UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF VITELLOGENIN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ASIAN SEABASS, *Lates calcarifer* (Bloch 1790)

NOOR FAZIELAWANIE BINTI MOHD RASHID

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

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

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July 2012

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Vitellogenin (vtg) is a major protein present abundantly in female fish undergoing oogenesis. In male and immature fish, vtg gene is normally suppressed. However, vtg synthesis can be induced by administration of estrogen hormone (E_2). This study was conducted to induce, purify and characterize vtg in E_2-treated immature *Lates calcarifer*. This is important for screening maturity status of this economically important species in a farm.

Two-year old immature *L. calcarifer* (n=10) were given three intraperitoneal injections of 17-β estradiol (E_2) at a dose of 2 mg/kg body weight at two days intervals to induce vitellogenesis. Control groups (vitellogenic female and matured male *L. calcarifer*, n=6) were injected with 0.9% saline only. Blood was collected three days after the last injection and plasma was purified through gel filtration chromatography using Sepachryl HR-300 column, eluted with Tris-HCl pH 8.0. A broad, single symmetrical peak consisting of vtg

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molecule was produced. Protein concentration was 0.059 mg/ml as determined by Bradford assay using Bovine Serum Albumin (BSA) as standard. The purified protein was electrophoresed on Native PAGE to confirm the purity and determine the molecular weight of putative vtg. The protein appeared as one dimeric circulating form with molecular weight of 545 kDa. In SDS-PAGE under reducing conditions, two major bands appeared at 232.86 and 118.80 kDa and minor bands at 100.60, 85.80 and 39.92 kDa, respectively. The purified vtg was used to generate polyclonal antibody (Abvtg) against vtg and the specificity of antibody was assessed by Western blot analysis. Two major bands were immunoreacted, whereas no cross-reactivity was observed with plasma from non-induced males. *Lates calcarifer* vtg was found to be phosphoglycolipoprotein as it positively stained for the presence of lipid, phosphorus and carbohydrate using Sudan Black B, methyl green and periodic acid/Schiff reagent solution (PAS), respectively. The amino acid composition was analysed by high sensitivity Amino Acid Analysis (AAA) which showed high percentage of non-polar amino acids (~48%).

The polyclonal antibody generated was used to develop a competitive enzyme-linked immunosorbent assay (ELISA) for quantifying plasma vtg concentration. Working ranges of the assay were from 31.2 to 1000 ng/ml with a sensitivity of 6.9 ng/ml. The ELISA demonstrated precision with intra- (< 8.4, n=9) and inter-assay (<12.1, n=5) variations at 90, 80 and 50% of binding. Serial plasma dilutions from vitellogenic females and E_2-treated immature *L. calcarifer* were paralleled to the vtg standard curve (purified vtg)

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as analyzed by Analysis of Covarians (ANCOVA) (p > 0.05). No cross-reaction was detected from male plasma dilution, thus validating that the assay quantified plasma vtg in *L. calcarifer*.

The accuracy and sensitivity of vtg measurement by immunoassay has broad application in aquaculture for managing fish broodstock in captivity. The present study successfully isolated and characterized vtg in induced immature *L. calcarifer*. Analysis of vtg using Abvtg is proposed as indicator in determining female maturity stage.

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

PEMBANGUNAN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) VITELOGENIN UNTUK SIAKAP ASIA, *Lates calcarifer* (Bloch 1790)

Oleh

NOOR FAZIELAWANIE BINTI MOHD RASHID

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Vitelogenin (vtg) adalah protein utama yang hadir dengan banyaknya dalam ikan betina yang menjalani proses oogenesis. Dalam ikan jantan dan belum matang, gen vtg biasanya tidak aktif. Walau bagaimanapun, sintesis vtg boleh diaruh dengan memberi hormon estrogen. Kajian ini dijalankan untuk meransang, menulen dan mengkelaskan vtg dalam *L. calcarifer* yang belum matang, dirawat dengan E_2. Ini penting bagi menentukan status kematangan spesies yang penting dari segi ekonomi ini dalam ternakan.

Lates calcarifer (n=10) yang belum matang (berumur dua tahun) diberi tiga suntikan intraperitoneal 17-β estradiol (E_2) pada dos 2 mg/kg berat badan selang dua hari untuk meransang vitelogenesis. Kumpulan kawalan (betina vitellogenic dan jantan yang matang, n=6) disuntik dengan 0.9% larutan salina sahaja. Tiga hari selepas suntikan terakhir, darah dikumpul dan plasma ditulenkan melalui penurasan gel kromatografi menggunakan kolum Sepachryl HR-300, dielut dengan Tris-HCl pH 8.0. Satu puncak tunggal

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simetri yang luas, terdiri daripada molekul vtg telah dihasilkan. Kepekatan protein adalah 0.059 mg/ml sebagaimana ditentukan oleh asei Bradford menggunakan piawai Bovine Serum Albumin. Protein yang telah ditulen itu dielektroforesis menggunakan PAGE asli untuk mengesahkan ketulenan dan menentukan berat molekul vtg. Protein didapati muncul sebagai satu bentuk edaran dimer dengan berat molekul 545 kDa. Dalam keadaan pengurangan SDS-PAGE, dua jalur utama wujud iaitu 232.86 dan 118.80 kDa dan jalur kecil yang masing-masing adalah 100.60, 85.80 dan 39.92 kDa. Vitelogenin yang telah ditulenkan digunakan untuk menghasilkan antibodi poliklon (Abvtg) terhadap vtg dan spesifisiti antibodi dinilai menggunakan analisis "Western blot". Dua jalur utama bertindakbalas imun, manakala tiada tindak balas silang diperhatikan daripada plasma jantan yang tidak dirangsang. *Lates calcarifer* vtg didapati fosfoglikolipoprotein kerana ia positif apabila diuji untuk kehadiran fosforus, lipid, dan karbohidrat masing-masing menggunakan Sudan Black B, metil hijau dan larutan berkala asid / Schiff reagen (PAS). Komposisi asid amino telah dianalisis dengan menggunakan kepekaan tinggi - Analisis Asid Amino (AAA) yang menunjukkan peratusan asid amino bukan polar yang tinggi (~ 48%).

Antibodi poliklon yang dihasilkan telah digunakan untuk membangunkan satu Enzyme-Linked Immunosorbent Assay (ELISA) bersaing bagi menentukan kepekatan plasma vtg. Julat asei yang diguna ialah 31.2-1000 ng/ml dengan sensitiviti 6.9 ng/ml. ELISA menunjukkan ketepatan dengan variasi asei-intra (<8.4, n=9) dan inter (<12.1, n=5) pada ikatan 90, 80 dan 50%. Pencairan plasma bersiri daripada betina vitelogeni dan rawatan estradiol dalam *L.*

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calcarifer yang belum matang adalah selari dengan keluk piawai vtg (vtg yang ditulen) seperti yang dianalisis oleh *Analysis of Covarians* (ANCOVA) ($p > 0.05$). Tiada tindak balas silang dikesan daripada pencairan plasma jantan, justeru mengesahkan asei dapat mengukur plasma vtg dalam *L. calcarifer*.

Pengukuran vtg yang tepat dan sensitif oleh asei imun mempunyai aplikasi yang luas dalam bidang akuakultur untuk mengurus induk yang diternak. Kajian ini berjaya mengasingkan dan mengkelaskan vtg dalam *L. calcarifer* belum matang yang dirawat. Analisis vtg menggunakan Abvtg adalah sesuai dicadangkan sebagai petunjuk kematangan ikan betina.

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I certify that a Thesis Examination Committee has met on July 2012 to conduct the final examination of Noor Fazielawanie Binti Mohd Rashid on her thesis entitled "Isolation, purification and characterization of vitellogenin in Asian sea bass *Lates calcarifer*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which has been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any institution.

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NOOR FAZIELAWANIE BINTI MOHD RASHID

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TABLE OF CONTENTS

		Page
ABSTRACT		ii
ABSTRAK		v
ACKNOWLEDGEMENTS		viii
APPROVAL		x
DECLARATION		xii
LIST OF TABLES		xv
LIST OF FIGURES		xvi
LIST OF ABBREVIATIONS		xviii

CHAPTER

1 **INTRODUCTION** 1

2 **LITERATURE REVIEW**
- 2.1 Aquaculture industry 7
- 2.2 Biology of Asian sea bass (*Lates calcarifer*)
  - 2.2.1 Life cycle 9
  - 2.2.2 Taxonomy and classification 10
  - 2.2.3 Feeding behavior and feeds 10
  - 2.2.4 Maturation 11
  - 2.2.5 Distribution 11
  - 2.2.6 Ecology 12
- 2.3 Importance of hormone (17-β estradiol) 13
- 2.4 Vitellogenin
  - 2.4.1 Vitellogenin in general 14
  - 2.4.2 Vitellogenin uptake by oocyte 17
  - 2.4.3 Other functions of vitellogenin 17
- 2.5 Vitellogenesis
  - 2.5.1 Process of vitellogenesis 18
  - 2.5.2 Site of vitellogenin synthesis 19
  - 2.5.3 Vitellogenesis in male and juvenile fish 19
- 2.6 Methods for determination of vitellogenin
  - 2.6.1 Indirect 21
  - 2.6.2 Direct 22

3 **ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF VITELLOGENIN**
- 3.1 Introduction 26
- 3.2 Materials and Methods
  - 3.2.1 Experimental animals 28
  - 3.2.2 Induction of vtg 28
  - 3.2.3 Plasma preparation 29
  - 3.2.4 Purification of vtg 30
  - 3.2.5 Determination of protein concentration 31

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3.2.6 Characterization of purified vtg 31
3.2.7 Production of anti-vtg polyclonal antibody 33
3.2.8 Immunoblotting (Western blot) 34
3.2.9 Amino acid analysis 35
3.3 Results
3.3.1 Purification of vtg 37
3.3.2 Characterization of vtg 39
3.3.3 Amino acid analysis 43
3.4 Discussion 45
3.5 Conclusion 47

4 DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)
4.1 Introduction 48
4.2 Materials and Methods
4.2.1 Development of ELISA 51
4.2.2 ELISA procedure
Antigen coating 52
Pre-incubation of samples and standards 52
Primary antibody incubation 53
Secondary antibody incubation 53
Substrate development 53
Data analysis 54
4.2.3 Standard curve 54
4.2.4 Assay precision 55
4.2.5 Antibody specificity 55
4.2.6 Assay validation 56
4.3 Results
4.3.1 Determination of optimal concentration of *L. calcarifer* vtg and antiserum dilutions 57
4.3.2 Parallelism of standard curves 60
4.3.3 Assay precision 62
4.3.4 Assay sensitivity 63
4.3.5 Antibody specificity 63
4.3.6 Assay validation 63
4.4 Discussion 65
4.5 Conclusion 66
5 GENERAL DISCUSSION 67
6 GENERAL CONCLUSION AND RECOMMENDATION 71

REFERENCES 73
APPENDICES
Appendix A : Injection of hormone and blood sampling 87
Appendix B : Determination of protein concentration 88
Appendix C : ELISA 89
BIODATA OF STUDENT 90
LIST OF PUBLICATIONS 91
LIST OF TABLES

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