Effect of subunits of mrigal (*Cirrhinus mrigala*) Vg (HA-I) on catfish vitellogenesis

Raju Sahu[1,2*] and Panchanan Nath[1]

1. Department of Zoology, Visva-Bharati University, Santiniketan-731235.
2. Department of Zoology, Sarojini Naidu College for Women, Kolkata-700028

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Abstract

Two forms of mrigal (*Cirrhinus mrigala*) vitellogenin (HA-I and HA-II) were purified and HA-I was found to induce complete vitellogenesis when administered to the Indian catfish, *Clarias batrachus* (Nath and Maitra, 2001). Therefore, in the present study, two major subunits of HA-I (subunit-I or SUI: 160 kDa and SUII: 83 kDa) were purified and tested along with trypsin digested HA-I for their role in catfish vitellogenesis. 10-day treatment of female catfish @ 1 mg/fish/day revealed that in SUII and TDH-treated groups the fully formed yolky oocytes (SIII) were present in the ovarian section. Since SUII is of 83 kDa protein and TDH also contain similar molecular weight (~75 kDa) protein and are most effective in the formation of SIII oocytes the 83 kDa (SUII) protein may possibly the regulator of vitellogenesis.

Keywords: Subunits of mrigal Vg, catfish vitellogenesis

1. Introduction

Vitellogenin (Vg) is a female specific glycol-lipo-phosphoprotein which appears in the blood at the onset of oocyte growth phase. Vg is synthesized in the liver in response to gonadotropin (GtH)-induced ovarian estradiol-17β (E2), secreted into circulation and incorporated in the developing oocytes by receptor mediated endocytosis (Sundararaj et al., 1982; Nath, 1999). Vg is progressively sequestered by growing oocytes and processed as yolk proteins to constitute the reserve food stuff of the larvae (Specker and Sullivan, 1994; Nath, 1999 for reviews). It is well established that synthesis of Vg can be induced by E2 treatment in both male and female fish and the incorporation of Vg into oocytes can be stimulated by GtH (Nath and Sundararaj, 1981b). To understand the mechanism of vitellogenesis, Vg has been purified either in one or multiple (two or more) forms depending on the fish species studied.

*Corresponding author

Recently Hiramatsu et al. (2003) have purified three forms of female specific proteins identified as three distinct Vgs (Vg A, Vg B and Vg C) from the blood plasma of estrogen-treated white perch, *Monro americana*. The existence of multiple forms of Vg in fishes may suggest that each type of Vg may have different function during reproduction. For example, Vg may act as a carrier for protein and steroid hormones (Babin, 1992; Cyr and Eales, 1992, see Specker and Sullivan, 1994 for review). Reis-Henriques et al. (1997, 2000) demonstrated that Vg inside the oocyte alter the Vg production in liver by modulating the ovarian E2 production in rainbow trout, *Oncorhynchus mykiss*. Matsubara et al. (1999) have reported that there occurs molecular alteration of two forms of Vg- phosvitin and lipovitelline during vitellogenesis and oocyte maturation in barfin flounder, *Verasper moseri*a. Nath et al. (1997) have demonstrated in the catfish, *Clarias batrachus*, that Vg induces complete vitellogenesis (synthesis and incorporation of Vg). Recently two forms of (HA-I and HA-II) of purified Vg from the E2-treated blood plasma of Indian major carp, *Cirrhinus mrigala* and their administration separately into female catfish, *Clarias batrachus* during different reproductive phases, results of which revealed that HA-I induces complete vitellogenesis whereas HA-II could induce only Vg synthesis (Nath and Maitra, 2001). In a pond culture system mrigal Vg (containing HA-I and HA-II) pellet implantations in to female catla (*Catla catla*) not only induce complete vitellogenesis but also provide the signal to the oocyte to undergo maturation (Sahu and Nath, 2007, manuscript under preparation). All the above findings made us to think whether the functional unit is present in native molecule (whole) of HA-I or a part of it.

Keeping this in view attempts have been made to separate the subunits of HA-I and cleaved the HA-I by trypsin and then administered into the female catfish, *Clarias batrachus* to induce vitellogenesis.

2. Materials and Methods

Collection and care of fish

Sexually mature specimens of Indian major carp, the mrigal, *Cirrhinus mrigala*, (body wt. range: 0.15-0.25g) were collected from local ponds and farm around Santiniketan (Lat. $23^{o}41^{/}30^{//}$ N and Long. $87^{o}30^{/}47^{//}$ E) and maintained in the tank.

Estradiol-17β (E_2) injection for vitellogenin synthesis

For bulk preparation of mrigal HA-I, E_2 was injected at the dose level of 50 μg/100g body weight for 30 days by means of a 1-ml tuberculin syringe fitted with 24-gauge needle. Blood was collected to separate plasma for further study.

Estimation of Protein

Protein content in the plasma samples or in elutes obtained during different steps of purification was determined following the method of Lowry *et al.* (1951).

Isolation of subunits from mrigal HA-I

Mrigal Vg (HA-I) was purified by subjecting the plasma of E_2-treated mrigal to gelfiltration and adsorption chromatography (Maitra et al.2007) used for the preparation of subunits. The purified HA-I was first dialyzed against 0.125 M ammonium bicarbonate (pH 7.94) and then urea was added to the HA-I solution with constant stirring so as to obtain 8 M

concentration. The resulting mixture was incubated 24 h at 37^{o}C under constant stirring with the help of magnetic stirrer. The urea-treated HA-I was then loaded on Sephacryl S-200 column (size: 40 cm x 1 cm) pre equilibrated with 0.125 M ammonium bicarbonate buffer containing 6 M ureaand the protein was eluted in the same buffer at the flow rate of 6 ml/h. 1-ml fractions were collected, absorbance was read for each fraction and plotted. 30 mg of separately purified HA-I in 1.5 ml of 0.125 M ammonium bicarbonate (pH 7.94) was loaded on to the same column (urea free) and eluted with a 0.125 M ammonium bicarbonate buffer (pH 7.94) in a similar manner and plotted.

Cleavage of HA-I by Trypsin

HA-I was mixed with trypsin where the ratio between trypsin to HA-I was 1:10 and incubated for 12 h at 37^{o}C. The reaction was stopped by adding aprotinin, the trypsin protease inhibitor. The ratio between aprotinin to protein was 1:20.

Native PAGE

High molecular weight marker proteins (Pharmacia) which include thyroglobulin (669 kDa), ferritin (440kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa) as well as mrigal HA-I, peak 1(SUI) and 2 (SUII) of gelfiltration of urea-treated HA-I and trypsin digested HA-I (TDH) was subjected to native PAGE in 4-15% gradient gel. After the run gels were stained with Coomassie Brilliant blue R250. The relative fonts (Rf) of standard marker proteins, HA-I, SUI, SUII and TDH were calculated by the following formula:

$$Rf = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}}$$

While measuring the distance of protein migration the lower extremity of the bands were considered.

Western Blot Analysis

Native PAGE (4 -15% gradient gel) of purified HA-I along with subunits of HA-I (SUI and SUII) was done for western blotting with mrigal anti-HA-I antiserum. The proteins were electroblotted from the gel on to a nitrocellulose membrane using Hoefer transferring unit (Hoefer TE 22 mighty small transfer Tank Unit) following the method of Towbin et al. (1979) (for details of the procedure see Maitra et al., 2007).

Electrophoretic conditions

A pre-run was given at 70 volts for 30 min before sample loading. Samples were loaded (20μg of each protein in each lane) and electrophoresed at constant 70 volts until the dye front migrated within 0.5 cm of the wicks at the anodic end of the gel. During electro-blotting current was set at 400mA.

Induction of vitellogenesis in the female catfish, *Clarias batrachus*, by subunits (SUI, SUII and TDH) of mrigal HA-I

Mature female catfish were collected during preparatory period (April) and acclimatize for 7 days in the laboratory condition prior to the commencement of experiment. A group of five fishes were sacrificed on the day of commencement of experiment which served as initial control. Purified HA-I, SU I, SU II and trypsin digested HA-I (TDH) were injected to separate groups of female catfish at the dose level of 1 mg/100 g body weight / day for a period of 10 days. The pooled fractions of SU I and SU II were dialyzed against 40 % propylene glycol in 10 Mm PBS (pH 8) to remove urea and then used for injection.

Next day following the last injection autopsy was made. At the time of autopsy blood was collected, plasma separated and used immediately or diluted (1:10) with PBS containing 30 % glycerol and kept in aliquots at -30^{o}C until used for the estimation of Vg by catfish Vg1 ELISA as described by Nath and Maitra, 2001. After the blood sampling ovaries were extirpated, weighed and then fixed in aqueous Bouin's for histological study. Transverse section of ovaries were cut at 7 µm and stained with haematoxylin-eosin. Three different stages of oogenesis were identified in the ovarian section following the method described by Ghosh and Nath (2005). Catfish Vg 1 ELISA was performed following the procedure of Nath and Maitra (2001).

3. Results

Separation of mrigal, *Cirrhinus mrigala*, Vitellogenin (HA-I) subunits

The native mrigal Vg (HA-I) after passing through Sephacryl S-200 gave a single and homogenous peak, whereas the 8 M urea-treated HA-I cleaved into two protein peaks (peak-1 and Peak-2) which were eluted after the HA-I (Fig. 1).

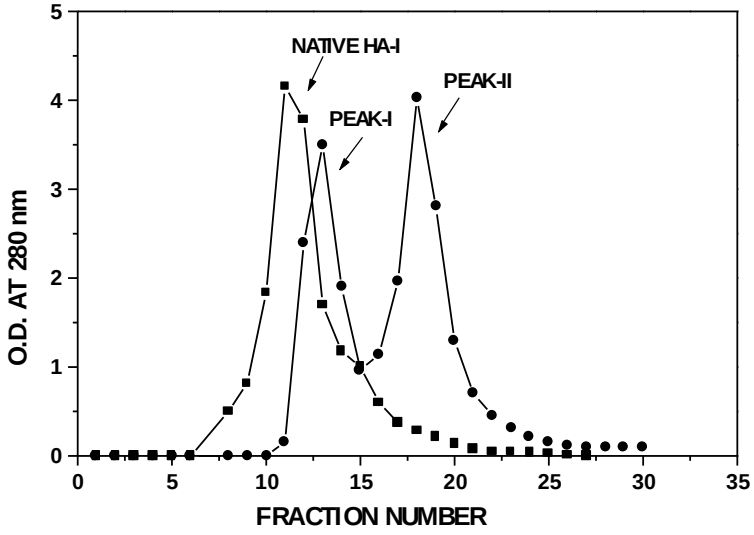

Fig. 1 Elution profile of 8M urea cleaved HA-I on Sephacryl S-200. 1 ml fraction was collected in each tube at the flow rate of 6 ml/h. The optical density of each tube was read at 280 nm.

Molecular weight estimation and Western Blotting

Fig. 2A (lanes 2 to 5) shows the electrophoretic patterns of purified HA-I; peak 1 (subunit I: SU I) and peak 2 (Subunit II: SU II) and trypsin digested HA-I (TDH) respectively on 4-15% gradient gel. HA-I, SU I and SU II on electrophoresis gave single protein band at Rf 0.157 (500 kDa, lane 2), Rf 0.328 (160 kDa, lane 3) and 0.43 (83 kDa, lane 4) respectively. TDH gave two protein bands at Rf. 0.21(364 kDa) and 0.44 (75 kDa) (see lane 5). The anti-HA-I antiserum was tested against the protein bands of HA-I, SU I and SU II by Western blotting and these bands were recognized by the anti-HA-I-antiserum (Fig. 2B).

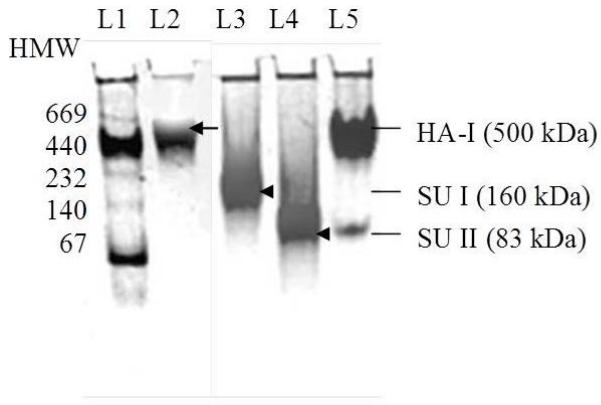

Fig. 2A

Fig. 2B

Fig. 2A: Native PAGE (4-15%) analysis of purified mrigal, *Cirrhinus mrigala*, HA-I and 8 M urea cleaved HA-I and trypsin cleaved HA-I along with high molecular weight marker proteins. Gels were stained with Coomassie Brillliant blue R250. Lane 1, molecular weight standards; Lane 2, purified HA-I and lane 3, peak 1of 8 M urea cleaved HA-I (SU I); Lane 4, peak 2 of 8 M urea cleaved HA-I (SU II); Lane 5, trypsin digested HA-I (TDH).

Fig. 2B: Western blot analysis of mrigal HA-I and peak I and peak II of 8 M urea cleaved mrigal HA-I. Samples were run on a 4 to 15 % gradient gel and were blotted on to a nitrocellulose membrane and incubated with anti-HA-I antiserum and peroxidase-anti peroxidase (PAP) (Anticorps anti IgG). Lane 2, HA-I; lane 3, SU I and lane 4, SU II. Lane numbers in Western blot analysis corresponds to the native PAGE lane numbers (see fig. 2A).

Induction of vitellogenesis by SUI, SUII and TDH of mrigal Vg

The results of the effects of HA-I, SU I, SU II and TDH on ovary and plasma Vg levels in the female catfish, *Clarias batrachus*, during preparatory period are presented in Table 1. In initial control the ovary was regressed (GSI: 0.23 ± 0.04) containing only stage-I (S-I) oocytes (Fig. 3A) and plasma Vg level was very low (0.26 ± 0.04 µg/ml). After 10 days of PBS (pH 8.0) 40 % propylene glycol treatment although GSI and plasma Vg levels were increased compared to initial control ovary contained only S-I oocytes (Fig. 3B). Ten days of treatment with HA-I, SU I, SU II and TDH resulted in an increase in GSI and plasma Vg levels (except in SU I treated group). Histological observation of ovaries revealed that S-II oocytes were present in the ovaries of all Vg-treated groups but S-III: the fully formed yolky oocytes were present only in SU II- (Fig. 3E) and TDH-treated groups (Fig. 3 D).

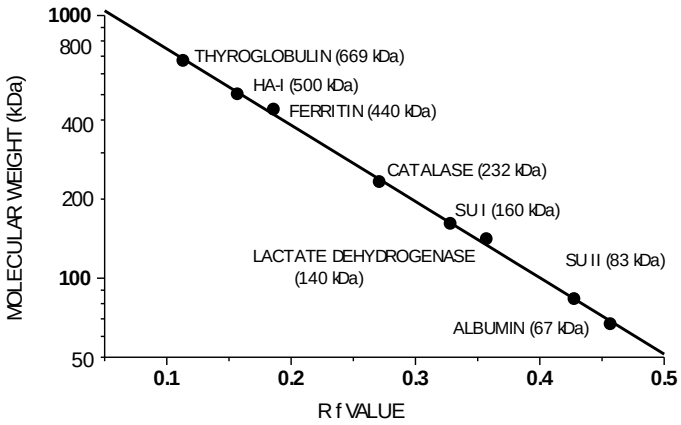

Fig. 2C: Calibration curve obtained after native PAGE (4-15%) of standard high molecular weight marker proteins, thyroglobulin-669 kDa, ferritin-440 kDa, catalase-232 kDa, lactate dehydrogenase-140 kDa and albumin-67 kDa. Note: The molecular weights of purified mrigal HA-I, SU I and SU II of 8 M urea cleaved HA-I, as determined from the calibration curve, are 500 kDa for HA-I, 160 kDa for peak 1 and 83 kDa for peak 2 of 8 M urea cleaved HA-I.

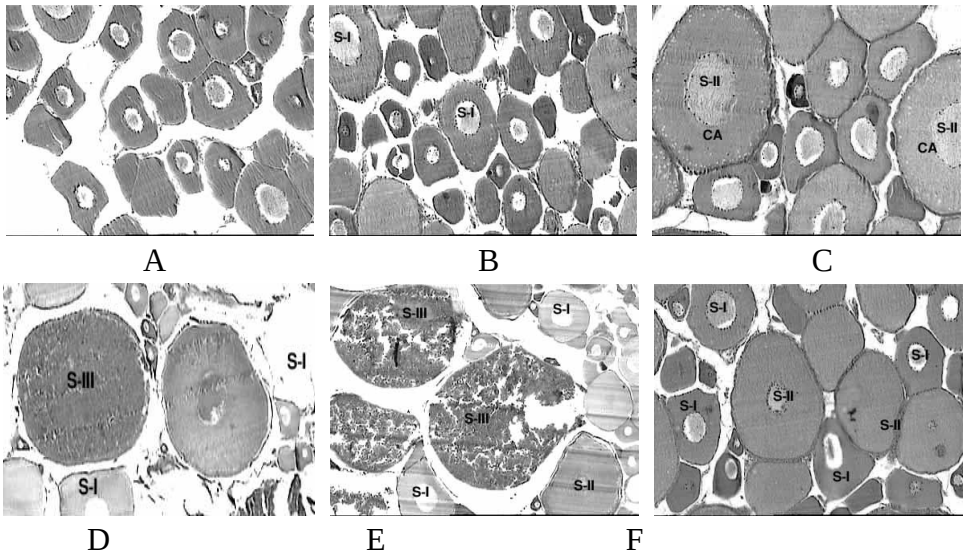

Fig. 3: Photomicrograph of transverse sections of ovaries of female catfish, *Clarias batrachus*, injected (a dose level of 1mg/100 g body weight) with HA-I (Fig. C); trypsin cleaved HA-I (Fig. D); peak I (Fig. E) and peak II (Fig. F). (A) Initial control: showing S-I oocytes (x 10); (B) Control: showing S-I oocytes (x 10); (C) HA-I treated: showing S-I and stage-II oocytes (x 10). (D) Trypsin cleaved HA-I treated: showing satge-I, stage-II & stage-III oocytes (x 4); (E) peak 2 treated: showing stage-III oocytes (x 4) and (F) peak 1 treated: showing stage-I oocytes (x 10).

Table 1

GROUPS	GSI	PLASMA Vg LEVEL	S-I	S-II	S-III
IC	0.232 ± 0.038	0.26 ± 0.038	100	-	-
CONTROL	1.19 ± 0.07	8.43 ± 0.44	100	-	-
HA-I TREATED	0.83 ± 0.115	17.5 ± 2.02	63.47 ± 1.68	36.41 ± 1.69	-
PEAK 1	0.57 ± 0.13	0.46 ± 0.38	86.9 ± 1.15	13.08 ± 1.15	-
PEAK 2	1.27 ± 0.84	126.6 ± 10.557	19.26 ± 3.72	20.96 ± 2.58	19.26 ± 3.72
TRYPSIN CLV HA-I	1.93 ± 0.5	18.9 ± 6.89	46.68 ± 1.9	36.12 ± 1.46	17.19 ± 1.21

Table 1: Effect of 10-day (C) injection (@ 1mg/100 body weight of fish) with HA-I, peak 1, peak 2 and trypsin cleaved HA-I on GSI, stage I, II and III oocytes and plasma Vg levels in female catfish, *Clarias batrachus*, during preparatory (April) period. The mean body weight from left to right were: for initial control: 101; for control: 91; for HA-I: 128; for peak 1: 91.6; for peak 2: 90 and for trypsin cleaved HA-I: 150 g.

4. Discussion

The data presented here indicates that mrigal Vg (HA-I) contains two hetero molecular subunits and the potencies of HA-I, subunit I (SU I), Subunit II (SU II) and trypsin digested HA-I (TDH) on induction of vitellogenesis in the catfish, *Clarias batrachus*.

In the present study purified HA-I gave a single peak when rechromatographed on sephacryl S-200 indicating its purity as it gave a single protein band (500 kD) on native PAGE. The HA-I is one form of Vg has been characterized in various ways (see Maitra et al., 2007).Western blot analysis revealed that anti-HA-I antiserum showed distinct reactivity with a single band corresponding to the single band demonstrated by Coomassie blue stains. Since this antiserum also shows cross reaction with egg yolk proteins (data not shown here) it is further strengthened the view that HA-I is mrigal Vg. The molecular weight estimates obtained for HA-I (500 kD) is comparable to those reported in the literature (550 kD) (see Specker and Sullivan, 1994).

Purified HA-I was dissociated into two subunits under 8 M urea and got separated into two protein peaks (peak I: SU I and peak II: SU II) on gel filtration. On native PAGE SU I and SU II gave single protein band of 160 kD and 83 kD respectively (see fig. 2C). On Western blotting the anti-HA-I antiserum recognized these two bands which are corresponding to those stained with protein stains (cf Figs. 2 A and 2B) and thus indicate that HA-I consists of two heterogenic peptides. The existence of two subunits of Vg has been reported in some teleost fishes (see Specker and Sullivan 1994 for references). SDS-PAGE analysis has also revealed that there occurs a single polypeptide or four identical polypeptides in several teleost species (see Utarbhand and Bunlipatanon, 1996 for references).

With respect to 160 kD peptide of HA-I it is comparable to that has been reported in channel catfish, *Ictalurus natalis* (150 kDa, Goodwin *et al*., 1992), striped bass, *Monro sexatilis* (170 kDa, Tao *et al*., 1993), Arctic charr, *Salvelinus alpinus* (158 kDa, Johnsen *et al*., 1999), English sole, *Pleuronectes vetulus* (130 kDa, Roubal *et al*., 1997), Gilheadseabream, *Sparus aurata* (Mosconi *et al*., 1998), Indian freshwater murrel, *Cahana punctatus* (175 kDa, Sehgal and Goswami, 2005) and with respect to 83 kDa it is in accordance with the findings of Hara *et al*. (1980) who demonstrated a 85 kDa peptide in Japanese eel, *Anguilla japonica*.

Trypsin digestion of HA-I (TDH) gave two protein bands, 364 kDa and 75 kDa (see Fig. 2A). It is already demonstrated that proteolytic enzyme like trypsin and cathepsin group cleaved Vg in the teleost fish studied so far (Kown *et al*. 2001; Carnevali *et al*.1999). The aim of cleaving Vg with trypsin in the present study to observe whether such cleaved Vg has the capacity to induce vitellogenesis in the fish.

HA-I administration induced synthesis and incorporation of Vg in female catfish and hence confirmed the earlier findings of Nath and Maitra (2001). Since HA-I is high molecular weight (500 kD) it was thought that the vitellogenesis inducing capacity may be present in one of its subunits. Interestingly it was found that both SU I and SU II could induce vitellogenesis but the potency of SU I is very less in comparison to SU II with respect to Vg synthesis and incorporation. SU II administration into female catfish not only induces higher plasma Vg level but also induces higher percentage of S II (vitellogenic) oocytes with the appearance of S III fully formed yolky oocytes (19%) in the ovaries. This is the first hand report in this regard. Interestingly TDH also induced complete vitellogenesis in female catfish and the response was at par with that of SU I and this may be due to the fact that TDH has a peptide (75 kD) similar to that (83 kD) of SU II as their electrophoretic mobility is almost same on native PAGE (Fig. 2A).

The findings thus suggest that the 83 kD peptide (SU II) may be the vitellogenesis inducing factor lying in the native molecule of HA-I. Since this is the first time report that a subunit of Vg could induce vitellogenesis, much more work is necessary in this regard to establish the peptide as vitellogenesis inducing factor.

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