ANNEXURE

to

PURIFICATION AND CHARACTERIZATION
OF VITELLOGENIN FROM
ASIAN CATFISH, *Clarias batrachus*

Ph.D. Thesis

of

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(Registration No. 15/ 2007/ Life sciences)
Examiner 1

Chapter I. Introduction

Comment: the 9 objectives could have been pooled to 3 or 4, covering the specific objectives.

Response: In thesis there are not 9 objectives, rather they are the technical programmes of the entire study. However, these may be concised to the followings:

1. To purify and biochemically characterize vitellogenin protein of *C. batrachus*.

2. To develop rabbit polyclonal antibodies to vitellogenin and to standardize immunological methods using the rabbit antibodies to detect/quantify vitellogenin in tissue and plasma samples.

3. To partially characterize vitellogenin gene in *C. batrachus* and to develop a molecular tool to quantify *Vtg* mRNA transcripts.

4. To study the kinetics of E$_2$ action on vitellogenin gene expression at transcription (mRNA) and translation (protein) levels.
Comment 1: Materials part appears to be incomplete without the mention of list of major chemicals (Brand name) and equipments (Brand and model) used in this study.

Response: The brand names of the major chemicals as well as equipments have been mentioned as and when it appeared in the written text, hence, a list was not given. However, a list of such items is provided below:

17β estradiol (E₂) (Sigma, St Louis, USA)
Heparin (Sigma, St Louis, USA)
Aprotinin (Sigma, St Louis, USA)
UV monitor (Bio-Rad, USA)
Syringe filter (Millipore, USA)
Centrifugal filter (Millipore, USA)
Commassie brilliant blue (R-250) (MP Biomedicals, France)
Commassie brilliant blue (G-250) (Merck, Germany)
Alcian blue (Himedia, Mumbai, India)
Methyl green (Himedia, Mumbai, India)
Sudan black B (Merck, Germany)
UV-Vis spectrophotometer (Varian, Colorado, USA)
Freund’s Complete Adjuvant (FCA) (Genei, India)
Freund’s Incomplete Adjuvant (FIA) (Genei, India)
PVDF membrane (Amersham Biosciences, UK)
Goat anti-rabbit alkaline phosphatase conjugate (Genei, India)
BCIP/NBT (Genei, India)
Goat anti rabbit horseradish peroxidise (HRPo) conjugate (Genei, India)
TMB substrate solution (Genei, India)
ELISA plate reader (Model MCC:340, Bio-rad, USA)
RNAlater (Sigma, USA)
Trireagent (Sigma, USA)
DEPC (Sigma, USA)
NANODROP (ND1000, thermo Scientific, Wilmington, USA)
Comment 2: Native gradient PAGE has been mentioned to be used in determination of molecular weight. It should be corrected to check the homogeneity and purity of vitellogenin (in native PAGE separation is based upon charge to mass ratio not on mass only).

Response: Native gradient PAGE (4-22.5%) was used in the our study to determine the molecular weight of native vitellogenin molecule. The gel was run for a long period of 10 h against decreasing pore size, that allows the samples to run as maximum as they can move. Therefore the samples move until it cannot move further. Hence charge did not come into play and the separation is based on mass only. This method also is known as Pore-limit electrophoresis and has been used to determine the molecular weight of native proteins by several authors.


Comment 3: Standard curve for Bradford protein estimation is missing

Response: Since protein estimation is a normal protocol, the standard curve hence, was not shown. However, the standard curve used in our study is given below.

![BSA standard curve used for protein estimation](image-url)
Comment 4: Principle of competitive ELISA is not very clear. It can be made better by making a flow diagram. Moreover, ELISA standard curve is shown in results but it should appear in materials and methods.

Response:

Flow diagram of competitive ELISA for *C. batrachus Vtg*:

1. Incubate with coating antigen (*C. batrachus Vtg*) in bicarbonate buffer for 3 hour at 25º C
2. Washing
3. Block with 1% BSA for 2 hr
4. Washing
5. Add Vtg standard or plasma samples in dilutions to respective wells.
6. Add rabbit anti-*C. batrachus* Vtg antibody to all the wells
7. Incubate overnight at 4º C
8. Washing
9. Add goat anti rabbit horseradish peroxidise (HRPo) conjugate
10. Washing
11. Add substrate solution (TMB/H₂O₂) and incubate for 10 min at room temperature in dark
12. Stop reaction by adding 1M H₂SO₄
13. Washing
14. Read plate in a ELISA reader at 450 nm

Along with the ELISA standard curve, serum dilution curves were also presented in same Fig. 11 to show the parallelism between curves. Hence, it was shown in results.
**Results**

**Comment 5:** For purification of vitellogenin selective precipitation by EDTA-MgCl2 and gel filtration was used but table for purification fold at various steps of purification is missing.

**Response:** Since the amount of Vtg present in initial plasma sample was unknown, the fold purification at precipitation step was not possible to obtain. Only the increase in protein concentration by hormone administration indicated the secretion of Vtg into blood. Hence, the fold purification at different steps has not been mentioned.

**Comment 6:** SDS PAGE under section 3.3.5 has mentioned minor bands of $< 29$ kDa but they are not visible in fig. 7.

**Response:** A good quality figure is given below.

![Fig. 7](image)

**Fig. 7.** Molecular weight determination of constituent polypeptides of *Clarias batrachus* vitellogenin in SDS-PAGE of 10% separating gel. Lane 1: Molecular weight markers and Lane 2: Purified vitellogenin.

**Comment 7:** No graph has been prepared to depict the molecular weight of vitellogenin polypeptides. (Rf vs log M.W).

**Response:** Molecular weight of the proteins and polypeptides were determined using software Quantity one (Bio rad, USA) and hence, graph was not drawn.
Comment 8: Figure 10 and 11 have shown log values vtg conc but actually they are simple increasing concentrations. Second part of figure 11 is not clear.

Response: Log values of Vtg conc were plotted in X- axis. The detailed data for Fig. 10 is given below.

<table>
<thead>
<tr>
<th>Conc. of Vtg (ng)</th>
<th>Log conc. of Vtg</th>
<th>Absorbance at 450 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rabbit serum dilutions</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>0.3815</td>
</tr>
<tr>
<td>500</td>
<td>2.7</td>
<td>0.6035</td>
</tr>
<tr>
<td>250</td>
<td>2.4</td>
<td>0.9675</td>
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<td>125</td>
<td>2.1</td>
<td>1.3545</td>
</tr>
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<td>62.5</td>
<td>1.8</td>
<td>1.7655</td>
</tr>
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<td>31.25</td>
<td>1.5</td>
<td>2.0855</td>
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<tr>
<td>15.625</td>
<td>1.2</td>
<td>2.254</td>
</tr>
<tr>
<td>7.8125</td>
<td>0.9</td>
<td>2.363</td>
</tr>
<tr>
<td>3.90625</td>
<td>0.6</td>
<td>2.403</td>
</tr>
<tr>
<td>1.953125</td>
<td>0.3</td>
<td>2.37</td>
</tr>
</tbody>
</table>

Fig. 10. Determination of optimal dilution of rabbit anti *Clarias batrachus* vitellogenin serum for use in competitive ELISA. Each rabbit antiserum dilution was reacted with various concentrations of Vtg.
The first part of Fig 11 shows the Vtg standard curve. Two fold serial dilutions of purified *C. batrachus* vitellogenin (2000 to 1.95 ng/ml corresponding to 3.3 to 0.3 log concentration as mentioned above for Fig. 10) were used to prepare the standard curve.

The second part of Fig.11 depicts the plasma dilution curve of vitellogenic female that showed parallelism with the Vtg standard curve (Fig. 11). Parallelism indicates that the developed system can be used to quantify Vtg concentration in the plasma samples. Further, no Vtg could be detected in the male plasma used at dilutions of 1:100 to 1:3200.

![Graph showing standard curve and plasma dilution curves](image)

**Fig. 11.** A representative standard curve of *Clarias batrachus* vitellogenin (left) and plasma dilution curves of *C. batrachus* vitellogenic female and normal male (right) in competitive ELISA.
**Comment 9:** for characterization of vitellogenin gene RNA isolation was carried out and its purity was checked as mentioned in 2.7.1 but no figure of purified RNA appeared in results.

**Response:** The quality of the isolated RNA was evaluated spectrophotometrically by A260/A280 ratio. RNA gel was not run.

**Comment 10:** Figure 20A RT-PCR has been shown in reverse mode (DNA bands should appear bright in black background).

**Response:** For better clarity and visibility the photos were taken in reverse mode.

**References**

**Comment 11:** Two references are missing.

Goodbred et al., 1997

Maltais and Roy, 2008

**Response:** Missing references are given below.


Maltais and Roy (2008): A typographical error and should be read as Maltais and Roy (2009), the full reference of which has already been mentioned in reference section.