Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

Journal of Fisheries and Aquatic Science 6 (7): 700-714, 2011
ISSN 1816-4927 / DOI: 10.3923/jfas.2011.700.714

Estrogen-Induced Vitellogenin in *Tor tambroides* (Bleeker, 1854): Purification, Characterization and ELISA Development

[1]N.A. Muhammad, [1]A. Christianus, [2]S.K. Daud, [1]C.R. Saad, [3]S.A. Harmin and [1]M.Y. Ina-Salwany

[1]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[2]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[3]Centre of Land and Aquatic Biotechnology, Faculty of Sciences and Biotechnology, UNISEL, 45600 Bestari Jaya, Selangor, Malaysia

Corresponding Author: A. Christianus, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia Tel: 03-8944884

ABSTRACT

Vitellogenin (vtg) is a high molecular weight glycophospholipoprotein synthesized in the liver under stimulation of estrogen. Basically found in sexually mature female, vtg being taken up by developing oocyte during maturation. It functioned as a nutrient storage for growing embryo. Having potential to be used as a maturation indicator, vtg helps to enhance fish fry production. *Tor tambroides* is one of the most sought after fish in Malaysia for having potential as a game fish yet so important economically due to high demand by farmers as well as consumers. Main problem in the mass production of *T. tambroides* fry in hatchery is in the availability and selection of matured broodstock. Identification of matured and ready females morphologically can only be done by experience workers. Therefore, establishment of simple technique to definite identify matured females is necessary. This has led to the identification, purification and development of enzyme linked immunosorbent assay (ELISA) to measure blood plasma vtg as maturation indicator. This will definitely contribute to the hatchery production of *T. tambroides* fry.

Key words: ELISA, purification, *Tor tambroides*, vitellogenin

INTRODUCTION

A number of studies on vitellogenin (vtg) focused on environmental concerns and reproductive physiology of fish. Vtg, an egg yolk precursor, is a high molecular weight protein normally found in sexually mature female vertebrates. It can also be detected in males as they carry the vtg genes. Vtg plays an important role in storing and supplying nutrients to developing oocyte. Vtg level is related to the maturation stage of eggs in female. Vtg has also been widely used as an indicator to detect estrogenic compound in the aquatic environment. Assays were developed to screen the existence of endocrine disruptors in environment that lead to reproductive impairment in vertebrates. Ngamniyom and Panyarachun (2011) investigated the expression of vtg genes in the livers of sex-undeterminable and intersex group of Thai medaka as reported by Williams *et al.* (2009) in which the exposure of fish to toxic compounds such as estrogen and steroid might result

in intersexuality of the fish. Several publications reviewed about the masculinization of female (Bortone and Davis, 1994) and also feminization of male fishes (Munkittrick *et al*., 1991). Vtg has been identified in fishes such as Chilean flounder (Leonardi *et al*., 2009), perch (Hennies *et al*., 2003), carp (Fukada *et al*., 2003), greenback flounder, rainbow trout and Atlantic salmon (Watts *et al*., 2003), turtles (Saka *et al*., 2008), crocodiles (Rainwater *et al*., 2008), toads (O'Brien *et al*., 2010) and lizards (Carnevali *et al*., 1991). Investigations related to vtg have become a major concern among scientists since endocrine disruptor chemicals may have adverse effects on vertebrates including fish species as well as humans (Colborn *et al*., 1993).

Vitellogenin, a large glycophospholipoprotein with molecular weight within a range of 250 kDa up to 600 kDa is synthesized in the liver in response to the presence of 17 β-estradiol (E2) (reviewed by Specker and Sullivan, 1994). This 17β-estradiol is synthesized by the gonads during sexual maturation in vertebrates of oviparous females. Vtg then transported into the bloodstream before being picked up through receptor-mediated endocytosis by the developing oocytes and cleaved into yolk proteins as a nutrient store (Tyler *et al*., 2000). Basically, vtg can be found in matured females and detected in a very small amount which can be considered as negligible in males and immature females.

Various methods have been developed to purify vertebrates' vtg. Magalhaes *et al*. (2004) developed one-step and non-denaturing purification method for *Cyprinus carpio* vitellogenin, while Wunschel *et al*. (2005) described the identification of vtg in fish using High Performance Liquid Chromatography (HPLC) separation coupled to MALDI mass spectrometry. During the process, proteins are separated either by size or charge of the molecule. Several methods applied are time-consuming and costly. Numerous purification steps are not preferred due to the probability of loss in protein purity. In this study, a simple flow method for purification was established and described.

Methods for the quantification of vtg in blood plasma of fish species developed include radioimmunoassay (Tyler *et al*., 1996), immunodiffusion, real time PCR (Celius *et al*., 2000) and Enzyme-linked Immune Sorbent Assay (ELISA). ELISA is the most favored quantification assay due to the ease for routine application. Anti-vtg antibodies are species-specific. It requires antibody production in animals such as rabbits and goats. However, the use of commercial antiserum is possible as long as it cross reacts against the target proteins, sensitive and reproducible through ELISA assay.

T. tambroides is one of the most sought after freshwater species in Malaysia. They can only be found in pristine rivers. Moreover, their numbers are declining due to overfishing and deforestation (Sungan *et al*., 2006). These species were selected since they are significant culturally and economically to Malaysia especially to indigenous people living in Sarawak. However in the Peninsula, species conservation is crucially critical and extinction of *T. tambroides* should be avoided. Documented report on this species has been suggested that this fish is able to reproduce throughout the year (Ismail *et al*., 2011). Identification of matured females through physical observation is insufficient to ensure the maturity stage of the fish.

This study was conducted with the purpose to characterize vtg in blood plasma of mahseer and to develop ELISA for measuring vtg concentration at plasma level. The lack of information available with regards to vtg in *T. tambroides* has led further investigation on this matter.

MATERIALS AND METHODS

Sample: Males and vitellogenic females *T. tambroides* used in this study were kept in outdoor freshwater ponds at Pangsun, Hulu Langat (3°12'39" N 101°52'51" E). The fish were fed with commercial pellet (Star feed) containing 42% protein.

Vtg induction: A total of 5 males with an average weight of 1.5±0.4 kg were induced with 5 mg kg^{-1} body weight of 17 β-estradiol (E2). E2 was dissolved in 0.05 mL ethanol diluted in 0.45 mL peanut oil (following ratio 1:9, ethanol: peanut oil). An anesthetic bath (MS222) was prepared to place fishes before injection in order to reduce handling stress on fish. Fishes were not fed 24 h prior to injection as well as blood sampling. Hormone mixture was intra-peritoneally injected into fish on day 1, 3 and 5. On day 9, blood sampling was carried out for each fish and pooled. Blood was allowed to clot at 4°C for few minutes and then immediately centrifuged at 4°C, 3,000 rpm for 15 min. The supernatant (plasma) was collected, re-centrifuged at 11,000 rpm for 10 min at the same temperature. Protease inhibitor (Complete Mini EDTA-free, Roche, Germany) was added half the volume of plasma aliquot into each centrifuge tube and stored at -80°C (to maintain protein stability) until further analysis.

Vtg screening: SDS-PAGE was performed as described by Laemmli (1970) using 7.5% resolving gel and 4% stacking gel. Plasma samples were diluted 1:2 to loading buffer (0.5M Tris HCl, glycerol, 10% SDS, β-mercaptoethanol, 0.5% (w/v) bromophenol blue) and boiled for about 10 min. Samples were loaded on the gel and electrophoresis was run at 80 V for 150 min. Molecular weight was estimated with PageRulerTM Plus Prestained Protein Ladder (Fermentas, EU). Coomassie Blue R-250 (Bio-Rad Laboratories, USA) was used to stain the gel overnight and later destained with methanol and acetic acid. The presence of vtg was screened by SDS-PAGE and viewed using AlphaImager® (AlphaInnotech, USA).

Purification of vtg by gel filtration chromatography: Vtg was isolated from plasma proteins by gel filtration chromatography. The column was equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) for at least two Column Volume (CV). All buffers were filtered through 0.45 μm filter paper to remove impurities. One milliliter of purified plasma was loaded into AKTAPrime plus Purification System (GE Healthcare, Sweden) through HiPrepTM 16/60 Sephacryl HR300 column (GE Bioscience, Sweden). At flow rate of 0.4 mL min^{-1} after several optimizations, five milliliter of peak-containing fractions were collected.

Ultrafiltration of fractions containing vtg: Each 5 mL of peak fractions were immediately transferred into Vivaspin 6 (GE Healthcare, Sweden) tubes. At 4°C, purified vtg was spin at 3000 rpm for 30 min. Immediately after stopped, the concentrated sample was transferred and aliquoted into centrifuge tubes and stored at -20°C until next analysis.

Native PAGE: The resolving gel contained 6% acrylamide and the stacking gel was 4%, the similar to that used in SDS-PAGE with the absence of SDS in both and β-mercaptoethanol in sample buffer. All the methods from casting the cassette until the end of polymerization of resolving and stacking gels were similar as in SDS-PAGE technique. Samples were diluted in 0.5 M Tris-HCl, pH 6.8, glycerol and 0.05% bromophenol blue (w:v) (without SDS and β-mercaptoethanol) and without sample heating. For native PAGE, vtg bulk was stained with four stains, Coomassie blue Laemmli (1970), Sudan black (Prat *et al.*, 1969), methyl green (Cutting and Roth, 1973) and periodic acid Schiff reagent (Covens *et al.*, 1988). The electrophoresis was run at 50 V for approximately 4 ½ h on ice. As the dye nearly reached the bottom, electrophoresis was stopped.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): During sample preparation, 200 μL of sample buffer was added to 50 μL of purified vtg sample (from female and

male *T. tambroides* blood plasma) and boiled at 100°C for 5 min before loading. Protein ladder was used for determination of polypeptides molecular weight by comparing the mobility of proteins with the markers of known molecular weight. SDS-PAGE was performed at 50V for 5 h on ice to maintain the protein stability. As the dye nearly reached the bottom, electrophoresis was stopped. Coomassie staining solution was used to stain and detect polypeptide appearance on the gel. After overnight of staining, the background was removed with methanol and acetic acid until the bands were visibled under a gel imager (AlphaImager® HP).

Immunodetection analysis: Proteins separated by SDS-PAGE were electro transferred onto polyvinylidene fluoride (PVDF) membrane as immobilized proteins which is detectable by specific antibodies. Before blotting, the gels were briefly immersed in freshly prepared 1x transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol) and PVDF membranes were first moistened with methanol before soaked in transfer buffer. Prior to immunodetection process , membranes were blocked with 1% bovine serum albumin in 1x phosphate-buffered saline containing 0.1% Tween-20 for 2 h and washed 4 times for 5 min each (4×5 times) before being incubated with carp vtg antiserum (1:850 diluted in blocking buffer) purchased from Biosense Laboratories (Norway) for 2 ½ h at RT. After washing for another 4×5 times in PBST, bound antibodies were detected with anti-mouse IgG-POD (Roche Diagnostics, Germany) at dilutions 1:1000 in blocking buffer with constant shaking. For the color reaction, membranes were soaked in Opti-4CN substrate (BioRad, USA) substrate for up to 30 min and rinsed with distilled water to stop the reaction. Blots were air-dried before documented for data analysis.

Antigen coating: A Nunc-Immuno Plate MaxiSorpTM microtiter plates of 96 wells were coated at 37°C for 2 h with 100 μL per well of purified mahseer vtg (253 ng mL^{-1}) diluted in coating buffer (50mM sodium bicarbonate buffer, pH 9.0).

Blocking: The coated plates were washed four times with 100 μL phosphate-buffered saline containing 0.1% Tween-20 (PBST) to remove unbound antigens and the remaining sites were blocked with 200 μL blocking buffer (1% skim milk in 1x PBST) for 1 h at 37°C.

Standard and samples preparation: The vtg standard and samples were thawed on ice. The standard was twofold serially diluted in blocking buffer (253-1.98 ng mL^{-1}) and the samples were tenfold serially diluted in blocking buffer (1:10-1:100 000). All diluted standard and samples were prepared in 15 mL centrifuge tubes.

Pre-incubation of standard and samples with antiserum: Anti-carp monoclonal antibody was diluted 1:500 in blocking buffer and was added 1:1 (v/v) into each standard and sample tube prepared earlier. Pre-incubation of this mixture took place for 2 h at 37°C.

Incubation with antiserum: The wells were washed four times in washing buffer as described previously. The pre-incubated standard/sample-antiserum mixture was added into wells (100 μL $well^{-1}$) and let incubation for another 2 h at 37°C.

Incubation with secondary antibody: The wells were washed four times as above before addition of 100 μL secondary antibody anti-mouse IgG-POD (Roche Diagnostics, Germany) diluted 1:2000 in blocking buffer. The wells were incubated for 1 h at 37°C.

Color development: The wells were washed four times as described above before the addition of 100 μL 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution (Calbiochem, USA). The incubation was performed in the dark at room temperature for up to 30 min. Once blue coloration is visible, the reaction was stopped by adding 50 μL 0.05 M sulfuric acid to all wells. The absorbance was measured at 405 nm using Thermo Scientific SkanIt® Software for Multiskan® FC (Thermo Scientific, Finland).

Expression of results and statistical analysis: The mean absorbance values of replicated wells were converted to percentage binding relative to analyte-free wells according to following equation (Lomax *et al*., 1998):

$$\frac{Bi}{B0\,(\%)} = \frac{OD\text{-}NSB}{OD0\text{-}NSB} \times 100$$

where, OD is the absorbance of a given sample or standard, OD0 is the absorbance of zero standard and NSB is the non-specific binding absorbance value. Binding percentage values were logit transformed (Rodbard and Lewald, 1970) using the following formula:

$$\text{logit}\,B = \frac{B}{100 - B}\,\text{loge}$$

and plotted against vtg concentration (ng mL^{-1}) for standard and log dose for plasma dilution curve. Parallelism between standard and plasma dilution curves was assessed by analysis of covariance (ANCOVA) using SPSS software.

RESULTS

Screening of vitellogenin by SDS-PAGE: The result obtained from screening of vtg polypeptides by SDS-polyacrylamide gel electrophoresis was shown in Fig. 1. SDS-PAGE analyses

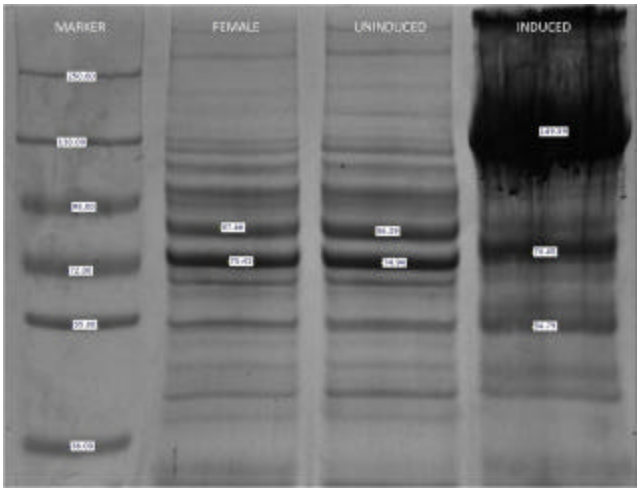

Fig. 1: Screening of plasma vtg in *T. tambroides* showing a high molecular weight band of 149.59 kDa which is not seen in female and untreated male plasma samples

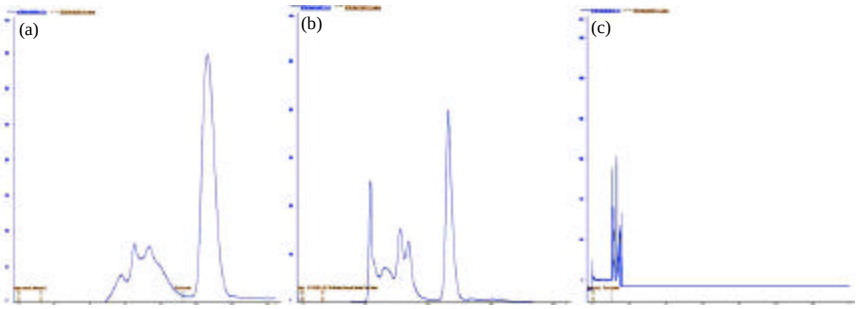

Fig. 2(a-c): Gel filtration elution profile of plasma from (a) E2-induced males, (b) vitellogenic female and (c) untreated males. The difference between (a), (b) with (c) and similarities demonstrated in (a) and (b) suggested that the Gaussian peak is vtg

of E2-treated male plasma proteins showed the presence of one major band with molecular mass of approximately 149 kDa and several minor bands of 78.85 and 54.79 kDa. The major band was not found in non-induced male and female plasma samples as shown in the figure above besides the polypeptides with molecular weight ranging from 72 k to 95 kDa. However, in this study, polypeptide was undetected in female plasma samples, only in E2-treated male plasma.

Chromatographic separation: The profiles of three different plasma samples from estrogen-induced males, sexually mature female and untreated male were shown in Fig. 2. A major peak was identified in the induced male and female plasma whereas absent in untreated sample.

Non-denaturing purification (native PAGE): We confirmed the properties of a glycophospholipoprotein in the sample by native PAGE (Fig. 3). Followed by staining with Coomassie blue, Sudan black, methyl green and periodic acid Schiff reagent, it revealed a non-denatured protein from E2-inducible fish fractions. A single protein band of approximately 700 kDa was visibled in males treated with 17β-estradiol and smear bands in vitellogenic female (Fig. 4) indicating the presence of other plasma protein other than vtg.

SDS-PAGE: Figure 5 showed the differences between commercial carp vtg, E2-treated male, vitellogenic female as positive control and untreated male as negative control when subjected to SDS-PAGE. A molecular weight of approximately 133 kDa was detected in carp vtg, E2-treated male and vitellogenic female plasma sample but absent in untreated males. There were several bands with slight similarity in molecular weight as shown by the positions of visible bands within the range of molecular weight of 72 to 133 kDa.

Immunodetection analysis: Figure 6 demonstrated that anti-carp vtg monoclonal antibody had recognized purified vtg of mahseer from E2-treated male plasma as well as vitellogenic female plasma. This indicated that the three polypeptides observed (133, 117 and 56 kDa) were vtg obvious from the compared samples. Untreated male plasma has no reactivity against antibody. This suggested that vtg was not present in untreated male sample. Carp antiserum reacted with E2-treated male and vitellogenic female plasma.

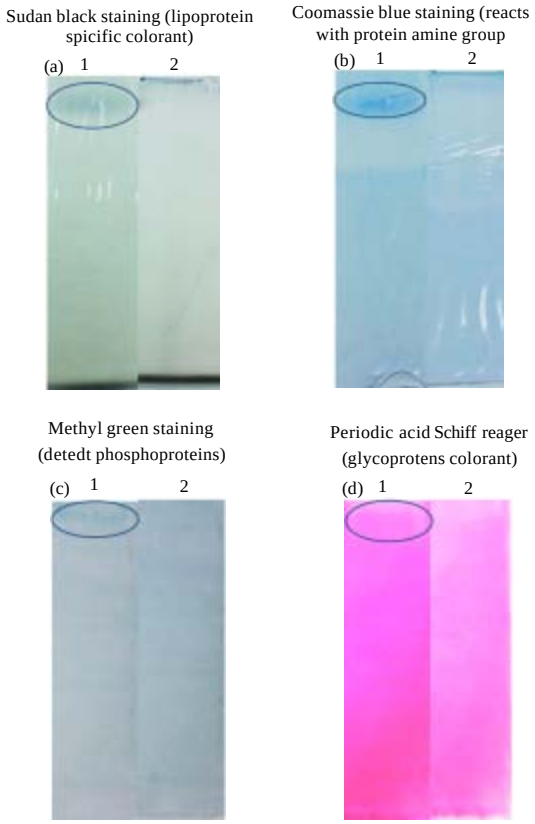

Fig. 3: Plasma protein composition by native PAGE of E2-treated (1) and untreated (2) male mahseer stained with Sudan black (a), Coomassie blue (b), methyl green (c) and periodic acid Schiff reagent (d)

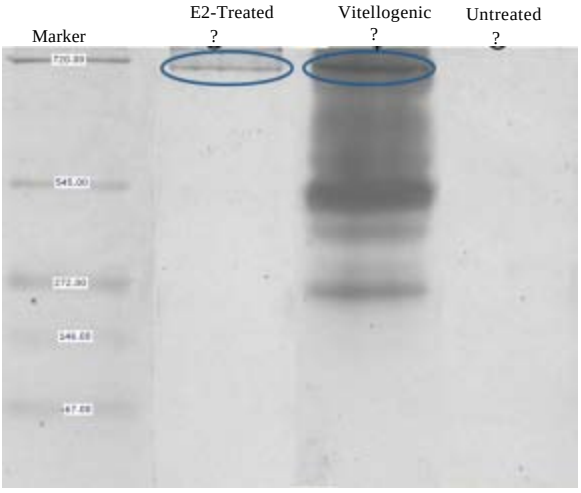

Fig. 4: Detection of a relatively high molecular weight of approximately 700 kDa revealed the presence of vtg polypeptides in E2-treated male and mature female plasma

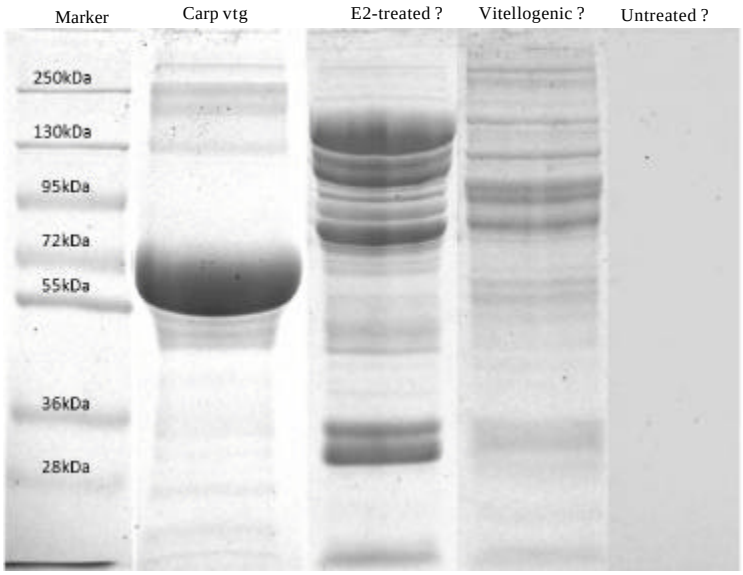

Fig. 5(a-d): Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of commercially available carp vtg, E2-treated and untreated male mahseer plasma and vitellogenic mahseer female plasma. Numbers at the left side indicate the molecular weight of protein markers (kDa)

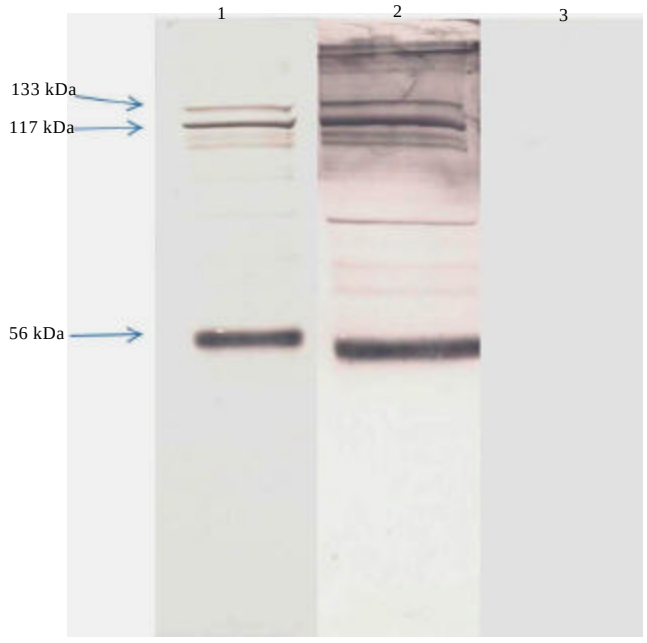

Fig. 6: Immunodetection analysis of purified mahseer vtg from E2-treated male (lane 1), female vitellogenic of mahseer (lane 2) and untreated male (lane 3). The numbers shown at the left side indicate the molecular mass of polypeptides present in E2-treated male and female but not in untreated male plasma

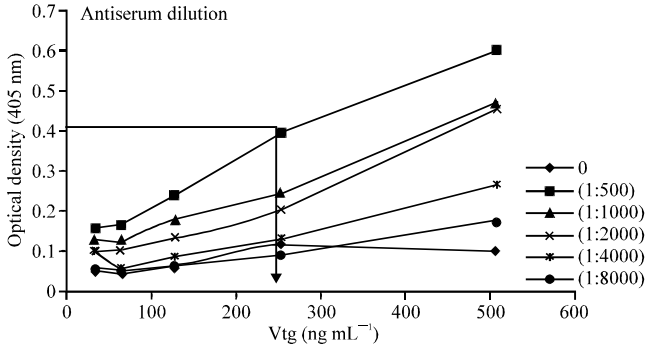

Fig. 7: The antigen-antibody dilution test was carried out to determine the optimal concentrations for ELISA. The chosen routine concentrations were 253 ng mL^{-1} for purified Vtg coating and 1:500 for anti-carp monoclonal antibody dilution

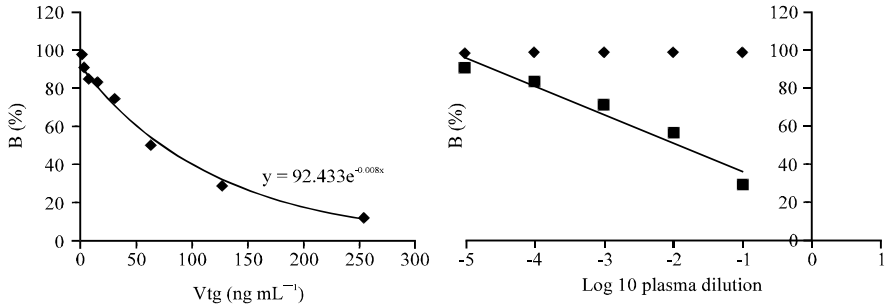

Fig. 8: (Left) Standard curve of binding percentages of purified mahseer vitellogenin against its concentration (ng mL^{-1}). The equation given above was used in the determination for the concentration at different binding percentages for inter- and intra-assay validation. (Right) Binding displacement curves obtained with serial dilutions (10-fold) of plasma from vitellogenic female and non-treated male *T. tambroides*. Control male plasma showed no significant cross reactivity with vtg detecting antibody

ELISA: A competitive ELISA for measuring vtg concentration in mahseer was developed through a series of optimization steps. The optimal concentration of purified vtg for coating is 253 ng mL^{-1} and the antiserum dilution use for full ELISA was 1:500 (Fig. 7). The minimum detectable vtg concentration in mahseer was 1.43 ng mL^{-1}.

Repeated measurements of the purified vtg sample at different binding percentages (80, 50 and 20%) for inter and intra-assay, were to determine the precision of this newly developed ELISA. The assays coefficients of variations were shown in Table 1.

The binding displacement curves for vitellogenic female mahseer against vtg standard were shown in Fig. 8. The specificity of the assay was determined by testing the parallelism of regression curves between the logit-transformed binding curves of standard and female mahseer plasma dilution (Fig. 9). Assessment of analysis of covariance (ANCOVA) using SPSS software showed that the slope for female mahseer is not statistically different from the standard ($F_{(12, 28)} = 1.678$, $p>0.05$).

Table 1: The coefficients of variations of mahseer Vtg ELISA assay at percentage bindings of 80%, 50% and 20%. The coefficient of variation (CV) was calculated and expressed as percentage: CV (%) = (standard deviation / mean)×100

	Percentage of maximum binding		
Coefficient of variations (CV %)	80	50	20
Inter-assay	9.64	11.32	12.69
Intra-assay	2.45	1.64	0.94

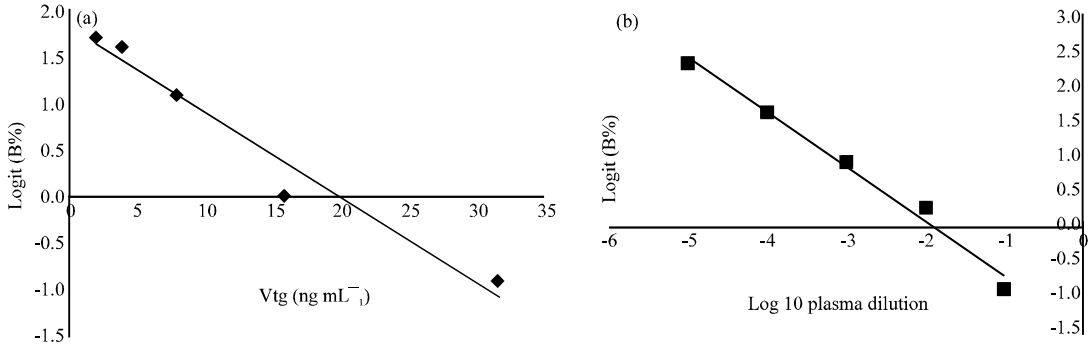

Fig. 9: Linearization of standard curve (left) and binding curve of male and female mahseer plasma (right) by logit transformation. This result indicated that anti-carp vtg antibody confirmed that a substance in female mahseer is antigenically similar to purified vtg identified in estrogen-treated male mahseer

DISCUSSION

As vtg is very unstable and degrades easily, the procedures for vtg purification and identification has been minimized and analysed to ensure that loss in purity for protein of interest will not be the major problem. This study confirmed the vtg synthesis upon administered with 17β-estradiol through SDS-PAGE screening. A protein band weighing 149 kDa appeared in E2-treated male plasma sample. In this study, gel filtration chromatography was performed to separate vtg from other plasma proteins. Some researchers used two steps purification which includes ion exchange followed by size exclusion chromatography (Hennies *et al*., 2003; Amano *et al*., 2009). The characteristics of vtg may differ in other species due to different techniques used during the procedures and also different identity of the vtg itself. In this study, the purification steps were minimized as vtg is a highly unstable protein. Vtg is being degraded into yolk proteins once taken up by the oocyte.

Prior to purification, mahseer plasma was subjected to gel filtration chromatography at a flow rate of 0.4 mL, 1 min, giving a major single peak found in E2-treated males and female plasma. The peak's absence in untreated male plasma sample indicated that the protein is estrogen inducible and it is reasonable to be identified as vtg. The skewed peaks appearance in all elution profiles may have resulted from the adsorption of proteins into the matrix. The Gaussian peak indicated that there are high amount of similar particles size detected in the same fraction. The peak fractions collected from chromatography were subjected to native and SDS-PAGE under optimized conditions.

Staining of mahseer vtg with methyl green, periodic acid Schiff reagent, Coomassie blue and Sudan black revealed that the isolated proteins are glycophospholipoproteins (Fig. 3) similar to

reports on other fish species (Maltais and Roy, 2009; Magalhaes *et al*., 2004). Even though the staining was not so clear, one single band was still visibled in all stains. Approximately 700 kDa protein band is stained in E2-treated male and vitellogenic female. However, this band did not appear single in female. This is due to the presence of other plasma proteins or the product of dissociation of protein structure in female plasma. Generally, vtg is an egg yolk precursor having molecular weight ranging from 250 to 600 kDa. However, in this study, 700 kDa was obtained. In *Cyprinus carpio* (Magalhaes *et al*., 2004), a 370 kDa phospholipoprotein with all vtg characteristics has been observed in estrogen-treated fish. Two bands were identified as vtg in redhorse (Maltais and Roy, 2009) with 520 and 260 kDa of molecular weight. Even though mahseer vtg has larger molecular mass, it is still recognized as glycophospholipoprotein due to the presence of the similar single band appeared in all stains. Many polypeptides are yielded by SDS-PAGE, however only three polypeptides were specified and recognized by anti-carp antibody in the immunodetection process. This strengthened the fact that other plasma proteins may also appear during the purification process.

The characteristic of mahseer vtg has been identified by the developed procedures of SDS-PAGE and Western blot in this study. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been widely used to purify vtg and in the determination of the molecular weight of the target proteins. Carp vtg (Biosense Laboratories, Norway) is subjected to SDS-PAGE in order to observe the similarity and difference when compared to E2-treated and female plasma as these two should technically have high molecular mass polypeptides. The negative control was collected from male *T. tambroides* without 17 β-estradiol injection and purified by gel filtration chromatography. E2-treated male plasma is collected from *T. tambroides*, which is a cyprinid, being treated with 17β-estradiol for 2 weeks with 2-days interval and first purified by gel filtration chromatography. The sample is collected from fractions containing Gaussian peak according to chromatogram layout as shown in Fig. 2a. The peak with higher absorbance showed the best immunoreactivity against antigen when stained with Western blot (Watts *et al*., 2003; Hennies *et al*., 2003; Amano *et al*., 2008; Leonardi *et al*., 2009).

Polypeptides were not detected in untreated male plasma. However, high molecular weight protein bands were observed in E2-treated male and positive control plasma. Protein bands seen in both estrogen-induced and vitellogenic female were slightly similar with molecular weight approximately 133 kDa, as seen in female, treated male and also carp vtg. Several slightly similar bands were present in female and estrogen-induced male plasma samples.

Commercially available vtg antiserum has been recently used to determine cross reactivity of vtg against specific antibodies and some researchers used the antiserum to validate assays for measurement of vtg concentration. Swart and Pool (2009) previously reported that vtg from *Oreochromis mossambicus* identified by commercial vtg antiserum produced from purified vtg of E2-treated Sea bream (*Sparus aurata*). In addition, commercially available anti-turbot vtg reported to confirm purified vtg from Chilean flounder (Leonardi *et al*., 2009). A number of studies showed that antibodies rose against carp (*C. carpio*) vtg cross reacted with purified vtg from other cyprinids such as fathead minnow (Nilsen *et al*., 2004), copper redhorse (Maltais and Roy, 2007) and shorthead redhorse (Maltais and Roy, 2009). In this experiment, commercially available anti-carp vtg monoclonal antibody (Biosense Laboratories, Norway) the cross reactivity against mahseer vtg was tested with Western blot. It showed that carp vtg antiserum recognized mahseer purified vtg from E2-treated male and mature female. The three specifically detectable polypeptides were identified as vtg since they were observed in both E2-treated male and sexually mature female

plasma but absent in untreated male (negative control). The cross-reactivity showed by mahseer vtg against anti-carp monoclonal antibody suggested that vtg gene is highly conserved (Maltais and Roy, 2007) between species within the same family.

In comparison, E2-treated male and positive control (mature female) plasma were observed to have polypeptides separated by SDS-PAGE. However, Western blot showed that carp antiserum detected lesser numbers of vtg. This showed that the proteins separated by SDS-PAGE earlier is a mixture, it is possible that the vtg was mixed with other protein found in mahseer plasma or it may be due to products resulted from protein degradation.

There were many protein bands observed on PAGE gel but after further identification by Western blot, the two major bands with the molecular weight of 133 and 117 kDa and a minor band of 56 kDa were observed. These bands were found to be within the same molecular weight range of vtg found in other freshwater fishes. Hennies *et al.* (2003) reported that vtg purified from carp (*C. carpio*) and perch (*Perca fluvialitis*) showed bands with high molecular weight, 180 and 160 kDa respectively. These vtg polypeptides are detected in female as well as in male plasma treated with E2. SDS-PAGE and Western blot revealed protein band with a molecular mass of 120 kDa in African sharptooth catfish (Braathen *et al.*, 2009) and Chilean flounder (Leonardi *et al.*, 2009). Meanwhile, in Chinese rare minnow, molecular masses of 170 and 140 kDa were observed when induced with E2 (Luo *et al.*, 2011). Similar bands were not observed in non-treated male plasma.

In this study, the development and validation of a competitive ELISA for measuring vtg in mahseer using a commercially-available anti-carp monoclonal antibody (Biosense Laboratories, Norway) is described. Anti-vtg antiserum is species specific and it requires antibody produced from each fish species (Hong *et al.*, 2009; Watts *et al.*, 2003; Shao *et al.*, 2005). However, this research showed that the commercially-available anti-carp used in ELISA recognized purified vtg of mahseer as shown by Western blot (Fig. 6). In this assay, pre-incubation of vtg standard and plasma dilution sample with antiserum (Lomax *et al.*, 1998; Shao *et al.*, 2005; Palumbo *et al.*, 2009) was performed in order to enhance the assay sensitivity. The free antigen on the plate and the soluble antigen in sample or standard compete for antibody binding. The minimum detectable vtg concentration, defined as the concentration correspondence to the mean absorbance values of 10 NSB wells plus three times the standard deviation, in mahseer plasma is 1.43 ng mL^{-1}. This is close to the detection limit reported in for other fish, for example, 2 ng mL^{-1} for Arctic charr (Johnsen *et al.*, 1999), 3 ng mL^{-1} for fathead minnow (Parks *et al.*, 1999) and 5.7 ng mL^{-1} for Chinese loach (Shao *et al.*, 2005). Inter (n = 6) and intra-assay (n = 5) were performed for validation purpose. Results revealed that this assay is reproducible and sensitive with coefficient of variations at binding percentages of 80, 50 and 20% are less than 15%. This is comparable to those reported in other fish species such as in Chinese rare minnow (Luo *et al.*, 2011), Chinese loach (Shao *et al.*, 2005), carp, zebrafish and medaka (Nilsen *et al.*, 2004). The parallelism between plasma dilution curve of female mahseer and Vtg standard when assessed statistically revealed that anti-carp antibody recognized antigen in a similar pattern.

The information on characteristics of vtg in mahseer plasma and the development of a sensitive assay for quantitation of plasma vtg in mahseer are useful for aquaculture and fisheries applications. Other than the role as bioindicator, vtg may also be used as a maturation indicator in female, sex determination of fish in the wild and captivity. Egg maturity is an important factor in broodstock selection for induce breeding purpose especially for mahseer. It is hope that this research will be able to contribute to the enhancement of breeding technology for the mass production of mahseer fry in Malaysia.

CONCLUSION

In conclusion, purification protocols and ELISA assay were successfully developed for the measurement of mahseer vtg. The commercially-available anti-carp vtg demonstrated cross reactivity against mahseer vtg, thus showing that this antibody can be used not only for carp, but also for other cyprinids.

ACKNOWLEDGMENT

Special thanks from the authors to Agri-Biotechnology Institute (ABI) under Ministry of Science, Technology and Innovation (MOSTI) of Malaysia for the financial aid to conduct this study, to Mr. Yee and his workers at Kampung Pangsun, Hulu Langat for the maintenance of fishes, last but not least to Mr. Muhammad Fadhil Syukri for guiding and assisting, and to all postgraduate students and staffs of Department of Aquaculture, UPM for their technical asistances during this study.

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