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Protein Profiling and Precursor-product Relationship between Vitellogenin and Lipovitellin in the African catfish, *Clarias gariepinus*

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Abstract:

Nutrients are deposited in the eggs as yolk to provide energy and to build embryonic structures. Lipovitellin, the major egg-yolk protein was purified from the oocytes of *Clarias gariepinus*. The amino acid sequences of lipovitellin (Lv) peptides obtained by Mass spectrometry were mapped on its precursor protein, Vitellogenin (Vg). Advanced proteomic tools, PAGE {one dimensional (1-D) andtwo-dimensional (2-D)} and mass spectrometry were employed to characterise Vg. Similarity in sequences of peptidesre-established the precursor-product relationship between Vg and Lv. Partial mRNA transcript of *vg* gene was translated into amino acid sequence that corresponded to conserved domains of Vitellogenin in NCBI database. Phylogenetic analysis of the nucleotide sequence of Africancat fish *vg* gene revealed a close similaritywith the fishes of the order Siluriformes.

Keywords:
Clarias gariepinus, Vitellogenin, Lipovitellin, Gel filtration chromatography, PAGE, MALDI-TOF/MS.

INTRODUCTION

A fish egg is a semi-closed system, which provides a well-protected environment and nutrition to the growing embryo [1]. The accumulation of yolk proteins during vitellogenesis and their mobilization during embryogenesis are the key processes for successful reproduction. Lipovitellin (Lv)is the major yolk-protein present in the fish oocytes. It consists of two polypeptides, the N-terminal lipovitellin heavy chain (LvH) and the C-terminal lipovitellin light chain (LvL). It mainly provides lipids and amino acids to the developing embryos as well as a reservoir of water till the drinking mechanism is developed[2].Some Lvs are degraded rapidly to release a pool of free amino acid (FAA) for immediate nutrition to the developing young ones while others are stored until larva finds suitable habitat [3-5].

Lipovitellin is derived from precursor protein, Vitellogenin (Vg), synthesized during gonadal growth in the liver of female fish under the influence of estrogens [6-9]. Vitellogenin is transported by blood, and gets incorporated in the growing oocytes via specific receptor-mediated endocytosis [10, 11]. In the oocytes, it is enzymatically cleaved into smaller yolk proteins: Lipovitellin, Phosvitin and β'-component [12,13]. Fish vitellogenin is a dimeric protein consisting of two identical subunits and several peripheral moieties such as carbohydrates, lipids and phosphates [14,15]. Multiple forms of vitellogenin gene, giving rise to multiple forms of Vitellogenin proteins *viz.* VgA, VgB and VgC have been characterized in many teleost fishes [16-20]. Advanced proteomic tools such as Two-dimensional electrophoresis and Mass spectrometry have been employed to identify and characterize different

forms of vitellogenins of fish [21-23].

VgA and VgB primarily proteolyzed into respective lipovitellins (LvHA and LvLA; LvHB and LvLB), while VgC remains intact during oocyte maturation. Differential enzymatic processing of lipovitellins is responsible for oocyte hydration and buoyancy of eggs [2]. Lipovitellins (LvHA and LvLA) derived from VgA, produce large pool of amino acids and responsible for pelagic nature of eggs while LvHB derived from VgB are partially cleaved and account for demersal nature of eggs [4]. The quantitative ratio of LvA to LvB in post-vitellogenic oocytes regulates the buoyancy of pelagic eggs by controlling the availability of FAA, which function as osmotic effectors during hydration. On the contrary, minimal proteolytic changes and limited hydration have been observed in the oocytes of fish spawning demersal eggs [24].

The African catfish, *Clarias gariepinus* is a demersal egg laying exotic species in India. It is a hardy carnivorous fish that predates on the young ones of other fishes and is therefore, a threat to native fish species of India. Basic research on reproductive biology of the African catfish may help to develop suitable strategies to keep a check on its numbers in the water bodies of India. Intact vitellogenin protein from catfish has been isolated and its molecular mass has been calculated [25-27]. The present study was undertaken to check the expression of vitellogenin gene in liver, characterize the vitellogenin protein purified from the blood of estradiol injected fishes and to isolate and characterize Lipovitellin from the oocytes of gravid fishes. Peptides of Vg and Lv will be mapped on translated *vg* mRNA transcripts and compared with information available in database.

MATERIAL AND METHODS

Animal and Ethics statement

The adult specimens of *C. gariepinus* (body wt. 150-200 g) were collected from the fish market around Delhi (Lat. 28°35′N, Long. 77°12′E). Males were identified by the presence of a muscular urogenital papilla. These fish were acclimatized to laboratory conditions (L:D::12:12) at 25±1°C for at least one week prior to the treatment. Fish were fed *ad libitum* every evening with minced meat. Water was renewed daily with de-chlorinated tap water. Fish procurement and maintenance protocol has been approved by the Institutional Animal Ethics Committee, Department of Zoology, University of Delhi.

Induction and Isolation of Vitellogenin

Five male catfish were administered with estradiol-17β (E_2) (50µg/100g body weight) intramuscularly for five consecutive days. Blood samples were collected twenty-four hours after the last injection of E_2. Fifteen minutes prior to bleeding, fish were injected with 4-5 TIU aprotinin, a protease inhibitor, intraperitoneally. Serum was separated from the blood by centrifugation at 3,000 g and the pooled serum samples were layered on Ultrogel AcA 34 packed in a column (H=100 cm, ID= 2.5 cm). Proteins were eluted with Tris-Cl buffer at 4°C at a flow rate of 20 ml/hr and absorbance was monitored at 280 nm [6, 28]. Vitellogenine luting at Ve/Vo = 1.48 [28] was further characterized.

Characterization of Vitellogenin protein

Native and SDS- Polyacrylamide Gel Electrophoresis (PAGE)
Peak sample eluting at Ve/Vo = 1.48 (Fig. 1A) was subjected to electrophoresis under native as well as under denaturing conditions [29-31]. Gels stained with Coomassie Brilliant Blue were analyzed using Image Analyzer software of the gel documentation system (Bio-Rad) (Fig. 1B, C).

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)
Isolated protein sample was also subjected to 2-D PAGE. The sample was mixed with equal volume of rehydration buffer (8M urea, 2% CHAPS [3-(3-Cholamidopropyl) Dimetylaminonio-1-propanesulphonate], 0.005% Bromophenol blue in 50% isopropanol) and dialyzed overnight against the same buffer at 4°C. The IPG strips (GE healthcare Immobiline Dry Strip, pH 3-10) were rehydrated and electrophoresed using Ettan IPG phor 3 Isoelectric Focusing System. For second dimension, the

strips were equilibrated in equilibration buffer (6M Urea, Tris (pH-8.8), 30% glycerol, 2% SDS, 0.005% Bromophenol blue in 50% isopropanol) along with 1 M DTT (Dithiothreitol) and were subjected to electrophoresis. Gel was stained with CBB G-250, destained and analyzedusing Image Analyzer software of the gel documentation system (Bio-Rad)

Mass Spectrometry Analysis

Peptide products, resolved by PAGE were subjected to Trypsin digestion (Promega, Gold Mass Spectroscopy Grade). Gel strips containing peptides were homogenized with 1 ml of 50 mM ammonium bicarbonate containing 50% acetonitrile and vortexed to remove the stain, followed by addition of acetonitrile (200-300 µL). Dried sample was digested with trypsin (1ng/slice), extracted with freshly prepared 50% acetonitrile and then chromatographed on a strong cationic exchange column followed by reversed phase separation. Separated peptides were subjected to MALDI-TOF/MS analyzer as well as online MS/MS using ion trap mass spectrometer (AB SCIEX TOF/TOF™ 5800). The data was analyzed using MASCOT search engine.

Characterization of vitellogenin Gene

RNA extraction and cDNA synthesis

After blood collection, the fish were decapitated and liver tissue was excised. For total RNA extraction, liver tissue from estrogenized fish were homogenized in TRIZOL (Sigma) and isolated by guanidinium thiocyanate-phenol-chloroform extraction method [32]. RNA integrity was checked by routine electrophoresis. The concentration and purity were analyzed by measuring the absorbance of RNA samples at 260 and 280 nm. Samples with DNA contamination were treated with DNase enzyme (Fermentas). The cDNA was synthesized from two micrograms of total RNA with RT (reverse transcriptase) and oligodT primer.

PCR amplification, cDNA cloning and Phylogenetic Analysis

Nucleotide sequences of *vg* mRNA from six teleosts were aligned using Clustal W software and degenerate primers were designed using OligoCalc software (Table 1). A portion of the *vg* gene was amplified by the polymerase chain reaction, which contained primers (15 pmol) in reaction buffer, Taq polymerase (Fermentas), dNTPs (BR-Biochem) and 2 µl of single-strandedcDNA. β-actin was used as a reference gene. The amplified product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega). The purified product was ligated with pGEMT-Easy Vector (Promega, Wizard, USA) overnight and transformed into electrocompetent *Escherichiacoli* DH5α cells by electroporation. Clones were screened by Blue-White screening method and positive clones were used for colony PCR to check the gene insert (Table 1).

Table 1: Nucleotide sequences of primers used for partial amplification of vitellogenin gene in African catfish. Degenerated primers were designed from the conserved regions of aligned vitellogenin sequences of *Clarias macrocephalus* (Accession no: EU138884.3), *Cyprinus carpio* (Accession no: AF414432.1), *Carassius auratus* (Accession no: DQ641252.1), *Danioreri o*(Accession no: NM_001045294.2), *Melanogrammus aeglefinus* (Accession no:AF284034.1) and *Morone saxatilis* (Accession no: HQ846510.1)

S. No.	Designed for	Primer name	Primer sequence (5´-3´)
1.	PCR	Forward primer 1(FA)	CTGGTTTCTGAGGCCGTAGTT
		Reverse primer 1 (RA)	TTAAGGCTGGCAGGTTGGCC
2.	PCR	Forward primer 2 (FB)	CTAGTGCTGTTTGAGACCAG
		Reverse primer 2 (RB)	CCAAGCTCAAGAACATCAGC

3.	Colony PCR	Forward Primer (M13)	TGTAAAACGACGGCCAG
		Reverse Primer (M13)	CAGGAAACAGCTATGACC

Plasmids were isolated from positive clones, purified by Wizard® Plus SV Miniprep DNA Purification System (Promega, Madison, WI, USA) and sequenced by Sanger's method. The nucleotide sequence was subjected to BLAST in NCBI and analyzed for sequence similarity with other fishes. The nucleotide sequence of vitellogenin of *C. gariepinus* was aligned with sequences of *vg* mRNA from other fishes, using Clustal W software. The phylogenetic tree was constructed using Neighbour-Joining method of MEGA 7.0 and 1000 bootstrap repetitions were used to check the reliability of the tree.

Isolation and Characterization of Egg- yolk protein

Gravid catfish, *C.gariepinus,* collected from market in the month of July were sacrificed by decapitation. Oocytes were separated and transferred to 0.9% saline containing 2mM Phenyl methyl sulphonyl fluoride (PMSF) and homogenized with an equal volume of 1M NaCl containing 3mM PMSF. The homogenate was passed through 100 µm sieve and centrifuged to obtain a clear supernatant, Egg yolk extract (EYE). Lipovitellin was precipitated from EYE by adding ammonium sulphate to 67% saturation followed by ultracentrifugation at 134,000 g for 1 h at 4^{o}C. Supernatant was discarded and pellet was dissolved in Tris-Cl buffer at 4^{o}C, dialyzed and fractionated on Sepharose-6B column. The eluted fractions were characterized by SDS-PAGE and MALDI TOF-MS/MS analysis.

RESULTS

Characterization of Vitellogenin

The serum sample from estrogenized male fish resolved into four peaks on size exclusion chromatography (Fig. 1A). The proteins eluting at Ve/Vo=1.48, resolved into three distinct forms α, β and γ under native conditions (Fig. 1B) and eight prominent peptides of varying molecular mass under denaturing conditions (Fig. 1C). Vitellogenin sample was also characterized by 2–D PAGE and the results illustrated that vitellogenin resolved into seven major spots (Fig. 1D). Peptides with high protein content *viz*. five peptides (i, ii, v, vii, viii; refer Fig. 1C) and one spot (refer Fig. 1D), were processed for mass spectrometry analysis.

Mass Spectrometric Analysis

Peptides digested with trypsin were subjected to MS/MS analysis. The probability-based Mowse score for each peptide was more than 80.The amino acid sequences and mass to charge (m/z) ratio of small trypsin-digested peptides (i, ii, v, vii, viii and spot 2; refer Fig. 1C, D) showed significant similarity with Vg protein of *C. macrocephalus, C. batrachus, Ictalurus punctatus, Daniorerio, Carassius auratus, Cyprinus carpio, Thunnus thunnus, Sparus aurata* and *Acanthogobiusflavimanus* (Table 2).

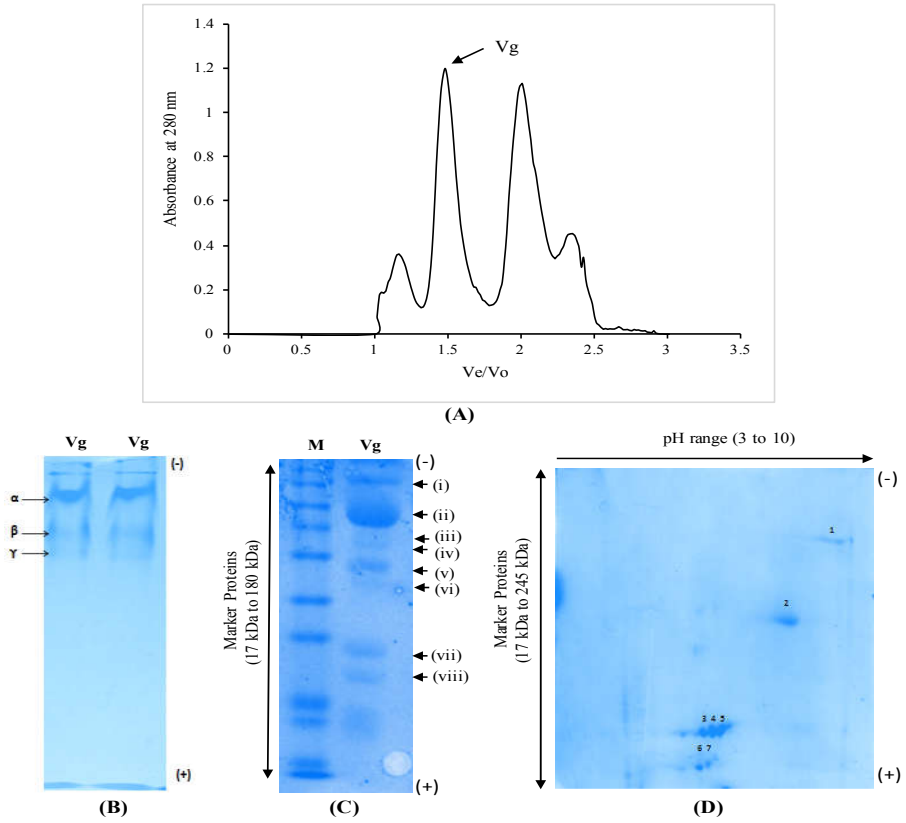


Figure 1: Gel filtration profile of ultracentrifuged serum from E_2-treated male catfish, *C. gariepinus* on UltrogelAcA 34 (A) Vitellogenin (Vg) eluting at Ve/Vo=1.48 (arrow in Fig 1A) was electrophoresed under native conditions on 5% resolving gel (B); under denaturing conditions on 8% resolving gel (C). Vitellogenin sample was subjected to 2 D-PAGE on 12.5% resolving gel (D).

Note: Vg resolved as three bands (α, βand γ) on Native PAGE; eight peptides (ito viii) on SDS PAGE and seven spots (1 to 7) on 2D PAGE

Gene (vg) Expression and Phylogenetic Analysis

The results of PCR suggest that *vg*gene was expressed in the liver of male catfish after estradiol administration. The nucleotide sequences cloned in plasmids were of 520 bp (Accession no. **MK280693**) and 278 bp (Accession no. **MH347894**). These sequences when subjected to BLAST on NCBI showed significant similarity with *vg* gene of other fishes. The amino acid sequences translated from the amplified products belong to the family of Vitellogenin_N; lipoprotein amino-terminal region (LPD_N) and Domain of unknown function (DUF 1943). These partial amino acid sequences of Vitellogenin were aligned with Vitellogenin sequence of *Clarias macrocephalus* to check the conserved regions. Peptides derived from purified Vg and Lv proteinsalso showed similarity with these sequences (Fig.2A, B)

Phylogenetic tree constructed for the partial *vg* sequences of *Clarias gariepinus* and other matching nucleotide sequences are illustrated in Fig. 2C. The amplified sequences of vitellogenin of *Clarias gariepinus* showed maximum similarity with the fishes, which belong to the order, Siluriformes followed by Characiformes and Cypriniformes.

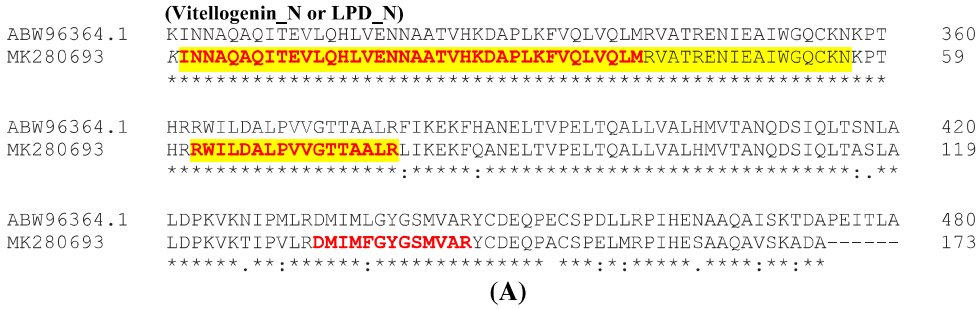


Figure 2: Alignment of deduced partial amino acid sequences of vitellogenin of *C. gariepinus* with vitellogenin of *C. macrocephalus* that code for

(A)N- terminal domain of vitellogenin

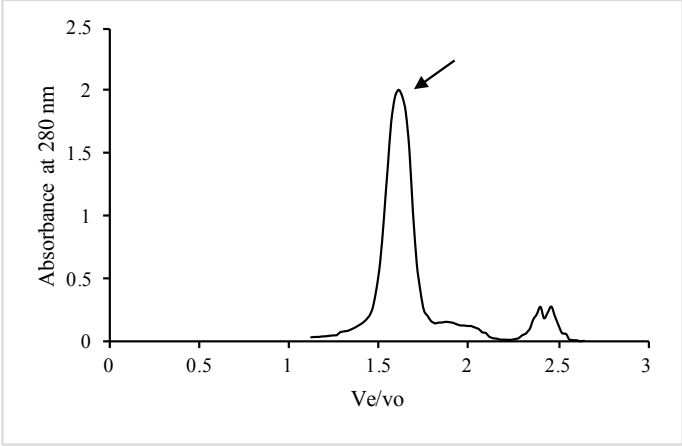
(B) Domain of unknown function.

Periods (.) and asterisks (*) below each pair of aligned amino acids indicate similarity and identity, respectively. Residue positions were indicated by the numbers on the right. Purified vitellogenin (red bold text) and lipovitellin (highlighted with yellow color) peptides are mapped on deduced primary Vg sequences

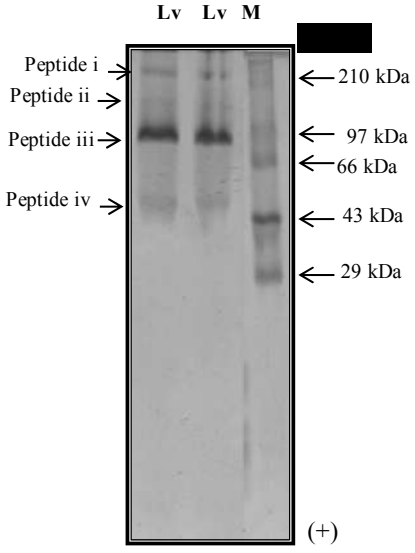
(C)Molecular phylogenetic tree based on partial nucleotide sequence of *C. gariepinus* vg with *vg* nucleotide sequences of fishes *viz. Clarias macrocephalus* (EU138884.3), *Ictalurus punctatus* (XM_017462806.1), *Ariopsis felis* (KJ804262.1), *Pygocentrus nattereri* (XM_017702244.1), *Carassius auratus* (DQ641252.1), *Misgurnus anguillicaudatus* (KF733655.1), *Astyanax mexicanus* (XM_022679416.1), *Gobiocypris rarus* (EU623080.1), *Ctenopharyngodon idella* (KT984759.1), *Rhinichthys cataractae* (EF202607.1) using MEGA 7.0 software.

Purification and Characterization of Egg-yolk protein

Proteins precipitated with ammonium sulphatere solved into only one major peak eluting at Ve/Vo= 1.6 by gel filtration chromatography on Sepharose 6B (Fig. 3A). Electrophoretic profile of the eluted proteins consisted of mainly four peptides ('i', 'ii', 'iii', 'iv') under denaturing conditions (Fig. 3B). These peptides were further digested with trypsin and subjected to MS/MS analysis. Their probability based Mowse score was more than 60. The amino acid sequence and mass to charge (m/z) ratio of the peptides thus obtained showed significant similarity with vitellogenin protein of *Clarias macrocephalus* and lipovitellin of *Ctenolabrus rupestris* (Table2).



(A)



(B)

Figure 3: (A) Elution profile of egg-yolk extract from vitellogenic ovarian follicles of *C. Gariepinus* precipitated with ammonium sulphate and chromatographed on Sepharose 6B

(B) Electrophoresis on SDS PAGE on 12% resolving gel
Lane Lv: Fraction obtained at Ve/Vo=1.66 (arrow in Fig 3A)
Lane M: Molecular weight markers
Note: Lv resolved as four bands (i to iv) on SDSPAGE

Table 2: Amino acid sequence and mass to charge ratio of Vitellogenin and Lipovitellin peptides of *C. gariepinus* showing similarity with peptides of Vitellogenin from other teleost species in NCBI database

Vitellogenin peptides of *Clarias gariepinus*			Teleost species having similar Vitellogenin peptide sequence
1D PAGE	**Amino acid sequences**	**M/Z ratio**	
(i, ii, v, vii, viii)	SLHPELR LSYYFSR QPLASIFVR FVQLVQLMR SSSSSKSGSSSR SMAPDYMHVAAAANVAIR SMAPDYMHVAAAANVAIRMLSPK GILNILQLNLK LDRLSYYFSR DMIMLGYGSMVARY VVSLAPPVPADAAQRY DAPLKFVQLVQLMR.V WILDDALPVVGTTAALR	851.4388 935.4251 1030.5663 1133.6115 1145.5330 1887.8887 2444.2295 1238.7430 1319.6379 1443.6473 1490.7936 1657.9049 1695.9076	*Clarias macrocephalus*
(i, ii, v, viii)	LEFEVQVGPK GSLQYEFATEILQTPIQLLI LVQPVALQLFMDK	1145.5743 2292.2144 1501.7941	*C. macrocephalus*
(i, ii, v)	IIDQAAQIAGR	1256.6558	*C. macrocephalus*
(i, ii, viii)	MLSPKLDR GDGPALERLEFEVQVGPK	959.5267 1940.9685	*C. macrocephalus* *C. batrachus*
(i, v, viii)	SLTGTATFSYIMKPTEK	1874.8772	*C. macrocephalus*
(ii, v, vii)	LHTVVPGK	850.4735	*C. macrocephalus*
(v, vii, viii)	NIENLPNEK	1070.5724	*C. macrocephalus*
(i, ii)	GKLHTVVPGK NIENLPNEKVVSLAPPVPADAAQR	1035.5892 2542.3259	*C. macrocephalus*
(i, v)	SLSSKQPLASIFVR LQDPQLFEYTGIWPK	1532.8281 1834.8531	*C. macrocephalus*
(vii, viii)	KILEGGLR FEFEWNR VPPAFAIIAR QANLLGDKVPPAFAIIAR VPGILTIAFPSFK	885.5164 1027.4253 1054.6031 1894.0732 1389.6433	*C. macrocephalus* *C. macrocephalus* *C. macrocephalus* *Cyprinus carpio* *C. macrocephalus*
(i)	RWILDALPVVGTTAALR	1851.9435	*C. macrocephalus*
(ii)	SGSSSRSGSK DIGLSYTETCAECQR ELMKETVK DLNNCHERVIK VVSLAPPVPADAAQRYK	939.4619 1816.9021 977.4891 1340.6770 1781.9551	*C. macrocephalus*

(v)	FEYANGVVGK TEGLQEALLK VSATVLNLHR VGDYISYVAPLAGVDADRAR INNAQAQITEVLQHLVENNAATVHK ETNMHVVSFAYSHIK	1083.5601 1101.5752 1109.4862 2057.9824 2755.4448 1762.8300	*Thunnus thunnus* *T. thunnus, Sparusaurata* *C. batrachus* *C. macrocephalus* *C .batrachus, C. macrocephalus* *Carassius auratus, C. carpio*
(vii)	IQNTIKLMDWR NINLNDQFMLFHK LGFQIAAYLDKSTPR	1488.7555 1648.8099 1679.9058	*Daniorerio* *C. macrocephalus* *Ictalurus punctatus*
(viii)	ADLQKSNVK FPEMTLSKR EPKLVQPVALQLFDK NFMDKIIDQAAQIATGR VPGILTIAFPSFKR VQVVFASISANDKWK	972.4937 1108.5343 1856.0259 1892.0089 1545.7831 1691.8302	*C. macrocephalus, C. batrachus* *C. macrocephalus* *C. carpio, Daniorerio* *C. macrocephalus* *C. macrocephalus* *C. macrocephalus*
2D PAGE			
('2')	R. ASQVIPAGLMVK. W K. ALGNAGHPRSLK. A R. QRGESVFSTVK. Y R. QMESRGNIVYK. V K. NWANVPIMMQK. L K. NWANVPIMMQK. L K. NWANVPIMMQK. L K. RASQVIPAGLMVK.	1214.6624 1221.6533 1237.6591 1325.7122 1331.7656 1333.7987 1347.7958 1369.7354	*Acanthogobiusflavimanus*
Lipovitellin peptides of *C. gariepinus*			
i', 'ii', 'iii'	QNGDGISVYAPSHGLHEVYFDR	2444.19	*Ctenolabrus rupestris*, Lipovitellin
	K.NTQNVYELHEAGPQGVCK.T SLTGTATFSYIMKPTEK R.GSLQYEFATEILQTPIQLLKV.I INNAQAQITEVLQHLVENNAATVHK R.VATRENIEAIWGQCK.N R.WILDALPVVGTTAALR.F VLGQEIAYANFDKNFMDK R.VGDYISIVAPLAGVDADRAR.N	1957.07 2671.31 1894.08 2066 2123.03 1695.94 2565.36 3179.67	*C. macrocephalus*, Vitellogenin
	DAPLKFVQLVQLMR GDGPALERLEFEVQVGPK	1657.91 1940	*C. macrocephalus*, Vitellogenin
'iv'	K.TFVYKYEGFLLSGLPQEGLVK.A QNGDGISVYAPSHGLHEVYFDR	1812 2444.19	*Ctenolabrus rupestris*, Lipovitellin
	K.FEYANGVVGKIFAPAR.V R.VATRENIEAIWGQCK.N R.WILDALPVVGTTAALR.F K.NIPLLRDMIMLGYGSMVAR.Y QANLLGDKVPPAFAIIAR R.VGDYISIVAPLAGVDADRAR.N R.NLCLSDALPIEQDGTIPALK.N	2427.14 3350.59 1695.94 1957 1894 3179.81 2536.29	*C. macrocephalus*, Vitellogenin

DISCUSSION

The present study attenuates the precursor-product relationship employing proteomic techniques in conjunction with biochemical and molecular analyses in fresh water catfish, C*larias gariepinus*. The fate of the precursor protein Vitellogenin processing in the oocytes varies depending upon the pelagic and benthic eggs. The study helps to understand the significance of variation between species in mechanisms of yolk formation and processing. In the present study, Vitellogenin from blood and its product—lipovitellin from vitellogenic oocytes of female catfish were isolated and characterized by Gel filtration chromatography and Electrophoretic techniques. Identification of these proteins was verified by Mass spectrometry.

One-step gel filtration chromatography is preferred to isolate vitellogenin from other serum proteins of the African catfish because this protein is highly susceptible to proteolysis and degradation. Aprotinin was used and all the analytical procedures were conducted at low temperature (4°C) to avoid proteolysis. Three forms of native vitellogenin and eight peptides of denatured vitellogenin (29 to 162 kDa) reflect heterogeneity in polypeptide pattern of catfish vitellogenin. Vitellogenin from this catfish having a molecular mass in the range of 385 kDa to 520 kDa has been isolated [25-27]. Review of literature suggests that charge and/or size isomers of vitellogenin protein exist in teleost fishes [11, 14, 33, 34].

Seven major spots resolved at different pH values and their molecular weight indicates the presence of charge as well as size isomeric forms of vitellogenin in circulation. Two-dimensional gel electrophoresis (2-D PAGE) combined with protein identification by mass spectrometry (MS), a reliable tool for proteomics, has been widely used for the identification and characterization of proteins in many fishes [23, 35, 36].

The molecular mass of purified yolk protein peptides ranges between 210kDa to 29kDa. On the basis of molecular mass of these peptides, it is proposed that lipovitellin in the *Clarias gariepinus* exists in heterogeneous forms as heavy chain (210kDa to 97kDa peptides) and light chain (43kDa to 29kDa peptides). In majority of teleosts, the heavy chain exists in the molecular weight range of 120kDa-90kDa while the light chain exists as 35kDa-14kDa [37-40].

MALDI TOF-MS/MS has been used to sequence and identify the peptides after electrophoresis. The spectra of these ions are analyzed by MS/MS software which provides the amino acid sequence of each [M+H] + precursor ion and the data set of their molecular masses in the form of peptide mass fingerprint (PMF) [41]. Major peaks from the spectra generated by MALDI-TOF for tryptic digests of five major peptides of protein isolated from serum of E_2 treated fish were analyzed by PMF search in MASCOT. From this analysis, number of amino acid sequences corresponding to a mass were compared and subjected to alignment by BLAST on NCBI (refer Table 2). Presence of congruent amino acid sequences in the peptides and the similarity of sequences with vitellogenin of other teleost fishes confirm the identity of protein as vitellogenin. The maximum similarity of these peptides with vitellogenin of *Clarias macrocephalus* further substantiates the fact that the peptides are from related species. Similarity of the peptide sequences of 2D spot with vitellogenin of *Acanthogobiusflavimanus*, corroborates the finding that protein is Vitellogenin. These techniques have been used to confirm the identity of vitellogenin protein in many teleost fishes such as *Salmosalar* [42], *Reinhardtius hippoglossoides* [43], *Epinephelus lanceolatus* [44], *Channa punctatus* [18, 19], *Pimephales promelas* and *Fundulus heteroclitus* [45].

Mass spectrometric results showed that the amino acid sequences of tryptic digests of four major peptides ('i', 'ii', 'iii', 'iv') of purified egg-yolk protein matched with vitellogenin region of *Clarias macrocephalus* as well as lipovitellin region of *Ctenolabarus rupestris* which confirmed that the isolated protein from egg yolk extract is lipovitellin (refer Table 2). Mapping of one common sequence (R. WILDALPVVGTTAALR. F, spanning region 363-379) in all the peptides with N-terminal domain of vitellogenin /lipoprotein of *Clarias macrocephalus* validatesthe identity of the purified protein in the present study as lipovitellin. It has been established that the lipovitellin heavy-chain (LvH) includes the N-terminus of vertebrate vitellogenin [46-48]. In addition, another sequence

(K.TFVYKYEGFLLSGLPQEGLVK.A, spanning region 29–49) mapped with N-terminal domain of Lipovitellin (Aa) of *Ctenolabrus rupestris* indicating the possibility of two isoforms of lipovitellin in the African catfish. Presence of two N-terminal domains in purified lipovitellin sample contemplates probability of two isoforms of vitellogenin A. In addition to *C. macrocephalus* and *Ctenolabrus rupestris*, these peptides are identical (80-90%) with other fish such as *Daniorerio, Labrusmixtus, Gadus chalcogrammus, Oncorhynchus nerka, Cirrhinus molitorella, Carrasius auratus* also.

In order to find out the resemblance between Vitellogenin and Lipovitellin isolated from catfish, the peptide sequences obtained from mass spectrometry were compared and homology was also checked with Vg of *C. macrocephalus* (Fig. 4). Sequence similarity between multiple peptides of vitellogenin and lipovitellin confirm the precursor-product relationship of vitellogenin and lipovitellin. Matching of peptide sequences of lipovitellin of *C. gariepinus* with the LvH and LvL region of Vitellogenin of *C. macrocephalus* further corroborates the fact that lipovitellin is cleaved product of vitellogenin. Like vitellogenin, lipovitellin is also conserved protein among fish.

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   1 MRAVVLALTL ALVASHQTNL DPEFAAGKTF VYKYEGFLLS GLPQEGLVKA GVKVSCKVFI
  61 SSVAQNTFLL KLQDPQLFEY TGIWPKDTFT PAAKLTSALY PQLVTPIKFE YANGVVGKIF
 121 APARVSATVL NLHRGILNIL QLNLKNTQNV YELHEAGPQG VCKTHYMISE DEKTHQIVVR
 181 KSKDLNNCHE RVIKDIGLSY TETCAECQQR LRSLTGTATF SYIMKPTEKG ALVSEAVVEE
 241 VHQFSLFNTE TGAAQMRAKQ TLNLLEVQNA PTAAAAGEYA ARGSLQYEFA TEILQTPIQL
 301 LKINNAQAQI TEVLQHLVEN NAATVHKDAP LKFVQLVQLM RVATRENIEA IWGQCKNKPT
 361 HRRWILDALP VVGTTAALRF IKEKFHANEL TVPELTQALL VALHMVTANQ DSIQLTSNLA
 421 LDPKVKNIPM LRDMIMLGYG SMVARYCDEQ PECSPDLLRP IHENAAQAIS KTDAPEITLA
 481 LKALGNAGQP ASLKTIMKVL PRFGSAAANI PMNVQIDAIL ALRNIAKKEP KLVQPVALQL
 541 FMDKSLHPEL RMVSCIVLFE TEPSVALMAT LAGALERETN MHVVSFAYSH IKSLTRSMAP
 601 DYMHVAAAAN VAIRMLSPKL DRLSYYFSRA THFDVYISPL MVGAAGSAYW INDASTILPR
 661 AIVAKARAYL AGESAAADVI EVGLRTEGLQ EALLKTSAAD QNADRATKIQ NTIKALMDWR
 721 SLSSKQPLAS IFVRVLGQEI AYANFDKNFM DKIIDQAAQI ATGRQARELM KETVKALQKG
 781 IAYHYAKPLM AAEMRRILPS SLGVPVEFSH YTAAVAAASL NVQAKTTPAL PENPETLTLD
 841 QLMKTDIQLQ AEARPSIALQ TFAVMGVNTA FIQAAVMARG KLHTVVPGKL SIRADLQKGN
 901 VKLEVLPAAA VPDHIVDVSF ETVAVSRNIE NLPNEKVVSL APPVPADAAQ RYKYSSVHKS
 961 LCSVCPYFNI KGCIEVNSQN AGYMESNPLY NLVGQHSARI TVARGDGPAL ERLEFEVQVG
1021 PKAAEKIRKE ISAGNDDNPE ESNILLKLRK ILEGGLRHTN SSSSSSSSS NSKSSSSSKS
1081 GSSSRSGSKS RSGSSSRSRN SSSRQSSSRN INLNDQFMKF HKDQGRSNAR GSDSMEAMWR
1141 QANLLGDKVP PAFAIIARAV RADRKLGFQI AAYLDKSTPR VQVVFASISA NDKWKICADA
1201 VLPSKHKVAA RFAIGEQCQD YSVTVKAETG LHESHPSTRF EFEWNRVPGI LTIAFPSFKR
1261 VGDYISIVAP LAGVDADRAR NNERAISLIV ALPTQKSLDI LLRFPEMTLS KRNLCLSDAL
1321 PIEQDGTIPA LKNMDIRVIV QNWINDYRKN
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Figure 4: Amino acid sequence of Vitellogenin of *C. macrocephalus*. Bold red amino acids (Vitellogenin peptides) and amino acids highlighted in yellow (Lipovitellinpeptides) indicate the sequences of *C. gariepinus* that matched with Vg sequence of *C. macrocephalus*. Many of these peptides are common in both vitellogenin and lipovitellin of *C. gariepinus*.

Specific gene(s) are expressed in hepatic tissue of fish [49-51]. Estradiol induced the expression of *vg* genes in the hepatocytes of catfish. Two partial nucleotide sequences of *vg*c DNA of *C. gariepinus*, matched (85% to 95%) with different segments of vitellogenin gene of *Clarias macrocephalus, Ictalurus punctatus and Ariopsis felis* (all of which belong to the order Siluriformes). This similarity is furtheraffirmed by phylogenetic analysis wherein evolutionary relationship of *C. gariepinus* with fishes from other orders of Ostariophysi (Siluriformes>Characiformes>Cypriniformes) has been observed.

The deduced amino acid sequences of catfish vitellogenin have conserved domains of Vitellogenin amino-terminal (accession no. pfam01347) or lipoprotein amino-terminal region (accession no. smart00638) and Domain of unknown function (accession no. pfam09172). Both Vitellogenin_N and LPD_N domains share the same lipoprotein amino-terminal region, which belongs to the large lipid transfer protein (LLTP) super family and involved in lipid interactions. DUF 1943 domain belongs to the family of proteins consisting of several large open beta-sheets and contributes to the function of

vitellogenin as an immunocompetent molecule and pattern recognition receptor [52,53]. Peptide sequences derived from isolated Vitellogenin and lipovitellin by mass spectrometry were mapped to the Vitellogenin amino acid sequences translated from partial cDNA sequence and similarity was observed (refer Fig. 3A, B). But the amino acid sequence of lipovitellin obtained by mass spectrometry showed homology only with the translated *vg* gene encoding lipoprotein amino-terminal region and not with the sequence encoding DUF 1943 domain. This mapping and structural similarity establish the relationship of yolk protein in the oocyte with vitellogenin in circulation which has been translated from *vg* mRNA in liver. The existence of precursor-product relationship of Vg and Lvhas been well documented by immunological techniques [37,38,48]. Mass spectrometry data and gene expression study suggest that primary proteolysis of vitellogenin leads to formation of lipovitellin, a major egg-yolk protein. The present investigation will lead to studysecondary proteolysis of yolk proteins into amino acids during maturation, which is essential for embryonic growth and development.

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CONFLICT OF INTEREST

There is no conflict of interest of any kind regarding this manuscript.

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