UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERIZATION OF MALAYSIAN MAHSEER (TOR TAMBROIDES BLEEKERS) VITELLOGENIN

NURUL ASHIKIN BINTI MUHAMMAD

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By

NURUL ASHIKIN BINTI MUHAMMAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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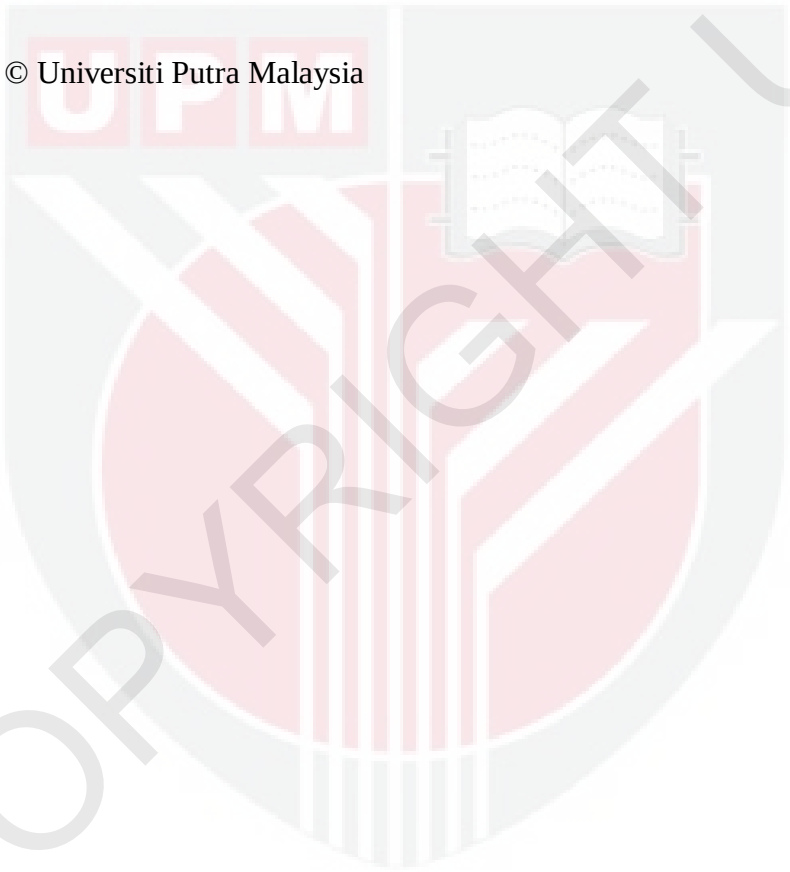

DEDICATIONS

This work is dedicated to my dearest parents,Muhammad bin Ismail and Hanisah binti Jaafar, and my loving siblings Nooraini Azizah, Nooraini Fazilah, Muhammad Munawwar, Nurul Aishah and Muhammad Shukri. Thank you for the never ending support and undeniable love. Without them, the completion of this study might be impossible.

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Thanks for the motivation, assistance and kindness you shared all these times.

Thank you,

Nurul Ashikin Muhammad

UNIVERSITI PUTRA MALAYSIA

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PURIFICATION AND CHARACTERIZATION OF MALAYSIAN MAHSEER (TOR TAMBROIDES BLEEKERS) VITELLOGENIN

By

NURUL ASHIKIN BINTI MUHAMMAD

February 2013

Chair: Annie Christianus, PhD

Faculty: Agriculture

Vitellogenin (vtg) is a high molecular weight glycophospholipoprotein synthesized in the liver under stimulation of estrogen. Basically found in sexually mature female, vtg is taken up by developing oocytes during maturation. It functions as a nutrient storage for growing embryo. Vtg has the potential as a maturation indicator for the successful production of fish fry. *Tor tambroides* is one of the most sought after fish in Malaysia, used as a game fish and economically important as cultured species. 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 identify matured females is necessary. Hence, in this current study, enzyme linked immunosorbent assay (ELISA) has been developed to measure blood plasma vtg as female maturation indicator. The

development of this indicator will definitely contribute to the hatchery production of *T. tambroides* fry.

Estrogen (17β-estradiol) was injected intra-peritoneally into five males (1.5 ±0.4kg). In order to confirm the synthesis of vitellogenin, raw plasma from E2-treated male, vitellogenic female and non-treated male were subjected to SDS-PAGE analysis. The presence of 149kDa protein band in E2-treated male plasma indicated the secretion of vitellogenin. Plasma samples were purified by size exclusion chromatography to separate protein particles according to molecular sizes. Fractions containing the bell-shaped major peaks were collected and subjected to native PAGE. Molecular weight of protein bulk was determined as 700kDa band.

Further reduction of the protein bulk by SDS-PAGE resulted in the appearance of protein bands with similar positions in E2-treated sample and female whereas non-treated male showed no similarity. Specificity of antibody in Western blot revealed that in purified E2-treated male plasma, only three bands (133kDa, 117kDa, 56kDa) were recognized by anti-carp monoclonal antibody and thus identified as vtg. Bands with similar positions were also detected in mature female plasma. Male plasma did not show any cross reactivity against antibody.

Enzyme-linked immunosorbent assay (ELISA) was developed for measurement of vtg concentration at plasma level. Purified vtg (253 ng/ml) was coated on 96-well microplate. Plasma samples were diluted with 1:500 antiserum to a final dilution of

1:1000. Linearization of binding displacement curves by logit transformation revealed that serial dilutions of mature female mahseer plasma slope was not statistically different from purified vtg of mahseer standard ($F_{0.05}$=1.678 $_{(12,28)}$, p>0.05). ELISA assay developed for *T. tambroides* vitellogenin was confirmed through inter- and intra-assay validation. At different binding percentages (20, 50 and 80%), the coefficient of variation (CV%) of both precision assays (inter- and intra-assay) were less than 15% which means ELISA developed for the measurement of plasma vtg concentration in *T. tambroides* is sensitive and repeatable.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENULENAN DAN PENCIRIAN VITELLOGENIN KELAH MERAH (TOR TAMBROIDES BLEEKERS)

Oleh

NURUL ASHIKIN BINTI MUHAMMAD

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Vitellogenin (vtg) ialah sejenis glikofosfolipoprotein dengan berat jisim molekular yang tinggi, dihasilkan di dalam hepar di bawah rangsangan hormon estrogen. Kebiasaannya ditemui di dalam haiwan betina yang matang, vtg diserap oleh telur yang sedang membesar semasa proses kematangan. Ia berfungsi sebagai penyimpan nutrien bagi tumbesaran embrio. Berpotensi sebagai penanda aras bagi kematangan telur, vtg membantu memperbaiki sistem penghasilan anak ikan. Kelah merah adalah salah satu ikan yang paling dicari di Malaysia, yang digunakan sebagai ikan permainan dan penting dari segi ekonomi sebagai spesies biakan. Masalah utama yang timbul dalam penghasilan benih ikan kelah merah yang banyak di tempat penetasan ialah pada ketersediaan dan pemilihan induk yang matang. Hanya pekerja yang mahir dan pakar sahaja yang berupaya mengenalpasti ikan betina yang matang dan bersedia untuk bertelur daripada aspek morfologi. Oleh itu, suatu kaedah yang

mudah untuk mengenalpasti ikan betina yang sesuai dijadikan sebagai induk adalah diperlukan. Maka, dalam kajian ini, ELISA telah dihasilkan untuk mengukur vtg dalam plasma ikan kelah merah sebagai penanda aras kematangan ikan betina. Penghasilan penanda aras ini pasti akan menyumbang kepada penghasilan pusat penetasan benih ikan kelah merah.

Estrogen (17β-estradiol) telah disuntik secara intra-peritoneal ke dalam lima ekor ikan jantan (1.5±0.4 kg). Untuk memastikan vtg telah terhasil, plasma yang belum melalui proses pengasingan, diambil dari ikan jantan yang telah disuntik, ikan betina yang matang dan ikan yang tidak disuntik telah diuji dan disaring untuk memerhati kehadiran jalur protein dengan jisim molekular yang tinggi menggunakan SDS-PAGE. Kemunculan jalur protein dengan jisim molekul 149 kDa dalam plasma ikan jantan yang telah disuntik dan ikan betina yang matang menandakan vtg telah terhasil dengan kehadiran hormon estrogen. Sampel plasma telah diasingkan menggunakan kromatografi pengasingan berdasarkan saiz molekul. Hasil sampel plasma pengasingan melalui kromatografi (protein bersaiz besar) yang mengandungi puncak simetri berbentuk loceng yang boleh dilihat pada graf yang tercetak bersama data pengasingan melalui kromatografi, telah dikumpulkan untuk analisa seterusnya. Melalui 'native PAGE', saiz protein yang terkumpul dianggarkan mempunyai saiz sebesar 700 kDa.

Pengurangan lanjut saiz protein kepada beberapa pecahan jalur protein oleh SDS-PAGE menunjukkan kehadiran protein jalur dengan kedudukan yang sama dalam sampel ikan jantan yang disuntik dengan estrogen dan ikan betina yang matang,

manakala sampel ikan jantan terkawal (yang tidak disuntik) menunjukkan tiada kesamaan pada kedudukan jalur protein tersebut. Di dalam analisis 'Western blot', plasma ikan jantan yang disuntik dengan estrogen menunjukkan tiga jalur polipeptida (133, 117 dan 56 kDa) dikesan oleh anti-kap antibody monoklon dan dikenalpasti sebagai vtg. Jalur-jalur pada kedudukan yang sama juga dikesan di dalam sampel ikan betina yang matang. Sampel ikan jantan yang tidak disuntik tidak menunjukkan sebarang tindakbalas terhadap antibodi.

ELISA telah pun berjaya dicipta dengan pengkhususan pada spesies ikan kelah merah, untuk pengiraan kepekatan vtg dalam plasma. Sampel vtg yang telah diasingkan (253 ng/ml) dilapis pada permukaan '96-well microplate'. Sampel plasma telah dicairkan dengan antiserum dengan nisbah 1:500 sehingga 1:1000. Pelinearan daripada lengkung anjakan mengikat oleh transformasi logit menunjukkan pencairan bersiri sampel ikan betina kelah merah mempunyai kecerunan yang secara statistiknya sama dengan sampel plasma vtg ikan kelah merah ($F_{0.05}$=1.678 $_{(12,28)}$, $p>0.05$). ELISA yang telah dicipta spesifik untuk *T. tambroides* terbukti boleh digunakan semula, mudah dianalisa dan sensitif sepertimana dibuktikan oleh hasil yang diperoleh melalui satu kaedah bagi mengukur ketepatan esei. Pada peratusan pengikatan yang berlainan (rendah, sederhana, tinggi), pekali variasi (CV %) bagi kedua-dua inter- dan intra-assay yang telah ditentukan adalah kurang daripada 15%. Ini bermakna ELISA yang telah berjaya dihasilkan melalui eksperimen ini yang bertujuan untuk mengukur kepekatan vtg dalam darah ikan kelah merah, adalah merupakan esei yang sangat berguna dan boleh diguna pakai pada masa hadapan.

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I certify that an Examination Committee met on 19th February 2013 to conduct the final examination of Nurul Ashikin binti Muhammad on her Master of Science thesis entitled "Purification and Characterization of Malaysian mahseer *Tor tambroides* (Bleeker, 1854) Vitellogenin" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree.

Member of the Examination Committee are follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follow:

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DECLARATION

I declare that the thesis is my original work for quotation and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at Universiti Putra Malaysia or other institutions.

NURUL ASHIKIN BT MUHAMMAD

Date: 19 February 2013

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LIST OF ABBREVIATIONS

ANCOVA	-	Analysis of covariance
APS	-	Ammonium persulfate
CV	-	Coefficient of variation
E2	-	17β-estradiol
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
MS222	-	Tricaine methanesulfonate
NSB	-	Non-specific binding
OD	-	Optical density
PBST	-	Phosphate buffered saline-Tween 20
PVDF	-	Polyvinylidene fluoride
SDS-PAGE	-	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TEMED	-	Tetramethylethylenediamine
TMB	-	Tetramethyl benzidine
vtg	-	vitellogenin

CHAPTER 1

INTRODUCTION

Vitellogenin (vtg), an egg yolk precursor, is a high molecular weight glycophospholipoprotein found in sexually mature female vertebrates. It is synthesized in the liver under estrogen stimulation. Plasma vtg level is related to the maturation stage of eggs in female. Vitellogenesis relates to the incorporation of vtg proteins and other molecules such as vitamins and lipids into growing oocyte. At the end of this process, the oocyte containing all molecules important for developing embryo becomes ready for fertilization. Therefore, information acquired on the characteristic and concentration of plasma vtg in female fish is can be used as an indicator for oocytes maturity.

1.1 Statement of Problem

Tor tambroides or locally known as kelah merah, can be found in pristine rivers. Their numbers are declining due to overfishing and deforestation (Sungan *et al*., 2006). Species conservation is crucially critical to avoid extinction of the species. Documented report suggested that this fish is able to reproduce throughout the year (Ismail *et al*., 2011). High demand for mahseer fry has urged researchers, farmers, and academicians to come up with reliable technique to enhance breeding and

spawning success. Identification of matured females through physical observation is inadequate to ascertain the maturity stage of the fish. Therefore, a study on vtg characteristics and concentration in mahseer were proposed as indicators for oocytes maturity.

1.2 Significance of Study

A number of studies on vitellogenin (vtg) focused on environmental concerns and reproductive physiology of fish. 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). Various methods have been developed to purify vertebrates' vtg. Magalhães *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 matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). 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 vtg 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 immuno 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.

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

1.3 OBJECTIVES

Therefore, the objectives of this study were:-

1. To determine the molecular weight of purified estrogen-induced vtg in mahseer by SDS-PAGE and Western blot

2. To characterize the specificity of commercial antibody against mahseer purified vtg by Western blot

3. To develop an enzyme-linked immunosorbent assay (ELISA) for the quantification of plasma vtg in mahseer

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