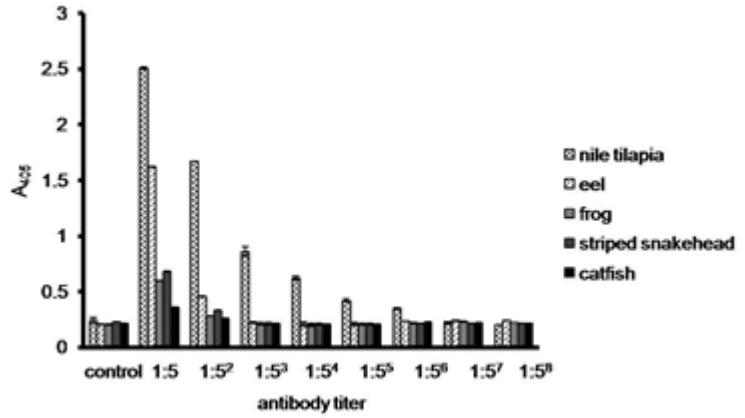

Fig. 3. Cross reactivity to mouse anti-Nile tilapia IgM antiserum using the ELISA technique. Sera of 5 species were fixed and probed with serial dilutions of the antiserum.

*Ontogeny of the antibody response in Nile tilapia serum*Whole blood from the Nile tilapia was collected at various days post hatch. Subsequently, the polyclonal mouse anti-Nile tilapia IgM antiserum was used as an immunological tool to investigate the onset of the antibody response in Nile tilapia serum ranging from 35 to 140 dph using WB techniques (Fig. 4A). The results of the SDS-PAGE corresponding with those of the WB analysis confirmed that antibody onset occurred in the Nile tilapia at 42 dph (Fig. 4B).

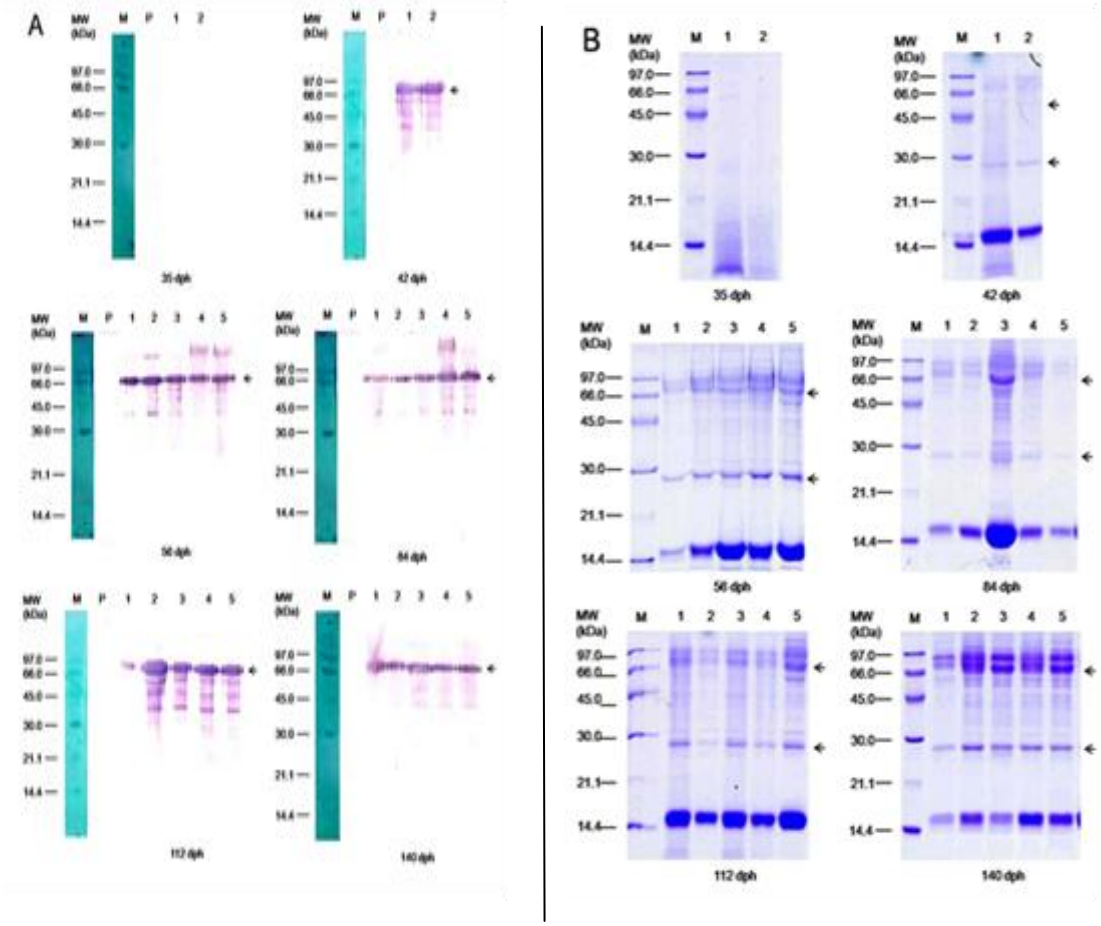

Fig. 4. Ontogeny of the Nile tilapia IgM antibody response. Sera from 35 to140 dph from 2 or 5 fish were separated using 13% SDS-PAGE and then subjected to WB (A) or stained with coomassie brilliant blue R (B). For WB, antigens were incubated with mouse anti-Nile tilapia IgM antisera diluted 1:2000 before detection. M, protein standard marker; P, preimmunized mouse serum as a negative control. The upper arrows indicate the heavy chain of IgM (A and B). The lower arrows indicate the light chain (B).

Discussion

Currently, at least one basic issue, the timing of the onset of antibody production for Nile tilapia farming, must be urgently addressed for the improvement of the vaccination processes. Although vaccination, including vaccinated offspring, is quite common among farmers, the appropriate time for vaccination is as yet unknown. However, due to the lack of an enzyme conjugated anti-Nile tilapia IgM antibody as a probe for detection, in this study, we developed "mouse anti-Nile tilapia IgM antibody". This development helped minimize at least 2 problems: (1) anti-Nile tilapia IgM antibody conjugated with an enzyme is not commercially available; and (2) suitable conjugation technology seems impossible in laboratories of third world countries.

This work was begun by purifying Ig from fish serum using protein G Sepharose. This is the first study to use protein G beads for the effective one-step purification of Nile tilapia IgM. Usually, protein G has a high affinity for the Fc region of IgG of many species, such as cows, horses, humans, pigs, rabbits, goats, mice, rats, etc. (Harlow and Lane, 1988), but no data have been reported for the efficiency for fish Ig. Fc part of fish IgM, bound to protein G with high affinity, resulting in dominance of one heavy chain and light chain after SDS-PAGE analysis. The amino acid sequence of the major purified protein was similar to the heavy chain of Ig type M of at least 2 fish species. Nile tilapia IgM is composed of 2 subunits; the larger one, or heavy chain is approximately 70 kDa, remarkably smaller than the IgM of many fish whereas the smaller subunit, or light chain is 27 kDa, approximately the same size as other fish IgM (Table 2).

Table 2. Size of representative fish IgM.

Species	*Common names*	*Heavy chains (kDa)*	*Light chains (kDa)*	*References*
Catla catla	Catla	88	26	Bag et al. (2009)
Cirrhinus mrigala	Mrigal	78	27	Bag et al. (2009)
Dicentrarchus labrax	Sea bass	88	26	Scapigliati et al. (1996)
Labeo rohita	Indian major carp	88	26	Bag et al. (2009)
Latris lineate	Striped trumpeter	86±7	28±3	Covello et al. (2009)
Neoditrema ransonneti	-	82	-	Nakamura et al. (2006)
Oreochromis niloticus	Nile tilapia	70	27	This study
Raja kenojei	Skate (cartilaginous fish)	45–50	-	Kobayashi et al. (1985)

Purified IgM from Nile tilapia was used as the antigen for mouse anti-Nile tilapia IgM antibody production. Five mice had an antibody titer higher than $1{:}10^4$ after only 3 booster inoculations, suggesting high antigenic properties of fish IgM in mice. The antibody was checked for cross reaction with Ig of many species. The cross reaction implied closed evolution. Moreover, this result indicated that the antibodies produced in this study could possibly be used as a detector for the ontogeny of the antibody response of the eel and striped snakehead fish.

IgM were positively detected in Carp (*Cyprinus carpio*) at 2 days (Romano et al., 1999) and channel catfish (*Ictalurus punctatus*) at 7 days (Magnadottiret al., 2005)), thus 42 dph observed in Nile tilapia seems relatively late. However, Nile tilapia antibody onset was similar to Zebra fish (*Danio rerio*), at 48 days (Lieschkeet al., 2001). Many freshwater fish must produce IgM. Their immune response must be accelerated for their survival since they are always raised in crowded conditions where pathogenic organisms thrive. Other factors, such as innate immunity, should be used for considering the survival of the specific types of fish in different environments. This information is particularly important for the culture of Nile tilapia and especially for the production of vaccinated offspring. The antibody produced has been effectively used for determining the ontogeny of antibody responses in Nile tilapia using WB. The onset of antibody

production observed in this study was 42 dph. Fish vary in their capacity for antibody production from 2 to 225 dph (Table 3).

Table 3. Antibody onset of fish.

Fish species	*Common names*	*Types*	*Antibody onset (time–post hatch)*	*References*
Anarhichas minor	Spotted wolfish	marine	1-10 weeks	Magnadottir et al. (2005)
Centropristis striata	Sea bass	marine	1-10 weeks	Magnadottir et al. (2005)
Cyprinus carpio	Carp	freshwater	2 days	Romano et al. (1999)
Danio rerio	Zebra fish	freshwater	48 days	Lieschke et al. (2001)
Gadus morhua	Atlantic cod	marine	28 days	Schroder et al. (1998)
Ictalurus punctatus	Channel catfish	freshwater	1 week	Magnadottir et al. (2005)
Latris lineate	Striped trumpeter	marine	225 days	Covello et al. (2009)
Oncorhynchus mykiss	Rainbow trout	marine	1 week	Magnadottir et al. (2005)
Oreochromis niloticus	Nile tilapia	freshwater	42 days	This study
Paralichthys olivaceus	Japanese flounder	marine	15 days	Liu et al. (2004)
Scophthalmus maximus	Turbot	marine	20-30 days	Padrós and Crespo (1996)
Sparus aurata	Gilthead seabream	marine	29 days	Jósefsson and Tatner (1993)
Synchiropus splendidus	Mandarin fish	marine	20 days	Tian et al. (2009)

The Western immunoblotting technique demonstrated predominant Nile tilapia IgM heavy chain with few cross reactions. Reactive bands appeared in similar patterns both in the native condition of whole serum and also after being purified, suggesting no degradation of heavy chains during the purification process. Heavy chain isotypes have been reported in a number of other fish species, including channel catfish (*Ictalurus punctatus*) rainbow trout (*Oncorhynchus mykiss*), redfin perch (*Perca fluviatilis*), and fugu (*Fugu rubripes*), after being investigated with denaturing condition (Savan et al., 2005). These isotypes or variants are believed to result from differential glycosylation. However, this report showed only one isotypes of Nile tilapia IgM heavy chain.

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