



## Protein Identification of Vitellogenin in River Catfish (*Hemibagrus nemurus*)

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### ABSTRACT

Mass production of fish broodstock with high quality eggs requires the knowledge on the chemical composition and physiochemical properties of vitellogenin (Vtg) during ovulation. Vtg is an egg yolk precursor phospholipoglycoprotein, and has been analysed to evaluate the reproductive conditions and determine the spawning period in captive and wild fish. In this study, Vtg was induced in male *H. nemurus* through three intramuscular injections of 17 $\beta$ -estradiol (E<sub>2</sub>). The Vtg was purified from the serum using gel filtration chromatography and the purified protein was reduced via SDS-PAGE. One major polypeptide corresponding to 130 kDa was observed. Vtg identification was done using peptide mass fingerprint (PMF) from the trypsin digestion of male *H. nemurus* Vtg induced with E<sub>2</sub>. The sequence homology of *H. nemurus* AYLAAADVLEVGVR matched the Vtg of other fish species when analysed using MALDI-TOF. Vtg was confirmed by MASCOT at 95% significant level. The potential protein that controls the reproductive process and oocyte development isolated from this study was discussed to understand the structure and function of Vtg.

### INTRODUCTION

*Hemibagrus nemurus*, known as the river catfish is an indigenous freshwater fish and is one of the most important cultured species of the Southeast Asia. Good meat quality makes it a favourite food among locals in Malaysia and other countries in Southeast Asia. Total catfish production in Malaysia has doubled over the last five years at 497,556 metric tonnes [1]. At present, these catfish are cultured in small cages, ponds, cement tanks, canvas tanks and ex-mining pools for local consumption and export. Unfortunately, the seed supply for catfish is seasonal and their inability to reproduce in captivity is a major hindrance to mass production. In natural habitat, this species is threatened by development, industrialisation, deforestation and overfishing. One of the ways to overcome this problem is by determining the spawning period of this species through the identification of Vitellogenin (Vtg), the specific protein related to oocyte growth.

At present, new proteomic technologies can be applied to identify the Vtg protein such as one or two-dimensional gel electrophoresis (2-DE), multidimensional high-performance liquid chromatography (HPLC) and mass spectrometry (MS). A multidimensional protein identification technology combining with tandem mass spectrometry is the most powerful approach to identify protein [2] for addressing important physiological questions. The sensitivity of analysis and the accuracy of results obtained through MS for protein identification have increased [3] since MS can tolerate protein mixtures and is amenable to high-throughput proteins. Furthermore, this information can be employed to identify the protein by searching protein databases via comparative proteomic studies.

Vtg is an egg yolk precursor protein found in most oviparous vertebrates and can be detected in males as they carry

the Vtg genes. It is a large glycopospholipoprotein that is synthesised in the liver and released into the bloodstream where it will be incorporated into developing oocytes [4]. In addition, Vtg was reported to have various molecular weights ranging from 300 to 640 kDa in different fish species belongs to different families and differ in their biochemical properties [5]. Many scientists have studied *H. nemurus* in the field of nutrition and genetics [6]. However, only few studies have been reported on the reproductive biology of this species [7,8,9] and none documented on Vtg. Hence, this study was conducted to characterise the Vtg in estradiol-induced males of *H. nemurus*. Being a great interest of local aquaculture field, confirmation of Vtg proteins through identification of the peptide via MALDI-TOF analysis is an obligatory step in the investigation of the Malaysian's *H. nemurus* reproductive biology. The Vtg studies would provide useful information for further analysis of reproductive physiology of the species, where the spawning period will be able to be predicted and maximum production of high quality eggs and fries is possible.

## MATERIALS AND METHODS

### Fish

Fifteen mature males, five vitellogenic females and three immature females were captured by local angler at Sungai Selangor, Bestari Jaya. The fishes were brought to the Aquaculture laboratory, Faculty Science and Biotechnology, Universiti Selangor and acclimatised for 2 weeks prior to the experiments. The fishes were kept in fibre tanks (1.2 m x 1.0 m x 2.5 m) equipped with filter system and aeration. The water parameters were maintained at a mean temperature of 29±2 °C, pH of 8.23 – 8.70 and dissolved oxygen at 6.5 mg/mL.

### Preparation of *H. nemurus* Vtg

Nine males of *H. nemurus* were injected with 17-β estradiol (2 mg E2/kg fish body weight) intramuscularly at three days intervals for two weeks. Three days after the last injection, blood was collected from the caudal vessels into heparinised syringes (5 mL) containing phenylmethylsulfonyl fluoride, PMSF (100 μL, 1 mM) and directly transferred into EDTA tube. The blood was maintained on ice, allowed to clot for one hour and centrifuged at 8000 rpm for 10 minutes at 5 °C. The plasma was withdrawn and immediately stored in the presence of PMSF at a ratio of 2:1 v/v (plasma:PMSF) to avoid proteolysis of Vtg and stored at -80 °C for vitellogenic purification.

### Gel filtration chromatography

Plasma Vtg was purified via gel filtration chromatography (GE Healthcare Bio-Science, Upsalla, Sweden) using Sepachryl HR 300 column (HiPrep 16/60) (GE Healthcare Bio-Science, Upsalla, Sweden). The column (120 mL) was equilibrated with two column volumes of 0.05 M Tris-HCl buffer pH 8.0. Samples were eluted with a flow rate of 0.4 mL/min at room temperature and 5 mL fractions were collected. One major peak was obtained from the column and immediately concentrated using Vivaspin centrifuge tube (30 kDa Molecular weight cut-off, GE Healthcare Bio Science, Upsalla, Sweden) at 4 °C and, 10000 rpm for 10

minutes. The Vtg protein was stored at -20 °C in aliquots of 200 μL before subjected to SDS-PAGE analysis.

### Protein assay

Protein concentrations of the purified samples were quantified via dye-binding method [10] using Bovine Serum Albumin BSA (Biorad) as a standard. The purified Vtg (200 μL, unknown concentration) was diluted in a ratio of 1:1 to 1:7 in Bradford reagent. Blank sample (0 μg/mL) was prepared using distilled water and Bradford reagent. The assay was measured using Biochrom Asys UVM340 Microplate Reader at absorbance wavelength of 595 nm. The standard curve of absorbance at 595 nm versus BSA concentration was plotted. The protein content of the unknown sample was estimated from the BSA standard curve.

### SDS-PAGE

SDS-PAGE was operated according to the method proposed by [11] on a BioRad Mini-PROTEAN II (Biorad, USA) electrophoresis gel. It was performed on 6.5% separating gel and 4% stacking gel. Prior to application on the gel, samples were diluted at a ratio of 1:1 in SDS sample buffer (125 mM Tris-HCl, 10% SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.02% bromophenol blue) (15 μg/mL) and heated to 95 °C for five minutes in a Thermomixer comfort 1.5 mL (Eppendorf). A 20 μL sample was loaded on to the gel. Molecular weight standards (Thermo Scientific PageRuler Plus Prestained Protein Ladder; 10-250 kDa) were included as reference. Electrophoresis was carried out on ice in a buffer (50 mM Tris, 192 mM glycine and 0.1% SDS) at a constant current of 100 mA, 50 V for three hours. It was then stained overnight in 0.1% Coomassie Brilliant Blue R250 (Hercules, Canada) with slow agitation on a shaker and immersed in destaining solution for 3 hours. Finally, results were viewed using gel imager (Alpha Innotech, Cell Bioscience, California).

### Molecular weight determination

The subunit of molecular mass of purified Vtg was estimated from the linear plot of log<sub>10</sub> MW versus mobility of the protein marker (relative migration distance). Band of protein markers (Thermo Scientific PageRuler Plus Prestained Protein Ladder) that represent the size of 10, 15, 25, 35, 55, 70, 100, 130 and 250 kDa were used to construct the calibration plot. Molecular weight of the samples were measured based on the values obtained of the bands in the standard.

### MALDI-TOF analysis

The protein of interest (major band) were excised from the stained SDS-PAGE using a clean, sharp scalpel and transferred into 1.5 mL capped micro tubes. The target band in each tube was labelled correctly according to the information required. Samples were dried and sent to Proteomics International (Perth, Australia) for MALDI-TOF analysis. Briefly, the purified protein samples were trypsin-digested and peptides-extracted according to the standard techniques [12]. The peptides were analysed by MALDI-TOF/TOF mass spectrometer using a 5800 Proteomic Analyzer. Bovine serum albumin was used as the standard. Generated mass spectra of the peptides were analysed using ProFound software, a tool for searching protein sequence collections with peptide mass maps.

## Identification of vitellogenin

The peptide mass fingerprints for the Vtg protein were searched in the corresponding organism database or Ludwig NR Database, in house database by using the MASCOT search engine with a probability-based scoring algorithm (<http://www.matrixscience.com>) and free-access internet search engine tools.

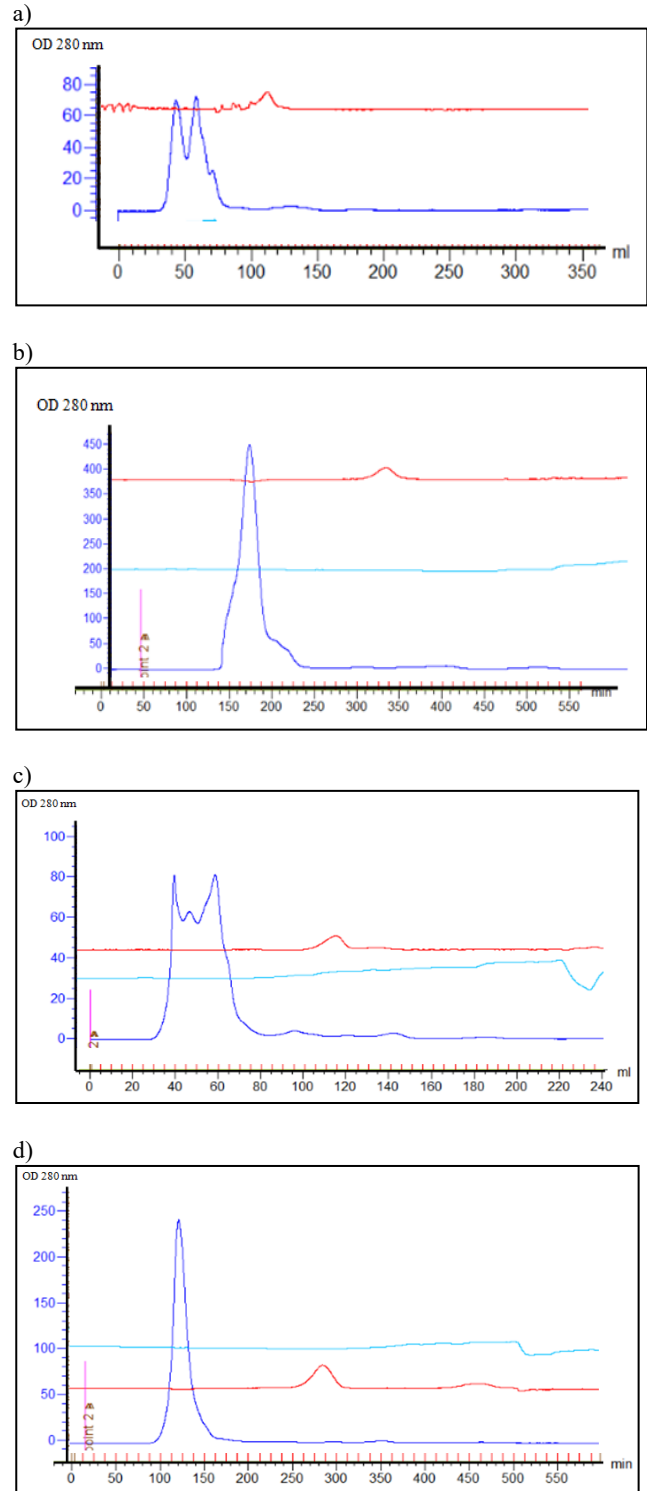
## RESULTS AND DISCUSSION

### Purification of Vtg

Vitellogenin was purified from the plasma of Estradiol-treated male *H. nemurus* via gel filtration chromatography using Sepachryl HR-300 column (GE Healthcare). **Fig. 1** shows the elution pattern for male *H. nemurus* before (a) and after treatment with 17- $\beta$  estradiol (b), immature female *H. nemurus* (c) and mature female (d). A single symmetrical peak was obtained from purified E2-treated fish indicating the presence of Vtg protein. This peak is very low in chromatographic profile of non-induced *H. nemurus*. Absorbance was read at OD 280 nm. Braathen et al. [13] reported that the best method to isolate Vtg from blood plasma is through chromatography technique. Table 1 shows the protein concentration of crude and purified plasma in male, treated male, mature and immature female of *H. nemurus*. The protein concentration of purified plasma was  $0.068 \pm 0.009$  mg/mL. Mosconi et al., [14] suggested that the degradation of purified protein could be avoided using purification process with added protease inhibitor (PMSF) to the samples, which contributes to the isolation of single Vtg molecule.

### Electrophoresis analysis

Electrophoresis of purified plasma from E2-treated *H. nemurus*, vitellogenic females, immature females and non-induced males under reducing condition was shown in **Fig. 2a** and **Fig. 2b**. The molecular weight of purified plasma from peak (b) (Purified plasma from E2-treated fish) and peak (d) (mature female) resulted in one major band (130 kDa) and other minor bands (80, 70, 60, 55 kDa : lane 1-6 (**Fig. 2a**) : lane 1-5 (**Fig. 2b**)). The major bands did not appear in control male plasma and immature female (lane 1-2, lane 5-6 (**Fig. 2a**)). According to the previous study done in catfish [15], the major band of Vtg recorded was 200 kDa on the SDS-PAGE. From the results obtained, this protein can be suggested as Vtg due to the similar range of molecular weight by SDS-PAGE stated in the previous study. This present study demonstrated that the induction of 17- $\beta$  estradiol in males *H. nemurus* had caused the appearance of high molecular weight protein that is expected to be Vtg. However, the same band position occurred in immature female (lane 5-6), with slightly fainter appearance because the samples were either tested during the non-reproductive period [16] or the fish is immature [17]. The purification of Vtg was successfully achieved by gel filtration chromatography using Sepachryl HR-300 column since the column was able to separate protein with high molecular weight. SDS-PAGE was reported to be the best methods for determining the molecular weight of Vtg [18].



**Fig. 1.** Gel filtration chromatography from plasma of immature male *H. nemurus* before (a), after treated with 17- $\beta$  Estradiol (b), immature female *H. nemurus* (c) and mature female (d) using Sepachryl (16/60) HR 300 Column (GE Healthcare Bioscience, Uppsala, Sweden).

Table 1. Protein concentration of crude and purified plasma in male, treated male and female of *H. nemurus*

	Crude protein concentration (mg/ml)	Purified plasma using gel filtration chromatography (mg/ml)
Treated male	0.25±0.010	0.068±0.009
Mature female	0.24±0.020	0.069±0.004
Male	0.21±0.010	0.050±0.004
Immature female	0.22±0.010	0.053±0.006

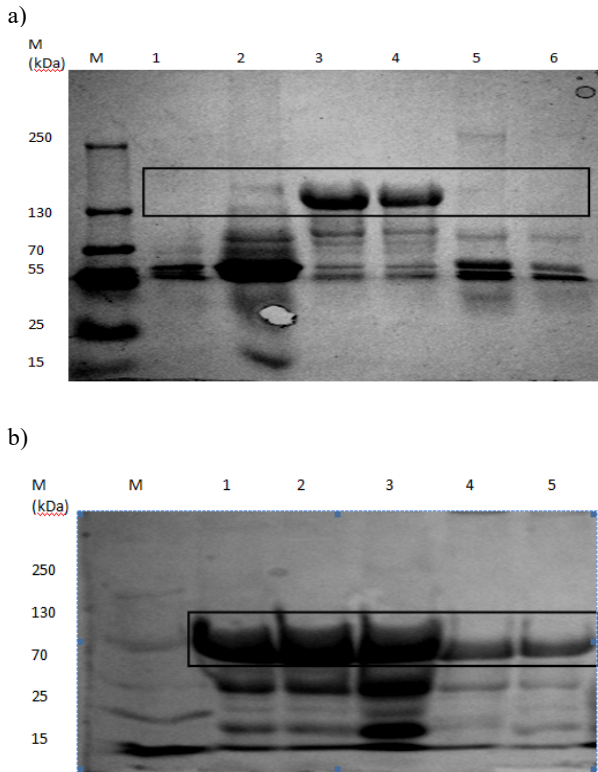


Fig. 2. SDS-PAGE result on vitellogenin. Lanes 1 and 2: Males plasma; Lane 3 and 4: Purified plasma from E2-treated fish; Lane 5 and 6: immature female. M: Molecular Weight Markers (Thermo Scientific) (a); SDS-PAGE result on vitellogenin. Lanes 1, 2 and 3: Purified plasma from E2-treated fish; Lane 4 and 5: Mature female (express vitellogenin properties) M: Molecular Weight Markers (Thermo Scientific) (b).

**Protein identification**

Protein identification of major bands was performed using MALDI-TOF analysis to determine the class of the subunit. The generated peptides were analysed by MALDI-TOF mass spectrometer and produced good readings. It has been confirmed that the major bands was Vtg through protein identification using peptide mass fingerprint (PMF) of trypsin-digested vitellogenin from male of *H. nemurus* that induced with E2. The Mascot Score Histogram showed that the significant level ( $P < 0.05$ ) confidant Vtg with the highest homology value (42%) is between *H. nemurus* (in this study) with the Vtg isoform of *Clupea harengus* fish from the Ludwig protein database (Fig. 3). Table 5 shows the most matching peptide of *H. nemurus* Vtg via the homology peptide massess generated, following the trypsin digestion with those available in the database (Ludwig, Swiss-Prot and MASCOT). It was confirmed that the molecular weight of river

catfish Vtg was 130 kDa and the peptide sequence was AYLAGAAADVLEVGVR, and only matched with four sequence homologies in the reference databases. The Table 6 shows the similarities between *H. nemurus* (used in this study) and the protein isoform in the Gene Bank (NCBI) with 100% identity and 50.3 maximum score.

Therefore, based on our current findings and data supported by previous literatures, it is suggested that the unidentified proteins obtained in this study is Vtg. The purified Vtg can be further investigated using immunological analysis, which is being widely applied in addition for fully characterised Vtg [19]. The technique uses antibodies that are very specific to Vtg and are utilised to distinguish Vtg from other fish and organisms. Hopefully the current finding will be beneficial for future studies in order to obtain better comprehension of *H. nemurus* Vtg. The implementation of local *H. nemurus* Vtg as a maturation biomarker is expected to be very attractive but more detailed research must be carried out to get better understandings.

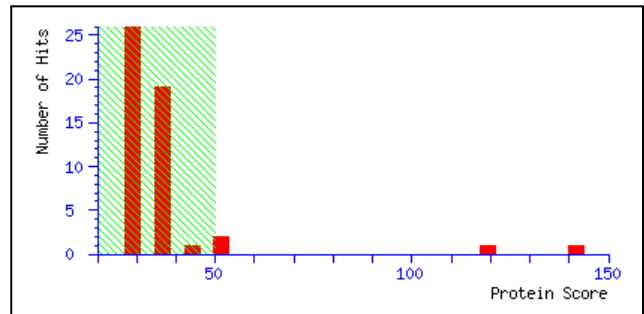


Fig. 3. Mascot Score Histogram (indicate identity or extensive homology  $p < 0.05$ ), where P is the probability the observed match is a random event.

Table 5. Peptide mass fingerprint (PMF) of the trypsin digestion of male *H. nemurus* Vitellogenin induce with E2.

Observed $m/z$	Calculated $m/z$	$\Delta m/z$	Peptide	Ions Score	Match
1574.6466	1573.8464	1	R.AYLAGAAADVLEVGVR.T (Major vitellogenin isoform 1 of <i>Clupea harengus</i> )	142	M
1574.6466	1573.8464	2	R.AYLAGAAADVLDIGVRT (Vitellogenin B2 of <i>Cyprinus carpio</i> )	122	M
1099.3989	1098.5417	1	R.RAEIDNPER.K (Disco-interacting protein 2 of <i>Ascaris suum</i> )	54	M
1099.3989	1098.5782	2	R.SSTVPRAPER.R (Uncharacterized protein of <i>Macaca mulatta</i> )	53	M
835.3831	834.5439	1	AIHLLLR	39	N.M
1746.7590	1745.8804	1	QLMPPASSMVTKATR + 2 Oxidation (M)	38	N.M
1466.6587	1465.8616	1	LIRIDEATGLPLR	38	N.M
843.4070	842.4974	1	ISNLQLR	33	N.M
1368.6440	1367.7772	1	SLSGLEPVINALR	33	N.M
816.3188	815.4323	1	VSI MPNR	32	N.M
1934.7975	1933.8687	1	ADGGIKKEEVNSAECEVR	30	N.M
1595.7423	1594.9698	1	ILGDVLVPSFPVLVK	29	N.M

Table 6. Data of protein blast of *H. nemurus* (in this study) and the Gene bank for sequences producing significant alignment.

Protein and species matching	Maximum score	Query cover (%)	Identity (%)	Accession
Vitellogenin, partial [ <i>Carassius auratus</i> ssp. 'PENGZE']	50.3	100	100	AFU35775.1
Lipovitellin [ <i>Ctenolabrus rupestris</i> ]	50.3	100	100	ABS53014.1
Vitellogenin B variant 1 [ <i>Carassius auratus</i> ssp. 'PENGZE']	50.3	100	100	AGZ80880.1
major Vitellogenin isoform 1 [ <i>Clupea harengus</i> ]	50.3	100	100	ACJ65208.1
Vitellogenin Aa [ <i>Centrolabrus exoletus</i> ]	50.3	100	100	ACK36963.1
Vitellogenin Aa [ <i>Labrus mixtus</i> ]	50.3	100	100	ACK36967.1
Vitellogenin Aa [ <i>Dicentrarchus labrax</i> ]	50.3	100	100	AFA26669.1
Vitellogenin Aa [ <i>Morone saxatilis</i> ]	50.3	100	100	ADZ57172.1
Vitellogenin A [ <i>Morone americana</i> ]	50.3	100	100	AAZ17415.1

## CONCLUSION

The present study successfully isolated and partially characterised Vtg in induced *H. nemurus* male by using gel filtration chromatography and SDS-PAGE. The protein was characterised as Vtg in *H. nemurus* as it fits all the characteristics of Vtg molecules described in previous studies. The results of the study facilitate a better understanding on Vtg as a biomarker for maturation status in river catfish (*H. nemurus*). This information can be used for improving broodstock management for the production of river catfish. However, further analysis needs to be carried out in order to strengthen and validate the current findings.

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